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IN VIVO METABOLISM AND REACTION WITH DNA OF THE CYTOSTATIC AGENT, 5-(3,3-DIMETHYL-1-TRIAZENO)IMIDAZOLE-4-CARBOXAMIDE (DTIC)

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Abstract—The cytostatic drug dacarbazine [DTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide] is strongly carcinogenic in rats. Bioactivation of DTIC yields a methylating intermediate but the extent of interaction with cellular macromolecules has not previously been reported. Following a single i.p. injection of [^{14}C-methyl]DTIC, exhalation of $^{14}CO_2$ occurred with a $t_{\frac{1}{2}max}$ of approximately 2 hr (0.95 mg/kg) and 2.5 hr (95 mg/kg). Of the total radioactivity administered, 8.5% was exhaled as $^{14}CO_2$; 54% was excreted via the urine, predominantly as unchanged DTIC. In liver, kidney and lung, formation of 7-[^{14}C]methylguanine in DNA and RNA was directly proportional with dose. DNA methylation by a single dose of DTIC (9.8 mg/kg; 5 hr survival time) was highest in liver (35 μmoles 7-methylguanine/mole guanine), followed by kidney (25 μmoles) and lung (20 μmoles). The remainder tissues showed 7-methylguanine concentrations approximately 50% of those in liver DNA, with the exception of the brain which had a very low extent of DNA modification (~1 μmole/mole guanine). At the specific radioactivity used (48 mCi/mmole), the promutagenic base O^6-methylguanine was only detectable in liver, kidney, lung, and stomach DNA (0.6–0.8 μmoles/mole guanine). Autoradiographic studies revealed a diffuse distribution of reaction products in rat liver. In contrast, *N*-nitrosodimethylamine and related carcinogens known to be bioactivated by the hepatic cytochrome P-450 system show a predominantly centrilobular distribution. This difference may be due to the greater stability of proximate carcinogens generated by α-C hydroxylation at one of the methyl groups of DTIC.

The clinical use of DTIC§ (dacarbazine, NSC 45388) has been documented in a series of reports which indicate that it shows a significant antitumor effect in metastatic cancer, in particular malignant melanoma [1–3]. In addition, it has been administered in chemotherapy regimens in combination with other drugs [4–6]. DTIC is mutagenic in eukaryotic and prokaryotic cells [7] and chronic bioassay studies in laboratory rodents have revealed a considerable carcinogenic potency [8]. Following chronic oral administration to rats, tumors were predominantly located in the mammary gland and the thymus.

Bioactivation of DTIC occurs by enzymatic hydroxylation of one of the methyl groups, leading to the formation of methanediazohydroxide [9] as the ultimate reactant species. Skibba *et al.* [10] have demonstrated the formation of 7-methylguanine in DNA and RNA following DTIC administration to rats but quantitative data on the extent of DNA alkylation have not been reported. We have determined the extent of nucleic acid methylation in various rat tissues over a wide dose range and found a linear increase with dose in liver, kidney and lung. In addition to 7-methylguanine, the promutagenic base, O^6-methylguanine was present in detectable amounts.

MATERIALS AND METHODS

Chemicals. DTIC (Dacarbazine) was purchased from Miles (Dome) GmbH (Frankfurt, F.R.G.). [^{14}C-methyl]DTIC was prepared by reaction of [^{14}C]dimethylamine with a four-fold molar excess of 5-diazoniumimidazole-4-carboxamide in methanol, followed by column chromatography of the product on basic alumina [11]. The radiochemical purity was determined by high pressure liquid chromatography on Lichrosorb RP-18 columns eluted with 40% (v/v) aqueous methanol containing 0.01% (v/v) perfluorobutyric acid, and found to be greater than 95%. Sephabsorb HP was purchased from Pharmacia Fine Chemicals (Sweden). Riatron and Kontrogel scintillation cocktails were from Kontron AG (Zürich, Switzerland) and DNA grade hydroxylapatite from Bio-Rad Laboratories AG (California, U.S.A).

Animals. Young adult female Fischer 344 rats (120–150 g body weight; Charles River Wiga, F.R.G.) were maintained on a standard laboratory diet with water *ad libitum*.

