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# MECHANISM OF ACTION OF 5-(3,3-DIMETHYL-1-TRIAZENO)IMIDAZOLE-4-CABOXAMIDE IN MAMMALIAN CELLS IN CULTURE*†

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**Abstract**—5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (DIC) is more lethal to Chinese hamster ovary cells and human malignant melanoma cells in culture in the presence than in the absence of light. Incubation of either cell line with DIC labeled at the side chain methyl groups with $^{14}C$ resulted in the association of radioactivity with DNA only if light was absent. However, with ring-labeled DIC-2-$^{14}C$, radioactivity was found in both DNA and RNA fractions regardless of the presence or absence of light during incubation. These results are essentially consistent with the current consensus regarding the mode of decomposition of DIC: on exposure to light, DIC undergoes decomposition to dimethylamine and 5-diazoimidazole-4-carboxamide which interacts with nucleic acids in an obscure manner. When light is excluded, however, an alternative pathway of decomposition is followed, with the ultimate formation of 5-aminoimidazole-4-carboxamide and a methyl carbonium ion, which then interacts with cell DNA.

5-(3,3-DIMETHYL-1-triazeno)imidazole-4-carboxamide (DIC, NSC-45388), a new antineoplastic agent, has shown activity comparable to that of 6-mercaptopurine and cyclophosphamide against experimental tumors.[1,2] Clinically, DIC is useful in the treatment of malignant melanoma[3] and several other solid tumors.[4] The mode of action of this interesting drug remains uncertain. In the present paper, we describe our attempts to determine the mechanism of action of DIC in mammalian cells in culture.

## MATERIALS AND METHODS

*Chemicals.* DIC, radioactive DIC labeled with $^{14}C$ at position-2 (DIC-2-$^{14}C$, 9·93 mc/m-mole) and the side chain dimethyl groups (DIC-DM-$^{14}C$, 5 mc/m-mole), 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MIC, NSC-407347), and 5-aminoimidazole-4-carboxamide-2-$^{14}C$ (AIC-2-$^{14}C$, 92·76 mc/m-mole) were supplied by Drug Development Branch, Drug Research and Development, National Cancer Institute.

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Thymidine-methyl-$^3$H ($^3$H-TdR, 1·9 c/m-mole) was obtained from Schwartz/Mann Bioresearch Inc., Orangeburg, N.Y. The chemical and radiochemical purity of these compounds was more than 99 per cent by chromatographic assay procedures. Other chemicals and reagents were purchased from regular sources of supply.

*Cell lines.* The Chinese hamster ovary (CHO) cells were supplied by Dr. R. M. Humphrey.[5] The strain of human malignant melanoma (M) cells capable of heavy pigment production was obtained from Dr. M. M. Romsdahl and Professor T. C. Hsu.[6] The growth medium was McCoy's 5a[7] supplemented with 20 per cent fetal calf serum.[8] The cells were subcultured as needed and grown in a 5% $CO_2$ humidified incubator.

*Radiochemical measurements.* Radioactivity was determined by standard techniques with a liquid scintillation spectrometer. In double-labeling experiments with $^{14}$C and $^3$H, the discriminator-ratio method[9] was applied to determine the actual counts per minute of each isotope.

*Colorimetric determination of DIC.* The rates of DIC decomposition in both 0·1 N HCl and McCoy's medium were determined in light and also in darkness at 37° by colorimetric assay of the unchanged DIC.[10]

*Cell survival.* As with other drugs, the cytotoxic action of DIC on cells in culture is quantitatively expressed by the inhibition of the capability of these cells to form colonies after drug exposure.[5,11] All experiments were performed with both M and CHO cells in the logarithmic phase of growth at an initial cellular density of about 2·4 to 3 $\times$ $10^4$ cells/sq. cm. The cells were treated with different amounts of DIC for various time intervals, either in the presence or absence of light.[12] In all light experiments, a fluorescent desk lamp (model P-2324, equipped with 2 15-W Ken-Rad F 15T8/CW bulbs, Dazor Mfg. Corp., St. Louis, Mo.) was placed in the incubator, 38 cm from the cells. After thorough washing and treatment with trypsin, known numbers of single cells were plated into plastic petri dishes and incubated at 37° for 6–8 days. The colonies formed were fixed, stained with crystal violet, and counted. A cell was considered viable if it divided to form a colony of 50 or more cells.

