ELSEVIER

European Journal of Pharmacology 415 (2001) 173–180

ejp

www.elsevier.nl/locate/ejphar

Failure of GPI compounds to display neurotrophic activity in vitro and in vivo

Arnaud Bocquet, Geneviève Lorent, Bruno Fuks, Renée Grimée, Patrice Talaga, Jean Daliers, Henrik Klitgaard *

Preclinical CNS Research, UCB S.A. Pharma Sector, Chemin du Foriest, B-1420 Braine-l'Alleud, Belgium

Received 2 November 2000; received in revised form 8 February 2001; accepted 13 February 2001

Abstract

The aim of this study was to evaluate the neurotrophic and neuroprotective properties of a series of immunophilin ligands and to assess the potential involvement of FK506 Binding Protein 12 kDa (FKBP12) rotamase inhibition in this activity. Both FK506 and rapamycin induced a potent inhibition of the FKBP12 rotamase activity (pIC_{50} values of 7.3 and 7.4, respectively) but only a modest inhibition was observed with 1-(3,3-dimethyl-2-oxo-pentanoyl)-pyrrolidine-2-carboxylic acid *S*-3-pyridin-3-yl-propyl ester (GPI 1046) (5.8), its N-oxide (5.4) and thioester (6.3) analogues. Compared to nerve growth factor, all these immunophilin ligands only induced marginal increases in neurite outgrowth of rat dissociated newborn dorsal root ganglia cells. Furthermore, systemic administration of GPI 1046 and its N-oxide and thioester analogues failed to prevent striatal dopamine depletion induced by acute or chronic i.p. treatment with 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP). These results suggest that inhibition of FKBP12 rotamase activity is not predictive for neurotrophic and neuroprotective properties of immunophilin ligands and question their therapeutic utility in neurodegenerative diseases like Parkinson's disease.

Keywords: Immunophilin; Rotamase; Dorsal root ganglia; Neuroprotection; MPTP (1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine); (Mouse)

1. Introduction

Immunophilin is the generic term for a binding protein of an immunosuppressive agent that belongs to the cyclophilin and/or FK506 Binding Protein 12 kDa (FKBP12) families. This binding protein has been identified as a potent peptidylprolyl *cis–trans* isomerase, also called rotamase, that catalyzes peptidyl proline *cis–trans* isomerization (Schreiber, 1991; Galat, 1993). Inhibition of this rotamase activity is considered an important mechanism through which immunosuppressant drugs provide beneficial effects in animal models of neurodegeneration (Hamilton, 1998). Indeed, neurotrophic effects of immunophilin ligands have been demonstrated in vitro using PC12 cells and cultured embryonic dorsal root ganglia treated with FK506 (Lyons et al., 1994; Steiner et al., 1997a). The neurotrophic effects were observed at low concentrations and with a potency comparable to that reported for neurotrophic proteins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Dragunow et al., 1997; Segal and Greenberg, 1996). Moreover, FK506 abolished the toxicity induced by glutamic acid on cortical cell cultures (Dawson et al., 1993) and displayed a neuroprotective effect in a rat model of ischemia (Butcher et al., 1997) as well as neurotrophic properties in the sciatic nerve crush model in the rat (Gold et al., 1997).

These findings have stimulated a search for small molecules mimicking the FKBP12 binding domain of FK506, thereby inhibiting the peptidylprolyl *cis–trans* isomerase activity and exerting neuroprotective or neurotrophic activities, but lacking immunosuppressive properties. 1-(3,3-dimethyl-2-oxo-pentanoyl)-pyrrolidine-2-carboxylic acid *S*-3-pyridin-3-yl-propyl ester (GPI 1046) has been reported to represent a very potent agent inducing neurite outgrowth in cultured chick dorsal root ganglia cells and showing protection in vivo in several animal models of neurodegeneration (Steiner et al., 1997a). In addition, GPI 1046 appears to improve spatial memory and reverse cholinergic fiber atrophy in aged mice (Sauer et al., 1999).

* Corresponding author. Tel.: +32-2-386-2660; fax: +32-2-386-3141.
E-mail address: henrik.klitgaard@ucb-group.com (H. Klitgaard).

0014-2999/01/$ - see front matter
PII: S0014-2999(01)00850-0

Recent experimental data appears to contrast these findings and thereby questions the therapeutic potential of immunophilin ligands. Thus, GPI 1046 induced only marginal effects in the chick dorsal root ganglia explant model of neurite outgrowth and revealed only a modest ability to enhance mesencephalic cell survival after [1-methyl-4-phenyl 1,2,3,6 tetrahydropyridinium ion (MPP^+)] induced injury (Harper et al., 1999). Furthermore, the same study reported no neurotrophic effects of GPI 1046 in the sciatic nerve crush model and no neuroprotective properties in the 6-hydroxydopamine lesioned rat. Finally, GPI 1046 was recently reported to be inactive against neuronal death in substantia nigra pars compacta following transection of the rat medial forebrain bundle (Winter et al., 2000) and in a rat model of spinal cord injury (Bavetta et al., 1999), whereas activity was observed with FK506 in these models.

These conflicting results stimulated the present study to perform experiments in which a series of immunophilin ligands were compared in vitro for their (1) binding affinity to FKBP12, (2) inhibition of FKBP12 rotamase activity and (3) neurotrophic effects on rat dorsal root ganglia cells. Selected immunophilin ligands were also tested in vivo for their neuroprotective properties in the rodent 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP) model of Parkinson's disease (Gerlach and Riederer, 1996). The aim was to evaluate the neurotrophic and neuroprotective properties of immunophilin ligands and to assess the potential involvement of FKBP12 rotamase inhibition in this activity.