Metabolic cage experiment. Two rats received a single i.p. dose of 0.95 mg/kg (0.005 mmole/kg; 48 mCi/mmole) or 95 mg/kg (0.52 mmole/kg; 0.41 mCi/mmole) of ^{14}C-DTIC. The exhaled $^{14}CO_2$ was determined using a metabolic cage (Jencons Metabowl, Hemel Hempstead, U.K.) by collecting

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§ Abbreviations used: DTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxaminde; [^{14}C-methyl]DTIC, 5-(3,3-[^{14}C]dimethyl-1-triazeno)imidazole-4-carboxamide; HMIC, 5-(hydroxymethyl-3-methyl-1-triazeno)imidazole-4-carboxamide; MTIC, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide.

samples (0.5 ml) from two serially connected Nilox columns (each containing 600 ml of 1 N NaOH) and counted for ^{14}C after addition of 1.0 ml H_2O and 10 ml of Kontragel (counting efficiency, 84–86%). The amount of [^{14}C-methyl]DTIC excreted into the urine was determined by high pressure liquid chromatography as described above. Collected feces were homogenized in 70 ml of water. To 1 ml of the suspension, 10 ml of Kontrogel were added (counting efficiency, 88%).

DNA methylation by DTIC in vivo. Three rats received a single i.p. injection of [^{14}C-methyl]DTIC (9.8 mg/kg, 48 mCi/mmole) and were killed 5 hr later. Liver, kidney, lung, brain, glandular stomach, small intestine, colon and spleen were rapidly removed, frozen in liquid N_2 and stored at −70°. DNA was isolated from the pooled tissues of three animals, using a modified hydroxylapatite method, as described earlier [12]. Immediately prior to analysis, DNA was depurinated in 0.1 M HCl at 80° for 30 min, neutralized to pH 5–6 and filtered through a 0.45 μm Gelman ACRO LC13 filter.

For the dose-response study on nucleic acid alkylation, [^{14}C-methyl]DTIC was diluted with unlabeled DTIC to give specific radioactivities ranging from 0.288 mCi/mmole (at 160 mg/kg) to 24 mCi/mmole (at 0.95 mg/kg). 7-Methylguanine in DNA and RNA was determined in liver, kidney and lung as described earlier [13]. Briefly, organs were homogenized at 3° in 10 vol. of water. After addition of 1 vol. of 10% TCA (w/v) to the homogenate, the precipitate was washed twice with 5% TCA, incubated in 2 ml 5% TCA for 45 min at 85° and neutralized by ether extraction to pH 5–6.

Radiochromatography. Purine bases were separated on Sephabsorb HP columns (1 × 50 cm) as previously described [14]. Samples were eluted with 10 mM phosphate buffer (pH 5.5) at a flow rate of 1.4 ml/min (fraction volume, 3.62 ml). Absorbance was determined in individual fractions at 260 nm, using a Shimadzu UV-240 spectrophotometer. Radioactivity was determined after the addition of 6.0 ml of Riatron scintillation cocktail (counting efficiency, 80–83%). Amounts of alkylated purines were expressed as μmole/mole guanine, assuming that their specific activity was the same as that of one of the methyl groups, i.e. half the specific radioactivity of the injected [^{14}C-methyl]DTIC.

Autoradiography. Liver samples from rats used in the DNA alkylation study (9.8 mg/kg [^{14}C-methyl]DTIC; 48 mCi/mmole) were fixed in buffered formaldehyde (4%), dehydrated and embedded in paraffin. Sections (6 μm) were exposed for 2–4 weeks in X-ray cassettes using an LKB 3H-Ultrofilm [15]. For comparison, liver samples from a rat that received a single i.p. dose of *N*-nitroso[^{14}C]dimethylamine (1.0 mg/kg; 51 mCi/mmole) were similarly processed for autoradiography.

RESULTS

The rate of DTIC metabolism was determined by monitoring exhaled $^{14}CO_2$ following a single i.p. injection of 0.95 or 95 mg/kg of [^{14}C-methyl]DTIC, corresponding to 0.005 and 0.52 mmole/kg respectively (Fig. 1). The time at which half the maximum amount of $^{14}CO_2$ was exhaled ($t_{\frac{1}{2}max}$) was 1 hr 54 min for the lower dose and 2 hr 36 min for the higher dose. The production of $^{14}CO_2$ reached a plateau after 10–12 hr. At this time, 8.3% (lower dose) and 8.9% (higher dose) of the total radioactivity administered had been exhaled as $^{14}CO_2$. An additional 53–55% of the injected radioactivity was excreted via the urine. Of this, approximately 85% was present as unchanged DTIC. The faeces contained 3.3 and 2.4% of the radioactivity administered at doses of 0.95 and 95 mg/kg respectively.

