The role of P-glycoprotein and breast cancer resistance protein (BCRP) in bacterial attachment to human gastrointestinal cells☆

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Abstract

Background and aims: Active efflux proteins such as P-glycoprotein (P-gp) are thought to have a protective role in the intestinal tract by preventing xenotoxin absorption. Some bacteria also need to adhere to the intestinal tract before causing disease through adhesin secretion. Thus, this study was initiated to examine whether any association exists between bacterial adhesion.

Methods: Three human cell lines (Caco2, RKO, and MCF7), and 6 species of bacteria were used in this study (Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Klebsiella pneumoniae, Clostridium sporogenes and Pseudomonas aeruginosa). Following incubation of our cells with active efflux inhibitors, bacteria incubated with a stable fluorescent dye were co-incubated at 37 °C for various times up to 240 min. Fluorescence intensity was used to compare bacterial attachment to these cell lines with either normal efflux protein expression or with induction or inhibition of efflux proteins.

Results: P-gp inhibition by either PSC-833 or GF120918 resulted in a significant increase of all bacterial attachment to Caco2 cells up to 3 fold. RKO cells and MCF7 cells did not alter their bacterial attachment with PSC-833. Fumitremorgen C, a dedicated BCRP inhibitor had no effect. In addition, rifampicin, a P-gp inducer, resulted in some limited reduction in Salmonella and Klebsiella attachment only.

Conclusions: These results indicate P-gp expression may contribute to the resistance of potential bacterial toxicity, by preventing them adhering to human enterocytes cells in the gastrointestinal tract, which may reduce the risk or intensity of gastrointestinal disorders.

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1. Introduction

The gastrointestinal tract is resident to over 300 different species of bacteria. The process of their bacterial adhesion has two-phases which are comprised of an initial, instantaneous, and reversible physical phase followed by a time-dependent and irreversible molecular and cellular phase. It is well recognized that the adhesion of bacteria to biological surfaces is a prerequisite to invasion and is the first step in pathogenesis. After attachment, in most cases pathogens will penetrate the epithelial layer, interacting with leukocytes and macrophages to induce an immune response. Bacterial adherence is also an important factor in the development of inflammatory bowel disease, as it has been shown that the number of bacteria attached to the intestinal tract is higher in IBD than in those of the normal population.

The etiology of IBD remains unresolved, however the persistent inflammation seen in IBD may be a result of enhanced immunological response to natural constituents of the gut or an autoimmune dysregulation or imbalance. Despite these proposed causes it remains evident that intestinal flora are an important co-factor in the pathogenesis of intestinal inflammation, given that broad-spectrum antibiotics can prevent initiation of IBD and reverse colitis in humans and animal models. Recent studies also confirm that patients with active IBD have reduced P-gp expression in the gastrointestinal tract.13

Intestinal epithelial cells are known to express the multi drug resistance (MDR1) protein also known as the ABCB1 gene product or P-glycoprotein. In humans, P-glycoprotein is concentrated at the apical surfaces of superficial columnar epithelial cells of the colon and the jejunum, the liver and pancreas. It is also found in the surface of the epithelium of the choroid plexus, which forms the blood-cerebrospinal fluid barrier as well as the blood brain barrier.

Panwala and colleagues showed that one quarter of mdr1a(−/−) knockout mice developed loose stools and had reduced growth rates. Furthermore these mice developed extra-intestinal manifestations of inflammation in the kidney, the liver and the spleen. These signs and symptoms have also been observed in human patients with inflammatory bowel disease. The similar characteristics of colitis in mdr1a(−/−) mice to that of patients with IBD suggested a link between reduced activity of P-glycoprotein in intestinal epithelial cells and pathogenesis of IBD. The prevention or reversal of this condition in these mice by antibiotic treatment further emphasized the link between bacteria and P-ggp. In addition, other research teams have started examining which bacteria are likely to induce this pathology in mdr1(−/−) mice, such as some species of Helicobacter. Recent studies also confirm that patients with active IBD have reduced P-gp expression in the gastrointestinal tract.

The purpose of this study was to investigate whether P-glycoprotein was able to alter bacterial adherence to Caco2 cells and then to compare Caco2 binding with a 2nd gastrointestinal cell line with no active P-gp expression or BCRP, or a human cell line with high BCRP expression, MCF7 with very low P-gp expression (although in this case a breast cancer cell line). Caco2 cells are commonly used models for the differentiated human intestine. The cells are derived from colon carcinoma and are able to differentiate spontaneously after confluence and show epithelial cell polarization and a fully developed apical brush-border membrane two weeks postconfluence. They are also able to form microvilli and express small intestinal like characteristics both biochemically and morphologically, including high P-glycoprotein expression.

The KRO colorectal carcinoma cell line has virtually no detectable P-gp activity and minimal BCRP activity, as shown from this study, and previously, while MCF-7 which is an estrogen receptor positive, tumor derived breast cancer cell model, that also shows no functional expression of P-gp, but does have high BCRP expression.

Six bacterial species were used for this project, from Gram negative rod shaped Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae and Pseudomonas aeruginosa to Gram positive rod shaped Clostridium sporogenes (anaerobe) and Gram positive cocci shaped Staphylococcus aureus.