2. Materials and methods

2.1. Drugs

GPI 1046 (1-(3,3-dimethyl-2-oxo-pentanoyl)-pyrrolidine-2-carboxylic acid *S*-3-pyridin-3-yl-propyl ester), its N-oxide analogue (1-(3,3-dimethyl-2-oxo-pentanoyl)-pyrrolidine-2-carboxylic acid *S*-3-(1-oxy-pyridin-3-yl)-propyl ester), and its thioester analogue (1-(3,3-dimethyl-2-oxo-pentanoyl)-pyrrolidine-2-carbothioic acid *S*-(3-phenyl-propyl) ester) were synthesized as described in published patents (WO 96/40633; WO 98/37885; WO 98/29116) (Fig. 1). These compounds were characterized by ^1H nuclear magnetic resonance (NMR), ^{13}C NMR, mass spectrometry, infrared and thin layer chromatography. FK506 and cyclosporin A were obtained from Sigma. Rapamycin was obtained from Calbiochem. All compounds were solubilized in dimethylsulfoxide (DMSO) (0.1%) for in vitro testing and in methylcellulose (1%)-DMSO (5%) for in vivo testing.

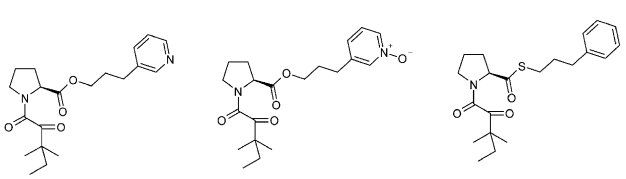

Fig. 1. Structures of GPI 1046 and its N-oxide and thioester analogues.

2.2. Animals

Dorsal root ganglia were excised for cell cultures from 3-day-old male Sprague–Dawley rats (IFFA Credo, Belgium). The in vivo experiments used 8-week-old male C57 Black/6 mice (IFFA Credo, France) weighing 20–22 g. The mice, fed ad libitum, were housed six to seven per cage in a room with controlled temperature, humidity and lighting. All experimental protocols were approved by the local Ethical Committee for the use of laboratory animals in Belgium.

2.3. FKBP12 binding assay

Binding of [^3H]dihydro-FK506 (Amersham) to the murine FKBP12 was performed as described by Siekierka et al. (1989) with minor modifications. The fusion protein glutathione S-transferase/FKBP12 was cloned into a glutathione S-transferase expression vector pGEX-5X-1 and over-expressed in *Escherichia coli* for further purification on a Glutathione Sepharose column (Amersham Pharmacia Biotech). Pure FKBP12 was recovered in the flow-through following Factor Xa digestion. The rotamase activity of the murine FKBP12 and its inhibition by rapamycin and FK506 was identical to that of the human FKBP12 (data not shown). Binding assays were performed at 25°C for 30 min in siliconized 96-well plates containing 20 mM Tris–HCl (pH 7.2), 0.015% (w/v) Triton X-100, 50 ng/ml FKBP12 and 2 nM [^3H]dihydro-FK506. Bound radioligand was separated from free radioligand by chromatography through Sephadex LH-20 microcolumns and counted in a Tri-Carb 2300 TR scintillation counter (Packard).

2.4. FKBP12 rotamase assay

The measurement of peptidyl-prolylisomerase (rotamase) activity of a recombinant human FKBP12 (Sigma) was based on a previously described method (Fischer et al., 1989). Briefly, the *cis–trans* isomerization of an alanine–proline bond in a model substrate, *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide (Bachem), was monitored spectrophotometrically in a chymotrypsin-coupled assay, which releases *para*-nitroanilide from the *trans* form of the substrate peptide. Under equilibrium conditions, about 85% of the peptide were in the *trans* form and were readily cleaved by chymotrypsin.

The remaining peptide having a *cis* conformation was cleaved upon enzymatic conversion to the *trans* form. In order to increase the proportion of the *cis* conformation,

the peptide was dissolved in 15 mM LiCl and trifluoroethanol to a final concentration of 100 μM. This solution was mixed with 50 mM HEPES (pH 7.8), 100 mM NaCl, 1 mM dithiothreitol 0.02% (w/v) Triton X-100 and 30 nM FKBP12. Various dilutions of compounds in 0.1% DMSO (final concentration) were added and the reaction mixture was held at 10°C for 10 min to reach thermal equilibrium and then transferred to the spectrophotometer (Uvikon). Chymotrypsin (Sigma) was added to the assay to initiate the enzymatic reaction and the change in absorbance at 390 nm was monitored for 120 s using a quartz cuvette. First-order rate constants were calculated from a non-linear regression analysis (GraphPad, Prism) and expressed as a function of inhibitor concentrations required to generate pIC_{50} values ($-\log$ of half-maximal enzyme inhibition, IC_{50}).

2.5. Culture of rat dorsal root ganglia neuronal cells

After dissection, dorsal root ganglia from rat new born (postnatal day 3 (P3)) were collected in phosphate buffered saline (PBS)/glucose at 4°C and incubated at 37°C for 45 min in 0.5% collagenase and 0.25% trypsine solution in PBS. The ganglia were then mechanically dissociated in N1 supplemented modified Eagle's medium (MEM) (Gibco). The cells were resuspended in MEM containing glucose 6 g/l and seeded on 96-well microculture plates pre-coated with poly-ornithine and laminin (Becton Dickinson) at a density of 3×10^3 cells per cm^2. The cells were incubated in a MEM + N1 cocktail in the presence of compounds. All cultures were maintained in a humidified atmosphere of 5% $C0_2$/95% air and 100% relative humidity for 24 h. NGF (Harlan) at 5 ng/ml (1.9 nM) was used as positive control.