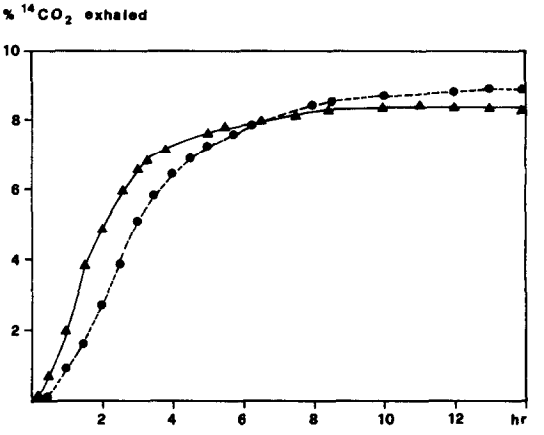

Fig. 1. Exhalation of $^{14}CO_2$ following a single dose of [^{14}C-methyl]DTIC. Adult rats received an i.p. injection of 0.95 mg/kg (▲) or 95 mg/kg (●). Exhaled $^{14}CO_2$ is expressed as cumulative percentage of the total radioactivity administered.

Formation of 7-methylguanine in DNA and RNA of liver, kidney and lung by a single i.p. injection of [^{14}C-methyl]DTIC (5 hr survival) showed linearity over a dose ranging from 4.5 to 160 mg DTIC/kg (Fig. 2). Highest concentrations were present in liver

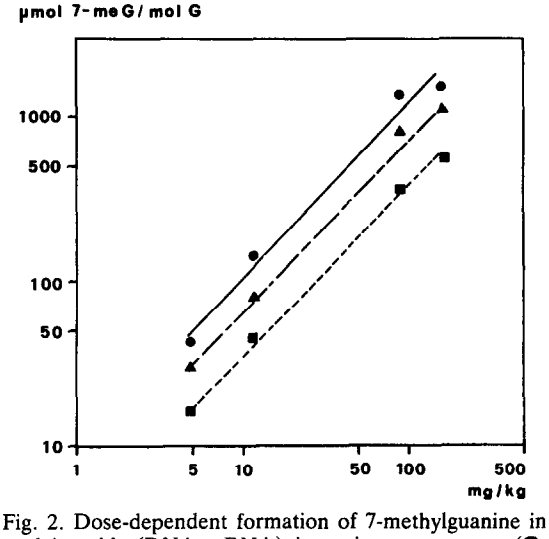

Fig. 2. Dose-dependent formation of 7-methylguanine in nucleic acids (DNA + RNA) in various rat organs (●, liver; ▲, kidney; ■, lung) after a single i.p. injection of [^{14}C-methyl]DTIC (one animal per dose; 5 hr survival time).

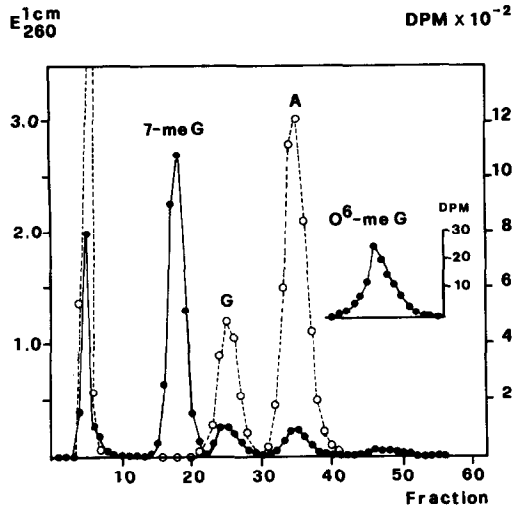

Fig. 3. Radiochromatographic profile of a kidney DNA hydrolysate from rats which received a single i.p. injection of [^{14}C-methyl]DTIC (9.8 mg/kg; 48 mCi/mmole; 5 hr survival). DNA isolation and Sephabsorb HP chromatography as described in the text. O---O, E_{260nm}; ●——●, DPM.

