Pharmaceutical and Biomedical Aspects of Topoisomerase I Inhibitors

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Pharmaceutical and Biomedical Aspects of Topoisomerase I Inhibitors

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Gelukkig zijn is de meest verwaarloosde plicht

Bertus Aafjes

Aan Jacqueline, Patrick en Sander

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Introduction

Pre-clinical and clinical pharmacokinetics plays an important role in the development of new anticancer agents and in the refinement of already existing therapies. In clinical studies, pharmacokinetic parameters, including area under the plasma concentration-time curve and/or time above a certain threshold concentration, have previously been shown to be related to the pharmacodynamic outcomes, such as myelosuppression or anti-tumor response. In order to obtain reliable pharmacokinetic parameters, analytical methodologies have to be developed and validated, enabling accurate determination of concentrations of anticancer drugs in biological matrices. These methodologies have to be validated in terms of selectivity, precision, accuracy and sensitivity, to obtain meaningful pharmacokinetic results.

During the last decade several analogues of the topoisomerase I inhibitor camptothecin have entered clinical practice. Topoisomerase I is a nuclear enzyme involved in the replication of DNA, by forming a cleavable complex, i.e. the covalent interaction between DNA and the enzyme. The cleavable complex results in a single strand break of the DNA, resulting in relaxation, followed by replication and resealing of the break. The camptothecin topoisomerase I inhibitors reversibly stabilize the cleavable complex, resulting in single-strand DNA breaks and thus termination of DNA replication, subsequently followed by cell death.

The camptothecin analogues share a pH-dependent reversible conversion between their pharmacologically active lactone form, which is able to diffuse across cell membranes, and their inactive ring-opened carboxylate form. The existence of the two forms of the camptothecin analogues requires quick sample handling at the site of the patient in order to acquire real-time pharmacokinetic data. In chapter 1, an overview of methodologies for the determination of camptothecin analogues is described and their applicability for pharmacokinetic analysis is discussed.

In this thesis, methodologies for the quantitative determination of the topoisomerase I inhibitors topotecan, 9-aminocamptothecin and lurtotecan are described. The applicability of these assays is shown in both clinical pharmacokinetic and *in vitro* studies.

Chapter 1

Determination of camptothecin analogues in biological matrices by high-performance liquid chromatography

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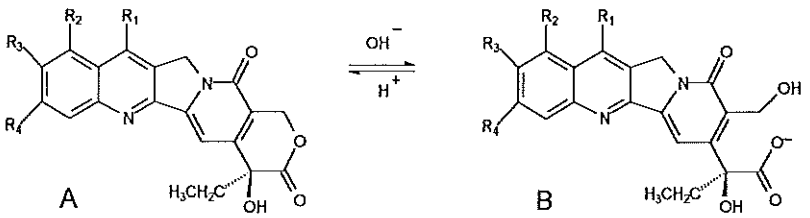
ABSTRACT

Several analogues of the topoisomerase I inhibitor camptothecin (CPT) have been introduced in clinical practice in the last decade. All CPT analogues are sensitive to a pH-dependent reversible conversion between a pharmacologically active lactone form and its inactive, lactone ring opened, carboxylate form. The reversible conversion is also depending on the, sometimes species dependent, protein binding properties of the two forms, resulting in different lactone to carboxylate plasma ratios for the various analogues. Pharmacokinetic analysis of the CPT analogues is helpful in understanding pharmacodynamic outcome of drug treatment, in clinical as well preclinical studies. Measurement of these analogues is habitually complicated by the chemical instability of the lactone moiety and necessitates a rapid centrifugation of the blood sample, preferable at the bedside of the patient, to collect the plasma supernatant. Since the lactone forms of these drugs are able to diffuse across cell membranes, including those of the red blood cells, rapid collection and processing is even necessary in case only the total concentrations of the CPT analogues are to be measured. Sample pretreatment procedures of the CPT analogues topotecan, irinotecan, 9-aminocamptothecin and lurtotecan are summarized and discussed in this review.

INTRODUCTION

The naturally occurring lactone form of camptothecin (CPT; Figure 1) is a poorly water-soluble inhibitor of DNA synthesis, by reversibly stabilizing the cleavable complex between topoisomerase I and DNA. This results in single-strand DNA breaks and thus termination of DNA replication, subsequently followed by cell death [1-4]. Since CPT itself was water-insoluble, the drug was formulated as the water-soluble sodium salt (NSC 100880). Unfortunately, due to this formulation, the delicate balance between the lactone and carboxylate forms was shifted toward the latter at neutral pH. Poor response rates in conjunction with severe toxicities were observed in early clinical trials with this agent, and the sodium salt of CPT was shown to yield only 10% of the activity of CPT against mouse leukemia while no anti-tumor activity was found in xenograft models [1]. During the last decade, various types of more or less water-soluble analogues of CPT, such as topotecan (TPT), irinotecan (CPT-11), 9-aminocamptothecin (9-AC) and lurtotecan (LRT) have been introduced into clinical practice (Figure 1).

CPT and its analogues share a pH dependent reversible conversion between the lactone and carboxylate form (Figure 1), from which the intact lactone form is able to diffuse across cell



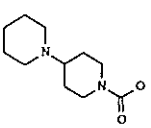
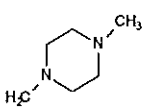
Compound	R_1	R_2	R_3	R_4
Camptothecin	H	H	H	H
Topotecan	H		OH	H
Irinotecan	CH_2CH_3	H		H
SN-38	CH_2CH_3	H	OH	H
9-Aminocamptothecin	H	NH_2	H	H
Lurtotecan		H		

Fig. 1: Chemical structures of the lactone (A) and ring-opened carboxylate (B) forms of camptothecin and analogues in clinical development.

membranes, while the pharmacologically inactive ring-opened carboxylate is trapped into the extra-cellular compartments, i.e. cell growth medium in the case of in vitro experiments and plasma water in in vivo studies [5]. The percentages of CPT analogues present in the lactone form at equilibrium in phosphate buffered saline are in the same order for the different CPT analogues, with values of 17.0 ± 2.0%, 15.3 ± 0.8%, 13.0 ± 2.0%, 15.3 ± 1.7% and 19.0 ± 1.0% for CPT, TPT, CPT-11, SN-38 (active metabolite of CPT-11) and 9-AC respectively. Addition of human serum albumin (HSA) at a concentration of 40 mg/mL, shifts the percentage of lactone at equilibrium for CPT and 9-AC below 2%, while for CPT-11 and SN-38 the percentage increased to respectively 24.0 ± 1.0% and 34.8 ± 1.7%. No change has been observed for topotecan in the presence of HSA, with 17.1 ± 0.4% in the lactone form at equilibrium. This phenomenon is caused by a preferential binding of the carboxylate forms of CPT and 9-AC to HSA, resulting in a shift of the equilibrium towards the carboxylate. In contrast, for TPT, CPT-11 and SN-38, the substituents at the R_1- and R_2-positions (Figure 1) hinder the binding of the carboxylate forms to HSA, and so stabilize the lactone form [6]. No data are available for LRT, from which we expect a stabilized lactone moiety, by the substitution at the R_1-position. However, the binding to serum albumin has been shown to be clearly species-dependent; In the case of 9-AC, which demonstrated high antitumor activity in preclinical mouse xenograft models [7], the lactone moiety is stabilized by murine serum albumin (MSA) but not by HSA, with 35.0 ± 6.2% in the pharmacologically active lactone form in the presence of MSA and only 0.63 ± 0.10 % in the presence of HSA [5]. Pharmacokinetic analyses of the camptothecins are thus important in clinical as well preclinical studies and are complicated by the chemical instability of the lactone moiety.

To ensure adequate measurements of the pharmacologically active lactone forms of the CPT analogues in kinetic studies, blood samples have to be processed directly after sampling at the site of the patient; either by (i) direct analysis of the samples, or by (ii) direct extraction of the lactone form from the plasma or by (iii) stabilizing the lactone to carboxylate ratio. Stabilization of the lactone to carboxylate ratio is preferable since this is the less laborious approach. In general, separations of the topoisomerase I inhibitors and endogenous compounds were performed by reversed-phase high-performance liquid chromatography (HPLC) methods, coupled with fluorescence detection. In this review we summarize the methods for sample treatment and detection of each CPT analogue in biological matrices, and the lower limit of quantitation (LLQ) or lower limit of detection (LLD) for each assay. The LLQ is of great importance for accurate pharmacokinetic analysis and is defined as the lowest concentration of the camptothecin, which can be measured accurate and precise. While the LLD, which is unreliable regarding accuracy and precision, is defined as the lowest detectable concentration that can be distinguished from the background noise [8].

SAMPLE TREATMENT OF CPT ANALOGUES FOR HPLC MEASUREMENTS

Topotecan

Topotecan (TPT, Hycamtin®, SKF 104864, NSC 609699, (S)-9-dimethylaminomethyl-10-hydroxycamptothecin; Figure 1) is a semisynthetic water-soluble CPT analogue, prepared by synthetic modification of 10-hydroxycamptothecin [1]. The intravenous formulation of TPT has been registered for the treatment of ovarian cancer in Europe and the USA [4]. The determination of the lactone, carboxylate and total (i.e., lactone plus carboxylate forms) concentrations of TPT in human plasma have been described in several publications. The plasma sample pretreatment in these published methods is based on a simple methanolic protein precipitation step immediately after the collection of the plasma according to the method of Beijnen et al. [9]. The ratios of the lactone to carboxylate concentrations in the methanolic extracts were found to be stable for at least 4 and 15 months when stored at a minimum of -70°C [10,11].

The first assay, published by Beijnen et al. [9], described the simultaneous determination of the lactone and carboxylate form of TPT with a LLD for both compounds of 0.2 ng/ml. A good base-line separation between the lactone form and endogenous material was achieved, however, in blank plasma samples an interfering peak for the carboxylate form was found. In order to get reliable results the chromatograms were reprocessed with subtraction of each corresponding blank chromatogram.

One of the assays described by Rosing et al. [12] is among the most sensitive with a LLQ of 0.05 ng/ml (Table 1) for the lactone and the total form of TPT. The total concentration of TPT was measured in a second analysis, where the samples were acidified with perchloric acid, which results in the conversion of the carboxylate form in the lactone form, followed by the determination of the lactone form. The amount of the carboxylate form was calculated as the difference between the total and lactone concentration. These authors have also described the impact of column temperature for the assay of topotecan in rat and dog plasma [13]. The sample treatment is based on the same method, while the column must be thermostated at 19-21°C to obtain sufficient baseline separations between peaks of endogenous compounds in rat and dog plasma and of TPT. The LLQs were established at 0.10 ng/ml for the lactone and lactone plus carboxylate concentrations in rat plasma and at 0.20 ng/ml for the concentrations of TPT in dog plasma (Table 1).

The method by Loos et al. [10] describes the simultaneous determination of the lactone and the carboxylate forms of TPT with sufficient separation between chromatographic peaks of

Table 1: HPLC-methods with corresponding LLQ values for the analysis of TPT

Ref	Year	Matrix	Sample Treatment	Detection		LLQ (ng/ml)		
				Ex (nm)	Em (nm)	Carbox	Lactone	Total
12	1995	HP	PP	361	527	-	0.05	-
			PP/AC	361	527	-	-	0.05
13	1996	RP	PP	361	527	-	0.10	-
			PP/AC	361	527	-	-	0.10
		DP	PP	361	527	-	0.20	-
			PP/AC	361	527	-	-	0.20
10	1996	HP	PP	381	525	0.10	0.10	-
		HU	AC	381	525	-	-	10
20	1997	HP	PP	390	520	0.25	0.50	-
				350-470	510-650	0.50	0.75	-
11[a]	1999	HP	PP	380	527	-	0.1	-
			PP/AC	380	527	-	-	0.1
		HU	AC	380	527	-	-	25
		HF[b]	E	380	527	-	-	0.3

Abbreviations: Ex = excitation wavelength, Em = emission wavelength, HP = human plasma, RP = rat plasma, DP= dog plasma, HU = human urine, HF = human feces, PP = protein precipitation, AC = acidification, E = extraction with acetonitrile/ammonium acetate, a = simultaneous determination of N-desmethyltopotecan, b = concentration in µg/g feces

endogenous materials and of the carboxylate and lactone forms of TPT, with the LLQ established at 0.10 ng/ml for both TPT forms (Table 1). In this manuscript, also a method for the determination of total TPT in urine is described. Total TPT, with a LLQ of 10 ng/ml, is measured after acidification of the urine sample with orthophosphoric acid, resulting in the conversion of the carboxylate form into the lactone form.

Warner et al. [14] describe non-validated HPLC methods for the simultaneous determination of the lactone and carboxylate forms of several camptothecin analogues in phosphate buffered saline and for topotecan also in human plasma. The only concentration tested is 2.5 ng/ml for both TPT forms, with a broad peak for the lactone form. Therefore, their application for simultaneous TPT lactone and carboxylate measurements in human plasma is not suitable for pharmacokinetic analysis in clinical trials after low intravenous dosages or oral administrations of TPT where low concentrations of the two forms of topotecan were expected [15-19]. Another publication of the same group [20] described an improved sensitivity of simultaneous determination of the lactone and carboxylate forms of topotecan in human plasma in comparison with already existing methods (Table 1). However, the LLQ for the lactone and carboxylate form were respectively 0.50 and 0.25 ng/ml using a tunable fluorescence detector with excitation and emission wavelengths of 390 nm and 520 nm respectively. Using a filter detector, with an excitation filter of 350 - 470 nm and an emission filter of 510 - 650 nm, the LLQ values were respectively 0.75 and 0.50 ng/ml, which are still much higher than described earlier [10,12].

Recently, an assay has been published for the simultaneous determination of TPT and N-desmethyltopotecan, one of the known metabolites of TPT, in human plasma, urine and feces [11]. For the determination of drug levels in plasma, two assays were developed, one for the determination of the lactone concentration and one for the determination of the total concentrations of TPT and its metabolite. The LLQ for the lactone as well as the total concentration of TPT and N-desmethyltopotecan was established at 0.1 ng/ml. In urine and feces only total levels of TPT and its metabolite were measured. The sample pretreatment for urine samples involved a dilution step in methanol, followed by acidification with phosphoric acid, resulting in LLQ values of 25 and 2.5 ng/ml of TPT and N-desmethyltopotecan, respectively. Fecal samples were homogenized in distilled water, followed by a double extraction with a mixture of acetonitrile and ammonium acetate pH=4. The LLQ for total topotecan in feces was 0.3 µg/g, while the LLQ for N-desmethyltopotecan was established at 0.03 µg/g feces.

Irinotecan

Irinotecan (CPT-11, 7-ethyl-10-{4-(piperidino)-1-piperidino}-carbonyloxycamptothecin; Figure 1) is a semisynthetic water-soluble analogue of CPT, with limited intrinsic cytotoxic activity. In biological systems, CPT-11 is converted by carboxylesterases into its 100 to 1000 fold more active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin; Figure 1). The drug has been marketed in the USA and Europe for the treatment of 5-fluorouracil-refractory (metastatic) colorectal cancer [1,4]. Over the last years, several HPLC methods have been reported for the determination of CPT-11 and its pharmacologically active metabolite SN-38 in plasma. The analysis of these compounds is rather complicated because of the existence of chromatographic peaks of other CPT-11 metabolites and the poor peak-shapes. The peak-shapes were optimized by using the cationic ion-pairing reagent tetrabutylammonium phosphate (TBAP) and analogues, which also enables the simultaneous determination the lactone and carboxylate forms of CPT-11 and SN-38 by increasing the retention times of the carboxylate forms on the analytical columns.

The first assay for the determination of total concentrations of CPT-11 and SN-38 in human plasma was described by Barilero et al. [21], with a LLD for both compounds of 1.0 ng/ml. In the described method, a good separation was achieved between the total drug in the lactone form for both CPT-11 and SN-38 after acidification and solid phase extraction of the plasma sample.

Rivory et al. [22] developed a HPLC method for the simultaneous determination of the lactone and carboxylate forms of CPT-11 and SN-38 in human plasma. The plasma clean up step involved a protein precipitation with a mixture of ice-cold methanol/acetonitrile (1:1, v/v). Adequate separation was achieved and the LLQs were established at respectively 10 and 2 ng/ml for both forms of CPT-11 and SN-38 (Table 2). The addition of mobile phase prior to injection to the protein-free supernatant was found to be an essential step in the assay. Omission of this buffer resulted in a complex of unresolved peaks. Two other metabolites were found under the chromatographic conditions, one of which has been identified as the ß-glucuronide form of SN-38. A second method for the simultaneous determination of the lactone and carboxylate forms of CPT-11 and SN-38 in human plasma has been developed and validated by Herben et al. [23]. The sample pretreatment was based on the same principle as described above, with the LLQs established at 1.0 ng/ml for CPT-11 lactone and carboxylate, and at 0.5 ng/ml for the lactone and carboxylate forms of SN-38 (table 2). A minor disadvantage of the latter published assay is the rather long overall run time of 20 min, which does not allow the analysis of large number of samples, since the protein-free extracts have to be injected directly after the addition of mobile phase.

Sumiyoshi et al. [24] developed a method for the simultaneous determination of total concentrations of CPT-11 and SN-38 in human plasma. The sample clean-up consisted of

precipitation of plasma proteins with methanol. Subsequently, the samples were evaporated and reconstituted in acidified (pH=2) mobile phase. The LLQs were established at 30 ng/ml for CPT-11 and 1 ng/ml for SN-38 (Table 2). The rather high LLQ of CPT-11 is due to the selected excitation and emission wavelengths of 380 nm and 556 nm, respectively, to obtain maximum sensitivity for the determination of the pharmacologically active metabolite SN-38. No other metabolites of CPT-11 than SN-38 were reported in this publication.

A method for the simultaneous determination of the carboxylate and lactone forms of SN-38 has been described by Kaneda et al. [25]. The described method is performed in rat plasma, with the LLQs of 5 ng/ml for both forms of SN-38 (Table 2). The sample preparation consists of a protein precipitation with cold (-80°C) methanol followed by addition of aquous zinc sulphate (10%, w/v), followed by centrifugation and direct injection into the HPLC-system.

The most sensitive assays available thusfar for the simultaneous determination of lactone and total levels of CPT-11 and SN-38 have been developed and validated by De Bruijn et al. [26], with a LLQ for the lactone of 0.5 ng/ml for CPT-11 and SN-38 (Table 2). The plasma sample clean-up for the lactone measurement consisted of a single liquid-liquid extraction technique with acetonitrile/n-butylchloride (1:4, v/v). The measurement for the determination of the total forms was carried out in a second analysis with LLQs of 2.0 ng/ml for both compounds (Table 2). The plasma samples were acidified and deproteinized with a mixture of perchloric acid and methanol, which resulted in the conversion of the carboxylate forms into the lactone forms, followed by determination of the lactone form. Six other peaks were found in the plasma samples of patients in the assay for the determination of the total forms of CPT-11 and SN-38. Two of them disappeared after incubation of a plasma sample with ß-glucuronidase, while the concentration of SN-38 increased, which is indicative for the presence of a ß-glucuronide conjugate (SN-38G) of SN-38 in plasma samples of cancer patients treated with CPT-11. This method was subsequently modified to allow analysis of other metabolites in plasma, urine and feces samples as well [27]. Two metabolites of CPT-11 were analyzed and validated in human plasma, known as SN-38G and 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]-carbonyloxy-camptothecin (also referred to as APC). The described method for the determination of both metabolites in human plasma was based on the assay described by De Bruijn et al. [26] with a slightly modified mobile phase and the plasma extract was necessarily diluted 2-fold with mobile phase prior to chromatography, because of unusual chromatographic behavior of compounds APC and SN-38G. The LLQ was established at 10 ng/ml APC and SN-38G. The change in mobile phase as compared to the earlier described method, resulted in poor accuracy and precision for CPT-11 and SN-38, due to severe tailing bands, particularly below 100 ng/ml. CPT-11 and SN-38 measurements in plasma samples were carried out by re-injection of the plasma supernatant using the earlier described method [26]. Urine and homogenized fecal samples were diluted (1:1, v/v) in blank plasma and further processed as

Table 2: HPLC-methods with corresponding LLQ values for the analysis of CPT-11/SN-38

Ref	Year	Matrix	Sample Treatment	Detection Ex (nm)	Detection Em (nm)	LLQ CPT-11 (ng/ml) Carbox	LLQ CPT-11 (ng/ml) Lactone	LLQ CPT-11 (ng/ml) Total
22	1994	HP	PP	355	515	10	10	-
24	1995	HP	PP/AC	380	556	-	-	30
25	1997	RP	PP	380	540	-	-	-
26	1997	HP	LL	355	515	-	0.5	-
			PP/AC	355	515	-	-	2.0
23	1998	HP	PP	375/385*	460/525*	1.0	1.0	-
27	1998	HU/HF	PP/AC	355	515	-	-	200
29	1998	HP	AC/SP	380	532	-	-	-
30	1999	HP	PP/AC/LL	380	556	-	-	-
31	1998	DP/RP	PP	362/375*	425/560*	4.8	5.9	-
32	1999	RP	AC/SP	373/380*	420/540*	-	-	5
33	1999	HS	PP	MS	-	-	-	10
			LL	MS	-	-	-	-

LLQ SN-38 (ng/ml)			Metabolites
Carbox	Lactone	Total	(No.)
2	2	-	1
-	-	1	-
5	5	-	-
-	0.5	-	-
-	-	2.0	1
0.5	0.5	-	-
-	-	100	2
-	-	0.004	-
-	-	0.005	-
1.6	2.4	-	-
-	-	5	1
-	-	-	-
-	-	0.5	-

Abbreviations: Ex = excitation wavelength, Em = emission wavelength, HP = human plasma, RP = rat plasma, DP= dog plasma, HU = human urine, HF = human feces, HS= human serum, PP = protein precipitation, AC = acidification, SP = solid phase extraction, LL = Liquid-liquid extraction, Metabolites = number of identified metabolites, others than SN-38, * = setting for CPT-11/SN-38 respectively, MS = detection using mass spectrometry.

described for human plasma samples, followed by a 10-fold dilution in mobile phase. The LLQs were established at 100 ng/ml for SN-38 and SN-38G and at 200 ng/ml for CPT-11 and APC (Table 2). The method was also validated for a second major oxidative metabolite of CPT-11, viz. NPC, in human plasma samples with similar validation characteristics [28].

Since the terminal disposition half-life of SN-38 in cancer patients treated with CPT-11 could not be estimated accurately in early pharmacokinetic studies, an assay for the determination of SN-38 at lower concentrations was needed. The first very sensitive assay was reported by Rivory et al. [29]. The plasma sample was acidified prior to solid-phase extraction and the LLQ for the total concentration of SN-38 was established at 10 pM (~4 pg/ml) (Table 2). However, the recovery of SN-38 was concentration dependent and ranged from 48 up to 92% and therefore log-log calibration curves with least-square linear regression were required. CPT-11 did not interfere with the assay. Compared to this method for the determination of SN-38 at low concentrations, a simplified method with comparable sensitivity has been described recently [30]. The method described by Rivory et al. consisted of a time consuming solid-phase extraction and showed concentration dependent recoveries. In the assay described by de Bruijn et al., protein precipitation followed by a one-step solvent extraction with chloroform, was used for sample clean up. The LLQ was established at 5 pg/ml (Table 2), with standard curves being linear over nearly three orders of magnitude. The use of acetonitrile as organic modifier in the mobile phase in stead of methanol, resulted in sharpening of the peaks and improved peak symmetry. No interference of CPT-11 was observed in the analytical runs.

Chollet et al. described a method for the simultaneous determination of the lactone and carboxylate forms of CPT-11 and SN-38 in rat and dog plasma [31]. This is yet another method, in which the lactone and carboxylate forms could be determined simultaneously, after cold methanol (-20°C) protein precipitation. The LLQs in dog and rat plasma were similar and were validated at 4.8 and 5.9 ng/ml for the carboxylate and lactone forms of CPT-11 respectively, and at 1.6 and 2.4 ng/ml for the carboxylate and lactone form of SN-38 respectively (Table 2).

Kurita et al. [32] have also described a method for the determination of total levels of CPT-11 and its metabolites SN-38 and SN-38G in rat plasma. The LLQs were established at 5 ng/ml for CPT-11 and SN-38 in rat plasma (Table 2). The method described determination of the compounds with a fully automated on-line solid-phase extraction system, which may have potential advantage for processing large numbers of samples simultaneously.

Recently, a non-fluorescence HPLC-method has been developed [33], using electrospray mass spectrometry, for the detection of CPT-11 and SN-38 concentrations in human serum. The sample clean up for the measurement for CPT-11 involved a protein precipitation with a LLQ of 10 ng/ml, while the LLQ for SN-38 was validated at 0.5 ng/ml after a liquid-liquid extraction (Table 2). The use of a mass spectrometer as detector, does not increase the sensitivities of the

determinations of CPT-11 and SN-38 compared to previously reported methods, using an ordinary fluorescence detector.

9-aminocamptothecin

9-aminocamptothecin (9-AC, NSC 603071; Figure 1) was the first synthetic analogue of CPT with promising antitumor efficacy in *in vivo* models. However, 9-AC was inappropriate for further clinical development, due to its poor water solubility. Eventually, the solubility problems were solved by the development of a colloidal dispersion formulation, and 9-AC has since been implemented in numerous clinical trials with the drug given either by bolus or prolonged continuous-intravenous infusion schemes or orally [1,4,34].

Up to now, 4 analytical methods have been published for the determination of the lactone and the lactone plus carboxylate form of 9-AC in human plasma. In the presence of HSA, the lactone form of 9-AC is rapidly converted to the carboxylate form with the equilibrium at the site of the carboxylate, which also necessitates a rapid processing of the blood samples for pharmacokinetic studies. In all methods, the blood sample was centrifuged directly, although the plasma was processed using totally different methods. The method published by Supko et al. [35] for the measurement of the intact lactone of 9-AC requires a rapid deproteinization of the plasma sample with methanol, followed by direct injection of diluted supernatant into the HPLC-system. For the measurement of the total drug concentration, the plasma sample was acidified using perchloric acid, followed by methanolic deproteinization. To increase the sensitivity of the assays, an inline postcolumn acidification of the eluent to pH 1.8-2.2 was necessary, which results in a LLQ of 5.0 ng/ml for the lactone and total concentration of 9-AC (Table 3).

A more sensitive assay was developed by Takimoto et al. [36] with a LLQ of 0.09 and 0.9 ng/ml (Table 3) for the lactone and total concentrations of 9-AC, respectively. Using a solid phase extraction for the determination of the lactone form immediately after collecting the plasma, which separated the lactone from the carboxylate, the sample could be stored for at least two months at -70°C prior to analysis. For the measurement of total 9-AC concentrations, the plasma samples were acidified prior to solid phase extraction by a 10-fold dilution with phosphoric acid (Table 3).

Another, more convenient and sensitive assay was developed later and does not require a direct sample clean-up step [37]. The lactone to carboxylate ratio of 9-AC was stabilized, for at least 4 months, by immediate freezing of the plasma samples at the site of the patient. After thawing the samples, the lactone form was extracted into an organic phase using liquid-liquid extraction, with a mixture of acetonitrile/n-butylchloride (1:4, v/v), while the carboxylate form remains in the water-phase. For the determination of the total 9-AC concentrations, the sample

clean-up consists of a simultaneous protein precipitation/ acidification step with a mixture of methanol and perchloric acid. The LLQs were established at, respectively, 0.05 and 0.10 ng/ml for the lactone and lactone plus carboxylate forms (Table 3).

Table 3: HPLC-methods with corresponding LLQ values for the analysis of 9-AC

Ref	Year	Matrix	Sample Treatment	Detection Ex (nm)	Detection Em (nm)	LLQ (ng/ml) Lactone	LLQ (ng/ml) Total
35	1992	HP	PP	352	418	5.0	-
			AC/PP	352	418	-	5.0
36	1994	HP	SP	365	440	0.09	-
			AC/SP	365	440	-	0.9
37	1997	HP	LL	370	450	0.05	-
			PP/AC	370	450	-	0.10
38	1998	HP	PP/SP	370	450	0.2	-
			PP/AC	370	450	-	0.2

Abbreviations: Ex = excitation wavelength, Em = emission wavelength, HP = human plasma, PP = protein precipitation, AC = acidification, SP = solid phase extraction, LL = liquid-liquid extraction

A sample clean-up procedure involving a direct deproteinization of the plasma at the site of the patient with cold methanol has been reported recently [38]. For the determination of the lactone form, the methanolic extract should be further processed within 48 h after sampling, using a solid phase extraction procedure, while for the determination of total drug levels the methanolic extract was acidified prior to injection into the HPLC-system (Table 3). The LLQs

for the lactone and total concentrations of 9-AC in human plasma were established at 0.2 ng/ml for both the lactone as well the total drug levels.

Lurtotecan

Lurtotecan, (LRT, GI147211, 7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin; Figure 1) is also a semisynthetic analogue of CPT and has recently been formulated as a liposomal preparation with the intent to stabilize the lactone moiety of the compound, and so improve the efficacy of LRT [39].

Only two methods were validated and published for the determination of the drug after administration of non-liposomal LRT; Stafford et al.[40] published the first of these, and describe the quantitation of the lactone and carboxylate forms of LRT in dog plasma using solid-phase extraction techniques. For the lactone-only determination the plasma was diluted with a buffer of pH 7.4 and applied on a solid phase cartridge, followed by a wash step which removes the carboxylate, while the lactone form remains at the cartridge and was eluted and concentrated before injection into the HPLC-system. For measurement of the total concentration of LRT, the plasma was acidified with hydrochloric acid before solid phase extraction. The excitation and emission wavelengths were set at 378 and 420 nm, respectively. The LLQ was established at 0.05 and 0.10 ng/ml for the lactone and total plasma concentrations, respectively, which was the most sensitive determination of any camptothecin analogue reported at that time.

The second method was developed and published by Selinger et al. [41], in which only a method for the determination of the lactone form of LRT is described, using human whole blood as matrix. The advantage of using whole blood instead of plasma, is the rapid and simple sample handling at the site of the patient. After drawing the blood sample, it can be kept for a maximum of 30 min in an ice-water bath, before freezing at -70°C. On the day of analysis the blood sample is further processed using a liquid-liquid extraction, with a mixture of acetonitrile/n-butylchloride (1:4, v/v), for the measurement of the lactone form. The LLQ has been validated at 0.15 ng/ml, using fluorescence detection as described above.

A method for the determination of total LRT levels in human plasma and urine after administration of NX211, i.e. liposomal LRT, has been recently developed and validated [42]. The sample clean up for the determination of total drug levels in plasma involved a deproteinization with 10% (w/v) aqueous perchloric acid-acetonitrile (2:1, v/v), while for the determination of the unchanged drug in urine a single solvent extraction with n-butanol-diethyl ether (3:4, v/v) was accomplished after acidification of the urine sample. Fluorescence detection in both assays was performed with excitation and emission wavelengths of respectively 378 and 420 nm. The LLQ in plasma was established at 1.0 ng/ml, which is sufficient for pharmacokinetic analysis of patient samples in an ongoing phase I trial. The fluorescence signal

of LRT in the urine assay was increased 14-fold prior to detection by post-column exposure of the eluent to UV-light, resulting in an LLQ of 0.50 ng/ml in the human urine samples.

CONCLUSIONS AND PERSPECTIVES

Camptothecins form a class of antineoplastic agents demonstrating significant antitumor activity against a broad range of human malignancies, including refractory ovarian and colorectal cancers. In recent years, a substantial amount of publications has yielded valuable insights into mechanisms of action and resistance, clinical pharmacodynamics and considerations of dosage and schedule, and route of drug administration. Many of these studies have been made possible by the development of selective analytical methodologies to specifically monitor the parent drugs and individual biotransformation products, with sufficient sensitivity to detect the compounds at levels achieved after therapeutic dosing.

The pH-dependent instability of the lactone moiety in the core structure of the camptothecins necessitates a rapid centrifugation of the blood sample, preferably at the site of the patient, to collect the plasma supernatant. Even when only total concentrations of the camptothecins are to be measured, this rapid collection of the plasma is necessary since the lactone forms of these drugs are able to diffuse across cell membranes, including those of the red blood cells, and thus a change in the lactone to carboxylate ratio has an effect on the total drug concentrations in the plasma compartment. To ensure adequate measurements of the lactone concentrations, the plasma samples have to be further processed immediately after centrifugation.

The most laborious methods for the determination of the lactone-only concentrations are those in which each individual plasma sample has to be analyzed or extracted directly after collection of the plasma. Clearly, the most convenient approach at the site of the patient is the one in which the lactone to carboxylate ratio is stabilized by direct freezing of the plasma or whole blood samples. For the lactone only measurements, the samples were further processed using either solid-phase or liquid-liquid extraction techniques. In both cases, only the lactone form is extracted, while the carboxylate form is eluted during the wash steps in the case of the solid-phase extractions, or remains in the water-phase in case of the liquid-liquid extractions. The total concentrations of the camptothecins in the directly frozen plasma samples were analyzed after acidification of the samples followed by solid-phase extractions of the total amount of the drugs in the lactone form, or by measurements of the compound in the lactone form after injection of supernatants of deproteinized and acidified samples. Another practically convenient approach to stabilize the lactone to carboxylate ratio is by methanolic deproteinization of plasma samples directly at the site of the patient. The methanolic extracts

should be stored upon analysis at a minimum of –70°C to prevent degradation of the lactone form. The advantage of this stabilization is the possibility of simultaneous measurement of the lactone and carboxylate forms of the camptothecins in one single run. However, this approach is not feasible for all CPT analogues, except for TPT and CPT-11, since the separation between the hydrophilic carboxylate forms and endogenous compounds (with similar fluorescence characteristics) in the reversed-phase HPLC methods are not sufficient enough for adequate determination of the carboxylate forms. Moreover, the overall run times have to be as short as possible to enable determination of complete runs of patient samples during day time, since the lactone to carboxylate ratio is not stable at 4°C [10], making automated injections overnight infeasible. For methods in which insufficient separation between the carboxylate form and endogenous compounds was achieved, the methanolic extracts were acidified and the total concentrations of the drugs were measured in a second analysis. Since the camptothecins have strong fluorescence characteristics, relatively low concentrations of these compounds could be measured in biological matrices, even after simple protein-precipitation extraction procedures without the need of any concentration step.

The new dimension in chemotherapy provided by TPT, CPT-11 and other analogues in the treatment of a variety of (solid) tumors assures growth in the area of camptothecin-related chemotherapeutic drugs. In general, with the continued application of clinical pharmacokinetic studies, coupled with new approaches in camptothecin drug design and formulation, more rational and selective chemotherapy should be possible in the future.

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Chapter 2

Topotecan

Chapter 2a

Sensitive high-performance liquid chromatographic fluorescence assay for the quantitation of topotecan (SKF 104864-A) and its lactone ring-opened product (hydroxy acid) in human plasma and urine

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ABSTRACT

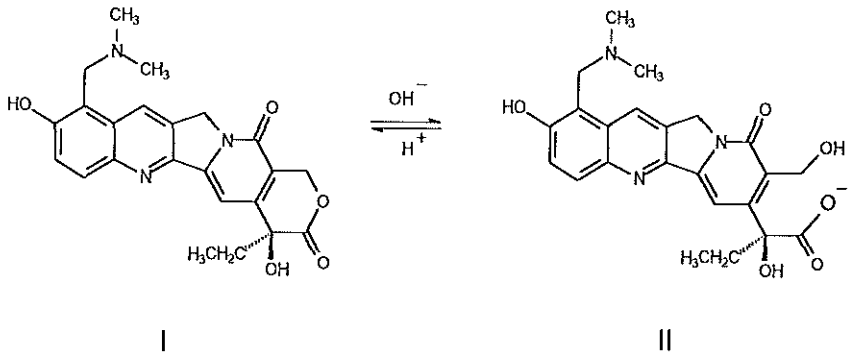
A sensitive reversed-phase high-performance liquid chromatographic fluorescence method is described for the simultaneous determination of topotecan (I) and the hydrolyzed lactone ring-opened product hydroxy-acid (II) in plasma and for the determination of I in urine. To 250 μl of plasma a volume of 750 μl of cold methanol was added to stabilize the pH dependent conversion of I into II. In plasma the lower limit of quantitation for both compounds was 0.10 ng/ml. The between-day variation for I at the LLQ was 7.1% and for II 5.5%. Prior to injection, urine samples were acidified with ortho-phosphoric acid and diluted with phosphate buffered saline (PBS). In urine the calibration curve was linear in the range of 10 to 250 ng/ml and the lower limit of quantitation was 10 ng/ml. The assay was developed to enable pharmacologic analysis of I in ongoing phase I and II studies in patients with solid tumors.

INTRODUCTION

Compound I [(S)-9-dimethylaminomethyl-10-hydroxy-camptothecin, SKF 104864-A] is a semisynthetic water-soluble analogue of camptothecin presently evaluated in clinical phase I and II trials. I is an inhibitor of the nuclear enzyme topoisomerase I. It stabilizes the cleavable complex between DNA and topoisomerase I, resulting in single-strand breaks of the DNA and finally cell death. Antitumor activity has been demonstrated in preclinical models and in phase I and II studies [1-6]. The results of preclinical and clinical studies indicate enhanced antineoplastic activity of I when administered daily for prolonged periods of time [2, 5-9].

Compound I is not stable at physiological pH in an aqueous solution. It is reversibly hydrolyzed from the closed-ring lactone (I) to an open-ring (II) in aqueous solution (figure 1). Compound II is not pharmacologically active [10,11].

An HPLC-assay for the analysis of I and II in human plasma has previously been developed by Beijnen et al. [11] with a lower limit of quantitation, for both compounds, of 1 ng/ml. The present methodology was developed because blank plasma samples revealed an interfering peak at almost the same retention time as II. In addition, the plasma concentrations in our clinical study, where I is administered orally for prolonged periods of time, were anticipated to be much lower than the LLQ of the previously developed methodology. Furthermore, an assay for I in urine was developed to determine the magnitude of the renal clearance of I in studies after i.v. administration.



I II

Fig. 1: pH-dependent interconversion of I and II.

EXPERIMENTAL

Chemicals and reagents

Compound I was obtained from Smith Kline Beecham Pharmaceuticals (King of Prussia, PA, USA). Methanol (HPLC-grade) was obtained from Rathburn and supplied by Brunschwig (Amsterdam, Netherlands). Triethylamine, potassium dihydrogenphoshate, sodium hydroxide and acetic acid (all analytical grade) were obtained from Baker (Deventer, Netherlands). Ortho-phosphoric acid (analytical grade) was obtained from Merck (Amsterdam, Netherlands). Phosphate buffered saline (PBS) was obtained from Oxoid and supplied by Boom (Meppel, Netherlands). PBS consisted of sodium chloride (8.0 g/l), potassium chloride (0.2 g/l), disodium hydrogenphospate (1.15 g/l) and potassium dihydrogenphosphate (0.2 g/l) and was supplied in tablets. One tablet was dissolved in 100 ml purified water. The water was purified with a Milli-Q-UF system (Millipore, Etten-Leur, Netherlands).

A stock solution of 1.0 mg/ml I was made by dissolving 50.0 mg I in 50.00 ml purified water. To 5.00 ml of the stock solution in water 45.00 ml of a 0.10% acetic acid solution were added. This solution contained 0.10 mg/ml of I. To another 5.00 ml of the stock solution 45.00 ml of a 0.10 N sodium hydroxide solution were added. This solution contained 0.10 mg/ml of II.

Chromatographic system

The HPLC-system consisted of a constaMetric 4100 pump (Thermo Separations), a Rheodyne 7125 injection port and a fluoriMonitor 4100 fluorescence detector (LDC Analytical). The data were

analyzed by the Chrom-Card data analysis system (Fisons). These apparatus were delivered by Interscience (Breda, Netherlands). The separation was achieved on a Shandon Hypersil BDS C18 column (100 mm * 3 mm ID, 3μm particle size), delivered by LC-service (Emmen, Netherlands). A model SpH 99 column oven, delivered by Spark Holland (Meppel, Netherlands), was set at 35°C for the assay in plasma and at 60°C for the assay in urine. The excitation wave length was set at 381 nm and the emission wave length at 525 nm.

For the assay in plasma the mobile phase consisted of a 10 mM potassium dihydrogenphosphate (filtered through a 0.45 HA Millipore filter, Millipore, Etten-Leur, Netherlands) with 25% methanol and 0.2% triethylamine. The pH was adjusted to pH 6.0 by addition of ortho-phosphoric acid. The mobile phase was degassed by ultrasonic and helium. The flow-rate was set at 0.70 ml/min.

For the assay in urine the mobile phase consisted of 20% methanol instead of 25%. The flow-rate was set at 1.00 ml/min.

Sample preparation and calibration curves in plasma

Immediately after preparing the standards 250 μl plasma were added to 750 μl of cold methanol of -20°C, according to the method of Beijnen et al. [11]. After mixing on a whirl mixer for 10 seconds the samples were centrifuged for 5 min at 4000g at 4°C and stored at -80°C. Plasma samples of patients were stored after mixing at -80°C and centrifuged on the day of analysis. Prior to analysis, 250 μl of the supernatant were added to 750 μl of PBS and mixed on a whirl mixer for 10 seconds. A volume of 200 μl was injected into the HPLC.

For the validation of the assay in plasma a nine-points calibration curve was processed in duplicate (table 1) and analyzed on 3 occasions. For the determination of the lower limit of quantitation (LLQ), 6 plasma samples of 6 independent individuals were taken and spiked with a concentration of 0.10 ng/ml of both compounds. Also 4 pools of quality control (QC) samples were prepared. Plasma pools

Table 1: Preparation of the calibration curves in plasma.

	Final concentration of I and II (ng/ml)								
	5.00	3.00	2.00	1.00	0.50	0.30	0.20	0.15	0.10
Plasma added (μl)	500	700	800	1800	500	700	800	850	900
10.0 ng/ml I and II added (μl)	500	300	200	200					
1.00 ng/ml I and II added (μl)					500	300	200	150	100

The solutions of 0.10 mg/ml I and 0.10 mg/ml II were separately diluted 100-fold with PBS and again 10-fold with plasma. A 200 μl volume of both solutions was added to 1600 μl of plasma. This solution contained 10.0 ng/ml I and II.

were spiked with 0.50, 2.00, 4.00 and 20.00 ng/ml for both compounds. The QC-sample of 20.00 ng/ml was used for dilution. Each run the QC-samples were analyzed 5 times.

The recovery of I and II was determined at concentrations of 2.00 and 4.00 ng/ml in plasma. The peak heights of 5 analyzed plasma samples were compared with the peak heights of 2 spiked concentrations of 2.00 and 4.00 ng/ml in PBS.

Calibration curves were made by linear regression analysis of peak heights versus concentration. For the concentration accepted as the LLQ, the %DEV of at least 80% of the samples assayed should be $\leq$ 20%. The average within- and between-run precision (%CV) for each concentration, excluding the LLQ, should be $\leq$ 15% and should be $\leq$ 20% for the LLQ. The average accuracy (%) for each concentration, including the LLQ should be within 85-115%.

Sample preparation and calibration curve in urine

In urine the total concentration of I was measured after conversion of II into I. To 250 µl urine 250 µl of 100-fold diluted pure ortho-phosphoric acid were added. After mixing on a whirl mixer for 10 seconds the mixture was incubated for at least 10 min at room temperature. Prior to injection 50 µl of this mixture were added to 950 µl of PBS and mixed on a whirl mixer for 10 seconds. A volume of 20 µl was injected into the HPLC.

Table 2: Preparation of the calibration curve in urine.

	Final concentration of I (ng/ml)						
	250	200	150	100	50	25	25
Urine added (µl)	750	800	850	900	500	500	900
1000 ng/ml I added (µl)	250	200	150	100			
100 ng/ml I added (µl)					500		100
50 ng/ml I added (µl)						500	

A solution containing 0.10 mg/ml of I was diluted 100-fold in urine. This results in a 1000 ng/ml solution of I.

For the validation of the assay in urine a seven-points calibration curve was prepared (table 2). The calibration curves were made in duplicate and analyzed on 3 occasions. For the determination of the LLQ, 10 urine samples of 10 independent individuals were taken and spiked with a concentration of 10 ng/ml of I. Pools of QC-samples were spiked with concentrations of 25, 100, 200 and 1000 ng/ml

I. The QC-sample of 1000 ng/ml was used for dilution. In each run the QC-samples were processed 5 times.

The recovery of I was determined at concentrations of 100 and 200 ng/ml in urine. The procedure is the same as the procedure described for plasma.

Calibration curves were constructed by linear regression analysis of peak heights versus concentration. The same acceptance criteria were applied as described for plasma samples.

Stability of I and II in plasma and urine

The stability of I and II was tested in plasma-extracts and in urine at different temperatures. In plasma the stability of both compounds was tested by incubating plasma-extracts with only I or only II for 24 h at room temperature (22°C), 4°C and -20°C. The stability at -80°C, the storage temperature for patient samples, was tested with methanolic plasma mixtures containing both compounds.

In urine the stability of I was tested by incubation of urine with I for 24 h at 4°C, 22°C, 37°C and in 1-fold with ortho-phosphoric-acid (1:100) diluted urine at 22°C. Also the stability of I in urine was tested at -80°C.

Human experiments

In an oral phase I study the starting dose was 0.15 mg/m². On day 1 and day 8 blood samples were collected up to 12 h. One of the first patients was treated with a dose of 0.4 mg. Immediately after sampling, the blood was centrifuged for 5 min at 3500g and the plasma was treated as outlined.

In another study where I is administered intravenously at a low daily dose of 0.5 mg/m² also urine samples were collected.

RESULTS

Assay in plasma

The calibration curves of I and II in plasma were linear in the range of 0.10 to 5.00 ng/ml with correlation coefficients of at least 0.9986. The retention time of II is 2.5 min and of I 6.5 min (figure 2). No significant interfering peaks were found in 6 independent blank plasma samples. The LLQ for both compounds in plasma was 0.10 ng/ml. The mean recovery in plasma of I was 99.3% and of II 100.6%. The within-run precision of the LLQ-samples of I was 4.4% and of II 9.7%. The accuracy was 93.2% and 106.6% respectively. The between-run precision of the LLQ was calculated with the

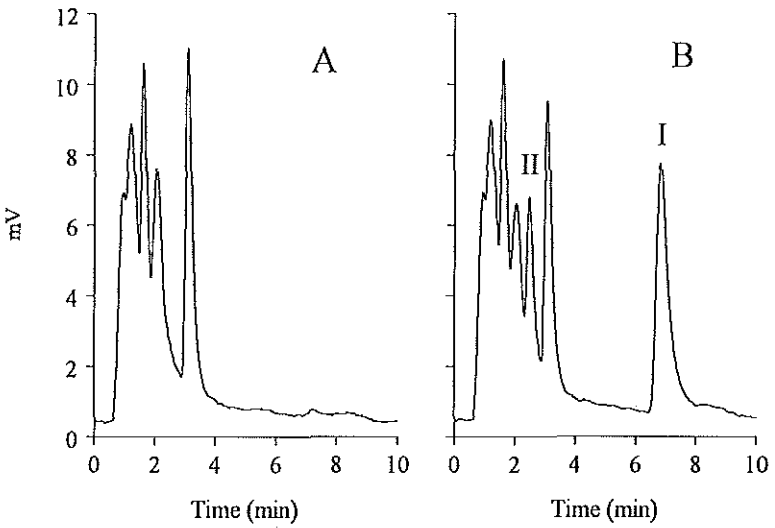


Fig. 2: Chromatograms of a blank blood sample (A) and of a blood sample containing 0.78 ng/ml of I and 0.24 ng/ml of II (B).

