2010

LACTONE-CARBOXYLATE INTERCONVERSION AS A DETERMINANT OF THE CLEARANCE AND ORAL BIOAVAILABILTY OF THE LIPOPHILIC CAMPTOTHECIN ANALOG AR-67

Eyob Debebe Adane
University of Kentucky, eyobdebebe@gmail.com

ABSTRACT OF DISSERTATION

EYOB DEBEBE ADANE

THE GRADUATE SCHOOL
UNIVERSITY OF KENTUCKY
2010

LACTONE-CARBOXYLATE INTERCONVERSION AS A DETERMINANT OF THE CLEARANCE AND ORAL BIOAVAILABILTY OF THE LIPOPHILIC CAMPTOTHECIN ANALOG AR-67

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

By

Eyob Debebe Adane

Lexington, Kentucky

Co-Directors: Dr. Mark Leggas, Assistant professor of Pharmaceutical Sciences
and Dr. Bradley D. Anderson, Professor of Pharmaceutical Sciences

Lexington, Kentucky
2010

ABSTRACT OF DISSERTATION

LACTONE-CARBOXYLATE NTERCONVERSION AS A DETERMINANT OF THE CLEARANCE AND ORAL BIOAVAILABILTY OF THE LIPOPHILIC CAMPTOTHECIN ANALOG AR-67

The third generation camptothecin analog, AR-67, is undergoing early phase clinical trials as a chemotherapeutic agent. Like all camptothecins it undergoes pH dependent reversible hydrolysis between the lipophilic lactone and the hydrophilic carboxylate. The physicochemical differences between the lactone and carboxylate could potentially give rise to differences in transport across and/or entry into cells. In vitro studies indicated reduced intracellular accumulation and/or apical to basolateral transport of AR-67 lactone in P-gp and/or BCRP overexpressing MDCKII cells and increased cellular uptake of carboxylate in OATP1B1 and OATP1B3 overexpressing HeLa-pIRESneo cells. Pharmacokinetic studies were conducted in rats to study the disposition and oral bioavailability of the lactone and carboxylate and to evaluate the extent of the interaction with uptake and efflux transporters. A pharmacokinetic model accounting for interconversion in the plasma was developed and its performance evaluated through simulations and in vivo transporter inhibition studies using GF120918 and rifampin. The model predicted well the likely scenarios to be encountered clinically from pharmacogenetic differences in transporter proteins, drug-drug interactions and organ function alterations. Oral bioavailability studies showed similarity following lactone and carboxylate administration and indicated the significant role ABC transporters play in limiting the oral bioavailability.

Key Words: AR-67, interconversion, lactone, carboxylate, P-gp, BCRP/Bcrp, OATP/Oatp, inverse Gaussian

Eyob Debebe Adane

Student's signature

December 15, 2010

Date

LACTONE-CARBOXYLATE INTERCONVERSION AS A DETERMINANT OF THE CLEARANCE AND ORAL BIOAVAILABILTY OF THE LIPOPHILIC CAMPTOTHECIN ANALOG AR-67

By

Eyob Debebe Adane

Mark Leggas, Ph.D.
Co-Director of Dissertation

Brad Anderson, Ph.D.
Co-Director of Dissertation

Jim Pauly, Ph.D.
Director of Graduate Studies

December 15, 2010
Date

RULES FOR THE USE OF DISSERTATIONS

Unpublished dissertations submitted for the Doctor's degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgments.

Extensive copying or publication of the dissertation in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky. A library that borrows this dissertation for use by its patrons is expected to secure the signature of each user.

Name Date

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

DISSERTATION

Eyob Debebe Adane

The Graduate School
University of Kentucky
2010

LACTONE-CARBOXYLATE INTERCONVERSION AS A DETERMINANT OF THE CLEARANCE AND ORAL BIOAVAILABILTY OF THE LIPOPHILIC CAMPTOTHECIN ANALOG AR-67

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

By

Eyob Debebe Adane

Lexington, Kentucky

Co-Directors: Dr. Mark Leggas, Assistant professor of Pharmaceutical Sciences
and Dr. Bradley D. Anderson, Professor of Pharmaceutical Sciences

Lexington, Kentucky
2010

DEDICATION

This work is dedicated to my late mother, Mulunesh Tekle-Mariam who could not share the happiness of this moment. I did it Mother!

ACKNOWLEDGEMENTS

I am deeply indebted to my advisor Dr. Mark Leggas for all the help he rendered throughout my studies. He has patiently guided and mentored me for the past five years. I also thank my co-advisor, Dr. Brad Anderson for his comments, questions and useful suggestions. My thanks also go to my committee members Dr. McNamara, Dr. Wedlund and Dr. Vore for their scientific advice and encouragement. I also thank Dr. Trevor Creamer for agreeing to be my external examiner. I acknowledge and give the sincerest gratitude to Dr. Jamie Horn for her instruction and help with instrumental methods.

My thanks also go to current and former Leggas lab members. I am happy to have made the acquaintances of Dr. Zhiwei Liu, Tamer Ahmed, Kuei-Ling Kuo, Eleftheria Tsakalozou, Dr. Dominique Talbert, Yali Liang and Dr. Mamta Goswami. I also thank Lipeng Wang and Dr. Philip Empey from Dr. McNamara's lab and Dr. Tian Xiang from Dr. Anderson's lab. Many thanks to Catina Rossoll, Graduate Program Student Affairs Coordinator at UK College of Pharmacy, for all her help in the past five years.

My family has been very supportive throughout my studies. I owe a lot to my wife, Lemlem Gizaw, for her patience, sacrifice and encouragement throughout the past five years. I also thank my children Ribka Eyob and Aryam Mulunesh Eyob for replenishing and sometimes draining my energy during the course of this project. I also thank my sister Guenet Samuel for instilling in me the value of education since early childhood. I appreciate the help and encouragement of my brother and friend Nebiyu Muleta and his family. Thanks be to God for giving me the patience to endure the ups and downs of graduate studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

BACKGROUND

1-1. Camptothecins

Camptothecin is a naturally occurring compound with anticancer properties. Since its isolation from the bark of *Camptotheca accuminata* by Wall and Wani in 1966, only two analogues, topotecan and irinotecan, have been approved by the Food and Drug Administration (FDA) for clinical use. However, recently there have been several other analogues that have entered preclinical and clinical development. The antitumor activity of camptothecins stems from their reversible interaction with the nuclear protein topoisomerase I (topo I) during cell replication. Topo I is an enzyme that facilitates DNA transcription by unwinding supercoiled DNA. During this process, topo I introduces a single strand DNA break allowing the DNA helix to rotate and relieve the torsional strain (1-3). Once the DNA helix unwinds, these single strand DNA breaks are religated and the enzyme repeats its action in the next coiled DNA segment. However, in the presence of a camptothecin, the enzyme–DNA complex is stabilized through weak non-covalent interactions between the camptothecin and topo I, leading to double strand breaks, and consequently, apoptosis and cell death (4, 5).

Camptothecin molecules depend on the E-ring lactone pharmacophore group for their pharmacological activity (6). **Figure 1-1** depicts the chemical structure and ring numbering of camptothecin as it undergoes a pH dependent, but reversible hydrolysis, between the labile lactone and ring open carboxylate forms. At acidic pH, the lactone form predominates, while at physiological pH and above, the carboxylate prevails (7). The presence of lipid bilayers protects the lactone (8). For camptothecin and some of the first generation analogues, such as 9-aminocamptothecin and 9-nitrocamptothecin, the stability of the lactone form is further compromised in vivo (9-12) due to the strong binding of the carboxylate to human serum albumin (13). The hydrolysis of camptothecins is purported to limit their clinical use because of presumed toxicity and lack of antitumor activity of the carboxylate (14).

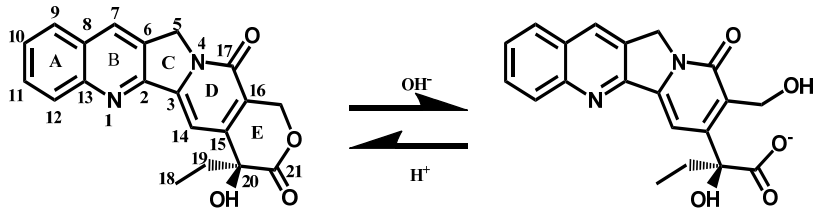


Figure 1-1. Chemical structure and ring numbering system of camptothecin and its pH dependent reversible hydrolysis from the lactone to the carboxylate.

The poor aqueous solubility of the parent compound camptothecin (2-3 μg/mL) (15) hinders its administration (16) and therefore its clinical utility. Efforts to increase the aqueous solubility of camptothecin by substitution of chemical groups on rings A and/or B have yielded second generation compounds with enhanced water solubility and stability. Topotecan, obtained by substitution of $-CH_2N(CH_3)_2$ at position 9 and an -OH group at position 10, is the first FDA approved water soluble analogue (17). The other camptothecin analog in clinical use, irinotecan, is a water soluble pro-drug of 7-ethyl-10-hydroxycamptothecin (SN-38).

Table 1-1 lists some camptothecin analogs in various stages of drug development while **Figure 1-2** presents their chemical structures. Efforts to further stabilize the lactone moiety of camptothecin have resulted in third generation camptothecin analogs (AR-67, gimatecan, chimmitecan, etc) that are highly lipophilic. It is likely that some of these analogs might advance to clinical use in the near future, although their poor aqueous solubility presents a challenge to administration through the oral or intravenous routes.

Among these analogues, 7-t-butyldimethylsilyl-10-hydroxycamptothecin (AR-67 or DB-67) displays elevated lactone levels in blood as a result of the weak binding of its carboxylate form to plasma proteins and the strong binding to lipid membranes and partitioning into red blood cells of the lactone form (11). Substitution of a tert-butylsilyl ($-Si(CH_3)_2C(CH_3)_3$) group at position 7 increases its lipophilicity and membrane partitioning, while the substitution of a hydroxyl (-OH) group at position 10 reduces the binding of the carboxylate with human serum albumin (11). **Figure 1-3** presents a comparison of the percent lactone concentrations at equilibrium of some camptothecin

analogues and AR-67 in whole blood (11). As the figure illustrates, percent lactone in whole blood is higher for AR-67 than for camptothecin or topotecan. In other studies, lipid membrane partitioning of AR-67 markedly decreased lactone hydrolysis and carboxylate formation (18, 19). The relatively higher percent lactone in blood makes AR-67 an attractive drug molecule since pharmacological activity is believed to be due to the lactone moiety.

Table 1-1. Camptothecin analogues in development or clinical use‡**.**

Analogue	Status	Route and indications
Topotecan	FDA approved	IV infusion in metastatic ovarian cancer (second line); SCLC (second line)
Irinotecan	FDA approved	IV infusion in metastatic colorectal cancer (first line with 5-FU/leucovorin)
Rubitecan (9-NC)	Phase II/III	Oral in pancreatic cancer (converted to 9-AC)
IDEC-132 (9-AC)	Phase II	IV infusion/oral/intraperitoneal in ovarian cancer
Exatecan	Phase II/III	IV infusion in various carcinomas
Lurtotecan	Phase II	IV infusion (liposomal) in ovarian and other carcinomas
Gimatecan	Phase I/II	Oral in glioblastoma, SCLC, solid tumors
PEG-camptothecin	Phase II	IV infusion (PEGylated) NSCLC and other solid tumors
Karenitecin	Phase II	Oral in glioblastomas, melanomas and NSCLC
Belotecan	Phase I/II	IV infusion in advanced SCLC, ovarian cancer, cervical cancer
Silatecan (AR-67)	Phase I	IV infusion in colorectal carcinoma, lung cancer

‡Adapted from references (20-22). NSCLC: non-small cell lung cancer; SCLC: small cell lung cancer; PEG: polyethylene glycol; IV: intravenous: 5-FU: 5-fluorouracil.

9-NC: 9-nitrocamptothecin

9-AC; 9-aminocamptothecin

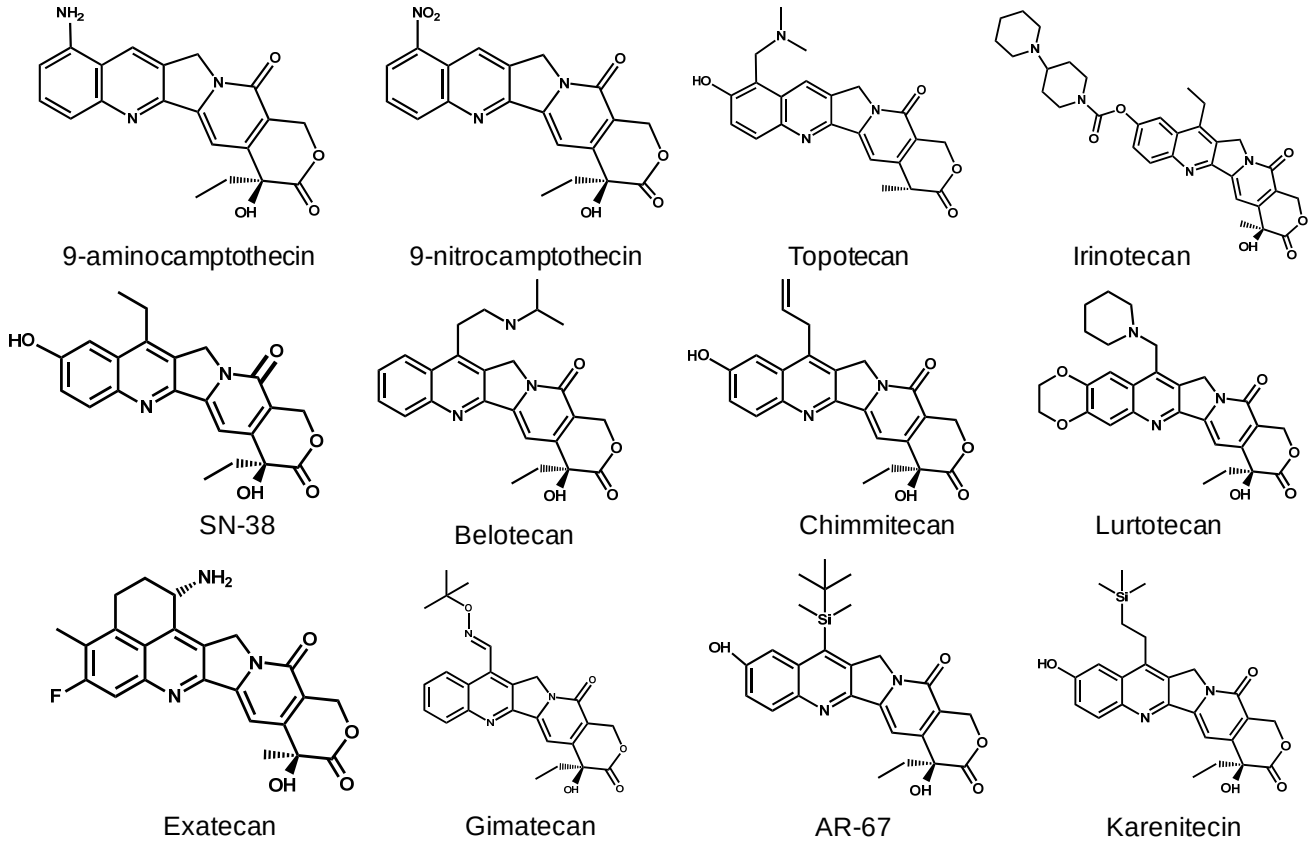


Figure 1-2. Chemical structures of camptothecins in development or clinical use.

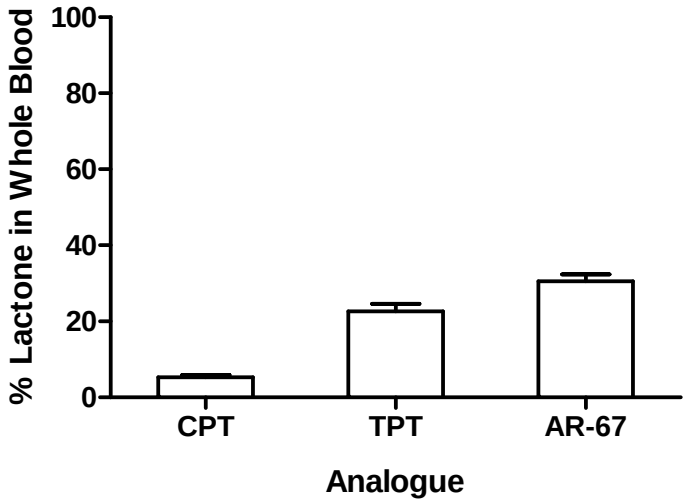


Figure 1-3. Percent lactone of camptothecin (CPT), topotecan (TPT) and AR-67 at equilibrium in human whole blood at pH 7.4 and 37^{o}C (Mean ± Standard deviation of n≥3 determinations). Adapted from references (9, 11).

AR-67 was further investigated for its antitumor effects in preclinical studies due to its improved lactone stabilityin the presence of lipid membranes (23). **Table 1-2** presents the median effective concentrations of camptothecin analogues for inhibition of proliferation in five glioma cell lines (23). As summarized in the table, AR-67 exerted potent antiproliferative effects at nanomolar concentrations and at ED50 values that were much lower than other camptothecin analogues considered in the study.

In U87 xenograft tumor bearing mice, a 5-day treatment with a 3 mg/kg subcutaneous dose of AR-67 resulted in a 61% reduction in tumor volume on day 28. Similar treatment with 10 mg/kg dose resulted in 73% reduction in tumor volume on day 28; however, continuation with 10 mg/kg dose for three cycles (1 cycle = 5 day treatment/21 days) arrested tumor progression for greater than 90 days. These data demonstrated that AR-67 exerted a dose and treatment duration-dependent antiproliferative effect (23). Similar results were obtained in our lab the details of which are presented in the appendix. Briefly, nude mice were first treated with different doses of AR-67 (0, 2, 5, 7.5, 10 and 15 mg/kg) via the intravenous or oral routes to determine the maximum tolerated dose. Weight was recorded everyday during treatment and every

other day following cessation of treatment. Blood counts were done on days 8, 11 and 21 after the first treatment. Our results indicated that the maximum tolerated dose was 37.5 mg/kg following the intravenous route whereas the oral route of administration was well tolerated, as no obvious signs of toxicity were noted. Subsequently, nude mice were implanted with H460 NSCLC tumor xenografts. Our results indicated that administration of the maximum tolerated dose (MTD) over a prolonged period of time was more efficacious in prolonging survival and delaying tumor growth than administration of the same dose over a shorter duration (Appendix II). Our toxicity and efficacy results favor the clinical use of AR-67.

Table 1-2. Comparison of the antiproliferative effect of AR-67, 9-AC, topotecan, and SN-38 in glioma cell lines‡.

Camptothecin analogue	ED50 (ng/mL) in glioma cell lines				
	U87	A172	SG388	LN-Z308	T98G
9-AC	300	300	1000	200	30
Topotecan	30	60	100	100	300
SN-38	30	20	100	20	100
AR-67	2	30	3	40	6

‡Adapted from reference (23). Cell proliferation was measured in five glioma cell lines (n= $2x10^4$ cells), which were plated and grown for 12 h before being treated for 4 days with various concentrations of each agent.

1-2. In vivo interconversion of camptothecins

Scott et al. (24) were the first to study the pharmacokinetics of the lactone and carboxylate forms of camptothecin. The lactone or the carboxylate forms of camptothecin were intravenously administered to anesthetized rats and their disposition characterized using a four compartment interconversion model (**Figure 1-4**) also proposed for hormones undergoing reversible biotransformation (25). The reversible and irreversible clearance parameters were obtained from areas under the plasma concentration versus time curves (AUC) of the parent (lactone) and metabolite (carboxylate) as indicated below by equations 1.1-1.4.

Systemic clearance of the parent (lactone)

$$Cl_{10} = \frac{Dose^{p} * AUC_{m}^{m} - Dose^{m} * AUC_{m}^{p}}{AUC_{p}^{p} * AUC_{m}^{m} - AUC_{m}^{p} * AUC_{p}^{m}} \qquad \text{Equation 1.1}$$

Systemic clearance of the metabolite (carboxylate)

$$Cl_{20} = \frac{Dose^{m} * AUC_{p}^{p} - Dose^{p} * AUC_{p}^{m}}{AUC_{p}^{p} * AUC_{m}^{m} - AUC_{m}^{p} * AUC_{p}^{m}} \qquad \text{Equation 1.2}$$

Parent to metabolite conversion clearance

$$Cl_{12} = \frac{Dose^{m} * AUC_{m}^{p}}{AUC_{p}^{p} * AUC_{m}^{m} - AUC_{m}^{p} * AUC_{p}^{m}} \qquad \text{Equation 1.3}$$

Metabolite to parent conversion clearance

$$Cl_{21} = \frac{Dose^{p} * AUC_{p}^{m}}{AUC_{p}^{p} * AUC_{m}^{m} - AUC_{m}^{p} * AUC_{p}^{m}} \qquad \text{Equation 1.4}$$

In the above equations the subscripts denote the compound that is measured (parent/metabolite) while the superscripts denote the compound that was administered (parent/metabolite), *p* denotes parent drug and *m* denotes the metabolite.

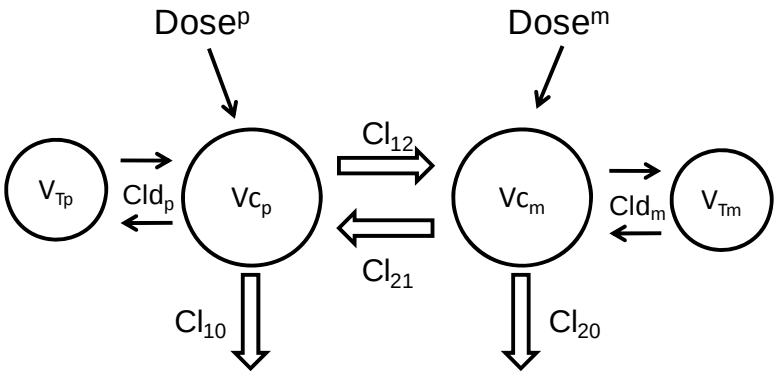


Figure 1-4. Four compartment model of reversible biotransformation from reference (25). V_{Cp} & V_{Cm}-Central volumes of distribution of parent and metabolite respectively.V_{Tp} & V_{Tm}-Peripheral volumes of distribution of parent and metabolite respectively. Cld_p and Cld_m-Distribution clearances of the parent and metabolite The studies indicated that the carboxylate form of camptothecin predominates in plasma following the administration of either the lactone or the carboxylate. However, exposure to one compound versus the other, i.e., lactone or carboxylate, depends on the compound administered despite the interconversion. Administration of camptothecin lactone provided a threefold higher lactone AUC compared to that provided by the same dose of camptothecin carboxylate. Lactone AUC represented 36.1% of the total AUC following camptothecin lactone administration and 6% following camptothecin carboxylate administration. Moreover, as illustrated in **Table 1-3**, lactone clearance (Cl_{10}) was fivefold higher than that of the carboxylate (Cl_{20}) while lactone to carboxylate conversion (Cl_{12}) was threefold higher than the reverse process (Cl_{21}) (24).

respectively. Cl_{12}, Cl_{21}, Cl_{10} and Cl_{20} are as described in the text.

The rapid lactone clearance and conversion to carboxylate coupled with the slow carboxylate clearance and conversion to the lactone may be responsible for the predominance of the carboxylate in plasma in vivo. In an in vivo open system, irreversible elimination, which occurs at different rates for the lactone and carboxylate forms, decreases the lactone and carboxylate concentrations in the body available for the

competing process, i.e, lactone to carboxylate interconversion. Biliary and urinary clearances of total camptothecin were three and 2.4 fold higher, respectively, after carboxylate administration than after lactone administration (26).

Similarly, the fastest clearance pathway of the second generation camptothecin analog, topotecan in dogs was the systemic clearance of the lactone (**Table 1-3**), which was 4.4 fold higher than that of the carboxylate (27). Clearance of lactone conversion to the carboxylate was about 3 fold higher than the reverse process suggesting that the driving forces in the disposition of topotecan are the rate of lactone elimination and lactone to carboxylate steady state concentration ratios.

Table 1-3. Clearances of the lactone (Cl_{10}, Cl_{12}) and carboxylate forms (Cl_{20}, Cl_{21}) of camptothecin (in rats) and topotecan (in dogs)‡.

	Lactone (ml/min)	Mean (SD)	Carboxylate (ml/min)	Mean (SD)	Ratio*
Camptothecin	CL10	47.8 (5.52)	Cl20	9.5 (3.3)	5
	Cl12	31.4 (4.18)	Cl21	10.4 (0.86)	3
	Lactone (ml/min)	Mean (SD)	Carboxylate (ml/min)	Mean (SD)	
Topotecan	CL10	176 (40.6)	Cl20	40.2 (9.00)	4.3
	Cl12	118 (48.0)	Cl21	60.2 (23.2)	2

‡Adapted from reference (24) (camptothecin data) and reference (27) (topotecan).
*Ratio values are (CL_{10}/CL_{20}) or (CL_{12}/CL_{21}).

As was seen with camptothecin, the form of topotecan administered determines the magnitude of exposure to the lactone and carboxylate forms (27). Administration of the lactone provided 47.4% lactone AUC and 1.6 fold higher lactone AUC than administration of the same dose of carboxylate, which provided 16.6% lactone AUC and 2.8 fold higher carboxylate AUC (27).

Irinotecan (CPT-11), another camptothecin analog in clinical use, is a water soluble pro-drug, which is acted upon by carboxylesterases primarily in the liver (28, 29) but also in other tissues such as the intestine (30) to irreversibly form 7-ethyl-10-hydroxycamptothecin (SN-38). As shown in **Figure 1-5**, in addition to lactone-

carboxylate interconversion, CPT-11 and SN-38 also undergo complex biotransformation mediated by cytochrome P450 (CYP450) and UDP-glucuronosyl-transferase (UGT) enzymes, respectively. Hence, exposure to irinotecan and its active metabolite, SN-38, is complicated not only by lactone hydrolysis, but also by multiple metabolism and transport pathways (31, 32) (33).

Conversion of CPT-11 to SN-38 is not complete and varies among species due to the differences in the presence and activity of their respective carboxylesterases (34-37). In mice, it was shown that the conversion of CPT-11 to SN-38 was saturable (36). As shown in **Figure 1-5**, CPT-11 conversion to SN-38 is also limited by CYP450 mediated biotransformation to two inactive metabolites. Although in-vitro studies demonstrate that the metabolites 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin (APC) and 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin (NPC) can be hydrolyzed to SN-38 by carboxylesterases (38), the catalytic efficiency is poor especially for the APC metabolite.

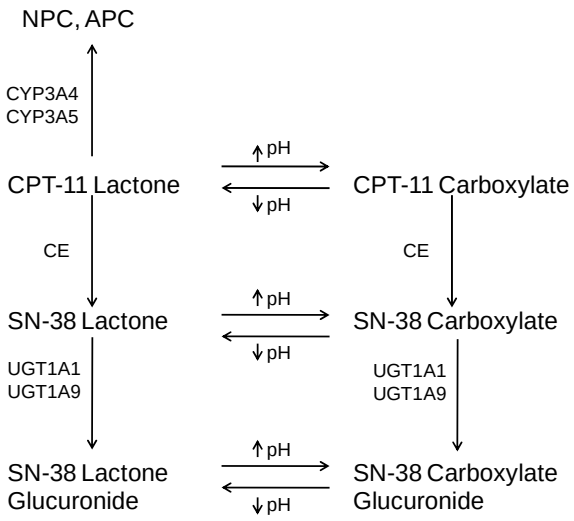


Figure 1-5. Metabolism and pH dependent interconversion of irinotecan (CPT-11) and its metabolite, SN-38. (Abbreviations used are CPT-11:-irinotecan; APC:-7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin; NPC:- 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin; CE:--carboxylesterase enzyme). Adapted from references (39, 40).

SN-38 is good substrate for hepatic phase-II enzymes, which mediate its glucuronidation, most likely at the phenolic hydroxyl at position 10 (see **Figure 1-1**). As shown in

Figure 1-6, the hydrophilic SN-38 glucuronide (SN-38-G) is then effluxed by Multidrug Resistance Protein 2 (MRP2) into the bile (33). The formation of glucuronide is thus the most important step in the detoxification process and individuals with genetic mutations, mainly in the UGT1A1 gene, are unable to efficiently glucuronidate SN-38 and therefore are more prone to toxicity following treatment with irinotecan (41, 42).

Once in the gastrointestinal tract, SN-38 glucuronide is subject to enzymatic hydrolysis and reconversion to SN-38 by bacterial β-glucuronidases (43, 44). Although the newly generated SN-38 has poor solubility and is a substrate for efflux transporters expressed in the gastrointestinal epithelium (43), some is likely to diffuse into these rapidly dividing cells causing cell death. This in turn manifests as the severe diarrhea associated with CPT-11 use (45). Although conversion to SN-38 represents a small fraction of the total CPT-11, antitumor activity and toxicity of CPT-11 is linked to the SN-38 generated. Therefore, understanding of factors affecting the disposition of SN-38 is essential from a pharmacological as well as a toxicological point of view. The third generation camptothecin analog, AR-67 shares structural similarities with SN-38 and may therefore share biotransformation and elimination pathways (**Figure 1-2**). The available literature indicates the presence of both the lactone and carboxylate forms in vivo in plasma, with the lactone form representing the majority of AR-67 (46). However, extensive studies on the disposition of the lactone and carboxylate forms of AR-67 and/or on the role of transporters in its disposition are lacking.

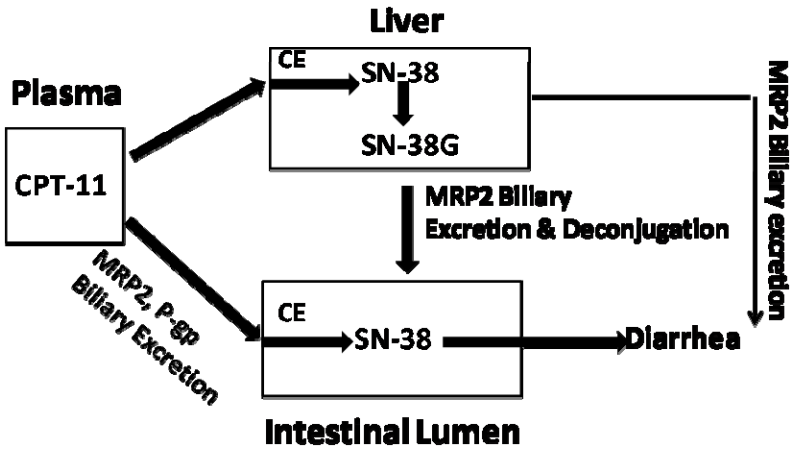


Figure 1-6. Carboxylestrase (CE)-mediated conversion of irionotecn (CPT-11) to SN-38 and the glucuronidation and biliary excretion of SN-38 and SN-38 Glucuronide (SN-38G) into the intestine (Redrawn and modified from (33).

1-3. Major transporters involved in the cellular uptake and efflux of camptothecins

As the target of camptothecins is the nuclear enzyme topo I, passage through the cell membrane could be seen as a prerequisite for cellular accumulation and pharmacological effect. One of the factors that affects the cellular accumulation and/or transport of camptothecins is interaction with uptake and efflux transporters. Interaction with uptake transporters likely facilitates cellular entry while interaction with efflux transporters will likely limit it. In general, camptothecins have been shown to interact with the ATP Binding Cassette (ABC) efflux transporters, P-gp, BCRP/Bcrp, and MRPs (47-50) as well as with the organic anion uptake transporter polypeptide OATP1B1 (51, 52). An extensive review of efflux and uptake transporters is available in the literature (53-56). A brief summary of major transporters relevant in cellular uptake and efflux of camptothecins is presented below.

1-3.1. ABC efflux transporters

ATP binding cassette (ABC) transporter proteins are expressed in nearly all organisms. The human genome encodes 49 ABC proteins, which are grouped into seven subfamilies (A, B, C, D, E, F and G). Common features of the architecture of ABC transporters are the presence of ATP binding subunits (also known as nucleotide binding

domains, NBDs) in the cytoplasm, and transmembrane domains (TMD), which are composed of six membrane spanning helices and embedded in the lipid bilayer (57). All ABC transporters contain at least two conserved cytoplasmic NBDs and at least two TMDs (57). The ATP binding subunits can bind and hydrolyze ATP, while the two transmembrane domains (TMDs) form a central cavity in the lipid bilayer and are believed to be sites for interaction with substrates (55, 58, 59). Secondary structures of ABC transporters are presented in **Figure 1-7**.

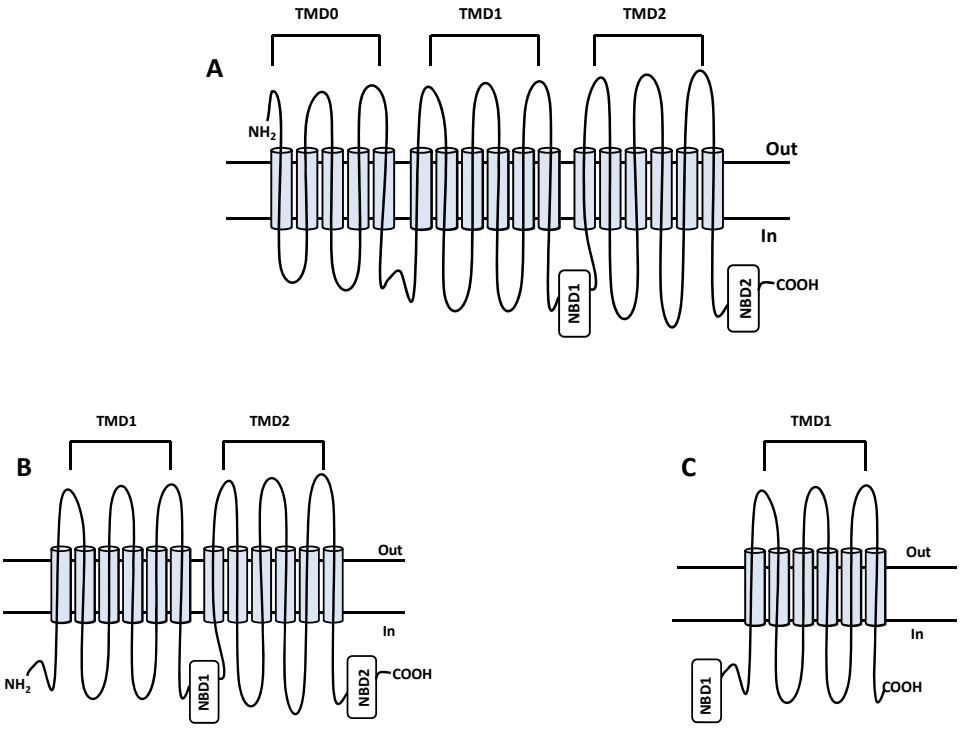


Figure 1-7. Representative secondary structures of ABC transporter proteins (A) MRP1, MRP2 & MRP3, (B) P-gp, (C) BCRP redrawn from references (54, 55, 58-60). Abbreviations used are TMD-Transmembrane Domain; NBD-Nucleotide Binding Domain.

P-glycoprotein (ABCB1)

P-gycoprotein is a 170 kDa ABC transporter protein that limits the intracellular accumulation of lipophilic substrates (61). It effluxes its substrates against a concentration gradient using the energy generated from the hydrolysis of ATP (62) and is believed to contribute to the development of multidrug resistance (61). It is widely

expressed in the endothelium and epithelium of most tissues, including the gastrointestinal tract, liver, kidney, brain, and placenta (63-65). Its expression in the apical face of gastrointestinal epithelium is associated with decreased oral bioavailability of substrates (66, 67). Expression in the liver and kidney contributes to the clearance of substrate drugs through biliary and renal pathways, respectively (68-71). Expression of P-gp at the blood-brain barrier limits the entry of substrates into the brain (72). Depending on the desired pharmacological outcome, the exclusion of drugs from the brain could be protective or undesirable (73, 74). Although P-gp physiological substrates have not been identified, P-gp transports several lipophilic and amphiphilic compounds. These compounds have widely varying structures and include many highly utilized clinical agents such as antiarrhythmics (e.g., quinidine, amiodarone), anti-retrovirals (e.g., saquinavir, ritonavir) and chemotherapeutic agents (e.g. doxorubicin, paclitaxel) (53, 58, 75, 76). Camptothecins have also been shown to be substrates of P-gp. For instance, P-gp contributed to the vectorial transport of camptothecin in MDCKII cell monolayersover expressing P-gp. In these cells, the efflux ratio, which is a the ratio of flux from the basolateral to the apical side to that of flux from the apical to the basolaateral side in was found to be 3.36. P-gp inhibition with GF120918 decreased the efflux ratio to 1.49 (47). In another study, P-gp overexpressing MDCKII cell lines were found to be 143 times more resistant to topotecan compared to control cells (48).

Multidrug resistance proteins (MRPs)

The multidrug resistant protein sub-family of ABC transporters has 12 members (MRP1-MRP12). MRPs 1-9 are transporters while MRP10 (ABCC7, CFTR) is a chloride channel. MRP11 (ABCC8, SUR1) and MRP12 (ABCC9, SUR2) are sulfonylurea receptors (54). Based on available data and relevance to the cellular efflux of camptothecins, a brief summary will be subsequently provided for MRP1, MRP2, MRP3, MRP4 and MRP5.

MRP1 (ABCC1)

MRP1 was the first member of MRPs to be identified in small cell lung cancer cell lines selected with doxorubicin (HL60/ADR) (77). It is a 190 kDa membrane glycoprotein (78-80) with three transmembrane domains (TMD0, TMD1 and TMD2)

and two ATP binding domains (NBD1 and NBD2). TMD0 possesses five transmembrane helices unlike TMD1 and TMD2, which possess six transmembrane helices (60, 81) (**Figure 1-7**).

MRP1 is expressed ubiquitously on the basolateral membrane of barrier forming epithelial cells and in the basolateral face of blood-brain barrier endothelium. Much like P-gp, MRP1 substrates vary in their structure, but are generally more hydrophilic and include several physiologically important molecules. The endogenous substrates include glutathione (GSH), oxidized glutathione (GSSG), glutathione conjugates of leukotrienes (LTC4, LTD4, LTE4) and prostaglandins (PGA_2), glucuronide conjugates of bilirubin and estradiol, and sulfate conjugates of estrone and taurocholate. It also transports a variety of drugs mostly as glutathione, glucuronide and sulfate conjugates (54). Some of the substrate drugs are methotrexate, doxorubicin, etoposide (glucuronide) and camptothecins (49). Thus, MRP1 appears to be an important efflux transporter that limits the intracellular accumulation of substrates and facilitates the efflux of endogenous and xenobiotic molecules that have been biotransformed.

MRP2 (ABCC2)

MRP2 is similar in size, membrane topology and substrate specificity to MRP1 (**Figure 1-7**). Unlike MRP1, however, MRP2 is expressed on the apical membrane of epithelial cells (60, 82) in the liver (83), kidney (84) and intestine (85). MRP2 transports leukotrienes (LTC4, LTD4 and LTE4), glutathione conjugates of prostaglandin A_1 and heavy metal ions, glucuronide and sulfate conjugates of endogenous substances (e.g., bilirubin, estriadiol, cholate, lithocholate) and xenobiotics (59). As such, it plays an important role the biliary secretion of drug metabolites into the bile (54, 60). CPT-11 and SN-38 glucuronide have been shown to be transported by MRP2 (33, 86). In humans, mutations in the MRP2 gene cause the Dubin-Johnson syndrome, an inherited hyperbilirubinemia in which the glucuronide conjugate of bilirubin is not secreted into bile (87).

MRP3 (ABCC3)

The secondary structure of MRP3 is similar to MRP1 and MRP2, but it is more closely related to MRP1 in terms of amino acid sequence, substrate specificity, and basolateral expression. It is found in liver, kidney, intestine, adrenal cortex, gall bladder and pancreas (88-90) and transports bile acids, glucuronide conjugates of bile acids and glutathione and glucuronide conjugates of xenobiotics (88, 89). A recent study showed that the mouse Mrp3 could transport SN-38 glucuronide (50), and given its basolateral expression in the liver, it may contribute to the high SN-38 glucuronide concentrations that are typically found in the blood following CPT-11 dosing.

MRP4 (ABCC4) and MRP5 (ABCC5)

Unlike MRP1-3, MRP4 and MRP5 lack one transmembrane domain (TMD0) and consist of two transmembrane domains and two nucleotide binding domains. This secondary structure is similar to P-gp. MRP4 expression could be apical or basolateral in different epithelial cells. It is expressed apically in the kidney (91) and in endothelial cells of brain capillaries (92, 93) and basolaterally in the prostate (94), choroid plexus (93) and hepatocytes (91). The substrates of MRP4 and MRP5 overlap and include cyclic and acyclic nucleotides (95). MRP4 prefers methylated thionucleotides, while MRP5 prefers unmethylated thionucleotides (96). MRP4 has been shown to confer resistance to topotecan and irinotecan (92, 93). At this time, no data on the interaction of MRP5 with camptothecins are available.

BCRP (ABCG2)

The Breast Cancer Resistance Protein (BCRP, ABCG2, MXR) was first cloned from MCF-7/AdrVp breast cancer cells rendered resistant to the P-gp inhibitor verapamil (97). Later on it was isolated from human placenta (98) and from mitoxantrone-resistant human colon carcinoma cell lines (99). As shown in **Figure 1-7**, BCRP is a 72-kDa half-transporter with one ATP binding domain and one transmembrane domain (TMD) consisting of six membrane spanning helices (59). It is believed to function after homodimerization of two monomers through disulfide bond formation (100). Studies also suggest that it can form a homotetramer (101) In contrast to other ABC transporters, its

ATP binding domain is at the N-terminus (97, 99). BCRP serves a protective purpose against xenobiotics/toxins in the gut (102), brain (103, 104), testis (104), and the placenta (102). It is also expressed at sites of reduced oxygen tension, liver and mammary gland (105-107). It transports a variety of substrates and their sulfate, glutathione and glucuronic acid conjugates. Some BCRP substrates include anthracyclines (e.g., doxorubicin, daunorubicin), anti-retrovirals (e.g., zidovudine, lamivudine) and proton pump inhibitors (e.g., pantoprazole) (39)(59). Several studies have indicated camptothecins to be BCRP substrates (48, 108, 109). BCRP overexpression decreased cellular accumulation and conferred resistance to 9-AC, but not to 9-NC. However, no differences were observed in the cytotoxicity of both 9-AC and 9-NC between control cells and cells that overexpress P-gp, MRP1 or MRP2. The authors suggested that the presence of a polar group at position 9 or 10 of camptothecin enhances interaction with BCRP (48).

Table 1-4 summarizes the 1 hour intracellular accumulation of the hydrophilic topotecan and the lipophilic gimatecan in sensitive (HT29) and resistant (Bcrp overexpressing HT29/MIT) colon carcinoma cells (110). HT29/MIT cells had significantly lower intracellular concentration of topotecan compared to HT29 cells. In contrast, cellular accumulation of the lipophilic gimatecan was similar in both HT29 and HT29/MIT cells. When compared to accumulation following incubation with the same concentration of topotecan (2 μg/ml), there was up to a 100 fold higher accumulation of gimatecan in both HT29 and HT29/MIT cells.The authors suggest that BCRP may not recongnize gimatecan. In another study the intracellular accumulation of topotecan in resistant T8 and MX3 ovarian cancer cells that overexpress BCRP was significantly lower than in non-BCRP expressing parental IGROV1 cell lines (109). The difference in accumulation was abolished by co-incubation with the efflux inhibitor GF120918. T8 cells were 111 and 259 times more resistant to topotecan and SN-38, respectively, compared to parental control cells. MX3 cells on the other hand, were 30 and 54 times more resistant to topotecan and to SN-38, respectively, compared to parental control cells. In the presence of GF120918, resistance to topotecan and SN-38 was diminished in T8 and MX3 cell lines (109). Moreover, the tyrosine kinase inhibitor imatinib reversed BCRP mediated resistance of Saos2 cells to topotecan and SN-38 (108).

Imatinib competes with ATP binding and is thus a functional inhibitor of BCRP. Reversal of resistance with imatinib could have resulted from the increased intracellular accumulation of topotecan observed (108). These results indicate the prominent role BCRP plays in limiting intracellular accumulation and/or in contributing to resistance to the cytotoxic effect of camptothecins. Therefore, it is expected it could play similar roles on AR-67.

Table 1-4. Intracellular accumulation of topotecan or gimatecan‡.

Camptothecin analogue	Concentration (μg/ml)	Mean drug content (ng/10^7 cells), (SD)	
		HT29	HT29/MIT
Topotecan	2	9 (5)	4 (3)
	20	195 (20)	80 (25)
Gimatecan	0.2	22 (4)	34 (3)
	2	250 (22)	390 (18)

‡Adapted From reference (110). Cells were incubated for 1 h with either topotecan or gimatecan. Values were obtained from quadruplicate determinations

1-3.2. Organic anion transporting polypeptides (OATPs)

Unlike ABC transporter proteins, OATPs do not require ATP for their function. As their name implies, their substrates are primarily organic anions and most importantly they function as uptake transporters with the potential for bidirectional transport (49, 111) . Given that the carboxylate forms of camptothecin analogues meet the physicochemical criteria of OATP substrates, interaction between carboxylate and OATPs has been the subject of investigation. The organic anion transporting polypeptides (OATPs-humans, Oatps-rodents) are members of solute carrier organic anion transporter family (SLCO) and are membrane transporters expressed in different species (e.g., humans, rats, mice, cows, quail, zebrafish) (40).

Eleven human OATPs have been identified and are categorized into six families, OATP1-6 with each family subdivided into subfamilies (56, 112). OATP1, which is the largest family, has three sub-families, OATP1A, OATP1B and OATP1C (47, 103). OATPs are predicted to have 12 membrane spanning domains (**Figure 1-8**) (56, 112). Uptake of substrates is sensitive to extracellular pH and is believed to be coupled with exchange of anions such as HCO^{3-}. The transport, however, is independent of sodium

(111). Among the OATPs, OATP1B1 (MW 84 kDa) and OATP1B3 (MW, 120 kDa) and the rodent homolog Oatp1b2 (MW ≈77 kDa) are believed to be liver-specific and are expressed basolaterally (111, 113-115). OATP1B1 and OATP1B3 share similar substrate specificity and are involved in the hepatic uptake of several endogenous compounds (e.g., bilirubin, estradiol 17-β-glucronide, LTC4) and drugs (e.g., statins, rifampin, SN-38, valsartan) (51, 56, 116, 117). OATP1B1 and/or OATP1B3-mediated hepatic uptake impacts drug concentration in the liver and contributes to the elimination of substrates either by metabolism or hepatobiliary efflux (115).

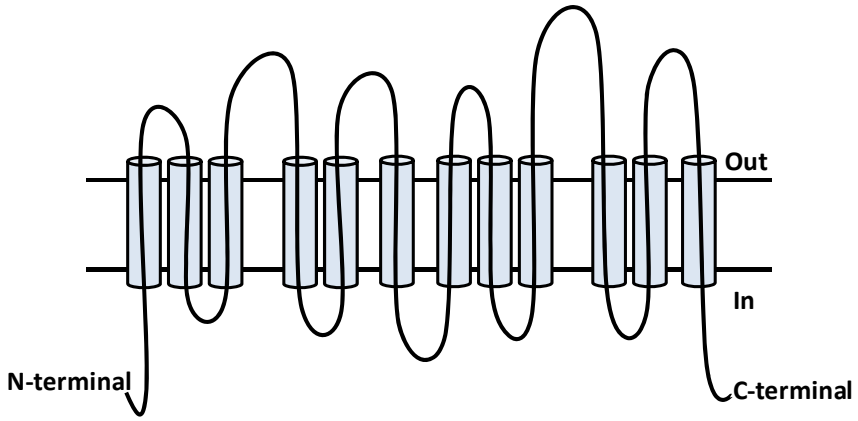


Figure 1-8. Predicted secondary structure of OATPs redrawn from reference (118).

The organic anion transporters OATP1B1 and/or OATP1B3 may play a role in the disposition of camptothecins as shown for irinotecan and SN-38 (51). Both irinotecan and SN-38 significantly inhibited OATP1B1-mediated uptake of the OATP1B1 substrate, [^{3}H]estrone-3-sulfate. Moreover, SN-38 displayed increased transport in HEK293 cells expressing OATP1B1 (51). The lipophilic camptothecin analogues gimatecan and BNP1350 were also transported by OATP1B1 in vitro as evidenced by trancellular studies in which basolateral to apical flux of gimatecan and BNP1350 in MDCKII-OATP1B1 cells was 2.7 fold higher than the apical to basolateral transport (52). Although these studies did not specifically assess the transport of the lactone or carboxylate forms, it is likely that the carboxylate forms of camptothecins, which are hydrophilic, are OATP substrates.

The hydrolysis of the lactone form to the carboxylate in the extracellular culture medium could also play a role in limiting intracellular accumulation in vitro (119). In IGROV-1, VERO and WIDR cell types, incubation of 9-AC carboxylate resulted in more than 18 fold lower intracellular accumulation than that done with the same concentration of 9-AC lactone (1.5-1.8 ng/mg protein with carboxylate vs. 22.3-32.5 ng/mg protein with lactone) (10). It is likely that the lipophilicity of the lactone allows enhanced intracellular accumulation by passive diffusion whereas the hydrophilicity or charge on the carboxylate limits it. Differential interactions with efflux and uptake transporters were noted for the lactone and carboxylate forms of statins (120), which like camptothecins undergo reversible lactone-carboxylate hydrolysis (120, 121). Thus, it is likely that such interactions would also be relevant for camptothecins in vitro and in vivo.

1-4. In vivo interaction of camptothecins with transporters

1-4.1. Disposition

The interconversion between lactone and carboxylate forms of camptothecins becomes important in their overall disposition as the lactone and carboxylate forms may differentially interact with efflux and uptake transporters. Thus, pharmacogenetic differences in transporters, changes in organ function, or drug-drug interactions may affect one or both types of transporters leading to changes in the predominant clearance pathway of the lactone and/or the carboxylate forms. In vitro studies have demonstrated that camptothecins are substrates of OATP and ABC efflux transporters (52, 109, 110). Moreover, this interaction has been shown to be relevant in the clearance, distribution and oral bioavailability of camptothecins using either chemical inhibitors or genetic knockout animals (122-124).

With the parent compound camptothecin, it has been shown that P-gp inhibition results in an almost two fold increase in brain exposure (122). The implication of a change in the MRP2-mediated clearance pathway of SN-38, i.e, biliary efflux of SN-38 glucuronide was illustrated in EHBR rats, which do not express MRP2. After intravenous administration of 10 mg/kg CPT-11 in EHBR rats, plasma AUC of SN-38 lactone glucuronide was 4 fold higher and the amount in the bile was about 7 fold lower

compared to wild type Sprague Dawley rats that received the same dose of CPT-11 (125). Another study showed that in the presence of the P-gp and MRP2 inhibitor cyclosporin, plasma concentration of irinotecan carboxylate, SN-38 lactone and SN-38 carboxylate were elevated in rats. It was also noted that biliary, intestinal exsorption and 'apparent' systemic clearance of CPT-11 lactone, CPT-11 carboxylate, SN-38 lactone and SN-38 carboxylate decreased significantly in the presence of cyclosporin (123). It appears therefore, that both P-gp and MRP2 are involved in the active efflux of ininotecan and SN-38 glucuronide.

Studies in Bcrp and/or mdr1 knockout animals also show the importance of ABC transporters in the clearance of topotecan (124). Topotecan clearance in Bcrp knockout animals was 73% of the value in wild type animals (15.4 ± 1.6 (SE) vs. 11.3± 2.2 L/hr/m^2, $p<0.05$). The clearance decreased to 54% of control values in the presence of the Bcrp transporter modulator gefitinib (15.4 ± 1.6 (SE) in wild type vs. 8.3 ± 2.5 L/hr/m^2 in Bcrp knockouts, $p<0.05$). Similarly in mdr1a/b knockout animals, topotecan clearance was 79% of that in wild type animals (15.5 ± 0.97 (SE) in wild type vs. 12.2 ± 1.5 L/hr/m^2 in knockout animals) and gefitinib decreased it to 65.8% of control values (124). Given that the lactone and carboxylate forms of camptothecins may be eliminated differently due to differential interaction with uptake and efflux transporters, alteration of the clearance of one but not of the other could take place. This was illustrated by probenecid, an organic anion transporter inhibitor, which predominantly decreased the clearance of the carboxylate in mice when co-administered with topotecan (126). Although in this study interconversion between the lactone and carboxylate forms was not taken into consideration during the estimation of pharmacokinetic parameters, "lactone" systemic clearance decreased 16.5% (16.3 without vs. 13.6 L/hr/m^2 with probenecid) while total drug systemic clearance decreased 31% in the presence of probenecid (10.9 without vs. 7.5 L/hr/m^2 with probenecid). Moreover, probenecid led to 1.2 and 2.2 fold increases in lactone and carboxylate AUCs, respectively, following administration of the lactone form. On the other hand, following carboxylate administration, probenecid led to 2.0 and 3.7 fold increase in lactone and carboxylate AUCs, respectively. Using equations 1-1 to 1-4 and areas under the plasma concentration versus time curve (AUC) of the lactone and carboxylate systemic and conversion

clearances of the lactone and carboxylate were calculated and are presented in **Table 1-5**. As shown in the table, probenecid appears to selectively decrease the clearance of the carboxylate (four fold reduction in carboxylate clearance). Although both lactone to carboxylate and carboxylate to lactone clearances decreased with probenecid, the ratios with respect to each other did not appreciably change.

Table 1-5. Effect of probenecid on the clearance of topotecan in mice‡.

Clearance ($L/hr/m^2$)	Without probenecid	With probenecid	Fold change
Lactone systemic clearance (Cl10)	3.33	3.09	1.06
Carboxylate systemic clearance (Cl20)	2.46	0.59	4.21
Lactone to carboxylate conversion (Cl12)	1.47	0.70	2.10
Carboxylate to lactone conversion (Cl21)	0.51	0.23	2.25

‡ Adapted from reference (126). Probenicid (600 mg/kg) was administered by oral gavage 30 minutes before and 3 h after administration of topotecan (1.25 mg/kg) by the intravenous route.

1-4.2. Oral bioavailability of camptothecins and role of efflux transporters

As shown in **Table 1-6**, the oral bioavailability of camptothecins is incomplete (127-131). For some, it can in part be attributed to their poor aqueous solubility. However, limited bioavailability is also a feature of more soluble analogs such as topotecan. Thus, interaction with ABC efflux transporters located in the gastrointestinal (GI) tract or metabolism in the GI epithelium are likely factors influencing the bioavailability of camptothecins. The oral bioavailability of camptothecins is reduced by ABC efflux transporters located in the gastrointestinal tract, while inhibition or absence of these transporters improves oral bioavailability (102, 124, 132, 133). Although it is likely that efflux transporters might also reduce the oral bioavailability of AR-67, the extent remains to be determined. Moreover, there is a scarcity of data with regard to the contribution of first pass metabolism in the intestine and/or the liver in reducing the bioavailability of camptothecins. Therefore, studies assessing first pass metabolism of

AR-67 are deemed necessary not only for AR-67 but potentially also for other camptothecins.

Table 1-6. The oral bioavailability (F%) of some camptothecin analogs.

Analogue	%	Species	Remark	Reference
9-NC	14.6	Rat	A solution of 9-NC in DMSO was diluted with a vehicle containing PEG-400 and sterile water (1:1, v:v) and acidified with phosphoric acid (pH 3.0-3.5)	(127)
Irinotecan	25%	Mice	Diluted in normal saline	(132)
Topotecan	30	Mice	Dissolved in water	(129)
	50	Dog	Dissolved in 0.9% saline (pH 3) and administered in size 12 gelatin capsules	(27)
	29.7	Rat	Dissolved in saline containing 5% d-glucose	(130)
	42	Humans	Administered as gelatin capsules containing anhydrous free base (topotecan)	(131)
Karenitecin	67	Mice	Solubilizer composed of N-methylpyrrolidone, PEG-300, Tween 80, ethanol and citric acid	(128)

1-5. Summary

AR-67 is a relatively lactone stable camptothecin analog undergoing early phase clinical trials as a chemotherapeutic agent. Its pH dependent interconversion allows the existence of the lactone and carboxylate forms in equilibrium. While the carboxylate prevails at physiological pH in vitro, it is the lactone that prevails in vivo. The reasons for this apparent discrepancy remain to be understood. The interaction of camptothecins with ABC efflux and OATP uptake transporters affects cellular accumulation, cytotoxcity and transcellular transport in vitro. On the other hand, ABC efflux transporters and OATPs have been shown to affect disposition and/or oral bioavailability. Because of their physicochemical differences, the lactone and carboxylate forms may preferentially interact with efflux and uptake transporters, and may therefore follow different elimination pathways in vivo. SNPs in transporter proteins, drug-drug interactions, and alterations in organ functions have the potential to alter pharmacokinetics and as a consequence the pharmacodynamics of the lactone and carboxylate forms. It is therefore essential to study the pharmacokinetics of the lactone and carboxylate forms. The reversibility of lactone and carboxylate conversion means that the study of the pharmacokinetics of camptothecins in general and of AR-67 in particular requires the separate administration and quantification of the lactone and carboxylate forms. Estimation of clearances that uniquely describe the disposition of the lactone and carboxylate forms of AR-67 as well as their interconversion will allow the identification of the driving force in AR-67 disposition and the prediction of likely outcomes of clearance changes.

Efficacy of protracted dosing of AR-67 in tumor bearing mice via the intravenous route and minimal signs of toxicity via the oral route make AR-67 a good candidate as an oral chemotherapeutic agent. However, its lipophilicity as well as its potential interaction with efflux transporters located in the gastrointestinal tract could limit its bioavailability. Studies are therefore required to investigate factors that affect the oral bioavailability of AR-67.

CHAPTER 2

HYPOTHESIS AND SPECIFIC AIMS

AR-67 is a potent third generation camptothecin analog that undergoes pH dependent but reversible hydrolysis between its lipophilic lactone and hydrophilic carboxylate forms. AR-67, like all camptothecin analogues exerts its effect through interaction with the nuclear enzyme Topoisomerase I (topo I). Therefore, it is essential that AR-67 enters the cell to exert its pharmacological action. The distinct physicochemical properties of the lactone and carboxylate forms could lead to differences in cellular accumulation and/or transport as a result of their differential interaction with uptake and efflux transporter proteins in vitro and in vivo. In this dissertation, the effect of ABC efflux transporters P-gp and BCRP and organic anion transporting polypeptides, OATP1B1 and OATP1B3, on cellular accumulation and transcellular transport of AR-67 was determined in vitro. The findings of in vitro studies were extended to the in vivo situation by studying the disposition and oral bioavailability of the lactone and carboxylate forms in the presence and absence of transporter inhibitors. The overall hypothesis is that interconversion between the lactone and carboxylate forms of AR-67 leads to differences in cellular accumulation and/or transport, systemic disposition and oral bioavailability.

Specific Aim 1

To determine the role of major efflux and uptake transporters on cellular efflux and uptake of AR-67. (Addressed in CHAPTER 3).

The lipophilic lactone and the hydrophilic carboxylate are expected to display differences in interaction with uptake (OATP1B1 and OATP1B3) and efflux transporters (P-gp and BCRP) in-vitro.

Rationale: Camptothecin analogues are substrates of P-gp and BCRP, which are ABC efflux transporters that facilitate drug removal from the cell. Due to the abundant expression of these two efflux pumps in organs primarily responsible for drug disposition (i.e., intestine, liver and kidney), these transporters have been shown to affect

elimination, distribution and oral bioavailability of various camptothecin analogues (102, 124, 134). On the other hand, there are studies that indicate the interaction of camptothecins such as irinotecan, SN-38 and gimatecan with the organic anion uptake transporter OATP1B1 (51, 52). OATP1B1 and OATP1B3 are organic anion uptake transporters that are preferentially expressed in hepatocytes and play an important role in drug elimination (56, 112). As a result of the labile nature of AR-67, both the lactone and carboxylate forms exist at physiological pH in vivo. It is likely that the more lipophilic AR-67 is a substrate of ABC efflux transporters, which may mediate its oral bioavailability, tissue distribution and systemic clearance in vivo. Conversely, the hydrophilic and charged carboxylate is more likely to be a substrate of the liver specific uptake transporters OATP1B1 and/or OATP1B3, which may mediate its hepatic uptake and biliary clearance.

Aim 1.1.

To determine the effect of P-gp and BCRP on intracellular accumulation of AR-67 lactone.

In this aim the intracellular accumulation of AR-67 was measured in control and P-gp or BCRP overexpressing cells. The effect of P-gp and BCRP inhibition on intracellular accumulation of AR-67 using GF120918 was also assessed.

Aim 1.2.

To determine the vectorial transport of AR-67 lactone by BCRP.

In this aim the apical to basolateral and basolateral to apical transport of AR-67 lactone was determined in control and BCRP overexpressing cells grown in transwell cell culture plates. Transport experiments were also carried out in the presence and absence of GF120918 and rifampin to assess effect of efflux transporter inhibition.

Aim 1.3.

To determine organic anion uptake transporter (OATP1B1 and/or OATP1B3) mediated-uptake of AR-67 lactone or carboxylate.

In this aim the intracellular accumulation of AR-67 lactone and carboxylate was measured in control and OATP1B1 or OATP1B3 overexpressing cell lines. The intracellular accumulation of AR-67 carboxylate was also measured in the presence and absence of known ABC efflux and/or uptake inhibitors, GF120918, bromosulphopthalein, and rifampin.

Specific Aim 2

To determine the in vivo disposition of the lactone and carboxylate forms of AR-67 in the presence and absence of efflux or uptake transporter inhibitors. (Addressed in CHAPTER 4).

Based on in vitro studies addressed in Aim 1, it was e hypothesized that ABC efflux transporters, P-gp and/or Bcrp likely affect the clearance of the lactone while Oatps likely affect the clearance of the carboxylate. It was hypothesized that inhibition of the predominant clearance term in the disposition of AR-67 wouldl lead to enhanced exposure to AR-67.

Rationale: Like all camptothecins, AR-67 undergoes a pH dependent reversible hydrolysis. Although it displays improved lactone stability in in blood, compared to other camptothecins, about 70% exists in the carboxylate form at physiological pH (11). However, in vivo the predominant species is the lactone. This discrepancy may be related to differences in the interconversion rate observed in vivo and may be compounded by the differential interaction of AR-67 lactone and carboxylate with efflux and uptake transporters, which could uniquely affect their respective clearances. Similar observations regarding differences in transporter interaction have previously been demonstrated with lactone and carboxylate forms of statin analogues(120, 121). Given that AR-67 displays reversible hydrolysis between the lactone and carboxylate, the potential for drug-drug interactions is increased. Furthermore, previous studies with statins and camptothecin analogues have shown that single nucleotide polymorphisms in OATP1B1 and BCRP transporters can significantly affect drug clearance. Therefore, using pharmacologic inhibitors the significance of efflux transporters (BCRP and P-gp) as well as OATPs on the pharmacokinetics of AR-67 was assessed.

Through the use of modeling and simulation and transporter inhibition studies in vivo, attempts are made to explain the apparent lactone stability of AR-67 and propose possible driving factor(s) in AR-67 disposition.

Aim 2.1.

To determine the pharmacokinetics of AR-67 lactone and carboxylate following intravenous administration of each form.

In this aim the lactone and carboxylate forms of AR-67 were separately administered to rats by the intravenous route. A 4-compartmental model allowing for interconversion between lactone and carboxylate was used to estimate lactone and carboxylate clearances by simultaneously fitting the plasma concentrations of the lactone and carboxylate forms following the administration of each form.

Aim 2.2.

To determine how clearance changes of either the lactone or carboxylate forms affect the disposition of AR-67.

In this aim the pharmacologic inhibitors GF120918 and rifampin to were used to decrease the clearance of the lactone and carboxylate forms, respectively. Modeling and simulation were also used to determine the effect of each inhibitor on the clearance of lactone and carboxylate.

Specific Aim 3

To determine the effect of efflux transporters P-gp and Bcrp on oral bioavailability of AR-67. (Addressed in CHAPTER 5).

Rationale: *In vitro* data from Aim 1 showed the dependence of intracellular accumulation and/or transcellular transport of AR-67 lactone on efflux transporters, P-gp and BCRP, and its independence on uptake transporters (OATP1B1 and OATP1B3). Furthermore, *in vivo* data from Aim 2 extended these findings and indicated the dependence of lactone clearance on efflux transport. However, the significance of gastrointestinal efflux transporters on oral bioavailability of AR-67 has not been studied.

The interconversion between the lactone and carboxylate forms of AR-67 in the gastrointestinal tract, the pH of which varies along its length, and the possible selective uptake of the carboxylate form into the liver by organic anion transporter protein mediated processes make the gastrointestinal absorption of AR-67 complex. Therefore, to fit the data from oral administration of AR-67 lactone and carboxylate and estimate oral bioavailability, a flexible input function was incorporated into the four compartment model used to fift the intravenous data.

Aim 3.1. To estimate oral bioavailability following lactone or carboxylate administration.

In this aim incremental doses of AR-67 lactone or carboxylate by were administered by the oral route to estimate bioavailability and its dependence on dose and form of AR-67 administered The 4-compartment pharmacokinetic model used in aim 2 was coupled with an inverse Gaussian input function to estimate the pharmacokinetic parameters of AR-67 following oral administration of either the lactone or carboxylate forms.

Aim 3.2. To determine the effect of efflux transporters P-gp and Bcrp on oral bioavailability.

In this aim animals were pretreated with the selective P-gp inhibitor zosuquidar and the dual P-gp and Bcrp inhibitor GF120918 prior to the administration of AR-67 in order to determine the effect of efflux transporters on oral bioavailability.

CHAPTER 3

INTERACTION OF THE LACTONE AND CARBOXYLATE FORMS OF AR-67 WITH ABC EFFLUX AND ORGANIC ANION TRANSPORTING POLYPEPTIDES (OATPS)

3-1. Introduction

Camptothecin analogs are substrates of ABC efflux transporters such as P-gp and BCRP (47, 102, 135). However, their interaction with organic anion transporting polypeptides (OATPs) was only recently recognized (51, 52). Whereas ABC efflux transporters have been implicated in development of resistance to the cytotoxic effect of camptothecins in vitro (109, 135, 136), in vivo studies have not yet clearly demonstrated this. However, oral bioavailability of camptothecins such as topotecan and irinotecan is reduced by P-gp and/or BCRP (105, 132), which pump substrates back into the lumen of the gastrointestinal tract. Studies conducted using ABC efflux transporter inhibitors and/or genetic knock-out animals further demonstrate the importance of ABC transporters in determining oral bioavailability (102, 132). Furthermore, ABC efflux transporters expressed in the biliary canalicular membrane pump substrate drugs into the bile and therefore contribute to the clearance of camptothecins through the biliary route (68, 69, 125, 137). On the other hand, organic anion transporting polypeptides, OATP1B1 and OATP1B3 are preferentially expressed in the liver (56). OATP-mediated uptake into the liver may represent a pathway for the elimination of hydrophilic compounds, which could be metabolized and/or effluxed into the bile once inside the hepatocyte (138). Therefore, ABC efflux and OATP transporters alone or in concert could affect pharmacokinetic and pharmacodynamic properties of camptothecins.

The camptothecin class of anticancer compounds undergo pH dependent reversible hydrolysis of the lactone moiety to a carboxylic acid (7). In vitro and at physiological pH, the carboxylate form predominates (11), while in vivo, the predominance of one or the other form depends on the analogue under consideration. For instance, the parent camptothecin (139) and first generation analogues such as 9-aminocamptothecin (140) are mainly in the form of the carboxylate while lipophilic

analogs, such as gimatecan (141), karenitecin (BNP1350) (142) and AR-67 (143) are mainly in the form of the lactone. Since the pharmacological activity of camptothecins is attributed to the lactone form (14), interconversion between the lactone and carboxylate forms is likely to impact pharmacological activity and cytotoxicity. Moreover, the existence of the lipophilic lactone and the hydrophilic carboxylate forms of camptothecins in equilibrium at physiological pH suggests that these two compounds could interact with uptake and efflux transporters differentially. This differential interaction could in turn lead to differences in the pharmacokinetic and pharmacodynamic properties of camptothecins. The third generation camptothecin analog, AR-67, is used in this study as a model compound to assess the role of efflux (P-gp & BCRP) and uptake transporters (OATP1B1 & OATP1B3) on cellular accumulation and transcellular transport of the lactone and carboxylate forms. The hypothesis is that AR-67 lactone is a substrate for P-gp and BCRP while the hydrophilic carboxylate but not the lipophilic lactone depends on OATP-mediated uptake for intracellular accumulation. The outcomes of these studies would serve as a basis for designing in vivo studies that will in turn assess the effect of these transporters on AR-67 disposition and/or oral bioavailability.

3-2. Methods

3-2.1. Chemicals

AR-67 was obtained from Novartis (East Hanover, NJ) and rifampin was purchased from Fisher Scientific (Fair Lawn, NJ). H^3 mannitol and BSP were from Perkin Elmer (Hebron, KY) Sigma Aldrich (St. Louis, MO) ?, respectively. GF120918 was a gift from GlaxoSmithKline (Research Triangle Park, NC). Minimum Essential Medium Eagle (MEM), Dulbeco's Modified Eagle Medium (DMEM) and reduced serum medium (Opti-MEM) and Fetal Bovine Serum (FBS) were from Invitrogen (Carlsbad, CA).

3-2.2. Intracellular accumulation of AR-67

Preparation of working solutions

A stock solution of AR-67 lactone in DMSO (1 mg/ml) was used for preparation of working solutions. For lactone accumulation studies, the stock solution was further diluted to 25, 50, 75 and 100 μM with DMSO. 10 μL of working solutions were added to 1 ml of incubation medium as described below for a final concentration of 0.25, 0.5, 0.75 and 1 μM. Working solutions for carboxylate uptake studies were prepared by dilution of DMSO stock solution with 0.005 N NaOH. For intracellular accumulation studies, a stock solution of GF120918 (10 mM) was prepared by dissolving 51 mg GF120918 in 10 ml of DMSO. This stock solution was further diluted to make a working solution (500 μM) by adding 6 μL of 10 mM stock solution to 114 μL in DMSO. For inhibition of MDR1 or BCRP , 10 μL of working GF120918 solution (500 μM) was added to 1 m of incubation medium. For trancellular flux studies. 10.1 mg of GF120918 was dissolved in 25 ml of DMSO to prepare ≈600 μM solution. This was further diluted in Opti-MEM (1:100).

Efflux studies

To examine the effect of human ABC efflux transporters on intracellular accumulation of AR-67, L-MDR1, MDCKII-BCRP or their respective vector transfected cells ($4x10^5$) were seeded in six well plates 24 hours before the experiment. MDCKII-

BCRP cells were grown in MEM containing 5% FBS while L-LCPK1 and L-MDR1 cells were grown in DMEM containing 10% FBS. On the day of the experiment cell culture medium was replaced with DMEM. Cells were incubated with 0.25, 0.5, 0.75 and 1 μM AR-67 lactone for 20 minutes. For ABC efflux transport inhibition studies, cells were preincubated with 5 μM GF120918 or control l solvent (10 μL DMSO) 10 minutes prior to the addition of 1 μM AR-67 lactone. At the end of 20 minutes the all the incubation medium was aspirated & discarded. Cells were then washed twice with ice cold 10% FBS in PBS (pH=7.4). Cells were then lysed with 200 μL of 0.5 N NaOH and placed on a rocker at 4^{o}C. Protein precipitation was carried out by adding 100 μL cell lysate to 400 μL of dry ice-cold methanol. The mixture was vortexed and centrifuged at $13x10^{3}$ g for 10 minutes. The resulting supernatant was poured into amber vials and stored at -80^{o}C until analysis by HPLC. The remainder of the cell lysate was used for protein quantification. Protein concentrations were determined with BCA reagents (Pierce, Rockford, IL) according to the manufacturer's instruction. For ABC efflux transport inhibition studies, cells were preincubated with 5 μM GF120918 prepared as above or control solvent 10 minutes prior to the addition of 1 μM AR-67 lactone.

Uptake studies

To measure the cellular uptake of AR-67 lactone or carboxylate, HeLa-pIRESneo control or cells expressing human organic anion transporting polypeptides (OATP1B1 or OATP1B3) were grown in six well plates (2 ml/well) in DMEM with 5% FBS. In order to minimize interconversion between the lactone and carboxylate forms of AR-67, the incubation medium (DMEM) was maintained at pH 7.4 for lactone uptake experiments and pH 8 for carboxylate uptake experiments. In addition, separate experiments were conducted to monitor hydrolysis of lactone at pH 7.2 and conversion of carboxylate to lactone at pH 8. Concentration dependence studies were conducted at 0.5, 1 & 5 μM AR-67 lactone (pH 7.2) or carboxylate (pH 8) for 10 minutes.

Carboxylate uptake inhibition studies were conducted at pH 7.4 medium (Opti-MEM) using the inhibitors GF120918 (5 μM), rifampin (100 μM) or bromosulfophthalein (50 μM) for 5 minutes. At the end of 5 minutes the incubation medium was aspirated and cells were washed twice with ice cold Opti-MEM. Cells were

then lysed with 200 µL of 0.5 N NaOH and placed on a rocker at 4°C. Extraction was carried out by adding 100 µL cell lysate to 400 µL of dry ice cold MeOH.

3-2.3. Transcellular flux of AR-67

MDCKII-pcDNA3 and MDCKII-BCRP cells were grown for 5-7 days in Corning Transwell 3414 membrane inserts (3.0-µm pore size, 24-mm diameter; Corning Glass works, Corning, NY) until the TEER was above 200 Ω·cm^{2}s. On the day of the experiment medium was aspirated and replaced with 1.8 ml of Opti-MEM with or without ≈5 µM GF120918 or ≈100 µM rifampin on the apical and basolateral sides. The cells were incubated at 37°C in humidified 5% CO_2 incubator for 1 hour. At the end of 1 hour, 0.2 ml of 50 µM AR-67 lactone prepared by dilution of a DMSO stock solution (1 mg/ml) in Opti-MEM immediately before the experiment was added to each well on either the apical or basolateral side (donor sides) to give a final concentration of 5 µM. An equal volume of control medium was added to the receiver side. The plates were then placed on a rocker and incubated at 37°C and 5% CO_2. 50 µL of sample was taken from the receiver and donor sides at 12 min, 24 min, 36 min and 48 min and added to 200 µL of dry-ice cold methanol and then centrifuged at 8.5x10^3 g for 3 minutes. The supernatant was transferred to empty amber vials and kept at -80°C until analysis by HPLC. Apparent permeability surface area product (PS) and other membrane flux parameters were calculated as described in the literature (47, 144) and as shown below.

$$PSapp = \frac{V}{C_0} * \frac{dC}{dt} \qquad \text{Equation 3.1}$$

$$ER_{\alpha} = \frac{\frac{dX^{BCRP}_{B\to A}}{dt}}{\frac{dX^{BCRP}_{A\to B}}{dt}} \qquad \text{Equation 3.2}$$

$$ER_{A} = \frac{\frac{dX^{BCRP}_{B\to A}}{dt}}{\frac{dX^{pcDNA3}_{B\to A}}{dt}} \qquad \text{Equation 3.3}$$

$$ER_B = \frac{\frac{dX_{A\rightarrow B}^{pcDNA3}}{dt}}{\frac{dX_{A\rightarrow B}^{BCRP}}{dt}}$$ Equation 3.4

where PSapp is the apparent permeability surface area product (both passive and transporter mediated), V is the volume of the receiver side, A is the surface area of the membrane, C_0 is the initial donor concentration and dX/dt, which is equal to V*dC/dt, is the slope of the plot of cumulative amount in the receiver side as a function of time. ER_α is asymmetry ratio, ER_A is apical efflux ratio and ER_B is basolateral efflux ratio. H^3 labeled mannitol was added on either the apical or basolateral side and was used to assess the integrity of the cell monolayers. Data from wells with <1% H^3 mannitol transfer per hour from the donor to the receiver side were considered for analysis. Fluxes in the A>B direction were compared with fluxes in the B>A direction analyzed using one-way ANOVA followed by a Bonerroni post-hoc t-test. A p-value <0.05 was considered a significant difference.

3-2.4. HPLC analysis

AR-67 concentrations in experimental samples from the receiver side were measured by HPLC as described in the literature (145). Concentrations of AR-67 lactone and carboxylate were summed to give total drug concentration. The concentration was normalized to protein content for samples obtained from cell lysates.

3-3. Results

3-3.1. Efflux of AR-67 lactone by BCRP and P-gp

Intracellular accumulation

To examine if AR-67 is a substrate of BCRP or MDR1, intracellular accumulation of AR-67 was studied in mock transfected cells and in cells that overexpress BCRP or MDR1. To measure differences in intracellular accumulation, we performed linear regression of the intracellular concentration of AR-67 as a function of the extracellular concentration. The slope of the regression line for L-LCPK cells was 2.6 fold higher than that of L-MDR cells (95% CI, 118.8 - 148.2 vs. 41.4 - 55.1). Similarly, the slope of the regression line for pCDNA3 cells was 7.6 fold higher than that of BCRP cells (95% CI, 106.1 - 166.0 vs. 9.1 - 26.9). These results indicate that at the range of extracellular AR-67 lactone concentrations studied (0.25, 0.5, 0.75 and 1 μM) efflux transporter overexpressing cells had significantly lower intracellular AR-67 concentration than their controls (Figure 3-1). To examine if decreased accumulation of AR-67 in transporter overexpressing cells was due to efflux by BCRP or P-gp, the intracellular accumulation of 1 μM AR-67 was measured in control and transporter overexpressing cells in the presence of the dual P-gp and BCRP inhibitor GF120918. In both BCRP and MDR1 overexpressing cells, preincubation with GF120918 resulted in significantly higher drug accumulation (Figure 3-1). Incubation with GF120918 abolished the difference in intracellular concentration between L-LCPK and L-MDR (unpaired two-tailed t-test, $p=0.21$) and between pCNDA3 and BCRP cells (unpaired two tailed t-test, $p=0.41$).

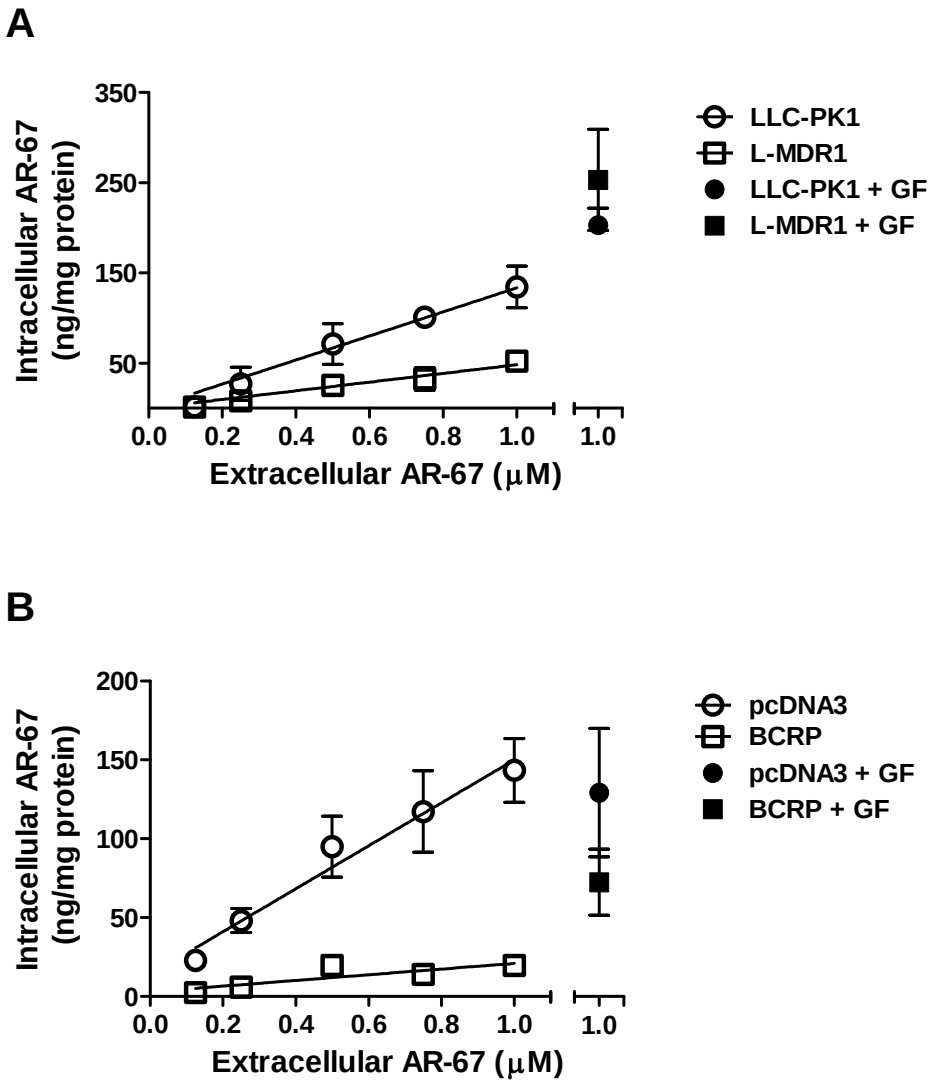


Figure 3-1. Efflux transporters (A) MDR1 and (B) BCRP limit the intracellular accumulation AR-67 lactone compared to control cells following 20 min incubation (open symbols). Pretreatment with 5 μM GF120918 reverses the effect of BCRP and MDR1 in cells incubated with 1 μM AR-67 lactone (closed symbols).

Transcellular transport of AR-67 lactone across cell monolayers

Based on the results of intracellular accumulation studies, which showed the interaction of AR-67 with P-gp and BCRP, vectorial transport of 5 μM AR-67 was assessed in MDCKII-pcDNA3 and MDCKII-BCRP cells. In MDCKII wild-type cells, apparent permeability surface area product (PS ($ml*sec^{-1}X10^{-5}$) of AR-67 from B to A (secretory transport) was slightly higher than from A to B direction (absorptive transport) ($PS_{B>A}$ 12.90 ± 0.57 vs. $PS_{A>B}$ 9.85 ± 1.17) (Table 3-1 and Figure 3-2A and Figure 3-2B). This difference was not statistically different in the presence or absence of transporter inhibitors. On the other hand, in BCRP-transfected cells, permeability surface area product (PS) was several fold higher ($p<0.05$) in the B to A direction compared with the A to B direction ($PS_{A>B}$ 0.34 ± 0.08 vs. $PS_{B>A}$ 31.02 ± 23.35; ER_{α} 90.82 ± 23.35) (Table 3-1 and Figure 3-2C and Figure 3-2D). GF120918 increased A to B transport and reduced B to A transport as shown by reduced efflux ratios (Table 3-1). However, it inhibited but did not completely abolish BCRP mediated efflux since A>B and B>A fluxes were still statistically different ($p<0.05$). Similar to GF120918, although not to the same extent, treatment with rifampin also reduced ($p<0.05$) BCRP mediated efflux as shown by reduced efflux ratios (Table 3-1).

3-3.2. Uptake of AR-67 lactone and AR-67 carboxylate by OATP1B1 and OATP1B3

To test the hypothesis that the hydrophilic carboxylate but not the lipophilic lactone requires uptake by OATP1B1 or OATP1B3 for entry into cells, we performed intracellular accumulation experiments in transporter overexpressing cells and in their controls. Our data show that interconversion was minimal for the duration of the experiments (Figure 3-3A.). For AR-67 lactone uptake studies, linear regression of the intracellular concentration as a function of the extracellular concentration showed no significant difference between the slopes of control and OATP1B1 or 1B3 overexpressing cells (95% confidence interval, 56.0 - 72.0 control vs. 54.0 - 67.0 OATP1B1 vs. 47.0 – 64.0 OATP1B3). These results suggest that lactone uptake is more likely dependent on diffusion than on uptake by OATPs (Figure 3-4**)**. For carboxylate uptake studies, the relationship between intracellular concentration and extracellular

concentration was not linear for OATP expressing cells. Carboxylate uptake was higher in OATP1B1 and OATP1B3 cells compared to control cells (Figure 3-4). OATP-mediated carboxylate uptake was confirmed using 50 μM BSP, 5 μM GF120918, and 100 μM rifampin, which inhibited carboxylate uptake in OATP1B1 and OATP1B3 cells to control (pIRESneo) levels (Figure 3-5).

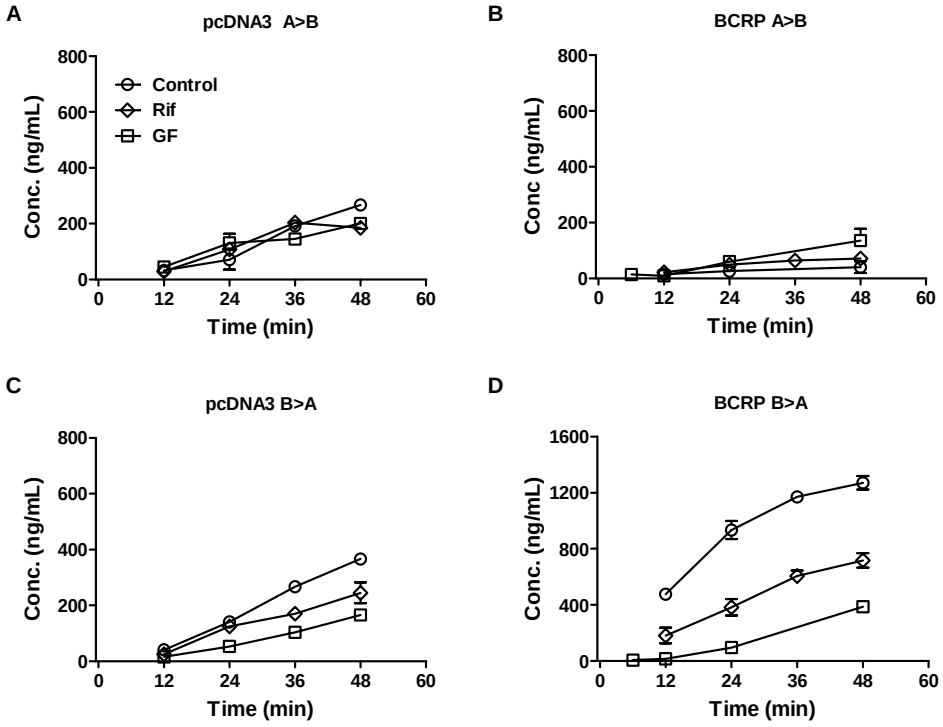


Figure 3-2. Apical to basolateral (A, B) and basolateral to apical (C, D) transport of 5 μM AR-67 lactone in pcDNA3 (A, C) and BCRP cells (B, D). Transport experiments were carried out in the presence and absence of 100 μM rifampin or 5 μM GF120918 as denoted in the figure. Data represent values obtained from n=3 wells.

Table 3-1. Permeability surface area product (PS) and efflux ratios of AR-67 lactone in the presence and absence of various transport inhibitors.

PS (ml/sec x 10^{-5})	MDCKII-pcDNA3			MDCKII-BCRP		
	Control Mean (SD)	Rifampin Mean (SD)	GF Mean (SD)	Control Mean (SD)	Rifampin Mean (SD)	GF Mean (SD)
A>B	9.85 (1.17)	6.29 (1.19)	6.01 (0.30)	0.34 (0.08)	2.44 (0.82)	6.55 (0.27)
B>A	12.90 (0.57)	8.11 (1.16)	5.93 (0.45)	31.02 (23.35)*	24.78 (2.30)*	14.85 (3.99)*
Efflux ratios						
	Formula		Control		Rifampin	GF
ER_{α} (Asymmetry Efflux Ratio)	$\frac{\frac{dX^{BCRP}_{B\to A}}{dt}}{\frac{dX^{BCRP}_{A\to B}}{dt}}$		90.82 (23.35)		10.15 (3.53)	2.27 (0.62)
ER_A (Apical Efflux Ratio)	$\frac{\frac{dX^{BCRP}_{B\to A}}{dt}}{\frac{dX^{pcDNA3}_{B\to A}}{dt}}$		2.41 (0.27)		3.06 (0.52)	2.50 (0.70)
ER_B (Basolateral Efflux Ratio)	$\frac{\frac{dX^{pcDNA3}_{A\to B}}{dt}}{\frac{dC^{BCRP}_{A\to B}}{dt}}$		28.84 (7.62)		2.58 (0.99)	0.92 (0.06)

*In MDCKII-BCRP cells, A>B fluxes in control, rifampin and GF groups were significantly different from their respective B>A fluxes ($p<0.05$). In MDCKII-pcDNA3 cells A>B fluxes were not found to be significantly different from their respective B>A fluxes.

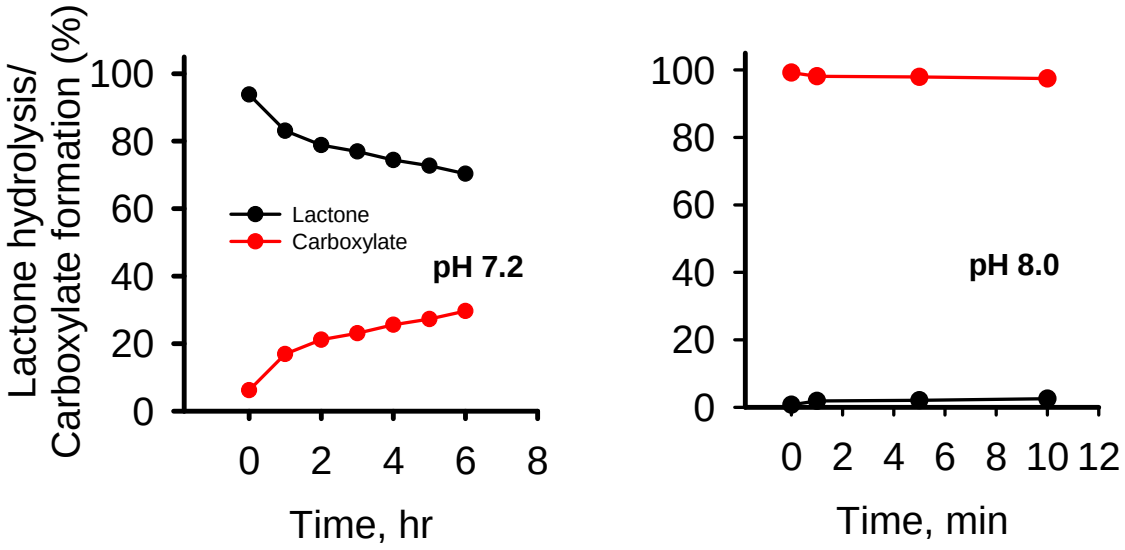


Figure 3-3. In vitro stability of (A) AR-67 lactone (pH 7.4) and (B) AR-67 carboxylate (pH 8) in transport medium.

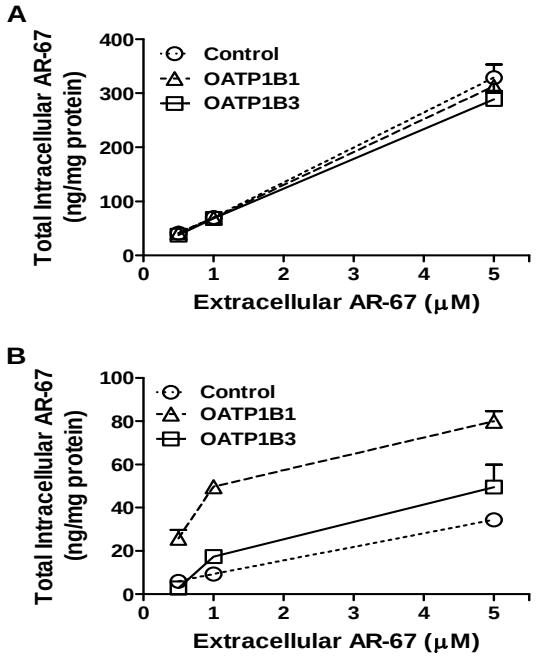


Figure 3-4**.** Uptake of AR-67 (A) lactone (1 µM) and (B) carboxylate (1 µM) after a 10 minute incubation in OATP1B1 and OATP1B3 transiently transfected HeLa cells.

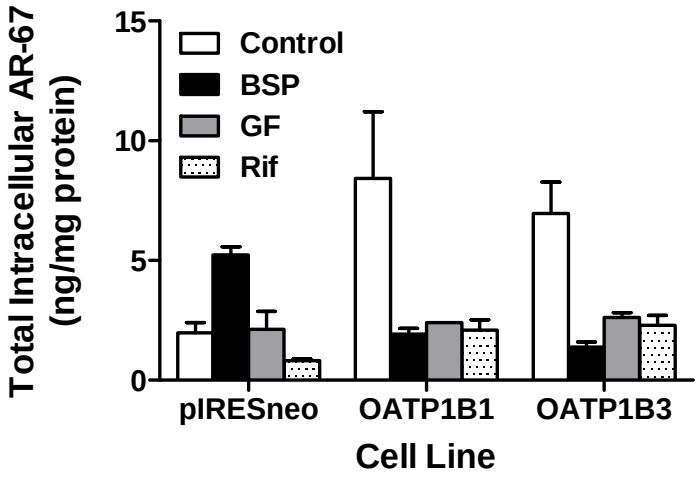


Figure 3-5. Inhibition of OAT1B1 and OAT1B3-mediated 5 minute carboxylate uptake in stably transfected HeLa cells with 50 μM BSP, 5 μM GF120918 (GF) or 100 μM Rifampin (Rif).

