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Design, Synthesis and Structure of Non-macrocyclic Inhibitors of FKBP12, the Major Binding Protein for the Immunosuppressant FK506

BY D. M. ARMISTEAD,* M. C. BADIA, D. D. DEININGER, J. P. DUFFY, J. O. SAUNDERS, R. D. TUNG, J. A. THOMSON, M. T. DECENZO, O. FUTER, D. J. LIVINGSTON, M. A. MURCKO, M. M. YAMASHITA AND M. A. NAVIA*

Vertex Pharmaceuticals Incorporated, 40 Allston Street, Cambridge, MA 02139-4211, USA

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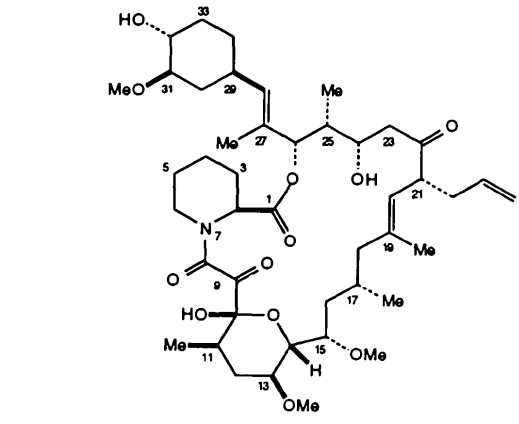
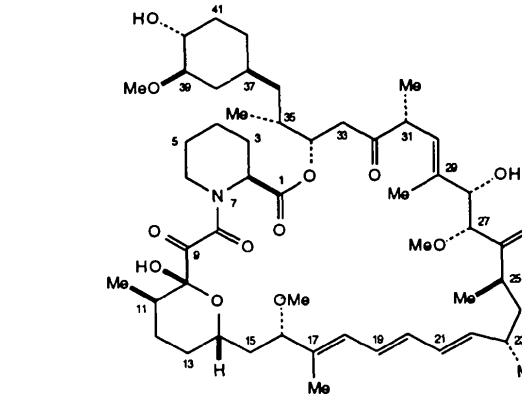
Abstract

We have synthesized a series of non-macrocyclic ligands to FKBP12 that are comparable in binding potency and peptidyl prolyl isomerase (PPIase) inhibition to FK506 itself. We have also solved the structure of one of these ligands in complex with FKBP12, and have compared that structure to the FK506–FKBP12 complex. Consistent with the observed inhibitory equipotency of these compounds, we observe a strong similarity in the conformation of the two ligands in the region of the protein that mediates PPIase activity. Our compounds, however, are not immunosuppressive. In the FKBP12–FK506 complex, a significant portion of the FK506 ligand, its 'effector domain', projects beyond the envelope of the binding protein in a manner that is suggestive of a potential interaction with a second protein, the calcium-dependent phosphatase, calcineurin, whose inhibition by the FKBP12–FK506 complex interrupts the T-cell activation events leading to immunosuppression. In contrast, our compounds bind within the surface envelope of FKBP12, and induce significant changes in the structure of the FKBP12 protein which may also affect calcineurin binding indirectly.

Introduction

The macrolide immunosuppressants FK506 (Fig. 1*a*) and rapamycin (Fig. 1*b*) are competitive inhibitors of the PPIase activity of the cytosolic FK506-binding protein, FKBP12 (Siekierka, Hung, Poe, Lin & Sigal, 1989; Harding, Galat, Uehling & Schreiber, 1989). Similarly, the peptidyl immunosuppressant cyclosporin A (CsA; Fig. 1*c*) is a potent PPIase inhibitor of its major cytosolic binding protein cyclophilin A (CyP-A) (Handschumacher, Harding, Rice, Drugge & Speicher, 1984). The inhibition of the PPIase activity of these two non-homologous immunophilins is not sufficient to account for the biological activity exhibited by these ligands. This has been demonstrated by the identification of synthetic non-immunosuppressive ligands that are potent inhibitors of FKBP12 (Bierer, Somers, Wandless, Burakoff & Schreiber, 1990; Sigal *et al.*, 1991; Dumont *et al.*, 1992; Holt, Luengo *et al.*, 1993; Holt, Konialian