Table 3: The average (av.) accuracy, the average within-run precision and the between-run precision of the QC-samples in plasma of I and II

QC-sample (ng/ml)	av. accuracy (%)		precision (%)			
			av. within-run		between-run	
	I	II	I	II	I	II
0.50	102.7	101.3	2.8	1.5	6.9	6.3
2.00	108.4	106.5	3.8	1.3	8.6	5.2
4.00	102.8	102.6	3.1	1.0	5.0	5.5
20.00	103.3	102.3	3.9	3.7	3.0	3.5

lowest concentration of the individual calibration curves used by the validation of the assay. The between-run precisions were respectively 7.1% and 5.5%. The values of the average accuracy, the average within-run precision and the between-run precision of the QC-samples are given in table 3.

Assay in urine

The calibration curves of I in urine were linear in the range of 10 to 250 ng/ml with correlation coefficients of at least 0.9984. Also for the assay in urine no significant interfering peaks for I were found. The LLQ was established at 10 ng/ml (concentrations in the clinical studies were not expected to be lower than 10 ng/ml). The mean recovery of I in urine was 101.9%. The within-run precision of the LLQ-samples was 7.2%. The between-run precision of the LLQ was 5.4%. The accuracy of the LLQ was 97.6%. The values of the average accuracy, the average within-run precision and the between-run precision of the QC-samples are given in table 4.

Table 4: The average (av.) accuracy, the average within-run precision and the between-run precision of the QC-samples in urine of I.

QC-sample (ng/ml)	av. accuracy (%)	precision (%)	
		av. within-run	between-run
25	99.7	4.4	3.3
100	97.5	4.1	0.6
200	97.8	5.3	1.8
1000	98.9	3.2	2.0

Stability of I and II in plasma and I in urine

The reversible hydrolysis of I in plasma-extracts is dependent of the temperature. I and II were found to be unstable at 4°C and 22°C. There was no hydrolyses at -20°C (figure 3). I and II were stable in methanolic plasma mixtures for at least 4 months at -80°C.

The stability of I in urine was also dependent of the temperature. At 37°C I was found to be unstable, at 22°C it was moderately stable and at 4°C I was stable for 24 h. I was stable at 22°C after dilution with ortho-phosphoric acid (figure 4). At -80°C I was stable for more than 3 months.

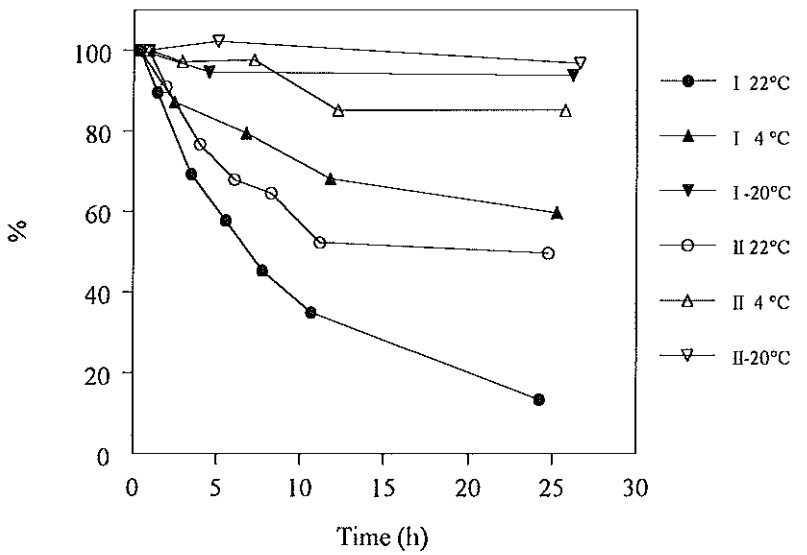


Fig. 3: Stability of I and II in plasma extracts at different temperatures.

Human experiment

The plasma concentration-time curves of I and II of the patient treated with 0.4 mg I are given in figure 5.

The concentration of I in the urine samples of patients who where treated in the intravenous protocol were all > 10 ng/ml (data not shown).

DISCUSSION

The described methodology for the assay in plasma with an LLQ of 0.10 ng/ml for I and II is appropriate for the measurement of plasma samples in ongoing clinical studies where low daily doses are administered. For the assay in urine the LLQ of 10 ng/ml was also satisfactory. Both compounds were unstable in plasma-extracts at 4°C and 22°C. In urine, I was found to be unstable at 37°C and

moderately stable at 22°C. Methanolic plasma mixtures and urine stored at -80°C were found to be stable for respectively at least 4 and 3 months.

In urine only I was measured after acidification to ensure total conversion of II into I.

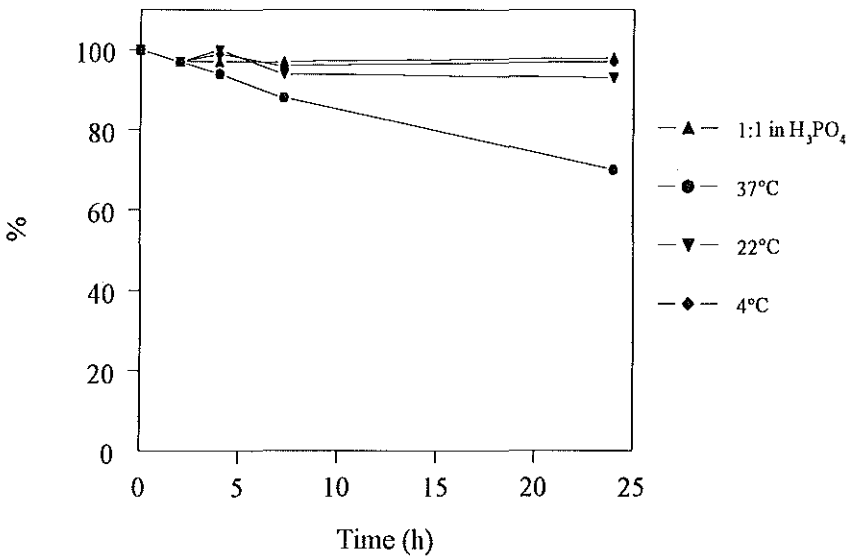


Fig. 4: Stability of I in urine at different temperatures and when diluted 1-fold with H_3PO_4.

CONCLUSION

A sensitive, selective, accurate and reproducible isocratic reversed phase HPLC method has been developed for the simultaneous analysis of I and II in plasma and the analysis of I in urine. Plasma sample pretreatment was carried out immediately after sample collection by deproteinizing with cold methanol as previously described [11]. Prior to injection the sample was diluted with PBS. The urine samples were analyzed after acidification with ortho-phosphoric acid and dilution with PBS. The methodology described for the measurement of plasma concentrations of both compounds and urine concentrations of I can be used to determine the pharmacokinetics in clinical studies with I administered at low doses.

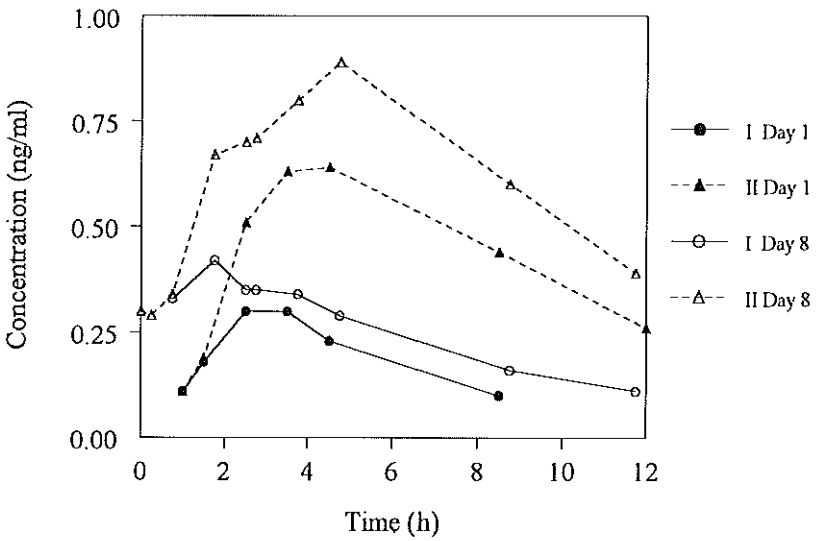


Fig. 5: Plasma concentration-time curves after oral administration of 0.4 mg on days 1 and 8.

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Chapter 2b

Topotecan lacks third space sequestration

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ABSTRACT

The objective of this study was to determine the influence of pleural and ascitic fluid on the pharmacokinetics of the antitumor camptothecin derivative topotecan. Four patients with histologic proof of malignant solid tumor received topotecan 0.45 or 1.5 mg/m^2 orally on several occasions both in the presence and absence of third space volumes. Serial plasma and pleural or ascitic fluid samples were collected during each dosing and analyzed by high-performance liquid chromatography for both the intact lactone form of topotecan and its ring-opened carboxylate form. The apparent topotecan clearance (CL/f) demonstrated substantial interpatient variability, but remained unchanged within the same patient in the presence [110±55.6 L/h/m^2 (mean±SD of 8 courses)] or absence of pleural and ascitic fluid [118±31.1 L/h/m^2 (7 courses)]. Similarly, terminal half-lives and AUC ratios of lactone to total drug in plasma were similar between courses within each patient. Topotecan penetration into pleural and ascitic fluid demonstrated a mean lag time of 1.61 h (range, 1.37 to 1.86 h) and ratios with plasma concentration increased with time after dosing in all patients. The mean ratio of third space topotecan total drug AUC to that in plasma was 0.55 (range, 0.26 to 0.87). These data indicate that topotecan can be safely administered to patients with pleural effusions or ascites, and that there is substantial penetration of topotecan into these third spaces that may proof beneficial for local antitumor effects.

INTRODUCTION

The increased risk of toxicity following chemotherapy in patients with pleural effusions and massive ascites is widely known, and has been well documented for several compounds including methotrexate [1,2] and fludarabine [3]. This phenomenon is most likely related to greater drug accumulation in the peripheral compartment and a slower transport back to the central compartment, ultimately resulting in prolonged drug exposure. For this reason, it is advised to evacuate large pleural and ascitic effusions prior to administration of these agents. On the other hand, penetration of the delivered chemotherapeutic agent should be sufficient to produce adequate drug distribution into the pleural or ascitic fluid to induce relevant local antitumor effects [4].

Diffusion of orally or systemically administered drugs into the peritoneum may be diminished by fibrous tissue due to prior surgery or prior regional i.p. chemotherapy, as reported for mitomycin C [4]. In addition, several other factors including molecular weight, hydrophobicity, blood and lymph flow and capacity of the capillary wall and intervening

interstitium have been shown to affect the peritoneal-blood barrier [5]. The same factors may also be applicable for pleural effusions and the pleural fluid-blood barrier, although only few paired plasma/pleural fluid pharmacokinetic data are available for antineoplastic agents [5-7].

In the absence of any pharmacokinetic data on third space sequestration for topotecan, a topoisomerase I inhibitor with substantial antitumor activity against various malignancies [reviewed in Ref. 8], we have prospectively evaluated the extent of penetration of this drug in pleural and ascitic fluid in cancer patients, and assessed the influence of these third spaces on topotecan plasma pharmacokinetics.

MATERIALS AND METHODS

Patients and treatment

A total of 4 patients with a histologically confirmed diagnosis of a malignant solid tumor that was refractory to standard forms of therapy was studied (Table 1). All patients had adequate hematopoietic, hepatic and renal functions [9]. The study drug topotecan was supplied as capsules containing either 0.25 or 1.0 mg of the active compound (SmithKline Beecham Pharmaceuticals Inc., Harlow, UK), and was administered orally once daily, after an overnight fast, either for 5 consecutive days and repeated every 3 weeks (3 patients) or for 2 consecutive days and repeated every week (1 patient). In all 4 patients, comedication was uniform and consisted of cisplatin (50 or 70 mg/m^2 administered as a 3-h i.v. infusion immediately before topotecan on day 1 of every course) and ondansetron (8 mg, i.v.) combined with dexamethasone (10 mg, i.v.) given 30 min before cisplatin. During therapy, the patients did not use any other medication that might have interfered with topotecan absorption and disposition. The clinical protocol was approved by the institutional review board, and patients signed informed consent before entering the study.

Sample collection

Material for pharmacokinetic analysis was collected during the first treatment course on days 1, 2 and 5 from patients on the 5-day schedule, and during courses 1, 2 and 3 on days 1 and 2 from the patient on the 2-day schedule. Blood samples were collected in 4.5-mL glass tubes containing lithium heparin as anticoagulant (Becton Dickinson, Meylan, France) and were obtained at the following time points: prior to dosing, and 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 12 h after topotecan administration. The blood samples were immediately placed in an ice-bath and centrifuged within 10 min at 3000xg for 5 min at 4°C, to separate the plasma. Subsequently, a volume of 250-µL of the plasma sample was added to 750-µl of ice-cold (-20°C) methanol in

2.0 mL polypropylene vials (Eppendorf, Hamburg, Germany). After vortex-mixing for 10 s, the samples were stored at -80°C until the day of analysis. Pleural and ascitic samples were obtained at the same time points as described for blood samples using a Medicut 16GA cannula (45x1.7mm internal diameter; Sherwood Medical, Tullamore, Ireland) and were collected in 4.5-mL polypropylene tubes, after discarding the first 10-mL of fluid. These samples were processed as described above for plasma.

Table 1: Patient characteristics

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4
Age (yrs)	41	65	40	35
Gender (M/F)	M	M	M	F
Carcinoma	ACUP[a]	rectum	ACUP	ovarian
Third space	pleural	pleural	ascites	ascites
Treatment schedule	d1-5 q3w[b]	d1-5 q3w	d1-5 q3w	d1-2 q1w[c]
Drug dose (mg/m^2/d)	1.50	1.50	1.50	0.45
Drug dose (mg/d)	3.00	2.75	3.25	0.75 (d1) 1.00 (d2)[d]

a: ACUP, adenocarcinoma of unknown primary origin; b: d1-5 q3w, once daily for 5 consecutive days, repeated every 3 weeks; c: d1-2 q1w, once daily for 2 consecutive days, repeated every week; d: As a result of body-surface area-based dosing, and given the availability of 0.25-mg and 1.0-mg topotecan capsules only, the calculated weekly dose was split into unequal daily doses.

Topotecan assay

The samples, plasma as well as pleural liquid and ascites, were analyzed using a reversed-phase HPLC assay with fluorescence detection, as described earlier [10], with minor modifications. In brief, samples were centrifuged for 5 min at 23,000 x *g* at 4°C, followed by a 5-fold dilution in phosphate-buffered saline prior to injection of 200-µL aliquots into the HPLC system. Chromatographic separations of topotecan carboxylate and lactone forms and endogenous compounds were achieved on a Hypersil BDS column (100x3 mm ID, 3 µm particle size; Shandon, Cheshire, UK), which was maintained at 35°C. The mobile phase, composed of 10 mM potassium dihydrogenphosphate-methanol-triethylamine (1750:500:4, v/v/v) with the pH adjusted to 6.0 (orthophosphoric acid), was delivered at a flow rate of 0.70 mL/min. The

excitation and emission wavelengths of the Jasco FP920 fluorescence detector (Tokyo, Japan) were set at 381 and 525 nm, respectively, with an emission band width of 40 nm. Chromatographic data analysis was performed based on peak height measurements relative to injected standards using the ChromCard system of Fisons (Milan, Italy).

Pharmacokinetic analysis

Individual plasma concentrations of topotecan lactone and carboxylate forms were fit to a linear two-exponential equation, using the software package Siphar version 4 (SIMED, Creteil, France), based on a variety of considerations including Akaike's and Schwarz' information criterion. The concentration-time profiles were obtained after zero-order input, with a weighted least-squares algorithm applying a weighting factor of $1/y$. The area under the concentration-time curve (AUC) were determined for both the lactone (AUC_L) and carboxylate forms (AUC_C) on the basis of the best fitted curves. The apparent plasma clearance of topotecan lactone (CL/f) was calculated by dividing the dose administered by the observed AUC. The apparent terminal disposition half-life ($T_{1/2}$) was calculated as $\ln 2/k_{el}$, where k_{el} is the observed elimination rate constant of the terminal phase. The peak plasma concentrations (C_{max}) were determined graphically from the observed experimental values. The ratio of the systemic exposure of topotecan lactone to total drug (L/T ratio) was defined as $AUC_L / (AUC_L + AUC_C)$. The fraction of drug penetrating into pleural or ascitic fluid was derived from the ratio of the topotecan total drug AUCs in the third space and plasma.

RESULTS

Plasma pharmacokinetics

Peak plasma concentrations and AUCs of topotecan lactone following an oral dose of 1.50 mg/m^2 to patients 1 and 2 were similar before and after pleural fluid was drained (fluid volumes, 3.1 and 1.1 L, respectively) (Table 2). Data from patient 3, who had recurrent ascites during all topotecan administrations with volumes of 8.4 and 9.4 L drained on days 2 and 6, respectively, indicated no difference in pharmacokinetic parameters between treatment days. Similarly, ascites (estimated to be 4.0, 1.0 and 1.0 L on 3 occasions by ultrasonography and percutaneous drainage) had no measurable effect on topotecan plasma pharmacokinetics in patient 4 (Table 2). Overall, the apparent topotecan clearance (CL/f) demonstrated substantial interpatient variability, but remained unchanged within the same patient in the presence [110±55.6 L/h/m^2 (mean±SD; 8 courses)] or absence of pleural or ascitic fluid [118±31.1 L/h/m^2 (7 courses)].

Topotecan L/T ratios in plasma were very similar between courses within each patient and averaged 40.0±3.89% (drained) and 40.0±6.11% (not drained), respectively.

Table 2: Summary of topotecan plasma pharmacokinetics in the presence or absence of pleura or ascitic fluid

Pat no.	Third space	No. of curves	AUC_L (ng.h/mL)	CL/f ($L/h/m^2$)	C_{max} (ng/mL)	$T_{1/2}$ (h)	L/T ratio (%)
1	pleural	1	12.9	136	2.06	1.83	44.7
	none	2	14.7, 18.3	119, 95.5	3.33, 6.50	2.17, 1.77	44.8,43.5
2	pleural	1	23.3	64.3	2.71	4.53	36.3
	none	2	18.7, 17.0	80.0, 88.1	2.04, 2.30	4.43, 5.90	34.6,35.8
3	ascites	3	22.1±1.92	68.2±5.82	2.43±0.32	4.38±0.36	40.3±2.4
4	ascites	3	5.80±1.82	155±61.2	1.51±0.65	3.00±0.58	39.2±5.3
	none	3	6.28±1.42	148±12.1	1.16±0.15	3.40±0.66	40.5±8.4

Data were obtained both in the presence and absence of third space fluids in each individual patient treated with topotecan doses and treatment schedules as given in Table 1. The AUC values were calculated by compartmental analysis, and data of patients 3 and 4 represent mean values ± SD. *Abbreviations:* AUC_L, area under the topotecan lactone plasma concentration-time curve; CL/f, apparent plasma clearance of topotecan lactone; C_{max}, peak plasma concentration of topotecan lactone; $T_{1/2}$, apparent terminal disposition half-life; L/T ratio, percent of total drug (lactone plus carboxylate forms) circulating as topotecan lactone.

Pleural and ascitic fluid penetration

Given the low plasma protein binding of topotecan [~35%; (11)] and the relatively high total protein content in pleural fluid and ascites of the patients (range, 38 to 45 mg/mL), no correction for protein binding was performed. Topotecan concentrations in pleural fluid and ascites peaked at ≥6 h after oral dosing, demonstrated a mean lag time of 1.61 h (range, 1.37 to 1.86 h; overall mean±SD in plasma, 0.63±0.28 h), and rose slowly to equal that in plasma by ~8 h (Figs. 1A to 1C). Topotecan disappearance from pleural fluid [$T_{1/2}$, 11.9 h (n=1)] and ascites [$T_{1/2}$, 7.94 h (n=1)] was slower than that from plasma. As a result, third space penetration, expressed as the ratio of concomitant pleural fluid or ascites and plasma concentration of total topotecan depended greatly on the sampling time point, and increased significantly with time in all

patients (Figs. 1D to 1F). Overall, the mean ratio of third space topotecan total drug AUC to that in plasma was 0.55 (range, 0.26 to 0.87). The hydrolysis of topotecan to the ring-opened form was rapid and L/T AUC ratios were 18.1% and 23.5% in pleural fluid and 29.2% in ascites. Measurement of topotecan in ascites from patient 3 indicated that less than 1% of the administered dose was present in ascites at 6 to 8 h after dosing, indicating lack of a sink effect.

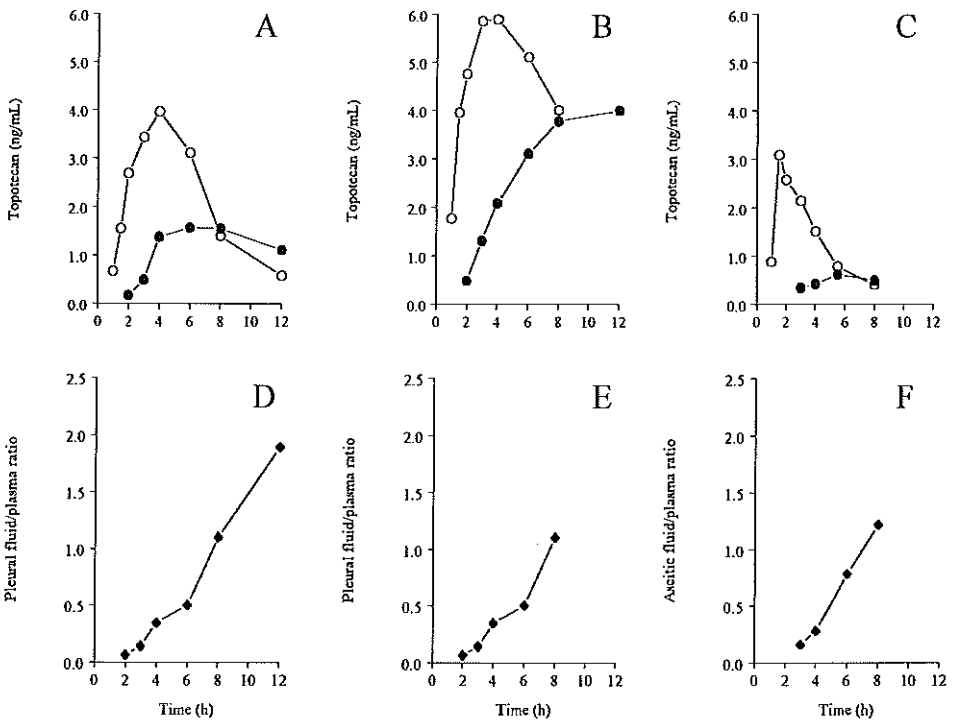

Fig. 1: Concentration *versus* time plots of topotecan expressed as total drug (lactone plus carboxylate) in plasma (open circles) and ascites (closed circles, patients 1 and 2) or pleural fluid (closed circles, patient 4) and corresponding topotecan third-space fluid:plasma concentration ratios in patients 1 (A and D), 2 (B and E), and 4 (C and F).

Toxicity

Overall, treatment was very well tolerated in the 4 patients. No severe hematological toxicity or other organ toxicity was observed following oral topotecan administration at these doses. The third patient experienced fatigue graded 2 on a 4-point scale (NCI-Common Toxicity Criteria), whereas the fourth patient had mild nausea and vomiting.

DISCUSSION

This study was performed to explore the influence of pleural and ascitic fluid on the pharmacokinetic behavior of topotecan in cancer patients. Although the topotecan administration was preceded by cisplatin infusion in this study in all patients, important pharmacokinetic interactions that may have influenced the generated data are not very likely; (*i*) comparison of the kinetics of topotecan in clinical combination therapy regimens with cisplatin to single agent therapy did not reveal an apparent interaction [12], and (*ii*) using a randomized cross-over design for the administration order, no statistically significant differences in clinical pharmacokinetics were observed between sequences of oral topotecan and i.v. cisplatin [9].

Topotecan concentrations in pleural fluid and ascites were initially less than in plasma, and several hours were required for equilibrium to be attained between these fluids and plasma. The limited surface area for topotecan diffusion relative to the volumes of fluid, and the fact that pleural fluid and ascites are not well stirred, in addition to the hydrophilic nature of the drug likely contributed to the slow equilibrium kinetics. Overall, both pleural fluid and ascites represented only a small additional compartment for topotecan distribution, particularly in view of the already large topotecan steady-state volume of distribution of 73-133 L [13]. Nonetheless, concentrations equivalent to that in plasma were achieved after 8 hours, and topotecan elimination was found to be more slowly from the pleural and peritoneal cavity than from plasma. This is in keeping with earlier findings indicating slow peritoneal clearance of topotecan and high peritoneal:plasma concentration ratios of >10 following i.p. drug administration [14, 15].

Topotecan has been detected previously in ascites of 2 patients treated with a combination of i.v. topotecan and oral etoposide [16]. However, the reported ascitic fluid:plasma concentration ratios were established by single point measurements at different times after administration. Since these concentration ratios were shown in our patients to be by no means constant parameters during the dosing interval (Figs. 1D to 1F), single-point data are clearly inappropriate to directly compare the extent of penetration by topotecan. Hence, the approach of using paired AUC values in third space fluids and plasma, as done in the current study, should

be considered the gold standard to report these ratios. Although the described data on topotecan accumulation is limited to only 4 patients, our results suggest that oral administration of topotecan can produce adequate drug distribution in pleural fluid and ascites at concentrations associated with significant antitumor activity in experimental models [7, 18]. In this context, it is of particular interest that topoisomerase I inhibitors were previously shown to be highly S-phase specific and that cytotoxicity is a function of the time to drug exposure above a certain threshold concentration [19]. The topotecan penetration and subsequent accumulation in the third spaces might thus offer a potential therapeutic advantage in that tumor cells in the thoracic and peritoneal cavity are exposed to high local drug levels for prolonged time periods. This concept has also been described recently for systemic therapy with the structurally-related camptothecin derivative, irinotecan (CPT-11), although in contrast to topotecan, concentrations appeared to decline in parallel with those in plasma [20]. The reason for this discrepant behavior is unknown, but it likely reflects intrinsic differences in physicochemical and/or pharmacokinetic properties of both compounds, including differential binding to (plasma) proteins.

The plasma pharmacokinetics of topotecan revealed a substantial degree of interindividual variability, in line with previous observations [9, 21]. By comparing topotecan plasma levels in the same patient before and after drainage of pleural or ascitic fluid, no differences in rate of absorption and elimination became apparent. The lack of increased systemic exposure to topotecan in patients with massive third space volumes was further substantiated by the lack of excess toxicity. Hence, in contrast to clinical information on irinotecan treatment that suggested an increased risk of severe toxicity in patients with large pleural effusions or ascites [20], there was no evidence that the severity of toxicity was different between study courses with and without third space volumes in our patients treated with topotecan.

In conclusion, we have shown that (*i*) topotecan plasma pharmacokinetics are unaltered in patients with third space volumes, (*ii*) it can be safely administered to patients with large pleural effusions or massive ascites, and (*iii*) there appears to be substantial penetration of topotecan into these third spaces that may proof beneficial for local antitumor effects.

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Chapter 2c

Phase I pharmacologic study of oral topotecan and intravenous cisplatin: sequence dependent hematologic side effects

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ABSTRACT

Background and Purpose In *in vitro* studies synergism and sequence dependent effects were reported for the combination of topotecan and cisplatin. Recently an oral formulation of topotecan became available. This phase I study was performed to assess the feasibility of the combination of oral topotecan and cisplatin, the pharmacokinetic interaction, and sequence dependent effects.

Patients and Methods Topotecan was administered orally daily for five days in escalating doses and cisplatin at a fixed dose of 75 mg/m² i.v. either before topotecan on day 1 (CT) or after topotecan on day 5 (TC) once every 3 weeks. Patients were treated in a randomized cross-over design.

Results Forty-nine patients entered the study; one patient was not eligible. The CT sequence induced significantly more severe myelosuppression than sequence TC, and resulted in MTD at a topotecan dose of 1.25 mg/m²/dx5. In sequence TC, the MTD was topotecan 2.0 mg/m²/dx5. DLT consisted of myelosuppression and diarrhea. Pharmacokinetics of topotecan and cisplatin were linear over the dose range studied, no sequence dependent effects were observed. In addition, topotecan did not influence the protein binding of cisplatin and the platinum-DNA adduct formation in peripheral leukocytes in either sequence.

Conclusion The recommended dose for phase II studies in patients, comparable with the patients studied, is oral topotecan 1.25 mg/m²/day x 5 preceded by cisplatin 75 mg/m² day 1 once every 3 weeks, and topotecan 2.0 mg/m²/day followed by the same dose cisplatin on day 5. No pharmacokinetic interaction could be discerned. The antitumor efficacy of both schedules should be evaluated in a randomized phase II study.

INTRODUCTION

Topotecan (9-dimethylaminomethyl-10-hydroxycamptothecin, Hycamtin®) is a water-soluble topoisomerase I inhibitor (camptothecin). DNA topoisomerase I is a nuclear enzyme, involved in cellular replication and transcription. Topotecan exists in two forms in a pH dependent dynamic balance between the closed lactone ring (active) form and the carboxy acid (inactive) form. By forming a covalent adduct between topoisomerase I and DNA, named the cleavable complex, topoisomerase I inhibitors interfere with the process of DNA breakage and resealing during DNA synthesis. The stabilized cleavable complex blocks the progress of the replication fork resulting in irreversible DNA double-strand breaks leading to cell death [1-3]. Based on their mechanism of action, synergy was suspected for the combination of topoisomerase I inhibitors and DNA damaging agents such as cisplatin. Preclinical studies confirmed this hypothesis. However, the observed interaction seemed to depend on the cell line studied and the schedule of administration of topotecan

and cisplatin used [4-17]. When topotecan was preceded by cisplatin, synergy was increased compared to concomitant incubation with both drugs in the IGROV-1 ovarian cancer cell line and the MCF7 cell line [5,6]. Also in the clinical setting drug sequencing seems to be important [17].

To date, topotecan has demonstrated prominent activity in several malignancies, most notable in ovarian [18-23], small cell lung carcinomas [24-26], and hematological malignancies [27-30], in which cisplatin is also highly active. Recently, an oral formulation of topotecan became available, which is a more convenient method of drug administration. The oral formulation has a bioavailability of 32-44% [31-33] with moderate intrapatient variability. The maximally tolerated dose for oral topotecan, administered for five days every 21 days as a gelatin capsule, has been determined as 2.3 mg/m²/day with myelosuppression, in particular neutropenia, as the dose limiting toxicity (DLT) [34]. Non-hematological toxicities were generally mild and not dose limiting, including fatigue, anorexia, nausea, vomiting and diarrhea. In ovarian- [35] and small cell lung cancer [36] randomized studies suggest the oral formulation is equivalent to the intravenous formulation.

Against this background, we initiated a phase I study in which patients were treated in a randomized cross-over design to determine the maximum tolerated dose of oral topotecan given daily for 5 days combined with cisplatin 75 mg/m² i.v. administered either on day 1 or day 5 every 21 days, to describe and quantitate the toxicities of the combination and to determine whether the sequence of topotecan and cisplatin administration has any influence on the observed toxicity or the pharmacokinetic interaction between the drugs.

PATIENTS AND METHODS

Patient selection

Patients with a histologically or cytologically confirmed diagnosis of a malignant solid tumor resistant to standard forms of therapy were eligible. Other eligibility criteria included the following: age between 18-75 years; Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2; no previous anticancer therapy for at least 4 weeks (6 weeks for nitrosoureas or mitomycin C); no previous therapy with topoisomerase I inhibitors; adequate hematopoietic function (absolute neutrophil count (ANC) $\geq 1.5 \times 10^9$/L and platelet count $\geq 100 \times 10^9$/L), renal function (creatinine clearance ≥ 60 mL/min) and hepatic (total serum bilirubin ≤ 1.25 x upper normal limit and serum aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) ≤ 2.0 x upper normal limits, in case of liver metastasis serum ASAT and ALAT ≤ 3.0 x upper normal limits) function. Specific exclusion criteria included the existence of gross ascites and/or any gastrointestinal condition that would alter absorption or motility. All patients gave written informed consent before study entry.

Dosage and dose escalation

Escalating doses of oral topotecan were combined with a fixed dose of cisplatin, 75 mg/m², administered intravenously over 3 hours once every 3 weeks. The starting dose of topotecan was 0.75 mg/m²/day for 5 consecutive days, which is 33% of the recommended dose of oral topotecan, when administered as a single agent. Dose escalation was based on the prior dose level toxicities. At least three patients were treated at each dose level. If one of three patients experienced dose limiting toxicity (DLT), three additional patients were entered at that dose level. The maximum-tolerated dose (MTD) was defined as one dose level below the dose that induced DLTs in 3 out of 6 patients during the first course in any sequence, which were defined as NCI-CTC grade 4 neutropenia lasting for five days or more, or complicated with fever requiring hospitalization, grade 4 thrombocytopenia and/or non-hematological toxicity ≥ grade 3 (grade 2 for renal toxicity), excluding nausea. Intrapatient dose escalation was not permitted. If a patient encountered DLT, the dose of topotecan was decreased one dose level at re-treatment. The treatment was resumed when the neutrophil count had recovered to ≥ 1.0×10^9/L and the platelet count to ≥ 100×10^9/L. A maximum of six cycles was administered to each individual patient.

Drug administration and sequencing

In the first part of the study, patients were randomly assigned at study entry to one of two treatment groups. Six patients were treated at each dose level.

Group A. In the first treatment course, patients received cisplatin as a 3-hour infusion diluted in 250 mL of hypertonic saline [3% (w/v) sodium chloride] on day 1, immediately followed by the oral administration of topotecan (sequence CT), which was given for five consecutive days on an empty stomach, at least 10 minutes before meals. In the second course, the sequence of administration of topotecan and cisplatin was reversed, starting with topotecan for 5 days and administering cisplatin on day 5, 3-hours after the last oral administration of topotecan at the same doses (sequence TC).

Group B. Patients received the two treatment courses in reversed order.

The third and following courses were administered using the least toxic sequence, with in the third course a 24 hour interval between the administration of cisplatin and topotecan to study the pharmacokinetics of both drugs to rule out the possibility of any pharmacokinetic interaction.

In the second part of the study, after determination of the MTD in the most toxic sequence, further dose escalation of topotecan was pursued in the reversed sequence. Patients were then enrolled to receive that single sequence with a 24-hour interval between the administration of topotecan and cisplatin in the second course only.

In all patients pre-medication consisted of ondansetron (8 mg i.v.) combined with dexamethasone (10 mg i.v.) administered 30 min before the start of the cisplatin infusion. To prevent cisplatin-induced renal damage, the administration of cisplatin was preceded by the

infusion of 1000 mL of a mixture of 5% (w/v) dextrose and 0.9% (w/v) sodium chloride over 4 hours, and followed by another 3000 mL with the addition of 20 mM potassium chloride and 2 g/L magnesium sulphate applied over 16 hours. Topotecan capsules containing either 0.25 or 1.00 mg of the active compound were supplied by SmithKline Beecham Pharmaceuticals (Harlow, UK). Cisplatin (Platosin®) was purchased as a powder from Pharmachemie (Haarlem, The Netherlands).

Treatment assessment

Before therapy a complete medical history was taken and a physical examination was performed. A complete blood count (CBC) including WBC and differential, and serum biochemistry, which included sodium, potassium, calcium, phosphorus, urea, creatinine, total protein, albumin, total bilirubin, alkaline phosphatase, aspartate aminotransferase (ASAT), alanine transferase (ALAT), γ-glutamyl transferase, glucose and uric acid, were performed, as was creatinine clearance. Weekly evaluations included history, physical examination, toxicity assessment according to the CTC criteria, and serum chemistry. CBC was determined twice weekly. Tumor evaluation was performed after three courses in the first part and after every two courses in the second part of the study according to the World Health Organisation (WHO) criteria for response. Duration of response was calculated from the start of treatment. Patients were treated for at least three cycles of therapy in the first part and two cycles in the second part of the study unless disease progression or unacceptable toxicity was encountered.

Sample collection for pharmacokinetics

Blood samples for pharmacokinetic analysis were obtained during the first 3 treatment courses until the MTD was reached in the most toxic sequence. Hereafter samples were only taken in the first 2 courses. Blood sampling for topotecan pharmacokinetics was performed on the first and fifth day of drug dosing, whereas for cisplatin pharmacokinetics sampling was performed on the day of administration (days 1, 5 or 6, dependent on the schedule). At the doses recommended for further study, additional topotecan pharmacokinetics were performed on day 2 of the first treatment course. Blood was withdrawn from a vein in the arm opposite to that used for drug infusion, and collected in 4.5-mL glass tubes containing lithium heparin as anticoagulant. For analysis of topotecan kinetics, samples were obtained at the following time points: prior to dosing, and 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 12 hours after administration of topotecan. Immediately after sampling, tubes were briefly immersed into an ice bath kept at the bedside, and plasma was separated within 10 min by centrifugation at 3000 x *g* for 5 min (4°C). Next, 250-μL aliquots of the plasma supernatant were added to 2.0-mL polypropylene vials (Eppendorf, Hamburg, Germany) containing 750-μL of ice cold (-20°C) methanol. After mixing on a vortex-mixer for 10 s, samples were stored at -80°C until the day of analysis.

Blood samples for measurement of cisplatin concentrations were obtained immediately before infusion; at 1, 2, and 3 hours after start of the infusion; and 0.5, 1, 2, 3, and 18 hours after the end of the infusion. Sample volumes were 4.5 mL each except at predose and 1 and 18 hours after infusion, which were 21 (3x7) mL each. Immediately after sampling plasma was separated by centrifugation at 3000 x *g* for 10 min. Next, 500-μL aliquots of the plasma supernatant were added to 1.0-mL of ice cold (-20°C) ethanol. After mixing on a vortex-mixer for 10 s, samples were stored at -80°C until the day of analysis. Blood samples to determine the cisplatin DNA-adduct levels were obtained immediately before infusion, and 1 and 18 hours after the end of the infusion.

Pharmacokinetic assays

Samples for topotecan kinetics were analyzed by a reversed-phase high-performance liquid chromatographic (HPLC) method, as described [37], that allowed simultaneous determination of the lactone and the hydrolyzed ring-opened carboxylate forms. Prior to drug analysis, samples were removed from the freezer and centrifuged for 5 min at 23000 x *g* (4°C), A volume of 100 μL was transferred to a clean microtube containing 400 μL phosphate buffer. Of this mixture, a 200-μL volume was used for analysis. The HPLC system consisted of a constaMetric 4100 solvent delivery system (LDC Analytical, Riviera Beach, FL), a Waters 717Plus autosampler (Bedford, MA), and a Jasco FP 920 fluorescence detector (Jasco). Chromatographic separations were achieved at 35°C on a Shandon Hypersil BDS column (100x3 mm, internal diameter; 3 μm particle size) from Applied Science (Breda, The Netherlands), with a mobile phase composed of 10 mM aqueous potassium dihydrogen phosphate containing 22% (v/v) methanol and 0.2% triethylamine, with the pH adjusted to 6.0 (orthophosphoric acid). The mobile phase was filtered [0.45-μm Millipore HA filters (Milford, MA)] and degassed by ultrasonication. The flow rate was set at 0.7 mL/min, and the column effluent was monitored at excitation and emission wavelengths of 381 and 525 nm, respectively with the emission band width set at 40 nm. Peak detection was performed with the Fisons ChromCard data analysis system (Milan, Italy). Drug concentrations in unknown samples were determined by interpolation on linear calibration curves, constructed in blank human plasma, by least-squares linear regression of peak heights *versus* $1/x$. The mean percentage deviation from nominal values (accuracy) and precision (within-run and between-run variability) were always <15%. The lower limit of quantitation for both the lactone and carboxylate forms were 100 pg/mL.

Non-protein bound and total cisplatin concentrations in plasma were determined by a validated analytical procedure based on measurement of platinum atoms by flameless atomic-absorption spectrometry (AAS) as described [38,39]. For measurement of unbound cisplatin, 500-μL aliquots of plasma were extracted with neat 1000 μL ice-cold ethanol in a 2-mL polypropylene vial. After a 2-hour incubation at -20°C, the supernatant was collected by centrifugation at 23,000x*g* for 5 min (4°C), and transferred to a clean vial. A volume of 600 μL was evaporated to dryness under nitrogen

at 60°C, and the residue reconstituted in 200 or 600 μL water containing 0.2% (v/v) Triton X-100 and 0.06% (w/v) cesium chloride by vigorous mixing. A volume of 20 μL was eventually injected into the AAS. For determination of total cisplatin, a 100-μL volume of plasma was added to 900 μL water containing 0.2% (v/v) Triton X-100 and 0.06% (w/v) cesium chloride, followed by vortex-mixing for 10 s. Of this solution, a volume of 20 μL was injected into the AAS. Samples were analyzed on a Perkin Elmer Model 4110 ZL spectrometer with Zeeman-background correction using peak area signal measurements at a wavelength of 265.9 nm and a slid width of 0.7 nm [38,39]. The injection temperature was set at 20°C. Platinum DNA adduct levels in peripheral leukocytes were determined as described [40], with modifications [41]. Following DNA isolation from buffy coat preparations, samples were digested with DNAse I and zinc chloride and injected into the furnace using a 4-times multiple sampling feature of the Perkin Elmer AAS. The cisplatin DNA-adduct levels were expressed as picogram platinum per microgram DNA (pg Pt/ μg DNA).

Pharmacokinetic data analysis

Individual plasma concentrations of topotecan were fit to a two-compartment model, using the software package Siphar v4.0 (SIMED, Creteil, France). The concentration-time profiles were obtained after zero-order input, with weighted least-squares analysis applying a weighting factor of $1/y$. The topotecan area under the plasma concentration-time curve (AUC) was determined for both the lactone ($AUC_{(L)}$) and carboxylate forms ($AUC_{(c)}$) on the basis of the best fitted curves. The apparent plasma clearance ($CL/f_{(L)}$) of topotecan lactone was calculated by dividing the dose administered (expressed in free base equivalents) by the observed AUC. The terminal disposition half-life [$T_{1/2}(z)$] of topotecan was calculated as $\ln 2/k$, where k is the terminal elimination rate constant (expressed in h^{-1}). The peak plasma concentrations (C_{max}) and the time to peak plasma concentration (T_{max}) were determined graphically from the (observed) experimental values. The ratio of the systemic exposure of topotecan lactone to total drug (L/T ratio) was defined as $AUC_{(L)}/[AUC_{(L)}+AUC_{(C)}]$.

Kinetic profiles of CDDP were obtained similarly using a two-compartment linear model with extended least-squares regression analysis as reported earlier [42]. The AUC of cisplatin was calculated to the last sampling time point with detectable drug levels (C_{last}) by the linear trapezoid method and extended to infinity by addition of C_{last}/k_{term}, where k_{term} is the slope obtained by log-linear regression of the final plasma concentration values.

Statistical considerations

Pharmacokinetic parameters for all compounds are reported as mean values ± S.D. The difference in pharmacokinetic parameters between sequences was evaluated statistically using a paired Student's

t-test. Probability values (two-sided) of less than 0.05 were regarded as statistically significant. All calculations were performed using the statistical packages NCSS version 5.X (J.L. Hintze, Kaysville, UT) and STATGRAPHICS Plus version 2.0 (Manugistics Inc., Rockville, MA).

RESULTS

Forty-nine patients entered this study between January 1997 and February 1999. Patient characteristics are listed in Table 1. One patient was not eligible due to reduced renal function at the time of study entry, 1 patient was not assessable for toxicity because of the occurrence of a cerebrovascular accident after two days of treatment with topotecan and was taken off study. Forty-seven patients were assessable for toxicity and 45 patients for response.

The majority of the patients was either asymptomatic or had only mild symptoms. Nineteen patients were female and 28 were male. Seventeen patients had received prior chemotherapy, one line only. No patients were pretreated with drugs known to be highly myelosuppressive i.e. carboplatin, mitomycin C, nitrosoureas or high dose cyclophosphamide. The most common tumor type was head and neck cancer. Dose levels of topotecan studied were 0.75, 1.0, 1.25, 1.5, 1.75, 2.0 and 2.3 mg/m²/day, respectively. The total number of assessable courses was 175. The median number of courses per patient was 4 (range 1-6).

Both myelosuppression and diarrhea were the principal DLTs of this regimen. Seven patients required dose reductions after experiencing dose-limiting toxicity. Once dose reduction had taken place, the courses in these patients were evaluated for toxicity at the lower dose level.

Hematologic toxicity and drug sequencing

The severity of the observed hematologic toxicity was clearly dependent on the sequence of drug administration. At each dose level studied, both neutropenia and thrombocytopenia were more severe when cisplatin administration preceded the administration of topotecan (CT sequence) (Table 2 and Figure 1) reflected in both a significantly lower nadir and percentage decrements in neutrophil and platelet counts in this sequence [P=<0.00001 (neutropenia), P=<0.00001 (thrombocytopenia)]. At the dose level of 1.0 mg/m² in the sequence CT, two out of six patients experienced neutropenia grade 4 lasting for 5 days or more. By protocol definition, these patients were judged as having DLT. At the next dose level combining topotecan 1.25 mg/m²/day with cisplatin 75 mg/m² in the sequence CT, of the six patients treated, one patient experienced neutropenic fever and another patient had neutropenia grade 4 lasting for more than 5 days in combination with diarrhea grade 3 and vomiting grade 4. Four additional patients were treated at this dose level. One of these patients, patient number 10, had

DLT because of a neutropenia grade 4 lasting longer than 5 days. Another patient, who formally was ineligible due to reduced renal function at study entry, experienced grade 4 neutropenia and thrombocytopenia in the second course, and died as a result of the complications of this toxicity.

