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Human DNA Topoisomerase I Inhibitory Activities of Synthetic Polyamines: Relation to DNA Aggregation

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Abstract—DNA aggregation by polyamines has acquired importance as a prerequisite for the cellular uptake of DNA for gene therapy. Intracellular polyamines are constitutive components of mammalian cells and their availability is critical for cell proliferation. Interference of polyamine biosynthesis by synthetic polyamines leads to cytotoxicity. Optimization of the polyamine structural parameters is necessary to control their DNA aggregation, cytotoxic or enzyme inhibitory activities. We designed two series of tetra- and hexamines and compared their human DNA topoisomerase I (top1) inhibitory effects with the DNA aggregation properties. We show that hexamines are more efficient inhibitors of DNA relaxation by top1 than tetramines and that they suppress the top1-mediated DNA cleavage while tetramines do not. The DNA aggregation abilities within two series of polyamines correlate with the length of their central methylene chain. By contrast, the top1 inhibition within two series does not show the same correlation but demonstrates a threshold inhibitory effect on going from the $(CH_2)_{12}$ to the $(CH_2)_{14}$ central chain. We show further that the structures of DNA aggregates formed by polyamines with the $(CH_2)_{10–12}$ or with the $(CH_2)_{14–16}$ chains are very different. The first are a fluid cholesteric-type phases, whereas the second are well-structured aggregates similar to columnar liquid crystals with high packing density of DNA duplexes. The structures of polyamines-induced DNA aggregates are proposed to be crucial for top1 catalysis. The structure–function correlation described here may serve as a guide for rational design of polyamines with desired DNA-aggregation or anti-top1 activities.

Introduction

DNA aggregation by polyamines has acquired significant importance as a prerequisite for the cellular uptake of DNA for gene therapy applications.[1] Otherwise, the intracellular polyamines putrescine, spermidine and spermine are present in all mammalian cells and their intracellular availability is critical for cell proliferation.[2] An important anticancer strategy based on the interference of synthetic polyamines with the biosynthesis of natural polyamines is emerging.[3] The net result of these two effects requires optimization of the polyamine structural parameters necessary for DNA aggregation, cytotoxic or enzyme inhibitory activities.

Despite a plethora of evidence on polyamine-induced DNA conformational changes, no thorough study of their effects on the activities of sequence-specific DNA binding proteins and, particularly, those being the targets of anticancer drugs, has been performed. At physiological concentrations, natural polyamines enhance the binding of several proteins to DNA (e.g., USF, TFE3, Ig/EBP, NF-IL6, YYI and ICP-4, a herpes simplex virus gene regulator), but inhibit others (e.g., Oct-1).[4]

DNA topoisomerase I is an intracellular enzyme involved in the relaxation of DNA during the processes of DNA replication, transcription and repair. Human DNA topoisomerase I (top1) was identified as a cellular target for anticancer drugs.[5] Top1 inhibitors can be grouped into two classes: top1 poisons and top1 suppressors.[6] The best characterized top1 poisons are camptothecin (CPT) derivatives which kill cells by trapping so-called cleavage complexes between top1 and

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DNA.[7] The typical DNA binders, including DNA intercalators (doxorubicin, aclacinomycin, etc.) and minor groove binders (distamycin, Hoechst33258, etc.) can suppress top1-linked DNA breaks due to prevention of the enzyme's access to DNA and/or distortion of the DNA structure.[8] The selectivity of the existing top1 suppressors remains questionable. Sensitivity to top1 poisons increases with top1 overexpression, whereas the opposite should be expected for top1 suppressors.

The natural polyamines are known to be able to stimulate the plasmid DNA relaxation by eukaryotic topoisomerases type I.[9,10] Spermidine stimulates calf thymus DNA topoisomerse I both under low salt and under physiological salt reaction conditions. Srivenugopal et al.[11] described that the synthetic spermidine analogues differentially modulate the activities of bacterial and mammalian type I topoisomerases on aggregated DNA in a manner reminiscent of the abilities of the polyamines to stimulate cell growth. To the best of our knowledge, there are no detailed studies of correlation between the structure of natural and synthetic polyamines and their effects on the human top1 activity.

Results

DNA precipitation by natural and synthetic polyamines under unsalted conditions

Natural multivalent cations spermidine and spermine are known to be able to induce condensation, aggregation and precipitation of DNA molecules.[18–20] Here, we compared effects of DNA precipitation by natural or by new synthetic polyamines differ in their total charge (tetra- or hexamines) and in the length of the central methylene chain (with the number of the CH_2-groups, $n = 10$–16). The new synthetic polyamines may be classified within two groups. The first one includes tetramines **1a** ($n = 10$), **1b** ($n = 12$), and **1c** ($n = 14$), whereas the Group 2 is represented by hexamines **2a** ($n = 10$), **2b** ($n = 12$), **2c** ($n = 14$), and **2d** ($n = 16$) (Fig. 1).

Figure 2 shows the DNA precipitation in the presence of spermidine and spermine. Our results correlate well with the literature data demonstrating the polyamine concentration-dependent increase of the DNA precipitation.[21] The relative potency of spermine and spermidine to precipitate DNA follows the expected tendency of tetravalent cations to be more effective then their trivalent counterparts. The calculated polyamines/DNA (phosphates) molar ratios (r_p) necessary for precipitation of the 50% of DNA were found to be 201 for spermidine and 19 for spermine.

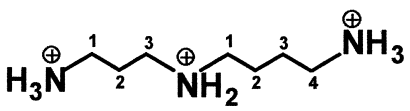
Under the same reaction conditions as in the study of DNA precipitation by natural polyamines spermine and spermidine, we have observed a strong dependence of the level of DNA precipitation on the structure and concentration of synthetic polyamines in solution (Fig. 3). The tetramines were found to have nearly the same DNA precipitation abilities being quite close to those of spermine with the r_p values equal to 17 for derivatives **1a** and **1b**, and 14 for derivative **1c** (compare with $r_p = 19$ for spermine). Our results show that for synthetic tetramines with the $n = 10$, 12, or 14, the abilities of DNA precipitation under unsalted conditions are quite similar and do not depend on the length of the polyamine central methylene chain. The synthetic hexamines are shown to be able to precipitate DNA at much lower concentrations than the tetramines. The r_p values for the hexamines were found to be 1.8 for **2a**, 0.8 for **2b**, 0.042 for **2c**, and 0.028 for **2d**. Figure 3 shows that the DNA precipitation potency of hexamines strongly depends on the length of central methylene chain of the molecule. Moreover, a clear correlation between hexamine structure and DNA precipitation potency was found. The polyamines **2c** ($n = 14$) and **2d** ($n = 16$) were more than 30-fold more potent in DNA precipitation than the polyamines **2a** ($n = 10$) and **2b** ($n = 12$), respectively. The increase of the central methylene chain of hexamines from $n = 12$ to 14 was therefore found to be critical for their DNA precipitation ability under unsalted conditions.

DNA precipitation by natural and synthetic polyamines under physiological salt conditions

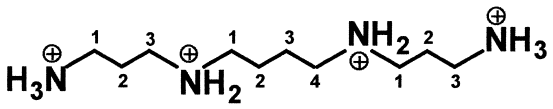
To study the effect of monovalent cations on the efficiency of DNA precipitation by natural and synthetic polyamines, we used a solution of 150 mM NaCl. Addition of NaCl dramatically decreases the DNA precipitation abilities of spermidine and spermine (Fig. 2) as previously described.[20,21] In this range of polyamines concentration, which corresponds to their intracellular physiological concentrations,[2] and in the presence of physiological salt concentrations, spermidine and spermine are not able to induce even 50% of the DNA precipitation at the r_p values up to 140 polyamine/DNA (compare with the $r_p = 19$ found for spermine under unsalted conditions).

When synthetic polyamines are concerned, the physiological salt concentration also decreases their DNA precipitation abilities. For the tetramines **1a**, **1b**, and **1c**, the r_p were not reached in the studied range of concentrations (Fig. 3). On the other hand, a clear difference in the DNA precipitation abilities of the tetramines has been revealed under physiological salt conditions. Lengthening of the central methylene chain within the tetramine molecule leads to an increase of their DNA precipitation abilities (Fig. 3): **1c** ($n = 14$) > **1b** ($n = 12$) > **1a** ($n = 10$).

For hexamines, the r_p values were equal to 1.25 for **2a**, 0.7 for **2b**, 0.35 for **2c**, and 0.28 for **2d**. Comparison of relative potencies of the DNA precipitation by hexamines under physiological salt conditions shows a linear dependence of DNA precipitation on the length of the hexamines central methylene chain (see Discussion). It appears that the presence of 150 mM NaCl has only a moderate effect on the DNA precipitation abilities of derivatives **2a** ($n = 10$) and **2b** ($n = 12$), whereas it dramatically suppresses DNA precipitation by the derivatives **2c** ($n = 14$) and **2d** ($n = 16$).

