Biochemical Pharmacology, Vol. 30, pp. 89–93.

0006-2952/81/0101-0089 $02.00/0

STUDIES OF THE MODE OF ACTION OF ANTITUMOUR TRIAZENES AND TRIAZINES—II. INVESTIGATION OF THE SELECTIVE TOXICITY OF 1-ARYL-3,3-DIMETHYLTRIAZENES

ANDREAS GESCHER, JOHN A. HICKMAN*, RICHARD J. SIMMONDS, MALCOLM F. G. STEVENS and KEITH VAUGHAN†

Cancer Chemotherapy Research Group, Department of Pharmacy, University of Aston in Birmingham, B4 7ET, U.K.

(*Received* 15 *July* 1980; *accepted* 12 *August* 1980)

Abstract—The hypothesis that 1-aryl-3-methyltriazenes (monomethyltriazenes) are the active species responsible for the *in vivo* cytotoxicity of 1-aryl-3,3-dimethyltriazenes (dimethyltriazenes) has been tested *in vitro* by a comparison of the toxicity of monomethyltriazenes to a tumour which is sensitive to the dimethyltriazenes *in vivo* (TLX5S lymphoma) and to one which is resistant *in vivo* (TLX5R lymphoma). The results indicate that monomethyltriazenes are non-selectively cytotoxic. *In vitro* activation of a dimethyltriazene by a liver homogenate and cofactors was found to produce metabolites which are more toxic to the TLX5S tumour than to the TLX5R whereas when a 1-aryl-3,3-diethyltriazene, which is not an antitumour agent, was activated in the same way, cytotoxic metabolites were generated but the TLX5S tumour showed no preferential sensitivity. This suggests that during metabolic activation of dimethyltriazenes a mixture of selective and non-selective metabolites are generated. In an *in vivo* test the TLX5R tumour was found to be resistant to a monomethyltriazene, and it is suggested that this may be metabolised to an active species by the host.

The aim of cancer chemotherapy is to bring about a selective inhibition of tumour cell proliferation with minimal effect to the host cells. At present most clinically used drugs effect cellular damage by indiscriminate cytotoxic effects on all proliferating cells. Typical of this type is the triazene 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC; I) which is used, with only limited success, in the treatment of malignant melanoma [1]. In the first paper of this series [2] the biomimetic oxidation and metabolism of triazenes of structure (II), where R_1 and R_2 are conjoined in a heteroalicyclic ring, was examined. Although these compounds exhibited unusual chemical and metabolic features they were, in general, devoid of antitumour properties. 1-Aryl-3,3-dimethyltriazenes (II; $R_1 = R_2 = Me$) on the other hand have pronounced antitumour properties in experimental systems [3, 4] and in this study we report renewed attempts to establish the nature of the agent(s) responsible for their selective toxicity towards the TLX5 lymphoma growing in the mouse.

A generally accepted hypothesis for the mechanism of action of dimethyltriazenes as antitumour agents is that they alkylate DNA [5]. This hypothesis parallels that for the mechanism of their carcinogenic action [6] where it is proposed that host-mediated oxidative demethylation of dimethyltriazenes occurs *in vivo* to generate monomethyltriazenes (III; $R_1 = Me$) [7]. The monomethyltriazenes are powerful methylating agents [8]. The requirement for a host-mediated metabolic activation of dimethyltriazenes to produce cytotoxic products was demonstrated by observations that the dimethyltriazenes have little toxicity *in vitro* but formed cytotoxic products when liver fractions and cofactors were added [3]. It was assumed that this system demethylated dimethyltriazenes to form monomethyltriazenes, compounds which are directly cytotoxic *in vitro*; it was further argued that the monomethyltriazenes are the species responsible for the *in vivo* antitumour activity of dimethyltriazenes [3].

There is however, evidence which does not fully support this hypothesis and much of it has been summarised by Hansch *et al.* [9]. Additional evidence relevant to this study, is that the dimethyltriazenes are active *in vivo* against the TLX5S lymphoma, a tumour which has a natural resistance to alkylating agents, and have optimal antitumour activity when used in a dosage schedule which resembles that for an antimetabolite rather than an alkylating agent—that is, when given in a daily dose schedule rather than as a single dose, although a single dose has some effect [4]. Furthermore, it is difficult to explain the *absolute* requirement for a methyl group at the terminal nitrogen of the triazene moeity (II; $R_1 = Me$, $R_2 = Alkyl$) in order to elicit antitumour activity since monoethyltriazenes (III; $R_1 = Et$) are also alkylating agents and their diethyltriazene precursors (II; $R_1 = R_2 = Et$) are de-ethylated by liver homogenates [4]. Although the diethyltriazenes have absolutely no antitumour activity against the TLX5S lymphoma *in vivo* they have a host toxicity similar to dimethyltriazenes [3, 4] and are carcinogens like the dimethyltriazenes [10]. This suggests that com-

* To whom reprint request should be addressed.

