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Isoform-Specific Inhibition of Cyclophilins[†]

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ABSTRACT: Cyclophilins belong to the enzyme class of peptidyl prolyl *cis*−*trans* isomerases which catalyze the *cis*−*trans* isomerization of prolyl bonds in peptides and proteins in different folding states. Cyclophilins have been shown to be involved in a multitude of cellular functions like cell growth, proliferation, and motility. Among the 20 human cyclophilin isoenzymes, the two most abundant members of the cyclophilin family, CypA and CypB, exhibit specific cellular functions in several inflammatory diseases, cancer development, and HCV replication. A small-molecule inhibitor on the basis of aryl 1-indanylketones has now been shown to discriminate between CypA and CypB in vitro. CypA binding of this inhibitor has been characterized by fluorescence anisotropy- and isothermal titration calorimetry-based cyclosporin competition assays. Inhibition of CypA- but not CypB-mediated chemotaxis of mouse $CD4^+$ T cells by the inhibitor provided biological proof of discrimination in vivo.

Cyclophilins (Cyps) are folding helper enzymes belonging to the class of peptidyl prolyl *cis*−*trans* isomerases (PPIases,[1] EC 5.2.1.8). They catalyze the *cis*−*trans* isomerization of peptidyl prolyl bonds in unfolded and native proteins (*1*). The most abundant member of the cyclophilin family in human tissues is cytosolic cyclophilin A (CypA, Cyp18), which is the major cellular target for and mediates the immunosuppressive actions of the cyclic undecapeptide cyclosporin A (CsA) (*1*, *2*). CsA binds to the active site of CypA, thereby interfering with its PPIase activity in a very potent manner (*1*, *2*). However, it is believed that the molecular basis of CsA-mediated immunosuppression is the ternary complex of CypA with CsA and the calcium-calmodulin-activated serine/threonine-specific protein phosphatase calcineurin; formation of this complex inhibits the protein phosphatase activity of calcineurin and thus prevents it from regulating cytokine gene transcription (*3*).

There are 20 different cyclophilins described in humans at the protein level underlining the importance of selectivity in pharmacological inhibition of a particular family member. The cyclophilin isoforms possess molecular masses ranging from 18 to 352 kDa, and all might catalyze prolyl bond *cis*−*trans* isomerization utilizing a highly conserved active site (*4*). Besides the prototypic CypA, seven other cyclophilins (CypB, CypC, PPIL1, CypD, USA-Cyp, CypJ, and PPIAL4) consist only of the cyclophilin domain, whereas the multidomain cyclophilins contain additional protein domains of different functionality, like RRM, TPR, or U-box domains (*4*). High susceptibility of the cyclophilin isoenzymes to CsA is determined by the presence of a tryptophan residue in the cyclophilin domain. Thus, the W121 residue of CypA was shown to be essential for CsA binding with a complex dissociation constant in the low nanomolar range (*5*, *6*).

Generally, cyclophilins have been shown to be involved in a multitude of cellular functions like cell growth, proliferation, and motility (*4*, *7*). Several of the isoforms are known to exhibit specific cellular functions, participate in specific interactions with other proteins, and are related to distinct pathophysiological processes. The mitochondrial CypD represents an important part of the mitochondrial permeability transition pore, which is an essential factor in apoptotic and necrotic cell death (*8*, *9*). Furthermore, interaction of CypD with mitochondrial amyloid-β protein increases mitochondrial, neuronal, and synaptic stress, and the absence of CypD protects neurons from Aβ- and oxidative stress-induced cell death (*10*). USA-Cyp and PPIL1 are part of the spliceosomal complex (*11*, *12*).

On the basis of our current knowledge, CypA and CypB are especially interesting members of the cyclophilin family. CypA was found to be overexpressed in many cancer cells (*13*), including human pancreatic cancer cells, oral squamous cancer cells (*14*), non-small cell lung cancer (*15*), and endometrial carcinoma (*16*). Both CypA and CypB were found to be

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[1]Abbreviations: ITC, isothermal titration calorimetry; pNA, 4-nitroanilide; PPIase, peptidyl prolyl *cis*−*trans* isomerase; wt, wild type; CsA, cyclosporin A; CypA, cyclophilin A; CypB, cyclophilin B; CF, carboxyfluorescein.

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associated with malignant progression of breast cancer (*17*, *18*). While CypA was shown to interact with the prolactin receptor (*19*), CypB was found to form a complex with prolactin and to increase the level of prolactin-induced proliferation (*20*). Besides their intracellular localization, both enzymes are found in the extracellular space and are thought to contribute to intercellular communication, inducing signaling responses in target cells (*21*). The presence of elevated levels of extracellular cyclophilins has been reported in several inflammatory diseases (*22*), including severe sepsis (*23*) and rheumatoid arthritis (*24*). Extracellular CypA and CypB were shown to contribute to inflammatory responses via their chemotactic activity which has been demonstrated for neutrophils, eosinophils, and T lymphocytes (*25*−*27*). The matrix metalloproteinase inducer CD147 mediates the signaling and chemotactic activities of both cyclophilins (*28*, *29*).

In addition, it has been shown that CsA suppresses HCV genome replication in HCV replicon cells and human hepatocytes infected with HCV (*30*, *31*). This anti-HCV activity is directly mediated by blocking the PPIase site of cyclophilin activity since the nonimmunosuppressive CsA analogues NIM811 and Debio 025, which do not show calcineurin inhibition via gain of function, attenuate the in vitro replication of HCV subgenomic replicons (*32*−*34*). Furthermore, the anti-HCV effect of Debio 025 was confirmed in patient studies, suggesting cyclophilin inhibition as a new therapeutic avenue for hepatitis C (*35*, *36*). Among the cyclophilin subfamily, by several lines of evidence, CypA and CypB have been identified as valid drug targets for hepatitis C treatment. Both cyclophilins were found to physically interact with the HCV RNA-dependent RNA polymerase NS5B (*37*, *38*), the essential catalyst for the HCV replication process (*39*). However, which PPIase, CypA or CypB or both, is involved in virus replication remains unclear. CsA inhibits both CypA and CypB in the lower nanomolar range, thus preventing discrimination between the cyclophilins (*40*). Different RNA interference approaches showed varying results in that either CypB (*37*), CypA (*38*), or both (*41*) were found to be essential for HCV replication.

