tive reverse transcription PCR with a 5-HTT cDNA–derived template containing a 172-bp deletion (base pairs 1635 to 1806) as internal standard. The PCR amplification (30 s at 95°C, 30 s at 61°C, 1 min at 72°C for 35 cycles) of 355- or 527-bp fragments was carried out with the amplimers se3 (5′-ATGCAGAAGCGATAGCCAACATG, base pairs 1437 to 1459 with respect to the transcription initiation site) and 3re (5′-AGATGAGGTTCCTATGCAGTAAC, base pairs 2147 to 2167). 5-HTT mRNA concentrations of lymphoblast cell lines with the *l/l* genotype were first titrated against incremental concentrations of competitive template ranging from 0.01 to 1.0 ng. The concentration of the competitive template at target/template equilibrium was then used to compare mRNA concentrations semiquantitatively in lymphoblast cell lines with different genotypes (*13*) before and after induction of 5-HTT gene transcription. To control for differences in the efficiency of reverse transcription of mRNA, we performed cDNA synthesis and subsequent competitive PCR in quadruplicate. The reaction products were electrophoresed through 2% agarose, visualized by ultraviolet illumination in the presence of ethidium bromide, and quantified by densitometric analysis.

16. Inhibitor binding to the 5-HTT protein was assayed by incubating membranes from different lymphoblast cell lines (*13*) with [^{125}I]RTI-55 (0.05 to 1 nM) for 1 hour at 37°C as described [J. D. Ramamoorthy *et al.*, *J. Biol. Chem.* **270**, 17189 (1995)]. Nonspecific binding was determined in the presence of 5 μM paroxetine. RTI-55 [3β-(4-iodophenyl)tropan-2β-carboxylic acid methyl ester tartrate] is a cocaine analog that potently inhibits 5-HT uptake and binds to 5-HTT with high sensitivity (*8*) [J. W. Boja *et al.*, in *Dopamine Receptors and Transporters*, H. B. Niznik, Ed. (Dekker, New York, 1994), pp. 611–644]. We determined 5-HT uptake by incubating 1×10^7 suspended lymphoblasts with 0.1 to 1 μM [^{3}H]5-HT for 30 min at 25°C in the absence or presence of 0.1 mM imipramine.
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18. Two independent groups of predominantly male siblings, other family members, and unrelated individuals were studied: (i) The NIMH sample (*17*) was recruited from the NIH and local college campuses by advertising for pairs of brothers and pairs of sisters for a study of personality traits and chromosomes. The sample consisted of 221 subjects, of whom 93% were male and 7% were female. The average age was 23.3 ± 6.8 years (range 18 to 64 years), the average educational level was 15.6 ± 2.1 years (range 12 to 20 years), and the average Kinsey score was 0.2 ± 0.7 (range 0 to 5.6, where 0 is exclusively heterosexual and 6 is exclusively homosexual). The ethnic composition was 79.1% white non-Hispanic, 10.0% Asian/Pacific Islander, 4.1% Hispanic/Latino, 4.1% African American/Black, and 2.7% other. The family structure of the NIMH sample was 208 siblings from 104 families and 13 unrelated individuals. (ii) The NCI sample [D. H. Hamer *et al.*, *Science* **261**, 321 (1993); S. Hu *et al.*, *Nature Genet.* **11**, 248 (1995)] was collected from NIH clinics and local and national homophile organizations for a study of sexual orientation, HIV progression, and psychological traits. The sample consisted of 284 subjects of whom 92% were male and 8% were female. The average age was 37.6 ± 9.7 years (range 18 to 72 years), the average educational level was 17.3 ± 2.6 years (range 12 to 20 years), and the average Kinsey score was 4.8 ± 2.0 (range 0 to 6). The ethnic composition was 93.6% white non-Hispanic, 5.3% Hispanic/Latino, 0.7% African American/Black, 0.4% Native American/Alaskan, and 0.4% other. The family structure of the NCI sample was 251 siblings from 106 families, 9 parents, and 24 unrelated individuals.
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34. We thank M. Schad, G. Ortega, and S. Jatzke for technical assistance, W. Davis and D. Drake for editorial assistance, and M. Altemus, J. Mizrahi, and A. Jaffe for logistical support. Supported by the Deutsche Forschungsgemeinschaft, the Bundesministerium für Bildung und Forschung, the European Commission, and the Intramural Research Programs of NIMH and NCI. K.P.L. is supported by the Hermann and Lilly Schilling Foundation.

