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

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Part of this work was presented as a poster presentation at the World Pharma Conference in Copenhagen, Demark in July 2010

Short title:

P-gp expression reduces bacterial attachment

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 adhesion secretion. Thus, this study was initiated to examine whether any association exists between bacterial adhesion and P-gp expression. **Methods.** Three human cell lines (Caco2, RKO, 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 upto 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.

Key Words: Caco2, MDR1, *E.coli,* BCRP, IBD

Introduction:

The gastrointestinal tract is resident to over 300 different species of bacteria [1]. 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 [2]. It is well recognized that the adhesion of bacteria to biological surfaces is a prerequisite to invasion and is the first step in pathogenesis [3]. 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 that in those of the normal population [4, 5].

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 [6]. 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 [7-9]. Moreover patients with IBD, appear to have an increased reactivity to their resident intestinal flora [7].

Intestinal epithelial cells are known to express the multi drug resistance (MDR1) protein also known as the ABCB1 gene product or P-glycoprotein [7]. 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 [10]. 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 [6].

Panwala and colleagues showed that one quarter of mdr1a(-/-) knockout mice developed loose stools and had reduced growth rates [7, 11]. 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-gp [7]. 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* [12]. Recent studies also confirm that patients with active IBD have reduced P-gp expression in the gastrointestinal tract [13].

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 [14]. They are also able to form microvilli and express small intestinal like characteristics both biochemically and morphologically, including high P-glycoprotein expression [15, 16]. The RKO colorectal carcinoma cell line has virtually no detectable P-gp activity and minimal BCRP activity, as shown from this study, and previously [17], while MCF-7 which is an oestrogen receptor positive, tumour derived breast cancer cell model [18], that also shows no functional expression of P-gp, but does have high BCRP expression [19].

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*.

Materials and Methods

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 (10,000U/mL), Streptomycin sulphate (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, 1mM non-essential amino acid (NEAA), 100U/mL Penicillin/Streptomycin and 2mM 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).

Bacterial culture

The aerobic bacteria were stored in bottles of nutrient broth. The aerobic bacteria (*Escherichia coli W* (ATCC 9637)*, Staphylococcus aureus* (ATCC 6538)*, Salmonella typhimurium* (ATCC 7823)*, Klebsiella pneumoniae var aerogenes* (NCDC 379-68) and *Pseudomonas aeruginosa* (NCTC 7244)) were inoculated onto agar plates and incubated for 48 hours at 37°C. *Clostridium sporogenes* (NCTC 533) was also inoculated onto agar plates and grown in anaerobic conditions for 72 hours.

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 minutes. Resuspending the pellet in 2x500µL cold PBS, 8µL of a 100µM 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 minutes at room temperature then centrifuged at 12000 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 hours (72 hours for *Clostridium sporogenes*) and were compared to controls to determine whether any

163 growth retardation has occurred either with colony counts or general growth
164 characteristics.

165 **Fluorescence method**

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

170

171 **Determining a rapid cell count**

172 In order to count the number of colony forming units on the same day as the
173 fluorescence study, standard curves were developed for each of the 6 bacteria.
174 Firstly a test was done to ensure that the absorbance measurement was not altered
175 by adding the fluorescent dye to the bacteria. Stained and unstained bacterial
176 suspensions in PBS were serially diluted in 96 well plates from stock, to 1/256th of
177 stock. Absorbance measurement was carried on these samples at 415nm
178 wavelength using a Tecan Sunrise 96 well plate reader using the Magellan 3
179 software for Windows 2000. There was no significant difference between the
180 absorbance for the bacterial suspensions with or without BacGreen Light staining.
181 Samples from each well of bacteria was diluted 1000 fold in PBS and spread over
182 4.7cm agar plates and allowed to grow for up to 48 hours. Dilutions of bacteria that
183 showed between 40 and 1000 colony forming units per plate were used for
184 generation of standard curves. After incubation, the colonies on each of the plates
185 were counted using a Chemilmager™ 4400 from AlphaInnotech (Cell Biosciences,
186 Santa Clara, CA, USA) with the assistance of the colony counting module with the
187 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 [20], which is a key characteristic needed to ensure no non-specific binding of our human cells during the attachment studies.

Human cell growth.

Caco-2 cells, RKO and MCF7 cells were grown in 25 cm^2 flasks. Cells were grown in 'growth medium' (high glucose DMEM with 25 mM Hepes (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 37ºC incubator with 5% CO_2.

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 1M 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 hour incubation in this buffered salt solution. A key difference between growth medium and assay medium was the absence of antibiotics in assay medium.

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 hours (Progesterone is a known P-gp inhibitor, however other research suggested that it could induce BCRP and P-gp over the long term) [21, 22]. Our data suggested that the only significant outcome of 48h 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).

