Design and synthesis of a rapamycin-based high affinity binding FKBP12 ligand‡

TK Chakraborty[a]*, HP Weber[b] and KC Nicolaou[c]*

[a]Organic Division III, Indian Institute of Chemical Technology, Hyderabad 500 007, India, [b]Preclinical Research, Sandoz Pharma Ltd, CH-4002 Basel, Switzerland and [c]Department of Chemistry, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA and Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA.

Background: The immunosuppressants rapamycin, ascomycin, FK506, and cyclosporin act by binding to a class of cytosolic proteins, the immunophilins. In the case of FK506, ascomycin and cyclosporin, the target of the immunophilin–immunosuppressant complex is calcineurin; in the case of rapamycin, the target is FRAP (TOR/RAFT1). Rapamycin, ascomycin and FK506 have a common domain responsible for binding to FKBP12, their cellular receptor, and different effector domains that determine the target of the complex. Both domains are necessary for signal transduction and biological activity.

Results: A hybrid molecule containing the rapamycin-FK506-ascomycin binding domain and a peptide tether has been designed, synthesized and biologically evaluated. The designed compound binds to FKBP12 with high afffinity but has no biological activity, as expected from its lack of an effector domain.

Conclusions: The designed rapamycin-based FKBP12 ligand exhibits powerful binding properties but, unlike rapamycin, shows no activity in IL-6 dependent B-cell proliferation and, in contrast to FK506, shows no activity in the IL-2 reporter assay. The modular nature of this designed molecule should make it possible to generate a series of compounds with effector domains for targeting either calcineurin or FRAP (TOR/RAFT1) or both, as potential biological tools and immunosuppressive agents.

Chemistry & Biology March 1995, **2**:157–161

Key words: FKBP12, FRAP (TOR/RAFT1), immunosuppression, rapamycin analogs

Introduction

Rapamycin (compound **1**, Fig. 1) [1], ascomycin (compound **2**) [2], FK506 (compound **3**) [3], and cyclosporin (compound **4**) [4] are naturally-occurring substances with potent immunosuppressive properties [5–8]. These compounds bind tightly to the immunophilins, a class of cytosolic proteins, forming complexes which in turn serve as ligands for other cellular targets involved in signal transduction [5–8]. Cyclosporin binds to cyclophilin [9], while ascomycin, FK506 and rapamycin all bind to FKBP12 [10,11]. The target of the cyclophilin–cyclosporin, FKBP12–ascomycin and FKBP12–FK506 complexes is calcineurin [12], whereas that of the FKBP12–rapamycin complex is a recently-characterized cellular target named FRAP [13], also called TOR [14] or RAFT1 [15]. Binding of the FKBP12–rapamycin complex to FRAP signals the arrest of the cell cycle.

The structures of FKBP12 and its ligands, FK506, ascomycin, and rapamycin have been elucidated by NMR spectroscopy [16,17] and X-ray crystallography [18,19]. Together with extensive biological results these studies established that each of these novel immunosuppressants bears two distinct domains, one that binds to the immunophilin receptor (the binding domain; pink segment, Fig. 1) and another that, together with the protein, serves as a composite surface to bind to the second protein target (the effector domain; blue segment, Fig. 1). Thus, these small organic molecules act as 'molecular adapters', noncovalently binding to the immunophilin via the binding domain, and forming a composite binding surface composed partly of the effector domain, partly of the immunophilin surface, which allows binding to the new target, exerting specific biological actions. This dual-domain model for the drug mechanism of action of these compounds provides unique opportunities and challenges in structure-based design of ligands for these proteins, and indeed a number of studies have already appeared [20–27].