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Discovering High-Affinity Ligands for Proteins: SAR by NMR

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A nuclear magnetic resonance (NMR)–based method is described in which small organic molecules that bind to proximal subsites of a protein are identified, optimized, and linked together to produce high-affinity ligands. The approach is called "SAR by NMR" because structure-activity relationships (SAR) are obtained from NMR. With this technique, compounds with nanomolar affinities for the FK506 binding protein were rapidly discovered by tethering two ligands with micromolar affinities. The method reduces the amount of chemical synthesis and time required for the discovery of high-affinity ligands and appears particularly useful in target-directed drug research.

Drugs are typically discovered by identifying active compounds from screening chemical libraries or natural products and optimizing their properties through the synthesis of structurally related analogs. This is a costly and time-consuming process. Suitable compounds with the requisite potency, compound availability, or desired chemical and physical properties cannot always be found. Furthermore, even when such compounds are found, optimization often requires the synthesis of many analogs.

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We now describe a method for identifying high-affinity ligands that can aid in the drug discovery process. The technique, which is called "SAR by NMR," is a linked-fragment approach wherein ligands are constructed from building blocks that have been optimized for binding to individual protein subsites (Fig. 1). In the first step of this process, a library of low molecular weight compounds (*1*) is screened to identify molecules that bind to the protein. Binding is determined by the observation of ^{15}N- or ^{1}H-amide chemical shift changes in two-dimensional ^{15}N-heteronuclear single-quantum correlation (^{15}N-HSQC) spectra (*2*) (Fig. 2) upon the addition of a ligand to

an ^{15}N-labeled protein. These spectra can be rapidly obtained, making it possible to screen a large number of compounds (*3*). Once a lead molecule is identified, analogs are screened to optimize binding to this site (Fig. 1, step 2). Next, a ligand is sought that interacts with a nearby site. Binding to a second pocket is determined by observing changes in a different set of amide chemical shifts in either the original screen or a screen conducted in the presence of the first fragment (Fig. 1, step 3). From an analysis of the chemical shift changes, the approximate location of the second ligand is identified. Optimization of the second ligand for binding to this site is then carried out by screening structurally related compounds (Fig. 1, step 4). When two "lead" fragments have been selected, their location and orientation in the ternary complex are determined experimentally either by NMR spectroscopy or by x-ray crystallography. Finally, on the basis of this structural information, compounds are synthesized in which the two fragments are linked together (Fig. 1, step 5) with the goal of producing a high-affinity ligand.

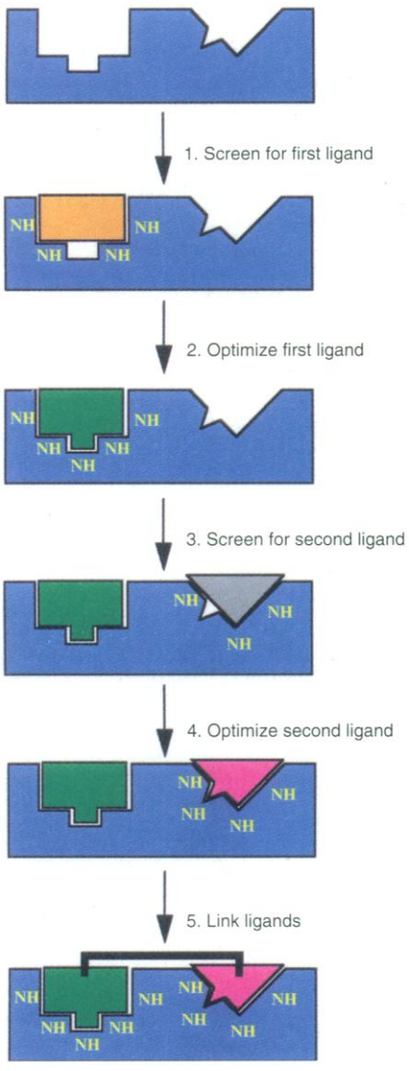
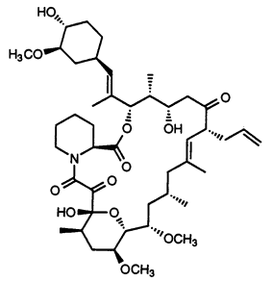


