Title:

*In vitro* predictability of drug-drug interaction likelihood of P-glycoprotein-mediated efflux of dabigatran etexilate based on $[I]_2 / IC_{50}$ threshold

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P-gp-mediated DDI potential of dabigatran etexilate

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AtoB, apical-to-basal, AUC, area under the curve, BtoA, basal-to-apical, CES, carboxylesterase, DDI, drug-drug interaction(s), DMEM, Dulbecco’s modified Eagle’s medium, EMA, European Medicines Agency, FBS, fetal bovine serum, FDA, Food and Drug Administration, high performance liquid chromatography, HPLC, NEAA, non-essential amino acids, Papp, apparent permeability coefficient, P-gp, P-glycoprotein, TEER, transepithelial electrical resistance.
Abstract:

Dabigatran etexilate, an oral, reversible, competitive and direct thrombin inhibitor, is an *in vitro* and *in vivo* substrate of P-glycoprotein (P-gp). Dabigatran etexilate was proposed as *in vivo* probe substrate for intestinal P-gp inhibition in a recent guidance on drug-drug interactions (DDI) from the European Medicines Agency (EMA) and the Food and Drug Administration (FDA). We conducted transcellular transport studies across Caco-2 cell monolayers with dabigatran etexilate in the presence of various P-gp inhibitors to examine how well *in vitro* IC₅₀ data in combination with mathematical equations provided by regulatory guidances, predict DDI likelihood. From a set of potential P-gp inhibitors, clarithromycin, cyclosporin A, itraconazole, ketoconazole, quinidine and ritonavir inhibited P-gp-mediated transport of dabigatran etexilate over a concentration range that may hypothetically occur in the intestine. IC₅₀ values of P-gp inhibitors for dabigatran etexilate transport were comparable to those of digoxin, a well-established *in vitro* and *in vivo* P-gp substrate. However, IC₅₀ values varied depending whether they were calculated from efflux ratios or permeability coefficients. Prediction of DDI likelihood of P-gp inhibitors using IC₅₀ values, the hypothetical concentration of P-gp inhibitors and cut-off value recommended by both the FDA and EMA, were in line with the DDI occurrence in clinical
studies with dabigatran etexilate. However, it has to be kept in mind that validity of the cut-off criteria proposed by the FDA and EMA depends on \textit{in vitro} experimental systems and the IC$_{50}$ calculation methods that are employed as IC$_{50}$ values are substantially influenced by these factors.
Introduction:

With the progress in the field of drug transporter research, transporter mediated drug-drug interactions (DDI) are being reported with increasing frequency, thus making it inevitable to study potential interaction during the process of drug development. P-glycoprotein (P-gp) is one of the drug transporters expressed in the gastrointestinal tract and is involved in the efflux of various kinds of drug into the lumen [Ambudkar et al., 2003; Schinkel et al, 2003]. Since local drug concentrations in the intestinal lumen may be high after oral administration and complete dissolution of the drug, intestinal P-gp can be effectively inhibited by drugs, which in return can result in increased exposure of any coadministered drug which is a substrate of P-gp. It is therefore essential with regard to safety and efficacy of drugs to recognize or predict potential DDIs in the intestine. Recently, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) issued guidances on DDI describing methods how to investigate DDIs in vitro and providing decision trees to judge about the necessity to conduct clinical DDI studies [FDA draft guidance, 2012; EMA guideline, 2012]. The ratio of the concentration of a putative inhibitor at DDI site (for gastrointestinal tract, [I]2: maximum oral dose taken at one occasion / 250 mL of assumed intestinal fluid volume) to the in vitro inhibition potency such as IC₅₀ and Ki value was
Dabigatran etexilate (PRADAXA®) has been approved in several countries for reducing the risk of stroke and systemic embolism in patients with non-valvular atrial fibrillation.

Dabigatran etexilate is a pro-drug and is rapidly hydrolyzed to active dabigatran via two short-lived intermediate metabolites after oral administration [Blech S et al., 2008]. In vitro studies showed that dabigatran etexilate is a substrate of P-gp, whereas the active drug dabigatran is not a substrate of P-gp [Ishiguro N, manuscript in preparation].

