Inhibition of P-glycoprotein mediated efflux of digoxin and its metabolites by macrolide antibiotics

Jeff Hughes and Andrew Crowe*

School of Pharmacy, Curtin University and Curtin Health Innovation Research Institute (CHIRI)

***to whom correspondence should be addressed.**

Dr. Andrew Crowe

School of Pharmacy, Curtin University

BLDG 306; Perth, Western Australia, 6102

Tel 61 8 9266 3423

Fax 61 8 9266 2769

Email A.P.Crowe@curtin.edu.au

Running title:

Digoxin and macrolide interactions

Abstract.

This study was conducted to determine the rate of P-glycoprotein (P-gp) mediated efflux of digoxin analogues and metabolites, and to assess the effects of macrolide antibiotics on this efflux. Bidirectional transport studies were conducted using our Caco-2 sub clone with high P-gp expression (CLEFF9). HPLC methods were employed to measure drug transport. All digoxin metabolites were P-gp substrates, although digoxin had the greatest efflux ratio. Erythromycin had no effect on the transport of digoxin, maintaining a Basolateral to apical efflux ratio of 14.8, although it did reduce the efflux ratio of dihydrodigoxin and digoxigenin by 34% and 43%, respectively. Azithromycin also had little effect on the transport of digoxin or any of its metabolites. In contrast, clarithromycin and roxithromycin almost eliminated basolateral targeted efflux. Using paclitaxel as a known P-gp substrate, erythromycin demonstrated only partial P-gp inhibitory capacity maintaining an efflux ratio over 100. In contrast, clarithromycin and roxithromycin were 10 fold greater P-gp inhibitors. Clarithromycin and roxithromycin are likely to exhibit drug interactions with digoxin via inhibition of efflux mechanisms. Azithromycin appears to have little influence on P-gp mediated digoxin absorption or excretion and would be the safest macrolide to use concurrently with oral digoxin.

Key words.

azithromycin, erythromycin, clarithromycin, P-glycoprotein, Caco-2,

Introduction.

Digoxin is one of the most commonly prescribed drugs for the management of atrial fibrillation and chronic congestive cardiac failure. Likewise, macrolide antibiotics (azithromycin, clarithromycin, erythromycin and roxithromycin) are commonly prescribed antibiotics used by tens of millions of patients every year. The high level of use of these agents means the chance of co-prescription is also high, and on the basis of recent case reports so is the potential for serious digoxin toxicity.

Digoxin improves the quality of life of patients with cardiac failure, however digoxin toxicity remains a common cause of hospital admissions (1-3). Abad-Santos and others reported that digitalis toxicity accounted for 3% of the mild adverse drug reactions in their hospital's emergency ward, 5% of moderate ones and 4% of serious ones, making it the second most common cause of drug-related hospital admissions (3). Due to digoxin's narrow therapeutic index, toxicity is common and often life-threatening (4). Hyperkalaemia, a hallmark of acute intoxication due to paralysis of the sodium-potassium ATPase pump, is often absent in chronic intoxication. In such cases hypokalaemia is more likely to occur due to chronic blockade of this ATPase in the nephrons, allowing renal excretion of excess extracellular potassium, in addition to the frequent concomitant use of potassium wasting diuretics (5).

Following intravenous administration, 50-70% of digoxin is excreted unchanged in the urine, thus a decrease in renal function predisposes to digitalis toxicity (6). Therapeutic levels are considered to range between 0.8-2.0 μg/L, although a lower range of 0.5-0.8 μg/L has been proposed for patients with heart failure (7), whilst levels greater than 3.0 μg/L are considered toxic. Several factors are reported to modify the sensitivity of the myocardium to digoxin,

which can enhance digitalis toxicity such as electrolyte imbalances, decreased lean body mass and co-administration of quinidine, amiodarone, verapamil, erythromycin and diuretics. All these factors may interact and their inter-relationships are likely to determine the presence and extent of digitalis toxicity (3,5).

The bioavailability of oral digoxin varies between 50% and 90% (8). It is influenced by drug formulation and gastrointestinal disorders such as celiac disease and radiation enteritis (9). Further, it is known that 10-15% of the population harbour the organism *Eubacterium lentum* within their intestinal tract (10,11). This is capable of degrading digoxin to dihydrodigoxin and its corresponding aglycone, dihydrodigoxigenin (12). These two metabolites, which are relatively inactive, are referred to as digoxin reduction products (DRPs). This biotransformation of digoxin significantly reduces the bioavailability of the drug in those individuals colonized with *E. lentum*. Lindenbaum and colleagues found that erythromycin or tetracycline given to three volunteers who produced large amounts of DRPs resulted in the disappearance of these from the stool and urine (13). This was accompanied by an increase in serum digoxin concentrations.

There have been a number of clinical cases of erythromycin, clarithromycin and roxithromycin related digoxin toxicity noted in the literature (5,10,11,14). In all cases cessation of digoxin and the macrolide resulted in a resolution of digoxin toxicity and a fall in digoxin levels. Reintroduction of digoxin in the absence of the macrolide did not result in further toxicity. Whilst most authors support inhibition of gut flora as the mechanism of this interaction, Wakasugi and colleagues from Japan suggested that clarithromycin's ability to inhibit the P-glycoprotein (P-gp) mediated tubular excretion of digoxin was the cause (15).

It is now known that digoxin renal tubular secretion does not involve the organic anion or cation system, nor does it involve its pharmacological receptor, membrane sodium-potassium ATPase (16). Rather, digoxin uses the apical membrane P-gp as its transporter (4). Toxic interactions between digoxin and quinidine, verapamil, amiodarone, cyclosporin, propafenone, spironolactone and itraconazole are all thought to originate from P-gp interactions (4).

Increasing use of macrolide antibiotics will result in greater exposure to digoxin-macrolide interactions. Given the seriousness of digoxin toxicity and the fact that it may arise even when digoxin concentrations are within the therapeutic range (17-20), it is important to quantify the clinical significance of these interactions and develop predictors of those patients who are at risk. It is also important to fully understand the mechanism of the interaction. This study was therefore undertaken to examine the role of P-gp mediated efflux on digoxin metabolites, as this has not been explored before, and to determine whether macrolide interactions with digoxin also extend to the metabolites generated in the gastrointestinal tract.

Materials and Methods.

Materials.

Digoxin , digitoxin and digoxigenin were all supplied from Fluka Biochemicals, while dihydrodigoxin and digoxigenin bis-digitoxoside were kindly donated by GlaxoSmithKline Australia Pty Ltd (Boronia, Vic, Australia). Cell culture reagents: Phosphate buffered saline (PBS), HBSS, HEPES and high glucose Dulbecco's Modified Eagle Medium (DMEM) were from Gibco BRL (Melbourne, Australia). Penicillin G, streptomycin and non essential amino acids were from Trace Biosciences (Castle Hill, NSW, Australia), while the foetal calf serum (FCS) was obtained from the Australian Commonwealth Serum Laboratories (Parkville, Vic, Australia).

Erythromycin, azithromycin and roxithromycin were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Clarithromycin was extracted from Klacid® tablets from Abbott Australasia (Kurnell, NSW, Australia), containing 250 mg clarithromycin, using acetone and nitrogen evaporation.

Cell culture.

Caco-2 sub clone cells, highly expressing P-gp, were seeded onto Millicell polycarbonate 0.6 cm^2 filter inserts in 24 well plates at 65,000 cells/cm^2, as described previously (21). 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% FCS) in a 37ºC incubator with 5% CO_2. Cells were incubated for 21-25 days to allow full maturation of the monolayer of cells. 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 400-800 $\Omega.cm^2$ for all cells in this

study. Resistance readings at the end of each experiment were not significantly different from initial values.

The studies were conducted using 'assay medium' consisting of Hanks balanced salt solution (HBSS) supplemented with both glucose (Ajax chemicals, NSW, Australia) and HEPES (Gibco BRL; Melbourne, Australia) 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.

Cells were incubated in pre-warmed assay medium with or without an efflux inhibitor for 30 min at the correct pH, and then rinsed in the same medium. TEER was measured and assay medium +/- inhibitors were placed in the receiver chambers. Paclitaxel, digoxin and related drugs at either 10 or 20 μM were added to the donor chamber of each well.was used as a known P-gp substrate. 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 3 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.

