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RESEARCH PAPER

Pharmacokinetic Modeling to Assess Factors Affecting the Oral Bioavailability of the Lactone and Carboxylate Forms of the Lipophilic Camptothecin Analogue AR-67 in Rats

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ABSTRACT

Purpose Camptothecin analogues are anticancer drugs effective when dosed in protracted schedules. Such treatment is best suited for oral formulations. AR-67 is a novel lipophilic analogue with potent efficacy in preclinical models. Here we assessed factors that may influence its oral bioavailability in rats.

Methods Plasma pharmacokinetic (PK) studies were conducted following administration of AR-67 lactone or carboxylate doses alone or after pre-dosing with inhibitors of the efflux transporters P-gp and Bcrp. A population PK model that simultaneously fitted to oral and intravenous data was used to estimate the bioavailability (F) and clearance of AR-67.

Results An inverse Gaussian function was used as the oral input into the model and provided the best fits. Covariate analysis showed that the bioavailability of the lactone, but not its clearance, was dose dependent. Consistent with this observation, the bioavailability of AR-67 increased when animals were pretreated orally with GF120918 or Zosuquidar.

Conclusion Absorption of AR-67 is likely affected by solubility of its lactone form and interaction with efflux pumps in the gut. AR-67 appears to be absorbed as the lactone form, most likely due to gastric pH favoring its formation and predominance. F increased at higher doses suggesting saturation of efflux mechanisms.

KEY WORDS BCRP· camptothecin · carboxylate · lactone · P-gp

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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

INTRODUCTION

AR-67 is a third generation camptothecin analogue that demonstrated potent antitumor activity in preclinical models (1). Thus, this congener is currently undergoing early phase clinical trials in patients with solid tumors (2). Camptothecins are a class of anticancer molecules that elicit their effect through interactions with topoisomerase I. This nuclear enzyme is typically expressed in all cells and performs its function during cell replication. As a direct consequence of this requisite interaction with topoisomerase I during cell replication, it has become apparent that camptothecin dosing schedules should be protracted to ensure that drug exposure is achieved, thereby increasing the potential for antitumor activity when different fractions of the tumor cell population enter the replication stage of their cell-cycle. Currently, there are only two camptothecin analogues, topotecan (Hycamtin®) and irinotecan (Camptosar®), available on the market while several others, including AR-67, are in various stages of development (2–4). In both rodents and humans, low dose protracted treatment by the intravenous route with topotecan or irinotecan was shown to be better tolerated and more efficacious than shorter and more intense therapies (5–7).

Although intravenous administration is typical for this class of compounds, an oral formulation may be more desirable for protracted dosing regimens. In addition, an oral formulation could reduce treatment cost while allowing for greater dosing flexibility (8). Oral topotecan has been shown to be as effective as intravenous docetaxel in patients with unresectable non-small-cell lung cancer (NSCLC) (9) and ovarian cancers (10). Similarly, in patients with solid tumors, oral irinotecan was well tolerated and advantageous in terms of providing enhanced tumor exposure to the active agent, SN-38, which is formed from irinotecan in a carboxylesterase-mediated presystemic conversion reaction (11–13).

A common feature of all camptothecins is the pH dependent reversible hydrolysis of the lipophilic lactone to the hydrophilic carboxylate (1,14). Following oral administration, the relative concentration of the lactone in relation to the carboxylate in the gastrointestinal tract is likely to be influenced by the local pH. Gastrointestinal regions with acidic pH are expected to maintain the lactone whereas those with physiological or alkaline pH should promote carboxylate formation. Since dissolution in the gastrointestinal tract influences absorption of orally administered solid dosage forms, differences in the aqueous solubility of the lactone and the carboxylate may in turn give rise to differences in oral bioavailability. Overall, the poor aqueous solubility of AR-67 lactone (0.11 μg/mL) (15) is expected to limit its oral bioavailability but improvement of AR-67 lactone solubility, through the use of excipients, could potentially overcome this limitation. A sulfobutylether-β-cyclodextrin based formulation of AR-67 that sustains a supersaturated solution concentration of the lactone *in vitro* (1–2 mg/mL) has previously been developed (15). Such a formulation could be potentially useful for oral and intravenous administration of AR-67. On the other hand, the more soluble carboxylate form may serve as an alternative solid oral dosage form with enhanced bioavailability because of its potentially superior dissolution in the gastrointestinal tract.

In addition to dissolution, efflux by ABC transporter proteins, such as P-gp and/or BCRP/Bcrp, located on the luminal side of gastrointestinal membrane is known to limit oral bioavailability of camptothecins (16,17). Published data on camptothecins (16,18) and previous *in vitro* studies in this laboratory (19) indicate that AR-67 and other camptothecins are substrates for P-gp and BCRP. This interaction is likely to limit the oral bioavailability of AR-67. Therefore, the objectives of the current study were to estimate the oral bioavailability of AR-67 lactone and AR-67 carboxylate and determine to what extent the major gastrointestinal efflux transporters (*i.e.*, P-gp and Bcrp) limit the oral bioavailability of AR-67.

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). Siliconized pipette tips were from Cole-Parmer (Vernon Hills, IL). Amber microcentrifuge tubes were from Crystalgen Inc. (Plainview, NY). Transparent siliconized microcentrifuge tubes, dimethylsulfoxide (≥99.7% DMSO) and glacial acetic acid were from Fisher Scientific (Fair Lawn, NJ). Magnesium- and calcium-free Dulbecco's phosphate buffered saline (PBS) was from Gibco Invitrogen (Carlsbad, CA). 5% Dextrose in water (D5W) was from Baxter Healthcare Corporation (Deerfiled, IL). Tween-80 and PEG-300 were from Sigma-Aldrich (St. Louis, MO). Sulfobutylether-β-cyclodextrin, sodium salt, with an average degree of substitution of 7 sulfobutyl ether residues per cyclodextrin molecule (SBE-β-CD, Captisol®) was from CyDex, Inc. (Overland Park, KS). AR-67 was provided from Novartis (East Hanover, NJ). The methods for preparation and lyophilization of the AR-67 formulations have been described previously (15,20). Briefly, a stock solution of AR-67 carboxylate was prepared by dissolving AR-67 lactone in NaOH (0.1 N). This solution was slowly added to 22.2% SBE-β-CD solution in water (w/v) buffered with 2 mM acetic acid to make AR-67 carboxylate of desired concentration. The SBE-β-CD to AR-67 ratio at was kept at 200:1 (w/w). AR-67 lactone solution was prepared by slow addition of HCl (0.1 N) (final pH≈4). AR-67 lactone or carboxylate solutions were lyophilized (15)and stored at −20°C until use. For animal studies, AR-67 lactone or carboxylate solutions (1–2 mg/mL) were prepared by reconstituting a lyophilized SBE-β-CD formulation of AR-67 (15) or from AR-67 powder as described above. GF120918 (Elacridar) was a gift from GlaxoSmithKline (Research Triangle Park, NC) and was solubilized in 10% Tween-80 and 40% PEG-300 in distilled water to make final concentrations of 0.03, 0.13, 0.33 and 2.7 mg/ml (21). The selective P-gp inhibitor, zosuquidar, was synthesized at the University of Kentucky following published procedures (22). The compound was dissolved in an aqueous solution of 20% SBE-β-CD. The purity of zosuquidar was 99.5% (23).

