Antisense Recognition of the HER-2 mRNA: Effects of Phosphorothioate Substitution and Polyamines on DNA·RNA, RNA·RNA, and DNA·DNA Duplex Stability†

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ABSTRACT: The HER-2 gene is overexpressed in a subset of breast, ovarian, lung, and pancreatic cancers. Antisense oligonucleotides suppress gene expression depending on the stability of the DNA·RNA hybrids formed at the target site. Polyamines, the cellular cations that interact with DNA and RNA, may influence hybrid stability in the cell. Therefore, we studied the ability of natural polyamines (putrescine, spermidine, and spermine) and a series of their structural analogues to stabilize DNA·RNA and RNA·RNA duplexes using melting temperature (T_m) measurements and circular dichroism (CD) spectroscopy. Phosphodiester (PO) and phosphorothioate (PS) oligonucleotides (ODNs) (15 nucleotides, 5′-CTCCATGGTGCTCAC-3′) targeted to the initiation codon region of the HER-2 mRNA, and complementary RNA and DNA ODNs, were used in this study. The relative order of thermal stability was as follows: RNA·RNA > PO−DNA·RNA > PO−DNA·PO−DNA > PS−DNA·RNA > PS−DNA·PO−DNA > PS−DNA·PS−DNA. The ability of polyamines to stabilize the duplexes improved with the cationicity of the polyamine, with hexamines being more effective than pentamines, which in turn were more effective than tetramines and triamines. However, chemical structural effects were clearly evident with isovalent homologues of spermidine and spermine. CD spectra showed B and A conformations, respectively, for the DNA and RNA helices. DNA·RNA hybrids adopted an intermediate structure between the B and A forms. These data help us to understand the role of endogenous polyamines in DNA·RNA hybrid stabilization, and provide information for designing novel polyamines to facilitate the use of antisense ODNs for controlling HER-2 gene expression.

Human epidermal growth factor receptor-2 (HER-2), a proto-oncogene, is amplified and overexpressed in 20−30% of breast tumors (*1*, *2*). The HER-2 positive status is associated with invasive disease, resistance to traditional therapeutic strategies, an increased relapse rate, and decreased survival. Herceptin is a humanized monoclonal antibody targeted to the HER-2/*neu* (*neu* being the rat equivalent of the human gene) gene product, p185 (*3*). Herceptin is used alone or in combination with chemotherapy to treat HER-2-overexpressing breast tumors, although there are reports of treatment-related cardiotoxicity (*4*, *5*). Several laboratories are investigating the use of sequence-specific ODNs[1] to inhibit the transcription and translation of the HER-2 gene (*6*−*8*).

Antisense ODNs refer to single-stranded DNA or RNA motifs that prevent the expression of a target gene by interfering with the translation of the mRNA (*9*). Some of the proposed mechanisms of action of antisense ODNs are (i) physical inhibition of the translation machinery, (ii) inhibition of the binding of translational factors, and (iii) cleavage of the RNA in the DNA·RNA hybrid by RNAseH,

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[1] Abbreviations: ODN, oligonucleotide; PS, phosphorothioate; PO, phosphodiester; T_m, melting temperature; BE, bis(ethyl); 3-4, spermidine; 3-4-3, spermine; 3-2, 1,6-diamino-3-azahexane; 3-3, 1,7-diamino-4-azaheptane; 3-5, 1,9-diamino-4-azanonane; 3-7, 1,11-diamino-4-azaundecane; 3-8, 1,12-diamino-4-azadodecane; 3-2-3, 1,10-diamino-4,7-diazadecane; 3-3-3, 1,11-diamino-4,8-diazaundecane (norspermine); 3-5-3, 1,13-diamino-4,10-diazatridecance; 3-6-3, 1,14-diamino-4,11-diazatridecance; 3-7-3, 1,15-diamino-4,11-diazapentadecane; 3-8-3, 1,16-diamino-4,13-diazahexadecane; 3-3-3-3, 1,15-diamino-4,8,12-triazapentadecane; 3-4-4-3, 1,17-diamino-4,8,14-triazaheptadecane; 3-3-3-3-3, 1,19-diamino-4,8,12,16-tetraazanonadecane; 3-4-3-4-3, 1,21-diamino-4,9,13,18-tetraazahenicosane.

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an enzyme that digests the RNA strand bound to the ODN (*10*). However, several practical difficulties have to be overcome to realize the potential benefits of antisense ODNs. These barriers include degradation of the ODN by nucleases, inefficient cellular uptake of the ODN and its nuclear transport, competition between specific target sites and nonspecific sites, and the stability of the DNA·RNA hybrid under cellular ionic and pH conditions (*11*−*14*). There have been many attempts to overcome these difficulties with varying degrees of success (*15*−*17*).

One of the most efficient structural modifications for increasing the nuclease resistance of ODNs is the replacement of an oxygen atom with a sulfur atom in the DNA backbone (*18*). The resulting phosphorothioate (PS) ODNs retain sequence-specific base pairing and are more resistant to cellular phosphodiesterases compared to phosphodiester (PO) ODNs, and elicit RNaseH activity when complexed with RNA (*14*, *15*). However, the thermodynamic stability of the hybrids formed from PS-bound ODNs (PS−ODNs) is lower than that formed from the PO-bound ODNs (PO−ODNs) (*19*). This is a major disadvantage of PS−ODNs, as effective antisense activity depends on the stability of the DNA·RNA hybrids *in vivo*. Positively charged counterions might help to stabilize these DNA·RNA hybrids by neutralizing the negative charge on the DNA or RNA phosphate groups. Among the positively charged molecules present in the cellular environment, polyamines {putrescine [$H_2N(CH_2)_4NH_2$], spermidine [$H_2N(CH_2)_3NH(CH_2)_4NH_2$] and spermine [$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$]} are unique in their prevalence and function (*20*). Polyamines stabilize DNA and RNA duplexes and DNA·RNA hybrids (*21*−*23*). However, a systematic investigation of the effectiveness of polyamines on the stability of DNA·RNA hybrids, especially those containing therapeutically important antisense oligonucleotides, is lacking at present.

We studied the ionic and structural effects of polyamines on DNA·RNA hybrid stability using melting temperature (T_m) measurements. Several novel polyamine analogues were synthesized, and their ability to alter the thermal helix−coil transition temperature of the duplexes was determined. The DNA part of the DNA·RNA hybrid is either a PS− or a PO−ODN. Our results show that polyamines can stabilize DNA·RNA hybrids in a concentration- and structure-dependent manner.