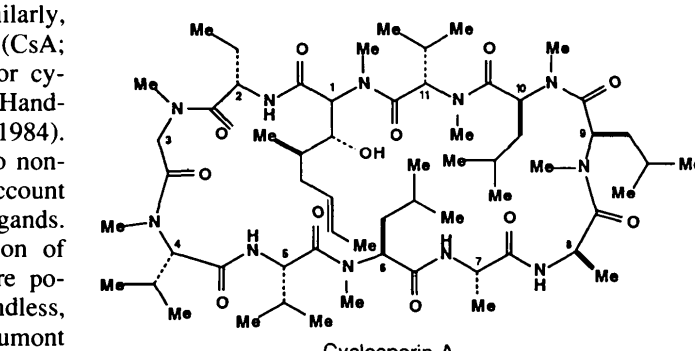


Fig. 1. Chemical structures and numbering scheme used to describe. (*a*) FK506 (USAN tacrolimus; Prograf®). (*b*) Rapamycin (AY-29890; USAN sirolimus; Rapamune®). (*c*) Cyclosporin A (Sandimmune®).

* Authors for correspondence.

et al., 1993; Yamashita *et al.*, 1994). In addition, FK506 and CsA have been shown to inhibit calcium-dependent signal-transduction events essential for the transcription of early T-cell activation genes such as those for IL-2, IL-3, IL-4 and GM-CSF (Tocci *et al.*, 1989; Sigal *et al.*, 1991). This mechanism of immunosuppression is distinct from that induced by rapamycin, which blocks calcium-independent fibroblast growth-factor stimulated proliferation of T cells at a later stage of the cell-cycle progression (Dumont, Staruch, Koprak, Melino & Sigal, 1990; Chung, Kuo, Crabtree & Blenis, 1992).

The auxiliary role of the immunosuppressants FK506, CsA and rapamycin in these pathways involves the formation of activated complexes with their respective cognate immunophilins, which then inhibit a downstream receptor protein involved in cytosolic signalling events. The data would also suggest that FK506 and CsA share a common target protein distinct from the target for rapamycin. The complexes of FK506/FKBP12 and CsA/CyP-A have been shown to be competitive inhibitors of the calcium-dependent serine–threonine phosphatase calcineurin, whereas the individual components of these complexes show no measurable affinity for this protein (Friedman & Weissman, 1991; Liu *et al.*, 1991). Nuclear factor of activated T cells (NFAT) has been identified as a substrate for calcineurin *in vitro* (Jain *et al.*, 1993) and the activation of this T-cell-specific transcription factor has been shown to be sensitive to FK506 and CsA (Mattila *et al.*, 1990) implicating the role of calcineurin as a pivotal enzyme in the T-cell receptor cascade. The rapamycin–FKBP12 complex, on the other hand, does not interact with calcineurin (Friedman &

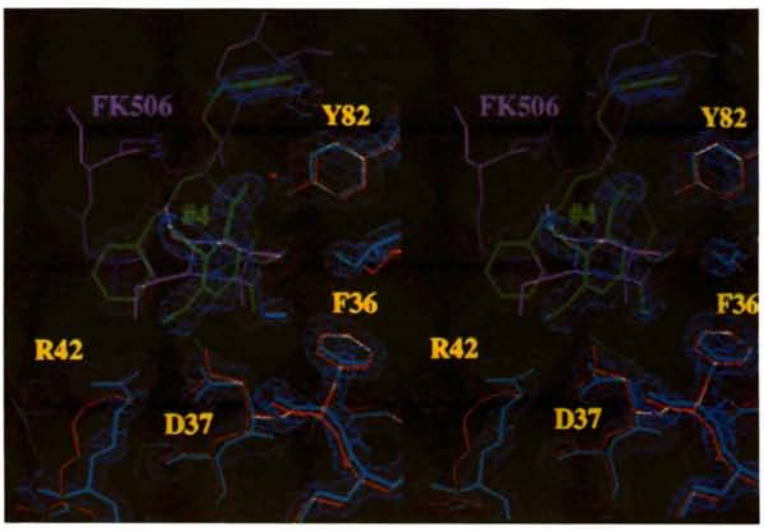


Fig. 2. Structure of ligand (4) (in green) in complex with FKBP12 (in light blue). The corresponding $2|F_o| - |F_c|$ electron-density map of the structure, contoured at 2 standard deviation units above background, is shown in dark blue. The structure of the FK506–FKBP12 complex is superimposed for reference; FK506 is shown in violet; the associated FKBP12 structure is shown in red.