Pharmacokinetic Studies

Female Harlan Sprague–Dawley rats weighing between 220 and 300*g* were used for these studies ($n=3$–6). To assess the dose dependence of oral bioavailability, animals were orally gavaged SBE-β-CD based solutions of either AR-67

lactone or AR-67 carboxylate at 2.5, 5, 10, 15 or 20 mg/kg doses (15). For estimation of absolute bioavailability, separate groups of animals (n=3–6) were also treated with 2.5 mg/kg doses of either AR-67 lactone or AR-67 carboxylate intravenously. To determine the effect of P-gp inhibition on oral bioavailability, rats were orally pretreated with zosuquidar (20 mg/kg, 7.5 mL/kg) 5 min before the oral or IV administration of AR-67 lactone (2.5 mg/kg, 2.5 mL/kg). To measure the effect of dual inhibition of P-gp and Bcrp on oral bioavailability, GF120918 was administered by oral gavage 5 min before the oral administration of AR-67 lactone or carboxylate (2.5 mg/kg, 2.5 mL/kg). Different doses of GF120918 (0.25, 1, 2.5 or 20 mg/kg, 7.5 mL/kg) were used to select a dose that provided maximal efflux inhibition. This dose of GF120918 was then used to measure its effect on systemic clearance following intravenous administration of either AR-67 lactone or carboxylate. In order to assess contribution of possible factors affecting oral bioavailability of AR-67, the hepatic extraction ratio (E_H) and the theoretical maximum oral bioavailability (F) were estimated as shown below (24),

$$E_H = \frac{Cl_{iv}}{Q_{H^*}\left(\frac{C_B}{C_P}\right)} \quad (1)$$

$$F = 1 - E_H \quad (2)$$

where, Cl_{iv} is the plasma clearance of AR-67 lactone, which is 1,800 ml/h/kg (25). Q_H is hepatic blood flow, which is 13.8 ml/min for a 250 g rat (26). C_B/C_P is the blood to plasma concentration ratio of AR-67.

Following AR-67 administration, blood (100 μL) was collected from the saphenous vein at 5–15 min, 30 min and at 1, 2, 4, 6, 8 and 12 h with heparinized hematocrit tubes and transferred into heparinized microcentrifuge tubes. Plasma was separated by centrifugation of blood at 8,500g and extracted 1:4 (v:v) with cold (−80°C) methanol (27). Samples were stored at −80°C until analysis by HPLC.

A separate group of animals (n≤3) receiving an oral dose of 2.5 mg/kg AR-67 lactone were euthanized at designated time points to examine the presence of unabsorbed drug in the gastrointestinal tract. Different segments of the gastrointestinal tract were excised after ligations were made with sutures between the lower esophageal and pyloric sphincters (for the stomach), at 20 cm from the stomach (for the duodenum) and at 20 cm proximal to the ileocecal junction (for the ileum). The region between the duodenum and the ileum was cut in half and the two halves were designated as the proximal and distal jejunum. The region beyond the ileo-cecal junction was designated the colon. The stomach and intestinal contents were gently expelled and washed with 10–20 mL of 20% human plasma in water and transferred into 50 mL screw cap conical tubes. The mixture was vortexed for 30 s and centrifuged for 10 min at 1,200 rpm. The supernatant was extracted 1:4 (v:v) with −80°C methanol. Samples were kept at −80°C until HPLC analysis.

HPLC Analyses

Lactone and carboxylate plasma concentrations were simultaneously quantified by HPLC using fluorescence detection at an excitation wavelength of 380 nm and emission wavelength of 560 nm based on a previously published method for analysis of AR-67 in mouse plasma (27). A partial assay validation using rat plasma as the matrix was carried out. The assay was linear in the range of 2.5–250 ng/mL for the carboxylate and 5–300 ng/mL for the lactone. Accuracy was determined as% of nominal value from the average of 5–10 injections of quality control samples on four different days. For both analytes, accuracy was within 15% of expected values at the low end of the calibration curve (7 ng/mL) and within 10% of expected values at middle (150 ng/mL) and high (250 ng/mL) concentrations. Assay precision (% relative standard deviation) was <6% across the calibration range. Both analytes were stable at 4°C for 6 h after the methanol extract was mixed with mobile phase buffer. This ensured stability during automated HPLC analyses. Extracted samples were stable at −80°C for 14 days. The lower limit of quantitation was 2.5 ng/mL for carboxylate and 5.0 ng/mL for lactone. Sample analysis was completed within 14 days after sample collection.

Pharmacokinetic and Statistical Analysis

Plasma concentrations of AR-67 lactone and carboxylate were analyzed using non-compartmental and compartmental methods. Non-compartmental analysis was conducted with WinNonlin v5.2 (Pharsight, Mountain View, CA) to estimate areas under the plasma concentration *versus* time curve (AUC). A compartmental model previously developed for the intravenous administration of AR-67 lactone and carboxylate (25) served as the basis for the model used to estimate pharmacokinetic parameters following oral administration of each AR-67 form. Parameter estimates were obtained by simultaneously fitting the lactone and carboxylate plasma concentrations resulting from the oral and intravenous administration of AR-67. Population pharmacokinetic modeling was performed using the Iterated Two Stage (ITS) algorithm implemented in ADAPT 5 assuming log-normal parameter distribution (28). The model building was performed at

several stages with increasing levels of complexity. A reduction of the negative log-likelihood by 3.84 ($p=0.05$, χ^2 distribution, one degree of freedom) was used to discriminate between models. To assess the dose dependence of oral bioavailability, linear regression was performed on dose-normalized AUCs whereas areas under the plasma concentration *versus* time curves (AUC) from efflux transporter inhibition studies were compared with ANOVA followed by a Bonferroni two-tailed post-hoc *t*-test using GraphPad Prism V5.02 for Windows. A *p*-value of less than 0.05 was considered significant.

RESULTS

Pharmacokinetics of Orally Dosed AR-67 Lactone and AR-67 Carboxylate

Female Sprague Dawley rats were dosed orally with AR-67 lactone or AR-67 carboxylate formulated in SBE-β-CD. Plasma samples were analyzed for AR-67 lactone and carboxylate. In order to probe for the existence of a dose-dependent increase in oral bioavailability due to saturation of efflux transporters and/or metabolizing enzymes, increasing doses of the lactone or carboxylate were administered. Pharmacokinetic studies were conducted in groups of 3–6 animals, which were administered escalating doses of either the lactone or the carboxylate forms of AR-67 (*i.e.*, 2.5, 5, 10, 15, and 20 mg/kg doses). Multiple plasma samples were collected from each animal over 12 h. The initial pharmacokinetic analyses were performed using non-compartmental methods to assess the relative change in AUC with increasing AR-67 dose. Plasma AR-67 concentrations were primarily in the lactone form, irrespective of the AR-67 form being administered, and lactone AUCs ranged between 80 and 95% of the total AUC (*i.e.*, lactone + carboxylate). Maximum plasma concentration (C_{max}) was observed within 30 min at most dosage levels. Time to reach maximum concentration in the plasma (T_{max}) did not differ between the lactone and carboxylate dosing. The results of oral bioavailability studies at different dose levels analyzed by non-compartmental methods are summarized in Table I. The plasma concentrations and AUCs of the predominant lactone form were dose normalized and are presented in Fig. 1a–d. For clarity, the minor carboxylate AUC is not shown but follows a similar pattern as the lactone. A trend towards an increase in dose normalized AUC with an increase in dose was observed following lactone administration suggesting some degree of saturation of efflux transporters and/or metabolizing enzymes at the high dose levels (Fig. 1a–b). On the other hand, oral carboxylate administration did not show such a trend (Fig. 1c–d). Linear regression of the dosed normalized AUC values obtained following the administration of multiple lactone dosage levels *versus* dose (Fig. 1b) demonstrates that the slope of the fitted line deviates significantly from zero ($p<0.05$). In contrast, the dose normalized lactone AUCs obtained following increasing doses of carboxylate (Fig. 1d) were variable and no significant deviation from zero could be ascertained.

