Quantitative prediction of oral bioavailability of a lipophilic antineoplastic drug bexarotene administered in lipidic formulation using a combined *in vitro* lipolysis/microsomal metabolism approach

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ABSTRACT

For performance assessment of the lipid-based drug delivery systems (LBDDS), *in vitro* lipolysis is commonly applied because traditional dissolution tests do not reflect the complicated *in vivo* micellar formation and solubilisation processes. Much of previous research on *in vitro* lipolysis have mostly focused on rank-ordering formulations for their predicted performances. In this study, we have incorporated *in vitro* lipolysis with microsomal stability to quantitatively predict the oral bioavailability of a lipophilic antineoplastic drug bexarotene (BEX) administered in LBDDS. Two types of LBDDS were applied: lipid solution and lipid suspension. The predicted oral bioavailability values ($F_{oral,predicted}$) of BEX from linking *in vitro* lipolysis with microsomal stability for lipid solution and lipid suspension were 34.2 ± 1.6% and 36.2 ± 2.6%, respectively, while the *in vivo* oral bioavailability (F_{oral}) of BEX was tested as 31.5 ± 13.4% and 31.4 ± 5.2%, respectively. The $F_{oral,predicted}$ corresponded well with the F_{oral} for both formulations, demonstrating that the combination of *in vitro* lipolysis and microsomal stability can quantitatively predict oral bioavailability of BEX. *In vivo* intestinal lymphatic uptake was also assessed for the formulations and resulted in <1% of the dose, which confirmed that liver microsomal stability was necessary for correct prediction of the bioavailability.

KEYWORDS

Lipid-based formulation; absorption; solubility; bioavailability; lymphatic transport

ABBREVIATIONS

BCS, biopharmaceutics classification system; BEX, bexarotene; ESI, electrospray ionisation; GI, gastrointestinal; HPLC, high performance liquid chromatography; LBDDS, lipid-based drug delivery systems; MRM, multiple reaction monitoring; PEG400, polyethylene glycol 400; SD, standard deviation; SEDDS, self-emulsifying drug delivery system.

INTRODUCTION

Modern drug discovery programmes have resulted in the development of increased number of drug candidates with low aqueous solubility [1]. It is a general concept that the drug must be solubilised in the gastrointestinal (GI) tract to be able to access the enterocytes for permeation [2]. Poor aqueous solubility limits the rate of dissolution and consequently the amount of the drug that can be absorbed following oral administration. In order to overcome such situations, a range of formulation approaches has been studied including the use of lipids, surfactants, solid dispersions and fabrication of nanoparticles [3]. Among them, the application of lipid-based drug delivery systems (LBDDS), including self-emulsifying drug delivery system (SEDDS), have been successful in increasing the solubility and oral bioavailability as well as reducing the variability of oral absorption [2, 4, 5].

The aim of most LBDDS is to solubilise poorly soluble drugs in the formulation and then maintain the drug in a solution as it is administered into the GI tract [2]. As a result of this solubilisation, the dissolution step of the drug in the GI tract can be avoided and therefore could promote absorption. For these reasons, it is a common practice to assess solubility of the drug in various lipids and surfactants during development of LBDDS [3, 6]. It has to be noted however that solubilisation of a drug during formulation processes does not always lead to solubilised drug under physiological conditions in the GI tract [7]. This is closely related to the complicated processes of LBDDS digestion and mixed micelles formation in the GI tract [1, 2]. Due to this complexity, *in vitro* lipolysis or digestion systems are recommended to assess and predict the performance of LBDDS at physiological conditions [8-10].

Traditionally, studies of performance assessment of LBDDS by *in vitro* lipolysis mostly provided rank-order of the formulations for further development or achieving certain level of *in vitro-in vivo* correlations [8, 11-15]. Recently, a novel approach of a combined *in vitro* lipolysis with microsomal metabolism was developed in our laboratory by Benito-Gallo *et al*, which provided an opportunity for quantitative prediction of oral bioavailability of drugs administered in LBDDS [8]. However, the concept of *in vitro* lipolysis/microsomal metabolism link was developed using only two model compounds, and warrants validation with additional compounds. In addition, the pharmacokinetic data used to develop the combined *in vitro* lipolysis/microsomal metabolism approach was obtained from literature, which was additional limitation of the previous work [8]. Therefore, in the current study, we show that *in vitro* lipolysis

