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EFFECTS OF 5-(3-METHYL-1-TRIAZENO)IMIDAZOLE-4-CARBOXAMIDE (NSC-407347), AN ALKYLATING AGENT DERIVED FROM 5-(3,3-DIMETHYL-1-TRIAZENO)IMIDAZOLE-4-CARBOXAMIDE (NSC-45388)

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Abstract—All experiments were performed in the absence of light. A 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MIC) concentration of less than 10^{-4} M had no effect on cell growth of L cells. At higher concentrations, the cells were inhibited to levels which were similar to those obtained with equimolar doses of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DIC). MIC inhibited the incorporation of ^{3}H-thymidine by DNA more than that of ^{3}H-uridine by RNA. Uptake of ^{3}H from ^{3}H-methyl-MIC by DNA was not influenced by the stage of the cell cycle. The greatest binding took place with DNA of the euchromatin fraction. MIC-treated DNA exhibited impaired template activity *in vitro* in the RNA polymerase system but not with that of DNA polymerase. Chromatography of DNA hydrolysate from ^{3}H-methyl-MIC-treated cells showed three major radioactive peaks, which corresponded to adenine, guanine and 7-methylguanine. Hydroxyurea markedly reduced the uptake of ^{3}H by adenine and guanine but had relatively little effect on the ^{3}H content of 7-methylguanine. Similarity of cytotoxic reactions of MIC to those of DIC supports the thesis that in the animal system DIC is metabolically converted to MIC, a potential methylating agent. Many of the effects of DIC can be accounted for by the action of MIC.

Previous studies have shown that the antitumor agent 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DIC, NSC-45388) was activated in animal tissues via oxidative *N*-demethylation to produce formaldehyde (HCHO) and 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MIC, NSC-407347) [1, 2]. MIC was unstable in aqueous solution at physiologic pH ($T_{1/2}$ = 8 min) [3] and decomposed to yield 4-aminoimidazole-5-carboxamide and methyl diazonium or carbenium ion [4, 5], which methylated nucleic acids. The methylating activity of MIC may play a prominent role in the biological and chemical action of DIC. DIC has also been shown to undergo decomposition under the influence of light to yield dimethylamine and 5-diazoimidazole-4-carboxamide [6]. Cytotoxicity of DIC to tissue culture cells was enhanced by exposure to light [7]; however, it remains unclear whether an analogous reaction occurs in the mammalian system *in vivo*. MIC has shown antitumor activity against L1210 and Walker 256 carcinoma [3]; however, results tended to be erratic, presumably due to instability of the drug in the aqueous medium used to administer it, and it was difficult to quantitatively assess the effects of MIC with those of DIC. The object of this investigation was to study the chemical and biologic effects of MIC and to compare them with those of DIC.

MATERIALS AND METHODS

5-(3-Methyl-1-triazeno)imidazole-4-carboxamide (MIC) was synthesized according to the procedure of Shealy and Krauth [3]. The compound was judged to be of high purity from its melting point, u.v. and infra red spectra. No 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DIC) was detected in the MIC preparation using thin-layer chromatography [8]. ^{3}H-methyl-MIC was prepared by the same technique by replacement of methylamine with its tritiated counterpart. The specific activity of the product was 22·5 mCi/mmole. DNA polymerase (EC 2.7.7.7, *Micrococcus lysodeikticus*, 90 units/mg of protein) and RNA polymerase (EC 2.7.7.6, *Escherichia coli* B, 284 units/mg of protein) were from Miles Laboratories, Elkhart, Ind. Methyl-^{3}H-thymidine (12 Ci/mmole), 2-^{14}C-thymidine-5′-tri-phosphate (45 mCi/mmole), ^{3}H-uridine-5′-triphosphate (27·2 Ci/mmole) and ^{14}C(GL)-lysine (230 mCi/mmole) were from ICN, Isotope and Nuclear Division, Cleveland, Ohio. 2-^{14}C-thymidine (5·26 mCi/mmole) was from New England Nuclear, Boston, Mass.

