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COMPARISON OF METABOLISM AND ACTIVITY OF AN ARYLDIMETHYLTRIAZENE AND AN ARYLDIETHYLTRIAZENE

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Abstract—The antitumoral activity and metabolism of 1-(4-acetylphenyl)-3,3-dimethyltriazene [pAc-$(CH_3)_2$] and 1-(4-acetylphenyl)-3,3-diethyltriazene [pAc-$(C_2H_5)_2$] were studied in mice. pAc-$(CH_3)_2$ showed significant antitumoral activity against M5076 ovarian reticular cell sarcoma, L1210 leukemia, EL 4 lymphoma in mice, but not against Lewis lung carcinoma. pAc-$(C_2H_5)_2$ was inactive in all these murine tumors and was much more toxic than pAc-$(CH_3)_2$. pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ were rapidly metabolized *in vitro* and *in vivo* to their respective monoalkyltriazenes and to 4-aminoacetophenone (pAc-NH_2). *In vitro*, 79% of the dimethyltriazene was metabolized to its monomethyl analogue, but only 27% of the diethyltriazene was metabolized to the monoethyltriazene. The monoalkytriazenes were almost completely biotransformed to pAc-NH_2 by a 9000 *g* liver fraction. The metabolic pattern in the *in vitro* study was comparable to that found *in vivo*.

The mechanism of the antineoplastic activity of 1-aryl-3,3-dimethyltriazenes (**I**, Fig. 1) is not well understood. They require metabolic activation in the liver [1, 2] through a pathway involving the formation of 1-aryl-3-methyltriazenes (II, Fig. 1), the cytotoxic species first proposed in 1969 [3]. Recently monomethyltriazene was positively identified by HPLC as a metabolite of a dimethyltriazene, 1-(4-acetylphenyl)-3,3-dimethyltriazene [4]. Though some doubts have been cast on the contention that monomethyltriazene (**II**) is responsible for the antitumoral activity of the dimethyltriazene [1, 5–8], the search for others, selectively cytotoxic metabolites of aryl-dimethyltriazenes has so far been unsuccessful.

Structure/activity studies of 1-aryl-3,3-dialkyltriazenes have confirmed that at least one *N*-methyl group is necessary for antitumoral activity and that substitutions in the phenyltriazene rings have no effect on activity against the TLX5 lymphoma [5]. Although 1-aryl-3,3-diethyltriazenes were readily dealkylated, they had no activity in the same model which is particularly sensitive to dimethyltriazenes [5]. However, L1210 leukemia responds to treatment with aryl-3,3-diethyltriazenes [9] and in Sarcoma 180 ascitic tumor a striking correlation was found between antitumoral activity and hydrolysis of aryltriazenes [10]. Interpretation of these conflicting results has been further complicated by the fact that antitumoral activity was assessed in different tumor models and 1-aryl-3,3-dialkyltriazenes with different substitutions were used [5, 9, 10].

In the present study the antitumoral effectiveness and toxicity of an aryldimethyltriazene [1-(4-acetylphenyl)-3,3-dimethyltriazene] and a diethyltriazene [1-(4-acetylphenyl)-3,3-diethyltriazene] were compared to establish whether the *N*-methyl moiety is necessary for activity on murine tumors. To test the hypothesis that there are pharmacokinetic or metabolic explanations for differences in activity between the methyl and ethyl analogues, a comparison was made of the *in vitro* and *in vivo* metabolism of the 1-(4-acetylphenyl)-3,3-dialkyltriazenes to the cytotoxic monoalkyltriazenes and to 4-aminoacetophenone.

MATERIALS AND METHODS

Animals and tumors. C57B male or female mice and CD2F1 male mice (20 ± 2 g body weight) from Charles River, Italy, were used. The tumor systems used were: (a) M5076/73A ovarian reticular cell sarcoma (M5) supplied by Mason Research Institute, DTC-Animal and Human Tumor Bank, Worchester, MA), implanted i.m. in C57B female mice (5×10^5 cells/mouse), which metastasizes to the liver, uterus and ovary; (b) L1210 leukemia transplanted i.p. in CD2F1 male mice (10^5 cells/mouse); (c) thymus-derived T-cell lymphoma EL-4 transplanted i.p. in C57B female mice (10^6 cells/mouse); and (d) Lewis lung carcinoma (3LL) implanted i.m. in C57B male mice (10^5 cells/mouse).