2. Materials and methods

2.1. Chemicals

PSC 833 was a kind donation from Novartis Pharmaceuticals (Basel, Switzerland), GF 120918 was kindly donated from GlaxoSmithKline (Victoria, Australia). MK571 and glyburide were purchased from Biomol International biochemicals. Fumitremorgin C was purchased from Alexis Biochemicals. BacLight Green was obtained from Molecular Probes (Eudene, Oregon, USA) while rifampicin, progesterone, Rhodamine 123 (Rh123), and most other fine chemicals used were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

Dulbecco’s Modified Eagles Medium (DMEM) and Dulbecco’s Phosphate buffered saline (PBS) were also purchased from Sigma Aldrich (Castle Hill, NSW Australia.) Penicillin G (10,000 U/mL), Streptomycin sulfate (10,000 μg/mL) and L-glutamine were from Gibco (Melbourne, Australia.) Non-essential amino acid solution (NEAA) was obtained from Trace Biosciences (Castle Hill, NSW, Australia,) while fetal calf serum (FCS) was supplied by Australian Commonwealth Serum Laboratories (Parkville, Vic, Australia.)

Caco2 cells growth medium contained 10% FCS, 1 mM non-essential amino acid (NEAA), 100 U/mL Penicillin/Streptomycin and 2 mM L-glutamine in DMEM.

Micro BCA protein assay chemicals were obtained in a kit from Pierce (Illinois, USA. Western blotting reagents (antibody wash and chemiluminescent substrate) were obtained from Invitrogen and were part of the Western Breeze detection kit. Ponceau S solution and protease Inhibitor tablets were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). The P-gp Mdr (G-1) mouse monoclonal, MRP1 (QCRL-1) mouse monoclonal, MRP2 (M2 III-6) mouse monoclonal antibodies were obtained from Santa Cruz Biotechnology. The BCRP (BXP-21) mouse monoclonal antibody was purchased from Chemicon (Invitrogen, Mulgrave, Vic, Australia).

Nutrient agar, nutrient broth and Thioglycate medium were from Oxoid Australia (Thebarton, Adelaide, South Australia).

2.2. Bacterial culture

The aerobic bacteria were stored in bottles of nutrient broth. The aerobic bacteria (E. coli W (ATCC 9637), S. aureus (ATCC 6538), S. typhimurium (ATCC 7823), K. pneumoniae var
2.3. BacLight Green preparation

Preliminary studies were conducted on BacLight Green stain (dissolved in DMSO to form 1 mM stock concentrations) to identify its potential to inhibit bacterial growth. Colonies of bacteria were scraped from agar plates and suspended in cold sterile PBS. The bacteria were then centrifuged at 3000 g for 10 min. Resuspending the pellet in 2×500 μL cold PBS, 8 μL of a 100 μM BacLight Green solution in DMSO was added to one tube for each of the bacteria to give a final dye concentration of 1.60 μM. These tubes were incubated for 45 min at room temperature then centrifuged at 12,000 g for 6 min, aspirated and washed three times with cold PBS. After the final wash, the pellets were re-suspended and plated on nutrient agar plates.

The nutrient agar plates were incubated at 37 °C for 24 h (72 h for C. sporogenes) and were compared to controls to determine whether any growth retardation has occurred either with colony counts or general growth characteristics.

2.4. Fluorescence method

A 96 well fluorescence plate reader (Fluostar Optima) from BMG LabTech (Mornington, Victoria, Australia) was used to detect fluorescent bacteria in black 96 well plates. A 485 nm Excitation filter and 520 nm Emission filter were used for detection using both top excitation and emission.

2.5. Determining a rapid cell count

In order to count the number of colony forming units on the same day as the fluorescence study, standard curves were developed for each of the 6 bacteria. Firstly a test was done to ensure that the absorbance measurement was not altered by adding the fluorescent dye to the bacteria. Stained and unstained bacterial suspensions in PBS were serially diluted in 96 well plates from stock, to 1/256th of stock. Absorbance measurement was carried on these samples at 415 nm wavelength using a Tecan Sunrise 96 well plate reader using the Magellan 3 software for Windows 2000. There was no significant difference between the absorbance for the bacterial suspensions with or without BacGreen Light staining. Samples from each well of bacteria was diluted 1000 fold in PBS and spread over 4.7 cm agar plates and allowed to grow for up to 48 h. Dilutions of bacteria that showed between 40 and 1000 colony forming units per plate were used for generation of standard curves. After incubation, the colonies on each of the plates were counted using a ChemiImager 4400 from Alphalnnotech (Cell Biosciences, Santa Clara, CA, USA) with the assistance of the colony counting module with the AlphaEaseFC program. This enabled linear regression analysis of the absorbance vs cell count and provided a formula to estimate cell count from absorbance to be determined for all individual bacterial species ensuring equivalent bacterial addition to the human cell lines. In addition, previous studies had already shown that BacLight Green accumulated inside the bacteria did not leak back out into the culture environment, which is a key characteristic needed to ensure no non-specific binding of our human cells during the attachment studies.

2.6. Human cell growth

Caco-2 cells, RKO and MCF7 cells were grown in 25 cm² flasks. Cells were grown in ‘growth medium’ (high glucose DMEM with 25 mM Hapes (pH 7.4), 2 mM glutamine, 1 mM non-essential amino acids, 100 U/mL penicillin-Streptomycin and 10% fetal calf serum (FCS)) in a 37 °C incubator with 5% CO₂.

Caco-2 cells were incubated for 19 days to allow full maturation of the monolayer of cells, including active P-gp expression, while the other cell lines were used 6–7 days after splitting at 20,000 cells per ml (2000 cells per well). The bacterial adhesion studies were conducted using ‘assay medium’ consisting of HBSS supplemented with both glucose (Ajax chemicals, NSW, Australia) and HEPES to give final concentrations of 25 and 10 mM respectively. The pH was adjusted to 7.4 using 1 M NaOH and 10% FCS was added to the medium to prevent the cells lifting off the base of 96 well plates during the extended 5 h incubation in this buffered salt solution. A key difference between growth medium and assay medium was the absence of antibiotics in assay medium.

