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A Proposed Molecular Model for the Interaction of Calcineurin with the Cyclosporin A–Cyclophilin A Complex

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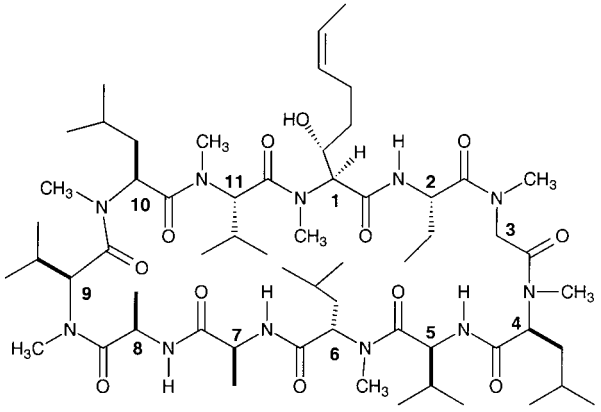
Abstract—Cyclosporin A (CsA) and FK506 are potent natural product immunosuppressants that induce their biological effects by forming an initial complex with cytosolic proteins termed immunophilins. These drug–immunophilin complexes then bind to and inhibit the serine/threonine protein phosphatase calcineurin (CN). Two classes of immunophilin have been identified with cyclophilins (CyP's) being proteins specifically binding CsA and FKBPs specifically binding FK506. Solution and crystal structures of various CsA–CyP and FK506–FKBP complexes have been determined and show no apparent structural similarity between the two classes of drug–protein complexes. These findings raise the question as to how, given their structural differences, these two complexes can both inhibit CN. While the crystal structure of the FK506–FKBP12–CN complex has been reported, no structure for a CsA–CyP–CN complex has been determined. Here are reported studies that use various modelling strategies to construct a model for the interaction of the cyclosporin A–cyclophilin A complex with calcineurin. The first stage of constructing this model consisted of using conformational comparison of CsA and FK506, GRID and GROUP analysis and restrained molecular dynamics to dock CsA into the FK506 binding site of the FK506–FKBP12–CN structure. An initial model for the CsA–CyPA–CN complex was then constructed by superimposing the structure of the CsA–CyPA complex onto the docked CsA molecule. This model was then optimised with molecular dynamics simulations run on sterically clashing regions. The validity of the model for the CsA–CyPA–CN complex was then examined with respect to the effect of chemical modifications to CsA and amino acid substitutions within CyPA on the ability of the drug–immunophilin complex to inhibit calcineurin.

Introduction

Cyclosporin A (CsA; **1**) is an undecapeptide that was first isolated from the fungi imperfecti *Tolypocladium inflatum* in 1976[1,2] and whose potent immunosupressive properties were first described by Borel et al.[3,49] CsA has subsequently been developed into a major pharmaceutical product (Sandimmune, neoral) marketed for the prevention of allograft rejection and used prophylactically for prevention of graft-versus-host disease following bone marrow transplantation.[4–6] Additionally, CsA in conjunction with the more recently discovered natural product macrolide immunosuppressant FK506 (**2**)[7] has greatly contributed to the elucidation of the signal transduction pathways that connect antigenic activation of the T-cell membrane receptor to lymphokine gene expression in the nucleus.[8]



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Both compounds effectively suppress the proliferative response of T-lymphocytes to antigen activation, primarily by inhibiting the transcription of early response cytokines including IL-2.[9] In recent years a detailed understanding of the mode of action of FK506 and CsA has been gained and extensively reviewed.[10–13] These

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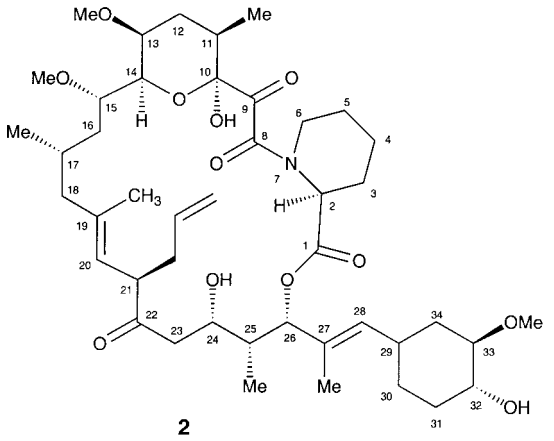
studies indicate that the modes of action of FK506 and CsA are very similar at the molecular level with each



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inducing its effect by interacting with two proteins. For FK506 the initial protein target has been called FKBP12[14,15] and is one of a group of proteins known as immunophilins characterised by strong affinity for natural product immunosuppressants and the ability to catalyse the *cis–trans* isomerisation of *cis*-proline residues in proteins and peptides (PPIase activity). Similarly, immunophilin targets have also been identified for cyclosporin A (CsA) which have been called cyclophilins and three isoforms cyclophilin A[16] (CyPA), cyclophilin B[17] (CyPB) and cyclophilin C[18] (CypC) all exhibiting PPIase activity have been shown to form immunosupressive complexes with CsA. FK506 and CsA potently inhibit the PPIase activity of their corresponding immunophilin targets, however, early hypotheses that the inhibition of the PPIase activity of these enzymes was the source of the immunosuppressive action of FK506 and CsA were discredited by studies with a synthetic FKBP12 ligand, 506BD, which also potently inhibited the PPIase activity of FKBP12 but did not have immunosuppressant activity.[19]

The above findings led to the search for a second protein target for CsA and FK506 which was subsequently identified using affinity columns containing either immobilised FK506–FKBP12 or CsA–CyPC complexes[18,20] as the serine/threonine protein phosphatase calcineurin (CN).[21] Thus it is seen that both FK506 and CsA induce their biological effects by forming an initial complex with an immunophilin protein with this complex then binding to and inhibiting the phosphatase activity of calcineurin. Subsequently the role of CN in cellular signal transduction has been determined as regulating the cellular location of a specific transcription factor called NF-AT.[22] Dephosphorylation of the cytoplasmic component of NF-AT by calcineurin allows its translocation to the nucleus and formation of a competent transcription factor for early stage growth factors.[22] Calcineurin is not inhibited by either drugs or immunophilins alone but only by the drug–immunophilin complexes which has been interpreted as indicating that it is the composite-surface of the drug–immunophilin complex that interacts with calcineurin. This hypothesis has been supported by the recent determination of structures for the complex of FK506–FKBP12–CN[23,24] which indicates that both FK506 and FKBP12 make extensive contact with CN. Comparison of the three-dimensional structures of FK506–FKBP12[25,26] and CsA in complex with CyPA,[27,28] CyPB,[29] CyPC[30] have not indicated any obvious similarities and there are no common structural features in evidence. These results suggest a puzzle as to how CsA and FK506 can induce the same biological effects through the same enzymic target given their structural differences in the active complexes. Even more intriguing is the amount of evidence suggesting that the CsA–CyP and the FK506–FKBP12 complexes bind to calcineurin in a similar fashion. This evidence includes observations that both FK506–FKBP12 and CsA–CyP are noncompetitive inhibitors of calcineurin.[31] Also competition experiments have shown that calcineurin bound to an affinity column by an immobilised FK506–FKBP12 complex can be eluted by solutions of the CsA–CyPA complex.[20] While the ultimate solution of this puzzle awaits the determination of the X-ray structure for the complex of CsA–CyP–CN this study proposes that sufficient information exists from structural studies, chemical modification and protein mutagenesis experiments to construct a model for the interaction of the CsA–CyPA complex with CN. This report outlines the construction of this model which is based on the hypothesis that CsA and FK506 both bind in a similar way to the same site on calcineurin.

Results and Discussion

Examining the interaction between FK506–FKBP12 and calcineurin

In attempting to construct a molecular model for the complex of CsA–CyPA–CN, the available structure for the complex of FK506–FKBP12–CN[23] was first examined to gain insight into how a drug–immunophilin complex has been observed to interact with calcineurin. A ribbon diagram for this complex is shown in Figure 1 (main body) and shows the calcineurin protein as a heterodimer made up of the catalytic A-subunit (CNA, red) and the regulatory B-subunit (blue). The A subunit interacts with the B-subunit through an extended segment of α-helix (B-subunit binding helix, BBH) which points like an appendage almost perpendicularly away from the main body of the catalytic subunit. The FK506 (purple)–FKBP12 (yellow) complex partially fills the space formed within the right angle bend between CNA and CNB and binds to calcineurin primarily through interaction with the head of the BBH region and some residues of the B-subunit. No atoms of either FK506 or FKBP12 are within 10 Å of the active site within the A subunit of calcineurin which is marked by a phosphate group (light pink) and metal ions (tan and grey). Looking in detail at the interaction of FK506 with CN (Fig. 1, inset) it is seen that FK506 binds to calcineurin by insertion of the hydrophobic region extending from C17 to C24 known as the 'effector loop' into a hydrophobic pocket defined primarily by residues Trp 352, Pro 355, Phe 356 from the BBH region of the A-subunit and

