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Ligand based NMR methods for drug discovery

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1. ABSTRACT

NMR has a long history in drug discovery and hit-to-lead optimization. Compared to many other methods NMR has the advantage of combining structural and functional parameters to characterize protein inhibitor interactions. NMR methods used in this context can be split into two categories; protein based experiments using isotopically labelled protein samples and a broad range of ligand based methods. Recently, there has been a strong emphasis on so-called ligand-based methods which offer a broad range of options to determine binding epitopes. Ligand-based methods are attractive because they are broadly applicable, impose few constraints on the composition of the target protein and don't require isotopic labeling of the protein or ligands. Such experiments include diffusion experiments, saturation transfer difference (STD-NMR), NOE pumping, waterLOGSY, SALMON, transferred-NOE and INPHARMA. Ligand-based NMR methods have been employed in screening and in lead optimization. One key advantage arises from their capability to pick up specific interactions for compounds of relatively low affinity and their ability to provide limited structural information without any need of crystallization or isotopic labeling.

2. INTRODUCTION

Structure-based drug design is commonly used in many industrial and research laboratories. Characterization of protein-ligand binding is of utmost importance for screening libraries of ligand fragments and for the optimziation of lead compounds. However, many of the technologies used for drug discovery are either too unspecific or too expensive or time consuming. While high-throughput screening (HTS) methods allow hundreds of thousands of scans in minimal time, there is an increasing need for smarter screening technologies which can detect compounds which slip through the broad filter of HTS. Such methods include plasmone resonance (Biacore), calorimetry (1) and Thermofluor (2) which provide a more robust assessment of protein ligand interactions. However, even these methods struggle to bridge the gap between HTS and structure based screening or lead optimization because physico-chemical characterizations can not be easily linked to structural parameters.

While X-ray analysis can determine protein structures in a short time without any limitation in protein size, the latter has a strong advantage for the analysis of protein-ligand interactions and does not require

Table 1. Summary of ligand based NMR methods for compound screening and lead optimization

NMR Method	Ligand vs Protein	Competition experiments possible?	Epitope mapping	High throughput	Description and references
STD NMR	excess	yes	yes	yes	Saturation transfer from protein to weakly binding ligands; binding epitope can be determined using build-up curves (22-24, 49-53).
WaterLOGSY	excess	yes	no	yes	Weakly binding com-pounds are identified through negative NOEs originating from the water resonance (25, 26).
SALMON	excess	yes	yes	yes	Similar experimental conditions to waterLOGSY; a solvent accessibility epitope can be derived through use of short mixing times (27, 28).
Transferred NOEs	excess	no	no	no	Information on protein/ligand interaction as well as conformation of bound ligand can be derived from cross peak analysis (19, 45, 47, 48).
INPHARMA	excess	Based on competitive binding	(yes)	no	Protein mediated inter-ligand NOEs of competitive binding ligands (29, 30).
SAR by ILOE	excess	no	(yes)	no	Direct inter-ligand NOEs of simultaneous binding ligands; binding constants can be enhanced by chemically linkage of the two ligands (55).
Pharmacophore by ILOE	excess	no	(yes)	no	Protein mediated inter-ligand NOEs of simultaneous binding ligands using pharmacophore-based search of bi-dentate molecules (54, 55).
FABS	excess	yes	no	yes	^{19}F chemical shift perturbation of a CF_3-group introduced into the ligand after conversion by the enzyme (56, 57).
FAXS	excess	no	no	yes	^{19}F chemical shift perturbation of a fluorinated medium to weakly binding reporter molecule upon binding of a high affinity ligand (56, 57).
T_2 and T_{1rho} relaxation	excess	yes	yes	yes	Resonance broadening upon ligand binding; binding epitope can be derived using build-up curves (31, 34).
Selective T_1 relaxation	excess	yes	yes	yes	Change in selective longitudinal relaxation rate upon ligand binding (31, 34, 37).
TINS	excess	yes	no	yes	Line broadening upon ligand binding to an immobilized target protein (35, 36).

crystallization which has often been the bottle-neck of structure based analysis. SAR by NMR (3) has been used most broadly to map ligand binding sites on proteins (4-9) it is limited by protein size (30-50kD) and the need to prepare isotopically labeled proteins of a similarly high concentration as required for crystal structures. In contrast, ligand-based NMR methods usually don't require isotopic labeling and can be used with an excess of ligand and a small concentration of protein. Such methods offer valuable binding parameters for ligands which interact with proteins such as a binding epitiope and an active conformation of a ligand. Here we review the most common ligand-based techniques used to characterize protein ligand interactions as summarized in Table 1. A detailed description of these methods is beyond the scope of this review article and can be found in various more detailed citations (10-18).

