Exo-Mechanism Proximity-Accelerated Alkylations: Investigations of Linkers, Electrophiles and Surface Mutations in Engineered Cyclophilin–Cyclosporin Systems

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Investigations on the scope and utility of exo-mechanism proximity-accelerated reactions in engineered receptor–ligand systems are reported. We synthesized a series of electrophilic cyclosporin (CsA) derivatives by varying electrophiles and linker lengths, prepared a series of nucleophilic cysteine mutations on the surface of cyclophilin A (Cyp), and examined their reactivity and specificity in proximity-accelerated reactions. Acrylamide and epoxide electrophiles afforded useful reactivity and high specificity for alkylation of engineered receptors in Jurkat cell extracts. We found that remote cysteines (> 17 Å from the ligand) could be alkylated with useful rates under physiological conditions. The results from mutations of the receptor surface suggest that the dominant factors governing the rates of proximity-accelerated reactions are related to the local environment of the reactive group on the protein surface. This study defines several parameters affecting reactivity in exo-mechanism proximity-accelerated reactions and provides guidance for the design of experiments for biological investigations involving proximity-accelerated reactions.

Introduction

Bioactive small molecules are useful both as experimental tools for investigating protein function and as therapeutic agents. As experimental tools, small molecules afford the advantages of precise temporal control of target function (onset of action ranges from seconds to minutes), control of the level of modulation through dose, reversibility of inhibition, and applicability to a broad range of experimental systems. Disadvantages of the small-molecule approach include a lack of specificity (families of related proteins are often inhibited to varying degrees) and limitations in the scope of targets that are readily modulated with small molecules, most notably protein–protein interactions.[1] Genetic approaches are the most common methods used to determine protein functions. Genetic mutation targets a single allele offering exquisite specificity. However, there are disadvantages to this approach: limitations of genetic protocols in many organisms, difficulty in achieving temporal control of inhibition, potential compensation by functionally related proteins, difficulty in identifying mutations that affect the functions of protein subdomains, and embryonic lethality when gene function is studied in whole organisms.[2, 3]

An emerging theme for solutions to these problems is to combine the advantages of small molecules and genetically encoded macromolecules, thereby allowing small molecules to target specific enzyme isoforms with temporal control. Selectivity can be imparted onto a noncovalent receptor–ligand system by introducing structural "bumps" onto the ligand and compensatory "holes" into the receptor.[4] Alternatively, reactive groups can be installed on the ligand and targeted to mutated amino acids on the receptor to obtain selectivity through the formation of covalent complexes. This approach has been successfully demonstrated for use in mapping receptor–ligand interfaces,[5] generating irreversible antibodies,[6] obtaining allele-specific modulators of target function[7] and for ligand-induced protein crosslinking.[8] These approaches provide the additional advantage of restricting the activity of the small molecule to the subset of cells or tissues expressing the genetically encoded effectors.[3, 9] We are interested in the use of engineered receptor–ligand pairs to specifically modulate the functions of engineered receptors through proximity-accelerated reactions. We previously reported the use of exo-mechanism proximity-accelerated reactions for the selective modulation of target function in the cyclophilin–cyclosporin (Cyp–CsA) receptor–ligand system.[7] An advantage of forming the covalent receptor–ligand bond outside the immediate ligand-binding site (exo-mechanism[10]) is the increased likelihood of identifying functionally silent receptor mutations.

In this study, we expand our investigations of proximity-accelerated alkylation by varying the nature of electrophiles, linker lengths, and receptor mutations to determine their ef-

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fects on the rate and specificity of alkylation. This study should provide guidance on the future development of ligands for proximity-accelerated reactions that rapidly modify their targets yet remain unreactive toward nonspecific cellular nucleophiles.

Results and Discussion

Synthesis of electrophilic CsA derivatives

The CsA derivatives have been given designations of CsA-**E#L#**, indicating the electrophile (**E#**) and linker (**L#**). Linkers and electrophiles were introduced into CsA through modification of the (4*R*)-4-[(*E*)-but-2-enyl]-4,*N*-dimethyl-L-threonine (MeBmt) residue of CsA by reductive ozonolysis generating a primary alcohol.[7] Reaction of the primary alcohol with 1,1′-carbonyldiimidazole provided active carbamate **1**. Displacement of imidazole with diamine linkers followed by addition of electrophiles provided the electrophilic CsA derivatives (Scheme 1) in moderate (unoptimized) yields. The precursor for electrophile **E6** was synthesized according to a published procedure.[11] Both epoxide ester precursors were saponified with potassium hydroxide giving potassium salts **E6** and **E7** that were used in the coupling reactions.

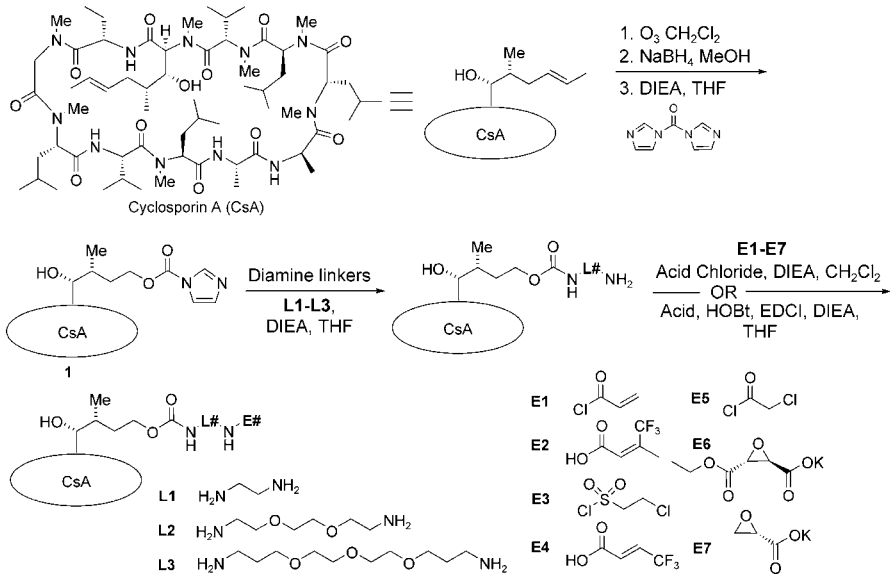
Selection, preparation and characterization of nucleophilic cyclophilin mutants

The reactivity of nucleophilic-receptor–electrophilic-ligand systems can be modulated through proximity and reactivity effects. To investigate proximity effects, we prepared a series of cyclophilin mutants in which surface residues at variable distances from the MeBmt side chain of CsA in the bound complex were mutated to cysteine (Table 1). The thiolate of cysteine serves as an excellent nucleophile in substitution and Michael addition reactions. The reactivity of cysteine residues with mild electrophiles is pH dependent, as the reactivity is derived from the thiolate form of cysteine. Additionally, the local environment influences the reactivity of a cysteine residue, through factors such as steric accessibility, hydrogen bonding, and residue mobility. For applications in living cells and organisms, where the pH cannot be experimentally altered, one approach for modulating the reactivity of cysteine residues is to alter their pK_a through second-site mutations. Lutolf and coworkers demonstrated that, for short peptides, introduction of charged amino acids adjacent to cysteines can alter pK_a values and modulate the reactivity of the thiol.[12] To investigate this approach, we prepared several double and triple mutants of Cyp containing charge altering arginine and histidine residues adjacent to the nucleophilic cysteine mutations (Table 1). We anticipated that the introduction of positively charged residues near cysteines would stabilize

Table 1. *Summary of expression and CsA binding ability of Cyp mutants.*

Cyp	Soluble Expression[a]	CsA Binding[b]
wild-type	yes	strong
P105C	yes	strong
N106C	yes	strong
A103C	yes	strong
G104C	yes	strong
E81C	yes	strong
K76C	yes	strong
F88C	yes	strong
R69C	yes	strong
T73C	yes	strong
G124C	yes	strong
K125C	yes	strong
K82C	no	n.d.
T107C	yes	strong
P105C/E84R	yes	strong
P105C/G104H	no	n.d.
G104C/P105H	no	n.d.
G104C/A103H	no	n.d.
P105C/N106H	yes	strong
P105C/E84R/D85H	yes	no
P105C/G104R	yes	weak
P105C/N106R	yes	weak
P105C/G104R/E84R	yes	no
P105C/N106R/E84R	yes	no

[a] Expression was conducted at 16 °C for 72 h. [b] CsA binding measured by using the fluorescence binding assay outlined in the Supporting Information. Binding in the low nM range typical for wt Cyp was classified as strong. Several mutants did not bind CsA while others bound weakly (~200–400 nM). n.d. = not determined.



