Stereoselective Interaction of Pantoprazole with ABCG2:

II. *In vitro* flux analysis

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Running title page

a) Stereoselective pantoprazole flux by ABCG2/rAbcg2

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d) Abbreviations:
   ABCG2/Abcg2: ATP binding cassette transporter family G member 2;
   PAN: pantoprazole
   P_{Ob}: observed permeability
   P_{SAE}, P_{SD} and P_{SPC}: permeability-surface area products for active apical efflux, passive diffusion and paracellular pathways, respectively
   T_{max} and K_{m}: maximum transport rate and the Michaelis constant, respectively
Abstract

(-) Pantoprazole (PAN) accumulated in rat milk stereoselectively and this accumulation was attributed to rAbcg2. In contrast, flux experiments at 25 µM showed that (+) PAN was preferentially transported by rAbcg2. The purpose of the current study was to comprehensively evaluate the transport of PAN isomers in empty-MDCKII and MDCKII cells expressing the human/rat (ABCG2/rAbcg2) isoforms at concentrations ranging from 3 to 200 µM. The apical to basolateral (A to B) and basolateral to apical (B to A) directional flux and the asymmetry efflux ratios (ER\textsubscript{a}) were virtually identical for both isomers in empty(mock transfected)-MDCKII monolayers, but were concentration dependent for both isomers in ABCG2 (human/rat)-MDCKII. Kinetic analysis using predicted cellular concentrations showed that (-) PAN had an 8-fold lower K\textsubscript{M} compared to (+) PAN for both rAbcg2 (0.25 vs 1.85 µM) and ABCG2 (0.6 vs 5.32 µM). (+) PAN had a 3-fold higher T\textsubscript{Max} compared to the (-) PAN for both rAbcg2 (7.86 vs 2.49 nmol/hr\text{*}cm\textsuperscript{2}) and ABCG2 (10.2 vs 3.29 nmol/hr\text{*}cm\textsuperscript{2}). Effective ABCG2 surface area permeability of (PS\textsubscript{AE,Eff}) of (-) PAN was 9,920 and 5,480 (µL/hr)/cm\textsuperscript{2} for rAbcg2 and ABCG2, respectively compared to the (+) PAN isomer (4,250 and 1,920 µL/hr\text{*}cm\textsuperscript{2}, respectively). These results indicate a stereoselective interaction of PAN with similar kinetic parameters for both human and rat ABCG2. (-) PAN is a better substrate than (+) PAN for ABCG2/rAbcg2 and provide a rationale for the preferential accumulation of (-) PAN into rat milk.
Introduction

Efflux transporters such as ABCB1, ABCC2 and ABCG2 are localized in numerous tissues throughout the body and play important roles in drug absorption, distribution, metabolism and excretion (Giacomini et al., 2010). These transporters can be major determinants of the pharmacokinetic, efficacy and safety profiles of drugs. Flux across a polarized cell monolayer, such as Caco-2 or Madin-Darby Canine Kidney II (MDCKII) cell line overexpressing transporters have become one of the more popular in vitro methods to characterize substrate – transporter interactions (Balimane et al., 2004; Balimane and Chong, 2005). However, a mechanistic understanding and quantitative description of transport or inhibition data from Transwells remains limited (Kalvass and Pollack, 2007; Sun and Pang, 2008).

ABCG2 is expressed on the apical surface of lactating mammary epithelial cells (Jonker et al., 2005) and facilitates the accumulation of substrate drugs in milk including: nitrofurantoin, cimetidine, topotecan, acyclovir, dietary carcinogens, ranitidine and ciprofloxacin (Oo et al., 1995; McNamara et al., 1996; Gerk et al., 2001; Oo et al., 2001; Jonker et al., 2005; Merino et al., 2005; Merino et al., 2006; van Herwaarden et al., 2007; Vlaming et al., 2009). Although diffusion plays a major role in drug appearance in milk, ABCG2 clearly contributes to the accumulation of its substrates in milk. Pantoprazole (PAN) is an ABCG2 substrate, and yet, one clinical report indicated that it has a very low milk to serum (M/S) ratio (Plante et al., 2004). There is no clear rationale for such a low ratio for a good ABCG2 substrate.

