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STIMULATION AND INHIBITION OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA-INDUCED STRAND BREAKS AND INTERSTRAND CROSS-LINKING IN Col E_1 PLASMID DEOXYRIBONUCLEIC ACID BY POLYAMINES AND INORGANIC CATIONS

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Abstract—The influence of various polyamines and metallic cations on 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-induced DNA single-strand breaks and DNA interstrand cross-linking was studied in Col E_1 plasmid using electrophoretic techniques. Spermidine and spermine (0.4 to 1.5 mM concentration range) markedly stimulated BCNU-induced DNA nicking, whereas putrescine had no effect on the nicking process. In contrast to the polyamines, BCNU-induced DNA nicking was decreased by the three inorganic cations, Na^+ (100 and 200 mM), Mg^{2+} (0.5 and 1.5 mM), and Co^{3+} $(NH_3)_6$ (0.2 and 0.4 mM), with the trivalent hexamminecobalt ions being most inhibitory. When the monofunctional *N*-methyl-*N*-nitrosourea (MNU) was used (instead of the bifunctionally active BCNU) to alkylate Col E_1 DNA, nicking of the DNA was inhibited by spermidine. Furthermore, the ability of chloroethylated Col E_1 DNA to form interstrand cross-links after treatment with BCNU was inhibited by 0.5 mM spermidine and 0.5 mM spermine, both concentrations within the intracellular range. Putrescine at 3–6 mM only marginally stimulated DNA cross-linking. In comparison, the inorganic cations all enhanced Col E_1 DNA cross-linking by BCNU, with the rank order of cross-link stimulation being Mg^{2+}, Na^+, and Co^{3+} $(NH_3)_6$. These results provide evidence that polyamines can interact with DNA to modulate chloroethylnitrosourea-induced DNA damage and that the interaction is not only a function of the charge on the polyamine molecule but also of the chemical structure of the polyamine.

The polyamines, spermidine and spermine, and the diamine, putrescine, are present in all living cells at millimolar concentrations and are involved in various aspects of cell proliferation [1–14]. The chemical nature of these cationic molecules confers upon them the ability to interact with nucleic acids, as is manifested by their ability to cause condensation of DNA, increase DNA melting temperature, induce DNA conformational transition, and protect nucleic acids against enzymatic degradation, ionizing radiation, and mechanical shear. These effects have been variously ascribed to the charge neutralization of the phosphate groups of DNA and the stabilization of DNA duplexes [5–8].

A large number of studies have explored the possibility that the alteration of DNA structure caused by polyamine depletion [9] could affect the cytotoxicity of DNA-directed chemotherapeutic agents, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) [10–15]. BCNU is a clinically active chloroethylnitrosourea (CENU) and is known to exert a significant part of its cytoxic action through the formation of DNA interstrand cross-links (ISC) in cells [16, 17]. A potentiation of BCNU cytotoxicity by polyamine depletion has been observed in many tumor cell lines in culture [12–15]; however, efforts to correlate the augmented cytotoxicity with an increased interstrand DNA cross-linking have not been successful in many of these studies [10, 12, 14, 15]. As a part of our continued efforts to understand the molecular mechanisms underlying CENU resistance of tumor cells, the present study was undertaken with Col E_1 plasmid DNA to determine whether polyamines, vis-a-vis inorganic cations, can modulate the induction of DNA strand breakage and DNA interstrand cross-linking by CENUs, and whether the charge and/or chemical structure of the cation is essential for the modulation of the DNA damage.

MATERIALS AND METHODS

Chemicals. Polyamines in their hydrochloride salt form were purchased from the Sigma Chemical Co. (St. Louis, MO), and aqueous stock solutions were neutralized to pH 7. Hexamminecobalt was obtained from the Aldrich Co. (Milwaukee, WI), and dissolved in water. BCNU (Bristol-Myers, Syracuse, NY) stock solutions were prepared by initially dissolving the drug in a small volume of ethanol and diluting with 5 mM sodium cacodylate buffer (pH 7.4), immediately prior to use. Calf thymus DNA, ethidium bromide and agarose were purchased from the Sigma Chemical Co.

