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CB 1954 Revisited

I. Disposition kinetics and metabolism

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Summary. Although it has been the subject of considerable interest for 15 years, originally as a cytotoxic agent and more recently as a radiosensitizer, there is very little pharmacokinetic information on CB 1954 (2,4-dinitro-5-aziridinylbenzamide). We have developed a rapid high-performance liquid chromatography assay for the drug and its metabolites and applied it to detailed examination of the pharmacokinetics of CB 1954 in mice and dogs. With IV administration a dose of 50 mg/kg gave peak blood concentrations of 100 μg/ml in mice, while 25 mg/kg gave peak plasma concentrations of 27 μg/ml in dogs. Peak concentrations were 3 to 5-fold lower for the IP route in mice and the oral route in dogs, and the bioavailabilities were 85% and 40%, respectively. Elimination $t_{½}$ values were 1.4–2 h in mice and 2.5–4 h in dogs and were independent of route of administration. Plasma protein binding was 57% but tissue penetration in mice was generally good. Tumour: plasma ratios were 50%–90%, while brain: plasma ratios were lower, at 37%–50%. The parent drug and several metabolites were identified and quantified in mouse urine, the total recovery being 24%–29%, of which 16%–25% was parent drug. The metabolites were also found in the circulation and in tissues. No changes in pharmacokinetics were seen with repeated dosing in mice or with administration of the protective agent phenyl AIC. Phenobarbitone pretreatment produced a small reduction in elimination $t_{½}$, mainly by accelerating aziridine ring removal. Allopurinol increased the blood levels of the 5-amino nitroreduction product. These studies provide a pharmacokinetic basis for interpreting the antitumour activity and toxicity of CB 1954, as well as for the development of new 'mixed-function' sensitizers.

Introduction

CB 1954 (2,4-dinitro-5-aziridinylbenzamide; Fig. 1) was synthesized by Khan and Ross at the Chester Beatty Research Institute, UK, as one of a series of tumour-inhibitory nitrophenylaziridines [12, 13]. It was found to be a highly potent and uniquely selective agent against the Chester Beatty Walker rat carcinosarcoma line WS [7, 12, 13], but comparatively inactive against the US Walker line WZ [15] and a number of other tumours originally tested [7, 8].

In the 15 years since the first report on CB 1954 was published interest in this enigmatic molecule has continued, principally because of the possibility that particular human tumours might be identified which would share the peculiar biochemical property presumed to render the UK Walker tumour so uniquely susceptible to it. The mechanisms of action of CB 1954 is still uncertain: though a monofunctional alkylating agent by virtue of the single aziridine group [12], it also exhibits characteristics of a purine antimetabolite [8–10]. The most recent evidence in favour of the latter mechanism is the demonstration of a powerful inhibition by CB 1954 of the DNA synthetic enzyme ribonucleotide reductase [18].

Clinical use of CB 1954 has been restricted to a small unpublished study by Dr Eve Wiltshaw at the Royal Marsden Hospital, where the disappointing results may have been due to its use against inappropriate tumour types. As well as the early demonstration of excellent activity against the Walker carcinosarcoma, later work showed CB 1954 to have intermediate activity against a human bladder carcinoma [18] and a human colon carcinoma (Tisdale, personal communication) in vitro.

Another reason for the maintenance of interest in this compound has been the recognition of its potent and unusual radiosensitizing properties [6, 16]. Investigation of CB 1954 as a lead compound in sensitizer development has resulted in the synthesis of a number of interesting mixed-function drugs, including RSU 1069 [2], which has just entered clinical trial.

