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Binding of pimecrolimus and tacrolimus to skin and plasma proteins:

Implications for systemic exposure after topical application

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AGP, α_1-acid glycoprotein; FKBP, FK506-binding proteins; fu, unbound fraction; HDL, high density lipoprotein; LDL, low density lipoprotein; LSC, liquid scintillation counting; VLDL, very low density lipoprotein.

Abstract

Pimecrolimus and tacrolimus are calcineurin inhibitors used for the topical treatment of atopic dermatitis. While structurally similar, they display specific differences including higher lipophilicity and lower skin permeation of pimecrolimus. The present study aimed at understanding the reason for the differences in skin permeation; in addition, plasma protein binding of the two drugs was analyzed side by side as a basis for comparison of systemic exposure to free drug. Permeation of pimecrolimus and tacrolimus through a silicon membrane was found to be similar, therefore we assumed that differences in skin permeation could be caused by differences in affinity to skin components. To test this hypothesis, we investigated binding of pimecrolimus and tacrolimus to a preparation of soluble human skin proteins. One binding protein of approximately 15 kDa, likely corresponding to macrophilin12, displayed a similar binding capacity for pimecrolimus and tacrolimus. However, less specific, non-saturating binding to other proteins was approximately 3-fold higher for pimecrolimus. Due to the high local drug concentration following topical administration, the unspecific, high-capacity binding is likely dominating the permeation through skin. In plasma both drugs bound predominantly to lipoproteins. This may impact disposition different to albumin binding. The unbound fraction of pimecrolimus in human plasma was about 9-fold lower compared to tacrolimus (0.4±0.1% versus 3.7±0.8%). In conclusion these results provide an explanation for the observed lower systemic exposure to pimecrolimus compared to tacrolimus after topical application and suggest that differences in systemic exposure to free drug might be even more pronounced.

Pimecrolimus (Elidel®) and tacrolimus (Protopic®) are calcineurin inhibitors used for the topical treatment of atopic dermatitis (Stuetz et al., 2006). The compounds bind to cytoplasmic proteins of the immunophilin family, in particular to macrophilin12 (Kissinger et al., 1995) (Grassberger et al., 1999) which is highly and ubiquitously expressed (Galat, 2003); inhibition of calcineurin occurs in a ternary calcineurin-immunophilin-drug complex. Despite a high degree of structural similarity (Figure 1), pimecrolimus and tacrolimus display characteristic differences in terms of pharmacological profile (Stuetz et al., 2001; Meingassner et al., 2003; Grassberger et al., 2004; Bavandi et al., 2006; Kalthoff et al., 2007), and physicochemical and pharmacokinetic properties. Regarding physicochemical properties, the higher lipophilicity of pimecrolimus is noteworthy: pimecrolimus features an 8-fold higher octanol-water distribution coefficient than tacrolimus (Billich et al., 2004). Another distinguishing feature between the two agents is their rate of skin permeation: the permeation rate from 1% solutions is approximately 10-fold lower for pimecrolimus compared to tacrolimus (Billich et al., 2004); also from the marketed 1% cream the permeation rate of pimecrolimus is about 6- and 4.3-fold lower than from 0.1% and 0.03% tacrolimus ointments, respectively, despite the much higher pimecrolimus concentration in the formulation (Meingassner et al., 2005). The reason for the pronounced difference in skin permeation of the two drugs has so far not been investigated in detail.

Low skin permeation is a favorable property for a topical drug, since it contributes to low systemic exposure levels and thus to a lower risk of systemic side effects. Indeed, topical pimecrolimus is associated with lower systemic drug exposure than tacrolimus (Draelos

et al., 2005). A comparison of systemic exposure levels should consider exposure to both total as well as unbound drug. The latter is relevant, since free rather than total drug concentrations may drive wanted or unwanted pharmacological effects. For tacrolimus very different unbound fractions in plasma of 1.2% and 27% have been reported based on different separation techniques (Piekoszewski et al., 1993; Zahir et al., 2001; Nagase et al., 1994); data on pimecrolimus have not been published so far.

Here we report on studies performed to better understand the cause of the difference in skin permeation between pimecrolimus and tacrolimus. In addition, we present data on comparative plasma protein binding, to allow for a comparison of systemic exposure to unbound drug and we identified the major binding partners in plasma for both drugs.

Methods and Materials

Permeation assay. Permeation was studied using static Franz-type diffusion cells where silicone elastomer membranes (Dow-Corning, Coventry, UK; #7-4107; 75 µm thick) were mounted. The exposed membrane area was 2.54 cm^2, and the volume of the receptor chamber was 5.8 mL. Phosphate buffered saline/ethanol 3:1 was used as receptor phase. All experiments were performed at 32°C in triplicates for 48 hrs. Pimecrolimus and tacrolimus were applied to the membranes either in solution (propylene glycol/oleyl alcohol 9:1) at a concentration of 1 % (w/v) in a volume of 300 µL, or in their marketed formulations (Elidel, 1% pimecrolimus cream, Novartis; Protopic, 0.1 or 0.03% tacrolimus ointment, Astellas; applied amount: 300 mg).

Samples of 100 µL were withdrawn from the receptor phase at 4 to 8 time points during the 48-hr experiment and replaced by fresh receptor fluid. After addition of an internal standard, and dilution with 0.1 % formic acid/acetonitrile 50:50, these samples were analyzed directly by HPLC MS/MS (see below).

Sample analysis. LC-MS/MS analysis was carried out with a Hewlett-Packard 1090 M HPLC coupled to a Finnigan LCQ mass spectrometer. A Phenomenex Luna C18 column (3 µm, 100x2 mm) equipped with a pre-column, was eluted isocratically with a flow rate of 200 µl/min at 60°C. The eluant was 0.1 % formic acid/acetonitrile 20:80. The sample injection volume was 10 µl. The effluent was delivered unsplit to the ESI ion-source used in positive mode. Under the chromatographic conditions used, pimecrolimus and tacrolimus yielded a strong sodium adduct. For MS/MS, parent ions were selected at 832.0 m/z with a band width of 4 m/z for pimecolimus, and at 826.2 m/z with a band width of 2 m/z for tacrolimus. Collision induced dissociation was carried out with a

collision energy of 28%, yielding a fragment ion at 604.2 m/z for pimecrolimus. A collision energy of 43% was applied to yield a fragment ion at 616.2 m/z for tacrolimus. The quantification of the parent ions was based on the area ratio of the fragment ions to the fragment ion of an internal standard. For calibration, receptor medium was spiked with variable amounts of the analytes resulting in concentrations of 1 to 1000 ng/ml. Calibration curves were set up both with fresh medium and with medium taken at 48 hrs from permeation assays with formulations only (i.e. without active compound), to control for possible interference by excipients, which, however, was not observed. The limits of quantification for pimecrolimus and tacrolimus were 10 ng/mL in receptor fluid. Calculation of flux was done as described (Schmook *et al.* 2001).

