Tether influence on the binding properties of tRNA$^{Lys}_3$ ligands designed by a fragment-based approach†

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A small library of 1,5-triazole derivatives linking a diaminocyclopentadiol and aromatic ketones has been prepared and screened using NMR and fluorescent techniques against tRNA$^{Lys}_3$, the HIV reverse transcription primer. The comparison of their binding properties to those of their 1,4-triazole isomers, previously discovered in a fragment-based approach, outlines the influence of the linker on affinity and binding selectivity in such an approach.

Introduction

A fragment-based approach has emerged over the last few years as a new method for lead identification and is now a well-established strategy for drug discovery.[1,2] The goal is to build drug leads in pieces, through the identification of weak binding fragments that are next, either expanded or linked together. The linking of two fragments that can simultaneously occupy non-overlapping regions into a larger compound can, under optimal conditions, give an additive effect on binding energies and moreover, entropic benefits will add to this energetic profit.[3] In principle, the linkers should not perturb the optimal binding geometry of the two fragments. However, it is difficult to link two fragments without distorting the binding mode of either and in some cases the new hybrid ligand may bind differently than the original unlinked fragments.[4] Plus, the linker itself can have unfavorable interactions[5] or develop conformational strain,[6] thus causing energetic penalties. These observations indicate that the linkage strategy is crucial and can dramatically influence the binding properties of the final compounds. As a consequence, many fragment-based ligands are suboptimal binders because they do not reach the expectation of additive binding energies of the fragments pieces. Thus, identifying a linker that allows the appropriate presentation of the two binding elements may be as important as the design of the fragments. This can only be achieved by iterative optimization and binding measurements. In several reported studies, the optimization of the length and geometry of the linker lead to a great enhancement of the compound potency.[7] However, due to limitations in linker chemistry, it is not always trivial to change these parameters without modifying the chemical nature of the tether.

RNA is becoming a valuable target for drug discovery, and of particular interest are those RNAs that fold into complex tertiary structures, since they are often involved in RNA–RNA or RNA–protein interactions that have an impact on important cellular processes.[8] If the fragment-based approach has demonstrated its potential in protein/ligand discovery, the use of such a strategy for the design of RNA ligands is still very limited.[9] We recently reported the identification of small ligands of tRNA$^{Lys}_3$, the HIV reverse transcription primer, in a fragment-based approach using NMR to identify ligands, on the basis of spectral changes induced by their binding to the target.[10] In a primary screen, diaminocyclopentanol (DACP) and kynuramine were identified as millimolar binders of the target (Fig. 1). The two fragments were evolved and connected *via* a 1,2,3-triazole moiety leading to a second generation of compounds, such as **1**, that are able to specifically bind the D stem of tRNA$^{Lys}_3$ with micromolar dissociation constant.

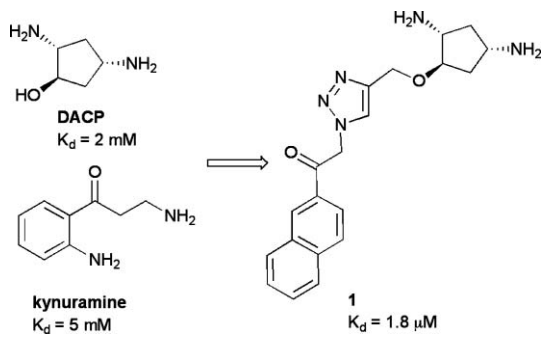

Fig. 1 Fragments identified as tRNA$^{Lys}_3$ ligand and compound **1** resulting from fragments evolution and linking by 1,2,3 triazole moiety.

The ligation of two molecules *via* a 1,2,3-triazole moiety is a very popular strategy in chemical biology.[11] Due to their high potential for association with biological targets through hydrogen bonding and dipole interactions, triazoles can generally be more than passive linkers and contribute to the binding energy of the compound to its target. In order to study more deeply the influence of the linker on the binding of our compounds, we were interested in changing the 1,4-triazole moiety for its 1,5-triazole isomer. Such a modification was expected to change the orientation between the two fragments without modifying the chemical nature or the size of the linker. Indeed, even though the presence of a methylene group between the fragments and the linker gives a partial motional

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freedom to the substituents, the 1,4-linkage induces a linear orientation while the 1,5-isomer forces the folding into an angular geometry, preventing a fully extended conformation from being reached. Thus, any difference obtained in the binding properties should result only from the way the two fragments are presented to the target. We report here the preparation of a small library of 1,5-disubstituted triazoles linking DACP and different aromatic ketones and the comparison of their affinities toward $tRNA^{Lys}_3$ to the 1,4-regioisomers previously reported (Fig. 2).[10] In this new family of analogues, due to the geometry of the tether, the two fragments are brought closer together compared to their 1,4-counterpart. The results show how the orientation of the two fragments can have a substantial impact on the location of the binding sites on the RNA target.