2.6. Immunohistochemistry on neurofilaments and neurite outgrowth process analysis

Immunohistochemistry on neurofilaments was performed 24 h after plating as described by Malgrange et al. (1994) with some modifications. Cultures were washed three times in Tris-buffered saline (TBS) and fixed in acetone/methanol for 10 min at −20°C. After three washes in TBS, the cells were incubated with 1% H_20_2 for 15 min at 37°C and then incubated for 30 min in a blocking buffer containing 0.05% Tween-20 and 1.5% reconstituted non-fat dry milk. The cells were then processed for immunocytochemistry by incubating for 45 min at 37°C with a mouse monoclonal antibody to neurofilaments (1:2000) (Sternberger Monoclonal, SMI 31, Affinity Research Products). They were then processed for visualization of tyrosine hydroxylase using mouse EnVision TM + HRP DAB system (Dako). The cultures were examined with bright field microscopy using a Zeiss Axiovert microscope. Counting and morphometric analyses of neurofilament positive cells were performed by using a computerized video image analysis system Zeiss KS-400. The percentage of cells bearing neurites and the number of neurofilament positive cells were determined in one field per well using an image analysis process previously described (Malgrange et al., 1994). Cells with processes that were at least twice the diameter of the cell body in length were counted as neurite bearing. The experiments were performed on three independent cultures and six wells per treatment.

2.7. Acute MPTP model

The mice received a single intraperitoneal (i.p.) injection of MPTP (Sigma) (25 mg/kg) or saline (10 ml/kg) on day 1. Vehicle or GPI 1046 (20 mg/kg), its N-oxide analogue (4 mg/kg) or thioester analogue (4 mg/kg) were administered i.p. 30 min prior to MPTP, followed by twice daily administration for 3 days. On day 4, mice received a last administration of vehicle/drug 5 h before sacrifice. The doses used of GPI 1046 and its N-oxide and thioester analogues were chosen based on published reports and patents showing activity with these doses in the acute MPTP model in mice after systemic administration (Steiner et al., 1997a; WO 96/40633; WO 98/37885; WO 98/29116).

2.8. Chronic MPTP model

MPTP (10 mg/kg) or saline was administered i.p. twice a week for 3 weeks. GPI 1046 (20 mg/kg i.p.), its N-oxide analogue (4 mg/kg i.p. or 100 mg/kg per os, (p.o.)) and thioester analogue (4 mg/kg i.p.) were administered once daily during this period after which the mice were sacrificed on day 22.

2.9. Dopamine concentration in the striatum

The mice were decapitated, the brains quickly removed and the striatum dissected and frozen instantaneously in liquid nitrogen. Striatal dopamine was extracted with a solution containing EDTA 0.3 mM, $HClO_4$ 0.1 N and Heparin 1400 IU/ml (1/20 w/v). Homogenized tissue was centrifuged 15 min at $12\,000 \times g$. The supernatant was analyzed by high-pressure liquid chromatography (Kontron 465) coupled with amperometric detection (Antec Decade), with a reverse phase column (Nucleosil® 300-5 C18 ET 125/2 mm, Macherey–Nagel). The mobile phase consisted of NaH_2PO_4 0.1 M, Pic B8 1 mM, EDTA 0.1 mM, NaCl 10 mM and pH adjusted to 3.0 with orthophosphoric acid. The results were calculated as nanogram of dopamine per milligram of tissue wet weight.

2.10. Statistical analysis

For neurite outgrowth and cell survival experiments, statistical significance was assessed by comparison of medians with a Kruskal–Wallis test followed by a non-para-

metric test corrected for multiple comparisons vs. control. For in vivo experiments, statistical significance was assessed by an unpaired Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. FKBP12 binding assay

The competitive binding assay in which [^{3}H]dihydro-FK506 bound to FKBP12 is separated from the free radioligand by size exclusion chromatography showed saturable and reversible binding of [^{3}H]dihydro-FK506 (data not shown). The radioligand binds to one single-site per protein with a K_d value of 0.35 ± 0.05 nM ($n = 3$). Competition-binding data (Fig. 2A) were analyzed with a single-site model. The pIC_{50} values for FK506 and rapamycin were calculated to be 9.4 ± 0.2 ($n = 3$) and 9.7 ± 0.1 ($n = 3$), respectively. This is in agreement with binding results reported previously (Siekierka et al., 1989). Cyclosporin A, at concentrations up to 10 μM, failed to compete with [^{3}H]dihydro-FK506 for binding. Surprisingly, similar results were obtained with GPI 1046, which also had no effect on the binding of [^{3}H]dihydro-FK506 at concentrations up to 10 μM.

3.2. FKBP12 rotamase activity

Assessment of the rotamase activity of FKBP12 revealed potent inhibition by FK506 and rapamycin (Fig. 2B). Calculations from concentration–response curves showed pIC_{50} values for rapamycin and FK506 of 7.4 ± 0.0 ($n = 3$) and 7.3 ± 0.1 ($n = 3$), respectively. In contrast, the cyclophilin inhibitor, cyclosporin A, had no effect on inhibition up to 10 μM, but GPI 1046, its N-oxide and

