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Absence of a requirement for long-range DNA torsional strain in the production of protein-associated DNA strand breaks in isolated mammalian cell nuclei by the DNA intercalating agent 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (m-AMSA)

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DNA intercalating agents, such as the clinically active antineoplastic drug 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (m-AMSA), produce in mammalian cells [1] or in isolated nuclei [2] DNA single- and double-strand breaks that appear to be associated with covalently linked proteins [3]. These protein-associated strand breaks were hypothesized to represent topoisomerase–DNA complexes which might be induced by the torsional strain generated in the DNA helix by the reduced helical twist which accompanies intercalation [3]. The topoisomerase–DNA complexes were proposed to act as swivels that would relieve this strain [4].

In the present study, the possible involvement of torsional strain was tested by examining the effects of X-ray-induced DNA breaks. X-ray-induced strand breaks can relieve torsional strain over DNA lengths of at least 10^{10} daltons, as indicated by the effects of low doses of X-ray on the sedimentation velocity of mammalian cell nucleoids [5]. If strand breaks can relieve torsional strain over long distances in nuclear chromatin and if this strain is required for the formation of protein-associated strand breaks, then it should be possible, by X-irradiation of cells or isolated nuclei prior to treatment with intercalator, to prevent the formation of the protein-associated strand breaks.

In practice, this experiment cannot readily be performed using intact cells, because cells would repair the X-ray-induced strand breaks during the subsequent treatment with intercalator. The experiment, however, can be conducted using isolated nuclei, because isolated nuclei do not repair X-ray-induced strand breaks, but do form protein-associated strand breaks in response to intercalators [2, 6]. Using isolated nuclei, we have studied the effect of X-rays on the production by m-AMSA of DNA single- and double-strand breaks and DNA–protein crosslinks. We conclude that long-range torsional strain does not play a role in the formation of protein-associated strand breaks by m-AMSA.

Materials and methods

Cells and radioactive labeling. L1210 mouse leukemia cells were grown in suspension cultures in RPMI 1630 medium plus 15% (v/v) fetal calf serum as described previously [1]. Cellular DNA was radioactively labeled in exponentially growing cells by incubation for 20 hr at 37° with [2-^{14}C]thymidine (0.02 μCi/ml) or with [*methyl*-^{3}H] thymidine (0.1 μCi/ml; 10^{-6} M unlabeled thymidine added) (New England Nuclear Corp., Boston, MA).

Isolation of L1210 cell nuclei. L1210 cells were centrifuged and resuspended in nucleus buffer [150 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM ethyleneglycolbis-(amino-ethylether)tetra-acetate (EGTA), 0.1 mM dithiothreitol, pH 6.4] at 0° as described previously [2, 6]. Cells were centrifuged and resuspended in nucleus buffer plus 0.27% Triton X-100, incubated for 10 min at 0°, and pelleted by centrifugation (1200 rpm for 5 min). The nuclei were then resuspended in nucleus buffer.

Drug treatment and irradiation of cells and nuclei. m-AMSA (NSC 249992), obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, was dissolved at 10 mM in dimethyl sulfoxide and stored frozen at −20°. m-AMSA treatments were for 30 min at 37° and were stopped by a 20-fold dilution of treated nuclei in drug-free nucleus buffer at 0°. Control nuclei were treated with dimethyl sulfoxide at the same final concentration as used in the m-AMSA treatments.

Isolated nuclei or L1210 cells in 0° nucleus buffer were irradiated with a 200 kV X-ray source as described previously [2, 7]. When irradiation preceded m-AMSA treatment, nuclei were warmed to 37° before drug treatment.

Filter elution assays. DNA single-strand breaks and DNA–protein crosslinks were quantified in nuclei by alkaline elution as described previously [1, 2, 7]. DNA double-strand breaks were measured by the elution method of Bradley and Kohn [8]. To extend the range of measurement of DSB frequencies to 20,000 DSB-rad-equivalents, no more than 2×10^5 nuclei were loaded per filter; the elution pump speed was increased four times to 0.12–0.16 ml/min [1]; and fractions were collected at 10-min intervals for 60 min.

