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MECHANISM OF ACTION OF ANTITUMOR 3-(2-HALOETHYL)ARYLTRIAZENES ON DEOXYRIBONUCLEIC ACID

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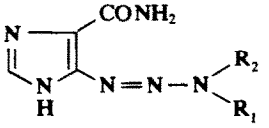
Abstract—A series of novel 3-(2-haloethyl)aryltriazenes, many of which exhibit marked antileukemic activity in animal test neoplasms, react readily with DNA under physiological conditions. With regard to a single strand scission (SSS), in contrast to the related 2-haloethylnitrosoureas which exhibit both Type I and Type II SSS (single strand scission) of DNA, the triazenes appear to react via Type II SSS of DNA by base alkylation followed by depurination or depyrimidination and subsequent hydrolysis of the apurinic site. The latter reaction was confirmed using apurinic site-specific endonuclease VI. A 3-(2-chloroethyl)aryltriazene readily degraded poly A by phosphate alkylation at a rate much faster than given by comparable nitrosoureas. Overall, the triazenes showed a preference for reaction at the more acidic phosphate sites in the DNA owing to their unique acid-promoted decomposition. This may, in part, account for the lack of detection of DNA interstrand cross-links and indicates a fundamentally different mechanism of action of the 3-(2-haloethyl)triazenes from the 2-haloethylnitrosoureas.

Substituted triazenes including 5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide (DTIC) (1a) and 5-[3,3-bis(2-chloroethyl-1-triazenyl] imidazole-4-carboxamide (BTIC) (1b) are clinically effective in the treatment of a range of neoplasms including



1(a) $R_1 = CH_3$, $R_2 = CH_3$
(b) $R_1 = CH_2CH_2Cl$, $R_2 = CH_2CH_2Cl$
(c) $R_1 = CH_3$, $R_2 = H$
(d) $R_1 = CH_2CH_2Cl$, $R_2 = H$

malignant melanoma, brain tumors and lymphomas [1–5]. Structure–activity relationships of triazenes showed that the anticancer activity arose from the alkyltriazene moiety [6, 7]. Recently we reported the synthesis of certain 3-(2-haloethyl)aryltriazenes and an examination of the chemical reactions involved in their aqueous decomposition [8]. In the present paper, we document their antileukemic properties.

While considerable evidence indicates that triazenes inhibit nucleic acid synthesis [9, 10], little is known about their mechanism of action, in particular of their chemical interaction with cell target sites such as DNA. It seemed probable that, like the clinically effective 2-haloethylnitrosoureas, they decompose under physiological conditions giving rise to electrophiles which attack biological macromolecules [1, 6, 9, 10].

We report an examination of the different types of reactions between 3-(2-haloethyl)aryltriazenes and DNAs and RNA under physiological conditions (and the contrast with the 2-haloethylnitrosoureas) and how these reactions may relate to their observed antileukemic activities.

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Chemistry

The 3-(2-haloethyl)aryltriazenes required in this study were prepared by synthetic procedures that have been reported [8]. Triazene (9) could be prepared by controlled condensation of ethyl magnesium bromide with *p*-cyanophenylazide without significant interference by attack on the nitrile group, in contrast to an earlier report of the inapplicability of this procedure [11]. The NMR spectrum of 10 shows two doublets at 5.05 and 5.3 δ at −45° but only one doublet at 5.15 δ at 30°, indicating rapid interconversion of the *syn* and *anti* triazene configurations at room temperature.

EXPERIMENTAL PROCEDURE

Materials

Ethidium bromide was purchased from the Sigma Chemical Co., St. Louis, MO, and λ-DNA (mol. wt 31×10^6) was obtained from Miles Biochemicals, Miles Laboratories, Inc., Elkhart, IN. Superoxide dismutase (EC 1.15.1.1) was obtained from the Sigma Chemical Co., and catalase (EC 1.11.1.6) (beef liver) was from the Aldrich Chemical Co., Milwaukee, WI. PM2-CCC (covalently closed circular) DNA was prepared as described previously [12]; the calf thymus topoisomerase was prepared according to the method of Herrick and Alberts [13].

Melting points were determined on a Fisher–Johns apparatus and are uncorrected. The IR spectra were recorded on a Nicolet 7199 F.T. spectrophotometer, and only the principal sharply defined peaks are

reported. The NMR spectra were recorded on Bruker WH 400 and WH 200 analytical spectrometers. The spectra were measured on approximately 10–15% (w/v) solutions in appropriate deuterated solvents with tetramethylsilane as standard. Line positions are reported in parts per million from the reference. Mass spectra were determined on an associated Electrical Industries MS-9 double-focussing high-resolution mass spectrometer. The ionization energy, in general, was 70 eV. Peak measurements were made by comparison with perfluorotributylamine at a resolving power of 15,000. Microanalyses were carried out by Mrs. D. Mahlow of this department. The triazenes required in this study were prepared following the general procedure described [8].

1-(p-*Cyanophenyl*)-3-(2-*hydroxyethyl*)*triazene* (**2**). This compound was obtained as a yellow solid which was purified by recrystallization from methylene chloride at −10°; m.p. (dec.) 100° (90% yield). Anal. Calc. for $C_9H_{10}N_4O$, 190.0855, M^+, 190.0853 (7.5%); Calc. for $C_9H_{10}N_2O$, 162.0793, M^+-N_2, 162.0793, (1.6%) (mass spectrum). PMR [$CDCl_3$] 2.15 (s, 1H, exch. OH), 3.9 (br, m, 4H, NCH_2CH_2O), 7.3–7.8 (m, 4H, aromatic), 9.5 (br, s, exch. NH). IR ν_{max} 3466, 3288, 3261, 1609 cm^{-1}.

1-(p-*Cyanophenyl*)-3-(2-*methoxyethyl*)*triazene* (**3**). The product was obtained as a solid which was purified by recrystallization from methylene chloride at −30° by adding petroleum ether dropwise; m.p. 81° (92% yield). Anal. Calc. for $C_{10}H_{12}N_4O$, 204.1012, M^+, 204.1012 (14%). Calc. for $C_{10}H_{12}N_2O$, 176.0949, M^+-N_2, 176.0951 (1.5%) (mass spectrum). PMR [CDl_3, −40°] 3.4 (s, OCH_3), 3.7 (t, 2H, CH_2O), 3.9 (t, 2H, NCH_2), 7.1–7.7 (m, 4H, aromatic), 8.6 and 9.6 (br, s, 2H, NH). IR ν_{max} ($CHCl_3$), 3182, 3160, 1609 cm^{-1}.

The following triazenes were obtained as viscous oils which were extracted in CH_2Cl_2, dried ($MgSO_4$), and precipitated from the filtered solution by cooling in dry ice–acetone and adding petroleum ether dropwise.

