Chapter 1

Introduction

1.1 Protein-Protein Interactions

The post-genomics era – a time when genomes are being sequenced and sequences are released with less flourish, has dawned. Since genome sequences do not always provide a direct link to biological activity, it is the complex interwoven pathways governed by proteins that are now in the focus of research. As the genomic era gives way to the proteomic era, biologists are asking less about how genes encode proteins and more about how proteins interact with each other.

1.2 Protein interaction domains

It is well known that the functioning of the majority of proteins within cellular structures depend on their highly specific, non-covalent interactions with other proteins. Protein-protein interactions (PPIs) are extremely important in wide range of biological activities. Understanding how these systems function or, more importantly malfunction could give insight into how to disrupt pathological processes. Also, interference with these interactions provides a means to control cellular processes. In many cases these interactions are mediated by small, modular, non-catalytic protein interaction domains like SH2 (Src homology 2), SH3 (Src homology 3), WW (named after 2 conserved tryptophan residues), PTB (phospo-tyrosine binding) and, PDZ (PSD95/Disc large/Zonula occludens-1).[1] These interaction domains may control not only the specificity of signal transduction, but also the kinetics with which cells respond to external and intrinsic signaling events and may therefore be involved in complex cellular behaviors.

Interaction domains are designed to recognize exposed features of their binding partners. A large family of domains like SH3, WW, EVH1[2,3] recognize proline rich segments in the target proteins, SH2 and PTB domains[4-9] specifically recognize phosphorylated tyrosine residues whereas PDZ domains mostly bind to the extreme C-termini of their target proteins.[10-12] In addition to interaction domains which mediate PPIs, growing families of domains are known to bind lipids, mostly phopsoinositides (PH and FYVE)[13,14] and DNA (Tubby).[15] The assembly of these domains within a protein creates a binding surface with varying specificities, which tend to place the contained catalytic domains in optimal position to modify their targets.

Given the ubiquitous nature of these interactions and the knowledge that mal-functioning of these protein interaction domains leads to diseased states, it is not surprising that PPIs are becoming attractive targets for scientists who are interested in developing inhibitors of these interactions as biological tools or as therapeutic agents.

1.2.1 Inhibition of PPIs – challenges

Inhibition of PPIs is a extremely challenging task. Natural small molecules that bind to the protein-protein interfaces are rare. Secondly, the protein-protein interaction interfaces may be large (~700-1500 A^2) and relatively flat thus unlikely to suit binding of small molecule ligands. Despite the above mentioned challenges the feasibility of using small molecules to inhibit PPIs has been demonstrated by a handful of published results.[16,17]

Palcitaxel (Taxol), a frontline anticancer drug, binds to the β-subunit of tubulin heterodimer thus triggering apoptosis. Several other natural products known to inhibit PPIs are therapeutically relevant. These include cyclosporine A, rapamycin and FK 506 which bind to specific protein targets activating signal transduction.[18] Motivated by tremendous therapeutic impact of these natural product based PPI inhibitors, several *in vitro* screening studies have been performed. The most well known inhibitors act on the anti inflammation target LFA-1. Three other protein-protein interaction targets, human double minute 2

(HDM2, known as MDM2 in mice), Bcl-2 and Bcl-XL, possess comparatively deep hydrophobic grooves, which accommodate α-helical peptides of their protein interaction partner. In > 30% of human sarcomas, the tumor suppressor p53 is thought to be inactivated through interaction with HDM2, which binds to a nine-residue amphiphatic helix of p53. In a study of this interaction, *in vitro* screening yielded 100- to 300-nM small-molecule inhibitors, the activity of which was demonstrated in xenograft models of cancer.[19] Bcl-2 and the related family member Bcl-X are anti-apoptotic proteins whose activity is regulated through interaction with approximately 16-residue α-helical peptides of pro-apoptotic proteins, such as BAK. Several chemical classes of 0.1- to 10-μM small molecule inhibitors have been identified by various research groups.[20]

1.3 PDZ domains

PDZ domains were originally recognized as ~100 amino acid long repeated sequences in the synaptic protein PSD-95/SAP90 (postsynaptic density the Drosophila septate junction protein) Discs-large, and the epithelial tight junction protein ZO-1 (Zona occludens). Initially they were also referred to DHR (Disc large homology repeats) or GLGF repeats based on a conserved GLGF sequence in most of the domains of this family. Since their discovery approximately 15 years ago, PDZ domains have emerged as one of the most important classes of protein interaction domains. They are fundamental in regulating the dynamic organization of the cell. PDZ domains play central role in organizing networks of signaling proteins and in targeting selected cellular proteins to multi-protein complexes.[10-12,21] These domains are also one of the most abundant protein domains in the *H. Sapiens* genome, with around 449 domains occurring in 250 proteins (Table 1.1). Along with vertebrates PDZ domains are wide spread in metazoans, plants and bacteria,[22] but are rare among viruses and yeast.

Domain	Number of occurrences
PTB	58
WW	142
SH2	170
PDZ	449
SH3	454
PH	469

Table 1.1: Most commonly occurring protein interaction domains in the *H. sapien* genome. Data obtained from the SMART database. (http://smart.embl-heidelberg.de)

1.3.1 Structure of the PDZ domains and their mechanism of peptide recognition

Like most of the protein domains, PDZ domains show compact globular structure with their `N´ and `C´ termini close to one another in their folded state. Thus the domains are highly modular and may be inserted into the proteins without significant structural distortion during the course of evolution. The PDZ domain fold typically consists of 6 β-strands (βA – βF) forming two opposing anti-parallel sheets flanked by 2 α-helices (αA and αB). (Figure 1.1) The peptide ligand binds within an extended surface groove lined by βB and αB in form of a β-strand. This mechanism of peptide binding is known as β–strand addition.[23] The peptide backbone is involved in extensive hydrogen bonding with the protein in a fashion similar to those observed in a β–sheet. The carboxylate group binds to the protein through a carboxylate-binding loop just preceding βB and has a conserved –GψGψ- motif (where ψ is a hydrophobic residue).