Results and discussion

Molecular design

With the aim of designing FKBP12 binding ligands whose complexes may or may not bind to the second cellular target, we considered structures **5a–c** (rapamycin-peptides, RAP-Pa–c, shown in Fig. 2). Such molecules, which contain the FKBP12-binding domain of rapamycin, FK506 and ascomycin, may be derived, in principle, from rapamycin (**1**) by semisynthesis through the common intermediate **8** (Fig. 3). The conformation of rapamycin's FKBP12-binding domain as found by X-ray crystallographic analysis of the FKBP12–rapamycin complex [19] was chosen as the most desired one for optimum binding. Operating on the three structures **5a–c** (Fig. 2), and using computer modeling, we undertook a computational search to determine the most suitable tether to constrain the binding domain of the designed molecules in this

*Corresponding authors. ‡This paper is dedicated to Professor AV Rama Rao on the occasion of his 60th birthday.

 ISSN 1074-5521

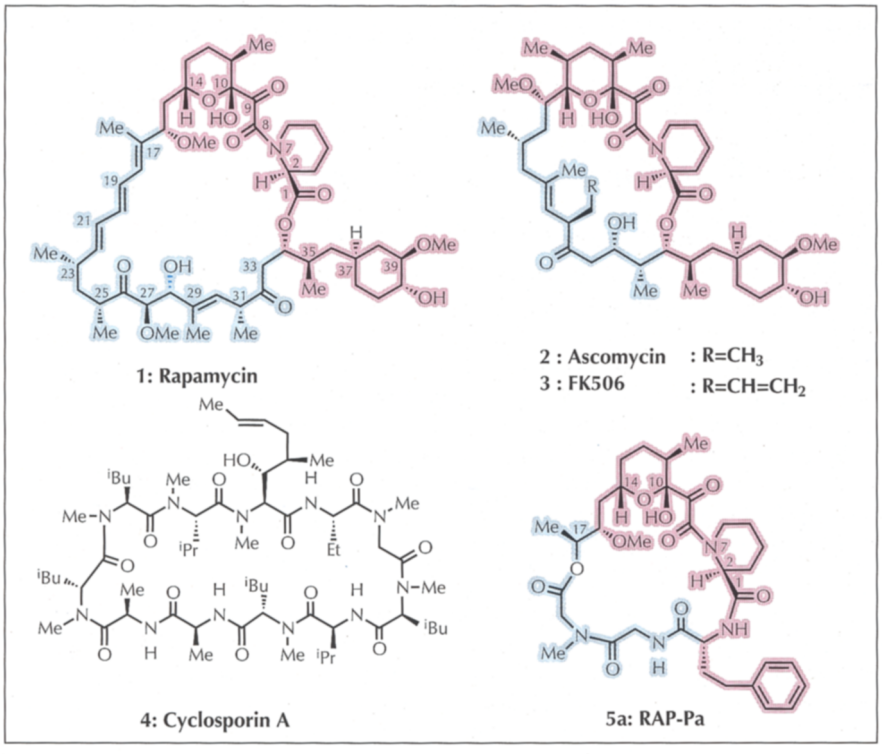

Fig. 1. Structures of the immunosuppressants rapamycin, ascomycin, FK506 and cyclosporin. The structure of the designed rapamycin-based FKBP12 ligand RAP-Pa is also shown. Pink, binding domain; blue, effector domain.

preferred conformation (see Materials and methods for details). The designed molecules (**5a–c**) contained the three peptide cassettes shown in Fig. 2. The D-homoPhe-Gly-sarcosine (Sar) cassette allows the formation of a 21-membered ring that is the same size as that found in FK506 and ascomycin, whereas the D-homoPhe-$(Gly)_3$ and D-homoPhe-$(Gly)_4$ cassettes lead to 24- and 27-membered rings, respectively. The D-homoPhe group was placed at the 'right' end of the peptide chain to substitute for the cyclohexyl appendage, the D-isomer providing the best fit for the C-26 stereocenter of ascomycin (or the C-34 center for rapamycin). The sarcosine residue in **5a** (RAP-Pa) was used to mimic the ethyl group at C-21 of ascomycin. A further advantage of the modular nature of the designed molecules is that it should allow the generation of libraries of compounds with effector domains that can bind to either calcineurin or FRAP (TOR/RAFT1) or both, as desired.

