Title	Gut microbiota-mediated bile acid transformations alter the cellular response to multidrug resistant transporter substrates in vitro: focus on P-glycoprotein
Authors	Enright, Elaine F.;Govindarajan, Kalaimathi;Darrer, Rebecca;MacSharry, John;Joyce, Susan A.;Gahan, Cormac G.
Publication date	2018-11-02
Original Citation	Enright, E. F., Govindarajan, K., Darrer, R., MacSharry, J., Joyce, S. A. and Gahan, C. G. M. (2018) 'Gut Microbiota-Mediated Bile Acid Transformations Alter the Cellular Response to Multidrug Resistant Transporter Substrates in Vitro: Focus on P-glycoprotein', Molecular Pharmaceutics, 15(12), pp. 5711-5727. doi: 10.1021/acs.molpharmaceut.8b00875
Type of publication	Article (peer-reviewed)
Link to publisher's version	https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.8b00875 - 10.1021/acs.molpharmaceut.8b00875
Rights	© 2018 American Chemical Society. This document is the Accepted Manuscript version of a Published Work that appeared in final form in Molecular Pharmaceutics, copyright © American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.8b00875
Download date	2025-06-01 21:48:48
Item downloaded from	https://hdl.handle.net/10468/7243

molecular pharmaceutics

Article

Gut microbiota-mediated bile acid transformations alter the cellular response to multidrug resistant transporter substrates in vitro: focus on P-glycoprotein

Elaine F. Enright, Kalaimathi Govindarajan, Rebecca Darrer, John MacSharry, Susan A. Joyce, and Cormac G.M. Gahan

Mol. Pharmaceutics, **Just Accepted Manuscript** • DOI: 10.1021/acs.molpharmaceut.8b00875 • Publication Date (Web): 02 Nov 2018

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Gut microbiota-mediated bile acid transformations alter the cellular response to multidrug resistant transporter substrates *in vitro*: focus on P-glycoprotein

Elaine F. Enright[1, 2], Kalaimathi Govindarajan[2], Rebecca Darrer[2], John MacSharry[2,4,5], Susan A. Joyce[2, 3†*] and Cormac G.M. Gahan[1, 2, 4†*]

1. School of Pharmacy, University College Cork, Cork, Ireland
2. APC Microbiome Institute, University College Cork, Cork, Ireland
3. School of Biochemistry and Cell Biology, University College Cork, Cork, Ireland
4. School of Microbiology, University College Cork, Cork, Ireland
5. School of Medicine, University College Cork, Cork, Ireland

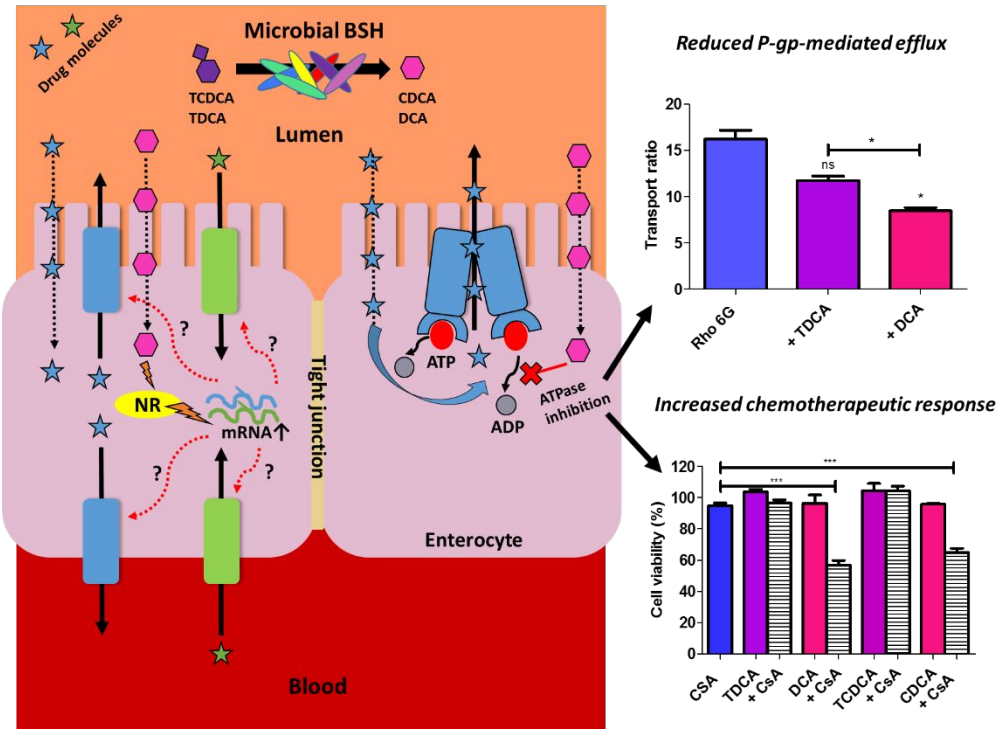
*authors contributed equally

†Corresponding authors:

Cormac G.M. Gahan, School of Microbiology & School of Pharmacy, University College Cork, Ireland. Telephone number: +353 21 4901363, Fax number: +353 21 4903101, Electronic address: c.gahan@ucc.ie

Susan A. Joyce, School of Biochemistry and Cell Biology, University College Cork, Ireland. Telephone number: +353 21 4901343, Electronic address: s.joyce@ucc.ie

Graphical Abstract



Abstract

Pharmacokinetic research at the host-microbe interface has been primarily directed towards effects on drug metabolism, with fewer investigations considering the absorption process. We previously demonstrated that the transcriptional expression of genes encoding intestinal transporters involved in lipid translocation are altered in germ-free and conventionalized mice possessing distinct bile acid signatures. It was consequently hypothesized that microbial bile acid metabolism, that is the deconjugation and dehydroxylation of the bile acid steroid nucleus by gut bacteria, may impact upon drug transporter expression and/or activity and potentially alter drug disposition.

Using a panel of three human intestinal cell lines (Caco-2, T84 and HT-29), that differ in basal transporter expression level, bile acid conjugation- and hydroxylation- status was shown to influence the transcription of genes encoding several major influx and efflux transporter proteins. We further investigated if these effects on transporter mRNA would translate to altered drug disposition and activity. The results demonstrated that the conjugation and hydroxylation status of the bile acid steroid nucleus can influence the cellular response to multidrug resistance (MDR) substrates, a finding that did not directly correlate with directionality of gene or protein expression. In particular, we noted that the cytotoxicity of cyclosporin A was significantly augmented in the presence of the unconjugated bile acids deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) in P-gp positive cell lines, as compared to their taurine/glycine-conjugated counterparts, implicating P-gp in the molecular response.

Overall this work identifies a novel mechanism by which gut microbial metabolites may influence drug accumulation and suggests a potential role for the microbial bile acid-deconjugating enzyme bile salt hydrolase (BSH) in ameliorating multidrug resistance through the generation of bile acid species with the capacity to access and inhibit P-gp ATPase. The physicochemical property of nonionization is suggested to underpin the preferential ability of unconjugated bile acids to attenuate the efflux of P-gp substrates and to sensitize tumorigenic cells to cytotoxic therapeutics *in vitro*. This work provides new impetus to investigate whether perturbation of the gut microbiota, and thereby the bile acid component of the intestinal metabolome, could alter drug pharmacokinetics *in vivo*. These findings may additionally contribute to the development of less toxic P-gp modulators, which could overcome MDR.

Keywords: Microbiota; bile acid metabolism; pharmacokinetics; drug absorption; multidrug resistance; drug transporter

Abbreviations: ABC, ATP-binding cassette; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BCRP, Breast Cancer Resistance Protein; Caco-2, human colorectal adenocarcinoma cell line; CA, cholic acid; CAR, constitutive androstane receptor; CDCA, chenodeoxycholic acid; CsA, cyclosporine A; CYP, cytochrome P450; DCA, deoxycholic acid; FXR, farnesoid X receptor; LCA, lithocholic acid; MRP, Multidrug Resistance Protein; mRNA, messenger RNA; OCT1, Organic Cation Transporter 1; PepT1, Peptide Transporter 1; P-gp, P-glycoprotein; PWSDs, poorly water-soluble drugs; PXR, pregnane X receptor; SLC, solute carrier transporters; TCDCA, taurochenodeoxycholic acid, TDCA, taurodeoxycholic acid;

1. Introduction

The human gastrointestinal tract can be viewed as a microbial ecosystem, harbouring several trillion microbial cells with varied functional repertoires. These collections of symbiotic bacteria have been widely reported to influence drug metabolism and thereby modulate clinical outcome.[1] Comparatively fewer studies have, however, investigated possible effects on the drug absorption process. The combination of an 'unstirred' water layer, tight junctions and an epithelial cell membrane expressing efflux proteins can in some instances severely limit drug transport across the intestinal barrier. We previously established that gut microbial bile acid metabolism can influence the solubilization capacity of simulated intestinal fluids and consequently the solubility of poorly water-soluble drugs (PWSDs).[2] Bile acids, at the millimolar micelle-invoking concentrations employed in this previous study, have been extensively investigated as emulsification and permeation enhancers with demonstrable roles in regulating lipophilic drug absorption.[3, 4] However, there remains a paucity of data examining the effects of bile acids on drug transport at micromolar signalling concentrations as endobiotic ligands for nuclear receptors or their influence on other cellular properties that may impact drug transport.[5]

The primary bile acids in humans, cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized in hepatocytes from cholesterol.[6] Subsequent to their biosynthesis, these primary bile acids are conjugated to either taurine or glycine, at a respective ratio of approximately 1:3, via an N-acyl amide bond. Amidation facilitates protonation at physiological pH and hence conjugated bile acids exist in an ionized form and are more correctly described as salts. Conjugated bile salts are stored and concentrated in the gallbladder for the interprandial period. Postprandially, the gallbladder is stimulated by cholecystokinin and bile salts enter the intestine wherein biotransformation by commensal bacteria occurs. Firstly, the microbial enzyme bile salt hydrolase (BSH) catalyses the hydrolysis of the taurine/glycine appendage liberating the free, unconjugated bile acid. Regenerated unconjugated CA and CDCA are then eligible for 7α-dehydroxylation, mediated by microbial 7α-dehydroxylase, yielding the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA), respectively (Figure 1). All of these deconjugated and dehydroxylated bile acid species can be recirculated to the liver via the portal blood where taurine/glycine reconjugation occurs. Although the genesis of the bile acid pool is synthesis by the host, its composition and physicochemical signature is thus significantly influenced by the microbiome.

In recent years, the role of endobiotic- (including bile acid) inducible nuclear receptors in regulating transporter expression has been uncovered.[7] These nuclear receptors display bile acid-specific sensitivities, such that subtle changes in local or systemic bile acid profiles could impact host physiology.[8] Previous studies within our group revealed that microbial re-colonization of germ-free mice significantly influences transcription of genes encoding intestinal transporters involved in lipid[9] and drug uptake (Figure 1, Supporting Information). As the bile acid pool composition of conventionalized mice is more chemically diverse than that of their germ-free counterparts,[10] we hypothesized that gut microbiota-mediated bile acid metabolism may impact upon the expression of drug transporters. Drug disposition is most often influenced by two superfamilies, the ATP-binding cassette (ABC) and solute carrier (SLC) transporters,[11] we thus focussed our efforts on elucidating the effect of bile acid deconjugation and dehydroxylation on the transcription and activity of 6 clinically relevant members (Figure 1, Supplemental Information, and Table 1).

Tumorigenic multidrug resistance has been associated with the overexpression of efflux transporter proteins of the ABC superfamily, including P-glycoprotein (P-gp, *ABCB1*) and Breast Cancer Resistance Protein (BCRP, *ABCG2*).[12] These transport proteins are also highly abundant at the apical membrane of the enterocytes and by returning a proportion of absorbed drug to the lumen, often pose a barrier to the uptake of susceptible substrates.[11, 13] Down-regulation, or inhibition of these multidrug resistance proteins by substrate competition, ATP depletion, or membrane perturbation may, therefore, augment intestinal absorption, as well as induce tumorigenic chemo-sensitivity.[14] Several lipophilic

drugs, such as verapamil, have proved useful as reversal agents *in vitro* and in preclinical animal models, [15, 16] however, their clinical applicability is likely to be limited by innate pharmacodynamic effects. Therefore, it is of interest to explore the endogenous factors that might influence the action of these transporters, and to identify reversing agents of low toxicity.

Cyclosporine A (CsA) is an immunosuppressive calcineurin inhibitor, which is typically prescribed in the setting of organ transplantation for the prophylaxis or treatment of allograft rejection.[17] Its clinical use is frequently challenged by highly variable pharmacokinetics, necessitating patient-specific dosage adjustments. In addition to CsA's susceptibility to metabolism by CYP3A4[18], several reports indicate that interpatient discrepancies in bioavailability can be partially ascribed to alterations in the intestinal expression of P-gp.[19, 20] In light of its reported curtailing effects on the growth of colon cancer cell lines,[21] CsA was selected for use in this study as a clinically relevant P-gp substrate with the potential to induce cytotoxicity. This latter characteristic affords the opportunity to assess variations in transporter functioning as changes in cellular toxicity. Similarly, SN-38, the active metabolite of the antineoplastic drug irinotecan, was chosen as a model substrate to evaluate the impact of bile acid conjugation and hydroxylation status on the activity of BCRP.[22]

Overall, this paper provides insight into the role of bile acid deconjugation and dehydroxylation (governed *in vivo* by the gut microbiota) in altering the multidrug resistant phenotype of Caco-2 and T84 cell lines, *in vitro* models of both the intestinal barrier and a tumorigenic colon. Firstly we examined the effect of conjugated/deconjugated and trihydroxy/dihydroxy bile acids on the messenger RNA (mRNA) transcripts of common enterocytic influx and efflux transporters. Secondly, we investigated whether these bile acid alterations to transporter mRNA could translate to altered drug disposition and activity using an *in vitro* cytotoxicity screen. In the absence of a correlation between altered drug activity and the directionality of gene/protein expression and considering reports of the non-transcriptional resistance reversing effects of surfactants,[23] other possible mechanisms by which bile acids could affect the absorptive potential of intestinal epithelial were investigated. As the composition of gut microbial communities, and thereby their metabolites, can vary greatly between individuals,[24] this work seeks to enhance the knowledge base of novel mechanisms by which the gut microbiome can contribute to pharmacokinetic variability.

