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Applications of NMR to structure-based drug design in structural genomics

Robert Powers

Department of Biological Chemistry, Wyeth Research, 87 Cambridge Park Dr., Cambridge, Massachusetts, 02140, U.S.A; tel: (617)-665-7997; fax: (617)-665-8993; e-mail: powersr@war.wyeth.com

Abstract

Structural genomics is poised to have a tremendous impact on traditional structure-based drug design programs. As a result, there is a growing need to obtain rapid structural information in a reliable form that is amenable to rational drug design. In this manner, NMR has been expanding and evolving its role in aiding the design process. A variety of NMR methodologies that cover a range of inherent resolution are described in the context of structure-based drug design in the era of structural genomics.

Introduction

Structure-based drug design has established itself as a fundamental and essential approach in most drug-development programs based on its continuing success in delivering novel drugs to clinical trials [1–8]. The design of drugs using structural information is an iterative procedure where each pass of the design cycle requires obtaining a new structure for the lead-compound(s) complexed with the protein of interest [6, 9–11]. This process is challenging in itself when applied to a single protein target but, in the realm of structural genomics, structure-based drug design becomes a formidable task with the definite possibility of being overwhelmed by the sheer magnitude of available structures [12]. Nevertheless, NMR [13, 14], X-ray [15, 16] and high-throughput screening (HTS) [17–22] are rising to the challenge of integrating structural genomics into a structure-based drug design program. In the ongoing evolution of structural genomics, NMR, X-ray and HTS will all continue to play critical and complementary roles.

Initiating the structure-based drug design cycle has traditionally depended on HTS for providing first-generation lead-compounds to obtain co-structures with the protein target of interest. Similarly, X-ray crystallography has historically been a major source for obtaining three-dimensional structures of protein–ligand complexes for the iterative drug design cycle [23]. The use of NMR for the structure elucidation of protein-ligand complexes is a relatively recent addition to the structure-based drug design approach [24] where a number of barriers exist that have sometimes limited its application. Utilizing current state-of-the-art methodologies, X-ray crystallography is generally more efficient in solving protein structures than NMR, where NMR is also limited to relatively low-molecular-weight proteins. Specifically, NMR requires extensive isotope labeling of the protein and may take six months to a year using standard methodology to determine a high-resolution structure for proteins <40 kDa. Conversely, X-ray crystallography routinely solves protein–ligand structures in weeks to months and in some cases as fast as a few days.

In spite of these limitations, the role of NMR in structure-based drug programs is continually expanding, where NMR is routinely being adapted to complement inherent limitations in X-ray crystallography and HTS. Specifically, NMR is routinely being used to identify and evaluate chemical leads [24–28]. One

reason for this development arises from the fact that results from HTS assays do not provide mechanistic information. The data obtained from an HTS screen does not readily establish if the observed activity results from the compound actually binding and inhibiting the protein target of interest. It is equally likely that the inactivation observed in an HTS screen is through another component of the assay or by some other physical means such as precipitation. Furthermore, the efficient and potentially rapid structure determination by X-ray is dependent on the prior identification of well-behaved stoichiometric binders.

Recent advances in probe technology [27], software development [29] and NMR methodology [14, 30–32] show exciting promise in significantly reducing the time requirement to determine a protein structure by NMR while simultaneously increasing the molecular-weight range of proteins amenable to NMR. Nevertheless, the present application of current NMR methodology in novel ways provides a plethora of information that is beneficial to the structure-design process. Based on the current state of NMR technology, a role for NMR in structural genomics may be described through a series of 'low resolution' alternatives in combination with traditional high-resolution solution structure determination (Figure 1).

NMR Screening

An unfortunate reality of screening large libraries of compounds in traditional HTS assays is that many of the identified 'hits' exhibit undesirable mechanisms of activity or poor physical properties. These poor properties of the compound may include insolubility, impurities, aggregation, instability and non-specific binding. As a result, depending on the nature of the assay and the protein target, only a small percentage of the identified hits actually bind the protein target of interest in a desirable manner. From experience, there is a high-correlation between 'good' behavior by a small-molecular-weight compound in the NMR and future success in obtaining a co-structure. 'Good' behavior is characterized by compounds that exhibit stoichiometric binding to the target protein without solubility or aggregation issues and/or any observable detrimental impact on the protein itself (precipitation, denaturation, etc.). The 1D NMR structure of the free compound readily indicates the relative aqueous solubility and stability of the compound, the compound's tendency to form high-molecular weight aggregates or micelle-like structures and, in addition, the accuracy of the structure.

Similar, a variety of NMR techniques may be utilized to verify binding, stoichiometry, and identification of the binding site on the protein [26, 27]. These include the 'SAR by NMR' [25] and SHAPES [33] approaches of screening libraries using NMR. 'SAR by NMR' identifies binders from chemical shift perturbations in 2D ^{1}H-^{15}N HSQC spectra. By mapping the chemical shift changes on the surface of the protein, the binding site may be identified which permits chemically linking compounds that bind proximal to each other. The SHAPES methodology utilizes a small-diverse library (<200) derived from known drug structures where binding to a target protein is identified from 1D-line-broadening or 2D-transfer NOEs experiments. Positive 'hits' are used as starting

