Selectivity of Polyamines on the Stability of RNA−DNA Hybrids Containing Phosphodiester and Phosphorothioate Oligodeoxyribonucleotides†

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Received January 26, 1999; Revised Manuscript Received May 17, 1999

ABSTRACT: RNA−DNA hybrid stabilization is an important factor in the efficacy of oligonucleotide-based antisense gene therapy. We studied the ability of natural polyamines, putrescine, spermidine, and spermine, and a series of their structural analogues to stabilize RNA−DNA hybrids using melting temperature (T_m) measurements, circular dichroism (CD) spectroscopy, and the ethidium bromide (EB) displacement assay. Phosphodiester (PO) and phosphorothioate (PS) oligodeoxyribonucleotides (ODNs) (21-mer) targeted to the initiation codon region of c-*myc* mRNA and the corresponding complementary RNA oligomer were used for this study. In the absence of polyamines, the T_m values of RNA−PODNA and RNA−PSDNA helices were 41 ± 1 and 35 ± 1 °C, respectively, in 10 mM sodium cacodylate buffer. In the presence of a hexamine analogue of spermine at a concentration of 25 μM, the hybrids were stabilized with T_m values of 80 and 78 °C, for RNA−PODNA and RNA−PSDNA, respectively. The d(T_m)/d(log[polyamine]) values, representing the concentration-dependent stabilization of hybrid helices by polyamines, increased from 10 to 24 for both the RNA−PODNA and RNA−PSDNA helices. Bisethyl substitution of the primary amino groups of the polyamines reduced the hybrid stabilizing potential of the polyamines. Among the homologues of spermidine [$H_2N(CH_2)_3NH(CH_2)_nNH_2$, where $n = 2-8$; $n = 4$ for spermidine] and spermine [$H_2N(CH_2)_3NH(CH_2)_nNH(CH_2)_3NH_2$, where $n = 2-8$; $n = 4$ for spermine], spermidine and spermine were the most effective agents for stabilizing the hybrid helices. At a physiologically compatible concentration of 150 mM NaCl, the hybrid helix formed from PODNA was more stable than that formed from PSDNA in the presence of polyamines. CD spectroscopic studies showed that the hybrids were stabilized in a conformation close to A-DNA in the presence of polyamines. The relative binding affinity of the polyamine homologues for the hybrid helices, as measured by the EB displacement assay, followed the same order in which they stabilized the hybrids. These results are important in the antisense context and in the general context of polyamine−nucleic acid interactions, and suggest that pentamine and hexamine analogues of spermine might be useful in improving the efficacy of therapeutic ODNs.

The formation of RNA−DNA hybrids by the Watson−Crick base pairing of complementary strands is involved in cellular processes such as the priming step in DNA replication, transcription, and reverse transcription (*1*−*4*). In addition, utilization of a DNA oligomer that selectively hybridizes to a target mRNA and represses gene expression, either by blocking mRNA translation or by facilitating the cleavage of the mRNA by RNAse H, has become an important gene therapy strategy, known as antisense therapeutics (*5*−*7*). Recent reports indicate there is much promise for this technology, and intense efforts are underway to develop therapeutic oligodeoxyribonucleotides (ODNs) to treat a diverse spectrum of diseases, including HIV, cancer, and herpes (*8*−*11*).

To realize the potential clinical utility of therapeutic ODNs, several practical difficulties have to be overcome. These include the sensitivity of ODNs to nucleases, inefficient cellular uptake of ODNs, and the stability of the hybrid helices under cellular ionic and pH conditions (*8*−*15*). Several strategies have been used in attempts to circumvent these problems. The biological stability of the ODNs has been significantly improved by replacing one of the nonbridging oxygen atoms with a sulfur atom, as in PS ODN (*16*, *17*). The modified ODN also exhibits significant ability

† This work was supported, in part, by Grants CA73058, CA80163, and CA42439 from the National Cancer Institute (T.J.T. and T.T.) and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan (A.S.).

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10.1021/bi990180t CCC: $18.00
Published on Web 07/29/1999

to elicit the activity of RNAse H, an enzyme which plays a key role in the antisense effect by cleaving the RNA strand bound to the ODNs (*18*). A variety of ODN delivery vehicles, such as lipofectin, polyaminolipids, branched polyamines, and cationic porphyrins, have been developed to facilitate the cellular uptake of ODNs (*19*−*22*).

Although many of the DNA modifications are focused on increasing the stability of the ODN against nuclease sensitivity, an important factor governing the success of the antisense therapeutic strategy is the thermodynamic stability of the hybrids formed from the ODN and its targeted mRNA (*23*−*27*). The correlation of thermodynamic stability with the ODN structure suggests the importance of primary and secondary structure and the overall conformation in hybrid stability (*28*). The secondary structure of the RNA−DNA hybrids is known to be intermediate between A- and B-form helices and, in the majority of them, closer to A-form, which is prevalent in RNA double helices (*25*). However, in many hybrids, the DNA part of the hybrid retains the B-form, which is prevalent in double-helical DNA (*23*, *25*). Lesnik and Frier (*25*) showed a direct correlation between the extent of A-DNA conformation in the secondary structure and the thermodynamic stability of the hybrid. The specificity of the RNA−DNA hybrid for interaction with RNAse H is suggested to arise from the conformation of the hybrid helices rather than from the nucleotide sequence (*28*). The stability of the RNA−DNA hybrids is governed in part by charge neutralization because the negative phosphate groups on the backbone of both RNA and DNA repel each other in the absence of counterions. Among the various biological cations involved in DNA and RNA helix stabilization in the cell, the polyamines appear to play a critical role because of their ubiquitous presence in all cells (*29*). The ability of natural and synthetic polyamines to cause structural and conformational alterations in DNA has been studied in detail, although the precise mode of binding of polyamines with DNA is still contentious (*30*−*33*). Previous studies using a series of spermidine and spermine homologues demonstrated polyamine structural specificity effects in the stabilization of duplex and triplex DNA structures (*2*, *34*−*36*). Despite these intriguing observations, investigations into the binding of polyamines on RNA−DNA hybrids are scant.

In this investigation, we evaluated the ionic and structural effects of polyamines on the RNA−DNA hybrid helix by synthesizing several novel analogues of the natural polyamines and determining their ability to alter the thermal helix−coil transitions of the hybrid. The DNA part of the helix consisted of either the phosphodiester or phosphorothioate ODN, comprising the complementary strand of the c-*myc* oncogene mRNA, including the AUG initiation codon. We used a synthetic RNA oligomer for our hybridization experiments. Our results indicate a dramatic increase in the T_m of RNA−DNA hybrids as the number of positive charges on the polyamine increased from two to six. However, spermidine and spermine, respectively, were the most effective tri- and tetravalent polyamines in stabilizing the RNA−DNA hybrids.