Detailed information on the pharmacokinetics of CB 1954 is lacking. Ross [16] reported some concentrations in biological fluids from animals and patients using a spectrophotometric assay, and Connors and Melzack [8] described the distribution of tritium-labelled CB 1954 in rat liver, spleen, and Walker tumour. Subsequently, Jarman et al. [11] isolated three metabolites from rat urine by lengthy procedures and identified them by mass spectrometry. In view of the continuing interest in this and related compounds, and of the paucity of pharmacokinetic and metabolic data on the drug, we have developed a high-performance liquid chromatography (HPLC) assay for CB 1954 and its metabolites. Here we describe this technique and its application to determination of the disposition kinetics and metabolism of CB 1954 in mice and dogs. We have also investigated pharmacokinetic interactions with allopuri-

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nol, an inhibitor of the enzyme xanthine oxidase which functions as a nitroreductase [3] and might reduce (and possibly activate) CB 1954, as well as interactions with phenyl AIC (4-amino-2-phenylimidazole-5-carboxamide), a purine precursor which protects against the cytotoxicity of CB 1954 [8]. In the companion paper to follow [30] we describe our studies on the antitumour activity and toxicity of this compound.

Material and methods

Drugs. CB 1954 and the following derivatives (Fig. 1) were kindly supplied by Prof. W. C. J. Ross, Dr D. E. V. Wilman, Dr M. Jarman and colleagues at the Chester Beatty Institute, London, and the Institute of Cancer Research, Sutton, UK: 5-(2-hydroxyethyl)-amino-2,4-dinitrobenzamide (CB 10-150); 4-amino-5-(1-aziridinyl)-2-nitrobenzamide (CB 7060); 2-amino-5-(1-aziridinyl)-4-nitrobenzamide (CB 10-236); and 5-amino-2,4-dinitrobenzamide (5-amino). Phenyl AIC was supplied by Dr I. J. Stratford of the MRC Radiobiology Unit, Harwell, UK. Allopurinol was obtained from Sigma and sodium phenobarbitone from Evans Medical or Thornton and Ross.

Animals and tumours. Adult inbred male BALB/c and CBA mice were obtained from OLAC and adult inbred C3H/He mice of both sexes from OLAC and our own breeding colony. Mice were housed in plastic cages on sawdust bedding made from soft white woods, and allowed laboratory chow and water ad libitum. They were used at 20–35 g body weight. Tumours of the EMT6 mouse mammary carcinosarcoma were grown intradermally in the flanks of BALB/c mice [19]. The mouse sarcomas RIF-1 and KHT were grown in C3H/He mice, intradermally in the flank and intramuscularly in the leg, respectively [20, 21]. Tumours of the HT29 human colonic adenocarcinoma were grown as xenografts in the flanks of CBA mice immunosuppressed by neonatal thymectomy and whole-body irradiation and reconstituted with syngeneic marrow [14, 22]. EMT6 tumours were grown in the contralateral flank of these mice as in their normal syngeneic host.

The dogs used were adult collie crossbreds weighing 11–22 kg. All were clinically normal, with hepatic and renal function and haematological parameters in the normal ranges. Food was witheld overnight before drug administration.

Drug administration. In mouse experiments CB 1954 was injected IP in arachis oil (0.01–0.04 ml/g), 10% acetone/arachis oil (0.01 ml/g), or 0.85% (w/v) saline (0.08 ml/g), and IV in 10% dimethyl acetamide/saline (0.01 ml/g). Phenyl AIC was injected IP in arachis oil (0.01 ml/g), and allopurinol and sodium phenobarbitone were given IP in saline (0.01 ml/g). For oral administration in dogs CB 1954 was packed into gelatin capsules, size no. 00, and for the IV route it was injected in up to 2 ml dimethyl sulphoxide.

Drug analysis. Procedures used for the collection of urine, blood and tissues were similar to those described in detail previously [23, 25, 26]. In mouse studies bleeding was by cardiac puncture with replicate animals sacrificed at each time-point, whereas in dogs serial samples were taken from the same animals. Urine was frozen on solid CO_2 as it was collected, and tissues were frozen in a solid CO_2/methanol bath immediately after removal. All samples were processed rapidly using cold reagents.

