Expedited Articles

Design and Synthesis of RNA-Specific Groove-Binding Cations: Implications for Antiviral Drug Design†

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As as initial step in the design of structure-specific RNA-interactive molecules as potential antiviral agents, we have focused on the synthesis of molecules that exhibit strong and preferential binding to duplex RNA. A series of polycationic ligands have been synthesized, and the degree of preferential binding to RNA has initially been determined by thermal denaturation (ΔT_m) with both RNA [poly(A)·poly(U)] and DNA [poly(dA)·poly(dT)] polymers at a variety of pH values. Seven compounds from the series exhibit a substantial degree of RNA-selective binding. The relatively high ΔT_m values obtained suggest a specific mode of interaction between these ligands and the RNA helix. By contrast, the much lower ΔT_m values with poly(dA)·poly(dT) DNA reflect a more nonspecific interaction mode. A viscometric titration study with poly(A)·poly(U) confirms that they do not bind by intercalation. The results, combined with the known structure and electronegative potential of duplex RNA, suggest that these molecules bind in the major groove via specific electrostatic and/or hydrogen-bonded interactions.

Introduction

A novel idea for the development of new drugs for the treatment of diseases such as AIDS, which are induced by RNA viruses, is the design of agents which bind to specific structures in the viral RNA genome. As an example, HIV-1 has two stem–bulge–loop RNA structures, TAR and RRE, that are essential for replication and gene expression of the virus[1] (Figure 1). Agents which bind to either of these specific RNA structures and inhibit their interactions with viral proteins will disrupt the reproductive cycle of the virus. The TAR and RRE receptor sites are not targeted by other known anti-HIV-1 drugs, and compounds which bind to them would, thus, be very attractive candidates for use against resistant strains of the virus or in combination chemotherapy.

The goal in the design of these agents is the specific targeting of the RNA structure, as opposed to the antisense method which requires disruption of the RNA structure for specific base pairing with the resulting single strand.[2] We have shown that specific bulges in the TAR structure can be selectively recognized by intercalator–base conjugates.[3] In a very exciting development, Green and coworkers[4] have shown that some natural aminoglycoside antibiotics can selectively target a structural loop in the RRE RNA of HIV-1 (Figure 1) and disrupt its interaction with viral proteins. The concept of designing RNA structure-specific antiviral agents is, thus, on a firm footing.

We are pursuing two separate lines in the development of structure-specific antiviral agents. In the first, we take advantage of the fact that TAR contains both single- and three-base bulges in the RNA stem structure (Figure 1). Due to its simplicity, we have concentrated first on the single-base A-bulge. Some intercalators specifically target

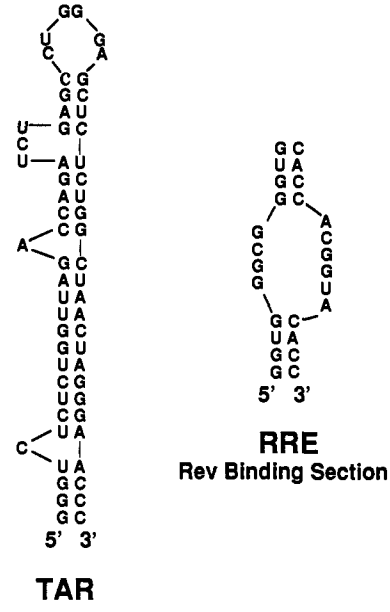

Figure 1.

bulges in RNA and bind to stem regions adjacent to the bulge site.[5–7] We have found that covalent attachment of RNA bases to the intercalator can yield compounds that bind selectively to specific TAR bulges.[3] In a second strategy, we are attempting to devise agents that selectively target the major groove in the TAR or RRE structures. Considerable evidence indicates that the tat regulatory protein of HIV-1 binds in the major groove of TAR.[8] Agents that bind in the major groove and disrupt the tat interactions should have useful antiviral activity. As a first step in this latter strategy, we have focused on the design of novel molecules that bind strongly and preferentially to RNA duplexes. The structure of double-stranded RNA differs dramatically from that of DNA. In particular, the 2'-OH group of the ribose ring forces the

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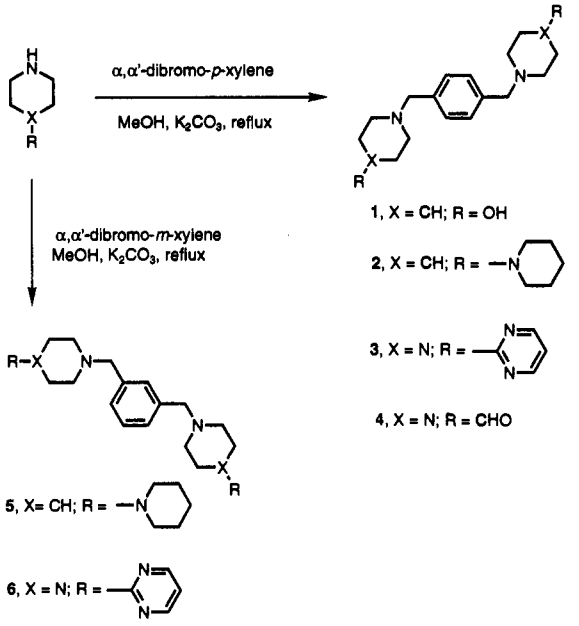
Scheme 1