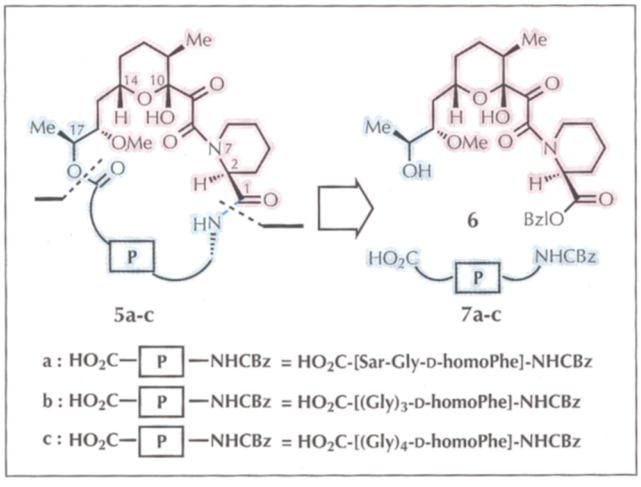

Fig. 2. Molecular design and retrosynthetic analysis of the designed FKBP12 ligands RAP-Pa-c (**5a–c**).

Chemical synthesis of designed rapamycin-based ligand 5a (RAP-Pa).

The synthesis of the hybrid compound **5a** (RAP-Pa) started with compound **8**, itself available in two steps [28] from rapamycin and proceeded along the lines summarized in Fig. 3. Diastereo- and chemoselective reduction of compound **8** with lithium selectride (Li-selectride) at −78 °C gave a single product, compound **6**, (96 % yield) which was assigned the S-configuration based on the non-chelation control Anh–Eisenstein model [29]. Silylation of compound **6** with trimethylsilyl triflate (TMSOTf) followed by brief exposure to dilute acid gave the hemiketal-protected derivative **9** in 92 % yield (~1:1 mixture of diastereoisomers). The latter compound was then coupled with the peptide chain shown as **7a** in Fig. 2 using 1,3-dicyclohexylcarbodiimide (DCC) and catalytic amounts of *N,N*-4-dimethylaminopyridine (DMAP) to give ester **10** in 85 % yield. Hydrogenolysis of both the Cbz and Bzl groups followed by macrolactamization using BOP reagent and diisopropylethylamine ($^iPr_2EtN$) gave the desired macrocycle ring in 45 % overall yield. Finally, desilylation of the latter compound with HF•pyridine furnished the targeted molecule **5a** in 92 % yield (mixture of two isomers, ~3:1 ratio).

Biological studies

The binding affinity of compound **5a** for FKBP12 was measured and compared to those of ascomycin and rapamycin using a competitive binding assay developed at Sandoz. The IC_{50} values for **5a**, ascomycin and rapamycin