Similar experiments were carried out with CHO cells and MIC.

*Isolation and analysis of nucleic acids.* After incubation with radioactive DIC (28–100 $\mu$g/ml, 1 to 4·8 $\mu$c/ml, 21–45 hr), the cells were harvested and lysed. The cellular nucleic acids were isolated by a standard procedure[13] with slight modifications.[14] In double-labeling experiments, the cells were incubated with $^3$H-TdR (0·2 to 1 $\mu$c/ml for 19–48 hr) before treatment with radioactive DIC. Similar incubation experiments have also been performed with CHO cells and AIC-2-$^{14}$C (10 $\mu$g/ml, 9·3 $\mu$/ml, for 21 hr). The nucleic acids were subjected to CsCl density gradient analysis in the usual manner.[13]

*Volatile* in vitro *metabolites of DIC.* Cultures of 12 $\times$ $10^6$ CHO cells in the logarithmic phase of growth were allowed to grow either in light or darkness in 1-l. Erlenmeyer flasks in which were placed as absorbants two 10-ml beakers containing 0·6 ml of 0·1 N HCl and 0·1 N NaOH. The flasks were plugged with cotton, and the cells were incubated with DIC-2-$^{14}$C separated for 72 hr. The radioactivity of the HCl and NaOH was measured before and after heating in a steam bath for about 30 sec to estimate the percentage loss due to heating. As a comparison, similar percentage loss of radioactivity was determined by heating solutions of $Na_2{}^{14}CO_3$ and $(^{14}CH_3)_2NH$, respectively, in dilute HCl and NaOH in an identical way.

## RESULTS

*Stability of DIC in medium.* The decomposition of DIC in medium follows first-order kinetics with a $T_{\frac{1}{2}}$ of 34 hr in the dark and 3·3 hr in the light. In other words, in the dark, 38 per cent of DIC remains unchanged after 47 hr; in contrast, essentially no DIC is detectable after 24 hr exposure to light.

*Effect of DIC on survival of cells in culture.* DIC had a slight effect on CHO cell survival at initial concentrations below 100 μg/ml. However, after continuous exposure to the drug at an initial concentration of 100 μg/ml in the dark for 48 hr, on the average 78 per cent of the cells survived. The drug was more inhibitory in the presence of light: 80 per cent of the cells survived after 6 hr, 63 per cent after 24 hr and 52 per cent after 48 hr (Fig. 1).

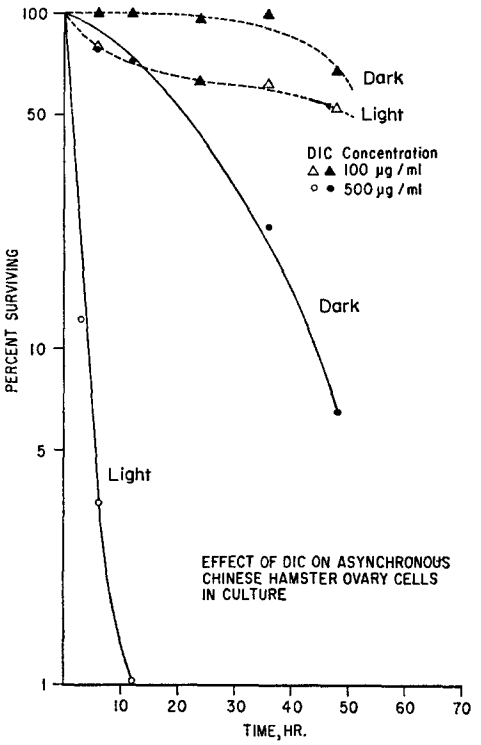


FIG. 1. Effect of DIC on asynchronous Chinese hamster cells in culture. Each point is the mean of three determinations. Typical of three experiments.

A more pronounced inhibition was seen at an initial DIC concentration of 500 μg/ml (Fig. 1). Again, the drug was more lethal to the cell in the light than in the dark. In darkness, 8 per cent of the cells survived after 48 hr, while in the light, a sharp exponential decrease in survival was observed, with 3·6 per cent of the cells surviving after treatment for only 6 hr.

