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TUMOUR INHIBITORY TRIAZENES: STRUCTURAL REQUIREMENTS FOR AN ACTIVE METABOLITE

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Abstract—A series of aryldialkyltriazenes and some related compounds have been investigated for their anti-tumour properties. Unlike the imidazole-triazenes in clinical use, aryltriazenes are stable in light and do not undergo photodecomposition to toxic diazonium salts. Some of the triazenes investigated had good anti-tumour activity yet did not form diazonium salts under physiological conditions, implying that this pathway is not important for anti-tumour action. Evidence has been obtained that only aryltriazenes that can be metabolized *in vivo* to an aryl-N^3-monomethyltriazene have anti-tumour properties. It was also found that the aryltriazenes were dose-schedule dependent in their anti-tumour action, probably a consequence of their short biological half-lives.

5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) is a clinically used anti-tumour agent [1]. A disadvantage of DTIC is that it is unstable and, in particular, it is prone to photodecomposition [2] which can lead to the formation of a more toxic diazonium ion. The nausea and vomitting caused by DTIC in man and which can be dose-limiting, could conceivably be due to such breakdown products of DTIC rather than DTIC itself. The use of DTIC in combination chemotherapy is limited because of its delayed myelosuppressive activity, and the short half-life of the presumed active metabolite of DTIC [5-(3-methyl-1-triazeno)imidazole-4-carboxamide] might also be one of the reasons why DTIC has been disappointing in man, since insufficient concentration of the active metabolite may reach distant tumour sites after injection. Some of these disadvantages associated with the clinical use of DTIC might be overcome by the use of analogues which are more stable and in a preliminary report [3], it was shown that aryltriazenes which do not undergo photodecomposition were as active against the TLX5 lymphoma as a series of imidazoletriazenes. This paper reports on the chemical stability, metabolism and anti-tumour properties of a series of aryldialkyltriazenes and some related compounds.

MATERIALS AND METHODS

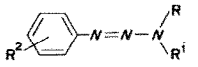
The TLX5 lymphoma was implanted subcutaneously in female CBA/LAC mice as previously described [3]. Anti-tumour effectiveness was measured by determining the maximum increase in life span of treated animals, the dose at which this occurred (optimum dose) and the toxic dose.

Groups of five animals were used for each dose level and their survival time compared with a group of ten control mice. An increase in life span of 20 per cent or greater was statistically significant. The triazenes were administered intraperitoneally as a suspension in arachis oil for 5 consecutive days, beginning 3 days after transplantation. The sensitivity of tumour cells *in vitro* to the various agents was measured by a bioassay procedure [4] and dealkylation by the method of Cochin and Axelrod [5]. Microsomes were obtained from the liver of rats which had been maintained for at least 3 days on sodium phenobarbitone (500 mg/l) in their drinking water. To demonstrate bioactivation of the triazenes, 1-(*p*-carbamoylphenyl)-3,3-dimethyltriazene (XI) was incubated with 10^6 TLX5 cells/ml with and without microsomes and co-factors. In previous experiments, activation of cyclophosphamide, as measured by a large increase in *in vitro* toxicity, could be obtained simply by incubating the cells with drug and microsomes in stoppered test tubes. In order to increase the toxicity of the aryltriazene (XI) however, it was necessary to increase the oxygenation of the medium by incubating in flasks so that a large surface area was exposed, and flushing out the flask with oxygen prior to the incubation.

The triazenes listed in Table 1 were prepared by the general method previously described [3]. The base used was varied, on occasions, excess of the aliphatic amine being used when readily available. The amines were either obtained commercially or prepared by published methods. A more detailed description of the syntheses involved is available in reference [6].