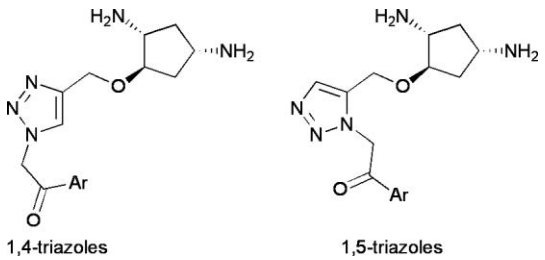


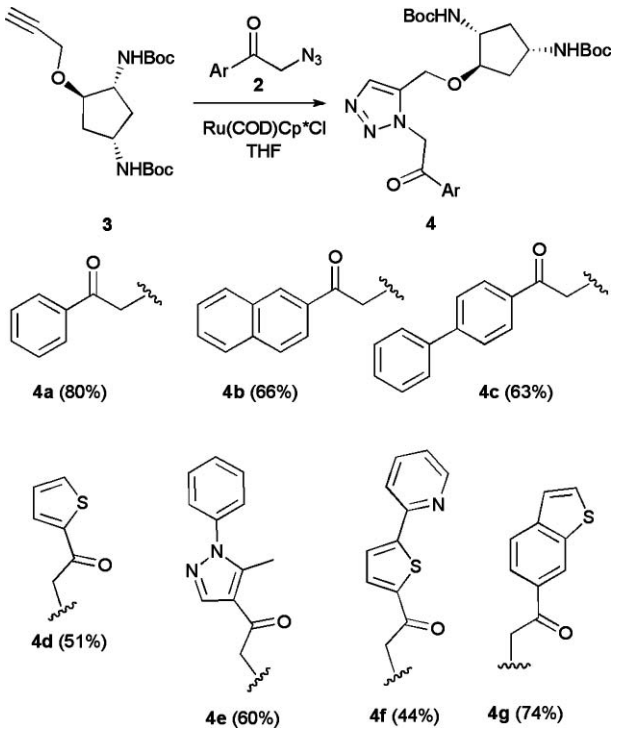
Fig. 2 Libraries of 1,4- and 1,5-triazoles linking DACP and aromatic ketones.

Results and discussion

Synthesis of a 1,5-triazole linked library

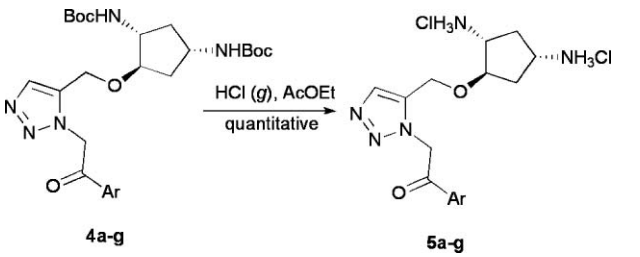
The popularity of 1,4-disubstituted triazoles for establishing connections in chemical biology is likely due to the ease of their preparation by the copper-catalyzed 1,3-dipolar cycloaddition of an azide and an alkyne (CuAAC).[12] On the other hand, 1,5-isomers have been only scarcely explored so far,[13] although their preparation can be easily accomplished by a ruthenium-catalyzed azide–alkyne cycloaddition reaction (RuAAC), recently reported by Fokin and coworkers.[14] This reaction furnishes the triazole derivatives with virtually a total 1,5-regioselectivity. The compatibility of the reaction with many functional groups suggests its possible application to the connection of complex functionalized molecules. Thus, we used these conditions for the preparation of the desired compounds.

