Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 15, pp. 1719-1724, 1995
Copyright © 1995 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0960-894X/95 $9.50+0.00

0960-894X(95)00290-1

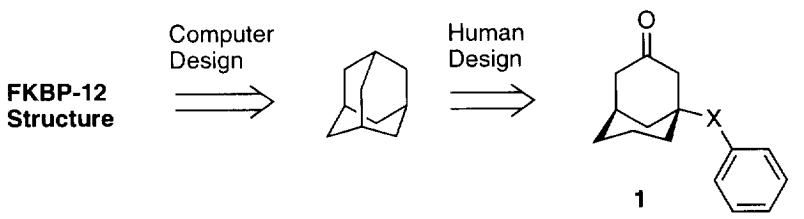
Design, Synthesis and X-ray Crystallographic Studies of Novel FKBP-12 Ligands

Robert E. Babine *, T. M. Bleckman, C. R. Kissinger, R. Showalter, L. A. Pelletier, C. Lewis, Kathleen Tucker, Ellen Moomaw, H. E. Parge and J. Ernest Villafranca

Agouron Pharmaceuticals, Inc.
3565 General Atomics Court
San Diego, CA 92121-1121

Abstract: Using the crystal structure of FKBP-12 a novel class of ligands were designed, prepared and evaluated. The crystal structure of the complex between **5** and FKBP-12 is reported.

The design of novel small molecule ligands to bind to proteins based upon knowledge of protein structure represents an important frontier in bioorganic and medicinal chemistry. A co-crystal structure of a protein-ligand complex allows one to gain new insights into the molecular recognition of protein-ligand complexes; in addition, it provides a starting point for design of more potent inhibitors.[1] FKBP-12 is an abundant protein found in species ranging from yeast to humans.[2] While FKBP-12 has activity as a rotamase enzyme[3] its natural function is not completely clear; however, it has been suggested that it has a role in protein folding and intracellular transport.[4] The immunosuppressive natural products FK506 and rapamycin exert their biological activity indirectly through binding to FKBP-12. It is the resulting protein-ligand complexes that inhibit distinct signal transduction pathways by binding to calcineurin[5] and FRAP[6] respectively. In addition to the role of FKBP-12 as a mediator of immunosuppression, an ingenious experiment has recently demonstrated that unnatural ligands to FKBP-12 can be used to regulate gene expression in appropriately genetically engineered cells.[7] Thus novel molecules that bind to FKBP-12 have potential as a starting point for the design of biologically useful compounds. We now describe the design and synthesis of novel FKBP-12 ligands and report a co-crystal structure of one member of this series.

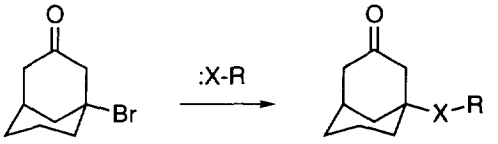


1

The design phase of this work used the crystal structure of the FKBP-FK506 complex[8] as a starting point. The ligand (FK506) was removed and the resulting empty active site examined. Ludi,[9] a suggestive program for the *de novo* design of ligands for proteins was then used to suggest complementary fragments for the hydrophobic

pipecolinic acid binding site of FKBP. Among the many fragments suggested, adamantane appeared to fill this site well and seemed well suited for further elaboration. Examination of the docked ligand for the formation of potential hydrogen bonds suggested the removal of a methylene bridge with the introduction of a carbonyl group. This should allow for a hydrogen bond to the backbone amide NH of Ile-56. Ludi was then used again on this modified ligand to suggest potential groups to fill the FK506 pyran binding site of FKBP-12. Among the many suggestions were aromatic groups appended through a linker to the bridgehead position of the new ring system. Finally, Ludi suggested a hydroxyl group in the meta position of an aromatic ring tethered by a methylene linker. This hydroxyl group has the potential to make favorable electrostatic contact with Asp-37. Thus, compounds of the generic structure **1** were *de novo* designed in an incremental approach using Ludi as a suggestive tool.

