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A combined cell based approach to identify P-glycoprotein substrates and inhibitors in a single assay

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Abstract

The objective of this project was to develop a cell based in vitro experimental procedure that can differentiate P-glycoprotein (P-gp) substrates from inhibitors in a single assay. Caco-2 cells grown to confluency on 12-well Transwell® were used for this study. The efflux permeability (B to A) of P-gp specific probe (viz., digoxin) in the presence of test compounds (e.g. substrates, inhibitors and non-substrates of P-gp) was monitored, and the influx permeability (A to B) of test compounds was evaluated after complete P-gp blockade. Radiolabelled digoxin was added on the basolateral side with buffer on the apical side. The digoxin concentration appearing on the apical side represents digoxin efflux permeability during the control phase (0–1 h period). After 1 h, a test compound (10 uM) was added on the apical side. The reduced efflux permeability of digoxin suggests that the added test compound is an inhibitor. The influx permeability of test compound is also determined during the 1–2 h study period by measuring the concentration of the test compound in the basolateral side. At the end of 2 h, a potent P-gp inhibitor (GF120918) was added. The increased influx permeability of test compound during the 2–3 h incubation period indicates that the added test compound is a substrate. Samples were taken from both sides at the end of 1–3 h and the concentrations of the test compounds and digoxin were quantitated. Digoxin efflux permeability remained unchanged when incubated with P-gp substrates (e.g., etoposide, rhodamine123, taxol). However, when a P-gp inhibitor was added to the apical side, the digoxin efflux (B to A permeability) was significantly reduced (ketoconazole = 51% reduction) as expected. The influx permeability of substrates increased significantly (rhodamine123 = 70%, taxol = 220%, digoxin = 290%) after the P-gp inhibitor (GF120918) was introduced, whereas the influx permeability of P-gp inhibitor and non-substrates was not affected by GF120918. Thus, this combined assay provides an efficient cell based in vitro screening tool to simultaneously distinguish compounds that are P-gp substrates from P-gp inhibitors.

Keywords: Permeability; Efflux; Caco-2 cell; High-throughput; P-glycoprotein; Inhibitor; Substrate

Abbreviations: A to B, apical to basolateral; ADME, absorption, distribution, metabolism, elimination; B to A, basolateral to apical; BCRP, breast cancer resistance protein; HBSS, Hank's balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IAM, immobilized artificial membrane; LSC, liquid scintillation counter; LRP, lung cancer resistance protein; MRP, multi-drug resistance protein; NCE, new chemical entity; PAMPA, parallel artificial membrane permeability assay; P-gp, P-glycoprotein; P_c, permeability co-efficient; TEER, trans-epithelial electrical resistance

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1. Introduction

Despite tremendous innovations in the drug delivery methods in the last few decades, the oral route still remains as the most preferred route of administration for most New Chemical Entities (NCE). The oral route is preferred by virtue of its convenience, low cost and high patient compliance compared to alternate routes. However, compounds intended for oral administration must have appropriate physico-chemical properties (i.e. adequate intestinal permeability without much efflux component) in order to achieve therapeutic concentrations. With the explosive growth in the field of genomics and combinatorial chemistry in the last few years, synthesizing a large number of potential drug candidates is no longer a bottleneck in the drug discovery process. Instead, the task of screening compounds simultaneously for biological activity and biopharmaceutical properties (e.g., solubility, permeability/absorption, stability, etc.) has become the major challenge. This has provided a great impetus within the pharmaceutical industry to implement appropriate screening models that are high capacity, cost-effective and highly predictive of in vivo permeability and absorption (Balimane et al., 2000; Hidalgo, 2001; Hillgren et al., 1995; Kerns, 2001; Lipinski et al., 2001).