Scheme 1. *Synthesis of electrophilic CsA derivatives.*

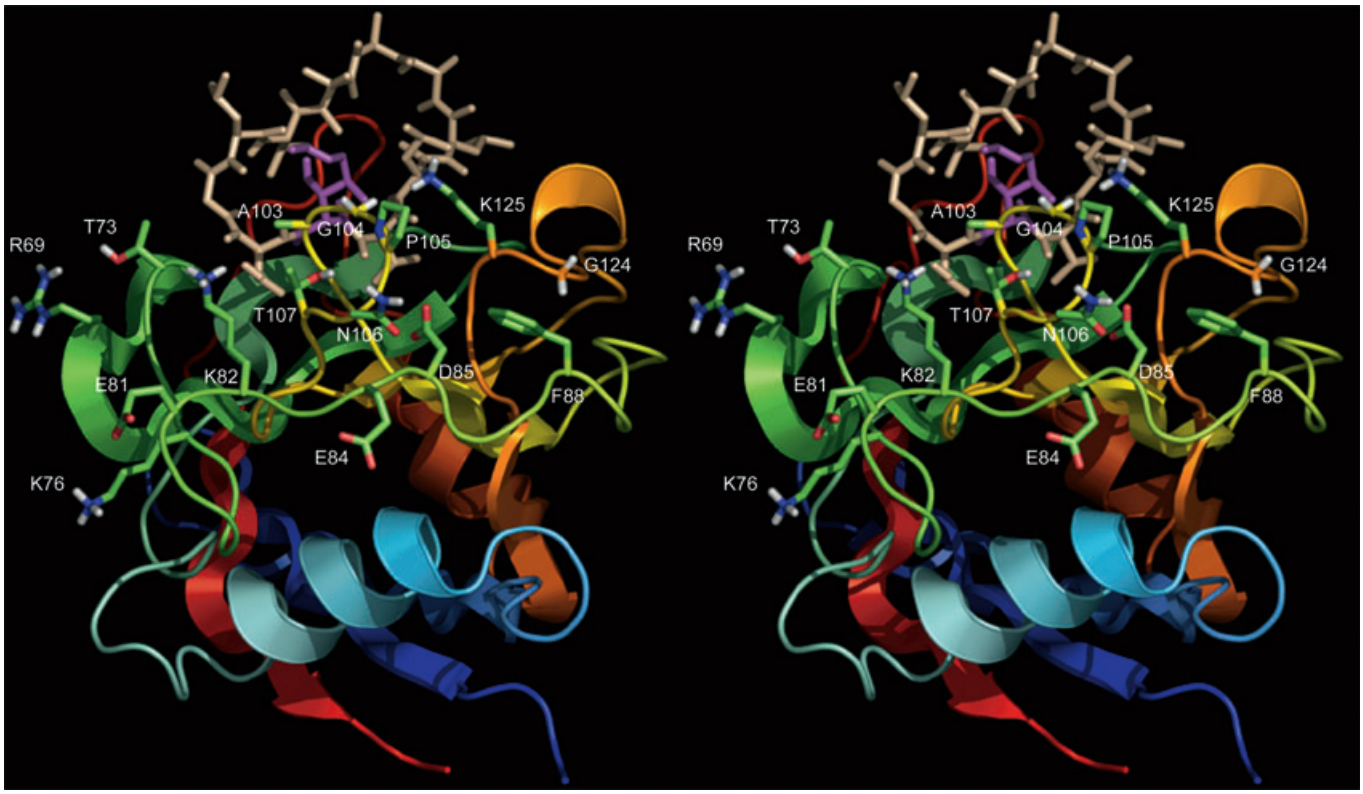


Figure 1. *Stereo representation of CsA (pink sticks) bound to Cyp (cartoon). The MeBmt side chain of cyclosporin is highlighted in purple, and the side chains of mutated residues at varying distances from the binding site are shown as sticks with labels. Graphics were created by using PyMOL (http://pymol.sourceforge.net/).*

the thiolate ion through coulombic effects and thereby accelerate alkylation reactions. The relative positions of CsA, its MeBmt residue, and the mutated residues of Cyp are depicted in Figure 1.

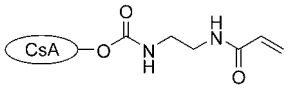
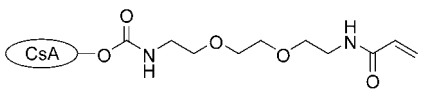
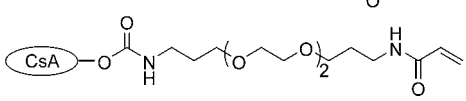
Cyp mutants were overexpressed in *E. coli*, purified and assayed for the ability to bind CsA by using a fluorescence binding assay. Mutants that bound CsA with K_d values in the low nanomolar range (~5 nM) were assumed to be properly folded and functional (Table 1). Mutant proteins were stored at −20 °C in the presence of glycerol and dithiothreitol (DTT) for up to a year without loss of activity. With the exception of Lys82, we were able to install single cysteine mutations without deleterious effects on protein folding or CsA binding in all cases.

Reactivity dependence on linker length and proximity of reactive groups

In our previous work we demonstrated that installing the nucleophilic cysteine outside the binding pocket afforded a protein competent for exo-mechanism proximity-accelerated alkylation while retaining near wild-type activity. To investigate the scope and utility of exo-mechanism proximity-accelerated reactions, we synthesized electrophilic CsA derivatives with variable linker lengths and assessed their ability to alkylate nucleophilic Cyp mutants both proximal to, and remote from the MeBmt residue (Table 2).

Cysteines were introduced into Cyp at varying distances and orientations relative to the MeBmt residue of CsA. Alkylation reactions were conducted at concentrations well above the K_d values (8 μM protein, 16 μM ligand) to ensure that the rates measured represent the relative reactivity of the bound complexes. Reactions were monitored by size separation on SDS-PAGE. A typical data set is shown in Figure 2. Several mutants depicted in Figure 1, namely K76C, F88C, G124C, and T107C

Table 2. *Reaction half-lives [min] of longer-linker electrophiles with various mutants.*[a]

Electrophile		Cyp P105C	E81C	T73C	K125C
E1L1		28	n.r.	n.r.	240
E1L2		55	n.r.	780	1320
E1L3		180	240	>1440	1440

[a] Reactions were carried out in phosphate buffered saline with 0.05 % Tween-20 and 1 mM glutathione pH 7.5 at 37 °C. n.r. = no reaction.

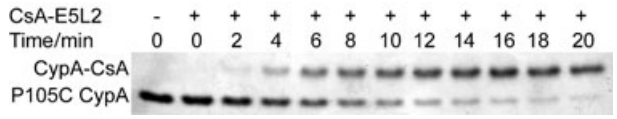
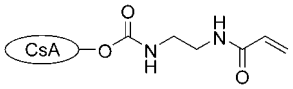
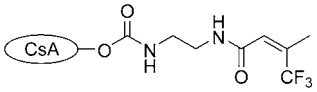
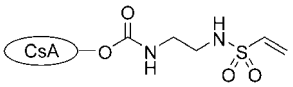
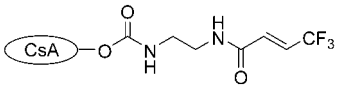
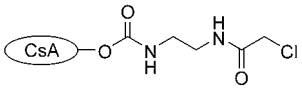
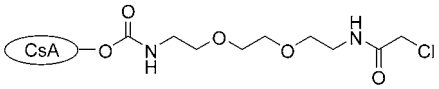
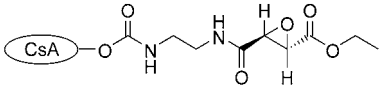
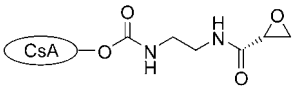
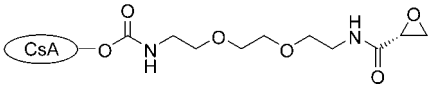


Figure 2. *Coomassie blue-stained SDS-PAGE of the reaction of CsA-**E5L2** (16 μM) with P105C-Cyp (8 μM) in 50 mM phosphate buffered saline with 0.05% Tween-20 and 1 mM glutathione pH 7.5 at 37°C.*

expressed well and bound CsA strongly, but were not alkylated by any of the electrophilic CsA derivatives. This lack of reactivity can arise in cases in which the linker on the ligand is too short to reach the nucleophile. Alternatively, it can result from local environment variables, such as steric accessibility, that render the cysteine unreactive. For example, Cys125 is readily alkylated, whereas Cys124 does not react at all. The small difference in distance between these two mutants implies that the local environment/pK_a of the cysteine is responsible for the lack of reactivity. We were pleasantly surprised to discover that extending the linker length only resulted in a sixfold decrease in the rate of reaction with the proximal and reactive P105C mutant. CsA-**E1L2** and CsA-**E1L3** were reactive on a relatively short time scale despite significantly extending the size of the ring formed. This finding suggests that long linkers will not significantly depress alkylation rates and that the local environment of the cysteine residue may dominate the slow reactivity seen for some mutants. In a striking example of remote alkylation, the E81C mutant was completely unreactive toward electrophilic CsA derivatives with short (**L1**, **L2**) linkers, yet was alkylated by CsA-**E1L3** with similar kinetics to the proximal P105C mutant despite having the ε-carbon of MeBmt and the β-carbon of residue 81 because not E81 but rather C81 reacts more than 17 Å apart in the crystal structure of Cyp as compared with 9.8 Å for the equivalent distance for residue 105.[13] A set of alkylation reactions were conducted at pH 8.5 for T73C giving half-life values of 70 and 120 minutes for CsA-**E1L2** and CsA-**E1L3**, respectively. This corresponds to an approximately tenfold increase over values measured at pH 7.5 and suggests that the pK_a of the T73C mutant may dominate its reactivity profile. An interesting case is presented by the R69C mutant, which is only 1 Å further from MeBmt than the T73C mutant and appears to be accessible for alkylation in models. However, it shows no reactivity for electrophilic CsA mutants at pH 7.5. Since this mutant was predicted to be within reach of CsA-**E1L3**, we conducted an alkylation experiment at pH 8.5 and alkylation occurred very slowly with a half-life above 48 h (data not shown). Again, the local environment of the cysteine appears to significantly affect its reactivity.