The synthesis of the target compounds was straightforward. The known racemic bromoketone **2**[10] was treated with various nucleophilic reagents **:X-R** (X = O^-, NHMe or S^- ; R = Ar or CH_2Ar) to give target compounds **3**, presumably through an intermediate bridgehead enone.[11] These compounds were evaluated for their ability to inhibit the rotamase activity[12] of FKBP-12. Table 1 shows the effect of the R group on activity as rotamase inhibitors (X=O) and Table 2 shows the effect of the X group on rotamase activity (R = C_6H_5).



2 **3**

Table 1
Ability of Compounds **3** to act as rotamase inhibitors (X = O)

Entry	R	Ki app (μM)
1	Ph	116.
2	(3-OH)Ph	16.7
3	(3-OMe)Ph	92.1
4	(2-Me)Ph	120.
5	(2,6-Me)Ph	461.
6	CH_2-Ph	85.8
7	H	no inhib.

Table 2
Ability of Compounds **3** to act as rotamase inhibitors (R = C_6H_5)

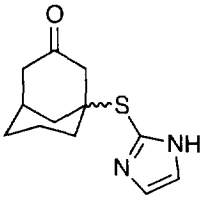
Entry	X	Ki app (μM)
1	O	116.
2	N(Me)	165.
3	S	9.2
4	SO_2	48.5

From the data in Table 1 it appears that small substituents on an aromatic ring can contribute about a factor of five to biochemical activity. The most potent example (entry 2) contains the meta phenol group suggested by Ludi. All of the data in Table 1 are consistent with the proposed binding mode (R group in pyran pocket); entries 1-6 show activity, entry 7 with no R group is inactive. Also consistent with the proposed binding mode, entry 5 was expected to be less potent than entry 4 due to steric reasons.

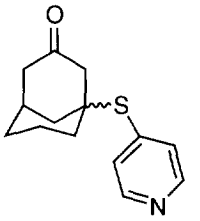
From the data in Table 2 it is clear that the X substituents can contribute over an order of magnitude to activity as rotamase inhibitors. The most potent example (entry 3) contains a sulfur atom. The sulfone (entry 4), was prepared to potentially mimic a twisted amide carbonyl; however, it loses activity relative to the sulfide. This result was somewhat surprising.

One additional piece of structure-activity data concerns the role of the ketone group. Sodium borohydride reduction of the ketone in compound **5** resulted in a significant loss of rotamase inhibition activity (261 μM). Thus, the ketone plays an important role in stabilizing the complex. This is consistent with it forming a hydrogen bond to the protein with a geometry that the alcohol cannot obtain. All of the above structure-activity data is consistent with the proposed binding model.

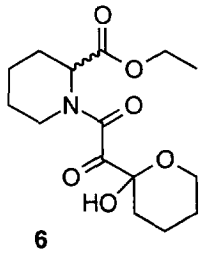
At this stage our goal was to obtain a crystal structure of the complex between one of these compounds and FKBP-12. To assist in this objective we prepared derivatives **4** and **5** of the sulfide that had water solubilizing groups on them. These compounds were evaluated as rotamase inhibitors and were found to have Ki app of 27.5 and 7.9 μM respectively. Compound **5** was chosen as the prime candidate for obtaining a co-crystal structure. For comparison, compound **6**, a racemic, simplified FK506 derivative was reported[13] to have a Ki app of 7 μM against FKBP-12.



