The role of P-glycoprotein in the absorption of novel antimalarial drugs

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Running head: In vitro anti-malarial P-glycoprotein efflux
Abstract

Bi-directional transport of four novel antimalarial compounds was determined using Caco-2 cell monolayers. P-glycoprotein mediated efflux was greatest for pyronaridine (5-20 µM) and low for naphthoquine (5 µM). At 20 µM naphthoquine, net efflux was blocked suggesting saturation of the transporter. Piperaquine and dihydroartemisinin were not transported by the system.

Key Words: MDR1, CLEFF9 subclone, piperaquine, pyronaridine, naphthoquine, dihydroartemisinin
Permeability-glycoprotein (P-gp) ATP-dependent transporters influence the passage of many drugs across epithelial barriers in the intestine, brain, liver and kidney (1) and may alter their pharmacokinetic and/or pharmacodynamic properties. Several antimalarial drugs are substrates and/or inhibitors of P-gp (9,10,15). In the intestine, P-gp has a secretory function which can contribute to low bioavailability and substrate-inhibitor interactions (14). The antimalarials dihydroartemisinin, naphthoquine, piperaquine and pyronaridine are highly lipid-soluble with LogP (log octanol:water partition coefficient) values of 2.6, 6.16, 6.16 and 4.98, respectively (ACD I Lab Software V 8.14 for Solaris®, 1994-2006 ACD/Labs, Toronto, Canada). Evidence suggests that dihydroartemisinin and piperaquine have oral bioavailabilities <50% (6,7) while pyronaridine may interfere with P-gp-mediated transport (10,11). We therefore investigated whether these four drugs are P-gp substrates and whether P-gp-mediated efflux explains the apparently low bioavailabilities of dihydroartemisinin and piperaquine.

Drug transport was studied in an in vitro gastrointestinal model using a monolayer of a CLEFF9 subclone of human Caco-2 cells with high P-gp-mediated efflux (4) and previously-reported experimental protocols (3-5). Briefly, cells were seeded onto 0.6 cm² polycarbonate filters in 24-well plates and grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) for 3 weeks. Following incubation in buffered Hank’s balanced salt solution (HBSS) with or without specific efflux inhibitors for 30 min at 37°C, trans-epithelial electrical resistance (TEER) was measured and assay medium/inhibitors were placed in the receiver chambers. Antimalarial drugs were added to the donor chamber of each well. The apical and basolateral chambers received 0.3 and 0.6 ml medium, respectively. TEER measurements were repeated at the conclusion of the studies to ensure continued monolayer integrity.
Naphthoquine was assayed using high performance liquid chromatography (HPLC) with UV detection at 260 nm after separation through an Eclipse XDB-C8 column (Agilent Technologies, Forest Hill, Australia) with a mobile phase of 22% acetonitrile, 10% methanol and 68% 20 mM KH$_2$PO$_4$ with 11 mM triethylamine and 0.1% trifluoroacetic acid pumped at 1.2 mL/min. The limit of detection (LOD) was 30 nM. Pyronaridine was assayed by HPLC with detection at 277 nm after separation through an XTerra C18, 100 x 4.6, 3.5 µm column (Waters Associates, Milford, MA) with acetonitrile:buffer (80 mM KH$_2$PO$_4$, 22 mM triethylamine HCl and 0.1% trifluoroacetic acid, pH 2.95) (18:82) pumped at 1.1 mL/min (LOD 60 nM). Piperaquine was assayed by HPLC with detection at 345 nm using the XTerra C18 column and a modified acetonitrile:buffer (8:92) (12) (LOD 120 nM). A dual-label β-counter was used to detect $^3$H-dihydroartemisinin and $^{14}$C-mannitol, the latter added during transport studies to monitor monolayer integrity.

Transport data are summarized in the Table. Naphthoquine (5 µM; pH 7.4 on both sides of monolayer) had a moderate 1.7-fold basolateral-to-apical efflux ($P<0.01$). The potent P-gp inhibitors 4 µM PSC-833 and 4 µM GF120918 abolished the net efflux gradient, suggesting a role for P-gp in naphthoquine efflux. At 20 µM, transport stabilized at 25 x 10$^{-6}$ cm/sec in both directions ($P>0.08$), suggesting saturation. Concentrations <5 µM were not tested due to the low naphthoquine LOD. A pH gradient was applied across the monolayer to simulate P-gp uptake in the upper gastrointestinal tract (10 mM 2-Morpholinoethanesulfonic acid (MES)-buffered HBSS, pH 6 in apical and 10mM HEPES buffered HBSS, pH 7.4 in basolateral chambers). Naphthoquine efflux increased by 83-fold that in the uptake direction. P-gp inhibitors only partially reduced the efflux gradient (to 30- to 40-fold), indicating that ionization factors and/or H$^+$ antiporter activity underlie rapid efflux with a pH 6.0/7.4 differential.
As the predicted pKa for naphthoquine is 8.02 (ACD I Lab Software), pH gradient
differences may reflect 88% and 99.5% ionization of the drug at pH 7.4 and 6.0,
respectively. The LogD (log octanol:water partition coefficient for a given pH) for
naphthoquine changes from 3.1 to 4.0 between pH 6.0 and 7.4, which should not
significantly alter lipid bilayer permeability. These differences in ionization and LogD
are unlikely to influence the efflux gradient.