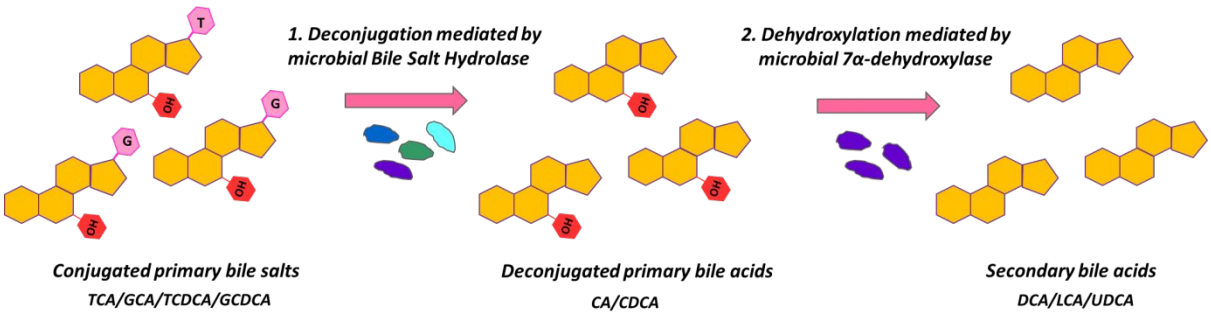


Figure 1. A schematic overview of microbial bile acid metabolism. Primary bile salts synthesized and glycine/taurine-conjugated by the host are released into the duodenum, functioning as physiological surfactants to aid the digestive process. Within the intestinal environment, these conjugated bile salts are substrates for microbial biotransformation, specifically glycine/taurine-deconjugation (mediated by the microbial enzyme bile salt hydrolase) and 7α-dehydroxylation (mediated by the microbial enzyme 7α-dehydroxylase). As a consequence of enterohepatic circulation, the human bile acid pool is comprised of free and conjugated forms of both host- (primary) and microbe- (secondary) derived bile acids.

2. Materials and Methods

Materials

Cyclosporine A (CsA) from *Tolypocladium inflatum* (purity ≥ 95 %), SN-38 (purity ≥ 98 %), verapamil hydrochloride (≥ 99 %), rhodamine 6G, dimethyl sulfoxide (DMSO, Hybri-Max, sterile-filtered, BioReagent, purity ≥ 99.7%), sodium taurocholate hydrate (≥ 95 %), cholic acid sodium salt hydrate (≥ 96%), sodium taurodeoxycholic acid hydrate (≥ 95 %), deoxycholic acid (≥ 99 %), sodium taurochenodeoxycholate (≥ 98 %), sodium chenodeoxycholate (≥ 97 %), thiazolyl blue tetrazolium bromide (MTT), Costar 3412 polycarbonate membrane cell culture transwell inserts (24 mm diameter, 0.4 μm pore size), Dulbecco's modified Eagle medium (DMEM), MEM non-essential amino acid solution (100 x), foetal bovine serum (FBS), penicillin-streptomycin, Ham's Nutrient Mixture F-12, phosphate buffered saline, T-175 flasks, tissue culture treated plates and other tissue culture reagents were sourced from Sigma-Aldrich, Ireland. RNeasy mini-kit was obtained from Qiagen Ltd., Ireland. TURBO DNA-free™ Kit was sourced from ThermoFisher Scientific, Ireland. Transcriptor reverse transcriptase and Universal Probe Library probes were procured from Roche LifeScience, Ireland. Primer "random", Protector RNase inhibitor and PCR Nucleotide Mix were purchased from Sigma-Aldrich, Ireland. SensiFAST™ Probe No-ROX Kit was sourced through MSC Ltd., Ireland. Primers were purchased from Eurofins Scientific, Ireland. Gels and buffers used for western blotting were sourced from BioSciences, Ireland. Anti-Sodium Potassium ATPase antibody, anti-P-glycoprotein antibody and anti-BCRP antibody were purchased from Abcam, Cambridge, UK. CellTiter Glo and P-gp-Glo assay systems were sourced through Promega, Ireland.

Cell line and culture

Caco-2 human epithelial colorectal adenocarcinoma cells, maintained by APC Microbiome Ireland and the School of Pharmacy cell culture collections and originally procured from the American Type Culture Collection (Rockville), were used at passage numbers 36-55 for the work described herein. Caco-2 cells were maintained in DMEM supplemented with 10 % (v/v) FBS, 1 % (v/v) non-essential amino acids and 100 U/ml penicillin and 100 μg/ml streptomycin. T84 human colon carcinoma (lung metastasis) cells, sourced from Sigma-Aldrich and maintained by APC Microbiome Ireland cell culture collection, were used as passage numbers 5-18. T84 cells were maintained in DMEM:F-12 with 10 % (v/v) FBS and 100 U/ml penicillin and 100 μg/ml streptomycin. HT-29 human colon adenocarcinoma cells, maintained by APC Microbiome Ireland, were used at passage numbers 13-25 for the work described herein. HT-29 cells were maintained in McCoy's 5A supplemented with 10 % (v/v) FBS and 100 U/ml penicillin and 100 μg/ml streptomycin. Cell lines were routinely grown in 175 cm^2 tissue culture flasks and passaged twice weekly at a dilution of 1:5 and incubated at 37 °C in a humidified chamber containing 5 % CO_2 and 95 % air.

RNA extraction, cDNA synthesis and quantitative PCR

Caco-2, T84 or HT-29 cells were plated in 6 well plates at a density of 50,000 cells/cm^2 in supplemented growth media (2.5 mL/well). 24 h later, cells were treated with 100 μM (Caco-2 and HT-29) or 50 μM (T84) test bile acids or an equivalent volume of DMSO (vehicle control) in DMEM:F-12 (Caco-2 and T84) or serum-free DMEM (HT-29). Following 24 h incubation, treatment media was aspirated, the cells were washed once with phosphate buffered saline (PBS) and RNA was isolated using the RNeasy mini-kit (Qiagen) according to the manufacturer's instructions. The quality and quantity of the purified (and TURBO DNase-treated) RNA was analysed using a NanoDrop Spectrophotometer. The samples were stored at – 80 °C pending the next steps outlined below.

cDNA synthesis was performed using 300-500 ng total RNA isolated from cells and mastermix consisting of a blend of 5 X transcriptor buffer (Roche), random primer (Roche), transcriptor reverse transcriptase (RT) enzyme (Roche), protector RNase inhibitor (Roche) and dNTP (Roche) in a total volume of 20 µL. cDNA was synthesized using a thermal cycler according to the following protocol steps: 1) primer annealing at 25 °C for 10 min, 2) cDNA synthesis at 55 °C for 30 min, 3) RT heat inactivation at 85 °C for 5 min, followed by cooling to 4 °C. The reaction mixture was then diluted to 100 µL with nuclease-free H_2O and stored at - 20 °C until analysis.

Specific primers for each gene (designed using the Universal Probe Library (Roche) and listed in Table 1) were used for quantitative PCR (qPCR) using a LightCycler® 480 Real-Time PCR system (Roche, Dublin, Ireland). A cycle consisted of: 10 seconds at 95 °C, 45 seconds at 60 °C, 1 second at 70 °C, and repeated for a total of 55 cycles. The expression of target genes in each sample was evaluated using the crossing-threshold value (Ct). The results were normalized to the β actin gene and relative mRNA expression was computed based on the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$). [25]

Cell viability assay

Cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), which enables the number of viable cells in culture to be determined based on ATP quantification. Cells were exposed to conjugated/deconjugated or primary/secondary bile acids in the absence and presence of model efflux substrates (such as SN-38 or CsA) to investigate the impact of microbe-mediated bile acid metabolism on cell viability and drug response.

Cells were plated at a density of 1.6 X 10^4 cells per well in 96-well plates and cultured for 24 h in 100 µL supplemented growth media. Following growth media aspiration, the cells were then exposed to serum-free DMEM:F-12 (Caco-2 and T84) or serum-free McCoy's (HT-29) media containing test bile acids and/or SN-38 or CsA, at concentrations indicated in the figures. All test substances were dissolved in DMSO at concentrations of 100 mM and 10 mM for bile acids and SN-38/CsA, respectively, and subsequently diluted with DMEM:F-12 (Caco-2 and T84) or serum-free McCoy's culture medium. Culture medium containing 0.05-0.2% DMSO, dependent upon the concentration or combination of test substances, served as negative controls. Subsequent to a 24 h treatment period, the plate was measured for luminescence using a BioTek Synergy 2 multi-mode microplate reader (Mason Technology, Ireland).

A tetrazolium-based MTT colorimetric assay was employed to verify the non-cytotoxicity of 100 µM unconjugated bile acids. Similar to the CellTiter Glo assay, Caco-2 cells were seeded at a density of 1.6 X 10^4 cells per well in 96-well plates in supplemented DMEM growth media. 24 h later, the media was removed and cells were treated with 100 µM test bile acids or an equivalent volume of DMSO vehicle (control) in DMEM:F-12. 3 h prior to the completion of the 24 h treatment period, 20 µL of a 5 mg/ml solution of MTT (3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) in phosphate buffered saline (PBS) was added to each well and the cells were further incubated for 3 h. The media was subsequently removed and the cells were washed twice with PBS. Solubilization of the formazan crystals was achieved by the addition of 200 µL DMSO to each well. Absorbance (OD) was measured with background subtraction at 570 nm using a VICTOR^2™ 1420 multilabel counter (Wallac Oy, Turku, Finland). Viability was calculated in comparison to vehicle-treated cells, representing 100 % viability. All viability data is presented as the mean ± SEM of 3-4 independent experiments performed in triplicate.

Western blot analysis

Cells were treated with either 100 μM (Caco-2 and HT-29) or 50 μM (T84) TDCA, DCA, or DMSO vehicle control for 24 h as outlined above for RNA isolation. Following treatment, the growth media was aspirated and cells were rinsed with cold PBS. Cells were subsequently lysed in RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate containing 1 X HALT protease and phosphatase inhibitors). Culture plates containing lysis buffer were left on ice for 10 min, after which lysate was collected using a cell scraper into pre-chilled Eppendorf tubes. Lysates were kept on ice for an additional 20 min. To remove debris, lysates were centrifuged at 14000 rpm for 15 min at 4 °C and the supernatants were collected. Total protein content was determined according to the BCA assay (Pierce). Proteins in the samples used for BCRP immunoblotting were denatured by boiling for 10 min. Samples used for Na^+/K^+-ATPase (loading control) and P-gp immunoblotting were not denatured. Total protein (13.5 μg) was resolved on 3-8% Tris-Acetate gels (NuPAGE) using NuPAGE Tris-Acetate SDS running buffer. 500 μL Bolt Antioxidant was added to the cathode chamber. Following electrophoresis (150 V until adequately resolved), proteins were transferred onto PVDF (0.45 μm) membranes (30 V for 90 min). Following transfer, membranes were blocked with 5% w/v non-fat dry milk in PBS-0.1% v/v Tween-20 for 1 h at room temperature. The membranes were then incubated with gentle shaking at 4 °C overnight with the relevant primary antibody in 15 mL PBS containing 1% w/v bovine serum albumin and 0.1% v/v Tween-20. Primary antibodies were used at a dilution of 1:10000, 1:1000 and 1:1000 for Na^+/K^+-ATPase, BCRP and P-gp, respectively. The following day, membranes were incubated for 1 h at room temperature with goat anti-rabbit HRPase-conjugated secondary antibodies at a 1:10000 dilution in 5% w/v non-fat dry milk in PBS-0.1% v/v Tween-20. Bands were visualized using the Advansta WesternBright Quantum kit. Protein expression levels were quantified using NIH Image J software, with the relative quantity of BCRP and P-gp protein expression calculated with respect to Na^+/K^+-ATPase.

Rhodamine 6G accumulation assay

The P-gp activity of conjugated (TCDCA and TDCA) and deconjugated (CDCA and DCA) bile acids was assessed by measuring the intracellular accumulation of rhodamine 6G (Rho 6G) in Caco-2 and T-84 cells with or without the co-presence of the test bile acid. Briefly, cells were incubated with 5 μM (Caco-2) or 2.5 μM (T84) Rho 6G (in DMEM:F-12) for 3 hr with/without 100 μM test bile acid. Following the incubation period, the cells were washed twice with serum-free culture media and then lysed using 100 μL 1% (w/v) Triton X-100 (Sigma-Aldrich, Ireland) at room temperature. Intracellular levels of Rho 6G were quantified spectroflourometrically using a VICTOR^2™ 1420 multilabel counter (Wallac Oy, Turku, Finland) with excitation (λ_{EX}) and emission (λ_{EM}) wavelengths set at 485 nm and 615 nm, respectively. Data was expressed as the percentage accumulation of Rho 6G in test cells relative to accumulation in untreated control cells, arbitrarily set at 100%. Increased intracellular accumulation of Rho 6G corresponds to reduced efflux activity.

In order to gain insight into the mechanism by which bile acids may modulate the increased cellular uptake of Rho 6G, accumulation assays were performed as outlined above at 4 °C, 21 °C and 37 °C. Experimental data is expressed as the mean ± SEM of four independent experiments (with n = 6 replicates per experiment).