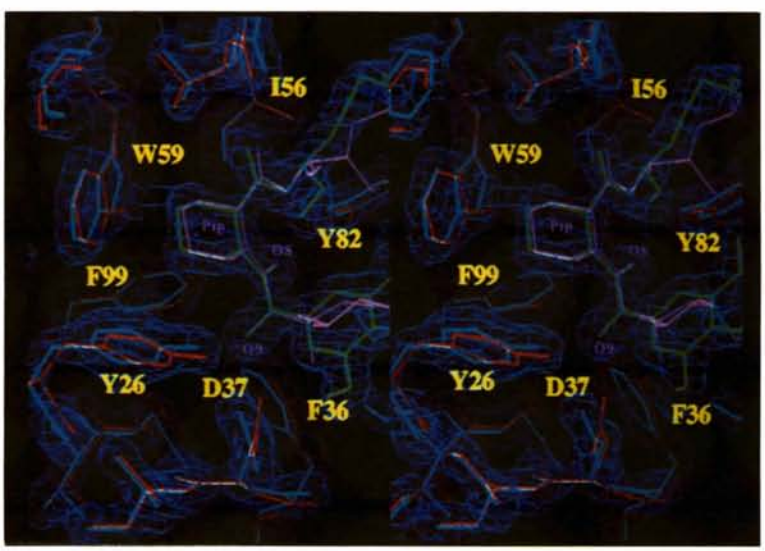


Fig. 3. Close-up view of the active site of FKBP12 showing the structures of the FKBP12 complexes with ligand (4) and with FK506. Electron density corresponding to the ligand (4) complex is superimposed. The color scheme used is as described for Fig. 2.

Weissman, 1991; Liu *et al.*, 1991), but rather has recently been shown to bind to a 220 kDa mammalian protein (Brown *et al.*, 1994). It has been suggested (Brown *et al.*, 1994) that this FKBP–rapamycin-associated protein (termed FRAP) may mediate the calcium-independent IL-2 receptor-linked signal transduction that activates p70 S6 kinase and the cyclin-dependent kinases. The activation of both p70 S6 kinase (Price, Grove, Calvo, Avruch & Bierer, 1992; Chung *et al.*, 1992; Kuo *et al.*, 1992; Calvo, Crews, Vik & Bierer, 1992) and the cyclin–Cdk complexes (Albers, Williams *et al.*, 1993; Albers, Brown *et al.*, 1993; Morice, Wiederrecht, Brunn, Siekierka & Abraham, 1993*a*,*b*) has been shown to be sensitive to rapamycin. Many reviews in this area have been published (Schreiber, 1991; Sigal & Dumont, 1992; Rosen & Schreiber, 1992; Schreiber & Crabtree, 1992; Liu, 1993*a*,*b*; Schreiber, Albers & Brown, 1993; Navia & Peattie, 1993*a*,*b*; Armistead & Harding, 1993).

The design of synthetic inhibitors of FKBP12 has been an area of intense interest (Bierer *et al.*, 1990; Hauske *et al.*, 1992; Holt, Luengo *et al.*, 1993; Holt, Konialian *et al.*, 1993; Teague & Stocks, 1993; Wang *et al.*, 1994; Yamashita *et al.*, 1994; Luengo *et al.*, 1994; Holt *et al.*, 1994; Stocks, Birkinshaw & Teague, 1994; Goulet, Rupprecht, Sinclair, Wyvratt & Parsons, 1994). As part of our ongoing immunosuppressive program, we sought to probe the binding site of FKBP12 by identifying small-molecule inhibitors of this immunophilin with affinity equal to or greater than FK506. Our strategy was to exploit binding to FKBP12 as a means of confining relatively flexible ligands to a predictable conformation which could be used as a platform to project ligand groups away from the surface of the protein, as observed for FK506 in the structure of its complex with FKBP12 (Van Duyne, Standaert, Karplus, Schreiber & Clardy, 1991, 1993; Wilson *et al.*, 1995).