2.7. P-gp and other transport inhibition/ enhancement

In studies where inhibition of P-gp, MRP1, MRP2 or BCRP were performed in conjunction with known substrates, cells were pre-incubated in HBSS containing the inhibitors for 30 min before initiation of the study. The P-gp inhibitors included 4 μM PSC-833 and 4 μM GF120918 (although this P-gp inhibitor is also known to inhibit BCRP). Fumitremorgin C at 10 μM was used as a potent BCRP inhibitor, and glyburide (200 μM) was used as broad MRP and ABCA1 inhibitor, although was subsequently discovered to inhibit P-gp also. The general MRP inhibitors 500 μM probenecid or for MRP1, MK571 at 25 μM were also used, to provide comparative non-Pgp efflux inhibition data.

In studies where induction of active efflux proteins was attempted, cells were incubated with 20 μM rifampicin, 10 μM β-estradiol, 50 μM carbamazepine or 10 μM progesterone for 48 h (Progesterone is a known P-gp inhibitor, however other research suggested that it could induce BCRP and P-gp over the long term). Data our suggested that the only significant outcome of 48 h pre-incubation with progesterone was binding of progesterone to P-gp that was not washed off with cold PBS, with little evidence of induction via western blotting (results not shown).

2.8. Confirmation of P-gp induction

To confirm that P-gp inducers such as rifampicin, methylprednisolone or progesterone increased the expression of active efflux proteins, western blotting was performed to measure protein levels.

Caco2 cells, RKO or MCF7 cells used for protein determination were incubated in 25 cm² flasks with potential inducers concurrently with the cells of the same passage number in the 96 well plates for bacterial adhesion studies.
tris HCl, 120 mM NaCl, 1% non-idet P40 substitute, 0.1% SDS and a protease inhibitor cocktail (SigmaAldrich) was added to the Human cell lines and left for 10 min. These samples were then placed in a sonicating water bath for 10 min, to assist with membrane disruption. Samples were centrifuged at 10,000 g for 10 min, and the supernatant collected for protein analysis. A modified Lowry assay using 96 well plates was used for total protein calculations. Standards using bovine serum albumin were prepared on the plate in duplicate between 10 and 1500 μg/mL.

2.9. Rhodamine 123 transport

Caco-2 cells were seeded onto Millicell polycarbonate 0.6 cm² filter inserts in 24 well plates at 65,000 cells/cm². Cells were grown in ‘growth medium’ (as above) in a 37 °C incubator with 5% CO₂.

Cells were incubated for 21 days to allow full maturation of the monolayer of cells, including active P-gp expression and increased trans-epithelial electrical resistance (TEER) formation. The TEER was measured both before and immediately after the study using an EVOM meter and the ENDOHM 12 chamber (World Precision Instruments, Sarasota, FL, USA) with readings between 400 and 600 Ω·cm² for all cells in this study. Resistance readings at the end of each experiment were not significantly different from initial values.

Filter inserts were transferred to fresh 24 well plates for the studies. The studies were conducted using ‘assay medium’ consisting of HBSS supplemented with both glucose (Ajax chemicals, NSW, Australia) and HEPES to give final concentrations of 25 and 10 mM respectively. The pH was adjusted to 7.4 using 1 M NaOH. For pH 6.0 studies, 10 mM Bis–Tris (USB, Cleveland, Ohio, USA) was used instead of HEPES and the pH adjusted with 1 M HCl. No Phenol red was present in the Assay medium, which was deemed important for fluorescence analysis of rhodamine 123 (Rh123).

Rifampicin was added at a 20 μM concentration to the Caco-2 cells every 24 h for 72 h prior to the commencement of the Rh123 transport study. Cells were washed 3 times in HBSS prior to the transport study to remove rifampicin from the solution. Other Caco-2 cells were incubated in pre-warmed assay medium +/− PSC-833 for only 30 min prior to the study. TEER was measured and assay medium +/− PSC-833 were placed in the receiver chambers. Rh123 were added to the donor compartment of each well. The apical (Ap) and basolateral (Bas) chambers received 0.3 and 0.6 mL of medium respectively. Sample was removed from the receiver chamber at various times over a three hour period. Constant volumes were maintained by adding pre-warmed medium to the receiver chambers in order to maintain an equilibrium pressure differential between the volumes in the donor and receiver chambers. Rh123 was detected using the FluoStar Optima 96 well fluorescence plate reader. The excitation filter was 485 nm and emission filter was 520 nm. Standard curves were generated using dilutions of the stock 10 μM Rh123 solution in HBSS using the same volumes collected in the experiment. Fluorescence was detected of aliquots collected in black fluorescence 96 well plates.

Drug transport through cell monolayers was calculated both as a simple amount passing the monolayer per min, which would vary depending on the concentration used in the donor compartment, and as an apparent permeability coefficient as calculated previously.24,25 Briefly, this calculation allows for a modification to the original Artursson equation,24 where the concentration in the donor compartment (C₀) is re-calculated after every 30 min time point to compensate for that already present in the receiver chamber to ensure a greater accuracy in calculating the rate of movement into the opposing chamber.

2.10. Western blotting for efflux protein detection

Forty micrograms of proteins were added to each well of 3–8% Tris–Acetate gels from Novex (Invitrogen) and electrophoresis conducted. Once the proteins had been transferred onto Immuno-blot PVDF membranes, they were washed in TBST (Tris buffered saline + 0.05% Tween 20).

Total protein was checked using the Ponceau S staining method, prior to blocking the membranes. Invitrogen’s Western Breeze western blotting kit was used for all subsequent protocols prior to viewing the immunoblots in an Alpha-Innotech ChemiImager 4400 with chemiluminescent detection and a CoolSnap HQ camera.