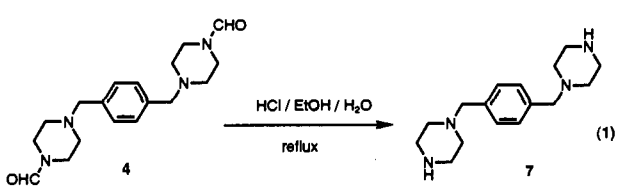


sugar to adopt the 3′-*endo* conformation in RNA duplex structures, and this results in an A-form double helix in comparison to the more standard B-form adopted by DNA.[9] Consequently, the narrow minor groove and wide major groove of B-form duplex DNA are replaced by a wide and very shallow minor groove and a deep and narrow major groove for double-helical RNA. The design of molecules that bind strongly and preferentially to duplex RNA, therefore, requires a new approach to that adopted with the more established field of DNA-interactive drugs.[10,11] To date, very few studies on RNA-interactive ligands have been undertaken. The preliminary structure–activity study presented here will (i) help to establish a library of important structural groups that will be of use in the future design process and (ii) lead to a greater understanding of the rules of RNA recognition.

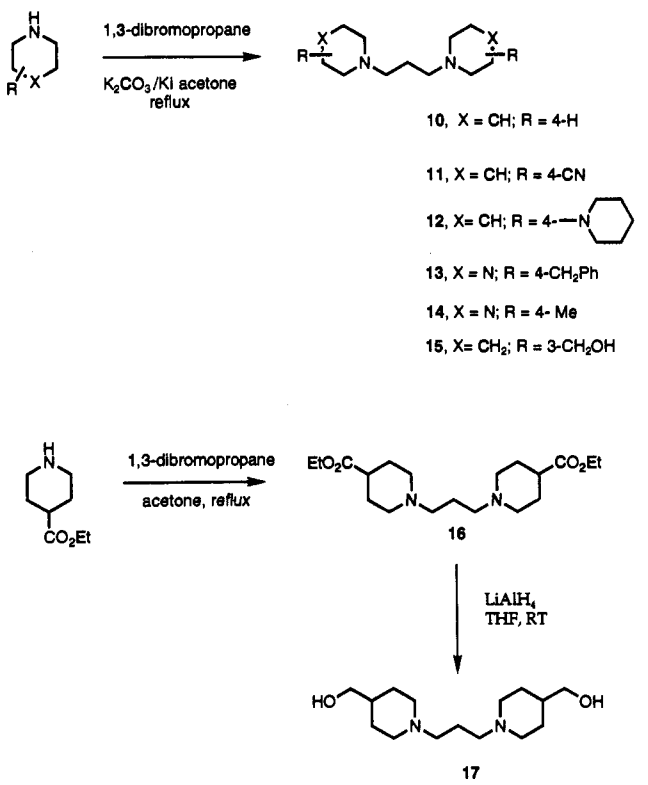
In this paper, we present the synthesis and biophysical evaluation of a group of polycationic ligands which exhibit a substantial degree of RNA-selective binding. Thermal melting (T_m) curves of the compounds complexed to both RNA [poly(A)·poly(U)] and DNA [poly(dA)·poly(dT)] polymers at a variety of pH values have been used as an initial assay for effective RNA-selective binding.

Chemistry

The three series of polycationic ligands (Schemes 1–3) were synthesized in a straightforward manner. Thus, simple nucleophilic substitution of either α,α′-dibromo-*p*-xylene or the *meta* derivative with the appropriate piperidine or piperazine compound in refluxing methanol afforded compounds **1–6** (series I) in moderate yield (Scheme 1). The bis-aldehyde compound **4**, prepared by this method, gave the secondary amine **7** upon complete acidic hydrolysis (equation 1) following a literature

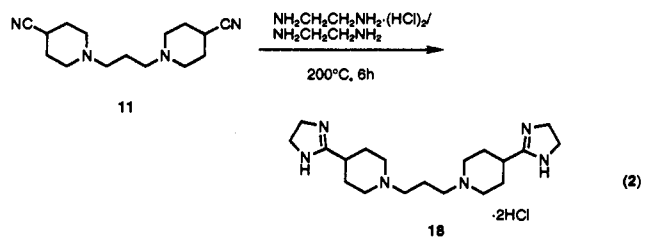


Scheme 2



procedure.[12] In addition, a bis-alkylation byproduct, identified as **8** (Table 2C), was isolated following acidic hydrolysis of the mother liquor after removal of **7**. The structurally related imidazoline **9** (Table 2A) was synthesized from the corresponding nitrile following procedures which will be described elsewhere.[13]

In the second series of molecules, the xylene-linking group was replaced by the more flexible propyl chain. The synthesis, as detailed in Scheme 2, involved facile nucleophilic substitution of 1,3-dibromopropane with both piperidine and piperazine derivatives in refluxing acetone, in the presence of K_2CO_3, to afford the amines **10–15** in moderate yield. In addition, reaction of 1,3-dibromopropane with an excess of ethyl isonipecotate in refluxing acetone afforded the bis-ethyl ester **16** which was reduced by $LiAlH_4$ to the corresponding alcohol **17** in high yield (Scheme 2). High-temperature fusion of nitrile **11**, obtained from 4-cyanopiperidine,[14] with a melt containing equimolar amounts of 1,2-diaminoethane and 1,2-diaminoethane dihydrochloride afforded the imidazoline **18** as a very hygroscopic hydrochloride salt in moderate yield (equation 2). The structurally related compound 4,4′-



trimethylenedipiperidine (**19**) (Table 2C) was obtained from the Aldrich Chemical Co. and used in the biophysical study without further purification.

