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# Labeled Ligand Displacement: Extending NMR-Based Screening of Protein Targets

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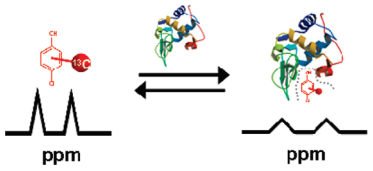
**ABSTRACT** NMR spectroscopy has enjoyed widespread success as a method for screening protein targets, especially in the area of fragment-based drug discovery. However, current methods for NMR-based screening all suffer certain limitations. Two-dimensional methods like "SAR by NMR" require isotopically labeled protein and are limited to proteins less than about 50 kDa. For one-dimensional, ligand-based methods, results can be confounded by nonspecific compound binding, resonance overlap, or the need for a special NMR probe. We present here a ligand-based method that relies on the exchange broadening observed for a $^{13}$C-labeled molecule upon binding to a protein target (labeled ligand displacement). This method can be used to screen both individual compounds and mixtures and is free of the artifacts inherent in other ligand-based methods.

**KEYWORDS** NMR, screening, fragment, labeling, ligand




Over the last few decades, nuclear magnetic resonance (NMR) spectroscopy has developed into a powerful tool for the study of biological molecules and systems.[1] The structure determination of small, well-behaved proteins and polynucleotides in solution using NMR has become routine. To complement this structural information, NMR spectroscopy can also provide information on the molecular dynamics of these macromolecules.[2] However, its most important contribution, especially in the area of pharmaceutical research, may be the ability of NMR spectroscopy to provide information about molecular interactions at the atomic level. This information includes, first and foremost, determining whether or not a particular small molecule binds to a protein of interest. Second, if the molecule does bind, which atoms of both the small molecule and the protein are involved in this interaction? Various NMR-based techniques have been developed to address these two points. These methods provide varying levels of detail and have been met with varying degrees of success.[3]

One of the earliest of these techniques to be developed was "structure−activity relationships (SAR) by NMR", which was the first method to be truly applied in a screening mode, that is, testing thousands of small molecules for binding to a protein target.[4] Since the initial publication describing SAR by NMR, the use of NMR in lead discovery and optimization has become widespread in the pharmaceutical industry. Most notably, SAR by NMR played an integral role in the discovery of the pro-apoptotic molecules ABT-737 and ABT-263, the latter of which is currently being tested clinically as an anticancer agent.[5]

SAR by NMR is a target-based method where the chemical shift of protein resonances, from either backbone amides or side chain methyl groups, is monitored as the protein is interrogated with a single small molecule or a mixture of small molecules. While the approach continues to be the most robust and reliable method for NMR-based screening of protein targets, it suffers from several disadvantages. One major limitation of traditional SAR by NMR is protein size. As the size of a protein increases, its rotational correlation time decreases, and subsequently, the resonance line width increases. The heteronuclear single quantum coherence (HSQC) spectrum upon which this method is based not only becomes more crowded due to a greater number of resonances, but the individual resonances become broader until the spectrum is no longer interpretable. In using this method, the realistic upper limit for the protein molecular mass is roughly 50 kDa. In addition, protein aggregation can rapidly degrade the quality of the HSQC spectrum regardless of protein size and can be a major hindrance to the SAR by NMR method. Finally, SAR by NMR requires isotopically labeled (either $^{15}$N or $^{13}$C) protein, which, in many cases, can be difficult to obtain.

In light of these limitations, other NMR-based methods have been developed, which, by contrast, monitor the change of a specific ligand property upon binding to a target protein. These ligand-based methods include saturation transfer difference (STD) spectroscopy,[6] waterLOGSY,[7] SLAPSTIC,[8] TINS,[9] FAXS,[10] one-dimensional direct competition methods,[11] and others.[3] Unfortunately, ligand-based methods also suffer from various limitations, the most common of which are interference from nonspecific binding of a small molecule to a target protein and resonance overlap in the one-dimensional spectra that are typically recorded for these ligand-based methods. The exception to this is the fluorine-based FAXS method. Therefore, there is a need for an improved and more reliable ligand-based

