32-Indolyl Ether Derivatives of Ascomycin: Three-Dimensional Structures of Complexes with FK506-Binding Protein

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32-Indole ether derivatives of tacrolimus and ascomycin retain the potent immunosuppressive activity of their parent compounds but display reduced toxicity. In addition, their complexes with the 12-kDa FK506-binding protein (FKBP) form more stable complexes with the protein phosphatase calcineurin, the molecular target of these drugs. We have solved the three-dimensional structures of the FKBP complexes with two 32-indolyl derivatives of ascomycin. The structures of the protein and the macrolide are remarkably similar to those seen in the complexes with tacrolimus and ascomycin. The indole groups project away from the body of the complex, and multiple conformations are observed for the linkage to these groups as well as for a nearby peptide suggesting apparent flexibility in these parts of the structure. Comparison of these structures with that of the ternary complex of calcineurin, FKBP, and tacrolimus suggests that the indole groups interact with a binding site comprising elements of both the calcineurin α- and *β*-chains and that this interaction is responsible for the increased stability of these complexes.

Introduction

The protein phosphatase calcineurin plays a key role in Ca^{2+}-dependent activation of lymphocytes, and it is the target through which the immunosuppressive drugs tacrolimus (FK506) and cyclosporin act.[1,2] These drugs are chemically distinct from each other, and each forms complexes with a distinct intracellular receptor protein: FK506-binding protein (FKBP) and cyclophilin, respectively. Each protein−drug complex is a potent inhibitor of calcineurin, although neither the drug nor its binding protein alone shows such activity.[2] For the FK506−FKBP complex, elements of both the protein and the drug interact directly with calcineurin. The three-dimensional structure of the calcineurin−FKBP−tacrolimus complex contains numerous and extensive contacts between calcineurin and both FKBP and tacrolimus,[3,4] and chemical modification of groups on either the protein or the drug has significant effects on the strength of calcineurin inhibition.[5−8] For example, the addition of a hydroxyl group at C18 of ascomycin produces no significant changes in the structure of the complex with FKBP, but this modification severely reduces the strength of the interaction with calcineurin and antagonizes the pharmacological effects of tacrolimus.[6,9] Similarly, drug complexes with yeast FKBP are structurally quite similar to their human homologues but afford stronger interactions with calcineurin.[8] L-732,-531, a 32-indolyl ether analogue of ascomycin (Figure 1), is as potent an immunosuppressive agent as tacrolimus but shows reduced toxicity.[10,11] In addition, the ternary complex of FKBP, L-732,531, and calcineurin is significantly more stable than the corresponding complex that contains tacrolimus.

To understand the structural basis for how FKBP complexes with such compounds interact with calcineurin, we have performed three-dimensional structural and in vitro functional analyses of two such complexes. We find that the FKBP complexes of two 32-indolyl ether derivatives of tacrolimus, like that of L-732,531, are potent inhibitors of calcineurin and that addition of the 32-indolyl ether overcomes the negative effect of the 18-hydroxyl group on calcineurin inhibition. We also find that the three-dimensional structures of these complexes differ only slightly from ascomycin complexes, beyond the addition of the indole group. This group does not interact directly with any part of the FKBP molecule but is fully exposed at the surface of the complex, in position to form extensive contacts with calcineurin. Because the asymmetric unit of each structure contains two FKBP−drug complexes, we have obtained four independent views of these molecules. In one of the four structures we find a change in the conformation of a peptide loop that makes direct contact with calcineurin. Comparison of these structures with the calcineurin−FKBP−tacrolimus complex suggests that the indole group binds in a hydrophobic site involving elements of the α- and *β*-chains of calcineurin and that the 18-hydroxyl group makes a close contact with part of the *β*-chain. These findings indicate that the alterations in the biochemistry and pharmacology of the 32-indolyl ether compounds are due primarily to the presence of the added moiety and not due to any change in the conformation of FKBP or the macrolide moiety of FK506.