The situation with their similar extracellular localization and secretion (*21*−*24*) as well as their involvement in HCV replication (*37*, *38*) makes it especially important to clarify their individual contributions in the respective physiological processes. Besides RNAi strategies, pharmacological downregulation of enzyme activity by specific inhibitors may be an effective approach in this field. Thus, compounds that interact selectively with either CypA or CypB and inhibit their PPIase activity are urgently required to clarify the function of these cyclophilins in vivo. In addition, those compounds might be applicable to the therapy of human diseases associated with the activity of a distinct cyclophilin. In this case, treatment with isoenzyme-specific inhibitors (i) would probably elicit fewer side effects than a global cyclophilin inhibitor like CsA and (ii) would not get dispersed into a cellular sink when sequestered by other cyclophilins.

Aryl 1-indanylketones have been reported to be inhibitory to the parvulin-type PPIase Pin1 by an interaction based on the twisted amide structure of these compounds, resembling the putative transition state of PPIase catalysis (*42*). Since the spatial arrangements of the active site residues of cyclophilins and Pin1 are similar (*57*), aryl 1-indanylketones were analyzed for their potential to inhibit the PPIase activity of cyclophilins.

In these studies, we describe small-molecule inhibitors on the basis of aryl 1-indanylketones that discriminate between CypA and CypB in vitro. Inhibition of CypA- but not CypB-mediated chemotaxis of mouse $CD4^+$ T cells provided biological proof of discrimination among the isoforms. CypA binding of this inhibitor has been characterized using fluorescence anisotropy- and isothermal titration calorimetry-based cyclosporin competition assays. Hydrophobic as well as polar interactions appear to be the driving forces for the active site binding of the inhibitor. Subtle differences in the spatial arrangement of subsites in the catalytic center of the cyclophilins might form the molecular basis of isoform selectivity.

EXPERIMENTAL PROCEDURES

Chemicals. Buffers were purchased from AppliChem (Darmstadt, Germany) or Merck (Darmstadt, Germany). Yeast extract and Peptone were purchased from Serva (Heidelberg, Germany). All other chemicals were purchased from Sigma (Munich, Germany) and of the highest purity available. Suc-Ala-Ala-Pro-Phe-pNA was purchased from Bachem (Heidelberg, Germany).

Chemical Synthesis. Aryl 1-indanylketones and benzofuranones were synthesized as described previously (*42*, *43*). The side chain carboxyfluorescein (CF)-labeled $[Ser]^8$-CsA derivative $[O\text{-}(CF\text{-}NH(CH_2)_2NHC(O)CH_2\text{-})\text{-}D\text{-}Ser]^8CsA$ was synthesized according to a procedure described by Zhang et al. (*44*).

Enzymes. Human CypA and CypB were expressed from vectors pQE70-CypA and pQE60-CypB, respectively in *Escherichia coli* M15 cells and purified as described previously (*45*). To obtain human PPIL1, the gene was PCR-amplified using gene-specific primers from an ORF encoding human PPIL1 (imaGenes, Berlin, Germany) and cloned into pET9a. After overexpression in *E. coli* BL21 cells, purification was performed in 10 mM Tricine (pH 7.0) through a fractogel DEAE 650 column (Merck). The flow-through was passed through a fractogel TSK AF-green matrix (Merck). Bound proteins were eluted with 3 M KCl. The PPIL1-containing fractions were pooled, dialyzed against HEPES buffer (10 mM, pH 6.5), and bound to a fractogel EMD SO_3^- 650 matrix (Merck). The elution was performed with a linear gradient up to 1 M NaCl. Finally, a size exclusion chromatography step was performed [10 mM HEPES (pH 7.8), 6 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT]. To obtain the expression plasmid of human $(His)_6$-CypD, the gene of the protein without the mitochondrial signal sequence was PCR-amplified using gene-specific primers from an ORF encoding human PPIL1 (imaGenes) and cloned into pET28a. The expression plasmid of mouse $(His)_6$-CypC (pET26b-mCypC) was obtained from G. Schwartz (Rochester, NY). The expression plasmid of human $(His)_{10}$-USA-Cyp (pET19-USA-Cyp) was a gift from D. Horowitz (Bethesda, MD). Purifications of His-tagged fusion proteins were performed using affinity chromatography on Ni-NTA resin followed by size exclusion chromatography in 10 mM HEPES (pH 7.8), 6 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT.

PPIase Activity Measurements and Inhibition Experiments. PPIase activity assays were performed at 283 K in quartz cuvettes with a path length of 1 cm under vigorous stirring with a Hewlett-Packard 8453A UV−vis spectrophotometer in 35 mM HEPES buffer (pH 7.8). PPIases were used at concentrations between 0.7 and 30 nM. Enzyme activities toward the substrate Suc-Ala-Ala-Pro-Phe-pNA were measured using the protease free assay according to Janowski et al. (*46*) in the additional presence of different concentrations of the compounds. A 30 mM

stock solution of the substrate in 0.5 M LiCl/TFE (anhydrous) was prepared fresh before the measurement (final substrate concentration of 60 μM). Prior to every measurement, all components except substrate were incubated for 300 s at 283 K. The measurement was started upon substrate addition, and the *cis*−*trans* isomerization kinetics of the substrate was followed at 330 nm.

Fluorescence Polarization-Based Ligand Displacement. Fluorescence polarization measurements according to ref (*47*) were performed on an Envision (PerkinElmer) fluorescence plate reader using black 96-well microtiter plates (PerkinElmer) filled with a total volume of 300 μL. Fluorescence anisotropy values were determined using a filter set for fluorescein polarization measurement (FP480/FP535). The anisotropy values (r) were calculated from the S- and P-polarized fluorescein fluorescence intensity after excitation with a flash lamp according to the relationship $r = (S - GP)/(S + G \times 2P)$ (G-factor $G = 1$) for every well.

