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Parallel artificial membrane permeability assay (PAMPA) combined with a 10-day multiscreen Caco-2 cell culture as a tool for assessing new drug candidates

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The parallel artificial membrane permeability assay (PAMPA) is extensively used for the evaluation of early drug candidates. It is high throughput, low cost and is amenable to automation. This method has been shown useful in assessing transmembrane, non-energy dependent, diffusion of drugs such that reasonable predictability with *in vivo* (passive) absorption is possible. Cell cultures mimicking the gastrointestinal tract such as the CACO-2 cultures have the advantage of taking into account other transport mechanism including paracellular and carrier-mediated uptake but are lower throughput and labor-intensive. In this study, the applicability of two high throughput permeability assays namely PAMPA (PSR4p, pION Inc.) and 96-well Caco-2 cell assay (MultiScreen, Millipore) were used to rank drug permeability as well as to predict passive and active drug absorption/secretion for a series of marketed drugs as well as a collection of structurally diverse drug candidates. CACO-2 cells were cultured using MultiScreen hardware over a period of 10 days with the integrity of the cells assessed using transepithelial electrical resistance (TEER) and by the ability of the monolayer to the transport a paracellular marker, sodium fluorescence. Effective permeability (P_{eff}) data were calculated using spectrophotometric data and were binned based on a pre-defined cut-off values as either highly and poorly permeable. A comparison of a well characterized drug training set indicate at least 85% concordance between the data generated from PAMPA and Caco-2 MultiScreen. The values obtained using the MultiScreen approach were also similar to data obtained from the literature using the conventional 21-day Caco-2 cell assay. Differences between PAMPA and CACO-2 ranking were useful indicators of either drug efflux (PAMPA (P_{eff}) > CACO-2 (P_{eff})) or absorptive transport (CACO-2 (P_{eff}) > PAMPA (P_{eff})). These results indicate that PAMPA combined with the MultiScreen Caco-2 cell culture may be a useful high throughput screening for predicting passive diffusion and active transport of new drugs.

1. Introduction

Drug discovery approaches using combinatorial chemistry have a number of advantages with regard to new drug identification but also provides a large array of compounds with undrug-like properties (Lipinski 1997, 2000, 2001, 2003, 2004). As *in vivo* drug absorption experiments are expensive and unlikely to be performed early in discovery based on limited drug substance availability, the ability to predict oral bioavailability for drug candidates at an early stage is a useful selection tool in the drug development process. Based on acceptable accuracy, such a prediction methodology could identify new entities with poor oral absorption properties prior to the expenditure of significant resources as well as lower the developmental risk for those compounds which do move forward. A number of efficient and reliable approaches have evolved during the discovery phases of development that attempt to select candidates with drug properties which are most conducive to the development of a useful, marketable agent. These tools are most efficiently used at the time of "Hit-to-Lead" profiling and include a number of computer-based and *in vitro* models to determine solubility, permeability, metabolic stability and toxicology of drugs (Lipinski 2000; Yamashita et al. 2002b; Stenberg et al. 2001; Avdeef and Testa 2002).

As indicated, two of the most important parameters for drug absorbability, a necessary but not sufficient condition for oral bioavailability, are drug solubility and permeability (Leuner and Dressman 2000). A system for classification of drugs based on their aqueous solubility and membrane permeability has been implemented by the Food and

