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STUDIES ON THE MECHANISM OF ACTION OF THE TUMOUR INHIBITORY TRIAZENES

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Abstract—A series of imidazole and phenyl dialkyl triazenes were synthesized and investigated for their anti-cancer activity in experimental animals. Active triazenes had a broad spectrum of anti-tumour action and like bischloroethylnitrosourea (BCNU) were active against tumours not sensitive to conventional alkylating agents. It was confirmed that at least one *N*-methyl group was necessary for anti-cancer activity but there was no correlation between dealkylation by liver microsomes and activity since a diethyl triazene was readily dealkylated but not active. A factor appears to be present in normal cell cytoplasm which can detoxify triazenes but which is absent from tumours sensitive to these agents.

3,3-DIMETHYLTRIAZENES were first shown to have anti-tumour activity in experimental animals some 17 years ago[1,2] but their clinical use originates from attempts to design antagonists of aminoimidazole carboxamide (AIC) whose ribotide is a precursor in purine biosynthesis.[3] 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (DIC) is of particular use in the treatment of malignant melanoma where regression rates of 20 per cent have been consistently obtained.[4]

It is now generally accepted that DIC does not act as an antagonist of AIC and that its anti-tumour action is a property only of its triazeno function. It has been shown that triazenes not containing the imidazole ring are just as effective anti-tumour agents.[5,6] Further studies on the mechanism of action have implicated alkylating metabolites, formed after enzymatic *N*-demethylation, as the actual anti-tumour agents or alkylating diazonium compounds formed by light or, in some cases, acid catalysed dissociation of the triazeno group.[7–12] Studies using animal tumours have shown the triazenes to be very effective against a wide range of transplanted tumours.[3,4,13,14] The broad spectrum of action shown by the triazenes and the finding that they may be converted *in vivo* into a variety of products makes a study of their mechanism of action important. Identification of the pathways by which the triazenes exert their anti-tumour effects may enable the design of agents for clinical use, which maintain the tumour specificity of the triazenes but do not break down into other products which may be toxic but not tumour inhibitory.

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MATERIALS AND METHODS

The ADJ/PC6 plasma cell tumour in Balb/C mice was passaged under sterile conditions by transplantation of tumour fragments. Drug effectiveness was measured by the method previously described[15] and expressed as a therapeutic index. The LD_{50} and the dose to cause 90 per cent tumour growth inhibition (ID_{90}) were calculated in one experiment on tumour bearing animals. Drugs were administered 3 weeks after transplantation when the tumours were about 1 g in weight. The TLX5 and R1 lymphomas were transplanted in CBA/LAC mice by subcutaneous injection of about 10^5 ascites cells, treatment commencing 3 days after transplantation. For these two tumours survival time was closely related to the number of tumour cells injected.[16] An assay of effectiveness was made by comparing the dose level (optimum dose) that gave the maximum extension in survival time with the "toxic" dose. The "toxic" dose was the first dose level above the optimum dose that gave no increase in survival time compared with controls.

A tumour line resistant to dimethyl triazenes was obtained by injecting animals bearing the TLX5 tumour with the optimum dose of an effective triazene [5-(3,3-dimethyl-1-triazeno)4-carbethoxy-2-phenyl imidazole]. When the tumour had re-grown it was transplated to a group of animals, which, 3 days later, were injected with the triazene. This procedure was repeated until the survival time of the treated animals was no longer greater than the controls. The resistant line obtained in this way has maintained resistance for fifty passages.

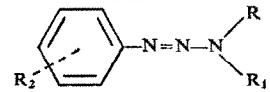
The sensitivity of tumour cells *in vitro* to the various agents was measured by a bioassay procedure previously described.[17] Washed tumour cells were suspended in TC 199/horse serum (60 : 40) at a concentration of 10^6 cells/ml. Aliquots were incubated with the drug at various concentrations for 2 hr at 37° together with cells similarly incubated but without the drug under test. Thereafter each incubation mixture was injected into groups of five animals at a concentration of 10^5 cells/ml. From the survival time of the control group and the survival time of animals injected with cells incubated with the drug, an estimate of the number of cells killed could be obtained.

