Pyrimidine–purine–pyrimidine triplex DNA stabilization in the presence of tetramine and pentamine analogues of spermine

T.J. Thomas, Carolyn Ashley, Thresia Thomas, Akira Shirahata, Leonard H. Sigal, and Jeremy S. Lee

Abstract: The formation and stability of triplex DNA were investigated in the presence of a number of tetramine (+4) and pentamine (+5) derivatives of spermine with altered spacing between the positive charges and bis(ethyl) substitution of pendant amino groups. Thermal denaturation profiles were measured for the duplex and triplex forms of poly[d(TC)]·poly[d(GA)] and poly(dA)·poly(dT); in both cases the pentamines were more effective than the tetramines in increasing the melting temperature (T_m) of the triplexes. Some structural effects were evident, although bisethylation of the polyamines had only a minor effect on the T_m of pyrimidine–purine–pyrimidine triplexes. Relative association constants to poly(dT)·poly(dA)·poly(dT) and poly[d(AT)] were measured by an ethidium competition assay. These results demonstrated tighter binding of the pentamines by a factor of up to 10-fold, but bisethylation consistently decreased the relative association constants to the triplex. A third assay involving transmolecular triplex formation between separated pyrimidine–purine tracts in plasmid DNA was also employed. Again the pentamines promoted triplex formation at lower concentrations than the tetramines but structural effects were very important in determining the degree of triplex formation. These results may be important for the design of suitable ligands to stabilize triplex DNA in antigene therapeutics and to elucidate the mechanism of action of polyamine analogues as antitumor drugs.

Key words: triplex DNA, polyamines, spermine analogues, triplex stability, antigene therapeutics.

Résumé : La formation et la stabilité de l'ADN triple brin ont été étudiées en présence de plusieurs tétramines (+4) et pentamines (+5) dérivées de la spermine ayant différents espacements entre les charges positives et les groupes amines biséthylés terminaux. Les profils de dénaturation par la chaleur des doubles et triples brins de poly[d(TC)]·poly[d(GA)] et de poly(dA)·poly(dT) ont été déterminés. Dans les deux cas, les pentamines augmentent plus la T_m des triples brins que les tétramines. Des effets structuraux sont évidents même si la biséthylation des polyamines a peu d'effet sur la T_m des triples brins pyrimidine–purine–pyrimidine. Les constantes relatives d'association au poly(dT)·poly(dA)·poly(dT) et au poly[d(AT)] ont été déterminées grâce à une méthode de compétition avec l'éthidium. Les résultats indiquent que les pentamines se lient jusqu'à 10 fois plus fortement et que la biséthylation diminue toujours les constantes relatives d'association au triple brin. Une troisième méthode mesurant la formation d'un triple brin transmoléculaire entre différentes portions pyrimidine–purine d'un ADN plasmidique a également été utilisée. De nouveau, les pentamines favorisent la formation de triples brins à des concentrations plus faibles que les tétramines, mais des effets structuraux sont très importants pour déterminer le taux de formation des triples brins. Ces résultats pourraient être importants pour la conception de ligands capables de stabiliser l'ADN triple brin au cours d'une thérapie dirigée contre un gène et pour l'élucidation du mécanisme d'action antitumoral d'analogues de polyamines.

Mots clés : ADN triple brin, polyamines, analogues de la spermine, stabilité de triples brins, thérapie dirigée contre un gène.

[Traduit par la rédaction]

Received February 10, 1997. Revised June 9, 1997. Accepted July 19, 1997.

Abbreviation: DFMO, difluoromethylornithine.

T.J. Thomas. Department of Medicine, University of Medicine and Dentistry of New Jersey – Robert Wood Johnson Medical School, New Brunswick, NJ 08903, U.S.A.
C. Ashley and J.S. Lee.[1] Department of Biochemistry, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada.
T. Thomas. Department of Environmental and Community Medicine, University of Medicine and Dentistry of New Jersey – Robert Wood Johnson Medical School, New Brunswick, NJ 08903, U.S.A.
A. Shirahata. Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-02, Japan.
L.H. Sigal. Deparment of Medicine and Department of Molecular Genetics and Microbiology and Pediatrics, University of Medicine and Dentistry of New Jersey – Robert Wood Johnson Medical School, New Brunswick, NJ 08903, U.S.A.

[1] Author to whom all correspondence should be addressed. e-mail: leejs@sask.usask.ca

Introduction

The polyamines spermine and spermidine are present in all eucaryotic cells. Their concentration is in the millimolar range but since the majority is bound to nucleic acids, the free concentration may be much lower (Sarhan and Seiler 1989). Since they bind tightly to DNA and RNA they are involved directly or indirectly in every cellular process that requires nucleic acids (Tabor and Tabor 1976, 1984, 1985). For example, topoisomerases are stimulated by spermine; the binding of some transcription factors is increased by polyamines whereas for others it is inhibited (Krasnow and Cozzarelli 1982; Panagiotidis et al. 1995).

The biosynthesis of polyamines varies through the cell cycle and is tightly regulated (Pegg 1988). In some tumor cells this regulation may be relaxed and polyamine concentrations are higher than in normal cells (Marton and Morris 1987). Therefore, polyamine biosynthesis is a potential target for cancer chemotherapy. Polyamines and their analogues are readily transported into cells, so inhibiting the biosynthesis can be accomplished in vivo (Feuerstein et al. 1992). For example, difluoromethylornithine (DFMO) inhibits ornithine decarboxylase, which is the first committed step in the polyamine biosynthetic pathway (Sjoerdsma and Schechter 1984). Other analogues such as bisethylspermine repress the biosynthetic enzymes and can lead to the depletion of polyamines in the cell (Porter and Sufrin 1986). In general, however, since all cells require polyamines for normal cell growth, these analogues tend to be nonspecific and toxic side effects are often encountered.

A major role for polyamines is in charge neutralization, allowing the DNA to be condensed more easily (Gosule and Schellman 1976; Thomas and Bloomfield 1983). More specific effects on DNA structure have also been observed and polyamines promote the conversion of B DNA to both A and Z forms (Thomas and Messner 1988; Thomas et al. 1991). Surprisingly, the mode of binding has proven difficult to define and sites within both minor and major grooves of DNA have been proposed (Feuerstein et al. 1986; Egli et al. 1991; Dickerson and Drew 1981). 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 (Tari and Secco 1995). This suggests that it is the spacing and distribution of phosphates in the backbone that determines the binding. Therefore, it is likely that the stabilization of A DNA, for example, is due to a narrower minor groove that the spermine can just span.