Monoclonal primary antibodies included P-gp Mdr1 (G-1) mouse monoclonal, MRP1 (QCRL-1) and MRP2 (M2 III-6) monoclonals were supplied from SantaCruz biotechnology (Santa Cruz, California, USA). BCRP monoclonal antibody (BXP-21) was purchased from abcam antibodies (Cambridge, UK) or Chemicon (Invitrogen, Mulgrave, Victoria, Australia). Monoclonal antibodies were diluted to a final concentration of 1 μg/mL (usually a 200 fold dilution) and added alongside mouse anti-β-actin (Sigma-Aldrich) at 1:10,000 dilution onto the membrane and rocked at room temperature for 1.5 h. After addition of the AP labeled 2nd antibody (supplied in WesternBreeze kit), Chemiluminescent reagent was added for 5 min before placing in the Chemilager dark environment. The membrane was left for 10–20 min while chemiluminescence was captured on the CoolSnap HQ 14bit 1.3 MP – 30 °C cooled digital camera. AlphaEase software was used to generate integrated density values of the proteins detected using chemiluminescence.

2.11. Bacterial attachment study

Prior to initiation of this study, fresh batches of bacterial broths were prepared and then plated to ensure no contamination has occurred. A number of these colonies were scraped from the culture plates, suspended in PBS and washed. BacLight Green was added to each of the bacterial solutions to produce a concentration of 800 nM and incubated for 45 min. After incubation, solution was centrifuged at 12,000 rpm for 6 min, washed 3 times with PBS and made up with PBS to a final volume of 1 mL. 100 μL of each sample was then diluted in 2 fold steps along a row of a 96 multi-well plate and absorbance measured using a Tecan sunrise plate reader with a 415 nm filter. From the readings, concentrations of each bacterial species could be calculated.

PSC 833 and GF 120918 inhibit P-gp function by blocking the ATPase pump in a competitive manner, therefore these inhibitors must be present at all times during the bacterial attachment study.27–29 Previous studies from our laboratory
have shown both of these P-gp inhibitors to be very effective at the concentrations used in this study to block P-gp activity in the same Caco2 cell line used here. The concentration of each of the bacteria calculated from the standard curves was then used to produce solutions with a final bacterial concentration of 10⁷ CFU/100 μL made up in DMEM containing either 4 μM of GF 120918 or PSC 833. Likewise, studies conducted with 25 μM MK571, 500 μM probenecid, 5 μM fumitremorgenc, 100 μM genistein, 50 μM quercetin and 200 μM glyburide were prepared in the same way.

Unlike our proposed inhibitors of active efflux proteins, the agents we considered as potential inducers needed 48 to 72 h preincubation. When rifampicin, carbamazepine, progesterone or β-estradiol were used our confluent human cell cultures needed to be pre-incubated with these inducers 48 h before the commencement of the bacterial attachment study as they had been shown in other studies, with different cells lines to increase P-gp function by enhancing its expression. However, our western blot results were not conclusive in illustrating increased P-gp expression (results not shown). Nevertheless, cells preincubated with these agents were washed free of the drugs before initiating our bacterial adhesion studies to reduce the possibility that some of the potential inducing agents could have also had short term functional blocking ability. Some of the inducers of P-gp protein do so by blocking function of the protein, which encourages cells to upregulate expression of P-gp to compensate for the loss of existing function. More recent studies with 1 μM digoxin, 3 mM Phenobarbital and 5 μM 1, 25 dihydroxyvitamin D have shown some increase in protein expression.

Human cells grown in black 96 multi-well plates were then prepared by aspirating and incubating them with Buffered salt solution containing efflux protein inhibitors for up to 30 min in the 5% CO₂ incubator. After the inhibitor solutions were aspirated, the cells were rinsed with PBS once and then fresh HBSS were loaded back into each of the wells. The bacterial solutions at 100 μL per well were then added at the corresponding time points ranging from 30 to 240 min and incubated at 37 °C in the 5% CO₂ incubator during this time period. Time points exceeding 4 h were not used to limit any bacterial influence on direct pathogenic effects on the human cells, as this study was focused on the attachment process of bacteria. Once the addition of bacterial solution to the Human cells at the required times had been completed, the cells were rinsed 3 times with cold PBS. A FLUOstar Optima fluorcent plate reader (BMG Labtechnologies) was used to measure fluorescence emitted and the data derived corresponded to the number of bacteria attached to the cell lines. The conditions set for fluorescence reading were as follows: A 485 nm excitation filter and a 520 nm emission filter, using an orbital detection protocol to average the measurement over 20 different locations in each well, and each assay was done with quadruplicate individual wells at each time point.

2.12. Test to confirm that P-gp inhibitors do not affect growth of bacteria

To exclude the possibility of the P-gp inhibitors having a direct effect on the growth of the bacteria, parallel studies were conducted where co-incubation with the active efflux inhibitors was followed by culturing on nutrient agar plates and placing at 37 °C overnight (72 h for Clostridium.) The visible colonies on the plates were counted and compared to the bacterial samples without inhibitors present. We did not observe any inhibition of bacterial growth with the concentration of efflux inhibitors used in this study in concert with the bacterial solutions.

2.13. Statistical analysis

Student two-tailed unpaired t-tests were carried out on each set of quadruplicate results for each time point. Significant differences were considered to have occurred with a P value of less than 0.05. One way ANOVA with Dunett post hoc analysis was also conducted to compare the significance of inhibitors and inducers for each bacterial species and each human cell line. Statistical significance was reached if P values were less than 0.05.