Table 3. *Reaction half-lives of CsA-**E#L#** with P105C Cyp.*[a]

E#L#	Electrophile	$t_{1/2}$ [min]
E1L1		28
E2L1		n.r.
E3L1		19
E4L1		14
E5L1		67
E5L2		7
E6L1		9
E7L1		75
E7L2		22

[a] Reactions were carried out in phosphate buffered saline with 0.05% Tween-20 and 1 mM glutathione pH 7.5 at 37°C. n.r. = no reaction.

Reactivity of electrophiles in proximity-accelerated alkylations

In order to investigate the utility of different electrophiles in proximity-accelerated reactions, we synthesized several CsA-**E#L1** derivatives with several types of electrophiles. Table 3

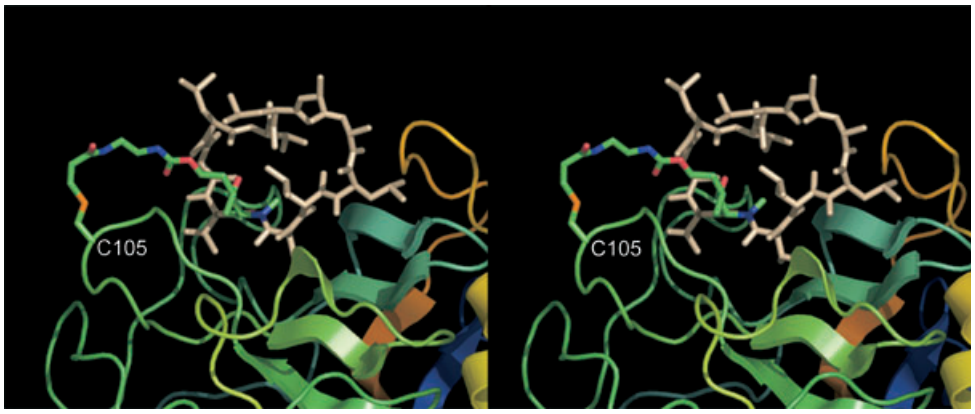


Figure 3. *Model of the CsA-**E1L1**/P105C-Cyp alkylation product. CsA-**E1L1** and P105C-Cyp were introduced into the structure by using Sybyl. The covalent bond was manually created, and the structure was energy minimized. CsA is represented as pink sticks, the relevant part of Cyp is represented as a cartoon, the MeBmt-C105 adduct is shown with sticks colored by element. Graphics were created by using PyMOL.*

summarizes the identities of the various electrophiles as well as their reaction half-lives with P105C-Cyp. We investigated several acrylamides with electron-withdrawing groups installed at their β-carbon, as well as a sterically demanding β-disubstituted acrylamide, a sulfonamide, an α-chloroacetamide and two epoxide-based electrophiles to assess the rate of alkylation with electrophiles of variable reactivity. Introduction of an electron-withdrawing *trans*-β-CF_3-acrylamide group in CsA-**E4L1** led to a twofold increase in rate. Additional modification of the β-carbon with a sterically hindering methyl group in CsA-**E2L1** abolished reactivity. The α-chloroacetamide of CsA-**E5L1** was expected to be the most reactive electrophile tested, yet was slow to react with P105C. Further examination of models based on Cyp–CsA crystal structures[14] simulating covalent adducts (Figure 3) suggested that the electrophilic carbon of CsA-**E5L1** was not able to effectively reach and alkylate P105C despite being only one carbon shorter than CsA-**E1L1**. We synthesized CsA-**E5L2** and found a greater than tenfold increase in alkylation rate with the longer linker thus supporting this hypothesis.

We also found that epoxides such as CsA-**E6L1**, which have been used in irreversible inhibitors of the cysteine proteases papain[11] and cruzain,[15] were found to be very reactive and selective. The related epoxide CsA-**E7L1**, however, was significantly less reactive than CsA-**E6L1**. Although monosubstituted α,β-epoxycarbonyl systems show similar reactivity at both the α- and β-positions,[16] it is possible that CsA-**E6L1** is more reactive due to the presence of two electron-withdrawing carbonyl groups on the epoxide or the differing absolute stereochemistry of the epoxides. Alternatively, the linker might be too short for optimal alkylation if the cysteine thiolate preferentially adds to the α-carbon of the epoxide, similar to the situation with CsA-**E5L#**. We synthesized and tested CsA-**E7L2** and found a threefold increase in reactivity, consistent with these length hypotheses.

Specificity of electrophilic alkylations

To investigate the behavior of the electrophilic CsA derivatives in a biological context, we tested their reactivity in Jurkat T-cell extracts containing P105C-Cyp. The alkylation of proteins in these cell extracts was monitored by Western blotting with an antibody that recognizes CsA (Figure 4). Close inspection of the nonspecifically alkylated proteins shows common bands among the various electrophiles and suggests that the labeled background proteins all interact with the electrophilic CsA derivatives through a common feature. However, the background bands are labeled in a CsA-independent manner, as CsA is unable to shut down the nonspecific labeling when added in excess (data not shown). The loss of background labeled