Fig. 1. An outline of the SAR by NMR method.

The SAR by NMR method is illustrated in the discovery of ligands that bind tightly to a protein that forms a complex with the potent immunosuppressant FK506 (**1**). This protein, which is called



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the FK506 binding protein (FKBP), inhibits calcineurin (a serine-threonine phosphatase) and blocks T cell activation (*4*) when it is complexed to FK506. Using NMR spectroscopy, we screened FKBP against our library of compounds (*5*). Many compounds were found that bound weakly to FKBP, including methyl-3-isoquinolinecarboxylate (K_d = 1.0 mM), 4-carbethoxy-3-methyl-2-cyclohexen-1-one (K_d = 0.5 mM), and 2-phenylimidazole (K_d = 0.2 mM). The trimethoxyphenyl pipecolinic acid derivative, **2**, showed the highest affinity for FKBP (K_d = 2.0 μM), which is consistent with earlier reports on the avidity of pipecolinic acid derivatives for FKBP (*6*). This compound was chosen for study without further optimization. The binding site for **2** is the same as that of the pipecolinic acid moiety of FK506 (*7*), as evidenced by the amide chemical shift changes of FKBP (Fig. 3).

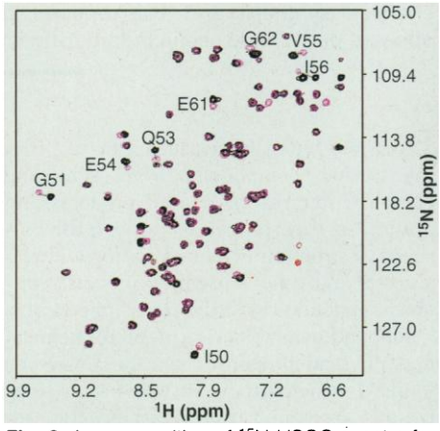

Fig. 2. A superposition of ^{15}N-HSQC spectra for FKBP in the absence (magenta contours) and presence (black contours) of compound **3**. Both spectra were acquired in the presence of saturating amounts of **2** (2.0 mM). Significant chemical shifts changes are observed for labeled residues.

To identify molecules that interact with FKBP at a nearby site (Fig. 1, step 3), we screened our library of compounds in the presence of saturating amounts of **2** (*8*). We found an initial candidate (**3**) that bound to FKBP with an affinity of 0.8 mM (Fig. 4) as measured from the changes in the ^{15}N-HSQC spectrum of uniformly ^{15}N-labeled FKBP upon binding **3** (Fig. 2). An analysis of these chemical shift changes indicated that the binding site for **3** is near the binding site for **2** (Fig. 3).

To probe the structural requirements for the binding of benzanilide derivatives to the second site (Fig. 1, step 4), we obtained analogs of 3 from our corporate sample collection and commercial sources and tested these compounds for binding to FKBP by NMR. From the structure-activity relationships (SAR) that are observed, it appears that both aromatic rings and the amide linkage are important for binding to FKBP, as evidenced by the decrease in affinity for analogs lacking these chemical moieties (*9*). The *para*-hydroxyl group (Fig. 4, R_1) on the aniline ring is also important for binding (**4**); whereas, the hydroxyl group on the benzoyl ring at R_2 does not contribute to the binding affinity (**5**). However, when R_3 (**6**) or R_4 (**7**) is a hydroxyl group, an increase in binding affinity is observed relative to the parent compound (**8**). Thus, both substituted *para*-hydroxyl substituents (R_1 and R_4) appear to contribute to the binding to FKBP. The benzanilide containing two *para* hydroxyls, **9**, was not commercially available