3-4. Discussion

In the current study, the effect of efflux (MDR1 and BCRP) and uptake (OATP1B1 and OATP1B3) transporters on cellular accumulation and/or transcellular transport of the lactone and carboxylate forms of AR-67 was measured. Cell lines overexpressing MDR1 or BCRP had markedly reduced intracellular accumulation following incubation with AR-67 lactone. The decreased intracellular accumulation in MDR1 or BCRP overepressing cells and increased accumulation in the presence of the dual MDR and BCRP inhibitor, GF120918 indicates that the decreased accumulation was due to efflux by these transporters. In transcellular flux studies, BCRP was found to limit the apical to basolateral transport (absorptive transport) and enhance the basolateral to apical transport of AR-67 further demonstrating the role of BCRP in the transport of AR-67. Consistent with efflux transporter inhibition, GF120918 and rifampin increased permeability in the apical to basolateral direction and reduced permeability in the basolateral to apical direction. These results are in agreement with the works of others. In a study that assessed the intracellular accumulation of topotecan in ovarian adenocarcinoma cells (IGROV1 and IGROV1-derived resistant T8 and MX3 cells), there was a threefold lower accumulation in resistant cell lines overexpressing BCRP which, upon co-incubation with GF120918, increased to levels observed in non-resistant cells (109).

The prominent role Bcrp plays in the transport of the water soluble topotecan was shown in LLC-PK1 and L-Bcrp cell lines (20). The apical to basolateral and basolateral to apical transport did not differ in the parental cell line (LLC-PK1) when residual P-gp activity was abolished with the specific P-gp inhibitor valspodar (PSC 833, 10 μM). The basolateral to apical transport was much more than in the apical to basolateral direction in Bcrp overexpressing cells under similar conditions. However, in the presence of the dual MDR and Bcrp inhibitor, GF120918 (10 μM), the difference in apical to basolateral and basalolateral to apical transport of topotecan was abolished (102).

The lipophilic camptothecin analog, gimatecan, shares similarities with AR-67 due to a bulky substitution at position 7 but differs from AR-67 by lack of an –OH group at position 10. According to Perego et al. (110) the cellular accumulation of gimatecan

was not affected by BCRP expression. In contrast, accumulation of topotecan was about two fold lower in BCRP overexpressing HT29/MIT (resistant) cell lines than in HT29 (sensitive) cell lines (110). Furthermore, Gounder et al. (146) showed that BCRP expression neither lowered the intracellular accumulation nor altered cytotoxicity of gimatecan. These findings suggested that enhanced lipophilicity might circumvent interaction with BCRP (110). However, a follow up study by Marchetti et al. (134) assessed the transport of gimatecan by Bcrp. The transcelluar transport of gimatecan across cell monolayers was more in the B to A than in the A to B direction in MDCKII-Bcrp1 cell lines but was similar in parental cell lines (Efflux ratio, B>A/A>B 3.1 in Bcrp vs. 0.94 in parental cell lines). This was observed after excluding the effect of P-gp with the selective P-gp inhibitor zosuquidar (5 μM). Differences in A to B and B to A transport of gimatecan in MDCKII-Bcrp1 cells were abolished by pantoprazole (500 μM) and GF120198 (5 μM) (134). This study demonstrated that gimatecan is a substrate for Bcrp. According to another study by Bates et al. (147), the parent compound camptothecin is a poorer substrate for BCRP than SN-38. Furthermore, based on efflux of homocamptothecins by BCRP, the authors propose that substitutions on A- and B-rings and stabilized E-ring contribute to recognition of camptothecins by BCRP. The latter raises the question of whether or not the carboxylate forms of camptothecins in general and that of AR-67 in particular are substrates of ABC efflux transporters. Further studies are needed to elucidate the mechanism of cellular exit for the carboxylate.

Enhanced uptake of AR-67 carboxylate in OATP1B1 and OATP1B3 overexpressing cells and its reversal by BSP and rifampin indicates OATP mediated uptake of the carboxylate. In addition, reversal of AR-67 carboxylate uptake by GF120918 indicates that GF120918 also inhibits OATPs as well as ABC transporters, MDR1 and BCRP. Both rifampin (10 μM) and GF120918 (5 μM) were recently shown to inhibit OATP mediated in vitro uptake of 17β estradiol 17β-D-glucuronide (E_2G), and the camptothecins gimatecan and BNP1350. Compared to the carboxylate form, AR-67 lactone accumulated much more but its accumulation was not affected by the expression of OATP1B1. This is likely related to diffusion of the lipophilic lactone (148). In one study, initial rate of uptake of irinotecan and SN-38 in isolated intestinal cells at pH 6.2 was more than threefold higher than at pH 8, where the carboxylate form would

predominate (148). Nozawa et al. (51) examined the cellular uptake of irinotecan, SN-38 and SN-38 glucuronide by OATP1B1 at pH 7.4 for up to 40 minutes. It was found that HEK293 cells expressing OATP1B1 accumulated more SN-38 than mock transfected cells. In this study, the drug solutions for the uptake studies were prepared in 50 mM phosphate buffer (pH 9.0) and left overnight before dilution with pH 7.4 uptake medium In PBS (pH 7.4) only 13% of irinotecan and SN-38 were found to exist as the lactone (149). It can be argued, therefore, that at pH 9.0, irinotecan, SN-38 and SN-38 glucuronide were predominantly in their carboxylate forms. Although dilution with a pH 7.4 uptake medium could favor the reformation of the lactone forms, the uptake measured could not necessarily be that of the lactone but also of the carboxylate. The study, nevertheless, demonstrates that an OATP1B1 mediated uptake process is involved in the intracellular accumulation of SN-38 (carboxylate).

In conclusion, these in vitro studies indicate that the lactone and carboxylate forms of AR-67 interact with uptake and efflux transporters. It is expected that such interactions will affect the pharmacokinetics and/or oral bioavailability of the lactone and carboxylate forms.

CHAPTER 4

FACTORS AFFECTING THE IN VIVO LACTONE STABILITY AND SYSTEMIC CLEARANCE OF THE LIPOPHILIC CAMPTOTHECIN ANALOGUE AR-67

4-1. Introduction

AR-67 (DB-67) is a highly lipophilic and potent third generation camptothecin analogue (11, 23) currently in early phase clinical trials as an anticancer agent (150). Previous in vitro experiments assessing percent lactone in whole blood, showed that equilibrium favors the carboxylate form of AR-67 as well as other camptothecin analogues. Nonetheless, percent lactone of AR-67 lactone is relatively hihger compared to other camptothecin analogues (11). This molecule was selected for further development among several analogues designed to have higher lactone fraction in the blood. Camptothecins owe their pharmacologic activity to their α-hydroxy-δ-lactone pharmacophore, which hydrolyses to the open ring or carboxylate form in a pH dependent, but reversible manner (2, 3, 7). Lower pH favors the lactone form, while plasma and alkaline pH favors the carboxylate (7). For many camptothecin analogues, the pH dependent lactone hydrolysis is strongly facilitated in plasma by carboxylate binding to serum proteins. As a result of the avid binding, sink conditions are established and the equilibrium shifts towards carboxylate formation (151). Thus, lactone concentrations reach lower levels than carboxylate in plasma at steady state (152), which is of concern because the latter is considered inactive. However, due to its capacity to revert to the lactone form in acidic environments, the carboxylate has also been associated with the increased toxicities observed in early camptothecin trials and with some 2nd generation analogues (153, 154). Comparatively, AR-67 was chosen for development based on its decreased interaction with albumin and its increased lipophilicity that facilitated partitioning in lipid membranes. Collectively, these physicochemical characteristics were believed to "protect" the lactone and minimize hydrolysis.

As compared to most drugs, cytotoxic anticancer agents have a fairly narrow efficacy-toxicity window and a good understanding of factors contributing to their disposition is essential for ensuring patient safety. The disposition of the lactone and

carboxylate forms of AR-67 is expected to vary due to differences in aqueous solubility, interaction with transporters and enzymes and distribution into tissues. Several studies have demonstrated that camptothecins are substrates of uptake and efflux transporters (47, 51, 134, 155). As would be expected from the physicochemical differences between lactone and carboxylate, we recently demonstrated that AR-67 lactone is a substrate for efflux transporters P-gp and BCRP, while the carboxylate is a substrate for the organic anion uptake transporters OATP1B1 and OATP1B3 *in vitro* (156). Although the overall drug disposition depends collectively on many factors, dissimilar transporter interactions will potentially lead to differences in lactone and carboxylate systemic clearances. The unique physicochemical properties of each camptothecin analogue also add an additional layer of complexity in estimating the reversible hydrolysis kinetic parameters and the lactone and carboxylate systemic clearances.

Although the in vitro interaction of camptothecins with transporters and the in vivo disposition of lactone and carboxylate forms of camptothecin (24, 26), topotecan (27) and irinotecan (157) have been studied, the pharmacokinetics of AR-67 have not been examined in detail. To accurately estimate lactone and carboxylate pharmacokinetic parameters, administration of both species is required (158, 159). In this study we used pharmacokinetic modeling and simulations to assess how clearance changes of either the lactone or carboxylate forms could affect overall drug disposition. A primary objective was to estimate the systemic and interconversion clearances of the lactone and carboxylate forms of AR-67. Furthermore, through simulation and in vivo pharmacologic inhibition of transporters, we examined the influence of AR-67 lactone clearance changes on drug disposition.

4-2. Methods

4-2.1. Chemicals

Ammonium acetate (Mallinckrodt Baker, Phillipsburg, NJ), HPLC grade acetonitrile and methanol (Burdick and Jackson, Muskegon, MI) were purchased from VWR (West Chester, PA). Tetrabutylammonium dihydrogen phosphate (TBAP; 1.0 M aq. solution), Tween-80 and PEG-300 were obtained from Sigma-Aldrich (St. Louis, MO). Dimethylsulfoxide (≥ 99.7% DMSO) and glacial acetic acid came from Fisher Scientific (Fair Lawn, NJ). Blank rat plasma, used in the preparation of calibrators and quality control solutions, was from Innovative Research (Novi, Michigan). Siliconized pipette tips were obtained from Cole-Parmer (Vernon Hills, IL) and amber and transparent siliconized microcentrifuge tubes were from Crystalgen Inc. (Plainview, NY) and Fisher Scientific (Fair Lawn, NJ) respectively. Magnesium- and calcium-free Dulbecco's phosphate buffered saline (PBS) was from Gibco Invitrogen (Carlsbad, CA). AR-67 was obtained from Novartis (East Hanover, NJ). Sulfobutylether-β-cyclodextrin (Captisol®) was received as a gift from CyDex, Inc. (Overland Park, KS). Rifampin for injection (USP) and diluent (5% dextrose in water, D5W) were from Baxter Healthcare Corporation (Deerfiled, IL) while GF120918 was a gift from GlaxoSmithKline (Research Triangle Park, NC).

4-2.2. Animal study design

Female Harlan Sprague-Dawley rats weighing between 240-270 g were used for the efflux inhibition studies. Animals were fasted during the experiment, but had free access to water. This was a four-week randomized crossover study. Treatment was allocated in a randomized scheme to each animal (n=6) such that each animal received the following four treatments: a) oral pretreatment with control vehicle (10% Tween-80, 40% PEG-300 in D5W) 5 minutes before intravenous AR-67 lactone (2.5 mg/kg), b) oral pretreatment with GF120918 (2.5 mg/kg solubilized in control vehicle) 5 minutes before intravenous AR-67 lactone (2.5 mg/kg), c) oral pretreatment with control vehicle 5 minutes before intravenous AR-67 carboxylate (at 2.5 mg/kg), d) oral pretreatment with

GF120918 (2.5 mg/kg solubilized in control vehicle) 5 minutes before intravenous AR-67 carboxylate (2.5 mg/kg).

The effect of uptake transporter inhibition on the pharmacokinetics of AR-67 was assessed in rats weighing 250-280 g using rifampin. Rifampin powder for intravenous administration (600 mg) was reconstituted with 10 ml of 5% dextrose in water. Animals were then pretreated with 50 mg/kg rifampin orally 1 h before the the intravenous administration of 2.5 mg/kg lactone or carboxylate solutions. AR-67 was administered through injection in the lateral tail vein. Following drug administration, about 100 μL of blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 12 h using heparinized hematocrit capillary tubes and was then transferred to heparinized microcentrifuge tubes.

4-2.3. HPLC analysis

Plasma was separated from blood cells by centrifugation at 8500 g for 3 minutes at room temperature. The plasma was extracted (1:4, v/v) with dry ice cold methanol (-80°C). The extracts were kept frozen at -80°C until analysis by HPLC with fluorescence detection for both AR-67 carboxylate and lactone forms as described previously (145). Assay accuracy and precision were validated in rat plasma and were found acceptable. Three quality control samples in the range of 2.5-250 ng/mL for carboxylate and 5-300 ng/mL for lactone demonstrated accuracy within 15% (85-115%) of nominal AR-67 concentrations. The relative standard deviation (RSD) was <6%. System suitability criteria were met prior to sample batch analysis. Samples were placed in an autoinjector (4°C) and injected within 6 hours to prevent lactone-carboxylate interconversion.

4-2.4. Pharmacokinetic analysis

Pharmacokinetic analysis was carried out using two approaches. The first utilized ADAPT-II to fit lactone-carboxylate interconversion (Figure 4-1A) and the in vivo disposition of each form using a 4 compartment model depicted in Figure 4-1B. The model was built using eight differential equations, four each for the lactone and carboxylate administration. Each set of four equations shared the same kinetic parameters, which were simultaneously fitted to the data obtained from the lactone and

carboxylate bolus doses using the maximum likelihood method (160). Micro-rate constants used in the model fitting process were converted into clearances using the volumes of the respective compartments. The alternate noncompartmental approach (NCA) utilized a method described in the literature for reversible biotransformation systems (161). This method was used as a basis for comparison of parameter estimates of the compartmental approach. In this analysis, areas under plasma concentration (AUC_{0-inf}) versus time curves of both the lactone and the carboxylate following administration of the lactone or the carboxylate were estimated with WinNonlin v.5.2. The elimination and apparent distribution half-lives of the lactone and carboxylate were calculated from the micro-rate constants estimated with ADAPT-II as described in the literature (162).

4-2.5. Simulations

The model depicted in Figure 4-1B was built in Stella® (High Performance System, Inc., Lyme, NH). Simulations were conducted to assess the clinical significance of clearance changes as they relate to alteration of exposure to either form of AR-67. In these simulations we estimated plasma concentrations while varying either the lactone or carboxylate clearance parameters that were estimated using compartmental modeling in ADAPT-II under control conditions (**Table 4-1**).

4-2.6. Statistical analysis

Differences between clearance parameters in the presence or absence of inhibitors were assessed using a paired two sample t-test (cross-over studies) and comparison of 95% confidence estimates of the parameters. The level of significance was $p<0.05$.

4-3. Results

4-3.1. Plasma pharmacokinetics of AR-67 lactone and carboxylate

The plasma pharmacokinetics of AR-67 were assessed in female Sprague Dawley rats following an intravenous bolus dose of 2.5 mg/kg. To obtain better estimates of the lactone –carboxylate interconversion kinetics, we administered AR-67 lactone and AR-67 carboxylate, separately, and measured the plasma concentration of both forms during the individual experiments. As shown in Figure 4-1A, the lactone and carboxylate

interconversion involves the hydrolysis of the lactone ring, but this reaction is reversible. To elucidate the role of each moiety in the overall drug disposition, we sought to selectively perturb the clearance of each AR-67 form. In previous studies we determined that AR-67 lactone is a substrate of BCRP and to lesser extent P-gp, while the carboxylate is a substrate of OATP1B1 (156). Therefore, the BCRP and P-gp pharmacologic inhibitor, GF120918, was used to decrease the lactone clearance and the OATP1B1 inhibitor, rifampin, was used to impair the carboxylate clearance. GF120918 and rifampin were administered orally 5 minutes and 1 hour, respectively, prior to the administration of the AR-67 intravenous bolus doses. Experiments with animals (six per group) receiving AR-67 alone (lactone or carboxylate) or AR-67 following GF120918 consisted of a four period randomized crossover design. In studies with animals receiving rifampin, each animal was only used once to avoid rifampin experimental artifacts via rifampin mediated metabolism and transporter induction (i.e., CYP450 and P-gp). Each animal was sampled at indicated time points via venipuncture of the saphenous vein. Pharmacokinetic analyses were carried out with ADAPT-II (160) following the implementation of the model depicted in Figure 4-1B. The data sets for lactone and carboxylate doses were analyzed simultaneously. The pharmacokinetic parameter estimates are presented in **Table 4-1** and the pharmacokinetic profiles are depicted in **Figure 4-2.** (A, B, C &D). When the lactone form was administered, the lactone concentrations (**Figure 4-2.**A) were much higher than the carboxylate (**Figure 4-2.**B). Interestingly, when the carboxylate dose was administered the carboxylate declined rapidly, and within 30 minutes the lactone reached similar concentration levels as the carboxylate (**Figure 4-2.**C and **Figure 4-2.**D). As expected, the administration of GF120918 prior to AR-67 lactone administration had a significant effect on the clearance of the lactone form, which decreased to approximately 60% of the clearance estimates obtained in animals not receiving the inhibitor prior to AR-67.

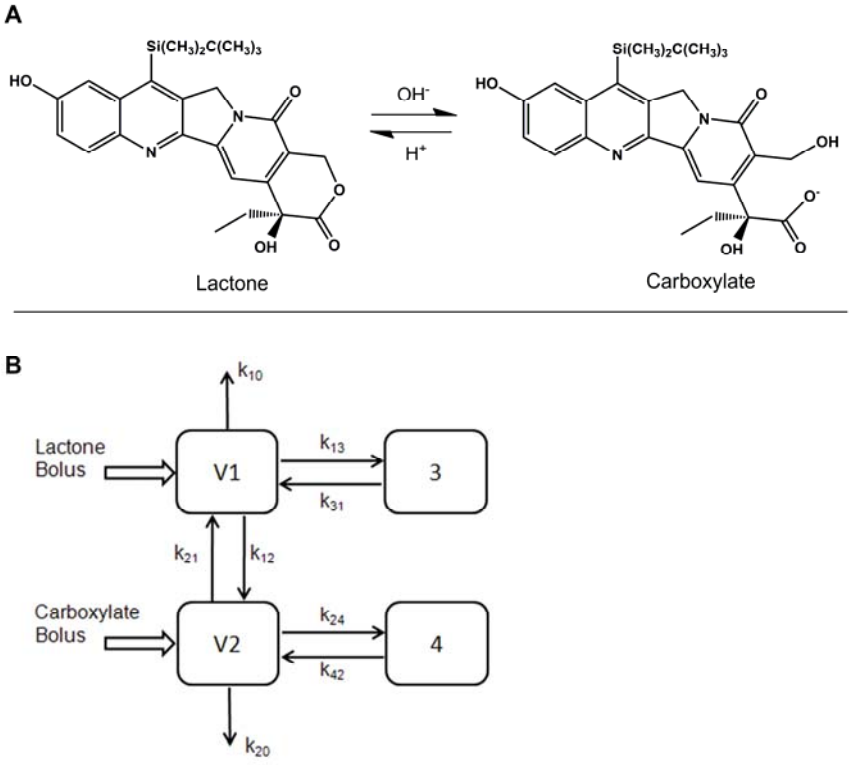


Figure 4-1. (A) AR-67 undergoes a pH dependent hydrolysis of the lactone and carboxylate moieties of AR-67. (B) A pharmacokinetic model allowing for AR-67 interconversion was used to fit the data.

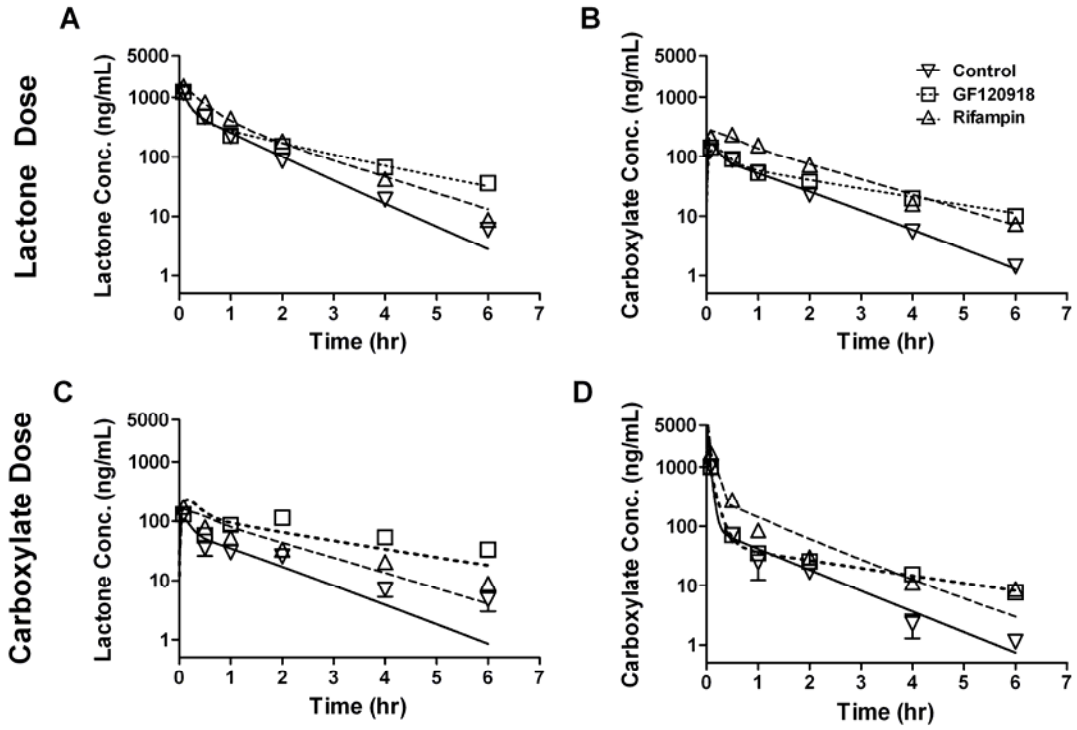


Figure 4-2. Plasma pharmacokinetics of AR-67 lactone (A, C) and carboxylate (B, D) following intravenous administration of 2.5 mg/kg lactone (A, B) or carboxylate (C, D). Each experiment represents mean ± standard deviation of 6 rats. Animals receiving AR-67 only (Control) or AR-67 following GF120918 pretreatment were used in a 4 period crossover experimental design. Animals receiving rifampin pretreatment were only used once. Lines represent the unweighted model estimated fits.

Although, the carboxylate concentrations were higher in the group receiving GF120918, the model predicted that the carboxylate clearance was unchanged and the higher concentrations were a result of the increased lactone concentrations. Rifampin pre-administration had significant effects on the clearance of the carboxylate, but notably it also had a significant effect on the clearance of the lactone, which decreased by approximately 60% and 45%, respectively, as compared to animals not receiving a pharmacologic inhibitor. In the case of the rifampin pre-treated animals, the model also predicted a significant decrease in the peripheral compartment distributional clearance of

the lactone, but not of the carboxylate. The central compartment volume estimate for the lactone (1.4 L/kg) was higher than the carboxylate moiety (0.5 L/kg) and there were no significant differences in its magnitude among the three experimental groups. Although differences were not statistically significant, the model predicted higher carboxylate volume when animals were pretreated with GF120918 and a lower volume when pretreated with rifampin (**Table 4-1**).

The apparent lactone stability of AR-67 in plasma is evident in Figure 4-3. Panels A and B depict the model predicted lactone to carboxylate ratios following administration of the lactone or carboxylate forms of AR-67, respectively. In all cases, following lactone administration, there was rapid conversion of the lactone to the carboxylate within the first 30-60 minutes. However, a second phase of slower conversion was observed at later time points with the ratio ranging between 2-fold and 4-fold (i.e., approximately 67-80% of AR-67 was in the lactone form) (Figure 4-3A). A similar rapid conversion was observed within 30 minutes after the carboxylate dose was administered followed by a steady state phase where the lactone and carboxylate concentrations were either equivalent or the lactone concentrations were higher, as was the case when GF120918 was administered (Figure 4-3D).

The effect of each inhibitor on the exposure to AR-67 is shown in Figure 4-3C and Figure 4-3D for the lactone and carboxylate dosages, respectively. When the lactone was administered, 84% of the total AUC was contributed by the lactone and pretreatment with GF120918 or rifampin significantly increased exposure to both forms (Figure 4-3C). Animals receiving GF120918 and rifampin pretreatment had 81% and 76%, respectively, of the total AUC in the lactone form. The slight decrease in lactone exposure (or increased carboxylate exposure) in the later group is consistent with the effect of rifampin in decreasing carboxylate clearance.

Table 4-1. Pharmacokinetic parameter estimates in rats gavaged with either the control vehicle or GF120918 prior to intravenous AR-67 administration. Parameters were estimated by fitting the model presented in **Figure 4-1** to the data or from areas under the plasma concentration versus time curves (NCA model) as previously described (24).

PK Parameter*	Control		GF120918		Rifampin	
	Estimate (95% CI)		Estimate (95% CI)		Estimate (95% CI)	
	ADAPT model	NCA model	ADAPT model	NCA model	ADAPT model	NCA model
Lactone systemic clearance (Cl_{10})	1.8 (1.4-2.1)	1.7 (1.3-2.1)	0.7** (0.4-1.1)	1.1 (0.8-1.5)	1.0** (0.8-1.2)	0.8 (0.5-1.1)
Carboxylate systemic clearance (Cl_{20})	6.3 (4.9-7.6)	4.9 (4.0-5.8)	6.1 (4.8-7.4)	3.5 (2.8-4.3)	2.6** (2.0-3.2)	2.7 (2.2-3.2)
Lactone to carboxylate conversion clearance (Cl_{12})	1.4 (1.0-1.8)	1.1 (0.8-1.2)	2.2 (1.6-2.9)	1.4 (1.2-1.6)	1.0 (0.7-1.3)	0.9 (0.7-1.1)
Carboxylate to lactone conversion clearance (Cl_{21})	0.9 (0.6-1.2)	1.3 (0.8-1.9)	3.6** (2.4-4.7)	3.5 (2.8-4.3)	0.6 (0.1-1.4)	0.5 (0.4-0.7)
Lactone distributional clearance (CL_D-L)	5.2 (4.5-5.9)	--	4.7 (1.2-8.2)	--	0.8 (0.1-1.7)	--
Carboxylate distributional clearance (CL_D-C)	2.4 (1.4-3.5)	--	3.2 (1.7-4.7)	--	1.9 (0.4-4.0)	--
Lactone central volume (V1)	1.4 (1.2-1.5)	--	1.6 (1.0-2.2)	--	1.4 (1.1-1.7)	--
Carboxylate central volume (V2)	0.5 (0.2-0.8)	--	1.4 (0.8-1.9)	--	0.20 (0.1-0.3)	--
Apparent lactone elimination half life (t ½ L)	--	82.3 (71.7-92.8)	--	194.0 (111.3-276.7)	--	100.3 (66.7-133.9)
Apparent carboxylate elimination half-life (t ½ C)	--	109.3 (50.6-167.9)	--	223.7 (182.5-264.9)	--	223.8 (113.3-334.2)

*Clearances are in L/hr/kg; half-lives are in minutes; volumes are in L/kg. **Parameter estimates were significantly different from the respective values obtained under control pretreatment conditions

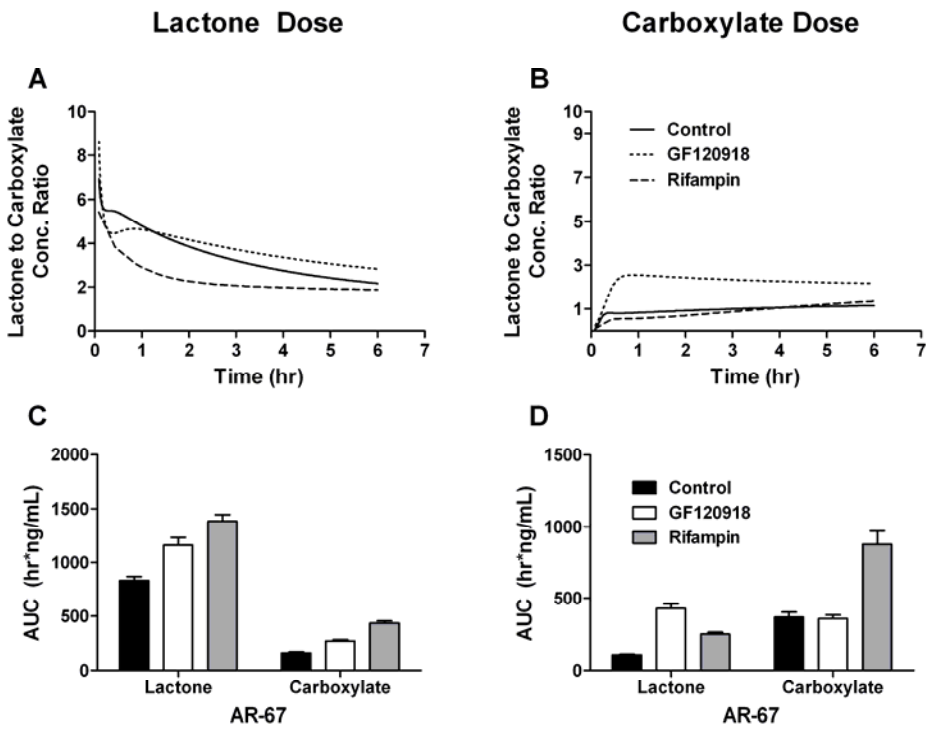


Figure 4-3. AR-67 exists primarily in the lactone form in the plasma of rats following lactone administration. The lactone to carboxylate concentration ratios were calculated based on the model fits to the experimental pharmacokinetic data following AR-67 lactone (A) or AR-67 carboxylate (B) administration alone or following the administration of GF120918 or rifampin. The area under plasma concentration versus time ($AUC_{0\text{-}24hr}$) for the lactone and carboxylate forms observed following administration AR-67 lactone (C) and AR-67 carboxylate intravenously (D).

In experiments where the carboxylate was administered, 22% of the total AUC was contributed by the lactone and again, pretreatment with GF120918 or rifampin significantly increased exposure to both forms (Figure 4-3D). Animals receiving GF120918 and rifampin pretreatment had 55% and 22%, respectively, of the total AUC in the lactone form.

4-3.2. Simulations assessing the effect of clearance changes on AR-67 exposure

The pharmacokinetic model fitted to the data obtained from the separate lactone and carboxylate administration suggests that the relative increases in the lactone and carboxylate concentrations in the GF120918 pretreated groups are consistent only with inhibition of the lactone clearance. In contrast, the model estimated that the observed increases in plasma concentrations of both lactone and carboxylate in the rifampin pretreated groups were due to the inhibition of both the lactone and carboxylate clearance. To better understand these results, we simulated the effects of decreasing lactone (Figure 4-4A, Figure 4-4B) or carboxylate (Figure 4-4C, Figure 4-4D) clearance on their respective AUC following lactone administration. The estimated AUCs are presented for clearance values ranging from 1-100% of the experimentally estimated clearance values. Thus, the axis was normalized between 0.01 and 1. The estimated AUC values are presented as the absolute estimates (Figure 4-4A, Figure 4-4C) or normalized to the AUC estimates obtained when there was no clearance inhibition (Figure 4-4B, Figure 4-4D). Simulations predicted that selective decrease in the lactone clearance would result in the increase of both the lactone and carboxylate AUC. The absolute magnitude of the increased exposure (Figure 4-4A) would be greater for the lactone, but the relative increase (Figure 4-4B) would be the same for either form. The selective decrease in the carboxylate clearance demonstrated that the magnitude of the AUC would increase for either form but the clearance has to decrease by more than 90% in order for the carboxylate AUC to be higher than the lactone one (Figure 4-4C). The relative increase, however, is more pronounced for the carboxylate resulting in a more rapid increase in carboxylate exposure with decreased carboxylate clearance (Figure 4-4D). Further analysis demonstrated that a decrease in the lactone or carboxylate clearance by

about 90% would yield similar increases in the overall exposure to AR-67 (Figure 4-5A). However, inhibition of the carboxylate clearance would also result in a significantly higher increase in carboxylate exposure (Figure 4-5B).

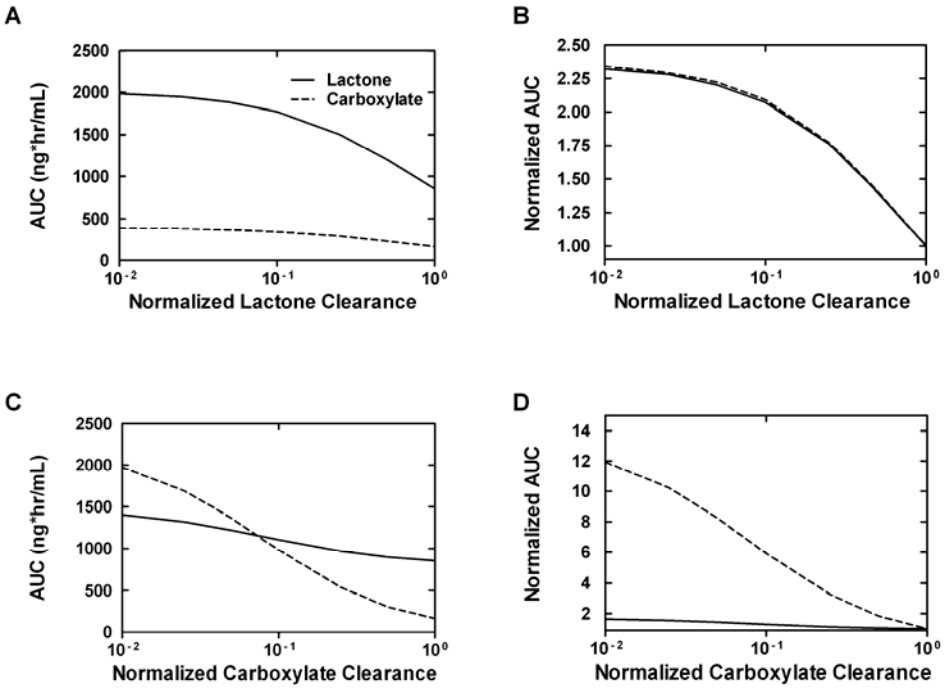


Figure 4-4. Simulations depicting the effect of lactone (A, B) or carboxylate (C, D) clearance inhibition following the intravenous bolus administration of AR-67 lactone.

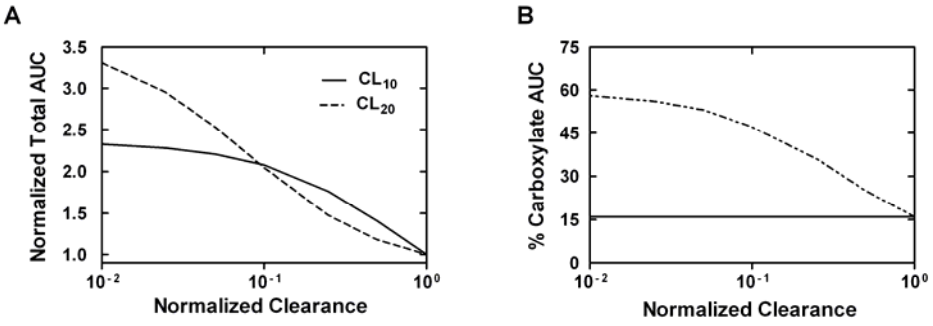


Figure 4-5. Simulation results depicting the effect of lactone (Cl_{10}) or carboxylate clearance (Cl_{20}) inhibition on total AR-67 AUC (A) and on the % carboxylate plasma AUC (B) following intravenous bolus administration of AR-67 lactone.

4-4. Discussion

In the current study we estimated the systemic and interconversion clearances of AR-67 by separately administering the lactone and carboxylate forms. According to our results, the predominant clearance term in AR-67 disposition was the systemic clearance of the carboxylate, which was more than threefold higher than the clearance of the lactone. Through transporter inhibition studies and simulations, we showed that decreased clearances of the lactone and carboxylate both led to elevated lactone and carboxylate plasma concentrations. Inhibition of carboxylate clearance led to a relatively enhanced carboxylate exposure.

The results of the compartmental modeling were corroborated by non-compartmental analyses (NCA) previously presented by Cheng and Jusko for metabolites undergoing interconversion (161). The NCA analyses estimated the systemic and interconversion clearances based on areas under the time-concentration curves, which were obtained by the trapezoidal rule method. Some differences were noted in the parameter estimates obtained by the two different analyses. In all cases but one, there was an overlap in the 95% confidence intervals around the parameter estimates (**Table 4-1**). In the case of the carboxylate clearance the means and 95% confidence intervals were different, but the relationship between the lactone and carboxylate clearances were similar for both methods (i.e., the carboxylate clearance was higher). Thus the parameter estimates from both methods lead us to the same conclusions. An advantage of the NCA analysis is its simplicity as it only requires the estimation of areas under the curve in the sampling compartment for parameter estimation (161). No assumption is required as to how many compartments are needed to fit the data. Furthermore, the NCA analysis can provide good initial parameter estimates for a more robust compartmental model analysis required to perform modeling and simulation of concentration-time profiles.

The pronounced difference in the systemic clearances of the lactone and the carboxylate suggest that the two moieties are eliminated via different pathways. Following lactone administration, the predominant form of AR-67 is the lactone as shown by a greater than 80% lactone AUC. This is most likely due to the lower systemic and interconversion clearance of the lactone compared to the systemic clearance of the

carboxylate. The carboxylate that is converted from the lactone is likely to be eliminated before it gets converted back to the lactone. Moreover, the effect of higher carboxylate clearance is more evident following the carboxylate dosing; the carboxylate rapidly declines from the plasma. In contrast, the pharmacokinetics of camptothecin and the camptothecin analog, topotecan, seem to be driven by the systemic clearance of the lactone and the lactone to carboxylate conversion clearance (24, 27). For camptothecin, the systemic clearance of the lactone was 5 fold higher than that of the carboxylate while lactone to carboxylate conversion clearance was three fold higher than the reverse process. Although the magnitudes of the various clearance parameters were different, a similar finding was also observed in the pharmacokinetics of topotecan. The systemic clearance of topotecan lactone was more than fourfold higher than the systemic clearance of the carboxylate and the conversion of the lactone to carboxylate was about threefold higher than the reverse process. Thus, rapid lactone clearance coupled with rapid lactone to carboxylate conversion and slow carboxylate elimination likely explains the fact that the predominant form of camptothecin and that of topotecan is the carboxylate. In addition to physiological pH, the strong binding of the carboxylate form to plasma proteins facilitates the hydrolysis of camptothecin and first generation analogs. (7) (163). In contrast, hydrolysis of AR-67 in blood in vitro occurred at a slower rate than SN-38 and camptothecin (11). In addition, the AR-67 lactone fraction at equilibrium was approximately 30% in whole blood as compared to 19.5% and 5% for SN-38 and camptothecin, respectively (11). Thus, it was hypothesized that the relatively higher AR-67 percent lactone in blood in vitro was a function of increased membrane partitioning in red blood cell membranes and decreased affinity of the carboxylate form for human serum albumin (11). The results in this chapter demonstrate that an additional mechanism, the relatively higher carboxylate clearance, contributes to the apparent in-vivo stability of AR-67 lactone.

The use of efflux and uptake transporter inhibitors allowed us to examine the effect of selective clearance changes on plasma concentration and overall exposure to AR-67. In the current study, the dual P-gp and BCRP/bcrp inhibitor GF120918 significantly decreased systemic clearance of the lactone, but not that of the carboxylate and led to elevated lactone and carboxylate concentrations. The effect of GF120918 on

lactone plasma concentration was quite pronounced following carboxylate administration, where lactone AUC increased 3.7 fold compared to that in control pretreated animals. The results indicate that lactone clearance depends on efflux by P-gp and Bcrp, consistent with our in vitro studies, which showed that the lactone is a substrate of P-gp and BCRP (156). GF120918 is widely used to assess the effect of P-gp and BCRP/Bcrp on drug disposition in vitro and in vivo. In both preclinical and clinical studies, pretreatment with GF120918 significantly increased the oral bioavailability of topotecan as a result of efflux inhibition leading to decreased clearance and increased gastrointestinal absorption (102, 133, 164) . In mice, pretreatment with GF120918 decreased plasma clearance and hepatobiliary excretion and increased fetal penetration and intestinal absorption of topotecan (102, 133, 164). Similarly, in clinical studies co-administration of GF120918 increased the apparent oral bioavailability of topotecan from 40% to 97% (133). In addition to inhibition of ABC transporters, P-gp and BCRP/Bcrp, GF1290918 was shown to inhibit the uptake of OATP substrates in vitro (165). The clearance of AR-67 carboxylate, which is a substrate of OATPs, did not significantly differ between control and GF120918 pretreatments. Nonetheless, it is possible that GF120918 could inhibit the OATP/Oatp-mediated hepatic uptake and subsequent biliary efflux of substrate drugs in vivo.

Our studies suggest that the increased AR-67 lactone and carboxylate exposure observed with rifampin pretreatment was related to a decrease in the clearance of both the lactone and the carboxylate. Thus, the inhibition of carboxylate clearance by rifampin is most likely due to inhibition of the hepatic uptake of the hydrophilic carboxylate, while inhibition of lactone clearance could have happened as a result of ABC transporter inhibition. There is literature evidence to support the effect of rifampin on both OATPs/Oatps and P-gp. Rifampin inhibited OATP1B1-mediated transcellular transport and intracellular accumulation of substrate drugs resulting in a 60% reduction in the intracellular accumulation of 17β-estradiol-17-(β-D-glucuronide) (E2G) and a significant reduction in the OATP1B1 mediated basolateral to apical transport of E2G, gimatecan and BNP1350 (52). In rats it was shown that rifampin pretreatment increased atorvastatin plasma concentration as a result of decreased Oatp-mediated hepatic uptake and metabolism by the liver. Decreased hepatic uptake in the presence of a single dose of

rifampin also led to decreased first-pass effect by the liver and therefore, increased oral bioavailability of atorvastatin from 5% to 14% (166). On the other hand, inhibition of P-gp by a single dose of rifampin was shown to lead to increased penetration of verapamil across the mouse blood-brain barrier (BBB) (167). This is consistent with previously discussed in-vitro results (Chapter 3), which demonstrated the carboxylate to be a substrate for uptake transporters OATP1B1 and OATP1B3, while the lactone form was transported by BCRP and to lesser extent by P-gp (156). The effect of rifampin on BCRP is currently under investigation.

Changes in clearance are likely to occur in clinical practice and may have implications in the clinical use and toxicity of camptothecin analogues. These changes could arise from pharmacogenetic differences in transporters between individuals and have been reported to lead to pharmacokinetic differences. In one study, plasma concentration of diflomotecan in 5 patients heterozygous for the ABCG2 421C>A allele, was 2.9 fold higher than that of 15 patients with wild-type alleles (168). Similarly an association was shown between high plasma pravastatin concentration and single nucleotide polymorphisms and haplotypes of organic anion transporting polypeptide (OATP1B1) (169). Lau et al have shown the major role organic anion uptake transporters play for the clearance of atorvastatin and its active metabolites by the hepatobiliary system and concluded that inhibition of hepatic uptake may have consequences on efficacy and toxicity of drugs mainly eliminated by the hepatobiliary system (116, 166).

In conclusion, lactone to carboxylate ratio of AR-67 is much higher in vivo than in vitro. This discrepancy can be explained by the fact that the in vivo system is not closed. The interconversion and the irreversible elimination of both lactone and carboxylate affect the plasma concentration of the lactone and carboxylate at any given moment. In addition, the carboxylate concentration is formation rate limited and the carboxylate moiety is eliminated as fast as it is formed. Because the lactone systemic clearance and the carboxylate to lactone conversion clearance is slower than the carboxylate systemic clearance, the lactone prevails. In summary, we studied the pharmacokinetics of the lactone and carboxylate forms of AR-67 and assessed through clearance inhibition studies and simulations the effect of clearance changes on plasma

concentrations and AUCs of AR-67. Our findings demonstrate that the carboxylate clearance is the predominant factor affecting the disposition of AR-67 and that lactone and carboxylate clearances are dependent on efflux and uptake processes respectively.

CHAPTER 5

ORAL BIOAVAILABILITY STUDIES OF THE LACTONE AND CARBOXYLATE FORMS OF AR-67

5-1. Introduction

AR-67 is a third generation camptothecin analogue with potent antitumor activity and enhanced lactone stability (18) that is currently undergoing early phase clinical trials in patients with solid tumors (150). Camptothecins are a class of anticancer molecules that elicit their effect through interactions with topoisomerase I. This nuclear enzyme is typically expressed in all cells and performs its function during cell replication. As a direct consequence of this requisite interaction with topoisomerase I during cell replication, it has become apparent that camptothecin dosing schedules should be protracted. This ensures that drug exposure is achieved and potential for antitumor activity increased when different fractions of the tumor cell population enter the replication stage of their cell-cycle. Currently, there are only two camptothecin analogues, topotecan (Hycamtin®) and irinotecan (Camptosar®), available on the market while several others, including AR-67, are in various stages of drug development (21, 150, 170). In both rodents and humans, low dose protracted treatment by the intravenous route with topotecan or irinotecan was shown to be better tolerated and to be more efficacious than shorter and more intense therapies (171-173).

Although the intravenous route is the typical administration modality, an oral formulation may be more desirable for protracted dosing regimens. In addition, an oral formulation could reduce treatment cost while allowing for greater dosing flexibility (174). Oral topotecan has been shown to be as effective as intravenous docetaxel in patients with unresectable non-small-cell lung cancer (NSCLC) (175) and ovarian cancers (176). Similarly, in patients with solid tumors, oral irinotecan was well tolerated and was advantageous in terms of enhanced exposure to the active agent, SN-38, due to carboxylesterase-mediated presystemic conversion from irinotecan (177-179).

A common feature of all camptothecins is the pH dependent reversible hydrolysis of the lipophilic lactone to the hydrophilic carboxylate (18, 180). Following oral administration, the relative concentration of the lactone and carboxylate in the

gastrointestinal tract is likely to be influenced by the local pH. Gastrointestinal regions with acidic pH are expected to maintain the lactone whereas those with physiological or alkaline pH should promote carboxylate formation. Since dissolution in the gastrointestinal tract influences absorption of orally administered drugs, differences in the aqueous solubility of the lactone and the carboxylate may in turn give rise to differences in oral bioavailability. Overall, the poor aqueous solubility of the AR-67 lactone (0.11 μg/mL) (181) is expected to limit its oral bioavailability but improvement of AR-67 lactone solubility, through the use of excipients, could potentially overcome this limitation. A sulfobutylether-β-cyclodextrin based formulation of AR-67 has previously been developed and sustains a supersaturated lactone solution (1-2 mg/mL) (181) and allows the oral and intravenous administration of AR-67. On the other hand, the carboxylate form may provide enhanced bioavailability due to its higher aqueous solubility and may therefore serve as an alternative formulation to the lactone, provided that it yields adequate bioavailability in the systemic circulation.

In addition to dissolution, efflux by ABC transporter proteins, such as P-gp and/or BCRP/Bcrp, located on the luminal side of gastrointestinal tract is known to limit oral bioavailability of camptothecins (102, 182). Published data on camptothecins (102, 124) and previous in vitro studies in this laboratory indicated that AR-67 is a substrate of P-gp and BCRP (183). This interaction is likely to affect the oral bioavailability of AR-67. Therefore, the objectives of the current study were 1) to estimate the oral bioavailability of AR-67 lactone and carboxylate and 2) to test the hypothesis that the efflux transporters, P-gp and Bcrp, limit the oral bioavailability of AR-67 by using pharmacological efflux transport inhibitors.

5-2. Methods

5-2.1. Chemicals

Ammonium acetate (Mallinckrodt Baker, Phillipsburg, NJ), HPLC grade acetonitrile and methanol (Burdick and Jackson, Muskegon, MI) were purchased from VWR (West Chester, PA). Alamethicin, uridine 5'-diphosphoglucuronic acid (UDPGA), NADP+, glucose-6-phosphate, glucose-6-phosphate dehydrogenase were from BD Biosciences (Woburn, MA). Siliconized pipette tips were from Cole-Parmer (Vernon Hills, IL). Amber microcentrifuge tubes were from Crystalgen Inc. (Plainview, NY). Transparent siliconized microcentrifuge tubes, dimethylsulfoxide (≥ 99.7 % DMSO) and glacial acetic acid were from Fisher Scientific (Fair Lawn, NJ). Magnesium- and calcium-free Dulbecco's phosphate buffered saline (PBS) was from Gibco Invitrogen (Carlsbad, CA). 5% Dextrose in water (D5W) was from Baxter Healthcare Corporation (Deerfiled, IL). Tween-80 and PEG-300 were from Sigma-Aldrich (St. Louis, MO). Sulfobutylether-β-cyclodextrin (SBE-β-CD, Captisol®) was from CyDex, Inc. (Overland Park, KS). AR-67 was provided from Novartis (East Hanover, NJ). AR-67 lactone or carboxylate solutions (1-2 mg/mL) were prepared in SBE-β-CD either by reconstituting a lyophilized SBE-β-CD based formulation of AR-67 lactone (181) or carboxylate or from AR-67 powder. GF120918 (Elacridar) was a gift from GlaxoSmithKline (Research Triangle Park, NC) and was solubilized in 10% Tween-80 and 40% PEG-300 in distilled water (184). Zosuquidar was synthesized at the University of Kentucky following published procedures (185) and was dissolved with slight modification of the published procedure in an aqueous solution of 20% SBE-β-CD (186).

5-2.2. Pharmacokinetic studies

Female Harlan Sprague-Dawley rats weighing between 220-300 *g* were used for these studies (n=3-6). To assess the dose dependence of oral bioavailability, animals received 2.5, 5, 10, 15 or 20 mg/kg of either AR-67 lactone or carboxylate by oral gavage. For estimation of absolute bioavailability, separate groups of animals (n=3-6) were also treated with 2.5 mg/kg doses of either the lactone or carboxylate intravenously. For all the studies the SBE-β-CD formulation of AR-67 was used (181). To determine the

effect of P-gp inhibition on oral bioavailability, rats were orally pretreated with zosuquidar (20 mg/kg, 7.5 mL/kg (dosing volume)) 5 minutes before the oral or IV administration of AR-67 lactone (2.5 mg/kg, 2.5 mL/kg). To measure the effect of dual inhibition of P-gp and Bcrp on oral bioavailability, GF120918 (0.25, 1, 2.5 or 20 mg/kg , 7.5 mL/kg dosing volume) prepared as described in the methods section were separately administered by oral gavage 5 minutes before the oral administration of AR-67 lactone or carboxylate (2.5 mg/kg, 2.5 mL/kg). Different doses of GF120918 were used to select a dose of GF120918 that provides maximal efflux inhibition. This dose of GF120918 was then used to measure its effect on systemic clearance following intravenous administration of either AR-67 lactone or carboxylate. In order to assess contribution of possible factors affecting oral bioavailability of AR-67, we estimated the hepatic extraction ratio (E_H) and the theoretical maximum oral bioavailability (F) as shown below.

$$E_H = \frac{Cl_{iv}}{Q_H * \left(\frac{C_B}{C_P}\right)}$$ Equation 5.1 (187)

$$F = 1 - E_H$$ Equation 5.2 (187)

Cl_{iv} is the plasma clearance of AR-67 lactone , which is 1800 ml/hr/kg (188). Q_H is hepatic blood flow, which is 13.8 ml/min for a 250 g rat (189). C_B/C_P is the blood to plasma concentration ratio of AR-67.

Following AR-67 administration, blood (100 µL) was collected from the saphenous vein at 5-15 min, 30 min and at 1, 2, 4, 6, 8 and 12 hr with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was separated by centrifugation of blood at 8,500 *g* and extracted 1:4 (v:v) with cold (-80°C) methanol (145). Samples were stored at -80°C until analysis by HPLC.

A separate group of animals (n≤3) receiving an oral dose of 2.5 mg/kg AR-67 lactone were euthanized at designated time points to examine the presence of metabolites and unabsorbed drug in the gastrointestinal tract. Different segments of the gastrointestinal tract were excised after ligations were made with sutures between the lower esophageal and pyloric sphincters (for the stomach), at 20 cm length from the

stomach (for the duodenum) and at 20 cm length proximal to the ileocecal junction (for the ileum). The region between the duodenum and the ileum was cut in half and was designated as the proximal and distal jejunum. The region beyond the ileo-cecal junction was designated the colon. The stomach and intestinal contents were gently squeezed out and washed off with 10-20 mL of 20% human plasma in water and transferred into 50 mL screw cap conical tubes. The mixture was vortexed for 30 seconds and centrifuged for 10 minutes at 1200 rpm. The supernatant was extracted 1:4 (v:v) with -80°C methanol. Samples were kept at -80°C until HPLC analysis.

5-2.3. AR-67 liver and intestinal metabolism

Microsome preparation

Intestinal and liver microsomes from non-fasting Sprague Dawley rats were prepared as described in the literature with minor modifications (190). Briefly, rats were euthanized with CO_2 and their left ventricle was perfused with 30 mL ice-cold saline. The duodenum, jejunum, ileum and the liver were excised. The intestinal segments were cut open longitudinally and washed with ice cold 1.15% KCl with gentle swirling. The mucosal layer was then gently scraped off with glass cover-slips. The liver was cut into small pieces and washed similarly to the intestinal segments. The samples obtained were immediately homogenized in 3 volumes of ice-cold homogenization buffer (50 mM Tris-HCl, 150 mM KCl, 1 mM EDTA, 20% (v/v) glycerol, trypsin and protease inhibitors) with a motor-driven Teflon pestle. The homogenate was centrifuged at 9,000 g for 20 minutes at 4°C. The supernatant was then centrifuged at 105,000 g for 60 min in Beckman L-90 K ultracentrifuge at 4°C. The supernatant was discarded and the pellet (microsome) was suspended in 3 mL of homogenization buffer. Protein contents of the samples were measured using modified Lowry protein assay kit according to the manufacturer's instructions (Pierce, Rockford, IL).

Microsomal incubation

Glucuronidation: Rat liver microsomes (RLM) and rat intestinal microsomes (RIM) reaction mixtures consisted of $MgCl_2$ (8 mM), alamethicin (63.6 μM), microsomal protein (1 mg/mL), and AR-67 (1 μM lactone) in 0.5 M Tris-HCl buffer (pH 7.5) containing 5 % DMSO. Incubation mixtures were prepared on ice, pre-incubated at 37°C for 2 min, and reactions were initiated by the addition of uridine 5'-diphosphoglucuronic acid (UDPGA, final concentration 2 mM).

Oxidation: Oxidative reaction incubations contained $MgCl_2$ (3.3 mM), NADP+ (1.3 mM), glucose-6-phosphate (3.3 mM), AR-67 (1 μM lactone) and microsomal protein (1 mg/mL) in 100 mM potassium phosphate buffer (pH 7.4). Mixtures were prepared on ice, pre-incubated at 37°C for 2 min, and reactions were initiated by the addition of glucose-6-phosphate dehydrogenase (final concentration 0.4 U/mL).

Reaction quenching and workup: RLM and RIM reactions were stopped at 0 and 60 min with methanol as outlined above for sample workup. Methanolic supernatants were analyzed by gradient HPLC methods as described below.

5-2.4. HPLC analyses

Lactone and carboxylate plasma concentrations were simultaneously quantified by HPLC using fluorescence detection at excitation wavelength of 380 nm and emission wavelength of 560 nm based on a previously published method for analysis of AR-67 in mouse plasma (145). A partial assay validation using rat plasma as the matrix was carried out. The assay was linear in the range of 2.5-250 ng/mL for the carboxylate and 5-300 ng/mL for the lactone. Accuracy for both analytes was within 15% of expected values at the low end of the calibration curve (7 ng/mL) and within 10% of expected values at middle (150 ng/mL) and high (250 ng/mL) concentrations. Assay precision (% relative standard deviation) was <6% across the calibration range. Both analytes were stable at 4°C for 6 hr in the methanol extract when mixed with mobile phase buffer. This ensured stability during automated HPLC analyses. Extracted samples were stable at -80°C for 14 days. The lower limit of quantitation was 2.5 ng/mL for carboxylate and 5.0 ng/mL for lactone. Sample analysis was completed within 14 days after collection.

A separate HPLC assay method using a mobile phase gradient was used for separation of AR-67 metabolites. Given the qualitatative purposes of the metabolite analyses and the lack of metabolite reference standards, a formal validation protocol was not performed. The mobile phase flow rate was 1 mL/min and was comprised by varying ratios of Solvent A (0.15 M ammonium acetate buffer containing 10 mM TBAP adjusted to pH 6.5) and Solvent B (acetonitrile). The initial A:B solvent ratio was set at 83:17 and was linearly ramped to 71:29 over the first 10 min. A 10 min linear ramp to 65:35 A:B immediately followed, dropping in a linear fashion to 60:40 A:B over the next 2 min. The ratio was increased to the initial 83:17 solvent ratio over 1 min, then held constant for 5 min.

5-2.5. Pharmacokinetic and statistical analysis

Non-compartmental pharmacokinetic analyses of the lactone and carboxylate plasma data were done with WinNonlin v5.2 (Pharsight, Mountain View, CA). A compartmental model previously developed for the intravenous administration of AR-67 lactone and carboxylate (143) served as the basis for the compartmental model used to estimate pharmacokinetic parameters following oral administration of each AR-67 form. Linear regression of dose-normalized AUC values was performed with GraphPad Prism V5.02 for Windows (GraphPad Software, San Diego, California). Compartmental pharmacokinetic modeling was also performed using algorithms implemented in ADAPT 5. Parameter estimates were obtained by simultaneously fitting the lactone and carboxylate plasma concentrations resulting from the oral and intravenous administration of AR-67. The lactone and carboxylate forms were administered separately by each route. Population pharmacokinetic modeling was performed using the Iterated Two Stage (ITS) algorithm implemented in ADAPT 5 assuming log-normal parameter distribution (191). The model building was performed at several stages with increasing levels of complexity. A reduction of the negative log-likelihood by 3.84 ($p = 0.05$, χ^2 distribution, one degree of freedom) was used to discriminate between models. Statistical analyses on areas under the plasma concentration versus time curve (AUC) were compared with ANOVA followed by Bonferroni two-tailed post-hoc t-test using GraphPad Prism V5.02 for Windows. A p-value of less than 0.05 was considered significant.

5-3. Results

Non-compartmental analyses

Female Sprague Dawley rats were dosed orally with AR-67 lactone or AR-67 carboxylate formulated in SBE-β-CD. Plasma samples were analyzed for AR-67 lactone and carboxylate. In order to assess if there were a dose-dependent increase in oral bioavailability due to saturation of efflux transporters and/or metabolizing enzymes, increasing doses of the lactone or carboxylate were administered. Pharmacokinetic parameters were assessed following administration of 2.5, 5, 10, 15, and 20 mg/kg doses to groups of 3-6 animals. Multiple plasma samples were collected from each animal over 12 hours. Plasma AR-67 concentrations were primarily in the lactone form, irrespective of the AR-67 form being administered, and lactone AUC ranged between 80 and 95% of the total AUC (i.e., lactone + carboxylate). Maximum plasma concentration (C_{max}) was achieved within 30 minutes at most dosage levels. Time to reach maximum concentration in the plasma (T_{max}) did not differ between the lactone and carboxylate dosing. The results of oral bioavailability studies at different dose levels analyzed by non-compartmental methods are summarized in Table 5-1. The plasma concentrations and AUCs of the predominant lactone form were dose normalized and are presented in Figure 5-1. For clarity the minor carboxylate AUC is not shown but it follows a similar pattern as the lactone. A trend towards an increase in dose normalized AUC with an increase in dose was observed following lactone administration and suggests some degree of saturation of efflux transporters and/or metabolizing enzymes at the high dose levels (Figure 5-1A-B). On the other hand, oral carboxylate administration did not show such a trend (Figure 5-1C-D). Linear regression of the dosed normalized AUC values obtained following the administration of multiple lactone dosage levels (Figure 5-1B) demonstrates that the slope of the fitted line deviated significantly from zero ($p=0.0001$). In contrast, the dose normalized lactone AUCs obtained following increasing doses of carboxylate (Figure 5-1D) were variable and no significant deviation from zero could be ascertained.

Table 5-1. Pharmacokinetic parameters obtained from noncompartmental analysis of plasma data in animals that received oral doses of the lactone or carboxylate.

Compound administered	Dose, mg/kg	Tmax, min#	Cmax, ng/mL# (SD)	$AUC_{0\text{-}inf}$, ng*hr/mL (SD)		% Lactone AUC
				Lactone	Carboxylate	
Lactone	2.5	30-60	19.3 (11.3)	61.8 (22.3)	3.2 (2.5)	95.5 (2.5)
	5	30	79.0 (32.0)	145.4 (32.1)	34.8 (7.8)	81.7 (1.1)
	10	30	120.9 (12.1)	370.2 (40.2)	71.5 (15.5)	81.1 (2.9)
	15	30	263.8 (53.5)	680.8 (129.2)	216.6 (32.7)	82.3 (14.6)
	20	30-120	272.0 (24.4)	966.8 (259.1)	232.2 (38.1)	80.4 (1.4)
Carboxylate	2.5	30	25.2 (9.3)	72.7 (21.3)	13.4 (7.7)	88.1 (6.2)
	5	30-60	58.6 (25.4)	152.9 (30.1)	34.6 (15.2)	86.0 (3.4)
	10	30	116.3 (20.2)	279.5 (35.5)	60.8 (6.3)	82.2 (2.9)
	15	30	380.99 (184.5)	956.7 (248.8)	233.8 (69.1)	80.0 (1.1)
	20	60	121.6 (83.4)	752.5(490.8)	67.1 (47.9)	91.5 (4.5)

[#]denotes value obtained for the predominant lactone form.

SD, standard deviation (n=3)

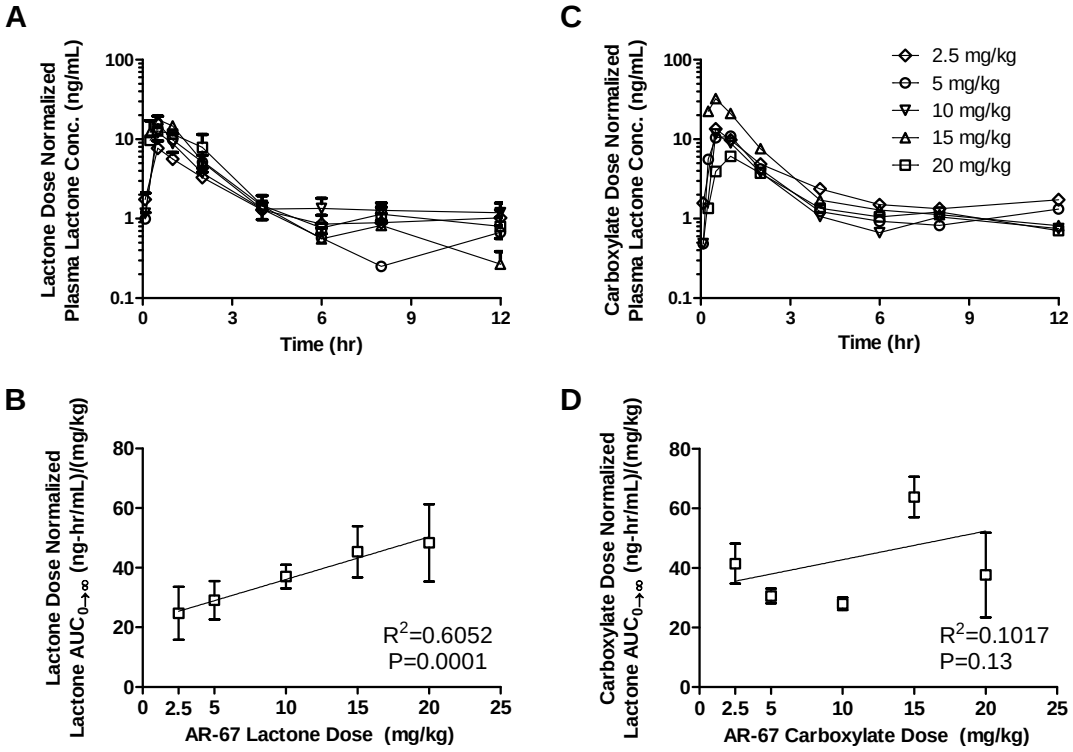


Figure 5-1. Assessment of dose-dependent increase in plasma concentration or AUC following oral administration of increasing doses of AR-67 lactone or carboxylate. Panels A & B represent plasma concentrations and AUCs normalized by dose of lactone while C & D represent similar data for the carboxylate dose. P-values indicate the significance of the slope's deviation from zero as obtained by linear regression (n=3).

Compartmental analyses

The positive slope of the line fitting the dose normalized AUCs obtained from increasing lactone AR-67 dosages suggested that increasing drug concentrations in the gastrointestinal tract saturate one or more of the processes that limit oral bioavailability. Thus, we sought to develop a pharmacokinetic model that would allow us to examine the effect of dose on oral bioavailability and clearance. The pharmacokinetic model would also allow the use of covariate models on the parameter estimates and a more robust statistical analysis when comparing the pharmacokinetic parameters obtained following pharmacologic inhibition of efflux transporters. First order absorption models were initially constructed to simultaneously model oral and intravenous data but did not adequately fit the data. Moreover, when the oral carboxylate dose input was into the carboxylate central compartment, the model fits were not satisfactory. Visual inspection of the lactone and carboxylate plasma concentrations over time suggested that AR-67 was mainly in the lactone form, irrespective of which form was orally administered. Therefore, based on this observation, a simplifying assumption was made to allow all oral inputs to be applied into the central lactone compartment (Figure 5-2). First order absorption models did not provide adequate fits. Therefore, a flexible input function was used instead to accommodate the apparently complex absorption processes. The data were modeled by incorporating an inverse Gaussian input (192, 193) into a four compartment disposition model which was previously used to model intravenously administered AR-67 lactone and carboxylate (143). Following extravascular administration, the model allows the estimation of absorption kinetic parameters for drugs with complex absorption behavior by decomposing the plasma concentration-time curve into input (absorption) and output (disposition) processes (192, 193). The inverse Gaussian input function IG(t) is described by:

$$IG(t) = Dose * F * \sqrt{\frac{MIT}{2\pi CVI^2 * t^3}} \exp[-\frac{(t - MIT)^2}{2CVI^2 * MIT * t}],$$

where F is oral bioavailability, MIT is the mean input time and CVI^2 is the variance or the relative dispersion of absorption times. Population data modeling was performed using the Iterated Two Stage (ITS) algorithm implemented in ADAPT 5 assuming a log-

normal parameter distribution (191). To estimate oral bioavailability of AR-67 at different dose levels, data from oral and intravenous inputs were simultaneously modeled using dose level and form administered orally, i.e. lactone or carboxylate, as covariates on F, MIT and CVI^2. To model the effect of efflux transporter inhibition with GF120918 on oral bioavailability and clearance, a similar modeling approach as above was followed. Oral and intravenous AR-67 data obtained from animals that were pretreated with either the control vehicle or the efflux inhibitor GF120918 were modeled using the presence of GF120918 and the form of AR-67 administered orally as covariates on clearance, F, MIT and CVI^2. The performance of alternative methods was judged by convergence of parameters, reduction in the negative log-likelihood, improvement in the error estimates of parameters and diagnostic plots. A reduction of the negative log-likelihood by 3.84 ($p = 0.05$, χ^2 distribution, one degree of freedom) was used as a criterion to include a covariate in the model.

Oral data at different dose levels (from this study) and intravenous data (from this and our previous study (143) were simultaneously analyzed using the Iterated Two Stage population algorithm in ADAPT 5 (191). This algorithm allows modeling of sparse and noisy population data (191) and was found appropriate for modeling our oral and intravenous data.

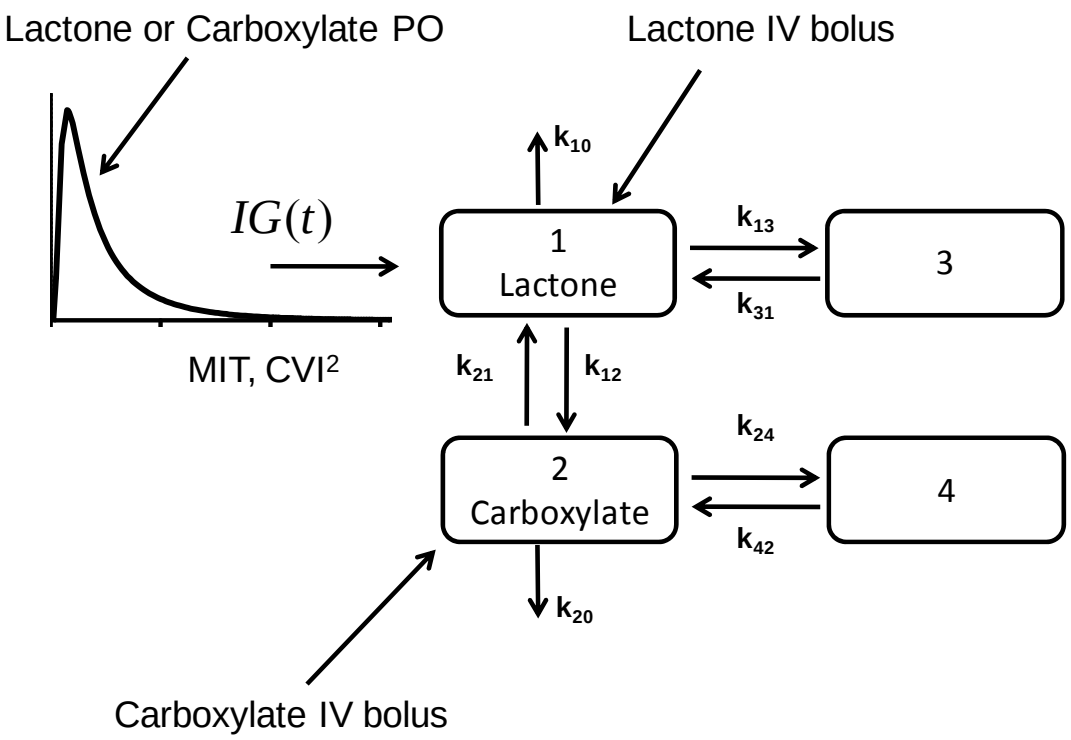


Figure 5-2. Schematic representation of the inverse Gaussian oral input of AR-67 linked to a four compartment disposition model with elimination occurring from the central lactone and carboxylate compartments.

We tested several models using dose level and AR-67 form administered, i.e., lactone or carboxylate, as covariates. The plasma concentrations of AR-67 lactone and carboxylate with all oral doses of AR-67 were much less than the concentrations from the intravenous dose of AR-67 lactone (2.5mg/kg) and carboxylate (2.5mg/kg) and are not likely to lead to saturation of clearance processes. Therefore, the clearances of AR-67 lactone and carboxylate were assumed to be the same for all oral doses of AR-67. Of the models tested, the model that allowed F, MIT and CVI^2 to be estimated uniquely at each dose level of lactone or carboxylate performed best based on convergence of iterations (finding of global minimum), decrease in negative log-likelihood and diagnostic plots. Plots of experimental and model fitted AR-67 plasma concentration as a function of time are shown in Figure 5-3 (lactone dose) and Figure 5-4 (carboxylate dose) while diagnostic plots are presented in Figure 5-5. As depicted in the figures, the model adequately predicted the observed data. Pharmacokinetic parameters are presented in

Table 5-2. Following the oral administration of lactone, bioavailability ranged between 5.8 and 10.4%. The highest bioavailability obtained was 10.4% at 15 mg/kg dose. The mean input time (MIT) ranged between 66.6 and 259.0 minutes while the dimensionless shape factor CVI^2 ranged between 1.9 and 8.8. Following carboxylate administration bioavailability ranged between 5.5 and 16.6%, MIT ranged between 141 and 200 minutes and CVI^2 ranged between 1.8 and 5.3. The 15 mg/kg carboxylate dose showed the highest bioavailability (F=16.6%).

Based on literature data on other camptothecin analogs (102, 124) and on oral bioavailability estimates of AR-67 of the current study, which increased with increasing dose, we speculated that intestinal efflux transporters, (i.e., P-gp and/or Bcrp may limit oral bioavailability of AR-67. In order to assess role of these efflux transporters, we used the selective P-gp inhibitor zosuquidar (194) and the dual P-gp and Bcrp inhibitor GF120918 (109, 195). Intravenous administration of 20 mg/kg zosuquidar was previously shown to effectively inhibit the function of P-gp at the blood-brain barrier as manifested by increased nelfinavir concentrations in the brain (185). Assuming that the same dose would provide much higher local concentrations in the GI and would effectively abolish P-gp function, we pretreated rats orally 5 minutes prior to AR-67 dosing. We observed that pretreatment with zosuquidar resulted in a statistically significant ($p<0.05$) threefold increase in lactone AUC (61.8±22.3 with 5% dextrose in water vs. 183.7±61.3 ng-hr/mL with zosuquidar). In the same animals, the carboxylate AUC increased nine-fold but these results were highly variable and given the sample size, this increase was not statistically significant (3.2±2.5 without vs. 28.0±13.0 ng-hr/mL with zosuquidar) (Figure 5-6A). Zosuquidar slightly increased the lactone AUC of intravenously administered AR-67 (1.2 fold, $p<0.05$) indicating that, at the dose administered, it exerted minimal but statistically significant effect on systemic clearance of AR-67 (Figure 5-6B).

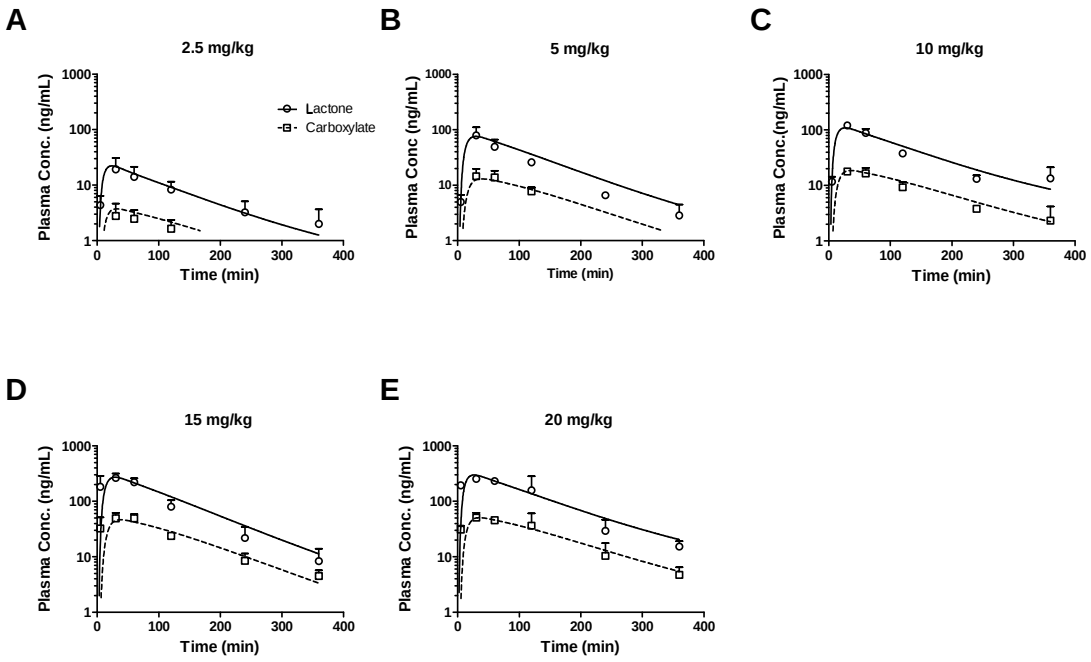


Figure 5-3. Plasma concentration of AR-67 lactone (-○-) and carboxylate (-□-) following oral doses of (A) 2.5, (B) 5, (C) 10, (D) 15 and (E) 20 mg/kg AR-67 lactone. The solid and dashed lines represent simulated population estimated lactone and carboxylate concentrations, respectively, which were generated by simulation using the mean population parameter estimates.

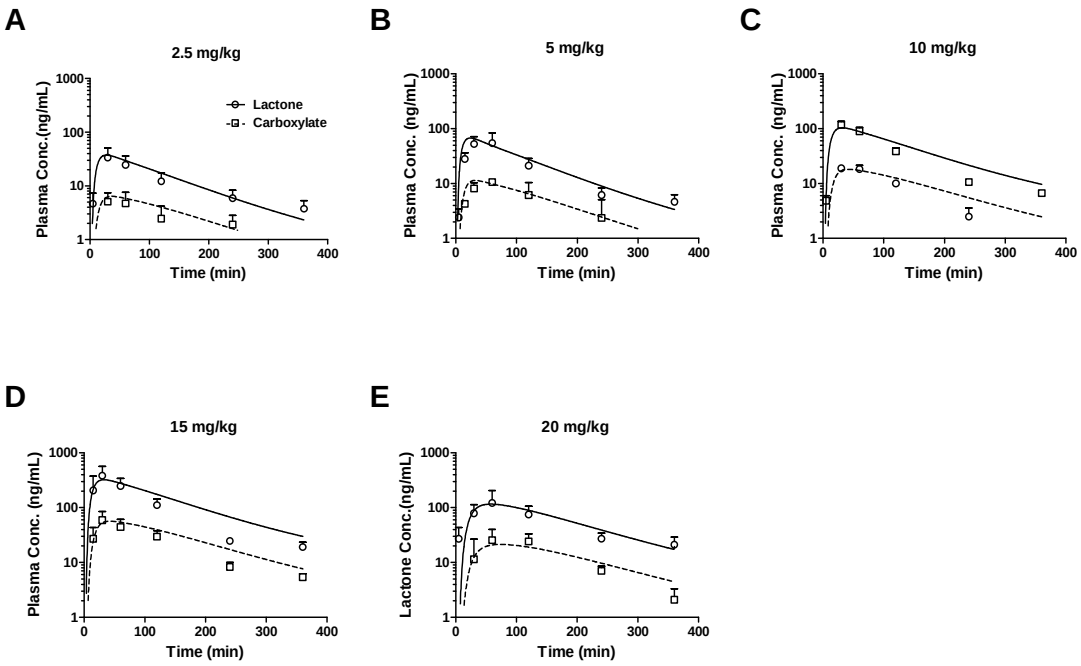


Figure 5-4. Plasma concentration of AR-67 lactone (⊖) and carboxylate (⊟) following oral doses of (A) 2.5, (B) 5, (C) 10, (D) 15 and (E) 20 mg/kg AR-67 carboxylate. The solid and dashed lines are the fitted lactone and carboxylate concentrations, respectively, which were generated by simulation using the mean population parameter estimates.

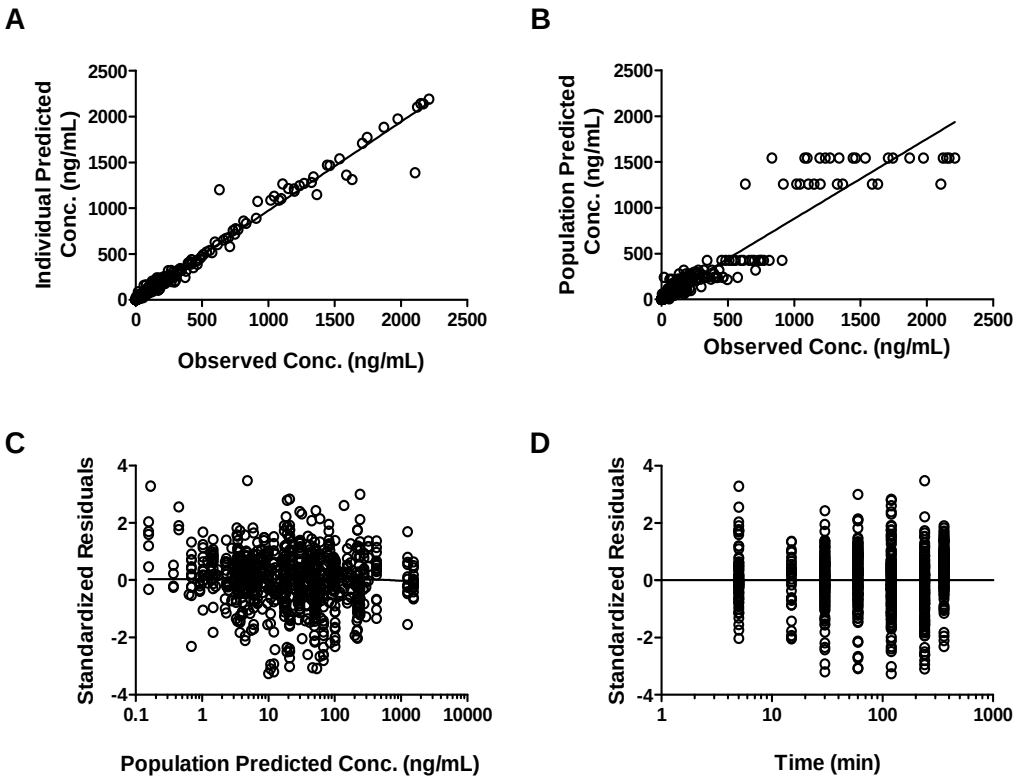


Figure 5-5. Goodness-of-fit and residual plots of the final covariate model. (A) Individual predicted versus observed concentrations, (B) population predicated vs. observed concentrations, (C) standardized residuals versus population predictions, D) standardized residuals vs. time. The solid line is the line of unity.

Table 5-2. Pharmacokinetic parameter estimates obtained by compartmental analysis of plasma data using the inverse Gaussian input.

Parameter	Lactone dose (mg/kg)					Carboxylate dose (mg/kg)					
	2.5	5	10	15	20	2.5	5	10	15	20	% CV
F %	5.8	9.6	7.9	10.4	9.9	9.5	7.8	8.1	16.6	5.5	20
MIT (min)	111.0	87.0	259.0	66.6	128.0	100.0	141.0	200.0	174.0	153.0	42.5
CVI^2	4.4	2.4	8.8	1.9	4.2	3.1	3.4	5.3	4.4	1.8	53.4
Cl_{Lact} (L/hr/kg)	1.53										54.0
Cl_{Carb} (L/hr/kg)	5.53										10.8
$Cl_{Lact \rightarrow Carb}$ (L/hr/kg)	1.39										12.0
$Cl_{Carb \rightarrow Lact}$ (L/hr/kg)	0.98										19.4

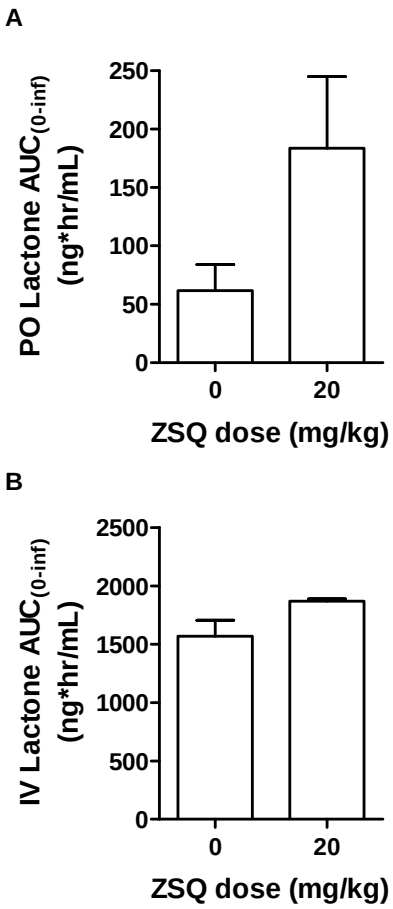


Figure 5-6. The effect of zosuquidar (ZSQ) (20 mg/kg PO) or control (5% dextrose in water) pretreatment on lactone AUCs following the oral (A) and intravenous (B) administration of 2.5 mg/kg AR-67 lactone.

To examine the effect of dual P-gp and Bcrp inhibition, we administered AR-67 lactone or carboxylate following pretreatment with different oral doses of GF120918 (0, 0.25, 1, 2.5 or 20 mg/kg). Due to poor aqueous solubility, GF120918 was formulated in 10% Tween-80 and 40% PEG-300 in distilled water, which allows the oral administration of GF120918 (184). The solution of 10% Tween 80 and 40% PEG-300 served as control for GF120918. As shown in Figure 5-7A, the 2.5 mg/kg dose of GF120918 yielded the highest increase in plasma AUC value. The increase was statistically significant ($p<0.05$) compared to control AUC values but not when compared to 1 mg/kg and 20 mg/kg GF120918 pretreatment doses. Pretreatment with 2.5 mg/kg oral dose of GF120918 five minutes before the oral administration of AR-67 lactone, resulted in a 5.5 fold increase in

lactone AUC (ng*hr/mL) (141.5±57.1 (Mean±SD) with control vs. 779.6±163.3 (Mean±SD) with GF120918) and about eleven fold increase in carboxylate AUC (13.2±5.6 (Mean±SD) with control vs. 142.4 ± 29.6 (Mean±SD) with GF120918). The increases in lactone and carboxylate AUCs were statistically significant ($p<0.05$). Similarly, pretreatment with 2.5 mg/kg oral dose of GF120918 five minutes before the oral administration of AR-67 carboxylate increased lactone AUC 4.2 fold (108.9±21.9 (Mean ± SD) with control vs. 457.7±96.1 (Mean±SD) with GF120918) and carboxylate AUC 5.2 fold (17.6±10.7 (Mean±SD) with control vs. 92.7±15.6 (Mean±SD). The increases in lactone and carboxylate AUCs were statistically significant ($p<0.05$). As was the case in the studies without an inhibitor (Figure 5-3 and Figure 5-4), following pretreatment with GF120918, the majority of AR-67 was in the form of the lactone, irrespective of which form of AR-67 was administered (percent lactone AUC 83-91%).

To assess if the increase in lactone and carboxylate AUCs due to pretreatment with GF120918 was also related to decrease in clearance, animals were pretreated with 2.5 mg/kg oral dose of GF120918 prior to the administration of 2.5 mg/kg AR-67 lactone or carboxylate intravenously. In agreement with our previous studies (143), GF120918 significantly increased lactone AUC (1.7 fold, $p<0.05$) and but not carboxylate AUCs (1.4 fold) following intravenous administration of AR-67 lactone (Figure 5-7B). Similarly, GF120918 significantly increased lactone AUC (≈3 fold, $p<0.05$) but not carboxylate AUC following intravenous administration of AR-67 carboxylate.

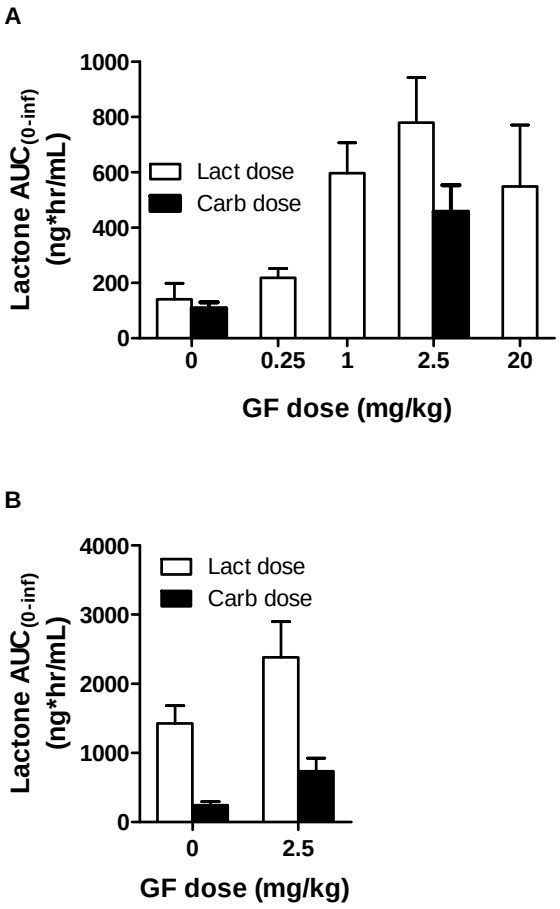


Figure 5-7. The effect of pretreatment with GF120918 (GF) or control vehicle on lactone AUCs following the oral (A) or intravenous (B) administration of 2.5 mg/kg AR-67. Unfilled bars and filled bars indicate lactone and carboxylate, respectively, as the administered forms.

Lactone and carboxylate plasma data from GF120918 inhibition studies were also fitted with the models outlined above. Models were constructed by including presence of GF120918 as a covariate on clearance, bioavailability, MIT and/or CVI^2. Model selection was based on convergence of iterations, decrease in negative log-likelihood and diagnostic plots. Incorporating GF120918 as a covariate on bioavailability and lactone clearance resulted in better model fits. Experimental data and simulated concentrations are shown in Figure 5-8 and pharmacokinetic parameters are presented in Table 5-3. The model was able to adequately fit the plasma concentrations of the lactone and carboxylate. The modeling results show that pretreatment with the dual P-gp and Bcrp

inhibitor GF120918 led to a threefold increase in bioavailability after taking the decrease in clearance into consideration (9.4±4.1% without GF120198 vs. 29.8±13.0% with GF120918 for the lactone dose and 9.1±3.9% without GF120198 vs. 27.4±11.9% with GF120918 for the carboxylate dose).

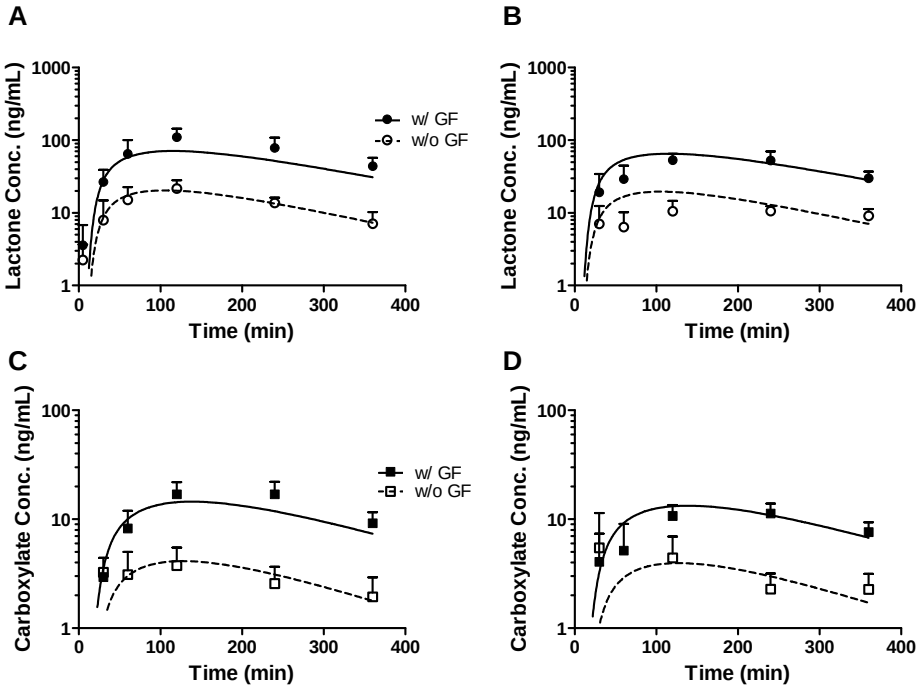


Figure 5-8. Plasma concentration of AR-67 lactone (A, B) or carboxylate (C, D) in the presence or absence of GF120918 (GF). (A) and (C) are the lactone and carboxylate concentrations, respectively, following lactone administration while (B) and (D) are the lactone and carboxylate concentrations, respectively, following carboxylate administration. The solid and dashed lines represent simulated concentrations obtained using the point estimates of the population pharmacokinetic parameters in the presence or absence of GF120918, respectively.

Table 5-3. AR-67 pharmacokinetic parameter estimates in rats orally pretreated with vehicle or 2.5 mg/kg GF120918 (GF) before the oral or intravenous administration of 2.5 mg/kg AR-67 lactone or carboxylate.