The current study was designed to investigate whether intestinal P-gp-mediated dabigatran etexilate transport is affected by P-gp inhibitors and to evaluate whether the DDI likelihood assessment for intestinal P-gp inhibition by in vitro data following regulatory guidances fits to the results found in clinic. For this, transcellular transport assays across the Caco-2 cell monolayers were conducted and the in vitro IC_{50} values were determined to assess the DDI likelihood of dabigatran etexilate with P-gp inhibitors and to evaluate the consistency of in vitro assessment to the clinical findings.
Materials and Methods:

Chemicals

\[^{14}C\]Dabigatran etexilate, dabigatran etexilate and BIBR 1087, ester cleavage of dabigatran etexilate [Blech S et al., 2008], and linagliptin were synthesized at Boehringer Ingelheim Pharma GmbH & Co. KG (Biberach, Germany). \[^{3}H\]Digoxin was obtained from PerkinElmer (Waltham, MA). Amiodarone, cyclosporin A, digoxin, itraconazole, ketoconazole, quinidine and tacrolimus were purchased from Sigma-Aldrich (St. Louis, MO). Clarithromycin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Nelfinavir and ritonavir were purchased from Toronto Research Chemicals (Toronto, Canada). Dulbecco’s modified Eagle’s medium (DMEM) with 3.7 g/L sodium bicarbonate was from BioChrom AG (Berlin, Germany). Fetal bovine serum (FBS), non-essential amino acids (NEAA), penicillin-streptomycin and L-glutamine were from Invitrogen (Carlsbad, CA). Collagen R solution was from Serva (Heidelberg, Germany). All other chemicals were of the highest reagent grade available from commercial sources.

Biological materials

Caco-2 cells were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Pooled human liver
microsomes were prepared in-house from human liver tissue. Liver tissue of 10 male and female donors was supplied by Tissue Transformation Technologies (Edison, NJ, USA). The tissue was homogenized with ice-cold 0.1 M phosphate buffer, pH 7.4, containing 1.15% potassium chloride followed by differential ultracentrifugation. Cytosol was removed, and the 100,000 g pellet was resuspended in 20 mM Tris buffer (pH 7.6 at ambient temperature) containing 0.25 M saccharose and 5.4 mM EDTA. The suspension was divided into aliquots, shock-frozen in liquid nitrogen, and stored at −80°C.

Caco-2 cell culture

Caco-2 cells were maintained at 37°C, 8% CO₂ and 90% relative humidity in 75 cm² cell culture flasks supplemented with 15 or 20 mL/flask of DMEM culture medium supplemented with 50 mL heat in-activated FBS, 5 mL NEAA, 5 mL penicillin-streptomycin and 5 mL L-glutamine. The cells were passaged once a week using 0.25% trypsin / 0.2% EDTA solution after having been washed with PBS(-) and 0.02% EDTA solution. About 1 x 10⁶ cells were seeded per flask. The culture medium was changed three times a week. Transwell filter inserts with a polycarbonate membrane (#3396, Corning, NY) were coated with 0.2 mL/well of collagen R solution. At 1 - 2 days after coating, the filter inserts were washed with phosphate buffered-saline (PBS) (+). Caco-2
cells were seeded at a density of 0.625 x 10^5 cells/filter on the collagen coated Transwell filter inserts. The inserts were placed on open reservoir plates. The inserts (apical compartment) were supplied with 0.25 mL and the outer wells with 32 mL/plate of DMEM culture medium. The cells were cultured at 37°C, 8% CO2 and 90% relative humidity in DMEM culture medium for 15 to 16 days where P-gp functionality and tightness of the monolayer was confirmed as comparable to those cultured for 21 days. The culture medium was replaced every 2 - 3 days.

Transcellular transport assay

Each transport experiment consisted of triplicate incubations using different filter inserts for both apical-to-basal (AtoB) and basal-to-apical (BtoA) direction. Experiments were conducted at 37°C and employed the use of a robotic workstation. To confirm confluence of the cell monolayer, transepithelial electrical resistance (TEER) was measured for each individual well before running the assay. Monolayers with TEER values greater than 500 Ω·cm^2 were used. The cells were equilibrated in the transport buffer (pH 7.2; 128 mM sodium chloride, 5.4 mM potassium chloride, 1 mM magnesium sulfate hexahydrate, 1.8 mM calcium chloride dihydrate, 1.2 mM disodium hydrogenphosphate 12-water, 0.41 mM sodium dihydrogenphosphate dihydrate, 15 mM HEPES, 4.2 mM sodium bicarbonate and
20 mM glucose) for 30 min. For the AtoB or BtoA transport experiment, the apical or basal side of the cell monolayer (donor compartment), respectively, was filled with donor solution containing the radiolabelled compound with or without P-gp inhibitor. The opposite chamber (receiver compartment) was filled with receiver solution supplemented with or without P-gp inhibitor. After preincubation with the radiolabelled compound with or without P-gp inhibitor for 30 min, the transport assay was started. Samples (50 μL) were taken at 0 and 90 min from the donor compartment, representing the actual start and end concentrations in the donor compartment. Samples were also taken at 0, 30, 60 and 90 min from the receiver compartment, for measurement of the amounts of compound that had passed through the monolayer. When each sample was withdrawn from the receiver compartment, it was immediately replaced with an equal volume of fresh receiver solution. Samples transferred into the vial were mixed with scintillation cocktail. Radioactivity was determined by means of a 3 min measurement in a liquid scintillation analyzer (TRI-CARB 2700 TR, 3100 TR, 3110 TR, PerkinElmer, Waltham, MA).

