MLSCN Probe Summary

Project: NMR-based chemical fragment screening for identification of novel FKBP12 inhibitors

Assay Submitter & Institution: Maurizio Pellecchia, Burnham Institute for Medical Research

Grant Number: XO1MH078942

Screening Center: San Diego Center for Chemical Genomics

Principal Investigator of Screening Center: John C. Reed

Assay Target: FKBP12

Probe PubChem Compound Identifier (CID): 16725062

I. Assay provider information:

Specific Aim*:* Identify novel chemical probes that target FKBP12

Significance: Peptidylprolyl isomerases (PPIases) are a group of cytosolic enzymes initially characterized by their ability to catalyze the *cis-trans* isomerization of cis-peptidylprolyl bonds. The *cis–trans* interconversion accelerated by PPIases is significant for protein folding because *cis* proline introduces critical bends within the protein conformation. FK506-binding proteins (FKBPs), including the prokaryotic FKBP-like trigger factor, represent one of the three families of enzymes sharing peptidyl–prolyl *cis–trans* isomerase activity. FKBPs, are found in all classes of organisms, some of them being highly conserved and others being more specific. These proteins have multiple roles in the cell, the best known being receptors for medically important immunosuppressors. However, most cellular functions of the different FKBPs are still unknown.

FKBP12 represents the minimal peptide sequence harboring the two properties of FKBPs (PPIase activity and immunophilin FK506 binding). The 3D-structure of hydrophobic core of the protein that forms the ligand-binding pocket is known, providing the basis for structure-based drug optimization. FKBP12 is a modulator of neurotrophic pathways. FK506, for example, promotes neuronal survival and stimulates neurite outgrowth via a FKBP12-dependent mechanism. We used unbiased NMR-based screening to identify novel chemical probes against FKBP12 with neurotrophic activity. FK506 and previously identified FK506-derived inhibitors (e.g. compound 1 in Table 1) require the presence of a α-keto amido group, a moiety that is critical for the ability of these compounds to bind FKBP12. Attempts to replace the α-keto amido group with more stable functional groups have been recently reported. We hope therefore to derive novel chemotypes that depart from the general FK506 structure and/or that lack this undesired functionality.

II. Screening Center information:

PubChem Bioassay Name: NMR Based -Screening Assay for FKBP12

PubChem bioassay identifier (AID): 608

***Assay description*:** The relatively small size of FKBP12 (10 kDa) makes it amenable to protein-NMR spectroscopy techniques, such as 2D [^{15}N, ^{1}H] or 2D [^{13}C, ^{1}H] experiments, designed for the detection of ligand binding. These approaches are preferential to transfer ligand-based techniques as they provide intrinsic structural binding site information and are largely void of false positives or false negatives. However, the use of 2D [^{15}N, ^{1}H] or 2D [^{13}C, ^{1}H] correlation spectra as a screening method requires relatively large amounts of protein per sample (> 100 μM) and relatively lengthy measurement times. To ameliorate these problems we propose a primary screen employing simple 1D ^{1}H NMR experiments, acquired in the presence and absence of a mixture of potential ligands. Eventual resonance overlap with resonance lines of test compounds is resolved by using ^{13}C-labeled samples and 1D ^{13}C-edited ^{1}H NMR experiments. This protocol has the intrinsic advantage of requiring relatively small protein concentrations (10-50 μM) and relatively short measurement times (typically from 15 minutes to 1 hour depending on the spectrometer used and the protein's molecular

weight). Because of the ^{13}C filter, signals arising from the test ligands or from solvent and buffers are effectively suppressed, thereby enabling the screening of compounds in mixtures without protein spectrum interference. Another advantage of this technique is that observables can also be detected in the aliphatic region of the protein spectrum (1 ppm and below), a region rarely populated by signals from organic molecules. In such cases, the use of ^{13}C labeling would not be necessary.