Radiolabels and stock solutions. Tritium-labeled pimecrolimus (543.3 MBq/mg) and tacrolimus (1035 MBq/mg; position of radiolabels are given in Figure 1) were prepared and supplied by the Isotope Laboratory of Novartis Pharma AG (Basel, Switzerland). Ethanolic stock solutions were prepared by serial dilution including unlabeled compound resulting in final specific activities of 10.9-543 MBq/mg and 20.7 – 207 MBq/mg for pimecrolimus and tacrolimus, respectively, and concentrations of 2 – 100 µg/mL (1000-times final assay concentrations).

Biological matrices. Human plasma (lithium heparin as anticoagulant, plasma pools from three healthy male donors) was delivered frozen from EFS-ALSACE, Strasbourg, France. Fibrin in plasma was removed by centrifugation for 10 min at 10000 g at room temperature and cleared plasma was frozen in aliquots at –20°C and defrosted before use. Human serum albumin (cat.no. A 1887), α_1-acid glycoprotein (G 9885) and γ-globulins (G 4386) were purchased from Sigma (St. Louis, MO, USA), and solutions were prepared

in PBS. Human high density lipoprotein (HDL, cat.no. 437641), low density lipoprotein (LDL, 437644) and very low density lipoprotein (VLDL, 437647) were purchased from Calbiochem (San Diego, CA, USA); these lipoproteins were delivered as solutions in 150 mM NaCl and 0.01% EDTA, pH 7.4. The concentrations of the lipoproteins as given by the manufacturer were adjusted to the used concentrations with PBS.

The water soluble protein fraction from human cadaver skin (skin extract) was prepared as follows: Skin specimens were obtained from NDRI (Philadelphia, PA, USA). Samples of similar weight from three different male donors were pooled and cut into small pieces (about 3x3 mm) using sharp scissors. The material was suspended in 10 volumes of ice-cold phosphate-buffered saline and homogenized in a Potter S homogenizer (B. Braun Bio-tech, Germany). The homogenate was first centrifuged at 3,500 *g*, 4°C for 5 min. The supernatant was then subjected to a second centrifugation (15,000 *g*, 1 hr, 4°C). The protein concentration in the supernatant (= skin extract) was determined using an assay from BioRad (cat. no. 500-0006) and bovine serum albumin as standard. The extract was frozen, stored at -80°C, and thawed before use. After thawing the solution was cleared by centrifugation (2000 *g*, 4 min, room temperature), before further use.

Protein binding. Binding of [^{3}H]pimecrolimus and [^{3}H]tacrolimus to proteins was analyzed by equilibrium gel filtration (Hummel et al., 1962) (Berger et al., 2003). Two systems were employed: (a) for analysis of total binding two 5 mL HiTrap Desalting columns (Amersham Biosciences) were used in series, here proteins (> 5 kDa) were not separated; or (b) for separation of binding proteins a Superose 6HR 10/300 (Amersham Biosciences) was used with a separation range of 5 – 5000 kDa. The gel filtration column was equilibrated with phosphate buffered saline containing the compound under

investigation at nominal concentrations of 2, 20, or, 100 ng/mL. The temperature was 37°C for the HiTrap Desalting columns and 22-24°C for the Superose 6 10/300, the flow rate was 0.2 mL/min in both cases. Protein containing solutions were injected at volumes chosen to ensure that a binding equilibrium was achieved on the column (e.g. 100 µL plasma, 0.2 mg skin protein, 0.2 – 4 mg of different plasma proteins). The eluate was analyzed for total protein by UV absorption (280 nm) and for total ^{3}H radioactivity in collected fractions by liquid scintillation counting (LSC) in a Packard Tricarb liquid scintillation counter. Pimecrolimus and tacrolimus concentrations in binding experiments were determined by LSC using the respective specific activities.

Data analysis. In contrast to the Superose 6 10/300 column the HiTrap Desalting column does not separate proteins (> 5 kDa) but the total protein runs faster than free [^{3}H]pimecrolimus or [^{3}H]tacrolimus, allowing to achieve binding equilibration on the column. The amount of compound bound (AB) per milliliter of plasma was calculated as: $(A_{PP}-C_{free}*V_{PP})/V_{Plasma}$, with A_{PP} being the total amount of compound in the protein peak fractions, C_{free} the actual free compound concentration on the column (average concentration in fractions before the protein peak), V_{PP} the total volume of the protein peak fractions and V_{Plasma} the injected plasma volume in milliliter. The fraction unbound in plasma (f_u) was calculated as: $1 – (AB/(AB+ C_{free})$. For purified plasma proteins, unbound fractions in plasma were calculated based on (a) the amount of compound bound per milligram of the used protein, (b) the measured free compound concentration and (c) a physiologically relevant concentration of the particular plasma protein (Table 3).

Molecular weight estimation of binding proteins. The Superose 6 10/300 column was calibrated using standard proteins (Amersham Biosciences: ribonuclease (15.6 kDa),

chymotrypsinogen (20.4 kDa), ovalbumin (48.1 kDa), albumin (63.5 kDa), aldolase (171 kDa), catalase (232 kDa), ferritin (391 kDa), thyroglobulin (725 kDa)); elution volumes of standard proteins (mean of two injections) were plotted over the molecular weight (log scale) and a calibration curve established by linear regression with an R^2 value of 0.958. The relative contributions of different separable proteins to the overall binding was roughly estimated by comparison of peak areas of radio signals.

Results

Permeation through a silicone membrane. We compared permeation of pimecrolimus and tacrolimus through a silicon elastomer membrane; such membranes are commonly used as artificial barriers for drug release studies from topical formulations. For both agents the permeation rate through the silicon membrane was nearly identical (Table 1) when applied as 1% solutions in propylene glycol/oleyl alcohol 9:1. Permeation was also tested for the marketed formulations of the two drugs. When applying Elidel (1% pimecrolimus cream), to the artificial membrane, permeation of pimecrolimus was 5.7-fold lower than from the 1% pimecrolimus solution, indicating somewhat lower release from the cream. Using Protopic (0.1% and 0.03% tacrolimus ointment) lower permeation as compared to Elidel was observed with the membrane. The differences in flux roughly reflected the differences in drug concentrations (Table 1). This suggests that the difference in drug concentration causes the difference in flux and confirms very similar permeation characteristics for pimecrolimus and tacrolimus through the artificial membrane. In contrast, as reported previously (Billich et al., 2004, Meingassner et al., 2005), the skin permeation of the two agents differs markedly, whether applied as 1% solutions or as commercial crème and ointments, respectively, and being 4-10 times lower for pimecrolimus compared to tacrolimus (Table 1).