All measurements were conducted in 35 mM HEPES buffer (pH 7.8) and 10 mM NaCl at room temperature. To prevent adsorption of the CsA derivatives to the walls of the wells, 1 μM CsH was added. CsH is almost inactive in binding CypA and consequently does not compete for the CypA active site. After manual addition of the assay components to the wells, the plates were shaken for 30 s prior to measurement. The binding of [O-(CF-NH(CH_2)$_2$NHC(O)CH_2-)-D-Ser]^{8}CsA to CypA was analyzed using a final [O-(CF-NH(CH_2)$_2$NHC(O)CH_2-)-D-Ser]^{8}CsA concentration of 50 nM and different concentrations of CypA between 1 nM and 1 μM. For ligand displacement experiments, final concentrations of 50 nM [O-(CF-NH(CH_2)$_2$NHC(O)-CH_2-)-D-Ser]^{8}CsA and 50 nM CypA were applied in the presence of various concentrations of CsA (from 10 nM to 10 μM) and the aryl 1-indanylketone compound **1** (Figure 1) (from 10 nM to 100 μM), respectively. We fitted the obtained anisotropy values to eq 1 to yield dissociation constant K_D for the [O-(CF-NH-(CH_2)$_2$NHC(O)CH_2-)-D-Ser]^{8}CsA−CypA complex.

$$r([\mathrm{R}]_t) = r_0 + c\left[\frac{K_D + [\mathrm{L}]_t + [\mathrm{R}]_t}{2} - \sqrt{\frac{(K_D + [\mathrm{L}]_t + [\mathrm{R}]_t)^2}{4} - [\mathrm{L}]_t[\mathrm{R}]_t}\right] \quad (1)$$

where $[L]_t$ and $[R]_t$ represent the total concentrations of [O-(CF-NH(CH_2)$_2$NHC(O)CH_2-)-D-Ser]^{8}CsA and CypA, respectively, and r_0 is the anisotropy value of [O-(CF-NH(CH_2)$_2$NHC(O)-CH_2-)-D-Ser]^{8}CsA in the absence CypA. The proportionality factor c relates the concentration of the complex to the anisotropy value. Ligand displacement experiments were evaluated according to the mathematical model of Wang (*48*) using Igor (Wavemetrics) for data fitting.

Isothermal Titration (ITC). ITC experiments were performed using a VP-ITC instrument (MicroCal). Prior to the experiment, all solutions and buffers were filtered through filter membranes with a pore size of 0.2 μm (Whatman) and degassed. Protein solutions were dialyzed against the assay buffer [35 mM HEPES (pH 7.8)]. Stock solutions (100 mM) of the inhibitors were prepared in DMSO and diluted into assay buffer. To maintain constant conditions in all samples, DMSO was also added to protein solutions at the appropriate concentration (<0.1%, v/v). In a typical experiment, 300 μL of a 20 μM solution of CypA was titrated in 15 μL steps into a 2 μM CsA solution at 293 K. Titrations were performed in the absence and presence of 200 μM **1**. By adding compound **1** to both the CsA and CypA solutions, we maintained a constant concentration of competing ligand throughout the experiment. The instrument stirring speed was set to 310 rpm, and the feedback gain mode was set to "high". Since the signal from the first injection can usually not be used for data analysis, only 2 μL was titrated in this step and the data point was omitted. Measured data were analyzed using Origin (MicroCal).

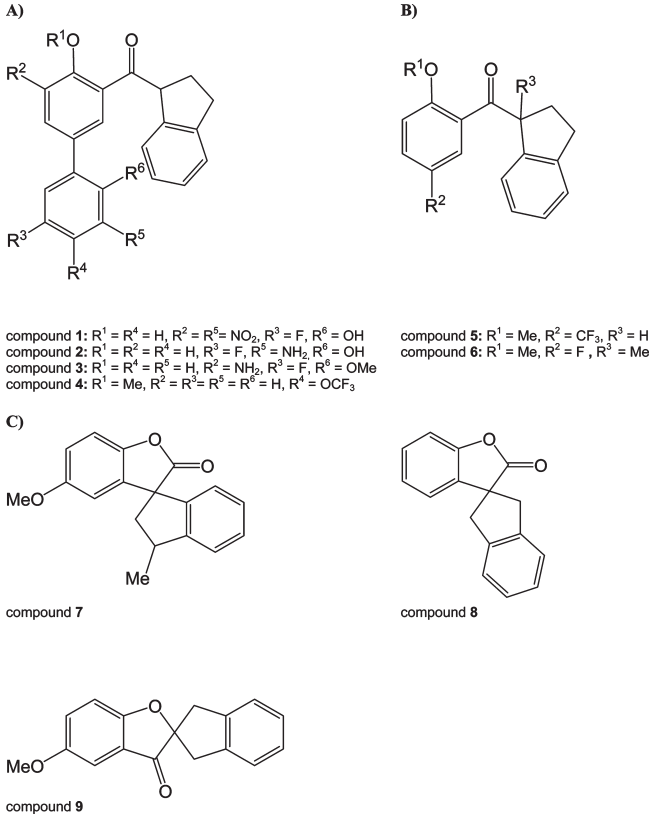

FIGURE 1: Chemical structure of aryl 1-indanylketones (A and B) and benzofuranones (C).

According to Zhang and Zhang (*49*), the thermodynamic parameters for the binding of a low-affinity ligand L_2 can be estimated by a competition ITC experiment. First, the protein P is titrated with a high-affinity ligand L_1 (ΔH_1 and K_1) which occupies the same binding site as L_2 to determine its binding parameters. Next, the titration is performed in the additional presence of the low-affinity ligand L_2 to obtain the apparent ITC parameters (ΔH_{app} and K_{app}). The ITC parameters for the low-affinity ligand (ΔH_2 and K_2) can be calculated according to the following equations (eqs 2 and 3).

$$K_2 = \left(\frac{K_1}{K_{app}} - 1\right)\frac{1}{L_{2tot}} \quad (2)$$

$$\Delta H_2 = (\Delta H_1 - \Delta H_{app})\left(1 + \frac{1}{K_2 L_{2tot}}\right) \quad (3)$$

Chemotaxis Assay. Activated CD4$^+$ T cells were generated by overnight stimulation of total mouse spleen cells at a density of 3×10^6 cells/well with the mitogen Concanavalin A (ConA) at 10 μg/mL in Click's medium containing 5% FCS in a 24-well tissue culture plate at 37 °C in the presence of 5% CO_2. The following day, the CD4$^+$ T cells were purified from the cultures

using a MACS (Miltenyi Biotec Inc., Auburn, CA) negative depletion kit. These purified $CD4^+$ T cells were then tested for chemotaxis in 48-well modified Boyden chambers (Neuroprobe, Gaithersburg, MD), with the two compartments separated by a 5 μm polycarbonate membrane (Neuroprobe). Medium (RPMI-1640 and 1% bovine serum albumin) containing 100 ng/mL recombinant human CypA (Calbiochem, San Diego, CA), 200 ng/mL recombinant CypB (generated at George Washington University), 1 ng/mL recombinant mouse RANTES (PeproTech, Rocky Hill, NJ), or nothing (medium alone) was placed in wells in the lower compartment of the Boyden chamber. Purified $CD4^+$ T cells in medium (10^4 per well) were placed in wells in the upper compartment. In some groups, compound **1** was added at a final optimized concentration of 2 μM to matching wells in the top and lower compartments. The loaded chamber was then incubated at 37 °C in the presence of 5% CO_2 for 50 min. Following incubation, the polycarbonate membrane was removed; nonmigrated cells were scraped off, and the membrane was stained with Wright-Giemsa (CAMCO, Fort Lauderdale, FL). A chemotactic index was generated for each well by dividing the number of cells migrating within each test well by the average number of cells migrating to medium alone.