PAN is marketed as a racemic mixture (Figure 1) and differences in the overall disposition of the two isomers have been reported both in man (Tanaka et al., 2001) and in rats (Xie et al., 2005). In a companion paper (Wang and McNamara, 2011), the disposition of PAN isomers in rat milk was explored and revealed a clear stereoselective difference associated with rAbcg2.
Milk to serum ratio of (-) PAN was 2.5 fold greater than that of (+) PAN and the M/S of (-) PAN was affected to a greater degree by the administration of GF120918 than was the M/S of (+) PAN. In contrast, *in vitro* experiments suggested that the rate of (+) PAN flux across a rAbcg2-MDCKII monolayer was greater than (-) PAN, at a donor concentration of 25 μM, which is lower than the previously reported Km estimated using Transwell flux experiments (Breedveld et al., 2004). The main purpose of the current paper is to fully explore this apparent contradiction by characterizing the transport of PAN isomers in monolayers consisting of rat (rAbcg2) and human ABCG2 overexpressing MDCKII cells. The first objective was to describe differences in the interaction between PAN isomers and rAbcg2/ABCG2. The second objective was to examine any interspecies difference between rAbcg2 and ABCG2. In analysis of this data, a functional model was developed to quantify kinetic parameters for flux experiments involving an apical efflux pump.
Material and methods

Chemicals and materials. PAN mixture (50:50) was purchased from Wyeth (Philadelphia, PA) and the isomers of PAN were a gift from Altana Pharma AG (Konstanz, Germany). Cell culture mediums were obtained from Invitrogen (Carlsbad, CA). All the organic solvents [high-performance liquid chromatography (HPLC) grade] were purchased from Thermo Fisher Scientific (Waltham, MA), and all of the other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless specified.

Over-expressing rAbcg2/ABCG2 in MDCK-II cell lines were used in the transport studies. The establishment of rat Abcg2 or empty vector (pcDNA3.1) alone in MDCKII was described previously (Wang et al., 2008). Human ABCG2 or empty vector (pcDNA3.1) alone in MDCKII was previously established in our lab. Briefly, the pcDNA3.1 plasmid construct alone (empty vector) and the pcDNA3.1 plasmid containing wild-type ABCG2 were prepared for transfection. MDCKII cell transfection was performed at 50% confluence with the lipid-based FuGENE 6® transfection reagent at a 3:1 ratio per manufacturer’s protocol. Transfected cells were then selected through the addition of 800 μg/mL genecitin to the parent cell line media (MEM containing glutamax, 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin). Further selection and functional studies were performed by flow-cytometry. The expression and localization of ABCG2 were confirmed by western blot and confocal microscopy.

PAN isomers transport in rat Abcg2 or human ABCG2 in MDCK-II cells. Briefly, rat/human ABCG2 or empty vector in MDCKII cells were seeded on microporous membrane filters (3.0-μm pore size, 24-mm diameter; Transwell 3414; Corning Inc., Corning, NY) at a density of 1.0 x 10^6 cells/well. Cells were grown for 4 days to achieve transepithelial electrical resistance >200 Ω·cm², and medium was replaced every other day. Before the experiment, the medium at both the apical and basolateral side of the monolayer was replaced with 2 ml of OptiMEM.
medium (Invitrogen) without serum and either apical or basolateral side was loaded with 3, 10, 30, 50 and 200 µM of individual PAN isomers as well as 0.2 µCi/ml [³H]mannitol (Perkin Elmer, Hebron, KY). Cells were incubated at 37°C in 5% CO₂. 50-µl aliquots were collected to assess the paracellular flux of [³H] mannitol into the opposite compartment; layers restricted mannitol transport to <1% of the total radioactivity per hour. For PAN isomer transport, 140-µl aliquots were taken at 0.5 and 1 h. Samples were stored at -80°C until the time of analysis by HPLC assay.

**PAN assay.** Concentrations of PAN were assayed by HPLC (system) using a Luna RP18 125 x 4.0-mm column (Phenomenex, Torrance, CA), elution with 40% acetonitrile/60% 10 mM potassium phosphate buffer (pH 7.2) at 0.5 ml/min, and UV absorption at 290 nm. Aliquots of PAN in Opti-MEM medium were directly injected onto the HPLC. The standard curve range was from 7.8 to 2000 ng/ml. All the standard curves showed an intraday and interday variability of <10% and $r^2 > 0.999$.