The chemical half-lives of the compounds in Table 6 were determined by a spectrophotometric method. Approximately 2 mg of the compound was dissolved in 1 ml dimethyl sulphoxide and diluted to 100 ml with 0·05 M pH 7·5 phosphate buffer. An aliquot was quickly introduced into a cuvette thermostated at 37° in a Pye Unicam SP 800 spectrophotometer and the spectrum scanned immediately. Repeat spectra were taken at intervals depending on the rate of decomposition of the sample. The half-life was determined from a logarithmic plot of the extent of decomposition on the ordinate against the time of hydrolysis on the abscissa.

Table 1. Physico-chemical Data of some novel 3,3-Dialkyl-1-aryl triazenes and related compounds



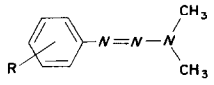
Number	R	Substituents R^1	R^2	M. Pt. (°C)	Crystal form*	Solvent †	Ref.	Analysis Requires C	H	N	Found C	H	N	Spectral data‡ λ_{max}	ϵ	λ_{max}	ϵ
I	CH_3	CH_3	H	Oil b.p. 130/20 mm			7							285	12,900	307	12,000
II	CH_3	CH_3	*o*-COOH	124	f.n.	A	7							240	13,500	323	19,400
III	CH_3	CH_3	*m*-COOH	120–123	n.	A	3							236	13,200	286	15,200
IV	CH_3	CH_3	*p*-COOH	172	n.	B	3							230	18,000	323	20,000
V	CH_3	CH_3	*o*-$COOCH_3$	Oil b.p. 197/20 mm			7	57·9	6·3	20·3	57·8	6·1	20·0	228	10,200	300	12,200
VI	CH_3	CH_3	*m*-$COOCH_3$	46	n.	C	8	57·9	6·3	20·3	57·9	6·3	20·5	237	13,900	287	16,300
VII	CH_3	CH_3	*p*-$COOCH_3$	102–104	rect. col.	A–C	8	57·9	6·3	20·3	58·0	6·3	20·4	228	8,900	325	22,000
VIII	CH_3	CH_3	*p*-$COOC_2H_5$	38–40	pl.	C		59·6	6·8	19·0	59·5	7·0	19·0	229	8,300	326	22,100
IX	CH_3	CH_3	*o*-$CONH_2$	135	n.	A	9							240	11,200	317	13,000
X	CH_3	CH_3	*m*-$CONH_2$	146–147	n.	A	8							235	13,500	287	15,400
XI	CH_3	CH_3	*p*-$CONH_2$	178	pr.	A	9							225	18,000	323	20,500
XII	CH_3	CH_3	*p*-$CONCH_2COOH$	158–159	n.	A		52·8	5·6	22·4	53·0	5·8	22·4	226	10,300	321	19,900
XIII	CH_3	CH_3	*p*-OCH_3		oil		10	60·3	7·3	23·5	60·6	6·8	23·4	288	14,400	323	13,000
XIV	CH_3	CH_3	*p*-NO_2	148	n.	A	7	49·5	5·2	28·9	49·2	5·2	29·3	239	8,000	364	24,300
XV	CH_3	CH_3	*p*-CF_3	67–68	n.	C		49·8	4·6	19·4	49·8	4·7	19·4	225	10,200	308	14,200
XVI	CH_3	CH_3	*p*-SO_2CH_3	131	n.	D		47·6	5·8	18·5	47·5	5·8	18·5	227	7,800	318	19,500
XVII	C_2H_5	C_2H_5	*p*-$CONH_2$	118–119	n.	A		60·0	7·3	25·4	59·7	7·3	25·3	225	10,300	325	21,300
XVIII	$CH(CH_3)_2$	$CH(CH_3)_2$	*p*-$CONH_2$	127–128	n.	A–C		62·9	8·1	22·6	62·7	7·9	22·8	225	9,600	327	20,400
XIX	CH_3	CH_2CH_3	*p*-$CONH_2$	133 134	n.	A–C		58·2	6·8	27·2	58·1	6·7	27·2	222	10,000	323	20,200
XX	CH_3	CH_2CH_2OH	*p*-$CONH_2$	142–144	n.	E		54·0	6·3	25·2	54·0	6·2	25·0	223	10,700	323	21,300
XXI	CH_3	$CH_2CH_2CH_2CH_3$	*p*-$CONH_2$	102–104	n.	A–C		61·5	7·7	23·9	61·6	7·6	24·0	218	9,200	324	19,600
XXII	CH_3	$CH_2CH_2CH_2CH_2CH_3$	*p*-$CONH_2$	98	n.	D		62·9	8·1	22·6	62·9	8·0	22·8	223	9,800	324	20,500
XXIII	CH_3	$CH(CH_3)_2$	*p*-$CONH_2$	148–149	n.	A–C		60·0	7·3	25·4	59·9	7·1	25·4	222	8,800	323	18,500
XXIV	CH_3	$CH_2C_6H_5$	*p*-$CONH_2$	139	pl.	D		67·1	6·0	20·9	67·0	6·0	21·2	212	11,900	327	20,400
XXV	CH_3	$C(CH_3)_3$	*p*-$CONH_2$	134–135	n.	A–C		61·5	7·7	23·9	61·5	7·6	23·8	222	9,700	321	19,800
XXVI	CH_3	OH	*p*-$CONH_2$	218	n.	F	11	49·5	5·2	28·9	49·2	5·3	28·7	215	6,100	318	19,400
XXVII	CH_3	H	*p*-$CONH_2$	147–149	n.	D		53·9	5·7	31·5	54·0	5·5	31·8	219	11,200	309	17,500
XXVIII	CH_3	H	*p*-SO_2CH_3	124–126	n.	D		45·1	5·2	19·8	44·8	5·1	19·4	220	8,200	290	17,300
XXIX	CH_3	C_2H_5	*o*-COOH	130–132	n.	A–C		58·0	6·3	20·3	58·0	6·3	20·3	241	10,500	321	14,800
XXX	CH_3	C_2H_5	*o*-$CONH_2$	76–77	n.	A		58·2	6·8	27·2	58·1	6·8	27·0	242	11,400	316	13,500
XXXI	CD_3	CD_3	*o*-COOH	127	n.	A	12	54·3		21·1	54·2		21·0	243	12,400	320	17,200
XXXII	CD_3	CD_3	*o*-$CONH_2$	135–136	n.	A	12	54·5		28·3	54·4		28·6	242	11,200	321	12,800
XXXIII	CH_3	$CH_2CH_2CH_2CH_3$	*o*-$CONH_2$	124	n.	G	9	61·5	7·7	23·9	61·2	7·7	24·0	242	11,700	319	15,100
XXXIV	CH_3	CH_2CH_2OH	*p*-$COOC_2H_5$	58–59	n.	A–C		57·3	6·8	16·7	57·1	6·9	16·7	230	9,700	326	24,000
XXXV	C_2H_5	H	*p*-$CONH_2$	145–147	n.	A–C		56·2	6·3	29·2	55·9	6·4	29·2	218	11,000	310	18,100