RESULTS

*Aryl 1-Indanylketone **1** Is a Specific Inhibitor of CypA among Cyclophilin Isoforms*. The chemical structures of aryl 1-indanylketones and benzofuranones used in this work are shown in Figure 1. Among these compounds, **1** was found to reversibly inhibit cyclophilin A (CypA, Cyp18) with a K_I value of 0.52 ± 0.15 μM (Figure 2A). Notably, CsA as the generic CypA inhibitor with a K_I value of 2.9 nM cannot markedly differentiate between six important human cyclophilins (Table 1). CypA is most sensitive to CsA, but the level of selectivity for CypB is in the range of 3-fold. In contrast, the aryl 1-indanylketone compound **1** is able to differentiate between the cyclophilins (Table 2). It was found that the PPIase activity of CypB, CypC, and USA-Cyp did not respond to compound **1** up to a concentration of 30 μM, indicating selectivity of at least 200-fold. CypB, CypC, and USA-Cyp share a high degree of homology with CypA (63, 56, and 55% identical amino acids with CypA in the PPIase domain, respectively). In assays using CypD and PPIL1, compound **1** had 4.6- and 56-fold selectivity for CypA inhibition, respectively. The K_I values were determined using recombinantly produced enzymes in a protease-free assay (*46*) with Suc-Ala-Ala-Pro-Phe-4-nitroanilide at a substrate concentration $[S] \ll K_M$. The reversibility of inhibition was thoroughly examined by dilution experiments of the CypA complex formed with **2**, which represents the tightest binding compound (Table 2). CypA (0.75 μM) and **2** (0.02−200 μM) were incubated for 30 min for gradual inactivation and subsequently diluted. The PPIase activity of CypA completely recovered from the inhibited enzyme when diluted 1:375 in 35 mM HEPES buffer (pH 7.8), regardless of whether the incubation time was prolonged or the concentration of **2** was further increased (Figure 2B).

Table 1: Properties of Prototypic Cyclophilins

enzyme	alias/ gene name[a]	molecular mass[a] precursor/ processed (Da)	identity with CypA (%)	k_{cat}/K_M[b] (M^{-1} s^{-1})	K_I[c] (CsA) (nM)
CypA	Cyp18/PPIA	18012	100	1.4×10^7	2.9
CypB	Cyp23/PPIB	22742/20289	63	5.6×10^6	8.4
CypC	Cyp23a/PPIC	22763	56	5.5×10^5	7.7
PPIL1	Cyp18.2a/PPIL1	18236	46	2.0×10^6	9.8
CypD	Cyp22/PPIF	22040/18897	75	1.3×10^7	6.7
USA-Cyp	Cyp19.2/PPIH	19208	55	6.9×10^5	91

[a] Data according to SwissProt. [b] k_{cat}/K_M values were determined with the protease-free PPIase assay according to ref (*46*) in 35 mM HEPES buffer (pH 7.8) at 283 K, using Suc-Ala-Ala-Pro-Phe-pNA (60 μM) as a substrate. [c] K_I values were determined with the protease-free PPIase assay in the presence of increasing concentrations of CsA. The standard deviations of the measurements of the kinetic constants were not larger than 10%.

Table 2: Inhibition of Prototypic Cyclophilins by Compounds **1** and **2**

	K_I[a] (μM)	
enzyme	**1**	**2**
CypA	0.52 ± 0.15	0.3 ± 0.1
CypB	> 100	12 ± 5
CypC	> 100	> 100
PPIL1	29 ± 7	> 100
CypD	2.42 ± 0.76	6.28 ± 0.73
USA-Cyp	> 100	> 100

[a] K_I values were determined with the protease-free PPIase assay according to ref (*46*) in 35 mM HEPES buffer (pH 7.8) at 283 K, using Suc-Ala-Ala-Pro-Phe-pNA (60 μM) as a substrate and increasing concentrations of aryl 1-indanylketones.

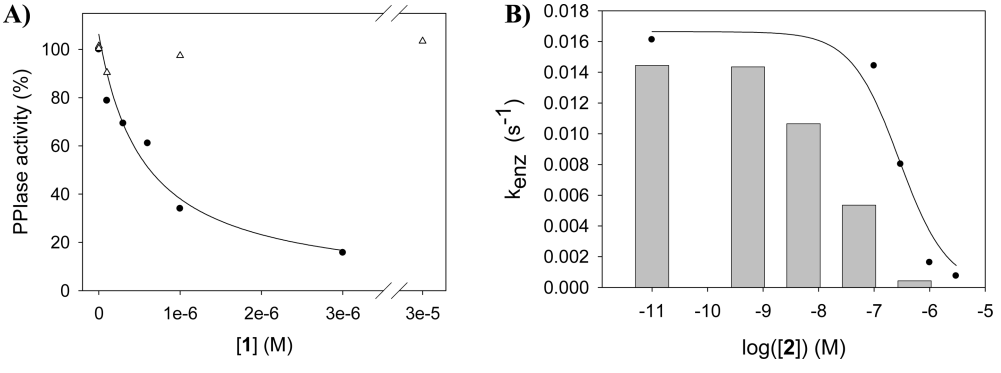

FIGURE 2: (A) Residual PPIase activity of CypA (●) and CypB (△) by compound **1**. (B) Reversibility of CypA inhibition by compound **2** analyzed by dilution experiments. CypA (0.75 μM) and **2** (0.02−200 μM) were incubated for 30 min. After 1:375 dilution in 35 mM HEPES buffer (pH 7.8), the PPIase activity was determined (bars) and compared with the PPIase activity of CypA at the indicated concentrations of compound **2**. The PPIase activity of CypA completely recovered from the inhibited enzyme. The PPIase activity was measured in 35 mM HEPES buffer (pH 7.8) at 283 K using Suc-Ala-Ala-Pro-Phe-pNA as a substrate. Each data point represents the mean of two measurements with a deviation of < 10%.

