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FK1706, a novel non-immunosuppressive immunophilin: neurotrophic activity and mechanism of action

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Abstract

Immunophilin ligands are neuroregenerative agents, characterized by binding to FK506 binding proteins (FKBPs), which stimulate recovery of neurons in a variety of injury paradigms. Here we report the discovery of a novel, non-immunosuppressive immunophilin ligand, FK1706. FK1706, a derivative of FK506, showed similarly high affinity for two FKBP subtypes, FKBP-12 and FKBP-52, but inhibited T-cell proliferation and interleukin-2 cytokine production with much lower potency and efficacy than FK506. FK1706 (0.1 to 10 nM) significantly potentiated nerve growth factor (NGF)-induced neurite outgrowth in SH-SY5Y cells, as did FK506. This neurite potentiation could be blocked by an anti-FKBP-52 antibody, as well as by specific pharmacological inhibitors of phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K), and the Ras/Raf/Mitogen-Activated Protein Kinase (MAPK) signaling pathway. FK1706 also potentiated NGF-induced MAPK activation, with a similar dose-dependency to that necessary for potentiating neurite outgrowth. Taken together, these data suggest that FK1706 is a non-immunosuppressive immunophilin ligand with significant neurotrophic effects, putatively mediated via FKBP-52 and the Ras/Raf/MAPK signaling pathway, and therefore that FK1706 may have therapeutic potential in a variety of neurological disorders.

Keywords: Immunophilin; Nerve growth factor; MAP kinase; Neurite outgrowth

1. Introduction

Nerve growth factor (NGF) and other members of the neurotrophin family are critical for the survival and differentiation of neurons within the peripheral and central nervous systems. Although the therapeutic potential of neurotrophins has generated much excitement in the past decade, inconvenient pharmacokinetics (necessity of injection and poor brain permeability), as well as non-specific effects and toxicity, have limited the clinical usefulness of systemic application of neurotrophic factors. Compounds that enhance neurotrophin signaling and overcome these other barriers might show greater therapeutic potential.

Tacrolimus (FK506; [3*S*-[3*R**[*E*(1*S**,3*S**,4*S**)], 4*S**,5*R**,8*S**,9*E*,12*R**,14*R**,15*S**,16*R**,18*S**,19*S**,26a*R**]]-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylethenyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(2-propenyl)-15,19-epoxy-3H-pyrido [2,1-*c*] [1,4] oxaazacyclotricosine-1,7,20,21(4H,23H)-tetrone) is a potent immunosuppressive drug used after organ transplantation. FK506 activity is mediated by binding to members of the FK506-binding proteins (FKBPs), creating

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a complex that binds to and inhibits the calcineurin phosphatase activity mediated by Ca^{2+}/calmodulin, thus preventing T-cell activation (Liu et al., 1992). In addition to its function in the immune system, FK506 also exhibits neuroprotective and neurotrophic effects, including stimulation of axonal regrowth and enhancement of functional recovery in a variety of neurodegenerative disease models (for review see Gold, 2000). Although the exact mechanism of this activity is not clear, binding to two members of the FKBP family, FKBP-12 and FKBP-52, are particularly important in mediating this effect (Costantini et al., 2001; Gold et al., 1997, 1999; Guo et al., 2001; Klettner et al., 2001; Steiner et al., 1997; Tanaka et al., 2002; for review see Gold and Villafranca, 2003). Despite the therapeutic potential of FK506 in ameliorating neuronal injury, its immunosuppressive activity limits chronic use in patients. Recently, however, it has become clear that non-immunosuppressive FKBP ligands can still show efficacy in neuronal injury paradigms, suggesting that this clinical limitation can be overcome (Gold et al., 1997; Guo et al., 2001; Zhang et al., 2001).

Immunophilin ligands have been shown to potentiate NGF-induced neurite outgrowth in SH-SY5Y neuroblastoma cells, providing a convenient model system for understanding the signaling interactions and neurotrophic potential of these compounds. Using this system, we evaluated the neurotrophic potential of a rationally-designed, non-immunosuppressive derivative of FK506 in which the calcineurin binding motif had been modified. Here we characterize this novel FKBP ligand, (1*R*,9*S*,12*S*,13*R*,14*S*,17*R*,18*E*,21*S*,23*S*,24*R*,25*S*,27*R*)-1,14-dihydroxy-12-{(*E*)-2-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl}-23,25-dimethoxy-13,19,21,27-tetramethyl-17-(2-oxopropyl)-11,28-dioxa-4-azatricyclo[22.3.1.$0^{4,9}$]octacos-18-ene-2,3,10,16-tetrone (FK1706, FR131706; chemical structure in Fig. 1). We also used specific inhibitors of signaling molecules from the NGF pathway to further delineate the signal transduction pathways responsible for the neurite potentiation properties of FK1706, and show that FK1706 potentiates NGF-induced MAPK activation.