Protocol summary:

a. Ligand binding was monitored by comparing the aliphatic region of 1D ^{1}H NMR spectra of a 32 μM FKPB12 solution (20 mM sodium phosphate buffer at pH 7.5 containing 90%/10% $H_2O/^{2}H_2O$ or 99.5% $^{2}H_2O$; T = 300 K) in the presence and in absence of compounds tested at a final concentration of 125 μM. Compounds were initially tested at mixtures of 10, and then individual compounds for those mixtures that caused significant perturbations in the spectrum (>0.08 ppm) were subsequently tested**.**

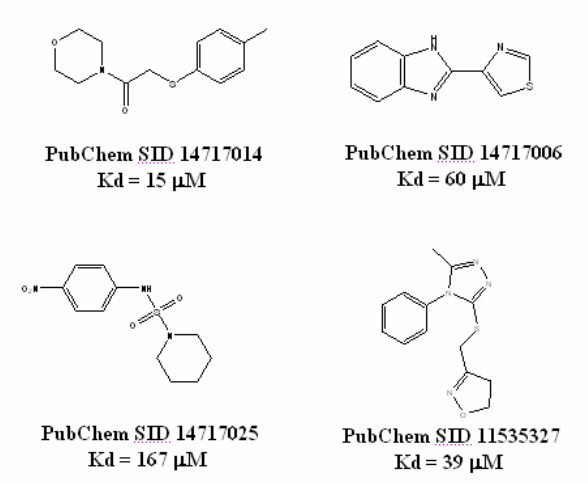


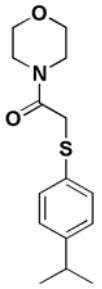
Figure 1: Hits from primary screen

b. Hit compounds with estimated Kd values of 100 μM or less are selected for further SAR studies. Analogues are selected and purchased directly from commercial sources and tested for binding to the target. SAR data are further analyzed to design additional analogues to be chemically synthesized.

By employing this strategy we identified the classes of novel FKBP12 ligands shown in **Figure 1**. Compound 14717006 and compound 14717025 resemble previously identified FKBP12 inhibitors, providing a further validation of this approach. Compound 14717014, while also displaying similarities to other FK506-derived inhibitors, presents novel structural features that could not have been easily anticipated by simply modifying these compounds.

Probe Compound:

Chemical name of probe compound: **2-(4-Isopropylphenylthio)-1-morpholinoethanone (16725062)**



The compound binds to FKBP12 with sub-micromolar affinity and stimulates neurite outgrowth at 0.1 to 1 μM concentration, similar to the bioactivity observed with FK506, while it is devoid of the metabolic inhibitory activity of FK506. Development of drug-like materials without the potential for adverse drug-drug interactions may hold promise for a new class of neuroprotective agents.

Chemistry:

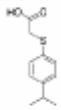
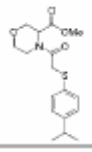
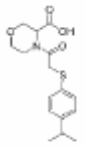
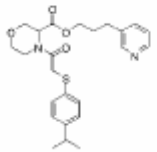
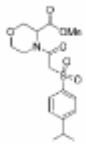
2-(4-Isopropylphenylthio)-1-morpholinoethanone. To a solution of 2-(4-isopropylphenylthio)acetic acid (210 mg, 1.0 mmol) in DMF (20.0 mL) was added EDC (228 mg, 1.2 mmol), HOBt (184 mg, 1.2 mmol), NEt_3 (300 mg, 3.0 mmol), and morpholine (174 mg, 2.0 mmol). After the reaction mixture was stirred at r.t. for 72 h, DMF was removed under reduced pressure. It was purified by silica gel flash chromatography (EtOAc-Hexanes = 1:7) to give as a colorless oil (100 mg, 36%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 (d, J = 8.4 Hz, 2 H), 7.17 (d, J = 8.1 Hz, 2 H), 3.69 (s, 2 H), 3.66-3.56 (m, 6 H), 1.48-3.42 (m, 2 H), 2.87 (sept, J = 6.9 Hz, 1 H), 1.22 (d, J = 6.9 Hz, 6 H). ^{13}C NMR (75 MHz, $CDCl_3$) d 167.5, 148.6, 131.4, 127.4, 66.9, 66.6, 46.9, 42.4, 37.0, 33.8, 24.0. HRMS m/z calcd for $C_{15}H_{22}NO_2S$ $[M + H]^+$ 280.1366, found 280.1361.

Results:

Structure-Activity Relationships

Initial structure-activity relationship data were obtained by selecting and testing commercially available derivatives of hit compounds from primary screen (**Figure 1**). Compound dissociation constants for FKBP12 were determined by 1D-Aliphatic ^{1}H NMR and isothermal titration calorimetry (ITC). Based on the SAR data obtained with **14717014,** the following chemical modifications were made: the synthesis of bi-dentate compounds derivatizing the morpholino group with secondary site binders and the modification of the linker region between the phenyl ring and the morpholino groups. In addition, SAR data relative to the phenyl ring of

14717014 suggests that a bulkier, hydrophobic group such as an isopropyl in the *para* position may be preferred. These strategies lead to the identification of sub-micromolar binders for FKBP12 (**Table 1**).

