Short Communication

Measurement of Binding of Basic Drugs to Acidic Phospholipids Using Surface Plasmon Resonance and Incorporation of the Data into Mechanistic Tissue Composition Equations to Predict Steady-State Volume of Distribution

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ABSTRACT:

Acidic phospholipid binding plays an important role in determining the tissue distribution of basic drugs. This article describes the use of surface plasmon resonance to measure binding affinity (K_D) of three basic drugs to phosphatidylserine, a major tissue acidic phospholipid. The data are incorporated into mechanistic tissue composition equations to allow prediction of the steady-state volume of distribution (V_SS). The prediction accuracy of V_SS using this approach is compared with the original methodology described by Rodgers et al. (J Pharm Sci 94:1259–1276), in which the binding to acidic phospholipids is calculated from the blood/plasma concentration ratio (BPR). The compounds used in this study [amlodipine, propranolol, and 3-dimethylaminomethyl-4-(4-methylsulfanyl-phenoxy)-benzenesulfonamide (UK-390957)] showed higher affinity binding to phosphatidylserine than to phosphatidylcholine. When the binding affinity to phosphatidylserine was incorporated into mechanistic tissue composition equations, the V_SS was more accurately predicted for all three compounds by using the surface plasmon resonance measurement than by using the BPR to estimate acidic phospholipid binding affinity. The difference was particularly marked for UK-390957, a sulfonamide that has a high BPR due to binding to carbonic anhydrase. The novel approach described in this article allows the binding affinity of drugs to an acidic phospholipid (phosphatidylserine) to be measured directly and demonstrates the utility of the binding data in the prediction of V_SS.

Introduction

Along with clearance, the volume of distribution at steady state (V_SS) is a key pharmacokinetic parameter that determines how long a drug remains in the body. Several approaches have been used to prospectively predict human V_SS, including scaling in vivo data from preclinical species, scaling in vitro data, and various in silico calculation methods (Obach, 2007).

An approach that has gained popularity in recent years is to use in vitro and physicochemical data for a particular compound to estimate its tissue/plasma partitioning and by accounting for the volumes of different tissues and their composition. This information is used to predict V_SS of a compound in an integrated physiologically based manner (eq. 1).

\[ V_{SS} = \sum (V_T \cdot K_p) + V_p \]  

where V is physical tissue volume, T is tissue, Kp is tissue to plasma partition coefficient, and p is plasma.

Prediction of Kp on the basis of tissue composition and the physicochemical properties of a compound was first described by Poulin and Theil (2000) for neutral compounds, with emphasis on lipophlicity and binding to neutral lipids. This approach has subsequently been used by various authors to predict V_SS for different compound datasets (Jones et al., 2006; De Buck et al., 2007). An extension to this mechanistic PBPK model that incorporates pH and binding of the ionized base to acidic phospholipids as an additional component of tissue binding was proposed by Rodgers et al. (2005a), initially for ionized bases. The affinity of a compound to acidic phospholipids was not measured directly but rather was estimated from the blood/plasma concentration ratio (BPR) (Rodgers et al., 2005a), taking advantage of the fact that the blood cells contain acidic phospholipids. The estimates of V_SS obtained using the approach described by Rodgers et al., although generally showing an improvement in accuracy for basic drugs, is sensitive to the accuracy of the BPR measurement and can be inaccurate if a compound binds significantly to components other than acidic phospholipids within blood cells (Rodgers et al., 2005b; Poulin et al., 2011).

With this limitation in mind, we investigated the feasibility of directly measuring the binding of basic compounds to acidic phospholipids using a surface plasmon resonance approach. The dissociation constant, K_D, obtained using this approach was incorporated into a mechanistic physiologically based pharmacokinetic model, and the accuracy of predicted V_SS was compared with the data obtained after intravenous administration of the compounds to humans. For comparison purposes, the predictions obtained using the approaches de-

ABBREVIATIONS: BPR, blood/plasma concentration ratio; UK-390957, 3-dimethylaminomethyl-4-(4-methylsulfanyl-phenoxy)-benzenesulfonamide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; SPR, surface plasmon resonance; AAFE, absolute average fold error.
Materials and Methods

Amlodipine and 3-dimethylanilinoethyl-(4-methylsulfanyl-phenoxy)-benzenesulfonamide (UK-390957) were synthesized at Pfizer Global Research and Development (Sandwich, UK). R,S-Propranolol HCI was purchased from Sigma-Aldrich (Gillingham, UK). EnantiomERIC purity R- and S-propranolol were provided by Professor Malcolm Rowland (University of Manchester).