◆ **Screen for Small Molecules that Bind the Protein Target**
  – MS/NMR Assay
  – 1D NMR (STD, tr-NOE, line-width)
◆ **Homology Models Utilizing NMR Structural Information**
  – Secondary structure identification.
◆ **NMR Structures Based on Minimal Restraints**
  – Deuterium labeled proteins.
  – Residual dipolar coupling constants.
◆ **Protein-Ligand Complexes**
  – free protein restraints & protein-ligand NOEs
◆ **High-Resolution Structures**
  – information on function and mechanistic details.
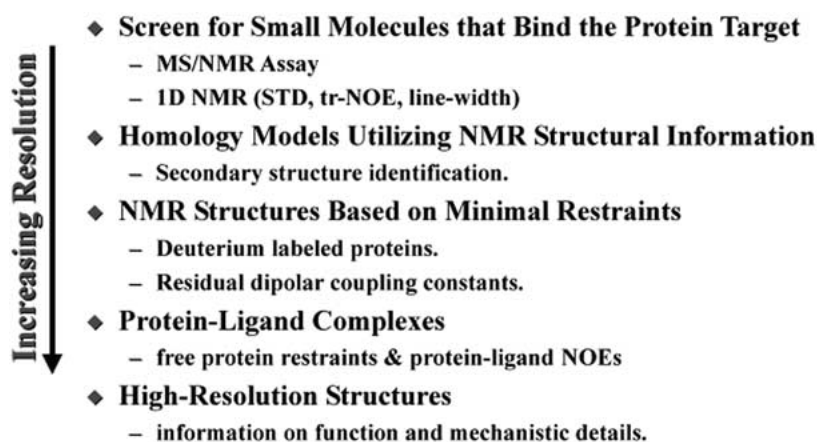
Increasing Resolution

Figure 1 Current application of low-resolution NMR methodologies in a structure-based drug design program that are applicable to structural genomics.

points for ligand design by using the compounds as the basis for structure-based searches of compound databases, directing the design of compound libraries for HTS assays or for guiding the synthesis of combinatorial libraries.

In general, initial chemical leads tend to be weak binders where the affinity is optimized through combined application of modeling, medicinal chemistry and structural information. The upper K_D limit ($\sim \mu$mol/L) for weak binders identified in standard HTS assays is usually defined by practical issues that affect protein and compound concentrations (solubility, availability, activity, etc.). Conversely, the NMR screening methodologies are equally suited for identifying both weak and tight binders ($K_D \sim$ nmol/L to mmol/L), depending on the nature of the experiment. NMR-based screens may readily identify very weak binders that may be missed in traditional HTS assays. Thus, NMR analysis provides critical information to evaluate the utility of the compound prior to initiating a structural effort (Figure 2).

Given the beneficial and versatile utility of NMR to evaluate chemical leads, NMR would appear to be a useful alternative to standard HTS techniques to screen small molecules for their ability to bind protein targets of interest [24–27]. Unfortunately, there are a number of disadvantages associated with a purely NMR-based screening approach. Since NMR is a relatively insensitive analytical technique, large quantities of protein (100 mg to >1 g) are required for a typical screen. Additionally, the NMR experiments used in a screening effort generally require long acquisition times (>10 min) and isotope enrichment of the protein (^{2}H, ^{13}C, ^{15}N).

Concurrent with NMR screening efforts, a number of methods using only mass spectrometric detection have been proposed for the screening of drug candidates by evaluating non-covalent complexes between the ligand and a targeted protein [34–36]. Mass spectrometry (MS)-based assays have some advantages compared with the NMR screening approaches, particularly in the significant increase in sensitivity (femtomoles) and speed ($\sim$ 1 min/sample). Also, MS screens eliminate the need to deconvolute since the MW of the compound can be used as a molecular tag for identification of 'hits'. Conversely, there are significant disadvantages with MS-based assays relative to the NMR methods. The main disadvantage of MS screens is the inability of the method to discriminate between specific and non-specific binding of the drug to the targeted protein. Also, because of the increase

in sensitivity, MS screen is more likely to identify weak non-specific binders. Additionally, screening by MS does not provide any direct information regarding the binding site of the ligand or the structure of the protein : ligand complex, which are fundamental strengths of NMR-based screens.

Based on the observation that NMR and MS screening efforts are complementary to each other, an MS/NMR screen was developed and previously described that takes advantage of and combines the inherent strengths of size-exclusion gel chromatography, mass spectrometry and NMR to identify bound complexes in a relatively universal high-throughput screening approach [28]. A diagram of the MS/NMR screen utilizing MMP-1 binding data is depicted in Figure 3. Briefly, size-exclusion gel chromatography is used to separate successful protein–ligand complexes from compounds that don't bind the protein target. This step of the assay may utilize mixtures where unique molecular weights for each compound in the mixture can be used as a molecular tag for deconvolution. Mass spectrometry is then used as a detector to analyze the eluent from the size-exclusion gel chromatography for the presence of a MW corresponding to a small molecule in the mixture. The sensitivity of the approach is probably dependent on the off-rate instead of the K_D since the gel-filtration size exclusion chromatograph occurs under non-equilibrium conditions and favors dissociation of the protein-ligand complex [37, 38]. Also, other factors such as concentration and loading volume on the column will affect the detection limit. Empirically, a good signal for binders with IC_{50}s in the ~ 100 μmol/L range was observed, suggesting that a 200 μmol/L binder should be detectable. Conversely, a compound with an IC_{50} of ~ 1 mmol/L was not detected, which suggests that the upper limit of detection is probably in the range of >200 μmol/L to 1 mmol/L.

