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ALTERATION OF DNA BY 5-(3-METHYL-1-TRIAZENO)IMIDAZOLE-4-CARBOXAMIDE (NSC-407347)

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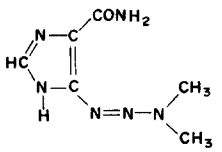
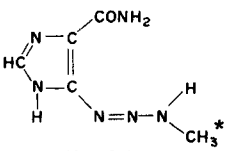
Abstract—5-(3-Methyl-1-triazeno)imidazole-4-carboxamide (NSC-407347, MTIC), an active intermediate in the metabolism of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388, DTIC), was investigated for its effect on DNA. When [^{3}H]MTIC was added to an aqueous solution of calf thymus DNA, it was rapidly hydrolyzed, and less than 0.5 per cent of the added ^{3}H was recovered in DNA as methyl groups attached to its base and phosphate constituents. It was estimated that the hydrolysis of phosphotriesters in methylated DNA released 0.5 per cent of total bound ^{3}H of DNA in the experiment *in vitro*, and 5.7 per cent *in vivo*. When MTIC was added to tissue culture cells which had been prelabeled with [^{3}H]thymidine, there was a large and immediate increase in acid-soluble radioactivity of both the cell media and the cells. At the same time, there was an accelerated drop in the specific activity of DNA, indicating that degeneration of DNA had occurred; MTIC at 10^{-3} M had a greater effect than at 10^{-5} M. Paper chromatographic studies showed that the acid-soluble radioactivity was predominantly in the form of thymidine. MTIC at 10^{-4} M induced repair synthesis of DNA but inhibited semi-conservative replication. The extent of repair synthesis was greater at 30 min than after 5 min. Treatment of cells with MTIC at 10^{-3} M irreversibly reduced the sedimentation of DNA in alkaline sucrose gradient. At 10^{-5} M, the reduction was reversible, and a normal pattern was restored in 30 min. Comparison of these results with those of sedimentation analysis of DNA in formamide gradient at neutral pH indicated that single strand breaks which were apparent in alkaline sucrose were probably the result of exposure of alkali-labile lesions in DNA to high pH.

The administration of the antitumor agent, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388, DTIC, DIC) to animals resulted in alkylation of DNA [1–3]. The mechanism of alkylation appeared to involve an enzymatic *N*-demethylation of DTIC to yield a monomethylated derivative, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (NSC-407347, MTIC), and formaldehyde. The structural formulae of the compounds appear below (an asterisk indicates the position of ^{3}H).



DTIC



MTIC

MTIC decomposed spontaneously in aqueous medium to form 5-amino-4-carboxamide (AIC) and an active methylating agent which was a methyl diazonium or carbenium ion [4]. The alkylation of nucleic acids by DTIC appeared to take place via the intermediate, MTIC [5]. DTIC also decomposed in the presence of light to produce 5-diazoimidazole-4-carboxamide and dimethylamine, the former cyclizing spontaneously to form 2-azahypoxanthine [6]. No alkylation of DNA or RNA was detected during the photodecomposition of DTIC [3]; hence, this pathway could not be invoked for its alkylating property. Both DTIC and MTIC induced tumor growth in rats [7, 8]. Both inhibited the uptake of thymidine by DNA and uridine by RNA [3, 5]. The methyl group from both DTIC [1, 2] and MTIC [3, 5] was bound to DNA *in vivo*. The similarity of the biologic and biochemical effects of MTIC to those of DTIC supported the view that MTIC was the active intermediate which is released *in vivo* [5]. Current investigation was undertaken to study the alterations in DNA which result from the action of MTIC. This paper deals specifically with the methylation of DNA by MTIC and the subsequent damage and repair of the altered DNA.

MATERIALS AND METHODS

Nonradioactive MTIC was synthesized according to Shealy and Krauth [9]. [Methyl-^{3}H]MTIC was prepared using the same procedure except for the replacement of methylamine with its methyl-tritiated counterpart. The specific activity of the product was 22.5 mCi/m-mole. [Methyl-^{3}H]thymidine (TDR) (12 Ci/m-mole) was purchased from Isotope and Nuclear Division, Cleveland, Ohio. Calf thymus DNA was from Worthington Biochemical Corp., Freehold, N.J.