Ligand-observed NMR methods are based on a fundamental difference between proteins and ligands. Small molecules used as protein inhibitors usually possess molecular masses below 1kDa whereas proteins have masses of typically larger than 10kDa. Associated with this difference in size are relaxation properties arising from slower or faster tumbling in solution. Ligand based NMR methods build on the transfer of protein relaxation properties arising from slow tumbling to the much smaller ligands.

In the presence of exchange between bound and free states relaxation properties can be transferred to ligands which bind to a protein. Therefore relaxation properties of small molecules not binding to proteins differ vastly from those which interact with a protein. While small molecules are characterized by small transverse relaxation rates, weakly positive cross-relaxation rates and large diffusion constants, the opposite is the case for large molecules. These differences cause line broadening, changes in the diffusion coefficient and an inversion of the sign of the nuclear Overhauser effect (NOE).

Since most ligand-observed NMR experiments used for drug design are recorded for small concentrations of the protein and a much larger concentration of the ligand, signal intensities also depend on exchange rates associated with ligand binding which influence the adoption of the protein relaxation properties by the ligand. Therefore the life time of the interaction which is inversely correlated to the off-rate becomes an important parameter which can be modulated by using a large excess of ligand. This does not apply to methods which require equal amounts of protein and ligand such as diffusion experiments (*vide infra*).

The transverse relaxation rate of a molecule is proportional to the line width of its NMR resonances. Signals of ligands bound to the protein will be broadened because the signals of the protein have a much larger line width (larger transverse relaxation rate, R_2) than those of small ligand molecules. The large line width of protein resonances is a direct consequence of the small autocorrelation time of large proteins in solution. In the same manner the transfer of other relaxation properties which are typical for large proteins have been used to study interactions and to screen for ligand binding. For example, diffusion properties are transferred from the protein-ligand complex to the ligand and can be used in screening. The most commonly used techniques which observe ligand resonances employ magnetization transfer by the nuclear Overhauser effect (NOE). These include the transferred NOE (19), NOE pumping (20) and reverse pumping (21), saturation transfer (STD-NMR) (22-24), waterLOGSY (25, 26), derivation of solvent accessibility epitopes from waterLOGSY experiments (SALMON) (27, 28) and inter-ligand NOEs between two competitively binding molecules (INPHARMA) (29, 30).

None of these techniques requires isotopic labeling of the protein, the required amount of protein is

usually very small (typical concentrations are 10-50 microM) and there is no upper limit size for the protein. Instead, relatively pure solutions must be used because any impurity contributes to the signal. Unfortunately ligands with very high affinities have low off-rates from the complex and can therefore score as non-binders. Competition experiments can help to circumvent this problem by measuring signals of a lower affinity ligand. Another limiting factor is the solubility of the ligand because most of these methods require an excess concentration of ligand. Some of these experiments are useful for high-throughput screening allowing spectra to be obtained in 5-10min, some provide more detailed information on ligand binding, some can be used to map the binding epitope of the ligand. Relaxation based methods will be covered briefly while techniques employing magnetization transfer will be described in greater detail.

3. RELAXATION METHODS

In NMR spectroscopy the term relaxation is used to describe the process which restores equilibrium magnetization and random phase. Two relaxation mechanisms must be distinguished, transverse and longitudinal relaxation with the respective relaxation rates R_2 and R_1. While (non-selective) longitudinal relaxation can be very similar for small and large molecules, transverse relaxation is quite different. NMR relaxation can be associated with fluctuating electrical fields which originate from the overall tumbling and internal motions of the molecule. The transverse relaxation rate of a nucleus in a molecule is to a first approximation proportional to its tumbling correlation time. Since large molecules tumble slowly in solution, the correlation time tau_C of their rotational motion is relatively long, causing large relaxation rates R_2 and therefore large line widths of NMR signals ($LW = R_2/pi$). Compounds interacting with a receptor which has a large molecular weight show broadened lines and increased R_2 values. Importantly, these transferred properties can vary across the small molecule depending on the time and localization of the interaction. The potential of relaxation-editing for screening has been demonstrated by Fesik and coworkers using FK506 binding protein (FKBP) and a nine-compound mixture from which one ligand with a dissociation constant of 200 microM was identified (31) T_2 and T_{1rho} filters have been commonly used to filter spectral components arising from short relaxation times (31-34).