Positive 'hits' from the MS stage are then further analyzed for binding by the presence of chemical-shift perturbations in a 2D ^{1}H-^{15}N HSQC NMR spectrum. Compounds that exhibit chemical shift changes that cluster in a biologically relevant region of the protein's surface are then used to determine a co-structure with the protein target. The MS/NMR assay was successfully used to screen $\sim$ 32,000 compounds against RGS4 as the target protein where a compound was identified that exhibited specific binding to RGS4 and inhibited the RGS4-Gα interaction [28].
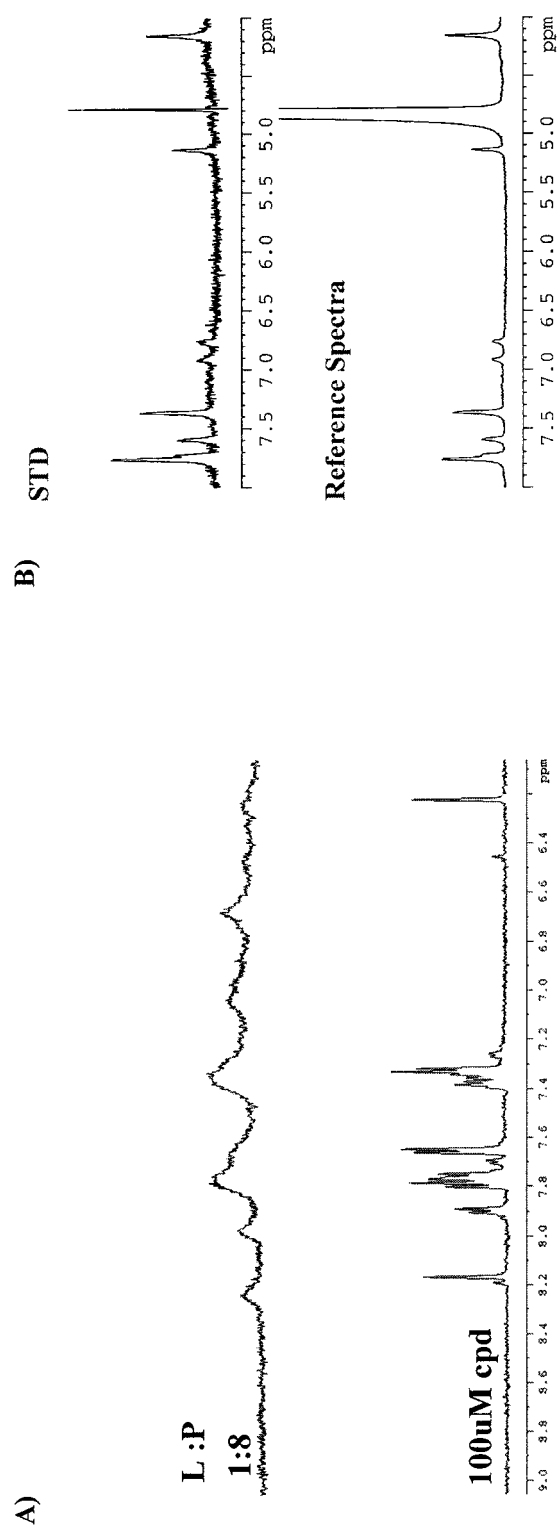
Figure 2 Examples of the utility of NMR to evaluate potential lead compounds. **(A) Bottom:** Reference 1D NMR spectra of free compound. **Top:** 1D NMR spectra of the compound in an 8 : 1 ratio to protein target. Extensive broadening exhibited by the compound in the presence of a small amount of protein is indicative of non-specific binding. **(B) Bottom:** Reference 1D NMR spectra of free compound. **Top:** 1D saturation transfer difference (STD) of compound in the presence of a protein target. The positive STD indicates that the compound binds the protein.
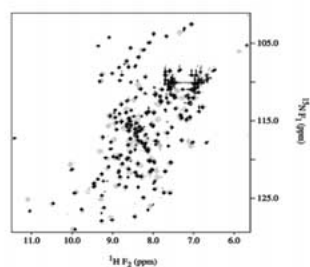
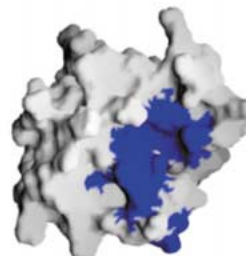Compound mixtures + protein passed through gel-filtration column
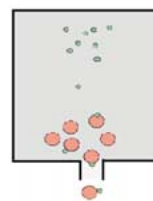
Mass Spectroscopy
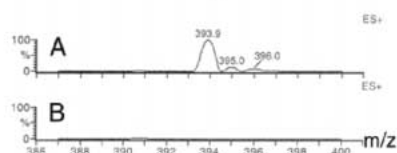
NMR chemical shift perturbations (2D ^{1}H-^{15}N HSQC)

Mapping of chemical shift perturbations on protein surface

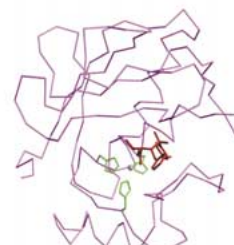High-resolution NMR structure of protein-ligand complex

Figure 3 Pictorial flow diagram of MS/NMR assay using data from the MMP-1 binding assay. (From reference [28] Copyright 2001 by the American Chemical Society.)

