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FKBP12-BINDING DOMAIN ANALOGUES OF FK506 ARE POTENT, NONIMMUNOSUPPRESSIVE NEUROTROPHIC AGENTS IN VITRO AND PROMOTE RECOVERY IN A MOUSE MODEL OF PARKINSON'S DISEASE

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Abstract: A series of simple N-(glyoxyl)pipecolate esters were synthesized as mimics of the FKBP12- binding domain portion of FK506. Compounds which were effective inhibitors of the prolyl isomerase activity of FKBP12 were extraordinarily potent neurotrophic agents in vitro, and were effective in a mouse model of Parkinson's Disease. These results suggest that FKBP12 ligands have therapeutic utility in neurodegenerative diseases.

The immunophilins FKBP12 and cyclophilin A are "receptors" for the immunosupressant drugs FK506 and cyclosporin A, respectively.[1] The drug-immunophilin complexes bind to target proteins-the calcium-calmodulin activated protein phosphatase calcineurin for cyclosporin A-cyclophilin and FK506-FKBP12,[2a] and a novel protein RAFT for rapamycin-FKBP.[2b,c] We have discovered that immunophilins are concentrated 10-40 fold more in the brain than in the immune tissues.[3] Within neural tissues, they influence neuronal process extension, and may have potential therapeutic applications in neurodegenerative disorders.[3] Recent studies established that immunosuppressant immunophilin ligands produce potent neurotrophic effects in vitro and in vivo.[4-6]

An elegant body of work directed towards elucidating the mechanism of immunosuppression by FK506 and related agents has been developed by a number of groups. Previously published work describing the structures of FK506[7] and nonimmunosuppressive analogs[8] bound to FKBP12 has established that immunosuppressant drugs such as FK506 may be considered as possessing two distinct binding domains (Figure 1): a) an FKBP12 binding domain that makes contact with the rotamase active site of FKBP12, functioning as an enzyme inhibitor and high-affinity ligand for the immunophilin, and b) an "effector" domain that extends beyond the surface of the protein and is capable of engaging in intermolecular interactions. It is this effector domain, together with the flanking loop regions of FKBP12, that comprises the calcineurin binding surface of the FK506/FKBP12 complex.

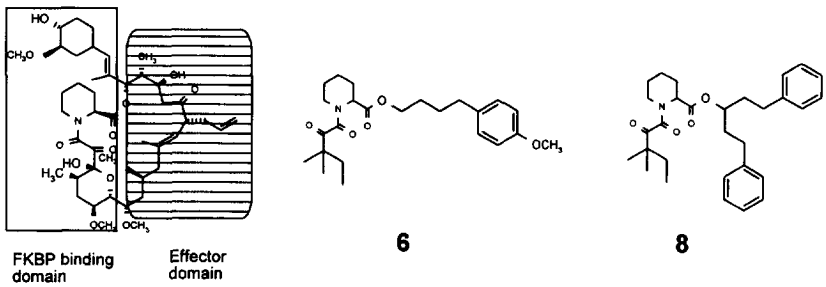


Figure 1. Binding domains of FK506 (left) together with representative FKBP binding domain analogues utilized in this study (right).

Nonimmunosuppressant analogues of FK506,[8,9] rapamycin,[10] and cyclosporin,[11] which possess modified effector

domains, have been described in the literature. We recently reported that several of these nonimmunosuppresant neuroimunophilin ligands elicite neurite outgrowth in PC12 cells and chick sensory neuronal cultures, suggesting that the neurotrophic effects of these drugs reside in their respective immunophilin binding domains. In addition, treatment of rats with nonimmunosupressive neuroimmunophilin ligands following sciatic nerve injury accelerated the rate of recovery assessed by both morphological and functional assays.[5,6] These data suggest that the neurotrophic actions of neuroimmunophilin ligands are independent of their immunosuppressive properties, and that it may therefore be possible to design small molecule neuroimmunophilin ligands which are potent neurotrophic agents but lack the immunosupressive effects of FK506 and rapamycin, a crucial consideration for the development of therapeutically useful agents for nervous system disorders.

