Pharm Res (2010) 27:1416–1425
DOI 10.1007/s11095-010-0137-3

RESEARCH PAPER

Factors Affecting the *In Vivo* Lactone Stability and Systemic Clearance of the *Lipophilic* Camptothecin Analogue AR-67

Eyob D. Adane • Zhiwei Liu • Tian-Xiang Xiang • Bradley D. Anderson • Markos Leggas

Received: 24 February 2010 / Accepted: 24 March 2010 / Published online: 29 April 2010

ABSTRACT

Purpose The narrow efficacy-toxicity window of anticancer agents necessitates understanding of factors contributing to their disposition. This is especially true for camptothecins as they exist in the lactone and carboxylate forms with each moiety differentially interacting with efflux or uptake transporters. Here we determined the disposition of the lactone and carboxylate forms of AR-67, a 3rd generation camptothecin analogue.

Methods Pharmacokinetic studies were conducted in rats given intravenous boluses of AR-67 lactone or carboxylate with or without pharmacologic inhibitor pretreatment (GF120918 or rifampin). Pharmacokinetic modeling was used to estimate clearances, while simulations assessed the impact of clearance changes on overall AR-67 exposure.

Results Our modeling showed that carboxylate clearance was 3.5-fold higher than that of the lactone. GF120918 decreased lactone clearance only, but rifampin decreased both lactone and carboxylate clearances. Simulations showed that decreasing carboxylate clearance, which controls the overall drug disposition, leads to significant increase in AR-67 exposure.

Conclusion The apparent *in vivo* blood stability of AR-67 is partly dependent on the increased carboxylate clearance. This may have clinical implications for populations with single nucleotide polymorphisms that impair the function of uptake transporter genes (e.g., SLCO1B1), which are potentially responsible for AR-67 carboxylate clearance.

KEY WORDS BCRP· carboxylate · lactone · OATP/Oatp · P-gp

E. D. Adane • Z. Liu • T.-X. Xiang • B. D. Anderson • M. Leggas (✉)
Department of Pharmaceutical Sciences, University of Kentucky
Lexington, Kentucky 40536, USA
e-mail: mark.leggas@uky.edu

ABBREVIATIONS

AUC	area under the plasma concentration *versus* time curve
BCRP/bcrp	breast cancer resistance protein
OATP/Oatp	organic anion transporting polypeptide
P-gp	p-glycoprotein
USP	United States Pharmacopeia

INTRODUCTION

AR-67 (DB-67) is a highly lipophilic and potent third-generation camptothecin analogue (1,2) currently in early phase clinical trials as an anticancer agent (3). Previous *in vitro* experiments, assessing lactone stability in whole blood, showed that equilibrium favors the carboxylate form of AR-67, as it does other camptothecin analogues, but AR-67 lactone is relatively more stable compared to other camptothecin analogues (1). This molecule was selected for further development among several analogues designed to be relatively more blood stable. Camptothecins owe their pharmacologic activity to their α-hydroxy-δ-lactone pharmacophore, which hydrolyses to the open ring or carboxylate form in a pH-dependent but reversible manner (4–6). Lower pH favor the lactone form, while plasma and alkaline pH favors the carboxylate (4). For many camptothecin analogues, the pH-dependent lactone hydrolysis is strongly facilitated in plasma by carboxylate binding to serum proteins. As a result of the avid binding, sink conditions are established, and the equilibrium shifts towards carboxylate formation (7). Thus, lactone concentrations reach lower levels than carboxylate in plasma at steady state (8), which is of concern because the latter is considered inactive. However, due to its capacity to revert to the lactone form in acidic environments, the carboxylate has also been associated with

the increased toxicities observed in early camptothecin trials and with some second-generation analogues (9,10). Comparatively, AR-67 was chosen for development based on its decreased interaction with albumin and its increased lipophilicity that facilitated partition in lipid membranes. Collectively, these physicochemical characteristics were believed to "protect" the lactone and minimize hydrolysis.

As compared to most drugs, cytotoxic anticancer agents have a fairly narrow efficacy-toxicity window, and a good understanding of factors contributing to their disposition is essential for ensuring patient safety. The disposition of the lactone and carboxylate forms of AR-67 is expected to vary due to differences in solubility, interaction with transporters and enzymes and distribution into tissues. Several studies have demonstrated that camptothecins are substrates of uptake and efflux transporters (11–14). As would be expected from the physicochemical differences between lactone and carboxylate, we recently demonstrated that AR-67 lactone is a substrate for efflux transporters P-gp and BCRP, while the carboxylate is a substrate for the organic anion uptake transporters OATP1B1 and OATP1B3 *in vitro* (15). Although the overall drug disposition depends collectively on many factors, dissimilar transporter interactions will potentially lead to differences in lactone and carboxylate systemic clearances. The unique physicochemical properties of each camptothecin analogue also add an additional layer of complexity in estimating the reversible hydrolysis kinetic parameters and the lactone and carboxylate systemic clearances.

Although the *in vitro* interaction of camptothecins with transporters and the *in vivo* disposition of lactone and carboxylate forms of camptothecin (16,17), topotecan (18) and irinotecan (19) have been studied, the pharmacokinetics of AR-67 have not been examined in detail. To accurately estimate lactone and carboxylate pharmacokinetic parameters administration of both species is required (20,21). In this study we used pharmacokinetic modeling and simulations to assess how clearance changes of either the lactone or carboxylate forms could affect overall drug disposition. A primary objective was to estimate the systemic and interconversion clearances of the lactone and carboxylate forms of AR-67. Furthermore, through simulation and *in vivo* pharmacologic inhibition of transporters, we examined the influence of AR-67 lactone clearance changes on drug disposition.

