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MECHANISM OF THE ANTILEUKEMIC EFFECTS OF 1-*p*-CARBOXAMIDOPHENYL-3,3-DIMETHYLTRIAZENE AND ITS *IN VITRO* METABOLITES*

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Abstract—DM-$CONH_2$, a dimethyltriazene active in prolonging the survival time of mice bearing TLX5 lymphoma, requires metabolic activation by liver homogenate supernatant and cofactors in order to exert *in vitro* cytotoxic effects on the same tumor cells, as determined by *in vivo* bioassay of their viability. From the examination of the metabolites produced during these *in vitro* experiments, it is found that *in vitro* cytotoxicity is attributable to the generation of MM-$CONH_2$ by oxidative *N*-demethylation of DM-$CONH_2$. Also the generation of DM-COO^- is observed, although this compound is not cytotoxic *in vitro*. The *in vivo* effects of DM-$CONH_2$ and CM-COOK on TLX5 lymphoma are not caused exclusively by cytotoxic effects of the drugs, since they are evident also when no reduction in the number or viability of peritoneal tumor cells is evident, whereas these parameters are significantly reduced by MM-$CONH_2$. The increase in survival time of mice bearing TLX5 lymphoma caused by the dimethyltriazenes used appears to be caused by the drugs without being subjected to metabolic activation, with a mechanism different from cytotoxicity for tumor cells.

DTIC is an imidazole dimethyltriazene which possesses antitumor activity against several experimental tumors [1], and which is clinically employed in the treatment of human malignancies [2–4]. Aryldimethyltriazenes, structurally related to DTIC, cause various biological effects, including antitumor activity in experimental systems [5–7], mutagenic effects on yeasts [8–10] and in *Drosophila melanogaster* [10, 11], and carcinogenic effects in rats [12] and mice [13]. These biological effects of aryldimethyltriazenes have been suggested to be caused by the generation of chemically reactive species from the parent dimethyl compound. Two different pathways have been identified, the former consisting of the hydrolysis of aryldimethyltriazenes to aryldiazonium cations. The second is the production, by microsomal oxidative *N*-demethylation, of arylmonomethyltriazenes, which decompose producing methylcarbonium ions. Both these pathways appear to participate in causing mutagenic and carcinogenic effects [8–10, 12].

As far as antitumor effects are concerned, it appears estabilished that dimethyltriazenes require metabolic activation in order to exert *in vitro* cytotoxic effects [5, 14]. Monomethyltriazenes produced *in vivo* by oxidative *N*-demethylation have been deductively considered candidates for the active species. The production of other species, such as 3-acetyl-3-methyl-1-phenyltriazene, acetanilide and other derivatives of aniline have been observed in the *in vitro* microsomal metabolism of 3,3-dimethyl-1-phenyltriazene [15], and the presence of 5-(3-hydroxymethyl-3-methyl-1-triazeno)imidazole-4-carboxamide has been shown in the urine of rats treated *in vivo* with DTIC [16]. However, the role of these metabolites in the effects of the parent compounds has not been unequivocally determined.

The aim of this investigation has been therefore to identify the metabolites produced by microsomal metabolism of 1-*p*-carboxamidophenyl-3,3-dimethyltriazene (DM-$CONH_2$)‖ in conditions which have been shown to activate this compound to products that are cytotoxic *in vitro* for TLX5 lymphoma cells [14]. The increase in life-span of mice bearing this tumor and the individual cytotoxicity of DM-$CONH_2$ metabolites for these tumor cells *in vitro* and *in vivo* have also been evaluated and are hereafter reported.

MATERIALS AND METHODS

Synthesis. DM-$CONH_2$, MM-$CONH_2$, DM-COOH, and its potassium salt, and [^{3}H]DM-$CONH_2$ (sp. act. 250 μCi/mmole) were synthesized following standard procedures [5, 17, 18]. NH_2-$CONH_2$ and formaldehyde were obtained from ICN-K & K Laboratories Inc., and from Hoechst Italia S.p.A., respectively.