linked with microsomal stability can quantitatively predict the oral bioavailability of bexarotene (BEX, structure shown in Figure 1), an antineoplastic compound, when administered orally in LBDDS in rats. In addition, the validation of the predictions was achieved in this work by conducting *in vivo* bioavailability and intestinal lymphatic transport studies. The information on lymphatic transport is important as drugs that have substantial intestinal lymphatic transport avoid liver at the first pass, and therefore hepatic microsomal metabolism element of quantitative prediction could be omitted.

MATERIALS AND METHODS

Materials

BEX was obtained from LC Laboratories (Woburn, MA, USA). Linoleic acid was purchased from Acros Organics (Loughborough, UK). Trizma maleate, $MgCl_2$, KH_2PO_4, K_2HPO_4, and reduced nicotinamide adenine dinucleotide phosphate (NADPH), porcine pancreatin powder (8 × USP specifications), sodium taurocholate (NaTc), NaCl, lecithin, tetrabromo-*o*-cresol and sunflower oil were from Sigma (Gillingham, UK). Calcium chloride was from Alfa Aesar (Lancashire, UK). Pooled male rat liver microsome was purchased from Gibco (Paisley, UK). Polyethylene glycol 400 (PEG400) was obtained from Fisher Scientific (Loughborough, UK). All solvents used were of HPLC grade or higher.

Solubility

Aqueous solubility of BEX at different pH was predicted by GastroPlus™ version 9.6.00015 with built-in ADMET Predictor™ v9.0.0.0. Reference solubility at pH 7.0 in water of 50 µM was given as input [16]. Solubility of BEX in various vehicles was measured following a previously reported method with minor modifications [17]. In glass vials, BEX (10 mg) was mixed with 1 mL of PEG400, linoleic acid or sunflower oil. The mixture was stirred magnetically for 72 h at 37 °C and then was filtered using a Costar Spin-X Centrifuge Tube (0.22 µm pore size, Fisher Scientific, Loughborough, UK) at 2400 *g* for 5 min. The filtrate was collected and subjected for analysis using HPLC-UV. The experiment was conducted in triplicate.

In vitro lipolysis

In vitro lipolysis was performed based on the method that was previously validated and reported [8, 18, 19]. The lipolysis digestion buffer was composed of the following: 50 mM tris maleate; 150 mM NaCl; 5 mM

$CaCl_2$, 5 mM NaTc; and 1.25 mM lecithin. The pH was adjusted to 6.8 prior to the experiment. BEX was formulated using linoleic acid or sunflower oil at 4 mg/mL and was added to the digestion buffer. The lipolysis was initiated by addition of the enzyme solution prepared from pancreatin extract and the pH of the reaction mixture was maintained at 6.8 using a pH-stat titrator (T50 Graphix with DG111-SC pH probe, Mettler Toledo Inc.) and stirred at 37 °C. Following completion of lipolysis, the mixture was subjected to ultracentrifugation at 268,350 *g* for 90 minutes at 37 °C (SORVALL® TH-641 Rotor, Thermo Fisher Scientific, UK). The lipid, micellar and sediment phases were collected and prepared for analysis using HPLC-UV. After analysis of samples from each phase, the fraction of drug found in each phase. The concentration of BEX in micellar phase was used to calculate the fraction predicted to be absorbed ($F_{abs,predicted}$) using equations reported previously [8]:

$$F_{abs,predicted} = [Drug]_{MP} \cdot \frac{40\ mL}{0.3\ mL\ \cdot 4\ mg/mL}$$

where, $[Drug]_{MP}$ is the drug concentration (mg/mL) found in micellar phase, 40 mL is the volume of *in vitro* lipolysis buffer and 0.3 mL of the 4 mg/mL BEX formulation was used. Experiment was performed in triplicate.

