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Modulation of Cyclosporin A/Cyclophilin Interactions by Drug Vehicles

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Abstract—Cyclosporin A (CsA) is a tight-binding inhibitor of the peptidyl-prolyl *cis/trans* isomerase (PPIase) activity of human cytosolic cyclophilin (Cyp18), the putative receptor for immunosuppressive effects of the drug. We examined the influence of cremophor EL (CEL), a surfactant that has found wide use for CsA formulation, on the kinetics of inhibition of the enzyme by CsA. Stock solutions of CsA in CEL administered into aqueous PPIase assays led to inhibition kinetics reminiscent to those of CsA dissolved in tetrahydrofurane, but caused an increase in the final K_i value of about sevenfold at 0.33% (v/v) CEL. The diminished drug affinity to Cyp18 obtained in experiments using CEL could also be established for analogues of cyclosporin A such as [Ala^2]-Cs, [Thr^2]-Cs, and [MeAla^6]-Cs, exhibiting K_i values 13–16-fold higher than in the absence of CEL. In addition, the time-dependent pattern of inhibition indicated only a minor population of bioactive conformation of CsA in bulky CEL. Conformational reshuffling of the bioinactive [*cis*-MeLeu^9-MeLeu^{10}]-Cs to create an inhibitory fraction of the drug was delayed in the presence of CEL micelles, despite potential ability of micelles exists to catalyze *cis/trans* isomerizations of N-alkyl peptide bonds. The pattern of inhibition when using cyclophilins distinct in their amino acid sequences to the human enzyme can be rationalized in terms of exceptional high structural requirements of human Cyp18 for the drug conformation. Copyright © 1997 Elsevier Science Ltd

Introduction

Cyclosporin A (CsA) is thought to exert its immunosuppressive action and many other physiological effects by binding to cyclophilins (Cyp), an enzyme family which belongs to the growing class of peptidyl-prolyl *cis/trans* isomerases (PPIases).[1-4] The human cytosolic enzyme Cyp18 isolated from T-lymphocytes and many other cells and tissues has been shown to be competitively inhibited by CsA with a K_i of 2.6 nM.[5] Formation of the drug/cyclophilin complex has been found to be the prerequisite for many biological effects of cyclosporins.[6-8] Binding of CsA and amino acid sequence of cyclophilins have been interrelated in that Trp^{121} was identified to function as the major determinant for affinity.[9] Even if enzyme inhibition has binding of the inhibitor as a prerequisite, and thus can function as a reliable tool to report about ligand–receptor affinities, there is considerable scattering in the values reported so far for cyclosporin A/Cyp18 binding constants (1.6–360 nM).[1,10,11] A further need for methodological clarification of evaluating cyclosporin-derived constants results from structure–activity relationships. Since the evaluation of cyclosporin derivatives makes use of both affinity-related constants (K_i, IC_{50}) and IC_{50} values of biological effects, misinterpretation can emerge from highly scattered constants. A good correlation between cyclophilin binding and anti-HIV activity was found by comparing the respective IC_{50} values for CsA and several analogues.[12] In contrast, a similar correlation could not be not found for cyclophilin binding and antimalarial action of cyclosporins[13] and for immunosuppressive effects of CsA analogues.[14]

Obviously, such kind of analysis has to correlate effects which should not be perturbed by competing conformational dynamics and preincubation artefacts of the ligands. Furthermore, if conformational heterogeneity is present, care must be taken to assure similar fractions of slowly interconverting conformers for both the biological assay and ligand binding test. Cyclic peptides are predestined to express such type of solvent-dependent heterogeneity to a considerable extent.

By using organic solvents, sometimes endowed with LiCl, an ingenious way became available to switch in solution between bioactive and bioinactive conformations of CsA, pointing to the conformation of the MeLeu^9–MeLeu^{10} peptide bond as major determinant for binding to Cyp18.[15,16] The interconversions involved have been found to be slow under conditions often encountered during receptor binding assays and the evaluation of cellular models, and can transmit conformational states adopted during storage in nonaqueous stock solutions to the assay.

