DMD Fast Forward. Published on April 25, 2013 as doi:10.1124/dmd.112.050021

Title Page:

The effect of BCRP, MDR1 and OATP1B3 on the antitumor efficacy of the lipophilic camptothecin AR-67 *in vitro*

Eleftheria Tsakalozou, Eyob D. Adane, Kuei-Ling Kuo, Abigail Daily, Jeffrey A. Moscow and Markos Leggas

Author affiliations:

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 S. Limestone St., Room 323, Lexington, KY 40536, USA (E.T., E.D.A., K.L.K., M.L.);

Department of Pediatrics, College of Medicine, University of Kentucky, 740 S. Limestone St. Room J457 Lexington, KY 40536, USA (A.D., J.A.M.)

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

Running title page:

Impact of transporters on the cytotoxicity of AR-67 *in vitro*

Corresponding author: Dr. Markos Leggas, Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 S. Limestone Ave., Room 323, Lexington, KY 40536, USA. Phone: 859-257-2633, Fax: 859-257-7564. E-mail: mlegg2@email.uky.edu

Number of Text pages: 18

Number of Tables: 0

Number of Figures: 9

Number of References: 56

Number of words in Abstract: 208

Number of words in Introduction: 505

Number of words in Discussion: 1385

ABBREVIATIONS:

ABC: ATP-binding cassette

BCRP: Breast Cancer Resistance Protein (ABCG2)

BSP: Bromosulfophthalein

Calcein-AM: Acetyloxymethyl 2-[[2-(acetyloxymethoxy)-2-oxoethyl]-[[3',6'-diacetyloxy-7'-[[bis[2-(acetyloxymethoxy)-2-oxoethyl]amino]methyl]-3-oxospiro[2-benzofuran-1,9'-xanthene]-2'-yl]methyl]amino]acetate

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

CCK-8: sulfated cholecystokinin octapeptide (l-aspartyl-l-tyrosyl-l-methionylglycyl-l-tryptophyl-l-methionyl-l-aspartyl-l-phenylalaninamide hydrogen sulfate ester)

CPM: Counts Per Min

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethysulfoxide

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

GF120918: N-4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide hydrochloride,

HCl: Hydrochloric Acid

Hoecht 33342: 2'-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole

HPLC-FL: High Performance Liquid Chromatography-Fluorescence

K_m: Michaelis-Menten rate constant

MDCKII: Madin-Darby Canine Kidney Strain II

MDR1: Multidrug Resistant protein 1 (ABCB1)

MEM: Minimum Essential Media

NaOH: Sodium Hydroxide

OATP: Organic Anion-Transporting Polypeptide

PBS: Phosphate-Buffered Saline

SN-38: 7-Ethyl-10-hydroxy-camptothecin

TEER: Transepithelial Electrical Resistance

V_{max}: Maximum transport rate

Abstract

AR-67 is a lipophilic camptothecin analogue, currently under early stage clinical trials. Transporters are known to have an impact on the disposition of camptothecins and on the response to chemotherapeutics in general due to their expression in tumor tissues. Therefore, the interplay between the BCRP, MDR1 and OATP1B1/OATP1B3 transporters and AR-67 and their impact on the toxicity profile of AR-67 was investigated. Using cell lines expressing the aforementioned transporters, we showed that the lipophilic AR-67 lactone form is a substrate for efflux transporters BCRP and MDR1. Additionally, OATP1B1 and OATP1B3 facilitated the uptake of AR-67 carboxylate in SLCO1B1 and SLCO1B3-transfected cell systems compared to the mock-transfected ones. Notably, both BCRPand MDR1, conferred resistance to AR-67 lactone. Prompted by recent studies showing increased OATP1B3 expression in certain cancer types, we investigated the effect of OATP1B3 expression on cell viability after exposure to AR-67 carboxylate. OATP1B3 expressing cells had increased carboxylate uptake as compared to mock transfected cells, but were not sensitized because the intracellular amount of lactone was fifty-fold higher than that of carboxylate and comparable between OATP1B3 expressing and non-expressing cells. In conclusion, BCRP- and MDR1-mediated efflux of AR-67 lactone confers resistance to AR-67, but OATP1B3-mediated uptake of the AR-67 carboxylate does not sensitize OATP1B3 expressing tumor cells.

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

AR-67 (7-t-Butyldimethylsilyl-10-hydroxycamptothecin, DB-67) (Fig. 1) is a third generation camptothecin analogue that belongs to the class of 7-silylcamptothecins (Bom et al., 2000; Curran et al., 2000). Similar to other camptothecins, AR-67 undergoes pH dependent, but reversible, hydrolysis of the lipophilic lactone to the hydrophilic carboxylate (Bom et al., 2000). Although both lactone and carboxylate forms interact with DNA (Staker et al., 2002), they have different transport characteristics. The lactone passively diffuses into the cell and is considered the pharmacologically active form. In contrast, the negatively charged carboxylate requires transporter-mediated uptake, and it is often considered an inactive form. Preclinical studies have demonstrated the high lipophilicity and an apparent blood stability of the lactone form of AR-67 compared to FDA-approved camptothecins (Bom et al., 2001).

A common link between drug disposition and drug efficacy are transporter proteins, which could play a pivotal role in both the disposition and efficacy or toxicity of camptothecin analogs. As AR-67 exists in equilibrium between the hydrophobic lactone and hydrophilic carboxylate forms, both influx and efflux transporters could potentially play roles in both metabolic clearance and tumor sensitivity. Intracellular drug concentration will be influenced by the balance between cellular efflux, potentially resulting in resistance, and cellular uptake, potentially resulting in sensitivity. Metabolic clearance, on the other hand, may result from vectorial transport, where both influx and efflux transporters contribute to clearance in the same direction.

The effect of transporters on the pharmacokinetic and pharmacodynamic profile of the FDA approved camptothecins topotecan and irinotecan has been demonstrated in previous studies. Topotecan and the active irinotecan metabolite, SN-38, have been identified as BCRP substrates (Nakatomi et al., 2001; de Vries et al., 2007) while MDR1-mediated transport has been reported for topotecan and irinotecan (Luo et al., 2002; de Vries et al., 2007). Notably,

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

expression of BCRP in established cancer cell lines and tumor biopsy samples has been associated with resistance to camptothecins (Kawabata et al., 2001; Candeil et al., 2004). Among the uptake transporters, OATP1B1 has been implicated in the transport of irinotecan and SN-38, which has also been identified as an OATP1B3 substrate (Nozawa et al., 2005; Yamaguchi et al., 2008). However, little is known about the potential interactions between AR-67 and transporters and the implications of these interactions on the antitumor activity of AR-67 and its pharmacokinetic profile.

In this study we explored the interaction of AR-67 with BCRP and MDR1 and with OATP1B3 and OATP1B1. First we determined if expression of the efflux transporters BCRP and MDR1 would have an impact on the cytotoxic profile of the lipophilic AR-67 lactone *in vitro*. Additionally, we examined the effect of OATP1B3 expression on the intracellular amounts of AR-67 lactone and carboxylate. Based on recent studies reporting increased expression of OATP1B3 in tumor tissues (Muto et al., 2007; Pressler et al., 2011), we tested whether increased intracellular AR-67 uptake, facilitated by OATP1B3, would potentiate the antitumor activity of AR-67 *in vitro*. To address these questions, we used established cancer cell lines that expressed functional forms of the BCRP, MDR1, OATP1B3 and OATP1B1 transporters.

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Cell Lines and Reagents.

Madin-Darby canine kidney II (MDCKII) cells were obtained from the European Collection of Cell Cultures. Cells were mock transfected with pcDNA3.1 vector or with pcDNA3.1-ABCG2 (Wang et al., 2012). OVCAR-8 and its derivative cell line expressing human MDR1 (NCI/ADR-RES) were from NCI Developmental Therapeutics. HeLa (cervical adenocarcinoma) cells were stably transfected with OATP1B3 cDNA and RKO (colon carcinoma) cells were stably transfected with OATP1B1 cDNA, inserted in the multiple cloning site of the pIRESneo2 vector (Clontech, Mountain View, CA) (Monks et al., 2007; Daily et al., 2010). MDCKII cells were cultured in Minimum Essential Media (MEM) supplemented with 5 % fetal bovine serum (FBS) and Geneticin (G418, 800 µg/mL) while OVCAR-8 and NCI/ADR-RES cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS. The HeLa and RKO cell lines were grown in DMEM supplemented with 10 % FBS and Geneticin (G418, 800 µg/mL). Additionally, non-essential amino acids (1 %) were added to the RKO growth media. Penicillin (100 U/mL) and streptomycin (100 µg/mL) were added in the media. All of the aforementioned materials were supplied by Gibco, Invitrogen Corporation (Carlsbad, CA). Transporter expression was validated by Western blot analysis and transporter function was verified with prototypical substrates (S*upplemental Material*).

Preparation of Drug Stock and Working Solutions.

AR-67 (DB-67; Novartis) was solubilized in dimethylsulfoxide (DMSO) at a concentration of 1 mg/mL (AR-67 lactone stock solution) and stored at -80 °C. AR-67 carboxylate working solutions were prepared by dilution of one volume of lactone stock solution with 9 volumes of 0.005 N NaOH and were allowed to convert to carboxylate overnight at 4 °C (AR-67 carboxylate

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

stock solution) (Horn et al., 2006). Working solutions were stored at -80 °C after their preparation and at 4 °C during the experimental procedure.

Intracellular Amount of AR-67 in BCRP and MDR1 Expressing Cell Lines.

To examine the effect of ABC efflux transporters BCRP and MDR1 on the intracellular amount of AR-67, cells were seeded in six-well plates. The next day, medium was replaced and the cells were incubated in serum-free medium (Opti-MEM) with 0.125, 0.25, 0.5, 0.75 and 1 µM AR-67 lactone for 5 min at 37°C. The incubation time was selected to ensure linearity in the transport process. At the end of the incubation, medium was aspirated and cells were washed twice with Opti-MEM (4 °C). Cells were lysed with 200 µL of 0.5 N NaOH and placed on a rocker (4 °C) for 10 min. Extraction and quantification of AR-67 was carried out as indicated in an HPLC-FL method under conditions that ensure system suitability (Horn et al., 2006). Protein was quantified using the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific). In inhibition studies, cells were pre-incubated with GF120918 (5 µM) (de Bruin et al., 1999; Evers et al., 2000) for 1 h prior to incubation with 1 µM AR-67 lactone (for 5 min).

Transepithelial Flux of AR-67.

MDCKII-pcDNA and MDCKII-BCRP cells were grown on Corning Transwell® 3414 membrane inserts (3.0-µm pore size, 24-mm diameter; Corning Glassworks, Corning, NY). When TEER exceeded 200 Ω·cm^{2}s in both cell lines, the medium was aspirated and replaced with 1.8 ml of serum-free Opti-MEM with or without GF120918 (5 µM) on both the apical and basolateral side. Cells were placed at 37°C in humidified 5 % CO_2 incubator for 1 h. Following, 0.2 ml of 50 µM AR-67 lactone was added to the donor side to a final concentration of 5 µM and 0.2 ml of medium was added to the receiver side. Cells were then placed on a rocker and incubated at 37

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

°C and 5 % CO_2. AR-67 amount was quantified in 50 µL samples using the HPLC method described previously. Apparent permeability and efflux were calculated as previously described (Kalvass and Pollack, 2007).

Intracellular Uptake of AR-67 Carboxylate.

The effect of the expression of OATP1B3 and OATP1B1 on the intracellular uptake of AR-67 was studied using the HeLa-pIRES/OATP1B3 and RKO-pIRES/OATP1B1 cell lines using serum-free medium (Opti-MEM). To evaluate the dose-dependent OATP-mediated uptake of AR-67, OATP transfected cell lines were incubated with 0.5, 1, 1.5, 2 and 3 µM AR-67 carboxylate for 1 min. The incubation time was selected to ensure linearity in the transport process as indicated by preliminary studies. The pH of carboxylate containing media was 7.2. In inhibition studies, Hela-pIRES/OATP1B3 and RKO-pIRES/OATP1B1 cells were incubated with 1 µM AR-67 carboxylate for 5 and 10 min, respectively. The inhibition of transporter-mediated uptake was studied by pre-incubation with bromosulfophthalein (BSP) (50 µM) (Seithel et al., 2007; Kalliokoski and Niemi, 2009) for 10 min and keeping the concentration of BSP constant during substrate incubation.

At the end of the incubation period, cells were washed twice with Opti-MEM (4 °C) before being lysed and AR-67, calculated as the sum of the two forms, was extracted from the cell lysate and quantified as described above (Horn et al., 2006).

When quantification of each of the AR-67 forms separately was required, cells were incubated with AR-67 carboxylate or lactone (1 µM) for 0.083, 0.5, 1, 3, 6, 12, 24 and 48 h at 37 °C and washed twice with Opti-MEM (4 °C) at the end of the incubation period. PBS was added; cells were scrapped off and lysed by sonication. AR-67 lactone and carboxylate were extracted and quantified using HPLC (Horn et al., 2006).

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

Drug Stability in Culture Media.

AR-67 interconversion was studied in cell culture media at pH 7.4 (37 ºC) and humidified atmosphere of 5 % CO_2. AR-67 carboxylate (1 µM) or lactone (1 µM) were added in the media at t=0 h. AR-67 carboxylate and lactone concentrations were quantified at 0, 0.5, 1, 2, 4 and 6 h using HPLC (Horn et al., 2006)

Cytotoxicity Assays.

Cells were seeded in 96-well plates at initial densities that ensured their exponential growth for the duration of the experiment. To study the effect of AR-67 lactone and AR-67 carboxylate on cell viability, cells were seeded and allowed to adhere overnight. Twenty-four hours later, the cell culture medium was replaced with drug-containing medium. The final concentration of DMSO in cell culture media never exceeded 1 %. Cells expressing BCRP and MDR1 were exposed to AR-67 lactone for 72 h. The effect of BCRP and MDR1 on AR-67 lactone-induced cytotoxicity was studied in the presence of the dual BCRP/MDR1 inhibitor GF120918. MDCKII-pcDNA/BCRP cells were pretreated for 30 min with GF120918 (1 µM) followed by co-treatment with GF120918 (1 µM) and AR-67 lactone. OVCAR-8 and NCI/ADR-RES cells were pretreated for 30 min with GF120918 (5 µM) followed by co-treatment with GF120918 (5 µM) and AR-67 lactone. The cytotoxic effect of AR-67 carboxylate on the OATP expressing cell lines after various exposure times was also evaluated. In these cases, cells were treated with AR-67 carboxylate for 5 and 30 min, 1 and 3 h. At the end of the incubation period, the drug-containing media was removed and the cells were washed once with ice cold PBS. Following, drug-free media was added in the wells and cells were allowed to grow for 48 h at which point their viability was assessed.

Cell viability was assessed with the CellTiter 96® AQeous Non-Radioactive Cell Proliferation Assay kit (Promega, Fisher Scientific) following the manufacturer's instructions. The viability of the HeLa- and RKO-OATP transfected cell lines was evaluated by using a resazurin assay (RSZ, Alamar Blue).

Data Analysis and Statistics.

GraphPad Prism (version 5.02 for Windows, GraphPad Software Inc., San Diego, USA) was employed for the graphical representation of the results, data analyses and statistical comparisons.