Results

DNA single-strand breaks (SSB). The effect of X-rays on the production of SSB in isolated nuclei in response to m-AMSA was determined by irradiating nuclei either before or after treatment with m-AMSA. One thousand rads produced a SSB frequency (0.9 per 10^6 nucleotides [9]) similar to that produced by 1 μM m-AMSA; the X-ray sensitivity of DNA in isolated nuclei and intact cells was similar. If the X-ray-induced SSB prevent the formation of m-AMSA-induced SSB, then irradiation prior to m-AMSA would result in a less than additive frequency of total SSB, whereas irradiation after m-AMSA treatment would produce an additive result. In fact, irradiation prior to m-AMSA produced close to an additive result, while irradiation after m-AMSA produced only a slightly lower value (Table 1). This result excludes the possibility that m-AMSA-induced SSB are stimulated by torsional strain transmitted over distances of the order of 5×10^5 base-pairs.

DNA double-strand breaks (DSB). The filter elution assay for DSB was calibrated by subjecting isolated nuclei to a series of X-ray doses. The elution curves had only modest degrees of curvature (Fig. 1), and the fraction of the DNA retained after 30 min of elution (the standard end-point) was linearly related to X-ray dose (Fig. 2). The calibration line (Fig. 2) was used to convert DNA retention to DSB frequency, expressed as the equivalent X-ray dose for that frequency of DSB (the units are "DSB-rad-equivalents").

Treatment of isolated nuclei with 5 μM m-AMSA produced a DSB frequency of approximately 8400 DSB-rad-equivalents. Additivity tests were carried out using 3,000, 5,000 or 10,000 rads of X-ray at 0°, followed by treatment with 5 μM m-AMSA at 37° for 30 min (Table 2). It is seen that these X-ray doses did not significantly decrease the m-AMSA-induced DSB. Since 10,000 rads of X-ray generates approximately 0.9 SSB/10^5 nucelotides, it is unlikely that the m-AMSA-induced DSB could be a response to torsional strain transmitted over distances of the order of 5×10^4 base pairs.

DNA–protein crosslinks (DPC). The effects of X-rays before or after treatment of isolated nuclei with 5 μM m-AMSA were compared with respect to the frequencies of the m-AMSA-induced DPC. The calculation of m-AMSA-induced DPC assumes that the frequency of X-ray-induced DPC is unaffected by the treatment with m-AMSA [7]. The 3,000 rad and 10,000 rad doses did decrease slightly the apparent frequency of m-AMSA-induced DPC when X-irradiation preceded m-AMSA, as compared to X-irradiation following m-AMSA (Table 3). The inhibition of m-AMSA-induced DPC, however, was small and did not increase when the X-ray dose was raised from 3,000 to 10,000 rads, as would have been expected if torsional strain was being relaxed. Intercalators are known to alter chromatin structure [10, 11]. The observed differences in Table 3 may be due to an effect of m-AMSA on chromatin such that protein–DNA contacts are altered so as to increase the ratio of DPC to SSB produced by X-rays. This would be consistent with the smaller difference observed at 10,000 rads than at 3,000 rads.

Table 1. Effect of pre-irradiation on m-AMSA-induced SSB*

	SSB frequency (SSB-rad-equivalents)	Number of determinations
m-AMSA (1 μM)	941 ± 152†	4
1000 Rads before m-AMSA	1805 ± 102	4
1000 Rads after m-AMSA	1693 ± 165	4

* Nuclei were either irradiated first and then treated with 1 μM m-AMSA for 30 min or treated with m-AMSA first and then irradiated. Drug treatments were stopped by adding drug-free nucleus buffer at 0° (1:20 dilution). SSB were determined by alkaline elution.

† Values are means ± S.D.

Table 2. Effect of pre-irradiation on m-AMSA-induced DSB*

X-ray dose (rads)	Measured DSB frequency (DSB-rad-equivalents)	Expected DSB frequency (DSB-rad-equivalents)	Number of determinations
0	8,388 ± 2,559†		7
3,000	12,900 ± 4,789	11,388	3
5,000	11,267 ± 4,197	13,388	3
10,000	24,100 ± 2,352	18,388	3

* Nuclei were irradiated at 0° and then warmed to 37° and treated with 5 μM m-AMSA for 30 min. Reactions were stopped by adding drug-free nucleus buffer at 0° (1:20 dilution). DSB frequency was determined by filter elution at pH 10.0.

† Values are means ± S.D.