Log D and pK_a. Log D was measured by a fully automated shake flask method that determines the partition of compound between octanol and phosphate buffer, pH 7.4 (Stopher and McClean, 1990). The pK_a of UK-390957 was determined according to the methods described by Gobry et al. (2000).

Measurement of Plasma Protein Binding and Blood/Plasma Ratio. Binding of UK-390957 to human plasma proteins was determined by equilibrium dialysis. Human plasma protein binding values for propranolol (Rodgers and Rowland, 2007) and amlodipine (Maddi et al., 2010) were taken from the literature. BPR measurements for all three compounds were made using heparinized human blood (for further details, see Allan et al., 2008). Measurements for amlodipine and propranolol were conducted at 1 μg/ml and over the concentration range of 50 to 5000 ng/ml for UK-390957.

Binding of Drugs to Phospholipid Liposomes. Interaction analysis was performed using a Biacore T100 instrument. All analyses were run at 25°C using an L1 sensor chip. Running buffer was 5% DMSO in phosphate-buffered saline (PBS) (0.01 M phosphate, 2.7 mM KCl, and 0.137M NaCl) with final pH 7.4. Before the start of each run, two 30-s consecutive pulses with 50 mM NaOH/propan-2-ol (3:2) (regeneration solution) were made at a flow rate of 100 μl/min to regenerate the chip surface.

Preparation of Liposomes. Lipids (phosphatidylserine and phosphatidylcholine) were purchased from Avanti Polar Lipids (Alabaster, AL) as lyophilized powders. Powders were dissolved in PBS to a nominal concentration of 10 mM phospholipid and shaken for at least 1 h at room temperature. When fully dissolved, lipid suspensions were extruded through a 100-nm polycarbonate filter using an Avanti Mini-Extruder kit. The lipid suspension was passed though the membrane 19 times to yield a clear solution of uniformly sized liposomes.

Capture of Liposomes. Liposomes were prepared for capture by dilution in running buffer to a concentration of 2 mM phospholipid. Liposomes were captured to saturation at a flow rate of 10 μl/min with an injection time of 180 s. One flow cell was left blank to act as a reference surface. The capture of the liposomes on the L1 chip is due to reversible, hydrophobic binding.

Drug Binding Analysis. Stock solutions of amlodipine, UK-390957, and propranolol were prepared in DMSO at a range of concentrations from 4 to 20 mM. Dilution of stock solution into PBS was made to yield a 5% DMSO in PBS solution with concentrations of 200 to 1000 μM. The top concentrations tested were 200 μM for amlodipine and 1000 μM for propranolol and UK-390957. These solutions were then further diluted in running buffer to give the final solutions tested. For each compound at least 12 different concentrations up to the maximum concentrations noted above were tested.

For each binding cycle a fresh liposome capture was made. Drugs were injected over lipid surfaces and reference surfaces in a single injection. The flow rate was 100 μl/min with a 45 s sample injection. Dissociation was monitored for 60 s. After each injection, the needle was washed with 50% DMSO in water to prevent compound carryover, followed by needle washes with running buffer before the next injection of test compound. At the end of each binding cycle the surface was regenerated using two 30 s pulses of regeneration solution and equilibrated with running buffer before the next injection of test compound.

Analysis of Phospholipid Binding Data. All data were double reference subtracted (reference cell and blank injection) to correct for instrumental artifacts and flow cell-specific variations. Binding affinities (K_d) for drug interactions with the phospholipid surface were estimated by plotting drug-binding response (RU), measured at steady state, against drug concentration (C). Binding affinity of propranolol to phosphatidylserine was estimated using a single-site binding isotherm.

\[ RU = (C \cdot R_{max}/C + (K_{D})) \]

where \( R_{max} \) is the maximal binding response estimated from the curve.

For amlodipine and UK-390957 a two-site binding isotherm was used to estimate \( K_{d2} \):

\[ RU = (C \cdot R_{max}/C + (K_{D1})) + (C \cdot R_{max}/C + (K_{D2})) \]

where \( R_{max} \) is the maximal binding response for the relevant binding site as estimated from the curve.