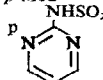
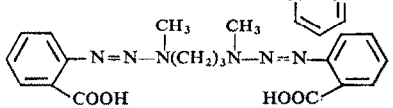
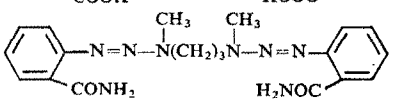
BCNU (*N'*,*N*-bischloroethyl-*N*-nitrosourea) and DIC were obtained through Dr. H. Wood from Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Washington. The imidazole triazenes were provided by Dr. J. Heyes of Beechams Ltd., and small samples of four of the phenyl triazenes by Dr. Ti Li Loo of the M. D. Anderson Hospital, Houston.

The triazene derivatives listed in Table 1 were prepared by the following general method: 0·1 mole of the aromatic amine was suspended in water (50 ml) at 0° and concentrated hydrochloric acid (25 ml) was added to the rapidly stirred solution. Sodium nitrite (0·1 mole) dissolved in the minimum quantity of water was added dropwise and the solution was stirred for a further 0·5 hr at 0°. Calcium carbonate (16 g) was then added followed by the appropriate amine (0·11 mole). After stirring for another 0·5 hr at 0° the product was isolated by filtration or by extraction with ether. When aromatic amino acids were used to produce the diazonium salt it was necessary to add glacial acetic acid (12·5 ml) in water (12·5 ml) before isolating the product. The dried product was crystallized from the solvents indicated in the table.

Since diazotization of *o*-aminobenzamides is not practicable the *o*-acid derivatives listed were prepared from the corresponding acids by the mixed anhydride method.

TABLE 1. PHYSICO-CHEMICAL DATA OF SOME NEW TRIAZENES



No.	Compound R	Compound R_1	Compound R_2	m.p.	Crystal form**	Solvent††	Analyses C	Analyses H	Analyses N	Analyses C	Analyses H	Analyses N	Spectral data‡‡			
1*	Me	Me	H	Oil (b.p. 130°/20 mm)									ϵ_{max}^{285nm}	12,900	ϵ_{max}^{307nm}	12,000
2	Me	Me	*o*-COOH	124° (lit* 126°)	f.n.	A–B							ϵ_{max}^{240nm}	13,500	ϵ_{max}^{323nm}	19,400
3	Me	Me	*o*-$CONH_2$	135° (lit† 127–131°)	n.	A–C	56·2	6·3	29·2	56·1	6·1	29·2	ϵ_{max}^{240nm}	11,200	ϵ_{max}^{317nm}	13,000
4	Et	Et	*o*-COOH	73–74°	f.n.	A–D	59·7	6·8	19·0	59·9	6·7	18·9	ϵ_{max}^{240nm}	8300	ϵ_{max}^{323nm}	14,700
5	Et	Et	*o*-$CONH_2$	78–80°	pr.	A–D	59·9	7·3	25·4	59·8	7·3	25·4				
6§	Et	Allyl	*o*-COOH	25°	‡		61·8	6·5	18·0	62·0	6·5	18·1				
7	Allyl	Allyl	*o*-COOH	55°	pr.	A	63·6	6·2	17·1	63·4	6·3	17·3				
8‡	Me	Me	*m*-COOH	120–123°	f.n.	E	56·0	5·7	21·8	55·9	5·8	22·1				
9‖	Me	Me	*m*-COOMe	46° (lit† 43–45°)	pr.	A	58·0	6·3	20·3	57·9	6·3	20·5				
10	Me	Me	*p*-COOH	§ 172°	pr.	F	56·0	5·7	21·8	55·6	5·8	21·6	ϵ_{max}^{230nm}	18,000	ϵ_{max}^{323nm}	20,000
11	Me	Me	*p*-$CONH_2$	178° (lit† 176–178°)	pr.	A–G							ϵ_{max}^{225nm}	18,000	ϵ_{max}^{323nm}	20,500
12	Me	Me	*p*-COOEt	38°–40	pl.	A	59·6	6·8	19·0	59·5	7·0	19·0				
13	Me	CH_2CH_2Cl	*p*-COOEt	68°	n.	A	53·4	6·0	15·6	52·8	5·8	15·5				
14¶		$-CH_2-CH_2-$	*p*-MeO	42–43°	pr.n.	A	61·0	6·2	23·7	60·7	6·2	23·9				
15	Me	Me	p- 	§ 235	pr.		47·0	4·6	27·4	46·5	4·5	27·0	ϵ_{max}^{220nm}	21,000	ϵ_{max}^{323nm}	21,500
16				160–161°	n.	G	57·3	5·6	21·1	57·6	5·7	21·2	ϵ_{max}^{240nm}	23,400	ϵ_{max}^{323nm}	31,500
17				181–183°	pr.n.	D–H	57·6	6·1	28·3	57·5	6·2	27·9	ϵ_{max}^{240nm}	23,460	ϵ_{max}^{323nm}	27,700