Parameter (Units)	Without GF	With GF
	Mean (SD)	Mean (SD)
$Cl_{Lactone}$ (L/hr*kg)[#]	1.23 (0.93)	0.82 (0.50)
$Cl_{Carboxylate}$ (L/hr*kg)	4.60 (3.15)	4.60 (3.15)
$Cl_{Lact \rightarrow Carb}$ (L/hr*kg)	1.41 (0.53)	1.41 (0.53)
$Cl_{Carb \rightarrow Lact}$ (L/hr*kg)	1.87 (1.33)	1.87 (1.33)
$F_{Lact\ dose}$ (%)[#]	9.4% (4.1)	29.8% (13.0)
$F_{Carb\ dose}$ (%)[#]	9.1% (3.9)	27.4% (11.9)
MIT (min)	152.0 (50.7)	152.0 (50.7)
CVI^2	1.2 (0.7)	1.2 (0.7)

[#]Presence of inhibitor was used as a covariate on these parameters.

Pretreatment of animals with the vehicle used to solubilize GF129018 (10% Tween-80 and 40% PEG-300 in distilled water) resulted in higher plasma AUC compared to animals pretreated with D5W (mean lactone AUC (ng*hr/mL) 141.5 with vehicle vs. 61.8 with D5W) indicating that excipients in the vehicle could also increase oral bioavailability. However, this excipient factor was taken into consideration by using the vehicle as a control when analyzing the GF120918 results.

Using equations 5.1 and 5.2, we calculated the hepatic extraction ratio (E_H) and the theoretical maximum oral bioavailability of AR-67 in order to assess the contribution of factors that would limit oral bioavailability. The hepatic extraction ratio (E_H) and bioavailability (F) were 0.54 and 0.46 respectively. These values are based on the minimum value of blood to plasma ratio of AR-67 (C_B/C_P=1), based on literature data that show AR-67 partitions into red blood cells (11). As was presented earlier, efflux transporter inhibition increased oral bioavailability to about 30%. Since the theoretical minimum bioavailability is 46%, we considered limited gastrointestinal solubility and/or metabolism as additional factors that would limit the oral bioavailability of AR-67.

In order examine the fate of the drug in the gastrointestinal tract and the presence of metabolites we dosed rats orally with 2.5 mg/kg AR-67 lactone. The % of AR-67 dose recovered in the washing fluid (20% human plasma) from contents ofthe stomach, small

intestine, colon as well as the cumulative % remaining in the GI tract is presented in Figure 5-9. The estimated half-life in the stomach was approximately 4.7 hours while in the small intestine the estimated half-life was approximately 1 hr. The total recovery at the initial time points was approximately 50% which suggests that the extraction efficiency of the washing buffer was limited and these values represent a potential 2-fold underestimation of the actual amount remaining in the gastrointestinal tract. Thus, it is possible that 5-10% of the dose still remained in the stomach at the 12 hr time point, while ~20-40% of the dose could be present in the GI tract between 6-12 hr after oral dosing.

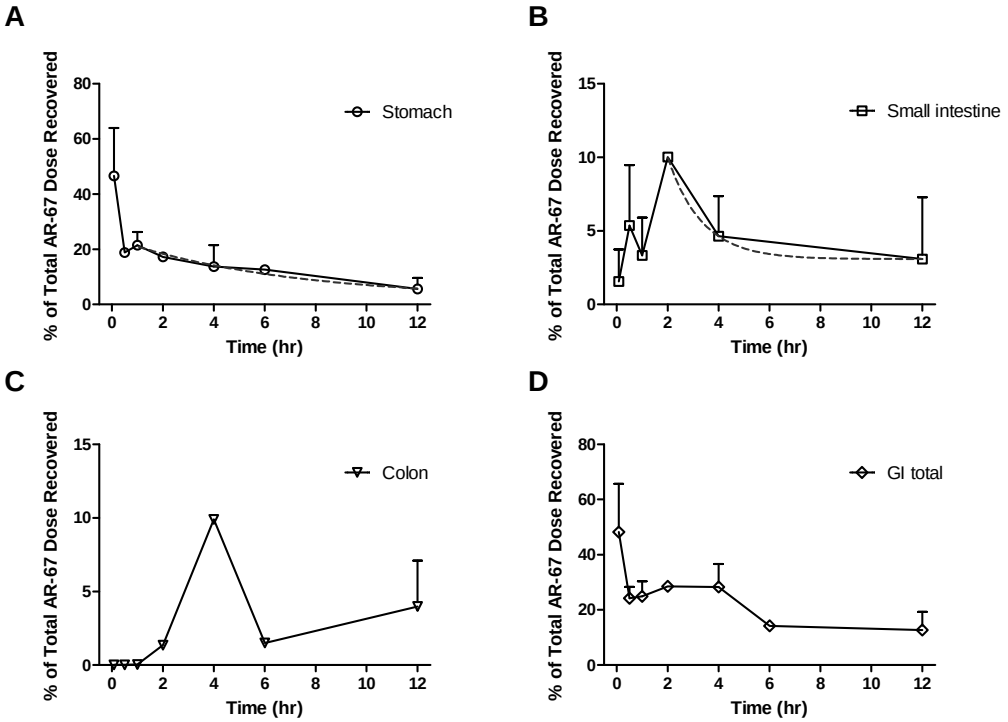


Figure 5-9. Total AR-67 recovered, as % of orally administered dose, from contents of (A) stomach, (B) small intestine, (C) colon and (D) the total dose recovered in the gastrointestinal tract (GI) following an oral dose of 2.5 mg/kg AR-67 lactone. Dotted lines represent the data fit with a monexponential decay model to estimate the half-life in the stomach and small intestine. (n=2-3 rats per time point).

In addition to assaying for AR-67, we also analyzed the gastrointestinal contents for the presence of oxidative and glucuronidation metabolites. To determine metabolite

formation we prepared rat liver and rat intestinal microsomes and demonstrated that they were functional by their capacity to degrade irinotecan and SN-38, which are known substrates of phase-I (i.e., CYP450s) and phase-II (i.e., UDP glucuronosyltransferases) metabolizing enzymes, respectively (data not shown). Samples were analyzed by gradient HPLC with fluorescence detection and early eluting peaks were considered metabolites given that their biotransformation would have rendered them relatively more hydrophilic than AR-67. Following AR-67 incubation with intestinal microsomes there was no evidence of significant oxidative or glucuronidation metabolite formation. However, following incubation of AR-67 lactone in rat liver microsomes under conditions that would promote glucuronidation revealed that AR-67 is glucuronidated and two additional hydrophilic peaks appeared in the chromatogram (Figure 5-10), which were considered as the carboxylate-glucuronide and lactone-glucuronide. The structures of these metabolites have not yet been characterized. In contrast, several additional peaks appeared in the chromatogram (Figure 5-10) following incubation with rat liver microsomes under conditions that would promote oxidative metabolism.

Figure 5-10 also shows a comparison of metabolites obtained in a sample from the jejunum at 2 h. These results indicate the presence of oxidative, glucuronide and other unidentified metabolites in intestinal contents, which could have arisen from gastrointestinal and liver metabolism.

Lack of metabolite reference standards did not allow us to make quantitative assessments of the metabolite concentrations but qualitative assessment based on relative peak heights demonstrated that although present, the metabolites were of low abundance in the GI contents as compared to the parent compound. As illustrated in Figure 5-11, both oxidative and glucuronide metabolites were observed in all segments considered.

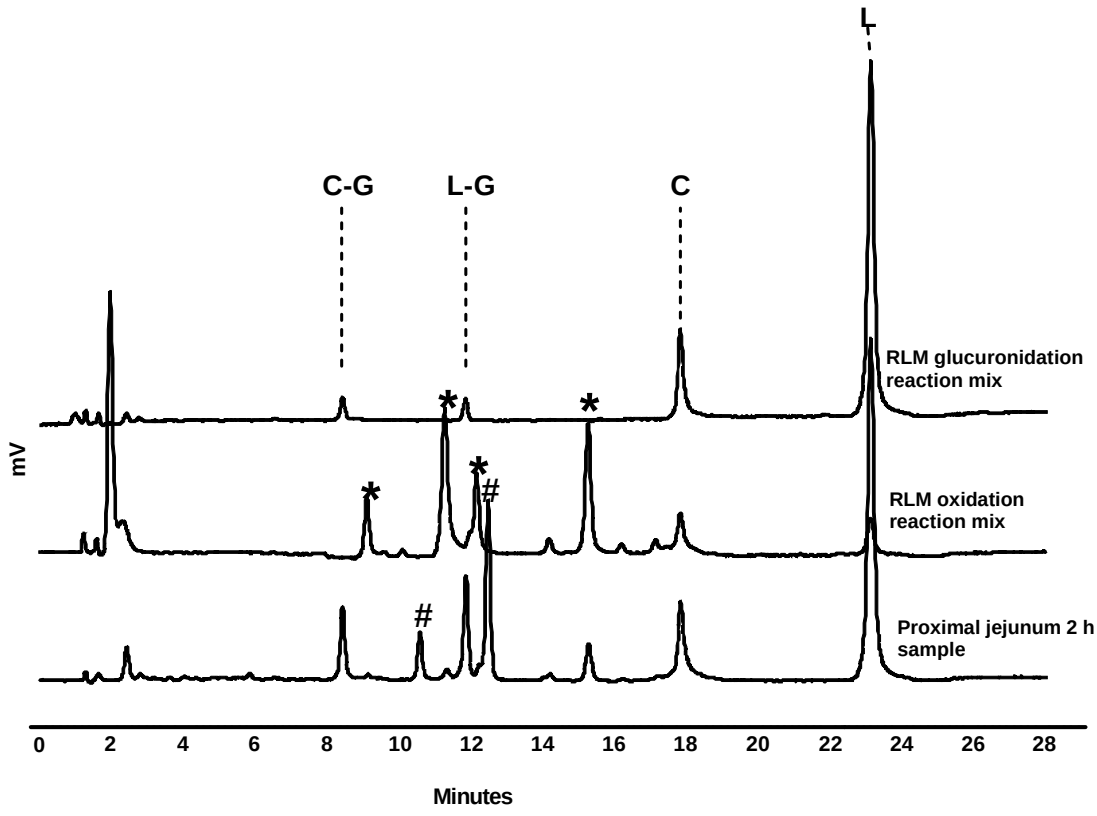


Figure 5-10. AR-67 and its metabolites in rat liver microsomes (RLM) and jejunum samples. Top and middle chromatograms show the presence of glucuronide and oxidative metabolites, respectively. Samples in the jejunum (bottom) indicate the presence of glucuronide, oxidative and other unidentified metabolites. C-G:-carboxylate glucuronide; L-G:-lactone glucuronide; C:-carboxylate; L:-lactone; *oxidative metabolites; # unidentified metabolites.

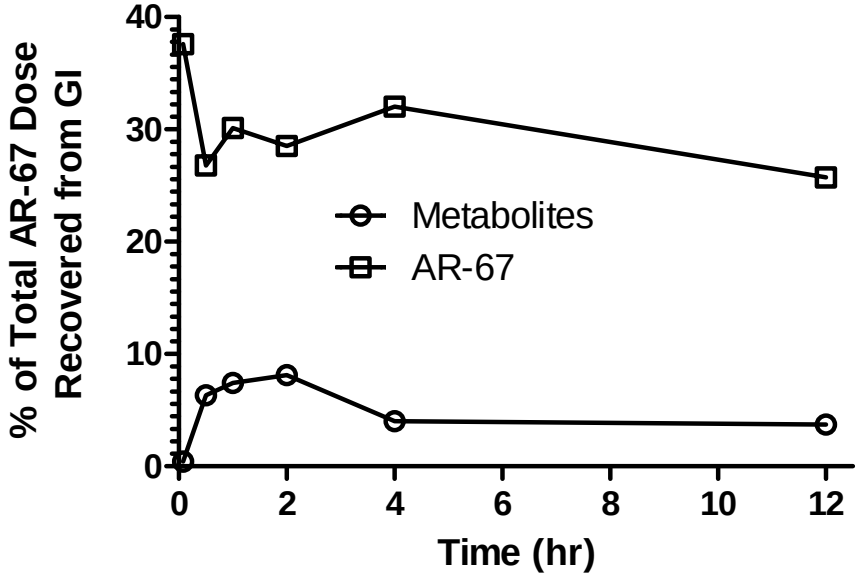


Figure 5-11. Amount of metabolites (-○-) and parent AR-67 (-□-) recovered (in nanogram (ng) equivalents[§]) from the gastrointestinal tract after an oral dose of 2.5 mg/kg AR-67 lactone. [§] Nanogram equivalents were calculated from the slopes and intercepts of AR-67 lactone and carboxylate standard curve and the volume of washing fluid used to empty the contents. Each time point in the graph above represents data from one animal.

5-4. Discussion

In this in vivo study the bioavailability of AR-67 following increasing doses of the lactone or carboxylate was compared. Furthermore, the effect of ABC efflux transporters P-gp and Bcrp on oral bioavailability was examined. Bioavailability estimates ranged between 4-17% and did not differ between lactone and carboxylate doses. The lactone form predominated in the plasma following lactone or carboxylate dosing (>83% lactone AUC). In contrast, it was shown in Chapter 4 that 84% of the plasma AUC was due to the lactone following intravenous lactone administration while following intravenous carboxylate administration, the lactone AUC accounted for only 22% (143). Percent lactone AUCs are therefore similar following the oral and intravenous administration of the lactone. A possible explanation for this discrepancy in percent lactone AUC between oral and intravenous carboxylate administration could be that only the lipophilic lactone form was absorbed from the gastrointestinal tract. In a study by Scott et al. (26) less than 1% of the administered dose was absorbed following intraduodenal administration of sodium camptothecin (carboxylate) dissolved in bile suggesting that carboxylate absorption is minimal. The carboxylate form is a substrate of the liver specific organic anion uptake transporters OATP1B1 and OATP1B3 (183). Therefore, the predominance of the lactone in the plasma could be also be related to the selective uptake of the carboxylate into the liver by these uptake transporters. Thus, if carboxylate is absorbed it would have been rapidly cleared by the liver and very little would have been available for conversion to carboxylate. This is consistent with the results in Chapter 4 that indicated that the carboxylate clearance is more than 5-fold higher than the carboxylate to lactone conversion clearance (5.5±0.6 vs. 0.98±0.19 L/hr/kg). Moreover, since the plasma exposure following lactone and carboxylate was practically identical, carboxylate to lactone conversion in the gut and subsequent lactone absorption is the most likely explanation for this observation across several doses, which spanned an order of magnitude (i.e., 2.5 mg/kg – 20 mg/kg).

Limited dissolution of lipophilic drugs in the gastrointestinal tract is known to limit bioavailability of orally administered drugs (196). The aqueous solubility of AR-67 is 0.11 μg/mL at pH 5.2 and ≈18 mg/mL at pH 10.2 (181). At pH 5.2 AR-67 is primarily

in the lactone form whereas at pH 10.2 it is predominantly in the carboxylate form (181). Based on aqueous solubility and membrane permeability alone, AR-67 lactone and carboxylate would belong to two different classes. Under the Biopharmaceutics Drug Disposition Classification (BCS) System, the lactone would be classified as a Class II drug given its low aqueous solubility and high membrane permeability and the carboxylate as a class III drug based on its high aqueous solubility and low membrane permeability (197). This means that under conditions that favor the predominance of the lactone in the gastrointestinal tract, bioavailability would be amenable to improvement by use of formulations that enhance dissolution. In this study, the formulation of Xiang & Anderson (181) that provides a supersaturated lactone solution was employed. While this formulation was able to maintain supersaturation in vitro, it may not have done so in vivo. This formulation is made through a pH regulated chemical conversion of the carboxylate in the presence of a sulfobutylether-β-cyclodextrin (SBE-β-CD). AR-67 lactone forms a predominantly 1:1 complex with SBE-β-CD, which involves inclusion of 7-t-butyldimethylsilyl residue in the SBE-β-CD core (181). The carboxylate also forms a 1:1 complex, the formation of which is an order of magnitude less than that of the lactone complex (181). It is highly likely that absorption AR-67 from this bulky complex occurs after dissociation form the complex. Therefore, the stability of the complex will have an effect on oral bioavailability. More studies are, however, needed to examine if and how much complexation affects the oral bioavailability of AR-67. If the charged carboxylate predominates in the gastrointestinal tract, formulation factors that enhance conversion to lactone may be relevant in improving bioavailability. Examination of luminal contents showed the presence of a significant portion of AR-67 in the stomach and in the colon. This could have resulted from efflux by ABC transporters and/or from the limited gastrointestinal solubility of AR-67. The contents also showed the presence of oxidized, glucuronidated and other unidentified metabolites. Taken together, these data indicate that ABC efflux limits the bioavailabilty of AR-67. The presence of metabolites and a significant portion of the administered dose unabsorbed in the gastrointestinal tract point that first pass metabolism and limited in vivo solubility could also reduce bioavailability of AR-67. However, CYP450 and UGT inhibition studies using oral grapefruit juice and valproic acid administration did not afford any improvement in oral bioavailability of

AR-67 (pilot studies, data not shown). More work is needed to quantify the contribution of intestinal metabolism on the oral bioavailability of AR-67.

Due to their apical expression in the lumen of the gastrointestinal tract, ABC efflux transporters P-gp and BCRP/Bcrp limit oral bioavailability of camptothecin analogs and their inhibition has been shown to increase oral bioavailability (102, 182). The threefold increase in oral bioavailability of AR-67 above the control vehicle with GF120918 demonstrates the involvement of efflux transporters P-gp and Bcrp in limiting the oral bioavailability of AR-67. Since we have previously demonstrated the lactone to be a substrate of P-gp and BCRP in vitro (183), the increase in bioavailability upon lactone administration in the presence of inhibitors is likely to be due to inhibition of P-gp and BCRP. On the other hand, the increase in bioavailability in the presence of GF120918 following carboxylate administration could have resulted from inhibition of lactone efflux and/or carboxylate efflux (provided the carboxylate is substrate of efflux transporters). Whether or not the carboxylate is also a substrate of P-gp and/or Bcrp has not yet been established and more work needs to be done in this regard.

Poor gut solubility might play a role and could magnify the effect of efflux transporter(s) in that enterocyte concentrations coming from the gut lumen will not be enough to saturate efflux transporters (198) partly explaining the increase in bioavailability we observed with efflux inhibition. The results of ABC transporter inhibition studies are in line with other studies demonstrating that oral bioavailability of camptothecin analogues is limited by ABC transporters and that inhibition of transporter function leads to improvement in oral bioavailability. Co-administration of topotecan and GF120918 by the oral route, increased plasma AUC of total topotecan more than six fold in P-gp knockout mice and greater than nine fold in wild-type mice compared with the wild type and P-gp knockout control mice (102). This increase is not only due to gastrointestinal efflux inhibition but also due to decreased systemic clearance (102). Similarly, in cancer patients GF120918 increased the bioavailability of topotecan 2.4 fold (40 to 97%) (133). The study, however, did not consider the effect of GF120918 on the clearance of topotecan. Therefore, the increase in bioavailability is likely to be due to decreased gastrointestinal efflux as well as decreased systemic clearance of topotecan. An

animal study using gefitinib as the ABC transporter inhibitor showed that a single dose of 100 mg/kg led to a 3.5 fold increase in the oral bioavailability of irinotecan in mice (25 in control versus 87% with gefitinib) (132). In another study, gefitinib (100 mg/kg) increased the bioavailability of topotecan in Bcrp knockout mice about 2.1 fold compared to wild-type animals (22 to 47%). Similarly, the same dose of gefitinib increased bioavailability about 1.7 fold (30 to 50%) in Mdr1 knockout animals compared to Mdr1 wild-type animals. The increase in bioavailability was related to both gastrointestinal efflux transporter inhibition and reduced systemic clearance (124). GF120918 was solubilized in an aqueous solution of 10% Tween 80 and 40% PEG-300. Both Tween 80 and PEG-300 increase oral bioavailability of lipophilic drugs through improved solubilization and/or inhibition of efflux transporters located in the gastrointestinal tract (8, 28, 29). However, as mentioned earlier in the results section, this excipient factor was taken into consideration by using the vehicle as a control when analyzing the GF120918 results.

The increase in oral lactone and carboxylate AUCs that was observed with GF120918 pretreatment (Table 5-3) was not solely due to the effect of the inhibitor. It is possible GF120918 formulation excipients may have improved in vivo solubility of AR-67, delayed its gastric emptying and inhibited Bcrp as was previously demonstrated in mice receiving topotecan (199). Inhibition of transport was found to be responsible for this increase as Tween-20 had little effect on oral AUC of topotecan in Bcrp knockout animals or on AUC of intravenously administered topotecan (199).

HPLC analysis of gastrointestinal contents indicated the presence of unabsorbed drug in the gastrointestinal tract long after drug administration. The presence of unabsorbed drug in the stomach long after drug administration is unlikely to have resulted from efflux by ABC transporters as the stomach is not believed to be a major site of drug absorption nor has it been shown to express ABC efflux transporters. More studies are needed to investigate the reason for the presence of unabsorbed drug in the stomach. First pass metabolism could also be responsible for limiting oral bioavailability of AR-67 as suggested by the presence of oxidative, glucuronide and other unidentified metabolites in the intestinal contents. Further studies are, however, required to quantify

the extent of first pass metabolism. Furthermore, we propose that pretreatment with an Oatp inhibitor prior to the oral administration of the carboxylate could help rule out the contribution of carboxylate uptake by Oatp as a reason for the predominance of the lactone form following oral administration of the carboxylate.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

The camptothecins represent an essential class of anticancer compounds that inhibit DNA replication by poisoning the nuclear enzyme Topoisomerase I (1-3). Camptothecins undergo pH dependent reversible hydrolysis from the lipophilic lactone to the hydrophilic carboxylate, which is favored at physiological and basic pH (7). The pharmacological activity of camptothecins is thought to be due to the ring-closed lactone form (6) although the ring-opened carboxylate also has the potential to interact with topo I (5). While the lipophilic nature of the lactone facilitates its diffusion through the plasma membrane, the hydrophilic nature of the carboxylate could limit its access to its site of action, i.e., the nucleus and may, therefore, be one reason for the reported lack of activity of the carboxylate. The poor aqueous solubility of camptothecin (15) and the rapid conversion of the lactone to carboxylate at physiological pH, especially in the presence of human serum albumin (9, 151), spurred research on water soluble and lactone stable analogues. First generation analogues still suffer from rapid lactone hydrolysis (9, 12) while second generation analogues, topotecan and irinotecan, possess improved water solubility and lactone stability and are currently in clinical use (200). Several third generation analogues are currently at various stages of preclinical and/or clinical investigation (20). Although these new generation analogues have improved lactone stability (11, 134), their poor aqueous solubility is a limiting factor for clinical use.

ABC efflux transporters and organic anion transporting polypeptides (OATPs/Oatps) play an essential role in the pharmacokinetics of substrate drugs in general and camptothecins in particular by regulating drug absorption, distribution and elimination (102, 116, 201-203). The existence of the lactone and carboxylate forms of camptothecins in equilibrium at physiological pH and the differences in their physicochemical properties (7) could contribute to differences in interaction with efflux and uptake transporters. This could in turn give rise to differences in cellular accumulation and/or transport in vitro and ultimately in pharmacokinetics. AR-67 is a third generation lactone stable camptothecin analog currently undergoing early phase clinical trials as a chemotherapeutic agent. AR-67 was chosen as a model compound for

this project based on its enhanced lactone stability and promising antiproliferative effects. Using this relatively lactone stable third generation camptothecin analog, the following hypotheses were tested:-1) Is the intracellular accumulation of AR-67 lactone reduced by ABC transporters P-gp and BCRP? and 2) Does AR-67 carboxylate or AR-67 lactone depend on uptake transporters, OATP1B1 or OATP1B3, for intracellular accumulation? It was speculated that these in vitro studies would provide essential information for the design of in vivo studies that will characterize the pharmacokinetics of the lactone and carboxylate forms as well as examine the role efflux and uptake transporters play in the disposition and oral bioavailability of AR-67 lactone and AR-67 carboxylate. In order to test the hypotheses stated above intracellular accumulation experiments were carried out in cell lines overexpressing ABC efflux (P-gp or BCRP) or uptake transporters (OATP1B1 or OATP1B3) using AR-67 lactone or AR-67 carboxylate. Transcellular transport experiments that assess the vectorial transport of AR-67 lactone across cell monolayers were also conducted in the presence or absence of efflux transporter inhibitors GF120918 and rifampin to examine the role of BCRP on the vectorial transport of AR-67 lactone. The results indicated that efflux by P-gp and BCRP decrease intracellular accumulation of AR-67 lactone. BCRP was shown to contribute to the vectorial transport of AR-67 by decreasing apical to basolateral transport and enhancing basolateral to apical transport across cell monolayers. Moreover, inhibition of P-gp and/or BCRP mediated efflux increased intracellular accumulation and reduced the vectorial transport of AR-67. OATP-mediated uptake was found to be responsible for the increased carboxylate uptake observed in OATP overexpressing cells. The wide expression of efflux transporters, P-gp and BCRP in organs involved in drug absorption, distribution, elimination (202) and the interaction of AR-67 lactone with these transporters indicates that the oral bioavailability, tissue distribution and systemic clearance of AR-67 is likely to be impacted by them. On the other hand, liver-specific uptake transporters, OATP1B1 or OATP1B3 (56) may represent a pathway for the elimination of the hydrophilic carboxylate. The interaction of the lactone with ABC efflux transporters and that of the carboxylate with organic anion transporting polypeptides (OATPs/Oatps) could lead to differences in the in vivo disposition of the lactone and carboxylate forms.Based on the results of in vitro studies the disposition of

the lactone and carboxylate forms of AR-67 were determined and the role of uptake and efflux transporters in the pharmacokinetics of AR-67 lactone and carboxylate was examined in rats (143). Single intravenous bolus doses of 2.5 mg/kg AR-67 lactone or carboxylate were given with or without oral transporter inhibitor pretreatment (GF120918 or rifampin). The results indicated that the majority of AR-67 was in the form of the lactone (% lactone AUC=84%) following lactone administration but in the form of the carboxylate following carboxylate administration (% carboxylate AUC=78%). Pharmacokinetic modeling and simulation was carried out to estimate reversible and irreversible (systemic) lactone and carboxylate clearances and assess the impact of clearance changes on overall AR-67 exposure. It was found that carboxylate systemic clearance was 3.5-fold higher than that of the lactone. Pretreatment with the dual P-gp and BCRP/Bcrp inhibitor, GF120918 decreased lactone clearance only, while pretreatment with rifampin decreased both lactone and carboxylate clearances. Simulations showed that decreasing carboxylate clearance significantly increases AR-67 exposure and suggested that the apparent in vivo blood stability of AR-67 is partly dependent on the increased carboxylate clearance. As the hydrophilic carboxylate is a substrate for OATPs, its clearance in vivo is likely to be mediated by these transporters. Changes in transporter function resulting from single nucleotide polymorphisms that impair the function of uptake transporter genes (e.g., SLCO1B1) or from drug-drug interactions could lead to altered exposure to AR-67 and lead to altered exposure to AR-67.

Several studies have indicated that the camptothecin class of anticancer compounds are better tolerated and more efficacious when administered at smaller but more frequent dosing schedules in what is known as protracted dosing (171-173). Previous preclinical studies in this lab have also demonstrated the efficacy of the third generation camptothecin analog, AR-67, when administered over a protracted schedule (Leggas lab unpublished data). Such dosing could easily be achieved using an oral dosage form as the oral route of administration offers many advantages ranging from flexibility of dosing regimen to minimization of cost. However, optimum bioavailability is a prerequisite to achieving the desired anticancer effects. It is therefore essential to study

factors that could potentially limit the oral bioavailability of AR-67 before advocating the clinical use of AR-67 via the oral route. The literature indicates that camptothecins are substrates of ABC efflux transporters and that their oral bioavailability is limited by P-gp and BCRP/Bcrp (47, 102, 124). First pass metabolism could also limit the amount of substrate drug reaching the systemic circulation following oral administration (204). Moreover, it has now been recognized that gastrointestinal efflux transporters and drug metabolizing enzymes could work in concert to limit the oral bioavailability of substrate drugs (198). In the case of most lactone stable camptothecins, such as AR-67, another limiting factor to bioavailability is their high lipophilicity. In these studies, the bioavailabilities of the lipophilic lactone and the hydrophilic carboxylate forms of AR-67 were determined. In addition, a pharmacokinetic model for the oral absorption of AR-67 was developed and the effect of efflux transporters P-gp and Bcrp on oral bioavailability was investigated, using the selective P-gp inhibitor, zosuquidar and the dual P-gp and BCRP/Bcrp inhibitor, GF120918. The presence of AR-67 metabolites and unabsorbed AR-67 in the gastrointestinal tract was also examined. Metabolism of AR-67 by intestinal and liver microsomes was also assessed to examine if first pass metabolism could impact oral bioavailability of AR-67.

The results of these studies indicated that the majority of AR-67 in the plasma was in the form of the lactone as represented by percent lactone AUCs ranging from 80-95% irrespective of whether the lactone or the carboxylate was orally administered. Dose normalized AUCs tended to increase with lactone dose suggesting the involvement of efflux transporters and/or metabolizing enzymes and saturation of either one or both of these processes at high doses of AR-67 lactone. Simultaneous modeling of the oral absorption of AR-67 following lactone and carboxylate inputs using the inverse Gaussian function and ITS algorithm in ADAPT5 provided estimates of bioavailability ranging from 6-17%. The pharmacokinetic model was able to adequately predict the plasma concentrations of lactone and carboxylate. Inhibition of P-gp with the selective P-gp inhibitor, zosuquidar, resulted in three fold increase in bioavailability over control (5% dextrose in water, D5W) pretreated animals. The increase resulted from GI efflux inhibition and minimal decrease in systemic clearance. On the other hand, pretreatment

with the dual P-gp and BCRP/Bcrp inhibitor, GF120918, led to increased bioavailability resulting from decreased gastrointestinal efflux and decreased systemic clearance. The modeling results indicated that inhibition of gastrointestinal efflux accounted for a threefold increase in bioavailability (F≈30) clearly indicating that both P-gp and Bcrp reduce the bioavailability of AR-67. However, based on the estimate of AR-67 extraction ratio, the oral bioavailability of AR-67 could be 46% suggesting efflux alone is not responsible for limiting bioavailability.

Examination of gastrointestinal contents indicated the presence of unabsorbed drug in the gastrointestinal tract, which could have resulted from the poor gastrointestinal tract (GI) dissolution of AR-67 and/or efflux by ABC transporters. Measurement of gastrointestinal contents in animals pretreated with ABC efflux inhibitors will with the accurate estimation of the contribution of poor GI dissolution in limiting oral bioavailability. At least 5% of the drug remained in the stomach at 12 h. The stomach is not believed to be a major site of drug absorption nor has it been shown to express ABC efflux transporters. Therefore, the presence of unabsorbed drug in the stomach long after drug administration is unlikely to have resulted from efflux by ABC transporters. More studies are needed to investigate the reason for the presence of unabsorbed drug in the stomach. Measurement of AR-67 in the feces and urine in addition to gastrointestinal contents will help with accurate estimation of the loss of AR-67 through the gastrointestinal tract or the urine. Intestinal and hepatic first pass metabolism could also be responsible for limiting oral BA of AR-67 as suggested by the presence of oxidative, glucuronide and other unidentified metabolites in the intestinal contents. It should be noted that the presence of metabolites in the gastrointestinal tract does not necessarily prove intestinal metabolism but provides an indication that first pass metabolism could limit oral bioavailability of AR-67. Incubation of AR-67 lactone with liver microsomes also suggests that AR-67 could be metabolized. Further studies are, however, required to quantify the extent of first pass metabolism as well as delineate if metabolism is either hepatic or intestinal.

The predominance of the lactone following oral administration of the lactone is in line with previous intravenous studies which also showed similar results (143). However,

the predominance of the lactone following oral administration of the carboxylate raised the question of which of the two forms is absorbed from the gastrointestinal tract. One explanation is that AR-67 is absorbed as the lactone form. Another explanation is that both could be absorbed from the gastrointestinal tract with selective uptake of the carboxylate into the liver by organic anion transporting polypeptides (Oatps). This however needs to be further studied. Pretreatment with an Oatp inhibitor prior to the oral administration of the carboxylate could help rule out the contribution of carboxylate uptake by Oatp as a reason for the predominance of the lactone form following oral administration of the carboxylate. In conclusion, the in vivo disposition of AR-67 and role of transporters in the clearance of the lactone and carboxylat forms is presented in Figure 6- 1.

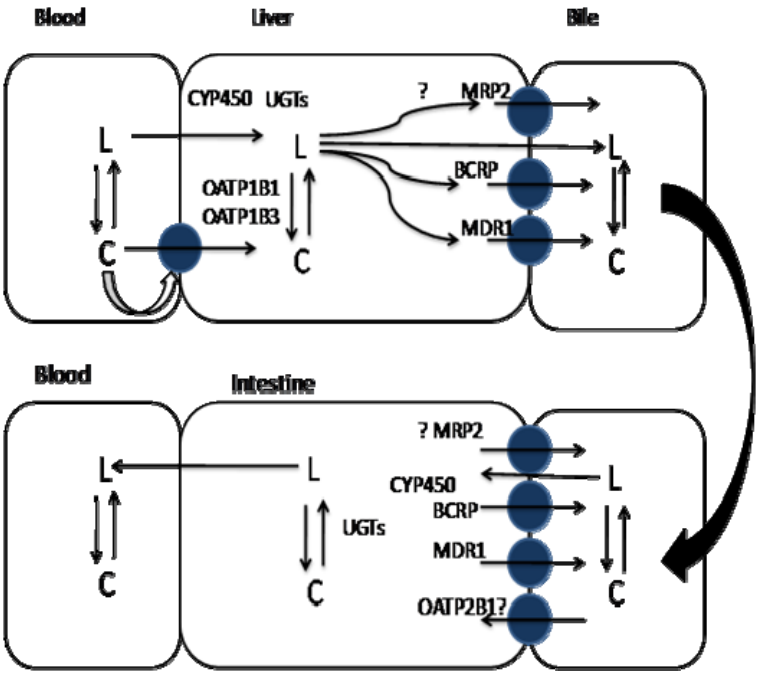


Figure 6- 1. AR 67 undergoes interconversion between lactone (L) and carboxylate forms in the blood and other tissues in vivo. The lipophilicity of the lactone is likely to favor its passive diffusion into the liver. The lactone is a substrate of efflux transporters BCRP and MDR1. Following its diffusion into the liver, the lactone could be 1) converted into the carboxylate, 2) metabolized by cytochrome P450 (CYP450) and/or UGT enzymes, or 3) effluxed by BCRP, MDR1 and possibly MRP2 into the bile. On the other hand, the

hydrophilic carboxylate is a substrate of OATPs and is likely taken up by OATP1B1 and/or OATP1B3 into the liver. Once inside the liver the carboxylate is reversibly converted to the lactone. It is possible that it could also be metabolized by CYP450s and/or UGTs and/or effluxed into the bile. The biliary contents are released into the gastrointestinal tract, from which the lipophilic AR-67 lactone could primarily be absorbed. It is possible that the carboxylate could also be absorbed through an uptake process (OATP2B1). Before AR-67 reaches the systemic circulation it is acted upon by efflux transporters MDR1, BCRP and possibly MRP2 and CYP450 and UGT enzymes.

REFERENCES

1. Y.H. Hsiang, Lihou, M.G., and Liu, L.F. . Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. . Cancer Res. 49 5077-5082 (1989).

2. Y.H. Hsiang, and Liu, L. F. Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. Cancer Res. 48:1722-1726 (1988).

3. C. Jaxel, K.W. Kohn, M.C. Wani, M.E. Wall, and Y. Pommier. Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: evidence for a specific receptor site and a relation to antitumor activity. Cancer Res. 49:1465-1469 (1989).

4. A.J. Ryan, S. Squires, H.L. Strutt, and R.T. Johnson. Camptothecin cytotoxicity in mammalian cells is associated with the induction of persistent double strand breaks in replicating DNA. Nucleic Acids Res. 19:3295-3300 (1991).

5. B.L. Staker, K. Hjerrild, M.D. Feese, C.A. Behnke, A.B. Burgin, Jr., and L. Stewart. The mechanism of topoisomerase I poisoning by a camptothecin analog. Proc Natl Acad Sci U S A. 99:15387-15392 (2002).

6. Y.H. Hsiang, R. Hertzberg, S. Hecht, and L.F. Liu. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J Biol Chem. 260:14873-14878 (1985).

7. J. Fassberg, and Stella, V.J. A kinetic and mechanistic study of the hydrolysis of camptothecin and some analogues. J Pharm Sci. 81:676-684 (1992).

8. E.D. Hugger, K.L. Audus, and R.T. Borchardt. Effects of poly(ethylene glycol) on efflux transporter activity in Caco-2 cell monolayers. J Pharm Sci. 91:1980-1990 (2002).

9. T.G. Burke, C.B. Munshi, Z. Mi, and Y. Jiang. The important role of albumin in determining the relative human blood stabilities of the camptothecin anticancer drugs. J Pharm Sci. 84:518-519 (1995).

10. W.J. Loos, J. Verweij, H.J. Gelderblom, M.J. de Jonge, E. Brouwer, B.K. Dallaire, and A. Sparreboom. Role of erythrocytes and serum proteins in the kinetic profile of total 9-amino-20(S)-camptothecin in humans. Anticancer Drugs. 10:705-710 (1999).

11. D. Bom, D.P. Curran, S. Kruszewski, S.G. Zimmer, J. Thompson Strode, G. Kohlhagen, W. Du, A.J. Chavan, K.A. Fraley, A.L. Bingcang, L.J. Latus, Y. Pommier, and T.G. Burke. The novel silatecan 7-tert-butyldimethylsilyl-10-hydroxycamptothecin displays high lipophilicity, improved human blood stability, and potent anticancer activity. J Med Chem. 43:3970-3980 (2000).

12. Z. Cao, N. Harris, A. Kozielski, D. Vardeman, J.S. Stehlin, and B. Giovanella. Alkyl esters of camptothecin and 9-nitrocamptothecin: synthesis, in vitro pharmacokinetics, toxicity, and antitumor activity. J Med Chem. 41:31-37 (1998).

13. T.G. Burke, and Mi, Z. H. Preferential Binding of the Carboxylate Form of Camptothecin by Human Serum Albumin. Analytical Biochemistry. 212:285-287 (1993).

14. R. Garcia-Carbonero, and Supko, J. G. Current perspectives on the clinical experience, pharmacology, and continued development of the camptothecins. Clin Cancer Res. 8:641-661 (2002).

15. B.A. Hanson, R.L. Schowen, and V.J. Stella. A mechanistic and kinetic study of the E-ring hydrolysis and lactonization of a novel phosphoryloxymethyl prodrug of camptothecin. Pharm Res. 20:1031-1038 (2003).

16. F. Zunino, and Pratesi, G. Camptothecins in clinical development. Expert Opin Investig Drugs. 13:269-284 (2004).

17. D.P. Curran, H. Josien, D. Bom, A.E. Gabarda, and W. Du. The cascade radical annulation approach to new analogues of camptothecins. Combinatorial synthesis of silatecans and homosilatecans. Ann N Y Acad Sci. 922:112-121 (2000).

18. D. Bom, D.P. Curran, J. Zhang, S.G. Zimmer, R. Bevins, S. Kruszewski, J.N. Howe, A. Bingcang, L.J. Latus, and T.G. Burke. The highly lipophilic DNA

topoisomerase I inhibitor DB-67 displays elevated lactone levels in human blood and potent anticancer activity. Journal of Controlled Release. 74:325-333 (2001).

19. T.X. Xiang, Z.Q. Jiang, L. Song, and B.D. Anderson. Molecular dynamics simulations and experimental studies of binding and mobility of 7-tert-butyldimethylsilyl-10-hydroxycamptothecin and its 20(S)-4-aminobutyrate ester in DMPC membranes. Mol Pharm. 3:589-600 (2006).

20. Y. Pommier. Topoisomerase I inhibitors: camptothecins and beyond. Nat Rev Cancer. 6:789-802 (2006).

21. E.J. Nam, J.W. Kim, J.H. Kim, S. Kim, S.W. Kim, S.Y. Jang, D.W. Lee, Y.W. Jung, and Y.T. Kim. Efficacy and Toxicity of Belotecan With and Without Cisplatin in Patients With Recurrent Ovarian Cancer. Am J Clin Oncol (2009).

22. S.J. Kim, J.S. Kim, S.C. Kim, Y.K. Kim, J.Y. Kang, H.K. Yoon, J.S. Song, S.H. Lee, H.S. Moon, J.W. Kim, K.H. Kim, C.H. Kim, B.Y. Shim, and H.K. Kim. A multicenter phase II study of belotecan, new camptothecin analogue, in patients with previously untreated extensive stage disease small cell lung cancer. Lung Cancer (2009).

23. I.F. Pollack, M. Erff, D. Bom, T.G. Burke, J.T. Strode, and D.P. Curran. Potent topoisomerase I inhibition by novel silatecans eliminates glioma proliferation in vitro and in vivo. Cancer Res. 59:4898-4905 (1999).

24. D.O. Scott, D.S. Bindra, and V.J. Stella. Plasma pharmacokinetics of lactone and carboxylate forms of 20(S)-camptothecin in anesthetized rats. Pharm Res. 10:1451-1457 (1993).

25. H.Y. Chengand W.J. Jusko. Mean interconversion times and distribution rate parameters for drugs undergoing reversible metabolism. Pharm Res. 7:1003-1010 (1990).

26. D.O. Scott, D.S. Bindra, S.C. Sutton, and V.J. Stella. Urinary and biliary disposition of the lactone and carboxylate forms of 20(S)-camptothecin in rats. Drug Metab Dispos. 22:438-442 (1994).

27. B.E. Davies, E.A. Minthorn, M.J. Dennis, H. Rosing, and J.H. Beijnen. The pharmacokinetics of topotecan and its carboxylate form following separate intravenous administration to the dog. Pharm Res. 14:1461-1465 (1997).

28. T. Yamagata, H. Kusuhara, M. Morishita, K. Takayama, H. Benameur, and Y. Sugiyama. Effect of excipients on breast cancer resistance protein substrate uptake activity. J Control Release. 124:1-5 (2007).

29. R.G. Strickley. Solubilizing excipients in oral and injectable formulations. Pharm Res. 21:201-230 (2004).

30. M. Takano, R. Yumoto, and T. Murakami. Expression and function of efflux drug transporters in the intestine. Pharmacol Ther. 109:137-161 (2006).

31. R. Xie, R.H. Mathijssen, A. Sparreboom, J. Verweij, and M.O. Karlsson. Clinical pharmacokinetics of irinotecan and its metabolites: a population analysis. J Clin Oncol. 20:3293-3301 (2002).

32. J.G. Slatter, L.J. Schaaf, J.P. Sams, K.L. Feenstra, M.G. Johnson, P.A. Bombardt, K.S. Cathcart, M.T. Verburg, L.K. Pearson, L.D. Compton, L.L. Miller, D.S. Baker, C.V. Pesheck, and R.S. Lord, 3rd. Pharmacokinetics, metabolism, and excretion of irinotecan (CPT-11) following I.V. infusion of [(14)C]CPT-11 in cancer patients. Drug Metab Dispos. 28:423-433 (2000).

33. Y. Sugiyama, Y. Kato, and X. Chu. Multiplicity of biliary excretion mechanisms for the camptothecin derivative irinotecan (CPT-11), its metabolite SN-38, and its glucuronide: role of canalicular multispecific organic anion transporter and P-glycoprotein. Cancer Chemother Pharmacol. 42 Suppl:S44-49 (1998).

34. S. Kawabata, M. Oka, K. Shiozawa, K. Tsukamoto, K. Nakatomi, H. Soda, M. Fukuda, Y. Ikegami, K. Sugahara, Y. Yamada, S. Kamihira, L.A. Doyle, D.D. Ross, and S. Kohno. Breast cancer resistance protein directly confers SN-38 resistance of lung cancer cells. Biochem Biophys Res Commun. 280:1216-1223 (2001).

35. R.M. Wadkins, C.L. Morton, J.K. Weeks, L. Oliver, M. Wierdl, M.K. Danks, and P.M. Potter. Structural constraints affect the metabolism of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) by carboxylesterases. Mol Pharmacol. 60:355-362 (2001).

36. C.F. Stewart, W.C. Zamboni, W.R. Crom, and P.J. Houghton. Disposition of irinotecan and SN-38 following oral and intravenous irinotecan dosing in mice. Cancer Chemother Pharmacol. 40:259-265 (1997).

37. P.D. Senter, K.S. Beam, B. Mixan, and A.F. Wahl. Identification and activities of human carboxylesterases for the activation of CPT-11, a clinically approved anticancer drug. Bioconjug Chem. 12:1074-1080 (2001).

38. S.P. Sanghani, S.K. Quinney, T.B. Fredenburg, W.I. Davis, D.J. Murry, and W.F. Bosron. Hydrolysis of irinotecan and its oxidative metabolites, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin and 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin, by human carboxylesterases CES1A1, CES2, and a newly expressed carboxylesterase isoenzyme, CES3. Drug Metab Dispos. 32:505-511 (2004).

39. M.C. Haaz, L.P. Rivory, C. Riche, and J. Robert. The transformation of irinotecan (CPT-11) to its active metabolite SN-38 by human liver microsomes. Differential hydrolysis for the lactone and carboxylate forms. Naunyn Schmiedebergs Arch Pharmacol. 356:257-262 (1997).

40. W. Yamamoto, J. Verweij, P. de Bruijn, M.J. de Jonge, H. Takano, M. Nishiyama, M. Kurihara, and A. Sparreboom. Active transepithelial transport of irinotecan (CPT-11) and its metabolites by human intestinal Caco-2 cells. Anticancer Drugs. 12:419-432 (2001).

41. K. Sai, M. Saeki, Y. Saito, S. Ozawa, N. Katori, H. Jinno, R. Hasegawa, N. Kaniwa, J. Sawada, K. Komamura, K. Ueno, S. Kamakura, M. Kitakaze, Y. Kitamura, N. Kamatani, H. Minami, A. Ohtsu, K. Shirao, T. Yoshida, and N. Saijo. UGT1A1 haplotypes associated with reduced glucuronidation and

increased serum bilirubin in irinotecan-administered Japanese patients with cancer. Clin Pharmacol Ther. 75:501-515 (2004).

42. J.Y. Han, H.S. Lim, E.S. Shin, Y.K. Yoo, Y.H. Park, J.E. Lee, I.J. Jang, D.H. Lee, and J.S. Lee. Comprehensive analysis of UGT1A polymorphisms predictive for pharmacokinetics and treatment outcome in patients with non-small-cell lung cancer treated with irinotecan and cisplatin. J Clin Oncol. 24:2237-2244 (2006).

43. K. Nakatomi, M. Yoshikawa, M. Oka, Y. Ikegami, S. Hayasaka, K. Sano, K. Shiozawa, S. Kawabata, H. Soda, T. Ishikawa, S. Tanabe, and S. Kohno. Transport of 7-ethyl-10-hydroxycamptothecin (SN-38) by breast cancer resistance protein ABCG2 in human lung cancer cells. Biochem Biophys Res Commun. 288:827-832 (2001).

44. K. Takasuna, T. Hagiwara, M. Hirohashi, M. Kato, M. Nomura, E. Nagai, T. Yokoi, and T. Kamataki. Involvement of beta-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res. 56:3752-3757 (1996).

45. E. Gupta, T.M. Lestingi, R. Mick, J. Ramirez, E.E. Vokes, and M.J. Ratain. Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. Cancer Res. 54:3723-3725 (1994).

46. W.C. Zamboni, L.L. Jung, S. Strychor, E. Joseph, B.A. Zamboni, S.A. Fetterman, B.J. Sidone, T.G. Burke, D.P. Curran, and J.L. Eiseman. Plasma and tissue disposition of non-liposomal DB-67 and liposomal DB-67 in C.B-17 SCID mice. Invest New Drugs. 26:399-406 (2008).

47. A.K. Lalloo, F.R. Luo, A. Guo, P.V. Paranjpe, S.H. Lee, V. Vyas, E. Rubin, and P.J. Sinko. Membrane transport of camptothecin: facilitation by human P-glycoprotein (ABCB1) and multidrug resistance protein 2 (ABCC2). BMC Med. 2:16 (2004).

48. R. Rajendra, M.K. Gounder, A. Saleem, J.H. Schellens, D.D. Ross, S.E. Bates, P. Sinko, and E.H. Rubin. Differential effects of the breast cancer resistance protein

on the cellular accumulation and cytotoxicity of 9-aminocamptothecin and 9-nitrocamptothecin. Cancer Res. 63:3228-3233 (2003).

49. G.D. Kruh, and Belinsky, M. G. The MRP family of drug efflux pumps. Oncogene. 22:7537-7552 (2003).

50. Y. Kitamura, H. Kusuhara, and Y. Sugiyama. Functional characterization of multidrug resistance-associated protein 3 (mrp3/abcc3) in the basolateral efflux of glucuronide conjugates in the mouse small intestine. J Pharmacol Exp Ther. 332:659-666.

51. T. Nozawa, H. Minami, S. Sugiura, A. Tsuji, and I. Tamai. Role of organic anion transporter OATP1B1 (OATP-C) in hepatic uptake of irinotecan and its active metabolite, 7-ethyl-10-hydroxycamptothecin: in vitro evidence and effect of single nucleotide polymorphisms. Drug Metab Dispos. 33:434-439 (2005).

52. R.L. Oostendorp, E. van de Steeg, C.M. van der Kruijssen, J.H. Beijnen, K.E. Kenworthy, A.H. Schinkel, and J.H. Schellens. Organic anion-transporting polypeptide 1B1 mediates transport of Gimatecan and BNP1350 and can be inhibited by several classic ATP-binding cassette (ABC) B1 and/or ABCG2 inhibitors. Drug Metab Dispos. 37:917-923 (2009).

53. G.L. Beretta, P. Perego, and F. Zunino. Mechanisms of cellular resistance to camptothecins. Curr Med Chem. 13:3291-3305 (2006).

54. S. Choudhuri, and Klaassen, C. D. Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. Int J Toxicol. 25:231-259 (2006).

55. P. Krishnamurthy, and Schuetz, J. D. Role of ABCG2/BCRP in biology and medicine. Annu Rev Pharmacol Toxicol. 46:381-410 (2006).

56. B. Hagenbuch, and Gui, C. Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. Xenobiotica. 38:778-801 (2008).

57. D.C. Rees, E. Johnson, and O. Lewinson. ABC transporters: the power to change. Nat Rev Mol Cell Biol. 10:218-227 (2009).

58. G. Szakacs, A. Varadi, C. Ozvegy-Laczka, and B. Sarkadi. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). Drug Discov Today. 13:379-393 (2008).

59. Q. Mao, and Unadkat, J. D. Role of the breast cancer resistance protein (ABCG2) in drug transport. Aaps J. 7:E118-133 (2005).

60. P. Borst, and Elferink, R. O. Mammalian ABC transporters in health and disease. Annu Rev Biochem. 71:537-592 (2002).

61. S.V. Ambudkar, S. Dey, C.A. Hrycyna, M. Ramachandra, I. Pastan, and M.M. Gottesman. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol. 39:361-398 (1999).

62. F.J. Sharom. The P-glycoprotein efflux pump: how does it transport drugs? J Membr Biol. 160:161-175 (1997).

63. F. Thiebaut, T. Tsuruo, H. Hamada, M.M. Gottesman, I. Pastan, and M.C. Willingham. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci U S A. 84:7735-7738 (1987).

64. I. Sugawara, I. Kataoka, Y. Morishita, H. Hamada, T. Tsuruo, S. Itoyama, and S. Mori. Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16. Cancer Res. 48:1926-1929 (1988).

65. A.H. Schinkel. The physiological function of drug-transporting P-glycoproteins. Semin Cancer Biol. 8:161-170 (1997).

66. A. Sparreboom, J. van Asperen, U. Mayer, A.H. Schinkel, J.W. Smit, D.K. Meijer, P. Borst, W.J. Nooijen, J.H. Beijnen, and O. van Tellingen. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. Proc Natl Acad Sci U S A. 94:2031-2035 (1997).

67. H. Kwon, R.A. Lionberger, and L.X. Yu. Impact of P-glycoprotein-mediated intestinal efflux kinetics on oral bioavailability of P-glycoprotein substrates. Mol Pharm. 1:455-465 (2004).

68. X.Y. Chu, Y. Kato, and Y. Sugiyama. Possible involvement of P-glycoprotein in biliary excretion of CPT-11 in rats. Drug Metab Dispos. 27:440-441 (1999).

69. X.Y. Chu, Y. Kato, K. Ueda, H. Suzuki, K. Niinuma, C.A. Tyson, V. Weizer, J.E. Dabbs, R. Froehlich, C.E. Green, and Y. Sugiyama. Biliary excretion mechanism of CPT-11 and its metabolites in humans: involvement of primary active transporters. Cancer Res. 58:5137-5143 (1998).

70. X. Tian, J. Li, M.J. Zamek-Gliszczynski, A.S. Bridges, P. Zhang, N.J. Patel, T.J. Raub, G.M. Pollack, and K.L. Brouwer. Roles of P-glycoprotein, Bcrp, and Mrp2 in biliary excretion of spiramycin in mice. Antimicrob Agents Chemother. 51:3230-3234 (2007).

71. M. Verschraagen, C.H. Koks, J.H. Schellens, and J.H. Beijnen. P-glycoprotein system as a determinant of drug interactions: the case of digoxin-verapamil. Pharmacol Res. 40:301-306 (1999).

72. J. Savolainen, J.E. Edwards, M.E. Morgan, P.J. McNamara, and B.D. Anderson. Effects of a P-Glycoprotein Inhibitor on Brain and Plasma Concentrations of Anti-Human Immunodeficiency Virus Drugs Administered in Combination in Rats. Drug Metab Dispos. 30:479-482 (2002).

73. J. Zongand G.M. Pollack. Morphine antinociception is enhanced in mdr1a gene-deficient mice. Pharm Res. 17:749-753 (2000).

74. S.P. Letrent, G.M. Pollack, K.R. Brouwer, and K.L. Brouwer. Effects of a potent and specific P-glycoprotein inhibitor on the blood-brain barrier distribution and antinociceptive effect of morphine in the rat. Drug Metab Dispos. 27:827-834 (1999).

75. A. Seelig, and Landwojtowicz, E. Structure-activity relationship of P-glycoprotein substrates and modifiers. Eur J Pharm Sci. 12:31-40 (2000).

76. C.B. Hendricks, E.K. Rowinsky, L.B. Grochow, R.C. Donehower, and S.H. Kaufmann. Effect of P-glycoprotein expression on the accumulation and cytotoxicity of topotecan (SK&F 104864), a new camptothecin analogue. Cancer Res. 52:2268-2278 (1992).

77. S.P. Cole, E.R. Chanda, F.P. Dicke, J.H. Gerlach, and S.E. Mirski. Non-P-glycoprotein-mediated multidrug resistance in a small cell lung cancer cell line: evidence for decreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II. Cancer Res. 51:3345-3352 (1991).

78. N. Krishnamachary, and Center, M. S. The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-kDa membrane bound glycoprotein. Cancer Res. 53:3658-3661 (1993).

79. R. Daoud, C. Kast, P. Gros, and E. Georges. Rhodamine 123 binds to multiple sites in the multidrug resistance protein (MRP1). Biochemistry. 39:15344-15352 (2000).

80. E.M. Leslie, R.G. Deeley, and S.P. Cole. Bioflavonoid stimulation of glutathione transport by the 190-kDa multidrug resistance protein 1 (MRP1). Drug Metab Dispos. 31:11-15 (2003).

81. P. Borst, R. Evers, M. Kool, and J. Wijnholds. A Family of Drug Transporters: the Multidrug Resistance-Associated Proteins. J Natl Cancer Inst. 92:1295-1302 (2000).

82. P.M. Gerkand M. Vore. Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. J Pharmacol Exp Ther. 302:407-415 (2002).

83. J. Madon, U. Eckhardt, T. Gerloff, B. Stieger, and P.J. Meier. Functional expression of the rat liver canalicular isoform of the multidrug resistance-associated protein. FEBS Lett. 406:75-78 (1997).

84. T.P. Schaub, J. Kartenbeck, J. Konig, H. Spring, J. Dorsam, G. Staehler, S. Storkel, W.F. Thon, and D. Keppler. Expression of the MRP2 gene-encoded

conjugate export pump in human kidney proximal tubules and in renal cell carcinoma. J Am Soc Nephrol. 10:1159-1169 (1999).

85. A.D. Mottino, T. Hoffman, L. Jennes, and M. Vore. Expression and localization of multidrug resistant protein mrp2 in rat small intestine. J Pharmacol Exp Ther. 293:717-723 (2000).

86. F.R. Luo, P.V. Paranjpe, A. Guo, E. Rubin, and P. Sinko. Intestinal transport of irinotecan in Caco-2 cells and MDCK II cells overexpressing efflux transporters Pgp, cMOAT, and MRP1. Drug Metab Dispos. 30:763-770 (2002).

87. J. Kartenbeck, U. Leuschner, R. Mayer, and D. Keppler. Absence of the canalicular isoform of the MRP gene-encoded conjugate export pump from the hepatocytes in Dubin-Johnson syndrome. Hepatology. 23:1061-1066 (1996).

88. G.L. Scheffer, M. Kool, M. de Haas, J.M. de Vree, A.C. Pijnenborg, D.K. Bosman, R.P. Elferink, P. van der Valk, P. Borst, and R.J. Scheper. Tissue distribution and induction of human multidrug resistant protein 3. Lab Invest. 82:193-201 (2002).

89. D.F. Ortiz, S. Li, R. Iyer, X. Zhang, P. Novikoff, and I.M. Arias. MRP3, a new ATP-binding cassette protein localized to the canalicular domain of the hepatocyte. Am J Physiol. 276:G1493-1500 (1999).

90. M. Kool, M. de Haas, G.L. Scheffer, R.J. Scheper, M.J. van Eijk, J.A. Juijn, F. Baas, and P. Borst. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res. 57:3537-3547 (1997).

91. R.A.M.H. Van Aubel, P.H.E. Smeets, J.J.M.W. van den Heuvel, and F.G.M. Russel. Human organic anion transporter MRP4 (ABCC4) is an efflux pump for the purine end metabolite urate with multiple allosteric substrate binding sites. Am J Physiol Renal Physiol. 288:F327-333 (2005).

92. J.D. Allen, Norris, M.D., Smith, J., Tobin, P., Tanabe K., Scheffer, G.L., Wielinga, P., Marshall, G.M., Haber, M. Expression of multidrug transporter

MRP4 is a marker of poor prognosis in neuroblastoma and confers resistance to CPT-11 in vitro. Proceedings of the 95th AACR Conference:506 (2004).

93. M. Leggas, M. Adachi, G.L. Scheffer, D. Sun, P. Wielinga, G. Du, K.E. Mercer, Y. Zhuang, J.C. Panetta, B. Johnston, R.J. Scheper, C.F. Stewart, and J.D. Schuetz. Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. Mol Cell Biol. 24:7612-7621 (2004).

94. K. Lee, A.J.P. Klein-Szanto, and G.D. Kruh. Analysis of the MRP4 Drug Resistance Profile in Transfected NIH3T3 Cells. J Natl Cancer Inst. 92:1934-1940 (2000).

95. J. Wijnholds, C.A. Mol, L. van Deemter, M. de Haas, G.L. Scheffer, F. Baas, J.H. Beijnen, R.J. Scheper, S. Hatse, E. De Clercq, J. Balzarini, and P. Borst. Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. Proc Natl Acad Sci U S A. 97:7476-7481 (2000).

96. L. Lai, and Tan, T. M. Role of glutathione in the multidrug resistance protein 4 (MRP4/ABCC4)-mediated efflux of cAMP and resistance to purine analogues. Biochem J. 361:497-503 (2002).

97. L.A. Doyle, W. Yang, L.V. Abruzzo, T. Krogmann, Y. Gao, A.K. Rishi, and D.D. Ross. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A. 95:15665-15670 (1998).

98. R. Allikmets, L.M. Schriml, A. Hutchinson, V. Romano-Spica, and M. Dean. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res. 58:5337-5339 (1998).

99. K. Miyake, L. Mickley, T. Litman, Z. Zhan, R. Robey, B. Cristensen, M. Brangi, L. Greenberger, M. Dean, T. Fojo, and S.E. Bates. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. Cancer Res. 59:8-13 (1999).

100. K. Kage, S. Tsukahara, T. Sugiyama, S. Asada, E. Ishikawa, T. Tsuruo, and Y. Sugimoto. Dominant-negative inhibition of breast cancer resistance protein as

drug efflux pump through the inhibition of S-S dependent homodimerization. Int J Cancer. 97:626-630 (2002).

101. J. Xu, Y. Liu, Y. Yang, S. Bates, and J.-T. Zhang. Characterization of Oligomeric Human Half-ABC Transporter ATP-binding Cassette G2. J Biol Chem. 279:19781-19789 (2004).

102. J.W. Jonker, J.W. Smit, R.F. Brinkhuis, M. Maliepaard, J.H. Beijnen, J.H. Schellens, and A.H. Schinkel. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. J Natl Cancer Inst. 92:1651-1656 (2000).

103. S. Cisternino, C. Mercier, F. Bourasset, F. Roux, and J.M. Scherrmann. Expression, up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood-brain barrier. Cancer Res. 64:3296-3301 (2004).

104. J. Enokizono, H. Kusuhara, A. Ose, A.H. Schinkel, and Y. Sugiyama. Quantitative investigation of the role of breast cancer resistance protein (Bcrp/Abcg2) in limiting brain and testis penetration of xenobiotic compounds. Drug Metab Dispos. 36:995-1002 (2008).

105. J.W. Jonker, M. Buitelaar, E. Wagenaar, M.A. Van Der Valk, G.L. Scheffer, R.J. Scheper, T. Plosch, F. Kuipers, R.P. Elferink, H. Rosing, J.H. Beijnen, and A.H. Schinkel. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc Natl Acad Sci U S A. 99:15649-15654 (2002).

106. J.W. Jonker, G. Merino, S. Musters, A.E. van Herwaarden, E. Bolscher, E. Wagenaar, E. Mesman, T.C. Dale, and A.H. Schinkel. The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. Nat Med (2005).

107. M. Maliepaard, G.L. Scheffer, I.F. Faneyte, M.A. van Gastelen, A.C. Pijnenborg, A.H. Schinkel, M.J. van De Vijver, R.J. Scheper, and J.H. Schellens. Subcellular

localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer Res. 61:3458-3464 (2001).

108. P.J. Houghton, G.S. Germain, F.C. Harwood, J.D. Schuetz, C.F. Stewart, E. Buchdunger, and P. Traxler. Imatinib Mesylate Is a Potent Inhibitor of the ABCG2 (BCRP) Transporter and Reverses Resistance to Topotecan and SN-38 in Vitro. Cancer Res. 64:2333-2337 (2004).

109. M. Maliepaard, M.A. van Gastelen, A. Tohgo, F.H. Hausheer, R.C.A.M. van Waardenburg, L.A. de Jong, D. Pluim, J.H. Beijnen, and J.H.M. Schellens. Circumvention of Breast Cancer Resistance Protein (BCRP)-mediated Resistance to Camptothecins in Vitro Using Non-Substrate Drugs or the BCRP Inhibitor GF120918. Clin Cancer Res. 7:935-941 (2001).

110. P. Perego, M. De Cesare, P. De Isabella, N. Carenini, G. Beggiolin, G. Pezzoni, M. Palumbo, L. Tartaglia, G. Pratesi, C. Pisano, P. Carminati, G.L. Scheffer, and F. Zunino. A novel 7-modified camptothecin analog overcomes breast cancer resistance protein-associated resistance in a mitoxantrone-selected colon carcinoma cell line. Cancer Res. 61:6034-6037 (2001).

111. S. Leuthold, B. Hagenbuch, N. Mohebbi, C.A. Wagner, P.J. Meier, and B. Stieger. Mechanisms of pH-gradient driven transport mediated by organic anion polypeptide transporters. Am J Physiol Cell Physiol. 296:C570-582 (2009).

112. B. Hagenbuch, and Meier, P. J. The superfamily of organic anion transporting polypeptides. Biochim Biophys Acta. 1609:1-18 (2003).

113. L.M. Satlin, V. Amin, and A.W. Wolkoff. Organic anion transporting polypeptide mediates organic anion/HCO3- exchange. J Biol Chem. 272:26340-26345 (1997).

114. S. Choudhuri, K. Ogura, and C.D. Klaassen. Cloning of the full-length coding sequence of rat liver-specific organic anion transporter-1 (rlst-1) and a splice variant and partial characterization of the rat lst-1 gene. Biochem Biophys Res Commun. 274:79-86 (2000).

115. H. Zaher, H.E. zu Schwabedissen, R.G. Tirona, M.L. Cox, L.A. Obert, N. Agrawal, J. Palandra, J.L. Stock, R.B. Kim, and J.A. Ware. Targeted disruption of murine organic anion-transporting polypeptide 1b2 (Oatp1b2/Slco1b2) significantly alters disposition of prototypical drug substrates pravastatin and rifampin. Mol Pharmacol. 74:320-329 (2008).

116. Y.Y. Lau, Y. Huang, L. Frassetto, and L.Z. Benet. effect of OATP1B transporter inhibition on the pharmacokinetics of atorvastatin in healthy volunteers. Clin Pharmacol Ther. 81:194-204 (2007).

117. W. Yamashiro, K. Maeda, M. Hirouchi, Y. Adachi, Z. Hu, and Y. Sugiyama. Involvement of transporters in the hepatic uptake and biliary excretion of valsartan, a selective antagonist of the angiotensin II AT1-receptor, in humans. Drug Metab Dispos. 34:1247-1254 (2006).

118. J. Konig, A. Seithel, U. Gradhand, and M.F. Fromm. Pharmacogenomics of human OATP transporters. Naunyn Schmiedebergs Arch Pharmacol. 372:432-443 (2006).

119. J. Cummings, G. Boyd, J.S. Macpherson, H. Wolf, G. Smith, J.F. Smyth, and D.I. Jodrell. Factors influencing the cellular accumulation of SN-38 and camptothecin. Cancer Chemother Pharmacol. 49:194-200 (2002).

120. C. Chen, R.J. Mireles, S.D. Campbell, J. Lin, J.B. Mills, J.J. Xu, and T.A. Smolarek. Differential interaction of 3-hydroxy-3-methylglutaryl-coa reductase inhibitors with ABCB1, ABCC2, and OATP1B1. Drug Metab Dispos. 33:537-546 (2005).

121. C. Chen, J. Lin, T. Smolarek, and L. Tremaine. P-glycoprotein has differential effects on the disposition of statin acid and lactone forms in mdr1a/b knockout and wild-type mice. Drug Metab Dispos. 35:1725-1729 (2007).

122. T.H. Tsai, C.H. Lee, and P.H. Yeh. Effect of P-glycoprotein modulators on the pharmacokinetics of camptothecin using microdialysis. Br J Pharmacol. 134:1245-1252 (2001).

123. K. Arimori, N. Kuroki, M. Hidaka, T. Iwakiri, K. Yamsaki, M. Okumura, H. Ono, N. Takamura, M. Kikuchi, and M. Nakano. Effect of P-glycoprotein modulator, cyclosporin A, on the gastrointestinal excretion of irinotecan and its metabolite SN-38 in rats. Pharm Res. 20:910-917 (2003).

124. M. Leggas, J.C. Panetta, Y. Zhuang, J.D. Schuetz, B. Johnston, F. Bai, B. Sorrentino, S. Zhou, P.J. Houghton, and C.F. Stewart. Gefitinib modulates the function of multiple ATP-binding cassette transporters in vivo. Cancer Res. 66:4802-4807 (2006).

125. X.-Y. Chu, Y. Kato, K. Niinuma, K.-I. Sudo, H. Hakusui, and Y. Sugiyama. Multispecific Organic Anion Transporter Is Responsible for the Biliary Excretion of the Camptothecin Derivative Irinotecan and its Metabolites in Rats. J Pharmacol Exp Ther. 281:304-314 (1997).

126. W.C. Zamboni, P.J. Houghton, R.K. Johnson, J.L. Hulstein, W.R. Crom, P.J. Cheshire, S.K. Hanna, L.B. Richmond, X. Luo, and C.F. Stewart. Probenecid Alters Topotecan Systemic and Renal Disposition by Inhibiting Renal Tubular Secretion. J Pharmacol Exp Ther. 284:89-94 (1998).

127. D.F. Zhong, K. Li, J.H. Xu, Y. Du, and Y.F. Zhang. Pharmacokinetics of 9-nitro-20(S)-camptothecin in rats. Acta Pharmacol Sin. 24:256-262 (2003).

128. A.H. Van Hattum, H.M. Pinedo, H.M. Schluper, F.H. Hausheer, and E. Boven. New highly lipophilic camptothecin BNP1350 is an effective drug in experimental human cancer. Int J Cancer. 88:260-266 (2000).

129. P.J. Houghton, P.J. Cheshire, L. Myers, C.F. Stewart, T.W. Synold, and J.A. Houghton. Evaluation of 9-dimethylaminomethyl-10-hydroxycamptothecin against xenografts derived from adult and childhood solid tumors. Cancer Chemother Pharmacol. 31:229-239 (1992).

130. S. Zhang, X. Wang, K. Sagawa, and M.E. Morris. Flavonoids chrysin and benzoflavone, potent breast cancer resistance protein inhibitors, have no

significant effect on topotecan pharmacokinetics in rats or mdr1a/1b (-/-) mice. Drug Metab Dispos. 33:341-348 (2005).

131. V.M. Herben, H. Rosing, W.W. ten Bokkel Huinink, D.M. van Zomeren, D. Batchelor, E. Doyle, F.D. Beusenberg, J.H. Beijnen, and J.H. Schellens. Oral topotecan: bioavailablity and effect of food co-administration. Br J Cancer. 80:1380-1386 (1999).

132. C.F. Stewart, M. Leggas, J.D. Schuetz, J.C. Panetta, P.J. Cheshire, J. Peterson, N. Daw, J.J. Jenkins, 3rd, R. Gilbertson, G.S. Germain, F.C. Harwood, and P.J. Houghton. Gefitinib enhances the antitumor activity and oral bioavailability of irinotecan in mice. Cancer Res. 64:7491-7499 (2004).

133. C.M. Kruijtzer, J.H. Beijnen, H. Rosing, W.W. ten Bokkel Huinink, M. Schot, R.C. Jewell, E.M. Paul, and J.H. Schellens. Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. J Clin Oncol. 20:2943-2950 (2002).

134. S. Marchetti, R.L. Oostendorp, D. Pluim, M. van Eijndhoven, O. van Tellingen, A.H. Schinkel, R. Versace, J.H. Beijnen, R. Mazzanti, and J.H. Schellens. In vitro transport of gimatecan (7-t-butoxyiminomethylcamptothecin) by breast cancer resistance protein, P-glycoprotein, and multidrug resistance protein 2. Mol Cancer Ther. 6:3307-3313 (2007).

135. M. Brangi, T. Litman, M. Ciotti, K. Nishiyama, G. Kohlhagen, C. Takimoto, R. Robey, Y. Pommier, T. Fojo, and S.E. Bates. Camptothecin resistance: role of the ATP-binding cassette (ABC), mitoxantrone-resistance half-transporter (MXR), and potential for glucuronidation in MXR-expressing cells. Cancer Res. 59:5938-5946 (1999).

136. U. Vanhoefer, M.R. Muller, R.A. Hilger, B. Lindtner, U. Klaassen, N. Schleucher, Y.M. Rustum, S. Seeber, and A. Harstrick. Reversal of MDR1-associated resistance to topotecan by PAK-200S, a new dihydropyridine analogue, in human cancer cell lines. Br J Cancer. 81:1304-1310 (1999).

137. X.Y. Chu, Y. Kato, and Y. Sugiyama. Multiplicity of biliary excretion mechanisms for irinotecan, CPT-11, and its metabolites in rats. Cancer Res. 57:1934-1938 (1997).

138. H. Kusuharaand Y. Sugiyama. Pharmacokinetic modeling of the hepatobiliary transport mediated by cooperation of uptake and efflux transporters. Drug Metab Rev.

139. B.C. Giovanella, N. Harris, J. Mendoza, Z. Cao, J. Liehr, and J.S. Stehlin. Dependence of anticancer activity of camptothecins on maintaining their lactone function. Ann N Y Acad Sci. 922:27-35 (2000).

140. M.J. de Jonge, J. Verweij, W.J. Loos, B.K. Dallaire, and A. Sparreboom. Clinical pharmacokinetics of encapsulated oral 9-aminocamptothecin in plasma and saliva. Clin Pharmacol Ther. 65:491-499 (1999).

141. S. Pace, F. Capocasa, C. Tallarico, R. Frapolli, M. Zucchetti, and A. Longo. Determination of total and lactone form of a new camptothecin derivative gimatecan (ST1481) and its metabolite ST1698 in human plasma by high-performance liquid chromatography with fluorimetric detection. J Pharm Biomed Anal. 50:507-514 (2009).

142. P.A. Thompson, S.L. Berg, A. Aleksic, J.Z. Kerr, L. McGuffey, R. Dauser, J.G. Nuchtern, F. Hausheer, and S.M. Blaney. Plasma and cerebrospinal fluid pharmacokinetic study of BNP1350 in nonhuman primates. Cancer Chemother Pharmacol. 53:527-532 (2004).

143. E.D. Adane, Z. Liu, T.X. Xiang, B.D. Anderson, and M. Leggas. Factors Affecting the In Vivo Lactone Stability and Systemic Clearance of the Lipophilic Camptothecin Analogue AR-67. Pharm Res.

144. J.C. Kalvassand G.M. Pollack. Kinetic considerations for the quantitative assessment of efflux activity and inhibition: implications for understanding and predicting the effects of efflux inhibition. Pharm Res. 24:265-276 (2007).

145. J. Horn, S.L. Jordan, L. Song, M.J. Roberts, B.D. Anderson, and M. Leggas. Validation of an HPLC method for analysis of DB-67 and its water soluble prodrug in mouse plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 844:15-22 (2006).

146. M.K. Gounder, A.S. Nazar, A. Saleem, P. Pungaliya, D. Kulkarni, R. Versace, and E.H. Rubin. Effects of drug efflux proteins and topoisomerase I mutations on the camptothecin analogue gimatecan. Invest New Drugs. 26:205-213 (2008).

147. S.E. Bates, W.Y. Medina-Perez, G. Kohlhagen, S. Antony, T. Nadjem, R.W. Robey, and Y. Pommier. ABCG2 mediates differential resistance to SN-38 (7-ethyl-10-hydroxycamptothecin) and homocamptothecins. J Pharmacol Exp Ther. 310:836-842 (2004).

148. K. Kobayashi, B. Bouscarel, Y. Matsuzaki, S. Ceryak, S. Kudoh, and H. Fromm. pH-dependent uptake of irinotecan and its active metabolite, SN-38, by intestinal cells. Int J Cancer. 83:491-496 (1999).

149. T.G. Burkeand Z. Mi. The structural basis of camptothecin interactions with human serum albumin: impact on drug stability. J Med Chem. 37:40-46 (1994).

150. S.M. Arnold, J.J. Rinehart, E. Tsakalozou, J.R. Eckardt, S.Z. Fields, B.J. Shelton, P.A. DeSimone, B.K. Kee, J.A. Moscow, and M. Leggas. A phase I study of 7-t-butyldimethylsilyl-10-hydroxycamptothecin in adult patients with refractory or metastatic solid malignancies. Clin Cancer Res. 16:673-680 (2010).

151. T.G. Burkeand Z. Mi. Preferential binding of the carboxylate form of camptothecin by human serum albumin. Anal Biochem. 212:285-287 (1993).

152. J.G. Liehr, A.E. Ahmed, and B.C. Giovanella. Pharmacokinetics of camptothecins administered orally. Ann N Y Acad Sci. 803:157-163 (1996).

153. P.J. Creaven, L.M. Allen, and F.M. Muggia. Plasma camptothecin (NSC-100880) levels during a 5-day course of treatment: relation to dose and toxicity. Cancer Chemother Rep. 56:573-578 (1972).

154. E.A. Natelson, B.C. Giovanella, C.F. Verschraegen, K.M. Fehir, P.D. De Ipolyi, N. Harris, and J.S. Stehlin. Phase I clinical and pharmacological studies of 20-(S)-camptothecin and 20-(S)-9-nitrocamptothecin as anticancer agents. Ann N Y Acad Sci. 803:224-230 (1996).

155. H. Li, H.E. Jin, W. Kim, Y.H. Han, D.D. Kim, S.J. Chung, and C.K. Shim. Involvement of P-glycoprotein, multidrug resistance protein 2 and breast cancer resistance protein in the transport of belotecan and topotecan in Caco-2 and MDCKII cells. Pharm Res. 25:2601-2612 (2008).

156. E.D. Adane, N. Monks, M.J. Roberts, J. Horn, J.A. Moscow, and M. Leggas. Transporter Pathways for the Novel Camptothecin Analog DB-67. AAPS Abstract M1032, *AAPS Workshop on Drug Transporters in ADME: From the Bench to the Bedside*, 2007.

157. L.P. Rivory, E. Chatelut, P. Canal, A. Mathieu-Boue, and J. Robert. Kinetics of the in vivo interconversion of the carboxylate and lactone forms of irinotecan (CPT-11) and of its metabolite SN-38 in patients. Cancer Res. 54:6330-6333 (1994).

158. W.F. Eblingand W.J. Jusko. The determination of essential clearance, volume, and residence time parameters of recirculating metabolic systems: the reversible metabolism of methylprednisolone and methylprednisone in rabbits. J Pharmacokinet Biopharm. 14:557-599 (1986).

159. H.Y. Chengand W.J. Jusko. Mean residence times and distribution volumes for drugs undergoing linear reversible metabolism and tissue distribution and linear or nonlinear elimination from the central compartments. Pharm Res. 8:508-511 (1991).

160. D.Z. D'Argenioand A. Schumitzky. ADAPT II User's Guide: Pharmacokinetic/Pharmacodynamic Systems Analysis Software, Biomedical Simulations Resource, Los Angeles, CA, 1997.

161. H. Chengand W.J. Jusko. Pharmacokinetics of reversible metabolic systems. Biopharm Drug Dispos. 14:721-766 (1993).

162. M. Rowland, and Tozer, T.N. Clinical Pharmacokinetics: Concents and Applications Williams & Wilkins 1995.

163. Z. Miand T.G. Burke. Differential interactions of camptothecin lactone and carboxylate forms with human blood components. Biochemistry. 33:10325-10336 (1994).

164. I.E. Kuppens, E.O. Witteveen, R.C. Jewell, S.A. Radema, E.M. Paul, S.G. Mangum, J.H. Beijnen, E.E. Voest, and J.H. Schellens. A phase I, randomized, open-label, parallel-cohort, dose-finding study of elacridar (GF120918) and oral topotecan in cancer patients. Clin Cancer Res. 13:3276-3285 (2007).

165. R.L. Oostendorp, E. van de Steeg, C.M.M. van der Kruijssen, J.H. Beijnen, K.E. Kenworthy, A.H. Schinkel, and J.H.M. Schellens. OATP1B1 mediates transport of gimatecan and BNP1350 and can be inhibited by several classical ABCB1 and/or ABCG2 inhibitors. Drug Metab Dispos:dmd.108.024901 (2009).

166. Y.Y. Lau, H. Okochi, Y. Huang, and L.Z. Benet. Multiple transporters affect the disposition of atorvastatin and its two active hydroxy metabolites: application of in vitro and ex situ systems. J Pharmacol Exp Ther. 316:762-771 (2006).

167. J. Zong, and Pollack, Gary M. Modulation of P-glycoprotein Transport Activity in the Mouse Blood-Brain Barrier by Rifampin. J Pharmacol Exp Ther. 306:556-562 (2003).

168. A. Sparreboom, H. Gelderblom, S. Marsh, R. Ahluwalia, R. Obach, P. Principe, C. Twelves, J. Verweij, and H.L. McLeod. Diflomotecan pharmacokinetics in relation to ABCG2 421C>A genotype. Clin Pharmacol Ther. 76:38-44 (2004).

169. M. Niemi, E. Schaeffeler, T. Lang, M.F. Fromm, M. Neuvonen, C. Kyrklund, J.T. Backman, R. Kerb, M. Schwab, P.J. Neuvonen, M. Eichelbaum, and K.T. Kivisto. High plasma pravastatin concentrations are associated with single nucleotide polymorphisms and haplotypes of organic anion transporting polypeptide-C (OATP-C, SLCO1B1). Pharmacogenetics. 14:429-440 (2004).

170. A.X. Zhu, N. Ready, J.W. Clark, H. Safran, A. Amato, N. Salem, S. Pace, X. He, N. Zvereva, T.J. Lynch, D.P. Ryan, and J.G. Supko. Phase I and Pharmacokinetic Study of Gimatecan Given Orally Once a Week for 3 of 4 Weeks in Patients with Advanced Solid Tumors. Clin Cancer Res. 15:374-381 (2009).

171. W.L. Furman, C.F. Stewart, C.A. Poquette, C.B. Pratt, V.M. Santana, W.C. Zamboni, L.C. Bowman, M.K. Ma, F.A. Hoffer, W.H. Meyer, A.S. Pappo, A.W. Walter, and P.J. Houghton. Direct translation of a protracted irinotecan schedule from a xenograft model to a phase I trial in children. J Clin Oncol. 17:1815-1824 (1999).

172. P.J. Houghton, P.J. Cheshire, J.D. Hallman, 2nd, L. Lutz, H.S. Friedman, M.K. Danks, and J.A. Houghton. Efficacy of topoisomerase I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. Cancer Chemother Pharmacol. 36:393-403 (1995).

173. V.M. Santana, W.C. Zamboni, M.N. Kirstein, M. Tan, T. Liu, A. Gajjar, P.J. Houghton, and C.F. Stewart. A pilot study of protracted topotecan dosing using a pharmacokinetically guided dosing approach in children with solid tumors. Clin Cancer Res. 9:633-640 (2003).

174. G. Liu, E. Franssen, M.I. Fitch, and E. Warner. Patient preferences for oral versus intravenous palliative chemotherapy. J Clin Oncol. 15:110-115 (1997).

175. R. Ramlau, R. Gervais, M. Krzakowski, J. von Pawel, E. Kaukel, R.P. Abratt, B. Dharan, K.M. Grotzinger, G. Ross, G. Dane, and F.A. Shepherd. Phase III study comparing oral topotecan to intravenous docetaxel in patients with pretreated advanced non-small-cell lung cancer. J Clin Oncol. 24:2800-2807 (2006).

176. D.L. Clarke-Pearson, L. Van Le, T. Iveson, C.W. Whitney, P. Hanjani, G. Kristensen, J.H. Malfetano, R.A. Beckman, G.A. Ross, S.R. Lane, M.H. DeWitte, and S.Z. Fields. Oral topotecan as single-agent second-line chemotherapy in patients with advanced ovarian cancer. J Clin Oncol. 19:3967-3975 (2001).

177. R.L. Drengler, J.G. Kuhn, L.J. Schaaf, G.I. Rodriguez, M.A. Villalona-Calero, L.A. Hammond, J.A. Stephenson, Jr., S. Hodges, M.A. Kraynak, B.A. Staton,

G.L. Elfring, P.K. Locker, L.L. Miller, D.D. Von Hoff, and M.L. Rothenberg. Phase I and pharmacokinetic trial of oral irinotecan administered daily for 5 days every 3 weeks in patients with solid tumors. J Clin Oncol. 17:685-696 (1999).

178. H. Dumez, A. Awada, M. Piccart, S. Assadourian, D. Semiond, G. Guetens, G. de Boeck, R.A. Maes, E.A. de Bruijn, and A. van Oosterom. A phase I dose-finding clinical pharmacokinetic study of an oral formulation of irinotecan (CPT-11) administered for 5 days every 3 weeks in patients with advanced solid tumours. Ann Oncol. 17:1158-1165 (2006).

179. O. Soepenberg, H. Dumez, J. Verweij, F.A. de Jong, M.J. de Jonge, J. Thomas, F.A. Eskens, R.H. van Schaik, J. Selleslach, J. Ter Steeg, P. Lefebvre, S. Assadourian, G.J. Sanderink, A. Sparreboom, and A.T. van Oosterom. Phase I pharmacokinetic, food effect, and pharmacogenetic study of oral irinotecan given as semisolid matrix capsules in patients with solid tumors. Clin Cancer Res. 11:1504-1511 (2005).

180. J. Fassbergand V.J. Stella. A kinetic and mechanistic study of the hydrolysis of camptothecin and some analogues. J Pharm Sci. 81:676-684 (1992).

181. T.X. Xiangand B.D. Anderson. Stable supersaturated aqueous solutions of silatecan 7-t-butyldimethylsilyl-10-hydroxycamptothecin via chemical conversion in the presence of a chemically modified beta-cyclodextrin. Pharm Res. 19:1215-1222 (2002).

182. A.H. Schinkel, U. Mayer, E. Wagenaar, C.A. Mol, L. van Deemter, J.J. Smit, M.A. van der Valk, A.C. Voordouw, H. Spits, O. van Tellingen, J.M. Zijlmans, W.E. Fibbe, and P. Borst. Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. Proc Natl Acad Sci U S A. 94:4028-4033 (1997).

183. M.H. Milewska, Jamie; Monks, Noel; Moscow, Jeffrey A.; Arnold, Susanne M.; Leggas, Markos. Metabolism and Transport Pathways of the Blood Stable Camptothecin AR-67 (7-t-butyldimethylsilyl-10-hydroxycamptothecin). Journal of Clinical Oncology. 27:(Abstract 2553) (2009).

184. W.-Q. Tong, and Wells, M. L. Parenteral pharmaceutical compositions containing GF120918A. Glaxo wellcomE inc;WO/1996/011007, 1996.

185. B.D. Anderson, M.J. May, S. Jordan, L. Song, M.J. Roberts, and M. Leggas. Dependence of nelfinavir brain uptake on dose and tissue concentrations of the selective P-glycoprotein inhibitor zosuquidar in rats. Drug Metab Dispos. 34:653-659 (2006).

186. A. Kaddoumi, S.U. Choi, L. Kinman, D. Whittington, C.C. Tsai, R.J. Ho, B.D. Anderson, and J.D. Unadkat. Inhibition of P-glycoprotein activity at the primate blood-brain barrier increases the distribution of nelfinavir into the brain but not into the cerebrospinal fluid. Drug Metab Dispos. 35:1459-1462 (2007).

187. J. Yang, M. Jamei, K.R. Yeo, A. Rostami-Hodjegan, and G.T. Tucker. Misuse of the well-stirred model of hepatic drug clearance. Drug Metab Dispos. 35:501-502 (2007).

188. E.D. Adane, Z. Liu, T.X. Xiang, B.D. Anderson, and M. Leggas. Factors affecting the in vivo lactone stability and systemic clearance of the lipophilic camptothecin analogue AR-67. Pharm Res. 27:1416-1425 (2010).

189. B. Daviesand T. Morris. Physiological parameters in laboratory animals and humans. Pharm Res. 10:1093-1095 (1993).

190. C. Emoto, H. Yamazaki, S. Yamasaki, N. Shimada, M. Nakajima, and T. Yokoi. Characterization of cytochrome P450 enzymes involved in drug oxidations in mouse intestinal microsomes. Xenobiotica. 30:943-953 (2000).

191. D.Z. D'Argenio, A. Schumitzky, and X. Wang. *ADAPT 5 User's Guide: Pharmacokinetic/Pharmacodynamic Systems Analysis Software.* Biomedical Simulations Resource, Los Angeles 2009.

192. M. Weiss. A novel extravascular input function for the assessment of drug absorption in bioavailability studies. Pharm Res. 13:1547-1553 (1996).

193. J. Wang, M. Weiss, and D.Z. D'Argenio. A note on population analysis of dissolution-absorption models using the inverse Gaussian function. J Clin Pharmacol. 48:719-725 (2008).

194. A.H. Dantzig, R.L. Shepard, J. Cao, K.L. Law, W.J. Ehlhardt, T.M. Baughman, T.F. Bumol, and J.J. Starling. Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. Cancer Res. 56:4171-4179 (1996).

195. J.D. Allen, R.F. Brinkhuis, J. Wijnholds, and A.H. Schinkel. The Mouse Bcrp1/Mxr/Abcp Gene: Amplification and Overexpression in Cell Lines Selected for Resistance to Topotecan, Mitoxantrone, or Doxorubicin. Cancer Res. 59:4237-4241 (1999).

196. R.L. Carrier, L.A. Miller, and I. Ahmed. The utility of cyclodextrins for enhancing oral bioavailability. Journal of Controlled Release. 123:78-99 (2007).

197. G.L. Amidon, H. Lennernas, V.P. Shah, and J.R. Crison. A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. Pharm Res. 12:413-420 (1995).

198. C.Y. Wuand L.Z. Benet. Predicting drug disposition via application of BCS: transport/absorption/ elimination interplay and development of a biopharmaceutics drug disposition classification system. Pharm Res. 22:11-23 (2005).

199. T. Yamagata, H. Kusuhara, M. Morishita, K. Takayama, H. Benameur, and Y. Sugiyama. Improvement of the oral drug absorption of topotecan through the inhibition of intestinal xenobiotic efflux transporter, breast cancer resistance protein, by excipients. Drug Metab Dispos. 35:1142-1148 (2007).

200. V.M. Herben, W.W. ten Bokkel Huinink, and J.H. Beijnen. Clinical pharmacokinetics of topotecan. Clin Pharmacokinet. 31:85-102 (1996).

201. A. Kalliokoski, and Niemi, M. Impact of OATP transporters on pharmacokinetics. Br J Pharmacol. 158:693-705 (2009).

202. S. Shugarts, and Benet, L. Z. The role of transporters in the pharmacokinetics of orally administered drugs. Pharm Res. 26:2039-2054 (2009).

203. G. Merino, J.W. Jonker, E. Wagenaar, A.E. van Herwaarden, and A.H. Schinkel. The Breast Cancer Resistance Protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion and milk secretion of the antibiotic nitrofurantoin. Mol Pharmacol:mol.104.010439 (2005).

204. C.Y. Wu, L.Z. Benet, M.F. Hebert, S.K. Gupta, M. Rowland, D.Y. Gomez, and V.J. Wacher. Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine. Clin Pharmacol Ther. 58:492-497 (1995).

APPENDIX I

PARTIAL VALIDATION OF AN HPLC METHOD FOR THE DETERMINATION OF THE LACTONE AND CARBOXYLATE FORMS OF AR-67 IN RAT PLASMA

I. Standards

AR-67 containing samples were prepared and/or stored in amber siliconized tubes. Aqueous solutions were kept on ice while all methanolic extracts were kept on dry ice. Measurements were made with siliconzed pipette tips. Extracts were kept frozen at -80°C.

A. AR-67 stock solution

A stock solution of AR-67 lactone was prepared using the analytical grade crystalline drug from Dr. Brad Anderson's lab (TOX LOT). A 1 mg/mL AR-67 stock solution was prepared by dissolving 10 mg of AR-67 powder with dimethylsulfoxide (DMSO) in a 10 ml volumetric flask. This stock was aliquoted in 40 µl volumes into amber siliconized microcentrifuge tubes and kept at –80°C.

B. Working standard solutions

Working AR-67 lactone and carboxylate solutions were prepared from the stock 1 mg/ml lactone solution as described in the literature for mouse data (REF) and outlined in Table 1. Lactone working solutions were prepared in 0.005 N HCl kept on ice while carboxylate solutions were prepared in 0.005 N NaOH kept on ice (Table 1). Working solutions were kept on ice for 1 h.

Table 1. Preparation of AR-67 lactone or carboxylate working solutions.

From solution	Aliquot	0.005 N HCl for lactone or 0.005 N NaOH for carboxylate (µL)	Final concentration
1000 µg/mL (stock solution)	10 µL	990 µL	10 µg/mL
1000 µg/mL (stock solution)	10 µL	QS to 10 mL	1 µg/mL
1000 µg/mL (stock solution)	10 µL	QS to 100 mL	0.1 µg/mL
1000 µg/mL (stock solution)	10 µL	990	10 µg/mL
10 µg/mL	100 µL	900	1 µg/mL
1 µg/mL	100 µL	900	0.1 µg/mL

C. Calibration and quality control standards

Lactone and carboxylate calibrators and quality control samples were prepared as outlined in Table 2 below. The concentrations of calibrators were 2.5, 5, 10, 20, 50, 100, 200 and 300 ng/mL. Samples were vortexed for 10 seconds after preparation and were extracted with cold methanol (-80^{o}C) (1:4, v/v). Mixtures were then vortexed for 10 seconds and centrifuged for 2 mins at 13,000 rpm (4ºC). The supernatant was poured into siliconized amber microcentrifuge tubes, and stored at -80°C. Three quality control samples:- a low (7 ng/mL), a medium (150 ng/mL) and a high (250 ng/mL)- were separately prepared, extracted and stored as described above for calibrators. Each quality control sample contained designated concentrations of both the lactone and carboxylate (Table 2).

Table 2. AR-67 standard curve and quality control sample preparation in rat plasma.