* Crystal form: f.n., flattened needles; n., needles; rect. col., rectangular columns; pl., plates; pr., prisms; c., cubes.

† Solvents: A, ethyl acetate; B, acetonitrile; C, light petroleum (b.p. 60–80°C); D, benzene; E, ethanol; F, methanol; G, cyclohexane.

‡ Spectra measured in ethanolic solutions.

Table 2. Anti-tumour activity of a series of 1-aryl-3,3-dimethyltriazenes against the TLX5 lymphoma



Number	Substituent R	Max. % I.L.S.	Optimal dose (mg/kg) (5 × daily)	Toxic dose (mg/kg) (5 × daily)
I	H	53	64	128
II	*o*-COOH	68	32	64
III	*m*-COOH	62	100	200
IV	*p*-COOH	72	25	200
V	*o*-$COOCH_3$	53	30	120
VI	*m*-$COOCH_3$	63	100	400
VII	*p*-$COOCH_3$	58	40	160
VIII	*p*-$COOC_2H_5$	61	50	200
IX	*o*-$CONH_2$	78	16	128
X	*m*-$CONH_2$	55	10	80
XI	*p*-$CONH_2$	55	25	200
XII	*p*-$CONHCH_2COOH$	46	400	>400
XIII	*p*-OCH_3	41	20	80
XIV	*p*-NO_2	39	100	400
XV	*p*-CF_3	61	200	400
XVI	*p*-SO_2CH_3	80	80	320

