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In Vitro P-glycoprotein Inhibition Assays for Assessment of Clinical Drug Interaction Potential of New Drug Candidates: A Recommendation for Probe Substrates.

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Running Title: Probe Substrates for In Vitro Pgp Inhibition Assays

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Nonstandard abbreviations: Pgp = P-glycoprotein, IC_{50} = concentration required for 50% inhibition; K = the concentration of inhibitor required for 50% increase in the prazosin A → B rate; A→B = Apical to basolateral; B→A = Basolateral to apical; B→A/A→B ratio = $P_{app\ B\rightarrow A}/P_{app\ A\rightarrow B}$; MDCK = Madin Darby canine kidney cells; P_{app} = apparent permeability

Abstract

As modulation of P-glycoprotein (Pgp) through inhibition or induction can lead to drug-drug interactions by altering intestinal, CNS, renal or biliary efflux, it is anticipated that information regarding the potential interaction of drug candidates with Pgp will be a future regulatory expectation. Therefore, to be able to utilize *in vitro* Pgp inhibition findings to guide clinical drug interactions studies, the utility of five probe substrates (calcein-AM, colchicine, digoxin, prazosin and vinblastine) was evaluated by inhibiting their Pgp-mediated transport across MDR1-MDCKII monolayers with 20 diverse drugs having various degrees of Pgp interaction (e.g., efflux ratio, ATPase, and calcein-AM inhibition). Overall, the rank order of inhibition was generally similar with IC_{50} values typically within 3- to 5-fold of each other. However, several notable differences in the IC_{50} values were observed. Digoxin and prazosin were the most sensitive probes (e.g., lowest IC_{50} values), followed by colchicine, vinblastine and calcein-AM. Inclusion of other considerations such as a large dynamic range, commercially available radiolabel, and a clinically meaningful probe makes digoxin an attractive probe substrate. Therefore, it is recommended that digoxin be considered as the standard *in vitro* probe to investigate the inhibition profiles of new drug candidates. Further, this study demonstrates that it may not be necessary to generate IC_{50} values with multiple probe substrates for Pgp as is currently done for cytochrome P450 3A4. Finally, a strategy integrating results from *in vitro* assays (efflux, inhibition and ATPase) is provided to further guide clinical interaction studies.

P-glycoprotein (Pgp) is a member of the ATP-binding cassette superfamily of transport proteins and is expressed in numerous tissues such as the luminal membrane of the small intestine and blood-brain barrier, and the apical membranes of excretory organs such as liver and kidney (Aryton and Morgan, 2001). Pgp has broad substrate recognition, which can affect the pharmacokinetics, efficacy, safety, and target organ specificity of drugs. As a result, drug-drug interactions due to inhibition or induction of Pgp are a recognized clinical concern (Englund et al., 2004; Balayssac et al, 2005) recently highlighted in the FDA concept paper on "Drug Interaction Studies - Study Design, Data Analysis, and Implications for Dosing and Labeling" (FDA, 2005).

Despite many years of investigation, considerable uncertainty remains about the number of drug binding sites within Pgp and their mutual relationships. It is postulated that the transmembrane (TM) regions of Pgp form a large binding pocket (Lugo and Sharom, 2005; Sharom et al, 1998) composed of amino acid residues from multiple TM segments (Loo and Clarke, 2001; Loo and Clarke, 2002). Recent experiments investigating drug-binding (Martin et al, 2000), fluorescent dye uptake (Shapiro and Ling, 1997; Lugo and Sharom, 2005), ATPase activity (Pascaud et al, 1998; Wang et al., 2000), and transport inhibition (Ayesh et al., 1996; Tang et al., 2004) are consistent with multiple (up to 4 have been speculated) drug binding / transport sites within the Pgp binding pocket. Thus, the Pgp macromolecule is very complex with respect to drug binding and transport.

The recent FDA concept paper on drug interactions recommends that new drug candidates be evaluated as substrates, inhibitors, and inducers of Pgp in order to assess the potential for clinical

drug-drug interactions. The existence of multiple drug binding / transport sites within Pgp raises the question whether multiple probe substrates will be needed to relate *in vitro* Pgp inhibition results to clinical drug interaction findings, as has been done for cytochrome P450 3A4 (Wandel et al., 1999; Yasuda et al., 2002). Therefore, the objective of this study was to determine whether multiple probe substrates are needed to assess *in vitro* Pgp inhibition potential by characterizing five potential probe substrates (calcein-AM, colchicine, digoxin, prazosin and vinblastine) that bind to different sites within the human Pgp protein (Martin et al., 2000; Shapiro and Ling, 1997). To meet this objective, IC_{50} values for 20 drugs having different interactions with Pgp based on efflux, ATPase and calcein-AM inhibition assay results (Polli et al., 2001) were determined for the five probes using MDR1-MDCK cell monolayers.