The azido-derivatives **2** required for the cycloaddition were obtained from the corresponding commercially available α-bromo-ketones. (Scheme 1) The alkyne derivative **3** was obtained by O-alkylation with propargyl bromide of racemic 2,4-diaminocyclopentanol, which was prepared by reductive opening of the corresponding bicyclic hydrazine.[15] Compounds **2** were mixed with alkyne **3** in the presence of Cp*RuCl(COD) in THF. The 1,5-substituted triazole derivatives **4** were obtained after chromatographic purification in moderate to good yields. The selective formation of the 1,5-regioisomer of **4** was unambiguously confirmed by the chemical shifts of C(4) and C(5) ranging between 132 and 135 ppm, which differ clearly from those of the corresponding 1,4-isomers (C(5) and C(4) in the range of 124–126 and 143–146 ppm respectively).[16]



Scheme 1 Synthesis of 1,5-disubstituted 1,2,3-triazole derivatives by RuAAC.

After cleavage of the Boc-protective group, diamines **5** were obtained as their hydrochloride salts and their binding properties towards $tRNA^{Lys}_3$ were evaluated. (Scheme 2)



Scheme 2 Deprotection of 1,5-disubstituted 1,2,3-triazole derivatives.

Analysis of the impact of the linker on $tRNA^{Lys}_3$ binding by fluorescence and NMR spectroscopies

Some of the prepared compounds being fluorescent, we used a fluorescence-quenching assay to estimate their dissociation constant (K_d) to $tRNA^{Lys}_3$, as previously reported. The assay was conducted in the presence of KCl (50 mM) in order to limit any non-specific electrostatic interaction. The observed binding curves are shown in Fig. 3. In general, the K_d values obtained for the 1,5-triazole derivatives are in the micromolar range, as for the 1,4-isomers[10] (Table 1). Thus, altering the orientation of the two fragments does not significantly alter the dissociation constant.

The binding sites of all the newly synthesized compounds were then analyzed by NMR spectroscopy in the window corresponding to the signals of the imino-hydrogen atoms of the RNA. The significant NMR chemical shift perturbations (CSP) induced by ligands **5a–e** reveal an interaction with the D stem of $tRNA^{Lys}_3$,

Table 1 Dissociation constant to $tRNA^{Lys}_3$ obtained by fluorescence-quenching titration. The value of K_d are indicated with the 95% confidence interval in parenthesis

Compound	K_d/μM	K_d/μM of the 1,4-triazole isomer[a]
5b	3.6 (1.9–6.7)	1.8 (0.6–2.2)
5c	5.3 (3.3–9.6)	1.2 (0.8–6.4)
5f	7.2 (3.7–12.7)	6 (1.2–9.3)

[a] From ref. 10

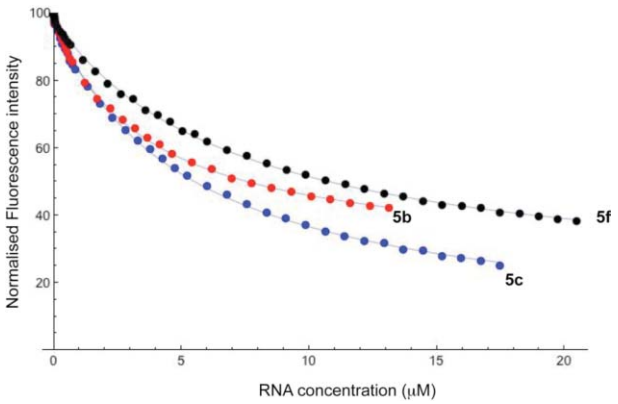


Fig. 3 Fluorescence titrations of compounds (a) **5b**, (b) **5c** and (c) **5f** with $tRNA^{Lys}_3$.

with an additional binding site appearing in the T stem for some of them (see the ESI†).

Compounds **5a–e** all bind to the D stem albeit with some discrepancies. For instance, compound **5e** binds very weakly with only small CSP, even for a 1 : 4 tRNA : ligand ratio, whereas the others induce the disappearance of the $tRNA^{Lys}_3$ D arm imino groups resonances, suggesting a different dynamic for the interaction of this compound. Compounds **5c** and **5g** have a clear second binding site in the T arm, as revealed by strong CSP or disappearance of some NMR peaks of the imino groups at high ligand : tRNA ratio (4 : 1 or over). Moreover, the interaction of compounds **5c** and **5g** involve all the imino groups of the T stem (Fig. 4). Interestingly, none of their corresponding 1,4-isomers bind to the T stem at a similar ligand : tRNA ratio.