**Incubation with human liver microsomes**

Hydrolytic activity of human liver microsomes towards [\(^{14}\)C]dabigatran etexilate was assessed by incubating 100 μM of [\(^{14}\)C]dabigatran etexilate with 0.05 mg/mL of human
liver microsomes in the absence of NADPH and in the presence of chemical inhibitors for 5 minutes. Incubations were initiated by addition of substrate and reactions were terminated by adding 200 μL of 0.2 M hydrochloric acid and vortex mixing. After centrifugation at +4°C, incubates were directly injected into the high performance liquid chromatography (HPLC) system. The employed HPLC method showed linearity \((r^2 \geq 0.99)\) over the range of 500 to 290,000 dpm (absolute amount injected on column) as assessed by quadruplicate injections of \([^{14}\text{C}]\)dabigatran etexilate at various concentrations. Inaccuracy and imprecision of the method were found as ≤15% and ≤10%, respectively.

**HPLC with radioactivity detection**

*In vitro* incubation samples were analyzed by a validated reversed phase HPLC with on-line radiodetection for quantification of \([^{14}\text{C}]\)dabigatran etexilate and \([^{14}\text{C}]\)BIBR 1087. A HPLC system was composed of an autosampler HTC PAL (Chromtech, Idstein, Germany) and HPLC pump PU-980, ternary gradient unit LC-980-02, 3-line-degaser DG-980-50 and UV/VIS detector UV-975 (Jasco, Gross-Umstadt, Germany). Chromatography was performed for total run time 33 min on LiChroCART Purospher RP 18-e 5 μm 125-2 analytical column with LiChroCART Purospher RP 18-e 5 μm 10-2 guard column (Merck, Darmstadt, Germany). Mobile phases were 0.05 M formic acid adjusted to pH 4.0 with
ammonia solution (A) and acetonitrile (B) using a programmed gradient (0 min: 10% (B), 21 min: 60% (B), 23 min: 80% (B), 24 min: 80% (B), 25 min: 10% (B)) at a flow rate of 0.4 mL/min. Analytes were on-line quantified on flow scintillation analyzer TR 525 (PerkinElmer, Waltham, MA) with addition of Ultima Flo M (3:1).

Data evaluation

Apparent permeability coefficient

The apparent permeability coefficient (Papp) is described by the following equation:

\[
Papp = \frac{1}{A \times C_{t0}} \times \frac{V_R \times \Delta C_R}{\Delta t}
\]

where Papp is the apparent permeability, C_{t0} is the radioactivity in the donor compartment at time 0, A is the area of the filter, V_R is the volume in the receiver compartment, \(\Delta C_R/\Delta t\) is the change in substance concentration over time in the receiver compartment. The transport rate (\(V_R \cdot \Delta C_R/\Delta t\)) was calculated from the linear part of the drug concentration versus time curve in the receiver compartment.

Efflux ratio

The efflux ratio is defined by the following equation:

\[
\text{Efflux ratio} = \frac{P_{\text{BtoA}}}{P_{\text{AtoB}}}
\]

where \(P_{\text{BtoA}}\) and \(P_{\text{AtoB}}\) represent the apparent permeability of the test compound.
from basal to apical and from apical to basal, respectively.

**Half-maximal inhibitor concentration**

The apparent IC\textsubscript{50} value was calculated by means of nonlinear least squares regression using the XLfit (IDBS, UK) according the equations (3a) for Papp\textsubscript{AtoB} and (3b) for Papp\textsubscript{BtoA} and efflux ratio.

\begin{align*}
\text{(3a)} & \quad T &= T_0 + \frac{(T_{\text{max}} - T_0) \cdot 1^h}{1^h + IC_{50}^h} \\
\text{(3b)} & \quad T &= T_0 - \frac{(T_0 - T_{\text{max}}) \cdot 1^h}{1^h + IC_{50}^h}
\end{align*}

where $T$ is the observed Papp or efflux ratio, $T_{\text{max}}$ is the Papp or efflux ratio at $I = \infty$, $T_0$ is the Papp or efflux ratio at $I = 0$, $I$ is the concentration of inhibitor applied, IC\textsubscript{50} is the inhibitor concentration for 50% inhibition and $h$ is the slope factor.
Results

**In vitro IC\textsubscript{50} determination on digoxin transport**

The *in vitro* inhibitory effect of various P-gp inhibitors on P-gp-mediated \(^3\text{H}\)digoxin (1 \(\mu\)M) transport was determined across the Caco-2 cell monolayers (Figure 1). Concentration ranges employed and IC\textsubscript{50} values obtained of 6 P-gp inhibitors and linagliptin, which was used as a negative control as it does not inhibit P-gp, are summarized in Table 1. Cyclosporin A was found to be the most potent inhibitor with IC\textsubscript{50} values of less than 1 \(\mu\)M, followed by itraconazole and ketoconazole. These *in vitro* IC\textsubscript{50} values determined from three different parameters, efflux ratio, Papp\textsubscript{AtoB} values and Papp\textsubscript{BtoA} values, were comparable to those published before [references in Table 1], although the IC\textsubscript{50} values determined from efflux ratio were lower than those determined from Papp\textsubscript{BtoA} and Papp\textsubscript{AtoB}.