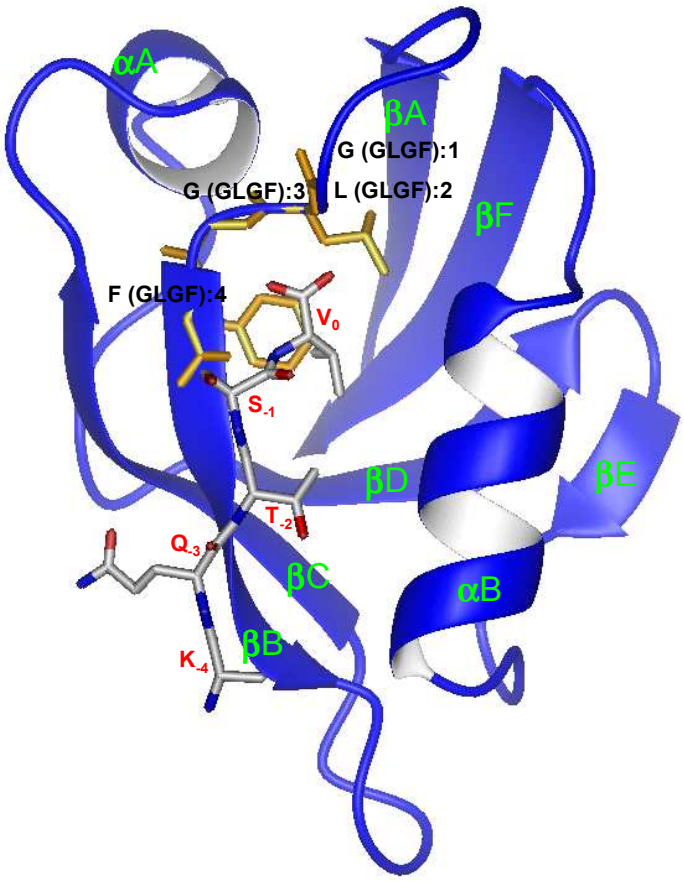

Figure 1.1: **Structure of a PDZ domain and its peptide recognition mode.** Ribbon model of the third PDZ domain of PSD95 (1BE9) in complex with C-terminal peptide ($KQTSV_{COOH}$) derived from cystine-rich-interactor of PDZ three (CRIPT). The residues in the conserved GLGF loop are colored yellow and the peptide residues are colored according to atom type.

1.3.2 Specificity of peptide recognition

Most of the PDZ domains specifically recognize 4-6 C-terminal residues of their target proteins. The nomenclature of the residues within the PDZ binding motif is as follows: the C terminal residue is referred to 0 and the preceeding residues towards the N terminus are referred to as -1, -2, -3 etc. This nomenclature will be used in all further discussions. Extensive peptide library screens in initial days revealed the specificities of the PDZ domains towards their target peptides.[24,25] These studies along with the structure of the PDZ peptide complexes suggested that the 0 and -2 residues in the peptide ligand are critical for the specificity of the protein-peptide interaction. Based on these studies, PDZ domains have been classified into 3 classes. Class 1 PDZ domains show preference to the motif S/T-X-ϕ-$_{COOH}$ (where ϕ is a hydrophobic amino acid and X is any amino acid). Class 2 PDZ domains recognize ϕ -X- ϕ-$_{COOH}$ and class 3 PDZ domains recognize X-X-C-$_{COOH}$ sequence motif. Despite this classification, many PDZ domains target a broad range of ligand sequences which does not match this classification scheme. [26-28]. The overlap of the recognition sequences suggest that the *in vivo* selectivity and specificity for the PDZ-target interaction is achieved by compartmentalization and by co-operativity of the accompanying domains.

Apart from C-terminal peptides of the target proteins PDZ domains have also been shown to recognize internal peptide modules.[29] [30] An example of the internal peptide recognition by a PDZ domain is that of the interaction between nNOS PDZ and syntrophin PDZ domain.[29] In this interaction a 30 residue extension of the nNOS PDZ domain adopts a β-finger orientation and docks in the syntrophin PDZ through its Phe(0) and Thr(-2) residues. In this interaction the C-terminus is replaced by a sharp β-turn. Thus, it is postulated that all PDZ domains may bind internal peptide motifs if they are presented within the correct structural context. Some PDZ domains are also known to interact with lipids.[31]

1.3.3 Functions of PDZ domains

PDZ domains are very important in dynamic organization of the cell. They play a central role in signaling by organizing network of receptors and in targeting cellular proteins to multi-protein complexes. The best known examples of PDZ organized multi-protein complexes occur in neuronal and epithelial cells. PDZ domains containing proteins appear to play a major role in organizing polar sites of cell-cell communication. PDZ domain containing proteins play a crucial role in organizing receptors and their downstream effectors as well as transporting and targeting appropriate proteins to the sites of signal transduction. Some of the best studied PDZ domain containing proteins involved in the above mentioned processes are discussed below:

At the dendritic side of the neuronal synapse there is a dense complex of proteins termed as postsynaptic density (PSD) which contain many of the signaling components. One of these proteins PSD-95 has three PDZ domains, a SH3 domain and a GuK domain is one of the best studied examples of PDZ scaffolds. The PDZ domains of PSD-95 bind to many of the members of the PSD. The first and the second PDZ domains of PSD-95 bind to the N-methyl-D-aspartic acid (NMDA) glutamate receptor different channels, thus suggesting a role of this protein in channel aggregation. The second PDZ domain of PSD-95 and the single PDZ domain of syntrophin bind the C-terminal PDZ domain of nNOS. These interactions, respectively, bring nNOS in close proximity to the NMDA receptor channel in neuronal synapses and mediate the association of nNOS with sarcolemmal membranes in skeletal muscle.[32] The binding of nNOS to the NMDA receptor couples NMDA receptor mediated Ca^{2+} influx to the activation of nNOS.