The third series of molecules examined involved the replacement of the propyl chain with a 1,4-bis(ethyl)-

Scheme 3

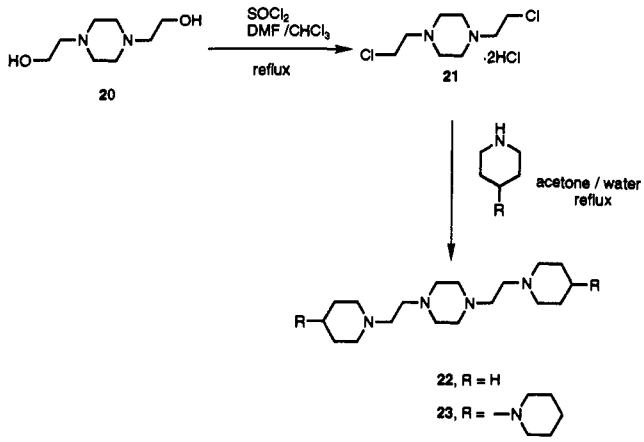


Table 1. Physical Properties of Polycationic Ligands

no.	mp,[a] °C	% yield[b]	method	formula[c]
1	163	75	A	$C_{18}H_{28}N_2O_2$
2	147–148	77	A	$C_{28}H_{46}N_4 \cdot 4HCl \cdot 0.4H_2O$
3	199–200 dec	48	A	$C_{24}H_{30}N_8 \cdot 4HCl \cdot 0.25H_2O$
4	164	77	A	$C_{18}H_{28}N_4O_2 \cdot 1H_2O$
5	67–68	45	A	$C_{28}H_{46}N_4 \cdot 4HCl \cdot 0.5H_2O$
6	152	51	A	$C_{24}H_{30}N_8 \cdot 2HCl \cdot 1H_2O$
7	150–152	77	A	$C_{16}H_{26}N_4 \cdot 0.1H_2O$
8	>300[d]	16		$C_{28}H_{42}N_6 \cdot 6HCl \cdot 0.25H_2O$
9	>300[d]	21		$C_{24}H_{36}N_6 \cdot 4HCl \cdot 1H_2O$
10	>200[d]	62	B	$C_{13}H_{26}N_2 \cdot 2HCl \cdot 1H_2O$
11	57.5–59	74	B	$C_{15}H_{24}N_4 \cdot 0.2H_2O$
12	60–62	92	B	$C_{23}H_{44}N_4 \cdot 4HCl \cdot 0.5H_2O$
13	59–60	39	B	$C_{25}H_{36}N_4$
14	288–290[d] dec	51	B	$C_{13}H_{28}N_4 \cdot 4HCl \cdot 1H_2O$
15	205–208[d]	57	B	$C_{15}H_{30}N_2O_2 \cdot 2HCl$
16		94		$C_{19}H_{34}N_2O_4 \cdot 0.5H_2O$
17	113–114	81		$C_{15}H_{30}N_2O_2 \cdot 0.4H_2O$
18	>250[d] dec	38		$C_{19}H_{34}N_6 \cdot 4HCl \cdot 1.75H_2O$
22	72–74.5	72		$C_{18}H_{36}N_4 \cdot 0.2H_2O$
23	174.5–176	54		$C_{28}H_{54}N_6 \cdot 0.5H_2O$

[a] Mp refers to free base unless otherwise stated. [b] Yields are not optimized. [c] C, H, N, and Cl analyses were within ±0.4% of the theoretical values. [d] Mp of hydrochloride salt.

piperazine group. Thus, chlorination of 1,4-bis(2-hydroxyethyl)piperazine (**20**) with $SOCl_2$ in a mixture of $CHCl_3$ and DMF afforded 1,4-bis(2-chloroethyl)piperazine (**21**) as the dihydrochloride salt in high yield (Scheme 3). Subsequent nucleophilic substitution of this bis-alkylating agent with both piperidine and 4-piperidinopiperidine afforded compounds **22** and **23** in moderate yield. Compound **22** was prepared using an excess of piperidine as solvent and sufficient water to maintain the salt of **21** in solution. In the case of **23**, an excess of 4-piperidinopiperidine (1,4′-bipiperidine) and **21** were mixed with acetone and a small volume of water was added to maintain a homogeneous solution.

Results and Discussion

The results of the thermal denaturation study of these compounds with both RNA [poly(A)·poly(U)] and DNA [poly(dA)·poly(dT)] polymer model systems at pH 6.3, 4.9, and 3.9 are illustrated in Tables 2A–C and 3.

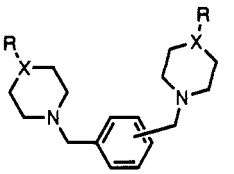
Of the initial 19 compounds studied, ligands **2**, **5**, **9**, **12**, and **18** all show a substantial degree of RNA-selective binding at pH 6.3 (Table 2A,B). Within the first series, compounds **1**, **3**, **6**, and **7**, containing the central xylene group, all exhibit very poor binding to both poly(A)·poly(U) and poly(dA)·poly(dT) at pH 6.3 (Table 2A). The low binding is undoubtedly due to their relatively low charge density at this pH; the pK_a of the tertiary nitrogens are substantially reduced by direct attachment to the electron-withdrawing benzylic group. Low charge density reduces the ability of these ligands to stabilize the nucleic acids by electrostatic and/or specific hydrogen-bonding contacts which have been shown to be of importance in drug–DNA interactions.[10,11] Incorporation of hydrogen-bond acceptor groups such as the pyrimidine rings in compounds **3** and **6** did not alter the binding affinity for either RNA or DNA. Replacement of the 4-hydroxyl group of **1**, however, with a second piperidine molecule (compound **2**) substantially increases the affinity for poly(A)·poly(U) while stabilizing poly(dA)·poly(dT) to a much lesser extent.

