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Linker dependent intercalation of bisbenzimidazole-aminosugars in an RNA duplex; selectivity in RNA vs. DNA binding

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Abstract

Neomycin and Hoechst 33258 are two well-known nucleic acid binders that interact with RNA and DNA duplexes with high affinities respectively. In this manuscript, we report that covalent attachment of bisbenzimidazole unit derived from Hoechst 33258 to neomycin leads to intercalative binding of the bisbenzimidazole unit (oriented at 64–74° with respect to the RNA helical axis) in a linker length dependent manner. The dual binding and intercalation of conjugates were supported by thermal denaturation, CD, LD and UV-Vis absorption experiments. These studies highlight the importance of linker length in dual recognition by conjugates, for effective RNA recognition, which can lead to novel ways of recognizing RNA structures. Additionally, the ligand library screens also identify DNA and RNA selective compounds, with compound **9**, containing a long linker, showing a 20.0 °C change in RNA duplex T_m with only a 9.0 °C change in T_m for the corresponding DNA duplex. Significantly, the shorter linker in compound **3** shows almost the reverse trend, a 23.8 °C change in DNA T_m , with only a 9.1 °C change in T_m for the corresponding RNA duplex.

Graphical Abstract

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Supplementary Material

Supplementary data (details of synthesis of conjugates **10**, **11**, experimental procedures and LD spectra) associated with this article can be found in the online version <http://dx.doi.org/XXXXXX>.

Keywords

Neomycin; Hoechst 33258; Intercalation; RNA duplex; linear dichroism

RNAs are highly diverse and dynamic¹ nucleic acid structures that contain duplex, loops, bulges and single stranded regions which play key regulatory roles in life processes. The functions of RNA go far beyond their repertoire of being one of the central components of translation process as evidenced by the emergence of catalytic RNAs,^{2,3} miRNAs⁴ and RNA G-quadruplexes⁵ in the past three decades. The establishment of non-coding RNA roles in cancer biology⁶ has brought renewed zeal in the study of RNA functions and their control for developing nucleic acid based therapeutics. Several classes of RNA binders have been reported.⁷ Many of these binders display promiscuity⁸ and non-specificity in RNA binding⁹ while some exhibit unanticipated modes of interaction.^{10,11} Thus, the development of new RNA interacting small molecules with defined mode of binding is of prime significance in nucleic acid based drug design. A majority of RNA interacting ligands¹² have been major groove binders,^{13–15} intercalators¹⁶/threading intercalators^{17,18}, binders of specific RNA loops and bulges^{19–24} as well as a few ligands that have been suggested to have mixed groove binding and intercalation properties.¹⁰ Although, the interaction of a peptide in the minor groove of the RNA has been reported²⁵, RNA minor groove recognition remains an area of limited success.²⁶

We have recently reported that enhanced recognition of RNA is indeed viable using a neomycin-Hoechst 33258 conjugate.²⁶ The premise of such dual RNA recognition, combining ligands with independent binding sites, is known to achieve multi-recognition of nucleic acids using engineered small molecules.²⁷ Such enhanced molecular recognition of nucleic acids can be achieved by dual/triple recognition agents made by covalent attachment of ligands that are independently capable of nucleic acid recognition at specific sites. The success of this strategy is dependent upon the choice of ligands for covalent attachment so that their nucleic acid binding sites do not overlap. The conjugation strategy has led to significant increase in the binding affinity of ligands towards the recognition of DNA triplexes,²⁸ quadruplexes,^{29,30,31} and DNA:RNA hybrid structures.³² Using this strategy, in certain cases, even a weak binder can be used to enhance the nucleic acid stability using a novel mechanism. An example of such enhancement is dual recognition of B-DNA by neomycin-Hoechst 33258 conjugates where a weak B-DNA binder neomycin can

significantly help in enhancing duplex DNA stability.³³ The B-DNA recognition was guided by tight binding of Hoechst 33258 in the minor groove of B-DNA and is assisted by neomycin, covalently attached by a suitably spaced linker.³⁴