Drug administration (FDA 2002). The biopharmaceutics classification system (BCS) as described in the Scale-up and Post-Approval Change (SUPAC) guidelines of the FDA is designed to reduce the amount of work required to validate a formulation or manufacturing change post-drug approval through the granting of biowaivers for certain clinical trials. For the purposes of these guidelines, drugs can be divided into 4 classes: highly soluble and highly permeable molecules are classified as BCS Class I compounds. BSC Class II compounds are poorly soluble but permeable with their solubility limiting absorption flux. BCS Class III compounds are considered soluble but poor permeable, meaning that their permeability is rate determining. BCS Class IV compounds are considered both poorly soluble and permeable. For this class of compounds, no *in vitro-in vivo* correlation is expected (Avdeef 2003; Bergstrom et al. 2003; Amidon et al. 1995; Dressman et al. 1998, Dressman et al. 2001; Wu and Benet 2005; Yu et al. 2002). Knowledge of the BCS gives direct information on the mechanism responsible for what limits oral bioavailability and what can be done to improve the drug fraction absorbed. We applied the BCS approach to early drug candidates and assessed their permeability using various approaches. The use of solubility and permeability data in this way has been suggested by several groups and can be considered a developmental classification systems (DCS) to distinguish it from the strict regulatory definition of the BCS (Dressman et al. 2001; Augustijns and Brewster 2007). To this end, PAMPA and Caco-2 monolayers have been used as a tool to predict passive, transcellular and active permeability (efflux or secretion) of drugs (Kerns et al. 2004). In this process, the calculated effective permeability data has been binned in high or low permeable categories and these data used to assess similarities in the two approach (passive uptake) as well as differences (active uptake).

The Caco-2 cell line, originally derived from a well differentiated human colon adenocarcinoma, is the most popular *in vitro* biological tool for predicting oral absorption of drug candidates in early drug discovery programs (Hidalgo et al. 1989; Artursson and Karlsson 1991; Artursson et al. 1994; Palm et al. 1996; Hillgren et al. 1995; Rubas et al. 1996; Balimane et al. 2000). Unfortunately, the culturing of fully differentiated Caco-2 monolayers, which traditionally requires at least 21 days, is time consuming and labor-intensive, and not suitable for high-throughput permeability measurements (Chong et al. 1997; Lentz et al. 2000; Yamashita et al. 2002a). This suggests that alternatives which provide similar information may be useful. The PAMPA, high-throughput Parallel Artificial Membrane Permeability Assay, is designed for testing permeability characteristics of molecules. This model is used within pharmaceutical companies to select out compounds with poor permeability properties as soon as possible in the development cycle (Wohnsland and Faller 2001; Kansy et al. 1998; Kerns 2001; Veber et al. 2002; Zhu et al. 2002). PAMPA involves non-biological artificial membranes and thus only focuses on the prediction of passive transcellular drug absorption.

In this present work, the preparation time of fully functional Caco-2 monolayers was shortened to 10 days to increase the efficiency for large scale screening. Compared to the conventional 21-day Caco-2 cells, the 10-day MultiSreen cell monolayers presented acceptable barrier properties as assayed using transepithelial electrical resistance and the transport of a fluorescent marker. The analysis of drug permeability was directly achieved using an UV spectrophotometer, avoiding the use of HPLC or LC/MS. These data were compared to the results generated using PAMPA. Thus, a series of well-characterized model drugs as well as a number Johnson & Johnson compounds were examined with regard to permeability using the two models and the data examined and analyzed. Since PAMPA contains neither active transporters nor metabolizing enzymes, the combination of a UV-based high throughput PAMPA with the MultiScreen Caco-2 cell culture may provide a useful, fast and high capacity approach for predicting passive diffusion and active transport of drugs.

2. Investigations and results

2.1. Transepithelial electrical resistance (TEER) of MultiScreen Caco-2 cell cultures

The monolayer characteristics, including the transepithelial electrical resistance of Caco-2 cells grown in MultiScreen devices for 10 days were controlled prior to the initiation of drug transport studies. In these studies, the TEER measurements were performed using Caco-2 cell monolayers seeded within a defined passage number (40 to 50). A large numbers of Caco-2 cell monolayers was assessed. Figure 1 indicates the inter-day TEER data variation of Caco-2 cells grown on MultiScreen support filters for 10 days. The means and the standard deviation of five representative experiments ranged between 364 ± 33 and $490 \pm 187\ \Omega \cdot cm^2$, respectively indicating that the variation of TEER values was reasonable.

