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A fluorescence polarization-based assay for peptidyl prolyl *cis*/*trans* isomerase cyclophilin A

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Abstract

Peptidyl prolyl *cis*/*trans* isomerase cyclophilin A (CypA) serves as a cellular receptor for the important immunosuppressant drug, cyclosporin A. In addition, CypA and its enzyme family have been found to play critical roles in a variety of biological processes, including protein trafficking, HIV and HCV infection/replication, and Ca^{2+}-mediated intracellular signaling. For these reasons, cyclophilins have emerged as potential drug targets for several diseases. Therefore, it is extremely important to screen for novel small molecule cyclophilin inhibitors. Unfortunately, the biochemical assays reported so far are not adaptable to a high-throughput screening format. Here, we report a fluorescence polarization-based assay for human CypA that can be adapted to high-throughput screening for drug discovery. The technique is based on competition and uses a fluorescein-labeled cyclosporin A analog and purified human CypA to quantitatively measure the binding capacity of unlabeled inhibitors. Detection by fluorescence polarization allows real-time measurement of binding ratios without separation steps. The results obtained demonstrated significant correlation among assay procedures, suggesting that the application of fluorescence polarization in combination with CypA is highly advantageous for the accurate assessment of inhibitor binding.

Keywords: Fluorescence polarization; Peptidyl prolyl *cis*/*trans* isomerase; Cyclophilin A; Cyclosporin A

Fluorescence polarization (FP)[1] measurements have long been a valuable biophysical research tool for investigating processes such as membrane lipid mobility, myosin reorientation, and protein–protein interactions at the molecular level [1–3]. Immunoassays represent the largest group of bioanalytical applications, having been developed and used extensively for clinical diagnostics [4,5]. The more recent advent of microplate readers equipped with polarizing optics has led to the adoption of fluorescence polarization as a readout mode for high-throughput screening (HTS) [6,7]. FP measurements provide information on molecular orientation and mobility and processes that modulate them, including receptor–ligand interactions, proteolysis, protein–DNA interactions, membrane fluidity, and muscle contraction. Because polarization is a general property of fluorescent molecules, polarization-based readouts are somewhat less dye dependent and less susceptible to environmental interferences such as pH changes than are assays based on fluorescence intensity measurements. Here, we report our FP assay development work for human peptidyl prolyl *cis*/*trans* isomerase cyclophilin A (CypA).

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[1] *Abbreviations used:* FP, fluorescence polarization; HTS, high-throughput screening; CypA, cyclophilin A; CsA, cyclosporin A; MeBmt, 4*R*-4-([E]-2-butenyl)-4-*N*-methyl-L-threonine; CN, calcineurin; PPIase, peptidyl prolyl *cis*/*trans* isomerase; Trp, tryptophan; hCypA, human CypA; DMSO, dimethyl sulfoxide; cps, counts per second; Cs-CMO, cyclosporine-carboxymethyloxime; 6-AMF, 6-aminomethylfluorescein; DMF, *N*,*N*-dimethylformamide; DIEA, *N*,*N*-diisopropylethylamine; THF, tetrahydrofuran; HOBt, *N*-hydroxybenzotriazole; EDAC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; TFA, trifluoroacetic acid; MALDI–TOF MS, matrix-assisted laser/desorption ionization time-of-flight MS.