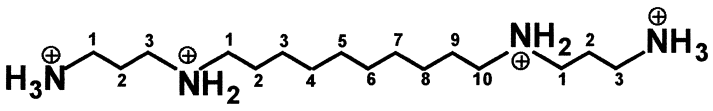


Spermidine
(3 – 4)

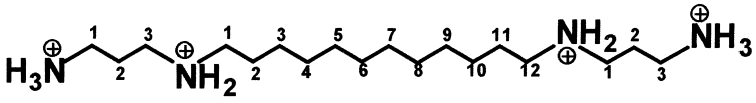


Spermine
(3 – 4 – 3, n = 4)

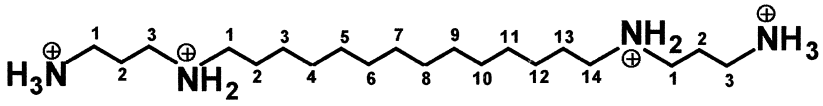
Tetramines:



1a
(3 –10– 3, n = 10)

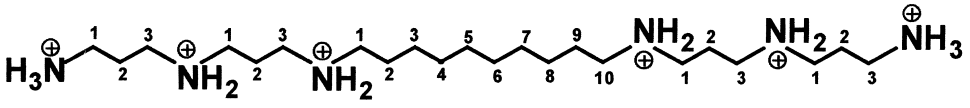


1b
(3 –12– 3, n = 12)

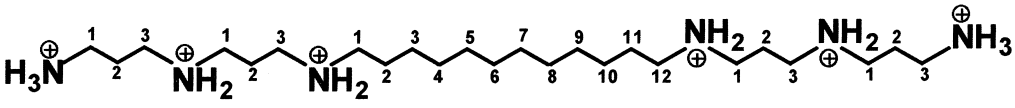


1c
(3 –14– 3, n = 14)

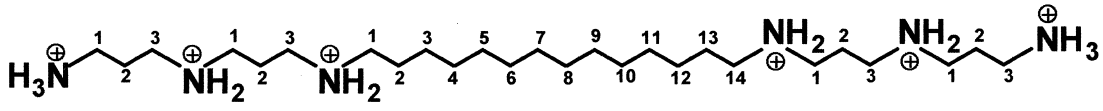
Hexamines:



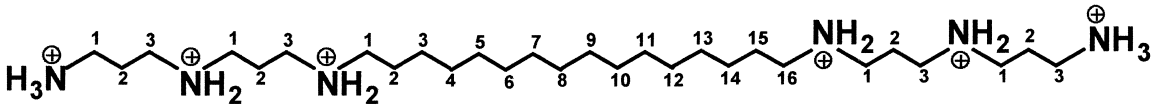
2a
(3 – 3 – 10 – 3 – 3, n = 10)



2b
(3 – 3 – 12 – 3 – 3, n = 12)



2c
(3 – 3 – 14 – 3 – 3, n = 14)



2d
(3 – 3 – 16 – 3 – 3, n = 16)

Figure 1. Structural representations of spermidine, spermine, and new synthetic tetramines and hexamines used in the present study. The polyamines within each group differ in the length of the central methylene chain expressed in the number of CH_2-groups (*n*).

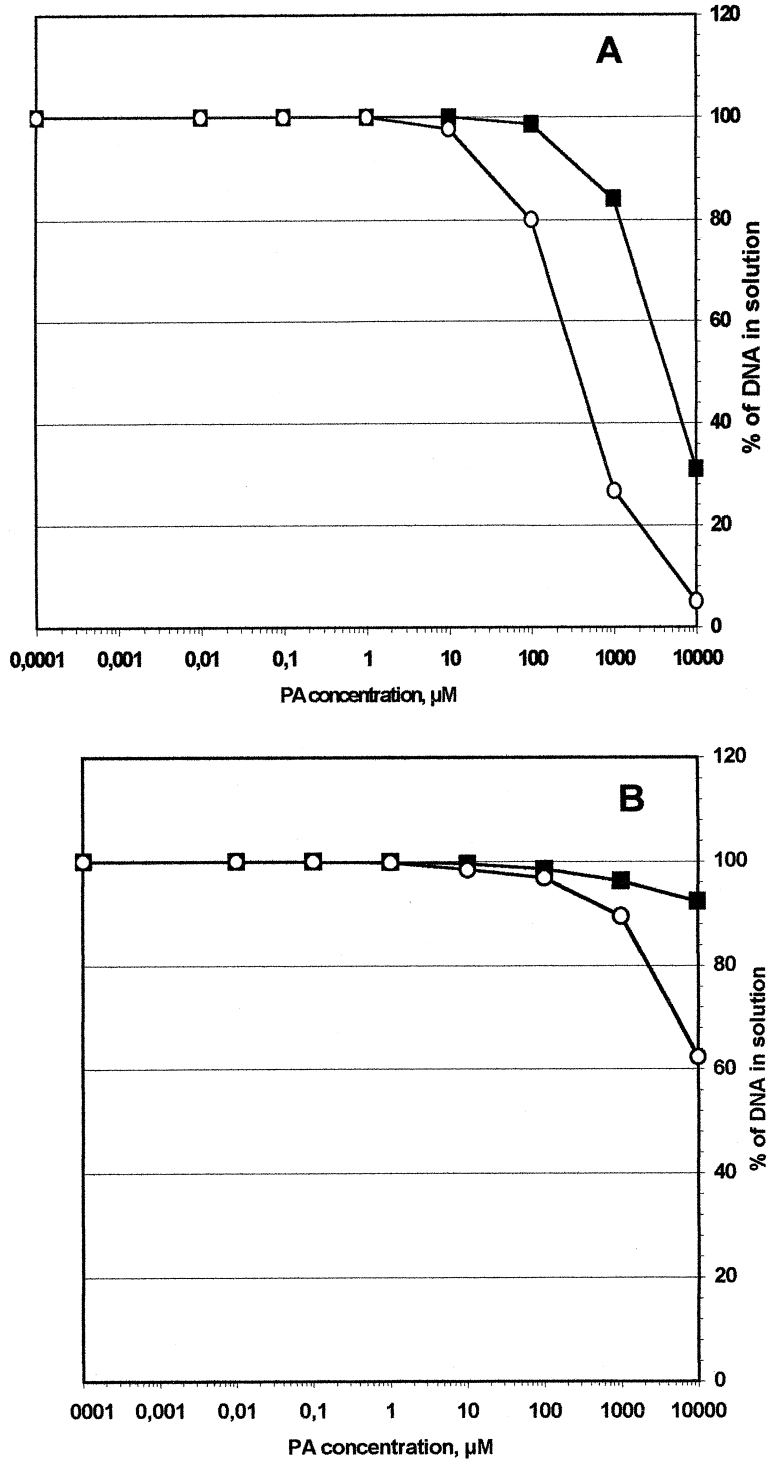


Figure 2. DNA precipitation induced by spermine (○—○) and spermidine (■—■) under unsalted and physiological salt conditions. Panel A: unsalted conditions. The DNA concentration was kept constant and equal to 7.2×10^{-6} M. Panel B: physiological salt (150 mM NaCl) conditions. The DNA concentration was kept constant and equal to 7.2×10^{-5} M.

Effects of synthetic polyamines on the plasmid DNA relaxation induced by human DNA topoisomerase I

We have analyzed the effects of the total charge and of the length of central methylene chain of synthetic polyamines on the enzymatic properties of top1. No stimulatory effects were found for polyamines derivatives of groups 1 or 2. In contrast, the presence of synthetic polyamines inhibited the supercoiled plasmid DNA relaxation by top1 in a concentration-dependent manner (Fig. 4).