Triplexes are also stabilized by polyamines and under physiological conditions triplexes may be the preferred conformation for pyrimidine–purine sequences even though the pK_a for the protonation of cytosine is 4.5 (Murray and Morgan 1973; Moser and Dervan 1987; Hampel et al. 1991). Spermine is more effective than spermidine in promoting triplex formation whereas acetylated polyamines and other analogues are generally less effective (Thomas and Thomas 1993). In both pyrimidine–purine–pyrimidine and pyrimidine–purine–purine triplexes the third strand binds in the major groove of the parent duplex. The precise details of these structures are not known but one would predict that the region of the major groove would have a very high negative charge density, which might form a tight binding site for positively charged polyamines. On the other hand, the minor groove may become more A-like and narrower in a triplex, which again might promote the binding of polyamines (Schmid and Behr 1991).

Triplex-forming oligonucleotides have attracted widespread attention because of their possible use in antigene therapies (Wagner 1995; Stein 1995). In principle, a specific oligonucleotide can bind to a single pyrimidine–purine tract in the regulatory region of a target gene and modulate its activity. Because triplex formation is sequence specific, the oligonucleotide can be designed so that it will only bind to a single site on the genome. To be effective the oligonucleotide must have a very high binding constant while retaining its specificity. Thus, a possible extension of this strategy is to attach a secondary ligand to the oligonucleotide to improve its triplex-forming potential (Thomas and Thomas 1993; Thomas et al. 1995). In this context polyamines may be useful secondary ligands.

For these reasons, we have studied the triplex-forming potential of a series of analogues of spermine. The compounds differ not only in their total charge (+4 or +5) but also in the charge separation; as well, the bis(ethyl) derivatives were tested because these compounds are resistant to catabolism within the cell and have been studied as possible antitumor agents (Porter and Sufrin 1986; Marton and Pegg 1995).

Materials and methods

Polyamines

Spermidine trihydrochloride and spermine tetrahydrochloride were purchased from Sigma Chemical Co. (St. Louis, Mo.). 1,11-Diamino-4,8-diazaundecane (norspermine, 3-3-3), N^1,N^{11}-bis(ethyl)norspermine (BE-3-3-3), N^1,N^{12}-bis(ethyl)spermine (BE-3-4-3), 1,14-diamino-5,10-diazatetradecane (homospermine, 4-4-4), N^1,N^{14}-bis(ethyl)homospermine (BE-4-4-4), 1,15-diamino-4,8,12-triazapentadecane (3-3-3-3), 1,15-bis(ethylamino)-4,8,12-triazapentadecane (BE-3-3-3-3), 1,19-diamino-5,10,15- triazanonadecane (4-4-4-4), and 1,19-bis(ethylamino)-5,10,15-triazanonadecane (BE-4-4-4-4) were synthesized according to procedures described earlier (He et al. 1994; Igarashi et al. 1995). The structures and purity of all polyamines were confirmed by elemental analysis, nuclear magnetic resonance, high-performance liquid chromatography, and gas chromatography – mass spectrometry. The chemical structures and abbreviations of these compounds are shown in Fig. 1. Concentrated solutions of the polyamines were prepared in 10 mM sodium cacodylate buffer, pH was adjusted to 7.2, and small volumes were added to oligonucleotide solutions to make up the necessary concentrations.

Polynucleotides

Poly[d(AT)] and poly[d(TC)]·poly[d(GA)] were synthesized with *Escherichia coli* DNA polymerase as described previously (Lee et al. 1979). Poly[d(TC)]·poly[d(GA)] was melted at a concentration of 40 μM (per nucleotide) in 10 mM HEPES, pH 7, with 5 mM NaCI (Lee et al. 1993). Poly(dA) and poly(dT) were purchased from Pharmacia Biotechnology (Piscataway, N.J.). The polynucleotides were dissolved in 10 mM sodium cacodylate buffer (pH 7.2) and 0.5 mM ethylenediaminetetraacetic acid (EDTA) and dialyzed extensively into the same buffer. Concentrations of poly(dA) and poly(dT) were measured using molar nucleotide extinction coefficients of 8900 at 257 nm for poly(dA) and 9000 at 265 nm for poly(dT). Poly(dA) and poly(dT) were mixed in a 1:2 molar ratio in 10 mM sodium cacodylate buffer and the appropriate concentrations of polyamines were added to prepare the triplex DNA solution. For melting temperature (T_m) measurements, polynucleotides were used in the concentration range of 15–25 μM (per nucleotide). The solutions were heated at 90°C for 5 min, cooled to room temperature (22°C), and then allowed

Fig 1. Structures of the polyamines.

STRUCTURE	ABBREVIATION
$H_3N^+(CH_2)_3N^+H_2(CH_2)_4N^+H_2(CH_2)_3N^+H_3$	Spermine
$EtN^+H_2(CH_2)_3N^+H_2(CH_2)_4N^+H_2(CH_2)_3N^+H_2Et$	BEspermine
$H_3N^+(CH_2)_3N^+H_2(CH_2)_3N^+H_2(CH_2)_3N^+H_3$	3-3-3
$H_3N^+(CH_2)_4N^+H_2(CH_2)_4N^+H_2(CH_2)_4N^+H_3$	4-4-4
$H_3N^+(CH_2)_3N^+H_2(CH_2)_3N^+H_2(CH_2)_3N^+H_2(CH_2)_3N^+H_3$	3-3-3-3
$H_3N^+(CH_2)_4N^+H_2(CH_2)_4N^+H_2(CH_2)_4N^+H_2(CH_2)_4N^+H_3$	4-4-4-4
$EtN^+H_2(CH_2)_3N^+H_2(CH_2)_3N^+H_2(CH_2)_3N^+H_2Et$	BE-3-3-3
$EtN^+H_2(CH_2)_4N^+H_2(CH_2)_4N^+H_2(CH_2)_4N^+H_2Et$	BE-4-4-4
$EtN^+H_2(CH_2)_3N^+H_2(CH_2)_3N^+H_2(CH_2)_3N^+H_2(CH_2)_3N^+H_2Et$	BE-3-3-3-3
$EtN^+H_2(CH_2)_4N^+H_2(CH_2)_4N^+H_2(CH_2)_4N^+H_2(CH_2)_4N^+H_2Et$	BE-4-4-4-4

to equilibrate for 16 h at this temperature before use in T_m experiments. These polymers are 200–1000 bases in length and thus give rise to sharp, highly cooperative transitions.