1.3.4 Regulation of interactions mediated by PDZ domains

Although the structures and mechanisms of a number of PDZ domains have been investigated, the regulation of the interactions is not fully understood. One of the mechanisms involved in regulation is phosphorylation of the residues

involved in the binding. A serine residue at position -2 of the inward rectifier K^+ channel Kir2.3 (IRK4), for example, can be phosphorylated by protein kinase A (PKA); phosphorylation of this residue by PKA abolishes IRK4 interaction with the PSD-95 PDZ domain. Similarly phosphorylation of a serine residue at -3 position of the α-amino-3-hydroxy-5-methyl-isoxazole-4- propionic acid (AMPA) receptor subunit GluR2 C-terminus by protein kinase C (PKC) abolishes its interaction with GRIP.

1.3.5 PDZ domains as a model for design of PPI inhibitors

PDZ domain containing proteins are involved in forming complex signal transduction networks. These proteins have been shown to play a major role in targeting, clustering and cycling of many membrane receptors and ion-channels. Provisional role of the PDZ domains in regulating these proteins which are involved in disease states make them prime targets for inhibitor design.[33] PDZ domains have a structurally well defined, but shallow binding surface that shows moderate affinity to their cognate peptide ligands. The discreet properties of the PDZ-peptide interaction make them a promising but challenging, barley druggable targets for modulation by low molecular weight compounds.[18,34-36]

1.4 The AF6 PDZ domain

The PDZ domain used as an example in this work was derived from the protein AF6 (*ALL-1* fusion partner on chromosome 6)[37]. AF6 contains two N-terminal Ras-assiociation domains,[38] a forkhead association domain (FHA)[39], a class V myosin homology repeat, also known as dilute domain (DIL), a class II PDZ domain and a praline rich sequence. The PDZ domain of AF6 mediates the interaction with a subset of ephrine receptor protein-tyrosine kinases,[40,41] the poliovirus receptor-related protein PRR2/nectin,[42] the junctional adhesion molecule (JAM)[43] and the breakpoint cluster region protein (BCR)[44]. The AF6 protein has been proven to be involved in Ras signaling pathway. AF6 acts a scaffolding protein which brings together BCR and Ras in a trimeric complex

(Figure 1.2). This trimeric complex might play an important role in down-regulating the Ras signaling pathway.[44]

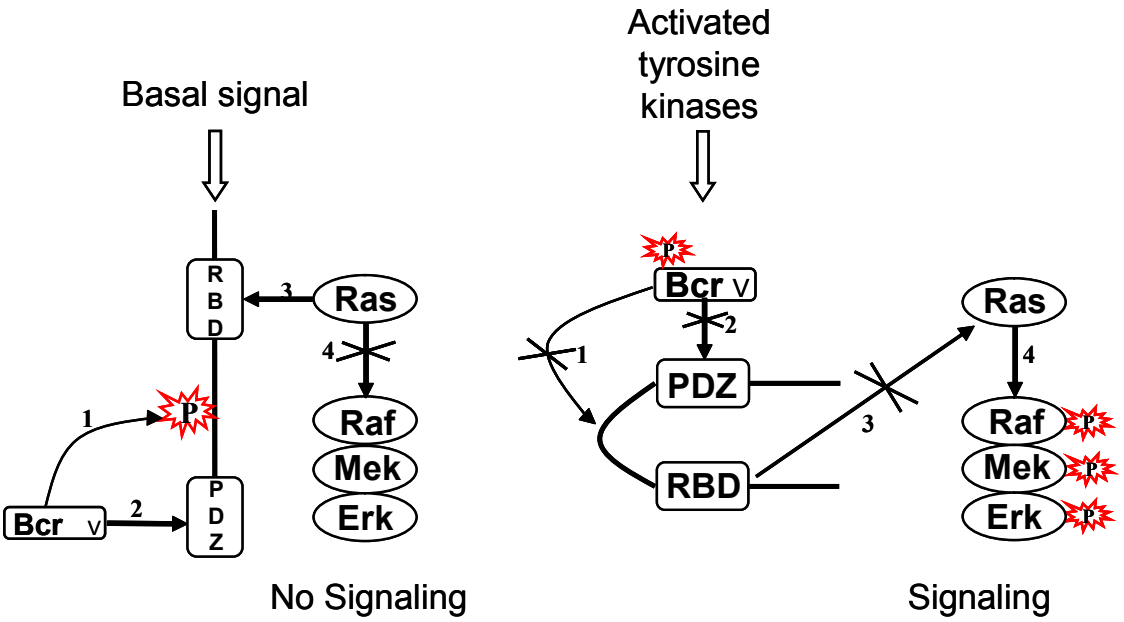

Figure 1.2: Effect of Bcr on Ras-dependent stimulation of ERK via AF6. a) In quiescent cells the constitutively active Bcr phosphorylates AF6 (step 1), which leads to the interaction of the PDZ domain of AF6 with the PDZ-binding motif of Bcr (step 2). This interaction increases the affinity of AF6 for Ras via the Ras binding domain (RBD) (step 3) and prevents binding of Raf to Ras (step 4). Under these conditions the protein kinase cascade composed of Raf, MEK, and ERK is not activated. b) Phosphorylation of Bcr on tyrosine residues inactivates its protein kinase activity. Therefore, Bcr cannot phosphorylate (step 1) and cannot bind to AF6 (step 2). Thus, AF6 does not compete with Raf for Ras (step 3) and does not interfere with the Ras-dependent activation of the protein kinase cascade (step 4). P represents phosphorylation of proteins.