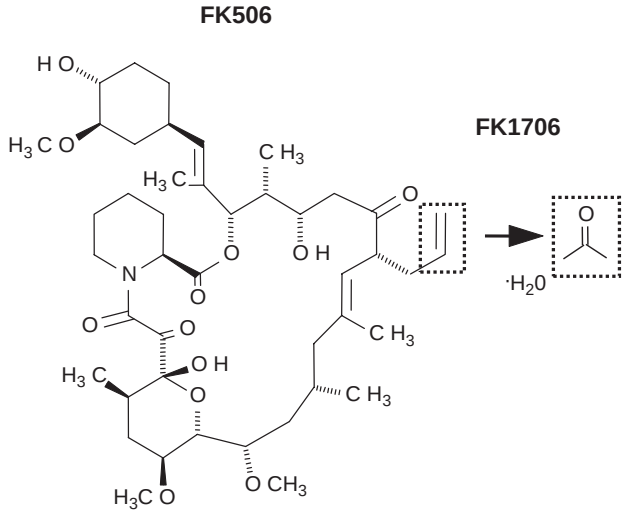

Fig. 1. Chemical structure of FK1706. Boxed area indicates changed moiety from FK506.

2. Materials and methods

2.1. Chemicals

FK1706 and FK506 were synthesized at Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO. NGF was purchased from Promega (Madison, WI). *N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) and aphidicolin were purchased from Sigma (St. Louis, MO). Lovastatin was purchased from Calbiochem (La Jolla, CA). 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene (U0126), 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), and 2-2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one (PD98059) were purchased from Cell Signaling Technology (Beverly, MA). All other inhibitors (Bisindolylmaleimide IX,methanesulfonate salt (Ro 31-8220); 1-[6-[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122); 1-(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione (U73343); anthra(1,9-cd)pyrazol-6(2H)-one 1,9-pyrazoloanthrone (SP600125); 3-(3,5-dibromo-4-hydroxybenzylidene)-5-iodo-1,3-dihydroindol-2-one (GW 5074); and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580)) were purchased from Biomol (Plymouth Meeting, PA).

2.2. FKBP binding assay

Dihydro-FK506, [propyl-^{3}H]([^{3}H]FK506, specific activity 76.9 Ci/mmol) was purchased from PerkinElmer (Wellesley, MA). The recombinant human FKBP-12 and FKBP-52 were produced in our laboratories. FKBP binding assays were carried out in siliconized 96-well plates in a final volume of 100 μl. Two nM of FKBP-12 or 10 nM of FKBP-52 was incubated for 120 min at room temperature with various concentrations of [^{3}H]FK506 in the presence or absence of 1 or 10 μM of unlabeled FK506, for FKBP-12 and FKBP-52, respectively. [^{3}H]FK506 stock solution was dried down in a siliconized tube and dissolved in assay buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), pH 7.2, containing 0.015% Triton X-100). Various concentrations of [^{3}H]FK506 were obtained by further dilutions of the stock solution in the assay buffer. Following incubation, 50 μl of the reaction mixture was applied to MicroSpin G-25 column (Amersham Biosciences, Piscateway, NJ), which had been equilibrated with the assay buffer, to separate bound radioligand from free radioligand. Radioactivities of the collected fractions

excluded from the MicroSpin G-25 columns were counted. Specific binding was defined as the portion of [^{3}H]FK506 binding that was displaced by 1 or 10 μM unlabeled FK506. Total incubated [^{3}H]FK506 was directly counted in aliquots (50 μl) of incubates. Data were analyzed by Scatchard plot to estimate K_d values.

For the competition experiments, binding assays were carried out using 2 nM (for FKBP-12) or 30 nM (for FKBP-52) [^{3}H]FK506 in the presence of various concentrations of FK506 or FK1706. The IC_{50} values of both drugs for each protein were corrected to the K_i values using the K_d values of [^{3}H]FK506 for each protein according to the following equation:

$$K_i = IC_{50}/(1 + [\text{radioligand}]/K_d)$$

where [radioligand] means concentration of the radioligand used.

2.3. Immunosuppression assays

2.3.1. Human peripheral blood mononuclear cells (PBMC) preparation

PBMCs were prepared from normal healthy volunteers by density gradient centrifugation using Ficoll-Paque plus (Amersham Pharmacia Biotech, Tokyo, Japan). Human blood was diluted with an equal volume of Roswell Park Memorial Institute medium (RPMI1640) and overlaid on Ficoll-Paque plus. After centrifugation for 30 min at room temperature, PBMCs were collected, washed with RPMI1640, and resuspended in RPMI1640 containing 2.5% human type AB serum, 2-mercaptoethanol, and antibiotics.

2.3.2. PBMC proliferation and cytokine assays

For the proliferation assay, PBMCs were stimulated with irradiated (2000R) PBMCs from distinct volunteers (1×10^5 cells/well) in 96-well flat bottom microplates. After 7 days in culture under 5% CO_2 in air, cells were labeled for 6 h with 10 kBq/well [^{3}H]thymidine (Moravek Biochemicals, Brea, CA). Cells were harvested and incorporated radioactivity was measured using a liquid scintillation counter. For the cytokine assay, PBMCs were stimulated with 10 μg/ml of concanavalin A (Sigma, St. Louis, MO) and cultured for 1 day in 96 well flat bottom microplates under 5% CO_2 in air. Cells were harvested by centrifugation and supernatants were collected. Human interleukin-2 concentrations in these supernatants were determined by enzyme-linked immunosorbent assay (ELISA; OptEIA™ set, BD-Biosciences, San Jose, CA). For these experiments, FK1706 and FK506 were dissolved in ethanol and diluted with RPMI-1640 media with 2.5% human type AB serum, 2-mercaptoethanol, penicillin and streptomycin. For the mixed lymphocyte reaction, weak responders