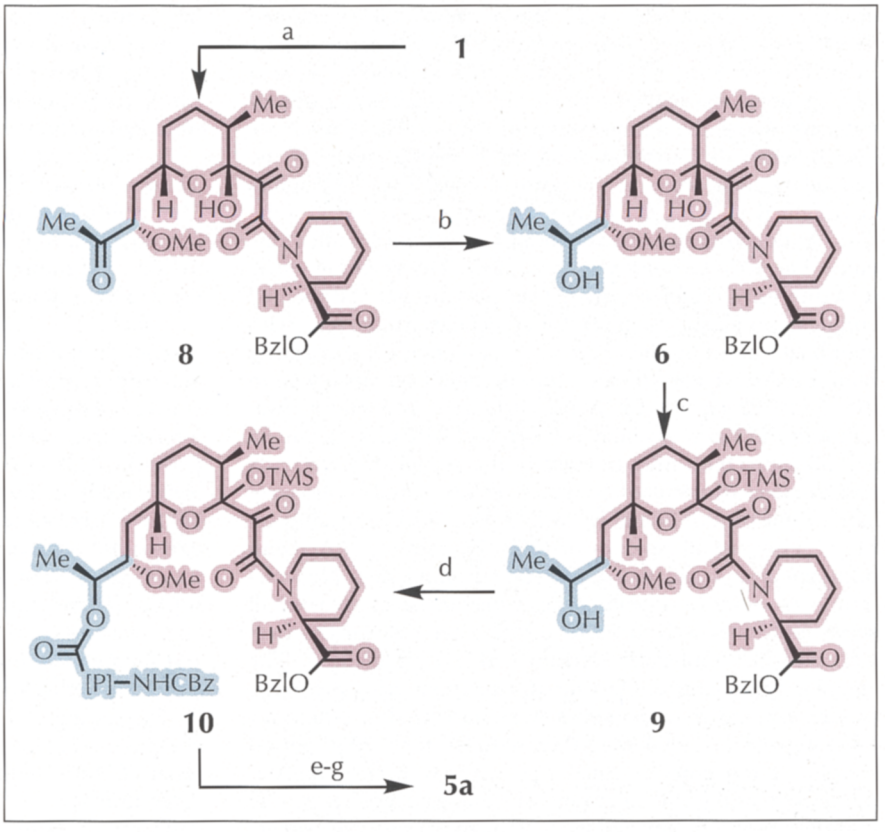

Fig. 3. Synthesis of the designed FKBP12 ligand RAP-Pa (compound **5a**). Reagents and conditions: **(a)** see [27], (two steps); **(b)** 3.0 equiv of Li-selectride, THF, –78 °C, 15 min, 96 %; **(c)** 2.2 equiv of TMSOTf, 4.0 equiv of 2,6-lutidine, CH_2Cl_2, 0 °C, 15 min, then 0.1 N HCl: THF (1:25), 25 °C 15 min 92 %; **(d)** 2.0 equiv of CBzNH-P-CO_2H, 2.0 equiv of DCC, 0.2 equiv of DMAP, CH_2Cl_2 0 °C, 48 h, 85 %; **(e)** H_2, 0.l equiv of 10 % palladium on carbon (Pd/C), MeOH, 25 °C, 1 h; **(f)** 3.0 equiv of BOP-reagent, 7.0 equiv of $^iPr_2EtN$, $CHCl_3$ (c=0.002 M), 25 °C, 4 h, 45 % (over 2 steps); **(g)** HF•pyridine, THF, 25 °C, 24 h, 92 %.

in this assay were found to be 9.6, 0.52 and 0.45 nM, respectively. In contrast to rapamycin, however, the compound showed no activity in IL-6-dependent B-cell proliferation and, in contrast to FK506, it exhibited no activity in the IL-2 reporter assay.

These results clearly demonstrate that it is possible to design, via molecular modeling, compounds with high binding affinity to FKBP12 using the binding domain of rapamycin as an anchor to bind to the protein receptor. Furthermore, these studies demonstrate the importance of the effector domain for biological activity and open the possibility of designing hybrid molecules with both FKBP12 binding sites and effector domains for either calcineurin or FRAP (TOR/RAFT1).

Significance

Immunosuppression is an important area of research, the medical benefits of which include the ability to transplant organs successfully and to treat a variety of immune diseases. The naturally-occurring immunosuppressive agents rapamycin, ascomycin, FK506 and cyclosporin are important clinical or potential clinical agents and are also useful biological tools in research into the mechanisms of signal transduction in T cells.

It has previously been suggested that the structures of these immunosuppressive agents can be divided into two domains, one responsible for binding to the cellular receptor, the other responsible for interacting with the target. In this study we have designed and synthesized a rapamycin-based compound that has only one of these functional domains, the domain responsible for binding to FKBP12, the cellular receptor for rapamycin, ascomycin and FK506. This designed compound does indeed bind tightly to FKBP12, but it does not have any of the biological activities of rapamycin, ascomycin or FK506. This observation confirms the prediction that both the binding domain and the effector domain are required to produce a biological effect.