Table 1: Patient characteristics

Characteristic		No. of Patients
No. Entered		49
No. Assessable		47
Age, years		
Median	57	
Range	28-70	
Sex		
Female		19
Male		28
Performance status		
Median	1	
Range	0-1	
Tumor type		
Head/Neck		11
(N)SCLC		10
ACUP		10
Cervical		3
Miscellaneous		13
Previous therapy		
Chemotherapy		9
Radiation		12
Chemotherapy and radiation		8
None		18

Table 2: Toxicity (worst per cycle) and drug sequencing

Topotecan Mg/m^2/day	Sequence	Nr pts/ Cycles	Neutropenia 3	4	Thrombopenia 3	4	Nausea 1	2	3	Vomiting 1	2	3	4
0.75	CT	6/9	1	1	1	0	7	0	0	3	0	0	0
0.75	TC	7/19	0	0	0	0	11	3	0	6	1	0	0
1.0	CT	7/7	2	3	1	0	4	2	1	1	5	0	1
1.0	TC	7/22	1	0	0	0	12	5	0	5	13	0	0
1.25	CT	9/17	7	5	4	1	6	6	1	2	3	0	1
1.25	TC	9/17	1	1	0	0	6	5	1	2	5	1	0
1.5	TC	3/13	3	1	0	0	4	2	0	0	0	0	0
1.75	TC	7/17	2	3	2	0	4	9	3	9	2	2	2
2.0	TC	12/36	11	2	1	2	15	12	3	10	9	3	2
2.3	TC	7/17	8	3	1	1	7	5	3	8	5	0	3

Diarrhea				Fatigue			DLT per pat.	DLT per cycle	
1	2	3	4	1	2	3		1st	all
0	0	0	0	5	2	0	1/6	0/6	1/9
0	0	0	0	6	4	0	0/7	0/6	0/19
1	0	0	0	0	3	0	2/7	2/6	2/7
4	1	0	0	7	2	0	2/7	0/6	2/22
7	3	1	0	5	2	0	4/9	3/9	5/17
4	0	0	0	7	2	0	3/9	1/9	3/17
2	0	0	0	8	1	0	1/3	0/3	1/13
4	0	0	0	3	6	0	3/7	2/6	4/17
8	6	1	2	9	13	1	7/12	4/10	8/36
2	1	0	3	4	2	1	4/7	4/7	5/17

Pts: patients; DLT: dose limiting toxicity; CT: cisplatin followed by topotecan; TC: cisplatin preceded by topotecan. Several patients experiencing DLT required dose reductions. Once dose reduction had taken place, patients were evaluated for toxicity at the lower dose level

Since 3 out of 9 (4 out of 10 taking into account the ineligible patient) patients experienced DLT no further dose escalation was pursued. The protocol defined MTD as the dose level below that level at which 3/6 patients experienced DLT. The dose level combining cisplatin 75 mg/m² day 1 with by topotecan 1.25 mg/m²/day day 1 to 5 is considered the recommended dose for this sequence, but only in non- or marginally pretreated patients in good physical condition and under strict conditions of control as the patients under study.

After the determination of the recommended dose in the sequence CT, dose escalation of topotecan continued in the reversed sequence. At the dose level 1.25 mg/m² 1 out of 6 patients experienced DLT consisting of neutropenic fever in this sequence. At the dose level 1.5 mg/m² no DLT was observed. One patient had a neutropenia grade 4 lasting for 5 days or more and vomiting grade 3 at dose level 1.75 mg/m² in the sequence TC. At the dose level combining topotecan 2.0 mg/m²/day with cisplatin, no DLT occurred in the initial 3 patients. It was decided to escalate the dose of topotecan to the dose recommended for use as a single agent, 2.3 mg/m²/day. Of the first three patients, only one patient developed DLT (diarrhea grade 4). However, three of the four additional patients treated at this dose level, were considered to have DLT, on the basis of vomiting grade 4 (1 patient), diarrhea grade 4 (1 patient), neutropenia grade 4 lasting for 5 days or more (2 patients) and thrombocytopenia grade 4 (1 patient). Thus, combining topotecan 2.3 mg/m²/day and cisplatin 75 mg/m² was not considered feasible. Seven additional patients were treated at dose level 2.0/75 mg/m². Four of these patients developed DLTs: vomiting grade 4 (2 patients), diarrhea grade 3 or 4 (3 patients) although manageable with loperamide therapy, neutropenia grade 4 lasting for 5 days or more (2 patients), and thrombocytopenia grade 4 (1 patient), resulting in DLT in 4 out of 10 patients. Thus, in full accordance to the recommendations for the sequence CT, the recommended dose of topotecan is 2.0 mg/m²/day combined with cisplatin 75 mg/m² for the sequence topotecan followed by cisplatin. Since it was felt that these doses are only feasible in non- or minimally pretreated patients in good physical condition under strict medical surveillance, it was decided to expand the dose level combining topotecan 1.75 mg/m² to six patients. One of these patients experienced grade 4 vomiting in the first course. No other DLTs were observed.

Overall, the hematologic toxicity was relatively mild (Table 2). Grade 3 to 4 neutropenia was observed in 55 of 175 courses (31%). It was complicated by neutropenic fever in only 4 patients. The onsets of neutropenia and thrombocytopenia were relatively late. The nadir of the neutrophils usually occurred around day 19 (range 4-30) after the start of the treatment and lasted for median 5 days (range 1-15). Thrombocytopenia was mild, being grade 3-4 in only 8% of the cycles, all in conjunction with neutropenia. Despite the limited severity of myelosuppression, treatment had to be delayed in 34% of the courses due to prolonged myelosuppression. A marked inhibition of erythropoiesis was observed. The percentage of patients requiring erythrocyte transfusions was 72%, in 74 of 175 courses.

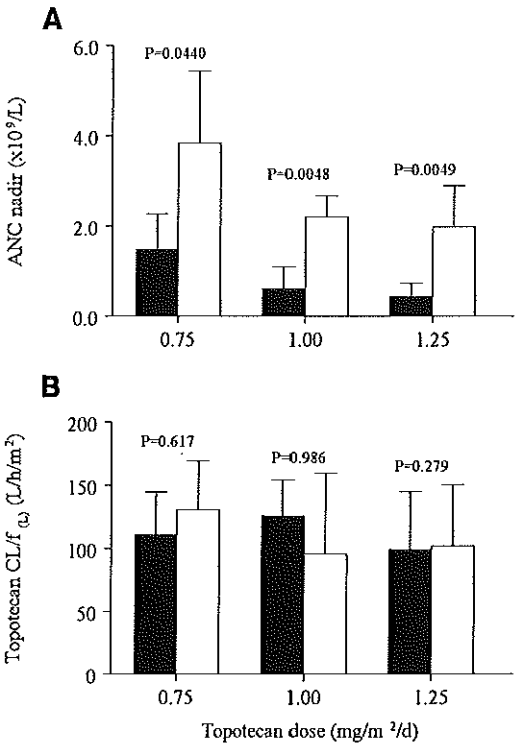

Fig. 1: (A) Absolute neutrophil count (ANC) nadirs and (B) topotecan clearance ($CL/f_{(L)}$) during the first and second courses in sequences CT (■) and TC (□). Data are expressed as mean (bars) ± SD (error bars).

Non-hematologic toxicity

Gastrointestinal toxicity was mild to moderate (Table 2) and generally comparable to the toxicities that result from similar single agent doses of topotecan and cisplatin. No sequence dependent effects were noted. Nausea grade 2 or 3 was observed in 60 of 175 courses (34%) and vomiting grade 3 or 4 in 15 of 175 courses (9%). Both were in time related to the administration of cisplatin. Diarrhea grade 3 or 4 was encountered in 7 cycles (4%) and had a median day of onset on day 8 (range 7-14) and a

median duration of 4 days (range 2-10). The diarrhea was self-limiting or resolved after low dose loperamide therapy in all but two patients, who were hospitalized for i.v. rehydration.

Consistent with the profile of cisplatin 75 mg/m², seventeen patients developed nephrotoxicity grade 1, and 4 patients grade 2 after a median of 2 cycles (range 1-6). Peripheral neurotoxicity grade 1 was encountered in 18 patients. Twenty patients had mostly reversible ototoxicity grade 2 (tinnitus) and 2 patients ototoxicity grade 3 after receiving median 2 cycles (range 1-6). One patient at dose level 2.3 mg/m² developed grade 4 bilirubinemia, due to obstruction of a biliary stent. One patient with a nasopharyngeal cancer, treated at dose level 2.0 mg/m² developed progressive dyspnoe accompanied by fever during the second course. An X-ray of the chest revealed interstitial enhancement with a reticulonodular pattern, especially more prominent at the bases. The pulmonary function demonstrated reduced lung volumes compatible with restrictive lung disease. Bronchoscopy revealed no abnormalities. Despite therapy with antibiotics and low dose corticosteroids, the patient's condition worsened and it was decided to perform an open lung biopsy. Pathologic examination revealed interstitial fibrosis with a marked infiltration with eosinophils, which was considered related to topotecan treatment. The patient was treated with high dose corticosteroids resulting in an amelioration of the symptoms.

Other side effects were mucositis (8 % of cycles), alopecia (19 patients grade 1, 7 patients grade 2), and fatigue.

Anti-tumor activity

Six patients achieved a partial response. The tumor types included, non-small cell and small cell lung cancer, ACUP, renal and pancreatic cancer. The patient with small cell lung cancer was pretreated twice with combination chemotherapy consisting of doxorubicin, etoposide and cyclophosphamide resulting in major responses of 30 and 25 weeks duration, respectively. Her response on cisplatin/topotecan chemotherapy lasted 22 weeks. The patient with pancreatic cancer was pretreated with 5-fluorouracil in combination with a DPD-inhibitor resulting in a partial response for 19 weeks. On therapy with cisplatin/topotecan he achieved a partial response for 26 weeks. The patient with renal cancer did not respond to prior therapy with a farnesyltransferase inhibitor. His response on cisplatin/topotecan lasted 20 weeks. The other 3 patients were not pretreated. The duration of their responses was 21 (NSCLC), 30 (ACUP) and 30^{+} (ACUP) weeks. Twenty-nine patients showed disease stabilization with a median duration of 20 weeks (range 8-68 weeks).

Topotecan and cisplatin pharmacokinetics

The possible effect of drug sequence on the pharmacokinetics of topotecan and cisplatin was investigated in the first 18 patients, that were randomized in a cross-over design for the administration sequence. These patients were all treated at the fixed cisplatin dose of 75 mg/m^2 and topotecan doses of 0.75, 1.0 or 1.25 mg/m^2/dx5. Table 3 lists the main pharmacokinetic parameters from a

compartmental analysis of the two drugs, with topotecan given at 0.75 mg/m^2/dx5. The sequence of drug administration did not significantly influence the disposition of topotecan lactone, indicating mean (±SD) AUC values of 7.52±2.51 and 6.18±1.56 ng.h/mL (P=0.31) using the CT and TC sequence, respectively (Fig. 2). The apparent clearance (CL/f) of topotecan lactone was clearly dose-independent in the range of 0.75 to 1.25 mg/m^2, similar to single agent data, and not significantly different between study courses (107±33.0 (CT) *vs* 109±53.5 L/h/m^2 (TC); P=0.38, paired Student's t-test) (Fig. 1). Similarly, the lactone to total drug AUC ratio (L/T ratio) was independent of the sequence and averaged 0.36±0.04 (CT) *vs* 0.33±0.08 (TC). Topotecan pharmacokinetic parameters obtained on the fifth administration day were essentially similar to the data from day 1 (Data not shown). In order to rule out a potential effect of the interval time between drug administration, kinetic data were obtained from the first 18 patients (3 receiving the CT sequence and another 3 receiving the reversed order at each dose level) treated with a topotecan dose of 0.75, 1.0 or 1.25 mg/m^2/dx5. Data of unpaired analysis in these patients indicated that a change of the interval time to 24 hours had no significant influence on any of the studied parameters (P>0.05, Mann-Whitney's U-test; Table 3).

Table 3: Effect of drug sequence and interval time on the pharmacokinetics of topotecan and cisplatin at a topotecan dose of 0.75 mg/m^2/dx5 and a single fixed cisplatin dose of 75 mg/m^2.

Parameter	*3-hour interval* (n=6)		*24-hour interval* (n=6)	
	CT (d1)	TC (d1)	CT (d1/2)	TC (d1/2)
Topotecan				
C_{max} (ng/mL)	2.05±0.96	1.97±0.55	3.01±1.85	2.86±1.10
$AUC_{(L)}$ (ng.h/mL)	7.52±2.51	6.18±1.56	9.20±3.45	9.89±3.09
$CL/f_{(L)}$ (L/h/m^2)	111±34.0	131±38.3	96.3±40.8	83.5±25.2
L/T ratio	0.36±0.04	0.33±0.08	0.33±0.03	0.33±0.02
Cisplatin				
AUC_{fu} (μg.h/mL)	2.30±1.17	2.29±0.20	3.24±0.27	3.07±0.51
CL_{fu} (mL/min)	817±463	747±177	823±52.2	765±139
AUC_{tot} (μg.h/mL)	33.3±10.5	29.6±4.12	46.5±1.26	45.3±4.08
A_{max} (pg/μg DNA)	2.91±2.33	1.93±1.28	2.41±0.81	3.20±2.78

Data are mean values ± S.D. Abbreviations: C_{max}, peak plasma level; AUC, area under the plasma concentration-time curve; CL/f, apparent clearance; L/T ratio, topotecan lactone to total drug AUC ratio; fu, unbound platinum fraction; tot, total platinum fraction; A_{max}, peak platinum DNA adduct levels in peripheral leukocytes.

The peak plasma levels and the plasma clearance of unbound cisplatin were also independent of the drug sequence with a 3-hour or a 24-hour interval time between administration (Table 3). Over the 3 dose levels studied, the cisplatin clearance was not dependent on the topotecan dose, and averaged 817±463 (CT) *vs* 747±177 mL/min (TC) (P=0.19) with the 3-hour interval time (Fig. 2). Similarly, sequence and topotecan dose had no influence on the protein binding of cisplatin (overall mean: 93.1±2.8%) and on the peak platinum DNA-adduct levels in peripheral blood leukocytes [4.58±4.12 (CT) *vs* 5.72± 4.66 pg Pt/μg DNA (TC) across all 3 dose levels; P=0.55].

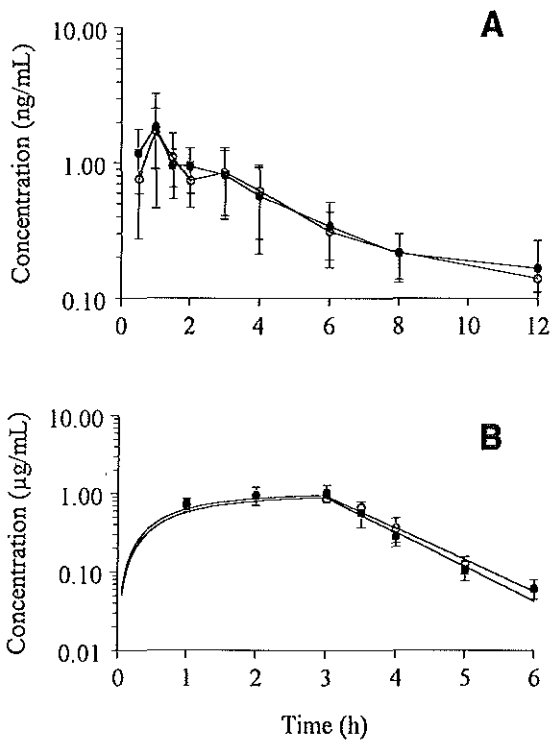

Fig. 2: Plasma concentration-time curves of topotecan lactone (A) and unbound cisplatin (B) (closed symbols: sequence CT; open symbols: sequence TC) in 6 patients treated with topotecan 0.75mg/m²/day and cisplatin 75 mg/m². Data represent mean values (symbol) ± S.D. (error bars).

Table 4: Summary of topotecan pharmacokinetics during the first course as a function of treatment cohort.

Topotecan ($mg/m^2/dx5$)	*n*	$C_{max(L)}$ (ng/mL)	$AUC_{(L)}$ (ng.h/mL)	$CL/f_{(L)}$ ($L/h/m^2$)	L/T ratio
CT sequence					
0.75	6	2.05±0.96	7.52±2.51	111±34.0	0.36±0.04
1.00	6	2.26±0.73	8.38±1.93	126±28.4	0.39±0.05
1.25	10	4.10±1.72	14.5±4.42	99.0±46.0	0.42±0.07
TC sequence					
0.75	6	1.97±0.55	6.18±1.56	131±38.3	0.33±0.08
1.00	6	3.88±2.43	8.27±4.47	95.8±63.8	0.35±0.16
1.25	6	4.33±2.43	14.7±5.64	102±48.3	0.38±0.05
1.50	3	6.87±5.74	18.2±6.11	106±29.0	0.37±0.05
2.00	6	5.92±3.10	18.4±6.74	131±64.6	0.38±0.04
2.30	7	3.72±1.00	18.4±6.15	145±60.1	0.39±0.07

Data are mean values ± S.D. Abbreviations: C_{max}, peak plasma level; AUC, area under the plasma concentration-time curve; CL/f, apparent clearance; L/T ratio, topotecan lactone to total drug AUC ratio; *n*, number of patients studied.

To further assess the effects of cisplatin administration and drug sequence on topotecan pharmacokinetics, all additional patients enrolled in the study had complete sampling performed, with the exception of 3 patients treated at the 2.0 mg/m^2/day topotecan dose and 1 patient (only second course missing) at the 2.3 mg/m^2/day dose. A summary of the topotecan pharmacokinetic data from the first course is provided in Table 4. In both sequence groups, substantial interpatient variability in kinetic parameters was apparent, with more than 2-fold variation in AUC values, although mean values were correlated to the administered dose (Spearman's ρ(rho)=0.76; TC sequence). There were no significant differences in any of the parameters between the topotecan dose levels (P>0.05, Kruskal-Wallis' test), consistent with a linear and dose-independent behavior of the compound. Pharmacokinetic parameters between sequences were again not significantly different. Parameters between the day of topotecan dosing were not significantly different as indicated by the ratio of the topotecan lactone AUC measured on days 1 and 5 (Data not shown), although the mean ratios slightly deviated from 1.0 probably as a result of a minor topotecan accumulation during the

consecutive treatment days. Pharmacokinetic data obtained during the second treatment course, again with sampling performed on days 1 and 5, were essentially similar to the first course (Not shown).

The effect of the topotecan dose on the disposition of unbound and total cisplatin in plasma during the first treatment course is shown in Table 5. None of the pharmacokinetic parameters between the sequences and the various topotecan dose levels was significantly different.

Table 5: Effect of topotecan dose on the pharmacokinetics of cisplatin during the first treatment course at a single fixed cisplatin dose of 75 mg/m^2.

Topotecan (mg/m^2/d$_x$5)	*n*	AUC_{fu} (μg.h/mL)	CL_{fu} (mL/min)	AUC_{tot} (μg.h/mL)	CL_{tot} (mL/min)
CT sequence					
0.75	6	2.30±1.17	817±463	33.3±10.5	88.1±45.1
1.00	6	2.64±1.27	623±310	41.5±6.51	58.6±13.5
1.25	10	2.97±1.12	962±660	37.9±4.39	56.3±7.72
TC sequence					
0.75	6	2.71±0.20	925±177	37.0±4.12	67.5±9.87
1.00	6	2.87±0.35	833±143	35.5±4.45	67.3±11.5
1.25	6	2.58±0.45	874±183	41.6±9.79	55.6±14.8
1.50	3	2.75±0.46	919±174	32.9±3.54	75.3±8.06
1.75	3	3.22±0.27	722±75.0	47.6±4.28	48.8±4.36
2.00	7	3.13±0.50	815±87.0	37.1±6.44	69.7±11.3
2.30	7	2.58±0.23	975±130	36.1±4.89	69.8±7.30

Data are mean values ± S.D. Abbreviations: AUC, area under the plasma concentration-time curve; CL, clearance; fu, unbound platinum fraction; tot, total platinum fraction; *n*, number of patients studied

At the recommended doses for further clinical studies, *viz.* 75 mg/m^2 cisplatin followed by 1.25 mg/m^2/day topotecan (CT) and 2.0 mg/m^2/day topotecan followed by 75 mg/m^2 cisplatin (TC), plasma sampling was also performed on day 2 to ensure that the topotecan disposition did not alter before day 5. Paired analysis showed that all relevant parameters were essentially similar between days of drug administration in both sequences (Table 6), although in the (less myelotoxic) TC sequence, the topotecan lactone peak plasma level and AUC values were slightly higher on day 2 as

compared to days 1 and 5. This is most likely caused by the small number of patients studied (n=4 on day 2), in combination with large intrapatient and interpatient variability in topotecan kinetics.

Table 6: Topotecan and cisplatin pharmacokinetics at the recommended doses during the first treatment course: (1) 75 mg/m^2 cisplatin followed by 1.25 mg/m^2/dx5 topotecan and (2) 2.00 mg/m^2/dx5 topotecan followed by 75 mg/m^2 cisplatin. Data are mean values ± S.D.

	(1) CT sequence (n=10)			*(2) TC sequence* [n=6 (T) or 7 (C)]		
Parameter	day 1	day 2	day 5	day 1	day 2	day 5
Topotecan						
C_{max} (ng/mL)	4.10±1.72	3.31±2.31	3.50±2.25	5.11±3.50	7.52±3.55	6.16±3.30
$AUC_{(L)}$ (ng.h/mL)	14.5±4.42	13.8±4.22	14.4±4.42	15.9±8.79	24.6±8.24	22.6±6.10
L/T ratio	0.40±0.03	0.39±0.07	0.40±0.08	0.36±0.02	0.37±0.04	0.41±0.05
Cisplatin						
AUC_{fu} (μg.h/mL)	2.97±1.12	-	-	-	-	3.13±0.50
CL_{fu} (mL/min)	962±660	-	-	-	-	815±87.0
AUC_{tot} (μg.h/mL)	37.9±4.39	-	-	-	-	37.1±6.44
A_{max} (pg/μg DNA)	3.12±6.52	-	-	-	-	2.34±2.67

Topotecan pharmacokinetic parameters on day 2 were only available from 4 patients. Abbreviations: C_{max}, peak plasma level; AUC, area under the plasma concentration-time curve; L/T ratio, topotecan lactone to total drug AUC ratio; fu, unbound platinum fraction; tot, total platinum fraction; CL, clearance; A_{max}, peak platinum DNA adduct levels in peripheral leukocytes

DISCUSSION

Both cisplatin and topotecan have broad antitumor activity. Because topoisomerase I inhibitors might interfere in the repair of cisplatin induced DNA interstrand cross-links, there has been considerable interest in the effects of combining these classes of drugs. Interaction of topoisomerase I inhibitors with platin derivatives have been studied *in vitro* and *in vivo*. The combination of topotecan and cisplatin was synergistic in teratocarcinoma [10], non-small cell lung cancer [5,11,14,17], ovarian cancer [5,9], esophageal cancer [16], breast cancer [5] and melanoma cell lines and in a human tumor xenograft of small cell lung cancer [13] and ovarian cancer [9]. In contrast, patterns of cross-resistance observed in studies with resistant small cell lung cancer cell lines suggested that topoisomerase I inhibitor-cisplatin combinations might be disadvantageous [12]. The

cytotoxicity of the combination of topotecan and cisplatin was also dependent on the schedule used. When V79 chinese hamster lung fibroblast were exposed to cisplatin early in the course of topotecan treatment synergy was most prominent [43]. This phenomenon was also confirmed in IGROV-1 ovarian cancer and MCF7 breast cancer cell lines. Incubation of these cells with cisplatin followed by topotecan resulted in optimal synergism [5]. However, in other cell lines variations in the scheduling of cisplatin and topotecan did not influence the observed interaction [5,17]. The potential importance of sequence dependence for the combination of cisplatin and the intravenous formulation of topotecan in the clinical setting was studied by Rowinsky et al., revealing enhanced myelosuppression when cisplatin administration preceded topotecan [17]. Recently, an oral formulation of topotecan with a bioavailability of 32-44% became available, which is a more convenient method of drug administration [31-33]. The reported phase I study was performed to explore the influence of alternate sequences of oral topotecan in a daily times five schedule and cisplatin on the observed side-effects and pharmacokinetic behavior of both drugs and to determine the maximum tolerated dose of topotecan in combination with cisplatin 75 mg/m² once every 3 weeks in both sequences.

Both neutropenia and diarrhea were the DLTs of oral topotecan combined with cisplatin in this schedule. Other toxicity was usually mild to moderate and consisted of nausea and vomiting, mucositis, fatigue, neuro- and nephrotoxicity and alopecia.

Myelosuppression was significantly more severe when cisplatin preceded topotecan administration. This observation is in accordance with the data reported for the combination of the intravenous formulation of topotecan and cisplatin [17]. The onset of the neutropenia was relatively late with a median day of onset of the nadir on day 19 (range 4-30). These data are in line with the data reported by Miller et al. [44]. The combination of topotecan, administered intravenously on day 1 to 5, with cisplatin on day 1 resulted in a neutrophil nadir around day 12 (range 8-25). Compared to the median time to neutrophil nadir of 12 days (range 9-15) for single agent oral topotecan [34] and day 9 (range 6-10) [19] for single agent intravenously administered topotecan, the nadir in our study was delayed. This resulted in treatment delay due to prolonged myelosuppression in 34% of the courses. Despite grade 3 or 4 neutropenia was observed in 31% of the courses, the incidence of neutropenic fever was only 2%.

The doses in this sequence, cisplatin followed by topotecan, we can recommend for phase II studies are oral topotecan 1.25 mg/m²/day day 1 to 5 and cisplatin 75 mg/m², but only in non- or minimally pretreated patients in good clinical condition and under strict medical surveillance, comparable to the patients treated in this study. In other circumstances dose adjustment of topotecan should be considered. In other phase I studies, combining cisplatin 50 mg/m² on day 1 with i.v. topotecan as a 30-min infusion daily for 5 consecutive days, neutropenia and thrombocytopenia constituted the principal toxicities. The recommended dose of topotecan for further trials was 0.75 to 1.0 mg/m²/day combined with cisplatin 50 mg/m², accounting for 50-66% of the single agent

intravenous dose of topotecan [17,44]. This percentage is similar to our results where the recommended dose of oral topotecan in this schedule is 54% of single agent oral topotecan in a daily times five schedule [34]. We realize that oral availability of topotecan is not taken into account. However, the relevance of bioavailability becomes questionable in view of the results of recent studies indicating that the oral administration of topotecan at a dose of 2.3 mg/m²/day is as effective as the intravenous administration of topotecan 1.5 mg/m²/day [35, 36] in both ovarian cancer and small cell lung cancer.

For the reversed sequence, the recommended doses are oral topotecan 2.0 mg/m²/day day 1 to 5 followed by cisplatin 75 mg/m². This constitutes a topotecan dose of 87% of the single agent dose. However, as indicated, it is recommended that the use of topotecan and cisplatin at these doses should be limited to patients similar to those studied in this trial, i.e., untreated or minimally pretreated patients with a good performance status under strict medical surveillance. In all other circumstances, topotecan dose reduction is recommended. This sequence of drug administration was also studied for the combination of the intravenous formulation of topotecan for 5 consecutive days in escalating doses and cisplatin 50 mg/m² in an alternating schedule with carboplatin, cisplatin, teniposide and vincristine in patients with small-cell lung cancer [45]. Preliminary data indicate that it is feasible to combine i.v. topotecan 1.5 mg/m²/day, the recommended dose of single-agent topotecan, with cisplatin 50 mg/m². Thus, the observed hematological toxicity is sequence dependent both for the intravenous and the oral formulation of topotecan in combination with cisplatin, resulting in a higher dose intensity of topotecan when administered before cisplatin.The observed pharmacokinetic parameters of the lactone and the carboxylate form of topotecan demonstrated linear and dose independent behavior over the total dose range studied and were similar to single agent data [46] and also comparable to the data obtained in the schedule with 24 hour interval between the administration of topotecan and cisplatin in our study, indicating no apparent pharmacokinetic interaction between topotecan and cisplatin. The sequence of drug administration also had no influence on the pharmacokinetics of topotecan at the dose levels administered, neither on day 1, 2 or 5. This is in contrast with the reported reduction of the clearance of intravenously administered topotecan observed after preceding cisplatin administration [17]. Sequence-dependent differences in toxicity and pharmacokinetics can be obscured by a large intra-patient variability in AUC. However, the intrapatient variability in AUC of topotecan lactone for oral topotecan expressed as coefficient of variation (CV) is 18.5% [34] and is comparable with the intrapatient variability in AUC observed after intravenous administration of topotecan (CV is 12.6%) [47]. Since patients were treated in a cross-over design, sequence dependent toxicological and pharmacological differences could be assessed as accurately as in an IV study.

Also the ratio of topotecan AUC of lactone to total drug correspond very well with data of a previous study in which oral topotecan was administered as a single agent [34] and did not vary with the sequence of drug administration.

The plasma clearance and volume of distribution of unbound cisplatin as well as the AUC up to the last measured time point of total cisplatin in plasma indicated no significant influence of topotecan on the protein binding and plasma disposition of cisplatin. Preclinical studies indicated that the reversal of cisplatin-induced DNA interstrand cross-links was delayed by concomitant incubation with a topoisomerase I inhibitor [48-50], without modifying their formation. However, in our study the values of the maximal platinum DNA-adduct formation in peripheral leukocytes and the area under the DNA-adduct versus time curve were consistent with single agent data [42], and were independent of the drug sequence. Although the preclinical observations might not be extrapolated to the clinical setting, it is possible that the extreme variability in platinum DNA-adduct values would not allow any small alteration in adduct formation to be observed even if it was present. It is also possible that other mechanisms may contribute to the enhanced toxicity observed for the sequence CT. In *in vitro* studies induction of topoisomerase I [51] and enhanced topoisomerase I inhibitory activity [52] were observed after incubation with cisplatin followed by the administration of a topoisomerase I inhibitor. Simultaneous incubation of platinum derivatives and topoisomerase I inhibitors resulted in enhanced S-phase arrest in human colon and ovarian cancer cell lines indicative of increased topoisomerase I inhibitor-induced cytotoxicity [48,49]. This observation might indicate that the synergistic toxicity observed for the combination of topoisomerase I inhibitors and platinum derivatives can partly be explained by a modification in cellular response to DNA damage.

Based on the available data, the importance of the sequence of drug administration and the enhanced toxicity observed when cisplatin is followed by topotecan can not simply be extrapolated to the antitumor activity of the combination. However, a sequence dependent effect on antitumor activity can not be ruled out. Further randomized phase II studies in patients with topotecan sensitive tumor types are needed to elucidate the importance of drug sequencing and possible cytotoxic interaction, and the potential relevance of the higher dose intensity of both drugs, that can be achieved when the less toxic sequence of drug administration is used.

In conclusion, the recommended dose for phase II studies in selected patients is oral topotecan 1.25 mg/m²/day for 5 consecutive days combined with cisplatin 75 mg/m² on day 1, once every 3 weeks, or topotecan 2.0 mg/m²/day day 1 to 5 followed by the same dose cisplatin on day 5. No pharmacokinetic interaction could explain the enhanced myelosuppression observed in the sequence CT.

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Chapter 2d

Inter- and intra-patient variability in oral topotecan pharmacokinetics: implications for body-surface area dosage regimens

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ABSTRACT

Anticancer drugs still are dosed based on the body-surface area (BSA) of the individual patient, while the BSA is not the main predictor of the clearance for the majority of drugs. The relevance of BSA-based dosing has not been evaluated for topotecan yet. A retrospective pharmacologic analysis was performed of kinetic data from 4 clinical phase I studies in which topotecan was administered orally as single agent combined with data from a combination study of topotecan and cisplatin. A strong correlation (r=0.91) was found between the area under the plasma concentration time curve of the lactone and carboxylate forms of topotecan by plotting 326 data sets obtained from 112 patients receiving oral topotecan at dose levels ranging from 0.15 - 2.70 mg/m^2. The intra-patient variability, studied in 47 patients sampled for 3 or more days, for the apparent lactone clearance (CL/F), ranged 7.4 - 69% (mean 24 ± 13%, median 20%). The inter-patient variabilities in the CL/F, calculated with the data of all studied patients, expressed in L/h/m^2 and in L/h were 38% and 42%, respectively. In view of the relatively high inter- and intra-patient variabilities in topotecan clearance, in contrast to a variability of only 12% in the BSA of the studied patients, no advantage of BSA-based dosing was found over fixed dose regimens.

INTRODUCTION

Most anticancer drugs are dosed based on the body-surface area (BSA) of the individual patient, with the aim of reducing inter-patient variability of drug exposure. However, this strategy failed for most drugs [1], except for docetaxel (Taxotere®), where BSA has been shown to be a main predictor of the clearance in a pharmacokinetic model [2]. The relevance of this concept of BSA-based dosing has not been evaluated for topotecan (Hycamtin®), one of the most promising agents that entered clinical practice in the recent years. Topotecan has antitumor activity against various human malignancies, and relationships have clinically been found between systemic exposure of topotecan and hematological toxicity [reviewed in 3-5], and with antitumor activity in preclinical models [6]. The bioavailability of oral topotecan in adult cancer patients ranged from 30 - 44% with inter-patient variabilities of 26 - 31% [7-9]. However, since pharmacokinetic analysis in most patients [10-12] has only been carried out for 2 days, a reliable estimate of the intra-patient variability of orally administered topotecan has not yet been established. The recommended dose for single agent oral topotecan is 2.3 mg/m^2/day for 5 days every 3 weeks. As alternative a fixed dose of 4 mg/day for 5 days every three weeks was proposed [10]. However, thusfar studies on oral topotecan were performed by dosing patients based on their body-surface area instead of using fixed dose regimens. Since most patients will

take their oral medication in an outpatient setting, it is important to keep dosing regimens as simple as possible and because of this it would be most practical to use fixed dosing regimens [13]. The aim of the present analysis of kinetic data from several phase I studies was to investigate, whether dosing of oral topotecan in the treatment of adult cancer patients based on body-surface area of individual patients has any advantage over fixed dose regimens.

PATIENTS AND METHODS

Patient selection

The patient selection criteria were fully described elsewhere for the oral phase I studies in which topotecan was administered as single agent [3] and for the study in which oral topotecan was combined with i.v. cisplatin [14]. In short, patients with a confirmed diagnosis of a malignant solid tumor resistant to standard chemotherapy regimens were eligible for these studies. Age should be between 18 and 75 years and performance status, defined by the Eastern Cooperative Oncology Group, had to be ≤ 2. No previous anticancer therapy for at least 4 weeks was allowed. Adequate hematopoietic and renal functions were required, patients with mildly impaired liver functions (i.e. total serum bilirubin $\leq$ 1.25 x upper normal limit, ASAT and ALAT ≤ 2 x upper normal limits and in case of liver metastases ≤ 3 x upper normal limits) were allowed to participate in the described studies. A specific exclusion criterion was the existence of any gastrointestinal circumstance, which could alter the absorption of topotecan. All patients signed informed consent.

Treatment schedules in the single agent phase I studies

Oral administration, using the i.v. formulation, of single agent topotecan was studied in 4 phase I studies [3]. The first study involved a twice-daily dosing of topotecan at dose levels of 0.15 - 0.60 mg/m^2 for 21 days, repeated every 28 days [3, 11]. In the second study, topotecan was administered twice-daily for 10 days, every 21 days, at dose levels of 0.50 - 0.80 mg/m^2 [3, 12]. In the third study the administration of topotecan was reduced to once a day for 10 days at dose levels ranging from 1.00 - 1.60 mg/m^2 [3, 12]. The final phase I study of single agent oral topotecan involved a once daily administration for 5 days, also repeated every 21 days, and included dose levels ranging from 1.20 - 2.70 mg/m^2 [3, 10]. In the daily times 5 schedule also patients were included who were treated with a fixed dose of 4 mg/day.

Treatment schedule in the combination phase I study

The latter mentioned regimen of oral topotecan, this time at dose levels ranging form 0.75 - 2.30 mg/m^2/day for 5 days, using the drug formulated in gelatin capsules, was also studied combined with a fixed dose of 75 mg/m^2 of i.v. cisplatin [14]. In the present report we also included pharmacokinetic-data of patients, treated with oral topotecan daily times 5, at dose levels of 1.50 and 1.75 mg/m^2 preceded by a 3 h cisplatin infusion at 50 mg/m^2 on day 1 of each course in an ongoing study using the same eligibility criteria as previously reported [14].

Pharmacokinetic sample collection and analysis

Blood samples were collected, upto 12 h after dosing [10-12, 14], in 4.5 ml glass tubes containing lithium heparin as anticoagulant and were centrifuged within 10 minutes to separate the plasma. Subsequently, the plasma was deproteinized by 4 fold dilution in ice-cold (-20°C) methanol, resulting in a stabilized lactone to carboxylate ratio [15], and stored at -80°C upon analysis. Simultaneous determination of the lactone and carboxylate form of topotecan was performed by a reversed-phase high-performance liquid chromatographic (HPLC) method as described [15], with minor modifications for the analysis of drug levels in the combination phase I study [14].

On the basis of the best fitted curves, two and three compartmental analysis models after zero-order input were used for the calculation of the area under the plasma concentration-time curves (AUC)$_{0\text{-infinity}}$ of the lactone as well the carboxylate form of topotecan as described [14]. The apparent oral clearance (CL/F) of topotecan lactone was calculated by dividing the dose per m^2 by the observed lactone AUC, expressed in L/h/m^2. The absolute CL/F, expressed in L/h, was calculated by dividing the absolute dose by the AUC of topotecan lactone.

Statistical analysis

Linear regression analysis was performed, using the NCSS package (Version 5.X; J.L. Hintze, East Kaysville, UT, 1992), to test potential relationships between evaluated parameters. One way analysis of variance was performed to evaluate statistically significant differences ($p<0.05$) between groups, using the same program.

RESULTS

In the 4 phase I studies on single agent oral topotecan 56 patients were evaluable for pharmacokinetic analysis for a total of 114 kinetic days (table 1). The majority of evaluable days

Table 1: Evaluable pharmacokinetic days in the 4 single agent phase I studies of orally administered topotecan.

Dose (mg/m^2)	twice daily	once daily	No. of pts	total number of evaluable kinetic courses				
				day 1	day 4	day 8	day 21	total
0.15	21 days		2	2		1		3
0.30	21 days		3	3		3		6
0.40	21 days		2	3		2	2	7
0.50	21 days		4	4		4		8
0.60	21 days		3	3		3		6
0.50	10 days		1	1		1		2
0.60	10 days		4	3		4		7
0.70	10 days		4	4		4		8
0.80	10 days		1	1				1
1.00		10 days	3	3		3		6
1.40		10 days	3	3		3		6
1.60		10 days	4	4		4		8
1.20		5 days	3	3	3			6
1.80		5 days	3	3	3			6
2.30		5 days	6	6	6			12
2.70		5 days	4	4	4			8
1.25 [a]		10 days	1	1		1		2
4.00 [a]		5 days	6	6	6			12
total [b]			56	57	22	33	2	114

a: Fixed dose

b: One patient in the twice daily 0.40 mg/m^2 was also sampled during course 2 and one patient in the once daily 1.40 mg/m^2 was also sampled during a course with a fixed dose of 1.25 mg

were obtained in the phase I trial studying the combination of oral topotecan and i.v. cisplatin, in which 56 patients were sampled for a total of 228 days (table 2).

By plotting 326 data sets (95% of total) of kinetic days for which both the lactone and the carboxylate AUC could be assessed, a strong linear relationship was found between the AUCs of the pharmacologic active lactone form of topotecan and its inactive ring-opened carboxylate form ($AUC_{carboxylate} = 1.62 + 1.57*AUC_{lactone}$, $r=0.91$, fig 1). For further estimation of inter- and intra-patient variabilities and their implication for BSA dosage regimens, pharmacokinetic parameters of the lactone form were used in this analysis.

Table 2: Evaluable pharmacokinetic days in the phase I study in which oral topotecan was combined with i.v. cisplatin.

Dose ($mg/m^{2)}$	No of pts	Total number of evaluable kinetic days										
		C1d1	C1d2	C1d5	C2d1	C2d5	C3d1	C3d5	C6d1	C6d2	C6d5	Total
0.75	6	6		6	6	6	6	6				36
1.00	6	6		6	6	6	6	6				36
1.25	11	10	4	11	5	5	4	4	2	2	1	48
1.50	9	9	5	8	3	3			1	1	1	31
1.75	10	10	6	9	3	3						31
2.00	8	6	3	7	4	4						24
2.30	7	7		7	4	4						22
Total	56[a]	54	18	54	31	31	16	16	3	3	2	228

a: 1 patient received 2.30 (Course 1) and 2.00 mg/m2 (Course 2)

Inter and intra-patient variability

Since the majority of the patients in the single agent oral phase I studies were sampled for pharmacokinetic analysis only for 2 days during 1 cycle, the intra-patient pharmacokinetic parameter variabilities for topotecan lactone were studied using the data obtained in the topotecan/cisplatin combination phase I study, in which pharmacokinetic sampling was performed during several cycles. As already reported, there was no pharmacokinetic interaction

between oral topotecan and i.v. cisplatin [14]. In view of this, all kinetic days (with and without cisplatin) were used for the present analysis. The intra-patient variability in AUC and CL/F of topotecan lactone was calculated as the standard deviation divided by the average, only using data of the 47 patients which at least had 3 evaluable kinetic courses of the AUC of topotecan lactone. The averaged intra-patient variability in the lactone AUC across all dose-levels was 24 ± 13% (median 20%, range 7.6 - 61%). The averaged intra-patient variability in the CL/F, expressed in L/h/m^2 and in L/h, was 24 ± 13% (median 20%, range 7.4 - 69%).

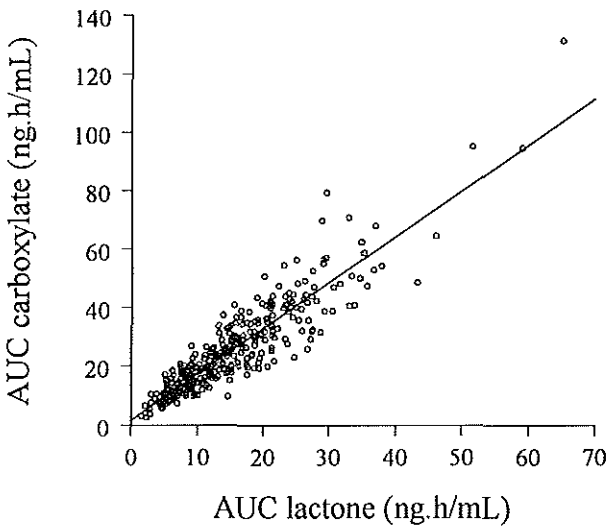

Fig 1: Relationship between the AUCs of the lactone and carboxylate form of topotecan in 326 evaluable data sets

The inter-patient variability in CL/F, expressed in L/h/m^2 as well as in L/h, was calculated using the data of all the patients, by using the averaged apparent CL/F of all kinetic days of each patient as single value. The averaged apparent CL/F was 103 ± 39.0 L/h/m^2 (CV=38%, n=107, fig 2a), with no significant difference (p=0.074) in the CL/F over the 19 studied dose-levels.

The averaged apparent CL/F, studied over 27 different individual dosages, was 194 ± 80.4 L/h (CV=42%, n=107, fig 2b) or 195 ± 81.1 L/h (CV=42%, n=114, fig 2b), by inclusion of the patients treated with a fixed dose.

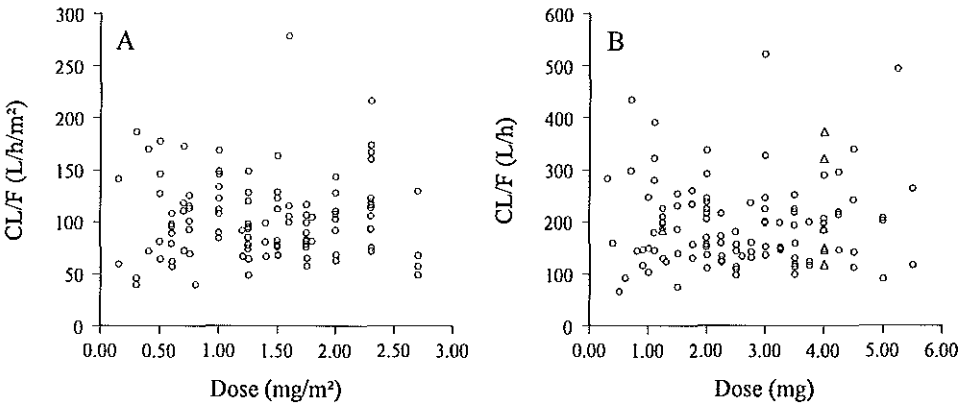

Fig 2: Plot of dose/m^2 (A) and absolute dose (B) and the averaged apparent CL/F (patients with a fixed dose are indicated by triangles).

In addition, no alteration in topotecan lactone kinetics was found (p=0.30) after multiple (up to 6) courses (fig 3), using the data of patients treated in the combination phase I study, in which patients were samples for multiple courses.

BSA as determinant for dose calculations

As shown in fig 2 and 3, the apparent CL/F was constant over the studied dose-ranges and courses. In fig 4 the BSA is plotted versus absolute apparent CL/F in L/h (mean ± S.D.), calculated with the actual dose given to each individual patient. A poor positive relationship was found between BSA and the average apparent CL/F (CL/F = 52.4 + 75.1*BSA, r=0.29), with large variabilities in the apparent CL/F across all studied BSA values in the 47 patients with 3 or more pharmacologically evaluable courses.

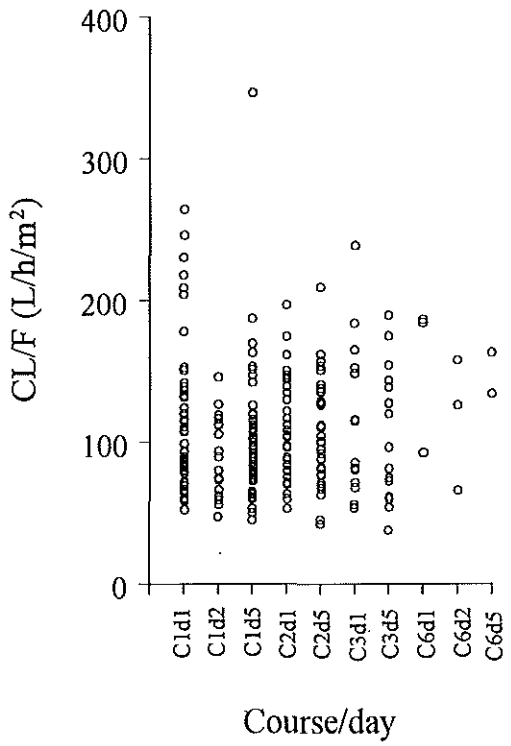

Fig 3: Apparent CL/F during multiple courses and days.

DISCUSSION

Dosing of most cytotoxic agents is commonly based on the BSA of patients, intending to reduce inter-patient pharmacokinetic variabilities of a compound. This, in turn, is based on an assumed relationship between the clearance of a compound and the BSA of the individual patient [1, 13]. However, calculation of the exact clearance of topotecan is not feasible since the compound has a reversible conversion from the lactone to the carboxylate form. Moreover, accurate dosing of oral topotecan based on BSA is also not feasible, since for oral use the drug is now only available as gelatin capsules containing 1.0 and 0.25 mg respectively, resulting in the necessity of rounding of the absolute dose to the nearest quartile mg. Since excretion by the kidneys is a major route of elimination of topotecan [reviewed in 4] and alterations in the pharmacokinetic parameters for topotecan have only been described in patients with a renal

dysfunction, i.e. creatinine clearance < 60 mL/min [16], in the set of studies we performed an altered topotecan clearance was not expected. The apparent CL/F of oral topotecan, in patients with a normal renal function, is highly dependent on the absorption of the lactone form from the gastrointestinal tract.