An extensive body of work from several groups in the immunosuppressant field has established N-(glyoxyl)pipecolate esters are capable of binding with high affinity to the FK506 binding site of FKBP12.[12-15] X-ray crystal structures of several such compounds complexed with FKBP12 have demonstrated that these simple compounds bind to the immunophilin in a manner very similar to the FKBP-binding portion of FK506.[12,15] In order to extend our observations regarding the neurotrophic effects of nonimmunosuppressive analogues of FK506, and further evaluate the hypothesis that the neurotrophic and immunosuppressant effects of FK506 could be structurally dissected, we prepared a series of small molecule ligands that possess affinity for FKBP12, but are devoid of immunosuppressive activity, and have evaluated the trophic effects of these structures in cultured sensory neurons from chick dorsal root ganglia, as well as central models of neurodegeneration. Compounds **1-14** (Table I) were prepared for the present study by known literature methods.[12,13] Compounds **3**,[12] **4**,[12] and **11**[15] have previously been reported in the literature. Compounds **1, 2, 5-10** and **12-14** are closely related analogues of these structures and were synthesized and tested for the purpose of exploring the SAR of the neurotrophic effects of these compounds. Compounds **1, 2, 5-7**, and **13** were prepared and tested as racemic mixtures; the others were evaluated as the (S)-pipecolates. All new compounds had ^{1}H NMR spectra and elemental analysis results in accord with the expected structures.

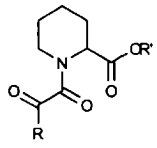
Compounds were evaluated for their ability to promote neurite outgrowth in cultured sensory neurons and for their ability to bind to FKBP12. Inhibition of the rotamase activity of FKBP-12 was assayed as described by Kofron,[16] using the peptide N-succinyl Ala-Leu-Pro-Phe p-nitroanilide as substrate. Apparent rotamase inhibition constants (K_i) were obtained and used as an approximate measure of ligand binding affinities. Dorsal root ganglia (DRGs) were dissected from embryonic chick, gestation day E8-E10, and explants of sensory neurons were cultured as described elsewhere.[6] Freshly isolated sensory neuronal cultures were treated with increasing concentrations of FKBP12 ligands and neurite outgrowth from the explant cultures was quantitated 48 hr post-treatment. Neurite outgrowth was assessed from photomicrographs of each explant culture and all processes whose lengths exceeded the explant's diameter were counted. Dose-response curves were generated from which ED_{50} values were obtained. The results of these experiments are summarized in Table 1.

We initially evaluated ligands **1-4** to test the hypothesis that FKBP12 binding, but not calcineurin inhibition, is necessary to achieve neurotrophic effects. These compounds contain the minimal structural elements for recognition of the rotamase enzymatic active site of FKBP12. Beginning with **1** as the minimal core structure, compounds **2-4** successively add in functionality to interact with the hydrophobic core of the immunophilin active site. Compound **1**, which contains only the minimal structural requirements for binding to FKBP12, is inactive as a rotamase inhibitor and is not neurotrophic. Compound **2** incorporates additional hydrophobic alkyl functionality to mimic part of the pyranose ring, as described by Holt et al., is slightly more potent as an FKBP12 inhibitor, and manifests extremely weak neurotrophic activity. Compound **3**, with the addition of the phenylpropyl ester group to mimic the cyclohexylethyl portion of FK506, manifests a significant increase in enzyme inhibitor activity, and elicits neurite growth effects in cultured neurons in a dose dependent manner. Compound **4**, with additional hydrophobic substitution on the aryl ring, is quite potent both as a rotamase inhibitor and neurotrophic agent.

These simple compounds are FKBP12 ligands which lack the effector domain functionality required for interaction with calcineurin, and thus should be incapable of eliciting immunosuppressive effects.[12,15] Compounds

3 and **4** were tested for their ability to inhibit calcineurin phosphatase activity and to block T-cell proliferation in standard in vitro assays. Neither compound inhibited either calcineurin activity or T-lymphocyte proliferation at concentrations greater than 10 μM. Compound **14** has likewise been reported to lack immunosupressive activity.[15] These results suggest that the immunosuppressant effects of FK506 may be cleanly separated from the neurotrophic effects in small molecule drug design.

Table 1.



