# Discovering novel chemical inhibitors of human cyclophilin A: Virtual screening, synthesis, and bioassay

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**Abstract**—Cyclophilin A (CypA) is a member of cyclophilins, a family of the highly homologous peptidyl prolyl *cis–trans* isomerases (PPIases), which can bind to cyclosporin A (CsA). CypA plays critical roles in various biological processes, including protein folding, assembly, transportation, regulation of neuron growth, and HIV replication. The discovery of CypA inhibitor is now of a great special interest in the treatment of immunological disorders. In this study, a series of novel small molecular CypA inhibitors have been discovered by using structure-based virtual screening in conjunction with chemical synthesis and bioassay. The SPECS_1 database containing 85,000 small molecular compounds was searched by virtual screening against the crystal structure of human CypA. After SPR-based binding affinity assay, 15 compounds were found to show binding affinities to CypA at submicro-molar or micro-molar level (compounds **1–15**). Seven compounds were selected as the starting point for the further structure modification in considering binding activity, synthesis difficulty, and structure similarity. We thus synthesized 40 new small molecular compounds (**1–6**, **15**, **16a–q**, **17a–d**, and **18a–l**), and four of which (compounds **16b**, **16h**, **16k**, and **18g**) showed high CypA PPIase inhibition activities with IC$_{50}$s of 2.5–6.2 μM. Pharmacological assay indicated that these four compounds demonstrated somewhat inhibition activities against the proliferation of spleen cells.
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## 1. Introduction

Cyclophilins were discovered originally for their high affinity against cyclosporin A (CsA), an immunosuppressive drug used to prevent allograft rejection.[1] The general biological function of cyclophilins is to catalyze the *cis–trans* isomerization of peptide bonds preceding proline in protein and peptides.[2] Of the 15 known human cyclophilins, cyclophilin A (hCypA) is one of the most important members and has been widely studied for mapping its biological functions.[3] In addition to a binding target of CsA, hCypA is able to enhance the rate of folding (or unfolding) of proteins via its peptidyl-prolyl isomerase (PPIase) activity.[4] hCypA performs an essential function in HIV-1 replication by binding specifically with the capsid domain (CA) of the Gag polyprotein.[5] hCypA may also render neuroprotective/neurotrophic effects[6] when presented at high levels in the brain.[7] Recently, it was discovered that the nucleocapsid (N) protein of SARS coronavirus (SARS-CoV) can bind to hCyPA, which may be associated with SARS-CoV infection.[8] CypA has attracted considerable attention not only for its novel catalytic activity, but also for its therapeutic significance. It is reported that CypA–CsA binding might inhibit the serine/threonine phosphatase activity of calcineurin, thereby blocking the production of cytokines including interleukin-2[9] and interferon γ.[10]

The discovery of CsA more than two decades ago heralded a new era in the field of organ transplantation. Since then, the number of transplanted organs has grown continuously and the search for novel immuno-

suppressants has intensified.[11] However, inhibitors of CypA are mainly derived from the natural sources (such as FK506,[12] rapamycin,[13] and sanglifehrin A[14]) and peptide analogues,[15] which are all large molecules, and little has been reported regarding the small molecule CypA inhibitors.

Although immunosuppressants like CsA have improved the rates of transplant success and prolonged patient survivals, some side effects with this agent have been sequentially found such as nephrotoxicity,[16] hypertension[17], and cardiotoxicity.[18] This thereby prompted us to discover novel small molecule CypA inhibitors in order to decrease the side effects. Structure-based ligand design has led to the identification of compounds that are currently in clinical trials or into the market.[19] The wealth of structural and functional information of CypA[15] has offered a solid starting point for the rational structure-based design of inhibitors. By using docking-based virtual screening approach in conjunction with surface plasmon resonance (SPR) determination, 15 novel small molecule CypA binders (hits) (compounds **1–15**) have been discovered. According to the binding potency, structural similarity, and synthetic complexity, seven hits (compounds **1–6** and **15**) were selected as the starting points for further structural optimization. Totally, 40 new compounds including **1–6** and **15** (the preparation methods for these seven compounds have not been reported) and their 33 analogues (**16a–q**, **17a–d**, and **18a–l**) have been synthesized and tested with biological assay. Finally, four compounds (**16b**, **16h**, **16k**, and **18g**) were found to show high CypA PPIase (peptidyl-prolyl isomerase) inhibition activities and one compound (**16h**) is active in inhibiting the proliferation of spleen cells.

## 2. Materials and methods

### 2.1. Small molecular database for virtual screening

SPECS database contains the structural information of 280,000 small molecules (http://www.specs.net). SPECS Company supplies all the compound samples collected from difference sources. First, SPECS database was evaluated using our own filter of druglikeness.[20] Non-druglike molecules were removed from the database, and finally 85,000 potentially druglike molecules were selected out for docking screening. These molecules were saved in the SPECS_1 database.

### 2.2. Virtual screening by molecular docking

The crystal structure of CypA in complex with sanglifehrin macrolide (SFM) (PDB entry 1NMK)[14] recovered from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb) was used as a target for virtual screening on the SPECS_1 database (http://www.specs.net). In the present study, the DOCK4.0 program[21] was employed for the primary screening. Residues of CypA around SFM at a radius of 6.5 Å were isolated for constructing the grids of the docking screening. The resulting substructure included all residues of the binding

pocket. During the docking calculation, Kollman-all-atom charges[22] were assigned to the protein and Gasteiger–Marsili partial charges[23] were assigned to the small molecules in the SPECS_1 database. Conformational flexibility of the compounds from the database was considered in the docking search.

The DOCK suite of programs is designed to find possible orientations of a ligand in a 'receptor' site.[21] The orientation of a ligand is evaluated with a shape scoring function and/or a function approximating the ligand–receptor binding energy. The shape scoring function is an empirical function resembling the van der Waals attractive energy. The ligand–receptor binding energy is taken to be approximately the sum of the van der Waals and electrostatic interaction energies. After the initial orientation and scoring evaluation, a grid-based rigid body minimization is carried out for the ligand to locate the nearest local energy minimum within the receptor binding site. The position and conformation of each docked molecule were optimized using single anchor search and torsion minimization method of DOCK4.0.[21] Thirty configurations per ligand building a cycle and 50 maximum anchor orientations were used in the anchor-first docking algorithm. All docked configurations were energy minimized using 100 maximum iterations and 1 minimization cycle.

The 5000 molecules with the highest score as obtained by DOCK search were re-scored by using the Consensus Score (CScore)[24] method encoded in Sybyl6.8.[25] The binding free energies and the binding modes of the top 1301 molecules with CScore ⩾4 to CypA were calculated and modeled using the FlexX[26] program encoded in Sybyl6.8.[25] FlexX is a flexible docking method that uses an incremental construction algorithm to place ligand into an active site. By dividing the ligand into small fragments and building incrementally, the conformational flexibility of the ligand can be considered in reasonable times. The Kollman-all-atom charge[22] and Gasteiger-Marsili partial charge[23] were used for cyclophilin A and candidate molecules, respectively, in the FlexX simulations.

Finally 82 compounds were distinguished and selected for bioassay on the basis of score by virtual screening and druglikeness analysis. The virtual screening was performed on a 128-processor SGI Origin 3800 supercomputer.

### 2.3. Chemistry

**2.3.1. Design of analogues of compounds 1–6, 15.** Compounds **1–6** and **15** (Fig. 1) bearing the highest binding affinities with CypA as obtained by the surface plasmon resonance (SPR) determination (see Section 4) for the 82 candidate compounds selected by virtual screening were used as lead compounds for designing new CypA inhibitors. Based on the structural and enzyme binding activity features of compounds **1–6** and **15**, 33 new analogues (**16a–q**, **17a–d**, and **18a–l**) (Tables 1 and 2) were designed and synthesized for the first round. Keeping the key groups of compounds **1–4**—2-substituted quinoline ring

and sulfanilamide moiety—we used various setric, electronic, and hydrophobic groups to substitute at position 2 of the quinoline ring in compounds **1**–**4** (Fig. 1) and obtained 10 analogues (**16a**–**j**) (Table 1). Replacing the quinoline ring with a pyridine ring, we obtained seven compounds (**16k**–**q**) (Table 1). Keeping the key groups of compounds **5** and **6**—phenyl alkyl acylamide and sulfanilamide moiety—we changed the length of the alkyl linkers, coupled with the sulfisoxazole of compound **1**, and obtained four compounds (**17a**–**d**) (Table 1). On the basis of the structural features of compound **15**, we designed compounds **18a**–**j** (Table 2), maintained dibenzoic acid or dibenzoic acid ester moiety, and replaced 3,5-dimethoxybenzamido group in compound **15** with other electronic and hydrophobic substituted benzamido groups. Substituting the methylene group of compound **15** with an ethylene group, we obtained compounds **18k**–**l** (Table 2).

**2.3.2. Synthetic procedures.** Scheme 1 depicts the sequence of reactions that led to the preparation of compounds **1**–**4** and **16a**–**j** using isatins (**19**) as the starting material. In general, the substituted 4-quinolinecarboxy-lic acids **20** were prepared by isatins (**19**) condensation with ketones in ethanol. Compound **20** was converted to the corresponding acyl chlorides (**21**) by refluxing with SOCl$_2$.[27] Then **21** was substituted by using different sulfanilamides, giving the target compounds **1**–**4** and **16a**–**j** (Table 1). The key intermediate **25** required by synthesizing compound **16j** was prepared from compound **23**[28] by sulfamation and deprotection.

Compounds **16k**–**q** were synthesized through the route outlined in Scheme 2. 2-Bromo-1-arylethanone (**26**) was reacted by refluxing with ammonium formate in 98% HCOOH, which afforded 4-aryloxazole (**27**).[29] Compound **28** was synthesized using the Usui Y. method[30] by compound **27** reacting with maleic acid at 110 °C for 15 min. Then, the target compounds **16k**–**q** were obtained after steps c–d of Scheme 1 were employed (Table 1).