Homology models based on NMR data

A major proposal of ongoing structural genomic programs is to limit the high-resolution structure determination effort to novel protein folds and to utilize homology modeling to determine the structures of the remaining proteins [39, 40]. In traditional homology models based on sequence alignment, the accuracy of the resulting homology model is strongly dependent on the sequence identity between the reference and target protein sequence. Typically, when the sequence identity falls below 30%, the homology models become less reliable where the atomic rms difference between the homology model and experimental structure may exceed 3 Å [41, 42]. Since a number of structural homologs have sequence identity significantly below 30%, standard homology modeling efforts may be limited. NMR may play a key role in this endeavor by incorporating readily obtainable experimental data in the homology modeling process [43, 44].

The secondary-structure elements present in a protein are generally identified rapidly by NMR as part of a standard sequence assignment procedure [45]. The regular secondary structure elements are identified from a qualitative analysis of sequential and inter-strand NOEs, NH exchange rates, $^3J_{HN\alpha}$ coupling constants and the $^{13}C\alpha$ and $^{13}C\beta$ secondary chemical shifts [46, 47]. The resulting secondary structure can then be used to generate a sequence alignment based on a correlation between the secondary structure elements between the target and reference proteins. Effectively, the NMR data permits a sequence alignment based on a higher information content then the primary sequence alone. Additional information, such as the location of cysteins involved in disulfide bonds and highly conserved residues can be used to aid the alignment. The application of NMR-derived structural information was used to determine a homology model for Oncostatin M (Figure 4) [48]. Combining mutational data with the Oncostatin M homology model generated a reasonable representation of the receptor-binding surface consistent with other interleukins and growth factors. This information provided support for the reliability of the Oncostatin M homology model. An additional utility of a structure-based sequence alignment was illustrated in the functional analysis of IL-13 [49] where a model of IL-13 complexed to the IL-4α receptor was generated from the IL-13 NMR structure and the IL-4 : IL-4α receptor X-ray structure. Furthermore, the generation of a homology model based on initial NMR information would provide a convenient structure as a starting point for the complete analysis of the NOE structural information and determination of a high-resolution structure.

NMR structures based on minimal restraints

Currently, the most time-consuming aspect of determining a protein structure by NMR is the manual interpretation and assignments of thousands of NOE-based distance restraints. It is well established that the relative precision and accuracy of a protein structure determined by NMR methodology is inherently dependent on the number and accuracy of the restraints used to determine the structure [50]. Clearly indicating that the thorough analysis of the NOESY experimental data is a crucial component for obtaining a high-quality NMR structure. The analysis of NOE data is an iterative process where an initial structure is used as a distance filter to aid in deciphering ambiguous NOE assignments. Obtaining a reliable initial structure is critical to the entire process where a high-quality homology model (see above) would expedite this process. A desirable alternative would be to determine an NMR structure of a protein amenable for a structure-based drug design program while avoiding the tedious analysis of NOESY data. Towards this end, a number of software approaches have been developed to expedite the NMR structure determination process, including ARIA [51], Auto-Structure [52], NOAH/DIAMOD [53, 54], and RossettaNMR [55, 56]

Recently, novel approaches have been established that provide a means to obtain structural information independent of traditional NOEs. Most notable is the measurement of residual dipolar couplings in partially oriented proteins dissolved in lipid bicelle solution [32]. Recent efforts have demonstrated that it is possible to reproduce the general fold of a protein using minimal NOE restraints and residual dipolar couplings where an rmsd of <1 Å between the structures calculated with the complete data set and a minimal number of restraints is obtainable [57, 58]. Based on this success, a number of software programs have been developed in attempts to determine protein structures based solely on residual dipolar coupling constants [44, 56, 59–61].