* See ref. 18 for further details.
† See ref. 5 for further details.
‡ Obtained as an oil which solidified on keeping.
§ Prepared by Mr. J. L. Everett.
‖ Prepared by Mr. D. E. Willman.
¶ C. S. Rondestvedt and S. J. Davies, *J. Org. Chem.* **22**, 200 (1957), obtained an impure form of this compound.
** Crystal forms; f.n., flattened needles; pr.n., prismatic needles; n., needles; pr., prisms; pl., plates.
†† Solvents: A, light petroleum (b.p. 40-60°); B, benzene; C, chloroform; D, ether; E, ethyl acetate; F, acetontrile; G, acetone; H, dimethylformamide; I, methanol.
‡‡ Spectra measured in ethanolic solutions.

This was essentially that described by Lin *et al*,[5] except that *sec*-butyl chloroformate was used and dry ammonia was passed into the freshly prepared anhydride solution for 2 hr at 0°.

For tests against tumour-bearing animals, compounds not soluble in saline were administered in 10% acetone–arachis oil and given by five daily intraperitoneal injections.

To assay the effect of drugs on the uptake of tritiated thymidine, uridine and leucine by tumour cells *in vitro*, washed tumour cells (3×10^6 cells/ml) were incubated with the drug in TC 199/horse serum (60 : 40) for 4 hr at 37°. Tritiated precursors were then added to aliquots of the incubate to a final concentration of 3 μCi/ml and the incorporation into DNA, RNA or protein followed at 20-min intervals for 80 min. Successive 1 ml aliquots taken at 20-min intervals were filtered through a Whatman GF/C glass fibre paper and washed successively in saline, 0·2 N perchloracetic acid and saline. After drying in air the filter paper was added to a vial containing 10 ml of scintillation fluid (AR toluene, 385 g; dioxan, 285 g; ethanol, 99·5%, 230 ml, naphthalene, 80 g/l. and butyl-PPO, 7 g/l.) and counted in a scintillation counter (Packard tricarb, model 3375).

The 10,000 *g* supernatant tissues fraction was prepared by removal of the appropriate organ and, after rinsing in ice-cold isotonic-KCl, homogenizing in 0·1 M potassium–phosphate buffer, pH 7·4 (1 g/4 ml buffer) at 0° in a Teflon-glass homogenizer for 3 min. The resultant homogenate was then centrifuged at 10,000 *g* and 0° for 20 min and the supernatant decanted.

Microsomes were prepared from the above supernatant by centrifugation at 100,000 *g* for 1 hr at 4°. The microsomal pellet was suspended in cold 0·1 M potassium–phosphate to produce a suspension containing microsomes from 1 g liver wet wt, in each 4 ml. The microsomes were always prepared from the liver of those rats given drinking water containing sodium phenobarbitone (500 mg/l.) for 3 days. The dry weight of the various fractions was obtained by evaporation to constant weight of 1 ml aliquots. The protein content was measured by the method of Lowry *et al.*[19] and the free SH content by the method of Ellman.[20]