RESULTS AND DISCUSSION

The first series of triazenes investigated for their anti-tumour activity contained varying substituents in the aromatic ring, whilst maintaining the methyl groups in the N^3 position. As shown in Table 2, all of these chemicals have anti-tumour activity, no matter whether the aromatic substituents are electron donating, such as *p*-methoxy (XIII) or electron withdrawing such as *p*-trifluoromethyl (XV) or *p*-methanesulphonyl (XVI). The presence of such groups can greatly alter the half-life of hydrolysis of the triazene to the diazonium ion (Fig. 1) and in the compounds listed in Table 2 this can vary from 28 min for the methoxy derivative (XIII) to 90 days in the case of the trifluoromethyl compound (XV). Since there is little difference in the anti-tumour activity of these compounds, it can be concluded that formation of the diazonium ion can play little part in the anti-tumour action of the aryltriazenes, although in some studies on the mechanism of action of DTIC, the diazonium ion has been claimed to be one of the active metabolites [13]. Diazonium salts formed from the

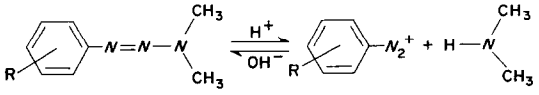
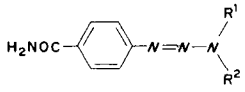


Fig. 1. Hydrolysis of 1-aryl-3,3-dimethyltriazene

Table 3. Anti-tumour activity of a series of 1-(*p*-carbamoylphenyl)-3,3-dialkyltriazenes against the TLX5 lymphoma



Number	Substituents R^1	R^2	Max. % I.L.S.	Optimal Dose (mg/kg) (5 × Daily)	Toxic Dose (mg/kg) (5 × Daily)	% Demethylation
XI	CH_3	CH_3	55	25	200	39
XVII	C_2H_5	C_2H_5	Inactive		400	NS
XVIII	$CH(CH_3)_2$	$CH(CH_3)_2$	Inactive		200	
XIX	CH_3	CH_2CH_3	46	40	160	13
XX	CH_3	CH_2CH_2OH	42	200	>400	NS
XXI	CH_3	$CH_2CH_2CH_2CH_3$	78	50	200	NS
XXII	CH_3	$CH_2CH_2CH_2CH_2CH_3$	102	40	160	
XXIII	CH_3	$CH(CH_3)_2$	52	25	200	
XXIV	CH_3	$CH_2C_6H_5$	86	160	320	
XXV	CH_3	$C(CH_3)_3$	Inactive		>200	39
XXVI	CH_3	OH	54	400	1600	NS
XXVII	CH_3	H	43	30	60	NS
XXXV	C_2H_5	H	Inactive		50	

NS = not significant.

aryltriazenes appear more toxic (that from XI for example is lethal at a single dose of 120 mg/kg), but have no anti-tumour activity *in vivo*. Triazenes which form a significant amount of the diazonium ion under physiological conditions may therefore not be suitable anti-tumour agents, since they may show increased toxicity with no gain in anti-tumour effect.

