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Chemical Interrogation of Drug/RNA Complexes: From Chemical Reactivity to Drug Design**

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Medicinal chemistry efforts oriented to the optimization of bioactive compounds have traditionally relied on the synthesis and evaluation of a large number of structurally related chemical derivatives. This procedure, which is in most cases expensive and time consuming, represents a major challenge for complex molecules containing numerous equivalent reactive positions. Unfortunately, such equivalent positions are not unusual among natural ligands, such as carbohydrates or polyamine RNA binders. Indeed, aminoglycosides (a family of antibiotic RNA-binding oligosaccharides) represent a typical example of this situation.

In a recent contribution, Herrmann and co-workers have proposed the use of RNA aptamers as protecting groups for the selective modification of these complex chemical structures.[1] Inspired by the principles of dynamic combinatorial chemistry,[2] and employing a fundamentally different approach, we hypothesized that the chemical reactivity of the aminoglycosides complexed with their target RNAs could provide valuable guidelines for the design of a reduced number of binders. Thus, the synthesis and evaluation process of the potential drug derivatives would be highly facilitated. To explore the scope and limitations of this concept, we have focused on a fairly simple chemical modification; the *N*-methylation of kanamycin B by reductive amination with formaldehyde (Figure 1a). This choice is fully justified by the current knowledge of RNA ligands.[3] In fact, these molecules frequently include several ammonium groups, that, in many occasions, are mono- or dialkylated, typically with methyl groups, as observed in aminoglycoside antibiotics and other recently described RNA binders. According to recent studies, this structural feature might be of general importance for the molecular recognition of nucleic acids (Supporting Information, Figure S1).[4,5]

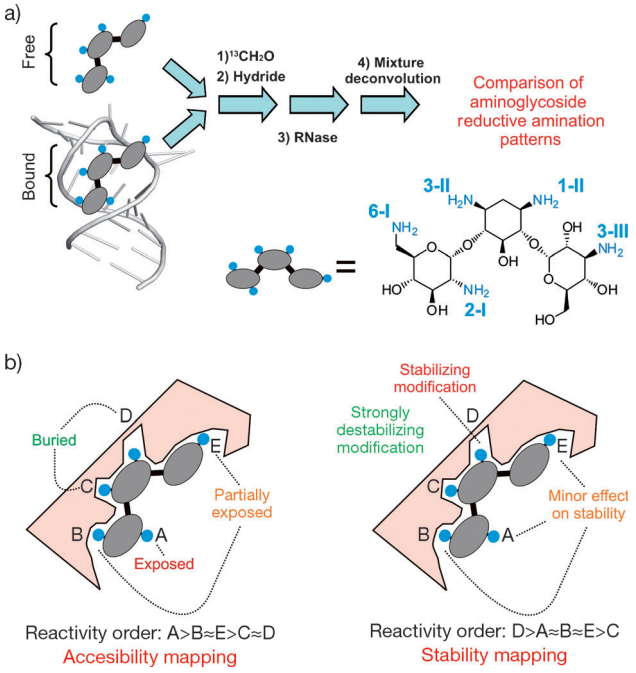

Figure 1. a) Schematic representation of the proposed method. The numbering of the different kanamycin B ammonium groups is shown. b) Information that can potentially be discovered by these experiments. More complex situations are also conceivable (see the main text).

In particular, the proposed method was based on a detailed comparison of the drug *N*-methylation patterns obtained from reductive amination reactions performed with both the free and RNA-bound drug (Figure 1a). We hypothesized that, in the most simple case, the observed changes could be kinetically dictated by the relative accessibility of the reactive amino groups within the complex, thus providing a simple way to chemically map the exposed or buried location of these key functions (left panel in Figure 1b). Alternatively, RNA might also alter the equilibrium populations of the different imine/enamine species leading to the enrichment of the most stable complexes (right panel in Figure 1b). Obviously, more complex situations could be conceived in which the reaction mixture would obey both, kinetic and thermodynamic constraints.

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Notably, reductive amination of kanamycin B can generate extremely complex mixtures formed by up to 243 highly similar derivatives. This fact, together with the lack of any chromophoric function, makes their deconvolution impossible by conventional methods, such as HPLC. Indeed, this objective represents a considerable challenge in itself. Thus, a different perspective has to be taken. Our concept is based on getting a simple assessment of the reaction output, by using the NMR-based method outlined in Figure 2.