A clear preference regarding the vehicles of CsA for in vitro studies was found with organic solvents such as ethanol.[17-19] The non-ionic surfactant cremophor EL (CEL) is often used clinically. CEL is a polyethoxylated castor oil that is obtained by reacting castor oil with ethylene oxide. Due to its micelle-forming properties the compound can be considered as a lipid membrane-mimicking vehicle. It already simulates an environment

Key words: cremophor EL, cyclosporin A, peptidyl-prolyl *cis/trans* isomerase, kinetics, cyclophilin

outside the cell that cyclosporins must encounter when crossing cell membranes. Because bioreactive conformation(s) of cyclosporin A were differentiated by a peptide bond isomerization, it is of considerable interest that N-alkyl peptide bonds were found to interact specifically with micelles. Both equilibrium constants and dynamics of interconversion were potential parameters to be influenced [20,21] (M. Kramer and G. Fischer, submitted). Indeed, several reports suggested that CsA can behave differently in vivo when either CEL or organic solvents were used as vehicles for the drug.[22,23] In this view the scope of the present work was to examine the effects of bulky CEL and CEL micelles on the bioreactivity of the drug. In addition, to gain access to open questions of the molecular mechanism of inhibition, other cyclosporins as well as enzymes homologous to recombinant human Cyp18 (rhCyp18) were utilized.

Results

Time-dependent inhibition of PPIase activity by CsA

The effect of bulky CEL, used to prepare the stock solution of CsA, on the time course pattern of inhibition of rhCyp18 was measured. Time-dependence of inhibition of PPIase activity of rhCyp18 by CsA was examined by incubating the enzyme with the inhibitor, which was added from different solvents. Incubation time was varied between 20 s and 1 h. Beside CEL the solvents DMSO, THF, 470 mM LiCl/THF, and 50% ethanol/water (v/v) were used. In simplification of the method presented earlier,[15] the slow decrease of PPIase activity of rhCyp18 due to time-dependent inhibition by CsA was described using eq (4) (see Experimental for details). The reaction progress curves shown in Figure 1 were measured by injecting CsA from stock solutions of different solvents. The signal utilized for monitoring the reaction was the decrease of PPIase activity, which is directly proportional to the concentration of the CsA/Cyp18 complex, at least for long incubation time. Already by visual inspection the time-dependent inhibition can be separated into several kinetic phases. For nonmicellar solvents this behaviour completely agrees with results obtained at slightly different conditions by Kofron and coworkers.[15] Table 1 gives a summary of the calculated amplitudes and rate constants. Significant inhibition of PPIase activity occurred during mixing time when CsA was added from LiCl/THF or polar solvents (DMSO, ethanol/water). When CsA was initially dissolved in THF or CEL only a very small, if any, extent of inhibition of rhCyp18 could be detected. This very fast decrease was regarded as the first phase. It is too fast to be resolved with manual mixing procedures, but its amplitude can be calculated by extrapolation.

With a half time of about 20 s the second kinetic phase of inhibition proceeds at a time scale sufficiently slow

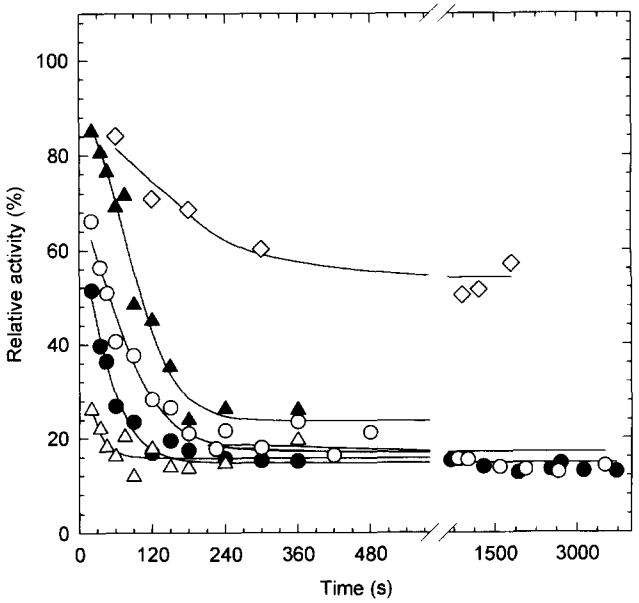

Figure 1. Decrease of relative activity of 2.1 nM rhCyp18 due to time-dependent inhibition by 20 nM CsA from CEL (◇), 50% ethanol/water (v/v) (○), DMSO (●), THF (▲) or 470 mM LiCl/THF (△). Each point was determined separately and represents the mean of three experiments. Curves were fitted as described in section Experimental. Determined parameters are presented in Table 1. Experiments were performed at 9.5 °C in 50 mM HEPES pH 8.0, 100 mM NaCl.