In comparison to the established binding of neomycin in the RNA major groove, Hoechst 33258 binding to RNA is poorly understood. A study, however, has suggested its RNA interaction through groove binding mode in a polymeric GC RNA duplex.³⁵ Hoechst 33258 has also been suggested to bind through intercalation in a short oligonucleotide derived from HIV-TAR RNA.³⁶ It has been reported that dual recognition of RNA by a neomycin-Hoechst 33258 conjugate leads to enhanced association constant (K_a) (compared to neomycin) by an order of magnitude. The exact binding mode of Hoechst 33258 moiety however was not determined.²⁶ Moreover, since neomycin drives the binding to RNA, linker length and composition is expected to play a significant role in optimization of binding of the two components. Another significant issue in the design of nucleic acid binding molecules is the DNA vs RNA selectivity. To address these questions, we now report the development of a series of novel neomycin-bisbenzimidazole conjugates (derived from Hoechst 33258) with varying linker lengths and compositions (Fig. 1). We investigate the RNA binding of these conjugates, with a focus on determining the exact binding mode of bisbenzimidazole moiety, and the influence of linker length in dual recognition of RNA vs. DNA duplex.

Figure 1 shows the chemical structures of ligands used in this study which were either commercially purchased or synthesized in our laboratory using click chemistry based conjugation approach reported previously by us.^{37,38} Briefly, alkyne functionalized Hoechst 33258 derivatives³⁹ were coupled with Boc protected neomycin azide under *in situ* Cu(I) catalytic conditions.⁴⁰ The resulting Boc protected neomycin-bisbenzimidazole conjugates were then deprotected under acidic conditions to provide the desired conjugates **3–11** which were further characterized by spectroscopic techniques. Complete synthesis and characterization details of two new conjugates **10**, **11** used in this study have been provided in the supplementary information (Scheme S1). The linkers joining the neomycin and Hoechst 33258 units in conjugates **3–11** are of increasing length (5–23 atoms) which can be further sub-classified according to the number of oxygen atoms present in them. Conjugates **3–5**, **6–10** and **11** contain one, two and seven oxygen atoms respectively between C-5 carbon of ribose ring III on neomycin and the bisbenzimidazole moiety (Fig. 1) and have thus been referred as monooxygen, bisoxygen and polyoxygen linker in the forthcoming sections.

To have a qualitative view of the binding affinities of ligands **3–11** towards RNA duplex, we performed FID displacement assay⁴¹ which has been used with several RNA and DNA oligomeric sequences to assess relative binding affinity of small molecules.^{42–44,44} FID experiment was done with a well characterized oligomeric duplex r(CGCAAUUUGCG)₂^{45,46} which is the RNA equivalent of A-tract DNA sequence d(CGCAAATTTGCG)₂⁴⁷ well known for Hoechst 33258 binding.⁴⁸ The changes in the fluorescence emission afforded by various ligands were plotted as percent change with respect to the linker lengths as shown on the X-axis of each bar in Figure 2. The changes in the fluorescence emission differentiate the ligands of high affinity from the weak binders. As shown in Figure 2, all conjugates showed better displacement (20.7–36.2%) of the intercalator probe in comparison to neomycin (20.4%) or Hoechst 33258 (8.2%). This data

shows that conjugates **3–11** result in a better binder *vis a vis* the binding of neomycin or Hoechst 33258 to the RNA duplex. In Figure 2, conjugates containing monooxygen (black bars), bisoxygen (red bars) and polyoxygen (blue bar) linkers show length dependent binding of the conjugates. In each of these subsets of conjugates **3–11**, as the linker length increases, a noticeable decrease in the percent fluorescence change was observed. The conjugate binding however increases with the polyoxygen linker (**11**) nearly matching the fluorescence change afforded by the best binder **5**. This result highlights the impact of linker length and composition (number of oxygen atoms) on the conjugate binding and shows better binding of conjugates **3–11** to the RNA duplex in comparison to neomycin (**1**) or Hoechst 33258 (**2**).