Binding to soluble skin proteins. To shed light on the observed differences in skin permeation of pimecrolimus and tacrolimus, binding to a preparation of soluble skin proteins was analyzed. For both drugs the amount bound per milligram of protein increased apparently linearly with the free drug concentration, in the concentration range covered, however, particularly at high concentrations the experimental scatter was

relatively large. This is likely due to the low protein concentration in the skin extracts, resulting in a limited binding capacity and thus a relatively small bound signal above the free concentration at high free concentrations. Some fluctuation in the free concentration on the column can contribute to the scatter. For pimecrolimus the slope of a linear fit was approximately 3-fold higher compared to tacrolimus (Figure 2A), suggesting a generally higher affinity of pimecrolimus in binding to soluble skin proteins.

To identify any major binding proteins in the skin preparation, the proteins were separated on a Superose 6 column equilibrated with pimecrolimus or tacrolimus. For both drugs only one major specific peak was identified. It eluted at a molecular weight of approximately 15 kDa (Figure 2B). The amount of compound in this peak was similar for both compounds: 13.5 ± 2.4 ng and 12.7 ± 3.5 ng per mg protein (total protein injected), for pimecrolimus and tacrolimus, respectively (n = 3). These values correspond roughly to the y-axis intercept, when total binding is plotted over the free drug levels (Figure 2A). This could result from saturation of the 15-kDa binding protein at all tested drug concentrations, due to a dissociation constant below the lowest tested concentration.

Plasma protein binding. Using equilibrium gel filtration on HiTrap Desalting columns we observed that at similar free concentrations of pimecrolimus and tacrolimus about 9-fold less of the latter was bound to plasma proteins (Figure 3A). Based on binding data at free concentrations of 0.48 to 93.3 ng/mL (pimecrolimus) and 3.3 to 80.2 ng/mL (tacrolimus), unbound fractions of pimecrolimus and tacrolimus in human plasma were estimated to be 0.4 ± 0.1% and 3.7 ± 0.8%, respectively (Table 2). No major concentration dependency of plasma protein binding was found in the tested concentration range. To identify the major binding proteins for pimecrolimus and

tacrolimus, the binding to purified human plasma proteins was analyzed (Table 3). At physiologically relevant concentrations of the different proteins, binding of pimecrolimus was estimated to be highest to lipoproteins, particularly to HDL. For tacrolimus, binding was highest to HDL, followed by VLDL and α_1-acid glycoprotein. Binding to α_1-acid glycoprotein and γ-globulins was similar for pimecrolimus and tacrolimus. In contrast, binding to human serum albumin and lipoproteins was 5 – 9 fold higher for pimecrolimus (Figure 4), which likely causes the overall higher binding of pimecrolimus in plasma.

To substantiate these results, binding to human plasma was analyzed on a Superose 6 gel filtration column. When plasma proteins were separated on a column equilibrated with pimecrolimus, the bulk of pimecrolimus eluted at the expected elution volumes of HDL ($\geq$ 170 kDa, major peak) and LDL (approximately 3500 kDa) (Figure 3B). The very broad peaks were in line with the variable molecular weight of lipoproteins, which are composed of apoproteins and lipids in somewhat varying ratios. The major protein peak at approximately 70 kDa (Figure 3B, UV trace), which corresponds mainly to albumin, was not linked to a pimecrolimus peak, confirming that albumin was less relevant for the overall plasma protein binding of pimecrolimus. In contrast to what could be expected from the experiment using purified plasma proteins (Table 3), no peak was found at the expected molecular weight of VLDL. This is likely due to removal of floating VLDL during plasma preparation for injection (removal of fibrin, see Methods).

Upon separation of human plasma proteins on a Superose 6 column equilibrated with tacrolimus, the highest and relatively slim tacrolimus peak concurred with the second half of the main protein peak (Figure 3B). This corresponds likely to α_1-acid glycoprotein bound tacrolimus. α_1-Acid glycoprotein is expected to elute slightly later than serum

albumin, due to its lower molecular weight (approximately 43 kDa), but can not be separated from serum albumin, since the molecular weight difference is insufficient. A broader peak was apparent at the expected elution volume of HDL and a small shoulder at the expected elution volume of LDL. This is in line with the experiments performed with isolated plasma proteins (Table 2); again a VLDL peak was missing, which may be attributed to separation of VLDL during plasma preparation.

Discussion

We recently reported that the rate of permeation through human skin is lower for pimecrolimus than for tacrolimus, when comparing 1% solutions as well as the marketed formulations of the two drugs (Table 1, Billich et al., 2004; Meingassner et al., 2005). This is in line with lower systemic exposure observed in patients treated with pimecrolimus cream as compared to those treated with tacrolimus ointment in atopic dermatitis (Draelos et al., 2005) and Netherton syndrom (Oji et al., 2005; Allen et al., 2001). In the present studies we found that permeation through an artificial membrane was similar for the two drugs (Table 1). The latter result indicates that the lower permeation of pimecrolimus through skin into the receptor fluid is not caused by a significantly slower release of the compound from the formulations as compared to tacrolimus, nor by a limited solubility of pimecrolimus in the receptor fluid. Rather it appears that the distribution equilibrium between skin and receptor fluid is more on the side of the skin in the case of pimecrolimus, leading to a markedly reduced permeation through skin compared to tacrolimus. These observations point to a stronger binding of pimecrolimus to components of the skin, compared to tacrolimus.

To test this hypothesis, we investigated binding of pimecrolimus and tacrolimus to a preparation of soluble skin proteins. It is important to note, that many major components of the skin, like e.g. collagen fibers or horny skin, are not covered by these experiments. In the skin protein preparation we identified one specific binding protein with a molecular weight of approximately 15 kDa, that displayed a similar binding capacity for pimecrolimus and tacrolimus. Likely this binding peak corresponds to a protein of the tacrolimus binding protein family (FK506-binding proteins, FKBP, also called

macrophilins) which comprises members with molecular weights close to 15 kDa, of which the FKBP12 or macrophilin12 is the best characterized and most prevalent (Galat, 2003). At very low free concentrations this low capacity and high affinity binding might result in similar amounts bound for pimecrolimus and tacrolimus. However, at higher free drug concentrations the protein preparation had approximately 3-fold more pimecrolimus bound as compared to tacrolimus, suggesting a higher affinity of pimecrolimus in this less specific binding. This result may explain the slower skin permeation of pimecrolimus: Due to the high local drug concentration following topical administration, the unspecific high-capacity binding is likely dominating binding in the upper skin layers. In deeper layers at low total concentrations the specific binding would be similar, resulting in a similar pull for both drugs from the deeper layers. Due to the higher binding capacity for pimecrolimus close to the site of application and a similar pull from deeper layers, the permeation of pimecrolimus would be slower and the concentration gradient in terms of total concentration steeper. Both pimecrolimus and tacrolimus bind to macrophilin12 with high affinity. Reported IC_{50} values based on different experimental setups are slightly lower for tacrolimus (0.88 ± 0.2 nM, Weiwad et al., 2006) compared to pimecrolimus (1.8 ± 0.3 nM; Grassberger et al., 1999). This suggests that at low total concentrations the free tacrolimus concentration would be lower and therefore the pull from deeper layers even somewhat higher for tacrolimus.