Pyronaridine showed greater flux with an apical-basolateral pH gradient of
6.0/7.4. The calculated pKa of pyronaridine is 10.22 with interpolated LogD of 0.6 and
1.7 at pH 6.0 and 7.4, respectively (ACD I Lab Software), suggesting that changes in
membrane partitioning could be structurally-related when the pH gradient conditions are
applied. Pyronaridine has been shown to inhibit P-gp-mediated efflux (10,11) but was
not thought to be transported itself (10). Our data suggest that pyronaridine is a weak P-
gp substrate, as the 1.8-fold efflux gradient for 20 µM ($P<0.001$) increased to 6.7-fold at
5 µM ($P<0.003$). Moreover, 20 µM completely abolished efflux during co-incubation
with the P-gp blockers PSC-833 or GF120918 (Figure, Table).

Neither piperaquine nor dihydroartemisinin exhibited P-gp-mediated transport.

While chloroquine, mefloquine, quinine and pyronaridine are weak P-gp substrates, they
inhibit the efflux of other P-gp substrates (8,9,11,14). Although our experiments did not
assess whether the present antimalarial drugs behave in the same way, our results
suggest limited P-gp-mediated pyronaridine and naphthoquine efflux at low micromolar
concentrations. As P-gp-mediated efflux appeared to increase as the drug concentration
was lowered, organs such as the brain with extensive P-gp expression might not
accumulate these drugs, as the greatest plasma concentrations after single dosing rarely
exceed 0.3-0.4 µM for either naphthoquine (13) or pyronaridine (2). Since oral doses of
each drug are 400-600 mg, concentrations at the gastrointestinal wall are likely to be at
least in the mid micromolar range, where initial absorption would occur based on the
physicochemical properties of the drugs and the fact that P-gp mediated efflux would be
saturated. As the luminal concentration falls to the low micromolar range, P-gp-
mediated efflux could attenuate continued oral absorption of these compounds,
especially pyronaridine.

In summary, it is unlikely that P-gp has a significant role in the gastrointestinal
absorption of piperaquine and dihydroartemisinin, while the effect of P-gp on the
absorption of naphthoquine will be limited due to saturation at low doses. Pyronaridine
may exhibit variable absorption on the basis of significant P-gp mediated efflux. It is
interesting that oral piperaquine has proved effective in clinical trials (6) despite our
inability to detect significant transport in our in vitro model. Lack of transport suggests
that a drug should not be given orally. Future piperaquine cell transport studies should
address this apparent inconsistency.

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References


with P-glycoprotein in an immortalised rat Brain capillary endothelial cell line, GPNT. Biochim Biophys Acta **1524**:212-219.


Figure caption: Bidirectional transport of 20 µM pyronaridine through the Caco-2 CLEFF9 subclone. Apical to basolateral direction (□, ■) and basolateral to apical direction (◊, ♦), with (■, ♦) and without (□, ◊) the presence of 4 µM PSC-833, a potent P-glycoprotein inhibitor, on both sides of the membrane.
Table: Transport rates of 5 and 20 µM antimalarial drugs through Caco-2 sub clone (CLEFF9) monolayers in both apical (Ap) to basolateral (Bas) and Bas to Ap directions. Each value represents the mean ± SEM of triplicate independent determinations.