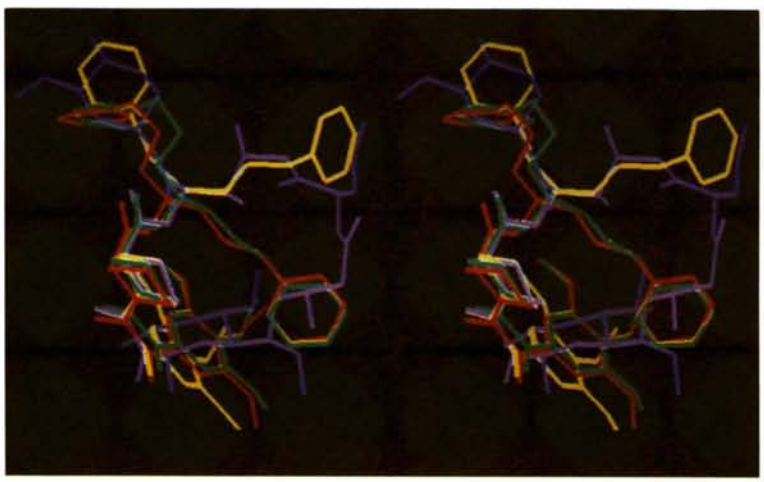


Fig. 4. Overlap of the conformation of FK506 (violet) taken from the crystal structure of its FKBP12 complex on the desired effector mimic conformation of compound (4) (in yellow). The predicted conformation of compound (4) as determined by molecular dynamics and Monte Carlo conformational analysis in *QUANTA* (red) and the experimentally determined X-ray conformation of (4) (green) in its FFKBP12 complex are also included.

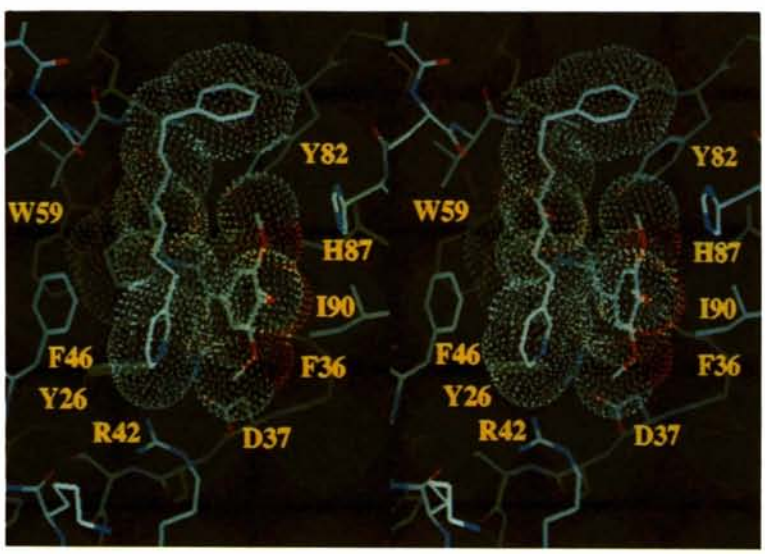
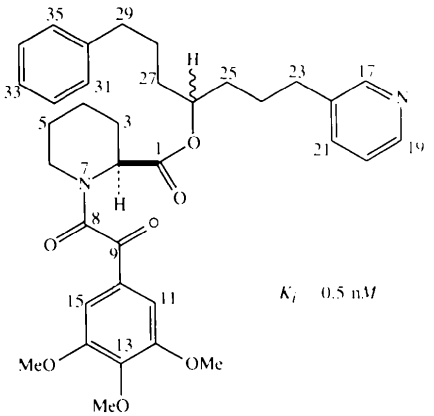


Fig. 5. Structure of the compound (4) complex with FKBP12, colored by element. The dot-surface representation of the ligand indicates the close fit of the ligand in the active site. Ligand–protein interactions are given in Table 1. A total of 734.0 Å^2 in solvent-accessible surface area is lost on binding the ligand.

