Published in final edited form as:
Bioorg Med Chem Lett. 2013 October 15; 23(20): 5689–5693. doi:10.1016/j.bmcl.2013.08.014.

Recognition of HIV-TAR RNA using Neomycin-Benzimidazole Conjugates

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Abstract

Synthesis of a novel class of compounds and their biophysical studies with TAR-RNA are presented. The synthesis of these compounds was achieved by conjugating neomycin, an aminoglycoside, with benzimidazoles modeled from a B-DNA minor groove binder, Hoechst 33258. The neomycin-benzimidazole conjugates have varying linkers that connect the benzimidazole and neomycin units. The linkers of varying length (5-23 atoms) in these conjugates contain one to three triazole units. The UV thermal denaturation experiments showed that the conjugates resulted in greater stabilization of the TAR-RNA than either neomycin or benzimidazole used in the synthesis of conjugates. These results were corroborated by the FID displacement and tat-TAR inhibition assays. The binding of ligands to the TARRNA is affected by the length and composition of the linker. Our results show that increasing the number of triazole groups and the linker length in these compounds have diminishing effect on the binding to TAR-RNA. Compounds that have shorter linker length and fewer triazole units in the linker displayed increased affinity towards the TAR RNA.

Keywords

TAR; Neomycin; Aminoglycosides; tat-TAR inhibition; Bnzimidazole

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Supplementary data

Details of experimental procedures and synthesis of DPA 123 is provided. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/XXXX

Since the first reports of HIV-AIDS in the United States in the early 1980s, research towards its cure have identified an RNA directed strategy which targets the interactions of tat protein with the Trans Activating Region (TAR) of the viral RNA.[1] A 29-mer oligonucleotide, which is a model of a full 59 mer TAR region of the viral RNA, contains two of the most commonly found structural features in the RNA-namely the hairpin loop and the short trinucleotide bulge (Figure 1a). The trinucleotide bulge region has a wide major groove that is accessed by the tat protein for viral replication and thus inhibition of this interaction has developed into a viable approach to stop viral growth.[2,3]

Several DNA and RNA binders have been investigated to inhibit tat-TAR interactions. These binders include polyamines (argininamide)[3], polyamides,[4] peptides,[5,6] peptidomimetics,[7] intercalators,[8] quinoline derivatives,[9,10] quinolones,[11] DNA minor groove binders,[2] aminoglycosides[12-14] and their derivatives.[15-17] Neomycin is an aminosugar (Figure 1b) that has been known for decades for its RNA binding.[18,19] Neomycin has been shown to inhibit the tat-TAR interaction by binding to the trinucleotide pyrimidine bulge of the TAR-RNA[14] and has the highest affinity among the selected aminoglycosides studied.[12] Neomycin has been observed to bind with the TAR-RNA making contacts in the minor groove present in the lower stem using its ring III and IV while rings I and II have been found to interact with the trinucleotide bulge (Figure 1a and 1b). The bis-benzimidazole Hoechst 33258, a known B-DNA minor groove binding molecule, has also been shown to interact with the TAR-RNA at a site opposite to the bulge region where it recognizes the helical region below the hairpin loop (Figure 1a). RNAase A footprinting analysis has suggested that Hoechst 33258 binds to GCUCU bases of the TAR RNA in the upper stem.[2]

Aminoglycosides have emerged as versatile nucleic acid binders over the past decade.[20-26] Several aminoglycoside conjugates have been synthesized[27-30] and shown to have enhanced binding to variety of DNA,[31-34] RNA,[35] and DNA:RNA hybrid[36] structures. We have recently reported recognition of TAR-RNA using a series of dimeric neomycin conjugates.[37,38] The neomycin dimers have shown significant enhancement in the protection of MT-2 cells from the cytopathic effects of HIV infection in comparison to neomycin alone.[37] These studies have opened new avenues for multi-valent approaches for the recognition of TAR RNA by aminoglycoside based small molecules.

