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Letters

SALMON: Solvent Accessibility, Ligand binding, and Mapping of ligand Orientation by NMR Spectroscopy

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Abstract: Quinone oxidoreductase 2 (NQO2) binds the prodrug tretazicar (also known as CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide), which exhibits a profound antitumor effect in human cancers when administered together with caricotamide. X-ray structure determination allowed for two possible orientations of the ligand. Here we describe a new NMR method, SALMON (solvent accessibility, ligand binding, and mapping of ligand orientation by NMR spectroscopy), based on waterLOGSY to determine the orientation of a ligand bound to a protein by mapping its solvent accessibility, which was used to unambiguously determine the orientation of CB1954 in NQO2.

Detection and characterization of specific protein–ligand binding is crucial in structure-based drug design. Several experiments have been proposed to screen for or to characterize protein–ligand interactions observing the NMR[a] signals of the ligand, including transferred NOE,[1–3] saturation transfer difference (STD),[4] pumped NOE,[5,6] waterLOGSY,[7,8] diffusion experiments,[9] and INPHARMA[10] which can determine relative ligand orientations. Unfortunately, only few experiments yield a binding epitope or allow the determination of the orientation of the ligand with respect to the protein. While STD-NMR has frequently been used to map binding epitopes, in particular for protein−oligosaccharide interactions,[11] its application to small ligand molecules has often been difficult to interpret. The waterLOGSY experiment[8] is commonly used for NMR-based ligand screening because it is often more sensitive than most of the other NMR methods used to probe protein–ligand binding, but it has to our knowledge not been used to derive binding epitopes.

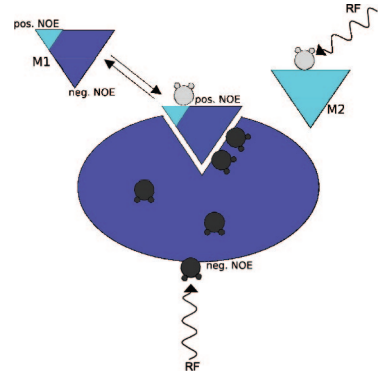

Figure 1. Schematic representation of the ePHOGSY-NOE principle. Selective radiofrequency irradiation of the solvent resonance followed by a NOESY transfer leads to signals with negative NOEs for ligands (M1) that bind to the protein (blue triangles). For molecules that do not bind to the protein (M2), opposite sign signals arise from a positive NOE polarization transfer (cyan). Parts of the protein-bound ligand may retain a positive NOE (cyan in M1) for solvent protons that remain solvent accessible. The polarization transfer may start from water molecules in the active site or on the protein surface or water exchanging with protons on the surface of the protein.

WaterLOGSY is based on the ePHOGSY-NOE experiment, which transfers magnetization via the nuclear Overhauser effect (NOE) and spin diffusion from bulk water molecules via the protein to the ligand. In addition, magnetization may be transferred to the ligand from water bound at the protein–ligand binding site. For small molecules that do not bind the protein, magnetization may also be directly transferred from bulk water to the ligand. Figure 1 summarizes the predominant relay pathways used in waterLOGSY to transfer water magnetization to the ligand. A ligand that binds to a protein experiences the

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[a] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; STD, saturation transfer difference; waterLOGSY, water ligand observed through gradient spectroscopy; ePHOGSY, enhanced protein hydration observed through gradient spectroscopy; INPHARMA, interligand NOE for pharmacore mapping; SALMON, solvent accessibility, ligand binding, and mapping of ligand orientation by NMR spectroscopy.