**In vitro IC\textsubscript{50} determination on dabigatran etexilate transport**

*In vitro* inhibitory effects of various drugs on the P-gp-mediated transport of \(^{14}\text{C}\)dabigatran etexilate was investigated across the Caco-2 cell monolayers and *in vitro* IC\textsubscript{50} values of various drugs were determined. P-gp-mediated efflux of dabigatran etexilate
in Caco-2 cells was markedly affected by the endogenous expression of carboxylesterases (CES) due to hydrolysis of the prodrug to its active moiety (Ishiguro N, manuscript in preparation). However, this study did not use a CES inhibitor, instead the impact of various drugs on CES-mediated hydrolysis of dabigatran etexilate to the intermediate metabolite BIBR 1087 was determined using human liver microsomes. The formation of BIBR 1087 from dabigatran etexilate was not inhibited by amiodarone, clarithromycin, digoxin, itraconazole, ketoconazole, quinidine and ritonavir (>85% of control at 50 µM of inhibitor). The IC₅₀ values were calculated from Papp_BtoA, because the IC₅₀ assay was conducted using [¹⁴C]dabigatran etexilate and LSC detection in the absence of CES inhibitor and dabigatran etexilate was reported to be stable only in the BtoA transport assay whereas profound hydrolysis was observed in the AtoB transport assay in the presence and absence of CES inhibitor [Ishiguro N, manuscript in preparation].

In Figure 2, the Papp_BtoA values of dabigatran etexilate in the presence of 10 drugs were plotted together with the IC₅₀ fitting curves. Concentration ranges employed and IC₅₀ values were summarized in Table 2. Cyclosporin A, itraconazole and tacrolimus showed the strongest inhibition with IC₅₀ values less than 1 µM. Ketoconazole and nelfinavir were
found less potent with IC$_{50}$ values between 1 µM and 10 µM. IC$_{50}$ values of clarithromycin, quinidine and ritonavir were greater than 10 µM. Digoxin showed weak inhibition with IC$_{50}$ values higher than 100 µM. Less than 50% inhibition was observed for amiodarone up to its solubility limit.

**Assessment of DDI likelihood**

The FDA and EMA released the guidances to evaluate the risk of DDI via drug transporters between known inhibitors and potentially coadministered drugs with new medical entities [FDA draft guidance, 2012; EMA guideline, 2012], where it is recommended to use the ratio of the concentration of a putative inhibitor at site of the DDI (e.g. [I]$_2$: maximum oral dose taken at one occasion / 250 mL of assumed intestinal fluid volume) to the *in vitro* inhibition potency such as IC$_{50}$ and K$_i$ value. In Table 1, the [I]$_2$ / IC$_{50}$ ratios of seven drugs tested in this study are shown together with the magnitude of area under the curve (AUC) increase in clinical interaction studies (AUC$_i$ / AUC) using digoxin as probe drug. Since these six of these drugs were known clinical P-gp inhibitors and the [I]$_2$ / IC$_{50}$ ratios determined from parameters either Papp$_{AtoB}$, Papp$_{BtoA}$ or efflux ratio exceeded the cut-off value of 10 that was set as threshold in these guidance. These assessments using in-house *in
vitro IC$_{50}$ data were consistent with findings of clinical interaction studies using digoxin as a P-gp probe drug in a way that AUC$_i$ / AUC for these six drugs was $> 1.25$. On the other hand, the [I]$_2$ / IC$_{50}$ ratio of linagliptin was less than 10 and this assessment was in agreement with the absence of P-gp-mediated clinical DDI between digoxin and linagliptin [Friedrich C et al., 2011].