Mouse fibroblast tissue culture cells (L929, Grand Island Biological Company, Grand Island, N.Y.) were grown in suspension in Joklik modified Eagle's minimal essential medium [9] containing 10% fetal calf

serum and 2 mM L-glutamine. All experiments with MIC and DIC were conducted in the absence of light. The cells were exposed to MIC or DIC at concentrations of 10^{-3} to 10^{-6} M. Cell counts were made in a hemocytometer at 24 and 48 hr after the addition of MIC (either as a dry powder or dissolved in dimethylsulfoxide) or DIC (dissolved in H_2O). Both drugs were used immediately after preparation.

In order to study the binding of ^3H from ^3H-MIC to DNA during various stages of the division cycle, cell division was synchronized by the method of Tobey and Ley [10]. Cells were brought to a state of reversible G_1 arrest by growth in an isoleucine-deficient medium for 96 hr. When isoleucine was restored to the cells, DNA synthesis was initiated with a high degree of synchrony, reaching a maximum at 12–16 hr. The generation time was 20–24 hr. Cells were removed at various intervals after the addition of isoleucine and were incubated with ^3H-MIC (10^{-3} M) for 30 min. DNA was isolated as previously described [2]. Specific activity was determined from ^3H counts, obtained by liquid scintillation counting [11], and μg DNA quantitated from absorbance measurements at 260 nm.

To study the binding of ^3H to DNA at various cellular sites, tissue culture cells were incubated with ^3H-MIC (10^{-3} M) for 30 min at 37°. The cells were homogenized, and nuclear and mitochondrial fractions were isolated according to Kasamatsu *et al.* [12]. The nuclear preparation was then sonicated for 70 sec (Bronson sonifier S-75, setting of 2), and heterochromatin, euchromatin and an intermediate fraction were isolated according to Yasmineh and Yunis [13]. DNA was isolated according to Kirby and Cook [14] and specific activities were determined. To obtain information about the site of binding, DNA from ^3H-MIC-treated cells was hydrolyzed in 1·0 N HCl at 100° for 1 hr. The hydrolysate was diluted until it was 0·05 N in HCl, and it was applied to a column (1 cm × 20 cm) of Dowex 50 × 4, 200–400 mesh, H^+ form. Elution was with 1 liter of a linear gradient (1–4 M HCl). Fractions of 10 ml were collected. The bases were located by absorbancy reading at 260 nm, and ^3H content of each fraction was determined with a liquid scintillation counter. Authentic samples of adenine, guanine and 7-methylguanine were used to establish the location of purine peaks, and each purine peak was identified by paper chromatography [15]. In addition, the identity of the 7-methylguanine peak was confirmed by mass spectrometry.

To study template activity of MIC-treated DNA, calf thymus DNA (0·5 mg in 1·0 ml) was reacted with MIC (2·5 to 250 μg) for 2 hr; then, the unbound drug was removed by chromatography on Sephadex G-50, using 0·01 M potassium phosphate buffer, pH 7·0, as eluant. Fractions containing DNA were pooled, lyophilized and dissolved in suitable volume for template assay. DNA polymerase was assayed according to Lynch *et al.* [16], using the purified preparation from *M. lysodeikticus*. RNA polymerase activity was determined after the method of Burgess [17] with the enzyme preparation from *E. coli* B.

