ESI-FTMS investigation of the binding modes of nucleic acid-tropic agents with RNA structures of the HIV-1 packaging signal

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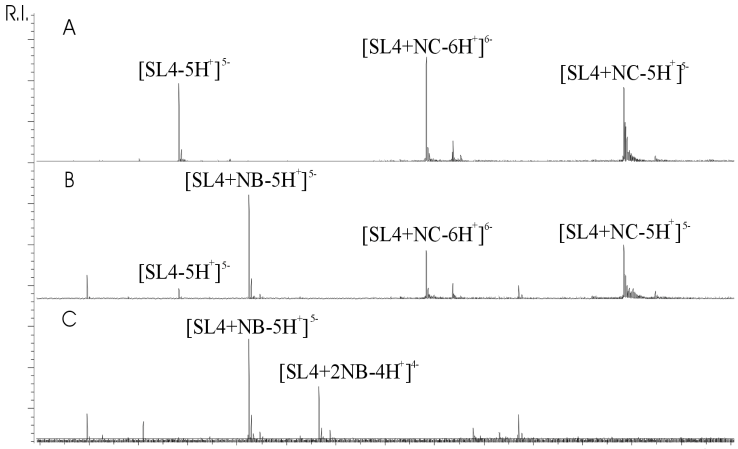
The gentle nature of electrospray ionization (ESI) combined with the high resolution provided by Fourier Transform mass spectrometry (FTMS) has recently enabled the characterization of the stoichiometry and binding affinity of non-covalent complexes formed by the interactions of the human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein p7 (NC) with individual stem-loops of the virus packaging signal (Ψ-RNA).[1] The same technique has allowed also for the detection of stable complexes formed by the Ψ-RNA stem-loops SL2, SL3, and SL4 with a series of aminoglycosidic antibiotics including neomycin B, paromomycin, and kanamycin B.[2] Due to the fact that these RNA structures are highly conserved across the different viral strains and, therefore, could constitute a possible target for antiviral therapy, we are now interested in investigating the determinants of the binding interactions with aminoglucosides and the possible effects on the binding with nucleocapsid protein *in vitro*.

A possible strategy for this investigation involves comparing the stoichiometry and binding affinity observed for neomycin B, a leading member of the aminoglycoside class, with those provided by other classes of nucleic acid-active agents, which present alternative binding mechanisms (e.g., minor groove vs. base intercalators, GC- vs. AT-binding drugs, etc.). The effects induced by the specific structural features of each substrate on the different binding modes can be investigated by performing competitive binding experiments that combine different types of binders, or by employing appropriately mutated RNA constructs.

RNA substrates corresponding to the intact HIV-1 packaging signal (Ψ-site), its individual stem-loop hairpins, and their mutants were obtained by *in vitro* transcription of the respective DNA templates by T7 RNA polymerase, or by chemical synthesis (Dharmacon, Inc., Lafayette, CO). Neomycin B, distamycin A, and mitoxantrone were purchased from Sigma Chemical Co. (St. Louis, MO) and utilized without further purification. Aliquots of drugs were mixed with RNA substrates in appropriate proportions in 10 mM ammonium acetate (pH 7.0) and incubated for 15 min. at room temperature. Analysis of each sample mixture was performed by direct infusion electrospray on a Bruker (Billerica, MA) Apex III Fourier transform mass spectrometer equipped with a 7T actively shielded magnet and an Apollo ESI interface with a home built heated metal capillary.

Preliminary data have shown that neomycin B binds to SL4 more tightly than the other stem-loops taken into consideration. Titration experiments performed by progressively increasing the amount of neomycin B in solution have also demonstrated that while equimolar additions can only lead to the formation of 1:1 complexes for each hairpin, further increases allow for the detection of a 2:1 complex only for SL4. The results suggest that, unlike the other hairpins, SL4 may contain a secondary binding site with lower binding affinity. No evidence of possible positive cooperativity could be inferred from a binding process that requires the first site to be fully saturated before the second site may become competitive.

More pointedly, experiments performed by adding neomycin B to preformed NC-stem-loops complexes have shown dramatic inhibitory effects on the binding of protein to the RNA constructs, which are very accentuated for SL4. In fact, addition of a five fold excess of neomycin B was found to cause the complete dissociation of the SL4-NC binding, which was replaced by the stable 2:1 neomycin B:SL4 complex. (Figure)



Inhibition of SL4:NC Complex. A) SL4+NC, B) SL4+NC+1xNB, C) SL4+NC+5xNB

These results have prompted at least two complimentary approaches to identify the location of the putative binding sites on the RNA hairpins, and SL4 in particular. The first approach is based on competitive binding between neomycin B and the GC-binder mitoxantrone, or the AT-binder distamycin. Preferential binding or reciprocal displacement can provide specific indications on the nature of the binding site(s). In the case of SL4, a maximum of four equivalents of mitoxantrone were found to bind to the construct in the presence of an excess of this drug, which is known to specifically target tandems of G-C pairs located in double-stranded regions. The fact that addition of 1x of neomycin B caused the complete displacement of mitoxantrone, with formation of a 1:1 neomycin B : SL4 complex, is another prove of the high affinity of these RNA substrate for this aminoglycoside and seems to suggest that the high-affinity binding site may be located in the double-stranded stem, rather than in the loop. An alternative explanation is based on the hypothesis that binding of neomycin B to the loop region may cause the destabilization of the contiguous stem and opening of the base pairs. This explanation is weakened by the observation that a tandem of G-C pairs, which enjoy high stabilization by three hydrogen bonds per pair, is actually located near the base of the stem, in a position that is not very likely to be affected by conformational changes in the loop.

The second approach makes use of specific mutants designed to eliminate or modify salient features of the hairpin structures. In an attempt to locate the binding site of neomycin B on SL3, we have prepared a construct in which the tetraloop bases were replaced by abasic nucleotides. A competitive binding experiment including the wild-type SL3 and the abasic analogue showed a much greater affinity toward the drug by the former, indicating that the single stranded loop region of SL3 plays an integral role in binding the aminoglycosidic antibiotic. This observation appears to be in stark contrast with the results discussed above for SL4. For this reason, similar experiments are underway with abasic SL4 to confirm the possible differences in binding modes for the two stem-loop structures.

References:
1. Hagan, N.A.; Fabris, D.; *Biochemistry* **2003** *42*, 10736.
2. Chilakuri, R.; Hagan, N.A.; Fabris, D; Proceedings of the 51st ASMS Conference on Mass Spectrometry and Allied Topics **200**), Montreal, Canada.

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