(13 μmoles/mole guanine/mg DTIC/kg), followed by kidney (7 μmoles) and lung (3.7 μmoles). The least squares regression equations were:

$$\log \text{7-methylguanine} = 1.038 \times \log \text{dose} + 0.995 \quad \text{(liver)},$$
$$= 1.034 \times \log \text{dose} + 0.8 \quad \text{(kidney)},$$
$$= 1.01 \times \log \text{dose} + 0.547 \quad \text{(lung)}.$$

A representative Sephasorb HP chromatogram of hydrolysed kidney DNA from rats treated with [^{14}C-methyl]DTIC is shown in Fig. 3. The major alkylated purines are 7-methylguanine and O^6-methylguanine. Metabolic incorporation into guanine and adenine was minimal. The extent of DNA alkylation in various rat tissues is shown in Table 1. Methylation was most extensive in liver and kidney. All other tissues had 7-methylguanine concentrations approximately half those of liver DNA, with the exception of brain DNA, which had methylpurine concentrations an order of magnitude lower than the other tissues. The O^6-methylguanine/7-methylguanine ratio was between 0.02 and 0.03.

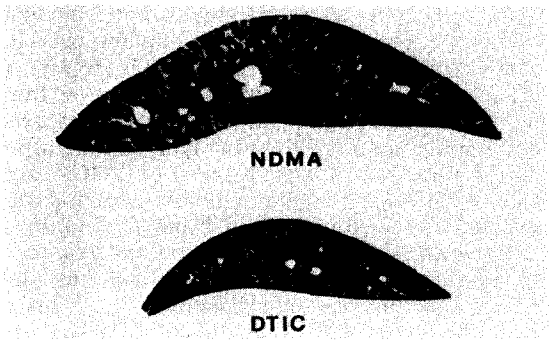

Fig. 4. Autoradiographs from rat liver showing the regional distribution of ^{14}C labelled macromolecules. Adult rats received a single i.p. dose of *N*-nitroso[^{14}C]dimethylamine (1 mg/kg) or [^{14}C-methyl]DTIC (9.8 mg/kg).

Autoradiographs (Fig. 4) from rats receiving a single i.p. dose of [^{14}C-methyl]DTIC revealed a diffuse distribution of reaction products in rat liver. In contrast, autoradiographs from rats treated with a single dose of *N*-nitroso[^{14}C]dimethylamine showed a patchy distribution, with preferential labelling of macromolecules in the centrilobular areas.

DISCUSSION

Bioactivation of DTIC requires hydroxylation of one of the methyl groups by a microsomal, NADPH-dependent, mixed-function oxidase, to form 5-(hydroxymethyl-3-methyl-1-triazeno)imidazole-4-carboxamide (HMIC). The latter metabolite was isolated from the urine and subsequently synthesized [19]. After the loss of formaldehyde [9], the remaining 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC) spontaneously tautomerizes and eliminates the methanediazonium ion as the ultimate methylating intermediate.

At a dose of 40 mg/kg the plasma clearance of DTIC in rats proceeds at a half-life of 30 min (Dr. C. J. Rutty, Institute of Cancer Research, Royal Cancer Hospital, Belmont, Sutton, Surrey SM2 5PX, U.K., personal communication). HMIC, the metabolite resulting from α-C hydroxylation of DTIC, was

Table 1. *In vivo* DNA methylation by [^{14}C-methyl]DTIC

Organ	7-meG (μmole/mole G)	% of liver	O^6-meG (μmole/mole G)	Ratio O^6-/7-meG
Liver	34.8	100	0.7	0.022
Kidney	24.6	71	0.8	0.033
Lung	20.1	58	0.7	0.035
Glandular stomach	18.4	53	0.6	0.033
Small intestine	17.5	50	n.d.	
Spleen	17.3	50	n.d.	
Large intestine	15.9	46	n.d.	
Brain	1.0	3	n.d.	