Materials and Methods

Materials. GlaxoSmithKline Chemical Registry supplied all test compounds, GF120918 (Elacridar) and [^{3}H]-Amprenavir (21 Ci/mmol). [G-^{3}H]-digoxin (5 Ci/mmol), [7-*methoxy*-^{3}H]-prazosin (70 Ci/mmol) and [G-^{3}H]-vinblastine sulphate were purchased from Amersham Biosciences, Inc. (Piscataway, NJ) and [ring C, *methoxy*-^{3}H]-colchicine (60-87 Ci/mmol) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO). Transwells (12-well, 11-mm diameter, 0.4 μm pores) were purchased from Corning Costar (Cambridge, MA). The calcein-AM assay kit (Vybrant Multidrug Resistance Kit) was purchased from Molecular Probes, Eugene, OR.

Monolayer Efflux Studies. Multidrug resistance-1 transfected MDCK type II (MDR1-MDCKII) cells expressing human Pgp were obtained from the Netherlands Cancer Institute (Amsterdam, Netherlands). Cell culture and transport studies were completed with slight modifications as previously described (Polli et al., 2001; Mahar Doan et al., 2002; Keogh and Kunta, 2006). Briefly, cells were split weekly at a ratio of 1:50 and grown in the absence of antibiotics or selection agent. For transport studies, cells were seeded onto polycarbonate Transwell filter membranes at a density of 300,000 cells/cm^2, media changed the following day, and transport assays completed 3 days later. Compounds were dissolved at 10 mM in 100% DMSO and diluted for studies in transport medium (Dulbecco's Modified Eagle Medium supplemented with L-glutamine, 25 mM HEPES, pyridoxine hydrochloride, 1% DMSO (v/v) but without sodium pyruvate, and phenol red). As part of the initial characterization, each probe substrate was tested at a number of concentrations (vinblastine, colchicine and prazosin 1 to 10

μM; digoxin 0.043 to 5 μM; n=4 test concentrations) and in both the apical-to-basolateral (A→B) and basolateral-to-apical (B→A) directions. Each probe substrate had linear flux across a concentration range bracketing the final test concentration used in the inhibition studies. The direction that provided the largest dynamic range was selected as the direction used in the inhibition studies. Based on these initial experiments, the probe substrates were tested at 1 μM (prazosin, vinblastine, colchicine) or 0.043 μM (digoxin) in apical-to-basolateral (A→B; prazosin) or basolateral-to-apical (B→A; digoxin, vinblastine, colchicine) directions.

Inhibitors were tested in triplicate at a minimum of eight concentrations generally spanning 0.3 to 100 μM. Monolayer studies were conducted at 37°C in a humidified incubator with shaking (90 rpm) for either 90 min (prazosin, digoxin, and vinblastine) or 240 min (colchicine). Markers for Pgp efflux ([^{3}H]-amprenavir- separate set of Transwells) and monolayer integrity (Lucifer yellow- every Transwell along with probe substrate) were included in each experiment. Radiolabeled probes were measured by liquid scintillation counting with Ultima Gold (Perkin Elmer, Boston, MA) scintillation cocktail using a TriCarb T2900 counter (Perkin Elmer, Boston, MA). The efflux ratio for [^{3}H]-amprenavir (test concentration of 3 μM) passed the assay criterion (≥ 12) and collapsed to unity in the presence of Pgp inhibitor GF120918 demonstrating the functional expression of human Pgp in the monolayers. Amprenavir is a substrate of Pgp but not BCRP or MRPs (Olson et al., 2002; Gupata et al., 2004). Lucifer yellow concentration in the receiver compartments was measured by a SpectraMax Gemini cytofluorimeter (Molecular Devices, Sunnyvale, CA) set to an excitation wavelength of 430 nm and an emission wavelength of 538 nm. Values of ≤ 20 nm/sec for Lucifer yellow (LY) permeability were considered acceptable for the assay.

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Calcein Inhibition Assay. The calcein-AM assay was optimized and performed using the Vybrant Multidrug Resistance Kit (Molecular Probes, Eugene, OR) and MDR1-MDCKII cells as described (Polli et al., 2001; Mahar Doan et al., 2002). Cells were seeded at 70,000 cells per well (200 μL culture medium) in 96 well black plates with clear bottoms (Packard Instrument Co., Meridian, CT). The medium was changed 24 hours after seeding, and the assay performed 48 hours later. On the day of the study, the medium was aspirated and monolayers washed three times with transport buffer. Test drugs were added to monolayers in 50 μL transport buffer containing 1% DMSO. Test concentrations of each drug (final concentrations of 0.1 to 100 μM, n = 7 except for GF120918 which was 0.001 to 10 μM) were selected based on previous work with this assay (Polli et al., 2001). DMSO concentration (1%) was constant in test and control wells (each n = 2). Plates were preincubated at 37^{o} for 10 minutes. Calcein-AM was added and plates were immediately placed in a SpectraMax Gemini cytofluorimeter (Molecular Devices, Sunnyvale, CA) for 60 minutes and read at 15-minute intervals at excitation and emission wavelengths of 485 and 530 nm, respectively. Pgp inhibition was quantified by use of the following equation:

$$Pgp\_Activity\_(\%\max)\_ = 100 - \frac{(RFU_{comp} - RFU_{background})}{(RFU_{GF120918} - RFU_{background})} \bullet 100$$

where RFU_{comp} = fluorescence in the presence of test compound (comp), $RFU_{GF120918}$ = fluorescence in the presence of 2 μM GF120918 (maximum inhibition), and $RFU_{background}$ = fluorescence in absence of the drug (typically 45 - 65 RFU).

Calculations. The transport rate of each probe was calculated using the following equation:

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$$J = \frac{V(\mathrm{d}C/\mathrm{d}t)}{A}$$

where J is the transport rate (nmol/cm^2/h), V is the receptor volume (ml), C is the receiver drug concentration (nmol/ml), t is time in hours, and A is the membrane surface area (cm^2). The permeability coefficient at pH 7.4 ($P_{7.4}$) for passive membrane transport of Lucifer yellow and probe substrates in the presence of GF120918 was determined using the following equation as previously described (Tran et al, 2004):

$$P_{7.4} = -\left(\frac{V_D V_R}{(V_D + V_R)At}\right)\ln\left\{1 - \frac{(V_D + V_R)C_R(t)}{(V_D C_D(t) + V_R C_R(t))}\right\} \text{ x } 10^7 \text{ nm/s}$$

where V_D, V_R are donor and receiver well volumes, respectively (mL), A is the membrane surface area (cm^2), t is the incubation time (seconds), $C_R(t)$ is the measured concentration in the receiver well at time t (nmol/ml), $C_D(t)$ is the measured concentration in the donor well at time t (nmol/ml).

The IC_{50} values, the concentration of inhibitor required for 50% inhibition of the B→A transport rates, were calculated with GraFit (version 5.06, Erithacus Software Limited, London, UK) using:

$$y = \frac{Range}{1 + \left(\frac{x}{IC_{50}}\right)^s} + \text{background}$$

where y = the rate of transport of an appropriate probe (expressed as a percentage of the uninhibited control), *Range* = the rate of probe in the absence of test compound minus the background transport rate, s = is the slope factor, x = the inhibitor concentration (μM), background = the uninhibited rate of probe transport (expressed as a percentage of the total rate).

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The *K* values, the concentration of inhibitor required for a 50% increase in the prazosin A→B rate, were calculated with GraFit (version 5.06, Erithacus Software Limited, London, UK) using the Hill equation:

$$v = \left(\frac{V_{\max}[\mathrm{S}]^n}{K^n + [\mathrm{S}]^n} \right)$$

where v = the rate of transport in nmol/cm^2/min, Vmax = the maximum rate of A → B transport of prazosin in nmol/cm^2/min, S = the concentration of inhibitor, n = the Hill coefficient, K = the inhibitor concentration that produces 50% of the rate for A → B transport of prazosin.

Mass balance was the percent of original drug mass accounted for at the end of the experiment (sum of the amount in the apical (A) and basolateral (B) chambers). Mass balance was calculated with the following equation:

$$\%\mathrm{MB} = \frac{\left[\left(C_{\mathrm{A}t} \cdot V_{\mathrm{A}}\right) + \left(C_{\mathrm{B}t} \cdot V_{\mathrm{B}}\right)\right]}{\left(C_0 \cdot V_{\mathrm{D}}\right)} \cdot 100$$

where $C_{\mathrm{A}t}$ and $C_{\mathrm{B}t}$ are the drug concentrations in the A and B chambers at time (t), C_0 is the concentration of the donor at time 0, V_{A} and V_{B} are the volumes of the A and B chambers, and V_{D} is the volume of the donor solution added to the appropriate chamber.

Results

Selection of Compounds. The probe substrates calcein-AM, colchicine, digoxin, prazosin and vinblastine were selected based on literature data suggesting possible differential binding to Pgp, availability of radiolabel material (colchicine, digoxin, prazosin and vinblastine) or fluorescence assays (calcein-AM), and efflux characteristics in the *in vitro* assay (Table 1). The 20 diverse drugs tested as inhibitors were selected to explore different interactions with Pgp (e.g., monolayer efflux, ATPase, and calcein-AM assays) and grouped according to the results reported by Polli et al. (2001) (Table 2). Of the selected 20 drugs, eight belong to category I in which the drugs exhibited agreement across the three assays; these drugs are inhibitors, unambiguous substrates or unambiguous nonsubstrates of Pgp. The remaining 12 drugs were selected from category II, which revealed differences among the assays related to membrane permeability and interaction with Pgp. Category II is further sub-divided based on the absence (groups IIA, n = 3, nontransported substrates) or presence (group IIB, n = 8, transported substrates) of monolayer efflux. Four of the five probe substrates (colchicine, digoxin, prazosin and vinblastine) were also included as test inhibitors and belong to either category I (prazosin and vinblastine) or IIB (digoxin and colchicine).