As opposed to a slope of zero, the positive slope of the line fitting the dose normalized AUCs obtained from increasing lactone AR-67 dosages suggests that increasing drug concentrations in the gastrointestinal tract saturate one or more of the processes that limit oral bioavailability.

Table I Pharmacokinetic Parameters Obtained from Non-Compartmental Analysis of Plasma Data in Animals that Received Oral Doses of the Lactone or Carboxylate

Compound administered	Dose, mg/kg	Tmax, min[a]	Cmax, ng/mL[a] (SD)	$AUC_{0\text{-}inf}$, ng*-*hr/mL (SD)		% Lactone AUC
				Lactone	Carboxylate	
Lactone	2.5	30–60	19.3 (11.3)	61.8 (22.3)	3.2 (2.5)	95.5 (2.5)
	5	30	79.0 (32.0)	145.4 (32.1)	34.8 (7.8)	81.7 (1.1)
	10	30	120.9 (12.1)	370.2 (40.2)	71.5 (15.5)	81.1 (2.9)
	15	30	263.8 (53.5)	680.8 (129.2)	216.6 (32.7)	82.3 (14.6)
	20	30–120	272.0 (24.4)	966.8 (259.1)	232.2 (38.1)	80.4 (1.4)
Carboxylate	2.5	30	25.2 (9.3)	72.7 (21.3)	13.4 (7.7)	88.1 (6.2)
	5	30–60	58.6 (25.4)	152.9 (30.1)	34.6 (15.2)	86.0 (3.4)
	10	30	116.3 (20.2)	279.5 (35.5)	60.8 (6.3)	82.2 (2.9)
	15	30	381.0(184.5)	956.7 (248.8)	233.8 (69.1)	80.0 (1.1)
	20	60	121.6 (83.4)	752.5(490.8)	67.1 (47.9)	91.5 (4.5)

[a] Denotes value obtained for the predominant lactone form

SD, standard deviation ($n=3$)

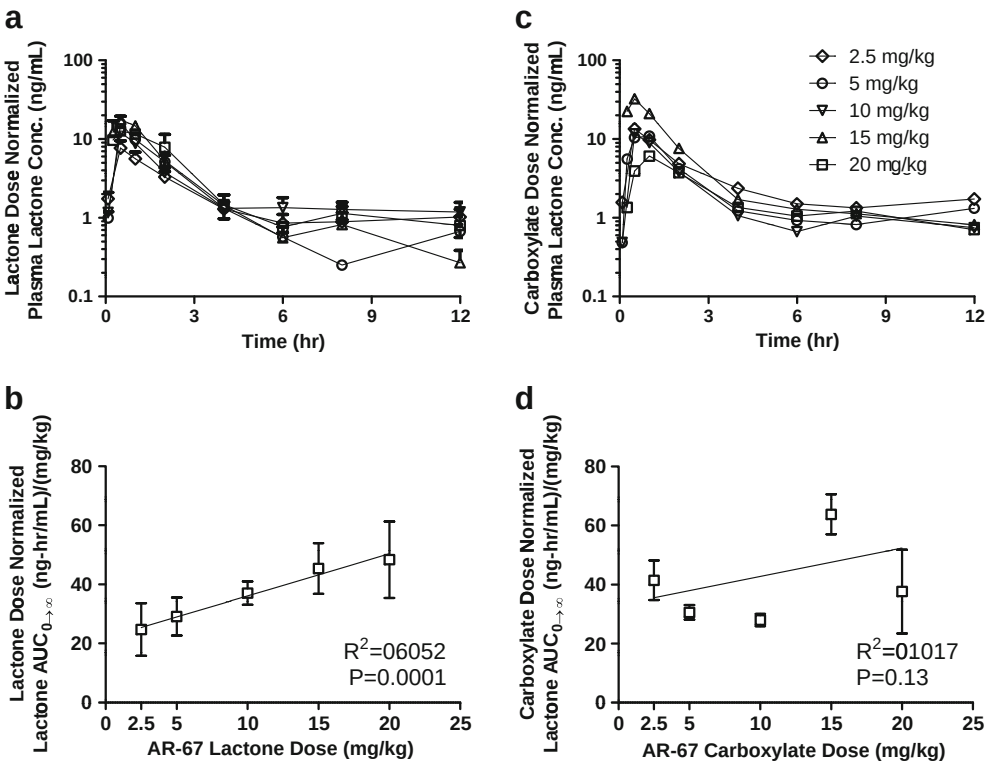

Fig. 1 Assessment of dose-dependence of plasma concentrations or AUCs following oral administration of increasing doses of AR-67 lactone or carboxylate. (**a**, **b**) Plasma concentrations and AUCs normalized by the dose of lactone administered. (**c**, **d**) Plasma concentrations and AUCs normalized by the dose of carboxylate administered. *P*-values indicate the significance of the slope's deviation from zero as obtained by linear regression ($n = 3$).

Thus, we sought to develop a pharmacokinetic model that would allow us to examine the effect of dose on oral bioavailability and clearance. The pharmacokinetic model would also allow the use of covariates to be imposed on the model parameters, which would provide a more robust statistical analysis when comparing the pharmacokinetic parameters obtained following pharmacologic inhibition of efflux transporters. First order absorption models were initially constructed to simultaneously model oral and intravenous data, but they did not adequately fit the data. Moreover, when the oral carboxylate dose was input into the carboxylate central compartment, the model fits were not satisfactory. Visual inspection of the lactone and carboxylate plasma concentrations over time suggested that AR-67 was mainly in the lactone form, irrespective of which form was orally administered. Therefore, based on this observation, a simplifying assumption was made to allow all oral inputs to be into the central lactone compartment (Fig. 2) and a flexible input function was used instead to accommodate the apparently complex absorption processes. The data were modeled by incorporating an inverse Gaussian input (29,30) into a four compartment disposition model, which was previously used to model intravenously administered AR-67 lactone and carboxylate (25). Following extravascular administration, the inverse Gaussian input model allows the estimation of absorption kinetic parameters for drugs with complex absorption behavior by decomposing the plasma concentration-time curve into input (absorption) and output (disposition) processes (29). The inverse Gaussian input function IG(t) is described by Eq. 3,

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

where F is oral bioavailability, MIT is the mean input time and CVI^2 is the variance or the relative dispersion of absorption times. Population modeling was performed using the Iterated Two Stage (ITS) algorithm implemented in ADAPT 5 assuming a log-normal parameter distribution (28). To estimate oral bioavailability of AR-67 at different dose levels, data from oral and intravenous inputs were simultaneously modeled using dose level and form administered orally, *i.e.* lactone or carboxylate, as covariates on F, MIT and CVI^2. To model the effect of efflux transporter inhibition with GF120918 on oral bioavailability and clearance, a similar modeling approach as above was followed. Oral and intravenous AR-67 data obtained from animals that were pretreated with either the control vehicle or the efflux inhibitor GF120918 were modeled using the