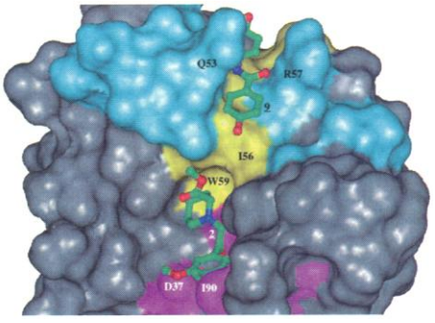

Fig. 3. A surface representation of FKBP showing the locations of **2** and **9**, as determined from ^{15}N-^{13}C-filtered NOE data (*11*). Residues that exhibited the largest chemical shift changes on the binding of **2**, **9**, or both **2** and **9** are colored in magenta, cyan, and yellow, respectively. Chemical shift changes for **9** (cyan and yellow) are those observed on the addition of **9** to FKBP in the presence of saturating amounts of **2** (2.0 mM). Weighted averaged chemical shifts were used ($\Delta\delta(^1H, ^{15}N) = |\Delta\delta(^1H)| + 0.2*|\Delta\delta(^{15}N)|$), and colored residues are those for which $\Delta\delta(^1H, ^{15}N)$ exceeded 0.15 and 0.05 ppm for **2** and **9**, respectively.

but was readily synthesized (*10*) and served as our best ligand for the second site (K_d = 100 μM).

A model of the ternary complex of **2**, **9**, and FKBP was generated on the basis of isotope-filtered NMR studies (Fig. 3) (*11*). In this model, the methyl ester of **2** is close to the hydroxyl group on the benzoyl ring of **9**. Thus, we designed linkers that would attach to these groups, span the distance between the two fragments, and have no steric clashes with the protein. Four compounds were synthesized (*12*) in which the pipecolinic acid moiety of **2** is connected to the hydroxyl group on the benzoyl ring of **9** with linkers of different lengths (**10** to **13**). In addition, a compound, **14**, in which the benzoyl ring of **9** is linked at a position *ortho* to the hydroxyl group was synthesized (*13*) to explore a different regiochemical linkage. The binding affinities of these five compounds for FKBP were measured in a fluorescence-based assay (*14*), and all of the compounds exhibit nanomolar affinities for binding to FKBP (Fig. 4).

To determine whether the linked compounds bind to the same site as the untethered compounds, we compared the intermolecular nuclear Overhauser effects (NOEs) observed in the NMR structure of the FKBP-**14** complex (*15*) (Fig. 5) to those observed in the ternary complex composed of **2**, **9**, and FKBP. The pipecolinic acid moieties and trimethoxyphenyl groups of **2** and **14** bind to the same FKBP binding site and form hydrophobic interactions with the protein (Fig. 5). In addition, the benzanilide moieties of **9** and **14** bind in similar locations in the linked and unlinked compounds (Fig. 5). However, several NOEs were observed between the benzoyl ring of **9** and Gln^{53}, Ile^{56}, and Arg^{57} which were not detected between the linked compound (**14**) and FKBP. This suggests that although the benzanilide moieties of **9** and **14** are in very similar locations in the two complexes, the introduction of the linker causes a small shift (approximately 1 to 2 Å) in the position of this group.

As demonstrated here, SAR by NMR is useful for discovering high-affinity ligands with experimentally derived information. Although the small untethered molecules might only bind in the micromolar to millimolar range to the target protein, the binding affinity of a linked compound is, in principle, the product of the binding constants of the individual fragments plus a term that accounts for the changes in binding affinity that are due to linking (*16*). Thus, molecules with submicromolar affinity can be obtained by linking two compounds that bind in the millimolar range. In the application that we describe, a compound that binds to FKBP with a K_d = 19 nM was synthesized by linking two molecules with binding affinities of 2 μM and 100 μM. These two fragments were rapidly identified and optimized (less than 2 months), and only five linked molecules were synthesized, all of which exhibit nanomolar affinities for FKBP.