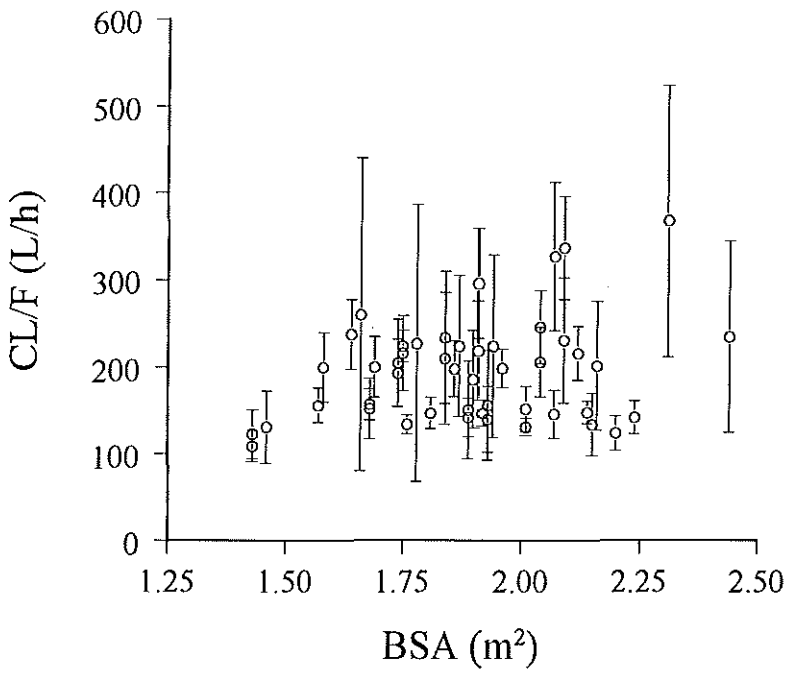

Fig 4: Body-surface area (BSA) versus absolute apparent CL/F expressed in L/h (mean ± S.D.).

The oral bioavailability of topotecan in adult cancer patients for drinking of the i.v. solution ranged from 30 ± 7.7% [7] to 44% [9] and was found to be 42 ± 13% for the drug formulated in gelatin capsules [8]. In these clinical studies inter-patient variabilities in the oral availability in adults ranged from 26 - 31% [7, 8], which is not dissimilar to the inter-patient variability of 38

and 42% for the apparent CL/F expressed in L/h/m^2 and in L/h, respectively, in our studies. Since the inter-patient variability was calculated with the averaged apparent CL/F of topotecan lactone, using the data of patients which were studied up to 6 times, this variability might even be underestimated.

Only limited information was available on the intra-patient variability of orally administered topotecan in adult cancer patients. Gerrits et al. [3] reported intra-patient variabilities in AUC of topotecan lactone of 25 ± 31% (n=22) and 35 ± 25% (n=10) in clinical phase I studies in which topotecan was administered either once daily times 5 or 10 respectively, and of 97 ± 70% (n=10) and 60 ± 51% (n=13) in twice daily times 10 and 21 schedules, respectively. Since samples were only collected for pharmacokinetic analysis on 2 treatment days during one cycle, an accurate estimation of the intra-patient variability was not possible. In this present analysis we assessed the intra-patient variability using data of 47 patients, who were sampled on 3 - 6 days each, resulting in an average intra-patient variability of the lactone AUC of 24 ± 13% (median 20%) with a range of 7.6 - 61%.

The broad range in the intra-patient variability in lactone AUC after oral administration of topotecan is probably related to the fact the carboxylate form is poorly absorbed from the small intestine, while the lipophilic pharmacologic active lactone form of topotecan is able to pass the membranes of the small intestine [4]. Since the pH in the small intestine ranges from pH 5 - 7 and the rate of interconversion between the lactone and carboxylate form of topotecan is pH dependent, the amount of topotecan which is available for absorption is related to a fluctuation in the pH.

We did not find saturation of the absorption, tissue distribution or elimination of orally administered topotecan over the studied dose-range of 0.15 - 2.70 mg/m^2 apparent from a lack in significant difference in the observed CL/F over the dose range studied. Also administration of multiple (up to 6) courses of orally administered topotecan did not alter the apparent topotecan lactone CL/F.

The inter-patient variability in the topotecan CL/F of 38 and 42%, expressed in L/h/m^2 and L/h respectively, is much larger than the 12% inter-patient variability in BSA of our patients (average BSA 1.9 ± 0.22 m^2, n=107). In view of the intra-patient variability of 24 ± 13% in the apparent lactone CL/F, with individual variabilities upto 69%, the inter-patient variability in the bioavailability of 26 - 31% and the poor relationship between the BSA and the average apparent CL/F, we feel that there is no scientific rationale for BSA-based dosing of orally administered topotecan in adult patients. This confirms our previous observation of similar pharmacokinetics after oral administration of either 2.3 mg/m^2 of topotecan or a fixed dose of 4 mg [10], which was already suggesting that fixed-dose regimens could be applied.

In conclusion, in view of the relatively high intra- and inter-patient variabilities in the AUC and CL/F of topotecan lactone and the relatively small range in observed BSA, oral topotecan can be added to the list of agents where BSA-adjusted dosing does not appear definitely better [1]. We recommend a fixed dose regimen for future use in clinical trials, which is more convenient for the oncologist and the pharmacist, is more cost-effective and last but not least, a fixed dose regimen is less cumbersome for the patients. Further randomized clinical studies in a large population are needed to fully explore the advantages of fixed dose regimens of orally administered topotecan, in which simultaneously the need for potential dosage adjustments at extreme BSA values have to be investigated.

A careful study of inter-patient variability of topotecan AUC in patients of the same BSA, renal and hepatic function, to look at the effects of factors as age, gender, protein binding, and inherited or acquired metabolic function in addition to expression of the MDR-1 P-glycoprotein and BCRP drug-transporting proteins [17] in intestinal tissues as an explanation for this variability, is currently being conducted.

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Chapter 2e

Gender-dependent pharmacokinetics of topotecan in adult patients

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ABSTRACT

Background Gender-dependent differences in the clinical pharmacokinetic behavior of various drugs have been documented previously. Most commonly, these differences are associated with differences in body composition, renal elimination, drug absorption or hepatic metabolism. Gender-dependent differences in the pharmacokinetics of topotecan (Hycamtin®) have not yet been described.

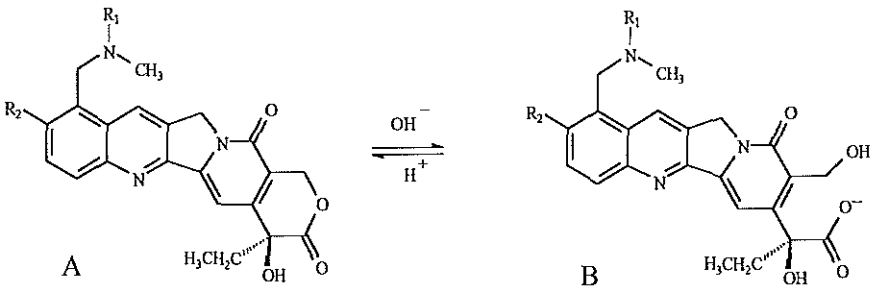
Patients and methods Pharmacokinetic data of the lactone and carboxylate forms of topotecan were derived from clinical studies in which topotecan was administered either orally or intravenously to a total of 55 males and 37 females.

Results A significant difference (P=0.0082) of 38% was found between the apparent clearance of topotecan lactone after oral administration in males (237±105 L/h) and females (163±62.5 L/h). When adjusted for body-surface area, this difference remained significant (P=0.031). Similarly, differences were noted in the percentage of topotecan in the lactone form (37.1±5.32% versus 41.7±6.51%, P=0.0076). Statistical analysis revealed that individual hematocrit values, which were consistently lower in females (P<0.023), were a significant predictor of the apparent topotecan lactone clearance. This was confirmed experimentally in *in vitro* incubation studies in whole blood using artificially altered hematocrit values and in blood samples from both male and female volunteers.

Conclusion Topotecan is thus subject to significant gender-dependent differences in pharmacokinetics that arise as a result of a physiological difference in hematocrit values between males and females. This finding may have significant implications for the interpretation of the relationships between pharmacokinetics and pharmacodynamic outcome of topotecan treatment, and may provide a basis for the development and refinement of future clinical protocols.

INTRODUCTION

Topotecan (Hycamtin®, fig 1), a water-soluble semisynthetic analogue of the topoisomerase I inhibitor camptothecin, is one of the most promising new anticancer agents. Single agent topotecan, administered intravenously (i.v.), has demonstrated antitumor activity against various solid tumors in adult cancer patients, including metastatic ovarian and small cell lung cancer. Most responses were achieved using a daily times 5 schedule in which topotecan was administered as a 30-min infusion [reviewed in 1]. Since daily i.v. administration of topotecan



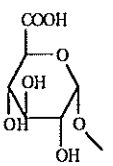
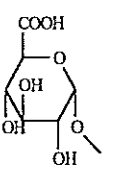
Compound	R1	R2
Topotecan	CH_3	OH
N-desmethyl topotecan	H	OH
Topotecan-*O*-glucuronide	CH_3	
N-desmethyl topotecan-*O*-glucuronide	H	

Fig. 1: Chemical structures of the lactone (A) and carboxylate (B) forms of topotecan and its known human metabolites.

for 5 days, repeated every 21 days, is inconvenient for patients, an oral formulation of topotecan has been developed with a bioavailability of 42 ± 13% [2]. Different administration schedules of oral topotecan have been evaluated in clinical studies, including once daily times 5 and 10 and twice daily times 10 and 21 administrations, from which the once daily times 5 schedule was recommended for future clinical studies [reviewed in 3]. The need for further clinical development of the oral topotecan formulation became even more important in view of recent findings that the oral formulation has similar efficacy as compared to the i.v. formulation, while less hematological toxicity was observed [4,5].

The pharmacokinetic profile of topotecan is quite complex since it can undergo a spontaneous pH-dependent interconversion between a pharmacologic active lactone form and an inactive carboxylate form (fig 1). Gender-dependent differences in pharmacokinetic behavior have been described for a wide variety of compounds over the last few decades. Most commonly, these have been shown to be associated with differences in body composition, renal elimination, drug absorption or hepatic function [reviewed in 6]. In this report, we describe gender-dependent differences in topotecan pharmacokinetics after both oral and i.v. administration, and present in vitro studies to provide a formal explanation of this phenomenon.

PATIENTS AND METHODS

Patient selection criteria

All patients included in the studies had a histologically or cytologically confirmed diagnosis of a malignant solid tumor, refractory to standard therapy or for which no recognized therapy was available. The patients participated in either a phase I study, in which oral topotecan was combined with i.v. cisplatin [7], or a phase II study of single agent topotecan administered as a 21-day continuous i.v. infusion [8]. The eligibility criteria, treatment plans and detailed clinical profiles have been fully described elsewhere [7,8].

Drug administration

SmithKline Beecham Pharmaceuticals (Harlow, UK) supplied capsules containing either 0.25 or 1.0 mg of topotecan lactone and a lyophilized vial preparation containing 5 mg of topotecan lactone. Orally administered topotecan was studied at dose levels of 0.75, 1.00, 1.25, 1.50, 1.75, 2.00 or 2.30 mg/m^2/day for 5 days, repeated every 3 weeks, in combination with a fixed dose of 75 mg/m^2 cisplatin by a 3 h infusion, in 49 patients [7]. A total of 10 patients, treated with oral topotecan daily times 5, at dose levels of 1.50 or 1.75 mg/m^2, preceded by i.v. cisplatin at a dose of 50 mg/m^2 on day 1 of each course, was also included in this study. In the i.v. phase II study, topotecan was

administered as a 21-days continuous infusion at dose levels of 0.50 and 0.60 mg/m^2/day at an infusion rate of 6 mL/24 h, using ambulatory pumps, repeated every 28 days [8].

Blood sample collection and analysis

Blood samples were collected in 4.5-mL glass tubes containing lithium heparin as anticoagulant. Following oral administration, samples were obtained prior to dosing, and 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 12 h after dosing on day 1 [7]. Similarly, in the i.v. trial blood was withdrawn prior to infusion, and at steady state on day 8 after start of dosing [8]. The blood samples were centrifuged immediately at the site of the patient to separate the plasma. The plasma samples were directly deproteinized by 4-fold dilution in ice-cold (-20°C) methanol, resulting in a stabilized lactone to carboxylate ratio [9], and stored at –80°C upon analysis. Simultaneous determination of the lactone and carboxylate form of topotecan was performed by a reversed-phase high-performance liquid chromatographic method as described [9].

Pharmacokinetic analysis

In the present pharmacokinetic analysis we used the pharmacokinetic data of day 1 of course 1 in the oral phase I study [7] and of day 8 in the continuous infusion phase II study [8]. The area under the plasma concentration-time curve (AUC) of total topotecan, i.e. lactone plus carboxylate, and the topotecan lactone and carboxylate forms in the oral phase I study were calculated by noncompartmental and two or three compartmental analysis models after zero-order input. The apparent clearance of topotecan lactone and the clearance of total topotecan in the oral phase I study were calculated by dividing the dose in mg/m^2 or the absolute dose in mg by the observed AUC and were expressed in L/h/m^2 and L/h, respectively. While in the i.v. study, the clearances were calculated by dividing the rate of infusion divided by the steady-state plasma concentration. The apparent terminal disposition half-life of topotecan lactone and carboxylate were calculated as ln2/k, in which k represents the rate constant of the terminal disposition phase. The lactone to total ratio in the oral phase I study was defined as the AUC_L/AUC_T, while the times to reach the maximum concentration for topotecan lactone and carboxylate were determined graphically. The lactone to total concentration ratio in the 21-days continuous infusion study was calculated as the concentration of the lactone form divided by the concentration of total topotecan.

In vitro experiments

From 5 male volunteers a volume of 12 ml heparinized blood was collected, from which 6 mL was centrifuged for 5 min at 2000*g* to separate plasma and blood cells. The plasma supernatants, combined with the buffy-coat, and the remaining red blood cell fractions were collected. Fractions of the whole blood, the red blood cells and the plasma were combined to

create different hematocrit values in blood of the same individual, ranging between 0.20 and 0.60 L/L. A volume of 1 mL of these samples was incubated simultaneously with 5 ng/mL of topotecan lactone and 5 ng/mL of topotecan carboxylate for 15 minutes at 37°C to study the impact of the hematocrit on the lactone to total concentration ratio in the plasma compartment. The blood samples were further processed as described above for the blood samples of the patients. The remaining fractions were used for the determination of hematocrit values. To confirm the gender-related difference in the topotecan pharmacokinetics, 1 mL of normal heparinized whole blood of 5 female and 5 male volunteers was incubated with 5 ng/mL of topotecan lactone and carboxylate and further processed as described above.

Statistical analysis

All parameters are reported as mean values ± SD. Two-tailed unpaired Student's t-tests were performed to evaluate statistically significant differences ($P<0.05$) in pharmacokinetic and biochemical parameters between males and females, using the NCSS package (Version 5.X; J.L. Hintze, East Kaysville, UT, 1992). Linear regression analysis was performed to test potential relationships between parameters, using the same program.

RESULTS

Clinical pharmacokinetics

A total of 54 patients (36 male and 18 females) enrolled in the oral phase I study was evaluated for pharmacokinetic analysis during day 1 of course 1. Since cisplatin has no effect on the pharmacokinetics of topotecan [7], courses without and in combination with i.v. administered cisplatin were used for the determination of gender-dependent differences in topotecan pharmacokinetics. The pharmacokinetic and biochemical characteristics of the evaluable patients are listed in table 1. The apparent clearance of topotecan lactone was significantly 1.4-fold faster in males as compared to females ($P=0.0082$), while after correction for the body-surface area the apparent clearance of the lactone form was remained significantly 1.3-fold faster in males ($P=0.031$). Interestingly, no significant differences were observed in the clearance of total topotecan. The lactone to total ratio of the AUC was significantly 1.3-fold higher in females ($P=0.0076$) and a significant correlation ($r=0.35$, $P=0.0086$) was found between the lactone to total AUC ratio and the apparent clearance of topotecan lactone (fig 2a). Linear regression analysis was performed between, respectively, the significantly different biochemical characteristics body-surface area and hematocrit and the apparent clearance of topotecan, expressed in L/h as well as in $L/h/m^2$. A significant relationship was found between

Table 1: Pharmacokinetic and biochemical characteristics

	Males	Females	p
Oral phase I study			
L/T (%)	37.1 ± 5.32 (n=36)	41.7 ± 6.51 (n=18)	0.0076
CL/F_L (L/h)	237 ± 105 (n=36)	163 ± 62.5 (n=18)	0.0082
CL/F_L (L/h/m^2)	123 ± 53.3 (n=36)	92.4 ± 33.3 (n=18)	0.031
CL/F_T (L/h)	85.0 ± 33.3 (n=36)	69.1 ± 29.9 (n=18)	NS
CL/F_T (L/h/m^2)	44.7 ± 17.9 (n=36)	38.5 ± 14.6 (n=18)	NS
$Tmax_L$ (h)	1.63 ± 1.25 (n=36)	1.92 ± 1.60 (n=18)	NS
$T½_L$ (h)	3.65 ± 1.05 (n=36)	3.47 ± 0.722 (n=18)	NS
BSA (m^2)	1.96 ± 0.204 (n=36)	1.77 ± 0.204 (n=18)	0.0003
Ht (L/L)	0.39 ±0.037 (n=30)	0.36 ± 0.041 (n=16)	0.015
CL_{creat} (mL/min)	89 ± 20 (n=21)	80 ± 19 (n=10)	NS
Albumin (g/L)	41 ± 4.0 (n=34)	41 ± 2.9 (n=15)	NS
Total protein (g/L)	76 ± 5.3 (n=34)	75 ± 4.8 (n=15)	NS
21-Days continuous intravenous infusion			
L/T (%)	29.6 ± 6.67 (n=18)	34.1 ± 5.70 (n=19)	0.034
CL_L (L/h)	73.9 ± 27.8 (n=19)	59.9 ± 13.6 (n=19)	NS
CL_L (L/h/m^2)	37.5 ± 14.3 (n=19)	34.3 ± 8.14 (n=19)	NS
CL_T (L/h)	21.3 ± 5.78 (n=18)	20.0 ± 3.85 (n=19)	NS
CL_T (L/h/m^2)	10.4 ± 3.88 (n=18)	11.5 ± 2.56 (n=19)	NS
BSA (m^2)	1.98 ± 0.139 (n=19)	1.76 ± 0.164 (n=19)	0.0001
Ht (L/L)	0.37 ±0.038 (n=12)	0.34 ± 0.028 (n=16)	0.023
CL_{creat} (mL/min)	98 ± 16 (n=9)	87 ± 22 (n=14)	NS
Albumin (g/L)	43 ± 4.0 (n=12)	41 ± 6.1 (n=16)	NS
Total protein (g/L)	70 ± 8.9 (n=12)	71 ± 16 (n=16)	NS

L/T (%) = lactone to total ratio, CL/F and CL = (apparent) clearance of topotecan lactone and total, Tmax = time to reach the maximal plasma concentration of topotecan lactone and carboxylate, T½ = terminal half life of topotecan lactone and carboxylate, BSA = body-surface area, Ht = hematocrit, CL_{creat} = creatinine clearance, NS = no significant difference.

the body-surface area and the absolute apparent clearance expressed in L/h (P=0.013), while no significant relationship was found after correction of the apparent clearance for the body-surface area. In contrast, the relationship between hematocrit and the absolute as well the corrected apparent clearance was significantly correlated (P=0.040 and P=0.030 respectively). In the

continuous i.v. phase II study, 38 patients (19 males and 19 females) had evaluable topotecan pharmacokinetics on day 8 of course 1, with a 1.2-fold higher lactone to total concentration in females (table 1). As in the oral study, a significant correlation (r=0.61, P<0.0001) between the lactone to total concentration and the apparent clearance of topotecan lactone was found (fig 2b).

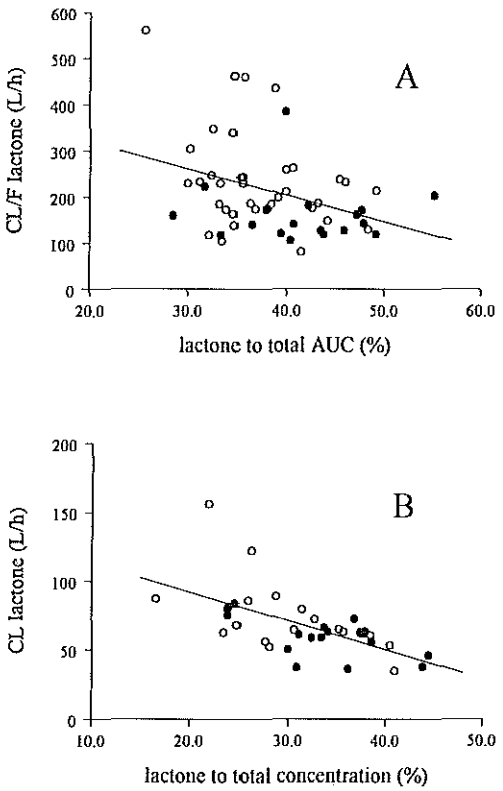


Fig. 2: Relationship between the lactone to total ratios and the apparent topotecan lactone clearance in patients treated with oral topotecan (A) or i.v. topotecan (B). Male patients are indicated by open circles and females by closed circles.

In vitro studies

We sought to define an in vitro model that would explain the differences between males and females in topotecan pharmacokinetics. In the experiments with the whole blood samples of the healthy male volunteers, with artificially altered hematocrit values, a strong significant correlation ($r=0.98$, $P<0.0001$) was found between the hematocrit value and the lactone to total concentrations after a 15-min incubation at 37°C with 5 ng/mL of both topotecan lactone and carboxylate (fig 3). By comparing normal heparinized whole blood samples of 5 male and 5 female volunteers, a significant difference ($P=0.0015$) in the hematocrit value was found, with mean values of 0.44 ± 0.014 L/L for males and 0.39 ± 0.019 L/L for females. After a 15-min incubation at 37°C with 5 ng/mL of topotecan lactone and carboxylate, the lactone to total concentration ratio in the plasma compartment was significantly higher ($P<0.0001$) in females as compared to males, with respective values of 27.8 ± 0.41 % and 25.2 ± 0.36 %. In addition, the change in the lactone to total concentration ratio was accompanied by a significantly ($P=0.010$) higher exposure of the lactone form in the plasma compartment of the blood samples of the female volunteers, with mean concentrations of 3.41 ± 0.103 and 3.22 ± 0.074 ng/mL for the blood samples obtained from the female and male volunteers.

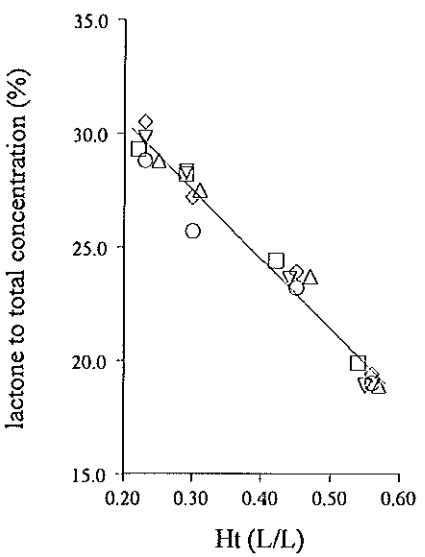


Fig. 3: Relationship between the hematocrit (Ht) and the lactone to total concentration ratio in plasma. Each symbol represents the created hematocrit value versus the lactone to total ratio in the plasma of 1 healthy volunteer.

DISCUSSION

In the present study, we have demonstrated for the first time that topotecan clearance is significantly slower in females as compared to males. These data complement previous knowledge of the clinical pharmacology of topotecan, and may have important clinical implications for its optimal use. Previous studies have revealed that major factors responsible for gender-dependent pharmacokinetics are related to differences in body composition, renal elimination, drug absorption and hepatic function [reviewed in 6]. Indeed, a significant difference in the body-surface area between male and female patients was found in the present study. However, the apparent clearance of topotecan lactone after oral administration was still 1.3-fold higher in male patients as compared to female patients after correction for body-surface area, indicating that body-surface area was not the major predictor for the gender-dependent clearance of topotecan lactone. Likewise, no significant linear relationship was found between body-surface area and the apparent clearance of topotecan lactone after correction of the apparent clearance for body-surface area.

Pharmacokinetic studies performed during previous clinical trials of topotecan have consistently failed to recognize the discrepant drug disposition in males and females. This is most likely caused by the fact that in most studies only a limited number of patients was sampled or combined measurement of topotecan lactone plus carboxylate was performed. Recently, gender-dependent differences in topotecan pharmacokinetics were not found in a population of children and adults in the range of 3 weeks to 22 years of age [10]. This is probably related to the fact that no gender differences in hematocrit values are reported in children under the age of 12 years and only marginal differences were observed between the 12 and 18 years [11].

Topotecan is mainly eliminated by the kidneys, with 40% (range 26-80%) of the dose excreted in the urine as parent compound within 24 h after a 30-min i.v. infusion [reviewed in 1]. One of the known metabolic pathways of topotecan is the loss of the methyl moiety linked to the nitrogen in the core structure of topotecan by the cytochrome P450 enzyme system, resulting in N-desmethyl topotecan (fig 1). Concentrations of this metabolite in plasma and urine were very low; after a 30-min i.v. infusion, peak plasma concentrations of N-desmethyl topotecan accounted for less than 1% of the maximal total drug concentration, and in urine only 1 to 4% of the delivered dose was excreted as N-desmethyl topotecan [12]. Recently, a new metabolic conjugation pathway has been described [13], resulting in the formation of topotecan-O-glucuronide and N-desmethyl topotecan-O-glucuronide (fig 1). Since relatively low amounts of these metabolites were excreted in the urine, with maximal concentrations of 10 and 3.5%, respectively, in comparison with urinary concentrations of the parent compound, coupled with the fact that altered topotecan clearance only has been described in patients with severely-

impaired renal function [14], gender-dependent differences in the known metabolic pathways of topotecan are unlikely to occur. However, minor gender-related differences in renal clearance could be expected, since the glomerular filtration rate of the kidneys is related to the body-weight, and thus higher topotecan clearance in males could be due to their higher body-weight [reviewed in 6]. Nevertheless, as described above, the apparent clearance of topotecan lactone was significantly higher in males, even after correction for the body-surface area (i.e. body-weight), while no significant difference was found for the clearance of total topotecan.

Differences in intestinal drug absorption between males and females after oral administration have been reported and shown to be possibly related to a slower gastric emptying rate in females, different levels of gut enzymes and differences in the hepatic first-pass effect. In the present analysis, however, differences in the gastric emptying rate are less likely, since the time to reach the maximum concentration of topotecan lactone after oral administration did not differ significantly. Moreover, previously we did not find differences in the oral bioavailability of the i.v. dosing solution between males and females of topotecan lactone, with bioavailabilities of 31 ± 8.4 % (n=7) and 30 ± 7.5% (n=5) for males and females, respectively [data compiled from 15].

Gender-related differences caused by different levels of liver and gut enzymes are not expected since metabolism is a minor route of elimination of topotecan. As described above, low amounts of the known metabolites of topotecan were detected in urine and plasma of patients. Likewise, in a phase I and pharmacologic study in patients with impaired hepatic function, similar topotecan pharmacokinetics were observed in patients with and without liver injury [16], also suggesting a minor role of liver enzymes in the overall elimination of topotecan.

A significant relationship was found following linear regression analysis of body-surface area versus the absolute apparent clearance of topotecan lactone, while after correction for body-surface area this relationship did not remain statistically significant. However, significant relationships were noted between the hematocrit and the absolute apparent clearance of topotecan lactone, as well as the apparent clearance corrected for body-surface area, indicating that hematocrit was a significant predictor for the apparent clearance of topotecan lactone.

To further evaluate the role of hematocrit in topotecan pharmacokinetics as a potentially important contributing factor to the observed gender-dependency, various additional in vitro studies were performed. Hematocrit values in healthy humans are known to be different in males and females, with respectively values of 0.44 ± 0.02 L/L and 0.39 ± 0.02 L/L [17]. Furthermore, erythrocytes are known to be carriers for a variety of endogenous compounds and drugs, including topotecan [18-20]. Drugs and endogenous compounds in the plasma compartment are in equilibrium between plasma proteins and plasma water, i.e. in a bound and unbound form. The plasma water is the central compartment, from which the unbound drug is able to move across cell membranes, including those of red blood cells. Topotecan has a plasma protein binding of approximately 35% [21], and hence 65% of the drug in principle is directly available for cellular uptake. To demonstrate the relationship between

hematocrit value and the lactone to total concentration ratio in plasma, whole blood of 5 male volunteers, with artificially altered hematocrit values, was incubated with topotecan lactone and carboxylate. The hematocrit appeared to be a principle predictor of the resulting topotecan lactone to total concentration ratio in the plasma compartment, with higher ratios at lower hematocrit values. This phenomenon was confirmed by in vitro incubation of topotecan in whole blood of males and females, showing significantly higher lactone to total topotecan concentrations in females as compared to males. The higher lactone to total topotecan ratios in blood with lower hematocrit values is most likely caused by the fact that the carboxylate form, which is charged, is not able to pass cell membranes and thus remains in the plasma compartment [22]. Hence, the absolute amount of the carboxylate form in the plasma compartment in the in vitro experiments is independent of the hematocrit value, resulting in lower carboxylate concentrations in blood samples with lower hematocrit values. In addition, we found significantly higher topotecan lactone concentrations in the plasma compartment of the blood samples of the female volunteers. This phenomenon is consistent with the in vivo finding of lower topotecan lactone clearance in females, as a result of higher exposure of the lactone form in females compared to males.

In conclusion, we have shown that topotecan is subject to significant gender-dependent differences in pharmacokinetic behavior that result from a physiologic difference in hematocrit values between males and females. This finding may have implications for interpretation of the relationship between pharmacokinetic parameters and pharmacodynamic outcome of topotecan treatment. A potential gender-dependent relationship between the pharmacokinetics and pharmacodynamics has to be investigated in a study using single agent topotecan at a fixed dose. Eventually, pharmacologic data generated in this investigation and the recognition of the gender-dependency in topotecan pharmacokinetics may provide a basis for the development and refinement of clinical protocols allowing more rational and selective treatment with topotecan.

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Chapter 2f

Phase I and pharmacological study of increased dose oral topotecan in combination with intravenous cisplatin

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ABSTRACT

Background A recent clinical study with cisplatin-topotecan chemotherapy has shown that the maximum tolerated dose was cisplatin 75 mg/m^2 on day 1 followed by oral topotecan 1.25 mg/m^2/day for 5 days [10]. Since preclinical studies have shown a relationship between topotecan exposure and antitumour response, we tried to increase the topotecan dose when given with a reduced fixed dose of cisplatin 50 mg/m^2.

Patients and Methods A total of 12 patients were treated with oral topotecan administered daily for 5 consecutive days in escalating doses starting at 1.50 mg/m^2/day and cisplatin was given as a 3-h infusion before topotecan. Serial plasma samples were obtained from all patients for pharmacokinetic analysis of the lactone and carboxylate forms of topotecan and unbound and total cisplatin.

Results Dose-limiting toxicity, consisting of thrombocytopenia, neutropenia and/or diarrhea, was encountered at the second dose level (topotecan 1.75 mg/m^2/day, cisplatin 50 mg/m^2). Hematological pharmacodynamics indicated a delayed onset of neutropenia with this combination as compared to single agent data. The apparent clearance of topotecan was independent of dose, with mean values of 109±65.8 and 73.5±14.4 L/h/m^2 (P=0.27) at the 1.50 and 1.75 mg/m^2/day doses, respectively. The topotecan dose did not affect any of the cisplatin pharmacokinetic parameters (P≥0.10), suggesting lack of a kinetic interaction.

Conclusion The maximum-tolerated dose of this combination is cisplatin 50 mg/m^2 on day 1 immediately followed by oral topotecan 1.50 mg/m^2/day for 5 days. A 33% reduction in cisplatin dose allowed only a 20% dose increase in oral topotecan. These results indicate that the studied regimen has no advantage as compared to the combination with cisplatin 75 mg/m^2.

INTRODUCTION

Topotecan [(S)-9-dimethylaminomethyl-10-hydroxycamptothecin, Hycamtin®] is a water-soluble semi-synthetic derivative of camptothecin, an inhibitor of the nuclear enzyme topoisomerase I known to interfere with the process of DNA breakage and resealing, resulting in irreversible DNA-single strand breaks and ultimately in cell death [1]. Since cisplatin is a DNA damaging agent, synergy was hypothesised for the combination with topotecan. This synergism was demonstrated in *in vitro* studies in various cancer cell lines [2]. *In vitro* studies indicated that topoisomerase I inhibitors delayed the reversal of cisplatin induced DNA interstrand cross links without modifying their formation, as a possible mechanism for the observed synergism. Also, simultaneous treatment prolonged the DNA and RNA synthesis inhibition induced by

either drug alone [3,4]. When observed, synergy seemed to be dependent on the sequence of administration and cytotoxicity increased when topotecan administration was preceded by cisplatin [5]. Driven by these promising *in vitro* data and by the clinical knowledge that topoisomerase I inhibitors and cisplatin have different toxicity profiles and share a broad spectrum of antitumour activity, several phase I studies combining i.v. topotecan and cisplatin were initiated [6-9]. All studies showed that a considerable dose reduction of i.v. topotecan was required as compared to the single agent dose, and that cisplatin given before topotecan produced a greater incidence of severe neutropenia in the studies in which the sequence dependent effects were studied [6,10].

Based on *in vitro* observations showing more pronounced antitumour efficacy with protracted exposure of topoisomerase I inhibitors at low concentration, several clinical studies on low dose continuous infusion of topotecan were performed [11-14]. Since oral delivery is a more convenient method for prolonged drug administration, and preferred by patients [15,16], an oral formulation of topotecan was developed. Topotecan, supplied in gelatin capsules, has an absolute bioavailability of 42±13% [17]. The maximally tolerated dose for oral topotecan, administered for 5 days every 21 days as a single agent was defined as 2.3 mg/m^2/day, with myelosuppression (in particular neutropenia) as the dose limiting toxicity [18]. Two randomised studies on single agent topotecan suggested that the oral formulation is equipotent to the intravenous formulation in patients with ovarian- and small cell lung cancer, whilst associated with less grade 3 and 4 neutropenia [19,20]. Recently, a phase I study combining oral topotecan (T) given for 5 days every 3 weeks with cisplatin (C) at 75 mg/m^2 on day 1 (sequence CT) or day 5 (sequence TC) was completed [10]. In congruency with the results of the study by Rowinsky with i.v. topotecan [6], the maximum tolerated dose (MTD) for topotecan in the CT sequence of 1.25 mg/m^2/day x 5 was considerably lower than that for the alternate sequence (2.00 mg/m^2/day x 5). Cisplatin was not observed to have an effect on the pharmacokinetics of topotecan[10].

Finally, topotecan systemic exposure was directly associated with antitumour activity in *in vivo* studies [21]. Given this apparent relationship between topotecan systemic exposure and clinical response, we tried to increase the topotecan dose in the combination cisplatin-oral topotecan by using a lower cisplatin dose. We performed a phase I study in patients with solid tumours with oral topotecan preceded by a fixed dose of i.v. cisplatin at 50 mg/m^2.

PATIENTS AND METHODS

Patient selection

Patients with a histologically or cytologically confirmed diagnosis of a malignant solid tumour refractory to standard forms of therapy, or for whom no better option than cisplatin-topotecan was available, were eligible. Other eligibility criteria included the following: age between 18-75 years; Eastern Cooperative Oncology Group performance status ≤ 2; no previous anticancer therapy for at least 4 weeks (6 weeks for nitrosoureas or mitomycin C); no previous therapy with topoisomerase I inhibitors; and adequate hematopoietic (absolute neutrophil count (ANC) $\geq 1.5 \times 10^9$/L and platelet count $\geq 100 \times 10^9$/L), renal (creatinine clearance ≥ 60 mL/min) and hepatic (total serum bilirubin ≤ 1.25 x upper normal limit and serum ASAT and ALAT ≤ 2.0 x upper normal limits, in case of liver metastasis: serum ASAT and ALAT ≤ 3.0 x upper normal limits) function. Specific exclusion criteria included the existence of gross ascites and/or any gastrointestinal condition that would alter drug absorption. The study protocol was approved by the institutional ethics board. All patients gave written informed consent before study entry.

Dosage and dose escalation

Escalating doses of oral topotecan were administered on day 1-5, every 21 days preceded by a fixed dose of 50 mg/m² cisplatin administered intravenously over 3-h given on day 1. The starting dose of topotecan was 1.50 mg/m²/day, which is 120% of the recommended dose for the combination with cisplatin at 75 mg/m^2 given day 1 in the same drug sequence [10]. Dose escalation was based on the prior dose level toxicities. At least 3 patients were treated at each dose level. If 1 of 3 patients experienced dose limiting toxicity (DLT), 3 additional patients were entered at that dose level. The maximum-tolerated dose (MTD) was defined as one dose level below the dose that induced DLTs in 3 out of 6 patients during the first course. DLTs were defined as NCI-CTC version 1994 grade 4 neutropenia lasting for 5 days or more, or complicated with fever requiring hospitalisation, grade 4 thrombocytopenia and/or non-hematological toxicity $\geq$ grade 3 (grade 2 for renal toxicity), excluding nausea. Intrapatient dose escalation was not permitted. If a patient encountered DLT, the dose of topotecan was decreased one dose level at retreatment. The treatment was resumed when the neutrophil count had recovered to $\geq 1.0 \times 10^9$/L and the platelet count to $\geq 100 \times 10^9$/L. A maximum of 6 cycles was administered to each patient.

Drug administration

All patients received cisplatin as a 3-h infusion diluted in 250 mL of hypertonic saline [3% (w/v) sodium chloride] on day 1, immediately followed by the oral administration of topotecan, which was given for five consecutive days on an empty stomach, at least 10 min before meals.

Pre-medication consisted of ondansetron (8 mg i.v.) combined with dexamethasone (10 mg i.v.) administered 30 min before the start of the cisplatin infusion. The administration of cisplatin was preceded by infusion of 1 L of a mixture of 5% (w/v) dextrose and 0.9% (w/v) sodium chloride over 4 h, and followed by another 3 L with the addition of 20 mM potassium chloride and 2 g/L magnesium sulphate applied over 16 h. Topotecan capsules containing either 0.25 or 1.00 mg of the active compound were supplied by SmithKline Beecham Pharmaceuticals (Harlow, UK). Cisplatin (Platosin) was purchased as a powder from Pharmachemie (Haarlem, The Netherlands).

Treatment assessment and pharmacokinetic data analysis

Treatment assessment and blood sampling was performed as described in the previous study [10]. Blood samples for pharmacokinetic analysis were taken at the following time points: for cisplatin before infusion; at 1, 2, and 3 h after start of the infusion; and at 0.5, 1, 2, 3, 4, and 18 h after the end of the infusion, and for topotecan prior to dosing; and at 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 12 hours after administration of topotecan.

Samples for topotecan kinetics were analysed by a validated reversed-phase high-performance liquid chromatographic method, that allowed simultaneous determination of the lactone and the hydrolysed ring-opened carboxylate forms, as described [22] with minor modifications [10]. Non-protein bound and total cisplatin concentrations in plasma were determined by a validated analytical procedure based on measurement of platinum atoms by flameless atomic-absorption spectrometry [23].

Individual plasma concentrations of topotecan were fit to a one- or two-compartment model, using the software package Siphar v4.0 (SIMED, Creteil, France). The concentration-time profiles were obtained after zero-order input, with weighted least-squares analysis applying a weighting factor of 1/y. Standard noncompartmental analysis of these data was also conducted. The topotecan area under the plasma concentration-time curve ($AUC_{0\text{-}INF}$) was determined for both the lactone ($AUC_{(L)}$) and carboxylate forms ($AUC_{(C)}$). The apparent plasma clearance ($CL/f_{(L)}$) of topotecan lactone was calculated by dividing the dose administered by the observed AUC. The terminal disposition half-life [$T_{1/2}(z)$] of topotecan was calculated as ln2/k, where k is the rate constant of the terminal disposition phase (expressed in h^{-1}). The peak plasma concentrations (C_{max}) and the time to peak plasma concentration (T_{max}) were determined graphically from the (observed) experimental values. The ratio of the systemic exposure of topotecan lactone to total drug (L/T ratio) was defined as $AUC_{(L)}/[AUC_{(L)}+AUC_{(C)}]$.

Model dependent pharmacokinetic analysis of CDDP was conducted similarly using a two-compartment linear model with extended least-squares regression analysis as reported earlier [10]. The noncompartmental analysis was also conducted in the same fashion, and $AUC_{0\text{-}INFs}$ of unbound and total cisplatin were calculated to the last sampling time point with detectable drug levels (C_{last}) by the linear trapezoid method and extended to infinity by addition of C_{last}/k_{term}, where k_{term} is the slope obtained by log-linear regression of the final plasma concentration values.

Table 1: Patients characteristics

Characteristic		No. of patients
Age, years		
Median	56	
Range	38-74	
Sex		
Female		4
Male		8
Performance status		
Median	1	
Range	0-2	
Tumour type		
Carcinoma of unknown primary		4
Mesothelioma		2
Non-small cell lung cancer		2
Head/Neck		1
Head/Neck+NSCLC		1
Cervical		1
Colon		1
Previous therapy		
Chemotherapy		2
Radiation		1
Chemotherapy and radiation		3
None		6

Statistical considerations

Pharmacokinetic parameters for all compounds are reported as mean values ± S.D. The difference in pharmacokinetic parameters was evaluated statistically using a Student's t-test and a Friedman's two-way analysis of variance test. Probability values (two-sided) of less than 0.05 were regarded as statistically significant. All calculations were performed using the statistical package NCSS version 5.X (JL Hintze, Kaysville, UT, 1992).

RESULTS

Twelve patients (8 males, 4 females) entered this study between March and September 1999. Patient characteristics are listed in Table 1. All patients were eligible, 11 patients were assessable for toxicity (1 patient died due to a suspected pulmonary embolism on day 11) and 10 patients were assessable for response (1 patient stopped treatment after one course at her own request and refused CT-evaluation). The majority of patients had mild symptoms. Fifty percent of patients, equally distributed over both dose levels, had received prior chemo- and/or radiotherapy. Overall, all patients were un- or minimally pretreated and in good clinical condition. The patient population was thus comparable to the 'highly selected' group of patients at the recommended dose level of topotecan 1.25 mg/m^2/day on day 1-5 in combination with cisplatin 75 mg/m^2 on day 1 in our previous study [10]. Dose levels of topotecan studied were 1.50 and 1.75 mg/m^2/day. The total number of assessable courses was 43, with a median number of courses per patient of 4 (range 1-6).

Gastrointestinal toxicity and myelosuppression were the principle DLTs of this regimen. Three patients, treated at dose level 1.75, required dose reductions after experiencing DLT, and 1 patient even had a second dose reduction to 1.25 mg/m^2/day.

Hematological toxicity

Hematological toxicity observed during all courses is shown in Table 2a. Neutropenia was the main hematological toxicity. The percentage of courses associated with grade 3 or 4 neutropenia (no DLTs) and grade 3 or 4 thrombocytopenia at the topotecan dose level of 1.50 mg/m^2 was respectively, 36% and 9%, and 32% for grade 1 or 2 thrombocytopenia. At the next dose level of topotecan 1.75 mg/m^2, grade 3 or 4 neutropenia occurred in 50% of courses, associated with grade 3 or 4 thrombocytopenia in 11% of courses. Grade 1 or 2 thrombocytopenia was observed in 22% of courses. At this dose level, 2 patients were judged as having experienced DLT: 1 had grade 4 neutropenia lasting for more than 5 days and 1 had neutropenic fever and grade 4 thrombocytopenia. The patients who had their topotecan dose

reduced due to hematological toxicity had a much higher incidence of grade 3-4 hematological toxicity in subsequent courses compared to patients who were initially treated at a lower dose level. All of these patients had had prior chemo- and/or radiotherapy. The percentage decrease in leukocyte, neutrophil and platelet count during the first course in 10 patients (1 patient was inassessable for toxicity and 1 patient did not have all blood samples taken exactly according to the protocol) is shown in Table 2b. The median day of onset of neutropenia during the first course was relatively late (day 18, range 14-21) as compared to topotecan single agent data, in contrary to thrombocytopenia nadir which occurred after an interval similar to the one of single agent nadir (day 14, range 12-18). An example of hematological pharmacodynamic-time profiles from 2 patients receiving topotecan at either 1.50 or 1.75 mg/m^2/day x 5 is shown in Fig 1.

Table 2a: Hematological toxicity (worst per cycle)

Topotecan mg/m^2/day	No. of pts/cycles	Neutropenia 3	4	Thrombocytopenia 3	4
1.50	6/16	2	1	0	0
1.75	6/18	3	6	1	1
1.50 (after dose reduction)	3/6	0	5	0	2
1.25 (after dose reduction)	1/3	0	3	2	1

Table 2b: Absolute hematological toxicity during course 1

Topotecan mg/m^2/day	No pts	Nadir WBC day	% decrease	Nadir ANC day	% decrease	Nadir platelets day	%decrease
1.50	5	18±3	74±16	19±2	84±11	15±3	62±20
1.75	5	15±1	83±13	16±2	93±10	13±1	83±6.9

Abbreviations: WBC, white blood cell count; ANC, absolute neutrophil count

Overall, at the recommended dose of topotecan 1.50 mg/m^2 on day 1-5, hematological toxicity was acceptable with 4 chemo- and/or radiotherapy pretreated patients experiencing neutropenia grade 4 for less than 5 days and grade 3-4 thrombocytopenia in only 2 of the 22 courses. Three patients with grade 2 anemia received blood transfusions. Only 1 course had to be delayed at this dose level for hematological reasons. Patients starting at the second dose level of topotecan 1.75 mg/m^2 and requiring dose reduction due to hematological toxicity were prone to have hematological toxicity at the lower dose levels. These patients accounted for 63% of all grade 3 or 4 neutropenia's at this dose level, and for all grade 3 or 4 thrombocytopenia's.

