Metabolic Pathways of the Camptothecin Analog AR-67

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ABSTRACT:

7-*tert*-Butyldimethylsilyl-10-hydroxycamptothecin (AR-67; also known as DB-67) is a novel lipophilic camptothecin analog in early-phase anticancer clinical trials. In support of these studies, we evaluated the metabolism of AR-67 in vitro and identified potential metabolites in patient samples. The lactone form of AR-67 was found to be preferentially metabolized over AR-67 carboxylate in human microsomes. Subsequently, the lactone form was tested as a substrate in a panel of CYP450 and UDP-glucuronosyltransferase (UGT) enzymes known to metabolize the majority of clinically approved molecules. AR-67 was metabolized by CYP3A5, CYP3A4, CYP1A1, and CYP1A2, in order of activity. Extrahepatic UGT1A8 and UGT1A7 possessed at least 6-fold higher metabolizing activity than UGT1A1 and other UGT en-

zymes tested. CYP1A1 and UGT1A7 displayed Michaelis-Menten kinetics, whereas CYP3A4, CYP3A5, and UGT1A8 displayed kinetics consistent with substrate inhibition. Chromatographic analysis of representative patient plasma and urine samples demonstrated the presence of AR-67 glucuronides and oxidized products in the urine but only in very minimal amounts. We conclude that limited in vivo metabolism of AR-67 by UGT1A1 may partly explain the absence of AR-67 glucuronides in plasma and hypothesize that UGT1A8- and CYP3A-mediated biotransformation within the gastrointestinal epithelium may provide protective mechanisms against AR-67 gastrointestinal toxicity.

Introduction

7-*tert*-Butyldimethylsilyl-10-hydroxycamptothecin (AR-67; also referred to as DB-67) is a third-generation camptothecin analog with improved lactone stability and cytotoxicity compared with the U.S. Food and Drug Administration-approved camptothecins, irinotecan (Camptosar) and topotecan (Hycamtin) (Bom et al., 2000, 2001). Camptothecins (CPTs) are S phase-specific topoisomerase I poisons with potent anticancer activity. Since the isolation of the camptothecin molecule from the bark of the oriental tree, *Camptotheca acuminata*, the clinical development of CPTs has been hampered by the need to preserve the E-ring hydroxylactone, which is required for cytotoxicity (Hsiang et al., 1989; Giovanella et al., 2000). For most CPTs, this lactone moiety undergoes reversible hydrolysis at physiological pH to form an apparently inactive carboxylate (Fig. 1). Thus, the overall anticancer activity of CPTs depends, in part, on the dynamics of their individual lactone-carboxylate equilibrium. To some extent, lactone stability of the analogs depicted in Fig. 1 has been credited to structural modifications of the camptothecin backbone. In particular,

the $-R_3$ hydroxyl group reduces carboxylate binding affinity to human serum albumin (Burke et al., 1995) and thus retards the hydrolysis process by eliminating this sink condition. In the case of AR-67, the increased lipophilicity afforded by its $-R_1$ *tert*-butyldimethylsilyl group also contributes to lactone protection via increased partitioning into lipid membranes (Burke et al., 1993). The cumulative result is a drug that displays potent anticancer activity (Pollack et al., 1999), with a high lactone AUC/total AUC ratio in vivo ($87.5 \pm 8.5\%$) (Arnold et al., 2010) compared with other clinically approved CPTs (lactone AUC ratios of 30–76%) (Zamboni et al., 1999; van Riel et al., 2002). Recent work from our laboratory has also demonstrated that the systemic clearance of the carboxylate form is 3.5-fold higher than that of the lactone, which ultimately leads to the apparently high lactone stability in plasma (Adane et al., 2010). Whether the high carboxylate clearance is mediated by enzymes or transporters remains to be investigated.

In addition to nonenzymatic lactone hydrolysis, a number of pathways have been established in the metabolism of clinically approved camptothecins in vivo. Primary and secondary *N*-demethylated and *O*-glucuronidated metabolites of topotecan have been isolated from the plasma and urine of patients at low levels but have not been implicated in its pharmacodynamics or toxicity (Herben et al., 1998; Rosing et al., 1998). In contrast, the metabolic pathways of CPT-11 (irinotecan) are more complex. CPT-11 can undergo metabolic activation by carboxylesterases to form the 100- to 1000-fold more potent

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ABBREVIATIONS: AR-67, 7-*tert*-butyldimethylsilyl-10-hydroxycamptothecin, DB-67; CPT, camptothecin; AUC, area under the curve; CPT-11, irinotecan; SN-38, 7-ethyl-10-hydroxycamptothecin; UGT, UDP-glucuronosyltransferase; P450, cytochrome P450; SPE, solid-phase extraction; DMSO, dimethyl sulfoxide; QC, quality control; HPLC, high-pressure liquid chromatography; MS, mass spectrometry; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; HLM, human liver microsome(s); HIM, human intestinal microsome(s); MS/MS, tandem mass spectrometry; GI, gastrointestinal; OAT, organic anionic transporting polypeptide; ABC, ATP-binding cassette; BCRP, breast cancer resistance protein.

FIG. 1. Structures of representative camptothecins and the reversible equilibrium of their E-ring hydroxylactones. The lactone forms of camptothecins exist in equilibrium with their carboxylates. This pH-dependent equilibrium is significantly shifted for those camptothecins with high carboxylate affinity for human serum albumin (Burke et al., 1995).

SN-38 metabolite (Kawato et al., 1991; Tanizawa et al., 1994). Moreover, CPT-11 undergoes sequential metabolism by members of the CYP3A family to form inactive ring-opened piperidine metabolites (Dodds et al., 1998). *O*-Glucuronidation by hepatic UDP-glucuronosyltransferases (UGTs), in particular UGT1A1, deactivates the potent SN-38 metabolite (Rivory and Robert, 1995; Rosing et al., 1998) and, together with hepatic organic anionic transport (Sugiyama et al., 1998) and diffusion of SN-38, provides the primary routes for SN-38 elimination. Accumulation of the highly cytotoxic SN-38 metabolite in the intestines and microfloral hydrolysis of the SN-38 glucuronide (Takasuna et al., 1996) is thought to be associated with the life-threatening diarrhea manifested in up to 25% of patients receiving moderate- to high-dose CPT-11 therapy (Kawahara, 2006; Hu et al., 2010). Thus, polymorphisms that impair the function of enzymes and transporters responsible for metabolizing and shuttling CPT-11 and its metabolites have an impact on the overall exposure of patients to the active SN-38 metabolite and contribute to interpatient variability in SN-38 kinetics, dynamics, and toxicity (Rouits et al., 2008; Cecchin et al., 2009; Innocenti et al., 2009).