Table 1. Effect of solvent of CsA (20 nM) on time-dependent inhibition of 2.1 nM rhCyp18

Solvent	1. Phase	2. Phase		3. Phase		Remaining activity
	Ampl.[a] (% activ.)	Ampl. (% activ.)	k_{obs} ($\times 10^{-2}$ s^{-1})	Ampl. (% activ.)	k_{obs} ($\times 10^{-3}$ s^{-1})	(% activ.)
EtOH/H_2O	28[b,c]	52	2.6±0.3	6	1.4±0.4	13
DMSO	34	48	3.6±0.5	7	1.8±0.8	14
THF	10	66	2.8±0.3	n.d.[d]		23
THF/LiCl	60	24	5.3±3.1	n.d.		16
CEL	12	34	1.1±0.3	n.d.		54

[a] Amplitudes (ampl.) are given as relative activities.
[b] Calculated as a difference to the activity at zero time.
[c] Amplitudes were determined with a mean error of 12%.
[d] n.d.: Not detected.

to be evaluated quantitatively. The observed first-order rate constants (k_{obs}) for this phase were similar for all solvents except of LiCl/THF. The higher value for k_{obs} in this experiment may be due to the relatively small amplitude of inhibition which results in a higher experimental error. As can be seen from Table 1 the amplitude of the second phase of inhibition depends on the solvent for CsA.

When CsA was added from DMSO or ethanol/water a third phase of decrease of activity was detected. The amplitude of this phase is relatively small and the observed rate constant is one order of magnitude lower than that of the second phase. The remaining activity represents the relative activity that was detected after the equilibrium of the inhibition reaction had established. No further change of PPIase activity could be found. While the remaining activities at a given CsA concentration in the experiments where the drug was added from organic solvents are similar, it is significantly higher when CsA was added from CEL (Table 1).

In order to assess the influence of the rhCyp18 concentration on the time-dependent inhibition, the amount of the enzyme was varied between 1.1 nM and stoichiometric amounts, whereas the CsA concentration was kept constant at 10 nM (CsA added from a stock solution in DMSO). However, no notable effect of enzyme concentration on k_{obs} ($k_{obs} = 2.1 \pm 0.5 \times 10^{-2}$ s^{-1}) was found, pointing out the monomolecularity of the process involved in the second kinetic phase of inhibition. The fraction of inactive enzyme produced during mixing time was also found to be independent of the enzyme concentration.

Effect of CEL on inhibition constant K_i

A K_i of 2.3 ± 0.5 nM has been determined for the inhibition of PPIase activity of rhCyp18 by CsA added from THF/LiCl in long-term incubation experiments (Table 2). Addition of CsA from other organic solvents (THF, DMSO, 50% ethanol/water) resulted in identical values for K_i (data not shown), indicating that the effect of the residual solvent in the assay was negligible. However, under the same conditions K_i is significantly higher for CsA added from CEL. In this experiment a K_i of 15.7 ± 1.9 nM was determined. Addition of CsA from THF/LiCl also showed this increase ($K_i = 13.4 \pm 0.6$) when CEL was present in the assay at the same concentration of 0.33% (v/v). The reduction in inhibitory potency caused by CEL depends on the cyclosporin structure as was found for immunosuppressive ([Ala^2]-Cs, [Thr^2]-Cs) and nonimmunosuppressive ([MeAla^6]-Cs) derivatives of CsA (Table 2). Using a constant ratio of Cyp18 and CsA in a concentration range of 0–0.66% (v/v) CEL the remaining activity was affected in a dose-dependent manner exhibiting a near linear relationship between activity and CEL concentration (data not shown). In this concentration range the PPIase activity itself is not influenced by CEL.

Table 2. Effect of CEL on K_i of cyclosporins

Cyclosporin	K_i (nM)	
	THF/LiCl	CEL
CsA	2.3 ± 0.5	15.7 ± 1.9
[Ala^2]-CsA (CsB)	12.2 ± 0.2	200.7 ± 19.9
[Thr^2]-CsA (CsC)	1.5 ± 0.2	21.0 ± 4.5
[MeAla^6]-CsA	5.8 ± 0.7	76.8 ± 18.3

