Second-Site NMR Screening with a Spin-Labeled First Ligand

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NMR spectroscopy in drug discovery research has recently widened its scope. Traditionally, biomolecular NMR has been primarily used to support lead optimization by providing structural information about lead compounds complexed to the target molecule (often a protein) under investigation. More recently, NMR has been recognized as a technique that is also valuable for lead finding. In a pioneering technique termed "SAR by NMR",[1] NMR screening[2] is used to identify a ligand for a first binding site on the target. At saturating concentrations of this first ligand, NMR screening is then used to discover a second ligand that binds to the target simultaneously and in the vicinity to the first ligand ("second-site screening"). The structure of the ternary complex is determined and used to guide chemistry to connect both ligands.[1,3] Due to additive binding energies and favorable entropic effects, the resulting linked compound may be a nanomolar ligand for the target, although both individual fragments exhibited only millimolar or micromolar affinity. Here we show that many of the exisiting problems in second-site screening, such as the occurrence of false positives, excessive protein demands, low sensitivity, insufficient compound solubility, and difficult automation, can be eliminated by using a spin-labeled analogue of the first ligand.

Spin−spin (R2) relaxation rates are proportional to the product of the squares of the involved spins' gyromagnetic ratio. The gyromagnetic ratio of protons is small, and R2 relaxation rates are thus relatively small. This is bliss for most aspects of NMR; however, the method presented here uses spin−spin relaxation to detect ligand binding, and thus spin−spin relaxation rates should be as large as possible. The gyromagnetic ratio of an unpaired electron is 658 times as large as that of a proton. Hence, R2 relaxation effects on protons by an unpaired electron, called paramagnetic relaxation enhancements, $R2_{para}$, are dramatically larger than the effects of a nuclear−nuclear interaction. Consequently, NMR studies employing reagents with unpaired electrons ("spin labels") have been successful in measuring long distances, dynamics, or surface accessibilities in proteins.[4] Here we introduce a new application of spin labels in biomolecular NMR to detect simultaneous binding of two ligands to a target protein. The method detects the paramagnetic relaxation enhancement on a second ligand caused by a spin-labeled first ligand, if and only if both ligands are bound to the target protein at the same time and at neighboring binding sites (Figure 1).

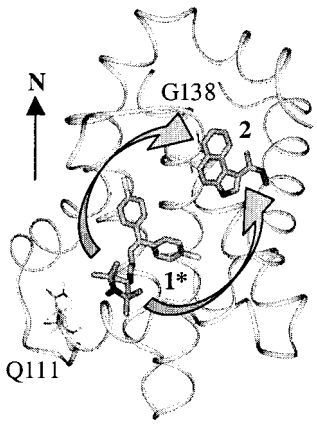
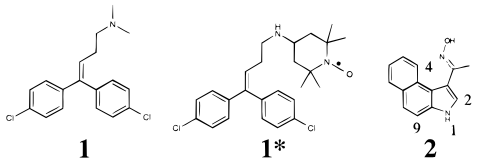


Figure 1. Principle of second-site screening using a spin-labeled first ligand. The spin-labeled first ligand (**1***) binds in the "southern" binding site of Bcl-xL. If and only if a compound binds simultaneously at a neighboring binding site, like compound **2**, does it experience a paramagnetic relaxation enhancement (thick arrows).

The method is illustrated with the anti-apoptotic protein Bcl-xL as an example. Bcl-2 and Bcl-xL are primarily responsible for the reduced susceptibility of cancer cells to undergo programmed cell death (apoptosis) upon chemotherapy, and are therefore interesting targets for cancer therapy.[5] In-house high-throughput screening using Bcl-2/Bax and Bcl-xL/Bax ELISA assays identified compound **1** which has an IC_{50} value of 140 μM for disruption of the Bcl-xL/Bax interaction. The affinity of this compound could not be optimized by traditional medicinal chemistry. The binding site of **1** on Bcl-xL was determined by NMR. It was found to overlap with the binding site of the N-terminal part of the Bak peptide, the natural antagonist.[5d,6] This binding site is in the "southern" part of the hydrophobic groove and is formed by the side chains of residues Leu 130, Leu 108, Ala 142, and Phe 105 (Figure 1). There is an adjacent binding site, termed the "northern" binding site, where the C-terminus of Bak peptide binds, and for which a ligand is sought by second-site NMR screening. Second-site screening using existing methods[2] was hampered by the inability to saturate the southern binding site with **1**, due to its low aqueous solubility.