Cpd. No.	R	R'	K_i, nM	ED_{50}, nM
1	OMe	Et	>10,000	>10,000
2	1,1-Dimethylpropyl	Et	1300	5000
3	1,1-Dimethylpropyl	3-Phenylpropyl	250	300
4	1,1-Dimethylpropyl	(3,4,5)-Trimethoxyphenylpropyl	25	80
5	1,1-Dimethylpropyl	1,7-Diphenyl-4-heptyl	30	5
6	1,1-Dimethylpropyl	4-(*p*-Methoxyphenyl)-1-butyl	60	43
7	1,1-Dimethylpropyl	1-(*p*-Methoxyphenyl)-6-phenyl-3-hexyl	15	0.17
8	1,1-Dimethylpropyl	1,5-Diphenyl-3-pentyl	20	0.016
9	tert-Butyl	3-Phenylpropyl	2	0.085
10	tert-Butyl	4-(*p*-Methoxyphenyl)-1-butyl	24	0.002
11	tert-Butyl	1,7-Diphenyl-4-heptyl	5	0.085
12	1,1-Dimethylpropyl	3,3-Diphenylpropyl	8.3	0.029
13	Phenyl	1,7-Diphenyl-4-heptyl	3	0.15
14	(3,4,5-Trimethoxy)-Phenyl	1,7-Diphenyl-4-heptyl	1	0.61

The ability of simple FKBP12 ligands to function as highly potent, nonpeptidic mimetics of proteinaceous growth factors is emphasized by the potent neurotrophic activities of compounds **7-14**. These simple molecules are potent inhibitors of FKBP12; more strikingly, they are effective neurotrophic agents in subnanomolar concentrations. These compounds consistently and in a dose-dependent fashion promote neurite extension from DRG cultures at picomolar concentrations, *in the absence of exogenously added growth factors*. Figure 2 shows photomicrographs of sensory neurons treated with varying concentrations of compound **11**. Figure 3 depicts the dose response curve for **11**. The maximal neurotrophic effect of **11** in these cultures, evaluated as the number of processes elicited from the explant, ranged from about 120-130 neurites. This value is comparable to the maximal neurite outgrowth elicited in these cultures by treatment with 25-50 ng/mL of nerve growth factor (NGF). (NGF at this dose typically elicits ca. 140-150 processes per explant in this assay.)

The efficacy of these compounds as neuroprotective and neuroregenerative agents has been demonstrated in vivo in an animal model of Parkinson's Disease. N-methyl-4-phenyl-tetrahydropyridine (MPTP) lesioning of dopaminergic neurons in mice was used as an animal model of Parkinson's Disease.[17] Four week old male CD1

white mice were dosed i.p. with 30 mg/kg of MPTP for 5 days. Compounds **8**, **9**, **10** and **11** (40 mg/kg), or vehicle, were administered s.c. along with the MPTP for 5 days, as well as for an additional 5 days following cessation of MPTP treatment. At 18 days following MPTP treatment, the animals were perfused and the brains were fixed, cryoprotected and sectioned. Immunostaining was performed on saggital and coronal brain sections using anti-tyrosine hydoxylase Ig to quantitate survival and recovery of dopaminergic neurons. In animals treated with MPTP and vehicle, a substantial loss of 60-70% of functional dopaminergic terminals was observed as compared to non-lesioned animals. Lesioned animals receiving neuroimmunophilin ligands showed a striking recovery of TH-stained striatal dopaminergic terminals, as compared with controls (Figure 4). Compounds **8**, **9**, **10**, and **11** produced recovery of striatal TH-immunostaining to 71%, 32%, 59%, and 44%, respectively, of control (non-lesioned) animals.

These experiments establish that ligands for the immunophilin FKBP12 exert powerful neuroprotective and neuroregenerative effects in vivo, and that these compounds comprise a novel class of systemically active small molecule growth factor mimetics. These effects are independent of the calcineurin interactions of FK506-FKBP12, and appear to reside in the FKBP-binding domain portion of FK506. It is thus possible to separate the immunosuppressant effects from the neurotrophic effects of FK506 on a clear structural basis, and to design compounds which are powerful neuroregenerative agents but lack the toxicity associated with the calcineurin-mediated effects of FK506.