Similarly, the *meta* derivative **5** exhibits a significant preference for the RNA helix. The presence of a protonatable group at this 4-position does, therefore, seem to be important for RNA-selective binding. Compound **9**, containing the highly basic imidazoline groups, shows the greatest preference for the RNA duplex within the first series with a ΔT_m for poly(A)·poly(U) approximately twice that observed for poly(dA)·poly(dT). The hydrogen-bonding array present within the protonated imidazoline moiety is undoubtedly involved in specific interactions with the RNA helix. In contrast, the poorer binding of **9** to poly(dA)·poly(dT) may be due to the presence of the piperidine molecules which (i) increase the overall steric bulk of the ligand, thus preventing a snug fit into the electronegative minor groove of the poly(dA)·poly(dT) helix, and (ii) lower the degree of ligand hydrophobicity; these features are characteristic of many DNA-binding polyaromatic imidazolines.[15] The structurally related piperazine ligand **8** does not show significant affinity for RNA and has very weak affinity for DNA at this pH value (Table 2C).

Within the second series (Scheme 2), compounds **10**, **11**, **13**, and **14** all exhibit a low binding affinity for both polynucleotides at pH 6.3 (Table 2B). As in the case of **1**, **3**, **6**, and **7**, a relatively low charge density prevents strong interactions with either nucleic acid at this pH. Similarly, the bis-secondary amine **19** (Table 2C) shows no appreciable binding to either the RNA or DNA duplex. In addition, the presence of two hydrogen-bond-donating/-accepting hydroxyl groups in compounds **15** and **17** does not increase the overall affinity for either duplex at this pH. As observed in the first series, however, the introduction of a second piperidine molecule in the 4-position of piperidine results in a substantial increase in the binding affinity for poly(A)·poly(U) RNA. Thus, **12** shows a significant degree of RNA binding, whereas the interaction with DNA is significantly weaker. The slightly higher ΔT_m value of **12** with RNA compared to either that of **2** or **5** suggests that the increased pK_a of the inner nitrogens, together with the more flexible propyl chain, combines to strengthen the overall RNA-interaction energy of **12**. A comparison of compounds **12**, **15**, and **17** further suggests that a protonatable group in the 4-position of the existing piperidine molecule is important for effective RNA-specific binding.

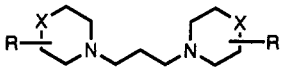
As observed for compound **9**, the introduction of an imidazoline group in the 4-position greatly enhances the RNA-binding affinity. Thus, **18** exhibits a very high ΔT_m for poly(A)·poly(U) at pH 6.3, whereas the binding to poly(dA)·poly(dT) is only approximately one-third of that observed for the RNA duplex. The relatively low ΔT_m for **18** complexed with poly(dA)·poly(dT) reflects a weaker,

Table 2. Thermal Denaturation Results

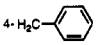


A. With Synthetic RNA and DNA Oligonucleotides

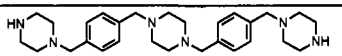
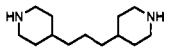
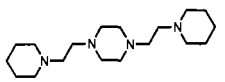
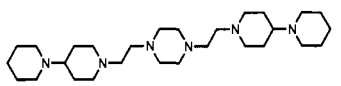
no.	X	R	substitution	ΔT_m,[a] °C (pH 6.3) poly(A)·poly(U)	ΔT_m,[a] °C (pH 6.3) poly(dA)·poly(dT)
1	CH	OH	1,4	0.0	0.0
2	CH		1,4	8.1	3.5
3	N		1,4	0.0	0.0
5	CH		1,3	8.0	3.3
6	N		1,3	0.0	0.0
7	N	H	1,4	0.0	0.0
9	CH		1,4	16.2	8.7



B. With Synthetic RNA and DNA Oligonucleotides

no.	X	R	ΔT_m, °C (pH 6.3) poly(A)·poly(U)	ΔT_m, °C (pH 6.3) poly(dA)·poly(dT)
10	CH	4-H	0.0	0.0
11	CH	4-CN	0.0	0.0
12	CH		9.8	3.3
13	N		0.0	0.0
14	N	4-CH_3	0.0	0.0
15	CH_2	3-CH_2OH	0.4	0.0
17	CH	4-CH_2OH	0.2	0.0
18	CH		18.1	6.1

C. For Miscellaneous Polycationic Ligands

no.	structure	ΔT_m, °C (pH 6.3) poly(A)·poly(U)	ΔT_m, °C (pH 6.3) poly(dA)·poly(dT)
8		0.0	2.3
19		0.0	0.1
22		0.0	0.0
23		7.6	6.5

[a] $\Delta T_m = T_m$ of the complex − T_m of the free nucleic acid.

primarily nonspecific, electrostatic binding mode. Interestingly, compound **18** shows a slightly greater degree of selectively for RNA compared to **9**, which again suggests that the more flexible propyl chain allows better complementary matching of the ligand and RNA duplex structures and is a better linking group for these polycationic ligands.

The 1,4-bis(ethyl)piperazine group was introduced in place of the propyl chain in an attempt to increase both the overall charge density and the steric bulk of the resulting ligands. Compound **22**, however, shows no significant binding to either RNA or DNA at pH 6.3, whereas **23** appears to bind with only slight preference for poly(A)·poly(U) (Table 2C). A comparison of **22** and **10** (with only two formal charges) suggests that the piperazine moiety does not significantly alter the degree of protonation at pH 6.3. A further comparison of the binding affinity of **23** and **12** for RNA indicates that the central 1,4-bis(ethyl)piperazine group exerts an overall destabilizing effect on the drug–RNA complex. The introduction of the piperazine molecule in place of the propyl chain may (i) induce an unfavorable conformation to the ligand which destabilizes the resulting specific complex or (ii) alter the overall degree and distribution of cationic charge such that specific drug–RNA interactions are weakened.

