Natural Products as Probes of Cellular Function: Studies of Immunophilins

By Michael K. Rosen and Stuart L. Schreiber*

One of the great mysteries of cell biology remains the mechanism of information transfer, or signaling, through the cytoplasm of the cell. Natural products that inhibit this process offer a unique window into fundamental aspects of cytoplasmic signal transduction, the means by which extracellular molecules influence intracellular events. Thus, natural products chemistry, including organic synthesis, conformational analysis, and methods of structure elucidation, is a powerful tool in the study of cell function. This article traces our understanding of a group of natural products from the finding that they inhibit cytoplasmic signaling to their current recognition as mediators of the interaction between widely distributed protein targets. The emphasis of the discussion is primarily structural. The interactions between the natural-product ligands and their protein receptors are analyzed at a molecular level in order to shed light on the molecular mechanisms of the biological functions of these compounds. In the process we hope to illustrate the power of chemical analysis as applied to biological systems. Through chemistry we can understand the molecular basis of biological phenomena.

1. Introduction

Signal transduction refers to the process by which extracellular molecules influence intracellular events. For example, the actions of the hormone insulin are initiated at the cell surface, when extracellular insulin binds to the membrane-bound insulin receptor. This binding triggers a series of membrane-associated events, followed by a cascade of poorly defined intracellular events that result in the transcription of genes that encode metabolic enzymes. Thus, a signal that originates outside the cell is transmitted through the cell membrane, along intermediate carriers in the cytoplasm, and into the nucleus, causing a change in the status of the cell. In recent years, much has been learned about the mechanisms of signal transduction at the membrane and in the nucleus of the cell. In contrast, very little is known about the mechanisms of signal transduction through the cytoplasm of the cell. Despite the importance of such processes, the detailed mechanisms of cytoplasmic signaling remain among the great mysteries of cell biology, a fact that has led the cytoplasm to be referred to as the "black box" of signal transduction.

Natural products chemistry has long played an important role in the elucidation of biological mechanisms. Pioneering synthetic and mechanistic studies of molecules such as steroids, prostaglandins, and porphyrins, to name only a few, have led to fundamental insights regarding the biological functions of these important classes of compounds. Certain natural products are also uniquely suited for the study of the mysterious processes of the cell, including cytoplasmic signaling, due to their interference with these processes. By studying inhibitory natural products bound to their biological receptors, we may gain a detailed understanding of the function of these receptors. Research in this area relies on a combination of the powerful, complementary tools of synthetic chemistry, molecular biology, cell biology, and methods of structure elucidation, including both NMR spectroscopy and X-ray crystallography. This review highlights recent advances in the structural and mechanistic understanding of cytoplasmic signaling that have arisen through application of these techniques to the study of the immunophilins, a family of cytosolic proteins that bind natural products. These advances have culminated in the identification and characterization of pentameric complexes composed of two normally noninteracting protein constituents and a natural product "glue" that binds the proteins together in a biologically significant manner. In the process, it has been discovered that a protein phosphatase is a key cytoplasmic component of a family of signal transduction pathways.

[*] Prof. Dr. S. L. Schreiber, M. K. Rosen
Department of Chemistry, Harvard University
Cambridge, MA 02138 (USA)

2. Background

FK506[1, 2] and cyclosporin A[3] (CsA) are fungal natural products (Fig. 1) that inhibit Ca^{2+}-dependent[**] signaling pathways in a variety of cell types.[4, 5] In T cells, both agents inhibit the transcription of a number of genes, including that encoding interleukin-2 (IL-2), which are normally activated by stimulation of (by binding of certain extracellular molecules to) the T cell receptor (TCR).[6] In mast cells they inhibit the exocytosis (i.e. movement to the cell surface and fusion with the cell membrane) of secretory vesicles that normally results from stimulation of the IgE receptor.[7] A variety of biochemical and biological data indicate that in both cases inhibition occurs within the cytoplasm and not at the cell surface or in the nucleus.