Rhodamine 6G apparent permeability assay

Bidirectional permeability assessments were performed using polycarbonate membrane cell culture transwell inserts (24 mm diameter, 0.4 μm pore size). Caco-2 cells were seeded at a density of 63,000 cells/cm^2 in supplemented DMEM and allowed to differentiate over a period of 21-23 days until transepithelial electrical resistance (TEER) reached ≥ 350 ohms. TEER values were measured periodically at the initiation of cell culture media changes (alternate days), as well as before and after

testing using an EVOM-Epithelial Voltohmmeter (World Precision Instruments, USA). During maintenance and testing, apical and basolateral chamber volumes were 1.5 ml and 2.6 ml, respectively. All transport experiments were carried out in triplicate. On experimental days, the mean TEER value was 408 ± 12 ohms following change to test media (DMEM:F-12). For test conditions, 100 µM test bile acids (TDCA/DCA) or verapamil (positive control) were added to both sides of the monolayer 1 h prior to initiation of the transport assay. Subsequent to this 1 h pre-incubation period, 5 µM Rho 6G (from a 5 mM DMSO stock) was added to the donor chamber and drug transport to the receiver chamber was analysed over 3 h at 37 °C. 100 µL samples were withdraw from the receiver chamber at 30, 60, 90, 120, 150 and 180 minutes and replaced with fresh media (containing test bile acid or verapamil, as appropriate). A 100 µL sample was also withdrawn from the donor chamber at the beginning (which was replenished with an equivolume of 5 µM Rho 6G-containing media) to confirm the initial donor concentration.

Rho 6G concentrations were determined on the basis of a standard curve with treatment solutions of known molarity ($R^2 \geq 0.99$). The cumulative amount of Rho 6G transported to the receiver chamber was calculated taking into consideration previously removed sample volumes.

The apparent permeability coefficient ($P_{app,}$ cm/s) of Rho 6G was calculated according to;

$$P_{app} = \frac{V_D}{[A \times M_D]} \times \frac{\Delta M_R}{\Delta t}$$

Where V_D is the donor chamber volume (cm^3); A is the surface area of monolayer (4.67 cm^2); M_D is the initial concentration of drug in the donor *chamber (mol); and $\frac{\Delta M_R}{\Delta t}$ is the change in receiver drug concentration over time* (mol/s).

In an effort to assess the impact of microbial bile acid metabolism on Rho 6G efflux, efflux ratios under the various test conditions were calculated by dividing the $P_{app,\ B\rightarrow A}$ by $P_{app,\ A\rightarrow B.}$ For B → A transport, the basolateral chamber is the donor chamber and the apical chamber is the receiver chamber. Conversely, for A → B transport, the apical chamber is the donor compartment and the basolateral chamber is the receiver compartment.

Data is expressed as the mean ± SD of 2 independent experiments performed in triplicate.

P-gp ATPase activity assay

The effect of test bile acids, as well as confirmation of P-gp substrate activity, on P-gp ATPase was examined using the P-gp-Glo assay system (Promega) according to the manufacturer's protocol with slight modification (a 3 h incubation period was utilized due to the low basal ATPase activity of the P-gp membranes). All data is presented as the mean ± SD (n =3).

Statistical analysis

A two-tailed, independent samples *t*-test, assuming normal distribution and equal variance, was used to assess the statistical significance for two-sample comparison. A P value < 0.05 was regarded to be statistically significant. All statistical analyses were conducted using GraphPad Prism 5 (San Diego, California).

Table 1. Primers for quantitative PCR analysis. Nucleotide sequences are presented for primer pairs of the genes of interest.

Transporter name	Gene name	Sequence (5'-3')
	ACTB (β actin)	(L) ATTGGCAATGAGCGGTTC (R) CGTGGATGCCACAGGACT
P-glycoprotein (P-gp)	*ABCB1*	(L) GAAATTTAGAAGATCTGATGTCAAACA (R) ACTGTAATAATAGGCATACCTGGTCA
Multidrug Resistance Protein 2 (MRP2)	*ABCC2*	(L) AGTGAATGACATCTTCACGTTTG (R) CTTGCAAAGGAGATCAGCAA
Breast Cancer Resistance Protein (BCRP)	*ABCG2*	(L) TGGCTTAGACTCAAGCACAGC (R) TCGTCCCTGCTTAGACATCC
Multidrug Resistance Protein 3 (MRP3)	*ABCC3*	(L) TGCTCTCCTTCATCAATCCA (R) TGGGGTTGGAGATAAACCTG
Peptide Transporter 1 (PepT1)	*SLC15A1*	(L) CAATGTTCTGGGCCTTGTTT (R) TTCCCGGACATAGTTGTTGC
Organic Cation Transporter 1 (OCT1)	*SLC22A1*	(L) TCCTCTTCCTGCTCTACTACTGG (R) TGGTCCATTATCTTTATTGCTTCA

3. Results

3.1 Impact of bile acid conjugation and hydroxylation state on intestinal drug transporter expression

In the liver, bile acid-dependent activation of FXR is known to induce the expression of transporters that function to extrude bile salts, cholesterol and phospholipids into the bile canaliculi.[26] While there is some evidence that bile acids can affect the expression of various ABC transporters in non-hepatic tissues [27, 28], to our knowledge, the effect of microbially biotransformed bile acids on enterocytic drug transporters and drug activity has not yet been considered. The impact of microbial BSH and 7α-dehydroxylase activity on the expression of six intestinal transporters commonly implicated in altered drug disposition *in vivo* was hence simulated in three intestinal cell lines with conjugated/deconjugated (Table 2) or primary/secondary (Table 3) bile acids. The bile acid concentrations employed were determined to be non-cytotoxic to the cell lines (Figure 2, Supporting Information) and fall within the range of that expected in the fecal water of healthy individuals following the consumption of a normal diet. [29]

In general, unconjugated DCA and CDCA increased, relative to vehicle control and their conjugated counterparts, the expression of genes encoding enterocytic drug transporters (Table 2). The inferred impact of microbial bile acid deconjugation on the expression of *ABCB1* (P-gp) and *ABCG2* (BCRP), however, differed from this generalizable trend across the three cell lines. Comparative expression profiles of CA- and DCA- treated cells were consistent with the findings of bile acid deconjugation, that is, the increased hydrophobicity of the steroidal nucleus of DCA was associated with increased expression of intestinal drug transporter mRNA (Table 3) in the three cell lines. Deviations from this general trend were again noted for *ABCB1* (P-gp) and *ABCG2* (BCRP) mRNA (Table 3).

In an effort to investigate if unconjugated, dihydroxy bile acids were likely to upregulate drug transporter mRNA through stimulation of FXR, effects on the expression of *FGF19*, a well-known FXR-regulated gene, were assessed. In general, unconjugated DCA and CDCA upregulated *FGF19* mRNA (Table 2, Supporting Information) in a manner similar to that observed for *ABCC2* and *ABCC3*, which are purportedly regulated by FXR. [30]

Cell line	Gene name	Relative mRNA expression following bile acid treatment			
		TCDCA	CDCA	TDCA	DCA
	ABCB1				
Caco-2[a]		0.55 ± 0.06 *	1.1 ± 0.14	0.96 ± 0.07	0.99 ± 0.05
T84[b]		1.07 ± 0.20	0.76 ± 0.02 ***	1.05 ± 0.10	0.9 ± 0.05
HT-29[a]		0.87 ± 0.10	3.19 ± 0.63 *	1.52 ± 0.32	2.47 ± 0.25 **
	ABCG2				
Caco-2[a]		1.8 ± 0.13 ***	4.7 ± 0.99 **	1.09 ± 0.11	3.69 ± 0.90 *
T84[b]		0.91 ± 0.07	0.79 ± 0.05 **	1.16 ± 0.10	0.81 ± 0.02 ***
HT-29[a]		1.04 ± 0.52	0.66 ± 0.33 **	1.13 ± 0.57	1.31 ± 0.66
	ABCC2				
Caco-2[a]		0.83 ± 0.09	2.29 ± 0.28 **	0.82 ± 0.13	1.96 ± 0.43
T84[b]		1.06 ± 0.07	2.21 ± 0.24 **	1.05 ± 0.07	2.25 ± 0.16 **
HT-29[a]		1.08 ± 0.05	6.55 ± 1.72 *	0.99 ± 0.11	8.99 ± 1.84 **
	ABCC3				
Caco-2[a]		0.59 ± 0.08 **	2.75 ± 0.33 **	0.93 ± 0.07	1.69 ± 0.24 *
T84[b]		0.99 ± 0.04	2.32 ± 0.28 **	1.03 ± 0.09	2.35 ± 0.16 **
HT-29[a]		1.13 ± 0.03 **	2.72 ± 0.29 **	1.08 ± 0.07	3.22 ± 0.27 ***
	SLC15A1				
Caco-2[a]		0.96 ± 0.16	2.43 ± 0.16 ***	0.93 ± 0.02 **	2.17 ± 0.21 **
T84[b]		1.05 ± 0.05	2.16 ± 0.16 **	1.06 ± 0.04	2.42 ± 0.12 ***
HT-29[a]		ND	ND	ND	ND
	SLC22A1				
Caco-2[a]		0.98 ± 0.06	2.23 ± 0.21 **	1.09 ± 0.22	1.18 ± 0.12
T84[b]		0.81 ± 0.08	1.37 ± 0.07 *	1.01 ± 0.26	1.52 ± 0.12 **
HT-29[a]		1.05 ± 0.18	2.17 ± 0.53	0.9 ± 0.09	2.98 ± 0.47 **

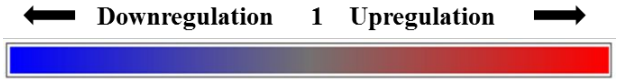


Table 2. Fold change in major intestinal drug efflux and uptake transporter gene expression (relative to vehicle control), examining the influence of bile acid conjugation status (governed by microbial BSH activity *in vivo*) on such transporters. Quantitative PCR was performed using total RNA extracted from 24 h 100 μM[a] or 50 μM[b] bile acid- or vehicle-treated cells. Gene expression was normalized to β actin. Data is expressed as the mean ± SEM of 4 independent experiments performed in duplicate for the Caco-2 and HT-29 cell lines and as the mean ± SEM of 3 independent experiments performed in triplicate for the T84 cell line. Asterisks denote a statistically significant difference in mRNA expression compared to vehicle controls. ND = not detected. Δ Ct values providing an indication of the basal expression level of each gene (relative to β-actin) are provided in the Supporting Information (Table 1).

Cell line	Gene name	Relative mRNA expression following bile acid treatment	
		CA	DCA
	ABCB1		
Caco-2[a]		0.83 ± 0.07	1.48 ± 0.30
T84[b]		0.93 ± 0.04	0.9 ± 0.05
HT-29[a]		1.65 ± 0.33	2.47 ± 0.25 **
	ABCG2		
Caco-2[a]		1.15 ± 0.08	4.34 ± 0.25 ***
T84[b]		0.97 ± 0.06	0.81 ± 0.02 ***
HT-29[a]		1.26 ± 0.21	1.31 ± 0.15
	ABCC2		
Caco-2[a]		1.09 ± 0.11	2.47 ± 0.38 **
T84[b]		0.99 ± 0.04	2.25 ± 0.16 **
HT-29[a]		1 ± 0.09	8.99 ± 1.84 **
	ABCC3		
Caco-2[a]		0.99 ± 0.02	1.98 ± 0.30 *
T84[b]		1 ± 0.05	2.35 ± 0.16 **
HT-29[a]		1.11 ± 0.10	3.22 ± 0.27 ***
	SLC15A1		
Caco-2[a]		1 ± 0.04	2.41 ± 0.25 **
T84[b]		1.1 ± 0.11	2.42 ± 0.12 ***
HT-29[a]		ND	ND
	SLC22A1		
Caco-2[a]		1.12 ± 0.23	3.81 ± 2.17
T84[b]		1.05 ± 0.10	1.52 ± 0.12 *
HT-29[a]		1.1 ± 0.12	2.98 ± 0.47 **

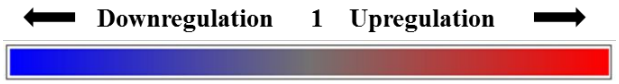


Table 3. Fold change in major intestinal drug efflux and uptake transporter gene expression (relative to vehicle control), examining the influence of bile acid hydroxylation status (governed by microbial 7α-dehydroxylase activity *in vivo*) on such transporters. Quantitative PCR was performed using total RNA extracted from 24 h 100 μM[a] or 50 μM[b] bile acid- or vehicle-treated cells. Gene expression was normalized to β actin. Data is expressed as the mean ± SEM of 4 independent experiments performed in duplicate for the Caco-2 and HT-29 cell lines and as the mean ± SEM of 3 independent experiments performed in triplicate for the T84 cell line. Asterisks denote a statistically significant difference in mRNA expression compared to vehicle controls. ND = not detected. Δ Ct values providing an indication of the basal expression level of each gene (relative to β-actin) are provided in the Supporting Information (Table 1).

3.2 Impact of bile acid deconjugation on the cellular response to therapeutics

To explore whether the observed alterations in intestinal drug transporter expression would translate to altered drug disposition and activity *in vitro*, initial work was undertaken in the Caco-2 cell line. DCA was determined to augment the cytotoxicity of CsA and SN-38, known substrates for P-gp- and BCRP-mediated efflux, respectively (Figure 2 (a), Figure 3 (a)). This ability of DCA to enhance the cytotoxicity of these MDR substrates did not correlate with the directionality of *ABCB1* (P-gp) or *ABCG2* (BCRP) expression (Table 2). Given the well investigated role of P-gp and BCRP in limiting the intestinal absorption of many clinically important therapeutics [11], we thus sought to further elucidate the underpinning mechanism. Three cell lines possessing distinct P-gp and BCRP phenotypes were employed for this purpose.

Deconjugation of the bile acid steroid nucleus was shown to differentially modulate the cytotoxicity of SN-38 and CsA in a manner contrary to that anticipated based on the expression of *ABCG2* (BCRP) and *ABCB1* (P-gp). DCA, but not its tauro-conjugate, significantly reduced Caco-2 cell viability (to 69.4 ± 2.07 %) compared to SN-38 alone (105.10 ± 1.86 %, P = 0.0002, Figure 2 (a)). Bile acid deconjugation did not affect the sensitivity of T84 or HT-29 cells to SN-38 (Figure 2 (b) and (c)).