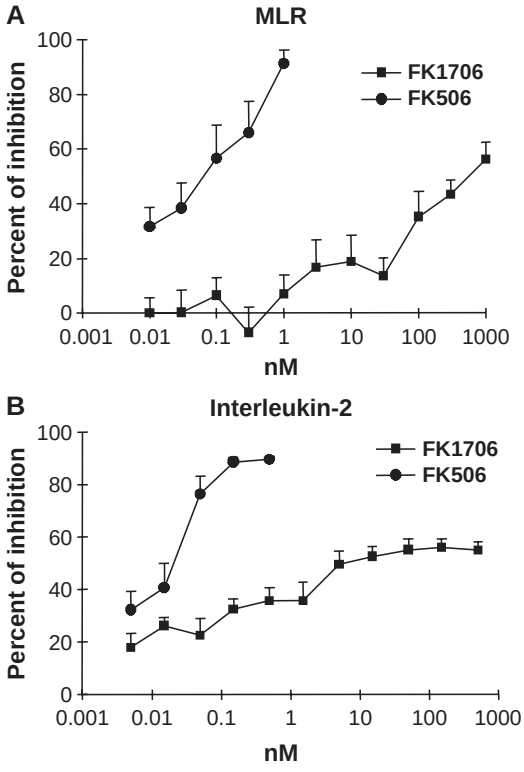

Fig. 2. FK1706 is a non-immunosuppressive immunophilin ligand. Measurement of FK506- and FK1706-mediated inhibition of T-cell activation using two assays: (A) measurement of proliferation using the mixed lymphocyte reaction (MLR) and (B) mitogen-stimulated cytokine production (interleukin-2). Data are mean±S.E.M.; n=12 (MLR) or n=6 (interleukin-2).

Table 1
FK1706 binds FKBPs with similar affinity to FK506, but inhibits T-cell proliferation and interleukin-2 cytokine production with much lower potency and efficacy

			FK1706	FK506
FKBP binding affinity	FKBP-12 (K_i, nM)		0.5	0.5
	FKBP-52 (K_i, nM)		14	15
Immunosuppression	Proliferation	IC_{50} (nM)	560	0.05
		Max % inhibition	56	91
		Max efficacy (nM)	1000	1
	Interleukin-2 production	IC_{50} (nM)	46	0.02
		Max % inhibition	56	90
		Max efficacy (nM)	150	0.5

In vitro FKBP binding and measurement of inhibition of T-cell activation using the mixed lymphocyte reaction (MLR) and mitogen-stimulated cytokine production (interleukin-2). Data represent means; n=3 (binding), n=11–12 (MLR), or n=6 (interleukin-2).

(defined as exhibiting a non-significant difference (Student's *t* or Aspin-Welch) between stimulated and non-stimulated conditions) were omitted.

2.4. Neurotrophic assay

2.4.1. Preparation of SH-SY5Y neuroblastoma cell cultures

SH-SY5Y human neuroblastoma cells (ATCC, Manassas, VA) were plated onto 6-well plates at 6×10^4 cells/well and treated with 0.4 μM aphidicolin (in DMEM/15% Fetal Bovine Serum/1% penicillin/streptomycin by volume) for 5 days beginning the day after plating. Cells were then treated with NGF (10 ng/ml) alone or with FK1706 or FK506 and maintained in media containing 0.4 μM aphidicolin. Pharmacological inhibitors were added 48 h later. Neurite length was measured 48 h later (i.e., 96 h after initial treatment with NGF). In the experiments with FKBP-52 antibody, the cells were permeabilized by co-treatment with the antibody and saponin (15 μg/ml) for 10 min followed by the addition of FKBP-52 antibody alone (50 ng/ml C-terminal antibody; Santa Cruz Biotechnology, Santa Cruz, CA) with FK1706 and afterwards neurite length was measured 96 h after the treatment. Controls (i.e., those cells treated with NGF alone) were also treated with saponin. Conditions for the non-immune immunoglobulin G (IgG) were identical, except that 50 ng/ml IgG was added instead of anti-FKBP-52 antibody.

2.4.2. Analysis of neurite length in SH-SY5Y neuroblastoma cells

SH-SY5Y neuroblastoma cells develop axonal-like processes upon treatment with NGF. For analysis of process length, cells (at least 20/well) were selected and photographed at 96 h after initiation of NGF treatment. Neurite lengths were measured using WinRoof software (Mitani Corporation, Fukui, Japan) running on an IBM XT computer; only processes more than twice the cell body length and only one process per cell were measured. Statistical analysis was performed using Student's *t*-test for comparison of two groups and one-way analysis of variance (ANOVA) followed by either Tukey's or Dunnett's multiple comparisons post-test for comparison of multiple groups. *P* value less than 0.05 was set as the level of significance.

