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Full Paper

Inhibition of P-glycoprotein–Mediated Efflux of Digoxin and Its Metabolites by Macrolide Antibiotics

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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.

Keywords: 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, but 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

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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). Furthermore, it is known that 10% – 15% of the population harbor 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 (Ap) 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 (Castle Hill, Australia), while dihydrodigoxin and digoxigenin bis-digitoxoside were kindly donated by GlaxoSmithKline Australia Pty., Ltd. (Boronia, Australia). Cell culture reagents: phosphate-buffered saline (PBS), Hanks balanced salt solution (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, Australia), while the foetal calf serum (FCS) was obtained from the Australian Commonwealth Serum Laboratories (Parkville, Australia).

Erythromycin, azithromycin, and roxithromycin were purchased from Sigma-Aldrich (Castle Hill, Australia). Clarithromycin was extracted from Klacid® tablets from Abbott Australasia (Kurnell, 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 37°C incubator with 5% CO_2. Cells were incubated for 21 – 25 days to allow full maturation of the monolayer of cells. The TEER (transendothelial electrical resistance) 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 $\Omega \cdot cm^2$ 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 HBSS supplemented with both glucose (Ajax chemicals, Taren Point, Australia) and HEPES (Gibco BRL) to give final concentrations of 25 and 10 mM respectively. The pH was adjusted to 7.4 by using 1M NaOH. For pH 6.0 studies, 10 mM Bis-Tris (USB, Cleveland, OH, 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 was 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. Paclitaxel was used as a known P-gp substrate. The 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-h 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-gp 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 P-gp inhibitors PSC-833 (4 μM) or GF120918 (4 μM), 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 (Agilent, Chatswood, Australia) 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 (Perkin Elmer, Melbourne, Australia) injected 60 μL of sample through a Zorbax Stable bonded (SB) C_{18} column, 5-μm pores, 15 cm × 4.6 mm I.D. with mated guard column (Agilent). 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 min. Limits of detection, using 60-μL injections into the column, were 50, 100, 50, and 70 nM for digoxin, digitoxin, digoxigenin, and digoxigenin bis-digitoxoside, respectively.

Alphascreen dihydrodigoxin detection

Dihydrodigoxin, with a reduced lactone ring, exhibited negligible chromatographic qualities. Instead, it was detected via the patented Alphascreen technology system by Perkin Elmer. A digoxin detection kit was purchased, which we showed to have adequate cross reactivity to dihydrodigoxin and to be quantitative for our requirements (Fig. 1). We used anti-digoxin acceptor beads with biotinylated-digoxin binding to these acceptor beads and streptavidin 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, which 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. Fifteen microliter volumes of all test samples in duplicate were added to 384 well white walled μClear flat-bottomed plates (Greiner Bio-One; Interpath Services, Perth, Australia). A 5-μL aliquot of acceptor beads was then added and incubated at room temperature for 30 min in the dark. A 5-μL aliquot of donor beads was subsequently

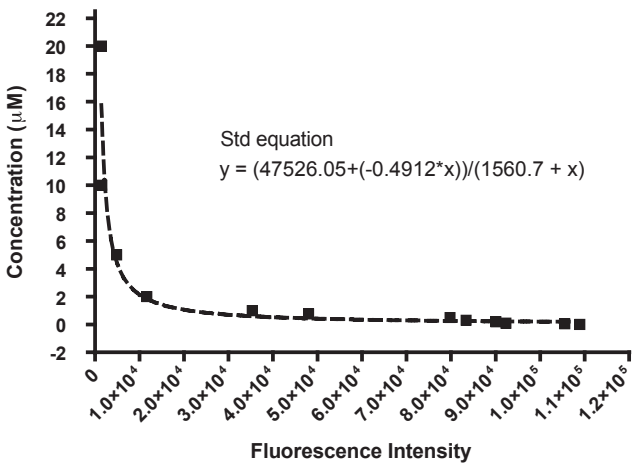

Fig. 1. Standard curve for competition assay using dihydrodigoxin to interfere with biotinylated digoxin in an Alphascreen digoxin detection kit after 60-min incubation of samples with donor beads.

