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NMR-based identification of peptides that specifically recognize the D-arm of tRNA.

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Abstract

Human tRNALys3 is used by HIV virus as a primer for the reverse transcription of its genome. The 18 nucleotides of at the 3'-end of the tRNALys3 are hybridized to a complementary sequence of the viral RNA called the primer binding site. A screen against the human tRNALys3 over a peptide library designed to target RNA has been performed. Of the 175 hexapeptides tested, three were found to bind to the D-stem of tRNALys3. Alanine-scanning was used to define the determinants of the interaction between the peptides and tRNALys3. They also bind to two other tested tRNAs, also at the level of the D-stem and loop, although the nucleotide sequence of the stem differs in one of them. These short peptides thus recognize specific structural features within the D stem and loop of tRNAs. Associated with other pharmacophores, they could be useful to design optimised ligands targeting specific tRNAs such as retroviral replication primers.

Keywords: screening, tRNA, peptide, NMR

Abbreviations: NOE: Nuclear Overhauser effect, trNOE, tranfer NOE

1. Introduction

The essential role of RNA in many biological processes makes RNA an attractive target in drug discovery. There are many potential RNA targets, including RNA that is involved in cellular proteins interaction such as transcription, splicing, and translation and, RNA that is involved in viral infection such as human immunodeficiency virus (HIV). Several systematic screens have been performed against fragments of the HIV-1 genomic RNA, but in all cases, the high affinity hits were strongly cationic [1-3] and their recognition was dominated by electrostatic effects that are often poorly selective. There is thus a need for strategies based on high selectivity rather than on high affinity. In this context, interaction screening by NMR can be an attractive technique, as it can both detect weak interactions and provide structural information on ligand-binding modes (for reviews on NMR-based screening, see [4-9]).

Using flow-injection NMR, we have screened against the human tRNALys3 over a peptide library designed to target RNA [10]. tRNALys3 is used by HIV-1 as a primer to the reverse transcription of its genome. The 18 nucleotides at the 3' end of tRNALys3 are hybridized to a complementary fragment of the viral RNA called the primer binding site (PBS). The aim of this study was to find molecules that bind specifically to tRNALys3 and that could provide leads for inhibitors of the formation of the HIV-1 reverse transcription initiation complex. In a first approach, a chemical library of short peptides was used, since these can be easily obtained and provide a rich variety of pharmacophore models for further development. Peptidomimetic modification of active peptides could then provide biostable analogs [11].

2. Materiels and Methods

2.1 Sample preparation

Human tRNALys3 was expressed *in vivo* from a recombinant plasmid and purified as previously described [12]. tRNAfMet and tRNAmMet were expressed and purified similarly [13, Wallis, 1995 #370]. The peptide library has been described [10], for NMR analyses, the peptide stocks (100 mM in DMSO-d6) into 5mM imidazole-HCl, pH7, prepared in 90% H_2O/10% ^2H_2O.

2.2 NMR experiments

Experiments were recorded on a Bruker Avance DRX 600 spectrometer equipped with a 3mm triple-resonance flow-injection probe. The probe was connected to a Gilson 215 liquid handler controlled by the NMR console (Bruker BEST system). For tRNA:peptide mixtures, solvent suppression was achieved by using the 'jump-and-return' sequence to avoid the saturation of imino protons [14]. Samples for 1D NMR screening contained 0.3 mM of tRNA and 1.2 mM of peptide in 5mM imidazole buffer pH 7, in a total volume of 200 µl in 96-well plates. The injected sample volumes were 160 µl. As imino chemical shifts in RNA are sensitive to pH, we used the spacing between the two aromatic NMR signals as an internal pH probe, which enabled us to eliminate false positives resulting from the pH shifts induced by ligand addition. For ligands identified in the primary screen, ^1H-^{15}N JR-HMQC spectra [15] were recorded using a sample containing 0.4 mM ^{15}N labelled tRNA and 1.6 mM peptide in 10mM phosphate buffer pH 6.5.

For the measurement of the dissociation constant (Kd) between peptide P1 and tRNALys3, a sample containing 0.7 mM of tRNALys3 in 10mM phosphate buffer pH 6.5 was titrated by increasing concentrations of P1 : 0.2, 0.4, 0.8, 1.5, 3, 4 and 7 mM. Kd values were extracted by non-linear least square fitting of the variation of imino proton chemical shifts to a single-site binding hyperbolic function. Confidence limits on the Kd were estimated by Monte-Carlo sampling using the MC-fit program [16].