2.5. Biochemical assay

2.5.1. Analysis of MAP kinase activation in SH-SY5Y neuroblastoma cells (ELISA)

SH-SY5Y cells were plated in 96-well plates at 2.4×10^4 cells/well. From the next day, cells were treated

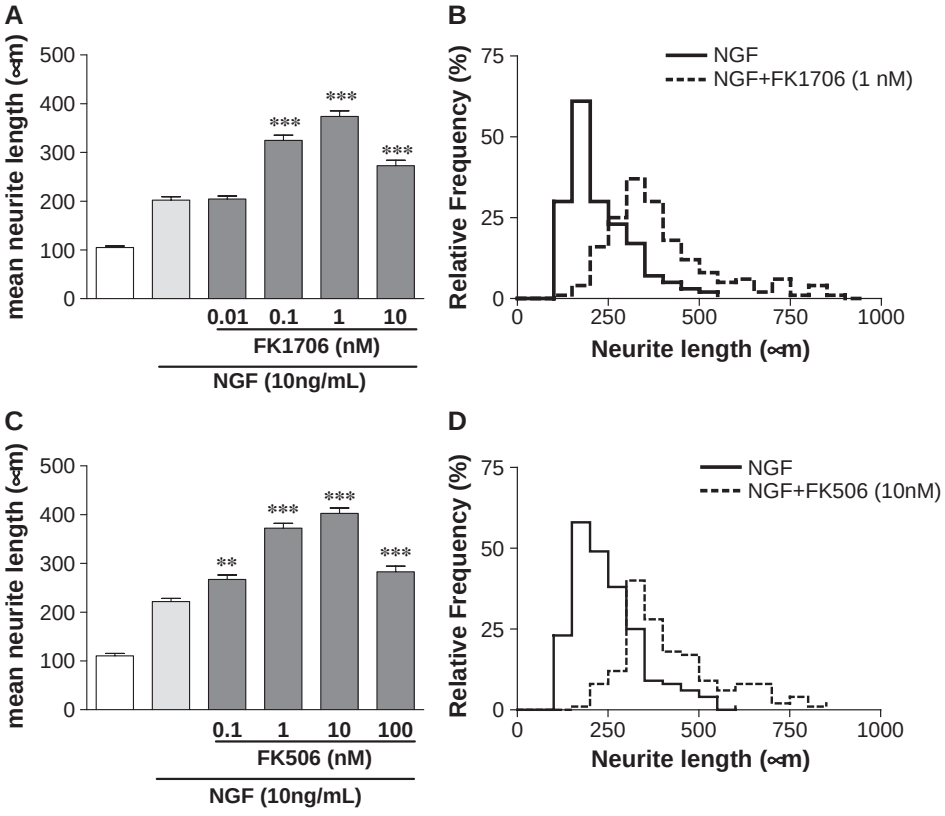

Fig. 3. Immunophilin ligands potentiate NGF-induced neurite outgrowth. FK1706 (A) and FK506 (C) potentiate NGF-induced neurite outgrowth. Neurite length was measured 96 h after NGF treatment was initiated. Data are mean±S.E.M. values of at least three independent experiments. **, ***P<0.01, 0.001 vs. NGF by one-way ANOVA with Tukey's post-test. (B, D) Relative frequency histogram comparing the distribution of individual neurite lengths following NGF treatment alone or with the maximally effective dose of immunophilin ligand.

in normal serum-containing media with 0.4 μM aphidicolin for 72 h, then washed and incubated for 16 h in reduced serum (1% FBS) media with 0.4 μM aphidicolin. Cells were stimulated for 30 min (37 °C) by the addition of NGF and FK1706 in reduced-serum media, and the reaction was terminated by removal of media and addition of 100 μl of 4% paraformaldehyde in PBS. Measurement of phosphorylated ERK-42/44 MAP kinase, total ERK, and cell counting was then conducted using the Fast Activated Cell-based ELISA (FACE) ERK-1/2 kit (Active Motif, Carlsbad, CA) as per the manufacturer's instructions.

3. Results

3.1. FK1706 and FK506 have similar binding affinity for FKBP-12 and FKBP-52

We first compared the binding affinity of FK506 and FK1706 for human FKBP-12 and FKBP-52. Scatchard analysis of the binding data estimated the K_d for [^{3}H]FK506 and human FKBP-12 or FKBP-52 to be 1.33 nM and 84.3 nM (n=3), respectively. FK1706 and FK506 displaced [^{3}H]FK506 in a concentration-dependent manner, and both showed similar binding affinities for FKBP-12 and FKBP-52 (Table 1).

3.2. FK1706 lacks immunosuppressive activity

We compared the immunosuppressive activity of FK1706 with FK506 in human PBMCs. FK506 showed strikingly potent immunosuppressive actions in this assay for both T-cell proliferation and interleukin-2 cytokine production. Despite a similar binding affinity for FKBP-12, which is known to mediate the immunosuppressive activity of FK506 (Steiner et al., 1992), FK1706 inhibited T-cell proliferation and interleukin-2 cytokine production with much lower potency and efficacy than FK506, indicating FK1706 possesses minimal immunosuppressive activity (Fig. 2, Table 1).

3.3. FK1706 potentiates NGF-induced neurite outgrowth in SH-SY5Y cells

We next determined the dose–response relationship of FK1706 for potentiating NGF-induced neurite outgrowth in SH-SY5Y cells, an established model system for studying immunophilin function (Gold et al., 1997, 1999; Price et al., 2003). NGF significantly potentiated neurite outgrowth (P<0.001), and FK1706 (0.1–10 nM) significantly increased NGF-induced neurite outgrowth with a bell-shaped dose–response relationship, with maximum efficacy at 1 nM (Fig. 3A). FK1706 and NGF co-treatment dramatically shifted the distribution of neurite lengths to the right compared with NGF alone, indicating growth of longer neurites (Fig. 3B). FK1706 had no effect on neurite outgrowth alone (data not shown). FK1706 was more potent at potentiating NGF-induced neurite outgrowth than FK506 (showed maximum efficacy at a lower dose, but with no difference in maximum efficacy; Fig. 3C,D).