Native Col E_1 DNA from *Escherichia coli* JC411 (Thy^-, Met^-, Leu^-, His^-, Arg^-) was prepared by chloramphenicol amplification followed by lysis with

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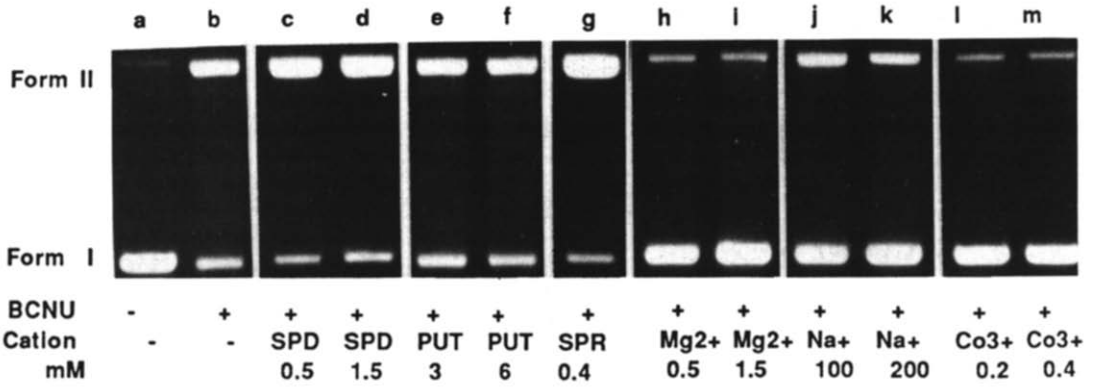

Fig. 1. Influence of polyamines and metal cations on strand scission induced by BCNU in Col E_1 DNA. Plasmid DNA exposed to polyamines (lanes a–g) or the metal cations (lanes h–m) was treated with 2 mM BCNU at 37° for 2 hr followed by agarose gel electrophoresis, as detailed in Materials and Methods. Form I band is closed circular superhelical DNA, and Form II is the nicked or single-strand broken form of Col E_1 DNA. Abbreviations: PUT, putrescine; SPD, spermidine; SPR, spermine; and Co^{3+} hexamminecobalt chloride.

lysozyme and Triton-100, and CsCl/ethidium bromide gradient centrifugation as previously described [18].

Assay for DNA strand break induction by BCNU. The conversion of negatively supercoiled Col E_1 DNA (Form I) to its nicked counterpart (Form II) by BCNU was followed by agarose gel electrophoresis. The reaction mixtures (total volume, 20 μL) containing 0.5 μg native Col E_1 DNA in 20 mM sodium cacodylate buffer (pH 7.4) were treated with the various cations and then with BCNU. The final BCNU concentration was 2 mM. After a 2-hr incubation at 37°, 3 μL of stop buffer/dye mix (10% sodium dodecyl sulfate, 100 mM EDTA: 20% Ficoll and 0.2% Bromophenol Blue) was added, and the samples were kept frozen. Electrophoresis (2 V/cm, overnight) was performed in 1.2% agarose submarine gels in a buffer (pH 8.0) of 50 mM Tris-phosphate and 1 mM EDTA. The gels were then stained for 30 min with 1 μg/mL ethidium bromide, and photographed under UV illumination.

Chloroethylation of Col E_1 DNA by BCNU. This procedure was modified [19] from a previously described method [20]. Twenty-five micrograms of native Col E_1 DNA in 250 μL of 20 mM sodium cacodylate buffer (pH 7.4) was treated with 2 mM BCNU for 10 min at 37°. The DNA was precipitated immediately with cold ethanol and left overnight at −40°. The DNA pellet was washed with 80% ethanol to remove BCNU decomposition products, then dried and, after redissolving, aliquots were taken for the reactions described below.

Agarose gel electrophoresis to detect interstrand cross-linking of Col E_1 DNA. An elegant method for analyzing interstrand cross-linking in closed circular supercoiled DNA molecules has been described by Robins *et al.* [20], based on earlier studies of phage DNA denaturation [21]. The principle involved is as follows: a brief exposure of plasmid DNA in its superhelical form (Form I) to strong alkali (pH > 12.6) results in its collapsing to a compact conformation with the strands out of register (Form IV). The Form IV structure cannot be generated from cross-linked circular DNA, because the latter has its complementary strands locked in register and renatures to the Form I conformation after alkali treatment. Agarose gel electrophoresis can resolve these various topological forms of DNA.

DNA interstrand cross-linking by BCNU was examined in these studies either by incubating chloroethylated Col E_1 for 4–6 hr in the presence or absence of cations, or by exposing native Col E_1 DNA to the various cations for 5 min at 37° and subsequently incubating it with 0.25 mM BCNU for 6 hr. DNA nicking at this BCNU concentration was minimal.