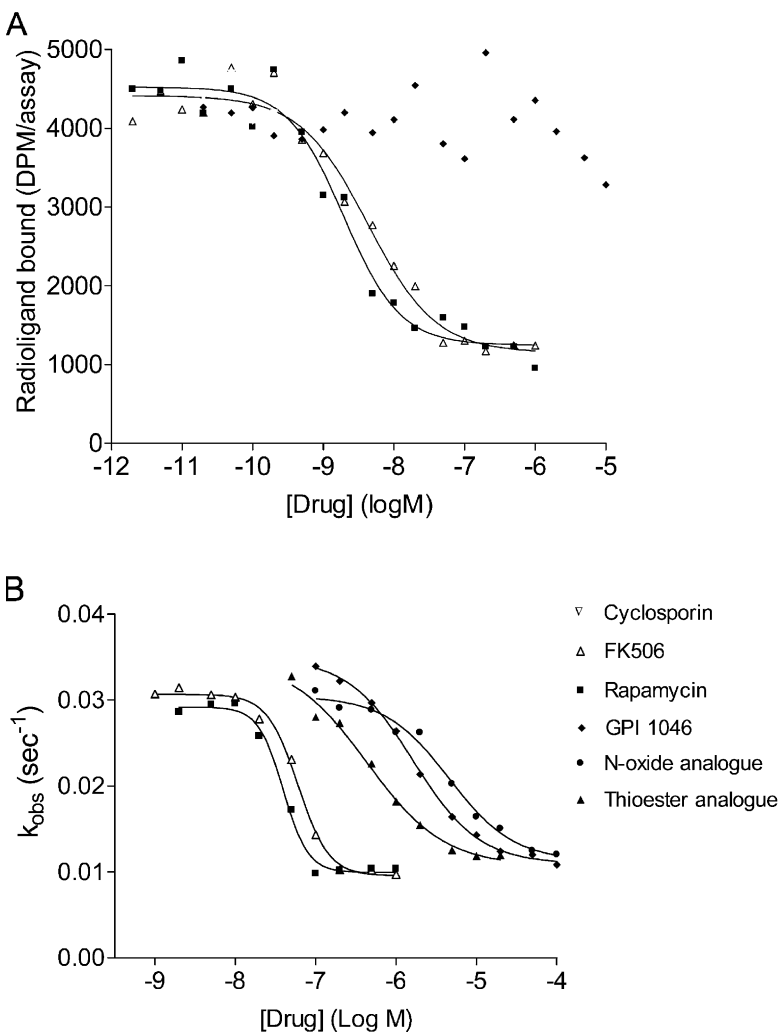

Fig. 2. Inhibition of [^{3}H]dihydro-FK506 binding to FKBP12 and its rotamase activity. (A) Competitive binding of [^{3}H]dihydro-FK506 with various concentrations of test drugs. The data points were fitted using a one-site model. (B) After incubation of FKBP12 along with increasing concentrations of various test drugs, chymotrypsin is added and rotamase activity determined. The data shown represent results from one typical experiment and were fitted using a sigmoidal function to yield the pIC_{50} values. Each experiment was repeated three times.

thioester analogues showed pIC_{50} values of 5.8 ± 0.1 ($n = 3$), 5.4 ± 0.1 ($n = 3$) and 6.3 ± 0.1 ($n = 3$), respectively.

3.3. Neurite outgrowth and cell survival studies on DRG cells

Putative neurotrophic activity of immunophilin ligands was examined in vitro on primary cultures of dissociated new born rat (P3) dorsal root ganglia cells. The culture was maintained in a serum-free medium supplemented with N1 in order to avoid any possible interaction of compounds with serum (Harper et al., 1999). The neurotrophic activity of immunophilin ligands was evaluated by determining the percentage of cells bearing neurites after 24 h (Table 1). At the same time, the effect of these putative neurotrophic agents on neuronal survival was assessed by counting the number of neurofilament-positive cells per captured field (Table 1).

Addition of NGF produced a concentration-dependent effect (data not shown) and markedly ($P < 0.001$) promoted neurite outgrowth at 5 ng/ml (1.9 nM) (Table 1).

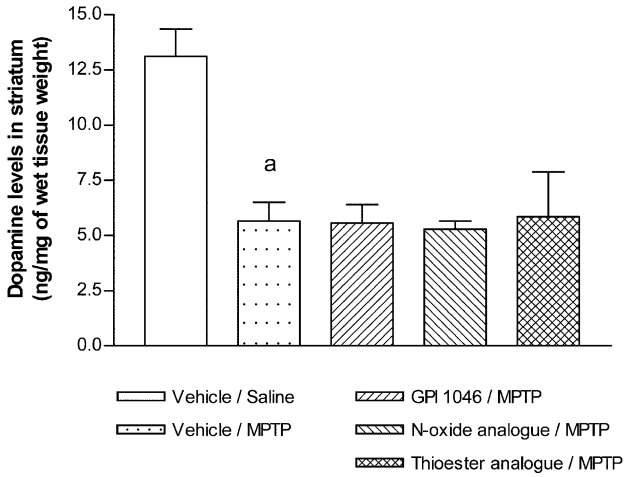

Fig. 3. Effect of immunophilin ligands on acute MPTP-induced dopamine depletion in the striatum of C57 Black/six mice. MPTP (25 mg/kg) or saline ($n = 7$) was administered once i.p. on day 1. The immunophilin ligand GPI 1046 (20 mg/kg, $n = 7$), and its N-oxide (4 mg/kg, $n = 7$) and thioester analogues (4 mg/kg, $n = 7$) or vehicle (methylcellulose 1%/DMSO 5%, $n = 6$) were administered i.p. twice daily for 3 days with the first administration 30 min before the MPTP injection and once on day 4 (5 h before sacrifice). All results are expressed as means ± S.D. ($n = 6$–7). [a]Indicates statistically significant difference of $P < 0.001$ between the vehicle/saline and vehicle/MPTP-treated groups.