Longitudinal relaxation rates are generally not a sensitive marker of ligand binding except if the protein resonances are kept in their equilibrium state while ligand resonances are disturbed. In this case ligands interacting with the receptor experience a change in their longitudinal relaxation rate which, to a first approximation, is proportional to the molecular tumbling correlation time of the protein ligand complex. The same principle underlies the reverse NOE pumping experiment by Chan and Shapiro (21).

A ligand bound to a protein adopts the relaxation properties of the complex. In the case of fast exchange free ligand molecules preserve the relaxation properties from the bound state for a short time. To observe signals of ligands which bind the target protein with dissociation constants K_D of typically 10^{-3} to 10^{-9}M an excess of ligand must be available. The broadening of resonances as a consequence of higher transverse relaxation rates is a typical property of spectra of small molecules in the presence of a large proteins. For this reason the line width and the transverse relaxation rate can be used as criteria for ligand screening. Broadening is more pronounced for large proteins or proteins bound to a matrix. Bound ligands can be identified using T_2 filter experiments (31). Usually spectra with and without protein must be compared to obtain meaningful results.

Broadening of ligand resonances induced by binding to a protein has been used frequently in the past, not only for drug design. Sykes and coworkers studied the interaction of the 85 kDa extracellular domain of the epidermal growth factor receptor (EGFR-ED) with the transforming growth factor TGF-α (32). By measuring line widths and transverse relaxation rates of methyl proton resonances of TGF-α for different TGF-α / EGFR-ED concentration ratios binding kinetics were determined. The line width of methyl protons showed a regional dependence indicating which parts of TGF-α were involved in the interaction. Selective broadening of lines has the potential to yield localized information in a ligand for regions interacting with a protein.

Siegal and coworkers recently described a fast, fragment based approach as *Target Immobilized NMR Screening (TINS) (*35, 36) where a target protein immobilized using a solid support (e.g. glass or sepharose beads) is in equilibrium with a solution containing small molecule compounds. A difference spectrum (beads without protein – beads with protein) shows ligands which bind the target protein owing to their line broadening arising form the interaction with the immobilized protein. This method is very sensitive and can be used to screen for ligands of very large proteins (e.g. membrane proteins). TINS can also be used to characterize competitive binding of different ligands.

Another relaxation based method has been developed by Jahnke *et al* (37). Here a paramagnetic spin label is introduced to the protein (*SLAPSTIC – Spin Labels Attached to Protein Side chains as a Tool to identify Interacting Compounds*) which in its vicinity (r < 15-20 Angstroem) will significantly broaden or quench all NMR signals. SLAPSTIC experiments are carried out in dilute solutions (< 100 microM) where intermolecular distances are much larger so that only ligand molecules interacting with the protein experience the line broadening.

4. DIFFUSION METHODS

Diffusion techniques rely on the dependence on the size of the molecule. According to the Stokes-Einstein equation $D = kT/6pXir$ where k is the Boltzmann constant,

T the temperature, Xi the viscosity and *r* the radius of the molecule, the diffusion coefficient *D* depends inversely on the size of a molecule. This principle has been used to analyze mixtures of compounds, for example in combinatorial chemistry (38, 39). Shapiro and coworkers combined diffusion editing techniques with TOCSY (total correlation spectroscopy) spectra to obtain two-dimensional spectra with little peak overlap and valuable information on the structure of the ligands (DECODES, diffusion encoded spectroscopy) (40).