Similar results were obtained with M cells.

*Effect of MIC on survival of CHO cells in culture.* The results of one of two experiments with CHO cells and MIC at an initial concentration of 100 μg/ml are shown in Fig. 2. After exposure to MIC for 48 hr there was no significant difference in cell

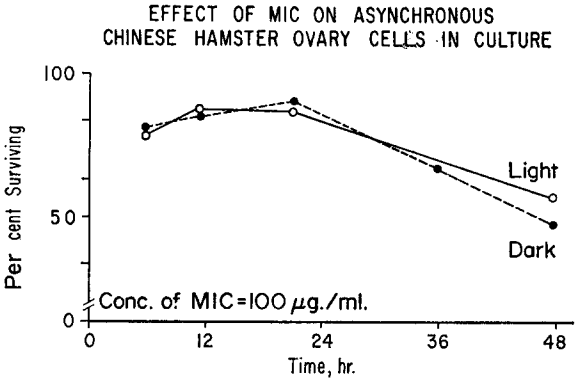


FIG. 2. Effect of MIC on asynchronous Chinese hamster cells in culture. Each point denotes the mean of three determinations. One of two experiments.

survival regardless of the presence of light. However, at a higher concentration of MIC, namely 500 μg/ml, cell viability was 16 per cent in the dark after 48 hr as compared with 31 per cent in the light.

*Labeling DNA by DIC-DM-$^{14}C$.* After exposure of CHO cells to DIC-DM-$^{14}C$ in darkness for 21 hr, analysis of the cell lysate showed a definite peak of radioactivity corresponding to the density of DNA (Fig. 3). A similar but more diffuse peak was also detected with M cells both sensitive and resistant to DIC.

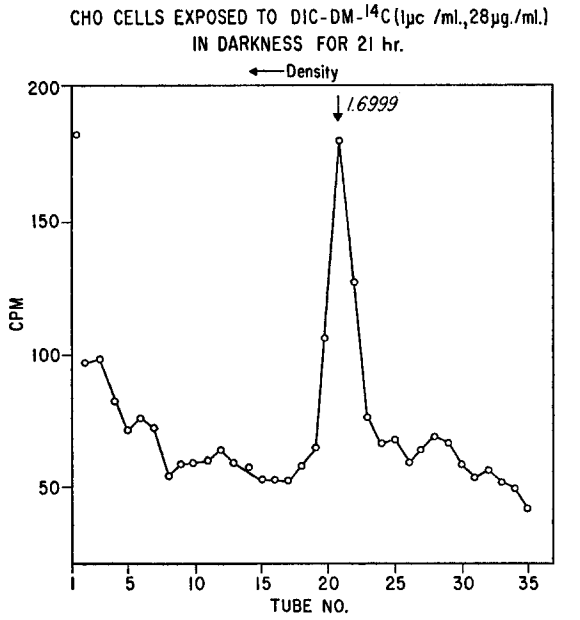


FIG. 3. Labeling of DNA of CHO cells with $^{14}C$ after exposure to DIC-DM-$^{14}C$ in darkness. One of three experiments.

To confirm that this peak of radioactivity was indeed located in the DNA fraction, double-labeling experiments were performed: CHO cells were treated with $^{3}$H-TdR before exposure to DIC-DM-$^{14}$C. Because $^{3}$H-TdR was taken up by the cells to a greater extent than was DIC-DM-$^{14}$C, the peak resulting from $^{14}$C appeared minute. Nevertheless, it coincided with the $^{3}$H peak at the density of DNA (Fig. 4).

