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Triazenes as Inhibitors of Mouse Sarcoma 180.* (22073)

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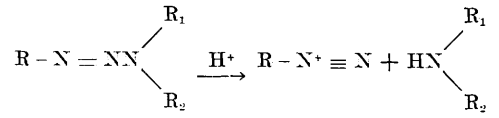
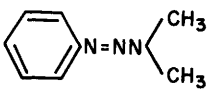
3,3-Dimethyl-1-phenyltriazene (Fig. 1) has recently been found to inhibit *in vivo* growth of the mouse sarcoma 180 (S-180). Tumor inhibitory activity has not previously been described for triazenes; thus a new biological activity is disclosed for derivatives containing the diazo moiety to which this activity might be ascribed. The triazenes are characterized by a high degree of lability in the presence of H-ions, resulting in the formation of a diazonium ion and an amine, thus:



The vigorous coupling activity of the diazonium ion is well known and this particular property of the triazenes ("diazoamines") has been utilized to advantage in the synthesis of azo dyes(1), for polymerization in the rubber industry(2), and for alkylation of dissimilar hydrocarbons in the synthesis of high-octane gasoline(3). It has heretofore been demonstrated that S-180 is sensitive to alkylating agents such as the mustard gases(4), triethylene melamine(5), and the polyfunctional phosphoramides(6). It therefore seemed reasonable to assume that a compound such as 3,3-dimethyl-1-phenyltriazene (hereafter to be referred to as "the triazene") could achieve systemic distribution intact and, at the cellular level, be subject to H-ion catalysis with liberation of benzenediazonium ion and dimethyl amine. The diazonium ion might then inhibit S-180 by reacting with elements essential for cell proliferation—a phenomenon postulated for other alkylating agents(7). This hypothesis will be examined below. It remains to be seen whether the triazenes are sufficiently selective for tumor cells over normal, rapidly proliferating tissue to be useful in higher mammals. In the mouse the therapeutic index is fairly low.



3,3-DIMETHYL-1-PHENYLTRIAZENE

FIG. 1.

Special emphasis will be given the triazene described above since it has proved so far to be the most interesting member of the series (Table I). A study of other diazoamines is continuing in an effort to establish a correlation between structure and tumor-inhibitory activity.

Materials and methods. Dimethyl-phenyltriazene† was synthesized by one of us (C. S. R., Jr.)(8). The compound is a pale, amber fluid with a specific gravity of 1.02.

* This investigation was supported in part by an institutional research grant from the American Cancer Society, by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of National Research Council and by a grant from the National Cancer Institute, U. S. Public Health Service.

TABLE I. Triazene Analogs Which Have Been Adequately Tested for S-180 Inhibition.

Compound	Source
Positive*	
3,3-Dimethyl-1-phenyltriazene	Dr. C. S. Rondestvedt, Jr. (U. Mich.)
3,3-Dimethyl-1-p-nitrophenyltriazene	"
3,3-Dimethyl-1-p-tolyltriazene	"
5(1-Naphthylazo) guanylurea	American Cyanamid Co.
1(5-Chloro-2-tolylazo)-5-(4-methoxy-2-sulfophenyl) biguanide	"
Negative	
3,3-Diethyl-1-phenyltriazene	Dr. R. K. Barclay (Sloan-Kettering)
Diazoaminobenzene	"
4,4'-(3,3'-Dimethoxy-4,4'-biphenylenebisazo)-morpholine	Eastman Kodak Co.
(3,3'-Dimethoxy-4,4'-biphenylene) bis-3,3-dimethyltriazene	"
1-[3,3-bis(2-Cyanoethyl)triazeno]-anthraquinone	"
1-(3,3-Dimethyltriazeno)-anthraquinone	"
1,1'-(4,4'-Biphenylene)bis(3,3-dimethyltriazene)	Parke, Davis & Co.
N^1-Phenylazo-N^2-phenylurea	Dr. Karl Pfister (Merck & Co.)
1-p-Nitrophenyl-3-phenyltetrazene	Remington Rand
1-p-Carboxyphenyl-3-phenyltetrazene	"
4-[3,3-bis(2-Hydroxyethyl)triazeno]benzenesulfonamide	Chem.-Bio. Coordination Center
1,3-di(4-Methoxyphenyl)triazene	Confidential
Diazo-1-amino-2-naphthol-4-sulfonic acid	National Aniline Co.
2,2'-Diazoaminofluorene	Dr. F. E. Ray (U. Florida)
4-(p-Nitrobenzenediazoamino)azobenzene	Purchased