Amongst the biopharmaceutical properties that need to be considered in early discovery, permeability assessment and P-glycoprotein (P-gp) interaction studies are critical in determining the fate of a compound. Transport of drug substances across the intestinal membrane is a complex and dynamic process. It includes the passage of compounds across various functional pathways in parallel. Passive transport occurs through the cell membrane of enterocytes (transcellular) or via the tight junctions between the enterocytes (paracellular). Various influx and efflux mechanisms (via carriers and transporters) are also functional. Drug efflux transporters such as P-gp may be a major determinant of absorption, distribution and elimination of a wide variety of drugs (Benet et al., 2003; Lin, 2003; Lin and Yamazaki, 2003; Malingre et al., 2001; Matheny et al., 2001; Polli et al., 1999; Sababi et al., 2001). P-gp is known to limit the oral absorption of drugs such as docetaxel and taxol; it can limit entry of drugs such as HIV protease inhibitors into brain and CNS; and it can actively facilitate excretion of drugs via biliary and urinary routes. Since P-gp interactions of a drug can play such a pivotal role in dictating their pharmacokinetics, increasing efforts are being made in early discovery and development to identify compounds that can potentially interact with P-gp.

Drug discovery scientists in evaluating permeability/absorption of drug candidates during the drug candidate selection process currently employ various techniques. The most pervasive pre-clinical methodologies currently used throughout the industry are: in vitro methods (ussing chamber, membrane vesicles, cell based Caco-2 cells, MDCK, etc., artificial lipid based PAMPA or IAM), in situ methods (single pass perfusion), in vivo methods (whole animal studies), and even in silico methods. These models provide information on permeability characteristics of test compounds but provide no information on their potential to interact with P-gp. An entirely different set of models is needed to gauge the potential of a test compound to interact with P-gp either as a substrate or as an inhibitor. There are literature reports of various in vitro and in vivo models that are used for assessing P-gp interactions with test compounds (Adachi et al., 2001; Balimane et al., 2004; Perloff et al., 2003; Polli et al., 2001; Yamazaki et al., 2001). In vitro assays such as ATPase assay, rhoadmine-123 uptake assay, calcein AM uptake assay, cell based bi-directional assay, radio-ligand binding assay along with in vivo models such as transgenic (knockout mice) and mutant animal models are most commonly used. However, all these models have a major drawback that they provide information regarding only one aspect of P-gp interaction: whether the test compound is a substrate or inhibitor of P-gp. In other words, two separate assays have to be performed, one for substrate and other for inhibitor identification. Keeping in mind the utility of these models as screening tools (i.e. early identification of the drug's potential to interact with P-gp) running these assays twice can lead to a significant loss of time and effort which can be highly counter-productive. In addition to the inefficiency, performing two assays lead to more compound requirement which can be a severe bottleneck in early discovery stage. Therefore, an ideal P-gp screening model would be the one that is efficient, cost-effective, predictive and provide two critical answers in one assay: (1) whether or not the compound is a P-gp substrate and (2) whether or not the compound is a P-gp inhibitor.

The current study describes the modified bi-directional cell based transport assay that can identify both substrates and inhibitors of P-gp in a single assay. There are certain limitations to this assay that must be borne in mind to prevent mis-interpretation of the data generated from these studies. If used judiciously, this assay has the potential to speed up screening of discovery compounds that might interact with P-gp.

2. Materials and methods

2.1. Chemicals

Caco-2 cells (passage # 17) were obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium, non-essential amino acids and antibiotics were purchased from JHR Biosciences (Lenexa, KS). Fetal bovine serum was obtained from Hyclone Lab. Inc. (Logan, UT). HTS-Transwell® inserts (surface area: 1 cm^2) with a polycarbonate membrane (0.4 μm pore size) were purchased from Costar (Cambridge, MA). Hank's balanced salt solution (HBSS) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents were analytical grade. ^{3}H-digoxin, ^{14}C mannitol and ^{14}C taxol were obtained from Perkin Elmer Life Sciences (Boston, MA). All other test compounds were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Caco-2 cell culture procedure

Caco-2 cells were seeded onto 12 well polycarbonate filter membrane at a density of 60,000 cells/cm^2. The cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% L-glutamine, 100 U/mL penicillin-G, and 100 μg/mL streptomycin. The culture medium was replaced every 2 days and the cells were maintained at 37 °C, 95% relative humidity, and 5% CO_2. Permeability studies were conducted with the monolayers cultured for approximately 21 days with the cell passage numbers between 50 and 80. Physiologically and morphologically well-developed Caco-2 cell monolayers with TEER values greater than 400 Ω cm^2 were used. Radiolabel mannitol was run as a control for monolayer integrity in all test runs.