DCA was similarly determined to enhance the cytotoxicity of CsA toward the Caco-2 cell line, with this combination yielding a cell viability of 59.52 ± 2.71 % versus 94.88 ± 1.66 % for CsA alone (P = 0.0004, Figure 3 (a)). CDCA was also found to augment the cytotoxicity of CsA (Figure 3 (a)). A reduced cell viability of 64.86 ± 2.70 % versus 94.88 ± 1.66 % was observed following dual treatment compared to CsA treatment alone (P = 0.0007, Figure 3 (a)). Again, these observations could not be explained by bile acid-induced changes to Caco-2 *ABCB1* (P-gp) mRNA transcripts. Glycine conjugated bile salts behaved similarly to taurine conjugates (Figure 3, Supporting Information). These findings were further confirmed in the T84 cell line (Figure 3 (b)), which is also P-gp positive. The HT-29 cell line, which is known to be P-gp negative [31], served as a control exhibiting no notable alterations to drug response (Figure 3 (c)) .

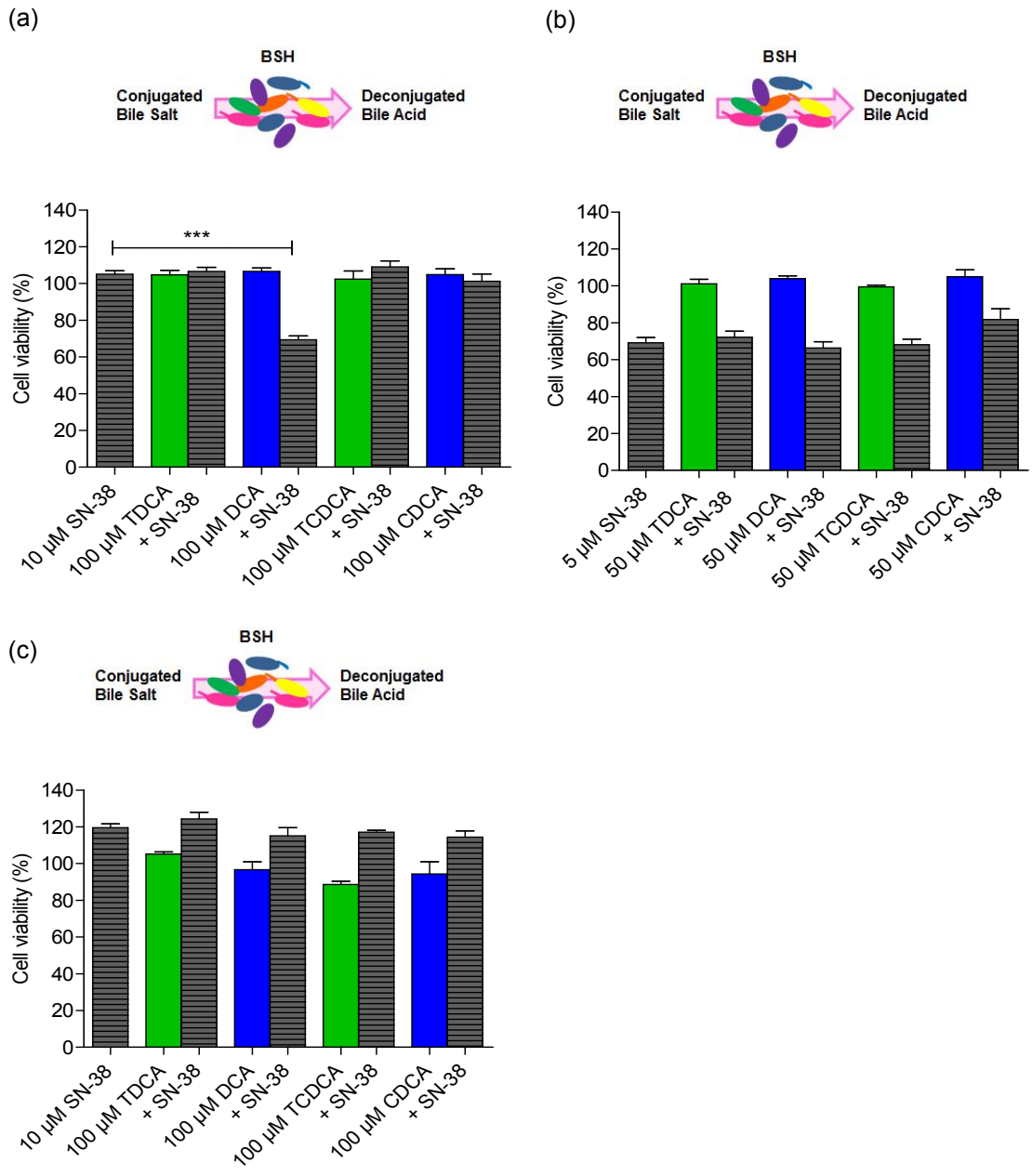


Figure 2. The effect of bile acid deconjugation (mediated by BSH *in vivo*) on Caco-2 (a), T84 (b) and HT-29 (c) cell viability (%) following 24 h exposure to the BCRP substrate SN-38. Unconjugated DCA was determined to increase the cytotoxicity of SN-38 in the Caco-2 (a) cell line, but had no impact upon the T84 (b) nor HT-29 (c) cell lines. Data is presented as the mean ± SEM of 3 independent experiments performed in triplicate.

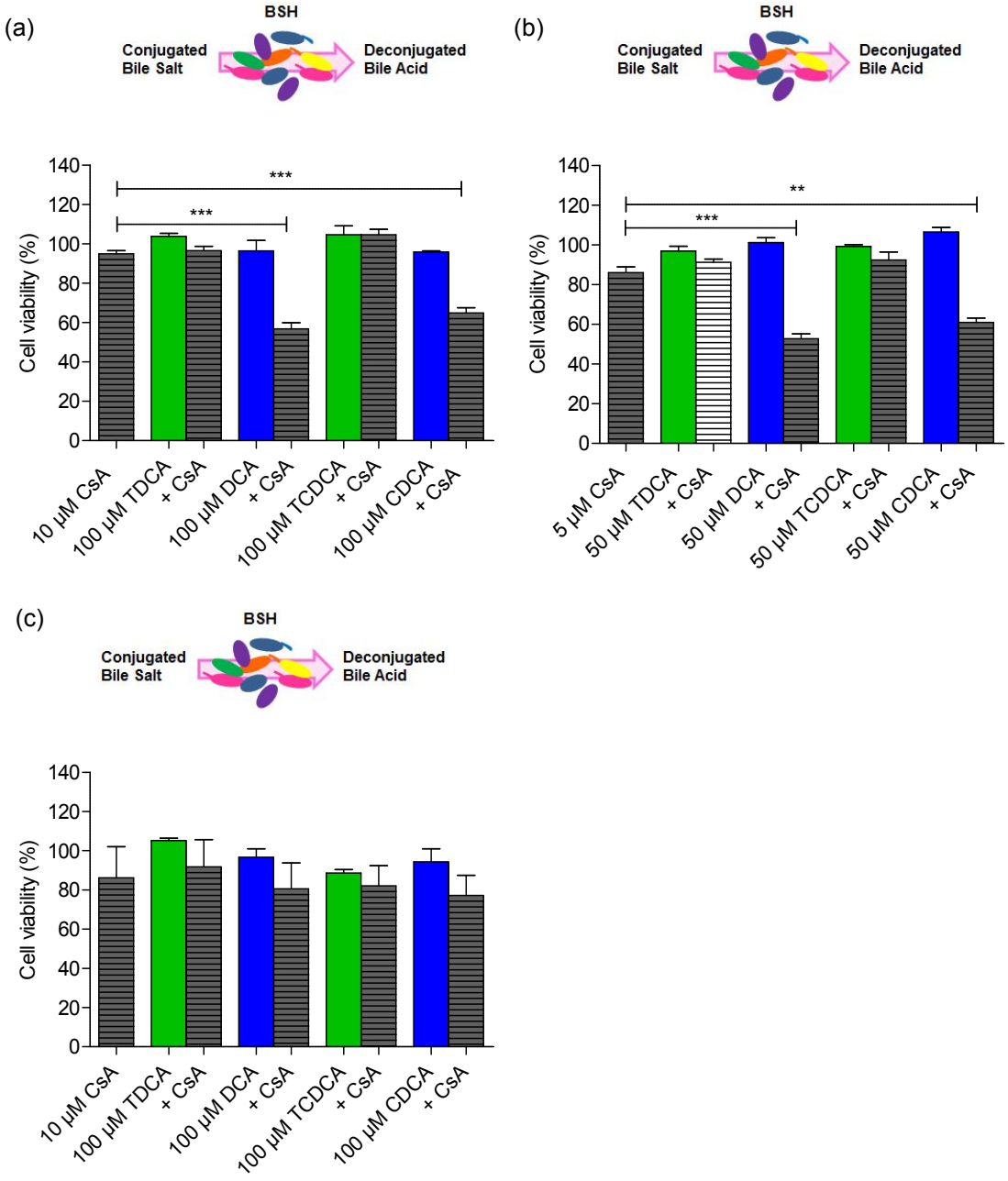


Figure 3. The effect of bile acid deconjugation (mediated by BSH *in vivo*) on Caco-2 (a), T84 (b) and HT-29 (c) cell viability (%) following 24 h exposure to the P-gp substrate CsA. Unconjugated DCA and CDCA were determined to increase the cytotoxicity of CsA in the Caco-2 and T84 cell lines ((a) and (b)), but did not influence cytotoxicity toward the HT-29 cell line (c). Data is presented as the mean ± SEM of 4 (Caco-2) or 3 (HT-29 and T84) independent experiments performed in triplicate.

3.3 Impact of bile acid dehydroxylation on the cellular response to therapeutics

The impact of microbial 7α-dehydroxylation of the cholate steroidal ring on the propensity of bile acids to adjust the cytotoxic action of SN-38 and CsA was simulated. Similar to the effect of deconjugation, bile acid dehydroxylation was associated with an increase in drug activity, illustrated by a diminution in cell viability following substitution of CA with DCA (Figure 4 (a)). Concurrent treatment of Caco-2 cells with SN-38 and DCA, resulted in a cell viability of 61.56 ± 3.47 % as compared to 90.52 ± 7.8 % for SN-38 alone (P = 0.0147, Figure 4 (a)). The bile acid hydroxylation state did not influence SN-38 cytotoxicity in BCRP-negative T84 (Figure 4 (b)) nor HT-29 (Figure 4 (c)) cell lines.

DCA, but not CA, was similarly shown to enhance the cytotoxicity of CsA (43.85 ± 2.47 % following combination treatment as compared to 91.24 ± 14 % for CsA alone, P = 0.0157, Figure 5 (a)). This effect was verified in the P-gp positive T84 cell line (Figure 5 (b)); cell viability was not affected in the HT-29 cell line (Figure 5 (c)). Similar to the impact of deconjugation, these findings cannot be correlated generally to transcriptional effects on *ABCB1* (P-gp) and *ABCG2* (BCRP). The results collectively indicate that unconjugated and secondary bile acids have a greater capacity to modulate the uptake/activity of common efflux substrates.

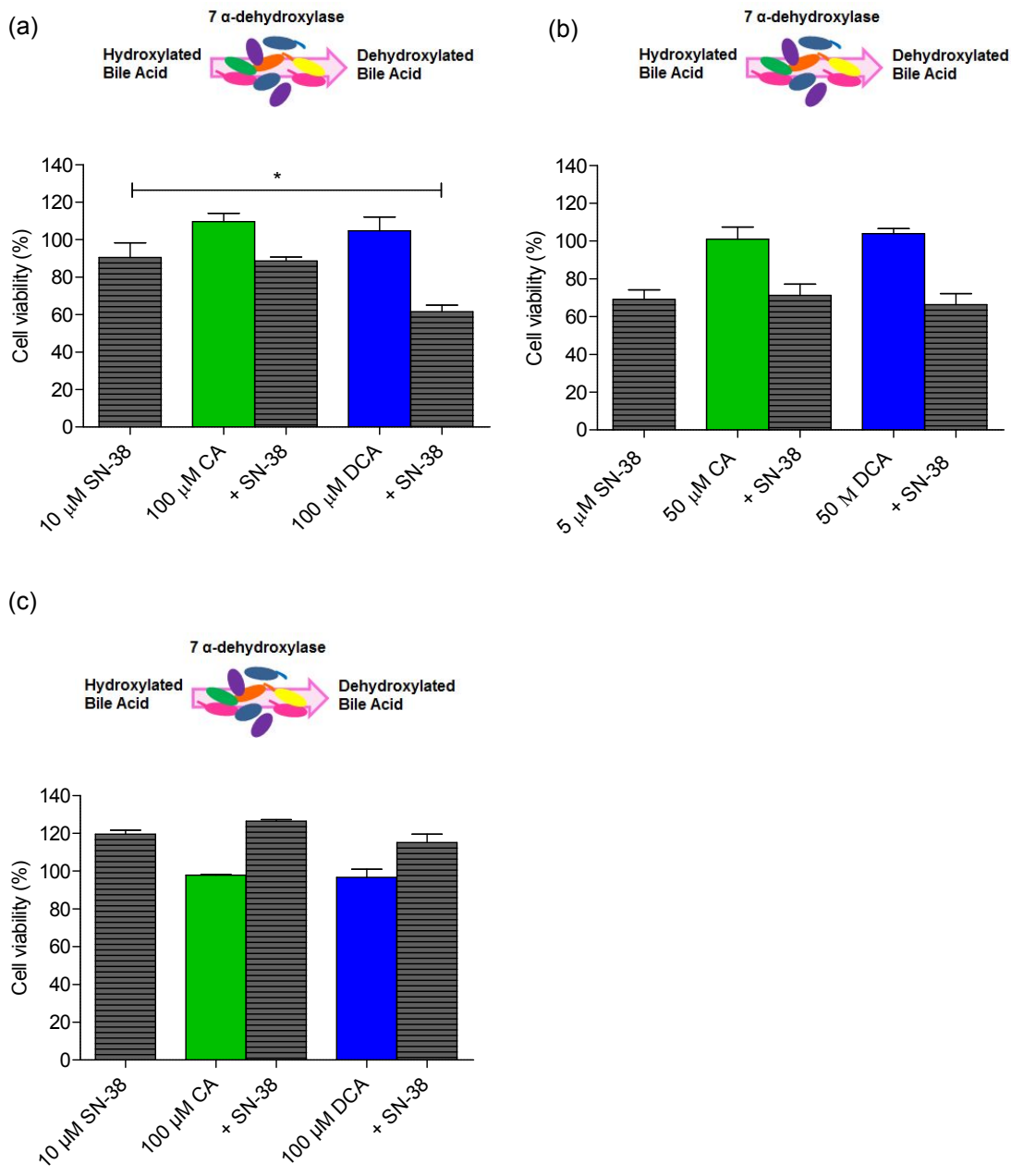


Figure 4. The effect of bile acid dehydroxylation (mediated by 7α-dehydroxylase *in vivo*) on Caco-2 (a) T84 (b) and HT-29 (c) cell viability (%) following 24 h exposure to the BCRP substrate SN-38. The dihydroxy bile acid DCA was determined to increase the cytotoxicity of SN-38 in the Caco-2 cell line ((a)), but did not influence cytotoxicity toward the T84 nor HT-29 cell lines (c). Data is presented as the mean ± SEM of 4 (Caco-2) or 3 (HT-29 and T84) independent experiments performed in triplicate.