The comparison of the binding properties of the 1,4-triazole **1**, the 1,5-triazole **5b**, and the previously reported ester-linked compound **6**,[10b] which all connect the same fragments, allows for further investigation of the impact of the linker on $tRNA^{Lys}_3$ binding (Fig. 5).

The ester-linked derivative **6** strongly binds to the D stem and has also a weak second site of interaction with the T stem that appears starting from a 1 : 4 tRNA : ligand ratio. The 1,4-triazole **1** is highly selective of the D stem, even at a high ligand : tRNA ratio, while the 1,5-triazole **5b** exhibits two binding sites located in the T and D stems. Nevertheless, the binding of **5b** to the T stem is different from that of compound **6**. Indeed, the intensities of the NMR signals relative to Ψ55, G65, T54 are more altered in the presence of **5b** than **6**. Since the imino protons are buried inside the helix, these observations suggest that the 1,5-triazole linkage allows compound **5b** to more deeply enter the T arm to come nearest to its imino groups.

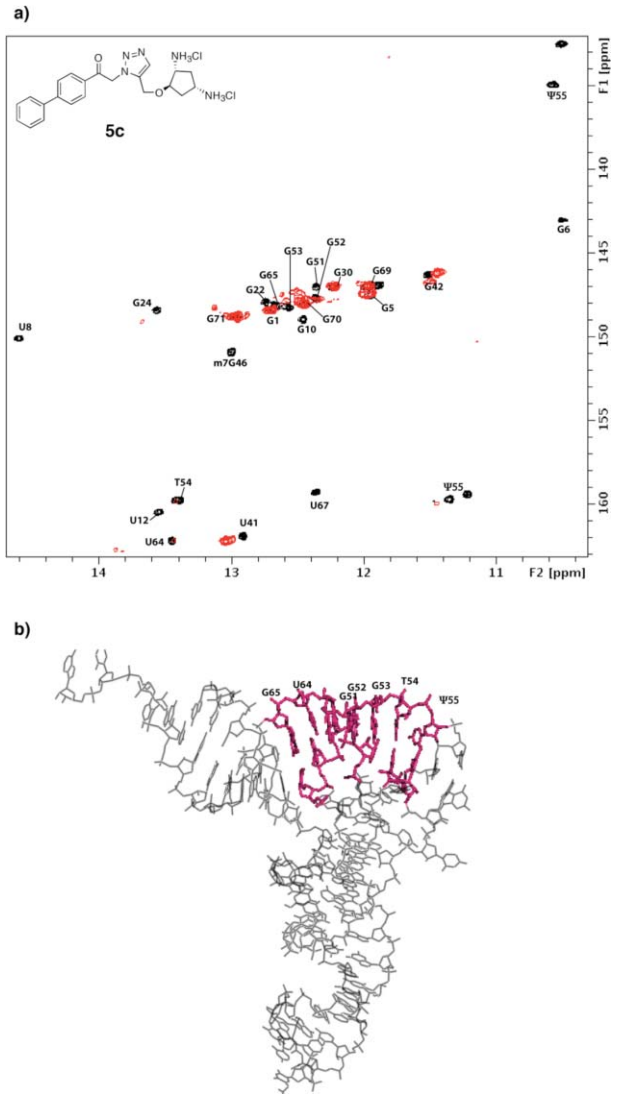


Fig. 4 NMR chemical shift mapping of the secondary binding site of compound **5c** on $tRNA^{Lys}_3$. (a) Reference TROSY spectra of ^{15}N-labelled $tRNA^{Lys}_3$ (0.2 mM) is in black whereas the spectrum in red is the TROSY spectra of $tRNA^{Lys}_3$ mixed with compound **5c**. (b) Mapping on $tRNA^{Lys}_3$ structure (PDB code 1FIR) of the T stem secondary binding site of compound **5c** in magenta.