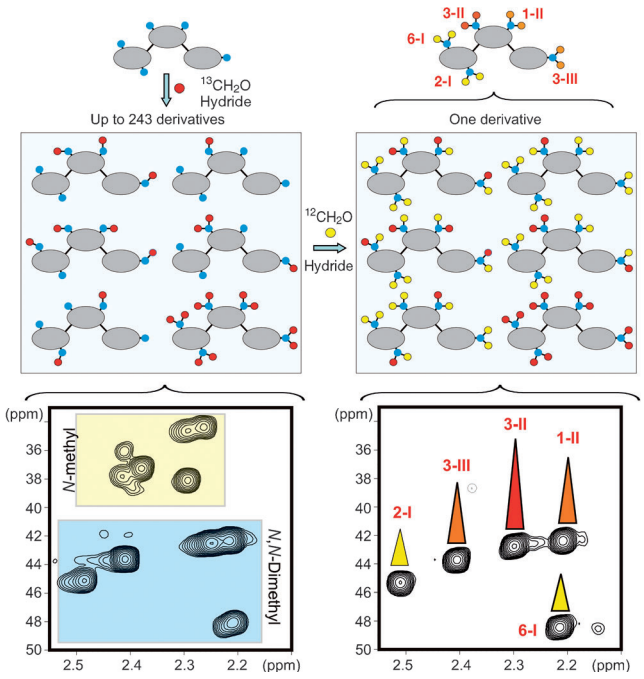

Figure 2. Schematic representation of the proposed NMR-based deconvolution method (see the main text). Red circles = labeled methyl groups; yellow circles = unlabeled methyl groups; orange circles = mixture of labeled/unlabeled methyl groups.

First, we performed reductive amination of the ligand with ^{13}C-labeled formaldehyde, both in the absence and presence of an excess of the RNA receptor. After digestion of the RNA with RNase B, the resulting mixture was analyzed by simply carrying out a second reductive amination reaction with an excess of ^{12}C formaldehyde. This isotopic dilution step reduces the library complexity down to a single permethylated derivative that incorporates distinct fractions of ^{13}C at the different positions. Standard NMR HSQC experiments of the reaction mixture acquired at pH 10.0 showed five well-resolved peaks (corresponding to the different $-NMe_2$ groups present in the modified aminoglycoside). These signals could be easily assigned by means of HMBC experiments and were integrated by using ^{13}C sodium acetate, present in the reaction buffer, as an internal reference. An interesting feature of this method is that, in contrast with conventional deconvolution methods, it does not allow the identification of individual components of the original mixture. Instead, the obtained data gives positional information related to the relative reactivity of the different amino groups of the drug.

As a proof of principle, we have analyzed kanamycin B methylation in the context of three different RNA fragments, whose structures in complex with this or closely related aminoglycosides have already been described: a tobramycin aptamer,[6] the ribosomal helix 69,[7,8] and the decoding A-site[9,10] (Figure 3). Preliminary methylation assays performed with free kanamycin B (Figure S2) showed that the reactivity of the different amino groups is strongly dependent on its degree of protonation. Taking this information into consideration, reductive amination of the different complexes was performed at pH 5.0. In this way, the protonation state of the drug in the free and receptor-bound states can be assumed to be roughly constant (Figure S3).

Next, we tested the reactivity of the complexes. The results are shown in Figure 3. The upper panels show HSQC experiments obtained following the method outlined in Figure 1 and 2 in the presence of RNA. Reference experiments obtained from samples with no RNA, are shown in the middle panels. Finally, HSQC-difference experiments obtained by subtracting the previous two data sets are shown at the bottom. In these spectra, drug positions whose methylation is enhanced by RNA binding, are represented as positive peaks (black), while those whose chemical modification is decreased are negative (red). Most importantly, these results conclusively prove that RNA affects the reactivity of the drug in a structure-dependent manner. Thus, *N*-methylation at position 6-I is enhanced (three- to fourfold) in the presence of the tobramycin aptamer, while a comparable effect is observed at position 2-I in the case of helix 69. Interestingly, this second effect is lost in a mutated version of helix 69 (Figure S4), which further supports the structural dependence of the signal amplifications/decreases detected. For the ribosomal A-site, smaller enhancements in the HSQC signals (1.8–1.9-fold) were detected for positions 6-I and 3-III. In addition, all three RNA fragments led to a significant decrease in the reactivity of the 3-II amino moiety.