On a biochemical level FK506 and CsA are also quite similar. Through synthesis of radiolabeled and immobilized derivatives of these agents for use in protein purification, it was discovered that both bind with high affinity to soluble, cytoplasmic receptor proteins[8] (immunophilins;[8, 9] this term is used to denote immunosuppressant binding proteins, as both FK506 and CsA are immunosuppressive drugs[2, 10]). The FK506 receptor has been named FKBP,[11] and the CsA receptor has been named cyclophilin[12] (CyP). Both proteins catalyze the isomerization of *cis* and *trans* amide-bond rotamers of peptide and protein substrates.[11, 12]

[**] The terms Ca^{2+}-dependent and -independent in this context refer to signaling pathways that are characterized by the presence or absence, respectively, of an immediate rise in the concentration of intracellular cytoplasmic Ca^{2+} following binding to the cell surface receptor.

The enzymatic activity of FKBP is potently inhibited by binding of FK506 (K_i = 0.4 nM), but not CsA,[11] and the activity of CyP is inhibited by CsA (K_i = 6 nM),[12] but not FK506.

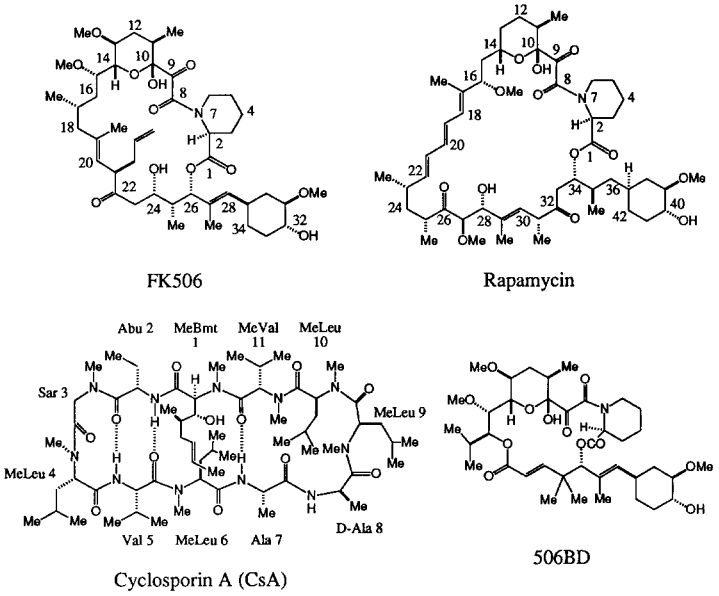

Fig. 1. Chemical formulas of the immunophilin ligands.

Although FK506 is approximately 100 times more effective than CsA in cellular assays, in virtually all other respects the two molecules behave identically in both T cells and mast cells. This similarity in biological function, coupled with the discovery that the molecules inhibit two distinct rotamase enzymes,[*] led to speculation that the biological effects of these agents were due to their rotamase inhibition. It was hypothesized that a necessary step in activation of unknown proteins required for IL-2 transcription in T cells was rotamase-catalyzed isomerization of a peptidyl–prolyl amide bond. Inhibition of this catalysis by FK506 or CsA would then result in inhibition of IL-2 transcription.[13]

Strong evidence against this "rotamase hypothesis" came from biochemical and biological studies of two structurally related molecules: rapamycin,[14, 15] an immunosuppressive fungal natural product and FKBP ligand, and 506BD,[16] a synthetic FKBP ligand (Fig. 1). Given the structural similarities between the three ligands, it is not surprising that, like FK506, rapamycin and 506BD also bind tightly to FKBP and inhibit the rotamase activity of the enzyme (K_i(rapamycin) = 0.2 nM; K_i(506BD) = 5 nM).[15, 16] In fact, NMR and crystallographic studies have demonstrated that FK506 and rapamycin interact with a common domain of FKBP through their common structural elements.[17] Fascinatingly, although FK506 and rapamycin both bind to FKBP and inhibit its rotamase activity, rapamycin does not inhibit the same TCR-mediated signaling pathway that is affected by FK506 and CsA. Rather, it blocks a later Ca^{2+}-independent pathway associated with T cell activation, which is mediated by the IL-2 receptor (IL-2R)[15, 18, 19] (Fig. 2). Even more striking is the fact that 506BD has no inhibitory effect on either of the signaling pathways inhibited by FK506 or rapamycin. Furthermore, it was shown that FK506, rapamycin, and 506BD can all block the actions of the others,

[*] The expression rotamase has the same meaning as PPIase (for peptidyl prolyl *cis–trans* isomerase) an acronym used by others for the description of proteins that catalyze the isomerization of *cis* and *trans* amide-bond rotamers of peptides and proteins.

