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Kinetics of lipid bilayer permeation of a series of ionisable drugs and their correlation with human transporter-independent intestinal permeability

Katharina F. Hermann[a], Claudia S. Neuhaus[a], Virgine Micallef[b], Björn Wagner[b], Maja Hatibovic[a], Hélène E. Aschmann[a,1], Franziska Paech[a,2], Rubén Alvarez-Sanchez[b], Stefanie D. Krämer[a,*], Sara Belli[b,**]

[a] Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, ETH Zurich, Vladimir-Prelog-Weg 4, CH-8093 Zurich, Switzerland
[b] Pharmaceutical Sciences, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd., Grenzacherstrasse 124, CH-4070 Basel, Switzerland

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ABSTRACT

For low molecular weight drugs, lipid bilayer permeation is considered the major route for *in vivo* cell barrier passage. We recently introduced a fluorescence assay with liposomes to determine permeation kinetics of ionisable compounds across the lipid bilayer by monitoring drug-induced pH changes inside the liposomes. Here, we determined the permeability coefficients (P_{FLipP}, FLipP for "Fluorescence Liposomal Permeability") across 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers of 35 ionisable drugs at pH 6.0 and compared them to available *in vivo* human jejunal permeability (P_{eff}) data. P_{FLipP} values were furthermore compared with published Caco-2 cell permeability coefficients ($P_{Caco\text{-}2}$), permeability coefficients determined with the parallel artificial membrane permeability assay (PAMPA) and with log *D* (pH 6.0). The log P_{FLipP}, corrected for predicted para-cellular diffusion, and log $P_{Caco\text{-}2}$ correlated best with log P_{eff}, with similar adjusted R^2 (0.75 and 0.74, n = 12). Our results suggest that transporter-independent intestinal drug absorption is predictable from liposomal permeability.

1. Introduction

In preclinical drug development, active compounds are evaluated at an early stage for their capability to cross biological barriers. This allows filtering compounds for favourable properties with respect to intestinal absorption and distribution to the pharmacological target and helps reducing the attrition rate later in development (Di et al., 2013). Common *in vitro* assays to predict barrier permeation are either cell-based, such as the Caco-2 cell permeability assay, or cell-free, such as the Parallel Artificial Membrane Permeability Assay (PAMPA). The enterocyte-like Caco-2 cells, a cell line originating from human epithelial colorectal adenocarcinoma, form monolayers in culture mimicking the intestinal drug barrier. For the permeability test, the compound of interest is added to the donor compartment of a two-chamber system separated by the cell monolayer cultured on a semi-permeable membrane and its appearance in the opposite compartment is determined (Hubatsch et al., 2007). Compound permeation across the cell layer is expressed as the apparent permeability coefficient (P_{app}), which is determined by the lipid-bilayer permeability, para-cellular diffusion, carrier-mediated uptake, active drug influx or efflux and diffusion across the aqueous boundary layer, alternatively called unstirred water layer (Krämer, 2016). Good correlations have been reported between Caco-2 cell logarithmic P_{app} (designated log $P_{Caco\text{-}2}$ in this work) and human intestinal permeability, log P_{eff} (Alsenz and Haenel, 2003; Sun et al., 2002). Major drawbacks are the 21 day-requiring cell culture and the high inter- and intra-laboratory variations due to heterogeneous properties of the cells. Furthermore, the extent of intestinal absorption of substrates of uptake carriers or efflux transporters may be under- or overestimated due to differences in transporter expression between *in vitro* and *in vivo* (Matsson et al., 2015; Olander et al., 2016). Drug-metabolizing enzymes are expressed in Caco-2 cells but in general at lower levels than in the human duodenum (Sun et al., 2002). Compound metabolism may thus vary between *in vitro* and *in vivo*. Although desirable for physiologically-based pharmacokinetic (PBPK) modelling and to predict permeability across non-intestinal barriers (Heikkinen et al., 2015), the individual permeation mechan-

* Correspondence to: S.D. Krämer, Inst. Pharm. Sciences, ETH Zürich, Vladimir-Prelog-Weg 4, CH-8093 Zurich, Switzerland.
** Correspondence to: S. Belli, Pharmaceutical Sciences, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd., Grenzacherstrasse 124, CH-4070 Basel, Switzerland.
E-mail addresses: stefanie.kraemer@pharma.ethz.ch (S.D. Krämer), sara.belli@roche.com (S. Belli).
[1] Present Address: University of Zurich, Epidemiology, Biostatistics & Prevention Institute (EBPI), Hirschengraben 84, CH-8001 Zurich.
[2] Present Address: Clinical Pharmacology and Toxicology, University of Basel, Hebelstrasse 20, CH-4056 Basel.