MATERIALS AND METHODS

Polyamines. Putrescine·2HCl, spermidine·3HCl (3-4), and spermine·4HCl (3-4-3) were purchased from Sigma Chemical Co. (St. Louis, MO). The structural homologues and analogues of spermidine and spermine were synthesized by procedures described previously (*24*−*27*). All polyamines are abbreviated by a numbering system, representing the number of methylene (CH_2) groups between the primary and secondary amino groups. For example, 3-4 is $NH_2(CH_2)_3NH(CH_2)_4NH_2$. Synthetic polyamines used in this study were 1,6-diamino-3-azahexane (3-2), 1,7-diamino-4-azaheptane (3-3), 1,9-diamino-4-azanonane (3-5), 1,11-diamino-4-azaundecane (3-7), 1,12-diamino-4-azadodecane (3-8), 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-diazatridecane (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), 1,17-diamino-4,8,14-triazaheptadecane (3-4-4-3), 1,19-diamino-4,8,12,16-tetraazanonadecane (3-3-3-3-3), 1,21-diamino-4,9,13,18-tetraazahenicosane (3-4-3-4-3), bis(ethyl)norspermine (BE-3-3-3), 1,15-bis(ethylamino)-4,8,12-triazapentadecane (BE-3-3-3-3), and bis(ethyl)spermine (BE-3-4-3). The purity of synthetic compounds was determined by elemental analysis, NMR, HPLC, and mass spectrometry. The chemical structures of natural and synthetic polyamines are shown in Figure 1. Polyamine analogue stock solutions (50 mM) were prepared in sterile, double-distilled water and appropriate dilutions made in 10 mM sodium cacodylate buffer [10 mM sodium cacodylate (pH 7.4) and 0.5 mM EDTA] prior to use.

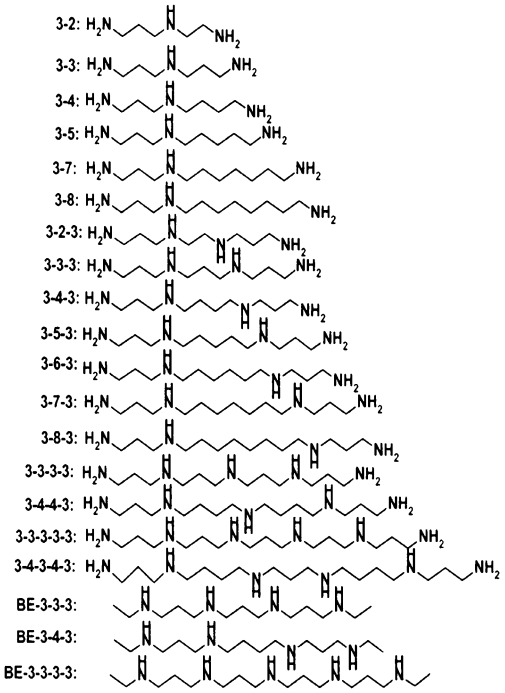

FIGURE 1: Chemical structures of the natural and synthetic polyamines used in this study.

Oligonucleotides. All ODNs were purchased from Oligos, Etc. (Wilsonville, OR). These were HPLC/gel purified and characterized by gel electrophoresis and/or capillary electrophoresis. The sequence of the ODNs used in this study is presented in Table 1. The RNA ODN is a part of the initiation codon of the HER-2 mRNA. The ODNs were dissolved in 10 mM sodium cacodylate buffer and aliquots stored at −70 °C until they were used. At the time of experiment, aliquots were thawed and absorbance was measured at 260 nm using a Beckman DU640 spectrophotometer.

DNA·RNA, DNA·DNA, and RNA·RNA duplexes were prepared by mixing complementary ODNs in a 1:1 molar ratio in 10 mM sodium cacodylate buffer in the presence or absence of appropriate concentrations of polyamines. For studies on the effect of the increased salt concentration on the stability of the hybrids, 140 mM NaCl was added to 10 mM sodium cacodylate buffer or 150 mM KCl was added to 1 mM sodium cacodylate buffer. The duplex/polyamine mixtures were vortexed for 10 s, heated in a boiling water bath for 10 min, allowed to equilibrate at room temperature (22 °C), and left at this temperature to attain equilibrium for 16 h before T_m measurements were taken.

Table 1: Nucleotide Sequences of ODNs Used in This Study

oligonucleotide	nucleotide sequence[a]	extinction coefficient (M^{-1} cm^{-1})
antisense PO−DNA/PS−DNA	5′-CTCACTGGTGCTCAC-3′	130 300
antisense RNA	5′-CUCCAUGGUGCUCAC-3′	126 100
complementary PO−DNA/PS−DNA	5′-GTGAGCACCATGGAG-3′	151 400
complementary RNA	5′-GUGAGCACCAUGGAG-3′	148 800
complementary RNA (mismatch 1)	5′-GUGAGCCCCAUGGAG-3′	143 600
complementary RNA (mismatch 2)	5′-GUGAGCCACAUGGAG-3′	148 800
complementary RNA (mismatch 3)	5′-GUGAGCCAGAUGGAG-3′	152 550

[a] The underlined bases in the RNA strands indicate mismatch bases, compared to the antisense DNA.

T_m Measurements. T_m measurements were carried out as described previously (*22*, *24*). The T_m block contained six cells, each with a volume of ~0.35 mL, of which the first was filled with buffer and used as a blank. The measurements were carried out at a heating rate of 0.5 °C/min, using a Beckman high-performance temperature controller, with the absorbance and temperature recorded every 30 s. The helix−coil transition temperature, which corresponds to half-dissociation of the duplex, was calculated as the T_m after pre- and post-transitional baselines had been constructed, corresponding to 100 and 0% hybrid, respectively (*22*). T_m values are the mean of three ot four measurements with a reproducibility within 1 °C.

Circular Dichroism (CD) Measurements. The CD spectra were recorded on an Aviv model 62DS circular dichroism spectrophotometer (Aviv Associates, Lakewood, NJ), fitted with a five-cell thermally regulated cell holder, and cells with a path length of 1 cm. The molar ellipticity was calculated using the equation $[\theta] = \theta/cl$, where θ is the observed ellipticity, c is the molar concentration of the ODN, and l is the path length. CD spectra were recorded in three separate experiments, and representative spectra are presented.

RESULTS

Effects of Spermine on the Stability of DNA·RNA, DNA·DNA, and RNA·RNA Helices. Figure 2 shows the melting profiles of duplex helices in the absence and increasing concentrations of spermine. The dissociation of the duplexes from single strands was seen as an increase in the absorbance at 260 nm, as the temperature increased from 20 to 90 °C. Compared to the other helices, the PO−DNA·PO−DNA and PS−DNA·RNA helices exhibited sharp cooperative melting transitions as the temperature increased. The DNA·RNA hybrid containing PS−DNA exhibited a sharper transition than the PO−DNA·RNA hybrid, indicating a highly co-operative transition.

A concentration-dependent increase in the T_m of the PO−DNA·RNA helix was observed with increasing concentrations of spermine. The PO−DNA·RNA hybrid had a T_m of 42.5 ± 0.5 °C in the absence of spermine (Table 2). Addition of 100 μM spermine increased the T_m of the hybrid to 73.9 °C. To measure the effectiveness of spermine in increasing the T_m, we further determined $dT_m/d(\log[\text{spermine}])$ by plotting T_m against log[spermine] (Figure 3). The $dT_m/d(\log[\text{spermine}])$ value for the PO−DNA·RNA hybrid was 13 (Table 4). This value is a quantitative measure of the ability of spermine to stabilize the hybrid.