Further thermal denaturation studies were performed on a selection of ligands at pH values of 4.9 and 3.9, and the results are illustrated in Table 3. Compounds **2**, **8**, **9**,

Table 3. Thermal Denaturation Results for RNA and DNA at Low pH Values

	ΔT_m, °C			
	poly(A)·poly(U)		poly(dA)·poly(dT)	
no.	pH 4.9	pH 3.9	pH 4.9	pH 3.9
2	15.4	17.7	6.7	5.6
3	0.0	nd	0.0	nd
6	0.0	nd	0.0	nd
8	15.7	29.3[a]	5.1	12.0
9	20.7	23.3	9.5	8.8
10	0.0	nd	0.0	nd
11	0.0	nd	0.0	nd
12	20.7	22.2	4.5	4.1
13	nd	2.4	nd	0.2
14	1.3	11.0	0.0	0.0
15	0.9	nd	0.0	nd
17	0.5	nd	0.5	nd
18	24.5	28.7	5.6	6.1
19	1.2	nd	0.7	nd
22	0.3	nd	0.2	nd
23	22.9	34.7	8.3	10.4

[a] Value for drug:RNA ratio of 0.1. nd: not determined.

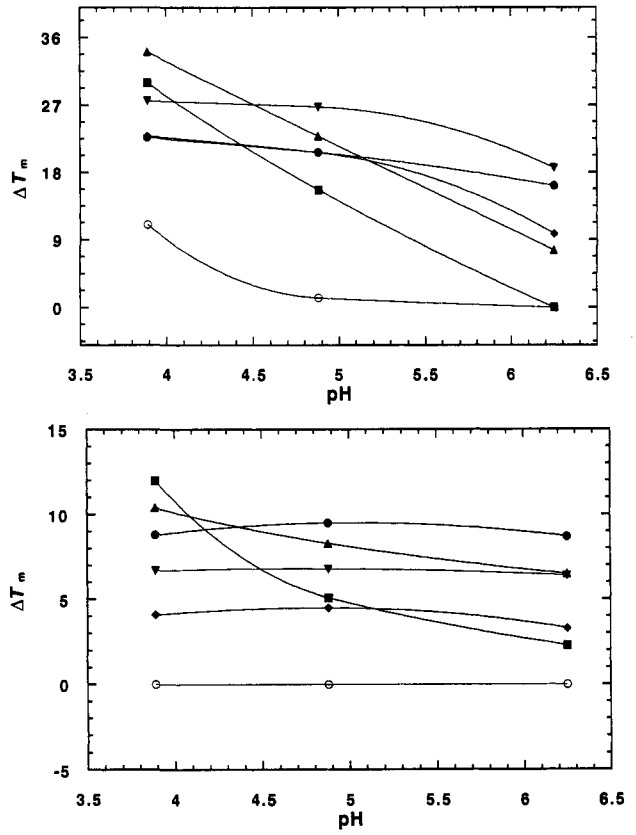


Figure 2. (Top) Plots of ΔT_m of poly(A)·poly(U) with compounds in MES10 buffer at a 0.3 ratio (drug:polymer phosphate) versus pH: **9** (●), **8** (■), **12** (◆), **23** (▲), **18** (▼), and **14** (○). (Bottom) Plots of ΔT_m of poly(dA)·poly(dT) with compounds in MES10 buffer at a 0.3 ratio (drug:polymer phosphate) versus pH: **9** (●), **8** (■), **12** (◆), **23** (▲), **18** (▼), and **14** (○).

12, **18**, and **23** all show very significant preferential binding to RNA. The dependence of ΔT_m on pH for selected ligands with both RNA and DNA is also illustrated graphically in Figure 2. For some compounds, the ΔT_m value for RNA increases dramatically with decreasing buffer pH, whereas the binding affinity with DNA appears to be only slightly pH dependent. The increase in RNA-binding affinity is undoubtedly due to enhanced protonation of the ligands which strengthens specific electrostatic and hydrogen-bonding interactions with the RNA helix. By contrast, the relatively small increase in ΔT_m observed for DNA reflects a more nonspecific electrostatic mode of interaction. Of particular note is compound **12** which shows a ΔT_m increase of over 10 °C for RNA from pH 6.3 to 4.9, whereas the increase in DNA stability is just over 1 °C. Interestingly, compound **8**, which binds weakly to RNA at pH 6.3, shows a dramatic increase in ΔT_m at low pH, whereas stabilization of poly(dA)·poly(dT) is increased to a smaller extent.

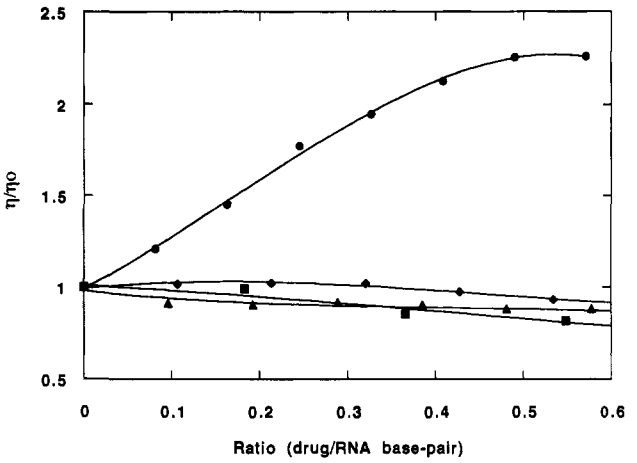


Figure 3. Viscometric titrations of poly(A)·poly(U) with ethidium (●), **9** (■), **2** (◆), and **12** (▲). Titrations were performed as described in the methods section.