The isocratic reversed-phase HPLC assay used was based on a modification of our earlier methods for misonidazole [28] and benznidazole [29]. The following procedures were found to be suitable for samples of urine, blood, plasma or tissue homogenate (20%–33% w/v). For most analyses 1 vol. sample was mixed thoroughly with methanol containing the internal standard 1-(2-nitroimidazol-1-yl)-3-fluoropropan-2-ol (Ro 07-0741, Fig. 1) kindly supplied by Dr C. E. Smithen, Roche, UK, and the mixture was then centrifuged (3000 *g* for 10 min at 4 °C). Where greater sensitivity was required the amount of methanol was reduced to 2 vol., and to aid protein precipitation the mixture was cooled in a solid CO_2/methanol bath before the centrifugation step was carried out at below 0 °C. In each case the clear supernatant was removed for injection directly into the HPLC apparatus . In other experiments, also to increase sensitivity, plasma was extracted with 1 or 2 vol. ethyl acetate; after centrifugation the ethyl acetate layer was removed, taken to dryness and resuspended in a small volume of methanol for injection into the HPLC apparatus. This consisted of a modular system from Waters. For most experiments it was fitted with a Radial Compression Module or Z-Module containing a Waters Radial-PAK C18 cartridge column (8 mm i.d.; 10 μm spherical particles loaded with octadecylsilane). For

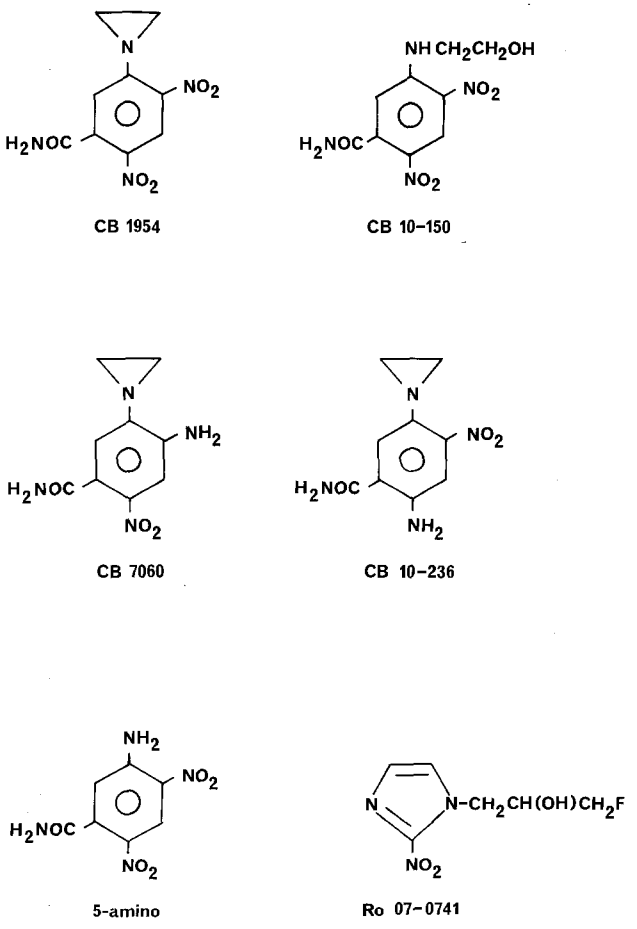

Fig. 1. Structures

optimal separation the mobile phase was 33% methanol/water and the flow rate was 2 ml/min. For more rapid analysis in the presence of fewer metabolites the methanol was increased to 40% and the flow rate to 3 ml/min. In addition, some analyses were performed with a Hichrom stainless steel column (25 cm × 4.9 mm i.d.) packed with Spherisorb S10 octadecylsilane. The injection volume was usually 10–15 µl. The absorbance of the effluent was monitored at 313 nm. CB 1954 and related compounds were identified by co-chromatography with authentic material and quantified by peak heights with reference to calibration curves which were linear over the concentration range studied.