Inhibitory activities of tetramines depend on the length of central methylene chain within the polyamine molecule. The r_{rlx} values which correspond to the polyamines/DNA (phosphate) ratios necessary for 50%-inhibition of DNA relaxation by top1 were found to be

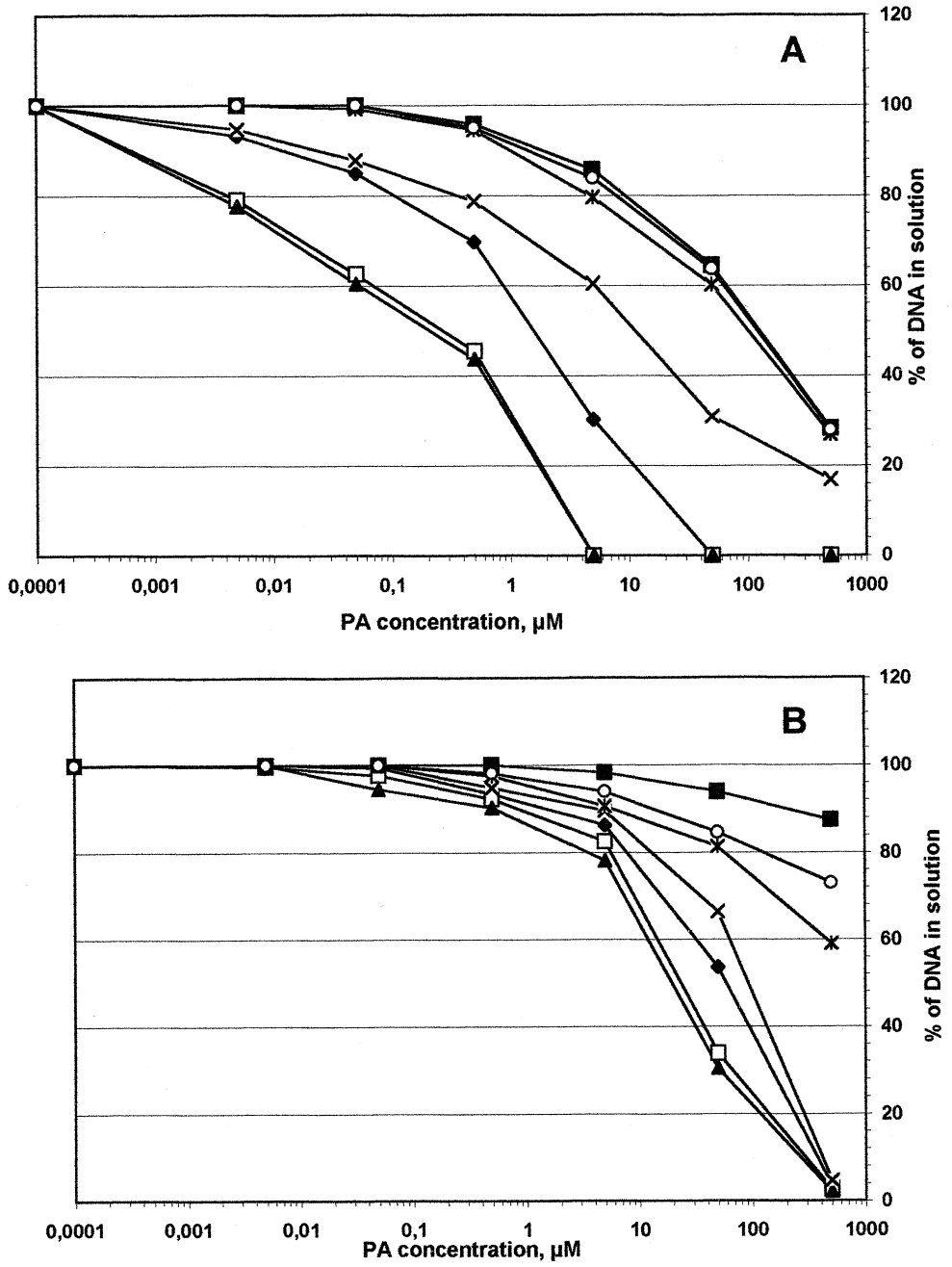


Figure 3. DNA precipitation induced by synthetic polyamines (**1a** ■—■; **1b** ○—○; **1c** *—*; **2a** x—x; **2b** ◆—◆; **2c** □—□; **2d:** ▲—▲) under unsalted and physiological salt conditions. Panel A: Unsalted conditions. The DNA concentration was kept constant and equal to 7.2×10^{-6} M. Panel B: Physiological salt (150 mM NaCl) conditions. The DNA concentration was kept constant and equal to $7{,}2\times10^{-5}$ M.

equal to 3.3 for **1c** ($n=14$) and higher than 100 for **1a** ($n=10$) and **1b** ($n=12$) (Fig. 4, panel B). So, the top1 inhibition activity of tetramines increases dramatically (more than 30-fold) with the increase of the length of their central methylene chain from $n=10$ and $n=12$ (analogues **1a** and **1b**, respectively) to $n=14$ (**1c**).

Hexamines exhibit much higher top1 inhibition activities compared with the derivatives of group 1. The corresponding r_{rlx} values were found to be 0.43; 0.5; 2.0, and 2.7 for hexamines **1d**, **1c**, **1b**, and **1a**, respectively (Fig. 4, panel A). So, the difference between the top1 inhibitory activities of derivatives **2d** and **2c** or **2b** and **2a** was found to be negligible, whereas it increases more than 4-fold on going from the derivatives with $n=10$ and 12 (**2a** and **2b**, respectively) to derivatives with $n=14$ and 16 (**2c** and **2d**, respectively) (Fig. 4, panel A).

Effect of synthetic polyamines on the human DNA topoisomerase I—mediated DNA cleavage

The other way to determine top1 catalytic activity involves an analysis of the DNA cleavage pattern. The restricted DNA fragment containing the inserted DNA sequences corresponding to top1 recognition specific sites[16] was used as an enzyme substrate.

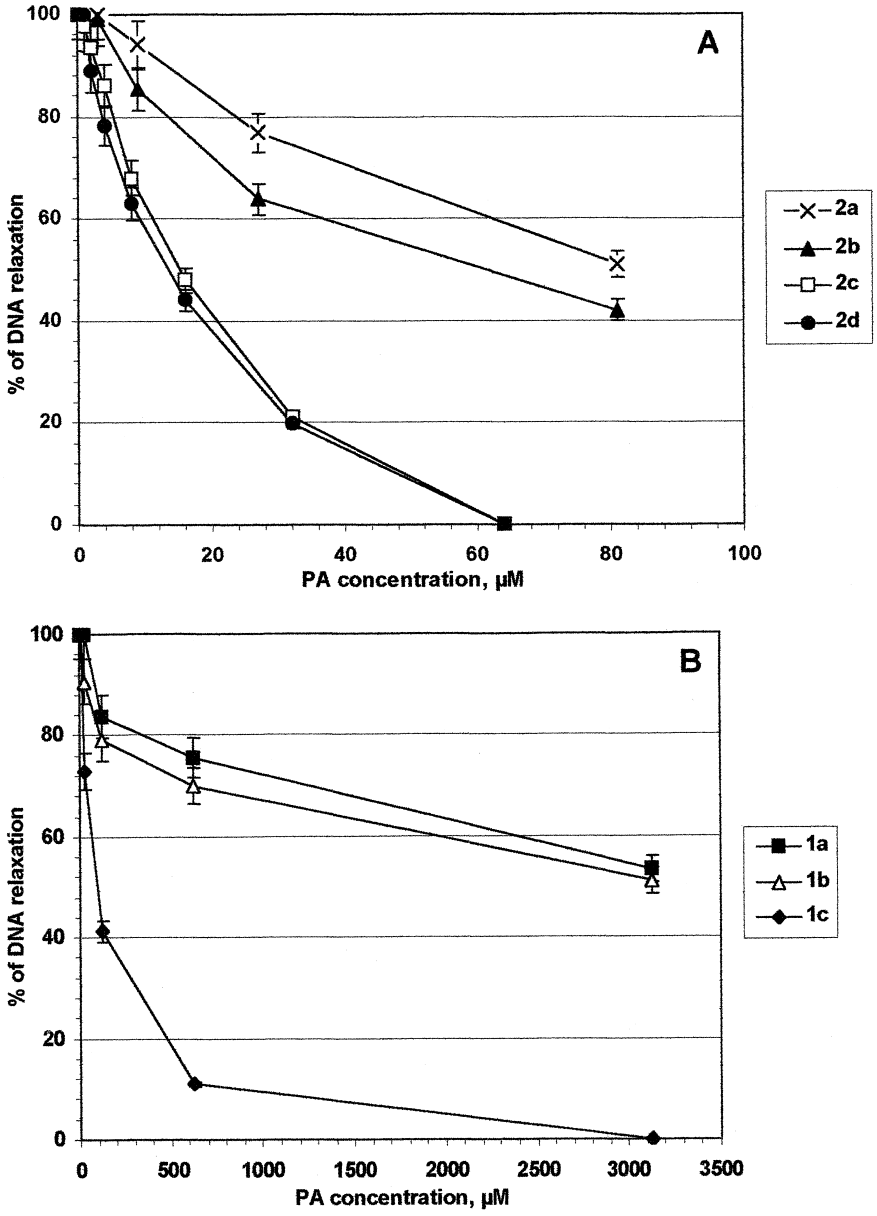


Figure 4. Inhibition of the human DNA topoisomerase I-mediated plasmid DNA relaxation. Panel A: synthetic hexamines **2a** (x—x); **2b** (▲—▲); **2c** (□—□); **2d** (●—●). Panel B: synthetic tetramines **1a** (■—■); **1b** (△—△); **1c** (◆—◆).