The DDI likelihood for putative P-gp inhibitors on the pharmacokinetics of dabigatran etexilate was assessed in same way (Table 2). The [I]$_2$ / IC$_{50}$ ratios of almost all drugs assessed were higher than 10. Exact [I]$_2$ / IC$_{50}$ ratio of amiodarone could not be determined due to its low solubility and/or low in vitro inhibitory potential. Among the drugs assessed, only digoxin had the [I]$_2$ / IC$_{50}$ ratio less than 10, which was in line with the 1.1-fold AUC increase observed clinically after coadministration of dabigatran etexilate with digoxin [Stangier J et al., 2012]. Although the [I]$_2$ / IC$_{50}$ ratio of clarithromycin was higher than 10, AUC$_i$ / AUC was $< 1.25$ [PI]. Ketoconazole and quinidine had high [I]$_2$ / IC$_{50}$ ratios (higher than 10) and these predictions were confirmed in clinic [PI].
Discussion

The evaluation of potential DDIs of new medical entities with marketed, coadministered drugs is of importance during drug development. The DDI likelihood assessment based on the ratio of expected local concentrations of the inhibitor at the DDI site and its \textit{in vitro} inhibitory potency, such as $K_i$ or $IC_{50}$. Therefore, \textit{in vitro} $IC_{50}$ value is one of the important parameters to assess the DDI likelihood. Nevertheless, the $IC_{50}$ values on P-gp activity have been determined by many different equations, data parameters and assay systems, and thereby it is well known that inter-laboratory variability of $IC_{50}$ values can be substantial. In order to clarify the causes of large inter-laboratory variability, \textit{in vitro} $IC_{50}$ values in this study were evaluated by several aspects.

Firstly, the impact of the use of different parameters, e.g. $P_{A\to B}$, $P_{B\to A}$ and efflux ratio from same data set on $IC_{50}$ values was assessed. As shown in Table 1, the $IC_{50}$ values determined from $P_{A\to B}$ or $P_{B\to A}$ were approximately three-times larger on average than those determined from efflux ratios. This difference may be derived from the complexities of transcellular transport assay. The transport across Caco-2 cell monolayers is determined by total 5 different permeability coefficients, viz. the influx and efflux transport across the apical and basal membrane in addition to P-gp-mediated efflux. As shown in
Figure 4, an efflux ratio is described as a ratio of a Papp for the P-gp-mediated efflux transport to a Papp for efflux transport across the apical membrane when an assumption that $PS_1 \times PS_3 = PS_2 \times PS_4$ is applied [Mizuno N et al., 2003]. At 50% of P-gp inhibition where $PS_{p-gp}$ is half of control condition, the ratio of efflux ratio minus one at 50% inhibited condition to that at control condition is exactly half, indicating that the IC$_{50}$ values determined from an efflux ratio represents 50% inhibition of P-gp-mediated efflux accurately. On the other hand, the ratio of CL$_{B_{to}A}$ which is determined by subtracting intrinsic passive permeability from the Papp$_{B_{to}A}$ at 50% P-gp inhibition is higher than half, when looking at an equation (Figure 4), suggesting that the IC$_{50}$ values determined from Papp$_{B_{to}A}$ are theoretically higher than intrinsic IC$_{50}$ values. The efflux ratio-based curve declines faster than Papp$_{A_{to}B}$-based curve since additional decline in the efflux ratio-based curve occurs as the Papp$_{A_{to}B}$ increases in the denominator. Since the IC$_{50}$ values were different among parameters employed for IC$_{50}$ calculation, a threshold value according to the data parameter employed is necessary for accurate assessment of DDI likelihood although the draft FDA guidance recommends the use of efflux ratios.

Secondary, the impact of the use of different probe substrate on IC$_{50}$ values was assessed. As well as digoxin, dabigatran etexilate is one of the in vivo P-gp probe substrates
recommended in the DDI guidances of the EMA and FDA for evaluating P-gp mediated DDI [FDA draft guidance, 2012; EMA guideline, 2012]. Although the impact of the use of different probe substrate should be evaluated by comparing mathematically accurate IC_{50} values determined from efflux ratios, only the IC_{50} values determined from Papp BtoA were compared between digoxin and dabigatran etexilate due to hydrolysis of dabigatran etexilate when used for Caco-2 transport assays in the AtoB direction. Since the IC_{50} values determined from same data parameter (Papp BtoA) were compared, there is no need to take the different data parameter-derived differences in the IC_{50} values into consideration (Figure 3 left). The IC_{50} values of 5 drugs, clarithromycin, cyclosporin A, itraconazole, ketoconazole and ritonavir were similar and within three-fold correlation range. IC_{50} value of quinidine was approximately 18-times higher when dabigatran etexilate was used as a P-gp probe substrate than that when digoxin was used. Shift of IC_{50} values of quinidine by changing *in vitro* P-gp probe substrate from digoxin to other probe substrates was previously reported [Ayesh S et al., 1996]. Therefore, P-gp seems to have different binding site for digoxin and dabigatran etexilate, which may explain more than 10-fold differences in IC_{50} values although exact mechanism needs to be elucidated.