Pharmacokinetic parameters. These were calculated as described in detail elsewhere [25, 26]. Brief particulars are as follows. Elimination half-life ($t_{½}$) was calculated from the equation $t_{½} = 1n2/k$, where k is the elimination rate constant given by the slope of 1n concentration plotted against time. Lines of best fit were fitted by least-squares linear regression analysis. Areas under plasma concentration × time curves were calculated in one of two ways, as appropriate:

1. From the expression $AIC_{0-\infty} = C_0/k$, where C_0 is the extrapolated concentration at time 0;

2. AUC from time 0 to time t was estimated by Simpson's rule. The remaining AUC was given by $AUC_{(t-\infty)}$ C_t/k, where C_t is the concentration at t. $AUC_{(0-\infty)}$ was then obtained by the sum of $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$.

Statistical analysis was by Student's *t*-test.

Physicochemical properties. The aqueous stability to CB 1954 was measured by the disappearance of the parent compound and the formation of the hydrolysis product CB 10-150 using HPLC. The octanol-water partition coefficient was obtained from the ratio of the concentrations in the two phases at equilibrium [17] with analysis by HPLC. Protein binding was determined as described previously [25], with benznidazole included as a positive control in all experiments.

Results

Physicochemical properties

CB 1954 is converted to the aziridine ring-opened hydrolysis product CB 10-150 (Fig. 1) in aqueous solution [16]. Hydrolysis of CB 1954 (100 µg/ml) was followed for 2 weeks in 180 m*M* phosphate buffer, pH 7.4, at 37 °C. CB 1954 and CB 10-150 were determined by HPLC (see below). Hydrolysis was very slow, with no more than 20% reaction at 2 weeks (estimated $t_{½}$ 30–50 days). However, in 90 m*M* HCl complete hydrolysis was almost immediate. The octanol-water partition coefficient was found to be 1.62 ± 0.04 (SE, $n = 4$), which compares with the value of 1.38 reported previously [17]. At a drug concentration of 30 µg/ml 53%–60% was bound to human plasma proteins in vitro. The one-electron reduction potential is −385 mV and is similar to that of misonidazole [17].

HPLC assay

Figure 2 illustrates a typical separation obtained with the most frequently used form of the HPLC assay (see legend and *Methods* for details). The chromatogram shown is for methanol-treated urine collected frozen over 24 h following an IP dose of 50 mg/kg CB 1954 to C3H/He mice. Urine samples consistently exhibited the maximum number of components amongst the biological samples analysed. Similar separations were obtained with blood, plasma, and tissue homogenates, but these usually contained fewer components.

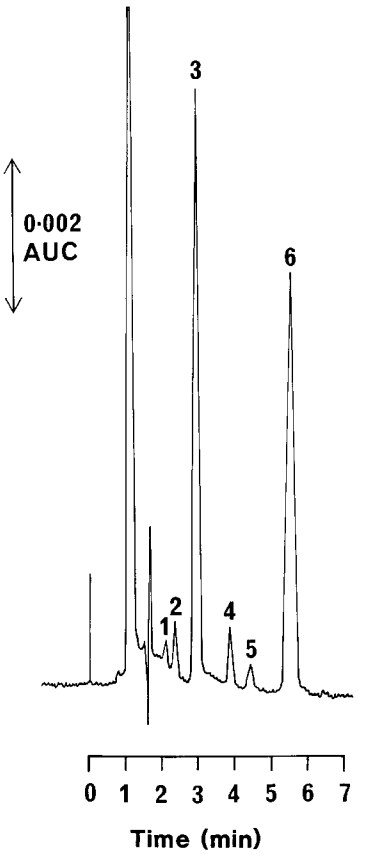

Fig. 2. HPLC of methanol-treated (1 :9) 24-h urine from C3H/He mice receiving 50 mg/kg CB 1954 IP. Peak *1*, unidentified polar metabolite; peak *2*, CB 7060; peak *3*, internal standard Ro 07-0741; peak *4*, CB 10-150; peak *5*, 5-amino metabolite; peak *6*, CB 1954. Chromatographic conditions: column, Waters Radial-PAK C18 Cartridge; mobile phase, 33% methanol/water; flow rate, 2 ml/min; column pressure, 750 psi; temperature, ambient; detection, absorbance at 313 nm; injection volume, 15 µl