Figure 5 shows specific pattern of cleavage of this DNA fragment by top1 with and without synthetic polyamines. In the absence of polyamines and in the presence of molar excess of enzyme, top1 is able to recognize its specific cleavage sites (A–H) which are clearly revealed after analysis of DNA cleavage products by PAG-electrophoresis (Fig. 5). The presence of some of the synthetic polyamines at the 10–1000 μM concentrations does not change the DNA sequence preference of enzyme but decreases the intensities of DNA cleavage in all top1 specific sites. The site by site quantitative analysis of DNA cleavage shows that the hexamines inhibit the cleavage in a concentration-dependent manner whereas the tetramines do not (Fig. 6). No inhibition of top1-mediated DNA cleavage by spermine or spermidine has been found under the same experimental conditions (data not shown).

The DNA cleavage inhibition effect of the hexamines is found to be determined by the length of central methylene chain of the molecule. Within group 2 of the polyamines, the derivative **2a** ($n = 10$) is found to be 12-fold less potent than the all other studied hexamines (Fig. 6).

Polarized microscopy of the DNA aggregates

DNA aggregates obtained in the presence of spermine or synthetic tetra- or hexamines have been observed by polarizing microscopy (Fig. 7). The photographs presented in panels 1–6 correspond to the DNA aggregation induced by polyamines under physiological salt conditions, at which the top1-mediated DNA relaxation assays have been done. The photographs presented in panels 7 and 8 correspond to the DNA aggregation

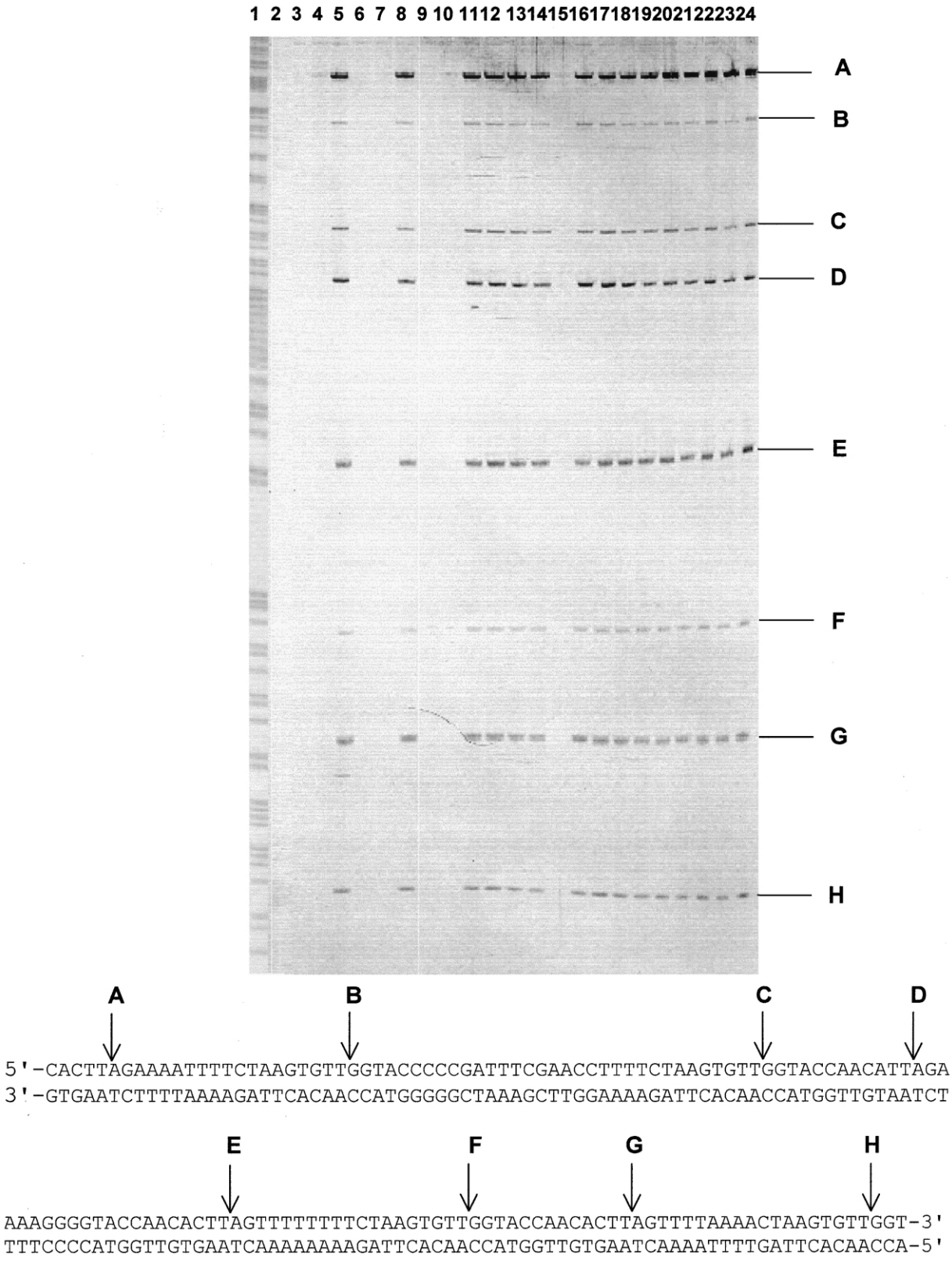


Figure 5. Site-specific DNA cleavage by human DNA topoisomerase I in the presence of synthetic polyamines. ^{33}P-labeled at the 3′-end DNA fragment was obtained by cleaving model plasmid pGEM7Zf(+)-1454 with *HindIII* and *ApaI* (lane 2) and incubated with the DNA topoisomerase I (lane 24, control). DNA cleavage by topoisomerase I was induced in the presence of synthetic polyamines **2b** (lanes 3–5), **2c** (lanes 6–8), **2d** (lanes 9–11), **1c** (lanes 12–14), **2a** (lanes 15–17), **1a** (lanes 18–20), or **1b** (lanes 21–23) at the 1000 μM (lanes 3, 6, 9, 12, 15, 18, and 21); 100 μM (lanes 4, 7, 10, 13, 16, 19, and 22), or 10 μM (lanes 5, 8, 11, 14, 17, 20, and 23) of the polyamines. DNA breaks were analyzed with 8% sequencing gels after treatment of DNA fragment by SDS and proteinase K and visualized by autoradiography. Horizontal lines (A–H) indicate positions of the topoisomerase I-specific DNA cleavage sites. Lane 1: chemical sequence A+G. The lower panel presents the sequence of the double-stranded DNA fragment used in this experiment. Vertical arrows (A–H) indicate again the positions of topoisomerase I cleavage sites within the sequence of the DNA fragment.

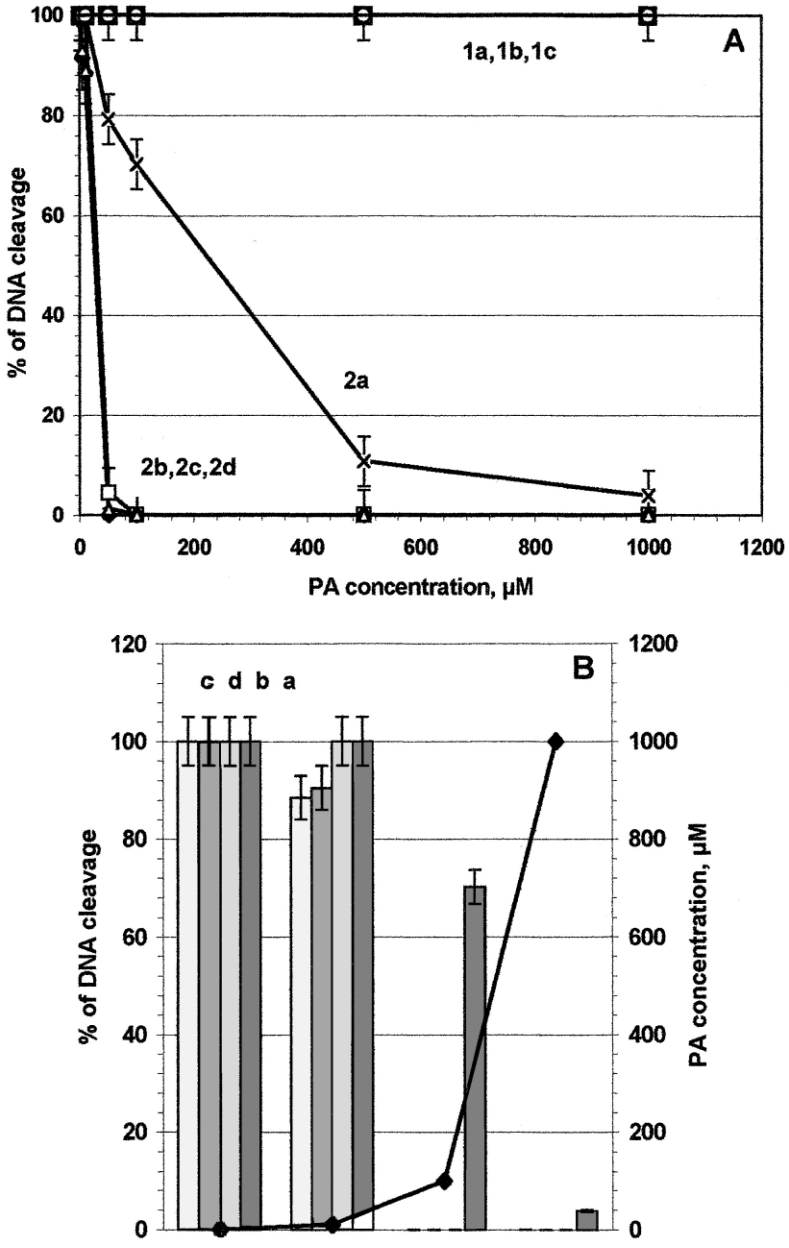


Figure 6. Inhibition of topoisomerase I-mediated DNA cleavage by synthetic polyamines (panel A). Panel B is the different presentation of the same data as in panel A in order to show the differences in the DNA cleavage inhibition activities for the hexamines.

under unsalted conditions, at which the top1-mediated DNA cleavage assays were proceeded. The polyamines/DNA ratios used here are equal to the r_p determined for each polyamine in the DNA precipitation assays.