HeLa (S-3) cells were maintained in Joklik-modified Eagle's Minimum Essential Medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum and 2 mM L-glutamine. The cells were grown in suspension in 225-ml centrifuge bottles in a drum rotating at 40 rev/min. The generation time was 20–24 hr.

All experiments with MTIC were performed in the absence of light, and solutions of MTIC in dimethylsulfoxide were used immediately after preparation.

For study of alkylation of DNA by MTIC *in vitro*, a solution of calf thymus DNA (2 mg/ml of 0.01 M phosphate buffer, pH 7.0) was incubated with 4.4 mg [methyl-^{3}H]MTIC for 30 min. DNA was dialyzed four times with 0.15 M NaCl–0.015 M sodium citrate, pH 7.0, and precipitated with 3% potassium acetate–95% ethanol. The process of dissolving and precipitating DNA was repeated two times. For study of alkylation of DNA *in vivo*, 160 ml of HeLa cell suspension (1×10^8 cells) was incubated at 37° with [^{3}H]MTIC (5×10^{-4} M) for 15 min. Cells were collected by centrifugation and kept frozen until use. DNA was isolated after the method of Koch and Stokstad [10] and distribution of ^{3}H was studied according to the procedure of Bannon and Verly [11]. DNA was depurinated by heating in 0.015 M NaCl–1.5 mM sodium citrate, pH 7.0, for 90 min at 100°. The depurinated DNA was precipitated with 4 vol. of 5% potassium acetate–95% ethanol, and after centrifugation the supernatant was concentrated and aliquots were used for chromatography. DNA was hydrolyzed in a pressure vessel in 7.5 N HCl at 100° for 6 hr. Carrier methanol was added and the mixture was evaporated under reduced pressure, trapping the volatile fraction with a liquid nitrogen trap. Crystals of 3,5-dinitrobenzoate of methanol were prepared from the volatile fraction [12] and compared with those of methanol. Its specific activity was determined after successive recrystallizations. The nonvolatile fraction was concentrated for further analyses. Chromatography was performed on Whatman 3MM paper with 2-propanol–cone. NH_3–H_2O (7:1:3, v/v) as solvent [13] or with ethyl acetate–HCOOH–H_2O (60:5:35, v/v) for development [14]. Fractions were also examined by thin-layer chromatography on Eastman 6065 sheets (Eastman Kodak Co., Rochester, N.Y.) with 2-methyl-1-propanol–0.8 M boric acid–conc. NH_3 (100:14:0.4, v/v) as the developing agent [15].

For studying the effects of MTIC on preformed DNA, cells were labeled with [methyl-^{3}H]TDR (final concentration: 1.0 μCi/ml) for 24 hr, then collected by centrifugation and suspended in fresh medium. Cells received either dimethylsulfoxide (control) or dimethylsulfoxide containing MTIC (final concentration: 10^{-3} or 10^{-5} M) and were incubated for 5 min, 30 min, 60 min and 4 hr. At the end of each incubation period, cell counts were made by counting in a hemacytometer, and cell viability was assessed by trypan blue staining. Cells were sedimented by centrifugation and the media removed. The acid-soluble fraction of the media was prepared by adding 9 vol. of 5% trichloroacetic acid to 1 vol. of media and filtering. The acid-soluble fraction of the cells was prepared by extracting the cells three times with cold 0.2 N perchloric acid. The residue was hydrolyzed with 0.3 N KOH for 1 hr at 37°, and DNA was precipitated by the addition of 1.0 N perchloric acid. DNA extract was obtained from the residue by heating it for 30 min at 100° in 10% NaCl–0.002 N Tris, pH 7.0. DNA was quantitated by absorbance readings at 260 nm and radioactivity was determined by adding 1.0 ml of the solution to 10 ml of PPO/POPOP/toluene/Triton X-100 (4.0 g/0.3 g/2300 ml/1000 ml) and counting in a liquid scintillation counter [16]*. Acid-soluble fractions of the cells were concentrated to a small volume for further chromatographic analysis as previously described.