Diffusion coefficients are measured in NMR by applying a linear magnetic field gradient for a short time. A gradient causes spatially dependent dephasing of the signal which can be reversed by applying the same spatially encoded gradient after a spin-echo. Rephasing fails for molecules which have "traveled" since the application of the first gradient. Consequently large molecules with small diffusion coefficients will be affected to a smaller degree by the diffusion filter than small molecules. For fast exchange the free ligand may carry diffusion properties from the complex. In principle two types of diffusion experiments have been used, pulsed field gradient spin echo (PFG-SE) (41) and pulsed field gradient stimulated echo (PFG-STE) (42). In the latter case loss of magnetization due to transverse relaxation is reduced although only half of the signal is observed. For this experiment the ligand and protein are commonly used in equimolar amounts. There has been some speculation whether diffusion NMR will also yield a binding epitope which is probably possible when stimulated echoes are used where longitudinal magnetization is subject to NOE cross relaxation inducing changes in signal intensity for long diffusion times (43).

Diffusion NMR can be used to directly determine the affinity of ligands without the need of titration with ligands. For free and bound ligand in fast exchange the observed diffusion coefficient represents a time-averaged mean of the free and bound species. The fraction of the bound ligand can be obtained from the diffusion constants of the protein D_{bound}, of the free ligand D_{free} and that of the the ligand in the mixture by $D_{obs} = x_{free}D_{free} + x_{bound}D_{bound}$ (x_i are mole fractions) assuming that the diffusion constants of the complex and free protein are the same.

For small molecules bound to larger partners the diffusion coefficient is reduced compared to non-binding molecules of a similar size. The observed diffusion coefficients are time-averaged according to the rate of exchange. Shapiro used the expression 'affinity NMR' for this approach because it is in some way reminiscent of separation by affinity chromatography (454). He reported applications of the method to study the binding of tetrapeptides to the glycopeptide vancomycine and to study the binding of small molecules to DNA.

To overcome problems arising from signal broadening of the ligand and from background signals of the protein Hajduk proposed to use differences between various spectra (31) where in the first step stimulated echo (STE) spectra of the compound mixture in the presence of the protein at high and low gradient strength are subtracted. This result is again subtracted from a stimulated echo (STE) spectrum of the chemical mixture in absence of the protein recorded at low gradient strength. The result is a spectrum which shows only signals of the small molecules that bind to the protein. This cumbersome approach which requires three different spectra has been reported for the identification of a 20 μM inhibitor for stromelysine from a mixture of 9 compounds. It is a disadvantage of this method that bound ligands may have different chemical shifts in fast exchange when equal amounts of protein and ligand are used.

Diffusion experiments are clearly a useful tool for drug screening and to a limited degree for epitope mapping. The main drawback is the relatively low sensitivity of the experiment which requires equimolar amounts of protein and ligand at concentrations of at least 50-100 μM. For low-affinity compounds even larger amounts of substances are needed as the diffusion effect becomes very small.

5. METHODS INVOLVING MAGENTIZATION TRANSFER

Methods using magnetization transfer represent the most popular NMR methods for drug discovery. They combine many advantages like applicability to large protein complexes, use of low protein concentrations and broad applicability. This class of methods includes many small variations of experiments which provide somewhat complementary answers to related problems.

5.1. TRANSFERRED NOE (Tr-NOE)

The transferred NOE (Tr-NOE) effect was originally described by Bothner-By (19, 45). Peters proposed to use the Tr-NOE for screening compound mixtures (46). The Tr-NOE is a nuclear Overhauser effect between adjacent spins in the ligand in presence of chemical exchange between the bound and unbound form (Figure 2). Small molecules experience small, positive cross-relaxation rates. These are negative for large molecules and have a much larger magnitude. Ligands in fast exchange between the bound and the free form experience transfer of the negative NOE from the protein complex to the population of the free molecules. As a consequence, in two-dimensional NOESY spectra positive cross peaks relative to diagonal peaks are observed for protein ligands while signals from free compounds are negative or disappear.

The theoretical framework of internuclear magnetization transfer via NOE for chemically exchanging molecules is well understood. The Tr-NOE effect benefits from large proteins which cause a strong negative NOE. Tr-NOEs are obtained from regular NOESY spectra. Signals arising from the protein are usually not observed for large proteins. Background signals arising from the protein can be suppressed by a T_2 or a T_{1rho} filter (spin-lock).