Calibrator /Quality control	AR-67 carb & lact. Conc (ng/mL)	From AR-67 carb & lact working solutions	Aliquot from working solution	PBS (pH 7.4)/ plasma (μL)
1	2.5	0.1	25 (carb)/25 (lact)	10/940
2	5	1	5	50/940
3	10	1	10	40/940
4	20	1	20	20/940
5	50	10	5	50/940
6	100	10	10	40/940
7	200	10	20	20/940
8	300	10	30	0
QC1	7	1	7	46/940
QC3	150	10	15	30/940
QC4	250	10	25	10/940

D. Short term matrix and extract stability

Short term stability of AR-67 quality control samples containing both lactone and carboxylate was determined in the matrix (rat plasma) or in the extract. The results of these studies are presented in Tables 3-5. For matrix stability studies plasma samples containing AR-67 lactone and carboxylate were kept on ice while for extract stability studies extracts were diluted with an equal volume of the mobile phase buffer and kept in the HPLC autoinjector chamber at 4^{o}C. AR-67 was found to be stable in the matrix for less than 3 hrs indicating that extraction should be performed immediately. The extract, on the other hand was stable for up to 6 hrs after mixing with the mobile phase buffer.

Table 3. Short term matrix stability-QC4.

250 ng/mL												
Time (hr)	Carboxylate (% initial)						Lactone (% initial)					
	1	2	3	Average	SD	RSD	1	2	3	Average	SD	RSD
0	100.0	100.0	100.0	100.0	0	0	100.0	100.0	100.0	100.0	0	0
1	113.0	103.5	114.5	110.3	6.0	5.4	89.1	81.6	90.4	87.0	4.8	5.5
3	126.6	120.7	127.2	124.8	3.6	2.9	64.4	62.7	63.7	63.6	0.9	1.4
6	126.6	120.7	127.3	124.8	3.6	2.9	44.4	42.3	47.0	44.5	2.3	5.2

Table 4a. Short term extract stability of AR-67 –QC1 trial 1.

7 ng/mL												
Time	Carboxylate (% initial)						Lactone (% initial)					
(hr)	1	2	3	Average	SD	RSD	1	2	3	Average	SD	RSD
0	100.0	100.0	100.0	100.0	0	0	100.0	100.0	100.0	100.0	0	0
1	93.7	90	103.1	95.6	6.8	7.1	99.9	96.1	103.1	99.7	3.5	3.5
3	86.4	85.6	87.7	86.7	0.9	1.1	102.8	103.5	97.4	101.3	3.3	3.3
6	85.0	86.7	82.2	84.6	2.3	2.7	109.0	118.0	112.0	113.0	4.6	4.0
24	87.1	79.3	72.1	79.5	7.5	9.4	165.4	150.6	135.9	150.6	14.7	9.8

Table 4b. Short term extract stability of AR-67- QC1 trial 2.

7 ng/mL												
Time	Carboxylate (% initial)						Lactone (% initial)					
(hr)	1	2	3	Average	SD	RSD	1	2	3	Average	SD	RSD
0	100	100	100	100	0	0	100	100	100	100	0	100
1	93.4	94.7	94.4	94.2	0.7	0.7	99.7	97.8	98.8	98.8	1.0	1.0
3	85.8	90.2	87.2	87.7	2.3	2.6	102.9	105.9	97.3	102.0	4.4	4.3
6	84.4	86.0	81.2	83.9	2.4	2.8	109.2	118.8	112.4	113.4	4.9	4.3
24	100.9	86.8	81.2	89.6	10.1	11.3	163.0	144.2	141.1	149.4	11.9	7.9

Table 5a. Short term extract stability of AR-67-QC4.

250 ng/mL												
Time	Carboxylate (% initial)						Lactone (% initial)					
(hr)	1	2	3	Average	SD	RSD	1	2	3	Average	SD	RSD
0	100.0	100.0	100.0	100.0	0	0	100.0	100.0	100.0	100.0	0	0
1	86.4	94.4	99.0	93.3	6.4	6.8	98.9	99.6	100.9	99.8	1.0	1.0
3	79.9	93.3	97.1	90.1	9.0	10.0	99.8	105.7	104.0	103.2	3.0	2.9
6	78.5	81.5	86.2	82.0	3.9	4.7	111.1	110.7	104.1	108.7	3.9	3.6
24	85.0	96.4	87.5	89.6	6.0	6.7	141.3	147.3	137.3	142.0	5.0	3.5

E. Long term matrix and extract stability studies

To determine the long term stability of AR-67 in the matrix and in methanolic extracts unextracted samples or extracted samples were kept at -80^{o}C for 0, 7 14 and 21 days before HPLC analysis. The results of these studies are shown in Tables 6 and 7. AR-67 lactone concentration in the matrix was less than 85% of the initial concentration by day 7 for both QC1 and QC4. Similarly, AR-67 carboxylate concentration was less than 85% of its initial concentration by day 21 for QC1. The carboxylate concentration of AR-67 in the maxtrix appears not to have changed for QC4. On the hand, the lactone concentration declined to ≤ 80% of the nominal concentration in the matrix within 7 days for both QC1 and QC4. In contrast, the extract was stable for up to 14 days (Tables 8 &9).

Table 6. Long term matrix stability of AR-67-QC1.

	7 ng/mL											
Day	Carboxylate (% initial)						Lactone (% initial)					
	1	2	3	Average	SD	RSD	1	2	3	Average	SD	RSD
0	100.0	100.0	100.0	0	0	0	100.0	100.0	100.0	100.0	0	0
7	93.7	99.0	98.9	97.2	3.1	3.1	75.9	80.9	82.7	79.8	3.5	4.1
14	81.8	101.8	96.4	93.3	10.4	11.1	65.1	80.5	78.0	74.5	8.2	11.0
21	75.9	88.6	86.4	83.7	6.8	8.1	72.1	85.9	87.1	81.7	8.3	10.2

Table 7. Long term matrix stability of AR-67-QC4.

250 ng/mL										
Day	Carboxylate (% initial)					Lactone (% initial)				
	1	2	Average	SD	RSD	1	2	Average	SD	RSD
0	100.0	100.0	100.0	0	0	100.0	100.0	100.0	0	0
7	110.8	106.1	108.4	3.3	3.1	78.0	75.5	76.7	1.8	2.3
14	105.2	91.5	98.4	9.7	9.8	63.6	72.8	68.2	6.5	9.5
21	103.9	109.4	106.6	3.9	3.6	88.9	95.6	92.2	4.8	5.2

Table 8. Long term extract stability-QC1.

7 ng/mL												
Day	Carboxylate (% initial)						Lactone (% initial)					
	1	2	3	Average	SD	RSD	1	2	3	Average	SD	RSD
0	100	100	100	100	0	0	100	100	100	100	0	0
7	103.8	103.4	108.3	105.1	2.7	2.6	111.8	89.8	95.6	99.1	11.4	11.5
14	85.9	83.7	84.5	84.7	1.1	1.3	95.9	93.1	98.5	95.8	2.7	2.8

Table 9. Long term extract stability-QC4.

QC4 (250 ng/mL): Long term extract stability										
Day	Carboxylate (% initial)					Lactone (% initial)				
	1	2	Average	SD	RSD	1	2	Average	SD	RSD
0	100.0	100.0	100.0	0	0	100.0	100.0	100.0	0	0
7	106.3	103.8	105.1	1.7	1.6	85.3	82.3	83.8	2.1	2.5
14	96.3	88.8	92.5	5.3	5.8	102.4	93.9	98.1	6.0	6.1

II. HPLC system and assay

A. Instrumentation and chromatographic conditions

Shimadzu HPLC system (Shimadzu Inc., Atlanta, GA)

Class-VP integrating software (Ver. 7.2.1.)

In-line degasser (DGU-14A)

LC-10ADVP pump

Refrigerated autoinjector (SIL-10ADVP) (rack temperature at 4°C)

Fluorescence detector (set at high sensitivity): excitation-380 nm and emission-560 nm

Reversed-phase C18 analytical column (Waters Nova-Pak C18 4μ; 3.9 x 150 mm)

Mobile phase buffer: 0.15 M ammonium acetate containing 10 mM tetrabutylammonium dihydrogen phosphate (TBAP; pH 6.5).

Mobile phase: 65% mobile phase buffer and 35% acetonitrile.

Flow rate: 1 mL/min

Retention times: carboxylate- ≈ 3 min and lactone- ≈ 9 mins.

B. Extract preparation and injection

Extracts were diluted with an equal volume of ice cold mobile-phase buffer and were briefly vortexed. Air bubbles were removed by gentle tapping on the walls of the vials and 50 μL injection was made.

C. Simultaneous calibration curves

Simultaneous carboxylate and lactone calibration curves were prepared as described above (Table 1). A list of the curves prepared and the parameters describing the curve are shown in Table 10. The parameters were generated by linear regression of peak lactone and carboxylate heights (x-axis) and analyte concentrations (y-axis).

Table 10. Simultaneous carboxylate and lactone calibration curves in rat plasma.

Carboxylate	Linear Regression Parameters			
Curve	Slope	y-intercept	R2	Weighing
1a	0.0003639	0.00000	0.99933	1/Response
1b	0.0004327	-1.06019	0.99985	None
1c	0.0004493	-0.19637	0.99864	1/Amount
2	0.0004175	0.01005	0.98889	1/Response
3a	0.0003858	-0.53943	0.99990	None
3b	0.0003528	0.20519	0.99973	None
3c	0.0003654	-0.15301	0.99797	None
4	0.0003775	0.00000	0.98967	None
3d	0.0003303	-0.21542	0.99812	None
3e	0.0002982	-0.38162	0.99963	None
4a	0.0002445	0.29259	0.99911	1/Response
4b	0.0002792	-0.21435	0.99099	1/Response2
5a	0.0003314	-0.25858	0.99967	1/Response
5b	0.0002414	-0.31928	0.99796	1/Response2
6a	0.0003501	-0.26273	0.99993	1/Response
6b	0.0002447	0.01827	0.99966	None
6c	0.0002740	-0.18674	0.99990	1/Response
7a	0.0003806	-0.0523507	0.999082	None
7b	0.0003454	-0.221892	0.999226	None
7c	0.0004053	0.16872	0.99871	None
7d	0.0002683	-0.343719	0.983075	1/Response
7e	0.0002603	0.368477	0.998514	1/Amount
7f	0.0002312	0.167203	0.991336	1/Response2
7g	0.0002589	-0.359501	0.997235	None
8a	0.0001986	-0.789946	0.999287	None
AVG	0.0003435	-0.1683572	0.9977976	
SD	0.0000629	0.2928403	0.0035012	
RSD	18.3082031	-173.9399113	0.3508967	

Table 10 Ctd

Table 10 Ctd

Lactone	Linear Regression Parameters			
Curve	Slope	y-intercept	R2	Weighing
1a	0.0005915	0.78068	0.99808	1/Response
1b	0.0007784	1.78870	0.99737	None
1c	0.0006923	0.82613	0.99579	1/Amount
2	0.0008396	-0.61582	0.98028	1/Response
3a	0.0006500	-0.09537	0.99614	1/Response
3b	0.0008215	-0.34830	0.99845	1/Response
3c	0.0008375	-0.63140	0.99615	1/Response
4	0.0007551	-0.31251	0.99905	1/Amount
3d	0.0006096	0.00013	0.99538	1/Response
3e	0.0004798	0.46941	0.99660	1/Response
4a	0.0004501	0.28835	0.99574	1/Response
4b	0.0004357	-0.16735	0.99856	1/Response
5a	0.0004784	0.31671	0.99853	1/Amount
5b	0.0003908	0.47596	0.99707	1/Amount
6a	0.0005673	0.31824	0.99892	1/Response
6b	0.0004577	0.21205	0.99839	1/Response
6c	0.0006683	0.19528	0.99869	1/Response
7a	0.0008204	-0.69046	0.99655	1/Amount^2
7b	0.0007718	-1.92884	0.99863	None
7c	0.0007676	-1.85451	0.99832	1/Response
7d	0.0005796	-0.85303	0.99880	1/Response
7e	0.0005647	-0.99484	0.99912	1/Response
7f	0.0005293	-0.78830	0.99825	1/Response
7g	0.0005498	-1.50909	0.99842	1/Amount
8a	0.0004234	-0.40877	0.99926	1/Response
AVG	0.0006432	-0.0486453	0.9966340	
SD	0.0001534	0.8562193	0.0040368	
RSD	23.8549161	-1760.1285629	0.4050393	

III. Accuracy and precision

A. Intraday precision.

Ten injections of QC3 and QC4 extracts were made in the same day to determine intraday precision of the assay. Table 11 shows the results of these studies. The CV values for both analytes in the QCs were < 15%.

Table 11. Intraday variability and accuracy.

Replicate	QC-3 (150 ng/mL)		QC-4 (250 ng/mL)	
	Carboxylate (ng/mL)	Lactone (ng/mL)	Carboxylate (ng/mL)	Lactone (ng/mL)
1	148.53	135.45	246.18	245.55
2	156.38	144.09	248.53	248.66
3	152.18	141.47	244.52	250.6
4	140.56	129.26	246.97	255.86
5	149.17	138.67	242.06	255.65
6	143.36	136.3	234.16	244.25
7	143.68	139.47	246.69	253.71
8	141.23	139.34	254.54	267.4
9	136.89	136.61	260.17	267.46
10	141.05	142.05	270.38	273.51
AVG	145.30	138.27	249.42	256.27
SD	6.05	4.18	10.09	10.02
CV	4.2	3.0	4.0	3.9
Accuracy (%)	96.9	92.9	99.08	102.5

B. Interday precision

The interday precision was determined as the %CV of average analyte values of 5-10 injections on four different days (Table 12). all CV values are < 15 %.

Table 12. Interday precision.

	QC-1 (7 ng/mL)		QC-3 (150 ng/mL)		QC-4 (250 ng/mL)	
	Avg. % of Nominal		Avg. % of Nominal		Avg. % of Nominal	
Day of Validation	Carb.	Lactone	Carb.	Lactone	Carb.	Lactone
Day 1	108.23 (n=5)	97.77 (n=5)	100.97 (n=5)	92.8 (n=5)	98.54 (n=5)	101.13 (n=5)
Day 2	110.57 (n=5)	96.49 (n=5)	96.87 (n=10)	92.5v (n=10)	93.41 (n=5)	98.33 (n=5)
Day 3	113.06 (n=5)	97.60 (n=5)	106.41 (n=5)	95.10 (n=5)	99.77 (n=10)	104.51 (n=10)
Day 4	117.77 (n=5)	99.94 (n=5)	111.00 (n=5)	96.97 (n=5)	105.14 (n=5)	105.27 (n=5)
Accuracy AVG	112.41	97.95	103.81	94.34	99.22	102.31
SD	4.1	1.4	6.2	2.1	4.8	3.2
CV%	3.6	1.5	6.0	2.2	4.9	3.1

IV. Lower limit of quantification (LLOQ)

The lower limit of quantification was determined from the AR-57 lactone and carboxylate calibration curves by comparison of the baseline (noise) and the peak signal of AR-67 lactone/carboxylate at 2.5 and 5 ng/mL concentrations (Table 13). The LLOQ defined as the concentration for which

1) S/N >= 5,

2) a mean estimated analyte concentration within 20 % of nominal, and

3) a RSD <= 20 %.

Table 13. Lower limit of quantification of AR-67 in rat plasma.

Curve	Conc (ng/mL)	Carboxylate Height (Signal, mm)	Baseline Height (noise, mm)	Lactone Height (Signal, mm)	Carboxylate S/N ratio	Lactone S/N ratio
2/21/2009		9.87	1.04	4.28	9.48	4.12
3/25/2009	2.50	8.66	0.69	4.16	12.52	6.01
	Average				11.0	5.97
	SD				2.15	1.34
	% RSD				19.5	22.4
3/25/2009		14.57	0.44	6.06	32.88	13.67
2/21/2009	5.00	17.97	0.66	9.62	24.43	14.69
	Average				28.7	12.05
	SD				5.97	0.72
	% RSD				20.9	6.0

The lower limit of quantitation is 2.5 ng/mL for the carboxylate analyte and 5 ng/mL for the lactone analyte.

Raw-data: short term matrix stability.

Data file name	Sample ID	Carb. (ng/mL)	Lact (ng/mL)	Carb. (% initial)	Lactone (% initial)
20AC-031608-Short term matrix & long term extact stability 005.dat	QC4a matrix - 0h-031608	260.76	248.27	100.0%	100.0%
20AC-031608-Short term matrix & long term extact stability 006.dat	QC4b matrix - 0h-031608	273.55	263.02	100.0%	100.0%
20AC-031608-Short term matrix & long term extact stability 007.dat	QC4c matrix - 0h-031608	259.41	252.52	100.0%	100.0%
20AC-031608-Short term matrix & long term extact stability 008.dat	QC4a matrix - 1h-031608	294.55	221.15	113.0%	89.1%
20AC-031608-Short term matrix & long term extact stability 009.dat	QC4b matrix - 1h-031608	283.07	214.61	103.5%	81.6%
20AC-031608-Short term matrix & long term extact stability 010.dat	QC4c matrix - 1h-031608	296.96	228.33	114.5%	90.4%
20AC-031608-Short term matrix & long term extact stability 011.dat	QC4a matrix - 3h-031608	330.08	159.84	126.6%	64.4%
20AC-031608-Short term matrix & long term extact stability 012.dat	QC4b matrix - 3h-031608	330.11	164.83	120.7%	62.7%
20AC-031608-Short term matrix & long term extact stability 013.dat	QC4c matrix - 3h-031608	329.91	160.88	127.2%	63.7%
20AC-031608-Short term matrix & long term extact stability 014.dat	QC4a matrix - 6h-031608	330.09	110.21	126.6%	44.4%
20AC-031608-Short term matrix & long term extact stability 015.dat	QC4b matrix - 6h-031608	330.15	111.25	120.7%	42.3%
20AC-031608-Short term matrix & long term extact stability 016.dat	QC4c matrix - 6h-031608	330.15	118.57	127.3%	47.0%

Raw data: Short term extract stability

Data Filename	Sample ID	Carb. (ng/mL)	Lact (ng/mL)	Carb. (% initial)	Lact. (%initial)
10AD-030108-Rat plasma short term extract stability 001.dat	QC1a-0h (prep 021308 Reext 030108)	8	6.97	100.0%	100.0%
10AD-030108-Rat plasma short term extract stability 002.dat	QC1b-0h (prep 021408 Reext 030108)	7.56	6.82	100.0%	100.0%
10AD-030108-Rat plasma short term extract stability 003.dat	QC1c-0h (prep 021408 Reext 030108)	7.88	7.28	100.0%	100.0%
10AD-030108-Rat plasma short term extract stability 004.dat	QC1a-1h (prep 021308 Reext 030108)	7.47	6.95	93.4%	99.7%
10AD-030108-Rat plasma short term extract stability 005.dat	QC1b-1h (prep 021408 Reext 030108)	7.16	6.67	94.7%	97.8%
10AD-030108-Rat plasma short term extract stability 006.dat	QC1c-1h (prep 021408 Reext 030108)	7.44	7.19	94.4%	98.8%
10AD-030108-Rat plasma short term extract stability 007.dat	QC1a-3h (prep 021308 Reext 030108)	6.86	7.17	85.8%	102.9%
10AD-030108-Rat plasma short term extract stability 008.dat	QC1b-3h (prep 021408 Reext 030108)	6.82	7.22	90.2%	105.9%
10AD-030108-Rat plasma short term extract stability 009.dat	QC1c-3h (prep 021408 Reext 030108)	6.87	7.08	87.2%	97.3%
10AD-030108-Rat plasma short term extract stability 010.dat	QC1a-6h (prep 021308 Reext 030108)	6.75	7.61	84.4%	109.2%
10AD-030108-Rat plasma short term extract stability 011.dat	QC1b-6h (prep 021408 Reext 030108)	6.5	8.1	86.0%	118.8%
10AD-030108-Rat plasma short term extract stability 012.dat	QC1c-6h (prep 021408 Reext 030108)	6.41	8.18	81.3%	112.4%
10AD-030108-Rat plasma short term extract stability 013.dat	QC1a-24h (prep 021308 Reext 030108)	6.92	11.69	100.9%	163.0%
10AD-030108-Rat plasma short term extract stability 014.dat	QC1b-24h (prep 021408 Reext 030108)	5.92	10.41	86.8%	144.2%

10AD-030108-Rat plasma short term extract stability 015.dat	QC1c-24h (prep 021408 Reext 030108)	5.58	9.99	81.2%	141.1%
10AD-021508-Rat plasma short term extract stability 002.dat	QC4a-0h-021508	248.85	238.81	100%	100%
10AD-021508-Rat plasma short term extract stability 003.dat	QC4b-0h-021508	239.31	228.35	100%	100%
10AD-021508-Rat plasma short term extract stability 004.dat	QC4c-0h-021508	265.1	255.58	100%	100%
10AD-021508-Rat plasma short term extract stability 005.dat	QC4a-1h-021508	215.07	236.14	86%	99%
10AD-021508-Rat plasma short term extract stability 006.dat	QC4b-1h-021508	225.8	227.37	94%	100%
10AD-021508-Rat plasma short term extract stability 007.dat	QC4c-1h-021508	262.41	257.81	99%	101%
10AD-021508-Rat plasma short term extract stability 008.dat	QC4a-3h-021508	198.94	238.38	80%	100%
10AD-021508-Rat plasma short term extract stability 009.dat	QC4b-3h-021508	223.19	241.35	93%	106%
10AD-021508-Rat plasma short term extract stability 010.dat	QC4c-3h-021508	257.54	265.72	97%	104%
10AD-021508-Rat plasma short term extract stability 011.dat	QC4a-6h-021508	195.34	265.41	78%	111%
10AD-021508-Rat plasma short term extract stability 012.dat	QC4b-6h-021508	195.01	252.86	81%	111%
10AD-021508-Rat plasma short term extract stability 013.dat	QC4c-6h-021508	228.42	266.15	86%	104%
10AD-021608-Rat plasma short term extract stability 003	QC4a-24h-021608	211.44	337.54	85%	141%
10AD-021608-Rat plasma short term extract stability 004	QC4b-24h-021608	230.68	336.31	96%	147%
10AD-021608-Rat plasma short term extract stability 005	QC4c-24h-021608	232.01	350.89	88%	137%

Raw data: Long term extract stability

Data file name	Sample ID	Carb. (ng/mL)	Lact. (ng/mL)	Carb. % initial	Lact. % initial
	Day 1				
20AC-041108-Rat PK Expt 111 & 112 056.dat	QC1a-day0-long term	7.73	7.14	100%	100%
20AC-041108-Rat PK Expt 111 & 112 057.dat	QC1b-day0-long term	7.35	8.65	100%	100%
20AC-041108-Rat PK Expt 111 & 112 058.dat	QC1c-day0-long term	7.01	7.22	100%	100%
	day 7				
20AC-041808-Rat plasma matrix & extract stability 001.dat	QC1a-extract-day 7-041808	8.02	7.98	103.8%	111.8%
20AC-041808-Rat plasma matrix & extract stability 002.dat	QC1b-extract-day 7-041808	7.6	7.77	103.4%	89.8%
20AC-041808-Rat plasma matrix & extract stability 003.dat	QC1c-extract-day 7-041808	7.59	6.9	108.3%	95.6%
	Day 14				
20AC-extract matrix stability-042508-002.dat	QC1a-Extract (041108)	6.64	6.85	85.9%	95.9%
20AC-extract matrix stability-042508-003.dat	QC1b-Extract (041108)	6.15	8.05	83.7%	93.1%
20AC-extract matrix stability-042508-004.dat	QC1c-Extract (041108)	5.92	7.11	84.5%	98.5%

Long term matrix stability

Data file	Sample ID	Carb (ng/mL)	Lact (ng/mL)	Carb % initial	Lact % initial
	Day 1				
20AC-041108-Rat PK Expt 111 & 112 059.dat	QC4a-day0-long term	262.58	261.12	100%	100%
20AC-041108-Rat PK Expt 111 & 112 061.dat	QC4c-day0-long term	272.44	279.4	100%	100%
	Day 7				
20AC-041808-Rat plasma matrix & extract stability 009.dat	QC4a-matrix-day 7-041808	290.91	203.6	110.8%	78.0%
20AC-041808-Rat plasma matrix & extract stability 010.dat	QC4b-matrix-day 7-041808	265.37	199.65		
20AC-041808-Rat plasma matrix & extract stability 011.dat	QC4c-matrix-day 7-041808	289.1	210.9	106.1%	75.5%
	Day 14				
20AC-extract matrix stability-042508-014.dat	QC4a-matrix (041108)	276.29	166.08	105.2%	63.6%
20AC-extract matrix stability-042508-015.dat	QC4b-matrix (041108)	330.01	202.5		
20AC-extract matrix stability-042508-016.dat	QC4c-matrix (041108)	249.37	203.44	91.5%	72.8%
	Day 21				
20AC-050208-Rat PK Expt 122 & 124 040.dat	QC4a-day 21-matrix	272.83	232.05	103.9%	88.9%
20AC-050208-Rat PK Expt 122 & 124 041.dat	QC4c-day 21-matrix	297.96	267.16	109.4%	95.6%

Inter and intraday variability and accuracy of Quality controls

Day 1	QC-1 (7 ng/mL)		QC-3 (150 ng/mL)		QC-4 (250 ng/mL)	
	Carb.	Lact.	Carb.	Lact.	Carb.	Lact.
	109.14	100.14	99.51	90.33	95.29	97.16
	107.43	95.43	98.80	90.37	102.22	103.76
	108.86	97.43	100.37	92.55	97.47	100.08
	109.00	100.14	101.92	94.29	98.53	101.58
	106.71	95.71	104.23	96.47	99.20	103.08
Average	108.23	97.77	100.97	92.80	98.54	101.13
SD	1.09	2.30	2.16	2.63	2.53	2.64
RSD	1.01	2.35	2.14	2.84	2.57	2.61
Day 2	QC-1 (7 ng/mL)		QC-3 (150 ng/mL)		QC-4 (250 ng/mL)	
	Carb.	Lact.	Carb.	Lact.	Carb.	Lact.
	116	98.57	99.02	90.30	95.19	100.00
	110.5714	95	104.25	96.06	91.21	96.56
	108.2857	94.45	101.45	94.31	93.40	98.67
	109.1429	96.71	93.71	86.17	92.16	96.33
	108.8571	97.71	99.45	92.45	95.07	100.07
			95.57	90.87		
			95.79	92.98		
			94.15	92.89		
			91.26	91.07		
			94.03	94.70		
Average	110.57	96.49	96.87	92.50	93.41	98.33
SD	3.15	1.76	4.04	1.58	1.76	1.81
RSD	2.85	1.82	4.17	1.70	1.88	1.84

Day 3	QC-1 (7 ng/mL)		QC-3 (150 ng/mL)		QC-4 (250 ng/mL)	
	Carb.	Lact.	Carb.	Lact.	Carb.	Lact.
	121	103.1429	105.78	89.94	98.47	98.22
	115.5714	100.5714	106.84	95.54	99.41	99.46
	108	92.28571	106.21	96.13	97.81	100.24
	114	97.71429	105.97	95.91	98.79	102.34
	106.7143	94.28571	107.23	97.97	96.82	102.26
					93.66	97.70
					98.68	101.48
					101.82	106.96
					104.07	106.98
					108.15	109.40
Average	113.06	97.60	106.41	95.10	99.77	104.51
SD	5.83	4.44	0.61	3.03	4.04	4.79
RSD	5.16	4.55	0.57	3.19	4.05	4.58
Day 4	QC-1 (7 ng/mL)		QC-3 (150 ng/mL)		QC-4 (250 ng/mL)	
	Carb.	Lact.	Carb.	Lact.	Carb.	Lact.
	126.2857	102	110.91	96.00	107.44	104.27
	122.8571	101.8571	110.61	96.39	106.77	106.96
	111.7143	95	109.70	96.02	105.25	105.07
	112.4286	96	111.15	97.30	106.50	107.10
	115.5714	104.8571	112.65	99.15	99.75	102.94
Average	117.77	99.94	111.00	96.97	105.14	105.27
SD	6.49	4.24	1.07	1.33	3.12	1.78
RSD	5.51	4.25	0.96	1.37	2.96	1.69

Intraday accuracy and precision.

	QC-3 (150 ng/mL)		QC-4 (250 ng/mL)	
	Carb.	Lact.	Carb.	Lact.
	99.02	90.30	98.47	98.22
	104.25	96.06	99.41	99.46
	101.45	94.31	97.81	100.24
	93.71	86.17	98.79	102.34
	99.45	92.45	96.82	102.26
	95.57	90.87	93.66	97.70
	95.79	92.98	98.68	101.48
	94.15	92.89	101.82	106.96
	91.26	91.07	104.07	106.98
	94.03	94.70	108.15	109.40
Average	96.87	92.50	99.77	104.51
SD	4.04	1.58	4.04	4.79
RSD	4.17	1.70	4.05	4.58

APPENDIX II.

MAXIMUM TOLERATED DOSE (MTD) FINDING EXPERIMENTS AND XENOGRAFT STUDIES IN FEMALE ATHYMIC NUDE MICE (NU/NU)

Maximum Ttolerated dose (MTD)

Animals were administered with D5W, 5 mg/kg, 7.5 mg/kg, 10 mg/kg and 15 mg/kg either orally or intravenously for five days (n=3/treatment, total n=30). Weight was recorded everyday during treatment and every other day following cessation of treatment. Blood counts were done on days 8, 11 and 21 after the first treatment. (The day treatment started was taken as day 0). Blood was withdrawn from the submandibular vein using lancets and the blood collected in hematocrit tubes. The count was made with Heska Vet Blood analyzer.

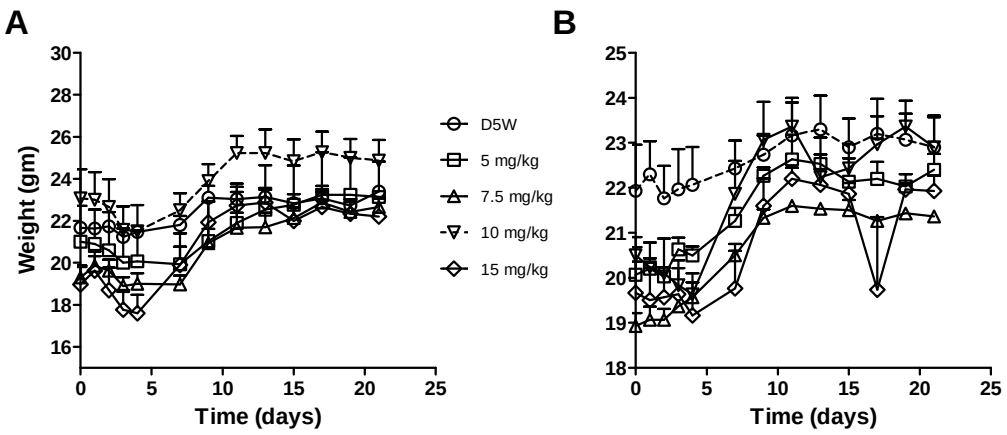


Figure A2- 1. Weights (gm) of nude mice treated with control (5% dextrose, D5W), 5, 7.5, 10 or 15 mg/kg AR-67 lactone A) intravenously and B) orally for five days. AR-67 was well tolerated following both oral and intravenous routes of administration.

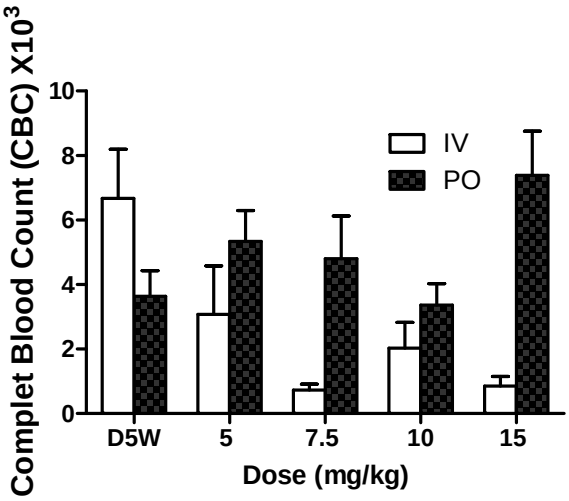


Figure A2- 2. Figure A2.2. Complete Blood counts (CBC $x10^3$) on day 8 in animals treated with control (D5W), 5, 7.5, 10 or 15 mg/kg AR-67 lactone either orally or intravenously for five days. Intravenous doses of AR-67 ≥ 7.5 mg/kg resulted in reduced CBC counts to below normal levels.

a) Xenograft studies

Non-small cell lung cancer (H460) tumor was implanted in the flank region of nu/nu mice (body weight 20-25 g). When tumors were palpable, mice (n=7 per treatment group) were randomized to four treatment groups and received

a). Control (5% dextrose in water (D5W) intravenously, b). 7.5 mg/kg/day intravenously for 5 days per cycle (1 cycle=21 days), c). 3.75 mg/kg/day intravenously for 10 days per cycle or d). 2.5 mg/kg/day intravenously for 15 days per cycle

The width and length of the tumors were measured using a caliper every other day for the duration of the study. Tumor volume was calculated using the following formula:

$V = PI * \frac{a * b^2}{6}$; V- is tumor volume in mm^3, PI=3.1416, a=size of the longest side in millimeters, b=size of the shortest side in millimeters. Mice were euthanized when their tumor volume reached 1500 mm^3 for humane reasons. Comparison between the median survivals of different treatment groups was done using Kaplan-Meir survival analysis.

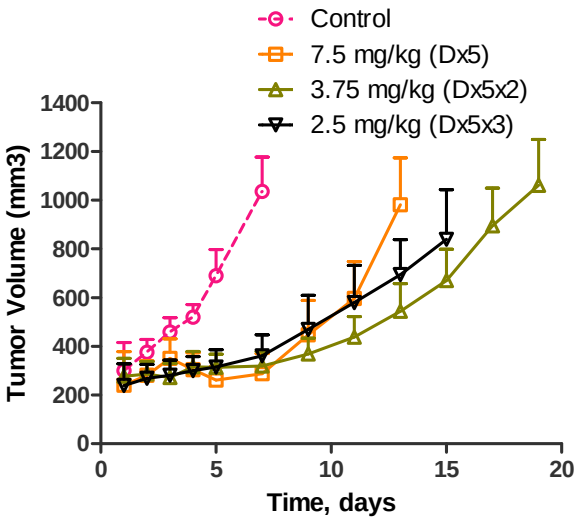


Figure A2- 3. Tumor volumes (mm^3) in animals nude mice treated with control or AR-67 intravenously (7.5 mg/kg for five days, 3.75 mg/kg for 10 days or 2.5 mg/kg for 15 mg/kg).

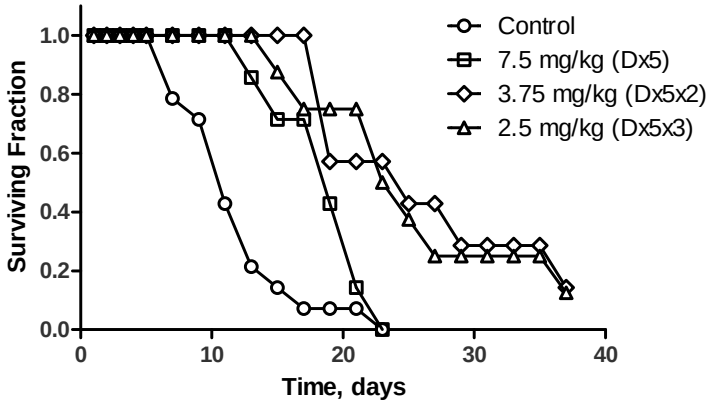


Figure A2- 4. Survival of H460 tumor bearing animals treated with control or AR-67 intravenously (7.5 mg/kg for five days, 3.75 mg/kg for 10 days or 2.5 mg/kg for 15 mg/kg). Median survival of mice receiving 3.75 mg/kg x10 days or 2.5 mg/kg x15 days was significantly longer than those receiving 7.5 mg/kg x5 days or control (5% dextrose in water).

APPENDIX III

ANIMAL EXPERIMENTS DATA

These studies were designed to assess 1) the effect of transporters on the AR-67oral bioavailability, 2) establish in-vivo/in-vitro correlations that would enable rapid screening of formulations in-vitro and allow for selection and further screening of formulations with potentially increased bioavailability, 3) to determine the bioavailability of selected formulations in capsule form. The studies were intended to be carried out in rats due to experimental limitations associated with administering capsules to mice. Initial studies in rats focused on the effect of transporters on the oral bioavailability of AR-67 and on establishing in-vivo/in-vitro correlations.

A. The effect of transporters on the oral bioavailability of AR-67 lactone and AR-67 carboxylate

Pharmacologic inhibition of efflux transporter proteins in the intestine was accomplished with GF120918 (N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide). This experimental agent has previously been shown to be an inhibitor of P-glycoprotein and BCRP. These proteins represent the two major efflux pumps expressed on the apical face of the intestinal epithelium and are known to transport xenobiotics, including other camptothecin analogs.

A1.Experimental design

To determine the effect of GF120918 (formulated in 40% PEG-300, 10% Tween-80 in D5W) on the oral bioavailability of AR-67 we administered the lactone or carboxylate forms formulated in SBE-β-CD (200:1, excipient: drug (w/w)). The dose of GF120918 was chosen following studies that determined the oral GF120918 dose required to obtain maximum area under the time-concentration curve (AUC) of total (lactone + carboxylate) AR-67 when AR-67 was administered orally as the lactone form.

To account for the increased liquid volume administered orally when animals were pretreated with GF120918, animals in control experiments received an equivalent volume of 5% dextrose in water (D5W) prior to receiving the drug. In addition, the effects of the vehicle/excipients used to solubilize GF120918 were assessed in separate studies administering the vehicle prior to AR-67. For proper assessment of bioavailability, the effect of GF120918 and its vehicle on the AUC was assessed following oral and intravenous administration of AR-67 as the lactone or carboxylate form.

Some additional experiments were carried out to determine the role of individual transporters and enzymes on the oral AUC of AR-67 lactone. Zosuquidar was used as an inhibitor of P-glycoprotein and novobiocin as an inhibitor of BCRP. Grapefruit juice and valproic acid pretreatment were used to assess the effects of CYP450 and UGT enzymes, respectively, on the oral AR-67 AUC.

The descriptions of the above experiments are listed in

Table 1. The experiment number can be used as a reference for experimental details and raw data.

A2. Summary of methods for pharmacokinetic experiments and sample analysis

All experiments were carried out in female Sprague-Dawley rats weighing approximately 300 grams. Each experiment was done in 3-6 rats. Drugs were administered via oral gavage or as an intravenous bolus injection in the tail vein. Blood samples (100-200 μL) were collected using saphenous vein bleeding and animals did not require anesthetization. Samples were collected at multiple time points from each animal and the concentration of AR-67 lactone and AR-67 carboxylate was assessed by HPLC after the system suitability criteria were met (i.e., three quality control standard solutions spanning a range of 2.5 – 300 ng/mL were prepared in plasma and analyzed. System suitability was met when their concentration was within 15% of the theoretical value)..

Table 1. Description of experiments designed to assess the effects of efflux transporters on oral bioavailability of AR-67 lactone and carboxylate in Sprague-Dawley rats (Table 1 continues on following page).

Exp#	Title/Objective	N	Notes
102	The effects of D5W (7.5 mL/kg) PO on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) PO in female SD rats		
100	The effects of GF120918 vehicle (40% PEG-300, 10% Tween-80 in D5W) PO pretreatment on bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO (2.5 mg/kg) in female SD rats	6	
101a	The effects of GF120918 vehicle (7.5 mL/kg) PO on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) PO in rats		Repeat of exp 100
106	Study on the effects of GF120918 (0.25 mg/kg) on the oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) PO in female SD rats		
127	Study on effects of GF120918 (0.25 mg/kg) on PK of SBE-β-CD based (200:1 E/D ratio) AR-67 lactone (2.5 mg/kg) PO in female SD rats		Repeat of exp 106
105	Study on the effects of GF (1.0 mg/kg) on the oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) PO in female SD rats		
101b	The effects of GF120918 (2.5 mg/kg) PO on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-		

	67 lactone (2.5 mg/kg) PO in female SD rats		
103a	The effects of GF120918 (2.5 mg/kg) PO on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) PO in female SD rats		Repeat of exp 101b
104b	Study the effects GF120918 (2.5 mg/kg) PO on PK profile following IV administration of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) in female SD rats		
103b	Study the effects of GF120918 (20.0 mg/kg) PO on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (20 mg/kg) PO in female SD rats		
104a	Study the effects of GF120918 vehicle (7.5 mL/kg) PO on PK profile following IV administration of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) in female SD rats		
107a	Study the effects of GF120918 vehicle (7.5 mL/kg) PO on PK profile following IV administration of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) in female SD rats		
107b	Study the effects GF120918 PO (2.5mg/kg) on PK profile following IV administration of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) in female SD rats		
Table 1 continued			
108	Study the effects of D5W (7.5 mL/kg) PO on PK profile following IV administration of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) in female SD rats		
131	Study on effects of GF120918 in 10% PEG-300 (2.5 mg/kg) on PK of SBE-β-CD based (200:1 E/D ratio) AR-67 lactone (2.5 mg/kg) PO in female SD rats		

135	Study on effects of GF120918 (2.5 mg/kg) IV on PK of SBE-β-CD based (200:1 E/D ratio) AR-67 lactone (2.5 mg/kg) PO in female SD rats		
152	Study on the effects of GF120918 (8.25 mg/kg) IV on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) in female SD rats		
113	Study on oral bioavailability of buffered SBE-β-CD (200:1, E:D) based AR-67 carboxylate (2.5 mg/kg) in female SD rats		
114	Study on oral bioavailability of unbuffered SBE-β-CD (200:1, E:D) based AR-67 carboxylate (2.5 mg/kg) in female SD rats		
119	The effect of GF120918 vehicle (7.5 mL/kg) PO on SBE-β-CD (200:1 E/D ratio) AR-67 carboxylate (2.5 mg/kg) PO in female SD rats		
125	The effect of GF120918 vehicle (7.5 mL/kg) PO on SBE-β-CD (200:1 E/D ratio) AR-67 carboxylate (2.5 mg/kg) PO in female SD rats		Repeat of exp 119
120	The effect of GF120918 vehicle (7.5 mL/kg) PO on SBE-β-CD (200:1 E/D ratio) AR-67 carboxylate (2.5 mg/kg) IV in female SD rats		
126	The effect of GF120918 vehicle (7.5 mL/kg) PO on SBE-β-CD (200:1 E/D ratio) AR-67 carboxylate (2.5 mg/kg) IV in female SD rats		Repeat of exp 120
117	Study on the effect of GF120918 (2.5 mg/kg) PO on bioavailability of SBE-β-CD based AR-67 carboxylate (2.5 mg/kg) PO in female SD rats		
128	Study on the effect of GF120918 (2.5 mg/kg) PO on bioavailability of SBE-β-CD based AR-67 carboxylate (2.5 mg/kg) PO in female SD rats		Repeat of exp 117

118	Study on effects of GF120918 (2.5 mg/kg) PO on PK of SBE-β-CD based (200:1 E/D ratio) AR-67 carboxylate (2.5 mg/kg) IV in female SD rats		
129	Study on effects of GF120918 (2.5 mg/kg) PO on PK of SBE-β-CD based (200:1 E/D ratio) AR-67 carboxylate (2.5 mg/kg) IV in female SD rats		Repeat of exp118
121	Study the effect of the selective P-gp inhibitor zosuquidar (20 mg/kg) PO on bioavailability of SBE-β-CD based AR-67 lactone (2.5 mg/kg) PO in female SD rats		
122	Study the effect of the selective P-gp inhibitor zosuquidar (20 mg/kg) PO on the kinetics of SBE-β-CD based AR-67 lactone (2.5 mg/kg) IV in female SD rats		
123	Study the effect of the selective Bcrp inhibitor novobiocin (50 mg/kg) PO on bioavailability of SBE-β-CD based AR-67 lactone (2.5 mg/kg) PO in female SD rats		
124	Study the effect of the selective Bcrp inhibitor novobiocin (50 mg/kg) PO on the kinetics of SBE-β-CD based AR-67 lactone (2.5 mg/kg) IV in female SD rats		
132	Study on effects of grapefruit juice double strength (10 mL/kg) on PK of SBE-β-CD based (200:1 E/D ratio) AR-67 lactone (2.5 mg/kg) PO in female SD rats		
134	Study on effects of UGT inhibition by valproic acid (200 mg/kg) on PK of SBE-β-CD based (200:1 E/D ratio) AR-67 lactone (2.5 mg/kg) PO in female SD rats		

A3.Data Analysis

Data were analyzed using non-compartmental analysis methods with WinNonlin v5.2 and GraphPad Prism 5.02. The area under the time-concentration curve was used to compare different formulations. Statistical analyses using t-test or ANOVA were carried out when appropriate.

A4.Results (effect of transporters on lactone and carboxylate)

In all cases the plasma concentration of AR-67 was primarily in the lactone form. A representative profile of AR-67 lactone and carboxylate pharmacokinetics is presented in

Figure 1. A summary of the treatment and resulting AUC and bioavailability is presented in

Table 1 and **Table 2**.

The oral bioavailability of AR-67 prepared in SBE-β-CD (200:1, E:D) was low in the rat (3.72% (±1.22%)). A dose dependent increase in the total AR-67 AUC was observed with increasing dose of GF120918. However, there was no significant difference in the oral AR-67 AUC between groups pretreated with 2.5 mg/kg and 20 mg/kg of GF120918 (886.72 (±155.95) vs. 1149.13 (±202.20 hr-ng/mL)). Inhibition of transporters with 2.5 mg/kg GF120918 significantly increased the total AR-67 bioavailability to 32.45% (±8.82%). However, some of that increase was a result of the GF120918 formulation, which alone increased the AR-67 bioavailability from 3.72% to 7.36% (±2.23%).

Given that GF120918 inhibits both P-glycoprotein and BCRP, some preliminary studies were conducted to assess the effect of single transporters. Zosuquidar (20 mg/kg) was used as an inhibitor of P-glycoprotein and novobiocin (50 mg/kg) as an inhibitor of BCRP. Zosuquidar treatment resulted in an increase of total AR-67 AUC, but its effect was relatively minor and resulted in an insignificant bioavailability increase of 6.70% (±2.41%) as compared to the control. The effect of novobiocin was also insignificant and pretreatment resulted in no apparent increase in the AUC.

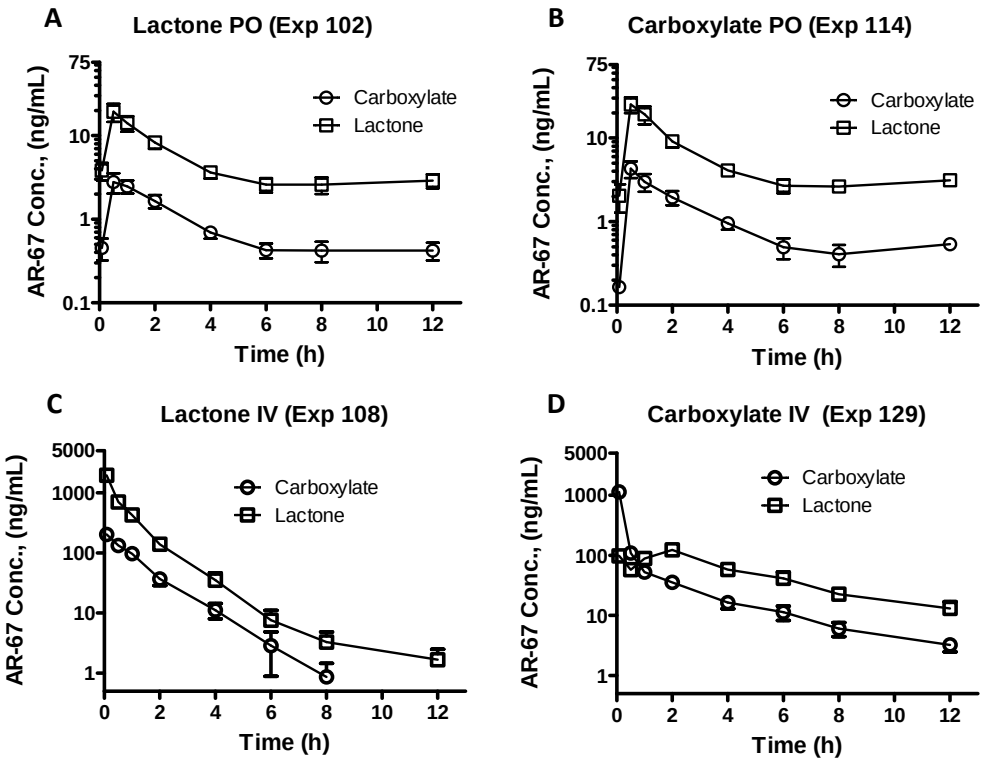


Figure 1. Typical AR-67pharmacokinetic profiles in plasma following (A, B) oral and (C, D) intravenous administration of (A, C) lactone and (B, D) carboxylate forms of AR-67.

Based on the low overall bioavailability, we also explored the potential contribution of metabolizing enzymes on the disposition of AR-67. Pretreatment with grapefruit juice is known to inhibit metabolizing enzymes of the CYP450 family while valproic acid has been shown to inhibit the effect of conjugating enzymes such as those of the UGT family. In these preliminary studies the effects of both inhibitors were minor.

The effect of transporter inhibition on AR-67 disposition following carboxylate administration was also assessed. The experimental treatments and the resulting AUC and bioavailability estimates are presented in **Table 3**. Carboxylate was administered in SBE-β-CD (200:1, E:D) and was either unbuffered or buffered with 0.5M sodium bicarbonate to sustain the carboxylate form in the rat stomach. The volume and buffer strength were

chosen based on preliminary studies that demonstrated that the stomach pH could be maintained ~pH 7 for 30-45 minutes (data not shown). The total bioavailability of oral carboxylate was 3.23% (±1.09%) when the carboxylate formulation was buffered with sodium bicarbonate to sustain the carboxylate form after oral administration vs. 4.33% (±1.29%) when the carboxylate solution was unbuffered. These bioavailability estimates were calculated using the intravenous lactone dose of AR-67. As with the lactone form, pretreatment with GF120918 (2.5 mg/kg) PO increased the oral bioavailability of total AR-67 to 36.48% (±5.15%) when bioavailability was estimated using the intravenous carboxylate dose (exp 118, 129) and 16.42% (±3.82%) when compared to intravenous lactone following GF120918 pretreatment. The latter is a rational comparison given that following oral administration of carboxylate the primary form measured in plasma was the lactone form (see

Figure 1and **Figure 2**, panel B).

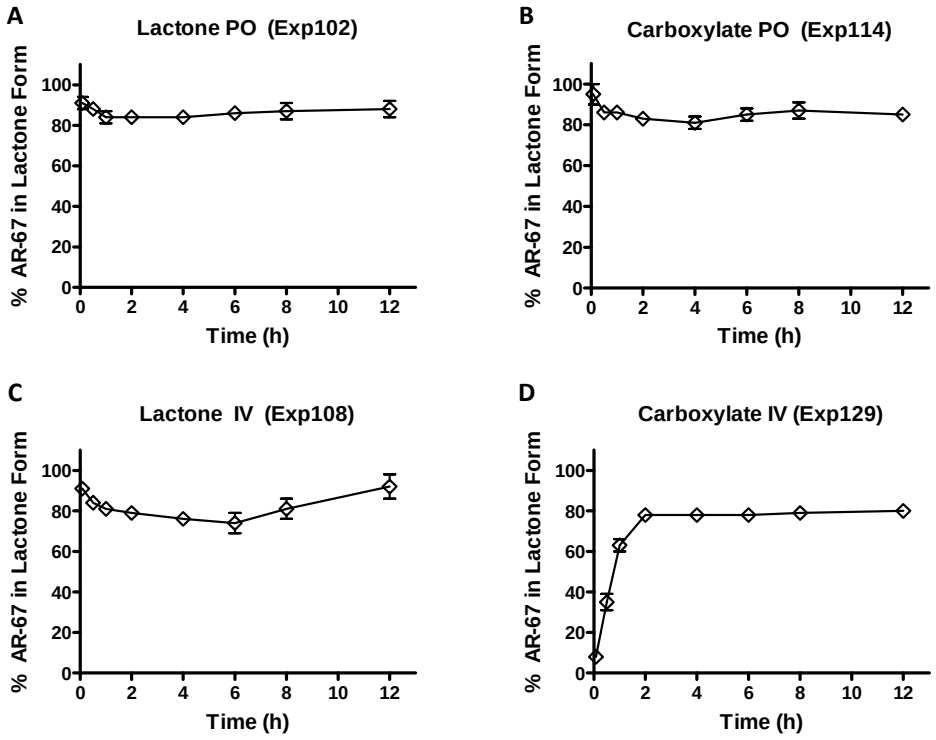


Figure 2. Typical lactone fraction profiles following (A, B) oral and (C, D) intravenous administration of (A, C) lactone and (B, D) carboxylate forms of AR-67.

A5.Conclusions

We conclude that AR-67 bioavailability in rats is low. AR-67 lactone is the major species observed in plasma after intravenous and oral administration. Following administration of the carboxylate form intravenously there is a rapid conversion to the lactone form within 2 hours. Although further work is needed to confirm this observation, it is likely that only the converted lactone is being absorbed after oral administration of carboxylate.

Pharmacologic inhibition of transporters increases the AUC when AR-67 is administered orally or intravenously. Although the bioavailability increases, it is only

~32%. This suggests that other unknown factors are more important contributors to the low bioavailability observed in the rat. Further studies are needed to determine the importance of BCRP and P-glycoprotein, but the minimal effect of zosuquidar, a validated P-glycoprotein inhibitor, suggests that P-glycoprotein may not be a major factor in the efflux of AR-67. An obvious implication for this result is the potential for increased partition in the brain, which is typically protected from xenobiotic entry via the action of P-glycoprotein expressed at the blood-brain barrier. This result along with the role of BCRP and intestinal metabolizing enzymes will have to be explored further in order to draw more definitive conclusions.

Table 2. The effect of transporter and metabolizing enzyme modulators on AR-67 lactone bioavailability.

Exp#	Pretreatment	Pretreatment Route	AR-67 Route	Average AUC (hr-ng/mL)	SE	F	SE	F estimated using exp#
102	D5W	PO	PO	68.64	21.61	3.72%	1.22%	108
108	D5W	PO	IV	1845.77	177.79			
100 , 101a	GF120918 vehicle	PO	PO	127.84	32.35	7.36%	2.23%	104a/107a
106, 127	GF120918 – 0.25 mg/kg	PO	PO	248.67	63.94			
105	GF120918 – 1.0 mg/kg	PO	PO	646.93	105.61			
101b, 103a	GF120918 – 2.5 mg/kg	PO	PO	886.72	155.95	32.45%	8.82%	104b/107b
103b	GF120918 – 20 mg/kg	PO	PO	1149.13	202.20			
104a, 107a	GF120918 vehicle	PO	IV	1736.02	288.80			
104b, 107b	GF120918 – 2.5 mg/kg	PO	IV	2732.88	566.53			
131	GF120918 (10% PEG-300)	PO	PO	289.01	28.67			
135	GF120918 – 2.5 mg/kg	IV	PO	244.14	57.99			
152	GF120918 – 8.25 mg/kg	IV	PO	324.85	63.32			
121	Zosuquidar – 20 mg/kg	PO	PO	155.68	55.95	6.70%	2.41%	122

122	Zosuquidar – 20 mg/kg	PO	IV	2323.42	49.04			
123	Novobiocin – 50 mg/kg	PO	PO	65.71	31.84	3.68%	1.94%	124
124	Novobiocin – 50 mg/kg	PO	IV	1784.77	373.85			
132	Grapefruit juice – 10 mL/kg	PO	PO	112.60	15.09	6.10%	1.01%	108
134	Valproic acid – 200 mg/kg	PO	PO	90.70	5.15	4.91%	0.55%	108

Table 3. The effect of transporter modulator GF120918 on AR-67 carboxylate bioavailability.

Exp#	Pretreatment	Pretreatment Route	AR-67 Route	Average AUC (hr-ng/mL)	SE	F	SE	F estimated using exp#
113	None	NA	PO (buffered)	59.67	19.31	3.23%	1.09%	108
114	None	NA	PO (unbuffered)	79.89	22.59	4.33%	1.29%	108
119, 125	GF120918 vehicle	PO	PO	104.14	12.31			
120, 126	GF120918 vehicle	PO	IV	833.48	120.44			
117, 128	GF120918	PO	PO	448.61	47.71	36.48%	5.15%	118, 129
						16.42%	3.82%	104b, 107b
118, 129	GF120918	PO	IV	1229.78	114.15			

B. Pharmacokinetic experiments to establish vitro/in-vivo correlations (IVIVC) in rats

Several formulations were prepared by Dr. Anderson's laboratory in order to test the capacity of different excipients to maintain supersaturation in-vitro by measuring the extent of AR-67 precipitation and relate that to oral bioavailability. All formulations were prepared by Dr. Anderson's lab and were provided on the day of the experiment.

B1. Experimental design

The descriptions of the above experiments are listed in **Table 4**. The experiment number can be used as a reference for experimental details and raw dataSeveral formulations were prepared in . order to achieve AR-67 supersaturation and maintain a solution. The extent of precipitation was to be compared with the bioavailability of each formulation. For animal experiments, the excipients were dissolved in simulated gastric fluid. Based on the results of the in-vitro studies the excipient concentrations were chosen to be 28:1 (E/D) as though to minimize the excipient that may be required to prepare capsules or tablets. A solution of AR-67 carboxylate was prepared in a basic solution and was mixed with simulated gastric fluid just prior to the gavage. Under these conditions, the carboxylate converts to the lactone and depending on the excipient it either stays in solution or precipitates. All animals received a dose of 2.5 mg/kg AR-67 lactone by oral gavage. Based on the results of experiments 109-112, the experiments with the best and worst excipient, in terms of maintaining supersaturation in-vitro, were repeated in male rats (exp 146 (Vitamin E-TPGS) and exp 147 (PEG-6000)).

Given the overall poor bioavailability, we also explored the potential for dose dependent bioavailability of the lactone and carboxylate forms. These experiments were performed using the SBE-β-CD (200:1 E/D ratio) formulation and the experiment number and descriptions are given in **Table 5**. The potential for using carboxylate as a means to prepare a spray-dried formulation was also explored by administering AR-67 carboxylate in Vit E-TPGS (28:1,E/D). As shown in **Table** 6 buffered and unbuffered

formulations of carboxylate in Vit E-TPGS were prepared. The buffer was included in an effort to maintain the carboxylate form once administered in the stomach via gavage.

B2. Summary of animal experiments and sample analysis

All experiments were carried out in Sprague-Dawley rats weighing approximately 300 grams. Please refer to section A2 for additional details.

B3. Data Analysis

Data were analyzed using non-compartmental analysis methods with WinNonlin v5.2 and GraphPad Prism 5.02. The area under the time-concentration curve was used to compare different formulations. Statistical analyses using t-test or ANOVA were carried out when appropriate.

Table 4. Description of experiments designed to establish in-vitro/in-vivo correlation (IVIVC) between degree of supersaturation/precipitation and oral bioavailability.

Exp#	Title/Objective		Notes
109	Study the oral bioavailability of PEG-6000 (28:1, E:D) based AR-67 lactone at 2.5 mg/kg in female SD rats		
110	Study the oral bioavailability of HP-CD (28:1, E:D) based AR-67 lactone at 2.5 mg/kg in female SD rats	3	
111	Study the oral bioavailability of SBE-β-CD (28:1, E:D) based AR-67 lactone at 2.5 mg/kg in female SD rats	3	
112	Study the oral bioavailability of vitamin E-TPGS (28:1, E:D) based AR-67 lactone at 2.5 mg/kg in female SD rats	3	
146	Study the oral bioavailability of E-TPGS (28:1 E/D ratio) based AR-67 lactone in male SD rats (2.5 mg/kg) PO	3	Repeat of exp112
47	Study the oral bioavailability of PEG-6000 (28:1 E/D ratio) based AR-67 lactone in male SD rats (2.5 mg/kg) PO	3	Repeat of exp109

Table 5. Effect of lactone or carboxylate dose on total AR-67 AUC.

Exp #	Title/Objective		Notes
151	Study the oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 carboxylate (2.5 mg/kg) PO in female SD rats	3	Carboxylate dose dependence
141	Study the oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 carboxylate (5 mg/kg) PO in female SD rats	3	Carboxylate dose dependence
138	Study the oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 carboxylate (10 mg/kg) PO in female SD rats	3	Carboxylate dose dependence
140	Study the oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (5 mg/kg) PO in female SD rats	3	Lactone dose dependence
137	Study the oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (10 mg/kg) PO in female SD rats	3	Lactone dose dependence

Note: Experiment 102 was used as the 2.5 mg/kg lactone dose.

Table 6. Effect of Vitamin E-TPGS on the bioavailability of AR-67 carboxylate.

Exp#	Title/Objective		Notes
115	Study the oral bioavailability of buffered Vitamin E-TPGS (28:1, E:D) based AR-67 carboxylate (2.5 mg/kg)		
116	Study the oral bioavailability of unbuffered Vitamin E-TPGS (28:1, E:D) based AR-67 carboxylate (2.5 mg/kg)		

B4. Results (IVIVC)

All formulations yielded low AUCs and consequently low oral bioavailability ranging from 2.38%-3.88% (see **Table 7**). Although there were some differences between the worst performing and best performing formulations (ranked from in-vitro results), these were minor and not as dramatic as would be expected based on the in-vitro behavior.

The potential for differential pharmacokinetics by gender was also explored because female rats have increased expression of some CYP450 enzyme isoforms. However, there was no significant difference in the oral bioavailability between male and

female rats administered the PEG-6000 and the Vitamin E-TPGS based formulations. The oral bioavailability in male rats was 3.04% for PEG-6000 and 3.52% for Vit E-TPGS based formulations.

The effect of dose on oral bioavailability was also examined (**Table 8**) but there was no significant difference with increasing dose of either the lactone or the carboxylate forms administered orally as determined by linear regression analysis of the bioavailability data (

and **Figure 3**).

The vitamin E-TPGS based carboxylate formulations (**Table 10**) yielded AUCs that were equivalent to the lactone formulation (exp 102) and the resulting bioavailability was 4.03% (±1.15%) for the unbuffered formulation and 3.63% (±0.59%) for the buffered one.

Table 7. Results of pharmacokinetic studies designed to establish in-vitro/in-vivo correlations. All excipients were in 28:1 (E/D) ratio.

Exp#	**Excipient**	**Rat gender**	**Average AUC (hr-ng/mL)**	**SE**	**F**	**SE**	**F estimated using exp#**
109	PEG-6000	Female	43.93	8.34	2.38%	0.51%	108 (AR-67 administered IV in SBE- β-CD, 200:1, [E/D])
110	HP-CD	Female	46.34	6.42	2.51%	0.42%	
111	SBE-β-CD	Female	52.46	11.11	2.84%	0.66%	
112	Vit E-TPGS	Female	71.69	7.47	3.88%	0.55%	
146	Vit E-TPGS	Male	64.91	5.58	3.52%	0.45%	
147	PEG-6000	Male	56.09	3.71	3.04%	0.36%	

Table 8. The effect of dose on AR-67 carboxylate and lactone bioavailability.

Exp#	AR-67 form/dose (mg/kg)	Average AUC (hr-ng/mL)	SE	F	SE	F estimated using exp#
151	Carboxylate (2.5)	135.57	39.49	7.34%	2.25%	108 (AR-67 administered IV in SBE-β-CD, 200:1, [E/D])
141	Carboxylate (5)	146.48	46.26	3.97%	1.31%	
138	Carboxylate (10)	323.11	39.66	4.38%	0.68%	
102	Lactone (2.5)	68.64	21.61	3.72%	1.22%	
140	Lactone (5)	183.97	41.78	4.98%	1.23%	
137	Lactone (10)	358.37	50.23	4.85%	0.83%	

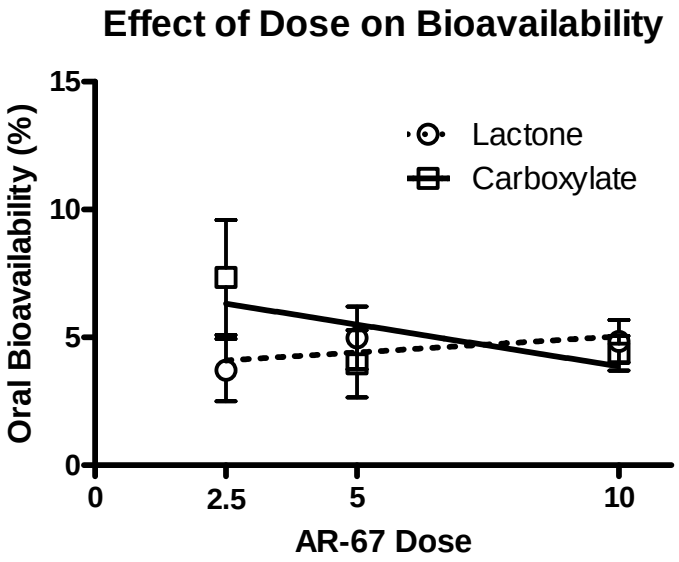


Figure 3. Effect of AR-67 dose on oral bioavailability. Lines represent linear regression (see

).

Table 9. Linear regression analysis of bioavailability data presented in Table 8.

Best-fit values	Lactone	Carboxylate
Slope	0.1254 ± 0.1311	-0.3266 ± 0.3538
Y-intercept when X=0.0	3.785 ± 0.8674	7.135 ± 2.340
X-intercept when Y=0.0	-30.18	21.85
1/slope	7.973	-3.062
95% Confidence Intervals		
Slope	-1.541 to 1.792	-4.822 to 4.169
Y-intercept when X=0.0	-7.236 to 14.81	-22.60 to 36.87
X-intercept when Y=0.0	-inf to +inf	-inf to +inf
Goodness of Fit		
R square	0.4777	0.4600
Sy.x	0.7082	1.911
Is slope significantly non-zero?		
F	0.9148	0.8518
DFn, DFd	1.000, 1.000	1.000, 1.000
P value	0.5142	0.5255
Deviation from zero?	Not Significant	Not Significant
Data		

Number of X values	3	3
Max number of Y replicates	1	1
Total number of values	3	3
Number of missing values	0	0

Table 10. Bioavailability of AR-67 carboxylate formulated in Vitamin E-TPGS.

Exp#	AR-67 form/dose (mg/kg)	Average AUC (hr-ng/mL)	SE	F	SE	F estimated using exp#
115	Carboxylate buffered	74.46	20.07	4.03%	1.15%	108 (AR-67 administered IV in SBE-β-CD, 200:1, [E/D])
116	Carboxylate unbuffered	67.03	8.71	3.63%	0.59%	

B5. Conclusions

The oral bioavailability of all formulations was extremely low. Establishing IV/IV correlations would be very difficult based on the narrow range of observed bioavailability among the different formulations. Interestingly, the Vit E-TPGS (28:1, E/D) formulation (exp 112) performed equivalently to the SBE-β-CD (200:1, E/D) formulation (exp 112).

There was no significant difference in the oral bioavailability between male and female rats administered the PEG-6000 and the Vitamin E-TPGS based formulations. There was no dose effect on the bioavailability in the doses tested (2.5 – 10 mg/kg).

The AR-67 carboxylate prepared in Vit E-TPGS (28:1, E/D) yielded similar bioavailability as the lactone formulation prepared in Vit E-TPGS (28:1, E/D) and SBE-β-CD (200:1, E/D) ratio. This was considered as a potential formulation for preparing solutions which could be spray dried. At this point, based on literature evidence, supersaturation was considered an important factor for increased bioavailability and the Vit E-TPGS was considered as a viable excipient to formulate AR-67carboxylate because of its capacity to maintain supersaturation of AR-67 at a lower excipient to drug ratio than SBE-β-CD.

In consultation with Arno Therapeutics we concluded that parallel studies in mice should also be considered because preliminary results from mouse pharmacokinetic

studies with cyclodextrin and cremophor/EL based formulations had indicated greater bioavailability. We concluded that mice could be used to establish IV/IV correlations and once we identify an optimum formulation, it could be tested in capsule form using the rat model.

C. Pharmacokinetic experiments to establish vitro/in-vivo correlations (IVIVC) in mice.

Formulations prepared in the laboratory of Dr. Anderson were tested for oral bioavailability in female C57BL/6 mice.

C1.Experimental design

The descriptions of the experiments are listed in **Table 11**. The experiment number can be used as a reference for experimental details and raw data. Several formulations were prepared in order to achieve AR-67 supersaturation and maintain a solution. The extent of precipitation was to be compared with the bioavailability of each formulation.

For animal experiments, the excipients were dissolved in simulated gastric fluid. Based on the results of the in-vitro studies the excipient concentrations were chosen to be 28:1 (E:D) as though to minimize the excipient that may be required to prepare capsules. Initial studies established baseline pharmacokinetics for the vitamin E-TPGS and SBE-β-CD formulations.

Excipients were chosen based on their capacity to maintain (Vit E-TPGS) or not (PEG-6000) supersaturation in-vitro. A propylene glycol/Vit E-TPGS (PG/TPGS) formulation was also tested due to its increased capacity to solubilize the lactone form. The effect of the excipient to drug ratio (Vit E-TPGS) on the bioavailability of AR-67 lactone was also studied.

C2.Summary of animal experiments and sample analysis

All experiments were carried out in female C57BL/6 mice weighing approximately 20-30 grams. Animals received a dose of 2.5 mg/kg or 5mg/kg AR-67 lactone by oral gavage. Please refer to section A2 for additional details.

C3.Data Analysis

Data were analyzed using non-compartmental analysis methods with WinNonlin v5.2 and GraphPad Prism 5.02. The area under the time-concentration curve was used to compare different formulations. Statistical analyses using t-test or ANOVA were carried out when appropriate.

Table 11. Description of pharmacokinetic studies in mice intended for in-vitro/in-vivo correlation (IVIVC) analysis.

Exp #	**Title/Objective**		**Notes**
159	Pharmacokinetics of SBE-β-CD based lactone (200:1, E:D) 5 mg/kg IV in female C57BL/6 mice	9	Baseline IV studies to establish oral bioavailability in mice
160	Pharmacokinetics of Vitamin E-TPGS based lactone (28:1, E:D) 5 mg/kg IV in female C57BL/6 mice		
158	Bioavailability of PG-TPGS based AR-67 lactone (3.2:28:1, E:D) 5 mg/kg PO in female C57BL/6 mice		
157	Bioavailability of PEG-6000 based AR-67 lactone (28:1, E:D) 5 mg/kg PO in female C57BL/6 mice		
162	Bioavailability of PEG-6000 based AR-67 lactone (28:1, E:D) 2.5 mg/kg PO in female C57BL/6 mice		
163	Bioavailability of Vit E-TPGS based AR-67 lactone (28:1, E:D) 2.5 mg/kg PO in female C57BL/6 mice		The effect of E:D (VitE-TPGS:AR-67)
167	Bioavailability of Vit E-TPGS based AR-67 lactone (56:1, E:D) 2.5 mg/kg PO in female C57BL/6 mice		
166	Bioavailability of Vit E-TPGS based AR-67 lactone (200:1, E:D) 2.5 mg/kg PO in female C57BL/6 mice		

C4.Results (IVIVC)

Intravenous administration of AR-67 lactone in mice yielded the typical biphasic pharmacokinetic profile of distribution/elimination that was also observed in rats. As shown in **Figure 4**, this was true for both the cyclodextrin and vitamin E based formulations, which yielded very similar AUCs (**Table 12**). **Figure 4** also demonstrates that regardless of the formulation, the lactone was the major form in plasma. Overall the total AR-67 concentrations were similar between the two formulations and the % lactone was 85-90% across all time points (**Figure 5**).

The oral bioavailability of the different formulations ranged between 9.7-15.7%. Detailed results are presented in **Table 12**. The oral bioavailability was estimated using the intravenous AUC obtained from the SBE-β-CD formulation (exp 159). This was decided because Vit E-TPGS yielded a slightly higher AUC, but that could be due to interaction between the excipient and efflux transporters in the kidney and liver (i.e., P-glycoprotein). As with the oral studies in rats, the lactone was the major species observed in plasma (refer to individual experiment reports. The pharmacokinetic profiles of total AR-67 resulting from administration of the PEG-6000 and Vit E-TPGS formulations (each 2.5 mg/kg, 28:1, E/D) are depicted in **Figure 6** and no apparent difference was observed between the two formulations, despite their significantly different capacity to maintain supersaturation.

There was an increase in the bioavailability of AR-67 formulated in Vit E-TPGS from ~11.5% in the 28:1 formulation to ~15.5% in the 56:1 and 200:1 E/D formulations (One-way ANOVA, with Bonferroni post-hoc correction for multiple comparisons).

Table 12. Results of pharmacokinetic studies designed to establish in-vitro/in-vivo correlations in mice administered AR-67 lactone in various formulations.

Exp#	**Excipient**	**E:D**	**Route/Dose (mg/kg)**	**Average AUC (hr-ng/mL)**	**SE**	**F**	**SE**	**F estimated using exp#**
159	SBE-β-CD	200:1	IV / 5.0	1325.08	104.25			
160	Vit E-TPGS	28:1	IV / 5.0	1500.97	22.25			
158	PG-TPGS	3.2:28:1	PO / 5.0	128.01	7.12	9.66%	0.93%	159
162	PEG-6000	28:1	PO / 2.5	70.39	9.22	10.62%	1.62%	
157	PEG-6000	28:1	PO / 5.0	194.83	25.02	14.70%	2.21%	
163	Vit E-TPGS	28:1	PO / 2.5	76.48	3.73	11.54%	1.07%	
167	Vit E-TPGS	56:1	PO / 2.5	103.91	5.62	15.68%	1.50%	
166	Vit E-TPGS	200:1	PO / 2.5	103.00	8.55	15.55%	1.78%	

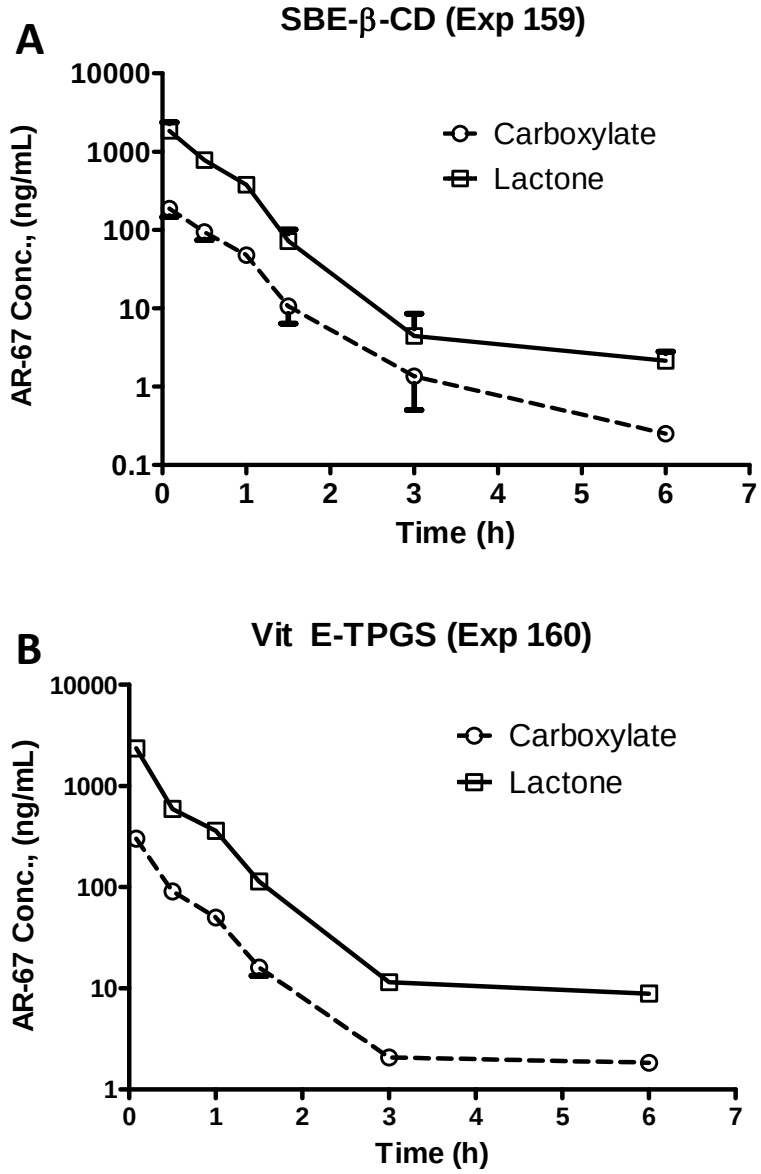


Figure 4. Pharmacokinetic profiles of AR-67 in mice following intravenous administration of AR-67 lactone formulated in (A) SBE-β-CD (200:1, E/D) and (B) Vitamin E-TPGS (28:1, E/D).

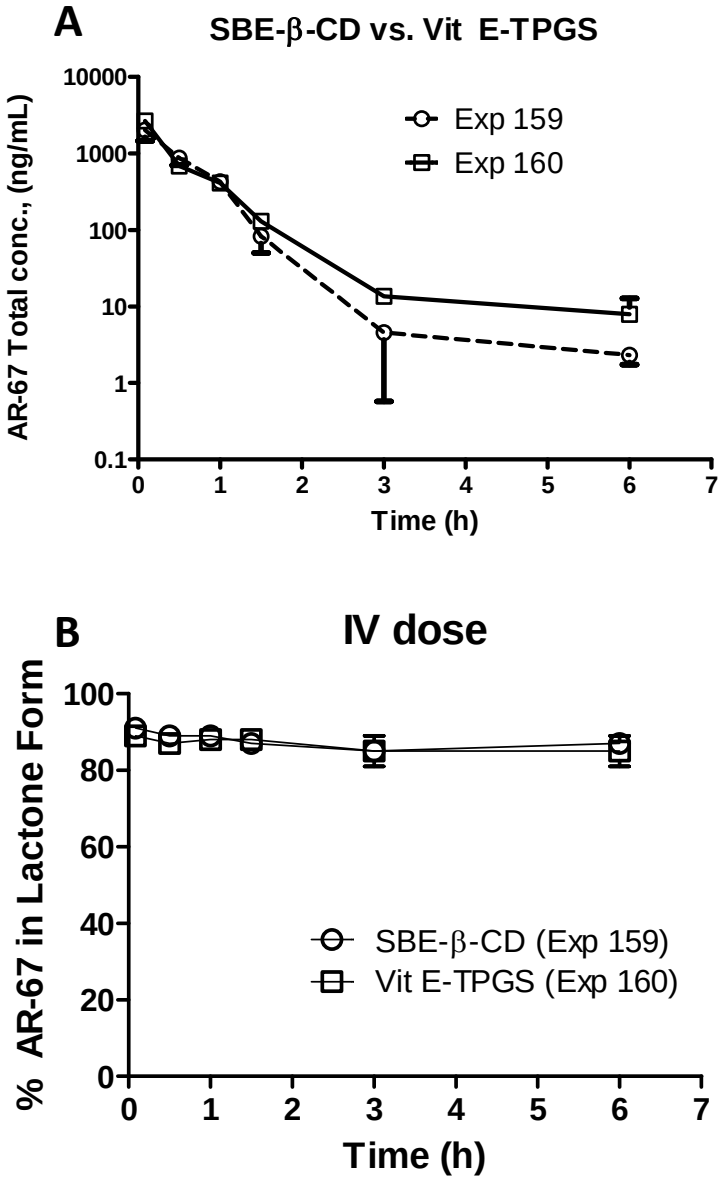


Figure 5. Comparative (A) total AR-67 pharmacokinetics and (B) lactone fraction of the SBE-β-CD and Vit E-TPGS formulations.

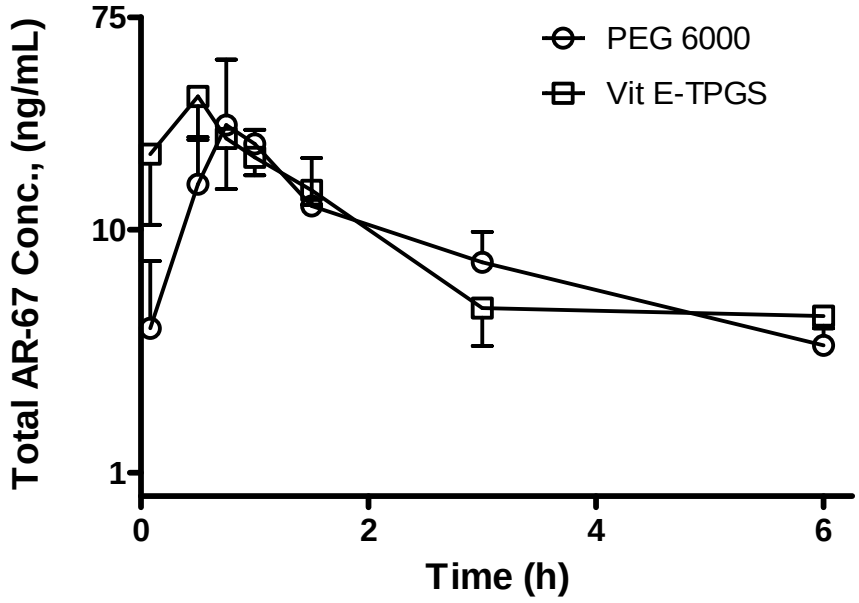


Figure 6. Pharmacokinetic profiles of AR-67 lactone administered orally (2.5 mg/kg) in PEG-6000 and Vit E-TPGS formulations (28:1, E/D).

C5.Conclusions

The Vitamin E-TPGS intravenous formulation appears to be equivalent to the SBE-β-CD formulation, but at a lower excipient to drug ratio. Additional studies to assess pharmacokinetic linearity must be done to ensure that the excipient does not influence AR-67 clearance at higher doses due to potential interactions between Vit E-TPGS and P-glycoprotein.

There was no obvious correlation between the in-vitro results (capacity to maintain supersaturation) and the oral bioavailability or AR-67 observed in mice. However, the bioavailability was 3 to 5-fold higher than that observed in rats (see section B). There was a significant increase in the oral bioavailability of AR-67 with the two formulations that had the higher E/D ratio of Vit E-TPGS:AR-67. However, additional studies will have to be done to verify these results given the importance of reducing the excipient in the formulation.

At this point, in consultation with Arno Therapeutics, we concluded that a vitamin E-TPGS based formulation, most likely based on carboxylate, was the formulation that we would pursue further.

Given the high lipophilicity of AR-67, we also considered the development of formulations that would facilitate the lymphatic transport and possibly increase oral bioavailability (see section D).

D. Pharmacokinetic experiments designed to assess bioavailability of formulations containing excipients that enhance lymphatic transport.

Several preliminary studies were carried out in mice using formulation prepared in Dr. Leggas' lab. Based on published evidence, the effects of oleic acid were tested in several experiments to assess its capacity to increase oral bioavailability through increased lymphatic transport of AR-67. However, in order to use this excipient with AR-67 carboxylate the sodium oleate had to be prepared. These latter formulations were prepared in the laboratory of Dr. Anderson. All formulations were tested for oral bioavailability in female C57BL/6 mice.

D1.Experimental design

The experiment number can be used as a reference for experimental details and raw data.

Baseline studies to assess the effect of food were carried out. Several lipid formulations were also prepared to test the effect of pretreatment and co-treatment on AR-67 carboxylate bioavailability. Finally, the effect of increasing amount of sodium oleate in Vit E-TPGS based formulations was tested using formulations that incorporated both excipients.

D2.Summary of animal experiments and sample analysis

All experiments were carried out in female C57/BL6 mice weighing approximately 20-30 grams. Animals received 2.5 or 5 mg/kg AR-67. Please refer to section A2 and to the experimental reports for additional details.

D3.Data Analysis

Data were analyzed using non-compartmental analysis methods with WinNonlin v5.2 and GraphPad Prism v5.02. The area under the time-concentration curve was used to compare different formulations. Statistical analyses using t-test or ANOVA were carried out when appropriate.

Table 13. Description of pharmacokinetic experiments designed to assess the effect of lipid excipients on the oral bioavailability of AR-67 in mice.

Exp #	Title/Objective	N	Notes
164	Bioavailability of SBE-β-CD based AR-67 lactone (200:1, E:D) 2.5 mg/kg PO in the absence of food during the experiment in female C57BL/6 mice	9	Determine food effects
165	Bioavailability of SBE-β-CD based AR-67 lactone (200:1, E:D) 2.5 mg/kg PO in the presence of food during the experiment in female C57BL/6 mice	9	
168	Study the effect of safflower oil pretreatment PO (5 mL/kg) on the absorption of E-TPGS based AR-67 lactone (2.5 mL/kg, 2.5 mg/kg) PO	9	Mr. Adane's formulations
169	Study the effect of oleic acid co-treatment PO (5 mL/kg) on the absorption of E-TPGS based AR-67 lactone (2.5 mL/kg, 2.5 mg/kg) PO	9	
170	(Control pretreatment) Study the effect of control (D5W) co-treatment PO (5 ml/kg) on the absorption of TPGS based AR-67 carboxylate (2.5ml/kg, 5 mg/kg) PO.	9	
171	Study the effect of oleic acid co-treatment PO (5 ml/kg) on the absorption of TPGS based AR-67 carboxylate (2.5ml/kg, 5 mg/kg) PO	9	
172	(Control pretreatment) Study the effect of control (D5W) co-	9	

	treatment PO (5 mL/kg) on the absorption of TPGS based AR-67 lactone (2.5 mL/kg, 5 mg/kg) PO		
173	Study the effect of oleic acid co-treatment PO (5 mL/kg) on the absorption of TPGS based AR-67 lactone (2.5mL/kg, 5 mg/kg) PO	9	
174	Study on the bioavailability of mixture of TPGS based AR-67 carboxylate (E:D ratio 56:1) premixed with oleic acid at 1:5, v/v (0.5 X) at dose of 5 mg/kg PO.	9	
175	Study the bioavailability of a mixture of TPGS based AR-67 carboxylate (E:D ratio 56:1) premixed with oleic acid at 2:1, v/v ratios (5X) at dose of 5mg/kg PO	9	
176	Study the absorption of 1:4 oleic acid:TPGS ratio (AR-67 carboxylate, 5 mg/kg) PO. [Formulation TX17-16-3]	9	Dr. Xiang's formulations
177	Study the absorption of 1:7 oleic acid:TPGS ratio (AR-67 carboxylate 5 mg/kg) PO. [Formulation TX17-15-2]	9	
180	Study the absorption of oleic acid:TPGS (1:7) based AR-67 carboxylate (5 mg/kg) PO. [Formulation TX17-16-2]	9	
181	Study the absorption of oleic acid:TPGS (1:10) based AR-67 carboxylate (5 mg/kg) PO [Formulation TX17-15-1]	9	

D4.Results (Sodium oleate / Vit E-TPGS)

As with the oral studies in rats, the lactone was the major species observed in mouse plasma. As shown in **Figure 7**, the effect of food was noticeable at the later time points, during which plasma concentrations were higher. However, the bioavailability was not significantly higher at 17.98% (±1.81%) in the fed group vs. 15.32% (±1.32%) in the fasted group.

For results of the preliminary experiments using oleic acid and safflower oil (168, 169, 170, 171, 172, 173, 174, 175). Although these data indicated that oleic acid had an effect on the oral bioavailability, the results from the preliminary experiments listed

above should be interpreted carefully because oleic acid was influencing the pH and perhaps altering the lactone/carboxylate ratio of AR-67 in the dosing solutions.

To overcome this limitation, formulations of carboxylate were prepared with various amounts of sodium oleate and Vit E-TPGS (50:1, E/D). The carboxylate was considered because of its higher solubility. These formulations yielded oral bioavailability in the order of ~20%. Although the amount of oleate varied from 9% (1:10, exp 181) to 20% (1:4, exp 176) of the total excipient weight the bioavailability was not significantly different among the four formulations tested. The pharmacokinetic profiles of these formulations are shown in

Figure 8.

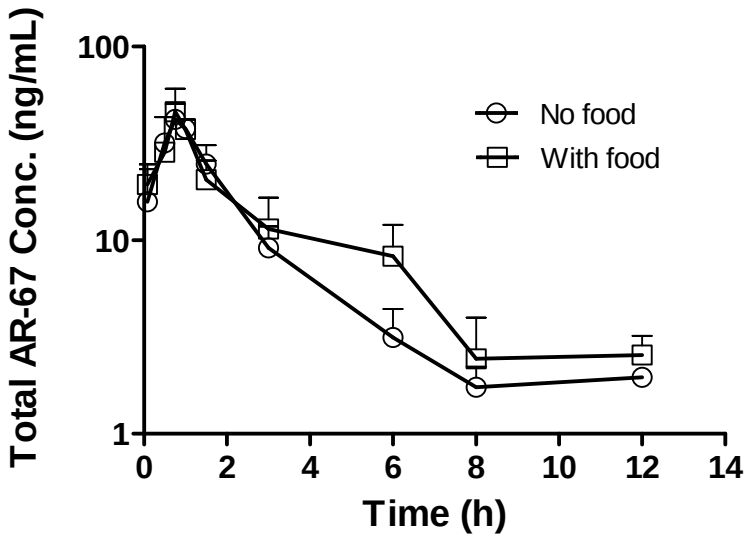


Figure 7. Pharmacokinetic profiles of AR-67 lactone administered orally to mice that were fasted or were allowed to feed throughout the experiment.

Table 14. Results of pharmacokinetic studies designed to establish the effect of food on the AUC of AR-67 lactone (SBE-β-CD, 200:1, E/D) administered orally to mice.

Exp#	**Food**	**Average AUC (hr-ng/mL)**	**SE**	**F**	**SE**	**F estimated using exp#**
164	No	101.48	3.56	15.32%	1.32%	159
165	Yes	119.13	7.43	17.98%	1.81%	159

Table 15. Results of pharmacokinetic studies designed to establish the effect of sodium oleate (formulated along with Vit E-TPGS, 50:1, E/D) on the bioavailability of AR-67 carboxylate administered orally to mice.

Exp#	**Sodium Oleate: Vit E-TPGS**	**Average AUC (hr-ng/mL)**	**SE**	**F**	**SE**	**F estimated using exp#**
176	1:4	285.00	15.90	21.51%	2.07%	159
77	1:7	269.20	56.30	20.32%	4.54%	
80	1:7	238.74	21.69	18.02%	2.17%	
81	1:10	270.44	18.62	20.41%	2.13%	

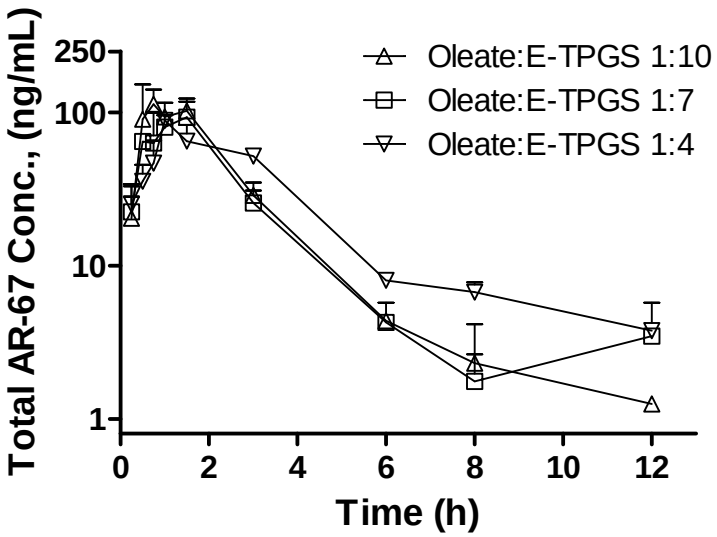


Figure 8. Pharmacokinetic studies in mice depicting plasma concentration profiles of total AR-67 after oral administration of AR-67 carboxylate formulated with sodium oleate and Vit E-TPGS.

D5.Conclusions

The effect of food has minimal contribution to the oral bioavailability of AR-67. Preliminary experiments indicated that oleic acid may facilitate increase in oral bioavailability. The carboxylate formulation was chosen because of its increased solubility. Sodium oleate increased the oral bioavailability, but there was not dose dependent effect. Although lipid excipients have been used to increase oral bioavailability via enhanced lymphatic transport, the mechanism by which oleate increased absorption is not clear. If the results were due to increased lymphatic transport we would have expected a low Cmax and delayed Tmax. However, in the oleate experiments the values of Tmax and Cmax were very similar to the formulations prepared without sodium oleate. Further studies will have to be undertaken to better understand this mechanism of increased absorption.

E. Pharmacokinetic experiments to assess bioavailability of amorphous solid and other miscellaneous formulations.

E1. Experimental design

Two amorphous solid formulations were provided to the University of Kentucky by Arno Therapeutics for assessment of bioavailability. These were coded as PMFD/1 and PMFD/2. Additional studies were undertaken to assess the bioavailability of formulations that contained either the exact composition of PMFD/1, but prepared at the University of Kentucky, or only select excipients found in PMFD/1. In addition, experiments to assess the bioavailability of AR-67 lactone, which was administered as a precipitate of carboxylate spiked into SGF or as suspended particles of different size were undertaken. The above experiments were carried out in mice.