Drugs. The various triazenes [pAc-$(CH_3)_2$,pAc-CH_3,pAc-$(C_2H_5)_2$, pAc-C_2H_5];§ were synthesised by published methods [11], pAc-NH_2 was purchased

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§ Abbreviation used: pAc-$(CH_3)_2$, 1-(4-acetylphenyl)-3,3-dimethyltriazene; pAc-CH_3, 1-(4-acetylphenyl)-3-methyltriazene; pAc-$(C_2H_5)_2$, 1-(4-acetylphenyl)-3,3-diethyltriazene; pAc-C_2H_5, 1-(4-acetylphenyl)-3-ethyltriazene; pAc-NH_2, 4-aminoacetophenone; AUC, area under the plasma concentration vs time curve.

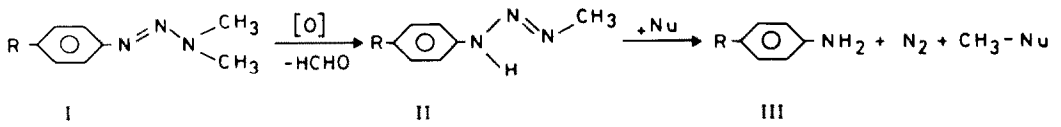

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Fig. 1. Metabolism of aryldimethyltriazenes; Nu,nucleophile.

from BDH Chemicals Ltd., and used as standard. Stock solutions were prepared in methanol every second week and stored at −20°. For pharmacokinetic studies and for investigating antitumoral activity, triazenes were dissolved in Tween 80, dimethylsulphoxide and saline (5:5:90).

Treatment and antitumoral activity. In the M5 system, dialkytriazenes were given i.p. (20 mg/kg) for 8 consecutive days, and (40 mg/kg) for 4 consecutive days starting on day 6 after tumor implant. On days 23 (20 mg/kg) and 27 (40 mg/kg) after tumor transplantation, corresponding to the median survival time of controls, autopsies were carried out; weights of tumor, liver and ovaries were recorded. Other groups of animals were used to establish the survival time as described by Geran *et al.* [12]. Each initial group consisted of 10 animals.

In the L1210 system, pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ (20 mg/kg); pAc-CH_3 and pAc-C_2H_5 (20 or 5 mg/kg) were given i.p. for 6 consecutive days starting on day 1 after tumor implant (7 mice per group). Mean and median survival time were calculated. In the EL-4 system, pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ (20 mg/kg); pAc-CH_3 and pAc-C_2H_5 (5 mg/kg) were given i.p. for 6 consecutive days starting on day 1 after tumor implant (10 animals per group). Mean and median survival time were calculated.

In the 3LL tumor system, the triazenes pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ were given i.p. (20 mg/kg) for 9 consecutive days, starting on day 6 after tumor implant. On day 24 after tumor transplant, corresponding to the median survival time of controls, autopsies were carried out and weights of tumor and lung metastases were recorded. Each initial group consisted of 10 animals.

For all tumor systems, deaths among treated mice occurring before the death of the first control mouse were considered toxic deaths and were not included in the evaluation. In some cases, when treatments were unexpectedly toxic they were stopped before the end of the proposed treatment schedule (as is explained in the legends of Table 1 and 3). Control animals received the vehicle only.

Haematological toxicity. Animals were treated for 8 days with pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ at the dose of 20 mg/kg and 4 days at the dose of 40 mg/kg. On the second day after completion of treatments ocular blood was taken from individual animals (5 mice per group) for white blood cell (WBC) counts; 20 μl blood were diluted with 0.38 ml Türker solution and cells were counted in a Bürker hemocytometer. For white blood cell counts (WBC), C57B female mice bearing M5 tumor were used and results were expressed as WBC/μl of blood (mean ± S.E. of 5 determinations).