To get insights into the origin of these effects, we used the available structural information (Figure 4). Thus, starting from the X-ray/NMR coordinates of the different aminoglycoside/RNA complexes, we carried out five nanosecond molecular-dynamic (MD) simulations and evaluated the solvent-accessible surface of the drug NH_3^+ groups from representative ensembles. These values are plotted in Figure 4 against the increase/decrease (%) detected for the HSQC signals. Fittingly, for the aptamer case, both data sets exhibit a very good linear correlation ($R = 0.96$). In fact, the comparison between the drug methylation patterns obtained for the free and complexed states allows for easy identification of the most exposed reactive moiety of kanamycin B, within the RNA binding pocket (the $-NH_3^+$ group at position 6-I).

A different result was obtained with helix 69 and the A-site receptors. In particular, for helix 69 the enhanced methylation detected at position 2-I of the RNA-bound drug did not correspond with its relatively modest solvent exposure. A similar lack of correspondence between accessibility and reactivity is also apparent for positions 6-I and 3-III in the kanamycin B/A-site complex. This observation could reflect the presence of alternative complexes, not considered in our analysis. Indeed, the co-existence of multiple binding modes constitutes a common feature for

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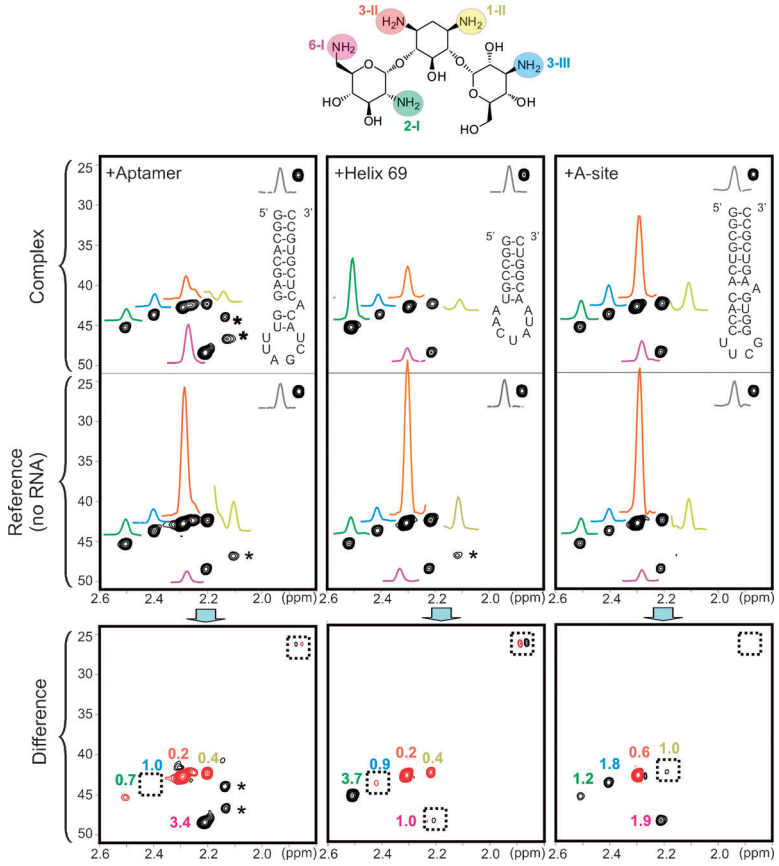

Figure 3. Reductive amination reactions performed with ^{13}C formaldehyde (200 μM), kanamycin B (200 μM), and three different RNA fragments (300 μM). The resulting mixtures were analyzed by the proposed NMR-based method (see the main text). To highlight changes in intensity, we have included cross sections of the different peaks. These are colored according to the code shown on the top of the figure. Increase/decrease factors for each signal (defined as $m \cdot I_o$, where I_o represents the peak intensities in the HSQC experiments obtained from the free drug) are shown in the bottom panels. The peak from ^{13}C-labelled AcONa, employed as internal intensity reference, is shown as a gray peak. This signal must be cancelled in the difference HSQC data shown at the bottom. * = impurity.

aminoglycoside recognition.[8,11] Alternatively, it could also result from dynamic motions of the RNA binding pocket, leading to transient increases in the water exposure of particular $-NH_3^+$ groups. Finally, our observations would also be consistent with a stabilizing influence of the *N*-methyl groups at positions 2-I in helix 69 and 6-I/3-III in the A-site complex.