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. Lysis buffer (20mM tris

HCl, 120mM 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 minutes. These samples were then placed in a sonicating water bath for 10 minutes, to assist with membrane disruption. Samples were centrifuged at 10,000g 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 10-1500 µg/mL.

Rhodamine 123 transport:

Caco-2 cells were seeded onto Millicell polycarbonate 0.6 cm^2 filter inserts in 24 well plates at 65,000 cells/cm^2. Cells were grown in 'growth medium' (as above) in a 37ºC incubator with 5% CO_2.

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 [23]. 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 400-600 $\Omega.cm^2$ 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 1M 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 hours for 72 hours 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 chamber 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 co-efficient as calculated previously [24, 25]. Briefly, this calculation allows for a modification to the original Artursson equation [26], where the concentration in the donor compartment (C_o) 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.

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 biotechnologyn(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 1µg/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 hours. After addition of the AP labelled 2^{nd} antibody (supplied in WesternBreeze kit), Chemiluminescent reagent was added for 5 min before placing in the ChemiImager dark environment. The membrane was left for 10-20 minutes 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.

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 minutes. After incubation, solution was centrifuged at 12000 rpm for 6 minutes, washed 3 times with PBS and made up with PBS to a final volume of 1mL. 100µL 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 [30-32]. The concentration of each of the bacteria calculated from the standard curves was then used to produce solutions with a final bacterial concentration of 10^7CFU/100µL 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 fumitremorgen C, 100 µM genestein, 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 hours preincubation. When rifampicin, carbamazepine, progesterone or β-estradiol were used our confluent human cell cultures needed to be pre-incubated with these inducers 48 hours 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

339 expression [33, 34]. However, our western blot results were not conclusive in illustrating
340 increased P-gp expression (results not shown). Nevertheless, cells preincubated with
341 these agents we washed free of the drugs before initiating our hour bacterial
342 adhesion studies to reduce the possibility that some of the potential inducing agents
343 could have also had short term functional blocking ability. Some of the inducers of P-
344 gp protein do so by blocking function of the protein, which encourages cells to
345 upregulate expression of P-gp to compensate for the loss of existing function [35, 36].
346 More recent studies with 1 μM digoxin, 3 mM Phenobarbital and 5 μM 1, 25
347 dihydroxyvitamin D have shown some increase in protein expression.
348
349 Human cells grown in black 96 multi-well plates were then prepared by aspirating
350 and incubating them with Buffered salt solution containing efflux protein inhibitors for
351 up to 30 minutes in the 5% CO_2 incubator. After the inhibitor solutions were
352 aspirated, the cells were rinsed with PBS once and then fresh HBSS were loaded
353 back into each of the wells. The bacterial solutions at 100μL per well were then
354 added at the corresponding time points ranging from 30 to 240 minutes and
355 incubated at 37°C in the 5% CO_2 incubator during this time period. Time points
356 exceeding 4 hours were not used to limit any bacterial influence on direct pathogenic
357 effects on the human cells [37], as this study was focused on the attachment process of
358 bacteria. Once the addition of bacterial solution to the Human cells at the required
359 times had been completed, the cells were rinsed 3 times with cold PBS. A FLUOstar
360 Optima fluorescent plate reader (BMG Labtechnologies) was used to measure
361 fluorescence emitted and the data derived corresponded to the number of bacteria
362 attached to the cell lines. The conditions set for fluorescence reading were as
363 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.

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 hours 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.

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.

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 (ABCB1) expressed (Figure 1). In addition, cells pre-incubated for 48 hours with rifampicin showed a small increased expression of P-gp when matched to cells passaged 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 hours were able to increase P-gp expression to a greater amount than 20 μM rifampicin. These Caco2 cells had relatively low expression of BCRP (ABCG2) (Figure 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 (Figure 1). The human breast cancer cell line MCF7 had the highest expression of BCRP protein from the three cell lines used in this study (Figure 1). Expression of BCRP appeared to be unaffected by 48 hour preincubation with rifampicin, or 72 hour incubation with vitamin D or Phenobarbital (Figure 1). It has also been shown in our laboratory that RKO cells show some MRP1 expression, Caco2 cells show MRP2 expression, while MCF7 cells have very little MRP1 or MRP2 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 hour studies shown here, and these values represent the bacteria unable to be washed from the human cell layers coating the wells (Figures 2 and 3). Depending on the