The role of cationicity of polyamines in the stability of the PO−DNA·RNA hybrid was next studied by measuring the T_m in the presence of different polyamines from the diamine, putrescine to the hexamine, 3-3-3-3-3, and the results are presented in Table 2. This table also presents the ΔT_m values (difference in T_m at 100 μM polyamine and T_m in the absence of polyamine) to indicate the effectiveness of different polyamines in increasing the T_m of the PO−DNA·RNA hybrid. Among the polyamines, hexamines had the strongest hybrid stabilizing ability, followed by pentamines and tetramines. While 100 μM pentamines and hexamines stabilized the hybrid with a T_m of ~78 °C, the T_m of the hybrid helix was only 52 °C in the presence of 100 μM putrescine. Thus, the relative hybrid stabilization effect of polyamines followed this order: hexamines > pentamines > tetramine > triamine > diamine.

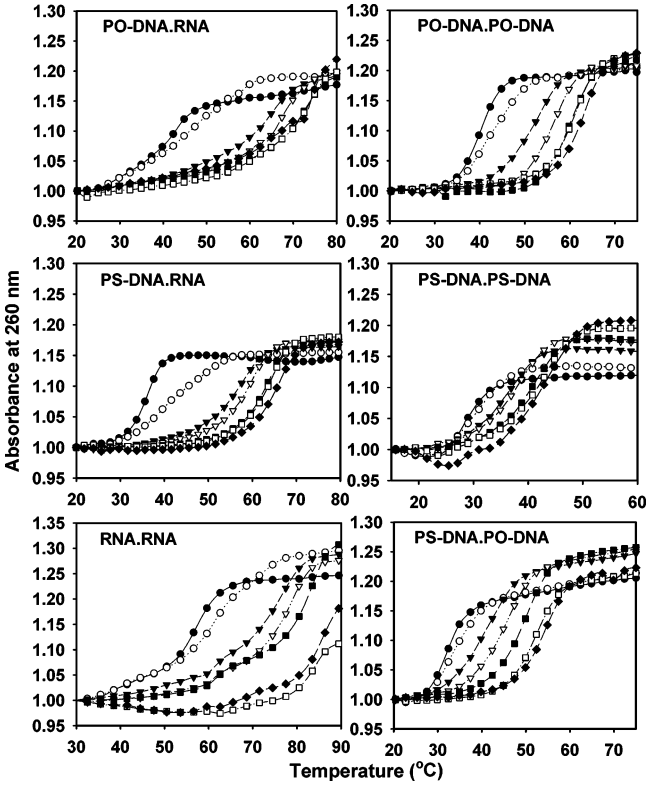

FIGURE 2: Melting profile of the PO−DNA·RNA, PS−DNA·RNA, PO−DNA·PO−DNA, PS−DNA·PS−DNA, PS−DNA·PO−DNA, and RNA·RNA helices. The concentrations of spermine that were used were 0 (●), 1 (○), 5 (▼), 10 (▽), 25 (□), 50 (■), and 100 μM (◆). The T_m measurements were carried out in 10 mM cacodylate buffer at a heating rate of 0.5 °C/min for all the hybrid helices.

The effect of polyamines on the stability of PS−DNA·RNA hybrid is summarized in Table 3. Figure 2B depicts the melting profile of the hybrid at different concentrations of spermine. The T_m value of the PS−DNA·RNA hybrid was ~10 °C lower than that of the PO−DNA·RNA hybrid. As in the case of the PO−DNA·RNA hybrid, a concentra-

Table 2: Effect of Polyamines on the Stability of the PO−DNA·RNA Hybrid

[polyamine] (μM)	T_m (°C)[a]						
	Put[b]	Spd[b]	Spm[b]	3-4-4-3[b]	3-3-3-3[b]	3-4-3-4-3[b]	3-3-3-3-3[b]
0	42.7	42.9	43.1	42.7	42.2	42.0	42.0
1	42.5	45.9	46.8	43.1	42.2	42.4	42.0
5	43.7	54.2	64.9	64.3	64.7	64.6	64.2
10	44.5	59.2	68.9	73.6	71.9	70.9	66.5
25	46.9	63.7	72.3	74.8	75.9	76.6	75.2
50	51.1	65.2	73.0	75.7	75.3	78.7	76.7
100	52.4	68.4	73.9	75.1	78.1	78.7	78.7
ΔT_m[c]	9.7	25.5	30.8	32.4	35.9	36.7	36.7

[a] The T_m measurements were conducted in a buffer containing 10 mM sodium cacodylate (pH 7.4) and 0.5 mM EDTA. The reproducibility of the T_m values was ±1 °C for three or four measurements. [b] Abbreviations: Put, putrescine; Spd, spermidine; Spm, spermine. Synthetic polyamines are abbreviated by a number system indicating the number of methylene ($-CH_2-$) groups between the primary and secondary amino groups. [c] $\Delta T_m = T_{m(100\ \mu M)} - T_{m(0)}$, where $T_{m(100\ \mu M)}$ is the T_m of the PO−DNA·RNA hybrid in the presence of 100 μM polyamine and $T_{m(0)}$ is the T_m of the hybrid in 10 mM sodium cacodylate buffer, in the absence of polyamine.

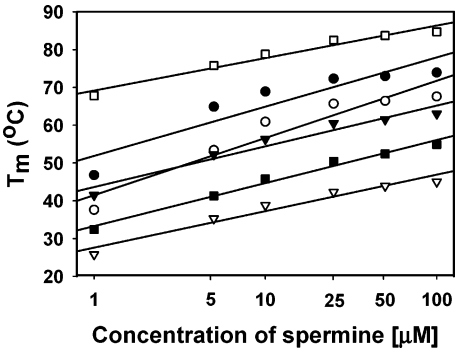

FIGURE 3: Plots of T_m vs log[spermine] showing the ability of spermine to increase the T_m of the PO−DNA·RNA (●), PS−DNA·RNA (○), PO−DNA·PO−DNA (▼), PS−DNA·PS−DNA (▽), PS−DNA·PO−DNA (■), and RNA·RNA hybrids (□).