In plasma we found that lipoproteins contributed strongly to the overall binding of pimecrolimus and tacrolimus, in line with published data for tacrolimus (Nagase et al., 1994). Using ultracentrifugation, an unbound fraction for both drugs of 20 to 30% in human plasma was determined ((Piekoszewski et al., 1993) and unpublished Novartis

internal data). However, while most of the total plasma protein can be separated by ultracentrifugation, lipoproteins can - depending on their characteristic density - either float or sediment very slowly or not at all (Olson, 1998), preventing a complete separation by ultracentrifugation. Therefore, plasma protein binding results based on ultracentrifugation are misleading in case of highly lipoprotein bound drugs, since they will underestimate the extent of protein binding. For tacrolimus a 20-fold lower unbound plasma fraction of 1.2% was measured employing ultrafiltration and equilibrium dialysis (Zahir et al., 2001; Nagase et al., 1994). This is closer to but approximately 3-times lower than the 3.7% determined in the present study by equilibrium gel filtration. These studies employing ultrafiltration and equilibrium dialysis may underestimate the concentration of tacrolimus in plasma water e.g. due to wall binding or binding to the membrane used for separation as suggested previously (Venkataramanan et al., 1995). Also with equilibrium gel filtration we experienced significant drug adsorption to the column. However, the actual free concentration could be determined accurately, due to use of radiolabeled drugs, large volumes available for measurement and lack of pipetting steps. Gel filtration separates molecules according to size by providing different bed volumes for differently sized molecules. When the system is equilibrated with the drug, the larger and faster moving proteins are always exposed to the same free drug concentration leading to equilibration (Hummel et al., 1962; Berger et al., 2003). Here, in addition to a protein separating gel filtration column, we used desalting columns, which only separate small molecules (≤ 1 kDa) from large molecules (≥ 5 kDa, group separation). This allows for short running times and an accurate determination of total binding in a complex mixture of proteins like e.g. plasma. The method is very powerful to determine differences in

binding of highly bound drugs, since the amount bound at a defined free concentration can be compared (Figure 3A), rather than very small free concentrations as in case of standard techniques. A possible source of error is the separation of low molecular weight plasma components like fatty acids and bilirubin, which can influence drug binding. On the other hand major sources of bias like wall or membrane binding of lipophilic drugs have little impact.

The unbound fraction of pimecrolimus in human plasma was determined here to 0.4%, i.e. about 9-fold lower than for tacrolimus. Reported clinical exposure levels for both drugs are based on blood concentrations (Draelos et al., 2005). Exposure to free drug can be derived using blood distribution data. In the relevant concentration range the fraction in plasma is 12% for pimecrolimus versus 2-5% for tacrolimus (Nagase et al., 1994, Zollinger et al., 2006). This partly compensates the observed difference in protein binding; therefore at similar total blood concentrations the free concentration would be 2 – 4 fold lower for pimecrolimus.

For pimecrolimus, binding to plasma lipoproteins was higher compared to binding to albumin and α_1-acid glycoprotein, the two main drug binding plasma proteins (Table 3). Binding of both drugs was highest to HDL, consistent with reported data for tacrolimus (Zahir et al., 2001; Nagase et al., 1994). α_1-Acid glycoprotein contributed substantially to the binding of tracrolimus in plasma (Table 3), which agrees with the finding that the unbound fraction of tacrolimus correlates with α_1-acid glycoprotein as well as HDL-cholesterol levels (Zahir et al., 2004). The binding of pimecrolimus to the different lipoproteins was higher as compared to tacrolimus, in line with the higher overall plasma protein binding of pimecrolimus and its higher lipophilicity (Figure 4). To understand the

possible impact of this high binding to lipoproteins on the disposition of these drugs, the function and disposition of lipoproteins needs to be considered. Plasma lipoproteins mediate lipid transport between tissues, e.g. HDL transports cholesterol to the liver. The liver is the central organ in lipoprotein metabolism and a major fraction of lipoproteins are eventually taken up by the liver via receptor mediated processes (Olson, 1998; Ginsberg, 1998). Pimecrolimus is mainly cleared by hepatic oxidative metabolism, followed by biliary excretion of metabolites (Zollinger et al., 2006) and also tacrolimus is mainly cleared by liver metabolism (Venkataramanan et al., 1995). While lipoprotein binding limits the free drug concentration and with it its liver and overall organ uptake e.g. by passive diffusion, high lipoprotein binding may on the other hand enhance drug uptake via lipoprotein-coupled transport into the liver. To clarify how strongly lipoprotein mediated uptake contributes to the overall liver uptake of pimecrolimus and tacrolimus would need further investigations. However, the effect might be more pronounced for the higher lipoprotein bound pimecrolimus, potentially contributing to a higher systemic clearance. A direct comparison of the systemic blood clearance of the two drugs is not possible, since for pimecrolimus no intravenous pharmacokinetic study was performed. The blood clearance of tacrolimus is 4 - 6 L/h, (Venkataramanan et al., 1995) which is likely lower than the blood clearance of pimecrolimus (CL/f: 72 L/h, (Zollinger et al., 2006)).

In conclusion, the current study highlights the importance of binding interactions and the interplay between specific high affinity and unspecific high capacity binding of topically applied drugs, for controlling drug exposure at the target site and in the systemic circulation. The presented in vitro data suggest that higher unspecific binding to skin

proteins is responsible for the lower skin permeation and the lower systemic exposure upon topical dosing of pimecrolimus compared to tacrolimus. In addition the side by side comparison of plasma protein binding of the two drugs suggests, that the difference in exposure to unbound drug is even more pronounced.

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Legend to Figures

Fig. 1. Structures of ^{3}H-labeled pimecrolimus (A) and tacrolimus (B).

The * denotes the position of 3H-label

Fig. 2. Binding of pimecrolimus and tacrolimus to human soluble skin proteins.

(A) Total binding to skin proteins analyzed on a HiTrap column equilibrated with either pimecrolimus or tacrolimus at different concentrations. The slope of the linear fit was 0.87 for pimecrolimus and 0.27 for tacrolimus. (B) Binding to human soluble skin proteins separated on a Superose 6 column equilibrated with either pimecrolimus or tacrolimus. The only major peak eluted for both drugs at a volume corresponding to a molecular weight of approximately 15 kDa. Elution profiles in (B) are from one representative out of three experiments.

Fig. 3. Binding of pimecrolimus and tacrolimus to human plasma proteins.