Compounds **5**–**6** and **17a**–**d** were synthesized by using the same process as compounds **1**–**4**, except compound **20** was replaced by compound **29**. The synthetic route is outlined in Scheme 3.
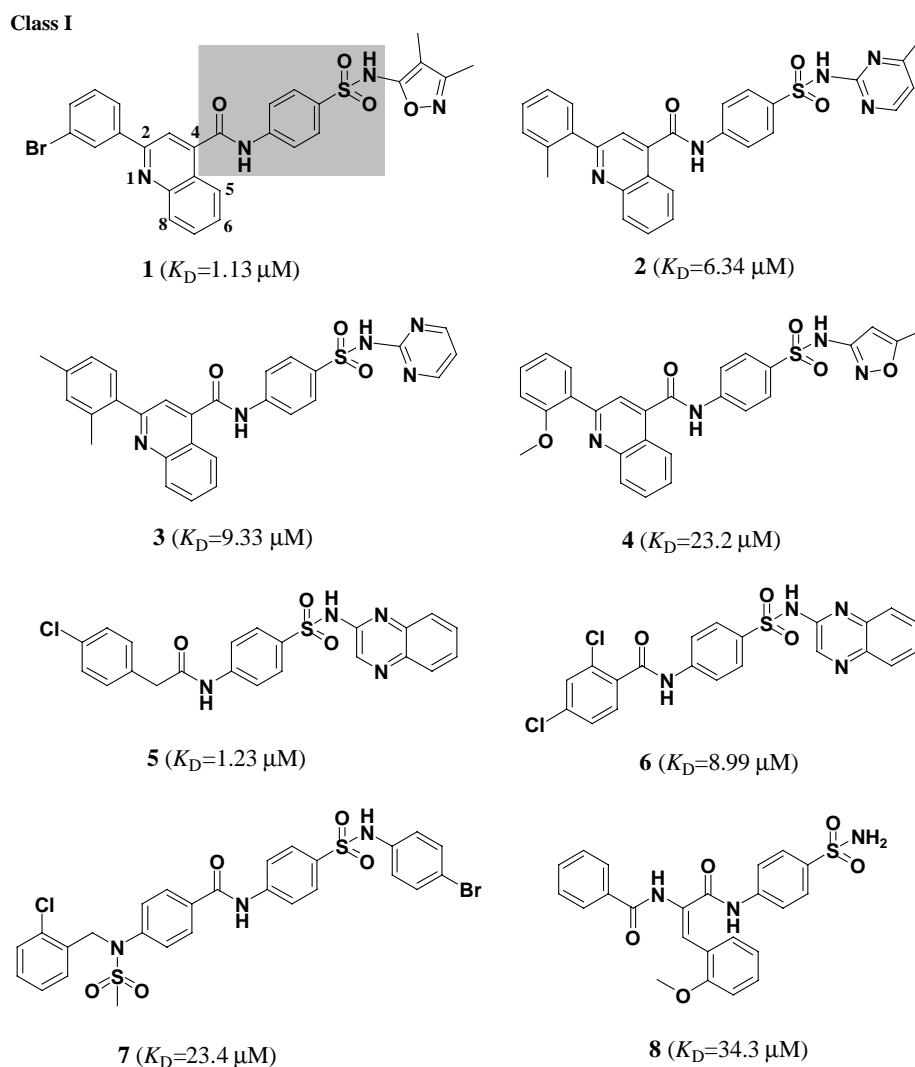


**Figure 1.** The structures of 15 binders (or hits) of CypA selected from the candidates by virtual screening and SPR-based binding affinity assay.
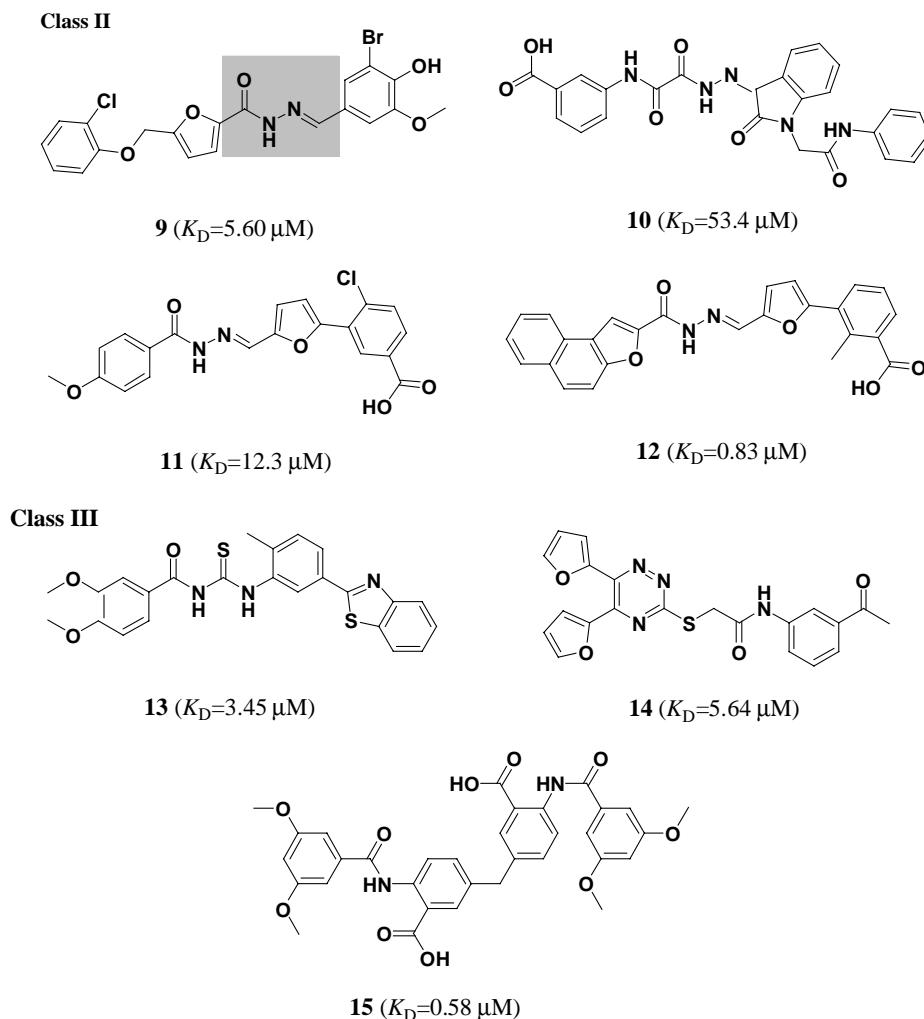
**Class II**



**9** ($K_D$=5.60 μM)



**10** ($K_D$=53.4 μM)



**11** ($K_D$=12.3 μM)



**12** ($K_D$=0.83 μM)

**Class III**



**13** ($K_D$=3.45 μM)



**14** ($K_D$=5.64 μM)



**15** ($K_D$=0.58 μM)

**Figure 1.** *(continued)*

Scheme 4 depicts the sequence of reactions that led to the preparation of compounds **15** and **18a–i** using anthranilic acid (**30**) as the starting material. Compound **31a**[31] was synthesized by heating anthranilic acid (**30**) with HCHO in 36% HCl. Refluxing **31a** with saturated HCl in methanol afforded compound **31b**. Acylation **31a–b** with various substituted benzoyl chlorides in pyridine afforded compounds **15** and **18a–f**. Compound **31b** reacted with benzyl bromide in refluxing NMP produced compound **18g**, which was hydrolyzed using LiOH at 25 and 80 °C to afford monoester **18h** and dicarboxylic acid **18i**, respectively.

Compounds **18j–l** were synthesized through the route outlined in Scheme 5. Refluxing 5-methyl-2-nitrobenzoic acid (**32**) with saturated HCl in methanol afforded compound **33**, which was then brominated by NBS in chloroform to afford compound **34**.[32] Compound **34** was coupled with compound **33**, giving the key intermediate **35** in the presence of *n*-BuLi at −78 °C, which was then reduced by Pd-C. Compound **18j** was obtained by N-acylation of **37** with 3,5-dimethoxybenzoyl chloride in pyridine. Compound **18k** was obtained under the same conditions as compound **18a**, and compound **18l** was quantitatively obtained by hydrolysis of **18k** using LiOH at room temperature.

### 2.4. Biological assay

#### 2.4.1. Binding assay.
CypA was expressed and purified according to our previously reported procedure.[8] The binding affinities of the virtual screening candidates to CypA in vitro were determined by employing the surface plasmon resonance (SPR) technology. The measurement was performed using the dual flow cell Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). Immobilization of the CypA to the hydrophilic carboxymethylated dextran matrix of the sensor chip CM5 (Biacore) was carried out by the standard primary amine coupling reaction wizard. The CypA to be covalently bound to the matrix was diluted in 10 mM sodium acetate buffer (pH 6.0) to a final concentration of 0.035 mg/mL. Equilibration of the baseline was completed by a continuous flow of HBS-EP running buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20, pH 7.4) through the chip for 1–2 h. All the Biacore data were collected at 25 °C with HBS-EP as running buffer at a constant flow rate of 30 μL/min. All the sensorgrams were processed by using automatic correction for non-specific bulk refractive index effects. The equilibrium constants ($K_D$ values) evaluating the protein–ligand binding affinities were determined by the steady state affinity fitting analysis of the Biacore data.

**Table 1.** Chemical structures of compounds **1–6** and **16–17**, and their activities



I

| Compound | R$_9$ | R$_3$ | $K_D$[a] (µM) | % inhibition at 10 µM[b] |
|---|---|---|---|---|
| **1** | | 3,4-Dimethyl-5-isoxazolyl | 1.13 | 3.20 |
| **2** | | 4-Methyl-2-pyrimidinyl | 6.34 | 11.2 |
| **3** | | 2-Pyrimidinyl | 9.33 | 10.2 |
| **4** | | 5-Methyl-3-oxazolyl | 23.2 | 26.5 |
| **16a** | | 3,4-Dimethyl-5-isoxazolyl | 10.2 | 18.0 |
| **16b** | | 3,4-Dimethyl-5-isoxazolyl | 52.3 | 67.3 |
| **16c** | | 3,4-Dimethyl-5-isoxazolyl | 4.11 | 10.0 |
| **16d** | | 3,4-Dimethyl-5-isoxazolyl | 223 | 15.0 |
| **16e** | | 3,4-Dimethyl-5-isoxazolyl | 10.6 | 40.0 |
| **16f** | | 3,4-Dimethyl-5-isoxazolyl | 91.3 | 30.0 |
| **16g** | | 3,4-Dimethyl-5-isoxazolyl | 1.79 | NA |
| **16h** | | 3,4-Dimethyl-5-isoxazolyl | 10.1 | 77.4 |
| **16i** | | 3,4-Dimethyl-5-isoxazolyl | 2.25 | 32.0 |
| **16j** | | 2-Quinoxalinyl | 3.13 | 36.0 |
| **16k** | | 3,4-Dimethyl-5-isoxazolyl | 46.0 | 74.7 |
| **16l** | | 3,4-Dimethyl-5-isoxazolyl | 89.5 | NA |

**Table 1** (*continued*)

| Compound | R_9 | R_3 | $K_D{}^a$ (μM) | % inhibition at 10 μM[b] |
|---|---|---|---|---|
| 16m | (4-methyl-2-phenylpyridinyl) | 3,4-Dimethyl-5-isoxazolyl | 14.5 | NA |
| 16n | (4-methyl-2-(4-chlorophenyl)pyridinyl) | 3,4-Dimethyl-5-isoxazolyl | 1.86 | NA |
| 16o | (4-methyl-2-(4-fluorophenyl)pyridinyl) | 3,4-Dimethyl-5-isoxazolyl | 42.4 | 26.0 |
| 16p | (4-methyl-2-(2-methylphenyl)pyridinyl) | 3,4-Dimethyl-5-isoxazolyl | 7.61 | NA |
| 16q | (4-methyl-2-(2-methylphenyl)pyridinyl) | 4-Methyl-2-pyrimidinyl | 340 | NA |
| 5 | (4-chlorophenylacetone) | 2-Quinoxalinyl | 1.23 | 3.2 |
| 6 | (2,4-dichloroacetophenone) | 2-Quinoxalinyl | 8.99 | 7.5 |
| 17a | (2,4-dichlorophenylacetone) | 2-Quinoxalinyl | 37.7 | NA |
| 17b | (acetophenone) | 2-Quinoxalinyl | 23.4 | 5.6 |
| 17c | (4-phenyl-2-butanone) | 2-Quinoxalinyl | 1.06 | NA |
| 17d | (4-chlorophenylacetone) | 3,4-Dimethyl-5-isoxazolyl | 0.35 | NA |

[a] Data obtained from SPR-based binding affinity assay. $K_D$ represents the equilibrium constant.
[b] Data are means of three independent experiments.