MATERIALS AND METHODS

Chemicals

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

Animal Study Design

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

The effect of uptake transporter inhibition on the pharmacokinetics of AR-67 was assessed in rats weighing 250–280 g using rifampin. Animals were pretreated with 50 mg/kg rifampin (USP) orally 1 h before the administration of 2.5 mg/kg lactone or carboxylate.

AR-67 was administered through injection in the lateral tail vein. Following drug administration, about 100 μL of blood was collected from the saphenous vein at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 12 h using heparinized hematocrit capillary tubes and was then transferred to heparinized microcentrifuge tubes.

HPLC Analysis

Plasma was separated from blood cells by centrifugation at 8,500 g for 3 min at room temperature. The plasma was

extracted (1:4, v/v) with dry ice cold methanol (−80°C). The extracts were kept frozen at −80°C until analysis by HPLC with fluorescence detection for both AR-67 carboxylate and lactone forms as described previously (22). Assay accuracy and precision were validated in rat plasma and were found acceptable. Three quality control samples in the range of 2.5–250 ng/mL for carboxylate and 5–300 ng/mL for lactone demonstrated accuracy within 15% (85–115%) of nominal AR-67 concentrations. The relative standard deviation (RSD) was <6%. System suitability criteria were met prior to sample batch analysis. Samples were placed in an autoinjector (4°C) and injected within 6 h to prevent lactone-carboxylate interconversion.

Pharmacokinetic Analysis

Pharmacokinetic analysis was carried out using two approaches. The first utilized ADAPT-II to fit the four-compartment model depicted in Fig. 1 to the data. A model was built using eight differential equations, four each for the lactone and carboxylate administration. Each set of four equations shared the same kinetic parameters, which were simultaneously fitted to the data obtained from the lactone and carboxylate bolus doses using the maximum likelihood method (23). The alternate approach utilized a method described in the literature for reversible biotransformation systems (24). In this latter method, areas under plasma concentration ($AUC_{0\text{-inf}}$) *versus* time curves of both the lactone and the carboxylate following administration of the lactone or the carboxylate were estimated with WinNonlin v.5.2. The elimination and apparent distribution half-lives of the lactone and carboxylate were calculated from the micro-rate constants estimated with ADAPT-II as described in the literature (25).

Simulations

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

Statistical Analysis

Differences between clearance parameters in the presence or absence of inhibitors were assessed using a paired two sample t-test. The level of significance was $p<0.05$.

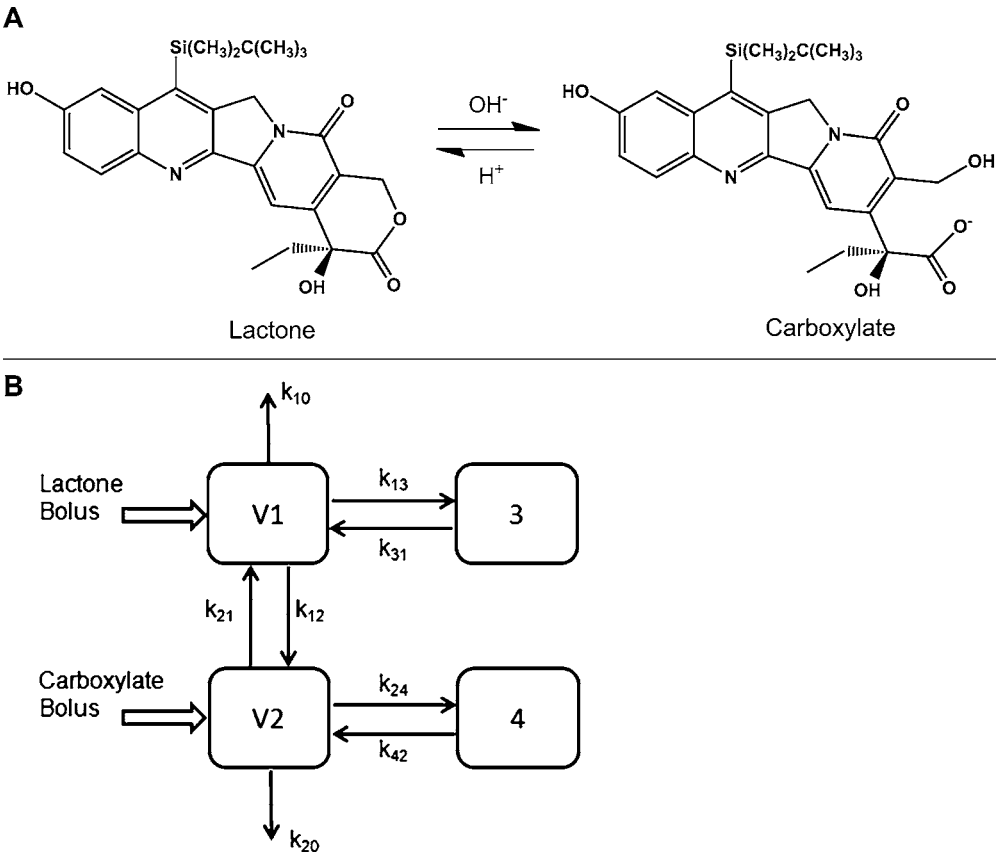

Fig. 1 (**A**) Chemical structures of AR-67 lactone and carboxylate. The two forms exist in a pH-dependent equilibrium. (**B**) A pharmacokinetic model allowing for AR-67 interconversion was used to fit the data.