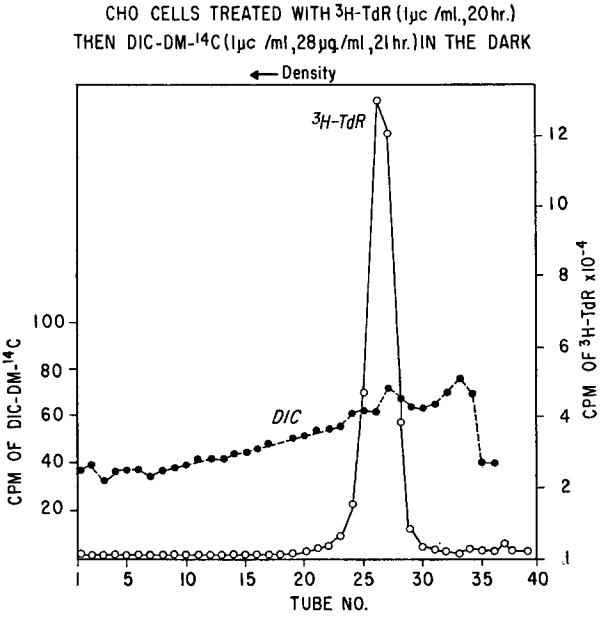


FIG. 4. Double-labeling of DNA of CHO cells with $^{3}$H and $^{14}$C after exposure to $^{3}$H-TdR followed by DIC-DM-$^{14}$C in darkness. Typical of three experiments.

However, when the above experiments were repeated in the light with either CHO or M cells, not even a slight peak of radioactivity of $^{14}$C was present in the DNA fraction. In fact, the radioactivity was distributed exactly like DIC-DM-$^{14}$C alone on a CsCl gradient (see below).

*Labeling of nucleic acids by DIC-2-$^{14}$C.* When CHO cells were incubated with DIC-2-$^{14}$C, the same results were obtained whether or not light was present (Figs. 5 and 6). The highest radioactivity was associated with the highest density corresponding to the position of RNA on the gradient. A smaller but unmistakable peak of radioactivity was also in the DNA fraction. This peak became more pronounced when a higher drug concentration (88·8 μg/ml, 4·8 μc/ml) was used (not shown). Pre-labeling with $^{3}$H-TdR confirmed that the peak occurred at the density of DNA (Fig. 6).

Similar observations have been made when M cells were used. No difference in the distribution of radioactivity was noted with M cells resistant to DIC.

*Labeling of nucleic acids by AIC-2-$^{14}$C.* The above experiments were repeated with AIC-2-$^{14}$C. The results were similar to those with DIC-2-$^{14}$C. Whether or not light was present, the highest counts were in RNA, the fractions of highest density; a distant large peak of radioactivity was also located in the DNA fraction.

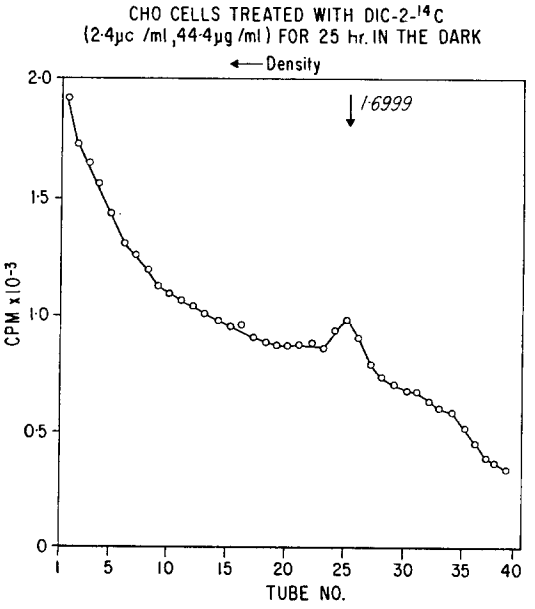


FIG. 5. Labeling of nucleic acids of CHO cells with $^{14}C$ after exposure to DIC-2-$^{14}C$ in darkness. Typical of three experiments.