3.4. FKBP-52 antibody blocks the neurite outgrowth potentiation of FK1706

We then determined the involvement of FKBP-52 in mediating this neurite potentiation using a monoclonal FKBP-52 antibody that does not interact with FKBP-12. The FKBP-52 antibody significantly blocked the ability of

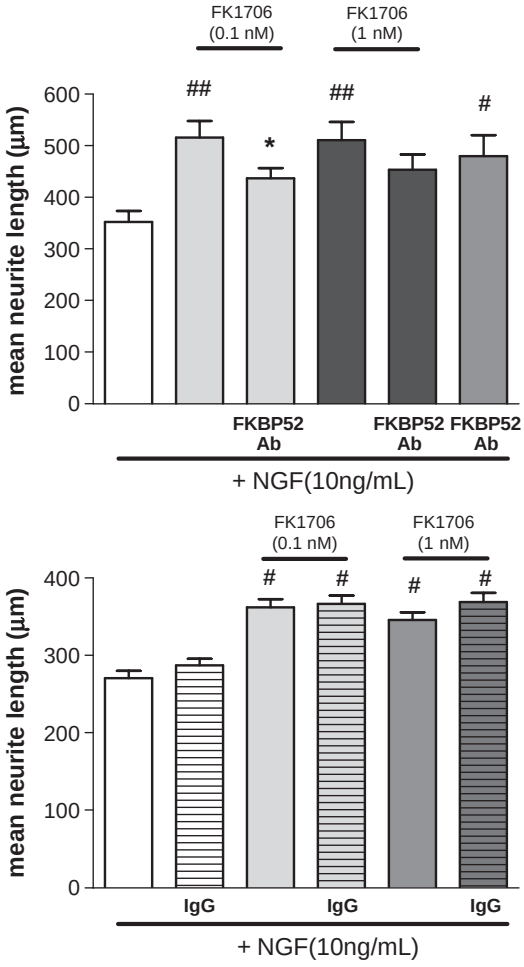

Fig. 4. FKBP-52 antibody blocks FK1706-potentiated neurite outgrowth. (A) Cells were permeabilized by co-treatment with saponin (15 μg/ml) for 10 min followed by the addition of FKBP-52 antibody (50 ng/ml) with FK1706, with neurite length measured 96 h after the treatment. Neurite lengths are decreased in cells treated with FKBP-52 antibody and FK1706 (0.1 or 1 nM) in the presence of NGF (10 ng/ml) compared with NGF plus FK1706 groups. (B) Non-immune IgG (50 ng/ml) does not affect NGF- or FK1706-induced neurite outgrowth. $^{\#, \#\#}P$<0.05, 0.01 vs. NGF by ANOVA followed by Dunnett's; *P<0.05 vs. FK1706 by Student's t-test.

FK1706 (0.1 nM) to potentiate NGF-induced neurite outgrowth from SH-SY5Y cells (Fig. 4A), whereas non-immune goat IgG had no effect on either NGF- or FK1706-induced neurite outgrowth (Fig. 4B).

3.5. The Raf/Ras/MAPK pathway is involved in FK1706-potentiated neurite outgrowth

We next wanted to determine the role of MAP kinase kinase (MEK1/2)/mitogen-activated protein kinase (ERK) in mediating FK1706 activity. We exposed differentiated SH-SY5Y cells to the MEK 1/2 inhibitors PD98059 and U0126, in the presence of NGF with or without FK1706. Both inhibitors (at 10 μM) blocked FK1706-potentiated neurite outgrowth with no effect on neurite length following NGF treatment alone (Fig. 5A, Table 2). However, higher concentrations of these inhibitors (20 μM) significantly inhibited NGF-induced neurite outgrowth (Price et al., 2003), confirming the importance of MEK 1/2 in NGF-induced neuritogenesis.

A number of signal transduction pathways converge on MEK/ERK. Therefore, to determine whether FK1706 interacted with the NGF signaling pathway upstream of MEK, we determined the involvement of the upstream signaling molecules Ras and Raf by using specific inhibitors for these molecules (GW5074 (1 μM) for Raf (Lackey et al., 2000); lovastatin (5 μg/ml) which inhibits Ras isoprenylation (Reusch et al., 1995)). Both inhibitors blocked the neurotrophic activity of FK1706 (Fig. 5B), without inhibiting NGF-induced neurite out-

