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THE METABOLISM OF THE ANTI-TUMOUR AGENT 5-(1-AZIRIDINYL)-2,4-DINITROBENZAMIDE (CB 1954)

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Abstract—The metabolism in the rat of 5-(1-aziridinyl)-2,4-dinitrobenzamide (CB 1954), an antitumour agent having a highly specific action on the Walker 256 carcinoma in rats, has been studied. Unchanged CB 1954 is the most abundant radioactive constitutent in the urine of rats treated with the tritium-labelled drug. The principal urinary metabolite, 4-amino-5-(1-aziridinyl)-2-nitrobenzamide, was also tumour inhibitory, though less so than CB 1954. The isomeric 2-amino-4-nitro derivative, which was not a urinary metabolite was much less active. Other urinary metabolites identified were 5-amino-2,4-dinitrobenzamide and 5-(2-hydroxyethyl)amino-2,4-dinitrobenzamide.

The results of this metabolic study are considered in relation to possible explanations for the specificity of CB 1954 towards the Walker tumour, and are compared with the earlier findings for the parent member of the series, 1-(1-aziridinyl)-2,4-dinitrobenzene (CB 1837).

5-(1-Aziridinyl)-2,4-dinitrobenzamide (CB 1954, Ia) was the most active member among a series of nitrophenylaziridines tested against the Walker 256 carcinoma in rats [1]. CB 1954 has a very narrow spectrum of antitumour activity, being virtually without effect on a wide range of other experimental tumours in the mouse and the rat [2] but having the highest therapeutic index* of any compound so far tested against the Walker tumour in this Institute.

As part of an investigation into the mode of action of the nitrophenylaziridines we report on the urinary metabolites of CB 1954 in the rat. This work is complementary to an earlier study [3] of the metabolism of the parent member of the series, 1-(1-aziridinyl)-2,4-dinitrobenzene (CB 1837, Ib).

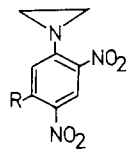
METHODS AND RESULTS

Details of animals used and of the Walker tumour assays were those reported previously in the related study of CB 1837 (Ib) and its metabolites [3], as was the method for determining the structures of metabolites by mass spectrometry, except that here the ion-source temperature was *ca* 200°.

Metabolism and synthesis.

Silicic acid for thin-layer chromatography (t.l.c.) on glass plates (20 × 20-cm) was Merck Kieselgel GF_{254}. Rotary evaporations were performed at 30° and 10 mm Hg and melting points (Kofler block) are corrected.

(i) *Identification and quantitative determination of CB 1954 and its metabolites in rat urine.* Four rats (each weighing *ca* 200 g) were injected i.p. with a solution in dimethyl sulphoxide of ^{3}H-labelled CB 1954 (5 mg/kg, sp. act. 1.9 mCi/m-mole, for preparation see below) and the urine (41 ml) was collected frozen for 24 hr. Recovery of radioactivity was 53 per cent of that administered. The urine was diluted with water to 50 ml and one-half was applied to a column



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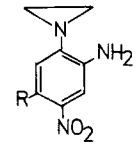

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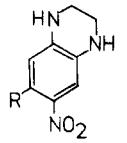
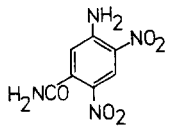


III

I-IIIa, R = $CONH_2$
I-IIIb, R = H



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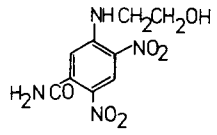

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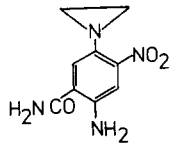

VI

* Therapeutic index, TI = $LD_{50}/ID_{90} \cdot LD_{50}$ = dose lethal to 50 per cent of animals in group, ID_{90} = dose producing 90 per cent tumour inhibition.