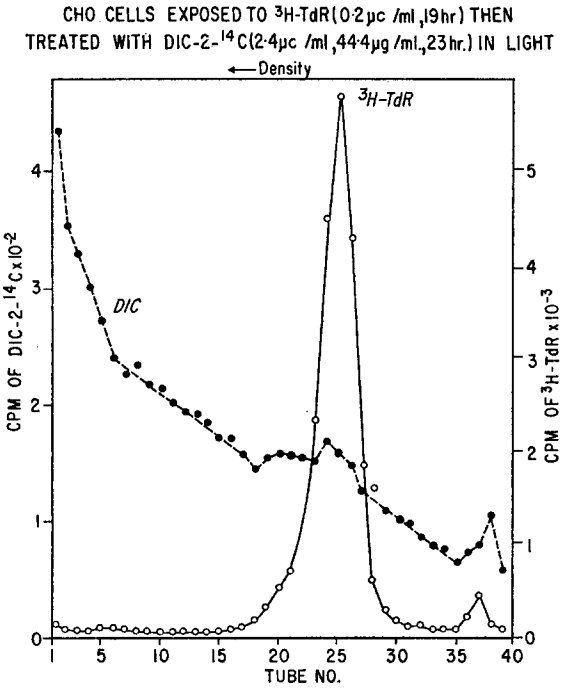


FIG. 6. Labeling of DNA of CHO cells with $^{3}H$ and both DNA and RNA with $^{14}C$ after exposure to $^{3}H$-TdR followed by DIC-2-$^{14}C$ in light. One of three experiments.

*Effect of DIC on DNA structure.* The effect of DIC on the structural integrity of DNA was determined by incubating CHO cells with $^{3}$H-TdR for 23 hr followed by DIC (500 $\mu$g/ml) for an additional 20 hr. CsCl density gradient analysis showed that there was no difference in the distribution of $^{3}$H in the DNA fraction of cells collected before and after DIC treatment. A marked peak of radioactivity resided in the DNA in both cases.

*Distribution of DIC on a CsCl gradient.* After DIC-DM-$^{14}$C and DIC-2-$^{14}$C had been incubated in McCoy's medium without cells under conditions of light and darkness, respectively, for 20 hr, an aliquot of the medium was mixed with the appropriate CsCl solution and centrifuged. Radioactivity increased linearly with decreasing density throughout the gradient. There was no peak of radioactivity.

*Volatile* in vitro *metabolites of DIC.* Brief heating in dilute NaOH caused a loss of 76 per cent of dimethylamine-$^{14}$C and 33 per cent of $^{14}CO_2$. In dilute HCl, the corresponding percentage losses were 24 per cent of dimethylamine-$^{14}$C and 86 per cent of $^{14}CO_2$. These data permitted us to estimate the relative amount of dimethylamine-$^{14}$C and $^{14}CO_2$ absorbed in the dilute NaOH and HCl during incubation of CHO cells with radioactive DIC, provided that these were truly the volatile *in vitro* metabolites of DIC.

With DIC-2-$^{14}$C, no significant radioactivity was detected in the absorbants.

With DIC-DM-$^{14}$C, however, radioactive volatile metabolites of DIC were found in both NaOH and HCl. In the light, 65–100 per cent of the absorbed material behaved like dimethylamine and 0–35 per cent like $CO_2$. In contrast, when light was excluded, only 23–50 per cent was "dimethylamine" and 50–77 per cent was "$CO_2$".

## DISCUSSION

The previous observation that the lethality of DIC to *Bacillus subtilis* is light-dependent[12] has now been extended to CHO and M cells in culture (Fig. 1). However, no enhancement of cytotoxicity by light was seen with MIC (Fig. 2). The initial concentrations of DIC were high because of the rapid decomposition of the drug in the culture medium, particularly in the light.

Although the exact mechanism of cytotoxic action of DIC remains to be elucidated, the interaction of DIC with DNA (Figs. 3 and 4) may be critical. This is consistent with the findings that at high concentrations DIC inhibits DNA synthesis in *B. subtilis*[12] and also that 7-methyl-$^{14}$C-guanine is excreted in the urine of rats and man dosed with DIC-DM-$^{14}$C.[15]

Like AIC-2-$^{14}$C, DIC-2-$^{14}$C confers its radioactivity to cellular RNA after incubation (Figs. 5 and 6). This and the fact that there was no labeling of DNA with DIC-DM-$^{14}$C in the light are in agreement with the current understanding of the decomposition of triazenes.[16] In the light, most of the DIC rapidly decomposes to dimethylamine and 5-diazomidazole-4-carboxamide (DZC). Though readily cyclizable to the relatively biologically inert 2-azahypoxanthine,[17] DZC is believed to be highly cytotoxic through interaction with nucleic acids. Upon exposure to light, therefore, DZC-2-$^{14}$C interacts with both DNA and RNA (Figs. 5 and 6). With DIC-DM-$^{14}$C and light, no labeling of nucleic acids is possible, since no DZC-2-$^{14}$C is formed. Nevertheless, it must be emphasized that this sequence of events involving DIC in the light may not necessarily have any counterpart *in vivo*.