2.3. Standard 2 h bi-directional transport procedure

The transport medium used for the bi-directional studies was HBSS buffer containing 10 mM HEPES. The pH of both the apical and basolateral compartments was 7.4. Prior to all experiments, each monolayer was washed twice with buffer and TEER was measured to ensure the integrity of the monolayers. The concentration of test compounds was 10–50 μM in this assay. The bi-directional permeability studies were initiated by adding an appropriate volume of buffer containing test compound to either the apical (for apical to basolateral transport; A to B) or basolateral (for basolateral to apical transport; B to A) side of the monolayer. Volume of the apical and basolateral compartment was maintained at 0.8 mL. The monolayers were then placed in an incubator for 2 h at 37 °C. Samples were taken from both the apical and basolateral compartment at the end of the 2 h period and the concentrations of test compound were analyzed by a high performance liquid chromatography method as described earlier (Chong et al., 1996). In case of mannitol, digoxin and taxol, radio labeled compound was used and the permeability was assessed by scintillation counting. Permeability coefficient (P_c) was calculated according to the following equation:

$$P_c = \frac{dA}{dt\, SC_0}$$

where dA/dt is the flux of the test compound across the monolayer (nmol/s), S the surface area of the cell monolayer (1 cm^2), and C_0 the initial concentration (50 μM) in the donor compartment. The P_c values were expressed as nm/s.

A set of well-characterized and well-studied compounds was selected to run the bi-directional assay. Mannitol (hydrophilic marker compound that is a P-gp non-substrate), standard P-gp substrates (saquinavir, rhodamine123, hoechst 33342, vincristine, digoxin, vinblastine, indinavir, etoposide, dexamethasone and taxol) (Ekin et al., 2002; Perloff et al., 2003; Polli et al., 2001; Tang et al., 2002; Wacher et al., 1995) and potent P-gp inhibitors (GF120918 and ketoconazole) (Donahue et al., 2003; Edwards et al., 2002; Kruijtzer et al., 2002; Polli et al., 1999) were studied.

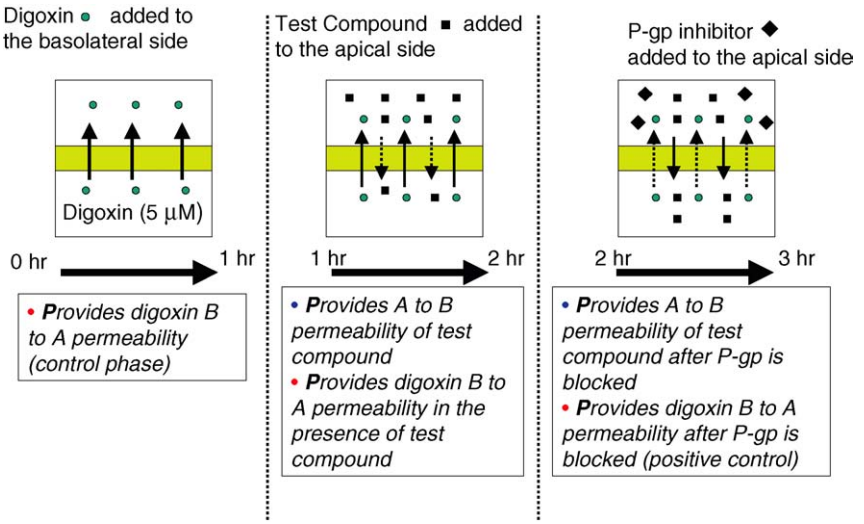

Fig. 1. Schematic of the combined P-gp substrate and inhibitor assay in the 12-well Caco-2 cell transwells. The total duration of the study is 3 h, with 0–1 h sample for digoxin control, 1–2 h sample for inhibitor assessment and 2–3 h sample for substrate assessment. Concentrations used of digoxin, test compound and GF120918 (potent P-gp inhibitor) were 5 μM, 10 μM and 4 μM, respectively. HPLC-UV and Liquid Scintillation Counting method were used for analytical measurement.