† Present address: Department of Chemistry, Saint Mary's University, Halifax, N.S., Canada B3H 3C3

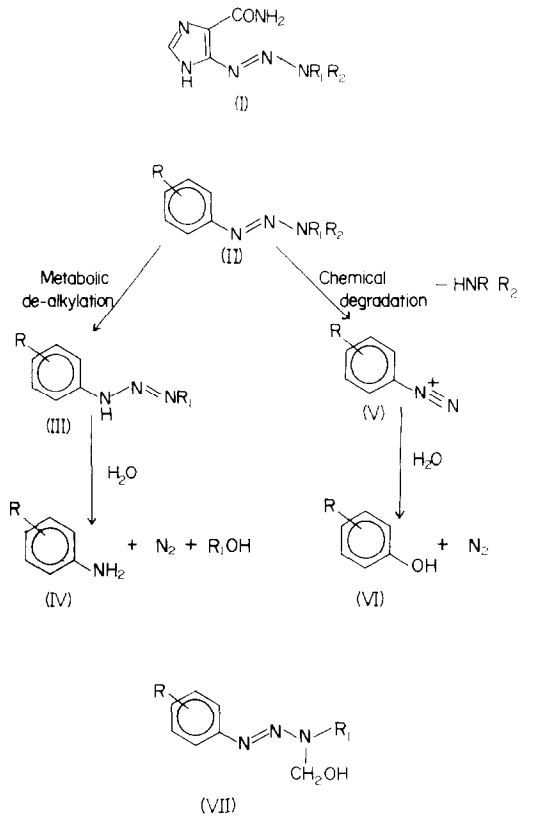

Fig. 1.

parisons of the mechanism of antitumour and carcinogenic activities may not be useful in this case.

The chemical and metabolic breakdown of dimethyltriazenes is complex and may involve the generation of a number of potentially cytotoxic species (Fig. 1), a situation which is also true for certain other antitumour drugs presently in use. In order to identify those species which are selectively cytotoxic we have compared the cytotoxicity of some of these breakdown products to two tumour cell lines *in vitro*: one tumour is sensitive to the dimethyltriazenes *in vivo* (TLX5S lymphoma); the counterpart has an induced resistance to the effects of dimethyltriazenes *in vivo* (TLX5R lymphoma). We argue that this, and other comparisons described below, allow for an estimation of whether an agent is non-selectively cytotoxic, and so will kill both TLX5S and TLX5R cells, or is selective and will kill only TLX5S cells.

MATERIALS AND METHODS

Materials. The triazenes used were synthesised by literature methods [8]. Nicotinamide adenine diphosphonucleotide, glucose-6-phosphate dehydrogenase and glucose-6-phosphate were purchased from Sigma (U.K.) Ltd. Media and serum were purchased from Gibco (Glasgow) Ltd.

Methods. The TLX5S lymphoma was passaged at seven day intervals by intraperitoneal injection of approximately 2×10^5 cells into 20 g male CBA/LAC mice. The TLX5R lymphoma was passaged in the same way; this tumour had been previously made resistant to ethyl 5-(3,3-dimethyl-1-triazeno)-2-phenylimidazole-4-carboxylate [4] and was found to be resistant to the optimum antitumour dose of the dimethyltriazenes used in the present study. Cells were counted with a Model ZBI Coulter Counter.

Antitumour test. Approximately 2×10^5 lymphoma cells were injected subcutaneously in the inguinal region of 20 g female CBA/LAC mice. After two days drugs were administered daily for five days by intraperitoneal injection of 0.1 ml of a solution or suspension of drug made by sonication in either 10% acetone–arachis oil or 10% dimethylsulphoxide–arachis oil. The day of death of the animals, which were in groups of five, was recorded and the survival time of treated animals compared with untreated controls. Survival time was shown to be proportional to the number of cells injected.