3. Results and Discussion

The human tRNALys3 is used by HIV-1 as a primer for the reverse transcription of its genome. Indeed, the 18 nucleotides at the 3' end of tRNALys3 are complementary to the primer binding site in the viral RNA (for reviews, see [17-19]). This tRNALys3-viral RNA duplex is then recognised by the reverse transcriptase (for reviews [17, 20]) which can start the elongation of the minus DNA strand. In order to prevent the formation of the initiation complex (tRNALys3/viral RNA), an attractive strategy would be to isolate ligands that bind to tRNALys3 and that could then hinder its recruitment by viral factors : the nucleocapsid protein, the reverse transcriptase or the viral RNA. For this purpose, we previously designed a library of hexapeptides to screen RNA ligands [10]. Briefly, we used a heuristic algorithmic approach to select *in silico* a set of 175 peptides, which simultaneously satisfies a set of physico-chemical constraints (charge, solubility, amino acid composition) and performs an optimised sampling of the peptide sequence space. In particular, strongly basic sequences (pI > 9) were systematically eliminated in order to avoid non-specific electrostatic binding to RNA.

In order to reduce the amounts of target tRNA used in the screening process, we first followed the 1D imino proton spectrum of the tRNA, since this only requires about 1mg (≈40 nmoles) of tRNA per point to obtain a decent quality spectrum in 20 minutes. The 1D imino proton spectrum offers two main advantages: (i) It is quite simple since it contains approximately one peak per base pair, *ie* about 25 peaks for tRNAs, and the corresponding protons are evenly distributed over the structure. (ii) The corresponding spectral region is essentially devoid of any signals originating from the peptides. In a second step, a more precise view of the interaction site of selected peptides was obtained using 2D heteronuclear experiments on ^{15}N-labelled tRNA. Indeed, we have previously reported the sequence-specific NMR assignments of ^{15}N and ^{1}H imino resonances involved in the secondary and tertiary base pairs of tRNALys3 [21]. Therefore, this second step enables us to define the binding footprint of the selected peptides at the level of each base pair in the tRNA. Over the peptide library previously designed to screen RNA ligands [10], three peptides were found to induce significant changes in the imino proton 1D spectra of tRNALys3. The sequence of the three

peptides are YHSRNN (P1), GDWHVR (P2) and WRHPDV (P3). Interestingly, all three peptides contain both arginine and aromatic residues, P1 and P2 have partially homologous sequences, *ie* ArHXR (Ar: aromatic residue, X: any residue). Figure 1 shows the binding footprint of peptide YHSRNN. The imino groups whose chemicals shifts are changed upon binding of the peptide are those of guanines 10, 22 and 24, the 7-methylated guanine 46, the 4-thiolated uridine at position 8 that forms a reverse Hoogsteen base pair with A14 and uridine 12. Interestingly, all the nucleotides in the D-stem of tRNALys3 appear involved in the binding of this peptide, plus m7G46 that makes a base triplet with the Watson-Crick G22-C13 base pair, also in the D-stem. Overall, similar footprints are observed for the three peptides, they all bind in the D-arm of tRNAlys3. For P2, the binding involves a supplementary base, G15, in the D-loop of tRNALys3 and the chemical shift variation for G24 and G10 upon binding of P2 is rather small. Therefore, P2 seems to bind near the D-loop and close to the three bases in the D-arm adjacent to the D-loop. Since P1 induces the strongest variation of chemical shift of the imino group of tRNALys3 upon binding, its interaction with tRNALys3 was extensively studied by NOE, trNOE, Kd measurement and alanine scanning. As a result, a dissociation constant (Kd) of 2mM was extracted from the measurement of the variation of the U8 imino proton NMR signal as the concentration of P1 increases. These magnitude of Kd are of the same order of one found for a peptide that bind to TAR RNA [22]. No NOE can be directly observed between the protons of the peptide and the imino groups of tRNALys3, probably due to the low affinity of P1 for tRNALys3. In the same way, no trNOE has been observed. In order to characterize the residues crucial to the binding to tRNALys3, each residue of the peptide P1 was sequentially mutated by an alanine. Then, the impact of the mutation for the binding to tRNALys3 was analysed by the observation of the 1D NMR spectra of the tRNALys3 imino region in presence of each modified peptide. Interestingly, the mutation by an alanine at position 1 or 4, *ie* AHSRNN and YHSANN, is sufficient to prevent the binding to tRNALys3. Therefore, both the tyrosine and the arginine are key residues for the binding to the D-arm of tRNALys3, possibly by contributing stacking, hydrogen bonding and electrostatic interactions. Finally, to test the specificity of the interaction of the peptides with tRNALys3, we have studied the binding of these three