In vitro *metabolism.* Mice were killed and their livers were immediately removed, weighed and homogenized in ice-cold 0.15 M KCl buffer solution (pH 7.4) in a ratio of 1:5 (w/v). The homogenates were centrifuged at 9000 *g* for 20 min. Incubations were started by adding 40 μg of the triazenes dissolved in 0.1 ml acetone to the supernatant fraction (1 ml) with NADPH (2 mg) as cofactor dissolved in 0.8 ml 0.15 M KCl buffer solution. Incubation were made at 37° in a shaking water bath in the presence of atmospheric oxygen.

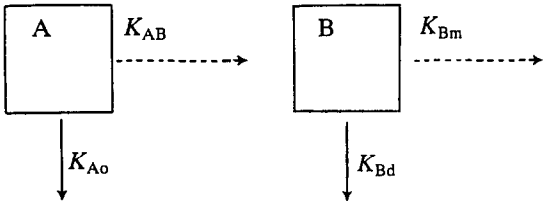
The triazene concentrations were 104.7 and 91.3 μM for pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ and 113 and 104.7 μM for pAc-$(CH_3)_2$ and pAc-(C_2H_5). Protein content was determined in 9000 *g* liver fractions by the method of Lowry *et al.* [13] using bovine serum albumin as the standard. In an analogous experiment using boiled 9000 *g* liver fraction, triazene chemical degradation was investigated. All experiments were run in triplicate.

At different intervals (t = 0, 5, 10, 20, 40 and 80 min), 1 ml aliquots of the incubation mixtures were deproteinized by addition of 1 ml ice-cold acetone. Samples were centrifuged at 600 *g* for 3 min then injected into the HPLC column.

In vivo *metabolism.* At 15, 30 and 60 minutes after single doses of 40 mg/kg of pAc-$(CH_3)_2$ or pAc-$(C_2H_5)_2$ four animals for each time were killed and blood was collected in heparinized containers. Plasma samples (0.1 ml) was added to the same volume of ice-cold acetone and centrifuged for 3 min at 600 *g*. Portions of the supernatant solution were injected into the HPLC column. The AUC values were calculated by the trapezoidal method.

Drug assay. Triazenes and pAc-NH_2 were chromatographed by HPLC separation on a Waters instrument equipped with a model 440 adsorbance detector. Separation of pAc-$(CH_3)_2$, pAc-CH_3 and pAc-NH_2 was achieved with an isocratic solvent system of 35% aqueous acetonitrile containing diethylamine (0.05%). The limits of detection were 0.2 μg/ml for the triazenes and 0.3 μg/ml for pAc-NH_2. This HPLC method was described in detail elsewhere [4]. Separation of pAc-$(C_2H_5)_2$, pAc-C_2H_5 and pAc-NH_2 was achieved with linear gradient solvent system of aqueous acetonitrile containing diethylamine (0.01%). The mobile phase initially contained 25% of acetonitrile and the final condition (60% of acetonitrile) was reached in 20 min. The limit of detection was 0.3 μg/ml for pAc-$(C_2H_5)_2$, pAc-C_2H_5 and pAc-NH_2. Both triazenes were separated at the mobile-phase flow-rate of 1.5 ml/min using a LiChrosorb RP 18 10 μm column (Merck, Darmstadt F.R.G.). Samples were detected at 340 nm.

Mathematical analysis. To describe the *in vitro* kinetics of pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ the following linear model was used:



where A = pAc-$(CH_3)_2$ or pAc-$(C_2H_5)_2$; B = pAc-CH_3 or pAc-C_2H_5; A(t) and B(t) are the amount of parent drugs and N-dealkylated metabolites present at time t;
K_{AB} and K_{Ao} are the first order rate constants of A metabolization to B or to unknown compounds respectively:
K_{Bd} and K_{Bm} are the first order rate constants for degradation and metabolization of B respectively.