A simple way to test this final hypothesis would be to estimate binding affinities of the five mono-*N*-methyl kanamycin B derivatives for the RNA fragments. Unfortunately, the regioselective modification of such a complex scaffold is an expensive, time-consuming, and challenging task. Thus, we decided to use a more straightforward approach based on the preparation of tailored aminoglycoside mixtures. As a first step, we performed the reductive amination of kanamycin B with ^{13}C-labeled formaldehyde, with a 20-fold excess of the aminoglycoside (Figure S5). Under these experimental conditions, the incorporation of two or more methyl groups to the aminoglycoside is seriously limited. Extensive chromatographic fractionation of the crude reaction mixture gave a mixture of the five mono-*N*-methyl compounds (labeled as **a**–**e** in Figure 5) in similar proportion, as judged by mass spectrometry and NMR.

Next, we performed binding experiments with the different RNA fragments. Figure 5 shows the evolution of this library (40 μM total aminoglycoside concentration) upon addition of the A-site and helix 69 fragments, as revealed by HSQC titration experiments. It should be mentioned that under these experimental conditions, the NMR signals for the bound species were basically undetectable, owing to a combination of line broadening and a low aminoglycoside concentration (in the 5–10 μM range for each derivative). Regarding helix 69, the RNA receptor promoted a gradual decrease in the intensity of all NMR signals (each corresponding to a well-defined simple derivative). Moreover, at receptor concentrations higher than 70 μM, all of the NMR signals become virtually undetectable. In conclusion, no selectivity, either for the compound modified at position 2-I or any other, is apparent from these experiments.

In contrast with this behavior, the A-site shows a clear preference for the ligand modified at position 3-III (derivative **e**). Indeed, it can be observed that the corresponding *N*-Me signal vanishes after addition of only 10 μM RNA. Moreover, the signal recovers only after displacement of the ligand by large concentrations of the natural aminoglycoside (greater than

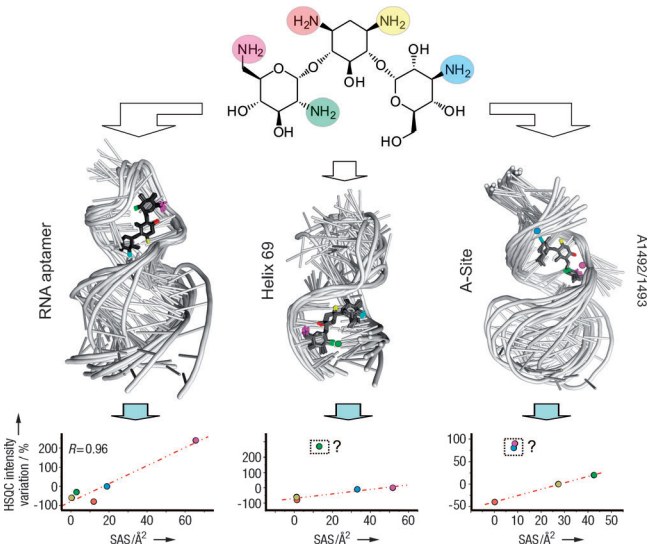

Figure 4. MD ensembles obtained for kanamycin B in complex with the aptamer, helix 69, and A-site receptors. Ammonium groups of the drug are colored according to the molecule shown above. Solvent accessible areas (SAS) of these reactive positions were evaluated from the ensemble and are plotted (at the bottom) against the change $(100 \cdot (I - I_o)/I_o)$ detected for the HSQC signals, using the method outlined in Figure 1 a and 2.