MATERIALS AND METHODS

Oligonucleotides. We used 21-mer antisense phosphodiester and phosphorothioate ODNs with the following sequence: 5′-GAAGTTCACGTTGAGGGGCAT-3′. This ODN

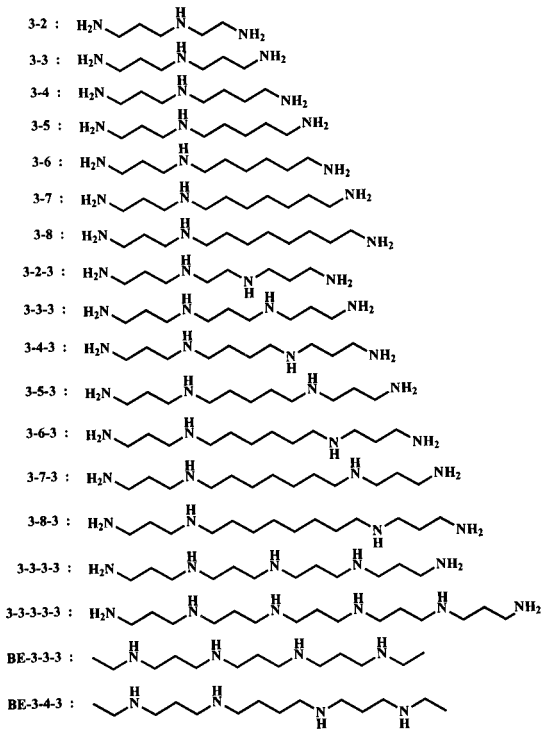

FIGURE 1: Chemical structures of natural and synthetic polyamines used in this study.

is targeted to c-*myc* mRNA, including its AUG translation initiation codon site, and is complementary to the antisense oligoribonucleotide, 5′-r(AUGCCCCUCAACGUGAACUUC)-3′. All ODNs were purchased from Oligos, Etc. (Wilsonville, OR), and were HPLC purified. The ODNs were dissolved in 10 mM cacodylate buffer [10 mM sodium cacodylate (pH 7.4) and 0.5 mM EDTA] and extensively dialyzed from the same buffer before use in physicochemical studies.

Polyamines. Putrescine·2HCl, spermidine·3HCl, and spermine·4HCl were purchased from Sigma Chemical Co. (St. Louis, MO). The structural homologues of spermidine, 1,6-diamino-3-azahexane (3-2), 1,7-diamino-4-azaheptane (3-3), 1,9-diamino-4-azanonane (3-5), 1,10-diamino-4-azadecane (3-6), 1,11-diamino-4-azaundecane (3-7), and 1,12-diamino-4-azadodecane (3−8) and spermine, 1,10-diamino-4,7-diazadecane (3-2-3) 1,11-diamino-4,8-diazaundecane (3-3-3, norspermine), 1,13-diamino-4,10-diazatridecane (3-5-3), 1,14-diamino-4,11-diazatetradecane (3-6-3), 1,15-diamino-4,11-diazapentadecane (3-7-3), 1,16-diamino-4,13-diazahexadecane (3-8-3), 1,15-diamino-4,8,12-triazapentadecane (3-3-3-3), and 1,19-diamino-4,8,12,16-tetraazanonadecane (3-3-3-3-3) were synthesized as described previously (*34*−*36*). The bis(ethyl)polyamine analogues, bis(ethyl)-spermine (BE-3-4-3) and bis(ethyl)norspermine (BE-3-3-3), were also synthesized according to previously described methods (*37*, *38*). The chemical structure and purity of the synthesized compounds were determined by elemental analysis, NMR, HPLC, and mass spectrometry. The chemical structures of these compounds are given in Figure 1. Polyamine analogue stock solutions (20 mM) were prepared in sterile, double-distilled water, and appropriate dilutions were made prior to use.

Preparation of RNA−DNA Hybrids. The RNA oligomer and the complementary phosphodiester and phosphorothioate oligomers were mixed in a 1:1 molar ratio in 10 mM

cacodylate buffer (pH 7.4) and the appropriate concentrations of polyamines and heated in a boiling water bath for 5 min, cooled to room temperature, and allowed to equilibrate for 16 h before performing the T_m measurements.

T_m Measurements. These measurements were carried out using a Beckman DU 640 spectrophotometer interfaced with an IBM computer. All the measurements were taken at a heating rate of 0.5 °C/min using a Beckman high-performance temperature controller (*35*). The measurements were carried out in 10 mM cacodylate buffer in the absence and presence of different concentrations of polyamines. The helix−coil transition temperature, which corresponded to half-dissociation of the hybrids, was calculated as the T_m after constructing pre- and post-transitional baselines corresponding to 100 and 0% hybrid, respectively (*39*).

Circular Dichroism (CD) Measurements. The CD spectra were recorded on an Aviv model 62DS circular dichroism spectrophotometer (Aviv Associates, Lakewood, NJ). Rectangular quartz cuvettes with a 1 cm path length were used for all measurements. The CD spectra were corrected for the buffer contribution. The molar ellipticity was calculated using the equation $[\theta] = \theta/cl$, where θ is the observed ellipticity, c is the molar concentration of the nucleotide, and l is the path length.

Ethidium Bromide Displacement Assay. The relative affinity of binding of spermidine and spermine homologues to RNA−PODNA and RNA−PSDNA hybrids was determined using an ethidium bromide (EB) displacement assay (*40*, *41*). Binding of polyamines to DNA helices causes a displacement of bound EB, resulting in a drop in the fluorescence emission intensity. The concentration of polyamine required to reduce the fluorescence intensity by 50% (EC_{50}) was calculated, and the reciprocal of this concentration was taken as a relative measure of the binding constant (*40*). Serial additions of polyamines were made to the hybrid−EB complex, and the emission intensity was measured at 590 nm (excitation at 510 nm) after an equilibration time of 1 min. The measurements were taken using a quartz microcuvette with a volume capacity of 350 μL and a 3 nm bandwidth for excitation and emission. The fluorescence in the absence of the polyamines was taken as 100% and that of free ethidium bromide as 0%. The concentration of hybrid was 5 μM. The binding affinity is expressed relative to that of spermine, for which the value was assigned as 1. All fluorescence measurements were performed using a SPEX (Edison, NJ) Fluoromax-2 spectrofluorometer.