4

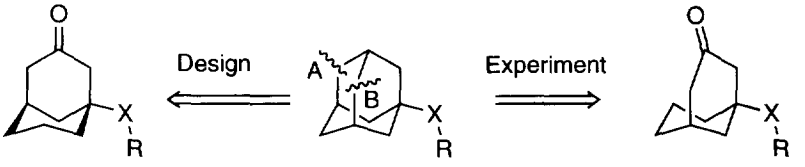


5



6

A 2.4 Å crystal structure was obtained for the complex of **5** and a mutant (P9Q, R13F, K17V, R18F) of FKBP-12 designed to promote crystallizability.[14] As expected, the specific activity of the mutant enzyme was identical to that of the wild type FKBP since the mutations were engineered to be distant from the active site. Figure 1 shows a "relaxed" stereo view of ligand **5** and the protein residues in close contact to the ligand. The major forces holding the complex together were, as expected, hydrophobic contact plus a hydrogen bond from the ketone carbonyl group. The pyridine ring makes van der Waals contact with the hydrophobic pocket that was filled by the pyran ring of FK506. The crystal structure did however reveal some surprises. First, while the fragmented adamantane ring occupied the pipecolinic binding site and the carbonyl group formed a hydrogen bond to Ile-56 it had achieved this with the enantiomeric bicyclic ring system. During modeling experiments methylene bridge **B** was removed; however, the observed bound conformer had methylene bridge **A** removed. Since **5** was prepared as a racemate the protein chose to selectively bind the enantiomer with broken bridge **A** over that with broken bridge **B**. Second, the sulfur atom was found to fill the same space (a small hydrophobic, electropositive



cavity formed by three aromatic ε-hydrogens, one each from Tyr-26, Phe-36 and Phe-99) that the ketone carbonyl of FK506 fills.[8] In FK506 derivatives, it has been shown that modifications to the ketone carbonyl results in decreased affinity of the ligand for FKBP.[13,15] This allows one to propose an important role for the sulfur atom. The binding mode of the observed enantiomer allows for the simultaneous filling of that cavity with the sulfur atom and hydrogen bond formation to the backbone NH of Ile-56 with good geometry. The observed position of the sulfur atom now provides an explanation for the five fold difference in activity between a sulfide and a sulfone (Table 2, Entries 3 vs. 4). In the observed bound conformation there is not sufficient room for a sulfone; therefore, a different higher energy binding mode must result.

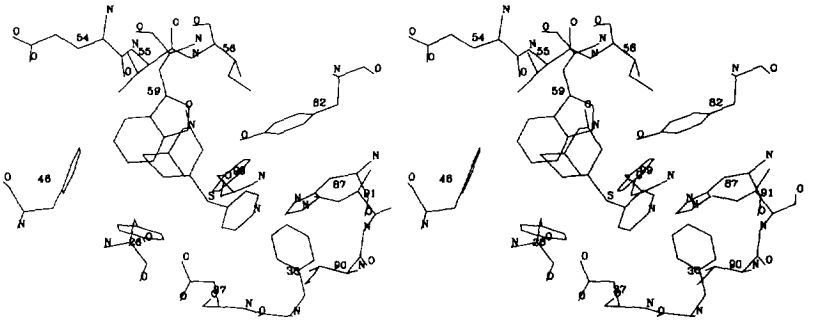


Figure 1: "Relaxed" stereo view of inhibitor **5** bound to FKBP-12. The bicyclic ring system makes VDW contact with the hydrophobic residues V55, I56, W59, F46, Y82 and F99. The carbonyl group makes an H-bond to the backbone NH of I-56. The sulfur atom fills a small hydrophobic, electropositive cavity formed by Y26, F36 and F99. The pyridine ring makes VDW contact with H87, I90, I91,Y82 and F99.

Prior to the crystallographic experiment, the possibility of the enantiomer of the modeled compound binding to FKBP-12 was considered a possibility. On the basis of qualitative and quantitative analysis of molecular dynamics simulations using Discover[16] and the CFF91 force field,[17] this possibility was considered unlikely. In a retrospective analysis it was observed that molecular dynamics simulations in aqueous solution using CFF91, CVFF, and AMBER, as implemented in Discover, were not capable of reproducing the crystal structure of the complex. However, molecular dynamics simulations in aqueous solution using CHARMm22[18] were able to reproduce the observed crystal structure.