The optimization of the linking strategy is an effective way to enhance the efficiency of a ligand discovered in a fragment-based approach and this strategy has been frequently outlined for the optimization of ligands for proteins bearing a single binding pocket.[1] The situation is different when working on ribonucleic targets, since topologically complex RNAs are known to potentially possess multiple binding sites. Selectivity is therefore highly desired, and generally more challenging to reach than affinity. As outlined by numerous crystallographic studies,[17] interactions between small molecules and RNA are known to involve a large number of direct or indirect water-mediated contacts, a reason why good binders are generally promiscuous ligands.[18] As a consequence, "good" fragments will probably lead to strong but rather "universal" binders if connected through flexible linkers. This work, as well as other recent publications in this field,[19] suggests that a rigid linker, able to lock the connected fragments

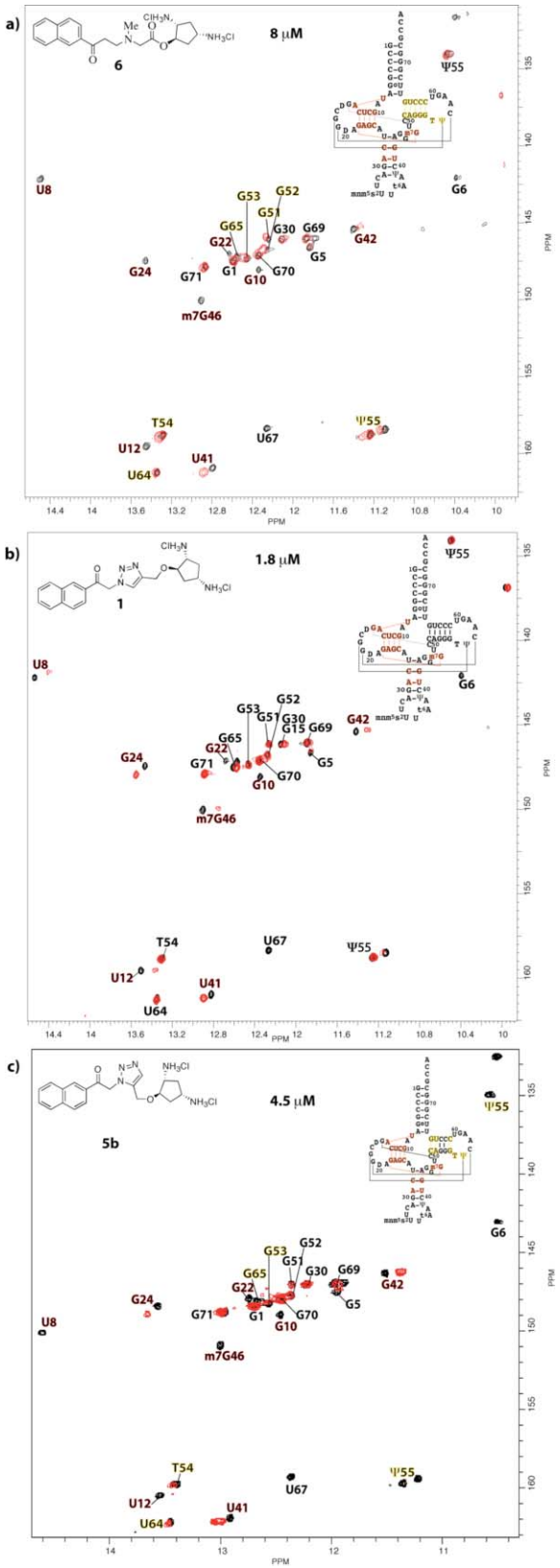
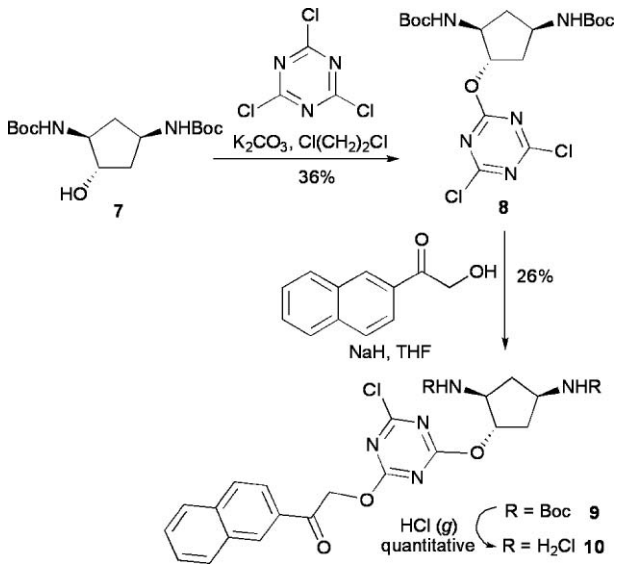