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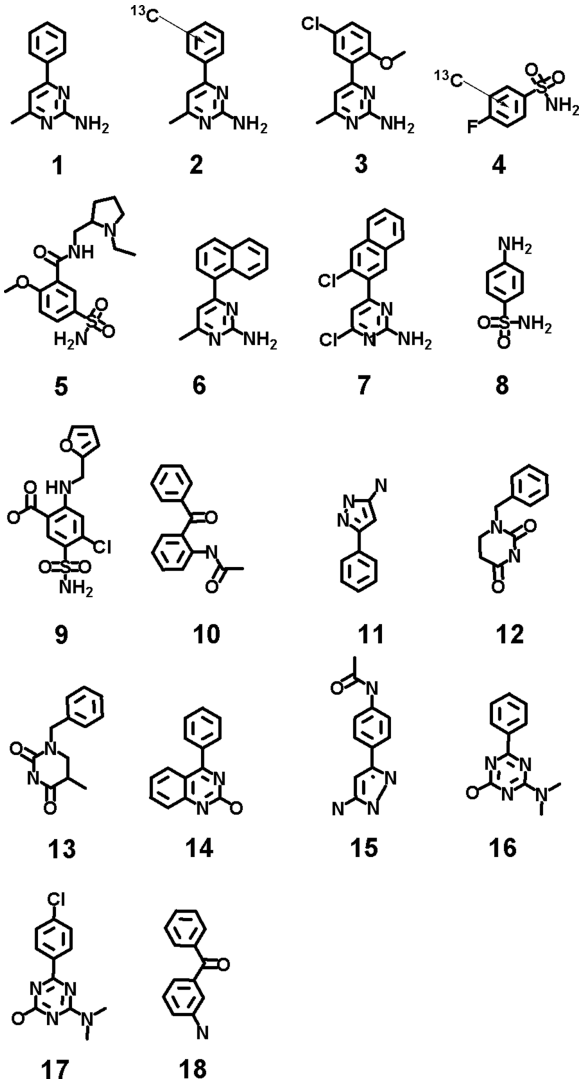


**Figure 1.** Various compounds employed in this study.

method that overcomes these limitations and can be executed using standard triple-resonance technology.

Herein, we describe a new ligand-based method that we have termed "labeled ligand displacement" (LLD), which is based on the displacement of an isotopically ($^{13}$C) labeled ligand from a protein target of interest upon addition of an individual compound or a mixture of compounds. The method is not limited by the size of the protein, is less sensitive to nonspecific compound binding than other ligand-based methods, and does not require preparation of isotopically labeled protein. It compares favorably to STD, the most widely employed ligand-based method, and offers some distinct advantages. Finally, LLD can be implemented with standard NMR technologies, including standard triple-resonance probes, and will greatly increase the number of protein targets for which NMR-based screening, especially fragment screening, can be applied.

The interaction of most small ligands with a protein target can be treated in terms of a simple two-site exchange

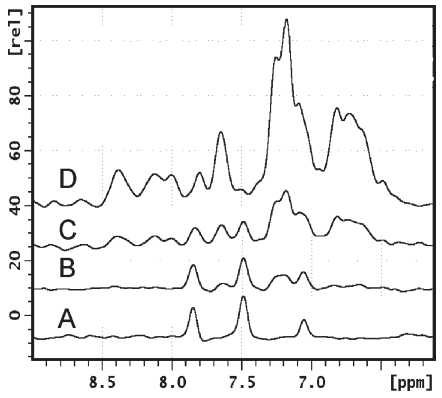


**Figure 2.** Effect of increasing protein (Hsp90, ATPase domain) concentration on the aromatic resonances of compound **1** (20 uM) in a proton NMR spectrum. (A) No protein, (B) 5 $\mu$M protein, (C) 10 $\mu$M protein, and (D) 30 $\mu$M protein.

between free and bound species. For a ligand that is in the fast to intermediate exchange regime with a protein target, the exchange-broadening contribution to the resonance line width at half height is given by:

$$\Delta\nu_{1/2} = (\tau_A \pi)^{-1}$$

where $\tau_A$ is the protein-bound lifetime of the ligand. Two factors affecting this bound lifetime are the inherent affinity of the ligand for the protein, which can be expressed as a dissociation constant, $K_D$ (equal to the ratio of $k_{on}$ to $k_{off}$), and the ratio of the protein to the ligand in solution. Thus, for a ligand with a given affinity for its protein target, there is a ratio of protein to ligand, which can be determined empirically, where this exchange-broadening contribution will be at its maximum.

This exchange-broadening effect could, in principle, be employed either directly or in competition mode, much like STD, to monitor ligand binding to a protein. To directly observe binding of a ligand to a protein, the ligand must have at least one resonance that does not overlap with signals from the added protein. However, for most ligands, this is most likely the exception rather than the rule. Similarly, to be used in a competition mode, the "probe" molecule must have at least one resonance that does not overlap with signals from the protein or with signals from potential binders. This overlap would likely preclude the screening of mixtures.