The solutions of the appropriate differential equation systems are:

$$A(t) = Ao.e^{-\alpha t} \quad (1)$$

$$B(t) = \frac{\alpha F Ao}{\alpha - \beta}(e^{-\beta t} - e^{-\alpha t}) \quad (2)$$

when A is incubated, and:

$$B(t) = Bo.e^{-\beta t}\,(a) \text{ or } B(t) = Bo.e^{-K_{Bd}.t}\,(b) \quad (3)$$

when B is incubated with or without cofactor respectively. Ao and Bo are amounts of triazenes and N-dealkyltriazenes present at time t = 0,

$$\alpha = K_{Ao} + K_{AB}$$

$$\beta = K_{Bd} + K_{Bm} \quad \text{and} \quad F = \frac{K_{AB}}{K_{AB} + K_{Ao}}$$

is the fraction of A converted to B. Experimental data obtained after incubation with cofactors were fitted simultaneously to equations (1), (2) and (3a) by a non-linear fitting program running on a microcomputer [14] minimizing the sum of squared errors; experimental points of the degradation of B were subsequently fitted to Eqn 3b using the same procedure.

Table 2. White blood cell counts after pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ treatments

	Drug dose (mg/kg) 20	40
	WBC/μl (mean ± S.E.)	
Controls	6,193 ± 592	7,160 ± 440
pAc-$(CH_3)_2$	6,238 ± 498	2,966 ± 366
pAc-$(C_2H_5)_2$	2,507 ± 416*	1,133 ± 400*†

Cells were counted the second day after completion of 8 days' treatment (20 mg/kg) and after 4 days' treatment (40 mg/kg). Groups of 5 animals were used.
* $P < 0.01$ against respective controls (Duncan's test).
† $P < 0.05$ against pAc-$(CH_3)_2$ 2 group (Duncan's test).

RESULTS

Antitumoral activity and toxicity

The antitumoral activity of pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ was comparatively evaluated in 4 rodent transplantable tumors. Table 1 shows the effects of repeated doses of 20 mg and 40 mg/kg of pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ on mice bearing the M5 ovarian reticular cell sarcoma. pAc-$(CH_3)_2$ had significant antitumoral activity: at 20 mg/kg it increased survival time by 52% (T/C = 152) and slightly inhibited primary tumor growth (by 33%). At both doses animals appeared to be free of metastases in the liver and ovaries. At the larger dose, 18% of the mice died of drug toxicity. pAc-$(C_2H_5)_2$ did not show any significant antineoplastic activity. The 20 mg/kg dose of the diethyltriazene caused the death of 11% of mice and 40 mg/kg killed all the animals. Table 2 shows that the diethyltriazene was significantly more leucopenic than the dimethyl compound on M5 bearing mice. As can be seen in Table 3, pAc-$(CH_3)_2$ was clearly more effective on EL-4 lymphoma than pAc-$(C_2H_5)_2$ and the same tendency was observed against

Table 1. Activity of 1-(4-acetylphenyl)-3,3-dimethyltriazene and 1-(4-acetylphenyl)-3,3-diethyltriazene against M5076/73A ovarian reticular cell sarcoma in mice

Drug	Treatment schedule* (mg/kg × days)	Tumor weight† (g ± S.E.)	Occurrence of metastases† (% of all animals) Liver	Ovaries	Toxic deaths (%)	T/C‡ (%)
Controls	—	4.91 ± 0.10	100	10	0	—
pAc-$(CH_3)_2$	20 × 8	3.92 ± 0.15§	0	0	0	152
pAc-$(C_2H_5)_2$	20 × 8	4.09 ± 0.31	90	0	11	122
Controls	—	6.36 ± 0.42	100	40	0	—
pAc-$(CH_3)_2$	40 × 4	4.27 ± 0.15‖	0	0	18	130
pAc-$(C_2H_5)_2$	40 × 3**	n.d.	n.d.	n.d.	100	33

* Treatment was given daily starting on day 6 after tumor implant;** a shorter treatment schedule was used when there was evidence of severe toxicity.
† On days 23 (20 mg/kg) and 27 (40 mg/kg) after tumor transplantation, corresponding to the median survival time of controls, autopsies were carried out. Each initial group consisted of 10 animals.
‡ Median survival time of treated mice/median survival time of untreated controls × 100. Groups of 10 animals were used to establish the survival time.
§ $P < 0.05$; ‖ $P < 0.01$ against respective controls (Duncan's test).
n.d. = not determinable.