To further explore the nature of RNA binding mode of conjugates **3–11**, we performed linear dichroism (LD) experiments with poly(rA)•(rU) duplex. The choice of polymeric RNA duplex was made since oligomeric nucleic acids cannot be studied by flow linear dichroism experiments (discussed later) used for the determination of binding mode. We initially probed the interaction of Hoechst 33258, neomycin and the best binder **7** from FID assay with poly(rA)•(rU) using CD spectroscopy. CD experiments show binding linked changes in the nucleic acid structure and also shed light on ligand-ligand/ligand-nucleic acid higher order structure formation during the binding process.^{49–51} Figures 3a–c represent the CD titration profiles of Hoechst 33258, conjugate **7** and neomycin respectively, while Figures 3d–f show the binding stoichiometry determined from these titrations. As shown in Figure 3a, in the absence of ligand, CD spectrum of poly(rA)•(rU) had the maximum absorption at 263 nm. Addition of increasing amounts of Hoechst 33258 resulted in CD intensity decrease at 263 nm while an induced CD was observed in the 320–380 nm wavelength region. The observed changes in CD intensity at 263 nm and the bisignate exciton CD spectrum (showing minimum at 332 nm and maximum at 371nm) indicate the interaction of the Hoechst 33258 (**2**) with the host RNA duplex. The appearance of such exciton CD bands during ligand-nucleic acid interaction is typically reflective of dimer or higher orders structure formation either in a groove binding or external stacking mode.^{10,11,49,51} The exciton CD band of Hoechst 33258 (**2**) is very similar to the changes observed when Berenil binds to poly(rA)•(rU)⁸ and carbocyanine dyes bind to higher order DNA structures⁵²; both of which were proposed to bind in a mixed groove binding/stacking fashion. Several other DNA duplex binding dyes have also shown such exciton CD signals in the presence of nucleic acids.⁵³ Therefore the observed exciton CD bands of Hoechst 33258 (**2**) in the presence of poly(rA)•(rU) is indicative of an higher order complex formation. Interaction of Hoechst 33258 (**2**) with a variety of AT rich B-DNA sequences have shown positive induced CD bands exclusively.^{54,34} Thus such bisignate exciton CD bands of Hoechst 33258 in the presence of RNA duplex also suggest a clear differences in its mode of binding, when compared to B-DNA duplex.

The changes in the nucleic acid CD intensity upon binding of a ligand can be used to obtain binding site size denoted by r_{bd} (ratio of bases per drug). The binding site size of Hoechst 33258 (**2**) to poly(rA)•(rU) duplex shows that it covers approximately five base pairs upon binding (Fig. 3d). This finding is in contrast to its binding site size for polymeric B-DNA duplex, which is reported to have a binding site size of ~10 base pairs.³⁴ The CD titration of

7 to poly(rA)•(rU) displayed noticeable changes in the CD intensity at 266 nm and 347 nm (Fig. 3b). As shown in Figure 3b, the changes in the CD signal are much pronounced at 266 nm than the same induced by either neomycin (Fig. 3c) or Hoechst 33258 alone (Fig. 3a). Furthermore, the induced CD at 347 nm is unidirectional and negative. This is in complete contrast to the exciton CD bands observed when Hoechst 33258 is bound to the same RNA duplex (Fig. 3a). Negative induced CD bands are typically seen when ligands bind in the intercalating fashion.⁵¹ Significant shape changes in the induced CD band of conjugate **7** in comparison to Hoechst 33258 demonstrate that the conjugate binds by a different mode to poly(rA)•(rU) than Hoechst 33258. The negative unidirectional induced CD in the bisbenzimidazole absorption region (347 nm) suggests intercalative binding of the bisbenzimidazole moiety. We then performed CD titration of poly(rA)•(rU) with neomycin (Figure 3c) which showed a binding site size of eight base pairs (Fig. 3f) as observed previously under similar conditions.⁵⁵