bacterial species, adhesion started to reach a plateau by 3 hours. Examples of *Escherichia coli* (Figure 2) and *Staphylococcus aureus* are shown here (Figure 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 hours 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 illustrates the effectiveness of PSC-833 at blocking P-gp function as evidenced by the increased apical to basolateral transport and decreased bacterial to apical transport of the known P-gp substrate rhodamine 123 [34]. Our previous studies with GF120918 also show similar effectivenesss 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 hour time course. However, RKO and MCF7 cells, with little to no P-gp expression, had much greater absolute fluorescence than the Caco2 cells (Figures 2 and 3). When P-gp was blocked with GF120918, as shown in figures 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 *Eschericia coli* associated with our human cell lines. Only one bacteria, *Staphylococcus aureus*, showing any

reduced binding to Caco2 cells with rifampicin pre-exposed cells (Figure 3, Table 1). Minimal increases in P-gp expression shown in Figure 1, and no change in bidirectional Rh123 transport (Figure 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 *Escherichia coli* and *Staphylococcus aureus*, but did not affect *Salmonella typhimurium* or *Clostridium sporogenes* in Caco2 cells (Table 1). Probenecid is a multiple MRP inhibitor, yet had no effect at all on any bacterial adhesion. Quercetin and genestein are also thought to modify the MRP family of transporters [38, 39], 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) [40] was tested at concentrations known to be effective against BCRP *in vitro* [41] 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 (Figure 1). Importantly, it did have some effect on bacteria remaining associated with MCF7 cultures (Table 2), which have much higher expression of BCRP (Figure 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 hour pre-incubations of the human cell lines as one report stated that long term exposure to 10 μM progesterone could increase BCRP in some oestrogen sensitive cells lines [22] 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 [43], 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 Caco-2 cells, mirroring the relative P-gp expression differences between these cells (Figures 2 and 3). Rifampicin is well known as a P-gp inducer [44] 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 [45], and that rifampicin does not have the same level of increase that can occur in cell lines with greater PXR levels [46]. Nevertheless, it was able to limit the binding of *Salmonella typhimurium* and *Klebsiella sporogenes* to a moderate extent in Caco2 cells, and these bacteria are both capable of inducing 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 *Staphylococcus aureus* to RKO gastrointestinal cells, although these cells have almost no expression of P-gp. MCF7 cells went in the other direction by

increasing binding of *Escherichia coli* with the use of 20 µM rifampicin for 48 hours (Table 2), but none of the other bacteria showed this effect, and it is not clear what the significance of the 24% increase in bacterial binding represents, given that much greater changes occurred with BCRP inhibition in the MCF7 cell line. In addition, the rhodamine 123 bidirectional transport study did not show any increased basolateral to apical transport after preincubation of rifampicin, which would have been expected if P-gp was working with greater effectiveness on this P-gp substrate (Figure 4). However, unlike functional blocking assays where drugs will have a relatively specific targets such as P-gp, when trying to induce expression using various drugs, it is likely that many parameters are being changed beyond induction of P-gp alone, 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.

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 hosts 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, 47]. 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 (polysaccharides, glycoproteins, glycolipid), cell capsules or fimbriae [2, 47]. It is here that we would expect any interactions between P-gp and bacterial adhesion to occur.

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 *Staphylococus aureus* were able to increase their foothold on Caco2 cells as active P-gp was depleted to a far greater extent than other bacteria. *Staphylococcus 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 as other bacteria known to invade the gastrointestinal environment, such as *Clostridium sporogenes* and *Salmonella typhimurium*, especially when compared to the more pulmonary infective agents such as *Pseudomonas* and *Klebsiella*. Interestingly, *Salmonella 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 *Salmonella typhimurium*, a research group recently showed canine MDCKII cells overexpressing human P-gp to internalise less than 20% of the bacteria compared to the same MDCKII cell line not overexpressing P-glycoprotein [50]. Their data matches the response of *Salmonella 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 internalised cells were extracted and plated for colony forming units to be counted, showing an 80% reduction in internalised *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 defence 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 MDR1 [50] 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 [2]. 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 mdr1a knockout mice studies [7]) 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 would provide up to 2 fold greater defence 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.

Conclusions:

In conjunction with a recent publication examining *Salmonella 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

631 colitis in mdr1(-/-) knock-out mice from studies conducted over a decade ago [7], and
632 providing potential insights into our understanding of gastrointestinal disorders
633 initiated by bacterial adhesion in humans.
634 The next step in the examination of this P-gp and bacterial adhesion association will
635 be to examine key adhesion molecules and their interaction with P-gp directly, with
636 bidirectional transport studies needed to elucidate the transporter affinity of these
637 peptides for active efflux transport. In addition, with current data showing low P-gp
638 expression in patients with active inflammatory bowel disease [13], time course studies
639 should be conducted to examine whether P-gp expression in these patients is lower
640 before or after the induction of an acute attack. This information would assist in
641 determining the causative nature of acute attacks, and whether P-gp is acting to
642 defend the colon from bacterial induced inflammation or not.
643

644 ## Acknowledgements:

645 The six species of bacteria used in this study were kindly initiated and supplied by
646 Angie Samec (Microbiology section; Curtin University, School of Pharmacy.)
647 The author wishes to thank the assistance of Ms Kristina Djunaedi, who assisted with
648 initial bacterial cell counting and early optimisation of the fluorescent protocols used
649 in this study.
650 PSC 833 was a kind donation from Novartis Pharmaceuticals (Basel, Switzerland),
651 GF 120918 was kindly donated from GlaxoSmithKline (Victoria, Australia)

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Figure legend:

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 hours. Wells 3-5 include 9 day old MCF7 cells with and without 20 μM rifampicin for 48 hours and 3.0 mM Phenobarbital exposure for 72 hours. Wells 6-7 include 7 day old RKO cells with or without 20 μM rifampicin exposure for 48h. 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 72h. Wells 11-12 have 0.25 and 2.0 μM digoxin for 48h, while Well 13 shows the effects of 2.0 μM digoxin exposure for 72h. Well 14 has Caco2 cells exposed to 3.0mM Phenobarbital for 72 h. β-actin was used as the loading marker protein for these blots. 40 μg of protein was loaded onto these blots. Densitometry analysis is shown under each well. Results have been normalised to Caco2 control well 8.

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 hour 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 $p<0.005$(**).

Figure 3.

Four hour time course study of *Staphylococcus aureus* attachment to B: Caco-2, C: RKO and D: MCF7 cells. *Staph. aureus* 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 hour 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 $p<0.005$(**).

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 (O). 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.

Table 1

Bacterial adherence to Caco2 cells:

The effect of modifiers to a range of known active efflux proteins on the fluorescence associated with bacterial adherence to Caco2 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 Caco2 cells exposed to the same fluorescently labelled bacterial cultures. The higher the ratio, the more bacteria have attached to the Caco2 cells in 180 min.

Modifier	Efflux protein modified	*Escherichia coli*	*Staphylococcus aureus*	*Salmonella typhimurium*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Clostridium sporogenes*
		Ratio to control	Ratio to control	Ratio to control	Ratio to control	Ratio to control	Ratio to control
None - control		1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.02
PSC833	P-gp blocked	**1.87 ± 0.03****	**2.82 ± 0.01****	**2.83 ± 0.05****	**1.71 ± 0.06****	**1.67 ± 0.02****	**3.2 ± 0.03****
GF120918	P-gp & BCRP blocked	**2.42 ± 0.02****	**2.55 ± 0.02****	**2.34 ± 0.03****	NA	**1.77 ± 0.08****	**2.85 ± 0.02****
Glyburide	ABCA1 and P-gp blocked	**1.30 ± 0.03****	**2.18 ± 0.04****	**1.49 ± 0.03****	**1.33 ± 0.02****	NA	NA
Fumitremorgin	BCRP blocked	**1.38 ± 0.02****	**1.45 ± 0.04****	1.05 ± 0.08	1.30 ± 0.08	1.23 ± 0.04	NA
MK571	MRP1 blocked	**1.31 ± 0.06***	**1.34 ± 0.13***	1.17 ± 0.03	NA	NA	1.12 ± 0.02
Probenecid	MRP family blocked	0.97 ± 0.05	1.06 ± 0.03	1.15 ± 0.04	NA	NA	1.01 ± 0.04
Quercetin	MRP family blocked	1.24 ± 0.05	NA	NA	1.15 ± 0.10	1.18 ± 0.07	NA
Genestein	MRP family	1.07 ± 0.05	NA	NA	0.97 ± 0.09	1.29 ± 0.08	NA

	blocked						
Rifampicin	PXR activation. P-gp induction	1.15 ± 0.09	0.86 ± 0.09	**0.61 ± 0.18***	1.11 ± 0.07	**0.72 ± 0.09***	0.85 ± 0.01
Carbamazepine	Weak PXR activation. P-gp induction	0.96 ± 0.05	NA	NA	0.96 ± 0.02	NA	NA
Progesterone	P-gp blocker	**1.47 ± 0.06****	**1.71 ± 0.18****	**1.52 ± 0.08****	**1.46 ± 0.23****	**1.64 ± 0.14****	NA

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 hours 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), Genestein (100 μM), Quercetin (50 μM): 48 hour incubations; Carbamazepine (50 μM), Rifampicin (20 μM) and Progesterone (10 μM). PXR = pregnane X receptor

All Caco2 results shown are in quadruplicate ± SEM.

Significant differences in the binding of each species of bacteria to Caco2 cells incubated or exposed to drugs compared to control Caco2 cells at 180 min are shown with $p<0.05$ (*) and $p<0.005$(**).

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 labelled bacterial cultures. The higher the ratio, the more bacteria have attached to the MCF7 or RKO cells in 180 min.