Given the structural similarities of AR-67 to other CPTs (Fig. 1), it was hypothesized that AR-67 would undergo metabolic glucuronidation and oxidation by the same enzymes responsible for their metabolism. Moreover, previous experience with SN-38 suggested that individual differences in AR-67 metabolic clearance, particularly via glucuronidation, may lead to severe delayed gastrointestinal toxicity in selected patients, beyond that typically exhibited by patients receiving CPT therapies. However, patients treated with AR-67 in the first phase I clinical trial did not experience diarrhea, even at doses exceeding the maximum tolerated dosage level, prompting us to evaluate the metabolism of AR-67. Thus, in these studies we investigated the *in vitro* metabolism of AR-67 by microsomes and select UGT and P450 enzymes. In addition, patient plasma and urine samples were analyzed for the presence of potential metabolites.

Materials and Methods

Materials. AR-67 of >98% purity was obtained from Novartis Pharmaceuticals Corporation (East Hanover, NJ). General reagents and solvents of ACS grade or higher were purchased from various laboratory supply vendors. Pooled human liver microsomes, human intestinal microsomes, Gentest insect and UGT control Supersomes, Gentest Supersomes of human UGTs (1A1, 1A3, 1A4, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17), and Supersomes of human P450s (1A1, 1A2, 2C8, 2C9*1, 2C19, 2D6*1, 3A4, and 3A5 containing cytochrome *b₅*, when available) as well as all buffers, cofactors, and reagents necessary for *in vitro* metabolism assays were obtained from BD Biosciences (San Jose, CA). β -Glucuronidase type B-I (1,240,000 units/g) was

acquired from Sigma-Aldrich (St. Louis, MO), and Waters Oasis HLB solid-phase extraction (SPE) columns were purchased from Thermo Fisher Scientific (Waltham, MA).

AR-67 Working Solution, Calibrator, and Quality Control Sample Preparation. Stock solutions of AR-67 were prepared in dimethyl sulfoxide (DMSO) and stored in aliquots at -80°C . Various concentrations of AR-67 lactone and carboxylate working solutions were separately prepared on ice by dilution with 0.005 M HCl or 0.005 M NaOH, respectively. In all cases, the lactone working solutions were prepared and used immediately, whereas carboxylate working solutions were equilibrated on ice for 1 h before use (Horn et al., 2006). The final DMSO concentration in working solutions was adjusted to 4%. AR-67 is a highly protein-bound, highly lipophilic drug with relatively low aqueous solubility (Bom et al., 2000; Xiang and Anderson, 2002). As a direct consequence, total protein content in all samples was kept constant at 0.25 mg/ml (except in range-finding enzymatic experiments), as was the total DMSO content in reaction mixtures (0.2%). The low solubility of AR-67 lactone limited the total AR-67 concentrations to 20 μM in enzymatic reactions.

For chromatographic analyses, calibrators and quality control (QC) samples were generated by the addition of working solutions of AR-67 lactone or carboxylate to buffered blank Supersomes (0.25 mg of total protein/ml), plasma, or urine. Calibrators contained 0, 5, 10, 30, 50, 100, 200, or 300 ng/ml amounts of each analyte, whereas QC samples contained 7, 150, or 250 ng/ml amounts of each analyte. Calibrators and QC samples were processed as indicated below and analyzed by HPLC.

Sample Processing and HPLC Analysis. To quench reactions and precipitate proteins, all samples were mixed with a 4 $\times$ volume of dry ice-cooled methanol (-80°C), vortexed (10 s), and centrifuged (2 min, 13,000 rpm, 4°C), and the methanolic supernatant was stored at -80°C . For HPLC analysis, methanolic supernatants were diluted with an equivalent volume of 0.15 M ammonium acetate buffer containing 10 mM tetrabutylammonium dihydrogen phosphate (pH 6.5) before a 50- μl injection (Horn et al., 2006).

HPLC analysis was performed on a Shimadzu liquid chromatography system (Shimadzu Inc., Atlanta, GA) equipped with a guard-protected Nova-Pak C18 column (4 μm ; 3.9 $\times$ 150 mm) using gradient elution. The mobile phase (1 ml/min flow rate) consisted of varying ratios of solvent A (0.15 M ammonium acetate buffer containing 10 mM tetrabutylammonium dihydrogen phosphate adjusted to pH 6.5) and solvent B (acetonitrile). The A/B solvent ratio started at 83:17 and was linearly adjusted to 71:29 over 10 min, to 65:35 over 10 min, to 60:40 A/B over 2 min, and to the initial 83:17 solvent ratio over 1 min and then was held constant for 5 min. For fluorescence detection, eluents were excited at 380 nm and then were monitored at optimized emission wavelengths for glucuronides (450 nm), oxidized metabolites (540 nm), or AR-67 (560 nm).

MS Analysis. A 0.21 mM working solution of AR-67 lactone was prepared by dilution of 4.2 mM methanolic AR-67 with 0.15 M formic acid (equilibrated 1 h on ice). A 2.1 mM working solution of AR-67 carboxylate was prepared from the same 4.2 mM AR-67 stock by dilution with 1 M NH_4OH (2-h equilibration on ice). Complete conversion was verified by HPLC, and the two working solutions were further diluted with methanol to create separate 20 μM solutions. These standard solutions were individually infused at 10 $\mu\text{l}/\text{min}$ through an atmospheric pressure chemical ionization (APCI) or an electrospray ionization (ESI) source into an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with all components controlled by Analyst version 1.4.2 software (Applied Biosystems). Compound-dependent parameters were optimized for signal intensities of the lactone and carboxylate molecular ions in both positive and negative modes using both ionization methods. On the basis of signal intensities after parameter optimization, flow injection analysis was conducted using negative mode ESI to estimate method detection limits. In brief, 50 μl of increasing concentrations of AR-67 lactone or carboxylate (0.1–100 $\mu\text{g}/\text{ml}$ analyte in methanol containing 9.5% of 0.15 M formic acid or 0.5% 1 M ammonium hydroxide for lactone or carboxylate analysis, respectively) were injected into a 0.5 ml/min flow of 65:35 water-acetonitrile for delivery to the ESI-MS system. Flow was analyzed using lactone or carboxylate optimized voltages for the respective 477 or 495 m/z molecular anions, and signal/noise ratios were calculated.

Human Liver Microsome and Human Intestinal Microsome Metabolism. *Glucuronidation.* Human liver microsome (HLM) and human intestinal

microsome (HIM) reaction mixtures consisted of MgCl_2 (8 mM), alamethicin (63.6 μM), microsomal protein (0.25 mg/ml), and AR-67 (0.2 μM lactone or carboxylate) in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2% DMSO. Incubation mixtures were prepared on ice and preincubated at 37°C for 2 min, and reactions were initiated by the addition of uridine 5'-diphosphoglucuronic acid (final concentration 2 mM).