Fig. 5 Study of the impact of the linker chemistry on the binding to $tRNA^{Lys}_3$. TROSY experiment of ^{15}N-labelled $tRNA^{Lys}_3$ alone (0.2 mM) in black and $tRNA^{Lys}_3$ mixed with compound (a) **6**, (b) **1** and (c) **5b** at 0.8 mM, in red. Dissociation constants measured by fluorescence spectroscopy are indicated on each spectrum.

into a spatial arrangement, might introduce some site selectivity on a complex structured ribonucleic target such as transfer RNAs. In our case, interaction with the T stem seems to be disabled by linkers imposing a long distance between the two fragments, whereas a closer spatial proximity between the two fragments introduced by the 1,5-triazole makes interaction with the T stem possible. To confirm this hypothesis, compound **10** was prepared using a triazine as a linker. The longer distance and higher angle between the two fragments imposed by this linker allow the substituents to point opposite one to the other in an extended conformation.

Thus, cyanuric chloride was treated with DACP in the presence of K_2CO_3 in dichloroethane under reflux. Compound **8** was obtained in rather a modest yield of 36%. This can be explained by the instability of the product in the reaction mixture, due to the substitution of its triazine moiety by the *in situ*-generated chloride ion, a side reaction that proved to be difficult to control (Scheme 3). α-Hydroxynaphtylacetophenone was selected as an aromatic ketone fragment. The corresponding alcohol was prepared according to a known method[20] and introduced on compound **8** after deprotonation with sodium hydride in THF, giving the corresponding triazine derivative **9**, in 26% yield. Finally, the Boc-protective groups were cleaved using a saturated solution of hydrogen chloride in ethyl acetate.



Scheme 3 Synthesis of 1,3-triazine-linked derivative from cyanuric chloride.

The NMR TROSY experiments showed that 1,3,5-triazine **10** interacts selectively with the D stem, with a dissociation constant of 1.4 μM, and targets the same part of the RNA as the same imino proton involved in the interaction (Fig. 6). This result confirms the role of the linker as a key player for the binding selectivity, showing therefore, that a linear geometry is required for the selectivity to the D arm whereas the orientation of the fragments imposed by the 1,5-triazole linkage allows its binding to the T arm.

The first step of HIV reverse transcription is the base pairing of $tRNA^{Lys}_3$ to the viral genomic RNA, at its primer-binding site (PBS), which is able in turn to recruit the reverse transcriptase specifically and start the viral replication. The interaction site of