2.4. Combined P-gp assay procedure

The same transport media as well as the pre-study procedures as listed in the previous section were used for the combined assay. Contrary to the standard 2 h bi-directional assay, the combined assay was a 3 h procedure. The addition of the blank buffer on the apical compartment and ^{3}H-digoxin (5 μM) on the basolateral compartment initiated the study. The monolayers were then placed in an incubator at 37 °C. After 1 h incubation, 25 μL sample was taken out from the apical compartment and analyzed by liquid scintillation counter (LSC). Immediately after sampling, 0.8 μL of the test compound (10 mM in DMSO) was added to the apical compartment followed by gentle mixing to obtain a uniform concentration of 10 μM in transport buffer. About 50 μL of sample was removed immediately from the apical compartment and used for HPLC analysis to serve as the starting concentration of test compound. The monolayers were then returned back into the incubator at 37 °C. After an additional 1 h of incubation, small volumes (25 μL for LSC and 50 μL for HPLC analysis) of samples were removed both from the apical and basolateral compartment (HPLC analysis of apical sample provided the new starting concentration of test compound). Immediately after the sampling, 0.7 μL of the GF120918 (4 mM in DMSO) was added to the apical compartment followed by gentle mixing to obtain a uniform concentration of 4 μM in the compartment. Again the monolayers were returned back into the incubator at 37 °C. Finally, after additional 1 h of incubation, the monolayers were taken out and small volumes (25 μL for LSC and 50 μL for HPLC analysis) of samples were removed both from the apical and basolateral compartment. Fig. 1 illustrates the various processes and the steps that are involved in the combined P-gp assay. The proof-of-principle of the combined P-gp assay was performed by using some of the same standard compounds as used earlier in the standard bi-directional assay.

3. Results and discussions

3.1. Bi-directional assay results for standard compounds

To test the validity of the Caco-2 cell based bi-directional transport assay model, a set of well-characterized and well studied compounds were selected and studied. The permeability values for these compounds across the Caco-2 cell monolayers

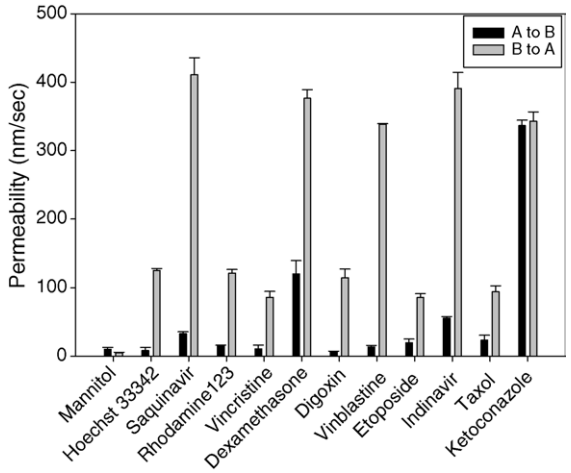

Fig. 2. Bi-directional permeability values for controls and P-gp substrates/inhibitors in the 12-well Caco-2 cell transwells. All the compounds were studied at 50 μM concentration at 37 °C for 2 h. Each column represents the mean ± standard deviation of three to six data points.

in the standard bi-directional assay are shown in Fig. 2. Mannitol permeability values were very low (<25 nm/s) in both directions suggesting that the cells had intact tight junctions. As expected, the well established P-gp substrates such as hoechst 33342, saquinavir, rhodamine123, vincristine, digoxin, vinblastine, indinavir, etoposide, dexamethasone and taxol demonstrated much higher efflux (B to A) permeability as compared to influx (A to B) permeability. The efflux ratio (ratio of efflux/influx permeability) for these compounds varied from 4 to 25. This demonstrated that the cells expressed adequate level of P-gp thus leading to a sufficiently adequate efflux ratio for these compounds. Digoxin (a well established P-gp substrate) demonstrated an efflux ratio of ∼10–15. However, ketoconazole, a potent P-gp inhibitor, demonstrated similar permeability values in both directions. Compounds that have very high intrinsic permeability values (e.g. verapamil, quinidine, ketoconazole) can significantly dominate over the efflux transporter mechanism leading to the loss of bi-directional transport. Ketoconazole lacks any bi-directional transport because it is both a high intrinsic permeability compound as well as a potent P-gp inhibitor (which would inhibit any efflux taking place). Mannitol, a P-gp non-interactor, used as a control in these studies also demonstrated similar permeability values in both directions. Therefore, the conventional cell based bi-directional assay successfully identifies P-gp substrates but fails to unequivocally identify P-gp inhibitors. To accurately identify P-gp inhibitors, an entirely different assay using ^{3}H-digoxin needs to be performed. The potential of the test compound to either inhibit the efflux (B to A) permeability and/or enhance the influx (A to B) permeability of ^{3}H-digoxin could be used to identify P-gp inhibitors.