* At tolerated doses tumor avg diameters of treated mice were 75% or less of the avg diameters of control tumors.

It is characterized by a pungent, aromatic odor and is irritating to mucous membrane when applied undiluted. Uniformity and stability of the various samples used were evaluated by ultraviolet spectrophotometry. The triazene is stable in alkali, but is rapidly decomposed by acid. It is, however, but sparingly soluble in water. For tumor inhibition studies, 18-22 g Swiss albino mice (Millerton Research Farms) were employed. Except where possible sex differences were to be explored, it was customary to use female mice. Single trocar implants of S-180 weighing *ca* 5 mg (wet weight) were made subcutaneously in the right axillary region. The implants were cut from tissue excised from donor mice after 7 days of growth. Usually therapy was initiated by direct oral intubation or by intraperitoneal injection 24 hours post-implantation and was continued for 7 successive days. Preliminary experiments established that it was immaterial whether the daily dose was given at one time or divided equally and the 2 half doses given at 7-hour intervals. Therefore the daily dose, uniformly contained in 0.1 ml of peanut oil/20 g mouse, was given as a single injection. Control mice received peanut oil alone by the appropriate route. All animals were weighed on the day of implantation and the day following the last injection. Tumors were measured through the skin with calipers. Two perpendicular diameters were determined and the mean obtained for the purpose of expressing tumor size as an "average diameter." In some experiments, the mice were sacrificed immediately thereafter and the tumors excised and weighed for the purpose of demonstrating the relationship between mass and average diameter.

Results and discussion. Intraperitoneal doses of the triazene employed in this study

† Original sample was supplied through the Chemical-Biological Coordination Center, Washington, D. C., as also was the case with 3,3-dimethyl-1-p-nitrophenyltriazene.

TABLE II. 3,3-Dimethyl-1-phenyltriazene Inhibition of S-180. Treatment given intraperitoneally and continued for 7 days.*

Daily dose, mm^3/kg	Avg wt change, g	Avg tumor diameter, mm	Avg tumor wt (wet) mg
62.5	−3	7.0	89.5
31.25	0	7.9	153.6
15.63	+1	9.3	234.2
0.00	+2	10.8	389.5

* Composite data from 3 separate experiments; a total of 60 mice/dose level.

TABLE III. 3,3-Dimethyl-1-phenyltriazene Inhibition of S-180 and Toxicity to Host following Oral Intubation. Therapy continued for 7 days.

Daily dose, mm³/kg	Avg tumor diameter, mm	Toxicity	
		Avg wt change, g	Mortality
250	6.4*	−5.3*	16/20
125	6.4	−4.0	0/20
62.5	8.5	−2.0	"
0.0	12.0	−1.0	"

* Based on 4 surviving animals only.

have ranged between 62.5 and 15.63 cm/kg/day; with these 2 extremes and an intermediate dose of 31.25 cm, a 3-point curve may be constructed from wet weight data (Table II) which is a straight-line function when the *log* of the dose is plotted against per cent inhibition of tumor growth. Thus, a linear dose-response relationship exists between the inhibitor and its effects on S-180 growth with a 50% effective dose of approximately 22 cm; this was found to be the case, irrespective of sex of the host. Table II also shows the relationship between "average diameter" and the actual mass of tumor tissue. In the tumor-bearing Swiss mouse, repeated daily doses of the triazene greater than 62.5 cm/kg proved lethal to most animals prior to completion of a 7-day course (Table III). Indeed, the 62.5 cm regimen resulted in delayed lethality not attributable to tumor growth (Table IV).