In vitro *cytotoxicity.* For the determination of *in vitro* cytotoxic effects of metabolically activated

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‖ Abbreviations: DM-$CONH_2$, 1-*p*-carboxamidophenyl-3,3-dimethyltriazene; MM-$CONH_2$, 1-*p*-carboxamidophenyl-3-methyltriazene; [^{3}H]DM-$CONH_2$, 1-*p*-carboxamidophenyl-3,3-[^{3}H]dimethyltriazene; DM-COOH, *p*-(3,3-dimethyl-1-triazeno)benzoic acid; DM-COOK, *p*-(3,3-dimethyl-1-triazeno)benzoic acid potassium salt; NH_2-$CONH_2$, *p*-aminobenzamide.

DM-$CONH_2$, a postmitochondrial liver supernatant, obtained by centrifuging a liver homogenate at 10,000 *g* for 30 min, was used. The livers were obtained from Sprague–Dawley rats which had received phenobarbitone in drinking water (0.5 g/l.) for 4 days before being killed, and were homogenized in 2 vol. of 0.1 M K-phosphate buffer, pH 7.4, using a Potter–Elvehjem tissue grinder. Protein concentration, determined by the method of Lowry *et al.* [19] using bovine serum albumin as standard, was adjusted to 60 mg/ml. The incubation mixture consisted of 100 μl of ethanol containing the drug, 2.9 ml of Dulbecco culture medium containing 4×10^6 TLX5 lymphoma cells and 4% fetal calf serum, and 1 ml of 0.1 M K-phosphate buffer, pH 7.4, containing 30 mg of protein of liver postmitochondrial supernatant, 12 μmoles of $MgCl_2$, 0.8 μmoles of NADP, 16 μmoles of glucose-6-phosphate and 0.35 U of glucose-6-phosphate dehydrogenase [NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Boehringer (Mannheim, West Germany)]. After incubation for 2 hr at 37° with gentle shaking tumor cell viability was bioassayed by injecting i.p. 0.1 ml aliquots into a group of five animals whose survival time was determined. The same experiments were also carried out in the absence of metabolic activation, omitting the inclusion of liver supernatant and cofactors in the *in vitro* incubation mixture.

Preparation of in vitro *metabolites of DM-$CONH_2$.* The incubation mixture was the same as earlier with the exception that the cell suspension was replaced by 0.1 M K-phosphate buffer, pH 7.4. After incubation at 37° for 2 hr, the reaction was stopped by freezing and the mixture lyophilized; the metabolites were solubilized from the residual solid by shaking with 1 ml of ethanol and the resulting solution was concentrated under reduced pressure.

Separation and identification of metabolites. The mixture of the solubilized metabolites was submitted to TLC on alumina plates (DC-Karten ALF, Hoechst). Co-chromatography with authentic samples of the triazene and NH_2-$CONH_2$ was also carried out. Plates were sprayed with the Ehrlich reagent for detection of aromatic amines [20] and with a specific reagent for detection of triazenes [21]. When [^{3}H]DM-$CONH_2$ was used, the chromatogram was also analyzed using a Radiochromatogram Scanner, Packard Model 7201. Alternatively, the metabolites isolated by TLC were scraped off the plate, solubilized using acetone–water (9:1) and were analyzed by mass spectrometry. Mass spectrometry was carried out using 70–70 VG Micromass linked with a VG 2035 Data System. Samples were introduced to the source via the direct solid probe or via a capillary gas chromatography column in a Pye Unicam Series 204 gas chromatograph. The column used (dimensions 40 m × 0.32 mm) was coated with OV 101 stationary phase. Helium was used as carrier gas at a flow rate of 2 ml/min; the injector and transfer line from the column to the mass spectrometer source were maintained at 250°. The column temperature conditions were 100° for the first 3 min, followed by an increase to 250° at 16°/min. The mass spectrometer operating conditions for E.I. were: accelerating voltage 4 kV, source temperature 200°,