Thermal denaturation profiles

T_m measurements were made at 260 nm on a Gilford 600 or Perkin–Elmer Lambda 2 spectrophotometer equipped with a thermoprogrammer. Melting profiles were obtained by increasing the temperature at a rate of 0.5°C/min with the absorbance and temperature being recorded every 30 s. T_m was taken as the temperature corresponding to half dissociation of the complexes and the reproducibility was within ±0.5°C. The first derivative, d*A*/d*T* (where *A* is the absorbance and *T* is the temperature), of the melting curve was computer generated and was also used for determining the T_m . T_m values obtained from both methods did not differ by >0.5°C.

Relative binding constants from ethidium competition

The method was originally described by Morgan et al. (1979). Briefly, ethidium binds well to poly(dT)·poly(dA)·poly(dT) in 2 mM $MgCl_2$, which is required for triplex stability (Morgan et al. 1979; Scaria and Shafer 1991). Upon addition of a competitor, ethidium is removed from the DNA and the level of binding can be estimated from the drop in fluorescence. With emission at 590 nm and excitation at 510 nm, the fluorescence of 1 μg/mL ethidium was measured in 2 mL of a buffer of 2.5 mM Tris HCI, pH 8.0, with 2 mM $MgCl_2$. The DNA concentration (in nucleotides) was 7 μM for the triplex poly(dT)·poly(dA)·poly(dT) and 5 μM for the duplex poly[d(AT)]. Serial additions of a concentrated solution of the polyamine were made and the fluorescence was measured after 1 min, which was found to be sufficient time to reach equilibrium. The total polyamine added did not exceed 50 μL and thus, no correction was made for dilution. The initial fluorescence was taken as 100%. The concentration of polyamine required to reach 50% fluorescence is inversely proportional to the binding constant (Morgan et al. 1979).

T-loop formation

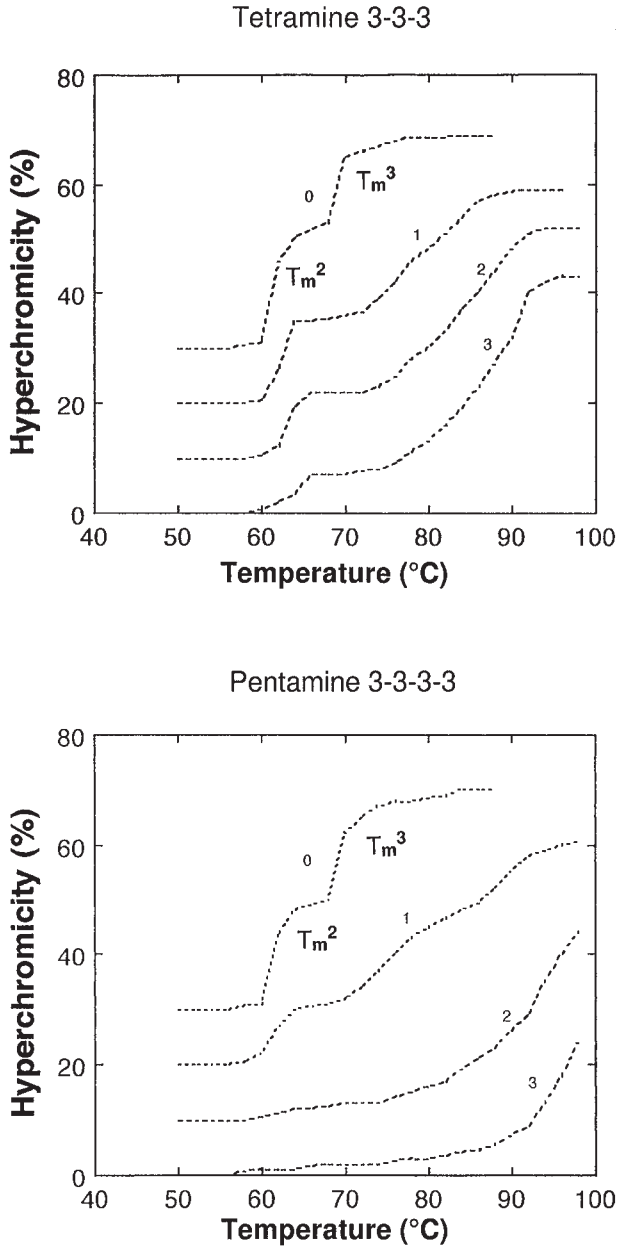
Plasmid pKHa3PYL/PUL contains two separated asymmetrical pyrimidine–purine tracts, which can form a transmolecular triplex with each other (Hampel et al. 1994; Lee et al. 1995). Nicked (OC) plasmid was prepared by γ-irradiation at 1500 rad (1 rad = 10 mGy) for 7 min. T-loops were formed by incubating 1.25 μg (300 μM in nucleotides) of OC plasmid for 24 h at 21°C in 40 mM sodium acetate (pH 4.0), 0.1 mM EDTA, together with added polyamine in a total volume of 10 μL. After addition of 2 μL of tracking dye (0.25% bromophenol blue in 30% glycerol) the total sample was added to a 0.85% agarose gel and electrophoresed at 3.6 V/cm for 6 h at 4°C in a pH 8 buffer of 40 mM Tris-acetate, 20 mM sodium acetate, and 0.1 mM EDTA.

Results

Thermal denaturation profiles provide a simple method to assess the binding of drugs to duplexes and triplexes. In general, the magnitude of the change in T_m is dependent on the amount of drug bound and the binding site size (McGhee 1976). Thus, for closely related drugs that have similar binding site sizes the change in T_m gives a measure of the relative binding strengths. As well, for a triplex to duplex transition, the T_m will increase if the drug binds to the triplex in preference to the duplex or decrease if the duplex is preferred (McGhee 1976). For poly(dT)·poly(dA)·poly(dT) the first T_m is for the melting of the triplex to duplex plus a pyrimidine strand (T_m^3). On the other hand, for poly[d(TC)]·poly[d(GA)] the first transition represents the melting (or dismutation) of duplex to triplex plus a purine strand (T_m^2) and the second T_m is the melting of the triplex to single strands (T_m^3) (Lee et al. 1984; Hampel et al. 1991; Lee et al. 1993). It should be noted that aggregation would have resulted in anomalous values for absorbance at 260 nm (A_{260}), which were not observed under the conditions of these experiments.