3.2. Validation of the 3 h combined assay set-up

Studies were carried out with radiolabelled digoxin to test the hypothesis that its influx and efflux permeability remained unchanged during the 3 h incubation. These quality control studies were essential to ensure that in the extended duration (3 h) of this assay, the cells maintained their integrity as well as adequate levels of P-gp. Fig. 3 demonstrates that the bi-directional

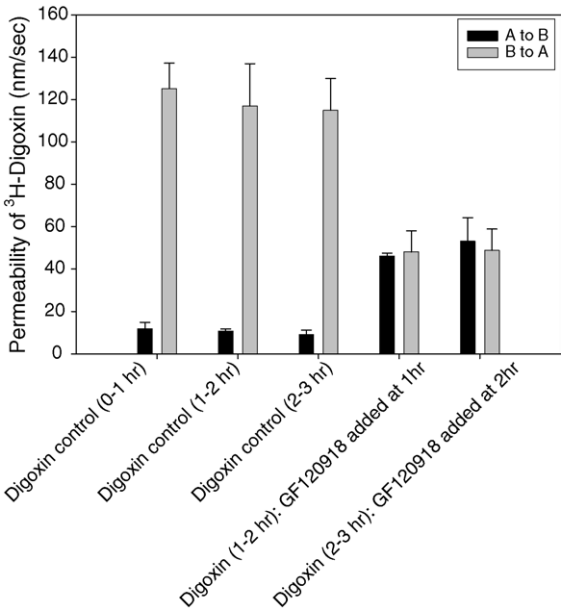

Fig. 3. Validation studies performed using ^{3}H-digoxin in the 12-well Caco-2 transwells. The total duration of the study was 3 h and the digoxin bi-directionality was assessed for three 1 h intervals. The A to B and B to A permeability of digoxin remained unchanged for the 3 h. Moreover, the effect of addition of GF120918 (significant increase in A to B permeability and significant decrease in B to A permeability) was similar whether it was added at 1 or 2 h time point.

permeability values of digoxin (P-gp substrate) were similar for the study duration from 1 to 3 h. Digoxin, used as a positive control in this assay, had an efflux ratio of ∼10-fold. There was little change in the absolute permeability values (both influx and efflux direction) within the duration of the study. The effect of potent P-gp inhibitor, GF120918, on the permeability of digoxin was also assessed. When GF120918 (4 μM) was added, the influx permeability (A to B) of digoxin more than doubled and the efflux permeability (B to A) decreased significantly as shown in Fig. 3. The effect of P-gp inhibitor was studied by adding the inhibitor either at the end of 1 h or at the end of 2 h in a 3 h study (permeability calculations were performed for 1 h duration, i.e., from 1 to 2 h or from 2 to 3 h). Permeabilities of digoxin were similar irrespective of whether the inhibitor was added at the 1 or 2 h time point.

3.3. Combined P-gp assay results for the test compounds

The test compounds used in this assay were mannitol (negative control that does not interact with P-gp), P-gp substrates such as digoxin, rhodamine123, taxol and etoposide and P-gp inhibitors such as ketoconazole and GF120918. In this 3 h study, the two most important parameters monitored were the following (1) changes in digoxin efflux (B to A) permeability in the presence of test compounds and (2) changes in the influx (A to B) permeability of test compounds when the potent P-gp inhibitor (GF120918) is added into the system. The decrease in digoxin efflux permeability in the presence of test compound was assessed by comparing the digoxin efflux permeability from 0 to 1 h (only digoxin present in the system) to the digoxin efflux permeability from 1 to 2 h (when digoxin was present along with 10 uM test compound). Fig. 4 illustrates the results obtained for the test compounds studied. Since mannitol does not interact with P-gp, as expected, digoxin efflux (B to A) permeability remained unchanged in the presence of mannitol. Similarly, digoxin efflux did not change significantly in the presence of classical P-gp substrates (i.e. pure substrates not inhibitors) such as taxol, etoposide and rhodamine123. These compounds are P-gp substrates with K_m values much higher than 10 μM (Gao et al., 2001) to show noticeable inhibition at the test