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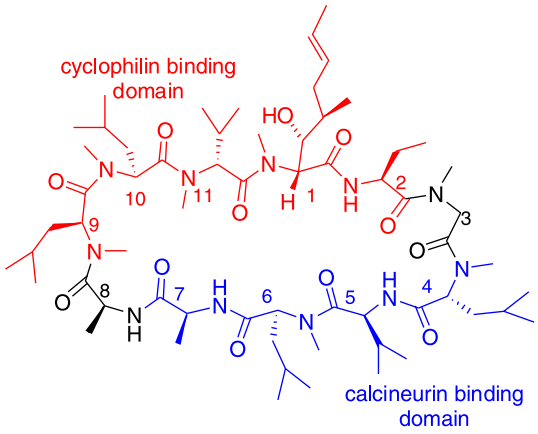
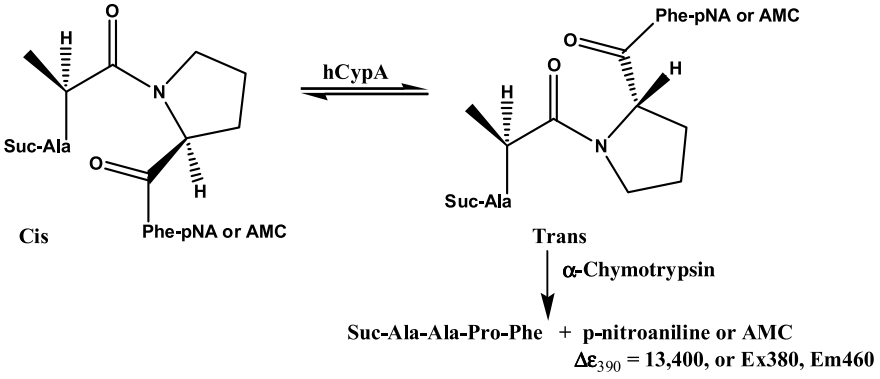
The immunosuppressant drug cyclosporin A (CsA) is a cyclic hydrophobic undecapeptide that contains seven *N*-methyl amino acid residues and the unusual amino acid 4*R*-4-([E]-2-butenyl)-4-*N*-methyl-L-threonine (MeBmt) in position 1 (Scheme 1). CsA is produced by the fungus *Tolypocladium inflatum* and was first isolated in 1976 by workers at Sandoz [8]. The structure of CsA has been confirmed by total synthesis [9]. The conformation of CsA (free in solution and bound to cyclophilin) has been solved by NMR [10] and X-ray crystallography [11]. CsA exerts its immunosuppressive effect by binding to CypA [12]. The binding of CsA to CypA involves extensive interactions from the amino acid residues at positions 9, 10, 11, 1, and 2, as highlighted in red in Scheme 1. The resulting CsA–CypA complex binds to calcineurin (CN), a Ca^{2+}/calmodulin-dependent phosphatase, and inhibits its enzyme activity [13]. The amino acid residues making contact with CN are highlighted in blue in Scheme 1. CsA is widely used to prevent and treat organ transplantation rejection and has potential therapeutic applications in the treatment of diseases such as asthma, psoriasis, atopic dermatitis, and rheumatoid arthritis [14]. However, CsA at doses used to produce a clinical effect is associated with side effects such as nephrotoxicity, hypertension, hepatotoxicity, anemia, and gastrointestinal intolerance [15–17]. There is also evidence to suggest a correlation between CN inhibition and many CsA-induced toxicities [18,19]. This mechanism-based toxicity presents unique challenges for improving the therapeutic index of CsA and underscores the strong need for identifying new nonimmunosuppressant small molecule cyclophilin ligands. To this end, we report here an FP assay that, for the first time, makes HTS efforts for CypA possible.



Scheme 1. CsA structure and its amino acid residues interacting with CypA and CN.

The enzymatic peptidyl prolyl isomerase (PPIase) activity of CypA is generally assessed by assays that are based on chymotrypsin-coupled isomer-specific proteolysis using the tetrapeptide derivatives Suc-Ala-Xaa-Pro-Yaa-para-nitro-anilides by UV–Vis spectroscopy or using Suc-Ala-Xaa-Pro-Yaa-amino-methyl-coumarins by fluorescence spectroscopy, as illustrated in Scheme 2 [20]. An uncoupled protease-free assay of PPIase activity based on minor absorption coefficients of *cis* and *trans* conformers of tetrapeptide anilides remote from far UV peptide absorption has also been described [21]. However, the uncatalyzed spontaneous *cis*/*trans* isomerization rate is extremely fast, with half lives of 4.6 s at 37 °C, 74 s at 10 °C, and 346 s at 0 °C [22], making these activity-based assays not useful for HTS. A fluorescence competition assay using [D-Lys (Dsn)]^8-CsA as probe has been reported for determining the equilibrium constant of the spectroscopically invisible ligand [23]. The intrinsic tryptophan (Trp) fluorescence of CypA increases two- to threefold on CsA binding and can also offer a good measurement of equilibrium binding constant of the spectroscopically invisible ligand binding to the CsA pocket. Unfortunately, most of the small molecule compound libraries contain a significant number of colored compounds and make these fluorescence intensity-based assays not optimal for HTS efforts. Recently, a surface plasmon resonance-based assay was also reported for human CypA (hCypA) [24]. However, this assay again does not meet the criteria for an HTS assay.

