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Effects of an inducer and an inhibitor of hepatic metabolism on the antitumor action of dimethyltriazenes*

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Summary. To investigate the role of monomethyltriazenes as the active metabolites of antitumor dimethyltriazenes, the in vivo simultaneous treatment with an inducer (phenobarbital, PB) or an inhibitor (carbon tetrachloride, CCl_4) of hepatic drug metabolism was examined in mice bearing Lewis lung carcinoma. Treatment with PB or CCl_4 with the dosage and schedules employed proved to be effective in markedly modifying the *N*-demethylation of the three dimethyltriazenes tested, as had been determined in vitro. No unambiguous increase by PB, or decrease by CCl_4, which might theoretically be expected if metabolic conversion to monomethyltriazenes was involved, was observed for the antitumor and antimetastatic activity of dimethyltriazenes. At the same time, a difference was noted between the effects on primary tumors and those on metastases. These data support the view that generalizations on the relevance of monomethyltriazenes as the active metabolites responsible for the antitumor and antimetastatic activity of dimethyltriazenes may not be valid.

Introduction

The biological properties of imidazole and benzenoid dimethyltriazenes include antitumor [2, 11, 13, 14, 20, 21], mutagenic [3, 25, 36, 37], carcinogenic [4, 27] and xenogenizing effects [28]. The existing evidence indicates that dimethyltriazenes undergo in vitro and in vivo (bio)transformation with generation of chemically reactive species [8]. Besides chemical hydrolysis to diazonium cations, which have been shown to be responsible for local carcinogenic effects [25, 27], the production via oxidative *N*-demethylation of monomethyltriazenes is generally considered to be responsible for the causation of the biological effects of dimethyltriazenes [8, 19, 25–27, 37]. Monomethyltriazenes thus produced quickly decompose by way of proton-catalyzed hydrolysis, generating methyl-carbocations capable of methylating cellular components including nucleic acids [6, 8, 16].

Since oxidative *N*-demethylation has been reported to be required for the antitumor action of dimethyltriazenes [1, 2, 17, 38], we thought it worthwhile examining the in vivo antitumor and antimetastatic effects of dimethyltriazenes in mice treated with an inducer or an inhibitor of hepatic drug metabolism, including *N*-demethylation. Consequently, the antitumor and antimetastatic effects of DM-CH_3, DM-NO_2, and DM-COOK were determined in mice bearing Lewis lung carcinoma and treated simultaneously with PB or CCl_4. In order to obtain additional information, the in vitro demethylation of the tested triazenes by liver preparations from the treated animals was also determined.

Materials and methods

Compounds used and animal treatment. The dimethyltriazenes *p*-(3,3-dimethyl-1-triazeno)benzoic acid potassium salt (DM-COOK), *p*-tolyl-3,3-dimethyltriazene (DM-CH_3) and *p*-nitrophenyl-3,3-dimethyltriazene (DM-NO_2) were synthesized according to procedures already reported elsewhere [9, 15, 18]. Phenobarbital (PB) and carbon tetrachloride (CCl_4) were purchased from Fluka (Buchs, Switzerland) and from Farmitalia Carlo Erba (Milan, Italy), respectively. DM-CH_3 and DM-NO_2 were suspended by sonication in olive oil and were administered i.p. in volumes of 0.05 ml per 10 g body weight; DM-COOK was given i.p. as a solution in 0.1 *N* $NaHCO_3$ using volumes of 0.1 ml per 10 g body weight. PB was administered as a fresh solution (0.1% w/v) each day in the drinking water plus i.p. injections of 80 mg/kg in 0.1 ml isotonic sodium chloride per 10 g body weight; CCl_4 (800 mg/kg) was diluted in olive oil and administered p.o. in a final volume of 0.1 ml per animal. The treatment schedule employed for each experiment is indicated in the footnote to the appropriate table.

Toxicity. The LD_{50} of DM-COOK, DM-CH_3 and DM-NO_2 has been determined according to the method of Litchfield and Wilcoxon [23] using tumor-bearing animals. The values of $LD_{0.05}$ employed for the evaluation of the antitumor and antimetastatic activity have been extrapolated from the plots relating log dose vs probit lethality.