Rivaroxaban, a factor Xa inhibitor used for anticoagulant therapy, is a P-gp substrate and its
in vivo pharmacokinetics is affected by P-gp inhibitors [Gnoth J et al., 2011; Mueck W et al., 2013]. IC$_{50}$ values of P-gp inhibitors on rivaroxaban transcellular transport were determined from MDR1-overexpressing LLC-PK1 cells [Gnoth J et al., 2011]. In order to assess the impact of different cell systems and substrates on IC$_{50}$ under conditions where no parameter effect is anticipated, the IC$_{50}$ values of those P-gp inhibitors calculated from efflux ratios of rivaroxaban transport were plotted over those of digoxin transport determined based on efflux ratio in this study (Figure 3 right). The IC$_{50}$ values on rivaroxaban transport obtained from MDR1-overexpressing LLC-PK1 cells was higher than those on digoxin transport obtained from Caco-2 cells except for itraconazole and plots except for itraconazole deviated from the upper boarder of three fold correlation. Km values vary among cell types and correlate with P-gp protein expression levels [Shirasaka Y et al., 2008]. Therefore, IC$_{50}$ values may be also vary with assay systems and P-gp expression. Furthermore, it was reported that different IC$_{50}$ values were given from different laboratories at the same P-gp inhibitor [Bentz J et al., 2013]. Hence, the cell systems for the determination of in vitro IC$_{50}$ values should be also taken into consideration when the DDI likelihood is assessed. Since the IC$_{50}$ values of itraconazole determined from the assay using dabigatran etexilate as substrate and from the assay using digoxin as
substrate are in a similar range, it is reasonable to argue that itraconazole may affect rivaroxaban transcellular transport across cell monolayer of P-gp-expressing LLC-PK1 cells differently, leading far lower IC\textsubscript{50} values compared to that obtained using digoxin as substrate. As the reason behind this low IC\textsubscript{50} value of itraconazole when rivaroxaban is used as substrate is unclear, further studies to elucidate the mechanism would be needed to address this phenomenon.

Our assessments of the DDI likelihood of dabigatran etexilate based on the proposed equations and the cut-off value of 10 by the FDA and EMA and \textit{in vitro} IC\textsubscript{50} values obtained (Table 1 and Table 2) yielded three true positive, one true negative and one false positive prediction. The latter relates to the prediction for clarithromycin that was formally not correct because the 1.2-fold AUC change of dabigatran by clarithromycin observed in clinics, was within bioequivalence criteria [PI]. In this case, however, solubility limitations of clarithromycin may have influenced the outcome of the clinical interaction study. In general, the prediction of DDI likelihood of digoxin with several P-gp inhibitors well correlates to AUC change of digoxin (four true positive, one true negative prediction). The cut-off value recommended by the FDA and EMA seems to fit to our \textit{in vitro} Caco-2 cells.
system when dabigatran etexilate and digoxin are used as *in vitro / in vivo* probe substrate of P-gp. However, it has to be kept in mind that clinical data showing no DDI via intestinal P-gp are very limited and therefore more clinical DDI studies to confirm validity of the cut-off value is highly desirable for a more accurate DDI assessment.

In conclusion, clear *in vitro / in vivo* correlation between *in vitro* IC₅₀ values using dabigatran etexilate as *in vitro* P-gp substrate and clinical AUC change of dabigatran suggests that dabigatran etexilate is one of suitable *in vitro* and *in vivo* P-gp probe substrate. In addition, it was found that many factors such as the differences in data parameter employed, *in vitro* probe substrate used and cell system used produce substantial difference for determining IC₅₀ values although this conclusion was lead out from six P-gp inhibitors on the transport of digoxin and dabigatran etexilate as *in vitro* P-gp probe substrate. For accurate assessment of DDI likelihood, it is suggested from this study that the threshold should be set depending on the *in vitro* experimental tools and method for data analysis as well as consistency of *in vitro* assessment to the clinical findings regarding DDI occurrence.
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Author contributions

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Wrote or contributed to the writing of the manuscript: Wataru Kishimoto, Naoki Ishiguro, Thomas Ebner and Olaf Schaefer
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Linagliptin NDA application (http://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/201202s000ClinPharmR.pdf)


Footnotes

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Figure legends

**Figure 1** Inhibitory effect of P-gp inhibitors on the AtoB and BtoA Papp values and efflux ratios of digoxin.

$[^{3}H]$Digoxin (1 μM) was incubated with Caco-2 cells in the absence or presence of clarithromycin (A), cyclosporin A (B), itraconazole (C), ketoconazole (D), linagliptin (E), quinidine (F) and ritonavir (G). Mean ± S.D. Papp values and efflux ratios from n=3 filters were presented. Open and closed points indicate the observed Papp$_{BtoA}$ and Papp$_{AtoB}$ values, respectively (upper), and closed points indicate the observed efflux ratio (lower). Solid line indicate the fitting curve.