Compound **1***, an adduct of **1** with a TEMPO[7] spin label, was synthesized by treatment of 4-bromo-1,1-di(4-chlorophenyl)-1-

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(6) Compound **1** was found to also bind weakly to the "northern" binding site. Although this feature is interesting, it is not relevant in the present context since relay or spin diffusion effects via the protein can be excluded. Compound **2** binds exclusively to the northern binding site.

(7) TEMPO, 2,2,6,6-tetramethyl-1-piperidine-1-oxyl. TEMPO is commercially available with a variety of functional groups.

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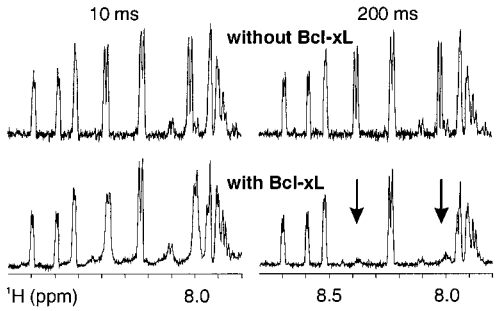

Figure 2. ^{1}H spectra of a library of 8 aromatic compounds in the presence of spin-label **1***, and in the absence (upper trace) and presence (lower trace) of Bcl-xL. The active compound **2** can be easily identified since its two resonances (arrows) are broader and vanish in the presence of Bcl-xL. Concentrations were 500 μM for library compounds, 50 μM for spin label **2**, and 100 μM for Bcl-xL (lower trace only). Spin lock durations were 10 (left column) and 200 ms (right column).

butene with 4-amino-TEMPO.[8] Compound **1*** approximately retains the affinity of **1**, which confirms earlier SAR studies that showed that the amine substituents can be altered without reducing binding affinity. The binding mode of **1*** on Bcl-xL was determined by NMR experiments that detect the paramagnetic enhancement, $R2_{para}$, of the R2 relaxation rate on amide groups of ^{15}N-labeled Bcl-xL. The paramagnetic center was found to be very close to the NH_2 side chain protons of Gln 111 (Figure 1). Using spin label **1***, a library of 8 compounds was screened for simultaneous binding to Bcl-xL. R2 (R1ρ) relaxation rates were measured by recording one-dimensional ^{1}H NMR spectra after a 90° pulse and a spinlock of variable duration (10−200 ms). Paramagnetic relaxation enhancement of the second-site ligand is evidence for simultaneous binding. Indeed, protons of some ligands experienced a drastic increase in their R2 relaxation rate (Figure 2), which proves binding to Bcl-xL simultaneously with and in the vicinity of the spin-labeled first ligand.[9] Among the hits was compound **2**, an aromatic ketoxime. Chemical shift analysis of ^{15}N-labeled Bcl-xL in the presence and absence of **2**, and intermolecular protein−ligand NOE effects unambiguously confirmed binding of **2** to the northern binding site, formed by residues Phe 97, Gly 138, Val 141, and Tyr 195. The affinity to Bcl-xL of **2** is approximately 1 mM, yet it could be easily detected with spin-labeled ligand **1***. In control experiments with compound **2** in the presence of spin label, but without Bcl-xL, no paramagnetic enhancement could be detected within experimental error, indicating that only simultaneous binding to Bcl-xL brings compound **2** and the spin label close enough together to convey paramagnetic relaxation enhancement to **2**.[10]

Paramagnetic relaxation enhancements were analyzed for each individual proton in **2**. It was found that protons around the indole nitrogen (protons 9, 1, 2, and 4) experience a larger paramagnetic relaxation enhancement than protons on the other side of **2**. This indicates that the lower part of **2**, in the orientation as displayed in Figure 1, is oriented toward the spin label. Thus, the spin label experiment gives information about the relative orientation of the second ligand, which is valuable for the design of a linker between both ligands.