Stuart L. Schreiber was born in 1956 and was raised in Virginia (USA). He studied as an undergraduate in Charlottsville, Virginia, where he obtained a B.A. in chemistry in 1977 at the University of Virginia. Following Ph.D studies under the direction of R. B. Woodward and Yoshito Kishi at Harvard University, he began his independent career in 1981 in the Department of Chemistry at Yale University. In 1988, he accepted a Professorship at the Department of Chemistry at Harvard. His group is engaged in the study of biological processes through the combined application of synthetic organic chemistry and molecular and structural biology.

Michael Rosen was born in 1965 in Philadelphia, Pennsylvania (USA). He received B.S. degrees in chemistry and chemical engineering from the University of Michigan in 1987. As a Winston Churchill Scholar during 1987–1988 he earned a C.P.G.S. in the Natural Sciences from the University of Cambridge (England) under the direction of Professor Alan R. Battersby. Since that time he has been a graduate student in the research group of Professor Stuart L. Schreiber at Harvard University. His primary interests lie in the structural analysis of proteins and protein–ligand complexes by NMR.

presumably through competitive binding to a common receptor, FKBP. In contrast, the actions of FK506 and rapamycin are unaffected by CsA, which does not bind FKBP; the actions of CsA are also unaffected by FK506, rapamycin, and 506BD, which do not bind CyP.[15, 16, 19, 20] If the biological properties of FK506 and rapamycin were simply due to inhibition of the rotamase activity of FKBP, then they should both interfere with the same pathways. 506BD should also inhibit these same pathways and thus be biologically active. Because this is not the case, it implies that FK506 and rapamycin do *not* act by *eliminating* a function of FKBP; rather they act by *adding* a function to the protein. In a sense, FK506 and rapamycin are prodrugs that are activated by binding to FKBP. CsA is similarly inactive until it binds CyP. This explanation of the immunophilin–ligand complexes as the species responsible for signal inhibition has been termed the "active complex" hypothesis.[8, 9, 15, 16, 21] Genetic studies have also provided strong support for this idea by demonstrating that FKBP is both necessary and sufficient to mediate the actions of rapamycin in yeast,[22, 23] and that CyP mediates the actions of CsA in this organism.[21, 24] Paradoxically, although FK506 and rapamycin are structurally similar and bind to a common protein, FKBP, their FKBP complexes have different biological actions. Furthermore, while CsA is structurally dissimilar to FK506 and binds to CyP, which is unrelated to FKBP, the biological actions of the CyP–CsA complex are indistinguishable from those of the FKBP–FK506 complex. It is interesting to speculate that upon binding to CyP a yet-to-be discovered CyP ligand may provide a complex that has properties indistinguishable from those of the FKBP–rapamycin complex.

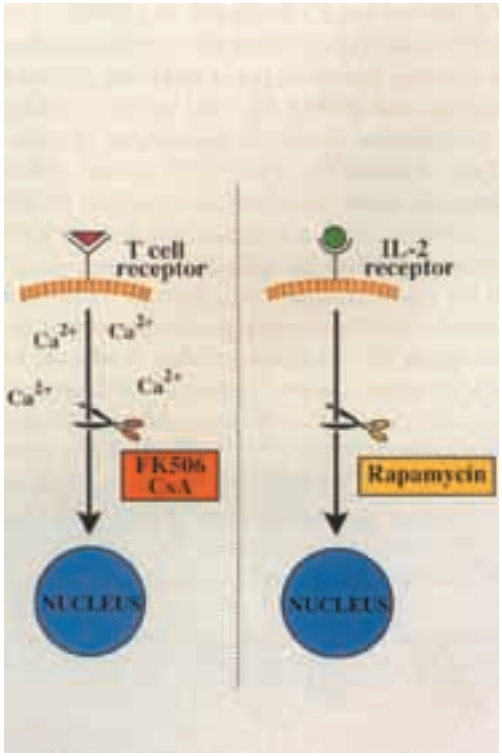

Fig. 2. FK506 and CsA inhibit Ca^{2+}-dependent signaling pathways, while rapamycin inhibits Ca^{2+}-independent pathways. These are illustrated in the T cell by the pathways emanating from the T cell receptor and the IL-2 receptor, respectively, but include signaling pathways in a variety of cell types, including mast cells and neurons.