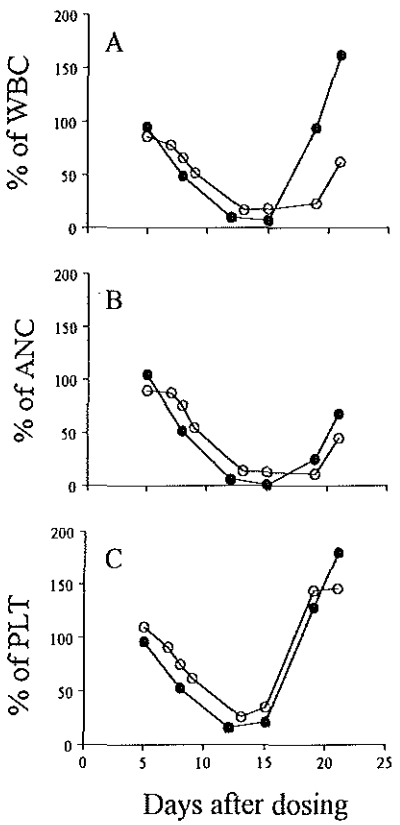

Fig 1: An example of hematological pharmacodynamic-time profiles from 2 patients receiving topotecan at either 1.50 (open circles) or 1.75 (closed circles) mg/m^2/day x 5.

Non-hematological toxicity

Fatigue and gastrointestinal toxicity were the most frequently reported non-hematological side-effects (data shown in Table 3). One patient treated with topotecan 1.50 mg/m^2 experienced grade 3 diarrhea in the first course, which was considered DLT as defined according to the protocol. The diarrhea occurred on day 7 and resolved within one day with loperamide treatment, therefore topotecan dose was not reduced. The patient had no diarrhea in the second course. On the next dose level of topotecan at 1.75 mg/m^2, 3 out of 6 patients experienced DLT, 1 had grade 3 mucositis and vomiting, 1 had grade 3 diarrhea and was treated with loperamide from day 14-16 (this patient also had grade 4 neutropenia lasting for more than 5 days), and 1 had diarrhea grade 3 on day 12 (and neutropenic fever). One extensively pretreated patient with progressive metastatic colon cancer died 11 days after the first administration of cisplatin and topotecan, presumably due to a pulmonary embolism, which was not considered related to the treatment. Since the patient died at home, the clinical diagnosis was not confirmed by additional investigations.

Other side effects were alopecia (grade 1 or 2 in 50% of patients) and peripheral neurotoxicity (grade 1 or 2 in 16% patients). No nephrotoxicity other than grade 1 was encountered.

Overall, non-hematological toxicity at the recommended dose level was infrequent. Other than the patient described with grade 3 diarrhea on day 7, who had grade 3 nausea in the second course, no toxicity graded > 2 was observed.

Table 3: Non-hematological toxicity (worst per cycle)

Topotecan mg/m^2/day	No. pts/cycles	Nausea 1	2	3	Vomiting 1	2	3	Diarrhea 1	2	3	Fatigue 1	2	3
1.50	6/16	5	2	1	2	1	0	1	0	1	9	0	0
1.75	6/18	6	4	1	1	2	1	5	3	2	6	2	0
1.50 (after dose reduction)	3/6	2	0	0	1	0	0	1	0	0	1	0	0
1.25 (after dose reduction)	1/3	0	0	0	0	0	0	0	0	0	1	0	0

Antitumour activity

One patient with a mesothelioma achieved a partial response, which is currently still ongoing after course 6. Another patient with pulmonary metastases of a head and neck tumour and a simultaneous secondary primary non-small cell lung cancer also achieved a partial response, which was not confirmed since she had radiotherapy for consolidation of the response. Six patients showed disease stabilisation. Two patients were not evaluable for response, including the one patient that presumably died due to a pulmonary embolism.

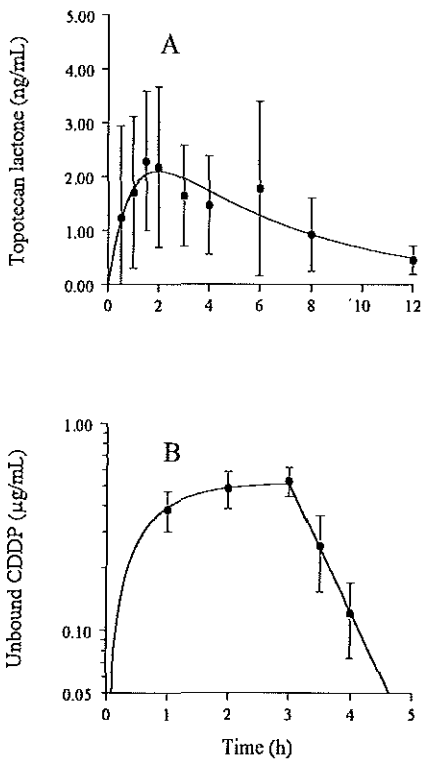

Fig 2: Average plasma concentration-time curves of (A) topotecan lactone and (B) unbound cisplatin (CDDP) in 6 patients on day 1 at the recommended dose level of topotecan at 1.50 mg/m^2.

Topotecan and cisplatin pharmacokinetics

Pharmacokinetic analysis was performed on data obtained from 11 out of 12 patients in course 1 and in 1 patient also in course 6. Table 4 and 5 list the main pharmacokinetic parameters of the two drugs with topotecan given at 1.50 or 1.75 mg/m^2/day x 5 in combination with a fixed dose of cisplatin 50 mg/m^2 day every 3 weeks. The average plasma concentration-time curves of topotecan lactone and unbound cisplatin on day 1 at the recommended dose level of topotecan at 1.50 mg/m^2 are presented in Fig 2.

Table 4: Summary of topotecan lactone pharmacokinetics of course 1 day 1 (data present mean values ± SD)

	Topotecan (mg/m^2/day)		
	1.50 (n=6)	1.75 (n=5)	p
C_{max} (ng/mL)	3.42 ± 1.36	5.08 ± 3.52	0.31
T_{max} (h)	2.61 ± 2.12	2.59 ± 2.14	0.99
AUC (ng.h/mL)	17.0 ± 6.83	24.6 ± 4.68	0.065
CL/f (L/h/m^2)	109 ± 65.8	73.5 ± 14.4	0.27
$t_{½(z)}$ (h)	3.53 ± 1.18	3.23 ± 1.24	0.69
L/T ratio	0.38 ± 0.034	0.42 ± 0.058	0.19

Abbreviations: C_{max}, peak plasma level; T_{max}, time to maximum concentration; AUC, area under the plasma concentratio-time curve; CL/f, apparent clearance; $t_{½(z)}$, half-life of the terminal disposition phase; L/T ratio, topotecan lactone to total drug AUC ratio.

The apparent clearance (CL/f) of the pharmacologically active topotecan lactone and the lactone to total drug AUC ratio (L/T ratio) were not dependent on dose and were similar to previous reported values obtained from studies where topotecan was administered as a single agent [18]. The interpatient variability in AUC was 40% at 1.50 mg/m^2 and 19% at 1.75 mg/m^2. The apparent clearance of topotecan (Cl/f) on days 1, 2 and 5 averaged 93.1±51.0 (n=11), 81.0±30.8 (n=9) and 76.7±14.4 L/h/m^2 (n=9), and were not significantly different (P = 0.20, Friedman's two way analysis of variance test), suggesting that topotecan does not accumulate following multiple dosing (Fig 3). All data were consistent with a dose-independent and linear behavior of topotecan.

Table 5: Summary of ciplatin pharmacokinetics of course 1 (data present mean values ± SD)

	Topotecan (mg/m^2/day)		
	1.50 (n=6)	1.75 (n=5)	p
Cisplatin total			
C_{max} (µg/mL)	1.87 ± 0.197	1.93 ± 0.318	0.71
AUC (µg.h/mL)	25.2 ± 3.55	23.2 ± 4.86	0.45
CL (mL/min)	65.3 ± 11.5	72.1 ± 24.5	0.56
$t_{½(z)}$ (h)	58.4 ± 32.2	34.6 ± 16.5	0.17
Cisplatin unbound			
C_{max} (µg/mL)	0.55 ± 0.087	0.62 ± 0.13	0.31
AUC (µg.h/mL)	1.35 ± 0.271	1.51 ± 0.305	0.38
CL (mL/min)	1221 ± 192.9	1068 ± 184.9	0.21
$t_{½(z)}$ (h)	0.77 ± 0.29	0.52 ± 0.11	0.10

C_{max}, peak plasma level; AUC, area under the plasma concentration-time curve; CL, clearance; $t_{½(z)}$, half-life of the terminal disposition phase.

The effect of the topotecan dose on the pharmacokinetics of total and unbound cisplatin in plasma during the first treatment course is shown in Table 5. Cisplatin clearance was not effected by concomitant administration of topotecan dose, with mean values of 65.3 and 72.1 mL/min when given in combination with 1.50 and 1.75 mg/m^2/day of topotecan, respectively.

Pharmacokinetic data for topotecan and cisplatin obtained during the sixth treatment course in one patient were similar to the first course, indicating no alteration in topotecan and cisplatin pharmacokinetics, topotecan CL/f on 3 separate days was 159±65.4 ng/h/m^2 and 168±14.0 ng/h/m^2, during the first and sixth course, respectively. Overall, pharmacokinetics of both compounds were dose independent and similar to single agent data [18].

DISCUSSION

The combination of i.v. cisplatin and oral topotecan is attractive given their broad anti-tumour activity, preclinical synergism, their different toxicity profiles and patient's preference of oral chemotherapy (when at least equally effective). In all phase I studies combining cisplatin and topotecan, the topotecan dose at MTD was considerately reduced as compared to single

agent topotecan regimens. Since preclinical studies suggest existence of a topotecan systemic exposure-response relationship, and while clinical studies in topotecan-sensitive tumour types still have to confirm this issue, we tried to increase the topotecan dose by decreasing the cisplatin dose to 50 mg/m^2. Although cisplatin at this dose is effective in combination regimens in non-small-cell lung cancer [24] and ovarian cancer [25], a reduced cisplatin dose is considered to be related with a loss of response and survival in, for example, ovarian cancer [26].

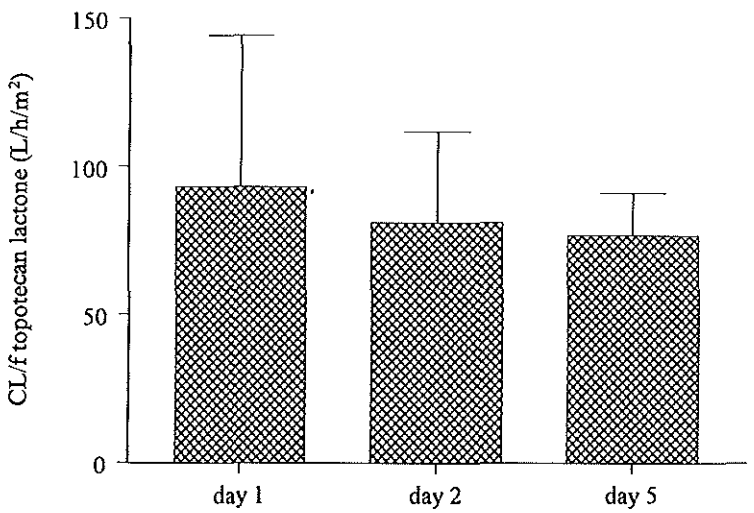

Fig. 3: The apparent clearance (CL/f) of topotecan lactone obtained on the first, second and fifth administration day in the first course of respectively 11, 9 and 9 patients treated with cisplatin 50 mg/m^2 on day 1 and topotecan 1.50 or 1.75 mg/m^2/d on days 1-5. Data represent mean values (bars) ± standard deviation (error bars).

In the present phase I study both myelosuppression and gastrointestinal toxicity were dose limiting in 3 out of 6 patients treated with topotecan at 1.75 mg/m^2 administered on day 1-5 every 3 weeks, preceded by i.v. cisplatin 50 mg/m^2 on day 1. Other toxicities such as nephrotoxicity, neurotoxicity, fatigue and alopecia were mild and infrequent. All patients who were treated at the 1.75 dose level and had their topotecan dose reduced due to dose limiting toxicity, had prior chemo- and/or radiotherapy, and experienced significant hematological toxicity at the lower dose levels, while the 6 patients who were initially treated at the recommended dose level of topotecan 1.50 mg/m^2 experienced less toxicity. Despite the late onset of the neutrophil nadir, compared to single agent data, only one course was delayed at the 1.50 mg/m^2 dose level. No other important toxicity was encountered at this dose level, except for one patient experiencing grade 3 diarrhea for one day only and grade 3 nausea.

The observed pharmacokinetic parameters of the lactone and the carboxylate form of topotecan demonstrated linear and dose independent behavior over the total dose range studied and were similar to data obtained from studies of topotecan administered orally as a single agent. Furthermore, topotecan dose had no influence on the unbound fraction of cisplatin, as suggested previously [10].

In conclusion, the MTD of topotecan is 1.50 mg/m^2 on day 1-5, preceded by cisplatin 50 mg/m^2 on day 1. This regimen seems to have no advantage as compared to our previous recommended schedule of topotecan 1.25 mg/m^2, preceded by cisplatin 75 mg/m^2 in a similar patient population [10], since the dose reduction of 33% of cisplatin only allows a minor dose escalation of topotecan.

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Chapter 3

9-Aminocamptothecin

Chapter 3a

Determination of the lactone and lactone plus carboxylate forms of 9-aminocamptothecin in human plasma by sensitive high-performance liquid chromatography with fluorescence detection

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ABSTRACT

Two sensitive reversed-phase high-performance liquid chromatographic fluorescence methods, with simple sample handling at the site of the patient, are described for the determination of the lactone and lactone plus carboxylate forms of 9-aminocamptothecin (9-AC). For 9-AC lactone, the sample preparation was a liquid-liquid extraction with acetonitrile-*n*-bulylchloride (1:4, v/v), whereas the sample preparation for 9-AC total (lactone plus carboxylate) was a simple deproteinization with 5% perchloric acid-methanol (1:1, v/v), which results in the conversion of the carboxylate into the lactone form. The lower limits of quantitation were 50 pg/ml and 100 pg/ml for 9-AC lactone and 9-AC total, respectively. The within-run precisions at four tested concentrations were ≤6.3% for 9-AC lactone and ≤5.3% for 9-AC total. The between-run precisions were ≤8.9% and ≤5.6%, respectively. The assays were developed to enable pharmacological analysis of 9-AC in a bioavailability and oral Phase I study in patients with solid tumors.

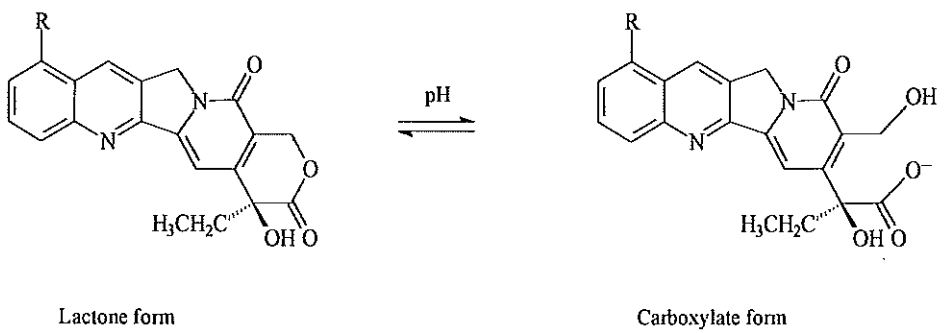
INTRODUCTION

20(*S*)-Camptothecin (CPT; NSC 94600) (fig. 1) is a cytotoxic plant alkaloid, that was first extracted from the wood and bark of the oriental tree *Camptotheca acuminata*, Decaisne (*Nyssaceae*) [1,2]. In spite of promising antitumor activity *in vitro* and in animal tumor models, severe toxicity, including diarrhea and dose-limiting myelosuppression precluded further clinical testing (reviewed in [3]). After the identification of DNA topoisomerase I as the molecular target of CPT [4,5], interest in new structure development was accelerated, with various derivatives of CPT (semi)synthesized to enhance the aqueous solubility. Two compounds of this type, *viz.* topotecan and irinotecan are currently undergoing clinical evaluation and have shown impressive antitumor activity [6]. Another derivative that is not soluble in water, 9-amino-20(*S*)-camptothecin (9-AC; NSC 603071; fig. 1) was recently also introduced into clinical trials because of its unprecedented antitumor activity against solid tumor xenografts [7,8].

Pharmacokinetic studies of the camptothecins, including 9-AC, are complicated by a chemical, pH-dependent instability of the lactone ring (fig. 1) [9], generating an open-ring carboxylate, which is over 1000-fold less active as an inhibitor of DNA topoisomerase I [10]. The importance of this nonenzymatic hydrolysis reaction of the lactone moiety in the pharmacology and toxicology of 9-AC is largely unknown. To address this question, development of analytical methodologies enabling the quantitation of both forms are required.

Various authors recently described assays for the determination of 9-AC lactone and/or total drug levels (lactone plus carboxylate; 9-AC total) applicable to plasma samples obtained from patients [11-13]. Major drawbacks of these procedures are the necessity for immediate analysis by high-performance liquid chromatography (HPLC), postcolumn acidification and/or laborious solid-phase extraction techniques.

We now describe novel assays for the analysis of 9-AC lactone and 9-AC total in human plasma, which only require a one-step solvent extraction for sample clean-up prior to reversed-phase HPLC with fluorescence detection. The presented methods have been thoroughly validated in terms of accuracy and precision, and are now successfully implemented in studies on the pharmacology of 9-AC in patients with solid tumors.



Lactone form

Carboxylate form

Fig. 1: Chemical structures and pH-dependent interconversion of the lactone and carboxylate forms of 9-AC ($R=NH_2$) and CPT (R=H).

EXPERIMENTAL

Chemicals and reagents

9-AC (lot #93L07A, containing the base with a purity of 100%) and CPT (lot #93K05A, containing the base with a purity of 100%) were obtained from Pharmacia Inc. (New Mexico, USA). Methanol, acetonitrile (both HPLC-grade), dimethyl sulfoxide (DMSO) and *n*-

butylchloride (both analytical grade) were purchased from Rathburn (Walkerburn, Scotland). Perchloric acid (70-72%, v/v; analytical grade) was obtained from Baker (Deventer, The Netherlands). The water was filtered and deionized with a Milli-Q-UF system (Milford, USA) and was used in all aqueous solutions. Drug-free human plasma originated from healthy donors and was delivered by the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

Stock solutions and standards

Separate stock solutions of 9-AC and CPT were made by dissolving 10.0 mg of 9-AC or CPT in 10.00 ml of DMSO. A volume of 5.00 ml of these solutions was accurately transferred to 50 ml volumetric flasks and diluted to the mark with DMSO, resulting in solutions containing 100,000 ng/ml of 9-AC or CPT, respectively. Working solutions of 9-AC lactone were prepared daily by serial dilution in a mixture of methanol-water-perchloric acid (500:500:1, v/v/v) from the primary stock solution. Similarly, working solutions of 9-AC total were made in methanol-water (1:1, v/v). Spiked plasma samples used as calibrations standards were prepared daily by addition of 50 µl of the working solutions to 950 µl of drug-free human plasma, resulting in calibration standards of 50, 100, 500, 1000, 1500 and 2000 pg/ml for 9-AC lactone and of 100, 200, 1000, 5000, 10000, 15000 pg/ml for 9-AC total.

Four pools of quality control (QC) samples were prepared in human plasma in the concentrations of 250, 900, 1600 and 150000 pg/ml for 9-AC lactone and of 500, 7500, 12500 and 250000 pg/ml for 9-AC total. The QC samples containing the highest concentrations were used as diluted control and/or for low volume injection. To minimize differences in the composition between clinical and QC samples, also the carboxylate form of 9-AC was added to the QC samples of 9-AC lactone. The 9-AC carboxylate was spiked at the same concentrations as 9-AC lactone and was prepared by adding 9-AC in methanol-water (1:1, v/v) to plasma, followed by incubation at 37°C for 20 hrs.

Sample preparation for the lactone form of 9-AC

To 1000 µl of human plasma a volume of 100 µl of 2.5 ng/ml of CPT in methanol-water-perchloric acid (500:500:1, v/v/v) and 0.8 g of NaCl were added in a glass tube supplied with PTFE-covered screw caps. For the extraction, 7.5 ml of acetonitrile-*n*-butylchloride (1:4, v/v) was added and the sample was vortex-mixed for 5 min, which resulted in the formation of a gel. Subsequently, the sample was centrifuged for 2 min at 4000 x *g* at ambient temperature. The sample was shaken once to break the gel and centrifuged for 5 min at 4000 x g. The supernatant was collected in a glass tube containing 50 µl of DMSO and evaporated at 50°C under a gentle stream of nitrogen, until a residue of approximately 50 µl was left over. To the residue, 50 µl of

methanol and 150 µl of perchloric acid-water (1:500, v/v) was added. The sample was transferred to a low volume insert of glass, and a volume of 150 µl was injected into the HPLC system.

Sample preparation for total 9-AC

A volume of 250 µl of 5% perchloric acid-methanol (1:1, v/v) was added to 250 µl of human plasma in 1.5 ml polypropylene-microcentrifuge tubes, followed by vortex-mixing for 10 min. Subsequently, the sample was centrifuged for 5 min at 24,000 x *g* at ambient temperature. The clear supernatant was transferred to a low volume insert of glass and 200 µl were injected into the HPLC system.

Chromatographic system

The HPLC system consisted of a constaMetric 3200 pump from LDC Analytical, a subsidiary of Thermo Instruments Systems Inc. (Riviera Beach, USA). The samples were injected by a Waters 717plus autosampler (division of Millipore, Milford, USA). A fluoriMonitor 4100 fluorescence detector from LDC Analytical was used for the detection. Chromatographic separations were achieved on a Inertsil ODS-80A column (150 * 4.6 mm ID, 5µm particle size) from GL Science Inc. (Tokyo, Japan), protected by a Lichrospher 100 RP-18 endcapped guard column (4 * 4 mm ID, 5 µm particle size) obtained from Merck (Darmstadt, Germany).

For the assay of the lactone form of 9-AC in plasma, the mobile phase was a mixture of methanol-water (40:60, v/v). The pH was adjusted to 2.20 by addition of perchloric acid. The mobile phase for the assay of 9-AC total in plasma was composed of methanol-water (32.5:67.5, v/v), with the pH adjusted to 2.10 using perchloric acid. The mobile phases were degassed by ultrasonication and were delivered at a flow rate set at 1.00 ml/min for both assays. The column was maintained at 40°C, also for both assays, using a model SpH99 column oven (Spark Holland, Meppel, The Netherlands), and the eluents were monitored at an excitation wavelength of 370 nm and an emission wavelength of 450 nm, with a bandwidth of 40 nm.

Calibration

Acquisition and integration of chromatographic data was performed with the Chrom-Card data analysis system (Fisons, Milan, Italy) running on an ICW workstation. Calibration curves were made by linear regression analysis of peak heights versus 1/concentration using the software package Lotus 2.4 (Lotus Development Corporations, New York, NY, USA).

Validation

For the validation of the assays of 9-AC lactone and 9-AC total in plasma, the six-points calibration curves were processed in duplicate and analyzed on four occasions with the lower limit of quantitation (LLQ) and the QC samples in quintuplicate. For the determination of the LLQ, plasma samples of five independent individuals were taken and spiked to contain 50 pg/ml for 9-AC lactone and 100 pg/ml for 9-AC total. For the concentration accepted as the LLQ, the percent deviation of at least 80% of the samples assayed should be ≤20%. The average within-run and between-run precision (expressed as the coefficient of variation (%CV) for each concentration, excluding the LLQ, should be ≤ 15% and should be ≤ 20% for the LLQ. The average accuracy (%) for each concentration, including the LLQ, should be within 85-115% of the nomuinal values.

The absolute recoveries were calculated on the basis of the concentrations of the calibration curves. For 9-AC lactone the peak heights of processed plasma samples were compared with the peak heights of two samples with a concentration equivalent to 1000 pg/ml 9-AC and 250 pg/ml CPT in plasma extracts with 100% recovery. For this purpose, 50 µl of 20 ng/ml of 9-AC and 100 µl of 2.5 ng/ml of CPT in methanol-water-perchloric acid (500:500:1, v/v/v) were evaporated in a tube containing 50 µl of DMSO, and were dissolved as plasma extracts. For 9-AC total, the peak heights of processed plasma samples were compared with the peak heights of two samples with a concentration equal to 5000 pg/ml of 9-AC in plasma extracts with 100% recovery; 25 µl of 100 ng/ml of 9-AC in methanol-water (1:1, v/v) was added to 975 µl blank plasma extract.

The stability of 9-AC total was tested by incubation of the four QC samples at 37°C for 20 hours and during 3 freeze-thaw cycles. At the freeze-thaw cycles the QC samples were put at room temperature for 1 hour before freezing again. The stability of 9-AC lactone was only tested during three quick freeze-thaw cycles. The samples were thawed quickly in cold water and restored at -80°C within 5 to 10 min. The long term stability of 9-AC total and 9-AC lactone at -80°C was also tested at the concentrations of the QC samples.

The following potentially co-administered drugs were tested for interference with the analytical methods: Paracetamol at a concentration of 0.50 mg/ml; alizapride, codeine, domperidon, morphine and ranitidine at a concentration of 0.10 mg/ml; and dexamethason and metoclopramide at concentrations of 0.05 and 0.01 mg/ml, respectively.

Human experiments

To demonstrate the applicability of the analytical methods, blood samples from a patient were collected at 0, 20 and 40 min, and at 1, 1.5, 2, 3, 5, 11, 24, 28, 31, 35, 48, 52 and 55 hrs after oral administration of 2.7 mg of 9-AC. Immediately after sampling, the blood was

centrifuged, at the site of the patient, for 5 minutes at 3000 x g at 4°C. The plasma was collected and directly placed on dry-ice at -20°C. Within a few hours the samples were stored at -80°C.

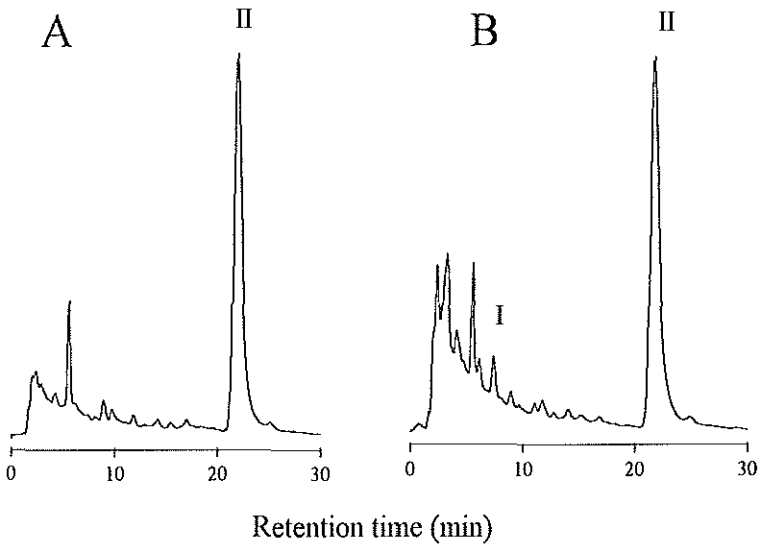


Fig. 2: Chromatograms of a blank human plasma sample containing 250 pg/ml of CPT, used as internal standard (A), and a human plasma sample containing 171 pg/ml of 9-AC and 250 pg/ml of CPT (B) in the assay for 9-AC lactone. Peaks I and II correspond to 9-AC and CPT, respectively.

RESULTS AND DISCUSSION

Calibration curves of the lactone and total (lactone plus carboxylate) forms of 9-AC in human plasma were linear in the range of 50 to 2000 pg/ml and 100 to 15000 pg/ml, respectively. The regression correlation coefficients were ≥0.992 for 9-AC lactone and ≥0.997 for 9-AC total, by

using weighted (1/concentration) linear least-squares regression analysis to prevent domination of the highest concentrations. Intercept values of the standard curves for both 9-AC lactone and 9-AC total were not significantly different from zero by Student's *t*-test (P>0.05). The retention times of 9-AC and CPT in the assay of the lactone form were 7.5 and 22 min (fig. 2), while the retention time of 9-AC in the assay of 9-AC total was 12.5 min (fig. 3), with overall chromatographic run times of 30 and 20 min, respectively. The selectivity for the analytes is shown by the sharp resolution of the peaks and no significant interfering peaks for both assays in blank plasma samples from five independent donors. All the tested potentially co-administered drugs did not give interfering peaks for 9-AC and CPT in the assay for 9-AC lactone, and for 9-AC in the assay for the 9-AC total.

Table 1: The mean accuracy, the mean within-run precision and the between-run precision of the QC samples (n=5) in human plasma of 9-AC lactone.

QC-sample (pg/ml)	accuracy (%) (mean)	precision (%) within-run (mean)	between-run
250	101.6	1.0	3.5
900	91.5	2.4	2.9
1600	90.3	1.7	5.2
150 000	98.9	4.1	8.9

The LLQ for 9-AC in the assays for the lactone and total form were 50 and 100 pg/ml, respectively. The lower value of the LLQ for the determination of the lactone was a consequence of concentration of the sample as compared to dilution of the sample in the assay for 9-AC total. The extraction recoveries of 9-AC and CPT in the assay for 9-AC lactone were 78.2 ± 7.98% and 92.4 ± 7.96%, and the recovery for 9-AC in the total assay was found to be 89.6 ± 6.87%. In both assays, extraction recoveries were independent of the spiked concentration. The within-run precisions of the LLQ samples of 9-AC lactone and 9-AC total were ≤4.2% and ≤10.2%, respectively. The range of accuracy for 9-AC lactone was 95.6% to 112.0%. For 9-AC total, the accuracy ranged from 95.2% to 107.6%. The average accuracy in both assays showed values ranging within 10% of the nominal values (Tables 1 and 2). The average within-run and between-run precisions for 9-AC lactone and 9-AC total in human plasma varied upto 5.5% and 8.6%, respectively (Tables 1 and 2).

9-AC total was found to be stable for 20 hours at 37°C, and during three freeze-thaw cycles. However, 9-AC lactone was not stable during three quick freeze-thaw cycles; there was a loss of the amount of 9-AC lactone of approximately 10% in comparison with the mean of a set of QC samples analyzed at the same time. This instability necessitates quick freezing of the samples after blood sampling in order to stabilize the ratio between 9-AC lactone and 9-AC carboxylate. 9-AC total and lactone forms were stable for at least 4 months when stored at -80°C (data not shown).

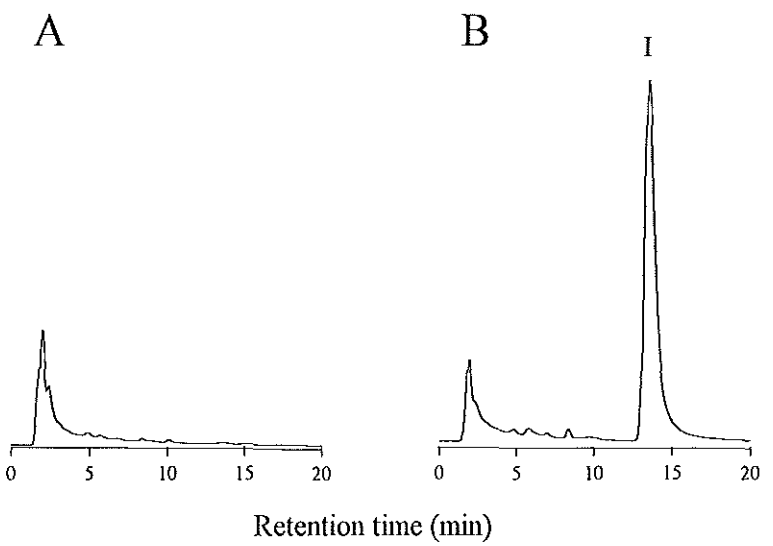


Fig. 3: Chromatograms of a blank human plasma sample (A), and a 10-fold dilute human plasma sample containing 89 264 pg/ml of 9-AC (B) in the assay for 9-AC total. The peak labeled I corresponds to 9-AC.

Table 2: The mean accuracy, the mean within-run precision and the between-run precision of the QC samples (n=5) in human plasma of 9-AC total.

QC-sample (pg/ml)	accuracy (%) (mean)	precision (%) within-run (mean)	between-run
500	102.6	2.5	3.6
7500	103.1	1.4	5.6
12 500	103.5	1.5	5.0
250 000	98.4	2.4	2.2

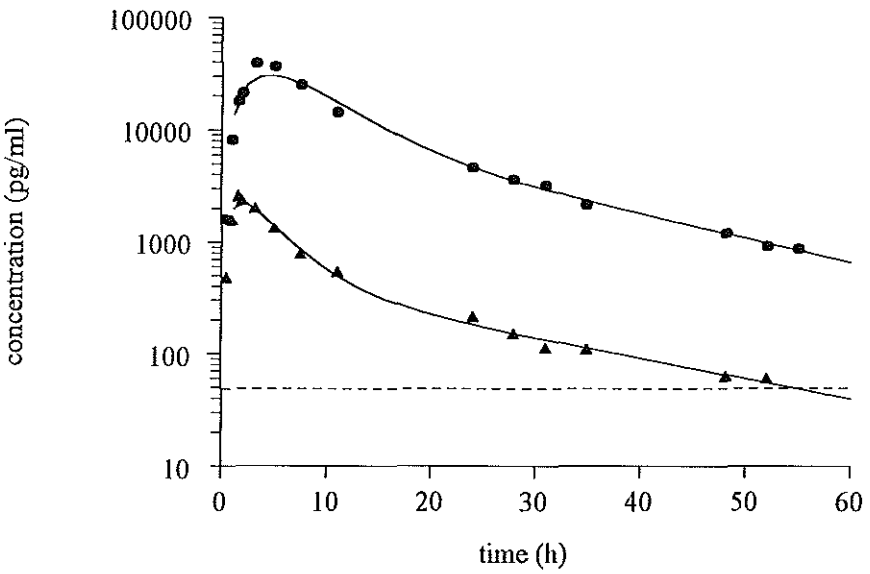


Fig. 4: Plasma concentration-time curves of 9-AC lactone (triangles) and 9-AC total (circles) in a patient after oral administration of 2.7 mg of 9-AC. The dotted line indicates the lower limit of quantitation for 9-AC lactone. Both curves were fitted to a two-compartment model using the MW/Pharm software package (Medi-Ware Groningen, Netherlands).

Plasma collected from a patient prior to the drug administration did not reveal the presence of endogenous peaks. Concentrations of 9-AC lactone could be readily estimated from protein-free extracts, whereas acidification of plasma samples, re-converting the carboxylate into the corresponding lactone, enabled determination of 9-AC total. The plasma concentration-time curves of 9-AC lactone and 9-AC total forms of a patient treated orally with 2.7 mg of 9-AC, are given in fig. 4. The data indicate that the LLQ's of 50 and 100 pg/ml for 9-AC lactone and 9-AC total, respectively, are sufficient for monitoring drug-plasma levels in samples obtained from patients treated at low oral doses.

In conclusion, two sensitive, selective, accurate and reproducible isocratic reversed-phase HPLC methods have been developed for the analysis of 9-AC lactone and 9-AC total in human plasma. The sample pretreatment procedures are based on single solvent extractions, thereby eliminating the need of laborious solid-phase extraction techniques [12,13]. Compared to previously described assays for 9-AC, our new methods provide equivalent to superior sensitivity with LLQ's of 50 pg/ml for 9-AC lactone and 100 pg/ml for 9-AC total (lactone plus carboxylate). The methodologies described permit the analysis of patient samples, and will be implemented in future investigations on the clinical pharmacology of 9-AC administered at low (oral) doses.

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Chapter 3b

Role of erythrocytes and serum proteins in the kinetic profile of total 9-amino-20(S)-camptothecin in humans

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ABSTRACT

9-Amino-20(*S*)-camptothecin (9-AC) is a water-insoluble topoisomerase-I inhibitor with evident schedule-dependent antitumor activity in preclinical studies. The pharmacokinetic behavior of 9-AC given as a bolus i.v. infusion (1.0 mg/m^2 over 5 min) was recently characterized in 12 patients in a bioavalability study. Remarkable rebound concentrations of 9-AC total drug (i.e. lactone *plus* carboxylate forms) were observed at about 2-3 hours after dosing. *In vitro* experiments indicated that this phenomenon was associated with a substantial uptake of 9-AC lactone by erythrocytes immediately after dosing, and its subsequent release followed by accumulation of 9-AC carboxylate in the plasma compartment mediated by a pH-dependent hydrolysis of the lactone form, which is unable to diffuse across cell membranes. The preferential binding of 9-AC carboxylate to human serum albumin shifts the equilibrium between the lactone and carboxylate forms of 9-AC to the pharmacological inactive carboxylate form.

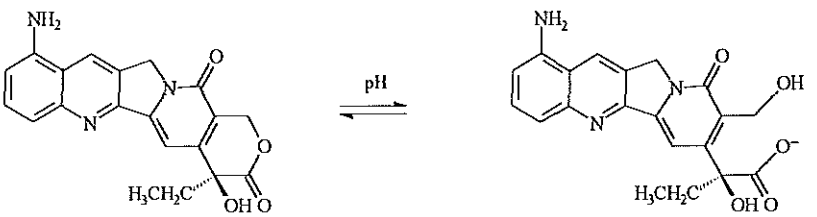
INTRODUCTION

9-Amino-20(*S*)-camptothecin (9-AC, NSC 603071) is a semisynthetic derivative of the naturally occurring plant alkaloid camptothecin that does not produce hemorrhagic cystitis associated with the parent compound [1]. The mechanism of action of 9-AC is based on inhibition of topoisomerase-I, an intranuclear enzyme which relaxes supercoiled DNA by creating single strand DNA breaks which are subsequently religated [2]. In preclinical studies, 9-AC demonstrated significant activity, (including cures) in mice xenografted with human carcinomas resistant to common antineoplastic agents [3]. These animal studies further demonstrated that drug efficacy is critically dependent on the duration of exposure, frequency of administration and plasma levels of the drug. On the basis of these data, several clinical trials have been performed with the drug administered using various continuous i.v. infusion schedules [4-9]. Currently, there is considerable interest to explore alternative routes and schedules of 9-AC administration, e.g. oral [10-12] or daily i.v. bolus administration [13], to facilitate the development of more prolonged dosing that may be required to optimize antitumor activity.

Pharmacokinetic studies with camptothecin analogues, including 9-AC, were previously shown to be complicated by a chemical, pH-dependent instability of the terminal α-hydroxy-δ-lactone ring (Fig. 1), generating a ring-opened carboxylate, which is over 1000-fold less active as an inhibitor of topoisomerase-I [14]. The clinical pharmacokinetics of 9-AC has been studied extensively in patients receiving the drug by i.v. infusion over 24 or 72 hours [4,5,7,9,15]. These studies showed that only about 10% of the total plasma 9-AC circulated in the active lactone form, which is considerably lower than that reported for topotecan [16] and the irinotecan metabolite SN-38 [17]. It has been

suggested, that this is related to differential affinity of the carboxylate forms of camptothecins for human serum albumin, causing a shift in the equilibrium hydrolysis within the systemic circulation [18].

Recently, we reported the pharmacokinetics of 9-AC lactone and carboxylate after bolus i.v. administration in 12 cancer patients participating in a bioavailability study [19]. In the present work, we evaluate the kinetic profile of the total 9-AC concentration, revealing a new feature of 9-AC disposition in humans, which is characterized by a significant rebound peak in the plasma profile. The role of erythrocytes and serum proteins in the kinetic profile of total 9-AC is described in this report.



Lactone form Carboxylate form

Fig. 1: Chemical structures and pH-dependent interconversion of the lactone and carboxylate forms of 9-aminocamptothecin.

MATERIALS AND METHODS

Chemicals and reagents

Pure reference standards of 9-AC (batch: 93L07A) and camptothecin (batch: 93K05A were provided by Pharmacia & Upjohn (Milan, Italy). Perchloric acid was obtained from Baker (Deventer, The Netherlands) as a 70% (v/v) aqueous solution. Human and murine serum albumin were purchased as essentially fatty acid free lyophilized powders from Sigma Chemicals Co. (St. Louis, MO, USA). All other reagents were of analytical grade or higher, and originated from Rathburn (Walkerburn, UK). Samples of human plasma and whole blood were obtained from healthy volunteers. HPLC grade water was prepared in-house using the Milli-Q UF Plus system from Millipore (Bedford, MA, USA).

Clinical pharmacokinetics

The pharmacokinetic profiles of the lactone and carboxylate forms of 9-AC after bolus i.v. administration were previously described by Sparreboom et al [19]. Briefly, 12 patients enrolled in a bioavalability study in which they received the lactone form as a single i.v. bolus of 1.0 mg/m^2. Quantitative determination of the lactone and total drug concentrations were performed in serial plasma samples obtained upto 55 h after dosing using a reversed-phase HPLC method as described earlier by Loos et al [20].

***In vitro* stability of 9-AC**

The rate of hydrolysis of 9-AC lactone was monitored in PBS, 4% (w/v) solutions of human and murine serum albumin in saline, and in heparinized human whole blood. All matrices were incubated in triplicate with 1.0 μg/ml of 9-AC lactone at 37°C, and 250-μl samples were taken at 0, 0.5, 1, 2, 4, 7.5, and 24 h after start of the incubation. The samples were diluted 2.5 to 50-fold in a pool of drug-free human plasma and analyzed for the intact 9-AC lactone and 9-AC total forms by HPLC as described by Loos et al. [20].

In a separate series of experiments, extracellular 9-AC total concentration-time profiles were monitored in samples of human whole blood, erythrocyte-rich PBS, a leukocyte buffy coat suspension, and platelet-rich plasma. Platelet-rich plasma with very little contamination from erythrocytes and leukocytes was prepared by centrifugation at 200×*g* for 20 min at room temperature [21]. Kinetic runs were initiated by addition of 9-AC lactone at 37°C to provide an initial concentration of 100 ng/ml. Aliquots were withdrawn periodically, processed to cell free samples by centrifugation at 15,000×*g* for 2 min (4°C), and analyzed by HPLC as described above.

Accumulation of 9-AC in cell cultures.

The human ovarian and colon carcinoma cell lines IGROV-1 and WIDR, and the non-malignant African green monkey kidney cell line VERO were cultured in Dulbecco's modified Eagle's Medium (DMEM; GibcoBRL, Life Technologies B.V., Breda, The Netherlands), supplemented with 10% of heat-inactivated fetal calf serum (Hyclone, Logan, UT), 10 m*M* of sodium carbonate, 2 m*M* of L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Life Technologies) in a humidified atmosphere of 5% CO_2 at 37°C. All cell lines were grown to 80 to 90% confluence in 6-well culture plates (Greiner, Alphen a/d Rijn, The Netherlands), and incubated in triplicate with 1.0 μg/ml of 9-AC lactone or 9-AC carboxylate for 10 min. The cells were washed rapidly three times with ice-cold saline, and harvested by scraping in 500 μL of water. An aliquot of 250 μl was used for determination of 9-AC total drug concentrations by HPLC, and 10 μl were used for the determination of the total protein content by a modification of the Bradford dye-binding method [22].

RESULTS

Clinical pharmacokinetics

As described earlier [19], the plasma concentration-time profiles of the lactone and carboxylate forms of 9-AC were remarkably similar for the 12 patients studied, with a very short initial half-life time of the lactone form of approximately 6.5 min. The overall estimated lactone to total drug ratio in plasma was 9.1±3.4%, indicating a rapid and substantial conversion to the carboxylate species.

Evaluation of the plasma profile of total 9-AC (i.e. lactone plus carboxylate) revealed a very pronounced secondary peak in all 12 patients, with a maximum of the total occurring at 2-3 hours after drug administration (Fig. 2).

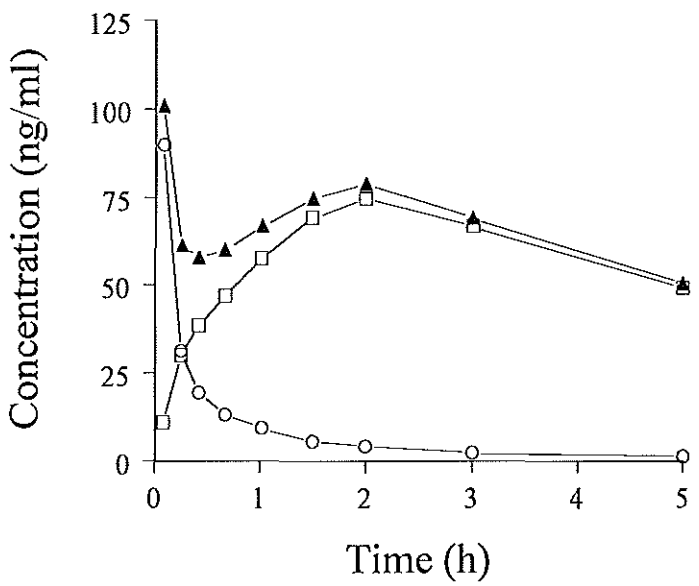


Fig. 2: Representative plasma concentration-time profile of 9-AC lactone (○), carboxylate (□) and total drug (i.e. lactone plus carboxylate forms;▲) in a single patient after i.v. drug administration of 1.0 mg/m^2

***In vitro* studies**

In order to gain insight into the pharmacologic mechanisms involved in this rebound peak phenomenon, various *in vitro* stability studies of 9-AC were performed. Equilibrium distribution ratios for accumulation of 9-AC into erythrocytes were found to change dramatically with time for 9-AC lactone concentrations in the therapeutically relevant range of 0.1 to 1.0 μg/ml (Fig. 3).

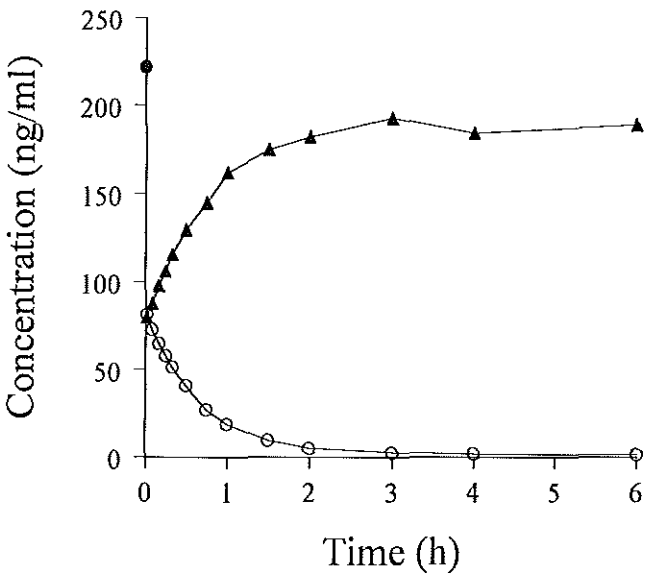


Fig. 3: Extracellular concentration-time profiles of 9-AC lactone lactone (○) and 9-AC total drug (▲) following incubation of 9-AC lactone at 100 ng/ml in human whole blood. The closed circle on the ordinate indicates the initial 9-AC total extracellular concentration estimate, based on a hematocrit of 0.45.