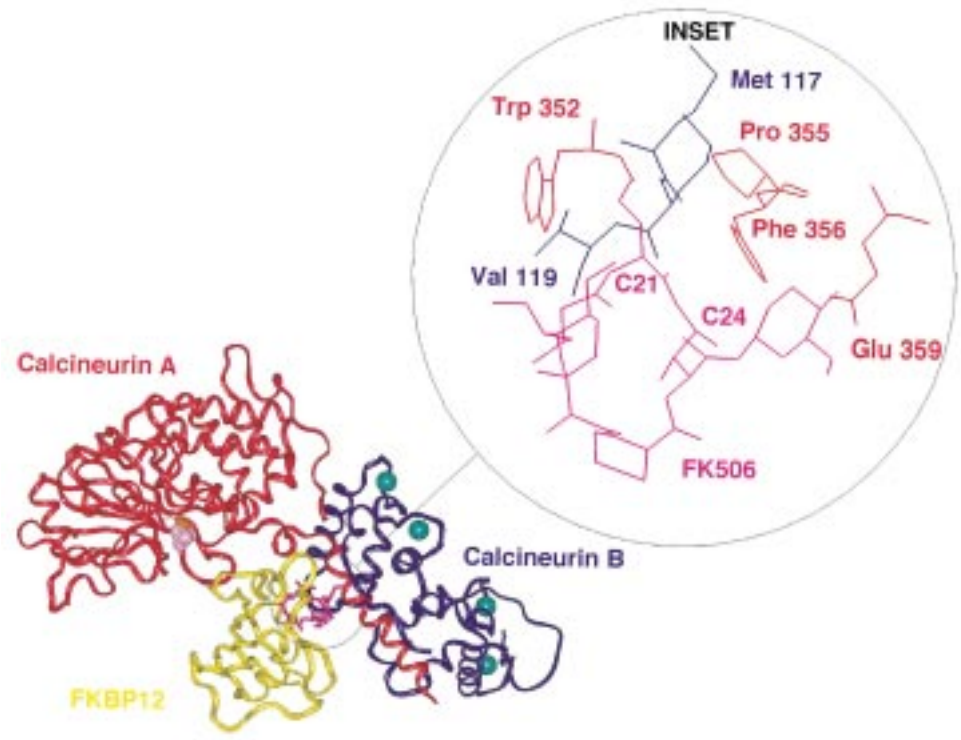


Figure 1. Molecular representations of the crystal structure of the FK506–FKBP12–calcineurin (CN) complex. Main body: ribbon diagram of the full FK506–FKBP12–CN structure, calcineurin A (CNA) is shaded red, calcineurin B (CNB) is shaded dark blue, FKBP12 is yellow and FK506 is purple. Solid turquoise spheres in the CNB subunit represent calcium ions and the active site in the CNA subunit is marked by a light pink phosphate ion and tan and white metal ions. Inset: expanded view of the interaction of FK506 (purple) with calcineurin. Residues of the calcineurin A subunit (CNA) are coloured red with residues of calcineurin B (CNB) coloured dark blue.

residues Met 117, Met 118, Val 119 from the B-subunit. Additionally, the cyclohexyl moiety of FK506 lies with one edge in a shallow groove lying between Phe 356, Pro 355 and Glu 359.

Strategy for constructing a model for the CsA–CyP–CN complex

In constructing a model of the interaction between CsA–CyPA–CN it is logically feasible to obtain the structure of the CN component of the complex from the above structure of FK506–FKBP12–CN and to use one of the available structures for the CsA–CyPA complex for this part of the complex. Implicit in this approach is the assumption that the structure of calcineurin when bound to FK506–FKBP12 is similar to that when calcineurin is bound to CsA–CyPA and that the CsA–CyPA structure does not change significantly on binding to CN. Some insight into the first of these assumptions can be made by comparing the unbound structure of calcineurin[24] with its structure in the FK506–FKBP12–CN structure discussed above. If an RMS overlay comparison is done for the BBH helix regions of the two structures for CN from residue 352 to 371 (results not shown) very good agreement is obtained with RMS deviations for all atoms of 0.87 and 0.35 Å for backbone atoms, while comparison of the complete B-subunits has an RMS deviation of 1.24 Å for all atoms and 0.72 Å for the backbone atoms. This suggests that the region of calcineurin binding the FK506–FKBP12 complex does not change significantly during the binding process and it seems reasonable to assume that this may also be the case for calcineurin binding the CsA–CyPA complex.

For the second assumption overlay analysis for all atoms in residues 26, 37, 42, 46, 48, 54, 56, 99, 87, 90 of FKBP12 and all atoms in FK506 for the FK506–FKBP12[26] structure compared to the same atoms in the FK506–FKBP12–CN[23] structure gave an RMS deviation of 0.92 A and when a ribbon diagram for FKBP12 in each of the structures in the above overlay (data not shown) were compared they were found to have a virtually identical folding pattern. This suggests that the FK506–FKBP12 complex is structurally arranged for interaction with calcineurin which also implies this could reasonably be assumed to be the case for the interaction of the CsA–CyPA complex with calcineurin. With the above discussion in mind the problem of constructing a model for the CsA–CyPA–CN complex reduces to a question of determining a suitable relative orientation of the two available structural fragments (CN from FK506–FKBP12–CN[23] structure and CsA–CyPA[28]) of the desired structure (CsA–CyPA–CN) which does not contain undue steric strain between these fragments and is consistent with the available experimental data regarding the interaction of CsA–CyPA with CN.

Conformational comparison of FK506 and cyclosporin A

The starting point for determining the appropriate orientation of the CsA–CyPA complex when bound to calcineurin was a comparison of the immunophilin bound conformations of FK506 and CsA. The aim of these comparisons was to identify an appropriate region of CsA for binding to the FK506 binding site of calcineurin. In light of the above discussion of the interaction of FK506 with CN the region of CsA chosen should contain a hydrophobic loop to be inserted into the binding site. Some comparisons of the conformations of FK506 when bound to FKBP12 with the conformation of CsA when bound to CyPA have previously been performed by Denesyuk and co-workers[32] who showed that superposition of the C17–C22 effector loop of FK506 with the peptide backbone of CsA from Sar^3 (N) to Meleu^4 (C) gave an RMS deviation of only 0.33 Å. Similarly, superposition of C17–C22 of FK506 with Val^5 (Cβ) to MeLeu^6 (C) gave an RMS deviation of 0.13 Å. At the time of Denesyuk's studies, structures of the CsA–CyP complex were unavailable and it was still unclear as to which residues of CsA may be interacting with CN. With this information now available comparison of the bound conformations of FK506 and CsA was performed concentrating on a comparison of the dihedral angles for the bonds of FK506 in the region C17–C24 and for CsA extending from Sar^3 (N) to Val^5 (Cα). The structures of FK506 and CsA used for this comparison were taken from crystal structures of FK506–FKBP12–CN[23] and CsA–CyPA[28] complexes, respectively. This comparison is illustrated in Figure 2 which is a plot of dihedral angle for the above segments of FK506 and CsA and shows very good agreement between the two molecules with no difference in dihedral angle in this region of greater than 30°. The similarity of the conformations of FK506 and CsA in these regions is further illustrated in Figure 3(a) which shows the result of superposition analysis where the atoms of these regions of FK506 and CsA were overlaid in pairs beginning with C17 of FK506 and Sar^3 (N) of CsA. This figure shows very good agreement between the two molecules within this region with an RMS deviation of only 0.24 Å and the overall space occupied by the two molecules being very similar. In particular the macrocyclic ring of FK506 and the peptide ring of CsA are seen to lie in the same plane with the cyclohexyl side chain of FK506 lying in the same plane as, and in the centre of, the peptide ring of CsA. However, the distance between the cyclohexyl group of FK506 and the peptide chain of CsA of ~3 Å suggest that interaction with the same protein surface may require some different orientation in this region.

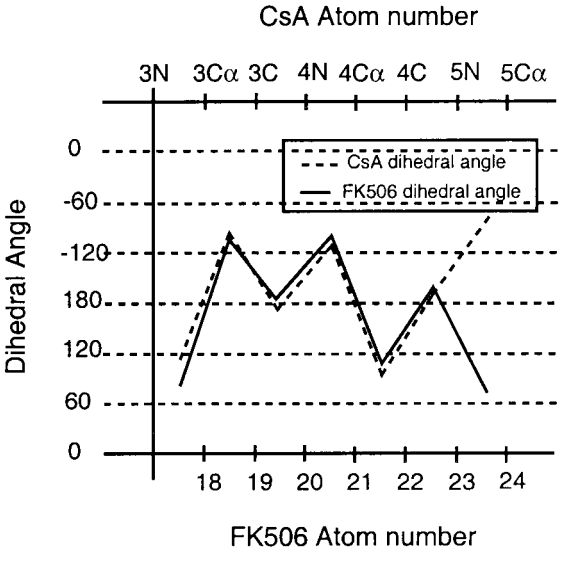


Figure 2. Plot of dihedral angle versus atom number for FK506 (solid line) and cyclosporin A (CsA: dashed line). The left-hand dihedral angle scale is drawn to reflect the equivalence of −180 and 180 degree angles. Atom numbers at the bottom are for FK506 and at the top for cyclosporin A. Each point in the graph is positioned between the atoms of the central bond of the dihedral angle.