(13 × 2-cm) of Amberlite XAD-2 resin which was washed with water (100 ml) then with methanol (100 ml). Recovery of the applied radioactivity from the column was 96 per cent of which 29 per cent was in the aqueous eluate and 71 per cent in the methanol eluate. One-tenth of the methanol eluate was reserved for subsequent quantitation of the components. The remainder was subjected to t.l.c. using benzene–ethanol (4:1) as irrigant (three developments). The components were visualised as yellow bands or as dark bands under u.v. radiation (Hanovia chromatolite) and also by scanning for radioactivity using a Berthold radiochromatogram scanner. CB 1954 and three mobile products (Table 1) were recovered by elution of the silicic acid in the appropriate regions with methanol. Mass spectrometry afforded evidence for their structures, and these were confirmed as IIa, IV and V by further t.l.c. alongside authentic samples. In the solvent mixture benzene–ethanol (4:1) compound IV had a mobility identical with that of the aminonitro-derivative (VI) which was isomeric with the identified metabolite IIa. However, after further t.l.c. of the metabolite having the mass spectrum and mobility of IV, using the solvent mixture benzene–ethanol, (19:1) in which IV (R_f 0.12) and VI (R_f 0.15) are separable, all of the radioactivity was at the R_f value corresponding to IV. Compound IV was also identical in benzene:ethanol (4:1) with the quinoxaline (IIIa) prepared by acid-catalyzed cyclization of IIa, but further t.l.c. of the metabolite, this time in chloroform–methanol (9:1) which separated IV (R_f 0.25) from IIIa (R_f 0.32) demonstrated the absence of IIIa among the metabolites.

The remaining one-tenth of the methanol eluate from the XAD-2 column was applied as a 5-cm wide strip to a t.l.c. plate and developed (twice) in benzene–ethanol (4:1) alongside the appropriate synthetic marker compounds (Ia, IIa, IV and V). After development, the appropriate region of the plate was divided into 0.5-cm wide strips and the silicic acid in these areas was removed into counting vials containing 0.1 N hydrochloric acid (0.5 ml), diluted with scintillation fluid (naphthalene, POP and POPOP in ethanol, dioxan and toluene) (10 ml) and assayed for radioactivity using a Packard Tricarb 3375 scintillation counter. Results were corrected for counts present in silicic acid removed from parallel "blank" areas of the plate. Eighty-five per cent of the radioactivity occurred in regions corresponding with the synthetic marker compounds (Table 1), and 6% at the origin (presumably polar metabolites).

(ii) *Synthesis.* ^{3}H-labelled CB 1954 was prepared using the method for unlabelled CB 1954 [1] from ethyleneimine and 5-chloro-2,4-dinitrobenzamide (0.67 g) which had been catalytically tritiated in the aromatic nucleus (Procedure TR 1, Radiochemical Centre, Amersham, Bucks). Column chromatography (Kieselgel G, 20 × 2-cm column) of the product with ethyl acetate as eluant afforded material of 99 per cent radiochemical purity as judged by t.l.c. (ethyl acetate, R_f 0.30) followed by radiochromatogram scanning.

4-Amino-5-(1-aziridinyl)-2-nitrobenzamide (IIa): CB 1954 (Ia, 5g) was added to a solution of sodium sulphide ($9H_2O$, 20 g) in water (400 ml). After stirring for 20 min, residual solid (0.77 g, unchanged CB 1954) was filtered off and the filtrate was allowed to stand for 1 hr, during which IIa (0.88 g, 24 per cent based on unrecovered CB 1954) was deposited as orange plates, m.p. 225° (decomp). (Found: C, 48.65; H, 4.6; N, 25.0%. $C_9H_{10}N_4O_3$ requires C, 48.65; H, 4.55; N, 25.0%). In the test against the Walker 256 carcinoma in rats the product gave: LD_{50}, 570 mg/kg; ID_{90}, 35 mg/kg; TI 16.