Oxidation. Oxidative incubations contained MgCl_2 (3.3 mM), NADP^+ (1.3 mM), glucose 6-phosphate (3.3 mM), AR-67 (0.2 μM lactone or carboxylate), and human liver or human intestine microsomal protein (0.25 mg/ml) in 100 mM potassium phosphate buffer at pH 7.4 (2C9 reactions were buffered with 100 mM Tris at pH 7.5), with a final DMSO concentration of 0.2%. Mixtures were prepared on ice, preincubated at 37°C for 3 min, and initiated by the addition of glucose-6-phosphate dehydrogenase (final concentration 0.4 units/ml).

Reaction quenching and workup. Microsomal reactions were stopped at 0, 1, 2, 5, 10, or 20 min with methanol as outlined above for sample workup. The resulting supernatants were analyzed by HPLC for the formation of AR-67 glucuronides or major oxidized metabolites.

Enzymatic Metabolism. In vitro metabolism studies were conducted using Supersomes prepared from baculovirus-infected insect cells (BTI-TN-5B1-4) expressing membrane-bound P450 or UGT enzymes. Oxidation and glucuronidation reactions were performed as outlined previously for microsomal incubations, except that protein levels were initially varied between 0.1 and 1 mg of total protein/ml (1 μM AR-67 lactone) to determine linearity. Time-dependent studies followed, in which recombinant enzymes (20 pmol/ml P450 or 0.25 mg/ml UGT Supersomes) were diluted as necessary with blank Supersomes to maintain 0.25 mg/ml total protein levels and then were exposed to 0.2 μM AR-67 lactone for 0, 1, 2, 5, 10, or 20 min in the presence of the appropriate cofactors ($n = 2$). Time points within the linear kinetic range were chosen for the highest active enzymes for use in concentration-dependent studies. Final conditions for the concentration-dependent studies were 0.25 mg/ml total protein (containing 20 pmol/ml P450 when applicable) incubated for 2 and 5 min (P450 and UGT enzymes, respectively) with 0.05 to 20 μM AR-67 lactone ($n = 3$).

Reaction quenching and workup. Reactions were incubated at 37°C and were stopped by sample workup procedures outlined above. The resulting supernatants were analyzed by HPLC for the formation of metabolites.

AR-67 Nonspecific Binding. The percentage of free AR-67 lactone in enzymatic incubation mixtures was calculated by mixing P450 or UGT Supersomes (0.25 mg/ml protein) with 1 μM AR-67 lactone without the addition of cofactors ($n = 3$). A 40- μl aliquot was processed according to the general procedure, with the remaining reaction mix (260 μl) applied to a Centrifree filtration device (30,000 molecular weight cutoff). The samples were centrifuged for 20 min at 3345g (4°C), and then were assayed for total AR-67 following general procedures. The percentage of unbound AR-67 was estimated after adjustment for AR-67 binding to filtration device surfaces.

Characterization of AR-67 Glucuronides. AR-67 glucuronide metabolites were prepared by scaled-up reactions of AR-67 lactone (5 μM) with UGT1A8 (1 mg/ml) but contained 5% DMSO.

β -Glucuronidase treatment. After incubation for 60 min, a 100- μl aliquot of the UGT1A8 reaction mix was added to 100 μl of β -glucuronidase (2000 units/ml in 200 mM phosphate buffer at pH 5) and incubated an additional 120 min. Quenched reaction solutions were analyzed by HPLC.

Glucuronide lactone and carboxylate interconversion. After incubation for 60 min, UGT1A8 reactions were quenched and treated according to the general workup procedure. Aliquots (40 μl) of the methanolic supernatant were reacted with equal volumes of 1 M HCl or 1 M NaOH for an additional 60 min before HPLC analysis to assess the interconversion of the glucuronide between lactone and carboxylate analytes at pH 3 and pH 11, respectively.

Excitation/emission scans. After a 60-min incubation reaction, samples were quenched and processed for HPLC analysis. Gradient flow was halted upon analyte detection within the fluorescence detector flow cell so that peak specific emission (excitation at 380 nm) and excitation (emission set at 450 nm for putative glucuronide analytes and at 560 nm for AR-67 carboxylate and lactone) spectra could be collected. After scan completion, flow was resumed, and the process was repeated for all fluorescing analytes within the sample.

Mass spectral analysis. For MS and MS/MS analysis, the putative glucuronide metabolites were isolated by SPE. After overnight incubation, the reaction

mixture was applied to a 1-ml Waters Oasis HLB SPE column (30 mg), which had been previously conditioned and equilibrated (1 ml of methanol then water, respectively). After loading, the glucuronides were eluted with 1 ml of 5% methanol in water (fraction 1), and AR-67 carboxylate and lactone were eluted with 1 ml of methanol (fraction 2). The fractions were dried at room temperature under N_2 and reconstituted in 1 ml of methanol. Reconstituted fractions were analyzed by HPLC for content. Fraction 1 was then treated with an equal volume of 0.15 M formic acid on ice for 1 h, and an aliquot was analyzed by HPLC to ensure complete conversion to the lactone form of the analyte. The acidified sample was then infused at 10 $\mu\text{l}/\text{min}$ through the ESI source into the API 2000 spectrometer, and the parameters were optimized for detection of the 653 m/z molecular anion of AR-67 lactone glucuronide. Subsequently, 100 to 900 m/z mass spectra and 653 m/z product ion spectra were collected.

Carboxylate and lactone glucuronide stability and recovery. AR-67 glucuronides were generated and isolated as described above for spectral analysis. The fraction 1 residue was dissolved in 500 μl of 0.005 M NaOH. A 250- μl aliquot was treated with 2 volumes of 0.005 M HCl to prepare the lactone glucuronide; whereas an additional aliquot was treated with 2 $\times$ volumes of 0.005 M NaOH to maintain the carboxylate glucuronide (both equilibrated for 2 h on ice). Analysis of these equimolar solutions allowed a relative response ratio between the two glucuronides forms to be calculated from their respective peak areas. These solutions were then used to spike methanol, human plasma, and human urine with either the lactone or the carboxylate glucuronide ($n = 3/\text{analyte}/\text{matrix}$). Abiding by the clinical procedures used in the collection and storage of in vivo samples (Arnold et al., 2010), the spiked plasma samples were immediately processed. The resulting methanolic supernatant was analyzed for initial levels of AR-67 glucuronide and then was stored at -80°C with repeated HPLC analysis 3 days, 1 week, 3 weeks, and 4 weeks after storage. Aliquots of the spiked urine samples were processed and analyzed immediately for comparison with remaining amounts, which were kept at ambient temperature for 24 h before processing and analysis. Differences in analyte levels between spiked methanol samples and processed plasma samples were used to estimate individual analyte recovery.