Besides Pin1 inhibition (*42*), compound **1** does not influence Par14 and only modestly affects FKBP12 (K_I = 38 ± 8 μM). The similar inhibitory properties of compound **1** toward Pin1 and CypA prompted us to investigate a series of recently reported Pin1 inhibitors of the aryl 1-indanylketone and spiroannulated benzofuranone type (*42*, *43*) to establish if there is a further enhanced selectivity for CypA over other cyclophilins, especially CypB (Table 3). CypA and CypB can be weakly inhibited by benzofuranones **7** and **9**, in a range that was already found for Pin1. Because of its low potency, the spirocyclic benzofuranone ring may not be suitable for the design of cyclophilin-directed inhibitors. In the aryl 1-indanylketone series, the most active inhibitor against CypA was compound **2** (K_I = 0.3 ± 0.1 μM). However, compound **2** inhibited CypB with a K_I of 12 ± 5 μM, thereby discriminating between both by a factor of only 40. Thus, compound **1** remained the compound with the greatest ability to discriminate between CypA and CypB by a factor of > 200.

A considerable difference in the inhibition of CypA and CypB was found when the two enantiomers of **6** were analyzed. The inhibitory (*R*) enantiomer had a 40-fold selectivity for CypA. Notably, the (*S*) configuration in the 1-methyl position totally compromised the inhibition of both CypA and CypB.

Table 3: Inhibition of CypA and CypB by Aryl 1-Indanylketones and Benzofuranones

	K_I[a] (μM)	
compound	CypA	CypB
1	0.52 ± 0.15	> 100
2	0.3 ± 0.1	12 ± 5
3	1.7 ± 0.5	8.6 ± 0.9
4	1.2 ± 0.4	2.1 ± 0.3
5	10 ± 2	> 100
(*S*)-**6**	> 100	> 100
(*R*)-**6**	7.5 ± 1.5	40 ± 10
7	21 ± 4	> 100
8	> 100	> 100
9	> 100	63 ± 12

[a] K_I values were determined with the protease-free PPIase assay according to ref (*46*) in 35 mM HEPES buffer (pH 7.8) at 283 K, using Suc-Ala-Ala-Pro-Phe-pNA (60 μM) as a substrate and increasing concentrations of aryl 1-indanylketones.

Aryl 1-Indanylketone ***1*** *Binds to the Active Site of CypA*. Next we analyzed whether compound **1** binds to CypA at the active site, which is composed of residues from four of the eight β-strands and a loop of aromatic residues forming a hydrophobic pocket at one side of the hydrophobic core of CypA (*50*). We used a CsA competition assay because this inhibitor was shown to bind to the active site of CypA (*47*). Displacement of a side chain carboxyfluorescein (CF)-labeled [Ser]^8-CsA derivative ([O-(CF-NH$(CH_2)_2$NHC(O)CH_2-)-D-Ser]^{8}CsA) from the CypA active site was determined at increasing concentrations of compound **1** using fluorescence anisotropy measurements. This compound inhibited the PPIase activity of CypA with a K_I value of 2.4 ± 0.3 nM.

The anisotropy change associated with the binding of [O-(CF-NH$(CH_2)_2$NHC(O)CH_2-)-D-Ser]^{8}CsA by increasing concentrations of CypA is shown in Figure 3A. When using various concentrations of CypA at an invariable concentration of the fluorescent derivative (50 nM), we obtained a saturating binding curve with a K_D of 4.0 ± 1.5 nM (Figure 3A). According to these data, the fluorescence anisotropy competition assay was set up to use 50 nM [O-(CF-NH$(CH_2)_2$NHC(O)CH_2-)-D-Ser]^{8}CsA and 50 nM CypA. As a reference competition assay, CsA was ap plied at various concentrations to the preformed [O-(CF-NH$(CH_2)_2$NHC(O)CH_2-)-D-Ser]^{8}CsA−CypA complex. As expected, the anisotropy value of the probe decreased with an increase in the concentration of CsA, providing a K_D of 12 ± 2 nM. Considering the different protein concentrations in the assays, this value agrees within the limits of error with the value determined by enzyme inhibition (Figure 3B and Table 1). When various concentrations of compound **1** were applied to compete for CypA of the preformed complex, the anisotropy value of the probe decreased with an increase in the concentration of compound **1** and provided a K_D of 0.54 ± 0.13 μM (Figure 3B).

Thermodynamic Parameters of the Interaction. Isothermal titration calorimetry was used to determine the thermodynamic parameters of the association reaction of inhibitor compound **1** with CypA (Table 4). Initially, the titration of 50 μM compound **1** with 200 μM CypA and the titration of