Table I Pharmacokinetic Parameter Estimates. Animals were Gavaged with Either the Control Vehicle or GF120918 Prior to Intravenous AR-67 Administration. Parameters were Estimated by Fitting the Model Presented in Fig. 1 to the Data or from areas Under the Plasma Concentration Versus Time Curves (NCA model) as Previously Described (24)

PK Parameter[a]	Control		GF120918		Rifampin	
	Estimate (95% CI)		Estimate (95% CI)		Estimate (95% CI)	
	ADAPT model	NCA model	ADAPT model	NCA model	ADAPT model	NCA model
Lactone systemic clearance (Cl_{10})	1.8 (1.4–2.1)	1.7 (1.3–2.1)	0.7 (0.4–1.1)	1.1 (0.8–1.5)	1.0 (0.8–1.2)	0.8 (0.5–1.1)
Carboxylate systemic clearance (Cl_{20})	6.3 (4.9–7.6)	4.9 (4.0–5.8)	6.1 (4.8–7.4)	3.5 (2.8–4.3)	2.6 (2.0–3.2)	2.7 (2.2–3.2)
Lactone to carboxylate conversion clearance (Cl_{12})	1.4 (1.0–1.8)	1.1 (0.8–1.2)	2.2 (1.6–2.9)	1.4 (1.2–1.6)	1.0 (0.7–1.3)	0.9 (0.7–1.1)
Carboxylate to lactone conversion clearance (Cl_{21})	0.9 (0.6–1.2)	1.3 (0.8–1.9)	3.6 (2.4–4.7)	3.5 (2.8–4.3)	0.6 (0.1–1.4)	0.5 (0.4–0.7)
Lactone distributional clearance (CL_D-L)	5.2 (4.5–5.9)	–	4.7 (1.2–8.2)	–	0.8 (0.1–1.7)	–
Carboxylate distributional clearance (CL_D-C)	2.4 (1.4–3.5)	–	3.2 (1.7–4.7)	–	1.9 (0.4–4.0)	–
Lactone central volume (V1)	1.4 (1.2–1.5)	–	1.6 (1.0–2.2)	–	1.4 (1.1–1.7)	–
Carboxylate central volume (V2)	0.5 (0.2–0.8)	–	1.4 (0.8–1.9)	–	0.20 (0.1–0.3)	–
Apparent lactone elimination half life (t 1/2 L)	–	82.3 (71.7–92.8)	–	194.0 (111.3–276.7)	–	100.3 (66.7–133.9)
Apparent carboxylate elimination half-life (t 1/2 C)	–	109.3 (50.6–167.9)	–	223.7 (182.5–264.9)	–	223.8 (113.3–334.2)

[a] Clearances are in L/hr/kg; half-lives are in minutes; volumes are in L/kg

RESULTS

Plasma Pharmacokinetics of AR-67 Lactone and Carboxylate

The plasma pharmacokinetics of AR-67 were assessed in female Sprague Dawley rats following an intravenous bolus dose of 2.5 mg/kg. To obtain better estimates of the lactone-carboxylate interconversion kinetics, we administered AR-67 lactone and AR-67 carboxylate separately and measured the plasma concentration of both forms during the individual experiments. As shown in Fig. 1A, the lactone and carboxylate interconversion involves the hydrolysis of the lactone ring, but this reaction is reversible. To elucidate the role of each moiety in the overall drug disposition, we sought to selectively perturb the clearance of each AR-67 form. In previous studies, we determined that AR-67 lactone is a substrate of BCRP and to a lesser extent P-gp, while the carboxylate is a substrate of OATP1B1 (15). Therefore, the BCRP and P-gp pharmacologic inhibitor, GF120918, was used to decrease the lactone clearance, and the OATP1B1 inhibitor, rifampin, was used to impair the carboxylate clearance. GF120918 and rifampin were administered orally 5 min and 1 h, respectively, prior to the administration of the AR-67 intravenous bolus doses. Experiments with animals (six per group) receiving AR-67 alone (lactone or carboxylate) or AR-67 following GF120918 consisted of a four-period randomized crossover design. In studies with animals receiving rifampin, each animal was only used once to avoid rifampin experimental artifacts via rifampin-mediated metabolism and transporter induction (i.e., CYP450 and P-gp). Each animal was sampled at indicated time points via venipuncture of the saphenous vein. Pharmacokinetic analyses were carried out with ADAPT-II (23) following the implementation of the model depicted in Fig. 1B. The data sets for lactone and carboxylate doses were analyzed simultaneously. The pharmacokinetic parameter estimates are presented in Table I, and the pharmacokinetic profiles are depicted in Fig. 2. When the lactone form was administered the lactone concentrations (Fig. 2A) were much higher than the carboxylate (Fig. 2B). Interestingly, when the carboxylate dose was administered the carboxylate declined rapidly, and within 30 min the lactone reached similar concentration levels as the carboxylate (Fig. 2C and D). As expected, the administration of GF120918 prior to AR-67 lactone administration had a significant effect on the clearance