For alkaline sucrose gradient centrifugation study of DNA, cells were incubated for 5 min or 30 min with either dimethylsulfoxide (5 μl/ml, control) or with MTIC in equal volume of dimethylsulfoxide (final concentration: 10^{-3} to 10^{-5} M). At the end of the incubation period, the cells were collected by centrifugation and suspended in 0.075 ml lysing solution (0.1 M EDTA–0.5 M NaOH). The mixture was sheared by passage five times through a No. 25 hypodermic needle [17] and then layered over an alkaline sucrose gradient (5-20% sucrose in 0.1 M NaOH–0.9 M NaCl–0.01 M EDTA). After standing at room temperature for 16 hr, it was centrifuged in a 50 Ti rotor (Beckman Instruments, Inc., Palo Alto, Calif.), at 20° for 270 min at 30,000 rev/min. About 30 fractions were collected from the top of the gradient using Fluorinert (Instrumentation Specialties Co., Lincoln, Neb.) as the displacement medium. Carrier DNA (50 μg of calf thymus DNA) was added to each fraction, and DNA was precipitated by the addition of 1.0 ml of 5% trichloroacetic acid. The precipitates were collected on glass filter discs (Whatman GF/C) and then washed in succession with 5% trichloroacetic acid (two times), 95% ethanol (one time) and acetone (one time). The discs were dried, placed in vials containing 10 ml PPO/POPOP/toluene (5.0 g/0.5 g/1000 ml) and counted in a liquid scintillation counter. To distinguish between single strand breaks and alkali-labile lesions in DNA, centrifugation was performed in 25–50% formamide gradient in H_2O [18]. Centrifugation was at 20° for 1 hr at 45,000 rev/min in a 50 Ti rotor. Fractions were collected and analyzed as before.

For study of DNA repair synthesis, a modification of the method of Walker and Ewart [19] was used. Cells were preincubated with 5-bromo-2′-deoxyuridine (BUDR) (0.5 μg/ml) and 5-fluro-2′-deoxyuridine (FUDR) (10^{-7} M) for 2 hr. Then the cells were exposed to MTIC in dimethylsulfoxide (final concentration in medium: 10^{-4} M) or to dimethylsulfoxide alone (control) for 5 and 30 min. Cells were separated by centrifugation and suspended in fresh medium containing BUDR (0.5 μg/ml), FUDR (10^{-7} M) and [^{3}H]TDR (final concentration: 20 μCi/ml). Cells were incubated for 3 hr at 37°, then collected by centrifugation and frozen. DNA was isolated as before, and buoyant density analysis was made by centrifugation at 20° in alkaline cesium chloride gradient in a 50 Ti rotor at 35,000 rev/min for 64 hr. About 40 fractions were collected from the top of the tube. Density of the individual fractions was determined from the refractive index readings [20]. Odd-numbered fractions were acidified; absorbance readings were made at 260 nm, and radioactivity was measured as before. Even-numbered fractions were used for rebanding. Appropriate fractions were pooled and the density of the combined fraction was adjusted to 1.78 g/cm^3. The volume was brought to 6.5 ml, and centrifugation

* PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)-benzene.

fractionation and quantitative procedures were repeated as before.