To calculate the kinetic parameters for the OATP1B3 and OATP1B1 transporters, AR-67 intracellular uptake was determined as described above. Transporter mediated uptake was corrected by subtracting intracellular amount in mock transfected cells and error propagation was applied. Data were modeled using the Michaelis-Menten equation which provided us with a satisfactory fitting:

$V = V_{max} \times C/(K_m + C)$

where V (ng of total AR-67/µg protein/min) was the rate of transport of AR-67 lactone or carboxylate, C (µM) is the concentration of AR-67 carboxylate added in the well and K_m (µM) was the concentration of AR-67 lactone or carboxylate at which the transporter activity was equal to its maximum value (V_{max}).

Dose-response data were also fitted using non-linear regression analysis. The viability of the treated cells was normalized with the viability of untreated (control) cells. IC_{50} values were estimated as the dose of the cytotoxic drug that induces response equal to half of the maximum response observed.

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

Results

AR-67 Lactone Is a Substrate of BCRP and MDR1.

BCRP and MDR1-expressing cell lines with confirmed expression and function (Supplemental Figures 1 and 2, respectively) were used to assess the interaction of AR-67 lactone with these efflux transporters. Initial studies assessed time-dependent intracellular AR-67 amount (data not shown), which demonstrated a time-dependent increase in intracellular amounts within the first 10 minutes. The intracellular amount of total AR-67 was then measured following 5 min incubation with a range of AR-67 lactone concentrations. Intracellular AR-67 amounts in MDCKII-BCRP (Fig. 2A) and NCI/ADR-RES cell lines (Fig. 2B) were lower than those in MDCKII-pcDNA and OVCAR-8 cells, respectively. The transporter effect was reversed when cells were pretreated with GF120918 (Fig. 2C and D), which is known to inhibit the drug efflux mediated by the ABCG2 and ABCB1 gene products (de Bruin et al., 1999; Evers et al., 2000). BCRP-mediated efflux of AR-67 lactone, in the presence and absence of GF120918, was also evaluated using polarized MDCKII transfected cells grown on Transwell® inserts. GF120918 had no effect on drug transfer from the apical to the basolateral side in MDCKII-pcDNA cells (Fig. 3A), but increased the transfer in MDCKII-BCRP cells (Fig. 3B). This is consistent with the apical expression of BCRP in polarized MDCKII cells (Grube et al., 2007; Wang et al., 2012). GF120918 also attenuated AR-67 transfer from the basolateral to the apical side in MDCKII-pcDNA (Fig. 3C). However, the effect on MDCKII-BCRP cells was more significant (Fig. 3D). This is consistent with the determination of the apparent permeability and efflux ratio values estimated (Supplemental Table 1) for transport across BCRP expressing cell monolayers. Collectively, these data suggest that AR-67 lactone is a substrate for MDR1 and BCRP.

Our attempt to perform similar studies with AR-67 carboxylate yielded insignificant intracellular AR-67 amounts in monolayers and drug transfer across polarized cells (data not shown).

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

AR-67 Carboxylate is Transported by OATP1B1 and OATP1B3.

Previous *in vivo* pharmacokinetic studies demonstrated that AR-67 carboxylate has a relatively higher clearance than the lactone form (Adane et al., 2010). Given the anionic nature of the carboxylate we reasoned that rapid clearance may be through its increased uptake in the liver by organic anion transporters. Here, we assessed the uptake of AR-67 carboxylate in cells transfected with either SLCO1B1 or SLCO1B3. The expression of the OATP1B1 and OATP1B3 transporters was confirmed using RT-PCR analysis and immunohistochemistry while their function was evaluated using prototype substrates (Supplemental Figure 3). Initial time-dependent studies showed rapid uptake of AR-67 carboxylate in both transporter expressing cell lines, but not in the mock transfected cells (data not shown). Subsequently, concentration-dependent uptake studies were conducted after incubation with AR-67 carboxylate for 1 min. The intracellular amount of total AR-67 was significantly higher in the OATP1B3 (HeLa-OATP1B3) and OATP1B1 (RKO-OATP1B1) expressing cells than that in the mock-transfected ones (Fig. 4). The concentration-dependent uptake of AR-67 carboxylate by OATP1B3 (Fig. 4A) is consistent with saturable kinetics. The transporter mediated uptake was diminished by the addition of BSP (Fig. 4C, D) (Seithel et al., 2007; Kalliokoski and Niemi, 2009). Kinetic analysis also demonstrated higher affinity of AR-67 carboxylate for OATP1B3, than OATP1B1, with estimated K_m values of 0.32 μM and 2.58 μM, respectively. Additionally, incubation with the AR-67 lactone form did not result in significant uptake differences between OATP1B3 or OATP1B1 and mock transfected cells (data not shown).

BCRP and MDR-1 Confer Resistance to AR-67 Lactone.

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

To evaluate the significance of the interaction between AR-67 lactone and the efflux transporters BCRP and MDR1, we assessed their potential for conferring drug resistance in the context of *in vitro* cytotoxicity studies. We found that BCRP confers a 10-fold resistance as measured by the differences in the estimated IC_{50} values between MDCKII-pcDNA and MDCKII-BCRP cells (Fig. 5A). The BCRP effect was negated in the presence of the transporter inhibitor GF120918 (Fig. 5B). Similarly, AR-67 lactone was significantly more cytotoxic to the OVCAR-8 than the NCI/ADR-RES cells as demonstrated by a 100-fold lower IC_{50} values (Fig. 5C). The transporter effect on the cytotoxic profile of AR-67 lactone was eliminated in the presence of GF120918 (Fig. 5D). To determine if sensitivity differences between transporter expressing and non-expressing cells were due to variability in the expression of the Top1 enzyme, we assessed its expression in the MDCKII-pcDNA/BCRP and OVCAR-8/NCI/ADR-RES cell lines (Supplemental Figure 4). Our results indicated comparable Top1 protein expression between the control and transporter-expressing cell lines.

OATP1B3 Expression Does Not Sensitize Cells to AR-67 Carboxylate.

Our study showed that OATP1B3 and OATP1B1, which are highly expressed in the liver (Abe et al., 1999; Konig et al., 2000b; Konig et al., 2000a; Abe et al., 2001), have the potential of transporting AR-67 carboxylate (Fig. 4). However, several recent studies have reported that OATP1B3 is also expressed in tumor tissues (Monks et al., 2007; Muto et al., 2007; Lee et al., 2008; Lockhart et al., 2008; Pressler et al., 2011). To determine whether active uptake of the carboxylate could sensitize OATP1B3 expressing cells, we conducted cytotoxicity studies (Fig. 6). The equivalent Top1 expression in HeLa-pIRES and HeLa-OATP1B3 was verified by Western blot (Supplemental Figure 4). Initially the cytotoxicity of AR-67 carboxylate was assessed after 48 h of continuous drug exposure (Fig. 6D), but due to the reformation of the

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

lactone upon incubation (Fig. 7A) we also determined the cytotoxicity after 0.5, 1, and 3 h (Fig. 6A-C) of drug treatment, which allows for relatively greater carboxylate exposure. As expected by the action of camptothecins during S-phase, longer incubation resulted in lower IC_{50} values (Fig. 6E). However, OATP1B3 expressing cells were not more sensitive than mock transfected cells.

Time-Dependent Intracellular AR-67 Uptake after Exposure to Lactone and Carboxylate.

To gain a better understanding of the apparent lack of sensitivity of OATP1B3 cells to AR-67 carboxylate (Fig. 6), we determined the intracellular exposure to each AR-67 form. The intracellular uptake of AR-67 was assessed over 48 h after exposing cells to carboxylate (1 μM, Fig. 8A) or lactone (1 μM, Fig. 8B). The lactone form was found to dominate the intracellular compartment regardless of which form was added in the cell culture media (Fig. 8). As expected, the intracellular AR-67 carboxylate amount was higher in the HeLa-OATP1B3 cells than in the HeLa-pIRES cells at all times and irrespective of the extracellular AR-67 form. However, the intracellular lactone amount was not different between the two cell lines, but it was significantly higher than the respective carboxylate one. The ratio of lactone to carboxylate increased from about 1, at 5 min, to 34 in the HeLa-pIRES and to 24 in the HeLa-OATP1B3 cell line after 48 h of incubation with AR-67 carboxylate (Fig. 8A). As expected, when AR-67 lactone was added in the cell culture media, it diffused rapidly into both cell lines and was about 100 times higher than the intracellular carboxylate at the 5-min time point (Fig. 8B). Although the amount of the intracellular carboxylate increased by almost 5 fold during the 48 h incubation, lactone dominated and lactone amounts were significantly higher than the carboxylate respective ones (Fig. 8B). Importantly, at steady state the intracellular lactone and carboxylate reached the same ratio regardless of which form was added in the cell culture media (Fig. 8).

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

Although carboxylate is the form that prevails in cell culture medium, after carboxylate (Fig. 7A) or lactone addition (Fig. 7B), this study demonstrates that the cells are exposed to significantly higher lactone amounts.

Notably, after 5 min of incubation with AR-67 carboxylate, intracellular AR-67 carboxylate accounted for 42 % and 55 % of the total drug in HeLa-pIRES and HeLa1B3 cells, respectively. This is the only time point with high intracellular AR-67 carboxylate amounts in the OATP1B3 cells compared to the respective lactone ones (Fig. 8A). To determine if this difference would be adequate to sensitize the OATP1B3 cells to carboxylate, we studied the cytotoxic effect of AR-67 carboxylate in Hela-pIRES and HeLa-OATP1B3 after drug exposure for 5 min, which was followed by 48 h growth. Although the IC_{50} values of carboxylate and lactone were lower in HeLa-OATP1B3 cells, as compared to HeLa-pIRES transfected cells, the differences were not statistically significant. As shown in Fig. 9A, the relatively increased exposure of OATP1B3 expressing cells to AR-67 carboxylate over the 5 min drug exposure did not result in significant sensitization. To verify this result we then assessed the effect of drug exposure on DNA damage by the induction of γ-H2AX (Furuta et al., 2003; Huang et al., 2004). In accordance with the lack of a transporter effect on cell viability (Fig. 9A), the degree of γ-H2AX activation after treatment with AR-67 carboxylate (Supplemental Figure 5) did not differ between the HeLa-pIRES and the HeLa-OATP1B3 transfected cells. For comparison, the cytotoxic effect of lactone was also studied under the same experimental conditions with similar results in both cell lines (Fig. 9B). Overall, HeLa-pIRES and HeLa-OATP1B3 were more sensitive to the lactone rather than the carboxylate when the IC_{50} values were used to evaluate the cytotoxic effect of AR-67 (Fig. 9).

Discussion

In this study, we demonstrated that BCRP and MDR1 transport AR-67 lactone. The MDCKII-BCRP cell line was found to be more resistant to AR-67 lactone treatment than the mock-transfected cells and MDR1 expression conferred resistance to the same anticancer agent using OVCAR-8 and NCI/ADR-RES cells. Additionally, we studied the interaction between AR-67 carboxylate with the uptake transporters OATP1B3 and OATP1B1 and showed that these transporters mediate its transport. However, the OATP1B3-expressing cell line did not show increased sensitivity to AR-67 carboxylate in *in vitro* systems. In an effort to study the factors that play a role on the antitumor activity of AR-67 *in vitro*, we quantified both AR-67 forms intracellularly after treatment with either AR-67 lactone or carboxylate. Interestingly, our intracellular uptake data indicated that the AR-67 lactone was favored intracellularly and overcame the OATP1B3-mediated increased intracellular uptake of AR-67 carboxylate by fifty-fold. This may explain why the uptake of AR-67 carboxylate does not sensitize OATP1B3 expressing cells.

Based on the physicochemical properties of the AR-67 lactone and carboxylate, we expected that both efflux and uptake transporters would interact with AR-67. Studies have shown that MDR1, and possibly BCRP, transport substrates that have the potential for partitioning into the lipid bilayer and are mostly lipid-soluble moieties with planar aromatic rings (Sharom, 2008), like AR-67 lactone. Conversely, OATP1B1 and OATP1B3 proteins selectively transport anions (Kalliokoski and Niemi, 2009), such as AR-67 carboxylate. Therefore, in our studies we adjusted the pH to ensure the exposure of cells to AR-67 lactone or carboxylate, as needed. Furthermore, the exposure and incubation times for transport and cell viability assays were considered in the context of lactone/carboxylate conversion kinetics.

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

Our studies showed that AR-67 lactone is a substrate of BCRP and MDR1 (Fig. 2 & 3). This is in accord with previous publications showing similar results with topotecan (de Vries et al., 2007; Sharom, 2008). Other camptothecin analogs have also been shown to be substrates of BCRP, which was reported to transport irinotecan, SN-38 and SN-38 glucuronide (Maliepaard et al., 1999; Nakatomi et al., 2001; Luo et al., 2002). Our studies also showed that AR-67 carboxylate is transported by OATP1B1 and OATP1B3 (Fig. 4). Previous studies have shown that irinotecan and its metabolite SN-38 are substrates of OATP1B1 (Nozawa et al., 2005). However, Yamaguchi et al showed that SN-38, but not irinotecan, can be taken up by OATP1B3 (Yamaguchi et al., 2008). Finally, *in vitro* studies have shown that among the lipophilic camptothecins, gimatecan and karenitecin, but not lurtotecan, are substrates of OATP1B1 (Oostendorp et al., 2009). However, the authors did not indicate which form was transported.

After identifying AR-67 lactone as a BCRP substrate, we used the MDCKII-pcDNA/BCRP cell lines to demonstrate that BCRP expression confers resistance to treatment with the lactone form (Fig. 5A). Our findings agree with findings from other studies on camptothecins. BCRP expressing lung cancer cell lines appeared to be more resistant to SN-38, than the parent cell line (Kawabata et al., 2001). Moreover, studies in metastatic hepatic tumor tissue indicated that BCRP might be implicated in the development of resistance specifically to irinotecan-based chemotherapy *in vivo* (Candeil et al., 2004). Despite the significant expression of BCRP in certain cancer types and especially in a small subpopulation of primitive stem cells (Katayama et al., 2009), definitive clinical relevance of BCRP expression in tumor tissues has yet to be proven. Additionally, as a result of the MDR1-mediated efflux of AR-67 in NCI/ADR-RES cells (Fig. 2B and D), as compared to OVCAR-8 cells, a resistance phenotype was observed (Fig. 5C). Previous studies have been suggestive of the prognostic role of MDR1 in leukemia patients treated with anthracyclines (Guerci et al., 1995; Smeets et al., 1997), established substrates of

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

MDR1. However, further studies are warrant to evaluate the impact of an MDR1-AR-67 interaction on clinical response in cancer patients.

