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Fragment based search for small molecule inhibitors of HIV-1 Tat-TAR

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ABSTRACT

Basic molecular building blocks such as benzene rings, amidines, guanidines, and amino groups have been combined in a systematic way to generate ligand candidates for HIV-1 TAR RNA. Ranking of the resulting compounds was achieved in a fluorimetric Tat-TAR competition assay. Although simple molecules such as phenylguanidine are inactive, few iteration steps led to a set of ligands with IC_{50} values ranging from 40 to 150 μM. 1,7-Diaminoisoquinoline **17** and 2,4,6-triaminoquinazoline **22** have been further characterized by NMR titrations with TAR RNA. Compound **22** is bound to TAR at two high affinity sites and shows slow exchange between the free ligand and the RNA complex. These results encourage investigations of dimeric ligands built from two copies of compound **22** or related heterocycles.

TAR, the transactivation response element of HIV is a noncoding RNA sequence important for viral replication.[1,2] It is located at the 5′ end of all viral transcripts. In the case of HIV-1, TAR forms a stem loop structure of 59 nucleotides with an UCU bulge close to the hexanucleotide loop (Fig. 1). Upon binding of Tat, a viral regulatory protein expressed in the early stage of virus replication, the protein–RNA complex recruits the human positive transcription elongation factor (P-TEFb) which increases gene expression several hundred-fold.[3,4] This mechanism requires the formation of a ternary complex of high affinity and specificity between two proteins and the RNA. Random mutations of TAR, that are frequent due to the low accuracy of the reverse transcriptase, have a high probability to abolish its function. On this basis TAR has been an attractive drug target for some time.[5,6] However, almost two decades of research have introduced many types of TAR ligands but none of them met the criteria to enter a drug development process yet, despite the fact that the ternary complex of Tat, TAR, and P-TEFb is still considered a promising site for antiviral intervention.[7]

Our previous work was focussed on peptides composed of non-natural aromatic amino acids and lysine or arginine.[8–12] Although nanomolar target affinities could be achieved, the specificity for TAR remained insufficient.

The study presented here originated from the idea to define elementary ligand fragments and to combine them unbiased from any structural information about TAR. Such fragments are: The benzene ring able to stack and to undergo hydrophobic interactions and amines, amidines, or guanidines as H-bond donors that can be protonated under physiological conditions. All resulting compounds were subjected to a fluorimetric competition assay using the 31 mer TAR model and the dye labeled Tat peptide shown in Figure 1.[13] The dicationic arginine amide, the simplest TAR ligand known to induce similar structural changes in TAR as longer Tat peptides,[14] shows an IC_{50} value in this assay of 1500 μM. Fragment based search strategies for RNA ligands[15] have been successfully applied not only to TAR[16] but also to other RNA targets such as Hepatitis C virus IRES,[17–19] ribosomal decoding site,[20–22] Tetrahymena group I intron,[23] $tRNA^{Lys}$,[24–26] riboswitches,[27] and RNA quadruplexes.[28] All compounds shown in Figure 2 were either purchased or synthesized as described in the Supplementary data. The fluorimetric competition assay afforded a fast identification and ranking of potent ligands.[29] Neither unsubstituted guanidinium chloride **1** nor simple derivatives like phenylguanidine **2**, benzylguanidine **3**, and its heterocyclic analogue **4** are able to displace the reference ligand from TAR. Heteroaromatic guanidine **5** and amidine **6** are inactive as well. In contrast, 2-aminoimidazole **7** looks promising at first glance. However, the IC_{50} value of this compound improves steadily when aqueous solutions are kept under air. This effect was also found with other compounds, for example tetraaminoquinazoline **23**. The electron rich heterocycles in particular have the tendency to produce false positive results, presumably by forming multiply charged oligomers. Such samples

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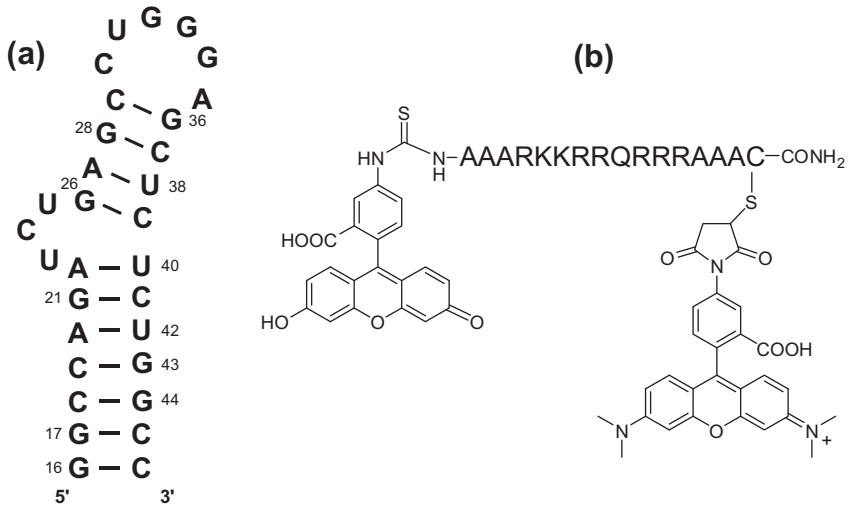

