DOSE-DEPENDENT PHARMACOKINETICS OF CYCLOSPORIN A IN RATS: EVENTS IN TISSUES

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ABSTRACT:

Cyclosporin A (CyA) tissue distribution kinetics was extensively studied after single 1.2-, 6-, and 30-mg/kg CyA doses (via 2-min i.v. infusion) to rats. Drug concentrations in blood and various tissues were measured using a specific radioimmunoassay. Based on total blood concentration data alone, CyA systemic pharmacokinetics appeared essentially linear. However, after taking the saturable, nonlinear blood cell binding into account, multiple nonlinear factors were identified. Intrinsic clearance at 30 mg/kg was about half the value at the two lower doses. Tissue distribution was also dose-dependent, with evidence of saturable binding in many tissues. In general, blood binding saturation (dissociation constant \( K_D = 0.18 \mu g/ml \)) occurred at a lower dose (concentration) than saturation of tissue binding (\( K_D, 0.005-0.77 \mu g/g \)), such that the volume of distribution at steady state first increased as the dose increased from 1.2 to 6 mg/kg, and then decreased as the dose increased to 30 mg/kg. Tissue binding was further investigated by various graphical analyses. Some organs showed a monophasic (single site) Scatchard plot of the tissue data at steady state, with high \( K_D \) values. In other organs, biphasic binding characteristics were observed with the \( K_D \) values of the high-affinity site in the same range as the \( K_D \) reported for the binding of CyA with cyclophilin, the putative target. Saturable tissue binding may therefore influence not only the pharmacokinetics but also the efficacy of CyA.

Cyclosporin A (CyA),\(^1\) an immunosuppressive polypeptide, is commonly used for the prevention of allograft rejection. Systemic disposition of CyA has been widely investigated in both animals and humans (Follath et al., 1983; Gupta et al., 1987; Wagner et al., 1987; Kawai et al., 1998). In general, blood concentrations of CyA in rats and humans show a pronounced multieponential decay after single i.v. doses, which suggests extensive but time-dependent distribution of CyA between blood and tissues. Some peripheral organs, such as adipose, into which CyA partitions extensively (Follath et al., 1983; Bernareggi and Rowland, 1991), are primarily responsible for the relatively large whole body volume of distribution of CyA (3–5 l/kg). CyA also binds with high affinity to a class of intracellular proteins, collectively called cyclophilin (CyPh), that exist in lymphocytes residing in essentially all organs throughout the body. This CyA-CyPh complex blocks the phosphatase activity of the enzyme calcineurin, thereby preventing cytokine induction (Schreiber and Crabtree, 1992), which is the most probable mode of CyA action for preventing grafted organ rejection. The extent of CyA binding to CyPh is therefore considered to be relevant to its efficacy.

Various CyPh classes have been reported (Schneider et al., 1994), and dissociation constants of CyA with these CyPhs (\( K_D, 0.012-0.036 \mu g/g \)) are within the therapeutic range of unbound CyA concentrations. Indeed, Bernareggi and Rowland (1991) suggested that saturation of tissue distribution occurs in some organs in a 6-day continuous i.v. infusion study in rat. More recently, Kawai et al. (1998) developed a physiologically based pharmacokinetic (PBPK) model for CyA in which they attempted to describe a time-dependent shift in tissue-to-blood drug partition in rat, assuming saturable tissue binding. In their PBPK model analysis, the estimated dissociation constant varied over a wide range (\( K_D, 0.0002-0.06 \mu g/g \)); however, the range overlapped with those reported for CyA-CyPh binding in vitro, as described above. An obvious limitation in their interpretation was that only one CyA dose (6 mg/kg, a therapeutic dose) was studied.

In this study, blood and tissue kinetics of CyA have been extensively investigated after single, short i.v. infusions covering a wide dose range, including a subtherapeutic (1.2 mg/kg), therapeutic (6 mg/kg), and supertherapeutic (30 mg/kg) dose in rats. The data were then analyzed by various graphical methods with the object of estimating specific tissue distribution parameters, as well as thoroughly assessing the multiple kinetic factors contributing to the complex and nonlinear CyA disposition.