1-(p-*Carboethoxyphenyl*)-3-(2-*hydroxyethyl*)*triazene* (**4**). A yellow solid; m.p. 88–90° (60% yield). Anal. Calc. for $C_{11}H_{15}N_3O_3$, 237.1113, M^+, 237.1106 (1%); Calc. for $C_{11}H_{15}NO_3$, 209.1052, M^+-N_2, 209.1047 (0.5%) (mass spectrum). PMR [CD_2Cl_2] 1.35 (t, 3H, CH_3), 2.1 (br, s, 1H, exch. OH), 3.85 (br, s, 4H,—NCH_2CH_2O—), 4.3 (5, 2H, OCH_2), 7.2–8.1 (m, 4H, aromatic), 9.2 (br, s, exch. NH). IR ν_{max} ($CHCl_3$), 3504, 3229, 3188, 3162, 1715, 1688 cm^{-1}.

1-(p-*Carboethoxyphenyl*)-3-(2-*methoxyethyl*)*triazene* (**5**). A yellow solid; m.p. 61–63° (92% yield). Anal. Calc. for $C_{12}H_{17}N_3O_3$, 251.1270, M^+, 251.1268 (20%); Calc. for $C_{12}H_{17}NO_3$, 223.1208, M^+-N_2, 223.1206 (5%) (mass spectrum). PMR [$CDCl_3$, −20°] 1.3 (t, 3H, CH_3), 3.4 (s, 3H, OCH_3), 3.75 (t, 2H, CH_2O), 3.95 (t, 2H, NCH_2), 4.35 (q, 2H, OCH_2), 7.2–8.2 (m, 4H, aromatic), 8.7, 9.9 (br, s, 1H, each, exch. NH). IR ν_{max} ($CHCl_3$) 3225, 3190, 3171, 1702, 1687 cm^{-1}.

1-(p-*Acetylphenyl*)-3-*ethyltriazene* (**6**). Yellow solid; m.p. 82–83° (82% yield). Anal. Calc. for $C_{10}H_{13}N_3O$, 191.1058, M^+, 191.1056 (43%). Calc. for $C_{10}H_{13}NO$, 163.0997, M^+-N_2, 163.0966 (7.5%) (mass spectrum). PMR [$CDCl_3$, −45°] 1,3 (t, 3H, CH_3), 2.6 (s, 3H, OCH_3), 4.6 (q, 2H, NCH_2), 7.1–8.1 (m. 4H, aromatic), 8.2 and 10.2 (br, 2s, exch. NH). IR ν_{max} ($CHCl_3$) 3216, 1667 cm^{-1}.

1-(p-*Cyanophenylazo*)*aziridine* (**7**). Triazene (**7**) was prepared by following the general procedure described for the 2-haloalkyltriazenes [8]. The neutralized diazonium salt solution was filtered and the filtrate cooled to −5°. To the stirred solution was added aziridine, in slight excess, dropwise. After stirring the mixture for 5 min the solid product was collected by filtration, washed with cold water and purified by rapid recrystallization from CH_2Cl_2 by cooling in a solid CO_2-acetone bath and adding petroleum ether dropwise. This yielded the triazene as an off-white solid; m.p. 45° (60% yield). PMR [$CDCl_3$, −20°], 2.3 (s, 4H, aziridine ring protons), 7.2–7.7 (m, 4H, aromatic).

1-(p-*Cyanophenyl*-Δ^2-1,2,3-*triazoline* (**8**). The azoaziridine (**7**) was isomerized following the procedure of Heine and Tomalia [14]. The resulting solid product was purified by recrystallization from methylene chloride and petroleum ether at low temperature as an off-white solid; m.p. 124° (95% yield). Anal. Calc. for $C_9H_8N_4$, 172.0749, M^+, 172.0757 (0.7%); Calc. for $C_9H_8N_2$, 144.0688, M^+-N_2, 144.0685 (78%) (mass spectrum). PMR [$CDCl_3$] 3.55 and 4.5 (2s, 4H,—CH_2CH_2—), 7.25–7.7 (m, 4H, aromatic). IR ν_{max} ($CHCl_3$) 3233, 1610 cm^{-1}.

1-(p-*Cyanophenyl*)-3-*ethyltriazene* (**9**). This triazene was prepared following an adapted procedure of Dimroth [15]. A solution of 1.44 g (10 mmoles) of *p*-cyanophenylazide in 2 ml of anhydrous ether was added slowly to a solution of 11 mmoles of ethylmagnesium bromide in 10 ml of anhydrous ether. After stirring the reaction mixture for 10 min, a saturated solution of ammonium chloride was added dropwise under cooling until the solid dissolved. The ether layer was removed, washed with water, and dried ($CaCl_2$). After removal of the solvent the solid residue was purified by recrystallization from methylene chloride: petroleum ether at low temperature affording the triazene (**9**); m.p. 95–97° (76% yield). Anal. Calc. for $C_9H_{10}N_4$, 174.0905, M^+, 174.0905, M^+, 174.0907 (34%); Calc. for $C_9H_{10}N_2$, 146.0843, M^+-N_2, 146.0843 (3%) (mass spectrum). PMR [CD_2Cl_2, −45°] 1.3 (m, 3H, CH_3), 3.7 (m, 2H, CH_2), 7.45–7.75 (m, 4H, aromatic), 8.35 and 9.65 (br, 2s, exch. NH). IR ν_{max} ($CHCl_3$) 3184, 3160, 2219, 1608 cm^{-1}.

1-(p-*Cyanophenyl*)-3-*ethyl*-3-(*hydroxymethyl*)*triazene* (**10**). This triazene was prepared by adapting a procedure due to Vaughan and co-workers [16]. *p*-Aminobenzonitrile (1.18 g, 10 mmoles) was diazotized at 0–5° in a mixture of 2.5 ml of concentrated hydrochloric acid and 10 ml of water using 800 mg of sodium nitrite dissolved in the minimum volume of water. The excess acid was neutralized with 1.6 g of $CaCO_3$ and the aqueous solution of the diazonium salt was added to a cold, premixed solution of 0.5 ml of ethylamine and (5 ml) of 40% aqueous formaldehyde. After stirring for 15 min, the resulting precipitate was collected, washed with cold water, dried under vacuum, and recrystallized from CH_2Cl_2/petroleum ether to afford triazene (**10**) as a white crystalline solid; m.p. 65–66° (42% yield).

Anal. Calc. for $C_{10}H_{12}N_4O$, N, 27.45%; Found, 27.15%. Calc. mol. wt, 204.1047, M^+, 204.1007 (0.4%), 174.0906, M^+-CH_2O, 146.0842, M^+-CH_2O-N_2, (mass spectrum). PMR [CD_2Cl_2, −45°] 1.1–1.45 (m, 3H, CH_3), 3.65–4.0 (m, 2H, CH_2), 4.4–4.7 (m, 1H, OH), 5.0–5.3 (2d, NCH_2O), 7.3–7.7 (m, 4H, aromatic), [CD_2Cl_2, 30°], 1.3 (t, 3H, CH_3), 2.95 (br, s, 1H, exch. OH), 4.85 (q, 2H, CH_2), 5.15 (d, 2H, NCH_2O), 7.3–7.7 (m, 4H, aromatic). IR ν_{max} ($CHCl_3$) 3432, 2228 cm^{-1}.