Both *in vitro* and clinical studies have indicated that OATP1B3 facilitates the cytotoxic effect of paclitaxel (Smith et al., 2007; Svoboda et al., 2011). Here, we tested if OATP1B3 would sensitize cells after exposure to the anionic AR-67 carboxylate. Our studies showed that the observed cytotoxic effect was not different between HeLa-pIRES and HeLa-OATP1B3 cells irrespective of the exposure time to the carboxylate (Fig. 6). However, the cytotoxic effect of AR-67 carboxylate increased in a time-dependent manner consistent with its mechanism of action targeting cells primarily in their S-phase. In addition, comparable activation of the DNA damage marker γ-H2AX (Supplemental Figure 5) in HeLa-pIRES and HeLa-OATP1B3 cells after 5 min exposure to AR-67 carboxylate was consistent with the absence of OATP1B3 effect on cell viability (Fig. 9A). The quantification of the intracellular levels of both AR-67 forms suggest that the lipophilic lactone diffused freely in the cell while the transfer of the carboxylate form into the cytoplasm was a slow and transporter-mediated process, limited by saturable kinetics (Fig 8). Ultimately, the intracellular lactone/carboxylate steady state amounts, independent of the extracellular lactone/carboxylate kinetics (Fig. 7), suggested that the AR-67 carboxylate-induced cytotoxic effect was restricted due to its physicochemical properties. Notably, the same intracellular lactone-carboxylate amount ratio was observed when cells were exposed to either lactone or carboxylate suggesting that the interconversion between the two forms depended strictly on intracellular factors (Fig. 8). Rapid conversion of carboxylate to the lactone form in the cytoplasm due to protein-binding cannot be excluded. Moreover, although the intracellular pH would favor carboxylate formation, the AR-67 intracellular distribution pattern could be similar to the one observed for third generation lipophilic camptothecin gimatecan, which partitions into lysosomes where the lactone form is favored due to low pH conditions (Croce et al., 2004).

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

The microenvironment of solid tumors is characterized by hypoxia and low pH as a result of disorganized vasculature and underdeveloped lymphatic system (Gillies et al., 2002; Cardone et al., 2005). Previous studies have shown increased transport activity of BCRP and MDR1 at acidic conditions (Thews et al., 2006; Breedveld et al., 2007) and pH-sensitive transport activity of OATP1B3 (Leuthold et al., 2009). Therefore, further studies on the AR-67 cytotoxic effect under low pH conditions are necessary.

The first-in-human study showed that approximately 87 % of total AR-67 quantified in the blood was in the lactone form (Arnold et al., 2010). However, pharmacokinetic modeling of preclinical data demonstrated that the apparent stability was due to increased carboxylate clearance (Adane et al., 2010). Our findings suggest that OATP1B1 and OATP1B3 hepatic expression is the likely mechanism for this clearance and thus it may play a key role in the disposition of AR-67. These studies, therefore, demonstrate how transporters can play a key role in drug disposition without having a significant role in cellular uptake and tumor responsiveness, which is a static system.

Multiple studies have underlined the importance of efflux and uptake transporters in the disposition of anticancer agents. Furthermore, genetic polymorphisms in ABCG2 and ABCB1 that were identified in patients receiving irinotecan and diflomotecan, respectively, resulted in altered pharmacokinetics and pharmacodynamics (Sai et al., 2003; de Jong et al., 2004; Sparreboom et al., 2004; Zhou et al., 2005). Additionally, SLCO1B1 polymorphisms possibly related with the toxicity profile of irinotecan and other cytotoxic agents have been reported (Xiang et al., 2006; Han et al., 2008; Innocenti et al., 2009). Similarly, the clinical impact of *in vitro* identified OATP1B3 polymorphisms need to be explored further in well-designed large-scale human trials (Letschert et al., 2004; Smith et al., 2007). In line with these studies and our results, future studies should explore further the potential role of BCRP, MDR1, OATP1B1 and OATP1B3 polymorphisms on the transport of AR-67.

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

In conclusion, our study demonstrated that AR-67 lactone is a substrate of the ATP-binding cassette transporters MDR1 and BCRP. Additionally, both BCRP- and MDR1-mediated efflux of AR-67 lactone conferred resistance using *in vitro* models. The clinical significance of these findings need to be explored further. Although our *in vitro* work suggests that OATP1B3 expression might not increase the efficacy of AR-67 given to cancer patients expressing the transporter in the tumor tissue, we demonstrated that AR-67 carboxylate can be transported by the OATP1B1 and OATP1B3 transporters. Taking into consideration the liver-specific expression of these uptake transporters (Abe et al., 1999; Konig et al., 2000a) and the impact of carboxylate clearance on exposure to AR-67 (Adane et al., 2010), we reason that they may be playing a key role in the elimination of AR-67.

Authorship contributions

Participated in research design: Tsakalozou, Adane and Leggas

Conducted experiments: Tsakalozou, Adane, Kuo, Daily

Performed data analysis: Tsakalozou, Adane, Kuo, Daily and Leggas

Wrote or contributed to the writing of the manuscript: Tsakalozou, Adane, Kuo, Daily, Moscow and Leggas

References

Abe T, Kakyo M, Tokui T, Nakagomi R, Nishio T, Nakai D, Nomura H, Unno M, Suzuki M, Naitoh T, Matsuno S, and Yawo H (1999) Identification of a novel gene family encoding human liver-specific organic anion transporter LST-1. *J Biol Chem* **274:**17159-17163.

Abe T, Unno M, Onogawa T, Tokui T, Kondo TN, Nakagomi R, Adachi H, Fujiwara K, Okabe M, Suzuki T, Nunoki K, Sato E, Kakyo M, Nishio T, Sugita J, Asano N, Tanemoto M, Seki M, Date F, Ono K, Kondo Y, Shiiba K, Suzuki M, Ohtani H, Shimosegawa T, Iinuma K, Nagura H, Ito S, and Matsuno S (2001) LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers. *Gastroenterology* **120:**1689-1699.

Adane ED, Liu Z, Xiang TX, Anderson BD, and Leggas M (2010) Factors affecting the in vivo lactone stability and systemic clearance of the lipophilic camptothecin analogue AR-67. *Pharm Res* **27:**1416-1425.

Arnold SM, Rinehart JJ, Tsakalozou E, Eckardt JR, Fields SZ, Shelton BJ, DeSimone PA, Kee BK, Moscow JA, and Leggas M (2010) A phase I study of 7-t-butyldimethylsilyl-10-hydroxycamptothecin in adult patients with refractory or metastatic solid malignancies. *Clin Cancer Res* **16:**673-680.

Bom D, Curran DP, Kruszewski S, Zimmer SG, Thompson Strode J, Kohlhagen G, Du W, Chavan AJ, Fraley KA, Bingcang AL, Latus LJ, Pommier Y, and Burke TG (2000) The novel silatecan 7-tert-butyldimethylsilyl-10-hydroxycamptothecin displays high lipophilicity, improved human blood stability, and potent anticancer activity. *J Med Chem* **43:**3970-3980.

Bom D, Curran DP, Zhang J, Zimmer SG, Bevins R, Kruszewski S, Howe JN, Bingcang A, Latus LJ, and Burke TG (2001) The highly lipophilic DNA topoisomerase I inhibitor DB-67 displays elevated lactone levels in human blood and potent anticancer activity. *J Control Release* **74:**325-333.

Breedveld P, Pluim D, Cipriani G, Dahlhaus F, van Eijndhoven MA, de Wolf CJ, Kuil A, Beijnen JH, Scheffer GL, Jansen G, Borst P, and Schellens JH (2007) The effect of low pH on breast cancer resistance protein (ABCG2)-mediated transport of methotrexate, 7-hydroxymethotrexate, methotrexate diglutamate, folic acid, mitoxantrone, topotecan, and resveratrol in in vitro drug transport models. *Mol Pharmacol* **71:**240-249.

Candeil L, Gourdier I, Peyron D, Vezzio N, Copois V, Bibeau F, Orsetti B, Scheffer GL, Ychou M, Khan QA, Pommier Y, Pau B, Martineau P, and Del Rio M (2004) ABCG2 overexpression in colon cancer cells resistant to SN38 and in irinotecan-treated metastases. *Int J Cancer* **109:**848-854.

Cardone RA, Casavola V, and Reshkin SJ (2005) The role of disturbed pH dynamics and the Na+/H+ exchanger in metastasis. *Nat Rev Cancer* **5:**786-795.

Croce AC, Bottiroli G, Supino R, Favini E, Zuco V, and Zunino F (2004) Subcellular localization of the camptothecin analogues, topotecan and gimatecan. *Biochem Pharmacol* **67:**1035-1045.

Curran DP, Josien H, Bom D, Gabarda AE, and Du W (2000) The cascade radical annulation approach to new analogues of camptothecins. Combinatorial synthesis of silatecans and homosilatecans. *Ann N Y Acad Sci* **922:**112-121.

Daily A, Monks NR, Leggas M, and Moscow JA (2010) Abrogation of microcystin cytotoxicity by MAP kinase inhibitors and N-acetyl cysteine is confounded by OATPIB1 uptake activity inhibition. *Toxicon : official journal of the International Society on Toxinology* **55:**827-837.

de Bruin M, Miyake K, Litman T, Robey R, and Bates SE (1999) Reversal of resistance by GF120918 in cell lines expressing the ABC half-transporter, MXR. *Cancer Letters* **146:**117-126.

de Jong FA, Marsh S, Mathijssen RH, King C, Verweij J, Sparreboom A, and McLeod HL (2004) ABCG2 pharmacogenetics: ethnic differences in allele frequency and assessment of influence on irinotecan disposition. *Clin Cancer Res* **10:**5889-5894.

de Vries NA, Zhao J, Kroon E, Buckle T, Beijnen JH, and van Tellingen O (2007) P-glycoprotein and breast cancer resistance protein: two dominant transporters working together in limiting the brain penetration of topotecan. *Clin Cancer Res* **13:**6440-6449.

Evers R, Kool M, Smith AJ, van Deemter L, de Haas M, and Borst P (2000) Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. *Br J Cancer* **83:**366-374.

Furuta T, Takemura H, Liao ZY, Aune GJ, Redon C, Sedelnikova OA, Pilch DR, Rogakou EP, Celeste A, Chen HT, Nussenzweig A, Aladjem MI, Bonner WM, and Pommier Y (2003) Phosphorylation of histone H2AX and activation of Mre11, Rad50, and Nbs1 in response to replication-dependent DNA double-strand breaks induced by mammalian DNA topoisomerase I cleavage complexes. *J Biol Chem* **278:**20303-20312.

Gillies RJ, Raghunand N, Karczmar GS, and Bhujwalla ZM (2002) MRI of the tumor microenvironment. *J Magn Reson Imaging* **16:**430-450.

Grube M, Reuther S, Meyer Zu Schwabedissen H, Kock K, Draber K, Ritter CA, Fusch C, Jedlitschky G, and Kroemer HK (2007) Organic anion transporting polypeptide 2B1 and breast cancer resistance protein interact in the transepithelial transport of steroid sulfates in human placenta. *Drug Metab Dispos* **35:**30-35.

Guerci A, Merlin JL, Missoum N, Feldmann L, Marchal S, Witz F, Rose C, and Guerci O (1995) Predictive value for treatment outcome in acute myeloid leukemia of cellular daunorubicin accumulation and P-glycoprotein expression simultaneously determined by flow cytometry. *Blood* **85:**2147-2153.

Han JY, Lim HS, Shin ES, Yoo YK, Park YH, Lee JE, Kim HT, and Lee JS (2008) Influence of the organic anion-transporting polypeptide 1B1 (OATP1B1) polymorphisms on irinotecan-pharmacokinetics and clinical outcome of patients with advanced non-small cell lung cancer. *Lung Cancer* **59:**69-75.

Horn J, Jordan SL, Song L, Roberts MJ, Anderson BD, and Leggas M (2006) Validation of an HPLC method for analysis of DB-67 and its water soluble prodrug in mouse plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **844:**15-22.

Huang X, Okafuji M, Traganos F, Luther E, Holden E, and Darzynkiewicz Z (2004) Assessment of histone H2AX phosphorylation induced by DNA topoisomerase I and II inhibitors topotecan and mitoxantrone and by the DNA cross-linking agent cisplatin. *Cytometry A* **58:**99-110.

Innocenti F, Kroetz DL, Schuetz E, Dolan ME, Ramirez J, Relling M, Chen P, Das S, Rosner GL, and Ratain MJ (2009) Comprehensive pharmacogenetic analysis of irinotecan neutropenia and pharmacokinetics. *J Clin Oncol* **27:**2604-2614.

Kalliokoski A and Niemi M (2009) Impact of OATP transporters on pharmacokinetics. *Br J Pharmacol* **158:**693-705.

Kalvass JC and Pollack GM (2007) Kinetic considerations for the quantitative assessment of efflux activity and inhibition: implications for understanding and predicting the effects of efflux inhibition. *Pharm Res* **24:**265-276.

Katayama R, Koike S, Sato S, Sugimoto Y, Tsuruo T, and Fujita N (2009) Dofequidar fumarate sensitizes cancer stem-like side population cells to chemotherapeutic drugs by inhibiting ABCG2/BCRP-mediated drug export. *Cancer Sci* **100:**2060-2068.

Kawabata S, Oka M, Shiozawa K, Tsukamoto K, Nakatomi K, Soda H, Fukuda M, Ikegami Y, Sugahara K, Yamada Y, Kamihira S, Doyle LA, Ross DD, and Kohno S (2001) Breast cancer resistance protein directly confers SN-38 resistance of lung cancer cells. *Biochem Biophys Res Commun* **280:**1216-1223.

Konig J, Cui Y, Nies AT, and Keppler D (2000a) Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide. *J Biol Chem* **275:**23161-23168.

Konig J, Cui Y, Nies AT, and Keppler D (2000b) A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. *Am J Physiol Gastrointest Liver Physiol* **278:**G156-164.

Lee W, Belkhiri A, Lockhart AC, Merchant N, Glaeser H, Harris EI, Washington MK, Brunt EM, Zaika A, Kim RB, and El-Rifai W (2008) Overexpression of OATP1B3 confers apoptotic resistance in colon cancer. *Cancer Res* **68:**10315-10323.

Letschert K, Keppler D, and Konig J (2004) Mutations in the SLCO1B3 gene affecting the substrate specificity of the hepatocellular uptake transporter OATP1B3 (OATP8). *Pharmacogenetics* **14:**441-452.

Leuthold S, Hagenbuch B, Mohebbi N, Wagner CA, Meier PJ, and Stieger B (2009) Mechanisms of pH-gradient driven transport mediated by organic anion polypeptide transporters. *Am J Physiol Cell Physiol* **296:**C570-582.

Lockhart AC, Harris E, Lafleur BJ, Merchant NB, Washington MK, Resnick MB, Yeatman TJ, and Lee W (2008) Organic anion transporting polypeptide 1B3 (OATP1B3) is overexpressed in colorectal tumors and is a predictor of clinical outcome. *Clin Exp Gastroenterol* **1:**1-7.

Luo FR, Paranjpe PV, Guo A, Rubin E, and Sinko P (2002) Intestinal transport of irinotecan in Caco-2 cells and MDCK II cells overexpressing efflux transporters Pgp, cMOAT, and MRP1. *Drug Metab Dispos* **30:**763-770.

Maliepaard M, van Gastelen MA, de Jong LA, Pluim D, van Waardenburg RC, Ruevekamp-Helmers MC, Floot BG, and Schellens JH (1999) Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. *Cancer Res* **59:**4559-4563.

Monks NR, Liu S, Xu Y, Yu H, Bendelow AS, and Moscow JA (2007) Potent cytotoxicity of the phosphatase inhibitor microcystin LR and microcystin analogues in OATP1B1- and OATP1B3-expressing HeLa cells. *Mol Cancer Ther* **6:**587-598.

Muto M, Onogawa T, Suzuki T, Ishida T, Rikiyama T, Katayose Y, Ohuchi N, Sasano H, Abe T, and Unno M (2007) Human liver-specific organic anion transporter-2 is a potent prognostic factor for human breast carcinoma. *Cancer Sci* **98:**1570-1576.