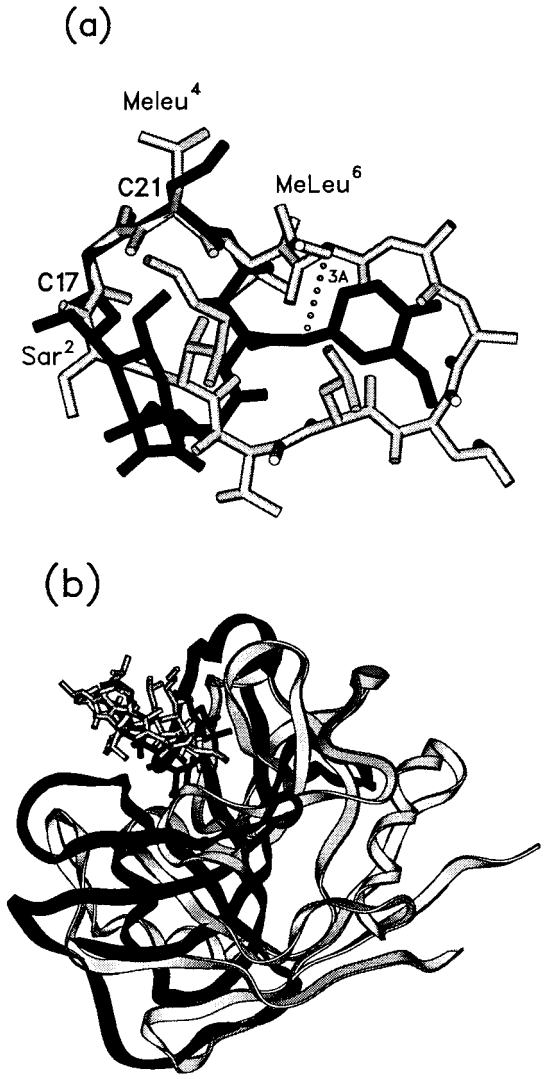


Figure 3. (a) Result of RMS overlay for the C17–C24 fragment of the FK506 molecule edited from the FK506–FKBP12–CN structure and the Sar^3 (N)-Val^3 (Cα) fragment of CsA molecule edited from the CsA-cyclophilin A (CyPA) structure. The root mean square deviation (RMS) for this overlay is 0.24 Å. Cyclosporin A is shaded grey and FK506 is black. (b) The same overlay as in (a) but with both FKBP12 and CyPA also displayed. The cyclosporin A–CyPA complex is shaded grey and the FK506–FKBP12 complex is black.

Using the above regions of FK506 and CsA as references a model (Fig. 3(b)) comparing the conformations of the FK506–FKBP12 and CsA–CyPA complexes was then constructed. The model was constructed by the same superposition analysis carried out above but includes the immunophilin protein components of the structures within the display. The aim of this model was not to identify specific areas of conformational similarity between the two proteins but to get an initial indication as to whether it is feasible for the CsA–CyPA complex to fill the same space on the surface of calcineurin as that occupied by the FK506–FKBP12 complex. In the figure the CsA–CyPA complex is shaded light grey and the FK506–FKBP12 is black and while no real similarity in secondary structure is apparent the structures do have a similar spatial arrangement with both showing the drug molecules projecting toward calcineurin and the protein surface expected to interact with CN occupying similar space. The extra residues of CyPA compared to FKBP12 are positioned in a region that would not be expected to interact with calcineurin.

Initial model for the cyclosporin A–CyPA–calcineurin complex

Given the favourable comparison of FK506–FKBP12 and CsA–CyPA in Figure 3(b) an initial model of the interaction of CsA–CyPA–CN complex was constructed by repeating the above superposition process using the structures of FK506–FKBP12–CN[23] and CsA–CyPA.[28] (Fig. 4, main body, see Experimental) This model shows the CsA–CyPA complex approximately filling the space in between CNA and CNB, which suggests that a model with CsA–CyPA in this approximate orientation is feasible. However, clearly some steric clashes are in evidence and with 32 residues of CN having at least one heavy atom within 2.5 Å of CsA or CyPA significant further optimisation of this model is obviously required. The strategy for optimising the model was to start with optimisation of the position of CsA within the FK506 binding site of calcineurin. In Figure 4 (inset) a zoomed in view of the initial orientation of CsA within the FK506 binding site of CN is shown. This shows the *iso*-butyl sidechain of the Meleu[4] residue of CsA positioned in the same hydrophobic binding site as the C21-allyl side chain of FK506 (Fig. 1, inset). The loop formed by the residues Sar[3]-Val[5] of CsA occupies a similar position as the effector loop of FK506 shown in Figure 1 (inset). However, at the α-carbon of Val[5] the branching to continue on to Meleu[6] results in positioning of the *iso*butyl sidechain of Meleu[6] in a severe steric clash with Pro 355 of CN. This also results in positioning the Ala[7] residue in steric clash with Glu 359. Significant steric clashing is also seen between the butenylmethylthreonine[1] (Bmt[1]) residue of CsA and Trp 352 residue of CN. These results are significant in that they suggest that the final model requires specific orientation of Meleu[4], Meleu[6], and Bmt[1] of CsA which is consistent

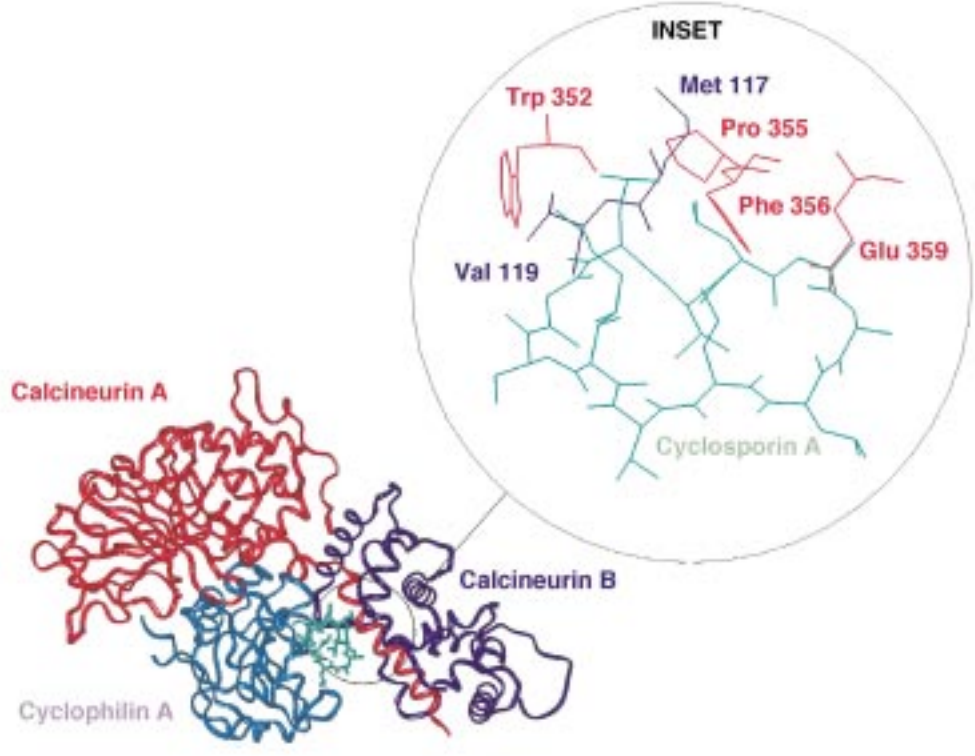


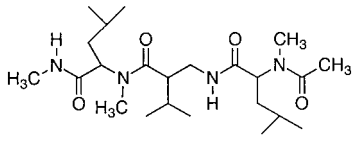
Figure 4. Main body: ribbon diagram representing the initial model structure of the CsA–CyPA–CN complex (see Experimental). Calcineurin A (CNA) is shaded red, calcineurin B is dark blue, cyclosporin A (CsA) is turquoise, and cyclophilin A (CyPA) is light blue. Inset: a zoomed-in view of the initial orientation of CsA within the FK506 binding site of CN. Residues defining the binding site are labelled and relevant residues of CsA are labelled. Residues of CNA are coloured red, residues of CNB are coloured dark blue and CsA is turquoise.

with structure–activity studies indicating slight modifications to these residues have a great effect on the activity of CsA.[33,34]

Docking cyclosporin A into the FK506 binding site of calcineurin

Figure 4 (inset) shows that the interaction between CsA and CN in the model structure is predominantly hydrophobic in nature and critically dependant on the specific orientation of the terminal methyl groups of the Meleu[4] and Meleu[6] sidechains. To gain insight into how these groups might be positioned within the defined binding site two related computational approaches were adopted. The GRID program package now in version 15 was originally developed in 1985[35] and is aimed at calculating a matrix of interaction energies between a probe molecule and a macromolecular target. This matrix of interaction energies can be used to indicate positions at which the small probe would be expected to interact favourably with the macromolecular target. Various probes are available and for this study the C3 probe representing a methyl carbon C–H was utilised (see Experimental) to survey a cutdown structure file of CNA and CNB which included all residues of these two subunits of calcineurin within 10 Å of the initial docked position of CsA. Comparison of the results of this calculation (results not shown) with the position of FK506 within the FK506 binding site of calcineurin shows clearly that FK506 is oriented to position itself exactly within the favourable interaction areas. Particularly, the C21-allyl side chain is positioned deeply within the hydrophobic cleft bordered by Trp 352 and Phe 356 (see Fig. 1, inset). This area has the strongest favourable interaction with a calculated energy of interaction between the probe and macromolecule in this region of −5.3 kcal/mol at the bottom of the cleft. A second region of favourable interaction lies in a shallow groove between Pro 355 and Glu 359, which is occupied in the model by the edge of the cyclohexyl group.