This dilemma is illustrated in Figure 2 for the binding of compound **1** (Figure 1) to the ATPase domain of heat-shock protein 90 (Hsp90).[12] Hsp90 is an intracellular molecular chaperone protein that consists of three domains and is widely expressed in various cells.[13] Targeting of the amino-terminal ATPase domain (MW ~ 28 kDa) in cancer cells by various molecules has been shown to have an antiproliferative effect.[14] Figure 2A shows the aromatic region of a one-dimensional proton spectrum of compound **1** in solution. This compound binds to the ATPase domain of Hsp90 with an inhibition constant ($K_i$) of 14 $\mu$M as determined in a fluorescence resonance energy transfer assay (TR-FRET).[12] As shown in Figure 2B–D, the addition of increasing amounts of protein causes broadening of the ligand signals. However, it is virtually impossible to monitor





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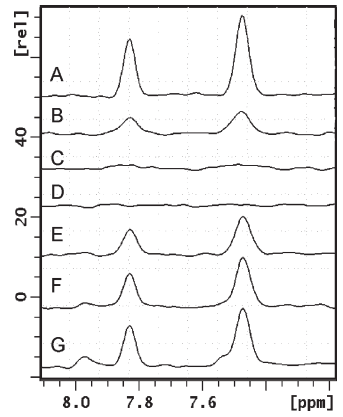


**Figure 3.** (A−D) Effect of increasing protein (Hsp90, ATPase domain) concentration on the aromatic resonances of compound **2** (20 $\mu$M) in a $^{13}$C-selected, proton NMR spectrum. (A) No protein, (B) 10 $\mu$M protein, (C) 20 $\mu$M protein, and (D) 30 $\mu$M protein. (E−G) LLD spectra for compound **2** (20 $\mu$M) in the presence of 30 $\mu$M protein plus increasing concentrations of compound **3**: (E) 100 $\mu$M, (F) 200 $\mu$M, and (G) 400 $\mu$M.

this broadening at higher protein concentrations due to resonance overlap with signals from the protein itself.

To overcome this limitation, a version of compound **1**, which contains a uniformly $^{13}$C-labeled phenyl ring, was prepared by coupling 4-chloro-6-methylpyrimidin-2-amine with $^{13}C_6$-labeled phenyl boronic acid (Supporting Information). Figure 3A shows a one-dimensional $^{13}$C-selected proton spectrum of the $^{13}$C-labeled ligand in buffer alone. This spectrum is essentially the first slice of a $^{13}$C-HSQC spectrum. In panels B−D is shown the effect of increasing concentrations of the unlabeled ATPase domain of Hsp90 on the ligand resonances. In contrast to the case with unlabeled ligand, it can clearly be seen that at a 1:1 molar ratio of protein to ligand, the ligand resonances are completely broadened out (Figure 3C). Upon addition of a competing ligand (compound **3**, $K_i \sim 11$ $\mu$M), signals from the labeled probe are recovered in a dose-dependent manner (Figure 3E−G). In spectra F and G, where compound **3** is at 200 and 400 $\mu$M, respectively, additional small signals appear at about 7.5 and 8.0 ppm from the 1.1 % natural abundance of $^{13}$C in compound **3**.

A further illustration of the utility of isotopic labeling to cleanly monitor exchange broadening is shown for the binding of $^{13}$C-labeled 4-fluorobenzenesulonamide (compound **4**, Figure 1) to bovine carbonic anhydrase. Carbonic anhydrases are ubiquitous enzymes that catalyze the conversion of carbon dioxide to bicarbonate ion and play an important role in various physiological processes including respiration and certain biosynthetic reactions.[15] Bovine carbonic anhydrase is a ~30 kDa protein that is inhibited by various sulfonamide-based ligands. Using surface plasmon resonance, the affinity of an unlabeled version of compound **4** was found to be about 1 $\mu$M for bovine carbonic anhydrase.[16] Compound **4** was made by treating uniformly $^{13}$C-labeled 4-fluorosulfonylchloride with ammonia in methanol (Supporting Information). Figure 4 illustrates the reduction in intensity of the aromatic resonances from the ligand in response to increasing protein concentration. At a 2:1 molar ratio of protein to ligand, the resonances from the free

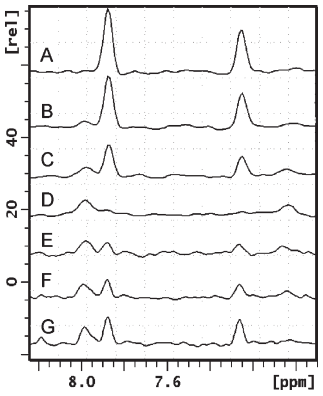


**Figure 4.** (A−D) Effect of increasing protein (carbonic anhydrase) concentration on the $^{13}$C-selected, proton NMR spectrum of compound **4** (20 $\mu$M). (A) No protein, (B) 10 $\mu$M carbonic anhydrase, (C) 20 $\mu$M carbonic anhydrase, and (D) 40 $\mu$M carbonic anhydrase. (E−G) LLD spectra for compound **4** (20 $\mu$M) in the presence of 40 uM protein plus increasing concentrations of compound **5**: (E) 100 $\mu$M, (F) 200 $\mu$M, and (G) 400 $\mu$M.