In the presence of spermine there is a separation of phases (Fig. 7, panel 1): a cholesteric-type fluid phase with small regions of a more concentrated phase. This effect of spermine-induced DNA aggregation was described previously.[22] DNA condensed in the presence of hexamines **2b** ($n=12$) (panel 2) or **2a** ($n=10$) (not shown) and tetramines **1b** ($n=12$) (panel 5) or **1a** ($n=10$) (not shown) yields similar results to the spermine-induced phase separation. In contrast, the regions of the concentrated phase are larger and tend to form liquid crystals type aggregates, as evidenced by their birefringence in polarized light. The hexamines **2c** ($n=14$) (panels 3 and 4) and **2d** ($n=16$) (not shown) or tetramine **1c** ($n=14$) (panel 6) yield very big and well-structured aggregates corresponding to the concentrated phase whereas the small regions of the fluid concentrated phase practically disappear. In these last figures, we note that between crossed polarizers, the intensity of the transmitted light from the concentrated structured phase is much more intense than from the concentrated phase induced by spermine, tetramine **1b** or hexamines **2a** and **2b**, which suggests a denser and regular packaging of the DNA helices. The concentrated phases of the DNA condensed in the presence of hexamines **2c** and **2d** and of tetramine **1c** are highly birefringent. The structure is similar to that of a columnar hexagonal liquid crystal (panel 4). Similar structures were described for the cobalthexamine-induced DNA aggregates by Pelta et al.[21]

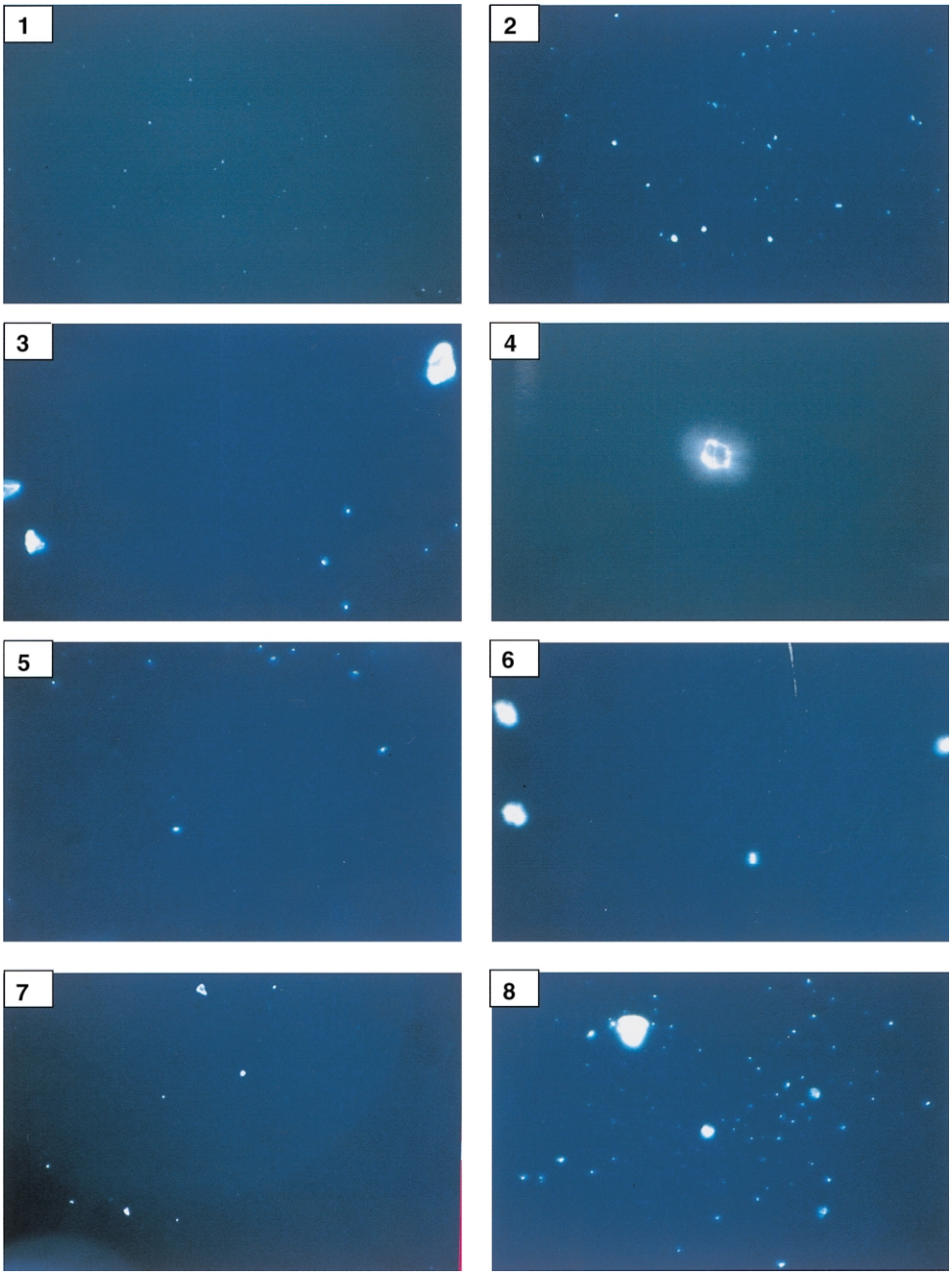


Figure 7. Polarized microscopy of the DNA aggregates. Polarizing microscopy of DNA aggregates formed by addition of spermine (panel 1); hexamines **2b** ($n=12$) (panel 2) and **2c** ($n=14$) (panels 3 and 4); tetramines **1b** ($n=12$) (panel 5) and **1c** ($n=14$) (panel 6) under physiological salt conditions. DNA aggregates formed by the hexamines **2a** ($n=10$) and **2b** ($n=12$) under unsalted conditions are presented in the panels 7 and 8, respectively. Magnification is ×400 for the panels 1–3 and 5–8, and ×800 for panel 4. DNA concentration: 1 mg/mL. The polyamines/DNA ratios used for DNA aggregation were equal to r_p (the calculated polyamines/DNA molar ratios necessary for precipitation of the 50% of DNA) determined for each polyamine in the DNA precipitation assays.

The structure of the DNA aggregates formed under unsalted conditions by the hexamines **2b** ($n=12$) (panel 8), **2c** ($n=14$), and **2d** ($n=16$) is shown to be very different from the aggregates formed by the hexamine **2a** ($n=10$) (panel 7). Much larger and more highly structured aggregates are found to be produced by hexamines with the $n=12$–16, than by hexamine **2a** ($n=10$).

Discussion

It is known, that polyamine-induced DNA aggregation increases upon an increase in the total charge of polycations and of the length of their central methylene chains.[10,22] Effect of electrostatic interactions is discussed in details in an excellent review of Bloomfield.[23]

An increase of the length of the central methylene chain within the polyamine molecule may play a role of important geometrical parameter enabling to make a cross-links between the different DNA molecules or between the different domains of the same DNA molecule in the process of DNA aggregation.[21] The variation of the length of the central methylene chain induces also the variations of the polyamines pKa values which may modify the DNA-aggregation potency of polyamines.[24]

All synthetic polyamines studied in our work demonstrate more potent DNA aggregation, compared with spermine or spermidine, and the DNA aggregation by hexamines was found to be much more potent than that by tetramines. Accordingly, we supposed that the relative DNA aggregation abilities within the each group of new synthetic polyamines (Fig. 1) should be determined by the length of their central methylene chains. Surprisingly, all synthetic tetramines studied under unsalted conditions demonstrated nearly the same DNA aggregation abilities, whereas addition of 150 mM NaCl induces the differences of potencies of DNA aggregation by tetramines. The hexamines relative potencies of DNA aggregation under both, unsalted and physiological salt, conditions were found to be quite different. Under unsalted conditions, the two shortest hexamines are nearly equipotent, whereas the longest hexamines are 20- and 30-fold more potent, respectively (Fig. 8, panel A, solid line). The physiological concentration of salt induces a differential effect on the relative DNA aggregation abilities of hexamines. The shortest hexamines show again nearly the same potencies of DNA aggregation, whereas the relative DNA aggregation by the longer hexamines **2c** and **2d** is strongly suppressed by salt (Fig. 8, panel A, dashed line).