To determine the exact binding mode of bisbenzimidazole moiety of the conjugates **3–11** with respect to the helical axis of the RNA duplex, we performed linear dichroism (LD) experiments. LD has been previously utilized to determine the binding mode of many established and novel ligands.^{56–60} In our experiments, we used microvolume couette flow LD⁶¹ which uses much lesser sample amounts to perform an experiment than the conventional setup. Figure 4 shows a representative LD spectrum of the poly(rA)•(rU) duplex in the absence and presence of **4**. The LD spectrum of RNA duplex shows negative absorption at 260 nm consistent with previous observations.^{62,63} In the presence of **4**, changes in the LD signal at both 260 nm and 348 nm are worthy of note. As seen in Figure 4, the chromophore binding shows an all-negative induced LD whose maximum is centered at 348 nm. Similar to **4**, all other conjugates showed negative induced LD (see supplementary information, Fig. S1) in the chromophore absorption region indicating the similarity of the binding mode of ligands **3–11**. Induced LD was then used to calculate the angle at which the bisbenzimidazole moieties were bound to the RNA duplex with respect to the helical axis.^{60,64,65}

The orientations of all the conjugates with respect to the RNA helical axis are summarized in Table 1. The data presented in Table 1 indicates that the bisbenzimidazole moiety of conjugates **3–11** binds by intercalation. The angle of inclination of bisbenzimidazole moiety with respect to the helical axis, in some cases, nearly matches the RNA base pair inclination to the helix (77°).⁶² The data shown in Table 1 and Figure 4b also reveals that as the linker length increases (for each sub-class of conjugates **3–11**), enhanced intercalation of the bisbenzimidazole moiety to the RNA duplex is observed (64–74 degrees). The increased intercalation could likely arise from the greater flexibility allowed by longer linkers towards the binding of bisbenzimidazole moiety during the intercalation process. The significant FID displacement (Fig. 2) and excellent intercalation by conjugate **11** may have its roots in the extra flexibility allowed by its linker and the lack of linker aggregation.

To probe the effects of dual binding, we performed UV thermal denaturation experiments using the best binder **7**. As shown in Table 2, in the absence of ligand, poly(rA)•(rU) melts at 58.1 °C. Addition of Hoechst 33258 and neomycin, which are the constituent units of conjugates **3–11**, afforded 0.4 °C and 10.9 °C thermal stabilization (T_m) respectively. A

control experiment containing neomycin and Hoechst 33258 together afforded T_m 10.6 °C, a value similar to the thermal stabilization by neomycin alone. However, conjugate **7**, at its saturating r_{bd} , showed a T_m of 19.0 °C. The large thermal stability afforded by conjugate **7** suggests dual RNA recognition, whereas neomycin and Hoechst 33258 controls showed much poorer T_m by themselves, as reported previously.²⁶ The negligible thermal stabilization afforded by Hoechst 33258 was surprising due to its considerable interaction observed by CD and UV-Vis experiments (Figures 3 and 5). However thermal stabilization based anomalies in the nucleic acid binding affinity have been observed before with Hoechst 33258 derived bisbenzimidazoles.⁶⁶ Such discrepancies can be attributed to temperature dependent binding of ligands to the nucleic acid.⁶⁶ To further gauge the strength of binding, we used an oligomeric RNA $r(CGCAAUUUGCG)_2$, and performed FID titration with one of the conjugates showing high displacement (Figure S5, supplementary information). The association constant (K_a) of conjugate **4** with $r(CGCAAUUUGCG)_2$ was determined to be $8.8 \times 10^6 \text{ M}^{-1}$, a value close to the K_a obtained with NH1, a neomycin-bisbenzimidazole conjugate, similar to the chemical structure of **4** ($K_a = 6.5 \times 10^6 \text{ M}^{-1}$).²⁶

Additionally, we performed UV-Vis absorption experiments to assess interaction of conjugate **7** with poly(rA)•(rU) duplex as differences in the absorption spectrum of ligand upon nucleic acid binding have long been studied to understand ligand-nucleic acid interactions. In this experiment, we observed the changes in the absorbance spectrum (chromophore absorption) of conjugate **7** upon binding to poly(rA)•(rU) and compared it to the binding of Hoechst 33258 to the same RNA duplex (Fig. 5). The binding of Hoechst 33258 to RNA duplex (Figure 5a) leads to hypochromism (32.2%) in the bisbenzimidazole absorption region and is accompanied by an insignificant red shift (1 nm) as well as a non-distinct isobestic point. Contrary to this observation, binding of conjugate **7** to RNA duplex (Fig. 5b) leads to a greater hypochromicity (34.1%) in the bisbenzimidazole absorption region and a larger red shift (10 nm) of the absorption maximum of the bisbenzimidazole moiety (Table 3). Besides, a clear isobestic point was observed at 362 nm. These results corroborate the data from CD, LD and thermal denaturation studies on the dual binding of conjugate **7** with the RNA duplex. The observation of large hypochromicity change and red shift in the absorption maximum of ligand upon RNA binding are also indicative of intercalation.^{67,68} The hypochromicity (34.1%) observed in the case of **7**, is similar to the values obtained with classical nucleic acid intercalator ethidium bromide.^{68–70}