**Apparent Permeability.** The observed permeability ($P_{obs}$, (µL/hr)/cm²) of PAN isomers or paracellular marker was determined by calculating its initial transfer rate ($\frac{\Delta X}{\Delta t}$, pmol/hr) across the cell layer and dividing by the surface area ($A$, cm²) of the Transwell and the initial concentration ($C_0$, pmol/mL) in the donor chamber (Eq.1). PAN isomers transport data in rat Abcg2 or human ABCG2 were expressed as mean ± S.D.

$$PS_{obs} = \frac{\Delta X}{A * C_0}$$

**Eq. 1**

**Theoretical and mathematical analysis.** Assuming the substrate does not interact with endogenous transporters, passive diffusion permeability ($PS_D$) is the same across both basolateral and apical membranes, no cellular metabolism, and rapid intracellular diffusion; a three
compartment kinetic model of transcellular flux across a monolayer system (ABCG2–MDCKII) is established in Figure 2. Briefly, the driving force for drug efflux from basolateral to apical side includes passive diffusion ($PS_D$) across both basolateral and apical membranes, paracellular transport ($PS_{PC}$) between cells and active transport efflux on apical side ($PS_{AE}$) associated with the transporter. The model assumes the substrate does not interact with any endogenous transporters, passive diffusion permeability ($PS_D$) is the same across both membranes, there is no cellular metabolism, and rapid intracellular diffusion. Substrate flux rates in three compartments can be described by the following set of differential equations (Eq.2-4).

$$\frac{dX_B}{dt} = C_C(PS_D) - C_B(PS_D) + (C_A - C_B)PS_{PC}$$ \hspace{1cm} \text{Eq. 2}

$$\frac{dX_A}{dt} = C_C(PS_D + PS_{AE}) - C_A(PS_D) + (C_B - C_A)PS_{PC}$$ \hspace{1cm} \text{Eq. 3}

$$\frac{dX_C}{dt} = C_A(PS_D) + C_B(PS_D) - C_C(2PS_D + PS_{AE})$$ \hspace{1cm} \text{Eq.4}

where $X_B$, $X_A$ and $X_C$ are the mass of drug in the basolateral, apical and cellular compartment; $C_C$, $C_B$ and $C_A$ are the concentration of drug in the cellular, basolateral and apical compartment, respectively; $PS_{AE}$, $PS_D$ and $PS_{PC}$ are the permeability-surface area products for active apical efflux, passive diffusion and paracellular pathways, respectively.

Equation 2 and 3 can be further refined to describe the initial permeability surface area product from B to A ($PS_{OBS,B\rightarrow A}$) or A to B ($PS_{OBS,A\rightarrow B}$)

$$PS_{OBS,B\rightarrow A} = \frac{dX_{A,B\rightarrow A}}{dt}C_B^0 = \frac{(PS_D)(PS_D + PS_{AE})}{(2PS_D + PS_{AE})} + PS_{PC}$$ \hspace{1cm} \text{Eq. 5}

$$PS_{OBS,A\rightarrow B} = \frac{dX_{B,A\rightarrow B}}{dt}C_A^0 = \frac{(PS_D)(PS_D)}{(2PS_D + PS_{AE})} + PS_{PC}$$ \hspace{1cm} \text{Eq. 6}
Paracellular permeability of mannitol was used as a surrogate for PAN PS<sub>PC</sub>.

PS<sub>D</sub> was estimated from the PAN flux in parent MDCKII (PS<sub>AE</sub>=0) by rearranging Equation 5 or 6 as

\[
PS_D = 2 \left[ PS_{obs,B \rightarrow A} or \frac{PS_{obs,A \rightarrow B}}{} - PS_{PC} \right] \tag{Eq. 7}
\]

Permeability associated with ABCG2 apical efflux (PS<sub>AE</sub>) was estimated from B to A or A to B PAN flux in rAbcg2/ABCG2-MDCKII rearranging 5 or 6 to yield 8 or 9, respectively.

\[
PS_{AE,B \rightarrow A} = \frac{PS_D^2 - 2PS_D(PS_{obs,B \rightarrow A} - PS_{PC})}{(PS_{obs,B \rightarrow A} - PS_{PC}) - PS_D} \tag{Eq. 8}
\]

\[
PS_{AE,A \rightarrow B} = \frac{PS_D^2 - 2PS_D(PS_{obs,A \rightarrow B} - PS_{PC})}{PS_{obs,A \rightarrow B} - PS_{PC}} \tag{Eq. 9}
\]

Initial transfer flux of from B to A (assuming C<sub>A</sub>=0) can be rewritten (Eq.10) to estimate C<sub>C</sub>.

\[
C_C = \frac{C_B(PS_D)}{(2PS_D + PS_{AE})} \tag{Eq. 10}
\]

where PS<sub>AE</sub> is approximated as in Eq. 8 and 9.