When light is excluded during incubation, the above mode of decomposition is

retarded sufficiently to permit an alternative pathway of DIC decomposition to assume importance. By the alternative pathway, DIC loses a methyl group from the side chain as $CO_2$ by oxidative demethylation with the simultaneous formation of MIC. Our observations and those of others that the side chain methyl group has partially found its way as $^{14}CO_2$ in the expired air of animals injected with DIC-DM-$^{14}$C are compatible with this concept.[18,19] MIC tautomerizes to "iso-MIC", 5-(3-methyl-2-triazeno)imidazole-4-carboxamide, which readily cleaves to AIC and the precursor of a methyl carbonium ion. The elevated excretion of AIC in the urine of patients treated with DIC[20] can thus be explained. Since the tautomerization of MIC is not catalyzed by light, hence the cytotoxicity of MIC is not light-dependent (Fig. 2). Furthermore, as a highly reactive alkylating agent, the methyl carbonium ion ensuing from DIC easily interacts with DNA. Therefore, with DIC-DM-$^{14}$C, cellular DNA was not labeled except in the dark, because only then were radioactive methyl carbonium ions produced. In contrast, with DIC-2-$^{14}$C, regardless of light or darkness, there was either DZC-2-$^{14}$C or AIC-2-$^{14}$C generated to impart radioactivity to the nucleic acids. In the dark, the cytotoxicity of DIC must be caused in part, at least, by the methyl carbonium ion. Apparently, while DZC interacts with both DNA and RNA, the methyl carbonium ion limits its attack to DNA only. The nature of the interaction between DZC and nucleic acids is not well understood at the present.

We have assumed that the volatile *in vitro* metabolites of DIC were $CO_2$ and dimethylamine. This is reasonable in view of the proposed decomposition mechanism of this drug. Admittedly, the experimental design does not allow us to evaluate adequately the capacity of the cells to metabolize DIC by *N*-methylation.

TABLE 1. MODE OF DECOMPOSITION OF RADIOACTIVE DIC AND LABELING OF NUCLEIC ACIDS OF CHO CELLS

| Compound | Light | DZC-2-$^{14}$C | AIC-2-$^{14}$C | $^{14}CH_3^+$ | DNA | RNA |
|---|---|---|---|---|---|---|
| DM-$^{14}$C | + | − | − | − | − | − |
| DM-$^{14}$C | − | − | − | + | + | − |
| 2-$^{14}$C | + | + | − | − | + | + |
| 2-$^{14}$C | − | + | + | − | + | + |

In Table 1 we summarize our finding regarding the mode of decomposition of radioactivite DIC and the labeling of nucleic acids of CHO cells.

The radioactivity peak of $^3$H-TdR at the DNA fraction of the CHO cells lysate remains unchanged after DIC treatment clearly indicates that this drug does not cause extensive breaks in the helical structure of DNA. Moreover, when either DIC-DM-$^{14}$C or DIC-2-$^{14}$C was placed on a CsCl gradient, a linear increase in counts from highest to lowest density was found. These findings leave little doubt that with DIC-DM-$^{14}$C in the dark, the peak radioactivity at the DNA density, though slight as compared with the $^3$H peak resulting from the uptake of $^3$H-TdR, is not an artifact. The unambiguous $^{14}$C peak in the DNA after incubation of the CHO cells with DIC-DM-$^{14}$C alone in darkness further strengthens this argument. The low $^{14}$C radioactivity associated with DNA precluded the isolation of any DNA fragments containing $^{14}$C.

Generally, in darkness M cells behaved like CHO cells. M cells resistant to DIC were not different from sensitive cells with respect to the labeling of nucleic acids by the radioactive drug. Evidently the mechanism of resistance to DIC must be sought elsewhere.

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