Characterization of Oxidized AR-67 Metabolites. For the following assays, CYP3A5 reaction mixtures were used to generate the oxidized metabolites. AR-67 lactone (5 μM) and recombinant protein (1 mg/ml) were reacted at 37°C in incubations containing 5% DMSO.

Excitation/emission scans. After 60 min, reaction samples were quenched and treated according to the general workup procedure. Stop-flow excitation/emission scans, as described earlier, were performed during gradient analysis to determine optimized excitation/emission wavelengths.

Mass spectral analysis. After overnight reaction, the mixture was applied to a 1-ml Waters Oasis HLB SPE column (30 mg) and conditioned and equilibrated as described earlier. After loading, 1 ml of 5% methanol in water (fraction 1) was applied, followed by 1 ml of methanol (fraction 2). The fractions were dried at room temperature under N_2 , reconstituted in methanol at one-fifth of the original applied volume, and analyzed by HPLC. Fraction 2 contained all fluorescing components. The reconstituted sample was then infused (10 $\mu\text{l}/\text{min}$) through the ESI source using optimized voltages.

Stability and recovery of major oxidized metabolites. Oxidized AR-67 metabolites were generated and isolated as described above for spectral analysis. The fraction 2 residue was dissolved in 500 μl of DMSO and used to spike methanol, human plasma, and human urine ($n = 3/\text{analyte}/\text{matrix}$). Samples were processed, stored, and analyzed in a time-dependent manner as described above for glucuronide stability. Likewise, recovery of the major CYP3A5 metabolite was calculated from differences in analyte levels between spiked methanol samples and processed plasma samples.

Metabolite Identification in Clinical Samples. A phase I study to determine the maximum tolerated dose of AR-67 was completed recently wherein qualified consenting patients received intravenous treatment with 1.67 to 12.4 mg/ m^2 of AR-67 for 5 consecutive days at the beginning of 21-day cycles (Arnold et al., 2010). Cycle 1, day 1, and day 4 plasma samples (predose, 5 min, 45 min, 65 min, 1.5 h, 2 h, 4 h, 6 h, 8 h, and 24 h after start of infusion) were collected for AR-67 pharmacokinetic analysis, as well as day 1 and day 4 total urine. Plasma samples were processed at the bedside, and the methanolic supernatants were stored at -80°C . Urine, which was collected for 24 h after infusion, was processed upon receipt, and the methanolic supernatant was

stored at -80°C . Interested readers are referred to the published phase I study for patient demographics (Arnold et al., 2010).

To investigate the metabolism of AR-67 in vivo, the supernatants from the 45-min plasma samples (i.e., AR-67 C_{max}) and the urine samples derived from 13 patients receiving doses of 7.5 mg/m^2 or higher were analyzed by gradient HPLC for the presence of peaks chromatographically similar to those obtained in AR-67 metabolic reactions with UGT and P450 enzymes. On the basis of the single-sample analyses results, one patient was determined to possess "non-representative" levels of AR-67 metabolites. All of the plasma sample supernatants from this patient were analyzed by gradient HPLC to generate time versus concentration plots for the glucuronide metabolites.

Quantitation, Kinetic, and Statistical Analysis. Weighted regression of peak areas to nominal matrix-matched calibrator concentrations yielded linear calibration curves for the lactone and carboxylate analytes, which were used to interpolate AR-67 lactone and carboxylate quantities in experimental samples. Total AR-67 was derived by addition of these two values. In instances in which experimental analyte concentrations exceeded 300 ng/ml , the original experimental methanolic supernatant was diluted with blank Supersome methanolic supernatant and reanalyzed as a $50\text{-}\mu\text{l}$ injection after mixing 1:1 with mobile phase buffer.

Because no commercial standards were available, metabolite peak areas were used to quantitate relative enzymatic activities, kinetic parameters, metabolite stability, and metabolite recovery. In quantitating carboxylate and lactone glucuronides, a molar response ratio (lactone/carboxylate) of 0.54 was estimated using the peak areas of equimolar carboxylate and lactone glucuronide solutions. After chromatographic analysis with fluorescence detection, this ratio was used to convert the carboxylate glucuronide peak areas to the lactone glucuronide peak areas, and a total area was reported. When the presence of glucuronides in patient samples was assessed, the "nanograms per milliliter equivalent" values for these metabolites were reported and were calculated from respective peak areas using the AR-67 lactone or carboxylate calibration curves.

Microsomal and enzymatic activities were calculated from early time points exhibiting linear kinetics for metabolite formation. Recombinant enzyme kinetics are described by the Michaelis-Menten (eq. 1) or the substrate inhibition models (eq. 2) as indicated by the plots of their metabolic velocity (V) against free substrate concentrations (S):

$$V = V_{\text{max}} \cdot S / (K_m + S) \quad (1)$$

$$V = V_{\text{max}} \cdot S / (K_m + S \cdot (1 + S/K_{si})) \quad (2)$$

These plots were fitted using nonlinear least-squares regression analysis (Prism version 5.02; GraphPad Software Inc., San Diego, CA) to estimate values for the Michaelis-Menten constant (K_m), the maximum velocity (V_{max}), and the substrate inhibition constant (K_{si}), when applicable (Houston and Kenworthy, 2000).

Results

Analytical Methods. Attempts to create a liquid chromatography-MS method for identifying both lactone- and carboxylate-derived AR-67 metabolites demonstrated a low sensitivity of this type of analysis for AR-67. APCI methods were found to be ≈ 10 times more sensitive than ESI methods for detection of AR-67 lactone; however, the carboxylate underwent near complete fragmentation and condensation within the APCI source regardless of ionization mode. Negative mode ESI was subsequently chosen for further investigation because this mode resulted in the least amount of carboxylate condensation. Individual analyte-dependent ESI-MS parameters were separately optimized for 477 (lactone) and 495 (carboxylate) m/z anion signal intensity. With use of these optimized voltages, flow injection analysis revealed a lower limit of quantitation for AR-67 lactone to be near 50 ng , with no improvement for the carboxylate, which underwent close to a 30% conversion to lactone within the ESI source. Thus, the majority of qualitative and all quantitative analyses were performed using gradient HPLC with fluorescence detection, which

TABLE 1

Metabolic activities of microsomal enzyme systems toward AR-67

Microsome-Substrate Combination	Phase I Oxidation Activity	Phase II Glucuronidation Activity
	<i>peak area/min/mg protein</i>	
HIM-AR-67 lactone	316.0 ± 8.5	302.1 ± 14.9
HLM-AR-67 lactone	241.2 ± 10.7	32.2 ± 7.4
HIM-AR-67 carboxylate	BDL	41.4 ± 21.1
HLM-AR-67 carboxylate	BDL	30.8 ± 9.4

BDL, beneath detection limits.

was accurate in quantitating 50 pg of material (on column). Interday accuracy for the HPLC analysis of total AR-67 in Supersomes, as determined from repeat analysis of 7, 150, and 250 ng/ml quality control samples was 108.87, 103.95, and 111.01% of the nominal values, with relative standard deviations of 19.0, 2.95, and 5.81%, respectively ($n = 9$). However, MS and MS/MS analysis was used for identifying molecular ions and producing fragmentation patterns for species isolated from UGT1A8 or CYP3A5 reaction incubations.