Bioassay. Ascites cells from a passage of the lymphoma were harvested on the seventh day, washed with saline and resuspended in RPMI 1640 media (6 parts) and horse serum (4 parts) at 2×10^6 cells per ml. 2 ml aliquots were incubated with drugs in

Table 1. Results of *in vivo* tests*

Experiment number	Compound Number	R	R_1	R_2	Tumour (TLX5S or TLX5R)†	Per cent ILS‡	Optimum dose (mg/kg, 5 × daily)	Toxic dose (mg/kg, 5 × daily)
1	II	*p*-Me	Me	Me	S	80	25	200
	II	*p*-Me	Me	Me	R	18	100	—
2	II	*p*-CO_2Me	Me	Me	S	58	40	160
	II	*p*-CO_2Me	Me	Me	R	0	40‖	—
3	III	*p*-Me	Me	—	S	42	12.5¶	25
4	III	*p*-CO_2Me	Me	—	S	87	5	80
	III	*p*-CO_2Me	Me		R	4	20	—

* 2×10^5 TLX5 cells were injected s.c. into the inguinal region on day 0. Animals were dosed daily from day 3 to day 7 and the increase in life span of the treated animals compared with untreated controls.

† TLX5S = tumour sensitive to dimethyltriazenes; TLX5R = tumour resistant to dimethyltriazenes.

‡ Per cent ILS = per cent increase in life span compared with controls.

‖ Tested only at optimal dose for the sensitive (TLX5S) tumour.

¶ Only active at this dose level.

Table 2. Bioassay results of triazenes and possible degradation products*

Experiment number	Compound Number	R	R_1	R_2	Tumour (TLX5S or TLX5R)†	Concentration (μg/ml)	Per cent ILS†
1	II	*p*-Me	Me	Me	S	1000	0
	II	*p*-Me	Me	Me	R	1000	0
2	II	*p*-CO_2Me	Me	Me	S	1000	0
	II	*p*-CO_2Me	Me	Me	R	1000	0
3	III	*p*-Me	Me	—	S	250	30
						50	31
						10	16
	III	*p*-Me	Me	—	R	250	45
						50	31
						10	24
4	III	*p*-CO_2Me	Me	—	S	250	52
						50	28
						10	15
	III	*p*-CO_2Me	Me	—	R	250	Cures‡
						50	20
						10	9
5	III	*p*-Me	Et	—	S	250	Cures‡
						50	50
						10	10
6	IV	*p*-Me	—	—	S	1000	0
	IV	*p*-CO_2Me	—	—	S	1000	0
	V	*p*-CO_2Me¶	—	—	S	50	8
		Sodium tetrafluoroborate			S	50	0
	VI	*p*-Me	—	—	S	1000	0

* 2×10^6 TLX5 cells/ml were incubated with the stated concentrations of drugs for 2 hr at 37°. 2×10^5 cells were then injected into animals and the increase in life span compared with animals which received untreated cells.

† For definitions see Table 1.

‡ Survival for > 30 days (approx. ILS = 200%) when experiment terminated.

¶ Tetrafluorobate salt.

Table 3. Bioassay* of triazenes with mouse liver 9,000 *g* homogenate and cofactors

Experiment number	Compound Number	R	R_1	R_2	Tumour (TLX5S or TLX5R)†	Concentration (μg/ml)	Per cent ILS†
1	II	*p*-CO_2Me	Me	Me	S	500‡	49, 22, 58, 83
	II	*p*-CO_2Me	Me	Me	R	500‡	5, 2, 22, 40
	II	*p*-CO_2Me	Me	Me	S	1000	80, Cures†
	II	*p*-CO_2Me	Me	Me	R	1000	57, 88
2	II	*p*-CO_2Me	Et	Et	S	500	40, 47, 38
	II	*p*-CO_2Me	Et	Et	R	500	59, 44, 79¶
3	III	*p*-CO_2Me	Me		S	20	20
	III	*p*-CO_2Me	Me		S	20‖	40

* For details of method see Table 2 and Materials and Methods Section.

† For definitions see Table 1.

‡ Results for four separate parallel experiments. By Mann-Witney non parametric estimation of variance $P < 0.029$.

‖ Without liver and cofactors mixtures.

¶ Four out of five animals with tumour: one animal either a 'cure' or a 'no-take'.

the dark, with shaking, for 2 hr at 37°. Drugs were added in 50 μl of dimethylsulphoxide. 0.1 ml of the cell suspension was injected i.p. into 20 g female CBA/LAC mice in groups of five and the day of death of animals receiving treated cells compared to those which had received solvent alone. Equal cell numbers per ml were used when the toxicity of a drug was compared on the TLX5S and TLX5R lymphomas.