Liver microsomal stability

Liver microsomal metabolic stability assay was performed using rat liver microsome following previously reported methods with minor modifications [8, 20]. The reaction mixture was composed of the followings: 0.5 mg microsomal protein per mL; 10 mM $MgCl_2$; 1 mM NADPH; and 84.7 mM potassium phosphate buffer at pH 7.4. BEX was tested at 1 µM and the reaction was initiated with the addition of NADPH. Samples were withdrawn at predetermined time points and reaction was terminated by excessive volume of acetonitrile. Samples were analysed by HPLC-UV and performed in triplicate.

Half-life ($t_{1/2}$) of BEX was obtained from the semi-log plot of concentration-time profile:

$$t_{1/2} = -\frac{0.693}{k}$$

where, *k* is the slope obtained by plotting natural log percentage of BEX versus time. The intrinsic clearance (CL_{int}) was then obtained by the following equation [21, 22]:

$$CL_{int} = \frac{0.693}{t_{1/2}} \cdot \frac{mL\ incubation}{mg\ microsomes} \cdot \frac{mg\ microsomes}{g\ liver} \cdot \frac{g\ liver}{kg\ body\ weight}$$

where, *mg microsomes/g liver* and *g liver/kg body weight* values were 44.8 and 40.0, respectively, for rats [23]. The hepatic clearance (CL_h) was then obtained by utilising parallel-tube model [21, 22]:

$$CL_h = Q \cdot (1 - e^{\left(-CL_{int}/Q\right)})$$

where, Q is hepatic blood flow rate of 55.2 mL/min/kg for rats [23]. The fraction that escapes hepatic metabolism (F_h) was then calculated using the following equation [21, 22]:

$$F_h = 1 - \frac{CL_h}{Q}$$

The F_h obtained from the above equation also represents the fraction that escapes hepatic first-pass effect during oral absorption.

Calculation of predicted oral bioavailability

By incorporating *in vitro* lipolysis and *in vitro* metabolic stability results, predicted oral bioavailability ($F_{oral,predicted}$) was calculated using the following equation [8]:

$$F_{oral,predicted} = F_{abs,predicted} \cdot F_h$$

Animal experiments

Animals

Procedures and protocols of all animal experiments in this study were approved by the Animal Care Committee of Sungkyunkwan University (School of Pharmacy) and performed in accordance with National Institutes of Health guidelines (NIH publication No. 86-23, revised 1985). Male Sprague-Dawley rats (8 weeks of age, body weight 238-274 g) were purchased from Samtako Co. (Osan, Gyeonggi-do, South Korea). Rats were kept in clean plastic cages with freely accessible standard rat diet (Samtako Co., Osan, Gyeonggi-do, South Korea) and water. The animals were housed at a temperature of 22 ± 2 °C with a 12 h light-dark cycle and a relative humidity of 55 ± 10% and were acclimatised for at least 1 week prior to any procedures.

In vivo plasma pharmacokinetics

The pharmacokinetics of BEX was characterised in rats after intravenous and oral administrations. Prior to surgery, the animals were anaesthetised by intraperitoneal injection of Zoletil® 50 (Virbac Laboratories, Carros, France) (22.5 mg/kg) and cannulated with a polyethylene tubing (0.58 mm i.d., 0.96 mm o.d., Natume, Tokyo, Japan) in the femoral and jugular veins for intravenous administration group or in the jugular vein only for oral administration group. Following the surgery, animals were kept in warm, clean cages for recovery for 24 h. For intravenous administration, BEX dissolved in PEG400 was injected into the femoral vein cannula at a dose of 5 mg/kg with injection volume of 1 mL/kg. For oral administration, BEX formulated in linoleic acid or sunflower oil (4 mg in 1 mL for both formulations) was administered by oral gavage at a dose of 10 mg/kg with dosing volume of 2.5 mL/kg. Blood samples (0.1 mL) were collected from the jugular vein cannula at predetermined time points and plasma samples were harvested by centrifugation at 16000 *g* for 5 min at 4 °C and stored at -20 °C until analysis.