To overcome the difficulties of current assay procedures and further facilitate the drug discovery effort for CypA, we have developed a fluorescence polarization-based assay for hCypA that can be adapted to HTS. The technique is based on competition and uses a fluorescein-labeled CsA analog and purified hCypA to quantitatively measure the binding capacity of nonlabeled inhibitors. Detection by fluorescence



Scheme 2. Chymotrypsin-coupled PPIase-catalyzed *cis*/*trans* isomerase assays.

polarization allows real-time measurement of binding ratios without separation steps. The FP assay uses a fluorescein-labeled CsA analog as probe. The parallel and perpendicular intensities of the probe are linear from 0.01 to 2 nM. The mP signals are between 240 and 70 and reach a plateau at 2 nM. The probe binds to CypA at a K_d value of 500 nM. The FP competition assay provides an accurate activity determination for CypA inhibitors and gives results comparable to those of the coupled spectrophotometric, coupled fluorogenic, and Trp fluorescence enhancement assays. More important, for the first time, the FP competition assay would allow the identification of weaker inhibitors in an HTS format that could serve as scaffolds for further rational structure-based drug design.

Materials and methods

hCypA was cloned and purified as described previously [25]. The CypA substrates Suc-AAPF-2, 4-difluoroanilide for direct spectrophotometric assay, and Suc-ALPF-AMC for coupled fluorogenic assay were obtained from Bachem, and their purities were greater than 95% as judged by HPLC analysis. Cyclosporins B, C, D, and H were obtained from Fujian Kerui Pharmaceutical, and their purities were greater than 95% as judged by HPLC.

Coupled fluorogenic assay for IC_{50} measurement

The reactions were started by the addition of 4 μl of 2.5 mM Suc-ALPF-AMC to 200 μl of 50 mM Hepes (pH 7.8), 0.15 mg/ml α-chymotrypsin, 3–6 nM hCypA, and various concentrations of inhibitor in 10% dimethyl sulfoxide (DMSO) at 4 °C. The reactions were monitored at an excitation wavelength of 380 nm and an emission wavelength of 460 nm for 5 min on an ICN Titertek Fluoroskan II 96-well microtiter fluorescence plate reader at ambient temperature using white microfluor U-bottom plates obtained from Dynex. k_{obs} rates were determined from the first-order curve fitting using Deltasoft data collecting and analyzing software (BioMetallics), and IC_{50} values were determined from the equation of percentage inhibition at various inhibitor concentrations (inhibition% = 100[I]/([I] + [IC_{50}])).

Direct spectrophotometric assay for IC_{50} measurement

The reactions were started by the addition of 5 μl of 20 mM Suc-AAPF-2,4-difluoroanilide to 500 μl of 50 mM Hepes (pH 7.8), 13 nM hCypA, and various concentrations of inhibitor in 10% DMSO at 4 °C. The reactions were monitored at a wavelength of 246 nm for 5 min on a Beckman DU640 spectrophotometer. The data were then exported into KaleidaGraph software. The k_{obs} rates were determined from first-order decay, and IC_{50} values were determined from the equation of percentage inhibition at various inhibitor concentrations (inhibition% = 100[I]/([I] + [IC_{50}])).

Trp quenching assay for K_d measurement

Four hundred microliters of 50 mM Hepes (pH 7.8), 10% DMSO, and 328 nM hCypA was titrated with increasing amount of inhibitors. The Trp fluorescence was excited at 280 nm, and an emission scan was taken from 300 to 400 nm on a SPEX FluoroMax. The fluorescence intensities at 350 nm at various inhibitor concentrations were fit to the quadratic equation $(F^0 + ((F - F^0)/[I])*(([CypA] + K_d + [I]) - sqrt(([CypA] + K_d + [I])-)^\wedge 2–4*[CypA]*[I])/2)$ to provide the K_d using KaleidaGraph software.