To both samples was added 12 M NaOH stock to achieve a final concentration of 0.5 M NaOH (total reaction volume 25 μL), and denaturation of the DNA was allowed to occur over 3 min. The samples were then neutralized with 3 M potassium acetate (pH 4.5) stock solution. Three microliters of dye-mix/stop buffer (described above) was added, and electrophoresis in neutral agarose gels was performed as detailed earlier.

RESULTS

Influence of polyamines on BCNU-induced strand scission in Col E_1 DNA. An optimal amount of strand scission (nicking) of Col E_1, plasmid DNA was found to occur, in our experimental conditions, at a 2 mM BCNU concentration, which was chosen for further studies. The results of polyamine influence on drug-induced DNA nicking are shown in Fig. 1, lanes a–g. Putrescine at 3 and 6 mM did not affect the induction of DNA strand breaks. However, 0.5 and 1.5 mM spermidine and 0.4 mM spermine (all concentrations within intracellular range) enhanced the nicking of Col E_1 DNA. Polyamines alone did not change the electrophoretic pattern of the DNA.

Effect of inorganic cations on BCNU-induced DNA strand breaks. The influence of mono-, di- and trivalent metallic cations, namely Na^+, Mg^{2+} and $Co^{3+}(NH_3)_6$, on DNA strand break induction as measured by the conversion of native Col E_1 DNA to its Form II by BCNU is represented in Fig. 1,

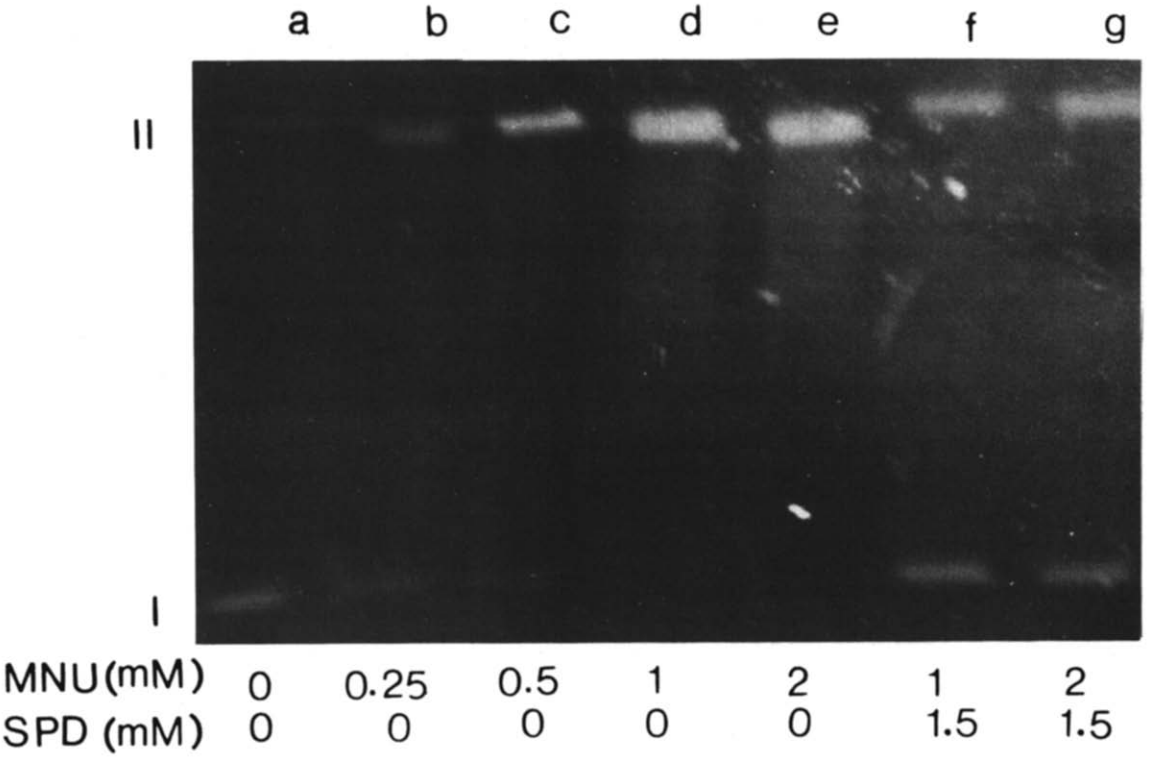

Fig. 2. Induction of single-strand breaks in Col E_1 DNA by *N*-methyl-*N*-nitrosourea (MNU) and its inhibition by spermidine. Supercoiled Col E_1 DNA was treated with MNU (lanes b–g) with (lanes f and g) and without (lanes a–e) spermidine, for 2 hr at 37°, and then electrophoresed to resolve the nicked (Form II) and supercoiled (Form I) forms. Lane a represents just DNA substrate.