P-glycoprotein and other transport inhibition

In studies where inhibition of active efflux proteins were performed in conjunction with known P-gp substrates or inhibitors, cells were pre-incubated in HBSS containing the inhibitors on both sides of the cells for 30 min before initiation of the study. The inhibitors included the following P-gp inhibitors, 4 μM PSC-833 or 4 μM GF120918, as used previously (21). The general MRP inhibitor, probenecid (at 500 μM) was also used.

Protein determinations

Protein concentrations were determined using a micro-Lowry method adapted for use with multiwell plates on a TECAN Sunrise 96 well plate spectrophotometer with a 750 nm filter, using Magellan 3 software for Windows 2000 professional.

HPLC analysis

HPLC methods were used to determine the concentrations of digoxin, digitoxin, digoxigenin and digoxigenin bis-digitoxoside: The mobile phase consisted of acetonitrile [EM Science, Gibbstown, NJ, USA] and water (31:69 v/v – for digoxin, 47:53 v/v for digitoxin, 25:75 v/v for digoxigenin and 27:73 v/v for digoxigenin bis-digitoxoside). The HPLC system consisted of an Agilent 1100 series system run though the Agilent PC package 'ChemStation' for Windows 2000. The quaternary pump ran at 1.2 mL/min and a Perkin Elmer Series 200 autosampler injected 60 μL of sample through a Zorbax Stable bonded (SB) C_{18} column, 5μm pores, 15 cm x 4.6 mm I.D. with mated guard column [Agilent, NSW, Australia]. The effluent was detected on an Agilent 1100 variable wavelength UV detector. Digoxin, digitoxin, digoxigenin and digoxigenin bis-digitoxoside were all measured at 215 nm, with typical retention times of 4.0, 4.4, 3.7 and 5.4 minutes. Limits of detection, using 60 μL injections into the column were 50 nM , 100 nM, 50 nM and 70 nM for digoxin, digitoxin, digoxigenin and digoxigenin bis-digitoxoside respectively.

Alphascreen dihydrodigoxin detection

Dihydrodogoxin, with a reduced lactone ring, exhibited negligible chromatographic qualities. Instead, it was detected via the patented Alphascreen technology system by Perkin-Elmer (Melbourne, Vic, Australia). A digoxin detection kit was purchased, which we showed to have adequate cross reactivity to Dihydrodigoxin, and to be quantitative for our requirements

(Figure 1). We used anti-digoxin acceptor beads with biotinylated–digoxin binding to these acceptor beads, and strepavidin donor beads, that would bind to the biotin residues protruding from the conjugated digoxin, now bound to the acceptor beads. All of this, including the buffer solutions was included in the digoxin detection kits.

The nature of bead-binding meant that instead of directly detecting the dihydrodigoxin, this was a competitive inhibition study that resulted in reduced fluorescence the greater the interference from dihydrodigoxin in the solution, that prevented biotinylated digoxin binding to the acceptor beads. Biotinylated digoxin was diluted in AlphaScreen buffer from its stock concentration to a working concentration of 0.45 nM. Acceptor and donor beads were both diluted to 100 μg/mL each. 15 μL volumes of all test samples in duplicate were added to 384 well white walled μClear flat bottomed plates (Greiner Bio-one, Interpath services, Perth). 5 uL of acceptor beads were then added and incubated at room temperature for 30 min in the dark. 5 uL of donor beads were subsequently added and the reaction allowed to proceed for a further 60 min before reading the individual wells in an En-Vision MultiPlate reader (Perkin Elmer Life Sciences, Melbourne, Vic). This approach to detect dihydrodigoxin is novel, and has not been used previously in the literature.

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 in our laboratory previously (22). Briefly, this calculation allows for a modification to the original Artursson equation (23), 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 (24).

Results in this study are presented as the mean ± SEM of between 3 to 5 individual studies, standardised on individual protein concentrations. Significant differences between values were examined using Student's two-tailed unpaired t-test or one way ANOVA with Dunnett's post hoc analysis. Results were considered significant if $P < 0.05$.

RESULTS

Basolateral to apical transport was 15 fold greater than transport in the apical to basolateral directions for digoxin using 20 μM drug concentrations (Figure 2a; Table 1). As both PSC-833 and GF120918 were able to neutralise the basolateral to apical flow of drug, this indicated P-gp as the likely cause of the efflux. Although Both PSC-833 and GF120918 can inhibit other ion channels and transporters in addition to P-gp, they are less potent inhibitors of other transporters (especially PSC-833) (25,26) and the only commonality between both PSC-833 and GF120918 is P-gp inhibition. Thus, when similar inhibition occurs through the use of two separate P-gp inhibitors, the probability of P-gp being involved is very high.

The metabolites of digoxin also had significant P-gp mediated efflux. Digoxin had the greatest efflux potential of the glycosides tested, however, removal of one monosaccharide unit to create digoxigenin bis-digitoxiside did not have a dramatic effect on the P-gp affinity, with the efflux ratio dropping only 10%, from 15.2 to 13.7. However, the apparent permeability rates (Papp) dropped from 3.1 to 0.9 x 10^{-6} cm/sec, which equated to a probable drop in absorption from 50% to 13%, based on comparison with drugs of known human absorption profiles (27) (Figure 3). Removal of all sugar moieties to generate digoxigenin, increases diffusion close to the levels of parent digoxin, yet affinity for P-gp was reduced, with only a 3.5 fold efflux ratio, which implies inherently better absorption of digoxigenin *in*

vivo. These metabolites occur through hydrolysis in the stomach, and are common (28). Dihydrodigoxin, created through bacterial metabolism in the gastro-intestinal tract (28), has the highest Papp of the metabolites tested, although active efflux was a significant factor in reducing this high Papp from allowing dihydrodigoxin to cross the cells (Table 2).

Digitoxin was also shown to be a P-gp substrate in this system, although basolateral to apical transport was only 4 fold higher than apical to basolateral transport using 20 μM digitoxin (Figure 2b: Table 2). The apparent permeability results for digitoxin were much higher than digoxin ($24x10^{-6}$ cm/sec compared to 3×10^{-6} cm/sec) (Tables 1 and 2). From previous studies in our laboratory on passive permeability (22), we can estimate that this represents no more than 50% absorption for digoxin, while the higher value for digitoxin shows 100% absorption is likely for this glycoside (Figure 3).

Dihydrodixogen had the greatest basolateral to apical efflux at almost 61×10^{-6} cm/sec (Table 2) with digoxigenin bis-digitoxiside a distant second with 15×10^{-6} cm/sec (Table 1). When examined as the net amount of drug transported in the basolateral to apical direction, after removal of the amount of drug transported in the apical to basolateral direction, as shown in figure 4, this reinforced the notion that digoxigenin has more physical drug effluxed in this *in vitro* setting compared with the other metabolites or parent digoxin drug with a net transport of over 35 ng/cm^2/min (Figure 4). When compared to digoxigenin, with only 1.4 ng/cm^2/min net efflux, then irrespective of its 3.5 fold higher efflux compared to its apical to basolateral transport direction, there is only a small amount of drug that can be affected by blocking P-gp. However, if dihydrodigoxin is created in the gastrointestinal tract, then co-administration with a P-gp inhibitor would allow significantly greater dihydrodigoxin to enter the body than other metabolites, or parent digoxin (Figure 4).

Using 100-200 μM macrolides on both sides of our Caco-2 cell monolayers, we showed erythromycin to have no effect on the transport of digoxin, maintaining an efflux ratio of 15 (Table 1). However, erythromycin was able to reduce the net basolateral direction transport of dihydrodigoxin and digoxigenin by 34% and 43%, respectively, and reduced digitoxin efflux by over 40% (Figure 5). Azithromycin was also able to reduce efflux for digitoxin, but unlike erythromycin, had a very limited ability to decrease the net basolateral to apical directed transport of digoxin or any of its metabolites, making it the weakest of the inhibitors studied here (Table 1, 2: Figure 5). y In contrast, both clarithromycin and roxithromycin were potent P-gp inhibitors able to increase apical to basolateral transport of digoxin from $0.9 \pm 0.1 \times 10^{-6}$ cm/sec to $3.3 \pm 0.3 \times 10^{-6}$ cm/sec (Table 1), while also reducing basolateral to apical transport from $13.6 \pm 0.8 \times 10^{-6}$ cm/sec to $7.5 \pm 0.4 \times 10^{-6}$ cm/sec in the case of clarithromycin, and $4.4 \pm 0.2 \times 10^{-6}$ cm/sec in the case of roxithromycin. This resulted in clarithromycin and roxithromycin reducing the efflux ratio for digoxin to 2.3 and 1.3 respectively. Using paclitaxel as our positive P-gp substrate, erythromycin demonstrated limited P-gp inhibitory capacity, reducing its efflux ratio from 390 ± 12 to a still very large 111 ± 8. In contrast, clarithromycin reduced efflux to 11 ± 1 and roxithromycin to 3.8 ± 0.3 confirming them as potent P-gp inhibitors (Table 1), and indicating that clarithromycin and roxithromycin are likely to promote drug interactions with digoxin via inhibition of efflux mechanisms. Azithromycin appeared to increase the efflux of digoxigenin bis-digitoxiside (Table 1), as did probenecid, and although both of these results were moderately significant, this was largely as a result of very low apical to basolateral transport. However, due to these results being close to detection limits of our HPLC system, changes in results from 0.1 to 0.2 can have a 2 fold impact on efflux ratios. Under these circumstances care needs to be taken with interpretation of efflux ratios, and we consider any affect of azithromycin or probenecid to be relatively

minor. Future research would need to be conducted to explore any significance of this interaction.