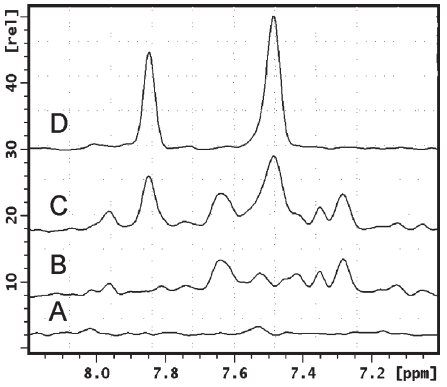


**Figure 5.** LLD method applied to screening mixtures. (A) Reference spectrum of Hsp90, ATPase domain plus probe (30 $\mu$M protein and 20 $\mu$M compound **2**). (B) Plus mixture of nine compounds (**10**−**18**), at 200 $\mu$M each, which do not bind to the protein. (C) Addition of 200 $\mu$M compound **3** ($K_i \sim 6$ uM). (D) Probe alone (20 $\mu$M).

ligand have completely broadened out. The two broad signals in spectrum D presumably arise from bound ligand. As shown for the Hsp90, ATPase domain, the addition of a competing ligand (compound **5**, $K_i \sim 48$ uM) results in recovery of the probe signals. In addition to utilizing specifically designed and labeled small molecules as probes, the broadening effect can also be observed for the binding of natural products, such as nucleotides and peptides, to proteins (Supporting Information, Figures 1S and 2S).[17,18]

Besides the screening of individual compounds, LLD can be used to screen mixtures of compounds against a protein target. Figure 5A shows a spectrum of the Hsp90, ATPase domain plus probe (compound **2**). In spectrum B, a mixture of nine compounds (**10**−**18** in Figure 1), which do not bind to the protein, has been added. These ligands show no perturbation of the protein $^{13}$C-methyl resonances in a $^{13}$C-HSQC spectrum when added at an approximately 10-fold molar excess. As can be seen, no recovery of the probe signals





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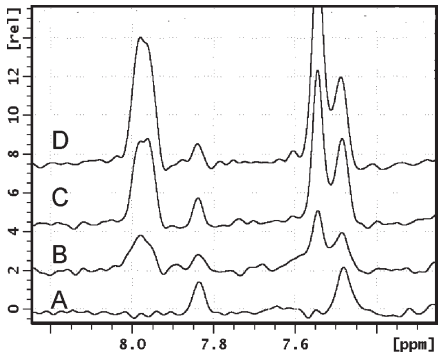


**Figure 6.** STD−competition spectra for the Hsp90, ATPase domain. (A) STD spectrum of probe molecule (compound **1**) at 100 $\mu$M in the presence of 10 $\mu$M protein, (B) plus 100 $\mu$M compound **3**, (C) plus 200 $\mu$M compound **3**, and (D) plus 400 $\mu$M compound **3**.

is observed. The broad background signals that appear between 7.2 and 8.0 ppm are from the 1.1% natural abundance of $^{13}$C in these ligands, which are each at 200 $\mu$M. Upon addition of 200 $\mu$M compound **6** ($K_i \sim 5$ $\mu$M), enough probe signal is recovered above the background that this compound could clearly be flagged as a binder.

Of all of the ligand-based screening methods using NMR, STD spectroscopy continues to be the most broadly applied ligand-based method. It has been applied in both direct and competitive modes and has been used not only to identify protein ligands but also in mapping the binding epitopes of protein-bound ligands.[6,19] The basis for the STD-NMR experiment is the transfer of magnetization from a protein to a bound ligand. While it is a very simple and versatile tool for analyzing protein−ligand interactions, it does suffer from a few significant drawbacks, among which are interference from nonspecific binding and signal overlap.