When drugs were bioassayed with the lymphoma cells in the presence of liver homogenate 1.1 ml of 4×10^6 lymphoma cells per ml were incubated with 0.5 ml of 9,000 *g* supernatant of a 20% homogenate of CBA/LAC mouse liver in Hank's Buffer (pH 7.4) and 0.4 ml of a solution of cofactors to give a final concentration of 5 mM $MgCl_2$ and to produce 1 mM NADPH. Incubations were done in closed tubes with air. When the liver preparation was boiled before its addition it was shown to be inactive with respect to both the production of formaldehyde [3] from a dimethyltriazene, and the formation of cytotoxic metabolites in the bioassay. All bioassays were performed aseptically.

RESULTS AND DISCUSSION

It was the purpose of the experiments described here to attempt to dissect the overall cytotoxicity into elements of selective and non-selective cytotoxicity. In agreement with the results of other workers we have found that whilst 1-aryl-3,3-dimethyltriazenes are active *in vivo* (Table 1, experiments 1 and 2) they are without significant toxicity when incubated with the TLX5S tumour *in vitro* (Table 2, experiments 1 and 2). When incubated with TLX5S cells and a mouse liver 9,000 *g* supernatant (with cofactors) in air, metabolism occurred to form cytotoxic species (Table 3, experiment 1). When *in vitro* bioactivations were performed simultaneously with TLX5S or TLX5R cells the toxicity was consistently higher to the TLX5S cells and the results shown in Table 3 (experiment 1) suggest that the percentage increase in survival time (% ILS) seen with the TLX5R cells represent the action of non-selective cytotoxic products whereas the greater % ILS seen with TLX5S cells suggests that the bioactivation system is capable of producing metabolites to which TLX5R cells are resistant.

The probable breakdown products after oxidative *N*-demethylation in the dark of a dimethyltriazene are shown in Fig. 1, reactions (II) → (III) → (IV). An obvious candidate that may be considered responsible for the selective *in vivo* and *in vitro* cytotoxicity of the dimethyltriazenes is the monomethyltriazene (III; R_1 = Me). However, if this is the active selective species generated in, and emerging from the liver to act at a distal site then it would be expected to show cytotoxicity to the TLX5S tumour but to have no effect on the TLX5R tumour *in vitro*. This was not the case (Table 2, experiments 3 and 4) and the almost equivalent toxicity to both tumours suggests that whilst the monomethyltriazenes are cytotoxic to the TLX5S lymphoma *in vitro*, possibly because of their chemical reactivity as methylating agents, their cytotoxicity is not responsible for the *selective* antitumour action of the dimethyltriazenes.

The *in vitro* cytotoxicity of the monomethyltriazenes may be due to some of their metabolites or chemical degradation products (Fig. 1). Two arylamines (IV), a diazonium salt (V) and a phenol (VI) related to the triazenes under test were bioassayed but had no toxicity at concentrations considered likely to be generated *in vitro* (Table 2, experiment 6). It is anyway unlikely that these compounds are involved in the antitumour effects of the triazenes when there is the absolute requirement for a methyl group in the molecule and when these degradation products may also be generated from ethyltriazenes.

The finding that the monomethyltriazenes are equally toxic *in vitro* to TLX5S and TLX5R cells did not extend to the *in vivo* test (Table 1, experiment 4). Here it was found that the TLX5R tumour was resistant to the monomethyltriazene, a puzzling result which supports the hypothesis that this is the active species responsible for selective cytotoxicity. Possible explanations for this result, which contradicts the aforementioned results, are that either the *in vitro* bioassay is not a valid system capable of allowing comparison between *in vivo* and *in vitro* cytotoxicity and selectivity, or that *in vivo* the monomethyltriazenes are yet further metabolised to selectively active species.

It is important to comment upon the first possibility since this type of bioassay system has been widely used in studies of cytotoxic drug action. Its use may be inappropriate when studies are made of the mechanism of action of compounds which, like the triazenes, require regimes of repeated administration for optimal effect *in vivo*, since in the bioassay only acute toxicity is measured. With respect to the present study a comparative analysis of the results for the activities of ethyltriazenes both *in vivo* and *in vitro* is relevant.

As mentioned in the Introduction, 1-aryl-3,3-diethyltriazenes and their monoethyltriazene metabolites have no activity on the TLX5S lymphoma *in vivo* [4]. This suggests that this tumour is naturally resistant to ethyltriazenes *in vivo*. (An alternative hypothesis is that the shorter half-lives of monoethyltriazenes in comparison to their methyl homologues may prevent them from attaining a sufficiently high concentration in a tumour; we consider this unlikely since amongst the dimethyltriazenes which are active there are monomethyltriazene analogues which, if generated *in vivo*, have half-lives comparable to monoethyltriazenes [11].) In the bioassay, monomethyl- and monoethyltriazenes were found to have similar toxicity (Table 2, experiments 3, 4 and 5) which suggests again that either the bioassay system is incapable of differentiating between selective and non-selective agents or that monoalkyltriazenes in general are non-selective agents and when generated *in vivo* are responsible only for whole animal toxicity and not for antitumour effects.