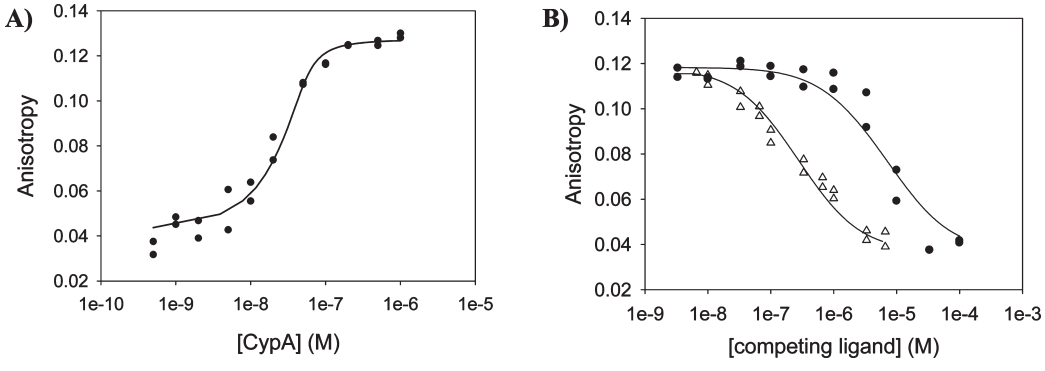

FIGURE 3: Aryl 1-indanylketone **1** and CsA are able to displace [O-(CF-NH$(CH_2)_2$NHC(O)CH_2-)-D-Ser]^{8}CsA from the complex with CypA. (A) Determination of the dissociation constant for the [O-(CF-NH$(CH_2)_2$NHC(O)CH_2-)-D-Ser]^{8}CsA−CypA complex. [O-(CF-NH$(CH_2)_2$NHC(O)-CH_2-)-D-Ser]^{8}CsA (50 nM) in 35 mM HEPES buffer (pH 7.8), 10 mM NaCl, and 1 μM CsH was titrated with increasing concentrations of CypA, and fluorescence anisotropy values were recorded. The anisotropy values obtained were fitted according to a quadratic equation (eq 1) to yield a dissociation constant (K_D) of 4.0 ± 1.5 nM for the CypA−[O-(CF-NH$(CH_2)_2$NHC(O)CH_2-)-D-Ser]^{8}CsA interaction. (B) Titration of the [O-(CF-NH$(CH_2)_2$NHC(O)CH_2-)-D-Ser]^{8}CsA−CypA complex with compound **1** (●) or CsA (△). The preformed complex of 50 nM CypA and 50 nM [O-(CF-NH$(CH_2)_2$NHC(O)CH_2-)-D-Ser]^{8}CsA in 35 mM HEPES buffer (pH 7.8), 10 mM NaCl, and 1 μM CsH was titrated with increasing amounts of compound **1** or CsA, and fluorescence anisotropy values were recorded. Ligand displacement experiments were evaluated according to the mathematical model of Wang (*48*), resulting in dissociation constants (K_D) of 540 ± 130 nM for the CypA−compound **1** complex and 12 ± 2 nM for the CypA−CsA complex.

Table 4: Thermodynamic Parameters for the Interactions of CypA with CsA and Compound **1**[a]

syringe	cell	ΔH (kcal mol^{-1})	$T\Delta S$ (kcal mol^{-1})	ΔG (kcal mol^{-1})	K_A (M^{-1})	N
CypA	CsA	-13.2 ± 0.1	-3.0	-10.2	$(4.05 \pm 0.94) \times 10^7$	1.3
CypA, **1**	CsA, **1**	-10.8 ± 0.1	-1.9	-8.9	$(4.12 \pm 1.59) \times 10^6$	1.6
1	CsA	ns[b]	ns[b]	ns[b]	ns[b]	ns[b]

[a] A solution of 2 μM CsA (initial concentration) in 35 mM HEPES buffer (pH 7.8) was titrated at 293 K with 20 μM CypA (initial concentration) in the absence and presence of 200 μM compound **1**. The error of each parameter represents the error of fitting. [b] No signal of heat formation.

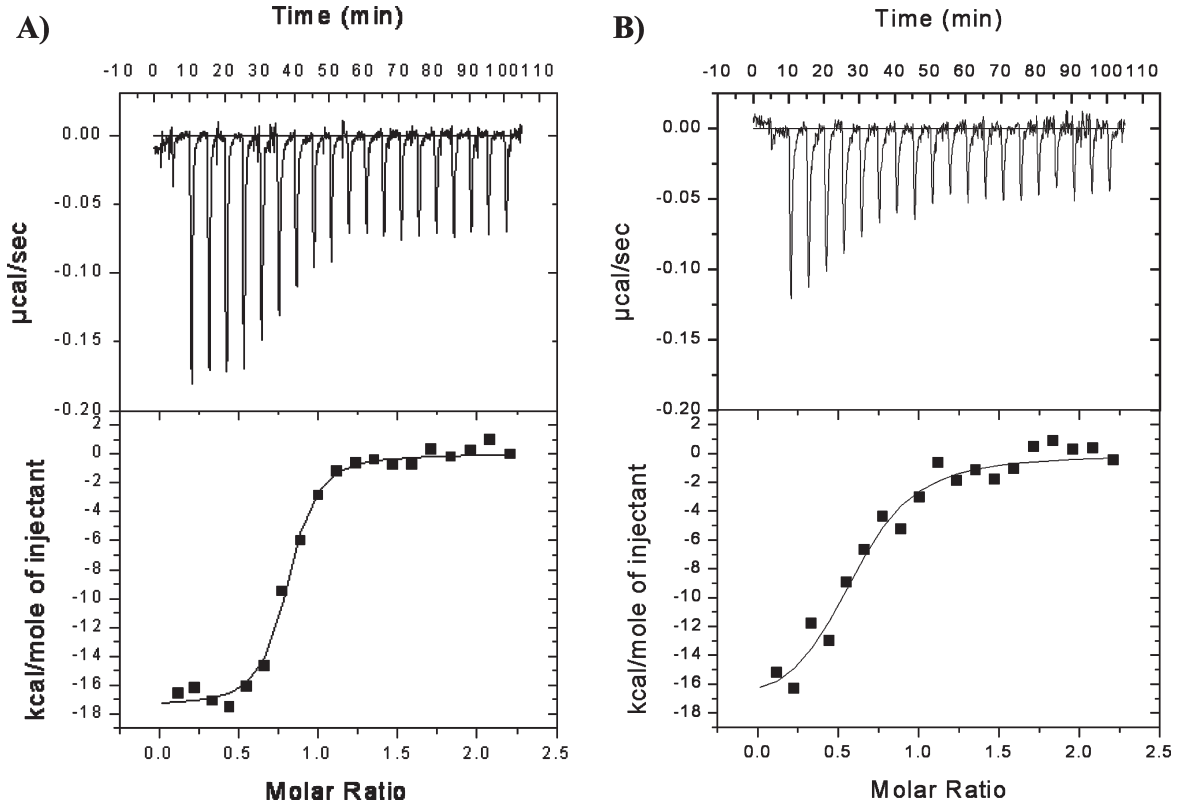

FIGURE 4: Isothermal titration of CsA with CypA in the absence (A) and presence (B) of compound **1**. A solution of 2 μM CsA (initial concentration) in 35 mM HEPES buffer (pH 7.8) was titrated at 293 K with 20 μM CypA in the absence and presence of 200 μM compound **1**. Via the addition of compound **1** to both the CsA and CypA solutions, a constant concentration of the competing ligand was maintained throughout the experiment.