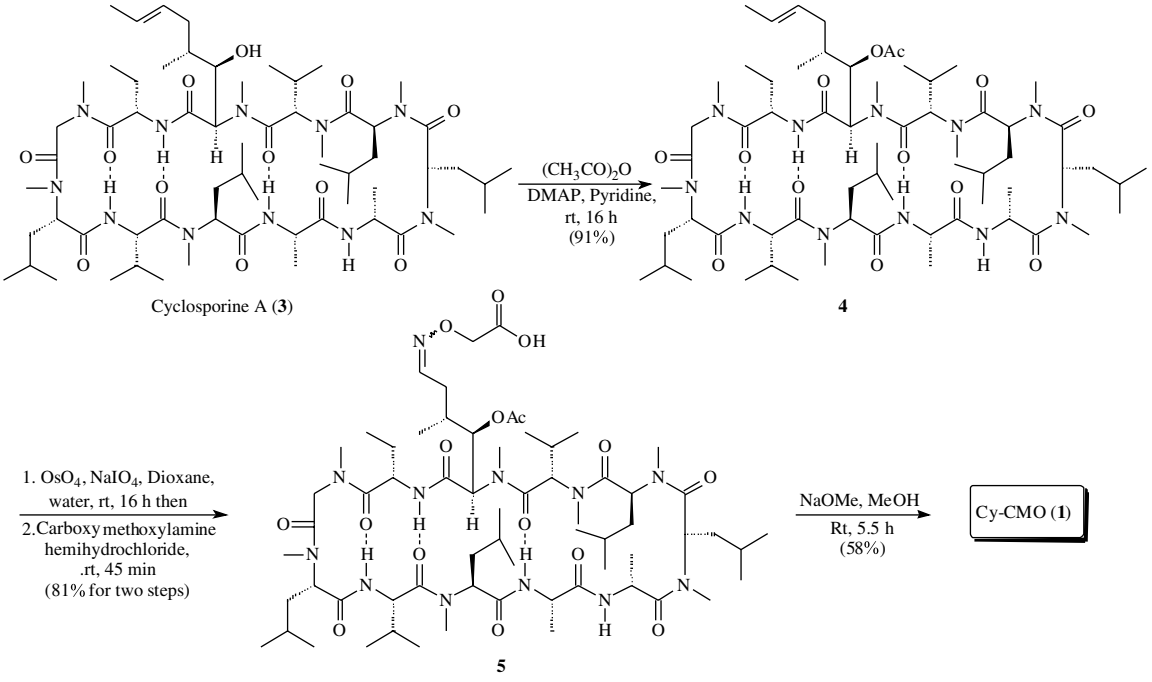
Fluorescence polarization competition assay for K_d measurement

FP measurements were performed on an Analyst AD Assay Detection System (Molecular Devices) using a continuous high-intensity, xenon-arc lamp as light source with filter settings suitable for fluorescein excitation (485 nm) and emission (530 nm). As a standard reading configuration, the excitation polarization filter was set in the S (static) position, whereas the emission polarization filter was dynamically polarizing the light in either the S or P (perpendicular) orientation. A fluorescein dichroic mirror (505 nm) was used to direct the polarized light into the assay well. Emitted polarized light was detected by the fluorescence photomultiplier tube with the SmartRead, sensitivity 2 setup option in counts per second (cps). Two intensity measurements were collected for each well: one when the dynamic polarizer was in the S position and one when the polarizer was in the P position. For a standardized assay setup, each individual well of a black 96-well LJL HE PS microplate (Molecular Devices) was loaded with 200 μl of 50 mM Hepes (pH 7.8), 0.5–1 μM hCypA (3–6 μl of 32.8 μM stock), 1–10 nM of fluorescein-labeled CsA analog, and various inhibitor concentrations in 10% DMSO. The reactions were read in an LJL instrument from Molecular Devices for 100 min. The average mP signals at various inhibitor concentrations were fit to the equation for competitive binding [26] in Excel to obtain the K_d value.