Nakatomi K, Yoshikawa M, Oka M, Ikegami Y, Hayasaka S, Sano K, Shiozawa K, Kawabata S, Soda H, Ishikawa T, Tanabe S, and Kohno S (2001) Transport of 7-ethyl-10-hydroxycamptothecin (SN-38)

by breast cancer resistance protein ABCG2 in human lung cancer cells. *Biochem Biophys Res Commun* **288:**827-832.

Nozawa T, Minami H, Sugiura S, Tsuji A, and Tamai I (2005) Role of organic anion transporter OATP1B1 (OATP-C) in hepatic uptake of irinotecan and its active metabolite, 7-ethyl-10-hydroxycamptothecin: in vitro evidence and effect of single nucleotide polymorphisms. *Drug Metab Dispos* **33:**434-439.

Oostendorp RL, van de Steeg E, van der Kruijssen CM, Beijnen JH, Kenworthy KE, Schinkel AH, and Schellens JH (2009) Organic anion-transporting polypeptide 1B1 mediates transport of Gimatecan and BNP1350 and can be inhibited by several classic ATP-binding cassette (ABC) B1 and/or ABCG2 inhibitors. *Drug Metab Dispos* **37:**917-923.

Pressler H, Sissung TM, Venzon D, Price DK, and Figg WD (2011) Expression of OATP family members in hormone-related cancers: potential markers of progression. *PLoS One* **6:**e20372.

Sai K, Kaniwa N, Itoda M, Saito Y, Hasegawa R, Komamura K, Ueno K, Kamakura S, Kitakaze M, Shirao K, Minami H, Ohtsu A, Yoshida T, Saijo N, Kitamura Y, Kamatani N, Ozawa S, and Sawada J (2003) Haplotype analysis of ABCB1/MDR1 blocks in a Japanese population reveals genotype-dependent renal clearance of irinotecan. *Pharmacogenetics* **13:**741-757.

Seithel A, Eberl S, Singer K, Auge D, Heinkele G, Wolf NB, Dorje F, Fromm MF, and Konig J (2007) The influence of macrolide antibiotics on the uptake of organic anions and drugs mediated by OATP1B1 and OATP1B3. *Drug Metab Dispos* **35:**779-786.

Sharom FJ (2008) ABC multidrug transporters: structure, function and role in chemoresistance. *Pharmacogenomics* **9:**105-127.

Smeets M, Raymakers R, Vierwinden G, Pennings A, van de Locht L, Wessels H, Boezeman J, and de Witte T (1997) A low but functionally significant MDR1 expression protects primitive haemopoietic progenitor cells from anthracycline toxicity. *Br J Haematol* **96:**346-355.

Smith NF, Marsh S, Scott-Horton TJ, Hamada A, Mielke S, Mross K, Figg WD, Verweij J, McLeod HL, and Sparreboom A (2007) Variants in the SLCO1B3 gene: interethnic distribution and association with paclitaxel pharmacokinetics. *Clin Pharmacol Ther* **81:**76-82.

Sparreboom A, Gelderblom H, Marsh S, Ahluwalia R, Obach R, Principe P, Twelves C, Verweij J, and McLeod HL (2004) Diflomotecan pharmacokinetics in relation to ABCG2 421C>A genotype. *Clin Pharmacol Ther* **76:**38-44.

Staker BL, Hjerrild K, Feese MD, Behnke CA, Burgin AB, Jr., and Stewart L (2002) The mechanism of topoisomerase I poisoning by a camptothecin analog. *Proc Natl Acad Sci U S A* **99:**15387-15392.

Svoboda M, Wlcek K, Taferner B, Hering S, Stieger B, Tong D, Zeillinger R, Thalhammer T, and Jager W (2011) Expression of organic anion-transporting polypeptides 1B1 and 1B3 in ovarian cancer cells: relevance for paclitaxel transport. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **65:**417-426.

Thews O, Gassner B, Kelleher DK, Schwerdt G, and Gekle M (2006) Impact of extracellular acidity on the activity of P-glycoprotein and the cytotoxicity of chemotherapeutic drugs. *Neoplasia* **8:**143-152.

Wang L, Leggas M, Empey PE, and McNamara PJ (2012) Stereoselective interaction of pantoprazole with ABCG2. II. In vitro flux analysis. *Drug Metab Dispos* **40:**1024-1031.

Xiang X, Jada SR, Li HH, Fan L, Tham LS, Wong CI, Lee SC, Lim R, Zhou QY, Goh BC, Tan EH, and Chowbay B (2006) Pharmacogenetics of SLCO1B1 gene and the impact of *1b and *15 haplotypes on irinotecan disposition in Asian cancer patients. *Pharmacogenet Genomics* **16**:683-691.

Yamaguchi H, Kobayashi M, Okada M, Takeuchi T, Unno M, Abe T, Goto J, Hishinuma T, and Mano N (2008) Rapid screening of antineoplastic candidates for the human organic anion transporter OATP1B3 substrates using fluorescent probes. *Cancer Lett* **260**:163-169.

Zhou Q, Sparreboom A, Tan EH, Cheung YB, Lee A, Poon D, Lee EJ, and Chowbay B (2005) Pharmacogenetic profiling across the irinotecan pathway in Asian patients with cancer. *Br J Clin Pharmacol* **59**:415-424.

Figures:

Fig. 1.

pH-dependent interconversion between the lactone and carboxylate form of the camptothecin analogue AR-67.

Fig. 2

Effect of BCRP and MDR1 expression on the intracellular AR-67 amounts in MDCKII-pcDNA/BCRP and OVCAR-8/NCI/ADR-RES cells. (A) MDCKII-pcDNA/BCRP and (B) OVCAR-8/NCI/ADR-RES cells were incubated with 0.125, 0.25, 0.5, 0.75 and 1 µM of AR-67 lactone for 5 min and the AR-67 intracellular amount was evaluated. The effect of the dual BCRP/MDR1 inhibitor GF120918 was tested by treating (C) MDCKII-pcDNA/BCRP and (D) OVCAR-8/NCI/ADR-RES cells without (open bars) and with (solid bars) GF120918 (5 µM for 1 h) prior to incubating with 1 µM AR-67 lactone for 5 min. At the end of the incubation periods, the cells were lysed and the intracellular AR-67 was quantified using HPLC as mentioned in the Materials and Methods section. Data are represented as mean (n=3) ± SD. Statistical analysis was performed using unpaired t-test, statistical significance for ** $p<0.01$ and *** $p<0.001$.

Fig. 3.

Effect of BCRP on the vectorial transport of AR-67 in MDCKII-pcDNA/BCRP cells. Transepithelial transport of 5 µM AR-67 lactone in MDCKII-pcDNA and MDCKII-BCRP cells apical to basolateral (A, B) and basolateral to apical (C, D) in the presence (open squares) and absence (open inverted triangles) of the inhibitor GF120918 (5 µM). AR-67 was quantified using HPLC as determined in the Materials and Methods section. Data are represented as mean (n=3) ± SD.

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 4.

Effect of OATP1B3 and OATP1B1 expression on the intracellular AR-67 amounts in HeLa-pIRES/OATP1B3 and RKO-pIRES/OATP1B1 cells. (A) HeLa-pIRES/OATP1B3 and (B) RKO-pIRES/OATP1B1 cells were incubated with 0.5, 1, 1.5, 2 and 3 µM of AR67 carboxylate for 1 min to study the dose-dependent uptake of the carboxylate form. The estimated K_m values for OATP1B3 and OATP1B1 were 0.32 (0.0-0.8316) µM and 2.58 (0.0-6.253) µM, respectively. They were obtained by using the Michaelis-Menten equation to fit the transporter mediated uptake of AR-67 as described in the Materials and Methods section and are reported as mean (95 % confidence interval). (C) HeLa-pIRES/OATP1B3 and (D) RKO-pIRES/OATP1B1 cells were incubated with 1 µM of AR-67 carboxylate for 5 and 10 min, respectively (open bars). The inhibitory effect of BSP (50 µM) was studied by pretreating for 10 min prior to exposure to AR-67 carboxylate as described previously for all cell lines (solid bars). Intracellular AR-67 was quantified using HPLC as determined in the Materials and Methods section. Data are represented as mean (n=3) ± SD. Statistical analysis was performed using unpaired t-test, statistical significance for ** $p<0.01$ and *** $p<0.001$.

Fig. 5.

BCRP and MDR1 decrease the cytotoxicity of AR-67 lactone. (A) Following 72 h drug incubation, IC_{50} values were estimated in MDCKII-pcDNA (0.21 µM, [0.11-0.40]) and MDCKII-BCRP (2.37 µM, [1.41-4.00], ** $p<0.01$, unpaired Student t-test). (B) After pretreatment (30 min) and in the presence of GF120918 and AR-67 lactone (72 h), IC_{50} values were obtained in MDCKII-pcDNA (0.88 µM, [0.72-1.09]) and MDCKII-BCRP (1.02 µM, [0.79-1.32]. (C) Similarly, IC_{50} values were estimated in OVCAR-8 (0.027 µM, [0.018-0.043]) and NCI/ADR-RES (1.16 µM,

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

[0.79-1.70], *** p<0.0001, unpaired Student t-test). (D) After pretreatment (30 min) and in the presence of GF120918 and AR-67 lactone (72 h), IC_{50} values were obtained in OVCAR-8 (0.053 µM, [0.044-0.064]) and NCI/ADR-RES cells (0.095 µM, [0.044-0.21]. The AR-67 lactone doses ranged from 10^{-7} to 21 µM. IC_{50} parameters and best fit lines were estimated by nonlinear regression analysis. Data are plotted as mean ± SD (n=3) and IC_{50} values (µM) are reported as (mean, [95 % confidence interval]).

Fig. 6.

Time-dependent cytotoxic effect of AR-67 carboxylate on HeLa-pIRES and HeLa-OATP1B3 cell lines. (A-D) HeLa-pIRES and HeLa-OATP1B3 cell lines were exposed to AR-67 carboxylate for 30 min (A), 1 (B), 3 (C) and 48 (D) h, as described in the Materials and Methods section. The AR-67 carboxylate doses ranged from 10^{-3} to 210 µM. (E) Summary of the cytotoxicity of AR-67 carboxylate on HeLa-pIRES and HeLa-OATP1B3 cells studied under the experimental conditions described in panels A to D. IC_{50} values to reflect the effect of AR-67 on cell viability were used. Cell viability was assessed at the end of the treatment as described in the Materials and Methods section. Nonlinear regression analysis was performed to model the data (solid line). Data points on the graphs are reported as mean (n=3) ± SD and IC_{50} values (µM) are reported as mean, (95 % confidence interval).

Fig. 7.

Stability of AR-67 in cell culture media. The time course of AR-67 interconversion was studied in cell culture media (pH 7.4, 37 ºC, 5 % CO_2) after the addition of 1 µM of AR-67 carboxylate (A) or 1 µM of AR-67 lactone (B). Data are represented as the % ratio concentration of carboxylate or lactone over total drug.

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 8.

Time course of AR-67 carboxylate and lactone intracellular amount in HeLa-pIRES and HeLa-OATP1B3 cells after incubation with AR-67 carboxylate or lactone. HeLa-pIRES and HeLa-OATP1B3 cells were incubated with 1 μM of the AR-67 forms, carboxylate (A) and lactone (B). AR-67 forms intracellularly were quantified by HPLC as described in the Materials and Methods section. Data are represented as mean (n=3) ± SD.

Fig. 9.

Effect of AR-67 carboxylate and lactone on HeLa-pIRES and HeLa-OATP1B3 cell lines. (A) HeLa-pIRES and HeLa-OATP1B3 cells were treated with AR-67 carboxylate for 5 min, cells were washed, drug-free media was added in the wells and cells were allowed to grow for 48 h before assessing cell viability. The estimated IC_{50} values were 40.32 μM (16.36-99.33) and 13.08 μM (8.29-20.62) for HeLa-pIRES and HeLa-OATP1B3 cells, respectively. (B) HeLa-pIRES and HeLa-OATP1B3 cells were treated with AR-67 lactone for 5 min, cells were washed and drug-free media was added in the wells. The estimated IC_{50} values were 1.08 μM (0.20-5.70) and 0.23 μM (0.028-1.93) for HeLa-pIRES and HeLa-OATP1B3 cells, respectively. Data analysis to obtain IC_{50} values was performed using nonlinear regression (solid line). Data are presented as mean (n=3) ± SD and IC_{50} values (μM) are reported as mean (95 % confidence interval).

DMD Fast Forward. Published on April 25, 2013 as DOI: 10.1124/dmd.112.050021
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1