DISCUSSION

Digoxin has a narrow therapeutic window making it a drug of concern when given chronically, whenever other pharmaceuticals are introduced for treatment of acute ailments. In addition, although digoxin is not widely metabolized in the human body, there are some active metabolites created, and these may have differing rates of P-gp mediated efflux compared to the parent digoxin, which could influence clinical outcomes when P-gp inhibitors alter gastro-intestinal absorption of digoxin and any metabolites created. Up to 10% of people are significant metabolisers of digoxin to dihydro-digoxin, which is subsequently excreted in the urine (28). Our study has shown that dihydro-digoxin is also a P-gp substrate, with an efflux ratio of 8.4 versus 15.2 for the parent digoxin. In addition one study showed that dihydrodigoxin and digoxigenin bis-digitoxoside are present at higher concentrations in the urine when administered orally rather than intravenously (29). This suggests that microflora degradation may be adding to the generation of metabolites with subsequent greater systemic absorption. Our study shows that once the metabolites are generated, either endogenously or via microflora, then a greater proportion of the drug would be absorbed, due to the metabolites having less of an affinity to P-gp and having greater inherent passive permeability across cell membranes (22,27).

Another digitalis analogue, digitoxin, is known to have complete human absorption, greater retention in the body, and greater control of supraventricular tachyarrhythmias than digoxin (30). It was shown here that digitoxin had higher passive permeability than digoxin, and its

affinity for P-gp was 4 fold less than digoxin, resulting in less competitive inhibition with other substrates, reinforcing clinical results (30).

About 10% of the population are high excretors of digoxin reduction products (DRPs), and these are thought to arise due to the anaerobic bacterium *Eubacterium lentum*, a common constituent of the intestinal microflora (13). However, it has subsequently been found that the presence of *E. lentum* could also be isolated in high concentrations from the stools of individuals who did not excrete DRPs when given digoxin orally (31). Further eroding the credibility of *E. lentum's* role in macrolide-digoxin interactions are recent studies that have shown that *E. lentum* is killed by a variety of current antimicrobials that have little influence on whole body digoxin pharmacokinetics (32). These reports put more emphasis on macrolides having an influence on the pharmacokinetics of digoxin rather than the bacteria they affect.

To that end, we were able to show that not only did clarithromycin and roxithromycin have strong P-gp inhibitory action against digoxin and its metabolites, but also paclitaxel, an antineoplastic drug with strong affinity to P-gp, had efflux inhibited by all of the macrolides. Azithromycin and erythromycin were very weak inhibitors though, when compared to clarithromycin and roxithromycin. These results comply with those of a previous study by Eberl and colleagues who also examined P-gp inhibition by macrolides in Caco-2 cells (33). Interestingly, in our study, roxithromycin was more potent than clarithromycin, especially with regards to the parent digoxin, while Eberl's study had clarithromycin at approximately double the potency of roxithromycin. Surprisingly, in our study erythromycin had only a minor role as a P-gp inhibitor, which suggested that changes to metabolism through erythromycin's other effects such as forming nitrosoalkenes and subsequent complexation with CYP3A4 and other cytochromes (34) may be the causative factors by which

Formatted: Font: Times New Roman, 12 pt

Formatted: Font: Times New Roman, 12 pt, Italic

Formatted: Font: Times New Roman, 12 pt

Formatted: Font: Times New Roman, 12 pt

Formatted: Font: Times New Roman, 12 pt

Formatted: Font: Times New Roman, 12 pt

Formatted: Font: Times New Roman, 12 pt

erythromycin increases plasma levels of digoxin, even though digoxin is not directly metabolised using these processes. Thus, with regards to erythromycin, it is still unclear the exact mechanism by which it increases digoxin concentrations. Eberl's 2007 publication does show erythromycin having an inhibitory function on digoxin transport with an inhibitory potency of 23 μM. As our study used over four times this concentration, we should have observed P-gp mediated efflux inhibition, which we did not, although a closer inspection of their data shows 500 μM erythromycin still having some digoxin efflux, such that their inhibition curves have some line fitting variability (33). Some reduction in efflux characteristics of some digoxin metabolites was noted, but not of digoxin itself. A greater disparity between these two related studies was regarding azithromycin. Apart from digitoxin, none of the digoxin related molecules were affected by azithromycin in our study, yet there was a weak, but observable inhibitory effect on digoxin transport from Eberl's 2007 study. The only clear difference between the two studies, was the measurement of tritiated hydrogen appearance on the opposite chamber in the previous work, while in our study we measured the digoxin directly with HPLC separation technology (33).

Wakasugi's laboratory demonstrated that high μM concentrations of clarithromycin reduced digoxin transcellular transport and increased cellular accumulation using kidney epithelial cell line monolayers (15). Further they were able to demonstrate reduced renal clearance of digoxin in a patient who was taking clarithromycin (200mg orally twice daily). In our study clarithromycin was almost as effective as PSC-833 at inhibiting P-gp mediated efflux of digoxin and all of the metabolites tested. The suggestion that the major contributor is increased bioavailability is supported by the work of Tsutsumi and colleages. (35). In their study intravenous digoxin was used, and no effect was seen on serum digoxin concentration-time curves. In fact these authors reported that renal excretion of digoxin was enhanced by the

co-administration of both clarithromycin and erythromycin. This is contrary to what Wakasugi and colleagues from Japan suggested regarding clarithromycin's ability to inhibit the P-gp -mediated tubular excretion of digoxin (15). In an additional report of six patients with end-stage renal disease who suffered digoxin toxicity following the administration of clarithromycin, it was suggested that inhibition of P-gp in the gut and/or bile capillary ducts was likely, as renal clearance in these patients was already grossly compromised (36). The fact that efflux of digoxin metabolites was also inhibited, suggests that their bioavailability would also be increased, and the presence of drug in their stools reduced.

Azithromycin had very weak P-gp inhibitory action in our study, with either digoxin or paclitaxel. This finding is consistent with the lack of case reports of digoxin-azithromycin drug interactions. However, as our study only looked at inhibitory action and not whether the macrolide was a substrate, there is still the possibility of some interaction with P-gp for azithromycin, as a pure substrate only. Some studies have suggested that azithromycin is a P-gp substrate (37,38), while Pachot's work in 2003 goes further to suggest that all of the macrolide antibiotics at very low micromolar concentrations do exhibit P-gp mediated efflux (37). In addition, a recent report found 5 μM concentrations of clarithromycin and erythromycin had efflux ratios of 22 and 8 each respectively in MDR1 transfected cells (39), but as we used high concentrations between 100-200 μM it may be expected that some competitive inhibition occurred even if the macrolides were substrates rather than pure inhibitors. High micromoloar concentrations were used in our study to reflect expected doses available to the gut wall, rather than in the circulation, so we do not believe that the concentrations used in our study are clinically unrealistic. The potency of P-gp inhibition in our study by clarithromycin and roxithromycin does suggest that these two macrolides have true inhibitory action against P-gp mediated efflux that may be separate to any competitive transport action. Gomes and others recently reported that in a 15 year population-based,

Formatted: Font: Times New Roman, 12 pt

nested case-control study, which investigated the association between hospitalization for digoxin toxicity and recent exposure to individual macrolide antibiotics that clarithromycin was associated with the highest risk of digoxin toxicity (40). Our finding would support this increased risk with concurrent clarithromycin use. They also reported that erythromycin and azithromycin were associated with much lower risk, which is again consistent with our findings, although we would not have predicted such an increased risk with azithromycin, based on P-gp inhibition.

