Enzyme Inhibitors

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A Cyclosporin Derivative Discriminates between Extracellular and Intracellular Cyclophilins**

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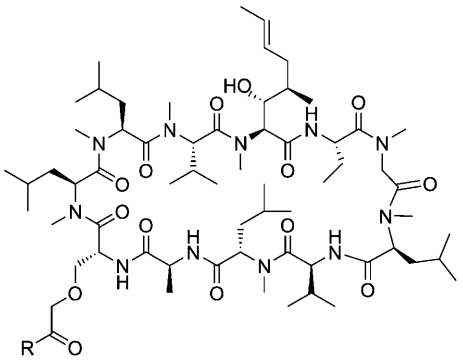
One of the challenges in the pharmacological down-regulation of enzyme activity is to assure selectivity in terms of the molecular nature and intraorganismic localization of the inhibitor target. Cyclosporin A (CsA) exemplifies a rather promiscuous tight-binding inhibitor of the cyclophilin(Cyp)-like peptidyl prolyl *cis*/*trans* isomerases (PPIases, EC 5.2.1.8) that is unable to distinguish between extracellular and intracellular Cyp, nor between the various human isoforms. In addition, physiological functions of CsA have been noted that are consistent with at least two separate modes of action: 1) blocking catalyzed conformational interconversions of prolyl bonds in substrate proteins, and 2) inhibiting the protein phosphatase calcineurin (CaN) when present as a CypA/CsA binary complex.[1,2] The latter pathway is thought to be the mechanism mediating the therapeutic effects of CsA in transplantation medicine and autoimmune diseases.[3] When applied to biological materials CsA undergoes rapid cellular uptake at 37 °C,[4,5] preferably accumulating as intracellular cyclophilin/CsA complexes.[6] Cyclophilin A (CypA) is a particularly abundant PPIase (0.19 μM in human whole blood[7]) and is thought to be the major intracellular binder of CsA.

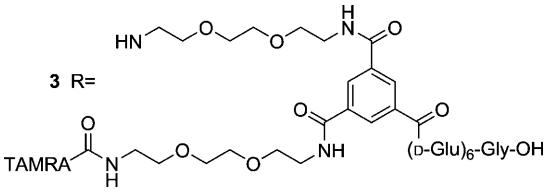
Herein, we present a strategy to restrict the enzyme inhibition of the extracellular fraction of cyclophilins based on a compound consisting of a CsA analogue as the molecular warhead and two specialized functional moieties.

In our search for an efficient cell-impermeable CypA inhibitor we were guided by the idea that the side chain of the [D-Ser[8]]-CsA would provide a structural platform for the synthesis of a bifurcated analogue containing both a fluorescent label and a moiety mediating cell-impermeability. As a positive control, compound **1** demonstrated high cell permeability of the [D-Ser[8]]-CsA moiety. Our approach for generating a cell-impermeable analogue of compound **1** was based on the hypothesis that distantly located functional groups on residue 8 of CsA, which flanks its CypA and CaN binding domains, will not interfere with the high CypA-inhibiting potency of [D-Ser[8]]-CsA.[8]

Trimesic acid amide constitutes the central part of analogue **3**, where the side chains are functionalized with a 5(6)-carboxytetramethylrhodamine (5(6)-carboxy-TAMRA)



1 R=NH(CH$_2$)$_5$NHC(=O)TAMRA
2 R=(D-Glu)$_6$-Gly-OH



and a side-chain-extended [D-Ser[8]]-CsA analogue. To compensate for the affinity of the cyclosporin moiety for the phospholipid membrane, the highly negatively charged H-(D-Glu)$_6$-Gly-OH moiety was N-terminally coupled as an amide to the remaining third carboxylate arm. We used it in the context of the highly lipophilic CsA side chains, although reports have shown that covalently attached oligo-Glu residues increase cell permeation of peptides.[9,10]

Here we report our studies on the generation of the cell-impermeable cyclosporin analogue **3** and demonstrate its functional abilities. Specifically, we show that although analogue **3** no longer mediates the immunosuppressive function of CsA, it retains the capacity to inhibit extracellular CypA-mediated chemotaxis of concanavalin(ConA)-activated mouse CD4$^+$ T cells.

Starting from [O-(NH$_2$(CH$_2$)$_5$NHC(O))CH$_2$-D-Ser[8]]-CsA,[8] preactivation of 5(6)-carboxy-TAMRA by HATU generated **1**. For the synthesis of **3**, a [H-(D-Glu(O*t*Bu))$_6$-Gly] Wang resin was synthesized using standard solid-phase

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peptide chemistry. An orthogonally Trt- and Fmoc-protected trimesic acid derivative[11] was preactivated with PyBOP and coupled to the peptide; the Fmoc groups were removed and the 5(6)-carboxy-TAMRA unit was attached. This construct was cleaved from the resin, purified, and finally the [*O*-carboxymethyl D-serine^8]-CsA[12] was attached to provide **3**.