In our continuing effort to develop novel small molecules for TAR RNA recognition, we herein report a series of dye Hoechst 33258. Hoechst 33258 has been shown to interact with the TAR RNA through intercalative binding.[39] However, the planar surface of Hoechst 33258 is wider than that of RNA base pairs. Therefore, a smaller benzimidazole was hypothesized to be a more complimentary surface that favors facile entry between the helical bases in addition to reducing the molecular weight and polarity of the conjugated ligand.

The monobenzimidazole derivatives modeled from Hoechst 33258 (referred to as benzimidazoles henceforth), like its parent structure, penetrates easily in the cell and can be neomycin–benzimidazole conjugates modeled from the bisbenzimidazole fluorescently detected (unpublished results). The benzimidazoles (**DPA 101** and **DPA 102,** Scheme 1) lack one benzimidazole unit in comparison to Hoechst 33258 which is a bisbenzimidazole

dye. These smaller benzimidazoles are much easier to synthesize and purify than the larger and more polar bisbenzimidazoles. The synthesis of neomycin-benzimidazole conjugates was achieved using a divergent strategy. The neomycin units (azide[40,41]/alkyne units) and the benzimidazole units (containing the complementary alkyne/azide) were prepared separately (Scheme 1, supporting information Scheme S1). Scheme 1 shows the synthesis of alkyne/azide terminated benzimidazoles. The synthesis of clickable benzimidazoles rested on selective incorporation of the terminal alkyne or azide units. **DPA 101** was synthesized in three steps from commercially available 5-chloro-2-nitroaniline (**1**) as shown in Scheme 1. This transformation was achieved by substitution of the chloro substituent in **1** with N-methyl piperazine in the presence of K_2CO_3. The synthesized compound 5-(4-methylpiperazin-1-yl)-2-nitroaniline was then hydrogenated to its corresponding diamine[42]**2** followed by condensation with 4-(prop-2-ynyloxy) benzaldehyde (**3**)[43] in the presence of $Na_2S_2O_5$ to afford the desired ligand **DPA 101**. Similarly, **DPA 102** was synthesized by condensation of the above mentioned diamine (**2**) with 4-(2-azidoethoxy) benzaldehyde (**4**)[44] in the presence of the oxidant sodium metabisulfite[45]. **DPA 101** and **DPA 102** were then reacted with various bisazides (**5a-d**) or bisalkynes (**6a-e**) to afford the azide or alkyne terminated benzimidazole derivatives (**DPA 103-DPA 112**) containing various linker lengths. The ligands **DPA 103-DPA 112** were then reacted with Boc protected neomycin azide (**7**) or Boc protected neomycin alkyne (**8**) (see supporting information S2a,b for the reaction scheme) under click chemistry conditions leading to the formation of Boc protected neomycin benzimidazole conjugates (**DPA 113-123**). Deprotection of the Boc groups was achieved with 4M HCl in dioxane (Scheme 2, S2a,b). The purity of new compounds was checked with TLC and ^{1}HNMR; the identity of the synthesized compounds was further corroborated by (^{1}H/^{13}C) NMR and mass (MALDI-TOF) spectroscopic analysis.. A reaction scheme for the synthesis of **DPA 123** and the structures of all ligands synthesized are displayed in Scheme 2.

These neomycin–benzimidazole conjugates (**DPA 113-123**) were then evaluated for their affinity towards TAR RNA.

The relative affinities of these ligands were evaluated by two methods. In the FRET analysis,[46] a tat peptide containing fluorescein and rhodamine as FRET pair was complexed with TAR RNA. The tat-TAR complex was incubated with ligand of interest to displace bound tat protein which is reflected by the quenching of fluorescein emission. The tat-TAR inhibitory affinity can be expressed as IC_{50} which reflects a ligand's ability to displace 50% bound tat peptide from TAR. In another assay based on fluorescent intercalator displacement assay (FID),[47] a TAR bound intercalator (EtBr) was successively displaced by the addition of ligand of interest. The resulting binding isotherm can then be analyzed to obtain DC_{50} values (DC_{50} corresponds to the concentration of the ligand required to displace 50% of the bound intercalator). The results of these assays are summarized in Table 1 and a representative plot is shown in Figure 2. The data shown in Table 1 reveals that affinity of these ligands towards TAR is dependent on both linker length and their composition. In general, ligands with shorter linker and fewer triazoles (eg **DPA 123, DPA 120**) displayed a stronger affinity to TAR RNA in comparison to conjugates with longer linker length and more triazole units (**DPA 113-DPA117**) as displayed by their DC_{50} and IC_{50} values. **DPA**