The above tolerated dosage schedule of the triazene was also used to treat animals with "established" tumors. Intraperitoneal therapy was begun 96 hours after tumor implantation and was continued for 7 days thereafter. The relative growth retardation produced by each dose level was comparable with that seen when therapy was started at the earlier post-implantation interval.

Since the triazene is acid-labile it was anticipated the agent would prove ineffective when administered by the oral route unless precautions were taken to neutralize gastric juice. The triazene, however, not only produced tumor-growth inhibition but also host intoxication in the same order of magnitude of dose when administered either orally or by intraperitoneal injection (Table III). Other than dissolving in peanut oil, no precautions were taken to protect the compound from attack by gastric acidity.

S-180 grew, but failed to resume *rapid* growth, following an inhibitory course of the triazene. This observation suggested viability of tumor cells, or their proliferative capacity, had been seriously impaired by the agent. Gross examination of treated tumors revealed a firm, compact consistency with no evidence of extensive necrosis, suggesting that necrotizing activity by the agent was not responsible for sluggish growth. To explore this point further, a series of mice implanted with S-180 was treated by intraperitoneal injection for 7 days with 62.5 cm/kg/day of the triazene. Pieces from treated and control tumors were implanted into normal mice the day following the last injection. One week later the treated S-180 had attained an average diameter of 4.7 mm whereas untreated tumor had grown to an average diameter of 12.8 mm (20 mice/group). There were no instances of failure to "take" among the reimplanted, treated tumors which indicated that some, if perhaps not all, cells remained viable.

Alkylating agents such as nitrogen mustard and triethylene melamine (TEM) characteristically cause depletion of the cellular elements of the marrow in intoxicated animals (9). Since there existed a reasonable expec-

TABLE IV. Relationship between S-180 Inhibitory Doses and Delayed Lethality to Host of 3,3-Dimethyl-1-phenyltriazene. Seven successive days of intraperitoneal administration.

Daily dose, mm³/kg	Tumor-bearing mice			Non-tumor-bearing mice	
	End of therapy		Mortality 7 days later	End of therapy mortality	Mortality 7 days later
	Avg tumor diam., mm	Mortality			
62.5	7.2	1/30	16/30	0/30	11/30
31.25	8.9	0/30	1/30	"	0/30
15.63	11.8	1/29	9/29	"	2/30
0	13.5	0/30	3/30	"	0/30

TABLE V. Effects of Nicotinamide and Nicotinic Acid on Activity of 3,3-Dimethyl-1-phenyltriazene in S-180-Bearing Mice. Therapy was given I.P. for 7 days; uniformly, when combined, the vitamin derivatives preceded the triazene.

Agent and dose/kg/day	Avg diam. of tumors, mm	Avg wt change, g	Mortality
Triazene, 100 mm³	—	—	10/10
" , 50 "	10.1	−4.5	2/10
Nicotinamide, 500 mg	14.8	+ .5	0/10
" " + triazene, 100 mm³	—	—	10/10
" " + " 50 "	8.3	−2.5	0/10
Nicotinic acid, 500 mg*	15.5	.0	"
" " + triazene, 100 mm³	7.8	−4.5	3/10
" " + " 50 "	10.1	−2.0	0/10
Control	15.4	.0	"

* Prepared as the sodium salt immediately prior to injection.

tation that the biological activity of the triazene might reflect the alkylating properties of the benzenediazonium ion, experiments were undertaken to learn if marrow depression might characterize triazene intoxication. Groups of non-tumor-bearing mice were treated with intoxicating doses of TEM (1 mg/kg/day) and the triazene (62.5 cm/kg/day) for 5 days. Another group, serving as control mice, received daily injections of peanut oil, the vehicle used for the 2 agents. Although the triazene-treated mice lost more weight (avg −3.1 g) than did those receiving TEM (avg −1.6 g), the total femoral nucleated cell count for the TEM-treated mice was 33% of the control count, whereas the count for triazene-treated mice was 50% of control values.‡ Thus, the triazene did depress bone marrow and this, undoubtedly, contributes to the overall intoxication of the mouse. However, it is distinctly less potent in this respect than is TEM under the conditions of this experiment.