To measure dealkylation, the reaction mixture consisted of 3 ml of microsomal suspension, 1·5 μM, NADP; 35 μM, glucose-6-phosphate; 25 μM, $MgCl_2$; 100 μM, nicotinamide; 1 μl, glucose-6-phosphate dehydrogenase (Boehringer, 140 μ/ml) and 75 μM of semicarbazide HCl (previously neutralized with 10% NH_4OH). The substrate concentration was 5 μM, dissolved in DMSO (0·1 ml). The total volume of the incubation mixture was 8·0 ml. All substrates were placed in light protected reaction vessels in air at 37° for 1 hr with shaking. The reactions were terminated by the addition of 4 ml of 10% sulphuric acid in an ice-bath. A blank reaction mixture without substrate was employed in each experiment to correct for endogenous aldehyde formation.

To measure demethylation the incubation acid mixture was diluted to 25 ml with water and distilled. 5·0 ml aliquots of the distillate were reacted with 2·0 ml of the Nash reagent and the coloured complex assayed for formaldehyde by the method of Cochin and Axelrod.[21] It was observed that the imidazole triazenes produced an intense yellow non-formaldehyde complex which interfered with the yellow formaldehyde complex in the Nash-developed reaction of the whole incubate. This made it necessary to distil the formaldehyde from the reaction mixture.

To measure de-ethylation the incubation acid mixture was diluted to 25 ml and distilled. 1·0 ml Aliquots were reacted with the *p*-hydroxy biphenyl reagent and the colorimetric estimation of acetaldehyde determined according to the method of Stotz.[22]

RESULTS

Table 2 shows the result of a typical test on animals bearing the TLX5 lymphoma. Controls die on days 10 and 11 following subcutaneous injection of approx. 10^5 cells 0·1 ml. Significant extension of survival time is obtained with at least three, 2-fold spaced dose levels. Toxicity is first seen at 200–400 mg/kg when the survival time is reduced far below that obtained at the optimum dose of 50 mg/kg. All the active triazenes so far tested (Table 5) have caused significant extension of survival time at three dose levels except for the derivative of sulphadiazine (compound No. 5, Table 5) which was effective over five dose levels.

TABLE 2. EFFECT OF A TRIAZENE (1-*p*-CARBETHOXYPHENYL-3,3-DIMETHYL-TRIAZENE) ON THE TLX5 LYMPHOMA

Dose level (mg/kg × 5)	Days of death	Mean survival time (days)	% IST
No treatment	10, 10, 10, 10, 10 10, 10, 11, 11, 11	10·3	
12·5	11, 11, 11, 11, 12	11·2	8·7
25·0	14, 14, 16, 17, 17	15·6	51·4
50·0	16, 16, 16, 17, 17	16·4	60·7
100·0	14, 14, 14, 16, 16	14·8	43·6
200·0	11, 12, 12, 12, 12	11·8	14·5
400·0	6, 7, 7, 7, 7	6·8	

Using 2-fold spaced-dose levels, the optimal dose is 50 mg/kg and the "toxic dose" 200 mg/kg. The majority of the tumour inhibitory triazenes showed significant extension of survival time over three dose levels except for one compound (Table 5, no. 5) which was active over five dose levels.

Table 3 compares the selectivity of action of a triazene and BCNU with a typical alkylating agent, platinum compound and anti-metabolite against three transplated tumours. Table 4 shows the response of the TLX5 tumour and a line derived from it with acquired resistance to triazenes to different classes of anti-tumour agent. The structure activity relationship of a number of triazenes against the TLX5 lymphoma is shown in Table 5. In Fig. 1, the effect of alkylating agents, anti-metabolites and triazenes on the uptake of labelled precursors of nucleic acid and proteins by the TLX5 tumour *in vitro* are compared. Table 6 shows a typical bioassay experiment to measure the toxicity of triazenes to TLX5 tumour cells *in vitro*. Although incubations were carried out in the dark, the mixtures were prepared in the light which could have lead to partial dissociation of the triazene to the corresponding diazonium compound. The toxicity of cells *in vitro* of the diazonium compound formed from the triazene used in Table 6 is shown in Table 7. Table 8 shows the ability of the 10,000 *g* supernatant fraction of various tissues to reduce the toxicity of supralethal doses of a triazene *in vitro*.