RESULTS

Stabilization of RNA−DNA Hybrids by Natural and Synthetic Polyamines. Figure 2 shows the absorbance−temperature profiles of the RNA−PODNA hybrid helix in the presence of different concentrations of spermine. The dissociation of the RNA−PODNA hybrid to the corresponding single strands was observed as an increase in the absorbance at 260 nm (A_{260}) as temperature increased from 20 to 90 °C. In the absence of spermine, the T_m of this hybrid was 41 ± 1 °C. Addition of spermine to the hybrid solution caused a concentration-dependent increase in the T_m of the hybrid, with a maximum T_m of 74 °C at 100 μM spermine.

To study the role of cationicity of polyamines on hybrid stabilization, we conducted T_m measurements of RNA−PODNA in the presence of different polyamines, from the diamine putrescine to the hexamine 3-3-3-3-3. The T_m values were determined in a concentration-dependent manner, and the measured values are presented in Table 1. Putrescine was less effective than spermidine and spermine in stabilizing the hybrid duplex. The T_m values of the RNA−PODNA hybrid at 100 μM spermidine and spermine were 67 and 74 °C, respectively. The pentamine and hexamine analogues of spermine, 3-3-3-3 and 3-3-3-3-3, respectively, exhibited higher hybrid stabilizing potential than spermine, and required much lower concentrations for hybrid stabilization. For example, 100 μM pentamine and hexamine increased the T_m of the RNA−PODNA hybrid to 82.5 and 85 °C, respectively. Thus, the relative efficiency of the polyamines in stabilizing the hybrid helix was in the following order: 3-3-3-3-3 > 3-3-3-3 > spermine > spermidine > putrescine.

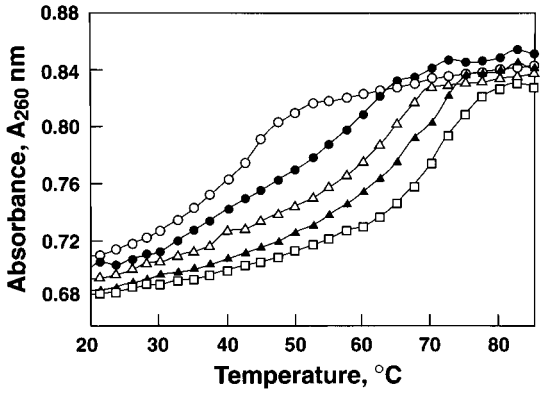

FIGURE 2: Melting profile of the RNA−PODNA hybrid in the presence of spermine. The concentrations of spermine were 0 (○), 5 (●), 10 (△), 25 (▲), and 50 μM (□). The T_m measurements were carried out in 10 mM cacodylate buffer [10 mM sodium cacodylate (pH 7.4) and 0.5 mM EDTA] at a heating rate of 0.5 °C/min.

Table 1: Effects of Polyamines on the T_m of the RNA−PODNA Hybrid[a]

concentration (μM)	T_m (°C)				
	putrescine	spermidine	spermine	3-3-3-3	3-3-3-3-3
0	42.0	41.5	41.5	42.0	41.5
1	42.0	44.3	45.5	49.9	56.4
5	42.5	52.3	54.0	64.9	73.8
10	43.5	56.7	63.7	68.9	74.3
25	46.6	62.3	66.7	71.0	79.9
50	48.7	65.0	71.0	73.0	81.7
100	53.0	67.0	74.0	82.5	85.0

[a] The T_m measurements were conducted in a buffer containing 10 mM sodium cacodylate (pH 7.4) and 0.5 mM EDTA.

To quantify the ability of each of the polyamines to increase the T_m of the RNA−DNA hybrid in a concentration-dependent manner, we plotted T_m against log[polyamine] (Figure 3). The slope of these plots, $d(T_m)/d(\log[\text{polyamine}])$ (Table 2), was the lowest for putrescine (10) and highest for 3-3-3-3-3 (22). The $d(T_m)/d(\log[\text{polyamine}])$ values represent a measure of the efficacy of a polyamine to increase T_m over a concentration range at which a linear increase in T_m is observed with polyamine concentration. Therefore, our results show a remarkable increase in hybrid stability with cationicity of the polyamines.

In the next set of experiments, we determined the ability of the polyamines to stabilize the RNA−PSDNA hybrid. The

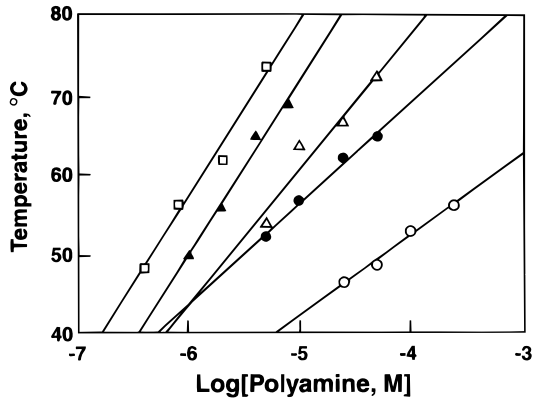

FIGURE 3: Plots of T_m vs log[polyamine] showing the ability of polyamines to increase the T_m of the RNA−PODNA hybrid in a concentration-dependent manner. T_m data plotted here are below the concentrations at which these values plateaued. The symbols represent T_m values for putrescine (○), spermidine (●), spermine (△), 3-3-3-3 (▲), and 3-3-3-3-3 (□).

Table 2: d(T_m)/d(log[Polyamine]) Values for Hybrid Stabilization

polyamine or cation	concentration range ($\times 10^6$ M)[a]	d(T_m)/d(log[M^{z+}])	
		RNA−PODNA	RNA−PSDNA
putrescine^{2+}	25−250	10	10
spermidine^{3+}	10−100	13	13
spermine^{4+}	10−100	19	17
3-3-3-3^{5+}	1−25	22	22
3-3-3-3-3^{6+}	0.4−25	22	24
Na^+	25000−150000	18.5	18
Mg^{2+}	250−2500	21.5	22.9

[a] A straight line was obtained upon plotting T_m against log[M^{z+}], where M^{z+} refers to either the polyamine or inorganic cation.