Formatted: Font: Times New Roman, 12 pt

Formatted: Font: Times New Roman, 12 pt

Formatted: Font: Times New Roman, 12 pt

This study demonstrated that roxithromycin, clarithromycin and to a lesser extent, erythromycin, were able to inhibit P-gp efflux both for digoxin and number of its metabolites or derivatives. In doing so it provides an alternative explanation for the observed reduction in excretion of DRP in the stools and urine of high DRP excreters administered macrolides. Inhibition of P-gp in the gut would result in an increase in the bioavailability of both digoxin and it reduction products, at the same time inhibition of P-gp in the kidney decreases the clearance of digoxin and its reduction products. The net result would be an increase in digoxin serum levels and a fall in excretion of DRPs in both the stools and urine.

From the perspective of choosing a macrolide antibiotic administrable to patients on digoxin, based on our results azithromycin would be the drug of choice and it has previously be reported not to effect cytochrome P-450, like the other macrolides (34). However, in light of the findings of Gomes and coworkers (40), it would seem prudent to monitor the serum levels of all patients commenced on a macrolide.

Acknowledgements.

Digoxigenin bis-digitoxoside and dihydrodigoxin were kindly donated by GlaxoSmithKline Australia. The Cyclosporin D derivative PSC-833, was kindly donated by Novartis Pharma, Basel, Switzerland. We thank the West Australian Medical Research institute (WAIMR) for allowing use of their Perkin Elmer Envision multi-plate reader during development of the dihydrodigoxin assay method.

Figure Legends.

Figure 1:

Std curve for competition assay using Dihydrodigoxin to interfere with biotinylated digoxin in an Alphascreen digoxin detection kit after 60 min incubation of samples with donor beads.

Figure 2:

Bidirectional transport of 20 μM digoxin (A) or digitoxin (B) through the Caco-2 CLEFF9 subclone. Apical to basolateral direction (squares) and basolateral to apical direction (diamonds), without (closed symbols) and with (open symbols) the presence of 4 μM PSC-833, a potent P-glycoprotein inhibitor, on both sides of the membrane.

Figure 3:

In vitro permeability co-efficient as an estimate of human intestinal absorption. Once P-gp mediated efflux was eliminated by use of P-gp blocking agents, the remaining apical to basolateral values for digoxin and its analogues could be plotted on our previously established Caco-2 permeability (22) vs human absorption comparison curve, as determined by Artursson and Karlsson (27) providing some indication to the inherent permeability of these molecules across the human gastro-intestinal tract when compared to other drugs previously transported across Caco-2 cell monolayers that have known human intestinal absorption percentages.

Figure 4:

Net transport rates in the efflux direction for 20 μM digoxin and metabolites / analogues using transport rates from Ap to Bas and Bas to Ap directions over a three hour period in Caco-2 monolayers grown on Millicell polycarbonate filter membranes. Results shown equate to transport in the Bas to Ap direction after removal of the Ap to Bas direction component for identical Drug and inhibitor concentrations. PSC 833 and GF120918 were each used at 4 μM, with probenecid at 500 μM. Solid columns = digoxin, white = digoxigenin, upwards angled lines = digoxigenin bis-digitoxoside, dotted = digitoxin and downward angled lines = dihydrodigoxin. Significance of inhibitor use reducing efflux compared to glycoside alone is shown (#) where $p<0.05$.

Figure 5:

Net transport rates in the efflux direction for 20uM digoxin and metabolites / analogues using transport rates from Ap to Bas and Bas to Ap directions over a three hour period in Caco-2 monolayers grown on Millicell polycarbonate filter membranes. Results shown equate to transport in the Bas to Ap direction after removal of the Ap to Bas direction component for identical Drug and inhibitor concentrations. Erythromycin and roxithromycin were each used at 200 μM, while clarithriomycin and azithromycin were used at 100 μM. Solid columns = digoxin, white = digoxigenin, upwards angled lines = digoxigenin bis-digitoxoside, dotted = digitoxin and downward angled lines = dihydrodigoxin. Significance of inhibitor use reducing efflux compared to glycoside alone is shown (#) where $p<0.05$.

Table 1. Efflux ratios (transport in the Bas to Ap direction compared to transport in the Ap to Bas direction) for the Cardiac Glycosides, Digoxin, digoxigenin and digoxigenin bis-digitoxoside after 3 hour transport studies in a Caco-2 sub clone (CLEFF) over-expressing P-glycoprotein.

Inhibitor	**Digoxin**			**Digoxigenin**			**Digoxigenin-bis-digitoxoside**		
	Ap to Bas $x10^{-6}$ cm/sec	Bas to Ap $x10^{-6}$ cm/sec	**Ratio (B-A)**	Ap to Bas $x10^{-6}$ cm/sec	Bas to Ap $x10^{-6}$ cm/sec	**Ratio (B-A)**	Ap to Bas $x10^{-6}$ cm/sec	Bas to Ap $x10^{-6}$ cm/sec	**Ratio (B-A)**
None	0.9 ± 0.1	3.6 ± 0.8	**15.2 ± 2.0**	1.9 ± 0.1	6.7 ± 0.2	**3.5 ± 0.2**	1.1 ± 0.5	15.2 ± 0.8	13.7 ± 6.7
PSC-833	3.4 ± 0.1$^{\#\#}$	2.9 ± 0.2$^{\#\#}$	**0.9 ± 0.1**$^{\#\#}$	2.5 ± 0.1$^{\#}$	2.6 ± 0.1$^{\#\#}$	**1.0 ± 0.1**$^{\#\#}$	1.1 ± 0.0	1.0 ± 0.0$^{\#\#}$	0.9 ± 0.1$^{\#\#}$
GF120918	2.9 ± 0.2$^{\#\#}$	2.7 ± 0.1$^{\#\#}$	**0.9 ± 0.1**$^{\#\#}$	2.5 ± 0.1$^{\#}$	2.5 ± 0.1$^{\#\#}$	**1.0 ± 0.1**$^{\#\#}$	1.4 ± 0.1	1.3 ± 0.1$^{\#\#}$	0.9 ± 0.2$^{\#\#}$
Probenecid	0.9 ± 0.1	2.9 ± 0.5	**13.8 ± 1.2**	2.1 ± 0.1	8.7 ± 0.2$^{\#\#}$	**4.1 ± 0.3**	0.4 ± 0.2	12.5 ± 0.2$^{\#}$	35.7 ± 6.1$^{\#}$
Erythromycin	1.0 ± 0.1	5.0 ± 2.0	**14.8 ± 1.1**	2.1 ± 0.1	4.2 ± 0.3$^{\#\#}$	**2.0 ± 0.2**$^{\#\#}$	1.1 ± 0.2	10.8 ± 0.6$^{\#\#}$	10.4 ± 2.8
Clarithromycin	3.3 ± 0.2$^{\#\#}$	7.5 ± 0.4$^{\#\#}$	**2.3 ± 0.3**$^{\#\#}$	3.2 ± 0.2$^{\#\#}$	3.7 ± 0.1$^{\#\#}$	**1.1 ± 0.1**$^{\#\#}$	0.9 ± 0.3	3.1 ± 0.2$^{\#\#}$	3.3 ± 1.3$^{\#\#}$
Roxithromycin	3.3 ± 0.3$^{\#\#}$	4.4 ± 0.2$^{\#\#}$	**1.3 ± 0.2**$^{\#\#}$	2.3 ± 0.1	3.0 ± 0.2$^{\#\#}$	**1.3 ± 0.2**$^{\#\#}$	0.7 ± 0.2	2.5 ± 0.1$^{\#\#}$	3.3 ± 1.1$^{\#\#}$

Azithromycin	1.2 ± 0.0[#]	20.5 ± 0.5	**17.6 ± 0.9**	2.1 ± 0.1	5.3 ± 0.3[#]	**2.6 ± 0.3**[#]	0.3 ± 0.1	12.1 ± 0.4[#]	44.1 ± 21.7[#]

Significance of inhibitor use in drug transport in either apical to basolateral or the reverse direction is indicated with # for ($p<0.05$) and ## for ($p<0.005$).

Table 2. Efflux ratios (transport in the Bas to Ap direction compared to transport in the Ap to Bas direction) for the Cardiac Glycosides, Dihydrodigoxin, digitoxin and our rapidly transported p-glycoprotein substrate, the antineoplastic agent, paclitaxel, after 3 hour transport studies in a Caco-2 sub clone (CLEFF) over-expressing P-glycoprotein.