TABLE 3.

	PC6	R1	TLX5
Methotrexate	0	65	55
Endoxan	136	0	0
$Pt(NR)_2Cl_2$	267	0	0
Triazene	86	98	67
BCNU	96	"Cures"	197

The effect of different classes of anti-tumour agents on the plasma cell tumour (results expressed as a therapeutic index) and the R1 and TLX5 lymphomas (results expressed as the percentage increase in survival time at the optimum dose). The triazene in this example was 5-(3,3-dimethyl-1-triazeno)-4-carbethoxy-2-phenyl imidazole and the platinum compound *cis*-dichlorobis (cyclohexylamine) platinum (II).

TABLE 4. EFFECT OF DIFFERENT CLASSES OF ANTI-TUMOUR AGENT ON THE TLX5 LYMPHOMA AND A TRIAZENE RESISTANT SUBLINE

	TLX5 % ILS	TLX5 (R)* % ILS
Triazene	67	0
BCNU	197	19
Methotrexate	55	84
Cytosine arabinoside	39	51

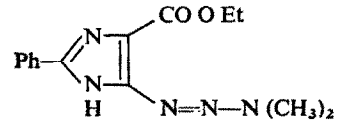
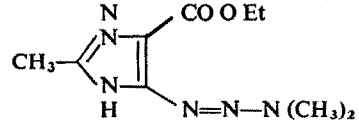
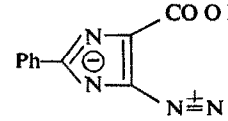
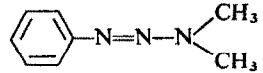
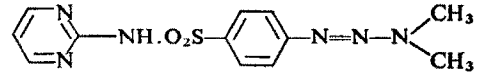
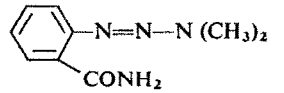
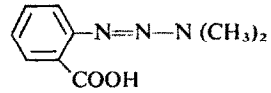
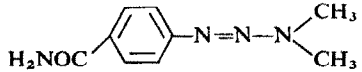
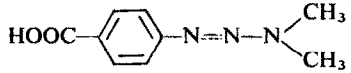
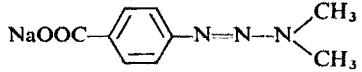
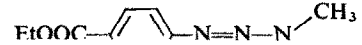
* Tumour with acquired resistance to a triazene, 5-(3,3-dimethyl-1-triazeno)-4-carbethoxy-2-phenyl imidazole, and cross-resistant to all triazenes. The triazene used in this experiment was 5-(3,3-dimethyl-1-triazeno)-4-carbethoxy-2-methyl imidazole.

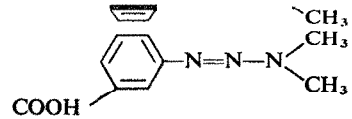
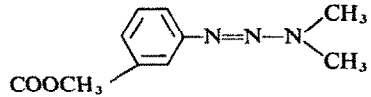
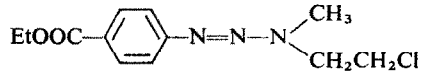
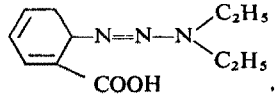
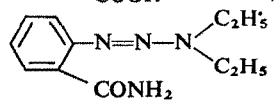
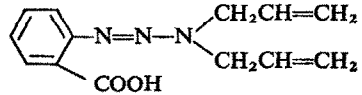
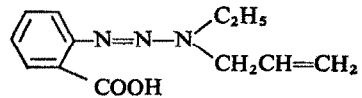
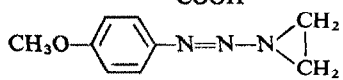
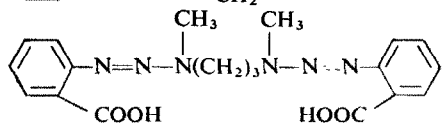
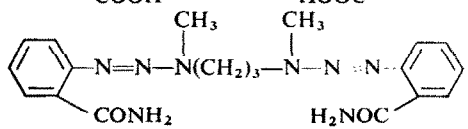
DISCUSSION