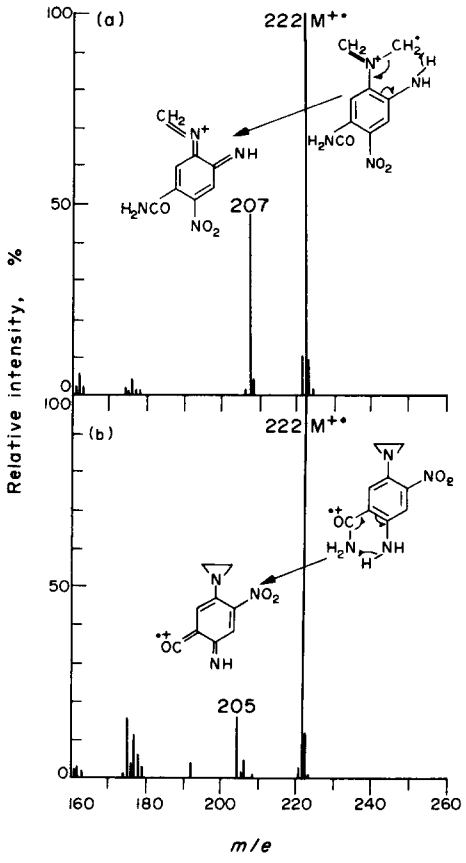

Fig. 1. Mass spectra: (a) 4-amino-5-(1-aziridinyl)-2-nitrobenzamide (IIa); (b) 2-amino-5-(1-aziridinyl)-4-nitrobenzamide (VI).

Purity of IIa used for the Walker tumour test: an aliquot (100 µl) of a solution of IIa (10 mg/ml) in acetone was concentrated onto the direct insertion probe of the mass spectrometer and the mass spectrum (A) determined under carefully recorded conditions with a source temperature of 200°.

Table 1. Quantitative determination of CB 1954 and urinary metabolites in the rat

Component	R_f value (benzene–ethanol, 4:1)	% of total urinary radioactivity
Ia	0.49	64.5
IIa	0.19	15.5
IV	0.31	2.2
V	0.24	2.8
unidentified	0.00	6

The mass spectrum (B) of a second aliquot of the IIa solution to which 1 μl of a solution of CB 1954 of the same concentration had been added was recorded using identical instrumental parameters. The peak height ratios *m/e* 222 (molecular ion $M^{+\cdot}$ for IIa): *m/e* 252 ($M^{+\cdot}$ for Ia) were measured. In spectrum A, there was no signal for *m/e* 252 above background levels and the ratio of the signal at *m/e* 222 to the background signal at *m*/e 252 was 1000:1. In spectrum B the appropriate ratio was 100:2.0. Since this ratio corresponded to a sample of IIa containing 1% w/w of Ia it can be concluded that the upper limit for CB 1954 contamination in the sample of IIa used for test was 0.05% w/w.

Formation of a quinoxaline (1,2,3,4-tetrahydro-6-nitroquinoxaline 7-carboxamide, VI) from IIa: a solution of IIa (50 mg) in 0.5 ml of a solution made by adding 60% perchloric acid (0.1 ml) to dimethyl sulphoxide (6 ml) was heated on a steam bath for 40 min. The solution was diluted with water, neutralized ($NaHCO_3$) and extracted with ether to give a homogeneous product (R_f 0.31 on t.l.c. in benzene–ethanol (4:1); IIa had R_f 0.19). The ultraviolet absorption spectrum (pH 7, phosphate) showed λ_{max} 278, 419 nm; IIa showed λ_{max} 262.5, 308.5, 374 nm. 2-Amino-1-(1-aziridinyl)-4-nitrobenzene [3] (IIb) when treated exactly as for IIa above afforded a product identical (t.l.c., u.v.) with the known [4] 1,2,3,4-tetrahydro-6-nitroquinoxaline (IIIb), λ_{max} 292, 436 nm; IIb showed λ_{max} 262, 311, 380 nm.

2-Amino-5-(1-aziridinyl)-4-nitrobenzamide (VI): CB 1954 (Ia, 5 g) and $NaHCO_3$ (5 g) were added to acetone (300 ml) and stirred until CB 1954 dissolved. Sodium dithionite ($Na_2S_2O_4$, 10 g) in water (100 ml) was added and stirring was continued for 30 min at 25–30°. The mixture was filtered and the filtrate concentrated under reduced pressure. The product (VI, 0.7 g, 16%) separated as an orange solid which afforded red-orange prisms (decomp. 215°) on recrystallization from aqueous acetone (Found: C, 48.7; H, 4.7; N, 25.1%). In the test against the Walker 256 carcinoma in rats, VI gave 37.7% reduction in tumour weight at 6 mg/kg (LD_{50}, 10.5 mg/kg).