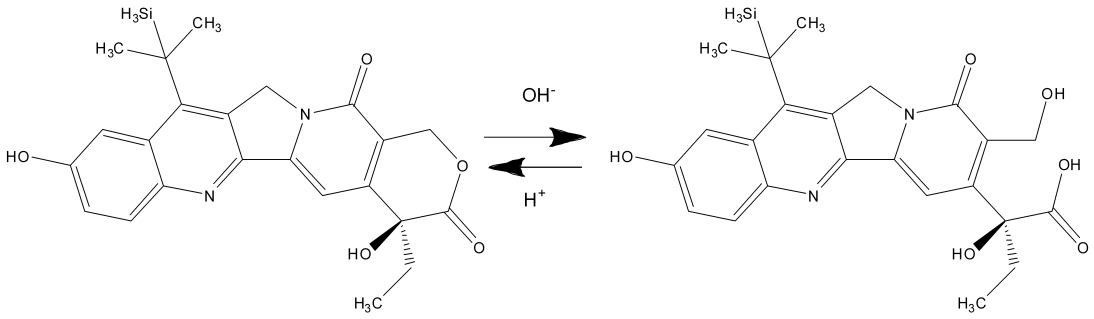
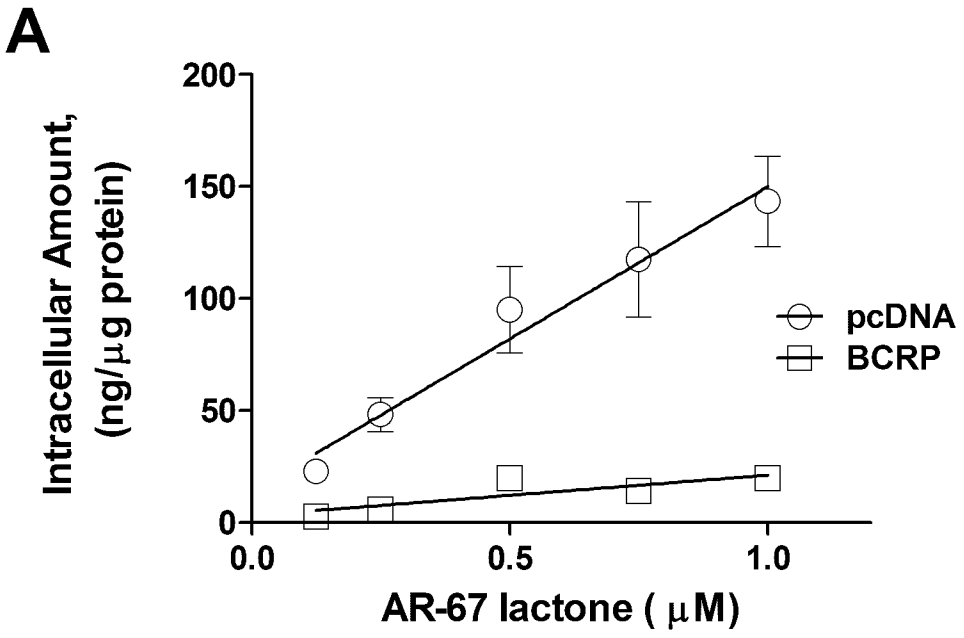
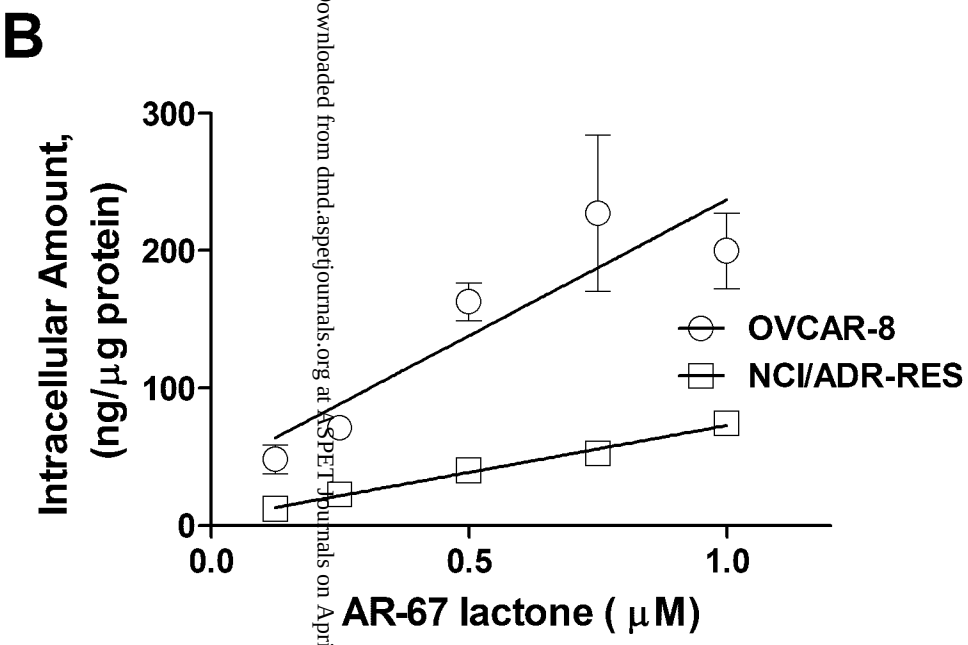
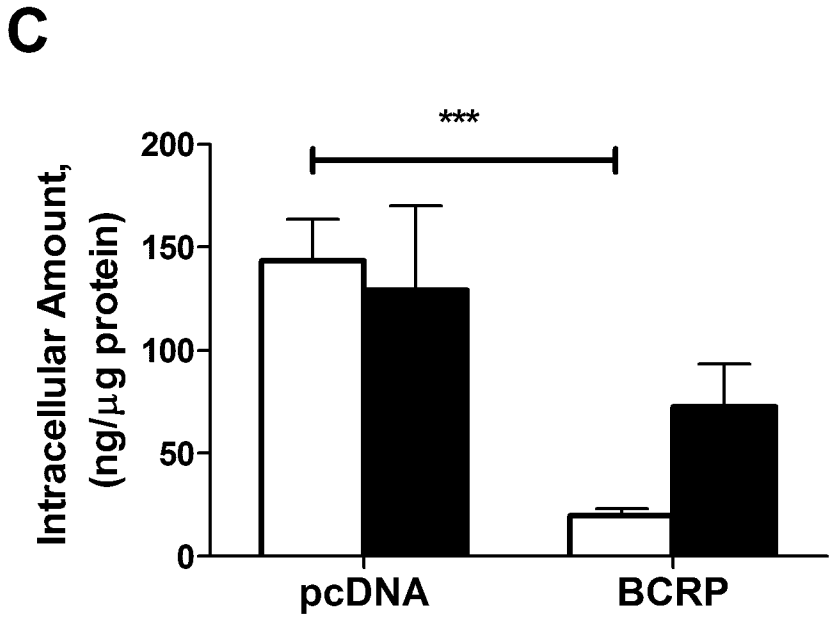


Figure 2







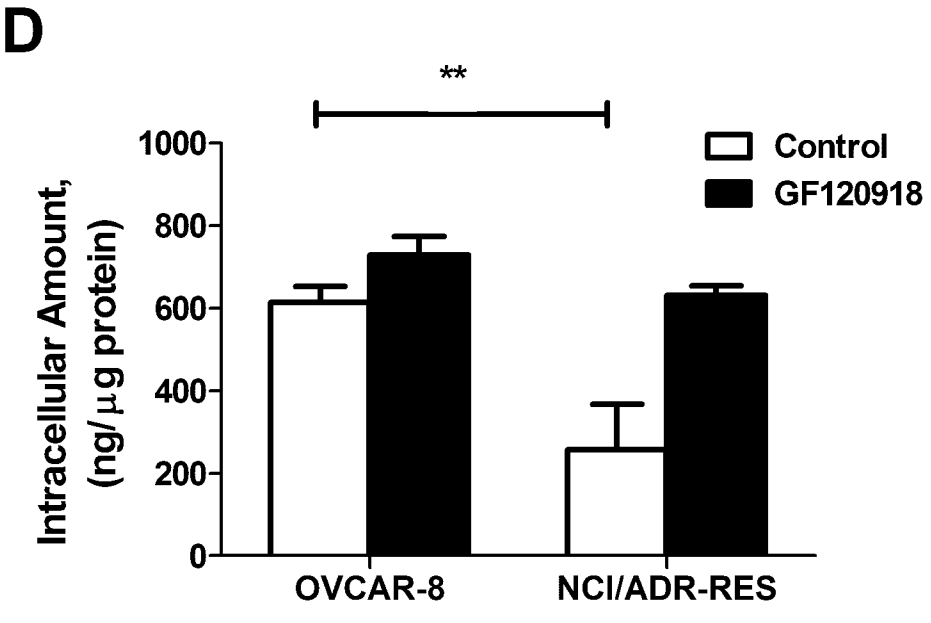
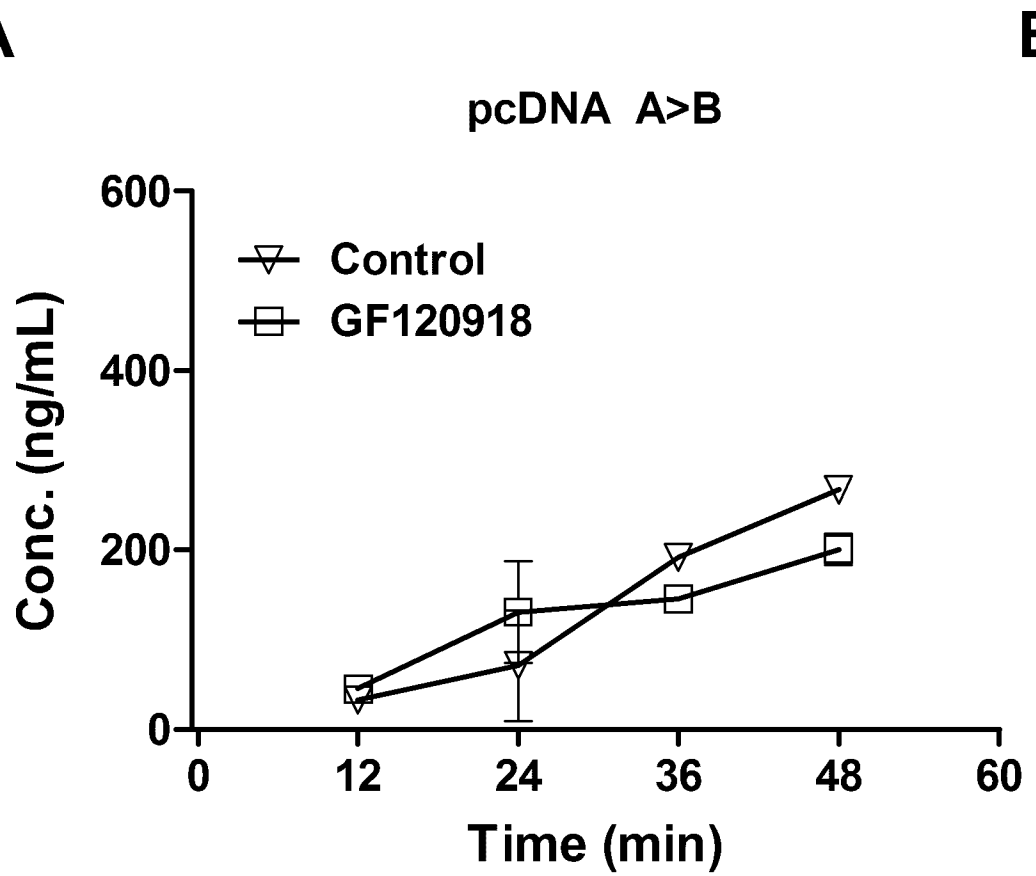
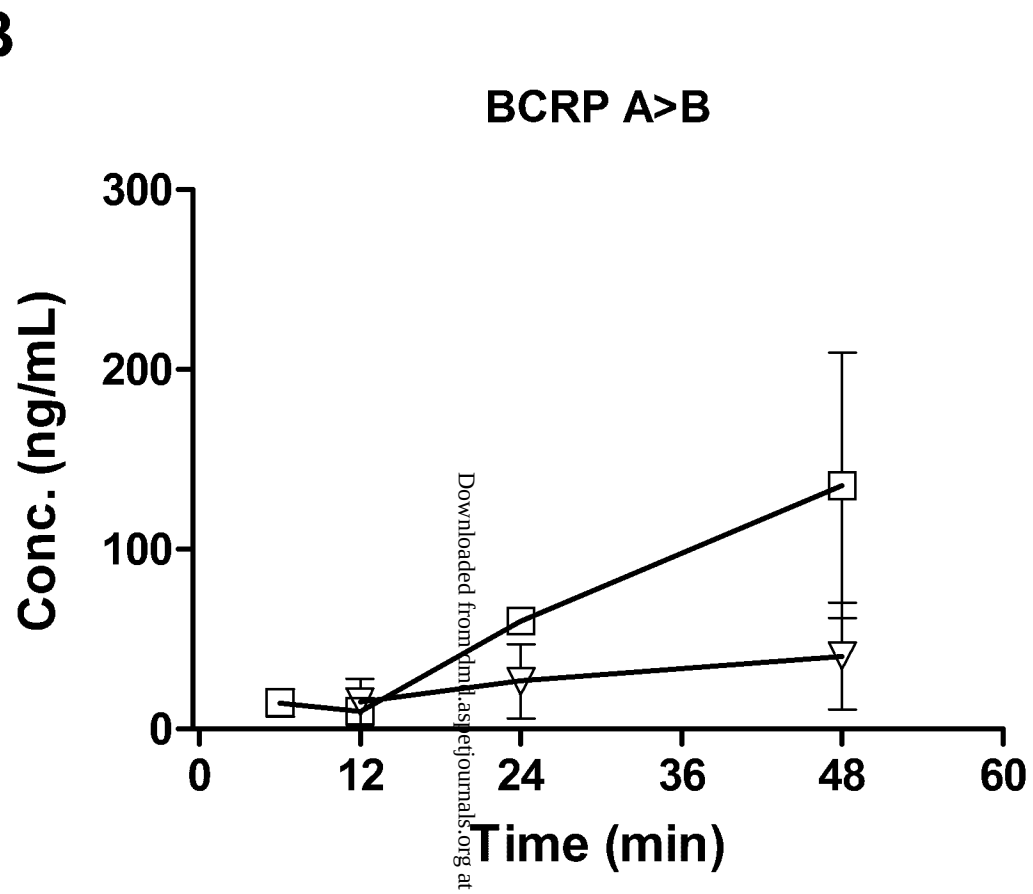
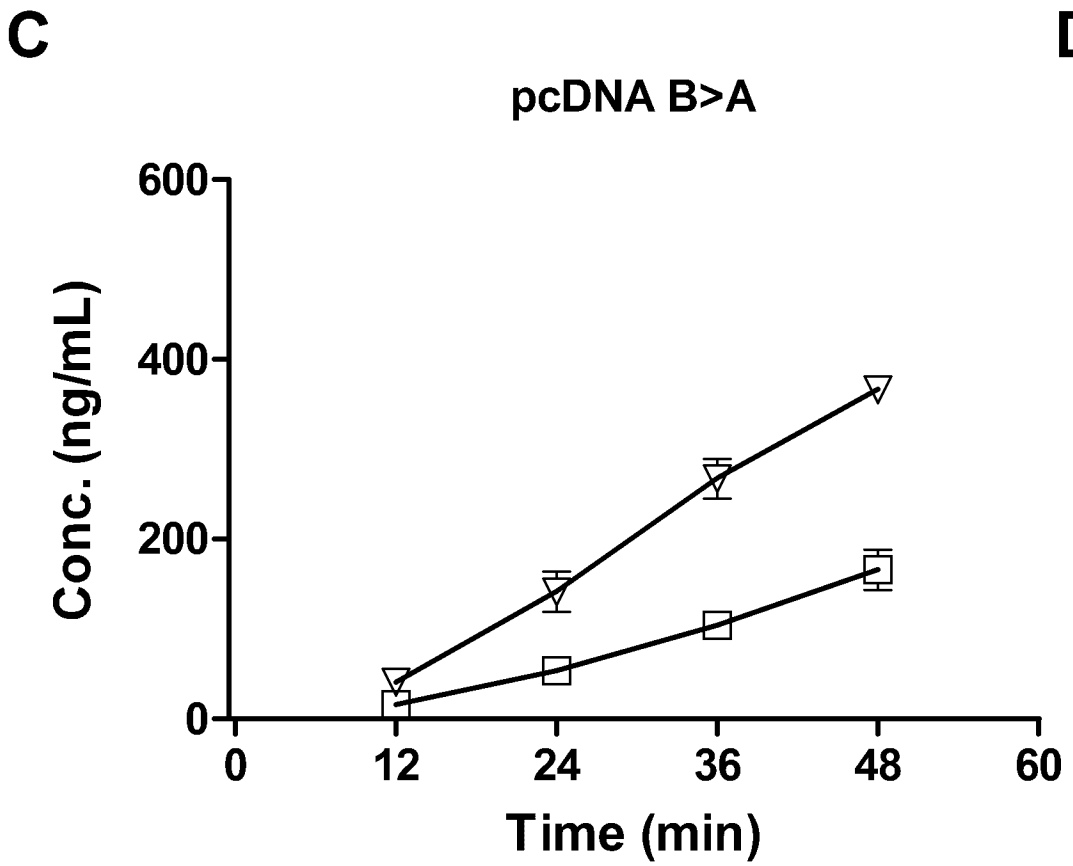