Table 1. Thin-layer chromatographic analysis of DM-$CONH_2$ metabolites

Eluent	R_f	Reactivity to reagents for: Triazenes	Aromatic amines	Identified as	Designated as
a	0.00	+	−	—	—
	0.25	−	+	*p*-Aminobenzamide (NH_2-$COHN_2$)	Metabolite **2**
	0.47	+	−	1-*p*-Carboxamidophenyl-3,3-dimethyltriazene (DM-$CONH_2$)	Metabolite **1**
b	0.18	+	−	*p*-(3,3-Dimethyl-1-triazeno)benzoic acid (DM-COOH)	Metabolite **3**
	0.87	−	+	*p*-Aminobenzamide (NH_2-$CONH_2$)	Metabolite **2**
	0.95	+	−	1-*p*-Carboxamidophenyl-3,3-dimethyltriazene (DM-$CONH_2$)	Metabolite **1**

a: acetone/ethyl acetate 4/1 (v/v); b: acetone/ethyl acetate/water 4/1/1.5 (v/v/v).

scan speed 1 sec/decade, electron energy 70 eV and trap current 200 μA. For C.I. operation the electron energy was 50 eV and the emission current 500 μA and isobutane was introduced to the source to a pressure of approximately 1 torr.

In vivo *experiments*. 10^5 TLX5 tumor cells were transplanted i.p. into CBA/Lac mice; drug treatment was performed daily on days 3–7 after tumor transplantation, by i.p. injection of the drugs in 0.05 ml per 10 g of animal weight of olive oil in the case of DM-$CONH_2$ and MM-$CONH_2$, and of 0.1 N $NaHCO_3$ in the case of DM-COOK. The survival time of the animals was recorded. In separate experiments the total number of peritoneal tumor cells was measured at the end of treatment, after their careful collection by repeated washing of the peritoneal cavity; cells were counted in a Coulter Counter Model ZF. The viability of peritoneal tumor cells at the end of treatment was also evaluated by transplanting 10^5 tumor cells obtained from treated donors i.p. into normal recipients. In this case the survival time of the normal recipients was recorded and compared with that of control animals that had received a similar transplant from donors that had not been treated with drugs; this provides an indirect indication of the viability of tumor cells present in the bioassayed sample [22].

RESULTS

Identification of DM-$CONH_2$ metabolites produced in vitro

The results of the chromatographic analysis of the metabolites obtained from *in vitro* activation of DM-$CONH_2$ are reported in Table 1. Three u.v. absorbing substances can be detected on thin-layer plates after chromatography. The R_F of the two substances having the higher chromatographic mobility are equal to those of DM-$CONH_2$ and NH_2-$CONH_2$; these substances are positive to spray reagents for triazenes and anilines, respectively. The third metabolite has a low chromatographic mobility, is positive only to the spray for triazenes and has the same R_F as DM-COOH (Table 1). The relative yield of the metabolites, estimated on the basis of their u.v. absorbance and of their radioactivity in the case of metabolites **1** (starting DM-$CONH_2$ recovered from the chromatographic plate) and **3**, indicates that metabolite **2** is produced in a quantity proportional to the demethylation of the starting material (about 12%), and that metabolite **3** is produced in slightly smaller amounts. The use of [^{3}H]DM-$CONH_2$ as the starting material, and the subsequent radiochromatographic analysis of the incubation mixture, show the presence in metabolites **1** and **3** of methyl groups (Fig. 1).