lanes h–m. All three inorganic cations inhibited the induction of DNA single-stand breaks by BCNU with an apparent correlation between the extent of inhibition and the valency of the cations. The trivalent hexamminecobalt ion, which mimicks the effect of spermidine in DNA aggregation [5], was in contrast to spermidine, a strong inhibitor of strand break induction by BCNU (compare Fig. 1, lanes c and l). The cations used here are all capable of binding DNA; whether the global ionic strength has any role in strand scission is not clear.

Effect of spermidine on methylnitrosourea-induced strand breaks in Col E_1 DNA. The monofunctional alkylating agent *N*-methyl-*N*-nitrosourea (MNU) can alkylate phosphotriester groups and bases in DNA and generate alkali labile sites, but cannot form DNA–DNA cross-links like BCNU [22]. We, therefore, used MNU as a model agent to investigate the role of alkali-labile sites on the induction of strand breaks in Col E_1 DNA and how this is affected by the polyamines. As shown in Fig. 2, spermidine effectively inhibited MNU-induced DNA strand breaks. The effects of spermidine on MNU and BCNU induction of single-strand breaks in DNA, as demonstrated in Fig. 2, were diametrically opposite.

DNA interstrand cross-linking: Effect of polyamines and inorganic cations. It was necessary to decrease the BCNU concentration to 0.25 mM in the cross-linking experiments as compared to the 2 mM concentration used in the strand break assays. The drug at a lower concentration supported a detectable amount of DNA cross-linking in the assay employed with minimal nicking activity.

The influence of cations on DNA cross-linking was examined by incubating native Col E_1 DNA with the cations followed by treatment with 0.25 mM BCNU for 6 hr. When chloroethylated Col E_1 DNA was used in these experiments to eliminate the possibility of BCNU decomposition products reacting with the cations, particularly the polyamines, essentially no difference in the results was observed. As shown in Fig. 3B, spermine and spermidine decreased the intensity of the Form I (cross-linked DNA) band, whereas putrescine slightly enhanced it. All of the inorganic cations that had inhibited DNA nicking by BCNU (see Fig. 1) stimulated the formation of DNA cross-links, with Mg^{2+} showing the highest stimulatory effect, followed by Na^+ and then hexamminecobalt. Controls of non-BCNU treated and spermidine-treated Col E_1 DNA are shown in Fig. 3A. Similar controls for chloroethylated Col E_1 are shown in Fig. 3C.

DISCUSSION

Polyamines play a significant, regulatory role in normal cell proliferation [1, 3, 23, 24], and are intimately involved in the neoplastic process [4, 25, 26]. Furthermore, oxidation of polyamines by polyamine oxidases has been shown to result in products that inhibit the clonal proliferation of human tumor stem cells *in vitro* [27]. Several studies [11, 15] have attempted to exploit the known effects of polyamines in cell proliferation and of the interaction of polyamines with cellular DNA to potentiate the antitumor action of chloroethylnitrosoureas. BCNU has