Effect of CEL micelles on time-dependent inhibition

To further characterize the interaction of CEL with CsA, the effect of CEL micelles on time-dependent inhibition of PPIase activity was examined. While in the previous experiments, using CsA in stock solutions of bulky CEL, time was sufficient to induce CEL-specific conformations to CsA, the drug was now added from other solvents to a mixture of rhCyp18 and preformed CEL micelles at 0.33% (v/v) CEL. The experimental conditions match exactly those applied to detect micellar catalysis of peptide bond isomerization in linear proline peptides (M. Kramer and G. Fischer, submitted). Indeed, 1.6-fold acceleration of the *cis* to *trans* isomerization in the same type of linear proline peptides was detected when applying micelles formed at 1.2% (v/v) CEL. Concomitantly, in these experiments the *cis/trans* ratio decreased 1.1-fold in favour of the *trans* conformer. However, we did not yet attempt to investigate whether the CEL-related rate enhancement meets all criteria of micellar catalysis. In the inhibition experiments using CsA in the presence of preformed micelles, enzymatic activity was measured with a sampling time ranging between 30 s and 10 min. CEL micelles had no effect on the amplitudes of fast phase of inhibition since for addition of CsA from both LiCl/THF and THF the magnitudes were similar to those determined without CEL in the assay mixture (Table 3). While the amplitudes of the second phase are also not affected by the micelles, the rate constants are distinct from the values measured in the absence of CEL. In the presence of CEL micelles PPIase activity decreased slightly faster when CsA was dissolved in THF/LiCl prior to addition to the assay opposite to a two to threefold lower value for k_{obs} when CsA was added from THF. However, in the latter case the k_{obs} measured for CsA dissolved in bulky CEL was similar to that of the THF stock solution (*Table 3*).

Dissociation of the rhCyp18/CsA-complex by micelles

In order to assess the influence of CEL micelles on the rhCyp18/CsA-complex already formed in a reversible reaction, 2.1 nM enzyme and 5 nM inhibitor were incubated for 5 min without CEL. After this time there was no detectable change of PPIase activity indicating that equilibrium was reached. Following addition of CEL [final concentration 0.33% (v/v)] the reaction mixture was sampled at different incubation times (30 s–15 min) to measure the PPIase activity. This procedure allows the reequilibration of the reversible

Table 3. Effect of CEL on time-dependent inhibition of 2.1 nM rhCyp18 by 5 nM CsA from different stock solutions

Solvent	CEL	1. Phase	2. Phase		Remaining activity
		Ampl.[a] (% activ.)	Ampl. (% activ.)	k_{obs} ($\times 10^{-2}$ s^{-1})	(% activ.)
LiCl/THF	–	33[b,c]	24	3.2±0.7	43
LiCl/THF	+	15	32	5.9±2.1	53
THF	–	4	44	3.2±0.3	52
THF	+	11	33	1.1±0.1	56
CEL	+	4	28	1.2±0.4	68

[a]Amplitudes (ampl.) are given as relative activities.
[b]Calculated as a difference to the activity at zero time.
[c]Amplitudes were determined with a mean error of 19%.

enzyme/inhibitor reaction. Slow increase of enzyme activity could be obtained that gave a rate constant of $2.1 \pm 0.8 \times 10^{-3}$ s^{-1}. This value is lower than that found for the formation of the enzyme/inhibitor complex in the presence of CEL ($k_{obs} = 1.2 \pm 0.4 \times 10^{-2}$ s^{-1}, see Table 3) by a factor of 6.

Time-dependent inhibition of cyclophilins from other species

Cyclophilins altered in their amino acid sequences at the CsA binding site can serve as probes to indicate any rearrangement of the primarily formed CsA/Cyp18 complex that tightens the interaction. The loss of the Trp^{121} determinant and the consequent reduction in CsA affinities of prokaryotic cyclophilins prompted us to measure the time-dependent inhibition of cytoplasmic cyclophilins from *Escherichia coli* (ecCyp18) and *Legionella pneumophila* (lpCyp18). The K_i values were reported to be 3.4 μM [24] and 1.3 μM (B. Schmidt, personal communication) for ecCyp18 and lpCyp18, respectively. In our experiments the concentration of CsA was adjusted to the respective K_i to yield the inhibitory complex to about 50 %, a level comparable to the experiments with rhCyp18. In addition to these cytosolic enzymes lacking the Trp, the mature rat mitochondrial Cyp18 (rCyp18mit) was used because it combines Trp-specific high CsA affinity with considerable sequence variations in the remaining part of the protein chain.[25]

The inhibition of rCyp18mit and both prokaryotic enzymes were found to be time-dependent when CsA was injected from a THF stock solution. The estimated rate constants differ only slightly from the values obtained for rhCyp18. As demonstrated by the values in Table 4 a higher extent of inhibition by CsA from the THF stock solution during mixing time was observed for lpCyp18 compared with the human enzyme.

What is most striking for a CsA application from THF/LiCl stock solutions is the lack of a subsequent change in the remaining enzyme activity after the initial drop of inhibition to 35% remaining activity occurred (Fig. 2). Apparently, for bacterial cyclophilins, being more promiscuous in their conformational requirements for binding, conformational reshuffling to further enhance binding affinity of the drug did not take place. The slight increase of PPIase activity with increasing incubation times is likely to be due to the sequestering of the hydrophobic CsA by the glass surface of the sample tube.