123, with a short linker and a single triazole, emerges as the best ligand in binding to the TAR with DC_{50} and IC_{50} values of 33 nM and 257 nM, respectively. To study the selectivity of DPA123 for TAR over other HIV RNA targets, we investigated its ability to affect the Rev-RRE (rev response element) complexation. We observed that **DPA 123** displays much better ability to affect tat-TAR binding, when compared to its ability to displace Rev from RRE, another RNA target of HIV virus. The IC_{50} value for inhibition of RRE RNA-HIV-1 Rev peptide complex was (768 ± 309) nM (see supporting information), significantly higher than IC_{50} value for inhibition of tat-TAR complexation.

The nucleic acid binding of these ligands was also evaluated using thermal denaturation experiments. The thermal denaturation temperatures are listed in Table 1. In the presence of benzimidazole **DPA 101** alone, the thermal stabilization afforded was 1.0 °C while the same with neomycin was 0.2 °C. All conjugates afforded higher thermal stabilization of TAR RNA than any of the building blocks used to build the conjugates, with **DPA 123** providing the highest thermal stabilization (5.6 °C). The highest thermal stabilization afforded by **DPA 123** also corroborated the results obtained from FID experiments and the tat-TAR inhibition assay. In some cases, however, we did not observe correlation between the thermal binding data and the DC_{50}/IC_{50} values. As these DC_{50}/IC_{50} values are obtained by evaluating two entirely different assays, the lack of correlation could be attributed to the differences in the binding sites of the two probes bound TAR in the two assays. EtBr intercalates nonspecifically whereas the fluorescein tagged tat protein binds more specifically in a 1:1 fashion. The thermal denaturation profiles of the RNA, on the other hand, reflect a more complex temperature dependence of ligand:RNA equilibrium constants and are not expected to always correlate with isothermal room temperature K_d or IC_{50} measurements.

We also performed circular dichroism studies with **DPA 123** to investigate the ligand induced changes in the conformation of the TAR RNA. As shown in Figure 3, the titration of **DPA 123** led to increasingly diminished signal at 210 nm and 262 nm which also displayed the presence of isobestic points at 231 nm and 272 nm. The changes in the CD intensity upon ligand binding are indicative of the complexation of ligand with the host RNA duplex that is accompanied by slight structural alterations of the nucleic acid structure.[48] However, the CD spectrum displays the characteristic A-form features (a negative band at 210 nm and a positive band at 263 nm)[49] during the entire course of titration suggesting that overall structural features of the RNA are retained upon binding of **DPA 123.**

Hoechst 33258 binding to TAR has previously been reported to occur in an intercalative way[39] and thus a similar mode of binding is likely for the benzimidazoles. We, however, did not observe any induced CD in the benzimidazole chromophore absorption at low drug:RNA ratios and the use of higher than 1.6 equivalents of **DPA 123** led to the precipitation which prevented us from studying the induced CD at higher drug concentrations.

In conclusion, our studies show that conjugation of neomycin to benzimidazoles modeled from Hoechst 33258 results in improved RNA binding and improved inhibition of RNA-protein interactions. Conjugates with short linkers (**DPA 123**) displayed a better and

selective binding (TAR vs. RRE) than compounds with longer linkers, and the binding was affected by the number and placement of triazole units. Effect of these conjugates on HIV inhibition is currently being explored and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support to this work was provided by National Institute of Health grants (R15CA125724 and 1R41GM100607).