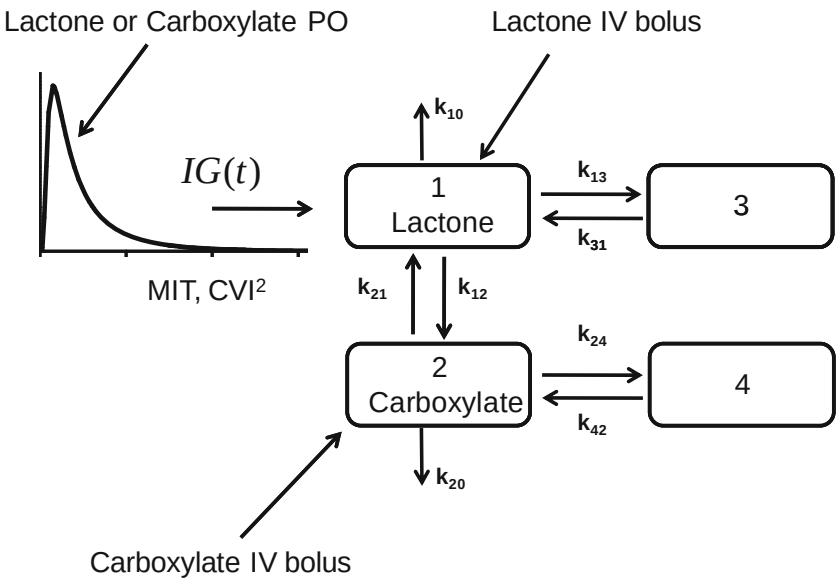

Fig. 2 Schematic representation of the inverse Gaussian oral input of AR-67 linked to a four compartment disposition model with elimination occurring from the central lactone and carboxylate compartments.

presence of GF120918 and the form of AR-67 administered orally as covariates on clearance, F, MIT and CVI^2. The performance of alternative methods was judged by convergence of parameter estimates, reduction in the negative log-likelihood, improvement in the error estimates of parameters and diagnostic plots. A reduction of the negative log-likelihood by 3.84 ($p=0.05$, χ^2 distribution, one degree of freedom) was used as a criterion to include a covariate in the model.

Oral data at different dose levels from this study and intravenous data from this and a previous study (25) were simultaneously analyzed using the Iterated Two Stage population algorithm in ADAPT 5 (28). This algorithm allows modeling of sparse and noisy population data (28) and was found appropriate for modeling the oral and intravenous data.

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

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

Literature data on other camptothecin analogs (16,18) and the dose dependent increase in oral bioavailability of AR-67 in the current study, suggested that intestinal efflux transporters, (*i.e.*, P-gp and/or Bcrp) may limit oral bioavailability of AR-67. In order to assess role of these efflux transporters, the selective P-gp inhibitor zosuquidar (31) and the dual P-gp and Bcrp inhibitor GF120918 (32,33) were used. Intravenous administration of 20 mg/kg zosuquidar was previously shown to effectively inhibit the function of P-gp at the blood–brain barrier as manifested by increased nelfinavir concentrations in the brain (23). Assuming that the same dose would provide much higher local concentrations in the GI and would effectively abolish P-gp function, rats were pretreated orally 5 min prior to AR-67 dosing. Pretreatment with zosuquidar resulted in a statistically significant ($p<0.05$) threefold increase in lactone AUC (ng-hr/mL) (61.8 ± 22.3 (Mean ± SD) with 5% dextrose in water *vs.* 183.7 ± 61.3 ng-hr/mL (Mean ± SD) with zosuquidar) (Fig. 6a). In the same animals, the

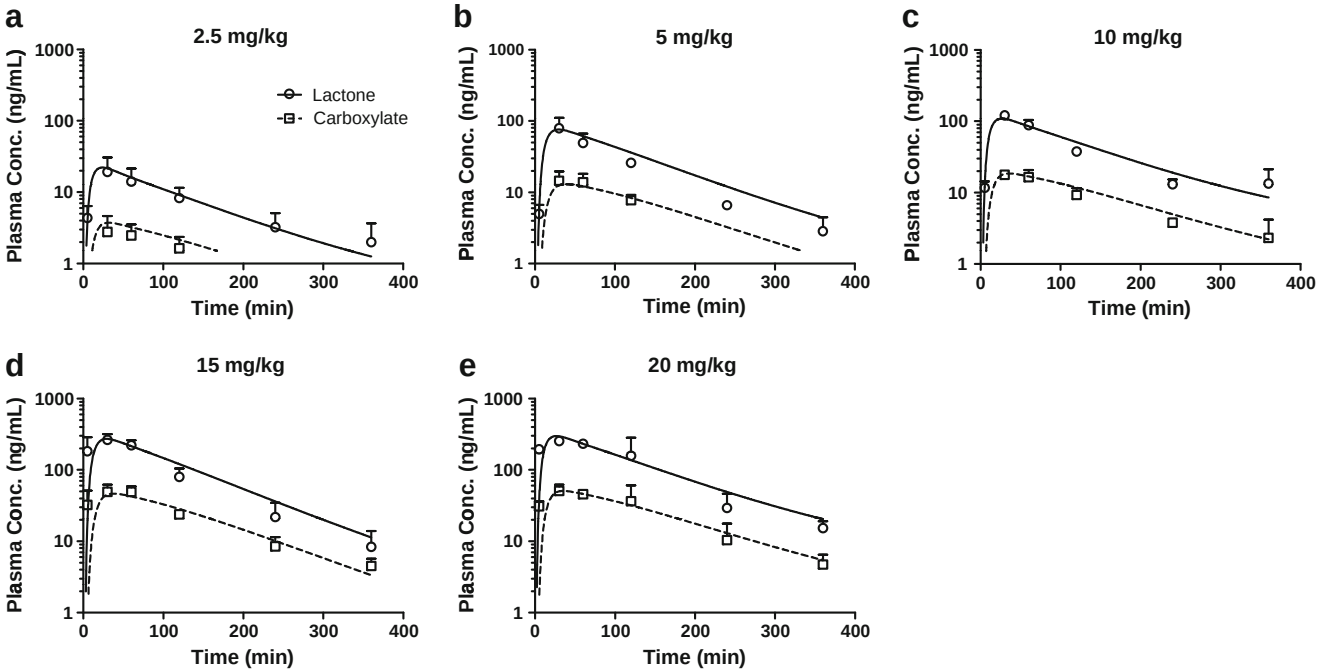

Fig. 3 Plasma concentrations of AR-67 lactone (—⊖) and carboxylate (—⊟—) following oral doses of (**a**) 2.5, (**b**) 5, (**c**) 10, (**d**) 15 and (**e**) 20 mg/kg AR-67 lactone. The *solid* and *dashed lines* represent simulated population estimated lactone and carboxylate concentrations, respectively, which were generated by simulation using the mean population parameter estimates.

carboxylate AUC (ng-hr/mL) increased nine-fold but these results were highly variable and given the sample size, this increase was not statistically significant (3.2±2.5 (Mean ± SD) without *vs.* 28.0±13.0 ng-hr/mL (Mean ± SD) with zosuquidar). Zosuquidar slightly increased the lactone AUC of intravenously administered AR-67 (1.2 fold, $p<0.05$)