Inhibitor	**Dihydrodigoxin**			**Digitoxin**			**Paclitaxel**		
	Ap to Bas $x10^{-6}$ cm/sec	Bas to Ap $x10^{-6}$ cm/sec	**Ratio (B-A)**	Ap to Bas $x10^{-6}$ cm/sec	Bas to Ap $x10^{-6}$ cm/sec	**Ratio (B-A)**	Ap to Bas $x10^{-6}$ cm/sec	Bas to Ap $x10^{-6}$ cm/sec	**Ratio (B-A)**
None	7.2 ± 0.4	60.8 ± 0.4	**8.4 ± 0.5**	9.5 ± 0.5	36.1 ± 1.3	**3.8 ± 0.4**	0.1 ± 0.0	78.2 ± 2.5	**391 ± 13**
PSC-833	18.2 ± 1.3$^{\#\#}$	18.0 ± 0.8$^{\#\#}$	**1.0 ± 0.1**$^{\#\#}$	25.8 ± 0.4$^{\#\#}$	22.2 ± 1.0$^{\#\#}$	**0.9 ± 0.1**$^{\#\#}$	9.7 ± 0.8$^{\#\#}$	12.0 ± 0.2$^{\#\#}$	**1.2 ± 0.1**$^{\#\#}$
GF120918	22.8 ± 1.2$^{\#\#}$	15.4 ± 0.6$^{\#\#}$	**0.7 ± 0.1**$^{\#\#}$	19.0 ± 0.4$^{\#\#}$	21.8 ± 1.3$^{\#\#}$	**1.1 ± 0.1**$^{\#\#}$	19.9 ± 0.2$^{\#\#}$	38.9 ± 0.6$^{\#\#}$	**2.0 ± 0.1**$^{\#\#}$
Probenecid	7.0 ± 0.4	63.0 ± 1.5	**9.0 ± 0.8**	12.5 ± 0.2$^{\#}$	35.6 ± 1.3	**2.8 ± 0.2**	0.5 ± 0.2	66.5 ± 1.0$^{\#\#}$	**136 ± 49**$^{\#}$
Erythromycin	8.6 ± 0.6	47.8 ± 3.3$^{\#}$	**5.6 ± 0.8**$^{\#}$	13.4 ± 1.2$^{\#}$	27.7 ± 2.0$^{\#}$	**2.1 ± 0.3**$^{\#\#}$	0.4 ± 0.0	47.4 ± 0.6$^{\#\#}$	**111.0 ± 8.8**$^{\#}$
Clarithromycin	39.1 ± 4.7$^{\#\#}$	37.0 ± 0.7$^{\#\#}$	**0.9 ± 0.1**$^{\#\#}$	31.7 ± 0.2$^{\#\#}$	25.3 ± 1.3$^{\#\#}$	**0.8 ± 0.0**$^{\#\#}$	3.8 ± 0.2$^{\#\#}$	42.5 ± 1.0$^{\#\#}$	**11.2 ± 0.8**$^{\#\#}$
Roxithromycin	42.7 ± 1.1$^{\#\#}$	34.4 ± 2.4$^{\#\#}$	**0.8 ± 0.1**$^{\#\#}$	29.0 ± 0.9$^{\#\#}$	25.2 ± 0.2$^{\#\#}$	**0.9 ± 0.0**$^{\#\#}$	14.6 ± 0.9$^{\#\#}$	55.4 ± 0.3$^{\#\#}$	**3.8 ± 0.2**$^{\#\#}$
Azithromycin	13.2 ± 0.7$^{\#}$	74.8 ± 6.4$^{\#}$	**5.7 ± 0.8**$^{\#}$	13.3 ± 1.1$^{\#}$	25.0 ± 1.6$^{\#\#}$	**1.9 ± 0.3**$^{\#\#}$	0.7 ± 0.0	23.6 ± 0.6$^{\#\#}$	**36.4 ± 1.6**$^{\#\#}$

Significance of inhibitor use in drug transport in either apical to basolateral or the reverse direction is indicated with # for ($p<0.05$) and ## for ($p<0.005$).

References.

1 Mahdyoon H, Battilana G, Rosman H, Goldstein S, Gheorghiade M. The evolving pattern of digoxin intoxication: observations at a large urban hospital from 1980 to 1988 American heart journal. 1990;120:1189-1194

2 Einarson TR. Drug-related hospital admissions Ann Pharmacother. 1993;27:832-840

3 Abad-Santos F, Carcas AJ, Ibanez C, Frias J. Digoxin level and clinical manifestations as determinants in the diagnosis of digoxin toxicity Ther Drug Monit. 2000;22:163-168

4 Koren G, Woodland C, Ito S. Toxic digoxin-drug interactions: the major role of renal P-glycoprotein Veterinary and human toxicology. 1998;40:45-46

5 Nordt SP, Williams SR, Manoguerra AS, Clark RF. Clarithromycin induced digoxin toxicity Journal of accident & emergency medicine. 1998;15:194-195

6 Marik PE, Fromm L. A case series of hospitalized patients with elevated digoxin levels The American journal of medicine. 1998;105:110-115

7 Rathore SS, Curtis JP, Wang Y, Bristow MR, Krumholz HM. Association of serum digoxin concentration and outcomes in patients with heart failure Jama. 2003;289:871-878

8 Lullmann H, Mohr K, Ziegler A, Bieger D. (2000) Color atlas of pharmacology., 2nd Ed. Ed., Thieme, Stuttgart

9 Grahame-Smith D, Aronson J. (1992) Oxford textbook of clinical phrmacology and drug therapy, 2nd Ed Ed., Oxford University Press, Oxford

10 Laberge P, Martineau P. Clarithromycin-induced digoxin intoxication Ann Pharmacother. 1997;31:999-1002

11 Corallo CE, Rogers IR. Roxithromycin-induced digoxin toxicity The Medical journal of Australia. 1996;165:433-434

12 Marcus FI. Pharmacokinetic interactions between digoxin and other drugs Journal of the American College of Cardiology. 1985;5:82A-90A

13 Lindenbaum J, Rund DG, Butler VP, Jr., Tse-Eng D, Saha JR. Inactivation of digoxin by the gut flora: reversal by antibiotic therapy The New England journal of medicine. 1981;305:789-794

14 Gooderham MJ, Bolli P, Fernandez PG. Concomitant digoxin toxicity and warfarin interaction in a patient receiving clarithromycin Ann Pharmacother. 1999;33:796-799

15 Wakasugi H, Yano I, Ito T, Hashida T, Futami T, Nohara R, et al. Effect of clarithromycin on renal excretion of digoxin: interaction with P-glycoprotein Clininal Pharmacology & Therapeutics. 1998;64:123-128

16 Koren G, Klein J, Giesbrecht E, Ben Dayan R, Soldin S, Sellers E, et al. Effects of quinidine on the renal tubular and biliary transport of digoxin: in vivo and in vitro studies in the dog J Pharmacol Exp Ther. 1988;247:1193-1198

17 Hobley A, Lawrenson J. Ocular adverse effects to the therapeutic administration of digoxin Ophthalmic Physiol Opt. 1991;11:391-393

18 Wolin MJ. Digoxin visual toxicity with therapeutic blood levels of digoxin American journal of ophthalmology. 1998;125:406-407

19 Mitchell G. Adverse reactions to digoxin in four patients with normal or low serum digoxin levels The Medical journal of Australia. 1997;167:111

20 Gomes T, Mamdani MM, Juurlink DN. Macrolide-induced digoxin toxicity: a population-based study Clininal Pharmacology & Therapeutics. 2009;86:383-386

21 Crowe A, Teoh YK. Limited P-glycoprotein mediated efflux for anti-epileptic drugs J. Drug. Target. 2006;14:291-300

22 Crowe A, Lemaire M. In Vitro and In Situ Absorption of SDZ-RAD using a Human Intestinal Cell Line (Caco-2) and a Single Pass Perfusion Model in Rats: Comparison with Rapamycin Pharm. Res. 1998;15:1666-1672

23 Artursson P. Epithelial Transport of Drugs in Cell Culture. I: A Model for Studying the Passive Diffusion of Drugs over Intestinal Absorbtive (Caco-2) Cells. J. Pharm. Sci. 1990;79:476-482

24 Youdim K, Qaiser M, Begley D, Rice-Evans C, Abbott N. Flavonoid Permeability Across An In Situ Model of the Blood-Brain Barrier Free Radical Biology & Medicine. 2003;36:592-604