Antitumor and antimetastatic activity. The tumor used is a locally maintained Lewis lung carcinoma line, originally provided by the tumor repository bank of NCI, NIH,

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Bethesda Md, USA; the tumor line is maintained by transplanting s.c. in C57BL mice tumor fragments, obtained from donors similarly inoculated 2 weeks before. Tumor fragments were prepared by mincing the tumor with scissors and mixing it with 2 volumes of Dulbecco's phosphate-buffered saline. Aliquots of 0.1 ml of tumor suspension were injected s.c. to C57BL (line maintenance) or to BD2F1 (tumor propagation for experiments) female mice weighing 18–20 g, purchased from Charles River SpA (Calco, Como, Italy). Primary tumor weight was measured at the end of treatment, and lung metastases, counted after sacrifice of the animals, as already reported in detail elsewhere [12].

Oxidative N-demethylation. In vitro oxidative *N*-demethylation of DM-COOK, DM-CH_3 and DM-NO_2 was determined using a postmitochondrial liver supernatant prepared from the livers of BD2F1 mice treated with PB, CCl_4 or with dimethyltriazenes. After sacrifice, the livers were homogenized in 4 vol. of 0.1 *M* potassium phosphate buffer, pH 7.4, using a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 10000 *g* for 30 min at 4° C, and the protein concentration of the supernatant was adjusted to 25 mg/ml, as determined with the method of Lowry et al. [24] using bovine serum albumin as standard. *N*-demethylation of dimethyltriazenes was determined by incubating 0.125–20 μmol of the drug for 15 min at 37° C in 100 μl ethanol (100 μl of 0.1 *M* potassium phosphate buffer, pH 7.4, for DM-COOK) with 1.9 ml of 0.1 *M* potassium phosphate buffer pH 7.4 containing 25 m*M* semicarbazide, 7.5 m*M* $MgCl_2$, 0.5 m*M* NADP, 5 m*M* glucose-6-phosphate, 0.25 units glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 5 m*M* nicotinamide as an NADPH-generating system (NADP, G6P and G6PDH were purchased from Boehringer Mannheim GmbH, FRG). The reaction was stopped by simultaneous addition under stirring of 1 ml saturated $Ba(OH)_2$ and 1 ml 20% $ZnSO_4$, and after centrifugation at 2000 g for 10 min the Nash colorimetric assay for formaldehyde was performed on the supernatant [5]. The optical density of the supernatant was read at 410 nm, providing, after subtraction of the reading of blank samples incubated at 0° C, a measure of demethylation. Demethylation was linear with time for at least 30 min, and proportional to enzyme concentration in the range used. Apparent K_m and V_{max} values were determined using experimental values for initial velocities of the reaction with increasing substrate concentrations, according to the method of Lineweaver and Burk [22].

Results

The effects of the combined treatment with PB or CCl_4 on the toxicity of DM-COOK, of DM-CH_3 and of DM-NO_2 are reported in Table 1. CCl_4 did not alter the toxicity of the triazenes used, whereas PB significantly increased the toxicity of DM-COOK and reduced that of DM-NO_2, leaving the toxicity of DM-CH_3 unaffected. The subsequently reported effects of the dimethyltriazenes on tumor growth and on the formation of spontaneous pulmonary metastases were determined using dosages equal to the $LD_{0.05}$ for each treatment combination used.

When combined treatments were performed and primary s.c. tumor growth was determined, the effects of DM-COOK were found to be reduced by PB and increased by CCl_4, whereas those of DM-CH_3 were abolished by CCl_4; the activity of DM-NO_2 was not significantly influenced in any of the treatment combinations examined. The combined treatment with DM-CH_3 and either PB or CCl_4 resulted in a more marked reduction in metastasis weight and number; the antimetastatic effects of DM-COOK and DM-NO_2 were increased by combination with CCl_4 (Table 2).