One obvious, if seemingly unlikely prediction from the above biological data is that the structurally unrelated FKBP–FK506 and CyP–CsA complexes should act, either directly or indirectly (through other proteins), upon a common target molecule that is distinct from the target acted upon by the FKBP–rapamycin complex. The FKBP–FK506 and CyP–CsA target should be a component of Ca^{2+}-dependent signaling pathways such as TCR-mediated transcription in T cells and IgE receptor-mediated exocytosis in mast cells; the FKBP–rapamycin target molecule should be a component of Ca^{2+}-independent signaling pathways such as IL-2R-mediated proliferation in T cells. In both cases, the target should be unaffected by either protein or ligand alone, since only the immunophilin–ligand complexes are active inhibitors (Fig. 3 top).

In fact, we have recently demonstrated that calcineurin (CN), also referred to as protein phosphatase 2B, a calmodulin-dependent serine/threonine protein phosphatase,[25] possesses all the predicted biochemical properties of a target common to both FKBP–FK506 and CyP–CsA.[26] CN is a heterodimeric protein composed of two subunits, calcineurin A (CNA), which contains the calmodulin-binding and phos-

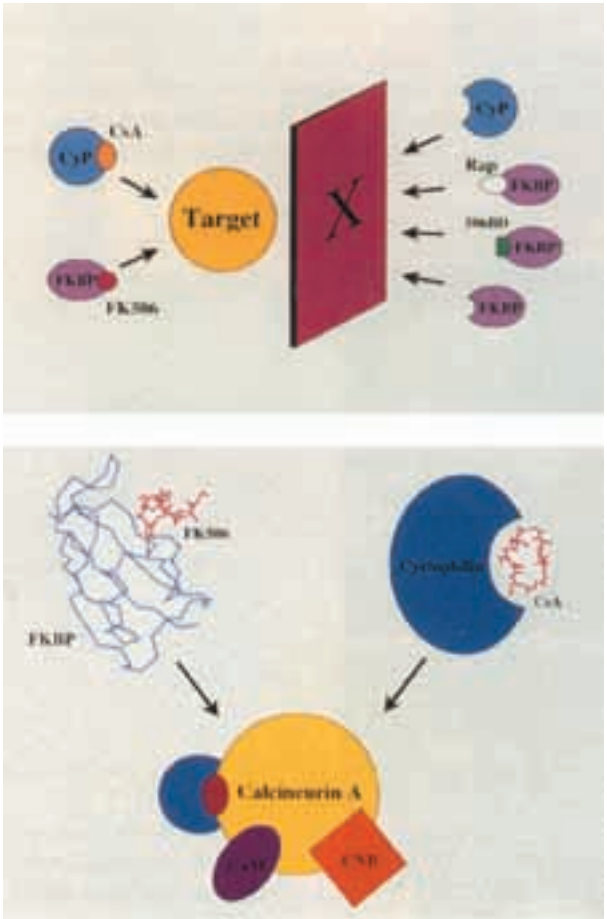

Fig. 3. Top: The CyP–CsA and FKBP–FK506 complexes act on a common target molecule that is not affected by FKBP or CyP alone, or by the FKBP–rapamycin or FKBP–506BD complexes. Bottom: Formation of the pentameric immunophilin-drug-CNA-CNB-calmodulin complex (see text).