Modifier	Efflux protein modified	*Escherichia coli*	*Staphylococcus aureus*	*Salmonella typhimurium*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Clostridium sporogenes*
MCF7		Ratio to control	Ratio to control	Ratio to control	Ratio to control	Ratio to control	Ratio to control
None - control		1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.05
PSC833	P-gp blocked	1.08 ± 0.05	1.08 ± 0.05	0.94 ± 0.02	NA	0.99 ± 0.02	1.10 ± 0.02
GF120918	P-gp & BCRP blocked	**1.60 ± 0.03****	1.14 ± 0.04	**1.57± 0.04****	**1.30 ± 0.05***	1.07 ± 0.04	**1.42 ± 0.03****
Fumitremorgin	BCRP blocked	1.19 ± 0.02	**1.29 ± 0.06***	1.21 ± 0.04	1.07 ± 0.05	1.14 ± 0.03	NA
Rifampicin	PXR activation. P-gp induction	**1.24 ± 0.09****	1.01 ± 0.07	0.87± 0.16	1.03 ± 0.03	0.92 ± 0.05	0.78 ± 0.10

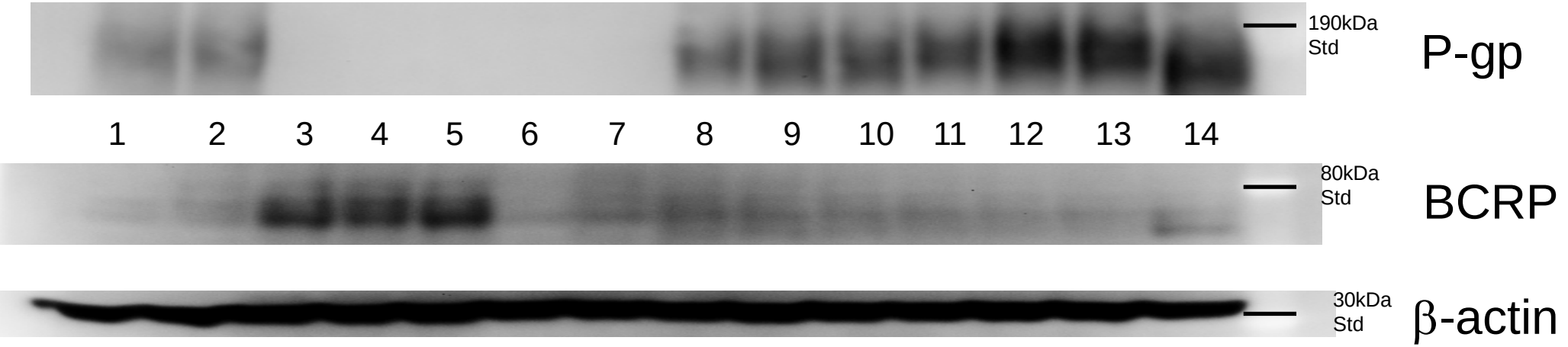
RKO							
None-control	MRP family blocked	1.00 ± 0.01	1.00 ± 0.01	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.02
PSC-833	P-gp blocked	1.03 ± 0.08	0.87 ± 0.03	1.01 ± 0.04	1.09 ± 0.02	1.01 ± 0.04	1.17± 0.01
Rifampicin	PXR activation. P-gp induction	1.04 ± 0.02	**0.71 ± 0.05****	0.94 ± 0.03	0.92 ± 0.01	0.86 ± 0.01	1.16 ± 0.03

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 hours 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), Genestein (100 μM), Quercetin (50 μM): 48 hour 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 1


190kDa
Std
P-gp
1 2 3 4 5 6 7 8 9 10 11 12 13 14
80kDa
Std
BCRP
30kDa
Std
β-actin