Preparation of diethyl 2-haloethylphosphates via esterification of diethyl phosphate with 2-haloethyltriazenes

Diethyl 2-fluorethylphosphate (**11**). A typical reaction required the addition of 450 mg (3 mmoles) of diethyl phosphate in 10 ml of ether to a suspension of 600 mg (3 mmoles) of 5-[3-(2-fluoroethyl)-1-triazenyl]imidazole-4-carboxamide in 50 ml of anhydrous ether. The reaction mixture was protected from light and the stirring was continued for 12 hr. After filtration and removal of the solvent *in vacuo*, the residue was chromatographed on florisil using chloroform as eluant and affording 60 mg (10% yield) of triester (**11**). Anal. Calc. for $C_6H_{15}FO_4P$, 201.0692, M^+, 201.0704 (mass spectrum). PMR [$CDCl_3$] 1.36 (t, 8H, $2CH_3$), 4.07–4.38 (m, 8H, OCH_2), 4.58 (2m, 2H, CH_2F). IR ν_{max} ($CHCl_3$) 1470, 1440, 1380, 1360, 1250 cm^{-1}.

2-Chloroethyl-diethyl phosphate (**12**). A solution of 1.5 g (10 mmoles) of diethyl phosphate in 10 ml of ether was added to a stirred suspension of 2.5 g (12 mmoles) of 1-(*p*-cyanophenyl)-3-(2-chloroethyl)triazene in 20 ml of anhydrous ether at room temperature. After 30 min the product was isolated and purified by chromatography on florisil using benzene/5% acetone as eluant yielding the triester **12**, 210 mg (8% yield). This material was identical to an authentic sample prepared by a literature procedure [17].

Endonuclease specific for apurinic sites of Escherichia coli *(endonuclease VI)*

Endonuclease VI was purified according to Verly and Rassart [18] from *E. coli* BATCC 11303; after the phosphocellulose chromatography, the enzyme was stored in 0.15 M NaCl, 0.04 M sodium phosphate (pH 6.5) with an equal volume of glycerol and kept at −20°. For the experiments, this preparation was diluted with a suitable buffer to the extent that it afforded a standard solution in which 10 μl contained sufficient endonuclease activity to completely cleave the apurinic PM2-DNA sample in 5 min at 37° (see Methods).

Methods

Ethidium fluorescence assay for Type I SSS of DNA. The fluorometric methods of measuring strand breakage of PM2-CCC DNA have been described [12, 19, 20]. The conversion of PM2-CCC DNA to PM2-OC (open circular) results in a 30% increase in fluorescence in the pH 11.8 ethidium assay solution [which was 20 mM potassium phosphate (pH 11.8), 0.4 mM EDTA and 0.5 μg/ml of ethidium bromide] owing to release of topological constraints.

The increase in fluorescence can be further enhanced by initially treating the PM2-CCC DNA with the calf thymus topoisomerase. Native PM2-CCC DNA contains negative supercoils. The topoisomerase, by acting as both an endonuclease and a ligase, removes the supercoils to relax the DNA. During this process the number of intercalation sites for ethidium (which itself unwinds the supercoiled PM2-CCC DNA) is decreased. The relaxation process can be monitored by a 25–30% decrease in fluorescence which results when all the supercoiled DNA is completely relaxed. The conversion of relaxed PM2-CCC DNA to PM2-OC DNA now results in 100% increase in fluorescence.

A 300-μl sample containing PM2-CCC DNA 1.0 A_{260}, 50 mM sodium cacodylate buffer (pH 7.0) and 400 mM NaCl was incubated at 37° with 2 μl of the topoisomerase solution. The fluorescence was monitored by transferring 20-μl aliquots into 2 ml of the pH 11.8 assay solution. When a 25–30% decrease in fluorescence had been observed (typically requiring a 30-min incubation [21]), a 2 mM concentration of the desired drug was introduced and the fluorescence was again monitored using 20-μl aliquots in 2 ml of the pH 11.8 assay solution. Readings were taken as soon as possible after addition of the aliquot to the assay medium (usually 60 sec) especially in those cases, e.g. compounds **2** and **4**, where strand scission is rapid, and also to minimize any contribution of apurinic site hydrolysis to the observation of Type I SSS. As explained below, the onset of Type II SSS is a much slower process under these conditions.

Ethidium fluorescence assay for Type II SSS of DNA. After the fluorescence reading of an aliquot of the reaction mixture had been taken to determine any Type I SSS, the pH 11.8 assay solution containing the 20-μl aliquot of the reaction mixture was incubated at 37°. At designated times, the solution was re-equilibrated to 22° for the fluorescence reading [21, 22]. Under these conditions, alkali-labile apurinic sites that are produced as a result of thermal loss of alkylated bases undergo a slow alkaline hydrolysis, resulting in single strand cleavage. The resulting open-circular PM2-DNA accepts more ethidium which is revealed by a slow progressive rise in the fluorescence readings.

An alternative and complementary method of detecting and quantifying Type II SSS takes advantage of the redundant information obtained from the before heat and after heat fluorescence readings. As we have seen, a rapid rise in fluorescence before heating indicates Type I SSS and, in the limit when topoisomerase-relaxed CCC DNA is completely nicked, a 100% rise is observed. Lesser extents of nicking are revealed by a smaller proportionate increase. After allowing for the small proportion of OC DNA (8%) already present, the extent of after heat fluorescence fall should be the same as the fluorescence rise before heating in the absence of competing reactions [e.g. Fig. 3, compounds **2** and **4**]. Any discrepancy [e.g. for **3** and **5** Fig. 3] is due to the additional scission caused by the Type II mechanism which can, therefore, be quantified (see Discussion).

Assay for endonuclease VI activity. The basis of the assay is that the enzyme cleaves PM2-CCC-DNA containing AP sites and thereby converts it to nicked

or open circular (OC) DNA which, since the latter is subject to no topological constraints, will accept more ethidium resulting in a characteristic 30% increase in fluorescence reading when measured at pH 8.0. As a further control to confirm that the apurinic sites have been cleaved, heat denaturation is performed at 96° for 3 min when the PM2-OC-DNA is converted into single strands followed by rapid cooling to 23°. The control for the assay consisted of a similar reaction substituting PM2-CCC-DNA which returns to register after the heating and cooling cycle thereby returning the fluorescence reading to its value. The reaction solution consisted of depurinated PM2-CCC-DNA [22] 1.0 A_{260} unit in 0.4 M NaCl and potassium phosphate buffer (pH 8.0). A 10-μl aliquot of the enzyme solution (0.47 A_{260} unit) was added, the reaction mixture was incubated at 37° for 15 min, and the fluorescence of the resulting PM2-OC-DNA was read using the standard pH 8.0 ethidium assay.