A final set of experiments was done in rats to assess the bioavailability of PMFD/1 in capsules or as a suspension in SGF.

Summary of animal experiments and sample analysis

Experiments were carried out in female C57BL/6 mice weighing approximately 20-30 grams. Please refer to section A2 and to the experimental reports for additional details. Experiments in rats were carried out as described in section A2.

E2. Data Analysis

Data were analyzed using non-compartmental analysis methods with WinNonlin v5.2 and GraphPad Prism v5.02. The area under the time-concentration curve was used to compare different formulations. Statistical analyses using t-test or ANOVA were carried out when appropriate.

Table 16. Description of experiments designed to assess the bioavailability of amorphous solid formulations in mice.

Exp #	Title/Objective	N	Notes
178	Study the oral bioavailability of PMFD 576/1 suspended in ddH2O	9	Fine precipitate was observed after mixing with water
179	Study the oral bioavailability of PMFD 576/2 suspended in ddH2O	9	Fine precipitate was observed after mixing with water
182	Study the oral bioavailability of PMFD 576/1 suspended in water (pH 4.85)	9	Repeated exp 178
183	Study the oral bioavailability of PMFD 576/1 suspended in S.G.F. (pH 1.2)	9	Repeated exp 178/182 (different diluent) Fine precipitate was observed after mixing with SGF.
184	Study the absorption of 5 mg/kg PO AR-67 in PVP K30:Tween-80:AR-67 (71:6:23) spiked in SGF (precipitate formed)	9	Prepared PVP/Tween80 formulation at UK. Fine precipitate was observed after mixing with SGF.
185	Study the absorption of 5 mg/kg PO AR-67 in Vit E-TPGS:Tween-80:AR-67 (71:6:23) in simulated gastric fluid (pH 1.2) (remained in solution)	9	Substituted Vit E-TPGS for PVP. Remained in solution.
187	Study the absorption of AR-67 lactone in SGF containing PVP (K30) (E/D 3:1)	9	PVP only prepared at UK (precipitate)
196	Study the absorption of freshly prepared AR-67 lactone in SGF containing 0.065% Tween-80	9	Tween-80 only prepared at UK (precipitate)
197	Study the absorption of AR-67 lactone in SGF containing 0.065% Tween-80 (administer 2 hr after mixing with SGF) at 5 mg/kg PO	9	Tween-80 only prepared at UK (allow more extensive precipitation)

Table 17. Description of experiments designed to assess the effect of drug particle size on oral bioavailability (suspensions of AR-67).

Exp#	Title/Objective		Notes
186	Study the absorption of AR-67 lactone in SGF at 5 mg/kg PO.		Car boxylate spiked into pH 1.2 SGF (PO)
194	Study the absorption of AR-67 lactone (particle size between 45 μm and 75 μm) in SGF (5 mg/kg, 2.5 ml/kg) PO	9	
195	Study the absorption of AR-67 lactone (particle size between 75 μm and 150 μm) in SGF (5 mg/kg, 2.5 ml/kg) PO	9	

Table 18. Description of experiments designed to assess the bioavailability of amorphous solid formulations administered as a suspension to rats.

Exp#	Title/Objective	N	Notes
192	Study on the oral bioavailability of PMFD 576/1 AR-67 lactone suspension at the dose of 5.0 mg/kg PO in rats		Dissolved in SGF.
193	Study on the oral bioavailability of capsule filled with PMFD 576/1 AR-67 lactone at the dose of 5.0 mg/kg PO in rats	3	

E3. Results

The oral bioavailability of the PMFD 576/1 formulation (exp 178) dissolved in ddH20 was among the highest observed yet (31.40±3.91%). The PMFD 576/2 formulation (exp 179) also displayed relatively good bioavailability (24.65±6.49%). These results of the PMFD 576/1 formulation were confirmed by a second experiment with bioavailability of 30.01±5.87% (exp 182). Subsequently, we sought to determine the bioavailability of a formulation that was prepared at the University of Kentucky using the exact composition of PMFD 576/1, but given as a solution in SGF (exp 184). As a control experiment we dissolved the amorphous solid (PMFD 576/1) in SGF (exp 183). The

results were practically identical with a biovailability of 28.22±2.88% for the University of Kentucky prepared formulation vs. 30.28±3.17% for the PMFD 576/1 dissolved in SGF.

To assess the importance of PVP, which in vitro had demonstrated poor capacity to maintain supersaturation, we substituted it with Vit E-TPGS in the PMFD/1 formulation (exp 185). The bioavailability of this formulation was 24.92±2.71%. Subsequently, we tested formulations that only contained PVP (3:1, E/D) or Tween-80 (0.065%). The PVP formulation gave a bioavailability of 22.83±2.37% (exp187). The Tween-80 formulation gave a bioavailability of 13.21±2.81% when administered immediately after mixing with SGF (exp 196) and 11.71±1.87% when allowed to stand for 2-hrs after mixing with SGF (exp 197). These results along with the AUC from each experiment are presented in **Table 19**.

The increased bioavailability results of formulations that formed a precipitate upon mixing with SGF prompted us to assess the bioavailability of lactone without any excipients. The lactone was prepared from a carboxylate solution, which was spiked into SGF and rapidly precipitated (exp 186). Interestingly, this formulation yielded a bioavailability of 24.38±2.89%. We also evaluated the bioavailability of solid AR-67 lactone particles, which were sieved from bulk drug available at the University of Kentucky. In these experiments we tested the bioavailability of particles that were 45-75 μm and 75-150 μm. The bioavailability of both of these suspensions (in SGF) was low (~ 5-6%). These results along with the AUC from each experiment are presented in

Table 20.

A final set of experiments were undertaken in rats to assess the bioavailability of PMFD/1 prepared as a suspension in SGF (exp 192) or filled into capsules (exp 193). Unfortunately, the bioavailability of both formulations was low (3.52±1.02% for the suspension and 4.72±2.35% for the capsules).

Table 19. Results of experiments designed to assess the bioavailability of amorphous solids formulations in mice.

Exp#	Excipient Dose (mg/kg)	Average AUC (hr-ng/mL)	SE	F	SE	NOTES
178	PMFD 576/1 – (5)	416.14	40.17	31.40%	3.91%	dd H2O
179	PMFD 576/2 – (5)	326.60	82.12	24.65%	6.49%	dd H2O
182	PMFD 576/1 – (5)	397.68	71.22	30.01%	5.87%	dd H2O
183	PMFD 576/1 – (5)	401.20	27.72	30.28%	3.17%	S.G.F. (pH 1.2)
184	PVP K30:Tween-80:AR-67 (71:6:23) – (5)	373.92	24.31	28.22%	2.88%	S.G.F. (pH 1.2)
185	Vit E-TPGS:Tween-80:AR-67 (71:6:23) – (5)	330.25	24.77	24.92%	2.71%	S.G.F. (pH 1.2)
187	PVP (K30) (E/D 3:1) – (5)	302.53	20.44	22.83%	2.37%	S.G.F. (pH 1.2)
196	0.065% Tween-80 – (5)	175.10	34.62	13.21%	2.81%	S.G.F. (pH 1.2)
197	0.065% Tween-80 – (5)	155.17	21.63	11.71%	1.87%	S.G.F. (pH 1.2)

Table 20. Results of experiments designed to assess the effect of drug particle size on oral bioavailability (suspensions of AR-67).

Exp #	Excipient / AR-67 Form	Average AUC (hr-ng/mL)	SE	F	SE	NOTES
186	None / [Carboxylate spiked into SGF (pH 1.2)] (5mg/kg)	323.06	28.57	24.38%	2.89%	Precipitates as lactone

194	None / 45 - 75 µm particles Lactone (5 mg/kg)	65.55	13.47	4.95%	1.09%	Sieved bulk material
195	None / 75 - 150 µm particles Lactone (5 mg/kg)	83.67	14.08	6.31%	1.17%	Sieved bulk material

Table 21. Results of experiments designed to assess the bioavailability of amorphous solid formulations administered as a suspension to rats.

Exp#	Formulation	Average AUC (hr-ng/mL)	SE	F	SE	NOTES
192	PMFD 576/1	130.02	35.42	3.52%	1.02%	Suspension in SGF (pH 1.2)
193	PMFD 576/1	174.20	85.28	4.72%	2.35%	Capsule

E4. Conclusions

The experiments demonstrate that there is no obvious in-vitro/in-vivo correlation in terms of the degree of supersaturation and bioavailability. The PMFD/1 formulation provided the highest bioavailability although it precipitated after mixing with pH 1.2 SGF. The combination of PVP and Tween-80 yielded the highest bioavailability. Studies with the carboxylate alone, which immediately precipitates to lactone in simulated gastric fluid, demonstrated that the bioavailability is in the order of 24%. Similarly studies with PVP alone demonstrated bioavailability of ~23%, while formulation of AR-67 with Tween-80 alone decreased the biovailability to ~12-13%. This suggests that PVP alone has no effect, whereas Tween-80 alone suppresses bioavailability.

Together these results suggest that the nature of the precipitate formed in the formulation that contains PVP/Tween-80 allows the highest bioavailability possibly due to the slow dissolution and a steady state availability of the lactone. The precipitate formed from the carboxylate spiked into SGF behaves similarly. Probably the lactone particles that precipitate provide a continuous source of lactone.Whereas when excipients are included to support supersaturation, the lactone may convert to the carboxylate, which is not likely to be absorbed due to its negative charge.

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Rat PK Study

(March 5th, 08)

Effects of vehicle pretreatment on oral bioavailability of SBE-β-CD based AR-67 lactone in rats

---

Expt # 100

12/02/2008

Objective

To determine the effects of vehicle (40% PEG-300, 10% Tween-80 in D5W) pretreatment on oral bioavailability and PK profile of SBE-β-CD based AR-67 lactone PO in female SD rats. This will serve as a control for GF120918 pretreatment group.

Expt 100: Study the effects of vehicle (40% PEG-300, 10% Tween-80 in D5W) PO pretreatment on bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO (2.5 mg/kg) in female SD rats.

Method

Animal treatment and sample processing

Expt 100, Rats (#100-#105, n=6) were dosed with 2.5 mg/kg SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO 5 min after vehicle administration at 7.5 ml/kg PO.

Dosing solution:

AR-67 lactone = 1.0 mg/mL

Vehicle (used to solubilizer GF):

40% PEG-300, 10% Tween-80 in D5W

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 100

Sequence name: 20AC-030708-Rat PK Expt 100 Reprocessing with 10AD method.seq

Method: 10AD-121807-Rat plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/AR-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/AR-67/Rat PK). Plots were made with Prism while nocompartmental analysis was made using Winnonlin v5.2.

Results

Table. Pharmacokinetic parameters estimated using the noncompartmental method in Winnonlin v5.2.

Expt	ID	Parameter	Units	Estimate	Average/ Median[1]	SD[2]/range
100	100	AUCall	h*ng/mL	118.0916	126.91	33.22
100	101	AUCall	h*ng/mL	85.6844		
100	102	AUCall	h*ng/mL	184.1765		
100	103	AUCall	h*ng/mL	114.0443		
100	104	AUCall	h*ng/mL	141.552		
100	105	AUCall	h*ng/mL	117.8906		
100	100	Cmax	ng/mL	37.59	27.09	7.34
100	101	Cmax	ng/mL	15.76		
100	102	Cmax	ng/mL	29.11		
100	103	Cmax	ng/mL	24.83		
100	104	Cmax	ng/mL	30.89		
100	105	Cmax	ng/mL	24.38		
100	100	Tmax	h	2	2	1--4
100	101	Tmax	h	4		
100	102	Tmax	h	2		
100	103	Tmax	h	2		
100	104	Tmax	h	1		
100	105	Tmax	h	2		

[1] Median was used for Tmax. All other parameters use the arithmetic mean.

[2] Standard deviation.

Figures

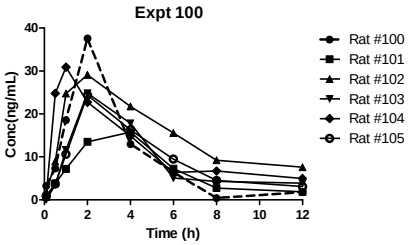


Fig. 1a. Plasma total DB-67 levels in SD rats treated with vehicle (PEG, tween80, D5W) PO 5 min before 2.5 mg/kg DB-67 lactone PO

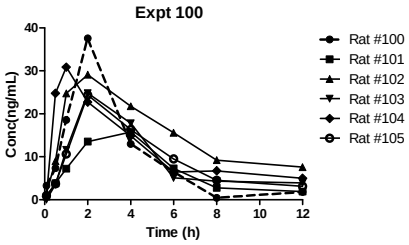


Fig. 1b. Semi-log plot of plasma total DB-67 levels in SD rats treated with vehicle (PEG, tween80, D5W) PO 5 min before 2.5 mg/kg DB-67 lactone PO

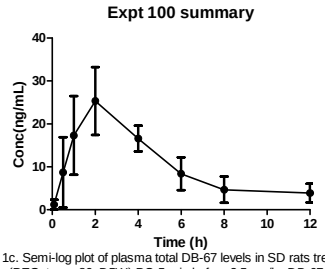


Fig. 1c. Semi-log plot of plasma total DB-67 levels in SD rats treated with vehicle (PEG, tween80, D5W) PO 5 min before 2.5 mg/kg DB-67 lactone PO

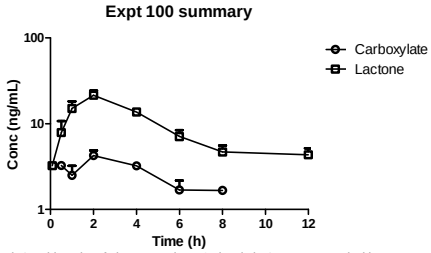


Fig. 1d. Semi-log plot of plasma total DB-67 levels in SD rats treated with vehicle (PEG, tween80, D5W) PO 5 min before 2.5 mg/kg DB-67 lactone PO

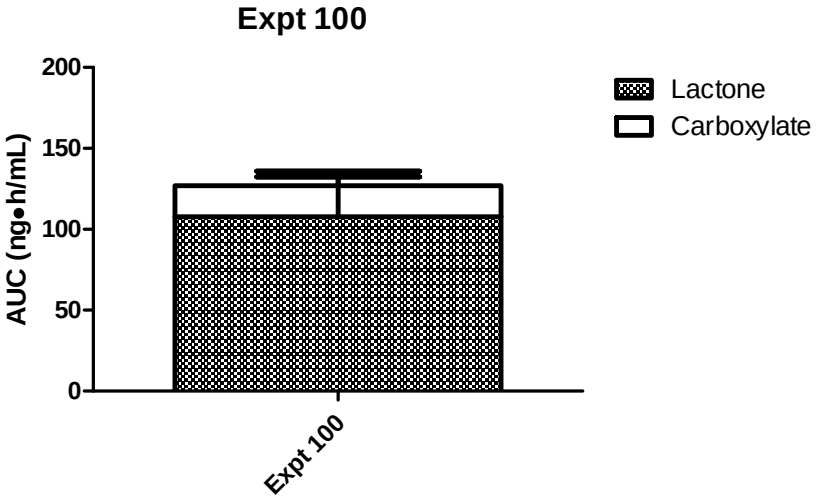


Fig. 2. AUCs of lactone and carboxylate forms of DB-67 in SD rats following PO administrtation of DB-67 lactone at 2.5 mg/kg 5 after vehicle pretreatment (7.5 ml/kg) PO

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 100

Raw Data

Expt #	Rat #	Time (h)	Carboxylate (ng/mL)	Lactone(ng/mL)	Total AR-67 (ng/mL)
100	100	0.083	0	3.24	3.24
100	100	0.5	0.6	6.71	7.31
100	100	1	2.13	16.41	18.54
100	100	2	6.62	30.97	37.59
100	100	4	2.22	10.75	12.97
100	100	6	0.76	5.7	6.46
100	100	8	0.31	0.09	0.4
100	100	12	0.01	1.78	1.79
100	101	0.083	0	0.7	0.7
100	101	0.5	0.03	3.78	3.81
100	101	1	0.68	6.55	7.23
100	101	2	1.75	11.75	13.5
100	101	4	2.43	13.33	15.76
100	101	6	0.97	6.3	7.27
100	101	8	0.21	2.54	2.75
100	101	12	0	1.89	1.89
100	102	0.083	0	1.52	1.52
100	102	0.5	0.7	8.24	8.94
100	102	1	2.98	21.77	24.75
100	102	2	3.08	26.03	29.11
100	102	4	3.51	18.25	21.76
100	102	6	2.63	12.95	15.58
100	102	8	1.67	7.54	9.21
100	102	12	0.89	6.69	7.58
100	103	0.083	0	0	0
100	103	0.5	0	3.51	3.51
100	103	1	1.23	10.37	11.6
100	103	2	3.77	21.06	24.83
100	103	4	3.81	14	17.81
100	103	6	0.9	4.2	5.1
100	103	8	0.51	3.8	4.31
100	103	12	0.34	3.53	3.87
100	104	0.083	0	0.71	0.71
100	104	0.5	3.26	21.51	24.77
100	104	1	5.06	25.83	30.89
100	104	2	4.38	18.34	22.72
100	104	4	2.8	12.09	14.89
100	104	6	1.02	5.41	6.43
100	104	8	1.06	5.67	6.73
100	104	12	0.69	4.28	4.97
100	105	0.083	0	0.96	0.96
100	105	0.5	0	3.72	3.72
100	105	1	1.12	9.5	10.62

100	105	2	3.4	20.98	24.38
100	105	4	2.82	13.5	16.32
100	105	6	1.41	8.07	9.48
100	105	8	0.63	3.9	4.53
100	105	12	0.25	2.86	3.11

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Rat PK Study

(March 11th, 08)

Effects of oral GF120918 on oral bioavailability of SBE-β-CD based AR-67 lactone in rats

---

Expt # 101

Objective

To determine oral GF120918 pretreatment on oral bioavailability of SBE-β-CD based AR-67 lactone in female SD rats. The vehicle pretreatment group will serve as a control for GF120918 pretreatment to study the effects of vehicle on AR-67 oral absorption. The vehicle pretreatment group is also a repeat of experiment 100 to see the reproducibility of the system.

Expt 101a: Study the effects of vehicle (7.5 mL/kg) PO on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) PO in rats

Expt 101b: Study the effects of GF120918 (2.5 mg/kg) PO on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) PO in rats

Method

Animal treatment and sample processing

Expt 101a, Rats (#121-#123, n=3) were dosed with 2.5 mg/kg SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO 5 min after vehicle pretreatment at 7.5 ml/kg PO.

Expt 101b, Rats (#124-#126, n=3) were dosed with 2.5 mg/kg SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO 5 min after GF pretreatment at 2.5 mg/kg PO.

Dosing solution:

AR-67 lactone = 1.0 mg/mL

GF120918 solution:

GF120918 was dissolved in vehicle (40% PEG-300, 10% Tween 80 in D5W) within 1 hour before experiment.

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 101a and 101b

Sequence name: 20AC-031408-Rat PK Expt 101 Reprocessing with 20AC method.seq

Method: 10AD-121807-Rat plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/AR-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/AR-67/Rat PK). Plots were made with Prism while nocompartmental analysis was made using WinNonlin v5.2.

Results

Tables: Pharmacokinetic parameters estimated using the noncompartmental method in Winnonlin v5.2

Expt 101

Expt	ID	Parameter	Units	Estimate	Average/ Median[3]	SD[4]/range
101a	121	AUCall	h*ng/mL	91.7871	128.77	55.51
101a	122	AUCall	h*ng/mL	192.6		
101a	123	AUCall	h*ng/mL	101.9193		
101a	121	Cmax	ng/mL	13.67	27.37	25.89
101a	122	Cmax	ng/mL	57.23		
101a	123	Cmax	ng/mL	11.21		
101a	121	Tmax	h	2	2	2--4
101a	122	Tmax	h	2		
101a	123	Tmax	h	4		
101b	124	AUCall	h*ng/mL	713.7297	738.63	68.21
101b	125	AUCall	h*ng/mL	686.3804		
101b	126	AUCall	h*ng/mL	815.7946		
101b	124	Cmax	ng/mL	136.02	139.20	9.35
101b	125	Cmax	ng/mL	131.86		
101b	126	Cmax	ng/mL	149.73		
101b	124	Tmax	h	2	2	2
101b	125	Tmax	h	2		
101b	126	Tmax	h	2		

Figures

[3] Median was used for Tmax. All other parameters used the arithmetic mean.

[4] Standard deviation.

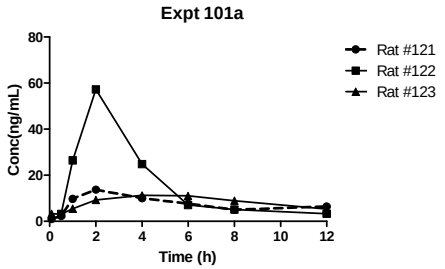


Fig. 1a. Plasma total DB-67 levels in SD rats treated with vehicle (PEG, tween80, D5W) PO 5 min before 2.5 mg/kg DB-67 lactone PO

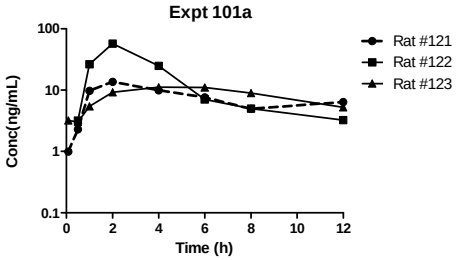


Fig. 1b. Semi-log plot of plasma total DB-67 levels in SD rats treated with vehicle (PEG, tween80, D5W) PO 5 min before 2.5 mg/kg DB-67 lactone PO

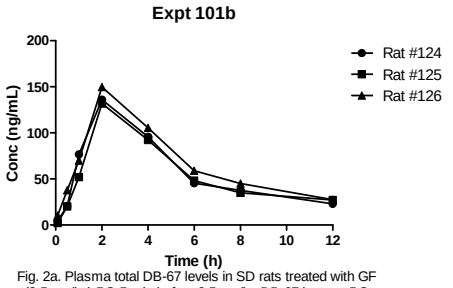


Fig. 2a. Plasma total DB-67 levels in SD rats treated with GF (2.5 mg/kg) PO 5 min before 2.5 mg/kg DB-67 lactone PO

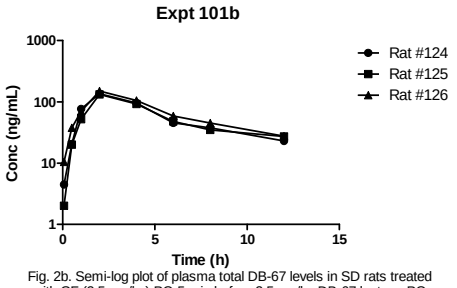


Fig. 2b. Semi-log plot of plasma total DB-67 levels in SD rats treated with GF (2.5 mg/kg) PO 5 min before 2.5 mg/kg DB-67 lactone PO

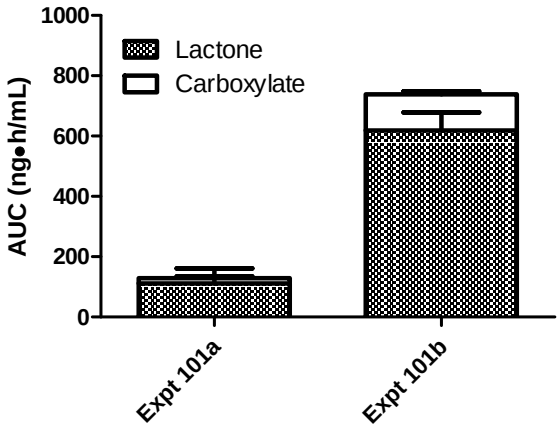


Fig. 3. AUCs of lactone and carboxylate forms of DB-67 in SD rats following PO administrtation of DB-67 lactone 5 min after administration of vehicle PO (expt 101a) and GF PO (expt 101b) at 2.5 mg/kg

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 101

Raw Data

Expt #	Rat #	Time (h)	Carboxylate (ng/mL)	Lactone (ng/mL)	Total AR-67 (ng/mL)
101	121	0.083	0	0.99	0.99
101	121	0.5	0.16	2.14	2.3
101	121	1	0.91	8.81	9.72
101	121	2	2.15	11.52	13.67
101	121	4	1.74	8.2	9.94
101	121	6	1.25	6.34	7.59
101	121	8	0.68	4.27	4.95
101	121	12	1.11	5.28	6.39
101	122	0.083	5.49	Null	Null
101	122	0.5	0	3.19	3.19
101	122	1	0	26.44	26.44
101	122	2	7.92	49.31	57.23
101	122	4	3.44	21.42	24.86
101	122	6	0.96	6.09	7.05
101	122	8	0.71	4.3	5.01
101	122	12	0.35	2.89	3.24
101	123	0.083	0.06	3.15	3.21
101	123	0.5	0.25	2.78	3.03
101	123	1	0.81	4.64	5.45
101	123	2	1.27	7.95	9.22
101	123	4	1.56	9.65	11.21
101	123	6	1.72	9.31	11.03
101	123	8	1.31	7.62	8.93
101	123	12	0.46	4.81	5.27
101	124	0.083	0.44	4.05	4.49
101	124	0.5	2.65	17.9	20.55
101	124	1	9	67.82	76.82
101	124	2	20.35	115.67	136.02
101	124	4	20.39	75.4	95.79
101	124	6	8.72	36.66	45.38
101	124	8	6.79	30.85	37.64
101	124	12	3.22	19.92	23.14
101	125	0.083	0.08	1.95	2.03
101	125	0.5	2.17	18	20.17
101	125	1	6.31	45.63	51.94
101	125	2	17.39	114.47	131.86
101	125	4	17.02	75.29	92.31
101	125	6	8.8	39.33	48.13
101	125	8	6.13	28.71	34.84
101	125	12	3.19	24.05	27.24
101	126	0.083	1.04	9.45	10.49
101	126	0.5	4.05	33.67	37.72
101	126	1	9	60.91	69.91

101	126	2	19.6	130.13	149.73
101	126	4	18.16	87.24	105.4
101	126	6	10.39	48.46	58.85
101	126	8	7.98	37.09	45.07
101	126	12	4.34	23.23	27.57

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Rat PK Study

(March 13th, 08)

Effects of D5W pretreatment on oral bioavailability of SBE-β-CD based AR-67 lactone in rats

---

Expt # 102

12/02/2008

Objective

To determine effects of D5W pretreatment on oral bioavailability of SBE-β-CD based AR-67 lactone in rats. This will serve as a control for GF120918 and vehicle pretreatment group.

Expt 102: Study the effects of D5W (7.5 mL/kg) PO on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) PO in female SD rats

Method

Animal treatment and sample processing

Expt 102, Rats (#131-#136, n=6) were dosed with 2.5 mg/kg SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO 5 min after administration of D5W at 7.5 ml/kg PO.

Dosing solution:

AR-67 lactone = 1.0 mg/mL

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 102

Sequence name: 20AC-031408-Rat PK Expt 102.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while nocompartmental analysis was made using WinNonlin v5.2.

Results

Tables

Expt 102

ID	Parameter	Units	Estimate	Average/ Median[5]	SD[6]/range
131	AUCall	h*ng/mL	88.1589	68.643	21.609
132	AUCall	h*ng/mL	76.0564		
133	AUCall	h*ng/mL	47.5229		
134	AUCall	h*ng/mL	40.205		
135	AUCall	h*ng/mL	93.8877		
136	AUCall	h*ng/mL	66.0274		
131	Cmax	ng/mL	25.75	22.218	12.913
132	Cmax	ng/mL	15.93		
133	Cmax	ng/mL	8.74		
134	Cmax	ng/mL	17.53		
135	Cmax	ng/mL	46.08		
136	Cmax	ng/mL	19.28		
131	Tmax	h	0.5	0.5	0.5-1
132	Tmax	h	0.5		
133	Tmax	h	1		
134	Tmax	h	0.5		
135	Tmax	h	0.5		
136	Tmax	h	0.5		

[5] Median was used for Tmax. All other parameters use the arithmetic mean.

[6] Standard deviation

Figures

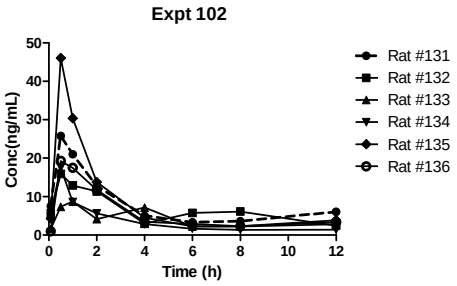


Fig. 1a. Plasma total DB-67 levels in SD rats treated with D5W PO 5 min before 2.5 mg/kg DB-67 lactone PO

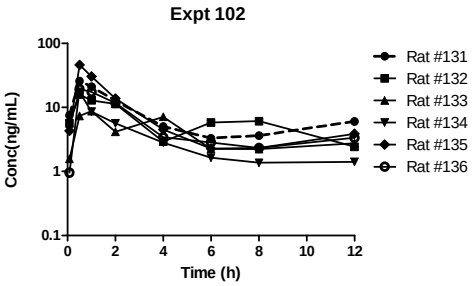


Fig. 1b. Semi-log plot of plasma total DB-67 levels in SD rats treated with D5W PO 5 min before 2.5 mg/kg DB-67 lactone PO

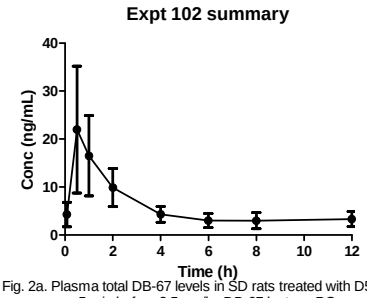


Fig. 2a. Plasma total DB-67 levels in SD rats treated with D5W PO 5 min before 2.5 mg/kg DB-67 lactone PO

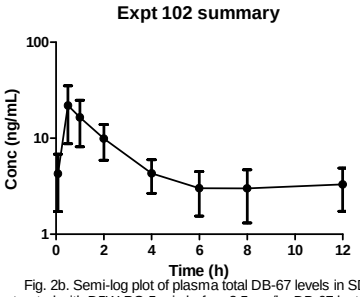


Fig. 2b. Semi-log plot of plasma total DB-67 levels in SD rats treated with D5W PO 5 min before 2.5 mg/kg DB-67 lactone PO

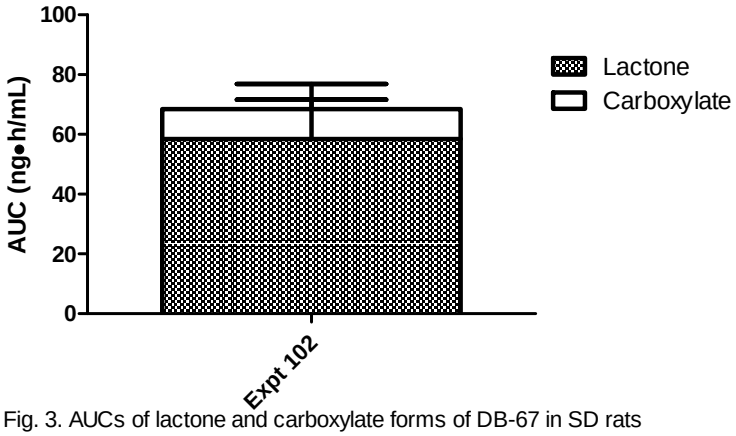


Fig. 3. AUCs of lactone and carboxylate forms of DB-67 in SD rats following PO administrtation of DB-67 lactone at 2.5 mg/kg 5 min after PO administration of D5W at 7.5 ml/kg

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 102

Raw Data

Expt #	Rat #	Time (h)	Carboxylate (ng/mL)	Lactone (ng/mL)	Total AR-67 (ng/mL)
102	131	0.083	0.75	6.76	7.51
102	131	0.5	2.84	22.91	25.75
102	131	1	2.66	18.38	21.04
102	131	2	2.28	10.45	12.73
102	131	4	0.89	4.15	5.04
102	131	6	0.47	2.83	3.3
102	131	8	0.51	3.11	3.62
102	131	12	0.83	5.2	6.03
102	132	0.083	0.74	4.86	5.6
102	132	0.5	1.97	13.96	15.93
102	132	1	1.97	10.92	12.89
102	132	2	1.73	9.62	11.35
102	132	4	0.46	2.46	2.92
102	132	6	0.82	4.97	5.79
102	132	8	0.91	5.21	6.12
102	132	12	0.36	2.08	2.44
102	133	0.083	0.1	1.47	1.57
102	133	0.5	0.92	6.42	7.34
102	133	1	1.87	6.87	8.74
102	133	2	0.62	3.54	4.16
102	133	4	1.06	6.09	7.15
102	133	6	0.39	1.85	2.24
102	133	8	0.34	1.89	2.23
102	133	12	0.39	2.36	2.75
102	134	0.083	0.69	4.98	5.67
102	134	0.5	2.43	15.1	17.53
102	134	1	1.41	7.17	8.58
102	134	2	0.99	4.64	5.63
102	134	4	0.4	2.43	2.83
102	134	6	0.26	1.38	1.64
102	134	8	0.1	1.27	1.37
102	134	12	0.06	1.35	1.41
102	135	0.083	0.4	3.9	4.3
102	135	0.5	6.34	39.74	46.08
102	135	1	4.46	25.92	30.38
102	135	2	2.47	11.29	13.76
102	135	4	0.79	3.72	4.51
102	135	6	0.24	2.03	2.27
102	135	8	0.22	2.1	2.32
102	135	12	0.45	3.42	3.87
102	136	0.083	0.04	0.92	0.96
102	136	0.5	2.14	17.14	19.28
102	136	1	2.39	15.08	17.47

102	136	2	1.77	9.96	11.73
102	136	4	0.54	2.88	3.42
102	136	6	0.37	2.48	2.85
102	136	8	0.44	1.89	2.33
102	136	12	0.44	2.92	3.36

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Rat PK Study

(March 18th, 08)

Dose dependence of GF120918 effect on oral bioavailability of SBE-β-CD based AR-67 lactone in rats

---

Expt # 103

12/02/2008

Objective

To determine the effects of GF120918 (GF) doses on oral bioavailability of SBE-β-CD based AR-67 lactone in female SD rats. This is a part of GF dose-response experiments.

Expt 103a: Study the effects of GF (2.5 mg/kg) PO on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) in rats PO

Expt 103b: Study the effects of GF (20.0 mg/kg) PO on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (20 mg/kg) in rats PO

Method

Animal treatment and sample processing

Expt 103a, Rats (#141-#143, n=3) were dosed with 2.5 mg/kg SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO 5 min after GF (2.5 mg/kg) PO.

Expt 103b, Rats (#144-#146, n=3) were dosed with 2.5 mg/kg SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO 5 min after GF (20 mg/kg) PO.

Dosing solution:

AR-67 lactone = 1.0 mg/mL

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 103a & 103b

Sequence name: 20AC-031808-Rat PK Expt 103.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while nocompartmental analysis was made using WinNonlin v5.2.

Results

Tables

Expt 103a & 103b

ID	Parameter	Units	Estimate	Average/ Median[7]	SD/range
141	AUCall	h*ng/mL	883.5052	1034.812	304.354
142	AUCall	h*ng/mL	906.0303		
143	AUCall	h*ng/mL	367.9726		
141	Cmax	ng/mL	146.85	786.781	59.637
142	Cmax	ng/mL	164.28		
143	Cmax	ng/mL	53.38		
141	Tmax	h	4	2.000	2--4
142	Tmax	h	2		
143	Tmax	h	2		
144	AUCall	h*ng/mL	739.3806	1149.130	202.195
145	AUCall	h*ng/mL	377.4737		
146	AUCall	h*ng/mL	402.1725		
144	Cmax	ng/mL	121.72	48.837	27.659
145	Cmax	ng/mL	66.8		
146	Cmax	ng/mL	100		
144	Tmax	h	2	2.000	0.5-2
145	Tmax	h	2		
146	Tmax	h	0.5		

[7] Median was used for Tmax. All other parameters use the arithmetic mean.

Figures

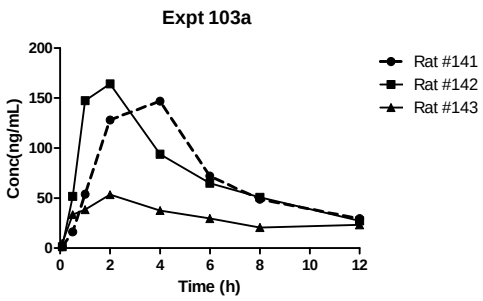


Fig. 1a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 PO 5 min after GF (2.5 mg/kg) PO

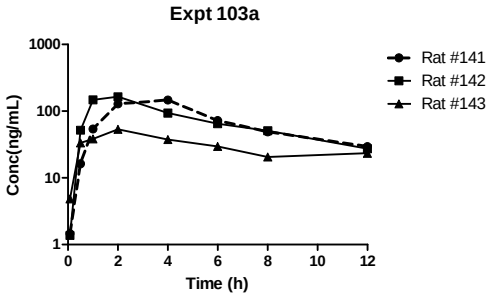


Fig. 1b. Semi-log plot of plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 PO 5 min after GF (2.5 mg/kg) PO

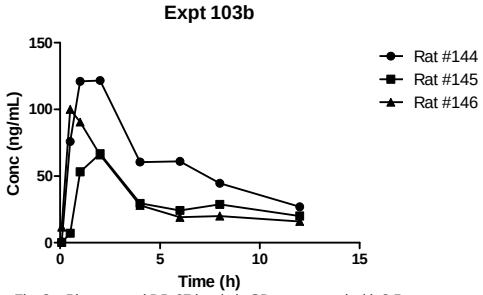


Fig. 2a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 PO 5 min after GF (20 mg/kg) PO

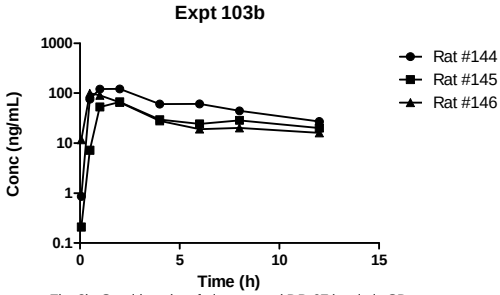


Fig. 2b. Semi-log plot of plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 PO 5 min after GF (20 mg/kg) PO

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 103

Raw Data

Expt #	Rat #	Time (h)	Carboxylate (ng/mL)	Lactone (ng/mL)	Total AR-67 (ng/mL)
103	141	0.083	0	1.46	1.46
103	141	0.5	2.06	14.2	16.26
103	141	1	5.66	48.26	53.92
103	141	2	15.83	112.22	128.05
103	141	4	21.56	125.29	146.85
103	141	6	11.51	60.64	72.15
103	141	8	8.58	40.17	48.75
103	141	12	5.73	23.73	29.46
103	142	0.083	0	1.37	1.37
103	142	0.5	4.86	46.88	51.74
103	142	1	14.88	132.58	147.46
103	142	2	20.98	143.3	164.28
103	142	4	17.56	76.3	93.86
103	142	6	10.85	54.07	64.92
103	142	8	8.18	42.77	50.95
103	142	12	4.79	22.48	27.27
103	143	0.083	0.69	4.14	4.83
103	143	0.5	3.6	29.77	33.37
103	143	1	4.61	33.73	38.34
103	143	2	7.54	45.84	53.38
103	143	4	7.14	30.44	37.58
103	143	6	4.8	24.78	29.58
103	143	8	3.11	17.45	20.56
103	143	12	3.95	19.37	23.32
103	144	0.083	0	0.86	0.86
103	144	0.5	9.46	66.43	75.89
103	144	1	13.89	107.23	121.12
103	144	2	19.18	102.54	121.72
103	144	4	8.82	51.8	60.62
103	144	6	10.61	50.43	61.04
103	144	8	6.53	38.04	44.57
103	144	12	4.12	22.84	26.96
103	145	0.083	0	0.21	0.21
103	145	0.5	0	7.2	7.2
103	145	1	6.32	46.84	53.16
103	145	2	10.5	56.3	66.8
103	145	4	5.53	24.03	29.56
103	145	6	4.62	19.71	24.33
103	145	8	5.11	23.64	28.75
103	145	12	3.71	16.3	20.01
103	146	0.083	1.58	10.08	11.66
103	146	0.5	11.75	88.25	100
103	146	1	14.57	75.94	90.51

103	146	2	12.72	53.03	65.75
103	146	4	5.15	22.96	28.11
103	146	6	4.07	15.06	19.13
103	146	8	3.26	16.86	20.12
103	146	12	2.85	13.18	16.03

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Rat PK Study

(March 20th, April 2nd and April 3rd, 08)

Effects of D5W, vehicle and GF120918 PO on PK profiles of SBE-β-CD based AR-67 lactone IV in rats

---

Expt # 104, Expt # 107 and Expt # 108

11/25/2008

Objective

To determine the effects of D5W, vehicle and GF120918 PO on PK profile of SBE-β-CD based AR-67 lactone IV in female SD rats. This experiment will determine the systemic effects of GF120918 and allow us to calculate absolute bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone following PO administration.

Expt 104, 107: Study the effects of vehicle and GF120918 PO on PK profile following IV administration of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) in female SD rats

Expt 108: Study the effects of D5W PO on PK profile following IV administration of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) in female SD rats

Method

Animal treatment and sample processing

Expt 104a, 107a, Rats (#151-#153, #171-#173, n=6) were dosed with SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) PO 5 min after vehicle administration at 7.5 mL/kg PO.

Expt 104b, 107b, Rats (#155-#156, #174-#176, n=6) were dosed with SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) PO 5 min after GF administration at 2.5 mg/kg PO.

Expt 108, Rats (#181-#186, n=3) were dosed with 2.5 mg/kg SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO 5 min after D5W administration at 7.5 mL/kg PO.

Dosing solution:

AR-67 lactone = 1.0 mg/mL

GF120918 was dissolved in vehicle (40% PEG300, 10% Teween-80 in D5W) within 1 hour before experiments.

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 μL:40 μL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 104, 107 & 108

Sequence name: 20AC-032008-Rat PK Expt 104.seq

20AC-040208-Rat PK Expt 107.seq

20AC-040308-Rat PK Expt 108.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while nocompartmental analysis was made using WinNonlin v5.2.

Results

Table 1. Pharmacokinetic parameters estimated using the noncompartmental method in Winnonlin v5.2

Expt 104a & 107a

Expt	ID	Parameter	Units	Estimate	Average/ Median[8]	SD[9]/range
104a	151	AUCall	h*ng/mL	1516.369	1736.020	288.804
104a	152	AUCall	h*ng/mL	1417.991		
104a	153	AUCall	h*ng/mL	1806.815		
107a	171	AUCall	h*ng/mL	2234.413		
107a	172	AUCall	h*ng/mL	1637.368		
107a	173	AUCall	h*ng/mL	1803.167		
104a	151	Cmax	ng/mL	1486.65	1848.380	385.513
104a	152	Cmax	ng/mL	1504.68		
104a	153	Cmax	ng/mL	2244.65		
107a	171	Cmax	ng/mL	2363.4		
107a	172	Cmax	ng/mL	1904.6		
107a	173	Cmax	ng/mL	1586.3		
104a	151	Tmax	h	0.083	0.083	0.000
104a	152	Tmax	h	0.083		
104a	153	Tmax	h	0.083		
107a	171	Tmax	h	0.083		
107a	172	Tmax	h	0.083		
107a	173	Tmax	h	0.083		
104a	151	Cl_obs	mL/h/kg	1646.418	1467.898	228.548
104a	152	Cl_obs	mL/h/kg	1759.938		
104a	153	Cl_obs	mL/h/kg	1381.384		
107a	171	Cl_obs	mL/h/kg	1112.829		
107a	172	Cl_obs	mL/h/kg	1524.243		
107a	173	Cl_obs	mL/h/kg	1382.576		
104a	151	Vss_obs	mL/kg	1677.758	1552.289	320.571
104a	152	Vss_obs	mL/kg	1993.347		
104a	153	Vss_obs	mL/kg	1075.022		

[8] Median was used for Tmax. All other parameters use the arithmetic mean.

[9] Standard deviation.

107a	171	Vss_obs	mL/kg	1361.343		
107a	172	Vss_obs	mL/kg	1472.479		
107a	173	Vss_obs	mL/kg	1733.785		

Expt 104b & 107b

Expt	ID	Parameter	Units	Estimate	Average/ Median	SD/range
104b	155	AUCall	h*ng/mL	2514.237	2732.880	566.527
104b	156	AUCall	h*ng/mL	3362.908		
107b	174	AUCall	h*ng/mL	2121.467		
107b	175	AUCall	h*ng/mL	2361.07		
107b	176	AUCall	h*ng/mL	3304.72		
104b	155	Cmax	ng/mL	1284.89	1859.448	478.310
104b	156	Cmax	ng/mL	2599.91		
107b	174	Cmax	ng/mL	1700.74		
107b	175	Cmax	ng/mL	1931.7		
107b	176	Cmax	ng/mL	1780		
104b	155	Tmax	h	0.083	0.083	0.000
104b	156	Tmax	h	0.083		
107b	174	Tmax	h	0.083		
107b	175	Tmax	h	0.083		
107b	176	Tmax	h	0.083		
104b	155	Vss_obs	mL/kg	2287.725	2160.307	431.934
104b	156	Vss_obs	mL/kg	1440.141		
107b	174	Vss_obs	mL/kg	2511.762		
107b	175	Vss_obs	mL/kg	2109.548		
107b	176	Vss_obs	mL/kg	2452.36		
104b	155	Cl_obs	mL/h/kg	971.643	920.354	190.222
104b	156	Cl_obs	mL/h/kg	728.1299		
107b	174	Cl_obs	mL/h/kg	1144.657		
107b	175	Cl_obs	mL/h/kg	1038.936		
107b	176	Cl_obs	mL/h/kg	718.4048		

Expt 108

Expt	ID	Parameter	Units	Estimate	Average/ Median	SD/range
108	181	AUCall	h*ng/mL	1755.983	1845.774	177.787
108	182	AUCall	h*ng/mL	1946.697		
108	183	AUCall	h*ng/mL	1785.454		
108	184	AUCall	h*ng/mL	1742.127		
108	185	AUCall	h*ng/mL	2160.657		
108	186	AUCall	h*ng/mL	1683.724		
108	181	Cmax	ng/mL	2349.6	2153.300	301.833
108	182	Cmax	ng/mL	2448.4		
108	183	Cmax	ng/mL	1923.7		
108	184	Cmax	ng/mL	2146.1		
108	185	Cmax	ng/mL	2375.8		
108	186	Cmax	ng/mL	1676.2		
108	181	Cl_obs	mL/h/kg	1422.477	1362.323	121.090
108	182	Cl_obs	mL/h/kg	1283.192		
108	183	Cl_obs	mL/h/kg	1398.782		
108	184	Cl_obs	mL/h/kg	1434.112		
108	185	Cl_obs	mL/h/kg	1154.951		
108	186	Cl_obs	mL/h/kg	1480.425		
108	181	Tmax	h	0.08	0.080	0.000
108	182	Tmax	h	0.08		
108	183	Tmax	h	0.08		
108	184	Tmax	h	0.08		
108	185	Tmax	h	0.08		
108	186	Tmax	h	0.08		
108	181	Vss_pred	mL/kg	1107.45	1290.360	224.124
108	182	Vss_pred	mL/kg	1197.219		
108	183	Vss_pred	mL/kg	1502.552		
108	184	Vss_pred	mL/kg	1125.87		
108	185	Vss_pred	mL/kg	1169.085		
108	186	Vss_pred	mL/kg	1639.984		

Figures

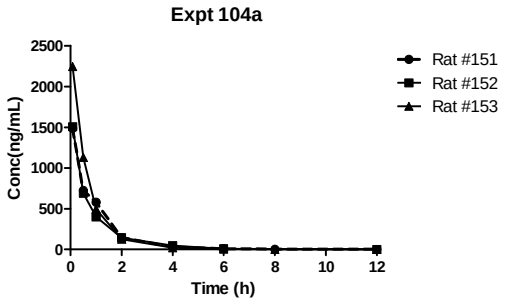


Fig. 1a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 IV 5 min after vehicle (PEG, tween80, D5W) PO

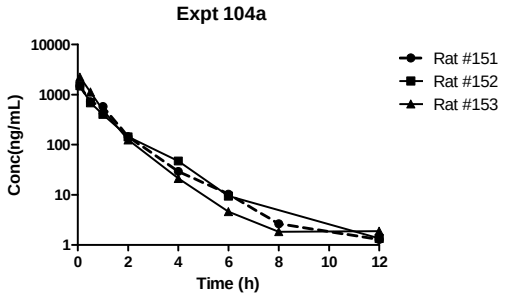


Fig. 1b. Plasma total DB-67 levels in SD rats treated with vehicle (PEG, tween80, D5W) PO 5 min before 2.5mg/kg DB-67 IV

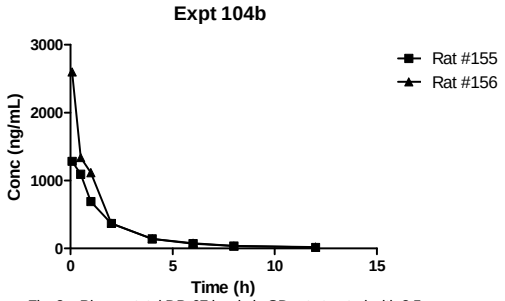


Fig. 2a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 IV 5 min after GF (2.5 mg/kg) PO

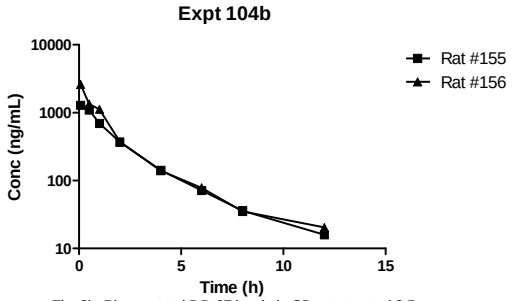


Fig. 2b. Plasma total DB-67 levels in SD rats treated 2.5 mg/kg DB-67 IV 5 min after GF (2.5 mg/kg) PO

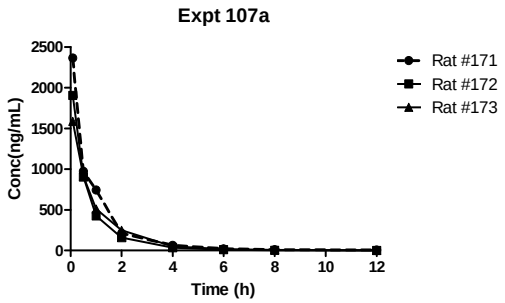


Fig. 1a. Plasma total DB-67 levels in SD rats treated with 2.5mg/kg DB-67 lactone IV 7 min after vehicle administration

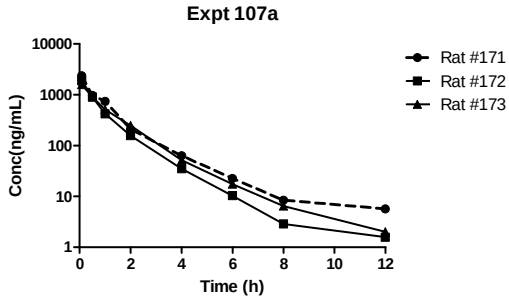


Fig. 1b. Semi-log plot of plasma total DB-67 levels in SD rats treated with 2.5mg/kg DB-67 lactone IV 7 min after vehicle administration

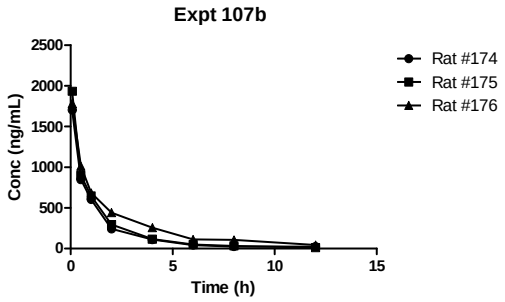


Fig. 2a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 IV 5 min after GF (2.5 mg/kg) PO

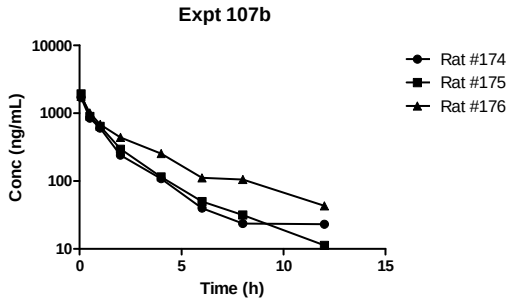


Fig. 2b. Semi-log plot of plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 IV 5 min after GF (2.5 mg/kg) PO

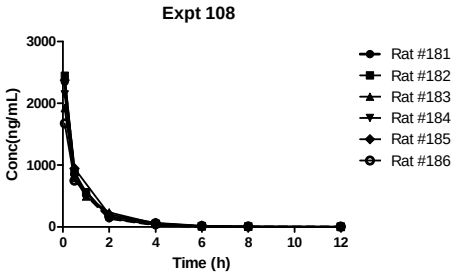


Fig. 1a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 IV 5 min after D5W PO

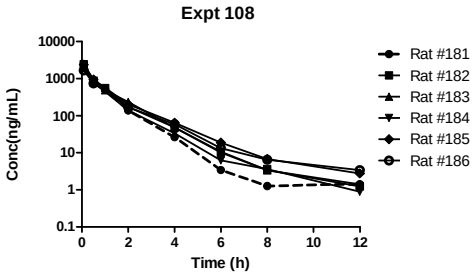


Fig. 1b. Semi-log plot of plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 IV 5 min after D5W PO

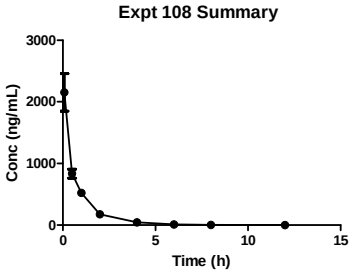


Fig. 2a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 IV 5 min after D5W PO

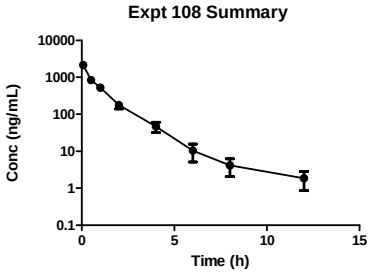


Fig. 2b. Semi-log plot of plasma total DB-67 levels in SD rats treated with 2.5mg/kg DB-67 IV 5 min after D5W PO

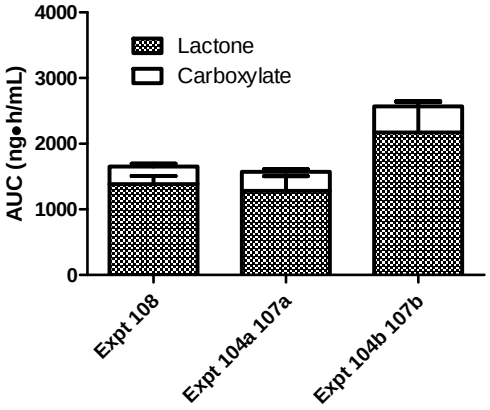


Fig. 3. AUCs of lactone and carboxylate forms of DB-67 in SD rats following IV administrtation of DB-67 lactone 5 min after PO administration of D5W (expt 108), vehicle (expt 104a 107a) and GF (expt 104b 107b) at 2.5 mg/kg

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 104

\\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 107

\\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 108

Raw Data

Expt	Rat	Time (h)	Carboxylate (ng/mL)	Lactone(ng/mL)	Total AR-67 (ng/mL)
104	151	0.083	292.53	1194.12	1486.65
104	151	0.5	125.69	598.02	723.72
104	151	1	115.15	464.50	579.64
104	151	2	32.82	111.76	144.58
104	151	4	7.88	21.59	29.47
104	151	6	2.85	7.48	10.33
104	151	8	0.46	2.18	2.64
104	151	12	0.18	1.10	1.28
104	152	0.083	268.85	1235.83	1504.68
104	152	0.5	131.40	557.01	688.41
104	152	1	71.49	330.45	401.94
104	152	2	31.40	112.36	143.76
104	152	4	13.23	33.97	47.20
104	152	6	2.06	7.36	9.42
104	152	8	Null	Null	Null
104	152	12	0.14	1.21	1.35
104	153	0.083	373.01	1871.63	2244.65
104	153	0.5	221.21	908.97	1130.19
104	153	1	85.75	409.58	495.34
104	153	2	27.81	97.65	125.46
104	153	4	5.73	15.49	21.22
104	153	6	1.14	3.49	4.63
104	153	8	0.39	1.45	1.84
104	153	12	0.78	1.10	1.88
104	155	0.083	105.98	1178.91	1284.89
104	155	0.5	162.69	930.92	1093.61
104	155	1	115.84	576.51	692.35
104	155	2	57.71	309.91	367.62
104	155	4	25.25	116.06	141.31
104	155	6	11.93	59.67	71.60
104	155	8	5.90	30.16	36.06
104	155	12	2.37	13.55	15.92
104	156	0.083	162.17	2437.74	2599.91
104	156	0.5	205.39	1133.00	1338.40
104	156	1	197.10	919.81	1116.91
104	156	2	63.11	307.55	370.66
104	156	4	23.71	116.31	140.02
104	156	6	13.95	64.30	78.25
104	156	8	6.40	28.68	35.08
104	156	12	2.62	17.72	20.34

Expt	Rat	Time (h)	Caboxylate	Lactone	Total AR-67
107	171	0.083	143.51	1557.23	1700.74
107	171	0.5	114	732.4	846.40
107	171	1	100.6	502.6	603.20
107	171	2	45.6	195	240.60
107	171	4	24.84	83.52	108.36
107	171	6	7.39	32.63	40.02
107	171	8	4.18	19.45	23.63
107	171	12	5.31	17.77	23.08
107	172	0.083	148.5	1783.2	1931.70
107	172	0.5	141.7	755.6	897.30
107	172	1	105.9	538.1	644.00
107	172	2	57.5	238.1	295.60
107	172	4	25.3	89.67	114.97
107	172	6	8.39	41.6	49.99
107	172	8	5.55	25.94	31.49
107	172	12	1.92	9.36	11.28
107	173	0.083	119.5	1660.5	1780.00
107	173	0.5	137.5	875	1012.50
107	173	1	107.8	575.8	683.60
107	173	2	78.1	363.4	441.50
107	173	4	51.13	204.16	255.29
107	173	6	21.87	90.05	111.92
107	173	8	16.3	89.47	105.77
107	173	12	5.98	37.1	43.08
107	174	0.083	105.98	1178.91	1284.89
107	174	0.5	162.69	930.92	1093.61
107	174	1	115.84	576.51	692.35
107	174	2	57.71	309.91	367.62
107	174	4	25.25	116.06	141.31
107	174	6	11.93	59.67	71.60
107	174	8	5.90	30.16	36.06
107	174	12	2.37	13.55	15.92
107	175	0.083	162.17	2437.74	2599.91
107	175	0.5	205.39	1133.00	1338.40
107	175	1	197.10	919.81	1116.91
107	175	2	63.11	307.55	370.66
107	175	4	23.71	116.31	140.02
107	175	6	13.95	64.30	78.25
107	175	8	6.40	28.68	35.08
107	175	12	2.62	17.72	20.34

Expt	Rat	Time (h)	Carboxylate	Lactone	Total AR-67
108	181	0.083	227.20	2122.40	2349.60
108	181	0.5	116.50	733.80	850.30
108	181	1	97.40	420.70	518.10
108	181	2	30.00	108.80	138.80
108	181	4	6.99	19.55	26.54
108	181	6	0.82	2.62	3.44
108	181	8	0.20	1.07	1.27
108	181	12	0	1.42	1.42
108	182	0.083	236.20	2212.20	2448.40
108	182	0.5	132.30	754.10	886.40
108	182	1	98.50	462.20	560.70
108	182	2	37.90	133.30	171.20
108	182	4	11.95	36.31	48.26
108	182	6	2.32	8.42	10.74
108	182	8	0.67	2.73	3.40
108	182	12.00	0.1	1.14	1.24
108	183	0.083	215.20	1708.50	1923.70
108	183	0.5	126.90	661.80	788.70
108	183	1	95.30	395.00	490.30
108	183	2	48.70	187.30	236.00
108	183	4	10.90	36.89	47.79
108	183	6	2.51	7.67	10.18
108	183	8	0.71	2.64	3.35
108	183	12.00	0.15	1.26	1.41
108	184	0.08	170.20	1975.90	2146.10
108	184	0.50	116.30	681.20	797.50
108	184	1.00	-	-	-
108	184	2.00	30.30	109.20	139.50
108	184	4.00	8.17	25.37	33.54
108	184	6.00	1.37	4.85	6.22
108	184	8.00	0.45	3.19	3.64
108	184	12.00	0.02	0.87	0.89
108	185	0.08	227.10	2148.70	2375.80
108	185	0.50	173.90	771.70	945.60
108	185	1.00	-	-	-
108	185	2.00	44.20	157.60	201.80
108	185	4.00	15.05	49.54	64.59
108	185	6.00	6.29	12.46	18.75
108	185	8.00	1.75	5.01	6.76
108	185	12.00	0.40	2.38	2.78
108	186	0.08	139.50	1536.70	1676.20
108	186	0.50	131.90	615.90	747.80
108	186	1.00	-	-	-
108	186	2.00	29.60	141.10	170.70
108	186	4.00	13.97	44.48	58.45

108	186	6.00	3.93	9.46	13.39
108	186	8.00	1.40	5.05	6.45
108	186	12.00	0.44	2.98	3.42

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Rat PK Study

(March 26th, 27th, 08)

Dose dependence of GF120918 effect on oral bioavailability of SBE-β-CD based AR-67 lactone in rats

---

Expt # 105 and Expt # 106

11/26/2008

Objective

To determine the effects of different doses of GF120918 (GF) on oral bioavailability of SBE-β-CD based AR-67 lactone in female SD rats. This is a part of GF (0.25, 1.0, 2.5, 20 mg/kg) dose-response experiments.

Expt 105: Study on the effects of GF (1.0 mg/kg) on the oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone in rats (2.5 mg/kg) PO

Expt 106: Study on the effects of GF (0.25 mg/kg) on the oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone in rats (2.5 mg/kg) PO

Method

Animal treatment and sample processing

Expt 105, Rats (#161-#163, n=3) were dosed with 2.5 mg/kg SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO 5 min after administration of GF (1.0 mg/kg) PO.

Expt 106, Rats (#164-#166, n=3) were dosed with 2.5 mg/kg SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO 5 min after administration of GF (0.25 mg/kg) PO.

Dosing solution:

AR-67 lactone = 1.0 mg/mL

GF120918 solution:

GF120918 was dissolved in vehicle (40% PEG-300, 10% Tween-80 in D5W) within 1 hour before experiments.

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 105 & 106

Sequence name: 20AC-040108-Rat PK Expt 105 & 106.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while noncompartmental analysis was made using WinNonlin v5.2.

Results

Tables. Pharmacokinetic parameters estimated using the noncompartmental method in Winnonlin v5.2

Expt 105 & 106

ID	Parameter	Units	Estimate	Average/ Median [10]	SD[11]/range
161	AUCall	h*ng/mL	578.184	646.933	105.608
162	AUCall	h*ng/mL	768.5319		
163	AUCall	h*ng/mL	594.0823		
161	Cmax	ng/mL	71.02	95.053	38.006
162	Cmax	ng/mL	138.87		
163	Cmax	ng/mL	75.27		
161	Tmax	h	6	2	2--6
162	Tmax	h	2		
163	Tmax	h	2		
164	AUCall	h*ng/mL	368.834	278.517	127.728
165	AUCall	h*ng/mL	188.1992		
164	Cmax	ng/mL	36.28	31.860	6.251
165	Cmax	ng/mL	27.44		
164	Tmax	h	2	2	2
165	Tmax	h	2		

[10] Median was used for Tmax. All other parameters use the arithmetic mean.

[11] Standard deviation

Figures

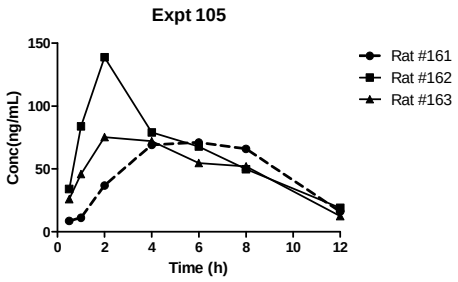


Fig. 1a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 PO 5 min after GF (1.0 mg/kg) PO

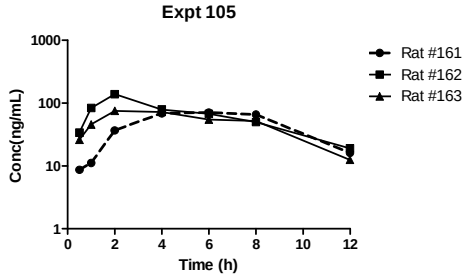


Fig. 1b. Semi-log plot of plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 PO 5 min after GF (1.0 mg/kg) PO

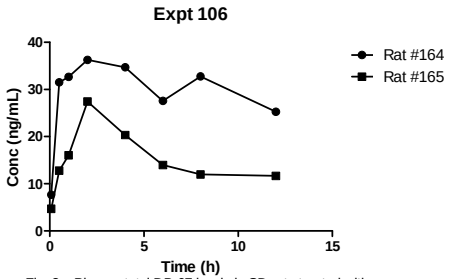


Fig. 2a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 PO 5 min after GF (0.25 mg/kg) PO

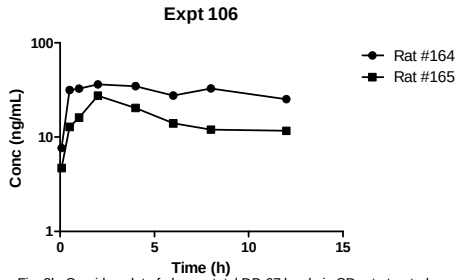


Fig. 2b. Semi-log plot of plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 PO 5 min after GF (0.25 mg/kg) PO

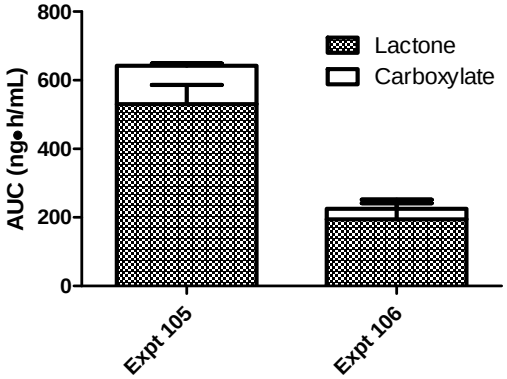


Fig. 3. AUCs of lactone and carboxylate forms of DB-67 in SD rats following PO administrtation of DB-67 lactone (2.5 mg/kg) 5 min after GF at 1.0 mg/kg (expt 105) and GF at 0.25 mg/kg (expt 106) PO

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 105 & 106

Raw Data

Expt #	Rat	Time (h)	Caboxylate (ng/mL)	Lactone (ng/mL)	Total (ng/mL)
105	161	0.083	0.00	0.90	0.90
105	161	0.5	1.27	7.43	8.70
105	161	1	1.73	9.45	11.18
105	161	2	3.91	32.84	36.75
105	161	4	12.28	56.79	69.07
105	161	6	16.09	54.93	71.02
105	161	8	14.78	51.18	65.96
105	161	12	3.41	12.79	16.20
105	162	0.083	0.00	0.57	0.57
105	162	0.5	2.99	31.06	34.05
105	162	1	8.96	74.87	83.83
105	162	2	19.06	119.81	138.87
105	162	4	12.20	66.92	79.12
105	162	6	12.84	55.00	67.84
105	162	8	9.36	40.49	49.84
105	162	12	3.35	15.73	19.08
105	163	0.083	0.00	0.16	0.16
105	163	0.5	3.64	22.45	26.09
105	163	1	5.96	39.95	45.90
105	163	2	11.07	64.20	75.27
105	163	4	12.62	59.44	72.06
105	163	6	9.70	44.96	54.66
105	163	8	9.11	42.98	52.09
105	163	12	2.20	10.32	12.52
106	164	0.083	1.13	6.54	7.67
106	164	0.5	4.76	26.77	31.53
106	164	1	5.01	27.67	32.68
106	164	2	5.53	30.75	36.28
106	164	4	6.50	28.21	34.71
106	164	6	4.88	22.70	27.58
106	164	8	5.14	27.65	32.79
106	164	12	4.29	21.00	25.29
106	165	0.083	0.40	4.30	4.70
106	165	0.5	1.37	11.42	12.79
106	165	1	2.05	13.99	16.04
106	165	2	4.01	23.43	27.44
106	165	4	3.36	16.98	20.34
106	165	6	2.48	11.50	13.98
106	165	8	2.09	9.90	11.99
106	165	12	1.70	9.98	11.68

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Rat PK Study

(April 8th - April 10th, 08)

Comparison of oral bioavailability of PEG, HP-CD, SBE-β-CD and Vitamin E-TPGS based AR-67 lactone formulations in rats

Expt # 109, Expt # 110, Expt # 111 and Expt # 112

12/03/2008

Objective

To compare the oral bioavailability of different excipients PEG, HP-CD, SBE-β-CD and vitamin E-TPGS based (E/D ratio 28:1) AR-67 lactone formulations in female SD rats and try to establish the correlation between in vivo bioavailability and in vitro solubility and precipitation data.

Expt 109: Study the oral bioavailability of PEG based AR-67 lactone at 2.5 mg/kg

Expt 110: Study the oral bioavailability of HP-CD based AR-67 lactone at 2.5 mg/kg

Expt 111: Study the oral bioavailability of SBE-β-CD based AR-67 lactone at 2.5 mg/kg

Expt 112: Study the oral bioavailability of vitamin E-TPGS based AR-67 lactone at 2.5 mg/kg

Method

Animal treatment and sample processing

Expt 109, Rats (#191-#193, n=3) were dosed with 2.5 mg/kg PEG based (28:1 E/D ratio) AR-67 lactone PO.

Expt 110, Rats (#194-#196, n=3) were dosed with 2.5 mg/kg HP-CD based (28:1 E/D ratio) AR-67 lactone PO.

Expt 111, Rats (#201-#203, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (28:1 E/D ratio) AR-67 lactone PO.

Expt 116, Rats (#204-#206, n=3) were dosed with 2.5 mg/kg vitamin E-TPGS (28:1 E/D ratio) AR-67 lactone PO.

Dosing solution: Stock AR-67 solution was mixed with excipients solution immediately before dosing to mimic the in vitro precipitation studies done in Dr. Anderson's lab. The AR-67 lactone in the dosing solution is 0.53 mg/mL.

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Expt 109 & 110

Sequence: 20AC-040908-Rat PK Expt 109 & 110.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Expt 111 & 112

Sequence: 20AC-041108-Rat PK Expt 111 & 112.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while noncompartmental analysis was made using WinNonlin v5.2.

Results

Tables:

Expt	ID	Parameter	Units	Estimate	Average/ Median[12]	SD[13]/range
109	191	AUCall	h*ng/mL	35.4258	43.93	8.34
109	192	AUCall	h*ng/mL	44.2584		
109	193	AUCall	h*ng/mL	52.0954		
109	191	Cmax	ng/mL	4.74	7.57	2.67
109	192	Cmax	ng/mL	7.93		
109	193	Cmax	ng/mL	10.05		
109	191	Tmax	h	0.5	0.50	0.50
109	192	Tmax	h	0.5		
109	193	Tmax	h	0.5		
110	194	AUCall	h*ng/mL	48.845	46.34	6.42
110	195	AUCall	h*ng/mL	39.0459		
110	196	AUCall	h*ng/mL	51.1408		
110	194	Cmax	ng/mL	10	13.50	3.06
110	195	Cmax	ng/mL	15.64		
110	196	Cmax	ng/mL	14.86		
110	194	Tmax	h	0.5	0.50	0.50
110	195	Tmax	h	0.5		
110	196	Tmax	h	0.5		
111	201	AUCall	h*ng/mL	39.6901	52.46	11.11
111	202	AUCall	h*ng/mL	59.9055		
111	203	AUCall	h*ng/mL	57.7961		
111	201	Cmax	ng/mL	5.66	10.17	3.96
111	202	Cmax	ng/mL	13.06		
111	203	Cmax	ng/mL	11.78		
111	201	Tmax	h	0.5	0.50	0.50
111	202	Tmax	h	0.5		
111	203	Tmax	h	0.5		
112	204	AUCall	h*ng/mL	65.7057	71.69	7.47

[12] Median was used for Tmax. All other parameters use the arithmetic mean.

[13] Standard deviation

112	205	AUCall	h*ng/mL	80.0592		
112	206	AUCall	h*ng/mL	69.296		
112	204	Cmax	ng/mL	29.02	30.92	3.93
112	205	Cmax	ng/mL	35.44		
112	206	Cmax	ng/mL	28.29		
112	204	Tmax	h	0.5	0.50	0.50
112	205	Tmax	h	0.5		
112	206	Tmax	h	0.5		

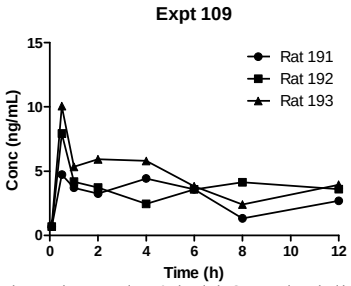


Fig. 1a. Plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg PEG based (28:1 E/D ratio) DB-67 lactone PO

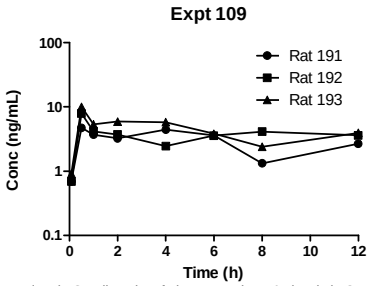


Fig. 1b. Semilog plot of plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg PEG based (28:1 E/D ratio) DB-67 lactone PO

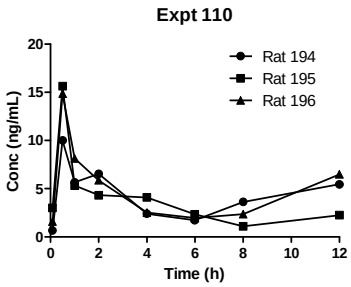


Fig. 2a. Plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg HP-CD based (28:1 E/D ratio) DB-67 lactone PO

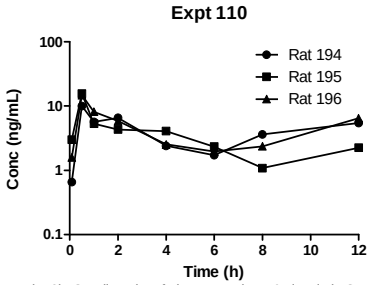


Fig. 2b. Semilog plot of plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg HP-CD based (28:1 E/D ratio) DB-67 lactone PO

Figures

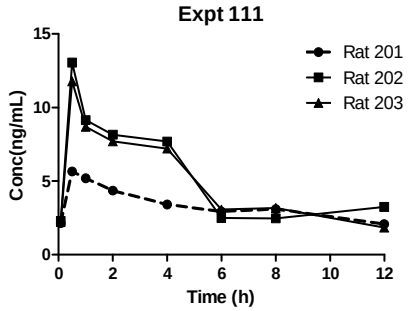


Fig. 3a. Plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg SBE-b-CD based (28:1 E/D ratio) DB-67 lactone PO

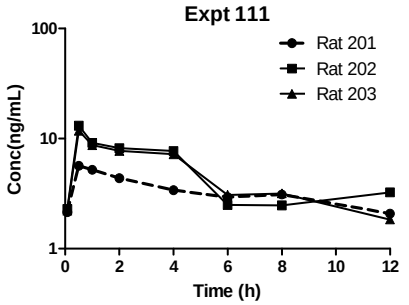


Fig. 3b. Semilog plot of plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg SBE-b-CD based (28:1 E/D ratio) DB-67 lactone PO

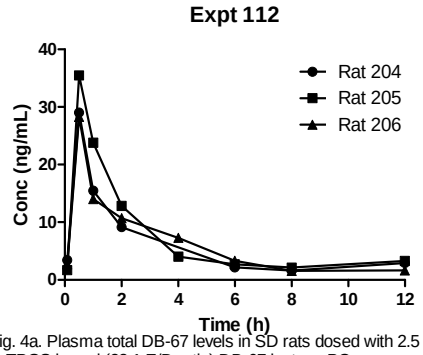


Fig. 4a. Plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg E-TPGS based (28:1 E/D ratio) DB-67 lactone PO

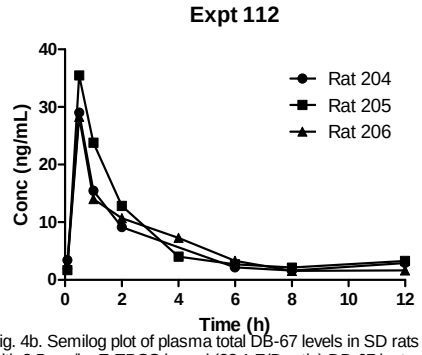


Fig. 4b. Semilog plot of plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg E-TPGS based (28:1 E/D ratio) DB-67 lactone PO

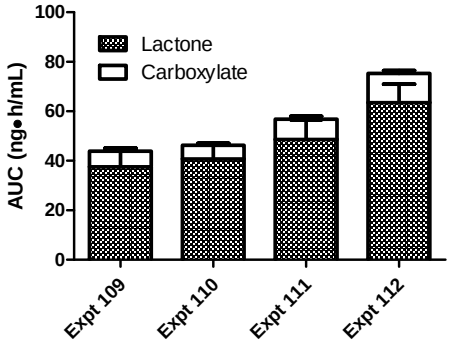


Fig. 5. AUCs of lactone and carboxylate forms of DB-67 in SD rats following PO administrtation of PEG (expt 109), HP-CD (expt 110), SBE-b-CD (expt 111) and E-TPGS (expt 112) based (28:1 E/D ratio) DB-67 lactone at 2.5 mg/kg

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 109 & 110

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 111 & 11

Raw Data

Expt	Rat ID	Time (h)	Carboxylate	Lactone	Total AR-67 (ng/mL)
109	191	0.08333	0	0.69	0.69
109	191	0.5	0.47	4.27	4.74
109	191	1	0.57	3.13	3.7
109	191	2	0.39	2.86	3.25
109	191	4	0.87	3.56	4.43
109	191	6	0.63	2.97	3.6
109	191	8	0.13	1.2	1.33
109	191	12	0.34	2.35	2.69
109	192	0.08333	0.16	0.53	0.69
109	192	0.5	0.8	7.13	7.93
109	192	1	0.76	3.42	4.18
109	192	2	0.52	3.21	3.73
109	192	4	0.34	2.12	2.46
109	192	6	0.49	3.09	3.58
109	192	8	0.64	3.5	4.14
109	192	12	0.34	3.27	3.61
109	193	0.08333	0	0.91	0.91
109	193	0.5	0.98	9.07	10.05
109	193	1	0.95	4.39	5.34
109	193	2	0.88	5.05	5.93
109	193	4	0.93	4.88	5.81
109	193	6	0.65	3.18	3.83
109	193	8	0.34	2.06	2.4
109	193	12	0.55	3.39	3.94
110	194	0.08333	0	0.66	0.66
110	194	0.5	1.24	8.76	10
110	194	1	0.75	4.91	5.66
110	194	2	0.89	5.65	6.54
110	194	4	0.36	2.05	2.41
110	194	6	0.15	1.57	1.72
110	194	8	0.37	3.25	3.62
110	194	12	0.58	4.88	5.46
110	195	0.08333	0.11	2.9	3.01
110	195	0.5	1.89	13.75	15.64
110	195	1	0.73	4.6	5.33
110	195	2	0.61	3.72	4.33
110	195	4	0.68	3.41	4.09
110	195	6	0.3	2.04	2.34
110	195	8	0.03	1.06	1.09
110	195	12	0.15	2.1	2.25
110	196	0.08333	0.05	1.54	1.59
110	196	0.5	1.76	13.1	14.86
110	196	1	1.25	6.89	8.14
110	196	2	0.83	5.04	5.87
110	196	4	0.29	2.24	2.53

110	196	6	0.16	1.79	1.95
110	196	8	0.26	2.1	2.36
110	196	12	0.72	5.77	6.49

Expt	Rat ID	Time (h)	AR-67 Carboxylate	AR-67 Lactone	Total AR-67 (ng/mL)
111	201	0.083333	0.29	2.14	2.14
111	201	0.5	0.72	5.66	5.66
111	201	1	0.94	5.2	5.2
111	201	2	0.75	4.36	4.36
111	201	4	0.61	3.4	3.4
111	201	6	0.44	2.93	2.93
111	201	8	0.68	3.1	3.1
111	201	12	0.31	2.08	2.08
111	202	0.083333	0.08	2.29	2.29
111	202	0.5	1.45	11.61	13.06
111	202	1	1.3	7.84	9.14
111	202	2	1.01	7.15	8.16
111	202	4	1.07	6.62	7.69
111	202	6	0.37	2.49	2.49
111	202	8	0.39	2.47	2.47
111	202	12	0.38	3.24	3.24
111	203	0.083333	0.2	2.22	2.22
111	203	0.5	1.29	10.49	11.78
111	203	1	1.27	7.43	8.7
111	203	2	1.2	6.51	7.71
111	203	4	1.24	5.97	7.21
111	203	6	0.71	3.07	3.07
111	203	8	0.57	3.17	3.17
111	203	12	0.17	1.84	1.84
112	204	0.083333	0.53	3.42	3.42
112	204	0.5	3.89	25.13	29.02
112	204	1	2.25	13.19	15.44
112	204	2	1.75	7.4	9.15
112	204	4	2.16	-	-
112	204	6	0.26	2.14	2.14
112	204	8	0.15	1.61	1.61
112	204	12	0.47	2.92	2.92
112	205	0.083333	0.1	1.67	1.67
112	205	0.5	4.32	31.12	35.44
112	205	1	3.88	19.91	23.79
112	205	2	2.13	10.69	12.82
112	205	4	0.81	4.02	4.02
112	205	6	0.44	2.67	2.67
112	205	8	0.34	2.12	2.12
112	205	12	0.44	3.29	3.29
112	206	0.083333	0.36	2.67	2.67
112	206	0.5	3.96	24.33	28.29
112	206	1	2.25	11.78	14.03
112	206	2	1.72	8.97	10.69

112	206	4	1.13	6.15	7.28
112	206	6	0.62	3.32	3.32
112	206	8	0.23	1.54	1.54
112	206	12	0.12	1.64	1.64

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Carboxylate Rat PK Study

(April 12th, 08-April 16th, 08)

Comparison of oral bioavailability of buffered/unbuffered SBE-β-CD and Vitamin E-TPGS based AR-67 carboxylate

4/23/2008

Objective

To compare the relative oral bioavailability of buffered/unbuffered SBE-β-CD and Vitamin E –TPGS based AR-67 carboxylate formulations in female SD rats

Expt 113: Study on oral bioavailability of buffered SBE-β-CD based AR-67 carboxylate (2.5 mg/kg)

Expt 114: Study on oral bioavailability of unbuffered SBE-β-CD based AR-67 carboxylate (2.5 mg/kg)

Expt 115: Study on oral bioavailability of buffered Vitamin E-TPGS based AR-67 carboxylate (2.5 mg/kg)

Expt 116: Study on oral bioavailability of unbuffered Vitamin E-TPGS based AR-67 carboxylate (2.5mg/kg)

Method

Animal treatment and sample processing

Buffered/unbuffered SBE-β-CD / Vitamin E-TPGS based AR-67 carboxylate was prepared by Dr. Xiang (pH ≈10). The buffer was made with 0.5M Na_2CO_3

Expt 113, Rats (#211-#213, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) buffered AR-67 carboxylate at 7mL/kg PO.

Expt 114, Rats (#214-#216, n=3) were dosed with 2.5mg/kg SBE-β-CD based (200:1 E/D ratio) buffered AR-67 unbuffered carboxylate at 7 mL/kg PO.

Expt 115, Rats (#221-#223, n=3) were dosed with 2.5mg/kg Vitamin E-TPGS based (28:1 E/D ratio) AR-67 buffered carboxylate at 7 mL/kg PO.

Expt 116, Rats (#224-#226, n=3) were dosed with 2.5mg/kg Vitamin E-TPGS based (28:1 E/D ratio) AR-67 unbuffered carboxylate at 7 mL/kg PO.

Dosing solution = 0.36 mg/mL.

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 μL:40 μL) and were analyzed.

Expt 113 & Expt 114:

Sequence: 20AC-041108-Rat PK Expt 113 & 114.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Expt 115 & Expt 116:

Sequence: 20AC-041708-Rat PK Experiment 115 &116.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK).

Results

Tables. Pharmacokinetic parameters estimated using the noncompartmental method in Winnonlin v5.2

Expt #113 PK Parameters

PK Parameters	Rat #211	Rat #212	Rat #213	Mean/Median[14]	SD[15]/Range
Tmax (h)	2.00	2.00	1.00	2.00	1.00-2.00
Cmax (ng/mL)	12.05	28.62	20.81	20.49	8.29
AUC0→t (ng*h/mL)	39.28	77.68	62.04	59.67	19.31

[14] Median was used for Tmax. All other parameters use the arithmetic mean.

[15] Standard deviation.

Expt #114 PK Parameters

PK Parameters	Rat #214	Rat #215	Rat #216	Mean/Median	SD/Range
Tmax (h)	0.50	0.50	0.50	0.50	0.50-0.50
Cmax (ng/mL)	39.12	17.50	31.68	39.12	17.5
AUC0→t (ng*h/mL)	92.86	53.8	93.01	79.89	22.59

Expt #115 PK Parameters

PK Parameters	Rat #221	Rat #222	Rat #223	Mean/Median	SD/Range
Tmax (h)	2.00	2.00	2.00	2.00	2.00-2.00
Cmax (ng/mL)	21.51	25.75	13.68	20.31	6.12
AUC0→t (ng*h/mL)	66.71	97.25	59.42	74.46	20.07

Expt #116 PK Parameters

PK Parameters	Rat #224	Rat #225	Rat #226	Mean/Median	SD/Range
Tmax (h)	0.50	0.50	1.00	0.50	0.50-1.00
Cmax (ng/mL)	24.29	17.16	32.73	24.29	17.16
AUC0→t (ng*h/mL)	59.68	76.65	64.75	67.03	8.71

Figures

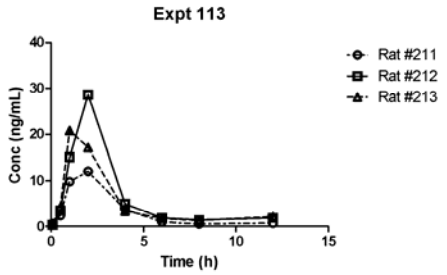


Fig. 1a. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg buffered SBE-β-CD based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

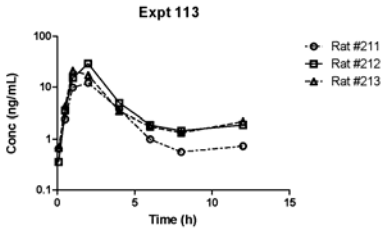


Fig. 1b. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg buffered SBE-β-CD based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

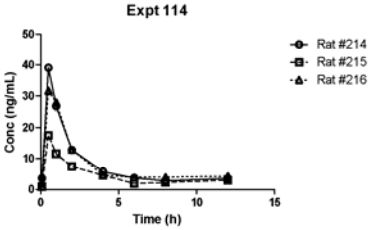


Fig. 2a. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg unbuffered SBE- β-CD based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

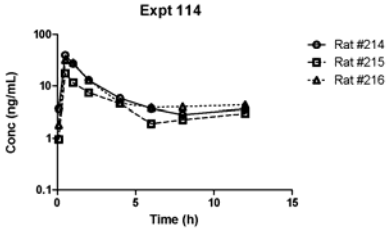


Fig. 2b. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg unbuffered SBE-β-CD based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\AUC Rat PK Expt 113, 114,115 & 116 (041708)

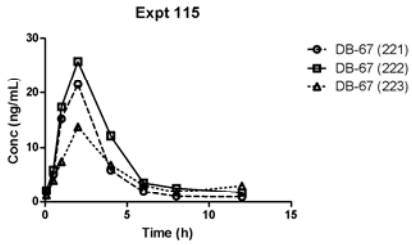


Fig. 3a. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg buffered Vit E-TPGS based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

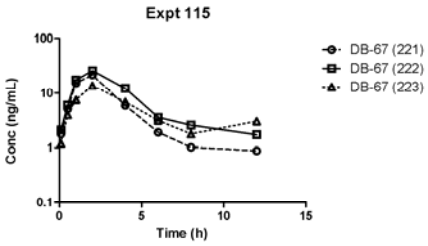


Fig. 3b. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg buffered Vit E-TPGS based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

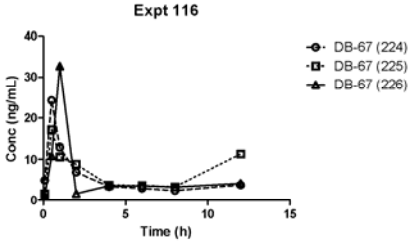


Fig. 4a. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg unbuffered Vit E-TPGS based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

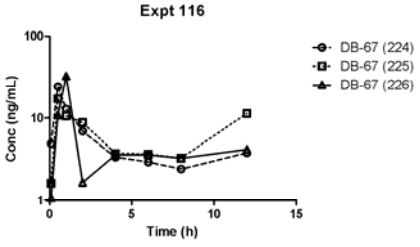


Fig. 4b. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg unbuffered Vit E-TPGS based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\AUC Rat PK Expt 113, 114,115 & 116 (041708)

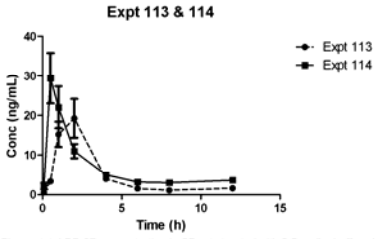


Fig. 5a. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg buffered (Expt 113) and unbuffered (Expt 114) SBE- β-CD based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

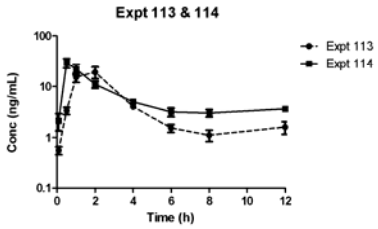


Fig. 5b. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg buffered (Expt 113) and unbuffered (Expt 114) SBE- β-CD based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

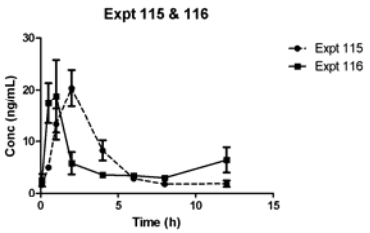


Fig. 6a. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg buffered (Expt 115) and unbuffered (Expt 116) Vit E-TPGS based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

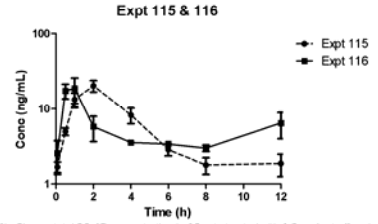


Fig. 6b. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg buffered (Expt 115) and unbuffered (Expt 116) Vit E-TPGS based (200:1 E/D) (pH=10.0) DB-67 carboxylate PO

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\AUC Rat PK Expt 113, 114,115 & 116 (041708)

Raw Data

Expt #	Rat ID	Time (h)	Carboxylate (ng/mL)	Lactone (ng/mL)	Total (ng/mL)
113	211	0.083	0.04	0.61	0.65
113	211	0.5	0.47	1.93	2.4
113	211	1	1.36	8.44	9.8
113	211	2	1.79	10.26	12.05
113	211	4	0.61	3.14	3.75
113	211	6	0.02	0.97	0.99
113	211	8	0	0.56	0.56
113	211	12	0	0.73	0.73
113	212	0.083	0.1	0.26	0.36
113	212	0.5	0.59	2.91	3.5
113	212	1	2.06	13	15.06
113	212	2	4.35	24.27	28.62
113	212	4	0.81	4.13	4.94
113	212	6	0.2	1.67	1.87
113	212	8	0.1	1.36	1.46
113	212	12	0.21	1.68	1.89
113	213	0.083	0.34	0.33	0.67
113	213	0.5	0.8	3.46	4.26

113	213	1	2.49	18.32	20.81
113	213	2	2.87	14.28	17.15
113	213	4	0.63	2.78	3.41
113	213	6	0.18	1.56	1.74
113	213	8	0.05	1.27	1.32
113	213	12	0.26	1.92	2.18
114	214	0.083	0.16	3.5	3.66
114	214	0.5	5.59	33.53	39.12
114	214	1	3.35	23.44	26.79
114	214	2	2.38	10.43	12.81
114	214	4	1.25	4.57	5.82
114	214	6	0.67	3.06	3.73
114	214	8	0.43	2.41	2.84
114	214	12	0.52	3.1	3.62
114	215	0.083	0	0.95	0.95
114	215	0.5	2.4	15.1	17.5
114	215	1	1.59	9.95	11.54
114	215	2	1.19	6.25	7.44
114	215	4	0.86	3.73	4.59
114	215	6	0.22	1.69	1.91
114	215	8	0.19	2.08	2.27
114	215	12	0.43	2.55	2.98
114	216	0.083	0.17	1.66	1.83
114	216	0.5	4.86	26.82	31.68
114	216	1	4.01	23.94	27.95
114	216	2	2.25	10.59	12.84
114	216	4	0.74	3.96	4.7
114	216	6	0.59	3.3	3.89
114	216	8	0.6	3.39	3.99
114	216	12	0.66	3.71	4.37

Expt# 115 & Expt# 116

Expt #	Rat ID	Time (h)	Carboxylate (ng/mL)	Lactone (ng/mL)		Total (ng/mL)
115	221	0.083	1.43		0.35	1.78
115	221	0.5	1.17		3.88	5.05
115	221	1	2.39		12.78	15.17
115	221	2	3.46		18.05	21.51
115	221	4	1.08		4.8	5.88
115	221	6	0.2		1.7	1.9
115	221	8	0.01		1.01	1.02
115	221	12	0.06		0.82	0.88
115	222	0.083	1.69		0.4	2.09
115	222	0.5	1.07		4.86	5.93
115	222	1	2.94		14.43	17.37
115	222	2	4.35		21.4	25.75
115	222	4	2.79		9.32	12.11
115	222	6	0.63		2.92	3.55
115	222	8	0.43		2.13	2.56
115	222	12	0.08		1.64	1.72
115	223	0.083	0.83		0.36	1.19
115	223	0.5	0.58		3.4	3.98
115	223	1	1.18		6.28	7.46
115	223	2	2.05		11.63	13.68
115	223	4	1.35		5.48	6.83
115	223	6	0.57		2.55	3.12
115	223	8	0.23		1.54	1.77
115	223	12	0.45		2.56	3.01
116	224	0.083	0.34		4.59	4.93
116	224	0.5	3.34		20.95	24.29
116	224	1	2.18		10.73	12.91
116	224	2	1.2		5.72	6.92
116	224	4	0.49		2.88	3.37
116	224	6	0.35		2.59	2.94
116	224	8	0.35		2.07	2.42
116	224	12	0.5		3.29	3.79
116	225	0.083	0.01		1.62	1.63
116	225	0.5	2.09		15.07	17.16
116	225	1	1.85		8.75	10.6
116	225	2	1.47		7.33	8.8
116	225	4	0.54		3.19	3.73
116	225	6	0.5		3.16	3.66
116	225	8	0.39		2.89	3.28
116	225	12	1.61		9.73	11.34
116	226	0.083	0.06		1.01	1.07

116	226	0.5	1.72	9.1	10.82
116	226	1	1.74	30.99	32.73
116	226	2	1.45	0.21	1.66
116	226	4	0.64	2.94	3.58
116	226	6	0.64	2.96	3.6
116	226	8	0.56	2.71	3.27
116	226	12	0.57	3.61	4.18

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Carboxylate Rat PK Study

(April 21st, 08-April 25th, 08)

Effects of GF120918 on oral bioavailability of SBE-β-CD based AR-67 carboxylate

---

Expt # 117, Expt # 118, Expt # 119 and Expt # 120

05/15/2008
Amendment:12/04/2008

Objective

To determine oral bioavailability of SBE-β-CD based (200:1 E/D ratio) AR-67 carboxylate and effect of GF120918 (GF) on oral bioavailability of AR-67 carboxylate in female SD rats

Expt 117: Study on the effect of GF (2.5 mg/kg) PO on bioavailability of SBE-β-CD based AR-67 carboxylate (2.5 mg/kg) PO

Expt 118: Study on the effect of GF (2.5 mg/kg) PO on SBE-β-CD based AR-67 carboxylate (2.5 mg/kg) IV

Expt 119: Study on oral bioavailability of SBE-β-CD based AR-67 carboxylate (2.5 mg/kg) PO

Expt 120: Study on PK profile of SBE-β-CD based AR-67 carboxylate (2.5 mg/kg) IV

Method

Animal treatment and sample processing

Expt 117, Rats (#231-#233, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) AR-67 carboxylate PO 5 min after GF (2.5 mg/kg) at 7.5 mL/kg PO.