Similarly, compound **23** exhibits a significant preference for RNA at low pH. Both results reflect a large increase in protonation which leads to a concomitant increase in RNA-binding affinity and confirm the importance of highly basic centers for effective RNA-binding ligands. Compound **14** appears to show more substantial binding to the RNA helix at the low pH value. Here, a significant change in the overall degree of protonation strengthens the RNA-interaction energy but appears to have little effect on the DNA-binding affinity. A viscometric titration study of compounds **2**, **9**, and **12** with poly(A)·poly(U) (Figure 3) indicates that they do not bind to the RNA duplex by intercalation. None of these compounds caused a significant increase in the viscosity, whereas ethidium caused a dramatic increase in viscosity, as expected for an intercalative binding mode. It therefore seems probable, in view of the high ΔT_m values for RNA, that these ligands bind in a specific manner via one of the helical grooves. Furthermore, since the negative potential is largest in the major groove of A-DNA (and therefore in the structurally related RNA),[16] the results indicate that binding occurs in the major groove via highly specific electrostatic and/or hydrogen-bonding interactions with phosphates and/or base atoms of the poly(A)·poly(U) helix.

The natural polycationic molecule spermine also binds selectively to poly(A)·poly(U) in preference to poly(dA)·poly(dT) as judged by thermal denaturation. In addition, X-ray crystallography,[17,18] molecular mechanics calculations,[19] and a recent study with spermine attached to a photoactivatable probe[20] have shown that spermine can form specific hydrogen-bonded contacts with both phosphates and base atoms within the major groove of RNA or the structurally related A-form DNA.

Conclusion

The preliminary data presented in this paper highlight the importance of basic residues in the design of effective RNA-selective ligands. Furthermore, the low-pH study

reveals that the degree of protonation can dramatically increase the degree of RNA-binding affinity while increasing the affinity for DNA to a much lesser extent. The presence of nonaromatic rings, such as piperidines, leads to an overall decrease in DNA binding compared to polyaromatic ligands bearing cationic groups.[15] This may be due to the increased steric requirements of the piperidine ring which prevent a close, hydrophobic fit into the DNA minor groove that is characteristic of a wide variety of well-documented DNA minor groove binders.[21]

It seems, therefore, that both charge and steric bulk are important requirements for strong preferential binding to the RNA duplex. The preliminary structure–affinity results presented here confirm that the concept of designing RNA-selective molecules is feasible. Further research is in progress to determine more fully the exact site(s) and nature of the interaction of these synthetic ligands with the RNA duplex using both the RNA footprinting technique and cleavage-based assays. In addition, the synthesis of a series of second-generation molecules that incorporate many of the important structural features that have been highlighted from this study is currently in progress.

Experimental Section

Chemistry. Melting points were recorded in a Thomas Hoover (Uni-melt) capillary melting point apparatus and are uncorrected. ^{13}C NMR spectra were obtained using a Jeol JNM-GX270 MHz spectrometer, and chemical shifts (δ) are in ppm relative to $CHCl_3$ (δ 77.00) or DMSO-d_6 (δ 39.50) for samples in D_2O. Mass spectra were recorded on a VG Instruments 70-SE spectrometer (Georgia Tech., Atlanta, GA). Elemental analyses were obtained from Atlantic Microlab Inc. (Norcross, GA) and are within ±0.4% of the theoretical values unless otherwise stated. IR spectra were recorded on a Michelson 100 (Bomem Inc.) FT-IR spectrometer. All chemicals and solvents were purchased from Fisher Scientific or Aldrich Chemical Co., unless otherwise stated, and used without further purification.

Biophysical Methods and Materials. Poly(dA)·poly(dT) from Pharmacia Inc. and poly(A)·poly(U) from Sigma Chemical Co. were prepared as previously described.[22] The experiments were conducted in MES buffer (0.01 M 2-(*N*-morpholino)-ethanesulfonic acid, 0.001 M EDTA, 0.1 M NaCl), and the pH was adjusted to 6.3, 4.9, and 3.9 using NaOH.

Thermal denaturation measurements were determined on a Varian Cary 4 spectrometer interfaced to a Dell/486 microcomputer as previously described[22] by following the absorption change at 260 nm as a function of temperature. The temperature was controlled by a Cary temperature controller that was programmed to raise the temperature at a rate of 0.5 °C/min. A thermistor fixed into a reference cuvette was used to monitor the temperature. T_m values were determined from first-derivative plots after the data were transferred to a Macintosh computer. Compounds are compared by the increase in T_m of the nucleic acid in the presence of the ligand of interest (ΔT_m = T_m of the complex – T_m of the free nucleic acid). The polymers were added to 1 mL of buffer in 1-cm path length reduced-volume quartz cells. Denaturation experiments were conducted at 1.0×10^{-4} M RNA and DNA in MES buffer. Stock solutions of compounds were added to the polymer solution to give a final drug:polymer phosphate ratio of 0.3.