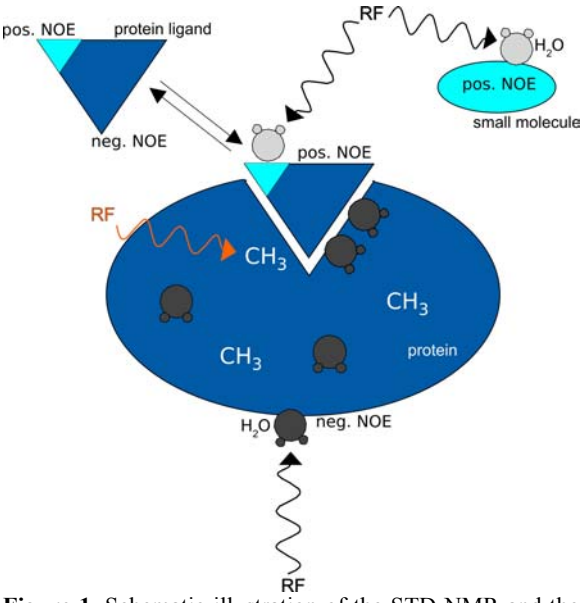

Figure 1. Schematic illustration of the STD-NMR and the waterLOGSY experiments. For the STD-NMR experiment protein resonances are saturated selectively without affecting resonances of the ligand. Increasing saturation is represented by darker blue tones. Compounds which interact with the protein (triangles) show the saturation effect. NMR signals from small molecules which are released from the protein in fast to intermediate exchange can be detected. Molecules which bind to the protein show reduced signal intensities as a consequence of saturation transfer. Non-binding molecules (oval circles) show no effect. In the waterLOGSY experiment magnetization is transferred from bulk water to the protons of the protein by NOE or by exchange of labile protons. The ligand in solution shows the transfer of magnetization from bulk water. The sign of the signal is different for molecules which bind the protein (+, dark blue color) and those which experience transfer of magnetization from bulk water without binding the protein (-, light blue). For short mixing times solvent exposed protons of the ligand partially retain their properties of the unbound ligand due to direct solvent interaction even when bound to the protein (SALMON). These parts of the bound ligand are also colored in light blue.

Peters demonstrated the potential of Tr-NOE to screen oligosaccharides for deglycosylated E-selectin/hIgG (47) and was able to select binding compounds and determine the bioactive conformation. To overcome severe overlap in mixtures of carbohydrates Peters also used three-dimensional TOCSY-Tr-NOESY experiments (48). Although this more time-consuming approach is useful to deconvolute mixtures of compounds it is not suitable for screening.

The Tr-NOE method is broadly applicable for large proteins with dissociation constants between 10^{-7}M and 10^{-3}M, requires only small amounts of protein (10-30microM, depending on its size) and has frequently been used to determine binding epitopes. Disadvantages of this method are connected with the need for relatively high ligand concentrations requiring highly soluble compounds, and the requirement to record two-dimensional NOESY spectra with long acquisition times. A large amount of spin diffusion limits epitope mapping.

5.2. SATURATION TRANSFER DIFFERENCE AND SATURATION TRANSFER DOUBLE DIFFERENCE

The saturation transfer difference method (STD-NMR) introduced by Meyer (24) is one of the most popular NMR methods to detect ligand binding. The sample condition requirements are the same as for the Tr-NOE experiment but a small variation allows for much faster one-dimensional spectra.

The principle of STD-NMR is illustrated in Figure 1. Protein proton resonances are selectively irradiated by a series of selective small flip-angle pulses to saturate the irradiated protons. Efficient cross-relaxation spin diffusion in large molecules spreads the saturation over the entire protein. This saturation will also be transferred to protons of ligand molecules bound to the protein, whereas protons of non-binding ligands will not experience the saturation transfer. It is therefore essential that the saturation pulse affects only protein resonances but not resonances of the ligand. Typically methyl resonances with unique chemical shifts in proteins between 0 and -2 ppm are saturated because low chemical shifts for methyl groups are typical for folded proteins. In a reference experiment the saturation pulse is applied at a frequency where none of the protein or ligand protons resonate (typically +30 ppm) and the reference spectrum is subtracted from the transfer spectrum.

Sample conditions for STD-NMR are fortunate for large proteins as the effect increases with the size of the protein and with the ratio of ligand to protein concentration.Fast dissociation rates also favor large STD effects which limits the method to lower affinity interactions. The necessity of a large ligand excess allows STD measurements with very low protein concentrations. The theory underlying the STD effect follows the same principles as that for the Tr-NOE method. Because it is very well understood it can be used for a quantitative analysis of the NMR spectra yielding binding epitopes (49) on the ligand molecule as well as binding constants if the complex structure is known.