RESULTS

When [^{3}H]MTIC was added to an aqueous solution of calf thymus DNA, only a minor fraction of the radioactivity was recovered from DNA. The major portion (96 per cent) of the label was hydrolyzed in the media and converted to a product which could be volatilized from the filtrate after precipitation of DNA. The radioactive compound in the distillate was identified as methanol, based on the similarity of the melting points of the 3,5-dinitrobenzoate isolated from this fraction (107–108°) with that of authentic methanol (107°) and on the stability of the specific activity of the derivative after repeated recrystallizations (50 cpm/mg). With experiments *in vivo*, the methanol produced by hydrolysis can be further metabolized by the cells to produce formaldehyde and formate [21] and thus enter the 1-carbon pool which is a potential source of C-2 and C-8 of the purine ring. Therefore, the ^{3}H uptake by DNA involved not only alkylation but *de novo* synthesis of purines as well. Less than 0.5 per cent of total ^{3}H added as MTIC was recovered in the DNA fraction. The distribution of ^{3}H in DNA was studied according to the procedure of Bannon and Verly [11]. Heating the alkylated DNA to 100° for 90 min at pH 7.0 resulted in the removal of 80–95 per cent of its radioactivity (Table 1). DNA was precipitated and the filtrate was analyzed by paper and thin-layer chromatography. The nonvolatile radioactivity was accounted for by 7-methylguanine, 3-methyladenine, adenine and guanine. No methyl phosphate was detected, and no volatile radioactivity was released after digestion with alkaline phosphatase. When the apurinic DNA was subjected to conditions which hydrolyzed the phosphotriesters (heating at 100° in 7.5 N HCl for 6 hr), part of the radioactivity could be volatilized. The volatile components were identified as methanol by comparison of the radioactive 3,5-dinitrobenzoate derivative isolated from the distillate with that of authentic methanol. From the methanol liberated by hydrolysis, it was estimated that phosphotriesters comprised 0.5 per cent of the total counts in the study *in vitro* and 5.7 per cent *in vivo*. Paper chromatographic analysis of the nonvolatile fraction showed two radioactive spots which co-chromatographed with 7-methylguanine or 3-methyladenine. Hence, alkylated purines were the major products of the methylating reactions. Release of additional methylated purines on acid hydrolysis may in part be due to strong adsorption of the bases to DNA [22].

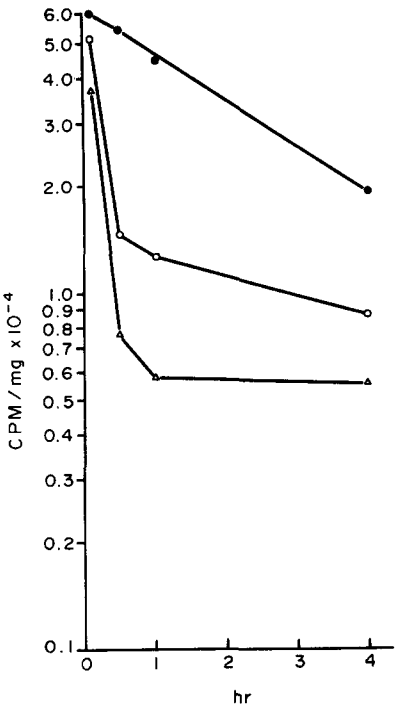


Fig. 1. Specific activity of DNA at various times after addition of MTIC to cells which had been prelabeled with [^{3}H]TDR. Key: control (●——●); 10^{-5} M MTIC (○——○); and 10^{-3} M MTIC (△——△).

When MTIC was added to tissue culture cells which had been preincubated with [^{3}H]TDR to label the DNA, there was a precipitous drop in DNA specific activity, indicating that a rapid degradation of preformed DNA had occurred (Fig. 1). MTIC at 10^{-3} M had a greater effect than at 10^{-5} M, and the rate of decline tapered off after 30 min. In the control cells, the DNA specific activity decreased exponentially with a half-life of about 2.5 hr, and the fall was attributable to dilution of labeled DNA with newly formed nonradioactive DNA. Figure 2a shows that the ^{3}H concentration of tissue culture media rose abruptly after the addition of MTIC to the cells. The rise was primarily in the acid-soluble fraction. The acid-soluble extract of the cells showed a similar increase, and the radioactivity was associated with thymine (panel b, in Fig. 2). MTIC had a greater effect at 10^{-3} M than at 10^{-5} M, and the increase in the rate of loss of ^{3}H from DNA correlated with the fall in DNA specific activity.