3. Results

Individual efflux proteins were determined by Western blot analysis. Of the three cell lines examined, Caco2 cells proved to be the only one with significant quantities of P-gp (ABC81) expressed (Fig. 1). In addition, cells pre-incubated for 48 h with rifampicin showed a small increased expression of P-gp when matched to cells passed at the same time (wells 1 and 2), although this was only a minor increase. 1, 25 dihydroxy vitamin D at both 0.75 μM and 5.0 μM concentrations for 72 h were able to increase P-gp expression to a greater amount than 20 μM rifampicin. These Caco2 cells had relatively low expression of BCRP (ABC2) (Fig. 1), with the P-gp inducers appearing to decrease expression of BCRP further in the Caco2 cell line. The RKO cell line was used as a 2nd human gastrointestinal cell line, however it was clear from these Western Blots that protein expression of both MDR1 and BCRP was very low in this cell line (Fig. 1). The human breast cancer cell line MCF7 had the highest expression of BCRP protein from the three cell lines used in this study (Fig. 1). Expression of BCRP appeared to be unaffected by 48 h preincubation with rifampicin, or 72 h incubation with vitamin D or Phenobarbital (Fig. 1). It has also been shown in our laboratory that RKO cells show some MR2 expression, Caco2 cells show MR2 expression, while MCF7 cells have very little MR1 or MR2 expression (results not shown). The attachment of bacteria to these three human cell lines was subsequently examined in our cell lines with different BCRP and P-gp expression to find an association with either BCRP or MDR1.

Bacterial fluorescence increased in intensity over the course of the 3 to 4 h studies shown here, and these values represent the bacteria unable to be washed from the human cell layers coating the wells (Figs. 2 and 3). Depending on the bacterial species, adhesion started to reach a plateau by 3 h. Examples of E. coli (Fig. 2) and S. aureus are shown here (Fig. 3), however the general trend was similar for all six species of bacteria examined. Bacterial attachment from blank wells without confluent cell layers was very low and did not increase with increasing incubation time. Bacteria were allowed to adhere for between 30 min to 4 h and the fluorescence determined after washing the cells three times in ice cold PBS. Initial studies were conducted within a 1 min
to 30 min time frame, but this period was found to be too short for adhesion to be meaningful in fluorescence analysis. The Pgp inhibitors PSC833 and GF120918 were incubated with Caco2 cells for only 30 min prior to the 1st bacterial incubation period to allow P-gp transport sites to be blocked prior to exposure to bacteria.

The Rhodamine 123 bidirectional transport study shown here (Fig. 4) illustrates the effectiveness of PSC-833 at blocking P-gp

Figure 1  Western blots and densitometry analysis of Caco2, RKO and MCF7 cells for ABCB1 (MDR1) and ABCG2 (BCRP) efflux proteins. A: Wells 1–2 include matched 19 day old Passage 80 Caco2 cells with and without 20 μM rifampicin exposure for 48 h. Wells 3–5 include 9 day old MCF7 cells with and without 20 μM rifampicin for 48 h and 3.0 mM Phenobarbital exposure for 72 h. Wells 6–7 include 7 day old RKO cells with or without 20 μM rifampicin exposure for 48 h. Caco2 cell data is contained in wells 8–14. Well 8 has passage 86 Caco2 cells as control data for inducers shown in wells 9–14. Wells 9–10 contains 0.75 μM and 5.0 μM 1, 25 dihydroxy vitamin D respectively for 72 h. Wells 11–12 have 0.25 and 2.0 μM digoxin for 48 h, while Well 13 shows the effects of 2.0 μM digoxin exposure for 72 h. Well 14 has Caco2 cells exposed to 3.0 mM Phenobarbital for 72 h. β-actin was used as the loading marker protein for these blots.

Figure 2  Four hour time course study of E. coli attachment to A: Caco-2, B: RKO and C: MCF7 cells. E. coli that absorbed the BacLight Green dye fluoresced with emission of 485 nm and excitation of 520 nm. Each of the human cells lines was tested in either normal culture conditions (squares) or with co-exposure with 4 μM GF120918 (diamonds) or with a 48 h pre exposure with 20 μM rifampicin (triangles), where rifampicin was removed before bacteria were allowed to attach. Results shown are the mean ± SEM of quadruplicate wells at each time point. Significant differences between active efflux modified cells and control cells at each time point are shown with p<0.05 (*) and p<0.005(**).
function as evidenced by the increased apical to basolateral transport and decreased bacterial to apical transport of the known P-gp substrate rhodamine 123. Our previous studies with GF120918 also show similar effectiveness of GF120918 in Caco-2 cells at blocking functional P-gp activity.16,30

It was evident that an increasing bacterial load occurred over a 3 to 4 h time course. However, RKO and MCF7 cells, with little to no P-gp expression, had much greater absolute fluorescence than the Caco2 cells (Figs. 2 and 3). When P-gp was blocked with GF120918, as shown in Figs. 2 and 3, this allowed bacterial attachment in Caco2 cells to start increasing to that of the other cell lines. Very similar results were obtained using PSC833, another common P-gp inhibitor, which is equally effective towards P-gp without affecting BCRP activity (Table 1). Rifampicin pre-incubation was not significantly effective at reducing E. coli associated with our human cell lines. Only one bacteria, S. aureus, showing any reduced binding to Caco2 cells with rifampicin pre-exposed cells (Fig. 3, Table 1). Minimal increases in P-gp expression shown in Fig. 1, and no change in bidirectional Rh123 transport (Fig. 4) indicate that little change had occurred with P-gp expression, so it was not surprising that irrespective of Caco2 exposure to rifampicin, bacterial adhesion results were similar in the majority of cases. Time course experiments were repeated with numerous co-incubated active efflux inhibitors to further the hypothesis that P-gp was responsible for altering bacterial adhesion. For clarity the 180 min data is shown due to the trend in adhesion differences being similar for all time points. MK571 is a specific MRP1 inhibitor and it did have a small effect on increasing accumulation of E. coli and S. aureus, but did not affect S. typhimurium or C. sporogenes in Caco2 cells (Table 1). Probenecid is a multiple MRP inhibitor, yet had no effect at all on any bacterial adhesion. Quercetin and genistein are also thought to modify the MRP family of transporters, yet they also had no effect on any bacterial adhesion of Caco2 cells here (Table 1). As GF120918 has the potential to block BCRP in addition to P-gp, a potent specific BCRP blocking agent (Fumitremorgin C) was tested at concentrations known to be effective against BCRP in vitro to elucidate the mechanisms of GF120918. This agent had no effect on bacterial binding to Caco2 cells (Table 1), although their BCRP protein level is low (Fig. 1). Importantly, it did have some effect on bacteria remaining associated with MCF7 cultures (Table 2), which have much higher expression of BCRP (Fig. 1), but this only resulted in 30 to 60% increases in bacterial loading, while blocking the activity of P-gp increased some of the bacterial association with Caco2 cells up to 3 fold higher.