Results

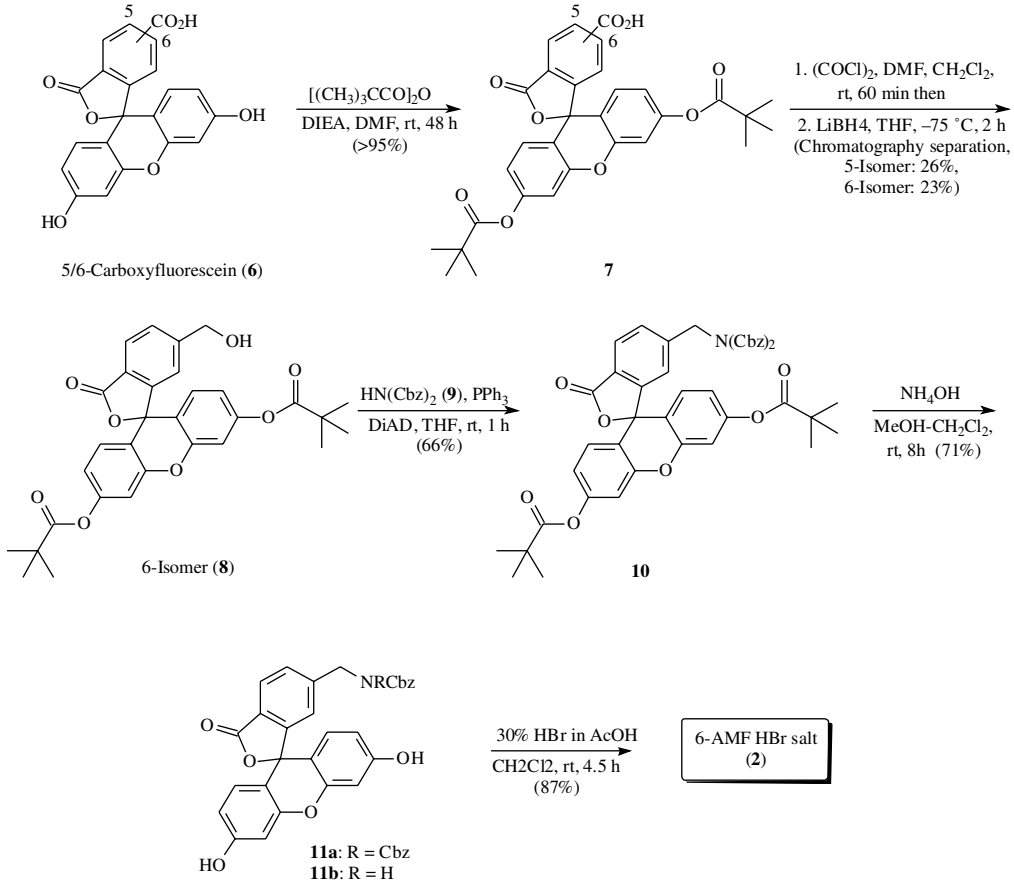
Synthesis of cyclosporine-carboxymethyloxime

Cyclosporine-carboxymethyloxime (Cs-CMO, **1**) was synthesized from CsA (**3**) in three steps, as described in Scheme 3 [27–30]. CsA (**3**) was treated with acetic anhydride in pyridine in the presence of 4-*N*,*N*-dimethylaminopyridine (DMAP) at room temperature for 16 h. The Cs-acetate (**4**) was isolated by flash chromatography (2% methanol in dichloromethane) in 91% yield. The Cs-acetate (**4**) was then treated with osmium tetroxide and sodium periodate in 1,4-dioxane-water (1:1 ratio) at room temperature for 16 h, followed by treatment of the intermediate aldehyde with carboxymethoxylamine hemihydrochloride at room temperature for 45 min. The compound (**5**) was



Scheme 3. Synthesis of Cy-CMO (**1**).

isolated by flash chromatography (5% methanol in dichloromethane) in 81% yield. Finally, the acetate group in compound **5** was cleaved by treatment with sodium methoxide in methanol at room temperature for 5.5 h, followed by HPLC purification and lyophilization to afford the desired Cs-CMO (**1**) in 58% yield and 96.3% purity.



Scheme 4. Synthesis of 6-AMF (**2**).