The structure of our designed FKBP-binding compound is modular. It is therefore now practical to generate a large number of these compounds, with or without the addition of domains that mimic the effector domain. It is conceivable that molecules may emerge from such libraries of related compounds that mimic or antagonize the biological actions of rapamycin or FK506/ascomycin, or even both. Such compounds may find useful applications in biology as tools, or in medicine as improved immunosuppressive agents.

Materials and methods

Molecular modeling

All molecular modeling studies were carried out using BIOSYM Insight II (2.2.0) from BIOSYM Technologies, Inc, San Diego, CA 92121 or Discover (2.9) on a Silicon Graphics terminal (4D-80) and on an Alliant F2800. The binding

domain of rapamycin was taken from the published FKBP12–rapamycin crystal structure. The structure of the 21-membered ring **5a** was constructed as follows. First a peptide bond was made between the C-1 carboxyl group of fragment **6** and the amino group of D-homoPhe-Gly-sarcosine. A series of torsions around the ϕ and ψ angles of the peptide fragment were then performed visually on the graphics of Insight II to bring the carboxyl group of sarcosine and the C-17 hydroxyl group of compound **6** to within bonding distance. The CO–O bond was then made. The resulting cyclic structure was minimized, first by steepest descent and then by conjugate-gradient methods using default parameters with coordinates fixed on the entire C_1/C_{17} segment. The resulting minimized structure was then superimposed on rapamycin in the FKBP12–rapamycin X-ray structure. Rapamycin was removed and a new assembly comprising FKBP12, structure **5a** and 53 water molecules (present in the crystal structure) was constructed. Molecular dynamics (MD) simulations were carried out on this assembly, putting positional constraints on the C atoms of all residues of FKBP12 within a 6 Å radius from the ligand and fixing all atoms beyond that. No constraints were set on ligand atoms, whereas the oxygens of all water molecules were fixed. The cvff. force field of Discover was used for molecular dynamics (MD) simulations. The assembly was minimized first for 100 iterations using the steepest descent and then dynamics were initialized at 300 K for 1000 steps of 1 fs. MD were then resumed for a 20 ps period sampling a trajectory at equal 1 ps intervals. Each frame was minimized using 100 iterations of conjugate gradients. The resulting twenty minimized ligand structures were then superimposed as shown in Fig. 4. The average root-mean-square deviation was about 0.1 Å. The analysis of the trajectory showed that the conformation of the domain remained almost fixed, although there were no constraints on it during the entire simulation process. Similar results were obtained when all constraints were removed from FKBP and the water molecules. The binding-domain conformation of compound **5a** remained almost unchanged even when FKBP12 was removed and the ligand was 'soaked' with water molecules in a sphere of 10 Å radius (a total of 104 water molecules were added) and dynamics were carried out on the assembly in a similar fashion as described above without any constraints for a 20 ps period.

Similar MD simulations were carried out compounds **5b** and **5c**, which contain 24- and 27-membered rings (Fig. 2). These simulations revealed considerable deviations in both the binding domain and the entire ligand geometry as the ring size and flexibility of the macrocycle increased. The greater entropic cost of binding such more flexible rings with FKBP12 would probably lead to weaker binding.

These molecular modeling studies led us to conclude that the 21-membered designed ring system **5a** was the best of the three compounds examined (**5a–c**) in terms of its rigidity and ability to 'lock' the rapamycin/FK506/ascomycin binding domain in the desired conformation for complexation with FKBP12.