Materials and Methods

Chemicals. CyA was supplied by Novartis Pharma AG (Basel, Switzerland). The vehicle for infusion was ethyl alcohol/polyethoxylated castor oil.

\(^1\)Abbreviations used are: CyA, cyclosporin A; \( V_{ss} \), volume of distribution at steady state; CyPh, cyclophilin; PBPK, physiologically based pharmacokinetic; \( C_u \), unbound drug concentration in plasma water; \( C_p \), drug concentration in plasma; \( f_u \), unbound fraction in plasma; \( f_p \), drug concentration in blood; \( f_u \), unbound fraction in blood; Hct, hematocrit; AUC, area under the concentration-time curve; CL, systemic clearance; \( C_T \), tissue concentration; \( K_p \), tissue-to-blood partition coefficient; \( K_{pu} \), tissue-to-unbound drug partition coefficient; \( K_{pu,app} \), tissue-to-unbound drug concentration ratios at each tissue sampling time; RIA, radioimmunoassay.

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(Cremophor EL; Sigma, St Louis, MO) (35:65). Sandimmune radioimmunoassay (RIA) kits (CYCLO-Trac) were purchased from INCSTAR Corp. (Stillwater, MN). All other chemicals were analytical grade.

In Vivo Experiments. Male Sprague-Dawley rats (277 ± 15 g) were divided into three dose groups (1.2, 6, and 30 mg/kg; n = 15 rats per group). Each dose group was subdivided into five time groups (2, 30 min, 2, 8, and 24 h: 3 rats per time group) for serial blood sampling (three time points per animal), terminal blood, and tissue sampling (Table 1). Either the femoral vein (for 2-, 8-, and 24-h groups) or both the femoral artery and vein (for 2- and 30-min groups) were catheterized (PE50, Clay Adams, Beckton Dickinson, Sparks, MD) under halothane anesthesia. CyA was administered to rats as a 2-min infusion via the femoral vein catheter under light halothane anesthesia; the halothane supply was discontinued when the infusion started. For the 2- and 30-min time groups, serial blood samples were collected via the arterial catheter until the animals were sacrificed. Animals in all other time groups had an additional treatment after the infusion. The catheter was removed, and the surgical site was allowed to recover; afterward, serial blood samples were collected via the jugular vein. Rats in the 2-min time group were sacrificed by decapitation; all other rats were sacrificed by bleeding from the aorta under ether anesthesia. A previous PBPK modeling study of CyA (Kawai et al. 1998) indicated that arterial-venous differences in CyA blood concentrations were negligible, except during the first few minutes after an i.v. dose. Therefore, blood concentration-versus-time profiles obtained in this study design are considered to be identical with those in arterial blood.

Blood samples were collected into tubes containing the anticoagulant EDTA, and organs (lungs, heart, kidneys, bone, skeletal muscles, spleen, liver, small intestine, skin, fat, thymus, and brain) were collected from individual rats after sacrifice. All samples were weighed and frozen at −20°C until analyzed for CyA.

Assay of CyA. Frozen samples were thawed at room temperature, and tissues were homogenized with an appropriate volume of water. Unchanged CyA concentration in blood and tissue homogenates was determined by a previously established and validated RIA (CYCLO-Trac; Ball et al., 1988; Bernareggi and Rowland, 1991). This method correlated very highly (r = 0.94–0.98) with a validated HPLC method for blood measurement, the result of which indicates that the potential overestimation of CyA concentration using the RIA is less than 5% for blood and all tissues. The limit of quantification of the RIA was 0.02 μg/ml in blood and 0.05 μg/g in tissues.

Data Analysis. Unbound drug concentration in blood. The unbound drug concentration in plasma (Cu) can be calculated as a product of the plasma concentration of the drug (Cp) and the unbound fraction in plasma (fu). The fu value of CyA is known to be constant under these study conditions (Lemaire and Tillement, 1982). In this study, however, this key concentration term, Cu, is related to the whole blood concentration (Cb), i.e.,

\[ Cu = fu \cdot Cp = fu \cdot (Cp/Cb) \cdot Cb = fu \cdot Cb \quad (1) \]