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). 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 ± S.E.M. of 3 – 5 individual studies, standardized on individual protein concentrations. Significant differences between values were examined by 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

Bas-to-Ap transport was 15-fold greater than transport in the Ap-to-Bas direction for digoxin using 20-μM drug concentrations (Fig. 2a and Table 1). As both PSC-833 and GF120918 were able to neutralize the Bas-to-Ap 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

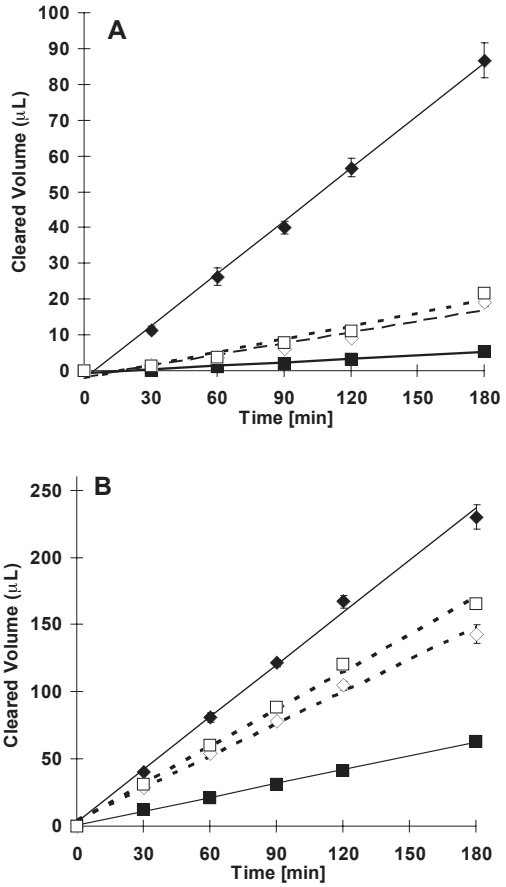

Fig. 2. Bidirectional transport of 20 μM digoxin (A) or digitoxin (B) through the Caco-2 CLEFF9 subclone. Ap-to-Bas direction (squares) and Bas-to-Ap direction (diamonds), without (closed symbols) and with (open symbols) the presence of 4 μM PSC-833, a potent P-gp inhibitor, on both sides of the membrane.

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-h transport studies in a Caco-2 sub clone (CLEFF) over-expressing P-gp

Inhibitor	Digoxin			Digoxigenin			Digoxigenin bis-digitoxoside		
	Ap-to-Bas $\times 10^{-6}$ cm/s	Bas-to-Ap $\times 10^{-6}$ cm/s	Ratio (B–A)	Ap-to-Bas $\times 10^{-6}$ cm/s	Bas-to-Ap $\times 10^{-6}$ cm/s	Ratio (B–A)	Ap-to-Bas $\times 10^{-6}$ cm/s	Bas-to-Ap $\times 10^{-6}$ cm/s	Ratio (B–A)
None	0.9 ± 0.1	13.6 ± 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	12.9 ± 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	15.0 ± 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$^{\#}$

Significant effect of inhibitor use in drug transport in either the Ap-to-Bas direction or the reverse direction is indicated with $^{\#}$ for $P < 0.05$ and $^{\#\#}$ for $P < 0.005$.

(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, but 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 (P_{app}) dropped from 3.1 to 0.9 × 10^{-6} cm/s, which equated to a probable drop in absorption from 50% to 13%, based on comparison with drugs of known human absorption profiles (27) (Fig. 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 gastrointestinal tract (28), has the highest P_{app} of the metabolites tested, although active efflux was a significant factor in reducing this high P_{app} from allowing dihydrodigoxin to cross the cell monolayers (Table 2).

Digitoxin was also shown to be a P-gp substrate in this system, although Bas-to-Ap transport was only 4-fold higher than Ap-to-Bas transport using 20 μM digitoxin (Fig. 2b and Table 2). The apparent permeability results for digitoxin were much higher than those for digoxin (24 × 10^{-6} cm/s compared to 3 × 10^{-6} cm/s) (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 (Fig. 3).

Dihydrodigoxin had the greatest Bas-to-Ap efflux at almost 61 × 10^{-6} cm/s (Table 2) with digoxigenin bis-digitoxiside a distant second with 15 × 10^{-6} cm/s (Table