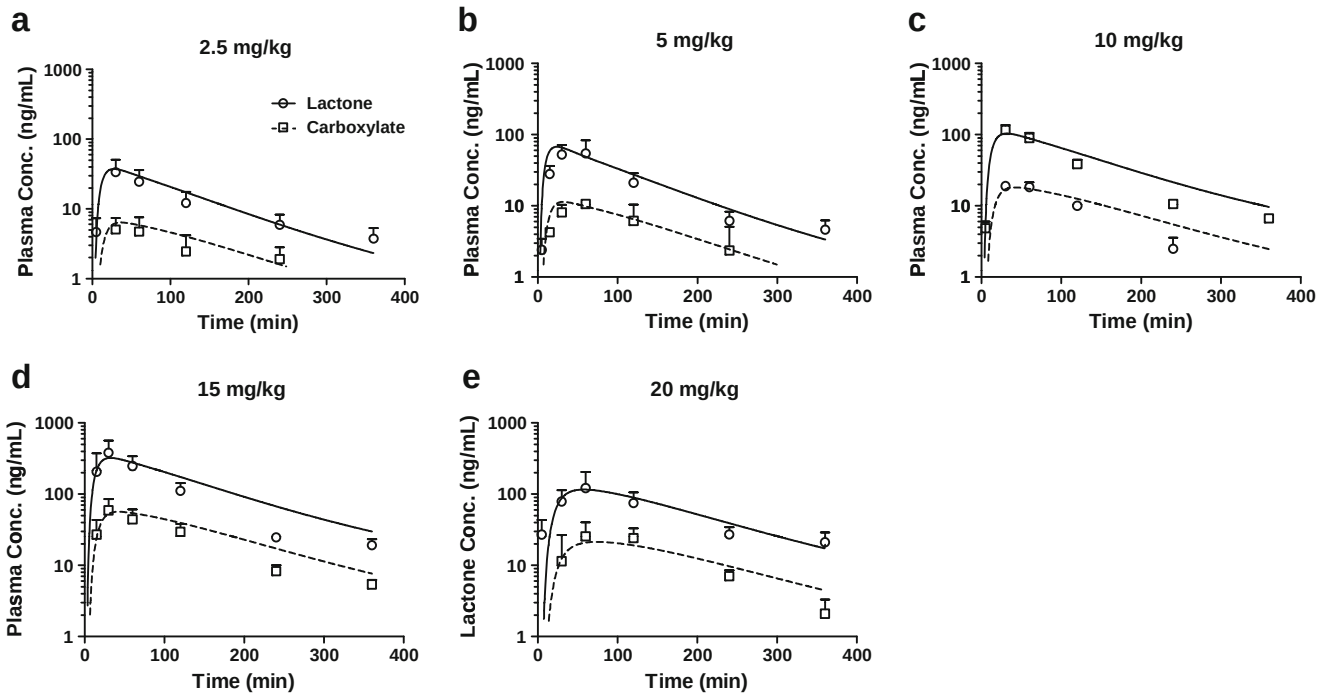

Fig. 4 Plasma concentrations of AR-67 lactone (⊖) and carboxylate (—⊟) following oral doses of (**a**) 2.5, (**b**) 5, (**c**) 10, (**d**) 15 and (**e**) 20 mg/kg AR-67 carboxylate. The *solid* and *dashed lines* are the fitted lactone and carboxylate concentrations, respectively, which were generated by simulation using the mean population parameter estimates.

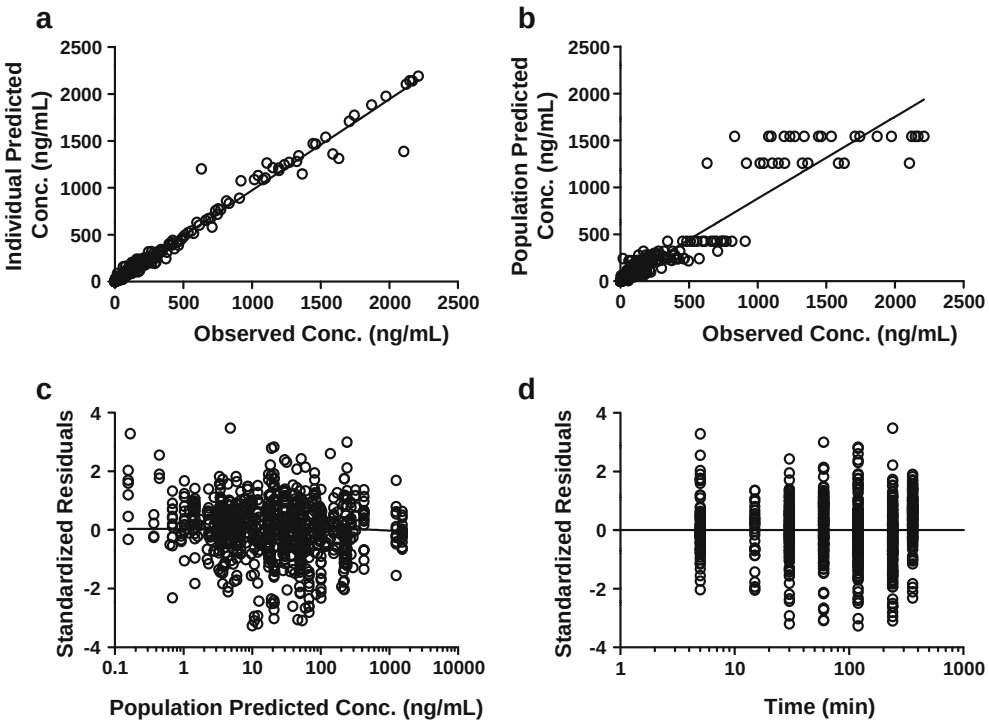

Fig. 5 Goodness-of-fit and residual plots of the final covariate model. (**a**) Individual predicted *versus* observed concentrations, (**b**) population predicted *vs.* observed concentrations, (**c**) standardized residuals *versus* population predictions, (**d**) standardized residuals *vs.* time. The *solid lines* in **a** and **b** represent the lines of unity.

indicating that, at the dose administered, it exerted a small but statistically significant effect on systemic clearance of AR-67 (Fig. 6b).

To examine the effect of dual P-gp and Bcrp inhibition, rats were pretreated with different oral doses of GF120918 (0.25, 1, 2.5 or 20 mg/kg) prior to the oral administration of AR-67 lactone or carboxylate. Due to poor aqueous solubility, GF120918 was formulated in 10% Tween-80 and 40% PEG-300 in distilled water (21). To avoid any excipient related effects in bioavailability, animals that did not receive GF120918 were predosed with an equal volume of 10% Tween 80 and 40% PEG-300 five min prior to receiving the AR-67 dose. As shown in Fig. 7a, the 2.5 mg/kg dose of GF120918 yielded the highest increase in plasma AUC value. The increase was statistically significant ($p<0.05$) compared to control AUC values, but not when compared to 1 mg/kg and 20 mg/kg GF120918 pretreatment doses. Pretreatment with 2.5 mg/kg oral dose of GF120918 5 min before the oral administration of AR-67 lactone, resulted in a 5.5 fold increase in lactone AUC (ng*-hr/mL) (141.5±57.1 *vs.* 779.6±163.3 (Mean ± SD) for control and GF120918, respectively) and about 11 fold increase in carboxylate AUC (13.2±5.6 *vs.* 142.4±29.6 (Mean ± SD) for control and GF120918, respectively). The increases in lactone and carboxylate AUCs were statistically significant ($p<0.05$). Similarly, pretreatment with 2.5 mg/kg oral dose of GF120918 5 min before the oral administration of AR-67 carboxylate increased lactone

Table II Pharmacokinetic Parameter Estimates Obtained by Compartmental Analysis of Plasma Data using the Inverse Gaussian Input

Parameter	Lactone dose (mg/kg)					Carboxylate dose (mg/kg)					% CV
	2.5	5	10	15	20	2.5	5	10	15	20	
F%	5.8	9.6	7.9	10.4	9.9	9.5	7.8	8.1	16.6	5.5	20.0
MIT (min)	111.0	87.0	259.0	66.6	128.0	100.0	141.0	200.0	174.0	153.0	42.5
CVI^2	4.4	2.4	8.8	1.9	4.2	3.1	3.4	5.3	4.4	1.8	53.4
Cl_{Lact} (L/h/kg)	1.53										54.0
Cl_{Carb} (L/h/kg)	5.53										10.8
$Cl_{Lact\rightarrow Carb}$ (L/h/kg)	1.39										12.0
$Cl_{Carb\rightarrow Lact}$ (L/h/kg)	0.98										19.4

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

AUC (ng-hr/mL) 4.2 fold (108.9±21.9 *vs.* 457.7±96.1 (Mean ± SD) for control and GF120918, respectively) and carboxylate AUC 5.2 fold (17.6±10.7 *vs.* 92.7±15.6 (Mean ± SD) for control and GF120918, respectively). The increases in lactone and carboxylate AUCs were statistically significant ($p<0.05$). As was the case in the studies without an inhibitor (Figs. 3 and 4), following pretreatment with GF120918, the majority of AR-67 was in the form of the lactone, irrespective of which form of AR-67 was administered (percent lactone AUC 83–91%).