Abbreviations

UV	Ultra Violet
FID	Fluorescent Intercalators Displacement
CD	Circular Dichroism
TAR	Trans Activating Region
NMR	Nuclear Magnetic Resonance
MALDI-TOF	Matrix Assisted Laser Desorption and Ionization- Time of Flight

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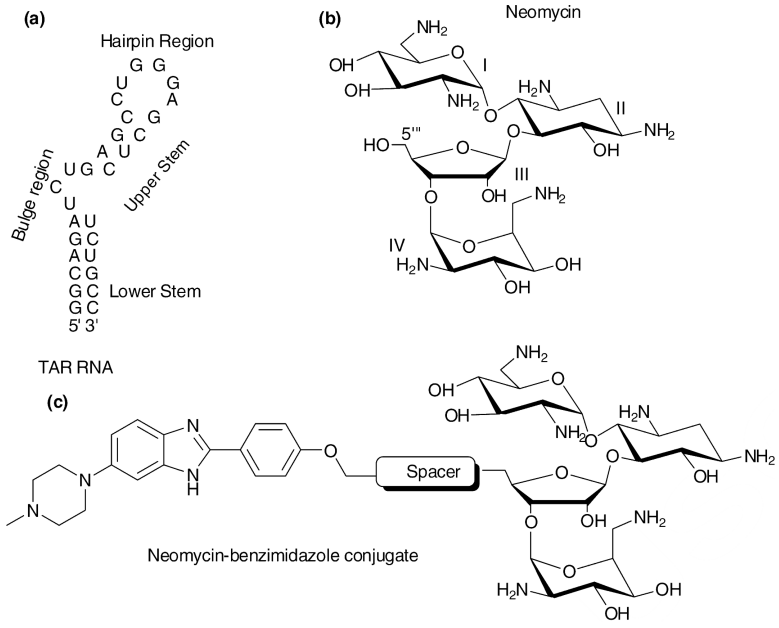
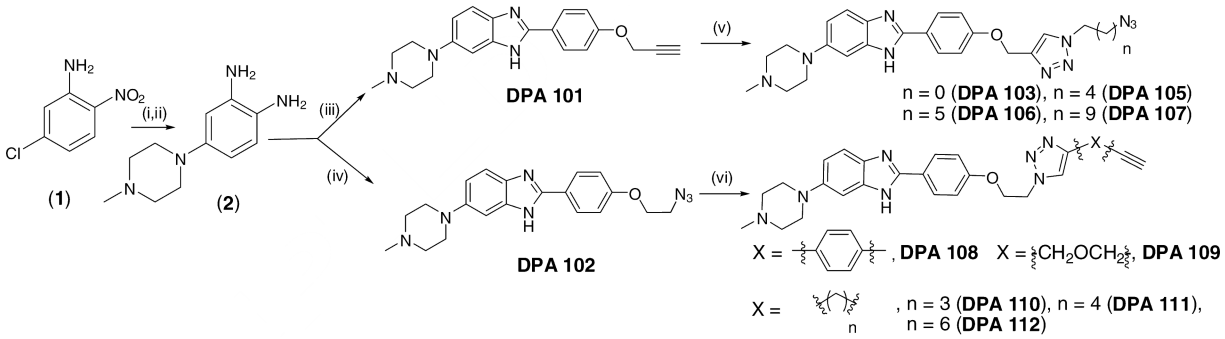