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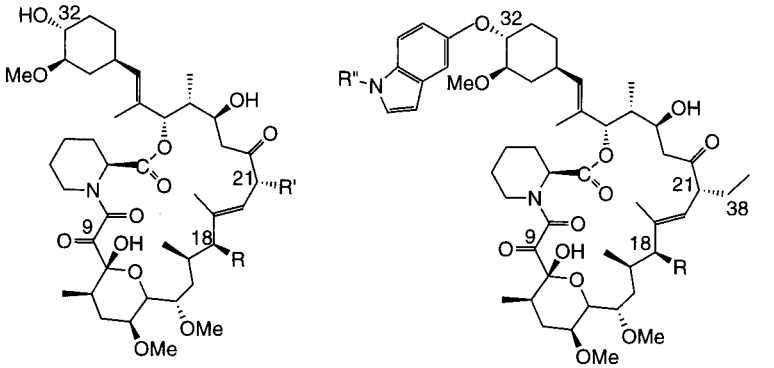

Compound	R	R'
Tacrolimus (FK-506)	H	allyl
Ascomycin (FK-520)	H	ethyl
L-685,818	OH	ethyl

Compound	R	R"
L-732,531	H	2-hydroxyl ethyl
L-709,858	H	ethyl
L-707,587	OH	methyl

Figure 1. Chemical structures of FKBP ligands. Atoms described in the text are identified explicitly.

Results and Discussion

The studies presented here reveal the structures of two new FKBP−ligand complexes. In each structure, the novel feature of the ligand, an indole group attached through an ether linkage to the 32 position, is extended away from the protein, fully exposed to solvent, and available to contact the target enzyme, calcineurin. Comparison of these structures with that of the FKBP−tacrolimus complex, both free and in the ternary complex with calcineurin, reveals that there is substantial similarity both in protein conformation and in the common parts of the bound ligands. There is, however, apparent conformational flexibility, both in the conformation of the linkage between the 32-indole ether moieties and the macrocycle and in the conformation of FKBP residues 86−89. The added group at C32 is an additional point of contact between the immunophilin−drug complex and calcineurin, and the conformation of particular groups in both the protein and ligand components of the complex may play a significant role in the inhibition of calcineurin, the molecular event at the center of tacrolimus- and cyclosporin-induced immunosuppression. In addition, comparison of the structures reported here with that of the ternary complex containing calcineurin suggests a structural explanation for both the reduction in biological activity associated with C18 substitution as well as the enhancement of binding associated with the C32-indolyl ethers.

The in vitro biochemical and pharmacological activities of the compounds studied here (Table 1) follow a pattern similar to that of L-732,531.[10] Both 32-indolyl ethers bind less strongly to FKBP than the corresponding 32-hydroxyl analogues, but both are potent inhibitors of T-cell activation through inhibition of the protein phosphatase activity of calcineurin. A comparison of L-685,818 and L-707,587 (Table 1) is particularly striking. Both compounds contain a hydroxyl group at C18 that, in L-685,818, causes a strong reduction in inhibition of calcineurin activity and of T-cell activation,[6] apparently because the hydroxyl group interferes with the interaction between calcineurin and the FKBP complex of this compound.[9] L-707,587, however, inhibits both of these activities, suggesting that the indole group at C32 provides sufficient calcineurin binding energy to overcome the effect of the hydroxyl group at C18.

The protein molecules in each of the structures presented here are generally quite similar to the protein portion of the FKBP complexes with tacrolimus and L-685,818. Each protein contains the 5-stranded antiparallel β-structure and short α-helix (Figure 2) seen in previous X-ray and NMR studies of FKBP−ligand complexes[9,12−17] as well as in the ternary complex with calcineurin.[3,4] When the α-carbon atoms of the two independent L-707,587 complexes are aligned with that of the ternary complex, they show root-mean-square (rms) deviations of 0.69 and 0.63 Å. A similar comparison using the L-709,858 complex shows rms deviations of 0.91 and 0.52 Å from the tacrolimus complex. The largest single deviation from the structure of the ternary complex involves FKBP residues 86−89 of one of the molecules in the L-709,858 complex, which differ by 2.7−4.7 Å. This region of this particular molecule also contains a bound molecule of heptyl β-D-glucopyranoside that lies between residues 86 and 89 of the protein and the bound ligand (Figure 3). The pyranose moiety of the detergent molecule lies roughly parallel to the indole group of the ligand at a distance of 4.1−5.5 Å. The oxygen atom of the pyranose ring makes a hydrogen bond to His87 $N^{\epsilon 2}$, and O6 makes hydrogen bonds to Thr85 O and His87 $N^{\epsilon 2}$. In all other parts of each protein molecule presented here, the deviations from the ternary complex are relatively small and associated with lattice contacts or flexible loops at the edge of the molecule.