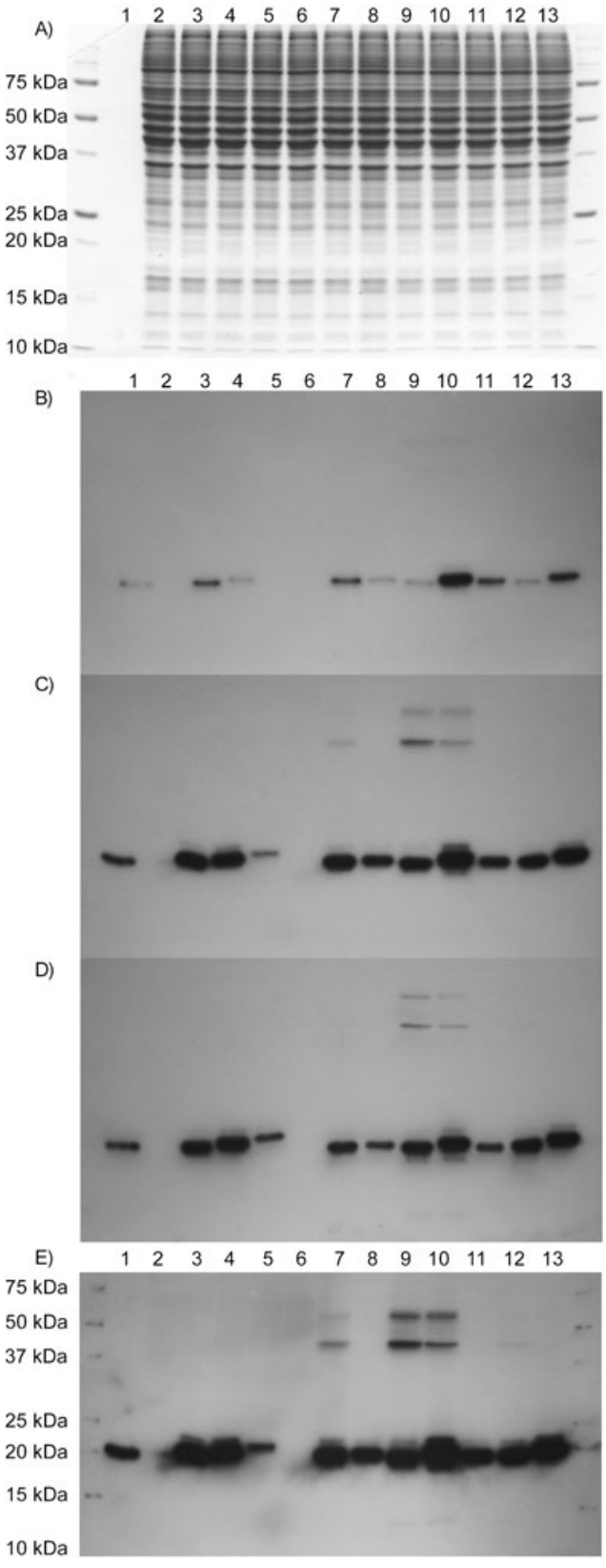


Figure 4. *Background reactivity of CsA derivatives in Jurkat T-cell extract. 1 μM P105C-Cyp was treated with 2 μM of indicated electrophile in PBS supplemented with 0.05% Tween-20 and 1 mM glutathione (lane 1) or cytosolic fraction of Jurkat T-cell extract (lanes 2–13). Proteins were visualized with A) Coomassie blue or B)–E) by Western blot probed with anti-CsA antibody for B) 1 hour, C) 4 hours, or D) 12 hours. Panel E is a longer exposure of the Western blot in panel (C) to reveal faint background bands. Lane 1: CsA-**E1L1**, lane 2: cell extract without electrophile to show that the antibody has no cross-reactivity, lane 3: CsA-**E1L1**, lane 4: CsA-**E1L2**, lane 5: CsA-**E1L3**, lane 6: CsA-**E2L1**, lane 7: CsA-**E3L1**, lane 8: CsA-**E4L1**, lane 9: CsA-**E5L1**, lane 10: CsA-**E5L2**, lane 11: CsA-**E6L1**, lane 12: CsA-**E7L1**, lane 13: CsA-**E7L2**.*

bands over time (from 4 to 12 h; Figure 4C–E) suggests that they are degraded in the extract, but, within the limits of detection, we do not see any degradation of the CsA-**E#L#**–P105C-Cyp products. Alkylation of Cyp with CsA derivatives containing longer linkers results in a greater mobility shift in SDS-PAGE separations (Figure 4). We previously reported that the acrylamide electrophile of CsA-**E1L1** shows high selectivity for alkylation of nucleophilic Cyp mutants in cell extracts and we confirm this here for derivatives with long linkers. We note that acrylamide CsA-**E4L1** and the epoxides CsA-**E6L1** and CsA-**E7L1** display excellent specificity and improved reactivity as compared with CsA-**E1L1**. Electrophile CsA-**E2L1** was found to be unreactive in vitro and in cell extracts. The sulfonamide CsA-**E3L1** reacted very quickly in vitro, but also showed significant levels of background labeling. As anticipated, the **E5** electrophiles were found to be the most reactive and the least specific. Compound CsA-**E6L1**, which is similar to epoxysuccinyl derivatives used to inhibit cysteine proteases,[11,15] shows no background labeling and high reactivity in vitro (9 min) as compared to CsA-**E1L1** (28 min) under identical conditions. Chen and co-workers[17] also found an epoxide electrophile to be very selective and reactive in protein labeling.

Second-site mutations and cysteine reactivity

Surface cysteine residues are expected to be partially deprotonated at physiological pH, and the local environment surrounding the cysteine side chain should influence its pK_a. In order to investigate the possibility of modulating the pK_a of cysteine residues and thus the reactivity of cysteine mutants, several second-site mutations were introduced. Histidines were installed flanking P105C, the most reactive cysteine ($t_{1/2}$ = 28 min at pH 7.5, Table 4), as well as adjacent to the less reactive G104C mutant ($t_{1/2} > 240$ min at pH 7.5) as the high reactivity of P105C might minimize the charge-altering effects of second-site mutations. However, our attempts to install histidines at positions 103–105 failed, as the mutants were insoluble. The P105C/N106H and P105C/E84R double mutants expressed well and bound CsA strongly. Residue 84 is predicted to be near P105C in the folded protein, although not in direct contact, possibly influencing P105C through N106 and D85. Further attempts to influence the local environment surrounding Cys105 with arginine mutations at residues 104 and 106 or histidine mutations at residue 85 resulted in mutants with low affinities for CsA (Table 1). The alkylation of the P105C, P105C/E84R and P105C/N106H mutants by CsA-**E1L1** as a function of pH is reported in Table 4. In the case of the P105C/E84R double mutant, we saw a twofold increase in the reaction rate at pH 8.0. To determine the origin of this change in reactivity, the pK_a values of the P105C, P105C/E84R and P105C/N106H mutants were determined to be 9.3 ± 0.1, 9.2 ± 0.1, and 9.4 ± 0.1, respectively. It is likely that the rate increase seen for the P105C/E84R mutant is due to accessibility or mobility changes rather than a pK_a effect. The local effects of charge-altering surface mutations may be shielded by the high dielectric constant of the medium (150 mM NaCl) for these exposed residues and perhaps would be more effective in systems in which the cysteine is located in a buried pocket as others have previously observed.[18] Another strategy, possibly worthy of investigation, would be to increase the accessibility and mobility of the surface cysteine through second-site mutations that reduce the steric demands of nearby residues.

Table 4. Reaction half-lives [min] of second-site mutants.[a]

pH	P105C	P105C/E84R	P105C/N106H
7.0	74	64	74
7.5	28	24	29
8.0	9	4.5	10
8.5	3	2	2
9.0	0.5	0.5	0.5

[a] Reactions carried out in PIPES/Bicine/borate buffered saline with 0.05% Tween-20 and 1 mM glutathione at 37 °C.

Implications for exo-mechanism proximity-accelerated reactions

Here, we have been working with engineered receptor–ligand systems, but the direct applications of proximity-accelerated reactions for affinity labeling of endogenous proteins are well known[19] and are even being exploited for the development of therapeutics with selective action.[20] The selective targeting of members of a receptor family through proximity-accelerated reactions with nonconserved surface nucleophiles might provide new reagents to investigate protein function. Our data show that even remote cysteines can be efficiently alkylated with long linkers; this suggests that this approach might be more fruitful than commonly assumed, as there is not a strict requirement for close positioning of reactive groups to enable selective reactions.

The use of long linkers and selective electrophiles might be particularly useful when applied to nondirected activity-based proteomic profiling experiments,[21] potentially expanding the range of proteins that can be selectively labeled by electrophilic probe molecules. For in vitro chemical-profiling reactions, incubation times and pH can be adjusted to maximize the reactivity of nucleophilic residues while maintaining selectivity to optimize the information content of these experiments.

Electrophilic small molecules have been utilized in drug discovery approaches.[22] In these tethering experiments, libraries of ligand fragments containing electrophilic disulfides are screened against receptors containing engineered cysteine residues to identify low-affinity ligand fragments and new ligand-binding pockets. Structural analysis of initially identified hits can then be used to guide the development of high-affinity reversible ligands. The use of long linkers and selective electrophiles might find utility in tethering experiments, thus allowing a greater portion of protein surface to be probed for potential binding pockets per engineered cysteine. Additionally, engineering cysteines further from the binding sites under investigation minimizes the chance that an engineered cysteine could perturb the binding pocket, thereby potentially biasing the identity of selected fragments.

In order to facilitate the synthesis of libraries of electrophilic ligands for use in applications such as proteomic profiling and tethering, we recently reported the development of the TRAM linker.[23] This linker enables the solid-phase synthesis of small-molecule libraries containing a tethered acrylamide electrophile upon cleavage from the solid support.

Proximity-accelerated reactions might also be useful in identifying small-molecule inhibitors of protein–protein interactions. The large and relatively flat protein–protein interaction interfaces are challenging targets for the identification of small molecules that successfully compete with natural binding partners. Through proximity-accelerated reactions the utility of small molecules to disrupt protein–protein interactions may be greatly extended for both natural and engineered systems.

Conclusion

We have investigated the effects of varying electrophiles, linkers, and receptor mutants on the reactivity and specificity of exo-mechanism proximity-accelerated alkylations in the Cyp–CsA receptor–ligand system. We found that electrophilic CsA derivatives containing acrylamide or epoxide electrophiles gave the most rapid and specific alkylations while vinylsulfonamide and α-chloroacetamides reacted rapidly with cysteine mutants but led to nonspecific labeling of proteins in Jurkat cell extracts. Even remote cysteines more than 17 Å away could be specifically alkylated with useful reaction rates under physiological conditions (pH 7.5, 150 mM NaCl) when long linkers were employed. The local environment largely determined the reactivity of surface cysteines. Attempts to increase their reactivity by lowering their pK_a values through second-site mutations were unsuccessful under physiological conditions. Our results collectively suggest that second-site mutations that increase the steric accessibility or mobility of reactive cysteines might provide a more effective approach for the modulation of cysteine reactivity. The results presented here should guide the design and implementation of experiments involving exo-mechanism proximity-accelerated reactions and suggest potential utility for exo-mechanism reactions in proteomic-profiling experiments, tethering approaches for drug discovery, and in the modulation of protein–protein interactions with small molecules.