2.2. Transport of a paracellular marker

At the end of transport experiments, toxicity of drugs on the tight junctions of the cell monolayers was investigated by an additional measurement of the transepithelial resistance and by assessment of the transport of a sodium fluorescein marker. Compared to the initial TEER values, multiple test ranges showed a significant increase of TEER following the 2 h incubation of the cell monolayers with drug substances and 1 h with sodium fluorescein (data not shown). Little or no sodium fluorescein permeated through the cell monolayers such that the overall transport of sodium fluorescein did not exceed 1%. Therefore, cells were considered appropriate for use if their TEER values were greater than $200\ \Omega \cdot cm^2$ and sodium

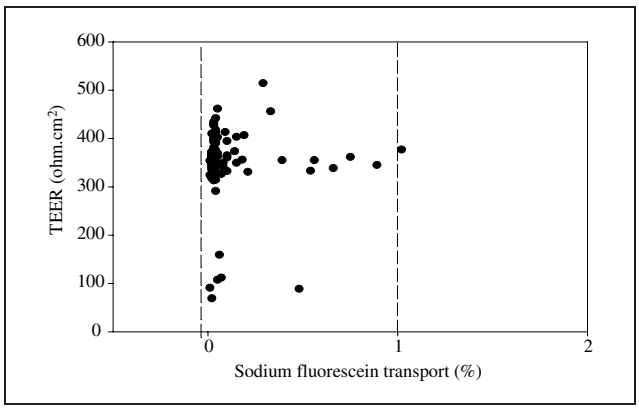

Fig. 1: Transepithelial electrical resistance (TEER) of CACO-2 cells seeded on MultiScreen inserts. The cells were cultivated for 10 days according to standard procedures as discussed in the Experimental section. Prior to drug transport experiments, the resistance of the call monolayers was controlled. Each dot represents an individual insert with the graph representing five individual experiments

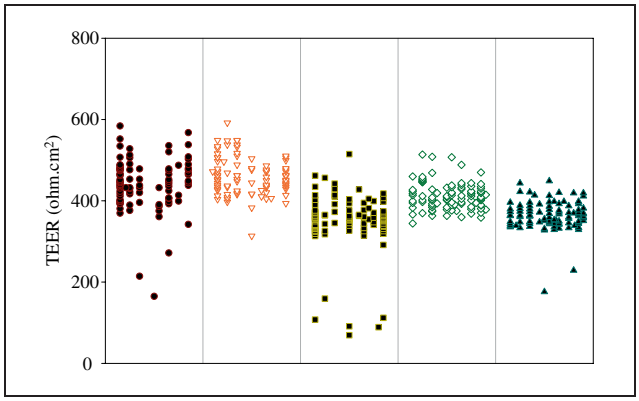

Fig. 2: Correlation between TEER and the transport of sodium fluorescein through CACO-2 cells grown for 10 days. Following the transport studies with drug s and drug candidates, the apical compartments containing the drug substance was replaced by sodium fluorescein. Cells were incubated for an additional 1 h at 37 °C. Fluorescence intensity was then recorded

fluorescein uptake was below 1% of Na-fuorescein as indicated in Fig. 2.

2.3. Permeability of model drugs across the artificial membrane and Caco-2 cells

In this assessment, the permeabilities of drugs were ranked into 2 classes, $>0.5 \times 10^{-6}$ and $<0.5 \times 10^{-6}$ cm/s for high and low permeable drugs, respectively. The permeability of 23 marketed drugs was first evaluated through PAMPA. The effective permeability coefficients of high permeable drugs were ranged between 0.51 to 16.11×10^{-6} cm/s and the P_{eff} values of low permeable model compounds, as calculated using the cut-off values, were less than 0.5×10^{-6} cm/s. In the CACO-2 monolayers, the transport of these drugs was observed from the apical to the basolateral compartments of Caco-2 cell monolayers grown in the MultiScreen devices for 10 days. The effective permeability coefficients obtained indicate that the P_{eff} of highly permeable drugs ranged from 0.9 to 1.9×10^{-6} to cm/s and the low permeable drugs were lower than 0.49×10^{-6} cm/s. The P_{eff} values of highly permeable were significantly higher in PAMPA compared to the analogous values measured in the Caco-2 cell system ($p < 0.05$), while the low permeable showed similar results in the two assays. The relationship between the rank order of drugs as evaluated using PAMPA and CACO-2 cells is described in Table 1. A comparison was made between the data obtained from the present study and the literature. As mentioned in the Table, the permeability rank order of 23 compounds obtained using Caco-2 cells grown in Multiscreen devices was similar to the their ranking from the traditional 21-day Caco-2 cells. In addition, 87% of the model compounds were similarly categorized as either highly or poorly permeability based on the Caco-2 Multiscreen and PAMPA assays.