Figure 1. Components of the fluorimetric competition assay. (a) 31 mer Model of HIV-1 TAR. (b) Structure of the dye labeled Tat mimetic. Conditions: 10 nM of peptide and of RNA; 50 mM Tris–HCl, pH 7.4, 20 mM KCl, 0.01% Triton-X 100, 37 °C. The K_d of the complex is 0.8 ± 0.3 nM.[9]

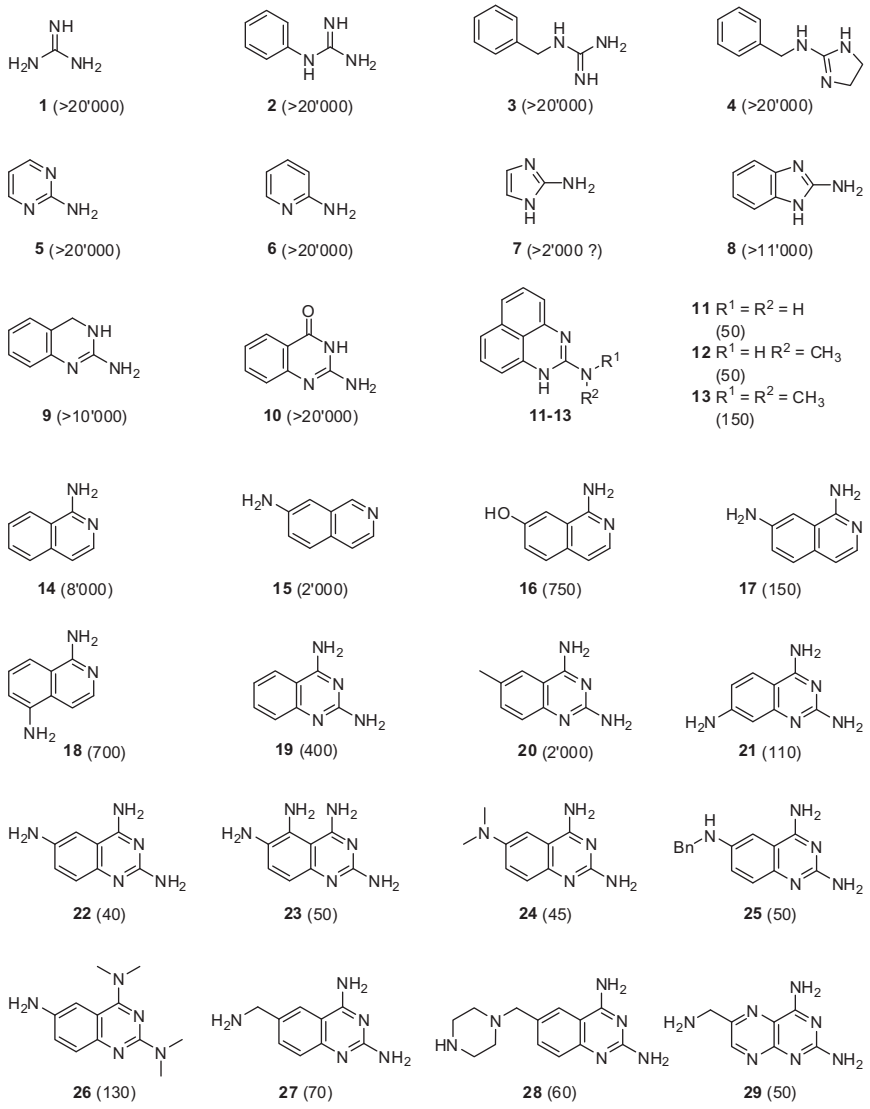

Figure 2. Compounds tested in the Tat-TAR competition assay. IC_{50} values (μm) are shown in brackets. Caution is required to eliminate errors due to decomposition or fluorescent emission of the ligands. See text and Figure 1 for details. Counterions: Cl^-: **1**, **3**, **4**, **9**, **12**, **21**, **23**, **24**, **25**, **27**, **28**; Br^-: **11**; I^-: **13**; nitrate: **2**; sulfate: **7**; trifluoroacetate: **26**; heterocycles **5**, **6**, **8**, **10**, **14**–**20**, **22**, and **29** were dissolved in buffer as neutral compounds.

must be carefully recrystallized and only freshly prepared solutions can be used in the assay. Most compounds, however, are either stable in solution or, after several days, show some decomposition forming inactive products.