Synthesis of 6-aminomethylfluorescein

6-Aminomethylfluorescein (6-AMF, **2**) was prepared from a mixture of 5/6-carboxyfluorescein (**6**) in six steps, as described in Scheme 4 [31,32]. First, the 5/6-carboxyfluorescein (**6**) was treated with trimethylacetic anhydride in *N*,*N*-dimethylformamide (DMF) in the presence of *N*,*N*-diisopropylethylamine (DIEA) at room temperature for 48 h. The bis-pivalyl derivative (**7**) was isolated as a mixture of 5- and 6-isomers by extractive workup in greater than 95% yield. The carboxylic acid group in bis-pivalyl derivative (**7**) was then converted to its acid chloride by treatment with oxalyl chloride in DMF and dichloromethane, which was then reduced to the corresponding alcohol using lithium borohydride in tetrahydrofuran (THF) at low temperature. The crude product was purified by flash chromatography (10% ether in dichloromethane), and the product (**8** as 5- and 6-isomers) was isolated in 26 and 23% yield, respectively. The 6-isomer of **8** was then treated with $HN(Cbz)_2$ (**9**, which was separately prepared from benzyl carbamate in two steps) in the presence of triphenylphosphine and diisopropyl azodicarboxylate in THF. The bis-Cbz compound (**10**) was isolated in 66% yield by flash chromatography (30% ethyl acetate in hexanes). The pivalyl groups in **10** were cleaved by treatment with ammonium hydroxide in methanol and dichloromethane at room temperature for 8 h to afford a mixture of **11a** and **11b** in 71% yield. Finally, the mixture of **11a** and **11b** was treated with 30% HBr in acetic acid to afford the desired 6-AMF (**2**) as its HBr salt in 87% yield and 96.8% purity.

Synthesis of fluorescein-labeled CsA analog

To Cs-CMO (**1**, 17.5 mg, 0.014 mmol) dissolved in anhydrous DMF (5 ml, Applied Biosystems) were added 6-AMF (**2**, 5.0 mg, 0.014 mmol), *N*-hydroxybenzotriazole (HOBt, 2.8 mg, 0.021 mmol, Aldrich), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDAC, 4 mg, 0.021 mmol, Sigma), and DIEA (15 mg, 0.056 mmol, Applied Biosystems). The reaction mixture was stirred at ambient temperature for 18 h, diluted with 1.0 ml of 90% DMSO in water, and purified by reverse-phase HPLC using Waters Delta Pak C_{18} stationary phase eluted with a linear gradient of 20–100% acetonitrile in 80 min with 0.1% trifluoroacetic acid (TFA) in water to give FP probe (Scheme 5) as a bright yellow powder (8.2 mg, 37%); matrix-assisted laser/desorption ionization time-of-flight MS (MALDI–TOF MS): $m/z = 1628.5$ (calculated 1628.9 $[M + Na]^+$).

FP probe identification

The challenges posed by the lack of functional groups and the structure complexity of CsA leave a rather limited opportunity for semisynthetic chemistry modifications. The intensity-based [D-Lys(Dns)]^8-CsA probe was accomplished through a total synthesis of D-Lys^8-CsA [33]. We were able to semisynthesize several CsA analogs through the chemical modification of the MeBmt group at position 1 of CsA and test them in the traditional coupled activity assay. The structure and activities for these three analogs are shown in Scheme 5. Modifications of the hydroxyl group of the MeBmt side chain in CsA analogs **1** and **2** were less favorable. The CsA analog **3** was found to inhibit the CypA PPIase activity at 130 nM. Therefore, CsA analog **3** was chosen to be linked with the fluorescein molecule to make the FP assay probe. When the FP probe was assayed in the activity assay, an IC_{50} of 0.49 μM (Fig. 1) was obtained.