ER<sub>α</sub> is defined as the ratio of the initial rate of B to A flux divided by the initial rate of A to B.

\[
ER_\alpha = \frac{\frac{dX_{obs,B \rightarrow A}}{dt}}{\frac{dX_{obs,A \rightarrow B}}{dt}} \tag{Eq. 11}
\]

**Kinetic Parameter (K<sub>M</sub> and T<sub>Max</sub>) Estimation.** The saturation transport kinetics of a drug efflux by rAbcg2 or ABCG2 at the apical surface can be modeled by Eq. 12; where T<sub>max</sub> is the maximum transport rate and K<sub>m</sub> the Michaelis constant. The PS<sub>Obs</sub>, PS<sub>D</sub>, PS<sub>PC</sub> of each PAN
isomer was estimated experimentally and used to estimate $PS_{AE}$ (Eq.8 and Eq.9). The intracellular concentrations ($C_C$) were estimated by Eq. 10. Non-linear regression using GraphPad Prism (5.04) was employed to estimate $K_m$ and $T_{max}$.

$$PS_{AE} = \frac{T_{Max}}{(K_M + C_C)} \quad \text{Eq. 12}$$

An effective apical efflux permeability ($PS_{AE,\text{Eff}}$), observed when $C_C \ll K_M$ (similar to intrinsic clearance) would be defined as

$$PS_{AE,\text{Eff}} = \frac{T_{Max}}{K_M} \quad \text{Eq. 13}$$

**Statistical Analysis.** A multivariate analysis (SPSS, IBM) was performed to assess the influence of concentration, direction and isomer on $PS_{Obs}$. A sum-of-squares F-test of kinetic parameters ($T_{Max}$ and $K_M$) was performed using GraphPad Prism (5.04).
Results

**Pantoprazole isomers transported in rAbcg2 or ABCG2.** The overall flux of PAN isomers was evaluated for both rAbcg2 and ABCG2 to assess stereoselectivity and species differences. The expression level and functional viability of stably expressing rat Abcg2 (Wang et al., 2008) and human ABCG2 (Empey et al., 2006) cDNA or vector alone in MDCKII cell lines has been established previously. The mass transport of PAN isomers was studied in empty-MDCKII and rAbcg2-MDCKII cells as shown in Figure 3. For both of the PAN isomers in empty-MDCKII monolayers, $P_{\text{OBS}}$ was slightly higher in the B to A direction and increasing with concentration ($p>0.05$). In contrast, the transport of PAN isomers exhibited a strong directional flux in rAbcg2-MDCKII cell line. There were marked differences between the PAN isomers transport at 30 and 50 μM for both directions. At lower (3 and 10 μM) concentrations, the difference between the isomers became negligible. At the highest donor concentration (200 μM), the flux of both isomers was equivalent to that observed in the empty-MDCKII cells. Parallel results were observed in empty-MDCKII and human ABCG2-MDCKII cell line transport study (Figure 4). The directional transport of the two isomers confirmed that PAN is a substrate for both rAbcg2 and ABCG2.

**Permeability parameters ($P_{\text{OBS}}, P_{\text{D}}$ and $P_{\text{PC}}$) and asymmetry efflux ratios ($E_{R_a}$).** To ascribe specific membrane properties to individual isomers, the apparent permeability parameters ($P_{\text{OBS}}, P_{\text{D}}, P_{\text{PC}}$) and asymmetry efflux ratios (Table 1 and 2) were obtained for both rAbcg2 and ABCG2. Assuming the transport permeability of mannitol is similar to PAN isomer transport across paracellular pathway ($P_{\text{PC}}$), values were small and consistent across studies (range 0.51 to 3.54 (μL/hr)/cm$^2$). $P_{\text{PC}}$ values were somewhat lower for ABCG2-MDCKII compared to empty-MDCKII. Using $P_{\text{OBS}}$ for PAN transport in empty-MDCKII, $P_{\text{D}}$ was
estimated from Equation 7 at different concentrations and for both directions (Table 1 and 2). The mean (±SD) $PS_D$ values were 244 (±18) and 287 (±16) (μL/hr)/cm² for the rat and human mock transfections, respectively.

$PS_{obs}$ of PAN isomers in empty-MDCKII cell lines were similar for both directions at different concentrations resulting in $ER_a$ values approximating unity for all of the experiments. By contrast, the $PS_{obs}$ of both PAN isomers in the transfected cell lines were markedly different with respect to direction, favoring strong basolateral to apical directional flux at lower PAN concentration. The apparent flux were similar for the two isomers, but with important differences. At the lowest PAN concentration, the $ER_a$ of (-) PAN was nearly double that of (+) PAN for both Abcg2-MDCKII (Table 1) and ABCG2-MDCKII (Table 2). As donor concentration increased, PAN flux decreased for the basolateral to apical direction and increased for the apical to basolateral direction in Abcg2/ABCG2-MDCKII (Table 1 and 2). As a result, the $ER_a$ for both PAN isomers in both rat and human ABCG2 transfected cell lines decreased with increasing donor concentration until approaching a value approaching unity (Table 1 and 2).