Experimental Section

General. Detailed experimental protocols and complete characterization of intermediates can be found in the supplementary information. All moisture-sensitive reactions were performed in oven-dried glassware under a stream of nitrogen. Bath temperatures were used to record reaction temperatures and all reactions were magnetically stirred. Flash-column chromatography (FCC) was performed on Silicycle silica gel 60 (230–400 mesh). Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 500 MHz by using a Varian UNITY 500 and INOVA 500 spectrometer. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded at 125 MHz by using a Varian UNITY 500 and INOVA 500 spectrometer with Shigemi Advanced Microtubes. High-resolution electrospray ionization mass spectra (HRESI-MS) were obtained on a Micromass LCT.

General procedure for the addition of a diamine linker to CsA. Diamine linker and base DIEA (4–8 equiv) were added to a solution of CsA and imidazole in dry THF. Upon completion, the reaction mixture was purified by FCC.

Cyclosporin A L2: ^{1}H NMR ([D$_8$]toluene): δ = 8.59 (d, J = 10 Hz, 1H), 8.13 (d, J = 6 Hz, 1H), 7.96 (d, J = 8 Hz, 1H), 7.71 (d, J = 9 Hz, 1H), 6.18 (m, 1H), 5.85 (dd, J = 11 Hz, 4 Hz, 1H), 5.60 (m, 2H), 5.47 (d, J = 10 Hz, 1H), 5.34 (t, J = 6 Hz, 1H), 5.06 (q, J = 8 Hz, 1H), 5.00 (d, J = 11 Hz, 1H), 4.93 (t, J = 10 Hz, 1H), 4.78 (m, 1H), 4.70 (m, 1H), 4.54 (m, 1H), 4.33 (m, 1H), 4.13 (d, J = 10 Hz, 1H), 4.08 (d, J = 11 Hz, 1H), 3.55–3.30 (m, 16H), 3.16 (s, 3H), 3.08 (s, 3H), 2.92 (s, 3H), 2.80 (s, 3H), 2.75–2.64 (m, 5H), 2.41–2.20 (m, 7H), 2.03–1.97 (m, 2H), 1.86–1.68 (m, 5H), 1.59–0.86 (m, 54H), 0.69 (d, J = 7 Hz, 3H); ^{13}C NMR ([D$_8$]toluene): δ = 174.49, 174.73, 173.29 (2C), 172.00, 171.49 (2C), 171.41, 170.84, 170.80, 169.21, 157.23, 73.29, 70.90, 70.68 (2C), 70.61, 63.99, 59.41, 59.31, 57.72, 55.74, 55.36, 54.51, 49.37, 48.91, 48.69, 48.19, 44.99, 41.35, 41.14, 39.92, 38.85, 37.53, 36.59, 32.54 (2C), 31.90, 31.58, 31.00, 30.95, 30.36, 29.91 (2C), 29.46 (2C), 28.58, 26.02, 25.47, 25.28, 25.12 (2C), 24.99, 24.55, 24.15, 24.02, 23.92, 23.74, 22.18, 21.97, 21.53, 18.79, 18.56, 18.18, 17.63, 15.52, 10.21; MS (HRESI-MS) calcd for $[C_{67}H_{123}N_{13}O_{16}+H]^+$: 1366.9289; found: 1366.9247.

Cyclosporin A L3. ^{1}H NMR ([D$_8$]toluene): δ = 8.30 (d, J = 10 Hz, 1H), 7.87 (d, J = 6 Hz, 1H), 7.70 (d, J = 7 Hz, 1H), 7.44 (d, J = 9 Hz, 1H), 5.87 (m, 1H), 5.58 (dd, J = 11 Hz, 4 Hz, 1H), 5.36 (dd, J = 11 Hz, 4 Hz, 1H), 5.30 (dd, J = 11 Hz, 3 Hz, 1H), 5.17 (d, J = 10 Hz, 1H), 5.07 (dd, J = 7 Hz, 7 Hz, 1H), 4.78 (m, 2H), 4.62 (t, J = 9 Hz, 1H), 4.51 (m, J = 7 Hz, 1H), 4.41 (m, 1H), 4.27 (m, 1H), 4.04 (td, J = 11 Hz, 4 Hz, 1H), 3.86 (d, J = 9 Hz, 1H), 3.81 (d, J = 13 Hz, 1H), 3.29–3.12 (m, 20H), 2.99 (m, 2H), 2.89 (s, 3H), 2.82 (s, 3H), 2.65 (s, 3H), 2.53 (s, 3H), 2.39 (m, 5H), 2.12–1.88 (m, 7H), 1.72 (m, 2H), 1.60–1.40 (m, 7H), 1.34–0.59 (m, 54H), 0.42 (d, J = 7 Hz, 3H); ^{13}C NMR ([D$_8$]toluene): δ = 173.59 173.34, 173.24, 172.98, 171.66, 171.24, 171.15, 170.90, 170.86, 170.68, 169.31, 157.03, 73.35, 70.55 (2C), 70.50, 70.21, 70.12 (2C), 69.62, 68.78, 63.25, 58.75, 58.69, 57.45, 55.46, 55.21, 54.31, 50.10, 48.60, 48.40, 48.00, 44.72, 40.33, 39.48, 39.31, 39.11, 38.05, 37.09, 36.19, 32.34, 32.21, 31.42, 31.37, 31.01, 30.13 (2C), 29.77, 29.58, 29.01 (2C), 25.28, 24.93 (2C), 24.68, 24.36, 23.85, 23.71, 23.65, 23.40, 21.86, 21.52, 21.25, 20.41, 19.83, 18.61, 18.29, 17.84, 17.65, 15.39, 9.85; MS (HRESI-MS) calcd for $[C_{71}H_{131}N_{13}O_{17}+H]^+$: 1438.9864; found: 1438.9836.

General procedure for the acylation of the CsA free amine linker with an acid halide. The acid chloride and base TEA (4–8 equiv) were added to a solution of the CsA primary amine in CH_2Cl_2 at 0 °C. The reaction mixture was warmed to room temperature and, upon completion of the reaction, was purified by FCC or by extraction and HPLC.

Cyclosporin A E1L2. ^{1}H NMR ([D$_8$]toluene): δ = 8.55 (d, J = 10 Hz, 1H), 8.14 (d, J = 6 Hz, 1H), 7.87 (d, J = 7 Hz, 1H), 7.73 (d, J = 8 Hz, 1H), 6.66 (s, 1H), 6.47 (d, J = 16 Hz, 1H), 6.17 (dd, J = 17 Hz, 10 Hz, 1H), 6.08 (s, 1H), 5.85 (dd, J = 11 Hz, 4 Hz, 1H), 5.60 (m, 2H), 5.47 (d, J = 10 Hz, 1H), 5.37 (d, J = 10 Hz, 1H), 5.32 (t, J = 7 Hz, 1H), 5.05 (q, J = 8 Hz, 1H), 4.93 (d, J = 10 Hz, 1H), 4.88 (t, J = 10 Hz, 1H), 4.78 (m, J = 7 Hz, 1H), 4.71 (m, 1H), 4.53 (m, 1H), 4.32 (td, J = 10 Hz, 4 Hz, 1H), 4.09 (d, J = 10 Hz, 1H), 4.04 (d, J = 13 Hz, 1H), 3.45–3.27 (m, 16H), 3.14 (s, 3H), 3.07 (m, 3H), 2.90 (s, 3H), 2.78 (s, 3H), 2.71–2.64 (m, 3H), 2.42–2.18 (m, 7H), 1.96 (m, 2H), 1.84–1.66 (m, 5H), 1.57–0.84 (m, 54H), 0.69 (d, J = 6 Hz, 3H); ^{13}C NMR ([D$_8$]toluene): δ = 173.63, 173.41, 173.24, 173.00, 171.67, 171.25, 171.14, 171.08,

171.00, 170.73, 169.25, 165.64, 157.06, 131.08, 126.21, 73.31, 70.50, 70.37, 70.24, 69.89 (2C), 63.54, 58.91, 58.58, 57.51, 55.47, 55.23, 54.25, 50.13, 48.61, 48.44, 47.97, 44.72, 40.67, 40.26, 39.35, 39.09, 37.09, 36.11, 32.26, 32.10, 31.56, 31.41, 31.03, 30.13, 29.81, 29.71, 29.59, 28.98, 28.86, 25.31, 24.90, 24.70, 24.34, 23.92, 23.75, 23.69, 23.66, 23.46, 21.87, 21.44, 21.22, 20.48, 19.87, 18.61, 18.30, 17.88, 17.69, 15.40, 9.88; MS (HRESI-MS) calcd for $[C_{70}H_{125}N_{13}O_{17}+Na]^+$: 1442.9214; found: 1442.9148.