The above result suggested that the GRID calculation procedure was capable of accurately representing the interaction of a hydrophobic loop with the FK506 binding site of calcineurin and this suggested that it may be possible to use the program GROUP[36] which is an extension of GRID for docking CsA into the FK506 binding site of calcineurin. GROUP determines possible orientations of probe molecules within a macromolecular target by sequentially fitting individual atoms of the fragment at energetically favourable positions using previously calculated grids of the interaction energies between probes representing individual atoms of the fragment and the macromolecular target. Previously, only polar atoms could be used in this fitting process but in the latest version of GROUP molecules can be tagged as being hydrophobic and used in the fitting process in conjunction with the DRY probe grid. The output of GROUP is the co-ordinates of the proposed binding site for the probe molecule. In this study GROUP was used to survey the proposed binding site of CsA on CN for possible binding orientations of a fragment (**3**) of CsA (see Experimental). This fragment is the Sar[3] to Meleu[6] region of CsA that has been modified at each terminus to more approximate the nature of this region in the full CsA molecule. This experiment indicated one energetically favourable orientation for **3** which is shown in Figure 5(a) positioned within the original C3 GRID map. This shows that the position of the Meleu[4] and Meleu[6] sidechains have been optimised by firstly withdrawing the Meleu[4] sidechain from the cleft between Trp 352 and Phe 356 and rotating the terminal methyl groups of Meleu[4] sidechain to align within the groove. The whole fragment has also been rotated to remove steric clashes between Meleu[6] and Pro 355 and place the Meleu[6] sidechain above the hydrophobic groove between Trp 352, Pro 355 and Phe 356.



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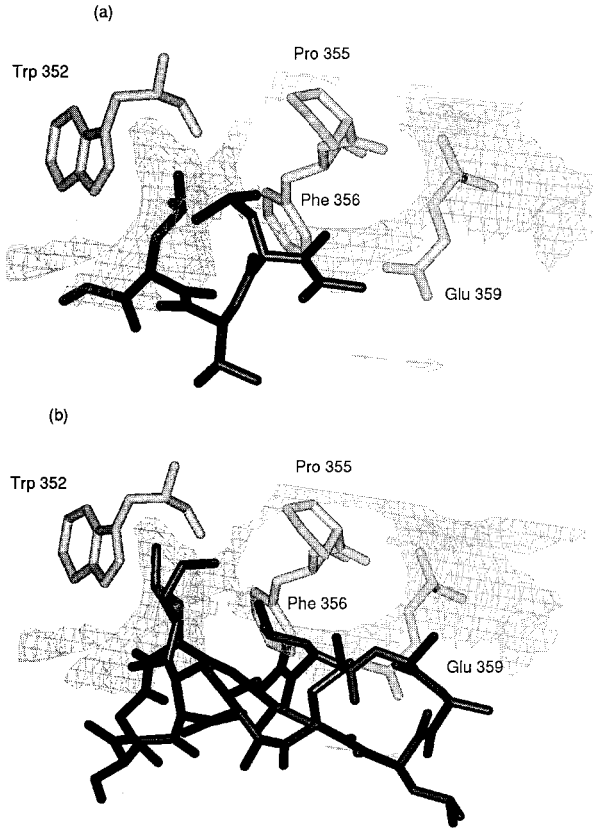

Figure 5. Contour map representations of the result of a GRID survey of the FK506 binding site of calcineurin (see Experimental). The C3 probe representing the C–H from a methyl carbon was used for the survey. In each figure a single contour is shown as light grey shading and represents favourable interactions of 2 kcal mol^{-1} or greater. Residues defining the FK506 and proposed CsA binding sites are displayed in grey and labelled. (a) Fragment **3** (black) oriented as indicated by GROUP analysis of binding site (see Experimental section). Note the modified positioning of the Meleu[4] sidechain (see Fig. 4, inset) and rotation of the whole fragment to remove steric clash between Meleu[6] sidechain and Pro 355 and to align the Meleu[4] sidechain with the region of favourable interaction; (b) final docked position of full CsA molecule (black) after molecular dynamics simulation.

Comparison of the conformation of the docked fragment of CsA (**3**) and the same region of CsA when bound to CyPA indicated little change in conformation of CsA during the docking process (data not shown). This then allowed the docking of the full CsA molecule with CN by superposition of the common atoms of **3** and CsA. To finally optimise the docking of CsA with CN a molecular dynamics simulation was conducted (see Experimental). The simulation was conducted using the above cutdown structure of CN with the protein residues restrained to their initial positions. To mimic the effect of CyPA on CsA during the simulation the conformation of the CyPA binding domain of CsA was maintained during the simulation by constraining the dihedrals of residues 1, 11, 10, 9 and 8 of the macrocyclic ring of CsA. The simulation was run for 100 ps and it was seen over this time span that the position of CsA with respect to CN remained relatively stable. This final optimised orientation of CsA with respect to calcineurin is shown in Figure 5(b) again positioned within the C3 GRID map and indicates that this orientation of CsA gives good complementarity between the surfaces of CsA and calcineurin.

Second stage model of the cyclosporin A–Cyclophilin A–Calcineurin (CsA–CyPA–CN) complex

A second stage model of the complex of CsA–CyPA–CN (see Experimental) could then be constructed by superposition of the full structure of CN and the structure of CyPA onto the optimised structure in Figure 5(b). This model (data not shown) showed much fewer steric clashes between calcineurin and either CyPA or CsA (14 residues of CN with any heavy atom within 2.5 Å of either CyPA or CsA). To finally optimise the model a molecular dynamics simulation was conducted on a subset of residues of the model that are within 5 Å of each other at the interface between CsA–CyPA and CN including a shell of weakly restrained residues 2 Å either side of the interface region (see Experimental). The results of this simulation constituting the final model of the CsA–CyPA–CN complex are shown as a space filling model in Figure 6(a).

Comparison of this model with that of the FK506–FKBP12-CN structure shows that while both small molecules are binding in the same site on calcineurin there are significant differences in how the small molecule links the two protein components of the complexes. In Figure 6(b) a space filling diagram of the complex of FK506–FKBP12–CN is displayed which shows that virtually all of the FK506 is sandwiched in between FKBP12 and CN with only 9% of the total surface area of FK506 solvent accessible in the FK506–FKBP12–CN complex. However, Figure 6(a) shows that much more of CsA in the complex is exposed to solvent (~23%). Comparing the surface area of contact between the two immunophilin–drug complexes and calcineurin it is seen that the FK506–FKBP12 complex has an area of contact with calcineurin of 1330 $Å^2$ and within this area only four residues of FKBP12 (Glu 54, Lys 44, Arg 42, and Lys 35) are oriented to suggest some type of hydrogen bonding to calcineurin residues. Also both the C13 and C15 methoxyl groups are oriented to form a possible hydrogen bond with Trp 352 sidechain nitrogen. For CsA–CyPA the area of contact with calcineurin is reduced to 1270 Å2 but eight residues of CyPA (Asp 66, Arg 69, Thr 73, Lys 81, Lys 82, Arg 148, Asn 149, Lys 151) are positioned to form possible hydrogen bonds with calcineurin. CsA also shows two possible hydrogen bonds with calcineurin between the backbone carbonyl of Sar^3 and sidechain amide of Asn B122 and between the backbone NH of Ala^8 and sidechain carboxylate of Glu 359.

Examination of the validity of the CsA–CyPA–CN complex model with respect to structure–activity relationships for chemical modifications to CsA

Both FK506 and CsA have been extensively modified to examine the relationship between their chemical structure and their biological activity.[33,34,37] The effect of these chemical modifications can broadly be separated into changes that affect the ability of the small molecules to bind their respective immunophilin and indirectly their immunosuppressant activity and changes that do not affect the affinity of the small molecule for the immunophilin but do affect the ability of the drug–immunophilin complex to inhibit calcineurin. As could be expected from the structure of the FK506–FKBP12–CN complex, modifications to the C21-allyl group[38] of FK506 were found to be in the latter category having minimal effect on the binding of FK506 to FKBP12 but substantially affecting the ability of the FK506–FKBP12 complex to inhibit calcineurin. In particular a trend was observed that reducing the size of the C21-allyl group by two carbons to a methyl group reduced activity by ~15-fold, however, increasing the size of the substituent by two carbons to a pentadiene group reduced activity by ~10,000-fold. In Figure 7 these structure–activity relationships for modification to FK506 are examined with respect to the position of the C21-allyl group within the binding site. In Figure 7(a) a zoomed view of the C21-allyl group within the FK506 binding site on CN shows this group binding deep within the site virtually filling the whole cleft. In Figure 7(b) the C21-allyl group has been modified to a pentadiene group which is clearly too large for the site with the end of the group coming within ~2 Å of the Leu 115 residue which forms the back of the cleft. In Figure 7(c) the C21-allyl group has been modified to a methyl group and can still reach into the site which is consistent with the above noted small reduction in the biological activity of this analogue of CsA.