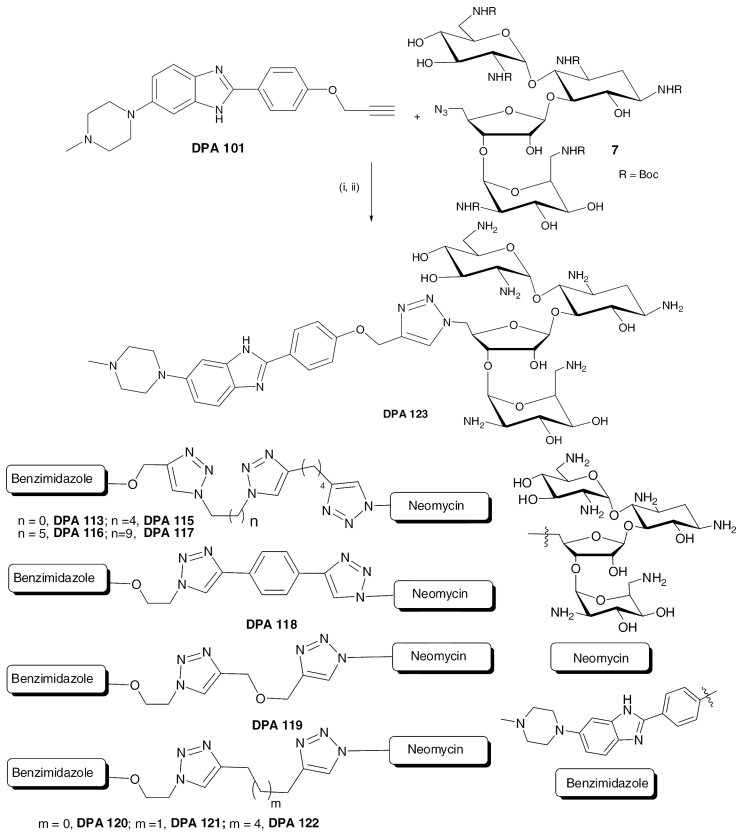
Figure 1.

(a) Illustration of 29 mer short oligonucleotide mimic of the TAR- RNA. (b,c) Chemical structures of neomycin and neomycin-benzimidazole conjugates.



Scheme 1.

Reagents and conditions (i) DMF, N-methylpiperazine, 100 °C, 5h, 60% (ii) Pd-C (10%), ethanol, H_2, 6h, qaunt. (iii) 4-(prop-2-ynyloxy) benzaldehyde (**3**), $Na_2S_2O_5$, H_2O, reflux, 12h, 65% (iv) 4-(2-azidoethoxy) benzaldehyde (**4**), $Na_2S_2O_5$, H_2O, reflux, 12h, 82% (v) sodium ascorbate, copper (II) sulfate, 50 fold excess bisazides [N_3-CH_2-$(CH_2)_n$-N_3; n = 0(**5a**); n = 4(**5b**), n = 5(**5c**), n = 9(**5d**)], ethanol, room temperature, overnight,65-80 % (vi) sodium ascorbate, copper (II) sulfate, 50 fold excess bisalkynes [1, 4 diethynyl benzene (**6a**), propargyl ether (**6b**), HC-$(CH_2)_n$-CH; n = 3(**6c**), n = 4(**6d**), n = 6(**6e**)] ethanol, temperature, overnight, 75-90 %.



Scheme 2.

(i) Sodium ascorbate, copper (II) sulfate, ethanol, room temperature, overnight (ii) Dichloromethane, 4M HCl in 1,4 dioxane, 49 % cumulative yield for two steps.

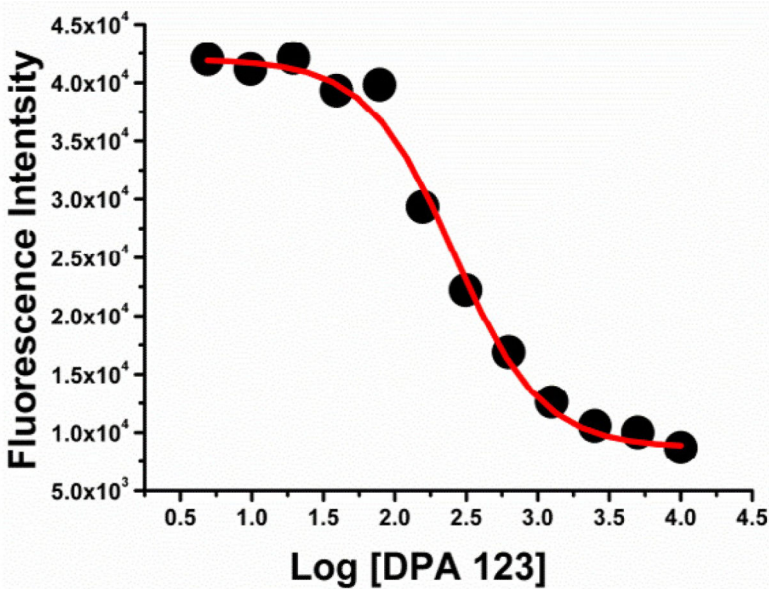


Figure 2.

