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Virus−Ligand Interactions: Identification and Characterization of Ligand Binding by NMR Spectroscopy

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In the search for new antiviral drugs, techniques for identifying and characterizing novel leads become increasingly important. Here, we describe the use of saturation transfer difference (STD) NMR spectroscopy[1−7] to identify and characterize the binding of an antiviral compound to native human rhinovirus serotype 2 (HRV2). The experiments demonstrate that it is well possible to subject targets of the size and complexity of whole viruses (for a model of an HRV2 particle cut open, cf. the table of contents) to STD NMR experiments. The principles of STD NMR have been known for many years,[3,4] but it was only recently that the potential of this technique for screening of libraries for compounds with binding activity toward protein receptors has been realized.[1,2] Probably even more important, the technique is also well suited to analyze binding epitopes of ligands bound to receptor proteins at atomic resolution.[7]

Previous NMR studies of virus−ligand interaction employing NMR chemical shift titrations have required very large quantities of the virus.[8] This approach is impractical, in particular when dealing with pathogenic viruses. On the basis of the example of a low molecular weight inhibitor[9] (Figure 1) that binds to an 8.5 MDa human rhinovirus, we demonstrate that solution state STD methodology not only reduces the amount of virus required by approximately 2 orders of magnitude but also allows for the identification and characterization of virus−ligand interactions at atomic resolution.

The very large size of viruses makes them particularly attractive for studies by STD NMR, as they inherently yield large line widths allowing for the irradiation of the virus without affecting the ligand protons. Furthermore, because of the larger correlation time of a virus in comparison to an average sized protein, spin diffusion and thus saturation transfer is very efficient. The large line width has additional benefits not just for STD-based NMR methods but also for transfer NOESY spectra, as protons from the virus capsid are invisible in the NMR spectra (for an example of a transfer NOESY spectrum, see the Supporting Information).

Human rhinovirus serotype 2 (HRV2), a member of the minor receptor group of the rhinovirus genus within the picornavirus family, contains a positive (messenger) sense 7.5kb RNA[10] with associated polyamine counterions.[11] The RNA is surrounded by an icosahedral protein capsid, consisting of 60 protomers, each comprising 4 polypeptides. The binding site for the antiviral compounds investigated in this study is a hydrophobic pocket within the viral coat protein VP1.[12] As each of the 60 pockets is available for occupation by a ligand, the virus concentration in the samples can be reduced accordingly (in this study to 0.02 μM).

The binding of an antiviral compound **1** to HRV2 was studied at an approximate 6000-fold molar excess over HRV2 virus particles. A series of STD spectra was obtained with saturation times ranging between 0.5 and 4 s. The signal-to-noise ratio in each of the spectra was determined and divided by the time taken to acquire the spectrum. From this, a saturation time of ca. 2 s was found to be the most efficient. Therefore, all subsequent spectra were obtained with this saturation time. The signals of the antiviral compound **1** were clearly observed (Figure 2), demonstrating that this ligand binds to HRV2. A second sample containing methyl-α-D-glucopyranoside and methanol at a ca. 10-fold excess over compound **1** only yielded STD signals from compound **1**, unambiguously discriminating binding and nonbinding compounds (data not shown here, cf. Supporting Information).

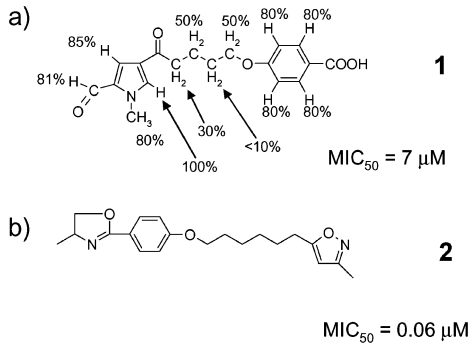

Figure 1. The structures of the antiviral compounds used in this study. (a) Compound **1**, REPLA 394[9] and (b) compound **2**, WIN 52084 (bottom). The MIC_{50} is the concentration resulting in protection of 50% of the cells against infection by HRV14. The percentages shown for REPLA 394 are the results of group epitope mapping, using the method of Mayer and Meyer[7] and the spectra shown in Figure 2. 100% is defined as the peak with the largest intensity in the STD spectrum (Figure 2d) with respect to its counterpart in the reference spectrum (Figure 2a). A larger percentage therefore denotes a closer proximity to the protein surface. Consequently, these protons are important in determining the binding epitope of the ligand, in this case, the protons of the benzene ring and the methyl group of the pyrrole.