Viscometric titrations were conducted in an Ubbelohde semimicro dilution viscometer (Cannon Series #75 viscometers) as previously described.[23] One milliliter of polymer solution, approximately 1.0×10^{-4} M poly(A)·poly(U) bases, was placed in the viscometer, and titrations were conducted in a constant-temperature water bath (Cannon Instrument Co.) by adding aliquots of a stock solution of the compounds. The additions were made directly into the poly(A)·poly(U) solution by using a Hamilton syringe modified to fit into the viscometer mixing chamber.[23]

Preparation of Symmetrical 1,1′-[1,4-Phenylenebis(methylene)]bisamines. General Procedure A. α,α'-Dibromo-*p*-xylene (0.01 mol), K_2CO_3 (0.01 mol), and an appropriate amine (0.022 mol) in MeOH (40 mL) were refluxed overnight with stirring. The progress of the reaction was monitored by TLC (44:8:1 $CHCl_3$–MeOH–NH_3, v/v/v). Upon completion, the mixture was concentrated under reduced pressure and the resulting solid washed with water. Recrystallization from EtOH afforded the product as a free base which was readily converted to the hydrochloride salt by means of ethanolic HCl.

1,1′-(1,3-Propanediyl)bis(4-cyanopiperidine) (11). General Procedure B. To a stirred solution of 4-cyanopiperidine[14] (4.00 g, 36.3 mmol) in acetone (25 mL) were added anhydrous K_2CO_3 (5.16 g, 37.3 mmol) and KI (0.40 g, 2.4 mmol) followed by a solution of 1,3-dibromopropane (3.67 g, 18.2 mmol) in acetone (20 mL). The resulting mixture was gently refluxed for 3.5 h, cooled to room temperature, and concentrated under reduced pressure. The crude solid was extracted with CH_2Cl_2/MeOH (9:1) and the yellow filtrate evaporated to an oil. Purification by column chromatography (silica gel, 52:7:1 $CHCl_3$–MeOH–NH_4OH, v/v/v) afforded a pale yellow oil which slowly crystallized to give 11 (3.57 g, 75%) as a cream solid: mp 57.5–59 °C; IR (KBr) 2236 cm^{-1} (CN); ^{13}C NMR ($CDCl_3$) δ 24.2, 26.1, 28.7, 51.3, 56.5, 121.7; MS (EI) *m/e* (relative intensity) 260 (M^+, 8), 150 ($M^+ - C_6H_{10}N_2$, 46), 135 ($M^+ - C_7H_{13}N_2$, 43), 123 ($C_7H_{11}N_2^+$, 100). Anal. ($C_{15}H_{24}N_4 \cdot 0.2H_2O$) C, H, N.

1,1′-(1,3-Propanediyl)bis(4-ethylpiperidinecarboxylate) (16). To a stirred solution of ethyl isonipecotate (15.58 g, 99.1 mmol) in acetone (50 mL) was added a solution of 1,3-dibromopropane (5.00 g, 24.8 mmol) in acetone (15 mL). After stirring at room temperature for 30 min, the mixture was refluxed for 3 h, cooled, and concentrated to a white crystalline solid. Purification by column chromatography (silica gel, 72:7:1 $CHCl_3$–MeOH–NH_4OH, v/v/v) afforded the bis-ester 16 (8.29 g, 94%) as a brown/red oil: ^{13}C NMR ($CDCl_3$) δ 13.7, 24.0, 27.7, 40.6, 52.5, 56.3, 59.6, 174.3; MS (EI) *m/e* (relative intensity) 354 (M^+, 12), 309 ($M^+ - C_2H_5O$, 10), 197 ($M^+ - C_8H_{15}NO_2$, 82), 182 ($M^+ - C_9H_{18}NO_2$, 56), 170 ($M^+ - C_{10}H_{18}NO_2$, 100). Anal. ($C_{19}N_{34}N_2O_4 \cdot 0.50H_2O$) C, H, N.

1,1′-(1,3-Propanediyl)bis[4-(hydroxymethyl)piperidine] (17). To a cooled solution of $LiAlH_4$ (40 mL of 1 M solution in THF, 0.04 mmol) in dry THF (40 mL) was added dropwise a solution of 16 (5.00 g, 14.1 mmol) in dry THF (25 mL), and the resulting mixture was stirred under an atmosphere of nitrogen at room temperature. After 1 h, a further aliquot of $LiAlH_4$ (10 mL of 1 M solution, 0.01 mol) was added and the mixture then left to stir under nitrogen for approximately 72 h. The reaction was then slowly quenched with $MgSO_4 \cdot 7H_2O$ followed by aqueous THF and the heavy precipitate filtered and washed with THF and $CHCl_3$/MeOH (9:1, v/v). The colorless filtrate was concentrated under reduced pressure and the slow moving oil chilled to a solid which was collected and washed with Et_2O to afford the alcohol 17 (3.10 g, 81%) as a cream solid: mp 113–114 °C; ^{13}C NMR (DMSO-d_6) δ 24.3, 28.8, 38.5, 53.3, 56.6, 66.0; MS (EI) *m/e* (relative intensity) 270 (M^+, 6), 155 ($M^+ - C_6H_{13}NO$, 52), 140 ($M^+ - C_7H_{16}NO$, 67), 128 ($C_7H_{14}NO^+$, 100). Anal. ($C_{15}H_{30}N_2O_2 \cdot 0.50H_2O$) C, H, N.