From the first set of compounds, a second series was generated by a formal ring closure converting guanidines **2** and **3** into compounds **8**–**10**. The planarization of the structures leads to minor gains in RNA affinity. 2-Aminobenzimidazole **8**, due to its enlarged stacking surface should bind more tightly to RNA than compound **7**. The IC_{50} value of **7** is therefore surprising and should be considered with some care. By fusing guanidine **9** with a second benzene ring, however, the first interesting RNA binder results: 2-aminoperimidine **11** (50 μM). Stepwise methylation of the amino group reduces the potential of such perimidines to form hydrogen bonds and should heavily interfere with RNA binding. The dimethyl derivative indeed shows a reduction in affinity. However, the effect is unexpectedly small (150 μM) suggesting nonspecific intercalation as the dominating binding mode of compounds **11**–**13**.

1-Aminoisoquinolines represent another structural class we have examined. The pK_a values of these compounds are in the range of 7–8, so they are approximately half protonated under physiological conditions. The 1-aminoisoquinoline scaffold can be found in a number of pharmaceutical agents and should have a good bioavailability. Combining the structural elements of the two moderately active isoquinolines **14** and **15** leads to 1,7-diaminoisochinoline **17** (150 μM), a compound with TAR affinity increased by a factor of 13 or 53, respectively. The position of the amino groups is relevant: A fivefold lower IC_{50} value is seen with 1,5-diamino derivative **18** (700 μM). The replacement of the 7-amino group by hydroxy (**16**, 750 μM) causes a similar change. Both, NH_2 and OH, can donate or receive hydrogen bonds but the potential to become protonated might be important for the higher affinity of compound **17**.

Although isoquinoline **14** and quinazolines **9** and **10** are only weak Tat-TAR inhibitors, a combination of their structural features gives 2,4-diaminoquinazoline **19**, a compound with significant affinity (400 μM). The scaffold of **19** is present in the antihypertensives prazosin, terazosin, and doxazosin, or in dihydrofolate reductase inhibitors such as methotrexate, and piritrexim. As in the case of isoquinoline **14**, the IC_{50} value of **19** improves by one order of magnitude by adding NH_2 into position 6 (**22**, 40 μM). 2,4,6-Triaminoquinazoline **22** has already been described as a Tat-TAR inhibitor.[30] The importance of the 6-amino groups is seen from the fact that its replacement by methyl causes a 50 fold reduction in affinity (**20**, 2000 μM). Decreased binding is also observed when the amino group is shifted from position 6–7 (**21**, 110 μM). Compound **21** shows faster decomposition than its isomer **22** leading to high-affinity polycationic products. The problem of low stability and false-positive assay data is most pronounced with tetraaminoquinazoline **23**. No significant change in IC_{50} values results when the 6-amino group of **22** is methylated or benzylated. From the similarity of **22** and **24** to methotrexate one might expect cytotoxic effects. Methylation of amino groups in the heterocyclic ring of methotrexate analogues, however, is known to weaken their interaction with dihydrofolate reductase.[31] We therefore tested compound **26** which still has considerable affinity to TAR. Replacement of the 6-amino group of **22** by aminomethyl in quinazolines **27**, **28**, and pteridine **29** slightly impairs the IC_{50} value although these compounds will form dications at pH 7 more readily. Interestingly, even the presence of multiple protonation sites in compound **28** is noneffective.

For representative ligands from Figure 2, we tested the interaction with RNA by ^{1}H NMR titrations[32] using the same 31 mer TAR construct as in the fluorimetric assay (see Fig. 1). The imino region from 12 to 14 ppm shows signals of guanosines and uridines involved in stable base pairs: G16, G17, G21, G26, G28, G36, U38, U42, G43, and G44. Due to the dynamic nature of base pair A22-U40, no signal is visible for U40 in the absence of ligands. Signal assignment was based on NOESY spectra and on comparison with published data. 7-Aminoquinoline **17** was titrated in steps of 0.25 equiv into a 0.2 mM solution of the RNA up to a final concentration of 0.4 mM. A continuous shift around 0.1 ppm for signals of G21, G26, G28, and U38 was observed (see Supplementary data). NMR signals shifted and line widths did not increase during the