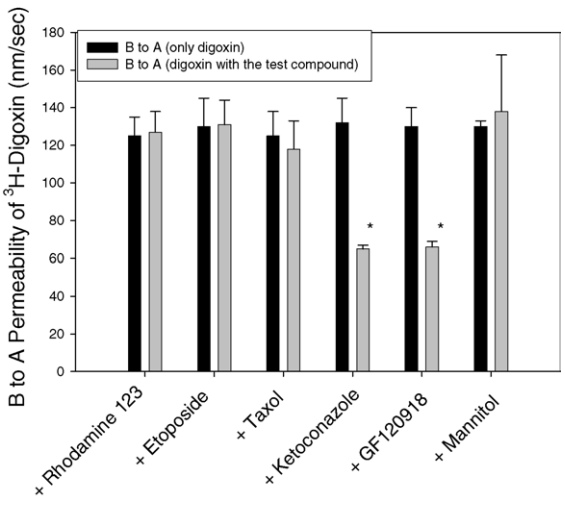

Fig. 4. Effect of various compounds on the efflux (basolateral to apical) permeability of ^{3}H-digoxin. Digoxin concentration was 5 μM and the test compounds were at 10 μM in the apical compartment. GF120918 (a specific and potent P-gp inhibitor) was used as a positive control and mannitol (paracellular marker) was the negative control. Each column represents the mean ± standard deviation of three to six data points. (* = B to A permeability for digoxin, with or without the test compound, was significantly different; $p < 0.01$).

concentration of 10 μM. Since the studies were performed at a single concentration of the test compound (10 μM), the affinity (K_m) of the compound to P-gp dictates whether or not inhibition will take place. Taken at high concentrations, even weak P-gp substrates are likely to show inhibition. Thus, at a reasonable concentration (10 μM) of test compounds, the assay discriminates between the P-gp substrates and potent P-gp inhibitors. In addition, the other interesting observation was a dramatic inhibition of digoxin efflux (B to A) permeability by potent P-gp inhibitors (ketoconazole and GF120918). Compared to the control wells, ketoconazole and GF120918 both caused ∼50% inhibition of digoxin efflux permeability. Thus, the model system can distinguish P-gp substrates from P-gp inhibitors.

The latter portion of the assay (from 2 to 3 h time duration) monitored the changes in test compounds influx (A to B) permeability in the presence and absence of GF120918 (a potent P-gp inhibitor). The basolateral samples obtained at 2 h (i.e. incubation from 1 to 2 h) provided the influx permeability of test compound by itself (i.e. no P-gp inhibitor present).

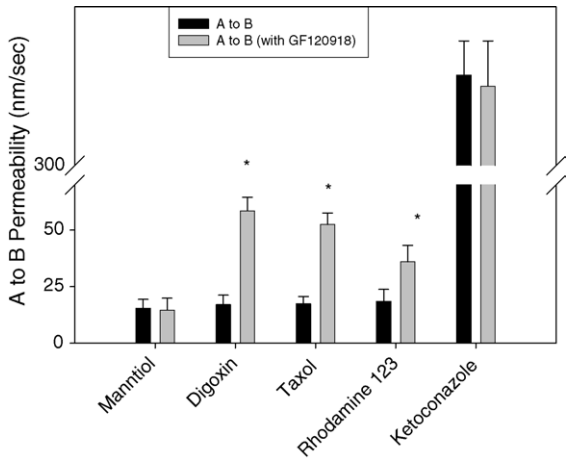

Fig. 5. Effect of addition of potent P-gp inhibitor (4 μM GF120918) on the influx permeability (A to B) of various test compounds. The test compounds were studied at 10 μM. Each column represents the mean ± standard deviation of three data points. (* = A to B permeability for the test compounds, with or without GF120918, was significantly different; $p < 0.02$).