Plasma (100 µL) was loaded onto a gel filtration column equilibrated with either pimecrolimus or tacrolimus. (A) Total binding to plasma proteins analyzed on a HiTrap column at actual free concentrations of 88 and 87 ng/mL for pimecrolimus and tacrolimus, respectively. (B) Binding to human plasma proteins separated on a Superose 6 column. The major UV peak at approximately 70 kDa corresponds mainly to albumin, and neither pimecrolimus nor tacrolimus co-elute strongly with this peak. The major peaks for pimecrolimus correspond to the expected elution volumes of HDL (≥ 170 kDa) and LDL (approximately 3500 kDa); the major peaks for tacrolimus correspond to the expected elution volumes of α_1-acid glycoprotein (approximately 43 kDa) and HDL (≥

170 kDa). Elution profiles in (B) are from one representative out of three experiments; the UV signal is in arbitrary units.

Fig. 4. Relative binding of pimecrolimus and tacrolimus to human plasma proteins

Binding of pimecrolimus to human plasma proteins relative to tacrolimus binding was calculated as ratio of the mean unbound fractions given in Table 3.

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Table 1**. Permeation of pimecrolimus and tacrolimus after application to a 75-µm silicone elastomer membrane**

Drug	**Formulation**	**Flux [a] (ng/cm^2/hr)**	
		Silicone	**Human skin [b]**
Pimecrolimus	1% in propylene glycol / oleyl alcohol 9:1	748 ± 41	91 ± 9
Tacrolimus		768 ± 66	816 ± 43
Pimecrolimus	Elidel, 1%	131 ± 21	1.5 ± 0.8
Tacrolimus	Protopic, 0.1%	15.5 ± 2.7	9.3 ± 1.6
Tacrolimus	Protopic, 0.03%	6.6 ± 1.7	6.6 ± 0.7

[a] Mean (n=3) and standard deviation is given; [b] from references Billich et al., 2004 and Meingassner et al., 2005.

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Table 2**. Binding of pimecrolimus and tacrolimus to human plasma proteins**

100 µL of plasma per run were loaded onto a gel filtration column equilibrated with either pimecrolimus or tacrolimus at different concentrations (n = 3 for each concentration).

Mean free concentration (ng/mL)	**Mean bound in plasma (ng/mL)**	**f_u (%), mean ± SD**
	Pimecrolimus	
0.48	99.5	0.5 ± < 0.1
9.64	2112	0.5 ± 0.1
93.3	26554	0.4 ± < 0.1
	Tacrolimus	
3.30	69.1	4.6 ± 0.4
16.9	512	3.2 ± 0.4
80.2	2384	3.3 ± 0.4

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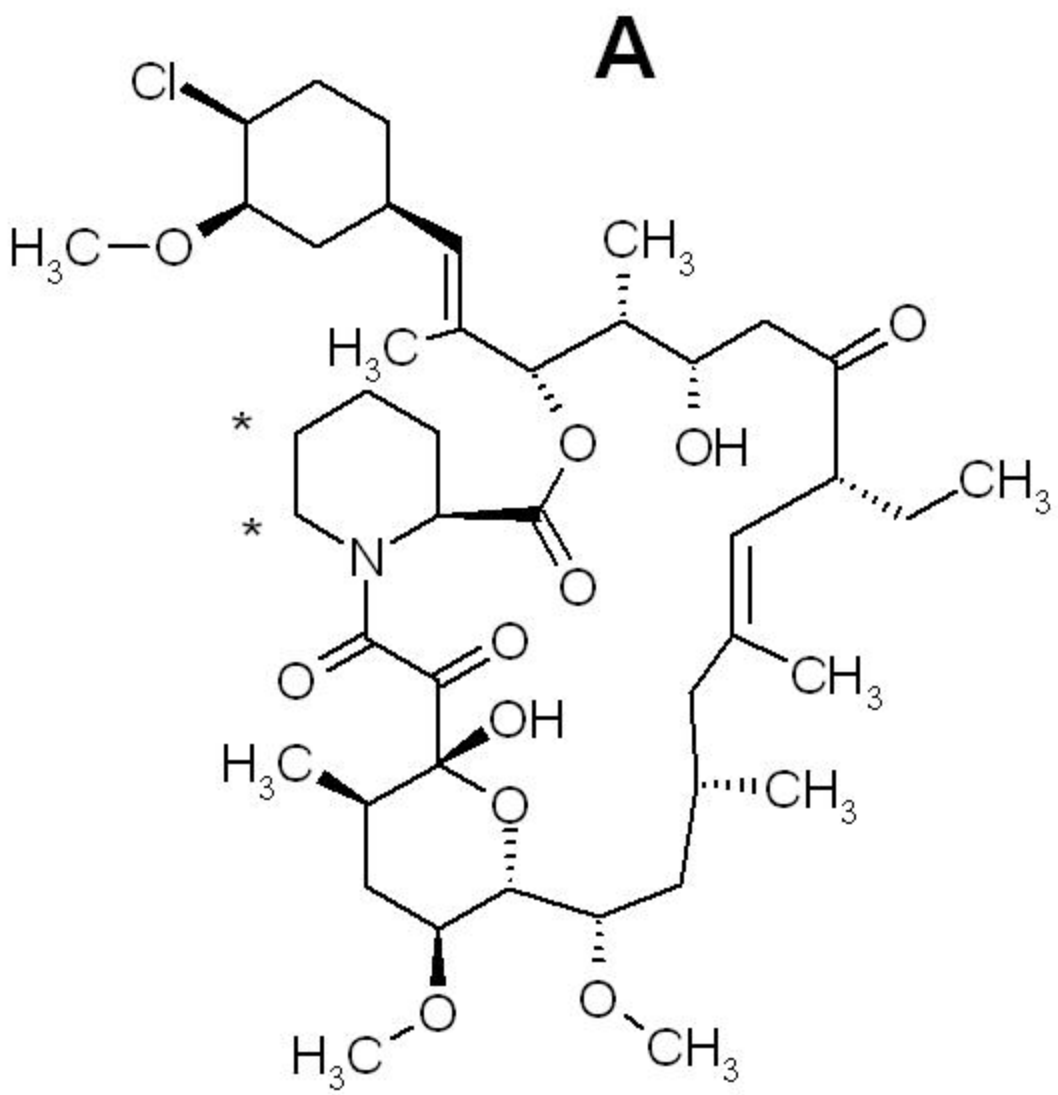
Table 3**. Binding of pimecrolimus and tacrolimus to isolated human plasma proteins**

Plasma protein concentrations in the physiologically relevant range were chosen for calculation of theoretical unbound fractions in absence of other plasma proteins.