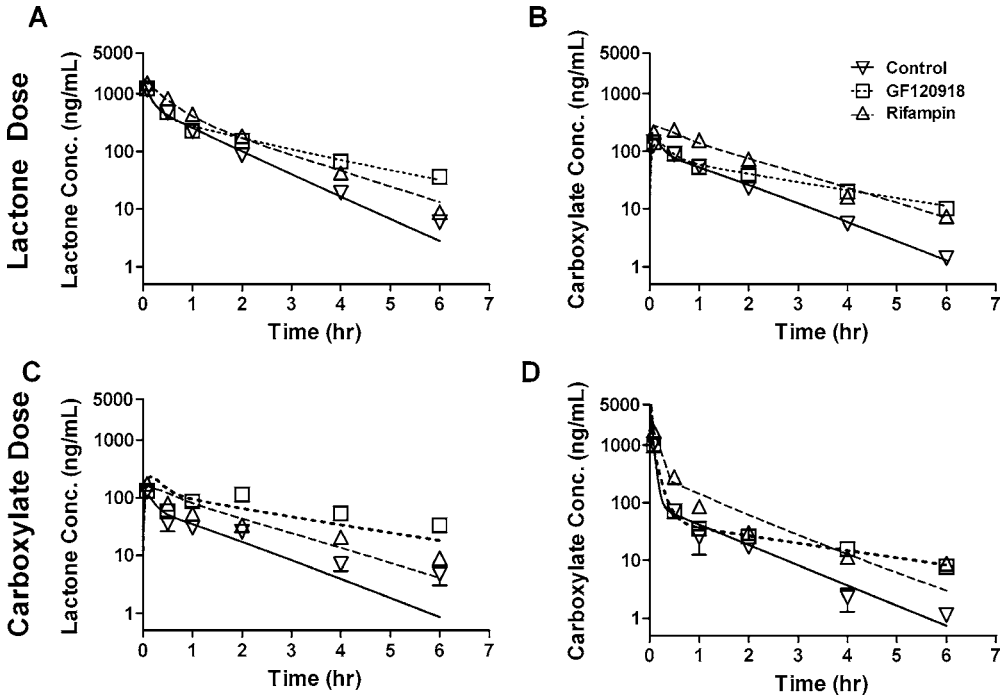

Fig. 2 Plasma pharmacokinetics of AR-67 lactone (**A**, **C**) and carboxylate (**B**, **D**) following intravenous administration of 2.5 mg/kg lactone (**A**, **B**) or carboxylate (**C**, **D**). Each experiment represents the average of 6 rats. Animals receiving AR-67 only (control) or AR-67 following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

of the lactone form, which decreased to approximately 60% of the clearance estimates obtained in animals not receiving the inhibitor prior to AR-67. Although the carboxylate concentrations were higher in the group receiving GF120918, the model predicted that the carboxylate clearance was unchanged, and the higher concentrations were a result of the increased lactone concentrations. Rifampin pre-administration had significant effects on the clearance of the carboxylate, but notably it also had a significant effect on the clearance of the lactone, which decreased by approximately 60% and 45%, respectively, as compared to animals not receiving a pharmacologic inhibitor. In the case of the rifampin pre-treated animals, the model also predicted a significant decrease in the peripheral compartment distributional clearance of the lactone, but not of the carboxylate. The central compartment volume estimate for the lactone (1.4 L/kg) was higher than the carboxylate moiety (0.5 L/kg), and there were no significant differences in its magnitude among the three experimental groups. Although differences were not statistically significant, the model predicted higher carboxylate volume when animals were pretreated with GF120918 and a lower volume when pretreated with rifampin (Table I).

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

The effect of each inhibitor on the exposure to AR-67 is shown in Fig. 3C and D for the lactone and carboxylate dosages, respectively. When the lactone was administered, 84% of the total AUC was contributed by the lactone, and pretreatment with GF120918 or rifampin significantly increased exposure to both forms (Fig. 3C). Animals receiving GF120918 and rifampin pretreatment had 81% and 76%, respectively, of the total AUC in the lactone form. The slight decrease in lactone exposure (or increased carboxylate exposure) in the later group is consistent with the effect of rifampin in decreasing carboxylate clearance. In experiments where the carboxylate was administered, 22% of the total AUC was contributed by the lactone, and again, pretreatment with GF120918 or rifampin significantly increased exposure to both forms (Fig. 3D). Animals receiving GF120918 and rifampin pretreatment had 55% and 22%, respectively, of the total AUC in the lactone form.

Simulations Assessing the Effect of Clearance Changes on AR-67 Exposure

The pharmacokinetic model fitted to the data obtained from the separate lactone and carboxylate administration suggests that the relative increases in the lactone and carboxylate concentrations in the GF120918 pretreated

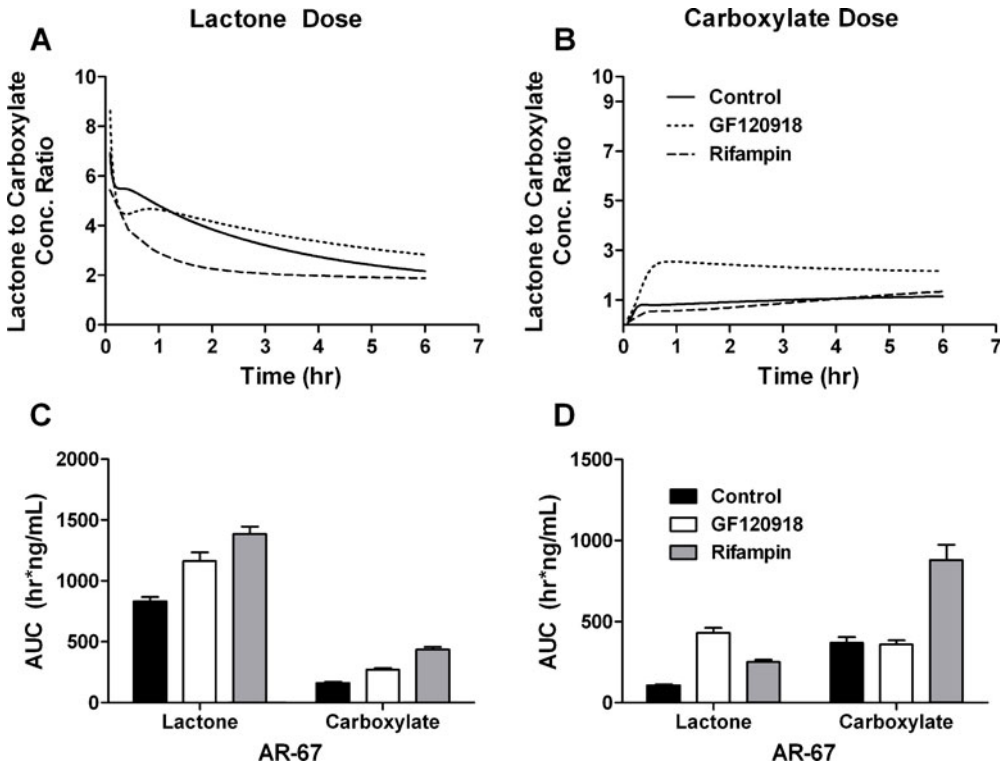

Fig. 3 AR-67 exists primarily in the lactone form in plasma following lactone administration. The lactone-to-carboxylate concentration ratios were calculated based on the model fits to the experimental pharmacokinetic data following AR-67 lactone (**A**) or AR-67 carboxylate (**B**) administration alone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