1.5 Ligand screening

There are three critical stages for the design of inhibitors in a drug/ligand discovery platform. The first stage is lead identification for the compounds that show moderate activity towards the target. The second step is *in vitro* lead optimization. This stage is an iterative process of design-synthesis-assay which is normally guided by 3D structural information of the complex. The final step is the optimization of *in vivo* potency of the obtained ligand. A modern ligand/drug discovery platform can be summarized as shown in Figure 1.3

Following sections summarize the NMR spectroscopy-based techniques for the identification of inhibitors for protein targets.

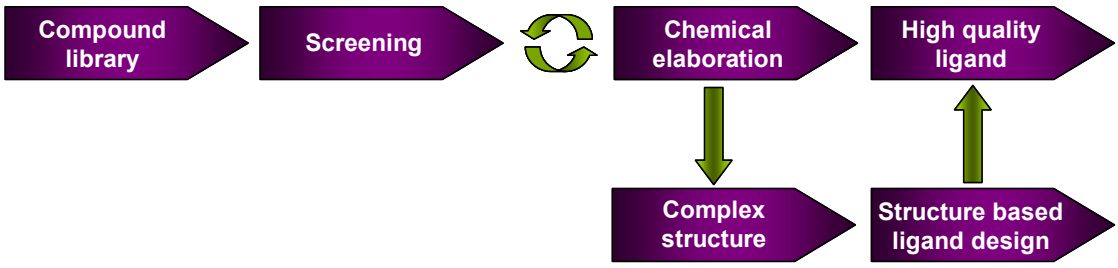

Figure 1.3: General work flow for ligand screening against protein targets.

Usually, high-throughput screening (HTS) methods are used for the purpose of ligand identification in the first step. HTS refers to a process of screening thousands of compounds against a given target in a relatively short period. The most commonly used ligand screening strategies utilize two basic approaches, the purely serendipity-based “brute-force” methods like combinatorial chemistry and high-throughput screening (HTS). Although these methods have been widely used in pharmaceutical industry for a long time, their use has had less impact in lead identification than originally predicted, and there is now a growing emphasis on more logical approaches like “knowledge-based” or focused screening. The “knowledge-based” approach, which makes use of literature and patent derived molecular entities, endogenous ligands or biological information.

A number of methods are available for ligand screening, including mass spectrometry,[45] fluorescence,[46] circular dichroism,[47] crystallography,[48] small molecule microarrays[49] and Nuclear magnetic resonance (NMR) spectroscopy.[50] Although conventional HTS methods, primarily fluorescence-based, have been successful in the pharmaceutical industry, suitable leads for ligand design are not always found using these methods. Today, NMR based techniques are being increasingly applied in ligand design and discovery programs for a number of reasons. Firstly, the changes detected in NMR based methods do not require a change in fluorescence upon ligand binding. Therefore, NMR screening can often identify the hits that are missed by traditional high-throughput screening approaches.[51] Secondly, depending on the protein and ligand concentrations used, NMR can detect weak ligand binding in the millimolar range. This can be useful in ligand screening approaches in which weakly binding ligands can sometimes be chemically linked to produce a tight binding compound.[52] Detection of weak binders can also be useful in the context of a search for functional ligand, in which a naturally occurring product inhibitor is likely to have a weak binding constant. Thirdly, unlike other spectroscopic methods, NMR provides details on the location of the binding site. This can then be compared with the known functional site, to determine whether the binding of a small molecule is physiologically relevant.

1.6 NMR spectroscopy as a tool for ligand screening and high resolution structure determination

1.6.1 NMR-based screening of ligands

The first case of NMR based screening for drug like molecules was reported in 1996.[53] Since then NMR spectroscopy has evolved into a powerful technique not only in the field of ligand discovery but also in the field of structure based ligand design.

Now, it is well established that NMR based screening techniques can be applied effectively in both, primary and secondary screenings. Primary screening refers to screening of a large compound libraries against the target, which leads to the discovery weak binding ligands (100 μM < Kd < 5mM), where as the secondary screening refers to a more detailed screening of compounds with moderate affinities which may be derived from the "hits" discovered by primary NMR screens or by any other biophysical technique.

Since the first case of NMR based lead discovery reported from Abbott Laboratories in 1996, a number of NMR based methods have been developed to identify ligands (Table 1.1). All these techniques take advantage of the fact that free ligand and protein in solution retain their characteristic NMR properties (like chemical shift, relaxation rates etc.). However, in presence of each other, depending on their mutual affinity both the molecules are in equilibrium between the free and the bound state. Therefore, the ligand transiently acquires the proteins NMR parameters, which are characteristic of a large molecule, and the presence of the ligand causes perturbations in the ligand binding site of the protein. It is this ability to experimentally monitor these changes which makes NMR based screening possible. These perturbations can be used qualitatively to detect ligand binding or quantitatively to assess the strength of the binding interaction.