The broad spectrum of action already seen with the triazenes in tests against a number of transplanted animal tumours is confirmed in the results listed in Table 3. Although the plasma cell tumour is very sensitive to alkylating agents and compounds acting by similar mechanisms (e.g. dicyclohexylamine-*cis* dichloro platinum II) it is quite insensitive to anti-metabolites like methotrexate or cytosine arabinoside. The R1 and TLX5 lymphomas on the other hand, while they are very sensitive to these anti-metabolites do not respond to nitrogen mustards, e.g. Endoxan, even though they are rapidly growing tumours. The dimethyltriazenes and BCNU, however, are highly effective against both the plasma cell tumour and the two lymphomas.

Two most common theories advanced for the mechanism of action of the triazenes involve the formation of methylating agents after enzymatic monodemethylation or dissociation of the parent compound to form alkylating diazonium compounds.[7–12] It is difficult to see how either mechanism, both of which involve monofunctional

TABLE 5. EFFECTS OF VARIOUS TRIAZENES ON THE TLX5 LYMPHOMA AND THEIR DEALKYLATION BY LIVER MICROSOMES

	Compound formula	Optimal dose	% IST	Toxic dose	Substrate % dealkylation
1		25	56	200	11·3 ± 3·2
2		25	67	200	5·3 ± 1·1
3		Inactive		16	0
4		16	53	128	41·6 ± 8·2
5		12·5	71·4	400	51 ± 6·8
6		16	79	128	21·4 ± 9·1
7		32	68	64	0·8 ± 1·3
8		25	55	400	
9		25	72	400	0·4 ± 4·1
10		100	56	400	
11		50	61	400	

12		100	61	200	
13		100	63	400	
14		Inactive		400	0
15		Inactive		200	
16		Inactive		200	46 ± 9·0
17		Inactive		400	
18		Inactive		400	
19		Inactive			
20		200	18	400	
21		Inactive		> 1000	

alkylation as the cytotoxic reaction, can account for the selectivity of action of the triazenes against the tumours listed in Table 3. Although the plasma cell tumour is very sensitive to difunctional alkylating agents, monofunctional alkylating agents including methylating agents such as *N*-methyl-*N*-nitrosoguanidine are completely witnout effect.* Furthermore the lymphomas are completely insensitive to difunctional agents and therefore not likely to be killed by monofunctional alkylating compounds which are generally considered to be less cytotoxic.[23] Formation of alkylating diazonium compounds from triazenes usually takes place in the light[7] and would not be expected to play a role *in vivo*. While 5-(3,3-dimethyl-1-triazeno)4 carbethoxy-2-phenylimidazole is highly active against the TLX5 tumour *in vivo* (Table 5, compound 1), the diazonium compound formed from it is completely inactive under the same conditions (Table 5, compound 3) although highly toxic.

Nevertheless, the importance of the methyl group in the triazene is shown in the structure activity relationships of Table 5. As has previously been reported, the presence of at least one methyl group is necessary for anti-tumour activity. However, inactive compounds such as the diethyl triazene (Table 5, compound 16) are dealkylated just as readily by liver microsomes as the corresponding dimethyltriazene (Table 5, compound 6) and are, furthermore, just as toxic to whole animals.