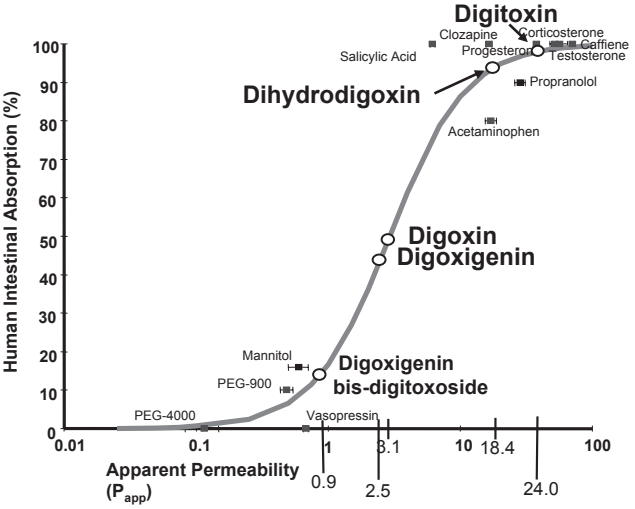

Fig. 3. In vitro permeability co-efficient as an estimate of human intestinal absorption. Once P-gp–mediated efflux was eliminated by use of P-gp–blocking agents, the remaining Ap-to-Bas values for digoxin and its analogues could be plotted on our previously established Caco-2 permeability (22) vs. human absorption comparison curve, as determined by Artursson and Karlsson (27), providing some indication of 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.

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 and digitoxin, and our rapidly transported P-gp substrate, the antineoplastic agent paclitaxel, after 3-h transport studies in a Caco-2 sub clone (CLEFF) over-expressing P-gp

Inhibitor	Dihydrodigoxin			Digitoxin			Paclitaxel		
	Ap-to-Bas ×10^{-6} cm/s	Bas-to-Ap ×10^{-6} cm/s	Ratio (B–A)	Ap-to-Bas ×10^{-6} cm/s	Bas-to-Ap ×10^{-6} cm/s	Ratio (B–A)	Ap-to-Bas ×10^{-6} cm/s	Bas-to-Ap ×10^{-6} cm/s	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$^{\#\#}$

Significant effect of inhibitor use in drug transport in either the Ap-to-Bas direction or the reverse direction is indicated with $^{\#}$ for $P < 0.05$ and $^{\#\#}$ for $P < 0.005$.

1). When examined as the net amount of drug transported in the Bas-to-Ap direction, after removal of the amount of drug transported in the Ap-to-Bas direction, as shown in Fig. 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 per min (Fig. 4). When compared to digoxigenin, with only 1.4 ng/cm^2 per min net efflux, then irrespective of its 3.5-fold higher efflux compared to its Ap-to-Bas 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 (Fig. 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 Bas direction transport of dihydrodigoxin and digoxigenin by 34% and 43%, respec-

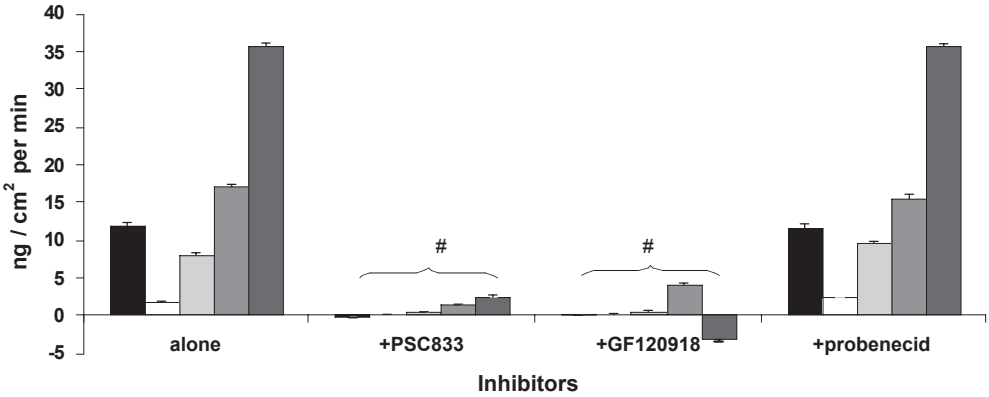

Fig. 4. Net transport rates in the efflux direction for 20 μM digoxin and metabolites/analogues using transport rates from Ap-to-Bas and Bas-to-Ap directions over a 3-h period in Caco-2 monolayers grown on Millicell polycarbonate filter membranes. Results shown equate to transport in the Bas-to-Ap direction 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. 1st column: digoxin, 2nd: digoxigenin, 3rd: digoxigenin bis-digitoxoside, 4th: digitoxin, 5th: dihydrodigoxin. Significance of inhibitor use in reducing efflux compared to glycoside alone is shown ($^{\#}$) where $P < 0.05$.