One of the advantages of SAR by NMR is the use of ^{15}N-HSQC spectra to detect the binding of small, weakly bound ligands to an ^{15}N-labeled target protein. Because of the ^{15}N spectral editing, no signal from the ligand is observed. Thus, binding can be detected even at high compound concentrations. This is an important advantage over conventional screening assays, such as fluorimetric or colorimetric assays, where high compound concentrations can give rise to large background signals. Another advantage of the use of ^{15}N-HSQC spectra is the ability to rapidly determine the different binding site locations of the fragments, which is critical for interpreting structure-activity relationships and for guiding the synthesis of linked compounds.

A number of linked-fragment based approaches that utilize molecular modeling have been described (*17*). However, computer-based methods have difficulties in reliably predicting which fragments will interact with the protein and the manner in which they bind. This kind of task is challenging because of (i) possible conformational changes of the protein and ligand when complexes are formed, (ii) the difficulties in treating bound water and counter ions, (iii) the neglect of entropy, and (iv) the use of relatively simple force fields (*17*). To circumvent these limitations, the SAR by NMR method uses experimentally derived information to guide fragment selection, optimization, and linking. An experimental approach for mapping the binding surface of proteins has been described (*18*), which in-

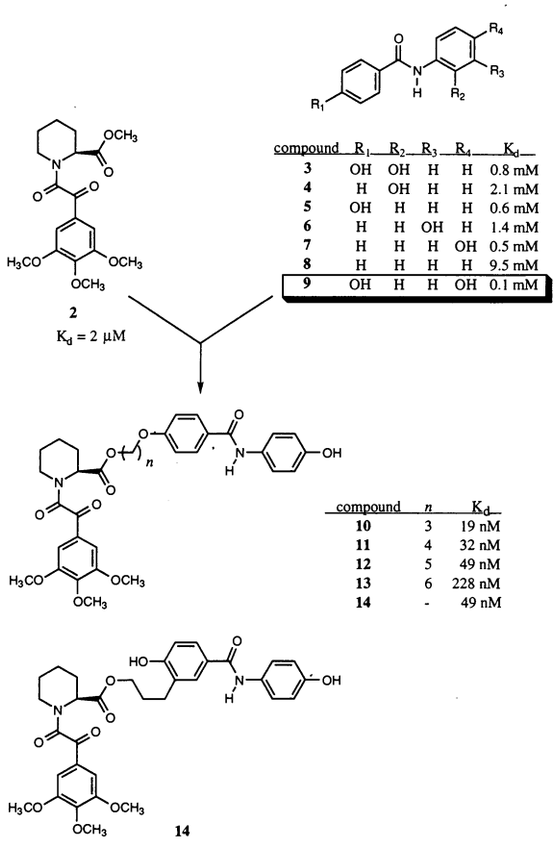

Fig. 4. Summary of the SAR by NMR method as applied to FKBP. Compounds with affinities from 19 to 228 nM were obtained from two fragment leads with affinities of 2 and 100 μM.

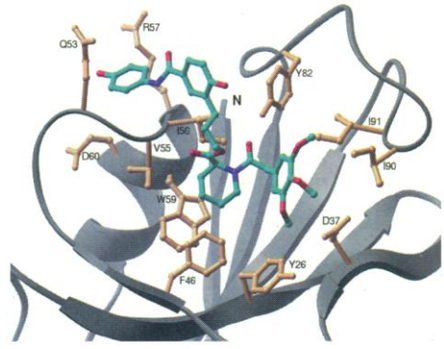

Fig. 5. Ribbon (*23*) depiction of the structure of FKBP (gray) when complexed to 14 (green carbon atoms). Shown in yellow are those residues that have NOEs to the ligand.

volves solving multiple x-ray crystal structures of a protein in different solvents. With this method, however, only organic solvents are examined as potential ligands, and the binding affinities are not measured.