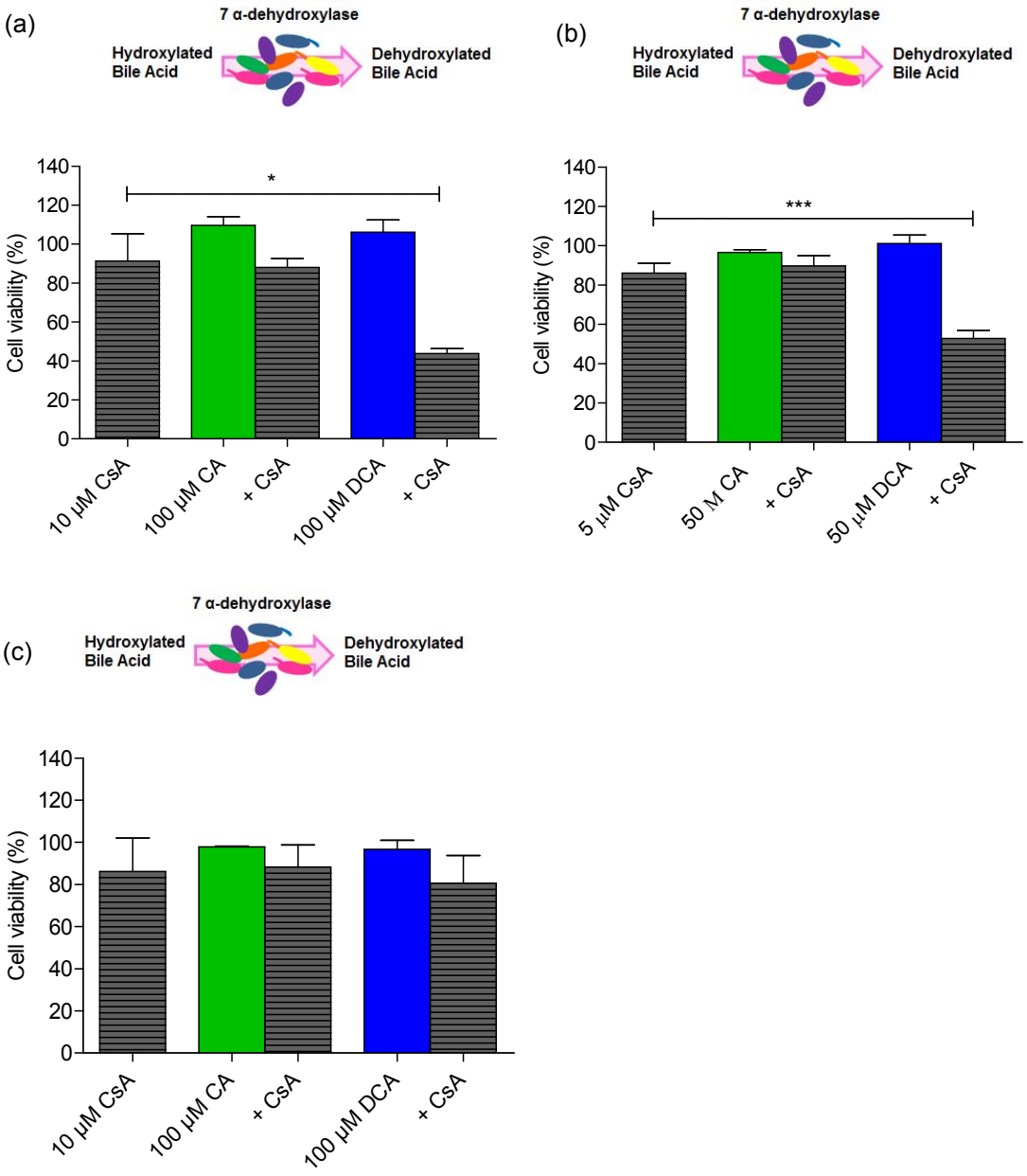


Figure 5. The effect of bile acid dehydroxylation (mediated by 7α-dehydroxylase *in vivo*) on Caco-2 (a), T84 (b) and HT-29 (c) cell viability (%) following 24 h exposure to the P-gp substrate CsA. The dihydroxy bile acid DCA was determined to increase the cytotoxicity of CsA in the Caco-2 and T84 cell lines ((a) and (b)), but did not influence cytotoxicity toward the HT-29 cell line (c). Data is presented as the mean ± SEM of 4 (Caco-2) or 3 (HT-29 and T84) independent experiments performed in triplicate.

In order to ascertain whether unconjugated, secondary bile acids could potentially increase SN-38 and CsA cytotoxicity through down-regulation of BCRP and P-gp proteins, respectively, western blotting was performed. No alteration to P-gp protein expression was observed in the P-gp positive cell lines Caco-2 and T84 (Figure 6 (a) and (b)). The HT-29 cell line was confirmed to be negative for P-gp protein expression (Figure 6 (a) and (c)). These mRNA and protein expression data collectively indicate that unconjugated and 7α-dehydroxylated bile acids modulate P-gp efflux by a mechanism distinct to transcription and protein translation. Similarly, regarding BCRP, no alteration to protein expression was noted in the BCRP-positive cell line Caco-2 (Figure 6 (a) and (c)). The T84 cell line was determined to be negative for BCRP hence supporting its utility as a negative control. Unconjugated DCA reduced BCRP protein expression relative to its conjugated counterpart in the HT-29 cell line, but this was not statistically significant to the vehicle control (Figure 6 (c)).

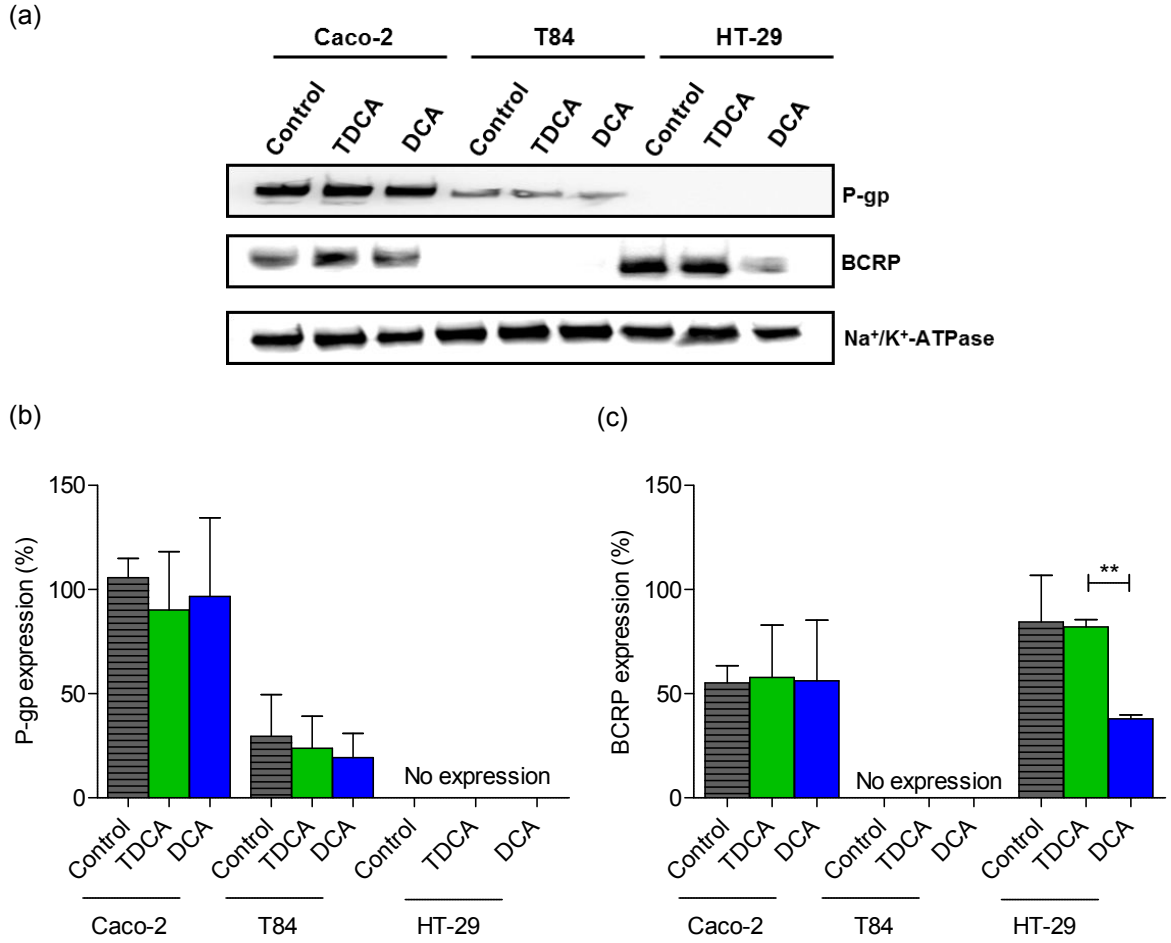


Figure 6. Representative western blots of P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP) in Caco-2, T84 and HT-29 cells (a). Caco-2 and T84 cells are P-gp positive, while Caco-2 and HT-29 cells are BCRP positive. Quantitated data from immunoblots for P-gp and BCRP are shown in (b) and (c), respectively. Cells were treated with TDCA, DCA or DMSO vehicle control for 24 h. Total cell lysate was harvested and subjected to immunoblotting with antibodies against indicated proteins. Bile acid treatments did not influence the expression of P-gp (b) or BCRP (c) relative to control cells. Unconjugated DCA was determined to down-regulate the expression of BCRP in HT-29 cells, as compared to its taurine-conjugated counterpart. Data is expressed as mean ± SD (n = 2).

3.4 Structure-activity relationship between bile acids and their ability to modulate the cellular response to a P-gp substrate

Unconjugated DCA and CDCA were shown to modulate the cytotoxicity of CsA in two P-gp positive cell lines (Caco-2 and T84). In an effort to investigate whether a structure-activity relationship underpinned bile acid-induced inhibition of P-gp functioning, simple linear regressions between the predicted octanol/water partition coefficient (LogP) of the test bile acids and cell viability were performed. Strong relationships were observed in both the Caco-2 and T84 cell lines (Figure 7), suggesting that the tendency of bile acids to inhibit P-gp activity is related to their hydrophobicity.

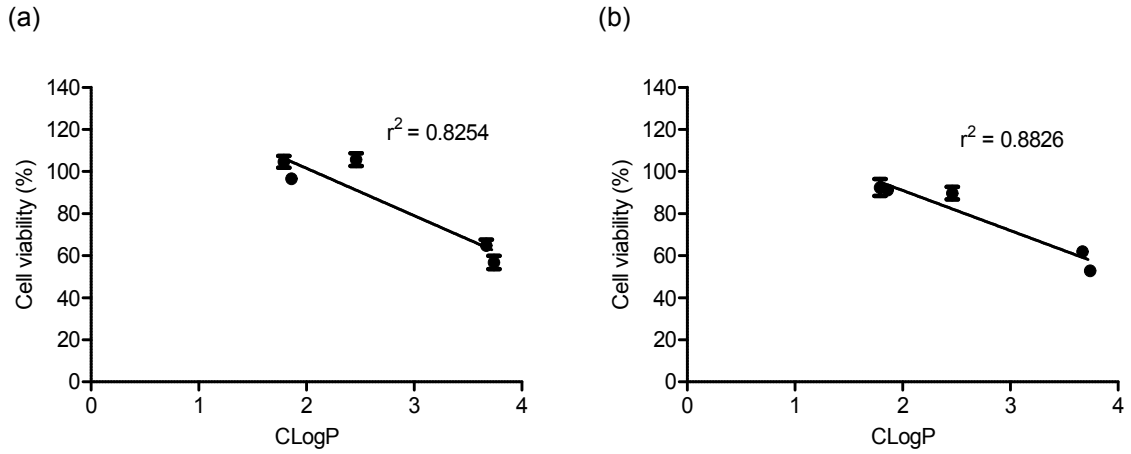


Figure 7. The impact of bile acids on CsA-induced cytotoxicity toward the Caco-2 (a) and T84 (b) cell lines as a function of their octanol/water partition coefficient (predicted values were obtained using MarvinSketch version 17.1.30.0, ChemAxon (http://www.chemaxon.com). Simple linear regression analysis was performed in GraphPad Prism 5.