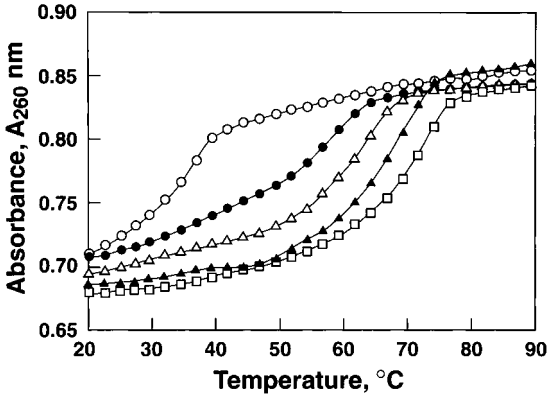

FIGURE 4: Melting profile of the RNA−PSDNA hybrid in the presence of spermine. The concentrations of spermine were 0 (○), 5 (●), 10 (△), 25 (▲), and 50 μM (□). T_m measurements were conducted in 10 mM cacodylate buffer.

melting profile of the RNA−PSDNA hybrid at various concentrations of spermine is depicted in Figure 4. In the absence of polyamines, the RNA−PSDNA hybrid melted at 35 ± 1 °C. As observed with the RNA−PODNA hybrid, T_m increased with the addition of the polyamines. T_m values of RNA−PSDNA in the presence of these polyamines are presented in Table 3. They showed the same order of efficacy in stabilizing the RNA−PSDNA hybrid that was found with RNA−PODNA (3-3-3-3-3 > 3-3-3-3 > spermine > spermidine > putrescine). In general, T_m values for the RNA−PSDNA hybrid were lower than that of the RNA−PODNA hybrid at the same polyamine concentrations. However, the patterns of T_m versus log[polyamine] plots were similar for

Table 3: Effect of Polyamines on the T_m of the RNA−PSDNA Hybrid

concentration (μM)	T_m (°C)				
	putrescine	spermidine	spermine	3-3-3-3	3-3-3-3-3
0	35.0	35.5	35.5	35.0	35.5
1	35.5	38.9	39.8	50.0	45.0
5	36.0	46.9	52.1	58.0	60.0
10	37.0	49.9	55.0	66.5	71.7
25	39.1	55.4	61.0	73.8	78.1
50	43.0	59.9	65.0	77.0	82.5
100	43.6	62.9	71.8	78.1	84.2

Table 4: T_m Values of RNA−DNA Hybrids in the Presence of NaCl and $MgCl_2$[a]

[NaCl] (mM)	T_m (°C)		[$MgCl_2$] (mM)	T_m (°C)	
	RNA−PODNA	RNA−PSDNA		RNA−PODNA	RNA−PSDNA
0	42	35.5	0	42	31.5
25	47.6	48.0	0.5	50.7	45.3
50	53.7	56.0	1	59.1	55.8
100	58.5	59.2	2.5	59.9	60.8
150	62.1	61.0	5	60.9	61.7

[a] All T_m measurements were conducted in 10 mM sodium cacodylate buffer. Small volumes of concentrated solutions of NaCl and $MgCl_2$ were added to the ODN solution to increase the cation content.

both hybrids (result not shown), and the d(T_m)/d(log[polyamine]) values were comparable (Table 2), despite the backbone modifications on the ODN.

Effect of Na^+ and Mg^{2+} on the Stability of the RNA−DNA Hybrid. We next examined the effects of two inorganic cations, Na^+ and Mg^{2+}, on the stability of both RNA−PODNA and RNA−PSDNA hybrids. Like polyamines, both Na^+ and Mg^{2+} stabilized the hybrids in a concentration-dependent manner (Table 4). With increasing concentrations of these cations, the T_m values of both hybrids increased, with d(T_m)/d(log[Na^+]) values of 18.5 and 18.0 for RNA−PODNA and RNA−PSDNA, respectively (Table 2). In the presence of Mg^{2+}, the corresponding d(T_m)/d(log[Mg^{2+}]) values were 21.5 and 22.9, and comparable to that of spermine and other higher polyamine analogues. The d(T_m)/d(log[M^{z+}]) (where M is the cation and z is the number of positive charges) values are related to the enthalpy changes (ΔH) and thermodynamic degree of ion dissociation (Δi) for the melting transition:

$$\Delta i = [\mathrm{d}(T_m)/\mathrm{d}(\log[M^{z+}])]\{[\Delta H/2.3R(T_m)^2]\}$$

where R is the gas constant. Therefore, it is difficult to speculate about the relative importance of d(T_m)/d(log[M^{z+}]) values of different cations in the absence of the corresponding ΔH values for the hybrid helix−coil transition in the presence of these cations. However, the concentration range at which a linear increase in T_m occurred with Na^+ is 1500−6000-fold higher than that of the tetra-, penta-, and hexavalent polyamines. It is also interesting to note here that putrescine^{2+} is a better divalent cation for increasing the T_m of the hybrid than Mg^{2+}. For example, the concentration of putrescine^{2+} which increases the T_m of the RNA−PODNA hybrid to 62 °C is 1 mM, while a 10-fold higher concentration of Mg^{2+} is required to exert the same effect.

Table 5: Effect of Bis(ethyl) Substitution of Polyamines on the T_m of RNA−DNA Hybrids[a]

concentration (μM)	T_m (°C)							
	3-3-3		BE-3-3-3		3-4-3		BE-3-4-3	
	PODNA	PSDNA	PODNA	PSDNA	PODNA	PSDNA	PODNA	PSDNA
0	41.3	36.0	42.0	35.0	41.5	35.5	41.5	35.0
5	53.0	50.0	51.0	48.8	54.0	52.1	51.7	48.8
10	63.0	53.0	59.5	52.7	63.7	55.0	54.3	55.7
25	64.5	59.0	63.0	58.5	66.7	61.0	66.5	63.0
50	70.0	64.0	68.5	63.5	71.0	65.0	70.5	65.1
100	73.0	68.5	71.0	65.5	74.0	71.8	71.0	67.3

[a] All T_m measurements were conducted in 10 mM sodium cacodylate buffer.

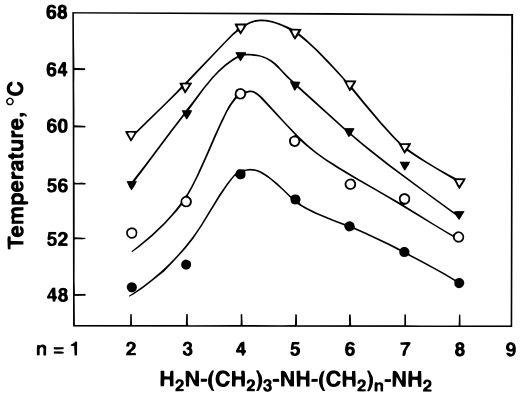

FIGURE 5: Structural specificity effects of spermidine homologues on the T_m of the RNA−PODNA hybrid. The number of methylene groups (n) on the variable arm of spermidine homologues is plotted against the corresponding T_m values at homologue concentrations of 10 (●), 25 (○), 50 (▼), and 100 μM (▽).