The SAR by NMR method is conceptually similar to combinatorial chemistry (*19*) in that both techniques utilize a building block approach in the construction of molecules. However, in practice, the two are quite different. In combinatorial chemistry, ligand discovery requires the preparation and testing of a large number of linked compounds. Unfortunately, the development of synthetic protocols and assays to identify active compounds is frequently difficult and time-consuming. Furthermore, the need for relatively uniform and straightforward coupling conditions limits the range of useful molecular building blocks and compound diversity. In contrast, when SAR by NMR is used, only a few compounds need to be synthesized because the untethered ligands are optimized prior to linking. In principle, the linked compounds are selected from a large virtual library composed of all combinations of the fragments. For example, for a protein with two independent binding sites, a compound collection containing 10^4 fragments represents a virtual library of more than 10^8 members (*20*). Actual chemistry, however, is highly focused on linking only those molecules that have been shown to bind to the protein and is guided by structural information on their relative locations and orientations. Furthermore, unlike combinatorial chemistry, the range of small molecules in the screening library in SAR by NMR is limited only by the requirement for aqueous solubility at millimolar concentrations, resulting in a diverse sample collection of compounds.

Although the SAR by NMR method is only applicable to small biomolecules (MW < 30 kD) that can be obtained in large quantities (≥200 mg) (*21*), many small proteins or protein domains fit these criteria and may serve as drug targets (*22*). When applied to these proteins, SAR by NMR can play an important role in drug discovery. Indeed, preliminary use of this technique with other proteins indicates that the method will be applicable to a variety of protein targets and an extremely valuable tool in drug research.