Table 3. Activity of 1-(4-acetylphenyl)-3,3-dimethyltriazene,1-(4-acetylphenyl)-3,3-diethyltriazene and their *N*-dealkylate metabolites against L1210 leukemia and EL-4 lymphoma of the mouse

Drug	Treatment schedule* (mg/kg × days)	L1210 S† (days ± S.E.)	L1210 T/C‡ (%)	EL-4 S† (days ± S.E.)	EL-4 T/C‡ (%)
Controls	—	8.3 ± 0.2	—	12.6 ± 0.3	—
pAc-$(CH_3)_2$	20 × 6	10.0 ± 0.2§	125	18.2 ± 0.6‖¶	150
	5 × 6	9.4 ± 0.2	113	15.8 ± 0.8‖	125
pAc-CH_3	20 × 6	10.2 ± 0.3§	131		
pAc-$(C_2H_5)_2$	20 × 5**	7.6 ± 0.5	87		
	20 × 4**			6.7 ± 0.1	58
pAc-C_2H_5	5 × 6	8.4 ± 0.5	113	12.7 ± 1.0	117
	20 × 6	8.7 ± 0.3	106		

* Treatments were given daily for 6 consecutive days, starting on day 1 after tumor implant;** a shorter treatment schedule was used when there was evidence of severe toxicity. Groups consisted of 7 animals (L1210) and 10 animals (EL-4).

† Mean survival time (days ± S.E.).

‡ Median survival time of treated mice/median survival time of untreated controls × 100.

§ $P < 0.05$; ‖ $P < 0.01$ against respective controls (Duncan's test).

¶ $P < 0.05$ against pAc-CH_3 group (Duncan's test).

L1210 leukemia in which pAc-$(CH_3)_2$ had marginal activity. pAc-CH_3, but not pAc-$(C_2H_5)_2$ showed some activity against EL-4 lymphoma and L1210 leukemia (see Table 3). Neither pAc-$(CH_3)_2$ nor pAc-$(C_2H_5)_2$ (20 mg/kg, days 1–9) had any antitumoral or antimetastatic activity against Lewis lung carcinoma of the mouse (3LL) (data not shown). As in the other murine tumors investigated in this study, also in 3LL bearing mice pAc-$(C_2H_5)_2$ was much more toxic than pAc-$(CH_3)_2$ (70% vs 10% toxic deaths).

In vitro *metabolism*

Figure 2 illustrates the metabolism of 104.7 μM pAc-$(CH_3)_2$ (2A) and 91.3 μM pAc-$(C_2H_5)_2$ (2B) on incubation with 9000 *g* liver fraction. Both substrates disappeared rapidly and the monoalkyltriazenes and pAc-NH_2 were detected as metabolites (or degradation products). The areas under the curve (AUC) obtained by plotting the drug concentration in the incubation medium against time were 756 nmoles/ml min for the pAc-$(CH_3)_2$ and only 82 nmoles/ml min for the pAc-$(C_2H_5)_2$. The monoalkyltriazenes appeared rapidly in the incubation medium, and from the peak concentrations disappeared at rates which seemed to follow first-order kinetics. For the pAc-CH_3 a disappearance rate constant (β) of $0.046 \pm 0.007\ min^{-1}$ was calculated and for the pAc-C_2H_5 $0.096 \pm 0.013\ min^{-1}$ (mean of three fittings ± S.E.). Altogether 79% ± 2 of the initial substrate concentration of the pAc-$(CH_3)_2$ was metabolized to the pAc-CH_3, against only 27% ± 1