groups are consistent only with inhibition of the lactone clearance. In contrast, the model estimated that the observed increases in plasma concentrations of both lactone and carboxylate in the rifampin pretreated groups were due to the inhibition of both the lactone and carboxylate clearance. To better understand these results, we simulated the effects of decreasing lactone (Fig. 4A, B) or carboxylate (Fig. 4C, D) clearance on their respective AUC following lactone administration. The estimated AUCs are presented for clearance values ranging from 1–100% of the experimentally estimated clearance values. Thus, the axis was normalized between 0.01 and 1. The estimated AUC values are presented as the absolute estimates (Fig. 4A, C) or normalized to the AUC estimates obtained when there was no clearance inhibition (Fig. 4B, D). Simulations predicted that selective decrease in the lactone clearance would result in the increase of both the lactone and carboxylate AUC. The absolute magnitude of the increased exposure (Fig. 4A) would be greater for the lactone, but the relative increase (Fig. 4B) would be the same for either form. The selective decrease in the carboxylate clearance demonstrated that the magnitude of the AUC would increase for either form but the clearance has to decrease by more than 90% in order for the carboxylate AUC to be higher than the lactone one (Fig. 4C). The relative increase, however, is more pronounced for the carboxylate, resulting in a more rapid increase in carboxylate exposure with decreased carboxylate clearance (Fig. 4D). Further analysis demonstrated that a decrease in the lactone or carboxylate clearance by about 90% would yield similar increases in the overall exposure to AR-67 (Fig. 5A). However, inhibition of the carboxylate clearance would also result in a significantly higher increase in carboxylate exposure (Fig. 5D).

DISCUSSION

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

The results of the compartmental modeling were corroborated by non-compartmental analyses previously

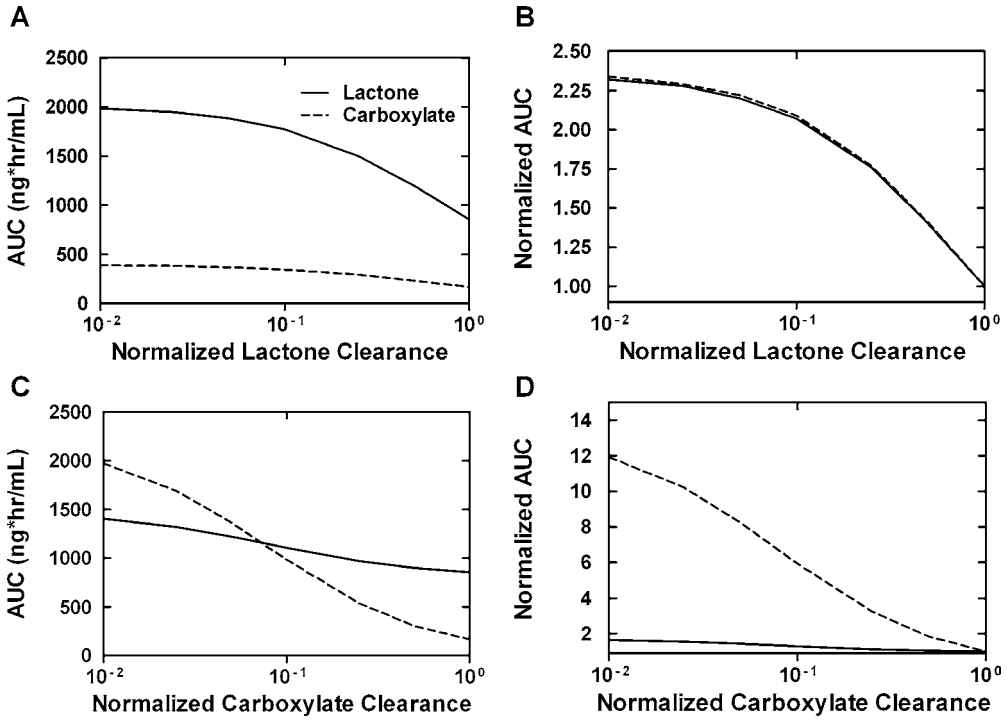

Fig. 4 Simulated lactone and carboxylate AUCs depicting the effect of lactone (**A**, **B**) or carboxylate (**C**, **D**) clearance inhibition following the intravenous bolus administration of AR-67 lactone.

presented by Cheng and Jusko for metabolites undergoing interconversion (24). The NCA analyses estimated the systemic and interconversion clearances based on areas under the time-concentration curves, which were obtained by the trapezoidal rule method. Some differences were noted in the parameter estimates obtained by the two different analyses. In all cases but one there was an overlap in the 95% confidence intervals around the parameter estimates (Table I). In the case of the carboxylate clearance, the means and 95% confidence intervals were different, but the relationship between the lactone and carboxylate clearances were similar for both methods (i.e., the carboxylate clearance was higher). Thus, the parameter estimates from both methods lead us to the same conclusions. An advantage of the NCA analysis is its simplicity, as it only requires the estimation of areas under the curve in the sampling compartment for parameter estimation (24). No assumption is required as to how many compartments are needed to fit the data. Furthermore, the NCA analysis can provide good initial parameter estimates for a more robust compartmental model analysis required to perform modeling and simulation of concentration-time profiles.