A representative plot showing the decrease in the fluorescence of fluorescein labeled tat-TAR complex upon ligand (**DPA 123**) binding of tat-TAR RNA complex. A 1:1 mixture of tat peptide (100 nM) and TAR RNA (100 nm) were mixed in Tris buffer containing 50 mM Tris, 20 mM KCl at pH 7.4. The resulting complex was titrated with DPA 123 until no change in fluorescence was observed. The resulting binding isotherm was fitted with a dose response curve using Origin 5.0 (see supporting information for fitting details).

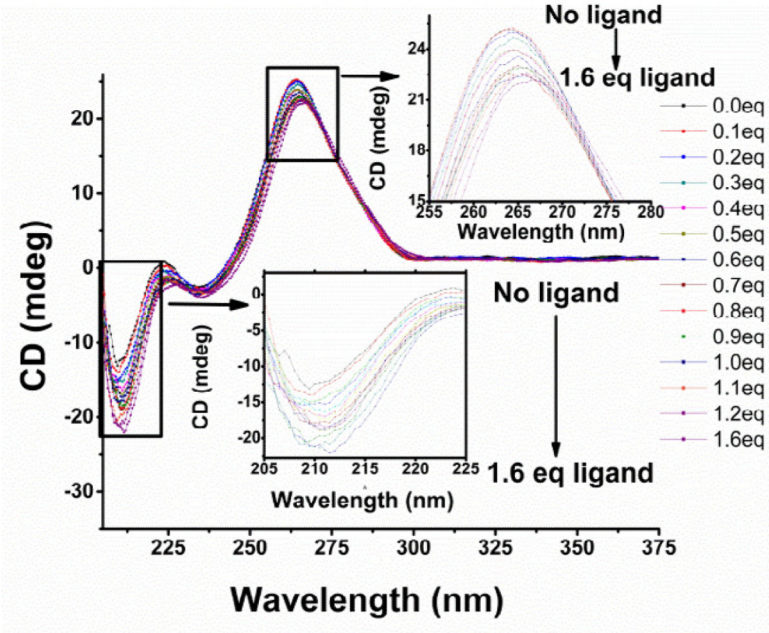


Figure 3.

CD titration profile of TAR RNA with DPA 123. The ligand was added serially to the RNA solution in small increments as displayed on the graph. After each successive addition, the nucleic acid ligand-complex was stirred and equilibrated for five minutes before the spectrum was recorded. Each spectrum shown in the figure is an average of three scans. The experiments were performed in cacodylate buffer containing 10 mM sodium cacodylate, 0.5 mM EDTA, 100 mM KCl at pH 6.8. (T = 20 °C). The boxed regions have been expanded for clarity.

Table 1

A table showing the DC_{50}, IC_{50} and thermal stabilization afforded by ligands used in the study.

Ligand	Linker Length[a]	No. of triazoles	DC_{50}(nM)	IC_{50}(nM)	ΔTm (°C)
Neomycin	NA	NA		1474 ± 79	0.2
DPA 101	NA	NA	ND	ND	1.0
DPA 123	5	1	33	272 ± 8	5.6
DPA 119	11	2	78	1285 ± 155	3.0
DPA 120	12	2	83	337 ± 43	3.4
DPA 121	13	2	140	507 ± 9	4.1
DPA 118	13	2	180	379 ± 20	3.0
DPA 122	15	2	81	949 ± 92	5.4
DPA 113	17	3	184	411 ± 51	2.5
DPA 115	20	3	150	851 ± 80	2.1
DPA 116	21	3	200	1368 ± 50	3.8
DPA 117	23	3	180	1380 ± 150	4.0

[a]The linker length was assigned by counting the number of main chain atoms between the 5’”-carbon on ribose ring (ring III, see Figure 1) of neomycin and oxygen atom on the phenolic end of the benzimidazole.