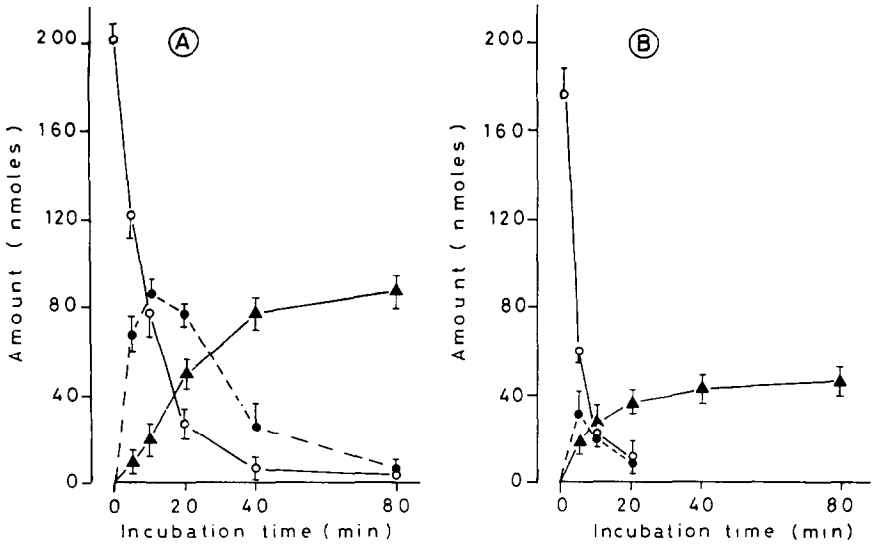


Fig. 2. Metabolism of pAc-$(CH_3)_2$ (A) and pAc-$(C_2H_5)_2$ (B) (○) by 9000 *g* mouse liver fraction. The metabolites formed are pAc-CH_3, pAc-C_2H_5 (●) and pAc-NH_2 (▲). The initial substrate concentrations were 104.7 μM pAc-$(CH_3)_2$ and 91.3 μM pAc-$(C_2H_5)_2$. Values are the means ± S.E. of three experiments and are expressed as amount (nmoles) of parent drugs and metabolites formed in 2 ml of homogenate mixture. Amounts missing at 40 or 80 min were lower than the limit of the sensitivity of the assay.

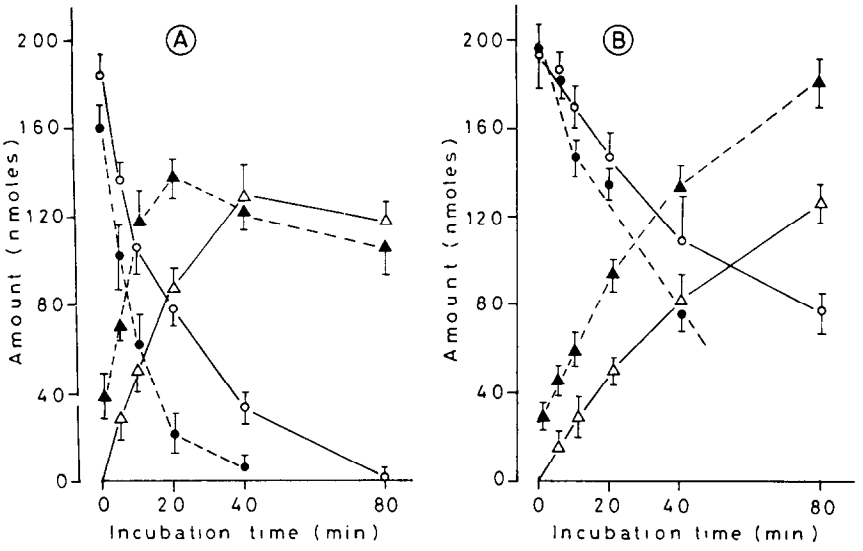


Fig. 3. Metabolism (A) and chemical degradation (B) of pAc-CH_3 (○) and pAc-C_2H_5 (●) by 9000 *g* mouse liver fraction (A) and 9000 *g* homogenate inactivated by heating (B). The amount of pAc-NH_2 formed from the pAc-CH_3 is marked by △; the amount of pAc-NH_2 formed from the pAc-C_2H_5 by ▲. Initial substrate concentrations were 113 μM pAc-CH_3 and 104.7 μM pAc-C_2H_5. Values are the means ± S.E. of three experiments and are expressed as amount (nmoles) of parent drugs and metabolites formed in 2 ml of homogenate mixture. Amounts missing at 80 min were lower than the limit of sensitivity of the assay.

of the pAc-$(C_2H_5)_2$ to the pAc-C_2H_5. Figure 3 shows the biotransformation (due to metabolism plus chemical-degradation) (A) and chemical degradation (B) of both monoalkyltriazenes under the same experimental conditions reported for the parent compounds. The chemical degradation of the monoalkylderivatives appeared to be described by first order kinetics with a rate constant (K_{Bd}) of $0.013 \pm 0.002\ min^{-1}$ for the monomethyl- and $0.023 \pm 0.001\ min^{-1}$ for the monoethyltriazene (mean of three fittings ± S.E.). Both monoalkyltriazenes appeared to be converted almost completely to pAc-NH_2. Under these conditions the dialkyltriazenes were stable.