10 μM CypA with 100 μM compound **1** at 293 K were performed. These direct titrations did not allow reliable determination of the thermodynamic parameter. Therefore, we have used a competitive method to obtain the thermodynamic parameters and association constant for the binding of compound **1** (*49*). This method is based on the thermodynamic coupling of two ligands that bind to the same site of a protein. The thermodynamic parameters for the low-affinity ligand can be obtained by conducting ITC experiments with a high-affinity ligand in the absence and presence of the low-affinity ligand. In this experiment, compound **1** constituted the low-affinity ligand and CsA was used as the high-affinity ligand. First, the titration of 2 μM CsA with 20 μM CypA at 293 K was performed (Figure 4A). It revealed a binding enthalpy ΔH_{ITC} of -13.2 ± 0.1 kcal mol^{-1}, a $T\Delta S_{ITC}$ of -3.0 kcal mol^{-1}, and a ΔG_{ITC} of -10.2 kcal mol^{-1} which results in an association constant K_A of $(4.05 \pm 0.94) \times 10^7$ M^{-1} (Table 4). These values agree well with the values published previously (*45*). In the presence of 200 μM compound **1**, the apparent binding constant for CsA was $(4.12 \pm 1.59) \times 10^6$ M^{-1}, which is ~10-fold smaller than that determined in the absence of compound **1** (Figure 4B). The association constant and the ΔH for the binding of compound **1** to CypA were obtained by using eqs 2 and 3, respectively. Thus, using the competition ITC approach, compound **1** revealed a binding enthalpy ΔH of -2.8 kcal mol^{-1}, a $T\Delta S$ of 3.5 kcal mol^{-1}, a ΔG of -6.3 kcal mol^{-1}, and an association constant K_A of 4.4×10^4 M^{-1}. The positive $T\Delta S$ showed that the association of CypA with compound **1** was entropically driven. The positive entropic contribution hints at a burial of solvent-accessible surface area on binding, since the release of ordered water molecules often contributes extensively and positively to the entropy of an interaction (*51*). The enthalpic contribution suggests that the enzyme−inhibitor complex is stabilized to some extent by hydrogen bonds or van der Waals interactions (*51*).

Remarkably, a dissociation constant K_D of 22.6 μM as determined by the ITC probe of CsA−compound **1** competition is much higher than the K_I value determined by the fluorescence anisotropy competition assay using a CsA derivative. This may be caused by differences in the experimental setup of the respective measurements. In ITC, the final CypA concentration was $\gg 10^3$-fold higher than the protein concentration applied in the other binding assays.

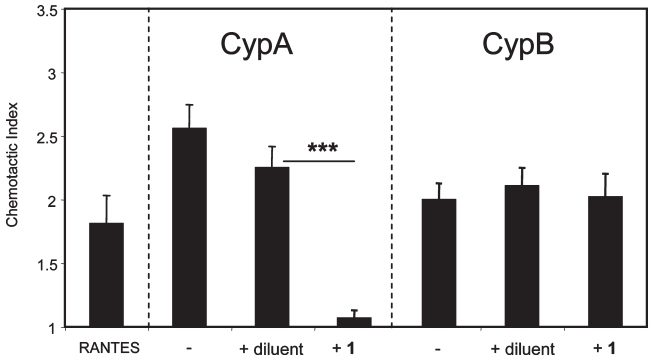

FIGURE 5: Aryl 1-indanylketone **1** inhibits the chemotactic activity of CypA but not CypB. Activated $CD4^+$ T cells were purified using MACS separation after overnight stimulation of spleen mononuclear cells with ConA. Chemotaxis assays were conducted using a 48-well modified Boyden chamber. RANTES was used as a positive control for cell migration. CypA and CypB were used at concentrations of 100 and 200 ng/mL, respectively. Compound **1** was used at a concentration of 2 μM. Data show mean (±standard error) chemotactic indices for each group with six wells per group. A chemotactic index was generated for each well by dividing the number of cells migrating within each well by the average number of cells migrating to medium alone.

*Aryl 1-Indanylketone **1** Inhibits the Chemotactic Property of CypA but Not of CypB*. Next we asked whether the application of the CypA-specific inhibitor compound **1** would elicit an influence on a cellular function already described to be associated with inhibition of the PPIase activity of cyclophilins by CsA. CypA and CypB have been shown to function as chemotactic agents for leukocytes (*26*, *28*, *29*). This function is based on the activity of CD147 as a signaling receptor for extracellular cyclophilins (*21*). The chemotactic activity of these cyclophilins can be inhibited by CsA (*22*).

To investigate the influence of compound **1** on cyclophilin-induced chemotaxis, we used CypA and CypB to induce the migration of mouse $CD4^+$ T cells (Figure 5). Groups of $CD4^+$ T cells were set up in Boyden chambers in the presence of a previously optimized dose of recombinant CypA or CypB. The chemotactic index represents the ratio of cells migrating within each test well to the average number of cells migrating to medium alone. On the basis of published studies and previous experience, a chemotactic index of >1.2 was considered as significant chemotaxis (*52*). As already described in the literature (*26*, *28*, *29*), CypA as well as CypB induced the migration of the T cells (Figure 5). Migration was also observed in response to a positive control chemokine, RANTES, which we had previously established was not sensitive to the indanylketone (data not shown). The addition of compound **1** inhibited the leukocyte chemotaxis induced by CypA almost completely, indicating the presence of an active site mediating the chemotactic properties of CypA. As expected from the inability of compound **1** to inhibit the PPIase activity of CypB, CypB-induced chemotaxis was barely impacted by addition of compound **1**. The diluent had no significant effect on the migration induced by either CypA or CypB. Taken together, these findings indicate that the ability of compound **1** to selectively inhibit CypA in vitro is mirrored by a distinct response of a relevant biological system.

DISCUSSION

Here we report aryl 1-indanylketones as the first PPIase inhibitors found to exhibit a potent inhibitory effect on CypA along with a considerable degree of selectivity among isoenzymes of the human family of cyclophilins. This structural template gave rise to the phenolic biphenyle ketone **1** which not only enabled a clear discrimination between CypA and CypB activities but also left FKBP12 and Par14 activities nearly unchanged at concentrations inhibitory to CypA. Previously published biochemical data for compound **1** reported a comparable inhibitory action on Pin1 (*42*). The proposed model for the interaction of aryl 1-indanylketones with Pin1 is based on the twisted amide structure of compound **1** which resembles the putative transition state of PPIase catalysis (*42*). The torsion angle between the aromatic-carbonyl and the indanyl moiety of 92° shown in the X-ray structure of an aryl 1-indanylketone corresponds with the features of a twisted amide supposed to form the transition state (*42*). However, the features which make the ring substitution pattern of compound **1** especially favorable for Pin1 inhibition remained open. Obviously, similar stereochemical inhibition characteristics for CypA and Pin1 as revealed by the relative inertness of the (*S*)-**6** enantiomer (Table 3) support the hypothesis of a transition state inhibitor, since stereochemical selectivity forms an integral part of transition state binding. Other structure−activity relationships are not evident from the limited set of compounds for both selectivity and potency (Table 3). The tight binding of CsA to cyclophilins might indicate its ability to mimic the conformation of the transition state of the reaction. However, structural comparison of the CypA−CsA complex with unligated CypA and the CypA−Ala-Pro complex suggested that CsA is unlikely to be a transition state analogue of the substrate (*66*), in contrast with an original proposal that CsA mimics a transition state of the PPIase reaction (*67*). Designed inhibitory compounds developed for CypA have been ground state analogues such as alkene isosteres of prolyl bonds or bicyclic *cis*-Pro mimics or transition state analogues such as phosphoamides (*53*−*56*).