The E.I. mass spectrum of metabolite **1** (direct insertion probe) is identical to that of an authentic sample of DM-$CONH_2$, which shows a molecular ion at *m/z* (%): 192 (27.1), 148 $[(M - N(CH_3)_2)]^+$ or $[M - CONH_2]^+$ (76.2), 120 $[M - N{=}N{-}N(CH_3)_2]^+$ (92.3) and 103 (100). The spectrum of the appropriate blank sample associated with unchanged triazene shows only weak peaks. The mass spectrum of authentic NH_2-$CONH_2$ shows the most intense peaks at *m/z* (%): 136 $[M]^+$ 74.4, 120 $[M - NH_2]^+$ (100), 92 $[M - CONH_2]^+$ (37.2) and 65 (31.3). The metabolite with the same chromatographic properties as NH_2-$CONH_2$ shows its prominent ions at *m/z* (%): 192 (12.2), 148 (33.5), 136 (55.3), 120 (100), 103 (34.2), 92 (28.1) and 65 (32). It appears therefore that the material which gives this spectrum is a mixture of NH_2-$CONH_2$ with contaminating DM-$CONH_2$. The appropriate blank spectrum is weak and shows no prominent ions at the *m/z* values mentioned earlier.

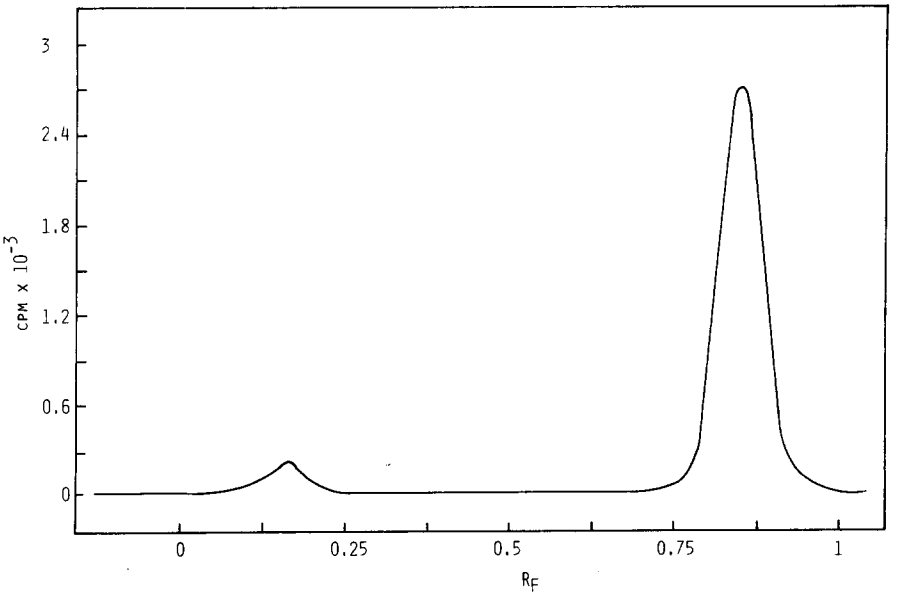

Fig. 1. Radiochromatographic analysis of DM-$CONH_2$ metabolites. TLC has been performed using eluent b (see Table 1). The operating conditions are: scan speed 0.5 cm/min, HV 1.2 kV, time constant 30 sec, helium–isobutane flow 150 ml/min.

Authentic DM-COOK was dissolved in N/100 hydrochloric acid and the free acid extracted into ethyl acetate. Samples of biologically derived DM-COO^- were treated similarly although an initial extraction at neutral pH with ethyl acetate was used to remove non-polar contaminants. The E.I. mass spectrum of the authentic benzoic acid shows a molecular ion at *m/z* (%): 193 (17.6), 149 [M − $N(CH_3)_2]^+$ or [M − $CO_2]^+$ (43), 121 [M − N=N-$N(CH_3)_2]^+$ (87.4) and 65 (100). A smaller extent of fragmentation is seen in the C.I. spectrum and the protonated molecular ion (*m/z* 194) is now the base peak, with fragments at *m/z* 149 (12.5%) and 123 (51.5%). The acid was also reacted with diazomethane in an ether solution at room temperature for 10 min, and the resulting methyl ester was chromatographed on the OV 101 column (retention time 10.8 min) and subjected to C.I. mass spectrometry. This spectrum also shows the MH^+ ion (*m/z* 208) as the base peak.