**Table 2.** Chemical structures of compounds **15** and **18** and their activities



III

| Compound | n | R_10 | R_11 | R_12 | $K_D{}^a$ (μM) | % inhibition at 10 μM[b] |
|---|---|---|---|---|---|---|
| 15 | 1 | COOH | COOH | 3,5-Dimethoxy-benzoyl | 0.58 | 12.4 |
| 18a | 1 | CO_2CH_3 | CO_2CH_3 | 3,5-Dimethoxy-benzoyl | NA | NA |
| 18b | 1 | COOH | COOH | Benzoyl | NA[c] | NA[c] |
| 18c | 1 | CO_2CH_3 | CO_2CH_3 | Benzoyl | 32.3 | 11.2 |
| 18d | 1 | COOH | COOH | 4-Methoxy-benzoyl | NA[c] | NA[c] |
| 18e | 1 | CO_2CH_3 | CO_2CH_3 | 4-Methoxy-benzoyl | NA | NA |
| 18f | 1 | CO_2CH_3 | CO_2CH_3 | 4-Cl-Benzoyl | 140 | 10.2 |
| 18g | 1 | CO_2CH_3 | CO_2CH_3 | Benzyl | 414 | 86.0 |
| 18h | 1 | COOH | CO_2CH_3 | Benzyl | 0.23 | 6.5 |
| 18i | 1 | COOH | COOH | Benzyl | 184 | NA |
| 18j | 1 | — | — | 3,5-Dimethoxy-benzoyl | 3.3 | 43.0 |
| 18k | 2 | CO_2CH_3 | CO_2CH_3 | 3,5-Dimethoxy-benzoyl | 87.5 | 15.6 |
| 18l | 2 | COOH | COOH | 3,5-Dimethoxy-benzoyl | NA[c] | NA[c] |

[a] Data obtained from SPR-based binding affinity assay. $K_D$ represents the equilibrium constant.
[b] Data are means of three independent experiments.
[c] These compounds cannot be assayed for poor solubility in DMSO.
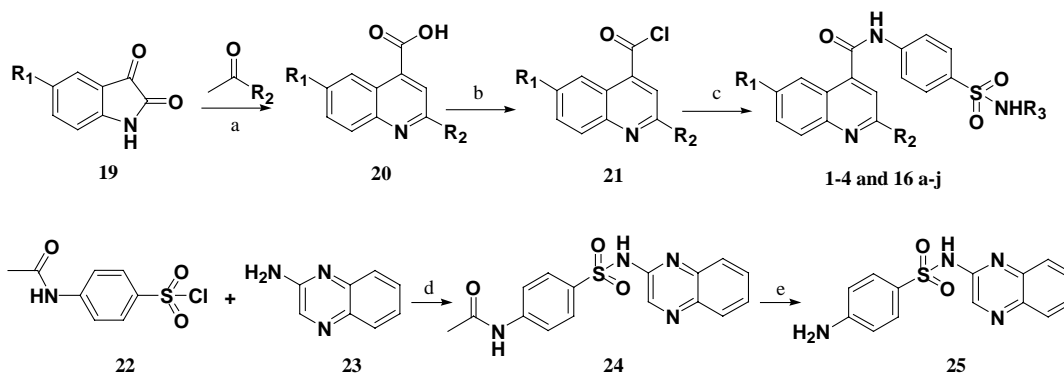
**Scheme 1.** Reagents and conditions: (a) (i) KOH, $C_2H_5OH$, $H_2O$, reflux, (ii) HCl, $H_2O$; (b) $SOCl_2$, reflux; (c) $R_3NHSO_2C_6H_6NH_2$-*p*, pyridine, 25 °C; (d) pyridine, 25 °C; (e) (i) NaOH, $H_2O$, reflux, (ii) HCl.
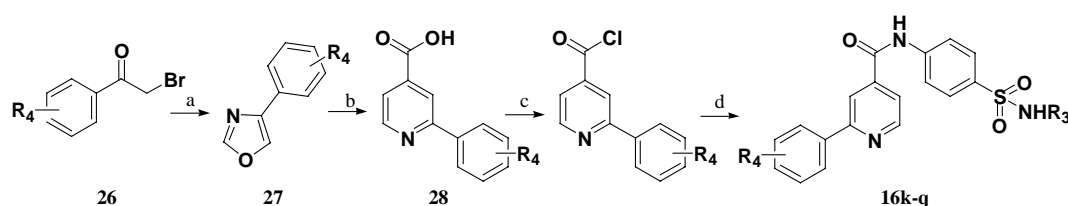


**Scheme 2.** Reagents and conditions: (a) HCOOH (98%), $HCOONH_4$, reflux; (b) maleic acid, 110 °C; (c) $SOCl_2$, reflux; (d) $R_3NHSO_2C_6H_6NH_2$-*p*, pyridine, 25 °C.
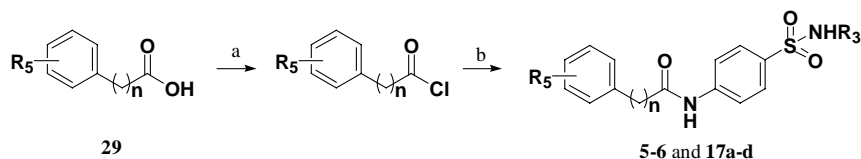


**Scheme 3.** Reagents and conditions: (a) $SOCl_2$, reflux; (b) $R_3NHSO_2C_6H_6NH_2$-*p*, pyridine, 25 °C.
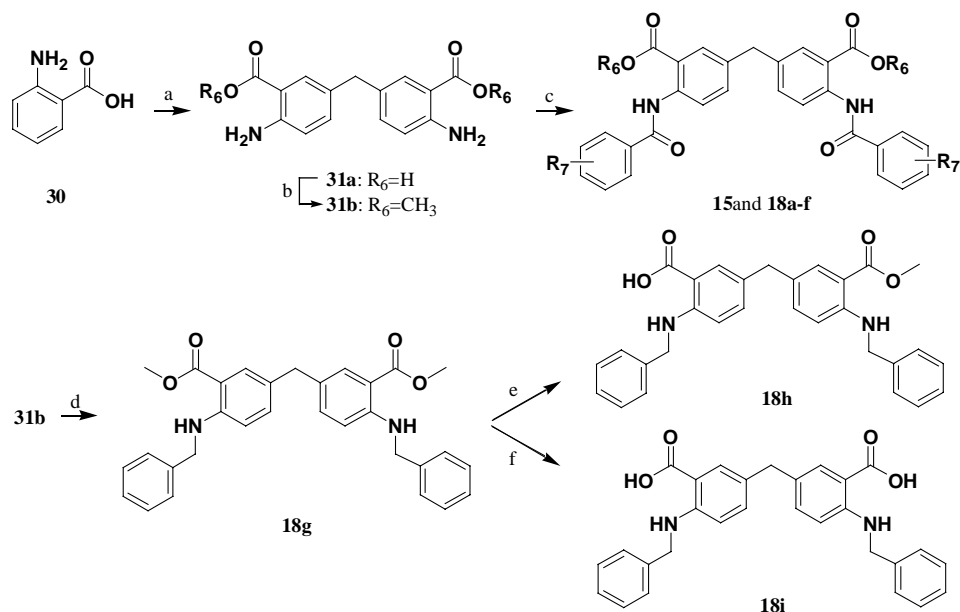


**Scheme 4.** Reagents and conditions: (a) 3% HCHO, HCl, $H_2O$; (b) $CH_3OH$, HCl; (c) substituted benzoyl chlorides, pyridine, 25 °C; (d) benzyl bromide, NMP, $Na_2CO_3$, $H_2O$; (e) LiOH, $THF/CH_3OH/H_2O$ = 3:1:1, 25 °C; (f) LiOH, $THF/CH_3OH/H_2O$ = 3:1:1, 80 °C.
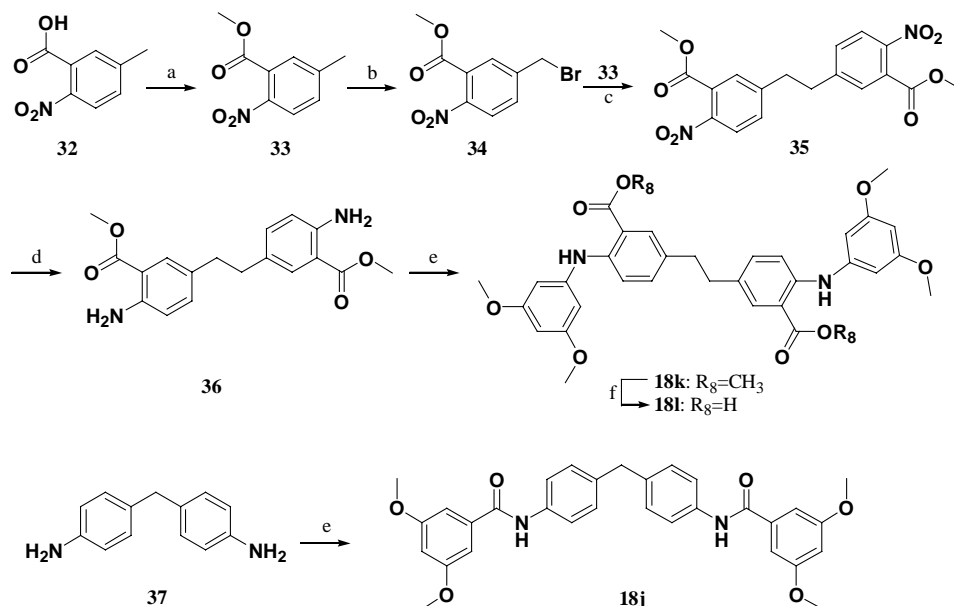
**Scheme 5.** Reagents and conditions: (a) $CH_3OH$, HCl; (b) NBS, $CHCl_3$, BzooBz; (c) *n*-BuLi, THF, $-78$ °C; (d) Pd-C, $H_2$, THF; (e) 3,5-dimethoxybenzoyl chloride, pyridine, 25 °C; (f) LiOH, $THF/CH_3OH/H_2O = 3:1:1$, 25 °C.

**2.4.2. Enzymatic activity assay.** CypA PPIase activity was measured at 4 °C by using the standard chymotrypsin-coupled assay.[33] The assay buffer (50 mM Hepes, 100 mM NaCl, pH 8.0) and CypA (500 nM stock solution) were pre-cooled to 4 °C, to which then was added 15 μL of 3 mg/mL chymotrypsin in 1 mM HCl. The reaction was initiated by adding 12 μL of 3.8 mM peptide substrate (Suc-Ala-Ala-cis-Pro-Phe–*p*NA) in LiCl/THF solution with rapid inversion. After a delay from the onset of mixing (usually 6 s), the absorbance of *p*-nitroaniline was followed at 390 nM until the reaction was complete (1 min). The final concentration of LiCl in the assay was 9.6 mM; THF was present at a concentration of 2% (v/v). Absorbance readings were collected every 0.1 s by a U-2010 spectrophotometer. The progress curves were analyzed by non-linear least-squares fit.

The inhibition assays of compounds were performed in the same manner as mentioned above. A 0.6 μL aliquot of the compounds in DMSO was added to the CypA solution in the assay buffer. After being pre-incubated for 1 h at 4 °C, the assay was started by the addition of chymotrypsin and the substrate. To calculate the half-maximal inhibitory concentration ($IC_{50}$), the percent of remaining PPIase activity is plotted against the common logarithm of the compound concentration, and the data were fitted using the sigmoidal fitting model by the Origin7.0 software.

**2.4.3. T cell viability assay.** Female ICR strains of mice, 6–8 weeks old and $20 \pm 2$ g, were purchased from the Experimental Animal House of China Pharmaceutical University (Nanjing, China) and were maintained in plastic cages with free access to pellet food and water (12 h light/dark cycle, $21 \pm 2$ °C). This study complied with the current ethical regulations on animal research in Nanjing University and the mice used were treated humanely.