Technique	Target requirement	Signal observed	Binding site info?	Kd (Molar)	Potential artifacts
$^{15}N/^{1}H$ Chemical shifts	^{15}N Labeled MW<40 kDa	Protein	Yes	$< 10^{-6}$	Insolubility Overlap
Diffusion edited	None	Ligand	No	$< 10^{-3}$	Insolubility Aggregation Overlap
Transfer NOE	None	Ligand	No	$\sim 10^{-6}$ -10^{-3}	Insolubility Aggregation
NOE Pumping	None	Ligand	No	$\sim 10^{-6}$-10	Insolubility Aggregation
STD NMR	None	Ligand	No	$\sim 10^{-7}$-10^{-3}	Insolubility Aggregation
WaterLOGSY	None	Ligand	No	$\sim 10^{-7}$-10^{-3}	

Table 1.2: NMR based screening techniques

1.6.2 Ligand-based NMR screening methods

High sensitivity of NMR spectroscopy to detect changes in the ligand NMR parameters lies behind the idea of the ligand-based or ligand observed NMR screening methods. As most of the ligand used in NMR based screening have molecular weights <1000 Da, the ligand´s properties like relaxation rate can be exploited to detect binding. Most of the ligand-based methods are based on: 1) transfer of 1H magnetization to the bound ligand from the protein, and 2) differential mobility of ligand in the free and bound states. Some of these methods are described below:

1.6.2.1 Transverse relaxation

Monitoring the transverse relaxation (T2) of the ligand signals is one of the most common methods to detect ligand binding by NMR. The transverse relaxation rate (1/T2) of a small molecule tumbling rapidly in the solution is typically longer than those of the protein molecules and ligands bound to protein, which are tumbling much slower in the solution. Thus by using a T2 relaxation filter, signals of the bound ligand can be selectively filtered out. In this method, two relaxation edited spectra are obtained, one with and one without the ligand, upon differencing these spectra the binding ligand can be obtained.

1.6.2.2 Nuclear Overhauser Effect (NOE) based methods

The methods based on the NOE include transferred NOE, NOE-pumping (and reverse NOE-pumping) and saturation transfer difference (STD) NMR.

a. Transfer NOE[54]: One physical parameter which distinguishes low molecular weight ligand and a protein, is the rotational correlation time τc. Low molecular weight compounds have a very short τc and thus exhibit positive NOEs, no NOEs or very small negative NOEs. Large protein molecules, which tumble slowly in the solution, have longer τc and exhibit large negative NOEs. Thus when a small molecule is liganded to a large protein the relaxation of the ligand is governed by the rotational correlation time of the protein. Thus the bound ligand

exhibit large negative NOEs which may be detected as transfer NOEs. The transfer NOE method yields information on the conformation of the ligand when bound to the protein.[55] From a structure-based ligand design perspective this information is very valuable for the further design of compounds of improved affinity. The detection of transfer NOEs, however, is not easy for high affinity ligands (Kd < 10μM), where the ligand resonances are no longer in fast exchange on the NMR timescale. Another potential problem is that compounds with low solubility in aqueous solution tend to aggregate and exhibit large and negative NOEs even in the absence of a macromolecular target. Furthermore, the transfer NOE method is not suited for high-throughput analysis because the 2D ^{1}H-^{1}H NOESY experiment on which it is based is relatively insensitive, requiring relatively long acquisition times to obtain spectra of sufficient quality.

b. NOE-pumping[56] and reverse NOE-pumping[57]: In contrast to the transfer NOE method described above which monitors the change in intra-molecular NOEs of the ligand, the NOE pumping experiment relies on NOE to transfer the signals of the protein to the bound ligand. In the NOE pumping experiment, a diffusion filter is used before the NOE experiment which destroys all of the ligand coherence, thus any ligand signal detected at the end of the NOE experiment arises from the polarization transferred from the protein to the bound ligand which is preserved after the diffusion filter. As the ligands dissociate from the target protein the transferred magnetization is carried over. The signals observed are not only from the ligands those are currently bound but also from the signals pumped to the ligands free in the solution.

The reverse NOE pumping (RNP) method, as the name suggests, detects the signals transferred to the protein from the ligand. In a RNP experiment a relaxation or isotope filter is employed to first attenuate the protein signals while preserving the signals from the ligand. The ligand then can loose its signal either by NOE pumping of relaxation. To detect signal loss because of NOE pumping, signal loss because of relaxation is measured by recording a reference spectra by using the same filter after the NOE experiment. Comparison of the two

spectra reveals the ligands that are involved in binding. Since the two spectra are recorded on the same sample by interleave acquisition, subtraction artifacts because of experimental conditions are minimized.

c. Saturation transfer difference (STD) NMR[58]: Saturation Transfer Difference (STD) spectroscopy has become the most popular method for screening due to small amounts of receptor required and the relative ease of implementation. STD as the name suggests is carried out by subtracting the spectrum obtained when irradiation is placed on a protein resonance from the spectrum obtained when irradiation is off resonance. When a protein is selectively irradiated it causes saturation of the entire protein as well as that of any ligands that may bind to the protein because of the efficient spin diffusion mechanisms of the large molecular weight of the protein (Figure 1.4).