Basolateral sampling at 3 h (i.e. incubation from 2 to 3 h) provided the influx permeability of test compound in the presence of GF120918. As shown in Fig. 5, mannitol influx (A to B) permeability remained unchanged in the presence of GF120918. However, classical P-gp substrates (i.e. pure substrates not inhibitors) such as taxol, etoposide and rhodamine123 demonstrated a dramatic increase in influx (A to B) permeability in the presence of GF120918. Compared to the control, the influx permeability of rhodamine123, taxol and digoxin increased significantly (increase of 70, 220 and 290% for rhodamine123, taxol and digoxin, respectively). This significant increase was primarily due to inhibition of the P-gp efflux activity resulting in a substantially higher influx permeability of these compounds. The significant increase in their influx permeability also suggested that P-gp potentially plays a major role in limiting the oral absorption of these compounds. Once the P-gp is knocked-out the higher intrinsic permeability of the compounds would no longer limit their absorption as exhibited by some clinical studies (Lin, 2003; Lin and Yamazaki, 2003; Malingre et al., 2001; Matheny et al., 2001). Contrary to these results, the influx (A to B) permeability of P-gp inhibitors had a very different outcome. Ketoconazole did not show any change in the influx permeability in the presence of GF120918. This lack of change could be due to the fact that these compounds have such high affinity for the transporter (either as an inhibitor or a very high affinity substrate/competitive inhibitor) that they start inhibiting their own efflux transport. Therefore, even when they are assessed singly in the Caco-2 cell system, they fail to provide an adequate efflux ratio (i.e. their A to B permeability is similar to B to A permeability).

4. Discussion and conclusions

One of the most important challenges facing the pharmaceutical industry at present is to develop high-throughput, cost-effective and predictive screening models that can be used during the decision making process early in drug discovery. Most discovery organizations have established a tiered approach with primary screening models (in silico and high throughput in vitro approaches) for screening of libraries of compounds followed by lower throughput screening models (cell culture and in vivo approaches) for selection and optimization of chemical leads. Increasingly, in addition to permeability assessment studies, P-gp interaction studies need to be performed at early stages of discovery. Various in vitro and in vivo models can be used for assessing the potential of a test compound to interact with P-gp. Normally, one or multiple model systems are utilized to identify substrates and/or inhibitors of P-gp. The modified bi-directional cell based transport assay described above can identify both substrates and inhibitors of P-gp in a single assay. In addition to providing two critical pieces of data in a single assay, one more distinct advantage of this model is that it minimizes the inter-individual well variability. Typically, the cell culture models often suffer from a large variability brought about due to different levels of expression of P-gp. These differences can either be passage-to-passage variability or even inter-well variability within a particular passage. Studies are conducted in different wells and it is often difficult to normalize the results for the variability between the wells. In the combined assay, performing the entire study in the same set of Caco-2 cells normalizes the differences between the wells. Both the control study (i.e. digoxin bi-directional values) and the follow-up study are done in the same unique well. Thus,

factors that can contribute to variability such as: level of expression of P-gp, integrity of the monolayers or any other unknowns gets minimized. Therefore, this modified assay provides a significant improvement over the traditional bi-directional protocol. Digoxin efflux ratio can vary from low of 8 to a high of 20 in different wells of the same passage of Caco-2 cells, highlighting the variability in efflux transporter expression. Thus, a combined assay described here, which normalizes for variability within the wells, can perform much more accurately and efficiently than the traditional assay. About 10 μM concentration of test compounds was used in this study and it is critical to keep that in mind when interpreting the results and making conclusions. The assay distinguishes the P-gp substrates from the P-gp inhibitors. However, certain compounds such as saquinavir, nelfinavir, etc. that are commonly identified as P-gp substrates have such high affinity for P-gp that at high enough concentrations they start functioning as competitive P-gp inhibitors. The modified assay utilizes the test compound at 10 μM and thus, is capable of distinguishing potent P-gp inhibitors from P-gp substrates (which do not have extremely potent affinity to P-gp and thus continue to act as substrates even at high concentrations). Since experimental factors such as: cell type (Caco-2, MDCK, LLC-PK1), expression level of P-gp in the cells used, incubation time, media, transport buffers, etc. can be significantly different in different labs, initial optimization of the assay conditions with regards to test drug concentration and incubation intervals is recommended prior to implementation.

In conclusion, the combined P-gp assay that can differentiate substrates as well as inhibitors in a single assay has been developed and validated. With the advantages described above over the traditional methods of P-gp studies in early discovery, the combined assay can be easily integrated into drug discovery settings providing a screening model, which is efficient, low cost and amenable to higher throughput.

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