Spirocyclic helical compounds as binding agents for bulged RNA, including HIV-2 TAR

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Based on fluorescence binding studies and 1D ^{1}H NMR studies, designed synthetic analogues of NCSi-gb bind specifically with two-base bulged RNA, including HIV-2 TAR RNA, making them potential lead compounds for antiviral drug development.

Bulges (unpaired bases) affect the secondary and tertiary structures of various types of RNA and appear to be involved in many biological control processes.[1,2] For example, they have been shown to act as intermediates in RNA splicing, and as binding motifs for the regulatory proteins involved in viral replication including the TAR region of HIV-1 and HIV-2.[2–4] It has also been shown that binding of 5 S rRNA to protein L5 allows 5 S rRNA to be transported to the nucleolus to start ribosome synthesis during vitellogenesis, and a highly conserved two-adenosine bulge appears to be particularly important for the L5 binding.[5] Therefore, bulge-binding agents could have important therapeutic potential. There are limited reports on small molecules binding specifically to bulged RNA and those reports are focused on HIV-1 TAR RNA binding agents.[6–11] Considering the universal distribution of bulged structures in all types of structurally functional RNAs,[2] it is important to develop small molecule binding agents for RNA bulged structures not limiting to HIV-1 TAR RNA.

NCSi-gb (**1**), a metabolite of the enediyne antitumor antibiotic NCS-chrom,[12] has been found to bind specifically to DNA containing a two-base bulge (Fig. 1).[13,14] However, NCSi-gb is labile due to its spiro-lactone ring system and possesses a bulky cyclic carbonate moiety that limits its bulge-binding properties.[14,15] Hence, DDI (**2**) was initially designed and synthesized as an analogue of NCSi-gb.[14] DDI and other analogues (**2–5**) share the same key structural feature that NCSi-gb has: a wedge-shaped aglycon moiety consisting of two aromatic ring systems held rigidly by a spirocyclic ring with a right-handed helical twist of about 35° and a pendant aminosugar moiety (Fig. 1).[14] Affinity of **2** for two-base DNA bulges is more than 10-fold weaker than that of NCSi-gb, and the weaker affinity was attributed to the difference in the aminoglycoside positions (entry 1, Table 1).[14,16] Therefore, analogues **3** and **4**, with aminoglycoside linkages on the aromatic ring systems, which are similar to that of NCSi-gb, were prepared.[17,18] The affinities of **3** and **4** for DNA two-base bulges are still much weaker than that of NCSi-gb and similar to that of **2**.[17,18] One major difference between analogues **2–4** and NCSi-gb is that these analogues have a β-aminoglucosyl linkage whereas NCSi-gb has an α-aminofucosyl moiety. Analogue **5**, which has an α-aminofucosyl moiety and is the closest analogue to NCSi-gb so far, was therefore prepared.[19] The binding affinity of **5** (0.13 μM) with the best two-base DNA bulge substrate is close to that of NCSi-gb (0.033 μM).[19] Analogue **6**, an isomer of **5** but with a left-handed twist of the two aromatic ring system, surprisingly, binds the two-base DNA bulge as strongly (0.08 μM) as **5**.[19]

Studies of NCSi-gb and its analogues have mostly focused on their interactions with DNA bulges. RNA bulged structures, by contrast, have been found to be very poor binding substrates for NCSi-gb.[20] Further, very weak cleavage at the three-base bulge site of HIV-1 TAR RNA was obtained with native NCS-chrom.[21] The NMR structure of NCSi-gb and a two-base bulged DNA indicates that the bulky cyclic carbonate moiety of NCSi-gb disrupts the in-plane alignment of the base pair 3′ next to the bulge.[15] The disruption could be more serious for two-base RNA bulges, as two-base RNA bulges might have a smaller pocket, as suggested by the shorter distance of the two flanking residues on the opposite strand of an RNA two-base bulge than that of the analogous DNA.[5] Therefore, affinity of the analogues for RNA bulges might be improved with the removal of the bulky cyclic carbonate moiety.[15]