Finally, comparison of the data on DNA aggregation by synthetic polyamines under unsalted and physiological salt conditions leds to the following conclusions: (i) all synthetic tetramines induce nearly the same DNA aggregation under unsalted conditions, whereas in 150 mM NaCl, tetramines with longer central methylene chain are more potent; (ii) DNA aggregation by hexamines under physiological salt conditions shows a linear dependence on the length of the central methylene chain (Fig. 8), whereas more than 20-fold increase of the DNA aggregation potency is found under unsalted conditions when going from the derivative **2b** ($n=12$) to derivative **2c** ($n=14$). So, an addition of salt strongly affects the relative abilities of DNA aggregation by hexamines with the longer central methylene chains, but induces much less effect on hexamines with the shorter chains.

It is assumed that multivalent cations bind electrostatically near the DNA surface, and cause DNA condensation by their correlated fluctuations and effects on water polarization.[23] The precipitate can be either solid (a true crystal) or can be in an amorphous state (either solid (a flocculate) or liquid (a coacervate)). The term 'complex coacervation' has been introduced in order to describe the phase separation

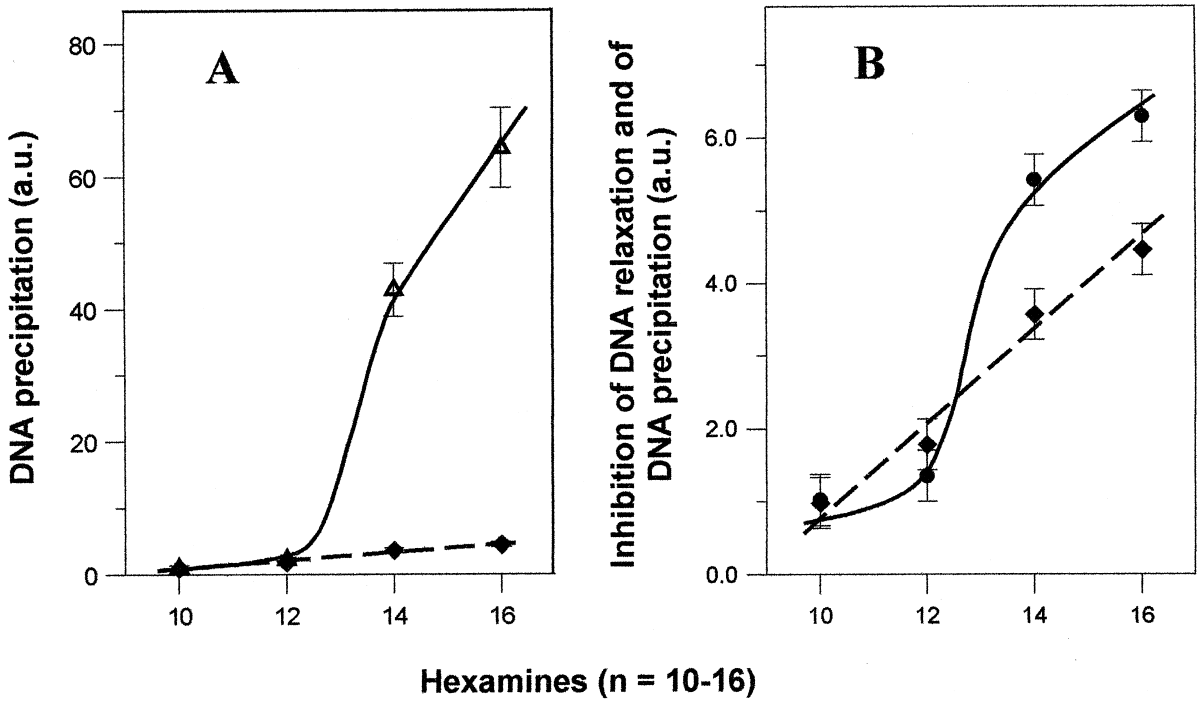


Figure 8. DNA precipitation abilities and topoisomerase I-mediated plasmid DNA relaxation inhibitory activities of synthetic hexamines. Panel A: relative DNA precipitation abilities of hexamines **2a** ($n=10$), **2b** (12), **2c** (14) and **2d** (16) under unsalted (solid line) and physiological salt (dashed line) conditions. Panel B: comparison of the relative DNA precipitation abilities (dashed line) and topoisomerase I-mediated plasmid DNA relaxation inhibitory activities (solid line) of synthetic hexamines under physiological salt conditions. The effects are expressed in terms of r_p (calculated polyamines/DNA molar ratios necessary for precipitation of 50% of DNA) or r_{rlx} (calculated polyamines/DNA molar ratios necessary for 50%-inhibition of plasmid DNA relaxation by human DNA topoisomerase I) and these values are normalized on DNA precipitation ability or DNA relaxation inhibitory activity, respectively, of derivative **2a** ($n=10$)—the shortest hexamine.

occurring in the process of polyelectrolytes precipitation in the presence of micro-ions or polycations. Several forces are supposed to be implicated in the stabilization of the DNA aggregates: van der Waals' interactions,[25] cross-links,[26] and hydration forces.[27] The role of electrostatic interactions was found to be a principle one in stabilizing the DNA aggregates,[23,28,29] whereas an existence of cross-links is supposed to be incompatible with the fluidity of the condensed phase.[21] The detailed polarized microscopy analysis of DNA aggregates induced by natural polyamines and by the inorganic cation cobalthexamine has been performed by Pelta et al.[21] If spermine and spermidine condense DNA into highly fluid anisotropic structures, described as a complex coacervate, cobalthexamine yields only densely packed non-fluid and well-organized anisotropic DNA structures.[21]

Our polarizing microscopy data demonstrate the clear differences of the structures of DNA aggregates produced by polyamines with the different length of central methylene chain (Fig. 7). DNA aggregation by spermine yields a highly fluid cholesteric phase with only microscopic insertions of clusters corresponding to a more concentrated phase (panel 1), very similar to this described by Pelta et al.[21] DNA aggregation by synthetic tetramines and hexamines with the shortest central methylene chain produces more clusters of the concentrated phase with the tendency to form a crystal (panels 2 and 5). Finally, the polyamines with the longest central chain show a threshold effect in yielding very big and well-organized aggregates with a high packing density of DNA molecules. These aggregates are strongly birefringent in polarized light and their structure is similar to the columnar hexagonal liquid crystal (panel 4). The aggregates produced by the polyamines with the longest central chain are very similar to those yielded by cobalthexamine,[21] where a high packing density of the DNA molecules is in agreement with interhelical spacing measured by X-ray diffraction: 27.5[26] or 28.2 Å[27] for cobalthexamine compared with 29.2 Å for spermine.[26]

The similarity of polarized microscopy images of aggregates produced by polyamines with the longest central chains (Fig. 7) with those reported for cobalthexamine[21] suggests that the longest polyamines are producing well-structured and densely packed 'non-fluid' DNA aggregates. The general thermodynamic considerations make evident that creation of the cross-links between the different DNA molecules as well as between the distinct domains of the same DNA molecule should be much more sensitive to the competitive salt effect than that for the other forces contributing to DNA aggregation. Together with the polarized microspectroscopy data, this effect may explain the differential modulation of the polyamine aggregation potencies by the physiological salt concentration.

It is interesting to note that cobalthexamine, in contrast to monovalent cations or spermine and spermidine, completely inhibits top1.[9] The effect of the structure of DNA aggregates on the DNAse I catalytic activity was described by Baeza et al.[30] Comparative electronic microscopy and enzymatic studies demonstrated direct correlation between the presence of highly regular condensed DNA structures and the DNA resistance to the action of DNAse I.