The prior analyses of the utility of residual dipolar coupling constants in structure determinations were
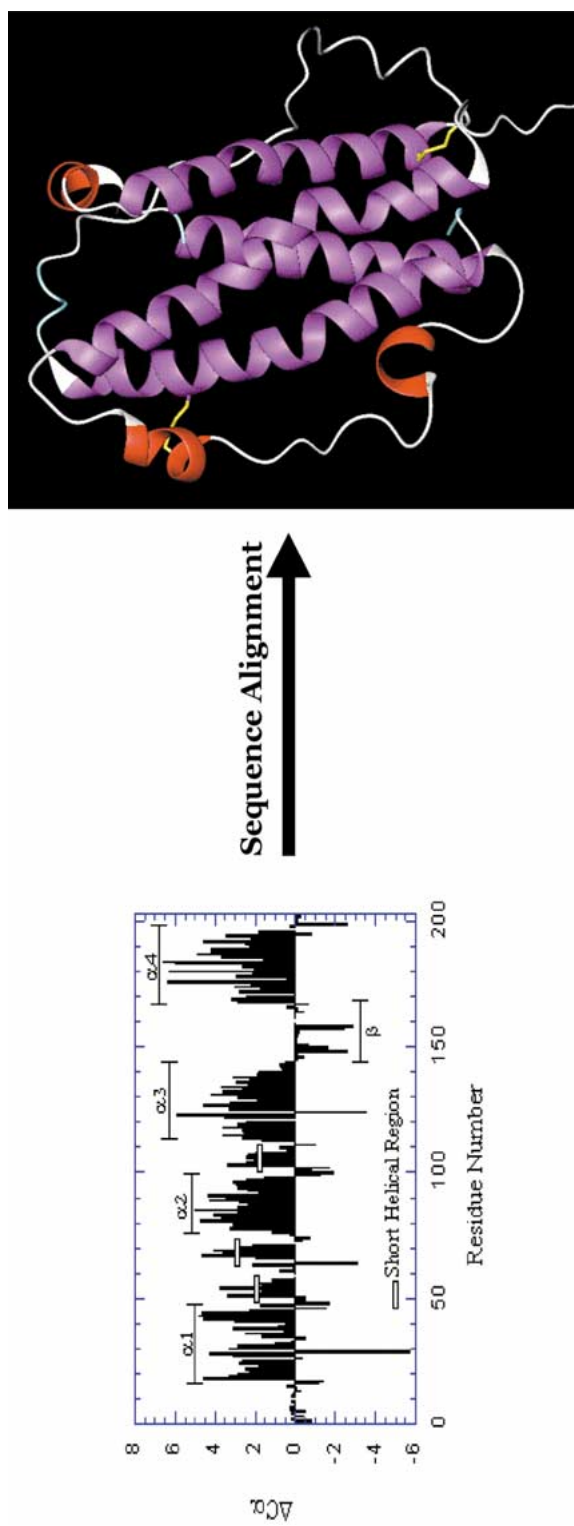
Figure 4 Illustration of a homology model based on NMR data. The sequence-specific secondary structure assignments based on carbon chemical shifts and the homology model of Oncostatin M. (From reference [48].)
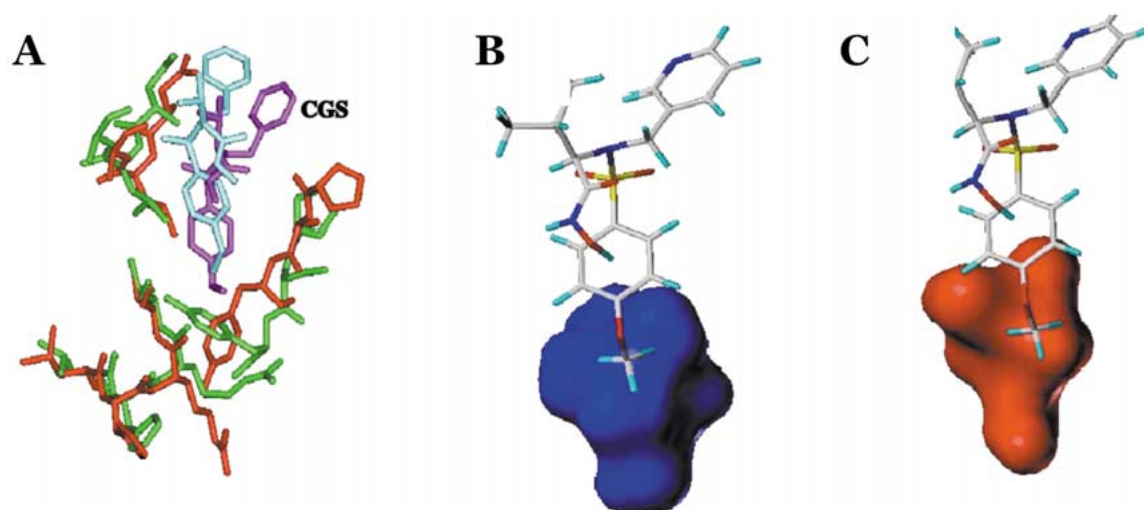
Figure 5 Reproduction of the overall structure and active-site of MMP-1 complexed with CGS-27023A using minimal NOE restraints. (From reference [62].) **(A)** Best-fit superposition of the backbone of MMP-1 structure for residues in active site together with CGS-27023A. The active residues displayed are 80–82, 112–115, and 138–140 that play a critical role in MMP-1 activity. MMP-1 : CGS-27023A complex calculated with a complete set of restraints is colored green and the structure based on the minimal restraint set is colored red with CGS-27023A (magenta) docked in. The S1′ pocket of MMP-1 calculated from a minimal set (**B**) and a complete set (**C**) of restraints for MMP-1 with CGS-27023A docked in for comparison.

expanded to evaluate the utility of a protein structure determined from minimal restraints in a structure-based drug design program. Particularly, would the active site of a protein be reasonably reproduced in a structure determined from minimal restraints such that it would be useable in a drug design effort [62]? The overall structure and active site of the MMP-1 : CGS-27023A complex was analyzed using a minimal set of NOEs (NH, methyl and aromatic), residual dipolar coupling constants and other readily obtainable restraints (chemical shifts, H-bonds, J-coupling). Comparison of the MMP-1 structure calculated with the minimal restraint set with the structure based on the complete data set reveals similar structures that maintain the fundamental characteristics of the active site. The rmsd for the active-site backbone atoms was 0.67 Å where the overall shape and size of the S1′ pocket were consistent (Figure 5). More importantly, the binding interaction of CGS-27023A in the structure based on minimal restraints is strikingly similar to the structure based on the complete data set. This analysis indicates that, in lieu of a high-resolution structure, an NMR structure based on minimal restraints would be a viable starting point to initiate a drug design process.