Monolayer Efflux Studies of Probe Substrates. MDR1-MDCKII cells have been shown to be a good *in vitro* model for determining if compounds are Pgp substrates (Polli et al., 2001, Tang et al., 2002; Taub et al, 2005). For each probe selected for use in the efflux assay (colchicine, digoxin, prazosin and vinblastine), a bidirectional concentration-dependent experiment was performed across MDR1-MDCKII cell monolayers (Table 3). The B→A/A→B ratios for each probe were greater than 1.0 at each of the four test concentrations between either 1 and 10 μM

(colchicine, prazosin and vinblastine) or 0.043-5 μM (digoxin) indicating Pgp-mediated efflux of each probe (Table 3 and data not shown). Due to the low efflux ratio of colchicine under the standard assay conditions, the effect of incubation time on the efflux ratio was further evaluated. An increase in the incubation time from 90 to 240 min increased the B→A/A→B ratio from 1.5 to 3.5 (data not shown). Since the efflux ratio was larger at 240 min, this time was used in all subsequent inhibition studies with colchicine. The efflux ratios for digoxin and vinblastine were greater than 50, while that for prazosin and colchicine were less than 4. Addition of 5 μM GF120918, a potent Pgp inhibitor, reduced the B→A/A→B ratios of colchicine, prazosin and digoxin to unity. In contrast, the efflux ratio of vinblastine was reduced to 2.3 in the presence of GF120918 suggesting that a second efflux transporter is present in the MDR1-MDCKII cells. The probe substrates had a range of permeability values ($P_{app\ B\rightarrow A}$) with digoxin having the lowest permeability (15.9 nm/s), followed by colchicine (53.4 nm/s), vinblastine (97.4 nm/s), and prazosin (283 nm/s). Finally, mass balance for all probes was acceptable (>80%), suggesting that there was minimal loss of substrate to plastic surfaces and cells under the experimental conditions.

Monolayer Pgp-mediated Inhibition by GF120918. The concentration dependent inhibitory effect of GF120918 on transport of each probe across MDR1-MDCKII monolayers was tested (Figure 1). Inhibition studies for digoxin, vinblastine, and colchicine were completed in the B→A direction because this direction had a large dynamic range due to the contribution of Pgp efflux (Table 3). The addition of GF120918 decreased the rate of digoxin, vinblastine, and colchicine B→A transport (test concentration 1 μM for vinblastine and colchicine, and 0.043 μM

for digoxin) by 86, 75 and 85%, respectively. The GF120918 IC_{50} values were similar across the three probe substrates ranging from 0.027 to 0.055 μM (Table 2 and Figure 1).

The Pgp inhibition assay of prazosin was completed in both the A→B and B→A directions due to the compound's high permeability and modest efflux ratio. Addition of GF120918 increased the A→B rate of prazosin (test concentration 1 μM) by 1.7-fold (Table 3) and resulted in the IC_{50} value of 0.05 μM for GF120918 (Table 2); an IC_{50} value of GF120918 similar to that for the other three probe substrates. In the B→A direction, GF120918 only inhibited the B→A rate of prazosin by 51%, yielding an estimated IC_{50} value of 0.025 μM, similar that for the A→B direction and the other probes (Tables 2 and 4). This demonstrates that the IC_{50} values for prazosin are not influenced by the direction of the assay. Of the four probe substrates tested in the monolayer efflux assay, prazosin had the smallest dynamic range (~ 3-fold), which is related to its high intrinsic membrane permeability. However, as this compound has been used extensively to characterize Pgp drug binding / transport pocket (Isenberg et al., 2001; Wang and George, 1997), it was examined further as a potential probe substrate. Finally, the IC_{50} value for GF120918 in calcein-AM was of 0.10 μM, a value similar to that observed for the other probe substrates.

Inhibition of Pgp-mediated Efflux of Probe Substrates by a Panel of Inhibitors. The inhibition of Pgp-mediated efflux of the five probe substrates by 20 diverse drugs was measured by determining either the basolateral to apical (B→A; colchicine, digoxin and vinblastine) transport across MDR1-MDCKII monolayers, the apical to basolateral (A→B; prazosin) transport across MDR1-MDCKII monolayers, or the increase in calcein fluorescence (calcein-