The endonuclease VI also exhibits exonuclease III activity. However, at the levels of enzyme employed, the very rapid rise in fluorescence, characteristic of the endonuclease VI activity, and the constant level of the resulting fluorescence reading over a period of 15 min ensure that the slower and subsequent exonuclease activity does not interfere with the enzymatic detection of apurinic sites. This serves as an internal control [21].

Detection of apurinic or apyrimidinic sites. A 300-μl solution containing 2 mM drug, 50 mM sodium cacodylate (pH 7.0), and relaxed PM2-CCC-DNA 1.0 A_{260} was allowed to react for 60 min while monitoring for Type I SSS. Ten microliters of the AP endonuclease solution was then added (the amount was determined by previous experiments with low pH depurinated PM2-CCC as described above). The fluorescence was then monitored as described in the beginning of Methods. The percentage of fluorescence increase with respect to the fluorescence at time 0 min was corrected for dilution by the enzyme solution [22].

Fluorescence determination of alkylation of PM2-CCC-DNA by triazenes. A 20-μl aliquot was taken at intervals from the reaction mixture [50 mM potassium phosphate (pH 7.2), 1.2 A_{260} units of PM2-CCC-DNA (90% CCC), 1 mM triazene in a total volume of 200 μl at 37°] and was added to 2 ml of the standard assay mixture [which was 20 mM potassium phosphate (pH 11.8), 0.4 mM EDTA, and 0.5 μg/ml of ethidium]. The fluorescence after heating at 96°/3 min followed by rapid cooling was compared with the initial value.

Under these conditions, unreacted PM2-CCC-DNA returns to register after heat denaturation because of topological constraints. Alkylated PM2-CCC-DNA shows a decrease in fluorescence because of thermally induced depurination or depyrimidination followed by alkaline strand scission of the apurinic site in the assay medium [20]. In contrast to the control PM2-CCC-DNA, the open-circular form, PM2-OC-DNA, is subject to denaturation under these conditions and, provided the pH of the assay is sufficiently high (11.8) to prevent formation of regions of accidental self-complementarity, the fluorescence falls. In the limit where all the PM2-DNA is alkylated, the fluorescence falls to zero. This assay has the particular advantage that competing processes may readily be detected. For example, Type I SSS (or other processes of single strand scission) will be revealed by the characteristic rise in fluorescence before the heating and cooling cycle as described previously. Similarly, if interstrand cross-links are formed, then they will serve as the nucleation sites for subsequent renaturation after the heat denaturation and cooling cycle. This will cause a characteristic recovery of the fluorescence readings [23]. In the absence of such competing processes and after allowing for the small and constant proportion of open circular (OC)-DNA present in the DNA (the identical batch of PM2-CCC-DNA, 92% CCC, was used for all the experiments), then the ratio of the decrease in fluorescence (after the heating and cooling cycle) to that of the control value may be taken as a measure of the extent of alkylation [20]. In a control experiment it was also shown that none of the components interfered with the ethidium fluorescence. This technique can be used to measure levels of alkylation not readily observed using, for example, calf thymus or λ-DNA. PM2-CCC-DNA has the added advantage of increased stability to a fairly wide pH range (compared with, for example, calf thymus DNA) [24] permitting the extension of this assay for alkylation to cover the effects of pH changes (see Table 2).

Detection of phosphate alkylation by RNA degradation. A 150-μl solution containing 4 mg/ml Poly A (Sigma mol. wt 162,000), 150 mM sodium cacodylate buffer (pH 7.0) and 150 mM 1-(*p*-cyanophenyl)-3-(2-chloroethyl)triazene (**13**) was incubated for 1 hr. The reaction was quenched in ice and dialyzed against 50 mM potassium phosphate (pH 7.2), 100 mM NaCl, 1 mM EDTA in triple distilled water at 4° for 24 hr. The dialysate was then diluted with the dialysis solution to 1.0 A_{260} and the sedimentation velocity determined on a Beckman analytical ultracentrifuge [21].

General method for the aqueous decomposition of 3-(2-haloethyl)aryltriazenes. The decompositions were performed at 37° in phosphate buffered (0.1 M, pH 7.2) aqueous solution in gas-tight reactivials, and the volatile products were analyzed by gas chromatography (GC) and identified by gas chromatography–mass spectrometry (GCMS). Each product was identified by its retention time compared with a standard and by its characteristic mass spectrum. The involatile products were separated by chromatography on silica gel using a mixture of acetone–benzene (1:10) as eluant, and the individual products were identified by their NMR and absorption spectra.

RESULTS AND DISCUSSION

Alkylation of DNA by 3-(2-haloethyl)aryltriazenes

The 3-(2-haloethyl)aryltriazenes alkylated PM2-CCC-DNA readily under physiological conditions as demonstrated by the ethidium fluorescence assay (Fig. 1, Table 1). The results are in accord with previous reports of *in vitro* alkylation of nucleic acids and nucleotides by phenylmonomethyl triazenes [25, 26] and by *in vitro* treatment of calf thymus

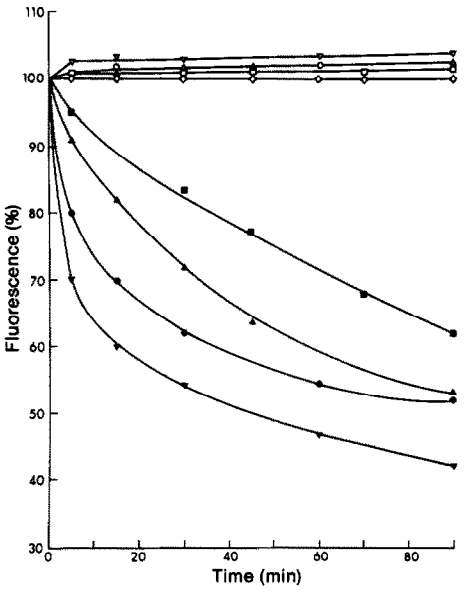
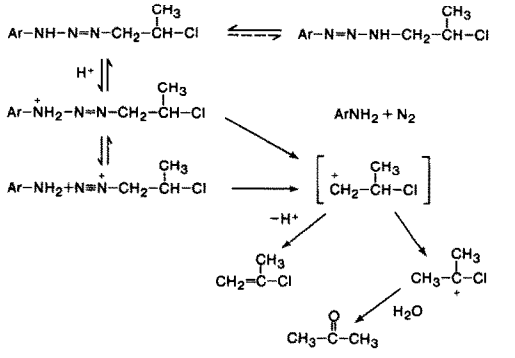