Examination of conformational changes of CsA by circular dichroism

A kinetic probe for conformational interconversions of CsA independent of enzyme activity can help to differentiate between conformational reshuffling of the CsA/rhCyp18 complex and CsA free in solution. As far UV circular dichroism is a useful tool to characterize conformational changes of peptides, CD spectra of CsA in different solvents were recorded. Indeed, subtle changes were detected between 220 and 260 nm with an isosbestic point at 228 nm (data not shown). Solvent jump experiments were performed to follow the time-dependent decrease in ellipticity at 232 nm after

Table 4. Time-dependent inhibition of different cyclophilins by CsA dissolved in THF

CsA (nM)	Cyp (nM)		1. Phase	2. Phase		Remaining activity
			Ampl.[a] (% activ.)	Ampl. (% activ.)	k_{obs} ($\times 10^{-2}$ s^{-1})	(% activ.)
10	3.0	rCyp18mt	9[b,c]	63	2.0±1.3	28
5	2.1	rhCyp18	4	44	3.2±0.3	52
1000	10.0	lpCyp18	41	27	4.4±2.0	32
1500	1.0	ecCyp18	15	20	3.2±1.3	65

[a]Amplitudes (ampl.) are given as relative activities.
[b]Calculated as a difference to the activity at zero time.
[c]Amplitudes were determined with a mean error of 12%.

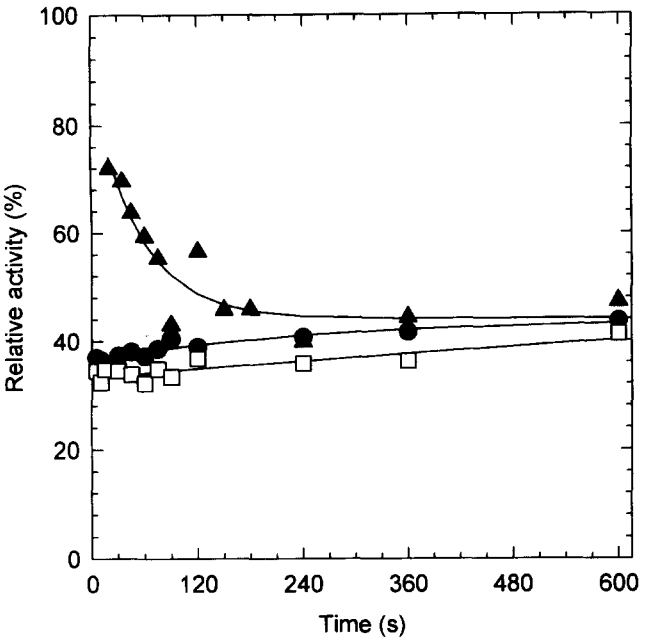

Figure 2. Time-dependent inhibition of different cyclophilins by CsA dissolved in THF/LiCl. Each point represents the mean of three independent measurements. Concentrations of enzyme and inhibitor were varied to take particular differences in K_i and k_{cat}/K_m into account. 2.1 nM rhCyp18 were inhibited by 5 nM CsA (▲), 10 nM lpCyp18 by 1 μM CsA (●), and 1 nM ecCyp18 by 1.5 μM CsA (□), respectively. Experiments were carried out in 50 mM HEPES pH 8.0, 100 mM NaCl at 9.5 °C.

addition of CsA from a LiCl/THF stock solution into bulky ethanol. Organic solvents were used exclusively in the experiments to avoid perturbation of measurements due to transient precipitation and resolubilization of CsA in aqueous solution. A slow conformational change of CsA inferred from the development of a CD signal ($\Delta^{\theta=35,000} \pm 14{,}000$ deg cm^2 dmol^{-1}) according to a first-order law with a rate constant of $k_{obs} = 4.0 \pm 0.8 \times 10^{-2}$ s^{-1} at 10 °C.

Discussion

Although detailed studies gave insight into the considerable conformational differences between CsA free in organic solutions and the drug bound to rhCyp18,[26–28] the reaction sequence that accompanies complex formation cannot be described completely. As a consequence of the low solubility of CsA its three-dimensional structure in water was not explored hence preventing detailed description of the reactant state. The knowledge on bioactive structures of CsA in aqueous buffer was advanced indirectly by Kofron et al. [15] utilizing solvent jumps during the inactivation of the enzyme by CsA. The results could be modelled by a two step mechanism of inhibition including a *cis* to *trans* isomerization of the *cis*-MeLeu^{9,10} peptide bond and a reshuffling of an initially formed [*trans*-MeLeu^{9,10}]-CsA /rhCyp18 complex, both occurring monomolecularly. A common value of 79 kJ mol^{-1} was estimated for $\Delta G^{\#}$ of the two respective processes, perhaps rendering them similar to each other in the molecular origin. Furthermore, results depicted in Figure 1 confirmed the requirement for more than two molecular events when starting the inhibition experiment from either DMSO or ethanol/water. However, the involvement of the enzyme in the slow first-order inactivations[15] may be a matter of controversy since the [D-MeSer^3, D-Ser-(*O*-Gly)^8]-Cs analogue locked in a fully developed, bioreactive structure already in water does not show any slow onset of inhibition, even if injected from DMSO solution.[29]