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AM) in MDR1-MDCKII cells (Table 2). Further, the inhibition of B→A transport of prazosin was determined for three inhibitors to confirm consistency of the IC_{50} values between the directions of the assay. Overall, the rank order of the probe substrates were generally similar across the 20 drugs (Tables 2 and 4), with probe substrates having a notable inhibition by six to nine of the drugs. As expected, GF120918 (category I inhibitor) was the most potent inhibitor (IC_{50} values below 0.10 μM), followed by cyclosporine A (category IIB3), ketoconazole (category IIA) and verapamil (category IIA); all these drugs are well established Pgp inhibitors. For GF120918, cyclosporine A and ketoconazole, there was little difference (within 3- to 5-fold of each) in the IC_{50} values across the probe substrates (Table 3). In contrast, there were notable differences in the IC_{50} values of verapamil across the probe substrates. The verapamil IC_{50} value for digoxin and prazosin was ≤11 μM, between 17 and 34 μM for colchicine and vinblastine, and was 60.9 μM for calcein-AM. It is of interest that diphenhydramine, the other category IIA drug, did not show any significant inhibition of the five probe substrates (Table 2), although calcein-AM fluorescence increased 15% at 100 μM of diphenhydramine.

Further evaluation revealed that category I non-substrates and IIB1/IIB2 transported substrates had little inhibitory activity towards any of the five probe substrates (Table 2). In contrast, there were some notable differences in the inhibition of the five probe substrates by category I substrate drugs. Amprenavir and prazosin had weak inhibition (IC_{50} values >70 μM) against all the probe substrates. In contrast, the IC_{50} values for quinidine and vinblastine ranged from 14.0 to >100 μM across the five substrates and fell into two groupings. For quinidine, the IC_{50} values were 14 to 23 μM for digoxin, prazosin and vinblastine, and ~50 μM for calcein-AM and colchicine. The vinblastine IC_{50} values for colchicine, digoxin and prazosin were 17 to 30 μM,

and those of calcein-AM and vinblastine >89 μM. It is of note that of the four probe substrates used as inhibitors, only vinblastine was able to demonstrate inhibition of its own transport (IC_{50} value = 89.7 μM), albeit rather weakly compared to the other positive inhibitors.

Discussion

The increased interest in Pgp and potential drug interactions is driving the need to validate the predictability of *in vitro* Pgp studies against *in vivo* data. Such *in vitro* assays will require the identification of specific probe substrates and inhibitors for Pgp (Ayrton and Morgan, 2001). Because a large number of substrates and modulators interact with Pgp, it has been speculated that Pgp has multiple drug binding / transport sites (Shapiro et al., 1999; Martin et al. 2000; Tang et al., 2004; Wang et al., 2001), which makes selection of probe substrates a key consideration when establishing an *in vitro* inhibition assay. In the present study, the substrates calcein-AM, colchicine, digoxin, prazosin, and vinblastine were selected based on different binding / transport by human Pgp (Shapiro and Ling, 1997; Shapiro et al., 1999; Martin et al. 2000), efflux characteristics, and availability of fluorescent / radiolabel drug. The 20 drugs used as inhibitors were selected based on behavior in the monolayer efflux, ATPase, and calcein-AM assays (see Selection of Compounds in Results). Overall, the rank order profiles of the five probe substrates were generally similar (Table 4), with probe substrates having interaction with six to nine of the drugs; however, there were several notable differences in the IC_{50} values.

Detailed examination of the relationship between drug category and IC_{50} values provides further insight into the selection of probe substrates and when a compound may be a potent inhibitor of Pgp. Category I nonsubstrate and category IIB1/2 substrate drugs had little to no inhibition of the probe substrates; consistent with previous calcein-AM and ATPase results showing little to no interaction with Pgp (Polli et al., 2001). This highlights the observation that “competitive” inhibition is not always seen for good Pgp substrates (Lugo and Sharom, 2005; Taub et al, 2005). This is further illustrated through the poor inhibition of the probe substrates on themselves in this

study (e.g., colchicine, digoxin, prazosin, and vinblastine). In contrast, category IIA and IIIB3 drugs were good Pgp inhibitors (IC_{50} values <15 μM) with the rank order of inhibition being GF120918 (I) > cyclosporine A (IIIB3) ≅ ketoconazole (IIA) ≥ verapamil (IIA). There were notable differences in the verapamil IC_{50}'s across the five probes. The IC_{50} values for digoxin and prazosin were ≤11 μM and those for calcein-AM, colchicine and vinblastine ≥17 μM, which may suggest differential binding of verapamil to Pgp relative to the other inhibitors in this group. This may be explained by GF120918, cyclosporine A and ketoconazole binding to a central modulatory site (M site, Table 1), thus inhibiting all substrates (Martin et al, 2000). In contrast, verapamil may exert its inhibitory effect at multiple drug transport / binding sites (R and/or P sites, Martin et al., 2000), which is consistent with the "two affinity" model (biphasic binding) proposed for [^{3}H]-verapamil binding to Caco-2 membranes over expressing Pgp (Doppenschmitt et al., 1999).