Fig. 1. Thermally induced DNA strand scission resulting from alkylation by 3-(2-haloethyl)aryltriazenes. Reaction of 1 mM drug in 10% aqueous acetonitrile for times indicated with 1.0 A_{260} unit of supercoiled PM2-CCC-DNA (pH 7.0, 37°) and assayed in the pH 11.8 ethidium medium. Open symbols are fluorescence readings before heat denaturation. Filled symbols are fluorescence readings after heat denaturation at 96°/3 min followed by cooling to 22° (■–■) 1-(*p*-cyanophenyl)-3-(2-bromoethyltriazene) (**25**); (▲–▲) 1-(*p*-carboethoxyphenyl)-3-(2-fluoroethyl)triazene (**16**), ± 0.8 mg/ml of superoxide dismutase or 0.8 mg/nl of catalase or 1.6 mM sodium benzoate; (●–●) 1-(*o*-nitrophenyl)-3-(2-fluorethyl)-triazene (**18**); (▼–▼) 5-[3-(2-fluorethyl)-1-triazenyl]imidazole-4-carboxamide (**17**); and (◇–◇) control experiment with untreated PM2-CCC-DNA.

DNA with tritium-labeled MTIC (**1d**) [27]. With the exception of those two compounds run in aqueous dimethylsulfoxide, one may discern in Table 1 a trend favouring greater DNA alkylation for triazenes that decompose more rapidly. Electrophiles generated from **16** may be removed to a greater extent by, for example, proton loss and hydride transfer (Scheme 1).

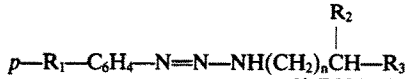
Scheme 1



The relative stabilities of *ortho* nitro-substituted triazenes (where NMR evidence indicates preference of one tautomer by intramolecular hydrogen bonding) and the *para* nitro-substituted isomer were also reflected in their relative rates of DNA alkylation (Fig. 1).

The 3-(2-haloethyl)aryltriazenes are generally labile compounds and (in contrast to the corresponding nitrosoureas [27] their rates of decomposition increase with decreasing pH of the medium

Table 1. Relative extents of alkylation of PM2-CCC-DNA determined by loss of ethidium fluorescence and rates of aqueous decomposition of 3-(2-haloethyl)aryltriazenes



Compound	n	R_1	R_2	R_3	% DNA alkylation 37°, pH 7.2, in 15 min*	$T_{\frac{1}{2}}$†
23	1	CH_3CO	H	F	23	38 min 24 sec
16	1	EtOCO	H	F	29	31 min 3 sec
16	1	EtOCO	H	F	19‡	25 min 22 sec‡
14	1	CN	CH_3	Cl	9	24 min 54 sec
15	1	CN	H	F	19	23 min 12 sec
28	3	CN	H	Cl	21	16 min 12 sec
17		(Imidazole)		F	40‡	11 min 44 sec‡
27	2	CN	H	Cl	24	6 min 36 sec
26	2	CH_3CO	H	Cl	33	4 min 24 sec
24	1	EtOCO	H	Cl	48	2 min 44 sec
13	1	CN	H	Cl	30	1 min 44 sec
22	1	CH_3CO	CH	Cl	41	<1 sec

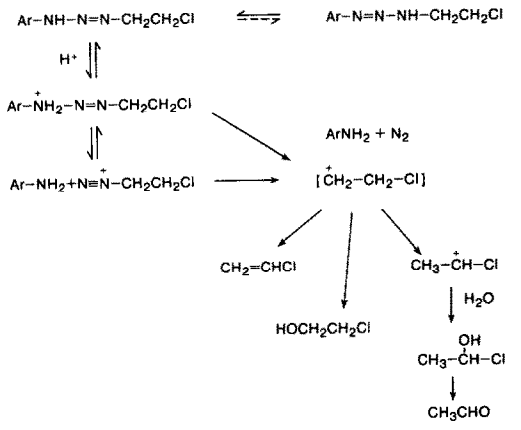
* Represents the percentage loss of ethidium fluorescence as a result of the heating (96°/3 min) and cooling (22°) cycle compared with the control which consisted of untreated PM2-CCC-DNA. The reaction medium contained 1 mM drug in 10% aqueous CH_3CN unless otherwise stated.

† $T_{\frac{1}{2}}$ indicates time for 50% decomposition determined polarographically.

‡ In 10% aqueous dimethylsulfoxide.

(Scheme 2). The concomitant increased rate of generation of electrophiles was reflected in a parallel increase in the rate of DNA alkylation in the pH range 10 to 6 (Table 2).

Scheme 2



DNA single strand breaks induced by 3-(2-haloethyl)aryltriazenes

Production of SSS was observed when a triazene-treated PM2-CCC-DNA was incubated at pH 7, 37° and assayed in pH 11.8 buffer, which quenched further reaction of the triazene with the DNA (Fig. 1). Control experiments with the untreated PM2-CCC-DNA demonstrated that it was stable to the pH 11.8 ethidium medium during the times necessary to perform the assays [21]. Under certain conditions (e.g. in the presence of adventitious traces of metal ions), triazenes can undergo decomposition to give free radicals [11, 28]. However, in the present case the slow strand scission assayed at pH 11.8 was unaffected by prior treatment at pH 7.0 with superoxide dismutase, by catalase, or by free radical scavengers such as isopropyl alcohol or sodium benzoate, in the case of a representative triazene [1-(*p*-carboethoxyphenyl)-3-(2-fluoroethyl)triazene (**16**) (Fig. 1). These observations rule out a free radical mechanism for the DNA strand scission such as that which operates for bleomycin or the anthracyclines in the presence of reducing agents [29, 30].

Origin of single strand breaks

Having excluded a free radical process for the SSS of DNA, it was necessary to consider Type I SSS which would involve primarily alkylation of the DNA phosphate residues with concomitant rapid hydrolysis of the resulting phosphate triesters in the pH 11.8 ethidium assay, resulting in strand breaks made evident by a consequent rise in fluorescence before heat denaturation. No such phenomenon was observed in the case of the 3-(2-haloethyl)-aryltriazenes examined (Fig. 1) although there was evidence for this type of mechanism in the special cases of the 3-(2-hydroxyethyl)aryltriazenes discussed below. In the absence of any evidence for Type I SSS for the 3-(2-haloethyl)aryltriazenes, it then seemed possible that they were giving rise to strand breaks primarily as a result of the Type II SSS mechanism. This consists of base alkylation, followed by depurination or depyrimidination and subsequent hydrolysis of the resulting apurinic site. The postulate of Type II SSS induced by triazenes was confirmed by using the apurinic site-specific enzyme endonuclease VI [22] on the triazene-treated DNA. A prompt and extensive production of SSS was observed (Fig. 2), confirming apurinic sites produced by the 2-haloethyltriazenes. A similar but less specific and more gradual increase in fluorescence resulted from exposure of the triazene-treated relaxed PM2-DNA by alkali at pH 11.8. For a given triazene the final levels of the fluorescence readings by either endonuclease VI or alkali treatment reached the same value (Fig. 2).