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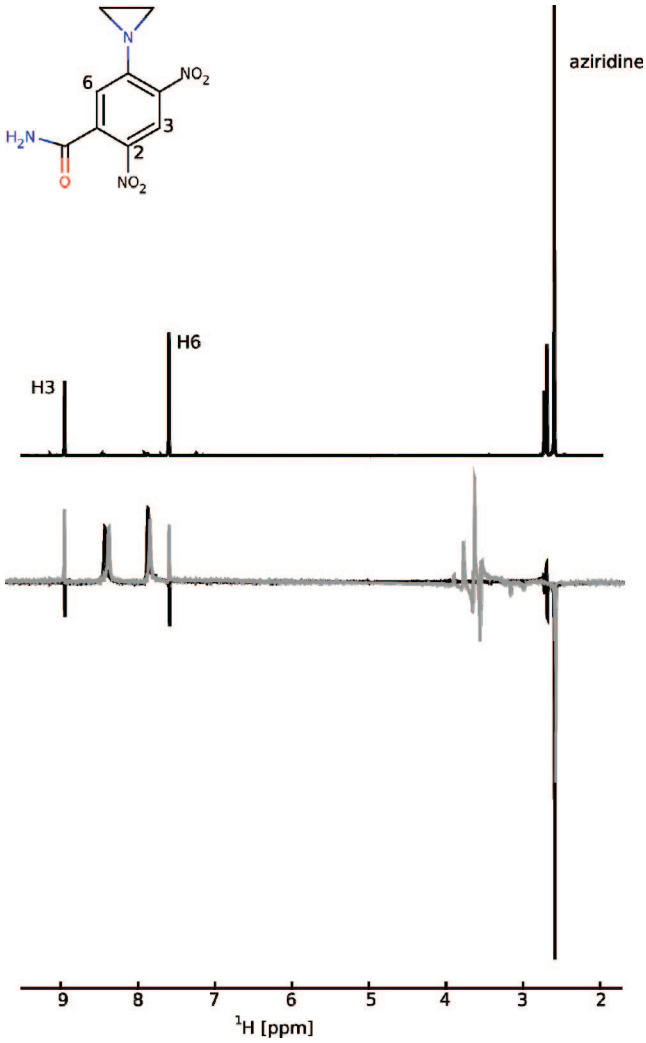

Figure 2. Structure and 1D spectrum (top) of CB1954 and waterLOGSY spectra (bottom) of free CB1954 (black) and of CB1954 bound to NQO2 (gray). The three spectra were recorded in 90% H_2O/10% D_2O at 800 MHz proton frequency. The concentration of CB1954 was 1 mM, whereas the protein concentration was approximately 30 μM; the NOESY mixing time was 1.2 s.

tumbling correlation time of the protein and therefore shows negative NOEs for the transfer of magnetization from the protons of water molecules bound to the protein surface. For sufficiently fast off-rates and with an excess of ligand, the resonances of the ligand in solution will show the effect of the negative NOE from the protein-bound state. In contrast, small molecules that only interact with bulk water experience much faster tumbling and therefore show a positive NOE for the interaction with water molecules. This causes opposite sign for signals from free vs protein-bound ligands.

Here we show that waterLOGSY can be used to probe for bulk water accessibility to the ligand and how the orientation of the ligand can be derived. Using this approach, we were able to determine the orientation of the prodrug tretazicar (5-(aziridin-1-yl)-2,4-dinitrobenzamide, also known as CB1954) in its NQO2 (quinone oxidoreductase 2, which originally was believed to use NAD(P)H as a cofactor) binding pocket. The orientation of CB1954 could not be obtained from X-ray analysis because electron densities allowed for two possible orientations of CB1954 (Figure 3).[12]

CB1954 has been shown to have a profound antitumor effect in human cancers when administered together with caricotamide

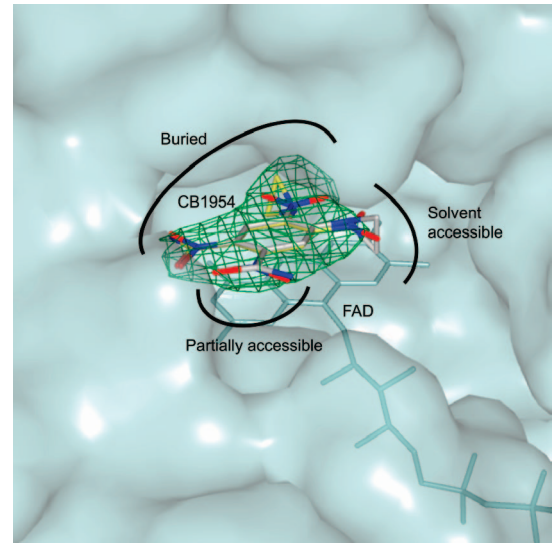

Figure 3. Surface representation of the CB1954 binding pocket of human NQO2. The experimentally determined electron density for CB 1954 ($F_o - F_c$ omit map, contured at 3σ) is shown as green mesh. The two most likely CB1954 poses are indicated, either with white carbon atoms (final model, as verified by this study) or in yellow. The FAD cofactor is shown as light blue stick model. The structure was determined at 2.1 Å resolution (PDB ID 1zx1).