Table 1
Effect of rapamycin, FK506, GPI 1046, its N-oxide and thioester analogues on neurite outgrowth and number of neurofilament-positive cells in dorsal root ganglia cells

Treatment	Percentage of cells-bearing neurites	Number of neurofilament positive cells (count/field)
Experiment 1		
Control	3.1 (1.6–6.8)	52.0 (32.5–70.0)
FK-506 1 nM	8.9 (3.1–14.8)	56.0 (49.0–67.5)
FK-506 100 nM	9.5 (4.0–14.5)	57.5 (42.5–72.0)
FK-506 10 μM	6.1 (2.8–10.6)	55.5 (39.0–68.5)
GPI 1046 1 nM	7.3 (5.5–9.9)	55.5 (45.5–73.0)
GPI 1046 100 nM	6.5 (3.9–10.8)	50.0 (40.5–60.5)
GPI 1046 10 μM	7.9 (2.9–11.1)	50.5 (42.0–67.0)
NGF 1.9 nM (5 ng/ml)	30.1 (25.0–45.4)[a]	103.5 (88.0–116.5)[a]
Experiment 2		
Control	8.4 (4.4–10.1)	55.5 (46.5–66.0)
Rapamycin 1 nM	12.3 (9.6–15.7)[b]	76.0 (57.5–86.0)[b]
Rapamycin 100 nM	11.2 (6.4–14.8)	67.5 (56.5–81.5)[b]
Rapamycin 10 μM	11.1 (7.8–12.9)	61.5 (48.0–75.5)
Thioester analogue 1 nM	12.5 (7.0–14.6)	64.0 (54.5–81.5)
Thioester analogue 100 nM	9.5 (5.1–13.4)	71.5 (62.0–80.0)[b]
Thioester analogue 10 μM	8.4 (7.1–13.7)	63.0 (53.0–78.0)
N-oxide analogue 1 nM	11.3 (7.3–15.3)[b]	72.5 (60.0–87.0)[b]
N-oxide analogue 100 nM	14.8 (11.8–19.9)[b]	72.0 (62.5–85.5)[b]
N-oxide analogue 10 μM	14.0 (8.4–18.8)[b]	70.0 (62.0–84.5)[b]
NGF 1.9 nM (5 ng/ml)	39.1 (32.8–44.2)[a]	111.0 (97.0–125.5)[a]

Concentrations of 1 nM, 100 nM and 10 μM of the compounds were added to the culture medium for 24 h. The results are expressed as medians (lower-upper quartiles) of three separate cell cultures ($n = 8$ fields per culture).

[a]Indicate statistically significant difference from control of $P < 0.001$.

[b]Indicate statistically significant difference from control of $P < 0.05$.

The percentage of cells bearing neurites was 30–39% after 24 h exposure whereas control cultures only showed 3–8% of cells with neurites. The number of neurofilament-positive cells was confirming the trophic activity of NGF. In contrast, the immunophilin ligands only marginally increased the percentage of dorsal root ganglia cells bearing neurites and the survival of neurofilament-positive cells after 24 h (Table 1).

3.4. Acute MPTP model

A single i.p. injection of MPTP (25 mg/kg) induced a significant decrease (47%) in dopamine levels in the striatum when compared to basal levels (Fig. 3). GPI 1046 (20 mg/kg), its N-oxide analogue (4 mg/kg) and thioester analogue (4 mg/kg), administered i.p. failed to prevent this decrease in dopamine levels (Fig. 3).

3.5. Chronic MPTP model

Administration of MPTP i.p. (10 mg/kg) twice a week for 3 weeks produced a progressive decrease of 31%, 36%, and 42% of basal striatal dopamine levels, respectively, after 1, 2 (data not shown) and 3 weeks of MPTP treatment (Fig. 4). GPI 1046 (20 mg/kg), its N-oxide analogue (4 mg/kg) and thioester analogue (4 mg/kg) administered i.p. or p.o. administration of the N-oxide analogue (100

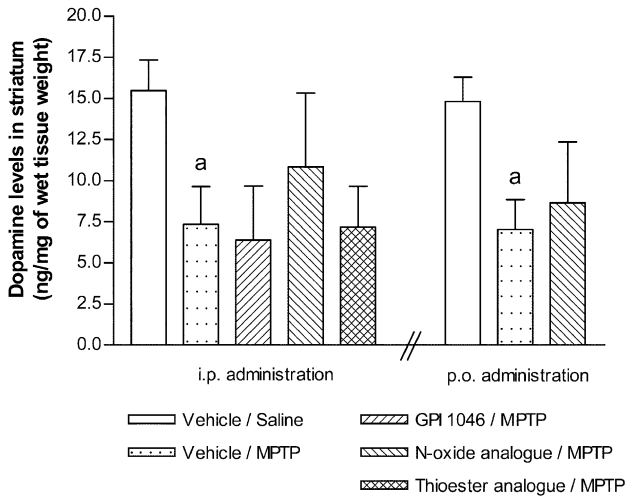

Fig. 4. Effect of daily administration of immunophilin ligands on chronic MPTP-induced dopamine depletion in the striatum of C57 Black/six mice. MPTP (10 mg/kg) or saline ($n = 12$) was administered i.p. twice a week for 3 weeks. The immunophilin ligands GPI 1046 (20 mg/kg, $n = 5$), and its N-oxide (4 mg/kg, $n = 5$), and thioester analogues (4 mg/kg, $n = 6$) or vehicle ($n = 9$) were administered i.p. daily for 21 days. The N-oxide analogue (100 mg/kg, $n = 6$) or vehicle ($n = 6$) was also administered p.o. daily for 21 days in a separate experiment. All results are expressed as means ± S.D. ($n = 5$–12). [a] Indicates statistically significant difference of $P < 0.001$ between the vehicle/saline and vehicle/MPTP-treated groups.

mg/kg) daily during 21 days did not counteract this reduction in dopamine levels (Fig. 4).