Monoalkyltriazenes, in general, alkylate acidic sites on DNA more effectively than basic sites [11]. Our observation that 3-(2-haloethyl)aryltriazenes readily esterify diethylphosphate indicates that the alkylation of the phosphate residues of nucleic acids by these triazenes could be a significant event both *in vivo* and *in vitro*.

The anilines released from the aqueous decomposition of aryltriazenes are without exception those containing electron-withdrawing groups, e.g. *p*-nitroaniline which is without effect on apurinic sites [22]. It appears then that the anilines produced from these 3-(2-haloethyl)aryltriazenes are unlikely to compete with the specific apurinic endonucleases in this type of cleavage.

The generation of a 2-hydroxyethylating species from 3-(2-haloethyl)aryltriazenes was conceivable

Table 2. Effects of pH of the medium on the extent of alkylation of PM2-CCC-DNA determined by loss of ethidium fluorescence and the rate of aqueous decomposition of 3-(2-fluoroethyl)-*p*-phenyltriazene (**15**)

Medium pH	Integrity of PM2-CCC-DNA (relative % ethidium fluorescence, 37%, 15 min)	PM2-CCC-DNA* (% DNA alkylation, 37%, in 15 min)	T_i† (26°)
10	102	0	>300 min 0 sec
8	98	10	168 min 0 sec
7	100	19	125 min 49 sec
6	97	29	52 min 55 sec

* Represents the percentage loss of ethidium fluorescence as a result of the heating (96°/3 min) and cooling (22°) cycle compared with the control which consisted of untreated PM2-CCC-DNA. The reaction contained 1 mM drug in 10% aqueous CH_3CN.

† T_i indicates time for 50% decomposition of the triazene determined polarographically.

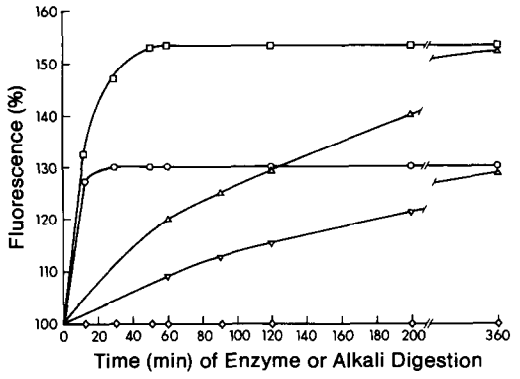


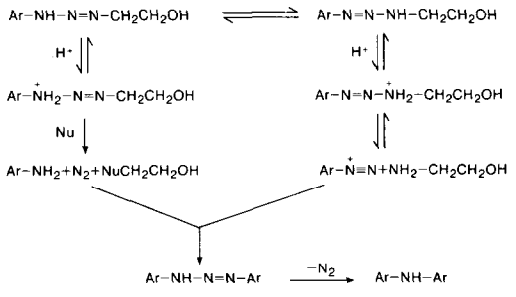
Fig. 2. Confirmation of Type II SSS induced in DNA by 3-(2-haloethyl)aryltriazenes with site-specific repair enzyme and with alkali incubation. Reaction of 1 mM drug with 1.0 A_{260} unit of topoisomerase relaxed PM2-CCC-DNA at pH 7.0, 37°. Open symbols are fluorescence readings before heat denaturation taken after 60 min of incubation with the drug followed by treatment with either endonuclease VI or alkali assay at pH 11.8. The time scale denotes time for nuclease digestion or alkaline treatment at pH 11.8. Key: (□–□) 1-(*p*-cyanophenyl)-3-(2-chloroethyl)triazene (**13**) and incubation with endonuclease V1; (△–△) triazene (**13**) and incubation at 37° at pH 11.8; (○–○) 1-(*p*-carboethoxyphenyl)-3-(2-fluoroethyl)triazene (**16**) and incubation with endonuclease VI; (▽–▽) triazene (**16**) and incubation at 37° at pH 11.8; and (◇–◇) control reaction.

via a previously identified 1,2,3-triazoline intermediate [8]. This was, therefore, examined by synthesis of the aryl 1,2,3-triazoline (**8**) and the corresponding aryl 3-(2-hydroxyethyl)triazene (**2**). The triazoline (**8**) was incubated at 37° and at pH 7 in the presence of sodium chloride for 24 hr, and the products were separated by silica gel chromatography and identified as *p-N*-(2-chloroethyl)aminobenzonitrile and *p-N*-(2-hydroxyethyl)aminobenzonitrile. Since these products are also obtained directly from the corresponding triazene (**13**), this, together with observation of scrambling of isotope labeling in specifically deuterium labeled 3-(2-chloroethyl)aryltriazenes [8], supports the intermediacy of the triazoline in the decomposition, as shown in Scheme 3.

When 1-(*p*-cyanophenyl)-3-(2-hydroxyethyl)triazene (**2**) was allowed to decompose at 37° in the presence of chloride ion, acetaldehyde and 2-chloroethanol (1:2) were identified by GCMS. The third anticipated product, ethylene glycol, was not detectable by GC from aqueous solution (as has been found by other workers [31, 32].

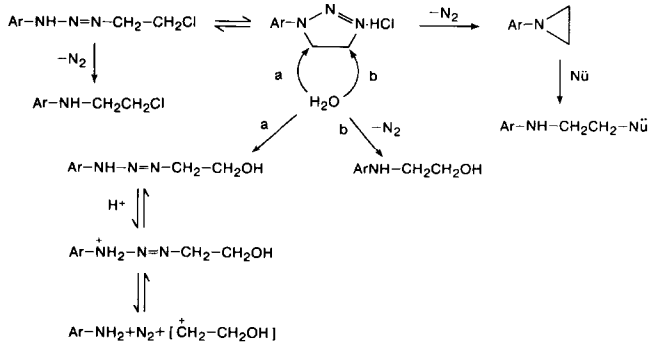
Similar aqueous decomposition at pH 7.2, 37° of 1-(*p*-carboethoxyphenyl)-3-(2-hydroxyethyl)triazine (**4**) in the presence of chloride ion afforded acetaldehyde and 2-chloroethanol in a ratio of 1:2. The involatile products included ethyl *p*-aminobenzoate and the diarylamine. The latter products, accounting for 30% of the involatiles, may arise as shown in Scheme 4.