Preparation of compound 5a

Standard synthetic methods and procedures were used to convert rapamycin (compound **1**) to compound **10** via the intermediates shown as **8**, **6** and **9** in Fig. 3. The amino acid (7.6 mg, 0.01 mmol) generated from compound **10** by hydrogenolysis was dissolved in anhydrous $CHCl_3$ (5 ml, 0.002 M) and stirred under argon with BOP reagent (13 mg, 0.03 mmol) and diisopropyl ethyl amine ($^iPr_2EtN$, 12 µl, 0.07 mmol) at 25 °C for 4 h. The reaction mixture was diluted with EtOAc (10 ml) and washed with brine (2 x 5 ml). The water layer was re-extracted with EtOAc (2 x 5 ml), dried ($MgSO_4$) and filtered. Concentration and flash chromatography (silica, 80→100 % ethyl acetate in petroleum ether) gave trimethylsilyl-**5a** (3.4 mg, 4.5 µmol, 45 %) as an amorphous solid. This compound (3.4 mg, 4.5 µmol) was dissolved in dry tetrahydrofuran (THF) (100 µl) and treated with HF•pyridine (10 µl). After thin layer chromatographic analysis revealed complete desilylation (24 h), the reaction mixture was quenched with aqueous saturated $NaHCO_3$ (5 ml) and extracted with EtOAc (3 x 5 ml). The combined organic layers were dried ($MgSO_4$), filtered, concentrated and subjected to flash chromatography (silica, EtOAc→5 % MeOH in EtOAc) to afford pure **5a** (2.8 mg, 4.1 µmol, 92 %) as a white amorphous solid. **5a**: mixture of isomers in DMSO (ca. 3:1) at room temperature; R_f = 0.12 (major), 0.10 (minor) (silica, EtOAc); high-pressure liquid chromatography (HPLC; analytical, Lichrospher 100 RP-18, 125 x 4 mm, 5 µM column): Retention time (RT) (min), 10.08 (major), 9.2 (minor) (gradient, 70:30→30:70 H_2O:CH_3CN in 20 min, flow rate 1.5 ml min^{-1}, at 40 °C); ^1H NMR (500 NHz, DMSO-d_6, mixture of isomers, ~3:1) δ 8.11 (major, dd, *J*=4.8 Hz) and 7.73 (minor, d, *J*=6.6, 3.0 Hz) (total 1 H, GlyN*H*); 7.4 (minor, d, *J*=7.8 Hz) and 6.67 (major, d, *J*=7.8 Hz) (total 1 H, homoPheN*H*); 7.3–7.1 (m, 5 H,

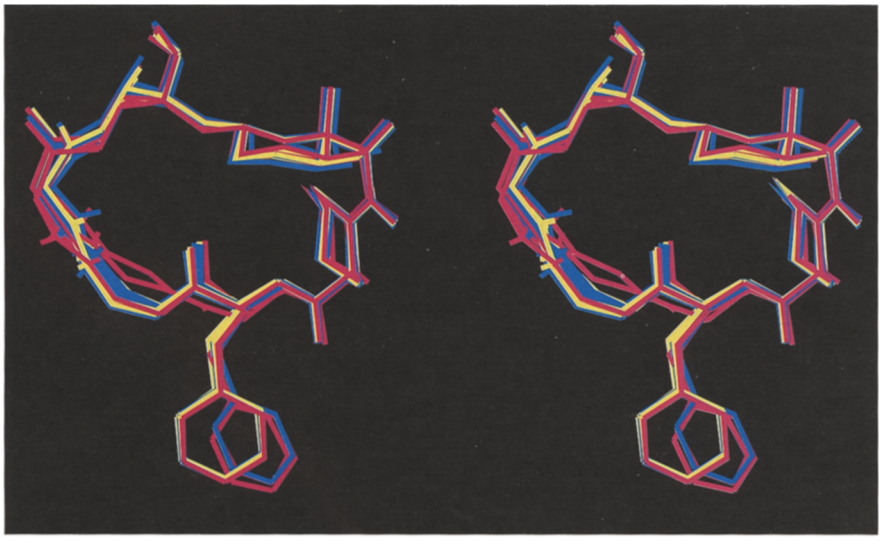

Fig. 4. Stereoview of the 20 superimposed energy-minimized structures of RAP-Pa (compound **5a**) sampled during 20-ps molecular dynamics simulations on an assembly comprising FKBP12, RAP-Pa and 53 water molecules present in the FKBP12–rapamycin crystal structure. For clarity FKBP12 and water molecules are not shown.