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isms cannot easily be assessed with this assay.

PAMPA aims to assess exclusively passive, transporter-independent transmembrane drug permeation (Avdeef et al., 2007; Kansy et al., 2004; Tsinman et al., 2011). Unlike the Caco-2 cell permeability assay where a cell-monolayer separates two aqueous compartments, in PAMPA a layer of lipids dissolved in an organic phase forms a lipophilic permeation barrier. Otherwise the principle is similar as for the Caco-2 cell permeability assay: the test compound is added to the donor compartment and permeability is determined from the mass transfer to a receiver compartment and expressed as log P_{app} (designated log P_{PAMPA} in this work). Since PAMPA is a cell-free assay, it offers a fast and cost-effective method that can be fully automated leading to a higher throughput than Caco-2 cell permeability assays.

Both PAMPA and Caco-2 cell permeability assays require sampling and quantification of the test compounds by a suitable method such as UV absorbance measurements. The presence of an unstirred water layer adjacent to the aqueous-barrier interface can be a confounding factor in both assays as it adds an additional diffusion barrier for the solute (Youdim et al., 2003). Although an aqueous boundary layer with low convection is also present adjacent to the intestinal mucosa, its 30–100 µm thickness (Lennernäs, 1998) is minor compared to the 1500 µm or more in Caco-2 cell permeability and PAMPA assays in the absence of stirring (Avdeef et al., 2004; Karlsson and Artursson, 1991). Experimental P_{app} values, therefore, result from a combination of permeation across the barrier itself and diffusion across the unstirred water layer. For high permeability compounds, the latter can be the rate-limiting step for permeation. Stirring can reduce the thickness of the water layer in the PAMPA assay to better mimic the *in vivo* situation (Avdeef et al., 2004).

Liposomes offer an ideal barrier model to determine log P_{app} across lipid bilayers (see *e.g.*, Tejwani and Anderson (2008)). Liposomes as barrier models reduce the maximal possible thickness of the unstirred water layer to the ratio of outer aqueous volume to barrier surface area, which is in the single-digit micrometer range or below at lipid concentrations above 0.4 mg/mL (Krämer, 2005). Liposomes can be prepared easily as homogenous vesicles with the desired lipid composition and their bilayer structure resembles the cell membrane lipid part more closely than PAMPA barriers, which contain a significant fraction of solvent. We recently developed an *in vitro* assay to study the kinetics of lipid bilayer permeation of basic drugs making use of liposomes containing the fluorescent pH-sensitive probe 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS, pK_a 7.0) in the aqueous lumen (Supplementary Fig. 1 and Eyer et al. (2014)). Once the neutral species of the basic test compound permeates across the lipid bilayer and reaches the inner lipid leaflet or the aqueous lumen, it immediately adapts its pH-dependent protonation state, taking up a proton. This results in a change of the ionisation state of the fluorescent probe, and consequently its fluorescence intensity (Eyer et al., 2014). Permeation of a weak base, therefore, results in an increase of HPTS fluorescence which can be monitored with a stopped-flow apparatus. The kinetics of fluorescence change reflect the kinetics of drug equilibration across the membrane. We designate the assay "Fluorescence Liposomal Permeability" (FLipP) in this work. Permeation kinetic parameters such as permeability coefficients (P_{FLipP}) can be calculated from the kinetics of fluorescence change and the liposomal hydrodynamic diameter (Eyer et al., 2014). Compounds without ionisable functionality as well as compounds with negligible ionised fraction at the experimental pH cannot be measured by the liposomal fluorescence assay due to their lack of ionisation change during the permeation process and, thus, lack of change in HPTS fluorescence emission.