Scheme 4



The triazoline (**8**) when incubated with PM2-CCC-DNA at 37°, pH 7, induced no Type I SSS over a period of 2 hr but only a very slow alkylation (Fig. 3). In contrast, the (2-hydroxyethyl)triazenes (**2**) and (**4**) caused rapid and extensive Type I SSS when incubated with PM2-CCC-DNA. Under similar conditions and as a control the corresponding 2-methoxyethylaryltriazenes (**3**) and (**5**) gave a much smaller rise in fluorescence before heat denaturation but a proportionately greater loss of fluorescence after heat denaturation. There is redundancy of information in Fig. 3 since, upon nicking a CCC-DNA containing a known amount of OC-DNA (in this case 8%), both the before heat and after heat readings can individually be used to calculate the percentage of additional nicking [21]. This is a useful property of the pH 11.8 assay solution since it effectively provides an internal control, i.e. any discrepancy between the before and after heat values suggests that some other process (e.g. additional

Scheme 3



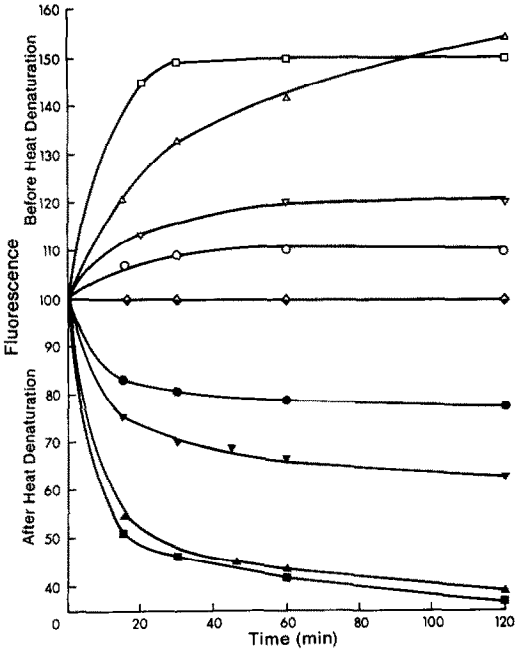


Fig. 3. Type I and Type II SSS induced in DNA by different 3-(2-haloethyl)aryltriazenes. Reaction of 1 mM drug in 10% aqueous acetonitrile with 1.0 A_{260} unit of topoisomerase relaxed PM2-CCC-DNA at pH 7.0, 37°. Open symbols are fluorescence readings before heat denaturation and indicate rapid Type I SSS. Closed symbols are fluorescence readings after heat denaturation at 96°/3 min followed by cooling to 22° and reveal Type II SSS. Key: (□–□) 1-(*p*-cyanophenyl)-3-(2-hydroxyethyl)triazene (**2**); (△–△) 1-(*p*-carboethoxyphenyl)-3-(2-hydroxyethyl)-triazene (**4**); (○–○) 1-(*p*-cyanophenyl)-3-(2-methoxyethyl)-triazene (**3**); (▽–▽) 1-(*p*-carboethoxyphenyl)3-(2-methoxyethyltriazene (**5**); and (◩–◩) triazoline (**8**) or control reaction.

mechanism of nicking Type II or cross-linking) was occurring simultaneously with the initial (Type I) nicking. Topoismerase relaxed PM2-CCC-DNA (100% CCC) should give a 100% increase in fluorescence before heating if all the DNA present is nicked at least once. The corresponding value for the sample used (92% CCC) is expected to be 92%. Compound **2** gave a 50% rise after 30 min corresponding to (50/92) ≡ 54% DNA scission. The corresponding after heat reading at 30 min was virtually the same at 53% signifying that for **2** one mechanism of scission predominates. In contrast, compound **5** showed a 15% fluorescence increase in 30 min, corresponding to (15/92) = 16% single strand scission. The corresponding after heat fluorescence, however, was 30% ≡ (30/92) = 32% SSS. The 17% discrepancy signifies an additional mode of strand scission which is only revealed after heating in the alkali medium, i.e. Type II SSS. A similar argument applies to compound **3**. A plausible interpretation, therefore, is that for **3** and **5** the much smaller extent of Type I SSS permits the detection of the slower Type II SSS. Interstrand cross-linking by the 3-(2-chloroethyl)aryltriazenes, e.g. **16**, **17**, **18** or **25**, would have been revealed by a recovery of the after heat fluorescence readings [33] observed in Fig. 1.

The differences in behavior of **2** and **4** compared with **3** and **5** cannot be attributed to difference in rates of decomposition and thereby of electrophile generation since structurally related pairs of (2-hydroxyethyl)- and (2-methoxyethyl)triazenes decompose at comparable rates as measured polarographically (Table 3). The results indicate that 3-(2-haloethyl)aryltriazenes decompose in part via an intermediate triazoline. The latter, while giving rise to a 2-hydroxyethylaniline by ring opening of the triazoline and loss of nitrogen, did not generate an appreciable concentration of 3-(2-hydroxyethyl)aryltriazene.

Assessment of nucleic acid phosphate group alkylation by 3-(2-haloethyl)aryltriazenes employing RNA

RNA internucleotide linkages are much less stable, and the glycosidic linkages are much more stable, than those of DNA [34]. This property permits the observation of RNA degradation by alkylating agents to be used as a diagnostic test for phosphotriester formation [35, 36]. The average molecular weight of poly A was determined before and after incubation with a 150 mM concentration of the 3-(2-chloroethyl)aryltriazene **13** at 37° at pH 7.0 using sedimentation velocity changes measured on a Beckman analytical ultracentrifuge. A maximum time period of 1 hr was chosen for this particular reaction after consideration of the $T_{\frac{1}{4}}$ value for triazene **13** and for comparison with data obtained in a similar experiment using 2-chloroethylnitrosoureas [22].

	Average molecular weight of Poly A	
Time (min)	Reaction with 150 mM **13**	Control
0	162,000	162,000
60	87,000	162,000

Table 3. Polarographic behavior of selected aryltriazenes

Compound	*p*—R_1—C_6H_4—N=N—NR_2—$CH_2CH_2R_3$ R_1	R_2	R_3	$E_{\frac{1}{4}}$ (V)* vs S.C.E.	$T_{\frac{1}{4}}$*
2	CN	H	OH	−0.925	9 min 36 sec
3	CN	H	OCH_3	−0.945	12 min 36 sec
4	CO_2Et	H	OH	−0.926	21 min 42 sec
5	CO_2Et	H	OCH_3	−0.942	27 min 30 sec
9	CN	H	H	−0.930	6 min 37 sec
10	CN	(CH_2OH)	(H)	−0.922	6 min 39 sec

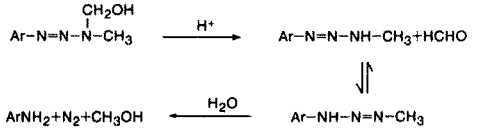
* Determined in 10% aqueous acetonitrile.

The results show an approximately 45% loss in molecular weight over a period of 1 hr. In contrast, the nitrosourea BCNU [1,3-bis(2-chloroethyl)nitrosourea] at 150 mM under similar conditions affords only 15% decrease in the molecular weight of poly A in 60 min [22]. The results suggest that extensive 2-chloroethylation of the phosphate residues of nucleic acids may contribute significantly to the mode of action of 3-(2-chloroethyl)aryltriazenes.