To demonstrate specific binding of compound **1** to the hydrophobic pockets of HRV2, a STD titration series was performed using a more potent viral inhibitor, compound **2**[13] (structures and antiviral potencies toward HRV14 are summarized in Figure 1). The addition of compound **2** to a sample containing compound **1** and HRV2 results in a concentration-dependent reduction in the intensity of the STD signals observed for compound **1** (cf. Supporting Information). In contrast, the binding of compound **2** to HRV2 cannot be seen in the STD spectrum at the concentrations employed in this study. The reduction in the STD signal intensities of compound **1** upon addition of **2** can thus be used to infer the presence of another compound competing for the same binding site. In general, this is particularly useful for the (indirect) detection of binding of any ligand with a very small K_D value (less than ca. 1−10 nM), where the binding kinetics lead to very small STD effects.[7,14] It has been demonstrated that competitive STD titration experiments can be used to determine the K_D value of a ligand.[7] Similar strategies to determine K_D values from competition NMR

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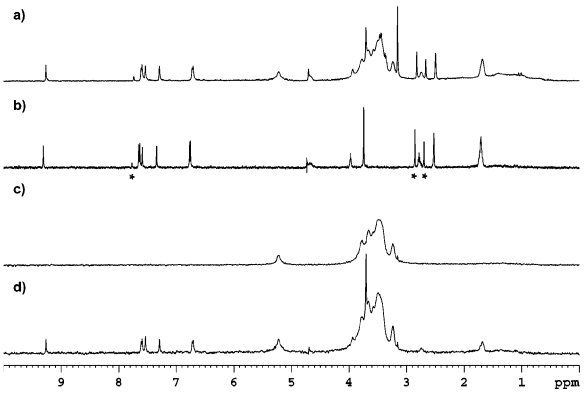

Figure 2. (a) Reference spectrum (with off-resonance irradiation only) for compound **1** and HRV2, (b) A 1D NMR spectrum of compound **1** alone,[20] (c) STD spectrum for HRV2 in the absence of compound **1**, and (d) STD spectrum, for compound **1** (120 μM) with HRV2 (20 nM) at a molar ratio of 6000:1 (100:1 over the binding sites).[20] The difference spectra (c,d) show only peaks for compounds that bind to HRV2. The only peaks visible in (c) correspond to the polyamines that serve as counterions for the RNA in the center of the virion. The spectrum shown in (d) shows additional resonances for compound **1**, which is assumed to bind in the hydrophobic pocket in VP1. The asterisks denote peaks arising from residual dimethylformamide.[20]

experiments have been discussed in the literature recently.[15,16] Because of the low solubility, we were not able to collect any data points at high concentrations of inhibitor **2**. Therefore, we only estimated the order of magnitude of the ratio of K_D values of **1** and **2** on the basis of the protocol of Mayer and Meyer.[7] The estimated ratio of dissociation constants is approximately 1 order of magnitude lower than the corresponding ratio of MIC_{50} values (cf. Figure 1). Nevertheless, we believe that such an estimate is useful because the determination of dissociation constants of small molecule ligands binding to native viruses applying other experimental techniques is not without problems (for the estimation of the range of relative dissociation constants, see the Supporting Information).

To further substantiate that binding of the ligand was specific, HRV2 was thermally denatured. Heating the sample to above 343 K leads to structural changes of the viral capsid, loss of the RNA and VP4, and the formation of subviral particles with the hydrophobic pockets collapsed.[17,18] The sample was thus incubated at 343 K until changes were observed in the NMR spectrum. These changes occurred in about 5 min. The sample was then rapidly cooled to 288 K, and STD spectra in the presence of compound **1** were recorded. The complete absence of STD signals from compound **1** is in accordance with the lack of binding to the deformed pockets.

Analysis of the STD spectra using the group epitope mapping method[7] allows for the determination of the binding epitope of compound **1**. The observed STD amplification factors (cf. Figure 1) are in good agreement with X-ray structures[19] of HRV14 cocrystallized with compound **2**, where the two aromatic moieties of the ligand were found to be involved in a stronger interaction with the protein surface than the aliphatic linker.[13] This demonstrates convincingly that STD NMR methods can considerably speed up the determination of the binding epitope for potential antiviral lead compounds.

As shown here, simple STD NMR experiments are able to provide significant information on the binding of ligands to native viruses requiring only very small amounts of virus and measurement times in the range of tens of minutes. This allows for a high throughput of ligand samples without significant consumption of virus, because it remains unaffected by the experiments and is easily separated from the low molecular weight ligands by ultra filtration. In addition to the detection of binding, a complete mapping of the ligand binding epitope was achieved. To date, cocrystallization of the virus and inhibitor was required to obtain this kind of information. Where sample production, solubility, or viral containment is a problem, the requirement of only small quantities and concentrations is an enormous advantage over other screening methods.

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Supporting Information Available: STD spectra of compound **1** in the presence of HRV2 and nonbinding ligands. Transfer NOESY spectrum of **1** in the presence of HRV2. Data analysis of the competitive inhibition of binding of **1** to HRV2 by compound **2** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(20) REPLA 394 (compound **1**) was synthesized according to Artico and coworkers[9] and further purified using preparative thin-layer chromatography. The final preparation includes a small quantity of dimethylformamide, which gives rise to the peaks marked with asterisks in Figure 2b. The NMR spectra were obtained from a sample in 50 mM potassium phosphate, 150 mM sodium chloride, D_2O buffer containing 5% d_6-DMSO at pH 7.4. Saturation was performed at −10 ppm for the reference spectrum and at −10 and −1 ppm for the difference spectrum. The spectra shown Figures 2 and 3 were obtained with a field strength of 12.1 T (500 MHz). The titration experiment with compound **2** was performed at a field strength of 16 T (700 MHz).

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