Expt 118, Rats (#234-#236, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) AR-67 carboxylate IV 5 min after GF (2.5 mg/kg) at 7.5 mL/kg PO.

Expt 119, Rats (#241-#243, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) AR-67 carboxylate PO 5 min after vehicle (40% PEG, 10% Tween 80, D5W) at 7.5 mL/kg PO.

Expt 120, Rats (#244-#246, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) AR-67 carboxylate IV 5 min after vehicle (40% PEG, 10% Tween 80, D5W) at 7.5 mL/kg PO.

Dosing solution = 1.0 mg/mL.

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit capillary tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Expt 117 & Expt 118:

Sequence: 20AC-042308-Rat PK Expt 117 & 118.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Expt 119 & Expt 120:

20AC-042408-Rat PK Expt 119 & 120.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK).

Results

Tables. Pharmacokinetic parameters estimated using the noncompartmental method in Winnonlin v5.2

Expt #	ID	PK Parameter	Units	Estimate	Average/ Median[16]	SD/range
117	231	AUCall	hr*ng/mL	372.5378	480.632	94.618
117	232	AUCall	hr*ng/mL	520.9203		
117	233	AUCall	hr*ng/mL	548.4368		
117	231	AUC inf_pred	hr*ng/mL	444.7429	595.625	143.451
117	232	AUC inf_pred	hr*ng/mL	611.8705		
117	233	AUC inf_pred	hr*ng/mL	730.2615		
117	231	Cmax	ng/mL	50	74.807	21.778

[16] Median was used for Tmax. All other parameters use the arithmetic mean.

117	232	Cmax	ng/mL	83.64		
117	233	Cmax	ng/mL	90.78		
117	231	Tmax	hr	4	4.000	4-4
117	232	Tmax	hr	4		
117	233	Tmax	hr	4		
119	241	AUCall	hr*ng/mL	94.56	94.974	11.823
119	242	AUCall	hr*ng/mL	106.9984		
119	243	AUCall	hr*ng/mL	83.3629		
119	241	AUC inf_pred	hr*ng/mL	110.356	117.933	12.823
119	242	AUC inf_pred	hr*ng/mL	132.7386		
119	243	AUC inf_pred	hr*ng/mL	110.7037		
119	241	Cmax	ng/mL	13.77	13.467	1.547
119	242	Cmax	ng/mL	14.84		
119	243	Cmax	ng/mL	11.79		
119	241	Tmax	hr	2	2	2-4
119	242	Tmax	hr	2		
119	243	Tmax	hr	4		

Expt #	ID	PK Parameter	Units	Estimate	Average/ Median	SD/ range
118	234	AUCall	h*ng/mL	1206.524		
118	235	AUCall	h*ng/mL	1360.368	1283.446	108.784
118	234	AUCINF_pred	h*ng/mL	1228.367		
118	235	AUCINF_pred	h*ng/mL	1378.094	1303.230	105.873
118	234	Cmax	ng/mL	1160.2		
118	235	Cmax	ng/mL	1244.4	1202.300	59.538
118	234	Tmax	h	0.083		
118	235	Tmax	h	0.083	0.083	0.083-0.083
118	234	Vss_obs	mL/kg	6279.421		
118	235	Vss_obs	mL/kg	5279.921	5779.671	706.753
120	244	AUCall	h*ng/mL	621.6544		
120	245	AUCall	h*ng/mL	798.4906		
120	246	AUCall	h*ng/mL	1075.162	831.769	228.578
120	244	AUCINF_pred	h*ng/mL	621.7071		
120	245	AUCINF_pred	h*ng/mL	798.7154		
120	246	AUCINF_pred	h*ng/mL	1075.208	831.877	228.562
120	244	Cmax	ng/mL	1245.7		
120	245	Cmax	ng/mL	1203.1		
120	246	Cmax	ng/mL	2282.4	1577.067	611.208
120	244	Tmax	h	0.083		
120	245	Tmax	h	0.083		
120	246	Tmax	h	0.083	0.083	0.083-0.083
120	244	Vss_obs	mL/kg	3316.46		
120	245	Vss_obs	mL/kg	5823.333		
120	246	Vss_obs	mL/kg	5169.809	4769.868	1300.411

Figures

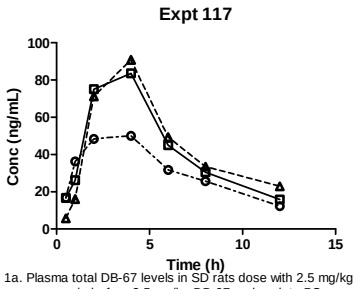


Fig. 1a. Plasma total DB-67 levels in SD rats dose with 2.5 mg/kg GF PO 5 min before 2.5 mg/kg DB-67 carboxylate PO

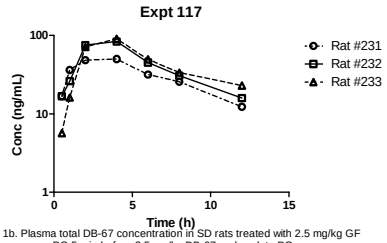


Fig. 1b. Plasma total DB-67 concentration in SD rats treated with 2.5 mg/kg GF PO 5 min before 2.5 mg/kg DB-67 carboxylate PO

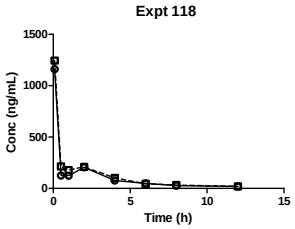


Fig. 2a. Plasma total DB-67 concentration in SD rats GF 2.5 mg/kg PO 5 min before DB-67 carboxylate (2.5 mg/kg) IV

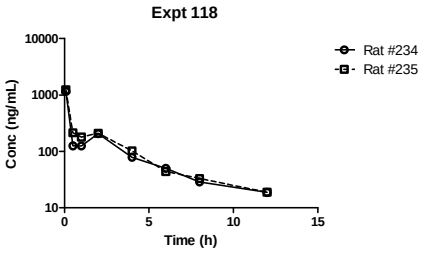


Fig. 2b. Plasma total DB-67 concentration in SD rats GF 2.5 mg/kg PO 5 min before DB-67 carboxylate (2.5 mg/kg) IV

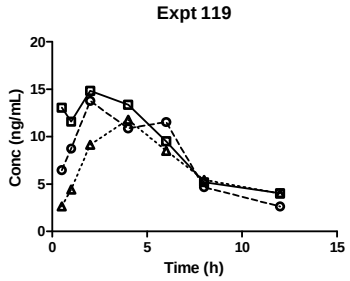


Fig. 3a. Plasma total DB-67 concentration in SD rats treated with vehicle (40% PEG, 10% Tween 80) PO 5 min before 2.5 mg/kg DB-67 carboxylate PO

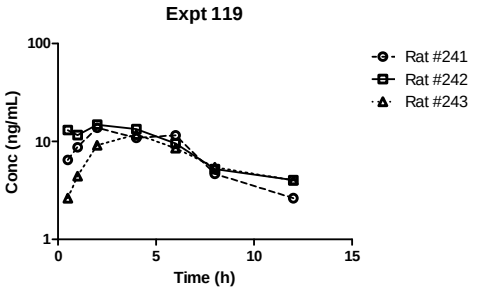


Fig. 3b. Plasma total DB-67 concentration in SD rats treated with vehicle (40% PEG, 10% Tween 80) PO 5 min before 2.5 mg/kg DB-67 carboxylate PO

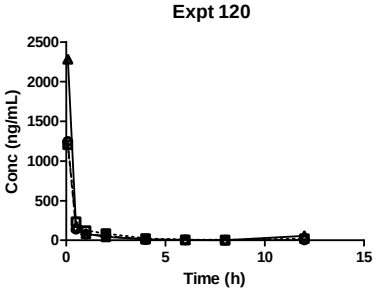


Fig. 4a. Plasma total DB-67 concentration in SD rats treated with vehicle (40% PEG, 10% Tween 80) PO 5 min before 2.5 mg/kg DB-67 carboxylate IV

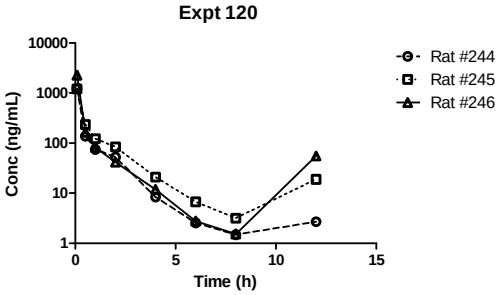


Fig. 4b. Plasma total DB-67 concentration in SD rats treated with vehicle (40% PEG, 10% Tween 80) PO 5 min before 2.5 mg/kg DB-67 carboxylate IV

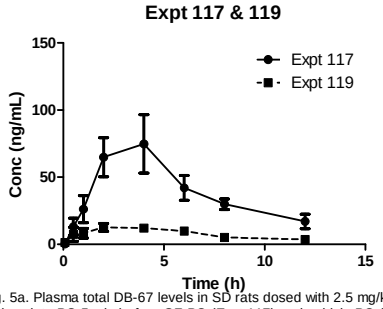


Fig. 5a. Plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg DB-67 carboxylate PO 5 min before GF PO (Expt 117) and vehicle PO (expt 119)

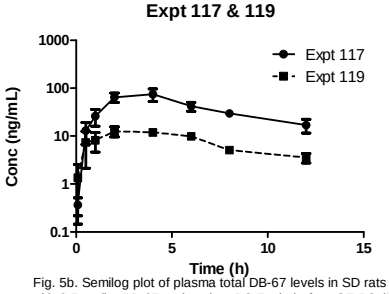


Fig. 5b. Semilog plot of plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg DB-67 carboxylate PO 5 min before GF PO (Expt 117) and vehicle PO (expt 119)

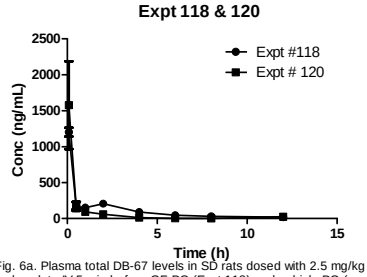


Fig. 6a. Plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg DB-67 carboxylate IV 5 min before GF PO (Expt 118) and vehicle PO (expt 120)

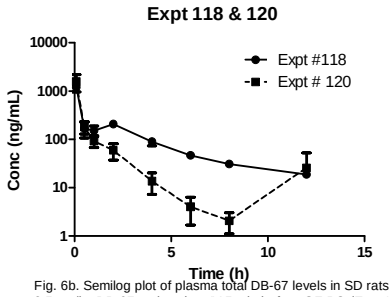


Fig. 6b. Semilog plot of plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg DB-67 carboxylate IV 5 min before GF PO (Expt 118) and vehicle PO (expt 120)

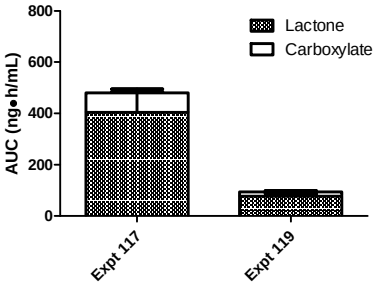


Fig. 7. AUCs of lactone and carboxylate forms of DB-67 in SD rats following PO administrtation of DB-67 carboxylate at 2.5 mg/kg 5 min after GF PO at 2.5 mg/kg (expt 117) and vehicle PO (expt 119)

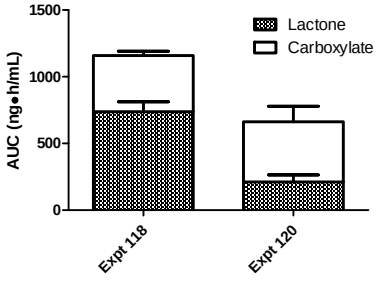


Fig. 8. AUCs of lactone and carboxylate forms of DB-67 in SD rats following IV administrtation of DB-67 carboxylate at 2.5 mg/kg 5 min after GFat 2.5 mg/kg PO (expt 118) and vehicle PO (expt 120)

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 117,118,119 & 120 (042408)

Raw Data

Expt #	Rat #	Time (h)	Carboxylate (ng/mL)	Lactone (ng/mL)	Total (ng/mL)
117	231	0.083	0	0.24	0.24
117	231	0.5	1.8	15	16.8
117	231	1	4.15	32.11	36.26
117	231	2	7.7	40.64	48.34
117	231	4	7.24	42.76	50
117	231	6	5.83	25.91	31.74
117	231	8	4.62	21.07	25.69
117	231	12	1.88	10.38	12.26
117	232	0.083	0.13	0.4	0.53
117	232	0.5	1.88	14.86	16.74
117	232	1	2.85	23.34	26.19
117	232	2	9.5	65.62	75.12
117	232	4	14.38	69.26	83.64
117	232	6	7.95	37.13	45.08
117	232	8	5.02	25.53	30.55
117	232	12	3.66	12.19	15.85
117	233	0.083	0	0.33	0.33
117	233	0.5	0.69	4.99	5.68
117	233	1	1.67	14.45	16.12
117	233	2	9.72	61.54	71.26
117	233	4	12.85	77.93	90.78
117	233	6	9.41	39.96	49.37
117	233	8	5.97	27.54	33.51
117	233	12	3.28	19.69	22.97
118	234	0.083	1068.3	91.9	1160.2
118	234	0.5	64.89	60.29	125.18
118	234	1	31.48	93.5	124.98
118	234	2	35.65	170.58	206.23
118	234	4	14.44	64.28	78.72
118	234	6	9.77	40.14	49.91
118	234	8	5.17	23.53	28.7
118	234	12	2.83	15.99	18.82
118	235	0.083	1126.4	118	1244.4
118	235	0.5	114.94	99.49	214.43
118	235	1	44.75	134.32	179.07
118	235	2	32.63	176.66	209.29
118	235	4	18.33	83.84	102.17
118	235	6	7.39	36.62	44.01
118	235	8	6.24	26.87	33.11
118	235	12	2.07	16.8	18.87

Expt #	Rat	Time (h)	AR-67 Carboxylate	AR-67 Lactone	Total
119	241	0.083	outlier	outlier	outlier
119	241	0.5	1.09	5.39	6.48
119	241	1	1.36	7.38	8.74
119	241	2	2.31	11.46	13.77
119	241	4	1.88	8.99	10.87
119	241	6	4.53	7.01	11.54
119	241	8	0.63	4.04	4.67
119	241	12	0.32	2.32	2.64
119	242	0.083	0.24	1.97	2.21
119	242	0.5	2.31	10.74	13.05
119	242	1	2.12	9.47	11.59
119	242	2	2.56	12.28	14.84
119	242	4	2.41	10.96	13.37
119	242	6	1.91	7.62	9.53
119	242	8	0.74	4.47	5.21
119	242	12	0.47	3.57	4.04
119	243	0.083	0	0.5	0.5
119	243	0.5	0.28	2.36	2.64
119	243	1	0.74	3.69	4.43
119	243	2	1.16	7.99	9.15
119	243	4	1.88	9.91	11.79
119	243	6	1.46	7.07	8.53
119	243	8	0.78	4.66	5.44
119	243	12	0.36	3.65	4.01
120	244	0.083	1150.5	95.2	1245.7
120	244	0.5	89.71	48.24	137.95
120	244	1	28.24	46.74	74.98
120	244	2	16.66	35.89	52.55
120	244	4	2.41	5.98	8.39
120	244	6	0.5	2.07	2.57
120	244	8	0.22	1.27	1.49
120	244	12	0.27	2.44	2.71
120	245	0.083	1107.6	95.5	1203.1
120	245	0.5	183.12	51.81	234.93
120	245	1	81.66	40.58	122.24
120	245	2	33.88	50.3	84.18
120	245	4	6.32	14.58	20.9
120	245	6	1.68	5.07	6.75
120	245	8	0.6	2.58	3.18
120	245	12	4.81	14.24	19.05
120	246	0.083	2106.7	175.7	2282.4
120	246	0.5	114.95	50.83	165.78
120	246	1	37.46	44.78	82.24
120	246	2	10.19	31.53	41.72

120	246	4	2.79	9.06	11.85
120	246	6	0.35	2.44	2.79
120	246	8	0.07	1.47	1.54
120	246	12	11.53	43.85	55.38

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Lactone Rat PK Study

(April 28th, 08-May 2th, 08)

Effects of the selective P-gp inhibitor zosuquidar and the selective BCRP inhibitor novobiocin on oral bioavailability of SBE-β-CD based AR-67 lactone

---

Expt # 121, Expt # 122, Expt # 123, and Expt # 124

05/12/2008

Objective

To determine the effects of selective P-gp and BCRP inhibition on oral bioavailability of SBE-β-CD based AR-67 lactone in rats

Expt 121: Study the effect of the selective P-gp inhibitor zosuquidar (20 mg/kg) PO on bioavailability of SBE-β-CD based AR-67 lactone (2.5 mg/kg) PO

Expt 122: Study the effect of the selective P-gp inhibitor zosuquidar (20 mg/kg) PO on the kinetics of SBE-β-CD based AR-67 lactone (2.5 mg/kg) IV

Expt 123: Study the effect of the selective Bcrp inhibitor novobiocin (50 mg/kg) PO on bioavailability of SBE-β-CD based AR-67 lactone (2.5 mg/kg) PO

Expt 124: Study the effect of the selective Bcrp inhibitor novobiocin (50 mg/kg) PO on the kinetics of SBE-β-CD based AR-67 lactone (2.5 mg/kg) IV

Method

Animal treatment and sample processing

Expt 121, Rats (#251-#253, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) AR-67 lactone PO 5 min after zosuquidar (20 mg/kg) at 7.5 mL/kg PO.

Expt 122, Rats (#254-#256, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) AR-67 lactone IV 5 min after zosuquidar(20 mg/kg) at 7.5 mL/kg PO.

Expt 123, Rats (#261-#263, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) AR-67 lactone PO 5 min after novobiocin (50 mg/kg) at 7.5 mL/kg PO.

Expt 124, Rats (#264-#266, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) AR-67 lactone IV 5 min after novobiocin (50 mg/kg) at 7.5 mL/kg PO.

Dosing solution concentrations

AR-67 lactone = 1.0 mg/mL.

Zosuquidar[17]= 2.67 mg/ml

Novobiocin[18]= 6.67 mg/ml

[17] 5g of SBE-b-CD was dissolved in D5W to a final volume of 25ml (20% solution). 66.7mg of zosuquidar was dissolved in 25ml of the SBE-b-CD solution.

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 μL:40 μL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank plasma.

Expt 121 & Expt 122:

Sequence name:20AC-050108-Rat PK Expt 121 & 122.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Expt 123 & Expt 124:

Sequence name: 20AC-050208-Rat PK Expt 122 & 124.seq

Method: 20AC-031208-reinjection of 121807 rat plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while noncompartmental analysis was made using WinNonlin v5.2.

[18]166.7 mg of novobiocin sodium was dissolved in 25ml D5W.

Results

Tables. Pharmacokinetic parameters estimated using the noncompartmental method in Winnonlin v5.2

(Expt 121 & 123)

Expt	ID	Parameter	Unit	Estimate	Average/ Median [19]	SD[20]/ range
123	261	AUCall	hr*ng/mL	99.7921	65.71	
123	262	AUCall	hr*ng/mL	36.7306		
123	263	AUCall	hr*ng/mL	60.5979		31.84
123	261	AUCINF_pred	hr*ng/mL	106.1287	77.81	28.72
123	262	AUCINF_pred	hr*ng/mL	48.7064		
123	263	AUCINF_pred	hr*ng/mL	78.5944		
123	261	Cmax	ng/mL	35.37	21.20	12.30
123	262	Cmax	ng/mL	13.31		
123	263	Cmax	ng/mL	14.93		
123	261	Tmax	hr	0.5	0.50	0.5-1
123	262	Tmax	hr	1		
123	263	Tmax	hr	0.5		
121	251	AUCall	hr*ng/mL	121.9123	155.68	55.95
121	252	AUCall	hr*ng/mL	124.8569		
121	253	AUCall	hr*ng/mL	220.2647		
121	251	AUCINF_pred	hr*ng/mL	126.9403	180.14	85.35
121	252	AUCINF_pred	hr*ng/mL	134.89		
121	253	AUCINF_pred	hr*ng/mL	278.5849		
121	251	Cmax	ng/mL	71.03	71.01	9.91
121	252	Cmax	ng/mL	61.1		
121	253	Cmax	ng/mL	80.91		
121	251	Tmax	hr	0.5	0.5	Same Tmax
121	252	Tmax	hr	0.5		
121	253	Tmax	hr	0.5		

[19] Median was used for Tmax. All other parameters use the arithmetic mean.

[20] SD: standard deviation

Expt 122 & 124

Expt	ID	Parameter	Units	Estimate	Average/ Median	SD /range
124	264	AUCall	hr*ng/mL	1566.039	1784.770	373.854
124	265	AUCall	hr*ng/mL	2216.446		
124	266	AUCall	hr*ng/mL	1571.823		
124	264	Cmax	ng/mL	1936.700	2153.900	499.566
124	265	Cmax	ng/mL	2725.300		
124	266	Cmax	ng/mL	1799.700		
124	264	Tmax	hr	0.083	0.083	
124	265	Tmax	hr	0.083		
124	266	Tmax	hr	0.083		Same Tmax
124	264	Vss_obs	mL/kg	1134.692	1208.065	291.935
124	265	Vss_obs	mL/kg	959.817		
124	266	Vss_obs	mL/kg	1529.687		
125	254	AUCall	hr*ng/mL	2357.721	2323.415	49.040
125	255	AUCall	hr*ng/mL	2267.245		
125	256	AUCall	hr*ng/mL	2345.278		
125	254	Cmax	ng/mL	2013.800	2061.233	47.104
125	255	Cmax	ng/mL	2061.900		
125	256	Cmax	ng/mL	2108.000		
125	254	Tmax	hr	0.083	0.083	
125	255	Tmax	hr	0.083		Same Tmax
125	256	Tmax	hr	0.083		
125	254	Vss_obs	mL/kg	1484.891	1535.702	55.156
125	255	Vss_obs	mL/kg	1594.362		
125	256	Vss_obs	mL/kg	1527.854		

Figures

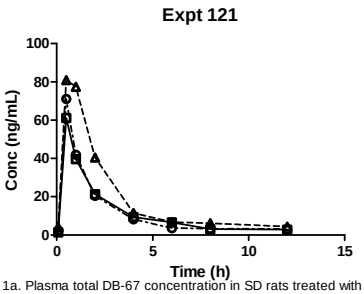


Fig. 1a. Plasma total DB-67 concentration in SD rats treated with 20 mg/kg Zosuquidar PO 5 min before 2.5 mg/kg DB-67 lactone PO

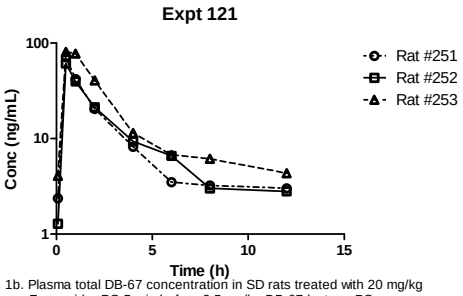


Fig. 1b. Plasma total DB-67 concentration in SD rats treated with 20 mg/kg Zosuquidar PO 5 min before 2.5 mg/kg DB-67 lactone PO

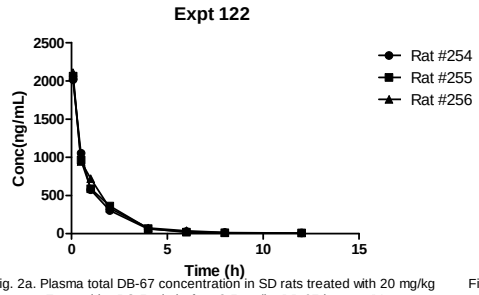


Fig. 2a. Plasma total DB-67 concentration in SD rats treated with 20 mg/kg Zosuquidar PO 5 min before 2.5 mg/kg DB-67 lactone IV

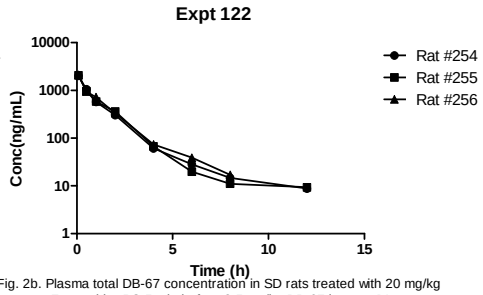


Fig. 2b. Plasma total DB-67 concentration in SD rats treated with 20 mg/kg Zosuquidar PO 5 min before 2.5 mg/kg DB-67 lactone IV

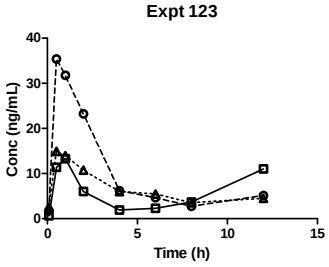


Fig. 3a. Plasma total DB-67 concentration in SD rats treated with 50 mg/kg Novobiocin PO 5 min before 2.5 mg/kg DB-67 lactone PO

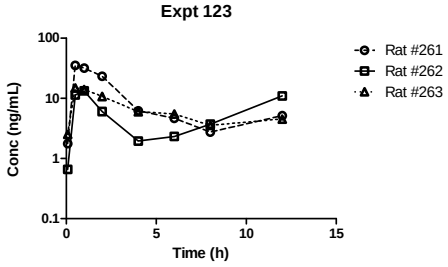


Fig. 3b. Plasma total DB-67 concentration in SD rats treated with 50 mg/kg Novobiocin PO 5 min before 2.5 mg/kg DB-67 lactone PO

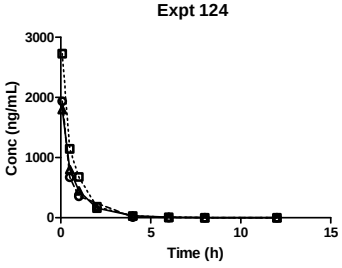


Fig. 4a. Plasma total DB-67 concentration in SD rats treated with 50 mg/kg Novobiocin PO 5 min before 2.5 mg/kg DB-67 lactone IV

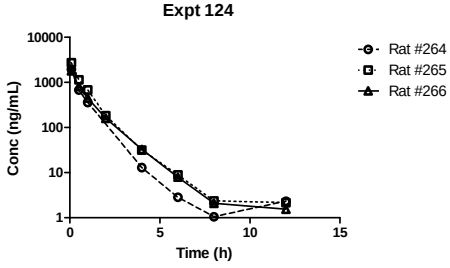


Fig. 4b. Plasma total DB-67 concentration in SD rats treated with 50 mg/kg Novobiocin PO 5 min before 2.5 mg/kg DB-67 lactone IV

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\AUC Rat PK Expt 121, 122,123 & 124(050208)

Raw Data

Sample ID	Expt #	Rat #	Time (h)	AR-67 Carboxylate	AR-67 Lactone	Total AR-67
L251-5min	121	251	0.083	0.05	2.32	2.37
L251-30min	121	251	0.5	11.74	59.29	71.03
L251-1h	121	251	1	7.69	34.3	41.99
L251-2h	121	251	2	4.31	16.11	20.42
L251-4h	121	251	4	1.51	6.69	8.2
L251-6h	121	251	6	0.55	2.95	3.5
L251-8h	121	251	8	0.45	2.78	3.23
L251-12h	121	251	12	0.46	2.55	3.01
L252-5min	121	252	0.083	0.09	1.19	1.28
L252-30min	121	252	0.5	9.27	51.83	61.1
L252-1h	121	252	1	7.4	32.19	39.59
L252-2h	121	252	2	4.54	16.74	21.28
L252-4h	121	252	4	1.89	7.47	9.36
L252-6h	121	252	6	1.24	5.35	6.59
L252-8h	121	252	8	0.49	2.52	3.01
L252-12h	121	252	12	0.4	2.4	2.8
L253-5min	121	253	0.083	0.32	3.75	4.07
L253-30min	121	253	0.5	12.17	68.74	80.91
L253-1h	121	253	1	14.4	63.02	77.42
L253-2h	121	253	2	10.28	30.15	40.43
L253-4h	121	253	4	2.62	8.81	11.43
L253-6h	121	253	6	1.35	5.39	6.74
L253-8h	121	253	8	1.28	4.86	6.14
L253-12h	121	253	12	0.87	3.47	4.34
L254-5 min Dilu (1:10)	122	254	0.083	169.4	1844.4	2013.8
L254-30 min Dilu (1:10)	122	254	0.5	198.6	855.4	1054
L254-1 h Dilu (1:10)	122	254	1	129	445	574
L254-2 h Dilu (1:10)	122	254	2	63.3	239.8	303.1
L254-4h	122	254	4	14.41	46.74	61.15
L254-6h	122	254	6	6.93	21.39	28.32
L254-8h	122	254	8	3.16	11.63	14.79
L254-12h	122	254	12	1.89	6.89	8.78
L255-5 min Dilu (1:10)	122	255	0.083	181.5	1880.4	2061.9
L255-30 min Dilu (1:10)	122	255	0.5	169.6	793.8	963.4
L255-1 h	122	255	1	111.2	476.8	588

Dilu (1:10)						
L255-2 h Dilu (1:10)	122	255	2	83.3	278.9	362.2
L255-4h	122	255	4	15.71	51.61	67.32
L255-6h	122	255	6	4.41	15.41	19.82
L255-8h	122	255	8	2.35	8.72	11.07
L255-12h	122	255	12	2.24	7.11	9.35
L256-5 min Dilu (1:10)	122	256	0.083	273.3	1834.7	2108
L256-30 min Dilu (1:10)	122	256	0.5	206.8	738.9	945.7
L256-1 h Dilu (1:10)	122	256	1	183.2	535.8	719
L256-2 h Dilu (1:10)	122	256	2	82.2	251.1	333.3
L256-4h	122	256	4	20.48	53.45	73.93
L256-6h	122	256	6	12.16	26.79	38.95
L256-8h	122	256	8	4.41	12.69	17.1
L256-12h	122	256	12	Will be reanalyzed		
L261-5min	123	261	0.083	0.07	1.7	1.77
L261-30min	123	261	0.5	3.98	31.39	35.37
L261-1h	123	261	1	5.47	26.28	31.75
L261-2h	123	261	2	4.17	19.03	23.2
L261-4h	123	261	4	0.99	5.24	6.23
L261-6h	123	261	6	0.87	3.79	4.66
L261-8h	123	261	8	0.35	2.39	2.74
L261-12h	123	261	12	0.68	4.46	5.14
L262-5min	123	262	0.083	0	0.66	0.66
L262-30min	123	262	0.5	1.92	9.45	11.37
L262-1h	123	262	1	2.47	10.84	13.31
L262-2h	123	262	2	1.34	4.72	6.06
L262-4h	123	262	4	0.28	1.68	1.96
L262-6h	123	262	6	0.41	1.91	2.32
L262-8h	123	262	8	0.68	3.04	3.72
L262-12h	123	262	12	1.75	9.27	11.02
L263-5min	123	263	0.083	0.17	2.37	2.54
L263-30min	123	263	0.5	1.72	13.21	14.93
L263-1h	123	263	1	1.91	12.06	13.97
L263-2h	123	263	2	1.89	8.85	10.74
L263-4h	123	263	4	1.04	4.96	6
L263-6h	123	263	6	0.88	4.61	5.49
L263-8h	123	263	8	0.52	3.03	3.55
L263-12h	123	263	12	0.7	3.83	4.53
L264-5 min	124	264	0.083	160.7	1776	1936.7

Dilu (1:10)						
L264-30 min Dilu (1:10)	124	264	0.5	124.8	549.1	673.9
L264-1 h Dilu (1:10)	124	264	1	69.8	289	358.8
L264-2 h Dilu (1:10)	124	264	2	Run stopped. Sample will be renalyzed		
L264-4h	124	264	4	3.65	9.26	12.91
L264-6h	124	264	6	0.76	2.09	2.85
L264-8h	124	264	8	0.24	0.82	1.06
L264-12h	124	264	12	0.38	1.95	2.33
L265-5 min Dilu (1:10)	124	265	0.083	235.5	2489.8	2725.3
L265-30 min Dilu (1:10)	124	265	0.5	186.1	960.5	1146.6
L265-1 h Dilu (1:10)	124	265	1	131.1	547	678.1
L265-2 h Dilu (1:10)	124	265	2	37	145.5	182.5
L265-4h	124	265	4	7.31	24.4	31.71
L265-6h	124	265	6	2.21	6.71	8.92
L265-8h	124	265	8	0.36	2.01	2.37
L265-12h	124	265	12	0.3	1.87	2.17
L266-5 min Dilu (1:10)	124	266	0.083	169.1	1630.6	1799.7
L266-30 min Dilu (1:10)	124	266	0.5	155.6	646.6	802.2
L266-1 h Dilu (1:10)	124	266	1	92.6	354.8	447.4
L266-2 h Dilu (1:10)	124	266	2	34.3	125.7	160
L266-4h	124	266	4	9.54	23.16	32.7
L266-6h	124	266	6	2.32	5.68	8
L266-8h	124	266	8	0.41	1.69	2.1
L266-12h	124	266	12	0.23	1.33	1.56

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on DB-67 Carboxylate Rat PK Study

(May 6th, 08)

Oral bioavailability of SBE-β-CD based DB-67 carboxylate

Expt # 125
Expt # 126

11/20/2008

Objective

To determine the oral bioavailability of SBE-β-CD based DB-67 carboxylate in female SD rats.

Expt 125: Study on PK of SBE-β-CD based (200:1 E/D ratio) DB-67 carboxylate (2.5 mg/kg) PO

Expt 126: Study on PK of SBE-β-CD based (200:1 E/D ratio) DB-67 carboxylate (2.5 mg/kg) IV

Method

Animal treatment and sample processing

Expt 125, Rats (#271-#273, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 carboxylate PO 5 min after vehicle administration at 7.5 mL/kg PO.

Expt 126, Rats (#274-#276, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 carboxylate IV 5 min after vehicle administration at 7.5 mL/kg PO.

DB-67 dosing solution:

DB-67 carboxylate = 1.0 mg/mL. \

Vehicle:

40% PEG-300, 10% Tween-80 in D5W

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 125 & Expt 126:

Sequence name: 20AC-050808-Rat PK Expt 125 & 126.seq

Method: 20AC-052108-reinjection of 051208 calibration curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while noncompartmental analysis was made using WinNonlin v5.2.

Results

Table 1. Pharmacokinetic parameters estimated using the noncompartmental method in Winnonlin v5.2 -- Expt 125 & 126

Expt #	Rat ID	Parameter	Units	Estimate	Average/ Median[21]	SD/ Range[22]
125	271	AUCall	hr*ng/mL	89.7		
125	272	AUCall	hr*ng/mL	132.1		
125	273	AUCall	hr*ng/mL	118.1	113.3	21.6
125	271	Cmax	ng/mL	14.8		
125	272	Cmax	ng/mL	18.7		
125	273	Cmax	ng/mL	17.3	16.9	2.0
125	271	Tmax	Hr	6		
125	272	Tmax	Hr	2		
125	273	Tmax	Hr	2	2	2-6
126	274	AUCall	hr*ng/mL	752.0		
126	275	AUCall	hr*ng/mL	900.9		
126	276	AUCall	hr*ng/mL	852.6	835.2	76.0
126	274	Cmax	ng/mL	1478.6		
126	275	Cmax	ng/mL	1813.0		
126	276	Cmax	ng/mL	1781.9	1691.2	184.8
126	274	Tmax	hr	0.083		
126	275	Tmax	hr	0.083		Same as
126	276	Tmax	hr	0.083	0.083	Tmax
126	274	Vss_pred	mL/kg	2872.3		
126	275	Vss_pred	mL/kg	3061.5		
126	276	Vss_pred	mL/kg	1764.5	2566.1	700.6

[21] Median was used for Tmax. All other parameters use the arithmetic mean

[22] SD: standard deviation

Figures

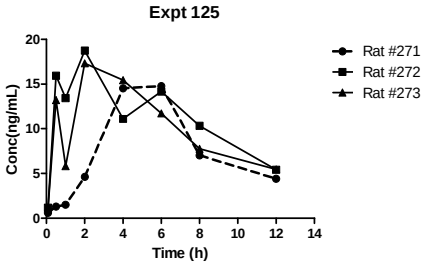


Fig. 1a. Plasma total DB-67 levels in SD rats treated with 200 mg/kg valproic acid PO 5 min before 2.5mg/kg DB-67 PO

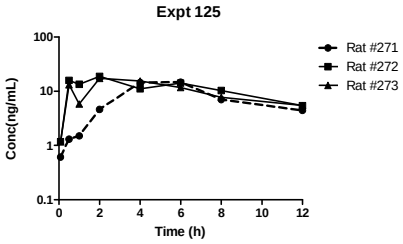


Fig. 1b. Semilog plot of plasma total DB-67 levels in SD rats treated with valproic acid (200 mg/kg) PO 5 min before 2.5mg/kg DB-67 PO

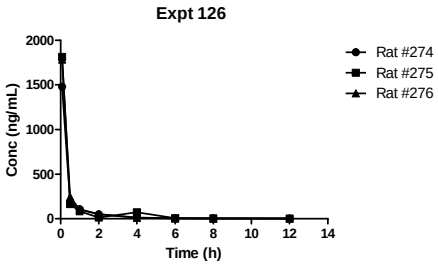


Fig. 2a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg GF IV 5 min before 2.5mg/kg DB-67 PO

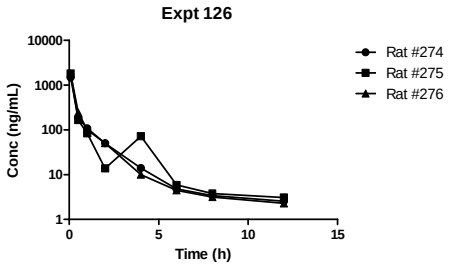


Fig. 2b. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg GF IV 5 min before 2.5mg/kg DB-67 PO

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 125 & 126

Raw Data

Sample ID	Expt #	Rat #	Time (h)	AR-67 Carboxylate (ng/mL)	AR-67 Lactone (ng/mL)	Total AR-67 (ng/mL)
L271-5min-050708	125	271	0.083	0	0.61	0.61
L271-30min-050708	125	271	0.5	0.08	1.23	1.31
L271-1h-050708	125	271	1	0.18	1.32	1.5
L271-2h-050708	125	271	2	0.48	4.14	4.62
L271-4h-050708	125	271	4	1.97	12.56	14.53
L271-6h-050708	125	271	6	2.66	12.11	14.77
L271-8h-050708	125	271	8	1.13	5.9	7.03
L271-12h-050708	125	271	12	0.57	3.84	4.41
L272-5min-050708	125	272	0.083	0.09	1.08	1.17
L272-30min-050708	125	272	0.5	1.68	14.26	15.94
L272-1h-050708	125	272	1	1.79	11.65	13.44
L272-2h-050708	125	272	2	2.62	16.12	18.74
272-4h-	125	272	4	2.67	8.44	11.11

050808						
272-6h-050808	125	272	6	2.9	11.27	14.17
272-8h-050808	125	272	8	2.32	8.01	10.33
272-12h-050808	125	272	12	1	4.43	5.43
273-5min-050808	125	273	0.083	0.32	0.86	1.18
273-30min-050808	125	273	0.5	9.68	3.54	13.22
273-1h-050808	125	273	1	1.03	4.79	5.82
273-2h-050808	125	273	2	6.18	11.15	17.33
273-4h-050808	125	273	4	2.91	12.54	15.45
273-6h-050808	125	273	6	2.39	9.33	11.72
273-8h-050808	125	273	8	1.77	5.99	7.76
273-12h-050808	125	273	12	1.14	4.32	5.46
274-5min-050808	126	274	0.083	1325.111	153.4848	1478.595
274-30min-050808	126	274	0.5	126.64	60.64	187.28
274-1h-050808	126	274	1	42.93	65.3	108.23
274-2h-	126	274	2	14.12	36.08	50.2

050808						
274-4h-050808	126	274	4	5.75	8.3	14.05
274-6h-050808	126	274	6	1.39	3.36	4.75
274-8h-050808	126	274	8	0.85	2.55	3.4
274-12h-050808	126	274	12	0.61	1.94	2.55
275-5min-050808	126	275	0.083	1631.592	181.44	1813.032
275-30min-050808	126	275	0.5	98.53	66.31	164.84
275-1h-050808	126	275	1	31.39	52.71	84.1
275-2h-050808	126	275	2	3.77	10.04	13.81
275-4h-050808	126	275	4	15.04	57.29	72.33
275-6h-050808	126	275	6	1.54	4.35	5.89
275-8h-050808	126	275	8	0.9	2.9	3.8
275-12h-050808	126	275	12	0.7	2.38	3.08
276-5min-050808	126	276	0.083	1588.487	193.4016	1781.888
276-30min-050808	126	276	0.5	172.45	72.89	245.34
276-1h-	126	276	1	35.52	65.81	101.33

050808						
276-2h-050808	126	276	2	13.2	37.37	50.57
276-4h-050808	126	276	4	2.83	7.23	10.06
276-6h-050808	126	276	6	1.09	3.36	4.45
276-8h-050808	126	276	8	0.78	2.36	3.14
276-12h-050808	126	276	12	0.54	1.75	2.29

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on DB-67 Rat PK Study

(May 6th, 08)

Effects of GF120918 on oral bioavailability of SBE-β-CD based DB-67 lactone and carboxylate in rats

---

Expt # 127, Expt # 128 and Expt # 129

11/19/2008

Objective

To determine the effects of GF120918 at low dose of 0.25 mg/kg on oral bioavailability of SBE-β-CD based DB-67 lactone in female SD rats

To determine the effects of GF120918 at 2.5 mg/kg on oral bioavailability of SBE-β-CD based DB-67 carboxylate in female SD rats

Expt 127: Study on effects of GF120918 (0.25 mg/kg) on PK of SBE-β-CD based (200:1 E/D ratio) DB-67 lactone (2.5 mg/kg) PO

Expt 128: Study on effects of GF120918 (2.5 mg/kg) on PK of SBE-β-CD based (200:1 E/D ratio) DB-67 carboxylate (2.5 mg/kg) PO

Expt 129: Study on effects of GF120918 (2.5 mg/kg) on PK of SBE-β-CD based (200:1 E/D ratio) DB-67 carboxylate (2.5 mg/kg) IV

Method

Animal treatment and sample processing

Expt 127, Rats (#281-#282, n=2) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 lactone PO 5 min after GF120918 (2.5 mg/kg) administration at 7.5 mL/kg PO.

Expt 128, Rats (#283-#285, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 carboxylate PO 5 min after GF120918 (2.5 mg/kg) administration at 7.5 mL/kg PO.

Expt 129, Rats (#286-#288, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 carboxylate IV 5 min after GF120918 (2.5 mg/kg) administration at 7.5 mL/kg IV.

Dosing solution:

DB-67 lactone = 1.0 mg/mL.

DB-67 carboxylate = 1.0 mg/mL.

GF120918 solution:

GF120918 was dissolved in vehicle (40% PEG-300, 10% Tween-80 in D5W) within 1 hour before experiment.

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 126, Expt 127 & Expt 128

Sequence name: 20AC-052108-Rat PK Expt 127, 128 & 129.seq

Method: 20AC-052108-reinjection of 051208 calibration curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while noncompartmental analysis was made using WinNonlin v5.2.

Results

Tables

Expt 127, 128 & 129

Expt	ID	Parameter	Units	Estimate	Average/ Median	SD/range
127	281	AUCall	h*ng/mL	214.548	218.813	6.031
127	282	AUCall	h*ng/mL	223.0773		
127	281	Cmax	ng/mL	32.18	32.850	0.948
127	282	Cmax	ng/mL	33.52		
127	281	Tmax	h	2	3.000	1.414
127	282	Tmax	h	4		
127	281	Cl_F_obs	mL/h/kg	10322.21	9771.833	778.358
127	282	Cl_F_obs	mL/h/kg	9221.451		
128	283	AUCall	h*ng/mL	430.8436	416.591	12.345
128	284	AUCall	h*ng/mL	409.2437		
128	285	AUCall	h*ng/mL	409.6845		
128	283	Cmax	ng/mL	71.95	68.443	5.363
128	284	Cmax	ng/mL	62.27		
128	285	Cmax	ng/mL	71.11		
128	283	Tmax	h	2	2.667	1.155
128	284	Tmax	h	4		
128	285	Tmax	h	2		
128	283	Cl_F_obs	mL/h/kg	4646.13	4888.916	216.822
128	284	Cl_F_obs	mL/h/kg	5063.252		
128	285	Cl_F_obs	mL/h/kg	4957.366		

Expt	ID	Parameter	Units	Estimate	Average/ Median	SD/range
129	286	AUCall	h*ng/mL	956.8197	1176.112	200.709
129	287	AUCall	h*ng/mL	1220.817		
129	288	AUCall	h*ng/mL	1350.699		
129	286	Cmax	ng/mL	1217.005	1226.252	280.125
129	287	Cmax	ng/mL	1510.886		
129	288	Cmax	ng/mL	950.8644		
129	286	Tmax	h	0.083	0.083	0.000
129	287	Tmax	h	0.083		
129	288	Tmax	h	0.083		
129	286	Vss_obs	mL/kg	6893.898	6584.311	870.879
129	287	Vss_obs	mL/kg	5600.936		
129	288	Vss_obs	mL/kg	7258.099		
129	286	Cl_obs	mL/h/kg	2489.785	2044.452	405.593
129	287	Cl_obs	mL/h/kg	1947.344		
129	288	Cl_obs	mL/h/kg	1696.228		

Figures

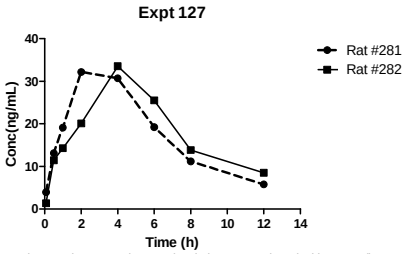


Fig. 1a. Plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg DB-67 lactone PO 5 min after GF (0.25 mg/kg) PO administration

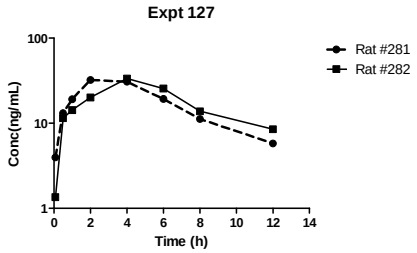


Fig. 1b. Semilog plot of plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg DB-67 lactone PO 5 min after GF (0.25 mg/kg) PO administration

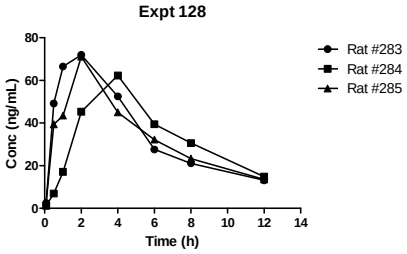


Fig. 2a. Plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg DB-67 carboxylate PO 5 min after GF (2.5 mg/kg) PO administration

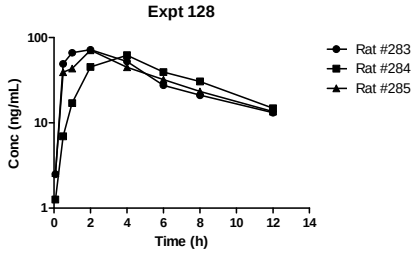


Fig. 2b. Semilog plot of plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg DB-67 carboxylate PO 5 min after GF (2.5 mg/kg) PO administration

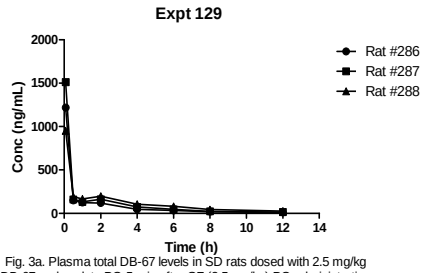


Fig. 3a. Plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg DB-67 carboxylate PO 5 min after GF (2.5 mg/kg) PO administration

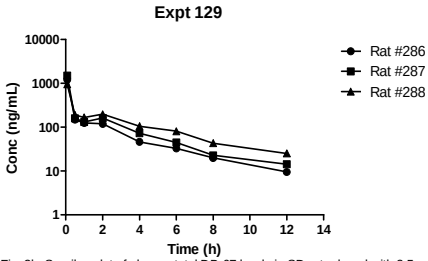


Fig. 3b. Semilog plot of plasma total DB-67 levels in SD rats dosed with 2.5 mg/kg DB-67 carboxylate PO 5 min after GF (2.5 mg/kg) PO administration

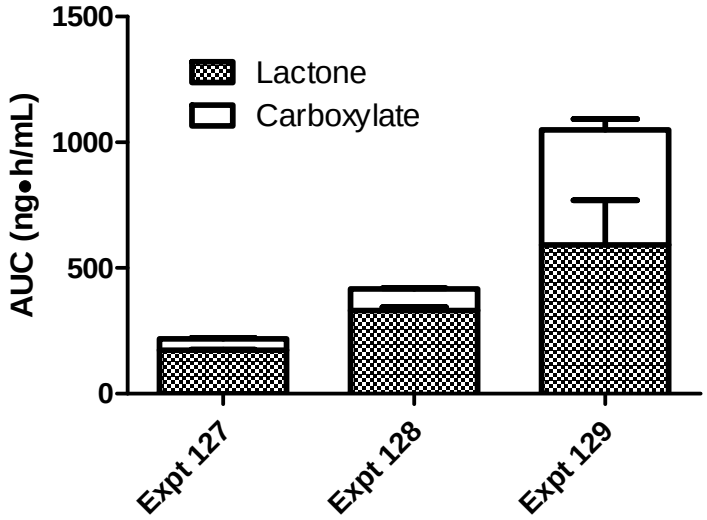


Fig. 7. AUCs of lactone and carboxylate forms of DB-67 in SD rats following PO administrtation of DB-67 lactone at 2.5 mg/kg 5 min after GF PO at 0.25 mg/kg (expt 127), PO (expt 128) and IV (expt 129) administration of DB-67 carboxylate at 2.5 mg/kg 5 min after GF PO at 2.5 mg/kg

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 127, 128 & 129

Raw Data

Expt #	Rat #	Time (h)	DB-67 Carboxylate	DB-67 Lactone	Total DB-67 (ng/mL)
127	281	0.083	0.43	3.53	3.96
127	281	0.5	2.49	10.63	13.12
127	281	1	3.59	15.52	19.11
127	281	2	6.02	26.16	32.18
127	281	4	6.12	24.58	30.7
127	281	6	3.86	15.36	19.22
127	281	8	2.78	8.4	11.18
127	281	12	1.22	4.56	5.78
127	282	0.083	0.36	0.99	1.35
127	282	0.5	2	9.45	11.45
127	282	1	3.03	11.25	14.28
127	282	2	4.46	15.64	20.1
127	282	4	6.61	26.91	33.52
127	282	6	6.05	19.46	25.51
127	282	8	3.27	10.58	13.85
127	282	12	1.65	6.86	8.51
128	283	0.083	0.42	2.08	2.5
128	283	0.5	7.56	41.63	49.19
128	283	1	12.02	54.46	66.48
128	283	2	13.39	58.56	71.95
128	283	4	11.36	41.16	52.52
128	283	6	5.55	22.06	27.61
128	283	8	4.05	17.05	21.1
128	283	12	2.65	10.51	13.16
128	284	0.083	0.49	0.77	1.26
128	284	0.5	1.15	5.81	6.96
128	284	1	3.22	13.89	17.11
128	284	2	8.93	36.4	45.33
128	284	4	12.41	49.86	62.27
128	284	6	9.75	29.71	39.46
128	284	8	7.17	23.44	30.61
128	284	12	3.04	11.77	14.81
128	285	0.083	1.38	1.21	2.59
128	285	0.5	6.2	33.15	39.35
128	285	1	7.38	36.1	43.48
128	285	2	14.95	56.16	71.11
128	285	4	9.47	35.53	45
128	285	6	7.18	25.03	32.21
128	285	8	4.98	18.42	23.4
128	285	12	2.31	11.24	13.55
129	286	0.083	1123.019	93.9862	1217.006
129	286	0.5	100.68	48.21	148.89

129	286	1	45.61	78.61	124.22
129	286	2	25.66	92.8	118.46
129	286	4	10.69	35.44	46.13
129	286	6	7.65	25.18	32.83
129	286	8	4.22	15.72	19.94
129	286	12	1.99	7.5	9.49
129	287	0.083	1398.05	112.8358	1510.886
129	287	0.5	96.77	62.14	158.91
129	287	1	44.99	86.33	131.32
129	287	2	32.35	128.75	161.1
129	287	4	15.82	56.84	72.66
129	287	6	8.9	35.79	44.69
129	287	8	4.66	18.21	22.87
129	287	12	3.12	11.19	14.31
129	288	0.083	864.994	85.8704	950.8644
129	288	0.5	128.39	62	190.39
129	288	1	66.68	99.9	166.58
129	288	2	48.37	148.66	197.03
129	288	4	22.6	82.22	104.82
129	288	6	17.36	63.48	80.84
129	288	8	9.17	33.91	43.08
129	288	12	4.58	20.57	25.15

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on DB-67 Lactone Rat PK Study

(May 14th, 08)

Effects of GF120918 and grapefruit juice on bioavailability of SBE-β-CD based DB-67 lactone

---

Expt # 131 and Expt # 132

11/21/2008

Objective

To determine the effects of GF (in 10% PEG-300) on oral bioavailability of SBE-β-CD based DB-67 lactone in rats

To determine the effects of grapefruit juice on oral bioavailability of SBE-β-CD based DB-67 lactone in rats

Expt 131: Study on effects of GF in 10% PEG-300 (2.5 mg/kg) on PK of SBE-β-CD based (200:1 E/D ratio) DB-67 lactone (2.5 mg/kg) PO

Expt 132: Study on effects of grapefruit juice double strength (10 mL/kg) on PK of SBE-β-CD based (200:1 E/D ratio) DB-67 lactone (2.5 mg/kg) PO

Method

Animal treatment and sample processing

Expt 131, Rats (#291-#293, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 lactone PO 5 min after GF120918 (2.5 mg/kg) administration at 7.5 mL/kg PO.

Expt 132, Rats (#294-#296, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 lactone PO 1 hour after grapefruit juice (double strength) administration at 10 mL/kg PO.

Dosing solution:

DB-67 lactone = 1.0 mg/mL

GF120918 solution:

GF120918 was dissolved in 10% PEG-300 in D5W, prepared freshly before using.

Grapefruit juice:

Double strength, Kroger brand, thawed right before using.

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 131 & 132

Sequence name: 20AC-051908-Rat PK Expt 131 & 132.seq

Method: 20AC-052108-reinjection of 051208 calibration curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while nocompartmental analysis was made using Winnonlin v5.2.

Results

Tables

Expt 131 & 132

ID	Parameter	Units	Estimate	Average/ Median	SD/range
291	AUCall	h*ng/mL	278.4716	289.007	28.669
292	AUCall	h*ng/mL	267.0952		
293	AUCall	h*ng/mL	321.4531		
291	Cl_F_obs	mL/h/kg	3573.39	5555.672	2541.509
292	Cl_F_obs	mL/h/kg	8420.896		
293	Cl_F_obs	mL/h/kg	4672.73		
291	Cmax	ng/mL	35.18	39.490	10.866
292	Cmax	ng/mL	51.85		
293	Cmax	ng/mL	31.44		
291	Tmax	h	2	2.000	2--6
292	Tmax	h	2		
293	Tmax	h	6		
294	AUCall	h*ng/mL	106.3305	112.595	15.086
295	AUCall	h*ng/mL	101.6499		
296	AUCall	h*ng/mL	129.8031		
294	Cl_F_obs	mL/h/kg	16673.44	16545.277	1352.975
295	Cl_F_obs	mL/h/kg	17829.61		
296	Cl_F_obs	mL/h/kg	15132.78		
294	Cmax	ng/mL	30.47	29.450	4.920
295	Cmax	ng/mL	24.1		
296	Cmax	ng/mL	33.78		
294	Tmax	h	0.5	0.500	0.500
295	Tmax	h	0.5		
296	Tmax	h	0.5		

Figures

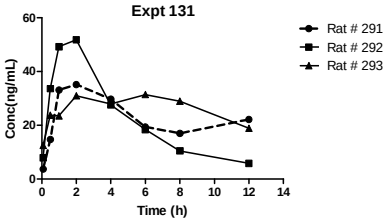


Fig. 1a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/mL GF in 10% PEG300 5 min before 2.5 mg/kg DB-67 lactone PO

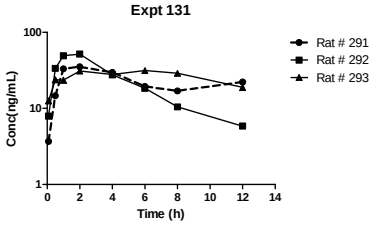


Fig. 1b. Semilog plot of plasma total DB-67 levels in SD rats treated with 2.5 mg/kg GF in 10% PEG300 5 min before 2.5 mg/kg DB-67 lactone PO

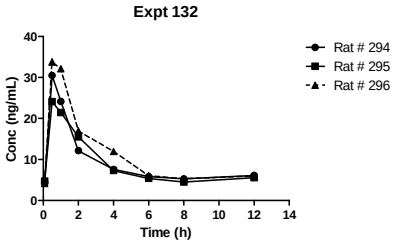


Fig. 2a. Plasma total DB-67 levels in SD rats pretreated with double strengh grapefruit juice (10 mL/kg) twice for 2 days and once 1 h before of 2.5 mg/kg DB-67 lactone PO

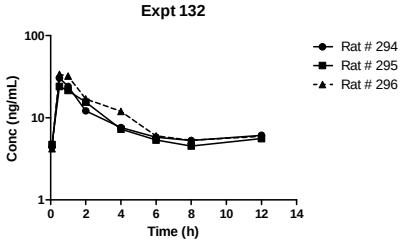


Fig. 2b. Semilog plot of plasma total DB-67 levels in SD rats pretreated with double strengh grapefruit juice (10 mL/kg) twice for 2 days and once 1 h before 2.5 mg/kg DB-67 lactone PO

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 131 & 132

Raw Data

Sample ID	Expt #	Rat #	Time (h)	Carboxylate	Lactone	Total AR-67
L291-5min-051908	131	291	0.083	0.07	3.61	3.68
L291-30min-051908	131	291	0.5	1.52	13.14	14.66
L291-1h-051908	131	291	1	4.18	28.9	33.08
L291-2h-051908	131	291	2	5.6	29.58	35.18
L291-4h-051908	131	291	4	4.58	25.12	29.7
L291-6h-051908	131	291	6	3.22	16.13	19.35
L291-8h-051908	131	291	8	2.92	14.03	16.95
L291-12h-051908	131	291	12	2.41	19.74	22.15
L292-5min-051908	131	292	0.083	0.83	7.06	7.89
L292-30min-051908	131	292	0.5	5.33	28.28	33.61
L292-1h-051908	131	292	1	7.1	42.11	49.21
L292-2h-051908	131	292	2	9.03	42.82	51.85
L292-4h-051908	131	292	4	5.17	22.42	27.59
L292-6h-051908	131	292	6	3.09	15.29	18.38
L292-8h-051908	131	292	8	1.59	8.88	10.47
L292-12h-051908	131	292	12	0.61	5.23	5.84
L293-5min-051908	131	293	0.083	1.4	11.1	12.5
L293-30min-051908	131	293	0.5	4.48	19.24	23.72
L293-1h-051908	131	293	1	3.94	19.59	23.53
L293-2h-051908	131	293	2	6.41	24.56	30.97
L293-4h-051908	131	293	4	6.05	21.9	27.95
L293-6h-051908	131	293	6	6.06	25.38	31.44
L293-8h-051908	131	293	8	5.2	23.75	28.95
L293-12h-051908	131	293	12	2.9	15.96	18.86
L294-5min-051908	132	294	0.083	0.22	4.2	4.42
L294-30 min-051908	132	294	0.5	3.6	26.87	30.47
L294-1 h-051908	132	294	1	3.58	20.58	24.16
L294-2 h-051908	132	294	2	1.8	10.35	12.15
L294-4 h-051908	132	294	4	0.94	6.66	7.6
L294-6h-051908	132	294	6	0.6	5.21	5.81
L294-8h-051908	132	294	8	0.48	4.81	5.29

L294-12h-051908	132	294	12	0.64	5.47	6.11
L295- 5min-051908	132	295	0.083	0.28	4.46	4.74
L295-30 min-051908	132	295	0.5	2.76	21.34	24.1
L295- 1h-051908	132	295	1	3.96	17.48	21.44
L295-2 h-051908	132	295	2	2.98	12.53	15.51
L295-4 h-051908	132	295	4	1.15	6.15	7.3
L295-6h-051908	132	295	6	0.64	4.73	5.37
L295-8h-051908	132	295	8	0.37	4.16	4.53
L295-12h-051908	132	295	12	0.6	4.97	5.57
L296-5min-051908	132	296	0.083	0.17	4.02	4.19
L296-30min-051908	132	296	0.5	3.45	30.33	33.78
L296- 1h-051908	132	296	1	4.63	27.5	32.13
L296-2 h-051908	132	296	2	2.65	14.29	16.94
L296-4 h-051908	132	296	4	1.94	10	11.94
L296-6h-051908	132	296	6	0.63	5.38	6.01
L296-8h-051908	132	296	8	0.49	4.83	5.32
L296-12h-051908	132	296	12	0.57	5.38	5.95

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on DB-67 Lactone Rat PK Study

(May 22nd, 08)

Effects of valproic acid (PO) and GF120918 (IV) on bioavailability of SBE-β-CD based DB-67 Lactone (PO)

Expt # 134 and Expt # 135

11/21/2008

Objective

To determine the effects of UGT inhibition by valproic acid on oral bioavailability of SBE-β-CD based DB-67 lactone in rats

To determine the effects of GF IV on oral bioavailability of SBE-β-CD based DB-67 lactone in rats

Expt 134: Study on effects of UGT inhibition by valproic acid (200 mg/kg) on PK of SBE-β-CD based (200:1 E/D ratio) DB-67 lactone (2.5 mg/kg) PO

Expt 135: Study on effects of GF (2.5 mg/kg) IV on PK of SBE-β-CD based (200:1 E/D ratio) DB-67 lactone (2.5 mg/kg) PO

Method

Animal treatment and sample processing

Expt 134, Rats (#301-#303, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 lactone PO 5 min after valproic acid (200 mg/kg) administration at 7.5 mL/kg PO.

Expt 135, Rats (#304-#306, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 lactone PO 5 min after GF (2.5 mg/kg) at 7.5 mL/kg IV.

Dosing solution:

DB-67 lactone = 1.0 mg/mL

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 134 & 135

Sequence name: 20AC-050308-Rat PK Expt 134 & 135.seq

Method: 20AC-052108-reinjection of 051208 calibration curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while nocompartmental analysis was made using Winnonlin v5.2.

Results

Tables

Expt 131 & 132

ID	Parameter	Units	Estimate	Average/ Median	SD/range
301	AUCall	h*ng/mL	94.1298	90.701	5.154
302	AUCall	h*ng/mL	84.7738		
303	AUCall	h*ng/mL	93.1988		
301	Cmax	ng/mL	18.86	21.460	3.554
302	Cmax	ng/mL	25.51		
303	Cmax	ng/mL	20.01		
301	Cl_F_obs	mL/h/kg	13984.2	12710.256	5616.915
302	Cl_F_obs	mL/h/kg	6565.784		
303	Cl_F_obs	mL/h/kg	17580.78		
301	Tmax	h	0.5	0.500	0.5--1
302	Tmax	h	0.5		
303	Tmax	h	1		
304	AUCall	h*ng/mL	192.3323	244.139	57.989
305	AUCall	h*ng/mL	306.7813		
306	AUCall	h*ng/mL	233.3036		
304	Cmax	ng/mL	26.21	40.703	25.607
305	Cmax	ng/mL	70.27		
306	Cmax	ng/mL	25.63		
304	Cl_F_obs	mL/h/kg	8207.239	6118.561	1808.882
305	Cl_F_obs	mL/h/kg	5063.222		
306	Cl_F_obs	mL/h/kg	5085.221		
304	Tmax	h	0.5	0.500	0.5--2
305	Tmax	h	0.5		
306	Tmax	h	2		

Figures

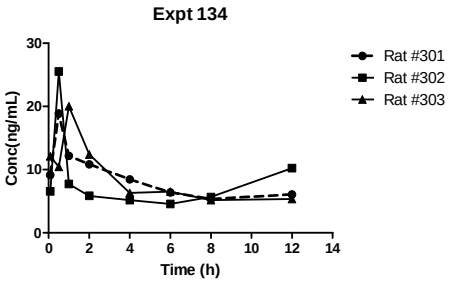


Fig. 1a. Plasma total DB-67 levels in SD rats treated with 200 mg/kg valproic acid PO 5 min before 2.5mg/kg DB-67 PO

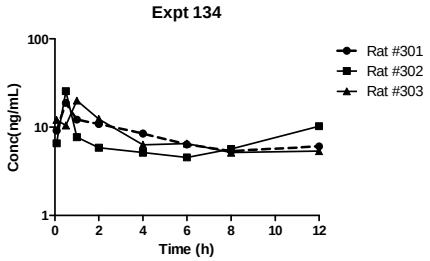


Fig. 1b. Semilog plot of plasma total DB-67 levels in SD rats treated with valproic acid (200 mg/kg) PO 5 min before 2.5mg/kg DB-67 PO

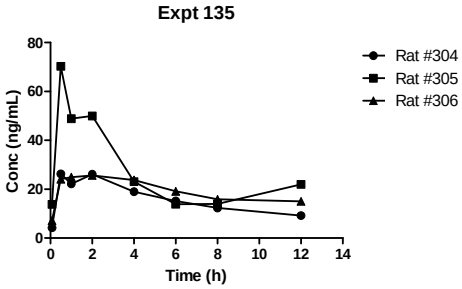


Fig. 2a. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg GF IV 5 min before 2.5mg/kg DB-67 PO

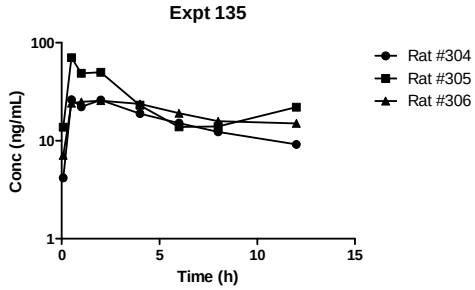


Fig. 2b. Plasma total DB-67 levels in SD rats treated with 2.5 mg/kg GF IV 5 min before 2.5mg/kg DB-67 PO

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 134 & 135

Raw Data

Sample ID	Expt #	Rat #	Time (h)	Carb.	Lact	Total AR-67
L301-5min-052308	134	301	0.083	0.63	8.51	9.14
L301-30min-052308	134	301	0.5	2.35	16.51	18.86
L301-1h-052308	134	301	1	1.91	10.26	12.17
L301-2h-052308	134	301	2	1.76	9.08	10.84
L301-4h-052308	134	301	4	1.28	7.17	8.45
L301-6h-052308	134	301	6	0.51	5.87	6.38
L301-8h-052308	134	301	8	0.53	4.82	5.35
L301-12h-052308	134	301	12	0.58	5.47	6.05
L302-5min-052308	134	302	0.083	0.33	6.23	6.56
L302-30min-052308	134	302	0.5	3.41	22.1	25.51
L302-1h-052308	134	302	1	1.03	6.68	7.71
L302-2h-052308	134	302	2	0.65	5.2	5.85
L302-4h-052308	134	302	4	0.58	4.58	5.16
L302-6h-052308	134	302	6	0.34	4.21	4.55
L302-8h-052308	134	302	8	0.57	5.09	5.66
L302-12h-052308	134	302	12	1.38	8.86	10.24
L303-5min-052308	134	303	0.083	1.09	10.99	12.08
L303-30min-052308	134	303	0.5	1.45	9	10.45
L303-1h-052308	134	303	1	3.13	16.88	20.01
L303-2h-052308	134	303	2	2.03	10.35	12.38
L303-4h-052308	134	303	4	0.69	5.62	6.31
L303-6h-052308	134	303	6	0.7	5.82	6.52
L303-8h-052308	134	303	8	0.41	4.74	5.15
L303-12h-052308	134	303	12	0.44	4.91	5.35
L304-5min-052308	135	304	0.083	0.18	4	4.18
L304-30min-052308	135	304	0.5	2.59	23.62	26.21
L304-1h-052308	135	304	1	2.63	19.55	22.18
L304-2h-052308	135	304	2	3.6	22.47	26.07
L304-4h-052308	135	304	4	2.88	16.08	18.96
L304-6h-052308	135	304	6	1.89	13.26	15.15
L304-8h-052308	135	304	8	1.27	11.04	12.31

L304-12h-052308	135	304	12	1.05	8.14	9.19
L305-5min-052308	135	305	0.083	2.45	11.31	13.76
L305-30min-052308	135	305	0.5	7.24	63.03	70.27
L305-1h-052308	135	305	1	7.01	41.8	48.81
L305-2h-052308	135	305	2	7.21	42.76	49.97
L305-4h-052308	135	305	4	3.69	19.32	23.01
L305-6h-052308	135	305	6	1.92	11.89	13.81
L305-8h-052308	135	305	8	1.58	12.4	13.98
L305-12h-052308	135	305	12	2.55	19.44	21.99
L306-5min-052308	135	306	0.083	0.72	6.37	7.09
L306-30min-052308	135	306	0.5	3.41	20.72	24.13
L306-1h-052308	135	306	1	4.33	20.58	24.91
L306-2h-052308	135	306	2	4.21	21.42	25.63
L306-4h-052308	135	306	4	3.6	20.17	23.77
L306-6h-052308	135	306	6	3.2	15.91	19.11
L306-8h-052308	135	306	8	2.61	13.21	15.82
L306-12h-052308	135	306	12	1.92	13.14	15.06

OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on DB-67 Rat PK Study

(May 29th, 08)

Bioavailability of SBE-β-CD based DB-67 lactone and carboxylate (10 mg/kg) administered PO

Expt # 137 and Expt # 138

11/22/2008

Objective

To determine the oral bioavailability of SBE-β-CD based DB-67 in lactone and carboxylate forms at 10 mg/kg in rats. This is a part of dose-dependent experiments (2.5, 5.0 10.0 mg/kg) to determine the linear range between DB-67 dose and AUC of plasma total DB-67 levels. To keep the total dosing volume consistent in all 3 groups at 10.0 mL/kg, the rats in 10 mg/kg groups were co-treated with no D5W.

Expt 137: Study on the oral bioavailability of SBE-β-CD based DB-67 lactone 10 mg/kg in SD female rats PO

Expt 138: Study on the oral bioavailability of SBE-β-CD based DB-67 carboxylate at 10 mg/kg in SD female rats PO

Method

Animal treatment and sample processing

Expt 137, Rats (#311-#313, n=3) were dosed with 10 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 lactone PO.

Expt 138, Rats (#314-#316, n=3) were dosed with 2.5 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 carboxylate PO.

Dosing solution:

DB-67 lactone = 1.0 mg/mL

DB-67 carboxylate = 1.0 mg/mL

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 137 & 138

Sequence name: 20AC-060908-Expt 137 & 138.seq

Method: 20AC-052108-reinjection of 051208 calibration curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while nocompartmental analysis was made using Winnonlin v5.2.

Results

Tables

Expt 137 & 138

ID	Parameter	Units	Estimate	Average/ Median	SD/range
311	AUCall	h*ng/mL	391.9731	358.374	50.234
312	AUCall	h*ng/mL	382.5226		
313	AUCall	h*ng/mL	300.6262		
311	Cmax	ng/mL	123.54	138.700	13.748
312	Cmax	ng/mL	150.36		
313	Cmax	ng/mL	142.2		
311	Cl_F_obs	mL/h/kg	4482.995	5028.643	531.551
312	Cl_F_obs	mL/h/kg	5544.876		
313	Cl_F_obs	mL/h/kg	5058.058		
311	Tmax	h	0.5	0.500	0.500
312	Tmax	h	0.5		
313	Tmax	h	0.5		
314	AUCall	h*ng/mL	319.2915	323.105	39.685
315	AUCall	h*ng/mL	364.5588		
316	AUCall	h*ng/mL	285.4643		
314	Cmax	ng/mL	146.59	135.163	21.060
315	Cmax	ng/mL	148.04		
316	Cmax	ng/mL	110.86		
314	Cl_F_obs	mL/h/kg	6988.664	6813.151	531.318
315	Cl_F_obs	mL/h/kg	6216.282		
316	Cl_F_obs	mL/h/kg	7234.507		
314	Tmax	h	0.5	0.500	0.500
315	Tmax	h	0.5		
316	Tmax	h	0.5		

Figures

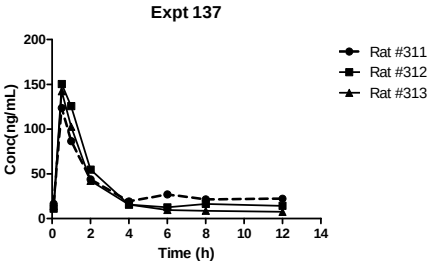


Fig. 1a. Plasma total DB-67 levels in SD rats treated with 10 mg/kg DB-67 lactone PO

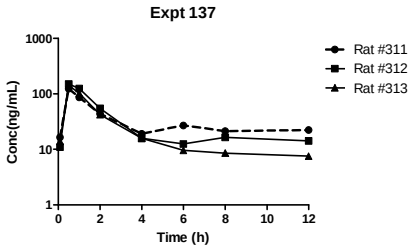


Fig. 1b. Semilog plot of plasma total DB-67 levels in SD rats treated with 10 mg/kg DB-67 lactone PO

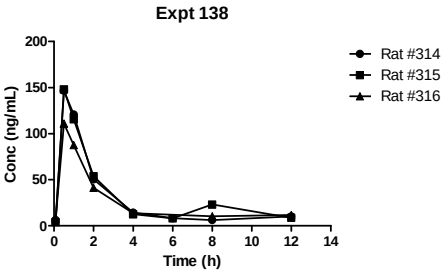


Fig. 2a. Plasma total DB-67 levels in SD rats treated with 10 mg/kg DB-67 carboxylate PO

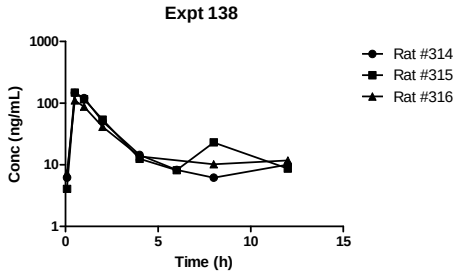


Fig. 2b. Plasma total DB-67 levels in SD rats treated with 10 mg/kg DB-67 carboxylate PO

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 137 & 138

Raw Data

Sample ID	Expt #	Rat #	Time (h)	Carboxylate (ng/mL)	Lactone (ng/mL)	Total (ng/ml)
L311-5 min	137	311	0.083	2.16	14.33	16.49
L311-30 min	137	311	0.5	15.42	108.12	123.54
L311- 1 h	137	311	1	11.49	74.9	86.39
L311- 2 h	137	311	2	7.61	36.1	43.71
L311- 4 h	137	311	4	3.5	15.59	19.09
L311- 6 h	137	311	6	4.5	22.48	26.98
L311- 8 h	137	311	8	3.66	17.71	21.37
L311- 12 h	137	311	12	3.68	18.62	22.3
L312-5 min	137	312	0.083	1.99	8.98	10.97
L312-30 min	137	312	0.5	18.06	132.3	150.36
L312- 1 h	137	312	1	19.9	105.74	125.64
L312- 2 h	137	312	2	11.81	42.87	54.68
L312- 4 h	137	312	4	3.99	11.83	15.82
L312- 6 h	137	312	6	2.38	10.15	12.53
L312- 8 h	137	312	8	3.01	13.46	16.47
L312- 12 h	137	312	12	3.04	11.2	14.24
L313-5 min	137	313	0.083	2.19	11.76	13.95
L313-30 min	137	313	0.5	20	122.2	142.2
L313- 1 h	137	313	1	17.96	84.56	102.52
L313- 2 h	137	313	2	8.3	33.94	42.24
L313- 4 h	137	313	4	3.91	11.92	15.83
L313- 6 h	137	313	6	1.83	7.78	9.61
L313- 8 h	137	313	8	1.55	7.02	8.57
L313- 12 h	137	313	12	1.86	5.69	7.55
L314-5 min	138	314	0.083	0.95	5.24	6.19
L314-30 min	138	314	0.5	18.78	127.81	146.59
L314- 1 h	138	314	1	21.8	99.15	120.95
L314- 2 h	138	314	2	9.81	40.61	50.42
L314- 4 h	138	314	4	3.03	11.43	14.46
L314- 6 h	138	314	6	1.64	6.65	8.29
L314- 8 h	138	314	8	1.04	5.15	6.19
L314- 12 h	138	314	12	2.21	7.85	10.06
L315-5 min	138	315	0.083	0.54	3.54	4.08
L315-30 min	138	315	0.5	19.96	128.08	148.04
L315- 1 h	138	315	1	17.27	98.38	115.65
L315- 2 h	138	315	2	10.53	43.3	53.83
L315- 4 h	138	315	4	2.49	10.02	12.51
L315- 6 h	138	315	6	1.48	6.67	8.15
L315- 8 h	138	315	8	4.57	18.59	23.16
L315- 12 h	138	315	12	2.27	6.42	8.69
L316-5 min	138	316	0.083	1.49	5.78	7.27

L316-30 min	138	316	0.5	17.91	92.95	110.86
L316- 1 h	138	316	1	15.92	71.71	87.63
L316- 2 h	138	316	2	9.76	31.45	41.21
L316- 4 h	138	316	4	3.19	10.47	13.66
L316- 6 h	138	316	6	miss	miss	miss
L316- 8 h	138	316	8	2.44	7.76	10.2
L316- 12 h	138	316	12	3.54	8.21	11.75

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on DB-67 Rat PK Study

(June 10th, 08)

Bioavailability of SBE-β-CD based DB-67 lactone and carboxylate (10 mg/kg) administered PO

Expt # 140 and Expt # 141

Objective

To determine the oral bioavailability of SBE-β-CD based DB-67 in lactone and carboxylate form at 5.0 mg/kg in rats. This is a part of dose-dependent experiments (2.5, 5.0 10.0 mg/kg) to determine the linear range between DB-67 dose and AUC of plasma total DB-67 levels. To keep the total dosing volume consistent in all 3 groups at 10.0 mL/kg, the rats in 5.0 mg/kg groups were co-treated with 5.0 mL/kg D5W.

Expt 140: Study on the oral bioavailability of SBE-β-CD based DB-67 lactone in rats at 5 mg/kg PO

Expt 141: Study on the oral bioavailability of SBE-β-CD based DB-67 carboxylate in rats at 5 mg/kg PO

Method

Animal treatment and sample processing

Expt 140, Rats (#321-#323, n=3) were dosed with 5.0 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 lactone PO.

Expt 141, Rats (#324-#326, n=3) were dosed with 5.0 mg/kg SBE-β-CD based (200:1 E/D ratio) DB-67 carboxylate PO.

Dosing solution:

DB-67 lactone = 1.0 mg/mL

DB-67 carboxylate = 1.0 mg/mL

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 137 & 138

Sequence name: 20AC-061108 Rat PK Exp 140 &141.seq

Method: 20AC-052108-reinjection of 051208 calibration curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while nocompartmental analysis was made using Winnonlin v5.2.

Results

Tables

Expt 140 & 141

Expt	ID	Parameter	Units	Estimate	Average/ Median	SD/range
140	321	AUCall	h*ng/mL	159.8737	183.97	41.78
140	322	AUCall	h*ng/mL	232.2123		
140	323	AUCall	h*ng/mL	159.8232		
140	321	Cmax	ng/mL	72.26	93.52	37.13
140	322	Cmax	ng/mL	136.39		
140	323	Cmax	ng/mL	71.91		
140	321	Tmax	h	0.5	0.50	0.50
140	322	Tmax	h	0.5		
140	323	Tmax	h	0.5		
140	321	HL_Lambda_z	h	2.7774	2.36	0.38
140	322	HL_Lambda_z	h	2.0383		
140	323	HL_Lambda_z	h	2.2683		
141	324	AUCall	h*ng/mL	199.7589	146.48	46.26
141	325	AUCall	h*ng/mL	123.1403		
141	326	AUCall	h*ng/mL	116.5495		
141	324	Cmax	ng/mL	66.64	49.16	20.43
141	325	Cmax	ng/mL	54.15		
141	326	Cmax	ng/mL	26.7		
141	324	Tmax	h	1	0.500	0.5--1
141	325	Tmax	h	0.5		
141	326	Tmax	h	0.5		
141	324	HL_Lambda_z	h	4.0737	4.65	0.79

141	325	HL_Lambda_z	h	4.3081		
141	326	HL_Lambda_z	h	5.5547		

Figures

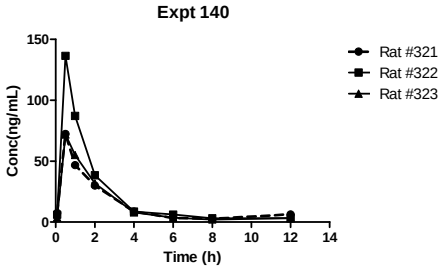


Fig. 1a. Plasma total DB-67 levels in SD rats treated with 5 mg/kg DB-67 lactone PO

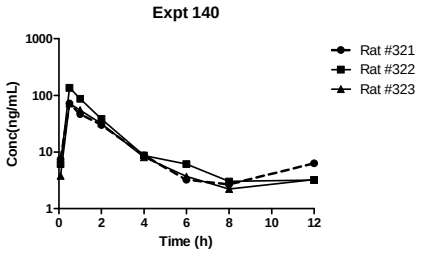


Fig. 1b. Semilog plot of plasma total DB-67 levels in SD rats treated with 5 mg/kg DB-67 lactone PO

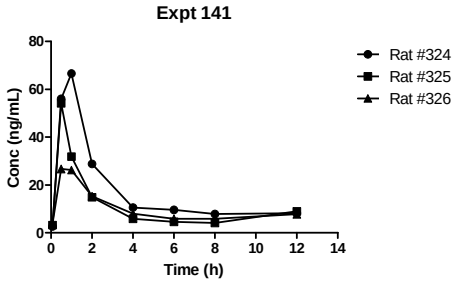


Fig. 2a. Plasma total DB-67 levels in SD rats treated with 5 mg/kg DB-67 carboxylate PO

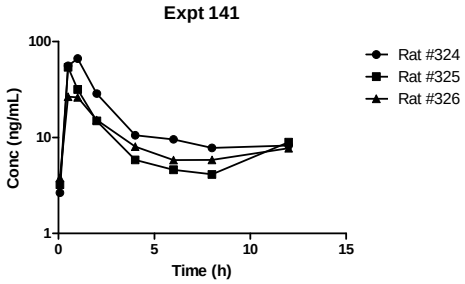


Fig. 2b. Semilog plot of plasma total DB-67 levels in SD rats treated with 5 mg/kg DB-67 carboxylate PO

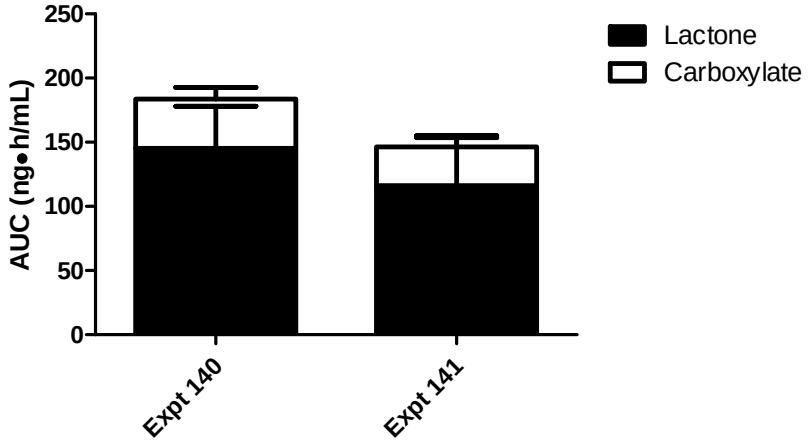


Fig. 3. AUCs of lactone and carboxylate forms of DB-67 in SD rats following PO administrtation of DB-67 lactone (expt 140) and carboxylate (expt 141) at 5 mg/kg

File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 140 & 141

Raw Data

Sample ID	Expt #	Rat #	Time (h)	Carboxylate	Lactone	Total DB-67 (ng/mL)
L321-5 min	140	321	0.083	0.92	6.56	7.48
L321-30 min	140	321	0.5	10.7	61.56	72.26
L321-1 h	140	321	1	10.81	35.96	46.77
L321-2 h	140	321	2	7.16	22.93	30.09
L321-4 h	140	321	4	1.87	6.87	8.74
L321-6 h	140	321	6	0.88	2.37	3.25
L321-8 h	140	321	8	0.56	2.12	2.68
L321-12 h	140	321	12	1.55	4.77	6.32
L322-5 min	140	322	0.083	0.96	5.15	6.11
L322-30 min	140	322	0.5	20.38	116.01	136.39
L322-1 h	140	322	1	18.91	68.31	87.22
L322-2 h	140	322	2	9.41	28.98	38.39
L322-4 h	140	322	4	2.23	6.45	8.68
L322-6 h	140	322	6	1.66	4.49	6.15
L322-8 h	140	322	8	0.68	2.35	3.03
L322-12 h	140	322	12	0.58	2.62	3.2
L323-5 min	140	323	0.083	0.63	3.19	3.82
L323-30 min	140	323	0.5	12.41	59.5	71.91
L323-1 h	140	323	1	11.55	43.52	55.07
L323-2 h	140	323	2	6.61	25.22	31.83
L323-4 h	140	323	4	1.67	6.43	8.1
L323-6 h	140	323	6	0.91	2.8	3.71

L323-8 h	140	323	8	0.39	1.84	2.23
L323-12 h	140	323	12	0.58	2.69	3.27
L324-5 min	141	324	0.083	0.44	2.22	2.66
L324-30 min	141	324	0.5	7.97	48.08	56.05
L324-1 h	141	324	1	13.61	53.03	66.64
L324-2 h	141	324	2	6.62	22.19	28.81
L324-4 h	141	324	4	2.53	8.04	10.57
L324-6 h	141	324	6	2.05	7.52	9.57
L324-8 h	141	324	8	1.46	6.34	7.8
L324-12 h	141	324	12	1.47	6.79	8.26
L325-5 min	141	325	0.083	0.43	2.79	3.22
L325-30 min	141	325	0.5	9.21	44.94	54.15
L325-1 h	141	325	1	6.46	25.37	31.83
L325-2 h	141	325	2	3.69	11.22	14.91
L325-4 h	141	325	4	1.22	4.64	5.86
L325-6 h	141	325	6	0.9	3.71	4.61
L325-8 h	141	325	8	0.72	3.41	4.13
L325-12 h	141	325	12	1.95	7.02	8.97
L326-5 min	141	326	0.083	0.59	3.2	3.79
L326-30 min	141	326	0.5	4.89	21.81	26.7
L326-1 h	141	326	1	5.89	20.33	26.22
L326-2 h	141	326	2	3.19	12.08	15.27
L326-4 h	141	326	4	2.3	5.75	8.05
L326-6 h	141	326	6	1.08	4.75	5.83
L326-8 h	141	326	8	1.22	4.63	5.85
L326-12 h	141	326	12	1.75	5.99	7.74

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on DB-67 Rat PK Study

(June 24th, 08)

Bioavailability of E-TPGS and PEG-6000 based DB-67 lactone in male SD rats

Expt # 146 and Expt # 147

11/24/2008

Objective

To determine the oral bioavailability of E-TPGS (28:1 E/D ratio) and PEG-6000 (28:1 E/D ratio) based DB-67 lactone in male SD rats

To determine whether there is gender-difference in the oral bioavailability of DB-67 oral formulations

Expt 146: Study on the oral bioavailability of E-TPGS (28:1 E/D ratio) based DB-67 lactone in male SD rats (2.5 mg/kg) PO

Expt 147: Study on the oral bioavailability of PEG-6000 (28:1 E/D ratio) based DB-67 lactone in male SD rats (2.5 mg/kg) PO

Method

Animal treatment and sample processing

Expt 146, Rats (#361-#363, n=3) were dosed with 2.5 mg/kg E-TPGS (28:1 E/D ratio) based DB-67 lactone PO.