Table 1: Permeability rank order for model drugs across both PAMPA and MultiScreen CACO-2 cell monolayers compared with CACO-2 assessed using standard culturing protocols

Compound	Caco-2(96 wells)	PAMPA	Literature
Antipyrine	H	H	H
Caffeine	H	H	H
Carbamazepine	H	H	H
Chlorpromazine	H	H	H
Griseofulvin	H	H	H
Indomethacin	H	H	H
Metoprolol	H	H	H
Piroxicam	H	H	H
Propranol	H	H	H
Quinidine	H	H	H
Acebutolol	L	L	L
Acycloguanisine	L	L	L
Amoxicillin	L	L	L
Atenolol	L	L	L
Cephalexin	L	L	L
Chloramphenicol	H	L	H
Chlorothiazide	L	L	L
Doxorubicin	L	L	L
Furosemide	L	L	L
Ranitidine	L	L	L
Salicylic acid	H	L	H
Sulfasalazine	L	L	L
Theophilline	H	L	H

H: $P_{eff} > 0.5 \times 10^{-6}$ cm/s
L: $P_{eff} < 0.5 \times 10^{-6}$ cm/s

Table 2: Permeability ranking concordance between PAMPA and a MultiScreen CACO-2 assay in a two class (high/low) binning system for a series of J & J discovery candidates

Comparison between PAMPA and Caco-2 cells (96 wells)		
Compounds tested	143	
Compounds identical classified	130	91%
Compounds 1 class higher in Caco-2 than in PAMPA	5	3%
Compounds 1 class Lower in Caco-2 than in PAMPA	8	6%

2.4. Ranking of Johnson & Johnson compounds

The uptake of 143 J & J compounds covering a large chemical space was investigated. The data generated from

Table 3: Drug retention (%) in either PAMPA or MultiScreen CACO-2 monolayers after drug transport studies

	PAMPA	CACO-2
Caffeine	0	39
Chloropromazine	64	58
Amoxicillin	0	0
Carbamazepine	6	39
Indomethacin	4	38
Cephalexin	4	22
Griseofulvin	25	36
Propanolol	19	39
Cimetidine	29	70
Acyclovir	0	15
Antipyrine	3	58
Methotrexaat	1	17
Ranitidine	5	22
Atenolol	0	0
Salicylic acid	0	26
Labetalol	11	55
Sulfasalazine	2	15
Furosemide	0	21
Metoprolol	2	15
Nadolol	0	13
Theophilline	0	32
Chlorothiazide	0	33
Quinidine	14	30
Doxorubicin	14	13
Piroxicam	1	44
Chloramphenicol	0	31
Acetobutol	0	36

PAMPA were compared to those obtained from the Multi-Screen Caco-2 cell assay. The results of the compounds tested indicate that 91% were identically classified as low or high permeable in Multiscreen Caco-2 cells and PAMPA while there was 9% discordance (Table 2).

2.5. Membrane retention of drugs

To avoid underestimation of permeability data, the membrane retention of model drugs or J & J compounds was taken into account during the assessment of drug permeability through PAMPA or Caco-2 cells. The data suggested significant drug retention in the Caco-2 cell monolayers with lower drug residing in PAMPA lipid membranes. Table 3 indicates the percentage of model compounds retained in the artificial or biological membranes. Chlorpromazine showed 64% membrane retention while most of the standard drugs assessed through PAMPA were less retained (up to 26%). High membrane retentions of model compounds were however obtained in Caco-2 cell model with values ranging from 0 to 70%.