To assess if the increase in lactone and carboxylate AUCs due to pretreatment with GF120918 was also related to a decrease in clearance, animals were pretreated with a 2.5 mg/kg oral dose of GF120918 prior to the intravenous administration of 2.5 mg/kg AR-67 lactone or carboxylate. In agreement with a previous study (25), GF120918 significantly increased ($p<0.05$) the AUC of lactone (1.7-fold and ~3-fold), but not that of the carboxylate; following intravenous administration of AR-67 lactone or carboxylate, respectively (Fig. 7b).

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

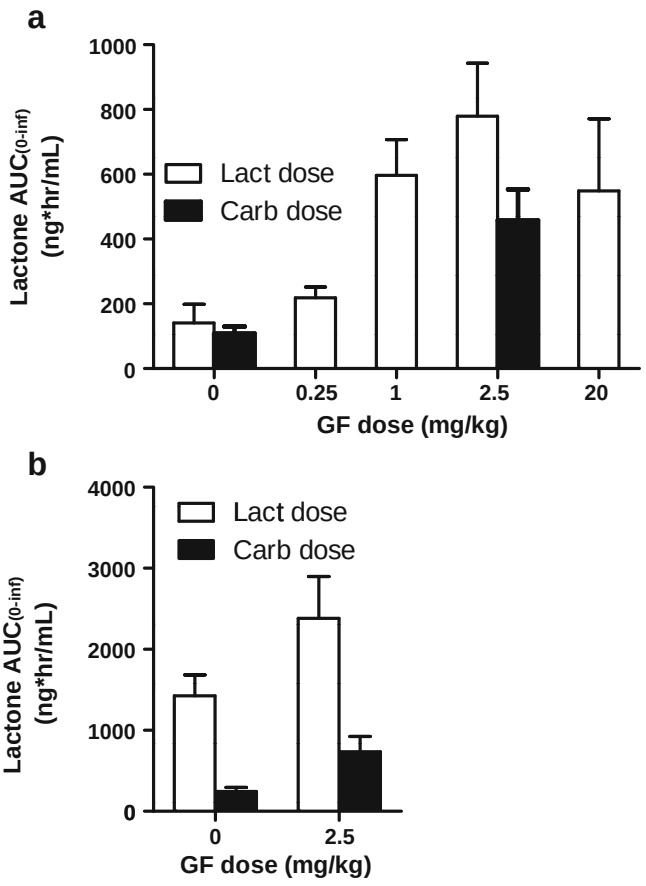

Fig. 7 The effect of pretreatment with GF120918 (GF) or control vehicle on lactone AUCs following the oral (**a**) or intravenous (**b**) administration of 2.5 mg/kg AR-67. *Unfilled bars* and *filled bars* indicate lactone and carboxylate, respectively, as the administered forms.

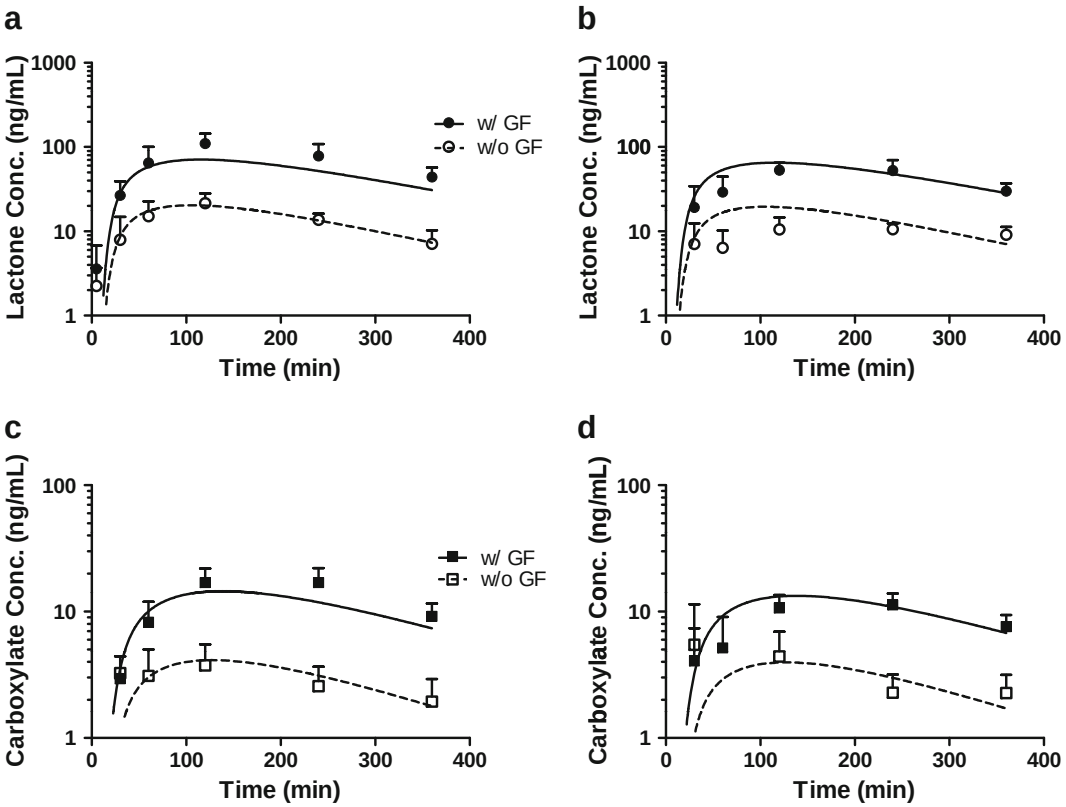

Fig. 8 Plasma concentration of AR-67 lactone (**a**, **b**) or carboxylate (**c**, **d**) in the presence or absence of GF120918 (GF). (**a**, **c**) Lactone and carboxylate concentrations, respectively, following lactone administration. (**b**, **d**) Lactone and carboxylate concentrations, respectively, following carboxylate administration. The *solid* and *dashed lines* represent simulated concentrations obtained using the point estimates of the population pharmacokinetic parameters in the presence or absence of GF120918, respectively.

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

Table III AR-67 Pharmacokinetic Parameter Estimates in Rats Orally Pretreated with Vehicle or 2.5 mg/kg GF120918 (GF) Before the Oral or Intravenous Administration of 2.5 mg/kg AR-67 Lactone or Carboxylate

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

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

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

Using Eqs. 1 and 2, the hepatic extraction ratio (E_H) and the theoretical maximum oral bioavailability of AR-67 were calculated in order to assess the contribution of factors that would limit oral bioavailability. The hepatic extraction ratio (E_H) and bioavailability (F) were 0.54 and 0.46 respectively. These values are based on a minimum value of blood to plasma ratio of AR-67 (C_B/C_P = 1), based on literature data showing that AR-67 partitions into red blood cells (34). As was presented earlier, efflux transporter inhibition increased oral bioavailability to about 30%. Since the theoretical minimum bioavailability is 46%, limited gastrointestinal solubility and/or metabolism were considered as additional factors that would limit the oral bioavailability of AR-67.