Phillips(10) observed that mice, intoxicated with the triazene, developed a syndrome characterized by unsteady stance and gait, choreaform movements of the head, and retropulsion (the "waltzing" syndrome). The behavior pattern so closely resembled that described by Goldin *et al.*(11) for mice intoxicated with certain alkylating agents that the "swimming test" applied by the latter investigators to detect the presence of this neurological disturbance was applied in this instance. The results were positive. In separate studies, Dagg *et al.*(12) demonstrated a teratogenic response to the triazene in the developing chick embryo which could be prevented by the administration of nicotinamide. The effects of nicotinic acid or its amide were therefore examined in tumor-bearing mice treated with the triazene. The results of a typical experiment are presented in Table V. It is evident tumor-inhibitory activity of the triazene was not altered by prior administration of nicotinic acid or the amide. However, mice treated with nicotinic acid and surviving the 100 cm triazene dose failed to exhibit the "waltzing" syndrome. In other experiments, 2 groups of non-tumor-bearing mice were treated with nicotinic acid (500 mg/kg/day, as the sodium salt) for 3 consecutive days, and a third group received control injections of isotonic NaCl (10 mice/group). One hour after the last intraperitoneal injection, the group injected with NaCl and one of the groups which had received sodium nicotinate received a single injection of the triazene. The dose of the triazene used (180 cm/kg) was one which previously had been shown capable of inducing the swimming deficiency mentioned above within 24 hours in the majority of mice without subsequent lethality. Examined the day following the triazene injection, 70% of the mice which had received isotonic NaCl and the triazene showed the

‡ The method of quantitating total nucleated cells of mouse femurs has been perfected at Sloan-Kettering Institute by Dr. Vincenzo Grifoni, a Visiting Fellow from the U. of Milan, Italy. Details of the method and some of its applications will be the subject of a future publication.

swimming defect (as well as the behavior symptoms of the "waltzing" syndrome) but none of those mice pretreated with nicotinic acid were so affected. There was no change in this situation for the following 3 days of observation. Any relationship between induction of teratogeny and induction of the "waltzing" syndrome by the triazene, other than that both abnormalities may be prevented by nicotinic acid derivatives, is obscure.

Consideration was given the possibility that chemical interaction in the peritoneal cavity between nicotinic acid and the triazene might account for the alteration in biological activity of the triazene. Appearing to negate this possibility were the results of an experiment wherein the nicotinic acid derivatives were administered by oral intubation 45 minutes prior to intraperitoneal injections of the triazene. Doses of agents were similar to those described in Table V and the results were essentially identical. Or, in another experiment with similar end-results, the vitamin derivatives were injected intraperitoneally 60 minutes before doses of 100, 75, and 50 cm/kg/day of the triazene. Finally, *in vitro* experiments were conducted wherein dilute solutions of nicotinic acid and its amide were added to buffered (pH 6.8) aqueous solutions of the triazene. These preparations were examined for possible changes in the ultraviolet absorption patterns. No significant changes were detected, suggesting failure of interaction under these conditions.

It may be adduced from the above that one of the features contributing to intoxication of the Swiss mouse by the triazene is a central nervous system lesion, the overt manifestations of which correspond with those of the genetically recessive characteristics described for Waltzer or Shaker mice(13). These manifestations may be prevented by the prior administration of nicotinic acid. The precise identity of this lesion with that described by Goldin *et al.*(11) remains to be established. Nevertheless, there is similarity between the circumstances leading to development of the lesion under the 2 experimental conditions which tends to support the notion that the triazene, as was found by Golden *et al.* for specific chlorinated tertiary amines, exerts a part of its biological activity by presenting the mouse with 1) an essential structural configuration and 2) an alkylating group. Failure to protect against triazene-induced tumor inhibition with nicotinic acid, however, suggests a bifunctional capacity of the triazene or, possibly a triazene-catabolite, farther removed from the diazonium ion, possessing tumor-inhibitory properties. Indeed, it is clear from the data in Table V that the higher doses of triazene were lethal to some individuals despite the administration of nicotinic acid but none of those animals, though obviously ill, demonstrated overt manifestations of the central lesion prior to terminus; this is further support for perceiving separable activities for the triazene.