In the binding studies, it is also important to consider the size of the bulge. Based on the best DNA bulge binding substrate,[14,19] HU3AGUU, HU3AGU, HU3AGUCC and HU3AU were designed as potential two-base bulge, one-base bulge, three-base bulge and duplex RNA binding substrates, respectively (Fig. 2). HIV-1 and HIV-2 TAR RNA were also included in the binding studies. The HIV-2 TAR RNA, the structure of which has been determined to possess a two-base bulge,[22] differs from HIV-1 TAR RNA by the deletion of the non-critical C24 residue in the bulge (Fig. 2). As TAR RNA is highly conserved and its interaction with Tat protein is required for efficient viral replication, it is a superb antiviral drug target.[23] Although there is more than one possible structure for the RNAs shown in Table 1, the use of the MFOLD program[20] clearly shows that preferred low energy structures are those with two-base bulges. The synthetic RNAs (entries 2–4 and 8) are designed in a way that other conformations will have either multiple bulges or mismatched base-pairs, which will result in lower stability. Also as shown clearly for DNA bulges,[12,15] these small molecules select out or actually induce two-base bulged structures in nucleic acids. The results of fluorescence binding studies are shown in Table 1. In all cases, binding was not observed for HU3AU, the duplex RNA with a loop but no bulge. NCSi-gb binds poorly or not at all with all RNA bulged structures with the

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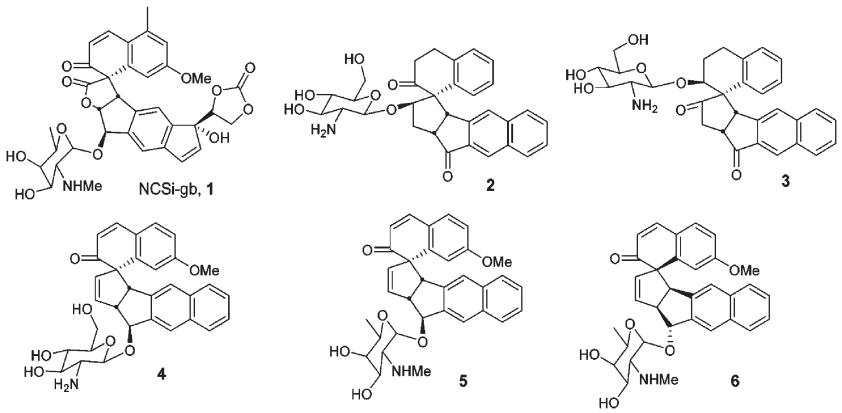

Fig. 1 Structures of NCSi-gb and its analogues.

Table 1 Dissociation constants (μM) of the drugs with DNA/RNA (Fig. 1) determined *via* emission spectra (λ_{exc} 390 nm; λ_{emm} 500 nm for compound **1**, λ_{exc} 310 nm; λ_{emm} 460 nm for compounds **2** and **3**, λ_{exc} 360 nm; λ_{emm} 480 nm for compounds **4–6** in cases of fluorescence stimulation and λ_{exc} 360 nm; λ_{emm} 500 nm for compounds **4–6** in cases of fluorescence quenching) at 5 °C. Stimulation of drug fluorescence is indicated in bold, whereas quenching is shown in standard type. Values in the parentheses represent standard deviations. Data in entry 1 are reported results[14,17–19]

Entry	Sequence code	**1**	**2**	**3**	**4**	**5**	**6**
1	HT3AGTT	0.033	0.46	0.28(0.05)	**0.55**	**0.13(0.03)**	**0.08(0.02)**
2	HU3AGUU	NAB[a]	21(9)	22(11)	NAB	**1.3(0.2)**	**1.1(0.2)**
3	HU3AGU	NAB	19(1)	68(22)	NAB	NAB	NAB
4	HU3AGCUU	NAB	20(5)	NAB	**5(1)**	**4.9(0.6)**	**12(1)**
5	HU3AU	NAB	NAB	NAB	NAB	NAB	NAB
6	HIV-1 TAR	NAB	NAB	NAB	NAB	NAB	NAB
7	HIV-2 TAR	14(4)	14(3)	13(3)	NAB	**8(4)**	9(2)
8	HU4AGUU					**1.6(0.5)**	**2.8(0.5)**

[a] NAB: no apparent binding.

only substrate showing very weak affinity being HIV-2 TAR RNA (14 μM). DDI binds extremely weakly to all RNA bulged structures tested except for HIV-1 TAR RNA. Compound **3** binds very weakly with one-base and two-base bulged structures but not with those containing a three-base bulge, while compound **4** binds only with HU3AGCUU, which has a three-base bulge.

	Sequence Code	Conformation
1.	HT3AGTT	5'-GTCCGATGCGTG T T 3'-CAGGCTACGCAC T TG
2.	HU3AGUU	5'-GUCCGAUGCGUG U 3'-CAGGCUACGCAC U U UG
3.	HU3AGU	5'-GUCCGAUGCGUG U 3'-CAGGCUACGCAC U U G
4.	HU3AGCUU	5'-GUCCGAUGCGUG U 3'-CAGGCUACGCAC U U UG C
5.	HU3AU	5'-GUCCGAUGCGUG U 3'-CAGGCUACGCAC U U
6.	HIV-1 TAR	24 C 22 UU 31 5'-CCAGAGAGC CU G 3'-GGUCUCUCG AG G 40
7.	HIV-2 TAR	21 UU 26 5'-GGCCAGAGAGC CU G 3'-CCGGUCUCUCG AG G 43 38
8.	HU4AGUU	5'-GUCCGAUGCGUG U U 3'-CAGGCUACGCAC U U UG

Fig. 2 Structures of oligonucleotides.