**Cellular concentration profile.** Recognizing that the driving force of transport of an apical efflux transporter (i.e., Abcg2/ABCG2) is cellular and not donor concentration, simulated cellular concentrations of PAN isomers corresponding to donor concentrations (Figure 5) were estimated using Equation 10 and based on initial estimates of permeability parameters ($PS_{obs}$, $PS_D$ and $PS_{PC}$, Table 1 and 2) of PAN isomers and apparent $PS_{AE}$ values. At low donor concentration (3 μM), cellular concentration were estimated to be 3.2% of donor concentration for (-) PAN and 5.6% for (+) PAN for rAbcg2 and 5.5% for (-) PAN and 11% for (+) PAN for
ABCG2. At high donor concentration (above 50 μM), cellular concentration was predicted to approach ½ of donor concentration (Figure 5).

**Kinetic parameters \(K_m\) and \(T_{\text{max}}\) for rat Abcg2 and human ABCG2.** To ascribe specific kinetic parameters for ABCG2/rAbcg2 for the individual isomers, the profile of apparent \(PS_{AE}\) (both directions) as a function of estimated cellular PAN concentration (Figure 5) was analyzed as in Figure 6. These estimates of \(PS_{AE}\) become less accurate as \(PS_{Obs}\) approaches its boundaries (very high or low donor concentrations). Kinetic parameters for the interaction of each isomer with Abcg2 and ABCG2 are shown in Table 3. The (-) isomer of PAN has an 8-fold lower \(K_m\) compared to the (+) PAN for both rAbcg2 (0.25 vs 1.85 μM, \(p<0.01\)) and ABCG2 (0.6 vs 5.32 μM, \(p<0.01\)), indicating a higher affinity of rAbcg2/ABCG2 for the (-) isomer. The (+) isomer of PAN has a roughly 3-fold higher \(T_{\text{max}}\) compared to the (-) PAN for both rAbcg2 (7.86 vs 2.49 nmol/hr*cm², \(p<0.01\)) and ABCG2 (10.2 vs 3.29 nmol/hr*cm², \(p<0.01\)). rAbcg2 had a lower \(K_m\) (a higher affinity) for the PAN isomers compared to human ABCG2, but comparable \(T_{\text{max}}\) values. The overall permeability associated with rAbcg2 and ABCG2 (\(PS_{AE}\), effective transport clearance at low PAN concentrations) was calculated as the ratio of \(T_{\text{max}}\) over \(K_m\). \(PS_{AE}\) was twice that for the (-) PAN (9,920 and 5,480 (μL/hr)/cm² for rAbcg2 and ABCG2, respectively) compared to the (+) PAN (4,250 and 1,920 L/hr*cm² for rAbcg2 and ABCG2, respectively).

**Modeling overall Permeability (\(PS_{Obs}\)).** To assess the validity of the deconvolution process, the overall \(PS_{Obs}\) was modeled as a function of donor concentration (Figure 7) by utilizing the \(PS_D\) values from the empty–MDCKII (Table 1 and 2) as well as \(PS_{pc}\) from the transfected cell lines (Table 1 and 2) and the ABCG2 related kinetic parameters (Table 3). The comprehensive relationships (Equations 5 and 6) were effective in modeling PAN flux in both
directions of (-) PAN ($r^2 = 0.979$) and (+) PAN ($r^2 = 0.988$) in ABCG2-MDCKII and (-) PAN ($r^2 = 0.985$) and (+) PAN ($r^2 = 0.986$) in rAbcg2-MDCKII.
Discussion

Our laboratory and others have demonstrated the importance of ABCG2 in drug accumulation in milk. In a companion paper (Wang and McNamara, 2012), we examine the transfer of PAN into rat milk that clearly demonstrated ABCG2 associated accumulation of (-) PAN in milk, however, initial *in vitro* result showed that (+) PAN had greater transport than (-) PAN in Abcg2-MDCKII cell line at 25 µM. In current study, the flux of individual PAN isomers were examined at concentrations from 3 to 200 µM to more rigorously characterize the stereoselectivity of ABCG2.