In contrast to the natural polyamines spermidine and spermine,[9,10,31] the synthetic polyamines, studied in our work, are not able to stimulate the top1 catalysis but demonstrate differential top1 inhibition activity depending on the total charge of the molecules and on the length of their central methylene chain. Comparison of experimental conditions used at the construction of phase diagrams for spermidine and spermine published before[20,32] with our data on the abilities of spermidine and spermine to induce the DNA aggregation shows, that the natural polyamines stimulate top1 when the DNA is aggregated. So, the DNA aggregation itself may not be the factor leading to top1 inhibition. Stewart et al.[9] have analyzed the potential mechanisms whereby the cations could effect a stimulation of top1 activity. For example, the cations are known to shield the negative charge of the phosphate backbone of duplex DNA, which in addition to allowing the two strands to wind tighter also reduces the effective diameter of the double helix,[33] making it more favorable for two duplexes to lie on top of each other to form a node. Since it has been shown that top1 has a preference for binding to nodes,[34,35] it could be envisioned that the presence of cations facilitated node recruits top1 to supercoiled DNA, thereby effectively increasing activity.

Our enzymatic studies show that all the analyzed synthetic polyamines do not induce any top1 stimulatory effect under the same DNA aggregation conditions when the natural polyamines do. Moreover, the clear structure-related top1 inhibitory effects have been identified within both groups of synthetic polyamines. The top1-mediated plasmid DNA relaxation inhibitory activities of the tetramines under physiological salt conditions are strongly dependent on the length of central methylene chain of the molecules. The r_{rlx} values for the shortest tetramines are nearly the same, whereas they are more than 30-fold higher for the longest derivative. So, an increase of the length of the tetramines central methylene chain from 12 to 14 CH_2-groups induces the threshold effect in the anti-top1 activity. A similar effect has been detected for the hexamines, which were found to be much more potent inhibitors of the top1-mediated plasmid DNA relaxation, compared with tetramines (compare the $r_{rlx} = 2{,}7$ for hexamine **2a** ($n = 10$) with $r_{rlx} > 100$ for tetramine **1a** ($n = 10$), for example). If the relative potencies of hexamines are compared, the top1 inhibitory activities are increased more than 4-fold on going from the derivative **2b** ($n = 12$) to derivative **2c** ($n = 14$), whereas hexamine **2b** ($n = 12$) is only 1.3-fold more potent than **2a** ($n = 10$) and derivative **2d** ($n = 16$) is only 1.2-fold more potent than **2c** ($n = 14$). Finally, our data show that the top1 inhibitory activities of polyamines do not correlate with their DNA aggregation potencies measured at the same experimental conditions.

Comparison of the effects of natural and synthetic polyamines on the pattern of top1-mediated DNA cleavage does not reveal any effects of spermine or

spermidine or of tetramines at the polyamine concentrations used. By contrast, hexamines induce pronounced structure-dependent effect of DNA cleavage inhibition: the longest hexamines show nearly equipotent DNA cleavage inhibition activities, whereas the shortest one (**2a** ($n = 10$)) was found to be 12-fold less active. So, an inhibition of DNA cleavage by hexamines does not depend on their DNA aggregation abilities, but is determined by the length of their central methylene chain.

One possible explanation of the difference between tetra- and hexamines DNA cleavage inhibition activities under unsalted conditions is as follows. Top1 is able to bind the exposed top1-specific DNA sequences within the spermine or tetramine-formed DNA aggregates and to cleave and to religate DNA without dissociation due to the processive character of the catalysis under unsalted conditions.[32] It is likely, that at the same extent of DNA aggregation by hexamines, these compounds are much more potent in suppression of the recognition of the DNA specific sites by top1 and/or in their ability to change the DNA topology—an effect certainly influencing the affinity of enzyme binding to its substrate.[36] In contrast to the natural polyamines spermine and spermidine, the new synthetic tetramines and hexamines used in our studies induce top1 inhibition. Moreover, the potencies of top1 inhibition do not correlate with the degree of DNA aggregation, but depend on the polyamines structure. This means that the types of structures of the DNA aggregates formed by the natural or by the different synthetic polyamines at the same extent of the DNA aggregation may play a key role in the top1 catalysis.

Comparative enzymatic and microscopic studies of synthetic tetra- and hexamines and natural polyamine spermine demonstrate that a threshold top1 inhibitory effect by polyamines with the longest central methylene chain may be explained by their ability to form highly condensed non-fluid aggregates with a columnar hexagonal liquid crystal structure. It is not clear if the appearance of these structures blocks an access of the enzyme to its specific recognition sites, or if the top1 binds DNA but is not able to cleave and transfer the very densely packed DNA strand. It is possible, that upon an increase of the length of the central methylene chain of the polyamine molecule from $n = 12$ to 14 (Fig. 1) under physiological salt conditions or from $n = 10$ to 12 under unsalted conditions, polyamine becomes able to make much more cross-links between the different DNA molecules or between the distinct domains of the same DNA molecule. This should lead to DNA aggregation within a densely-packed highly condensed phase and to induce a threshold increase of top1 inhibition—an effect demonstrated in our study.

Conclusions

We designed two homologous series of tetramines (**1a**–**c**) and hexamines (**2a**–**d**) differing in the length of their central methylene chain (Fig. 1) and compared their effects within the DNA aggregation and top1 inhibition assays. Hexamines are shown to be much more efficient inhibitors of the DNA relaxation by top1 than tetramines and inhibit the top1-mediated DNA cleavage while the tetramines do not.

Although the DNA aggregation abilities within two series of polyamines correlate with the length of their central methylene chain, the top1 inhibition does not show the same correlation. The threshold top1 inhibitory effect was found within both groups of synthetic polyamines on going from analogues with the $(CH_2)_{10–12}$ to analogues with the $(CH_2)_{14–16}$ central methylene chains (Fig. 1). When compared under conditions of equal DNA aggregation potency, the structure of polyamine-induced DNA aggregates was found to depend on the polyamine central methylene chain's length. DNA aggregation by spermine as well as by synthetic polyamines with the shortest ($(CH_2)_{10–12}$) central methylene chains yields a highly fluid cholesteric-type DNA aggregate. By contrast, the polyamines with the longest central chains ($(CH_2)_{14–16}$) produce very different non-fluid aggregates with a high packing density of DNA molecules being very similar to hexagonal columnar liquid crystals. Polyamines yielding aggregates of the second type demonstrate a threshold top1 inhibitory effect, whereas polyamines yielding the fluid aggregates do not.

Described structure–function correlation within these polyamines' series may serve as a guide for the synthesis of new polyamines with desired DNA-aggregation or anti-top1 activities.

Experimental

Materials

Natural polyamines spermine and spermidine were purchased from Sigma (St Quentin Fallavier, France). New synthetic polyamines (Fig. 1) were synthesized as described.[12] Concentrated 10 mM polyamines stock solutions were prepared in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) and diluted to desired concentration immediately before use. The polycations concentration range used in our study was 0–1 mM for the synthetic polyamines and 0–10 mM for the natural polyamines.

Calf thymus (CT) DNA was purchased from Sigma (St Quentin Fallavier, France), dissolved in TE buffer to 5 mg/mL stock solution and dialyzed against the same buffer. The purity of DNA was estimated by calculation of the A_{260}/A_{280} ratio being equal to 1.89. The DNA molar concentration (in phosphate) was determined by applying molar extinction coefficient $\varepsilon = 6600\ M^{-1}\ cm^{-1}$.[13]

pGEM-7Z(+f) plasmid and restriction endonucleases were purchased from Promega (Charbonnières, France) and *E. coli* strain "Sure" was purchased from Stratagene (Amsterdam, The Netherlands). Bovine pancreatic DNase I and Klenow fragment of *Escherichia coli* DNA polymerase I were purchased from Sigma (St Quentin Fallavier, France) and Boehringer (Blanquefort, France), respectively.

Recombinant 68-kDa human DNA top1 was purified to homogeneity from insect cells using a two-step procedure as described.[14,15] Specific activity of top1 used in our assays was found to be 1.8×10^6 units/mg, where one unit of activity is determined as an amount of enzyme yielding 100% of relaxation of 300 ng of supercoiled pGEM-7Z + (f) plasmid DNA in 30 min at 37 °C.

DNA relaxation assay

0.3 μg (0.15 pmol) of supercoiled pGEM7Z + (f) plasmid DNA was incubated for 5 min at 25 °C with two units of human DNA top1 in 30 μL of reaction mixture containing 10 mM Tris–HCl, pH 7.8, 5% glycerol, 0.5 mM EDTA, 0.3 mM 2-ME, and 0.15 M NaCl. Modulation of the top1-mediated plasmid DNA relaxation by the polyamines was tested as follows: the aliquots of the polyamine stock solutions were added to the reaction mixture and incubated during 20 min at room temperature before addition of the enzyme. The reaction of DNA relaxation by the top1 was stopped by addition of 0.5% SDS and the products were analyzed by electrophoresis in a 0.8% agarose gel running in TAE buffer. Gels were stained with ethidium bromide and photographed under UV. The percent of relaxed form of DNA was determined by densitometry analyses of gels using BioRad software. The polyamines potencies of inhibition of the DNA relaxation by human DNA top1 were expressed in r_{rlx} (the calculated polyamines/DNA (phosphate) ratios necessary for 50%-inhibition of DNA relaxation by human DNA top1).