Under the same experimental conditions, there was no substantial drug accumulation in human platelets or peripheral leukocytes that may have affected the kinetic behavior of 9-AC (total drug) in human plasma (data not shown). Measurement of extracellular 9-AC total drug following incubation of 9-AC lactone in human whole blood demonstrated a rapid fall in concentration, caused by drug

accumulation in erythrocytes, followed by a continuous rise until an apparent steady state was established at approximately 2-3 hours (Fig. 3), similar to that observed in our patients. At steady-state, the 9-AC (total drug) plasma to erythrocyte concentration ratio was estimated to be about 100:1. This result can be understood by considering that the large fraction of 9-AC lactone taken up initially into erythrocytes (~25-30%) will redistribute to the plasma water, followed by dissociation of the lactone moiety due to the physiologic pH and the presence of serum proteins (Table 1). Under *in vitro* conditions in PBS, 10.7±0.4% of 9-AC was in the lactone form at equilibrium. The addition of human serum albumin, however, was found to further shift the lactone to carboxylate equilibrium dramatically in favor of the latter due to a higher affinity of the carboxylate form, with less than 1% of total drug remaining as lactone. In whole blood, 0.8±0.2% of 9-AC was in the lactone form at equilibrium, which is not significantly different from human serum albumin solution and human plasma. The fact that only the intact lactone form of 9-AC can diffuse across cell membranes further contributed to the subsequent accumulation of the carboxylate species (Table 2). Drug accumulation in the various cell lines tested after incubation with the carboxylate form was only approximately 5% in comparison with the accumulation during exposure to the lactone form. However, the cellular accumulation is seriously influenced by the lactone-carboxylate interconversion during incubation, as only 3.5±0.15% (mean±SD) of extracellular carboxylate is converted into lactone after 10 min at 37°C, compared to 38.2±2.07% of lactone into carboxylate.

Table 1: Stability of 9-AC lactone at equilibrium in various media[a]

Matrix	$t_{1/2}$[b] (min)	% as lactone at equilibrium	pH
Phosphate buffered saline	25.8±0.31	10.7±0.42	7.4
Human serum albumin	37.9±1.9	0.63±0.10	7.0
Murine serum albumin	244±17.5	35.0±6.2	7.0
Human whole blood	23.6±0.48	0.81±0.21	7.4
Human plasma	12.2±0.72	1.3±0.50	7.4

a: All matrices were incubated in triplicate with 1.0 µg/ml of 9-AC lactone at 37°C, with serial samples taken upto 24 h.
b: Abbreviation: $t_{1/2}$, half-life.

Table 2: Cellulair accumulation of 9-AC lactone and 9-AC carboxylate in various cell lines[a]

Cell line	9-AC lactone (ng/ml protein)	9-AC carboxylate (ng/ml protein)	C/L[b] (%)
IGROV-1	29.9 ± 1.97	1.5 ± 0.24	5.1
VERO	22.3 ± 1.65	1.2 ± 0.30	5.4
WIDR	32.5 ± 1.08	1.8 ± 0.10	5.5

a: All cell lines were incubated in triplicate with 1.0 µg/ml of 9-AC lactone or 9-AC carboxylate for 10 min at 37°C.
b: Carboxylate to lactone concentration ratio

DISCUSSION

Recently we have described the pharmacokinetics of 9-AC in a cohort of patients that received the drug by bolus i.v. administration [19]. The lactone hydrolysis was rapid with greater than 90% conversion to the pharmacologically inactive, ring-opened carboxylate form within 3 hours following a 5-min infusion, which is similar to earlier findings [5,15]. The pharmacokinetic profile of 9-AC total drug (this report) was characterized by the presence of a major secondary peak, and was shown to be caused by an unusual mechanism involving initial uptake of 9-AC lactone in erythrocytes, followed by the progressive accumulation of the carboxylate form of the drug in plasma. Pharmacokinetic studies performed during clinical trials of 9-AC administered as a continuous i.v. infusion have consistently failed to recognize this behavior [4,5,7,9,15]. This can be explained by the fact that the frequency of sampling during the first several hours after infusion was not only less intensive, but the steady state levels of total drug at the maximum tolerated dose were more than 10-fold lower than the peak levels in the present report.

The rate of uptake of 9-AC lactone by erythrocytes *in vitro* was too rapid to be reliably estimated, with an initial uptake half-life in the order of 30 s or less from phosphate buffered saline (to a hematocrit of ~0.45) or human whole blood. This indicates that the rate of exchange between red cells and plasma water is sufficiently rapid to be effectively instantaneous on the time scale of disposition of 9-AC. However, the rate of 9-AC lactone uptake *in vivo* is clearly influenced by the extensive binding of 9-AC carboxylate to proteins, principally serum albumin, causing a gradual depletion of drug available for cellular distribution. Thus, the apparent contradiction between the

high plasma to blood cell concentration ratio of 9-AC total drug and the high initial accumulation of the lactone form into erythrocytes is due to a compensating effect of protein binding of the carboxylate form following hydrolysis of the α-hydroxy-δ-lactone function, thereby trapping the drug in the plasma compartment. The net effect is presumably that early after drug dosing, erythrocytes act as a depot from which the plasma 9-AC lactone is supplied, followed by accumulation of the 9-AC carboxylate in plasma. While we did not test this hypothesis directly, our *in vivo* data would appear to support this notion. In particular, our finding that 9-AC carboxylate did not diffuse across cell membranes is consistent with the significantly reduced volume of distribution for this species in our patients [19], and suggests a preferential cellular uptake of the lactone accompanied by accelerating predominance of the carboxylate in plasma.

Previous studies indicated that this unusual disposition feature may not be unique for 9-AC, as this rebound phenomenon has also been described for the related compound, irinotecan. The initial observation of this behavior was described by Rivory et al. [17], and similar data have been generated from numerous patients treated with irinotecan, although the authors surmized that it might be related to enterohepatic recirculation [23-25]. However, the observation of rebound concentrations of irinotecan and its metabolite SN-38 are not as distinct as those seen with 9-AC in the present study, and are apparently only observed with the drug administered to patients using short i.v. infusion schedules (i.e. ≤30 min). This is probably because drug concentrations will often not be significant at low plasma concentrations to ascertain the secondary peak, and it is only as the plasma concentration increases locally, e.g. following an i.v. bolus injection, that erythrocytes carry a physiologically relevant load [26]. In case of SN-38, the situation is also complicated by the occurrence of enterohepatic cycling following biliary secretion of the hydrophilic C10-glucuronic acid conjugate, which is hydrolyzed by bacterial β-glucuronidase in the intestines [27]. Another possible explanation for the discrepancy between camptothecins in the rebound phenomenon may come from the differences in protein binding of the lactone and carboxylate species for the different camptothecins. It has been described previously that the binding affinity of both drug forms for human serum albumin is an important determinant in the marked differences in the lactone to carboxylate ratios between drugs [18]. For 9-AC and the parent drug camptothecin, serum protein binding of the carboxylate form is highly favored over the closed ring form, and an equilibrium favoring the carboxylate form is rapidly established. In case of 9-AC, this is probably the main reason for the extremely low lactone to total drug AUC ratio (i.e. <10%). For topotecan, irinotecan and SN-38 these ratios are 36%, 38% and 53%, respectively [16,17]. Therefore, it is reasonable to assume that similar effects will be less pronounced in case of topotecan and irinotecan. The absence of the secondary peak in the 9-AC total plasma profile in rodents [28] most likely also relates to differences in lactone to carboxylate ratios compared to humans; at equilibrium, the amount of 9-AC remaining in the lactone form in human plasma is 1.30±0.50%, whereas in mouse plasma or murine serum albumin solution, approximately 35% is present as the lactone form (Table 1).

In conclusion, we have shown that a major rebound peak in the plasma profile of 9-AC arises as the result of a balance between extensive erythrocyte uptake of the lactone form and extensive serum protein binding of the carboxylate form within the systemic circulation, resulting in a shift in equilibrium over time. These data support the idea that 9-AC is an exceptionally dynamic drug in biological systems, with its pharmacokinetic profile influenced strongly by hydrolytic processes as well as by differential cellular distribution and protein binding of the lactone and carboxylate forms.

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Chapter 3c

Clinical pharmacokinetics of encapsulated oral 9-aminocamptothecin in plasma and saliva

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ABSTRACT

Objective To study the pharmacokinetics and pharmacodynamics of the novel topoisomerase I inhibitor and antitumor agent, 9-amino-20(*S*)-camptothecin (9-AC), in patients with solid tumors after repeated oral dosing.

Methods Thirty-two cancer patients received oral 9-AC formulated in capsules with polyethylene glycol-1000 as excipient at doses that ranged from 0.25 to 1.5 mg/m^2/day. Serial plasma and saliva samples were obtained on days 1 and 6 or 8 of the first cycle and analyzed for the lactone and carboxylate forms of 9-AC by HPLC.

Results 9-AC demonstrated linear and dose-independent pharmacokinetics, with extremely small intrapatient kinetic variability (coefficient of variation: <10%). However, interpatient variability in plasma pharmacokinetics was large (coefficient of variation: 99%). The relative extent of lactone to carboxylate interconversion was large (>90%), and predictable from individual pretreatment serum albumin values (p=0.0099). The 9-AC concentration ratio in plasma and saliva was strongly patient dependent, and highly variable around a mean value of 1.4 , suggesting that saliva is an unreliable matrix for kinetic monitoring. The area under the curve of the lactone form of 9-AC was significantly correlated with the dose-limiting hematological toxicity (p<0.001).

Conclusion Our data indicate that the large interindividual pharmacodynamic variability in response to 9-AC is mainly caused by a variability in kinetic characteristics, suggesting that a kinetic-dynamic guided study design is warranted in future clinical investigations.

INTRODUCTION

In the early 1970s, camptothecin, a plant alkaloid extract from the bark and wood of the Chinese tree *Camptotheca acuminata*, was demonstrated to possess antineoplastic activity [1]. Analogs of camptothecin belong to a family of anticancer agents with a unique mechanism of action, which is based on reversible inhibition of DNA topoisomerase I [2,3].

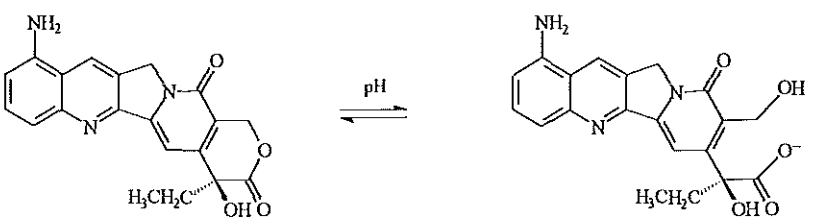
Despite the cytotoxicity of the compound further development was halted because of a number of severe and unpredictable side-effects observed in early clinical trials [4-6]. The subsequent search for less toxic analogs of camptothecin resulted in the discovery of irinotecan, topotecan, 9-amino-20(S)-camptothecin (9-AC), 9-nitrocamptothecin, DX-8951f and GI147211. The first two of these, irinotecan and topotecan, were recently registered for the treatment of colorectal and ovarian cancer, respectively.

In aqueous solutions, camptothecins are unstable and undergo a rapid, pH-dependent, non-

enzymatic hydrolysis of the terminal lactone ring to form the more water-soluble, ring-opened carboxylate form (Fig. 1) [7]. The presence of the intact terminal lactone ring is thought to be essential for the topoisomerase I inhibition [8]. The closed lactone ring predominates at acidic pH, whereas in human plasma, the equilibrium between these two species greatly favors formation of the carboxylate form, partly because of the physiologic pH and the preferentially binding of this form to albumin [9,10]. The ratio of the lactone form to the total drug concentration at steady state in plasma is different for each camptothecin analog, which might have important pharmacokinetic and pharmacodynamic implications.

In preclinical studies 9-AC demonstrated activity against human colon, breast, prostate, non-small cell lung cancer and melanoma xenografts 11-14]. Preclinical *in vivo* data suggested that duration of exposure to 9-AC lactone above a certain threshold concentration (10 n*M*) and frequency of administration were essential for antitumor activity [15,16].

Based on its preclinical activity, 9-AC appeared to merit evaluation as an antineoplastic agent. To mimic the preclinical studies, initial Phase I studies using the intravenous formulation of 9-AC focused on schedules with prolonged infusion duration of 24-120 hr [17-21], or a continuous infusion for 21 days every 4 weeks [22]. Pharmacokinetic data obtained during these studies showed marked interpatient variability. Steady state plasma concentrations of 9-AC lactone greater than 10 n*M* were achieved only in the Phase I study of the 24-hour infusion of 9-AC at the dose recommended for further Phase II studies (i.e. 1.65 mg/m^2). When 9-AC was administered as a 72-hr infusion once every 2 or 3 weeks, the maximal tolerated dose was 35-54.2 μg/m^2/hr. The dose limiting toxicity consisted of neutropenia in combination with thrombocytopenia and correlated to the steady state 9-AC lactone concentration.



Lactone form Carboxylate form

Fig. 1: Chemical structures and pH-dependent interconversion of the lactone and carboxylate forms of 9-aminocamptothecin.

Recently, we reported that 9-AC demonstrated rapid absorption in humans after oral delivery with an overall bioavailability of approximately 50% [23]. In the present report, we present a comprehensive analysis of the plasma pharmacokinetics of the lactone and carboxylate forms of 9-AC in cancer patients receiving the drug orally over a wide range of dose levels, with special focus on pharmacokinetic-pharmacodynamic characteristics. In order to assess the clinical usefulness of salivary monitoring of 9-AC for kinetic modeling, paired plasma and coinciding unstimulated saliva samples were collected in a limited number of patients.

MATERIALS AND METHODS

Patient population

The patients, from whom pharmacokinetic curves were obtained, participated in an oral bioavailability study of 9-AC and/or in a Phase I trial of oral 9-AC administered daily for 7-14 consecutive days every 3 weeks. Treatment plans and detailed clinical profiles have been documented elsewhere [23]. Eligibility criteria included a histologically or cytologically confirmed diagnosis of a solid malignant tumor not amenable to established forms of treatment. All patients had an adequate hematopoietic (absolute peripheral granocyte count $\geq 2.0 \times 10^9$/L and platelet count $\geq 100 \times 10^9$/L), hepatic (bilirubin within normal limits, and serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase ≤ 2.5 times normal limit) and renal (serum creatinine <133 μM) function. Other eligibility criteria included the following: age between 18 and 75 years; Eastern Cooperative Oncology Group performance status ≤ 2; estimated life expectancy ≥ 12 weeks; no previous anticancer therapy for at least 4 weeks (6 weeks for nitrosoureas or mitomycins); no previous therapy with other camptothecins and/or intensive ablative regimens. Specific exclusion criteria included significant gastrointestinal dysfunction that could alter absorption or motility, and chronic treatment with corticosteroids. Concomitant administration of H_2-antagonists, antacids, proton-pump inhibitors and non-steroidal anti-inflammatory drugs were avoided. All patients gave written informed consent before study entry.

Treatment plan and dose escalation

The oral formulation of 9-AC was supplied by Pharmacia & Upjohn (Nerviano, Italy) as hard gelatin capsules that contained 0.10, 0.25 or 1 mg of the active drug and polyethylene glycol-1000 (PEG1000) as excipient [23], and were stored at room temperature.

A detailed description of the preparation and rationale for composition of the formulation has been described earlier [23]. Patients received 9-AC orally with 150-200 mL of water at dose levels of 0.25, 0.40, 0.60, 0.84, 1.0 or 1.1 mg/m^2/day for 7 or 14 consecutive days, or as a single dose of 1.5 mg/m^2.

Intrapatient dose escalation in the phase I trial was not permitted. Weekly evaluation of the patients included a clinical history, physical examination, toxicity assessment according to common toxicity criteria (CTC), and serum chemistry. A complete blood cell count with differentiation was determined twice weekly.

Sample collection and drug analysis

Serial blood samples were collected in heparinized tubes from an indwelling venous catheter at 0, 0.33, 0.67, 1, 1.5, 2, 3, 5, 7.5, 11 and 24 hours after administration on days 1 and 6 or 8 of the first treatment course. In a limited number of patients, unstimulated saliva samples were obtained at coinciding time points. In one patient with a malignant pleural effusion additional pleural fluid sampling was performed to assess the influence of the pleural effusion on the plasma pharmacokinetics. Immediately after sampling, aliquots of plasma (separated at 4°C), pleural fluid and saliva were frozen at -80°C, and analyzed later for 9-AC lactone and 9-AC total drug (i.e. lactone *plus* carboxylate) with use of a validated reversed-phase high-performance liquid chromatographic method as described previously [24]. Drug concentrations in patient plasma samples were calculated using interpolation of the corresponding regression analysis. Specimens with drug levels exceeding the upper range of the calibration curve were reanalyzed upon appropriate dilution with drug-free plasma. Saliva and pleural fluid samples were diluted 4-fold in drug-free plasma and analyzed with use of the same analytical assay, with minor modifications. The lower limits of detection of the assays were 0.05 ng/mL in plasma and 0.4 ng/mL in pleural fluid and saliva.

Data analysis

Plasma-concentration data were analyzed by both non-compartmental and compartmental analysis using the Siphar version 4.0 software package (SIMED, Créteil, France). The model-independent pharmacokinetic parameters included the maximum plasma concentration (C_{max}) and the time to reach the peak concentration (t_{max}). Initial parameter estimates were obtained by an automated peeling algorithm based on the Powell method to three compartments, which yielded the best statistical fit as determined by Akaike's information criterion and the *F*-test. The AUC values were calculated based on the best fitted curve, as were the disposition half-lives ($t_{1/2}$). The apparent absorption rate constant (k_a) was obtained through numerical point-area deconvolution. Relationships between the AUC and pharmacodynamic outcome were evaluated with (log-)linear and (non-)sigmoidal-maximum effect modeling using Siphar and NCSS version 5.0 (Dr.Jerry Hintze, East Kayesville, UT).Within individual patients, myelosuppression was described as the continuous variable, consisting of percentage decrease in white blood cell count (WBC), absolute neutrophil count (ANC) and platelet count (PLT). The relative hematological toxicity was defined as : % decrease = (pretherapy value-nadir value)/(pretherapy value)*100. Only the first course of each patient was taken into consideration

to avoid potentially confounding bias due to cumulative toxicity. All data were fitted to a sigmoidal maximum effect (E_{max}) model based on the modified Hill equation, as follows: $E=E_0+E_{max}*[(KP^{\gamma})/(KP^{\gamma}+KP_{50}^{\gamma})]$. In this equation, E_0 is the minimum reduction possible, fixed at a value of 0, E_{max} is the maximum response, fixed at 100, KP is the pharmacokinetic parameter of interest, KP_{50} the value of the pharmacokinetic parameter predicted to result in half of the maximum response, and γ is the Hill constant describing the sigmoidicity of the curve. Models were evaluated for goodness of fit by minimisation of sums of the squared residuals and by reduction of the estimated coefficient of variation for fitted parameters. Significance of the relationships were assessed by construction of contingency tables with subsequent χ^2 analysis.

Table 1: Patient characteristics

Characteristic		No. of patients
No. entered		32
No. assessable for toxicity		30
Age, years		
Median	59	
Range	29-74	
Sex		
Female		14
Male		16
Performance status		
Median	1	
Range	0-2	
Tumor type		
Colorectal		14
Ovarian		5
Sarcoma		2
Mesothelioma		2
Lung (non-small cell)		2
Miscellaneous		5
Previous treatment		
Chemotherapy		16
Radiation		2
Chemotherapy and radiation		10
None		2

RESULTS

Demographic characteristics of all 32 patients who had blood sampling for pharmacokinetic analysis are shown in Table I. Ten patients completed the oral bioavailability study and subsequently participated in the phase I and pharmacokinetic study of oral 9-AC. Hence, pharmacokinetic data were obtained in 42 courses. Plasma pharmacokinetics could not be determined in 1 course on day 1 and in 2 courses on day 8 as a result of limited sample availability or significant chromatographic interference in the drug assay by an unknown compound. Twenty-seven patients were assessable for pharmacodynamic analysis and 30 patients were assessable for toxicity.

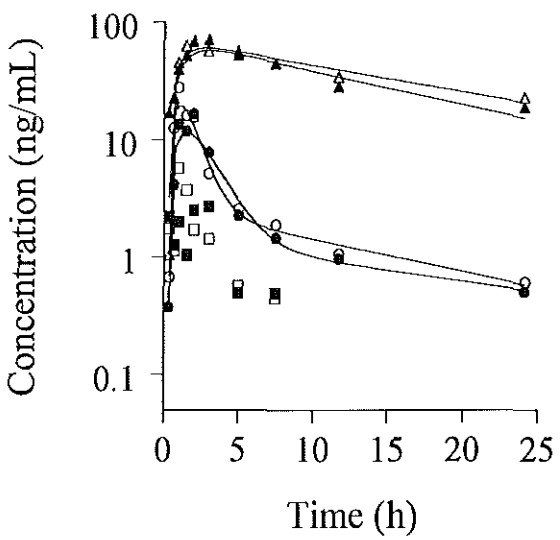


Fig. 2: Representative concentration-time profiles of 9-AC lactone (circles) and 9-AC total (triangles) in plasma and in saliva (rectangle) measured on day 1 (open symbols) and day 8 (closed symbols) of the first treatment course in a single patient following oral administration of 9-AC at a dose level of 0.84 mg/m^2/day in a daily-times fourteen schedule. All pharmacokinetic curves were fitted to a tri-exponential equation assuming a three-compartment modal for the distribution and elimination of the drug.

The plasma concentration-time profiles of 9-AC lactone and 9-AC carboxylate were similar for all patients studied, with representative examples shown in Fig. 2. The pharmacokinetics of both species of 9-AC could be best described with a three-compartment model. The kinetic parameters obtained by means of this model are presented in Tables II and III. The absorption of 9-AC lactone after oral administration was rapid with a lag time of 0.29±0.07 hr (mean ± SD; n=41), maximum peak drug levels at 0.99±0.12 hr, and a mean absorption rate constant of 3.03±0.90 h^{-1}. In the first plasma samples the ring-opened carboxylate form of 9-AC was already detectable. Maximal plasma concentrations of 9-AC carboxylate were reached at 2.41±0.64 hr after dosing. Eventually, the 9-AC carboxylate accounted for 91.1±2.11% of 9-AC total drug concentrations, indicating a clear predominant conversion of lactone to carboxylate. Elimination of 9-AC from the central plasma compartment was characterized by a decay in an apparent tri-exponential manner based on conventional compartment modeling using weighed least-squares analysis with a weighting factor of $1/Y$. The mean values for the linear segments of 9-AC lactone were $t_{1/2}$ (α): 0.26 hr (range, 0.13-0.38 hr), $t_{1/2}$(β): 0.78 hr (range, 0.54-1.13 hr) and $t_{1/2}$(γ): 7.47 hr (range, 3.66-12.6 hr). No significant quantitative differences were observed between the decay kinetics of 9-AC lactone and 9-AC carboxylate.

Table 2: Model-dependent pharmacokinetics of 9-AC_{lac} after oral drug administration of 9-AC.

Dose (mg/m^2/day)	0.25	0.40	0.60	0.84
n	6	3	4	6
k_a (1/h)	ND	ND	ND	ND
t_{lag} (h)	0.33±0.13	0.32±0.05	0.38±0.14	0.28±0.05
t_{max} (h)	1.06±0.35	0.79±0.15	0.94±0.11	0.98±0.27
C_{max} (ng/mL)	2.09±0.82	7.94±7.39	3.43±0.60	4.61±1.51
$t_{1/2}(\alpha)$ (h)	0.25±0.20	0.13±0.11	0.23±0.07	0.24±0.11
$t_{1/2}(\beta)$ (h)	0.68±0.40	0.82±0.33	0.54±0.20	0.73±0.33
$t_{1/2}(\gamma)$ (h)	5.22±4.22	8.65±1.25	3.66±0.55	10.3±6.77
$AUC_{0-\infty}$ day 1 (ng.h/mL)	6.88±4.76	16.2±14.5	8.15±0.76	12.9±3.97
$AUC_{0-\infty}$ day 8 (ng.h/mL)	9.48±3.85	17.4±15.5	8.69±0.70	11.7±2.98
intra %CV of AUC	7.31	7.31	4.44	9.74
inter %CV of AUC	69.2	89.5	89.2	30.7
% of 9-AC_{total} AUC	7.18±2.33	12.9±4.20	8.76±3.49	6.98±1.72

The kinetic data and recorded AUC values for the following days of administration were similar to those achieved the first day in the same patient (Tables 2 and 3). Hence, the resulting intrapatient variability in AUC and peak drug levels, expressed as the coefficient of variation, was extremely small and averaged 8.67% for 9-AC lactone and 10.9% for 9-AC carboxylate. The interpatient variability in the observed pharmacokinetics, however, was large, with coefficients of variation in AUC values as high as 89.5% for 9-AC lactone and 99.0% for 9-AC carboxylate.

Over the total dose range studied, 9-AC lactone and 9-AC total demonstrated linear and dose-independent pharmacokinetics (Figs. 3*A* and *B*, respectively). No significant relationship was observed between the AUCs of 9-AC total and that of the pharmacologically active species, 9-AC lactone (not shown). The interpatient variation in the equilibrium ratio of 9-AC lactone and 9-AC carboxylate could be explained in part by a individual differences in pretreatment serum albumin levels, for which a significant correlation with the AUC ratio of 9-AC lactone and 9-AC carboxylate could be demonstrated (r=0.471, p=0.0099). This finding clearly indicates that separate monitoring of 9-AC lactone and 9-AC carboxylate concentrations is mandatory to relate drug levels to pharmacodynamic outcome in patients treated with oral 9-AC.

1.0	1.1	1.5
7	3	12
ND	ND	3.03±0.90
0.29±0.05	0.17±0.13	0.24±0.13
0.99±0.29	1.00±0.01	1.17±0.33
11.0±7.46	12.2±6.95	6.82±2.98
0.24±0.09	0.36±0.07	0.38±0.21
0.76±0.51	0.82±0.16	1.13±0.59
5.10±2.68	6.76±2.26	12.6±4.20
31.2±19.1	49.3±29.7	31.9±14.3
31.2±27.0	48.5±23.8	ND
4.96	6.48	ND
61.1	60.3	44.8
10.2±4.94	8.67±3.22	7.30±3.22

Abbreviations: n, number of patients; k_a, absorption rate constant; t_{lag}, lag time; t_{max}, time to peak plasma levels; C_{max}, maximum plasma concentrations; $t_{1/2}(i)$, half-life of the i-th disposition phase; AUC, area under the plasma concentration *versus* time curve; CV, coefficient of variation.

Table 3: Model-dependent pharmacokinetics of 9-AC$_{car}$ after oral drug administration of 9-AC.

Dose (mg/m^2/day)	0.25	0.40	0.60	0.84
n	6	3	4	6
t_{lag} (h)	0.41±0.20	0.34±0.04	0.39±0.13	0.31±0.02
t_{max} (h)	2.60±1.19	2.20±0.601.6	3±0.23	2.01±0.49
C_{max} (ng/mL)	9.43±3.50	11.8±5.76	14.5±6.93	15.2±4.88
$t_{1/2}(\alpha)$ (h)	0.55±0.50	0.40±0.30	0.20±0.20	0.18±0.26
$t_{1/2}(\beta)$ (h)	1.78±0.67	1.12±0.44	1.00±0.69	1.07±0.55
$t_{1/2}(\gamma)$ (h)	7.50±3.96	6.59±2.82	4.98±1.74	9.61±3.73
$AUC_{0-\infty}$ day 1 (ng.h/mL)	90.8±48.2	89.0±49.4	102±45.1	174±39.4
$AUC_{0-\infty}$ day 8 (ng.h/mL)	177±127	137±105	83.9±34.1	162±36.3
intra %CV of AUC	13.9	27.7	1.63	8.52
inter %CV of AUC	53.1	55.5	44.3	22.6
% of 9-AC$_{total}$ AUC	92.8±2.33	87.1±4.20	91.2±3.49	93.0±1.72

Salivary drug monitoring was evaluated as an option for determining the AUC of 9-AC lactone. The 9-AC lactone concentration ratio in plasma and unstimulated saliva was strongly patient-dependent and highly variable around a mean value of ~1.4, suggesting that saliva is an unreliable matrix for pharmacokinetic analysis of this drug (Fig. 2).

To determine the impact of a pleural effusion on the pharmacokinetics of 9-AC, plasma and pleural effusion samples were obtained for drug analysis in a single patient with a malignant pleural effusion. The mean pleura versus plasma concentration ratio of 9-AC lactone was 4.95%±2.32 (mean ± SD; range, 0.7-6.8%).These data indicate that pleural effusion does not constitute a major pharmacokinetic compartment for this drug.

The pharmacokinetic data obtained from 27 patients were plotted against the percentage decrease in white blood cell count (WBC), platelet count (PLT) and absolute neutrophil count (ANC), at nadir relative to the pretreatment value. Four different models, based on linear, log-linear, maximum effect (E_{max}), and sigmoidal E_{max} fitting, were compared for their ability to describe the data. Using sigmoidal E_{max} modeling of the pharmacokinetic and hematological toxicity data significant correlations between the AUC of 9-AC lactone and the percentage decrease in WBC (r=0.86; p<0.001; Fig. 4*A*), percentage decrease in PLT (r=0.83; p<0.001; Fig. 4*B*) and percentage decrease in ANC (r=0.66; p<0.001; Fig. 4*C*) could be demonstrated. In addition, the worst observed

1.0	1.1	1.5
7	3	12
0.35±0.15	0.34±0.06	0.31±0.11
2.36±1.19	3.67±0.94	2.39±1.05
25.5±12.7	41.8±19.6	35.4±19.2
0.24±0.38	0.96±0.18	1.04±0.78
0.78±0.51	4.26±1.60	2.83±1.96
8.62±3.57	10.1±2.29	12.4±6.48
357±265	578±311	510±354
315±327	592±304	ND
6.49	10.5	ND
99.0	53.9	69.4
89.8±4.94	91.3±3.22	92.7±4.15

Abbreviations: n, number of patients; t_{lag}, lag time; t_{max}, time to peak plasma levels; C_{max}, maximum plasma concentrations; $t_{1/2}(i)$, half-life of the i-th disposition phase; AUC, area under the plasma concentration *versus* time curve; CV, coefficient of variation.

myelotoxicity grade according to common toxicity criteria (CTC) in the entire patient population correlated with the AUC of 9-AC lactone (r=0.93; p<0.001; not shown). The development of any myelotoxicity grade 2 or worse was associated with an AUC of 9-AC lactone ≥17.3 ng*h/mL, using the Hill equation and data shown in Fig. 3*B*. Pharmacokinetic/pharmacodynamic relationships based on (log-)linear and non-sigmoidal E_{max} models were less predictive, as were models based on 9-AC carboxylate or 9-AC total (not shown).

DISCUSSION

Topoisomerase I inhibitors are of great clinical interest because of their unique mode of action, their important antitumor activity and the high expression of the enzyme in various human tumor types. 9-AC, a semisynthetic analog of camptothecin revealed a broad antitumor activity in preclinical studies. Initial Phase I studies focused on schedules with prolonged infusion duration. In order to facilitate the prolonged drug administration, an oral formulation was developed. 9-AC can be administered orally as a colloid dispersion (CD) or as gelatine capsules in PEG1000. In dogs the mean oral bioavailability of the CD formulation was 13% (range, 4.5-26%), as compared to 10% of

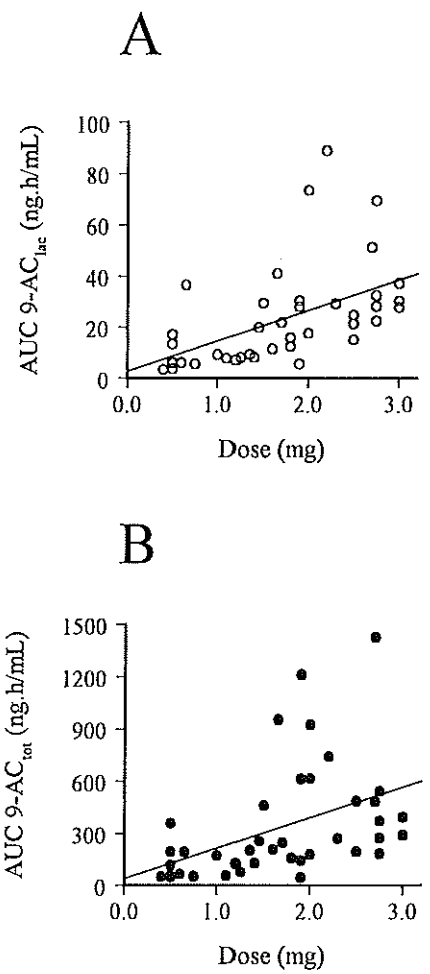


Fig. 3: Absolute dose of 9-AC plotted versus AUC of 9-AC lactone (A) and 9-AC total (B).

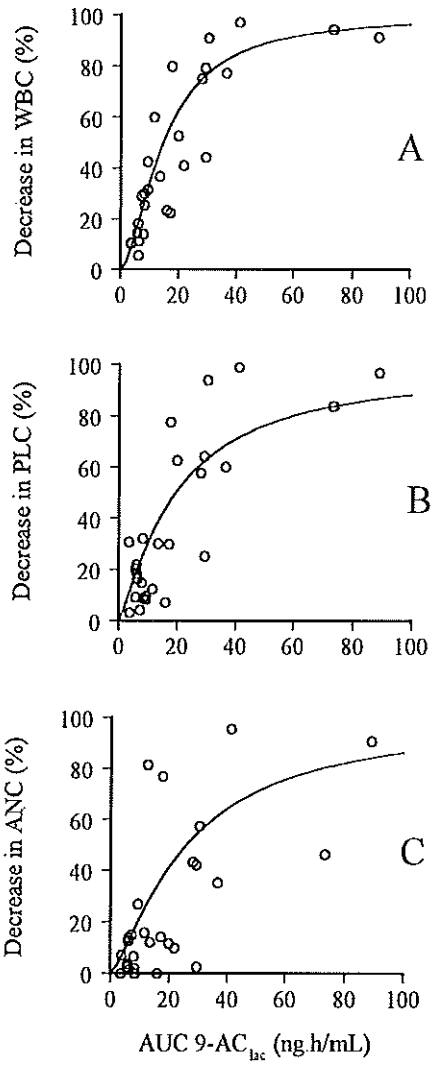


Fig. 4: Correlation between the area under the plasma concentration-time curve (AUC) of 9-AC lactone and the percentage decrease in white blood cells (WBC) at nadir of the first treatment course (A), in platelets (PLC) (B) and in neutrophiles (ANC) (C). The lines represent the fitting of the data to a sigmoidal maximum-effect model.

the PEG1000. Both formulations retained their antitumor activity after oral administration. Recently the phase I study on the oral administration of the CD formulation of 9-AC, 5 days per week, every 2 weeks was completed [25]. Diarrhea was the dose limiting toxicity at a dose level of 0.2 mg/m^2. PEG1000 9-AC was previously shown to demonstrate rapid intestinal absorption in patients after oral delivery, with an overall bioavailability (F) of 48.6±17.6%. This compares favorable to other camptothecin analogs, including topotecan (F=30.0%) [26], 7-(4-methyl-piperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin (GI147211; F=11.3%) [27], and irinotecan (F=12-21%) [28]. The terminal half-life ($t_{1/2}$) of 9-AC was shown to be 12.6±4.20 hr, which is substantially longer than that of topotecan (range, 2.35-5.91 hr) [26] and GI147211 (range, 6.85±3.13 hour) [27]. Another difference between topoisomerase I inhibitors constitutes the AUC ratio of the active lactone and the total drug of the parent drug. The conversion of 9-AC lactone into the ring-opened species in plasma could be demonstrated from the first sample acquired (i.e. at 0.33 hr). At equilibrium, the 9-AC carboxylate accounted for 91.1±2.11% of 9-AC total drug concentrations This compares unfavorable to the percentages present in the ring-opened form at equilibrium observed in patients for topotecan (~50%) [26], GI147211 (~60%) [27], and irinotecan and its active metabolite SN-38 (~65% and ~35%, respectively) [29]. These figures underscore the important differences in pharmacokinetics between camptothecin analogs.

Our results of the Phase I study with oral 9-AC capsules indicated that the drug could be administered in a 14-day schedule repeated every 3 weeks with tolerable and manageable toxicity [30]. The dose-limiting toxicities were a combination of thrombocytopenia *plus* neutropenia complicated by fever and diarrhea occurring at a dose level of 1.1 mg/m^2/day. Other side effects were mild to moderate (CTC grade 1 to 2) and consisted of nausea, vomiting, alopecia, mucositis and fatigue. Although 9-AC demonstrated a linear pharmacokinetic behavior over the entire dose range studied, we observed that the AUC of 9-AC lactone was a better indicator for the observed hematological toxicity than the dose. The intrapatient variability in AUC and peak drug levels was extremely small and averaged less than 10% for 9-AC lactone. However, the interpatient variability in the concentrations of 9-AC at each of the sample-time points as well as in the AUC was large, with values for the coefficient of variation as high as 99%. In this study, the high variability in lactone to carboxylate interconversion was significantly related to individual differences in pretreatment serum albumin levels. Although our results need to be confirmed in a larger number of patients, they tend to indicate that higher protein levels will result in a more profound binding of 9-AC carboxylate, thereby further diminishing the effective concentration of the active species of the drug. In all, these data indicate that classical drug dosing based on body-surface area alone is unlikely to be effective in minimizing interpatient differences in systemic exposure to oral 9-AC.

The pharmacokinetics of 9-AC were clearly related to the pharmacodynamic outcome, (i.e. hematological toxicity). The sigmoidal E_{max} model was found most appropriate to fit the kinetic data to the observed myelosuppression. The best correlation was obtained with the AUC of 9-AC lactone,

the exposure to the active drug. Considering this pharmacokinetic-pharmacodynamic relationship, a target AUC for 9-AC lactone can be defined according to the grade of toxicity that is considered to be acceptable in future studies. If hematological toxicity graded 2 or less is defined as acceptable, then the target AUC of 9-AC lactone is 17.3 ng*h/mL, using the Hill equation and data shown in Fig 3*A*.

For pharmacokinetic and pharmacodynamic analysis frequent blood sampling is inevitable. In order to evaluate salivary drug monitoring of 9-AC as an alternative to drug monitoring in plasma, the concentrations and AUC of 9-AC total and lactone were measured in unstimulated saliva samples in 5 patients during this study. The 9-AC concentration ratio in plasma and unstimulated saliva proved to be strongly patient-dependent and highly variable, suggesting that saliva is an unreliable matrix for pharmacokinetic analysis of 9-AC. Similar results were obtained recently for pharmacokinetic analysis of CPT-11 and SN-38 in saliva demonstrating large interpatient variability in plasma/saliva ratios [31].

Recently, we developed a limited-sampling model for reliable and accurate prediction of the systemic exposure to 9-AC after oral drug administration, using only one time blood sample taken at 3 hr after drug dosing [32]. In order to further diminish the interpatient variability in drug exposure in future studies, a pharmacokinetic guided approach may be considered. After oral administration of an appropriate starting dose of 9-AC (i.e. 1 mg/m^2), the 9-AC lactone plasma concentration can then be measured at 3 hr after drug dosing. Using the limited-sampling model and the linear-regression relationship between drug dose and AUC (Fig. 3), the optimal dose leading to the target AUC, determined according to the toxicity considered acceptable, can be calculated.

This procedure may prove valuable in reducing interpatient variation in exposure to 9-AC, and will enable us to optimize the treatment for any given patient by combining maximally achievable doses with tolerable toxicity during treatment. This strategy seems to be interesting both in further phase II studies using the 14 day administration schedule and in Phase I studies with different schedules of administration.

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Chapter 4

Liposomal lurtotecan (NX 211)

Chapter 4a

Liposomal lurtotecan (NX 211): determination of total drug levels in human plasma and urine by reversed-phase high-performance liquid chromatography

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ABSTRACT

Lurtotecan (GI147211; LRT) is a semisynthetic and water-soluble analogue of the topoisomerase I inhibitor camptothecin. To determine whether the therapeutic efficacy of LRT in patients could be improved, the drug was encapsulated in liposomes (NX 211; Gilead Sciences). In order to allow accurate description of the pharmacokinetic behavior of NX 211 in cancer patients, we have developed sensitive RP-HPLC assays with fluorescence detection (λ_{ex}=378 nm; λ_{em}=420 nm) for the determination of total LRT levels in human plasma and urine. Sample pretreatment involved deproteinization with 10% (w/v) aqueous perchloric acid-acetonitrile (2:1, v/v), and chromatographic separations were achieved on an Inertsil-ODS 80A analytical column. The lower limit of quantitation (LLQ) was established at 1.00 ng/ml in plasma (200-µl sample) and at 100 ng/ml in urine (200 µl of 40-fold diluted sample). The within-run and between-run precisions were <7.5%. LRT concentrations in urine <100 ng/ml were determined by a modified procedure comprising a single solvent extraction with *n*-butanol-diethyl ether (3:4, v/v). In this assay, the fluorescence signal of LRT was increased 14-fold prior to detection by post-column exposure to UV light (254 nm) in a photochemical reaction unit. The LLQ of this assay was 0.500 ng/ml (150-µl sample) and the within-run and between-run precisions were <10%.

INTRODUCTION

Lurtotecan (7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(*S*)-camptothecin; also known as GI147211; LRT) (Fig. 1) is a novel semisynthetic analogue of camptothecin, a cytotoxic plant alkaloid that was first extracted from the wood and bark of the oriental tree, *Camptotheca acuminata* [1]. The mechanism of action of camptothecin derivatives is based on stabilization of the cleavable complex formed by the intranuclear enzyme topoisomerase I and DNA, and on induction of single-stranded DNA breaks [2]. LRT has previously been shown to have significant activity in both *in vitro* cytotoxicity assays and *in vivo* tumor model systems [3-5], and was recently introduced into clinical trials [6-8].

Clinical pharmacokinetic studies of camptothecin derivatives, including LRT, are complicated by a chemical, pH-dependent instability of the α-hydroxy-δ-lactone moiety in the core structure of the compounds, generating a ring-opened carboxylate form. This lactone functionality undergoes rapid hydrolysis in aqueous solution under physiological conditions, i.e. at pH 7 or above, and results in a virtually complete loss of biological activity [9]. In recent years, considerable effort has been put in the development of alternative formulations that would allow prolonged systemic

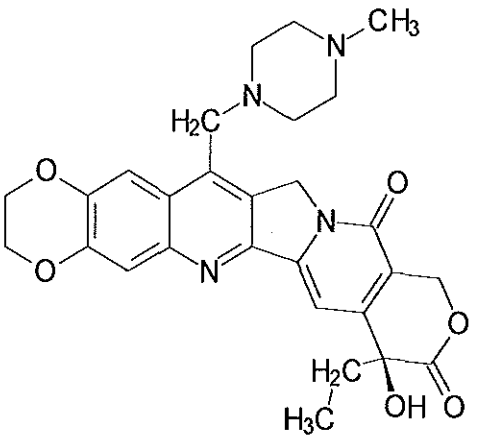

Fig. 1: Chemical structure of lurtotecan (LRT).

exposure to the pharmacologically active drug form. One of these approaches is the incorporation of the lactone forms of camptothecins in liposomal particles. Indeed, recent preclinical studies indicated that liposomal encapsulation of the topoisomerase I inhibitors topotecan [10,11], camptothecin [12,13] and irinotecan [14,15] proved to be very efficient against lactone ring opening, increased antitumor activity in experimental tumor models and dramatically enhanced tissue distribution and the systemic availability. Efficacy studies performed in nude mice bearing human colon and head and neck tumor xenografts have also indicated improved therapeutic efficacy for a new liposomal formulation of LRT (NX 211; Gilead Sciences) as compared to nonliposomal LRT [16].

Based on these favorable results, we recently started a project to study the safety profile and clinical pharmacokinetics of NX 211 in patients with advanced solid tumors. In the context of this study, we have now developed sensitive RP-HPLC methods with fluorescence detection using a sample clean-up procedure that disrupts the liposomes, thus enabling determination of total drug levels in plasma and urine samples following NX 211 administration. The methods have been validated in terms of sensitivity, accuracy and precision [17], and have been used in a pharmacokinetic experiment in a patient to investigate their applicability *in vivo*.

EXPERIMENTAL

Chemicals and reagents

LRT dihydrochloride monohydrate (lot: U2044/164/1, containing 78.11% of the free base) and NX 211 (liposomal LRT, lot: 181801F, containing 0.49 mg LRT as free base/ml) were delivered by Gilead Sciences (San Dimas, CA, USA). The internal standard (IS) 6,7-dimethoxy-4-methylcoumarin (lot 79F3652) was obtained from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), diethyl ether, *n*-butanol, methanol and acetonitrile were purchased from Rathburn (Walkerburn, UK). Perchloric acid (70-72%, v/v, in water), neat acetic acid and sodium hydroxide were supplied by Baker (Deventer, The Netherlands). Ammonium acetate was delivered by Roth (Karlsruhe, Germany) and sodium chloride by Merck (Darmstadt, Germany). All water used in the study was filtered and deionized with a Milli-Q-UF system (Millipore, Milford, MA, USA). Drug-free human plasma for the construction of calibration curves and quality control (QC) samples originated from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

Stock solutions

Stock solutions of LRT were made in triplicate by dissolving X mg LRT in (X x 0.7811) ml DMSO, resulting in a solution containing 1.00 mg/ml LRT (free base). The working stock solution of LRT, containing 0.100 mg/ml free base, was prepared by a 10-fold dilution of the stock solution in DMSO. A stock solution of the IS at a concentration of 1 mg/ml was prepared by dissolving 50 mg IS in 50 ml DMSO.

Standards for total LRT in plasma and urine

Spiked plasma samples used as calibration standards were prepared daily in duplicate by addition of 10 µl of serial dilutions in methanol-water (1:1,v/v) from the working solution of LRT to 240 µl of drug-free human plasma. This resulted in calibration standards of 1.00, 2.50, 5.00, 10.0, 25.0, 50.0 and 100 ng/ml LRT (free base) in plasma. Four pools of QC samples for LRT were prepared in human plasma at concentrations of 4.00, 20.0, 75.0 and 750 ng/ml, by addition of the appropriate volume of the LRT working solution or dilutions in 10 mM aqueous sodium hydroxide-methanol (1:1, v/v) (to shift the equilibrium to the carboxylate form) to human plasma. The QC containing 750 ng/ml LRT was used to investigate the suitability of small-volume (20 µl) injections. Lower limit of quantitation (LLQ) samples in plasma were prepared daily in separate blank plasma samples obtained from 5 healthy volunteers at a concentration of 1.00 ng/ml. To minimize a potential difference with clinical samples, 2 pools of recovery control (RC) samples containing 20.0 and 750 ng/ml NX 211 were also prepared by addition of an aliquot of the NX 211 stock solution (in phosphate-buffered saline) to human plasma. Two pools of QC samples were prepared in human urine at concentrations of 250 and 2500 ng/ml, which were diluted 40-fold in blank human plasma prior to extraction. The

LLQ samples for urine were prepared daily at a spiked concentration of 100 ng/ml in quintuplicate using 5 different drug-free urine samples.