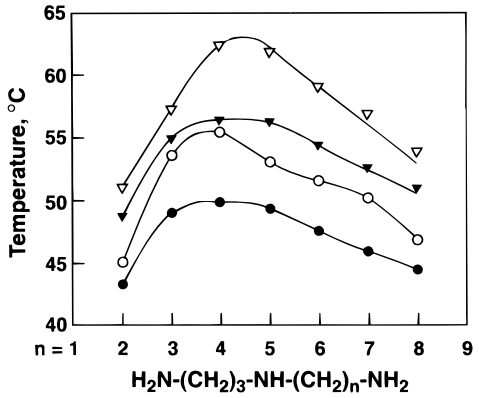

FIGURE 6: Structural specificity effects of spermidine homologues on the T_m of the RNA−PSDNA hybrid. The number of methylene groups (n) on the variable arm of spermidine homologues is plotted against the corresponding T_m values at homologue concentrations of 10 (●), 25 (○), 50 (▼), and 100 μM (▽).

Effect of Bis(ethyl) Substitution of Polyamines on the Stability of RNA−DNA Hybrids. Bis(ethyl) analogues of polyamines are known for their potential chemotherapeutic effects in cancer cells (*42*). To study the possibility of using these compounds in hybrid stabilization, we determined the T_m values of both RNA−PODNA and RNA−PSDNA hybrids in the presence of two bis(ethyl) polyamine analogues: BE-3-3-3 and BE-3-4-3. Both of them stabilized the hybrid DNA, and the melting profiles were similar to that of spermine (profiles not shown). However, T_m values were lower than that in the presence of unsubstituted polyamines (Table 5).

Structural Specificity of Spermidine Homologues in Stabilizing RNA−DNA Hybrids. In the next set of experiments, we recorded the melting profiles of both RNA−PODNA and RNA−PSDNA hybrids in the presence of spermidine homologues. These triamines differed in the number of CH_2 groups on the variable arm of spermidine (Figure 1) and thus provided us with an opportunity to disentangle charge effects from that of polyamine structure. The T_m values of both RNA−PODNA and RNA−PSDNA hybrids increased with polyamine concentration in the presence of all the analogues that were studied. Plots of the T_m values of RNA−PODNA and RNA−PSDNA hybrids versus the number of CH_2 groups in the variable arm of the triamine (Figures 5 and 6) exhibited the highest T_m with spermidine ($n = 4$) at all the concentrations that were studied (10, 25, 50, and 100 μM). In general, the efficacy of these compounds in stabilizing the hybrid helices was in the following order: 3-4 (spermidine) > 3-3 > 3-5 > 3-6 > 3-7 > 3-8 > 3-2. In previous studies involving DNA−DNA duplex stabilization, the efficacy of 3-2 was closer to that of putrescine^{2+} than to that of spermidine^{3+} (*34*). A possible reason for the reduced efficacy of 3-2 might be related to its reduced charge (lower pK_a).

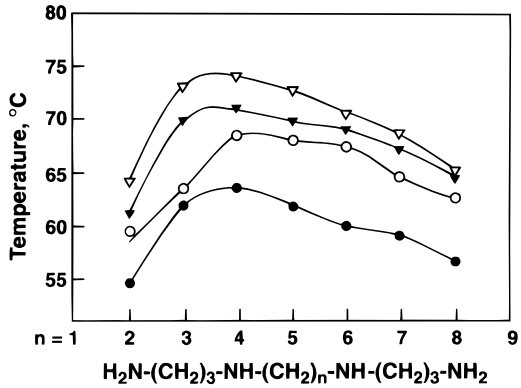

FIGURE 7: Structural specificity effects of spermine homologues on the T_m of the RNA−PODNA hybrid. The number of methylene groups (n) on the variable arm of spermine homologues is plotted against the corresponding T_m values at homologue concentrations of 10 (●), 25 (○), 50 (▼), and 100 μM (▽).

Structural Specificity of Spermine Homologues in RNA−DNA Hybrid Stabilization. In the next set of experiments, we investigated the effects of spermine homologues in stabilizing RNA−PODNA and RNA−PSDNA hybrids (Figures 7 and 8). These compounds increased the T_m of both hybrid helices in a concentration-dependent manner; however, their efficacy in increasing T_m varied with the structural geometry of the compounds. These plots also show that the natural polyamine spermine had the highest stabilizing potential, closely followed by its nearest homologues, 3-3-3

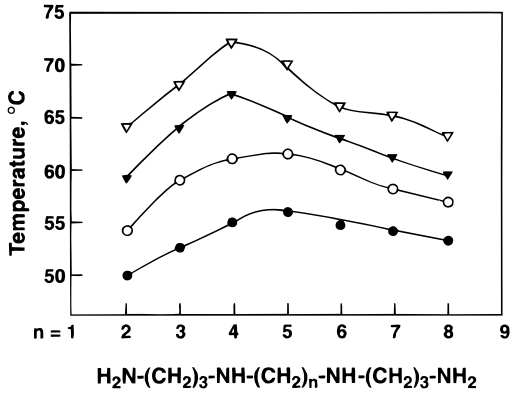

FIGURE 8: Structural specificity effects of spermine homologues on the T_m of the RNA−PSDNA hybrid. The number of methylene groups (n) on the variable arm of spermine homologues is plotted against the corresponding T_m values at homologue concentrations of 10 (●), 25 (○), 50 (▼), and 100 μM (▽).

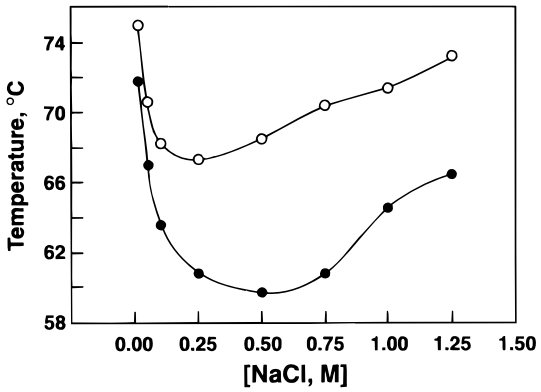

FIGURE 9: Effect of NaCl on the stabilization of RNA−PODNA (○) and RNA−PSDNA (●) in the presence of 100 μM spermine.

and 3-5-3. However, a dramatic decrease in T_m occurred with 3-2-3, although this compound differed from spermine by two methylene groups only in the central bridging region. This could be a consequence of its reduced cationicity (please see the Discussion). Similarly, the higher homologues were less effective than spermine in stabilizing RNA−DNA hybrids. The ability of the analogues to stabilize the hybrid helices was in the following order: 3-4-3 > 3-5-3 > 3-3-3 > 3-6-3 > 3-7-3 > 3-8-3 > 3-2-3. These findings demonstrate that the chemical structure of the polyamines has a major effect on RNA−DNA hybrid stabilization.