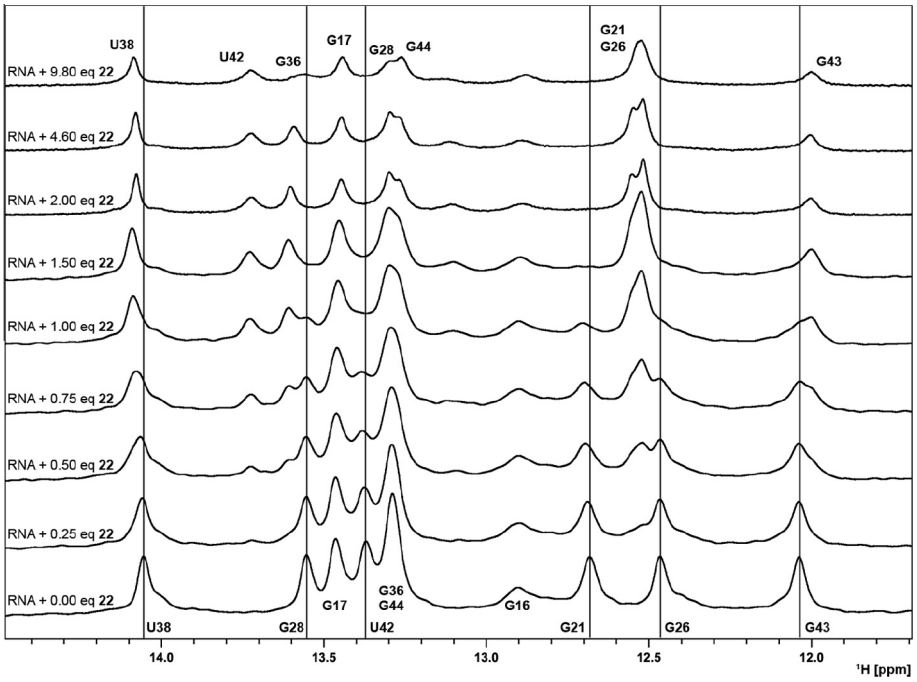

Figure 3. 1D ^{1}H NMR spectrum (600 MHz, 283 K) of the HIV-1 31 mer TAR RNA (0.2 mM RNA H_2O/D_2O 9:1, pH 6.2). The signals of imino protons are shown as a function of added quinazoline **22**.

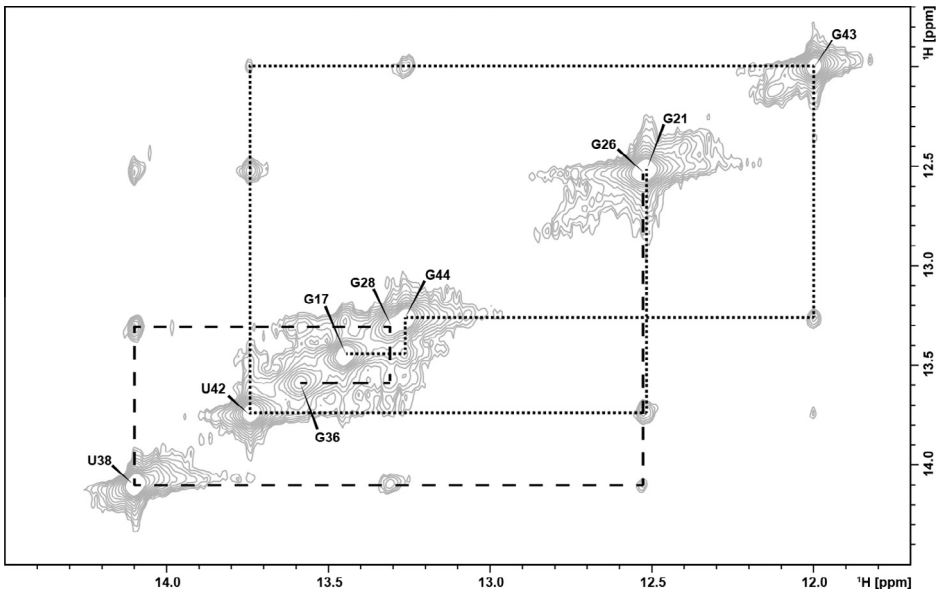

Figure 4. ^{1}H, ^{1}H NOESY spectrum (700 MHz, 283 K, 150 ms mixing time) of the imino protons of HIV-1 31 mer TAR RNA (0.5 mM RNA, H_2O/D_2O 9:1, pH 6.2) in the presence of 2 equiv of compound **22**. Through space connectivities for the lower stem (G17-G44-G43-U42-G21) are highlighted by a dotted line. The dashed line shows the correlation of spins belonging to the upper stem (G26-U38-G28-G36).