In this work, we addressed the question whether this liposomal assay can predict intestinal drug permeability. We chose 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) as lipid to prepare the liposomes as it is abundant in the intestinal barrier and is in its liquid crystalline phase at temperatures above 0 °C, mimicking the fluidity of the permeable domains of the cell plasma membranes (Bernhard et al., 1995; Ehehalt et al., 2004). A pH of 6.0 was chosen as an approximation to the microclimate pH at the mucosal surface of the small intestine where most drugs are absorbed (Dahlgren et al., 2015; Shiau et al., 1985). We compared log P_{FLipP} values with published human intestinal log P_{eff} values (Dahlgren et al., 2015). These log P_{eff} values result from direct *in vivo* determinations in the human gastrointestinal tract with single-pass perfusion systems Loc-I-Gut. We evaluated our assay in comparison with the Caco-2 cell permeability assay, PAMPA, drug physico-chemical properties, and predicted log P_{eff} from an *in silico* model for quantitative structure-ADME property relationships. The liposomal assay was originally designed for basic compounds (Eyer et al., 2014). In this work, we further optimized it to allow the inclusion of acids and complex ampholytes in the test set (Table 1). The test set furthermore contained some drugs beyond Lipinski's rule of 5 (Lipinski et al., 2001).

2. Material and Methods

2.1. Materials

Amoxicillin, antipyrine, chlorpromazine, cimetidine, desipramine, domperidone, doxycycline hyclate, emetine di-HCl hydrate, erythromycin, furosemide, gentamycin sulfate, hydrochlorothiazide, irinotecan, isotretinoin, labetalol HCl, loperamide, (±)-metoprolol tartrate, salicylic acid, ranitidine HCl, (±)-verapamil HCl and HPTS were purchased from Sigma-Aldrich (Buchs, Switzerland) and atenolol, carbamazepine, methotrexate hydrate, nadolol, oxytetracyclin, (±)-propranolol HCl, rifampicin, tetracyclin from Fluka (Buchs, Switzerland). Penicillin V and piroxicam were from abcr (Karlsruhe, Germany). Terbutaline was from Prestwick Chemicals (Illkirch, France). Amiloride, cefazolin, enalapril maleate, josamycin, ketoprofen, naproxen, nelfinavir, novobiocin, saquinavir were synthesized at F. Hoffmann - La Roche with > 93% purity. POPC was purchased from Anatrace Products, Maumee, OH, USA. All other chemicals were of analytical grade (> 95% purity) from Sigma-Aldrich, Fluka or as indicated.

2.2. Preparation of Vesicles

Liposomes containing HPTS were prepared by lipid film hydration and extrusion as described previously (Eyer et al., 2014) with some modifications. In brief, a POPC solution in methanol/chloroform (1/1) was evaporated at 37 °C under vacuum to form a lipid film in a round flask. The lipid film was flushed with nitrogen before 200 mM NaCl containing 10 mM HPTS if not stated otherwise was added to result in a POPC concentration of 10 mg/mL. The lipids were hydrated at 37 °C for 30 min to form multilamellar vesicles. These were submitted to 10 cycles of freeze-thaw in isopropranol/dry ice and a 37 °C water bath and subsequently to 15 extrusion cycles through two stacked polycarbonate membranes with pore size 200 nm (Nucleopore Track-Etch Membrane, Whatman, UK) by means of a 10 mL thermobarrel extruder (LipexTM, Northern Lipids, Canada) at 37 °C. The outer aqueous phase of the liposomes was exchanged to 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)/145 mM NaCl, pH 6.0 by means of an equilibrated size exclusion column (PD-10 SephadexTM G-25M, GE Healthcare, UK). The average mean hydrodynamic diameter of the final liposomes was 167.7 ± 10.7 nm with a polydispersity index of 0.10 ± 0.02 as determined by dynamic light scattering with a Zetasizer 3000HSA (Malvern Instruments, Malvern, UK). The liposomes were stored at 4 °C in the dark for up to one week prior to the kinetic measurements.

The luminal HPTS concentration of the liposomes prepared with 10 mM HPTS was estimated to 6.3 ± 0.6 mM. It was determined in triplicates from one liposome preparation by lysing the liposomes with 1% triton-X 100 and comparing the fluorescence signal measured in a plate reader (Synergy HT, BioTek Instruments, Luzern, Switzerland) with standard solutions containing known concentrations of HPTS, HPTS-free liposomes, triton-X 100 and buffer at the respective con-