Upon incubation with recombinant hCypA and hCypB, **3** reversibly inhibited PPIase activity, with K_i values of (1.8 ± 0.6) and (1.3 ± 0.5) nM, respectively. Efficient inhibition was also achieved by **1**, with K_i values of (4.3 ± 0.5) and (12.0 ± 2.8) nM. (Figure S1 in the Supporting Information). Under the experimental conditions used here CsA exhibited K_i values of (8.4 ± 2.5) and (6.9 ± 2.1) nM, respectively. We subsequently synthesized a compound that lacked the [Ser^8]-CsA part of **3**. As expected, this compound did not show any influence on cyclophilins up to the limiting assay concentration of 1 μM. CypA/**1** and CypA/**3** complexes also both inhibited CaN in the RII phosphopeptide dephosphorylation assay[8] in vitro, with IC_{50} values of (0.8 ± 0.1) and (26.2 ± 1.2) μM, respectively (Figure S2 in the Supporting Information).

These findings led us to predict that the high Cyp affinities of **1** and **3** would predispose them to be sequestered into intracellular spaces. Indeed, confocal laser-scanning microscopy of Jurkat cells incubated with medium containing 500 nM **1** showed a strong TAMRA fluorescence signal inside, but not outside, the cells. This distribution is typical of the expected CypA-driven enrichment of **1** in the cytosol (Figure 1b). In

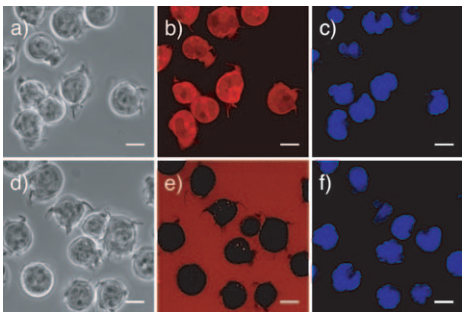

Figure 1. Jurkat cells were incubated for 3 h with 500 nM **1** (a–c) or 500 nM **3** (d–f) in a humidified chamber with 5% CO_2 at 37°C and examined by confocal laser scanning microscopy (b,c,e,f) and transmitted light microscopy (a,d). Nuclei were stained with Hoechst 33342 (c,f). Scale bars: 10 μm.

contrast, **3** remained fully outside of the cells, with almost no signal for intracellular localization (Figure 1e). CypA-deficient cells showed a lower intracellular accumulation of **1** but did not show a change in the distribution pattern of **3** (Figure 2). Uptake measurements using a flow cytometer showed no significant difference in fluorescence levels between control Jurkat cells and cells treated with **3**, limiting any uptake to $\ll 1\%$ of the concentration in the medium (Figure S3 in the Supporting Information).

To understand whether the positively charged rhodamine residue is essential for the blocked cellular uptake of **3**, we synthesized compound **2**, in which the terminal amino group of $(D\text{-Glu})_6$-Gly-OH moiety is directly attached to the carboxyl group of [*O*-carboxymethyl D-Ser^8]-CsA. With a K_i

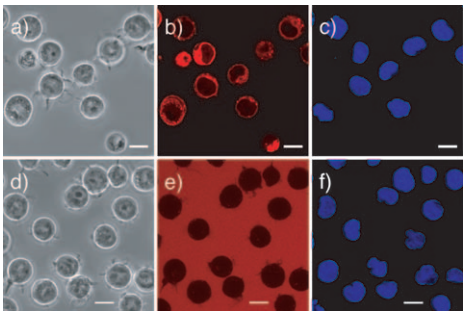

Figure 2. Human CypA^{-}/$^{-}$ Jurkat cells were incubated for 3 h with 500 nM **1** (a–c) or 500 nM **3** (d–f) in a humidified chamber with 5% CO_2 at 37°C and examined by confocal laser scanning microscopy (b,c,e,f) and transmitted light microscopy (a,d). Nuclei were stained with Hoechst 33342 (c,f). Scale bars: 10 μm.

value of (1.3 ± 0.2) nM this compound also exhibited a high affinity for CypA. Uptake was determined by a competition assay using Jurkat cells presaturated with **1**. Even when **2** was present in 100-fold excess relative to **1**, compound **2** did not displace the fluorescent analogue. This indicates that the presence of highly negatively charged residues alone is sufficient to mediate the cell-impermeable property of **2** and **3**.