Cyclosporin A E1L3. ^{1}H NMR ([D$_8$]toluene): $\delta=8.55$ (d, $J=9$ Hz, 1H), 8.13 (d, $J=8$ Hz, 1H), 7.94 (d, $J=6$ Hz, 1H), 7.76 (d, $J=8$ Hz, 1H), 6.54 (s, 1H), 6.46 (dd, $J=17$ Hz, 2 Hz, 1H), 6.15 (m, 2H), 5.85 (dd, $J=11$ Hz, 3 Hz, 1H), 5.63–5.56 (m, 2H), 5.46 (d, $J=10$ Hz, 1H), 5.42 (dd, $J=10$ Hz, 2 Hz, 1H), 5.33 (t, $J=7$ Hz, 1H), 5.05 (q, $J=8$ Hz, 1H), 4.95 (d, $J=11$ Hz, 1H), 4.84 (m, 1H), 4.80–4.68 (m, 2H), 4.53 (m, 1H), 4.31 (m, 1H), 4.12 (d, $J=9$ Hz, 1H), 4.05 (d, $J=14$ Hz, 1H), 3.53–3.40 (m, 16H), 3.32–3.23 (m, 4H), 3.15 (s, 3H), 3.07 (s, 3H), 2,91 (s, 3H), 2,79 (s, 3H), 2.71–2.64 (m, 4H), 2,39–2.18 (m, 8H), 1.98 (m, 2H), 1.84–1.60 (m, 8H), 1.50–0.84 (m, 53H), 0.68 (d, $J=6$ Hz, 3H); ^{13}C NMR ([D$_8$]toluene): $\delta=174.01$, 173.30, 172.89, 172.78, 171.53, 171.19, 170.91, 170.39, 170.28, 168.67, 164.39, 156.84, 132.09, 72.79, 70.48, 70.42, 70.24, 69.91, 69.60, 68.57, 63.31 (2C), 59.09, 58.81, 57.34, 55.31, 55.04, 54.02, 48.93 (2C), 48.42, 48.23, 47.76, 44.54, 40.67 (2C), 39.47, 38.41, 38.04, 37.70, 37.11 (2C), 36.29, 32.04, 31.29, 31.29, 30.98, 30.56, 30.48, 30.41, 29.43, 29.10, 29.01, 28.90, 28.03 (2C), 25.58, 25.07, 24.84, 24.67, 24.54, 24.06, 23.66, 23.53, 23.46, 23.24, 21.72, 21.56, 21.11, 18.31, 18.08, 17.62, 15.04, 9.71; MS (HRESI-MS) calcd for $[C_{74}H_{133}N_{13}O_{18}+Na_2]^{2+}$: 768.9844; found: 768.9809;

Cyclosporin A E3L1: ^{1}H NMR ($CDCl_3$): $\delta=8.32$ (d, $J=10$ Hz, 1H), 7.83 (d, $J=7$ Hz, 1H), 7.89 (d, $J=7$Ha, 1H), 7.44 (d, $J=10$ Hz, 1H), 6.54 (dd, $J=17$, 10 Hz, 1H), 6.24 (d, $J=17$ Hz, 1H), 5.99 (m, 1H), 5.91 (d, $J=10$ Hz, 1H), 5.76 (m, 1H), 5.68 (dd, $J=11$ Hz, 4 Hz, 1H), 5.37 (dd, $J=11$ Hz, 3 Hz, 1H), 5.32 (d, $J=9$ Hz, 1H), 5.24 (dd, $J=11$ Hz, 4 Hz, 1H), 5.10 (t, $J=7$ Hz, 1H), 5.01 (m, 1H), 4.95 (d, $J=11$ Hz, 1H), 4.86 (m, $J=8$ Hz, 1H), 4.68 (m, 2H), 4.46 (m, $J=7$ Hz, 1H), 4.37 (td $J=10$ Hz,6 Hz, 1H), 4.00 (m, 2H), 3.48 (s, 3H), 3.43 (s, 3H), 3.28 (s, 3H), 3.21–3.17 (m, 7H), 3.10 (s, 3H), 2.66 (s, 6H), 2.47 (m, 1H), 2.33 (s, 1H), 2.19–2.04 (m, 4H), 2.00 (m, 2H), 1.89 (m, 2H), 1.63 (m, 1H), 1.47–1.12 (m, 15H), 1.06–0.84 (m, 40H), 0.76 (d, $J=7$ Hz, 3H); ^{13}C NMR ($CDCl_3$): 173.91, 173.79, 173.61, 173.33, 173.30, 173.06, 171.74, 171.38, 171.15, 170.94, 169.36, 157.34, 136.54, 126.29, 73.25, 63.86, 59.19, 58.77, 57.70, 55.60, 55.49, 54.41, 50.32, 48.84, 48.61 (2C), 48.20, 44.95, 43.56, 41.40, 40.52, 39.52, 39.33, 37.26, 36.38, 32.39, 31.88, 31.72, 31.61, 31.31, 30.37, 30.02, 29.83, 29.17, 28.81, 25.48, 25.11, 25.03, 24.91, 24.55, 24.11, 23.95, 23.87, 23.68, 22.07, 21.66, 21.39, 20.62, 20.00, 18.82, 18.47, 18.12, 18.04, 15.58, 10.04; MS (HRESI-MS) calcd for $[C_{65}H_{117}N_{13}O_{16}S+Na]^+$: 1390.8360; found: 1390.8309.

Cyclosporin A E5L1: ^{1}H NMR ($CDCl_3$): $\delta=8.37$ (d, $J=10$ Hz, 1H), 7.85 (d, $J=7$ Hz, 1H), 7.77 (d, $J=9$ Hz, 1H), 7.51 (m, 1H), 7.45 (d, $J=8$ Hz, 1H), 5.93 (m, 1H), 5.68 (dd, $J=11$ Hz, 4 Hz, 1H), 5.38 (dd, $J=11$ Hz, 3 Hz, 1H), 5.32 (d, $J=9$ Hz, 1H), 5.24 (dd, $J=11$ Hz, 4 Hz, 1H), 5.09 (t, $J=7$ Hz, 1H), 5.01 (q, $J=9$ Hz, 1H), 4.95 (d, $J=11$ Hz, 1H), 4.86 (m, $J=7$ Hz, 1H), 4.68 (m, 2H), 4.45 (m, $J=7$ Hz, 1H), 4.37 (m, 1H), 4.04–3.95 (m, 4H), 3.53–3.38 (m, 9H), 3.26 (s, 3H), 3.20–3.17 (m, 4H), 3.10 (s, 3H), 2.66 (s, 6H), 2.47 (m, 1H), 2.22–2.05 (m, 4H), 2.02–1.89 (m, 2H), 1.83–1.59 (m, 6H), 1,43 (m, 2H), 1.32–1.09 (m, 11H), 1.05–0.84 (m, 40H), 0.75 (d, $J=7$ Hz, 3H); ^{13}C NMR ($CDCl_3$) 173.89, 173.59, 173.36, 173.11, 171.82, 171.70, 171.38, 171.27, 171.13, 170.98, 169.34, 166.73, 157.78, 73.32, 63.95, 59.16, 58.76, 57.70, 55.67, 55.46, 54.42, 50.33, 48.86, 48.63, 48.24, 44.96, 42.78, 41.12, 40.55, 40.40, 39.54, 39.37, 37.30, 36.43, 32.36, 32.07, 31.69, 31.63, 31.31, 30.38, 30.02, 29.84, 29.20, 28.76, 25.54, 25.14, 25.04, 24.93, 24.60, 24.08, 23.95, 23.88, 23.86, 23.66, 22.10, 21.70, 21.42, 20.62, 20.06, 18.85, 18.50, 18.05, 18.00, 15.60, 10.06; MS (HRESI-MS) calcd for $[C_{65}H_{116}N_{13}O_{15}Cl+Na]^+$: 1376.8300; found: 1376.8236.