Finally, to determine the DNA versus RNA selectivity of conjugates **3–11**, we performed UV thermal denaturation experiments with an AT rich dodecamer sequence $d(CGCAAATTTGCG)_2$ and its RNA equivalent $r(CGCAAUUUGCG)_2$. The maximum thermal stabilization obtained with conjugates **3–11** were 23.8 °C and 21.9 °C for DNA and RNA duplexes respectively (Table 4). While the maximum thermal stabilization temperatures were numerically similar, a linker length based DNA versus RNA selectivity was notably evident. For the DNA duplex, there was a concomitant decrease in the thermal stabilization as the linker length increased for conjugates **3–11** in each sub-classification (monooxygen, bisoxygen). In stark contrast, the thermal stabilization increased with an increase in linker length for each sub-class of conjugates **3–11** for RNA duplex. These results clearly represent that neomycin-bisbenzimidazole conjugates with long linkers are

better RNA duplex stabilizers than DNA duplex. For example, compound **9** shows a 20.0 °C change in RNA duplex T_m with only a 9.0 °C change in T_m for the corresponding DNA duplex. Significantly, the shorter linker in compound **3** shows almost the reverse trend, a 23.8 °C change in DNA T_m , with only a 9.1 °C change in T_m for the corresponding RNA duplex. The inability of DNA minor groove binders, including sequence specific polyamides,⁷¹ to have RNA minor groove shape complementarity has been attributed as the prime reason for their DNA versus RNA selectivity.⁷¹ In this case, the linker length dependent RNA selectivity is expected to have its origins in greater stacking of bisbenzimidazole with the bases in the case of RNA since the expected mode of bisbenzimidazole interaction here is intercalation, if the binding to the polymer RNA and oligomer RNA follow similar modes of interaction. We have previously reported that a similar neomycin-bisbenzimidazole conjugate (NH1) displays sequence-specific binding to both RNA and DNA duplexes where the A-tract break in the nucleic acid sequence led to diminished binding of the conjugate.^{26,34} Conjugates **3–11**, with a similar composition, can be expected to show similar specificities in binding.

In conclusion, we have demonstrated that conjugation of neomycin (**1**) to Hoechst 33258 (**2**) results in an improved duplex RNA recognition by a dual binding mode. The bisbenzimidazole moiety of all the conjugates studied show intercalative mode of binding. The intercalation of the bisbenzimidazole moiety was found to be significantly linker length dependent. As the linker length increases from 5–23 atoms, the inclination of bisbenzimidazole moiety with respect to the RNA helical axis increased from 64° to 74°. The dual binding was also reflected by large UV thermal stabilization of the RNA duplex by conjugate **7**. It was further supported by UV-visible absorption experiments which displayed stronger complexation of conjugate **7** with RNA duplex in comparison to Hoechst 33258. These studies show that dual recognition of nucleic acids is a powerful tool not only for enhanced macromolecular targeting but also for altering and optimizing the binding mode of ligands. A unique linker dependent DNA versus RNA selectivity was also observed where conjugates with longer linkers stabilized RNA duplex better than DNA duplex, and vice versa. These observations offer promise that dual recognition by covalently attached ligands with independent binding sites can be used to derive motifs for selectivity and precision in binding of nucleic acids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.