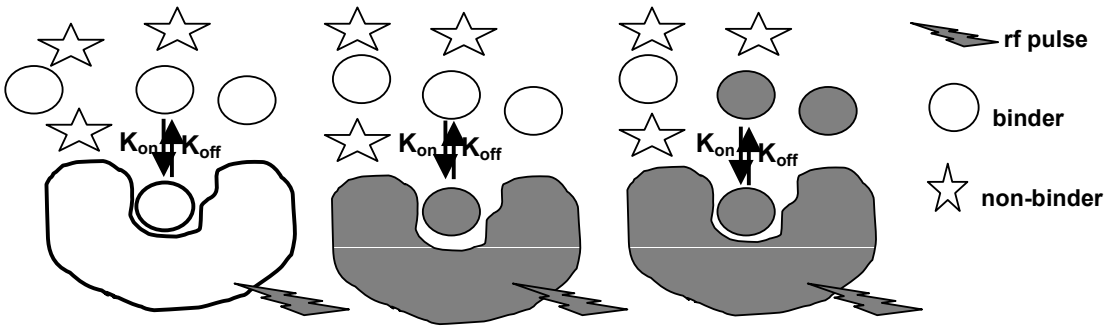

Figure 1.4: **Detection of binders using the Saturation Transfer Difference (STD) experiment.**
Frequency selective irradiation (lightning bolt) cause selective 1H saturation (shading) of the target protein. The irradiation is applied for a sustained interval during which saturation spreads throughout the entire receptor via $^{1}H–^{1}H$ cross-relaxation (spin-diffusion). Saturation is transferred to binding compounds (circles) during their residence in the receptor binding site. The number of ligands having experienced saturation transfer increases as more ligand exchanges on and off the receptor during the sustained saturation period. Non-binding compounds are unaffected (stars).

A complementary reference experiment is then recorded by irradiating off-resonance with respect to protein using the same rf pulse train. Because of the off-resonance pulses no perturbation in the resonances occurs. The on-

resonance and off-resonance experiments are recorded on same sample in interleaved fashion and the subtracted. The resulting difference spectrum has peaks only of the species which have experienced the saturation. This includes the protein and the binding compound resonances. The protein resonance will not be visible because of their relatively low concentration in the sample or they can be filtered out using a diffusion filter.

d. WaterLOGSY[59,60]**:** Water-Ligand observed via gradient spectroscopy (WaterLOGSY) is a method closely related to STD NMR. As in STD NMR the protein-ligand complex is selectively 'tagged' by a pulse scheme. Instead of direct perturbation of the protein resonances, WaterLOGSY indirectly tags the ligand by selectively perturbing the bulk magnetization of water. The transfer of energy in a waterLOGSY experiment is water-protein-ligand. The selective inversion of the water magnetization can be achieved by number of methods[61,62] but the most popular method is the selective inversion using e-PHOGSY[63]. Inverted water magnetization is transferred to the bound ligands via three simultaneous strategies. One strategy involves direct $^{1}H–^{1}H$ cross-relaxation between the bound ligand and 'bound' water molecules within the binding site. A second strategy is direct cross-relaxation with exchangeable receptor NH/OH protons within the binding site. Chemical exchange of these protons with those of bulk water inverts their magnetization. The third strategy involves indirect cross-relaxation with remote exchangeable NH/OH protons via spin diffusion. The inverted magnetization is then relayed to other non-labile spins via spin-diffusion. The above magnetization transfer schemes allow binding compounds to pick up the magnetization while residing in the receptor binding site. Binding compounds are distinguished from non-binding compounds by their differential cross-relaxation properties with water. In the magnetization transfer schemes above, the binders interact directly or indirectly with inverted water spins to yield negative cross-relaxation rates. Thus when the spectrum is phased the bound

ligands having received magnetization from the water will have opposite phase to the non-binding ligands.

1.6.2.3 Diffusion

Translation diffusion rate, which can be accurately measured using gradient assisted NMR spectroscopy[64], can be used for detection of protein-ligand interactions. In the case of a ligand in fast exchange between the free and receptor-bound states, the observed translational diffusion coefficient (D_{obs}) is given by:

$$D_{obs} = D_{free}\, M_{free} - D_{bound}\, (1 - M_{free}),$$

where, M_{free} is the mole fraction of the ligand in the free state and D_{free} and D_{bound} are the translational diffusion coefficients for the free and receptor-bound ligand respectively. Thus, the diffusion coefficient of a small molecule will change upon binding to a protein, and this change can be used to detect ligand binding. To monitor binding, parameters need to be chosen for the diffusion filter (gradient strength and diffusion delay time) that efficiently suppress the signals of compounds which do not bind to the target receptor but allow the observation of the signals of the receptor and receptor-bound ligands. The use of diffusion-edited NMR spectroscopy for screening compound libraries was first illustrated by Lin *et al*.[65,66]

1.6.3 Protein-based NMR screening methods

The other approach to detect interaction between a ligand and a target is to analyze the chemical shift perturbations observed in the target when the ligand interacts with its surface. Traditionally crowded spectra and challenges in resonance assignments of large proteins have limited the utility of this approach to relatively small monomeric proteins (> 30,000 Da). Recent advances in isotope labeling strategies and development of new pulse programs (TROSY[67,68] etc) have somewhat relaxed the molecular weight restriction of the target protein extending the number of targets those can be screened by these methods.

The methods that observe protein resonances as proof of ligand binding are discussed below.

1.6.3.1 Chemical shift mapping

Of the NMR sensitive parameters chemical shifts are the ones that have been used most extensively in detecting protein ligand interactions. 2D ^{1}H-^{15}N HSQC or ^{1}H-^{13}C HSQC[69] experiments are recorded in presence and absence of the ligand. By comparing the chemical perturbations in the spectrum after addition of the ligand, binding can be detected. While the former allows detection of changes in the amide protons and nitrogen nuclei of the backbone and Asn and Gln side chains and requires the protein sample to be enriched in ^{15}N, the latter requires ^{13}C enrichment but yields information on chemical shift changes in all side chains. Although ^{13}C labeling allows the chemical shift perturbation approach to sample hydrophobic patches on the surface of the protein, the ^{15}N experiment is normally preferred because it requires neither the relatively costly ^{13}C enrichment nor the often lengthy process of side chain assignment. In both cases, by measuring the chemical shift changes as a function of ligand concentration the affinity constant between the ligand and the target can be accurately measured. A typical chemical shift perturbation experiment is shown in Figure 1.5. The most important feature of this method is the structural information that it delivers; the binding site can be mapped on the surface of the protein.