As seen from this equation, fu, which is the unbound fraction in blood and relates Cb to Cu, becomes concentration-dependent when blood cell binding is saturated, i.e., when the ratio Cp/Cb varies over the in vivo concentration range, despite the linear plasma protein binding. Such nonlinear blood cell distribution of CyA was studied formerly (Kawai and Lemaire, 1993) and is characterized by the relationship

\[ Cb = \left( \frac{Cu}{fu} \right) \cdot \left( 1 + nPF/(K_D + Cu) \right) \cdot Hct \quad (2) \]

where K_D and nPF are the dissociation constant and binding capacity, respectively, of the saturable blood cell binding, and Hct is the hematocrit (given as a fractional value). In this study, only whole blood CyA concentrations and the value of Hct were measured. The values of Cu were calculated from the corresponding quadratic solution, eq. 3, using the parameter values fu0 (0.062), K_D (0.185 μg/ml), and nPF (4.64 μg/ml), determined previously in vitro (Kawai and Lemaire, 1993).

\[ Cu = 0.5 \cdot \left[ Cb - \left( 1 - Hct \right) \cdot fu_0 + Hct \cdot K_D - Hct \cdot nPF + \left( Cb - \left( 1 - Hct \right) \cdot fu_0 + Hct \right) \right] \quad (3) \]

and where appropriate, fu0 was calculated from the ratio Cu/Cb.

Blood kinetic analysis. A triexponential equation was fitted to the concentration-time data for both Cb and Cu

\[ Cb(or Cu) = C_1 \cdot e^{-\lambda_1 t} + C_2 \cdot e^{-\lambda_2 t} + C_3 \cdot e^{-\lambda_3 t} \quad (4) \]

where t denotes time after the end of the 2-min i.v. phase. The parameters C_1, C_2, and C_3 denote constant coefficients, and λ_1, λ_2, and λ_3 denote the exponential coefficients.

The terminal elimination half-life (t_1/2) was calculated from the smallest exponential coefficient, λ_1, as 0.693/λ_1. The area under the blood concentration-time curve from the time of dosing until infinite time (AUC_0–∞) was calculated by the linear trapezoidal rule with extrapolation beyond the last measurable concentration, according to

\[ AUC_0–∞ = AUC_{0–3} + C_{int}/\lambda_1 \quad (5) \]

where AUC_{0–3} is the area under the blood concentration-time curve from the time of dosing until the last measurable blood concentration (C_{int}). Systemic clearance (CL) and the volume of distribution at steady state (Vss) were calculated according to

\[ CL = \text{Dose}/AUC_{0–∞} \quad (6) \quad Vss = CL \cdot AUMC_{0–∞}/AUC_{0–∞} \quad (7) \]

where AUMC_{0–∞} is the area under the first moment curve. AUMC_{0–∞} was calculated as

\[ AUMC_{0–∞} = AUMC_{0–t} + t_{last} \cdot C_{int}/\lambda_3 \quad (8) \]

where t_{last} is the time corresponding to C_{last}.

Tissue distribution kinetic analysis. The measured tissue concentrations were analyzed without correction for the drug in residual blood because either the tissue-to-blood concentration ratio of CyA was high, the vascular space relative to tissue volume was low, or a combination of both conditions existed, such that there was no more than 4%, and often less, of the drug in the tissue blood of all the organs studied. Tissue-to-unbound drug concentration ratios at each tissue sampling time (Kpu,app) were determined by dividing the tissue concentrations (C_t) by the corresponding Cu. More universal tissue distribution parameters, i.e., tissue-to-blood partition coefficients (K) and tissue-to-unbound-drug concentration partition coefficients (Kpu), were calculated by an AUC method (Gallo et al., 1987) using finite AUC values (AUC_{0–24h}) for Cu and Cu, respectively, versus those for individual tissue concentrations. A graphical analysis (Patlak and Blasberg, 1983) was also applied by plotting Kpu,app versus normalized time for Cu; the normalized time was calculated using

\[ \text{Normalized time (h)} = \frac{[\text{AUC}_{\text{unbound}}]}{[\text{Cu}]} \quad (9) \]

that is, the ratio of AUC (calculated by a linear trapezoidal method) to the concentration at time t for unbound drug. Kpu,app itself varies with time, due