Microsomal Metabolism of AR-67. To gain a preliminary understanding of the metabolic fate of AR-67, the lactone and carboxylate forms were individually incubated with HIM or HLM in the presence of cofactors required for glucuronidation or oxidation. These initial studies showed that the lactone was selectively metabolized over the carboxylate, regardless of the microsomal or cofactor systems used (Table 1). Under conditions optimal for phase II metabolism, only the intestinal microsomes demonstrated significant metabolic activity toward AR-67 lactone (activity of $302,100 \pm 14,900$ total metabolite peak area/min/mg protein). HPLC analysis of this reaction mixture demonstrated the appearance of two fluorescing analytes, chromatographically separable from AR-67 lactone and its carboxylate form. These two metabolites were thought to correspond to the glucuronides of AR-67 carboxylate and lactone, respectively, because they exhibited greater corresponding polarity than either the lactone or its hydrolysis product. Under phase I oxidative conditions both HLM and HIM metabolized AR-67 lactone to chromatographically similar fluorescing products (activity of $241,200 \pm 10,700$ and $316,000 \pm 8500$ primary metabolite peak area/min/mg protein, respectively), which included an early eluting product associated with the presence of NADPH, as well as at least one major and one minor polar fluorescing metabolite. Thus, qualitative assessment of the in vitro oxidation and glucuronidation of AR-67 lactone in HLM and HIM showed that oxidation is likely to be the major biotransformation pathway for AR-67 in the liver but that both AR-67 oxidation and glucuronide conjugation may occur in the intestine.

Enzymatic Metabolism of AR-67 in Supersomes. Given that AR-67 was glucuronidated in microsomal systems, further investigations were conducted using single recombinant enzymes to identify the proteins involved in the biotransformation process. In time-dependent studies, AR-67 lactone ($0.2 \mu\text{M}$) glucuronidation was studied in incubations containing human recombinant UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, or 2B17. Among these, only UGT1A1, 1A3, 1A7, 1A8, 1A9, and 2B4 were capable of conjugating AR-67. The activities of these enzymes for total metabolite formation are listed in Table 2, with UGT1A8 and UGT1A7 displaying greater than 6 times the velocity of the other active UGT enzymes under linear conditions. Qualitatively, all active UGT enzymes yielded the same two polar peaks, which were observed in HIM and HLM reactions.

The oxidative metabolism of AR-67 was also studied in incubations containing human recombinant CYP1A1, 1A2, 2C8, 2C9*1, 2C19, 2D6*1, 3A4, or 3A5. Time-dependent studies consisting of recombi-

TABLE 2

Metabolic activities of recombinant microsomal enzyme systems toward AR-67

P450 Enzyme	Phase I Oxidation Activity	UGT Enzyme	Phase II Glucuronidation Activity
	peak area/min/pmol P450/1000		peak area/min/mg protein/1000
1A1	11.5 ± 1.2	1A1	15.7 ± 6.2
1A2	0.2 ± 0.1	1A3	47.3 ± 16.6
2C8	BDL	1A4	BDL
2C9*1	BDL	1A6	BDL
2C19	BDL	1A7	321.7 ± 63.2
2D6*1	BDL	1A8	1311.6 ± 295.5
3A4	9.8 ± 0.2	1A9	5.9 ± 15.0
3A5	36.0 ± 2.1	1A10	BDL
Blank	BDL	2B4	0.3 ± 7.5
		2B7	BDL
		2B15	BDL
		2B17	BDL
		Blank	BDL

BDL, beneath detection limits.

nant P450 (20 pmol of P450/ml with 0.25 mg of total protein/ml) incubated with AR-67 lactone (0.2 μ M), demonstrated that only CYP1A1, 1A2, 3A4, and 3A5 were capable of converting AR-67 lactone to more polar, fluorescing species in a time-dependent manner. Again, these phase I reactions also gave rise to an early eluting peak, which was present in control reactions of blank Supersomes containing an NADPH-regenerating system but absent in those devoid of oxidative cofactors. The relative activity of these enzymes toward the lactone, as assessed by product formation under linear conditions, was CYP3A5 $\gg$ CYP1A1 $\approx$ CYP3A4 $>$ CYP1A2 (Table 2). The appearance of the major and minor metabolite peaks resulting from the reactions of CYP1A incubations were qualitatively similar, but they were relatively more hydrophilic than the metabolites arising from CYP3A4, CYP3A5, and microsomes.

Subsequently, we conducted concentration-dependent studies with AR-67 concentrations ranging between 0.05 and 20 μ M. Time points of 5 and 2 min, which were within the linear range, were selected for UGT and P450 incubations, respectively. Under these conditions, AR-67 lactone underwent limited chemical hydrolysis ($\leq 6\%$ conversion to carboxylate), 81.3 $\pm$ 0.1% binding to Supersomes, and conversion to a single metabolite in phase I reactions. Substrate concentrations greater than 20 μ M were not investigated because of the limited solubility of AR-67 lactone in the 4% DMSO working solutions. Plots of the metabolite formation rates against the free (unbound) lactone substrate concentrations are displayed in Fig. 2. UGT1A8, CYP3A4, and CYP3A5 displayed plots characteristic of substrate inhibition (R^2 values of 0.979, 0.987, and 0.910, respectively), whereas UGT1A7 and CYP1A1 displayed classic Michaelis-Menten kinetics (R^2 values of 0.930 and 0.893, respectively). Estimates of K_m , V_{max} , and K_{si} , where applicable, are reported in Table 3.