The pronounced difference in the systemic clearances of the lactone and the carboxylate suggest that the two moieties are eliminated via different pathways. Following

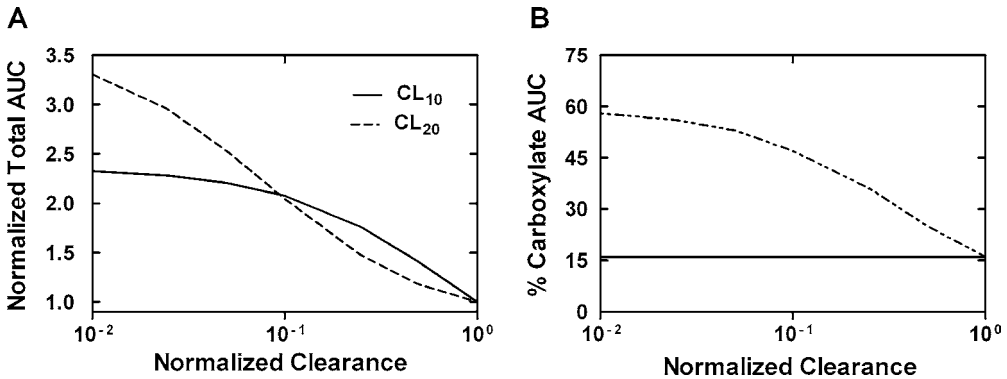

Fig. 5 Simulation results depicting the effect of lactone or carboxylate clearance inhibition on total AR-67 AUC (**A**) and on the % carboxylate plasma AUC (**B**) following intravenous bolus administration of AR-67 lactone.

lactone administration, the predominant form of AR-67 is the lactone as shown by a greater than 80% lactone AUC. This is most likely due to the lower systemic and interconversion clearance of the lactone compared to the systemic clearance of the carboxylate. The carboxylate that is converted from the lactone is likely to be eliminated before it gets converted back to the lactone. Moreover, the effect of higher carboxylate clearance is more evident following the carboxylate dosing, which rapidly declines from the plasma. In contrast, the pharmacokinetics of camptothecin and the camptothecin analog, topotecan, seem to be driven by the systemic clearance of the lactone and the lactone-to-carboxylate conversion clearance (16,18). For camptothecin, the systemic clearance of the lactone was 5-fold higher than that of the carboxylate, while lactone-to-carboxylate conversion clearance was three-fold higher than the reverse process. Although the magnitudes of the various clearance parameters were different, a similar finding was also observed in the pharmacokinetics of topotecan. The systemic clearance of topotecan lactone was more than four-fold higher than the systemic clearance of the carboxylate, and the conversion of the lactone to carboxylate was about three-fold higher than the reverse process. Thus, rapid lactone clearance coupled with rapid lactone-to-carboxylate conversion and slow carboxylate elimination likely explains the fact that the predominant form of camptothecin and that of topotecan is the carboxylate. The rapid *in vivo* lactone-to-carboxylate conversion in the case of camptothecin is in line with *in vitro* results that show rapid hydrolysis of camptothecin in plasma and physiological pH buffer (4). The avid binding of the carboxylate to serum proteins is known to further facilitate lactone hydrolysis (26). In contrast, *in vitro* results demonstrated that the AR-67 lactone hydrolysis occurred at a slower rate than SN-38 and camptothecin (1). In addition, the AR-67 lactone fraction at equilibrium was approximately 30% in whole blood as compared to 19.5% and 5% for SN-38 and camptothecin, respectively (1). Thus, it was hypothesized that the relatively higher AR-67 lactone stability *in vitro* was a function of increased membrane partitioning in red blood cell membranes and decreased affinity of the carboxylate form for human serum albumin (1). Our results demonstrate that an additional mechanism, the relatively higher carboxylate clearance, contributes to the apparent *in vivo* stability of AR-67 lactone.

The use of efflux and uptake transporter inhibitors allowed us to examine the effect of selective clearance changes on plasma concentration and overall exposure to AR-67. In the current study, the dual P-gp and BCRP/bcrp inhibitor GF120918 significantly decreased systemic clearance of the lactone, but not that of the carboxylate, and led to elevated lactone and carboxylate concentrations. The effect of GF120918 on lactone plasma concentration was quite pronounced following carboxylate administration where lactone AUC increased 3.7-fold compared to that in control pretreated animals. The results indicate that lactone clearance depends on efflux by P-gp and Bcrp consistent with our *in vitro* studies, which showed that the lactone is a substrate of P-gp and BCRP (15). GF120918 is widely used to assess the effect of P-gp and BCRP/Bcrp on drug disposition *in vitro* and *in vivo*. In both preclinical and clinical studies, pretreatment with GF120918 significantly increased the oral bioavailability of topotecan as a result of both decreased clearance and increased gastrointestinal absorption (27–29). In mice, pretreatment with GF120918 decreased plasma clearance and hepatobiliary excretion and increased fetal penetration and intestinal absorption of topotecan (27). Similarly, in clinical studies, co-administration of GF120918 increased the apparent oral bioavailability of topotecan from 40% to 97% (28).

Our studies suggest that the increased AR-67 lactone and carboxylate exposure observed with rifampin pretreatment was related to a decrease in the clearance of both the lactone and the carboxylate. Thus, the inhibition of carboxylate clearance by rifampin is most likely due to inhibition of the hepatic uptake of the hydrophilic carboxylate, while inhibition of lactone clearance could have happened as a result of ABC transporter inhibition. There is literature evidence to support the effect of rifampin on both OATPs/Oatps and P-gp. Rifampin inhibited OATP1B1-mediated transcellular transport and intracellular accumulation of substrate drugs, resulting in a 60% reduction in the intracellular accumulation of 17β-estradiol-17-(β-D-glucuronide) (E2G) and a significant reduction in the OATP1B1-mediated basolateral-to-apical transport of E2G, gimatecan and BNP1350 (30). In rats, it was shown that rifampin pretreatment increased atorvastatin plasma concentration as a result of decreased Oatp-mediated hepatic uptake and metabolism by the liver. Decreased hepatic uptake in the presence of a single dose of rifampin also led to decreased first-pass effect by the liver and, therefore, increased oral bioavailability of atorvastatin from 5% to 14% (31). On the other hand, inhibition of P-gp by a single dose rifampin was shown to lead to increased penetration of verapamil across the mouse blood-brain barrier (BBB) (32). This is consistent with our *in vitro* results, which demonstrated the carboxylate to be a substrate for uptake transporters OATP1B1 and OATP1B3, while the lactone form was transported by BCRP and to a lesser extent by P-gp (15). The effect of rifampin on BCRP is currently under investigation.