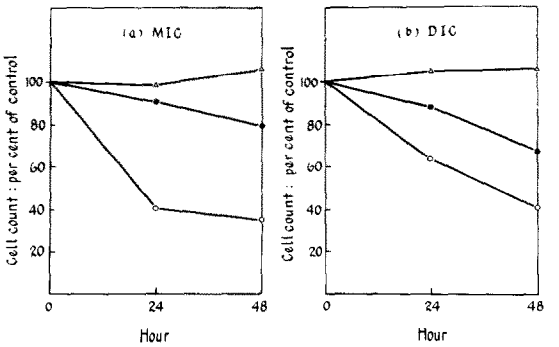

Fig. 1. Effect of MIC (a) and DIC (b) on viable cell count. Key: 10^{-3} M (○—○); 10^{-4} M (●—●); and 10^{-5} M (△—△). MIC was used either as a dry powder or a solution in dimethylsulfoxide. DIC was dissolved in H_2O. Both drugs were used immediately after preparation. Trypan blue was used for determination of cell viability.

All incubation mixtures were run in duplicate, and the same experiments were carried out at least twice. Uptake of ^3H-TTP by acid-insoluble fraction was proportional to the amount of DNA (up to 15 μg/assay with DNA polymerase and up to 75 μg/assay with RNA polymerase). Glass filter discs were used for filtration and washing of DNA precipitates [18]. Radioactivity was determined by dissolving DNA on the filter discs with 0·25 ml NCS solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.). After standing overnight, 10 ml of a Triton–PPO–POPOP–toluene* mixture [11] was added and the samples were counted in a liquid scintillation counter.

For sucrose density gradient centrifugation studies, DNA was isolated from cells which had been treated with MIC (10^{-3} M) at 37° for 1 hr. It was layered on 4·5 ml of sucrose gradient (5–20%, w/v) in 0·1 M NaCl–0·9 M NaOH–1·0 mM EDTA, pH 7·0 or pH 12·7 [19]. The tubes were centrifuged at 83,000 *g* at 20° for 3 hr in a No. 40 Spinco rotor. Tubes were punctured, 3-drop fractions were collected and, after adding 0·5 ml H_2O to each fraction, the absorbance was measured at 260 nm.

RESULTS AND DISCUSSION

When counts were made of L cells at 24 and 48 hr after exposure to various concentrations of MIC, there was no difference from the control values when the drug concentration was less than 10^{-4} M (Fig. 1). Reduction of cell growth was found at 10^{-4} M or greater, the extent of which resembled those observed with equimolar doses of DIC. At 10^{-4} M or greater, MIC inhibited the incorporation of ^3H-uridine by RNA. The incorporation of ^3H-thymidine by DNA was more sensitive and was inhibited at 10^{-5} M (Fig. 2). MIC had no effect on the incorporation of ^{14}C-

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

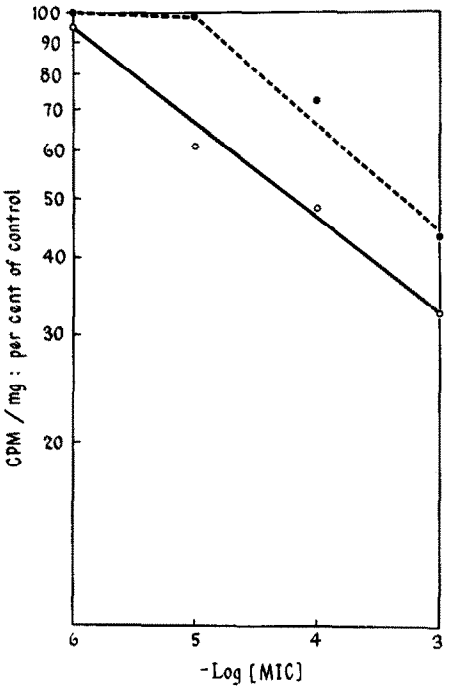

Fig. 2. Effect of varying the concentration of MIC on incorporation of ^{3}H-thymidine by DNA (○——○) and ^{3}H-uridine by RNA (●– – –●).