4. Discussion

The main finding of the present study was the lack of relationship between inhibition of FKBP12 rotamase activity and neurotrophic effects in vitro among a series of immunophilin ligands. Furthermore, the neurotrophic effect observed was only modest and none of the immunophilin ligands showed any neuroprotection in vivo in the MPTP model of Parkinson's disease. This questions both inhibition of FKBP12 rotamase activity as the mechanism for neurotrophic activity of immunophilin ligands and the potential therapeutic utility of these compounds for neurodegenerative diseases like Parkinson's disease.

Several in vitro studies have observed an impressive promotion of neurite outgrowth with NGF and other growth factors like BDNF and GDNF (Mocchetti and Wrathall, 1995; Gavazzi et al., 1999). However, the therapeutic utility of these peptides is hampered by their poor oral absorption and lack of brain penetration. Intrastriatal or intracerebroventricular injection of GDNF in the rat provides almost complete protection of 6-hydroxydopamine lesioned nigral dopaminergic neurons (Rosenblad et al., 1999). Furthermore, GDNF improves the symptoms of pharmacologically induced Parkinson's disease in monkeys (Walton, 1999). These results support the idea of developing small orally active non-peptide molecules that possess neuroprotective and neurotrophic properties able to stimulate recovery in neurodegenerative diseases. This highlights the interest in FKBP12 activity as a drug discovery target for the identification of non-immunosuppressive analogues of FK506 with neurotrophic and neuroprotective properties.

Contrasting reports exist regarding the potency of immunophilin ligands as neurotrophic compounds. A number of published articles report that FK506 and GPI 1046 display a neurotrophic potency equal to NGF (Lyons et al., 1994; Steiner et al., 1997a). In contrast, marginal neuritogenic effects of GPI 1046 on cultured chick dorsal root ganglia explants have also been reported (Harper et al., 1999). These discrepancies may relate to different models (dissociated dorsal root ganglia vs. explants; embryonic vs. postnatal dorsal root ganglia; chick vs. rat) and/or to different culture conditions giving rise to various growth factor requirements as well as to different levels of endogenous growth factors in the culture. The fact that dorsal root ganglia cells in culture consist of a mixture of cells coming from different segmental levels with distinct subpopulations responding differently to a variety of trophic factors may also explain this discrepancy (Carreau et al., 1997; Gavazzi et al., 1999).

It has been reported that GPI 1046 promotes re-growth and functional recovery of dopaminergic neurons after subcutaneous and oral administration in animal models of Parkinson's disease (Steiner et al., 1997a). Another series of immunophilin ligands also appear to protect striatal dopaminergic innervation in the MPTP mouse model of Parkinson's disease after oral administration (Costantini et al., 1998). The present study contrasts these reports by a lack of activity of immunophilin ligands after i.p. and p.o. administration in both the acute (Fig. 3) and chronic (Fig. 4) MPTP mouse models, which reproduce the biochemical events that occur in Parkinson's disease, in particular the loss of dopamine content in striatum. Thus, despite a weak but significant activity in vitro on neurite outgrowth observed with GPI 1046 N-oxide analogue, neither GPI 1046 nor its two analogues did protect against the decrease in dopamine levels induced by MPTP, even after chronic administration for 3 weeks. It should be noted that previous studies (Costantini et al., 1998; Steiner et al., 1997a) measured the density of the tyrosine hydroxylase positive fibers in the striatum, but this is thought to correlate with striatal dopamine levels that were measured in the present study (Costantini et al., 1998). In the MPTP mouse model, 85% and 40% protection were obtained with subcutaneous injections of GPI 1046 (20 mg/kg) before and after administration of MPTP, respectively. Significant protection of striatal tyrosine hydroxylase-immunostaining was also observed in MPTP-treated mice after oral administration of 10–100 mg/kg of GPI 1046 (Steiner et al., 1997b). Similar results were subsequently reported for another

immunophilin ligand (V-10,367) administered orally at doses of 100–400 mg/kg (Costantini et al., 1998). The absence of any neuroprotective action of GPI 1046 in our in vivo model could be explained by a rapid hydrolysis of the ester function, conferring a very short half-life to this compound (WO 98/37885). However, the N-oxide analogue was also inactive despite a markedly longer half-life and improved bioavailability (WO98/37885). This suggests that the absence of in vivo activity may reflect the minor neurotrophic effects observed in vitro. Thus, it appears that future studies, involving more potent ligands with sufficient bioavailability, are required in order to fully elucidate the therapeutic potential of immunophilin ligands in animal models of Parkinson's disease.

Inhibition of FKBP12 rotamase activity by FK506 and analogues like rapamycin involves a complex interaction between those ligands and certain domains of the FKBP12 protein (see Hamilton and Steiner, 1998 for review). It has been shown that the pipecolinyl and the pyranose rings, the keto-amide linkage as well as a portion of the cyclohexylpropenyl ester side chain found in the structure of FK506 are crucial elements allowing it to bind to FKBP12. The pipecolinyl-keto-amide part of the molecule is considered to represent a transition-state that mimics the natural peptidylprolyl substrate. Since GPI 1046 contains bioisosteric elements of all the above mentioned chemical features necessary for binding to this "FKBP12 binding portion of FK506" (prolyl ring instead of pipecolinyl ring, keto-amide linkage, and a pyridine-propenyl ester side chain instead of the cyclohexylpropenyl ester) (Fig. 5), it was expected that this molecule would displace the binding of [^{3}H]dihydro-FK506 to FKBP12. Furthermore, X-ray crystal structure analysis of high affinity FKBP12 ligands (FK506, rapamycin and small non-immunosuppressive pipecolyl ester derivatives) showed that all molecules display an analogous binding site (Holt et al., 1993). The binding results obtained in the present study with GPI 1046 deviate from this assumption and confirms a recent report showing that GPI 1046 had a marked lower (10.000 fold) affinity for FKBP12 in a similar binding assay (Rabinowitz et al., 2000). This suggests that GPI 1046 may inhibit the rotamase activity of FKBP12 via a different binding site than that of FK506.

