

Passive permeability is a crucial parameter in choosing the right assay to detect the interaction of compounds with ABCB1 (Pgp) and ABCG2 (BCRP)

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INTRODUCTION

The Pgp (MDR1, ABCB1) and BCRP (MXR, ABC-P, ABCG2) are two ABC transporters that are similar in many respects. Both proteins are expressed in several (and similar) pharmacological barriers, both transport a wide variety (and partially overlapping) substrates and both transporters are involved in the multidrug resistance phenomenon. *In vitro* transporter assays are routinely used to detect the interaction of compounds with ABCB1 (Pgp), while the importance of ABCG2 (BCRP) is just gaining wider acceptance. There are a plethora of tools available, however, these assays often give conflicting results. We hypothesized that some of the contradicting data are caused by the different passive permeability of the test compounds. The substrate binding site of ABC transporters is located within the plasma membrane or the cytoplasm. Therefore, the low permeability compounds would not reach the site of interaction in the cellular assays. Also, in case of highly permeable substrates in the monolayer assay or in the vesicular transport assay the rate of the passive process might be significantly higher than the active transport thereby making the transporter interactions undetectable.

METHODS and RESULTS – ABCB1 (Pgp) studies

We screened a diverse chemical library of 1296 compounds and selected high ($P_{app} > 50 \cdot 10^{-6}$ cm/s) and low ($P_{app} < 5 \cdot 10^{-6}$ cm/s) permeability ones as detected by HDM-PAMPA that interacted with ABCB1 as detected by the ATPase assay. We then assayed the compounds with the Calcein assay and the Caco-2 monolayer efflux assay using GF120918 as inhibitor of ABCB1. Table 1. shows the assay systems used, assay conditions and determined parameters. We calculated the ratio of Calcein IC_{50} and ATPase K (Calcein IC_{50} /ATPase K) and the ratio of A-B permeability for Caco-2 cells in the presence and absence of GF120918 (Caco-2 $P_{app} +GF/-GF$). We correlated these calculated parameters to passive permeability (HDM-PAMPA P_{app}).

High permeability compounds did not exhibit GF120918 sensitive A-B permeability in the Caco-2 assay indicating that the passive permeability significantly exceeded the rate of the active transport for these molecules. Yet, these "false negative" compounds all inhibited the transport of calcein AM in the Calcein assay highlighting the possibility of ABCB1 mediated drug-drug interactions caused by these compounds. As most ABCB1 interacting compounds have high passive permeability the frequency of "false negatives" could be very high for ABCB1 monolayer efflux assays.

Most of the low permeability compounds (10 out of 11) exhibited GF120918 sensitive A-B permeability in the Caco-2 assay. The apparent affinity of these compounds was significantly greater in the calcein assay than in the ATPase assay indicating that in this cellular assay the intracellular concentration of these compounds was lower than the concentration applied to the test buffer (figure 1).

METHODS and RESULTS – ABCG2 (BCRP) studies

We selected known ABCG2 substrates (estrone-3-sulfate, prazosin, topotecan, sulfasalazine, methotrexate, leflunomide) and inhibitors (Ko143 and Ko134). The ATPase assay utilizing mammalian membranes detected the interaction of the compounds with ABCG2. All substrates stimulated, while the inhibitors inhibited the baseline vanadate sensitive ATPase activity of the membrane preparations (figure 2). We could detect the ATP dependent transport of estrone-3-sulfate, methotrexate and sulfasalazine (figure 3), while we could not detect the transport of leflunomide (data not shown). All compounds tested showed interaction in the indirect vesicular transport assay utilizing methotrexate as transported substrate at 100 μ M. In indirect cellular assays (Hoechst assay and prazosin accumulation assay) prazosin, leflunomide, Ko143 and Ko134 showed up as inhibitors while no inhibition was observed for topotecan, methotrexate, sulfasalazine or estrone-3-sulfate. We measured or calculated the apparent permeability of these compounds and found that similarly to the ABCB1 studies the results of the different assays showed strong correlation with passive permeability (table 2).

| Assay | Test compound concentration(s) | Determined parameters |
|--|---|--|
| HDM-PAMPA | 100 μ M | HDM-PAMPA P_{app} |
| ATPase assay | 8 concentration points (3-fold dilution) starting at 100 or 300 μ M | Apparent affinity to primary binding site (ATPase K) |
| Calcein assay | 8 concentration points (3-fold dilution) starting at 100 or 300 μ M | IC_{50} (Calcein IC_{50}) |
| Caco-2 (A-B) assay in the absence of GF120918 | 10 μ M or 100 μ M | P_{app} Caco-2 (A-B) -GF |
| Caco-2 (A-B) assay in the presence of 1 μ M GF120918 | 10 μ M or 100 μ M | P_{app} Caco-2 (A-B) +GF |

Table 1. Assays used and determined parameters in ABCB1 *in vitro* correlation studies.