5-Amino-2,4-dinitrobenzamide (IV) [5] and 5-(2-hydroxyethyl) amino-2,4-dinitrobenzamide (V) [1] were prepared by published methods. Major peaks in the mass spectra were: for IV, *m/e* 226 (100% relative intensity, $M^{+\cdot}$), *m/e* 210 (17%) and *m/e* 196 (13%); for V, *m/e* 270 (11%, $M^{+\cdot}$), *m/e* 239 (100%, $[M\text{-}CH_2OH]^+$), *m/e* 223 (30%) and *m/e* 190 (15%). These spectra were identical with those obtained for the corresponding metabolites (Table 1) to which these structures were assigned.

DISCUSSION

Three metabolites were identified in the urine of rats treated with CB 1954. The principal metabolite was a half-reduced product, subsequently shown to be IIa. The *ortho* relationship between the amino and aziridino functions in synthetic IIa was established by demonstrating a change in u.v. absorption characteristics on treatment with perchloric acid which was analogous to that observed when the corresponding CB 1837 analogue (IIb) was converted into the known quinoxaline (IIIb). The mass spectra (Fig. 1) of synthetic IIa and its isomer (VI), which was likewise synthesised by partial reduction of CB 1954, were also consistent with the proposed relative orientations of the amino and nitro functions. This isomer (VI) had previously been suggested [6] as a metabolite of CB 1954 which could be responsible for its activity against the Walker tumour. Thus AIC (4-aminoimidazole-5-carboxamide) [7] and related compounds [8] completely protect against the anti-tumour effect of CB 1954 and the structural resemblance between this purine precursor and VI, in particular the *vicinal* relationship between the amino and carboxamido functions in both molecules provides a rationale for this observation. However, for VI to be the compound responsible for the antitumour activity of CB 1954 *in vivo* it must clearly be a metabolite of CB 1954 and be highly active against the Walker tumour. The present results show that neither condition is fulfilled. Compound VI was not detected in the urine of rats treated with CB 1954, and it exhibited only a slight inhibitory action on the growth of the Walker tumour *in vivo*. An alternative explanation is therefore needed for the observed protective effects. Additional metabolites of CB 1954 were 5-amino-2,4-dinitrobenzamide (IV) and 5-(2-hydroxyethyl)amino-2,4-dinitrobenzamide (V), the hydrolysis product of CB 1954. Compound (V) was previously stated to be the sole urinary metabolite of CB 1954 [2] but the discrepancy between this earlier report and the present results was largely resolved by the discovery that synthetic IIa, which was not available at that time, had a very similar mobility to V in the t.l.c. system (water-saturated ethyl acetate) employed in the previous study.

Although the metabolism of CB 1954 resembled that of CB 1837 inasmuch as the half-reduced products (IIa and IIb respectively) were the principal non-polar urinary metabolites, the metabolic profiles of the two compounds were otherwise markedly different. Thus recovery of radioactivity in urine was much higher for CB 1954 and unchanged drug accounted for most of the excreted radioactivity, whereas CB 1837 (0.9 per cent of total urinary radioactivity) was largely metabolised. Non-polar components (Ia, IIa, IV and V) accounted for at least 85 per cent of the urinary radioactivity from CB 1954-treated rats, whereas polar components (76%) were the major products in urine from rats receiving CB 1837. Whereas a polar metabolite of CB 1837 (the mercapturic acid conjugate) was the principal metabolite, and was abundantly recovered from urine following chromatography on Amberlite XAD-2 resin, this method yielded only minor quantities of polar metabolites of CB 1954, and these have not yet been identified.