The effect of 3-3-3 and 3-3-3-3 on the T_m of poly[d(TC)]·poly[d(GA)] is shown in Fig. 2. As the concentration of 3-3-3 increased, the T_m^3 (owing to melting of the triplex) increased by up to 17°C and there was also some increase in the hyperchromicity of the transition. Particularly at 1 μM of 3-3-3 the triplex transition appeared to be biphasic or at least very broad. As discussed by McGhee (1976), this does not necessarily

Fig. 2. The effect of 3-3-3 and 3-3-3-3 on the thermal denaturation profile of poly[d(TC)]·poly[d(GA)]. The % hyperchromicity is defined as 100(A_{260} – initial A_{260})/(initial A_{260}). For clarity each curve has been offset by 10%. The total change in hyperchromicity is about 40% for melting of this triplex and is independent of polyamine. The DNA was melted in the presence of 0, 1, 2, and 3 μM of the polyamine as indicated. T_m^2 and T_m^3 refer to the melting of the duplex and triplex, respectively.



imply cooperative binding but is often observed even for simple intercalators. In contrast, T_m^2 (owing to melting of the duplex) increased by only 3°C at 3 μM 3-3-3 and the magnitude of the hyperchromicity decreased. Thus, as with spermine described previously, 3-3-3 preferentially bound to the triplex and promoted dismutation of duplex to triplex. For the pentamine 3-3-3-3 (Fig. 2*b*) the change in T_m^3 was even more pronounced and increased by 26°C at 3 μM. (Although the melting was not complete, the value of T_m^3 could be estimated assuming that the total hyperchromicity was about 40% and independent of polyamine concentration.) Above 1 μM of 3-3-3-3 there was no discernible first transition, and therefore the pentamine is a very strong promoter of triplex formation.

The profile for 3-3-3 is typical of the other tetramines and that for 3-3-3-3 is typical of the other pentamines. In all cases the ΔT_m for the duplex transition was less than 4°C and for the triplex the results are summarized in Table 1. In the tetramine series of compounds, spermine gave the largest increase in T_m but amongst the bisethylated compounds BE-3-3-3 was the most effective and had a higher ΔT_m than 3-3-3. In the pentamine series, 3-3-3-3 had the greatest increase in T_m but that for BE-3-3-3-3 was smaller than for both 4-4-4-4 and BE-4-4-4-4. Thus, it is very difficult to discern any obvious trends in these results except that the pentamines always have a higher ΔT_m than the tetramines.

Similarly, the effect of the polyamines on the T_m of poly(dT)·poly(dA)·poly(dT) and poly(dA)·poly(dT) was assessed and the results are summarized in Table 2. Under these conditions, no triplex transition was observed in the absence of polyamines. Thus, for example, 5 μM spermine was required to promote triplex formation whereas this occurred at 1 μM for the bisethylated derivative. Similarly, BE-3-3-3 was more effective than 3-3-3 but 4-4-4 promoted triplex formation at 5 μM whereas BE-4-4-4 did not. As expected for the pentamines, the increases in T_m values for both duplex and triplex were larger. All of them promoted triplex formation at 1 μM and 3-3-3-3 gave the largest increase in T_m as was found for the poly[d(TC)]·poly[d(GA)]·poly[(C^+T)] triplex.

Another rapid method for assessing binding to DNA was to measure the loss of binding of ethidium as a function of added competitor. As shown in Fig. 3 for 4-4-4-4, 3-3-3-3, spermine, 3-3-3, and BE-3-3-3, the polyamines compete with ethidium for binding to poly(dT)·poly(dA)·poly(dT) at millimolar concentrations but there is 50-fold range in their effectiveness. The concentration required to give 50% fluorescence is inversely proportional to the binding constant, and thus relative binding constants can be calculated with spermine given the value of unity. These are listed in Table 3 together with results for poly[d(AT)] also in a buffer containing 2 mM $MgCl_2$. It was not possible to compare the binding to duplex poly(dT)·poly(dA) because the binding of ethidium was too weak in the presence of 2 mM $MgCl_2$. Similarly, ethidium did not bind to the poly[d(TC)]·poly[d(GA)]·poly[d(C^+T)] triplex (Lee et al. 1984). As expected, the pentamines were better competitors than the tetramines. For poly(dT)·poly(dA)·poly(dT), bisethylation significantly reduced the relative binding constant compared with the unmodified compound. This was not the case for poly[d(AT)], for which only BEspermine had a binding constant lower than the unmodified compound. For both DNAs, it is interesting that spermine had the highest binding constant of the tetramines and that of 4-4-4-4 was higher than that of 3-3-3-3. Therefore, the spatial distribution as well as the total charge is important for determining the strength of binding.

If triplexes are present within chromosomes, then they may form between separated pyrimidine–purine tracts (Burkholder et al. 1988, 1991). This process can be mimicked in a plasmid such as pKHa3 PYL/PUL, which will form a transmolecular triplex or T-loop at low pH in the presence of polyamines (Lee et al. 1995). Since T-loops have an altered mobility their

Table 1. Effect of polyamines on the T_m of triplex poly[d(TC)]·poly[d(GA)]·poly[d(C^+T)].

Concn. (μM)	Spermine	BEspermine	3-3-3	BE-3-3-3	4-4-4	BE-4-4-4	3-3-3-3	BE-3-3-3-3	4-4-4-4	BE-4-4-4-4
0	71	70.5	70	70.5	70	70.5	70.5	71	70	70.5
1	78.5	78.5	79.5	81	79	80	82.5	80.5	81	83.5
2	86	83.5	85	86.5	85	85	93	88.5	89	89.5
3	90.5	88	87.5	90	88	89	96.5	91.5	94	94

Table 2. Effect of polyamines on the T_m of poly(dA)·2poly(dT) and poly(dA)·poly(dT).