In vivo *metabolism*

Mice were injected with 40 mg/kg of either pAc-$(CH_3)_2$ or pAc-$(C_2H_5)_2$ and plasma levels of drug and metabolites were measured. The AUC obtained by plotting the concentration of the monoalkyltriazenes against time (up to 1 hr) after administration of the dialkyltriazenes were 550 nmoles/ml min for pAc-CH_3 and only 49 nmoles/ml min for pAc-C_2H_5; the AUC of pAc-NH_2 formed from pAc-CH_3 were 432 nmoles/ml min and 274 nmoles/ml min from pAc-C_2H_5 (Table 4). The AUC values after the parent compound were 440 nmoles/ml min for pAc-$(CH_3)_2$ and 169.5 nmoles/ml min for pAc-$(C_2H_5)_2$ (Table 4).

Table 4. Plasma levels (mean of four values ± S.E.) of pAc-$(CH_3)_2$ and metabolites pAc-CH_3 and pAc-NH_2 and of pAc-$(C_2H_5)_2$ and metabolites pAc-C_2H_5 and pAc-NH_2 in mice treated with the two drugs at the same dose (40 mg/kg i.p.)

Drug	Plasma concentration (nmoles/ml) Time after treatment (min) 15	30	60	AUC (nmoles/ml × min)
pAc-$(CH_3)_2$	13.1 ± 1.5	9.8 ± 1.5	1.8 ± 0.3	440.0
pAc-CH_3	14.8 ± 1.7	13.6 ± 3.4	1.5 ± 0.4	550.5
pAc-NH_2	8.7 ± 1.0	9.7 ± 1.7	5.6 ± 0.5	432.7
pAc-$(C_2H_5)_2$	5.9 ± 1.4	2.6 ± 0.1	1.5 ± 0.3	169.5
pAc-C_2H_5	2.9 ± 0.8	0.7 ± 0.1	n.d.	48.8
pAc-NH_2	10.4 ± 1.6	4.6 ± 0.4	1.0 ± 0.1	274.5

n.d. = not detectable.

DISCUSSION

pAc-$(CH_3)_2$ shows antineoplastic activity against three murine transplantable tumors, M5 reticular cell sarcoma, EL-4 lymphoma and L1210 leukemia, whereas pAc-$(C_2H_5)_2$ was inactive. This finding is in agreement with previous reports by Audette *et al.* [5] and by Connors *et al.* [1] that 1-(*o*-carboxamidophenyl)-3,3-dimethyltriazene and 1-(*p*-carboxamidophenyl)-3,3-dimethyltriazene have anticancer effect against TLX5 lymphoma whereas their diethyl analogues were inactive.

In all experiments we found that pAc-$(C_2H_5)_2$ was much more toxic than pAc-$(CH_3)_2$. This was observed in C57Bl mice transplanted with M5 or 3LL or EL-4 and in CD2F1 mice transplanted with L1210. Even though pAc-$(C_2H_5)_2$ did not inhibit the growth of the tumors investigated, it was cytotoxic against white blood cell precursors; in M5 bearing mice it caused more severe leucopenia than that observed after pAc-$(CH_3)_2$. The much greater antitumor selectivity of pAc-$(CH_3)_2$ compared to pAc-$(C_2H_5)_2$ stimulated us to undertake detailed comparative studies of these two compounds, aimed at elucidating whether differences in the metabolism and pharmacokinetics of pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ explained their different biological properties. These two compounds were chosen as models of dimethylphenyltriazenes and diethylphenyltriazenes. Previous comparative studies on the metabolism of dimethylphenyltriazenes and diethylphenyltriazenes were in fact inadequate since formaldehyde and acetaldehyde had been determined as a measure of *N*-dealkylation of dimethylphenyl- and diethylphenyltriazenes respectively [1, 3, 5]. Apart from the variability shown in these studies [1, 3, 5] the specificity of the methods is questionable. Since this measurement does not distinguish the *N*-dealkylation of the substrate from that of other metabolites [15].