**Figure 2** Inhibitory effect of P-gp inhibitors on the Papp$_{BtoA}$ values of dabigatran etexilate.

$[^{14}C]$Dabigatran etexilate (1 μM) was incubated with Caco-2 cells in the absence or presence of amiodarone (A), clarithromycin (B), cyclosporin A (C), digoxin (D), itraconazole (E), ketoconazole (F), nelfinavir (G), quinidine (H), ritonavir (I) and tacrolimus (J). Mean ± S.D. Papp values from n=3 filters were presented. Closed points and solid line indicate the observed Papp$_{BtoA}$ and the fitting curve, respectively.
Figure 3  Correlation analysis of the *in vitro* IC$_{50}$ values of P-gp inhibitors between two different probe substrates.

*In vitro* IC$_{50}$ values determined from Papp$_{B_{to}A}$ values (A) and efflux ratios (B) were plotted. Solid, fine dotted and coarse dotted line represent 1:1, 3:1 and 10:1 correlation line, respectively. 1: cyclosporin A, 2, itraconazole, 3: ketoconazole, 4: quinidine, 5: ritonavir, 6: clarithromycin.

Figure 4  Ratios of efflux ratio- and Papp$_{B_{to}A}$-based data parameters at 50% inhibition of P-gp to those at control condition.

PS$_1$ and PS$_2$ represent the permeability-surface area products for the influx and non-P-gp-mediated efflux across the apical membrane of the cell monolayers, respectively. PS$_3$ and PS$_4$ represent the permeability-surface area products for the efflux and influx across the basal membrane of the cell monolayers, respectively. PSP$_{gp}$ represents the permeability-surface area products for P-gp-mediated efflux across the apical membrane. A represents the area of the filter and Papp$_{int}$ represents the intrinsic passive permeability coefficient.
Table 1  *In vitro* IC$_{50}$ values and [I]$_2$/IC$_{50}$ ratios of P-gp inhibitors on digoxin transport in Caco-2 cells and reported AUC increase of digoxin after coadministration of P-gp inhibitors.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. range used (µM)</th>
<th>Efflux ratio</th>
<th>IC$_{50}$ value (µM)</th>
<th>[I]$<em>2$/IC$</em>{50}$ ratio</th>
<th>AUC$_i$/AUC ratio of digoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Efflux</td>
<td>Papp</td>
<td>Papp</td>
<td>Published data</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ratio</td>
<td>AtoB</td>
<td>BtoA</td>
<td></td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>1 - 100</td>
<td>7.0</td>
<td>13</td>
<td>17</td>
<td>4.1 – 66</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>0.1 - 12</td>
<td>0.29</td>
<td>0.72</td>
<td>0.54</td>
<td>1.3 – 3</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.1 - 10</td>
<td>0.46</td>
<td>1.1</td>
<td>0.83</td>
<td>2 – 6</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.3- 30</td>
<td>0.42</td>
<td>1.2</td>
<td>1.2</td>
<td>0.99* - 4.6</td>
</tr>
<tr>
<td>Linagliptin</td>
<td>10 - 300</td>
<td>9.4</td>
<td>32</td>
<td>33</td>
<td>55d - 66.1*e</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.03 - 10</td>
<td>0.60</td>
<td>3.0</td>
<td>1.9</td>
<td>2.2 – 21</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>1 - 100</td>
<td>1.5</td>
<td>3.6</td>
<td>4.5</td>
<td>3.8 – 10</td>
</tr>
</tbody>
</table>

[^H]Digoxin was incubated at 1 µM. Drugs added as putative P-gp inhibitor were present in both donor and receiver compartment. na: not available. The IC$_{50}$ values from publication were determined from efflux ratio based parameters except for indicating asterisk (from Papp$_{BtoA}$ based parameters). a: Eberl S et al., 2007; Cook A et al. (efflux ratio & net efflux ratio), 2010, b: Choo F et al. (net efflux ratio), 2000; Cook A et al. (efflux ratio & net efflux ratio), 2010, c: Faessel M et al. (Papp$_{BtoA}$), 2008; Cook A et al. (efflux ratio & net efflux ratio), 2010, d: Linagliptin NDA application, e: Ishiguro N et al (Papp$_{BtoA}$), 2013, f: Choo F et al. (net efflux ratio), 2000; Fenner S et al. (net efflux ratio), 2009, g: Gurley J et al., 2006; Gurley J et al., 2008, h: Jalava M et al., 1997; i: Friedrich C et al., 2011, j: Pedersen E et al., 1983; Rameis H 1985, k: Penzak R et al., 2004; Kirby J et al., 2012.
Table 2  *In vitro* IC₅₀ values and [I]₂ / IC₅₀ ratios of P-gp inhibitors on dabigatran etexilate transport in Caco-2 cells and reported AUC increase of dabigatran after coadministration of P-gp inhibitors.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. range used (µM)</th>
<th>IC₅₀ value (µM)</th>
<th>[I]₂ / IC₅₀ for dabigatran etexilate</th>
<th>AUCᵢ / AUC of dabigatran</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiodarone</td>
<td>1 - 10</td>
<td>&gt;10</td>
<td>&lt;470</td>
<td>1.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.3 - 30</td>
<td>28</td>
<td>36</td>
<td>1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>0.1 - 12</td>
<td>0.69</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Digoxin</td>
<td>1 - 100</td>
<td>125</td>
<td>0.0080</td>
<td>1.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.3 - 10</td>
<td>0.41</td>
<td>1100</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.1 - 6</td>
<td>3.4</td>
<td>880</td>
<td>2.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>0.3 - 10</td>
<td>3.6</td>
<td>2100</td>
<td></td>
</tr>
<tr>
<td>Quinidine</td>
<td>1 - 60</td>
<td>33</td>
<td>150</td>
<td>1.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.3 - 30</td>
<td>13</td>
<td>260</td>
<td></td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>0.1 - 6</td>
<td>0.66</td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>