Progesterone was used for 48 h pre-incubations of the human cell lines as one report stated that long term exposure to 10 μM progesterone could increase BCRP in some estrogen sensitive cells lines and P-gp levels in other cell lines by 2 fold.21,42 However, progesterone is one of the few potent P-gp blocking agent that is not transported by P-gp, and thus, is likely to block using mechanisms that do not involve the active site, and may persist for some time, and although the Caco2 cells in this study were washed 3 times in cold PBS before initiating bacterial adhesion, it is likely that much of the progesterone bound to the P-gp receptor remained attached and therefore continued to act as a blocking agent while adhesion studies were conducted, and this was the likely reason significant increases in bacterial adhesion were observed in this particular section of the study (Table 1). The RKO cell line, which had almost no P-gp expression, also had much higher total fluorescence, and thereby total bacterial association with the RKO cells compared to Caco2 cells, mirroring the relative P-gp expression differences between these cells (Figs. 2 and 3). Rifampicin is well known as a P-gp inducer through activation of the pregnane X receptor (PXR) and was successful at limiting the binding of some bacteria to Caco2 cells. It is known that Caco2 cells have only low level PXR expression, and that rifampicin does not have the same level of increase that can occur in cell lines with greater PXR levels. Nevertheless, it was able to limit the binding of S. typhimurium and K. sporogenes to a moderate extent in Caco2 cells, and these bacteria are both capable of inducing

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**Figure 3** Four hour time course study of *Staphylococcus aureus* attachment to B: Caco-2, C: RKO and D: MCF7 cells. *S. aureus* that absorbed the BacLight Green dye fluoresced with emission of 485 nm and excitation of 520 nm. Each of the human cell lines was tested in either normal culture conditions (squares) or with co-exposure with 4 μM GF120918 (diamonds) or with a 48 h pre exposure with 25 μM rifampicin (triangles), where rifampicin was removed before bacteria were allowed to attach. Results shown are the mean ± SEM of quadruplicate wells at each time point. Significant differences between active efflux modified cells and control cells at each time point are shown with p<0.05 (*) and p<0.005(**).
gastrointestinal infections. Further work is needed with other P-gp inducers such as Vitamin D, digoxin or Phenobarbital to examine definitive reduction in bacterial adhesion as a consequence of increased P-gp activity. Incubation with rifampicin was able to limit the binding of *S. aureus* to RKO gastrointestinal cells, although these cells have almost no P-gp expression, and possibly BCRP expression to some degree, which makes examination of reduced binding of bacteria as a response of P-gp more challenging to prove into the future, than the evidence of increased binding through functional P-gp blockage which is a much clearer conclusion from the data of this study.

### 4. Discussion

This study has shown compelling evidence that P-gp expression, and possibly BCRP expression to some degree, has a significant ability to prevent close association of bacteria with human gastrointestinal cells. This is the first stage of pathogenic bacterial cellular invasion by creating an environment from which to cause an infection, and thus low P-gp expression may allow an increased risk of pathological outcomes.

There are two phases of bacterial adhesion to host surfaces. Phase one involves overcoming physical forces such as Brownian motion, van der Waals attraction forces, gravitational forces, surface electrostatic charge and hydrophobic interactions.\(^2,4^7\) Phase two of the adhesion process requires the union of bacterial surface adhesins with the complementary human gastrointestinal cell receptors. Adhesins can take the form of bacterial cell wall components...
Our results showed the P-gp blocking agents PSC 833, GF 120918, progesterone and to a limited extent, glyburide, all increase bacterial attachment to Caco2 cells, which indicates the involvement of P-gp in limiting the attachment of bacteria to these cells. We have used PSC-833 and GF120918 in many previous bidirectional drug transport studies with Caco2 cell monolayers and find them to both give excellent P-gp blocking ability at the concentrations used in this study. In addition, rhodamine 123 bidirectional studies were repeated in this study to show the effectiveness of PSC-833 as a potent P-gp blocking agent.25,30,31

Glyburide was initially used in other labs as an ABCA1 inhibitor,48 however, it was clear from other publications that this agent has moderate P-gp blocking ability as well.49 Thus, although glyburide was not as potent as PSC-833, or GF120918 in this study at increasing bacterial attachment, this would be keeping within its likely potency range against P-gp.

Some of the bacterial species, such as S. aureus were able to increase their foothold on Caco2 cells as active P-gp was depleted to a far greater extent than other bacteria. S. aureus incubation resulted in much higher cell fluorescent counts compared to the other five examined, suggesting better binding to the human cells, and P-gp blockage also produced the greatest fold increases for this bacteria as well other bacteria known to invade the gastrointestinal environment, such as C. sporogenes and S. typhimurium, especially when compared to the more pulmonary infective agents such as Pseudomonas and Klebsiella. Interestingly, S.