The spleen was aseptically taken from mice, crushed gently, and separated into single cells by squeezing in 5 mL D-Hanks' solution (Gibco-BRL). The cells obtained were passed through eight layers of gauze and centrifuged at 1000 rev/min for 5 min at 4 °C. Pellets were added into 10 mL of sterile 0.17 M Tris-(hydroxymethyl) aminomethane containing 0.75% $NH_4Cl$ (pH 7.5) followed by centrifugation to remove erythrocytes. After washing twice with RPMI 1640 containing 100 U $mL^{-1}$ penicillin, 100 U $mL^{-1}$ streptomycin, and 10% fetal calf serum (FCS) (RPMI 1640 medium), they were re-suspended in the RPMI 1640 medium and used for cell culture.

Spleen cells were cultured in 96-well flat-bottomed microplates (Falcon) at a density of $5 \times 10^5$ cells per well in RPMI 1640 medium (0.2 mL) and stimulated with 5 μg $mL^{-1}$ Con A for 72 h at 37 °C in 5% $CO_2$-air in the presence or absence (control group) of various concentrations of compounds. Then the cell growth was evaluated with modified MTT assay.[34] Briefly, 20 μL of 5 mg $mL^{-1}$ MTT in RPMI 1640 was added for a further 4 h incubation. After removing the supernatant, 200 μL of DMSO was added to dissolve the formazan crystals. The plate was shaken for 10 min and then read on an ELISA reader (Sunrise Remote/Touch Screen; TECAN, Austria) at 540 nm. All assays were run in triplicate and the effect of compounds on the proliferation of mouse spleen cells induced by Con A was calculated by Eq. 1

$$\text{Inhibitory rate } (\%) = (\text{control } (OD_{540}) - \text{compounds } (OD_{540})) / \text{control } (OD_{540}). \quad (1)$$

Data were expressed as means ± SD (significance of differences between groups). Statistical analysis was evaluated by one-way analysis of variance, followed by Student's two-tailed *t* test for the evaluation of the differ-

ence between two groups and Dunnett's *t* test between control group and multiple dose groups. One-way analysis of variance revealed a significant effect at *P* < 0.05.

## 3. Results and discussion

### 3.1. Identification of binders (hits) by virtual screening

Targeting the crystal structure of CypA (PDB entry 1NMK),[14] we searched the SPECS_1 database by using DOCK4.0.[21] The small molecules were ranked according to their scores calculated by using the scoring function of DOCK. The top 5000 candidate molecules were obtained with the best scores by a shape complementarity scoring function in DOCK.[21] These compounds were re-estimated using Cscore.[24] A total of 1301 compounds with Cscore = 4 or 5 (the best score of Cscore is 5) were subsequently docked and ranked using the FlexX[26] program. Finally, 82 compounds were selected according to the score of FlexX for biological assay. Because enzymatic assay is time-consuming, therefore, surface plasmon resonance (SPR) measurements were used for the primary screening, determining the binding affinity of these 82 candidate molecules to CypA. Immobilization of CypA resulted in a resonance signal at about 5370 resonance units (RUs). Among the 82 compounds, the biosensor RUs of 15 compounds were concentration-dependent. The collected data indicated that these 15 compounds can bind to CypA in vitro and the binding affinities to cyclophilin A are in the submicro- or micro-molar range ($K_D$ = 53.4–0.58 μM). These compounds could be designated as binders (or hits) of CypA, their chemical structures and binding affinities shown in Figure 1.
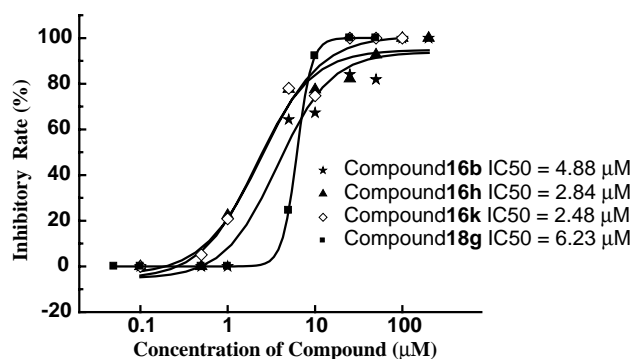


**Figure 2.** Concentration dependence of PPIase activity by **16b**, **16h**, **16k**, and **18g**, the concentration of CyPA was kept constant at 25 nM, while the concentration of compounds ranged from 0.1 to 200 μM.

### 3.2. Analogue design and synthesis

Structurally, the 15 hits can be divided into three classes: class I includes eight compounds containing sulfanilamide moiety (compounds **1**–**8**); class II compounds are acylhydrazone derivatives (compounds **9**–**12**); class III is comprised by compounds **13**–**15**, which have no distinct structural character (Fig. 1). Considering the structural similarity and the binding affinity with CypA, we took compounds **1**–**6** in class I and compound **15** in class III as leaders for structural optimization. Totally, 40 compounds (**1**–**6**, **15**, **16a**–**q**, **17a**–**d**, and **18a**–**l**) were designed and synthesized, and their chemical structures are shown in Tables 1 and 2. These compounds were synthesized through the routes outlined in Schemes 1–5, and the details for synthetic procedures and structural characterizations are described in the Experimental section.

### 3.3. Biological activities

**3.3.1. The PPIase activity of CypA.** For the primary assay, the percent inhibitions of the compounds at 10 μM were measured. The results are listed in Tables 1 and 2. Four compounds, i.e., **16b**, **16h**, **16k**, and **18g**, can remarkably inhibit the PPIase activity of CyPA (percent inhibition at 10 μM > 50%), indicating that these four compounds are CypA inhibitors. Therefore, we determined their $IC_{50}$ values, which are, respectively, 4.88, 2.84, 2.48, and 6.23 μM (Fig. 2). In addition, the binding affinities of the 33 analogues were determined by using the SPR technology, the results also listed in Tables 1 and 2. The PPIase activities of the compounds do not consistently correlate with the SPR binding affinities. The reason may be that the protein was immobilized to a sensor chip in the SPR assay, which affects the conformational flexibility of the protein. Accordingly, good binders identified by the SPR assay may not be good inhibitors. To find CypA inhibitors rather than binders, the effects of compounds on the PPIase activity of CypA have to be determined.

**3.3.2. Inhibition activity against the proliferation of spleen cells.** To test the immunosuppressive effects of **16a**, **16h**, **16k**, and **18g**, anti-proliferation activities on spleen cells in vitro of the four inhibitors have been evaluated. The result is listed in Table 3, indicating that compounds **16a**, **16h**, and **16k** show partial influence on the viability of the naïve spleen cells. It is remarkable that the effective concentration of compound **16h** is ~10 μM in inhibition of the proliferation of mouse spleen cells induced by Con A, which is slightly lower than that of CsA. This indicates that compound **16h** is a good lead for designing more potent immunosuppressive agents.

**Table 3.** Effect of compounds **16b**, **16h**, **16k**, **18l**, and CsA on the proliferation of mouse spleen cells induced by Con A in vitro

| Dose (μM) | Inhibitory rate (%)[a] | | | | |
|---|---|---|---|---|---|
| | CsA | 16b | 16h | 16k | 18g |
| 100 | 84.31 ± 4.88[b] | 47.35 ± 4.98[b] | 46.74 ± 5.39[b] | 49.05 ± 4.69[b] | 28.83 ± 3.39[b] |
| 10 | 82.57 ± 5.20[b] | 29.78 ± 5.00 | 34.26 ± 5.60[b] | 22.71 ± 7.06 | 7.46 ± 4.13 |
| 1 | 56.52 ± 1.60 | 14.13 ± 9.97 | 20.76 ± 8.41 | 15.33 ± 7.81 | 1.20 ± 6.41 |

[a] Each value indicates the mean ± SD of three experiments using three mice with triplicate sets in each assay.
[b] One-way analysis of variance revealed a significant effect at *P* < 0.05.
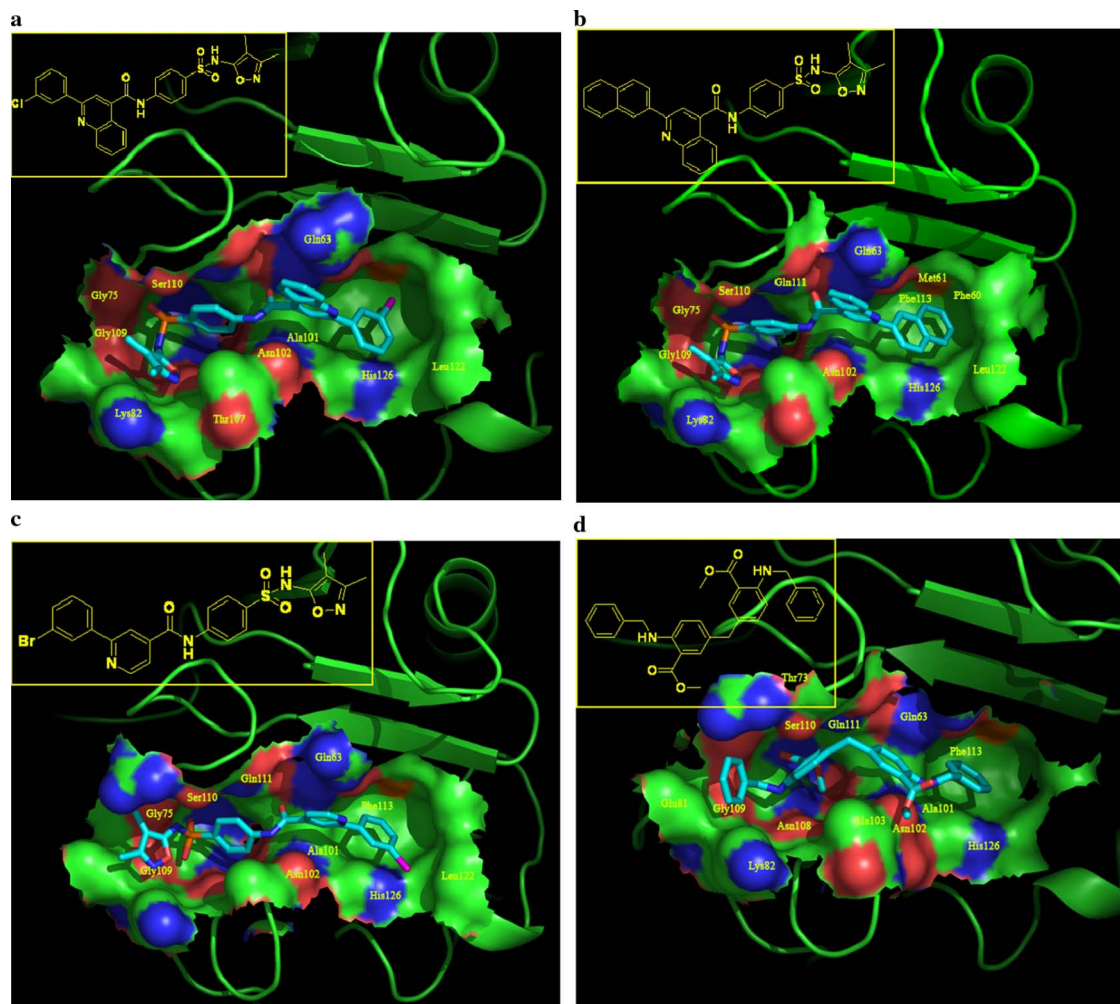
**Figure 3.** Three-dimensional structural modes of inhibitors **16b** (a), **16h** (b), **16k** (c), and **18g** (d) to CypA derived from the docking simulations. These four images were generated using the Pymol program (http://www.pymol.org/). The CypA surface was colored by electrostatic potential.