In our investigations it became apparent that the complex binding behaviour of CsA is a matter of rhCyp18 but failed to affect bacterial cyclophilins. In the THF experiments the CsA/enzyme ratio was 100–1500, exceeding that used with the mammalian enzymes greatly. Thus, the marked amplitudes of the fast phase of inhibition may result from either a minor amount of a very stable, highly bioreactive CsA isomer or less severe requirements for ligand binding of the bacterial enzymes. In addition, the small differences in the rate constants of the second kinetic phase between both variants of cyclophilin may signal a reaction proceeding independently of the enzyme. Furthermore, rate constants of the second phase did not reveal marked changes in the CsA/rhCyp18 ratio indicating that free enzyme may not contribute to the rate limiting step under our conditions. In support of the above notion a slow conformational change of CsA in free solution being added from THF/LiCl into hydroxylic solvents became obvious by CD-spectroscopy. The first-order rate constant of $k_{obs} = 4.0 \pm 0.8 \times 10^{-2}$ s^{-1} is quite similar to those found for the second phase of inhibition when monitored by the decrease of enzyme activity (Table 1).

It was further shown that the THF/LiCl experiments applied to the bacterial enzymes did not reveal a slow phase but expressed full inhibition immediately after mixing. The detected decrease to 35% remaining activity during mixing time corresponds to K_i values of 1.1 μM and 0.5 μM for ecCyp18 and lpCyp18, respectively. This is in good agreement with the reported values of $K_i = 3.4$ μM [24] for ecCyp18 and $K_i = 1.3$ μM for the enzyme from *L. pneumophila* (B. Schmidt, personal communication). Thus, both prokaryotic enzymes are inhibited by [*trans*-MeLeu^{9,10}]-CsA in a very rapid process, which does not exhibit slow binding typical for rhCyp18.

Next, the unambiguous demonstration of the capacity of polar solvents in inducing one kind of bioactive conformation of CsA[29] remained open for drug vehicles like CEL used for therapeutic purposes. Even if systematic investigations are lacking, a dependence of biological effects of CsA on the vehicle used for the drug has already been reported. The inhibition of oxidative phosphorylation in mitochondria from rat kidney cortex by CsA was found to be more pronounced comparing ethanol to DMSO or CEL as vehicles.[23] In a rabbit perfusion model CsA was shown to inhibit vascular release of prostacyclin when dissolved in CEL but not when dissolved in ethanol.[22]

Starting with CsA dissolved in CEL two kinetic effects became obvious in our inhibition experiments. (1) The shape of the inhibition curve closely resembled that of CsA dissolved in THF that also lacked both a very fast and a very slow phase, and (2) there was a considerable decrease of the rate constant of the second phase. From (1) it follows that only a minor amount of bioactive conformation of CsA is present in CEL. Thus, at the low inhibitor/enzyme ratio used here CsA cannot markedly inhibit enzyme activity immediately after mixing (Table 1). On the other hand, preformed micelles were not able to alter rapidly the large portion of the bioreactive conformation present in THF/LiCl toward the bioinactive conformation present in bulky CEL (Table 3). In contrast, preformed micelles were found to decrease the rate constant of the slow phase resulting from THF stock solutions to a level typical of CsA dissolved in CEL. Obviously, micellar catalysis of the slow process thought to result from the *cis* to *trans* isomerization of the $MeLeu^9$—$MeLeu^{10}$ peptide bond is lacking. The mechanism by which the observed retardation may occur must include micelle bound, uncomplexed drug. The micellar attack of the CsA/rhCyp18 complex can be expected to be diffusion-controlled, as in the presence of CEL micelles no delay of decrease of PPIase activity was detected for inhibition by CsA dissolved in THF/LiCl.