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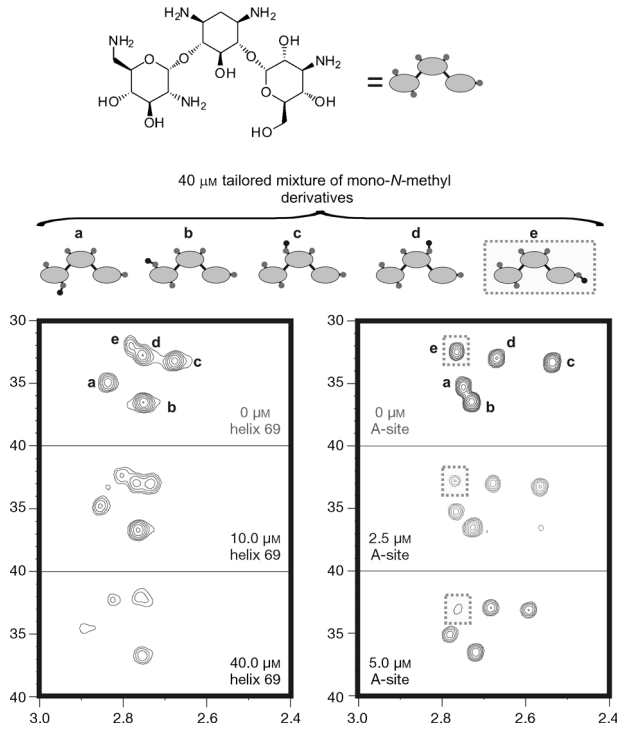

Figure 5. Evolution of a ^{13}C labeled mixture of mono-*N*-methyl kanamycin derivatives (**a**–**e**) after addition of helix 69 (pH 5.5) and the A-site (pH 7.0), as revealed by HSQC experiments. RNA concentrations are indicated.

100 μM). These observations are consistent with the extra stability of the complex between the A-site and derivative **e** and provide an explanation for the enhanced *N*-methylation of kanamycin at position 3-III in the complexed state. In addition, this conclusion is in agreement with the presence of *N*-Me groups at this site in several natural aminoglycosides (Figure S6).[11a]

Regarding the unusual reactivity detected for position 2-I in the kanamycin/helix 69 complex and 6-I in the kanamycin/A-site complex, according to our data, they do not reflect any stabilizing influence for the respective *N*-methyl modifications. In our opinion, this behavior most likely results from the dynamic character of the receptor in the proximity of the modified $-NH_3^+$ groups (Figure S6).

Notably, position 2-I of the aminoglycoside interacts with the two terminal base-pairs of the helix 69 fragment,[8] a region of the receptor that is affected, to some extent, by fraying. This process might largely enhance its solvent exposure and, consequently, its reactivity. In a similar way, position 6-I of the drug is known to interact with a highly dynamic region of the A-site; adenines 1492 and 1493 (Figure 4 and S6). The reported structures show that both residues are flipped out of the helix axis upon binding of the aminoglycoside.[9,10] However, the extent of the conformational change, detected by X-ray and NMR, differs greatly. Moreover, the dynamic behavior of both residues seem to depend on the bound aminoglycoside.[12] According to our short MD simulations, a more pronounced flipping out of A1492/A1493 (consistent with the X-ray structure) would lead to a large enhancement in the solvent exposure of position 6-I of the drug, which could explain its preferred methylation (Figure S6).

In summary, we have shown that the reductive amination of aminoglycoside/RNA complexes can provide information from both the molecular-recognition and medicinal-chemistry perspective. In particular cases, as in the aptamer/kanamycin complex, the observed reactivities show a strong correlation with the buried/exposed character of the different reactive $-NH_3^+$ positions. In contrast, a lack of correlation between reactivity and accessibility can reflect the dynamic character of the drug environment within the complex or the presence of alternative binding modes. Finally, in favorable cases, the modification of particular positions of the drug within the complexes might point to a stabilizing influence of such modification. From a methodological perspective, we have set up a very simple experimental method, based on a combination of NMR and isotopic labeling, which is able to analyze extremely complex aminoglycoside mixtures to provide "positional information" about the different reactive drug moieties. This concept may be exploited and adapted to a variety of examples within the field of DNA/RNA-targeted drug design.

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