Concn.	Spermine		BEspermine		3-3-3		BE-3-3-3		4-4-4		BE-4-4-4		3-3-3-3		BE-3-3-3-3		4-4-4-4		BE-4-4-4-4	
(μM)	T_m^1	T_m^2	T_m^1	T_m^2	T_m^1	T_m^2	T_m^1	T_m^2	T_m^1	T_m^2	T_m^1	T_m^2	T_m^1	T_m^2	T_m^1	T_m^2	T_m^1	T_m^2	T_m^1	T_m^2
0	—	52.7	—	53	—	52.5	—	52.7	—	53.4	—	53.5	—	52.5	—	53.2	—	53.2	—	53
1	—	57	57.4	66.5	—	58.5	—	62.6	—	61	—	62	60	73.8	57	70	54.5	62.2	55.4	61
2.5	—	62	62.9	68.7	—	63.5	62.6	67	—	69.1	—	69.2	70.9	86.4	60	70.6	62.9	75.3	65	75
5	69.5	72	—	77.1*	—	73	65.5	74.1	68.6	78.7	—	74.4	—	89.4	—	75.3*	76.1	86.7	81	83

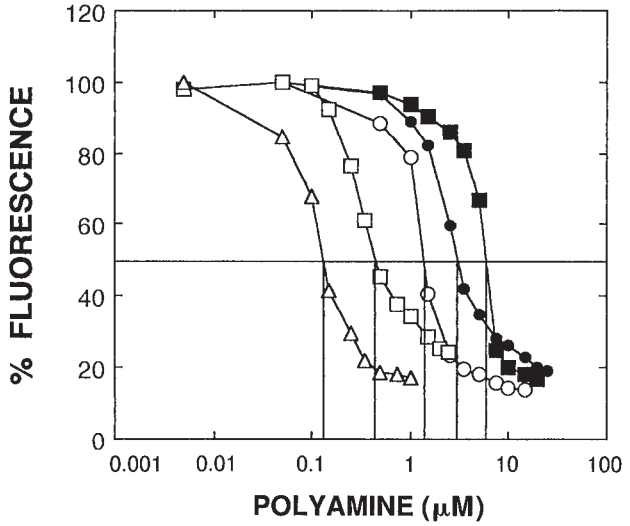
Note: —, no triplex DNA melting was observed.

*Both triplex and duplex melting temperatures merged into a single T_m, indicating the melting of triplex DNA to single strands.

Table 3. Relative binding constants measured by ethidium competition method.

DNA	Spermine	BEspermine	3-3-3	BE-3-3-3	4-4-4	BE-4-4-4	3-3-3-3	BE-3-3-3	4-4-4-4	BE-4-4-4-4
Poly(dA)·2poly(dT)	1	0.68	0.43	0.22	1.1	0.65	2.9	1.9	10	6.2
Poly[d(AT)]	1	0.65	0.72	0.57	3.2	3.2	5.7	5.7	46	43

Fig. 3. Ethidium competition assay. Upon addition of polyamine, the ethidium is released from the DNA and the fluorescence decreases. △, 4-4-4-4; □, 3-3-3-3; ○, spermine; ●, 3-3-3; ■, BE-3-3-3. The concentration of polyamine at 50% fluorescence is inversely proportional to the binding constant.

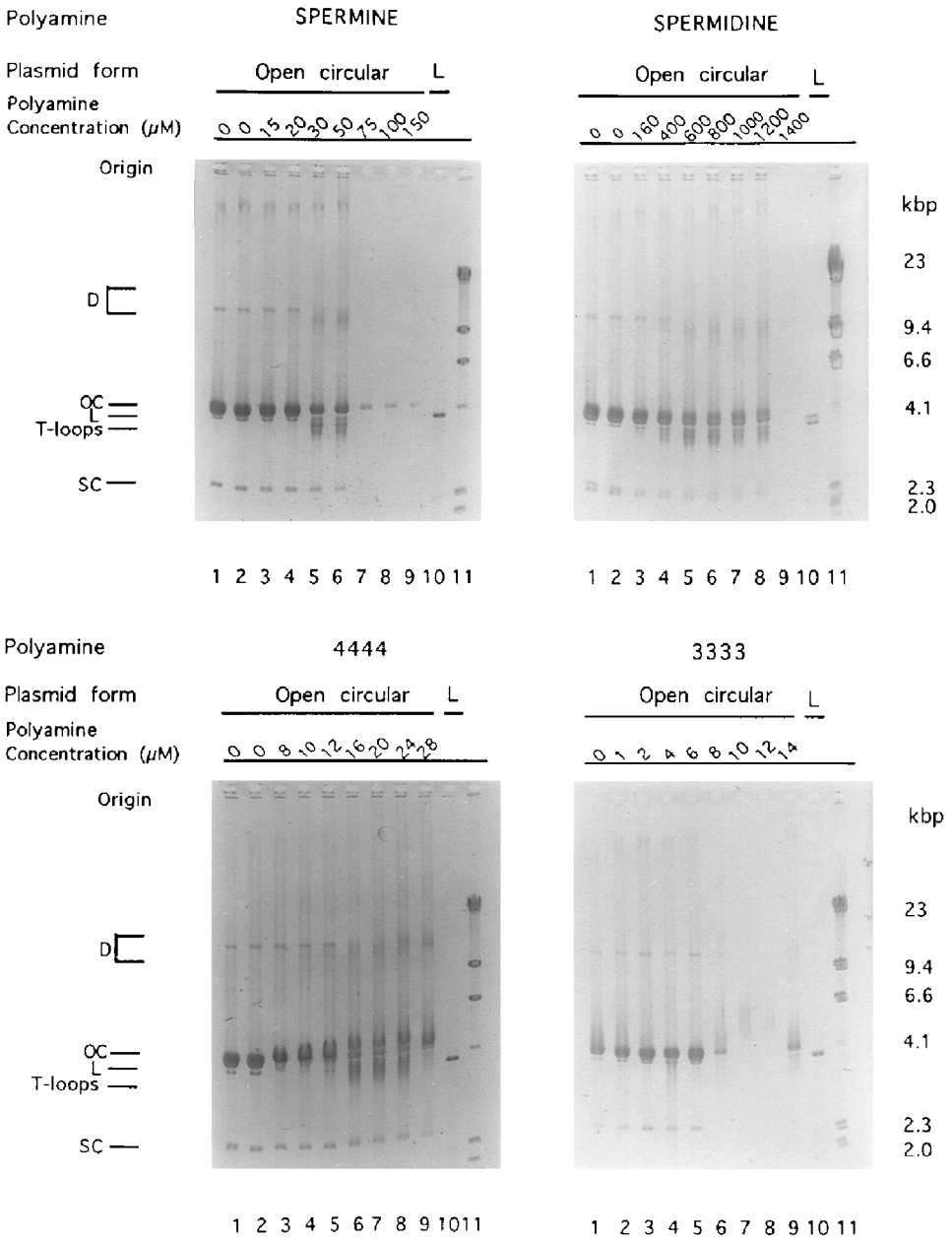


formation can be assessed by agarose gel electrophoresis. Therefore, the ability of the polyamine analogues to promote T-loops was assessed (Fig. 4). For spermine, T-loops became evident at a concentration of 30 μM and about 30% of the open circles were converted to the faster mobility form. At concentrations above 50 μM, precipitation occurred and not all of the DNA entered the gel. For spermidine, much higher concentrations were required (400 μM), although the level of T-loop formation was similar. For the pentamine 4-4-4-4, some T-loops were formed at only 10 μM and at the optimal concentration of 16 μM the majority of the DNA had been converted to T-loops. The other pentamine 3-3-3-3 formed T-loops at even lower concentrations (4 μM) but the percentage conversion was very poor (as judged from the smear migrating ahead of the linear band) and precipitation of the DNA occurred above 6 μM. Thus, the level of T-loop formation was dependent on the spatial distribution of the charges in the polyamine. The results for the other analogues are summarized in Table 4. In general, bisethylation is less important than total charge in promoting T-loop formation.