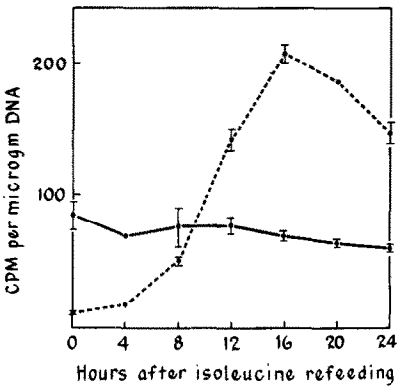

Fig. 3. Binding of ^{3}H from ^{3}H-methyl-MIC to DNA during the cell cycle. Every 4 hr after isoleucine addition to the culture medium, aliquots of cells were removed and incubated with ^{3}H-MIC (10^{-3} M) and ^{14}C-thymidine ($9{\cdot}5 \times 10^{-4}$ M) for 30 min. DNA was isolated and specific activity was determined using the dual label technique. DNA, cpm/μg: ^{3}H (——); ^{14}C (– – –). Each point represents a mean of two separate experiments.

lysine by the protein fraction. Similar results were also observed with DIC in tissue slice experiments [2]; hence, no significant difference has been demonstrated between the effects of MIC and DIC with the above criteria.

When cells which had been synchronized by isoleucine starvation were reacted with ^{3}H-methyl-MIC at various times during the cell cycle and DNA isolated for specific activity determination, the results showed a uniform binding to DNA throughout the cell cycle (Fig. 3); hence, phase specificity did not appear to influence this reaction.

DNA was extracted from mitochondrial, euchromatin, heterochromatin and intermediate chromatin fractions from ^{3}H-MIC-treated cells and the specific activities were determined. The highest level was found to be associated with the euchromatin fraction (Table 1). Labeling of DNA of other cell organelles also took place, although to a diminished amount ($\frac{1}{4}$ to $\frac{1}{2}$ less). It was reported that no significant difference existed in the buoyant densities of the main peak DNA isolated from euchromatin and heterochromatin fractions, indicating that base compositions were similar [20]. No phase specificity was observed in the binding of ^{3}H to DNA; hence, there was no evidence that alkylation of euchromatin fluctuated during the cell cycle. This may be related to the fact that over 90 per cent of the cell DNA is in the heterochromatin fraction (Table 1); therefore, any change in a relatively small per cent of the total, as would be represented by the euchromatin fraction, would be masked in the over-all effect. The preferential binding to DNA of the euchromatin fraction may be related to its relatively diffuse fibrillar state which may play a role in the accessibility of the DNA to the action of alkylating agents.

A previous study showed that when MIC was added to an aqueous solution of calf thymus DNA, the methyl group was transferred intact to the 7-position of the guanine residue [4, 5]. When ^{3}H-MIC was

Table 1. Binding of MIC of DNA to different cell fractions*

Cell fraction	Per cent of total DNA	MIC bound to DNA (dis./min/μg)
Mitochondria	5·49	6490
Euchromatin	1·21	10,130
Intermediate chromatin	2·13	6350
Heterochromatin	91·06	6520

* Cells were incubated with ^{3}H-methyl-MIC (10^{-3} M) for 1 hr at 37°. Cells were removed by centrifugation, and DNA was isolated from cell fractions as described in the text.

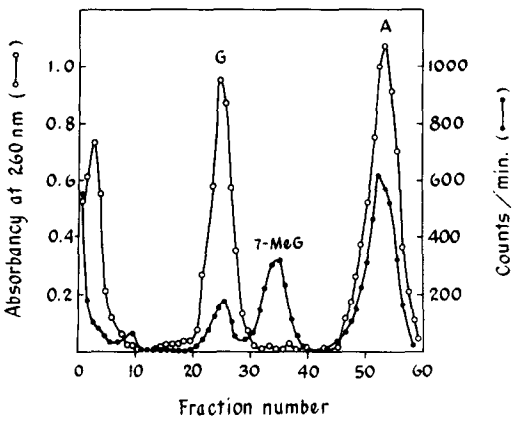

Fig. 4. Chromatography of acid hydrolysate of DNA from L cells which had been reacted with ^{3}H-MIC (10^{-3} M) for 4 hr. Abbreviations: A, adenine; G, guanine; and 7-MeG, 7-methylguanine.