1.6.3.2 Selective active site isotope labeling:

Although advent of new pulse sequences like TROSY (Transverse Relaxation Optimized Spectroscopy)[67,68] and CRIPT (Cross Relaxation Induced Polarization Transfer) have increased the molecular weight limit of the proteins suitable for NMR above 100 kDa, spectra obtained from such systems can none the less be extremely complicated, making protein-ligand interaction studies tedious. One

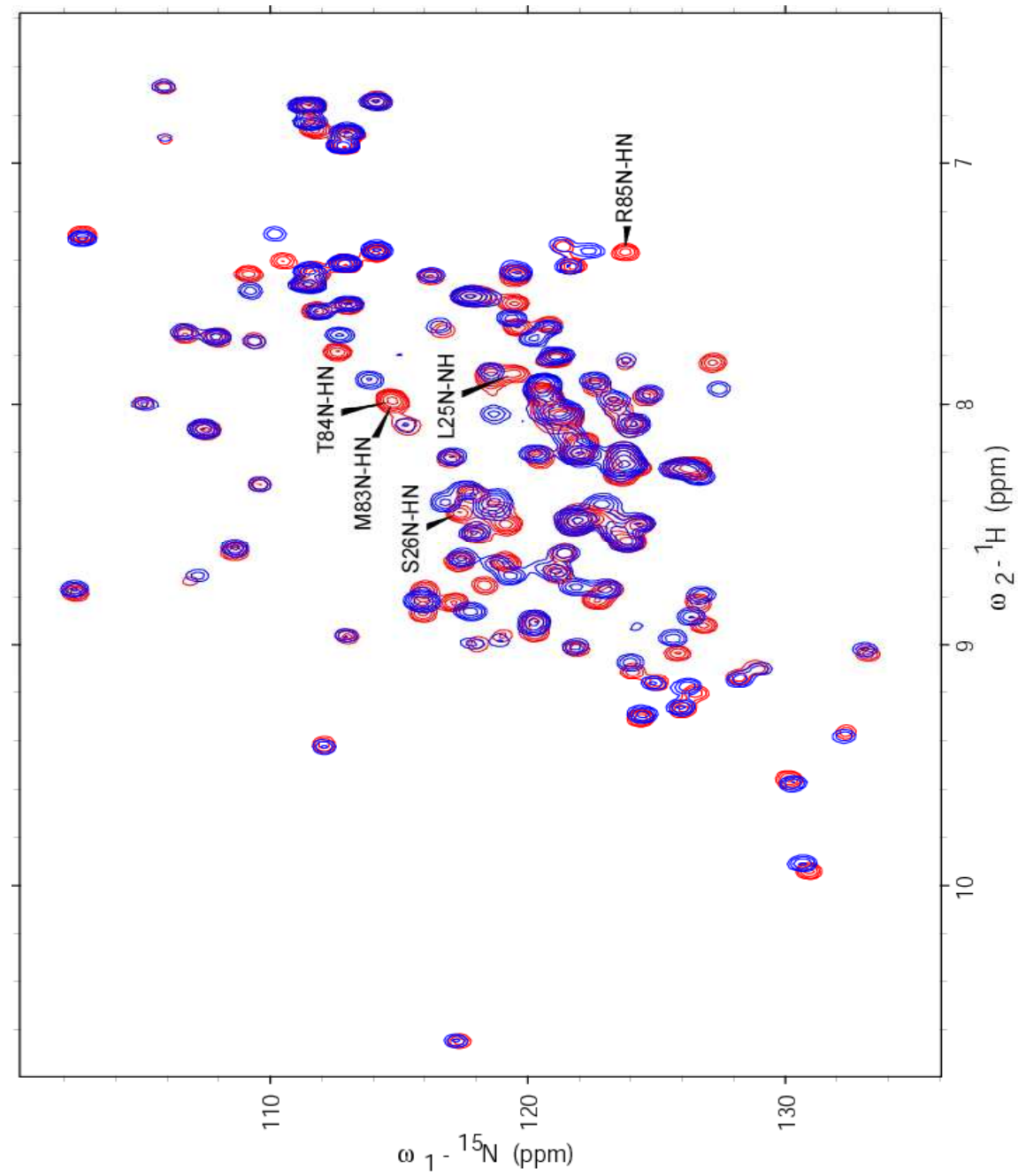

Figure 1.5: Detection of protein ligand interaction by Chemical shift mapping. 1H-15N HSQC spectra of the AF6 PDZ domain in presence of a small molecule ligand (Blue) and in absence of the ligands (Red) are shown. The residues showing chemical shit perturbations are labeled.

way of reducing the complexity of the spectra is to selectively label the acitive site of the protein under study. Recently a labeling scheme that selectively labels pair of residues lying in the active has been demonstrated.[70,71] In this case the authors selectively labeled one residue with ^{13}C and the other by ^{15}N so that these two residues will be the only source of the signal in a multi-resonance experiment. Compounds can be then tested against such selectively labeled proteins and those that bind to the protein in the active site will cause chemical shift perturbation of the labeled amino acids.