Discussion

It has been suggested that polyamines might be useful secondary ligands for antigene strategies involving triplex formation (Thomas and Thomas 1993). The polyamines could be used as free ligands or attached to the end of the oligonucleotide or possibly to the 5′ position of pyrimidine bases (Barawkar et al. 1996; Tung et al. 1993). Several benefits can be envisaged, including increased resistance to nucleases, increased cellular uptake by decreasing the net charge, and increased triplex formation and stability at the target sequence. It has been shown previously that spermine is more effective than spermidine in stabilizing triplexes (Hampel et al. 1991); therefore, higher analogues such as the pentamines 3-3-3-3, BE-3-3-3-3, 4-4-4-4, and BE-4-4-4-4 might be yet more successful in promoting triplex formation. From the results presented above this would indeed appear to be the case. As a group the pentamines increased the T_m of both poly(dT)·poly(dA)·poly(dT) and poly[d(TC)]·poly[d(GA)]·poly[d(C^+T)] triplexes to a

Fig. 4. Polyamine-induced formation of T-loops in pKHa3PYL/PUL assessed by electrophoresis in agarose gels. The mobility of the T-loops is slightly faster than the linear (L) and open circular (OC) forms of the plasmid. The supercoiled (SC) DNA has the highest mobility and the open circular dimers are the slowest. In each gel, lanes 1–9 have increasing concentrations of polyamine (shown at the top of the gel), lane 10 is a linear standard, and lane 11 contains molecular weight markers.



greater extent than the tetramines (Tables 1 and 2). The relative binding constants to poly(dT)·poly(dA)·poly(dT) were also higher than those of the tetramines by about an order of magnitude (Table 3). Similarly, the pentamines induced T-loop formation at 2- to 5-fold lower concentrations. However, structural specificity effects were exerted by the pentamines: 3-3-3-3 gave the largest increase in T_m of both triplexes but 4-4-4-4 had the highest relative binding constant. As well, 4-4-4-4 formed a higher percentage of T-loops, although this occurred at a higher concentration than for 3-3-3-3. BE-3-3-3-3 was less effective than 3-3-3-3 in all three types of assay whereas BE-4-4-4-4 was as good as or better than 4-4-4-4 in increasing the T_m of triplexes. These results indicate the interplay of ionic and structural effects in polyamine–DNA interactions and polyamine-mediated triplex DNA stabilization.

For the tetramines, the different assays suggest different trends. For example, the relative binding constant for BEspermine is lower than that of spermine, yet BEspermine induces the formation of poly(dT)·poly(dA)·poly(dT) at a concentration of 1 μM compared with 5 μM for spermine. This pattern is also found with BE-3-3-3 and 3-3-3. Of course, the two assays are measuring different properties. The T_m assay is measuring the ability of the polyamine to influence the equilibrium:

$$\text{poly(dA)·poly(dT)} + \text{poly(dT)} \rightleftharpoons \text{poly(dT)·poly(dA)·poly(dT)}$$

Therefore, the strength of binding of the polyamine to the duplex and poly(dT) is important whereas the ethidium competition assay only involves binding of the polyamine to the triplex. On the other hand, the T_m assay may be more representative of the situation in vivo where both duplexes and single-stranded nucleic acids will be present.

Previous determinations of association constants of polyamine binding with double-helical (K_h) and single-stranded (K_c) calf thymus DNA showed a 100- to 500-fold difference between K_h and K_c values; the duplex DNA had a higher affinity for polyamines than single-stranded DNA (Basu et al. 1990). The difference between K_h and K_c roughly correlates with the increased negative charge density of duplex DNA compared with that of single-stranded DNA: the linear charge spacing of duplex and single-stranded DNA is 4.3 and 1.7 Å (1 Å = 0.1 nm), respectively. Polyamine–triplex DNA association constants (K_t) have not as yet been estimated; however, on the basis of the decreased charge spacing (1.1 Å) of this form of DNA structure, imposed by the association of the third strand of DNA onto the major groove of the duplex DNA, a stronger association of polyamines to triplex DNA than that to duplex and single-stranded forms could be expected. In general the relative binding constants of bisethylated polyamines are lower than that of their unethylated parent compounds (Table 3), suggesting that steric hindrance of the bulky ethyl groups on the pendant amino groups may play a role. Similarly the K_h values of bisethylated polyamines are 10–50 times lower than those of their unethylated analogs, further suggesting the importance of steric factors in polyamine–DNA interactions (Basu et al. 1990). Plum and Bloomfield (1990) also reported that when a methyl group is substituted at one of the pendant amino groups of spermidine the K_h is 4 times lower than that of spermidine. These reports indicate that substitution of ethyl or methyl groups on the pendant amino groups of polyamines affects their binding affinity to duplex DNA and is consistent with our findings, as presented in Table 3.

Singleton and Dervan (1993) attempted to analyze the ionic effects in the association constants of oligonucleotide-directed triplex formation at single DNA sites in the presence of Na^+, Mg^{2+}, and spermine^{4+} using the counterion condensation theory developed by Manning (1978) and Record et al. (1978). Wilson and Bloomfield (1979) and Thomas and Bloomfield (1983) modified and utilized this theory to explain polyamine- and $Co(NH_3)_6^{3+}$-mediated collapse of DNA. According to this theory, counterion binding is explicitly considered only as delocalized binding of point-charge ligands in the negative field of polyion backbone. However, polyamines are cations with definite structure and different charge separation and charge density, dependent on the chemical structure of the molecule. If all bonds are in the *trans* conformation in these polyamines, the charge separation between the amino and imino nitrogens in the three methylene parts of the molecules is 4.97 Å (e.g., 3-3-3-3). This distance becomes 6.25 Å between the amino and imino nitrogen when separated by a tetramethylene bridging region (e.g., 4-4-4-4). Some bending back of these molecules could also be expected, depending on the length of the molecule as well as the length of the methylene bridge. In molecular models considered by Feuerstein et al. (1986), the tetramethylene bridging is amenable to make intermolecular hydrogen bonding contacts between DNA phosphate groups. This would explain the higher relative binding constants of 4-4-4 (K_a = 1.1) compared with 3-3-3 (K_a = 0.43) and of 4-4-4-4 (K_a = 10) compared with 3-3-3-3 (K_a = 2.9), as determined from the ethidium competition method (Table 3). Steric effects are demonstrated by a 2-fold decrease in the relative binding constants of bis(ethyl)polyamines to triplex DNA compared with their unsubstituted parent molecules. Interestingly, these steric effects are negligible in the binding of polyamines to the duplex form of poly[d(AT)] (Table 3).