Competition of Na^+ and Polyamines for RNA−DNA Hybrid Stabilization. In the next set of experiments, we examined the ability of Na^+ to alter the T_m values of RNA−DNA hybrids stabilized with spermine (Figure 9). At 100 μM spermine, the T_m values for RNA−PODNA and RNA−PSDNA hybrids were 74 and 71.8 °C, respectively. With the addition of NaCl, the T_m of hybrid helices decreased. At a physiologically compatible Na^+ concentration of 150 mM, the T_m values of RNA−PODNA and RNA−PSDNA hybrids were 67 and 60 °C, respectively, in the presence of 100 μM spermine. The corresponding T_m values were 62.1 and 61 °C, respectively, in the presence of 150 mM Na^+ alone. In the case of the RNA−PODNA hybrid, the T_m values gradually increased at concentrations of >250 mM and reached a value of 73 °C at 1250 mM Na^+. In contrast, the T_m of the RNA−PSDNA hybrid remained repressed up to 500 mM Na^+, and then increased to a value of 66 °C at 1250 mM NaCl. Thus, RNA−PODNA was more stable than the RNA−PSDNA hybrid in the presence of combinations of spermine and NaCl.

Table 6: T_m Values of RNA−DNA Hybrids in the Presence of 150 mM NaCl and 1 mM Polyamines

	T_m (°C)	
polyamine	RNA−PODNA	RNA−PSDNA
none	62.1	61.0
putrescine	65.2	56.3
spermidine	69.2	62.8
spermine	72.0	71.0
3-3-3-3	79.0	73.0
3-3-3-3-3	82.0	75.5

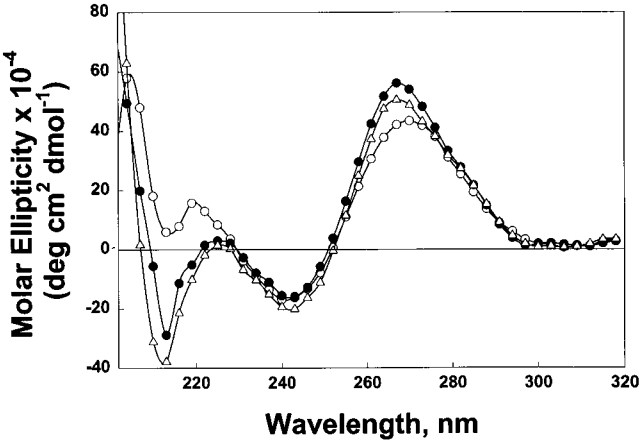

FIGURE 10: CD spectra of the RNA−PODNA hybrid in the presence of 0 (○), 10 (●), and 25 μM spermine (△). The CD spectra were recorded in 10 mM cacodylate buffer.

The intracellular concentration of polyamines has been reported to be in the millimolar range (*43*), although the "free" or active concentration is probably lower. Therefore, we next examined the ability of different 1 mM polyamines to increase the stability of both hybrid helices in the presence of 150 mM Na^+ (Table 6). The T_m values thus determined were 72 and 69.7 °C, respectively, for spermine-stabilized RNA−PODNA and RNA−PSDNA hybrids. As observed in the low-ionic strength medium, the efficacy of the polyamines in increasing the T_m of the hybrid helices increased with cationicity and the values were in the following order: 3-3-3-3-3 > 3-3-3-3 > spermine > spermidine > putrescine.

CD Spectroscopic Studies. RNA−DNA hybrids are known to have a structure close to A-form nucleic acid (*25*). To examine whether polyamine-mediated stabilization of the hybrid is due to a change in the conformation toward A-form DNA, we recorded the CD spectra of the hybrid in the presence of spermine between 205 and 320 nm. The CD spectrum of the RNA−PODNA hybrid (Figure 10) has two positive bands at 220 and 270 nm and a negative band at 240 nm. This CD spectrum is different from the typical B-DNA spectrum that has a conservative band with negative and positive peaks around 245 and 280 nm, respectively, or a typical A-DNA spectrum that has a strong negative band about 210 nm, a weak negative band about 245 nm, and a very intense positive band about 265 nm (*24*, *25*, *44*). In the CD spectrum of RNA−PODNA, the intensity at 270 nm is ~3-fold higher than that of the negative band, suggesting a structure closer to that of A-DNA than B-DNA. However, no negative band was seen at ~210 nm. The presence and

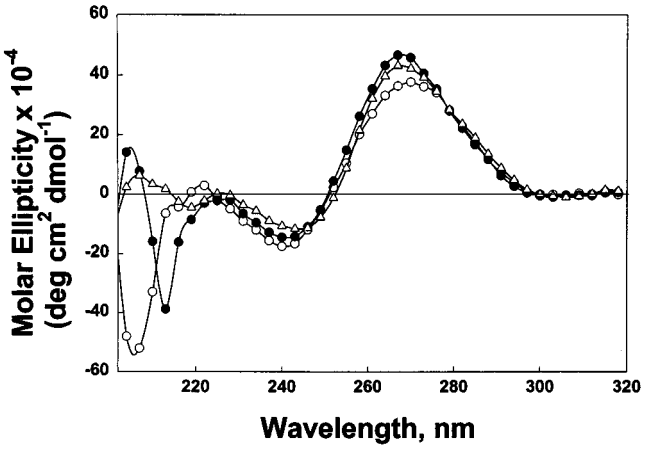

FIGURE 11: CD spectra of the RNA−PSDNA hybrid in the presence of 0 (○), 10 (●), and 25 μM spermine (△). The CD spectra were recorded in 10 mM cacodylate buffer.

the intensity of the negative 210 nm band in the hybrid CD are known to be a function of the base sequence (*26*, *45*). With the addition of spermine, the intensity of the positive band increased with the simultaneous development of a strong negative band at 210 nm, suggesting that the degree of A-form increases with polyamine addition. The ellipticity of the positive band showed a small decrease at spermine concentrations of >25 μM.

We also recorded the CD spectrum of the RNA−PSDNA hybrid in the presence and absence of spermine (Figure 11). In the absence of spermine, the CD spectrum of this hybrid was different from that of the RNA−PODNA hybrid in the low-wavelength region (200−220 nm), but similar at wavelengths above 220 nm. It exhibited a strong negative band at ∼207 nm, closer to the 210 nm band characteristic of A-DNA. With the addition of spermine, the ellipticity decreased and the band maximum red shifted to 212 nm at 10 μM and the ellipticity further decreased at 25 μM. However, the spectral changes were similar to that of RNA−PODNA at higher wavelengths (>220 nm), and stabilized at >25 μM spermine.