The biological extracts were studied by C.I. mass spectrometry as it was found that less contamination was seen in the resulting spectra. The C.I. probe spectrum of metabolite **3** shows the presence of DM-COO^- (*m/z* 194, 149 and 123). Some minor contaminating peaks of higher mass are also seen but these are due to an impurity from the isolation procedure as they are also present in the spectrum of a similar sample isolated following an incubation with no triazene present. A portion of the metabolite was methylated and subjected to GC–MS. A component was eluted at the retention time of the methyl ester of DM-COOH. Although only a weak mass spectrum was obtained the MH^+ ion at *m/z* 208 and a fragment ion at *m/z* 137 are present. There is thus strong evidence that metabolite **3** is DM-COO^- and that the structures DM-$CONH_2$ and NH_2-$CONH_2$ can be assigned to metabolites **1** and **2**, respectively.

In vitro *cytotoxicity*

DM-$CONH_2$ does not exert direct cytotoxic effects on tumor cells; on the contrary, however, its metabolic activation results in significant cytotoxic effects observed when the treated tumor cells are injected into normal mice. MM-$CONH_2$ and formaldehyde have also been examined in these conditions. The concentration used (0.26 μmoles/ml) is the same as that generated during metabolic activation of DM-$CONH_2$, as measured colorimetrically in separate experiments. MM-$CONH_2$ causes cytotoxic effects comparable to those of metabolically activated DM-$CONH_2$, whereas formaldehyde is devoid of any effect. DM-COOK, tested at concentrations up to the initial concentration of DM-$CONH_2$ (2.1 μmoles/ml) is similarly devoid of cytotoxic effects (Fig. 2).

In vivo *effects*

DM-$CONH_2$, MM-$CONH_2$ and DM-COOK significantly increase the survival time of mice bearing TLX5 lymphoma. The activity of MM-$CONH_2$ appears more pronounced than that of DM-$CONH_2$, since it causes an increase in the life-span greater than 30% at four dose levels, as compared to three dose levels for DM-$CONH_2$, and because it causes a greater increase in the life-span; the activity of DM-COOK appears to be of the same magnitude as that of DM-$CONH_2$ and MM-$CONH_2$. The number of peritoneal tumor cells is decreased by DM-$CONH_2$ and MM-$CONH_2$ in a dose-dependent way; however, at 130 μmoles/kg, DM-$CONH_2$ significantly increases the survival time of the treated