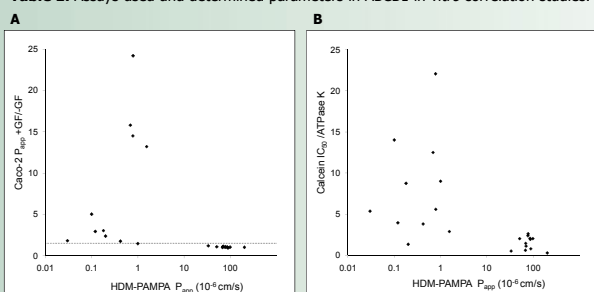


Figure 1. (A) Correlation between HDM-PAMPA P_{app} and Caco-2 $P_{app} +GF/-GF$. Cutoff (1.5) is also shown. (B) Correlation between HDM-PAMPA P_{app} and Calcein IC_{50} /ATPase K. See METHODS and RESULTS for further details.

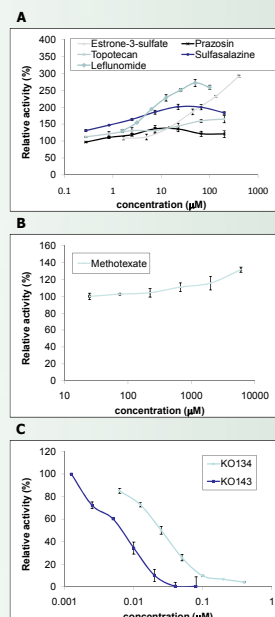


Figure 2. Stimulation and inhibition of the ABCG2 ATPase activity by substrates (A and B) and inhibitors (C) of ABCG2.

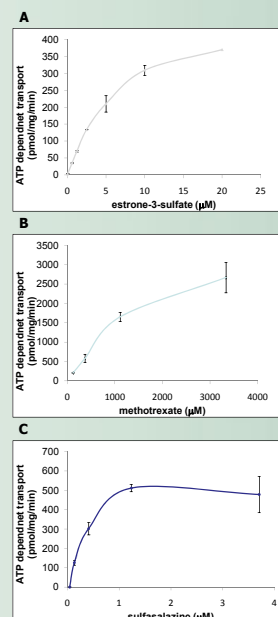


Figure 3. Direct ABCG2 vesicular transport of estrone-3-sulfate (A), methotrexate (B) and sulfasalazine (C).

| Compound (concentration) | Indirect vesicular transport assay | Indirect cellular accumulation assays | | |
|---------------------------------|------------------------------------|---|---|--|
| | | Inhibition of ATP dependent MTX transport (%) | Inhibition of Hoechst 33342 efflux (% of Ko143) | Inhibition of Prazosin efflux (% of Ko143) |
| Prazosin (100 μ M) | 99.6 +/- 7.9 | 104 +/- 11.5 | N.A. | 3.13 |
| Leflunomide (100 μ M) | 92.4 +/- 3.6 | 61.1 +/- 3.5 | N.D. | 363.1 [#] |
| Ko143 (1 μ M) | 98.8 +/- 4.8 | 100 +/- 3.7 | 100 +/- 4.7 | 60.25 [#] |
| Ko134 (1 μ M) | 94.8 +/- 7.8 | 98 +/- 4.8 | 97 +/- 5.3 | 75.85 [#] |
| Topotecan (100 μ M) | 80.2 +/- 14.7 | 1.1 +/- 5.8 | 4.1 +/- 1.5 | 0.61 |
| Methotrexate (100 μ M) | N.A. | 4.5 +/- 3.9 | 2.4 +/- 1.6 | 0.02 |
| Sulfasalazine (100 μ M) | 100 +/- 7.5 | -4.3 +/- 7.3 | 1.3 +/- 1.2 | 0.03 |
| Estrone-3-sulfate (100 μ M) | N.D. | 1.7 +/- 6.3 | 2.4 +/- 3.2 | 0 |

Table 2. Inhibition of ATP dependent MTX transport, Hoechst 33342 efflux, prazosin efflux and passive permeability measured in the HDM-PAMPA assay or estimated from molecular structure ([#]) for the test compounds. False negative results are shaded.

CONCLUSIONS

- The ATPase assay and the Calcein assay readily detected the ABCB1 interaction with highly permeable compounds, while the monolayer efflux assay did not.
- The monolayer efflux assay and the ATPase assay readily detected the ABCB1 interaction with low permeability compounds, while there was an increase in apparent affinity in the Calcein assay.
- The ATPase assay and the indirect vesicular transport assay readily detected both low and high permeability ABCG2 interacting compounds.
- The transport of very low permeability (charged) ABCG2 substrates can be detected in the direct vesicular transport assay, while we could not detect the transport of high permeability substrates using this method.
- Cellular assays used did not indicate the interaction of low permeability ABCG2 substrates with the transporter.

References:

- 1: Oliver von Richter, Hristos Glavinas, Peter Krajcsi, Stephanie Liehner, Beate Siewert, Karl Zech (2007) A novel screening strategy to identify ABCB1 substrates and inhibitors *Manuscript submitted*
- 2: Glavinas H, Kis E, Pal A, Kovacs R, Jani M, Vagi E, Molnar E, Banskagi S, Kele Z, Janaky T, Bathori G, von Richter O, Koomen GJ, Krajcsi P (2007) ABCG2 (BCRP/MXR) ATPase assay – a useful tool to detect drug – transporter interactions *Drug Metab Dispos.* 35(9):1533-42.