A structural understanding of the composite surface of these inhibitors in complex with FKBP12 would then allow for the *de novo* design of small-molecule immunophilin-mediated calcineurin inhibitors. In this report we describe the identification of a first generation of subnanomolar inhibitor of FKBP12 [compound (4)], and the detailed analysis of the interactions that this molecule makes with this protein as determined by X-ray crystallographic analysis of the immunophilin–ligand complex.



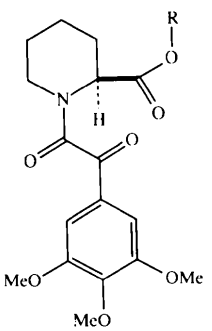
(4)

Methods

The purification of bovine FKBP12 and the protocol used to form ligand complexes with the protein have been described in the accompanying paper (Wilson *et al.*, 1995). Crystals of bovine FKBP12 in complex with ligand (4) were grown by vapor diffusion. 5 µl drops containing 110 mg ml^{-1} solutions of the complex were mixed with an equal volume of reservoir solution containing 30% PEG 6000, 1 *M* LiCl, 0.1 *M* sodium acetate, pH 6.5. High-quality crystals grew at room temperature in two weeks after macroseeding. Crystals of the complex diffract to 1.8 Å resolution, in space group *R*3, with unit-cell dimensions $a = b = c = 41.98$ Å, and $\alpha = \beta = \gamma = 83.98°$. 7897 unique reflections were obtained from diffraction data collected on a Siemens multiwire area detector. This represents 90% of all the available reflections, and 66% of the reflections in the 1.9–1.8 Å shell of data. Reflections were processed using the *XENGEN* system of programs (Howard, Gilliland, Finzel & Poulos, 1987), as provided by the manufacturer. Initial phase information was obtained by Patterson correlation molecular-replacement methods (Brünger, 1990*a*,*b*) using protein coordinates from the structure of the FKBP12–FK506 complex (Van Duyne *et al.*, 1991, 1993; Wilson *et al.*, 1994). The model was improved by map interpretation using the program *QUANTA* (Version 3.3, Molecular Simulations Inc., 1993), and by cycles of simulated-annealing refinement using *X-PLOR* (Brünger, 1990*b*; Brünger, Krukowski & Erickson, 1990). Difference electron-density maps allowed the placement of 42 solvent molecules. Further refinement and map interpretation allowed for the unambiguous placement of the ligand (4) in the structure (Fig. 2). The current *R* factor for the structure of the FKBP12 complex with ligand (4) stands at 18.2% at 1.8 Å resolution, with good geometry (r.m.s. bond distances = 0.014 Å; r.m.s. bond angles = 2.84°).

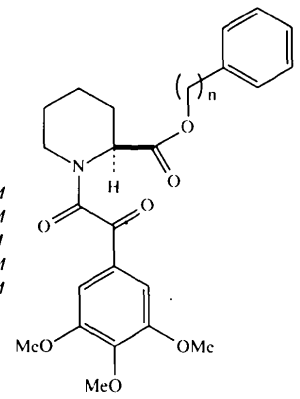
Results and discussion

An analysis of the solution NMR (Moore, Peattie, Fitzgibbon & Thomson, 1991; Michnick, Rosen, Wandless, Karplus & Schreiber, 1991) and X-ray (Wilson *et al.*, 1995) structures of uncomplexed FKBP12, and of the X-ray structure of the FK506–FKBP12 complex (Van Duyne *et al.*, 1991, 1993; Wilson *et al.*, 1995) reveals that the binding pocket of FKBP12 is largely hydrophobic, containing six of the nine aromatic side chains in the protein. We chose to explore the FKBP12-binding site (Fig. 3) with a flexible hydrophobic probe tethered to the fixed binding core (1). Molecules containing the generalized binding core (1) had been previously identified as potent inhibitors of FKBP12 (Armistead *et al.*, 1995) and this core was chosen as the basis molecule for this study.