Table 3: Effect of Polyamines on the Stability of the PS−DNA·RNA Hybrid

[polyamine] (μM)	T_m (°C)[a]						
	Put[b]	Spd[b]	Spm[b]	3-4-4-3[b]	3-3-3-3[b]	3-4-3-4-3[b]	3-3-3-3-3[b]
0	33.1	33.4	33.6	33.6	34.0	33.5	33.5
1	34.9	37.8	37.6	33.9	34.0	33.0	33.5
5	35.6	46.4	53.4	54.7	58.9	55.0	55.0
10	36.8	54.3	60.9	65.2	67.4	56.0	57.7
25	38.8	57.1	65.7	67.9	69.4	69.0	68.7
50	40.8	58.4	66.4	68.1	70.2	69.1	70.7
100	44.4	62.2	67.6	68.2	70.2	69.7	70.4
ΔT_m[c]	11.3	28.8	34.0	34.6	36.2	36.2	36.9

[a] The T_m measurements were conducted in a buffer containing 10 mM sodium cacodylate (pH 7.4) and 0.5 mM EDTA. The reproducibility of the T_m values was ±1 °C for three or four measurements. [b] Abbreviations: Put, putrescine; Spd, spermidine; Spm, spermine. Synthetic polyamines are abbreviated by a number system indicating the number of methylene ($-CH_2-$) groups between the primary and secondary amino groups. [c] $\Delta T_m = T_{m(100\ \mu M)} - T_{m(0)}$, where $T_{m(100\ \mu M)}$ is the T_m of the PS−DNA·RNA hybrid in the presence of 100 μM polyamine and $T_{m(0)}$ is the T_m of the hybrid in 10 mM sodium cacodylate buffer, in the absence of polyamine.

tion-dependent increase in the T_m was observed with increasing concentrations of polyamines. The $dT_m/d(\log[\text{spermine}])$ value was 15.1 (Table 4). A ΔT_m of 34 °C was observed in the presence of 100 μM spermine (Table 3). The order of efficacy of polyamines in stabilizing this hybrid was similar to that of the PO−DNA·RNA hybrid.

Table 4: Slope Values of $dT_m/d(\log[\text{Spermine}])$ of the Different Hybrids

type of hybrid	$dT_m/d(\log[\text{spermine}])$[a]
PO−DNA·RNA	13.04
PS−DNA·RNA	15.1
PO−DNA·PO−DNA	10.7
PS−DNA·PO−DNA	11.3
PS−DNA·PS−DNA	9.6
RNA·RNA	8.59

[a] $dT_m/d(\log[\text{spermine}])$ values were determined from the slopes of T_m vs log[spermine] plots (Figure 3).

To evaluate the effect of antisense ODN binding to mismatch RNA targets, we conducted T_m measurements on PO−DNA and PS−DNA ODNs hybridized to RNA ODNs with one, two, or three mismatch nucleotides. (These hybrids are represented in the text as PO−DNA/PS−DNA·RNA M1, PO−DNA/PS−DNA·RNA M2, or PO−DNA/PS−DNA·RNA M3, respectively.) In the absence of any polyamine, the T_m values of PO−DNA·RNA M1, PO−DNA·RNA M2, and PO−DNA·RNA M3 hybrids were 28.4, 23.7, and 21.7 °C, respectively, compared to a T_m value of 42.5 °C for the PO−DNA·RNA hybrid without any mismatch base pairs. In the presence of 100 μM spermine, T_m values of the three mismatch hybrids were 58.7, 53.8, and 47.8 °C, respectively. However, the T_m values were 15, 20, and 26 °C, respectively, lower than that of the PO−DNA·RNA hybrid without any mismatch in the presence of 100 μM spermine. The T_m value of PS−DNA·RNA M1 in the absence of spermine was 25 °C, and the T_m increased to 51.4 °C in the presence of 100 μM spermine. However, this increase in the T_m was 16.2 °C smaller than that of the PS−DNA·RNA hybrid without any mismatch [T_m = 67.6 °C (Table 3)]. There was no cooperative melting profiles in the case of PS−DNA·RNA M2 and PS−DNA·M3. Taken together, these results show that the hybrid stability was reduced remarkably with the introduction of a mismatch base in the target RNA, and the increase in T_m of mismatch hybrids is much lower than that of the hybrid with perfectly complementary bases.

We next conducted T_m measurements of PO−DNA·PO−DNA, PS−DNA·PS−DNA, PS−DNA·PO−DNA, and RNA·RNA duplexes in the presence of putrescine, spermidine, and spermine. Figure 2 shows the melting profiles of these duplexes in the presence of increasing concentrations of spermine. There was a 14 °C reduction in the T_m of the PS−DNA·PS−DNA duplex compared to the T_m of the PO−DNA·PO−DNA hybrid (Table 5). The T_m value of the PS−DNA·PS−DNA hybrid (26.5 °C) was also significantly lower than that of the PS−DNA·RNA hybrid (33.6 °C). The PS−DNA·PO−DNA helix, which comprised the modified antisense ODN and its complementary sequence, had a T_m intermediate between the T_m of PO−DNA·PO−DNA and PS−DNA·PS−DNA hybrids.

As in the case of other helices, 100 μM spermine increased the T_m of the PS−DNA·PO−DNA hybrid from 32 to 54.9 °C. The RNA duplex had a higher T_m value than that of the DNA·DNA duplex and DNA·RNA hybrid (Figure 2 and Table 6). The stability of the RNA duplex increased with spermine in a concentration-dependent manner, with a ΔT_m of 27.1 °C at 100 μM spermine. The pentamine 3-4-4-3 and

Table 5: Effect of Polyamines on the Stability of the PO−DNA·PO−DNA, PS−DNA·PS−DNA, and PS−DNA·PO−DNA Hybrids

[polyamine] (μM)	T_m (°C)[a]								
	PO−DNA·PO−DNA			PS−DNA·PS−DNA			PS−DNA·PO−DNA		
	Put[b]	Spd[b]	Spm[b]	Put[b]	Spd[b]	Spm[b]	Put[b]	Spd[b]	Spm[b]
0	39.9	40.1	40.5	26.0	26.5	25.8	32.3	32.1	31.9
1	41.2	41.3	41.5	26.0	27.4	25.8	32.3	33.0	32.4
5	42.0	46.6	52.2	26.0	31.0	35.3	32.9	37.9	41.3
10	42.5	49.8	56.3	26.5	33.0	38.8	32.9	40.4	45.8
25	43.9	53.8	60.5	27.0	37.4	42.3	34.3	44.9	50.4
50	45.9	56.5	61.5	29.9	40.4	43.9	36.4	47.4	52.4
100	48.4	59.5	63.0	32.4	42.9	45.0	38.9	50.4	54.9
ΔT_m[c]	8.5	19.4	22.5	6.4	16.4	19.2	6.6	18.3	23

[a] The T_m measurements were conducted in a buffer containing 10 mM sodium cacodylate (pH 7.4) and 0.5 mM EDTA. The reproducibility of the T_m values was ±1 °C for three or four measurements. [b] Abbreviations: Put, putrescine; Spd, spermidine; Spm, spermine. Synthetic polyamines are abbreviated by a number system indicating the number of methylene ($-CH_2-$) groups between the primary and secondary amino groups. [c] $\Delta T_m = T_{m(100\ \mu M)} - T_{m(0)}$, where $T_{m(100\ \mu M)}$ is the T_m of the various DNA·DNA duplexes in the presence of 100 μM polyamine and $T_{m(0)}$ is the T_m of the hybrid in 10 mM sodium cacodylate buffer, in the absence of polyamine.