For the interaction of CsA with calcineurin the proposed binding model suggests the *iso*-butyl side chain of $Meleu^4$ plays the same role in the interaction between CsA and CN as the C21-allyl group of FK506. As expected modifications to this group[34] did not affect the ability of these CsA analogues to bind cyclophilins but did effect the ability of the complex of these analogues with CyPA to induce immunosuppression. However, in contrast to the data for FK506, modestly increasing the size of the $Meleu^4$ sidechain had minimal effect on the immunosuppressant activity of CsA analogues whereas shortening the sidechain and particularly introducing

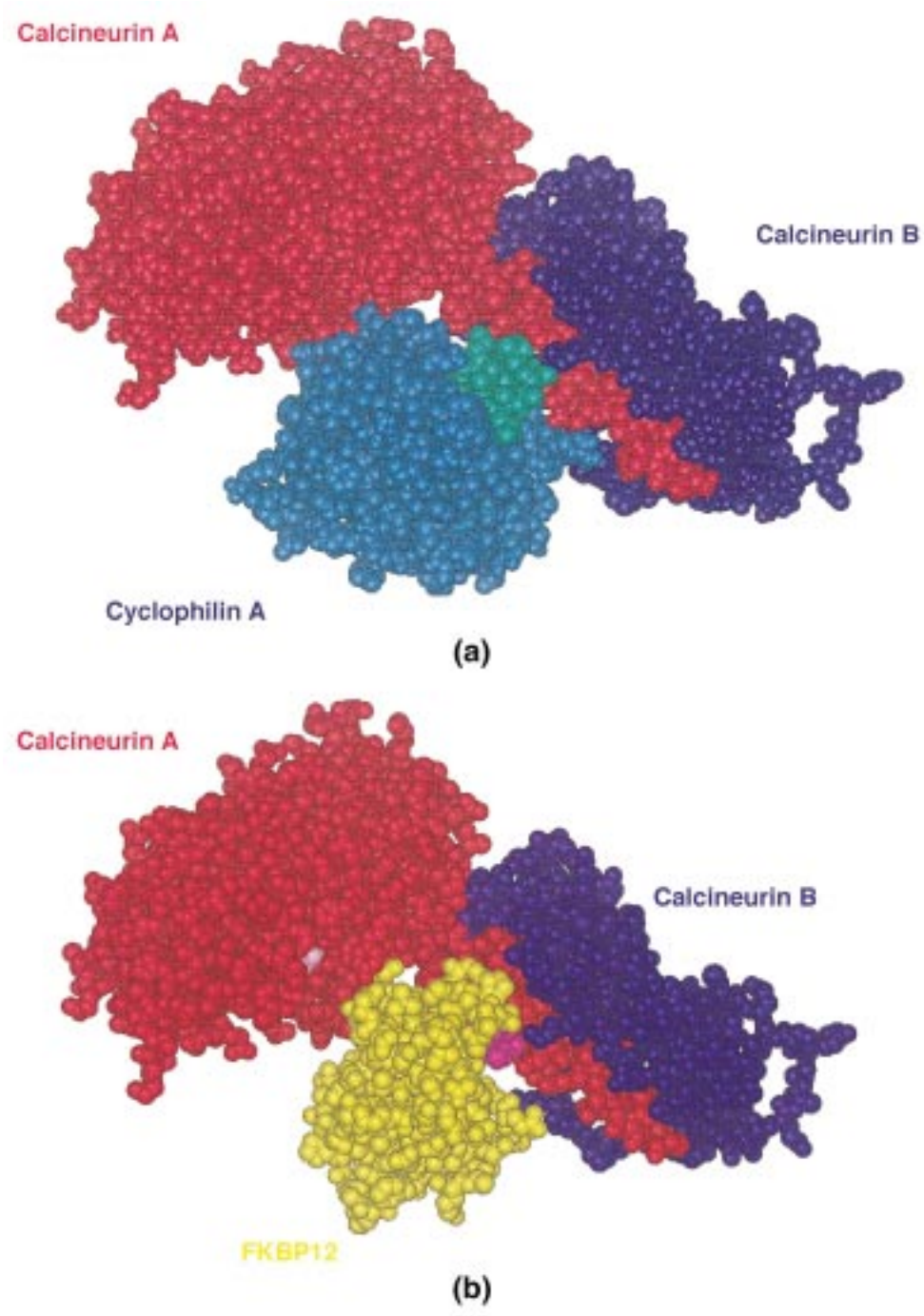

Figure 6. (a) Space filling diagram of the final model of the CsA–CyPA–CN structure with calcineurin A shaded red, calcineurin B shaded dark blue, cyclophilin A shaded light blue and CsA is turquoise. Note the much greater exposed surface area of CsA compared to FK506 in Figure 6(b). (b) Space filling diagram of the FK506–FKBP12–CN structure with calcineurin A shaded red, calcineurin B shaded dark blue, FKBP 12 shaded yellow and FK506 is purple. Note the very small amount of exposed surface area for FK506.

branching closer to the peptide backbone significantly reduced activity. These contrasting findings for CsA and FK506 can be explained by the proposed model for binding of CsA with calcineurin as shown in Figure 8. In Figure 8(a) a zoomed view of the orientation of the Meleu[4] sidechain within the binding site is displayed and this shows that the *iso*-butyl sidechain binds in the mouth of the binding site such that there is still significant space in the site to accommodate larger groups such as an iso-amyl sidechain (reduced activity for analogue only ~ninefold) (Fig. 8b). However, when the sidechain is decreased in size and branched, this results in the sidechain not reaching the binding site and also placing both methyl groups in the narrow mouth of the binding site, thus resulting in greatly reduced (~2500-fold) activity (Fig. 8(c)). Thus, the differences in orientation of FK506 and CsA within their binding site on calcineurin proposed by our docking experiments are in agreement

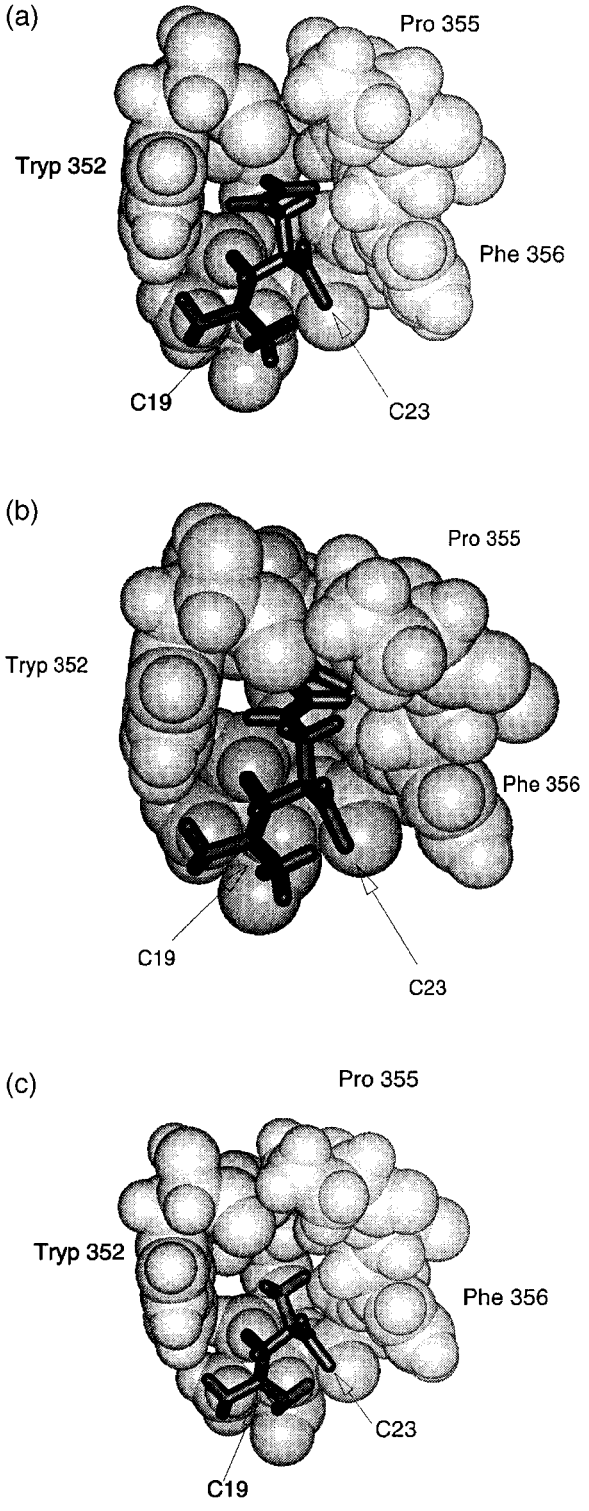

Figure 7. Examination of the structural basis for observed structure–activity relationships for modifications to FK506. Zoomed view of the FK506 binding site of calcineurin with residues of CN represented as space filling spheres. CNA is shaded light grey, CNB is shaded dark grey and FK506 black. (a) Unmodified view of binding site showing C21 allyl group from FK506. Note that sidechain binds positioned deeply within binding site. (b) View of binding site with the C21-allyl group modified to a pentadiene group. Note this sidechain extends to within ~2 AÅ of the back of the binding pocket. (c) View of binding site with the C21-allyl group modified to a methyl group. Note that shorter methyl group still extends some distance into the binding pocket.

with the observed structure–activity relationships for these two compounds.

Examination of the validity of the CsA–CyPA–CN complex model with respect to the effect of amino acid substitutions within CyPA on the biological activity of the drug–immunophilin complex

As in chemical modifications to FK506 and CsA, the effect on the biological activity of the drug–immunophilin complex by amino acid substitutions in the immunophilin can be divided into substitutions that affect the ability of the mutant enzyme to bind the drug and thus indirectly the immunosuppression activity of the complex or substitutions which do not affect the mutants affinity for drugs but do affect the immunosuppressant activity of the complex.