(1)

The distance from a terminal hydrophobic phenyl group [*R* = Ph in (1)] and the binding core was varied by the sequential addition of methylene spacers. The effect of this variation on the binding to FKBP12 was monitored by the measurement of the K_i value for a particular ligand as an inhibitor of the PPIase activity of FKBP12. [Details of the assay used have been described by Park, Aldape, Futer, DeCenzo, & Livingston (1992).] We assumed that the flexible arm of the inhibitor, linked to the core (1) bound in the protein, would have access to protein interactions involving van der Waals contacts between the terminal aromatic ring of the ligand and the hydrophobic binding pocket of the enzyme. K_i values for these molecules vary [see (I)] from the benzyl ester (2*a*) to the 6-phenylhexyl ester (2*e*), with the most potent compound in this series being the 4-phenylbutyl ester

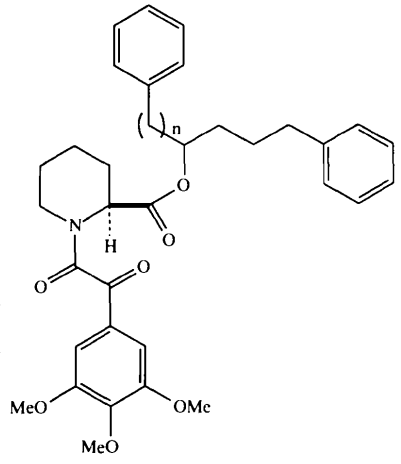


(2a) $n = 1$; $K_i = 100$ n*M*
(2b) $n = 2$; $K_i = 110$ n*M*
(2c) $n = 3$; $K_i = 19$ n*M*
(2d) $n = 4$; $K_i = 70$ n*M*
(2e) $n = 5$; $K_i = 100$ n*M*

(I)

(2*c*). This compound is approximately 50-fold less potent than FK506 as a PPIase inhibitor.

The next iteration in this process was to append a second, analogous, flexible aromatic tether onto (2*c*) to further probe the FKBP12-binding pocket and to identify additional binding sites, in an effort to increase the potency of this class of inhibitors. Once again, the distance between the terminal aromatic ring and the core was varied by the addition of methylene groups to produce a second generation of compounds [see (II)]. Of these, the optimal symmetrical bis-phenylpropyl ester (3*d*) is only twofold less potent than FK506 as an inhibitor of FKBP12, with a K_i of 1.0 n*M*. We refer to these compounds as 'semaphores', with reference to the two-armed signaling devices they resemble.



(3a) $n = 0$; $K_i = 80$ n*M*
(3b) $n = 1$; $K_i = 32$ n*M*
(3c) $n = 2$; $K_i = 83$ n*M*
(3d) $n = 3$; $K_i = 1$ n*M*
(3e) $n = 4$; $K_i = 20$ n*M*
(3f) $n = 5$; $K_i = 45$ n*M*

(II)

With a potent small-molecule PPIase inhibitor in hand, our next goal was to co-complex a member of the semaphore class of inhibitors to FKBP12 for X-ray crystallographic studies. To improve the solubility characteristics of these relatively hydrophobic compounds, we chose to replace one of the phenyl groups in (3*d*) with a more hydrophilic pyridine ring. The diastereomeric 3-pyridylpropylphenylpropyl ester (4) was essentially equipotent to FK506 as a FKBP12 inhibitor [K_i = 0.5 *versus* 0.4 n*M*, respectively (Park *et al.*, 1992)] and was chosen as our initial candidate for structural analysis by molecular modeling and X-ray crystallography.

Table 1. *Contacts between the semaphore inhibitor* (4) *and FKBP*12

Distances were calculated from the coordinates of the refined structure of the complex using the program *X-PLOR* (Brünger, 1990*b*).

FKBP12	Ligand 4	Distance (Å)
Tyr26 OH	C9-carbonyl O atom	3.36
Tyr26 OH	C6	3.49
Tyr26 OH	N18	3.70
Tyr26 CE1	C9-carbonyl O atom	3.52
Tyr26 CZ	C5	3.56
Phe36 CE2	C9-carbonyl O atom	3.46
Asp37 OD1	C15	3.50
Asp37 OD1	C14-methoxy methyl	3.57
Asp37 OD1	C9-carbonyl O atom	3.65
Arg41 NH1	N18	3.41
Arg42 NH1	C19	3.67
Phe46 CE1	C4	3.69
Glu54 O	C28	3.48
Val55 CA	C1-carbonyl O atom	3.19
Ile56 N	C1-carbonyl O atom	3.02
Trp59 CD2	C6	3.49
Trp59 NE1	C3	3.54
Tyr82 OH	C8-carbonyl O atom	2.73
Tyr82 OH	C12-methoxy methyl	3.18
Tyr82 OH	C1-ester O atom	3.26
Tyr82 CE2	C12-methoxy methyl	3.49
His87 CD2	C12-methoxy O atom	3.68
Ile90 CD1	C14	3.50
Ile90 CD1	C15	3.60