Table 6: Effect of Polyamines on the Stability of the RNA·RNA Duplex

[polyamine] (μM)	T_m (°C)[a]				
	Put[b]	Spd[b]	Spm[b]	3-4-4-3[b]	3-4-3-4-3[b]
0	56.7	56.9	57.6	56.3	56.8
1	57.7	58.8	67.8	74.6	71.8
5	58.7	67.8	75.8	79.7	78.7
10	59.7	70.9	78.8	83.2	84.2
25	60.9	74.8	82.5	87.2	ND[d]
50	61.9	76.8	83.7	ND[d]	ND[d]
100	64.9	79.8	84.7	ND[d]	ND
ΔT_m[c]	8.2	22.9	27.1	30.9	27.4

[a] The T_m measurements were conducted in a buffer containing 10 mM sodium cacodylate (pH 7.4) and 0.5 mM EDTA. The reproducibility of the T_m values was ±1 °C for three or four measurements. [b] Abbreviations: Put, putrescine; Spd, spermidine; Spm, spermine. Synthetic polyamines are abbreviated by a number system indicating the number of methylene ($-CH_2-$) groups between the primary and secondary amino groups. [c] $\Delta T_m = T_{m(\text{highest conc})} - T_{m(0)}$, where $T_{m(\text{highest conc})}$ is the T_m of the RNA·RNA duplexes in the presence of the highest concentration of polyamine that was used and $T_{m(0)}$ is the T_m of the hybrid in 10 mM sodium cacodylate buffer, in the absence of polyamine. [d] Not detected. The T_m measurements appeared to be >95 °C and could not be determined in our study.

hexamine 3-4-3-4-3 also increased the T_m of the RNA·RNA duplex in a concentration-dependent manner. We were unable to measure T_m values above a critical concentration (50 μM for 3-4-4-3 and 25 μM 3-4-3-4-3) because the RNA·RNA duplex melted at >95 °C.

The hybrid stabilizing effect of polyamines was also quantified by plotting T_m against log[spermine] (Figure 3). Slope values of these plots, dT_m/d(log[spermine]), can be related to the efficacy of polyamines in stabilizing the hybrids (Table 4). Thus, spermine has the strongest stabilizing effect on PS−DNA·RNA hybrid and the weakest effect on the RNA·RNA duplex.

Effect of Na^+ on the Stability of the DNA·RNA Hybrids and DNA·DNA/RNA·RNA Duplexes. We next determined the effects of polyamines on the T_m of hybrids at a physiologically compatible ionic concentration of 150 mM Na^+ (Table 7). There was a 20 °C increase in the T_m of PO−DNA·RNA hybrids on addition of 140 mM NaCl, as compared to the T_m of the hybrids in 10 mM sodium cacodylate buffer (Tables 2, 3, and 5). In addition, the T_m values of the PO−DNA·RNA hybrid and the PO−DNA·PO-DNA·duplex were nearly identical in the presence of 150 mM NaCl. Putrescine had no major effect on the stability of the duplexes even at a concentration of 1 mM. Spermidine (1 mM) increased the T_m values of PO−DNA·RNA, PS−DNA·RNA, and RNA·RNA helices by 5, 6, and 10 °C, respectively. However, the effect of spermidine on the T_m of DNA duplexes was only marginal (ΔT_m = 2 °C). A similar trend was observed in the presence of spermine. The T_m values of the PO−DNA·RNA hybrid increased by 10 °C in the presence of 1 mM spermine, whereas the ΔT_m was 15 °C for the RNA duplex. Interestingly, spermidine and spermine had a higher stabilizing effect on RNA-containing duplexes compared to that of DNA·DNA duplexes.

Effect of K^+ and Mg^{2+} on the Stability of DNA·RNA Hybrids. Since potassium is the major monovalent cation present within the cells, we also conducted T_m measurements in the presence of 150 mM KCl. The T_m values obtained for the PO−DNA·RNA hybrid in the presence of KCl (60.1 °C) were similar to the T_m values obtained in the presence of 150 mM NaCl (61.5 °C). There was a near-identical trend in the increase in T_m values of the PO−DNA·RNA hybrids in the presence of 150 mM K^+ (72.2 °C) or 150 mM Na^+ (72 °C) in the presence of 1 mM spermine. The T_m value of the PS−DNA·RNA hybrid was 51.2 °C in the presence of 150 mM K^+. In comparison, the T_m value was 54.2 °C in the presence of 150 mM Na^+. Thus, the PS−DNA·RNA hybrid was slightly more stable in the presence of 150 mM Na^+ than in 150 mM K^+.

To determine the effects of physiological concentrations of divalent cations on polyamine-mediated hybrid stability, we conducted T_m measurements of the PO−DNA·RNA hybrid in the presence of 2 mM $MgCl_2$ and 0−1 mM spermine. We obtained a T_m of 65.8 °C in the presence of Mg^{2+} alone. The T_m value increased to 76.8 °C (ΔT_m = 12 °C) upon addition of 1 mM spermine. This result indicates that spermine and Mg^{2+} acted in concert to increase the T_m of the PO−DNA·RNA hybrid. A similar trend was also found for the PS−DNA·RNA hybrid. The T_m of the PS−DNA·RNA hybrid increased from 57.8 °C in the absence of spermine to 69.7 °C in the presence of 100 μM spermine.

Effect of Bis(ethyl) Substitution on the Stability of the DNA·RNA Hybrid. Bis(ethyl)polyamine analogues are being developed as potential cancer therapeutic agents (*28*). We examined how this structural change affected the ability of

Table 7: Effect of Polyamines on the Stability of the Various Hybrids in the Presence of 150 mM Na^+

[polyamine] (mM)	T_m (°C)[a]					
	PO−DNA·RNA	PS−DNA·RNA	PS−DNA·PO−DNA	PO−DNA·PO−DNA	PS−DNA·PS−DNA	RNA·RNA
			Putrescine			
0	62.0	53.4	52.8	61.4	45.8	72.8
0.5	62.5	54.0	53.3	62.9	45.8	74.4
1	63.0	54.4	53.8	62.9	45.8	75.8
ΔT_m[b]	1.0	1.0	1.0	1.5	0	3.0
			Spermidine			
0	61.9	53.4	52.4	60.9	44.9	73.9
0.5	65.0	57.9	53.4	62.0	45.9	78.9
1	67.5	59.5	54.4	63.0	46.9	82.4
ΔT_m	5.6	6.1	2.0	2.1	2.0	8.5
			Spermine			
0	61.5	54.2	52.4	61.4	44.9	72.8
0.5	69.9	63.7	54.3	63.5	46.4	85.3
1	72.0	65.2	55.9	64.9	47.4	87.4
ΔT_m	10.5	11	3.5	3.5	2.5	14.6

[a] The T_m measurements were carried out in 150 mM sodium cacodylate buffer (140 mM NaCl added to 10 mM sodium cacodylate buffer). The reproducibility of the T_m values was ±1 °C for three or four measurements. [b] $\Delta T_m = T_{m(1\ mM)} - T_{m(0)}$, where $T_{m(1\ mM)}$ is the T_m of the various hybrids in the presence of 1 mM polyamine and $T_{m(0)}$ is the T_m of the hybrid in 150 mM sodium cacodylate buffer, in the absence of polyamine.