<table>
<thead>
<tr>
<th>Time Group</th>
<th>Sampling Time</th>
<th>Sampling Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min</td>
<td>−1, 0, 1, 2 min</td>
<td>Arterial catheter</td>
</tr>
<tr>
<td>30 min</td>
<td>5, 10, 20, 30 min</td>
<td>Arterial catheter</td>
</tr>
<tr>
<td>2 h</td>
<td>0.5, 1, 1.5, 2 h</td>
<td>Jugular vein</td>
</tr>
<tr>
<td>8 h</td>
<td>2, 4, 6, 8 h</td>
<td>Jugular vein</td>
</tr>
<tr>
<td>24 h</td>
<td>8, 12, 18, 24 h</td>
<td>Jugular vein</td>
</tr>
</tbody>
</table>

* Terminal sampling from aortal bleeding except for 2-min group (arterial catheter).
The blood kinetics of CyA exhibited multiexponential decay characteristics after a 2-min i.v. infusion (Fig. 1). The profiles for the three different doses were similar, despite different magnitudes of concentrations. Because CyA blood cell uptake is saturable and concentration-dependent (Bernareggi and Rowland, 1991; Kawai and Lemaire, 1993; Kawai et al., 1998), the 

\[ C_T = B_{\text{max}} \cdot Cu(K_{0,T} + Cu) + \alpha \cdot Cu \]

(10)

where \( B_{\text{max}} \) is the tissue-binding capacity, \( K_{0,T} \) is the dissociation constant, and \( \alpha \) is a linear binding coefficient.

The solid lines represent simulations with best-fit parameters generated from fitting a triexponential equation to the data, and the symbols represent average values from two to six rats with standard deviations (vertical bar). Unbound fractions were generated by the blood concentration data and blood distribution isotherm previously obtained from an in vitro study (Kawai and Lemaire, 1993), not only to saturable tissue binding but also to differences in the timescale for approach to steady state caused by different blood decay profiles associated with the various doses. The normalized time corrects for these differences in timescale. Using tissue concentration measurements (\( C_T \)) at steady state, tissue-binding parameters were estimated using

\[ V_{\text{T,D}} \]

Results

The blood kinetics of CyA exhibited multiexponential decay characteristics after a 2-min i.v. infusion (Fig. 1). The profiles for the three different doses were similar, despite different magnitudes of concentrations. Because CyA blood cell uptake is saturable and concentration-dependent (Bernareggi and Rowland, 1991; Kawai and Lemaire, 1993; Kawai et al., 1998), the \( f_{\text{int}} \) of CyA was calculated (Fig. 1, inset). Between the 1.2- and 30-mg/kg doses, \( f_{\text{int}} \) ranged from 0.050 to 0.094, suggesting that saturation in blood cell binding alters \( f_{\text{int}} \) as much as 90% in the dose range studied, which might have a significant impact on the CL as well as tissue distribution.

The global parameters (AUC, \( V_{\text{ss}} \), CL, and \( t_{\text{1/2}} \)) are summarized graphically in Fig. 2. The effect of dose, between 1.2 and 30 mg/kg, on these parameters was seen to be quite complicated due to saturable blood cell binding. It was thus necessary to also calculate the parameters for unbound drug in blood. For example, dose-normalized \( \text{AUC}_{\text{p,u}} \) values for total blood concentration ranged from 6 to 8.8 \( \mu g/h/ml \) (per mg/kg) with only a slight tendency toward the higher value at 30 mg/kg, which may lead to the conclusion that CyA pharmacokinetics is linear over this dose range. However, when based on unbound drug, there was a clear dose-dependence, with the 30-mg/kg dose yielding a dose-normalized AUC (0.68 \( \mu g-h/ml \)) approximately twice that for the lower two doses (0.37 and 0.36 \( \mu g/h/ml \)), which were very similar. Not unexpectedly, the same dose-response findings applied to clearances of total and unbound drug in blood, as these values are merely reciprocals of the corresponding dose-normalized AUCs. These results suggested that the systemic blood clearance of CyA was constant within the dose range 1.2 to 6 mg/kg (142–165 mL/h/kg) and slightly lower at 30 mg/kg (114 mL/h/kg), presumably due to saturation of the metabolic enzymes (CL for unbound drug was 2.7, 2.8, and 1.5 h/kg at 1.2, 6, and 30 mg/kg, respectively), and that saturable blood cell binding partly masked this fact when analysis was based on total blood concentration-time data.