Reactions of a 3-(α-hydroxymethyl)aryltriazene, a plausible triazene metabolite, with DNA

1-Aryl-3,3-dimethyltriazenes have been shown to undergo enzymatic primary metabolism by oxidative demethylation in their antitumor action [3, 37]. To gain insight into possible metabolic pathways the 3 - (hydroxymethyl - 3 - ethyl)*p* - cyanophenyltriazene (**10**) and the 3-ethyl-*p*-cyanophenyltriazene (**9**) were prepared. Compound **10** is a plausible primary oxidative metabolite of (3-ethyl-3-methyl)-*p*-cyanophenyltriazene (**10a**) and **10** in turn, could give rise to **9**. While **10a** would be inactive towards DNA in the absence of enzymatic activation, compounds **9** and **10** generate electrophiles and at comparable rates in accord with general Scheme 5. The half-lives for decomposition in 10% aqueous CH_3CN of **9** and **10** are identical at 6 min 37 sec (Table 3). The volatile products of decomposition of **10** contained formaldehyde and ethanol (82% of volatiles). The involatile products of **10** contained *p*-(*N*-ethylamino)benzo-nitrile. Similar aqueous decomposition of triazene (**9**) gave ethanol (100% of volatile products) and *p*-(*N*-ethyl)aminobenzonitrile and *p*-aminobenzonitrile as the involatile products.

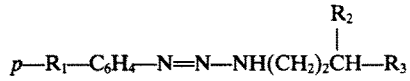
Scheme 5

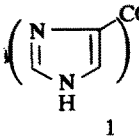


Consideration of DNA interstrand cross-linking by 3-(2-haloethyl)aryltriazenes

By analogy with 2-chloroethylnitrosoureas [21, 38], one might anticipate the detection of DNA interstrand cross-links by the 3-(2-chloroethyl)aryltriazenes (**13**). Although, as has been pointed out above, extensive alkylation of both the bases and the phosphate residues of DNA by the triazenes is apparent, the interstrand cross-links were not detectable. As described in the preceding section, the redundancy of information obtained from the ethidium fluorescence assays would have permitted the detection of interstrand cross-linking if it had competed successfully or had not been masked by the concomitant alkylation and strand scission.

Table 4. Activity of 2-haloethylaryltriazenes against P388 leukemia*



Compound	n	R_1	R_2	R_3	Dose (mg/kg)/day) Q01D × 09	Weight diff. (T–C)	CNTL body change‡	Survivors on day 5	Mean survival time/days Test	Contl.	% T/C§
19	1	NO_2	H	F	25	−1.7	2.1	5/5	13.5	10.5	128
16	1	COOEt	H	F	25	−5.3	1.0	6/6	30.0	11.7	256
23	1	CH_3CO	H	F	12.5	−2.5	2.1	5/5	28.5	10.7	271
22	1	CH_3CO	H	Cl	100	−0.9	2.1	5/5	11.0	10.5	104
14	1	CN	CH_3	Cl	20	−2.5	1.5	5/6	12.3	10.3	119
26	2	CH_3CO	H	Cl	50	−0.9	2.1	5/5	13.3	10.5	125
9	1	CN	H	H	50	−2.4	2.1	3/3	13.0	10.5	123
17	1		H	F	3.13	−2.8	1.7	6/6	19.0	10.6	179
6	1	CH_3CO	H	H	100	−3.7	2.1	5/5	14.5	10.5	138
2	1	CN	H	OH	20	−3.6	2.3	6/6	13.8	10.7	128
3	1	CN	H	OCH_3	25	−1.5	2.3	6/6	11.3	10.7	105
4	1	COOEt	H	OH	20	−5.1	2.3	6/6	12.7	10.7	118
5	1	COOEt	H	OCH_3	100	−5.5	2.3	6/6	14.8	10.7	138
15	1	CN	H	F	25	−5.1	1.0	4/6	16.0	11.7	136
13	1	CN	H	Cl	50	−2.7	2.1	5/5	15.0	10.5	142
27	2	CN	H	Cl	100	−2.3	1.3	5/6	13.7	11.3	121
28	3	CN	H	Cl	50	−6.5	1.0	6/6	12.3	11.7	105
29	4	CN	H	Cl	25	−0.8	1.0	6/6	12.3	11.7	105

* Assays for activity against lymphoid leukemia P388 were performed according to specifications established by the Cancer Chemotherapy National Service Centre [40]. Suspensions of the compounds in saline and Tween 80 were administered intraperitoneally within 5 min of preparation of suspensions. P388 cells (10^5) were implanted intraperitoneally in mice on day 0. The drugs were administered daily from day 1 on a 1- to 9-day schedule.

† Average weight change of treated mice minus average weight change of control mice for a given dose.

‡ Average weight change of control animals in grams (weight day 2 minus weight day 1).

§ The % T/C values were true maxima in multiple dose assays.

A number of factors seem to be involved here. First, under comparable conditons the triazenes decompose much more readily and thus give rise to a greater flux of electrophiles than the comparable nitrosoureas, e.g. $T_{\frac{1}{2}}$ for triazene (**13**) is 1 min 44 sec compared with 79 min for bis(2-chloroethyl)nitrosourea [21]. Second, it is the 2-chloroethylation of the DNA bases by 2-haloethylnitrosoureas which results in cross-links [33, 38]. However, the triazenes owing to their acidic activation [11] show a preferential reactivity towards the phosphate residues of nucleic acids. This is reflected in the more extensive degradation of RNA by triazene (**13**) (50% in 1 hr) compared with that produced by BCNU (16% in 1 hr). Third, the onset of interstrand cross-linking by 2-chloroethylnitrosoureas can normally be observed after about 2 hr of incubation at 37° [33]. During this time period, the extensive amounts of alkylation of the phosphate residues of the nucleic acids (indicated by both the RNA and DNA experiments) leading to strand scission may prevent the detection of any interstrand links that might be formed by the aryltriazene.

In conclusion, the evidence suggests that 3-(2-haloethyl)aryltriazenes generally decompose very rapidly under physiological conditions to generate electrophiles including the 2-chloroethyl cation and, as a minor pathway, the l-aryltriazoline. Because of the unique acid-promoted decomposition of triazenes which may lead to preferential reaction in tumor tissue (which has lower pH than normal tissue [23, 39]), this results in preferential and extensive alkylation of the DNA phosphate residues (although the bases are also alkylated), resulting in single strand scission largely of Type II. In contrast to the base-activated 2-chloroethylnitrosoureas which produce interstrand DNA-base cross-links, the analogous triazenes generate more phosphate alkylation which may prevent the detection of interstrand cross-links. Several of these new types of aryl triazenes, especially the 2-fluoroethyl derivatives (**16**, **17** and **23**), exhibit promising antileukemic properties in animal test systems (Table 4).

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