3. Discussion

More and more, the pharmaceutical industry is automating permeability assays in order to increase the throughput of drug screenings and compound selection. Automated systems for Caco-2 cell permeability assays are commercially available through Tecan/BD Bioscience and Bohdan Mettler Toledo (van de Waterbeemd et al. 2003). In these automated systems, 14- to 18-day old Caco-2 cell monolayers are used for drug screening however information on the effectiveness of these methodologies is limited (Garberg et al. 1999). As cell-based permeability assays are relatively expensive, mainly due to the cost of maintaining the cell cultures, alternatives based on artificial membranes have been considered. These non-cell based permeability assays, including PAMPA, are fully automated allowing for the assessment and quantitation of drug permeability and membrane retention. This is possible through an integrated 96-well plate UV spectrophotometer. The assay is relatively fast, inexpensive, and straightforward.

In this study, PAMPA was used to rank compounds with regard to their ability to penetrate membranes. When penetrating biological barriers, drugs may have simultaneous access to several different mechanisms of transport, including the paracellular and transcellular routes as well as active and facilitated transport via molecular carriers. The majority of drugs (>80%) have been reported to enter the blood stream by passive diffusion through the intestinal epithelium (Mandagere et al. 2002). Consequently, permeability assays that measure passive transport through lipophilic barriers can be correlated with human drug absorption values realizing that absorption is only one of many process that have to occur in order for a material to be bioavailable (van de Waterbeemd and Gifford 2003). Caco-2 cells grown in MultiScreen devices were characterized in the present study. The monolayer formation was confirmed based on results from transepithelial electrical resistance and the permeability of sodium fluorescein. The TEER values remained constant over a period of 21 days, suggesting that the 10-day Caco-2 cells were fully differentiated and appropriate for use in drug transport experiments.

Generally speaking, the PAMPA assay provides the benefits of a biologically relevant membrane and the ability to tailor that membrane to a specific target such as the blood-brain barrier. Unlike Caco-2 systems, uptake assays are compatible with pH profiling over a fairly broad range. Due to the high variability of pH in the lumen of the G.I. tract, pH profiling can help better predict drug behavior. For most models, a binning approach is favored over linear correlations. Using high- and low-permeability classes, both PAMPA and CACO-2 can provide data that categorically agree with the human *in vivo* jejunum permeabililty.

As described, a number of different mechanisms are involved in drug transport across cell barriers. The most important of these are transcellular passive diffusion, paracellular diffusion, active transport with a transporter and vesicular and other types of transcytosis. In addition, the drug can be metabolised at the cell wall by CYP3A4 and/or effluxed by P-gp or Multi Resistance Protein (MRP) (van de Waterbeemd et al. 2003). PAMPA provides insight into the passive diffusion of drugs. The PAMPA method can complement the more complicated mechanistic assays which are mainly of interest in examining the active transport of molecules. It has been demonstrated that the outliers in PAMPA assay are solutes known to be actively transported (Avdeef 2003; Kerns et al. 2004). Unfortunately results from Caco-2 cells for actively transported compounds may incorrectly predict the extent of oral absorption, due to possible quantitative under- or overexpression of the active transporter system. In our study, the low concentration of drugs used for transport experiments may overcome problems related to the saturation of the transporters. The PAMPA setup used in this study allows calculation of drug retention in the membrane. The difference between the percentage of retention in Caco-2 compared to PAMPA is likely explained by the composition of the membrane lipids. The most common lipid components of membranes are phosphotidylcholine (PC) and phosphotidylethanolamine (PE). Phospholipids account for 46% of the outer leaflet membrane constituents with PC and sphingomyelin (SP) about equal the amount (Rawn 1989) It has been shown that in PAMPA, the composition of lipid bilayers can influence the retention of drugs in the membrane with a 2% DOPC showing low retention compared to a systems with 10% or higher lecithin content. Cell culture assays are also subject to drug retention by the lipid membranes. High retention has been reported in Caco-2 cells (Sawada et al. 1994). As long as the retention is less than 90%, most drugs have sufficient UV absorptivity to be adequately characterized when the initial concentration is $\sim 50\ \mu M$.