Evidently, CEL micelles were found to be formed rapidly after injecting the CEL/CsA mixture into the aqueous phase, which, in turn, provides the basis for the similarity of the rate constants of the slow phase for preformed micelles and additions from bulky CEL. Moreover, micelles and rhCyp18 compete for CsA in the equilibrated reaction mixture as well, leading to a considerable reduction in the concentration of the CsA/rhCyp18 complex. This change is reflected in an increase of the inhibition constant. Reasoning that this competition liberates active enzyme by prior micelle incubation, dissociation kinetics of the complex became available. Assuming diffusion-controlled bimolecular complex formation the consequent dissociation of the already formed CsA/rhCyp18 complex by CEL micelles exhibited rate constants ($k = 2.1 \pm 0.8 \times 10^{-3}\ s^{-1}$) much too low to be explained simply by the decay of the complex. In effect, partitioning of CsA between micelles and rhCyp18 may include conformational interconversions of the drug. In a similar manner the CEL influence on the K_i values depends on minor structural variations of the cyclic peptide. Thus, the effect of the micelles cannot be accounted for by the overall hydrophobicity alone (Table 2). One possible explanation for the micellar effects is that CEL micelles and rhCyp18 might preferably bind to distinct CsA conformations.

Conclusions

This study demonstrates that the micellar drug vehicle cremophor EL modulates the binding of rhCyp18 to cyclosporins in both kinetics and binding affinity in aqueous buffer at 9.5 °C. The processes responsible for the time-dependence of inhibition of rhCyp18 by CsA are expected to be much faster in homogenous solutions in vivo at physiological temperatures, but may be decelerated when CsA has to traverse lipid membranes. In view of these results, a CEL-induced shift of the equilibrium between the different conformations of CsA would be of particular relevance when monitoring biological activities. The kinetic pattern of inhibition of various cyclophilins suggests a plausible reaction sequence consisting of conformational interconversions that occur at the level of cyclosporins free in solution.

Experimental

Chemicals

CsA, [Ala^2]-Cs, [Thr^2]-Cs and cremophor EL were a gift of H. Pötter, Arzneimittelwerk Dresden GmbH (Dresden, Germany). [$MeAla^6$]-Cs was kindly given by Dr A. Lawen, Monash University (Clayton, Australia). Recombinant human Cyp18 was from Boehringer-Mannheim (Mannheim, Germany). Bovine α-chymotrypsin, trifluoroethanol, DMSO and ethanol were purchased from Merck, Darmstadt. Buffer salt 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) was from Serva (Heidelberg, Germany). Succinyl-AAPF-4 nitroanilide and pyrene were obtained from Sigma (Deisenhofen, Germany). Tetrahydrofurane (anhydrous) (THF) was from Aldrich (Steinheim, Germany). LiCl and NaCl (analytical grade) were from Roth (Karlsruhe, Germany).

CD-measurements

CD measurements were performed with a Jasco (Tokyo, Japan) J-710 spectrometer equipped with a Neslab RTE 100 thermostat. The spectrometer was calibrated using ammonium D-10-camphorsulphonate (Jasco, Tokyo, Japan) for standard. The temperature was set to 10 °C. A 1 mm quartz cuvette was used. Spectral bandwidth was adjusted to 1 nm. Spectra were recorded from 270 to 190 nm and averaged over eight accumulations. For kinetic measurements the wavelength was set to 232 nm. CsA was used in a final concentration of 110 μM. CsA from a stock solution in LiCl/THF was injected into the cuvette containing EtOH and the change of CD signal was recorded.

Measurement of PPIase activity

PPIase activity of rhCyp18 was determined using a protease coupled UV–vis spectrometric assay using chymotrypsin and the tetrapeptide substrate succinyl-AAPF-4-nitroanilide at 9.5 °C in 50 mM HEPES, pH 8.0, 100 mM NaCl. Stock solns of the substrate were prepared in 470 mM LiCl/TFE. Final concentration of substrate was usually 20–40 μM with a *cis*-conformer fraction of 50%. Concentration of TFE in the assay was less than 0.1% (v/v).

The enzyme rhCyp18 was used from a 100 μM stock soln which was diluted to yield a 210 nM soln in

aqueous buffer. The fraction of active enzyme was determined fluorimetrically as described previously.[30] Enzyme concentrations of stock solutions of *E. coli* cytoplasmic cyclophilin (ecCyp18) and *L. pneumophila* cytoplasmic cyclophilin (lpCyp18) were determined spectrophotometrically using the absorption coefficient at 280 nm.[31] Authentic proteins were purified as described elsewhere.[32] Stock solns of cyclosporins were prepared in DMSO, 50% aq EtOH (v/v), THF, 470 mM LiCl/THF or CEL in concentrations 1000-fold (organic solvents) and 300-fold (CEL) higher than final concentrations, respectively. In the relative activity scale the 100% value was determined using corresponding solvent blanks with similar incubation time as were applied to the inhibitor experiments. Final concentration of organic solvents in the assay was less than 0.2% (v/v). Inhibition of rhCyp18 by these solvents is negligible under these conditions. The contribution of added LiCl to ionic strength can be neglected. Fast mixing of CEL in aq buffer was reached using an ultrasonic processor Bioblock B72442 (Vibra Cell, Strasbourg, France) with five to seven pulses (7 W, 0.2 s pulse, 0.6 s break). Sonication did not affect the PPIases.