COMPOUND	STRUCTURE	Kd (μM)
13		>4000
10		0.3*
11		426
12		5.6
6 Pubchem ID **16725062**		0.2*
16		505

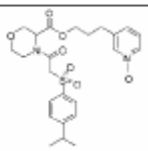
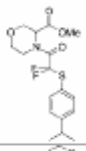
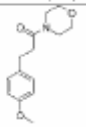
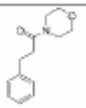
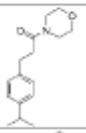
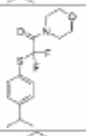
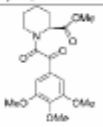
COMPOUND	STRUCTURE	Kd (μM)
17		1285
8		190
9		204
Compound **71** (BI-69B9)		578
18		2.3
7		2.8
1		3.0

Table 1. SAR data relative to the synthesized compounds

Cell-Based Evaluation

To test the hypothesis that the purported FKBP12 binders would stimulate neurite outgrowth similar to that seen with FK506, we treated primary cortical neurons with either 0.1 μM or 1 μM of test compound for 72 hours and then calculated average neurite length. As expected, FK506 treatment resulted in an increase in average neurite length. Treatment with several of the compounds identified in this study resulted in stimulation of neurite outgrowth. In comparison to control treatment, most compounds resulted in robust neurite outgrowth. In most cases, neurite outgrowth was stimulated to a level comparable to that seen with FK506. Thus we feel that these compounds have biological activity similar to FK506 in this assay.

While these data strongly suggest FKBP12 is the relevant target of the compounds, we cannot rule out the possibility that the molecules could interact with other closely related targets (e.g. FKBP12.6, FKBP13, FKBP25, FKBP38, and FKBP38). However, when tested against Pin1, another proline *cis-trans* isomerase, the compounds did not show any appreciable binding. The immunosuppressive activity of these compounds has not been established.

Metabolic Stability Studies

The novel FK506 binding compounds **8**, **9**, **10**, **11**, **12**, and **6** (**Table 1**) as well as FK506 and compound **1** were assayed for their metabolic stability in the presence of pooled human liver microsomes and an NADPH generating system. With the exception of **11** and FK506, that showed half-lives of greater than 60 minutes, the compounds exhibited half lives of about 40 minutes. We do not attribute a significant difference to the measured half-lives because the metabolic stability of FK506 might be due to inhibition of its own metabolism, as is commonly observed for carbon-carbon double bond-containing compounds. To investigate this aspect for FK506 and compounds of Table 1, their ability to inhibit the conversion of the marker substrate testosterone to its prominent metabolite 6-hydroxy testosterone by human liver microsomes at concentrations of 10 μM was examined. At 10 μM, FK506 completely inhibited the formation of 6-hydroxy testosterone whereas the other compounds examined showed only ~50% inhibition at 10 μM. Detailed determination of the IC_{50} of FK506 for inhibition of testosterone hydroxylase showed it possessed an IC_{50} value of 0.81 μM, compared to an IC_{50} value of approximately 10 uM for compound **6** Hence, the smaller molecular weight compounds have a significantly decreased metabolic inhibitory activity compared to FK506. The smaller molecular weight compounds are anticipated to possess acceptable metabolic stability to study in vivo. For FK506, it is likely that the apparent metabolic stability is due to inhibition of its own metabolism. Development of drug-like materials without the potential for inhibition of metabolism and contribution to adverse drug-drug interactions may hold promise for a new class of neuroprotective agents.

III. Appendices:

Design, methods of preparation and characterization, as well as comparative data on probe, similar compound structures and prior probes, biological activity and in vitro ADME properties compared to existing compounds are reported in the enclosed manuscript (John L. Stebbins, Ziming Zhang, Jinhua Chen, Bainan Wu, Aras Emdadi, Megan E. Williams, John Cashman, and Maurizio Pellecchia. Nuclear Magnetic Resonance Fragment-Based Identification of Novel FKBP12 Inhibitors. *J. Med. Chem. 2007*, in press.)