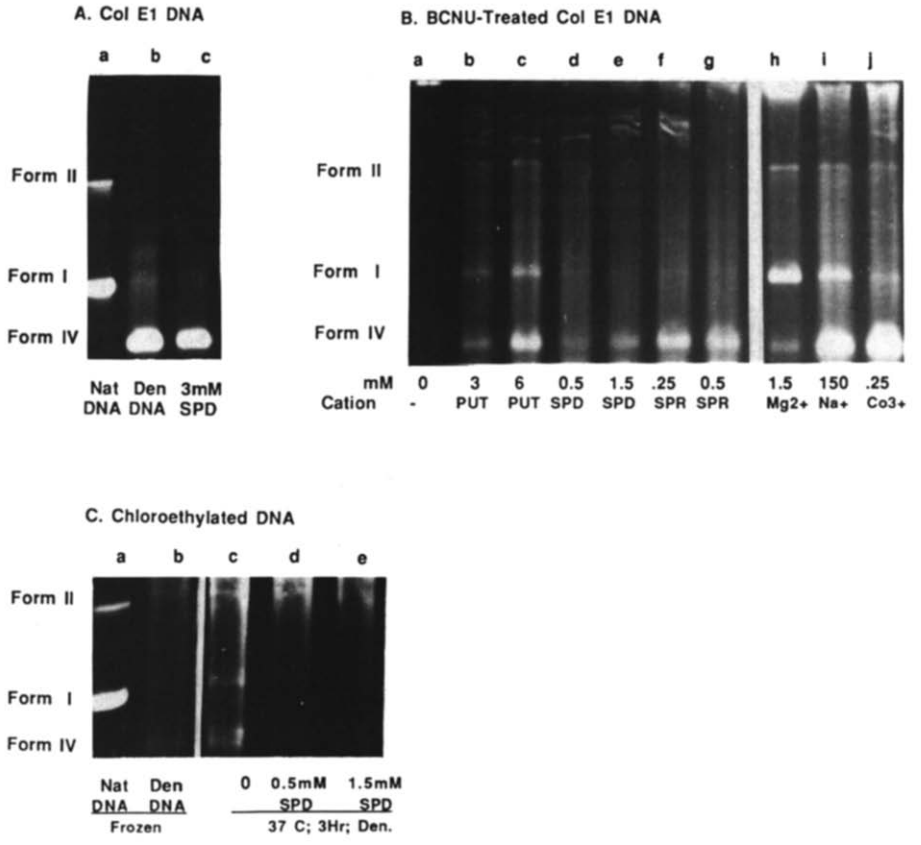

Fig. 3. Effect of polyamines and metal ions on BCNU-induced interstrand cross-linking in Col E_1 DNA. (A) Control runs with Col E_1 DNA that had not been treated with alkylating agent. Lane a: Col E_1 native DNA. Lane b: denatured Col E_1 DNA. Lane c: Col E_1 DNA denatured in the presence of 3 mM spermidine. (B) DNA was incubated with 0.25 mM BCNU and cations at 37° for 4 hr, denatured with NaOH, neutralized, and electrophoresed in 1.2% agarose. (C) Chloroethylated Col E_1 DNA was incubated in the absence (lanes a–c) or presence (lanes d and e) of spermidine. After a 3-hr incubation, samples b–e were denatured + neutralized, and all samples were electrophoresed. Lane a contained just native Col E_1 DNA. Chloroethylated DNA was frozen to prevent formation of DNA cross-links. *Migration of DNA* in panels A–C: Form I—closed circular supercoiled and cross-linked DNA; Form II—nicked and nicked-cross-linked DNA; and Form IV—alkali denatured closed circular DNA.

been shown to induce an increased tumor cell kill in rats and in a number of human and rodent tumor cell lines that are made polyamine deficient by treatment with α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase [12–15, 28]. Based on evidence from viscoelastometric studies in polyamine-depleted 9L brain tumor cells [9], it was suggested that an increased accessibility of DNA to BCNU could result in increased cross-linking of DNA and hence the augmented cytotoxicity [11]. However, evidence in support of this hypothesis has been limited, and the results point to the operation of additional mechanisms of BCNU cytotoxicity in polyamine-deficient cells [12–15].

In the present study, we used plasmid DNA to investigate whether DNA interstrand cross-linking and DNA strand breakage induced by BCNU can be modified by polyamines. The studies were also aimed at determining whether the ions which curtailed DNA strand breakage by BCNU also affected BCNU-induced DNA interstrand cross-linking. Our results demonstrated that polyamine which accentuated DNA nicking also inhibited DNA cross-linking by BCNU. In contrast, the bivalent metallic cation, Mg^{2+}, enhanced the formation of cross-links but inhibited DNA single-strand breakage. The results of the effects of the trivalent hexamminecobalt ion and of spermidine on BCNU-induced DNA nicking and cross-linking were diametrically opposed. This was interesting because not only does hexamminecobalt behave like spermidine and exhibit polyamine-like effects in several enzymatic reactions involving DNA [7, 29] but it also, like spermidine, causes B → Z transition and DNA compaction as a result of polynucleotide charge neutralization [5]. Thus, the modification of DNA damage by spermidine, that we observed, cannot be attributed solely to the charge on spermidine (since the same effects would have been seen with hexamminecobalt), but also would appear to be related to the chemical structure of spermidine. Such structure-dependent effects of polyamines on condensation of DNA [30], DNA melting [31] and aggregation [7] have been well studied with the aid of spermidine analogs. The ability of spermidine analogs to influence the properties of DNA were correlated with the longer methylene chain length of the polyamine molecule.