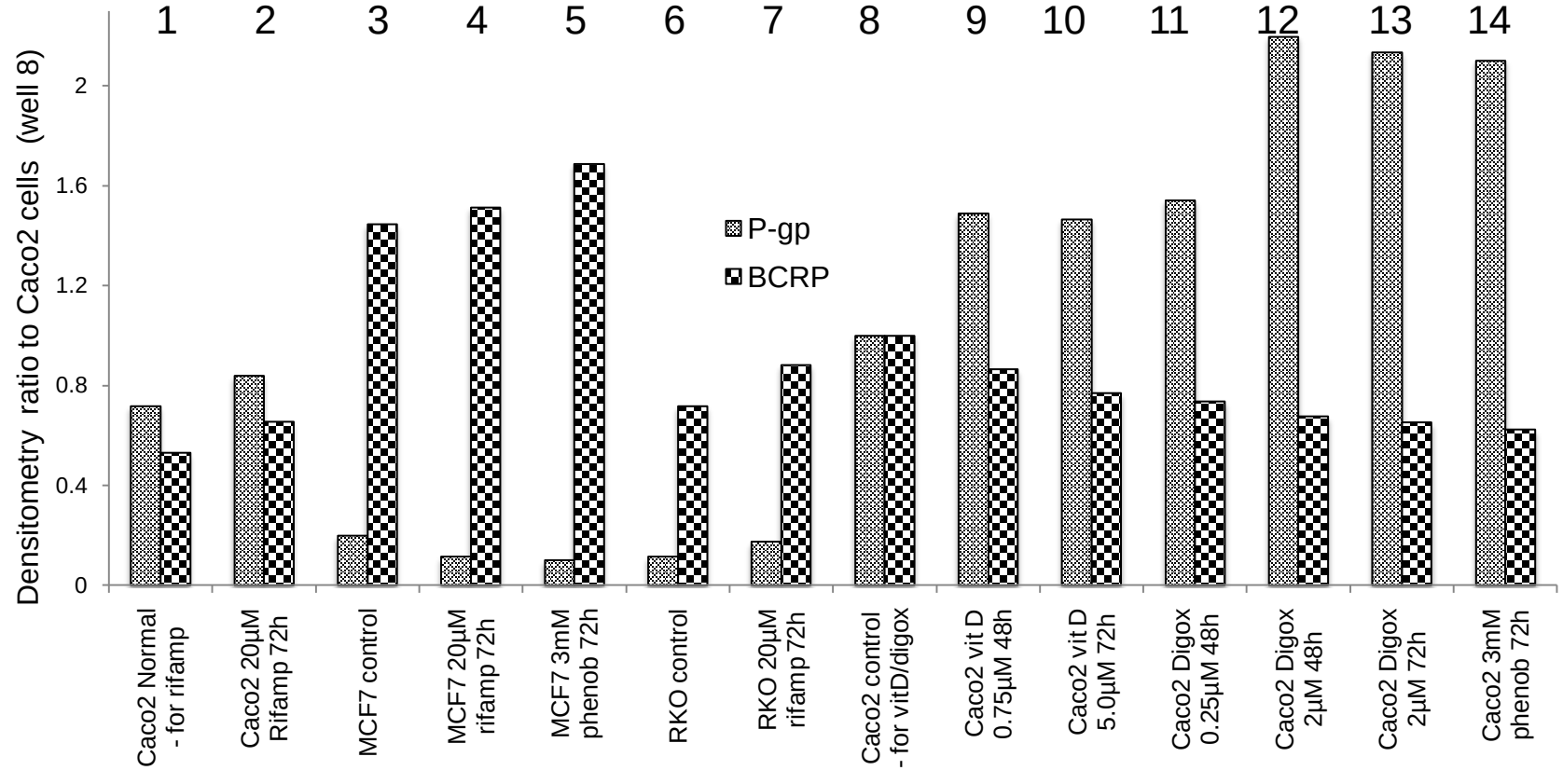

1 2 3 4 5 6 7 8 9 10 11 12 13 14
Densitometry ratio to Caco2 cells (well 8)
0
0.4
0.8
1.2
1.6
2
P-gp
BCRP
Caco2 Normal - for rifamp
Caco2 20μM Rifamp 72h
MCF7 control
MCF7 20μM rifamp 72h
MCF7 3mM phenob 72h
RKO control
RKO 20μM rifamp 72h
Caco2 control - for vitD/digox
Caco2 vit D 0.75μM 48h
Caco2 vit D 5.0μM 72h
Caco2 Digox 0.25μM 48h
Caco2 Digox 2μM 48h
Caco2 Digox 2μM 72h
Caco2 3mM phenob 72h