### 3.4. Binding models

To gain structural information for further structural optimization, the 3D binding models of the designed compounds to CypA were generated based on the docking simulation, Figure 3 shows the interaction models of four potent inhibitors (**16a**, **16h**, **16k**, and **18g**) to CypA. From Figure 3, one can see that there are two binding sites in the surface binding groove of CypA: site A is a small binding pocket formed by Lys82, Met100, Ala101, Asn102, Ala103, Thr107, Asn108, Gly109, Ser110, Gln111, and Phe112; near site A, there is a larger pocket, which is called site B, formed by Phe60, Met61, Gln63, Ala101, Asn102, Ala103, Phe113, Leu122, and His126; there is a 'saddle' between the two sites. The binding models (Figs. 3a–c) indicate that class I compounds (Table 1) interact with CypA in a similar way: the common sulfanilamide moiety occupies the polar binding site (site B), forming three to four hydrogen-bonds (H-bonds) with residues Gln63, Gly75, Asn102, Thr107, Ser110, and Gln111 (Fig. 4); the 2-substituents of quinoline rings (**1–4** and **16a–j**) and pyridine rings (**16k–q**) or aryl rings (**5**, **6** and **17a–d**) fill in the hydrophobic pocket (site A); and the quinoline moiety (**1–4** and **16a–j**), the

pyridine moiety (**16k–q**) or alkyl groups (**5**, **6** and **17a–d**) act as linkers interacting with the saddle part between sites A and B. The interacting models for class III compounds to CypA are mostly similar to those for class I compounds, except that compounds **15**, **18a**, **18d–f**, and **18j–l** extend from site B due to their longer linkers and/or substituents.

In summary, we have discovered four potent CypA inhibitors (**16a**, **16h**, **16k**, and **18g**) by using a structure-based virtual screening approach in conjunction with chemical synthesis and bioassay. One of them (compound **16h**) is active with very close potency to CsA in inhibiting the proliferation of spleen cells, demonstrating that this compound may be a good lead for discovering new immunosuppressive agents. Discovering small molecule CypA inhibitors is an urgent need, so the new chemical structures produced in this study are of significance.

### 4. Experimental

The reagents (chemicals) were purchased from Lancaster, Acros, and Shanghai Chemical Reagent Company,
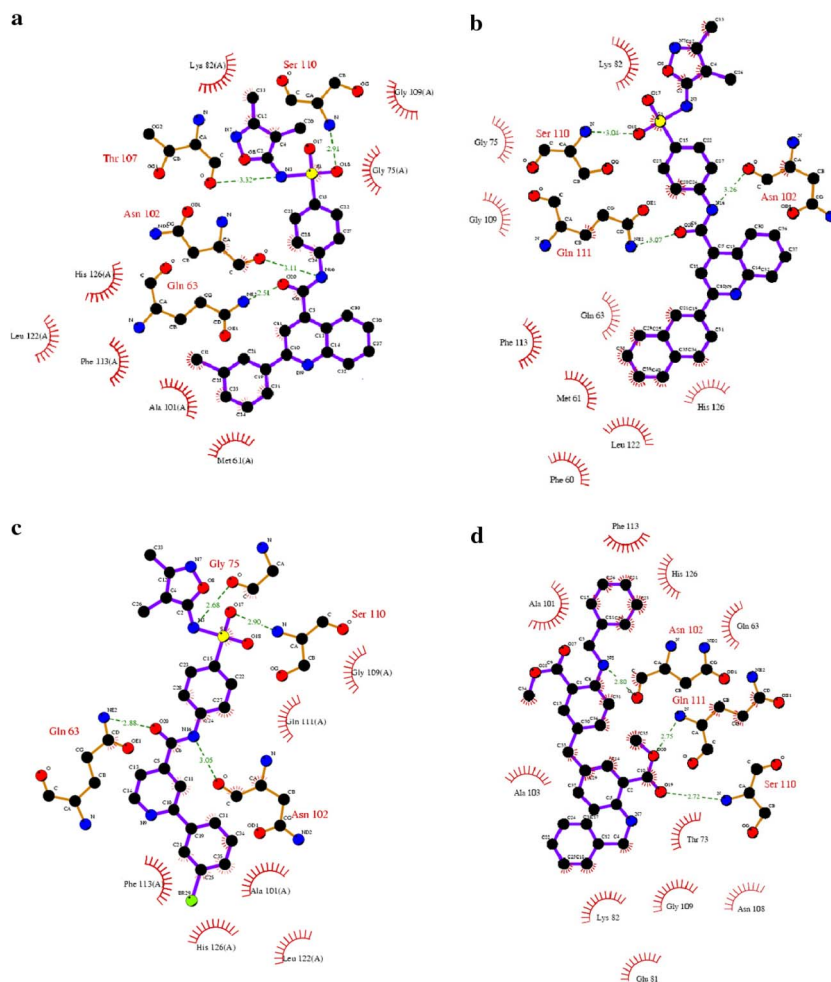
a

b

c

d

**Figure 4.** Two-dimensional representation for the interacting mode of compounds **16b** (a), **16h** (b), **16k** (c), and **18g** (d) with CypA. It is drawn using the LIGPLOT program.[35] A distance between donor and acceptor of less than 3.4 Å is considered as a hydrogen-bond, and a 4.1 Å distance between two hydrophobic atoms is considered a hydrophobic interaction.

and used without further purification. Analytical thin-layer chromatography (TLC) was HSGF 254 (150–200 μm thickness, Yantai Huiyou Company, China). Yields were not optimized. Melting points were measured in capillary tube with a SGW X-4 melting point apparatus without correction. Nuclear magnetic resonance (NMR) spectra were given on a Brucker AMX-400 NMR (IS as TMS). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane. Proton coupling patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br). Low- and high-resolution mass spectra (LRMS and HRMS) were given with electric, electrospray, and matrix-assisted laser desorption ionization (EI, ESI, and MALDI) produced by Finnigan MAT-95, LCQ-DECA spectrometer, and IonSpec 4.7 T.

### 4.1. 4-Amino-*N*-quinoxalin-2-yl benzenesulfonamide (25)

A mixture of 4-acetylamino-benzenesulfonyl chloride **22** (2.34 g, 10 mmol) and compound **15**[28] (1.45 g, 10 mmol) in pyridine (20 mL) was stirred overnight at 25 °C, poured into H$_2$O (50 mL), and extracted with EtOAc. The combined organic layer was washed, dried, filtered, and condensed. The residue was purified by flash chro-

matography on silica gel, eluted with a mixture of EtOAc/petroleum ether (1:2, v/v), to afford **24** (1.08 g, 31.6%) as a pale yellow solid; ^{1}H NMR (DMSO): δ 2.03 (s, 3H), 7.61 (t, 1H), 7.78 (m, 4H), 7.90 (d, 1H), 8.02 (d, 2H), 8.62 (s, 1H); EI-MS *m/z* 342 (M$^+$), 277 (100%).

A mixture of compound **24** (1 g, 3 mmol) and NaOH (1.5 g, 37.5 mmol) in H$_2$O (15 mL) was refluxed for 2 h to generate a yellow solution, which was acidified to pH 5.5 at 70–80 °C. After cooling to room temperature, the precipitate was collected, washed with H$_2$O, and dried, to afford compound **25** (0.8 g, 91.2%) as a yellow solid: mp 258–260 °C; EI-MS *m/z* 300 (M$^+$), 236 (100%).

### 4.2. General procedures for preparations of 20[27] are described as those for 2-(3-fluorophenyl)-quiniline-4-carboxylic acid (20a)

As illustrated in Scheme 1, isatin **19** (34.0 mmol), 3-fluoroacetophenone (40.8 mmol), and 85% KOH pellets (102 mmol) were dissolved in EtOH (40 mL), and the reaction mixture was stirred at 80 °C for 24 h. The mixture was condensed, diluted with water, and acidified with concd HCl, and the precipitate was collected,

washed, and dried to afford **20a**; yield 80%; mp 232–234 °C; ^{1}H NMR (DMSO): $\delta$ 7.33–7.42 (m, 3H), 7.55 (d, 1H), 7.75 (m, 1H), 7.87 (m, 1H), 8.02 (s, 1H), 8.13 (d, 1H), 8.71 (d, 1H); EI-MS *m/z* 266 (M[+]−1), 222 (100%).

### 4.3. General procedures for preparations of 28 are described as those for 2-phenylpyridine-4-carbonic acid (28m)

A mixture of $\alpha$-bromoacetophenone **26m** (10 g, 50 mmol), HCOONH$_4$ (11 g, 174 mmol), and 98% HCOOH (53 mL) was refluxed under stirring for 2.5 h. The reaction mixture was poured into H$_2$O, alkalinized with concentrated NaOH, and extracted with Et$_2$O. The combined organic layer was washed, dried, filtered, and condensed. The crude product obtained was purified by flash chromatography on silica gel, eluted with a mixture of EtOAc/petroleum ether (1:10, v/v), to afford **27m** (1.2 g, 16.7%) as a yellow oil; ^{1}H NMR (CDCl$_3$): $\delta$ 7.33 (d, 1H), 7.42 (d, 2H), 7.75 (d, 2H), 7.94 (m, 2H).

A mixture of 4-phenyloxazole **27m** (1 g, 6.9 mmol) and maleic acid (0.83 g, 7.1 mmol) was heated at 110 °C until melting for 2 h. The solid obtained after cooling was triturated with Et$_2$O, collected by suction filtration. The filter cake was recrystallized with 95% EtOH to yield **28m** (0.2 g, 14.6%) as a yellow solid: mp 263–264 °C; ^{1}H NMR (DMCO): $\delta$ 7.56 (m, 3H), 7.85 (d, 1H), 8.20 (d, 2H), 8.38 (s, 1H), 8.88 (d, 1H). EI-MS *m/z* 199 (M[+]) (100%), 155.

### 4.4. General procedures for preparations of 4-substituted carboxamido sulfanilamides 1–6, 16a–q, and 17a–d are described as those for 1

#### 4.4.1. 4-(2-(3-Bromophenyl)-quinoline-4-carboxamido)-N-(3,4-dimethyl-isoxazol-5-yl)benzene sulfonamide (1). A suspension of 3-bromophenyl-quinoline-4-carboxylic acid (0.33 g, 1 mmol) in redistilled SOCl$_2$ (6 mL) was refluxed gently for 3 h. A clear solution resulted. The SOCl$_2$ was then evaporated under reduced pressure. The yellow solid thus obtained was used in the next step without further purification.

A mixture of yellow solid thus obtained, sulfisoxazole (0.27 g, 1 mmol), and pyridine (5 mL) was stirred overnight at 25 °C, poured into H$_2$O (30 mL), and extracted with EtOAc. The combined organic layer was washed, dried, filtered, and condensed. The residue was purified by flash chromatography on silica gel, eluted with a mixture of EtOAc/petroleum ether (1:4, v/v), to afford **1** (120 mg, 20.8%) as a white solid: mp 254–256 °C; ^{1}H NMR (DMSO): $\delta$ 1.71 (s, 3H), 2.10 (s, 3H), 7.57 (t, 1H), 7.75 (m, 2H), 7.84 (d, 2H), 7.90 (m, 1H), 8.03 (d, 2H), 8.22 (m, 2H), 8.40 (d, 1H), 8.48 (s, 1H), 8.58 (t, 1H); EI-MS *m/z* 578 (M[+]+1), 203 (100%); HRMS (EI) *m/z* calcd C$_{27}$H$_{21}$BrN$_4$O$_4$S (M[+]) 576.0473, found 576.0467.