Therefore the present study represents the first attempt at a quantitative comparative metabolic investigation of a dimethylphenyl- and a diethylphenyltriazene in which the parent compounds and their metabolites are determined by specific analytical methods.

We found that only 27% of pAc-$(C_2H_5)_2$ was converted to its monoalkylderivative as compared to 79% for pAc-$(CH_3)_2$. The finding that pAc-$(C_2H_5)_2$ was transformed, in small amount, to its monoethyltriazene together with the observation of its rapid disappearance in 9000 *g* mixture raises the hypothesis that pAc-$(C_2H_5)_2$ might follow a metabolic pathway different from *N*-dealkylation. In fact after 5 min (see Fig. 2B) almost all pAc-$(C_2H_5)_2$ had disappeared, but the amounts of pAc-C_2H_5 and pAc-NH_2 were relatively low.

It is worth noting that monoethyltriazene is less stable than the monomethyltriazene, their chemical half-lives being about 30 and 53 min respectively. Both appear to be converted almost completely to pAc-NH_2.

This conversion appears accelerated in the presence of 9000 *g* liver fraction, the half-lives becoming 7 min for pAc-C_2H_5 and 15 min for pAc-CH_3.

In contrast to a previous hypothesis [16], aryldiazonium ions do not appear to be formed by spontaneous hydrolysis of these triazenes. In fact we found that pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ were stable, at least for the duration of the experiment (3 hr). Almost all the pAc-CH_3 or pAc-C_2H_5 was converted to pAc-NH_2, which presumably is not a reductive product of the *p*-acetylphenyldiazonium ion as it has been shown that the phenyldiazonium ion is not reduced to aniline [3]. Therefore diazonium salts cannot be considered responsible for the toxicity of these compounds, particularly for the much greater toxicity of pAc-$(C_2H_5)_2$ versus pAc-$(CH_3)_2$. The marked differences in the metabolism of pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ observed *in vitro* were confirmed *in vivo*, in mice treated with these two drugs. Though our *in vivo* studies do not permit any pharmacokinetic analysis, as the sampling intervals were limited to three points, they clearly indicated that *in vivo* pAc-C_2H_5 is formed after pAc-$(C_2H_5)_2$ treatment much less than pAc-CH_3 after pAc-$(CH_3)_2$. We therefore suggest that the different pharmacological activities of pAc-$(CH_3)_2$ and pAc-$(C_2H_5)_2$ could, at least in part, be due to different metabolism. The activity of dialkyltriazene is thought to be mediated by *N*-dealkylation with the formation of monoalkyltriazene that contributes to alkylating DNA or RNA bases [17–19]. The capacity of monomethyl or monoethyltriazenes to form adducts with guanine has been known since 1971 [20].

Therefore we could hypothesize that the lower antitumoral activity of pAc-$(C_2H_5)_2$ could be partly due to the fact that there is less of the monoethyl derivative than the monomethyl derivative from pAc-$(CH_3)_2$. What seems interesting is that the better antitumoral activity of pAc-$(CH_3)_2$ is accompanied not by higher toxicity but by much less, suggesting that the toxicity is not mainly due to the monoalkyl derivative (which was always considered responsible for the antitumoral activity and toxicity), but presumably to other still unidentified metabolites.

In this respect we thought that pAc-$(C_2H_5)_2$ formed 1-[4-(1-hydroxyethyl)-phenyl]-3,3-diethyltriazene on the basis of our recent finding that pAc-$(CH_3)_2$ could be biotransformed to 1-[4-(1-hydroxyethyl)phenyl]-3,3-dimethyltriazene [15] but this was not the case.

Further investigations are therefore needed in order to elucidate the full fate of pAc-$(C_2H_5)_2$. Studies on the mechanism by which pAc-$(CH_3)_2$, pAc-$(C_2H_5)_2$ and their metabolites act are warranted, with the aim of dissociating antitumoral activity and toxicity.

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