Protein–ligand complexes

A fundamental component of the structure-based drug design protocol is the iterative structure determination process. As each new lead candidate is identified, a new complex structure is required. Clearly this aspect of the process is critically dependent on a very rapid determination of the protein–ligand complex, where a turn-around time of days to weeks is typically required to support the drug design effort. This rapid need for protein–ligand complexes precludes solving a high-resolution structure by standard NMR methodology. In lieu of a high-resolution structure, a structure of a protein–ligand complex can be obtained by augmenting a complete restraint list that defines a protein structure with intermolecular NOEs obtained between the protein and the new ligand [63, 64]. As an example, the solution structure of CL-82198 complexed to MMP-13 was determined using the structural restraints from the MMP-13 : WAY-151693 NMR structure [65] appended with the intra- and inter-molecular NOEs for CL-82198 complexed to MMP-13 obtained from 2D-^{12}C,^{12}C-filtered-NOESY and 3D-^{13}C-filtered/^{13}C-edited-NOESY spectra. The original structural restraints for the MMP-13 : WAY-151693 complex was edited to remove the WAY-151693 restraints and MMP-13 intra-molecular NOEs that were inconsistent with the new MMP-
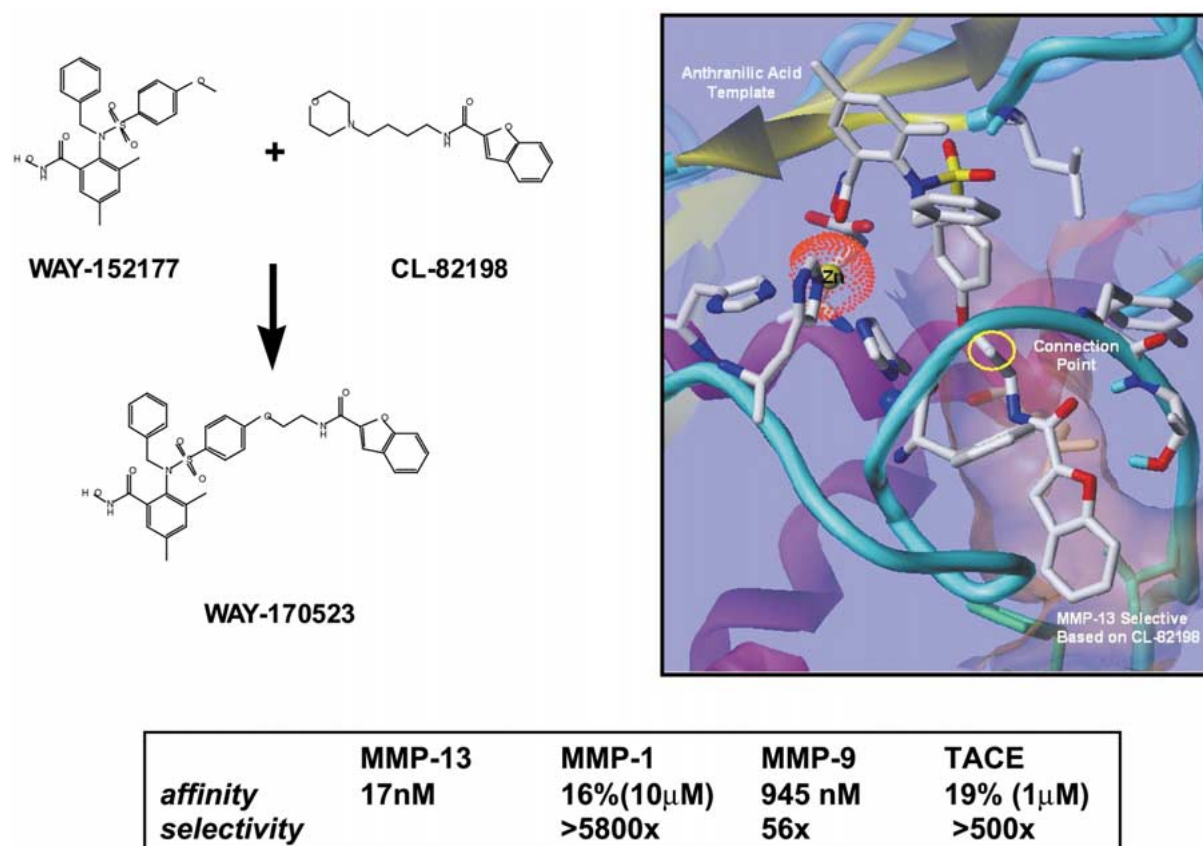
	MMP-13	MMP-1	MMP-9	TACE
affinity	17nM	16%(10μM)	945 nM	19% (1μM)
selectivity		>5800x	56x	>500x

Figure 6 Rapid determination of a protein–ligand complex by NMR by appending a complete restraint data set with ligand specific intra- and inter-molecular restraints (from [64]). (A) Design scheme showing the flow from CL-82198 and WAY-159062 to WAY-170523. (B) Expanded view of the NMR MMP-13 : CL-82198 complex overlayed with the MMP-13 : WAY-152177 model demonstrating approach to forming the hybrid inhibitor WAY-170523 where the MMP-13 active site is shown as a grid surface with CL-82198 and WAY-152177 shown as liquorice bonds. View is looking at the S1′ pocket. The table lists the observed IC_{50} and selectivity for the hybrid compound.