#### 4.4.2. 4-(2-(2-Methylphenyl)-quinoline-4-carboxamido)-N-(4-methyl-pyrimidin-2-yl) benzenesulfonamide (2). This compound was purified by flash chromatography with

EtOAc/petroleum ether (1:4, v/v), yield 31.3%: mp 291–293 °C; ^{1}H NMR (DMSO): $\delta$ 2.29 (s, 3H), 2.44 (s, 3H), 6.91 (d, 1H), 7.21 (m, 3H), 7.62 (d, 1H), 7.71 (t, 1H), 7.87 (t, 1H), 7.89 (s, 1H), 7.95 (d, 2H), 8.02 (d, 2H), 8.18 (q, 2H), 8.32 (d, 1H); EI-MS *m/z* 509 (M[+]), 336 (100%); HRMS (EI) *m/z* calcd C$_{28}$H$_{23}$N$_5$O$_3$S (M[+]) 509.1518, found 509.1521.

#### 4.4.3. 4-(2-(2,4-Dimethylphenyl)-quinoline-4-carboxamido)-N-pyrimidin-2-yl benzenesulfonamide (3). This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 40.0%: mp 240–241 °C; ^{1}H NMR (DMSO): $\delta$ 2.36 (s, 3H), 2.42 (s, 3H), 7.05 (t, 1H), 7.21 (m, 2H), 7.57 (d, 1H), 7.71 (t, 1H), 7.87 (m, 2H), 7.96 (d, 2H), 8.02 (d, 2H), 8.17 (q, 2H), 8.52 (d, 2H); EI-MS *m/z* 509 (M[+]), 350 (100%); HRMS (EI) *m/z* calcd C$_{28}$H$_{23}$N$_5$O$_3$S (M[+]) 509.1515, found 509.1521.

#### 4.4.4. 4-(2-(2-Methoxyphenyl)-quinoline-4-carboxamido)-N-(5-methyl-oxazol-2-yl) benzenesulfonamide (4). This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 46.9%: mp 266–268 °C; ^{1}H NMR (DMSO): $\delta$ 2.31 (s, 3H), 3.82 (s, 3H), 6.15 (s, 1H), 7.17 (t, 1H), 7.22 (d, 1H), 7.56 (t, 1H), 7.67 (t, 1H), 7.92 (m, 4H), 8.02 (d, 2H), 8.10 (s, 1H), 8.18 (t, 2H); EI-MS *m/z* 514 (M[+]), 234 (100%); HRMS (EI) *m/z* calcd C$_{27}$H$_{22}$N$_4$O$_5$S (M[+]) 514.1314, found 514.1311.

#### 4.4.5. 4-(4-Chlorophenylacetamido)-N-quinoxalin-2-yl benzenesulfonamide (5). This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 33.2%: mp 263–265 °C; ^{1}H NMR (DMSO): $\delta$ 3.65 (s, 2H), 7.35 (m, 4H), 7.62 (t, 1H), 7.78 (m, 4H), 7.92 (d, 1H), 8.05 (d, 2H), 8.62 (br, 1H); EI-MS *m/z* 452 (M[+]), 387 (100%); HRMS (EI) *m/z* calcd C$_{22}$H$_{17}$ClN$_4$O$_3$S (M[+]) 452.0711, found 452.0710.

#### 4.4.6. 4-(2,4-Dichlorobenzamido)-N-quinoxalin-2-yl benzenesulfonamide (6). THis compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 43.8%: mp 217–219 °C; ^{1}H NMR (DMSO): $\delta$ 7.51 (dd, 1H), 7.68 (m, 3H), 7.78 (t, 1H), 7.89 (d, 1H), 7.99 (m, 3H), 8.21 (d, 2H), 8.72 (s, 1H); EI-MS *m/z* 472 (M[+]), 173 (100%); HRMS (EI) *m/z* calcd C$_{21}$H$_{14}$Cl$_2$N$_4$O$_3$S (M[+]) 472.0163, found 472.0164.

#### 4.4.7. 4-(2-(3-Fluorophenyl)-quinoline-4-carboxamido)-N-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16a). This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 26.7%: mp 257–258 °C; ^{1}H NMR (DMSO): $\delta$ 1.85 (s, 3H), 2.12 (s, 3H), 7.40 (t, 1H), 7.67 (q, 1H), 7.73 (t, 1H), 7.84 (d, 2H), 7.92 (t, 1H), 8.05 (d, 2H), 8.25 (m, 4H), 8.47 (s, 1H); EI-MS *m/z* 516 (M[+]), 222 (100%); HRMS (EI) *m/z* calcd C$_{27}$H$_{21}$FN$_4$O$_4$S (M[+]) 516.1261, found 516.1268.

#### 4.4.8. 4-(2-(3-Chlorophenyl)-quinoline-4-carboxamido)-N-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16b). This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 30.5%: mp 237–238 °C; ^{1}H NMR (DMSO): $\delta$ 1.70 (s, 3H), 2.10 (s,

3H), 7.62 (m, 2H), 7.72 (t, 1H), 7.86 (d, 2H), 7.89 (t, 1H), 8.04 (d, 2H), 8.22 (t, 2H), 8.35 (d, 1H), 8.45 (s, 1H), 8.48 (s, 1H); EI-MS *m/z* 532 (M⁺), 266 (100%); HRMS (EI) *m/z* calcd $C_{27}H_{21}ClN_4O_4S$ (M⁺) 532.0969, found 532.0972.

**4.4.9.** **4-(2-(4-Bromophenyl)-quinoline-4-carboxamido)-*N*-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16c).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 35.2%: mp 248–250 °C; ¹H NMR (DMSO): δ 1.70 (s, 3H), 2.10 (s, 3H), 7.67 (t, 1H), 7.79 (d, 2H), 7.82 (d, 2H), 7.88 (t, 1H), 8.02 (d, 2H), 8.18 (d, 2H), 8.33 (d, 2H), 8.42 (s, 1H); EI-MS *m/z* 578 (M⁺), 203 (100%); HRMS (EI) *m/z* calcd $C_{27}H_{21}BrN_4O_4S$ (M⁺) 576.0431, found 576.0467.

**4.4.10.** **4-(2-(2-Bromophenyl)-quinoline-4-carboxamido)-*N*-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16d).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 40.3%: mp 243–244 °C; ¹H NMR (DMSO): δ 1.74 (s, 3H), 2.15 (s, 3H), 7.47 (t, 1H), 7.57 (t, 1H), 7.75 (m, 2H), 7.81 (t, 3H), 7.90 (t, 1H), 8.00 (m, 3H), 8.17 (d, 1H), 8.22 (d, 1H); EI-MS *m/z* 578 (M⁺), 203 (100%); HRMS (EI) *m/z* calcd $C_{27}H_{21}BrN_4O_4S$ (M⁺) 576.0464, found 576.0467.

**4.4.11. 4-(2-Pyridin-4-yl-quinoline-4-carboxamido)-*N*-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16e).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 37.7%: mp 238–239 °C; ¹H NMR (DMSO): δ 2.04 (s, 3H), 2.35 (s, 3H), 7.97 (m, 1H), 8.14 (m, 3H), 8.32 (d, 2H), 8.47 (d, 1H), 8.52 (dd, 2H), 8.59 (d, 1H), 8.77 (s, 1H), 9.01 (dd, 2H); EI-MS *m/z* 499 (M⁺), 205 (100%); HRMS (EI) *m/z* calcd $C_{26}H_{21}N_5O_4S$ (M⁺) 499.1316, found 499.1314.

**4.4.12. 4-(2-Thiophen-2-yl-quinoline-4-carboxamido)-*N*-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16f).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 24.3%: mp 234–235 °C; ¹H NMR (DMSO): δ 1.70 (s, 3H), 2.10 (s, 3H), 7.25 (t, 1H), 7.64 (t, 1H), 7.76 (d, 1H), 7.82 (m, 3H), 8.11 (m, 5H), 8.34 (s, 1H); EI-MS *m/z* 504 (M⁺), 210 (100%); HRMS (EI) *m/z* calcd $C_{25}H_{20}N_4O_4S_2$ (M⁺) 504.0916, found 504.0926.

**4.4.13. 4-(2-Cyclohexanyl-quinoline-4-carboxamido)-*N*-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16g).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 21.6%: mp 222–223 °C; ¹H NMR (DMSO): δ 1.35 (m, 1H), 1.48 (m, 2H), 1.75 (m, 6H), 1.87 (m, 2H), 1.99 (m, 2H), 2.10 (s, 3H), 2.96 (m, 1H), 7.61 (t, 1H), 7.66 (s, 1H), 7.80 (m, 3H), 7.99 (d, 2H), 8.03 (d, 1H), 8.07 (d, 1H); EI-MS *m/z* 504 (M⁺), 210 (100%); HRMS (EI) *m/z* calcd $C_{27}H_{28}N_4O_4S$ (M⁺) 504.1822, found 504.1831.

**4.4.14. 4-(2-Naphthalen-2-yl-quinoline-4-carboxamido)-*N*-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16h).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:3, v/v), yield 37.1%: mp 264–266 °C; ¹H NMR (DMSO): δ 1.71 (s, 3H), 2.05 (s, 3H), 7.60 (m, 2H), 7.70 (t, 1H), 7.88 (m, 3H), 8.00 (m, 1H), 8.05 (d, 2H), 8.11 (d, 2H), 8.19 (d, 1H), 8.24 (d, 1H), 8.59 (m, 2H), 8.95 (s, 1H); EI-MS *m/z* 548 (M⁺), 254

(100%); HRMS (EI) *m/z* calcd $C_{31}H_{24}N_4O_4S$ (M⁺) 548.1509, found 548.1518.

**4.4.15. 4-(2-(3-Bromophenyl)-6-fluoro-quinoline-4-carboxamido)-*N*-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16i).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 34.9%: mp 267–269 °C; ¹H NMR (DMSO): δ 1.70 (s, 3H), 2.10 (s, 3H), 7.56 (t, 1H), 7.74 (d, 1H), 7.87 (m, 3H), 7.96 (d, 1H), 8.04 (d, 2H), 8.29 (q, 1H), 8.38 (d, 1H), 8.56 (m, 2H); EI-MS *m/z* 596 (M⁺), 221 (100%); HRMS (EI) *m/z* calcd $C_{27}H_{20}FBrN_4O_4S$ (M⁺) 594.0376, found 594.0373.

**4.4.16. 4-(2-(3-Bromophenyl)-quinoline-4-carboxamido)-*N*-quinoxalin-2-yl benzenesulfonamide (16j).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 38.2%: mp 285–286 °C; ¹H NMR (DMSO): δ 7.53 (t, 1H), 7.67 (m, 2H), 7.76 (m, 2H), 7.86 (m, 2H), 7.95 (d, 1H), 8.00 (d, 2H), 8.14 (d, 2H), 8.18 (d, 2H), 8.35 (d, 1H), 8.40 (s, 1H), 8.53 (t, 1H), 8.72 (br, 1H); EI-MS *m/z* 611 (M⁺), 203 (100%); HRMS (EI) *m/z* calcd $C_{30}H_{20}BrN_5O_3S$ (M⁺) 609.0452, found 609.0470.

**4.4.17. 4-(2-(3-Bromophenyl)-pyridine-4-carboxamido)-*N*-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16k).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 38.7%: mp 240–242 °C; ¹H NMR (DMSO): δ 1.70 (s, 3H), 2.10 (s, 3H), 7.54 (t, 1H), 7.71 (d, 1H), 7.82 (d, 2H), 7.86 (d, 1H), 8.03 (d, 2H), 8.21 (d, 1H), 8.38 (t, 1H), 8.46 (s, 1H), 8.90 (d, 1H); EI-MS *m/z* 528 (M⁺), 260 (100%); HRMS (EI) *m/z* calcd $C_{23}H_{19}BrN_4O_4S$ (M⁺) 526.0292, found 526.0311.