In contrast, \( V_{\text{ss}} \) seemed to peak at the middle dose, regardless of any correction for unbound drug in blood (2.0 mL/kg for total and 30 mL/kg for unbound drug). The terminal \( t_{1/2}, \) a hybrid parameter of clearance and distribution volume, increased moderately with increase in dose, with or without the correction for unbound fraction in blood (from 7.4 to 12 h for total and from 6.5 to 10 h for unbound). Dose- or concentration-nonlinear tissue distribution was also likely. However, this was not readily observed when assessment was based on blood pharmacokinetic data alone.

Tissue concentrations of CyA in various organs are shown in Fig. 3. CyA tissue concentration-versus-time profiles varied considerably among the organs and were not always similar in shape to the blood concentration-time profiles (see Fig. 1). Overall tissue profiles seemed to roughly parallel each other for the three doses, with a slight tendency toward a slower decay at the higher doses. Brain displayed a distinctive dose-response, i.e., the highest dose showed a concave downward profile, the middle dose was concave upward, whereas all of the measurements following the lowest dose were under the limit of quantification except for that at 2 min. The last value corresponded to the concentration calculated from drug in the capillary blood and vascular volume in brain, suggesting no substantial tissue distribution.

\( K_{\text{pu}} \), app values were calculated as the ratio of measured tissue concentrations versus calculated \( C_u \) (see Materials and Methods). The values of \( K_{\text{pu}} \), app were plotted against the normalized time (Patlak plot, see Materials and Methods). The results of six major organs are shown in Fig. 4. For most organs, \( K_{\text{pu}} \), app increased rapidly with normalized time and approached a certain level within the study period, at least for the lowest dose, indicating that equilibrium of drug between blood and tissues was achieved in these organs. For skin, in contrast, \( K_{\text{pu}} \), app increased progressively until the last sampling point, suggesting that equilibrium had not been achieved within the study period (24 h); fat, muscle, and thymus showed profiles similar to that of skin.

Regardless of whether equilibrium was achieved by the end of the sampling period, \( K_p \) and \( K_{\text{pu}} \) values (Table 2) were also calculated by an AUC method (Gallo et al., 1987). These latter time-averaged distribution parameters decreased with increasing dose for all tissues except for the brain. \( V_{\text{ss}} \) values were calculated from these parameters, i.e., the sum of \( K_p \) or \( K_{\text{pu}} \) multiplied by the actual mass of the corresponding organ. Comparison of these volumes of distribution (calculated from actual tissue distribution data) clearly demonstrated that an increase of dose reduces overall tissue distribution of CyA within the dose range studied. This effect occurred to a lesser extent when based on whole blood data (\( K_p \)) than when based on unbound concentration data (\( K_{\text{pu}} \)), due to the saturable blood cell binding. From these data analyses, it was concluded that the distribution...
volume of unbound CyA decreased on increasing dose, presumably due to greater saturation of tissue binding. Saturation in blood cell binding occurred over the same concentration range and counteracted the saturation in tissue binding when the volume of distribution was assessed on the basis of whole blood data.

Additional graphical analyses were performed to characterize this saturable tissue binding. As shown in Fig. 5, $K_{pu,app}$ values in various organs were plotted against the respective tissue concentrations, assuming steady-state conditions. Data from all doses were pooled. However, only those values associated with steady-state conditions, based on the Patlak plot (Fig. 4), were used. For some organs (e.g., lung, heart, liver, and skin), this Scatchard-type plot portrayed a biphasic binding isotherm consisting of high- and low-affinity sites, whereas plots for other organs (e.g., kidney and bone) were either monophasic or too scattered to discriminate different phases. In either case, nonlinear tissue distribution was obvious for all organs, giving the negative slopes observed in the Scatchard plots.