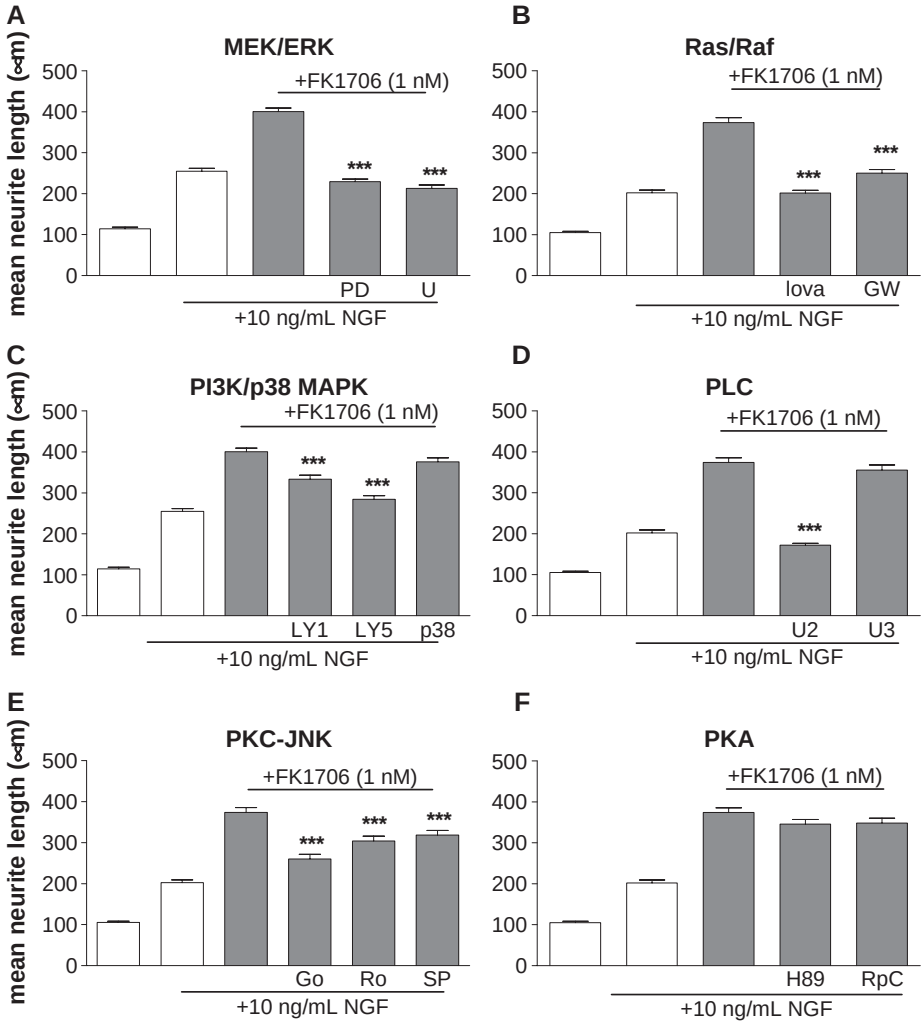

Fig. 5. FK1706 potentiates NGF-induced neurite outgrowth via the Ras/Raf/MAP kinase pathway and involves PLC and PI3K signaling. (A) The MEK 1/2 inhibitors, U0126 (U; 10 μM) or PD98059 (PD; 10 μM), block the neurotrophic activity of FK1706. (B) Inhibitors of Raf (lovastatin, lova, 5 μg/ml) or Ras (GW5074, GW, 1 μM) block the neurotrophic activity of FK1706. (C) The PI3K inhibitor LY294002 (LY1, 1 μM; LY5, 5 μM) blocks FK1706-potentiated neurite outgrowth, but the p38 MAP Kinase inhibitor SB203580 (SB; 10 μM) has no effect. (D) The PLC inhibitor U73122 (U2; 1 μM), but not the inactive analogue U73343 (U3; 1 μM), blocks FK1706-potentiated neurite outgrowth. (E) Inhibitors of PKC, Go6983 (Go; 1 μM) and Ro31-8220 (Ro; 1 μM), as well as the JNK inhibitor SP600125 (SP; 1 μM), modestly inhibited FK1706-potentiated neurite outgrowth. (F) Inhibitors of PKA, H89 (5 μM) and RpcAMPs (RpC; 10 μM), do not block FK1706 activity. Neurite length was measured 96 h after NGF treatment was initiated, with inhibitors present during the final 48 h. Data are mean±S.E.M. values of three independent experiments. ***P<0.001 vs. NGF+FK1706 by one-way ANOVA with Tukey's post-test.

growth (Table 2). Taken together, these data suggest that the Ras/Raf/MAPK pathway is necessary for FK1706-potentiated neurite outgrowth.

3.6. Signaling molecules proximal to the neurotrophic tyrosine kinase receptor (trkA) are involved in FK1706-potentiated neurite outgrowth

In addition to the Ras/Raf/MAPK pathway, other signaling molecules are also activated upon addition of NGF, including phospholipase C (PLC), protein kinase C (PKC), protein kinase A (PKA) and c-Jun N-terminal kinase (JNK) (Sofroniew et al., 2001). Using the same pharmacological inhibitor strategy, we tested whether these molecules were involved in the mechanism of action of FK1706. The PI3K inhibitor, LY294002 (Vlahos et al., 1994), but not the p38 MAP Kinase inhibitor, SB203580 (Cuenda et al., 1995), blocked FK1706-potentiated neurite outgrowth (Fig. 5C). The PLC inhibitor, U73122, effectively blocked FK1706 activity, whereas the inactive analog, U73343, had no effect (Fig. 5D). Two inhibitors of PKC, Ro 31-8220 and Go6983, and the JNK inhibitor, SP600125 (Han et al., 2001) slightly but significantly inhibited FK1706 activity (Fig. 5E). Two inhibitors of PKA, H-89 and RpcAMPs, did not affect FK1706 activity (Fig. 5F).