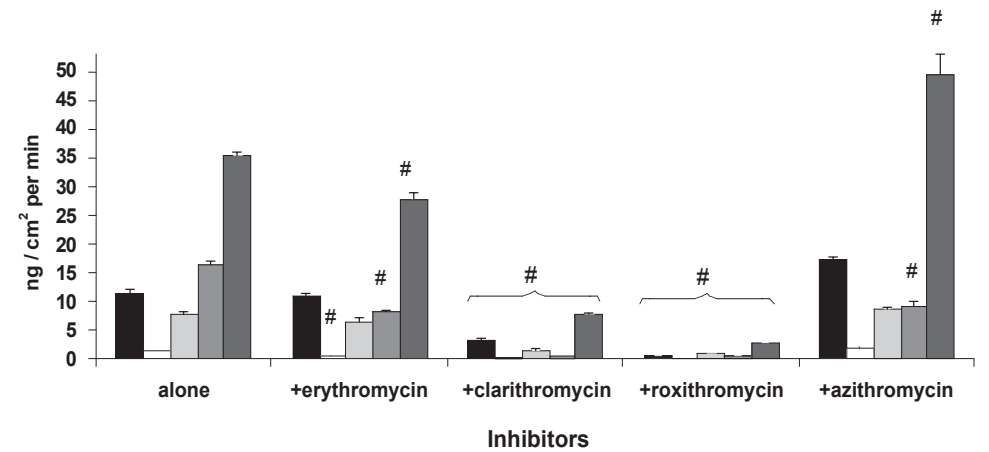

Fig. 5. Net transport rates in the efflux direction for 20 μM digoxin and metabolites/analogues using transport rates from Ap-to-Bas and Bas-to-Ap directions over a 3-h period in Caco-2 monolayers grown on Millicell polycarbonate filter membranes. Results shown equate to transport in the Bas-to-Ap direction 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 clarithromycin and azithromycin were used at 100 μM. 1st column: digoxin, 2nd: digoxigenin, 3rd: digoxigenin bis-digitoxoside, 4th: digitoxin, 5th: dihydrodigoxin. Significance of inhibitor use in reducing efflux compared to glycoside alone is shown ($^{\#}$) where $P < 0.05$.

tively, and reduced digitoxin efflux by over 40% (Fig. 5). Azithromycin was also able to reduce efflux for digitoxin, but unlike erythromycin, had a very limited ability to decrease the net Bas-to-Ap–directed transport of digoxin or any of its metabolites, making it the weakest of the inhibitors studied here (Tables 1 and 2, Fig. 5). In contrast, both clarithromycin and roxithromycin were potent P-gp inhibitors able to increase Ap-to-Bas transport of digoxin from $0.9 \pm 0.1 \times 10^{-6}$ to $3.3 \pm 0.3 \times 10^{-6}$ cm/s (Table 1), while also reducing Bas-to-Ap transport from $13.6 \pm 0.8 \times 10^{-6}$ to $7.5 \pm 0.4 \times 10^{-6}$ cm/s in the case of clarithromycin and $4.4 \pm 0.2 \times 10^{-6}$ cm/s 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 reduced it 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 Ap-to-Bas transport. However, due to these results being close to the 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 metabolizers of digoxin to dihydrodigoxin, which is subsequently excreted in the urine (28). Our study has shown that dihydrodigoxin 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, 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 DRPs, and these are thought to arise due to the anaerobic bacterium *E. 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 erythromycin increases plasma levels of digoxin, even though digoxin is not directly metabolized using these processes. Thus, the exact mechanism by which erythromycin increases digoxin concentrations is still unclear. 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 micromolar concentrations of clarithromycin reduced digoxin transcellular transport and increased cellular accumulation using kidney epithelial cell line monolayers (15). Furthermore, they were able to demonstrate reduced renal clearance of digoxin in a patient who was taking clarithromycin (200 mg 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 colleagues (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 that clarithromycin and erythromycin, each at 5 μM, had an efflux ratio of 22 and 8, 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. In a 15-year population-based, nested case-control study that investigated the association between hospitalization for digoxin toxicity and recent exposure to individual macrolide antibiotics, Gomes and others recently reported 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.

This study demonstrated that roxithromycin, clarithromycin, and to a lesser extent, erythromycin were able to inhibit P-gp efflux both for digoxin and a 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, while 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, our present

results indicate that azithromycin would be the drug of choice and it has previously been reported not to affect cytochrome P-450, unlike 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 commencing administration of a macrolide.

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