Expt 147, Rats (#364-#366, n=3) were dosed with 2.5 mg/kg PEG-6000 (28:1 E/D ratio) based DB-67 lactone PO.

Dosing solution:

DB-67 lactone = 0.53 mg/mL

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 146 & 147

Sequence name: 20AC-062508-Rat PK expt146.seq

Method: 20AC-052108-reinjection of 051208 calibration curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/DB-67/Rat PK). Plots were made with Prism while nocompartmental analysis was made using Winnonlin v5.2.

Results

Tables

Expt 146 & 147

Expt	ID	Parameter	Units	Estimate	Average/ Median	SD/range
146	361	AUCall	h*ng/mL	67.557	64.911	5.582
146	362	AUCall	h*ng/mL	58.4979		
146	363	AUCall	h*ng/mL	68.679		
146	361	Cl_F_obs	mL/h/kg	6940.735	12098.437	5249.082
146	362	Cl_F_obs	mL/h/kg	17434.36		
146	363	Cl_F_obs	mL/h/kg	11920.22		
146	361	Cmax	ng/mL	11.7	11.957	1.928
146	362	Cmax	ng/mL	10.17		
146	363	Cmax	ng/mL	14		
146	361	Tmax	h	0.5	0.500	0.000
146	362	Tmax	h	0.5		
146	363	Tmax	h	0.5		
147	364	AUCall	h*ng/mL	58.6448	56.090	3.712
147	365	AUCall	h*ng/mL	51.8324		
147	366	AUCall	h*ng/mL	57.7942		
147	364	Cl_F_obs	mL/h/kg	13239.37	15094.742	2623.887
147	365	Cl_F_obs	mL/h/kg	Missing		
147	366	Cl_F_obs	mL/h/kg	16950.11		
147	364	Cmax	ng/mL	8.56	7.810	2.607
147	365	Cmax	ng/mL	4.91		
147	366	Cmax	ng/mL	9.96		
147	364	Tmax	h	0.5	1.833	1.893
147	365	Tmax	h	4		
147	366	Tmax	h	1		

Figures

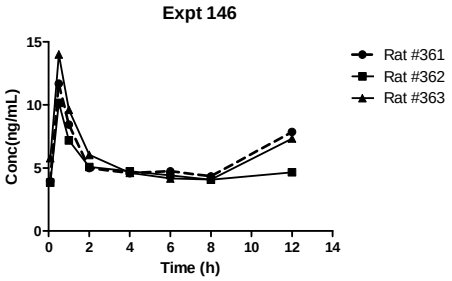


Fig. 1a. Plasma total DB-67 levels in male SD rats treated with 2.5 mg/kg DB-67 PO

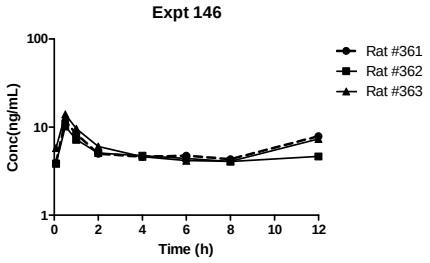


Fig. 1b. Semilog plot of plasma total DB-67 levels in male SD rats treated 2.5 mg/kg DB-67 PO

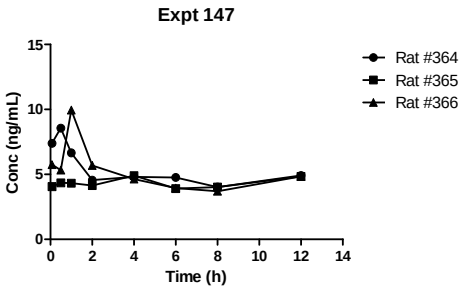


Fig. 2a. Plasma total DB-67 levels in male SD rats treated with 2.5 mg/kg DB-67 PO

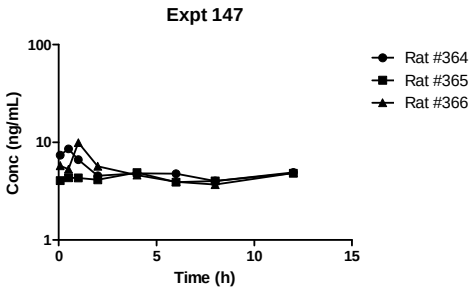
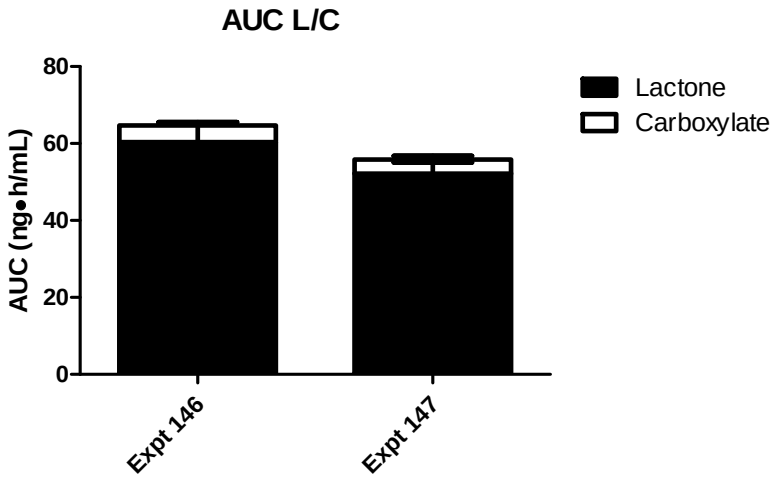


Fig. 2b. Plasma total DB-67 levels in male SD rats treated with 2.5 mg/kg DB-67 PO



File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 146 & 147

Raw Data

Expt #	Rat #	Time	Carboxylate	Lactone	Total DB-67 (ng/mL)
146	361	0.083	0.14	3.81	3.95
146	361	0.5	1.07	10.63	11.7
146	361	1	0.94	7.5	8.44
146	361	2	0.35	4.64	4.99
146	361	4	0.27	4.33	4.6
146	361	6	0.25	4.49	4.74
146	361	8	0.22	4.11	4.33
146	361	12	0.2	7.66	7.86
146	362	0.083	0.16	3.69	3.85
146	362	0.5	1	9.17	10.17
146	362	1	0.79	6.4	7.19
146	362	2	0.39	4.69	5.08
146	362	4	0.34	4.4	4.74
146	362	6	0.27	4.15	4.42
146	362	8	0.26	3.82	4.08
146	362	12	0.13	4.52	4.65
146	363	0.083	0.56	5.22	5.78
146	363	0.5	1.86	12.14	14
146	363	1	1.5	8.12	9.62
146	363	2	0.61	5.43	6.04
146	363	4	0.33	4.29	4.62
146	363	6	0.27	3.9	4.17
146	363	8	0.25	3.85	4.1
146	363	12	0.21	7.12	7.33
147	364	0.083	1.78	5.61	7.39
147	364	0.5	1.53	7.03	8.56
147	364	1	0.87	5.78	6.65
147	364	2	0.32	4.23	4.55
147	364	4	0.41	4.4	4.81
147	364	6	0.34	4.43	4.77
147	364	8	0.17	3.85	4.02
147	364	12	0.23	4.69	4.92
147	365	0.083	0.17	3.88	4.05
147	365	0.5	0.23	4.11	4.34
147	365	1	0.37	3.95	4.32
147	365	2	0.25	3.89	4.14
147	365	4	0.39	4.52	4.91
147	365	6	0.24	3.67	3.91
147	365	8	0.19	3.83	4.02
147	365	12	0.16	4.68	4.84
147	366	0.083	0.43	5.33	5.76
147	366	0.5	0.41	4.91	5.32
147	366	1	0.78	9.18	9.96

147	366	2	0.48	5.21	5.69
147	366	4	0.37	4.26	4.63
147	366	6	0.18	3.76	3.94
147	366	8	0.12	3.57	3.69
147	366	12	0.23	4.62	4.85

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Rat PK Study

(July 1st, 08)

Bioavailability of SBE-β-CD based AR-67 carboxylate in rats &
effect of GF120918 IV on bioavailability of SBE-β-CD based AR-67 lactone (PO) in rats

Expt # 151 and Expt # 152

11/24/2008

Objective

To determine the oral bioavailability of SBE-β-CD based AR-67 carboxylate in female SD rats

To determine the effects of GF IV on the oral bioavailability of SBE-β-CD based AR-67 lactone in female SD rats.

Expt 151: Study on the oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 carboxylate in rats (2.5 mg/kg) PO

Expt 152: Study on the effects of GF (8.25 mg/kg) IV on oral bioavailability of SBE-β-CD (200:1 E/D ratio) based AR-67 lactone (2.5 mg/kg) in rats PO

Method

Animal treatment and sample processing

Expt 151, Rats (#401-#403, n=3) were dosed with SBE-β-CD (200:1 E/D ratio) based AR-67 carboxylate PO.

Expt 152, Rats (#404-#406, n=3) were dosed with 2.5 mg/kg SBE-β-CD (200:1 E/D ratio) based AR-67 lactone PO 5 min after GF (8.25mg/kg) IV.

Dosing solution:

AR-67 lactone = 1.0 mg/mL

Blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed. For samples needing dilution (5min, 30min, 1h & 2h after intravenous doses), a 1:10 dilution was carried out using 1:4 extract of blank rat plasma.

Expt 146 & 147

Sequence name: 20AC-070208-Rat PK animal 401-406.seq

Method: 20AC-052108-reinjection of 051208 calibration curve.met

Data were exported and saved in Leggas Lab/Experimental Results/AR-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/AR-67/Rat PK). Plots were made with Prism while noncompartmental analysis was made using WinNonlin v5.2.

Results

Tables. Pharmacokinetic parameters estimated using the noncompartmental method in Winnonlin v5.2

Expt 151 & 152

ID	Parameter	Units	Estimate	Average/ Median[23]	SD/range
401	AUCall	h*ng/mL	180.7498	135.569	39.491
402	AUCall	h*ng/mL	107.6384		
403	AUCall	h*ng/mL	118.3181		
401	Cl_F_obs	mL/h/kg	11525.04	15405.491	3642.343
402	Cl_F_obs	mL/h/kg	18750.43		
403	Cl_F_obs	mL/h/kg	15941.01		
401	Cmax	ng/mL	73.62	48.283	22.396
402	Cmax	ng/mL	40.1		
403	Cmax	ng/mL	31.13		
401	Tmax	h	0.5	0.500	0.500
402	Tmax	h	0.5		
403	Tmax	h	0.5		
401	HL_Lambda_z	h	3.1535	3.852	0.659
402	HL_Lambda_z	h	3.9399		
403	HL_Lambda_z	h	4.4637		
404	AUCall	h*ng/mL	355.4199	324.850	63.324
405	AUCall	h*ng/mL	252.04		
406	AUCall	h*ng/mL	367.089		
404	Cl_F_obs	mL/h/kg	5561.148	5395.049	702.121

[23] Median was used for Tmax. All other parameters use the arithmetic mean

405	Cl_F_obs	mL/h/kg	5999.228		
406	Cl_F_obs	mL/h/kg	4624.772		
404	Cmax	ng/mL	61.23	42.973	16.308
405	Cmax	ng/mL	29.85		
406	Cmax	ng/mL	37.84		
404	Tmax	h	2	2.000	1-6
405	Tmax	h	6		
406	Tmax	h	1		
404	HL_Lambda_z	h	5.8779	6.423	0.525
405	HL_Lambda_z	h	6.9264		
406	HL_Lambda_z	h	6.4648		

Figures

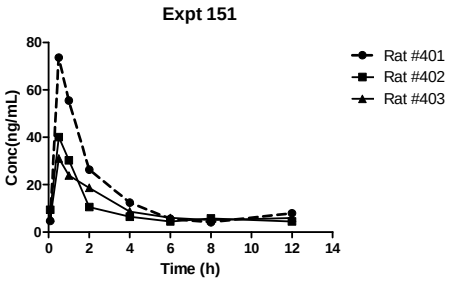


Fig. 1a. Plasma total DB-67 levels in SD rats 2.5 mg/kg DB-67 carboxylate PO

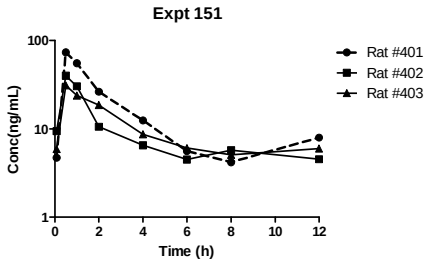


Fig. 1b. Semilog plot of plasma total DB-67 levels in SD rats treated with 2.5 mg/kg DB-67 carboxylate PO

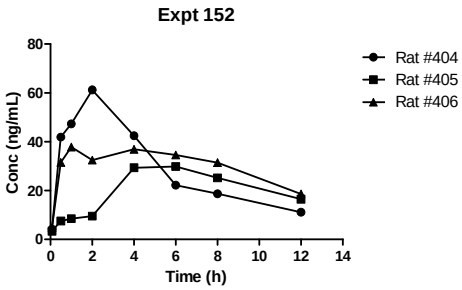


Fig. 2a. Plasma total DB-67 levels in SD rats treated with 8.25 mg/kg GF IV 5 min before 2.5mg/kg DB-67 PO

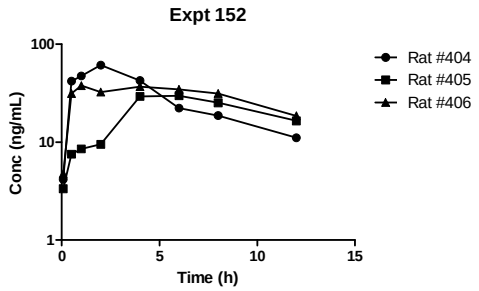
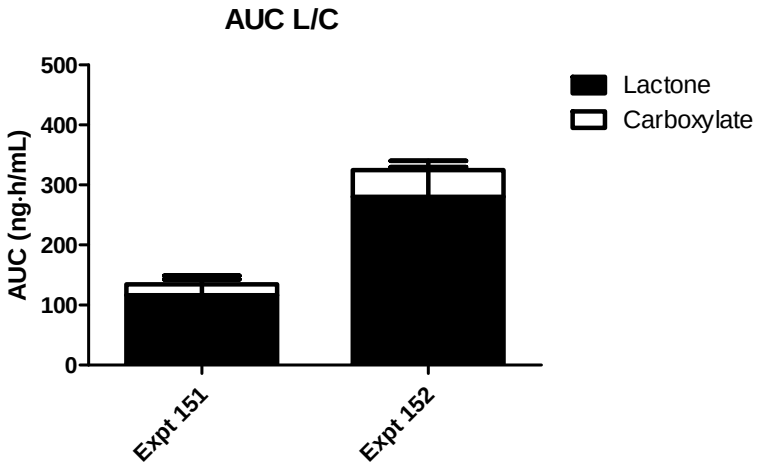


Fig. 2b. Plasma total DB-67 levels in SD rats treated with 8.25 mg/kg GF IV 5 min before 2.5 mg/kg DB-67 lactone PO



File path: \\pharm\public\PS\Leggas Lab\EXPERIMENTAL RESULTS\DB67 Oral PK\REPORTS\Prism data Files\ AUC Rat PK Expt 151 & 152

Raw Data

Data ID	Expt #	Rat ID	Time (h)	Carboxylate (ng/ml)	Lactone (ng/ml)	Total AR-67 (ng/ml)
401 5 min	151	401	0.083	0	4.7	4.7
401 30 min	151	401	0.5	8.36	65.26	73.62
401 1 hour	151	401	1	9.89	45.63	55.52
401 2 hour	151	401	2	5.02	21.34	26.36
401 4 hour	151	401	4	2.56	9.9	12.46
401 6 hour	151	401	6	0.6	5.01	5.61
401 8 hour	151	401	8	0	4.2	4.2
401 12 hour	151	401	12	1	6.95	7.95
402 5 min	151	402	0.083	0.87	8.57	9.44
402 30 min	151	402	0.5	6.23	33.87	40.1
402 1 hour	151	402	1	5.54	24.79	30.33
402 2 hour	151	402	2	1.58	9.01	10.59
402 4 hour	151	402	4	0.72	5.79	6.51
402 6 hour	151	402	6	0	4.49	4.49
402 8 hour	151	402	8	0.52	5.22	5.74
402 12 hour	151	402	12	0.34	4.18	4.52
403 5 min	151	403	0.083	0.53	5.36	5.89
403 30 min	151	403	0.5	4.05	27.08	31.13
403 1 hour	151	403	1	4.29	19.58	23.87
403 2 hour	151	403	2	3.45	15.23	18.68
403 4 hour	151	403	4	1.24	7.45	8.69
403 6 hour	151	403	6	0.64	5.41	6.05
403 8 hour	151	403	8	0.37	4.7	5.07
403 12 hour	151	403	12	0.53	5.45	5.98
404 5 min	152	404	0.083	0	4.17	4.17
404 30 min	152	404	0.5	3.78	38.15	41.93
404 1 hour	152	404	1	5.8	41.53	47.33
404 2 hour	152	404	2	7.33	53.9	61.23
404 4 hour	152	404	4	5.57	36.93	42.5
404 6 hour	152	404	6	3.15	19.09	22.24
404 8 hour	152	404	8	2.53	16.18	18.71
404 12 hour	152	404	12	1.28	9.82	11.1
405 5 min	152	405	0.083	0	3.34	3.34
405 30 min	152	405	0.5	0	7.53	7.53
405 1 hour	152	405	1	0	8.53	8.53
405 2 hour	152	405	2	0	9.51	9.51
405 4 hour	152	405	4	2.04	27.33	29.37
405 6 hour	152	405	6	4.36	25.49	29.85
405 8 hour	152	405	8	3.6	21.63	25.23
405 12 hour	152	405	12	2.52	13.96	16.48
406 5 min	152	406	0.083	0	4.78	4.78

406 30 min	152	406	0.5	4.14	27.33	31.47
406 1 hour	152	406	1	5.81	32.03	37.84
406 2 hour	152	406	2	5.18	27.35	32.53
406 4 hour	152	406	4	5.46	31.41	36.87
406 6 hour	152	406	6	6.21	28.35	34.56
406 8 hour	152	406	8	5.3	26.11	31.41
406 12 hour	152	406	12	3.21	15.39	18.6

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY
UNIVERSITY OF KENTUCKY (LEGGAS LAB)

Report on AR-67 Rat PK Study

(Feb 19th, 2009)

Bioavailability of amorphous PMFD576/1 suspension and capsule filled with PMFD576/1 in female SD rats

---

Expt # 192 and Expt # 193

03/24/2009

Objective

It has been showed that PMFD 576/1 (PVP (K30)-Tween-80-lactone) gave the highest oral bioavailability, which is about 2-fold in comparison with that given by supersaturated AR-67 lactone solution with excipients SBE-β-CD or E-TPGS in mice. We further examined the oral bioavailability of the same formulation and capsules filled with PMFD 576/1 in female SD rats in the following 2 experiments:

Expt 192: Study on the oral bioavailability of PMFD 576/1 AR-67 lactone suspension in SGF (pH1.2) at a dose of 2.5 mg/kg PO in rats

Expt 193: Study on the oral bioavailability of capsule filled with PMFD 576/1 AR-67 lactone at a dose of 2.5 mg/kg PO in rats

Method

Formulation

The PMFD 576/1 powder was weighted in glass vial by Dr. Xiang and reconstituted in SGF (pH 1.2) to make 2 mg/mL AR-67 suspension, and the capsule were filled with PMFD 576/1 powder using filling apparatus by Dr. Xiang.

Animal treatment and sample processing

Expt 192, Rats ((#801-#803, n=3) were dosed with PMFD 576/1 suspension in SGF (pH 1.2) PO at the dose of 5 mg/kg.

Expt 193, Rats (#804-#806, n=3) were dosed with capsules filled with PMFD 576/1 at the dose of 5 mg/kg.

Blood was collected from the saphenous vein at 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 μL:40 μL) and were analyzed.

Expt 192 & 193

Sequence name: 20AC-022109-rat plasma PK Expt 192 & 193.seq

Method: 20AC-022209-reinjection of rat plasma curve prep 062008.met

Data were exported and saved in Leggas Lab/Experimental Results/AR-67 Oral PK folder. (A backup copy of HPLC data was also saved in Leggas Lab/Jamie's project/AR-67/Rat PK). Plots were made with Prism while noncompartmental analysis was made using WinNonlin v5.2.

Results

1. **Prism file: ..\Prism data Files\AUC Rat PK Expt 192 & 193.pzf**
2. **Tables**

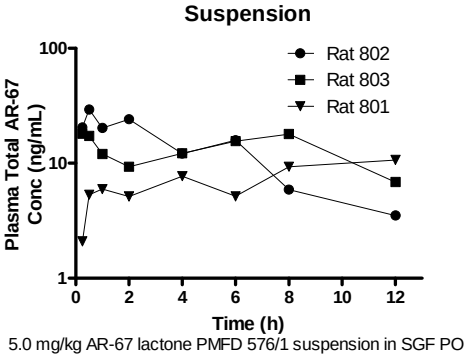
Table 1. Summary of pharmacokinetic parameters

Expt #192	5 mg/mL AR-67 lactone PMFD 576/1 suspension						
AUC (ng·h/mL)	Mean	SD	Tmax (h)	Median	Cmax (ng/mL)	Mean	SD
Carb	26.40	8.56	Car		Car		
Lactone	103.65	27.39	Lactone		Lactone		
Total	130.02	35.42	Total	0.50	Total	19.36	9.40

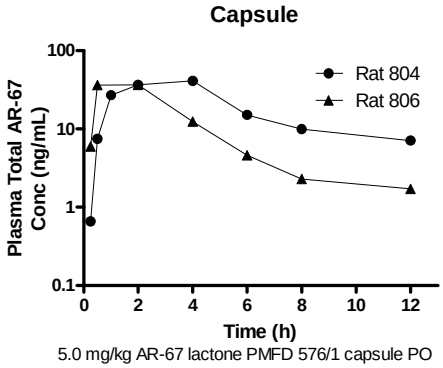
Expt #193	5 mg/mL AR-67 lactone PMFD 576/1 in capsule						
AUC (ng·h/mL)	Mean	SD	Tmax (h)	Median	Cmax (ng/mL)	Mean	SD
Carb	33.65	11.47	Car		Car		
Lactone	140.57	73.86	Lactone		Lactone		
Total	174.20	85.28	Total	2	Total	38.80	3.36

Expt #140	5 mg/mL AR-67 lactone in SBE-β-CD 200:1						
AUC (ng·h/mL)	Mean	SD	Tmax (h)	Median	Cmax (ng/mL)	Mean	SD
Carb	38.31	9.17	Car		Car		
Lactone	145.40	32.67	Lactone		Lactone		
Total	183.77	41.77	Total	0.5	Total	93.52	37.13

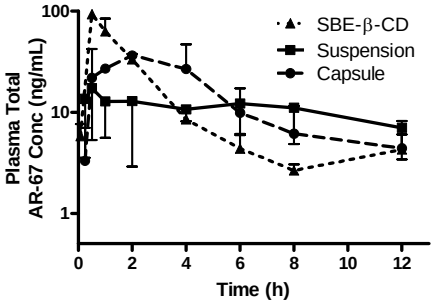
3. Figures



5.0 mg/kg AR-67 lactone PMFD 576/1 suspension in SGF PO



5.0 mg/kg AR-67 lactone PMFD 576/1 capsule PO



Comparison of 5 mg/kg oral AR-67 lactone in SBE- β-CD (200:1) solution, suspensions in SGF (pH 1.2) and capsule (n=3)

Raw Data

Expt	Rat ID	Time (h)	Carboxylate (ng/mL)	Lactone (ng/mL)	Total (ng/mL)
192	801	0.25	0.39	1.7	2.09
192	801	0.5	0.69	4.62	5.31
192	801	1	1.09	4.87	5.96
192	801	2	1.14	4	5.14
192	801	4	1.39	6.33	7.72
192	801	6	1.22	3.93	5.15
192	801	8	1.7	7.6	9.3
192	801	12	2.13	8.56	10.69
192	801	24	0.26	1.07	1.33
192	802	0.25	2.48	18.09	20.57
192	802	0.5	4.15	25.2	29.35
192	802	1	3.83	16.5	20.33
192	802	2	4.03	20.1	24.13
192	802	4	2.64	9.51	12.15
192	802	6	3.22	12.68	15.9
192	802	8	1.2	4.7	5.9
192	802	12	0.6	2.91	3.51
192	802	24	0	0.4	0.4
192	803	0.25	2.07	15.96	18.03
192	803	0.5	2.46	14.85	17.31
192	803	1	2.47	9.59	12.06
192	803	2	2.09	7.24	9.33
192	803	4	4.86	7.33	12.19
192	803	6	2.79	12.79	15.58
192	803	8	3.5	14.45	17.95
192	803	12	1.25	5.6	6.85
192	803	24	0.24	1.52	1.76
194	804	0.25	0.06	0.6	0.66
194	804	0.5	0.79	6.67	7.46
194	804	1	3.46	23.54	27
194	804	2	5.83	30.7	36.53
194	804	4	8.11	33.06	41.17
194	804	6	2.98	12.15	15.13
194	804	8	1.76	8.18	9.94
194	804	12	1.33	5.76	7.09
194	804	24	0.33	1.6	1.93
194	805	0.25	0.05	0.2	0.25

194	805	0.5	0.06	0.46	0.52
194	805	1	12.93	58.83	71.76
194	805	2	0.11	0.26	0.37
194	805	4	0.08	0.26	0.34
194	805	6	0.14	0.38	0.52
194	805	8	0.17	0.96	1.13
194	805	12	2.44	9.52	11.96
194	805	24	2.47	9.55	12.02
194	806	0.25	0.07	5.87	5.94
194	806	0.5	4.94	31.36	36.3
194	806	1	0.13	0.51	0.64
194	806	2	7.67	28.75	36.42
194	806	4	3.61	8.83	12.44
194	806	6	1.16	3.45	4.61
194	806	8	0.56	1.74	2.3
194	806	12	0.43	1.28	1.71
194	806	24	1.14	5.27	6.41

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE EFFECTS OF PEG-6000 AND PG-TPGS ON THE ORAL ABSORPTION OF AR-67 LACTONE IN MICE

(PERFORMED 7/16/2008-7/17/2008)

BIOAVAILABILITY PEG-6000 AND PG-TPGS BASED AR-67 LACTONE FORMULATIONS IN FEMALE C57BL/6 MICE

EXPT # 157 AND EXPT # 158

Objective

Two excipients or mixtures of excipients were separately assessed for their effects on the oral bioavailability of AR-67. These experiments were done following in vitro solubility studies that showed that PEG-6000 was unable to maintain AR-67 lactone in solution at an excipient to drug ratio of 28:1 while the presence of propylene glycol (PG) improved the solubilzing capacity of vitamin E-TPGS. We therefore, assessed the relationship between in vitro dissolution and oral bioavailability.

Expt 157: Study the absorption of PEG-6000 based AR-67 lactone (5 mg/kg, 9.43 ml/kg) PO.

Expt 158: Study the absorption of PG-TPGS based AR-67 lactone (5 mg/kg, 9.43 ml/kg) PO.

Method

Formulation

a) SGF (pH 1.2) containing PEG-6000 was spiked with carboxylate stock solution to prepare 0.53 mg/mL dosing solution (28:1, E:D) and was dosed immediately after mixing.

b) SGF (pH 1.2) containing PG-TPGS was spiked with carboxylate stock solution to prepare 0.53 mg/mL dosing solution (3.2:28:1, E:D) and was dosed immediately after mixing

AR-67 carboxylate stock solution was prepared by Dr. Xiang.

Animal treatment and sample processing

Expt 157: Mouse# 451-459 were treated with AR-67 lactone suspension in PEG-6000 containing simulated gastric fluid (pH 1.2) right after mixing.

Expt 158: Mouse# 461-469 were treated with AR-67 suspension in PG-TPGS containing simulated gastric fluid (pH 1.2) right after mixing.

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h and 12 h. Each animal was sampled at 3-4 different time points. The blood sample of about 50 μL was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma of 25 μL was collected by

centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 µL,-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name:
20AC-071808-Mouse PK ext (PEG vs PG-TPGS).seq
20AC-071708-mouse PK expt.seq

Method:
20AC-070307-Mouse plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/AR-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

4. Prism file:..\ ..\Prism files\Mouse PK 5mg PO Expt 157 & 158.pzf
5. WinNonlin file: ....\Winnonlin files\Mouse PK Expt 157 & 158.wsp
6. Tables

Table 1. Basic pharmacokinetic parameters

Expt #157

	AUC(ng*h/mL) Mean (SE)	Tmax (h) Median	Cmax (ng/mL) Mean (SE)
Carboxylate	19.10 (2.43)	0.50	6.06 (1.24)
Lactone	175.72 (22.68)	0.50	58.38 (9.96)
Total	194.83 (25.02)	0.50	64.44 (11.20)

Expt #158

	AUC (ng*h/mL) Mean (SE)	Tmax (h) Median	Cmax (ng/mL) Mean (SE)
Carboxylate	13.55 (0.75)	0.75	6.89 (0.72)
Lactone	114.47 (0.17)	0.17	55.86
Total	128.01 (0.17)	0.17	60.66

7. Figures

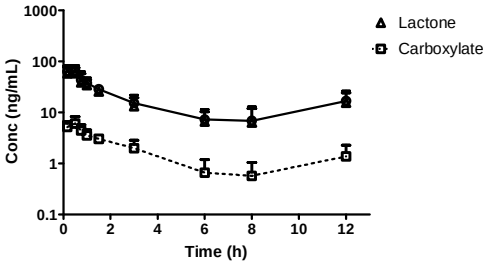


Figure 1a. Plasma concentration of AR-67 carboxylate and lactone and AR-67 in C57BL/6 mice treated with 5mg/kg PEG-6000 based AR-67 lactone PO.

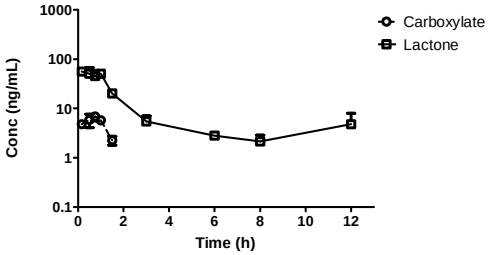


Figure 1b. Plasma concentration of AR-67 carboxylate and lactone in C57BL/6 mice treated with 5mg/kg PG-TPGS based AR-67 lactone PO.

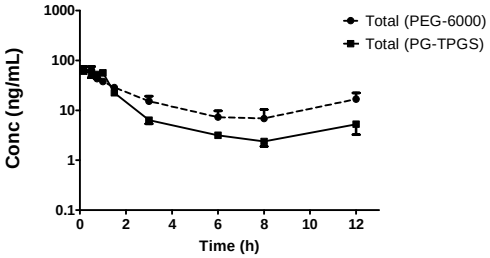


Figure 1c. Comparison of plasma concentration of total AR-67 in C57BL/6 mice treated with 5mg/kg PEG-6000 or PG-TPGS based AR-67 lactone PO

File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\Mouse PK 5mg PO Expt 157 & 158.

Raw Data

Sample ID	Expt	Time (h)	Carb. (ng/mL)	Lact. (ng/mL)	Total (ng/mL)
451-10min	157	0.167	5.24	63.93	69.17
452-10min	157	0.167	3.72	36.91	40.63
453-10min	157	0.167	6.56	73.29	79.85
454-30min	157	0.5	3.82	40.68	44.5
455-30min	157	0.5	8.1	75.14	83.24
456-30min	157	0.5	6.26	59.33	65.59
457-45min	157	0.75	2.93	21.11	24.04
458-45min	157	0.75	5.43	58.57	64
459-45min	157	0.75	4.91	36.39	41.3
451-1h	157	1	3.74	41.45	45.19
452-1h	157	1	2.49	23.58	26.07
453-1h	157	1	4.4	37.65	42.05
454-1.5h	157	1.5	3.02	24.99	28.01
4551.5h	157	1.5	2.96	25.84	28.8
456-1.5h	157	1.5	3.09	26.02	29.11
454-3h	157	3	1.23	7.15	8.38
455-3h	157	3	2.94	19.07	22.01
456-3h	157	3	1.81	13.51	15.32
451-6h	157	6	0.29	5.11	5.4
452-6h	157	6	0.41	3.84	4.25
453-6h	157	6	1.28	10.9	12.18
457-8h	157	8	0.15	1.31	1.46
458-8h	157	8	1.09	12.35	13.44
459-8h	157	8	0.47	5.19	5.66
457-12h	157	12	0.6	7.54	8.14
458-12h	157	12	1.14	13.71	14.85
459-12h	157	12	2.37	25.07	27.44
461-10min	158	0.167	4.8	55.86	60.66
464-30min	158	0.5	7.35	66.65	74
465-30min	158	0.5	6.38	51.51	57.89
466-30min	158	0.5	3.88	33.06	36.94
467-45min	158	0.75	8.02	57.85	65.87
468-45min	158	0.75	7.1	48.19	55.29
469-45min	158	0.75	5.55	30.25	35.8
461-1h	158	1	5.74	50.47	56.21
464-1.5h	158	1.5	2.75	22.31	25.06
465 1.5h	158	1.5	2.2	20.38	22.58
466-1.5h	158	1.5	1.84	17.9	19.74
464-3h	158	3	0.95	7.07	8.02
465-3h	158	3	0.75	3.93	4.68
466-3h	158	3	0.95	5.41	6.36
461-6h	158	6	0.35	2.81	3.16
467-8h	158	8	0.13	1.97	2.1

468-8h	158	8	0.19	1.52	1.71
469-8h	158	8	0.38	2.94	3.32
467-12h	158	12	0.37	3.91	4.28
468-12h	158	12	0.79	8.32	9.11
469-12h	158	12	0.24	2.12	2.36

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON PHARMACOKINETICS OF SBE-β-CD AND VITAMIN E-TPGS BASED AR-67 LACTONE

(PERFORMED 3/10/2009-3/13/2009)

PHARMACOKINETICS OF AR-67 LACTONE (SBE-B-CD, 200:1, E:D;TPGS, 200:1, E:D) IN FEMALE C57BL/6 MICE

EXPT # 159 AND EXPT # 160

Objective

We performed pharmacokinetic studies on the SBE-β-CD (200:1, E/D) and TPGS based AR-67 lactone formulations. The data generated will be used as references for estimation of oral bioavailability of AR-67.

Expt 159: Pharmacokinetics of IV SBE-β-CD based AR-67 lactone (200:1, E:D) at a dose of 5 mg/kg.

Expt 160: Pharmacokinetics of IV TPGS based AR-67 lactone (200:1, E:D) at a dose of 5 mg/kg.

Method

Formulation

a) A dosing solution of 1 mg/mL AR-67 containing SBE-β-CD was prepared (200:1, E:D) by reconstituting lyophilized powder.

b) A dosing solution of 0.53 mg/mL AR-67 containing TPGS was prepared (200:1, E:D) from 5.3 mg/mL of carboxylate stock solution.

AR-67 carboxylate stock solution as well as the excipient solution was prepared by Dr. Xiang.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 159: mouse#471-479 were treated IV SBE-β-CD based AR-67 lactone (200:1, E:D) at a dose of 5 mg/kg.

Expt 160: mouse#481-489 were treated IV TPGS based AR-67 lactone (28:1, E:D) at a dose of 5 mg/kg. (The stock AR-67 carboxylate solution precipitated when stored at 4°C overnight.

Groups of three mice were sampled at 5 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h and 12 h. Each animal was sampled at 3-4 different time points.

The blood sample of about 50 μL was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes.

Plasma of 25 μL was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 μL,-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 μL:40 μL) and were analyzed.

Sequence name: 20AC-072708-Mouse PK Expt SBE-β-CD vs TPGS IV.seq AND 20AC-072508-Mouse PK expt SBE-CD vs TPGS IV.seq

Method: 20AC-070307-Mouse plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

1. Prism file..\Prism files\Mouse PK 5mg-kg IV SBE-CD vs TPGS (Expt 159 & 160).pzf
2. WinNonlin file: ..\Winnonlin files\Mouse PK Expt 159 & 160.wsp
3. Tables

Table 1. Basic pharmacokinetic parameters

Expt 159: 5.0 mg/kg Lactone (SBE-β-CD,200:1, E:D) IV

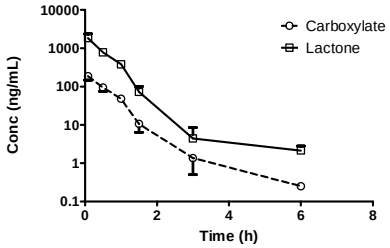
AUC	AUC (ng*hr/mL) Mean (SE)	Tmax (h) Median	Cmax (ng/mL) Mean (SE)
Carb	137.47 (6.69)	0.08	187.44 (23.42)
Lactone	1187.63 (94.97)	0.08	1836.64 (305.12)
Total	1325.08 (104.25)	0.08	2024.08 (328.54)

Expt 160: 5.0 mg/kg Lactone (TPGS, 28:1, E:D) IV

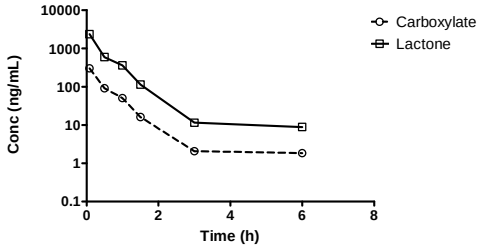
	AUC (ng*hr/mL) Mean (SE)	Tmax (h) Median	Cmax (ng/mL) Mean *
Carb	180.84 (2.17)	0.08	301.36
Lactone	1320.19 (20.44)	0.08	2355.84
Total	1500.97 (22.25)	0.08	2657.20

***single point**

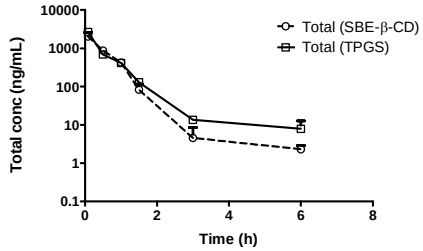
4. Figures



Expt 159: Plasma concentration of carboxylate and lactone in female C57BL/6 mice treated intravenously with 5 mg/kg SBE-β-CD based AR-67 lactone (E:D ratio 200:1)



Expt 160: Plasma concentration of carboxylate and lactone in female C57BL/6 mice treated intravenously with 5 mg/kg TPGS based AR-67 (E:D ratio 28:1)



Expt 159 vs 160: Plasma concentration of total AR-67 in female C57BL/6 mice treated intravenously with 5 mg/kg SBE-β-CD or TPGS based DB-67

File path: \\pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files.

Raw Data

Expt	Sample ID	Time (h)	Carb. (ng/mL)	Lact. (ng/mL)	Total (ng/mL)
159	471	0.083	147.12	1306.32	1453.44
159	472	0.083	186.96	1840.32	2027.28
159	473	0.083	228.24	2363.28	2591.52
159	474	0.5	75.68	666.4	742.08
159	475	0.5	115.44	952.56	1068
159	476	0.5	91.6	716.8	808.4
159	471	1	44.8	364.8	409.6
159	472	1	49.28	378.16	427.44
159	473	1	49.28	394.56	443.84
159	474	1.5	5.73	39.12	44.85
159	475	1.5	12.51	92.13	104.64
159	476	1.5	13.57	85.71	99.28
159	474	3	1.91	9.14	11.05
159	475	3	1.8	1.41	3.21
159	476	3	0.37	2.76	3.13
159	471	6	0.25	1.39	1.64
159	472	6	0.39	2.6	2.99
159	473	6	0.29	2.41	2.7
159	477	8	0	0.09	0.09
159	478	8	0	0.8	0.8
159	479	8	0.1	0.62	0.72
159	477	12	0	2.17	2.17
159	478	12	0	0.56	0.56
159	479	12	0.08	0.78	0.86
160	483	0.083	301.36	2355.84	2657.2
160	484	0.5	82.56	578.08	660.64
160	485	0.5	103.36	669.52	772.88
160	486	0.5	87.52	533.28	620.8
160	481	1	47.6	342.56	390.16
160	482	1	48.4	339.68	388.08
160	483	1	54.64	403.52	458.16
160	484	1.5	18.72	130.05	148.77
160	485	1.5	13.14	100.78	113.92
160	486	1.5	16.46	110.34	126.8
160	484	3	2.26	12.77	15.03
160	485	3	1.98	10.88	12.86
160	486	3	1.97	10.84	12.81
160	481	6	0.26	1.95	2.21
160	482	6	1.65	9.32	10.97
160	483	6	2.03	8.48	10.51

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE BIOAVAILABILITY OF PEG-6000 (28:1, E:D) AND VITAMIN E-TPGS BASED AR-67 LACTONE (28:1, E:D) IN MICE

(PERFORMED 7/30/2008-7/31/2008)

BIOAVAILABILITY OF PEG-6000 (28:1, E:D) AND VITAMIN E-TPGS BASED AR-67 LACTONE (28:1, E:D) IN FEMALE C57BL/6 MICE

EXPT # 162 AND EXPT # 163

Objective

The oral bioavailability of PEG-6000 based AR-67 lactone (28:1, E:D) and Vitamin E-TPGs based AR-67 lactone (28:1, E:D) were determined in the following experiments, which is a series of studies to screen excipients for better formulations with higher bioavailability.

Expt 162: Study the bioavailability of PEG-6000 based AR-67 lactone (28:1, E:D) at 2.5 mg/kg PO in female C57BL/6 mice.

Expt 163: Study the bioavailability of Vitamin E-TPGs based AR-67 lactone (28:1, E:D) at 2.5 mg/kg PO in female C57BL/6 mice.

Method

Formulation

The 2 formulations were prepared at 1 mg/mL by Dr. Xiang.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 162: mice (#501-509) were orally treated with PEG-6000 based AR-67 lactone (28:1, E:D) at 2.5 mg/kg PO in female C57BL/6 mice.

Expt 163: mice (#511-519) were orally treated with Vitamin E-TPGs based AR-67 lactone (28:1, E:D) at 2.5 mg/kg PO in female C57BL/6 mice.

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h and 12 h. Each animal was sampled at 3 different time points.

The blood sample of about 50 μL was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma of 25 μL was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 μL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name: 20AC-080108-Mouse plasma curve & QCs prep 073108.seq

20AC-080408-Mouse PK Expt 162 & 163 ctd from 080108.seq

Method:

20AC-080108 mouse plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/AR-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

1. Prism file: .\Prism files\Mouse PK 2.5 mg PO Expt 162 & 163.pzf
2. WinNonlin file: .\Winnonlin files\Mouse PK Expt 162 & 163.wsp

3. Tables

Table 1. Basic pharmacokinetic parameters

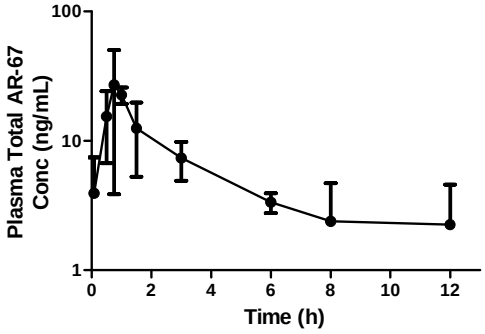
Expt #162: 2.5 mg/kg Lactone (PEG-6000, 28:1, E:D) PO

	AUC (ng*h/mL) Mean (SE)	Tmax (h) Median	Mean (SE)
Carboxylate	7.69 (1.17)	0.75	3.05 (1.72)
Lactone	62.70 (8.09)	0.75	23.94 (11.64)
Total	70.39 (9.22)	0.75	26.99 (13.36)

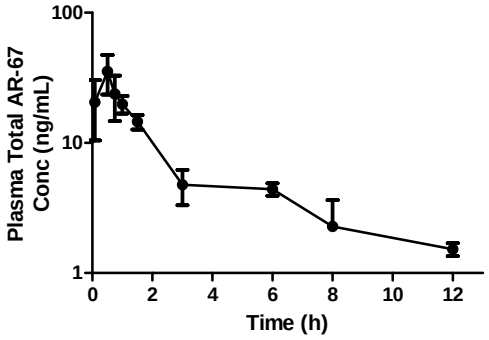
Expt #163: 2.5 mg/kg Lactone (TPGS, 28:1, E:D) PO

	AUC (ng*h/mL) Mean (SE)	Tmax (h) Median	Cmax (ng/mL) Mean
Carboxylate	9.21 (1.74)	0.08	9.78 (5.95)
Lactone	67.27 (2.63)	0.50	31.49 (5.97)
Total	76.48 (3.73)	0.50	35.39 (6.89)

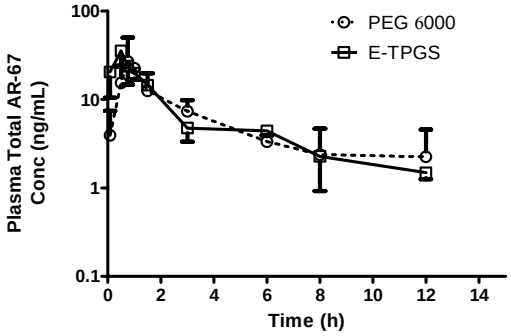
4. Figures



2.5 mg/kg PEG-6000 based DB-67 (28:1,E:D) PO



2.5 mg/kg Vit E-TPGS based DB-67 (28:1, E:D) PO



2.5 mg/kg Vit E-TPGS (28:1, E:D) versus
PEG 6000 (28:1, E:D) based DB-67 PO

Raw Data

Expt	Mouse ID	Time (h)	Carb. (ng/mL)	Lact. (ng/mL)	Total (ng/mL)
162	501	0.083	0.78	7.2	7.98
162	502	0.083	0.14	1.89	2.03
162	503	0.083	0.22	1.57	1.79
162	504	0.5	3.27	21.18	24.45
162	505	0.5	1.79	12.86	14.65
162	506	0.5	0.76	6.36	7.12
162	507	0.75	2	18.06	20.06
162	508	0.75	6.41	46.4	52.81
162	509	0.75	0.74	7.37	8.11
162	501	1	2.61	20.79	23.4
162	502	1	2.84	22.47	25.31
162	503	1	2.04	16.95	18.99
162	504	1.5	2.36	17.69	20.05
162	505	1.5	1.63	10.23	11.86
162	506	1.5	0.77	4.86	5.63
162	504	3	1.32	8.84	10.16
162	505	3	0.86	5.38	6.24
162	506	3	0.67	4.99	5.66
162	501	6	0.3	2.78	3.08
162	502	6	0.37	3.64	4.01
162	503	6	0.36	2.58	2.94
162	507	8	0.44	4.57	5.01
162	508	8	0	0.65	0.65
162	509	8	0	1.48	1.48
162	507	12	0	0.97	0.97
162	508	12	0	0.84	0.84
162	509	12	0.34	4.6	4.94
163	511	0.083	21.67	9.21	30.88
163	512	0.083	3.91	6.95	10.86
163	513	0.083	3.75	16	19.75
163	514	0.5	2.24	20.61	22.85
163	515	0.5	5.42	41.18	46.6
163	516	0.5	4.06	32.67	36.73
163	517	0.75	1.93	16.95	18.88
163	518	0.75	1.74	16.47	18.21
163	519	0.75	3.82	30.35	34.17
163	511	1	2.84	18.11	20.95
163	512	1	2.61	19.64	22.25
163	513	1	2.01	14.35	16.36
163	514	1.5	1.71	14.29	16
163	515	1.5	1.74	13.44	15.18
163	516	1.5	1.21	11.24	12.45
163	514	3	0.49	5.66	6.15

163	515	3	0.55	4.3	4.85
163	516	3	0	3.28	3.28
163	511	6	0.44	4.11	4.55
163	512	6	0.44	4.37	4.81
163	513	6	0.36	3.52	3.88
163	517	8	0.29	3.46	3.75
163	518	8	0.12	1.9	2.02
163	519	8	0	1.07	1.07
163	517	12	0.19	1.46	1.65
163	518	12	0	1.59	1.59
163	519	12	0	1.32	1.32

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE EFFECTS OF FEEDING DURING EXPERIMENT ON BIOAVAILABILITY OF AR-67 LACTONE IN MICE

(PERFORMED 8/7/2008-8/8/2008)

EFFECTS OF FEEDING DURING EXPERIMENT ON THE BIOAVAILABILITY AR-67 LACTONE IN FEMALE C57BL/6 MICE

EXPT # 164 AND EXPT # 165

Objective

Because AR-67 lactone is highly lipophilic, we tested the effects of presence of food during experiment on bioavailability of AR-67 lactone PO.

Expt 164: Study the bioavailability of SBE-β-CD based AR-67 lactone (200:1, E:D) at 2.5 mg/kg PO WITHOUT food during experiment.

Expt 165: Study the bioavailability of SBE-β-CD based AR-67 lactone (200:1, E:D) at 2.5 mg/kg PO WITH food during experiment.

Method

Formulation

The lyophilized powder SBE-β-CD based AR-67 lactone (200:1, E:D) was reconstituted in D5W to make 1 mg/mL dosing solution.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 164: mice (#521-529) were orally treated SBE-β-CD based AR-67 lactone (200:1, E:D) at the dose of 2.5 mg/kg WITHOUT food during experiment

Expt 165: mice (#531-539) were orally treated SBE-β-CD based AR-67 lactone (200:1, E:D) at the dose of 2.5 mg/kg WITH food during experiment

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h and 12 h. Each animal was sampled at 3-4 different time points.

The blood sample of about 50 μL was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma of 25 μL was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 μL,-80^{o}C). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name:

20AC-080808-Mouse PK Expt 164 & 165 (SBE-β-CD fed vs non-fed).seq

Method:

20AC-080808-mouse plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

1. Prism file: ..\Prism files\Mouse PK 2.5 mg PO Expt 164 & 165 food effect.pzf
2. WinNonlin file: ..\Winnonlin files\Mouse PK Expt 164 & 165.wsp

3. Tables

Table 1. Basic pharmacokinetic parameters

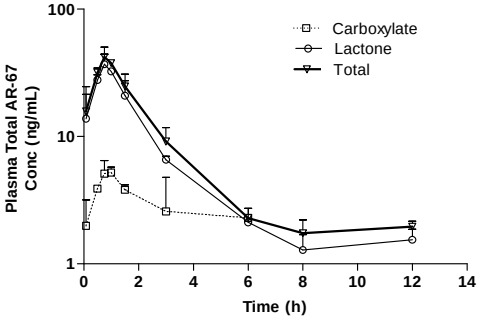
Expt 164: 2.5 mg/kg Lactone (SBE-β-CD 200:1) PO without food during expt

	AUC (ng*h/mL) Mean (SE)	Tmax (h) Median	Cmax (ng/mL) Mean (SE)
Carboxylate	19.42 (4.22)	1.00	5.22 (0.31)
Lactone	82.06 (5.11)	0.75	36.99 (4.14)
Total	101.48 (3.56)	0.75	42.11 (4.91)

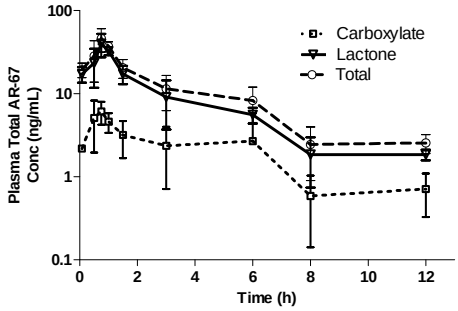
Expt 165: 2.5 mg/kg Lactone (SBE-β-CD 200:1) PO with food during expt

	AUC (ng·h/mL) Mean (SE)	Tmax (h) Median	Cmax (ng/mL) Mean (SE)
Carboxylate	23.85 (5.68)	0.75	6.11 (1.09)
Lactone	95.28 (4.75)	0.75	40.04 (7.25)
Total	119.13 (7.43)	0.75	46.15 (8.31)

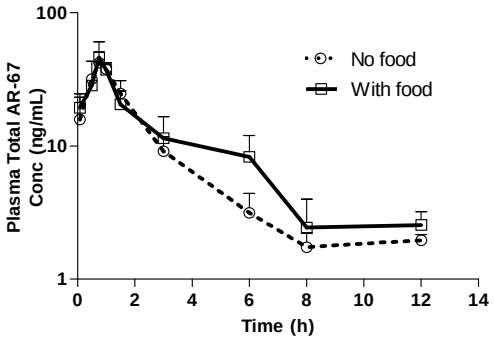
4. Figures



2.5 mg/kg oral AR-67 lactone SBE- β-CD (200:1, E:D)
WITHOUT food during experiment



2.5 mg/kg oral AR-67 lactone SBE- β-CD (200:1, E:D)
WITH food during experiment



2.5 mg/kg oral AR-67 lactone SBE- β-CD (200:1, E:D)
WITH versus WITHOUT food during experiment

Raw Data

Expt	Animal #	Time (h)	Carb. ng/mL	Lact. (ng/mL)	Total (ng/mL)
164	521	0.083	3.35	22.63	25.98
164	522	0.083	1.36	9.57	10.93
164	523	0.083	1.25	9.17	10.42
164	524	0.5	3.83	26.04	29.87
164	525	0.5	3.95	29.74	33.69
164	526	0.5	Missing	Missing	Missing
164	527	0.75	4.26	33.61	37.87
164	528	0.75	4.41	32.14	36.55
164	529	0.75	6.67	45.23	51.9
164	521	1	5.12	31.35	36.47
164	522	1	4.74	33.94	38.68
164	523	1	5.79	32.05	37.84
164	524	1.5	4.07	16.27	20.34
164	525	1.5	3.56	25.56	29.12
164	526	1.5	Missing	Missing	Missing
164	524	3	4.13	6.87	11
164	525	3	1.02	6.25	7.27
164	526	3	Missing	Missing	Missing
164	521	6	2.29	2.1	4.39
164	522	6	0.35	1.51	1.86
164	523	6	0.45	2.73	3.18
164	527	8	0.88	1.05	1.93
164	528	8	0.16	1.04	1.2
164	529	8	0.33	1.76	2.09
164	527	12	0.73	1.18	1.91
164	528	12	0.16	1.63	1.79
164	529	12	0.36	1.82	2.18
165	531	0.083	2.34	21.16	23.5
165	532	0.083	2.16	16.63	18.79
165	533	0.083	2.04	13.92	15.96
165	534	0.5	7.74	34.6	42.34
165	535	0.5	5.95	24.07	30.02
165	536	0.5	1.63	11.41	13.04
165	537	0.75	4.57	32.87	37.44
165	538	0.75	5.55	32.7	38.25
165	539	0.75	8.22	54.54	62.76
165	531	1	5.05	33.79	38.84
165	532	1	3.22	28.51	31.73

165	533	1	5.53	35.53	41.06
165	534	1.5	4.48	16.49	20.97
165	535	1.5	3.5	22	25.5
165	536	1.5	1.54	13.5	15.04
165	534	3	4.16	5.7	9.86
165	535	3	0.97	6.27	7.24
165	536	3	1.92	15.28	17.2
165	531	6	6.26	6.06	12.32
165	532	6	0.73	4.21	4.94
165	533	6	1.06	6.44	7.5
165	537	8	1.1	3.05	4.15
165	538	8	0.3	0.85	1.15
165	539	8	0.36	1.66	2.02
165	537	12	0.98	2.04	3.02
165	538	12	Missing	Missing	Missing
165	539	12	0.44	1.65	2.09

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE EFFECT OF EXCIPIENT TO DRUG RATIO ON THE BIOAVAILABILITY OF E-TPGS BASED AR-67 LACTONE IN MICE

(PERFORMED 8/19/2008-8/20/2008)

COMPARISON OF E:D RATIOS OF 56:1 AND 200:1 ON THE BIOAVAILABILITY OF E-TPGS-BASED AR-67 LACTONE IN C57BL/6 MICE

EXPT # 166 AND EXPT # 167

Objective

Expt 166: Study the E:D ratio of 200:1 on the absorption of E-TPGS based AR-67 lactone (2.5ml/kg, 2.5 mg/kg) PO.

Expt 167: Study the E:D ratio of 56:1 on the absorption of E-TPGS based AR-67 lactone (2.5ml/kg, 2.5 mg/kg) PO.

Method

Formulation

The excipient solution and AR-67 carboxylate stock solution (20 mg/mL) were prepared by Dr. Xiang, the excipient solution were spiked with carboxylate stock solution to make 2 mg/mL dosing solution. The dosing solutions were made freshly before dosing.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 166, mice (#541-# 549, n=9, three per time point) were dosed with 2.5 mg/kg TPGS based AR-67 lactone (E:D ratio 200:1) PO.

Expt 167, mice (#551-# 559, n=9, three per time point) were dosed with 2.5 mg/kg TPGS based AR-67 lactone (E:D ratio 56:1) PO.

Dosing solution was prepared by Sagar Rane to give a solution concentration of 1 mg/mL lactone after mixing 1 part of AR-67 stock solution (10 mg/mL carboxylate) with 9 parts of simulated gastric fluid containing TPGS.

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 4 h, 6 h, 8 h and 12 h. Each animal was sampled at three different time points.

Blood (about 50 μL) was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma (about 25 uL) was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 uL,-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name:
20AC-082008-Mouse Expt 166 & 167.seq &
20AC-082108-mouse Exp 166 & 167.seq

Method:
20AC-082208-mousecurve.met

Data were exported and saved in Leggas Lab/Experimental Results/AR-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and Winnonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

1. Prism files: \Prism files\Mouse PK Expt 166 & 167.pzf
2. Winnonlin files: \Winnonlin files\Mouse PK Expt 166 & 167.wsp

3. Tables

Table 1. Summary of basic pharmacokinetic parameters

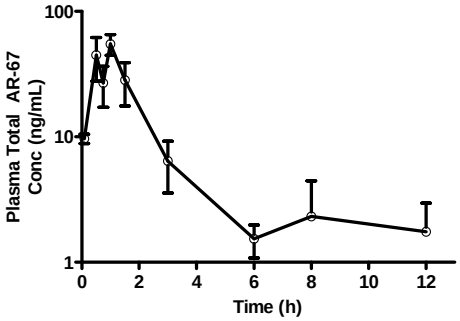
Expt #166: 2.5 mg/kg Lactone (TPGS 200:1) PO

	AUC(ng*h/mL) Mean	Tmax (h) Median	Cmax (ng/mL) Mean (SE)
Carboxylate	10.16 (0.93)	1.00	4.94 (1.2)
Lactone	92.84 (7.7)	1.00	49.90 (6.10)
Total	103.0 (8.55)	1.00	54.83 (7.29)

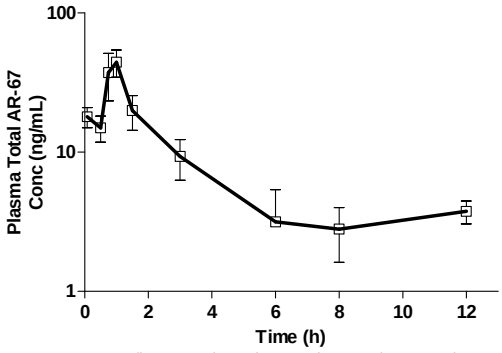
Expt # 167: 2.5 mg/kg Lactone (TPGS 56:1) PO

AUC (ng*h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean	SE
Carb	9.92	0.56	Carb	0.75	Carb	4.11	0.30
Lactone	93.99	5.15	Lactone	1.00	Lactone	40.43	5.35
Total	103.91	5.62	Total	1.00	Total	44.29	5.66

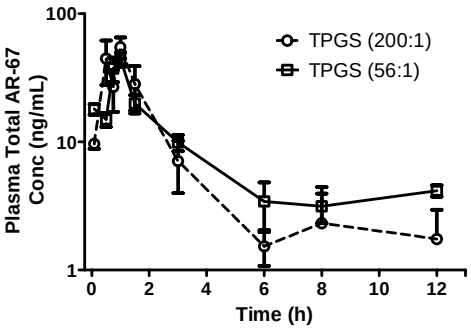
4. Figures



2.5 mg/kg TPGS based DB-67 lactone (E:D ratio 200:1) PO.



2.5 mg/kg TPGS based DB-67 lactone (56:1, E:D) PO.



2.5 mg/kg TPGS (200:1, E:D) versus TPGS (56:1, E:D) based AR-67 lactone PO

Raw Data

Sample ID	Expt	Animal #	Time (h)	Carb. (ng/mL)	Lact. (ng/mL)	Total (ng/mL)
541-5min	166	541	0.083	0.85	9.38	10.23
543-5min	166	543	0.083	0.86	8.21	9.07
544-30min	166	544	0.5	3.71	29.34	33.05
545-30min	166	545	0.5	6.26	57.97	64.23
546-30min	166	546	0.5	4.14	32.63	36.77
547-45min	166	547	0.75	2.36	25.15	27.51
548-45min	166	548	0.75	3.67	32.34	36.01
549-45min	166	549	0.75	1.73	15.05	16.78
541-1h	166	541	1	3.74	43.8	47.54
543-1h	166	543	1	6.13	55.99	62.12
544-1.5h	166	544	1.5	4.03	33.77	37.8
545-1.5h	166	545	1.5	3.16	27.07	30.23
546-1.5h	166	546	1.5	1.54	15.15	16.69
544-3h	166	544	3	0.42	3.23	3.65
545-3h	166	545	3	0.97	8.67	9.64
546-3h	166	546	3	0.68	7.26	7.94
541-6h	166	541	6	0.21	1.64	1.85
543-6h	166	543	6	0.1	1.11	1.21
547-8 h	166	547	8	0.43	4.28	4.71
548-8 h	166	548	8	0.14	0.54	0.68
549-8 h	166	549	8	0.16	1.4	1.56
547-12 h	166	547	12	0	1.2	1.2
548-12 h	166	548	12	0.12	0.79	0.91
549-12 h	166	549	12	0.33	2.8	3.13
551-5 min	167	551	0.083	1.1	13.45	14.55
552-5 min	167	552	0.083	1.94	17.95	19.89
553-5 min	167	553	0.083	1.83	17.65	19.48
554-30 min	167	554	0.5	1.66	16.9	18.56
555-30 min	167	555	0.5	1.01	11.49	12.5
556-30 min	167	556	0.5	1.51	12.3	13.81
557-45 min	167	557	0.75	4.36	36.71	41.07
558-45 min	167	558	0.75	4.45	44.64	49.09
559-45 min	167	559	0.75	3.51	18.38	21.89
551-1 h	167	551	1	4.31	48.93	53.24
552-1 h	167	552	1	4.01	41.8	45.81
553-1 h	167	553	1	3.26	30.55	33.81
554-1.5 h	167	554	1.5	1.33	12.43	13.76
555-1.5 h	167	555	1.5	2.29	22.24	24.53
556-1.5 h	167	556	1.5	1.99	19.42	21.41
554-3 h	167	554	3	0.84	8.08	8.92
555-3 h	167	555	3	1.16	11.58	12.74
556-3 h	167	556	3	0.91	7.1	8.01
551-6 h	167	551	6	0.16	1.94	2.1

552-6 h	167	552	6	0.19	1.81	2
553-6 h	167	553	6	0.49	5.72	6.21
557-8 h	167	557	8	0.22	2.13	2.35
558-8 h	167	558	8	0.21	2.1	2.31
559-8 h	167	559	8	0.57	4.18	4.75
557-12 h	167	557	12	0.42	4.06	4.48
558-12 h	167	558	12	0.36	2.95	3.31
559-12 h	167	559	12	0.45	4.25	4.7

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE EFFECT OF LIPID/FREE FATTY ACID PRETREATMENT ON THE BIOAVAILABILITY OF E-TPGS BASED AR-67 LACTONE IN MICE

(PERFORMED 8/28/2008-8/29/2008)

EFFECT OF PRETREATMENT WITH SAFFLOWER OIL AND CO-TREATMENT WITH OLEIC ACID ON THE BIOAVAILABILITY OF TPGS-BASED AR-67 LACTONE (E:D 200:1) IN FEMALE C57BL/6 MICE

Objective

Long chain unsaturated free fatty acids and lipids composed of long chain unsaturated fatty acids facilitate the lymphatic transport of lipophilic drugs administered by the oral route. In order for lipids to enhance lymphatic transport, however, they first need to be digested into the corresponding free fatty acid. The purpose of these mouse PK studies was to facilitate the lymphatic transport of AR-67 lactone by pretreatment with a lipid (safflower oil, containing triglycerides of linoleic acid) and by co-treatment with oleic acid.

Expt 168: Study the effect of safflower oil pretreatment PO (5 mL/kg) on the absorption of E-TPGS based AR-67 lactone (2.5 mL/kg, 2.5 mg/kg) PO.

Expt 169: Study the effect of oleic acid co-treatment PO (5 mL/kg) on the absorption of E-TPGS based AR-67 lactone (2.5 mL/kg, 2.5 mg/kg) PO

Method

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 168, Mice (#561-# 569, n=9, three per time point) were dosed with 2.5 mg/kg TPGS based AR-67 lactone (E:D ratio 200:1) PO 1 hour after safflower oil pretreatment (5 mL/kg) PO.

Expt 169, Mice (#561-# 569, n=9, three per time point) were dosed with 2.5 mg/kg TPGS based AR-67 lactone (E:D ratio 200:1) PO 5 minutes after oleic acid pretreatment (5 mL/kg) PO.

Dosing solution was prepared as described in Dr. Xiang's protocol (Refer to L0050 page 28 for protocol) to give a solution concentration of 1 mg/ml lactone after mixing 1 part of AR-67 stock solution (10 mg/mL carboxylate) with 9 parts of simulated gastric fluid containing TPGS.

Groups of three animals were sampled at 5 min, 30 min, 45 min, 1 h, 1.5 h, 4 h, 6 h, 8 h and 12 h. Each animal was sampled at three different time points.

Blood of about 50 µL was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma (about 25 µL) was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 µL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name:
20AC-090208-Mouse PK Expt 168 & 169.seq
Method:
20AC-082208-mousecurve.met
Data were exported and saved in Leggas Lab/Experimental Results/AR-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and Winnonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

1. Prism files: \Prism files\Mouse PK expt 168 & 169.pzf
2. Winnonlin files: \Winnonlin files\Mouse PK expt 168 & 169a.wsp

3. Tables (from Prism)

Table 1. Summary of basic pharmacokinetic parameters

2.5 mg/kg Lactone (TPGS 200:1) PO Safflower seed oil pretreatment with food during expt			
Expt #168	AUC (ng*h/mL)	Tmax (h)	Cmax (ng/mL)
Carboxylate	14.93	3	2.633
Lactone	95.29	1.5	21.05
Total	110.2	1.5	23.04
F (Bioavailability)	0.18		

2.5 mg/kg Lactone (TPGS 200:1) PO Oleic acid co-treatment with food during expt			
Expt #169	AUC (ng*h/mL)	Tmax (h)	Cmax (ng/mL)
Carboxylate	15.04	8	2.053
Lactone	125.4	8	17.3
Total	140.4	8	19.36
F (Bioavailability)	0.23		

4. Figures

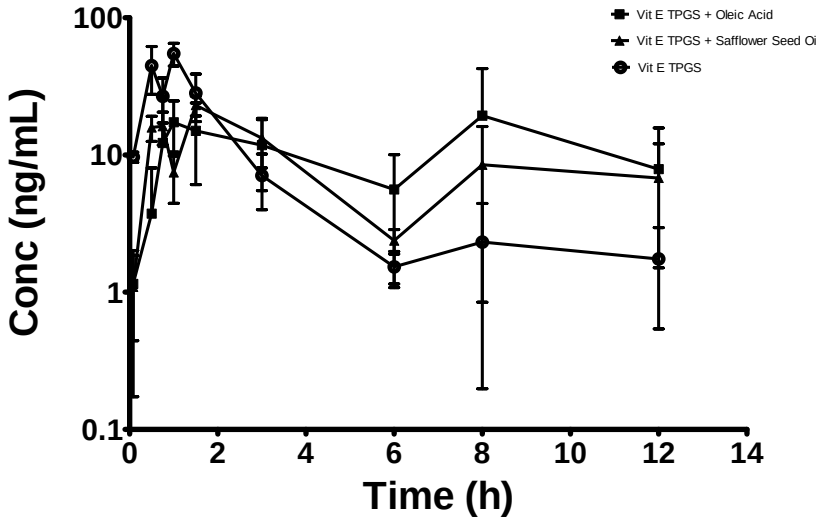


Fig.1.: Semi-logarithmic plot of the plasma concentration of total AR-67 (Mean±SD) in female C57BL/6 mice treated with 2.5 mg/kg AR-67 PO with or without co-treatment/pretreatment with oleic acid or safflower seed oil (TPGS E:D 200:1) File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\ Mouse PK expt 168 & 169.

Raw Data

Expt	Animal #	Time (h)	Carb. (ng/mL)	Lact. (ng/mL)	Total (ng/mL)
168	561	0.083	0.13	0.93	1.06
168	561	1	1.23	9.71	10.94
168	561	8	2.65	11.22	13.87
168	562	0.083	0	0.19	0.19
168	562	1	0.37	5.41	5.78
168	563	0.083	0.23	1.8	2.03
168	563	1	0.31	5.35	5.66
168	563	8	0.24	2.84	3.08
168	564	0.5	1	12.78	13.78
168	564	1.5	2.77	19.22	21.99
168	564	3	4.28	14.6	18.88
168	565	0.5	0.81	13.28	14.09
168	565	1.5	1.83	25.43	27.26
168	565	3	2.69	5.85	8.54
168	566	0.5	1.4	18.26	19.66
168	566	1.5	1.37	18.51	19.88
168	566	3	0.93	11.48	12.41
168	567	0.75	1.06	12.87	13.93
168	567	6	0.27	1.87	2.14
168	567	12	0.24	1.73	1.97
168	568	0.75	0.9	12.51	13.41
168	568	6	0.26	2.67	2.93
168	568	12	0.45	5.51	5.96
168	569	0.75	1.46	19.76	21.22
168	569	6	0.2	1.86	2.06
168	569	12	1.06	11.38	12.44
169	571	0.083	0.21	1.09	1.3
169	571	1	1.23	11.09	12.32
169	571	8	5.16	40.94	46.1
169	572	0.083	0.71	1.06	1.77
169	572	1	1.93	23.96	25.89
169	572	8	0.64	6.23	6.87
169	573	0.083	0	0.38	0.38
169	573	1	1.1	12.63	13.73
169	573	8	0.36	4.74	5.1
169	574	0.5	1	7.7	8.7
169	574	1.5	2.36	21.84	24.2
169	574	3	3.08	16	19.08
169	575	0.5	0.09	1.23	1.32
169	575	1.5	0.44	5.95	6.39
169	575	3	0.55	7.8	8.35
169	576	0.5	0.1	1.09	1.19
169	576	1.5	1	13.4	14.4

169	576	3	0.63	7.34	7.97
169	577	0.75	0.23	3.21	3.44
169	577	6	0.92	3.53	4.45
169	577	12	2.5	14.28	16.78
169	578	0.75	0.2	4.41	4.61
169	578	6	0.72	9.81	10.53
169	578	12	0.25	4.71	4.96
169	579	0.75	1.87	26.8	28.67
169	579	6	0.17	1.67	1.84
169	579	12	0.23	1.69	1.92

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE EFFECT OF FREE FATTY ACID PRETREATMENT ON THE BIOAVAILABILITY OF TPGS BASED AR-67 CARBOXYLATE IN MICE

(PERFORMED 9/11/2008-9/12/2008)

EFFECT OF CO-TREATMENT WITH OLEIC ACID ON THE BIOAVAILABILITY OF TPGS-BASED AR-67 CARBOXYLATE (E:D 56:1) IN FEMALE C57BL/6 MICE

EXPT # 170 AND EXPT # 171

Objective

Long chain unsaturated free fatty acids facilitate the lymphatic transport of lipophilic drugs administered by the oral route. This is a continuation of similar experiments done previously (Expt 168 & 169) with the lactone. The purpose of these mouse PK studies was to facilitate the lymphatic transport of AR-67 carboxylate by co-treatment with oleic acid. The carboxylate was chosen for further studies because of its enhanced water solubility.

Expt 170: (Control pretreatment) Study the effect of control (D5W) co-treatment PO (5 ml/kg) on the absorption of TPGS based AR-67 carboxylate (2.5ml/kg, 5 mg/kg) PO.

Expt 171: Study the effect of oleic acid co-treatment PO (5 ml/kg) on the absorption of TPGS based AR-67 carboxylate (2.5ml/kg, 5 mg/kg) PO.

Method

Formulation

The formulation of AR-67 carboxylate in TPGS (E:D ratio 56:1) was prepared at 2 mg/ml by Sagar Rane on 092308 and was analyzed by HPLC in Leggas lab on 09/23/08 and 09/24/08 prior to use. The formulation had 0.01M sodium bicarbonate.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 170, Mice (#581-# 589, n=9, three per time point) were dosed with 5 mg/kg TPGS based AR-67 carboxylate (E:D ratio 56:1) PO 5 min after D5W (5 ml/kg) PO.

Expt 171, Mice (#591-# 599, n=9, three per time point) were dosed with 5 mg/kg TPGS based AR-67 carboxylate (E:D ratio 56:1) PO 5 minutes after oleic acid pretreatment (5 ml/kg) PO.

Groups of three mice were sampled at 5 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h and 12 h, 18 h, 31 h and 51h. Each animal was sampled at three different time points.

Blood (about 50 uL) was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma (about 25 uL) was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 uL,-80^{o}C). Samples were kept at -80^{o}C until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed**.**

Sequence name:
20AC-092508.seq

Method:
20AC-082208-mousecurve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and Winnonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

1. Prism files:..\Prism files\Mouse PK Expt 170 & 171.pzf
2. Winnonlin files: ..\Winnonlin files\Mouse PK Expt 170 & 171.wsp

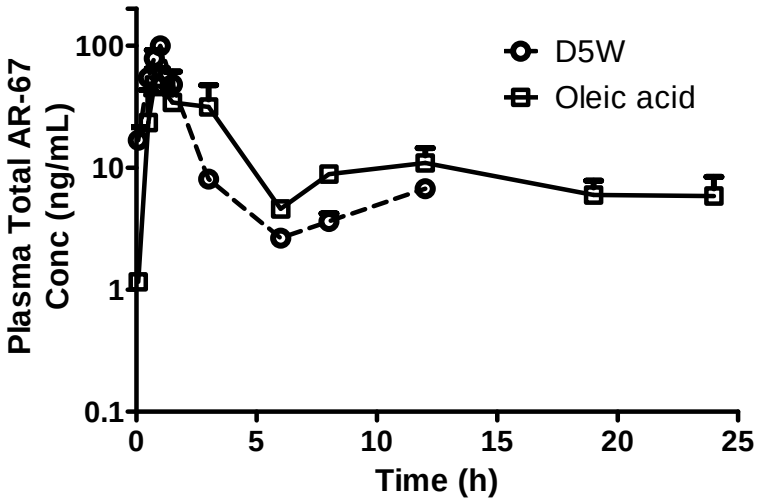
3. Tables (from Prism)

Table 1. Summary of basic pharmacokinetic parameters

Expt #170	5 mg/kg carboxylate (TPGS 56:1) PO + D5W co-treatment					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean (SE)
Total	176.48	7.48	Total	1.00	Total	99.72 (11.33)

Expt #171	5 mg/kg Carboxylate (TPGS 56:1) PO + Oleic acid co-treatment					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean (SE)
Total	202.29	68.33	Total	0.75	Total	47.54 (17.73)

4. Figures



5 mg/kg oral E-TPGS based AR-67 carboxylate (56:1,E/D)
5 min after administration of D5W or oleic acid (5 mL/kg) PO

File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\ Mouse PK expt 170 & 171

Raw Data

Expt	ID	Time (h)	Carboxylate (ng/mL)	Lactone (ng/mL)	Total (ng/mL)
170	581	0.083	3.72	12.55	16.27
170	581	1	8.75	78.16	86.91
170	581	8	0	4.83	4.83
170	581	19	1.17	0	1.17
170	582	0.083	1.85	23.37	25.22
170	582	1	6.11	83.84	89.95
170	582	8	0	2.99	2.99
170	583	0.083	0	8.71	8.71
170	583	1	11.9	110.41	122.31
170	583	8	0	3.03	3.03
170	584	0.5	3.84	56.53	60.37
170	584	1.5	4.14	48.33	52.47
170	584	3	0	7.78	7.78
170	585	0.5	4.09	54.33	58.42
170	585	1.5	3.73	45.77	49.5
170	585	3	0	9.88	9.88
170	586	0.5	2.98	41.96	44.94
170	586	1.5	3	39.17	42.17
170	586	3	0	6.52	6.52
170	587	0.75	4.86	71.83	76.69
170	587	6	0	2.58	2.58
170	587	12	1.86	4.87	6.73
170	588	0.75	7.09	95.15	102.24
170	589	0.75	3.91	52.24	56.15
170	589	6	0	2.71	2.71
171	591	1	6.46	73.4	79.86
171	591	8	0	7.52	7.52
171	591	19	1.57	4.58	6.15
171	592	1	1.16	11.66	12.82
171	592	8	0	7.74	7.74
171	592	19	0	2.64	2.64
171	593	0.083	1.16	0	1.16
171	593	1	4.64	41.47	46.11
171	593	8	0	11.26	11.26
171	593	19	1.79	7.32	9.11
171	594	0.5	2.96	40.31	43.27
171	594	1.5	6.97	81.51	88.48
171	594	3	5.26	55.76	61.02
171	594	24	1.06	0	1.06

171	595	1.5	0	5.94	5.94
171	595	3	0	6.87	6.87
171	595	24	0	9.81	9.81
171	596	0.5	0	3.46	3.46
171	596	1.5	0	8.28	8.28
171	596	3	1.77	24.82	26.59
171	596	24	0	6.7	6.7
171	597	0.75	3.07	44.29	47.36
171	597	6	0	4.41	4.41
171	597	12	6.56	10.72	17.28
171	598	0.75	5.98	72.35	78.33
171	598	6	0	5.17	5.17
171	598	12	0	5.19	5.19
171	599	0.75	1.22	15.7	16.92
171	599	6	0	4.22	4.22
171	599	12	0	10.32	10.32

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE EFFECT OF FREE FATTY ACID PRETREATMENT ON THE BIOAVAILABILITY OF TPGS BASED AR-67 LACTONE IN MICE

(PERFORMED 9/11/2008-9/12/2008)

EFFECT OF CO-TREATMENT WITH OLEIC ACID ON THE BIOAVAILABILITY OF TPGS-BASED AR-67 LACTONE (E:D 56:1) IN FEMALE C57BL/6 MICE

EXPT # 172 AND EXPT # 173

Objective

Long chain unsaturated free fatty acids facilitate the lymphatic transport of lipophilic drugs administered by the oral route. This is a continuation of similar experiments done previously (Expt 168, 169, 170, 171) with the lactone (2.5 mg/kg) & carboxylate (5 mg/kg). The purpose of these mouse PK studies was to facilitate the lymphatic transport of AR-67 lactone by co-treatment with oleic acid and to compare with results obtained from carboxylate (Expts 170 & 171).

Expt 172: (Control pretreatment) Study the effect of control (D5W) co-treatment PO (5 mL/kg) on the absorption of TPGS based AR-67 lactone (2.5 mL/kg, 5 mg/kg) PO.

Expt 173: Study the effect of oleic acid co-treatment PO (5 mL/kg) on the absorption of TPGS based AR-67 lactone (2.5mL/kg, 5 mg/kg) PO.

Method

Formulation

The formulation of AR-67 lactone in TPGS (E:D ratio 56:1) was prepared at 2 mg/ml by E. Adane on 100208 and was analyzed by HPLC in Leggas lab on 10/06/08 prior to use. The formulation concentration was 1.72 mg/ml.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 172, Mice (#601-# 609, n=9, three per time point) were dosed with 5 mg/kg TPGS based AR-67 lactone (E:D ratio 56:1) PO 5 min after D5W (5 mL/kg) PO.

Expt 173, Mice (#6111-# 619, n=9, three per time point) were dosed with 5 mg/kg TPGS based AR-67 lactone (E:D ratio 56:1) PO 5 minutes after oleic acid pretreatment (5 mL/kg) PO.

Groups of three mice were sampled at 5 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h and 12 h, 18 h, 31 h and 51h. Each animal was sampled at three different time points.

Blood (about 50 μL) was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma (about 25 μL) was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 μL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 μL:40 μL) and were analyzed.

Sequence name: 20AC-101008-Mouse PK expt 172 & 173.seq
Method: 20AC-082208-mousecurve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and Winnonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

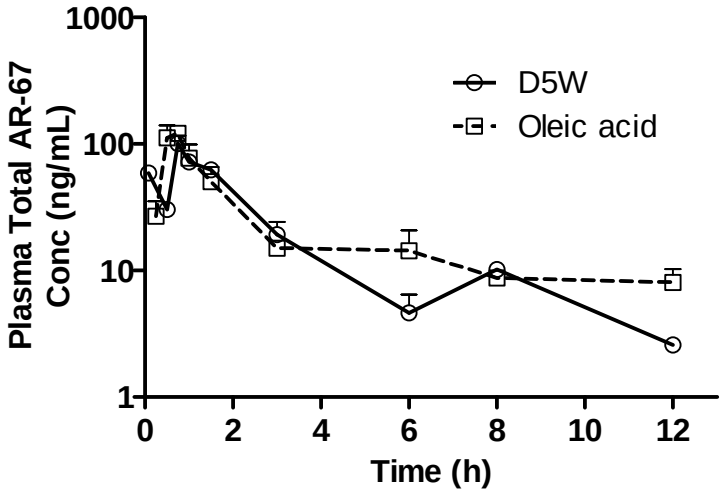
1. Prism files:..\Prism files\Mouse PK Expt 172 & 173.pzf
2. Winnonlin files: ..\Winnonlin files\Mouse PK Expt 172 & 173.wsp
3. Tables

Table 1. Summary of basic pharmacokinetic parameters

Expt #172	5 mg/kg Lactone (TPGS 56:1) PO: D5W retreatment					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean (SE)
Total	230.24	18.62	Total	0.75	Total	100.67 (16.09)

Expt #173	5 mg/kg Lactone (TPGS 56:1) PO: Oleic acid pretreatment					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean (SE)
Total	256.47	30.45	Total	0.75	Total	121. 57 (8.34)

4. Figures



5 mg/kg oral AR-67 lactone in TPGS (56:1 E/D) 5 min after treatment with D5W or oleic acid (5 ml/kg)

File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\ Mouse PK expt 172 & 173.

Raw Data

Expt	Time (h)	ID	Carboxylate (ng/mL)	Lactone (ng/mL)	Total (ng/mL)
172	0.083	601	4.81	68.49	73.3
172	0.083	602	4.1	50.5	54.6
172	0.083	603	3.98	44.54	48.52
172	0.5	604	2.83	29.51	32.34
172	0.5	605	1.79	26.54	28.33
172	0.75	607	9.5	75.22	84.72
172	0.75	608	11.86	120.99	132.85
172	0.75	609	7.43	77.02	84.45
172	1	601	6.69	69.27	75.96
172	1	602	7.33	79.59	86.92
172	1	603	5.25	47.83	53.08
172	1.5	604	6.22	57.94	64.16
172	1.5	605	5.83	68.31	74.14
172	1.5	606	4.09	45.18	49.27
172	3	604	5.28	19.18	24.46
172	3	605	2.25	21.76	24.01
172	3	606	1.09	8.34	9.43
172	6	607	8.3	2.22	8.3
172	6	608	0.46	2.63	2.63
172	6	609	0.43	2.92	2.92
172	8	601	4.46	3.73	8.19
172	8	602	1.18	9.6	10.78
172	8	603	1.2	10.44	11.64
172	12	607	9.35	2.58	11.93
172	12	608	0.52	1.21	1.73
172	12	609	0.33	1.43	1.76
172	14	604	5.05	2.87	7.92
172	14	605	0.53	1.11	1.64
172	14	606	0.33	1.56	1.89
173	0.25	611	1.18	15.2	16.38
173	0.25	612	1.41	19.2	20.61
173	0.25	613	3.64	39.86	43.5
173	0.5	615	13.17	127.36	140.53
173	0.5	616	8.62	74.98	83.6
173	0.75	617	10.87	119.02	129.89
173	0.75	619	9.22	104.02	113.24
173	1	612	5.66	50.4	56.06
173	1	613	9.1	89.83	98.93
173	1.5	614	2.12	20.05	22.17
173	1.5	615	8.14	66.05	74.19
173	1.5	616	4.89	50.23	55.12
173	3	615	1.87	14.17	16.04

173	3	616	1.47	12.46	13.93
173	6	617	2.97	8.97	11.94
173	6	618	2.45	24.04	26.49
173	6	619	0.63	4.62	4.62
173	8	611	1.34	6.59	7.93
173	8	612	1.14	9.95	11.09
173	8	613	0.82	7.12	7.12
173	12	617	4.66	4.95	9.61
173	12	618	1.03	9.79	10.82
173	12	619	0.49	3.67	3.67
173	14	614	9	10.6	19.6
173	14	615	0.68	2.65	2.65
173	14	616	0.61	14.46	14.46
173	24	611	1	4.8	5.8
173	24	612	0.2	1.66	1.86
173	24	613	0.13	1.11	1.24
173	28	617	2.68	0.77	3.45
173	28	618	0.42	0.61	1.03
173	28	619	0.53	0.48	1.01

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE EFFECT OF FREE FATTY ACID ON THE BIOAVAILABILITY OF TPGS BASED AR-67 CARBOXYLATE IN MICE

(PERFORMED 10/16/2008-10/17/2008)

EFFECT OF OLEIC ACID AMOUNT IN THE FORMULATION ON THE ORAL BIOAVAILABILITY OF TPGS-BASED AR-67 CARBOXYLATE (E:D, 56:1) IN FEMALE C57BL/6 MICE

Objective

Long chain unsaturated free fatty acids facilitate the lymphatic transport of lipophilic drugs administered by the oral route. The previous experiments have shown that pretreatment and co-treatment with oleic acid increased the oral bioavailability of AR-67 by about 1.5 fold, and this is a continuation of this set of experiments to incorporate the oleic acid in the final formulation. The purpose of these mouse PK studies was to determine the bioavailability of mixture of E-TPGS based formulation premix with oleic acid.

Expt 174: Study on the bioavailability of mixture of TPGS based AR-67 carboxylate (E:D ratio 56:1) premixed with oleic acid at 1:5, v/v (0.5 X) at dose of 5 mg/kg PO.

Expt 175: Study the bioavailability of a mixture of TPGS based AR-67 carboxylate (E:D ratio 56:1) premixed with oleic acid at 2:1, v/v ratios (5X) at dose of 5mg/kg PO.

Method

Formulation

The formulation of AR-67 carboxylate in TPGS (E:D ratio 56:1) was prepared at 2 mg/ml by E. Adane and was analyzed by HPLC in Leggas laboratory.

Animal treatment and sample processing

Expt 174: mice (#621-629) were treated with mixture of TPGS based AR-67 carboxylate (E:D ratio 56:1) premixed with oleic acid at 1:5, v/v (0.5 X) at dose of 5 mg/kg PO

Expt 175: mice (#631-639) were treated orally with a mixture TPGS based AR-67 carboxylate (E:D ratio 56:1) premixed with oleic acid at 2:1, v/v ratios (5X) at dose of 5mg/kg PO.

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h, 12 h and 24 h. Each animal was sampled at three different time points.

Blood (about 50 μL) was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma (about 25 uL) was

collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 uL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name: 20AC-102008-Mouse PK expt 174 & 175.seq

Method: 20AC-070307-Mouse plasma curve.met
Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and Winnonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

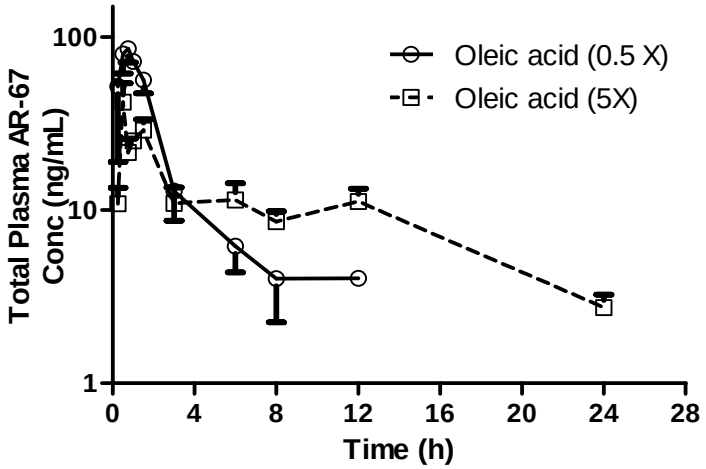
1. Prism files: ..\Prism files\Mouse PK Expt 174 & 175.pzf
2. Winnonlin files: ..\Winnonlin files\Mouse PK Expt 174 & 175.wsp
3. Tables

Table 1. Summary of basic pharmacokinetic parameters

Expt #174	5 mg/kg Lactone (TPGS 56:1) PO:Winnonlin Oleic acid 0.5x					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean (SE)
Total	198.24	11.32	Total	0.75	Total	85.92 (8.34)

Expt #175						
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean (SE)
Total	158.54	11.41	Total	0.50	Total	42.03 (12.16)

4. Figures



5 mg/kg oral AR-67 carboxylate mixture of oleic acid and TPGS based DB-67 carboxylate (E:D ratio 56:1) at 1;5, v/v (0.5 X) or 2:1, v/v ratios (5X)

File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\ Mouse PK expt 174 & 175.

Raw data

Expt	Time (h)	ID	Carb. (ng/ml)	Lact. (ng/ml)	Tot. (ng/ml)	Filter >=1 Carb. (ng/ml)	Filter >=2.5 Lact. (ng/ml)	Filter Total
174	0.25	621	3.03	46.81	49.84	3.03	46.81	49.84
174	0.25	622	5.33	80.5	85.83	5.33	80.5	85.83
174	0.25	623	1.48	18.63	20.11	1.48	18.63	20.11
174	0.5	624	5.41	73.93	79.34	5.41	73.93	79.34
174	0.5	625	6.89	91.34	98.23	6.89	91.34	98.23
174	0.5	626	8.45	53.47	61.92	8.45	53.47	61.92
174	0.75	627	8.2	89.64	97.84	8.2	89.64	97.84
174	0.75	628	8.28	81.8	90.08	8.28	81.8	90.08
174	0.75	629	7.13	62.72	69.85	7.13	62.72	69.85
174	1	621	6.29	66.6	72.89	6.29	66.6	72.89
174	1	622	9.04	62.29	71.33	9.04	62.29	71.33
174	1.5	624	5.06	49	54.06	5.06	49	54.06
174	1.5	625	6.35	59.97	66.32	6.35	59.97	66.32
174	1.5	626	4.84	43.87	48.71	4.84	43.87	48.71
174	3	627	4.53	12.03	16.56	4.53	12.03	16.56
174	3	628	0.65	7.71	8.36	0.5	7.71	8.21
174	3	629	1.4	12.26	13.66	1.4	12.26	13.66
174	6	624	0.34	3.93	4.27	0.5	3.93	4.43
174	6	625	0.56	7.35	7.91	0.5	7.35	7.85
174	6	626	0.52	5.91	6.43	0.5	5.91	6.41
174	8	627	1.71	0.17	1.88	1.71	1.25	2.96
174	8	628	0.24	2.53	2.77	0.5	2.53	3.03
174	8	629	0.51	4.77	5.28	0.5	4.77	5.27
174	12	621	0.27	3.67	3.94	0.5	3.67	4.17
174	12	622	0.16	0.82	0.98	0.5	1.25	1.75
174	12	623	0	4.14	4.14	0	4.14	4.14
174	24	624	5.13	0.21	5.34	5.13	1.25	6.38
174	24	625	0	0.4	0.4	0	1.25	1.25
174	24	626	7.24	0.59	7.83	7.24	1.25	8.49
175	0.25	631	0.64	7.65	8.29	0.5	7.65	8.15
175	0.25	632	0.98	15.13	16.11	0.5	15.13	15.63
175	0.25	633	0.62	7.65	8.27	0.5	7.65	8.15
175	0.5	634	5.18	57.45	62.63	5.18	57.45	62.63
175	0.5	635	2.01	18.51	20.52	2.01	18.51	20.52
175	0.5	636	3.39	39.55	42.94	3.39	39.55	42.94
175	0.75	637	1.32	15.66	16.98	1.32	15.66	16.98
175	0.75	638	2.48	27.8	30.28	2.48	27.8	30.28
175	0.75	639	1.64	15.72	17.36	1.64	15.72	17.36

175	1	631	3.02	21.03	24.05	3.02	21.03	24.05
175	1	632	2.15	22.93	25.08	2.15	22.93	25.08
175	1	633	3.06	23.18	26.24	3.06	23.18	26.24
175	1.5	634	2.61	17.74	20.35	2.61	17.74	20.35
175	1.5	635	3.91	30.47	34.38	3.91	30.47	34.38
175	1.5	636	3.02	29.38	32.4	3.02	29.38	32.4
175	3	637	0	9.42	9.43	0	9.42	9.42
175	3	638	0.62	6.66	7.28	0.5	6.66	7.16
175	3	639	1.29	14.77	16.06	1.29	14.77	16.06
175	6	634	1.95	14.26	16.21	1.95	14.26	16.21
175	6	635	0.84	5.42	6.26	0.5	5.42	5.92
175	6	636	1.15	10.72	11.87	1.15	10.72	11.87
175	8	637	0.72	9.09	9.81	0.5	9.09	9.59
175	8	638	0.46	5.61	6.07	0.5	5.61	6.11
175	8	639	0.95	8.96	9.91	0.5	8.96	9.46
175	12	631	0.76	10.96	11.72	0.5	10.96	11.46
175	12	632	1.06	13.54	14.6	1.06	13.54	14.6
175	12	633	0.57	6.83	7.4	0.5	6.83	7.33
175	24	634	0	0.97	0.97	0	1.25	1.25
175	24	635	0.36	2.89	3.25	0.5	2.89	3.39
175	24	636	0	2.22	2.22	0	1.25	1.25

Note: LLOQ of carboxylate is 1.0 ng/mL; LLOQ of lactone is 2.5 ng/mL; if the concentration is larger than 0 and smaller than LLOQ, half of LLOQ value was taken.