It was considered appropriate to apply a model with two binding parameters for these compounds because there was some “retention” on the phospholipid surface, seen as a lack of return of the sensorgrams to baseline at the end of an injection. It is probable that each binding event consists of binding of compound to both the ionized acidic head group and to the lipid tail of the acidic phospholipid to varying degrees. For these two compounds, only the high-affinity \( K_d \) was used in the calculation of \( V_{bc} \).

Incorporation of Acidic Phospholipid Binding Data into Tissue Composition Equations to Predict \( V_{bc} \). The equation developed by Rodgers et al. (2005a) for the estimation of tissue/unbound plasma concentration ratio (Kpu) for moderately strong bases is shown in eq. 2:

\[ Kpu = f_{lw} + \left( \frac{X}{Y} \cdot f_{lw} \right) + \left( \frac{P \cdot f_{lw} + (0.3P + 0.7) \cdot f_{lw}}{Y} \right) + \frac{K_{AAP} \cdot [AP] \cdot Z}{Y} \]

where \( X, Y, \) and \( Z \) are expressions accounting for drug ionization (Rodgers et al., 2005a), \( P \) is the octanol/water partition coefficient, \( f_{lw} \) is the fractional tissue volumes, \( K_{AAP} \) is the association constant of drug for acidic phospholipids (milligrams per gram) and \([AP] \) is the concentration of acidic phospholipid in the tissue (milligrams per gram), \( iw \) is intracellular water, \( ew \) is extracellular water, NL is neutral lipid, and NP is neutral phospholipids.

The value of \( K_{AAP} \) is unknown and is calculated from the BPR by appropriate rearrangement of eq. 2. Each tissue Kpu value is then calculated, by substitution of the appropriate parameters into eq. 2 and subsequent correction for the fraction unbound in plasma (Fu) to obtain the associated Kp (Fu × Kpu). Coupled with the associated tissue volumes, \( V_{bc} \) is then calculated as shown in eq. 1.

The in vitro data used for these calculations are shown in Table 1. The tissue composition and tissue volume data were taken from Brown et al. (1997), Poulin and Theil (2000) and Rodgers et al. (2005a).

Predictions of \( V_{bc} \) were made under four scenarios: 1) the Rodgers and Rowland method (using BPR to estimate acidic phospholipid affinity) (Rodgers et al., 2005a); 2) the Poulin and Theil method (Poulin and Theil, 2000); 3) modifications to the Poulin and Theil method suggested by Berezhkovskiy (2004); and 4) the Rodgers and Rowland method (using SPR data to estimate acidic phospholipid affinity). For the last method, the measured \( K_d \) was corrected for the compound and acidic phospholipid molecular weights to produce a \( K_{d}^{2} \) in milligrams per gram, ensuring stoichiometry, \( K_{d}^{2} \) was then calculated by taking the reciprocal of the \( K_{d}^{2} \). This \( K_{d}^{2} \) was then inserted into eq. 2.

Absolute Average Fold Error. The accuracy of the four methods to predict \( V_{bc} \) was assessed by calculating the absolute average fold error (AAFE) (eq. 3) for each of the prediction methods relative to the actual observed \( V_{bc} \).

\[ AAFE = \frac{10}{n} \sum \frac{log(FE)}{n} \]

TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mol. Wt.</th>
<th>LogD</th>
<th>pK_a</th>
<th>Fraction Unbound in Human Plasma</th>
<th>Human Blood: Plasma Concentration Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amlodipine</td>
<td>408.9</td>
<td>1.95</td>
<td>9.1</td>
<td>0.05</td>
<td>1.48 ± 0.1a</td>
</tr>
<tr>
<td>Propranolol</td>
<td>259.3</td>
<td>1.95</td>
<td>9.45</td>
<td>0.11</td>
<td>0.68, 0.75</td>
</tr>
<tr>
<td>UK-390957</td>
<td>352.5</td>
<td>1.78</td>
<td>8.4</td>
<td>0.068 ± 0.017b</td>
<td>3.8, 3.5, 2.7b</td>
</tr>
</tbody>
</table>

*a Mean ± S.D., n = 3 determinations.

*b Mean ± S.D., determined in n = 20 individuals.

 UK-390957 concentration was 50, 500, and 5000 ng/ml, respectively.