Table 4. Concentration of polyamine required for the formation of T-loops.

Polyamine	Concentration (μM)
Spermine	30
Spermidine	400
BEspermine	20
3-3-3	20
BE-3-3-3	20
4-4-4	25
BE-4-4-4	30
3-3-3-3	4
BE-3-3-3-3	10
4-4-4-4	10
BE-4-4-4-4	12

The structural effect of polyamine binding to triplex DNA is less evident in the polyamines' efficacy in provoking T-loop formation (Table 4) and stabilizing triplex and duplex structures (Tables 1 and 2). In both of these cases 3-3-3-3 is the most efficient molecule, inducing T-loop formation at 4 μM concentration and stabilizing triplex DNA to a T_m of 95.5°C for poly[d(TC)]·poly[d(GA)]·poly[d(C$^+$T)] and 89.4°C for poly(dT)·poly(dA)·poly(dT). Bisethylation had no major effect on the relative ability of different polyamines to stabilize triplex DNA. Cation charge and structural effects were thus exerted to different degrees in T_m, T-loop formation, and polyamine–DNA association, suggesting multiple modes of polyamine–DNA interactions, as mentioned in the introduction (Feuerstein et al. 1986; Egli et al. 1991; Dickerson and Drew 1981; Tari and Secco 1995).

In conclusion, we determined the effects of a series of higher valent polyamines on triplex DNA stabilization in a model system involving disproportionation of polynucleotides and direct binding of a polynucleotide to duplex DNA. We also determined the effects of these polyamines on T-loop formation using plasmid DNA that could undergo transmolecular triplex DNA formation. Our results suggest that pentamines are more efficacious than tetramines in increasing the T_m of triplex DNA and T-loop formation, although structural effects are clearly evident. Bisethylation has only a minor effect on the T_m of pyrimidine–purine–pyrimidine triplex DNA formation; however, steric hindrance imposed by the ethyl substitution decreases the relative association constants of the polyamines with triplex and duplex forms of poly(dT)·poly(dA)·poly(dT) and poly[(dAT)], respectively. These results might be important in the design of suitable ligands to stabilize triplex DNA in antigene therapeutics and to elucidate the mechanism of action of polyamine analogs as antitumor drugs.

Acknowledgement

These investigations were supported by the Medical Research Council of Canada (J.S.L.), National Institutes of Health grants CA42439 and CA73058 (T.T.), a New Jersey State Commission on Cancer research grant (T.T. and T.J.T.), and a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture, Japan (A.S.).

References

Barawkar, D.A., Rajeev, K.G., Kumar, V.A., and Ganesh, K.N. 1996. Triplex formation at physiological pH by 5-Me-dC-N^4-(spermine) [X] oligonucleotides. Nucleic Acids Res. **24**: 1229–1237.

Basu, H.S., Schwietert, H.C.A., Feuerstein, B.G., and Marton, L.J. 1990. Effects of variation in the structure of spermine on the association with DNA and the induction of DNA conformational changes. Biochem. J. **267**: 329–334.

Burkholder, G.D., Latimer, L.J.P., and Lee, J.S. 1988. Immunofluorescent staining of mammalian nuclei and chromosomes with a monoclonal antibody to triplex DNA. Chromosoma, **97**: 185–192.

Burkholder, G.D., Latimer, L.J.P., and Lee, J.S. 1991. Immunofluorescent localization of triplex DNA in polytene chromosomes of *Chironomus* and *Drosophila*. Chromosoma, **101**: 11–18.

Dickerson, R.E., and Drew, H.R. 1981. Structure of a B-DNA dodecamer II. J. Mol. Biol. **208**: 535–556.

Egli, M., Williams, L.D., Gao, Q., and Rich, A. 1991. Structure of the pure spermine form of Z-DNA at 1 Å resolution. Biochemistry, **30**: 11 388 – 11 402.

Feuerstein, B.G., Pattabiraman, N., and Marton, L.J. 1986. Spermine–DNA interactions: a theoretical study. Proc. Natl. Acad. Sci. U.S.A. **83**: 5948–5952.

Feuerstein, B.G., Szollsi, J., Basu, H.S., and Marton, L.J. 1992. Difluoromethylornithine alters calcium signalling in platelet-derived growth factor-stimulated A172 brain tumour cells in culture. Cancer Res. **52**: 6782–6789.

Gosule, L.C., and Schellman, J.A. 1976. Compact form of DNA induced by spermidine. Nature (London), **259**: 333–335.

Hampel, K.J., and Lee, J.S. 1993. Two-dimensional pulsed-field electrophoresis of yeast chromosomes. Biochem. Cell Biol. **71**: 190–196.

Hampel, K.J., Crosson, P., and Lee, J.S. 1991. Polyamines favor DNA triplex formation at neutral pH. Biochemistry, **30**: 4455–4459.

Hampel, K.J., Ashley, C., and Lee, J.S. 1994. Kilobase-range communication between polypurine–polypyrimidine tracts in linear plasmids mediated by triplex formation. Biochemistry, **33**: 5674–5681.

He, Y., Suzuki, T., Kashiwagi, K., Kusama-Eguchi, K., Shirahata, A., and Igarashi, K. 1994. Correlation between the inhibition of cell growth by bis(ethyl)polyamine analogues and the decrease in the function of the mitochondria. Eur. J. Biochem. **221**: 391–398.

Igarashi, K., Koga, K., He, Y., Shimogori, T., Ekimoto, H., Kashiwagi, K., and Shirahata, A. 1995. Inhibition of the growth of various human and mouse tumour cells by 1,15-bis(ethylamine)-4,8,12-triazapentadecane. Cancer Res. **55**: 2615–2619.

Krasnow, M.A., and Cozzarelli, N.R. 1982. Catenation of DNA rings by topoisomerases. J. Biol. Chem. **257**: 2687–2693.