aromatic); 6.73 (major, s) and 6.57 (minor, s) (total 1 H, $C_{10}OH$); 5.04 (major, dq, J=6.6, 3 Hz) and 4.92 (minor, dq, J=6.6 Hz) (total 1 H, $C_{17}H$); 4.96 (m, 1 H, C_2H); 4.49 (major, d, J=18.0 Hz) and 3.86 (minor, d, J=16.8 Hz,) (total 1 H, Sarα-H): 4.32 (m, 2 H, $C_{26}H$, $C_{16}H$); 4.14 (major, m) and 3.85 (minor, m) (total 2 H, Glyα-H); 3.95 (m, 2 H, Sarα-H, $C_{14}H$); 3.54 (major, br d, J=15.0 Hz) and 3.47 (minor, br d, J=15.0 Hz) (total 1 H, C_6H); 3.39 (major, dd, J=15.0 Hz) and 3.31 (minor, dd, J=15.0 Hz) (total 1 H, C6H'); 3.21 (major, s) and 3.19 (minor, s) (total 3 H, OCH_3); 3.04 (minor, s) and 2.77 (major, s) (total 3 H, SarN–CH_3); 2.58 (m, 2 H, PhCH_2); 2.2–1.2 (m, 15 H, CH_2 and CH); 1.11 (major, d, J=6.6 Hz) and 1.0 (minor, d, J=6.6 Hz) (total 3 H, $C_{17}CH_3$); 0.73 (d, J=6.6 Hz, 3 H, $C_{11}CH_3$); MS(FAB): Calc'd. for $C_{34}H_{48}N_4O_{10}$+Li (M+Li)$^+$: 679, found m/z 679 (M+Li)$^+$.

Acknowledgements: We thank Drs R Sedrani, S Cottens, H Fliri, MD Walkinshaw and RM Wenger for useful discussions and suggestions. We also wish to acknowledge the assistance from M Ponelle (NMR spectroscopy), C Quiquerez (mass spectroscopy), and Drs G Zenke and W Schuler (biological assays).