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3. A two-dimensional (2D) HSQC spectrum of a uniformly ^{15}N-labeled protein can be acquired in about 10 minutes on a 500-MHz NMR spectrometer, allowing 100 2D spectra to be recorded in 1 day with an automatic sample changer. If compounds are tested in mixtures of 10 (as in our strategy), it is possible to robotically screen 1000 compounds per day.
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5. At the time of this screening our library consisted of approximately 1000 compounds.
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8. At the time of this screening, our library consisted of approximately 1600 compounds.
9. The binding of benzamide, *N,N*-dimethylbenzamide, 4-hydroxyacetanilide, and 4-hydroxyphenylbenzoate to FKBP was negligible at compound concentrations up to 10 mM.
10. *p*-Anisoyl chloride was treated with *p*-anisidine, and the methyl groups were removed with boron tribromide.
11. The NMR samples of the ternary complex were composed of uniformly ^{15}N-, ^{13}C-labeled FKBP (2.0 mM), **2** (2.0 mM), and **9** (5.0 mM) in a D_2O or a mixture of H_2O and D_2O (9 to 1) phosphate buffered solution (50 mM, pH 6.5) containing 100 mM NaCl, and 0.05% sodium azide. The ^1H, ^{13}C, and ^{15}N backbone and side-chain resonances of FKBP in the complex were assigned from an analysis of several three-dimensional (3D) ^{15}N- and ^{13}C-edited NMR experiments [G. M. Clore and A. M. Gronenborn, *Methods Enzymol.* **239**, 349 (1994)]. A total of 17 intermolecular restraints were used to dock **9** to the known structure of FKBP (*7*). Compound **2** was placed in a location similar to that observed in the ascomycin complex, which was consistent with the chemical shift changes observed on binding of **2** (Fig. 3).
12. Methyl-4-hydroxybenzoate was treated with base and each of four haloalkyl acetates [$X(CH_2)_nOAc$, n = 3 to 6], and the products were hydrolyzed with aqueous NaOH. The resulting benzoic acid derivatives were coupled with *p*-(*t* -butyldimethylsilyloxy)aniline. The primary alcohols were coupled with *N*-FMOC-L-pipecolinic acid, the FMOC group was removed with piperidine, and the resulting amines were treated with 3,4,5-trimethoxybenzoylformyl chloride. The silyl groups were then removed with tetrabutylammonium fluoride to provide the desired compounds **10** to **13**.
13. Methyl-4-hydroxybenzoate was treated with base and allyl iodide, and the methyl ester was hydrolyzed with aqueous NaOH. The resulting benzoic acid derivative was coupled with *p*-(*t* -butyldimethylsilyloxy)aniline, and the allyl ether was then converted to the corresponding allyl phenol via Claisen rearrangement. The resulting hydroxyl group was protected with *t* -butyldimethylsilyl chloride, and the terminal alkene was converted to the desired primary alcohol by hydroboration and oxidation. This alcohol was converted to the desired compound **14** as described for **10** to **13** (*12*).
14. Fluorescence measurements were performed on a SPEX Fluorolog 2-1-2 instrument. Titrations were carried out by following the decrease in intrinsic fluorescence of FKBP on sequential addition of the compounds as perdeuterated DMSO stock solutions. DMSO concentrations did not exceed 1% of the total volume.
15. The NMR samples of the binary complex consisted of uniformly ^{15}N-, ^{13}C-labeled FKBP (2.0 mM) and **14** (2.0 mM) in a D_2O or in a mixture (9:1) of H_2O and D_2O phosphate buffered solution (50 mM, pH 6.5) containing 100 mM NaCl and 0.05% sodium azide. The ^1H, ^{13}C, and ^{15}N backbone and side-chain resonances of FKBP in the complex were assigned by standard methods [G. M. Clore and A. M. Gronenborn, *Methods. Enzymol.* **239**, 349 (1994)]. Intra- and intermolecular distance restraints were obtained from ^{13}C- and ^{15}N-resolved NOESY spectra and 2D ^{15}N-^{13}C-filtered NOESY spectra. Distance restraints were classified into three categories on the basis of the NOE cross-peak intensity and given a lower bound of 1.8 Å and upper bounds of 3.0 Å, 4.0 Å, and 5.0 Å. Hydrogen bonds, identified for slowly exchanging amides, were each defined by two restraints: 1.8 to 2.3 Å for the H-O distance and 2.8 to 3.3 Å for the N-O distance. Structures were calculated with the X-PLOR 3.1 program [A. T. Brünger, *X-PLOR 3.1 Manual* (Yale Univ. Press, New Haven, CT, 1992)] on Silicon Graphics computers with a hybrid distance geometry-simulated annealing approach [M. Nilges *et al.*, *FEBS Lett.* **229**, 317 (1988)]. A total of 877 approximate interproton distance restraints were derived from the data, which included 34 hydrogen bonds, 63 intermolecular distance restraints, and 19 intra-ligand distance restraints. After simulated annealing, 21 structures were obtained with good covalent geometry, no distance violations greater than 0.2 Å, and an overall root-mean-square deviation (rmsd) to the averaged structure of 1.0 Å for backbone atoms and 1.7 Å for all heavy atoms. Final energies (E) for the averaged, minimized structure were E_{tot} = 192.9, E_{bond} = 15.6, E_{ang} = 124.7, E_{impr} = 19.4, E_{vdw} = 22.8, and E_{noe} = 10.3 kcal/mol.
16. In principle, the binding energy for the linked compound (ΔG_{AB}) should be the sum of the intrinsic binding energies for the unlinked compounds (ΔG_A and ΔG_B) plus a term to account for the linking of the two fragments (ΔG_{link}): $\Delta G_{AB} = \Delta G_A + \Delta G_B + \Delta G_{link}$ [W. P. Jencks, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4046 (1981)]. In terms of binding affinities, this translates to $K_d(AB) = K_d(A)^*K_d(B)^*L$, where L is the linking coefficient derived from ΔG_{link}. For example, for two compounds with $K_d(A) = 10^{-3}$ M^{-1} and $K_d(B) = 10^{-3}$ M^{-1} and L less than 1.0, the expected affinity for the linked compound would be $K_d(AB) < 10^{-6}$ M^{-1}.
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20. In general, the number of potential linked ligands in a virtual library is n^*D^w, where D is the number of fragments contained in the database, w is the number of adjacent, independent binding sites in the protein, and n is the number of linkers that could be used to join the fragments.
21. To reduce the amount of protein required by the technique, the protein is recycled by removing the ligands with dialysis after the NMR experiment.
22. The SAR by NMR method is even applicable to proteases, provided that the first ligand inhibits the enzyme.
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