Although the substitution of the aromatic ring is relatively unimportant in the aryltriazene series, the variation of the N^3-alkyl groups is extremely important. Using the *p*-carbamoylphenyl group as the basic structure, various dialkyltriazenes have been tested for their anti-tumour activity (Table 3). It can be seen that the presence of at least one N^3-methyl group is essential for anti-tumour activity. Thus, both the diethyl- (XVII) and the di-isopropyltriazenes (XVIII) are inactive, although there is little alteration in the lethal dose. On the other hand, compounds XIX to XXVII which contain a methyl group at N^3 are active against the TLX5 lymphoma (with the exception of compound XXV). These results were of particular interest since various triazenes have been investigated for their carcinogenic activity and while there are variations in site of tumour incidence, no such clear distinction between methyl and other alkyl triazenes was seen, 3,3-diethyltriazenes being as carcinogenic as corresponding 3,3-dimethyl analogues [14]. 3-Methyl-3-*tert*-butyltriazene (XXV) is an apparent exception in Table 3 since, although it contains an N^3-methyl group it has no anti-tumour properties. The reason for this anomaly becomes clear when the dealkylation of the triazenes is considered (Table 3). The 3,3-dimethyltriazene (XI) loses approximately one methyl group when incubated with liver microsomes and co-factors, and presumably the monomethyltriazene is formed. Under similar conditions the diethyl analogue undergoes 46 per cent de-ethylation to form the corresponding monoethyltriazene [3], which has no anti-tumour activity. Little demethylation occurred with the compounds XIX, XX and XXI, but since it is known that microsomal *N*-dealkylation occurs preferentially in the longest chain [15, 16], it can be assumed that where dealkylation occurs, it will lead in each case to the formation of the monomethyltriazene. 3-Methyl-3-*tert*-butyltriazene (XXV) no longer occupies an anomalous position since having no α-hydrogen in the higher alkyl chain, this group cannot be removed. As a consequence, demethylation occurs as indicated in Table 3 and the resulting product is the mono-*tert*-butyltriazene rather than the monomethyl analogue. These results suggest that where metabolism can occur to form a monomethyltriazene, the agent is a tumour inhibitor, but where this is not possible (even if dealkylation can take place) the agent is not a tumour inhibitor.

Compound XXVI, the 3-hydroxy-3-methyltriazene [17] (Table 3) is of interest as it does not undergo demethylation, is not toxic to TLX5 lymphoma cells *in vitro*, yet is active against the same cells *in vivo*. It is known from studies on the metabolism of a number of carcinogens, that *N*-hydroxy compounds formed by microsomal metabolism may be subsequently dehydroxylated [18]. If this occurs in the case of XXVI, then the monomethyltriazene would again be formed. Thus, in all cases, anti-tumour activity can be ascribed to the generation of a monomethyltriazene.

Further evidence that monomethyltriazenes are the active metabolites comes from studies on their toxicity to TLX5 lymphoma cells *in vitro* (Table 4). The dimethyltriazene (XI) is quite non-toxic to cells, a concentration of >2000 μg/ml being required to cause significant loss of cell viability. When the same triazene is incubated with the cells in the presence of liver microsomes, co-factors and well-oxygenated, a 10-fold increase in toxicity is observed. The monomethyl analogue (XXVII) on the other hand, is quite toxic when incubated alone with these cells, but this toxicity is reduced when liver microsomes and co-factors are added to the incubation medium with the result that under these conditions the lethal doses of the mono- and dimethyltriazenes are of the same order. It thus appears that the dimethyltriazene can be activated by liver supernatant to the monomethyltriazene, but that further metabolism may then take place resulting in loss of toxicity. It had previously been observed in experiments with imidazoletriazenes, that supernatant from liver and kidney (but not

Table 4. Effect of microsomal metabolism on the toxicity of 1-(*p*-carbamoylphenyl)-3,3-dimethyltriazene and its metabolite 1-(*p*-carbamoylphenyl)-3-methyltriazene to TLX5 lymphoma cells *in vitro*

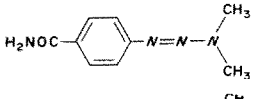
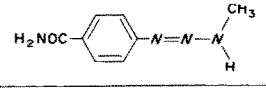
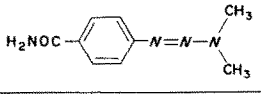
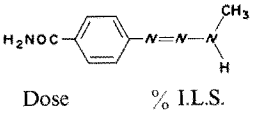
		Toxic Dose (γ/ml) Before metabolism	After metabolism
XI		>2000	200
XXVII		25	100