peptides to two others tRNA, *E. coli* tRNAfMet that has the same sequence as human tRNALys3 in the D-arm and *E. coli* tRNAmMet that has a different sequence (see [23] for the sequences). All three peptides were found to bind to tRNAfMet and tRNAmMet (data not shown) and the binding also occurs in the D-arm of these tRNAs. Therefore, these peptides appear to be specific for the structure of the D stem and loop of tRNA, independently of the nucleotide sequence within the D-stem.

4. Conclusion

The present study demonstrates that flow injection NMR screening of a small optimized library of short peptides is a viable approach for isolating weak but specific ligands of a structured RNA. The three identified peptides are structure-specific, and could provide leads for the design of high affinity ligands. Such short peptides could be further optimised and/or coupled with other RNA-binding pharmacophores, such as polyamines, aminoglycoside fragments or intercalating groups and provide useful tools for studying the structure or interfering with the metabolism of such structured RNA.

Acknowlegments

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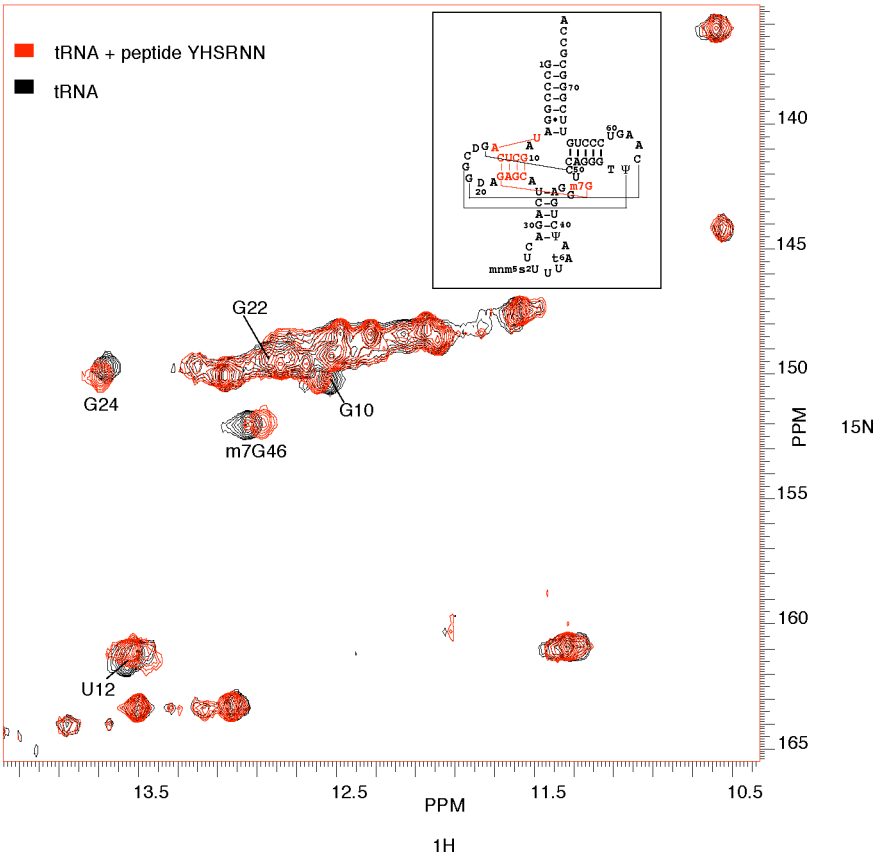
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Figure Legends

Figure 1 : Superimposition of two HMQC experiments in the region of the imino groups of the ^{15}N labelled tRNALys3 with (blue) and without (black) the peptide YHSRNN. The bases that are indicated on the spectra and that are in blue in the secondary structure of tRNALys3 are those for which the chemical shifts of the imino proton change of more than 0.04 ppm.


tRNA + peptide YHSRNN
tRNA
G22
G24
G10
m7G46
U12
140
145
150
155
160
165
PPM
15N
13.5
12.5
11.5
10.5
PPM
1H