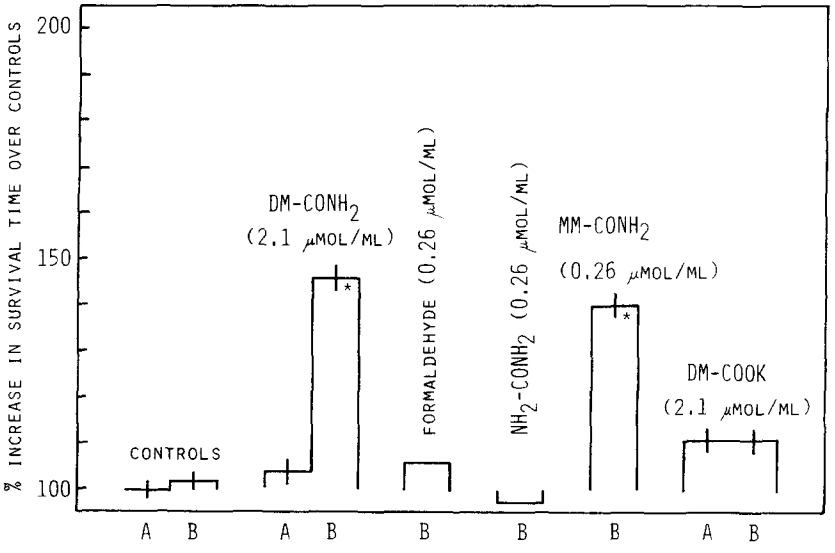

Fig. 2. *In vitro* cytotoxicity of DM-$CONH_2$ and its metabolites on TLX5 lymphoma cells. Tumor cells were incubated *in vitro* in the absence (A) or in the presence (B) of a metabolically activating system. 10^5 tumor cells were then injected into normal recipients whose survival time, expressed as a percentage of that of mice injected with tumor cells incubated in the absence of the drug, is indicated in the ordinates. Each value is the mean ± S.E. obtained using groups of five mice; *means significantly different from controls, Student–Newmann–Keuls test [29], $P < 0.05$. The actual value in the control group is 10.4 ± 0.2 days.

Table 2. Effects of DM-$CONH_2$, MM-$CONH_2$ and DM-COOK on the survival time and on the number and viability of tumor cells in mice bearing i.p. TLX5 lymphoma

Compound	Daily dose (μmoles/kg)	Survival time (days) Mean ± S.E.	Number of peritoneal tumor cells when killed Average × 10^6 ± S.E.	Survival time of mice inoculated with 10^5 peritoneal tumor cells (days)
—	—	10.0 ± 0.2	1355 ± 122	10.1 ± 0.2
DM-$CONH_2$	65	10.7 ± 0.3	1071 ± 208	10.4 ± 0.2
	130	15.0 ± 0.0*	1195 ± 295	10.5 ± 0.3
	260	15.0 ± 0.6*	309 ± 89*	13.5 ± 0.8*
	520	14.1 ± 1.0*	88 ± 30*	14.2 ± 0.2*
MM-$CONH_2$	17.4	12.0 ± 0.6	768 ± 192*	11.0 ± 0.6
	34.8	15.7 ± 0.3*	661 ± 162*	11.5 ± 0.9
	69.6	15.3 ± 0.3*	140 ± 33*	13.4 ± 0.8*
	139.2	17.7 ± 0.9*	115 ± 26*	15.3 ± 0.2*
	278.4	15.0 ± 1.0*	116 ± 38*	16.1 ± 0.3*
DM-COOK	56	10.3 ± 0.3	1002 ± 184	10.9 ± 0.3
	108	16.7 ± 1.2*	914 ± 172	11.8 ± 0.2
	216	16.8 ± 0.9*	942 ± 208	12.2 ± 0.2
	432	14.5 ± 0.2*	1148 ± 154	13.0 ± 0.2*

Groups of 4–10 tumor-bearing animals were treated i.p. on days 3–7 after tumor transplantation; 24 hr after the last drug administration the animals were killed and their peritoneal tumor cells were transplanted into normal recipients whose survival time is reported. The highest dosage used for each compound is the maximum tolerated one.

* Means significantly different from that of controls, Student–Newmann–Keuls test [29], $P < 0.05$.

animals without significantly decreasing the number of peritoneal tumor cells, whereas the number of tumor cells is significantly lowered by MM-$CONH_2$ at all the dosages examined including the lower inactive one (Table 2). A dose-dependent reduction in viability, resulting from the bioassay of *in vivo* treated tumor cells, is observed for DM-$CONH_2$ and MM-$CONH_2$, which is absent at their lowest active dosage. For DM-COOK the number of peritoneal tumor cells is not significantly reduced at any dosage active in increasing survival time, and cell viability is decreased only at the highest dosage (Table 2).

DISCUSSION

Dimethyltriazenes have been reported to be active *in vivo* as antineoplastic agents because of their conversion by oxidative *N*-demethylation by hepatic microsomes to monomethyltriazenes, which produce by hydrolysis the alkylating species methyl carbocations [23, 24]. Indeed, DM-$CONH_2$ is cytotoxic *in vitro* only when metabolically activated [5, 14]. However, the view that dimethyltriazenes cause their antineoplastic activity via *N*-demethylation can be questioned since no evident correlation appears between *in vitro* oxidative *N*-demethylation and *in vivo* antitumor activity [25, 26]. This fact suggests that their antineoplastic activity could be due to metabolites different from the monomethyltriazene.