Cyclosporin A E5L2: ^{1}H NMR ([D$_8$]toluene): $\delta=8.59$ (d, 10 Hz, 1H), 8.14 (d, $J=7$ Hz, 1H), 7.93 (d, $J=7$ Hz, 1H), 7.73 (d, $J=8$ Hz, 1H), 6.10 (s, 1H), 5.85 (dd, $J=11$ Hz, 4 Hz, 1H), 5.59 (m, 2H), 5.46 (d, $J=10$ Hz, 1H), 5.33 (t, $J=7$ Hz, 1H), 5.06 (q, $J=8$ Hz, 1H), 4.96 (d, $J=12$ Hz, 1H), 4.89 (t, $J=10$ Hz, 1H), 4.78 (m, $J=7$ Hz, 1H), 4.70 (m, 1H), 4.53 (m, $J=7$ Hz, 1H), 4.33 (m, 1H), 4.12 (d, $J=10$ Hz, 1H), 4.07 (d, $J=14$ Hz, 1H), 3.71 (s, 2H), 3.54 (m, 4H), 3.44–3.29 (m, 14H), 3.17 (s, 3H), 3.08 (s, 3H), 2.91 (s, 3H), 2.68 (m, 4H), 2.41–2.16 (m, 7H), 1.97 (m, 2H), 1.85–1.66 (m, 5H), 1.60–1.45 (m, 6H), 1.38 (m, 2H), 1.31–0.85 (m, 47H), 0.69 (d, $J=7$ Hz, 3H); ^{13}C NMR ([D$_8$]toluene): $\delta=174.83$, 174.12, 173.66, 173.60, 172.37, 171.98, 171.81, 171.70, 171.20, 171.12, 169.44, 165.70, 157.57, 73.61, 71.28, 71.13, 70.98, 70.16, 64.38, 59.89, 59.52, 58.14, 56.10, 55.77, 54.80, 49.73, 49.22, 49.05, 48.54, 45.34, 43.18, 41.73, 41.46, 40.32, 40.28, 39.22, 37.88, 36.97, 32.74, 32.25, 32.12, 31.84, 31.37 (2C), 31.22, 30.25, 29.82, 29.73, 28.74, 26.39, 25.83, 25.64, 25.48, 25.34, 24.91, 24.47, 24.38, 24.28, 24.08, 22.52, 22.32, 21.87, 21.40, 19.12, 18.89, 18.51, 17.98, 15.83, 10.52; MS (HRESI-MS) calcd for $[C_{69}H_{124}N_{13}O_{17}Cl+Na_2]^{2+}$:743.9361; found: 743.9335.

General procedure for the acylation of the CsA free amine linker with a carboxylic acid. The acid and EDC (1.5 equiv), HOBt (1 equiv), and base (DIEA; 4 equiv) were added to a solution of the CsA primary amine in THF. Upon completion of the reaction, the mixture was extracted and purified by FCC or HPLC.

Cyclosporin A E2L1. ^{19}F NMR ($CDCl_3$): -70.83; ^{1}H NMR ($CDCl_3$): $\delta=$ 8.38 (d, $J=10$ Hz, 1H), 7.87 (m, 2H), 7.75 (m, 1H), 7.45 (d, $J=8$ Hz, 1H), 6.39 (s, 1H), 5.69 (dd, $J=11$ Hz, 4 Hz, 1H), 5.47 (m, 1H), 5.41 (dd, $J=12$ Hz, 4 Hz, 1H), 5.28 (m, 2H), 5.10 (t, $J=7$ Hz, 1H), 5.01 (q, $J=9$ Hz, 1H), 4.93 (d, $J=11$ Hz, 1H), 4.87 (m, $J=7$ Hz, 1H), 4.66 (m, 2H), 4.45 (m, 7 Hz, 1H), 4.38 (m, 1H), 4.00 (m, 2H), 3.60 (m, 1H), 3.49 (s, 3H), 3.45 (s, 3H), 3.37 (m, 1H), 3.22 (s, 3H), 3.18 (m, 4H), 3.11 (s, 3H), 2.66 (m, 6H), 2.49 (m, 1H), 2.27 (m, 2H), 2.19–2.07 (m, 3H), 1.99 (m, 1H), 1.86–1.80 (m, 3H), 1.76–1.54 (m, 12H including integration of H_2O), 1.43 (m, 2H), 1.33–1.12 (m, 16H), 1.06–0.84 (m, 37H), 0.75 (d, $J=7$ Hz, 3H); ^{13}C NMR ($CDCl_3$): 173.92, 173.63, 173.29, 172.95, 171.96, 171.72, 171.43, 171.35, 171.14, 171.01, 169.18, 164.77, 157.38, 138.05 (q, $J=30$ Hz), 124.63 (q, $J=5$ Hz), 123.68 (q, $J=272$, CF3), 76.67, 73.05, 64.06, 60.62, 59.75, 59.30, 58.67, 57.71, 55.54, 54.27, 50.29, 48.80, 48.60, 48.15, 44.89, 40.54, 40.49, 40.16, 39.53, 39.35, 38.37, 37.20, 36.40, 32.16, 31.58, 31.46, 31.30, 30.53, 30.40, 30.02, 29.91, 29.58, 29.17, 28.45, 25.50, 25.16, 24.98, 24.94, 24.54, 24.18, 23.97, 23.91, 23.86, 23.67, 22.07, 20.66, 19.92, 18.21, 18.01, 15.46, 12.06, 10.05; MS (HRESI-MS) calcd for $[C_{68}H_{118}N_{13}O_{15}F_3+Na]^+$: 1436.8720; found: 1436.8770.

Cyclosporin A E4L1: ^{1}H NMR ($CDCl_3$): $\delta=8.39$ (d, $J=10$ Hz, 1H), 7.99 (m, 1H), 7.88 (d, $J=7$ Hz, 2H), 7.47 (d, $J=7$ Hz, 1H), 6.77 (m, 1H), 6.65 (d, $J=16$ Hz, 1H), 5.68 (dd, $J=10$ Hz, 3 Hz, 1H), 5.47 (m, 1H), 5.42 (dd, $J=11$ Hz, 3 Hz, 1H), 5.29 (d, $J=9$ Hz, 2H), 5.10 (t, $J=6$ Hz, 1H), 5.01 (m, 1H), 4.92 (d, $J=12$ Hz, 1H), 4.86 (m, 1H), 4.66 (m, 2H), 4.45 (m, 1H), 4.38 (m, 1H), 4.00 (m, 2H), 3.63 (m, 1H), 3.49 (s, 3H), 3.45 (m, 4H), 3.2 (m, 7H), 3.11 (s, 3H), 2.66 (s, 6H), 2.50 (m, 1H), 2.19–2.07 (m, 3H), 1.98 (m, 1H),1.83 (m, 2H), 1.75–1.63 (m, 3H), 1.44 (m, 4H), 1.34–1.18 (m, 14H), 1.06–0.85 (m, 39H), 0.74 (d, $J=6$ Hz, 3H); ^{13}C NMR ($CDCl_3$): 173.85, 173.57, 173.21, 172.80, 171.96, 171.59, 171.39, 171.26, 171.07, 170.95, 169.07, 162.95, 157.47, 131.66 (d, $J=5$ Hz), 127.90 (q, $J=35$ Hz), 123.91 (q, $J=$

218 Hz), 72.94, 64.13, 59.24, 58.57, 57.63, 55.45, 54.16, 50.21, 48.73, 48.51, 48.06, 44.82, 40.69, 40.41, 40.32, 39.44, 39.27, 37.14, 36.34, 32.06, 31.58, 31.52, 31.48, 31.25, 30.46, 30.32, 29.94, 29.84, 29.09, 28.33, 25.44, 25.00, 24.86, 24.45, 24.13, 23.90, 23.84, 23.78, 23.60, 21.97, 21.42, 21.27, 20.59, 19.86, 18.73, 18.24, 18.15, 17.91, 15.38, 9.97; ^{19}F NMR ($CDCl_3$): $\delta = -61.52$; MS (HRESI-MS) calcd for $[C_{67}H_{116}N_{13}O_{15}F_3+Na]^+$: 1422.8564; found: 1422.8600.

Cyclosporin A E6L1: ^{1}H NMR ($CDCl_3$): $\delta = 8.38$ (d, $J = 9$ Hz, 1H),7.86 (d, $J = 7$ Hz, 1H), 7.78 (d, $J = 9$ Hz, 1H), 7.46 (d, $J = 8$ Hz, 1H), 7.21 (m, 1H), 5.88 (t, $J = 6$ Hz, 1H), 5.68 (dd, $J = 11$ Hz, 4 Hz, 1H), 5.38 (dd, $J = 11$ Hz, 3 Hz, 1H), 5.30 (d, $J = 9$ Hz, 1H), 5.24 (dd, $J = 10$ Hz, 4 Hz, 1H), 5.09 (t, $J = 7$ Hz, 1H), 5.01 (q, $J = 8$ Hz, 1H), 4.95 (d, $J =$ 12 Hz, 1H), 4.86 (m, $J = 8$ Hz, 1H), 4.69 (m, 2H), 4.45 (m, $J = 8$ Hz, 1H), 4.37 (m, 1H), 4.25 (ABq, $J_{AB} = 14$ Hz, $J_q = 7$ Hz, 2H), 4.00 (m, 2H), 3.66 (d, $J = 2$ Hz, 1H), 3.59 (d, $J = 2$ Hz, 1H), 3.48 (s, 3H), 3.44–3.37 (m, 5H), 3.26 (m, 3H), 3.20–3.17 (m, 5H), 3.10 (s, 3H), 2.66 (s, 6H), 2.47 (m, 1H), 2.36 (m, 1H), 2,18–2.05 (m, 3H), 1.99 (m, 1H), 1.90 (m, 1H), 1.84–1.56 (m, 8H), 1.43 (m, 2H), 1.33–1.17 (m, 13H), 1.14–0.84 (m, 39H), 0.75 (d, $J = 7$ Hz, 3H); ^{13}C NMR ($CDCl_3$): $\delta =$ 173.68, 173.38, 173.11, 172.83, 171.58 (2C), 171.16, 171.12, 170.91, 170.77, 169.05, 166.85, 166.27, 157.47, 72.99, 63.75, 62.12, 59.01, 58.54, 57.48, 55.40, 55.20, 54.09, 54.00, 52.71, 50.09, 48.61, 48.39, 47.94, 44.67, 40.28 (2C), 40.12, 39.34, 39.13, 37.02, 36.18, 32.06, 31.76, 31.42 (2C), 31.12, 30.16, 29.80, 29.61, 28.97, 28.35, 25.28, 24.90, 24.79, 24.71, 24.31, 23.92, 23.76, 23.70, 23.65, 23.45, 21.86, 21.38, 21.17, 20.44, 19.82, 18.62, 18.21, 17.82 (2C), 15.30, 14.08, 9.85; MS (HRESI-MS) calcd for $[C_{69}H_{121}N_{13}O_{18}+Na]^+$: 1442.8850; found: 1442.8862.