Protein	**Assumed plasma concentration of protein (mg/mL)**	**Mean free concentration (ng/mL)**	**Mean bound to plasma protein (ng/mL plasma)**	**Mean f_u (%), individual values in brackets**
		pimecrolimus		
HSA	40	7.1	83	**7.9** (8.2/7.6)
AGP	1.0	6.3	44	**13** (14/11)
γ-globulins	12	6.4	2.7	**71** (65/77)
HDL	3.9	7.6	787	**1.0** (0.9/1.0)
LDL	3.6	7.9	316	**2.5** (2.6/2.4)
VLDL	1.3	8.2	616	**1.3** (1.3/1.3)
		tacrolimus		
HSA	40	22	44	**42** (27/64/35)
AGP	1.0	29	199	**13** (15/11)
γ-globulins	12	25	16	**62** (58/65)
HDL	3.9	31	395	**7.3** (7.3/7.3)
LDL	3.6	33	127	**21** (21/20)
VLDL	1.3	30	216	**12** (12/12)

Figure 1



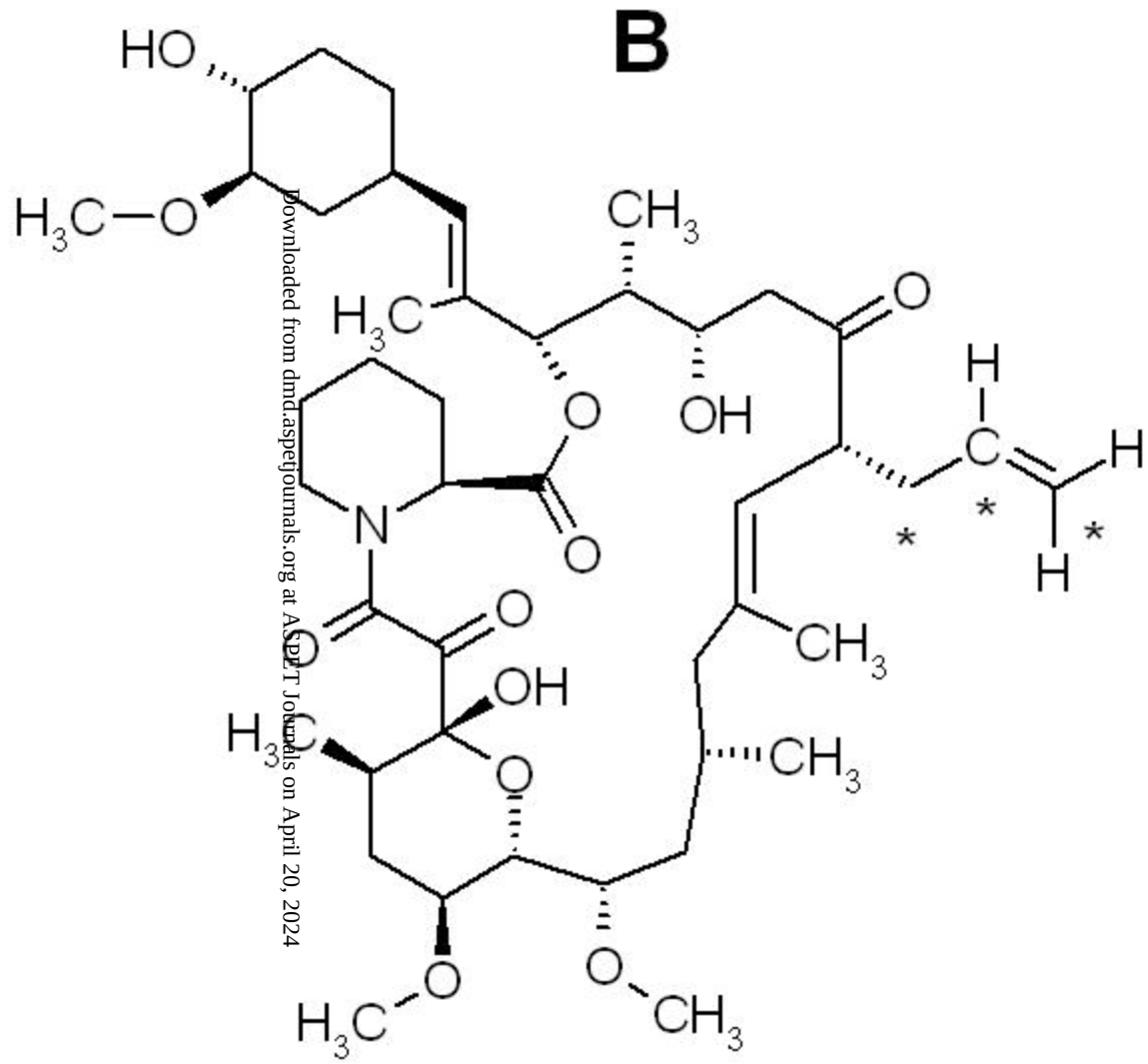


Figure 2A

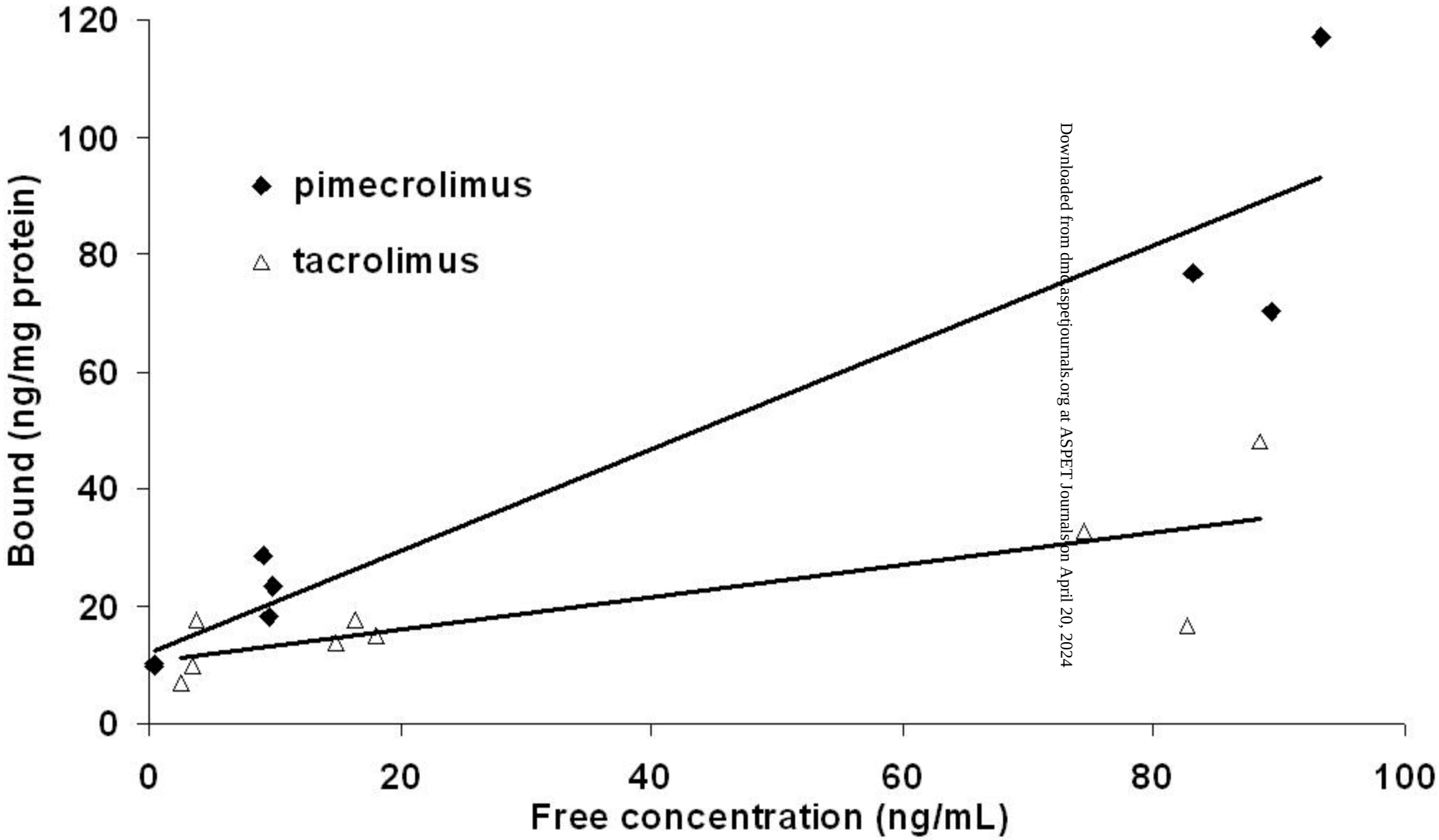


Figure 2B

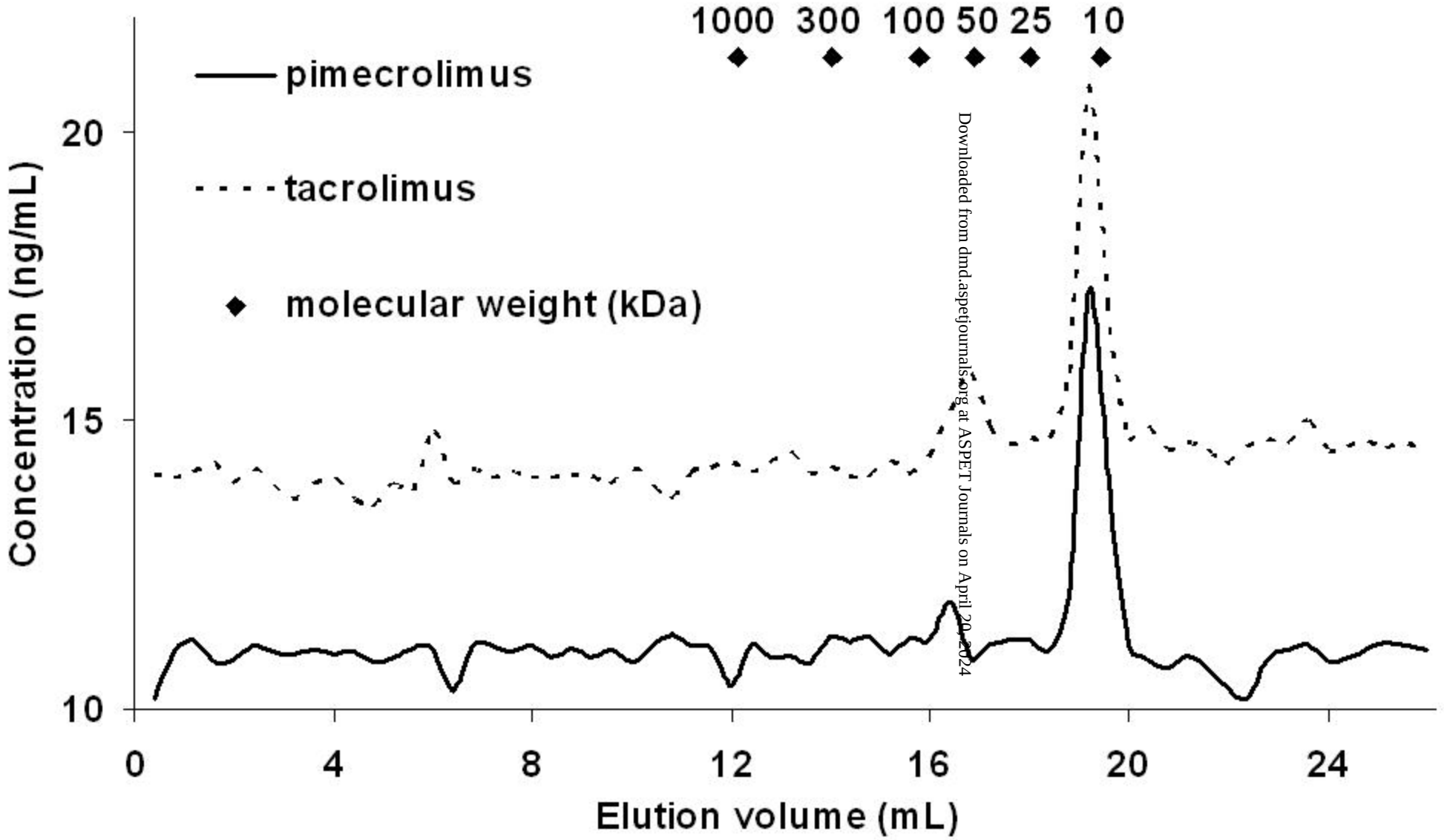


Figure 3A

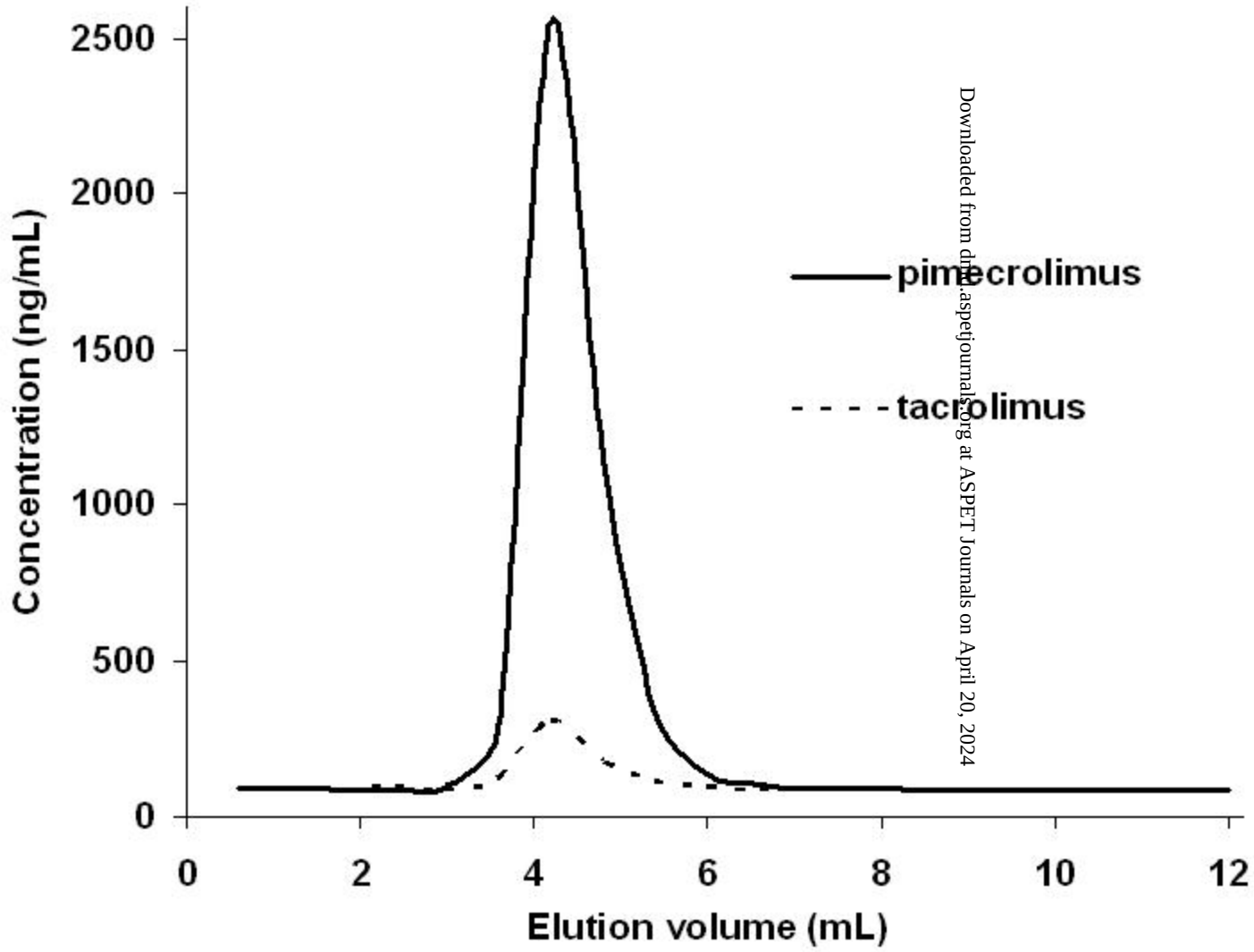


Figure 3B

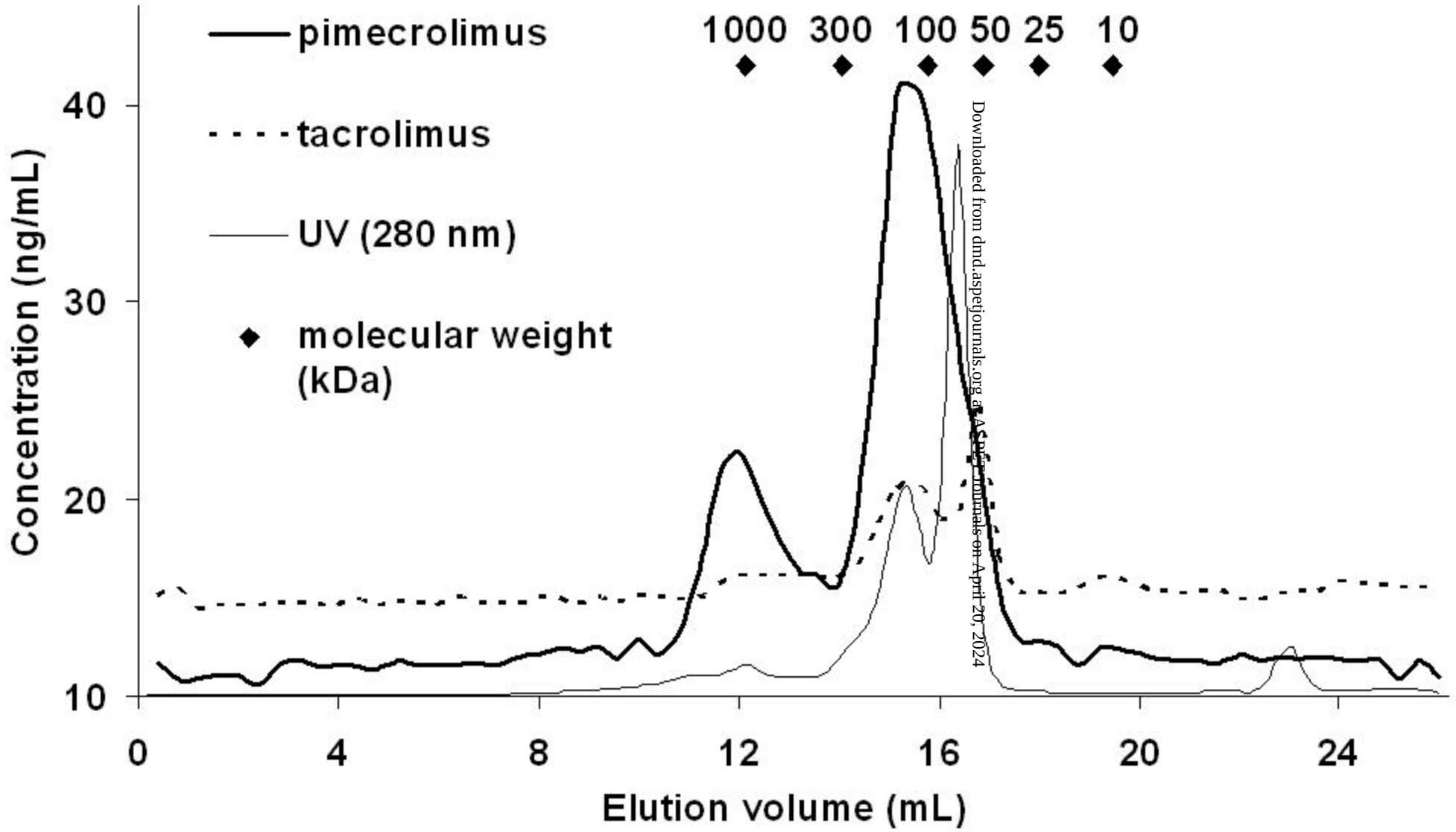


Figure 4