Figure 3







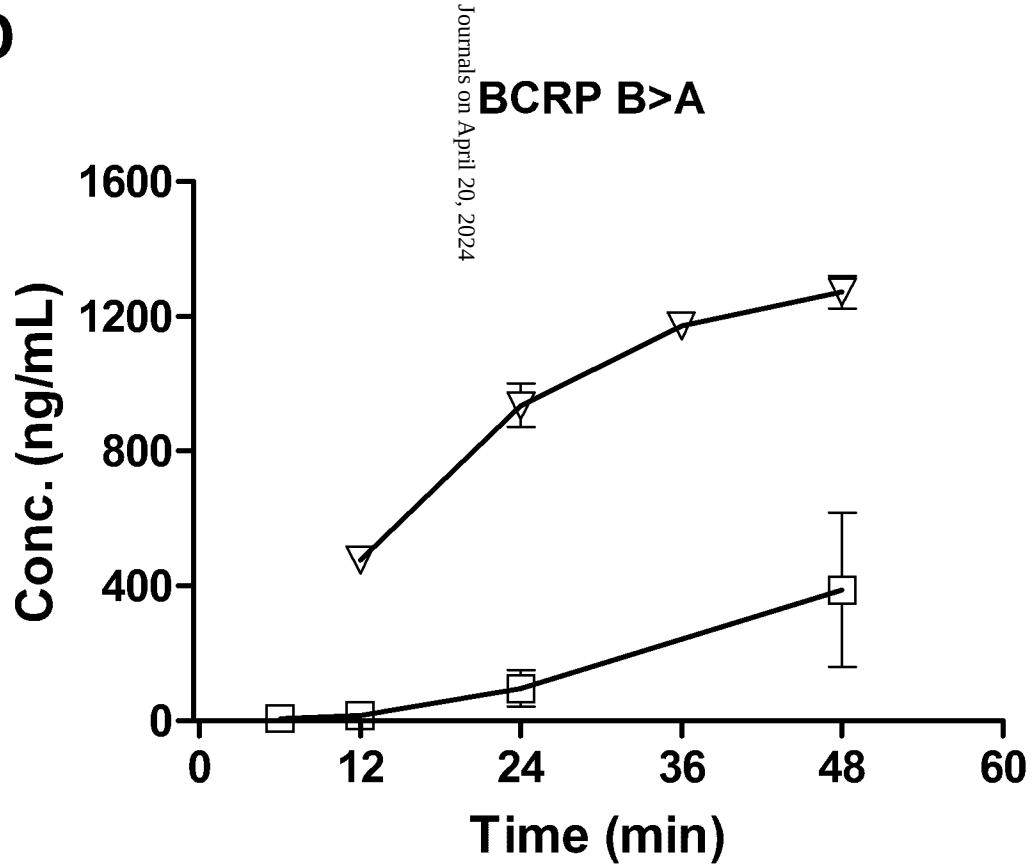


Figure 4

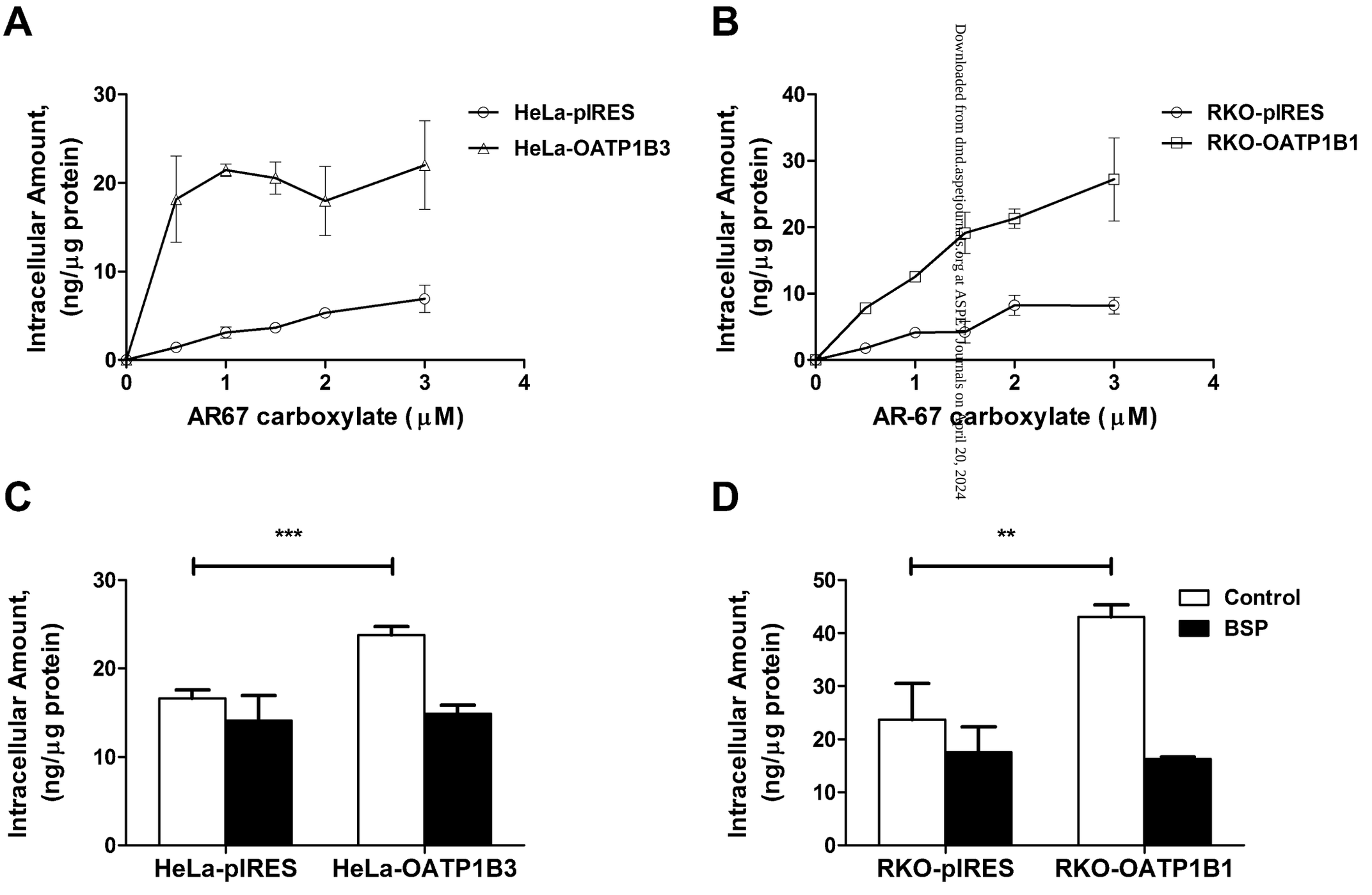


Figure 5

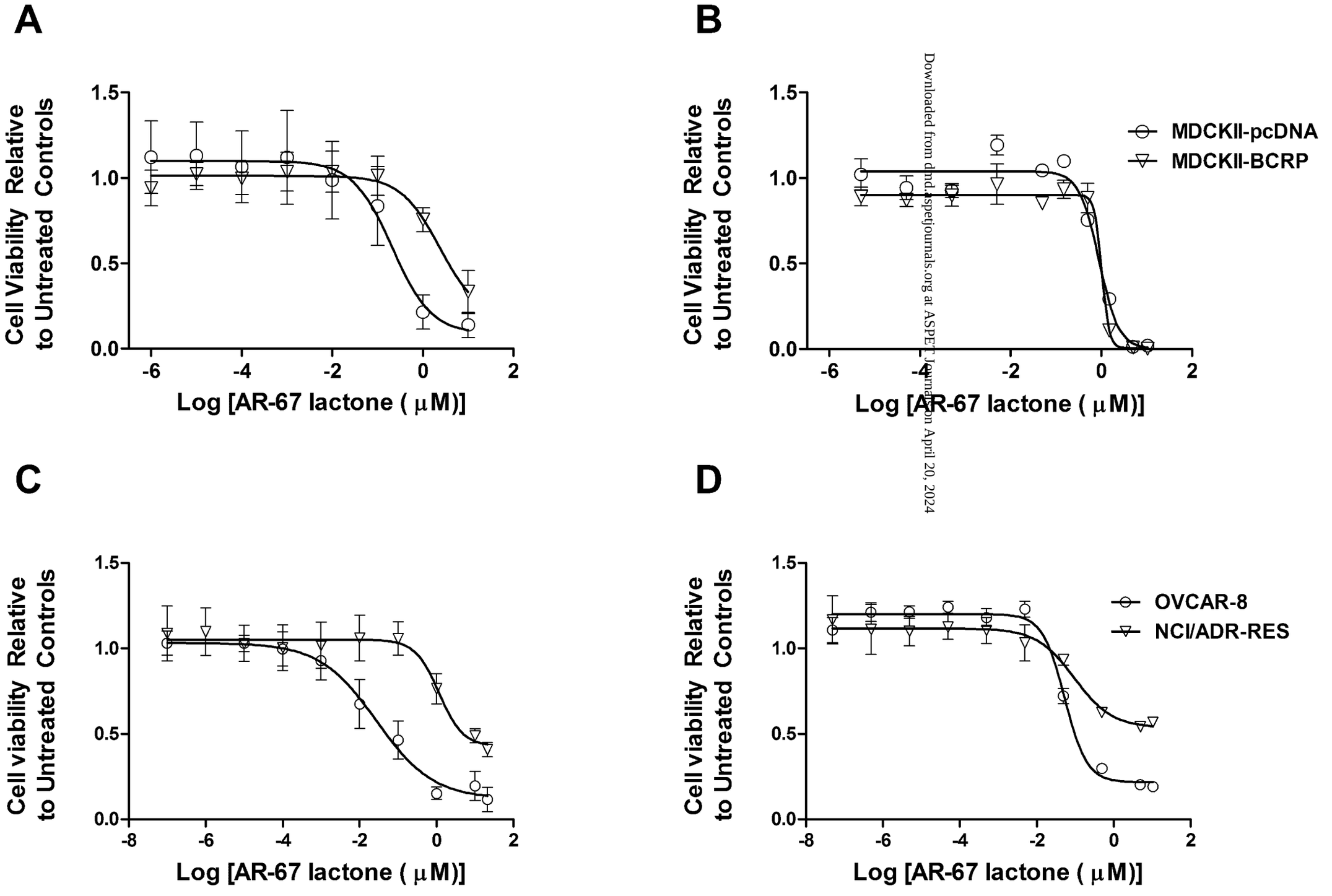


Figure 6

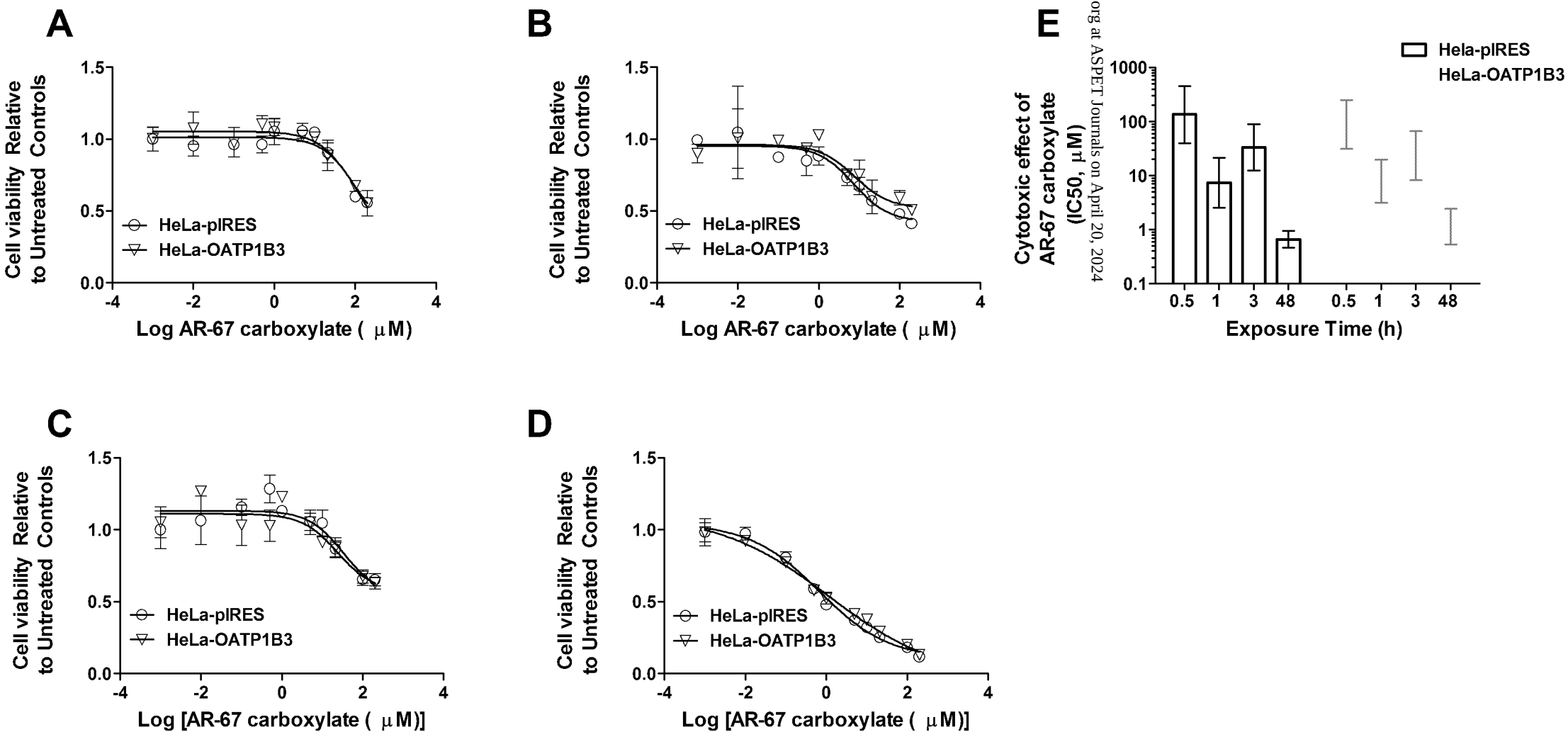
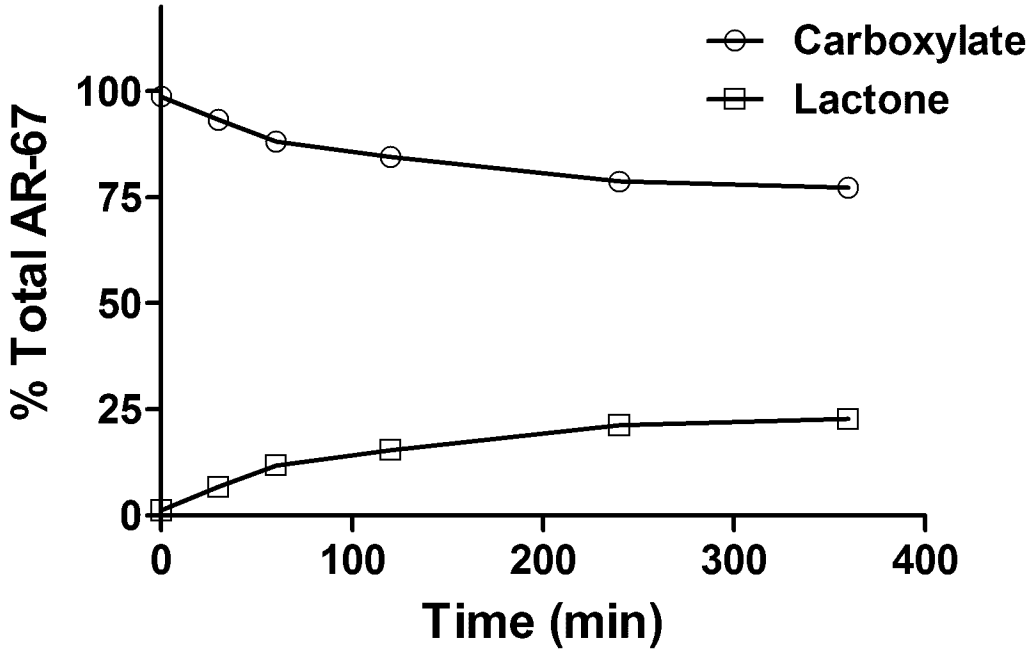