Characterization of the AR-67 Glucuronides. Several assays were used to identify and characterize the carboxylate and lactone glucuronides, which were observed after incubation of AR-67 lactone with UGT1A8. The formation of AR-67 glucuronide was confirmed by subsequent disappearance of the polar peaks after treatment with β -glucuronidase (type B-I) to regenerate AR-67 carboxylate and lactone (Fig. 3A). To gain additional information regarding the structural position of the glucuronide moiety, AR-67 glucuronides obtained after exposure to UGT1A8 were acidified or alkylated by the addition of 1 M HCl or 1 M NaOH, respectively. As demonstrated in Fig. 3B, exposure of the glucuronide mixture to acidic conditions converts the glucuronide to the lactone form, whereas exposure to basic pH leads

FIG. 2. Enzyme kinetics plots of UGT1A7, UGT1A8, CYP1A1, CYP3A4, and CYP3A5 activity toward AR-67 lactone. Top, Michaelis-Menten and substrate inhibition curve fitting of total glucuronide formation velocities (total metabolite peak area per minute per milligram of protein) against the free lactone substrate concentration in incubations with UGT1A8 and UGT1A7. Bottom, similar fitting for velocities (primary metabolite peak area per minute per picomole of P450) of major metabolite formation resulting from CYP3A4-, CYP3A5-, and CYP1A1-catalyzed oxidation of AR-67 plotted against free substrate concentration. Each point represents the mean, with error bars depicting S.D. of three determinations.

to the conversion to the carboxylate form. In both cases, the parent AR-67 lactone and carboxylate peaks are minor. This result corroborates the identity of the "3" labeled peak as an AR-67 carboxylate *O*-glucuronide and the "4" labeled peak as an AR-67 lactone *O*-glucuronide. The capacity of the glucuronide to interconvert between the lactone and carboxylate forms while maintaining the glucuronide moiety limits conjugation to sites not directly involved in carboxylate condensation. In other words, the presence of either an acyl glucuronide (i.e., 21-*O*-glucuronide) or a 22-*O*-glucuronide would necessarily undergo cleavage during condensation under acidic conditions to form AR-67 lactone. However, a trace amount of AR-67 lactone is noted within the chromatogram of the acidified sample and suggests that either a C21 or C22 glucuronide may be formed on AR-67 carboxy-

TABLE 3

Enzyme kinetic parameters of recombinant P450 and UGT enzymes toward AR-67 lactone

Data are means $\pm$ S.E.

Enzyme	K_m μ M	V_{max} peak area/min/pmol P450/1000	K_{si} μ M
CYP1A1	0.098 $\pm$ 0.016	48 $\pm$ 1.5	N.A.
CYP3A4	0.63 $\pm$ 0.11	622 $\pm$ 63	3.0 $\pm$ 0.58
CYP3A5	0.21 $\pm$ 0.053	275 $\pm$ 33	2.0 $\pm$ 0.56
		peak area/min/mg protein/1000	
UGT1A7	0.19 $\pm$ 0.026	6.6 $\pm$ 0.21	N.A.
UGT1A8	1.0 $\pm$ 0.43	48 $\pm$ 15	1.2 $\pm$ 0.58

N.A., not applicable.

FIG. 3. Identification and characterization of AR-67 glucuronides. A, representative chromatographic traces of UGT1A8 incubations analyzed immediately (0 min) or 120 min after β -glucuronidase treatment are shown. Peaks labeled 1, 2, 3, and 4 correspond to AR-67 carboxylate, AR-67 lactone, AR-67 carboxylate glucuronide, and AR-67 lactone glucuronide, respectively. B, representative traces of the pH-dependent equilibrium between the putative carboxylate and lactone glucuronide metabolites of AR-67, shown at pH 7.4, 3, and 11. Peaks are labeled according to the system described for A. C, excitation (left) and emission (right) scans obtained for AR-67 lactone glucuronide (—) and AR-67 lactone (---) during chromatographic analysis. D, mass spectra of the isolated glucuronide fraction after acidification to pH 3, demonstrate the presence of a 653.2 m/z anticipated for AR-67 lactone glucuronide. The inset shows the product ion scan of the 653.2 m/z species. E, structural properties of the AR-67 lactone glucuronide, with primary fragmentation.

late, which could have arisen from lactone hydrolysis. To assess the spectral characteristics of the four peaks observed in Fig. 3A, stop-flow HPLC gradient analysis of the UGT1A8 reaction mixture supernatant was used to allow collection of excitation and emission scans. Both glucuronide forms possessed excitation maxima at 390 nm and emission maxima at 440 nm, compared with AR-67 carboxylate and lactone, which possess 380 nm excitation and 560 nm emission maxima wavelengths. This distinction is depicted in Fig. 3C, which highlights the differences between the lactone and lactone glucuronide. The spectral shift is further evidence that the formation of the glucuronide is most likely on the phenolic hydroxyl group.

Solid-phase extraction was used to separate the presumed glucuronide peaks, which are labeled 3 and 4 in Fig. 3, A and B. Treatment of this fraction with formic acid converted the mixture to only the 4 metabolic product. Subsequent MS and MS/MS analysis of this acidified fraction revealed a prominent 653 m/z and an MS/MS fragmentation pattern consistent with an AR-67 lactone glucuronide (Fig. 3D).

Collectively, these data suggest that the AR-67 lactone glucuronide is formed at the phenolic hydroxide with some minor fraction being an acyl glucuronide (Fig. 3E).

Characterization of Oxidized AR-67 Metabolites. Emission and excitation scans collected on CYP3A5 products displayed 380 nm excitation maxima and 540 nm emission maxima. These were not significantly shifted from AR-67 with 380 and 560 nm excitation and emission maxima, respectively. Processing incubation reactions through an SPE column did not allow for separation of oxidized metabolites from AR-67 carboxylate and lactone. Thus, MS analysis was essentially conducted on an SPE-purified reaction mixture. Molecular and fragment ions of AR-67 lactone (substrate with molecular weight = 478 g/mol) and carboxylate (hydrolysis product with molecular weight = 496 g/mol) were prominent amid the 400 to 700 m/z range expected for oxidized AR-67 (Fig. 4A). Product ion scans of 493.2 and 465.1 m/z revealed evidence for AR-67 hydroxylation and subsequent dealkylation products (Fig. 4, B and C).

FIG. 4. Characterization of oxidized AR-67 metabolites. A, mass spectra from AR-67 lactone (5 μ M) incubated with CYP3A5 (1 mg/ml) are shown. Letters with arrows refer to additional figure panels. B, the 493.2 m/z product ion spectrum suggests formation of a hydroxylated AR-67 metabolite, which may account for the presence of 493.2 and 449.3 m/z ions within the AR-67 lactone-CYP3A5 reaction mixture extract. Low concentrations coupled with high ionization within Q1 may account for the notable absence of the 493.2 m/z molecular ion within the 493.2 m/z product ion scan. C, the 465.1 m/z product ion spectra suggests subsequent dealkylation of hydroxylated AR-67 lactone may occur and would account for the 465.1 and 421.1 m/z ions within the AR-67 lactone-CYP3A5 reaction mixture extract.