phatase active sites, and calcineurin B (CNB), which is a Ca^{2+}-binding protein with, as yet, unknown function. The binding of calmodulin to CNA results in a tenfold increase in the phosphatase activity of the enzyme. In direct binding assays, CN binds to both the FKBP–FK506 and CyP–CsA complexes, but not to FKBP or CyP alone, or to the FKBP–rapamycin complex. In addition, the binding of CN to FKBP–FK506 is inhibited by CyP–CsA (but not by CyP or CsA alone), suggesting that the two complexes compete for the same, or two interacting binding sites. The phosphatase activity of calcineurin, when measured with a phosphopeptide substrate, is potently inhibited by the FKBP–FK506 and CyP–CsA complexes, but is unaffected by FKBP, CyP, FK506, rapamycin, 506BD, or the FKBP–rapamycin complex. Interestingly, it was recently reported that the translocation of a cytosolic component of the transcription factor NF-AT, which regulates IL-2 transcription and is sensitive to FK506, into the cell nucleus due to a rise in intracellular calcium levels is blocked by both FK506 and CsA.[27] The common dependence on intracellular calcium, location in the cytosol, and sensitivity to FK506 and CsA have led us and others to speculate that this component of NF-AT may be a substrate for CN, and that its location in the cell may be dependent on its phosphorylation state.[9, 28, 29] Thus, the inhibition of IL-2 transcription by FK506 and CsA may be the result of their indirect inhibition of the dephosphorylation of a component of NF-AT.

An important question arises from this body of data: How does the binding of FK506 and rapamycin to FKBP and of CsA to CyP change both the ligands and the receptors, enabling the complexes to perform functions the individual components are incapable of performing alone? The remainder of this review will focus on the aspects of ligand–receptor interactions most relevant to this question. In particular, we will discuss the structures of both the natural products and the proteins, and the changes that occur to each upon binding. The pentameric immunophilin-drug-CNA-CNB-calmodulin complexes (Fig. 3 bottom) will then be analyzed in light of the known biochemical data on the interactions between the immunophilin–ligand complexes and calcineurin.

3. Structural Studies of FKBP and its Complexes with FK506 and Rapamycin

3.1. Initial Work

Early structural studies of the FKBP–FK506 and CyP–CsA complexes centered on analysis of the interactions between ligand and receptor. These studies were motivated, in part, by two conflicting proposed mechanisms of rotamase catalysis, and hence of rotamase inhibition by the ligands[30] (Fig. 4). One mechanism involved initial formation of a tetrahedral enzyme–substrate adduct similar to that formed during the hydrolysis of the amide bond by serine or cysteine proteases. Rotation about the C–N bond in the adduct, followed by expulsion of the enzyme nucleophile would result in amide-bond isomerization. This mechanism was supported by studies of Fischer et al.,[12b] who showed that in CyP modification of a cysteine in the active site with *p*-hydroxymercuribenzoic acid eliminated the rotamase activity of the enzyme. Fischer et al.[31] also found an inverse secondary deuterium-isotope effect with substrates containing [α,α-^{2}H]Gly-Pro, indicating a change in hybridization ($sp^2 \rightarrow sp^3$) and thus formation of a covalent bond in the transition state of the reaction. These kinetic data were, however, disputed by Harrison and Stein,[32] who found a normal secondary deuterium-isotope effect using the same substrates. Site-directed mutagenesis studies further demonstrated that none of the cysteine residues in CyP are required for catalysis.[33] These results, coupled with measurement of thermodynamic activation parameters for CyP[32] and FKBP,[34, 35] led to an alternative mechanism of rotamase activity involving binding of a transition-state structure that contains a twisted, or distorted amide bond. In this view, the energy needed to overcome amide bond resonance and isomerize the C–N bond would come from favorable noncovalent interactions between the enzyme and a peptide substrate.

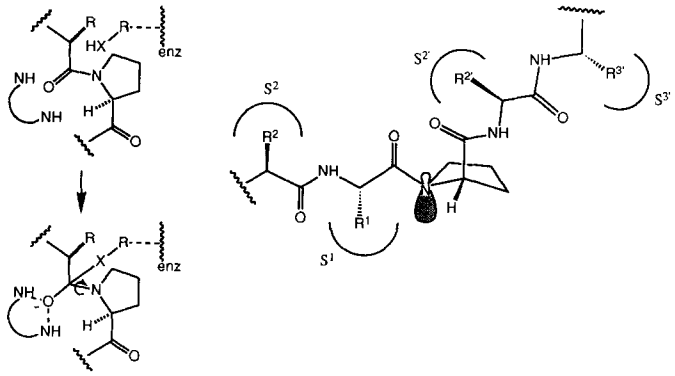

Fig. 4. Two proposed mechanisms of rotamase catalysis. Left: Initial formation of a tetrahedral intermediate. Right: Catalysis by stabilization of a twisted amide bond. enz = enzyme.