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The fold error (FE) was calculated by dividing the predicted $V_{ss}$ by the observed $V_{ss}$. The $\log_{10}$ of the absolute values of FE (disregarding sign) were summed and divided by the number of observations ($n$). The AAFE was calculated as the antilog$_{10}$ of this value.

Clinical Data. The $V_{ss}$ values of propranolol and amlodipine after intravenous dosing in humans were taken from a compilation of human pharmacokinetic data (Obach et al., 2008). The $V_{ss}$ of UK-390957 was determined after intravenous dosing in humans. The protocol for this study was reviewed and

FIG. 1. Representative examples of sensorgrams collected across phosphatidylserine surfaces; data are compiled from three separate sample runs. Insets show response (RU) at equilibrium plotted against compound concentration from which $K_D$ is estimated.
approved by an independent ethics committee, and all subjects gave written informed consent. Subjects (n = 15) received doses of UK-390957 (3, 10, and 15 mg) by intravenous infusion each over 45 min in a crossover design such that all subjects received all doses. PK parameters were found to be independent of dose and a mean value of \(V_{ss}\) was calculated for each subject using the data at all three doses.

### Results and Discussion

The physicochemical, fraction unbound in plasma, and BPR data for amlodipine, UK-390957, and propranolol are listed in Table 1. Binding of all three compounds to phosphatidylserine was observed using SPR when solutions of the compounds were flowed over a sensor chip on which liposomes of the lipid had been immobilized. Data from three independent experiments at a number of concentrations were analyzed to estimate the binding affinity of the compound for phosphatidylserine (Fig. 1). In contrast, although binding of all three compounds was observed when phosphatidylcholine was immobilized on the sensor chip, the binding was not of sufficient affinity to accurately determine a \(K_D\) (data not shown).

The measured \(K_D\) for binding of propranolol (117 ± 11 \(\mu\)M; mean ± S.E.M.) and amlodipine (6.3 ± 1.8 \(\mu\)M) to the acidic phospholipid, phosphatidylserine, was lower (higher affinity) than that calculated from the BPR data (1092 and 45 \(\mu\)M, respectively). The converse was seen for UK-390957 (\(K_D\) 28.1 \(\mu\)M BPR versus 72.1 ± 10 \(\mu\)M SPR method).

The binding of the individual \(R\) and \(S\)-enantiomers of propranolol to phosphatidylserine was investigated to determine whether there was any stereospecificity in the interaction of the enantiomers with the acidic phospholipid. The binding of the two enantiomers to phosphatidylserine was similar (\(K_D\) 117 ± 10 and 122 ± 12 \(\mu\)M for the \(R\)- and \(S\)-enantiomer, respectively). This result is in line with literature data showing only small differences in the blood/serum concentration ratio, unbound volume of distribution at steady state (\(V_{ss}\)) (\(V_u\) fraction unbound in plasma), and calculated acidic phospholipid binding for the two enantiomers in white and African-American subjects (Sowinski et al., 1996). The blood/serum concentration ratio was 0.87 and 0.86 for \(R\)- and \(S\)-propranolol, respectively, in African-American subjects and 0.89 and 0.86, respectively, in white subjects. \(V_{ss}\) for \(R\)- and \(S\)-propranolol was 2220 and 1960 liters, respectively, in African-American subjects and 2110 and 1960 liters, respectively, in white subjects. The calculated acidic phospholipid binding (\(K_D\)) based on BPR for \(R\)- and \(S\)-propranolol was 751 and 901 \(\mu\)M, respectively, in African-American subjects and 610 and 625 \(\mu\)M, respectively, in white subjects.

#### Table 2

**Human \(V_{ss}\) after intravenous dosing and estimates of human \(V_{ss}\) from tissue composition approaches**

<table>
<thead>
<tr>
<th>Compound</th>
<th>(V_{ss}) ((\mu)M)</th>
<th>Predicted (V_{ss}) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amlodipine</td>
<td>17</td>
<td>8.1</td>
</tr>
<tr>
<td>Propranolol</td>
<td>3.1</td>
<td>1.3</td>
</tr>
<tr>
<td>UK-390957</td>
<td>5.2(^a)</td>
<td>25.5(^b)</td>
</tr>
<tr>
<td>AAPE</td>
<td>2.9</td>
<td>2.1</td>
</tr>
</tbody>
</table>

\(^a\) \(V_{ss}\) for UK-390957 was 398 ± 75 liters (mean ± S.D.; n = 15 individuals); mean body weight for the individuals was 76.7 kg.