For FKBP12 extensive studies[39,40] on the effect of amino acid substitution on biological activity have been conducted. These studies were performed before the availability of structural information for the complex of FK506–FKBP12–CN so most mutations concentrated on charged surface residues which were postulated to contact calcineurin. Three residues were found to have no significant effect on the ability of the mutant enzymes to bind FK506 but significantly reduced the ability of the complexes to inhibit calcineurin. Mutation of His 87 to a valine resulted in a reduction of affinity of the FK506–H87V–FKBP12 complex for calcineurin of ~fivefold, mutation of Arg 42 to a lysine gave a reduction in activity of ~170-fold and the double mutation His87Val, Arg42Lys gave a reduction in activity of 680-fold. While mutation of Ile 90 to residues ranging from alanine, valine and lysine resulted in reductions in affinity to calcineurin for the mutant enzyme–FK506 complex of approximately 10-, 100-, and 2000-fold, respectively. Recently,[41] we have performed modelling studies on the interaction of FK506 and FKBP12 that suggest that at least some of the importance of these residues to the activity of the FK506–FKBP12 complex is due to the interaction of these residues with FK506 during formation of the FK506–FKBP12 complex. We postulated that this interaction results in a conformational change in FK506 leading to a composite surface for the FK506–FKBP12 complex which is appropriate for interaction with calcineurin. However, examining the position of Ile 90 and His 87 residues within the structure of FK506–FKBP12–CN (Fig. 9(a)) also shows that they are positioned at the mouth of the hydrophobic cleft formed by Trp 352 and Phe 356 which is the FK506 binding site of calcineurin. These residues could then be viewed as a hydrophobic clamp sealing FK506 into the binding site and excluding water from the binding site and thus intensifying the strength of the interaction. The hydrophobic nature of the interaction between CN, FK506 and FKBP12 is highlighted by the very large effect of mutating Ile 90 to a lysine, this places the charged terminal amine of the lysine directly into the hydrophobic cleft which resulted in the very large loss of affinity noted above. Interestingly, when the binding orientation of CsA within the binding site of CN is examined (Fig. 9(b)) it is seen that the sidechains of Meleu[6] and Bmt[1]

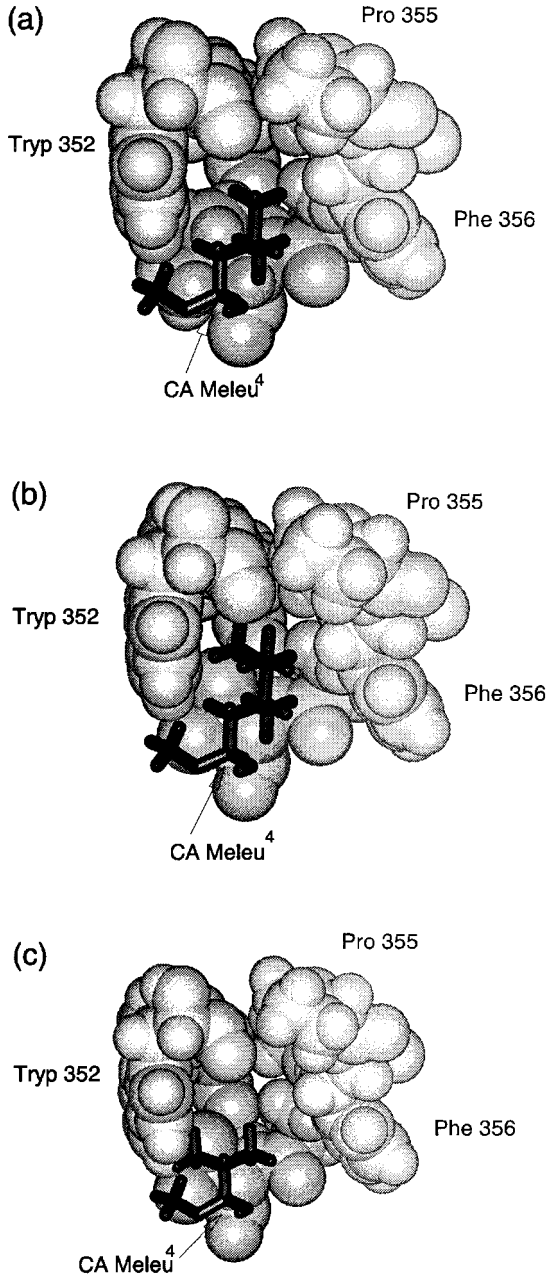

Figure 8. Examination of the consistency of the proposed model for the CsA–CyPA–CN complex with known structure–activity relationships for modifications to CsA. Zoomed in view of CsA binding site of calcineurin from model structure with residues of CN represented as space filling spheres. CNA is shaded light grey, CNB is shaded dark grey and CsA is black. (a) View of Meleu[4] sidechain in CsA binding pocket of calcineurin. Note that the Meleu[4] sidechain is positioned in the mouth of the cavity. (b) View of binding site with Meleu[4] sidechain modified to an *iso*-amyl group. Note that group is still relatively easily accomodated within the pocket. (c) View of binding site with Meleu[4] modified to a valine sidechain. Note that sidechain does not extend at all into binding

occupy very similar positions to those of the Ile 90 and His 87 in FKBP12. Modification to the sidechains of Meleu[6] and Bmt[1] is also known to greatly reduce the affinity of the CsA analogue–CyPA complex for CN.[33] These findings again support the docked orientation of CsA within the FK506 binding of calcineurin and suggest that CsA can be viewed as mimicking both parts of FK506 and FKBP12 in interacting with CN.

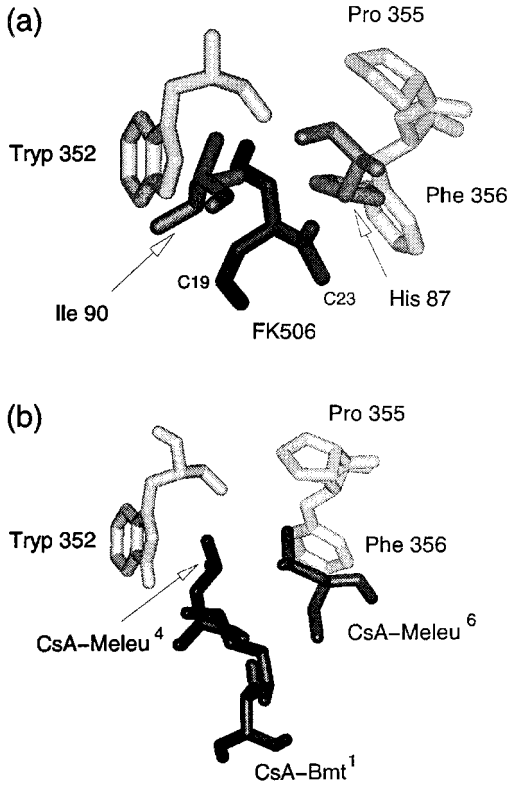

Figure 9. Examination of the validity of the proposed model for the CsA–CyPA–CN complex with respect to known biological effects of amino acid substitutions within FKBP12. (a) A zoomed in view of the FK506 binding site of the FK506–FKBP12–CN structure showing a fragment of FK506 (black) positioned between CNA residues (light grey) Trp 352, Pro 355, Phe 356. Ile 90 and His 87 (black) two residues of FKBP12 that have been shown by protein mutagenesis experiments to play a role in the interaction of the FK506–FKBP12 complex and CN are displayed. Note how these residues appear to act as a clamp sealing off hydrophobic binding site from solvent water. (b) A zoomed in view of CsA binding site of the proposed model of the CsA–CyPA–CN complex showing the Meleu[4] (black) residue of CsA positioned between CNA residues (light grey) Trp 352, Pro 355, Phe 356. Also shown are Meleu[6] and Bmt[1] residues of CsA (black). Note that these residues appear to adopt a very similar position to those of Ile 90 and His 87 in (a) suggesting they perform a similar function to these residues.

For CyPA studies with mutant enzymes[31,43] have also identified several residues that significantly affect the ability of the CyPA–CsA complex to inhibit calcineurin. In particular substitution of Arginine 69 by a glutamate residue resulted in a reduction of activity of 13-fold, substitution of Lysine 125 with a tryptophan reduced activity by 6 fold and substitution of Arginine 148 by a glutamate increased activity by ~16-fold. Examining the position of these residues within the model of the interaction CsA–CyPA–CN shows that consistent with the above biological data Arg 69 and Arg 148 are positioned on the interface between CyPA and calcineurin this is consistent with the above described biological effects of mutation of these residues. The above results suggest that in interacting with calcineurin it is preferable for the CsA–CyPA complex to have certain residues at particular positions. One means of determining whether these residues are in their appropriate positions within the proposed model is to examine what residues occupy these same positions in the known structure of

FK506–FKBP12–CN complex. To achieve this comparison the structures for our model of the CsA–CyPA–CN complex and the crystal structure of the FK506–FKBP12–CN complex were overlaid using the calcineurin molecules as reference (data not shown). Firstly looking at the modification of Arg 69 it is seen that FKBP12 has in this position a lysine residue (Lys 44) with its terminal amino nitrogen only ~2 Å from the terminal nitrogen of the guanidine group of Arg 69 in CyPA. The terminal guanidine of Arg 69 of CyPA hydrogen bonds to the backbone carbonyl of Leu 123 of calcineurin while the terminal amine of Lys 44 of FKBP12 hydrogen bonds to the sidechain carbonyl of Asn 121 of calcineurin. These observations are quite consistent between the two structures and seem to support the positioning of Arg 69 of CyPA in our model of the CsA–CyPA–CN complex. However, while, as pointed out above, mutation of Arg 69 in CyPA to a glutamate results in a significant loss of activity for the complex, Aldape et al.[40] has reported that mutation of Lys 44 to glutamate in FKBP12 resulted in no loss of activity for the FK506–FKBP12 complex. This may reflect the ability of an asparagine residue to hydrogen bond with either a lysine or glutamate using either the carbonyl oxygen or the amide nitrogen of the side chain, respectively.