### Table 2: Bacterial adherence to MCF7 and RKO cells: The effect of modifiers to a range of known active efflux proteins on the fluorescence associated with bacterial adherence to MCF7 and RKO cells after 180 min of exposure in 96 well plates. Fluorescence is reported as a ratio of the fluorescence attained in concurrent control cultures of these human cell lines exposed to the same fluorescently labeled bacterial cultures. The higher the ratio, the more bacteria have attached to the MCF7 or RKO cells in 180 min.

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Efflux protein modified</th>
<th>Escherichia coli</th>
<th>Staphylococcus aureus</th>
<th>Salmonella typhimurium</th>
<th>Pseudomonas aeruginosa</th>
<th>Klebsiella pneumoniae</th>
<th>Clostridium sporogenes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ratio to control</td>
<td>Ratio to control</td>
<td>Ratio to control</td>
<td>Ratio to control</td>
<td>Ratio to control</td>
<td>Ratio to control</td>
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<tr>
<td>MCF7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None-control</td>
<td></td>
<td>1.00 ± 0.04</td>
<td>1.00 ± 0.05</td>
<td>1.00 ± 0.04</td>
<td>1.00 ± 0.05</td>
<td>1.00 ± 0.03</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>PSC833</td>
<td>P-gp blocked</td>
<td>1.08 ± 0.05</td>
<td>1.08 ± 0.05</td>
<td>0.94 ± 0.02</td>
<td>NA</td>
<td>0.99 ± 0.02</td>
<td>1.10 ± 0.02</td>
</tr>
<tr>
<td>GF120918</td>
<td>P-gp and BCRP blocked</td>
<td>1.60 ± 0.03**</td>
<td>1.14 ± 0.04</td>
<td>1.57 ± 0.04**</td>
<td>1.30 ± 0.05*</td>
<td>1.07 ± 0.04</td>
<td>1.42 ± 0.03**</td>
</tr>
<tr>
<td>Fumitremorgin</td>
<td>BCRP blocked</td>
<td>1.19 ± 0.02</td>
<td>1.29 ± 0.06*</td>
<td>1.21 ± 0.04</td>
<td>1.07 ± 0.05</td>
<td>1.14 ± 0.03</td>
<td>NA</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>PXR activation. P-gp induction</td>
<td>1.24 ± 0.09**</td>
<td>1.01 ± 0.07</td>
<td>0.87 ± 0.16</td>
<td>1.03 ± 0.03</td>
<td>0.92 ± 0.05</td>
<td>0.78 ± 0.10</td>
</tr>
<tr>
<td>RKO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None-control</td>
<td>MRP family blocked</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.01</td>
<td>1.00 ± 0.04</td>
<td>1.00 ± 0.04</td>
<td>1.00 ± 0.04</td>
<td>1.00 ± 0.02</td>
</tr>
<tr>
<td>PSC-833</td>
<td>P-gp blocked</td>
<td>1.03 ± 0.08</td>
<td>0.87 ± 0.03</td>
<td>1.01 ± 0.04</td>
<td>1.09 ± 0.02</td>
<td>1.01 ± 0.04</td>
<td>1.17 ± 0.01</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>PXR activation. P-gp induction</td>
<td>1.04 ± 0.02</td>
<td>0.71 ± 0.05**</td>
<td>0.94 ± 0.03</td>
<td>0.92 ± 0.01</td>
<td>0.86 ± 0.01</td>
<td>1.16 ± 0.03</td>
</tr>
</tbody>
</table>

Drugs known to inhibit the activity of active efflux proteins were incubated with the confluent Caco2 cell cultures for 30 min prior to the bacterial co-incubation, while drugs known to induce efflux proteins were incubated for 48 to 72 h prior to the bacterial co-incubation. Concentrations of each of the drugs used was as follows: Inhibitors; PSC833 (4 μM), GF120918 (4 μM), MK571 (25 μM), probenecid (500 μM), fumitremorgin C (10 μM), glyburide (200 μM), genistein (100 μM), quercetin (50 μM): 48 h incubations; carbamazepine (50 μM), rifampicin (20 μM) and progesterone (10 μM). PXR = Pregnane X receptor.

All MCF7 and RKO results shown are in quadruplicate ± SEM. Significant differences in the binding of each species of bacteria to either MCG7 or RKO cells incubated or exposed to drugs compared to control MCF7 or RKO cells at 180 min are shown with p<0.05 (*) and p<0.005(**).

Figure 4  Bidirectional transport of 10 μM Rhodamine 123 through Caco2 monolayers. Apical to basolateral direction (□, ■) and basolateral to apical direction (◇, ◇), with (□, ◇) and without (■, ◇) the presence of 4 μM PSC-833, a potent P-glycoprotein inhibitor, on both sides of the membrane. In addition, 72 h preincubation with 20 μM Rifampicin was conducted (□). Rifampicin was washed off and Rh123 was added alone without any other drugs. The dashed line shows apical to basolateral transport and the small/large dashed line shows basolateral to apical transport.

(polysaccharides, glycoproteins, and glycolipid), cell capsules or fimbriae.2,47 It is here that we would expect any interactions between P-gp and bacterial adhesion to occur.
typhimurium has been shown previously to interact with P-gp, downregulating its expression,50 which has been suggested can increase its own infectiveness. Other bacteria used in this study, such as E. coli W are not overtly pathogenic,51 and this may have explained why the fold increase of bacterial fluorescence was not up to the level of the other gastrointestinal infective bacteria. The use of more virulent E. coli bacteria in more stringent PC3 lab facilities may assist in providing answers in this area.