25 de Bruin M, Miyake K, Litman T, Robey R, Bates S. Reversal of resistance by GF120918 in cell lines expressing the ABC half-transporter, MXR Cancer Lett. 1999;146:117-126

26 Aszalos A, Thompson K, Yin J, Ross D. Combinations of P-Glycoprotein Blockers, Verapamil, PSC833, and Cremophor Act Differently on the Multidrug Resistance Associated Protein (MRP) and on P-Glycoprotein (Pgp) Antican. Res. 1999;19:1053-1064

27 Artursson P, Karlsson J. Correlation between Oral Drug Absorption in Humans and Apparent Drug Permeability Coefficients in Human Intestinal Epithelial (Caco-2) Cells. *Biochem. Biophys. Res. Comm*. 1991;**175**:880-885

28 Schentag J, Bang A, Kozinski-Tober J. (2006) Digoxin. In: Burton, M., Shaw, L., Schentag, J., and Evans, W. (eds). *Applied Pharmacokinetics & Pharmacodynamics: Principles of Therapeutic Drug Monitoring*, 4th Ed., Lippincott Williams & Wilkins, Philadelphia

29 Hinderling P, Hartmann D. Pharmacokinetics of Digoxin and Main Metabolites/Derivatives in Healthy Humans Therapeut Drug Monit. 1991;13:381-401

30 Belz G, Breithaupt-Grogler K, Osowski U. Treatment of congestive heart failure--current status of use of digitoxin European Journal of Clinical Investigation. 2001;31 Suppl 2:10-17

31 Dobkin J, Saha J, Neu H, Lindenbaum J. Digoxin-Inactivating Bacteria: Identification in Human Gut Flora Science. 1983;220:325-327

32 Citron D, Merriam C, Warren Y, Fernandez H, Goldstein E. In Vitro Activities of Ramoplanin, Teicoplanin, Vancomycin, Linezolid, Bacitracin, and four other Antimicrobials against Intestinal Anaerobic Bacteria Antimicrob. Agents. Chemother. 2003;47:2334-2338

33 Eberl S, Renner B, Neubert A, Reisig M, Bachmakov I, Konig J, et al. Role of p-glycoprotein inhibition for drug interactions: evidence from in vitro and pharmacoepidemiological studies Clin Pharmacokinet. 2007;46:1039-1049

34 Periti P, Mazzei T, Mini E, Novelli A. Pharmacokinetic Drug Interactions of Macrolides Clin. Pharmacokinet. 1992;23:106-131

35 Tsutsumi K, Kotegawa T, Kuranari M, Otani Y, Morimoto T, Matsuki S, et al. The Effect of Erythromycin and Clarithromycin on the Pharmacokinetics of Intravenous Digoxin in Healthy Volunteers Journal of Clinical Pharmacology. 2002;42:1159-1164

36 Hirata S, Izumi S, Furukubo T, Ota M, Fujita M, Yamakawa T, et al. Interactions between clarithromycin and digoxin in patients with end-stage renal disease Int J Clin Pharmacol Ther. 2005;43:30-36

37 Pachot J, Botham R, Haegele K, Hwang K. Experimental Estimation of the Role of P-Glycoprotein in the Pharmocaokinetic Bahaviour of Telithromycin, a Novel Ketolide, in Comparison with Roxithromycin and other Macrolides Using the Caco-2 Cell Model. J Pharm Pharmaceut Sci. 2003;6:1-12

38 Sugie M, Asakura E, Zhao Y, Torita S, Nadai M, Baba K, et al. Possible Involvement of the Drug Transporters P Glycoprotein and Multidrug Resistance-Associated Protein Mrp2 in Disposition of Azithromycin Antimicrobial Angents and Chemotherapy. 2004;48:809-814

39 Takeuchi T, Yoshitomi S, Higuchi T, Ikemoto K, Niwa S, Ebihara T, et al. Establishment and characterization of the transformants stably-expressing MDR1 derived from various animal species in LLC-PK1 Pharm Res. 2006;23:1460-1472

40 Gomes CM, van Paassen H, Romeo S, Welling MM, Feitsma RI, Abrunhosa AJ, et al. Multidrug resistance mediated by ABC transporters in osteosarcoma cell lines: mRNA analysis and functional radiotracer studies Nuclear medicine and biology. 2006;33:831-840

Figure 1

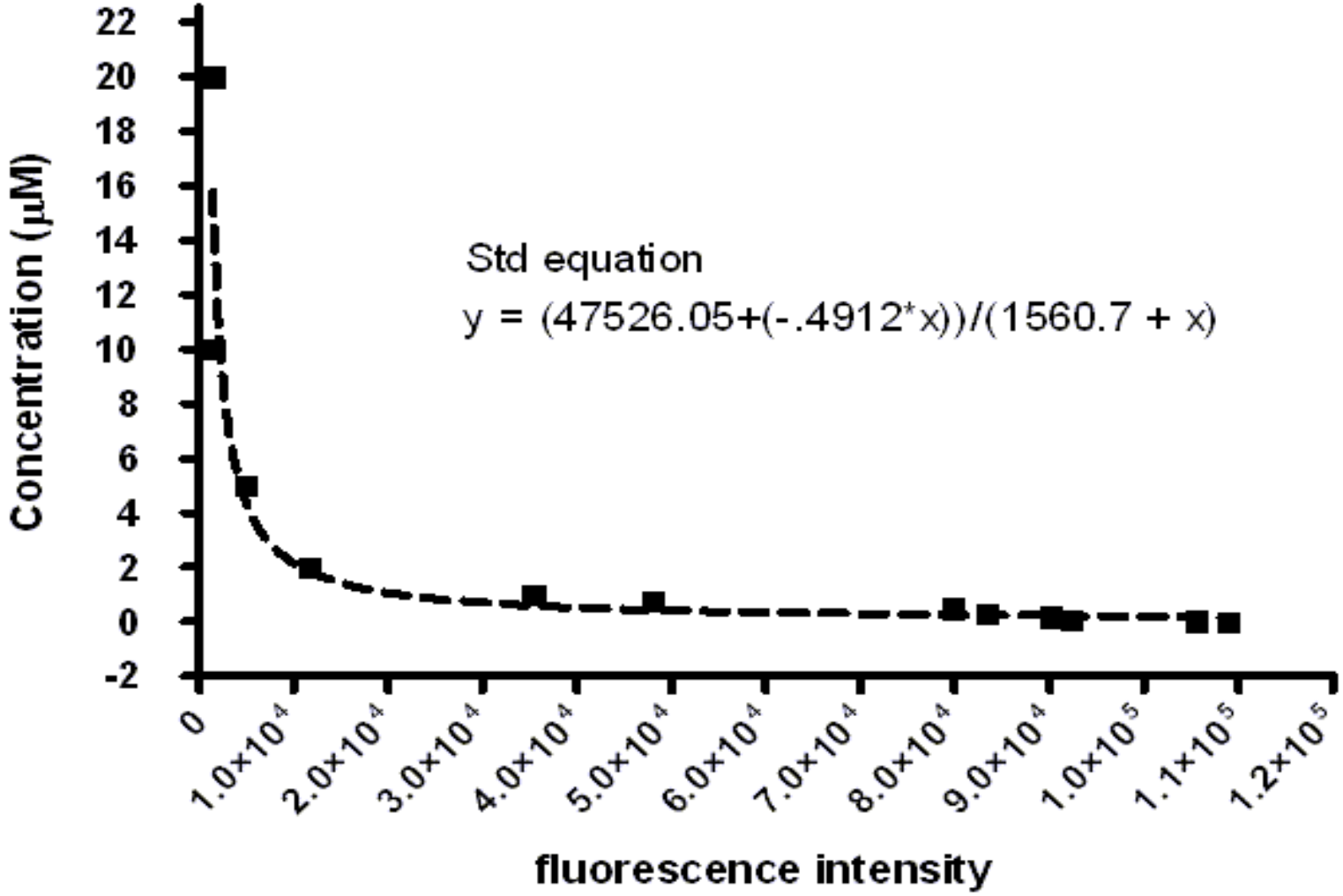
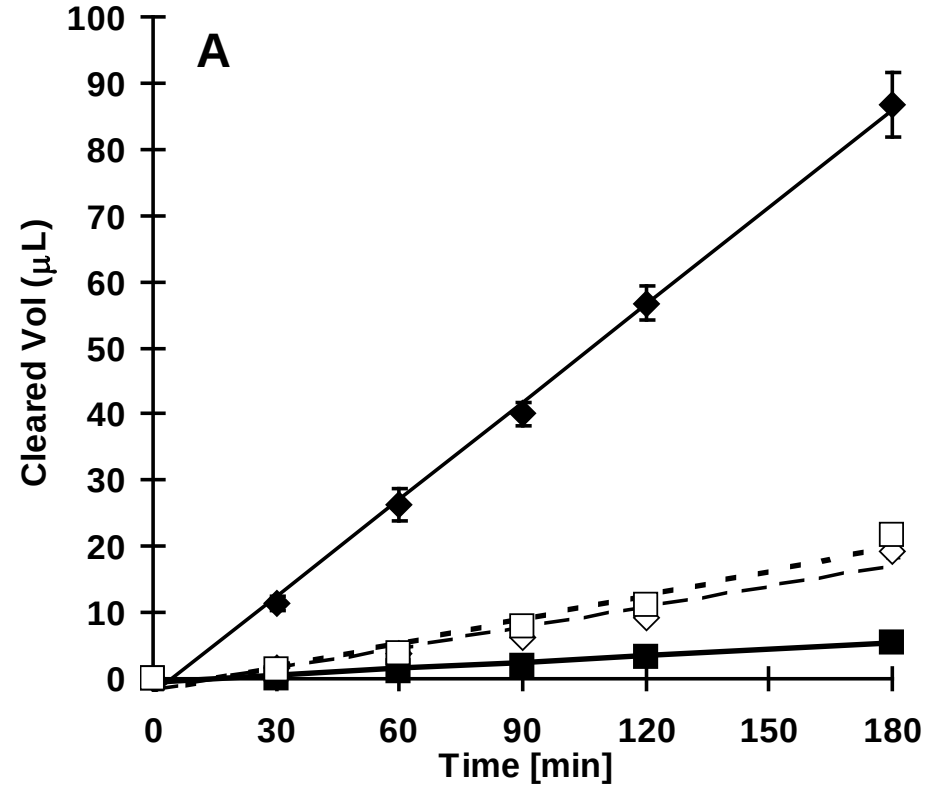