In order to examine the fate of the drug in the gastrointestinal tract and the presence of metabolites, we dosed rats orally with 2.5 mg/kg AR-67 lactone. The% of AR-67 dose recovered in the washing fluid (20% human plasma) from the contents of the stomach, small intestine, and colon as well as the cumulative% remaining in the GI tract is presented in Fig. 9. The estimated half-life in the stomach was approximately 4.7 h while in the small intestine the estimated half-life was approximately 1 h. The total recovery at the initial time points was approximately 50%, which suggests that the extraction efficiency of the washing buffer was limited and these values represent a potential 2-fold underestimation of the actual amount remaining in the gastrointestinal tract. Thus, it is possible that 5–10% of the dose still remained in the stomach at the 12 h time point, while ~20–40% of the dose could be present in the GI tract between 6 and 12 h after oral dosing.

DISCUSSION

In this *in vivo* study the bioavailability of AR-67 following administration of increasing doses of the lactone and carboxylate forms were compared. Furthermore, the effect of ABC efflux transporters, P-gp and Bcrp, on the oral bioavailability of AR-67 lactone and AR-67 carboxylate was examined. Bioavailability estimates ranged between 4 and 17% and did not differ between lactone and carboxylate doses. The lactone form predominated in the plasma following the oral administration of both AR-67 lactone and AR-67 carboxylate as shown by plasma lactone AUCs, which accounted for greater than 83% of the total plasma AUC. When the lactone was dosed intravenously, lactone AUC accounted for 84% of the total AUC, while it accounted for only 22% of the total AUC following intravenous carboxylate administration (25). Percent lactone AUCs are, therefore, similar following the oral and intravenous administration of the lactone form but different when the carboxylate is administered. A possible explanation for this discrepancy in percent lactone AUC between oral and intravenous carboxylate administration could be that only the lipophilic lactone form was absorbed from the gastrointestinal tract. In a study by Scott *et al.* (35) less than 1% of the administered dose was absorbed following intraduodenal administration of sodium camptothecin (carboxylate) dissolved in bile suggesting that carboxylate absorption is minimal. The carboxylate form of AR-67 is a substrate of the liver specific organic anion uptake transporters OATP1B1 and

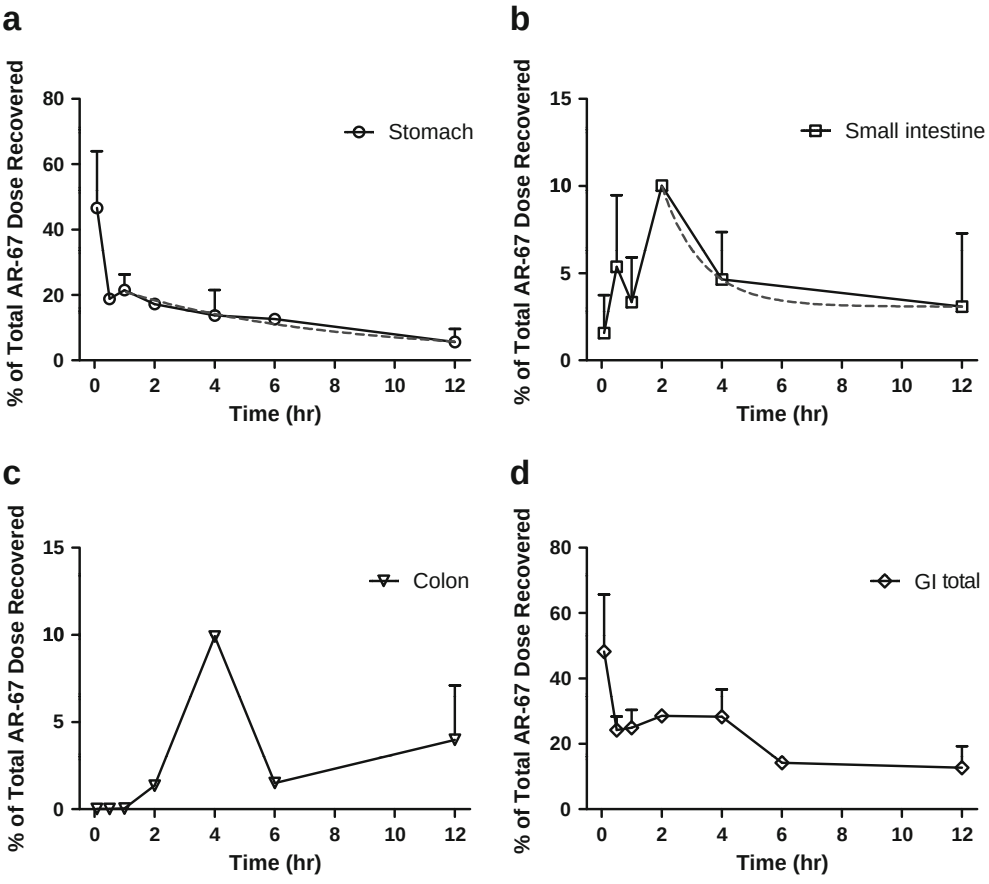

Fig. 9 Total AR-67 recovered, as a% of orally administered dose, from contents of (**a**) stomach, (**b**) small intestine, (**c**) colon and (**d**) the total dose recovered in the gastrointestinal tract (GI) following an oral dose of 2.5 mg/kg AR-67 lactone. *Dotted lines* represent the data fit with a monexponential decay model to estimate the half-life in the stomach and small intestine. ($n = 2$–3 rats per time point). (Recommend removing the *dotted lines*).

OATP1B3 (19). Therefore, the predominance of the lactone in the plasma following oral carboxylate administration could also be related to the selective uptake of the carboxylate into the liver by these uptake transporters. Thus, if carboxylate was absorbed, it would have been rapidly taken up and then cleared by the liver and very little would have been available in the systemic circulation in the form of the carboxylate. This is plausible based on results from a previous study, which determined that carboxylate clearance is more than 5-fold higher than the carboxylate to lactone conversion clearance (5.5 ± 0.6 *vs.* 0.98 ± 0.19 L/h/kg). However, since the plasma exposures (AUCs) following oral lactone and oral carboxylate administration were practically identical, carboxylate to lactone conversion in the gut, especially at the acidic pH in the stomach, and subsequent lactone absorption, is the most likely explanation for this observation across several doses, which spanned an order of magnitude (*i.e.*, 2.5 mg/kg–20 mg/kg). As shown by Xiang and Anderson (15) the half-life for the carboxylate to lactone conversion is ~0.5 h (first-order k parameter for ring closing is estimated to be 1.19 h^{-1}) at pH 4.0 and even faster conversion may occur in the rat stomach (pH 3) in the fed and 4 in the fasted states (36). Thus, it is plausible that >90% of carboxylate is converted to the lactone in the stomach, since the transit time in this compartment is about 4.7 h. Although, the carboxylate derived lactone in the stomach should convert back to carboxylate as it transits into the slightly basic pH environment of the small intestine, this process may be sufficiently slow to allow for lactone absorption.