Changes in clearance are likely to occur in clinical practice and may have implications in the clinical use and toxicity of camptothecin analogues. These changes could arise from pharmacogenetic differences in transporters between individuals and have been reported to lead to pharmacokinetic differences. In one study, plasma concen-

tration of diflomotecan in 5 patients heterozygous for the ABCG2 421C>A allele was 2.9-fold higher than that of 15 patients with wild-type alleles (33). Similarly an association was shown between high plasma pravastatin concentration and single nucleotide polymorphisms and haplotypes of organic anion-transporting polypeptide (OATP1B1) (34). Lau *et al.* have shown the major role organic anion uptake transporters play for the clearance of atorvastatin and its active metabolites by the hepatobiliary system and concluded that inhibition of hepatic uptake may have consequences on efficacy and toxicity of drugs mainly eliminated by the hepatobiliary system (35,36).

In conclusion, AR-67 stability is much higher *in vivo* than *in vitro*. This discrepancy can be explained by the fact that the *in vivo* system is not closed. The interconversion and the irreversible elimination of both lactone and carboxylate affect the plasma concentration of the lactone and carboxylate at any given moment. In addition, the carboxylate concentration is formation rate limited, and the carboxylate moiety is eliminated as fast as it is formed. Because the lactone systemic clearance and the carboxylate-to-lactone conversion clearance are slower than the carboxylate systemic clearance, the lactone prevails. In summary, we studied the pharmacokinetics of the lactone and carboxylate forms of AR-67 and assessed, through clearance inhibition studies and simulations, the effect of clearance changes on plasma concentrations and AUCs of AR-67. Our findings demonstrate that the carboxylate clearance is the predominant factor affecting the disposition of AR-67 and that lactone and carboxylate clearances are dependent on efflux and uptake processes respectively.

ACKNOWLEDGMENTS

This work was supported in part by the National Institutes of Health (RCA123867A) and research grants from Arno Therapeutics.

REFERENCES

1. Bom D, Curran DP, Kruszewski S, Zimmer SG, Thompson Strode J, Kohlhagen G *et al.* The novel silatecan 7-tert-butyldimethylsilyl-10-hydroxycamptothecin displays high lipophilicity, improved human blood stability, and potent anticancer activity. J Med Chem. 2000;43:3970–80.
2. Pollack IF, Erff M, Bom D, Burke TG, Strode JT, Curran DP. Potent topoisomerase I inhibition by novel silatecans eliminates glioma proliferation *in vitro* and *in vivo*. Cancer Res. 1999;59:4898–905.
3. Arnold SM, Rinehart JJ, Tsakalozou E, Eckardt JR, Fields SZ, Shelton BJ *et al.* A phase I study of 7-t-butyldimethylsilyl-10-hydroxycamptothecin in adult patients with refractory or metastatic solid malignancies. Clin Cancer Res. 2010;16:673–80.
4. Fassberg J, Stella VJ. A kinetic and mechanistic study of the hydrolysis of camptothecin and some analogues. J Pharm Sci. 1992;81:676–84.
5. Jaxel C, Kohn KW, Wani MC, Wall ME, Pommier Y. Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: evidence for a specific receptor site and a relation to antitumor activity. Cancer Res. 1989;49:1465–9.
6. Hsiang YH, Liu LF. Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. Cancer Res. 1988;48:1722–6.
7. Burke TG, Mi Z. Preferential binding of the carboxylate form of camptothecin by human serum albumin. Anal Biochem. 1993; 212:285–7.
8. Liehr JG, Ahmed AE, Giovanella BC. Pharmacokinetics of camptothecins administered orally. Ann N Y Acad Sci. 1996; 803:157–63.
9. Creaven PJ, Allen LM, Muggia FM. Plasma camptothecin (NSC-100880) levels during a 5-day course of treatment: relation to dose and toxicity. Cancer Chemother Rep. 1972;56:573–8.
10. Natelson EA, Giovanella BC, Verschraegen CF, Fehir KM, De Ipolyi PD, Harris N *et al.* Phase I clinical and pharmacological studies of 20-(S)-camptothecin and 20-(S)-9-nitrocamptothecin as anticancer agents. Ann N Y Acad Sci. 1996;803:224–30.
11. Lalloo AK, Luo FR, Guo A, Paranjpe PV, Lee SH, Vyas V *et al.* Membrane transport of camptothecin: facilitation by human P-glycoprotein (ABCB1) and multidrug resistance protein 2 (ABCC2). BMC Med. 2004;2:16.
12. Nozawa T, Minami H, Sugiura S, Tsuji A, Tamai I. Role of organic anion transporter OATP1B1 (OATP-C) in hepatic uptake of irinotecan and its active metabolite, 7-ethyl-10-hydroxycamptothecin: *in vitro* evidence and effect of single nucleotide polymorphisms. Drug Metab Dispos. 2005;33:434–9.
13. Marchetti S, Oostendorp RL, Pluim D, van Eijndhoven M, van Tellingen O, Schinkel AH *et al. In vitro* transport of gimatecan (7-t-butoxyiminomethylcamptothecin) by breast cancer resistance protein, P-glycoprotein, and multidrug resistance protein 2. Mol Cancer Ther. 2007;6:3307–13.
14. Li H, Jin HE, Kim W, Han YH, Kim DD, Chung SJ *et al.* Involvement of P-glycoprotein, multidrug resistance protein 2 and breast cancer resistance protein in the transport of belotecan and topotecan in Caco-2 and MDCKII cells. Pharm Res. 2008;25:2601–12.
15. Adane ED, Monks N, Roberts MJ, Horn J, Moscow JA, Leggas M. Transporter Pathways for the Novel Camptothecin Analog DB-67. AAPS Abstract M1032, AAPS Workshop on Drug Transporters in ADME: From the Bench to the Bedside (2007).
16. Scott DO, Bindra DS, Stella VJ. Plasma pharmacokinetics of lactone and carboxylate forms of 20(S)-camptothecin in anesthetized rats. Pharm Res. 1993;10:1451–7.
17. Scott DO, Bindra DS, Sutton SC, Stella VJ. Urinary and biliary disposition of the lactone and carboxylate forms of 20(S)-camptothecin in rats. Drug Metab Dispos. 1994;22:438–42.
18. Davies BE, Minthorn EA, Dennis MJ, Rosing H, Beijnen JH. The pharmacokinetics of topotecan and its carboxylate form following separate intravenous administration to the dog. Pharm Res. 1997;14:1461–5.
19. Rivory LP, Chatelut E, Canal P, Mathieu-Boue A, Robert J. Kinetics of the *in vivo* interconversion of the carboxylate and lactone forms of irinotecan (CPT-11) and of its metabolite SN-38 in patients. Cancer Res. 1994;54:6330–3.
20. Ebling WF, Jusko WJ. The determination of essential clearance, volume, and residence time parameters of recirculating metabolic systems: the reversible metabolism of methylprednisolone and methylprednisone in rabbits. J Pharmacokinet Biopharm. 1986;14:557–99.
21. Cheng HY, Jusko WJ. Mean residence times and distribution volumes for drugs undergoing linear reversible metabolism and