The bound ligands are also similar in conformation. The common portions of these ligands, the macrolide and cyclohexane rings, adopt similar conformations and

Table 1. Biochemical and Biological Properties of Immunosuppressive Agents

compound	calcineurin inhibition[a] (nM)	calcineurin off-rate[b] (min)	T-cell[c] (nM)	FKBP binding[d] (nM)
tacrolimus	11.1	15.1	0.30	0.39
L-732,531	11.4	254	0.19	5.06
L-709,858	8.21		0.78	27
L-685,818	2500		antagonist	0.7
L-707,587	79		5.1	3.1

[a] Inhibition of calcineurin phosphatase activity by FKBP−drug complexes[23] expressed as IC_{50}'s. [b] Off-rate of FKBP−drug complexes from their complexes with calcineurin.[10] [c] Inhibition of T-cell proliferation induced by PMA−ionomycin.[24] [d] Relative affinities of compounds for FKBP expressed as IC_{50}'s in a competitive binding assay versus tacrolimus.[10]

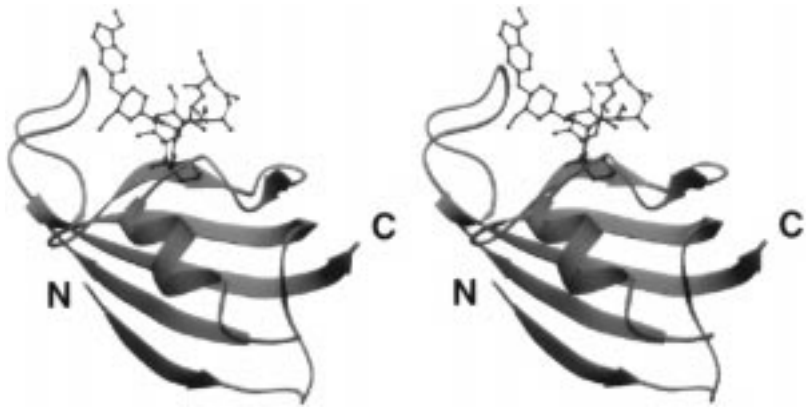

Figure 2. Schematic representation of the FKBP−L-707,587 complex. The protein is represented by a cartoon displaying the secondary structures and the bound ligand as a ball-and-stick model. The indole group projects away from the body of the structure at the top left.

make similar interactions with their protein partners. The comparable atoms in the two L-707,587 molecules both show rms differences of 0.39 Å when aligned with tacrolimus in the ternary complex; in L-709,858, the two molecules show differences of 0.22 and 0.23 Å.

In contrast to this general pattern of similarity, there are two aspects in which these structures show significant variation. First, the geometrical relationship between the bound drug and the FKBP molecule varies among the various complexes, and second, the conformations adopted by the indolyl ether groups show some variation. When the protein molecules of the four complexes reported here are aligned with the protein part of the FKBP−tacrolimus molecule, the bound drugs are tilted with respect to one another; that is, the C9 atoms, which are deep in the FKBP binding site, are all within 0.4 Å of one another, while the C38 atoms on the opposite side of the molecule span a range of 1.5 Å, corresponding to a tilt of approximately 8°. This tilting of the bound ligand within the FKBP binding site is similar to that observed when the FKBP−tacrolimus complex is compared with the ternary complex with calcineurin.[3] In that comparison as well, the bound tacrolimus molecule is tilted approximately 8° between the two structures. When the ligands rather than the proteins are aligned, however, the conformations of the macrocyles in all four molecules are very similar. The indole groups of the two L-707,587 molecules are similar to each other and to one of the L-709,858 molecules. In the L-709,858 molecule associated with the bound detergent molecule and the altered conformation of residues 86−89, however, this conformation is different (Figure 4). The two dihedral angles describing the ether linkage are −106 ± 15° and −57 ± 9° for the three similar conformations (Figure 3A) and −63° and +147° for the other (Figure 3B).