reacted with the cells and the DNA extracted, hydrolyzed and chromatographed on Dowex 50, the radioactivity was found predominantly in three peaks which corresponded to adenine, guanine and 7-methylguanine (Fig. 4). When hydroxyurea, which inhibits semiconservative replication of DNA [21], was added to the medium, the ^{3}H content of adenine and guanine was markedly reduced without a corresponding effect on 7-methylguanine so that the latter now accounted for about $\frac{2}{3}$ of the total (Table 2). This indicated that there were at least two modes of uptake of ^{3}H by DNA and that the methylation reaction was relatively insensitive to the action of hydroxyurea. The uptake of ^{3}H by adenine and guanine, which became labeled during the *de novo* synthesis of the purines, was preferentially inhibited by hydroxyurea. The study of effects of DIC on nucleic acid syntheses was variable. It was

Table 2. Binding of ^{3}H-MIC to purine bases of DNA

Fraction	(cpm/peak) − Hydroxyurea	+ Hydroxyurea
Adenine	883	100
Guanine	215	70
7-Methyl-guanine	457	320

reported to adversely affect RNA and protein syntheses more than that of DNA in L1210 cells [22] and *E. coli* [23]. In the latter system, complete reversal of inhibition could be obtained by the addition of cysteine and homocysteine; therefore, a more complicated mechanism of inhibition may be involved than merely alteration of template activity. In other experiments, DIC was found to selectively inhibit DNA synthesis in rat tissues [24] and sarcoma 180 slices [2]. The similarity of these results to those which were obtained with MIC in the current study is a further corroboration that effects of DIC can be largely accounted for by the action of its metabolite, MIC. In order to determine whether the adverse effect of MIC on nucleic acid synthesis was the result of impaired template activity, MIC-treated DNA from which unbound drug was removed by chromatography on Sephadex was assayed for RNA and DNA polymerase activities. Table 3 shows that MIC-treated DNA had reduced template activity with RNA polymerase but not with DNA polymerase. However, in studies with L cells, MIC was found to inhibit both RNA and DNA syntheses with greater effect on the latter. These results suggested that impairment of DNA synthesis in MIC-treated cells was not due to diminished template activity but to some other factors. It may be possible that alkylation *per se* caused the initial error, which then led to additional inactivation resulting from the production of nonfunctional RNA and/or protein.

Table 3. Effect of MIC binding on template activity of DNA

DNA*	MIC bound to DNA (pmoles/μg)	DNA polymerase† (cpm)	DNA polymerase† (% of Control)	RNA polymerase‡ (cpm)	RNA polymerase‡ (% of Control)
Untreated	0	220	100	2280	100
Treated with MIC (5 μg/mg DNA)	0·28	200	91		
Treated with MIC (500 μg/mg DNA)	2·88	205	92	680	30

* DNA (0·5 mg/ml) was reacted with MIC for 2 hr in the dark. It was separated from unbound MIC by chromatography on Sephadex G-50 using 0·01 M phosphate buffer, pH 7·0, as eluant. Control DNA was similarly treated. The specific activity of ^{3}H-MIC was used to estimate the extent of binding of MIC to DNA.

† For DNA polymerase, the reaction mixture (0·5 ml) contained 0·1 M Tris, pH 7·4, 4×10^{-3} M 2-mercaptoethanol, $1{\cdot}6 \times 10^{-2}$ M KCl, 2×10^{-3} M ATP, 8×10^{-5} M each of dGTP, dCTP and dATP, $1{\cdot}6 \times 10^{-5}$ M ^{3}H-TTP, 8–12 μg DNA and 8·6 units DNA polymerase. Incubation was for 10 min at 37°.