3.5 Impact of bile acid conjugation status on rhodamine 6G uptake and bidirectional transport

To further examine the hypothesis that unconjugated bile acids possess a greater capacity to modulate the intracellular accumulation of P-gp substrates by inhibiting efflux activity, a series of uptake and transport studies involving a fluorescent rhodamine dye, rhodamine 6G (Rho 6G), were performed. Rho 6G was confirmed to be a P-gp substrate by its ability to stimulate basal P-gp ATPase activity (Figure 4, Supporting Information).

In the Caco-2 cell line, unconjugated DCA and CDCA, but not their tauro-conjugated counterparts, increased the intracellular accumulation of Rho 6G following a 3 h co-incubation period at 37 °C (Figure 8 (a)). CsA 100 µM, used as a positive control to validate the assay, significantly increased the intracellular accumulation of Rho 6G (data not shown). Although not statistically significant, a similar trend was observed in the T84 cell line (Figure 8 (b)). The less pronounced phenotype observed in T84 cells may reflect lower levels of P-gp expression in this cell line (Figure 6 (a)). To further prove that this observed enhancement in Rho 6G uptake in the presence of unconjugated bile acids was via a transporter-mediated, energy-dependent process, parallel experiments were performed at 4 °C and at room temperature (approximately 21 °C). The accumulation of Rho 6G in the absence and presence of test bile acids did not differ at 4 °C or 21 °C, indicating that an energy-dependent process is responsible for the enhancement conferred by unconjugated bile acids at 37 °C.

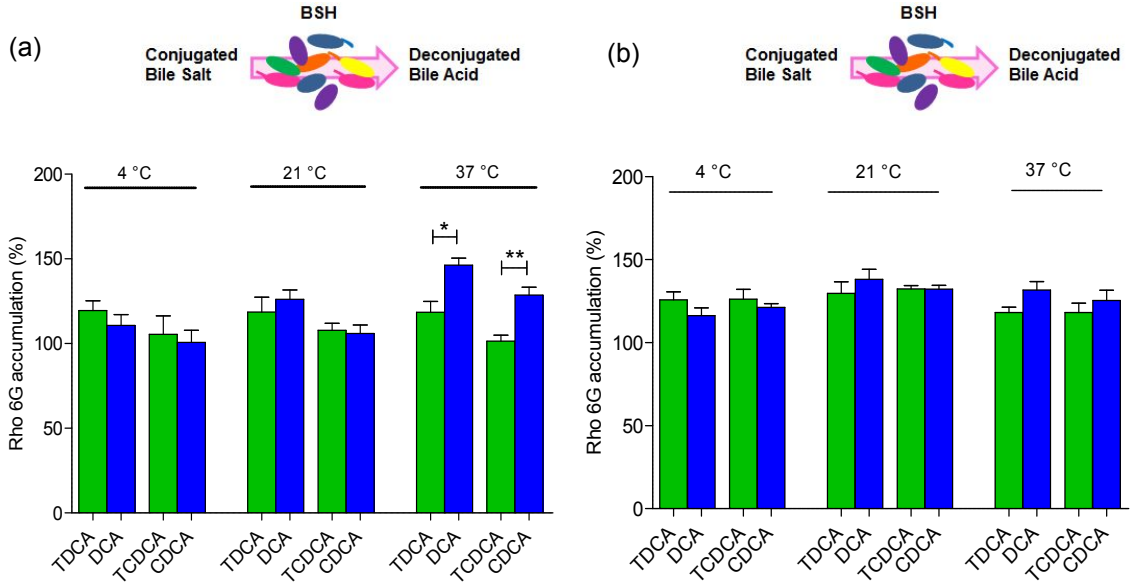


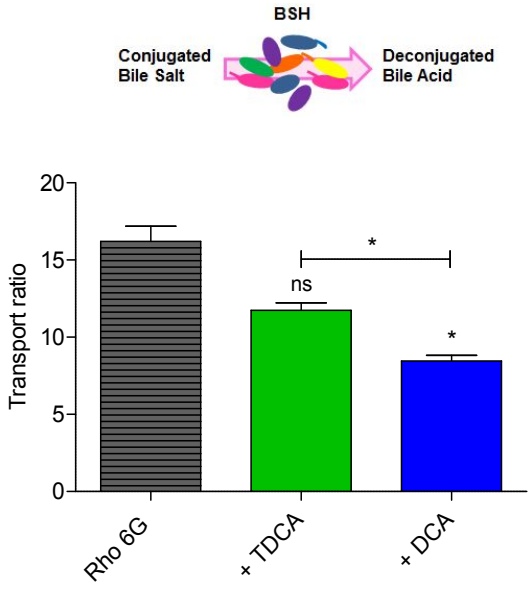
Figure 8. The effect of test bile acids on Rho 6G accumulation in Caco-2 (a) and T84 (b) cells following 3 h co-incubation. The Caco-2 37 °C experimental data illustrates that unconjugated DCA and CDCA increase the accumulation of Rho 6G to a greater extent than their tauro-conjugated counterparts (a). Although not significant, a similar trend was observed in the T84 cell line (b). A comparison of Rho 6G accumulation assays performed at 4 °C, 21 °C and 37 °C indicates that an active transporter or carrier-mediated process is implicated. The data is expressed as % fluorescent dye accumulation in control cells exposed solely to Rho 6G, arbitrarily set at 100%, and represents the mean ± SEM of four independent experiments (n = 6 for each assay condition).

Caco-2 monolayers are the most frequently used cellular model to simulate the intestinal epithelial barrier and to predict the flux of drugs across human intestinal tissue.[32] The flux of Rho 6G across Caco-2 monolayers in both absorptive (apical to basolateral) and secretory (basolateral to apical) directions was investigated in the presence of conjugated (TDCA) and deconjugated (DCA) bile acids. These polarized flux measurements were employed to confirm decreased P-gp efflux activity as the cause of the increase in Rho 6G accumulation following concurrent treatment with unconjugated bile acids.

Involvement of P-gp-mediated efflux is signified by a transport ratio of greater than 2,[33] essentially indicating that the net flux of the drug *in vivo* would be towards the gastrointestinal, as opposed to the serosal, side. The transport ratio of Rho 6G was determined to be 16.20 ± 1.39 (Figure 9), confirming that it is subject to efflux in the Caco-2 monolayer model. Verapamil was used as a positive control, and significantly reduced the basolateral to apical permeability of Rho 6G and consequently its transport ratio (2.17 ± 0.29) across the Caco-2 monolayers (Figure 9) by competitively saturating the pump. Following a pre-incubation period of 1 h and further continuous exposure for 3 h, DCA, but not TDCA, significantly reduced the secretory transport of Rho 6G ($P < 0.05$). This alteration in apparent permeability significantly impacted upon the transport ratio of Rho 6G, indicating an inhibition of efflux activity (Figure 9).

The addition of TDCA and DCA to the apical and basolateral compartments of the transwell setup was associated with a reduction in TEER (Table 3, Supporting Information). This observation is not unexpected. Araki *et al.* demonstrated that micromolar concentrations of bile acids decreased the TEER of Caco-2 monolayers, but that this was not a consequence of permanent cellular insult.[34] Similarly in this study, Caco-2 monolayer viability was not affected by the presence TDCA or DCA (Figure 5, Supporting Information). Considering that Rho 6G is a lipophilic dye and that TDCA and DCA induced

comparable reductions in TEER, we do not anticipate significant or differential impacts upon flux through the paracellular route.



Transport substrate	P-gp modulator	Test bile acid	$P_{app}A \rightarrow B$ (cm/s x 10^{-6})	$P_{app}B \rightarrow A$ (cm/s x 10^{-5})	Transport ratio
Rhodamine 6G (5 µM)			1.43 ± 0.18	2.31 ± 0.1	16.20 ± 1.39
Rhodamine 6G (5 µM)	Verapamil (100 µM)		4.80 ± 1.52	1.02 ± 0.19*	2.17 ± 0.29
Rhodamine 6G (5 µM)		TDCA (100 µM)	1.72 ± 0.11	2.01 ± 0.01 (*)	11.74 ± 0.69
Rhodamine 6G (5 µM)		DCA (100 µM)	2.01 ± 0.06	1.75 ± 0.06* (*)	8.47 ± 0.51

Figure 9. Transport ratios ($P_{app}B \rightarrow A / P_{app}A \rightarrow B$) of Rho 6G (5 µM) across Caco-2 monolayers in the absence and presence of P-gp modulators (100 µM test bile acids TDCA or DCA). Statistical significance in apparent permeability was assessed relative to control cells exposed to Rho 6G only ($P < 0.05$). (*) indicates a statistically significant difference between bile acid treatments ($P < 0.05$). Data is expressed as the mean ± SD of 2 independent experiments performed in triplicate. Data represents the mean ± SD of 2 independent experiments performed in triplicate.

3.6 Exploring the mechanism by which bile acids inhibit P-glycoprotein functioning

P-gp functions as an ATP-dependent drug efflux pump. Compounds that interact with P-gp can be classified as either substrates or inhibitors according to their tendency to stimulate or inhibit the ATPase activity of the transporter (i.e. its ability to hydrolyse ATP), respectively. In order to elucidate the possible mechanisms underpinning the observed enhancement in CsA toxicity and accumulation of Rho 6G in the presence of unconjugated bile acids, the effect of the test bile acids on P-gp ATPase activity was assessed using artificial human P-gp membrane vesicles.

In general, both conjugated and deconjugated bile acids inhibited basal P-gp ATPase activity (Figure 10 (a)). The test bile acids were also shown to inhibit substrate (verapamil or Rho 6G) stimulated P-gp ATPase, though this was not a generalizable effect (Figure 10 (b) and (c)).

The effects of test bile acids at a 5 μM concentration were also examined as it is unclear what percentage of the treated bile acid concentration (100 μM) is available to interact with P-gp in the cellular system. A 5 μM concentration of the test bile acids was also found to inhibit P-gp ATPase activity (Figure 6, Supporting Information).

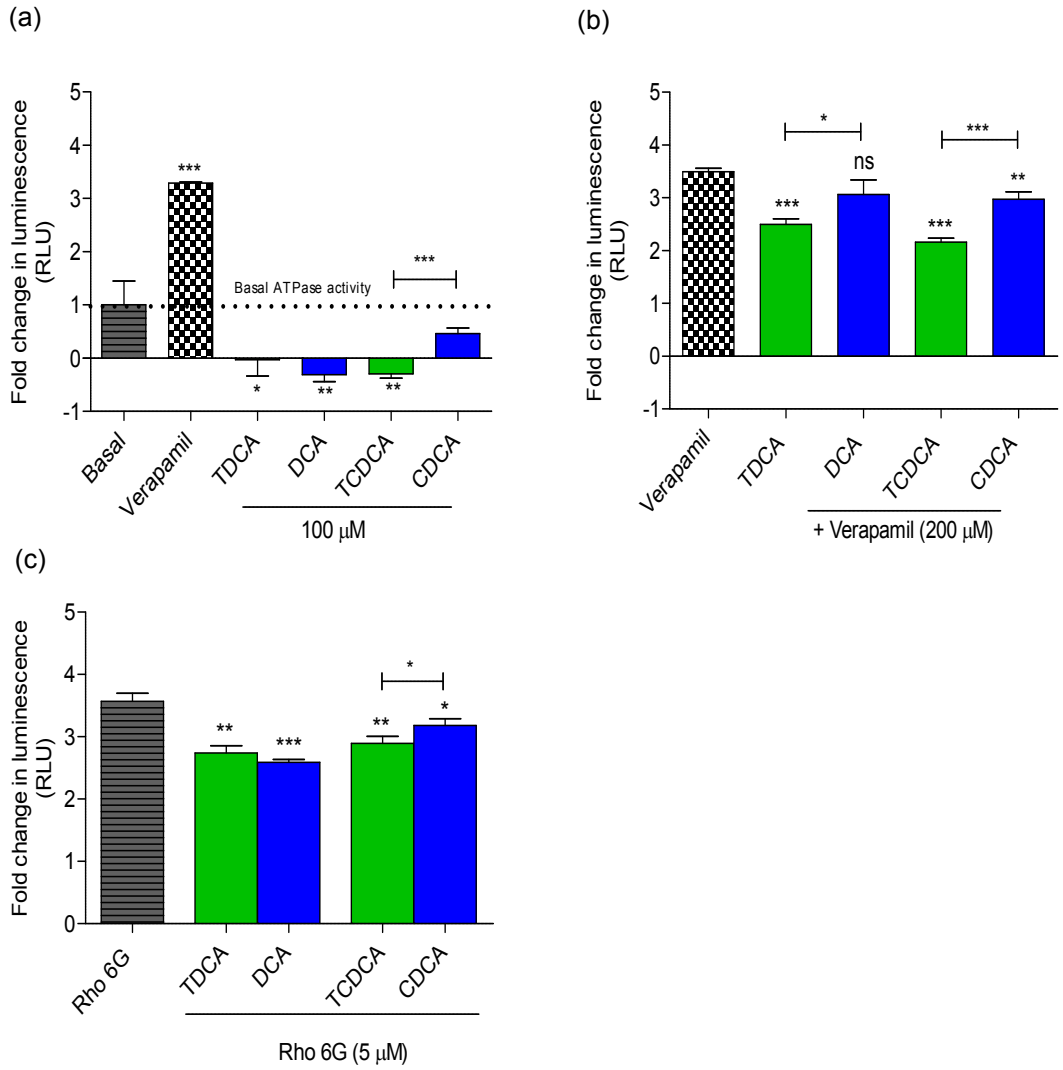


Figure 10. The effect of 100 μM bile acids on (a) basal P-gp ATPase activity, (b) 200 μM verapamil-stimulated and (c) 5 μM Rho 6G-stimulated P-gp ATPase activity. RLU = relative light units. Horizontal bars are indicative of a statistically significant difference between conjugated and unconjugated bile acids (n = 3, mean ± SD).