Modeling studies showed that it was possible to overlap one of the semaphore arms of compound (4) with the effector domain of FK506, and indeed it was our hope that such a conformation would enable us to mimic the exposed region of FK506. This overlap is shown in Fig. 4 with FK506 shown in violet, and the initial effector mimic conformation in yellow. However, molecular dynamics and other conformational analysis carried out with *QUANTA* and *CHARMm* suggested that strong aromatic–aromatic interactions between one semaphore arm of the ligand and the side chain of Phe46 would pull the inhibitor deeper into the binding pocket. Consequently inhibitors in this class would entirely miss the effector domain (Fig. 4, red). This prediction was later confirmed by the X-ray structure (Fig. 4, green), where the r.m.s. deviation between the modeled and X-ray structures is ~0.5 Å. The large difference between the initial and experimentally determined conformations is possible because of the many rotatable bonds in the semaphore inhibitors. This flexibility makes these compounds non-optimal as platforms for the design of immunosuppressive FK506 mimics.

The structure of the FKBP12 complex with ligand (4), and the corresponding $2|F_o| - |F_c|$ electron-density map, are shown superimposed in Fig. 2. One of the two semaphore arms of ligand (4) occupies roughly the same volume in the binding cavity that corresponds to the 'northwest corner' (atoms C27–C34 in Fig. 1*a*) of FK506

in its complex with FKBP12. The other semaphore arm fills a previously unidentified 'southeast' binding site in the vicinity of residue Phe46 and Tyr26, as defined in Table 1 and Figs. 2 and 5. The assignment of the pyridyl semaphore arm to the southeast binding site is made on the basis of chemical consistency, since the identity of the phenyl and pyridyl arms is indistinguishable structurally. This interaction may be responsible for the tight binding – comparable to that of FK506 itself – that is observed for this compound.

Fig. 3 shows the structure of the complex in the immediate vicinity of the active site of FKBP12. The pipecolate and dicarbonyl components common to FK506 and to ligand (4) are seen to overlap quite closely, suggesting that the macrocyclic structure of FK506 is not required to drive tight binding to FKBP12. Instead, the tight binding seen for compound (4) may result from its snug fit to the structure of FKBP12, as seen in Fig. 5. Somewhat more quantitatively, the solvent-accessible area excluded (Le Grand & Mertz, 1993) on binding compound (4) is calculated to be 734.0 $Å^2$.

Concluding remarks

The non-macrocyclic semaphore inhibitors described here have been shown to be tight binders to FKBP12 and potent inhibitors of the PPIase activity of that protein. In spite of that, concurrent calcineurin inhibitory activity is not observed for those molecules in complex with FKBP12. One obvious reason for this is the absence of an equivalent to the effector domain of FK506 (backbone atoms C18–C23 and the C21 allyl group) projecting into the space proximal to FKBP12 that might well be occupied by calcineurin (Fig. 2). A second possibility is that the phenyl group involved in the northwest corner interaction is not mimicking correctly the C29–C33 unsaturated hexyl ring of FK506. A third effect on the structure of the complex may be due to the 3,4,5-trimethoxy benzene ring of ligand (4), which is not in the plane of the pyran moiety in FK506. This substituent occupies a volume that is not filled in the FKBP12–FK506 complex (Fig. 2). In addition, the substituent seriously distorts the position of the flexible loop (Wilson *et al.*, 1995) containing residues His87, Ile90 and others as compared to the conformation in the FK506–FKBP12 complex (Fig. 2). Mutations at Ile90 in particular have been shown to significantly affect the ability of the mutant FKBP12 complex with FK506 to bind calcineurin (Aldape *et al.*, 1992; Yang, Rosen & Schreiber, 1993, Itoh *et al.*, 1995).

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