Table 8: Effect of Polyamines and Their Analogues on the Stability of the PO−DNA·RNA and PS−DNA·RNA Hybrids

[polyamine] (μM)	T_m (°C)[a]											
	3-3-3		BE3-3-3		3-4-3		BE3-4-3		3-3-3-3		BE3-3-3-3	
	PS[c]	PO	PS	PO	PS	PO	PS	PO	PS	PO	PS	PO
0	33.7	42.0	33.5	41.8	33.6	41.8	33.4	42.7	34.0	42.2	33.6	42.1
1	35.0	42.9	35.0	42.7	37.6	46.8	35.3	42.5	34.0	42.2	33.6	42.1
5	55.0	63.9	49.4	60.8	53.4	64.9	50.6	58.4	58.9	64.7	33.5	42.4
10	61.0	67.5	56.4	63.8	60.9	68.9	57.4	63.4	67.4	71.9	34.3	43.4
25	64.0	71.0	61.4	66.8	65.7	72.3	61.4	66.1	69.4	75.9	54.0	62.9
50	66.0	72.5	62.1	68.2	66.4	73.0	62.2	68.5	70.2	75.3	60.2	67.1
100	68.0	74.4	63.6	69.3	67.6	73.9	63.7	70.0	70.2	78.1	64.2	70.8
ΔT_m[b]	34.3	32.4	37.1	27.5	34	32.1	30.3	27.3	36.2	35.9	30.6	28.7

[a] The T_m measurements were conducted in a buffer containing 10 mM sodium cacodylate (pH 7.4) and 0.5 mM EDTA. The reproducibility of the T_m values was ±1 °C for three or four measurements. [b] $\Delta T_m = T_{m(100\ \mu M)} - T_{m(0)}$, where $T_{m(100\ \mu M)}$ is the T_m of the PO−DNA·RNA or PS−DNA·RNA hybrid in the presence of 100 μM polyamine and $T_{m(0)}$ is the T_m of the hybrid in 10 mM sodium cacodylate buffer, in the absence of polyamine. [c] PS and PO refer to PS−DNA·RNA and PO−DNA·RNA hybrids, respectively.

polyamines to stabilize DNA·RNA hybrids. For this purpose, we conducted T_m measurements in the presence of BE-3-3-3, BE-3-4-3, and BE-3-3-3-3 (Table 8). For comparison, we have included the T_m values in the presence of the non-(bisethyl)polyamines in Table 8. We obtained smooth melting profiles when absorbance was plotted against temperature (figures not shown). The ability of bis(ethyl)polyamine analogues to stabilize DNA·RNA hybrids was lower than that of the corresponding non-bisethylated polyamines. For example, T_m values of the PO−DNA·RNA hybrid were 74.4 and 69.3 °C in the presence of 100 μM 3-3-3 and BE-3-3-3, respectively. However, there was no significant difference in the hybrid stabilizing capacity of BE-3-3-3 and BE-3-4-3. Although 3-3-3-3 stabilized the DNA·RNA hybrids to a greater extent than 3-3-3, the stabilizing effects of BE-3-3-3 and BE-3-3-3-3 on hybrid helices were comparable.

Structural Specificity of Spermidine and Spermine Homologues in Stabilizing DNA·RNA Hybrids. To separate charge effects from structural effects of polyamines, we next conducted T_m measurements of PO−DNA·RNA and PS−DNA·RNA hybrids in the presence of isovalent spermidine and spermine homologues. Spermidine homologues had the general structure $H_2N(CH_2)_3NH(CH_2)_nNH_2$, where $n = 2-8$. Figure 4 shows the effects of spermidine homologues on the T_m of these hybrids. Spermidine ($n = 4$) was the most effective trivalent polyamine to stabilize the hybrids, with ΔT_m values of 26 and 20 °C, respectively, for PO−DNA·RNA and PS−DNA·RNA helices at 100 μM spermidine. The lower ($n = 2$ and 3) and higher ($n = 5$, 7, and 8) homologues were less effective than spermidine in stabilizing the hybrids. This result shows that trivalent polyamines exert structural specificity effects on stabilizing DNA·RNA hybrids.

In the next set of experiments, we studied the stabilizing effect of spermine homologues of the general structure $H_2N(CH_2)_3NH(CH_2)_nNH(CH_2)_3NH_2$, where $n = 2-8$, on the T_m of PO−DNA·RNA and PS−DNA·RNA hybrids (Figure 5). We found that the extent of stabilization of the PO−DNA·RNA hybrid by 3-3-3, 3-4-3, and 3-5-3 was comparable, with a T_m value of 74.4 ± 0.1 °C, in the presence of 100 μM homologues. As with spermidine homologues, the lower ($n = 2$) and higher ($n = 7$ and 8) spermine homologues were less effective than spermine in stabilizing the hybrids. This result further confirms the structural specificity effect of isovalent polyamines.

CD Spectra of the Duplexes in the Presence of Spermine. DNA and RNA exist in the B and A conformations, respectively. However, DNA·RNA hybrids are known to

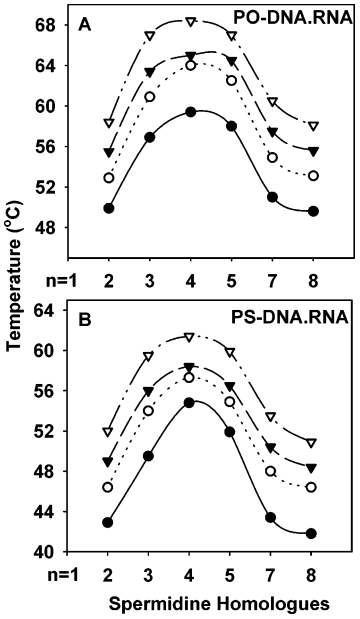

FIGURE 4: (A and B) Structural specificity of spermidine homologues on the PO−DNA·RNA and PS−DNA·RNA hybrid helices, respectively. All T_m measurements were carried out in 10 mM sodium cacodylate buffer (pH 7.4). The concentrations of spermidine homologues were 10 (●), 25 (○), 50 (▼), and 100 μM (▽). *n* is the number of methylene groups on the variable arm of spermidine.