Table 5. Effect of a monomethyl and a dimethylaryltriazene against the TLX5 lymphoma *in vivo*

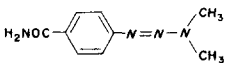
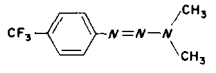
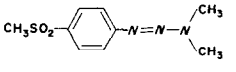
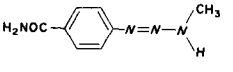
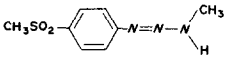
XI 

Dose (mg/kg)	% I.L.S.	Dose (mg/kg)	% I.L.S.
5 × 12·5	14	1 × 12·5	4
5 × 25	55	1 × 25	18
5 × 50	51	1 × 50	18
5 × 100	47	1 × 100	24
5 × 200	4	1 × 200	22
5 × 400	−61	1 × 400	−61

XXVII 

Dose (mg/kg)	% I.L.S.
5 × 3·75	− 3
5 × 7·5	6
5 × 15	32
5 × 30	43
5 × 60	− 5
5 × 120	−58

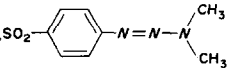
Table 6. Half-life of hydrolysis at 37° in pH 7·5 phosphate buffer of a number of triazenes

Number	Structure	Half-life (mins.)
XI		$1{\cdot}24 \times 10^5$
XV		$1{\cdot}33 \times 10^5$
XVI		No apparent decomposition
XXVI		11·25
XXVIII		25

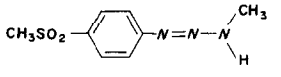
tumour) could protect against their toxicity to TLX5 lymphoma cells *in vitro*, but there was no evidence in this case that it was an enzyme mediated reaction [3].

Although this evidence implicates the monomethyltriazenes (and possibly the alkylating products into which they decompose) as the active metabolites, they are not better as tumour inhibitors than their dimethyl analogues and sometimes less effective. Table 5 shows that the dimethyltriazene is more effective than the monomethyltriazene which it generates *in vivo*. In recent work it has been shown that this may be related to the biological half-life of the two agents, the mono-methyl compound having a shorter half-life than the dimethyl [19] (Table 6). A monomethyltriazene with greater stability has now been prepared (XXVIII) and its anti-tumour activity is comparable to that of its dimethyl analogue (Table 7).

Table 7. Effect of a monomethyl and a dimethylaryltriazene against the TLX5 lymphoma

XVI 

Dose (mg/kg)	% I.L.S.
5 × 10	2
5 × 20	35
5 × 40	74
5 × 80	80
5 × 160	43
5 × 320	−43

XXVIII 

Dose (mg/kg)	% I.L.S.
5 × 12·5	4
5 × 25	37
5 × 50	71
5 × 100	38
5 × 200	48
5 × 400	−62

Table 5 shows that, as previously observed with DTIC in some tumour-systems, fractionated doses of triazenes are more effective than single injections. The reason for this dose-schedule dependency is not clear since these agents are thought to act by covalent binding to macromolecules, and other classes of agent that act in a similar way (e.g. alkylating agents, nitrosoureas and platinum complexes), are as effective by a single injection as by daily injections. Dose schedule dependency is usually a property of anti-tumour agents that act specifically at some stage of the cell cycle such as the anti-metabolites.

These results provide evidence that the active metabolites of the aryldialkyltriazenes are monomethylaryltriazenes and that triazenes that form other than a monomethyl derivative on metabolism, are not anti-tumour agents. Similar results have been reported for analogues of DTIC [20]. The reason for this is not known, although it may be associated with differences in chemical stability and of biological half-life. The tumour inhibition seen with the aryltriazenes has been shown to be dose-schedule dependent, and if they are used clinically they will, like DTIC, probably be most effective if given by daily injections.

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