titration. Accordingly, free compound **17** is in fast exchange with the TAR-**17** complex. From analysis of the chemical shifts, we predict the binding site to be close to G26, similar to the complex TAR forms with arginine amide.[14] This assumption is consistent with the effects on G26, G28, and U38 and also the absence of shifts for G36. Binding at G26 is expected to stabilize the A22-U40 base pair, which also affects the resonance of G21.

In the same way, a titration with quinazoline **22** was performed. Above 0.4 mM, **22** was added in steps of 1.3 equiv up to a final ligand-RNA ratio of 9.8:1. Significant changes occur only in the range from 0 to 2 equiv of **22**. Interestingly, most signals do not shift continuously. They gradually fade away and reappear in their new position: such response during ligand titration is characteristic for slow exchange of free compound **22** and the TAR-**22** complex (Fig. 3). From NMR, triaminoquinazoline **22** shows higher affinity than compound **17**, in agreement with fluorimetric assay data.

Resonance assignments for the newly arising signals were obtained from NOESY spectra in the presence of 2 equiv of **22** (Fig. 4). With the exception of G16 G17, and G44, all other signals are significantly displaced. Effects are most pronounced for G36, G28, U42, and G21.

TAR not only binds the arginine rich domain of Tat but also a number of arginine-derived ligands. Arginine amide[14] and other guanidinium compounds[33] have been shown by NMR to form complexes with G26 resembling a G-C Hoogsteen base pair. Based on these findings, we tested whether combining guanidines, amines and aromatic rings would yield high affinity TAR ligands. In fact, this hypothesis proved to be effective. Just a few iteration steps led from inactive molecules to several candidates with IC_{50} values below 200 μM. From our NMR data we propose that heterocycles containing the substructure of amidines and guanidines may behave like arginine amide and interact with G26. Indeed, the NMR titration of TAR with isoquinoline **17** is consistent with such interpretation. The best ligand found in this study is triaminoquinazoline **22**. Both structures have in common the distance between the amidine part and the isolated amino group. In spite of this similarity, the interaction with TAR is quite different: **22** not only is a much better ligand, showing slow exchange in NMR. It also has two high affinity binding sites in TAR. Compound **22** and its tetraamino analog **23** both have been identified previously as potent Tat-TAR inhibitors in an industrial drug screening program.[30,34] From chemical probing with diethyl pyrocarbonate, a binding site of **23** in the loop region close to A35 was assumed. Compound **23** also protects G36 against methylation by dimethyl sulfate.[34] Given the similarity between compounds **22** and **23**, this observation coincides with the strong influence compound **22** exerts on the signal of G36. The second binding site of **22** must be in the lower stem around G21. This observation opens a new strategy for the development of drug-like heterocyclic Tat-TAR inhibitors by combining two units of compounds **17** and **22** with appropriate linkers.

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Supplementary data

Supplementary data (Synthetic procedures and characterization data for all new compounds or new synthetic pathways. NMR titration of the 31 mer TAR RNA with compound **17**. ^{1}H and ^{13}C NMR spectra of compounds depicted in Fig. 2.) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.11.004.

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29. *Fluorimetric binding assay:* Experiments were performed as described previously[9] in 96-well microplates by titration of compounds **1**–**29** into a mixture of 31 mer TAR RNA (10 nM; BioSpring, Frankfurt, Germany) and Tat peptide (10 nM; Thermo Electron Corporation, Ulm, Germany) in TK buffer (50 mM Tris–HCl, 20 mM KCl, 0.01% Triton-X 100, pH 7.4, 37 °C). Different to previous reports, the excitation wavelength was optimized here (Reader: Tecan safire[2]; λ_{ex} = 489 nm, λ_{em} = 590 nm). Contact quenching of the two dyes, not FRET, is the dominant effect explaining the fluorescence change in this assay. An independent titration into buffer free of RNA and Tat peptide was conducted for each compound. Fluorescent emission of ligands thus could be subtracted from the emission value of the Tat peptide. Titrations were repeated with ligand solutions kept under air for 1–3 days. In most cases no change or a minor decline of RNA affinities was observed. In contrast, higher affinities were found for compounds **7** and **23**. We attribute these false positives to the formation of oligomeric decomposition products of the heterocycles.
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