<sup>[¹⁴C]Dabigatran etexilate was incubated at 1 µM. Drugs added as putative P-gp inhibitor were present in both donor and receiver compartment. a: Package Insert, b: Stangier J et al., 2012.</sup>
FIGURE 1

(A) $P_{app}$ (x10^-6 cm/sec) vs. Clarithromycin (µM)

(B) $P_{app}$ (x10^-6 cm/sec) vs. Cyclosporin A (µM)

(C) $P_{app}$ (x10^-6 cm/sec) vs. Itraconazole (µM)

(D) $P_{app}$ (x10^-6 cm/sec) vs. Ketoconazole (µM)

(E) Efflux ratio vs. Clarithromycin (µM)

(F) Efflux ratio vs. Cyclosporin A (µM)

(G) Efflux ratio vs. Itraconazole (µM)

(H) Efflux ratio vs. Ketoconazole (µM)

(E) $P_{app}$ (x10^-6 cm/sec) vs. Linagliptin (µM)

(F) $P_{app}$ (x10^-6 cm/sec) vs. Quinidine (µM)

(G) $P_{app}$ (x10^-6 cm/sec) vs. Ritonavir (µM)

(Efflux ratio vs. Linagliptin (µM)

Efflux ratio vs. Quinidine (µM)

Efflux ratio vs. Ritonavir (µM)
FIGURE 3

(A) 

(B) 

\( \text{IC}_{50} (\mu M) \) on BtoA transport of Dabigatran etexilate in Caco-2 cells vs. \( \text{IC}_{50} (\mu M) \) on BtoA transport of Digoxin in Caco-2 cells

\( \text{IC}_{50} (\mu M) \) on efflux ratio of Rivaroxaban in P-gp-expressing LLC-PK1 cells vs. \( \text{IC}_{50} (\mu M) \) on efflux ratio of Digoxin in Caco-2 cells
## FIGURE 4

<table>
<thead>
<tr>
<th>Data parameter</th>
<th>Papp(_{BtoA})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
</tr>
<tr>
<td>Efflux ratio</td>
<td>( \frac{PS_4 \times (PS_2 + PS_{P-gp})}{PS_1 + PS_3} )</td>
</tr>
<tr>
<td>Efflux ratio - 1</td>
<td>( \frac{PS_{P-gp}}{PS_2} )</td>
</tr>
<tr>
<td>( (Papp_{BtoA} - Papp_{int}) \times A = CL_{BtoA} )</td>
<td>( PS_4 \times (PS_2 + PS_{P-gp}) )</td>
</tr>
<tr>
<td>( CL_{BtoA} = PS_4 + PS_{P-gp} + PS_3 )</td>
<td>( PS_2 + PS_{P-gp} + PS_3 )</td>
</tr>
<tr>
<td><strong>Efflux ratio at 50% P-gp inhibition / Efflux ratio at 100% P-gp activity</strong></td>
<td></td>
</tr>
<tr>
<td>( \frac{0.5 \times PS_{P-gp}}{PS_2} / \frac{PS_{P-gp}}{PS_2} = 0.5 )</td>
<td>( \frac{PS_4 \times (PS_2 + 0.5 \times PS_{P-gp})}{PS_2 + 0.5 \times PS_{P-gp} + PS_3} / \frac{PS_4 \times (PS_2 + PS_{P-gp})}{PS_2 + PS_{P-gp} + PS_3} )</td>
</tr>
<tr>
<td>( 0.5 \times \left(1 + \frac{PS_2 \times PS_3 + (PS_2 + PS_{P-gp}) \times (PS_2 + 0.5 \times PS_{P-gp})}{(PS_2 + PS_{P-gp}) \times (PS_2 + PS_3 + 0.5 \times PS_{P-gp})}\right) &gt; 0.5 )</td>
<td></td>
</tr>
</tbody>
</table>