To identify those structural features that underlie the different biochemical properties that these ligands show as calcineurin inhibitors, it is useful to compare these structures with the ternary complex of FKBP, tacrolimus, and calcineurin. As noted above, addition of the 32-indole ether group results in FKBP complexes that interact more strongly with calcineurin. In the L-707,-587 complex, the indole group provides sufficient binding energy to overcome the strong negative effect of the hydroxyl group at C18 that is usually associated with very weak calcineurin activity. If the complexes described here are aligned to the ternary calcineurin complex using the α-carbon atoms of FKBP as guides, the indole groups fit easily into a groove in the surface of the calcineurin molecule (Figure 3). This groove is defined by residues 355−362 of the calcineurin α-chain and 161−165 of the calcineurin β-chain, which are both components of helices. The closest contacts are between the edge of the indole and the peptide between Gly358 and Glu359, the Glu359 side chain of the calcineurin α-chain, and the side chain of Thr362 of the calcineurin β-chain. This model suggests that noncovalent interactions between the indole and this site account for the increased stability and slower off-rates associated with the 32-indole immunophilins.[10] The site in which these groups bind is, in fact, large enough to accommodate groups larger than an indole, and it is possible that this volume can be exploited to increase the interactions with calcineurin further or to introduce chemical groups with more desirable pharmacological properties. In fact, the groove is large enough to accommodate the bound detergent molecule of the L-709,858 complex (Figure 3B).

The region near C18 of the macrocycle in the ternary complex reveals a possible structural basis for the reduced affinity for calcinuerin when this site is derivatized. When the L-707,587 complexes are aligned to the ternary complex, the hydroxyl group at C18 makes a close contact with residues 119−122 of the calcineurin β-chain, in particular with the C^{β} of Asn122. This contact probably results in the reduced affinity of L-685,818, while the increased interactions of the indole groups appear to provide enough binding energy to overcome most of this negative effect.

The observed flexibility in the residues 86−89 of FKBP is similar to that seen in the ternary complex with calcineurin.[3] In that complex, a displacement of approximately 1 Å is observed in the position of these residues, a significant change but less than the 2−4 Å movement seen in the L-709,858 complex. Several studies have shown that alteration of His87, Gly89, and Ile90 can have profound effects on the ability of the FKBP−tacrolimus complex to interact with calcineurin.[5,7,8,18] Previous NMR and dynamics studies on FKBP and FKBP complexes indicate that there is considerable flexibility in the loops of FKBP near residues 42 and 89 when no ligand is bound and that this flexibility is significantly reduced by binding of