Table 1. LD_{50} of DM-COOK, DM-CH_3 and DM-NO_2 in mice treated with PB or CCl_4[a]

Treatment	–	PB	CCL_4
DM-COOK	94 (82–107)	67 (59– 76)*	105 (71–146)
DM-CH_3	50 (40– 62)	47 (38– 59)	66 (43–100)
DM-NO_2	210 (167–246)	310 (288–333)*	195 (142–267)

[a] Following s.c. implantation of Lewis Lung carcinoma, groups of 5 BD2F1 mice were treated daily with dimethyltriazenes i.p. on days 1–14, starting 24 h after tumor implantation. PB was administered in drinking water on days 1–14 plus one i.p. injection on each of days 1 and 8; CCl_4 was administered p.o. on days 1, 6, and 11. Treatment with PB and CCl_4 was always given 1 h before dime thyltriazene administration. Deaths occurring within 1 week after last drug administration were considered for evaluation. Each value is expressed as mg/kg/per day, with confidence limits for $P = 0.05$

* Significantly different from that obtained in mice treated only with the triazene (Litchfield and Wilcoxon test [23], $P = 0.05$)

Examination of the oxidative *N*-demethylation in vitro indicates for DM-CH_3 and for DM-NO_2 K_m and V_{max} values in the same order as those of aminopyrine, whereas no appreciable demethylation was detected for DM-COOK (Table 3). The *N*-demethylation of DM-CH_3 and DM-NO_2 is significantly increased (13- and 6-fold, respectively) by the action of a liver postmitochondrial supernatant prepared from mice treated with PB. After treatment with CCl_4 the demethylation of DM-CH_3 is completely abolished and that of DM-NO_2 is reduced to 14%. When liver postmitochondrial supernatants are prepared from animals treated with DM-NO_2 or DM-CH_3, the demethylation of DM-CH_3 is no different from that observed using untreated liver donor mice, whereas the demethylation of DM-NO_2 is increased two fold (Table 4).

Discussion

The aim of this work was to investigate the role of in vivo production of monomethyltriazenes in the antitumor action of dimethyltriazenes, by means of the simultaneous treatment of the animals with an inducer (PB) or an inhibitor (CCl_4) of hepatic drug metabolism [7, 34]. A first series of experiments was carried out to determine the dosage and treatment schedule of PB and CCl_4 that would be effective in modifying hepatic drug metabolism. The dosages and schedules employed in the experiments reported were found to be capable of markedly modifying the *N*-demethylation of the tested dimethyltriazenes, as determined in vitro using liver preparations obtained from the treated animals.

If monomethyltriazenes were the active metabolites responsible for the antitumor action of dimethyltriazenes, it could theoretically be expected that the administration of the inducer of *N*-demethylation would increase, and that

Table 2. Antitumor and antimetastatic activity of DM-COOK, DM-CH_3 and DM-NO_2 in mice treated with PB or CCl_4

Animal treatments			Primary tumor[a] weight	Lung metastases[b]	
Inducer or inhibitor	Dimethyltriazene			Number	Weight
	Compound	mg kg^{-1} day^{-1}			
–	–	–	100 ± 13 (1294 ± 162)[c]	100 ± 10 (50.6 ± 5.0)[e]	100 ± 19 (277 ± 52)[d]
–	DM-COOK	62	42 ± 5	13 ± 2	7 ± 2
–	DM-CH_3	26	61 ± 8	26 ± 5	13 ± 2
–	DM-NO_2	105	51 ± 6	57 ± 16	36 ± 11
PB	–	–	100 ± 9 (842 ± 77)[c]	100 ± 11 (50.1 ± 5.3)[e]	100 ± 19 (256 ± 50)[d]
PB	DM-COOK	47	65 ± 8*	27 ± 8	12 ± 5
PB	DM-CH_3	24	73 ± 11	6 ± 1*	4 ± 1*
PB	DM-NO_2	245	63 ± 6	47 ± 8	29 ± 7
CCl_4	–	–	100 ± 22 (1245 ± 274)[c]	100 ± 14 (42.1 ± 5.8)[e]	100 ± 28 (279 ± 77)[d]
CCl_4	DM-COOK	53	28 ± 14*	8 ± 1*	2 ± 0*
CCl_4	DM-CH_3	26	92 ± 15*	18 ± 7	6 ± 2*
CCl_4	DM-NO_2	97	47 ± 17	19 ± 7*	11 ± 7*