In Vivo Identification of AR-67 Metabolites in Patients. A gradient HPLC with fluorescence detection assay was used to analyze plasma and urine samples collected from patients enrolled in a phase I study (Arnold et al., 2010). Assay performance was assessed using UGT1A8 and CYP3A5 formed metabolites, which were spiked into human plasma and urine. Recovery of both the carboxylate and lactone glucuronides from spiked plasma by methanolic quenching was $100 \pm 11.7\%$. Stability of the glucuronides stored as methanolic supernatants at -80°C was $92.62 \pm 1.76\%$ and $92.26 \pm 7.84\%$ over 28 days for the carboxylate and lactone form, respectively. The carboxylate glucuronide in urine at room temperature for 1 day underwent $95.44 \pm 0.31\%$ conversion to the lactone glucuronide with $87.79 \pm 3.05\%$ total glucuronide remaining. Under similar conditions, the lactone glucuronide underwent little conversion ($1.29 \pm 1.14\%$) with $100.74 \pm 4.05\%$ total glucuronide intact after 24 h. The recovery of the major CYP3A5 oxidative metabolite from spiked plasma was found to be $88.95 \pm 3.34\%$ with stability of $100.65 \pm 4.09\%$ over 28 days in methanolic supernatants stored at -80°C . Stability in urine kept at room temperature for at least 24 h was $91.04 \pm 6.78\%$.

Plasma from patients receiving 7.5 to 12.4 mg/m² AR-67 did not demonstrate the presence of circulating glucuronide metabolites or CYP3A-dependent oxidation products, except in the case of one patient we designated as nonrepresentative (Fig. 5A). The carboxylate and lactone glucuronides in plasma of the nonrepresentative patient were semiquantitated as nanogram per milliliter equivalents of carboxylate or lactone. The sum of these equivalents was plotted against time to show an approximate total AR-67 glucuronide exposure for this patient (Fig. 5B). There were no apparent differences in the plasma concentrations of total AR-67 or total *O*-glucuronidated metabolites between days 1 and 4 in this patient. On the basis of the HPLC analyses, the total amount of AR-67 in the 24-h urine samples from representative patients was calculated to be $3.02\% (\pm 1.28\%)$ of the dose administered, whereas the nonrepresentative patient excreted 11.17% of dose. All urine samples from these patients contained various, but limited, amounts of oxidized and glucuronidated AR-67 metabolic products (Fig. 5A).

Discussion

AR-67 is a novel lipophilic camptothecin analog. This third-generation congener is currently in early-phase clinical trials administered on a five times daily intravenous dosing schedule, every 21 days. Typical dose-limiting toxicities of patients receiving CPTs include low peripheral blood counts and diarrhea. The latter toxicity is related to the intrinsic cytotoxic activity of CPTs toward the rapidly proliferating GI epithelium and is exacerbated in individuals with low metabolizing efficiency. Although in the course of the first AR-67 phase I study, the dose-limiting toxicities were of hematopoietic origin, diarrhea was not observed (Arnold et al., 2010). This finding prompted us to initiate studies to determine the pathways of AR-67 metabolism, as well as to analyze patient samples for evidence of potential metabolites.

HLM and HIM studies demonstrated that under experimental conditions AR-67 lactone, but not carboxylate, could undergo oxidative metabolism (Table 1). This finding confirmed previous reports of NADPH-dependent AR-67 metabolism by mouse and human liver microsomes (Yeh et al., 2010). However, this selectivity is not unique to AR-67 because preferential lactone biotransformation was observed in liver microsomes reacted with CPT-11 (Lokiec et al., 1996; Haaz et al., 1998; Rosing et al., 1998; Santos et al., 2000). The products resulting from HLM and HIM reaction with AR-67 lactone were attributed primarily to CYP3A metabolism, because of their chromatographic similarity with products formed after AR-67 incu-

FIG. 5. AR-67 glucuronidation and oxidation in clinical samples. A, chromatographic traces from enzymatic and standard samples (top) for comparison to plasma (middle) and urine (bottom) samples collected from a nonrepresentative and a representative metabolizing patient enrolled in a phase I AR-67 clinical trial. B, concentration versus time curves for day 1 (filled symbols) and day 4 (open symbols) plasma levels of total AR-67 (squares) and AR-67 glucuronides (circles) in the nonrepresentative patient. Total AR-67 glucuronides are estimated as nanogram per milliliter equivalents of AR-67. AR-67 carboxylate and lactone are indicated within traces as peaks labeled 1 and 2, respectively, putative carboxylate and lactone glucuronides are labeled as peaks 3 and 4, respectively, the CYP3A oxidized AR-67 metabolites are labeled as peaks 5 and 6, and a CYP1A metabolite is labeled as peak 7.

bation with recombinantly expressed CYP3A4 and CYP3A5. Although we did not attempt to estimate relative activity factors in this work, we anticipate that the subpopulation of patients lacking CYP3A5 activity may have lower overall oxidative clearance (Thelen and Dressman, 2009). Nevertheless, metabolites were observed after incubation in liver and intestinal microsomal systems, suggesting the potential for oxidative metabolic clearance in both hepatic and GI tissue.

HLM and HIM reactions optimized for glucuronidation demonstrated that HIM enzymes had the highest activity toward AR-67 lactone, which was almost 10-fold higher than that of HLM. The activity toward AR-67 lactone in HIM was 7-fold higher than the activity toward AR-67 carboxylate, but the activities observed in HLM were the same (Table 1). This result is in contrast with previous findings with SN-38 that demonstrated no difference in the glucuronidation of lactone and carboxylate in HLM (Haaz et al., 1997). In all cases, two glucuronide products were formed and were chromato-

graphically identical to those produced by recombinant UGT enzymes. These two products were later identified as the carboxylate and lactone glucuronides of AR-67. The lactone glucuronide was the predominant product in all reactions, even with AR-67 carboxylate as the substrate, suggesting that enzymatic interaction occurred through formation of the AR-67 lactone in AR-67 carboxylate incubations. Although relative activity factors were not calculated, the observed in vitro activity and expression patterns of the active UGT enzymes allowed us to infer that UGT1A8 may account for a majority of the HIM activity (Tukey and Strassburg, 2000). In contrast, the HLM activity must be the cumulative effect of UGT1A1, 1A3, 1A9, and 2B4. Collectively, the data suggest the potential for low-level glucuronidation-mediated clearance in the liver with additional capacity within the GI tract.