13 : CL-82198 complex. Essentially, the new intra- and inter-molecular NOEs for CL-82198 are given precedence in defining the structure of the active site for MMP-13. The utility of the MMP-13 : CL-82198 structure was verified by the success of designing a potent and selective inhibitor of MMP-13 (Figure 6). Thus, it is routinely feasible to determine a protein–ligand complex structure by NMR in a few weeks and with the implementation of cyroprobes this time-period may be reduced to a few days [27].

Conclusions

Using currently available NMR methodology, it is routinely feasible to provide rapid structural information that will play critical roles in a structure-based drug-design effort. With the evolving integration of structural genomics into traditional structure-based drug design programs, swift structure determination becomes an essential requirement. The NMR approaches demonstrated run the full gambit of complexity and cover a range of resolution to address a number of key issues that are encountered in a drug design program. NMR is now routinely used to evaluate and identify lead-compounds prior to initiating a structural effort. Rapidly obtained NMR data may be applied to improve the quality of a homology model as part of the structural genomics paradigm. Additionally, NMR structures that can be quickly determined from a minimal number of restraints are amenable to structure-based design efforts. Furthermore, the process of generating iterative protein–ligand complex structures for the design cycle can be accomplished swiftly by appending complete NMR restraint information that describes a protein structure with the

specific ligand-based intra- and inter-molecular restraints. Finally, with recent advances in probe technology, software development and NMR methodology the time commitment for solving a high-resolution structure by NMR will continue to decrease and the molecular-weight limitations for proteins amenable for NMR will continue to increase.

Acknowledgements

References

1. Morris, P. E., Jr. and Omura, G. A. (2000) *Curr. Pharm. Des.* **6,** 943–959.

2. Maignan, S. and Mikol, V. (2001) *Curr. Top. Med. Chem.* **1,** 161–174.

3. Craik, D. J. and Scanlon, M. J. (2000) *Annu. Rep. NMR Spectrosc* **42,** 115–174.

4. Colacino, J. M., Staschke, K. A. and Laver, W. G. (1999) *Antiviral Chem. Chemother.* **10,** 155–185.

5. Gubernator, K. and Boehm, H. J. (1998) *Methods Princ. Med. Chem* **6,** 15–36.

6. Kubinyi, H. (1998) *Curr. Opin. Drug Discovery Dev.* **1,** 4–15.

7. Mihelich, E. D. and Schevitz, R. W. (1999) *Biochim. Biophys. Acta* **1441,** 223–228.

8. Sintchak, M. D. and Nimmesgern, E. (2000) *Immunopharmacology* **47,** 163–184.

9. Blundell, T. L. (1996) *Nature* **384,** Suppl.), 23–26.

10. Babine, R. E. and Bender, S. L. (1997) *Chem. Rev.* **97,** 1359–1472.

11. Bohacek, R. S., McMartin, C. and Guida, W. C. (1996) *Med. Res. Rev.* **16,** 3–50.

12. Burley, S. K. (2000) *Nat. Struct. Biol.* **7,** 932–934.

13. Montelione, G. T., Zheng, D., Huang, Y. J., Gunsalus, K. C. and Szyperski, T. (2000) *Nat. Struct. Biol.* **7,** 982–985.

14. Prestegard, J. H. (2001) *Polym. Prepr.* **42,** 60.

15. Lamzin, V. S. and Perrakis, A. (2000) *Nat. Struct. Biol.* **7,** 978–981.

16. Abola, E., Kuhn, P., Earnest, T. and Stevens, R. C. (2000) *Nat. Struct. Biol.* **7,** 973–977.

17. Armstrong, J. W. (1999) *Am. Biotechnol. Lab.* **17,** 26, 28.

18. Gonzalez, J. E. and Negulescu, P. A. (1998) *Curr. Opin. Biotechnol.* **9,** 624–631.

19. Oldenburg, K. R. (1998) *Annu. Rep. Med. Chem* **33,** 301–311.

20. Fernandes, P. B. (1998) *Curr. Opin. Chem. Biol.* **2,** 597–603.

21. Kenny, B. A., Bushfield, M., Parry-Smith, D. J., Fogarty, S. and Treherne, J. M. (1998) *Prog. Drug Res* **51,** 245–269.

22. Silverman, L., Campbell, R. and Broach, J. R. (1998) *Curr. Opin. Chem. Biol.* **2,** 397–403.

23. Oakley, A. J. and Wilce, M. C. J. (2000) *Clin. Exp. Pharmacol. Physiol.* **27,** 145–151.

24. Roberts, G. C. K. (2000) *Drug Discovery Today* **5,** 230–240.

25. Shuker, S. B., Hajduk, P. J., Meadows, R. P. and Fesik, S. W. (1996) *Science* **274,** 1531–1534.

26. Moore, J. M. (1999) *Biopolymers* **51,** 221–243.

27. Shapiro, M. J. and Wareing, J. R. (1999) *Curr. Opin. Drug Discovery Dev.* **2,** 396–400.

28. Moy, F. J., Haraki, K., Mobilio, D., Walker, G., Powers, R., Tabei, K., Tong, H. and Siegel, M. M. (2001) *Anal. Chem.* **73,** 571–581.