Figure 2

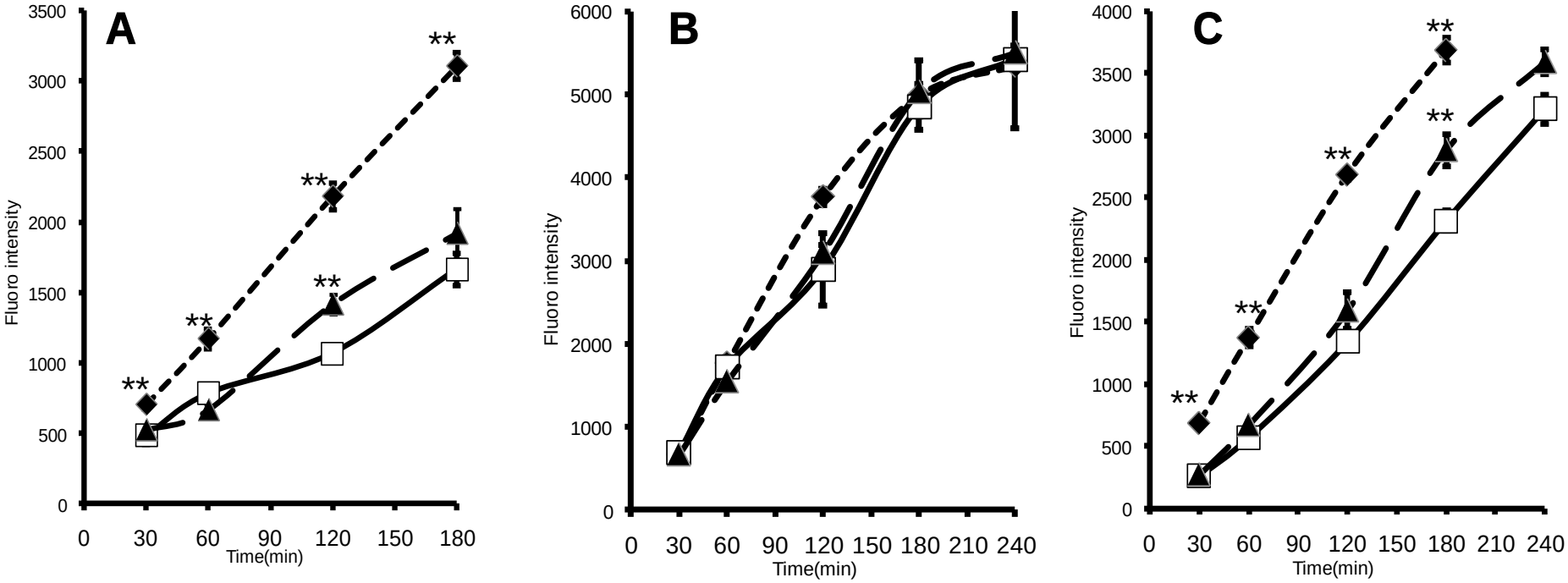

A
B
C
Fluoro intensity
Time(min)
**
0
500
1000
1500
2000
2500
3000
3500
4000
5000
6000
0 30 60 90 120 150 180
0 30 60 90 120 150 180 210 240