Table 1. Distribution of ^{3}H in DNA treated with [^{3}H]MTIC

Experiment	Fraction	Total cpm	Per cent of total
In vitro	DNA	1,454,000	100.0
	Depurinated DNA	77,640	5.3
	Volatile, 7.5 N HCl hydrolysate	7,500	0.5
	Nonvolatile, 7.5 N HCl hydrolysate	72,500	5.0
In vivo	DNA	49,600	100.0
	Depurinated DNA	9,800	19.0
	Volatile, 7.5 N HCl hydrolysate	2,830	5.7
	Nonvolatile, 7.5 N HCl hydrolysate	3,890	7.8

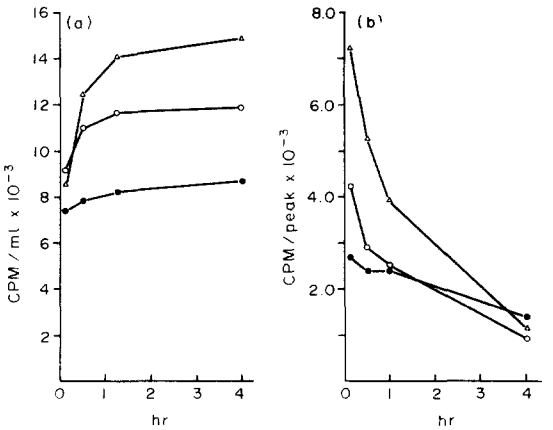


Fig. 2. Panel a: release of radioactivity into cell medium at various times after addition of MTIC to cell culture in which the DNA had been prelabeled with [^{3}H]TDR. Panel b: in thymine fraction from the acid-soluble fraction of the cells. Key: control (●——●); 10^{-5} M MTIC (○——○); and 10^{-3} M MTIC (△——△).

DNA replication was examined using equilibrium density gradient centrifugation of DNA in alkaline cesium chloride. Cells were preincubated with BUDR and FUDR, then exposed to MTIC, after which they were incubated in media containing [^{3}H]TDR, BUDR and FUDR. DNA was extracted and analyzed by buoyant density gradient centrifugation in alkaline cesium chloride. Newly synthesized semi-conservative synthesis was associated with radioactivity in the peak of daughter DNA and was evident only in control DNA (Fig. 3a). The existence of repair synthesis of DNA in MTIC-treated cells was manifested by a peak of radioactivity which coincided with the peak for parental DNA (Fig. 3b). Control DNA showed little or no radioactivity in this region. Essentially the same results were obtained by rebanding of parental peaks. Repair synthesis was greater at 30 min after MTIC (10^{-4} M) treatment than after 5 min. On the other hand, semi-conservative synthesis of DNA was inhibited by this concentration of MTIC.

Treatment of the HeLa cells for 5 min with MTIC at 10^{-5} M significantly reduced the rate of sedimentation of DNA in alkaline sucrose gradient (Fig. 4).

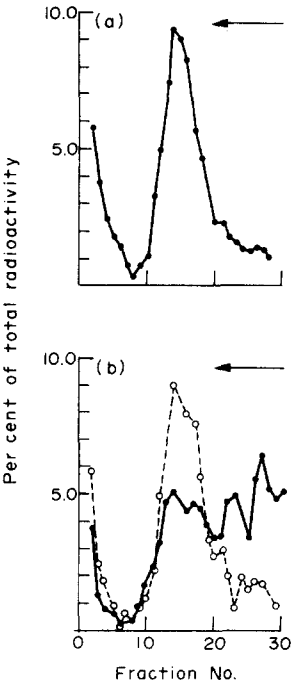


Fig. 4. Alkaline sucrose gradient sedimentation profile of DNA. Panel a, control; panel b, 10^{-5} M, 5 min (——); 30 min (– – –). Arrow designates the direction of sedimentation.

At this concentration of MTIC, the reaction was reversible, and the normal sedimentation profile of DNA was restored after 30 min (panel b in Fig. 4), indicating that some rejoining of DNA strands had taken place. Similar results were observed with MTIC at 10^{-4} M, but at 10^{-3} M no recovery was obtained, even after 1 hr. The decreased sedimentation rate has been ascribed to reduction in molecular weight of DNA due to single strand breaks in the denatured DNA. When the samples were analyzed by sedimentation in formamide gradient at neutral pH, there was no difference between the control and MTIC-treated DNA. These results indicated that single strand breaks were produced during the analytical procedure probably by hydrolysis of DNA apurinic sites at alkaline pH. However, it was evident that alkali-labile