The STD principle can be combined with two-dimensional homonuclear (24) and heteronuclear (50) experiments. STD-TOCSY was presented by Meyer and coworkers employing a mixture of seven oligosaccharides and wheat germ agglutinin. Competition studies can make STD-NMR accessible to ligands with nanomolar dissociation constants and slow dissociation rates. STD-NMR has also been used in combination with HR-MAS solid state NMR spectroscopy (51).

The potential of STD-NMR for epitope mapping has been demonstrated for many examples, including large proteins such as the 120 kDa lectin agglutinin binding methyl beta-D-galactoside (23). More recently Peters

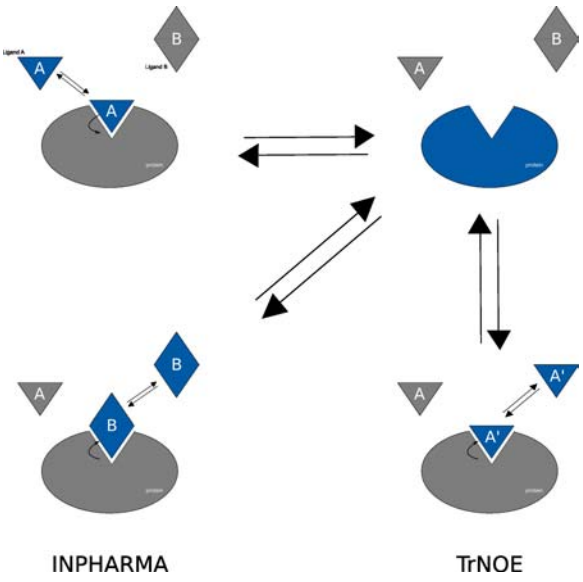

Figure 2. Schematic illustration of the Tr-NOE and INPHARMA experiment. Magnetization is transferred from ligand A via the protein back to itself (Tr-NOE) or to another competitively binding ligand B (INPHARMA). In both cases the cross peaks in a 2D experiment are used to either determine the conformation of the bound ligand (Tr-NOE) or to derive the relative orientation of the two ligands when bound to the protein (INPHARMA).

demonstrated the applicability to study receptor recognition by a calicivirus showing binding epitopes for several histo-blood group antigens. The same study also used fragments of the virus and showed that L-fucose is a minimal structural requirement for specific molecular recognition by virus particles (52).

One major limitation of STD-NMR is that very tight binders (K_D <= 10nM) score as non-binders causing false negatives in screening. Also, the necessity to have a large excess of ligand limits STD-NMR to reasonably soluble compounds (> 500 μM) although addition of compounds in DMSO helps to overcome this limitation.

Saturation transfer double difference (STDD-NMR) is an extension of the STD-NMR approach which allows observation of ligand binding to membrane proteins in entire cells. Two STD-NMR spectra of the sample are acquired, one with addition of the ligand and a second one without the ligand. Finally the difference of the two spectra is used. This approach requires extreme care for sample preparation and is very demanding in terms of instrument stability owing to its nature as a double difference method. Meyer and coworkers have used this approach to study binding properties of a pentapeptide to integrin $alpha_{IIb}alpha_3$ in living cells and liposomes (53).

5.3. NOE PUMPING

NOE pumping is closely related to STD-NMR. In a NOE pumping experiment magnetization is transferred by an NOE from the protein to the ligand. Protein magnetization is selected by a diffusion filter which cancels the signals of the ligand and is transferred to the ligand by intermolecular cross-relaxation. Ligand molecules in fast exchange which experience magnetization transfer from the protein are detected. All other small molecules will not show signals owing to their large diffusion coefficient. NOE pumping has been demonstrated using human serum albumin and a mixture of the three compounds salicylic acid, L-ascorbic acid and glucose where only the signals of salicylic acid were observed in the NOE pumping spectrum. The same ligand could not be detected in a pure diffusion edited experiment without NOE transfer (20, 21).