Figure 6 illustrates the LLD competition experiment of Figure 3E−G but carried out in STD mode. In Figure 6A is the aromatic region of the STD spectrum for compound **1** bound to the Hsp90, ATPase domain. The ligand to protein ratio in this case is 10:1, which is typical for an STD competition experiment. Spectra B−D show the effect of adding increasing amounts of a competing ligand, compound **3**. With compound **3** at 100 $\mu$M (spectrum B), there is a clear decrease in the STD signal. However, as additional compound is added, there is no further decrease in the STD signal (spectra C and D). We have observed this with several other systems in our lab and have attributed it to nonspecific interaction of the STD probe molecule with the protein. This nonspecific binding gives rise to a certain "background" STD signal, which remains even upon further addition of competing ligand. Figure 6 also illustrates the second drawback of the STD−competition method, which is the potential overlap of signals from a competing ligand with the signals from the STD probe molecule. In this particular case, there is only a slight overlap of a resonance from added compound **3** (~7.59 ppm) with a resonance from the probe. However, it is clear from this figure how resonance overlap could be a serious drawback, especially for screening mixtures of compounds. Ideally, one can choose a probe with a resonance well-removed from signals of potential binders, but this may not always be possible.

Resonance overlap is also a potential drawback of the one-dimensional competition method described by Siriwardena et al.[11] In this method, the intensity of an unlabeled probe molecule in the presence of its protein target is monitored as potential binders are added. As with STD, a probe molecule must be found whose NMR signals do not overlap with those from the probe, a limitation that is overcome with the LLD method.

In choosing the $^{13}$C-labeled ligand (probe) for an LLD experiment, two factors need to be considered. The first is the affinity of the probe for the protein. In our experience, probe molecules should ideally have an affinity of between 1 and 100 $\mu$M. For probes that bind more weakly, a large excess of protein may need to be added to observe sufficient exchange broadening. In contrast, it may be difficult to compete away a probe molecule that binds too tightly to its protein target, especially with weakly binding fragments. The second factor to consider is the ease with which an isotopically labeled probe molecule can be obtained. Isotopically labeled amino acids, peptides, and nucleotides are, in general, easily obtained from commercial sources. Synthetic ligands present more of a challenge, as the appropriate isotopically labeled starting materials must be found. However, because only part of a synthetic ligand needs to be labeled, that is, a single phenyl moiety or methyl group, in order for the molecule to work as a probe, this aspect of the method is not as limiting as it may appear.

Screening a protein target for potential binders is carried out by first preparing a stock solution of the isotopically labeled probe and unlabeled protein at the appropriate, experimentally determined, protein to ligand ratio. Compounds can then be added to this stock solution individually or as mixtures. Compound binding will result in displacement of the probe and subsequent recovery of signal. For a series of ligands added at the same concentration, the amount of signal recovered will be proportional to the affinity of each ligand for the protein (Supporting Information, Tables 1 and 2). The sensitivity of this method as a screening tool can be adjusted in two ways, either by varying the concentration of potentially competing ligands or by varying the concentration of the target protein. Higher ligand concentrations can be used to increase the sensitivity of the method for screening weakly binding fragments, while lower concentrations can be used to increase the selectivity of the method. Analogously, the sensitivity/selectivity of the method can be adjusted by varying the amount of target protein. The addition of protein above the amount needed to observe maximal broadening will increase the selectivity of the method as the additional protein will act as a "sink" for competing ligands.

In addition to simple binding conformation, this method could be used to quickly obtain a rough estimate of the $K_D$ value for a given ligand, provided that a few ligands of known and varying affinity are available. These known ligands could be used to derive a calibration curve by plotting the percent recovered intensity for each ligand against its affinity. Recovered intensity values for ligands of unknown affinity could then be estimated from this curve.

We have developed a new ligand-based method for NMR screening that we have named LLD as it is based on the displacement of a carbon-13-labeled probe molecule from a



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protein target. It is a very simple method to apply and has advantages over both the traditional protein-based screening methods such as "SAR by NMR" as well as other ligand-based methods such as STD. As compared to protein-based methods, LLD can be applied to any size protein, and in fact, the broadening effect on which the method is based should be enhanced for larger proteins. While the amounts of protein needed are about the same as for protein-based methods, isotopically labeled protein is not needed, and thus, protein from sources other than recombinant *Escherichia coli* grown in minimal media can be used. As compared to STD methods, LLD allows one to filter out signals from competing ligands, which is especially advantageous for the screening of mixtures. In addition to the utility of LLD for traditional small molecule or fragment screening, it could also, in principle, be used as an orthogonal assay for essentially any soluble protein target.

**SUPPORTING INFORMATION AVAILABLE** Experimental procedures along with two figures showing the exchange-broadening effect for a $^{13}$C-labeled peptide bound to the protein Bcl-$x_L$ and for $^{13}$C-labeled ATP bound to Hsp90 and two tables that show the amount of signal recovered upon addition of competing ligands for both Hsp90 and carbonic anhydrase. This material is available free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

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