Summary. 1. 3,3-Dimethyl-l-phenyltriazene possesses inhibitory activity for growth of the mouse tumor, sarcoma 180. Certain other triazenes have similar activity. 2. Dimethyl-phenyltriazene produces comparable tumor inhibition whether administered orally or intraperitoneally. 3. The triazene induces the "waltzing" syndrome in the Swiss albino mouse used in this study. 4. Nicotinic acid administered prior to the triazene prevents the "waltzing" syndrome but does not modify tumor inhibition. 5. In doses intoxicating to the Swiss mouse, the triazene is a moderate bone marrow depressant.

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Effects of Certain Triazenes on Chick Embryos and on Tumors Explanted to the Chorioallantois.* (22074)

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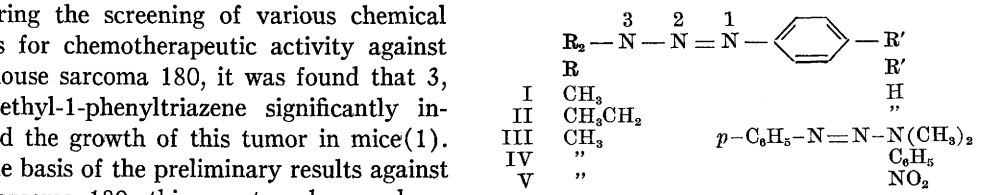
During the screening of various chemical agents for chemotherapeutic activity against the mouse sarcoma 180, it was found that 3, 3-dimethyl-1-phenyltriazene significantly inhibited the growth of this tumor in mice(1). On the basis of the preliminary results against the sarcoma 180, this agent and several related compounds were selected for more extensive study in secondary systems used in the tumor screening program(2-4).

This report is concerned with the effects of certain triazenes on the development of the chick embryo and on tumors growing on the chorioallantois. In the studies reported here it was found that the anomalies induced in the chick embryo by triazenes were similar to those caused by other, unrelated, teratogens whose effects are preventable or reversible by nicotinamide(5-9). Consequently, nicotinamide was tested for its ability to prevent the teratogenic effects of the triazenes.

Materials and methods. Commercially obtained White Leghorn eggs, incubated at 38°, were used for all experiments. The compounds tested for teratogenic activity against the chick embryo have the following structures:



	R	R′
I	CH_3	H
II	CH_3CH_2	"
III	CH_3	p-C_6H_5-N=N-N$(CH_3)_2$
IV	"	C_6H_5
V	"	NO_2

I: 3,3-dimethyl-1-phenyltriazene
II: 3,3-diethyl-1-phenyltriazene
III: 1,1′-(4-4′-biphenylene) bis [3,3-dimethyltriazene]
IV: 1-(4-biphenylyl)-3,3-dimethyltriazene
V: 3,3-dimethyl-1-*p*-nitrophenyltriazene

I is an amber-colored liquid with a specific gravity of 1.02. II is also a liquid, while III, IV, and V are solids. Because of their low solubility, these compounds were prepared as suspensions in isotonic saline or in 0.5% sodium carboxymethylcellulose in isotonic saline. The freshly prepared suspensions were injected into the yolk sac of 4-day-old embryos. The eggs were sacrificed after the 18th day of incubation. The embryos were weighed, examined for gross abnormalities, and selected embryos were cleared and stained with alizarin to show skeletal defects. In the protection experiments, nicotinamide in isotonic saline was injected immediately following the triazene. The tumors used were mouse sarcoma 180 and Toolan's human epidermoid carcinoma #3 (H.Ep.#3)(10,11). Preparatory to chemotherapy trials, triazene-I was injected into the yolk sac of 12-day-old embryos to determine tolerated dose levels

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