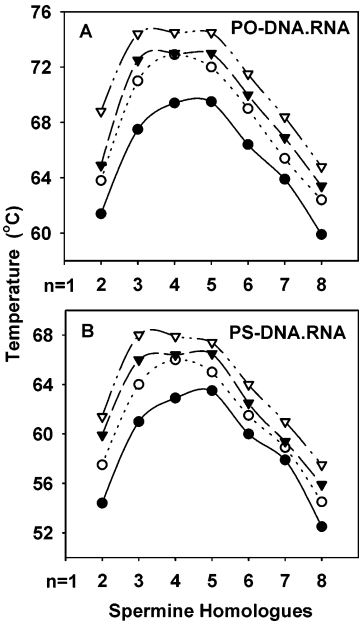

FIGURE 5: (A and B) Structural specificity of spermine homologues on the PO−DNA·RNA and PS−DNA·RNA hybrid helices, respectively. All T_m measurements were carried out in 10 mM sodium cacodylate buffer (pH 7.4). The concentrations of spermine homologues were 10 (●), 25 (○), 50 (▼), and 100 μM (▽). *n* is the number of methylene groups on the variable arm of spermine.

exist in an intermediate state between the B and A forms (*29*, *30*). To examine the effects of polyamines on the conformations of different forms of duplexes, we recorded the CD spectra of the duplexes in the absence and presence of increasing concentrations of spermine (Figure 6). Both the phosphodiester and phosphorothioate DNA duplexes have the conservative B-DNA spectra, with a positive peak at 270 nm and a negative peak at 250 nm. No significant differences

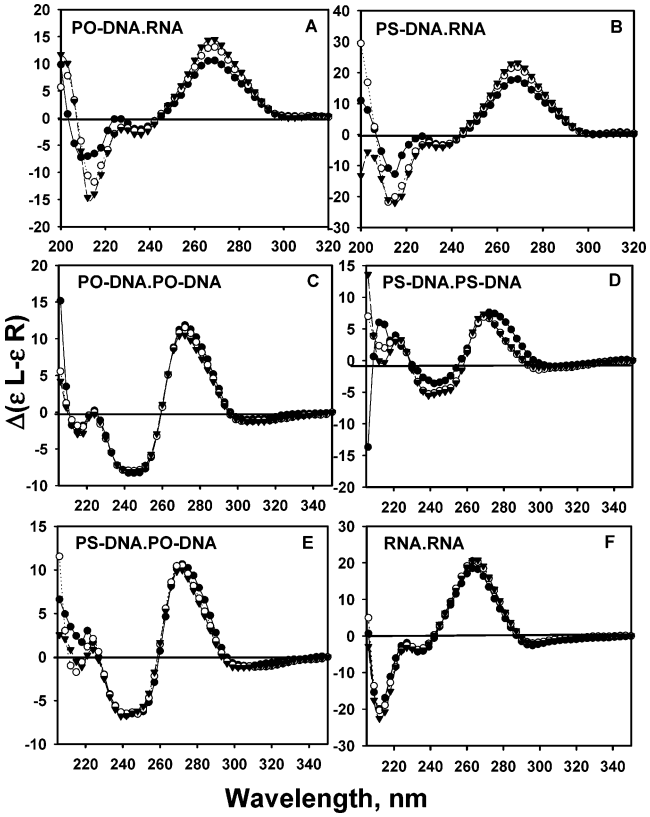

FIGURE 6: (A−F) CD spectra of the PO−DNA·RNA, PS−DNA·RNA, PO−DNA·PO−DNA, PS−DNA·PS−DNA, PS−DNA·PO−DNA, and RNA·RNA hybrids, respectively, in the presence of 0 (●), 10 (○), and 25 μM spermine (▼). All spectra were recorded in 10 mM sodium cacodylate buffer and corrected to the blank. The experiments were repeated at least three times, and representative spectra are shown here.

were found in the CD spectral pattern of either the PO−DNA·PO−DNA or PS−DNA·PO−DNA hybrid in the presence of spermine. The RNA·RNA duplex has the classical A form conformation, with an intense negative band at 210 nm and a large positive band around 260 nm. There was a slight increase in the intensity of the 260 nm peak, suggesting a stabilizing effect of the existing A-DNA conformation by spermine. The RNA hybrids had a conformation closer to the A form (panel F), with a positive peak at ~270 nm. The intensity of the positive peak was ~4-fold higher than that of the negative peak at 230 nm. Addition of spermine slightly increased the positive peak intensity, indicating a stabilization of the A form conformation. In addition, the intensity of the negative band at 210 nm also increased with spermine. A small increase in the intensity of the positive band at 270 nm was also observed in the presence of spermine.

DISCUSSION

Our results show that natural and synthetic polyamines are capable of stabilizing nucleic acid helices formed from a 15-mer antisense ODN targeted toward the initiation codon of the HER-2/neu mRNA. The duplex stabilizing effect of polyamines was observed with DNA, RNA, and hybrid helices. Among the natural polyamines, spermine was most efficient in stabilizing the hybrids. Spermine (100 μM) stabilized the PO−DNA·RNA hybrid with ΔT_m values of 29 and 10 °C in the presence of 10 and 150 mM Na^+,

respectively (Tables 2 and 7). Among the synthetic polyamines, the hexamines with six positive charges exerted stronger duplex stabilizing effect on the PS−DNA·RNA hybrid than pentamines and tetramines. The trend in increasing the T_m is comparable for hybrids containing PO− and PS−DNA counterparts, although the $dT_m/d(\log[\text{spermine}])$ value is higher for the PS−DNA·RNA hybrid (Table 4). The phosphorothioate modification had an adverse effect on the stability of the hybrid in the absence or presence of polyamines (Tables 2, 3, and 5). Our results show lower T_m values of the PS−DNA·PS−DNA duplex in the presence of polyamines, as compared to the T_m values of PS−DNA·RNA or PS−DNA·PO−DNA helices.

We found that the relative stabilities of the duplexes under low-ionic strength conditions (10 mM cacodylate buffer) were in the following order: RNA·RNA > PO−DNA·RNA > PO−DNA·PO−DNA > PS−DNA·RNA > PS−DNA·PO−DNA > PS−DNA·PS−DNA. In the presence of 150 mM NaCl, the thermal stability of the PO−DNA·RNA and PO−DNA·PO−DNA hybrids is comparable. Furthermore, 1 mM spermine had a higher stabilization effect on the PO−DNA·RNA hybrid (ΔT_m = 10.5 °C) compared to that of the PO−DNA·PO−DNA duplex, which exhibited a ΔT_m of only 3 °C. This differential effect suggests that polyamines can be used to stabilize DNA·RNA hybrids, without altering the stability of the genomic DNA in a cell. Previous studies have shown a sequence dependence in the relative stabilities of DNA·DNA, RNA·RNA, and DNA·RNA duplexes (*31*−*33*). Hybrid helices in which the DNA and RNA strands consist of purine and pyrimidine bases, respectively, are less stable than helices containing a purine RNA strand and a pyrimidine DNA strand (*31*, *32*). In studies using random sequences, the observed trend of relative stability is as follows: RNA·RNA > DNA·DNA > DNA·RNA (*33*). In the HER-2 antisense ODN, the DNA strand has 33% purine and 66% pyrimidine, and this ratio is favorable for the formation of stable hybrids with potent biological activity. The stability difference between the RNA duplexes and the other hybrids may be attributed to the presence of a 2′-hydroxyl group in the ribose molecule in RNA and of a C-5 methyl group in the thymine base of the DNA (*34*). NMR studies indicate A·U base pairs of RNA are stronger than A·T base pairs of DNA (*35*).