Limited dissolution of lipophilic drugs in the gastrointestinal tract is known to limit bioavailability of orally administered drugs (37). The aqueous solubility of AR-67 is 0.11 μg/mL at pH 5.2 and ≈18 mg/mL at pH 10.2 (15). At pH 5.2 AR-67 is primarily in the lactone form whereas at pH 10.2 it is predominantly in the carboxylate form (15). Based on aqueous solubility and membrane permeability alone, AR-67 lactone and carboxylate would belong to two different classes. Under the Biopharmaceutics Drug Disposition Classification (BCS) System, the lactone would be classified as a Class II drug given its low aqueous solubility and high membrane permeability and the carboxylate as a class III drug based on its high aqueous solubility and low membrane permeability (38). This means that under conditions that favor the predominance of the lactone in the gastrointestinal tract, bioavailability would be amenable to improvement by use of formulations that enhance dissolution. In this study, the formulation of Xiang & Anderson (15) that provides a supersaturated lactone solution was employed. While this formulation was able to maintain supersaturation *in vitro*, it may not have done so *in vivo*. This formulation is prepared through a pH regulated chemical conversion of the carboxylate in the presence of a sulfobutylether-β-cyclodextrin (SBE-β-CD). AR-67 lactone forms a predominantly 1:1 complex with SBE-β-CD, which involves inclusion of the 7-t-butyldimethylsilyl residue in the SBE-β-CD core (15). The carboxylate also forms a 1:1 complex, the formation constant for which is an order of magnitude less than that of the lactone complex (15). It is highly likely that membrane absorption of AR-67 upon administration in the GI occurs after dissociation from the complex as a result of dilution of the drug formulation by the fluid content of the gastrointestinal tract (7.8 ± 1.5 ml (fed) and 3.2 ± 1.8 ml (fasted) rats) (36). Therefore, the stability of the complex is likely to have an effect on oral bioavailability. This study did not assess the membrane transport of the lactone and carboxylate forms of AR-67 and should not be interpreted as suggesting that only the lactone form undergoes membrane transport. Infusion studies in isolated gastrointestinal segments that measure membrane transport as a function solution pH could examine this. More studies are, however, needed to examine if and how much complexation affects the oral bioavailability of AR-67.

Examination of luminal contents showed the presence of a significant portion of AR-67 in the stomach and in the colon. This could have resulted from efflux by ABC transporters and/or from the limited gastrointestinal solubility of AR-67 lactone. Taken together, these data indicate that ABC efflux limits the bioavailability of AR-67. Due to their apical expression in the lumen of the gastrointestinal tract, ABC efflux transporters P-gp and BCRP/Bcrp limit oral bioavailability of camptothecin analogs and their inhibition has been shown to increase oral bioavailability (16, 17). The threefold increase in oral bioavailability of AR-67 when animals were predosed with GF120918 demonstrates the involvement of efflux transporters P-gp and Bcrp in limiting the oral bioavailability of AR-67. Since we have previously demonstrated the lactone to be a substrate of P-gp and BCRP *in vitro* (19), the increase in bioavailability upon lactone administration in the presence of inhibitors is likely to be due to inhibition of P-gp and BCRP. On the other hand, the increase in bioavailability in the presence of GF120918 following carboxylate administration could have resulted from inhibition of lactone efflux and/or carboxylate efflux (provided that the carboxylate is a substrate of efflux transporters). Whether or not the carboxylate is also a substrate of P-gp and/or Bcrp has not yet been established and more work needs to be done in this regard.

The absorption of AR-67 is a complex process due to the pH dependent lactone to carboxylate interconversion and interaction with efflux transporters, P-gp and Bcrp. The predominant form of AR-67 depends on the local pH as

well as the membrane binding affinity of AR-67 lactone and AR-carboxylate. However, this study did not measure the local concentration of AR-67 lactone and carboxylate nor the site of predominant AR-67 absorption. The expression of P-gp and Bcrp increases along the gastrointestinal tract (39). Thus, the effect of P-gp on the lactone form, which is likely to have a higher permeability, may be minimal in the upper GI tract. However, the effect of transporter mediated efflux is the sum of P-gp and/or Bcrp efflux taking place at all potential absorption sites in the gastrointestinal tract. Therefore, the low P-gp expression in the upper gastrointestinal tract, did not lead to absence of transporter effect overall when determining oral bioavailability of AR-67 lactone.

Poor gut solubility might play a role and could magnify the effect of efflux transporter(s) in that enterocyte concentrations coming from the gut lumen would not be sufficient to saturate efflux transporters (40) partly explaining the increase in bioavailability observed with efflux inhibition. The results of ABC transporter inhibition studies are in line with other studies demonstrating that oral bioavailability of camptothecin analogues is limited by ABC transporters and that inhibition of transporter function leads to improvement in oral bioavailability. Co-administration of topotecan and GF120918 by the oral route, increased plasma AUC of total topotecan more than six fold in P-gp knockout mice and greater than nine fold in wild-type mice compared with their respective control treated P-gp knockout and wild type mice (16). This increase is not only due to gastrointestinal efflux inhibition but also due to decreased systemic clearance (16). Similarly, in cancer patients GF120918 increased the bioavailability of topotecan 2.4 fold (40 to 97%) (41). The study, however, did not consider the effect of GF120918 on the systemic clearance of topotecan. Therefore, the increase in bioavailability is likely to be due to decreased gastrointestinal efflux as well as decreased systemic clearance of topotecan. An animal study using gefitinib as the ABC transporter inhibitor showed that a single dose of 100 mg/kg led to a 3.5 fold increase in the oral bioavailability of irinotecan in mice (25% in control *versus* 87% with gefitinib) (42). In another study (18), gefitinib (100 mg/kg) increased the bioavailability of topotecan in Bcrp knockout mice about 2.1 fold compared to wild-type animals (22% to 47%). Similarly, the same dose of gefitinib increased bioavailability about 1.7 fold (30% to 50%) in Mdr1 knockout animals compared to Mdr1 wild-type animals (18). The increase in bioavailability was related to both gastrointestinal efflux transporter inhibition and reduced systemic clearance (18).

The increase in oral lactone and carboxylate AUCs that was observed with GF120918 pretreatment (Table III) was not solely due to the effect of the inhibitor. GF120918 was solubilized in an aqueous solution of 10% Tween 80 and 40% PEG-300. Both Tween 80 and PEG-300 increase oral bioavailability of lipophilic drugs through improved solubilization and/or inhibition of efflux transporters located in the gastrointestinal tract (43–45). However, as mentioned earlier in the results section, this excipient factor was taken into consideration by using the vehicle as a control when analyzing the GF120918 results. The AUCs resulting from pretreatment with the GF120918 vehicle were higher than those in animals that were not pretreated with excipients. These higher AUCs may have resulted from the effect of these formulation excipients on AR-67 solubility, delayed gastric emptying, and/or inhibition of Bcrp; as was previously demonstrated in mice receiving topotecan (46). In that study (46), Tween-20 increased the topotecan AUC in wild-type animals, but had little effect on oral AUC of topotecan in Bcrp knockout animals or on the AUC of intravenously administered topotecan (46). Inhibition of Bcrp mediated efflux in the gastrointestinal tract was found to be responsible for the increase (46).

The magnitude of oral bioavailability observed in rats may not necessarily reflect the bioavailability in other models. For example, pharmacokinetic studies of AR-67 in mice have shown that the bioavailability is ~25% (Adane and Leggas, unpublished data), which is similar to the bioavailability of topotecan in mice (16,18). Interestingly, these preclinical estimates of the oral bioavailability of topotecan represent an underestimation of the oral bioavailability (34–45%) observed in pediatric patients and in adults (41, 47). Although our preclinical data may not ultimately correlate with the magnitude of bioavailability in other species, our rat studies have provided an understanding of factors affecting the oral bioavailability of AR-67 and in relative terms these factors should be similar across species.

In summary, the oral bioavailability of AR-67 appears to be limited by several factors. In addition to efflux by ABC transporters, HPLC analysis of gastrointestinal contents indicated the presence of AR-67 in the gastrointestinal tract long after its oral administration, which suggests that the drug may have precipitated. In a recent *in vitro* study, AR-67 was shown to be a substrate of CYP450 and UGT enzymes (48). Therefore, first pass metabolism could also be responsible for limiting oral bioavailability. Further studies are required to quantify the effect of first pass metabolism on the oral bioavailability of AR-67. Furthermore, pretreatment with an Oatp inhibitor prior to the oral administration of the carboxylate could help rule out the contribution of carboxylate uptake by Oatp as a reason for the predominance of the lactone form following oral administration of the carboxylate.

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