Table 3. Effect of pre-irradiation on m-AMSA-induced DPC*

		DNA retention on filter	Mean DPC frequency (rad-equivalents)	Number of determinations
3,000 rads	Before m-AMSA	0.351 ± 0.030†	1245	11
	After m-AMSA	0.438 ± 0.035	1982	8
10,000 rads	Before m-AMSA	0.204 ± 0.044	1574	11
	After m-AMSA	0.237 ± 0.027	1945	8

* Nuclei were either irradiated before or after m-AMSA treatment (5 μM for 30 min at 37°). Reactions were stopped by a 20-fold dilution in ice-cold nucleus buffer. The fraction of ^{14}C-labeled DNA retained on the polyvinyl chloride filters is a measure of DPC frequency [7].

† Mean ± S.D.

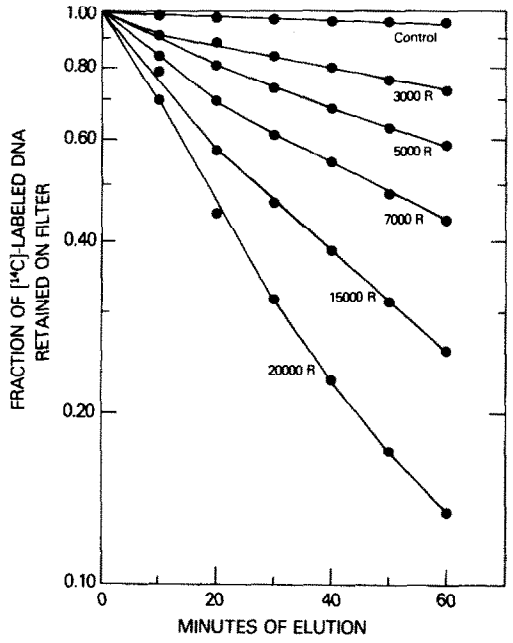

Fig. 1. Elution curves of double-stranded DNA from untreated and X-irradiated nuclei. Nuclei were irradiated in ice. DSB frequency was determined by filter elution assay at pH 10.0 using 2×10^5 nuclei per filter. Elutions were carried out at a pump speed of 0.12–0.16 ml/min and 10-min fractions were collected over a 60-min period.

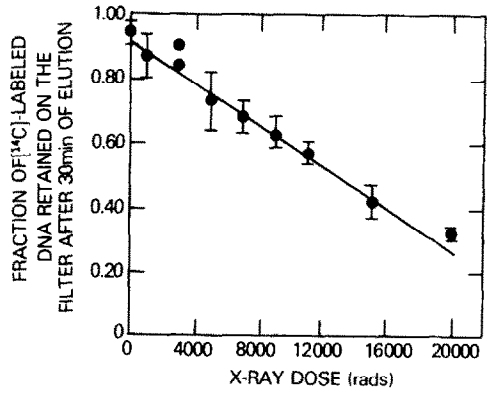

Fig. 2. DNA double-strand break calibration curve of X-irradiated L1210 cell nuclei. Nuclei were X-irradiated in ice and then eluted at pH 10.0 at a pump speed of 0.12–0.16 ml/min over a 60-min period. The fraction of [^{14}C]-thymidine-labeled DNA retained on the filter after 30 min of elution (Y) was determined and plotted against the administered X-ray dose (X) (40 independent determinations; regression line, $Y = 0.911 - [3.23 \times 10^{-5}]X$; correlation coefficient, −0.95). Error bars; standard deviations of at least three independent determinations.

Discussion

The present findings limit the possible distance over which torsional strain might be transmitted in DNA in an assumed stimulation of protein-associated strand breaks by DNA intercalators. Other work [12] has indicated that these protein-associated strand breaks represent an effect of intercalators on a topoisomerase II enzyme which may become covalently bound to DNA in a complex in which either one or both DNA strands are broken. The question addressed in the current work is whether torsional strain stimulates the formation of these complexes. The answer can be given within limits of distance over which any such stimulus might be transmitted.

The role of torsional strain was tested by using X-rays to introduce SSB which would act as swivels to relax any such strain that might be transmitted far along the DNA. X-ray-induced SSB did not inhibit significantly the formation of m-AMSA-induced SSB, DSB or DPC. The maximum X-ray-induced SSB frequencies that could be employed in these experiments set upper limits to the distance over which any assumed torsional strain might be transmitted. This upper limit, in the assays for m-AMSA-induced SSB, was approximately 5×10^5 base pairs; in the assays for DSB and DPC, it was approximately 5×10^4 base pairs. The possibility that torsional strain acts as a stimulus over distances shorter than these is not excluded, but in this case any relaxing effect of the protein-associated strand breaks also could not be transmitted over distances longer than 5×10^4 base pairs.