29. Moseley, H. N. B. and Montelione, G. T. (1999) *Curr. Opin. Struct. Biol.* **9,** 635–642.

30. Pervushin, K. (2000) *Q. Rev. Biophys.* **33,** 161–197.

31. Fowler, C. A., Tian, F., Al-Hashimi, H. M. and Prestegard, J. H. (2000) *J. Mol. Biol.* **304,** 447–460.

32. Tjandra, N. and Bax, A. (1997) *Science* **278,** 1111–4.

33. Fejzo, J., Lepre, C. A., Peng, J. W., Bemis, G. W., Ajay, Murcko, M. A. and Moore, J. M. (1999) *Chem. Biol.* **6,** 755–769.

34. Dunayevskiy, Y. M., Lai, J.-J., Quinn, C., Talley, F. and Vouros, P. (1997) *Rapid Commun. Mass Spectrom.* **11,** 1178–1184.

35. Siegel, M. M., Tabei, K., Bebernitz, G. A. and Baum, E. Z. (1998) *J. Mass Spectrom.* **33,** 264–273.

36. Siegel, M. M. (2002) *Curr. Topics Med. Chem.* **2,** 13–33.

37. Blom, K. F., Larsen, B. S. and McEwen, C. N. (1999) *J. Comb. Chem.* **1,** 82–90.

38. Patapoff, T. W., Mrsny, R. J. and Lee, W. A. (1993) *Anal. Biochem.* **212,** 71–8.

39. Sanchez, R., Pieper, U., Melo, F., Eswar, N., Marti-Renom, M. A., Madhusudhan, M. S., Mirkovic, N. and Sali, A. (2000) *Nat. Struct. Biol.* **7,** 986–990.

40. Skolnick, J. and Fetrow, J. S. (2000) *Trends Biotechnol.* **18,** 34–39.

41. Sanchez, R. and Sali, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13597–13602.

42. Sanchez, R. and Sali, A. (1999) *J. Comput. Phys.* **151,** 388–401.

43. Chou, J. J., Li, S. and Bax, A. (2000) *J. Biomol. NMR* **18,** 217–227.

44. Meiler, J., Peti, W. and Griesinger, C. (2000) *J. Biomol. NMR* **17,** 283–294.

45. Clore, G. M. and Gronenborn, A. M. (1991) *Annu. Rev. Biophys. Biophys. Chem* **20,** 29–63.

46. Clore, G. M. and Gronenborn, A. M. (1989) *Crit. Rev. Biochem. Mol. Biol.* **24,** 479–564.

47. Wishart, D. S. and Sykes, B. D. (1994) *Meth. Enzymol* **239,** 363–92.

48. Kitchen, D., Hoffman, R. C., Moy, F. J. and Powers, R. (1998) *Biochemistry* **37,** 10581–10588.

49. Moy, F. J., Diblasio, E., Wilhelm, J. and Powers, R. (2001) *J. Mol. Biol.* **310,** 219–230.

50. Clore, G. M. and Gronenborn, A. M. (1991) *Science* **252,** 1390–9.

51. Linge, J. P., O'Donoghue, S. I. and Nilges, M. (2001) *Meth. Enzymol.* **339,** 71–90.

52. Greenfield, N. J., Huang, Y. J., Palm, T., Swapna, G. V. T., Monleon, D., Montelione, G. T. and Hitchcock-DeGregori, S. E. (2001) *J. Mol. Biol.* **312,** 833–847.

53. Xu, Y., Jablonsky, M. J., Jackson, P. L., Braun, W. and Krishna, N. R. (2001) *J. Magn. Reson.* **148,** 35–46.

54. Xu, Y., Wu, J., Gorenstein, D. and Braun, W. (1999) *J. Magn. Reson.* **136,** 76–85.

55. Bowers, P. M., Strauss, C. E. M. and Baker, D. (2000) *J. Biomol. NMR* **18,** 311–318.

56. Rohl, C. A. and Baker, D. (2002) *J. Am. Chem. Soc.* **124,** 2723–2729.

57. Gardner, K. H., Rosen, M. K. and Kay, L. E. (1997) *Biochemistry* **36,** 1389–1401.

58. Clore, G. M., Starich, M. R., Bewley, C. A., Cai, M. and Kuszewski, J. (1999) *J. Am. Chem. Soc.* **121,** 6513–6514.

59. Delaglio, F., Kontaxis, G. and Bax, A. (2000) *J. Am. Chem. Soc.* **122,** 2142–2143.

60. Andrec, M., Du, P. and Levy, R. M. (2001) *Journal of the American Chemical Society* **123,** 1222–1229.

61. Hus, J.-C., Marion, D. and Blackledge, M. (2001) *J. Am. Chem. Soc.* **123,** 1541–1542.

62. Huang, X., Moy, F. and Powers, R. (2000) *Biochemistry* **39,** 13365–13375.

63. Moy, F. J., Chanda, P. K., Chen, J. M., Cosmi, S., Edris, W., Skotnicki, J. S., Wilhelm, J. and Powers, R. (1999) *Biochemistry* **38,** 7085–7096.

64. Chen, J. M., Nelson, F. C., Levin, J. I., Mobilio, D., Moy, F. J., Nilakantan, R., Zask, A. and Powers, R. (2000) *J. Am. Chem. Soc.* **122,** 9648–9654.

65. Moy, F. J., Chanda, P. K., Chen, J. M., Cosmi, S., Edris, W., Levin, J. I. and Powers, R. (2000) *J. Mol. Biol.* **302,** 671–689.