The common tissue-binding parameters, assuming a two-site binding model (eq. 10), were estimated for all organs that showed attainment of steady state (Fig. 4). Typical examples of data fitting, for liver and kidney, are shown in Fig. 6, and estimated binding parameters for these and various other organs are summarized in Table 3. The estimated $K_{D,T}$ values ranged from 0.005 to 0.77 $\mu$g/g, and the specific binding potential ($B_{min}/K_{D,T}$) was larger than the linear binding coefficient ($\alpha$), indicating that saturable binding is a dominant feature of overall tissue distribution. In this parameter estimation, the value of $\alpha$ was set to 1 for kidney, spleen, bone, and gut. Scatchard plots (Fig. 5) for these organs were monophasic, and thus the organ volume was assigned to the linear term (i.e., $\alpha = 1$) for spatial distribution of the unbound drug.

**Discussion**

This wide-ranging (1.2–30 mg/kg) i.v. dose study in rats confirms earlier suggestions (Bernareggi and Rowland, 1991; Kawai et al., 1998) that individual elements involved in the pharmacokinetics of CyA, such as tissue distribution, distribution within blood, and unbound clearance, are dose-nonlinear. Evidently, however, these nonlinearities interact in such a way that the systemic pharmacokinetic parameters, such as clearance and volume of distribution, based on whole blood measurements and assessed in the conventional way, appear to be essentially independent of dose. That is, events assessed in whole blood mask the underlying nonlinearities.

Detailed data interpretation, taking into account the concentration dependence of binding within blood ($fu_B$), reveals many insights. First, unbound CyA clearance, which approximates to intrinsic clearance because CyA is a low-clearance compound dominated by the hepatic enzyme system (Vickers et al., 1992), was obviously lower at the highest dose (Fig. 2). However, because of a corresponding diminished blood binding (i.e., higher $fu_B$), blood clearance remained virtually unchanged with dose. Second, tissue distribution comprised two parallel nonlinear components. The dose-dependent nature of these components was determined by their $K_D$ values, which were 0.18 $\mu$g/ml for blood cell binding (Kawai and Lemaire, 1993) and ranged from 0.005 to 0.77 $\mu$g/ml (Table 3) for various organs. Although some organs (e.g., lung, liver, and heart) possessed a smaller $K_D$ value than blood cells, for the majority of organs that contributed the greatest to body mass, such as muscle and skin, the $K_D$ value was much larger. Accordingly, saturation of blood cell binding occurred at lower doses than saturation of some tissue binding sites, thereby altering the whole body tissue distribution space of CyA in a dose-dependent manner. These effects explain why the estimated $V_{ss}$ increased as the dose increased from 1.2 to 6 mg/kg, and then subsequently decreased as the dose increased to 30 mg/kg (Fig. 2). However, this fails to explain why an initial increase was also observed for the $fu_B$-corrected volume of distribution, which may be due to experimental limitations, such as intersubject variation.

Certainly, the alternative form of analysis to estimate the distribution volume, based on the AUC (moment) approach (Gallo et al., 1987), showed a consistent decrease in $V_{ss}$ for blood unbound CyA with doses increasing from 1.2 to 30 mg/kg (Table 2). The $V_{ss}$ estimated from $K_p$ values for the lowest dose (1.2 mg/kg) was 2.4 l/kg, which is somewhat lower than the reported distribution volume.
of CyA (3–5 l/kg). The immunoassay method, a highly specific assay (see Materials and Methods), is unlikely to be the explanation. The answer may be due, at least in part, to the limited duration of the study (24 h). In addition, the assumption of linear or nonlinear distribution appears to be important. For example, $K_{pu}$ values estimated by the linear AUC method for liver and kidney at the lowest dose were 218 and 142, respectively. These values are, respectively, 60 and 30% smaller than those estimated from the present nonlinear model analysis (Table 3); i.e., $a + B_{max}/K_{D,T}$ which yielded values for $K_{pu}$ of 543 (liver) and 204 (kidney), assuming that linear conditions prevail after administration of the lowest dose.