Although none of the metabolites of CB 1837 tested showed appreciable activity against the Walker tumour *in vivo*, the most effective, IIb, having a therapeutic index of only 1.8, the corresponding CB 1954 metabolite (IIa) had a TI of 16, which compares favourably with that of agents in clinical use e.g. chlorambucil (TI = 10, [9]), cyclophosphamide (TI = 22, [9]), and melphalan (TI = 20, [2]). Nevertheless, the ID_{90} was only 0.7 per cent that of CB 1954 itself, and prompted a rigorous check on the purity of the sample of IIa used for the Walker test. This

sample was deliberately treated with 1 per cent of its weight of CB 1954 and the mass spectrum of this mixture compared with that of the test sample. No CB 1954 was detected in the latter, and the maximum level which would have excaped detection, 0.05 per cent, was far below that required for the observed ID_{90} value for IIa to be attributable to a CB 1954 impurity.

Evidence [3] based on a discrepancy between *in vivo* and *in vitro* toxicity towards the Walker tumour suggested that CB 1837 may require metabolic activation, and that the half-reduced analogue (IIb) might be the toxic metabolite. It is unlikely, however, that IIa plays a similar role in relation to CB 1954. Thus there is no corresponding discrepancy between the *in vivo* and *in vitro* toxicities of CB 1954 [2]. Also, although IIa was somewhat more toxic in the bioassay (Table 2) than might have been predicted [3] on the basis of an ID_{90} of 35 mg/kg, it was still much less active than CB 1954, whereas IIb was more active *in vitro* than was CB 1837.

The results of the antitumour test on the principal metabolite (IIa) of CB 1954 dictate a revision of the previously-held view that an electron-withdrawing substituent *ortho* to the aziridine moiety is mandatory for appreciable activity against the Walker tumour [6]. Although some of the differences in chemotherapeutic index between related nitrophenylaziridines with the required structural features for antitumour activity could be correlated with the ether/water partition coefficient (P^E) which could reach an optimal value for CB 1954 [1], the present studies suggest additional factors which could contribute to the differences between CB 1954 and CB 1837. Thus the much less extensive metabolism of CB 1954 compared with CB 1837 would be consistent with the higher host toxicity (LD_{50} = 28 mg/kg) and antitumour activity (ID_{90} = 0.4 mg/kg) of CB 1954 [1] compared with CB 1837 (226 and 22 mg/kg respectively). Moreover, the principal metabolite of CB 1954, IIa, is both much less toxic (LD_{50} = 570 mg/kg) and less potent (ID_{90} = 35 mg/kg) than the parent drug, as well as being, on the evidence of its recovery from urine, relatively less abundant. This metabolite (IIa) therefore probably makes only a minor contribution to the toxic and tumour inhibitory effects of CB 1954. In contrast the corresponding metabolite (IIb) of CB 1837 is relatively more abundant than the parent drug, is more toxic, (LD_{50} 179 mg/kg) and less potent (ID_{90} = 100 mg/kg) and could therefore contribute to the lower selectivity (lower TI) of CB 1837 compared with CB 1954.

Thus whilst the metabolic studies on CB 1954, and the earlier results on CB 1837 have afforded interesting new insights into structure–activity relationships they have not provided explanations for the fundamental questions relating to the antitumour activity of the nitrophenylaziridines, namely the reason for their specificity for the Walker tumour, and the absence of the usual requirement for bifunctionality in an antitumour alkylating agent. There were no bifunctional alkylating agents (e.g. azo, azoxy or hydrazo-derivatives) identified among the urinary metabolites of CB 1954 or of CB 1837, but the possibility that these might be formed and excreted by another route is not excluded.

Table 2. Bioassay of 4-amino-5-(1-aziridinyl)-2-nitrobenzamide (IIa) against Walker cells

Drug concentration (γ/ml)	Number of tumour takes in 30 days	Mean survival time of tumour-bearing animals (days)
Controls	100%	6.6
6.25	100%	9.0
12.5	60%	13.0
25	20%	23
50*	no takes	—

* Three higher concentrations (100, 200 and 400 γ/ml) also gave no takes.

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