An assessment of the data is useful in the context of the work by Kerns et al. (2004). In their combination of PAMPA and CACO-2 data, a series of equalities and inequalities were defined such that:

- if PAMPA and CACO-2 correlate then passive diffusion is the likely uptake mechanism,
- if PAMPA $\ll$ CACO-2 then absorptive transport is assumed to occur,
- if PAMPA $\gg$ CACO-2 then secretory transport is assumed to occur.

In their screen of model compounds, theophylline, antipyrine and caffeine were deemed to undergo absorptive transport. In the current assessment, data from Table 1 suggests that theophylline can be similarly categorized however there is not a difference between the CACO-2 and PAMPA data sufficient enough to categorize antipyrine and caffeine in this way. Interestingly, in the work of Kerns et al. (2004), theophylline shows the greatest deviation from the diagonal meaning that the current method may not have

the bandwidth to pick up smaller differences. Likewise model compounds that are subject to secretory transport were not demonstrated in the training set in the current study while propranolol was cited as an example in the work of Kerns et al. In addition, as demonstrated in this work other compounds using transporters (i.e., acetyl salicylic acid) were poorly permeable in PAMPA (Yee 1997). For the J & J set of compounds, 130 were similarly classified and thus may be transported by simple membrane diffusion. Five (3%) appeared to be candidates for absorptive transport and 8 (6%) for secretory transport. Armed with this type of information, earlier intervention may be possible with regard to compound selection or approaches to improve biopharmaceutical properties of the candidate.

4. Experimentals

4.1. Chemicals

All chemicals were purchased from Sigma (Bornem, Belgium) unless otherwise stated. Sodium fluorescein was provided by Across (Geel, Belgium). All supplements and cell culture media were purchased from Invitrogen Inc. (Merelbeke, Belgium).

4.2. Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (ATCC), and were used between passage 40 and 50. The cultures were mycoplasma free (mycoplasma detection kit; Roche Gmbh, Mannheim, Germany). The cells were maintained in 175 cm^2-plastic culture flasks (BD Biosciences, Erembodegem, Belgium). The cells were subcultivated before reaching confluence. Caco-2 cells were harvested with 0.25% trypsin and 0.2% EDTA (5–15 min) at 37 °C and seeded in new flasks. The culture medium, Dulbecco's Modified Eagle Medium (DMEM), was supplemented with 1% non-essential amino acids (NEAA), 2 mM L-glutamine, 100 U/ml penicilline/streptomycine and 10% foetal bovine serum (FBS). The cells were seeded in 96-well MultiScreen Caco-2 (Millipore, Brussels) in 75 μL volumes at 4.6×10^5 cells/well for 10 days. They were fed basolaterally and apically with 250 μL and 75 μL of fresh medium every other day, and were incubated at 37 °C, 5% CO_2 for 10 days.

4.3. Measurement of membrane integrity

Measurement of transepithelial electrical resistance (TEER) was used to determine the integrity of the cell monolayers. The resistance of the cell monolayers grown on 96-well MultiScreen Caco-2 devices was measured using an Evom resistance voltohm meter (World Precision Instruments, Berlin, Germany).

4.4. Drug transport through 96-well MultiScreen Caco-2

Prior to the transport of model compounds, the apical compartment of the cell monolayers was washed with HBSS buffer and allowed to equilibrate for 30 min at 37 °C. For the apical to basolateral transport, the buffer was removed from the cells and replaced with 150 μl of the test compound (25–50 μM) on the apical side while the basolateral compartment was replaced with 250 μl of the transport buffer. The monolayers were incubated for 120 min at 37 °C, on a Vibrax shaker at 80 rpm. At 0 and 120 min, the resistance was measured. The transport experiments were performed under "sink" conditions, when the concentration of the drug in the receiver side (250 μl) was $<$ 10% of the dosing concentration. The amount of drug accumulated in the basal compartment was determined at different time points: 15, 30, 60, 90 and 120 min.