In a typical assay, buffer including chymotrypsin (1.7 mg ml^{-1}) was maintained at constant temperature for 5 min. After addition of 12 µl rhCyp18 and 1.2 µl CsA the mixture was incubated again (20 s–10 min). For variation of the enzyme concentration 6 and 24 µl of a 210 nM rhCyp18 solution were added, respectively. In every assay the final volume was fixed at 1.2 ml. When CEL was added separately, it was done before addition of CsA.

The assay was started with the PPIase substrate after incubating enzyme and inhibitor for a given period of time. Time course of reaction was followed with a HP8452 photodiodearray spectrometer. Release of 4-nitroaniline was detected at 390 nm. Because the condition $[S]_0 \ll K_M$ was met for all assays the progress curves can be described by first-order kinetics. From the observed rate constant k_1 a first-order rate constant k_{enz} for enzymatic catalysis of *cis* to *trans* isomerization can be calculated as

$$k_1 = k_{uncat} + k_{enz} \quad (1)$$

where k_{uncat} is the first-order rate constant for uncatalyzed *cis* to *trans* isomerization. The specificity constant k_{cat}/K_M was calculated from k_{enz}:

$$k_{enz} = (k_{cat}/K_M)\,[E]_0, \quad (2)$$

where $[E]_0$ is the total enzyme concentration. To take time-dependence of inhibition into account progress curves obtained for short incubation times were evaluated as described previously[15] using eq (3):

$$v_i = k_{uncat}[S] + \frac{1}{2}\frac{k_{cat}}{K_M}[S]([E]_0 - [-I]_0 - K_t + \sqrt{([E]_0 - [I]_0 - K_t)^2 + 4[E]_0 K_t}) \quad (3)$$

with

$$K_t = K_{if} + (K_{in} - K_{if})\, e^{-k_{obs}t},$$

where K_{in} and K_{if} are the initial and final inhibition constants, respectively, K_t is the inhibition constant at time t and k_{obs} is the observed first-order rate constant for their interconversion. Curves were fitted using the program NLSFIT written by K. Schittkowski (University Bayreuth, Germany). Initial velocities were calculated as described elsewhere.[15] Means of triple determinations of PPIase activity were plotted vs incubation time and fitted using eq (4):

$$RA = \frac{v_i}{v_0} 100, \quad (4)$$

where RA is the remaining PPIase activity in percent, v_i is derived from eq (3) by omitting the term $k_{uncat}[S]$ and v_0 is given by $k_{enz}[S]$. Estimated inhibition constants were converted to percent remaining activity using eq (4) by setting the respective constant equal to K_t.

Determination of K_i

To determine K_i values in the final state of inhibition, PPIase activity of rhCyp18 at different cyclosporin concentrations was measured. Enzyme and inhibitor were incubated at 9.5 °C for 10 min when cyclosporins were added from THF or 470 mM LiCl/THF and 30 min when added from CEL, respectively, to allow the mixture to equilibrate completely. Cyclosporins were added from stock solns that were 1000-fold (THF, 470 mM LiCl/THF) and 300-fold (CEL) more concentrated than the final concentration. The amount of the solvent was kept constant in the assay for each determination of K_i. Concentration of THF in the assay was less than 0.2% (v/v), CEL was used at 0.33% (v/v). K_i was calculated for different cyclosporins using the rate law for tight binding inhibition devised by Morrison.[33]

Determination of CMC of CEL

CMC was determined according to the method of Kalyanasundaram and Thomas.[34] The excitation wavelength was set to 310 nm. The emission spectra of pyrene in aq solns of CEL were recorded from 350 to 450 nm at 10 °C. Pyrene was used in a final concentration of 200 nM, CEL concentration was varied between 0.0001 and 1.2% (v/v). For CEL a CMC of 0.006% (v/v) was determined. With respect to the heterogeneous nature of CEL this result is in rather good agreement with the value published previously [0.009% (w/v) [35]]. Pyrene and CEL were incubated for times ranging from 45 s to 9 min. No change in emission spectra could be detected, indicating rapid formation of micelles. With the aid of sonication, complete solubilization of CEL was achieved during the mixing time of the PPIase assay (0–20 s).

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