Looking at the substitution of Arg 148 within CyPA it was seen that FKBP12 has a glutamate residue (Glu 53) in the corresponding position of the FK506–FKBP12–CN complex. This is consistent with the biological data as changing the Arg 148 to a glutamate resulted in increasing the activity of the CsA–CyPA complex to a similar level to that of the FK506–FKBP12 complex which also has a glutamate residue in this position. The substitution of a Lys 125 to a tryptophan as indicated above results in a ~sixfold reduction in activity of the complex for inhibition of calcineurin but does not affect the ability of the mutant CyPA to bind CsA. However, within the proposed model, Lys 125 is not positioned to interact with calcineurin but is adjacent to the CsA binding site. A plausible explanation for this observed behaviour may be that mutation of Lys 125 to the hydrophobic and bulkier tryptophan residue may produce a mutant CyPA which is still capable of binding CsA but in doing so alters the conformation of CsA such that the composite surface of the CsA–CyPA complex in the mutant is different from that in the wild-type complex. This conformationally altered complex may then be less efficient at interacting with calcineurin than the wild-type CyPA–CsA complex. Supporting this hypothesis is the observation that CsA is known to exist in multiple conformations in solution and CyPA selects only one of these conformations for binding[42] thus the above mutation may be altering the conformational selectivity of the mutant enzyme.

Conclusions

Here has been reported a study which has proposed a model for the complex of cyclosporin A–cyclophilin A and calcineurin. This model is based on the assumption that the CsA–CyPA complex binds to calcineurin in a similar manner to the FK506–FKBP12 complex. Docking experiments confirmed that irrespective of the structural differences between CsA and FK506, CsA can be positioned within the FK506 binding site of calcineurin in a structurally complementary fashion. The cyclophilin A structure could then be positioned relative to calcineurin using the docked position of CsA as reference. This resulted in a structure with a small number of steric clashes. The final model was obtained by using molecular dynamics simulations to remove the final steric clashes. This model was then shown to be consistent with observed changes in the biological activity of the CsA–CyPA complex induced by chemical modification of CsA and amino acid substitutions for CyPA. These results suggest that whether by chance or design, nature has developed two diverse solutions to the same structural problem of creating a non-active site directed ligand for calcineurin.

Experimental

General

Molecular modelling was performed on a Silicon Graphics Indigo running under the IRIX 5.3 operating system. Coordinates for the FK506–FKBP12,[26] CsA–CyPA,[28] CsA-CyPB,[29] FK506–FKBP12–CN,[23] and CN[24] were obtained from the Brookhaven protein data bank. The InsightII software package[44] was used for general geometric measurements on structures including dihedral angles and atomic distances and construction of molecular models including superposition of structures by RMS overlay. Molecular representations in Figures 1, 3 and 4 were prepared with the Setor program package[45] with other molecular representations prepared using InsightII.

Energy minimisations and stochastic molecular dynamics simulations were performed on model structures using the Batchmin program within Macromodel version 6.0.[46] The macromodel implementation of the Amber forcefield was used in all calculations. For energy minimisations the Polak–Ribiere conjugate gradient minimisation procedure was used. Solvent water was included in all calculations using the Macromodel implementation of the GB/SA model[47] which treats a solvent as a fully equilibrated analytical continuum starting near the van der Waals surface of the solute. The SHAKE[48] algorithm was invoked in all dynamics simulations with all bonds to hydrogen atoms constrained. All molecular dynamics simulations were conducted at an average temperature of 300 K.

Construction of molecular models

The initial model of the interaction of CsA–CyPA with calcineurin (Fig. 4(a)) was constructed by positioning the structure of the CsA–CyPA complex relative to the structure of the FK506–FKBP12–CN complex by superposition of conformationally similar regions of FK506 and CsA (see Results and Discussion) within the above two structures. The superposition analysis was

conducted using the InsightII superposition option within the transform menu of the viewer module at the atom pick level for atoms C17–C24 of the macrocyclic ring of FK506 and Sar^3 (N) to Val^5 (Cα) of the macrocyclic ring of CsA. After this overlay the FK506–FKBP12 components of the model were removed to leave a model of the interaction of the CsA–CyPA–CN complex. This model was then saved as a protein data bank file (.pdb) using the put option within the molecule menu option of InsightII. Construction of the second stage model for the complex of CsA–CyPA–CN began with the structure file for the finally docked orientation of CsA within the binding site of CN. This file contains CsA in its final docked position and residues of calcineurin within 10 Å of CsA and each of these were used as reference for construction of the model. Firstly, the calcineurin component of the complex was positioned by superposition of the common residues from the FK506–FKBP12–CN complex with those of calcineurin in the docked structure. Then the CyPA component of the complex was positioned by superimposing the CyPA binding domains of CsA in the docked structure and the same region in the CsA–CyPA structure. The final complex model was then completed by removing FK506–FKBP12 and CsA of the CsA–CyPA complex from the display. This structure was then saved as a .pdb file as above.

Docking of cyclosporin A within the FK506 binding site of calcineurin

GRID analysis of proposed CsA binding site of calcineurin. The GRID[35] analysis started with the .pdb file created above for the initial model of the CsA–CyPA–CN complex (see Fig. 4). Using the interface option of the subset menu of the InsightII viewer module a subset was created containing all residues of CN within 10 Å of CsA. This subset was then edited to add all required hydrogens and remove the CsA molecule and the resulting structure saved as a .pdb file. This file was used as input to the GRIN program[35] which prepares the appropriate input files for the GRID program. The GRID analysis of this file was then conducted using the C3 probe with a grid spacing of 0.5 A (directive NPLA = 2). The survey area was defined to include the whole surface of CN that interacts with CsA in the model (TOPX = 13.0, TOPY = 45.0, TOPZ = 26.5, BOTX = −10.0, BOTY = 30.0, BOTZ = 8.5). Other directive settings were as those in the standard grid.in command file. To prepare the resultant grid map for visual presentation using InsightII (see Figs. 5(a) and (b)) the output .kont file from the above GRID survey was converted to a .grd file using the k2i program supplied with GRID. To display the grid in relation to the surveyed region the macromolecular target file was read into InsightII and this was used as reference for reading in the above .grd file. Contours at various energy levels could then be created using the contour pull down menu of InsightII.

GROUP analysis of proposed CsA binding site of calcineurin. The GROUP[36] program fits a small molecule into a set of grid maps calculated for interaction between probes and a macromolecular target. The small molecule used for this analysis was a modified fragment of CsA shown in the Results and Discussion section as **3**, which was edited from the structure of CsA in the CsA–CyPA complex and then modified as shown using the Builder module of InsightII and then saved as a .pdb file. The macromolecular component for the docking was the same .pdb file used in the above GRID survey. For the GROUP analysis a set of GRID maps for each type of atom within the small molecule to be docked plus water are required thus GRID maps using the H_2O (water), H (hydrogen), N1 (sp3 NH with lone pair), N (sp3 N with lone pair), C3 (methyl CH_3 group), O (sp^2 carbonyl oxygen), and DRY (hydrophobic) probes were calculated. Each of these grids were calculated for the same survey area and using the same directive settings as above except that the spacing of the Grid points was 1 Å (NPLA = 1). Each of these grid maps was then prepared for a group run by processing with the k2m program to compress the data converting the .kont files of each of these grids to .mini files. Both the small molecule and macromolecular target files were then processed using GRIN to provide appropriate input files for GROUP. However, the small molecule probe file was modified by giving the three carbon atoms at the ends of the sidechains of $Meleu^4$ and $Meleu^6$ an atom type number of 91. This forces GROUP to include these atoms in the docking process by attempting to fit them into the DRY hydrophobic grid. The GROUP run was then performed with the output of this program the coordinates in .pdb file format for the optimum positioning of the small molecule within the GRID maps. To obtain the structure of the small molecule docked into calcineurin, the .pdb file of binding site used for both the GRID and GROUP surveys was loaded into InsightII and then the output file from the GROUP analysis loaded using the binding site file as reference. The combined display of the binding site protein residues of calcineurin and the docked position of **3** was saved as a .pdb file. Positioning of the full CsA molecule within the binding site was then achieved by superposition of the corresponding atoms of **3** within the above docked structure and CsA from the CsA–CyPA structure.[28] **3** Was then removed from the display and the docked position of CsA in the binding site saved as a .pdb file.