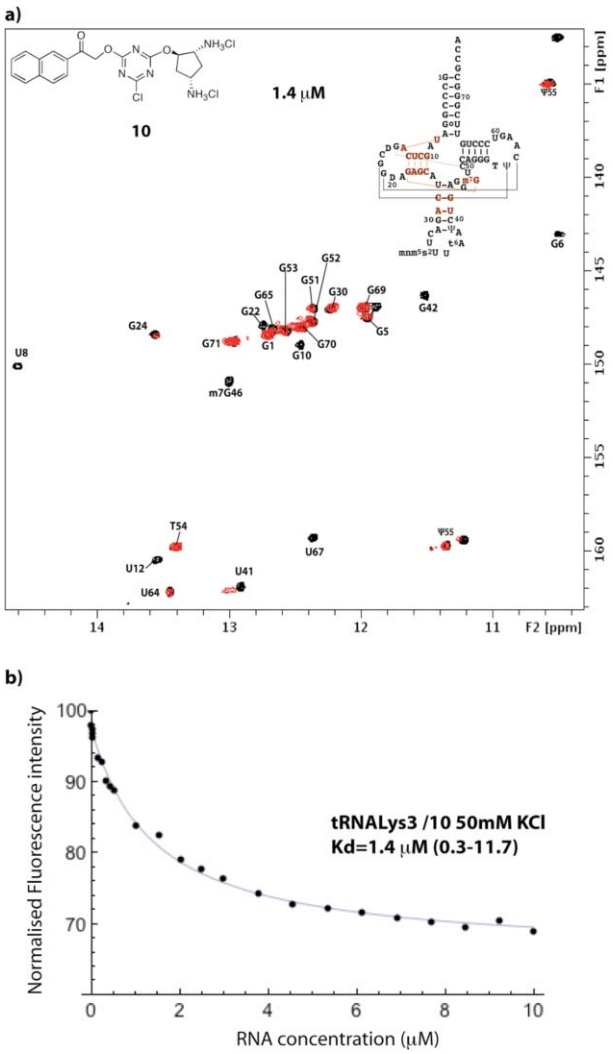


Fig. 6 Analysis of the binding properties of compound **10**. (a) NMR chemical shift mapping of the binding site of compound **10** on $tRNA^{Lys}_3$ imino groups at 1 : 4 molar ratio. (b) Fluorescence titration of compound **10** with $tRNA^{Lys}_3$. The value of K_d is indicated with the 95% confidence interval in parenthesis.

$tRNA^{Lys}_3$ with the viral RNA is located in its acceptor and T arm. The formation of this reverse transcription initiation complex requires the chaperone activity of the viral nucleocapsid protein that is known to bind to the D-arm of $tRNA^{Lys}_3$.[21] Thus, small molecules that bind to either the D arm or the acceptor and T arms are potential leads for the inhibition of the formation of the HIV-1 reverse transcription initiation complex. Within the ligands that we reported up to now, only few binders of the T stem were discovered, and the NMR CSP on the imino groups obtained with these compounds were rather weak, although the DACP fragment has a major binding site in this arm. We have therefore considered the T stem as much more challenging to target with small molecules than the D stem. Thus discovery that changing the geometry of the linker allows for directing the ligand to the T arm is valuable and bring new insight in order to further develop ligands of this stem.

Conclusion

In this work, we have shown that small fragments, able to interact weakly with the D or T stem of $tRNA^{Lys}_3$, can be connected by triazoles to furnish better ligands with improved affinity and, in some cases, selectivity. This improvement of selectivity is mainly controlled by the geometry of the linker, rather than its chemical nature. As D and T stems of $tRNA^{Lys}_3$ are both involved in the first steps of the HIV reverse transcription, the development of ligands able to bind to each or both sites is of interest, and might be feasible by a better exploration of conformational space accessible by rigid linkers. This work is ongoing in our laboratories.

Experimental

NMR Experiments

Experiments were recorded on a Bruker Avance DRX 600 spectrometer equipped with a cryofit system (60 μL) enabling us to connect the cryoprobe to a Gilson 215 liquid handler controlled by the NMR console (Bruker BEST system). All NMR experiments were conducted at 20 °C. TROSY spectra[22] with watergate sequence[23] for solvent suppression were recorded using samples containing 0.2 mM of ^{15}N-labelled $tRNA^{Lys}_3$, produced as previously reported,[24] for ligand concentration of 1 or 4 equivalents in a 10 mM phosphate buffer at pH 6.5 and 50 mM KCl. Samples were prepared in a total volume of 200 μL in 96-well plates and the injected sample volume was 180 μL.

Fluorescence titrations

Fluorescence titrations were conducted at 25.0 °C on a JASCO spectrofluorimeter. Excitation and emission wavelengths were 341 and 478 nm, respectively, for **5b** and **10**, 300 and 415 nm, respectively, for **5c**, and 336 and 403 nm, respectively, for **5f**. The excitation and emission bandwidths were 10 nm. Fluorescence titration experiments were performed by adding increasing concentrations of nucleic acid to a fixed amount of ligand (2 or 4 μM) in a phosphate buffer (10 mM, pH 6.5) for a salt concentration of 50 mM KCl. Fluorescence intensities were corrected for dilution and were fitted using eqn (1). Confidence limits on the K_d were estimated by Monte-Carlo sampling using the MC-Fit program.[22]

$$I = I_0 - \frac{I_0 - I_\infty}{2nN_t}\left(K_d + L_t + nN_t - \sqrt{\left(K_d + L_t + nN_t\right)^2 - 4L_t nN_t}\right) \quad (1)$$

where I_0: fluorescence intensity without RNA, I: fluorescence intensity at a given concentration of RNA, I_∞: fluorescence intensity at the plateau, n: number of RNA binding sites on the ligand, L_t: total concentration of RNA, N_t: total concentration of ligand.

Acknowledgements

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