Concentration (µM)
22
20
18
16
14
12
10
8
6
4
2
0
-2
Std equation
y = (47526.05+(-.4912*x))/(1560.7 + x)
0
1.0×10^4
2.0×10^4
3.0×10^4
4.0×10^4
5.0×10^4
6.0×10^4
7.0×10^4
8.0×10^4
9.0×10^4
1.0×10^5
1.1×10^5
1.2×10^5
fluorescence intensity

Figure 2


A
Cleared Vol (μL)
100
90
80
70
60
50
40
30
20
10
0
0
30
60
90
120
150
180
Time [min]

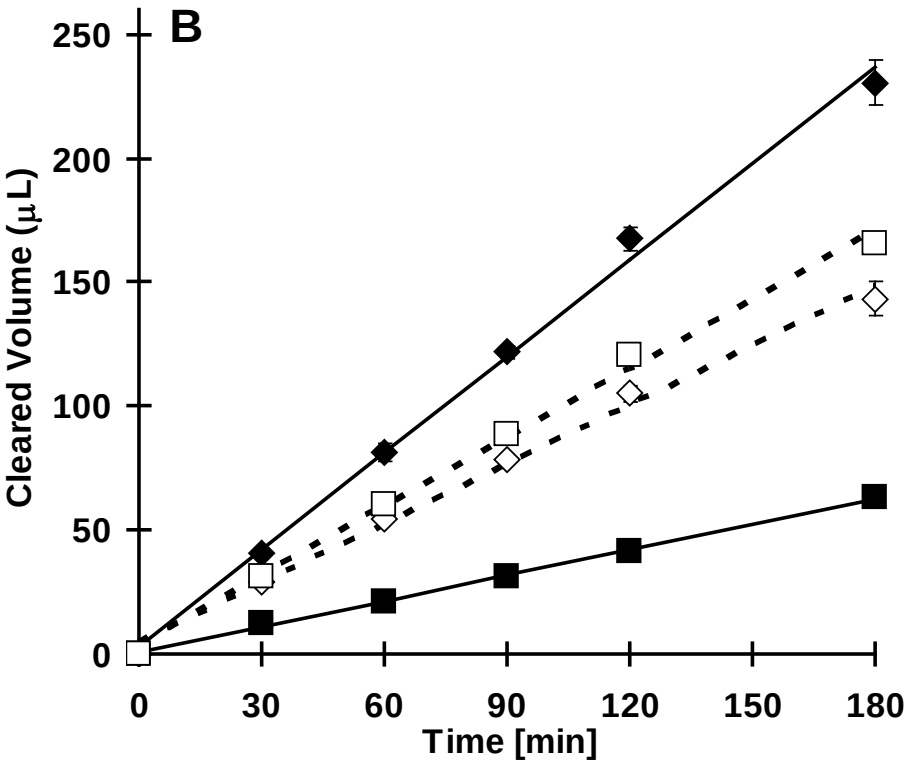

B
Cleared Volume (μL)
250
200
150
100
50
0
0
30
60
90
120
150
180
Time [min]

Figure 3

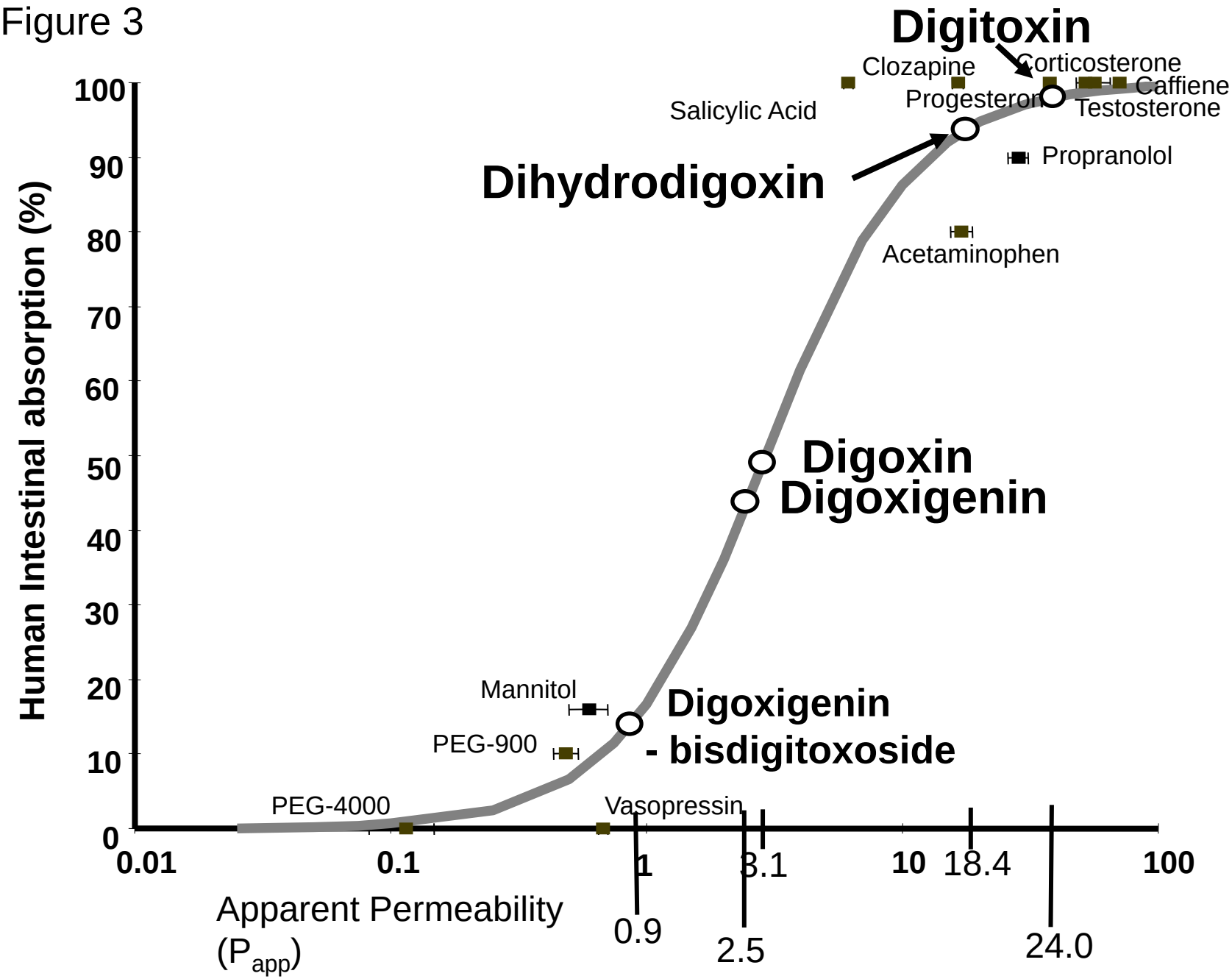

Digitoxin
Clozapine
Corticosterone
Caffiene
Progesteron
Testosterone
Salicylic Acid
Propranolol
Dihydrodigoxin
Acetaminophen
Digoxin
Digoxigenin
Mannitol
Digoxigenin
- bisdigitoxoside
PEG-900
PEG-4000
Vasopressin
Human Intestinal absorption (%)
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
3.1
10
18.4
100
Apparent Permeability
(P app)
0.9
2.5
24.0