In vivo lymphatic uptake

Lymphatic delivery of BEX was characterised in rats after oral administration. Prior to surgery, the animals were given corn oil (1 mL) by oral gavage to facilitate mesenteric lymph duct cannulation. Approximately 2 h later, the rats were anaesthetised by intraperitoneal injection of Zoletil® 50 (Virbac Laboratories, Carros, France) (22.5 mg/kg), and the right side of the flank was shaved by electric clipper and sterilised by 70% ethanol solution. The mesenteric lymph duct was exposed by incision of the right abdomen. After punctuation of the duct, a polyethylene tubing (0.58 mm i.d., 0.96 mm o.d., Natume, Tokyo, Japan) was cannulated. The cannula was fixed and adhered with the use of cyanoacrylate glue. After cannulation, the wound was closed by suture and surgical clips. The animals were then kept in warm, clean cages for recovery for 2 h. For oral administration, BEX dissolved in PEG400, linoleic acid or sunflower oil was administered to three groups of rats by oral gavage at a dose of 10 mg/kg with 2.5 mL/kg dosing volume. The lymph fluid was continuously collected from the cannula and the collection tube was changed at predetermined intervals. Collected lymph samples were stored at -20 °C until analysis.

Analytical methods for determination of concentration levels

Determination of BEX in samples from in vitro experiments

Samples from *in vitro* experiments were analysed based on a previously reported HPLC-UV method [24] with minor modifications. Modifications included using flow rate of 0.4 mL/min and the use of hexane (3 mL) as the extraction solvent. The range of calibration curves was also adjusted to 500 – 20000 ng/mL.

Determination of BEX in samples from in vivo experiments

An API 2000 mass spectrometer coupled with a Waters 2690 separation module was used for sample analysis. Separation was achieved on a Kinetex biphenyl column (100 × 2.1 mm, 2.6 µm, Phenomenex, Torrance, CA, USA). The column oven temperature was 40 °C and the flow rate was 0.25 mL/min. The total run time was 8 min and the data were processed by analyst version 1.4.0 (AB Sciex, Framingham, MA, USA).

The electrospray ionisation (ESI) source was operated in negative mode. The multiple reaction monitoring (MRM) parameters and MS/MS conditions were as follows: *m/z* 347.1 → 303.4 for BEX; *m/z* 422.78.9 for tetrabromo-*o*-cresol (internal standard, IS); curtain gas: 25 psig; collision gas: 5 psig; ion spray voltage: -4500 V; ion source temperature: 400 °C; ion source gas 1: 20 psig; ion source gas 2: 40 psig; declustering potential: -41 V; focusing potential -350 V; entrance potential: -12 V; collision energy: -30 eV; collision cell exit potential: -28 eV.

Both plasma and lymph samples were prepared by protein precipitation with acetonitrile. Samples (50 µL) were added with IS solution (100 µL, 500 ng/mL tetrabromo-*o*-cresol in acetonitrile) and additional acetonitrile of 100 µL. The mixture was vortex-mixed for 10 sec and then centrifuged for 5 min at 16000 *g*. The supernatant (70 µL) was then mixed with 130 µL of water and transferred to a HPLC vial. A portion (15 µL) of the mixture was injected into the LC-MS/MS.

Statistical analyses

All data were presented as mean ± SD (standard deviation). Two-tailed unpaired t-test was applied to determine statistical significance and a *p*-value of <0.05 was considered significant. When more than two groups were compared, a one-way ANOVA followed by Tukey's multiple comparisons test was utilised. GraphPad Prism version 7.01 (GraphPad Software, Inc., La Jolla, CA, USA) was used for

statistical analysis. Non-compartmental analysis using Phoenix WinNonlin 6.3 software (Pharsight, Mountain View, CA, USA) was applied to calculate the pharmacokinetic parameters from plasma concentration-time profiles.

RESULTS

The predicted pH-dependent aqueous solubility profile of BEX is shown in Figure 1. Although it was predicted to be slightly higher in basic pH conditions, the solubility in overall was predicted to be <0.1 mg/mL. It was in agreement with the fact that BEX is a class II drug of the Biopharmaceutics Classification System (BCS) and therefore BEX would benefit with application of LBDDS [16, 25].

Solubility assessment results of BEX in linoleic acid and sunflower oil are shown in Figure 2. The solubility of BEX in linoleic acid was 6.2-fold higher than in sunflower oil. It should be noted that the solubility in sunflower oil was <4 mg/mL, and hence the formulation of BEX in sunflower oil used for *in vitro* lipolysis and *in vivo* pharmacokinetic experiments was a lipid suspension. Solubility of BEX in linoleic acid was >4 mg/mL and therefore the formulation tested in the experiment was a clear solution.