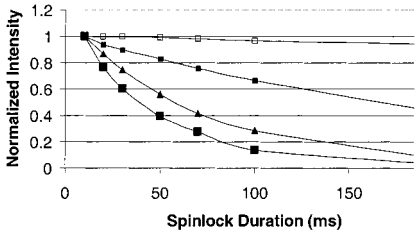

Figure 3. Normalized intensities of proton 4 from compound **2** as a function of spin lock duration. Bcl-xL concentrations were 0 (open squares), 10 μM (small filled squares), or 100 μM (triangels). Spin label **1*** was present in 50 μM concentration, except for one data series (large filled squares, 100 μM concentration). The concentration of **2** was 500 μM.

A severe drawback of NMR screening has been the requirement for large protein quantities, typically in the range of 50 to 500 mg per screen, since existing NMR screening methods require protein concentrations of up to 100−250 μM.[1] Figure 3 shows that concentrations of 10 μM (unlabeled) Bcl-xL are sufficient to unambiguously identify compound **2**. The measuring time even at 10 μM Bcl-xL concentration was only 10 min. Model calculations show that a compound with 10-fold higher affinity (K_D = 0.1 mM) can be detected within the same time using 1 μM Bcl-xL solutions. The protein concentration can be even lower if the second ligand binds closer to the TEMPO moiety than in the present case. Commercially available cryoprobes will further reduce it to the nM range. This represents a breakthrough reduction in protein demands by 1 or 2 orders of magnitude.

Second-site screening with a spin-labeled first ligand has a great number of advantages. First, it is the only currently available method that detects only true second-site ligands, i.e., ligands that bind to the target protein simultaneously with and in the vicinity of the first ligand. Other existing methods[2] detect false positives if the protein is not fully saturated with the first-site ligand. Full saturation with the first ligand requires high aqueous solubility for weakly binding ligands, which is often not given. Second, it is extremely sensitive and reduces the required protein amounts by 1 or 2 orders of magnitude. The protein can be unlabeled, only partially purified, and there are no limits for its molecular size. Third, it is extremely easy to set up and analyze, and thus highly amenable to automation. Fourth, it is insensitive to slight variations in pH or solvent viscosity, which can easily lead to false positives with other currently available screening methods. Last, the orientation of the second ligand with respect to the first ligand is a byproduct of the method, and provides critical information for optimal linkage of both ligands. A drawback of the method is the need for a spin-labeled first ligand, where the spin label is attached to solvent-exposed regions. Once this synthetic design hurdle is passed, however, the advantages of second-site screening with a spin-labeled first ligand are clear. The new method has a strong potential to become a standard NMR screening procedure.[11]

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(8) 4-Bromo-1,1-di(4-chlorophenyl)-1-butene was synthesized in two steps by condensating 4,4′-dichlorobenzophenone with the Grignard reagent derived from cyclopropyl bromide to yield the corresponding cyclopropyl alcohol in 80% yield. The alcohol, on treatment with hydrogen bromide in acetic acid, underwent a homoallylic rearrangement to give 4-bromo-1,1-di(4-chlorophenyl)-1-butene in 90% yield.

(9) $R2_{para}$ is much larger than the increase in R2 that is simply due to the higher average molecular weight.

(10) Nonspecific effects are expected only at mM spin label concentrations (ref 4).

(11) If the protein itself is spin-labeled using well-established chemical derivatisation techniques (ref 4), this method also appears suitable to identify ligands for a first binding pocket (manuscript in preparation)