Examination of category I substrate drugs revealed differences in probe substrate inhibition profiles. Amprenavir and prazosin (as an inhibitor) were weak Pgp inhibitors across the probes; only IC_{50} values for colchicine was determined. On the other hand, quinidine markedly inhibited digoxin, prazosin and vinblastine efflux (IC_{50} values of 11 to 23 μM), but was up to 3-fold less potent towards colchicine and calcein-AM (IC_{50} values near 50 μM). Vinblastine (as an inhibitor) inhibited colchicine, digoxin and prazosin transport with similar IC_{50} values (17 to 31 μM); however, inhibition of itself and calcein-AM was up to 5-fold weaker. These data suggest that category I substrate drugs may have different interactions with the Pgp drug binding / transport sites. This is not surprising as previous work has demonstrated differential interactions between Pgp substrates (rhodamine, Hoechst 33342, vinblastine and prazosin) such that different

combinations of substrates can yield stimulation or inhibition of efflux (Dey et al., 1997; Martin et al., 2000; Shapiro and Ling, 1997, Wang, et al, 2001; Lugo and Sharom, 2005; Taub et al, 2005). Like [^{3}H]-verapamil binding, [^{3}H]-vinblastine binding to Caco-2 membranes also fits a "two affinity" model, which may explain the differences in IC_{50} values for the probe substrates.

Considering the importance of Pgp in drug disposition, it is evident that Pgp-mediated drug transport can play a central role in drug-drug interactions (Ayrton and Morgan, 2001; Balayssac et al., 2005). Data derived from the present study and reported by Kunta and Keogh (2006) can serve as a guide to design clinical drug interaction strategies for Pgp. Digoxin proved to be a sensitive probe and is our recommendation as the probe substrate for *in vitro* Pgp inhibition assays. Its advantages are a large *in vitro* efflux ratio (>50), limited passive membrane permeability, good mass balance, collapse to unity in the presence of GF120918 and availability of radiolabel drug commercially; these characteristics give digoxin a sufficient dynamic range over which to measure inhibition of Pgp-mediated transport *in vitro*. Further, IC_{50} values reported here for GF120918, cyclosporine A, vinblastine, quinidine and verapamil are in agreement with Ki values reported in Caco-2 or MDCK-MDR1 cells using digoxin as a probe substrate (Tang et al., 2002), highlighting the consistency of digoxin as a probe substrate. Finally, digoxin offers the advantage of being a useful clinical probe substrate, which is mainly eliminated in humans as unchanged drug in the urine, therefore minimizing any confounding metabolism issues.

Vinblastine and colchicine are recommended as alternate *in vitro* probes. These probes have inhibition profile similar to, but less sensitive, than that of digoxin. One advantage of vinblastine

is that it has a larger efflux range than colchicine. Even though prazosin is as sensitive as digoxin, prazosin is not recommended as a probe substrate due to its high membrane permeability and limited dynamic range of the assay (3-fold efflux). Surprisingly, calcein-AM proved to be the least sensitive probe (Table 4). In particular, calcein-AM transport was insensitive to verapamil and quinidine inhibition, relative to the other probe substrates tested. No clear pattern or explanation is available for this behavior. There are conflicting reports in the literature on the IC_{50} values of these drugs on calcein-AM transport and our data are consistent with a number of these studies (Tiberghien and Loor, 1996; Wang et al., 2001; Schwab et al, 2003). Finally, translation of *in vitro* data for these alternative probe substrates to clinical drug interaction studies may be more difficult compared to digoxin due to the limited experience with these agents as clinical Pgp probe substrates, and the associated complication of metabolism. However, results reported here can serve as a bridge between the use of these alternate *in vitro* probes and that of digoxin as a clinical probe substrate.

A comparison of the digoxin *in vitro* IC_{50} data from this study with clinically relevant drug-drug interactions (Lanoxin® Tablets product information; Englund et al., 2004) revealed a close relationship between the clinical and *in vitro* findings. For example, quinidine (Angelin et al., 1987; Mordel et al., 1993; Fromm et al., 1999), verapamil (Pedersen et al., 1983; Verschraagen et al., 1999), and cyclosporine A (Okamura et al., 1993) have well-documented effects on digoxin pharmacokinetics *in vivo* and have established Pgp inhibitory effects *in vitro*. These Pgp inhibitors belong to categories I (unambiguous substrate), IIA (nontransported substrates) and IIB3 (transported substrates), and have IC_{50} values between 1 and 15 μM for digoxin. In contrast, none of the category I unambiguous nonsubstrates or category IIB1/2 substrates had

interaction with digoxin *in vitro*, consistent with the *in vivo* literature. Therefore, as a guide, potential Pgp-mediated drug-drug interactions should be taken into consideration with compounds that belong to categories I (unambiguous substrates only), IIA (nontransported substrates) and IIB3 (transported substrates) that have *in vitro* IC_{50} values less than 15 μM (Table 5). Further, the free fraction of drug in plasma and dose (in particular if > 100 mg) are other important parameters to consider when evaluating the need for a clinical drug interaction study. As discussed in the FDA Guidance and in the review by Sahi, J (2005), drug-drug interaction potential can be estimated using [I]/Ki, where I = inhibitor concentration and Ki = the inhibition constant (note that use of total concentration is recommended in the FDA Guidance because this provides the most conservative estimate). If the [I]/Ki ratio is <0.02, the chance of an interaction is remote. In contrast, as the [I]/Ki ratio increases, the chance of an interaction increases. More detailed investigations are required to address the utility of this approach to drug transporters.