**4.4.18. 4-(2-(4-Bromophenyl)-pyridine-4-carboxamido)-*N*-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16l).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 35.2%: mp 248–249 °C; ¹H NMR (DMSO): δ 1.67 (s, 3H), 2.08 (s, 3H), 7.76 (d, 2H), 7.84 (m, 3H), 8.02 (d, 2H), 8.16 (d, 2H), 8.41 (s, 1H), 8.89 (d, 1H); EI-MS *m/z* 526 (M⁺), 126 (100%); HRMS (EI) *m/z* calcd $C_{23}H_{19}BrN_4O_4S$ (M⁺) 526.0308, found 526.0310.

**4.4.19. 4-(2-Phenyl-pyridine-4-carboxamido)-*N*-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16m).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 48.2%: mp 219–220 °C; ¹H NMR (DMSO): δ 1.66 (s, 3H), 2.08 (s, 3H), 7.56 (m, 3H), 7.80 (m, 3H), 8.02 (d, 2H), 8.15 (d, 2H), 8.17 (s, 1H), 8.87 (d, 1H); EI-MS *m/z* 448 (M⁺), 57 (100%); HRMS (EI) *m/z* calcd $C_{23}H_{20}N_4O_4S$ (M⁺) 448.1214, found 448.1206.

**4.4.20. 4-(2-(4-Chlorophenyl)-pyridine-4-carboxamido)-*N*-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16n).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 38.1%: mp 244–245 °C; ¹H NMR (DMSO): δ 1.66 (s, 3H), 2.08 (s, 3H), 7.62 (d, 2H), 7.82 (m, 3H), 8.02 (d, 2H), 8.22 (d, 2H), 8.41 (s, 1H), 8.87 (d, 1H); EI-MS *m/z* 482 (M⁺), 216 (100%); HRMS (EI) *m/z* calcd $C_{23}H_{19}ClN_4O_4S$ (M⁺) 482.0829, found 482.0816.

**4.4.21. 4-(2-(4-Fluorophenyl)-pyridine-4-carboxamido)-N-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16o).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 55.6%: mp 232–234 °C; $^1$H NMR (DMSO): $\delta$ 1.68 (s, 3H), 2.08 (s, 3H), 7.39 (t, 2H), 7.82 (m, 3H), 8.03 (d, 2H), 8.26 (m, 2H), 8.39 (s, 1H), 8.87 (d, 1H); EI-MS $m/z$ 466 (M$^+$), 200 (100%); HRMS (EI) $m/z$ calcd C$_{23}$H$_{19}$FN$_4$O$_4$S (M$^+$) 466.1107, found 466.1111.

**4.4.22. 4-(2-(2-Methylphenyl)-pyridine-4-carboxamido)-N-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (16p).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 25.1%: mp 202–203 °C; $^1$H NMR (DMSO): $\delta$ 1.66 (s, 3H), 2.08 (s, 3H), 2.35 (s, 3H), 7.36 (m, 3H), 7.48 (d, 1H), 7.79 (d, 2H), 7.84 (d, 1H), 8.01 (m, 3H), 8.87 (d, 1H); EI-MS $m/z$ 462 (M$^+$), 167 (100%); HRMS (EI) $m/z$ calcd C$_{24}$H$_{22}$N$_4$O$_4$S (M$^+$) 462.1345, found 462.1361.

**4.4.23. 4-(2-(2-Methylphenyl)-pyridine-4-carboxamido)-N-(4-methyl-pyrimidin-2-yl) benzenesulfonamide (16q).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 28.9%: mp 242–243 °C; $^1$H NMR (DMSO): $\delta$ 2.31 (s, 3H), 2.35 (s, 3H), 6.90 (d, 2H), 7.35 (m, 3H), 7.47 (d, 1H), 7.82 (d, 1H), 8.01 (q, 4H), 8.32 (m, 1H), 8.87 (d, 1H); EI-MS $m/z$ 459 (M$^+$), 286 (100%); HRMS (EI) $m/z$ calcd C$_{24}$H$_{21}$N$_5$O$_3$S (M$^+$) 459.1369, found 459.1365.

**4.4.24. 4-(2,4-Dichlorophenylacetamido)-N-quinoxalin-2-yl benzenesulfonamide (17a).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 35.2%: mp 235–236 °C; $^1$H NMR (DMSO): $\delta$ 3.83 (s, 2H), 7.38 (m, 1H), 7.42 (d, 1H), 7.55 (d, 1H), 7.62 (t, 1H), 7.79 (m, 4H), 7.92 (d, 1H), 8.04 (d, 2H), 8.62 (br, 1H); EI-MS $m/z$ 486 (M$^+$), 236 (100%); HRMS (EI) $m/z$ calcd C$_{22}$H$_{16}$Cl$_2$N$_4$O$_3$S (M$^+$) 486.0309, found 486.0321.

**4.4.25. 4-Benzamido-N-quinoxalin-2-yl benzenesulfonamide (17b).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 45.4%: mp 258–259 °C; $^1$H NMR (DMSO): $\delta$ 7.63 (m, 4H), 7.75 (t, 1H), 7.82 (m, 1H), 7.93 (m, 3H), 8.03 (d, 2H), 8.13 (d, 2H), 8.65 (br, 1H); EI-MS $m/z$ 404 (M$^+$), 105 (100%); HRMS (EI) $m/z$ calcd C$_{21}$H$_{16}$N$_4$O$_3$S (M$^+$) 404.0943, found 404.0943.

**4.4.26. 4-(3-Phenyl-propionamido)-N-quinoxalin-2-yl benzenesulfonamide (17c).** This compound was purified by flash chromatography with EtOAc/petroleum ether (1:2, v/v), yield 39.3%: mp 213–214 °C; $^1$H NMR (DMSO): $\delta$ 2.65 (t, 2H), 2.90 (t, 2H), 7.16 (t, 1H), 7.26 (m, 4H), 7.62 (t, 1H), 7.79 (m, 4H), 7.93 (d, 1H), 8.03 (d, 2H), 8.63 (br, 1H); EI-MS $m/z$ 432 (M$^+$), 367 (100%); HRMS (EI) $m/z$ calcd C$_{23}$H$_{20}$N$_4$O$_3$S (M$^+$) 432.1261, found 432.1256.

**4.4.27. 4-(4-Chlorophenylacetamido)-N-(3,4-dimethyl-isoxazol-5-yl) benzenesulfonamide (17d).** This compound was purified by flash chromatography with EtOAc/petro-

leum ether (1:2, v/v), yield 49.3%: mp 193–194 °C; $^1$H NMR (DMSO): $\delta$ 1.60 (s, 3H), 2.07 (s, 3H), 3.69 (s, 2H), 7.39 (q, 4H), 7.69 (d, 2H), 7.78 (d, 2H); EI-MS $m/z$ 419 (M$^+$), 125 (100%); HRMS (EI) $m/z$ calcd C$_{19}$H$_{18}$ClN$_4$O$_3$S (M$^+$) 419.0704, found 419.0706.

**4.4.28. 4,4′-Methylenebisanthranilic acid (31a).**[31] To a stirred mixture of anthranilic acid **30** (6.85, 50 mmol), H$_2$O (63 mL), and 36% HCl (13 mL) at 50 °C was added 3% aqueous formaldehyde (18 mL). The resulting solution was heated at 70 °C and stirred for 4 h. Upon neutralization with ammonia, the precipitate was filtered, washed successively with H$_2$O and hot acetic acid, followed by drying to afford **31a** (4.8 g, 33.6%) as yellow solid: mp 258–260 °C.

**4.4.29. 4,4′-Methylenebisanthranilic acid dimethyl ester (31b).** A mixture of **31a** (1 g, 3.5 mmol) in methanol (30 mL) saturated with HCl was refluxed for 5 h, poured into crushed ice, and pH adjusted to 9 with 2 N NaOH. The precipitate was collected, dried, and purified by flash chromatography on silica gel, eluted with a mixture of EtOAc/petroleum ether (1:10, v/v), to afford **31b** (0.30 g, 27.5%) as a white solid: mp 144–145 °C; $^1$H NMR (CDCl$_3$): $\delta$ 3.80 (s, 2H), 3.88 (s, 6H), 5.60 (br, 2H), 6.62 (d, 2H), 7.08 (dd, 2H), 7.65 (d, 2H).

**4.4.30. 6,6′-Bis-(3,5-dimethoxybenzamido)-3,3′-methanediyl-di-benzoic acid (15).** A mixture of **31a** (0.2 g, 0.7 mmol), 3,5-dimethoxybenzoyl chloride (0.31 g, 1.54 mmol), and pyridine (5 mL) was stirred at 0 °C for 5 h. The resulting mixture was poured into H$_2$O, and the precipitate was collected, dried, and purified by flash chromatography on silica gel, eluted with a mixture of CH$_3$OH/CHCl$_3$ (1:15, v/v), to afford **15** (70 mg, 16.3%) as a white solid: mp > 300 °C; $^1$H NMR (DMSO): $\delta$ 3.80 (s, 12H), 3.90 (s, 2H), 6.68 (s, 2H), 7.14 (s, 4H), 7.30 (d, 2H), 7.92 (s, 2H), 8.58 (d, 2H); MALDI-MS $m/z$ 637 [M+Na]$^+$; HRMS (MALDI) $m/z$ calcd C$_{33}$H$_{30}$N$_2$O$_{10}$Na [M+Na]$^+$ 637.17927, found 637.18080.

**4.4.31. 6,6′-Bis-benzamido-3,3′-methanediyl-di-benzoic acid (18b).** In the same manner as described for **15**, **18b** was prepared from benzoyl chloride, yield 20.0%: mp 288–289 °C; $^1$H NMR (CDCl$_3$): $\delta$ 4.24 (s, 2H), 7.58 (m, 4H), 7.61 (m, 2H), 7.67 (s, 4H), 8.09 (s, 2H), 8.32 (m, 4H); EI-MS $m/z$ 494 (M$^+$), 105 (100%). HRMS (EI) $m/z$ calcd C$_{29}$H$_{22}$N$_2$O$_6$ (M$^+$) 494.1487, found 494.1479.

**4.4.32. 6,6′-Bis-(4-methoxybenzamido)-3,3′-methanediyl-di-benzoic acid (18d).** In the same manner as described for **15**, **18d** was prepared from 4-methoxybenzoyl chloride; yield 20.0%: mp 280–282 °C; $^1$H NMR (DMSO): $\delta$ 3.86 (s, 6H), 4.07 (s, 2H), 7.05 (d, 2H), 7.13 (d, 2H), 7.30 (d, 1H), 7.62 (d, 1H), 7.79 (d, 1H), 7.99 (m, 4H), 8.13 (d, 2H), 8.62 (d, 1H); EI-MS $m/z$ 554 (M$^+$), 135 (100%). HRMS (EI) $m/z$ calcd C$_{31}$H$_{26}$N$_2$O$_8$ (M$^+$) 554.1678, found 554.1690.