4. Discussion

4.1 The effect of bile acid conjugation and hydroxylation status on the expression of genes encoding drug transporters

Several recent studies have sought to investigate the role of the gut microbiome in influencing the expression of intestinal drug transporters. [35, 36] Previous studies within our research group involving germ-free and conventionalized mice, possessing distinct bile acid profiles, identified altered transcription of intestinal transporters involved in lipid transport.[9] Reanalysis of this data set (for the purpose of this study) additionally revealed altered transcription of enterocytic transporters involved in drug translocation (Figure 1 (a) and (b), Supporting Information). To the best of our knowledge, the effect of microbe-mediated bile acid transformations on the expression of human intestinal drug transporters has not been elucidated to date.

A panel of three human intestinal cell lines were employed to examine the effects of individual bile acids on the transcriptional expression of drug transporters. As the disposition of both endogenous molecules, such as bile acids, and drugs is most often related to two superfamilies, the ABC and SLC transporters, we focussed our efforts on elucidating the effect of bile acid transformations on the transcription and activity of several clinically relevant members (Figure 1, Supporting Information). In general, bile acid deconjugation and dehydroxylation were associated with transcriptional upregulation of genes encoding these transporters (in concurrence with in-house murine data). It appears that an unconjugated, dihydroxy bile acid signature is fundamental to the induction of common human intestinal transporters as exemplified by the ability of dihydroxy CDCA and DCA, but not trihydroxy CA, to generally upregulate mRNA transcripts (Table 2 and 3). These findings may explain, at least partially, recent reports of altered drug transporter expression in dysbiotic (germ-free and antibiotic-induced) animal models. [35] [37]

The expression of drug transporters is a major determinant of local and systemic drug exposure and resulting pharmacodynamic effects. Therefore, interindividual variation in drug response could be related to alterations in transporter expression. Taking into consideration our findings in gnotobiotic mice and in cultured enterocytes, we suggest that perturbation of the gut microbiota and therefore the bile acid pool may hence have implications for drug disposition and warrants further investigation. Such variation in transporter gene expression is likely to be determined by bile acid specific-stimulation of nuclear receptors such as FXR, PXR and the constitutive androstane receptor (CAR). In support of this, we report that unconjugated DCA and CDCA upregulate *FGF19* (a well-known FXR-regulated gene, Table 2, Supporting Information) in a manner similar to that observed for *ABCC2* and *ABCC3*, which are also purportedly regulated by FXR. [30] The mechanism underpinning the differential expression of *ABCB1* (P-gp) and *ABCG2* (BCRP) across the 3 cell lines following bile acid treatment, however, remains to be definitively elucidated. It is also challenging to decipher which of these commonly used cell lines provides information that is most relevant to the *in vivo* milieu and which would be the best candidate for transcriptional studies. Bourgine *et al.* report similar correlation coefficients between all 3 cell lines and human small intestinal (ileal) tissue biopsies (0.672 (Caco-2), 0.708 (T84) and 0.679 (HT-29)). The best correlation, however, was observed for human colonic tissue biopsies and T84 and HT-29 cell lines. [38] It is also important to note that mRNA gene expression data do not always correlate with the expression pattern of encoded proteins.

4.2 The effect of bile acid conjugation and hydroxylation status on the cytotoxicity of multidrug resistance transporter substrates

Guided by the knowledge that bile acids can significantly affect the expression of drug transporter mRNA in mice and in cultured human enterocytes, we investigated the possible implications for drug uptake using an *in vitro* drug cytotoxicity screen. Alterations to drug toxicity, which must succeed

changes to cellular accumulation, was used as a preliminary marker to assess the effect of bile acid treatment on the uptake and activity of a range of drugs.

Among the bile acids tested, unconjugated dihydroxy bile acids modulated the cytotoxicity of the model MDR substrates SN-38 and CsA, although these effects appeared to be drug-specific. We reason that the transporter specificity of these substrates (BCRP[39] and P-gp[40], respectively) may explain the differential tendencies of unconjugated, dihydroxy bile acids to modulate their cytotoxicity (Figure 2-5). However, such bile acid-dependent drug toxicities could not be explained by bile acid-induced changes to *ABCG2* (BCRP) and *ABCB1* (P-gp) mRNA expression (Table 2 and Table 3) nor to expressional changes in BCRP or P-gp proteins (Figure 6).

Cellular energy consumption constitutes one source of multidrug resistance inhibition as drug efflux is an energy-dependent process. Neither conjugated nor deconjugated bile acids at working concentrations induced any appreciable changes in cellular ATP levels (Figure 2, Supporting Information, cell viability based on ATP quantification). Hence, it is unlikely that a bile acid-induced depletion of intracellular ATP underpins the chemo-sensitization of Caco-2 and T84 cells as has been previously reported for non-physiological surfactants such as Pluronic P85.[41, 42] We reason that unconjugated bile acids must sensitize cells to SN-38 and CsA through alternative, functional effects on these transporter proteins.[14]

4.3 The effect of bile acid conjugation status on the cellular uptake of P-gp substrates

The P-gp activity of cell lines has been routinely investigated by monitoring the accumulation and efflux of small, fluorescent molecules, such as rhodamine dyes. Rho 6G is reportedly a relatively hydrophobic P-gp substrate, [43] which was verified herein by its capacity to stimulate basal P-gp ATPase (Figure 2, Supporting Information). Previous studies by Zastre *et al.*, indicate that the P-gp transporter is responsible for limiting the intracellular accumulation of Rho 6G in Caco-2 cells, with little or no contribution from multidrug resistance protein isoforms.[43] Unconjugated DCA and CDCA significantly increased Caco-2 cellular accumulation of Rho 6G ($P < 0.05$), an effect that was not evident in the presence of tauro-conjugated equivalents (Figure 8 (a)). Parallel studies performed at 4 °C and 21 °C confirmed that the effect of bile acid deconjugation on the accumulation of Rho 6G is an energy-dependent process (Figure 8 (a)). In T84 cells, although not statistically significant, a similar trend was observed (Figure 8 (b)).

This phenomenon supports the hypothesis that unconjugated bile acids can positively modulate the accumulation of P-gp substrates, thereby pointing to a potential role for microbial BSH in influencing the uptake and activity of molecules susceptible to P-gp exclusion. We note reports in non-intestinal cell lines, which suggest that in addition to susceptibility to P-gp efflux, Rho 6G may be subject to uptake by organic cation transporters (OCT1 and OCT2). [44] While the basal mRNA expression of SLC22A1 (encoding OCT1) in the intestinal cell lines employed in this study is low (Table 1, Supporting Information), the contribution of these transporters to Rho 6G may warrant further consideration.

4.4 The effect of bile acid conjugation status on the transport of P-gp substrates across Caco-2 monolayers: a model of the gastrointestinal barrier

Bidirectional transport studies (with polarized Caco-2 monolayers) were employed to confirm and quantify the impact of bile acid deconjugation on the translocation of Rho 6G, specifically enabling assessment of alterations to absorptive and secretory apparent permeability. Bile acids were added to both the apical and basolateral compartments as bile acids are present both intraluminally and in the portal circulation *in vivo*. This experiment is the first in the literature to demonstrate that micromolar concentrations DCA can preferentially decrease (relative to its tauro-conjugate) the basolateral to apical secretion of Rho 6G and to consequentially identify a possible role for microbial BSH in modulating the uptake of P-gp substrates (Figure 9). Lo *et al.* previously demonstrated that 1.2 mM DCA reduced

the secretion of epirubicin, a P-gp substrate, across Caco-2 cell monolayers.[14] Lo and colleagues speculated that the membrane perturbation induced by DCA, and identified by Sakai *et al*.,[45] may invoke changes in the fluidity of the membrane and hence inhibit the activity of P-gp. Given the surfactant nature of bile acids and observed reductions in TEER (Table 3, Supporting Information), changes in membrane fluidity may partially underpin the ability of certain bile acids to circumvent P-gp-mediated efflux. However, no statistically significant difference in TEER reduction was noted following treatment with TDCA and DCA, we hence sought to further investigate the preferential capacity of unconjugated DCA to attenuate the active efflux of Rho 6G.

4.5 The effect of bile acid conjugation status on P-gp ATPase activity

ABC transporters contain ATP-binding domains that possess ATPase activity, that is, the ability to hydrolyse ATP to ADP (adenosine diphosphate), providing the energy for substrate translocation. Many P-gp modulators such as verapamil and CsA, are substrates themselves, competitively inhibiting its functioning without interrupting the catalytic turnover of the pump.[46] Unconjugated DCA and CDCA were determined to modulate the cytotoxicity of CsA (Figures 3 (a-b)) and the accumulation of Rho 6G (Figure 8), despite neither bile acid invoking changes to basal P-gp expression (Figure 6). It was therefore postulated that unconjugated bile acids may be substrates for P-gp efflux or inhibitors of P-gp ATPase, thereby preferentially mitigating the active efflux.

Both conjugated and deconjugated bile acids were generally found to inhibit basal, as well as substrate (verapamil and Rho 6G) stimulated P-gp ATPase activity (Figure 10). This inability of the P-gp-Glo assay system to identify a role for bile acid conjugation status in differentially modulating P-gp ATPase activity most likely reflects the artificial nature of the membrane vesicles. Physiological membranes are relatively impermeable to ionized (conjugated) bile salt species,[47] and hence their transport is dependent upon carrier proteins. In contrast, a significant proportion of unconjugated bile acids exist in the unionized form at intestinal pH and are consequently available for passive, non-ionic diffusion. In general, the unionized form of a weak acid will permeate the intestinal membrane much quicker than its ionized form.[48] In a cellular model, the cytosolic leaflet of the plasma membrane, which contains the substrate binding site and two nucleotide binding domains, must be approached by the passive diffusion of a molecule across the lipid bilayer, whereas in a cell membrane fraction (such as the P-gp-Glo system) it is directly accessible from aqueous phase.[49] We propose that in the cellular system unconjugated bile acids, such as DCA and CDCA, are thus most likely to interact with P-gp due to an elevated hydrophobic character. To the best of our knowledge, this is the first study to report the ability of bile acids to modulate P-gp activity through an inhibition of P-gp ATPase. Telbisz *et al.* have, however, demonstrated that glycocholic acid (GCA), taurocholic acid (TCA) and CA can significantly reduce basal BCRP (*ABCG2*) ATPase activity.[50] Whilst the bile acids included in our study were not considered by Telbisz *et al.*, we suggest that the differential inhibition of BCRP ATPase by DCA, as compared to TDCA, may perhaps partially explain the increased cytotoxicity of the BCRP (*ABCG2*) substrate SN-38 (Figure 2 (a)) and warrants further investigation. Overall, the possibility that bile acids may compete with P-gp substrates for competitive extrusion via the active site was excluded due to their tendency to inhibit, rather than stimulate, basal P-gp ATPase.

While our findings consistently indicate that unconjugated bile acids preferentially modulate the uptake/activity of specific P-gp and BCRP transporter substrates *in vitro*, the contribution of other transport processes (additional active transporters or indeed passive diffusion mechanisms) cannot be ruled out entirely. In interpreting these findings, it is important to be cognizant that many drug transporter proteins exhibit significant overlap in substrate specificity. [51]

Conclusion

In concurrence with in-house observations of altered intestinal transporter mRNA transcripts in germ-free and conventionalized mice, we demonstrated that unconjugated/dihydroxy bile acids can similarly alter the expression of genes encoding human drug transporters *in vitro*. Future work should be directed toward investigating possible implications for drug disposition *in vivo*.

This study has identified that the conjugation and hydroxylation status of a bile acid dictates its capacity to modulate the uptake of efflux transporter substrates and thereby circumvent multidrug resistance *in vitro*. This study has hence indirectly identified a role for microbial bile acid metabolism in modulating MDR transporters, which in the case of P-gp appears to be mediated by inhibition of ATPase activity. The generalizable attenuating effect of deconjugation on P-gp functionality can be attributed to the more favourable physicochemical property of nonionization, pointing to a structure-activity relationship between the bile acid steroid nucleus and reversal of multidrug resistance.

While future work is necessary to decipher whether this phenomenon could persist in the more complex *in vivo* milieu in presence of fat-soluble vitamins and lipophilic xenobiotics; this study provides additional impetus to consider microbial metabolites, in addition to the host, as possible factors influencing drug pharmacokinetics. Perturbation of the human gut microbiota, and consequently the bile acid metabolome[52], could contribute to altered drug uptake across the intestinal barrier and therefore potentially constitute a source of pharmacokinetic variability.

Supporting Information

Supporting information is available as additional figures and tables as noted in the text. Schematic indicating polarized expression of common intestinal drug transporter proteins; cell viability data outlining selection of non-cytotoxic, working concentrations of bile acids; average cycle threshold values of drug transporter genes considered in this study relative to β-actin; cell viability data investigating the impact of glycine-deconjugation on the ability of bile acids to circumvent multidrug resistance; P-gp Glo assay data of Rho 6G; TEER of Caco-2 monolayers; cell viability of Caco-2 monolayers; P-gp Glo assay data of 5 μM bile acids.

Acknowledgements

Elaine F. Enright is a recipient of a Government of Ireland Postgraduate Scholarship from the Irish Research Council (grant number GOIPG/2015/3261). The authors acknowledge the funding of APC Microbiome Ireland by the Science Foundation of Ireland Centres for Science, Engineering and Technology (CSET) programme (Grant Number SFI/12/RC/2273). The authors wish to kindly acknowledge the expertise and support of Dr. Maria Angeles Nuñez Sanchez with western blotting.

References

[1]. Enright, E. F.; Gahan, C. G.; Joyce, S. A.; Griffin, B. T. The impact of the gut microbiota on drug metabolism and clinical outcome. *The Yale journal of biology and medicine* **2016,** *89*, (3), 375-382.

[2]. Enright, E. F.; Joyce, S. A.; Gahan, C. G.; Griffin, B. T. Impact of gut microbiota-mediated bile acid metabolism on the solubilization capacity of bile salt micelles and drug solubility. *Mol Pharm* **2017,** *14*, (4), 1251-1263.