The ability of the bioassay system to differentiate between selective and non-selective metabolites was confirmed by a comparison of the toxicity of a dimethyl- and a diethyl- triazene *in vitro* when metabolically activated (Table 3, experiments 1 and 2). When the dimethyltriazene was activated by the liver homogenate the TLX5S tumour proved to be more sensitive than the TLX5R, although, as men-

tioned above, the TLX5R is affected by what must be non-selective cytotoxic metabolites (Table 3, experiment 1). In the case of the activation of the diethyltriazene cytotoxicity to *both* tumour lines was observed but there was no preferential toxicity to the TLX5S tumour (Table 3, experiment 2). This shows that activation of a diethyltriazene generates biologically-active species, as would be expected, but that they are not related to antitumour effects. Only a dimethyltriazene appears to be capable of forming selective antitumour metabolites and this is reflected in the greater sensitivity of the TLX5S tumour in the bioassay when compared to the TLX5R tumour.

The second possibility which may explain the *in vivo* resistance of the TLX5R tumour to a monomethyltriazene is that the monomethyltriazene is metabolised *in vivo*. Some evidence has been adduced to support this possibility [12]. However, it should be noted that we failed to activate a monomethyltriazene in the bioassay system (Table 3, experiment 3), and the reduction of cytotoxicity seen with the liver homogenate may be due to non-selective 'mopping up' of the monomethyltriazene.

In conclusion, the experiments reported here suggest that chemical species other than monomethyltriazenes may be responsible for the selective antitumour effects of dimethyltriazenes. We are currently analysing the metabolities of dimethyltriazenes which are formed *in vitro* under the conditions which give rise to selective cytotoxicity. In addition, we have recently focussed our attention on 1-aryl-3-alkyl-3-hydroxymethyltriazenes (VII; R = range of electron-attracting substituents, R_1 = alkyl). This type of compound has long been postulated [7] as a crucial intermediate in the oxidative demethylation of 1-aryl-3,3-dimethyltriazenes (II; $R_1 = R_2 = Me$) and the precursor of monomethyltriazenes (III; R_1 = Me). Hitherto, hydroxymethyltriazenes have been considered as exceedingly fugitive species incapable of significant persistance in a cellular environment. This is not the case however: synthetic routes have been developed for the preparation and purfication of these compounds [13, 14] and we will report on their properties in the next paper in this series.

Acknowledgements—This work was generously supported by grants from the Cancer Research Campaign and the Medical Research Council.

REFERENCES

1. S. K. Carter and M. A. Friedman, *Eur. J. Cancer* **8**, 85 (1972).
2. M. F. G. Stevens, A. Gescher and C. P. Turnbull, *Biochem. Pharmac.* **28**, 769 (1979).
3. T. A. Connors, P. M. Goddard, K. Merai, W. C. J. Ross and D. E. V. Wilman, *Biochem. Pharmac.* **25**, 241 (1976).
4. R. C. S. Audette, T. A. Connors, H. G. Mandel, K. Merai and W. C. J. Ross, *Biochem. Pharmac.* **22**, 1855 (1973).
5. V. H. Bono Jr., *Cancer Treatment Rep.* **60**, 141 (1976).
6. P. Kleihues, G. F. Kolar and G. P. Margison, *Cancer Res.* **36**, 2189 (1976).
7. R. Preussman, A. von Hodenberg and H. Hengy, *Biochem. Pharmac.* **18**, 1 (1969).
8. K. Vaughan and M. F. G. Stevens, *Chem. Soc. Rev.* 377 (1978).
9. C. Hansch, G. J. Hatheway, F. R. Quinn and N. Greenberg, *J. Med. Chem.* **21**, 574 (1978).
10. S. Ivankovic, R. Port and R. Preussmann, *Z. Krebsforsch.* **86**, 307 (1976).
11. J. A. Hickman, J. K. Horton, M. F. G. Stevens and K. Vaughan, unpublished results.
12. B. L. Pool, *J. Cancer. Res. Clin. Oncol.* **93**, 215 (1979).
13. A. Gescher, J. A. Hickman, R. J. Simmonds, M. F. G. Stevens and K. Vaughan, *Tetrahedron Lett.* 5041 (1978).
14. M. Julliard, G. Vernin and J. Metzger, *Synthesis* 116 (1980).