By demonstrating that the active site of CypA but not of CypB resembles Pin1 in its affinity for compound **1**, we have revealed previously unidentified similarities and differences in the catalytic mechanisms of PPIases. Interestingly, despite the lack of a common global fold in cyclophilins and Pin1, the spatial arrangement of their active site residues is similar (*57*). The CypA active site residues superimposed with the active site of Pin1 show four CypA residues having identical counterparts in the human Pin1, and two residues exhibit mirror symmetry (*57*).

Elements contributing to the differential inhibition of CypA and CypB might become visible in X-ray structures of the respective Cyp−CsA complexes. However, the active sites of CypA and CypB are nearly identical (*58*). There are no obvious differences in sequence or structure in the X-ray structures of both proteins in complex with CsA for any residues of the binding pocket. Even the water-mediated contacts in these complexes were found to be essentially conserved (*58*). In contrast, comparison of structural parameters of the Cyp−CsA complexes in solution showed a small difference in the NMR chemical shifts of one of the NOE peaks involving the $MeVal^{11}$ residue of CsA, which occupies the prolyl binding pocket of the cyclophilins. Despite the strong similarities in the X-ray structures of the active sites of CypA and CypB, the catalytic efficiency of CypB is ~2.5-fold lower than that of CypA for the same tetrapeptide model substrate (*59*) (Table 1). This suggests that for both cyclophilins the active sites in the vicinity of the prolyl binding sites might exhibit functionally significant differences, probably in the transition state of catalysis (*60*). It follows that the discriminative power of compound **1** can result from the fine-tuning of the contacts at the P1−S1 subsite. Prolyl *cis*−*trans* isomerizations

undergo two dramatic steric changes due to the loss of the pseudo-double bond character of the peptide bond in the transition state: (a) adopting the perpendicular arrangement of the carbonyl group and the proline ring and (b) distortion of the proline nitrogen atom hybridization in direction of sp^3. Obviously, the degree of steric change in the cyclophilin-catalyzed reaction depends on the transition state structure, and any slight change in these parameters might alter the transition state binding of substrates or inhibitors. We hypothesize that the state of hybridization of the C1 atom of the indanyl residue of compound **1**, which resembles the hybridization pattern of the proline nitrogen in the transition state, is an especially sensitive parameter of transition state binding, and thus inhibitory power and selectivity factors of aryl 1-indanylketones. By examining Pin1 inhibitor constants using a panel of substrate-like compounds, a six-membered pipecolinyl residue was found to be far superior to the five-membered prolyl residue (*64*). Notably, the X-ray structures of two Pin1−pipecolinyl inhibitor complexes support the role of nitrogen hybridization for inhibition power (*65*).

Binding of compound **1** to the active site of CypA when measured by direct ITC of the components did not allow reliable determination of the thermodynamic parameters. Calculation of the thermodynamic parameter of this interaction using an ITC-based competition assay according to Zhang and Zhang (*49*) revealed enthalpic as well as entropic contributions to the free energy of ligand binding. The positive $T\Delta S$ calculated for the binding of compound **1** to the CypA active site indicates entropically favored binding. Favorable entropy changes are generally attributed to hydrophobic interactions, to an increase in solvent entropy from burial of hydrophobic groups, and to release of water upon binding (*51*). The favorable enthalpic term indicates that hydrogen bond formation or van der Waals interactions take place. Interestingly, polar substituents of the biaryl group were necessary to mediate the specific nanomolar interaction with CypA. Since hydrophobic interactions are generally considered to be inherently nonspecific, selectivity may be attained by shape complementarity of spatially separated polar groups (*61*).

The presence of specific polar substituents in compound **1** could also induce transition state complementarity of the extended hydrophobic binding patches of the aromatic rings via indirect effects on the indanyl C1 atom hybridization. In contrast, the less selective but potent binding of CsA to cyclophilins is mainly mediated through conserved hydrogen bonds within the cyclophilin family for which W121 of CypA, for example, provides a directing template. Direct hydrophobic interactions contribute to a smaller degree to the total binding energy (*45*). Thus, as revealed by ITC, formation of the CypA−CsA complex is completely enthalpically driven, and CsA binding is associated with unfavorable entropy. The finding that different ligands can bind by enthalpy- or entropy-driven mechanisms has been described in the case of other protein−ligand interactions. For example, the binding of biotin to streptavidin is entirely enthalpy driven, whereas the binding of azobenzene ligands to the same protein is entropically favored (*62*). Similarly, the development of HIV-1 protease inhibitors had demonstrated either enthalpically or entropically driven ligand binding against the same binding cavity (*63*).

Both extracellular CypA and CypB can function as chemotactic agents for leukocytes (*26*, *28*, *29*), with CD147 being the principal signaling receptor for this function (*28*, *29*). Elevated levels of extracellular cyclophilins have been observed in many inflammatory diseases, including severe sepsis, rheumatoid arthritis, and vascular smooth muscle cell disease (*22*−*24*). The application of compound **1** specifically inhibited the chemotaxis induced by CypA, whereas the CypB-induced chemotaxis was not influenced by the compound (Figure 5). The fact that cell migration is not impacted in the absence of exogenously provided CypA (for example, when using RANTES) demonstrates that only CypA is a target for compound **1** in these assays. These findings also suggest that inhibition of endogenous Pin1 by compound **1** has no effect on the chemotaxis of activated $CD4^+$ T cells. Alternatively, the inhibitor cannot reach the particular fraction of Pin1 required for mediating an effect.

In conclusion, we have shown that aryl 1-indanylketone **1** selectively blocks the catalytic activity of CypA without interfering with the PPIase activity of CypB. This inhibition inhibits the chemotactic function of CypA for $CD4^+$ T cells. These results emphasize the specificity of compound **1** for CypA in a physiological context and support the use of this compound as a biological probe in analyzing and dissecting CypA- and CypB-dependent pathways.

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