‡ For RNA polymerase, the reaction mixture (0·5 ml) contained 0·04 M Tris, pH 7·9, 0·15 M KCl, 0·01 M $MgCl_2$, 0·1 mM EDTA, 0·1 mM dithiothreitol, 0·25 mg bovine serum albumin, 0·15 mM each of UTP, GTP, CTP and ^{14}C-ATP, 75 μg DNA and 5 units RNA polymerase. Incubation was as above.

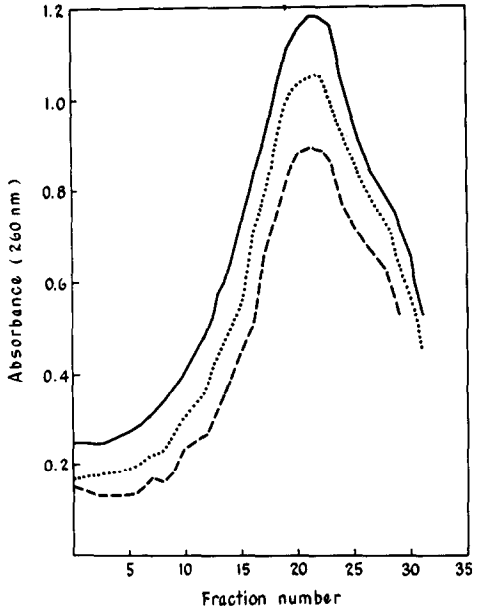

Fig. 5. Sedimentation profiles of DNA in alkaline sucrose gradient. DNA (0·4 mg) was layered on top of sucrose gradient (5–20%, w/v) in 0·9 M NaOH–0·1 M NaCl–1 mM EDTA, pH 12·7. Centrifugation was at 83,000 *g* for 3 hr at 20°. Key: control (——); MIC-treated (– – – –); and DIC-treated (· · · · ·).

The metabolic route of DIC via the MIC intermediate appears to be the major pathway in man and animals. When ^{14}C-methyl-DIC was administered to rats and man, labeled 7-methylguanine was found in the urine [25]. RNA and DNA isolated from various tissues of the rats were shown to contain the greatest proportion of the radioactivity in 7-methylguanine with lesser amounts in adenine and guanine. With microsomal preparations, ^{14}C-methyl-DIC was oxidatively *N*-demethylated to form ^{14}C-HCHO and MIC [1, 2]. The former entered the pool of one-carbon fragments and was thus incorporated into the purine precursors during *de novo* synthesis. The MIC intermediate possessed the properties of a potential methylating agent and when reacted with DNA, the methyl group was transferred mainly to N-7 of guanine [4, 5]. In the presence of light, DIC rapidly decomposed via 5-diazoimidazole-4-carboxamide to 2-azahypoxanthine, but no alkylation of DNA was detected [2]. When cells were reacted with ^{14}C-2-DIC, labeled DNA was observed [7], but this may have resulted from the metabolic formation of ^{14}C-2-AIC which became incorporated into the purine precursor pool.

A study of sedimentation profiles of DNA from MIC-treated cells in 5–20% sucrose gradient showed no change at either pH 12·7 (Fig. 5) or at pH 7·0. Thus, reduced template activity of RNA polymerase could not be attributed to single strand breaks or to significant reduction of molecular size of DNA. Similar results were found in DNA from DIC-treated cells.

It was shown that DIC has a carcinogenic potential, and when fed over an extended period to weanling rats, it induced the formation of mammary adenocarcinoma and thymic lymphosarcoma [26]. Mammary fibroadenoma and leiomyosarcoma were observed at lower doses given over a longer period of time [27]. Since the injection of MIC alone induced leiomyosarcoma, it was suggested that MIC may be involved in the induction of tumors by DIC.

The similarity of action of DIC with that of MIC supports the thesis that DIC is metabolized primarily to MIC in the animal system and that the latter can largely account for the toxic properties of DIC. This does not exclude the possibility that other reactions may also be involved.

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