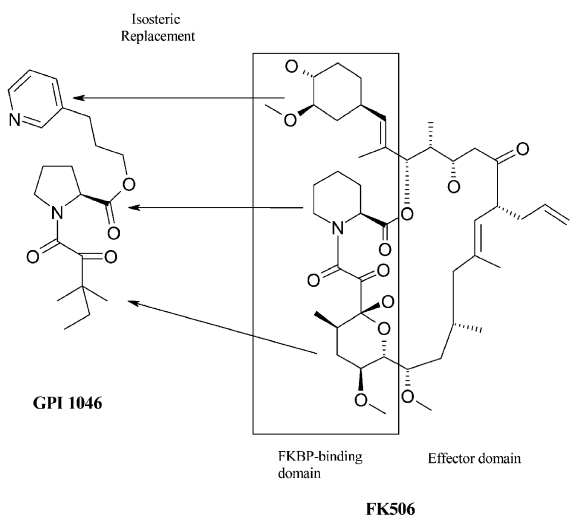

Fig. 5. Structural relationship between FK506 and GPI 1046.

The neuroprotective and neurotrophic actions of immunophilin ligands are believed to be mediated via the inhibition of the enzyme FKBP12 (Hamilton, 1998). However, neurotrophic activity with FK506 has been demonstrated on hippocampal primary cell cultures from FKBP12 knock out mice (Gold et al., 1999). Interestingly, recent studies have demonstrated that pretreatment with a monoclonal FKBP52 antibody abolishes the neurotrophic action of FK506 on neuroblastoma SH-SY5Y cells. This suggests that other receptors in the FKBP family, like FKBP52, may represent more relevant immunophilin binding sites. Indeed, it has been suggested that FKBP12 does not mediate the neurite outgrowth promoting effect of immunophilin ligands since their neurotrophic activity appears mediated by dissociation of the FKBP52/HSP90/steroid receptor complex augmenting the mitogen-associated protein signaling pathway (Gold et al., 1999).

In summary, the present study did not reveal neuroprotective and neuroregenerative properties of GPI 1046 and related analogues in mice models of Parkinson's disease and only minor neurotrophic effects were observed in vitro on dorsal root ganglia neurite outgrowth. Based on the findings obtained in the present study, there appears to be no relationship between the neuroprotective effects in vitro and inhibition of FKBP12 rotamase activity, suggesting that the latter is of minor relevance to the neurotrophic activity of immunophilin ligands. Thus, it remains for future studies to elucidate the precise neurotrophic mechanism that would permit a systematic search for new immunophilin ligands with improved neurotrophic properties relevant for the treatment of neurodegenerative diseases such as Parkinson's disease.

Acknowledgements

We thank Professor G. Moonen, University of Liège, Belgium, for the generous gift of a murine FKBP12 clone, Dr. B. Malgrange, University of Liège, Belgium, for her advice on DRG cell cultures, Drs. A. Marchand and S. Papot (working in the group of Professors G. Guillaumet and M.C. Viaud, University of Orleans, France), for the synthesis of GPI 1046 and its analogues, Dr. B. Bennett, UCB Pharma for critical reading of an early version of this manuscript and A. Matagne for typing of the manuscript. The skillful and committed technical assistance of N. Defresne, E. Gillent, A. Heremans, D. Joye, K. Leclercq, B. Mullier, is greatly appreciated.

References

Bavetta, S., Hamlyn, P.J., Burnstock, G., Lieberman, A.R., Anderson, P.N., 1999. The effect of FK506 on dorsal column axons following spinal cord injury in adult rats: neuroprotection and local regeneration. Exp. Neurol. 158 (2), 382–393.

Butcher, S.P., Henshall, D.C., Teramura, Y., Iwasaki, K., Sharkey, J., 1997. Neuroprotective action of FK506 in experimental stroke: in vivo evidence against an antiexcitotoxic mechanism. J. Neurosci. 17 (18), 6939–6948.

Carreau, A., Gueugnon, J., Benavides, J., Vigé, X., 1997. Comparative effects of FK506, rapamycin and cyclosporin A, on the in vitro differentiation of dorsal root ganglia explants and septal cholinergic neurons. Neuropharmacology 36, 1755–1762.

Costantini, L.C., Chaturvedi, P., Armistead, D.M., McCaffrey, P.G., Deacon, T.W., Isacson, O., 1998. A novel immunophilin ligand: distinct branching effects on dopaminergic neurons in culture and neurotrophic actions after oral administration in an animal model of Parkinson's disease. Neurobiol. Dis. 5 (2), 97–106.

Dawson, T.M., Steiner, J.P., Dawson, V.L., Dinerman, J.L., Uhl, G.R., Snyder, S.H., 1993. Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. Proc. Natl. Acad. Sci. U. S. A. 90 (21), 9808–9812.

Dragunow, M., MacGibbon, G.A., Lawlor, P., Butterworth, N., Connor, B., Henderson, C., Walton, M., Woodgate, A., Hughes, P., Faull, R.L., 1997. Apoptosis, neurotrophic factors and neurodegeneration. Rev. Neurosci. 8, 223–265.

Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., Schmid, F.X., 1989. Cyclophilin and peptidyl-prolyl *cis–trans* isomerase are probably identical proteins. Nature 341, 476–478.

Galat, A., 1993. Peptidylproline *cis–trans* isomerases: immunophilins. Eur. J. Biochem. 216, 689–707.

Gavazzi, I., Kumar, R.D.C., McMahon, S.B., Cohen, J., 1999. Growth responses of different subpopulations of adult sensory neurons to neurotrophic factors in vitro. Eur. J. Neurosci. 11, 3405–3414.