Cyclosporin A E7L1: ^{1}H NMR ($CDCl_3$): $\delta = 8.37$ (d, $J = 10$ Hz, 1H), 7.86 (d, $J = 7$ Hz, 1H), 7.75 (d, $J = 9$ Hz, 1H), 7.46 (d, $J = 8$ Hz, 1H), 7.02 (m, 1H), 5.88 (t, $J = 5$ Hz, 1H), 5.68 (dd, $J = 11$ Hz, 4 Hz, 1H), 5.38 (dd, $J = 12$ Hz, 4 Hz, 1H), 5.31 (d, $J = 9$ Hz, 1H), 5.23 (dd, $J =$ 11 Hz, 4 Hz, 1H), 5.09 (t, $J = 6$ Hz, 1H), 5.01 (q, $J = 8$ Hz, 1H), 4.95 (d, $J = 11$ Hz, 1H), 4.86 (m, $J = 7$ Hz, 1H), 4.73–4.69 (m, 2H), 4.45 (m, $J = 7$ Hz, 1H), 4.36 (m, 1H), 4.04–3.91 (m, 2H), 3.48 (s, 3H), 3.44–3.40 (m, 5H), 3.26 (s, 3H), 3.17 (s, 3H), 3.10 (s, 3H), 2.95 (dd, $J =$ 6 Hz, 5 Hz, 1H), 2.83 (dd, $J = 6$ Hz, 2 Hz, 1H), 2.66 (s, 6H), 2.47 (m, 1H), 2.18–2.04 (m, 4H), 2.02–1.90 (m, 2H), 1.83–1.56 (m, 12H), 1.43 (m, 2H), 1.32–1.25 (m, 9H), 1.23–0.84 (m, 39H), 0.75 (d, $J = 6$ Hz, 3H); ^{13}C NMR ($CDCl_3$): $\delta = 173.70$, 173.39, 173.16, 172.91, 171.65, 171.48, 171.20, 171.11, 170.96, 170.78, 169.15, 168.99, 157.41, 73.10, 63.67, 58.99, 58.58, 57.51, 55.46, 55.22, 54.17, 50.15, 49.56, 48.65, 48.43, 47.98, 47.34, 44.73, 40.04, 40.32, 39.63, 39.37, 39.14, 37.01, 36.19, 32.16, 31.89, 31.44, 31.49, 31.17, 30.17, 29.84, 29.61, 29.01, 28.52, 25.31, 24.94, 24.83, 24.74, 24.36, 21.94, 21.88, 21.49, 21.25, 21.17, 20.51, 20.43, 19.94, 19.82, 18.72, 18.60, 18.32, 18.24, 17.83, 15.38, 9.91; MS (HRESI-MS) calcd for $[C_{66}H_{117}N_{13}O_{16}+Na]^+$: 1370.8639; found: 1370.8640.

Cyclosporin A E7L2: ^{1}H NMR ([D_8]toluene): $\delta = 8.31$ (d, $J = 10$ Hz, 1H), 7.86 (d, $J = 7$ Hz, 1H), 7.67 (d, $J = 7$ Hz, 1H), 7.45 (d, $J = 8$ Hz, 1H), 6.27 (m, 1H), 5.83 (m, 1H), 5.58 (dd, $J = 11$ Hz, 4 Hz, 1H), 5.33 (td, $J = 11$ Hz, 3 Hz, 1H), 5.21 (d, $J = 10$ Hz, 1H), 5.06 (t, $J = 6$ Hz, 1H), 4.78 (q, $J = 8$ Hz, 1H), 4.68 (d, $J = 11$ Hz, 1H), 4.62 (dd, $J =$ 10 Hz, 9 Hz, 1H), 4.53–4.41 (m, 2H), 4.27 (m, $J = 7$ Hz, 1H), 4.06 (m, 1H), 3.84 (d, $J = 9$ Hz, 1H), 3.78 (d, $J = 14$ Hz, 1H), 3.26 (s, 2H), 3.17–2.98 (m, 14H), 2.91 (m, 1H), 2.88 (s, 3H), 2.81 (s, 3H), 2.64 (s, 3H), 2.52 (s, 3H), 2.39 (s, 3H), 2.15–1.92 (m, 8H), 1.69 (m, 2H), 1,58–1.40 (m, 4H), 1,29–1.18 (m, 5H), 1.15–0.57 (m, 52H), 0.42 (d, $J = 6$ Hz, 3H); ^{13}C NMR ([D_8]toluene): $\delta = 173.08$, 172.36, 171.90, 171.87, 170.61, 170.26, 170.02, 169.96, 169.43, 169.35, 167.74, 166.56, 155.78, 71.86, 69.52, 69.21, 68.51, 62.60, 58.14, 57.74, 56.39, 54.35, 54.01, 53.04, 48.36, 47.95, 47.46, 47.27, 46.78, 45.31, 43.58, 39.94, 39.70, 38.52, 37.57, 37.44, 36.09, 35.18, 30.99, 30.47, 30.35, 30.08, 29.61, 29.45, 28.96, 28.49, 28.06, 27.96, 27.06, 26.99, 24.64, 24.07, 23.88, 23.72, 23.58, 23.15, 22.70, 22.61, 22.52, 22.31, 20.76, 20.54, 20.10, 18.53, 17.35, 17.14, 16.73, 16.21, 14.07, 8.76; MS (HRESI-MS) calcd for $[C_{70}H_{125}N_{13}O_{18}+Na_2]^{2+}$: 740.9531; found: 740.9550.

Plasmids and culture conditions: Mutants were constructed by QuikChange (Stratagene) mutagenesis of plasmid pGEX2TK-Awt (K. Liu and S. L. Schreiber, unpublished results) expressing wild-type human cyclophilin A as a fusion with glutathione-S-transferase (GST-Cyp). Cultures of freshly transformed *E. coli* BL21(DE3) cells (Stratagene) were grown in LB medium at 37 °C supplemented with ampicillin (100 μg mL^{-1}) and 1% dextrose to an OD_{600} of 0.9, chilled to 16 °C, and induced with isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.5 mM. Cells were harvested 72 h later by centrifugation, and the protein was purified immediately.

General purification of Cyp proteins: All steps were performed at 4 °C. Cells were disrupted by sonication in PBS (50 mM potassium phosphate, pH 7.5, 150 mM NaCl) containing PMSF (1 mM), after 30 min incubation with EDTA (10 mM) and lysozyme (100 μg mL^{-1}). Cellular debris was removed by centrifugation and the supernatant was applied to GST-Bind resin (Novagen) column. The column was washed with PBS and thrombin (33 units mL^{-1}, 2 mL, Sigma) was applied to the column to initiate cleavage of Cyp from GST overnight. The protein was further purified on a 26 mm × 70 cm Superdex-200 FPLC column (Amersham Biosciences) in PBS supplemented with DTT (0.1 mM) to provide protein of greater than 95% purity as judged by SDS-PAGE. Purified protein was stored in aliquots in PBS with DTT (1 mM) and 25% glycerol at −20 °C.

General procedure for alkylation: Pure protein from a frozen stock was diluted into PBS containing reduced glutathione (GSH; 1 mM) and 0.05% Tween-20 to a final concentration of 8 μM. The protein mixture was preincubated at 37 °C for 5 min, and the alkylation reaction was initiated by addition of the appropriate electrophile in 50% ethanol to a final concentration of 16 μM. At designated time points, aliquots (10 μL) were withdrawn from the reaction mixture and quenched into loading buffer (4 μL; 280 mM Tris-HCl pH 6.8, 1% SDS, 0.5 M β-mercaptoethanol, 30% glycerol, and 0.0012% bromphenol blue) and run on a 8% SDS-PAGE. The alkylation product was quantified for each time point by densitometry analysis of Coomassie-blue stained SDS-PAGE by using the Image-J program (NIH).

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