Figure 7

A


Carboxylate
Lactone
% Total AR-67
100
75
50
25
0
0
100
200
300
400
Time (min)

B

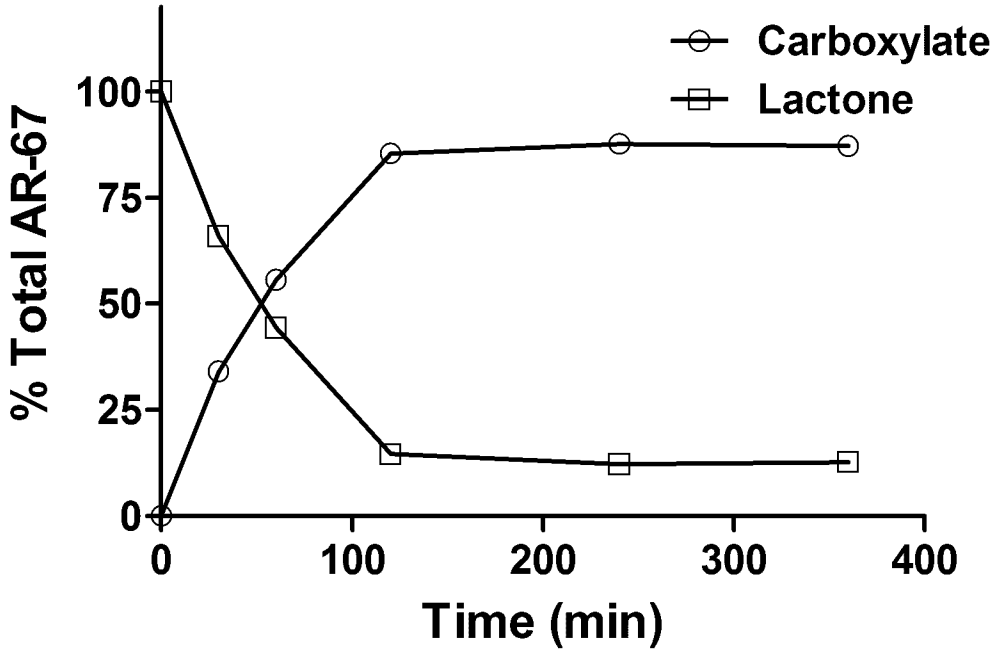
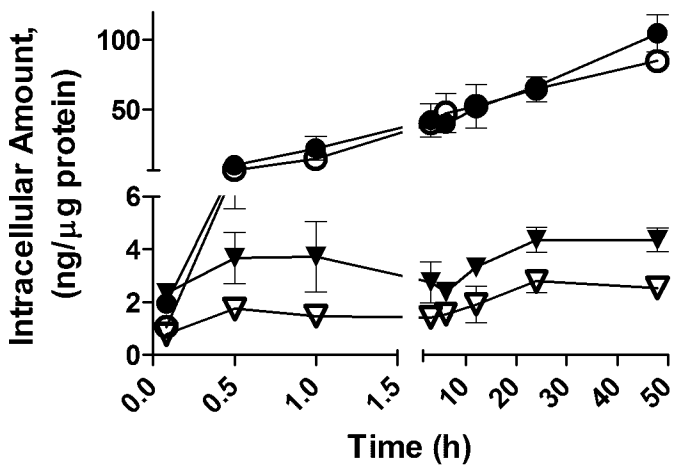

Carboxylate
Lactone
% Total AR-67
100
75
50
25
0
0
100
200
300
400
Time (min)

Figure 8

A



B

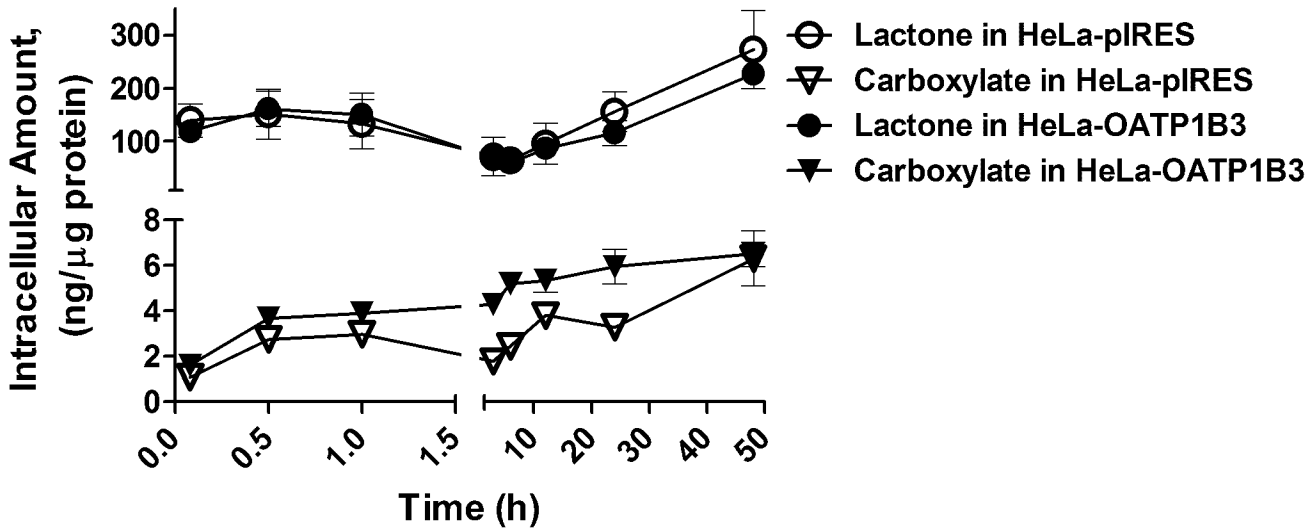
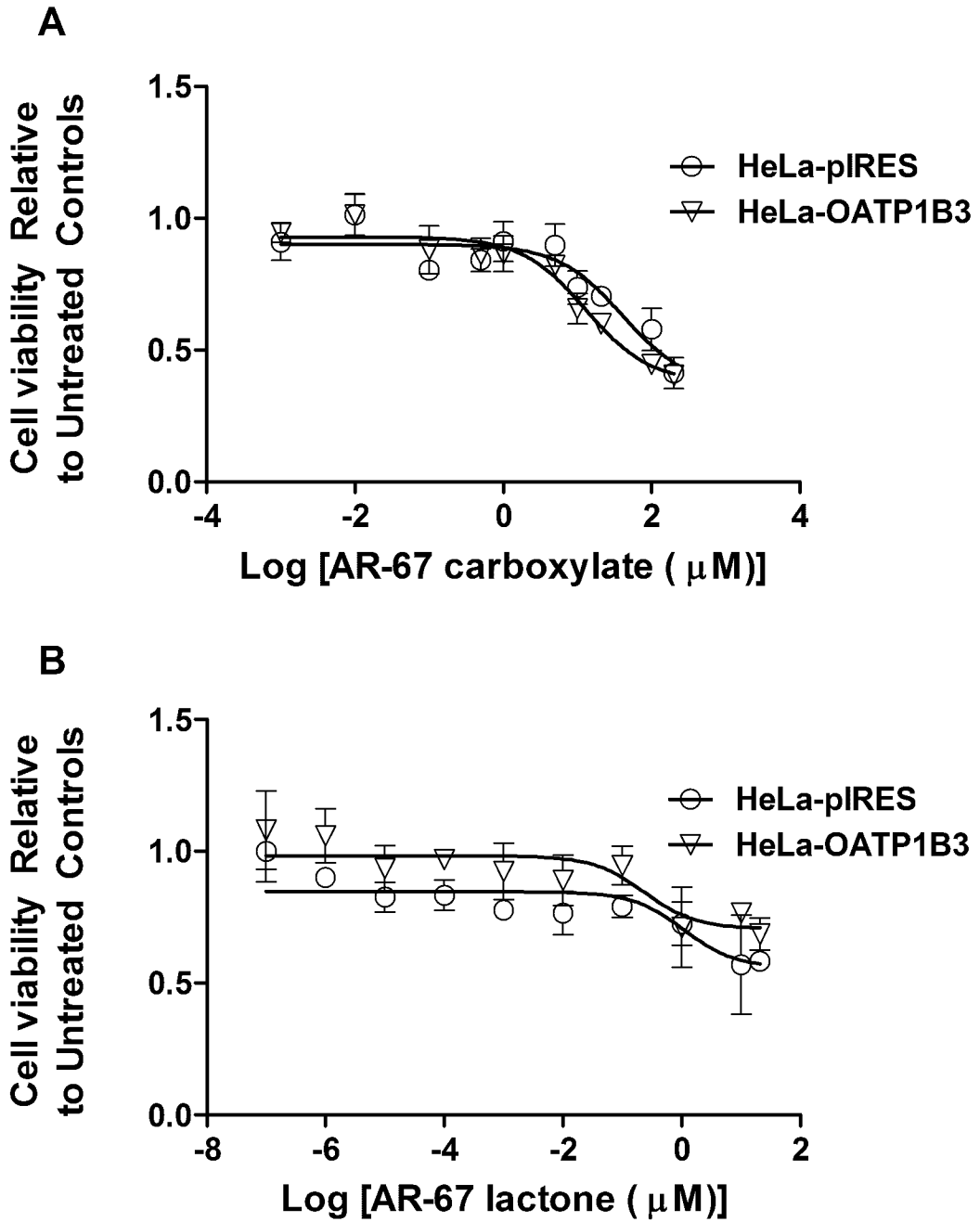


Figure 9


A
Cell viability Relative to Untreated Controls
1.5
1.0
0.5
0.0
-4
-2
0
2
4
Log [AR-67 carboxylate (μM)]
HeLa-pIRES
HeLa-OATP1B3
B
Cell viability Relative to Untreated Controls
1.5
1.0
0.5
0.0
-8
-6
-4
-2
0
2
Log [AR-67 lactone (μM)]
HeLa-pIRES
HeLa-OATP1B3

Supplemental Material

The effect of BCRP, MDR1 and OATP1B3 on the antitumor efficacy of the lipophilic camptothecin AR-67 *in vitro*

Eleftheria Tsakalozou, Eyob D. Adane, Kuei-Ling Kuo, Abigail Daily, Jeffrey A. Moscow and Markos Leggas

Drug Metabolism and Disposition

Materials and Methods

Immunoblot Analysis.

BCRP, MDR1 and Topoisomerase 1 (Top1) protein expression in transfected cell lines was confirmed by Western blot analysis using standard procedures. Commercially available antibodies clone BXP-21 and clone F4 (Kamiya Biomedical Company, Seattle, WA) and Top1 (Abcam, Cambridge, MA) were used for detection of BCRP (1:100), MDR1 (1:1000) and Top1 (1:500). Actin (Sigma-Aldrich, St. Louis, MO) was used as a loading control.

Hoechst 33342 and Resazurin Assays.

BCRP functional activity was demonstrated by measuring intracellular accumulation of (2 μM) Hoechst 33342 (Invitrogen, Carlsbad, CA) (Sharom, 2008) in the MDCKII-pcDNA and MDCKII-BCRP cells in the presence and absence of 4 μM GF120918 (de Bruin et al., 1999; Evers et al., 2000) (a gift from GlaxoSmithKline) as previously described (Wang et al., 2008). Fluorescence was measured in cell lysates using a microplate reader and values were normalized to protein concentration (Pierce BCA protein Kit, Fisher Scientific). MDR1 functional activity in OVCAR-8

and NCI/ADR-RES cell lines was evaluated using Vinblastine (Sigma-Aldrich, St. Louis, MO) and Vorinostat (SAHA) (Cayman Chemical Company, Ann Arbor, MI). The cell lines were incubated with either Vinblastine (10^{-9} – 1 μM) or SAHA (10^{-6} – 756 μM) for 72 hours and cell viability was assessed using a resazuring assay. IC_{50} values were obtained by performing non-linear regression analysis (GraphPad Prism, version 5.02 for Windows, GraphPad Software Inc., San Diego, USA) on the normalized to untreated cells as described in detail in the Materials and Methods section.

Efflux activity of BCRP.

The data collected when studying the BCRP-mediated transport of AR-67 lactone in polarized MDCKII transfected cells grown on Transwell® inserts (Fig. 3) were used to quantify the efflux activity of BCRP in the presence and absence of GF120918. Apparent permeability values and efflux ratios were estimated (Kalvass and Pollack, 2007; Sun and Pang, 2008). Apparent permeability values ($P_{app,A>B}$ and $P_{app,B>A}$) were obtained for surface of the transwell membrane S=4.5cm^2 and after quantifying the AR-67 concentration at the donor side at t=0 h using HPLC (Horn et al., 2006). Efflux ratios were equal to $P_{app,B>A}/P_{app,A>B}$. Standard deviation values were calculated with the propagation error theory.

RT-PCR Analysis.

Hela-pIRES, Hela-OATP1B3, RKO-pIRES and RKO-OATP1B1 were grown to confluence in growth medium. RNA was isolated using RNeasy Kit (Qiagen) following manufacturer's protocol including on-column DNA digestion. cDNA synthesis was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The PCR reaction was performed by SYBR Green-based qPCR using an iCycler Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The gene-specific primers for human SLCO1B3, SLCO1B1and 18s were as follows: SLCO1B3, 5'-

GTCCAGTCATTGGCTTTGCA-3' (forward) and 5'-CAACCCAACGAGAGTCCTTAGG-3' (reverse), SLCO1B1, 5'-TGCTGTGATGTCATTGTCCTT-3' (forward) and 5'-CATGACATGTGAGGTGCCTCCAAG-3' (reverse), 18s, 5'-CGCCGCTAGAGGTGAAATTCTT-3' (forward) and 5'-CGAACCTCCGACTTTCGTTCTT-3' (reverse). Amplification conditions were as follows: 95 °C (5 min), [95 °C (45 sec), 62 °C (1 min), 72 °C (1 min)] × 30 cycles, 95 °C (2 min). The PCR products were separated by 3 % agarose electrophoresis and visualized by UV in the presence of ethidium bromide.

^{3}H-BQ-123 and ^{3}H-CCK-8 Intracellular Uptake Assay.

Stable clones of RKO cells with plasmids containing the transporter OATP1B1 or empty vector pIRESneo2, were seeded and allowed to reach confluency. BQ-123, a substrate for the OATP1B1 transporter (Kullak-Ublick et al., 2001), was obtained from GE Healthcare. Cells were incubated with 5 μM of BQ-123 in transport buffer for 30 min (37°C). To validate the function of the OATP1B3 transporter, stable clones of Hela cells with plasmids containing the transporter OATP1B3 or empty vector pIRESneo2, were seeded and allowed to reach confluency. CCK-8 (GE Healthcare), a substrate specific for only the OATP1B3 transporter (Ismair et al., 2001), was incubated at a final concentration of 5 nM in transport buffer for 10 min (37°C). At the end of the incubation period, BQ-123 or CCK-8 was removed by aspiration, followed by three successive washes in PBS (4°C). Cells were then dried at 37°C and lysed by shaking at room temperature overnight in 0.2 N NaOH. Samples were neutralized with 0.2 N HCl. Intracellular radioactivity of the lysate was measured by a liquid scintillation counter from a lysate and scintillation cocktail mixture. Protein concentrations of lysates were measured using the Bio-Rad Protein Assay, allowing for normalization of samples in CPM/mg.

Immunohistochemistry.