Gerlach, M., Riederer, P., 1996. Animal models of Parkinson's disease: an empirical comparison with the phenomenology of the disease in man. J. Neural Transm. 103, 987–1041.

Gold, B.G., Zeleny-Pooley, M., Wang, M.S., Chaturvedi, P., Armistead, D.M., 1997. A nonimmunosuppressant FKBP12 ligand increases nerve regeneration. Exp. Neurol. 147, 269–278.

Gold, B.G., Densmore, V., Shou, W., Matzuk, M.M., Gordon, H.S., 1999. Immunophilin FK506-Binding Protein 52 (not FK506-Binding Protein 12) mediates the neurotrophic action of FK506. J. Pharmacol. Exp. Ther. 289, 1202–1210.

Hamilton, G.S., 1998. Immunophilin ligands for the treatment of neurological disorders. Exp. Opin. Ther. Pat. 8, 1109–1124.

Hamilton, G.S., Steiner, J.P., 1998. Immunophilins: beyond immunosuppression. J. Med. Chem. 41, 5119–5143.

Harper, S., Bilsland, J., Young, L., Bristw, L., Boyce, S., Mason, G., Rigby, M., Hewson, D., Smith, D., O'Donnell, R., O'Connor, D., Hill, R.G., Evans, D., Swain, C., Williams, B., Hefti, F., 1999. Analysis of the neurotrophic effects of GPI 1046 on neuron survival and regeneration in culture and in vivo. Neuroscience 88, 257–267.

Holt, D.A., Luango, J.I., Yamashita, D.S., Oh, H.J., Konialian, A.L., Yen, H.K., Rozamus, L.W., Brandt, M., Bossard, M.J., Levy, M.A., Eggleston, D.S., Liang, J., Schultz, L.W., Stout, T.J., Clardy, J., 1993. Design, synthesis and kinetic evaluation of high-affinity FKBP ligands and the X-ray crystal structures of their complexes with FKBP12. J. Am. Chem. Soc. 115, 9925–9938.

Lyons, W.E., George, E.B., Dawson, T.M., Steiner, J.P., Snyder, S.H., 1994. Immunosuppressant FK506 promotes neurite outgrowth in cultures of PC12 cells and sensory ganglia. Proc. Natl. Acad. Sci. U. S. A. 91, 3191–3195.

Malgrange, B., Delree, P., Rigo, J.M., Moonen, G., Baron, H., 1994. Image analysis of neuritic regeneration by adult rat dorsal root ganglion neurons in culture: quantification of the neurotoxicity of anticancer agents and of its prevention by nerve growth factor or basic fibroblast growth factor but not brain-derived neurotrophic factor or neurotrophin-3. J. Neurosci. Methods 53, 111–122.

Mocchetti, I., Wrathall, J.R., 1995. Neurotrophic factors in central nervous system trauma. J. Neurotrauma 12 (5), 853–870.

Rabinowitz, M., Seneci, P., Rossi, T., Dal Cin, M., Deal, M., Terstappen, G., 2000. Solid-phase/solution-phase combinatorial synthesis of neuroimmunophilin ligands. Bioorg. Med. Chem. Lett. 10, 1007–1010.

Rosenblad, C., Kirik, D., Devaux, B., Moffat, B., Phillips, H.S., Bjorklund, A., 1999. Protection and regeneration of nigral dopaminergic neurons by neurturin or GDNF in a partial lesion model of Parkinson's disease after administration into the striatum or the lateral ventricle. Eur. J. Neurosci. 11 (5), 1554–1566.

Sauer, H., Francis, J.M., Jiang, H., Hamilton, G.S., Steiner, J.P., 1999. Systemic treatment with GPI 1046 improves spatial memory and reverses cholinergic neuron atrophy in the medial septal nucleus of aged mice. Brain Res. 842, 109–118.

Schreiber, S.L., 1991. Chemistry and biology of the immunophilins and their immunosuppressant ligands. Science 251, 283–287.

Segal, R.A., Greenberg, M., 1996. Intracellular signalling pathways activated by neurotophic factors. Annu. Rev. Neurosci. 19, 463–489.

Siekierka, J.J., Hung, S.H.Y., Poe, M., Lin, C.S., Sigal, N.H., 1989. A cytosolic protein for the immunosuppressant FK506 has peptidyl–prolyl isomerase activity but is distinct from cyclophilin. Nature 341, 755–757.

Steiner, J.P., Hamilton, G.S., Ross, D.T., Valentine, H.L., Guo, H., Connolly, M.A., Liang, S., Ramsey, C., Li, J.J., Huang, W., Horworth, P., Soni, R., Fuller, M., Sauer, H., Nowotnik, A.C., Suzdak, P.D., 1997a. Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models. Proc. Natl. Acad. Sci. U. S. A. 94, 2019–2024.

Steiner, J.P., Ross, D.T., Valentine, H.L., Liang, S., Guo, H., Huang, W., Suzdak, P.D., Hamilton, G.S., 1997b. The orally active neuroimmunophilin ligand GPI 1046 promotes structural and functional recovery in the mouse MPTP model of Parkinson's disease. Soc. Neurosci. Abstr. 23 (2), 1728.

Walton, K.M., 1999. GDNF: a novel factor with therapeutic potential for neurodegenerative disorders. Mol. Neurobiol. 19 (1), 43–59.

Winter, C., Schenkel, K., Buger, E., Eickmeier, C., Zimmermann, M., Herdegen, T., 2000. The immunophilin ligand FK506, but not GPI 1046, protects against neuronal death and inhibits c-Jun expression in the substantia nigra pars compacta following transection of the rat medial forebrain bundle. Neuroscience 95 (3), 753–762.