In the *reverse NOE pumping experiment (RNP)* the ligand spectrum is selected employing a transverse relaxation filter which eliminates the signals of the protein and inverts the ligand signals. This filter exploits the much faster transverse relaxation rate (R_2) of the protein compared to the slower relaxation of the ligand. During a mixing time ligand magnetization is partially transferred to the protein via intermolecular cross-relaxation. A reference experiment in which no magnetization transfer takes place is subtracted. In the difference spectrum only signals of compounds which interact with the protein are observed. This experiment was used for human serum albumin with glucose and a mixture of unbranched fatty acids as putative ligands. Only the fatty acids were observed in the difference spectrum. Intensity ratios between different ligands were used to rank the affinities of different compounds.

NOE pumping and reverse NOE pumping are very sensitive experiments for primary NMR screening, the latter is considered to be more sensitive. As a consequence of spin diffusion epitope mapping is not feasible.

5.4. WaterLOGSY and SALMON

The WaterLOGSY (Water-Ligand Observed via Gradient SpectroscopY) experiment represents another variation of magnetization transfer between a protein and a ligand in which protein magnetization is selected through water molecules bound to the protein (26). This may include water bound at the protein ligand interface but also surface bound water. Magnetization from water molecules is selected using a selective pulse followed by a mixing time during which magnetization is transferred from water to the protein and to the ligand. At the same time magnetization can also be transferred directly from bulk water to the ligand without the pathway through the protein (see Figure 1). For the direct interaction between the water and the ligand the NOE is positive and small, leading to negative signals in the final spectrum whereas the NOEs arising from the magnetization pathway via the protein are negative (= positive signals in the spectrum) due to the larger auto-correlation time of the protein. It is important to note that most pharmaceutical compounds are very hydrophobic and therefore the direct interaction between the water and the ligand molecules is relatively inefficient. The interaction between the ligand and the protein is much tighter allowing a better magnetization transfer. This adds to the more favorable cross-relaxation rate for large molecules.

Because an excess of ligand is used, it is always the signal of the free ligand that is observed. Depending on the dissociation constant of the complex either the contribution of negative NOEs typical for the protein or positive NOEs for small molecules will prevail. This allows a fast distinction between molecules which bind to the protein and those which do not bind because NOEs of the two will have opposite signs in the spectra.

WaterLOGSY experiments have a great potential in primary NMR screening. The experiment is very sensitive and requires only very low protein and modest ligand concentrations. Signals of non-binding ligands have opposite sign compared to signals of ligands which bind with high affinity. The dissociation constant of complexes may be as low as 10 nM.

WaterLOGSY and epitope mapping. While the initial waterLOGSY experiment seemed to be limited to the identification of binders *vs* non-binders recent work showed that waterLOGSY can be used to probe for bulk water accessibility to derive the orientation of a bound ligand. This approach has been termed SALMON (*Solvent Accessibility and protein Ligand binding studied by NMR Spectroscopy*) (27, 28). To be able to derive a solvent accessibility epitope from waterLOGSY spectra shorter mixing times have to be chosen to avoid blurring of of the epitope by massive spin diffusion. SALMON also requires a slightly modified pulse sequence employing gradient selection of the transfer pathway rather than a phase cycle.

For short mixing times a solvent accessibility epitope can be derived when at least parts of the ligands are partially solvent accessible when bound to the protein by comparing signal intensities in the SALMON spectrum with those in a normal 1D spectrum.

The information content of these spectra is complementary to STD-NMR spectra. Moreover, other solvents than water or co-solvents have been used as a starting point for the magnetization transfer to probe for different polarities of the binding region (28).

5.5. INPHARMA

Magnetization can also be transferred from one ligand molecule to another via the protein if the exchange rate is sufficiently fast (see Figure 2). Carlomagno has used this effect to transfer magnetization between to different ligands which bind in the same binding pocket (29). Magnetization that is transferred from one ligand to the other can be seen as additional cross peaks in a 2D-NOESY spectrum. If the complex structure is known for one of the ligands, the ligand orientation of the other ligand can be derived from the additional cross peaks in the spectrum.

This method requires two ligands at a reasonable concentration to be present in a low concentration protein solution. While long instrument times are required it has great potential to map binding sites when the orientation of one ligand is known from prior structural analysis. This has been demonstrated for tubulin-bound epothilone A in competition with discodermolide or baccatin III (29, 30).