Rats received a single i.p. dose of [^{14}C-methyl]DTIC (9.8 mg/kg, 48 mCi/mmole) and were killed 5 hr later. Concentrations of 7-methylguanine (7-meG) and O^6-methylguanine (O^6-meG) are expressed as fraction of the parent base guanine (G).
n.d., not detectable.

found to be more stable than the parent compound ($t_{\frac{1}{2}}$, 53 min). The exhalation of $^{14}CO_2$ following DTIC administration does not directly reflect the rate of DTIC metabolism. This radioactivity is derived from the ^{14}C-formaldehyde that is formed during the decomposition of HMIC. In addition, methanediazonim ion, the ultimate reactant of DTIC, reacts with tissue water to yield methanol, which is subsequently oxidized to formaldehyde. This results in a ^{14}C labelling of the one-carbon pool and ultimately to the formation of $^{14}CO_2$. It is, therefore, not surprising that the rate of exhalation ($t_{\frac{1}{2}max}$, 2 hr) is somewhat slower than the half-life of HMIC, i.e. the most stable DTIC intermediate. The fraction of ^{14}C-DTIC exhaled within 12 hr amounted to 8%, which corroborates the value of 4% determined by Skibba *et al.* [9] 6 hr after DTIC administration.

The present study does not allow one to conclude in which tissues DTIC is enzymatically activated. *In vitro* studies on 3,3-dimethyl-1-phenyltriazene, a structural analogue of DTIC, suggest that only liver and kidney are actively engaged in dialkyltriazene metabolism [16, 17]. This is supported by our observation that the highest levels of DNA methylation were present in liver and kidney. In most of the other tissues, 7-methylguanine levels amounted to approximately 50% of those in rat liver, suggesting that these organs become alkylated by a DTIC metabolite. Exceptionally low levels of alkylation were present in brain and this is most probably due to the inability of DTIC metabolites (HMIC, MTIC) to cross the blood–brain barrier [18].

The formation of 7-methylguanine in nucleic acids was linear over a dose ranging from 4.5 to 160 mg/kg, indicating that the microsomal enzymes participating in the metabolism of DTIC were not saturated. Linearity over the entire dose range also suggests that the fraction of DTIC metabolites that is released into the systemic circulation and responsible for DTIC alkylation in extrahepatic tissues does not vary with dose. DTIC shares with *N*-nitrosodimethylamine the initial step in enzymatic bioactivation, i.e. hydroxylation of one of the methyl groups. This reaction is mediated by the microsomal cytochrome P-450 system which is predominantly located around the central vein of the liver lobule. If the half-life of the resulting proximate carcinogen is very short, methylation products with hepatic macromolecules are preferentially located in the centrilobular areas. This is exemplified for *N*-nitroso-[^{14}C]dimethylamine (Fig. 4, upper), which yields a monohydroxy derivative with a reported half-life of approximately 10 sec [20, 21]. In contrast, the distribution of reaction products derived from [^{14}C-methyl]DTIC was found to be homogeneous, with no detectable centrilobular accentuation (Fig. 4, lower). This is probably due to the fact that the bioactivation of DTIC yields a stable hydroxymethyl intermediate (HMIC), which can be redistributed within the liver [16, 21] and transported to other tissues via the systemic circulation.

Although 7-methylguanine contributes to the cytotoxicity of alkylating agents, other modified bases are apparently more important for its adverse biological effects [22]. This is particularly true for O^6-methylguanine, a promutagenic lesion assumed to be involved in the initiation of malignant transformation by methylating carcinogens. The ultimate carcinogen of methylating dialkyl-triazenes and nitrosamines is methanediazonium ion in equilibrium with methanediazohydroxide [23]. When the reaction takes place *in vitro*, i.e. in the absence of repair enzymes, the molar ratio of O^6-/7-methylguanine is 0.11. The ratios of 0.02–0.035 observed in the present experiments indicate that most of the O^6-methylguanine formed was already repaired during the 5 hr observation period. Rat liver has a remarkably high activity of O^6-methylguanine-DNA alkyltransferase [24]. Overloading of the hepatic alkyltransferase system [25] can only be expected when the amount of O^6-methylguanine exceeds the value of approximately 180 μmoles/mole guanine [26], i.e. at concentrations much higher than those observed in the present study. O^4-Methylthymine, although repaired at a much slower rate, was not detectable. *In vitro* studies [22] indicate that this promutagenic base is formed at concentrations 50–100 times lower than O^6-methylguanine.

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