Optimisation of molecular models

Energy minimisation and molecular dynamics simulation were used to remove steric clashes at two stages in the construction of the final model of the CsA–CyPA–CN complex. Firstly the final docked structure of CsA prepared above using GRID and GROUP analysis was energy minimised for 500 gradient steps. During this minimisation an atom restraint file was applied which constrained the position of all atoms within the protein with a flat well potential with a force constant of 100 kJ A^{-1}. Additionally the torsional angles of the peptide backbone of CsA from the Cα carbon of Bmt^1 through $Meval^{11}$, $Meleu^{10}$, $Meleu^9$ to the Cα carbon of $D\text{-}Ala^8$ were constrained with a flat well potential with a force constant of 1000 kJ A^{-1}. The rest of the CsA molecule

was left unrestrained. The output from this energy minimisation was then used as input for a molecular dynamics simulation. This was run for 100 ps with extended non-bonded cut-offs invoked and a time step of 3.0 fs. The simulation was sampled every 5.0 ps.

Optimisation of the second stage model of the CsA–CyPA–CN complex (Fig. 6(a)) began with preparing the model structure for use in the calculation, this required addition of hydrogens to all polar atoms. This was achieved by reading the .pdb file of the structure into the macromodel and using the H ADD, H DEL options for full molecule addition and deletion of hydrogens. This file was then saved as a macromodel dat file. Next it was necessary to define the areas of the structure to be included in the optimisation process. This was achieved by reading the .pdb file of the constructed model into InsightII then using the interface option of the subset menu to create subsets containing all residues of CyPA within 5 Å of calcineurin and all residues of calcineurin within 5 Å of CyPA. The residues within each of these subsets were then listed. Then using the above dat file within macromodel these residues and CsA were defined as the active subshell for calculation. Additionally, a further shell of residues within 2 Å of these unrestrained residues was defined which were weakly restrained during the optimisation by a flat well potential with a force constant of 10 kJ A^{-1}. Under these conditions this structure was then energy minimised for 500 gradient steps. The output from this energy minimisation was then used as input for 100 ps of molecular dynamics simulation. This was run with the same restraint file as above applied, extended nonbonded cutoffs were invoked and a time step of 1.5 fs was used. The simulation was sampled every 5 ps.

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References

1. Dreyfuss, M.; Haerri, E.; Hofman, H.; Kobel, H.; Pache, W.; Tscherter, H. *Eur. J. Appl. Microbiol.* **1976**, *3*, 125.
2. Ruegger, A.; Kuhn, M.; Lichti, H.; Loosli, H.-R.; Quiquerez, C.; von Wartburg, A. *Helv. Chim. Acta* **1976**, *59*, 1075.
3. Borel, J. F.; Faurer, C.; Gubler, H. U.; Stacholin, H. *Agents Actions* **1976**, *6*, 468.
4. Padova, F. E. *Persp. Drug Disc. Des.* **1994**, *2*, 49.
5. Hertenstein, B.; Arseniev, L.; Novotny, J.; Ganser, A. *Biodrugs* **1998**, *9*, 105.
6. Uberti, J. P.; Adams, P. T.; Jacobsen, P.; Scalzo, A. *Bone Marrow Transplantation* **1997**, *19*, 1233.
7. Taga, T.; Tanak, H.; Goto, T.; Tada, S. *Acta Cryst.* **1987**, *C43*, 751.
8. Crabtree, G. R.; Clipstone, N. A. *Annu. Rev. Biochem.* **1994**, *63*, 1045.
9. Hess, A. D.; Colombani, P. M. *Trans. Proc.* **1988**, *20* (Supp 2), 29.
10. Rosen, M. K.; Schreiber, S. L. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 384.
11. Thompson, A. W.; Starzl, T. E. *Immunology Rev.* **1993**, *136*, 71.
12. Schreiber, S. L.; Albers, M. W.; Brown, E. J. *Acc. Chem. Res.* **1993**, *26*, 412.
13. Clardy, J. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 56.
14. Harding, M. W.; Galat, A.; Uehling, D. E.; Schreiber, S. L. *Nature (Lond.)* **1989**, *341*, 758.
15. Sierkierka, J. J.; Hung, S. H.; Poe, M.; Lin, C. S.; Sigal, N. H. *Nature (Lond.)* **1989**, *341*, 755.
16. Handschumacher, R. E.; Harding, M. W.; Rice, J.; Drugge, R. J. *Science* **1988**, *226*, 544.
17. Price, E.; Zydowsky, L.; Jin, M.; Baker, C.; McKeon, F.; Walsh, C. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 1903.
18. Friedman, J.; Weissman, I. *Cell* **1991**, *66*, 799.
19. Bierer, B. E.; Somers, P. K.; Wandless, T. J.; Burakoff, S. J.; Schreiber, S. L. *Science* **1990**, *250*, 556.
20. Liu, J.; Farmer, J. D.; Lane, W. S.; Friedman, J.; Weissman, I.; Schreiber, S. L. *Cell* **1991**, *66*, 807.
21. Klee, C. B.; Krinks, M. H.; Crouch, T. H. *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 6270.
22. Rao, A. *Immunol. Today* **1994**, *15*, 274.
23. Griffith, J. P.; Kim, J. L.; Kim, E. E.; Sintchak, M. D.; Thompson, J. A.; Fitzgibbon, M. J.; Fleming, M. A.; Caron, P. R.; Hsiao, K.; Navia, M. A. *Cell* **1995**, *82*, 507.
24. Kissinger, C. R.; Parge, H. E.; Knighton, D. R.; Lewis, C. T.; Pelletier, L. A.; Tempczyk, A.; Kalish, V. J.; Tucker, K.; Showalter, R. E.; Moomaw, E. W.; Gastinel, L. N.; Habuka, N.; Chen, X.; Maldonado, F.; Barker, J. E.; Bacquet, R.; Villafranca, J. E. *Nature (Lond.)* **1995**, *378*, 641.
25. Van Duyne, G. D.; Standaert, R. F.; Karplus, P. A.; Schreiber, S. L.; Clardy, J. *Science* **1991**, *252*, 839.
26. Itoh, S.; Decenzo, M. T.; Livingston, D. J.; Pearlman, D.; Navia, M. A. A. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1983.
27. Theriault, Y.; Logan, T.; Meadows, R.; Yu, L.; Olejniczak, E. T.; Holzman, T. F.; Simmer, R. L.; Fesik, S. W. *Nature* **1993**, *361*, 88.
28. Mikol, V.; Kallen, J.; Pflugl, G.; Walkinshaw, M. D. *J. Mol. Biol.* **1993**, *234*, 1119.
29. Mikol, V.; Kallen, J.; Walkinshaw, M. D. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 5183.
30. Ke, H.; Zhao, Y.; Luo, F.; Weissman, I.; Friedman, J. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11850.
31. Etzkorn, F. A.; Chang, Z. Y.; Stolz, L. A.; Walsh, C. T. *Biochemistry* **1994**, *33*, 2380.
32. Denesyuk, A. I.; Korpela, T.; Lundell, J.; Sara, R.; Zav'yalov, V. R. *Biochem. Biophys. Res. Comm.* **1993**, *194*, 280.
33. Wenger, R. M. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 77.
34. Papageorgiou, C.; Borer, X.; French, R. R. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 267.
35. GRID *Version 15*; **1995**, Molecular Discovery Ltd; Goodford, P. *J. Med. Chem.* **1985**, *28*, 849.
36. GROUP, *Version 15*, **1995**, Molecular Discovery Ltd.
37. Goulet, M. T.; Rupprecht, M.; Sinclair, P. J.; Wyvratt, M. J.; Parsons, W. H. *Persp. Drug Disc. Des.* **1994**, *2*, 145.
38. Organ, H. M.; Holmes, M. A.; Pisano, J. M.; Staruch, M. J.; Wyvratt, M. J.; Dumont, F. J.; Sinclair, P. J. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 657.
39. Futer, O.; Decenzo, M. T.; Aldape, R. A.; Livingston, D. J. *J. Biol. Chem.* **1995**, *270*, 18935.
40. Aldape, R. A.; Futer, O.; Decenzo, M. T.; Jarrett, B. P.; Murcko, M. A.; Livingston, D. J. *J. Biol. Chem.* **1992**, *267*, 16029.
41. Ivery, M. T. G.; Weiler, L. *Bioorg. Med. Chem.* **1997**, *5*, 217.
42. Altschuh, D.; Braun, W.; Kallen, J.; Mikol, V.; Spitzfaden, C.; Thierry, L. C.; Vix, O.; Walkinshaw, M. D.; Wutherich, K. *Sructure* **1994**, *2*, 963.

42. Zydowsky, L. D.; Etzkorn, F. A.; Chang, H. Y.; Ferguson, S. B.; Stolz, L. A.; Ho, S. I.; Walsh, C. T. *Protein Science* **1992**, *1*, 1092.
44. InsightII Version 2. 3. 0 **1993**, Biosym Technologies.
45. Evans, S. J. *J. Mol. Graphics* **1993**, *11*, 134.
46. Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caulfield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440.
47. Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. *J. Am. Chem. Soc.* **1990**, *112*, 6127.
48. Ryckaert, J. P. *Mol. Phys.* **1985**, *55*, 549.
49. Borel, J. F.; Wiesinger, D. In *Regulatory Mechanisms on Lymphocyte Activation*; Lucas, D. O., Ed.; Academic: New York, 1977.