Figure 4

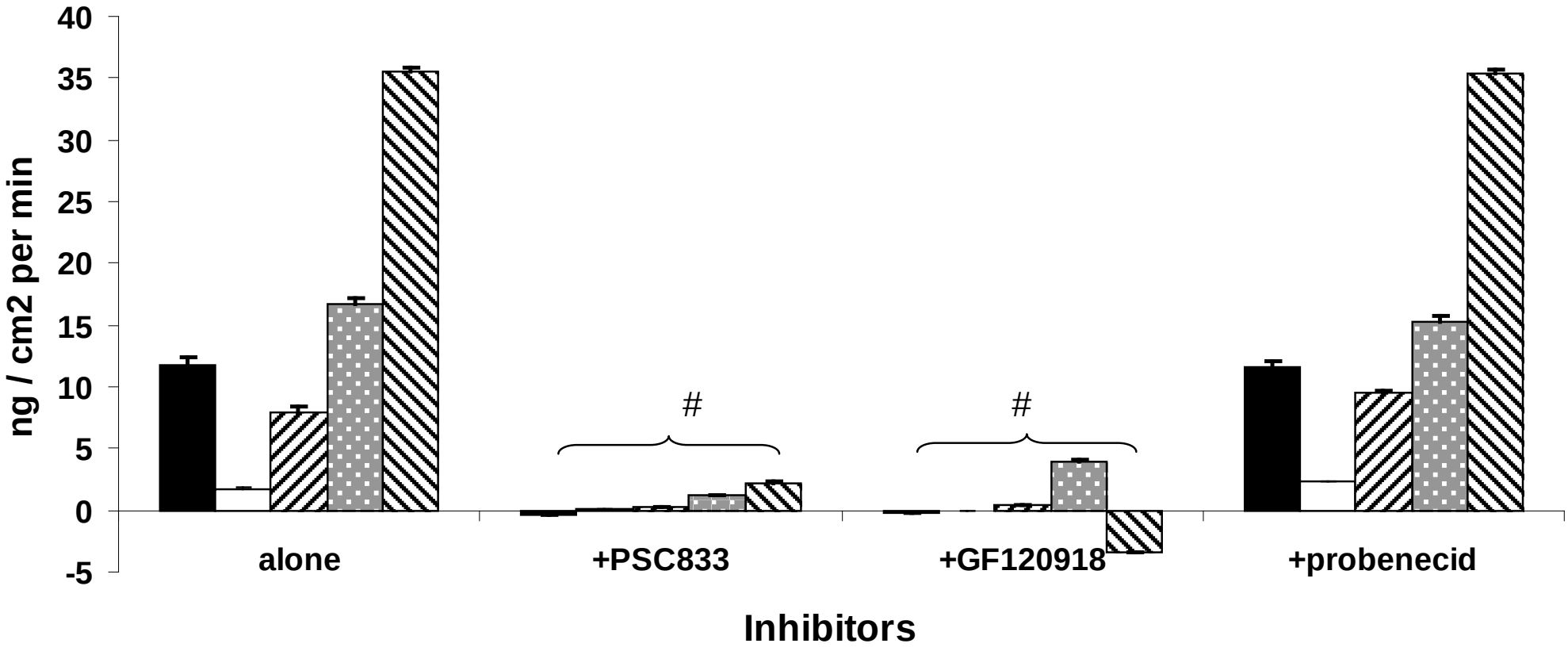
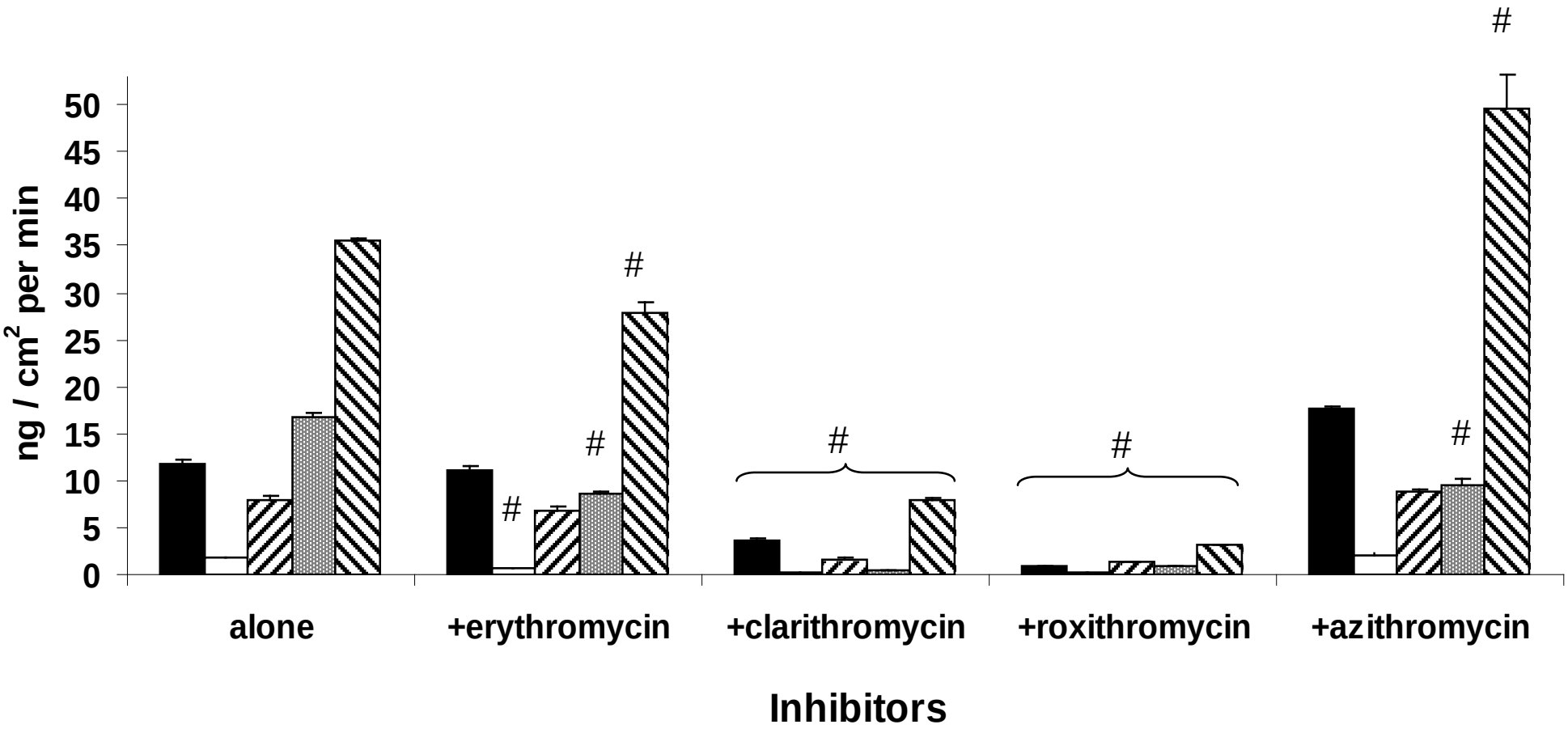

40
35
30
25
20
15
10
5
0
-5
ng / cm2 per min
#
#
alone
+PSC833
+GF120918
+probenecid
Inhibitors

Figure 5


ng / cm2 per min
0
5
10
15
20
25
30
35
40
45
50
#
alone
+erythromycin
+clarithromycin
+roxithromycin
+azithromycin
Inhibitors