The basic reactions involved in nitrosourea-induced DNA strand breakage include the alkylation of bases and phosphate groups on DNA, followed by depurination and strand breakage. Strand breakage resulting from base alkylations is a slow process *in vitro*, even at alkaline pH. On the other hand, the alkylated phosphodiesters are readily hydrolyzed at pH 11.8 to result in strand breakage [32, 33]. BCNU at 2 mM and pH 7.0 used in DNA strand break assays is expected to decompose predominantly into chloroethyldiazonium ions and chlorethylisocyanate besides acetaldehyde, dichloroethane, chloroethanol and vinyl chloride to varying extents [34]. The contribution of the minor decomposition products to DNA strand scission cannot be excluded; an example of this kind is the hydrolysis of isocyanates derived from the carbamoylating nitrosoureas to form amines, which in turn can interact with the apurinic sites to cause strand scission [32]. Supportive of this observation is the ability of the diamine putrescine to enhance the rate of chain breakage in depurinated DNA [35, 36] and alkylated DNA [37, 38]. The amines have been postulated to form Schiff base conjugates with the aldehyde form of deoxyribose residues at apurinic sites and lead to enhanced breakage [35, 39].

Our results draw attention to the differential effect of polyamines on alkylated lesions in DNA induced by nitrosoureas. The stimulatory and inhibitory effects of spermidine on BCNU- and MNU-induced strand breaks, respectively, are interesting and may be related both to the different alkylating species generated by the two agents, as well as the carbamoylating products that are produced from BCNU but not MNU. Polyamines ungergo carbamoylation when treated with BCNU [40] or chloroethylisocyanate (unpublished results). While the lability of the *N*-glycosidic bonds at N-7 and N-3 of purines and the O^2-position of pyrimidines in BCNU-treated DNA may be increased at the physiological pH by polyamines through a mechanism described above, the inhibition of MNU-induced breaks by spermidine may be mediated by the shielding of the phosphate groups leading to reduced phosphotriester formation. Consistent with this assumption is the generation of phosphotriesters at a higher frequency by the monofunctional MNU than by BCNU [41].

The mechanism of polyamine inhibition of *in vitro* DNA cross-linking is less clear. Our results with the cross-linking of chloroethylated DNA point to the conformational fluctuations as one of the possible causes for the spermidine-induced inhibition of interstrand cross-linking in the circular supercoiled plasmid DNA. A clear reduction in the single-strandedness of the negatively supercoiled DNA exposed to spermidine and a consequent decrease in S_1 nuclease and topoisomerase I activity was shown by us in a previous study [42]; a similar mechanism may occur in this non-enzymatic study. Clinical alkylators like BCNU exhibit a marked preference for the stretch of guanine sequences in DNA [43, 44]. Stabilization of double-stranded DNA by the spermidine molecule forming a bridge across the minor or major groove [6, 45] in supercoiled plasmid DNA may enhance or suppress the interaction of target sequences with nitrosoureas for alkylation; such a consideration cannot be ruled out in our study. It would be worthwhile to investigate the cross-linking of short DNA fragments by BCNU and the effect of cations thereon. This approach would eliminate the constraint of circularity present in plasmid DNA and may reflect the effect of spermidine solely on double-stranded DNA structure and its stabilization by the polyamine.

The curtailment of DNA cross-linking seen in the presence of spermidine is supportive of the enhanced interstrand cross-linking by BCNU in polyamine-deficient murine tumor cells [11]. However, other considerations such as the direct inactivation of the cytotoxic decomposition products of BCNU, i.e. the carbamoylation of polyamines by the alkylisocyanates [40], and a possible delay in the operation of DNA repair systems [46, 47] resulting in time-dependent increases in cross-linking are worth investigating in polyamine-deficient tumor cells. It is tempting to speculate the involvement of such alternative mechanisms in many human tumor cell lines where CENU-induced cell kill was augmented by α-difluoromethylornithine in the absence of increased DNA cross-linking [10, 12, 14, 15] at 6 hr after drug treatment. Finally, our results also provide an *in vitro* experimental system for testing the ability of the phosphorothioate or other derivatives of polyamines as selective protectors of normal tissues such as bone marrow against the cytotoxic action of CENUs.

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