**4.4.33. 6,6′-Bis-(3,5-dimethoxybenzamido)-3,3′-methanediyl-di-benzoic acid dimethyl ester (18a).** A mixture of **31b** (50 mg, 0.15 mmol), 3,5-dimethoxybenzoyl chloride (68 mg, 0.34 mmol), and pyridine (5 mL) was stirred at 0 °C for 5 h. The resulting mixture was poured into H$_2$O, and the precipi-

tate was collected, dried, and purified by flash chromatography on silica gel, eluted with a mixture of EtOAc/petroleum ether (1:4, v/v), to afford **18a** (23 mg, 22.5%) as a white solid: mp 194–196 °C; $^{1}$H NMR (DMSO): $\delta$ 3.85 (s, 12H), 3.88 (s, 6H), 4.05 (s, 2H), 6.78 (s, 2H), 7.07 (s, 4H), 7.59 (d, 2H), 7.88 (s, 2H), 8.44 (d, 2H); MALDI-MS $m/z$ 665 [M+Na]$^{+}$. HRMS (MALDI) $m/z$ calcd $C_{35}H_{34}N_2O_{10}Na$ [M+Na]$^{+}$ 665.21057, found 665.21140.

**4.4.34. 6,6′-Bis-benzamido-3,3′-methanediyl-di-benzoic acid dimethyl ester (18c).** In the same manner as described for **18a**, **18c** was prepared from benzoyl chloride, yield 20.3%: mp 214–216 °C; $^{1}$H NMR (DMSO): $\delta$ 3.88 (s, 6H), 4.06 (s, 2H), 7.65 (m, 8H), 7.90 (s, 2H), 7.97 (d, 4H), 8.50 (d, 2H); EI-MS $m/z$ 522 (M$^{+}$), 105 (100%); HRMS (EI) $m/z$ calcd $C_{31}H_{26}N_2O_6$ (M$^{+}$) 522.1781, found 522.1791.

**4.4.35. 6,6′-Bis-(4-methoxybenzamido)-3,3′-methanediyl-di-benzoic acid dimethyl ester (18e).** In the same manner as described for **18a**, **18e** was prepared from 4-methoxybenzoyl chloride, yield 28.0%: mp 234–236 °C; $^{1}$H NMR (DMSO): $\delta$ 3.84 (s, 6H), 3.88 (s, 6H), 4.04 (s, 2H), 7.14 (d, 4H), 7.58 (d, 2H), 7.93 (m, 6H), 8.51 (d, 2H); MALDI-MS $m/z$ 605 [M+Na]$^{+}$. HRMS (MALDI) $m/z$ calcd $C_{33}H_{30}N_2O_8Na$ [M+Na]$^{+}$ 605.18944, found 605.18960.

**4.4.36. 6,6′-Bis-(4-chlorobenzamido)-3,3′-methanediyl-di-benzoic acid dimethyl ester (18f).** In the same manner as described for **18a**, **18f** was prepared from 4-chlorobenzoyl chloride, yield 26.0%: mp 241–242 °C; $^{1}$H NMR (CDCl$_3$): $\delta$ 3.95 (s, 6H), 3.99 (s, 2H), 7.46 (d, 2H), 7.50 (d, 4H), 7.90 (s, 2H), 7.99 (d, 4H), 8.85 (d, 2H); EI-MS $m/z$ 590 (M$^{+}$), 139 (100%). HRMS (EI) $m/z$ calcd $C_{31}H_{24}Cl_2N_2O_6$ (M$^{+}$) 590.1030, found 590.1011.

**4.4.37. 6,6′-Bis-benzylamino-3,3′-methanediyl-di-benzoic acid dimethyl ester (18g).** To a stirred mixture of **31b** (100 mg, 0.32 mmol), NaCO$_3$ (80 mg, 0.75 mmol), and NMP (3 mL) was added benzyl bromide (0.09 mL, 0.75 mmol). The mixture was then heated at 90 °C for 10 h. The reaction mixture after cooling was transferred to a separatory funnel using a 1:1 mixture of H$_2$O and EtOAc. The product was extracted with EtOAc. The combined organic phases were washed, dried, filtered, and concentrated. The residue was purified by flash chromatography on silica gel, eluted with a mixture of EtOAc/petroleum ether (1:20, v/v), to afford **18g** (43 mg, 27.4%) as a white solid: mp 86–88 °C; $^{1}$H NMR (DMSO): $\delta$ 3.64 (s, 2H), 3.76 (s, 6H), 4.42 (d, 4H), 6.63 (d, 2H), 7.15 (d, 2H), 7.26 (m, 2H), 7.32 (m, 8H), 7.60 (d, 2H), 7.96 (t, 2H); MALDI-MS $m/z$ 517 [M+Na]$^{+}$. HRMS (MALDI) $m/z$ calcd $C_{31}H_{30}N_2O_4Na$ [M+Na]$^{+}$ 517.20978, found 517.21090.

**4.4.38. 6,6′-Bis-benzylamino-3,3′-methanediyl-di-benzoic acid monomethyl ester (18h).** A mixture of **18g** (50 mg, 0.1 mmol) and LiOH (20 mg, 0.8 mmol) in THF/Methanol/H$_2$O (3:1:1, v/v/v, 10 mL) was stirred at room temperature for 12 h. The resulting solution was acidified to pH 2 using 1 N HCl. The product was extracted into the organic phase using EtOAc (3 × 25 mL), dried, filtered, and concentrated. The residue was purified by flash chroma-

tography on silica gel, eluted with a mixture of EtOAc/petroleum ether (1:4, v/v), to afford **18h** (20 mg, 41.6%) as a white solid: mp 173–175 °C; $^{1}$H NMR (DMSO): $\delta$ 3.64 (s, 2H), 3.77 (s, 3H), 4.43 (t, 4H), 6.65 (q, 2H), 7.17 (q, 2H), 7.33 (m, 10H), 7.62 (q, 2H), 7.95 (t, 2H); MALDI-MS $m/z$ 503 [M+Na]$^{+}$. HRMS (MALDI) $m/z$ calcd $C_{30}H_{28}N_2O_4Na$ [M+Na]$^{+}$ 503.19413, found 503.19610.

**4.4.39. 6,6′-Bis-benzylamino-3,3′-methanediyl-di-benzoic acid (18i).** In the same manner as described for **18h** but room temperature was changed into refluxing temperature, **18i** was prepared as a white solid, yield 21.2%: mp 179–181 °C; $^{1}$H NMR (DMSO): $\delta$ 3.64 (s, 2H), 4.41 (s, 4H), 6.61 (d, 2H), 7.14 (d, 2H), 7.33 (m, 10H), 7.60 (s, 2H); MALDI-MS $m/z$ 489 [M+Na]$^{+}$. HRMS (MALDI) $m/z$ calcd $C_{29}H_{26}N_2O_4Na$ [M+Na]$^{+}$ 489.17848, found 489.17930.

**4.4.40. 5-Bromomethyl-2-nitro-benzoic acid methyl ester (34).**[32] A mixture of 5-methyl-2-nitro-benzoic acid **32** (1 g, 0.55 mmol) in methanol (30 mL) saturated with HCl was refluxed for 5 h, and most of solvent was evaporated, filtered, washed, and dried to afford **33** (0.91 g, 84.5%) as a white solid: mp 76–78 °C.

A mixture of **33** (0.9 g, 4.7 mmol), NBS (1.25 g, 7.0 mmol), benzoyl peroxide (0.05 g), and CH$_3$Cl (30 mL) was stirred for 24 h. The resulting solution was washed successively with H$_2$O, aqueous NaCO$_3$, brine, dried, filtered, and concentrated. The residue was purified by flash chromatography on silica gel, eluted with a mixture of EtOAc/petroleum ether (1:10, v/v), to afford **34** (0.43 g, 33.8%) as a white solid: mp 58–61 °C.

**4.4.41. 6,6′-Bis-nitro-3,3′-ethanediyl-di-benzoic acid dimethyl ester (35).** To a stirred mixture of **33** (0.5 g 2.5 mmol), n-BuLi (2.5 M in hexane) (2 mL, 5 mmol), and redistilled THF (10 mL) was added dropwise **34** (0.35 g, 1.3 mmol) in redistilled THF (5 mL) at −78 °C under N$_2$. The resulting mixture was stirred for 3 h at −78 °C and then warmed to room temperature overnight. The solution was poured into aqueous NH$_4$Cl, extracted with EtOAc. The combined organic phases were dried, filtered, and concentrated. The residue was purified by flash chromatography on silica gel, eluted with a mixture of EtOAc/petroleum ether (1:4, v/v), to afford **35** (0.11 g, 21.8%) as a yellow solid: $^{1}$H NMR (CDCl$_3$): $\delta$ 3.07 (s, 4H), 3.93 (s, 6H), 7.37 (d, 2H), 7.50 (d, 2H), 7.89 (d, 2H); EI-MS $m/z$ 388 (M$^{+}$), 194 (100%).

**4.4.42. 6,6′-Bis-amino-3,3′-ethanediyl-di-benzoic acid dimethyl ester (36).** A mixture of **35** (110 mg, 0.28 mmol), 10% palladium on charcoal (20 mg), and THF (20 mL) was stirred at 25 °C for 8 h in an atmosphere of hydrogen. The catalyst was filtered and the filtrate was concentrated to dryness. The residue was washed with a few methanol to afford **36** (90 mg) as a white solid: mp 216–218 °C.

**4.4.43. 6,6′-Bis-(3,5-dimethoxybenzamido)-3,3′-ethanediyl-di-benzoic acid dimethyl ester (18k).** In the same manner as described for **18a**, **18k** was prepared from **36**, yield 58.0%: mp 215–216 °C; $^{1}$H NMR (CDCl$_3$): $\delta$ 2.93 (s, 4H), 3.88 (s, 12H), 3.94 (s, 6H), 6.64 (s, 2H), 7.18 (s, 4H), 7.44 (d, 2H),

7.87 (s, 2H), 8.83 (d, 2H); MALDI-MS *m/z* 679 [M+Na]$^+$. HRMS (MALDI) *m/z* calcd $C_{36}H_{36}N_2O_{10}Na$ [M+Na]$^+$ 679.22622, found 679.22800.

**4.4.44. 6,6′-Bis-(3,5-dimethoxybenzamido)-3,3′-ethanediyl-di-benzoic acid (18l).** A mixture of **18k** (32 mg, 0.05 mmol) and LiOH (12 mg, 0.5 mmol) in THF/methanol/$H_2O$ (3:1:1, v/v/v, 20 mL) was stirred at room temperature for 12 h. The resulting solution was acidified to pH 2 using 1 N HCl. The product was extracted, dried, filtered, concentrated, and purified by flash chromatography using EtOAc/petroleum ether (2:1, v/v), to afford **18l** (18 mg, 58.1%) as a pale red solid: mp > 300 °C; $^1$H NMR (DMSO): δ 2.91 (s, 4H), 3.86 (s, 12H), 6.74 (s, 2H), 7.07 (s, 4H), 7.54 (d, 2H), 7.90 (s, 2H), 8.59 (d, 2H); ESI-MS *m/z* 627 [M-H]$^+$. HRMS (ESI) *m/z* calcd $C_{34}H_{31}N_2O_{10}$ [M−H]$^+$ 627.1979, found 627.1989.

**4.4.45. Bis-(4-(3,5-dimethoxybenzamido)-phenyl)-methane (18j).** In the same manner as described for **18a**, **18j** was prepared from 4,4′-methanediyl-bis-aniline **37**, yield 56.0%; mp 185–187 °C; $^1$H NMR (CDCl$_3$): δ 3.83 (s, 12H), 3.96 (s, 2H), 6.61 (t, 2H), 6.97 (d, 4H), 7.19 (d, 4H), 7.56 (d, 4H); ESI-MS *m/z* 525 [M−H]$^+$. HRMS (ESI) *m/z* calcd $C_{31}H_{29}N_2O_6$ [M−H]$^+$ 525.2026, found 525.2078.

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