We next performed a mixed-lymphocyte reaction using human peripheral blood mononuclear cells (PBMC)[13] (Figure 3a) and ConA stimulation of mouse splenic lymphocytes[14] (Figure 3b) to establish the potential immunosuppressive properties of our compounds. Whereas **1** was immunosuppressive, **3** demonstrated no immunosuppressive activity up to 10 μM. Efforts have previously been made to synthesize cell-impermeable cyclosporins by cross-linking CsA with macromolecules such as aminodextran beads or ovalbumin.[15] Although inhibition of IL-2 production in phorbol ester activated EL-4 cells and ConA-activated T-cell-enriched murine splenocytes was still observed, interpretation of the data was hampered by the potential release of cell-permeable cyclosporins from the macromolecular drugs.

HPLC profiling was used to evaluate the stability of **3** both in mouse and fetal calf serum (Figure S4 in the Supporting Information). The almost unchanged profiles after 48 h of incubation at 37°C indicated stability to chemical and enzymatic decomposition, ruling out the likelihood of immunosuppression effects resulting from fragmentation of **3**. This is in accordance with the lack of immunosuppression for **3** (Figure 3).

Extracellular cyclophilins have been found to be involved in neuroprotection,[16] ephithelial differentiation,[17] and signaling receptor functions including leukocyte migration by means of interaction with CD147 on the cell surface.[18] To test the capacity of **3** to inhibit leukocyte migration induced by extracellular cyclophilins, mouse $CD4^+$ T cells were purified and stimulated with CypA as previously described[14] in the presence of **3**. As shown in Figure 4, **3** inhibited CypA-mediated T-cell chemotaxis to almost basal levels. Importantly, **3** had no impact on leukocyte migration mediated by the chemokine RANTES, confirming the specificity of **3** for extracellular CypA.

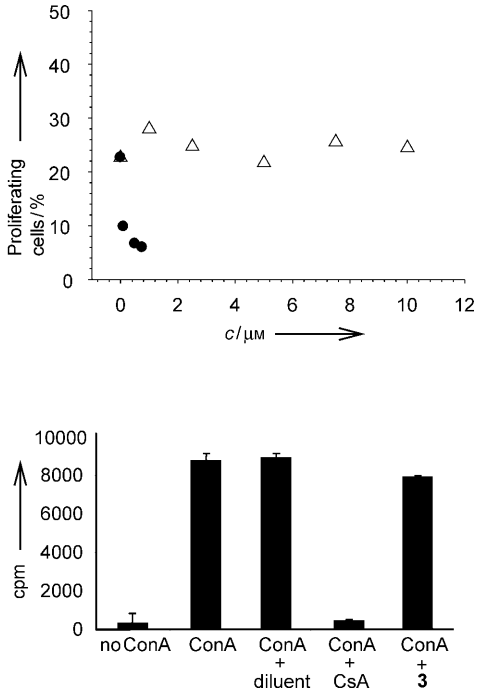

Figure 3. a) Mixed-lymphocyte reaction with human PBMCs. Human PBMCs from healthy donors (5×10^5 responder cells) labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) were incubated with **3** (triangle) or **1** (circles) and stimulated with allogenic PBMC (5×10^5 cells) from another individual that had been exposed to γ-irradiation (stimulator cells). After 5 days of culture the samples were analyzed by flow cytometry. The data show the percent of proliferating cells within the total population. b) Proliferation of ConA-stimulated splenic lymphocytes. Mouse spleen cells were activated for 48 h in medium alone (no ConA), with ConA without drug, with ConA plus diluent alone (1% ethanol), with 2 μM unmodified CsA, or with 2 μM **3**. [^{3}H]Thymidine was added for the final 6 h of culture. Data show level of proliferation as mean ± SE in counts per minute (cpm), with $n=6$ wells for each group.

In summary, a potent cyclophilin inhibitor has been synthesized which has trimesic acid amide as a central unit and is completely cell-impermeable. The compound contains a 6-mer D-glutamic acid moiety and 5(6)-carboxytetramethylrhodamine as a fluorescence probe attached to a modified cyclosporin warhead. Unlike clinically used cyclosporins, **3** is not sequestered inside cells by binding proteins. This should enable us in future studies to specifically address the capacity of extracellular cyclophilins to contribute to inflammatory responses.

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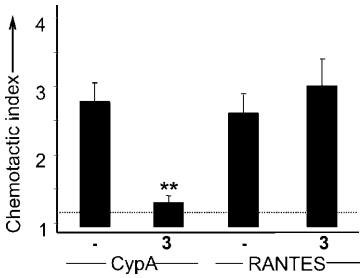

Figure 4. Mouse spleen cells were activated overnight with ConA and the activated CD4+ T cells were then isolated by MACS separation and set up in Boyden chamber chemotaxis assays. The cells were tested for migration either to CypA (100 ng mL^{-1}) or RANTES (1 ng mL^{-1}), with or without 2 mM of **3.** Data show the mean ± SE chemotaxis index for each group, with $n=5$ or 6 wells per group. The dashed line marks the level of significant chemotaxis (>1.2). ** = a statistical difference at the $p<0.01$ level as determined by Student's t-test.

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