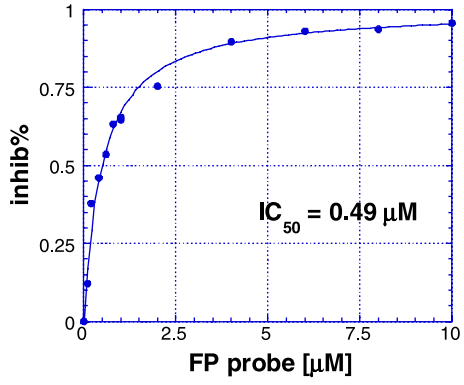
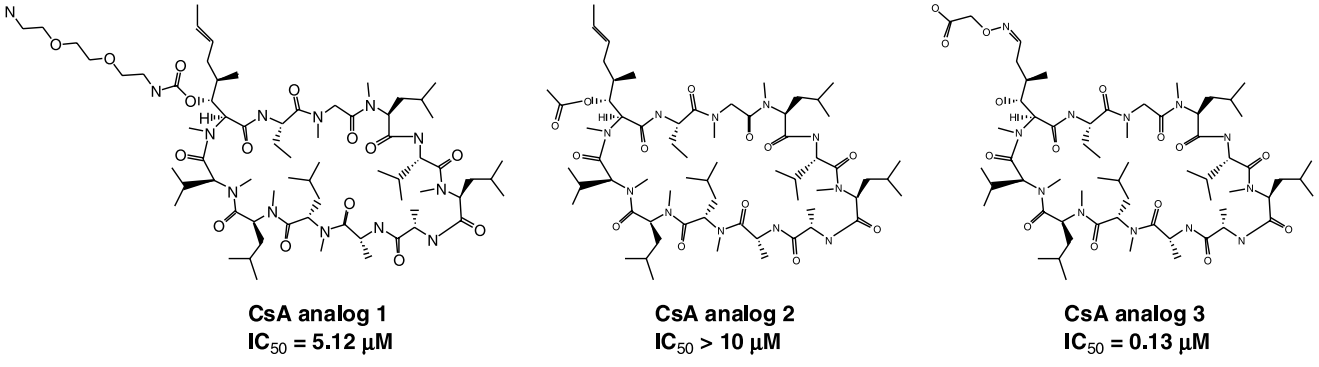


Fig. 1. IC_{50} plot of fluorescein-labeled CsA analog in the coupled fluorogenic assay. Inhib, inhibition.



Scheme 5. Structures and activities of several CsA analogs.

FP competition assay development

The parallel and perpendicular intensities of the FP probe were analyzed at various probe concentrations. The parallel and perpendicular intensities of the probe are linear from 0.01 to 2 nM. The mP signals are between 240 and 70 and reach a plateau at 2 nM (Fig. 2). When using 1 nM of the probe and various concentrations of hCypA, we obtained a saturating binding curve with a K_d of 0.5 μM (Fig. 3). The FP competition assay was set up to use 1 nM of the FP probe and 0.5 μM of the CypA. When various concentrations of CsA were included, the mP of the probe decreased with the increasing concentration of CsA and provided a K_d of 14 nM in this FP competition assay (Fig. 4).

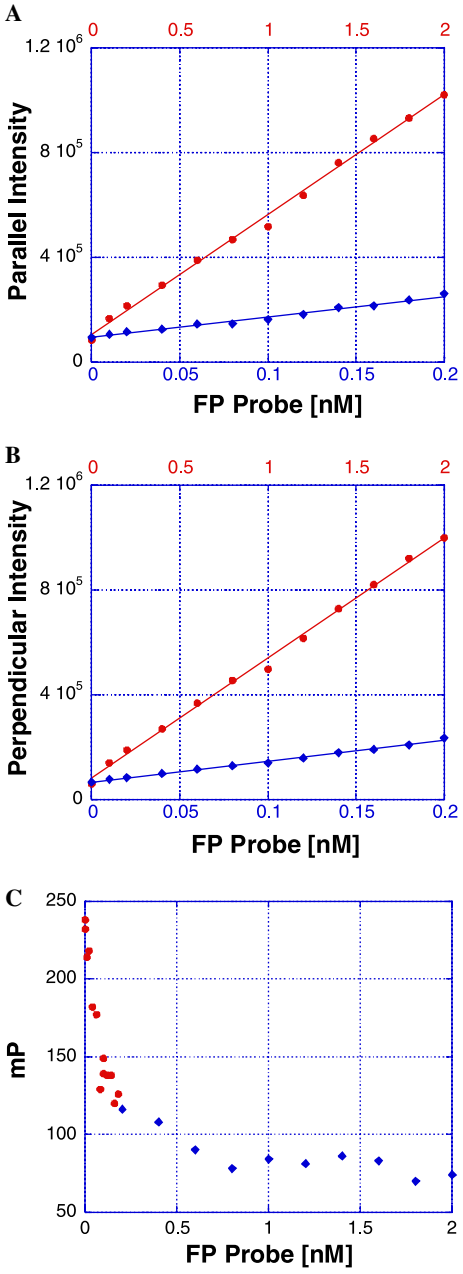


Fig. 2. Fluorescence intensities and mP of the fluorescein-labeled CsA analog at concentration ranges from 0.01 to 0.2 nM (●) and from 0.2 to 2 nM (◆). (A) FP probe parallel intensity versus probe concentration. (B) FP probe perpendicular intensity versus probe concentration. (C) FP probe mP versus probe concentration.