(1-carbamoylmethyl-3-carbamoyl-1,4-dihydro-pyridine), which acts as a cofactor in the reduction of the 4-nitro group of CB1954 to a 4-hydroxylamino group by the human reductase NQO2. In contrast to its rat homologue, NQO1, the human NQO2 cannot use biogenic cosubstrates such as NADH or NADPH as electron donors. However, in the presence of caricotamide, NQO2 can catalyze the two-electron reduction of quinones and the four-electron nitro reduction of CB1954. The coadministration of caricotamide and CB1954 to tumor-bearing mice (prostate and colorectal human cancer xenografts) produces a profound antitumor effect.[13]

Figure 2 shows the 1D spectrum of free CB1954 (upper panel) and the waterLOGSY spectra of CB1954 in absence of protein (lower panel, black) and in the presence of NQO2 (lower panel, gray). The waterLOGSY spectrum of CB1954 in the absence of protein shows five different signals of different sign. The resonances of H3, H6, and the aziridine protons give negative signals whereas two broader signals arising from exchange of the two amide protons with water yield an exchange signal of opposite sign in the waterLOGSY spectrum. The addition of a small amount of NQO2 caused a change of sign for the resonances of H3 and H6, whereas the resonances of the aziridine protons and the HN protons did not change sign. Clearly, the inversion of the H3 and H6 signals represents the typical observation in waterLOGSY spectra, where the ligand shows tumbling properties of the protein causing a negative NOE. The puzzling result for the aziridine protons can be attributed to the fact that this group is accessible to bulk water because it protrudes from the protein (Figure 3). Another possible explanation for this behavior may be free rotation of the aziridine group around the C−N bond. However, we expect that this would potentiate the observed effect because free rotation would be hindered inside the binding pocket. This result is in good agreement with X-ray data, which allowed the orientation derived from these results as one of two possible orientations of the ligand. Further support for our interpretation arises from the fact that the H6 proton shows a reduced intensity compared with H3 in the waterLOGSY spectrum, which is not

the case in the 1D spectrum. This can be attributed to the fact that this proton is closer to the surface than H3, which is completely buried in the protein.

To obtain a better understanding for the magnetization transfer mechanism underlying the waterLOGSY experiment, a NQO2 sample with all protons on nonexchangeable positions replaced by deuterium was prepared and dissolved in water (90% H_2O, 10% D_2O) to be protonated solely in exchangeable positions (some backbone H^N and side chain NH^x). Owing to the substantially reduced network of protons, which is required to transfer the magnetization from the surface through the deuterated protein to the ligand, only the signal that originates from direct contact with the water should be observed in the waterLOGSY spectrum. Figure S1 of the Supporting Information compares the waterLOGSY spectra for the protonated and the deuterated protein. The signal of the aziridine protons shows the same sign and intensity as that for the nondeuterated protein sample, whereas the resonances of the aromatic ring protons now have a very small signal intensity. These results further support the interpretation that the contribution to the aziridine signal in the waterLOGSY spectrum arises solely from the magnetization of the water protons that are in direct contact with CB1954. For the NQO2−CB1954 interaction, the main mechanism for the waterLOGSY magnetization transfer from water to the ligand in the presence of protein must be attributed to water molecules bound to the protein surface. Only a relatively small residual intensity of aromatic ring protons may arise from transfer via water molecules in the binding site or the amide protons on the protein surface near the binding site.

The information obtained from SALMON was used to determine the orientation of the final NQO2 structure.[12] With the aziridine protons pointing toward the solvent, the aromatic ring of CB1954 is oriented such that the side chain of asparagine 161 (N161) forms hydrogen-bonding contacts to the 2-nitro group of CB1954. The arrangement found in the ternary CB1954/NQO2/FAD+ complex is in acceptable agreement with a previously described ping-pong reaction mechanism[14] where the FAD molecule is reduced first by the cosubstrate and subsequently hydride transfer takes place from the reduced isoalloxazine ring to the nitrogen atom of the 4-nitro group of CB1954 to initiate the catalytic reaction sequence, which likely proceeds through a nitroso intermediate to the hydroxylamine.

The interpretation of the sign of signals in SALMON using the waterLOGSY experiment should be broadly applicable to determine binding epitopes for ligands with solvent-accessible protons. With the help of a protein structure, the solvent accessibility can be translated into the orientation of the ligand. Together with INPHARMA,[10] which yields a relative orientation compared with another ligand, this novel application of waterLOGSY will provide valuable information about the ligand orientation without any need for highly soluble and isotopically labeled proteins. We found that it can also be used to derive structural information in cases where proteins form soluble aggregates and are therefore not directly amenable to 2D NMR analysis (data not shown). These results open new avenues for the application of waterLOGSY not only for the identification of protein inhibitors or activators, but on their orientation as well. The SALMON method could be beneficial for directing the process of inhibitor screening, lead optimization, and structure-based drug design.

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Supporting Information Available: The waterLOGSY spectra for the protonated and the deuterated protein and a schematic of identification of solvent-accessible parts of a protein-bound ligand. This material is available free of charge via the Internet at http://pubs.acs.org.

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