Standards of LRT in the sensitive urine assay

Calibration standards in urine were also prepared daily in duplicate by addition of 10 µl of serial dilutions of LRT in methanol-water (1:1, v/v) to 240 µl blank human urine, at final concentrations of the free base of 0.500, 1.00, 2.50, 5.00 and 10.0 ng/ml. Three pools of QC samples were prepared at concentrations of 1.25, 7.50 and 250 ng/ml by addition of appropriate volumes of a dilution of the LRT working stock solution [in 10 mM aqueous sodium hydroxide-methanol (1:1, v/v)]. The QC sample containing 250 ng/ml, identical to that used for the assay of total LRT in plasma and urine, was diluted 10-fold in blank urine before extraction, and was further used to show the applicability of low volume injections (10 µl). The LLQ samples were prepared daily at a concentration of 0.500 ng/ml, again in quintuplicate using 5 different drug-free urine samples.

HPLC instrumentation and conditions

The HPLC systems consisted of constaMetric 3200 and 4100 solvent delivery systems (LDC Analytical, Riviera Beach, FL, USA), Waters 717plus autosampling devices (Milford, MA, USA), a Beam Boost photochemical reaction unit supplied with a coil of 25 m and an internal diameter of 0.3 mm (ICT-ASS-Chem, Bad Homburg, Germany), and Jasco 821-FP and FP-920 fluorescence detectors (Jasco Benelux B.V., Maarssen, The Netherlands). Separations were achieved on a stainless-steel analytical column (150x4.6 mm internal diameter) packed with Inertsil ODS-80A material (5 µm particle size), delivered by Alltech Applied Science (Breda, The Netherlands). The mobile phase was identical in both assays and was composed of 1.0 M aqueous ammonium acetate (pH 5.5)-water-acetonitrile (10:72.5:17.5, v/v/v) with the pH adjusted to 5.5 (acetic acid). The analytical columns were maintained at 60°C using a model SpH99 column oven (Spark Holland, Meppel, The Netherlands). A detailed composition of the various HPLC systems used for the two assays is provided in Table 1. Peak recording and integration were performed with the Chrom-Card data analysis system (Fisons, Milan, Italy). All calibration curves were fitted by weighted ($1/x$) least-squares linear regression analysis using the peak height ratios of LRT and the IS *versus* the nominal concentrations of the standards.

Sample treatment for total LRT in plasma and urine

Samples were prepared by addition of 100 µl IS solution [100 ng/ml in 10% perchloric acid-acetonitrile (2:1, v/v)] to 200 µl human plasma, or 40-fold diluted urine in plasma, in a 1.5-ml polypropylene vial (Eppendorf, Hamburg, Germany). Samples were mixed vigorously for 30 min on a multi-tube vortex mixer, followed by centrifugation at 23,000x*g* (5 min) at ambient temperature. A 250-µl volume of the clear supernatant was transferred to a low volume glass insert, from which 200

µl (or 20 µl in case of QC and RC samples containing 750 ng/ml LRT and NX 211, respectively) were injected into the HPLC system.

Table 1: Composition of the two HPLC systems.

Equipment	Sensitive urine	Total plasma/urine
Pump 4100	---	Flow-rate: 1.25 ml/min
Pump 3200	Flow-rate: 0.75 ml/min	---
Autosampler 717p	✔	✔
Column oven SpH99	T=60°C	T=60°C
Column ODS-80A	✔	✔
Beam Boost	Lamp: λ=254 nm	---
	Coil: 25 m; ID: 0.3 mm	
Detector FP-920	λ_{ex}=378 nm	---
	λ_{em}=420 nm	
	Em band: 40 nm	
Detector 821-FP	---	λ_{ex}=378 nm
		λ_{em}=420 nm
		Em band: 30 nm

Sample treatment for LRT in the sensitive urine assay

A 100-µl volume of IS solution (50 ng/ml in 25 mM aqueous ammonium acetate, pH 3.0) was added to a 12-ml glass tube supplied with a PTFE-covered screw cap containing 150 µl urine. After incubation for 30 min at room temperature, 0.8 g solid sodium chloride was added, followed by extraction with 2 ml *n*-butanol-diethyl ether (3:4, v/v) by vigorous vortex mixing for 5 min. Subsequently, the sample was centrifuged at 4000xg (5 min), followed by collection of 1 ml upper organic layer, which was evaporated to dryness under a gentle stream of nitrogen at 70°C for a period of 45 min. The dried residue was redissolved in 150 µl 25 mM aqueous ammonium acetate (pH 3.0) and transferred to a glass insert. A volume of 50 µl (or 10 µl for the QC sample containing 250 ng/ml LRT) was injected into the HPLC system.

Validation

Validation runs of LRT in plasma and urine and of LRT in the sensitive urine assay included a set of calibration samples assayed in duplicate, and LLQ and QC samples in quintuplicate, and was performed on 4 separate occasions. Precisions were calculated by one-way analysis of variance (ANOVA) for each test concentration, using the run-day as the classification variable. The accuracy of at least 80% of the samples assayed at each concentration should be in the range of 80 to 120%. The within-run (WRP) and between-run precisions (BRP) should be <20% at the concentration of the LLQ and <15% at the concentrations of the QC samples and the average accuracy (ACC) should be within 85-115% for each concentration, including the LLQ.

The extraction recoveries of LRT and the IS in the assay for total LRT in plasma and urine were calculated by comparing peak heights obtained from a sample containing 25.0 ng/ml LRT in phosphate-buffered saline that was extracted (as described for plasma), to those obtained in extracted QC samples prepared in the biological matrix. The extraction recoveries in the sensitive urine assay were calculated by comparing observed peak heights of the processed urine samples of the calibration curves to peak heights obtained from spiked samples containing 1.00 ng/ml LRT and 10.0 ng/ml IS in 25 mM aqueous ammonium acetate (pH 3.0).

The stability of LRT and the IS in plasma and urine was established (*i*) during 3 consecutive freeze-thaw cycles, in which the samples were put at room temperature for 30 min after thawing, and (*ii*) during an overnight incubation at 37°C. The concentrations used were 20.0 and 75.0 ng/ml for plasma and 250 and 2500 ng/ml for urine, and were analyzed using the assay for the determination of total LRT in plasma and urine.

The selectivity of the assays was tested by the degree of separation of the compounds of interest and possible other chromatographic peaks caused by endogenous components and/or potentially co-administered drugs. The interference from endogenous material for LRT and the IS in human plasma and urine was determined by visual inspection of HPLC profiles of 5 processed blank plasma and urine samples obtained from 5 healthy volunteers. Interference from potentially co-administered drugs was tested at a spiked concentration of 10 μg/ml in a blank plasma extract for the assay of total LRT in plasma and urine, and at 10 μg/ml in 25 mM aqueous ammonium acetate (pH 3.0) for LRT in the sensitive urine assay. The tested compounds included acetaminophen, alizapride, codeine, dexamethasone, domperidon, metoclopramide, morphine, leucovorin, lorazepam, paroxetine, and ranitidine.

RESULTS AND DISCUSSION

In approaching the present analytical procedures, we used our own previous RP-HPLC procedure for the quantitative determination of total nonliposomal LRT (lactone plus carboxylate forms) in human whole blood as a starting point [18]. Because of the pH-dependent instability of the α-hydroxy-δ-lactone moiety in the core structure of LRT, resulting in the ring-opened carboxylate from at hight pH and the ring-closed lactone form at low pH, we decided to focuss only on measurement of total concentrations in the present study, since disruption of liposomes while maintaining the physiologic lactone to carboxylate ratio may not be feasible. The choice of the IS, 6,7-dimethoxy-4-methylcoumarin, and the optimal fluorescence wavelength couple of LRT (378/420 nm) was based on earlier work descibed for determination of LRT in human blood and dog plasma by Selinger et al. [19] and Stafford and St. Claire [20], respectively.

For the purpose of assay validation, all QC samples were prepared with the carboxylate form of LRT, to ensure a quantitative conversion to the lactone species of the total amount of LRT, prior to measurement, present in plasma and urine of clinical samples.

Assay of total LRT in plasma and urine

Initially, the assay of total LRT in plasma and urine was validated with only LLQ and QC samples of nonliposomal LRT (free base) in plasma and urine, using an extraction time of 15 min. The calibration curves were linear in the range of 1.00 to 100 ng/ml with Pearson's regression correlation coefficients ranging from 0.9986 to 0.9997, by using weighted ($1/x$) linear least-squares regression analysis. The retention times of LRT and the IS were 11 and 15 min respectively, with an overall run time of 20 min.

One of the tested blank plasma sample specimens showed a minor (unknown) interfering peak in the chromatogram for LRT, and was replaced by a new blank plasma obtained from a healthy volunteer to enable accurate determination of the LLQ. No interfering peaks with retention times around the IS were found in the tested blank plasma samples. In the 5 (40-fold diluted) blank human urine samples, no interfering peaks were found for LRT; however, all tested urine samples showed a small peak with the same retention time as the IS, but this interference did not significantly alter the observed data. The tested drugs potentially co-administered with NX 211 did not interfere with the analytes of interest. Some minor peaks were found with retention times of 1 to 3 minutes, causing no problem for the determination of LRT in plasma and urine.

The LLQ was established in plasma at 1.00 ng/ml and in urine at 100 ng/ml, with 95% of the LLQ samples falling within the acceptable accuracy range of 80 to 120% [17]. The within-run and between–run precisions at the 5 tested concentrations in plasma, including the LLQ, were <7.5% and <4.1%, respectively, with the accuracy ranging from 96 to 110% (Table 2). The within-run and between-run precisions in urine at the 3 tested concentrations were <6.7% and <4.4%, respectively,

Table 2: Validation characteristics of total LRT in plasma and urine.

Matrix	Nom.conc. (ng/ml)	Mean (ng/ml)	WRP[a] (%)	BRP[a] (%)	ACC[a] (%)
Plasma	1.00 [b]	0.959	7.5	4.1	96
	4.00	3.84	7.4	c	96
	20.0	22.0	2.7	1.0	110
	75.0	81.4	2.3	2.1	109
	750	789	2.8	2.4	105
Urine	100 [b]	96.8	6.7	4.4	97
	250	269	3.0	0.12	108
	2500	2525	4.3	2.1	101

a: Abbreviations: WRP, within-run precision; BRP, between-run precision; ACC, average accuracy
b: Lower limit of quantitation sample
c: No additional variation was observed as a result of performing the assay in different runs

with an accuracy range of 97 to 108% (Table 2). The extraction recoveries, estimated by comparing peak heights obtained by direct injection of standard solutions containing 25.0 ng/ml LRT and IS in blank plasma extracts to those obtained in extracted plasma samples of the calibration curves, were 89±8.2% (mean ± standard deviation) and 67±4.8% for LRT and the IS, respectively. No loss of LRT was estimated at the tested concentrations after 3 freeze-thaw cycles or during overnight incubation of the samples at 37°C (data not shown).

Using an extraction time of 15 min, we noted that the extraction recovery of plasma samples containing NX 211 (liposomal LRT) was approximately 10% lower for LRT as compared to plasma samples spiked with nonliposomal LRT (free base). The extraction efficiency of the samples containing NX 211 was eventually increased by extending the vortex-mixing time to 30 min, at which maximum recovery was reached (data not shown). The assays of total LRT in plasma and urine were re-validated during 3 analytical runs (with a 30-min mixing time during sample extraction), and the calibration curves were assayed in duplicate with the 4 QC samples of LRT and 2 RC samples containing NX 211 spiked at concentrations of 20.0 and 750 ng/ml, both in plasma, in triplicate. The Pearson's regression correlation coefficients in the re-validation runs ranged from 0.9995 to 0.9998, and the range of the within-run and between-run precisions of the QC samples containing LRT were 1.8 to 3.6% and 0.73 to 2.5% respectively, with an overall accuracy between 99 and 104%. The within-run precisions of the RC samples were 1.9 and 2.5%, respectively, for the samples containing

20.0 and 750 ng/ml, whereas the respective between-run precisions were 2.3 and 7.3%. The extraction recoveries of LRT in the QC and RC samples containing 20.0 ng/ml LRT and NX 211 were 90±2.1% and 85±3.9%, respectively, and 83±3.0% (LRT) and 82±3.0% (NX 211) for the samples containing 750 ng/ml.

Representative RP-HPLC chromatograms derived from a blank human plasma pool and a plasma sample spiked to contain 10.0 ng/ml LRT (free base) are shown in figs. 2A and 2B. An additional chromatographic peak was found in the RC samples containing NX 211 with a retention time of approximately 48 min. This peak was later identified as a photochemical

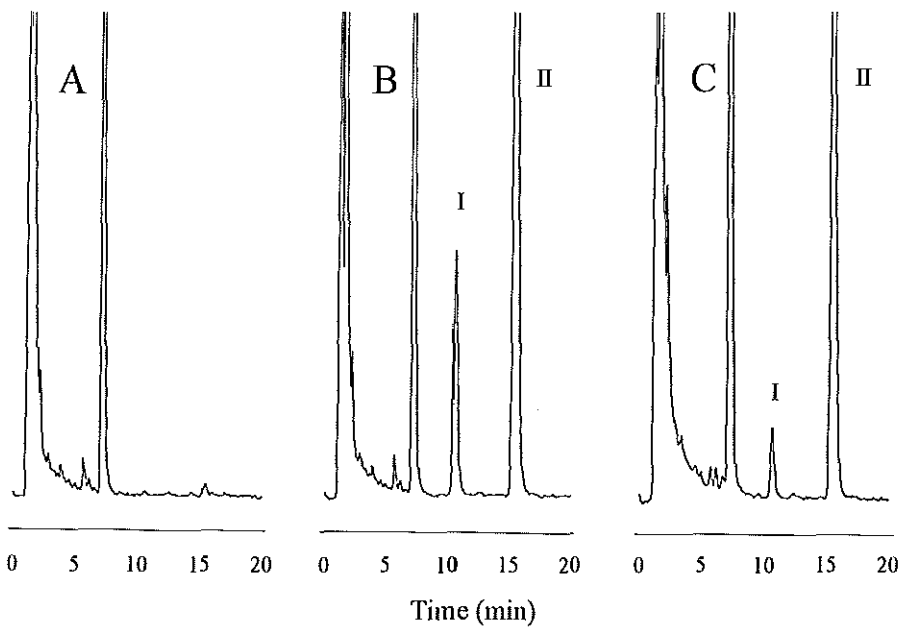

Fig. 2: Chromatograms of a blank human plasma sample (A), a plasma sample spiked with 10.0 ng/ml LRT free base (B) and a plasma sample obtained from a patient 8 h after the administration of NX 211 at a dose level of 0.8 mg (C). Peaks labeled I and II correspond to LRT and the IS, respectively.

degradation product of NX 211 by comparison of the compound's chromatographic behavior on the RP-HPLC column and spectroscopic properties with a pure reference standard. Isolation, purification and structural identification, in addition to the role of this compound in the overall drug disposition will be described separately.

Assay for the sensitive determination of LRT in urine

Previous studies have shown that renal clearance of LRT in patients treated with nonliposomal drug was low, with approximately 10-14% of the delivered dose excreted as unchanged parent drug in urine [6]. In order to allow determination of low concentrations of LRT in urine, which can be anticipated following treatment with NX 211 at low dosages, an assay was also required with increased sensitivity as compared to the assay described for total LRT concentrations in plasma and urine with an LLQ (for urine) of 100 ng/ml (see above). The sensitivity of this assay could theoretically be improved by decreasing the dilution factor used for urine samples prior to extraction. However, this will likely result in substantially prolonged run times in order to get sufficient separation between the peaks of interest (i.e. LRT and the IS) and those resulting from the presence of endogenous material, which would in turn compromise assay sensitivity.

Eventually, the assay sensitivity could be significantly improved, in part, by increasing the fluorescence intensity of LRT through a modification of the detection procedure. LRT is known to be slightly light sensitive [19] and since photochemical reactor units in combination with HPLC has been described for a wide variety of other compounds [21-25], where increased detector signal outputs have been described from 2 to 80 folds, we have evaluated the impact of post-column photodegradation on the fluorescence activity of LRT. Post-column exposure of LRT to UV light (254 nm) results in a loss of the piperazinomethylene moiety on C7 of the LRT molecule, as determined by electro-spray ion-trap mass spectrometry [m/*z*=409 (LRT-C7 side chain)]. The influence of the photochemical reaction unit on the fluorescence of LRT was estimated by injections of 50 µl of 5 ng/ml LRT in 25 mM ammonium acetate (pH 3.0) onto the HPLC system as described for this assay. The flow rate was varied from 0.50 to 2.00 ml/min, resulting in irradiation times of 300 to 75 s. At each flow rate, 2 injections were performed, one with the lamp of the photochemical reaction unit on and another one with the lamp switched off. The ratios of the peak heights obtained with the lamp on and off were calculated. As displayed in fig. 3, the fluorescence intensity of LRT increased 9 to 15-fold depending on the flow rate used (0.50 to 2.00 ml/min). The use of a flow rate set at 0.75 ml/min (i.e. and irradiation time of 200 s) resulted in a 14-fold increased fluorescence signal of LRT, and was associated with an acceptable total run time (35 min) with retention times of 19 and 24 min for LRT and the IS, respectively.

Fig. 4 shows representative chromatograms of a blank human urine sample and a sample spiked with 2.50 ng/ml LRT (free base). The calibration curves of LRT were linear in the range of 0.500 to 10.0 ng/ml, with Pearson's correlation coefficients ranging from 0.9954 to 0.9994, also using

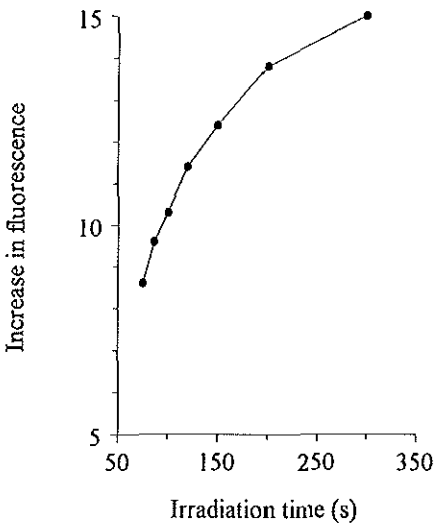

Fig. 3: Influence of the photochemical reaction unit on the fluorescence intensity of LRT.

weighted (1/x) least-squares linear regression analysis. No analytical interference was found between LRT or the IS and of the tested drugs potentially co-administered with NX 211. However, a number of three additional peaks were found with retention times of 6, 7 and 10 minutes. Since these elute in the big front of the chromatograms of urine and high concentrations of the drugs were spiked these peaks have no impact on the determination of low concentration of LRT in urine. Small peaks with the same retention time as LRT and the IS were found in all of the tested blank urine samples, so the LLQ could not be established below a concentration of 0.500 ng/ml, with 80% of all samples in the acceptable range of accuracy. The within-run and between-run precisions at the 4 tested concentrations were <5.5 and <10%, respectively, with the accuracy ranging from 97 to 108% (Table 3). Taking into consideration that only approximately half of the added volume of the organic layer was evaporated, the extraction recoveries were around 80 and 92% for LRT and IS respectively.

Table 3: Validation characteristics of total LRT urine (sensitive assay).

Nom.conc. (ng/ml)	Mean (ng/ml)	WRP[a] (%)	BRP[a] (%)	ACC[a] (%)
0.500[b]	0.539	5.5	c	108
1.25	1.31	3.0	3.1	105
7.50	7.27	2.8	6.7	97
250	243	4.2	10	97

a: Abbreviations: WRP, within-run precision; BRP, between-run precision; ACC, average accuracy
b: Lower limit of quantitation sample
c: No additional variation was observed as a result of performing the assay in different runs

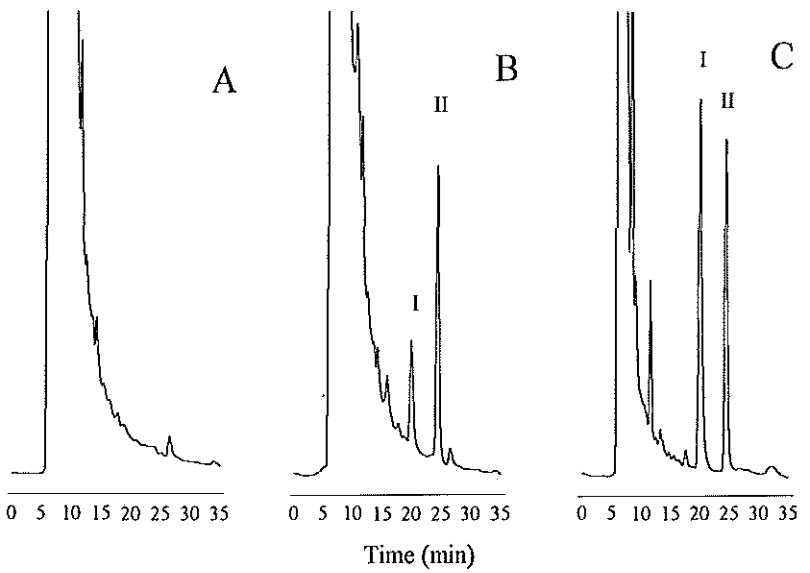

Fig. 4: Chromatograms of a blank human urine sample (A), a urine sample spiked with 2.50 ng/ml LRT free base (B) and a urine sample obtained from a patient, collected 12-24 h after the administration of NX 211 at a dose level of 0.8 mg (C). Peaks labeled I and II correspond to LRT and the IS, respectively.

Assay application

The suitability of the developed methods for clinical use was demonstrated by the determination of LRT in biological specimens obtained from a patient treated with NX 211 at a dose of 0.8 mg. Examples of the patient's sample trace are shown in Fig. 2C (plasma) and Fig. 4C (urine). Distinct peaks were obtained for LRT in both matrices that were well resolved from endogenous components.

CONCLUSION

In conclusion, we have developed and evaluated new liquid chromatographic methods for measuring total LRT levels in human plasma and urine. The primary elements of novelty described in this work are the use of human plasma as biological matrix containing liposomal LRT (NX 211) and the utilization of a photochemical reaction unit to increase the native fluorescence intensity of LRT in order to achieve sub-nanogram per milliliter determinations. The methods were shown to meet the current requirements as to validation of bioanalytical methodologies [17], providing excellent precision and accuracy. The described methods permit the analysis of patient samples, and will be implemented in an ongoing clinical trial to investigate the disposition of LRT in cancer patients receiving NX 211.

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Chapter 4b

Liposome-encapsulation significantly reduces lurtotecan (NX 211) clearance in cancer patients

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ABSTRACT

This study was designed to evaluate the disposition of liposome-encapsulated lurtotecan (NX 211) in 6 cancer patients receiving the drug as a 30-min i.v. infusion (dose, 1.6–4.3 mg/m^2). Serial plasma, whole blood, urine, and feces samples were collected for 96 h and analyzed by reversed-phase high-performance liquid chromatographic assays. The pharmacokinetic profile in plasma was characterized by a slow systemic clearance of 0.78±0.88 L/h/m^2 (mean±SD) with a mono-exponential decline, and a steady-state volume of distribution approximating the blood volume (3.5±2.6 L/m^2). In addition, the total lurtotecan blood:plasma concentration ratios averaged 0.66±0.13, indicating limited drug accumulation in blood cells. Urinary excretion was 8.2±4.7% of the delivered dose, indicating that renal clearance is a minor route of elimination of the unchanged drug. Total fecal excretion amounted to 9.7±6.2% of the dose, and 2 potential metabolites could be identified from fecal extracts. Overall, these data indicate that NX 211 administration results in significantly increased plasma exposure as compared to non-liposomal lurtotecan, which may be a potential advantage with pharmacodynamic importance.

INTRODUCTION

Lurtotecan is a totally synthetic analog of 20-(*S*)-camptothecin, a natural product isolated from *Camptotheca acuminata* [1]. Structurally, lurtotecan is unique among camptothecin drugs, because of a dioxolane moiety on the A-ring and a bulky 4-methyl-piperazinomethylene group on the C-7 position (fig. 1). These molecular modifications have resulted in enhanced aqueous solubility as compared to the original agent and increased affinity of the compound for DNA topoisomerase I, the cellular locus through which camptothecin analogues produce their antitumor activity. The cytotoxicity of these so-called topoisomerase I inhibitors is distinctly S-phase specific, and various preclinical studies with lurtotecan as well as other camptothecin drugs have suggested that prolonged exposure, achieved either by repeated doses or prolonged infusion, might be beneficial for efficacy profiles (reviewed in [2]). In order to exploit this apparent schedule dependency, several clinical trials have been conducted with lurtotecan focussing on intermittent (daily for 5 days every 3 weeks [3-5]) or prolonged i.v. dosing schedules (72-h infusion every 4 weeks [6] or 7-, 14-, or 21-day infusions [7]). These studies have shown that the pharmacokinetic behaviour of lurtotecan is influenced significantly by a chemical, pH-dependent hydrolysis of the lactone functionality in the core structure, generating a ring-opened carboxylate form, which is devoid of biological activity [8]. Based on this finding, considerable effort has been put recently in the development of alternative pharmaceutical vehicles that would allow prolonged systemic exposure to the active lactone form.

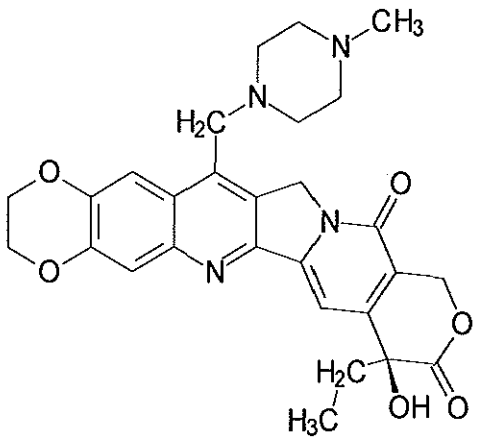

Fig. 1: Chemical structure of lurtotecan.

Among various approaches, liposomal encapsulation of camptothecin analogues was shown to be very efficient against lactone hydrolysis and, in fact, increased antitumor activity in mouse models by enhancement of tissue distribution and systemic drug availability [9-13]. Here, we have examined the disposition of a new liposomal formulation of lurtotecan (NX 211) in a group of cancer patients to investigate the clinical utility of this concept.

PATIENTS AND METHODS

Patients and treatment

Patients with a histologically confirmed diagnosis of advanced solid tumor refractory to standard therapy were eligible for the present study. Additional eligibility criteria included: age $\geq$18 years; Eastern Cooperative Oncology Group performance status $\leq$2; no previous treatment with antineoplastic agents for at least 3 weeks (4 weeks in case of carboplatin or any investigational agent and 6 weeks in case of nitrosureas or mitomycin C); adequate hematopoietic (absolute neutrophil count $>1.5\times10^9$/L, and platelet count $>100\times10^9$/L), renal (serum creatinine within normal limits), and hepatic function (total serum bilirubin within normal limits, and aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT)

levels ≤ 2.5 times upper normal limits); and no known hypersensitivity to systemic liposomal formulations or any drug chemically related to lurtotecan. The current clinical protocol was approved by the Rotterdam Cancer Institute Review Board, and all patients signed informed consent before study entry.

NX 211 was formulated as a sterile liposomal dispersion of lurtotecan in a buffer composed of 10-mM ammonium chloride and 9% sucrose and was obtained from Gilead Sciences Inc. (San Dimas, CA, USA). The drug product was supplied in 50-mL vials containing 5 mg lurtotecan. Each vial contained 80 mg hydrogenated-soy phosphatidylcholine, 19 mg cholesterol, 0.9 g sucrose, 2 mg citric acid, 5 mg ammonium chloride to a total volume of 10 mL. Dose solutions for administration were prepared in polyvinyl chloride-free infusion containers protected from light and under aseptic conditions by dilution of the pharmaceutical preparation with sterile 5% dextrose (D5W) to a volume of 25 mL. The NX 211 dose (1.6, 3.2 or 4.3 mg/m^2) was administered as a 30-min i.v. infusion.

Sample collection and preparation

Blood samples for pharmacokinetic analysis were drawn from a vein in the arm opposite to that used for drug infusion, and collected in 5-mL glass tubes containing lithium heparin as anticoagulant. Duplicate samples were obtained before drug administration and at 0.5 (end of infusion), 1, 1.5, 2.5, 4, 6, 8, 24, 48, 72, and 96 h after start of infusion. At each sampling time point, 1 aliquot of whole blood was immediately transferred to a polypropylene vial and stored at –80°C, while another was processed to plasma by centrifugation for 5 min at 3000xg (4°C), which was then also stored at -80°C until the time of analysis. Complete urine collections were obtained for the duration of the study in 12-h or 24-h portions, and aliquots were stored frozen in polypropylene vials. Complete collections of feces were also obtained in polystyrene containers, and stored immediately at –80°C. After thawing, these samples were homogenized individually in 4 volumes of phosphate buffered saline (PBS) using an Ultra-Turrax T25 homogenizer (IKA-Labortechnik, Dottingen, Germany).

Analytical methods

Lurtotecan dihydrochloride monohydrate (lot: U2044/164/1, containing 78.11% of the free base) was supplied by Gilead Sciences Inc. (San Dimas, CA, USA) and was used as standard for all reversed-phase high-performance liquid chromatographic assays (HPLC). Plasma and urine concentrations of lurtotecan were determined using validated HPLC assays as described previously [14]. The sample pretreatment for the analysis of total plasma concentrations (i.e., the total of lactone plus carboxylate levels) consists of protein precipitation with 10% (w/v) aqueous perchloric acid-acetonitrile (2:1, v/v). Lurtotecan was separated from endogenous compounds on an Inertsil-ODS

80A column, with a mobile phase composed of 1 M aqueous ammonium acetate-water-acetonitrile (100:725:175, v/v/v). The flow rate was set at 1.25 mL/min, the temperature was maintained at 60°C and the column effluent was monitored with excitation and emission wavelengths of 378 and 420 nm, respectively. Urine concentrations of lurtotecan were quantified after a solvent extraction with *n*-butanol-diethyl ether (3:4, v/v), using the same column and mobile phase. The flow rate was set at 0.75 mL/min and the fluorescence signal of lurtotecan was increased 14-fold prior to detection by exposure of the effluent to UV light (254 nm) in a photochemical reactor unit. The LLQs were 1.00 and 0.50 ng/mL for the determination of total lurtotecan concentrations in human plasma and urine, respectively.

For the determination of lurtotecan in whole blood and feces, the assay for total plasma concentrations was modified as outlined below. The HPLC systems consisted of a constaMetric 3200 solvent delivery system (LDC Analytical, Riviera Beach, FL), a Waters 717plus autosampler (Milford, MA), a Inertsil-ODS 80A analytical column (150x4.6 mm I.D., 5 µm particle size; Alltech Applied Sciences, Breda, The Netherlands) maintained at 60°C by a model SpH99 column oven (Spark Holland, Meppel, The Netherlands), a Beam Boost photochemical reactor unit supplied with a coil of 25m x 0.3mm I.D. (ICT-ASS-Chem, Bad Homburg, Germany), and a Jasco FP-920 fluorescence detector (Jasco, Maarssen, The Netherlands) operating at excitation and emission wavelengths of 378 and 420 nm (40 nm band width), respectively. The mobile phases consisted of 1 M aqueous ammonium acetate-water-acetonitrile (100:725:175, v/v/v), with the flow rates set at 1.25 and 0.75 mL/min for the determination of total lurtotecan levels in blood and fecal samples, respectively.

Aliquots of 50 µL heparinized whole blood were pretreated with 500 µL of 5% (w/v) aqueous perchloric acid-acetonitrile (5:1, v/v) in 1.5-mL polypropylene tubes (Eppendorf, Hamburg, Germany). The samples were vigorously vortex-mixed for 30 min on a multi-tube vortex mixer, followed by centrifugation for 5 min at 23,000x*g* at ambient temperature. A volume of 250 µL of the clear supernatant was transferred to a low volume insert of glass, from which 200 µL were injected into the HPLC system. The calibration curves were constructed in PBS in concentrations of 0.25, 0.50, 1.00, 5.00, 10.0 and 25.0 ng/mL, by serial dilutions of a lurtotecan working solution containing 0.10 mg/mL (expressed as free base). Three pools of quality-control samples were prepared in heparinized whole blood at concentrations of 0.40, 20.0 and 2000 ng/mL, by addition of appropriate volumes of lurtotecan in PBS to the whole blood. In addition, to minimize potential differences with clinical samples, a recovery control sample containing 7.50 ng/mL lurtotecan (as NX 211), was also analyzed simultaneously. The sample containing 2000 ng/mL was diluted 100-fold in PBS prior to extraction.

Aliquots of 100 µL feces homogenates were deproteinized and acidified with 1000 µL of 5% (w/v) aqueous perchloric acid-acetonitrile (5:1, v/v) containing 100 ng/mL 6,7-dimethoxy-4-methylcoumarin (Sigma, St. Louis, MO, USA), which was used as the internal standard.

Subsequently, the samples were vigorously vortex-mixed for 15 min on a multi-tube vortex mixer, followed by centrifugation at ambient temperature at 23,000xg for 5 min. A volume of 100 µL supernatant was transferred to a limited volume insert of glass, from which 10 µL were injected onto the analytical column. Spiked homogenized fecal samples used as calibration standards in concentrations of 10, 25, 50, 100 and 250 ng/mL were prepared by addition of 10 µL of serial dilutions in PBS from the lurtotecan working solution to 240 µL drug-free feces homogenates. Three pools of quality-control samples containing 40, 200 and 2000 ng/mL lurtotecan were prepared by addition of appropriate volumes of lurtotecan in PBS to blank human feces homogenates. The sample containing 2000 ng/mL was diluted 10-fold in a mixture of PBS and the extraction solution (1:10, v/v) prior to injection.

Validation of both assays included a set of calibration samples assayed in duplicate, with lower limit of quantitation and quality-control samples in quintuplicate, and was performed on 4 separate occasions. The lower limit of quantitation samples were prepared daily in drug-free heparinized whole blood samples and fecal homogenates of 5 healthy volunteers. Within-run and between-run precisions were calculated by one-way ANOVA for each concentration using the run-day as variable. The extraction recoveries for lurtotecan and the internal standard in the assay for lurtotecan in feces specimens were calculated by comparing peak heights obtained from an extracted sample containing 100 ng/mL lurtotecan in PBS to those obtained in extracted calibration samples. The extraction recoveries for lurtotecan in the assay in whole blood were calculated using the data of the quality and recovery-control samples in comparison to calibration standards.

Table 1: Characteristics of the studied patients

Patient No.	Dose (mg/m^2)	Gender	Age (yrs)	Tumor type	BSA (m^2)	Ht (L/L)
1	1.6	Female	61	NSCLC[a]	1.63	0.30
2	1.6	Female	74	sarcoma	1.56	0.28
3	3.2	Female	57	myosarcoma	2.09	0.40
4	3.2	Female	40	SCLC	2.06	0.37
5	3.2	Male	52	bile duct carcinoma	1.88	0.41
6	4.3	Male	55	bladder carcinoma	1.98	0.36

a: NSCLC, non-small cell lung cancer; SCLC, small-cell lung cancer; BSA, body-surface area; Ht, hematocrit on day 1

Pharmacokinetic analysis

Individual plasma and whole blood concentrations of lurtotecan were fit to a model with multi-exponential functions using the Siphar version 4.0 software package (SIMED, Créteil, France), using Powell's method. In all cases, concentration-time profiles were best fit to a mono-exponential equation after zero-order input with weighting according to y_{obs}^{-1} (not shown). The disposition half-life was calculated as $ln2/k$, in which k is the elimination rate constant in h^{-1}. The total plasma clearance of lurtotecan was calculated by dividing the dose (expressed in mg base equivalents per squared meter of body surface area) by the observed AUC. The volume of distribution at steady state was calculated using the same program.

RESULTS

Complete pharmacokinetic studies were performed in 6 patients entered onto a phase I clinical trial of liposomal lurtotecan (NX 211) given as a 30-min i.v. infusion [15]. Full clinical toxicities and treatment responses will be reported in detail elsewhere in due course. The group consisted of 4 females and 2 males ranging in age from 40 to 74 years (Table 1). The median clinical chemistry values for these patients included total bilirubin levels of 6 μM (range 5-11), serum creatinine levels of 90 μM (range 75-108), ASAT and ALAT of 27 units/L (range 19-47) and 20 units/L (range 8-57 units/L), respectively.

Analytical methods

The lurtotecan plasma concentration values for patients treated with NX 211 reported here are the sum of both encapsulated and non-encapsulated drug. In order to gain a preliminary insight into the disposition and elimination of NX 211 in humans, the assay procedures as described recently [14] were slightly modified for the determination of total lurtotecan concentrations in human whole blood and fecal homogenates. PBS was used as matrix for the construction of the calibration standards for the determination of total lurtotecan levels in human heparinized whole blood, since pools of drug-free heparinized whole blood were not available. Because of this, we focussed on an extraction procedure with quantitative (i.e., 100%) recovery. Eventually, the extraction recoveries for lurtotecan, using the described method, were 103, 100 and 97% for the quality-control samples containing 1.25, 20.0 and 2000 ng/mL of lurtotecan and 101% for the recovery-control sample containing 7.50 ng/mL of NX 211. However, the extraction recovery for the internal standard still did not reach 100% (data not shown), and, hence, no internal standard was used in this assay. The calibration curves were linear over the entire range studied, with the validation characteristics of the quality control

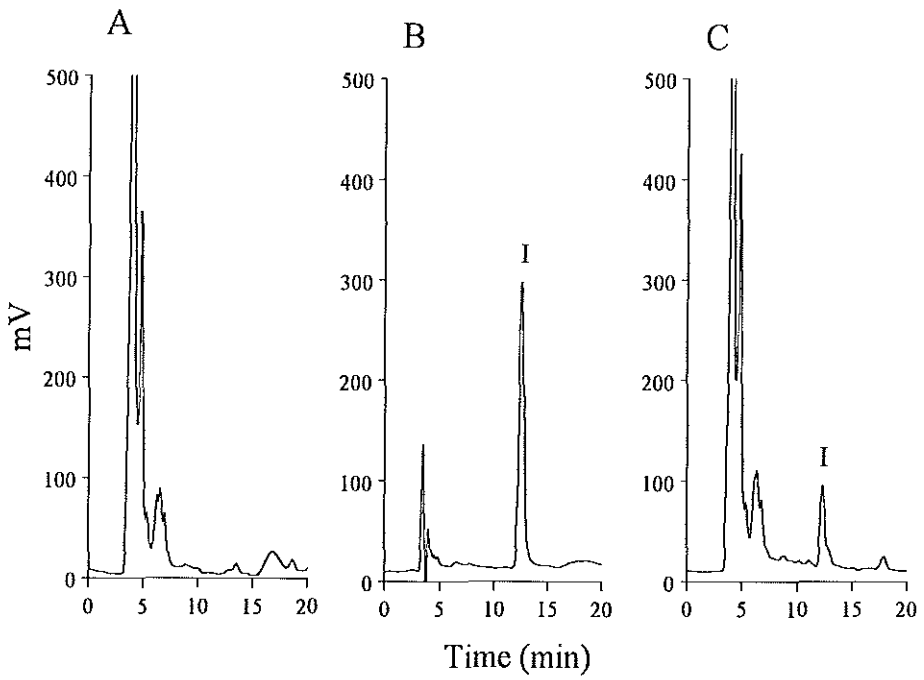

Figure 2: Chromatograms of whole blood samples of patient 6; blank (A), a 100-fold diluted sample obtained at the end of the infusion containing 1265 ng/mL lurtotecan (B) and a sample taken 96 h after the start of the infusion containing 3.7 ng/mL lurtotecan (C). Peak labeled I correspond to lurtotecan.

samples summarized in Table 2. Fig. 2 shows representative chromatograms of lurtotecan in whole blood samples of patient 6 after the administration of NX 211. Similar data were obtained for the determination of lurtotecan in feces homogenates (Table 2), with recoveries of lurtotecan and the internal standard of 100 and 98.5%, respectively. Representative chromatograms of lurtotecan in fecal extracts of the same patient are shown in fig 3.

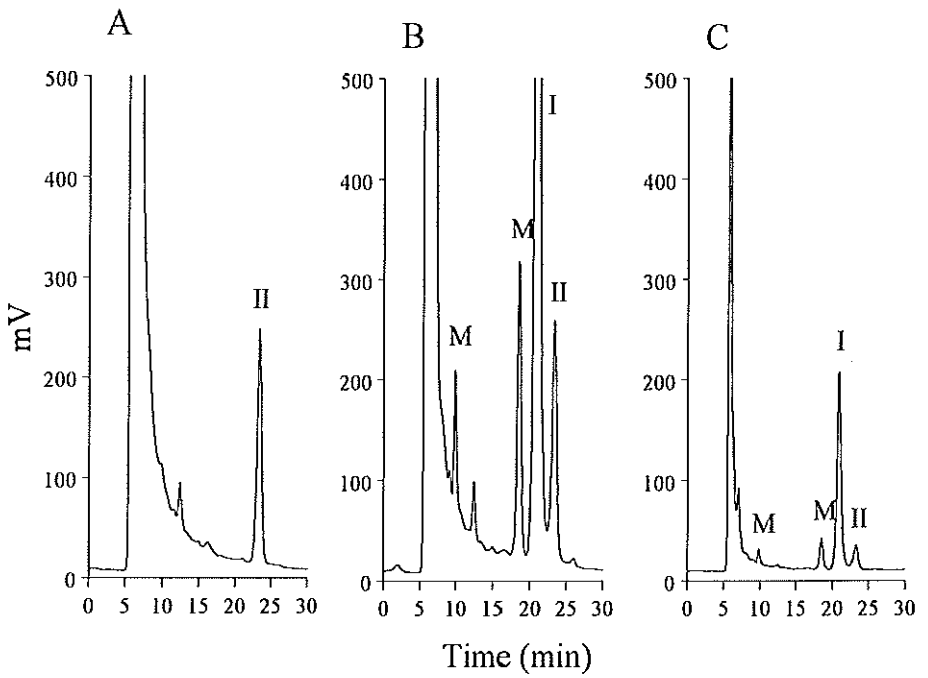

Figure 3: Chromatograms of fecal extracts of patient 6: blank (A), an undiluted fecal homogenate from a feces sample collected at 54 h after the start of the infusion with 2 potential metabolites (B) and the same sample 10-fold diluted after extraction containing 976 ng/mL lurtotecan (C). Peaks labeled I, II and M correspond to lurtotecan, internal standard and potential metabolites, respectively.

Disposition of lurtotecan

In table 3, the pharmacokinetic data are summarized of total lurtotecan, which could best be fitted with a mono-exponential decline, in plasma as well as in whole blood. Characteristic were the slow plasma clearances of the drug of 0.10 – 2.38 L/h/m^2, the mono-exponential decline with a half life of 2.42 – 11.2 h and the low steady state volume of distribution, ranging from 1.58 – 8.31 L/m^2. The mean blood:plasma total lurtotecan concentration ratio of 0.66±0.13 is

indicative for liposomal encapsulation of lurtotecan in the plasma compartment, since no or limited drug is accumulated into the red blood cells (fig 4). Figure 5 shows the kinetic profile of total lurtotecan of patient 6 in plasma and whole blood. An additional chromatographic peak was found in the plasma samples with a retention time of approximately 50 minutes (not shown), which had an equal retention time as the chromatographic peak in the recovery control samples as described recently [14]. Structural identification as well as pharmacologic properties of this compound will be discussed in a separate communication shortly.

The urinary and fecal excretions of the unchanged parent compound lurtotecan are listed in table 3. The total excretion of lurtotecan in urine and feces ranged from 8.5 – 38% in the 6 studied patients, suggesting extensive metabolism of lurtotecan. Inspection of the HPLC chromatograms from fecal extracts revealed 2 major additional peaks, which were absent in the blank fecal extracts, that might represent lurtotecan metabolites (fig 3). In fig 6, the cumulative excretions of lurtotecan in urine and feces of patient 6 are shown.

Table 2: Validation characteristics of quality-control samples for the two HPLC assays

Nominal (ng/mL)	Observed (ng/mL)	WRP[a] (%)	BRP (%)	ACC (%)
Lurtotecan in whole blood				
0.25[b]	0.24	7.8	12.4	94.9
1.25	1.29	11.4	5.1	103
20.0	20.2	8.7	7.5	101
2000	1950	13.2	c	98.0
Lurtotecan in feces homogenate				
10[b]	11	5.0	3.9	106
40	41	4.3	6.9	101
200	203	2.9	6.7	101
2000	1926	3.6	8.6	96.3

a: WRP, within-run precision; BRP, between-run precision; ACC, accuracy. b: Lower limit of quantitation samples. c: No additional variation was observed as a result of performing the assay in different runs.

DISCUSSION

In the present study, we have described for the first time the human pharmacokinetics of a liposome-encapsulated camptothecin analogue in plasma, whole blood, urine and feces.

Of the greatest importance for the antitumor activity of lurtotecan treatment is the disposition of the agent in plasma. Lurtotecan has shown to be an active compound in animal models as well as in clinical trials. In general, for topoisomerase I inhibitors, prolonged exposure to the agent has been associated with an increase in cytotoxicity. Of particular note, the phase I data for free lurtotecan suggest that antitumor activity may be enhanced with continuous infusion, since responses were observed in the 72-h and 21-day continuous infusion schedules [6,7] As far as toxicity is concerned, although prolonged infusion is associated with an increase in thrombocytopenia, it has not been associated with an increase in the incidence and severity of neutropenia. Therefore, the use of a liposomal formulation of lurtotecan, as administered here, may also improve efficacy by increasing exposure to the active (lactone) drug form and modifying the safety profile, thus enhancing the therapeutic index of the parent compound.

Table 3: Pharmacokinetic parameters of lurtotecan after NX 211 administration

Patient No.	CL[a] (L/h/m^2)	$V_{d,ss}$ (L/m^2)	$T_{1/2}$ (h)	Fe_u (%)	Fe_f (%)	Ratio bl/pl
1	2.38	8.31	2.65	5.4	9.1	0.80
2	0.67	3.94	3.99	4.9	3.6	0.70
3	0.10	1.58	11.2	8.2	6.4	0.54
4	0.28	1.76	4.18	6.1[b]	6.2	0.81
5	1.15	3.63	2.42	7.3	11.7	0.57
6	0.11	1.65	10.6	17.4	21.0	0.52
mean	0.78	3.48	5.84	8.6	9.7	0.66
SD	0.88	2.59	3.99	5.1	6.2	0.13
CV	113%	74%	68%	59%	64%	20%

a: CL, total plasma clearance; MRT, mean residence time; $V_{d,ss}$, volume of distribution at steady-state; $T_{1/2}$, half-life of the terminal disposition phase; Fe_u, fraction of the delivered dose excreted as unchanged drug in urine within 96 h; Fe_f, fraction of the delivered dose excreted as unchanged drug in feces within 96 h; %CV, coefficient of variation. b: Incomplete recovery (0-12 h urine sample missing), not used for calculations.