Compounds **5** and **6**, structurally closest to the natural product, which, among the NCSi-gb analogues, have the strongest affinity to two-base DNA bulges, also showed superior binding with the two-base RNA bulged structure with dissociation constants with HU3AGUU of about 1 μM. They showed poorer binding with the three-base bulge HU3AGCUU and none with the one-base bulge HU3AGU, which is in line with their binding patterns to DNA bulges. Modest binding was found with HIV-2 TAR RNA and none with HIV-1 TAR RNA. To confirm that two-base RNA bulge is the preferred binding site for compounds **5** and **6**, binding studies with HU4AGUU (entry 8, Table 1, Fig. 2) were conducted. HU4AGUU has a similar bulged structure as that of HU3AGUU but has a U4 loop instead of a U3 loop. Binding of HU4AGUU to compounds **5** and **6** is similar to that of HU3AGUU. The absence of any binding of HIV-1 TAR RNA with NCSi-gb and its analogues might be due to the ability of HIV-1 TAR RNA to form unusual structures, such as a U31A22U40 base triple,[24] which might preclude bulge binding.

^{1}H NMR 1D titration experiments were then conducted between compound **5** and HU3AGUU or HIV-2 TAR RNA. ^{1}H NMR signals in the imino proton region are more dispersed than those of the aromatic and the sugar regions, and therefore were used to monitor the change upon binding. ^{1}H NMR assignments of uncomplexed HIV-2 TAR RNA have been accomplished previously by Brodsky,[22] and several imino proton resonances are labeled in Fig. 3. Upon addition of compound **5**

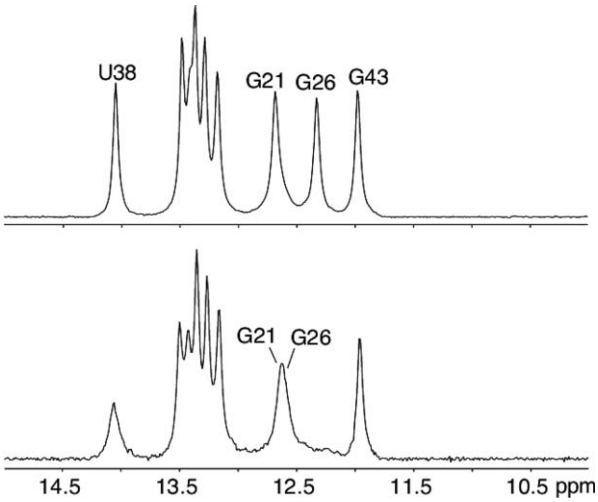

Fig. 3 ^{1}H NMR spectra (imino proton region) of HIV-2 TAR RNA before (upper spectrum) and after (lower spectrum) addition of compound **5**.

(1 equiv.) to a solution of HIV-2 TAR RNA, the NMR signal at δ 12.32, corresponding to the G26 imino proton, was downfield shifted to δ 12.66 (Fig. 3). This shift indicates that the putative bulge is the preferred binding site. Until the solution structure of the complex is obtained using 2D NMR, the 1D analysis provides the most compelling evidence for binding to the two-base bulge. When compound **5** was added to a solution of HU3AGUU oligomer, which has two AU base pairs next to the bulge, NMR shifts in the imino proton region were also observed. However, the changes are not resolvable due to overlapping of the NMR signals and no structural information was derived from the 1D NMR study. The AU base pair next to the bulge is usually less stable than the GC base pair. As a result, the ^{1}H signal for the imino proton of the AU base pair next to bulge is often broad and sometimes even disappeared due to the exchange with water solution.[25] This could explain the absence of striking change in the NMR studies of HU3AGUU, as signals for the imino protons of AU base pairs next to the bulge might not show up in the 1D ^{1}H NMR spectrum.

In conclusion, based on fluorescence binding studies and 1D ^{1}H NMR studies, designed synthetic analogues of NCSi-gb were found to bind specifically with two-base bulged RNA, including HIV-2 TAR RNA, making them potential lead compounds for antiviral drug development. Analogues having the same aminosugar (α-*N*-methylfucosamine) as of NCSi-gb and the same aminosugar position are the most promising candidates. To the best of our knowledge, this is the first report on small molecule binding agents specifically for two-base RNA bulges. 2D NMR structural studies of the drug-RNA complex are currently underway.

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