Table 2
Effect of pharmacological inhibitors on neurite outgrowth

Inhibitor treatment	Target	Concentration	Neurite length (μm)	
			Inhibitor alone	Inhibitor+ NGF
Experiment 1				
None			115.2±5.4	254.8±6.9
LY294002	PI3K	1 μM	117.7±5.2	236.8±7.5
		5 μM	109.2±3.3	252.6±8.5
PD98059	ERK	10 μM	116.7±4.0	230.7±7.2
U0126	ERK	10 μM	97.5[a]±3.0	231.9±9.3
SB203580	p38 MAPK	10 μM	124.2±4.3	232.2±7.1
Experiment 2				
None			105.0±3.3	202.1±6.8
Lovastatin	Ras	5 μg/ml	115.5±2.7	171.2±5.3
GW5074	Raf	1 μM	110.5±3.3	202.5±8.6
U73343	PLC	1 μM	108.2±2.9	193.0±6.9
U73122	PLC	1 μM	106.1±2.3	180.1±5.7
Ro 31-8220	PKC	1 μM	109.4±3.1	199.0±6.6
Go6983	PKC	1 μM	103.8±2.5	183.1±9.3
SP600125	JNK	1 μM	114.5±3.1	200.8±5.8
H-89	PKA	5 μM	118.7±3.5	270.6[b]±8.6
Rp-cAMPs	PKA	10 μM	126.3±4.7	260.5[b]±9.7

Data represent length of the longest neurite on SH-SY5Y human neuroblastoma cells treated with or without NGF for 96 h, with inhibitor treatments during the last 48 h. Data are mean neurite length (μm)±S.E.M. from three independent experiments. Adapted with permission from Price et al. (2003).

[a] Significantly different than no inhibitor treatment ($P<0.05$).

[b] Significantly different than NGF alone, ($P<0.05$).

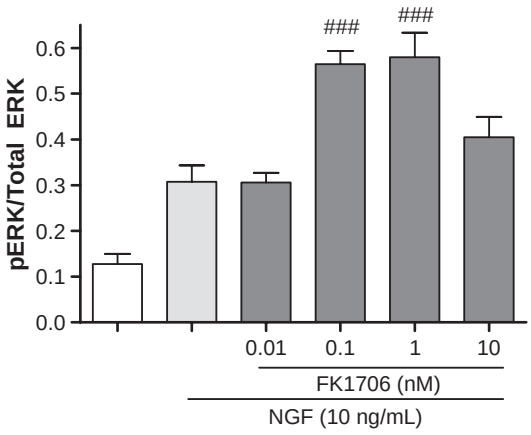

Fig. 6. FK1706 potentiates NGF-induced ERK-1/2 phosphorylation. ELISA data showing normalized (to total ERK-1/2) levels of phosphorylated (active) ERK-1/2. Both levels of p42/44 and total ERK were normalized to cell number within each well. Cells were incubated in reduced serum media overnight, and p42/44 ERK was measured 30 min after NGF treatment was initiated. Data are mean±S.E.M. values (n=5) of a single experiment representative of two independent experiments. $^{###}P<0.001$ vs. NGF by one-way ANOVA with Tukey's post-test.

3.7. FK1706 potentiates NGF-induced activation of MAP kinase

To directly confirm the role of MAP kinase in FK1706-mediated potentiation of NGF signaling, we measured the level of active (phosphorylated) MAP kinase following stimulation with NGF alone or with FK1706. Quantitative ELISA analysis demonstrated that FK1706 significantly potentiated p42/44 (ERK-1/2) MAP kinase phosphorylation relative to NGF alone, with a similar dose-dependency to that seen for potentiating neurite outgrowth (Fig. 6).

4. Discussion

Immunophilin ligands show significant promise as treatments for nerve injury and neurological disease. However, current clinically available immunophilins (e.g., FK506, cyclosporine A, and rapamycin) are immunosuppressive, thus restricting their long-term clinical use. The immunosuppressive effects of immunophilins are mediated by binding to FKBP-12 (Steiner et al., 1992). Subsequent inhibition of calcineurin by this immunophilin/FKBP-12 complex leads to inhibition of cytokine synthesis (Liu et al., 1992) and immunosuppression. However, neither calcineurin inhibition nor binding to FKBP-12 are necessary for the neurotrophic effects of immunophilins, suggesting that the nerve regeneration and immunosuppressive properties can be separated (Costantini et al., 2001; Gold et al., 1997, 1999; Guo et al., 2001; Klettner et al., 2001; Steiner et al., 1997; Tanaka et al., 2002). Thus, the goal of this work was to evaluate and characterize the novel non-immunosuppressive immunophilin ligand, FK1706.

Our finding that FK1706 did not dramatically inhibit lymphocyte proliferation or interleukin-2 production demonstrates that this compound is not immunosuppressive, at least at doses that potentiate neurite outgrowth. The observation that the NGF-potentiating activity of FK1706 can be disrupted by an anti-FKBP-52 blocking antibody suggests that this FKBP is important (but not exclusively involved) in mediating the neurotrophic effect of this compound, consistent with previous work with FK506 (Gold et al., 1999). Interestingly, the anti-FKBP-52 antibody also potentiated NGF-induced neurite outgrowth, as shown previously using a different anti-FKBP-52 antibody (Gold et al., 1999). Although the mechanism of this potentiation is unclear, the fact that the antibody is directed against a C-terminal sequences of FKBP-52, a portion of FKBP-52 that regulates interaction with hsp90 (Cheung-Flynn et al., 2003), suggests it is possible that the antibody directly blocks the interaction of FKBP-52 with hsp90, thus modulating the interaction of FKBP-52 and the cytoskeleton (e.g., Pratt et al., 1999).