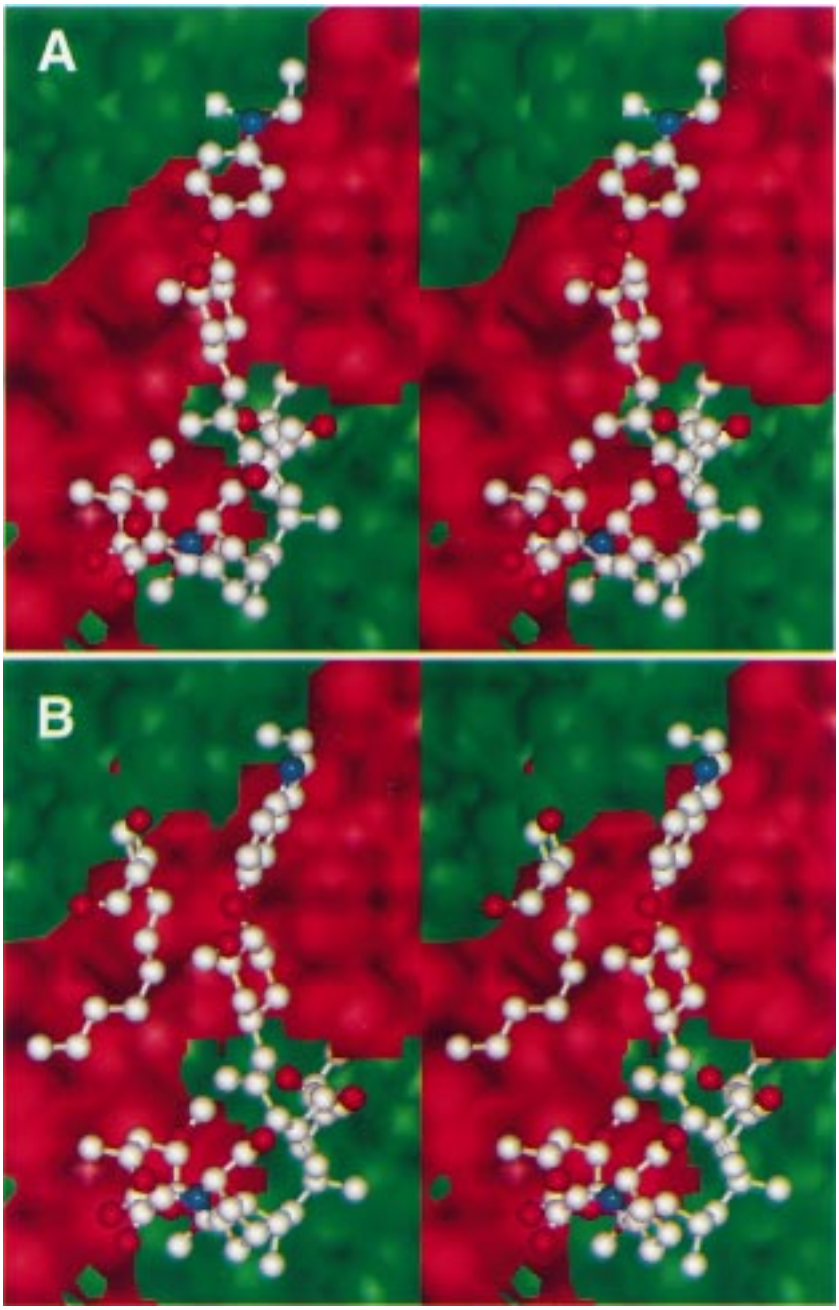

Figure 3. Model of the interaction of the FKBP−L-709,858 complex with calcineurin. The α-carbon atoms of the complexes were aligned with the FKBP portion of the ternary complex of calcinuerin, FKBP, and tacrolimus.[3] The bound ligands are shown as ball-and-stick models with white carbon atoms, and the calcineurin α- and *β*-chains are shown as a van der Waals surface. The α-chain is colored red, and the *β*-chain is green. (A) One of the two independent L-709,858 molecules. The indole group (top) can be accommodated by a cavity containing elements of both α- and *β*-chains. The side chain of Glu359 of the α-chain is at the right of the indole ring, and the side chain of Thr362 of the *β*-chain is to the left. (B) Same region of calcineurin with the aligned structure of the second L-709,858 molecule and the heptyl *β*-D-glucopyranoside molecule with which it cocrystallizes. The proposed binding site in calcineurin is large enough to accommodate both configurations of the indole group as well as the detergent molecule without significant rearrangement.

tacrolimus and related compounds.[19,20] The altered conformation of residues 86−89 seen in the present study is probably an artifact of crystallization: there is no evidence that any molecule related to heptyl *β*-D-glucopyranoside participates in any biologically significant interaction related to immunosuppression. However, the present findings, combined with the structure of the FKBP−tacrolimus−calcineurin complex, do indicate that both the chemical nature and the conformational flexibility of this loop play an important role in the inhibition of calcineurin and may be useful in the design of calcineurin inhibitors.

The results of this work enrich our view of the effector face of the FKBP−tacrolimus complex, the molecular surface that interacts directly with calcineurin. Figure 5 presents a solvent-accessible representation of the complex. At the top of the figure is the 32-indole ether group that delineates the class of compounds described here. This group is exposed to solvent in the FKBP complex and is free to adopt a variety of conformations.