However, using a virulent strain of S. typhimurium, a research group recently showed canine MDCKII cells over-expressing human P-gp to internalize less than 20% of the bacteria compared to the same MDCKII cell line not over-expressing P-glycoprotein.50 Their data matches the response of S. typhimurium in this current study using human Caco2 cells very closely. In their study all bacteria external to the MDCKII cells were killed, and the internalized cells were extracted and plated for colony forming units to be counted, showing an 80% reduction in internalized Salmonella. In this current study fluorescence associated with the cells was determined simply using a 96 well plate reader. Remarkably, their study and these current results come to the same conclusion, being that the expression of functional human P-gp will limit close association with bacteria capable of inducing potential infections.

Other human cell lines that expressed much lower amounts of P-gp, or expressed other efflux proteins indicated that the influence of other efflux proteins, such as BCRP, may have a small role to play, but it was P-gp that appeared to limit the bacterial adhesion when comparing the combination of cells lines examined and inhibitors used. For example, in the MCF7 cell line, which has BCRP as its efflux protein of greatest expression, the P-gp inhibitor PSC-833 had no effect, which was not surprising given the lack of P-gp in the cell line, while GF120918 was more effective, but not to the same level in Caco-2 cells. In these cells GF120918 would be blocking P-gp to a larger extent than BCRP, as Caco2 cells were shown here to have much less BCRP than the MCF7 cell line. In addition, the BCRP specific inhibitor Fumitremorgin was very limited in its effectiveness to increase bacterial adhesion. This evidence reinforces the role of P-gp in preventing bacterial attachment. Thus, when the use of inhibitors of different efflux proteins are compared in a cell line with high P-gp expression (Caco2), high BCRP expression (MCF7), and little expression of either (RKO), it became clear that only drugs known to inhibit P-gp in the cell line that expressed it showed vastly significant increases in binding. The lack of PSC-833 induced binding in the MCF7 and RKO cell lines confirmed that this drug itself was not primarily responsible for the increased bacterial attachment. Ideally, reduced binding with elevated P-gp levels would add more weight to the evidence for P-gp efflux associated with reduced binding of bacteria. Certainly, the Caco2 cells had less fluorescence, and thereby less bacteria attached at any given time than the MCF7 and RKO cell lines, which does conform with this hypothesis of P-gp enacted defense against gastrointestinal bacteria. Attempting to increase P-gp expression in the Caco2 cells with rifampicin exposure only slightly increase P-gp protein expression in Caco2 cells, with no additional bidirectional transport activity of Rh123, yet was also associated with a slight decrease in bacterial binding, for two of the 6 species. Nevertheless, the previous publication from McCormick’s group showing significantly decreased invasion of Salmonella with a transfected canine cell line with human MDR150 would support the notion that increased P-gp expression should decrease adhesion of the bacterial species examined here.

Bacteria have been shown to have multiple adhesins, especially peptide based molecules for attaching to different surfaces or receptors.5 It is known that some polysaccharides and proteins are P-gp substrates,52,53 which forms the basis of this study’s proposal that bacterial adhesion factors that are comprised of these materials may interact with P-gp and in turn the attachment process may be affected due to this interaction. P-gp is able to recognize foreign substrates, in this case the bacterial attachment factors, preventing the bacteria from attaching by dislodging these factors from the cell surface.

A recent clinical study showed decreased P-gp protein expression in the colon of patients, and the lower the expression, the greater the clinical inflammation from Ulcerative colitis. Their conclusion was that IL-8 and other cytokines had reduced the MDR1 protein.13 Our data in this current study conforms with this clinical experiment, except that our data would suggest that the clinical inflammation in IBD (similar to that seen in previous mdrla knockout mice studies7) may be a result of the lower P-gp expression, rather than the low P-gp expression coming from the increased clinical inflammation.13 Thus, time course studies that follow when low expression has occurred would be the next step to confirming this hypothesis.

The data in this study supports the concept that P-gp not only is protective of xenotoxic compounds, but also protects from the bacteria themselves getting close enough to initiate a cascade of toxic or inflammatory events that leads to clinical diseases such as ulcerative colitis. It has been suggested that in the gastrointestinal tract if a person has a homozygous CC polymorphism at position 3435 of the ABCB1 (MDR1) code this might provide up to 2 fold greater defense against xenobiotics, and that this is the rationale behind the TT homozygous allele only representing 6% of the African population.54,55 They are thought to have increased resistance to gastrointestinal illness, and our data would support this resistance being a combination of allowing bacteria to be flushed through the gut more rapidly and having delayed attachment that would otherwise lead to subsequent gastrointestinal damage, in addition to xenobiotic compound efflux from foods consumed in their diet.

5. Conclusions

In conjunction with a recent publication examining S. typhimurium interactions with P-gp expression, and the ability of P-gp to block uptake of Salmonella in MDCK cells,50 this current study shows that functional P-gp reduces adhesion of many species of bacteria directly to human cells endogenously expressing this efflux protein, suggesting a mechanism to explain increased intestinal colitis in mdr1(−/−) knock-out mice from studies conducted over a decade ago,7 and providing potential insights into our understanding of gastrointestinal disorders initiated by bacterial adhesion in humans.
The next step in the examination of this P-gp and bacterial adhesion association will be to examine key adhesion molecules and their interaction with P-gp directly, with bidirectional transport studies needed to elucidate the transporter affinity of these peptides for active efflux transport. In addition, with current data showing low P-gp expression in patients with active inflammatory bowel disease, time course studies should be conducted to examine whether P-gp expression in these patients is lower before or after the induction of an acute attack. This information would assist in determining the causative nature of acute attacks, and whether P-gp is acting to defend the colon from bacterial induced inflammation or not.

Acknowledgments

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References