tissue distribution and linear or nonlinear elimination from the central compartments. Pharm Res. 1991;8:508–11.
22. Horn J, Jordan SL, Song L, Roberts MJ, Anderson BD, Leggas M. Validation of an HPLC method for analysis of DB-67 and its water soluble prodrug in mouse plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2006;844:15–22.
23. D'Argenio DZ, Schumitzky A. ADAPT II User's Guide: Pharmacokinetic/Pharmacodynamic Systems Analysis Software. Biomedical Simulations Resource (1997).
24. Cheng H, Jusko WJ. Pharmacokinetics of reversible metabolic systems. Biopharm Drug Dispos. 1993;14:721–66.
25. Rowland M, Tozer TN. Clinical Pharmacokinetics: Concents and Applications, Williams & Wilkins (1995).
26. Mi Z, Burke TG. Differential interactions of camptothecin lactone and carboxylate forms with human blood components. Biochemistry. 1994;33:10325–36.
27. Jonker JW, Smit JW, Brinkhuis RF, Maliepaard M, Beijnen JH, Schellens JH *et al.* Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. J Natl Cancer Inst. 2000;92:1651–6.
28. Kruijtzer CM, Beijnen JH, Rosing H, ten Bokkel Huinink WW, Schot M, Jewell RC *et al.* Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. J Clin Oncol. 2002;20:2943–50.
29. Kuppens IE, Witteveen EO, Jewell RC, Radema SA, Paul EM, Mangum SG *et al.* A phase I, randomized, open-label, parallel-cohort, dose-finding study of elacridar (GF120918) and oral topotecan in cancer patients. Clin Cancer Res. 2007;13:3276–85.
30. Oostendorp RL, van de Steeg E, van der Kruijssen CM, Beijnen JH, Kenworthy KE, Schinkel AH *et al.* Organic anion-transporting polypeptide 1B1 mediates transport of Gimatecan and BNP1350 and can be inhibited by several classic ATP-binding cassette (ABC) B1 and/or ABCG2 inhibitors. Drug Metab Dispos. 2009;37:917–23.
31. Lau YY, Okochi H, Huang Y, Benet LZ. Pharmacokinetics of atorvastatin and its hydroxy metabolites in rats and the effects of concomitant rifampicin single doses: relevance of first-pass effect from hepatic uptake transporters, and intestinal and hepatic metabolism. Drug Metab Dispos. 2006;34:1175–81.
32. Zong J, Pollack GM. Modulation of P-glycoprotein transport activity in the mouse blood-brain barrier by rifampin. J Pharmacol Exp Ther. 2003;306:556–62.
33. Sparreboom A, Gelderblom H, Marsh S, Ahluwalia R, Obach R, Principe P *et al.* Diflomotecan pharmacokinetics in relation to ABCG2 421C>A genotype. Clin Pharmacol Ther. 2004;76:38–44.
34. Niemi M, Schaeffeler E, Lang T, Fromm MF, Neuvonen M, Kyrklund C *et al.* High plasma pravastatin concentrations are associated with single nucleotide polymorphisms and haplotypes of organic anion transporting polypeptide-C (OATP-C, SLCO1B1). Pharmacogenetics. 2004;14:429–40.
35. Lau YY, Huang Y, Frassetto L, Benet LZ. effect of OATP1B transporter inhibition on the pharmacokinetics of atorvastatin in healthy volunteers. Clin Pharmacol Ther. 2007;81:194–204.
36. Lau YY, Okochi H, Huang Y, Benet LZ. Multiple transporters affect the disposition of atorvastatin and its two active hydroxy metabolites: application of *in vitro* and *ex situ* systems. J Pharmacol Exp Ther. 2006;316:762–71.