Another focus of this work is the ways in which the specific CyA binding to tissue constituents in vivo can be linked with in vitro efficacy parameters. One of the major factors differentiating in vivo from in vitro systems is that drug distribution equilibrium is often not reached between physiologic and/or biologic compartments in vivo, whereas most in vitro (efficacy) studies are performed under steady-state conditions. In vivo tissue-to-blood CyA exchange is known to reach a pseudo steady state within the first few hours postdose for many but not all organs (Kawai et al., 1998). A graphical method (Patlak and Blasberg, 1983) was thus used to evaluate whether or when steady-state conditions prevail in vivo. This plot not only allows an estimate of the blood-to-tissue influx clearance from the initial slope but also provides a visual assessment as to what extent steady state, i.e., influx and efflux balance, is achieved. In this study, the normalized time was calculated based on $Cu$, considering unbound

![Graphical representation of tissue concentration-to-time profiles of CyA in various organs of rats after 1.2- (●), 6- (■), and 30- (▲) mg/kg doses.](https://i.imgur.com/3.png)
drug in systemic circulation to be the driving force for drug uptake. As shown previously (Kawai et al., 1998), the initial rapid increase in $K_{pu,app}$ ceased within the first few time points; beyond those time points, the values either remained constant or increased gradually.

Under linear conditions, the existence of a plateau denotes attainment of steady state. However, in the case of nonlinear distribution, the value of $K_{pu,app}$ may increase, even though the blood-to-tissue drug exchange is at steady state, because the drug concentrations in blood and tissues decrease at later time points. This was particularly so for the plots of the highest dose data, which generally showed a continual increase in $K_{pu,app}$ at later times. Even for those cases, however, drug distribution was assumed to be at steady state, if steady state had already been achieved at the lowest dose, because the distribution volume should then be the largest and the time taken to reach steady state should therefore be the longest (Kawai et al., 1991). Although there are obvious limitations, we considered this evaluation of steady state worthwhile for defining the following assessment of specific tissue binding.

**FIG. 4.** Tissue-to-unbound blood concentration ratios ($K_{pu,app}$) plotted against normalized time (Patlak and Blasberg, 1983) for various organs after 1.2- (●), 6- (□), and 30- (▲) mg/kg i.v. doses.

Each point represents the average value from three rats with S.D. (vertical bar).

**TABLE 2**

<table>
<thead>
<tr>
<th>Dose, mg/kg</th>
<th>$K_p$</th>
<th>$K_{pu}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>7.14</td>
<td>4.22</td>
</tr>
<tr>
<td>Heart</td>
<td>5.88</td>
<td>3.82</td>
</tr>
<tr>
<td>Kidney</td>
<td>7.69</td>
<td>6.70</td>
</tr>
<tr>
<td>Bone</td>
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<tr>
<td>Muscle</td>
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<tr>
<td>Spleen</td>
<td>7.38</td>
<td>5.47</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Gut</td>
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<td>3.87</td>
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<tr>
<td>Skin</td>
<td>4.17</td>
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</tr>
<tr>
<td>Fat</td>
<td>6.74</td>
<td>7.48</td>
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<tr>
<td>Thymus</td>
<td>3.62</td>
<td>3.39</td>
</tr>
<tr>
<td>Brain</td>
<td>N.D.</td>
<td>0.80</td>
</tr>
<tr>
<td>$V_{ss}$ (l/kg)</td>
<td>2.40</td>
<td>1.83</td>
</tr>
</tbody>
</table>

*Time- and subject-averaged values obtained from pooled concentration-time data in each dose group ($n=15$; three animals per time point).

**TABLE 2 (continued)**

<table>
<thead>
<tr>
<th>Dose, mg/kg</th>
<th>$K_p$</th>
<th>$K_{pu}$</th>
</tr>
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<tr>
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<td>41.9</td>
<td>19.6</td>
</tr>
<tr>
<td>Muscle</td>
<td>18.1</td>
<td>13.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>136</td>
<td>65.7</td>
</tr>
<tr>
<td>Liver</td>
<td>218</td>
<td>103</td>
</tr>
<tr>
<td>Gut</td>
<td>95.0</td>
<td>46.5</td>
</tr>
<tr>
<td>Skin</td>
<td>76.9</td>
<td>20.4</td>
</tr>
<tr>
<td>Fat</td>
<td>124</td>
<td>89.8</td>
</tr>
<tr>
<td>Thymus</td>
<td>66.7</td>
<td>40.7</td>
</tr>
<tr>
<td>Brain</td>
<td>N.D.</td>
<td>3.90</td>
</tr>
<tr>
<td>$V_{ss}$ (l/kg)</td>
<td>44.2</td>
<td>22.0</td>
</tr>
</tbody>
</table>

*N.D., not determined (because most brain measurements were below the limit of quantification).