A particularly striking feature of these FKBP12 ligands is their extraordinary neurotrophic potency. Compounds such as **8-13** have potencies to promote neurite outgrowth 10-100 times greater than their K_is to inhibit FKBP12 rotamase activity. We have observed this dichotomy in a number of structural series of FKBP12 ligands, although in all cases compounds that do not bind to FKBP12 and significantly inhibit its rotamase activity are ineffective as neurotrophic agents. Given the high cellular concentration of FKBP12 in neurons (ca. 500 nM), and the striking divergence of K_i and ED_{50} for the most potent compounds, it is unlikely that simply inhibiting FKBP12 rotamase activity is responsible for the observed neurotrophic effects. In the original studies of the mechanism of immunosuppression by FK506, the low drug concentrations at which immunosuppresant effects were elicited relative to FKBP12's cellular concentration in T-lymphocytes led to the hypothesis that FK506 induced a gain of function for FKBP12, culminating in the identification of calcineurin as the target of the drug-protein complex. Although it seems clear that calcineurin inhibition is not involved in the neurotrophic effects of these drugs, a similar gain of function for FKBP12 upon ligand binding may be responsible. It is known that, in addition to its enzymatic role in folding proteins, FKBP12 also functions as a modulatory member of a number of molecular complexes, including ion channels and steroid and growth factor receptors.[18] Binding of the ligands may be facilitating or disrupting protein-protein interactions in a neuronal signalling pathway. While we have not evaluated the present compounds with respect to binding to other receptors or FKBP isoforms, we have investigated these possibilities for a particularly potent member of a related series of FKBP12 ligands whose in vivo activity we have recently described. GPI1046 (3-(3-pyridyl)-1-propyl (2*S*)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinecarboxylate), a high affinity FKBP12 ligand and potent neurotrophic agent in vitro and in vivo, is at least 100-fold more selective for FKBP12 over FKBP13, -25 and -52. GPI 1046 was also found to have no appreciable activity in over 200 receptor-binding assays and over 30 enzyme assays. Taken together, these results suggest that liganding of FKBP12 is important for the neurotrophic effects of these compounds, but the subsequent events in the mechanistic pathway remain to be elucidated. The mechanism of action of these and related compounds is under extensive investigation in our laboratories.

The neurotrophic effects of these compounds are comparable in potency to neurotrophic growth factors such as NGF and related proteins. Small molecule FKBP ligands such as these hold promise as a new class of therapeutic agents for the treatment of degenerative disorders of the nervous system. Unlike proteinaceous growth factors, these molecules readily cross the blood brain barrier and possess systemic activity. The therapeutic utility of these and related compounds is under intensive investigation and will be described in due course.

Figure 2. Compound 11 promotes neurite outgrowth in chick sensory neurons.

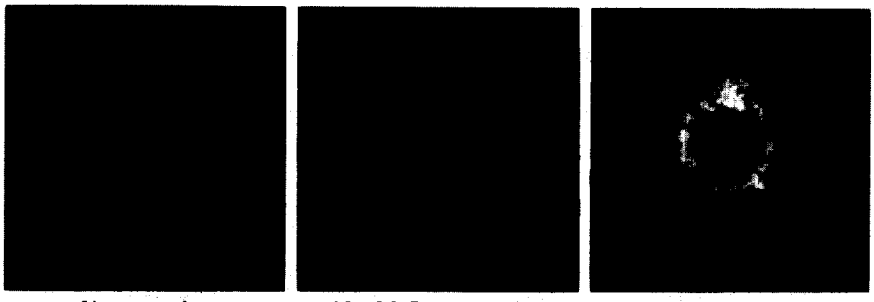


Figure 3. Compound 11 elicits neurite outgrowth in a dose dependent manner.

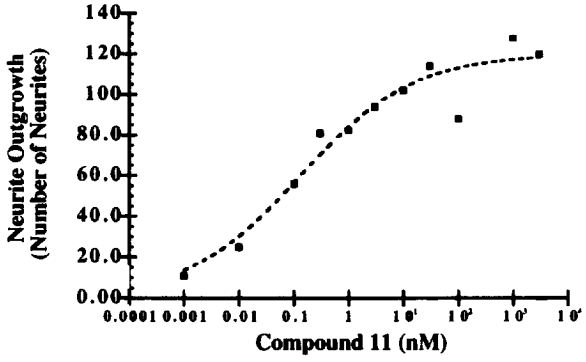
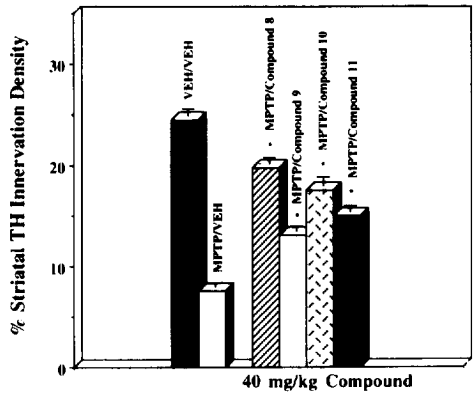


Figure 4. Protection of nigral striatal dopamine neurons from MPTP-toxicity by concurrent treatment with neuroimmunophilin ligands.



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