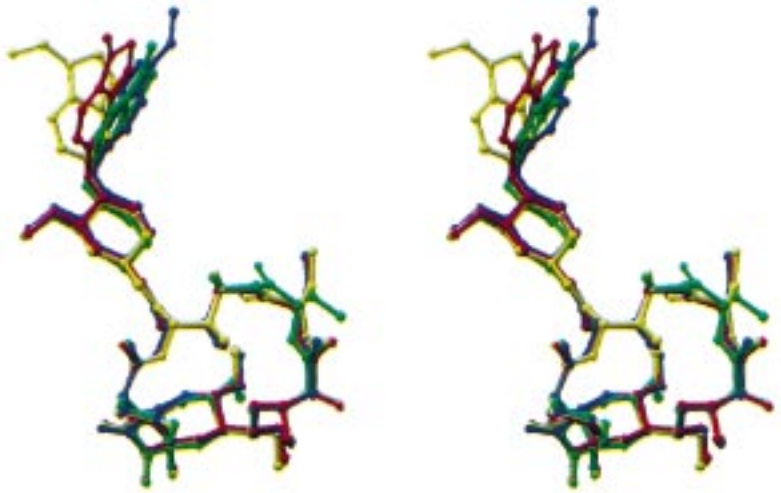

Figure 4. Conformations of FKBP ligands. The bound conformations of L-707,587 and L-709,858 are shown. The molecules have been aligned to one another, using the atoms of the macrolide as guides. The two L-709,858 molecues are colored yellow and blue; the two L-707,587 molecules are red and green.

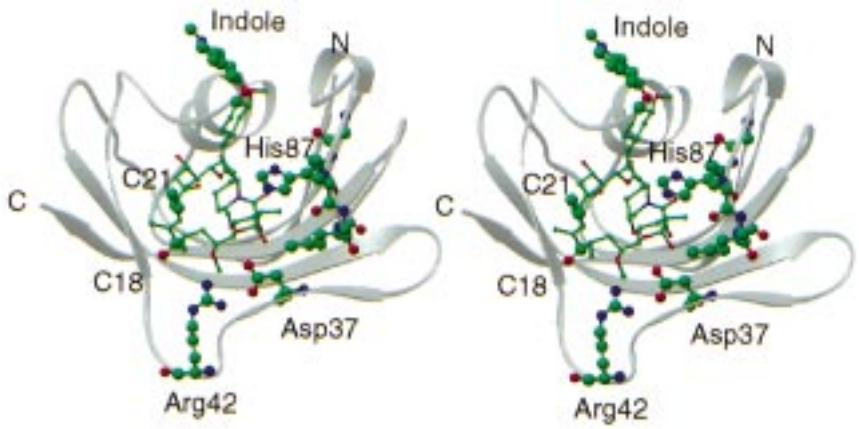

Figure 5. Effector face of an FKBP complex. The FKBP−L-707,587 complex is depicted as a protein cartoon and stick model. Regions of the protein where changes in the chemical structure produce significant changes in the interaction with calcineurin are depicted as a ball-and-stick model.

When the calcineurin complex is formed, however, this group apparently fits into a complementary site made up by both the α- and *β*-chains of calcineurin, and a particular conformation may be induced or selected. Surrounding this moiety and the bound ligand is a ring of residues that make contact with calcineurin and can be modified to modulate the biological effects of the FKBP−tacrolimus system. These include residue 87, a histidine in human FKBP. The His-to-Val mutation in the human protein significantly weakens the strength of the calcineurin interaction.[5] In contrast, the comparable residue in yeast FKBP is phenylalanine, and the yeast FKBP complexes have higher affinities for a mammalian calcineurin than the human homologue.[8] This residue also lies in the Gly-His-Pro-Gly sequence whose conformation can be altered by exogenous agents and may be altered in the calcineurin complex. In the same region, is the Gly-Ile sequence that also appears to make a crucial interaction.[7,18,21] Additional points of contact are at C21, where the allyl group of tacrolimus points into a hydrophobic subsite on calcineurin, and at C18, where addition of a hydroxyl group severely reduces this strength of the interaction, consistent with a close contact between tacrolimus and the enzyme. At the bottom of the figure are Asp37 and Arg42, and mutation of these residues causes a sharp reduction in ability to inhibit calcineurin activity.[5] The combined mutational, chemical, and structural studies delineate a large and complex intermolecular interface between calcineurin and FKBP complexes, over 30 Å in diameter and involving multiple related hydrophobic and hydrophilic subsites. Because chemical modifications over the entire span of the surface can produce such significant changes in the strength and nature of calcineurin inhibition, the combined protein and ligand groups not only mediate the intermolecular interactions of this system but also represent a structural foundation for the design of highly potent and specific immunosuppressive drugs.