5.6. SAR by ILOEs

The fragment based approach for drug discovery usually results in weakly binding compounds. Frequently the binding site on the protein allows for the simultaneous binding of more than one compound. In this case direct interligand NOEs (ILOEs) have been observed in two-dimensional NOESY spectra. Becattini and Pellecchia have used ILOEs to identify pairs of weakly binding compounds and chemically linked them to convert them into high affinity ligands (54). They termed this method SAR by ILOEs. The ligand/ligand interaction can then be used for a pharmacophore-based search for bi-dentate compounds (55). The advantage of this approach is that it is possible to screen for a library of weakly binding molecules and convert those into high affinity ligands.

6. EXPERIMENTS USING FLUORINATED REPORTER MOLECULES (FABS, FAXS)

Dalvit and coworkers developed two ^{19}F-observed methods for high throughput NMR screening (56, 57). One of them uses 3-FABS (*3 Fluorine Atoms for Biochemical Screening*), a substrate with a CF_3-group, and observes a change in chemical shift upon substrate conversion. While this method requires the introduction of a reporter group into a substrate it can be used to obtain kinetic and inhibition data.

The FAXS (*Fluorine chemical shift Anisotropy and Exchange for screening*) method uses ^{19}F-labeled reporter molecules. Here a known weak binder (the 'spy' molecule) and a second molecule (the 'control' molecule) which is known not to bind to the protein are labeled with fluorine. Any molecule that binds to the same binding pocket on the protein will compete with the spy molecule and will alter the amount of time the spy molecule spends on the protein molecule. When bound to the protein the spy molecule is not observed because slow tumbling broadens its signal substantially owing to the large chemical shift anisotropy of ^{19}F. When a competing inhibitor binds the signal of the reporter molecule is gradually restored. The advantages of this approach are that ^{19}F resonances of the spy molecule are recorded for which no water suppression is required and that high affinity ligands can be detected.

7. CONCLUSIONS

Ligand-based NMR methods offer a broad spectrum of possibilities for small molecule screening and lead optimization using binding epitopes. While many of these methods have now been available for several years, there are still gradual improvements. In the future these methods will be applied to increasingly complex proteins, including membrane proteins, proteins with relatively low solubilities and large protein complexes. The challenge is to integrate ligand-based NMR into an efficient drug screening and drug design process, at a sufficiently early stage to harness the best possible impact. In this context the preparation of proteins with limited solubility becomes the most limiting requirement which deserves further attention to apply these technologies to their full capacity.

8. ACKNOWLEDGEMENTS

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Abbreviations: 1D = one dimensional; 2D-NOESY = two-dimensional nuclear Overhauser enhancement spectroscopy; DECODES = diffusion encoded spectroscopy; DMSO = dimethylsulfoxide; EGFR-ED = epidermal growth factor receptor; FABS = fluorine atoms for biochemical screening; FAXS = fluorine chemical shift anisotropy and exchange for screening; FKBP = FK506 binding proteins, family of proteins that have prolyl isomerase activity; ILOE = interligand NOE; INPHARMA = inter-ligand NOEs between to competively binding molecules; KD = dissociation constants; NOE = nuclear Overhauser effect; NOESY = nuclear Overhauser enhancement spectroscopy; hIgG = chimeric immunoglubulin G; HMQC = heteronuclear multi-quantum correlation spectroscopy; HR-MAS NMR = high resolution – magic angle spinning NMR; HSQC = heteronuclear single-quantum correlation spectroscopy; HTS = high-throughput screening; PFG-SE = pulsed field gradient spin echo; PFG-STE = pulsed filed gradient stimulated echo; RF = radiation frequency; RNP = reverse NOE pumping; SALMON = solvent accessibility and protein ligand binding studied by NMR spectroscopy; SLAPSTIC = spin labels attached to protein side chains as a tool to identify interacting compounds; SAR by NMR = structure activity relationship by nuclear magnetic resonance; STD = saturation transfer difference; STDD-NMR = saturation transfer double difference NMR; STE NMR = stimulated echo; TGF-α = transforming growth factor; TINS = target immobilized NMR screening; TOCSY = total correlation spectroscopy; Tr-NOE = transferred NOE; TROSY = transverse relaxation optimized spectroscopy; waterLOGSY = water-ligand observed via gradient spectroscopy

Key Words: High-throughput screening, Structure Activity Relationship, Drug-Protein Interactions, Bioaffinity Study, Protein Relaxation, Protein Binding Sites, Small Molecule Screening, Review

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