Failure to act as a substrate for dealkylating enzymes cannot therefore explain in every case the specific requirement for a methyl group in the tumour inhibitory triazenes. Whether the formaldehyde produced from the active triazenes has any significance compared with the higher aldehydes produced by dealkylation of inactive triazenes is a matter for further investigation. Preliminary experiments using combinations of triazenes inactive as anti-tumour agents but readily dealkylated with compounds that generate formaldehyde have given negative results. The inactivity of the 3-methyl-3-chloroethyl triazene was at first surprising (Table 5, compound 14). If demethylation occurs a chloroethyl carbonium ion might be formed which would be similar to that believed to be generated from BCNU. This compound was quite inactive but it was subsequently shown not to be demethylated (Table 5). In most series of anti-tumour agents (e.g. the alkylating agents) one obtains a fairly wide variation in activity between individual members of the series. This is to be expected since anti-tumour effect is determined not only by the nature of the functional group but also by other properties of the molecule which may determine, among other things, its transport, intracellular distribution and reactivity. The effects of the dimethyltriazenes on the TLX5 lymphoma, however, were remarkably consistent. Despite wide variations in structure, providing the dimethyltriazene group was present in the molecule, extension of survival time was always between 53 and 71 per cent and with few exceptions the toxic dose between 200 and 400 mg/kg. The sulphadiazine derivative was prepared because it had been established that sulphadiazine concentrates in neoplastic tissue[24] and it was of interest that this triazene was the most selective compound tested, causing more than 50 per cent increase in survival time at dose levels from 6·25 to 100 mg/kg.

In their pharmacological properties the triazenes closely resemble BCNU. From Table 3, it can be seen that the spectrum of action of BCNU like the triazenes includes tumours naturally insensitive to either alkylating agents or anti-metabolites. The TLX5 tumour line with acquired resistance to a triazene (Table 4) shows almost a

* Unpublished work.

TABLE 6. BIOASSAY OF A TRIAZENE (5-3,3-DIMETHYL-1-TRIAZENE)-4-CARBETHOXY-2-PHENYL IMIDAZOLE) USING TLX5 LYMPHOMA CELLS *in vitro*

Concn of drug in incubate (μg/ml)	Survival time of recipient animals (days)	Mean survival time (days)
None	10, 10, 10, 10, 10	10·0
12·5	10, 10, 10, 10, 11	10·2
25·0	10, 10, 11, 11, 11	10·6
50·0	11, 11, 12, 12, 12	11·6
100	13, 13, 14, 14, 17	14·4
200*	14, 17, 17, 17	
400†	14, 17	

* One-fifth of the animals did not develop tumours.
† Three-fifths of the animals did not develop tumours.

TABLE 7. BIOASSAY OF A DIAZONIUM COMPOUND (5-DIAZO-4-CARBETHOXY-2-PHENYLIMIDAZOLE) USING TLX5 LYMPHOMA CELLS *in vitro*

Concn of drug in incubate (μg/ml)	Survival time of recipient animals (days)	Mean survival time (days)
None	10, 11, 11, 11, 11	10·8
1·0	11, 12, 12, 12, 12	11·8
2·0	11, 12, 12, 12, 12	11·8
4·0*	11, 11, 12, 13	
8·0†	13	
16	No tumours	

* One-fifth of the animals did not develop tumours.
† Four-fifths of the animals did not develop tumours.

TABLE 8. BIOASSAY OF A TRIAZENE (5-(3,3-DIMETHYL-1-TRIAZENO)-4-CARBETHOXY-2-PHENYL IMIDAZOLE) IN THE PRESENCE OF VARIOUS TISSUE EXTRACTS

Treatment	Days of death	Mean
None	11, 11, 11, 11, 12	11·2
Triazene only	No tumours	
Triazene + liver extract	11, 11, 11, 11, 11	11·0
Triazene + kidney extract	11, 11, 11, 12, 12	11·4
Triazene + spleen extract	11, 11, 11, 11, 11	11·0
Triazene + TLX5 extract	No tumours	
Triazene + PC6 extract	No tumours	

The concentration of the triazene in the absence of cell extracts is supralethal.

complete cross-resistance to BCNU. In contrast, the resistant tumour shows an increased or collateral sensitivity to methotrexate or cytosine arabinoside. Enhanced sensitivity in resistant tumour lines to compounds not acting by the same pathway has previously been reported.[25] A further similarity exists between the triazenes and BCNU in their effects on tumour cells *in vitro*. At dose levels of alkylating agents sufficient to kill about 90 per cent of tumour cells present, sensitive tumours show a selective inhibition of thymidine incorporation into DNA (Fig. 1). This is consistent with the proposed mechanism of action of these agents which involves DNA as the primary target site. Anti-metabolites such as 6-mercaptupurine at similar dose levels prevent the incorporation of precursors into DNA and RNA but not protein. This again is in agreement with the known inhibitory effect of 6-mercaptopurine on purine biosynthesis. At comparable dose levels, the triazenes equally inhibit the incorporation of all three precursors. Under the same conditions BCNU has identical effects.*