We consider the development of quantitative computational methods to distinguish between different enantiomers bound to a protein (diastereomeric complexes), and between different bound conformations of the same ligand to be a very important issue. These situations are somewhat simplified since one does not need to explicitly consider the energetics of either the protein alone or the ligand alone in aqueous solution, just the energetics of the complex. We are presently addressing this and related issues.[19]

A novel class of ligands for FKBP-12 have been described and the crystal structure of the complex of **5** has been solved. The bound complex contains the enantiomer of the designed compound. This result clearly demonstrates the significance of obtaining co-crystal structures as a prelude to further model based design.[20]

References and Notes

1. Appelt, K.; Bacquet, R. J.; Bartlett, C. A.; Booth, C. L. J.; Freer, S. T.; Fuhry, M. A. M.; Gehring, M. R.; Herrmann, S. M.; Howland, E. F.; Janson, C. A.; Jones, T. R.; Kan, C-C.; Kathardekar, V.; Lewis, K. K.; Marzoni, G. P.; Matthews, D. A.; Mohr, C.; Moomaw , E. W.; Morse, C. A.; Oatley, S. J.; Ogden, R. C.; Reddy, M. R.; Reich, S. H.; Schoettlin, W. S.; Smith, W. W.; Varney, M. D.; Villafranca, J. E.; Ward, R. W.; Webber, S.; Webber, S. E.; Welsh, K. M.; White, J. *J. Med. Chem.* **1991**, *34*, 1925.

2. For a recent review see: Fischer, G. *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1415.

3. (a) Siekierka, J. J.; Hung, S. H. Y.; Poe, M.; Lin, C. S.; Sigal, N. H. *Nature* **1989**, *341*, 755. (b) Harding, M. W.; Galat, A.; Uehling, D. E.; Schreiber, S. L. *Nature* **1989**, *341*, 758.

4. (a) Wiederrecht, G.; Etzkorn, F. *Persp. Drug Disc. Design* **1994**, *2*, 57. (b) Schmid, F. X.; Mayr, L. M.; Mucke, M.; Schonbrunner, E. R. In Lorimer, G. (Ed.), Advances in Protein Chemistry Series; Academic Press; San Diego, CA, 1993, Vol. 44, pp 25-66. (c) Jayaraman, T.; Brillantes, A.-M.; Timerman, A.; Fleischer, S.; Erdjument-Bromage, H.;Tempst, P.; Marks, A. *J. Biol. Chem.* **1992**, *267*, 9474. (d) Collins, J. *Biochem. Biophs. Res. Commun.* **1991**, *178*, 1288. (e) Timmerman, A.; Ogunbummi, E.; Freund, E.; Wiederrecht, G.; Marks, A.; Fleisher, S. *J. Biol. Chem.* **1993**, *268*, 22992. (f) See also Ref. 2 and 7.

5. Liu, J.; Farmer, J. D. Jr.; Lane, W. S.; Friedman, J.; Weissman, I.; Schreiber, S. L. *Cell* **1991**, *66*, 807.

6. (a) Brown, E. J.; Albers, M. W.; Shin, T. B.; Ichikawa, K.; Keith, C. T.; Lane, W. S.; Schreiber, S. L. *Nature* **1994**, *369*, 756. (b) Sabatini, D. M.; Erdjument-Bromage, H.; Liu, M.; Tempst, P.; Snyder, S. H. *Cell* **1994**, *78*, 35.