Here, we have shown that the disappearance of lurtotecan after NX 211 administration was characterized by a mono-exponential decline with a terminal disposition half-life in plasma of approximately 6 h. This is in contrast to the multiphasic elimination from plasma demonstrated by lurtotecan, which displays a terminal half-life, estimated as 9.6±4.8 h in a cohort of 14 patients [4]. The basis for the longer elimination half-life of non-liposomal lurtotecan is most likely due to the slow elimination of the larger fraction of drug initially distributed to tissue. With NX 211, a prolonged association of lurtotecan within circulating intact liposomes in the plasma compartment would be assumed to release free drug over a period of time, quite possible resulting in the same terminal half-life as lurtotecan, but with concentrations below the lower limit of quantitation of the assay. The total lurtotecan plasma clearance from NX 211, on average 0.78 L/h/m^2, is 25 times slower than the clearance of the free drug, which was established at 21±9.6 L/h/m^2 [4]. The observed steady

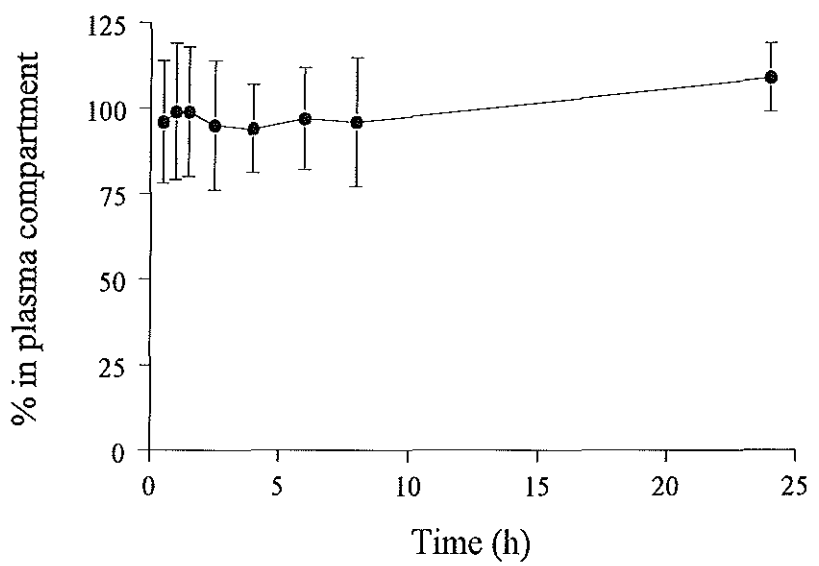

Figure 4: Disposition of lurtotecan in the blood compartment in the studied patients (mean ± S.D.).

state volume of distribution of 3.48±2.59 L/m^2 and the blood/plasma ratio of 0.66±0.13 are indicative for encapsulation of lurtotecan in the liposomes, which are mainly located in the plasma compartment.

The observed variability in the pharmacokinetic behavior of lurtotecan after the administration of NX 211 is slightly higher to that reported for the free drug, with an interpatient variability in the plasma clearance of 113% for NX 211, versus 46% [4] for free lurtotecan, while these values for the volume of distribution were 74% and 52% [4], respectively.

Urinary excretion of the unchanged drug was 8.6 ± 5.1% in the 6 studied patients, which is similar with earlier findings of 14.3 ± 7.0% [3] and 11% [4] after a 30 min infusion of free drug, indicating that renal clearance plays also a minor role in the elimination of NX 211. Since also the total amount

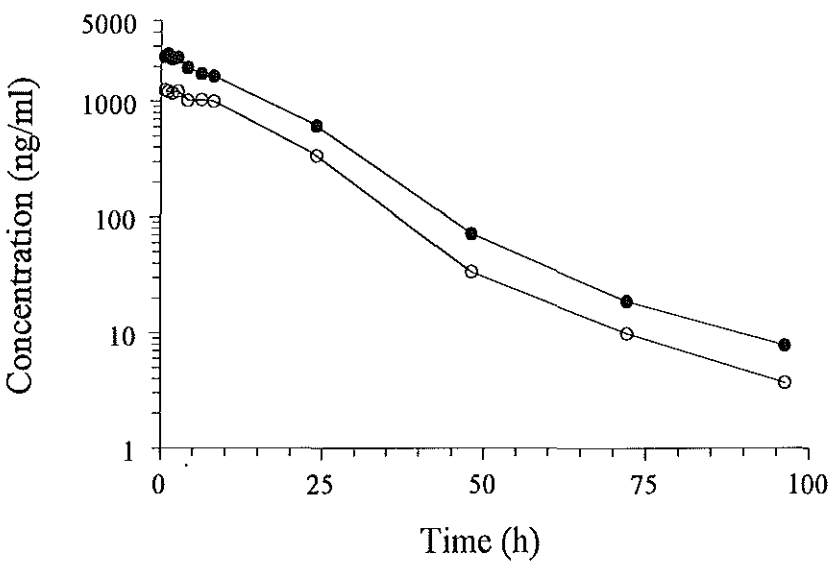

Figure 5: Kinetic profile of lurtotecan in plasma (closed circles) and whole blood (open circles) in patient 6 after administration of 8.5 mg NX211.

of lurtotecan in feces accounted for only 9.7% of the administered dose, lurtotecan is probably extensively metabolized. At least 2 potential metabolites of lurtotecan were observed in the chromatograms of fecal homogenates, which will be part of further research regarding the metabolism of NX 211.

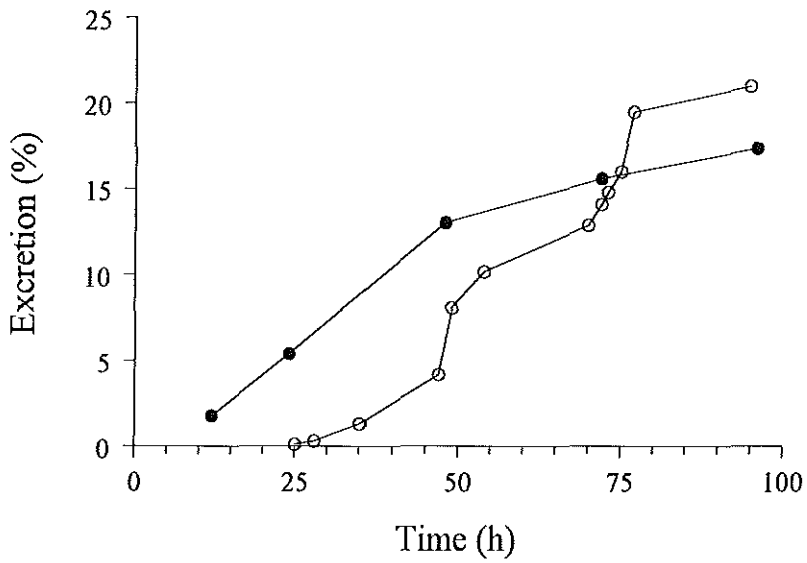

Figure 6: Cumulative excretion of unchanged drug in urine (open circles) and feces (closed circles) of patient 6 after administration of 8.5 mg NX211.

In conclusion, administration of liposome-encapsulated lurtotecan significantly reduces the plasma clearance of the drug, which in turn might prove beneficial for pharmacodynamic outcome. Toxicity and anti-tumor response in relation to the pharmacokinetics are currently under investigation in a clinical phase I trial.

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Summary and conclusions

Over the last decade, several analogues of the topoisomerase I inhibitor camptothecin, including topotecan, irinotecan, lurtotecan and 9-aminocamptothecin, have been introduced into clinical practice. Topoisomerase I is a nuclear enzyme involved in the replication of DNA, by forming a covalent binding with DNA, the cleavable complex. This cleavable complex is stabilized by these camptothecins, resulting in a single strand breakage of the DNA, which finally leads to cell death.

The camptothecin analogues share a pH-dependent reversible conversion between the pharmacologically active ring-closed lactone form and its inactive, lactone ring-opened, carboxylate form. A quantitative measurement of these analogues is rather complex because of the chemical instability of the lactone moieties of the camptothecin analogues.

Methodologies for the quantitative determination of the topoisomerase I inhibitors topotecan, 9-aminocamptothecin and lurtotecan are described and the applicability of these assays is shown in this thesis.

In **Chapter 1**, an overview is given of the methodologies currently used for the determination of topotecan, irinotecan, 9-aminocamptothecin and lurtotecan. Since these camptothecins have strong fluorescence characteristics, the analytes were quantified by fluorescence detection. As mentioned, the camptothecin analogues have a pH-dependent reversible conversion between the pharmacologically active lactone form and the inactive carboxylate form, which necessitates rapid centrifugation of the blood samples to collect the plasma. Also when only total concentrations, i.e. lactone plus carboxylate, are to be measured, this direct blood processing is crucial, since the lactone form is able to diffuse across the cell membranes of red blood cells, and thus a change in the lactone to carboxylate ratio has an effect on the total drug concentrations in the plasma compartment. Stabilization of the lactone to carboxylate ratio by direct freezing of the plasma sample is the most convenient approach for the determination of the lactone only concentrations. On the day of analysis all samples can be handled at once by solid-phase or liquid-liquid extraction techniques, in which only the lipophilic lactone form is extracted, while the carboxylate form is discarded. The total drug concentrations are measured in a second analysis after acidification of the samples. Another, simple way of stabilizing the lactone to carboxylate ratio is by cold methanolic deproteinization of plasma samples, immediately after collection of the plasma. The lactone and carboxylate concentrations are measured simultaneously in one analytical run. However, up to now, this approach has only been shown to be feasible for the determination of the two forms of topotecan and irinotecan.

Pharmaceutical and biomedical aspects of topotecan, a semisynthetic water-soluble camptothecin analogue, are described in **chapter 2**. The intravenous formulation of topotecan (Hycamtin®) has been registered for the treatment of ovarian cancer in Europe and the USA,

while an oral formulation is still under clinical investigation.

In **chapter 2a** the simultaneous determination of the lactone and carboxylate forms of topotecan in human plasma and of the total concentration in human urine is described. The plasma clean up is based on simple methanolic protein precipitation, which stabilizes the lactone to carboxylate ratio when stored at –80°C. The lower limit of quantitation for the lactone as well the carboxylate form is established at 0.10 ng/ml, with an overall run time of 10 min, which enables analysis of complete runs of patient samples during day time. Total topotecan concentrations in human urine samples, with a lower limit of quantitation of 10 ng/ml, are measured in the form of topotecan lactone, after acidification with orthophosphoric acid.

The influence of third spaces, such as pleural- and ascitic fluid, on the pharmacokinetics of topotecan and the penetration of topotecan into these fluids is described in **chapter 2b**, in 4 patients in the presence and absence of third space volumes. The apparent topotecan clearance, the terminal disposition half-lives and the AUC ratios of lactone to total drug in plasma remained unchanged within the same patient in the presence or absence of pleural or ascitic fluid. The penetration of topotecan into the pleural and ascitic fluid showed a mean lag time of 1.61 h and ratios with plasma concentration increased with time after dosing in all patients, with a mean ratio of third space topotecan total drug AUC to that in plasma of 0.55. So, topotecan can be safely administered to patients with pleural effusions or ascites, with substantial penetration of topotecan into these third spaces for local antitumor effects.

In **chapter 2c**, the pharmacokinetic interactions, and sequence dependent effects of orally administered topotecan in combination with i.v. cisplatin were studied in a phase I and pharmacological study. Cisplatin was given to a total of 49 patients at a fixed dose of 75 mg/m^2 as a 3-hour infusion, before topotecan on day 1 or after topotecan on day 5, in combination with oral topotecan at dose levels of 0.75 to 2.3 mg/m^2 for 5 days, repeated every 3 weeks. When topotecan was preceded by cisplatin, a more severe myelosuppression was induced, compared to the alternate sequence. The MTD for the most toxic sequence, cisplatin followed by topotecan, was established at a dose of 1.25 mg/m^2/dx5 of topotecan, while for the reversed sequence the MTD was found at a dose of 2.0 mg/m^2/dx5 of topotecan. The possible effect of drug sequence on the pharmacokinetics of topotecan and cisplatin was investigated in the first 18 patients, which were randomized in a crossover design for the administration sequence. No significant differences in the topotecan pharmacokinetics were found when topotecan was preceded or followed by cisplatin. As for topotecan, the pharmacokinetics of cisplatin were not influenced by the administration sequence. The antitumor efficacy at the MTD of both schedules should be evaluated in a randomized phase II study.

In the clinical practice of medical oncology, most anti-cancer drugs are commonly dosed based on the body-surface area of the individual patient, with the aim reducing inter-patient variability in drug exposure, which is based on an assumed relationship between the clearance of a compound and

the body-surface area of the individual patient. In **chapter 2d**, the relevance of body-surface area based dosing of orally administered topotecan in adult cancer patients is evaluated, by estimation of the intra- and inter-patient variabilities in topotecan lactone pharmacokinetics. The intra-patient variability in the topotecan lactone AUC, using the data of 47 patients who were sampled for 3 - 6 days, was 24 ± 13% (median 20%) with a range of 7.6 - 61%. The inter-patient variabilities in the apparent clearance of topotecan lactone, expressed in L/h/m^2 as well as in L/h, were 38 and 42%, respectively. In view of the relatively high intra- and inter-patient variabilities in the AUC and apparent clearance of topotecan lactone and the relatively small range in observed body-surface area of 12%, oral topotecan can be added to the list of agents where body-surface area adjusted dosing does not appear definitely better. Moreover, accurate dosing of oral topotecan based on body-surface area is also not feasible, since for oral use the drug is now only available as gelatine capsules containing 1.0 and 0.25 mg respectively, resulting in the necessity of rounding of the absolute dose to the nearest quartile mg. We recommend a fixed dose regimen for future use in clinical trials, which is more convenient for the oncologist and the pharmacist, is more cost-effective and last but not least, a fixed dose regimen is less cumbersome for the patients. Further randomized clinical studies are needed to fully explore the advantages of fixed dose regimens over body-surface area based dosing of orally administered topotecan in adult patients.

In **chapter 2e**, the gender-dependent differences in topotecan pharmacokinetics in adult cancer patients after oral as well i.v. administration and the explanation of this phenomenon is described. A significant 1.4-fold faster apparent clearance of topotecan lactone was found in males as compared to females treated with oral topotecan. After correction for the body-surface area, the apparent topotecan lactone clearance remains significantly 1.3-fold faster in males. In addition, a significant 1.3-fold higher lactone to total ratio of the AUC in females was found. Linear regression analysis between the significantly different biochemical characteristics body-surface area and hematocrit and the apparent clearance of topotecan lactone, expressed in L/h/m^2, resulted in a significant correlation of the apparent clearance with the hematocrit but not with the body-surface area. As in the oral study, female patients enrolled in a continuous i.v. study also had a higher lactone to total steady state plasma concentration ratio. In *in vitro* studies with altered hematocrit values, a strong significant correlation was found between the hematocrit value and the lactone to total concentrations. In addition, the lactone to total plasma concentration ratio, in *in vitro* experiments, was significantly higher in blood of female volunteers as compared to male volunteers, with significantly higher topotecan lactone concentrations in the plasma compartment of the blood samples of the female volunteers. The implications of this gender-related difference in topotecan pharmacokinetics on the pharmacodynamics and tumor responses have to be evaluated in a large study in which topotecan is administered at a fixed dose.

Chapter 2f is describing a clinical phase I study with increased oral topotecan dosages, in comparison with the dosages used in chapter 2c, in combination with a lower fixed dose of 50 mg/m^2 of i.v. administered cisplatin. The dose-limiting toxicities consisted of myelosuppression and gastrointestinal toxicity at a topotecan dose-level of 1.75 mg/m^2/d administered on day 1-5, preceded by 50 mg/m^2 cisplatin on day 1. The pharmacokinetics of topotecan and cisplatin were similar to earlier data. The MTD of i.v. cisplatin at a dose-level of 50 mg/m^2 on day 1, directly followed by 5 days of oral topotecan at a dose of 1.50 mg/m^2, has no advantage as compared to our previous recommended schedule for phase II studies of i.v. cisplatin at a dose of 75 mg/m^2 on day 1 in combination with 1.25 mg/m^2/d for 5 days of oral topotecan (chapter 2c), since only a minor dose escalation of topotecan could be achieved by a dose-reduction of 33% of cisplatin.

The poor water-soluble synthetic camptothecin derivative 9-aminocamptothecin, which has shown promising anti-tumor efficacy in xenograft models, is discussed in **chapter 3**. Due to the water solubility problems, 9-aminocamptothecin seemed to be inappropriate for further clinical development. Since a colloidal dispersion formulation has been developed, phase I and II clinical trials with i.v. administered 9-aminocamptothecin have been conducted, while gelatin capsules were developed for oral administration.

In **chapter 3a**, two high-performance liquid chromatographic methods for the determination of the lactone and total (i.e. lactone plus carboxylate) 9-aminocamptothecin concentrations in human plasma are described, with lower limits of quantitation of 50 and 100 pg/ml, respectively. The lactone form of 9-aminocamptothecin was extracted from the plasma by a single liquid-liquid extraction. The sample preparation for the determination of total plasma levels consists of a simple deproteinization/acidification step, in which the carboxylate form is converted to the lactone form. Both assays are sensitive, selective, accurate and reproducible and have been implemented in several clinical and *in vitro* studies.

The factors responsible for the phenomenon of rebound concentrations in the kinetic profile of total 9-aminocamptotheicn after a bolus i.v. infusion, at approximately 2-3 hours after dosing, are described in **chapter 3b**. After a 5-min bolus infusion, 9-aminocamptothecin is rapidly hydrolyzed, with less than 10% present in the pharmacologically active lactone form at 3 hours after dosing. In *in vitro* experiments, in which 9-aminocamptothecin was incubated in fresh human heparinized whole blood, the lactone instantaneously accumulated in the red blood cells, resulting in low plasma concentrations of 9-aminocamptothecin. Subsequently, the lactone form is hydrolyzed to the ring-opened carboxylate form, which is not able to pass cell membranes. So in time, higher concentrations of total 9-aminocamptothecin were detected in the plasma compartment. In addition, the carboxylate form of 9-aminocampthothecin has a high affinity for human serum albumin, resulting in a shift of the equilibrium towards the carboxylate form, which in turn is responsible for the rebound concentrations of total 9-aminocamptothecin in the

plasma compartment of cancer patients, after a i.v. bolus infusion. The absence of the rebound peak in preclinical murine xenograft models is most likely related to differences in the lactone to carboxylate ratios compared to humans, with percentiles of 9-aminocamptothecin present in the lactone form at equilibrium in solutions of human or murine serum albumin of respectively 0.63 and 35.0 %.

In **chapter 3c** the clinical pharmacokinetics of 9-aminocampthotecin in adult cancer patients are described in plasma and saliva after oral administration, and relationships have been made with the pharmacodynamics. In contrast to other camptothecin derivatives, such as topotecan, lurtotecan and irinotecan, the conversion of the lactone form into the carboxylate form appeared to be much faster, with the equilibrium favoring the carboxylate form. The pharmacokinetics were linear and dose-independent and the intra-patient variability in lactone AUCs were extremely low, while the inter-patient variability in the lactone AUCs were high. The 9-aminocampothecin lactone concentration ratio in plasma and unstimulated saliva were patient dependent and highly variable, indicating that unstimulated saliva is an unreliable matrix for pharmacokinetic analysis of 9-aminocamptothecin. From one patient, also pleural samples were collected and analyzed for 9-aminocamptothecin. As for topotecan (described in chapter 2b), pleural effusions are not a major compartment for 9-aminocamptothecin distribution. The AUC of the pharmacologically active lactone form significantly correlates with hematological toxicities like percentage decrease in total white blood cells, platelets and neutrophils. Since the intra-patient variability in drug exposure was very small, 9-aminocamptothecin could be individually dosed, using a target AUC as endpoint, in future clinical trials.

In **chapter 4**, pharmaceutical and biomedical aspects of lurtotecan, a semisynthetic camptothecin analogue are described. In order to improve the efficacy of lurtotecan in cancer patients, the drug was encapsulated in liposomes.

Chapter 4a, is describing reversed-phase fluorescence high-performance liquid chromatographic methods for the determination of total lurtotecan concentrations in human plasma and urine in samples of patients after administration of NX 211 (i.e. a liposomal formulation of lurtotecan). For the determination of total plasma concentrations, the sample pretreatment consists of a simple deproteinization/acidification step, resulting in a lower limit of quantitation of 1.00 ng/ml. This method has also been validated for the analysis of lurtotecan in urine samples, in which the urine was diluted 40-fold in blank human plasma, with the lower limit of quantitation established at 100 ng/ml urine. Lurtotecan concentrations in urine <100 ng/ml were determined by a modified procedure, using a single solvent extraction technique. The fluorescence signal of lurtotecan was increased prior to detection by post-column exposure to UV light (254 nm) in a photochemical reaction unit, in which the piperazinomethylene moiety on C7 of the molecule is lost, resulting in a 14-fold higher fluorescence signal compared to the parent

compound. The LLQ of this assay was 0.500 ng/ml. The methods meet the current requirements as to validation of bioanalytical methodologies and are implemented in an ongoing clinical phase I trial of NX 211.

The clinical applications of the assays described in chapter 4a, are described in **chapter 4b**, in which preliminary pharmacokinetics are shown of lurtotecan in patients treated with NX 211. In addition to the described assays in chapter 4a, in this chapter also assays and pharmacokinetics of lurtotecan in human whole blood and feces are described. As in the assay for the determination of total lurtotecan plasma concentrations, the sample treatment procedures of whole blood and feces samples consist of a simple protein precipitation/acidification step. In both assays, the photo-chemical reactor unit was used to increase the fluorescence signal of lurtotecan, resulting in lower limits of quantitation of 0.25 and 10 ng/ml in whole blood and feces, respectively. The pharmacokinetic profile in plasma was characterized by a clearance of 0.78 L/h/m^2, which is 25 times slower than these for the free drug, with a mono-exponential decline with a half-life of approximately 6 h, and a steady-state volume of distribution of approximately the blood volume. The low volume of distribution and the observed blood/plasma ratio of 0.66 are indicative for encapsulation of the drug in liposomes, which are mainly located in the plasma compartment. Urinary and fecal excretions of the parent compound lurtotecan were low, with mean values of respectively 8.6% and 9.7%, indicating that lurtotecan in probably extensively metabolized. In the chromatograms of the fecal homogenates 2 potential metabolites of lurtotecan were observed. The significantly reduced plasma clearance of lurtotecan by encapsulation of the drug in liposomes, might be beneficial for pharmacodynamic outcome. Toxicity and anti-tumor response in relation to the pharmacokinetics are currently under investigation in a clinical phase I trial.

Conclusions and future perspectives

The camptothecins belong to the class of topoisomerase I inhibitors and have shown antitumor activity against a broad range of human malignancies, including refractory ovarian and colorectal cancers. Over the last decades, several methodologies have been developed and validated for the determination of the lactone, carboxylate and total concentrations of camptothecin analogues in biological matrices. Since the lactone form is the pharmacological active form of these compounds, and the equilibrium between the lactone and carboxylate forms in plasma of the camptothecins differs from derivative to derivative and from species to species, the analysis of the lactone form is of great importance for pharmacokinetic-pharmacodynamic relationships.

In the future, the camptothecins will be increasingly combined with other drugs, because of which sensitive, selective, accurate and reproducible analytical methods for the determination of

camptothecin analogues, especially for the lactone forms of these drugs, are needed to get insight into possible pharmacokinetic interactions and their implications for the pharmacodynamics.

Generally, in the future, dosing of anti-cancer agents will hopefully be individualized, based on the pharmacokinetics of each individual patient, especially when more is known about pharmacogenetics, in order to get more efficient and less toxic therapies.

Samenvatting en conclusies

In de strijd tegen kanker worden steeds nieuwe methoden gezocht om de ongebreidelde groei van tumorcellen af te remmen. Eén van de nieuwste methoden is het blokkeren van het topoisomerase I enzym, dat betrokken is bij de deling van DNA en dus mede verantwoordelijk is voor de vermeerdering van cellen. DNA is een keten van nucleotiden dat in zich in een opgedraaide vorm in de celkern bevindt. Het topoisomerase I enzym rolt dit DNA af, waardoor andere enzymen voor de vermenigvuldiging kunnen zorgen, waarna het topoisomerase I enzym het DNA weer in zijn oorspronkelijke vorm terugbrengt. Door dit DNA-enzym complex te blokkeren, breekt het DNA, wat uiteindelijk resulteert in celdood. De laatste decennia zijn er diverse analoga van de topoisomerase I remmer camptothecine, zoals topotecan, irinotecan, lurtotecan en 9-aminocamptothecine ontwikkeld en getest in klinische studies.

Al de tot nu toe klinisch gebruikte derivaten van camptothecine hebben gemeen, dat er een zuurgraad afhankelijk evenwicht is tussen een farmacologisch werkzame lactonvorm en een niet werkzame carboxylaatvorm. Kwantitatieve bepalingen van deze topoisomerase I remmers worden door deze chemische instabiliteit van de lactonvorm bemoeilijkt.

Analytische methoden voor de concentratiebepaling van de topoisomerase I remmers topotecan, 9-aminocamptotecine en lurtotecan in plasma, bloed, urine en feces worden in dit proefschrift beschreven. Daarnaast worden er toepassingen van deze analyse methoden in zowel preklinische als klinische studies beschreven.

In **hoofdstuk 1**, wordt een overzicht gegeven van analyse methoden, zoals die momenteel wereldwijd voor concentratiebepalingen van topotecan, irinotecan, 9-aminocamptothecine en lurtotecan worden gebruikt. Aangezien deze camptothecinederivaten sterk fluorescerende eigenschappen hebben, worden deze stoffen gedetecteerd met behulp van fluorescentie, na te zijn gezuiverd en gescheiden van andere endogeen voorkomende stoffen met behulp van hogedruk vloeistofchromatografie. Hierbij worden componenten op grond van chemische en fysische eigenschappen gescheiden. Aangezien de camptothecinederivaten een zuurgraad afhankelijke omzetting kennen van de lacton- naar de carboxylaatvorm, en visa versa, is het noodzakelijk dat bloedmonsters direct na afname gecentrifugeerd worden om het plasma te verzamelen, waarin de concentraties van de camptothecinederivaten bepaald worden. Daar alleen de werkzame lactonvorm in staat is om celmembranen te passeren, inclusief die van de rode bloedcellen, is het tevens noodzakelijk het bloed direct af te draaien wanneer alleen de totale concentratie van de camptothecinederivaten wordt bepaald. Een verschuiving van het evenwicht tussen de lacton- en carboxylaatvorm in het bloed heeft namelijk een direct gevolg voor de totale concentratie van de camptothecine in het plasma. Nadat het plasma is verzameld kan dit het beste direct ingevroren worden, zodat de omzetting van lacton- naar carboxylaatvorm wordt gestopt. Voor de bepaling van de lactonconcentraties kunnen de plasmamonsters op de dag van analyse ontdaan worden van onder andere eiwitten en de carboxylaatvorm door middel van vaste fase- of vloeistofextracties. In beide gevallen wordt op grond van wateroplosbaarheid

de lacton- van de carboxylaatvorm gescheiden. De totale concentraties van de camptothecinederivaten worden in een tweede analyse gemeten door het aanzuren van het plasmamonster, waardoor de totale hoeveelheid van de camptothecine in de lactonvorm komt. Naast het direct invriezen van plasmamonsters kan het evenwicht tussen de lacton- en carboxylaatvorm ook gestabiliseerd worden door het plasma direct te onteiwitten met koude methanol en dit extract in te vriezen. De lacton- en carboxylaatconcentraties kunnen dan in één analytische bepaling gemeten worden. Doordat de diverse camptothecinederivaten verschillende chemische en fysische eigenschappen hebben, is de laatste methode in de praktijk alleen mogelijk gebleken voor topotecan en irinotecan.

In **hoofdstuk 2** worden de farmaceutische en biomedische aspecten van het wateroplosbare camptothecineanaloog topotecan beschreven. De intraveneuze formulering van topotecan (Hycamtin®) is voor de behandeling van eierstokkanker geregistreerd in Europa en de Verenigde Staten. De orale formulering van topotecan is nog in de fase van klinisch onderzoek.

De gelijktijdige bepaling van de lacton- en carboxylaatvorm van topotecan in humaan plasma, na stabilisatie van het evenwicht door middel van een extractie met ijskoude methanol, en van totale topotecanconcentraties in humane urine is beschreven in **hoofdstuk 2a**. De laagste concentratie die nog adequaat en precies bepaald kon worden, voor zowel de lacton- als de carboxylaatvorm, werd gesteld op 0.10 ng/ml. Hiermee mag deze methode tot één van de gevoeligste worden gerekend. Totale concentraties van topotecan in urine worden gemeten in de lactonvorm na aanzuring van het monster, met een laagste bepalingsgrens van 10 ng/ml.

Een toepassing van de in hoofdstuk 2a beschreven analytische methode is beschreven in **hoofdstuk 2b.** Hierin wordt de invloed van derde ruimtes, zoals ascites en pleuravocht, op de plasmakinetiek van topotecan beschreven bij 4 patiënten. De derde ruimtes fungeerden niet als 'sink' voor topotecan en hadden geen invloed op de farmacokinetiek van zowel de lacton- als carboxylaatvorm van topotecan. De ratio's tussen de concentratie in de derde ruimte en het plasma nam bij alle patiënten toe in de tijd na orale toediening, met een gemiddeld oppervlak onder de concentratie tijdcurve ratio van 0.55. Topotecan kan dus veilig aan patiënten met ascites en/of pleuravocht worden gegeven zonder dat de plasmakinetiek verandert, terwijl er wel farmacologisch actieve concentraties in de ascites en het pleuravocht gevonden werden.

Hoofdstuk 2c beschrijft een klinische fase I studie in 49 patiënten, waarin een vaste dosis cisplatin van 75 mg/m^2 werd gecombineerd met toenemende orale topotecan doseringen van 0.75 tot 2.30 mg/m^2. Cisplatin werd door middel van een 3-uur durend intraveneus infuus toegediend, vlak voor of na een 5-daagse orale toediening van topotecan, welke elke drie weken werd herhaald. Farmacokinetische interacties tussen de twee cytostatica en schema afhankelijke bijwerkingen werden bestudeerd. Cisplatin toegediend voor topotecan gaf aanzienlijk ernstigere bijwerkingen, in de vorm van beenmergschade, dan het omgekeerde schema. De hoogst haalbare

doseringen van topotecan waren respectievelijk 1.25 en 2.00 mg/m^2. In de eerste 18 patiënten werd een mogelijk farmacokinetische interactie van de twee cytostatica onder invloed van het schema onderzocht in een gerandomiseerde 'cross-over' studie. De farmacokinetiek van zowel topotecan als cisplatin werd niet beïnvloed door de volgorde van toediening. In een gerandomiseerde fase II studie zal moeten worden uitgezocht welke van de twee schema's de meeste antitumor-activiteit laat zien.

De meeste cytostatica worden vandaag de dag nog steeds aan de hand van het lichaamsoppervlak van de individuele patiënt gedoseerd. Dit heeft als doel de interpatiëntvariatie in de expositie aan het betreffende cytostaticum te verminderen. In **hoofdstuk 2d** wordt de relevantie van lichaamsoppervlak-gebaseerde dosering van oraal topotecan in volwassen patiënten bediscussieerd aan de hand van de intra- en interpatiëntvariatie in topotecan expositie. De intrapatiëntvariatie in de oppervlakte onder de plasmaconcentratie tijdcurve van topotecan lacton was 24 ± 13% (mediaan 20%, range 7.6 – 61%) in 47 patiënten. De interpatiëntvariatie in de plasmaklaring van de lactonvorm was 38 en 42%, respectievelijk uitgedrukt in L/h/m^2 en in L/h. De kleine variatie in het lichaamsoppervlak van de bestudeerde patiënten van 12% staat in schril contrast tot de gevonden intra- en interpatiëntvariaties in de plasma-expositie en -klaring. Een ander probleem voor exacte toediening van oraal topotecan is het feit dat er alleen capsules beschikbaar zijn met 0.25 en 1.00 mg, zodat alle doseringen sowieso moeten worden afgerond. Oraal toegediend topotecan kan worden toegevoegd aan de lijst met cytostatica, waarbij dosering gebaseerd op lichaamsoppervlak niet beter is dan gelijke dosering van iedere patiënt. Hierbij dient tevens te worden opgemerkt dat een gelijke dosering makkelijker is voor de apotheker en oncoloog, kostenefficiënter is en veel minder omslachtig is voor de patiënt. Om het nut van gelijke dosering bij iedere volwassen patiënt aan te tonen, zal er een gerandomiseerde klinische studie uitgevoerd moeten worden, waarbij de farmacokinetiek, bijwerkingen en tumorrespons worden onderzocht.

Geslachtsafhankelijke verschillen in de plasmakinetiek van topotecan en de verklaring voor dit verschil worden in **hoofdstuk 2e** beschreven. Na orale toediening werd er een significante 1.4 keer snellere plasmaklaring van de lactonvorm van topotecan geconstateerd bij mannelijke patiënten in vergelijking met vrouwelijke patiënten. Na correctie voor de lichaamsoppervlakken bleef de plasmaklaring significant 1.3 keer sneller bij mannen. Daarnaast werd er een significant 1.3 keer hogere ratio bij vrouwen gemeten in de oppervlakte onder de plasmaconcentratie tijdcurven van topotecan lacton tot deze van topotecan totaal. Zowel de hematocriet waarde als het lichaamsoppervlak waren significant verschillend tussen de bestudeerde mannen en vrouwen. Er werd een significante lineaire relatie gelegd tussen de plasmaklaring van topotecan gecorrigeerd voor het lichaamsoppervlak en de hematocriet waarde, terwijl deze relatie niet kon worden gelegd tussen de plasmaklaring en het lichaamsoppervlak. Ook in een studie waarbij topotecan gedurende 21 dagen met een continu infuus werd toegediend, hadden vrouwen een

significant hogere lacton tot totaal 'steady state' concentratieratio. Bij laboratorium-experimenten, waarbij de hematocriet waarde kunstmatig veranderd was, werd een zeer goede significante relatie gevonden tussen de hematocriet waarde en de lacton tot totaal topotecan concentratieratio in het plasmacompartiment van de bloedmonsters. Ook in bloedmonsters van vrouwelijke vrijwilligers werd een significant hogere lacton tot totaal topotecan plasmaconcentratieratio gemeten in vergelijking met bloedmonsters van mannelijke vrijwilligers na incubatie met topotecan. In het plasmacompartiment van de bloedmonsters van vrouwen werd ook een significant hogere concentratie van de lactonvorm gevonden in vergelijking met de lactonconcentratie in de bloedmonsters van mannen. De implicaties van deze geslachtsafhankelijke topotecan plasmakinetiek ten aanzien van de tumorrespons en toxiciteit zullen in een grote studie met geselecteerde patiënten moeten worden bestudeerd, waarbij alle mannen en vrouwen een gelijke dosis topotecan krijgen toegediend.

Hoofdstuk 2f beschrijft een klinische fase I studie met als doel: het ophogen van de dosis topotecan, in vergelijking met de studie die beschreven is in hoofdstuk 2c, in combinatie met een lagere dosering van 50 mg/m^2 cisplatin. De dosislimiterende bijwerkingen waren beenmergsuppressie en klachten betrekking hebbend op het maagdarmstelsel bij een dosis van 1.75 mg/m^2 topotecan gedurende 5 dagen, voorafgegaan door een enkelvoudige dosis cisplatin. Er werden geen verschillen in de plasmakinetiek van zowel topotecan als cisplatin gevonden in vergelijking met eerdere studies. De hoogst haalbare dosis van 1.50 mg/m^2 topotecan gedurende 5 dagen in combinatie met 50 mg/m^2 cisplatin op dag 1 is waarschijnlijk niet effectiever dan het geadviseerde schema van 75 mg/m^2 cisplatin op dag 1 gevolgd door 1.25 mg/m^2 topotecan gedurende 5 dagen. De cisplatin dosering dient namelijk met 33% verminderd te worden om een minimale verhoging in de topotecan dosering mogelijk te maken.

In **hoofdstuk 3** wordt het slecht wateroplosbare camptothecinederivaat 9-aminocamptothecine besproken. Aangezien 9-aminocamptothecine één van de meest effectieve middelen in preklinische modellen bleek te zijn, is er lang gezocht naar een goede formulering voor klinische toepassing. Sindsdien zijn er verscheidene intraveneuze fase I- en II- en orale fase I studies uitgevoerd.

Hoofdstuk 3a beschrijft hogedruk vloeistofchromatografie methoden voor de bepaling van de 9-aminocamptothecine lactonconcentraties en voor de totale concentraties in humaan plasma. De lactonvorm werd geëxtraheerd door middel van een vloeistofextractie, waarbij de laagst adequaat meetbare concentratie 50 pg/ml was. In een tweede analyse werd de totale concentratie bepaald na een simpele onteiwittingsstap, waarbij de totale hoeveelheid 9-aminocamptothecine in de lactonvorm werd gebracht. De laagst adequaat en precies meetbare concentratie bij de laatstgenoemde methode was 100 pg/ml.

Na een 5-minuten durende intraveneuze toediening van 9-aminocamptothecine werd er een zogenaamde 'rebound peak' in het kinetisch profiel van de totale concentratie van 9-aminocamptothecine in humaan plasma geconstateerd. De factoren die deze 'rebound peak' veroorzaken, worden in **hoofdstuk 3b** beschreven. De toegediende lactonvorm van 9-aminocamptothecine bleek in humaan bloed zeer snel omgezet te worden in de niet werkzame carboxylaatvorm, waarbij 3 uur na toediening minder dan 10% zich nog in de lactonvorm bevond. Bij laboratoriumexperimenten, waarbij 9-aminocamptothecine aan vers bloed werd toegevoegd, verdeelde het zich direct over de rode bloedcellen en het plasma. Aangezien de toegevoegde lactonvorm zeer snel werd omgezet in de carboxylaatvorm, die niet in staat is om passief de membranen van de rode bloedcellen te passeren, nam de concentratie van 9-aminocamptothecine in het plasmacompartiment in de tijd toe. In tegenstelling tot onder andere topotecan heeft de carboxylaatvorm van 9-aminocamptothecine een hoge affiniteit voor humaan serumalbumine. Hierdoor komt het evenwicht aan de kant van de carboxylaatvorm te liggen, zodat de totale plasmaconcentraties toenemen. Deze 'rebound peak' werd niet waargenomen bij preklinische studies in muizen. Dit kan verklaard worden doordat de percentages 9-aminocamptothecine die zich - na het instellen van het evenwicht - nog in de lactonvorm bevinden, in oplossingen van humaan- of muizen serumalbumine respectievelijk 0.63 en 35.0% zijn.

In **hoofdstuk 3c** wordt de kinetiek van 9-aminocamptothecine in plasma en speeksel na orale toediening beschreven. Tevens worden relaties gelegd tussen de plasmakinetiek en de bijwerkingen. De lactonvorm van 9-aminocamptothecine wordt, in tegenstelling tot deze andere bekende camptothecinederivaten, zoals topotecan, lurtotecan en irinotecan, zeer snel omgezet in de carboxylaatvorm. De plasmakinetiek was lineair met de dosis met zeer lage intrapatiëntvariaties in de expositie van de lactonvorm, terwijl de interpatiëntvariaties in deze expositie relatief hoog waren. Concentratieratio's tussen ongestimuleerd speeksel en plasma waren zeer variabel, zodat plasma niet vervangen kan worden door ongestimuleerde speeksel voor de bepaling van het kinetisch profiel van 9-aminocamptothecine. Tevens werd bij een patiënt met pleuravocht onderzocht welk effect deze derde ruimte op de plasmakinetiek heeft. Net als voor topotecan (beschreven in hoofdstuk 2b) hoopt 9-aminocamptothecine zich niet in deze derde ruimte op en kan 9-aminocamptothecine aan patiënten met pleuravocht worden gegeven zonder dat de expositie in het plasma afneemt. Ten aanzien van bijwerkingen correleert de oppervlakte onder de plasmaconcentratie tijdcurve van de lactonvorm met het percentage daling in witte bloedcellen en trombocyten. In toekomstige klinische studies met oraal toegediende 9-aminocamptothecine kunnen patiënten individueel gedoseerd worden met een vooraf bepaalde oppervlakte onder de plasmaconcentratie tijdcurve als farmacokinetisch eindpunt, aangezien de intrapatiëntvariatie extreem laag is.

In het één na laatste hoofdstuk van dit proefschrift worden analytische methoden beschreven voor de bepaling van de liposomaal geformuleerde topoisomerase I remmer lurtotecan, terwijl in het laatste hoofdstuk een toepassing van deze methoden wordt beschreven.

Hoofdstuk 4a beschrijft een hogedruk vloeistofchromatografische methode voor de bepaling van totale lurtotecan concentraties in plasma en urine van patiënten na de toediening van NX 211, de liposomaal geformuleerde lurtotecan. Totale plasmaconcentraties worden bepaald na het onteiwitten en aanzuren van het monster, waarbij de laagst adequaat meetbare concentratie op 1.00 ng/ml gesteld is. Deze methode werd ook gevalideerd voor de bepaling van lurtotecan concentraties in urine die hoger zijn dan 100 ng/ml, door de urine voor extractie 40 keer te verdunnen in lurtotecan-vrije plasmamonsters. Voor lurtotecan concentraties in urine lager dan 100 ng/ml werd een andere methode ontwikkeld die gebruik maakt van een vloeistofextractie. Tevens werd het fluorescerende vermogen van lurtotecan bij deze methode verhoogd door het eluaat voor detectie bloot te stellen aan ultraviolet licht, waarbij de piperazinomethyleen groep van het molecuul werd afgesplitst, resulterend in een 14 keer hoger fluorescerend signaal. Concentraties tot en met 0.500 ng/ml konden nog nauwkeurig en accuraat worden gemeten. Deze twee analytische bepalingsmethoden worden gebruikt in een lopende fase I studie met NX 211.

De toepassingen van de hierboven genoemde methoden worden beschreven in **hoofdstuk 4b**, waarin de kinetiek van lurtotecan wordt beschreven bij 6 patiënten die experimenteel NX 211 toegediend hebben gekregen. Tevens worden twee nieuwe methodieken beschreven voor de bepaling van lurtotecan concentraties in bloed- en fecesmonsters. De monstervoorbewerking bestaat voor beide methoden uit een onteiwittingsstap in combinatie met aanzuren. Doordat ook hier het fluorescerend vermogen werd verhoogd door middel van ultraviolet licht, werden de laagst adequaat meetbare concentraties in bloedmonsters en gehomogeniseerde fecesmonsters respectievelijk gesteld op 0.25 en 10 ng/ml. De plasmakinetiek van lurtotecan kon in alle gevallen het best worden beschreven met een mono-exponentiële afname, met een halfwaarde tijd van gemiddeld 6 uur. De plasmaklaring was gemiddeld 0.78 L/uur/m^2, wat 25 keer langzamer is dan de plasmaklaring van niet liposomaal toegediende lurtotecan. Het distributie volume op "steady state" was ongeveer net zo groot als het totale bloedvolume van de patiënten en de bloed tot plasma totaal lurtotecan concentratieratio was gemiddeld 0.66. Deze parameters zijn indicatief dat lurtotecan zich nog in de liposomen bevindt, die zich voornamelijk in het plasmacompartiment ophouden. Zowel de urinaire als de fecale excretie van onveranderd lurtotecan was laag met respectievelijk gemiddelde waarden van 8.6% en 9.7%. Een groot deel van lurtotecan zal dus waarschijnlijk omgezet worden in metabolieten. In de chromatogrammen van gehomogeniseerde fecesmonsters werden 2 extra pieken gevonden, die afwezig waren in de blanco fecesmonsters van de patiënten. Momenteel wordt in een lopende fase I studie gekeken naar de toxiciteit en mogelijke anti-tumor respons in relatie tot de farmacokinetiek.

Conclusies en toekomstdromen

De camptothecinederivaten behoren tot de klasse van de topoisomerase I remmers, die niet meer weg te denken zijn uit de kankergeneeskunde. Effectiviteit is onder andere aangetoond tegen eierstok- en dikkedarmkanker. Om de concentraties van deze topoisomerase I remmers in verschillende biologische monsters adequaat te kunnen bepalen zijn vele methodieken ontwikkeld die voornamelijk gebaseerd zijn op hogedruk vloeistofchromatografie in combinatie met fluorescentie detectie. Aangezien alleen de lactonvorm werkzaam is, en het evenwicht tussen de lacton- en carboxylaatvorm in het plasmacompartiment varieert tussen de verschillende analoga en kan variëren van mens tot proefdier, blijven goede analysemethoden voor de bepaling van de lactonvorm zeer belangrijk om goede relaties te kunnen leggen tussen de (plasma)kinetiek van een camptothecine en de bijwerkingen en/of tumorrespons.

Ter verhoging van de effectiviteit zullen in de toekomst de topoisomerase I remmers steeds vaker worden gecombineerd met cytostatica met andere werkingsmechanismen. Aangezien in combinatietherapieën de doseringen van de cytostatica meestal lager zijn, blijven gevoelige, selectieve, accurate en reproduceerbare bepalingsmethoden nodig om inzicht te krijgen in mogelijke farmacokinetische interacties en de hieruit voortvloeiende bijwerkingen en/of tumorrespons.

Ik droom van een toekomst waarin anti-kankermiddelen individueel worden gedoseerd, gebaseerd op het farmacokinetisch profiel van het betreffende cytostatica in iedere afzonderlijke patiënt, met het doel om de bijwerkingen te verlagen, terwijl de effectiviteit van het middel verbetert. Met het oog op de vorderende kennis van genetische verschillen in onder andere enzymsystemen, verantwoordelijk voor bijvoorbeeld detoxificatie van allerhande stoffen, zou de realiteit van deze droom toch niet heel ver weg hoeven te zijn

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 12 november 1968 te Rotterdam. Zijn jeugd heeft hij doorgebracht in Nieuw-Lekkerland, alwaar hij de lagere school doorliep. In 1986 behaalde hij het HAVO eindexamen aan de Lage Waard te Papendrecht. In Rotterdam werd in hetzelfde jaar begonnen aan de opleiding tot medisch microbiologisch analist aan de polytechnische faculteit van de Hoge School Rotterdam & Omstreken, welke in 1990 met goed gevolg werd beëindigd. In het kader hiervan was hij gedurende het laatste studiejaar werkzaam op de afdeling virologie van het Academisch Ziekenhuis Rotterdam (Prof. dr. N. Masurel). Onder leiding van de viroloog dr. Ph.H. Rothbarth en hoofdanalist P. Schrijnemakers is de liefde voor de research ontstaan. Na het vervullen van de militaire dienst is hij in november 1991 als research analist in dienst getreden van de afdeling interne oncologie (Prof. dr. G. Stoter) van Dr. Daniel den Hoed Kliniek op het laboratorium van de experimentele chemotherapie en farmacologie (Prof. dr. J.H.M. Schellens), alwaar hij in de gelegenheid is geweest zelfstandig onderzoek te verrichten. De laatste jaren was dit onderzoek, onder leiding van Prof. dr. J. Verweij en dr. A. Sparreboom, voornamelijk gericht op topoisomerase I remmers, wat uiteindelijk geresulteerd heeft in dit proefschrift.

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