Relative Binding Affinity of Spermidine and Spermine Homologues with Hybrid Helices. To confirm whether the structural specificity observed in RNA−DNA helix stabilization is due to the differences in the relative binding affinities of these polyamines for the hybrids, we determined their relative binding affinities using an ethidium displacement assay. Results of these experiments are presented in Table 7. Among the triamines, spermidine had the maximum binding affinity, which was very close to its next lower or higher homologue. On a relative scale of 1 for spermine, the binding affinity for binding to the RNA−PODNA hybrid decreased with alteration in the number of CH_2 groups in the aliphatic chain. Among spermine homologues, 3-3-3, with one less CH_2 group than spermine, had a binding affinity close to that of spermine. However, 3-2-3 had the lowest binding affinity, about 25-fold lower than that of spermine, showing that a decrease of more than one CH_2 group drastically reduced the binding affinity. Homologues with a higher number of CH_2 groups also had reduced affinity that decreased with increases in chain length. For 3-8-3, the binding affinity was 0.09. Compared to that of spermine, the binding affinity for spermidine was ∼50-fold lower than that of spermine. The relative binding affinities of spermidine and spermine homologues for binding to RNA−PODNA and RNA−PSDNA were similar (Table 7).

Table 7: Relative Binding Constants Measured by the Ethidium Competition Method

polyamine analogue	relative binding constant[a]	
	RNA−PODNA	RNA−PSDNA
3-2	0.005	0.003
3-3	0.02	0.02
3-4 (spermidine)	0.023	0.02
3-5	0.02	0.014
3-6	0.007	0.012
3-7	0.0056	0.006
3-8	0.002	0.004
3-2-3	0.04	0.06
3-3-3	0.89	0.9
3-4-3 (spermine)	1.00	1.0
3-5-3	0.35	0.81
3-6-3	0.24	0.18
3-7-3	0.31	0.09
3-8-3	0.09	0.07

[a] The binding constants were calculated as the reciprocal of EC_{50}, the concentration of polyamine required to displace 50% of the ethidium bound to RNA−DNA hybrids.

DISCUSSION

The main goal of this investigation was to examine the ability of natural and synthetic polyamines to stabilize RNA−DNA hybrids formed from a therapeutically important ODN in its phosphodiester and phosphorothioate forms. Both forms of this ODN have been shown to suppress the expression of the c-*myc* oncogene in cancer cell lines (*46*−*49*). It is possible that the natural polyamines might participate in RNA−DNA hybrid formation in the cell because the intracellular spermidine and spermine levels are known to be in the millimolar concentration range (*29*). A part of the "bound" polyamines may be existing in association with negatively charged macromolecular assemblies in the cell, including double-stranded DNA, folded RNA, ribosome, and chromatin. Since many of these supramolecular structures are associated with proteins and cellular cations other than polyamines, their affinity for "free" polyamines is likely to be lower than that of free ODN and nascent mRNA. Our studies demonstrate that spermine can stabilize RNA−DNA hybrids with a ΔT_m of about 33 °C at 100 μM in low-salt buffer. At a physiologically compatible ionic concentration of 150 mM Na^+, there was an increase of 10 °C in T_m in the presence of 1 mM spermine (Table 6). The phosphorothioate modification, specifically introduced to increase the biological stability of the ODN, had a deleterious effect on the thermodynamic stability of the hybrid. A similar destabilizing effect was observed in the case of triplex DNA formation when the phosphorothioate ODN was compared with its phosphodiester analogue (*50*). Although isovalent polyamine homologues were less efficient than the natural polyamines, spermidine and spermine, in stabilizing RNA−DNA hybrids, a novel series of pentamine and hexamine analogues were much more effective than spermine in stabilizing the hybrid helices, with a ΔT_m of up to 20 °C in the presence of 1 mM 3-3-3-3-3 in 150 mM NaCl.

The increased stability of RNA−DNA hybrid helices with the cationicity of the polyamines suggests that the overriding

factor in polyamine−hybrid helix interaction is electrostatic in origin. Bloomfield and colleagues (*51*−*53*) have successfully applied the counterion condensation theory, developed by Manning (*54*) and Record et al. (*55*), in theoretically interpreting polyamine−DNA interactions, especially as it applies to DNA condensation. According to this theory, polyamines are considered to be structureless point charges that form an ionic cloud around DNA and shield the negative charges of the phosphate groups, thereby reducing the repulsion between the phosphate groups. Since site-specific interactions are not envisioned as a part of this formalism, all isovalent molecules should behave similarly, irrespective of the geometric structure of these molecules. However, results of this study using a series of spermidine and spermine analogues show significant structural effects of these polyamines in stabilizing RNA−DNA hybrids. In both series, the natural polyamines (spermidine and spermine) have the highest stabilizing effect, indicating a natural selection process for synthesizing molecules ideally suited for interacting with DNA and RNA−DNA helices.

Several crystallographic studies have provided evidence for the interaction of spermine with DNA in its B, A, and Z conformations. The Drew−Dickerson model shows that the spermine is bound to the upper end of the major groove of the B-DNA dodecamer, d(CGCGAATTCGCG) duplex (*56*). The pendant NH_3^+ groups are bound to the nucleic acid phosphates, while one of the two NH_2^+ groups forms a hydrogen bond to a guanine O6 and, via a bridging water molecule, to a cytosine N4 on the next base pair down. It is also reported that spermine could assume different conformations yet form extensive van der Waals contacts with the same hydrophobic zone in a sequence-specific manner (*57*). Recently, the crystal structure of spermine bound to a distorted B-DNA hexamer was reported in which the spermine was bound across a narrowed minor groove rather than being embedded within it (*58*). Jain et al. (*59*) found extensive amine−phosphate interactions in B-DNA, whereas A-DNA lacked any such hydrogen bonding interactions. However, extensive hydrophobic contacts between the CH_2 groups and the nucleic acid bases, particularly thymine methyl groups, were seen, suggesting that hydrophobic interaction is a more important factor in the binding of polyamines with A-form DNA than with B-form DNA. In more recent studies, Wahl and Sundaralingam (*60*) found that spermine binds exclusively to either of the grooves, or to the phosphate groups of the backbone, or exhibits a mixed binding mode in A-DNA. However, Verdaguer et al. (*61*) found that the two nitrogen atoms in one-half of the spermine molecule are bound inside the major groove, while the two other nitrogen atoms are bound to phosphate groups across the borders of this groove of A-DNA. In the left-handed Z-DNA hexamer, d(CGCGCG), spermine was localized along the edge of the deep groove and also spanning the entrance to the groove (*62*). Theoretical studies (*63*) further suggested that the level of binding of spermine to A-form DNA is ∼1.5-fold higher than that to B-DNA.