7. Spencer, D. M.; Wandless, T. J.; Schreiber, S. L.; Crabtree, G. R. *Science* **1993**, *262*, 1019.

8. Van Duyne, G. D.; Standaert, R. F.; Karplus, P. A.; Schreiber, S. L.; Clardy, J. *Science* **1991**, *252*, 839.

9. (a) *Ligand_Design User Guide*, version 2.3.0, San Diego: Biosym Technologies, 1993. (b) Bohm, H. J. *J. Comp. Aided. Molec. Design* **1992**, *6*, 69. (c) Bohm, H. J. *J. Comp. Aided. Molec. Design* **1992**, *6*, 593-606

10. Heuman, A. *Synthesis* **1979**, 53.

11. House, H. O.; Kleschick, W. A.; Zaiko, E. J. *J. Org. Chem.* **1978**, *43*, 3653.

12. Compounds were assayed for inhibition of prolyl isomerase activity of human recombinant FKBP-12 (purified as described[21]) using 100 μM succinyl-ala-leu-pro-phe-*p*-nitroanilide (Bachem) as substrate. Assays were performed at 10 or 15°C as described[22] and initial velocities were determined by nonlinear least-squares fits of each progress curve to a single exponential. Apparent Ki's were determined by analyzing the concentration-dependence of each inhibitor on prolyl isomerase activity and fitting the data to an equation for competitive tight-binding inhibition with a correction for uncatalyzed isomerization.[22] All data were analyzed using the software program KineTic (BioKin, Ltd., Madison, WI).

13. Holt, D. A.; Konialian-Beck, A. L.; Oh, H-J; Yen, H-K.; Rozamus, L. W.; Krog, A. J.; Erhard, K. F.; Ortiz, E.; Levy, M. A.; Brandt, M.; Bossard, M. J.; Luengo, J. I. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 315.

14. A sample of **5** was dissolved in DMSO at a concentration of 0.5 M, mixed in a 5:1 molar ratio with a mutant of FKBP (P9Q,R13F,K17V,R18F) and stored at 4 °C. Crystals of the complex were obtained by vapor diffusion using hanging drops. The reservoir solution contained 1.7 M ammonium sulfate and 3% hexanediol in 100 mM Tris buffer at pH 8.5. The FKBP-ligand complex was spun on a desktop centrifuge at 15,000 rpm for 5 minutes to remove particulates prior to use. Three uL of the complex were mixed with three uL of reservoir solution to form the hanging drops. Small, single crystals appeared within one to three days and continued to grow for several weeks.

X-ray diffraction data were collected at room temperature from a single crystal with approximate dimensions 0.3 mm x 0.2 mm x 0.1 mm. Data were collected on a San Diego Multiwire Systems area detector using a Rigaku RU-200 copper rotating anode operating at 50 kV and 180 mA. The overall R_{sym} was 0.0819 for 47,494 observations of 10,045 reflections collected to a resolution of 2.4 Å. The space group was determined to be R3, with unit cell dimensions a=b=135.43, c=37.5, $\alpha=\beta=90°$, $\gamma=120°$ and two complexes in the asymmetric unit.

Phases were determined by molecular replacement using the coordinates of FKBP from the FKBP-FK506 complex structure (reference 8) as a search model. Molecular replacement and refinement calculations were carried out using the program X-PLOR, version 3.1 [Brünger, A.T. (1992) X-PLOR v3.1 Manual (New Haven, Connecticut: Yale University Press)]. A cross rotation search was carried out in 2.5° intervals using 15 to 4 Å data and a maximum cross-vector length of 40 Å. The rotations corresponding to the two highest rotation function peaks (7.0σ and 4.5σ) were optimized by Patterson correlation refinement and used to generate starting coordinates for translation searchs. Independent two-dimensional translation searchs over a 1.0 Å grid each produced a clear solution (peak heights of 7.1σ and 6.5σ). The two translation function solutions were related to a common origin by calculating the partial structure factor contribution from one solution and carrying out a one-dimensional translation search in the z direction with the second solution. The combined translation function solutions produced a model with an R-factor of 0.370 for data ($F>2\sigma_F$) in the resolution range 10 to 3.5 Å. Rigid-body refinement reduced the R-factor to 0.344 for data in the range 10 to 2.7 Å. Subsequent refinement of individual atomic positional and thermal parameters by congugate gradient minimization resulted in a model with an R-factor of 0.287 for all observed data in the 10 to 2.4 Å resolution range. At this point, electron density for the entire ligand was visible at a level of 2σ or higher in a difference map in both copies of the complex in the asymmetric unit. A model of **5** was built using the program Quanta, version 4.1 (Molecular Simulations, Inc., 16 New England Executive Park, Burlington, MA 01803-5297) and fit to the electron density. Display of electron density and manual adjustment of the model were carried out using the program X-FIT [McRee, D. E. *J. Mol. Graphics* **1992**, *10*, 44-47]. After the ligand was fit to density in both complexes, the model was subjected to several additional rounds of positional and restrained temperature factor refinement along with manual refitting of incorrectly positioned sidechains based on difference maps and annealed omit maps.