Long range torsional strain does not appear to be required for the induction of protein-associated DNA breaks by m-AMSA. It seems more likely that the intercalators interact directly with a topoisomerase–DNA complex and stabilize intermediate states of the complex, in which either one or both DNA strands are cleaved [12].

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Cepharanthine protection of Na^+,K^+-activated adenosinetriphosphatase of plasma membranes from rat cerebral synaptosomes against inhibition by ascorbate

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Cepharanthine, a biscoclaurine-type alkaloid obtained mainly from *Stephania cephalantha*, is known to protect a variety of tissues by stabilization of biological membranes that are under attack from bilirubin, phospholipase, X-ray irradiation, oncogenic agents, etc. There are two proposed mechanisms of membrane stabilization by the alkaloid: cepharanthine represses a fluidity change of the membrane lipid bilayer [1–3], and it prevents peroxidation of the membrane lipids [4, 5]. However, there could be another stabilization mechanism that involves membrane enzymes or proteins. For instance, the drug prevents K^+ loss of erythrocytes caused by noxious agents which inhibit Na^+, K^+-activated adenosinetriphosphatase (EC 3.6.1.3) (Na^+, K^+-ATPase) of the plasma membrane [1, 2]. One of the underlying mechanisms may possibly be cepharanthine protection of the Na^+, K^+ pump from the agents.

Plasmalemmal Na^+, K^+-ATPase has been shown to be inhibited by ascorbate, which is particularly abundant in the brain and adrenal medulla. Using synaptosomal plasma membranes of rat cerebrums, we reported that the inhibition mechanism may not involve lipid peroxidation but may be the direct attack of ascorbate radicals on the enzyme molecules [6], unlike the conventional belief that the inhibition is via lipid peroxidation. Our conclusion was based mainly on the facts that Na^+, K^+-ATPase was strongly inhibited, in spite of little peroxidation of the membrane lipids, in the presence of ascorbate even after the metal contamination had been very carefully removed from all the chemicals used, and that dimethylfuran (an active oxygen scavenger) did not prevent Na^+, K^+-ATPase inhibition but *p*-nitrosodimethylaniline (an eliminator of organic free radicals) did.

Thus, there is a possibility that cepharanthine prevents the free radical attack on Na^+, K^+-ATPase of the plasma membrane. The present work examined this possibility by the use of our determination of Na^+, K^+-ATPase activity of the plasmalemmal preparation from rat cerebral synaptosomes [6].

Materials and methods

Most of the materials and methods were as reported elsewhere [6]. Briefly, synaptosomal plasma membranes were prepared from the forebrains of male rats of the Wistar strain by a slight modification of the method of Lee and Phillis [7]. An ATP preparation (Sigma Chemical Co., Grade 1) was purified by a batch method with Chelex 100 to eliminate the contaminating heavy metals, and other chemicals used were also purified by column chromatography of the same chelating resin. The water preparation had a specific resistance over $18 \times 10^6\ \Omega$/cm. The standard assay medium for ATPase determination contained 120 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 4 mM ATP, 50 mM imidazol–HCl buffer (pH 7.4), plasmalemmal fraction, and the additions (specified in Table 1) in a final volume of 1 ml. Incubation was at 37° for 10 min. Na^+, K^+-ATPase activity was expressed as the difference between the amount of P_i liberated in the presence of Mg^{2+}, Na^+, and K^+ and that liberated in the presence of Mg^{2+} alone. The degree of lipid peroxidation was determined by the method of Ottolenghi [8]. For peroxidation determination the membrane fraction was incubated at 37° for 10 min in a medium (final volume, 1 ml) containing 50 mM Tris buffer (pH 7.4) and the additions as specified in Table 2. Cepharanthine (6′, 12′-dimethoxy-2, 2′-dimethyl-6, 7-[methylenebis(oxy)]oxyacanthan) was a gift of the Kaken Pharmaceutical Co., Japan. The drug was dissolved in a small amount of ethanol, and the ethanol solution was added to the incubation mixtures. The final ethanol concentration was 17.4 μM, which affected neither ATPase activity nor lipid peroxidation.

Results and discussion

Na^+-K^+-ATPase of synaptosomal plasma membrane was inhibited by over 50% in the presence of 0.1 mM ascorbate alone (Table 1a) as we have reported [6]. When cepharanthine (0.1 mM) was added together with ascorbate, the inhibition was about 10%. The presence of ascorbate and