The mechanism involving a tetrahedral intermediate led to the consideration that FK506 and rapamycin might bind to FKBP through nucleophilic attack of a side chain of the enzyme on one of the two electrophilic carbonyl carbon atoms of the ligand, C8 or C9. ^{13}C NMR studies of the complex of fully synthetic [8,9-^{13}C]FK506[36] and recombinant human FKBP,[37] however, showed no evidence for formation of a tetrahedral adduct.[30] These studies also demonstrated that FK506 binds to FKBP in a single conformation, although the unbound ligand exists in organic solution as a 2:1 mixture of *cis* and *trans* amide-bond rotamers.

The finding that FK506 and, by inference, rapamycin do not bind covalently to FKBP suggested a possible explanation for the strong interaction between the ligands and their receptor. In the solid state, both FK506 and rapamycin possess a dihedral angle of approximately 90° about the C8–C9 bond. This conformation is also maintained in the complexes of both ligands with FKBP. A dihedral angle of 90° between C8 and C9 and a planar N7–C8 amide group place the keto carbonyl roughly perpendicular to the plane of the pipecolinyl ring (pipecoline = methylpiperidine). Because the pipecolinyl ring most probably mimics the proline ring in natural peptide substrates, the keto carbonyl of FK506 or rapamycin is in the same position as would be a twisted amide carbonyl group of a peptide undergoing rotamase catalysis (Fig. 5). Thus, the perpendicular keto carbonyl groups of FK506 and rapamycin allow the ligands to mimic

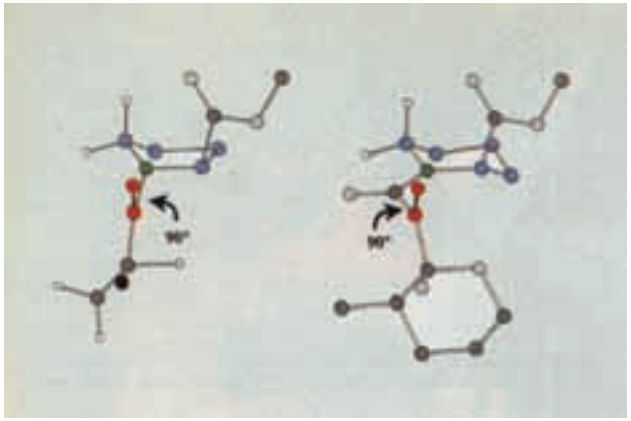

Fig. 5. FK506 and rapamycin may mimic a twisted peptido–prolyl amide bond in a peptide substrate. Left: Model of a twisted amide bond in a peptide substrate. Right: Portion of the crystal structure of free FK506. The analogous carbonyl groups in the two structures are colored red.

a transition-state structure involving a twisted amide bond.[30]

The view of FK506 and rapamycin as twisted amide peptidomimetics was further extended by studies of the substrate specificity of FKBP.[34] In the peptide series succinyl-Ala-Xaa-Pro-Phe-(*p*-nitro)anilide, it was found that peptides with branched hydrophobic amino acids Leu, Ile, and Val as Xaa were greatly favored (up to 1000-fold over peptides with Xaa amino acids with charged side chains as determined by measurement of $K_{cat}K_m^{-1}$ values) with Leu > Ile > Val. Analysis of the structures of FK506 and rapamycin suggested a possible rationale for these observations. Beginning with the pipecolinic acid moiety and proceeding in the "*N*-terminal" direction, both ligands possess a dicarbonyl group, a tertiary hydroxyl group, and a branched aliphatic chain. As illustrated in Figure 6, these can be mapped onto the amide carbonyl group, amide nitrogen atom, and the side chain of a branched aliphatic amino acid residue *N*-terminal to a proline. Thus, the binding of FK506 and rapamycin to FKBP was proposed to result from the ability of the ligands to mimic a Leu-Pro dipeptide with a twisted amide bond.[38]