[a] Determined at the end of treatment on day 15 after tumor implantation.
[b] Determined at sacrifice, on day 21
[c] Average tumor or weight (mg) per mouse determined in control groups
[d] Average metastasis weight (mg) per mouse determined in control groups
[e] Average number of metastases per mouse, determined in control groups
Each value is the mean percent ratio (treated over controls) ± SE obtained in groups of at least 10 BD2F1 mice following s.c. implantation of Lewis lung carcinoma and daily treatment on days 1 – 14 with dimethyltriazenes i.p. starting 24 h after tumor implantation, as described in footnote to Table 1. All the reported values are significantly different from the corresponding controls, with the exception of the primary tumor weight in mice concomitantly treated with DM-CH_3 and CCl_4
* Significantly different from the corresponding values obtained in the group treated with dimethyltriazenes only. The Student-Newman-Keuls test [35] was used for the statistical analysis; $P = 0.05$

Table 3. Apparent K_m and V_{max} of in vitro *N*-demethylation for dimethyltriazenes and aminopyrine

Substrate	K_m (mM)	V_{max} (nmol HCHO × mg $prot^{-1}$ × min^{-1})
DM-CH_3	0.06 ± 0.01	2.7 ± 0.6
DM-NO_2	0.11 ± 0.01	10.6 ± 0.6
DM-COOK	not measurable	
Aminopyrine	0.34 ± 0.05	4.3 ± 0.1

Each value is the mean ± SE obtained in three repeated experiments, in a range of dimethyltriazene concentrations of 0.03 – 1 m*M*. The correlation coefficients of the linear regressions made to calculate the intercepts in Lineweaver-Burk plots were in the range of 0.94 – 0.99

of the inhibitor of *N*-demethylation decrease, the antitumor effects of dimethyltriazenes. The results reported in this paper do not fit this hypothesis. In fact, the antitumor effects of the individual dimethyltriazenes are not unambiguously decreased by CCl_4 or increased by PB, with the exception of the unexpected finding of the effects on metastases, which are increased by CCl_4 for each of the three dimethyltriazenes examined. Other data available from the literature indicate a lack of correlation between in vitro cytotoxicity and *N*-demethylation for dimethyltriazenes [1, 30, 31] and a lack of correlation between *N*-demethylation measured in vitro and in vivo antitumor activity for dimethyltriazenes [10, 30, 32]. At the same time, contrasting data are available, which show, in other experimental condi-

Table 4. In vitro *N*-demethylation of DM-CH_3 and DM-NO_2 by postmitochondrial supernatant prepared from livers of animals treated in vivo with PB, CCl_4 or with the same dimethyltriazene

Substrate	Treatment				
	None	PB	CCl_4	DM-CH_3	DM-NO_2
DM-CH_3	0.17 ± 0.03	2.21 ± 0.19*	0.00	0.14 ± 0.05	–
DM-NO_2	1.48 ± 0.13	9.16 ± 0.94*	0.20 ± 0.03*	–	3.27 ± 0.20*

Groups of 5 mice were treated i.p. with dimethyltriazenes daily for 6 consecutive days. PB was administered in the drinking water for 6 days, plus one i.p. injection at the beginning of oral administration; CCl_4 was administered p.o. on days 1 and 6. Sacrifice and liver collection were performed 24 h after the last drug administration
Each value is the mean ± SE demethylation of the substrate, expressed as nmol HCHO × mg $prot^{-1}$ × min^{-1} obtained in five separate determinations. Substrate concentrations were 0.5 and 1 m*M* for DM-CH_3 and DM-NO_2, respectively (9-fold greater than the K_m values for each substrate)
* Significantly different from the corresponding values obtained for the untreated mice. The Student-Newman-Keuls test [35] was used for the statistical analysis; $P = 0.05$

tions, an apparent correlation between *N*-demethylation and antitumor activity [2, 17, 38]. In analysis of these data, it should be noted that *N*-demethylation in vitro does not necessarily match the production of monomethyltriazenes in vivo, as observed for DTIC [29]. Actual determination of monomethyltriazene concentrations in plasma, including the time-course, may be preferable, but was outside the aim of the present investigation.

The results presently reported do not allow clarification of the discrepancies in the role found to be played by monomethyl derivatives in the antitumor action of dimethyltriazenes. On the other hand, they indicate a difference between primary tumors and metastases in the effect exerted by the single and combined treatments performed, which has already been observed on several earlier occasions [21, 32, 33] and which further suggests different mechanisms for the antimetastatic and antitumor activity. In our opinion, these findings support the view that, although generalizations on the pathways leading to metabolic activation of dimethyltriazenes may be valid in some instances, individual variations not covered by such wide generalizations are revealed by a detailed study of specific biological activities of selected compounds.

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