The R-factor for the final model was 0.203 for all observed data (9854 reflections) in the resolution range of 10.0 to 2.4 Å (R=0.170 for 8280 reflections with $F>2\sigma_F$). In the final model, the root-mean-square (rms) deviation of bond lengths from ideal values was 0.016 Å and of bond angles was 2.958°. 74 well-ordered water molecules and one bound sulfate ion were included in the model. The rms coordinate difference between the two copies of the complex in the asymmetric unit was 0.496 Å for all atoms and 0.171 Å for alpha carbons. Only one significant conformational difference between the two copies of the complex was found among residues contacting the ligand: the orientation of the imidazole ring of His-87 differed by a rotation of approximatly 90°, apparently due to crystal packing interactions.

15. Goulet, M. T.; Rupprecht, K. M.; Sinclair, P. J.; Wyvratt, M. J.; Parsons, W. H. *Persp. Drug Disc. Design* **1994**, *2*, 145.

16. *Discover User Guide*, version 2.9, San Diego: Biosym Technologies, 1993.

17. Maple, J. R.; Hwang M.-J.; Stockfisch, T. P.; Dinur, U.; Waldman, M.; Ewig, C. S.; Hagler, A. T. *J. Comput. Chem.* **1994**, *15*, 162.

18. (a) CHARMm has been developed by Molecular Simulations Inc. (b) *Quanta Parameter Handbook*, version 4.0, Burlington, MA: Molecular Simulations Inc.,1994 (c) Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. *J. Comput. Chem.* **1983**, *4*, 187.

19. We have carried out preliminary quantitative computational analyses of molecular dynamics trajectories obtained with CHARMm22 for the observed and modeled enantiomers of **5** bound to FKBP-12. These preliminary results suggest that the observed enantiomer makes more favorable contact with FKBP than does the modeled enantiomer . While these results are encouraging we are now carrying out control experiments on this and other systems. Important issues to be addressed are convergence (length of simulation, type and length of cutoffs, frequency of sampling, size of active site, amount of waters), scaling factors for various terms (see, Åqvist, J.; Medena, C.; Samuelsson, J-E. *Protein Engineering* **1994**, *7*, 385.) and consistency from system to system. This work is in progress and will be reported in due course.

20. Reich, S. H.; Melnick, M.; Davies , J. F. II; Appelt, K.; Lewis, K. K.; Fuhry, M. A.; Pino, M.; Trippe, A. J.; Nguyem, D.; Dawson, H.; Wu, B-W.; Musick, L.; Kosa, M.; Kahil, D.; Webber, S.; Gehlhaar, D. K.; Andrada, D.; Shetty, B. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 3298.

21. Human recombinant FKBP was purified as described with elimination of the heat treatment step. Standaert, R. F.; Galat, A.; Verdine, G. L.; Schreiber, S. L. *Nature* **1990**, *346*, 671.

22. Kofron, J. L.; Kuzmic, P.; Kishore, V.; Colón-Bonilla, E.; Rich, D. H. *Biochemistry* **1991**, *30*, 6127.

23. Coordinates for the complex between **5** and FKBP-12 are available upon request by e-mail at babine@agouron.com.

(*Received in USA* 1 *June* 1995; *accepted* 27 *June* 1995)