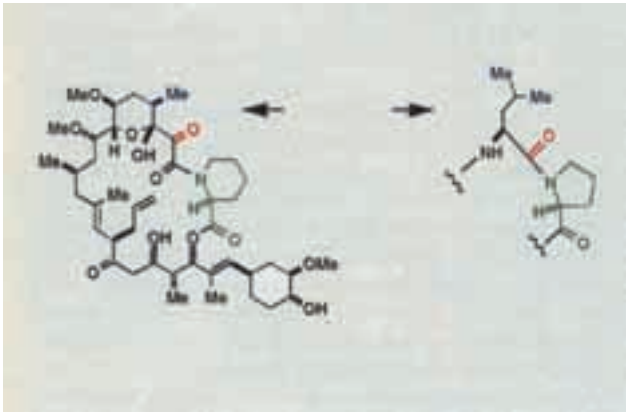

Fig. 6. FK506 (left) and rapamycin may mimic a leucine–proline substrate with a twisted amide bond (right). Analogous atoms are colored alike. Leu is the

3.2. Three-Dimensional Structures

In order to further explain the enzymatic, ligand-binding and biological properties of the immunophilins, we undertook the structure determination of free human FKBP by NMR.[39–41] Our success in this effort was due significantly to the cooperative nature of FKBP, which proved to be soluble and stable, and gave beautiful NMR spectra. Structural studies of the human FKBP–ligand complexes were also undertaken in a fruitful collaboration with Professor Jon Clardy's group at Cornell University. This work resulted in high-resolution crystal structures of both the FKBP–FK506 and FKBP–rapamycin complexes.[42–44] Analyses of the three FKBP structures, showing both the bound and free forms of the protein, have yielded new insights into many aspects of immunophilin function.

3.2.1. Overview of the Structures

All three FKBP structures show the same fold of the protein (Fig. 7 left). The structure is characterized by a five-stranded antiparallel β sheet with a novel +3, +1, −3, +1 loop topology. The strands of the sheet, which run roughly

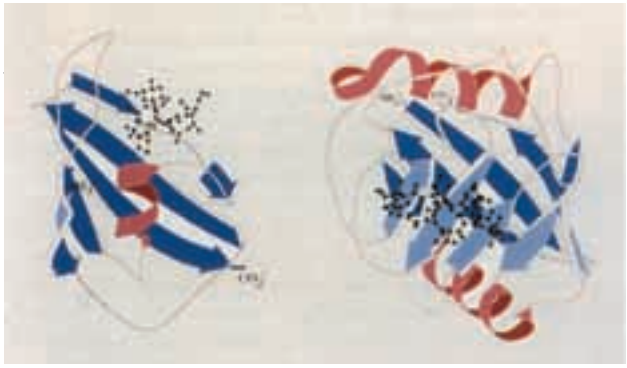

Fig. 7. Richardson diagrams of FKBP (left) and CyP (right). A ball-and-stick model of FK506 is positioned in the ligand-binding site of FKBP[42,44]. A similar model of CsA[69,70] is used to indicate *schematically* the location of the ligand-binding site in CyP[67,68]. We prepared an approximate model of the CyP–CsA complex by docking the structure of bound CsA[69] into the structure of free CyP[68] using reported intermolecular NOEs observed between MeLeu 9 of CsA and Trp 121 of CyP[73]. We thank Professor Ke for providing the CyP coordinates.

perpendicular to the long axis of the molecule, are composed of residues 2–8, 21–30, 35–38 with 46–49 (this strand is interrupted by a loop at residues 39–45), 71–76, and 97–106. A short amphipathic α helix containing residues 57–63 is aligned with the long axis of the protein and lies against the sheet, forming a tightly packed hydrophobic core. The core is composed entirely of aliphatic and aromatic residues, with all but one of the aromatic residues clustered at one end of the molecule. The conserved aromatic and aliphatic side chains of Tyr 26, Phe 36, Phe 46, Val 55, Ile 56, Trp 59, Tyr 82, and Phe 99 line a shallow cleft at the *N*-terminus of the α helix, forming the FK506 and rapamycin binding site. The

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.