Studies with all the commercially available recombinant UGT enzymes showed that UGT1A8 and UGT1A7 activity toward AR-67 was 6-fold higher than 1A3, 1A1, 1A9, and 2B4 (Table 2). However, it should be noted that in the recombinant enzyme reactions, we did not evaluate the effect of fatty acid-free human serum albumin, which has been shown to increase UGT1A9, UGT2B7, and CYP2C9 activities (Rowland et al., 2008a,b). Of interest, UGT1A8 and UGT1A7 are extrahepatic with distinct GI expression, as opposed to UGT1A3 and UGT1A1, which are expressed in liver and GI tract (Tukey and Strassburg, 2000; Nakamura et al., 2008). This pattern of activity reflected the microsomal observations and leads us to hypothesize that increased activity and localization of UGT1A8 in the GI tract might provide a protective mechanism against AR-67 cytotoxicity in GI epithelium. In contrast, the diminished activity of UGT1A1 and other UGT enzymes expressed in the liver could in part explain the absence of AR-67 glucuronide metabolites in the plasma of almost all patients. Concentration-dependent studies showed that UGT1A8 displayed

strong substrate inhibition kinetics ($K_{si} \approx K_m$), whereas UGT1A7 displayed classic Michaelis-Menten kinetics.

Given the presence of several hydroxyl groups on both forms of AR-67 (Fig. 1), there is potential for the formation of multiple glucuronide products. Incubation of AR-67 lactone with all active UGT enzymes resulted in two products with greater hydrophilicity than either AR-67 lactone or carboxylate. These products were tentatively identified as the carboxylate and lactone 10-*O*-glucuronides (Fig. 3) after studies of β -glucuronidase treatment, pH modulation, and emission wavelength shifts (Rosing et al., 1998). The glucuronide structures were subsequently confirmed with MS/MS analysis (Fig. 3D).

A panel of P450s, including those known to metabolize approximately 90% of drugs (Guengerich, 2006), as well as those known to metabolize topotecan and irinotecan (Lokiec et al., 1996; Haaz et al., 1998; Rosing et al., 1998; Santos et al., 2000), were used to evaluate AR-67 metabolism. Of these, only the CYP3A and CYP1A enzymes were found to transform AR-67 lactone (Table 2). The products from the CYP3A enzymes were distinctly more hydrophobic than the CYP1A products and matched those formed in microsomes. Our preliminary MS studies provide evidence for hydroxylated and dealkylated products of AR-67 from CYP3A reactions, which occur on the camptothecin ring system. However, further work is required to fully determine the structures of metabolites arising from CYP3A and CYP1A. Concentration-dependent studies showed that under experimental conditions the CYP3A enzymes displayed substrate inhibition kinetics, whereas CYP1A1 displayed classic Michaelis-Menten kinetics.

Chromatographic analysis demonstrated that 12 of 13 patients who received the maximum tolerated dosage or higher had no detectable metabolites in their plasma and only minimal metabolites in their urine (Fig. 5A). Quantitative AR-67 analysis in the urine from these representative patients indicated that 3.02% ($\pm 1.28\%$) of the admin-

FIG. 6. Schematic diagram of enzymes and transporters implicated in the disposition of AR-67. The lactone form of AR-67 readily diffuses into hepatocytes, whereas the carboxylate entry may be facilitated by uptake transporters (e.g., OATP1B1 and OATP1B3) expressed on the basolateral face of hepatocytes. Inside the hepatocytes AR-67 may undergo oxidative metabolism and phase-II conjugation and these metabolites may be excreted by active efflux transporters [e.g., P-glycoprotein (P-gp) and BCRP] into the bile. Intact AR-67 may also be effluxed into the bile. AR-67 glucuronides that are carried into the intestinal lumen via the bile may be hydrolyzed by bacterial enzymes [e.g., bacterial β -glucuronidase (BG)] to form AR-67. The lactone moiety may diffuse into the intestinal epithelium or may precipitate in the lumen. Inside the intestinal epithelium AR-67 may undergo oxidative metabolism or phase-II conjugation. Intact AR-67 and metabolites may be actively effluxed back into the intestinal lumen. Intact drug may also diffuse into the circulation. The lack of metabolites in the plasma of most patients but their presence in the urine indicates that low amounts of metabolites may be effluxed into plasma, but these are rapidly cleared by the kidney.

istered AR-67 dose was excreted in the urine as unchanged drug. A nonrepresentative patient displayed much higher amounts of AR-67 metabolites in the plasma and urine (Fig. 5A) and excreted 11.17% of dose as unchanged AR-67 in the urine. The metabolites present in this patient included AR-67 glucuronides, CYP3A products, a CYP1A product, and several minor unidentified analytes. The difference in the plasma and urine metabolite levels between this patient and the rest of the study subjects could not be attributed to metabolite stability or differences in sample processing.

Collectively, the data suggest that the bulk of AR-67 is processed through the liver and is efficiently transferred, as unchanged drug or as metabolites, into the bile for elimination. Little is known about AR-67 transport, because only a few transporters have been tested for their capacity to translocate AR-67 (Milewska et al., 2009). The organic anionic transporters OATP1B1 (SLCO1B1) and OATP1B3 (SLCO1B3) have been shown in transiently transfected cells to import AR-67 carboxylate in a saturable and inhibitable manner, whereas the presence of stably transfected ABC transporters, P-glycoprotein (ABCB1) and BCRP (ABCG2), reduce intracellular AR-67 levels. No studies have investigated the transport of phase I or phase II AR-67 metabolites, although studies with CPT-11 metabolites suggest that multidrug resistance-associated protein 2 (ABCC2) and BCRP (ABCG2) may transport AR-67 glucuronides (Smith et al., 2006). To help us construct a mechanistic framework for understanding AR-67 kinetics, we present a schematic diagram depicting our current knowledge of enzymes and transporters implicated in AR-67 disposition (Fig. 6). Because the primary clearance pathways appear to be hepatic, we have omitted potential interactions with kidney transporters. Further studies will be necessary to determine whether interpatient variability in the GI expression of CYP3A4, CYP3A5, UGT1A7, or UGT1A8 is a prognostic factor for increased gastrointestinal toxicities. However, this may be more relevant if oral formulations of AR-67 are sought or if dosing schedules exploring the efficacy of less frequent, but higher dosage levels are tested in the clinic.

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Authorship Contributions

Participated in research design: Leggas, Milewska, and Horn.
Conducted experiments: Milewska and Horn.
Contributed new reagents or analytic tools: Milewska.
Performed data analysis: Horn, Milewska, and Leggas.
Wrote or contributed to the writing of the manuscript: Horn, Milewska, Arnold, and Leggas.
Other: Arnold was responsible for patient recruitment; Arnold and Leggas acquired financial support for this research.

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