[3]. Mukaizawa, F.; Taniguchi, K.; Miyake, M.; Ogawara, K.-i.; Odomi, M.; Higaki, K.; Kimura, T. Novel oral absorption system containing polyamines and bile salts enhances drug transport via both transcellular and paracellular pathways across Caco-2 cell monolayers. *International Journal of Pharmaceutics* **2009,** *367*, (1), 103-108.

[4]. Meaney, C. M.; O'Driscoll, C. M. A comparison of the permeation enhancement potential of simple bile salt and mixed bile salt:fatty acid micellar systems using the CaCo-2 cell culture model. *International Journal of Pharmaceutics* **2000,** *207*, (1), 21-30.

[5]. Chiang, J. Y. Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocrine reviews* **2002,** *23*, (4), 443-63.

[6]. Begley, M.; Gahan, C. G.; Hill, C. The interaction between bacteria and bile. *FEMS microbiology reviews* **2005,** *29*, (4), 625-51.

[7]. Teng, S.; Piquette-Miller, M. Regulation of Transporters by Nuclear Hormone Receptors: Implications during Inflammation. *Molecular Pharmaceutics* **2008,** *5*, (1), 67-76.

[8]. Long, S. L.; Gahan, C. G. M.; Joyce, S. A. Interactions between gut bacteria and bile in health and disease. *Molecular Aspects of Medicine* **2017,** *56*, (Supplement C), 54-65.

[9]. Joyce, S. A.; MacSharry, J.; Casey, P. G.; Kinsella, M.; Murphy, E. F.; Shanahan, F.; Hill, C.; Gahan, C. G. M. Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. *Proceedings of the National Academy of Sciences of the United States of America* **2014,** *111*, (20), 7421-7426.

[10]. Sayin, Sama I.; Wahlström, A.; Felin, J.; Jäntti, S.; Marschall, H.-U.; Bamberg, K.; Angelin, B.; Hyötyläinen, T.; Orešič, M.; Bäckhed, F. Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring fxr antagonist. *Cell Metabolism* **2013,** *17*, (2), 225-235.

[11]. Estudante, M.; Morais, J. G.; Soveral, G.; Benet, L. Z. Intestinal drug transporters: An overview. *Advanced Drug Delivery Reviews* **2013,** *65*, (10), 1340-1356.

[12]. Szakacs, G.; Paterson, J. K.; Ludwig, J. A.; Booth-Genthe, C.; Gottesman, M. M. Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* **2006,** *5*, (3), 219-234.

[13]. Makhey, V. D.; Guo, A.; Norris, D. A.; Hu, P.; Yan, J.; Sinko, P. J. Characterization of the regional intestinal kinetics of drug efflux in rat and human intestine and in caco-2 cells. *Pharmaceutical Research* **1998,** *15*, (8), 1160-1167.

[14]. Lo, Y.-L.; Huang, J.-D. Effects of sodium deoxycholate and sodium caprate on the transport of epirubicin in human intestinal epithelial Caco-2 cell layers and everted gut sacs of rats. *Biochemical Pharmacology* **2000,** *59*, (6), 665-672.

[15]. Germann, U. A. P-glycoprotein—a mediator of multidrug resistance in tumour cells. *European Journal of Cancer* **1996,** *32*, (6), 927-944.

[16]. O'Brien, F. E.; O'Connor, R. M.; Clarke, G.; Dinan, T. G.; Griffin, B. T.; Cryan, J. F. P-glycoprotein inhibition increases the brain distribution and antidepressant-like activity of escitalopram in rodents. *Neuropsychopharmacology* **2013,** *38*, (11), 2209-2219.

[17]. Albring, A.; Wendt, L.; Harz, N.; Engler, H.; Wilde, B.; Kribben, A.; Lindemann, M.; Schedlowski, M.; Witzke, O. Relationship between pharmacokinetics and pharmacodynamics of calcineurin inhibitors in renal transplant patients. *Clinical transplantation* **2015,** *29*, (4), 294-300.

[18]. Wu, C.-Y.; Benet, L. Z.; Hebert, M. F.; Gupta, S. K.; Rowland, M.; Gomez, D. Y.; Wacher, V. J. Differentiation of absorption and first-pass gut and hepatic metabolism in humans: Studies with cyclosporine. *Clinical Pharmacology & Therapeutics* **1995,** *58*, (5), 492-497.

[19]. Masuda, S.; Goto, M.; Kiuchi, T.; Uemoto, S.; Kodawara, T.; Saito, H.; Tanaka, K.; Inui, K.-i. Enhanced expression of enterocyte P-glycoprotein depresses cyclosporine bioavailability in a recipient of living donor liver transplantation. *Liver Transplantation* **2003,** *9*, (10), 1108-1113.
[20]. Lown, K. S.; Mayo, R. R.; Leichtman, A. B.; Hsiao, H.-l.; Turgeon, D. K.; Schmiedlin-Ren, P.; Brown, M. B.; Guo, W.; Rossi, S. J.; Benet, L. Z.; Watkins, P. B. Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine. *Clinical Pharmacology & Therapeutics* **1997,** *62*, (3), 248-260.
[21]. Masuo, T.; Okamura, S.; Zhang, Y.; Mori, M. Cyclosporine A inhibits colorectal cancer proliferation probably by regulating expression levels of c-Myc, p21WAF1/CIP1 and proliferating cell nuclear antigen. *Cancer Letters* **2009,** *285*, (1), 66-72.
[22]. Mao, Q.; Unadkat, J. D. Role of the Breast Cancer Resistance Protein (BCRP/ABCG2) in Drug Transport—an Update. *The AAPS Journal* **2015,** *17*, (1), 65-82.
[23]. Dudeja, P. K.; Anderson, K. M.; Harris, J. S.; Buckingham, L.; Coon, J. S. Reversal of multidrug-resistance phenotype by surfactants: Relationship to membrane lipid fluidity. *Archives of Biochemistry and Biophysics* **1995,** *319*, (1), 309-315.
[24]. Lozupone, C. A.; Stombaugh, J. I.; Gordon, J. I.; Jansson, J. K.; Knight, R. Diversity, stability and resilience of the human gut microbiota. *Nature* **2012,** *489*, (7415), 220-230.
[25]. Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods (San Diego, Calif.)* **2001,** *25*, (4), 402-8.
[26]. de Aguiar Vallim, Thomas Q.; Tarling, Elizabeth J.; Edwards, Peter A. Pleiotropic roles of bile acids in metabolism. *Cell Metabolism* **2013,** *17*, (5), 657-669.
[27]. Chewchuk, S.; Boorman, T.; Edwardson, D.; Parissenti, A. M. Bile acids increase doxorubicin sensitivity in abcc1-expressing tumour cells. *Scientific Reports* **2018,** *8*, (1), 5413.
[28]. Inokuchi, A.; Hinoshita, E.; Iwamoto, Y.; Kohno, K.; Kuwano, M.; Uchiumi, T. Enhanced expression of the human multidrug resistance protein 3 by bile salt in human enterocytes. A transcriptional control of a plausible bile acid transporter. *The Journal of biological chemistry* **2001,** *276*, (50), 46822-9.
[29]. Kok, T. M. C. M. D.; Van Faassen, A.; Glinghammar, B.; Pachen, D. M. F. A.; Rafter, J. J.; Baeten, C. G. M. I.; Engels, L. G. J. B.; Kleinjans, J. C. S. Bile acid concentrations, cytotoxicity, and ph of fecal water from patients with colorectal adenomas. *Digestive Diseases and Sciences* **1999,** *44*, (11), 2218-2225.
[30]. Staudinger, J. L.; Woody, S.; Sun, M.; Cui, W. Nuclear-receptor-mediated regulation of drug- and bile-acid-transporter proteins in gut and liver. *Drug metabolism reviews* **2013,** *45*, (1), 48-59.
[31]. Schumacher, U.; Nehmann, N.; Adam, E.; Mukthar, D.; Slotki, I. N.; Horny, H.-P.; Flens, M. J.; Schlegelberger, B.; Steinemann, D. MDR-1-overexpression in HT 29 colon cancer cells grown in SCID mice. *Acta Histochemica* **2012,** *114*, (6), 594-602.
[32]. Artursson, P.; Palm, K.; Luthman, K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Advanced Drug Delivery Reviews* **1996,** *22*, (1), 67-84.
[33]. Polli, J. W.; Wring, S. A.; Humphreys, J. E.; Huang, L.; Morgan, J. B.; Webster, L. O.; Serabjit-Singh, C. S. Rational use of in vitro p-glycoprotein assays in drug discovery. *Journal of Pharmacology and Experimental Therapeutics* **2001,** *299*, (2), 620.
[34]. Araki, Y.; Katoh, T.; Ogawa, A.; Bamba, S.; Andoh, A.; Koyama, S.; Fujiyama, Y.; Bamba, T. Bile acid modulates transepithelial permeability via the generation of reactive oxygen species in the Caco-2 cell line. *Free Radical Biology and Medicine* **2005,** *39*, (6), 769-780.
[35]. Kuno, T.; Hirayama-Kurogi, M.; Ito, S.; Ohtsuki, S. Effect of intestinal flora on protein expression of drug-metabolizing enzymes and transporters in the liver and kidney of germ-free and antibiotics-treated mice. *Molecular Pharmaceutics* **2016,** *13*, (8), 2691-2701.
[36]. Fu, Z. D.; Selwyn, F. P.; Cui, J. Y.; Klaassen, C. D. RNA-seq profiling of intestinal expression of xenobiotic processing genes in germ-free mice. *Drug Metabolism and Disposition* **2017,** *45*, (12), 1225.
[37]. Zhang, Y.; Limaye, P. B.; Renaud, H. J.; Klaassen, C. D. Effect of various antibiotics on modulation of intestinal microbiota and bile acid profile in mice. *Toxicology and Applied Pharmacology* **2014,** *277*, (2), 138-145.
[38]. Bourgine, J.; Billaut-Laden, I.; Happillon, M.; Lo-Guidice, J. M.; Maunoury, V.; Imbenotte, M.; Broly, F. Gene expression profiling of systems involved in the metabolism and the disposition of

xenobiotics: comparison between human intestinal biopsy samples and colon cell lines. *Drug metabolism and disposition: the biological fate of chemicals* **2012,** *40*, (4), 694-705.
[39]. Nakatomi, K.; Yoshikawa, M.; Oka, M.; Ikegami, Y.; Hayasaka, S.; Sano, K.; Shiozawa, K.; Kawabata, S.; Soda, H.; Ishikawa, T.; Tanabe, S.; Kohno, S. Transport of 7-Ethyl-10-hydroxycamptothecin (SN-38) by breast cancer resistance protein ABCG2 in human lung cancer cells. *Biochemical and Biophysical Research Communications* **2001,** *288*, (4), 827-832.
[40]. Silva, R.; Vilas-Boas, V.; Carmo, H.; Dinis-Oliveira, R. J.; Carvalho, F.; de Lourdes Bastos, M.; Remião, F. Modulation of P-glycoprotein efflux pump: induction and activation as a therapeutic strategy. *Pharmacology & Therapeutics* **2015,** *149*, (Supplement C), 1-123.
[41]. Batrakova, E. V.; Li, S.; Alakhov, V. Y.; Elmquist, W. F.; Miller, D. W.; Kabanov, A. V. Sensitization of cells overexpressing multidrug-resistant proteins by pluronic p85. *Pharmaceutical Research* **2003,** *20*, (10), 1581-1590.
[42]. Kabanov, A. V.; Batrakova, E. V.; Yu. Alakhov, V. An essential relationship between ATP depletion and chemosensitizing activity of Pluronic® block copolymers. *Journal of Controlled Release* **2003,** *91*, (1), 75-83.
[43]. Zastre, J. A.; Jackson, J. K.; Wong, W.; Burt, H. M. P-Glycoprotein Efflux Inhibition by Amphiphilic Diblock Copolymers: Relationship between Copolymer Concentration and Substrate Hydrophobicity. *Molecular Pharmaceutics* **2008,** *5*, (4), 643-653.
[44]. Ugwu, M. C.; Oli, A.; Esimone, C. O.; Agu, R. U. Organic cation rhodamines for screening organic cation transporters in early stages of drug development. *Journal of Pharmacological and Toxicological Methods* **2016,** *82*, 9-19.
[45]. Sakai, M.; Imai, T.; Ohtake, H.; Azuma, H.; Otagiri, M. Effects of absorption enhancers on the transport of model compounds in caco-2 cell monolayers: Assessment by confocal laser scanning microscopy. *Journal of Pharmaceutical Sciences* **1997,** *86*, (7), 779-785.
[46]. Maki, N.; Hafkemeyer, P.; Dey, S. Allosteric modulation of human p-glycoprotein: Inhibition of transport by preventing substrate translocation and dissociation. *Journal of Biological Chemistry* **2003,** *278*, (20), 18132-18139.
[47]. Dietschy, J. M. Mechanisms for the intestinal absorption of bile acids. *Journal of Lipid Research* **1968,** *9*, (3), 297-309.
[48]. Schanker, L. S.; Tocco, D. J.; Brodie, B. B.; Hogben, C. A. M. Absorption of drugs from the rat small intestine. *Journal of Pharmacology and Experimental Therapeutics* **1958,** *123*, (1), 81.
[49]. Nervi, P.; Li-Blatter, X.; Aanismaa, P.; Seelig, A. P-glycoprotein substrate transport assessed by comparing cellular and vesicular ATPase activity. *Biochimica et biophysica acta* **2010,** *1798*, (3), 515-25.
[50]. Telbisz, Á.; Özvegy-Laczka, C.; Hegedűs, T.; Váradi, A.; Sarkadi, B. Effects of the lipid environment, cholesterol and bile acids on the function of the purified and reconstituted human ABCG2 protein. *Biochemical Journal* **2013,** *450*, (2), 387.
[51]. Schinkel, A. H.; Jonker, J. W. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* **2003,** *55*, (1), 3-29.
[52]. Enright, E. F.; Griffin, B. T.; Gahan, C. G. M.; Joyce, S. A. Microbiome-mediated bile acid modification: Role in intestinal drug absorption and metabolism. *Pharmacological research* **2018,** *133*, 170-186.