* Calculated from the sum of $V_{pu}K_p$ or $V_{pu}K_{pu}$ for all organs measured, including blood, where $V_{pu}$ is organ mass.
Regarding saturable CyA binding, two categories of organs were identified by Scatchard analysis of the in vivo steady-state tissue data. The first category was characterized by its biphasic tissue binding (Fig. 5), demonstrating both high- and low-affinity binding sites. $K_D,T$ values estimated from the high-affinity site varied within a narrow range (0.005–0.17 $\mu$g/g), which overlapped approximately with the reported $K_D$ for CyA-CyPh binding measured in vitro (0.012–0.048 $\mu$g/g; Dalgarno et al., 1986; Ryffel, 1993). It was thus probable that CyA-CyPh binding could also be measured in vivo. If this is the case, the approach used in this study allows an evaluation of in vivo efficacy based on target occupancy, referring to in vitro data. In contrast, the biological meaning of the second category of organs remains unclear. For these organs, the Scatchard plot was essentially monophasic, and the estimated $K_D$ values (0.42–0.77 $\mu$g/g) were one order of magnitude larger than those reported for CyA-CyPh binding. In these organs, saturable binding site(s) with high affinity probably exist but are undetectable based on the current in vivo data. A number of CyPh subclasses are currently known (Schneider et al., 1994); however, the affinity of CyA to all of these has not been systematically investigated. This leaves a possible explanation if they have different affinities for CyA and distribute in tissues in an organ-specific manner. In addition to different CyPh classes, other saturable binding sites with high capacity and low affinity may also exist in some organs.

Compared with other organs, brain revealed a unique dose-response in the distribution profile, showing a disproportionately higher tissue concentration at higher doses. The result, however, is consistent with the current knowledge of the blood-brain barrier at which P-glycoprotein efficiently pumps out substrates (Cordon-Cardo et al., 1989; Thiebaut et al., 1989), including CyA (Saeki et al., 1993; Sakata et al.,

**Fig. 5.** Tissue-to-unbound blood concentration ratios ($K_{pu,app}$) at steady state versus tissue concentration in major target organs for transplantation. Each point represents a value from an individual rat. The points from 1.2-, 6-, and 30- mg/kg dose groups were shown as ●, □, and ▲, respectively.

**Fig. 6.** Tissue CyA concentration profiles in liver and kidney as a function of unbound concentration in blood. The solid lines represent simulations using best-fit parameters generated from fitting a two-site binding model (eq. 10) to the data. The symbols represent average values from three rats with S.D. (vertical bar).
The present data suggest that such an efflux system limits CyA entry into the brain at subtherapeutic to therapeutic doses and that more than a dose-proportional increase in brain exposure occurs, once the efflux system is saturated.

Overall, we have described CyA disposition over a wide dose range by interpreting the data cautiously in terms of individual elements, i.e., tissue distribution, blood distribution, and clearance. This wide dose range, especially the highest dose (30 mg/kg), is not directly applicable to the therapeutic situation, e.g., the reported highest in peak blood concentration in human was 2 μg/ml after oral administration of a microemulsion formulation of CyA (Mueller et al., 1994). However, based on blood distribution modeling in human (Legg and Rowland, 1988), this concentration corresponds to 0.12 μg/ml unbound CyA, which is comparable with the K_D for blood cell binding (0.185 and 0.128 μg/ml in rat and human, respectively) as well as the K_D,T in some organs (i.e., lung, heart, and liver; Table 3). In addition, this study provides important insights into the pharmacokinetics of CyA, which appears simple but is actually a complex hybrid of various nonlinear kinetic processes. The study also suggests that, in vivo, nonlinear factors are likely to be involved in the delivery of CyA to the target protein cyclophilin. Once the location of the target cells has been identified, whether local (organ) or otherwise (e.g., lymph nodes), the approach used here should facilitate the integration of in vitro efficacy parameters (such as target occupancy) into the pharmacokinetic and pharmacodynamic evaluation of CyA in vivo.

References