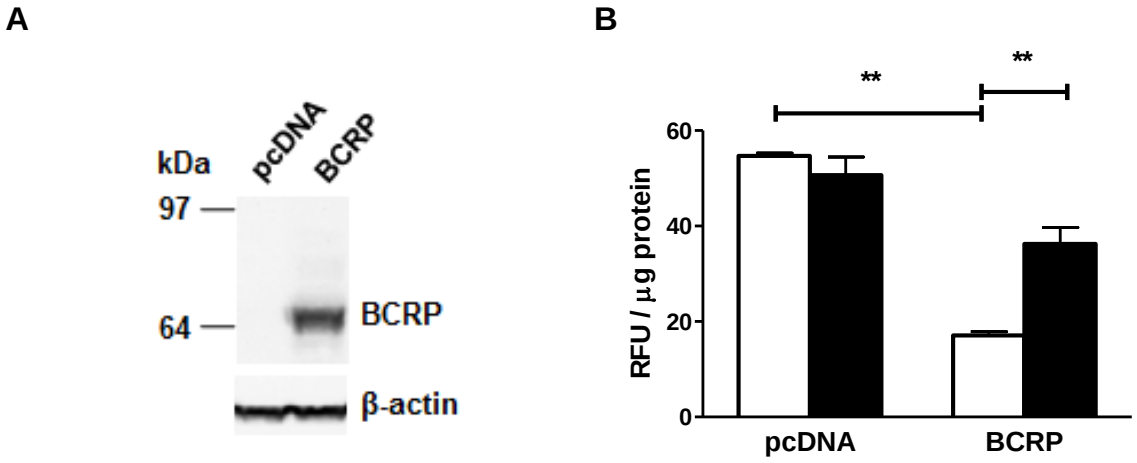
Immunohistochemical analysis was used to verify the expression of OATP1B3 and OATP1B1 in the HeLa and RKO cell lines and liver tissue. Cell pellets and tissues were processed with Histogel (Richard-Allan Scientific, Fisher Scientific) and fixed in 4 % formalin. Antigen retrieval was performed using citrate buffer (0.01M citrate buffer, 0.05 % Tween-20, pH 6.0) at 100 °C for 3 min. A 5 % hydrogen peroxide in PBS solution and an avidin/biotin blocking system were used for blocking of the endogenous peroxidase and the endogenous biotin. Following incubation with 5 % normal goat serum, slides were incubated with anti-OATP2 (OATP2, MDQ/2F260, Novus Biologicals, Littleton, CO) (1:100) overnight (4 °C). The secondary goat anti-mouse antibody (1:100, room temperature) was applied. Streptavidin-HRP conjugate (Dako, Carpinteria, CA) and the Vector NovaRED peroxidase substrate kit were used for visualization. Nuclei were counter-stained using hematoxylin. The expression of the aforementioned transporters was evaluated in human liver tissue. To exclude false positive results, slides were incubated with chromatographically purified mouse IgG (Zymed, San Francisco, CA). All supplies were obtained from Vector (Burlingame, CA) unless otherwise stated.

γH2AX activation in HeLa-pIRES and HeLa-OATP1B3 cells.

Western blot analysis was used to assess the phosphorylation of Histone 2AX (γH2AX) in HeLa-pIRES and HeLa-OATP1B3 cells as a result of exposure to AR-67 carboxylate (20 μM) for 5 min. At the end of the treatment period, AR-67 was removed and cells were allowed to grow in drug-free media for 3 hr. Cell lysate preparation and western blot analysis were performed following standard procedures. Anti-Phospho-Histone H2AX (Cell Signaling, Danvers, MA) (1:500) was used for γH2AX detection. Protein expression was quantified performing densitometry analysis using the Molecular Imaging Software (version 4.04, Eastman, Kodak, New Haven, CT) and Actin (Sigma-Aldrich, St. Louis, MO) was used as a loading control.

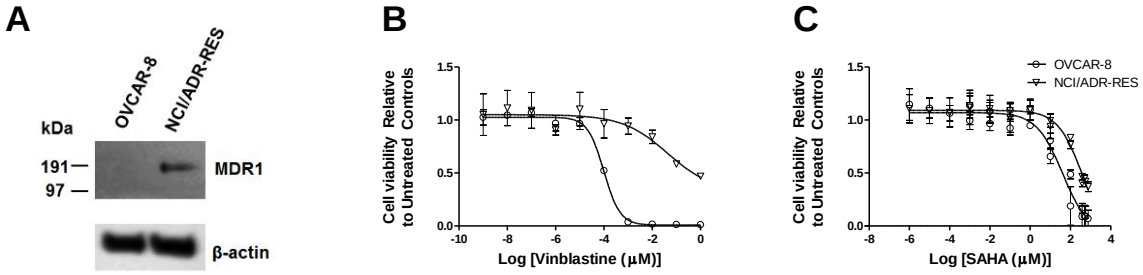
Supplemental Figure 1.

Expression and functional validation of BCRP transporter in MDCKII-pcDNA/BCRP cells. Immunoblotting analysis of lysates prepared from (A) MDCKII-pcDNA/BCRP cells to evaluate the BCRP expression. Actin was used as a loading control. (B) MDCKII-pcDNA/BCRP cells were incubated with Hoechst 33342 (2 µM) for 45 min (open bars). The inhibitory effect of 4 µM of GF120918 in the transporter-mediated efflux of (B) Hoechst 33342 was also assessed (solid bars) as described in the Materials and Methods section. Data are represented as mean (n=3) ± SD. Statistical analysis was performed using unpaired and paired t-test, statistical significance for * $p<0.05$ and ** $p<0.01$.



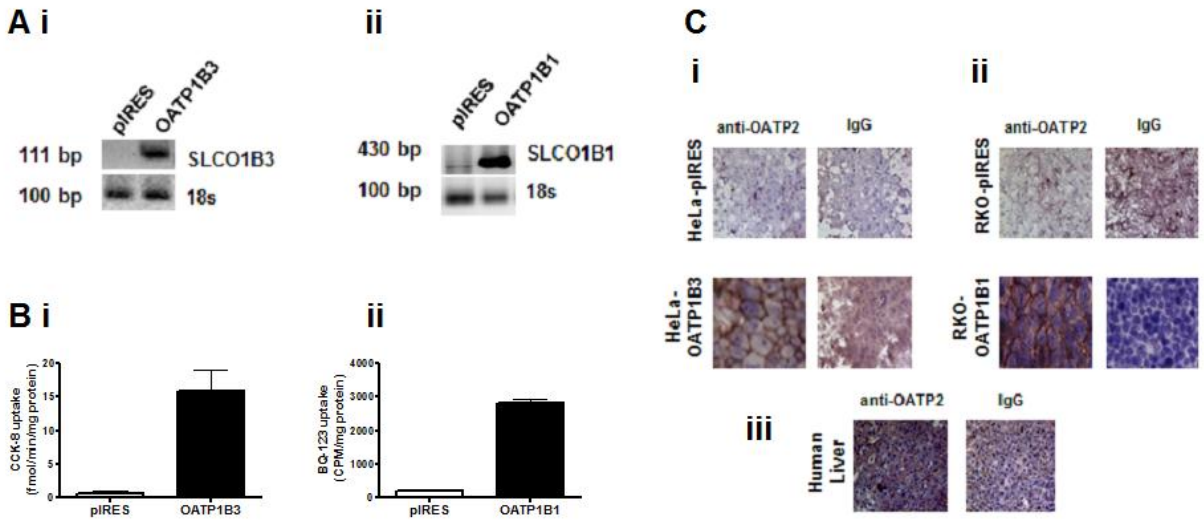
Supplemental Figure 2.

Expression and functional validation of MDR1 transporter in OVCAR-8/NCI/ADR-RES cells. Immunoblotting analysis of lysates prepared from (A) OVCAR-8/NCI/ADR-RES cells to evaluate the MDR1 expression. Actin was used as a loading control. (B) Effect of Vinblastine on OVCAR-8 and NCI/ADR-RES cell lines. Cells were treated with Vinblastine for 72 hours before assessing cell viability as described in the Materials and Methods section. The estimated IC_{50} values were 0.099 nM (0.086-0.116) and 43.9 nM (0.75-2,563) for OVCAR-8 and NCI/ADR-RES cells, respectively. (C) Effect of SAHA on OVCAR-8 and NCI/ADR-RES cell lines. Cells were treated with SAHA for 72 hours before assessing cell viability as described in the Materials and Methods section. The estimated IC_{50} values were 40.79 µM (21.78-76.38) and 418.5 µM (59.61-2939) for OVCAR-8 and NCI/ADR-RES cells, respectively when exposed to SAHA. Data are represented as mean (n=3) ± SD. Data analysis to obtain IC_{50} values was performed using nonlinear regression. IC_{50} values (µM) are reported as mean (95 % confidence interval).



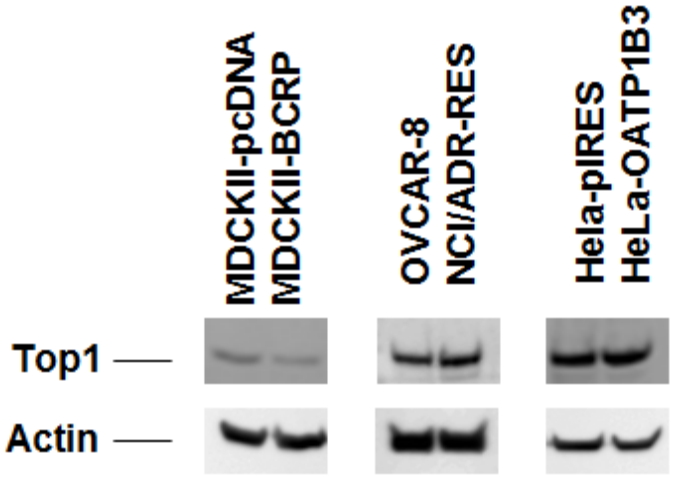
Supplemental Figure 3.

Expression and functional validation of OATP1B3 and OATP1B1 transporters in HeLa and RKO cell lines. (A) Quantitative RT-PCR analysis was used to validate (i) OATP1B3 and (ii) OATP1B1 mRNA expression in the stably OATP-transfected HeLa and RKO cells, respectively. (B) (i) Hela-pIRES and Hela-OATP1B3 cells were incubated with 0.005 µM of ^{3}H-CCK-8 for 10 min. (ii) RKO-pIRES and RKO-OATP1B1 cells were incubated with 0.5 µM of ^{3}H-BQ-123 for 30 min. The scintillation counting was determined in the cell lysate and normalized with the cell lysate protein concentration as described in the Materials and Methods section. Data are represented as mean ± SD (n=8-10). (C) Immunohistochemical staining for OATP2 was used to validate OATP1B1 and OATP1B3 transporter expression in the OATP stably transfected cell lines (i) HeLa-pIRES and HeLa-OATP1B3 and (ii) RKO-pIRES and RKO-OATP1B1, respectively. (iii) Human liver was used as positive control to confirm the specificity of the OATP2 antibody for the OATP1B1 and OATP1B3 uptake transporters.



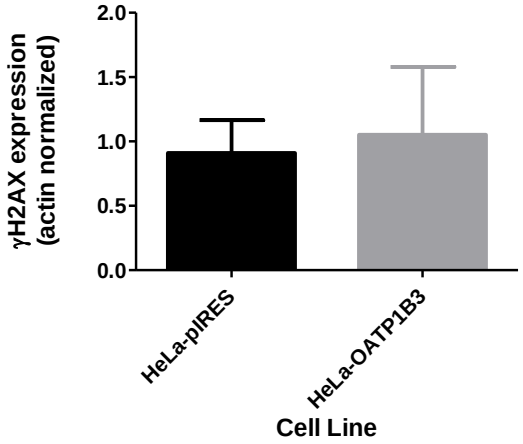
Supplemental Figure 4.

Expression of Top1 in MDCKII-pcDNA/BCRP, OVCAR-8/NCI/ADR-RES and HeLa-pIRES/OATP1B3 cell lines. Immunoblotting analysis was employed to evaluate the expression of Top1 in lysates prepared from MDCKII-pcDNA/BCRP, OVCAR-8/NCI/ADR-RES and HeLa-pIRES/OATP1B3 cells. Actin was used as a loading control.



Supplemental Figure 5.

Expression of γH2AX in HeLa-pIRES/OATP1B3 cell lines. Western blot analysis was used to evaluate γH2AX in lysates of mock- and OATP1B3-transfected cells treated with AR-67 carboxylate (20 μM) for 5 min. γH2AX to actin band intensity ratios were calculated and are presented as mean (n=3) ± SD. Statistical analysis was performed using unpaired t-test, p=0.669.



Supplemental Table 1.

P_{app} and efflux ratio values describing the efflux activity of BCRP for AR-67 lactone in polarized MDCKII cells, in the presence and absence of the BCRP inhibitor GF120918.

	Control		GF120918 (5μM)	
	MDCKII-pcDNA	MDCKII-BCRP	MDCKII-pcDNA	MDCKII-BCRP
$P_{app,A>B}$	2.18 ± 0.26	0.08 ± 0.02	1.33 ± 0.07	2.96 ± 0.12
$P_{app,B>A}$	2.85 ± 0.13	6.86 ± 0.7	1.31 ± 0.10	6.72 ± 1.81
Efflux ratio (B>A/A>B)	1.31 ± 0.17	90.82 ± 23.35	0.99 ± 0.09	2.27 ± 0.62

P_{app} is expressed in cm/sec x 10^5

Values are expressed as mean ± SD

References

de Bruin M, Miyake K, Litman T, Robey R and Bates SE (1999) Reversal of resistance by GF120918 in cell lines expressing the ABC half-transporter, MXR. *Cancer letters* **146**:117-126.

Evers R, Kool M, Smith AJ, van Deemter L, de Haas M and Borst P (2000) Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. *British journal of cancer* **83**:366-374.

Horn J, Jordan SL, Song L, Roberts MJ, Anderson BD and Leggas M (2006) Validation of an HPLC method for analysis of DB-67 and its water soluble prodrug in mouse plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **844**:15-22.

Ismair MG, Stieger B, Cattori V, Hagenbuch B, Fried M, Meier PJ and Kullak-Ublick GA (2001) Hepatic uptake of cholecystokinin octapeptide by organic anion-transporting polypeptides OATP4 and OATP8 of rat and human liver. *Gastroenterology* **121**:1185-1190.

Kalvass JC and Pollack GM (2007) Kinetic considerations for the quantitative assessment of efflux activity and inhibition: implications for understanding and predicting the effects of efflux inhibition. *Pharm Res* **24**:265-276.

Kullak-Ublick GA, Ismair MG, Stieger B, Landmann L, Huber R, Pizzagalli F, Fattinger K, Meier PJ and Hagenbuch B (2001) Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* **120**:525-533.

Sharom FJ (2008) ABC multidrug transporters: structure, function and role in chemoresistance. *Pharmacogenomics* **9**:105-127.

Sun H and Pang KS (2008) Permeability, transport, and metabolism of solutes in Caco-2 cell monolayers: a theoretical study. *Drug Metab Dispos* **36**:102-123.

Wang L, Leggas M, Goswami M, Empey PE and McNamara PJ (2008) N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dih ydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918) as a chemical ATP-binding cassette transporter family G member 2 (Abcg2) knockout model to study nitrofurantoin transfer into milk. *Drug metabolism and disposition: the biological fate of chemicals* **36**:2591-2596.