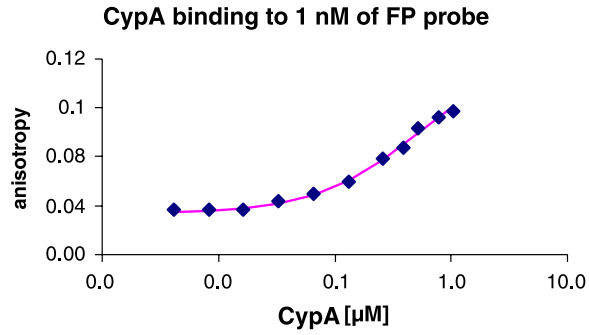


Fig. 3. Binding curve of fluorescein-labeled CsA analog to CypA.

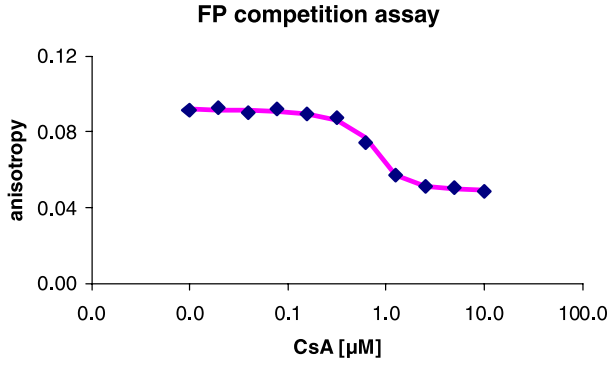


Fig. 4. Binding curve of CsA in the FP competition assay.

FP competition assay validation

Four CsA analogs—CsB, CsC, CsD, and CsH—and CsA itself were tested in FP competition assays, activity-based coupled fluorogenic assay, activity-based direct spectrophotometric assay, and binding-based Trp enhancement assays. The K_d and IC_{50} values are summarized in Table 1. As seen from the table, the K_d values of the CsA analogs obtained in the FP competition assay were in excellent agreement with IC_{50} or K_d values obtained by the other assays.

Conclusion

The identification of nonimmunosuppressive CypA binders has important implications for preventive and/or therapeutic applications in infectious diseases and other diseases. As such, a body of knowledge enhancing the ability to identify and design novel small molecules that bind to CypA has emerged [34,35,24]. In the absence of an HTS assay, a scientist must rely on other approaches, such as "virtual screening" and rational structure-based drug design, for identifying inhibitors of CypA [36,37]. In this article, we have reported a fluorescence polarization-based

Table 1
Comparison of IC_{50} values from the coupled and direct activity assays and K_d values from the Trp enhancement and FP competition assays

Cyclosporin	IC_{50} (Coupled fluorogenic) (μM)	IC_{50} (Direct spectrophotometric) (μM)	K_d (Trp enhancement) (μM)	K_d (Fluorescence polarization) (μM)
CsA	0.010 ± 0.004	0.013 ± 0.006	0.042 ± 0.050	0.011 ± 0.001
CsB	0.089 ± 0.007	0.086 ± 0.012	0.061 ± 0.076	0.107 ± 0.002
CsC	0.029 ± 0.008	0.018 ± 0.006	0.058 ± 0.120	0.035 ± 0.001
CsD	0.19 ± 0.03	0.078 ± 0.014	0.098 ± 0.091	0.186 ± 0.003
CsH	3.3 ± 0.6	11.7 ± 3.4	>10	>10

assay for hCypA that can be adapted to HTS for drug discovery. In general, competition assays measure the binding of a labeled ligand (also called reference or tracer peptide) in the presence of various concentrations of an unlabeled ligand (also called the competitor or inhibitor) to CypA. The advantage of this approach is that, because only a reference ligand is labeled, an adverse effect on affinity potentially caused by the labeling process does not affect the comparison of the unlabeled ligands. This type of assay is not only conceptually simple but also a sensitive and most viable alternative for high-throughput applications.

Acknowledgments

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