The current interest of the FDA and European regulatory agencies in Pgp drug transport is likely to increase expectations that new drug candidates be evaluated for inhibition of Pgp-mediated transport, in particular for specific therapies such as cardiovascular, oncology, and neurology. Therefore, based on the result of the present study, we recommend the use of digoxin as a standard probe substrate for *in vitro* Pgp inhibition studies to determine the inhibition potential of drug candidates.

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Footnotes

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Legends for Figure:

Figure 1. Inhibition of Pgp-mediated transport of probe substrates A) digoxin, B) vinblastine, C) colchicine, D-E) prazosin and F) calcein-AM. Inhibition was measured by determining the basolateral to apical (B→A; digoxin, colchicine, vinblastine, and prazosin) transport across MDR1-MDCKII monolayers, apical to basolateral (A→B; prazosin) transport across MDR1-MDCKII monolayers, or efflux of calcein-AM from MDR1-MDCKII cells. Symbols representing the inhibitors are: ○ for GF120918, ∇ for quinidine, Δ for verapamil, ◊ for cyclosporine A, and □ for ketoconazole. The values represent mean ± S.D. of at least three independent determinations.

Table 1. Properties of Pgp Probe Substrates and Inhibitor.

Probe	MW [1]	Apparent Permeability (P_{app} B → A; nm/s) [2]	Postulated Binding Site [3]	Comment	Reference
Calcein-AM	995	ND [4]	R, P	High throughput screening probe.	Tiberghien and Loor, 1996; Polli et al., 2001; Schwab et al., 2003
Colchicine	399	53	H	Single binding site in Pgp.	Shapiro and Ling, 1997, Martin et al., 2000
Digoxin	781	16	R	Clinically important probe.	Englund et al., 2004; Kunta and Keogh, 2006
Prazosin	382	283	P	Historical probe for Pgp binding studies.	Isenberg, et al., 2001, Shapiro et al., 1999
Vinblastine	811	97	R	Historical probe for multidrug resistance.	Doppenschmitt et al., 1999
GF120918	564	ND[4]	M	Clinically significant inhibitor and research tool.	Polli, et al., 2001; Tang et al., 2002

1. Molecular weight of the free form
2. Apparent permeability in the presence of the Pgp inhibitor GF120918 (5 μM)
3. H - Hoechst 33342 site; P - progesterone/prazosin site; R - rhodamine 123/anthracycline site; M - central modulation site (Shapiro and Ling, 1997; Martin et al., 2000 ; Lugo and Sharom, 2005)
4. ND = Not determined

Table 2. Inhibition of Pgp-mediated transport of probe substrates across MDR1-MDCKII cells [1].

Inhibitor	**EAC** [2]	**Class**	**Calcein -AM** IC_{50} value [3]	**Digoxin B → A** IC_{50} value	**Vinblastine B → A** IC_{50} value	**Colchicine B → A** IC_{50} value	**Prazosin A → B** K value [4]	**Prazosin B → A** IC_{50} value
GF120918	NNY	I Inhibitor	0.101 ± 0.014	0.055 ± 0.003	0.043 ± 0.004	0.027 ± 0.002	0.050 ± 0.027	0.025 ± 0.003
Ranitidine	NNN	I	no	no	no	no	no	-
Propranalol	NNN	Unambiguous	no	no	no	no	>100 [5]	-
Methotrexate	NNN	nonsubstrates	no	no	no	no	no	-
Triamterene	NNN		no	no	no	no	no	-
Amprenavir	YYY	I	>100	no	>100	91.3 ± 11.6	>100	-
Prazosin [6]	YYY	Unambiguous	>100 [5]	no [6]	no [6]	70.7 ± 3.8	no [6]	-
Quinidine	YYY	substrates	55.5 ± 2.34	14.9 ± 9.0	22.9 ± 3.4	51.7 ± 9.8	14.0 ± 1.23	-
Vinblastine	YYY		>100	17.8 ± 2.2	89.7 ± 15.6	30.1 ± 4.1	21.9 ± 11.7	-
Verapamil	NYY	IIA	60.9 ± 8.91	10.7 ± 4.1	33.5 ± 2.1	17.3 ± 1.9	1.18 ± 0.20	1.55 ± 0.56
Diphenhydramine	NYY	Nontransported	No	no	no	no	no	-
Ketoconazole	NYY	substrates	10.1 ± 1.6	3.07 ± 0.76	6.34 ± 1.98	5.49 ± 0.98	2.38 ± 0.18	0.65 ± 0.12
Daunorubicin	YNN		no	no	>100	>100	no	-
Colchicine	YNN	IIB1	no	no	no	no	no	-