PAN isomers have identical physiochemical properties; hence, diffusion properties of PAN isomers should be indistinguishable. The diffusion permeability (PS\(_D\)) of PAN isomers transport in empty MDCKII cell line (parent cell line) at five different concentrations was virtually identical (*Table 1 and 2*). There was essentially no directional transport difference observed indicating limited interaction of PAN isomers with endogenous transporters in the parent MDCKII cell line. Consistent with the literature (Breedveld et al., 2004), both PAN isomers are substrates for rAbcg2/ABCG2. Moreover, both isomers display a concentration dependent flux profile in both human and rat ABCG2-MDCKII cell line (Figure 3 and 4). The stereoselective interaction of PAN with rAbcg2/ABCG2 was clear at 30 and 50 µM, but not evident at lower or higher concentrations.

The model (*Figure 2*) provides a clear context for the interpretation of the isomer flux. At low concentrations, permeability across the apical membrane is so rapid (PS\(_{AE}\) is so large relative to PS\(_D\)), that the rate limiting steps for PAN flux are associated with diffusion (PS\(_D\)) across the basolateral membrane and paracellular flux (PS\(_{PC}\)). Under these concentrations, overall flux becomes independent of rAbcg2/ABCG2 associated PS\(_{AE}\). As rAbcg2/ABCG2 transport
becomes saturated at higher concentrations (PS_{AE} is decreased), the contribution of diffusion across the apical membrane to overall flux approaches transport across empty-MDCKII. Only at the intermediate donor concentrations (Figure 3 and 4) does the contribution and stereoselectivity of rAbcg2/ABCG2 to PAN transport become evident as measured by overall flux. Often flux studies, including those examining stereoisomer differences, are carried out at low substrate concentrations in high expressing clones to optimize the opportunity for detecting a response. Under such conditions, it is unlikely that stereoselective interactions would be reported since diffusion (i.e., across the opposite membrane or paracellular) is rate limiting and dominates the overall flux.

Taken together the flux data from empty and rAbcg2/ABCG2-MDCKII cell lines were consistent with the three compartment kinetic model shown in Figure 2. The model comprises apparent permeability due to passive diffusion on either apical or basolateral side (PS_{D}), paracellular permeability through tight junction between cells (PS_{PC}) and ABCG2-mediated active transport permeability on the apical side (PS_{AE}). In order to analyze the data several critical assumptions were made.

First, mannitol was assumed to be an adequate marker for PAN PS_{PC}. This assumption was particularly critical in establishing the limits of apical to basolateral flux at low PAN concentrations. Under low substrate concentrations in high expressing clones, good substrates can have PS_{AE} values that are very large (e.g., PS_{AE}>10^{*}PS_{D} as is the case for PAN). In practical terms, most of the substrate that enters the cell from the apical side is fluxed back out to the donor compartment and more of the drug reaching the basolateral side is associated with paracellular flux (PS_{PC}).
The second assumption was related to the estimation of cellular concentration. Ideally, intracellular unbound concentrations should be measured; however, this is technically very challenging. Relating kinetic parameters ($V_{\text{Max}}$ and $K_M$) to donor concentrations was problematic. Initial attempts to fit flux to permutations of the Michaelis–Menten equation as a function of donor concentration resulted in fits that did not mimic the observed data. Fitting flux to donor concentrations suggested an apparent cooperative interaction (i.e., Hill coefficient) with the transporter (Pan et al., 2007). The driving force for an apical efflux transporter, such as rAbcg2/ABCG2, is cellular and not donor concentration. The apparent cooperativity reflected the fact that there is relatively more cellular drug concentration as donor concentration increases due to saturation of the efflux transporter (Figure 5). Intracellular concentrations were not measured in the current study, but were predicted by the model (Figure 2) using the apparent $P_{\text{AE}}$ as well as estimates of $P_{\text{D}}$ and $P_{\text{PC}}$.

These intracellular concentrations were then used to estimate kinetic parameters. The estimated $K_M$ values ranged from 0.25 to 5.3 μM, whereas the donor concentrations around the donor concentration necessary to achieve these concentrations (Figure 7) were in the 20 to 100 μM range. The (-) PAN had a $K_M$ value that was 8-fold lower of the (+) isomer indicating (-) PAN had an 8-fold higher affinity for ABCG2 as well as rAbcg2. The capacity ($T_{\text{Max}}$) was 70% lower for (-) PAN for ABCG2 and rAbcg2. For typical therapeutic dosing, serum unbound concentrations are likely to be below 0.1 μM; hence, cellular concentrations would also be expected to be well below the $K_M$. The effective permeability ($P_{\text{AE, Eff}}$ listed in Table 3) would be more than two fold greater for (-) PAN compared to the (+) isomer. Therefore, (-) PAN is predicted to be a better substrate for rAbcg2/ABCG2 than (+) PAN. In the companion paper (Wang and McNamara, 2012), the M/S for (-) PAN in rats was 1.36 and decreased markedly in response to GF120918 (an inhibitor of rAbcg2/ABCG2) when compared to 0.54 for
(+.) PAN. Hence, the stereoselective effect of PAN with rAbcg2 \textit{in vitro} manifested itself in a measureable \textit{in vivo} difference in disposition, i.e., accumulation in rat milk.