The first step of the present investigation was thus aimed at isolating the metabolites of DM-$CONH_2$ produced *in vitro* in the presence of hepatic microsomes and cofactors, using the same conditions which activate the drug to cytotoxic products [14]. Other investigators have examined and reported the metabolites of aryldimethyltriazenes produced *in vitro* and *in vivo*. Pool [15] reported the generation of several species *in vitro* but did not indicate either the yield of each metabolite (which might be exceedingly small considering the experimental conditions used) or its role for the *in vivo* antitumor activity. The isolation by Kolar and co-workers of 5-(3-hydroxymethyl-3-methyl-1-triazeno)imidazole-4-carboxamide (H-MTIC) as a urinary metabolite of *in vivo* administered DTIC has also been reported, but in this case the *in vivo* role of H-MTIC, which could be a detoxification product, is not clarified. The presently reported data indicate that the dimethyltriazene employed, besides its transformation to the monomethyl derivative, is also converted to another dimethyltriazene, $DM\text{-}COO^-$; this conversion should be ascribed to the action of unspecific liver amidases [27]. $DM\text{-}COO^-$ does not appear to undergo a further conjugation with glucuronic acid, which is widely described for benzoic acids, since the pattern of DM-$CONH_2$ metabolites is not modified either by the presence of UDPGA in the incubation mixture or by the treatment after incubation with glucuronidase (unreported results). However, the isolation of a new metabolite, different from the monomethyltriazene does not explain the *in vitro* cytotoxicity of DM-$CONH_2$. In fact, DM-COOK is devoid of cytotoxic effects even at concentrations equal to the initial one of DM-$CONH_2$ and up to 8.4 μmoles/ml, while the latter produces *in vitro* effects only in the presence of a microsomal activating system. Furthermore, these effects, in agreement with previously reported observations [5], can be attributed to the production of the cytotoxic MM-$CONH_2$. Indeed, MM-$CONH_2$ at a concentration equal to that produced by metabolic activation of DM-$CONH_2$ causes cytotoxic effects of the same magnitude as those of metabolically activated DM-$CONH_2$.

The examination of the *in vivo* antitumor effects of DM-$CONH_2$, MM-$CONH_2$ and DM-COOK indicates that all these compounds are capable of significantly increasing the life-span of mice bearing

TLX5 lymphoma. However, the mechanism of the antileukemic action of the dimethyltriazenes is different from that of MM-$CONH_2$. In fact, the significant prolongation of the life-span of the animals treated with MM-$CONH_2$ is parallelled by a drastic reduction in the number of peritoneal tumor cells and in their viability at all the active dosages, thus indicating cytotoxic properties of this compound also *in vivo*. On the contrary, similar cytotoxic effects are not observed for DM-$CONH_2$ and in particular for DM-COOK at *in vivo* active dosages. In similar conditions, CCNU causes the absence of detectable peritoneal tumor cells when an increase in the survival time of the host identical to that presently reported for dimethyltriazenes is observed, as expected considering the growth kinetics of this tumor [22].

These data thus indicate that the oxidative *N*-demethylation of DM-$CONH_2$ to MM-$CONH_2$ cannot account alone for the *in vivo* antileukemic action of DM-$CONH_2$. The marginal role of activation via demethylation is further supported by the *in vivo* antileukemic action of DM-COOK, which is practically resistant to oxidative *N*-demethylation *in vitro* [28]. The relevance of the conversion of DM-$CONH_2$ to DM-COO^- for the antileukemic properties of the former seems unimportant because of the limited deamidation observed *in vitro*. In the light of the data presented and considerations made, the dimethyltriazenes examined might act also without being subjected to metabolic transformation, mainly increasing the survival time of leukemic mice with a mechanism different from cytotoxicity, whose nature is currently under investigation in our laboratory.

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