Both formulations of BEX in linoleic acid and sunflower oil were tested for their performance in *in vitro* lipolysis system (Figure 3). Interestingly, both formulations resulted in comparable fractions of the drug found in the micellar phase, although formulation of linoleic acid was a clear solution and that of sunflower oil was a suspension. It showed that the concentration of BEX in the micellar phase is comparable regardless of their solubilised state in the formulation.

The results of liver microsomal stability of BEX performed using rat liver microsome is shown in Table 1. The parameters of CL_{int}, CL_h and F_h were calculated from the half-life obtained from the stability test and the obtained F_h indicated that BEX would be classified as a moderately extracted compound [26].

In vivo plasma pharmacokinetic profiles were determined in rats following intravenous and oral administrations. The profiles are shown in Figure 4 and pharmacokinetic parameters derived from the profiles are shown in Table 2. Although the C_{max} differed between the two formulations following oral

administration, the overall exposure, determined by AUC_{inf}, did not differ significantly. Therefore, the oral bioavailability (F_{oral}) was comparable between the two formulations. The elimination half-life was also not significantly different between the formulations.

Following the method of the previously reported study [8], two predicted values were obtained: the fraction predicted to be absorbed ($F_{abs,predicted}$) and the predicted oral bioavailability ($F_{oral,predicted}$). The $F_{oral,predicted}$, which incorporates results of *in vitro* lipolysis and liver microsomal stability, resulted in comparable values to the *in vivo* experimental F_{oral} values (Table 2). It demonstrated that oral bioavailability of BEX achievable by LBDDS can be quantitatively predicted by application of *in vitro* lipolysis linked with microsomal stability.

The intestinal lymphatic transport of BEX resulting from the formulations was tested with mesenteric lymph duct cannulated rats (Figure 5). For this purpose, a lipid-free vehicle (PEG400) was also tested and it was shown that both formulations did not improve lymphatic uptake of BEX compared with the lipid-free vehicle.

DISCUSSION

In vitro lipolysis experiments are commonly utilised in assessment of LBDDS because the performance of LBDDS can be complicated by physiological processes of lipid digestion and therefore simple dissolution tests are often not applicable [1, 27]. In general, in the *in vitro* lipolysis studies, the amount of the drug in the micellar fraction is considered to have the most relevance to oral absorption [8]. This is because the micellar phase consists of mixed micelles with the solubilised drug which represents the fraction readily available for absorption. The lipid fraction contains the undigested lipids and the sediment fraction is what has precipitated during the lipolysis, therefore the drug in these two fractions is not readily available for absorption. Both formulations of linoleic acid and sunflower oil resulted in comparable fraction of BEX in the micellar phase following lipolysis (Figure 3), and therefore were predicted to have comparable fraction absorbed ($F_{abs,predicted}$, Table 2). These results indicate that the performance of the two formulations following oral administration would be at similar levels.

The $F_{abs,predicted}$ was then incorporated with F_h values from microsomal stability tests to predict oral bioavailability ($F_{oral,predicted}$), hence reflecting both absorption and hepatic first-pass effect. The $F_{oral,predicted}$ values for the two formulations shown in Table 2 corresponded to the F_{oral} values obtained from *in vivo* pharmacokinetic experiments, which demonstrates that the approach of linking *in vitro* lipolysis with microsomal stability can quantitatively predict the oral bioavailability of BEX following its administration in LBDDS.

The quantitative prediction of oral bioavailability was shown to be successful for two types of LBDDS in this study: lipid suspension (sunflower oil) and lipid solution (linoleic acid) (Table 2). Although sunflower oil was not able to fully solubilise BEX at 4 mg/mL, it interestingly resulted in comparable performance in *in vitro* lipolysis with linoleic acid formulation in which BEX was fully solubilised (Figure 3). Moreover, it was remarkable that the two formulations resulted in comparable *in vivo* F_{oral} (Table 2), although a suspension would have had an additional dissolution step included in the solubilisation processes of the drug. This highlights the fact that the *in vitro* lipolysis offers a more biorelevant performance assessment of LBDDS than traditional dissolution and that the *in vitro* lipolysis/microsomal metabolism link approach can be applied to different types of LBDDS.