<table>
<thead>
<tr>
<th>Anti-malarial drug</th>
<th>pH in Ap / Bas chambers</th>
<th>Concentration (µM) ± efflux modifiers</th>
<th>Ap → Bas (cm/s) (x 10^-6)</th>
<th>Bas → Ap (cm/s) (x 10^-6)</th>
<th>Fold difference (net flow direction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthoquine</td>
<td>7.4 / 7.4</td>
<td>5</td>
<td>13.3 ± 0.6</td>
<td>22.6 ± 1.9 *</td>
<td>1.7 (efflux)</td>
</tr>
<tr>
<td></td>
<td>7.4 / 7.4</td>
<td>5 + 4 µM PSC-833</td>
<td>22.3 ± 1.3 *</td>
<td>22.2 ± 2.8</td>
<td>1.0 (no net flux)</td>
</tr>
<tr>
<td></td>
<td>7.4 / 7.4</td>
<td>5 + 4 µM GF120918</td>
<td>15.1 ± 0.8 *</td>
<td>15.7 ± 0.6 *</td>
<td>1.0 (no net flux)</td>
</tr>
<tr>
<td></td>
<td>7.4 / 7.4</td>
<td>5 + 25 µM MK571</td>
<td>15.6 ± 0.8 *</td>
<td>25.7 ± 2.0 *</td>
<td>1.7 (efflux)</td>
</tr>
<tr>
<td></td>
<td>6.0 / 6.0</td>
<td>5</td>
<td>12.9 ± 0.8 *</td>
<td>37.0 ± 1.9 *</td>
<td>2.9 (efflux)</td>
</tr>
<tr>
<td></td>
<td>6.0 / 7.4</td>
<td>5</td>
<td>1.8 ± 0.1</td>
<td>151.5 ± 16.8 *</td>
<td>82.8 (efflux)</td>
</tr>
<tr>
<td></td>
<td>6.0 / 7.4</td>
<td>5 + 4 µM PSC-833</td>
<td>3.1 ± 0.1 *</td>
<td>94.8 ± 4.8 *</td>
<td>30.8 (efflux)</td>
</tr>
<tr>
<td></td>
<td>6.0 / 7.4</td>
<td>5 + 4 µM GF120918</td>
<td>2.2 ± 0.1</td>
<td>81.8 ± 4.8 *</td>
<td>38.1 (efflux)</td>
</tr>
<tr>
<td></td>
<td>7.4 / 7.4</td>
<td>20</td>
<td>27.3 ± 0.6</td>
<td>22.4 ± 2.0</td>
<td>0.8 (no net flux)</td>
</tr>
<tr>
<td></td>
<td>7.4 / 7.4</td>
<td>20 + 4 µM PSC-833</td>
<td>23.8 ± 0.4</td>
<td>18.8 ± 2.0</td>
<td>0.8 (no net flux)</td>
</tr>
<tr>
<td>Compound</td>
<td>pH 1</td>
<td>pH 2</td>
<td>pH 3</td>
<td>pH 4</td>
<td>pH 5</td>
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</tr>
<tr>
<td>Pyronaridine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4 / 7.4 5</td>
<td>4.2 ± 0.6</td>
<td>28.0 ± 3.4 #</td>
<td>6.7 (efflux)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4 / 7.4 5 + 4 μM PSC-833</td>
<td>1.7 ± 0.0 *</td>
<td>12.4 ± 1.2 *#</td>
<td>7.3 (efflux)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4 / 7.4 5 + 4 μM GF120918</td>
<td>3.7 ± 0.2</td>
<td>16.7 ± 0.5 *#</td>
<td>4.5 (efflux)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0 / 7.4 5</td>
<td>3.8 ± 0.1</td>
<td>52.9 ± 3.0 #</td>
<td>13.9 (efflux)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0 / 7.4 5 + 4 μM GF120918</td>
<td>4.0 ± 0.1</td>
<td>26.7 ± 0.5 *#</td>
<td>6.7 (efflux)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4 / 7.4 20</td>
<td>10.3 ± 0.3</td>
<td>18.8 ± 0.3 #</td>
<td>1.8 (efflux)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4 / 7.4 20 + 4 μM PSC-833</td>
<td>15.2 ± 0.3</td>
<td>16.3 ± 1.1</td>
<td>1.1 (no net flux)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4 / 7.4 20 + 4 μM GF120918</td>
<td>11.5 ± 0.6</td>
<td>13.2 ± 0.7</td>
<td>1.1 (no net flux)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4 / 7.4 20 + 500 μM probenecid</td>
<td>12.8 ± 0.6</td>
<td>22.4 ± 0.5 #</td>
<td>1.8 (efflux)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0 / 7.4 20</td>
<td>1.7 ± 0.0</td>
<td>32.1 ± 2.0 #</td>
<td>19.0 (efflux)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piperaquine</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>7.4 / 7.4 20</td>
<td>0.0 ± 0.6</td>
<td>0.1 ± 0.1 #</td>
<td>na (detection too low)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4 / 7.4 20 + 4 μM PSC-833</td>
<td>0.9 ± 0.1</td>
<td>0.4 ± 0.0 #</td>
<td>0.4 (uptake)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>7.4 / 7.4</td>
<td>1</td>
<td>32.0 ± 2.6</td>
<td>21.9 ± 1.7[^#]</td>
<td>0.7 (uptake)</td>
</tr>
<tr>
<td>---------------------</td>
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</tr>
<tr>
<td></td>
<td>7.4 / 7.4</td>
<td>1 + 4 µM PSC-833</td>
<td>30.8 ± 1.5</td>
<td>21.1 ± 2.4[^#]</td>
<td>0.7 (uptake)</td>
</tr>
<tr>
<td></td>
<td>7.4 / 7.4</td>
<td>20</td>
<td>38.6 ± 5.0</td>
<td>29.9 ± 0.6</td>
<td>0.8 (no net flux)</td>
</tr>
</tbody>
</table>

[^#]: significant difference between (Bas to Ap) and (Ap to Bas) transport for the particular set of drug transport conditions (p<0.05)

[^*]: significant difference between drug transport in a particular direction for a given concentration and the equivalent transport with the addition of an efflux pump inhibitor (P<0.05).