\(^b\) A blood/plasma concentration ratio of 3.5 was used in the calculation because plasma concentrations were <100 ng/ml in the clinical study with UK-390957.

In tissues, a number of different acidic phospholipids are present, in addition to phosphatidylserine, including cardiolipin, phosphatidylinositol, phosphatidylglycerol, and phosphatidic acid. For this study phosphatidylserine was chosen as the model acidic phospholipid on the basis of the known importance for binding of this constituent (Yata et al., 1990) and its ready availability, well defined composition, and relatively high abundance in mammalian tissues (Rodgers et al., 2005b).

For the calculation of \(V_{ss}\), it was assumed that phosphatidylserine was the predominant acidic phospholipid in human tissue. The actual and predicted \(V_{ss}\) for amlodipine, propranolol, and UK-390957 are shown in Table 2. Incorporating the \(K_D\) values (Table 3) measured by SPR into the mechanistic tissue composition equations described by Rodgers and Rowland led to an improvement in the prediction accuracy of \(V_{ss}\) for all three compounds. The improvement in accuracy was particularly marked for UK-390957. As with other primary sulfonamide compounds UK-390957 has a relatively high affinity for carbonic anhydrase (IC\(_{50}\) 92 nM; 33 ng/ml data on file at Pfizer Global Research and Development) and, thus, extensively partitions into red blood cells where more than 90% of the total body content of carbonic anhydrase resides (Hinderling, 1997). Binding similar to that for carbonic anhydrase and partitioning into red blood cells have been seen with other sulfonamide-containing compounds, such as acetazolamide, methazolamide, dorzolamide, and chlorothalidone (for review, see Hinderling, 1997).

There are some limitations to measuring the binding of drugs to acidic phospholipids using SPR techniques. The equipment and expertise to conduct these studies are not as widely available as those for liquid chromatography-tandem mass spectrometry, and it is an extra piece of data that has to be generated compared with estimates of \(K_D\) from BPR, which is often generated for other purposes (converting plasma clearance to blood clearance). As with any experimental approach, there will be compounds for which the SPR approach is not suitable, if, for instance, a compound showed preferential binding to acidic phospholipids other than phosphatidylserine, then the \(V_{ss}\) estimate based on binding to phosphatidylserine is likely to be lower than the actual in vivo \(V_{ss}\).

Phosphatidylserine is predominantly located in the cytosolic leaflet of cell membranes (Lüllmann and Wehling, 1979), and therefore the drug must have sufficient lipophilicity to cross into the membrane and reach the binding site. This restriction is removed in the SPR measurements because the drug has direct access to the acidic phospholipid. Thus, it is possible that for some compounds binding to acidic phospholipids may be observed in vitro but not in vivo, leading to an overestimation of the \(V_{ss}\) using the in vitro approach.

Despite these limitations, the utility of measuring the binding of basic drugs to acidic phospholipids by SPR techniques and incorporation of the SPR data into tissue composition equations to predict \(V_{ss}\) for basic compounds has been demonstrated. However, further studies will be needed to assess whether the method has general application. The technique appears to be particularly useful for compounds that distribute into red blood cells due to binding to components other than...
Acidic phospholipids (e.g., carbonic anhydrase, hemoglobin, or cyclophilin) or if increased partitioning occurs due to drug-induced phospholipidosis (Poulin et al., 2011).

Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research & Development, Sandwich, Kent, United Kingdom (H.S., I.G., H.M.J.); Clinical Pharmacology, Pfizer Global Research & Development, Sandwich, Kent, United Kingdom (J.D.); and Centre for Applied Pharmacokinetic Research, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, United Kingdom (M.R.)

Authorship Contributions

Participated in research design: Small, Gardner, Jones, and Davis.

Conducted experiments: Small and Davis.

Contributed new reagents or analytic tools: Rowland.

Performed data analysis: Small, Gardner, Jones, and Rowland.

Wrote or contributed to the writing of the manuscript: Small, Gardner, Jones, and Rowland.

References


Address correspondence to: Dr. Hannah Jones, Building 500 (IPC 664), Pfizer Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research & Development, Ramsgate Road, Sandwich, Kent CT 13 9NJ, UK. E-mail: hannah.jones@pfizer.com