A plot showing the screening of ligands **1–11** with oligomeric RNA duplex $r(\text{CGCAAUUUGCG})_2$ using the FID assay. The RNA concentration was $1\ \mu\text{M}/\text{duplex}$ while ethidium bromide concentration was $6\ \mu\text{M}$. The experiments were performed in buffer 10 mM sodium cacodylate, 0.5 mM EDTA 100 mM NaCl at pH 7.0 ($T = 23\ ^\circ\text{C}$).

Figure 3.

CD titration of poly(rA)•(rU) with (a) Hoechst 33258 (b) conjugate **7** (c) neomycin in buffer 10 mM sodium cacodylate, 0.5 mM EDTA and 100 mM NaCl at pH 7.0 ($T = 25\text{ }^{\circ}\text{C}$). A concentrated solution of ligand solution was added serially to a solution of poly(rA)•(rU) (40 μM / bp) after five minute equilibration time. Each measurement represents an average of two scans. Plots (d–f) show the binding site size of Hoechst 33258, conjugate **7** and neomycin respectively. r_{bd} is the ratio of RNA base pairs per drug molecule.

Figure 4.

(a) LD spectrum of poly(rA)•(rU) in the absence (black line) and presence of conjugate **4** (blue line). The RNA duplex (1 mM/bp) was mixed with conjugate **4** at r_{bd} 10 before the scan was taken for the RNA-ligand complex. The experiments were performed in buffer 10 mM sodium cacodylate, 0.5 mM EDTA and 100 mM NaCl at pH 7.0 at room temperature. Each spectrum is an average of three scans (b) A scatter plot showing linker length dependent intercalation of the bisbenzimidazole moiety in poly(rA)•(rU) RNA duplex for each sub-class of conjugates **3–11**.

Figure 5.

UV-Vis absorption profile of (a) poly(rA)•(rU)-Hoechst 33258 complex and (b) poly(rA)•(rU)-conjugate 7 complex. The concentration of the RNA duplex was 15 μM /bp while the concentration of Hoechst 33258 or conjugate 7 was 3 μM in each experiment. The experiments were performed at room temperature in buffer 10 mM sodium cacodylate, 0.5 mM EDTA and 100 mM NaCl at pH 7.0.

Table 1

A table listing the angles of the bound bisbenzimidazole moiety of conjugates 3–11 to the poly (rA)•(rU)duplex.

Conjugate	Sub-class	Linker length (atoms)	Angle (in degrees)
3	Monooxygen	5	64
4	Monooxygen	8	66
5	Monooxygen	12	71
6	Bisoxxygen	10	66
7	Bisoxxygen	11	65
8	Bisoxxygen	13	67
9	Bisoxxygen	15	67
10	Bisoxxygen	17	69
11	Polyoxxygen	23	74

Table 2

UV thermal denaturation temperatures of poly(rA)•(rU) in the absence and presence of ligands

Ligand	r _{bd}	T _m	T _m (°C)
None	--	58.1	--
Hoechst 33258	5	58.5	0.4
Neomycin	8	68.9	10.8
Neomycin+Hoechst 33258	8 and 5 respectively	68.6	10.5
Conjugate 7	6	78.1	19.0

Table 3

A table showing the optical properties of Hoechst 33258 and conjugate 7 upon poly(rA)•(rU) binding.

Ligand	λ_{max} (nm) Free	Bound	λ_{max} (nm)	% hypochromicity
Hoechst 33258	338	339	1	32.2
Conjugate 7	339	349	10	34.1

Table 4

A table showing the thermal denaturation temperatures and T_m (°C) values for DNA and RNA oligomers of the same sequence by conjugates **3–11**.

Ligand	d(CGCAAATTTGCG) ₂		r(CGCAAATTTGCG) ₂	
	T_m (°C)	T_m (°C)	T_m (°C)	T_m (°C)
None	53.8	0	51.9	0
3	77.6	23.8	61.0	9.1
4	76.6	22.8	67.0	15.1
5	67.0	13.2	68.1	16.2
6	78.4	24.6	66.9	15.0
7	76.0	22.2	67.7	15.8
8	75.5	21.7	73.8	21.9
9	72.8	13.0	73.0	20.3
10	72.0	18.2	ND ^a	ND ^a
11	70.2	16.4	59.1	7.2

^aND (Not determined due to broadness of thermal melting profile)