Isolating the various parameters (PS$_D$, PS$_{PC}$, T$_{Max}$ and K$_M$) allowed the overall PS$_{Obs}$ to be successfully modeled (\textbf{Figure 7}). PAN flux in the B to A direction at low PAN concentration was very rapid across the apical membrane and overall flux was limited by the rate of diffusional flux across the basolateral membrane plus diffusion via the paracellular pathway. PAN flux in the A to B direction was limited largely to paracellular diffusion since the counter flux associated with PS$_{AE}$ was so efficient that virtually none of the drug which entered the cell from the apical surface fluxed to the basolateral compartment through the cell itself.

The current \textit{in vitro} flux data for PAN isomers would suggest that the stereoselectivity in rat milk accumulation (Wang and McNamara, 2012) would also hold for human exposure as well since ABCG2 was also two fold difference in PS$_{AE, Eff}$ for PAN isomers (\textbf{Table 3}). The one case report did not examine individual isomers (Plante et al., 2004). The current work did not provide insights into the observed clinical report of a low M/S for pantoprazole (Plante et al., 2004).

In conclusion, there was stereoselective transport of PAN isomers in monolayers of both rat (rAbcg2) and human ABCG2 expressed in MDCKII cell line with the (-) isomer possessing a greater affinity. A mechanism based model was used to quantify differences in kinetic parameters between the two isomers which were not evident by examining flux at any single concentration. The unique characteristic of PAN isomers interaction with ABCG2 provided ideal experimental evidence to model drug transport across a cell monolayer. These kinetic parameters also provided insights as to the extent of differences that arise for \textit{in vivo} studies. The current work also provided insights into the species difference in ABCG2 transport.
Footnote

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Authorship Contributions

Participated in research design: Lipeng Wang and Patrick J. McNamara

Conducted experiments: Lipeng Wang

Contributed new analytic tools: Lipeng Wang

Performed data analysis: Lipeng Wang and Patrick J. McNamara

Wrote or contributed to the writing of the manuscript: Lipeng Wang, Markos Leggas, Philip E. Empey and Patrick J. McNamara
Reference


DMD #41616

Wang L, Leggas M, Goswami M, Empey PE, and McNamara PJ (2008) N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolino(ylethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918) as a chemical ATP-binding cassette transporter family G member 2 (Abcg2) knockout model to study nitrofurantoin transfer into milk. Drug Metab Dispos 36:2591-2596.


Figure 1 The structure of pantoprazole (PAN) isomers.

Figure 2 Theoretical model of PAN flux across rAbcg2/ABCG2-MDCKII cell monolayer in a Transwell system. PS_D: passive diffusion; PS_PC: permeability of paracellular transport; PS_AE: active efflux mediated by ABCG2 on apical side.

Figure 3 Mean (± SD, n=3) PAN isomer transport in pcDNA3.1-MDCKII (left panels) or rAbcg2-MDCKII cells (right panels) at initial donor PAN concentrations of 3, 10, 30, 50 and 200μM. The isomers were studied individually; squares indicate (+) PAN, circles represent (-) PAN, closed symbols indicate basolateral to apical flux and open symbols represent apical to basolateral flux.

Figure 4 Mean (± SD, n=3) PAN isomer transport in pcDNA3.1-MDCKII (left panels) or human ABCG2-MDCKII cells (right panels) at initial donor PAN concentrations of 3, 10, 30, 50 and 200μM. The isomers were studied individually; squares indicate (+) PAN, circles represent (-) PAN, closed symbols indicate basolateral to apical flux and open symbols represent apical to basolateral flux.

Figure 5 Predicted cellular concentration of PAN (Equation 10) relative to donor concentration based on PS_D and PS_PC described in Table 1 and 2 as well as approximated values of PS_AE (Equation 8 and 9) were used. Squares indicate (+) PAN, circles represent (-) PAN, open symbols indicate rAbcg2-MDCKII and closed symbols represent ABCG2-MDCKII. The line of identity is provided as a reference.