4.5. Transport of sodium fluorescein

The paracellular transport was assessed using sodium fluorescein. After an initial period of incubation with the test compound, the cell monolayers were rinsed with Hanks buffered saline (HBSS) and incubated with sodium fluorescein for an additional time of 60 min at 37 °C, on a Vibrax shaker at 80 rpm. The diffusion of sodium fluorescein into the basal compartment was determined by measuring the fluorescence intensity, excitation 485 nm, emission 535 nm, using a Tecan Genios (Tecan Benelux, Mechelen).

4.6. Parallel artificial membrane permeability assay (PAMPA)

The PAMPA system is a sandwich assay formed from a 96-well microplate and 96-well microfilter plate, such that each composite well is divided into two chambers: donor at the bottom and acceptor at the top. The two chambers are separated by a 125 μm-thick microfilter disc, coated with 2% wt/vol solution of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) in dodecane containing 0.1% BHT (2,6-di-tert-butyl-4-hydroxytoluene), under conditions such that multilamellar bilayers form inside the filter channels when the system is in contact with an aqueous buffer solution. The system uses 10 μl of a haystack solution of the drug candidate (in DMSO) in a 96-well microtiter plate, allowing up to 384 analyses (four plates) in a 24-hour period.

4.7. Drug transport through the artificial membrane

A Tecan Genesis robotic system was used to perform all solution transfers. The phosphate-free universal buffer system (*p*ION) was used as the system solution for the robotics system. Compounds were dissolved in DMSO to 5–10 mM concentration. A Millipore multiscreen®-IP clear plate with hydrophobic filter membrane (0.45 μm pores) was used as the acceptor plate. The 96 well custom donor plate was obtained from *p*ION and was filled with phosphate-free universal buffer system (*p*ION, Worburn, MA) and adjusted with 0.5 M KOH to pH 4 or 7.4. The test compounds were diluted in the buffer to a final concentration of 25–50 μM. The UV spectrum of this reference solution was taken scanning from 190–500 nm. The *p*ION plate was then filled with diluted drug solutions to prepare the 'donor' wells. The lipid DOPC as a 2% solution in dodecane, obtained in sealed ampules from *p*ION and maintained at −20 °C until use, was applied to the filter plate by the robotics system using 4 μl per well of the 2% DOPC solution. The wells defined as "receptor" were then filled with 200 μl of system solution buffer, covered and placed at the top of the donor plate, and incubated at room temperature for 18 h. After this incubation time the PAMPA sandwich of plates were separated. The amount of drugs in the donor and acceptor wells was determined by UV spectroscopy performed in 96-well Greiner Star UV plates using a Molecular Devices Spectramax 190 spectrophotomer. Mass balance was used to determine the amount of material retained in the membrane. Permeability was calculated as follows. All experiments were performed at room temperature and in triplicate.

4.8. Permeability analysis

The effective permeability Peff (cm/s) of a compound was calculated according to Eq. (1) (Avdeef et al. 2001):

$$C_A(t) = \left(\frac{M - m}{V_D + V_A}\right) + \left(C_A(0) - \frac{M - m}{V_D + V_A}\right) e^{-P_e A \left(\frac{1}{V_D} + \frac{1}{V_A}\right) t}$$

where $C_A(t)$ = is the concentration of the drug (mol/cm^3) in the acceptor well at time t, M = total amount of compound (mol), m = amount lost to membrane (mol), VD = volume of donor well, VA = volume of acceptor well (0.2 cm^3), A = effective area of membrane (0.3 cm^2), t = permeation time.

4.9. Statistical analysis

Statistical analysis was completed using analysis of variance (Anova) (Statgraphics Plus). Multiple range tests were applied to determine which means were significantly different from others.

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