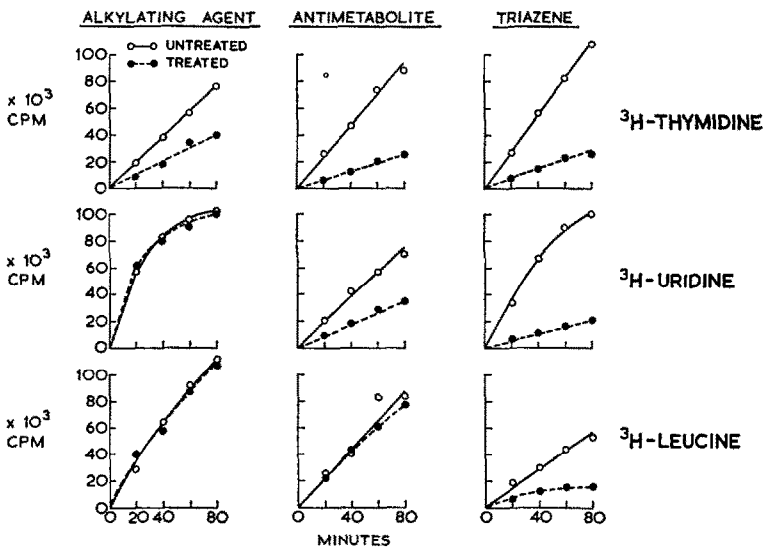


FIG. 1. Effect of an alkylating agent (5-aziridinyl-2,4-dinitrobenzamide) on the uptake of tritiated precursors into nucleic acid and proteins of Walker tumour cells *in vitro* and of an anti-metabolite, 6-mercaptopurine and triazene (5-(3,3-dimethyl-1-triazene)-4-carbethoxy-2-phenyl imidazole) on the uptake of tritiated precursors into TLX5 lymphoma cells *in vitro*.

Studies of the action of anti-tumour agents on cells *in vitro* can indicate the importance of host effects in determining the anti-tumour properties of an agent and particularly whether prior metabolism by liver is essential for activity.[26]

A typical bioassay is shown in Table 6. Cells incubated for 2 hr at 37° at a concentration of 10^6 cells/ml when re-injected into animals cause death at about 10 days showing that no cell death occurs during incubation. Incubations with increasing dose levels of a triazene shows a typical increase of cell kill as measured by survival time of recipient animals. The triazenes bioassayed in this way have proved to be fairly non-toxic and could indicate that some *in vivo* activation is required. In contrast, the diazonium compound (Table 7) derived from the triazene is extremely toxic killing lym-

* J. Hare, unpublished work.

phoma cells *in vivo* at very low dose levels. However, it is not an anti-tumour agent *in vivo* because it is also highly toxic to the host.

In experiments to determine whether triazenes required activation by the host, bioassays were carried out where sublethal doses of triazenes were incubated with cells in the presence of microsomes or liver supernatant in sufficient quantity to give extensive demethylation. It was observed during these experiments that, instead of activating the triazenes the liver 10,000 *g* supernatant afforded considerable protection against their *in vitro* toxicity. Extension of this work showed that extracts of liver, spleen and kidney can all protect against supralethal doses of triazenes *in vitro* (Table 8). However, equivalent amounts (adjusted to have either equal dry weight, protein or free SH content) of the 10,000 *g* supernatant of two triazene-sensitive tumours, are non-protective. This phenomenon requires further investigation but it may give a clue to the selectivity of anti-tumour action of the triazenes. Triazenes may break down or be enzymatically converted intracellularly to highly toxic products of an, as yet, unknown structure. These toxic products appear to be neutralized by factors in the cytoplasm of normal tissue but not in sensitive tumour cells.

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