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE EFFECT OF OLEATE TO TPGS RATIO ON THE ABSORPTION OF TPGS BASED AR-67 CARBOXYLATE IN MICE

(PERFORMED 12/9/2008-12/10/2008)

COMPARISON OF 1:4 AND 1:7 OLEATE TO TPGS RATIOS ON THE ABSORPTION OF TPGS-BASED AR-67 CARBOXYLATE (E:D~50:1) IN FEMALE C57BL/6 MICE

Objective

Long chain unsaturated free fatty acids facilitate the lymphatic transport of lipophilic drugs administered by the oral route. Prior experiments that assessed lymphatic transport were done by either by pretreating animals with oleic acid before administration of AR-67 or mixing oleic acid with AR-67 formulation right before dosing. We thought efficient mixing will be a problem when animals were pretreated with the oil before AR-67 administration; hence the reason for premixing oleic acid and AR-67 formulation prior to dosing. However, the latter approach was observed to decrease the pH drastically because of the oleic acid. Thus a series of studies were conducted to incorporate oleic acid in the formulation as the oleate. The purpose of the current mouse PK studies was to compare the absorption of AR-67 carboxylate formulations containing 1:4 and 1:7 oleate to TPGS ratios.

Expt 176: Study the absorption of 1:4 oleate:TPGS ratio (AR-67 carboxylate, 2.5ml/kg, 5 mg/kg) PO. [Formulation TX17-16-3]

Expt 177: Study the absorption of 1:7 oleate:TPGS ratio (AR-67 carboxylate,2.5ml/kg, 5 mg/kg) PO. [Formulation TX17-15-2]

N.B. The formulations were kept frozen at-20 for a week before use and were thawed overnight.

Method

Formulation

The formulations were prepared at 2 mg/ml by Dr. Xiang on --- (as the carboxylate)

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 176, Mice (#641-# 649, n=9, three per time point) were dosed with 1:4 ratio of oleate to TPGS (5 mg/kg PO, 2.5 ml/kg).

Expt 177, Mice (#651-# 659, n=9, three per time point) were dosed with 1:7 ratio of oleate to TPGS (5 mg/kg PO, 2.5 ml/kg).

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h, 12 h and 24 h. Each animal was sampled at 3-4 different time points.

Blood (about 50 uL) was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma (about 25 uL) was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 uL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name:

20AC-121208-Mouse Plasma PK Expt 176 & 177.seq

Method:

20AC-082208-mousecurve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and Winnonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

1. Prism files:..\Prism files\Mouse PK 5mg PO exps 176 & 177.pzf
2. Winnonlin files: ..\Winnonlin files\Mouse PK Expt 176.wsp
   ..\Winnonlin files\Mouse PK Expt 177.wsp

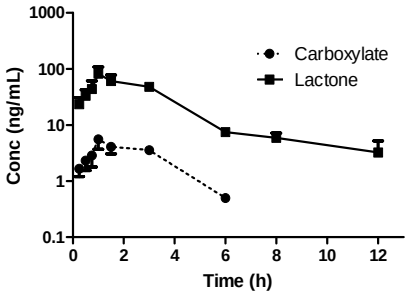
3. Tables

Table 1. Summary of basic pharmacokinetics parameters

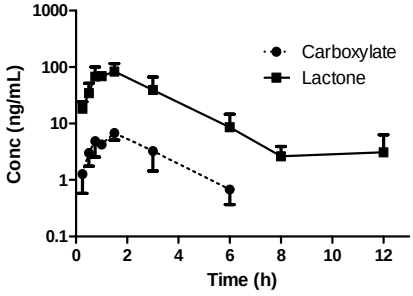
Expt #176	5.0 mg/kg Carboxylate PO Oleate:TPGS 1:4,					
AUC(ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean
Carb	22.81		Carb	1.00	Carb	5.55
Lactone	267.90		Lactone	1.00	Lactone	83.08
Total	285.00	15.90	Total	1.00	Total	88.62

Expt #177	5.0 mg/kg Carboxylate PO Oleate:TPGS 1:7					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean
Carb	19.46		Carb	1.50	Carb	6.80
Lactone	250.50		Lactone	1.50	Lactone	83.58
Total	269.20	56.30	Total	1.50	Total	90.38

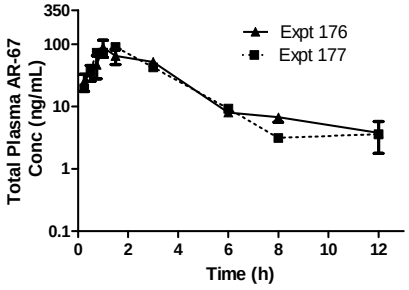
4. Figures



5 mg/kg oral AR-67 in TPGS (E:D, 50:1), Oleic Acid:TPGS 1:4



5 mg/kg oral AR-67 in TPGS (E:D, 50:1), Oleic Acid:TPGS 1:7



5 mg/kg oral AR-67, oleic acid:TPGS (1:4) (Expt 176) and oleic acid:TPGS (1:7) (Expt 177)

File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\ Mouse PK expt 176 & 177.

Appendix

Raw Data

Sample ID	Expt	Mouse #	Time (h)	Carb. (ng/ml)	Lact. (ng/ml)	Total (ng/ml)
641-15min	176	641	0.25	1.73	27.19	28.92
642-15min	176	642	0.25	1.18	15.15	16.33
643-15min	176	643	0.25	2.08	28.17	30.25
644-30min	176	644	0.5	3.17	41.18	44.35
645-30min	176	645	0.5	2.16	35.33	37.49
646-30min	176	646	0.5	1.64	22.95	24.59
647-45 min	176	647	0.75	3.89	61.04	64.93
648-45 min	176	648	0.75	1.72	25.71	27.43
649-45 min	176	649	0.75	3.04	44.97	48.01
641-1h	176	641	1	3.94	55.22	59.16
642-1h	176	642	1	7.56	105.14	112.7
643-1h	176	643	1	5.14	88.87	94.01
644-1.5h	176	644	1.5	5.19	76.53	81.72
645-1.5h	176	645	1.5	4	63.01	67.01
646-1.5h	176	646	1.5	3.11	43.25	46.36
644-3h	176	644	3	3.9	45.86	49.76
645-3h	176	645	3	3.4	54.41	57.81
646-3h	176	646	3	3.4	44.42	47.82
641-6h	176	641	6	0.77	8.32	8.32
642-6h	176	642	6	0.72	7.19	7.19
643-6h	176	643	6	0.83	7.04	7.04
647-8 h	176	647	8	0.53	5.22	5.22
648-8 h	176	648	8	0.73	7.36	7.36
649-8 h	176	649	8	1.41	5.16	6.57
647-12 h	176	647	12	0.48	2.39	2.87
648-12 h	176	648	12	0.53	5.22	5.75
649-12 h	176	649	12	0.41	3.31	3.72
641-24h	176	641	24	0.36	1.14	1.5
642-24h	176	642	24	0.4	0.74	1.14
643-24h	176	643	24	0.21	0.7	0.91
651-15min	177	651	0.25	0.91	13.16	14.07
652-15min	177	652	0.25	1.85	24.77	26.62
653-15min	177	653	0.25	1.48	17.22	18.7
654-30min	177	654	0.5	1.73	24.03	25.76
655-30min	177	655	0.5	3.07	25.06	28.13
656-30min	177	656	0.5	4.25	54.3	58.55
657-45 min	177	657	0.75	4.43	68.12	72.55
658-45 min	177	658	0.75	2.81	35.58	38.39

659-45 min	177	659	0.75	7.39	100.05	107.44
651-1h	177	651	1	3.94	68.12	72.06
652-1h	177	652	1	4.09	66.85	70.94
653-1h	177	653	1	4.69	72.24	76.93
654-1.5h	177	654	1.5	8.79	119.61	128.4
655-1.5h	177	655	1.5	5.69	66.22	71.91
656-1.5h	177	656	1.5	5.91	64.91	70.82
654-3h	177	654	3	5.29	70.3	75.59
655-3h	177	655	3	1.84	19.55	21.39
656-3h	177	656	3	2.65	28	30.65
651-6h	177	651	6	1.04	15.47	16.51
652-6h	177	652	6	0.57	6.09	6.66
653-6h	177	653	6	0.57	4.2	4.77
657-8 h	177	657	8	0.45	3.8	4.25
658-8 h	177	658	8	0.46	0.17	0.63
659-8 h	177	659	8	0.45	2.83	3.28
657-12 h	177	657	12	0.74	6.78	7.52
658-12 h	177	658	12	0.38	2.34	2.72
659-12 h	177	659	12	0.31	1.86	2.17
651-24h	177	651	24	0.52	4.28	4.8
652-24h	177	652	24	0.21	0.65	0.86
653-24h	177	653	24	0.42	0.9	1.32

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE BIOAVAILABILITY OF AMORPHOUS FORMULATIONS PMFD 576/1 AND PMFD 576/2 SUSPENDED IN DDH_2O IN MICE

(PERFORMED 1/17/2009-1/18/2009)

THE BIOAVAILABILITY OF AMORPHOUS FORMULATIONS PMFD 576/1 AND PMFD 576/2 DISSOLVED IN DDH2O IN FEMALE C57BL/6 MICE

Objective

The oral bioavailability of 2 amorphous formulations PMFD 576/1 and PMFD 576/2 provided by Arno was determined.

PMFD 576/1: PVP K30: Tween-80: AR-67 (71:6:23), spray dry power

PMFD 576/2: PVP K90: Tween-80: AR-67 (71:6:23), spray dry power

The purpose of the current mouse PK studies was to determine the bioavailability of amorphous formulations PMFD 576/1 and PMFD 576/2 suspended in water.

Expt 178: Study the oral bioavailability of PMFD 576/1 in ddH2O (pH 4.85)

Expt 179: Study the oral bioavailability of PMFD 576/2 in ddH2O (pH 6.00)

The amorphous powders were kept at -20 oC. The dosing suspensions were prepared freshly at 2 mg/mL AR-67 andwere used within 1 hour after preparation. They were vortexed vigoursly before gavage.

Method

Formulation

The powder was weighed by Dr. Xiang and certain volume of ddH2O was added to make 2 mg/mL AR-67 suspension.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 178, Mice #661-# 669 were dosed with PMFD 576/1 in ddH2O (pH 4.85) at 5 mg/kg PO.

Expt 179, Mice #671-# 679 were dosed with PMFD 576/2 in ddH2O (pH 1.2) at 5 mg/kg PO.

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h, 12 h and 24h. Each animal was sampled at 3-4 different time points.

At each sampling time point, blood samples of about 50 μL were collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma of 25 μL was collected by centrifugation of blood and then

extracted 1:4 (v/v) with cold MeOH (100 µL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name:
20AC-121908-Mouse PK expt 178 & 179 real.seq
Method:

20AC-122408-Mouse curve.seq

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

1. Prism file: ..\Prism files\Mouse PK 5mg PO exps 178 & 179.pzf
2. WinNonlin file: ..\Winnonlin files\Mouse PK Expt 178 & 179.wsp

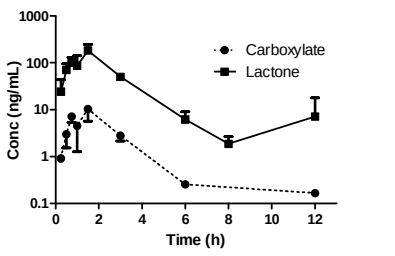
3. Tables

Table 1. Summary of basic pharmacokinetic parameters

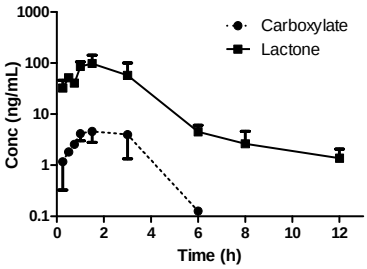
Expt #178	5.0 mg/kg lactone PO amorphous PMDF 576/1 dissolved in ddH2O					
AUC (ng·/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean
Carb	23.89		Carb	1.50	Carb	10.32
Lactone	392.85		Lactone	1.50	Lactone	183.31
Total	416.14	40.17	Total	1.50	Total	193.62

Expt #179	5.0 mg/kg lactone PO amorphous PMFD 576/2 dissolved in ddH2O					
AUC (ng·/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean
Carb	16.35		Carb	1.50	Carb	4.59
Lactone	312.54		Lactone	1.50	Lactone	98.46
Total	326.60	82.12	Total	1.50	Total	103.08

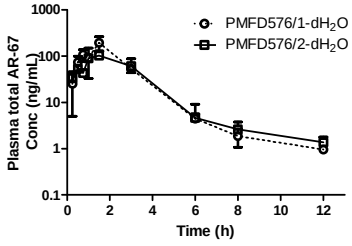
4. Figures



5 mg/kg oral AR-67 in (lactone, pH 4.9) in amorphous PMFD 576/1



5 mg/kg oral AR-67 in (lactone, pH 6.0) in amorphous PMFD 576/2



Expt 178: 5 mg/kg Oral AR-67 in (lactone, pH 4.7) in amorphous PMFD 576/1
Expt 179: 5 mg/kg Oral AR-67 in (lactone, pH 6.0) in amorphous PMFD 576/2

Raw Data

					Filter >=1	Filter >=2.5	Filter
Expt #	Mouse #	Time (h)	Carb. (ng/ml)	Lact. (ng/ml)	Carb. (ng/ml)	Lact. (ng/ml)	Total (ng/ml)
178	661	0.25	2.58	60.06	2.58	60.06	62.64
178	662	0.25	0.48	22.23	0.5	22.23	22.73
178	663	0.25	0.14	12.59	0.5	12.59	13.09
178	664	0.5	5.62	123.16	5.62	123.16	128.78
178	665	0.5	4.09	93.9	4.09	93.9	97.99
178	666	0.5	1.89	58.94	1.89	58.94	60.83
178	667	0.75	11.02	162.62	11.02	162.62	173.64
178	668	0.75	10.23	140.41	10.23	140.41	150.64
178	669	0.75	6.62	88.97	6.62	88.97	95.59
178	661	1	9.3	156.14	9.3	156.14	165.44
178	662	1	7.17	152.62	7.17	152.62	159.79
178	663	1	1.16	29.78	1.16	29.78	30.94
178	664	1.5	18.04	249.25	18.04	249.25	267.29
178	665	1.5	15.62	314.94	15.62	314.94	330.56
178	666	1.5	6.59	150.74	6.59	150.74	157.33
178	664	3	2.72	53.17	2.72	53.17	55.89
178	665	3	3.9	68.27	3.9	68.27	72.17
178	666	3	4.3	74.91	4.3	74.91	79.21
178	661	6	0	4.57	0	4.57	4.57
178	662	6	0.39	11.88	0.5	11.88	12.38
178	663	6	0.2	7.78	0.5	7.78	8.28
178	667	8	0	2.46	0	1.25	1.25
178	668	8	0	3.16	0	3.16	3.16
178	669	8	0	2.86	0	2.86	2.86
178	667	12	0	1.12	0	1.25	1.25
178	668	12	0	2.2	0	1.25	1.25
178	669	12	0.92	19.5	0.5	19.5	20
178	661	24	0	0.29	0	1.25	1.25
178	662	24	0	0.67	0	1.25	1.25
178	663	24	0	4.18	0	4.18	4.18
179	671	0.25	2.66	61.55	2.66	61.55	64.21
179	672	0.25	0.9	26.83	0.5	26.83	27.33
179	673	0.25	1.39	38.05	1.39	38.05	39.44
179	674	0.5	2.53	68.32	2.53	68.32	70.85
179	675	0.5	2.55	72.47	2.55	72.47	75.02
179	676	0.5	2	60.37	2	60.37	62.37
179	677	0.75	3.09	56.12	3.09	56.12	59.21
179	678	0.75	3.7	54.04	3.7	54.04	57.74

179	679	0.75	3.19	47.77	3.19	47.77	50.96
179	671	1	5.97	107.78	5.97	107.78	113.75
179	672	1	6.47	138.74	6.47	138.74	145.21
179	673	1	3.7	89.36	3.7	89.36	93.06
179	674	1.5	8.3	183.43	8.3	183.43	191.73
179	675	1.5	5.95	131.51	5.95	131.51	137.46
179	676	1.5	3.66	69.1	3.66	69.1	72.76
179	674	3	8.97	138.46	8.97	138.46	147.43
179	675	3	2.35	37.01	2.35	37.01	39.36
179	676	3	4.14	47.39	4.14	47.39	51.53
179	671	6	0.12	7.32	0.5	7.32	7.82
179	672	6	0	6.68	0	6.68	6.68
179	673	6	0	3.67	0	3.67	3.67
179	677	8	0	2.73	0	2.73	2.73
179	678	8	0	6.28	0	6.28	6.28
179	679	8	0	2.04	0	1.25	1.25
179	677	12	0	2.25	0	1.25	1.25
179	678	12	0	2.85	0	2.85	2.85
179	679	12	0	0.65	0	1.25	1.25
179	671	24	0	0.49	0	1.25	1.25
179	672	24	0	7.74	0	7.74	7.74
179	673	24	0	0.58	0	1.25	1.25

Note: LLOQ of carboxylate is 1.0 ng/mL; LLOQ of lactone is 2.5 ng/mL; if the concentration is larger than 0 and smaller than LLOQ, half of LLOQ value was taken.

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE EFFECTS OF OLEIC ACID TO E-TPGS RATIOS ON ABSORPTION OF TPGS BASED AR-67 CARBOXYLATE IN MICE

(PERFORMED 12/22/2008-12/23/2008)

COMPARISON OF 1:7 AND 1:10 OLEATE TO TPGS RATIOS ON THE ABSORPTION OF TPGS-BASED AR-67 CARBOXYLATE (E:D~50:1) IN FEMALE C57BL/6 MICE

Objective

Long chain unsaturated free fatty acids facilitate the lymphatic transport of lipophilic drugs administered orally. Previous experiments that assessed lymphatic transport were done by either pre-treating animals with oleic acid or mixing oleic acid with AR-67 formulation right before dosing. We thought efficient mixing will be a problem when animals were pretreated with the oil before AR-67 administration; hence the reason for premixing oleic acid and AR-67 formulation prior to dosing. However, the latter approach was observed to decrease the pH drastically because of the oleic acid. Thus a series of studies were conducted to.

a) Buffer the formulation to prevent pH change due to oleic acid.

b) Incorporate oleic acid in the formulation as the oleate.

The former approach was abandoned and the latter was studied further. The purpose of the current mouse PK studies was to compare the absorption of AR-67 carboxylate formulations containing 1:7 and 1:10 oleate to TPGS ratios.

Expt 180: Study the absorption of oleate:TPGS (1:7) based AR-67 carboxylatemg/kg, 2.5ml/kg) PO. [Formulation TX17-16-2]

Expt 181: Study the absorption of oleate:TPGS (1:10) based AR-67 carboxylate (5 mg/kg, 2.5ml/kg) PO [Formulation TX17-15-1]

N.B. The formulations were kept frozen at -20oC for a week before use and were thawed overnight.

Method

Formulation

The formulations were prepared at 2 mg/mL by Dr. Xiang on --- (as the carboxylate)

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 180: Mice (#681-# 689, n=9) were dosed with oleic acid:TPGS (1:7) based AR-67 carboxylate at 5 mg/kg PO

Expt 181, Mice (#691-# 699, n=9) were dosed with oleic acid:TPGS (1:10) based AR-67 carboxylate at 5 mg/kg PO

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h and 12 h, 18 h, 24h. Each animal was sampled at 3-4 different time points.

The blood sample of about 50 µL was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma of 25 µL was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 uL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name:

20AC-122708-Mouse Exp 180 & 181.seq

20AC-122408-mouse curve & Exp 180 & 181.seq

Method:

20AC-122408-mouse curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

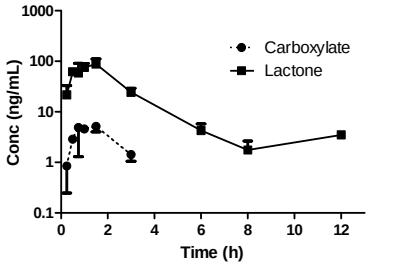
1. Prism file:..\Prism files\Mouse PK 5mg PO exps 180 & 181.pzf
2. WinNonlin file: ..\Winnonlin files\Mouse PK Expt 180 & 181.wsp
3. Tables

Table 1. Summary of basic pharmacokinetic parameters

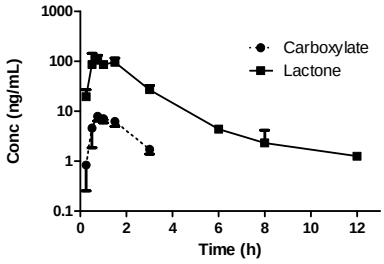
Expt #180	5.0 mg/kg Carboxylate PO Oleate : TPGS 1:7					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean
Carb	12.22		Carb	1.50	Carb	5.16
Lactone	229.40		Lactone	1.50	Lactone	87.86
Total	238.74	21.69	Total	1.50	Total	93.02

Expt #181	5.0 mg/kg Carboxylate PO Oleate :TPGS 1:10					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean
Carb	16.10		Carb	1.50	Carb	7.89
Lactone	255.00		Lactone	1.50	Lactone	105.10
Total	270.44	18.62	Total	1.50	Total	112.90

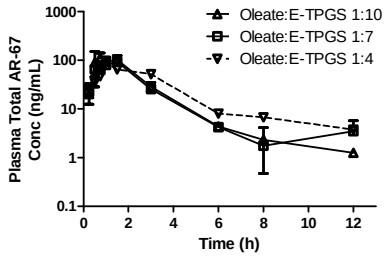
4. Figures



5 mg/kg Oral AR-67 in TPGS (E:D,50:1), Oleic Acid:TPGS 1:7



5 mg/kg Oral AR-67 in TPGS (E/D 50:1), Oleic Acid:TPGS 1:10



5 mg/kg oral AR-67 in Oleic Acid:TPGS

File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\ Mouse PK expt 180 & 181.

Raw Data

Expt	Mouse #	Time (h)	AR-67 Carboxylate	AR-67 Lactone	Filter >=1 AR-67 Carboxylate	Filter >=2.5 AR-67 Lactone	Filter total
180	681	0.25	1.54	33.36	1.54	33.36	34.9
180	682	0.25	0.66	11.42	0.5	11.42	11.92
180	683	0.25	0.87	20.03	0.5	20.03	20.53
180	684	0.5	3	63.12	3	63.12	66.12
180	685	0.5	2.84	58.98	2.84	58.98	61.82
180	686	0.5	2.79	63.7	2.79	63.7	66.49
180	687	0.75	5.47	86.23	5.47	86.23	91.7
180	688	0.75	8.14	67.35	8.14	67.35	75.49
180	689	0.75	1.04	20.76	1.04	20.76	21.8
180	681	1	5	76.24	5	76.24	81.24
180	682	1	4.88	90.26	4.88	90.26	95.14
180	683	1	3.79	60.36	3.79	60.36	64.15
180	684	1.5	3.91	63.64	3.91	63.64	67.55
180	685	1.5	5.35	89.69	5.35	89.69	95.04
180	686	1.5	6.21	110.25	6.21	110.25	116.46
180	684	3	1.01	18.63	1.01	18.63	19.64
180	685	3	1.56	27.19	1.56	27.19	28.75
180	686	3	1.73	26.96	1.73	26.96	28.69
180	681	6	0	2.71	0	2.71	2.71
180	682	6	0	4.41	0	4.41	4.41
180	683	6	0	5.64	0	5.64	5.64
180	687	8	0	2.27	0	1.25	1.25
180	688	8	0	2.78	0	2.78	2.78
180	689	8	0	2.19	0	1.25	1.25
180	687	12	0	3.34	0	3.34	3.34
180	688	12	0	3.93	0	3.93	3.93
180	689	12	0	3.16	0	3.16	3.16
180	681	24	0	0.34	0	1.25	1.25
180	682	24	0	3.06	0	3.06	3.06
180	683	24	0	0.23	0	1.25	1.25
181	691	0.25	0.41	11.14	0.5	11.14	11.64
181	692	0.25	0.82	22.25	0.5	22.25	22.75
181	693	0.25	1.51	25.26	1.51	25.26	26.77
181	694	0.5	7.4	150.19	7.4	150.19	157.59
181	695	0.5	4.45	73.54	4.45	73.54	77.99
181	696	0.5	1.92	33.73	1.92	33.73	35.65
181	697	0.75	8.77	126.7	8.77	126.7	135.47
181	698	0.75	8.73	113.27	8.73	113.27	122
181	699	0.75	6.16	75.18	6.16	75.18	81.34
181	691	1	5.73	84.01	5.73	84.01	89.74
181	692	1	7.44	83.49	7.44	83.49	90.93

181	693	1	8.02	91.58	8.02	91.58	99.6
181	694	1.5	7.7	118.98	7.7	118.98	126.68
181	695	1.5	5.44	83.62	5.44	83.62	89.06
181	696	1.5	5.54	86.05	5.54	86.05	91.59
181	694	3	1.47	24.69	1.47	24.69	26.16
181	695	3	1.57	22.79	1.57	22.79	24.36
181	696	3	2.11	33.64	2.11	33.64	35.75
181	691	6	0	4.26	0	4.26	4.26
181	692	6	0	4.87	0	4.87	4.87
181	693	6	0	3.99	0	3.99	3.99
181	697	8	0	1.51	0	1.25	1.25
181	698	8	0	2.18	0	1.25	1.25
181	699	8	0	4.43	0	4.43	4.43
181	697	12	0	1.83	0	1.25	1.25
181	698	12	0	1.69	0	1.25	1.25
181	699	12	0	0.14	0	1.25	1.25
181	691	24	0	0.44	0	1.25	1.25
181	692	24	0	0.24	0	1.25	1.25
181	693	24	0	0.22	0	1.25	1.25

Note: LLOQ of carboxylate is 1.0 ng/mL; LLOQ of lactone is 2.5 ng/mL; if the concentration is larger than 0 and smaller than LLOQ, half of LLOQ value was taken.

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE BIOAVAILABILITY OF AMORPHOUS FORMULATION OF AR-67 (PMFD 576/1) SUSPENDED IN DDH_2O AND S.G.F.IN MICE

(PERFORMED 1/14/2009-1/15/2009))

THE BIOAVAILABILITY OF AMORPHOUS FORMULATION OF AR-67 (PMFD 576/1) SUSPENDED IN DDH2O AND S.G.F. IN FEMALE C57BL/6 MICE

EXPT # 182 AND EXPT # 183

Objective

The previous studies have shown that Arno's amorphous formulation PMFD 576/1 (suspended in ddH2O) gave the best bioavailability among all the formulations we tested so far. The following studies were conducted to assess the reproducibility of our results and examine the effect of the suspending medium pH on oral bioavailability of PMFD 576/1.

PMFD 576/1: PVP K30: Tween-80: AR-67 (71:6:23), spray-dried power.

Expt 182: Study the oral bioavailability of PMFD 576/1 suspension in ddwater (pH 4.85)

Expt 183: Study the oral bioavailability of PMFD 576/1 suspension in S.G.F. (pH 1.2)

The amorphous powders were kept at -20oC. The dosing suspensions were prepared freshly and used within 1 hour of preparation and vortexed vigorously before gavage.

Method

Formulation

The powder was weighed by Dr. Xiang. Water or SGF was added to make 2 mg/mL AR-67 dosing suspensions.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 182, Mice #701-# 709 were dosed with PMFD 576/1 in H2O (pH 4.85) at 5 mg/kg PO.

Expt 183, Mice #711-# 719 were dosed with PMFD 576/1 in S.G.F. (pH 1.2) at 5 mg/kg PO.

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h, 12 h, and 24h. Each animal was sampled at 3-4 different time points.

At each sampling time point, blood samples of about 50 μL were collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma of 25 μL was collected by centrifugation of blood and then

extracted 1:4 (v/v) with cold MeOH (100 µL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name:

20AC-0111609-mouse PK expt 182 & 183.seq

Method:

20AC-010909-mouse curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

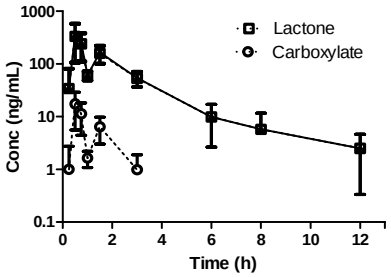
1. Prism file: ..\Prism files\Mouse PK 5 mg PO exps 182 & 183.pzf
2. WinNonlin file: ..\Winnonlin files\Mouse PK Expt 182 & 183.wsp
3. Tables

Table 1. Basic pharmacokinetic parameters

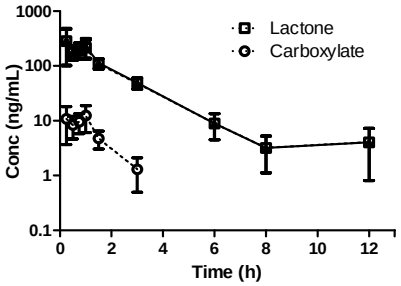
Expt #182	5.0 mg/kg lactone PMFD 576/1 in ddH2O PO, a repeat of Expt 178					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean
Carb	11.71		Carb	1.00	Carb	13.38
Lactone	384.83		Lactone	0.25	Lactone	256.62
Total	397.68	71.22	Total	0.25	Total	270.08

Expt #183	5.0 mg/kg lactone PO amorphous PMFD 576/1 in SGF					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean
Carb	13.57		Carb	1.00	Carb	9.58
Lactone	386.12		Lactone	0.25	Lactone	219.23
Total	401.20	27.72	Total	0.25	Total	227.62

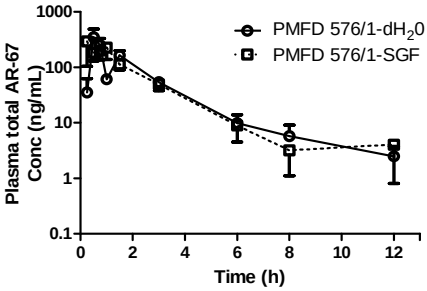
4. Figures



5 mg/kg oral AR-67 in PMFD 576/1 dissolved in ddH $_2$O



5 mg/kg oral AR-67 in PMFD 576/1 dissolved in S.G.F. (pH 1.2)



5 mg/kg oral PMFD 576/1 dissolved in water Vs in S.G.F.

Raw Data

Expt	Mouse #	Time (h)	Carb. (ng/ml)	Lact. (ng/ml)	Filter >=1 Carb. (ng/ml)	Filter >=2.5 Lact. (ng/ml)	Filter Total (ng/ml)
182	701	0.25	0	8.45	0	8.45	8.45
182	702	0.25	0	7.36	0	7.36	7.36
182	703	0.25	3.01	87.14	3.01	87.14	90.15
182	704	0.5	28.12	576.96	28.12	576.96	605.08
182	705	0.5	19.31	304.61	19.31	304.61	323.92
182	706	0.5	4.78	119.37	4.78	119.37	124.15
182	707	0.75	16.19	353.87	16.19	353.87	370.06
182	708	0.75	14.39	273.61	14.39	273.61	288
182	709	0.75	3.47	96.15	3.47	96.15	99.62
182	701	1	1.32	52.73	1.32	52.73	54.05
182	702	1	2.29	73.34	2.29	73.34	75.63
182	703	1	1.32	53.02	1.32	53.02	54.34
182	704	1.5	9.65	205.11	9.65	205.11	214.76
182	705	1.5	6.74	178.3	6.74	178.3	185.04
182	706	1.5	2.87	92.4	2.87	92.4	95.27
182	704	3	1.78	56.09	1.78	56.09	57.87
182	705	3	1.19	68.7	1.19	68.7	69.89
182	706	3	0	35.17	0	35.17	35.17
182	701	6	0	17.97	0	17.97	17.97
182	702	6	0	3.99	0	3.99	3.99
182	703	6	0	7.73	0	7.73	7.73
182	707	8	0	12.43	0	12.43	12.43
182	708	8	0	2.31	0	1.25	1.25
182	709	8	0	3.6	0	3.6	3.6
182	707	12	0	5	0	5	5
182	708	12	0	2.46	0	1.25	1.25
182	709	12	0	2.22	0	1.25	1.25
182	704	24	0	35.42	0	35.42	35.42
182	705	24	0	1.35	0	1.25	1.25
182	706	24	0	3.68	0	3.68	3.68
183	711	0.25	15.6	425.72	15.6	425.72	441.32
183	712	0.25	14.44	352.8	14.44	352.8	367.24
183	713	0.25	2.58	76.43	2.58	76.43	79.01
183	714	0.5	10.02	194.27	10.02	194.27	204.29
183	715	0.5	4.13	119.93	4.13	119.93	124.06
183	716	0.5	10.97	214.28	10.97	214.28	225.25
183	717	0.75	7.89	170.88	7.89	170.88	178.77
183	718	0.75	13.99	260.86	13.99	260.86	274.85
183	719	0.75	6.99	144.66	6.99	144.66	151.65
183	711	1	5.14	122.18	5.14	122.18	127.32

183	712	1	16.24	279.1	16.24	279.1	295.34
183	713	1	15.98	247.17	15.98	247.17	263.15
183	714	1.5	4.1	100.64	4.1	100.64	104.74
183	715	1.5	3.5	92.5	3.5	92.5	96
183	716	1.5	6.73	132.61	6.73	132.61	139.34
183	714	3	2.11	59.07	2.11	59.07	61.18
183	715	3	1.3	48.37	1.3	48.37	49.67
183	716	3	0.49	37.82	0.5	37.82	38.32
183	711	6	0	11.79	0	11.79	11.79
183	712	6	0	11.25	0	11.25	11.25
183	713	6	0	3.84	0	3.84	3.84
183	717	8	0	2.95	0	2.95	2.95
183	718	8	0	5.37	0	5.37	5.37
183	719	8	0	2.37	0	1.25	1.25
183	717	12	0	1.74	0	1.25	1.25
183	718	12	0	3.29	0	3.29	3.29
183	719	12	0	7.59	0	7.59	7.59
183	714	24	0	2.74	0	2.74	2.74
183	715	24	0	17.28	0	17.28	17.28
183	716	24	0	1.39	0	1.25	1.25

Note: LLOQ of carboxylate is 1.0 ng/mL; LLOQ of lactone is 2.5 ng/mL; if the concentration is larger than 0 and smaller than LLOQ, half of LLOQ value was taken.

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE BIOAVAILABILITY OF PVP K30 AND E-TPGS BASED AR-67 LACTONE IN MICE

(PERFORMED 1/29/2009)

THE BIOAVAILABILITY OF PVP K30:TWEEN-80:AR-67 (71:6:23) AND E-TPGS:TWEEN-80:AR-67 (71:6:23) BASED FORMULATIONS IN FEMALE C57BL/6 MICE

Objective

In previous mouse PK studies using Arno's amorphous formulations, we demonstrated that the amorphous formulation PMFD 576/1 had the highest AUC and gave an oral bioavailability of 33%. It suggested that keeping the drug in solution at supersaturated condition (at least as tested in vitro) might not be as important as keeping it in the amorphous form for absorption of AR-67 from GI tract. The amorphous form, and/or the combination of PVP K30 with Tween-80 might contribute to higher oral bioavailability by preventing crystallization of AR-67. To test this hypothesis, we designed two formulations to see the effects of excipients on oral bioavailability of AR-67.

a) PVP K30: Tween-80: AR-67 (71:6:23) in simulated gastric fluid (pH 1.2): lactone was made by spiking stock carboxylate solution with simulated gastric acid fluid containing PVP and Tween 80.

b) E-TPGS: Tween-80: AR-67 (71:6:23) in simulated gastric fluid (pH 1.2): lactone was made by mixing stock carboxylate solution with simulated gastric acid fluid containing E-TPGS and Tween 80.

The purpose of the current mouse PK studies was to compare the bioavailability AR-67 lactone formulations containing PVP K30: Tween-80: AR-67 (71:6:23) and E-TPGS: Tween-80: AR-67 (71:6:23).

Expt 184: Study the absorption of 5 mg/kg PO AR-67 in PVP K30: Tween-80: AR-67 (71:6:23) in simulated gastric fluid (pH 1.2)

Expt 185: Study the absorption of % mg/kg PO AR-67 in E-TPGS: Tween-80: AR-67 (71:6:23) in simulated gastric fluid (pH 1.2)

Excipients were mixed with simulated gastric fluid and stock AR-67 carboxylate solution before dosing. Because of lack of stability of TPGS at acidic pH all dosing suspensions were freshly prepared used within 1 hour after preparation. Suspension were vortexed every time before gavage.

Method

Formulation

Each solution was prepared by Dr. Xiang, and the final dosing solution was made by mixing them as sequence described.

Sequence	Solution	Volume (μL)
1	PVP K30 (or E-TPGS) with Tween-80	250
2	Simulated gastric fluid (pH 1.2)	2000
3	Carboxylate (20 mg/kg)	250

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 184, Mice (#721-# 729) were dosed with AR-67 in PVP K30: Tween-80: AR-67 (71:6:23) in SGF (pH 1.2) at 5 mg/kg PO.

Expt 185, Mice (#731-# 739) were dosed with AR-67 in E-TPGS: Tween-80: AR-67 (71:6:23) in SGF (pH 1.2) at 5 mg/kg PO.

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h, 12 h and 24h. Each animal was sampled at 3-4 different time points.

At each sampling time point, blood samples of about 50 μL were collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma of 25 μL was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 μL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 μL:40 μL) and were analyzed.

Sequence name:

20AC-012309-Mouse Plasma PK Expt 184 & 185.seq

20AC-012409-Mouse Plasma PK Expt 184 & 185.seq

Method:

20AC-010909-mouse curve.met

Data were exported and saved in Leggas Lab/Experimental Results/AR-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

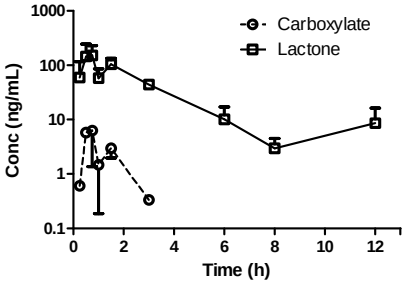
1. Prism file: ..\Prism files\Mouse PK 5 mg PO exps 184 & 185.pzf
2. WinNonlin file: ..\Winnonlin files\Mouse PK Expt 184 & 185.wsp
3. Tables

Table 1. Summary of basic pharmacokinetic parameters

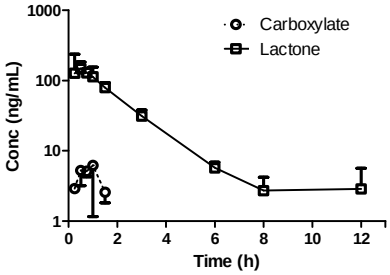
Expt #184	5.0 mg/kg lactone PO (PVP K30:Tween-80:AR-67 (71:6:23) in SGF pH 1.2)					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean
Carb	6.94	1.33	Carb	0.75	Carb	6.30
Lactone	366.49	23.36	Lactone	0.75	Lactone	152.4
Total	373.92	24.31	Total	0.75	Total	158.7

Expt #185	5.0 mg/kg lactone PO (E-TPGS:Tween-80:AR-67 (71:6:23) in SGF pH 1.2)					
AUC	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean
(ng·h/mL)						
Carb	6.30	1.69	Carb	1	Carb	6.24
Lactone	322.01	23.18	Lactone	0.5	Lactone	148.6
Total	330.25	24.77	Total	0.5	Total	153.8

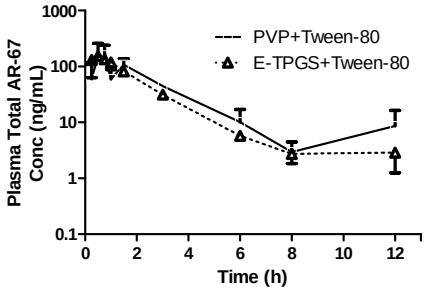
4. Figures



5 mg/kg oral AR-67 in PVP K30:Tween-80:AR-67 (71:6:23) in SGF (pH 1.2)



5 mg/kg oral AR-67 in E-TPGS:Tween-80:AR-67 (71:6:23) in SGF(pH 1.2)



5 mg/kg Oral AR-67 in PVP K30:Tween-80:AR-67 (71:6:23)
or E-TPGS:Tween-80:AR-67 (71:6:23) in simulated gastric fluid (pH 1.2)

Raw Data

Expt	Mouse #	Time (h)	Carb. (ng/ml)	Lact. (ng/ml)	Filter >=1 Carb. (ng/ml)	Filter >=2.5 Lact. (ng/ml)	Filter Total (ng/ml)
184	721	0.25	0	5.49	0	5.49	5.49
184	722	0.25	0	54.46	0	54.46	54.46
184	723	0.25	1.82	118.96	1.82	118.96	120.78
184	724	0.5	13.04	262.16	13.04	262.16	275.2
184	725	0.5	1.95	73.47	1.95	73.47	75.42
184	726	0.5	2.38	99.02	2.38	99.02	101.4
184	727	0.75	6.38	167.77	6.38	167.77	174.15
184	728	0.75	11.2	220.57	11.2	220.57	231.77
184	729	0.75	1.33	68.86	1.33	68.86	70.19
184	721	1	0	26.63	0	26.63	26.63
184	722	1	2.04	74.99	2.04	74.99	77.03
184	723	1	2.39	72.28	2.39	72.28	74.67
184	724	1.5	3.14	102.94	3.14	102.94	106.08
184	725	1.5	1.89	75.12	1.89	75.12	77.01
184	726	1.5	3.84	135.67	3.84	135.67	139.51
184	724	3	0	40.77	0	40.77	40.77
184	725	3	0.39	45.99	0.5	45.99	46.49
184	726	3	0.46	45.95	0.5	45.95	46.45
184	721	6	0	17.69	0	17.69	17.69
184	722	6	0	4.16	0	4.16	4.16
184	723	6	0	8.47	0	8.47	8.47
184	727	8	0	2.32	0	1.25	1.25
184	728	8	0	4.18	0	4.18	4.18
184	729	8	0	3.46	0	3.46	3.46
184	727	12	0	7.82	0	7.82	7.82
184	728	12	0	1.89	0	1.25	1.25
184	729	12	0	16.81	0	16.81	16.81
184	724	24	0	1.27	0	1.25	1.25
184	725	24	0	0.92	0	1.25	1.25
184	726	24	0	0.7	0	1.25	1.25
185	731	0.25	1.54	90.1	1.54	90.1	91.64
185	732	0.25	0	38.88	0	38.88	38.88
185	733	0.25	7.21	252.1	7.21	252.1	259.31
185	734	0.5	3.65	132.03	3.65	132.03	135.68
185	735	0.5	4.54	124.77	4.54	124.77	129.31
185	736	0.5	7.58	188.9	7.58	188.9	196.48
185	737	0.75	5.75	123.23	5.75	123.23	128.98
185	738	0.75	5.38	144	5.38	144	149.38
185	739	0.75	4.17	121.38	4.17	121.38	125.55
185	731	1	4.86	148.82	4.86	148.82	153.68
185	732	1	1.99	65.46	1.99	65.46	67.45
185	733	1	11.86	124.36	11.86	124.36	136.22

185	734	1.5	2.62	85.34	2.62	85.34	87.96
185	735	1.5	1.81	64.17	1.81	64.17	65.98
185	736	1.5	3.32	89.8	3.32	89.8	93.12
185	734	3	0	34.23	0	34.23	34.23
185	735	3	0	36.28	0	36.28	36.28
185	736	3	0	23.35	0	23.35	23.35
185	731	6	0	4.39	0	4.39	4.39
185	732	6	0	6.37	0	6.37	6.37
185	733	6	0	6.44	0	6.44	6.44
185	737	8	0	4.28	0	4.28	4.28
185	738	8	0	2.63	0	2.63	2.63
185	739	8	0	2.19	0	1.25	1.25
185	737	12	0	2.23	0	1.25	1.25
185	738	12	0	6.11	0	6.11	6.11
185	739	12	0	1.72	0	1.25	1.25
185	734	24	0	0.73	0	1.25	1.25
185	735	24	0	1.12	0	1.25	1.25
185	736	24	0	0.6	0	1.25	1.25

Note: LLOQ of carboxylate is 1.0 ng/mL; LLOQ of lactone is 2.5 ng/mL; if the concentration is larger than 0 and smaller than LLOQ, half of LLOQ value was taken.

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON BIOAVAILABILITY OF EXCIPIENT FREE (BLANK) AND PVP (K30) CONTAINING AR-67 LACTONE SUSPENSIONS IN MICE

(PERFORMED 2/3/2009-2/4/2009)

COMPARISON OF THE BIOAVAILABILITY OF EXCIPIENT FREE (BLANK) AND PVP (K30) SUSPENSIONS OF AR-67 LACTONE IN FEMALE C57BL/6 MICE

Objective

Previous experiments showed that the amorphous formulation of AR-67 (PMFD 576/1 (PVP (30 K)-Tween 80-lactone) gave 2-fold increase in oral bioavailability compared to supersaturated solutions of AR-67 lactone (e.g. SBE-β-CD or E-TPGS). To delineate if this increase in bioavailability is due to PVP or tween 80, we studied the bioavailability of AR-67 suspension in the in presence or absence PVP using the following two formulations:

a) Blank AR-67 lactone in SGF: simulated gastric fluid (SGF, pH 1.2) was spiked with carboxylate stock solution to make 2 mg/mL dosing suspension and dosed right after mixing.

b) PVP + AR-67 lactone in SGF: simulated gastric fluid containing PVP (K30) (pH 1.2) (E: D, 3:1) was spiked with carboxylate stock solution to make 2 mg/mL, and dosed right after mixing.

Expt 186: Study the bioavailability of blank AR-67 lactone suspension in SGF at 5 mg/kg (2.5 mL/kg) PO.

Expt 187: Study the bioavailability of AR-67 lactone suspension in SGF containing PVP (K30) (E/D 3:1) at 5 mg/kg (2.5 mL/kg) PO

Method

Formulation

The dosing solutions were prepared by mixing AR-67 carboxylate stock solution (20 mg/mL) with SGF or SGF containing PVP K30 (E: D, 3:1); then dosed right after mixing.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 186: Mouse# 741-749 were treated with blank AR-67 lactone suspension in simulated gastric fluid (pH 1.2) right after mixing.

Expt 187: Mouse# 751-759 were treated with AR-67 lactone suspension in simulated gastric fluid (pH 1.2) containing PVPK30 (E: D, 3:1) right after mixing.

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h, 12 h, and 24 h. Each animal was sampled at 3-4 different time points.

The blood sample of about 50 μL was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma of 25 μL was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 μL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name:

20AC-020509-mouse PK expt 186 & 187.seq

Method:

20AC-010909-mouse plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

1. Prism file:..\Prism files\Mouse PK 5 mg PO exps 186 & 187.pzf
2. WinNonlin file: ..\Winnonlin files\Mouse PK Expt 186 & 187 0-8 h.wsp

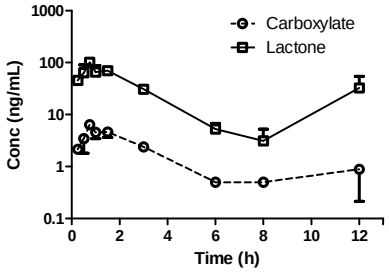
3. Tables

Table 1. Basic pharmacokinetic parameters

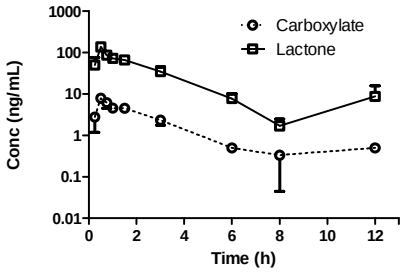
0-12 h Expt #186	5.0 mg/kg AR-67 lactone PO in SGF (pH 1.2)						
AUC (ng*h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean	SE
Carb	19.21	1.04	Carb	0.75	Carb	6.39	0.22
Lactone	303.85	28.54	Lactone	0.75	Lactone	101.31	1.99
Total	323.06	28.57	Total	0.75	Total	107.70	2.14

0-12 h Expt #187	5.0 mg/kg AR-67 lactone PO (PVP K30:AR-67 (71:23)) in SGF (pH 1.2)						
AUC (ng*h/mL)	Mean		Tmax (h)	Median	Cmax (ng/mL)	Mean	SE
Carb	18.99	0.76	Carb	0.50	Carb	7.92	0.86
Lactone	283.53	19.73	Lactone	0.50	Lactone	138.38	12.38
Total	302.53	20.44	Total	0.50	Total	146.29	13.23

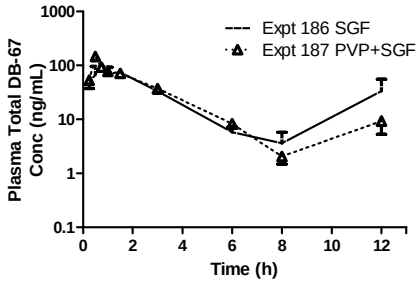
4. Figures



5 mg/kg Oral AR-67 in simulated gatric fluid (pH 1.2)



5 mg/kg Oral AR-67 in PVP K30:AR-67 (71:23) in simulated gatric fluid (pH 1.2)



5 mg/kg Oral AR-67 in SGF (Expt 186) and in PVP:AR-67 (71:23) (Expt 187) in SGF

File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\ Mouse PK 5 mg PO exps 186 & 187.

Raw Data

Expt	Mouse #	Time (h)	Carb. (ng/ml)	Lact. (ng/ml)	Filter >=1 Carb. (ng/ml)	Filter >=2.5 Lact. (ng/ml)	Filter Total (ng/ml)
186	741	0.25	2.31	50.5	2.31	50.5	52.81
186	742	0.25	1.79	40.39	1.79	40.39	42.18
186	743	0.25	2.34	45.62	2.34	45.62	47.96
186	744	0.5	5.14	90.66	5.14	90.66	95.8
186	745	0.5	3.33	64.38	3.33	64.38	67.71
186	746	0.5	1.88	36.61	1.88	36.61	38.49
186	747	0.75	6.48	104.75	6.48	104.75	111.23
186	748	0.75	5.97	97.86	5.97	97.86	103.83
186	749	0.75	6.72	101.32	6.72	101.32	108.04
186	741	1	3.8	51.81	3.8	51.81	55.61
186	742	1	5.86	90.65	5.86	90.65	96.51
186	743	1	4.05	54.28	4.05	54.28	58.33
186	744	1.5	4.03	61.52	4.03	61.52	65.55
186	745	1.5	5.71	76.99	5.71	76.99	82.7
186	746	1.5	4.08	70.85	4.08	70.85	74.93
186	744	3	2.11	26.26	2.11	26.26	28.37
186	745	3	2.48	32.83	2.48	32.83	35.31
186	746	3	2.56	33.26	2.56	33.26	35.82
186	741	6	0.63	6.26	0.5	6.26	6.76
186	742	6	0.28	3.57	0.5	3.57	4.07
186	743	6	0.44	5.95	0.5	5.95	6.45
186	747	8	0.31	1.94	0.5	1.25	1.75
186	748	8	0.47	5.4	0.5	5.4	5.9
186	749	8	0.26	2.72	0.5	2.72	3.22
186	747	12	1.67	31.48	1.67	31.48	33.15
186	748	12	0.65	54.98	0.5	54.98	55.48
186	749	12	0.66	11.06	0.5	11.06	11.56
186	747	24	0	0.39	0	1.25	1.25
186	748	24	0.11	0	0.5	0	0.5
186	749	24	0.21	0.28	0.5	1.25	1.75
187	751	0.25	1.2	21.5	1.2	21.5	22.7
187	752	0.25	2.74	57.85	2.74	57.85	60.59
187	753	0.25	4.41	71.59	4.41	71.59	76
187	754	0.5	6.31	114.07	6.31	114.07	120.38
187	755	0.5	8.19	146.46	8.19	146.46	154.65
187	756	0.5	9.25	154.6	9.25	154.6	163.85
187	757	0.75	7.75	107.7	7.75	107.7	115.45
187	758	0.75	6.5	85.92	6.5	85.92	92.42
187	759	0.75	4.41	66.72	4.41	66.72	71.13
187	751	1	4.71	75.16	4.71	75.16	79.87
187	752	1	4.63	78.45	4.63	78.45	83.08

187	753	1	4.37	63.99	4.37	63.99	68.36
187	754	1.5	5.23	84.5	5.23	84.5	89.73
187	755	1.5	3.58	54.21	3.58	54.21	57.79
187	756	1.5	4.75	60.5	4.75	60.5	65.25
187	754	3	2.77	45.34	2.77	45.34	48.11
187	755	3	2.56	35.93	2.56	35.93	38.49
187	756	3	1.71	23.85	1.71	23.85	25.56
187	751	6	0.96	9.67	0.5	9.67	10.17
187	752	6	0.66	8.67	0.5	8.67	9.17
187	753	6	0.4	5.06	0.5	5.06	5.56
187	757	8	0	2.39	0	1.25	1.25
187	758	8	0.28	2.47	0.5	1.25	1.75
187	759	8	0.26	2.63	0.5	2.63	3.13
187	757	12	0.23	1.93	0.5	1.25	1.75
187	758	12	0.91	14.83	0.5	14.83	15.33
187	759	12	0.45	10.31	0.5	10.31	10.81
187	757	24	0	0.47	0	1.25	1.25
187	758	24	0.11	0.34	0.5	1.25	1.75
187	759	24	0.14	0.72	0.5	1.25	1.75

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE ORAL BIOAVAILABILITY AND PHARMACOKINETICS OF AR-67 LACTONE AND CARBOXYLATE IN MICE

(PERFORMED 3/10/2009-3/13/2009)

ORAL BIOAVAILABILITY AND PHARMACOKINETICS OF AR-67 (SBE-B-CD, 200:1 E:D) LACTONE AND CARBOXYLATE IN FEMALE C57BL/6 MICE

Objective

We studied the oral bioavailability and systemic and interconversion clearances of the lactone and carboxylate forms of AR-67 in SBE-β-CD (200:1, E/D). Data from this panel of experiments will be used to build a pharmacokinetic model of interconversion for AR-67.

Expt 188: Study the bioavailability of AR-67 lactone in SBE-β-CD (200:1, E/D) at dose of 10 mg/kg (10 mL/kg) PO

Expt 189: Study the pharmacokinetics of AR-67 lactone in SBE-β-CD (200:1, E/D) at dose of 10 mg/kg (10 mL/kg) IV

Expt 190: Study the pharmacokinetics of AR-67 carboxylate in SBE-β-CD (200:1, E/D) at dose of 10 mg/kg (10 mL/kg) PO

Expt 191: Study the pharmacokinetics of AR-67 carboxylate in SBE-β-CD (200:1, E/D) at dose of 10 mg/kg (10 mL/kg) IV

Method

Formulation

The AR-67 lactone and carboxylate SBE-β-CD (200:1, E/D) lyophile powder were prepared by Dr. Xiang, and reconstructed to 1 mg/mL using D5W.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 188: mouse# 761-769 were treated with AR-67 lactone in SBE-β-CD (200:1, E/D) at dose of 10 mg/kg (10 mL/kg) PO

Expt 189: mouse# 771-779 were treated with AR-67 lactone in SBE-β-CD (200:1, E/D) at dose of 10 mg/kg (10 mL/kg) IV

Expt 190: mouse# 781-789 were treated with AR-67 carboxylate in SBE-β-CD (200:1, E/D) at dose of 10 mg/kg (10 mL/kg) PO

Expt 191: mouse# 791-799 were treated with AR-67 carboxylate in SBE-β-CD (200:1, E/D) at dose of 10 mg/kg (10 mL/kg) IV

Groups of three mice were sampled at 5 min (IV)/15 min (PO), 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h, 12 h and 24 h. Each animal was sampled at 3-4 different time points.

The blood sample of about 50 μL was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma of 25 μL was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 μL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 μL:40 μL) and were analyzed.

Sequence name:

20ac-021109-Exp 188 & 189.seq

Method:

20ac-021109-0109 mouse plasma curve reinjected.met

Sequence name:

20ac-021109-Exp 188 & 189.seq

Method:

Carboxylate:

20AC-061807-mouse plasma curve.met

Lactone

20ac-021109-0109 mouse plasma curve reinjected.met

Sequence name:

20ac-021109-Exp 188 & 189.seq

Method:

Carboxylate:

20AC-061807-mouse plasma curve.met

Lactone

20ac-021109-0109 mouse plasma curve reinjected.met

Sequence name:

20ac-021109-Exp 188 & 189.seq

Method:

20AC-021609-reinjection of 0109 mouse plasma curve.met

Sequence name:

20ac-021109-Exp 188 & 189.seq

Method:

20AC-021709-reinjection of 0109 mouse plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

Results

1. Prism file: ..\Prism files\Mouse PK 10 mg PO exps 188 & 190.pzf
   ..\Prism files\Mouse PK 10 mg IV exps 189 & 191.pzf
2. WinNonlin file: ..\Winnonlin files\Mouse PK Expt 188 & 190.wsp
   ..\Winnonlin files\Mouse PK Expt 189 & 191.wsp
3. Tables

Table 1. Basic pharmacokinetic parameters

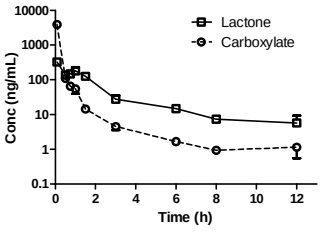
Expt #188	10 mg/kg lactone PO (SBE-β-CD 200:1)						
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean	SE
Carb	34.49	0.88	Carb	0.75	Carb	16.83	4.67
Lactone	323.95	13.00	Lactone	0.75	Lactone	160.52	40.38
Total	358.44	13.54	Total	0.75	Total	177.35	45.02

Expt #190	10 mg/kg carboxylate PO (SBE- β -CD 200:1)						
AUC (ng·h/mL)	Mean		Tmax (h)	Median	Cmax (ng/mL)	Mean	SE
Carb	36.68	2.40	Carb	0.50	Carb	19.43	1.98
Lactone	408.56	13.55	Lactone	0.50	Lactone	217.83	22.35
Total	445.24	14.74	Total	0.50	Total	237.25	24.08

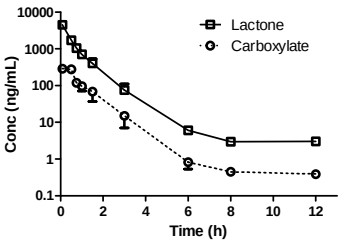
Expt #189	10 mg/kg lactone IV (SBE- β -CD 200:1)						
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean	SE
Carb	349.28	31.54	Carb	0.08	Carb	288.86	18.81
tone	3066.89	118.14	Lactone	0.08	Lactone	4515.65	27.39
Total	3414.43	130.27	Total	0.08	Total	4804.51	9.66

Expt #191	10 mg/kg carboxylate IV (SBE- β -CD 200:1)						
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Median	Cmax (ng/mL)	Mean	SE
Carb	1420.57	76.46	Carb	0.08	Carb	3926.42	292.29
Lactone	508.53	22.79	Lactone	0.08	Lactone	327.56	22.83
Total	1891.70	93.68	Total	0.08	Total	4253.98	310.31

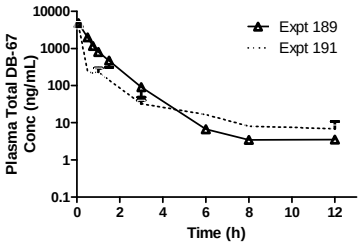
4. Figures



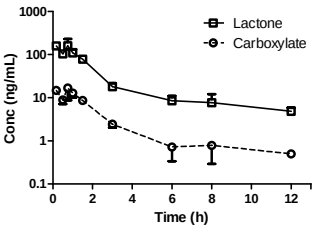
10 mg/kg IV AR-67 Carboxylate in SBE- β-CD (200:1)



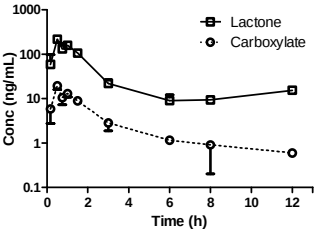
10 mg/kg IV AR-67 Lactone in SBE- β-CD (200:1)



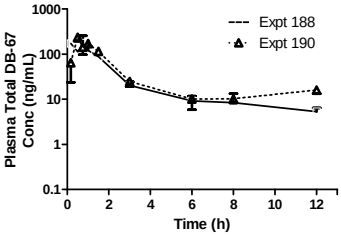
10 mg/kg IV AR-67 lactone (solid) and carboxylate (dashed) in SBE- β-CD (200:1)



10 mg/kg oral AR-67 lactone in SBE- β-CD (200:1)



10 mg/kg oral AR-67 carboxylate in SBE- β-CD (200:1)



10 mg/kg oral AR-67 lactone (solid) and carboxylate (dashed) in SBE- β-CD (200:1)

File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\ Mouse PK 10 mg PO exps 188 & 190
File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\ Mouse PK 10 mg IV exps 189 & 191

Raw Data

Expt	Mouse #	Time (h)	Carb. (ng/mL)	Lact. (ng/mL)	Filter >=1 Carb. (ng/mL)	Filter >=2.5 Lact. (ng/mL)	Filter Total
188	761	0.167	14.48	153.22	14.48	153.22	167.7
188	762	0.167	17.09	190.2	17.09	190.2	207.29
188	763	0.167	12.56	131.54	12.56	131.54	144.1
188	764	0.5	7.65	92.22	7.65	92.22	99.87
188	765	0.5	10.9	112.63	10.9	112.63	123.53
188	766	0.5	8.12	105.21	8.12	105.21	113.33
188	767	0.75	9.22	89.56	9.22	89.56	98.78
188	768	0.75	25.32	229.4	25.32	229.4	254.72
188	769	0.75	15.96	162.59	15.96	162.59	178.55
188	761	1	9.95	87.53	9.95	87.53	97.48
188	762	1	15.25	133.48	15.25	133.48	148.73
188	763	1	13.2	105.73	13.2	105.73	118.93
188	764	1.5	8.01	80.7	8.01	80.7	88.71
188	765	1.5	8.9	69.99	8.9	69.99	78.89
188	766	1.5	8.92	85.08	8.92	85.08	94
188	764	3	2.87	22.43	2.87	22.43	25.3
188	765	3	2.1	16.54	2.1	16.54	18.64
188	766	3	2.3	15.34	2.3	15.34	17.64
188	761	6	1.16	8.62	1.16	8.62	9.78
188	762	6	0.72	10.98	0.5	10.98	11.48
188	763	6	0.75	5.91	0.5	5.91	6.41
188	767	8	1.35	12.58	1.35	12.58	13.93
188	768	8	0.6	4.08	0.5	4.08	4.58
188	769	8	0.82	6.32	0.5	6.32	6.82
188	767	12	29.75	387.71	29.75	387.71	417.46
188	768	12	0.58	4.1	0.5	4.1	4.6
188	769	12	0.53	5.61	0.5	5.61	6.11
188	764	24	0.7	8.11	0.5	8.11	8.61
188	765	24	0.76	5.03	0.5	5.03	5.53
188	766	24	0.57	2.99	0.5	2.99	3.49
190	781	0.167	170.98	425.08	170.98	425.08	596.06
190	782	0.167	3.66	32.13	3.66	32.13	35.79
190	783	0.167	8.04	86.26	8.04	86.26	94.3
190	784	0.5	20.2	247.86	20.2	247.86	268.06
190	785	0.5	15.67	174.13	15.67	174.13	189.8
190	786	0.5	22.41	231.49	22.41	231.49	253.9

190	787	0.75	14.22	180.73	14.22	180.73	194.95
190	788	0.75	9.44	116.57	9.44	116.57	126.01
190	789	0.75	7.95	100.89	7.95	100.89	108.84
190	781	1	188.18	372.81	188.18	372.81	560.99
190	782	1	25.07	47.4	25.07	47.4	72.47
190	783	1	48.47	100.99	48.47	100.99	149.46
190	784	1	12.58	180.19	12.58	180.19	192.77
190	785	1	11.04	152.8	11.04	152.8	163.84
190	786	1	15.49	145.09	15.49	145.09	160.58
190	784	1.5	8.24	96.22	8.24	96.22	104.46
190	785	1.5	9.04	127.45	9.04	127.45	136.49
190	786	1.5	9.36	95.24	9.36	95.24	104.6
190	784	3	3.89	27.13	3.89	27.13	31.02
190	785	3	2.12	18.22	2.12	18.22	20.34
190	786	3	2.45	20.76	2.45	20.76	23.21
190	781	6	2.46	12.56	2.46	12.56	15.02
190	782	6	0.62	6.65	0.5	6.65	7.15
190	783	6	0.76	7.74	0.5	7.74	8.24
190	787	8	0.71	9.3	0.5	9.3	9.8
190	788	8	0.72	8.04	0.5	8.04	8.54
190	789	8	1.73	10.66	1.73	10.66	12.39
190	787	12	0.96	12.87	0.5	12.87	13.37
190	788	12	1.29	17.55	1.29	17.55	18.84
190	789	12	0	16.04	0	16.04	16.04
190	784	24	0.61	5.9	0.5	5.9	6.4
190	785	24	0.62	5.85	0.5	5.85	6.35
190	786	24	0.65	5.74	0.5	5.74	6.24

Expt	Mouse #	Time	Carb. (ng/mL)	Lact (ng/mL)	Filter >=1 Carb. (ng/mL)	Filter >=2.5 Lact. (ng/mL)	Filter Total (ng/mL)
189	771	0.083	251.394	4570.137	251.394	4570.137	4821.531
189	772	0.083	304.59	4483.479	304.59	4483.479	4788.069
189	773	0.083	310.596	4493.346	310.596	4493.346	4803.942
189	774	0.5	315.984	1657.1	315.984	1657.1	1973.084
189	775	0.5	296.689	1356.552	296.689	1356.552	1653.241
189	776	0.5	219.509	2127.671	219.509	2127.671	2347.18
189	777	0.75	129.941	1129.968	129.941	1129.968	1259.909
189	778	0.75	119.168	989.254	119.168	989.254	1108.422
189	779	0.75	109.592	1031.947	109.592	1031.947	1141.539
189	771	1	82.194	593.446	82.194	593.446	675.64
189	772	1	125.552	862.106	125.552	862.106	987.658
189	773	1	81.662	682.423	81.662	682.423	764.085
189	774	1.5	88.757	539.806	88.757	539.806	628.563
189	775	1.5	85.806	395.434	85.806	395.434	481.24
189	776	1.5	31.63	274.07	31.63	274.07	305.7
189	774	3	22.47	98.64	22.47	98.64	121.11
189	775	3	6.67	35.6	6.67	35.6	42.27
189	776	3	15.81	93.92	15.81	93.92	109.73
189	771	6	0.64	5.07	0.5	5.07	5.57
189	772	6	1.15	6.99	1.15	6.99	8.14
189	773	6	0.67	6	0.5	6	6.5
189	777	8	0.5	3.21	0.5	3.21	3.71
189	778	8	0.46	2.68	0.5	2.68	3.18
189	779	8	0.39	3.05	0.5	3.05	3.55
189	777	12	0	10.62	0	10.62	10.62
189	778	12	0.44	2.87	0.5	2.87	3.37
189	779	12	0.34	3.19	0.5	3.19	3.69
189	774	24	5.34	7.04	5.34	7.04	12.38
189	776	24	0.68	0.91	0.5	1.25	1.75
191	793	0.083	3964.328	358.66	3964.328	358.66	4322.988
191	792	0.083	4412.653	340.954	4412.653	340.954	4753.607
191	791	0.083	3402.276	283.069	3402.276	283.069	3685.345
191	794	0.5	130.28	133.71	130.28	133.71	263.99
191	795	0.5	94.53	139.47	94.53	139.47	234
191	796	0.5	103.1	139.4	103.1	139.4	242.5

191	797	0.75	60.8	147.57	60.8	147.57	208.37
191	798	0.75	79.4	169.96	79.4	169.96	249.36
191	799	0.75	54.8	129.78	54.8	129.78	184.58
191	791	1	40.37	125.3	40.37	125.3	165.67
191	792	1	71.25	195.88	71.25	195.88	267.13
191	793	1	53.65	222.65	53.65	222.65	276.3
191	794	1.5	14.44	125.12	14.44	125.12	139.56
191	795	1.5	15.48	127.67	15.48	127.67	143.15
191	796	1.5	13.66	125.05	13.66	125.05	138.71
191	794	3	3.84	23.59	3.84	23.59	27.43
191	795	3	5.61	36.7	5.61	36.7	42.31
191	796	3	4.09	23	4.09	23	27.09
191	791	6	1.5	12.59	1.5	12.59	14.09
191	792	6	1.45	17.12	1.45	17.12	18.57
191	793	6	2.04	15.16	2.04	15.16	17.2
191	797	8	0.76	6.03	0.5	6.03	6.53
191	798	8	1.1	8.92	1.1	8.92	10.02
191	799	8	0.97	7.13	0.5	7.13	7.63
191	797	12	1.28	3.17	1.28	3.17	4.45
191	798	12	0.62	4.19	0.5	4.19	4.69
191	799	12	1.66	9.85	1.66	9.85	11.51
191	794	24	0.7	1.67	0.5	1.25	1.75
191	795	24	0.2	0	0.5	0	0.5
191	796	24	0.22	0	0.5	0	0.5

Note: LLOQ of carboxylate is 1.0 ng/mL; LLOQ of lactone is 2.5 ng/mL; if the concentration is larger than 0 and smaller than LLOQ, half of LLOQ value was taken.

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON EFFECT OF CRYSTAL SIZE ON THE ORAL BIOAVAILABILITY OF AR-67 LACTONE IN MICE

(PERFORMED 2/24/2009-2/25/2009)

EFFECT OF CRYSTAL SIZE ON THE ORAL BIOAVAILABILITY OF AR-67 LACTONE IN FEMALE C57BL/6 MICE

Objective

Previous experiments showed that amorphous formulations such as PMFD 576/1 (PVP (30 K)-Tween 80-lactone) and blank AR-67 lactone (prepared by spiking simulated gastric fluid (SGF) with AR-67 carboxylate stock solution) gave higher oral bioavailability compared to supersaturated solutions of AR-67 lactone (e.g. SBE-β-CD or E-TPGS). Since that charge on the carboxylate might reduce absorption by passive diffusion, we hypothesized that conversion of lactone into the carboxylate at the alkaline pH of the small intestine is likely to be rapid from solution formulations and slow from suspensions (amorphous formulations). We speculated that lactone particles in suspension can serve as a drug reservoir due to relatively lower dissolution and conversion rates. Thus particles with optimal combination of dissolution and conversion rates are likely to give enhanced oral bioavailability. To test this hypothesis, we prepared a series of particles of different sizes by serial sieving and tested the bioavailability of suspensions of two different particle size distributions in mice.

Expt 194: Study the absorption of AR-67 lactone (particle size: 45 μm to 75 μm) in SGF (5 mg/kg, 2.5 ml/kg) PO.

Expt 195: Study the absorption of AR-67 lactone (particle size:75 μm to 150 μm) in SGF (5 mg/kg, 2.5 ml/kg) PO.

Method

Formulation

Dr. Xiang prepared AR-67 crystals in 5 different sizes by sieving:

- Size 1: ≥600 μm
- Size 2: 150 μm to 600 μm
- Size 3: 75 μm to 150 μm
- Size 4: 45 μm to 75 μm
- Size 5: ≤ 45 μm

Due to the limited amount of size 5 and the difficulty of making homogenous suspensions with sizes 1 and 2, sizes 3 and 4 were used to make 2 mg/mL suspensions for mouse PK experiments.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 194: mouse #811-819 were treated with AR-67 (particle size: 45 μm-75 μm) suspension in simulated gastric fluid (pH 1.2). The dosing solutions (2 mg/mL) were administered right after mixing.

Expt 195: mouse#751-759 were treated with AR-67 (particle size:75 μm-150 μm) suspension in simulated gastric fluid (pH 1.2), the dosing solution (2 mg/mL) were administered right after mixing.

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h,12 h, and 24 h. Each animal was sampled at 3-4 different time points.

The blood sample of about 50 μL was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma of 25 μL was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 μL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis

Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 μL:40 μL) and were analyzed.

Sequence name:

20AC-022709-mouse plasma PK expt 194 & 195.seq

20AC-030209-mouse plasma PK expt 194 &195.seq

Method:

20AC-022709 mouse plasma curve.met

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

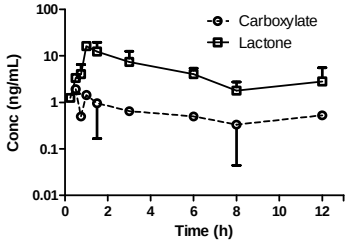
Results

1. Prism file: ..\Prism files\Mouse PK 5 mg PO exps 194 & 195.pzf
2. WinNonlin file: ..\Winnonlin files\Mouse PK Expt 194 & 195.wsp
3. Tables

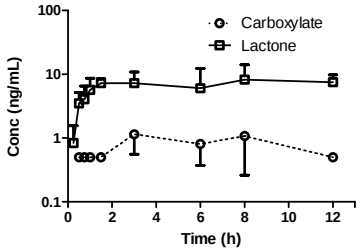
Table 1. Basic PK parameters						
0-12 h						
Expt #194	5 mg/kg AR-67 lactone PO (45 µm -75 µm crystals)					
AUC (ng*h/mL)	Mean	SE	Tmax (h)	Cmax (ng/mL)	Mean	SE
Carb	6.86	1.73	0.50	Carb	1.93	0.22
Lactone	58.68	11.93	1.00	Lactone	16.29	1.41
Total	65.55	13.47	1.00	Total	17.73	1.50

0-12 h						
Expt #195	5 mg/kg AR-67 lactone PO (75 µm -150 µm crystals)					
AUC (ng*h/mL)	Mean	SE	Tmax (h)	Cmax (ng/mL)	Mean	SE
Carb	9.76	2.03	3.00	Carb	1.15	0.34
Lactone	73.91	12.08	8.00	Lactone	8.23	4.16
Total	83.67	14.08	8.00	Total	9.31	4.74

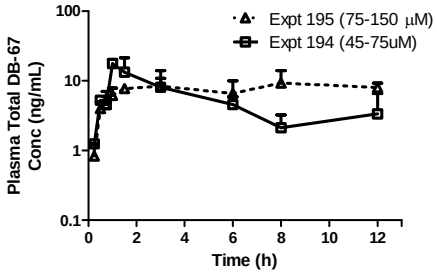
4. Figures



5 mg/kg oral AR-67 (particle size:45 μm-75 μm) in simulated gatric fluid (pH 1.2)



5 mg/kg oral AR-67 (particle size:75 μm-150 μm) in simulated gatric fluid (pH 1.2)



Comparison between 5 mg/kg oral AR-67 (Expt 194, 45 μm-75 μm)
and oral AR-67 (Expt 195, 75 μm-150 μm) in simulated gatric fluid (pH 1.2)

File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\ Mouse PK 5 mg PO exps 194 & 195

Raw Data

Expt	Mouse #	Time (h)	Carb. (ng/mL)	Lact. (ng/mL)	Filter >=1 Carb. (ng/mL)	Filter >=2.5 Lact. (ng/mL)	Filter Total (ng/mL)
194	811	0.25	0	1.04	0	1.25	1.25
194	812	0.25	0	1.05	0	1.25	1.25
194	813	0.25	0	0.82	0	1.25	1.25
194	814	0.5	1.73	3.42	1.73	3.42	5.15
194	815	0.5	2.38	3.74	2.38	3.74	6.12
194	816	0.5	1.69	2.91	1.69	2.91	4.6
194	817	0.75	0.87	5.03	0.5	5.03	5.53
194	818	0.75	0.48	5.91	0.5	5.91	6.41
194	819	0.75	0.23	1.8	0.5	1.25	1.75
194	811	1	1.58	18.94	1.58	18.94	20.52
194	812	1	1.47	15.81	1.47	15.81	17.28
194	813	1	1.27	14.11	1.27	14.11	15.38
194	814	1.5	0.73	8.22	0.5	8.22	8.72
194	815	1.5	0.93	8.19	0.5	8.19	8.69
194	816	1.5	1.87	20.82	1.87	20.82	22.69
194	814	3	0.98	6.83	0.5	6.83	7.33
194	815	3	0	2.66	0	2.66	2.66
194	816	3	1.44	12.91	1.44	12.91	14.35
194	811	6	0.55	4.53	0.5	4.53	5.03
194	812	6	0.49	2.51	0.5	2.51	3.01
194	813	6	0.55	5.15	0.5	5.15	5.65
194	817	8	0	1.45	0	1.25	1.25
194	818	8	0.81	2.88	0.5	2.88	3.38
194	819	8	0.74	1.79	0.5	1.25	1.75
194	817	12	0	0.48	0	1.25	1.25
194	818	12	1.08	5.99	1.08	5.99	7.07
194	819	12	0.52	2.09	0.5	1.25	1.75
194	814	24	0	0.49	0	1.25	1.25
194	815	24	0.18	0.47	0.5	1.25	1.75
194	816	24	0	0.22	0	1.25	1.25
195	821	0.25	0	0.37	0	1.25	1.25
195	822	0.25	0	0.51	0	1.25	1.25
195	823	0.25	0	0	0	0	0
195	824	0.5	0.79	5.44	0.5	5.44	5.94
195	825	0.5	0.53	2.55	0.5	2.55	3.05
195	826	0.5	0.33	2.5	0.5	2.5	3
195	827	0.75	0.2	2.06	0.5	1.25	1.75
195	828	0.75	0.5	5.06	0.5	5.06	5.56
195	829	0.75	0.39	5.87	0.5	5.87	6.37

195	821	1	0.91	8.93	0.5	8.93	9.43
195	822	1	0.6	5.15	0.5	5.15	5.65
195	823	1	0.41	2.96	0.5	2.96	3.46
195	824	1.5	0.59	7.01	0.5	7.01	7.51
195	825	1.5	0.74	8.57	0.5	8.57	9.07
195	826	1.5	0.61	6.2	0.5	6.2	6.7
195	824	3	1.65	10.95	1.65	10.95	12.6
195	825	3	1.29	7.16	1.29	7.16	8.45
195	826	3	0.59	3.67	0.5	3.67	4.17
195	821	6	14.96	13.06	14.96	13.06	28.02
195	822	6	0.33	1.1	0.5	1.25	1.75
195	823	6	1.11	3.82	1.11	3.82	4.93
195	827	8	0.83	4.07	0.5	4.07	4.57
195	828	8	10.16	9.17	10.16	9.17	19.33
195	829	8	1.65	12.39	1.65	12.39	14.04
195	827	12	0.33	8.62	0.5	8.62	9.12
195	828	12	0.62	8.99	0.5	8.99	9.49
195	829	12	0.53	4.98	0.5	4.98	5.48
195	824	24	0.15	0	0.5	0	0.5
195	825	24	0	0	0	0	0
195	826	24	0.63	17.77	0.5	17.77	18.27

Note: LLOQ of carboxylate is 1.0 ng/mL; LLOQ of lactone is 2.5 ng/mL; if the concentration is larger than 0 and smaller than LLOQ, half of LLOQ value was taken.

DEPARTMENT OF PHARMACEUTICAL SCIENCES, COLLEGE OF PHARMACY

UNIVERSITY OF KENTUCKY (LEGGAS LAB)

REPORT ON THE EFFECTS OF TWEEN-80 AND DEGREE OF CRYSTALLIZATION ON BIOAVAILABILITY OF AR-67 LACTONE IN MICE

(PERFORMED 3/4/2009-3/5/2009)

EFFECTS OF TWEEN-80 AND DIFFERENT DEGREE OF CRYSTALLIZATION ON THE BIOAVAILABILITY AR-67 LACTONE IN FEMALE C57BL/6 MICE

Objective

Previous experiments showed that amorphous formulations such as PMFD 576/1 (PVP (30 K)-Tween 80-lactone) and blank AR-67 lactone (prepared by spiking simulated gastric fluid (SGF) with AR-67 carboxylate stock solution) gave higher oral bioavailability compared to supersaturated solutions of AR-67 lactone (e.g. SBE-β-CD or E-TPGS). Since that charge on the carboxylate might reduce absorption by passive diffusion, we hypothesized that conversion of lactone into the carboxylate at the alkaline pH of the small intestine is likely to be rapid from solution formulations and slow from suspensions (amorphous formulations). We speculated that lactone particles in suspension can serve as a drug reservoir due to relatively lower dissolution and conversion rates. Thus particles with optimal combination of dissolution and conversion rates are likely to give enhanced oral bioavailability. To test this hypothesis, we examined the effect of tween-80 and degree of crystallization on bioavailability of AR-67 lactone.

a) SGF (pH 1.2) containing Tween-80 (0.065%) was spiked with carboxylate stock solution to prepare 2 mg/mL dosing suspension and was dosed immediately after mixing.

b) SGF (pH 1.2) containing Tween-80 (0.065%) was spiked with carboxylate stock solution and was dosed 2 hours after mixing (the suspension was incubated at 37oC for 2 hours to allow crystal to grow).

Expt 196: Study the bioavailability of freshly prepared AR-67 lactone in SGF containing 0.065% Tween-80 (5 mg/kg, 2.5 ml/kg) PO.

Expt 197: Study the bioavailability of AR-67 lactone in SGF containing 0.065% Tween-80 (2 hr after preparation) (5 mg/kg, 2.5 ml/kg) PO

Method

Formulation

The dosing suspension with concentration of 2 mg/mL were prepared by spiking SGF containing Tween-80 (0.065%) with AR-67 carboxylate stock solution (20 mg/mL); AR-67 carboxylate stock solution were prepared at 20 mg/ml by Dr. Xiang.

Animal treatment and sample processing

(Refer to \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\sampling time for details)

Expt 196: Mouse# 831-839 were treated with AR-67 lactone suspension in T-80 containing simulated gastric fluid (pH 1.2) right after mixing.

Expt 197: Mouse# 841-849 were treated with AR-67 suspension in T-80 containing simulated gastric fluid (pH 1.2) administered right 2 h after preparation.

Groups of three mice were sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 6 h, 8 h and 12 h, 24h. Each animal was sampled at 3-4 different time points.

The blood sample of about 50 µL was collected from the saphenous vein with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma of 25 µL was collected by centrifugation of blood and then extracted 1:4 (v/v) with cold MeOH (100 µL,-80oC). Samples were kept at -80oC until analysis.

HPLC analysis Before injection into the HPLC, samples were diluted with an equal volume of mobile phase buffer (40 µL:40 µL) and were analyzed.

Sequence name:

20AC-030609-mouse plasma PK expt 196 & 197.seq

20AC-030709-mouse plasma PK expt 196 & 197.seq

Method: 20AC-022709-mouse plasma curve

Data were exported and saved in Leggas Lab/Experimental Results/DB-67 Oral PK / Mouse oral PK folder. Areas under the curve were estimated with Prism v5 and WinNonlin v5.2 (sparse sampling noncompartmental analysis). Plasma concentration versus time plots were made with Prism v5.

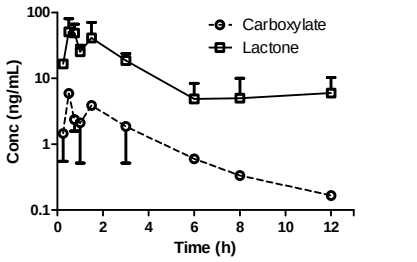
Results

1. Prism file:..\Prism files\Mouse PK 5 mg PO exps 196 & 197.pzf
2. WinNonlin file: ..\Winnonlin files\Mouse PK Expt 196 & 197.wsp
3. Tables

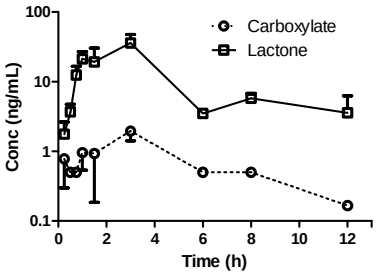
Table 1. Basic pharmacokinetic parameters						
Expt #196	5 mg/kg lactone PO (T-80 + SGF dosed immediately after preparation)					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Cmax (ng/mL)	Mean	SE
Carb	14.11	5.22	0.50	Carb	5.92	3.63
Lactone	160.99	29.71	0.50	Lactone	50.92	16.74
Total	175.10	34.62	0.50	Total	56.84	20.20

Expt #197	5 mg/kg lactone PO (T-80 + SGF dosed 2 hours after preparation)					
AUC (ng·h/mL)	Mean	SE	Tmax (h)	Cmax (ng/mL)	Mean	SE
Carb	9.20	1.12	3.00	Carb	1.95	0.31
Lactone	145.97	20.55	3.00	Lactone	36.01	6.72
Total	155.17	21.63	3.00	Total	37.96	7.04

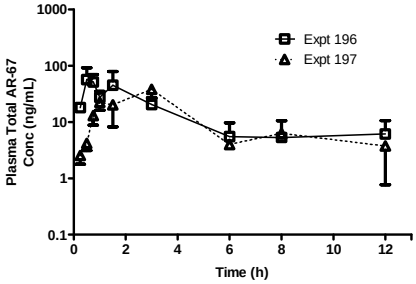
4. Figures



5 mg/kg oral AR-67 lactone suspension in
T-80 containing S.G.F. (pH 1.2) dosed at o h after preparation



5 mg/kg oral AR-67 lactone suspension in T-80 containing
simulated gatric fluid (pH 1.2, dosed at 2 h after preparation)



5 mg/kg oral AR-67 lactone suspensions dosed at o h (solid)
Vs 2 h (dashed) after preparation in T-80 containing SGF (pH 1.2)

File path: \\Pharm\public\PS\Leggas Lab\Experimental Results\DB67 Oral PK\Mouse oral PK\Prism files\ Mouse PK 5 mg PO exps 196 & 197.

Raw Data

Expt	Mouse ID	Time (h)	Carb. (ng/mL)	Lact. (ng/mL)	Filter >=1 Carb. (ng/mL)	Filter >=2.5 Lact. (ng/mL)	Filter Total (ng/mL)
196	831	0.25	0.88	19.3	0.5	19.3	19.8
196	832	0.25	2.3	15.06	2.3	15.06	17.36
196	833	0.25	1.57	15.23	1.57	15.23	16.8
196	834	0.5	13.1	80.35	13.1	80.35	93.45
196	835	0.5	3.29	50.02	3.29	50.02	53.31
196	836	0.5	1.37	22.38	1.37	22.38	23.75
196	837	0.75	1.47	30.82	1.47	30.82	32.29
196	838	0.75	2.95	65.81	2.95	65.81	68.76
196	839	0.75	2.67	50.17	2.67	50.17	52.84
196	831	1	1.03	18.52	1.03	18.52	19.55
196	832	1	3.94	30.91	3.94	30.91	34.85
196	833	1	1.36	26.62	1.36	26.62	27.98
196	834	1.5	8.55	74.54	8.55	74.54	83.09
196	835	1.5	1.16	18.58	1.16	18.58	19.74
196	836	1.5	1.92	30.49	1.92	30.49	32.41
196	834	3	3.4	24.47	3.4	24.47	27.87
196	835	3	1.08	14.14	1.08	14.14	15.22
196	836	3	1.08	17.41	1.08	17.41	18.49
196	831	6	0	2.1	0	1.25	1.25
196	832	6	1.3	8.26	1.3	8.26	9.56
196	833	6	0.31	5.17	0.5	5.17	5.67
196	837	8	0.56	10.73	0.5	10.73	11.23
196	838	8	0.19	2.08	0.5	1.25	1.75
196	839	8	0	2.98	0	2.98	2.98
196	837	12	0	9.76	0	9.76	9.76
196	838	12	0.4	6.96	0.5	6.96	7.46
196	839	12	0	1.82	0	1.25	1.25
196	834	24	0	0.9	0	1.25	1.25
196	835	24	0	0.5	0	1.25	1.25
196	836	24	0	0.35	0	1.25	1.25
197	841	0.25	0.79	1.34	0.5	1.25	1.75
197	842	0.25	0.34	2.78	0.5	2.78	3.28
197	843	0.25	1.33	1.62	1.33	1.25	2.58
197	844	0.5	0.32	3.45	0.5	3.45	3.95
197	845	0.5	0.14	2.74	0.5	2.74	3.24
197	846	0.5	0.28	4.84	0.5	4.84	5.34
197	847	0.75	0.82	16.09	0.5	16.09	16.59
197	848	0.75	0.68	13.47	0.5	13.47	13.97
197	849	0.75	0.53	7.92	0.5	7.92	8.42
197	841	1	0.84	14.82	0.5	14.82	15.32
197	842	1	1.04	25.28	1.04	25.28	26.32

197	843	1	1.33	23.95	1.33	23.95	25.28
197	844	1.5	1.79	32.12	1.79	32.12	33.91
197	845	1.5	0.78	13.38	0.5	13.38	13.88
197	846	1.5	0.75	12.15	0.5	12.15	12.65
197	844	3	2.39	45.39	2.39	45.39	47.78
197	845	3	2.12	39.66	2.12	39.66	41.78
197	846	3	1.34	22.98	1.34	22.98	24.32
197	841	6	0.23	3.47	0.5	3.47	3.97
197	842	6	0.27	3.25	0.5	3.25	3.75
197	843	6	0.25	3.79	0.5	3.79	4.29
197	847	8	0.25	4.91	0.5	4.91	5.41
197	848	8	0.45	6.87	0.5	6.87	7.37
197	849	8	0.44	5.56	0.5	5.56	6.06
197	847	12	0	1.71	0	1.25	1.25
197	848	12	0	2.94	0	2.94	2.94
197	849	12	0.48	6.52	0.5	6.52	7.02
197	844	24	0	0.35	0	1.25	1.25
197	845	24	0	0.69	0	1.25	1.25
197	846	24	0	1.07	0	1.25	1.25

Note: LLOQ of carboxylate is 1.0 ng/mL; LLOQ of lactone is 2.5 ng/mL; if the concentration is larger than 0 and smaller than LLOQ, half of LLOQ value was taken.

VITA

Eyob Debebe Adane

Date and place of birth: August 12, 1978, Ethiopia

EDUCATIONAL INSTITUTIONS ATTENDED

2005-to date	Ph.D (in progress)	University of Kentucky
2001-2003	MS (pharmacology)	Addis Ababa University
1995-2000	B.Pharm.	Addis Ababa University

PROFESSIONAL POSITIONS HELD

Sept 2010- to date Pharmacist, Appalachian Healthcare Regional Hospital, Hazard, KY

2009- Sept 2010 Pharmacist, Chandler Medical Center, University of Kentucky, Lexington, KY

2005-2010 Research assistant, Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY

2003 – 2005 Lecturer in Pharmacology, Faculty of Medicine, Addis Ababa University, Ethiopia

2001 – 2003 Assistant Lecturer in Pharmacology, Faculty of Medicine, Addis Ababa University, Ethiopia

2000 – 2001 Graduate Assistant in Pharmacology, Faculty of Medicine, Addis Ababa University, Ethiopia

PROFESSIONAL PUBLICATIONS

E.D. Adane, Z. Liu, T.X. Xiang, B.D. Anderson, and M. Leggas (2010). Factors Affecting the In Vivo Lactone Stability and Systemic Clearance of the Lipophilic Camptothecin Analogue AR-67. Pharm Res. (2010) 27:1416-1425

E. D. Adane et al. ABC efflux transporter inhibition and oral bioavailability of the lactone and carboxylate forms of AR-67 (manuscript under preparation)

Eyob Debebe, Eyasu Makonnen and Asfaw Debella (2007). Antinociceptive effect of the methanolic extract of roots of Andrachne aspera in three models of nociception. Pharmacologyonline 1: 41-48