Figure 6 Apparent apical efflux permeability (PS_AE, Equation 12) as a function of simulated cellular PAN concentration (Equation 10). Panel A: rat Abcg2; Panel B: human ABCG2-MDCKII cell monolayer; Squares indicate (+) PAN, circles represent (-) PAN, open symbols
indicate basolateral to apical \( PS_{AE} \) and closed symbols represent apical to basolateral \( PS_{AE} \). The initial estimates of \( PS_{AE} \) (Equations 8 and 9) were used to generate cellular concentration (Equation 10) and these concentrations were used in the final estimation process. Both axes were log transformed for easier visualization; fitting was performed using untransformed pooled data from both directional \( PS_{AE} \). The solid and dashed lines are the fitted results for the (−) PAN and (+) PAN isomers, respectively.

**Figure 7** Overall permeability (\( PS_{obs} \)) as a function of PAN donor concentration. **Panel A:** rat Abcg2; **Panel B:** human ABCG2-MDCKII cell monolayer; Squares indicate (+) PAN, circles represent (−) PAN, closed symbols indicate basolateral to apical flux and open symbols represent apical to basolateral flux. The simulation line represents Equation 5 and 6 for basolateral to apical and apical to basolateral flux, respectively. Fitted parameters \( T_{MAX} \) and \( K_M \) (Table 3) were used to generate cellular concentration (Equation 10) and simulation of overall \( PS_{obs} \) together with \( PS_D \) and \( PS_{PC} \) as described in Table 1 and 2. Model limits for basolateral to apical (\( PS_D + PS_{PC} \)), apical to basolateral (\( PS_{PC} \)) and empty cell line flux are presented for reference points. The small square box presents the range of PAN concentration in serum in rat or pharmacological concentration in human, respectively.
Table 1 The observed asymmetry efflux ratios ($\text{ER}_\alpha$), permeability parameters ($\text{PS}_{\text{PC}}$) of mannitol and permeability parameters ($\text{PS}_{\text{obs}}$ and $\text{PS}_D$) of PAN isomers transport in empty and rAbcg2-MDCKII as a function of PAN donor concentration (PS units: $\mu$L/hr/cm$^2$).

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<th>Isomer</th>
<th>Parameter</th>
<th>Direction</th>
<th>3μM</th>
<th>10μM</th>
<th>30μM</th>
<th>50μM</th>
<th>200μM</th>
<th>Mean</th>
<th>SD</th>
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Table 2 The observed asymmetry efflux ratios (ER_α), permeability parameters (PS_{PC}) of mannitol and permeability parameters (PS_{obs} and PS_{D}) of PAN isomers transport in empty and ABCG2-MDCKII as a function of PAN donor concentration. (PS units: (μL/hr)/cm²).

<table>
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<th>Isomer</th>
<th>Parameter</th>
<th>Direction</th>
<th>3μM</th>
<th>10μM</th>
<th>30μM</th>
<th>50μM</th>
<th>200μM</th>
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Table 3 The fitted mean (± SE from nonlinear fitting) kinetic parameters (K<sub>m</sub> and T<sub>max</sub>) in both rAbcg2 and ABCG2. Overall permeability associated with rAbcg2/ABCG2 (PS<sub>AE,Eff</sub>, effective transport permeability at low PAN concentrations) was calculated as the ratio of T<sub>max</sub> over K<sub>m</sub>.

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<th>(+) PAN</th>
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<td>Mean (±SE)</td>
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<td>7.86 ± 2.20</td>
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<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
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<td>1.85 ± 0.66</td>
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Figure 1

(-) PAN

(+ ) PAN
Figure 3

Empty-MDCKII

- (-) PAN A to B
- (+) PAN B to A
- (+) PAN A to B

PAN transferred (pmol)

Time (Hour)

3µM

PAN transferred (pmol)

Time (Hour)

10µM

PAN transferred (pmol)

Time (Hour)

30µM

PAN transferred (pmol)

Time (Hour)

50µM

PAN transferred (pmol)

Time (Hour)

200µM

PAN transferred (pmol)

Time (Hour)

rAbcg2-MDCKII

- (-) PAN A to B
- (+) PAN B to A
- (+) PAN A to B

PAN transferred (pmol)

Time (Hour)
Figure 4

Empty-MDCKII

ABCG2-MDCKII

3 μM

10 μM

30 μM

50 μM

200 μM
Figure 5

[Graph showing the relationship between Donor Concentration (µM) and Cellular Concentration (µM) for different groups indicated by symbols: (+) PAN rAeg2, (-) PAN rAeg2, (+) PAN ABCG2, (-) PAN ABCG2. Unity line is also shown.]
Figure 6

A. rAbcg2

B. ABCG2
Figure 7

A. rAbeg2

B. ABCG2

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