Dexamethasone	YNN	Transported	no	no	no	no	no	-
Fexofenadine	Y- N	substrates	no	no	no	no	no	-
Digoxin	Y- N		no	no	no	no	no	-
Erythromycin	YYN	IIB2	no	no	no	no	no	-
Indinavir	YYN	Transported	no	no	no	no	>50	-
Trimethoprim	YYN	substrates	no	no	no	no	no	-
Cyclosporin A	YNY	IIB3 Transported substrates	2.22 ± 0.02	1.6 ± 0.3	6.18 ± 1.90	1.36 ± 0.09	0.98 ± 0.33	0.74 ± 0.12

1. The inhibition of probe substrates by test compounds was determined as described under *Materials and Methods*. A minimum of 8 concentrations (n = 3) per test compound were used to determine the IC_{50} value (calcein-AM, digoxin, vinblastine, colchicine) using the full 4-parameter equation or K value (prazosin) using the Hill equation.
2. EAC: E, monolayer efflux; A, drug-stimulated ATPase; C, calcein-AM. Results are reported as yes (Y) or no (N). For efflux, yes = substrate and no = nonsubstrate; for ATPase assay, yes = stimulator and no = no activity; and for calcein-AM assay, yes = response >10% maximum fluorescence and no = response <10% maximum fluorescence. See Polli et al, 2001 for further details.
3. IC_{50} is the concentration of inhibitor required for 50% inhibition of probe transport.
4. *K* value is the concentration of inhibitor required for 50% increase in the prazosin A → B rate
5. Compounds with an IC_{50} value reported to be greater than a number represent that notable inhibition (>20%) was observed at the highest test concentration. However, an accurate IC_{50} value could not be determine from the dataset.
6. Prazosin at >50 μM increased Lucifer yellow passive permeability (>20 nm/s) suggesting a breach in the tight junctions between monolayers; therefore, data only up to 50 μM was used in the analysis of IC_{50} curves.

Table 3. Transport and permeability of probe substrates across the MDR1-MDCKII cells.

Compound	P_{app} A → B (*nm/s*)	Mass balance A → B	P_{app} B → A (*nm/s*)	Mass balance B → A	B → A/ A → B Ratio
Digoxin[1]	3.07 ± 0.30 [2]	88.5	159± 17	95.4	51.7
Digoxin + GF120918[3]	11.9 ± 0.6	81.0	15.9 ± 1.5	80.4	1.33
Prazosin[4]	165 ± 28	98.1	482 ± 56	102	2.95
Prazosin + GF120918	273 ± 29	85.4	283 ± 25	93.4	1.04
Vinblastine[4]	8.46 ± 3.66	121	474 ± 42	121	56.0
Vinblastine + GF120918	42.0 ± 4.2	113	97.4 ± 12.2	117	2.32
Colchicine[4,5]	63.4 ± 1.6	107	220 ± 8	105	3.46
Colchicine + GF120918	75.1 ± 4.0	101	53.4 ± 3.0	102	0.71

1. 0.043 μM concentration was used for probe substrate; time 90 min.
2. Average ± SD; n ≥ 3.
3. 5 μM concentration was used for GF120918.
4. 1 μM concentration was used for probe substrate; time 90 min.
5. Experiment time of 240 min was used for colchicine.

Table 4. Rank Order Comparison of Inhibitors on the Pgp-mediated Transport of Selected Probe Substrates across MDR1-MDCKII cells.

Probe	IC_{50} Value (μM)			
	<5	5-15	15-50	>50
Calcein-AM	918, CsA	K		V, Q, VB, P, A
Colchicine	918, CsA	K	V, VB	Q, P, A, Dau
Digoxin	918, CsA, K	V, Q	VB	
Prazosin	918, CsA, K, V	Q	VB	Prop, A, I
Vinblastine	918	CsA, K	V, Q	VB, A, Dau

Abbreviation: 918 – GF120918; A – Amprenavir, CsA – cyclosporine A; Dau – daunorubicin; I – indinavir; K – ketoconazole; P – prazosin; Prop – propranolol; Q – quinidine; VB – vinblastine; V – verapamil

Table 5. Categorizing Pgp Inhibition Potential by Ability to Inhibit Digoxin B→A Transport Across MDR1-MDCKII cells.

	IC_{50} value	**Pgp inhibition potential**
Inhibitor	< 1 μM	High
	1 – 15 μM	Modest
	> 15 μM	Low

Figure 1