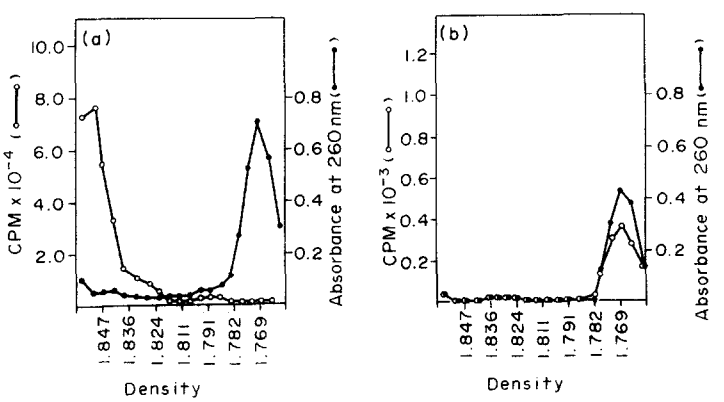


Fig. 3. Buoyant density analysis of DNA from HeLa cells. Panel a, control; panel b, 10^{-4} M MTIC, 5 min. DNA isolated from approximately the same number of cells was analyzed on each gradient. Difference in radioactivity scales between the two graphs is due to the low specific activity of DNA in cells exposed to MTIC.

lesions were produced in DNA by MTIC and that these alterations were reversible at low concentrations of MTIC.

DISCUSSION

Previous studies had shown that MTIC had properties of an alkylating agent, and its methyl group was transferred *in toto* to DNA [3–5]. When [methyl-^{3}H]MTIC was reacted with DNA *in vivo* or *in vitro*, most of it was hydrolyzed in the aqueous medium to form methanol. The major methylated product was 7-methylguanine. Other reaction products present in minor amounts were 3-methyladenine and methyl phosphotriesters. DNA phosphotriesters have recently been shown to be stable to heat at neutral pH [11]. This property has been employed to estimate the methyl groups attached to oxygen of phosphodiester linkages in DNA. It was estimated that 0.5 per cent of ^{3}H was derived from methyl groups in DNA phosphotriesters *in vitro* and 5.7 per cent *in vivo*. Phosphotriester formation in DNA has also been observed with the use of other alkylating agents [23, 24].

The addition of MTIC to cells containing DNA labeled with [^{3}H]thymidine caused an immediate release of radioactive thymine from DNA to the acid-soluble fraction of the cells and the media. At the same time, there was a rapid decline in the DNA specific activity, indicating that degradation of DNA had occurred. Alkylated purines are lost spontaneously from DNA, producing apurinic sites [25]. In addition, during the repair process, nonalkylated as well as alkylated bases are removed from DNA, depending on the size of the patch. Hence, the above loss of thymine from DNA could be a result of a repair process which was induced in the drug-treated cells. At alkaline pH, the apurinic sites can be converted into single strand breaks by hydrolysis of neighboring phosphodiester bonds [26], and the resultant DNA can show a reduction of molecular weight upon centrifugation in an alkaline sucrose gradient. The reduction of molecular weight was not evident when MTIC-treated DNA was centrifuged in a neutral formamide gradient; therefore, alkali-labile lesions, but not single strand breaks, were probably present *in situ*. At low concentrations of MTIC (10^{-4} and 10^{-5} M), the alkali-labile lesions were reversible, and a normal sedimentation pattern was re-established in 30 min. At a higher concentration (10^{-3} M), no recovery was observed. MTIC at 10^{-4} M inhibited semi-conservative replication of DNA, but induced repair synthesis of DNA to occur.

DTIC was first developed as a possible antitumor agent to be used for clinical trials [27]. Recently, it has been shown that both DTIC and MTIC were carcinogenic in rats [28]. Similarity of the reactions of MTIC to that of DTIC support the concept that MTIC is the active intermediate during the metabolism of DTIC, and further investigations on its effect on DNA may be of consequence not only in the understanding of alkylation but in the study of molecular mechanisms in carcinogenesis as well.

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