Figure 3

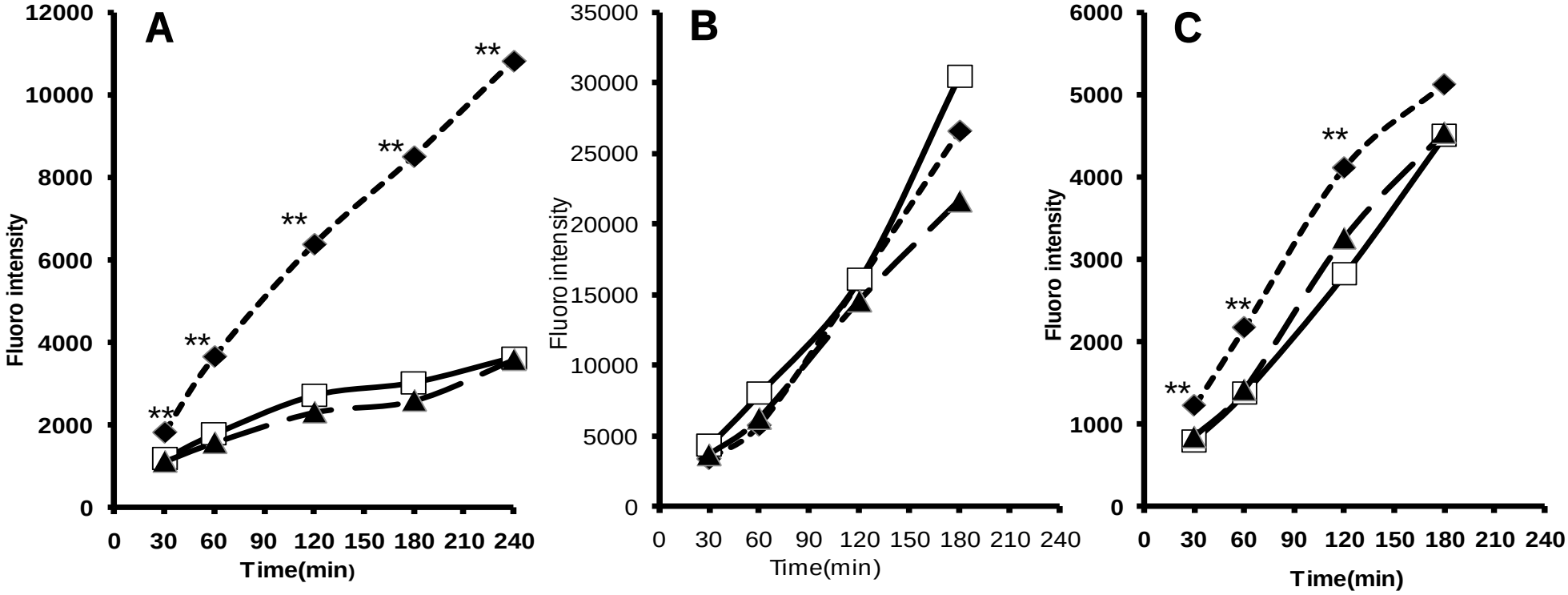
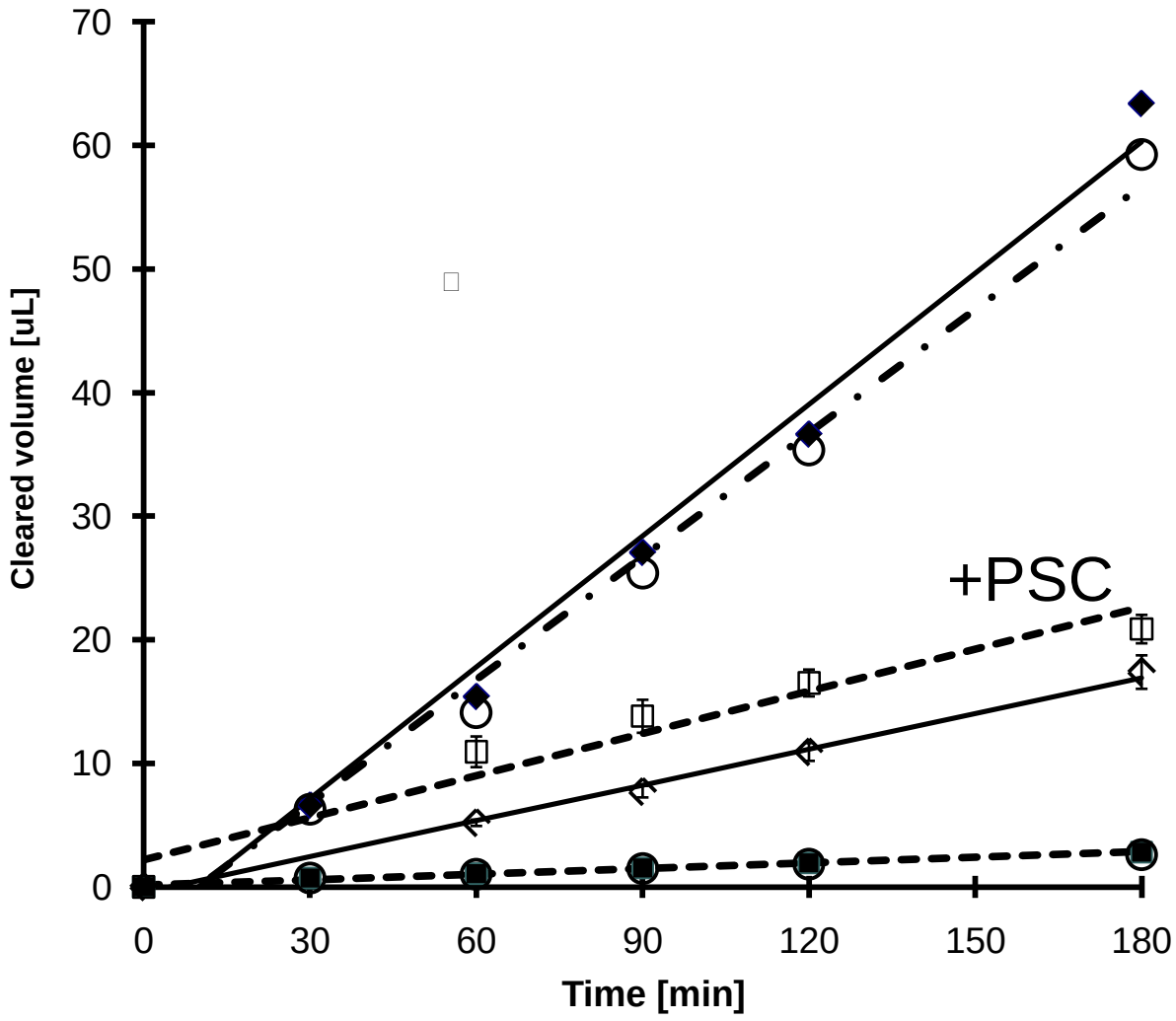

A
B
C
Fluoro intensity
Time(min)
**
0
30
60
90
120
150
180
210
240
2000
4000
6000
8000
10000
12000
5000
15000
20000
25000
30000
35000
1000
3000
5000

Figure 4


70
60
50
40
30
20
10
0
Cleared volume [uL]
+PSC
0
30
60
90
120
150
180
Time [min]