1.6.4 Screening of ligands for protein mixtures

As practiced today NMR based screening is a serial process. Multiplexing can increase the throughput of the screening process. NMR based screens have been multiplexed to screen multiple compounds together and upto 100 compounds have been tested simultaneously[72], but multiplexing number of proteins in a screen is more challenging because of the inherent complexity of the protein signals. Recently Zartler et.al described a method of screening several protein targets at once by Rapid Analysis and Multiplexing of Experimentally Discriminated Uniquely labeled Proteins by NMR (RAMPED-UP NMR).[73] In this method, the proteins to be screened are uniquely labeled with one amino acid type. Because of the unique labeling the spectra are greatly simplified, resonances that are most likely to be affected by binding are the only ones observed, and peaks that yield little or no information upon binding are eliminated, allowing the analysis of multiple proteins easily and simultaneously.

1.6.5 Comparison of protein-based and ligand-based methods

As discussed above screening can proceed by ligand-based or protein-based methods. Identifying the chemical shift perturbations in the protein-based methods, one not only identifies the binding ligands but can also localize their binding site. Such methods exploit the site specific information provided by the

assigned spectra of the protein of interest along with priori knowledge of its 3D structure. This suggests strategies for fragment based ligand design. As described by Shuker et.al., low affinity ligands binding to distinct sub-sites on the protein can be linked or extended to yield high affinity ligands (SAR-by-NMR).[53] By monitoring the chemical shift perturbations of a assigned protein one can also distinguish specific and non-specific binding. Also unlike ligand-based methods, protein-based screening methods do not rely on fast exchange of the ligand between bound and unbound states so is equally well suited to identify weak as well tight binding ligands.

The main problem facing the protein-based methods is the type of protein chosen for the screening purposes. Most of the pharmaceutically important targets can be challenging to realize in a cost effective manner. For example one of the pre-requisite of protein-based methods is expression of labeled (^{15}N, ^{13}C, ^{2}H) target in mg quantities which can become prohibitively expensive. Secondly, the most popular expression system for NMR purposes, *E. coli*, is not suitable for expressing mammalian proteins, whose expression may be toxic to the host cell. Even if the protein is expressed in large quantities resonance assignment of large proteins (> 30,000 Da) frequently encountered in pharmaceutical research, can be a tedious and time consuming process.

Ligand-based methods compare the NMR parameters of ligand in presence and absence of the protein. So the size of the protein used in this case is irrelevant. Secondly ligand-based methods do not require large quantities of isotopically enriched protein samples for screening purposes. Also no assignment of the target protein is necessary in this case allowing rapid screening of ligand for number of target proteins.

The main disadvantage of ligand-based methods is its inability to localize the binding ligands on the protein. Secondly as most of the ligand-based approaches rely on fast exchange of ligand between the bound and unbound states they are biased towards weakly binding ligands and large ligand molar excess. The

consequent risk is that at such high concentrations ligands may start binding to the secondary, low affinity, non-specific sites on the protein.

Clearly, both ligand and protein-based screening assays have distinct advantages and disadvantages but the method of choice for ones purpose should be decided after taking into consideration the above mentioned properties of the two methods.

1.7 High resolution structure determination by NMR spectroscopy

It has been more than 40 years since NMR spectroscopy hit the analytical scene, and yet its capabilities continue to evolve. Since its early days when NMR was primarily used to verify the structure of small organic molecules, the technology behind this technique has exploded and today NMR spectroscopy has become one of the most valuable tools to study protein structures. Standard experimental procedures for structure determination by NMR utilize experimentally derived distances and torsional angle restraints in a constrained MD run. Structures determined by using inter-proton distances based on NOE intensity, supplemented with torsional angles and chemical shift data are of reasonably high quality. Based on the development for NMR techniques it has been suggested that the structures determined by NMR will have similar resolution as compared to X-ray crystallography (~2.5 Å).[74]

1.8 Objectives of this research

Protein-protein interactions (PPIs), which are mediated by small modular domains, are extremely important in a wide range of biological activities but are extremely challenging pharmacological targets due to the difficulties in inhibitor design.

Here, we use PDZ domains as test cases for development for PPI inhibitors. PDZ domain containing proteins are involved in forming complex signal transduction networks. Prior to this study no reversible small molecule modulators for the PDZ domain family were known. Also, no 3D structures of the AF6 PDZ domain or the AF6 PDZ domain in complex with its ligands were determined earlier. The aim of this work is to identify low-molecular-weight, reversible ligands for PDZ domains using the AF6 PDZ domain as a representative for ligand development purpose. To achieve this goal, following work packages were designed:

1. NMR based screening of ligands for the AF6 PDZ domain to identify novel inhibitors for these previously untargeted protein domains. ^{1}H-^{15}N HSQC based screening method is applied to identify ligands from the FMP compound library.
2. Screening of analogues of the positive “hits” from the primary screens to identify ligands with higher affinity. The affinity of ligands is quantified by monitoring the chemical shift perturbations of backbone HN’s of the protein and also by determining binding constants by NMR titrations for few compounds.
3. Structure determination of the AF6 PDZ domain and AF6 PDZ domain in complex with the best binding ligand. Standard NMR derived structural restraints are applied in the calculation of the three dimensional structure of this domain.

Ligand screening and optimization is a cyclic procedure where successive rounds of screening and analogue synthesis alternate with each other. In this work the synthesis of ligands is guided by the screening results. New compounds

are synthesized by taking into consideration the chemical shift perturbations of the proteins backbone HNs to generate better binding ligands than those obtained from the primary screens. The structure of the AF6 PDZ domain in complex with the best binding ligand will be used as a starting point further structure-based ligand development.

1.9 References

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