DNA plasmid construct

The human top1 DNA substrate were prepared in the form of plasmid construct containing a number of specific top1 cleavage sites following the protocol described elsewhere.[16] The plasmid DNA construct was cleaved with *HindIII* and *ApaI* and their restricted fragment was 3′-end-labeled with [α-^{33}P]dATP in the presence of the Klenow fragment of DNA polymerase I, according to protocol.[17] The uniquely 3′-labeled DNA fragments were purified by electrophoresis on non-denaturing 5% (w/v) polyacrylamide gels and isolated by electroelution followed by ethanol precipitation.

Topoisomerase cleavage assays

DNA cleavage by the top1 was carried out by incubating 50 units of the enzyme with 5 μL of a radioactively labeled DNA fragment (3000–10,000 cpm) in 10 mM Tris–HCl (pH 7.8), 5% glycerol, 0.5 mM EDTA, 0.3 mM 2-ME within 20 μL final volume of reaction mixture.

Analysis of the inhibition of top1-mediated DNA cleavage in the presence of polyamines was carried out under the same experimental conditions, after addition of the various concentrations of polyamines and preincubation of the reaction mixture for 20 min at rt. These samples were further treated with top1 for 20 min at rt, followed by addition of the SDS and proteinase K at the 0.5% (w/v) and 1 mg/mL concentrations, respectively. After incubation for a further 45 min at 37 °C, DNA was purified by phenol extraction, precipitated with ethanol, washed with 70% ethanol and dried.

Gel electrophoresis

DNA samples were dissolved in 1.5 μL of the formamide/dye mixture (90% formamide containing 15 mM EDTA, pH 8, 0.05% bromphenol blue and 0.05% xylene cyanole FF dyes), heated 1 min at 90 °C and applied to 8% denaturing polyacrylamide gel. Electrophoresis was carried out for 65 min at 65 W (2500 V). Before exposure, the gel was fixed with 10% acetic acid and dried on glass pretreated with Bind-silane (LKB). Cleavage products were identified by comparison with 'A + G' Maxam-Gilbert sequencing ladder.

DNA precipitation assay

Equal aliquots of CT DNA were mixed with the diluted samples of polyamines in a buffer containing 1.5 mM Tris–HCl (pH 7.5), 0.5 mM EDTA in the presence or absence of NaCl to obtain various drug/DNA molar ratio in the 200 μL of final volume. The DNA concentration was kept constant and equal to 7.2×10^{-6} M in experiments under unsalted conditions and 7.2×10^{-5} M under physiological salt conditions.

The samples were extensively vortexed, incubated for 20 min at rt and centrifuged for 10 min at 14,000 rpm. The percentage of the nonprecipitated DNA in solution was determined by measurement of absorbance of the supernatant at 260 nm and comparison of the values with the control samples.

The polyamine potencies of DNA precipitation were expressed in r_p (the calculated polyamines/DNA (phosphate) molar ratios necessary for precipitation of the 50% of DNA).

Polarizing microscopy

CT DNA was precipitated from a 1 mg/mL solution in the presence of 150 mM NaCl by various polyamine concentrations. An aliquot of the pellet was recovered and deposited between slide and coverslip. The preparation was observed and photographed between linear crossed polarizers in a Nikon Optishot (Champigny Sur Marne, France) microscope.

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References

1. Cooney, M.; Czernuszewicz, G.; Postel, E. H.; Flint, S. J.; Hogan, M. E. *Science* **1988**, *241*, 456.
2. Tabor, C. W.; Tabor, H. *Annu. Rev. Biochem.* **1984**, *53*, 749.
3. Ha, H. C.; Woster, P. M.; Yager, J. D.; Casero, R. A., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11557 and references therein.
4. Panagiotidis, C. A.; Artandi, S.; Calame, K.; Silverstein, S. J. *Nucleic Acids Res.* **1995**, *23*, 1800.
5. Pommier, Y.; Pourquier, P.; Fan, Y.; Strumberg, D. *Biochim. Biophys. Acta* **1998**, *1400*, 83 and references therein.
6. Lock, R. B.; Ross, W. E. *Anticancer Drug Des.* **1987**, *2*, 151.
7. Jaxel, C.; Kohn, K. W.; Pommier, Y. *Nucleic Acids Res.* **1988**, *16*, 11157.
8. Chen, A. Y.; Yu, C.; Gatto, B.; Liu, L. F. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *90*, 8131.
9. Stewart, L.; Ireton, G. C.; Parker, L. H.; Madden, K. R.; Champoux, J. J. *J. Biol. Chem.* **1996**, *271*, 7593.
10. Srivenugopal, K. S.; Wemmer, D. E.; Morris, D. R. *Nucleic Acids Res.* **1987**, *15*, 2563.
11. Strivenugopal, K. S.; Morris, D. R. *Biochemistry* **1985**, *24*, 4766.
12. Bradley, J. C.; Vigneron, J. P.; Lehn, J. M. *Synth. Commun.* **1997**, *27*, 2833.
13. Wells, R. D.; Larson, J. E.; Grant, R. C.; Shortle, B. E.; Cantor, C. R. *J. Mol. Bol.* **1970**, *54*, 465.
14. Fleury, F.; Ianoul, A.; Kryukov, E.; Sukhanova, A.; Kudelina, I.; Wynne-Jones, A.; Bronstein, I. B.; Maizieres, M.; Berjot, M.; Dodson, G. G.; Wilkinson, A. J.; Holden, J. A.; Feofanov, A. V.; Alix, A. J.; Jardillier, J. C.; Nabiev, I. *Biochemistry* **1998**, *37*, 14630.
15. Bronstein, I. B.; Wynne-Jones, A.; Sukhanova, A.; Fleury, F.; Ianoul, A.; Holden, J. A.; Alix, A. J.; Dodson, G. G.; Jardillier, J. C.; Nabiev, I.; Wilkinson, A. J. *Anticancer Res.* **1999**, *19*, 317.
16. Sukhanova, A.; Grokhovsky, S.; Zhuze, A.; Roper, D.; Bronstein, I. *Biochem. Mol. Biol. Int.* **1998**, *44*, 997.
17. Sambrook, J.; Fritsch, E. F.; Maniatis, T. In *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1989.
18. Basu, H. S.; Marton, L. J. *Biochem, J.* **1987**, *244*, 243.
19. Sikorav, J.-L.; Pelta, J.; Livolant, F. *Biophys. J.* **1994**, *67*, 1387.
20. Saminathan, M.; Antony, T.; Shirahata, A.; Sigal, L. H.; Thomas, T.; Thomas, T. J. *Biochemistry* **1999**, *38*, 3821.
21. Pelta, J.; Livolant, F.; Sikorav, J. L. *J. Biol. Chem.* **1996**, *271*, 5656.
22. Basu, H. S.; Schwietert, H. C.; Feuerstein, B. G.; Marton, L. J. *Biochem. J.* **1990**, *269*, 329.
23. Bloomfield, V. A. *Biopolymers* **1997**, *44*, 269 and references therein.
24. Sumari, K.; Matsuoka, H.; Yamaoka, H. *J. Phys. Chem.* **1996**, *100*, 9000 and references therein.
25. Bloomfield, V. A.; Wilson, R. W.; Rau, D. C. *Biophys. Chem.* **1980**, *11*, 339.
26. Schellman, J. A.; Parthasaratary, N. *J. Mol. Biol.* **1984**, *175*, 313.
27. Rau, D. C.; Parsegian, V. A. *Biophys. J.* **1992**, *61*, 260.
28. Rouzina, I.; Bloomfield, V. A. *J. Phys. Chem.* **1996**, *100*, 9977.
29. Grønbech-Jensen, N.; Mashl, R. J.; Bruinsma, R. F.; Gelbart, W. M. *Phys. Rev. Lett.* **1997**, *78*, 2477.
30. Baeza, I.; Gariglio, P.; Rangel, L. M.; Chavez, P.; Cervantes, L.; Arguello, C.; Wong, C.; Montanez, C. *Biochemistry* **1987**, *26*, 6387.
31. McConaughy, B. L.; Young, L. S.; Champoux, J. J. *Biochim. Biophys. Acta* **1981**, *655*, 1.
32. Krasnow, M. A.; Cozzarelli, N. R. *J. Biol. Chem.* **1982**, *257*, 2687.
33. Rybenkov, V. V.; Cozzarelli, N. R.; Vologodskii, A. V. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5307.
34. Zechiedrich, E. L.; Osheroff, N. *EMBO J.* **1990**, *9*, 4555.
35. Madden, K. R.; Stewart, L.; Champoux, J. J. *EMBO J.* **1995**, *14*, 5399.
36. Pilch, D. S.; Xu, Z.; Sun, Q.; LaVoie, E. J.; Liu, L. F.; Breslauer, K. J. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 13565.