X-ray crystallographic analysis of spermine binding with yeast phenyl alanyl tRNA shows that one of the two strongly bound spermine is located at the major groove of the double-helical region, while the other is in a region where the polynucleotide chain turns sharply (*64*). NMR studies of the binding of putrescine, spermidine, and spermine to tRNA also point out that polyamines cannot be simply considered simple organic cations (*65*). Although the primary amino group (NH_3^+) charge density is higher, Frydman et al. (*65*) found that secondary amino groups (NH_2^+) bind with higher affinity than primary amino groups. Taken together, the mode of binding of spermine to DNA is difficult to define, and sites within both the major and minor grooves of nucleic acids might be involved, depending on the nucleic acid sequence and conformation, as well as the number of methylene groups separating the primary and secondary amino groups of polyamines, and the number and nature of alkyl substitution on the nitrogen.

Structural specificity effects of polyamines in RNA−DNA hybrid stability are clearly evident from our studies with spermidine and spermine homologues (Figures 5−8). In both homologous series, compounds differing by one methylene group had a negligible effect on ΔT_m compared to that of spermidine and spermine. A decrease of two methylene groups, as in 3-2 and 3-2-3, had a dramatic effect in reducing the ability of polyamines to stabilize RNA−DNA helices. This effect is partly due to the reduced level of protonation of 3-2 and 3-2-3 at the buffer pH of 7.4. For example, the reported pK_a values for 3-2-3 are 10.7, 10.0, 8.5, and 5.8, suggesting that this molecule might act as a triamine under the conditions of our experiment (pH 7.4) (*66*). In contrast, the pK_a values of spermine are 10.9, 10.1, 8.8, and 7.9, while those of spermidine are 10.8, 9.8, and 8.4 (*67*). The higher homologues of spermidine and spermine have pK_a values close to or higher than that of spermidine and spermine because of the increased charge separation (*66*, *67*). Thus, the observed reduction in the hybrid stabilizing effect of the higher homologues reflects structural specificity effects of polyamines.

Bis(ethyl) derivatives of polyamines are an emerging group of cancer chemotherapeutic agents and are believed to substitute for natural polyamines in some of their functions (*42*). Although the hybrid stabilizing potential of bis(ethyl) derivatives is relatively lower than that of the unsubstituted polyamines, their effects are potentially important, because they have the added advantage of acting as cell growth inhibitors. These compounds are easily transported by cancer cells utilizing the polyamine transporter(s) (*68*). They are known to deplete the intracellular level of natural polyamines, and disrupt the cellular functions of polyamines, including their ability to modulate gene regulatory protein−DNA interactions (*69*, *70*). Thus, RNA−DNA hybrid stabilization by bis(ethyl)polyamines, albeit the extent is lower than that of spermidine and spermine, may lead to synergistic cell growth inhibition by utilizing multiple targets.

The ion competition experiments (Figure 9) show distinct differences in the binding of spermine with RNA−PODNA and RNA−PSDNA in the presence of Na^+ ions, although both hybrids have similar T_m values in the presence of 150 mM Na^+ alone. The relative binding affinities of both spermidine and spermine homologues toward both hybrid helices were also similar (Table 7). The reduction of T_m with increases in Na^+ concentration at a constant polyamine concentration was previously observed for duplex DNA stabilization (*34*). An explanation for this phenomenon has been provided on the lines suggested by Manning (*54*) Record et al. (*55*, *71*) for divalent cations in the presence of

excess univalent cation. The duplex or hybrid is stabilized relative to the single strands by counterion condensation. The polyamines, which are more stabilizing than the univalent cations, dominate the condensation at low Na^+ concentrations, but are driven off as the Na^+ concentration increases. At the same time, there is a weaker destabilization by the diffuse Debye−Hückel ion atmosphere composed predominantly of univalent ions. The balance of these complex interactions results in an initial decrease in T_m with Na^+ concentration, reaching a minimum followed by a rise as the univalent cation comes to dominate the condensation. The differential effect of Na^+ on spermine−RNA−PODNA and spermine−RNA−PSDNA hybrid complexes further suggests different modes of spermine binding between the phosphodiester and phosphorothioate ODNs. This result is in agreement with previous findings showing a destabilizing effect of phosphorothioate-modified ODNs in DNA−DNA and DNA−RNA duplex stabilization (*17*, *72*). Musso and van Dyke (*50*) also found a similar effect on triplex DNA stabilization. These differences may arise from a combination of factors, such as entropy of single strands, water structure around the backbone, and altered stacking interactions. In a recent spectroscopic and thermodynamic analysis, Clark et al. (*73*) found that the destabilizing effect of the phosphorothioate substitution might arise from a difference in entropy upon forming the S-DNA−S-DNA duplexes, while the destabilizing effect in the S-DNA−RNA hybrids appeared to come from a difference in enthalpy.

Conformational studies suggest that RNA−DNA hybrids adopt a structure close to the A-form DNA even in the absence of polyamines. Significant structural changes were observed only at low polyamine concentrations. Hung at al. (*26*) compared the CD spectra of RNA−PODNA and RNA−PSDNA hybrids and found similarity in the spectra at wavelengths above 220 nm. Lesnik et al. (*25*) found a correlation between the ellipticity at the 210 nm band and hybrid stability (*25*). In the work presented here, although a correlation was observed between the intensity of the 210 nm band and the spermine-mediated stability of the RNA−PODNA hybrid, such a correlation was not observed with the RNA−PSDNA hybrid, albeit the similarities in spectral changes at higher wavelengths. These differences may be due to microstructural differences in the local conformation of the hybrid constituted from phosphodiester and phosphorothioate oligodeoxyribonucleotides.

In summary, our results show a remarkable stabilizing effect of polyamines on RNA−DNA hybrid helices, and identified pentamine and hexamine analogues of spermine as better hybrid stabilizing agents than natural polyamines. This study also demonstrated the structural specificity effects of isovalent polyamine analogues in stabilizing hybrid helices, suggesting the importance of site-specific interactions in a model polyamine−hybrid helix system. A detailed understanding of the ionic and structural requirements of polyamines for hybrid stabilization may be useful in the design and development of novel polyamine-based ligands for improving the efficiency of antisense applications. This line of research may also provide new insights into the biochemical events involved in the formation of hybrid helices, and advance our understanding of the mechanism of polyamine−nucleic acid interactions.

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BI990180T