1,1′-(1,3-Propanediyl)bis(4-imidazolinylpiperidine) (18). A mixture of 1,2-diaminoethane (0.26 mL, 3.9 mmol) and 1,2-diaminoethane dihydrochloride (0.51 g, 3.8 mmol) was heated under an atmosphere of nitrogen to 80 °C in an oil bath to form a melt. To this stirred mixture was introduced, in portions, 11 (1.00 g, 3.8 mmol), and the resulting mixture was heated to 180–200 °C under an atmosphere of nitrogen for 6 h. On cooling, the solid was dissolved in water and filtered and the aqueous phase concentrated under reduced pressure. To a solution of the crude material in EtOH was added an excess of Et_2O, and the organic phase was carefully decanted. A solution of the resulting pure material (as judged by TLC) in EtOH was stirred with an excess of ethereal HCl and the solid formed collected and dried briefly in air and then over P_2O_5 to afford the tetrahydrochloride of 18 (0.71 g, 38%) as a light brown hygroscopic solid: mp >250 °C dec; ^{13}C NMR (D_2O) δ 20.3, 27.0, 33.0, 45.8, 53.0, 55.0, 172.6; MS (EI) *m/e* (relative intensity) 346 (M^+, 7), 263 ($M^+ - C_4H_7N_2$, 4), 237 ($M^+ - C_6H_9N_2$, 5), 193 ($C_{11}H_{19}N_3^+$, 9), 180 ($C_{10}H_{18}N_3^+$, 5), 166 ($C_9H_{16}N_3^+$, 9), 97 ($C_6H_{11}N^+$, 100). A sample of product in MeOH

was boiled with decolorizing charcoal, filtered, concentrated, and dried over a high vacuum to give an analytical sample as a light brown hygroscopic solid. Anal. ($C_{19}H_{34}N_6 \cdot 4HCl \cdot 1.75H_2O$) C, H, N. HRCIMS obsd, 346.2836; calcd for $C_{19}H_{34}N_6$, 346.2845.

1,4-Bis(2-chloroethyl)piperazine (21). 1,4-Bis(2-hydroxyethyl)piperazine (**20**) (10.00 g, 57.4 mmol) was partially dissolved in $CHCl_3$ (100 mL) and DMF (20 mL) and cooled in an ice/water bath to approximately 5 °C. To this stirred mixture was slowly added a solution of $SOCl_2$ (21.0 mL, 287.9 mmol) in $CHCl_3$ (20 mL) to give a whitish precipitate. After complete addition, the mixture was refluxed for 6.5 h and then left overnight at room temperature. The solution was evaporated and toluene (50 mL) added to the crude product. The precipitated solid was collected and dried. Addition of cold MeOH to the crude solid gave a yellow filtrate which was removed to leave a white solid which was washed with cold MeOH (550 mL) and dried to afford the dihydrochloride salt of **21** (14.29 g, 88%) as a white powder: mp >200 °C dec (lit.[24] mp 250 °C dec); MS (EI) *m/e* (relative intensity) 210 ($M^+ - H$, 3), 174 ($M^+ - HCl$, 56), 161 ($M^+ - CH_2Cl$, 100). Anal. ($C_8H_{14}N_2 \cdot 4HCl$), C, H, N, Cl.

1,4-Bis[2-(1-piperidino)ethyl]piperazine (22). A mixture of **21** (0.50 g, 1.8 mmol) and piperidine (10.0 mL, 101.1 mmol) was stirred at room temperature for 30 min and then gently heated to 80–85 °C. After 1 h, water (approximately 1 mL) was added to give a yellow solution which was maintained at this temperature for 5 h. Concentration under reduced pressure afforded a waxy solid which was mixed with Et_2O and collected. The solid (piperidine hydrochloride?) was removed by filtration and the filtrate concentrated. Purification by column chromatography (silica gel, 52:7:1 $CHCl_3$–MeOH–NH_4OH, v/v/v) afforded a yellow oil which rapidly crystallized to furnish **22** (0.39 g, 72%) as a cream solid: mp 72–74.5 °C; ^{13}C NMR ($CDCl_3$) δ 24.2, 25.8, 53.5, 54.9, 55.9, 56.5; MS (EI) *m/e* (relative intensity) 307 ($M^+ - H$, 21), 223 ($M^+ - C_5H_{11}N$, 6), 210 ($M^+ - C_6H_{12}N$, 83), 196 ($M^+ - C_7H_{14}N$, 9), 180 (22), 127 (49), 112 ($C_6H_{12}N_2^+$, 49), 98 ($C_6H_{12}N^+$, 100). Anal. ($C_{18}H_{36}N_4 \cdot 0.2H_2O$) C, H, N.

1,4-Bis[2-(1,4′-bipiperidino)ethyl]piperazine (23). To a stirred solution of 4-piperidinopiperidine (1.48 g, 8.8 mmol) in acetone (10 mL) at room temperature were added **21** (0.50 g, 1.8 mmol) and sufficient water to dissolve the mixture completely (approximately 1.5 mL). The solution was refluxed for 4 h with water being occasionally added to maintain a solution, cooled to room temperature, and evaporated to dryness. Purification of the crude material by column chromatography (silica gel, 41:8:1 $CHCl_3$–MeOH–NH_4OH, v/v/v) afforded **23** (0.45 g, 54%) as a cream solid: mp 174.5–176 °C dec; ^{13}C NMR ($CDCl_3$) δ 24.7, 26.3, 27.6, 50.0, 53.5, 54.0, 55.8, 56.1, 62.6; MS (CI) *m/e* 475 (M^+, 100), 390 ($M^+ - C_5H_{11}N$, 7), 307 ($M^+ - C_{10}H_{19}N_2$, 10), 281 (46), 195 ($C_{12}H_{23}N_2^+$, 28), 169 ($C_{10}H_{21}N_2^+$, 72). Anal. ($C_{28}H_{54}N_6 \cdot 0.5H_2O$) C, H, N.

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