References

1. Swindells, D.C.N., White, P.S. & Findlay, J.A. (1978). The X-ray crystal structure of rapamycin, $C_{51}H_{79}NO_{13}$. *Can. J. Chem.* **56**, 2491–2492.
2. Morisak, M. & Arai, Y. (1992). Identity of the immunosuppressant FR-900520 with ascomycin. *J. Antibiotics (Tokyo)* **45**, 126–127.
3. Tanaka, H., *et al.*, & Taga, T. (1987). Structure of FK506: a novel immunosuppressant isolated from *Streptomyces*. *J. Am. Chem. Soc.* **109**, 5031–5033.
4. Rüegger, A., *et al.*, & von Wartburg, A. (1976). Cyclosporin A: ein immunsuppressiv wirksamer peptid metabolit aus *Trichoderma polysporum Rifai*. *Helv. Chim. Acta* **59**, 1075–1082.
5. Goto, T., *et al.*, & Imanaka, H. (1991). FK506: historical perspectives. *Transplant. Proc.* **23**, 2713–2717.
6. Schreiber, S.L. & Rosen, M.K. (1992). Natural products as probes of cellular function: studies of immunophilins. *Angew. Chem. Int. Ed. Engl.* **31**, 384–400.
7. Handschumacher, R.E., Harding, M.W., Rice, J., Drugge, R.J. & Speicer, D.W. (1984). Cyclophilin A: a specific cytosolic bonding protein for cyclosporin A. *Science* **226**, 544–547.
8. Snyder, S.H. & Sabatini, D.M. (1995). Immunophilins and the nervous system. *Nat. Med.* **1**, 32–37.
9. Schreiber, S.L. (1991). Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science* **251**, 283–287.
10. Siekierka, J.J., Hung, S.H.Y., Poe, M., Lin, C.S. & Sigal, N.H. (1989). A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-propyl isomerase activity but is distinct from cyclophilin. *Nature* **341**, 755–760.
11. Harding, M.W., Galat, A., Uehling, D.E. & Schreiber, S.L. (1989). A receptor for the immunosuppressant FK506 is a *cis–trans* peptidyl-propyl isomerase. *Nature* **341**, 758–760.
12. Liu, J., Farmer, J.D., Friedman, Jr, J., Weissman, I. & Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin–cyclosporin A and FKBP–FK506 complexes. *Cell* **66**, 807–815.
13. Brown, E.J., *et al.*, & Schreiber, S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin–receptor complex. *Nature* **369**, 756–758.
14. Kunz, J., Henriques, R., Schneider, U., Deuter-Reinhard, M., Movva, N.R. & Hall, M.N. (1993). Target of rapamycin in yeast, TOR2, is an essential phosphatidyl kinase homolog required for G1 progression. *Cell* **73**, 585–596.
15. Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P. & Snyder, S.H. (1994). RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **78**, 35–43.
16. Meadows, R.P., *et al.*, & Fesik, S.W. (1993). Three-dimensional structure of the FK506 binding protein/ascomycin complex in solution by heteronuclear three- and four-dimensional NMR. *Biochemistry* **32**, 754–765.
17. Wandless, T.J., Michnick, S.W., Rosen, M.K., Karplus, M. & Schreiber, S.L. (1991). FK506 and rapamycin binding to FKBP: common elements in immunophilin–ligand complexation. *J. Am. Chem. Soc.* **113**, 2339–2341.
18. Van Duyne, G.D., Standaert, R.F., Karplus, P.A., Schreiber, S.L. & Clardy, J. (1993). Atomic structures of the human immunophilin FKBP12 complexes with FK506 and rapamycin. *J. Mol. Biol.* **229**, 105–124.
19. Van Duyne, G.D., Standaert, R.F., Schreiber, S.L. & Clardy, J. (1991). Atomic structure of the rapamycin human immunophilin FKBP12 complex. *J. Am. Chem. Soc.* **113**, 7433–7434.
20. Ikeda, Y., Schultz, L.W., Clardy, J. & Schreiber, S.L. (1994). Structural basis for peptidomimicry by a natural product. *J. Am. Chem. Soc.* **116**, 4143–4144.
21. Holt, D.A., *et al.*, & 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.
22. Andrus, M.B. & Schreiber, S.L. (1993). Structure-based design of an acyclic ligand that bridges FKBP12 and calcineurin. *J. Am. Chem. Soc.* **115**, 10420–10421.
23. Teague, S.J. & Stocks, M.J. (1993). The affinity of the excised binding domain of FK-506 for the immunophilin FKBP-12. *Bioorg. Med. Chem. Lett.* **3**, 1947–1950.
24. Holt, D.A., *et al.*, & Badger, A. (1993). Structure–activity studies of nonmacrocyclic rapamycin derivatives. *Bioorg. Med. Chem. Lett.* **3**, 1977–1980.
25. Hauske, J.R., Dorff, P., Julin, S., DiBrino, J., Spencer, R. & Williams, R.J. (1992). Design and synthesis of novel FKBP inhibitors. *J. Med. Chem.* **35**, 4284–4296.
26. Goulet, M.T. & Hodkey, D.W. (1991). Construction of an FK-506 analog from rapamycin-derived materials. *Tetrahedron Lett.* **32**, 4627–4630.
27. Schreiber, S.L., *et al.*, & Somers, P.K. (1992). Molecular recognition of immunophilins and immunophilin–ligand complexes. *Tetrahedron* **48**, 2545–2558.
28. Goulet, M.T. & Boger, J. (1990). Degradative studies on the tricarbonyl-containing macrolide rapamycin. *Tetrahedron Lett.* **31**, 4845–4848.
29. Anh, N.T. & Eisenstein, O. (1977). Theoretical interpretation of 1,2 asymmetric induction — importance of antiperiplanarity. *Nouv. J. Chim.* **1**, 61–70.

Received: **17 Feb 1995**. Accepted **28 Feb 1995**.