Although *in vitro* lipolysis has been mainly used for digestible lipids, as the experimental system contains lipase enzyme, we here show that it can also be applied to formulations of lipid-digestion product (or indigestible lipid). Linoleic acid used in this study is a free fatty acid which is in fact one of the products of lipid digestion and the applicability of *in vitro* lipolysis is demonstrated by the $F_{oral,predicted}$ corresponding with the experimental *in vivo* F_{oral} (Table 2). The presence of lipids or digestive products, including free fatty acids, in the GI tract itself can induce release of the cholecystokinin, which stimulates secretion of pancreatic enzymes and bile acids [2, 28]. The *in vitro* lipolysis system mimics these endogenous components and therefore it better mimics the environment of the GI tract. Although indigestible lipids and lipid-digestion products would not benefit from the 'lipolysis' process, the *in vitro* lipolysis system as a whole provides more biorelevance in assessment of their ability to facilitate mixed micelle formation and hence drug solubilisation.

It should be noted that BEX has logP of 7.28 (ACD/Labs, Toronto, Canada) and belongs to class II of the BCS [25]. Accordingly, the oral bioavailability of BEX would be more dependent on solubility in the GI tract rather than permeability across membranes. Therefore *in vitro* lipolysis results were sufficient to predict the absorbed fraction without consideration of permeability which was in accordance with previous studies for similar compounds [8, 12, 13].

The results in Figure 5 show that the intestinal lymphatic uptake did not differ significantly between the two formulations, and in fact not different from a lipid-free vehicle. LBDDS are often employed to enhance intestinal lymphatic delivery of lipophilic drugs for the purpose of increasing the oral bioavailability and/or targeting the intestinal lymphatic system [3, 28-30]. It has been previously suggested that it is the inherent physicochemical properties of the drug that determines the association ability of the drug with chylomicrons which eventually governs intestinal lymphatic transport [17, 31]. The intestinal lymphatic transport of BEX, with its low chylomicron association reported previously [32], was not affected by LBDDS. It confirms the relevance of application of liver microsomal stability in bioavailability prediction as minimal lymphatic transport would mean that hepatic first-pass effect would be applied to BEX [8]. When hepatic first-pass effect is applied to the absorbed drug, *in vitro* lipolysis system alone would not be able to predict the oral bioavailability accurately. Therefore, it becomes evident that liver microsomal stability studies needed to be linked with *in vitro* lipolysis in order to quantitatively predict the oral bioavailability of BEX.

In conclusion, we have shown that oral bioavailability of LBDDS can be quantitatively predicted by incorporation of *in vitro* lipolysis and microsomal stability. The evaluations and predictions were applied to formulations of a lipid suspension and a lipid solution, which resulted in comparable *in vitro* and *in vivo* performance. The predictability of the approach was found to be acceptable for the two different types of LBDDS. In order to make a head-to-head comparison, experimental bioavailability obtained from our own *in vivo* pharmacokinetic studies was used. Additionally, intestinal lymphatic transport was assessed for the formulations to confirm that microsomal stability results need to be linked with *in vitro* lipolysis for the oral bioavailability prediction.

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Conflict of interest

The authors declare no conflict of interest.

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FIGURE CAPTIONS

Figure 1. Chemical structure of bexarotene (A) and pH-dependent solubility predicted by GastroPlus™ (B).

Figure 2. Solubility assessment of bexarotene in linoleic acid and sunflower oil (mean ± SD, n = 3). **, $p<0.05$.

Figure 3. *In vitro* lipolysis assessment of bexarotene in formulations of linoleic acid (solution) and sunflower oil (suspension). The amount of drug was analysed in lipid, micellar and sediment phases (mean ± SD, n = 3). N/S, not significant.

Figure 4. Plasma concentration-time profiles of BEX following intravenous administration at 5 mg/kg (in PEG400) and oral administration at 10 mg/kg (in linoleic acid or sunflower oil) in rats (mean ± SD, n = 5).

Figure 5. Cumulative intestinal lymphatic uptake of BEX from different formulations in 24 h following oral administration in mesenteric lymph duct cannulated rats (mean ± SD, n = 3). N/S, not significant.