We tested the specificity of antisense ODN recognition of the target mRNA using mismatch sequences. We observed that duplex stability decreased with an increasing number of mismatches. As the numbers of mismatch base pairs increased from 1 to 3 in the target RNA of the PO−DNA·RNA hybrid, T_m values reduced from 43 to 21.7 °C. Although spermine increased the stability of mismatch hybrids, the T_m values were much lower than that of target DNA·RNA hybrids complexed with spermine. The PS modification further destabilized the hybrids formed with mismatch RNAs.

CD spectra of nucleic acids yield unique signatures that are used to interpret the overall secondary structure of DNA, RNA, and their hybrids (*29*). The CD spectra of PO−DNA·RNA and PS−DNA·RNA hybrids closely resemble each other above 220 nm, indicating that phosphorothioate modification did not affect the conformation of the helix. Our study suggests that DNA·RNA hybrids adopt a conformation close to the A form even in the absence of polyamines, with a characteristic negative band at 210 nm and a broad positive band at 270 nm. The band at 210 nm is a characteristic feature of helices involving RNA (*36*−*39*). A substantial increase in the relative intensity of the negative band at 210 nm was observed with increasing concentrations of spermine, indicating that spermine promoted and/or stabilized the A-DNA conformation of DNA·RNA hybrids. The ability of DNA·RNA hybrids to be cleaved by RNaseH is suggested to arise from the conformation of the hybrid helices rather than the nucleotide sequence (*40*). The markedly different CD spectra of the PS−DNA·PS−DNA duplex might be due to a distortion of its secondary structure, attributed to the steric hindrance or less than optimum stacking interactions between the bases due to the presence of phosphorothioate group (*41*).

Although electrostatic binding plays a predominant role in duplex stabilization, the chemical structure of the polyamine is also important in the degree of stabilization (Table 6 and Figures 4 and 5). Substitution of an ethyl group at the primary amino groups reduced the duplex stabilization effect of polyamines. Since the ethyl substitution changes the size and not the charge of the polyamine, it should not alter electrostatic interaction of the polyamine with DNA. However, ethyl substitution introduces steric hindrance for site-specific interaction, including hydrogen bonding such that the ability of the terminal amino groups to form hydrogen bonds with the functional groups on DNA is hindered. Reduction in the T_m values indicates the importance of the terminal amino groups in stabilizing the duplex.

Chemical structural effects of polyamines on hybrid stabilization are also evident in studies using spermidine and spermine homologues (Figures 4 and 5). The reduced efficacy of 3-2 to stabilize the hybrids can be attributed to its reduced charge (lower pK_a) (*42*). In the case of spermine homologues, it was found that homologues lower than 3-3-3 and higher than 3-5-3 were less efficient in stabilizing hybrids. In the case of higher homologues, increased charge separation by methylene groups results in a molecule with a pK_a value closer to or higher than that of spermidine and spermine. Therefore, the difference in the hybrid stabilizing capacity of the higher homologues of spermidine and spermine reflects the structural specificity of polyamines in their interactions with DNA·RNA hybrids. Structural specificity effects of spermidine and spermine homologues have been previously reported in DNA condensation (*43*) and B-DNA to Z-DNA conformational transitions (*44*).

Molecular modeling and X-ray crystallographic studies indicate site-specific interactions of polyamines with DNA (*45*−*48*). Molecular mechanical calculations indicate binding of spermine to the major groove is energetically more favorable than that to the minor groove. Energy minimization calculations using the $(dG\text{-}dC)_5\cdot(dG\text{-}dC)_5$ model suggest the folding of the major groove of DNA around spermine with the widening of the major groove and compaction of the intrastrand phosphate distances (*45*). Recent molecular modeling studies, using a B-DNA model, indicate that spermine binding is highly mobile with binding sites across major and minor grooves, around the phosphates, and is capable of forming bridges between different helices (*46*). Infrared studies agree with the modeling studies by demonstrating that there could be interstrand attachment at both the major and minor grooves, although the major groove is

the preferred binding site of spermine (*47*). The extent of spermine binding and its exchange with other cations and hydration shell at any particular site depends on several parameters, including DNA sequence, geometry of grooves, and the nature of other cations and anions in the medium (*48*, *49*). Raman spectroscopic studies by different investigators have indicated base-specific as well as electrostatic interactions, independent of base composition (*50*−*52*). While delocalized polyamine electron density diffuses along DNA, the electrostatic potential gradient may interact at a perpendicular plane to the helix axis. The hydration shell around the polar groups of the bases and the phosphate groups make electrostatic and hydrogen bonding contacts with polyamines, retaining the mobility of these molecules around DNA (*48*).

The presence of flexible methylene groups in higher-valence polyamines increases the probability of intrastrand binding, leading to cross-linking between different DNA·RNA strands. Although there is a significant increase in the T_m of the hybrids on addition of polyamines at a physiologically compatible ionic condition of 150 mM Na^+ or 150 mM K^+, this increase was lower than that observed under low-salt conditions. However, polyamines acted in concert with $MgCl_2$ to increase the T_m of the DNA·RNA hybrids. The reduction in the overall stabilizing effect of the polyamines under high-salt conditions can be explained by the counterion condensation theory proposed by Manning (*53*) and Record et al. (*54*). According to this theory, as Na^+ increases, multivalent cations are displaced by Na^+ due to ion competition, thereby reducing the affinity of polyamine for the helices. Complex competition between these interactions and a sensitive balance, maintained by counterion stabilization and weak destabilization of the Debye−Huckel univalent ion atmosphere, is attributed to this effect. The stability of the DNA·RNA hybrid formed by the antisense ODN *in vivo* is an important factor affecting the therapeutic potential of antisense ODNs (*55*). Within the cell, it is possible that polyamines, such as spermidine and spermine, might influence the stability of such hybrids (*56*). Polyamine analogues can be designed to increase the stability of the DNA·RNA hybrid, although the effect of other counterions in competing with polyamines in DNA binding has to be considered.

In summary, our results show that natural polyamines have a remarkable ability to stabilize different nucleic acid duplexes and hybrids. Compared to the unmodified counterparts, phosphorothioate modification resulted in decreased duplex stability. The order of stability of the duplexes remained the same in the absence and presence of spermine, with the following order: RNA·RNA > PO−DNA·RNA > PO−DNA·PO−DNA > PS−DNA·RNA > PS−DNA·PO−DNA > PS−DNA·PS−DNA. The extent of stabilization increases with cationicity of polyamine analogues, with a hybrid stabilizing efficacy in the following order: hexamines > pentamines > tetramines > triamines > diamines. Bis(ethyl)-substituted polyamines are also able to stabilize the hybrids, albeit to a lesser extent than their unmodified counterparts. CD studies showed that spermine stabilized the hybrid helices by stabilizing the conformation toward the A form. These results might be useful in designing polyamine analogues for stabilizing DNA·RNA hybrids within the cell, especially when phosphorothioate ODNs are used as antisense drugs. We are extending the observations from this study and are evaluating the efficiency of polyamine−antisense ODN complexes to block HER-2 expression in HER-2-overexpressing human breast cancer cells.

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