Experimental Section

Chemical and Biochemical Methods. Ascomycin derivatives were synthesized as described.[22] Inhibition of calcineurin,[23] inhibition of PMA−ionomycin-induced T-cell proliferation,[24] inhibition of ionomycin-induced T-cell proliferation,[24] and binding of compounds to FKBP[10] were measured by published methods.

X-ray Methods. Recombinant FKBP12 was expressed in *Escherichia coli* and purified by standard procedures.[25] Although we were unable to crystallize the FKBP complex with L-732,531, we were able to crystallize several related species

Table 2. Crystallographic Data Summary

	L-707,587	L-709,858
Crystals and Data Collection		
space group	$I4$	$P6_122$
a (Å)	119.74	74.26
c (Å)	57.21	236.45
d_{min} (Å)	2.90	2.50
completeness—all (%)	98.9	81.4
$F > 2s$	93.7	76.2
highest shell	96.2	62.1
R_{sym}	0.0585	0.0565
Refinement Statistics		
R (20.0 Å−d_{min})	0.2231	0.2436
R_{free} (20.0 Å−d_{min})	0.3146	0.3096
rmsd bonds (Å)	0.007	0.009
angles (deg)	1.53	1.55
dihedrals (deg)	26.22	26.69
impropers (deg)	1.18	1.13
Ramachandran Statistics		
most-favored region (%)	79.7	87.2
additional-allowed (%)	19.8	11.6
generously allowed (%)	0.6	1.2

(Figure 1). FKBP complexes (1.2:1 drug−protein) were crystallized by vapor diffusion. The L-707,587 complex crystallized from 39% saturated ammonium sulfate, 0.10 M potassium phosphate, pH 6.1; the L-709,858 complex crystallized from 47% saturated ammomium sulfate, 0.2% β-heptyl D-glucopyranoside, 0.10 M potassium phosphate, pH 5.6. The L-707,-587 complex crystals belong to the tetragonal space group $I4$ with $a = 119.74$, $c = 57.21$ Å and usable diffraction extends to a resolution of 2.9 Å; the L-709,858 crystals belong to the hexagonal space group $P6_122$ with $a = 74.26$, $c = 236.45$ Å with a diffraction limit of 2.5 Å. Diffraction data were collected using a Siemens X-ray area detector and Cu Kα radiation from a Rigaku RU-200 rotating anode X-ray generator. Data were processed using the XENGEN[26] and PHASES[27] packages. Both structures were solved by molecular replacement using the protein portion of the FKBP−L-685,818 structure[9] as the probe molecule. The L-707,587 complex was solved using MERLOT,[28] and the L-709,858 complex was solved using X-PLOR.[29] Both structures were refined using X-PLOR. All observed data with Bragg spacings between 20.0 Å and the diffraction limit were used, and a bulk solvent mask was applied. In the final stages of refinement, each model was confirmed by simulated-annealing omit maps,[30] in which approximately 10% of the model was omitted from the calculation. The asymmetric unit of the current model of the FKBP−L-707,587 complex contains 2 protein−ligand complexes and 15 ordered water molecules; that of the FKBP−L-709,858 complex contains 2 protein−ligand complexes, 66 ordered water molecules, and 1 heptyl β-D-glucopyranoside molecule. The crystallographic data and results are summarized in Table 2. The coordinates and structures factors for these complexes have been deposited in the Protein Data Bank (ID codes 1qpf and 1qpl).

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