Lee, J.S., Johnson, D.A., and Morgan, A.R. 1979. Complexes formed by $(pyrimidine)_n \cdot (purine)_n$ DNAs on lowering the pH are three-stranded. Nucleic Acids Res. **6**: 3073–3091.

Lee, J.S., Woodsworth, M.L., Latimer, L.J.P., and Morgan, A.R. 1984. Poly(pyrimidine)·poly(purine) synthetic DNAs containing 5-methylcytosine form stable triplexes at neutral pH. Nucleic Acids Res. **12:** 6603–6613.

Lee, J.S., Latimer, L.J.P., and Hampel, K.J. 1993. Coralyne binds tightly to both T·A·T and $C \cdot G \cdot C^+$-containing DNA triplexes. Biochemistry, **32**: 5591–5597.

Lee, J.S., Ashley, C., Hampel, K.J., Bradley, R., and Scraba, D.G. 1995. A stable interaction between separated pyrimidine·purine tracts in circular DNA. J. Mol. Biol. **252**: 283–288.

Manning, G.S. 1978. The molecular theory of polyelectrolyte solutions with applications to the electrostatic properties of polynucleotides. Q. Rev. Biophys. **11**: 179–246.

Marton, L.J., and Morris, D.R. 1987. Polyamines in cell proliferation and differentiation. *In* Inhibition of polyamine metabolism. *Edited by* P.P. McCann, A.E. Pegg, and A. Sjoerdsma. Academic Press, New York. pp. 79–105.

Marton, L.J., and Pegg, A.E. 1995. Polyamines as targets for therapeutic intervention. Annu. Rev. Pharmacol. Toxicol. **35**: 55–91.

McGhee, J.D. 1976. Theoretical calculations of the helix-coil transition of DNA in the presence of large, cooperatively binding ligands. Biopolymers, **15**: 1345–1375.

Morgan, A.R., Lee, J.S., Pulleyblank, D.E., Murray, N.L., and Evans, D.E. 1979. Ethidium bromide fluorescence assays, part 1. Nucleic Acids Res. **7**: 547–569.

Moser, H.E., and Dervan, P.B. 1987. Sequence-specific cleavage of double helical DNA by triple helix formation. Science (Washington, D.C.), **238**: 645–650.

Murray, N.L., and Morgan, A.R. 1973. Enzymatic and physical studies on triplex $dT_n \cdot dA_n \cdot dT_n$. Can. J. Biochem. **51**: 436–449.

Panagiotidis, C.A., Artandi, S., Calame, K., and Silverstein, S.J. 1995. Polyamines alter sequence-specific DNA-protein interactions. Nucleic Acids Res. **23**: 1800–1809.

Pegg, A.E. 1988. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. Cancer Res. **48**: 759–774.

Plum, G.E., and Bloomfield, V.A. 1990. Structural and electrostatic effects on binding of trivalent cations to double-stranded and single-stranded poly[d(AT)]. Biopolymers, **29**: 13–27.

Porter, C.W., and Sufrin, J.R. 1986. Interference with polyamine biosynthesis and (or) function by analogs of polyamines and methionine as a potential anticancer chemotherapeutic strategy. Anticancer Res. **6**: 525–542.

Record, M.T., Jr., Anderson, C.F., and Lohman, T.M. 1978. Thermodynamic analysis of ion effects on the binding and conformational equilibria of proteins and nucleic acids. Q. Rev. Biophys. **11**: 103–178.

Sarhan, S., and Seiler, N. 1989. On the subcellular localization of polyamines. Biol. Chem. Hoppe-Seyler, **370**: 1279–1284.

Scaria, P.V., and Shafer, R.H. 1991. Binding of ethidium to a DNA triple helix. J. Biol. Chem. **266**: 5417–5423.

Schmid, N., and Behr, J.P. 1991. Location of spermine and other polyamines on DNA as revealed by photoaffinity cleavage with polyaminobenzenediazonium salts. Biochemistry, **30**: 4357–4361.

Singleton, S.F., and Dervan, P.B. 1993. Equilibrium association constants for oligonucleotide-directed triple helix formation at single sites: linkage to cation valence and concentration. Biochemistry, **32**: 13 171 – 13 179.

Sjoerdsma, A., and Schechter, P.J. 1984. Chemotherapeutic implications of polyamine synthesis inhibition. Clin. Pharmacol. Ther. **35**: 287–300.

Stein, C.A. 1995. Does antisense exist? Nat. Med. **1**: 1119–1121.

Tabor, C.W., and Tabor, H. 1976. Polyamines. Annu. Rev. Biochem. **45**: 285–306.

Tabor, C.W., and Tabor, H. 1984. Polyamines. Annu. Rev. Biochem. **53**: 749–791.

Tabor, C.W., and Tabor, H. 1985. Polyamines in microorganisms. Microbiol. Rev. **49**: 81–89.

Tari, L., and Secco, A.S. 1995. Base-pair opening and spermine binding. Nucleic Acids Res. **23**: 2065–2073.

Thomas, T.J., and Bloomfield, V.A. 1983. Collapse of DNA caused by trivalent cations: pH and ionic specificity effects. Biopolymers, **22**: 1097–1106.

Thomas, T.J., and Messner, R.P. 1988. Structural specificity of polyamines in left handed Z-DNA formation. J. Mol. Biol. **201**: 463–467.
Thomas, T., and Thomas, T.J. 1993. Selectivity of polyamines in triplex DNA stabilization. Biochemistry, **32**: 14 068 – 14 074.
Thomas, T.J., Gunnia, U.B., and Thomas, T. 1991. Polyamine-induced B-DNA to Z-DNA conformational transition of a plasmid with (dG-dC) insert. J. Biol. Chem. **266**: 6137–7141.
Thomas, T.J., Faaland, C.A., and Thomas, T. 1995. Suppression of c-myc oncogene expression by a polyamine-complexed triplex forming oligonucleotide in MCF-7 breast cancer cells. Nucleic Acids Res. **23**: 3594–3599.
Tung, C.-H., Breslauer, K.J., and Stein, S. 1993. Polyamine-linked oligonucleotides for DNA triple helix formation. Nucleic Acids Res. **21**: 5489–5494.
Wagner, R.W. 1995. The state of the art in antisense research. Nat. Med. **1**: 1116–1118.
Wilson, R.W., and Bloomfield, V.A. 1979. Counter-ion induced condensation of DNA. A light-scattering study. Biochemistry, **18**: 2192–2196.