It is interesting to note that both FK1706 and FK506 enhanced NGF-induced neurite outgrowth with a bell-shaped dose–response relationship, showing diminishing efficacy at higher doses, consistent with previous studies (Chang et al., 1995; Gold et al., 1999; Price et al., 2003). Importantly, FK1706 exhibits no neurotrophic effects alone but only enhances NGF-induced neurite outgrowth (FK1706-induced neurite extension is not observed in SH-SY5Y cells treated with aphidicolin alone, whereas aphidicolin and NGF act synergistically to induce differentiation, e.g., Jensen, 1987; LoPresti et al., 1992), suggesting that FK1706 functions through enhancing NGF signaling and not directly via activation of the NGF receptor (trkA). Furthermore, FK1706 also potentiated neurite outgrowth in hippocampal neurons (B.G. Gold and T. Yamaji, unpublished observation) suggesting that these effects are not cell-specific. Finally, FK1706 exhibited greater potency than FK506 (lower dose necessary for maximum efficacy), suggesting that FK1706 has similar potential for efficacy in animal models.

While our data suggests that FKBP-52 at least partly mediates the NGF-potentiating activity of FK1706, the molecules that serve as convergence points between FKBP-52 and the NGF signaling pathway are unknown. Therefore, we used specific pharmacological inhibitors of signaling molecules downstream of NGF stimulation to elucidate the mechanism by which FK1706 potentiates NGF-induced neurite outgrowth. At the concentrations tested with FK1706, these inhibitors did not inhibit neurite outgrowth after NGF treatment (Table 2), demonstrating the specificity of these signaling molecules in mediating the activity of FK1706. The critical role of these signaling molecules precludes complete inhibition under chronic conditions, and these concentrations reflect the highest non-cytotoxic dose in our chronic treatment conditions (data not shown). Furthermore, the fact that most inhibitors had functional consequences suggests that, even though inhibition might not have been complete, their target molecules play a role in FK1706 activity.

NGF binds to the high affinity protein tyrosine kinase receptor, TrkA, initiating several signaling pathways affecting both morphological and transcriptional targets (Huang and Reichardt, 2001). Our finding that inhibitors of the Ras/Raf/MEK/ERK pathway blocked FK1706-induced neurite potentiation demonstrates that this pathway is critical for FK1706 activity in SH-SY5Y cells, as has been shown for FK506 (Price et al., 2003). We also show that FK1706 potentiates NGF-induced ERK phosphorylation with a similar dose-dependency for potentiating neurite outgrowth, confirming the role that ERK signaling plays in FK1706 activity and demonstrating that the ability of immunophilin ligands to increase ERK activation occurs via a calcineurin-independent mechanism. The role of PLC-γ signaling in mediating the long-term activity of NGF is largely unknown, although it plays a role in NGF-induced gene expression (Choi et al., 2001). Our finding that the PLC inhibitor U73122, but not the inactive analog U73343, inhibited FK1706-potentiated neurite outgrowth suggests a role for PLC in differentiation downstream of FK1706 activity. NGF/TrkA stimulation of PI3K is involved in neurite outgrowth and differentiative signaling, and our results suggest that this protein is also important in the mechanism of FK1706-potentiated neurite outgrowth. Although the role of PKC in neurite outgrowth is complex, as both a PKC inhibitor (staurosporine) and activator (phorbol esters) induce differentiation and neurite outgrowth in SH-SY5Y cells (Leli et al., 1992), the apparently weak inhibitory effect that the PKC inhibitors Go6983 and Ro31-8220 had on FK1706 activity is in line with recent evidence that nuclear accumulation of active ERK requires functional PKC and is prevented by addition of the PKC inhibitor GF109203X (Olsson et al., 2000). However, the mechanism by which these inhibitors only partially inhibited FK1706-potentiated neurite outgrowth deserves further scrutiny. Inhibitors of JNK and p38 MAP kinases only weakly (JNK inhibitor) or did not affect (p38 MAPK inhibitor) FK1706 activity, consistent with findings that these kinases are stimulated by pro-inflammatory cytokines and cellular stress such as UV light and osmotic shock, but are not stimulated by neurotrophins (Mielke and Herdegen, 2000). Using two inhibitors of PKA activity with distinct mechanisms (Rp-cAMPS is a competitive inhibitor of cAMP binding, whereas H-89 is a competitive inhibitor of ATP binding), we show here that PKA activity is not required for FK1706-potentiated neurite outgrowth. Thus, while this data should be interpreted cautiously because of the indirect nature of inhibitor studies, these data suggest that FK1706 enhances neurite outgrowth through the Ras/Raf/MAP kinase signaling pathway downstream of PLC and PI3K.

Taken together, these data demonstrate that FK1706 represents a novel non-immunosuppressive immunophilin

ligand with significant neurotrophic activity. Our data indicate that FK1706 enhances NGF-induced neurite outgrowth via FKBP-52 and the Ras/Raf/MAP kinase signaling pathway downstream of PLC and PI3K. Furthermore, these results demonstrate FK1706 specifically enhances NGF signaling via a common growth factor signaling pathway (including activation of ERK), but has no neurotrophic properties alone, suggesting that non-specific stimulation of neurotrophin substrates would be reduced in vivo. Finally, these studies provide insight into the potential molecular mechanisms by which FK1706 might promote nerve regeneration in vivo, and further suggest that FK1706 has therapeutic potential for treating a variety of neurodegenerative diseases.

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