The parent drug was eluted as the final component in the chromatogram (peak 6). Four other peaks were eluted earlier, indicative of their greater hydrophilic character. Three of these were identified by co-chromatography as the metabolites characterized in rat urine by Jarman et al. [11]. In order of increasing polarity these were: the aziridine ring-removed 5-amino compound (peak 5), the aziridine ring-opened hydrolysis product CB 10-150 (peak 4), and the 4-amino reduction product CB 7060 (peak 2) (see Fig. 1 for structures). The synthetic isomeric 2-amino reduction product CB 10-236 eluted between the 5-amino metabolite and CB 1954, being fully resolved from them, but as in the rat studies [11] we could find no evidence of its presence as a metabolite in any of the materials analysed. We did, however, consistently observed in urine a peak eluting immediately before CB 7060, indicating the

presence of an additional, more polar metabolite (peak 1, Fig. 2). It should be mentioned that no interfering peaks were present in fluids and tissues from control animals, and that no peak was masked by the internal standard, which was chosen principally for this reason.

To illustrate the performance of the assay some details will be given for the most frequently used form (see *Methods*). At a concentration of 10 μg/ml in the mouse plasma the coefficient of variation for ten replicate analyses was 4.4% for the most lipophilic component CB 1954 and 4.7% for the most hydrophilic known component CB 7060. The lower limit of quantitation on-column was about 1–1.5 ng, representing a plasma concentration of about 1–1.5 μg/ml for a 15 μl injection. This was improved about 3-fold by using the lower volume of methanol (see *Methods*), and larger injection volumes can also be exploited. The extraction efficiency was 99.3% for CB 1954 and 100.2% for CB 7060.

Pharmacokinetics in mice and dogs

CB 1954 in blood and plasma. Figure 3 shows combined data from two experiments comparing the pharmacokinetics of CB 1954 in the blood of BALB/c mice following injections of 50 mg/kg either IP or IV. Biexpoential decay was observed after IV injection; the distribution phase was very short, with a t½ of 4.0 min (95% confidence limits, 3.1–9 min), whereas the elimination t½ was 1.4 h (1.2–1.7 h). Values for the volume of distribution parameters Vd_{ext} and Vd_{area} were 1.32–1.22 l/kg, respectively. CB 1954 was absorbed rapidly after IP injection, reaching peak levels of 20–30 μg/ml by 15–30 min, which were, however, lower than those of up to 100 μg/ml for the IV route. The elimination t½ was identical to that for the IV route ($P > 0.1$). The estimated AUC values were 83 and 71 μg ml^{-1} h for the IV and IP routes, respectively, and the IP bioavailability was therefore 85%. Figure 3C shows data for different IP doses (25, 50, and 100 mg/kg). There appeared to be a tendency for the elimination t½ to increase with dose (1.9, 2.0, and 2.2 h), but this was not significant ($P > 0.1$).

CB 1954 was administered to five dogs IV in up to 2 ml dimethyl sulphoxide and, on a separate occasion, orally in gelatin capsules. Plasma pharmacokinetics for one dog are shown in Fig. 4 and kinetic parameters for the group are summarized in Table 1. Doses were between 10 and 25 mg/kg, and as the kinetics were linear in this range the values in Table 1 were normalized to the higher dose. Following IV injection peak plasma concentrations were observed more or less immediately (usually 5–15 min) and drug elimination was monoexponential, with a mean t½ of 3.3 h (range 2.5–4.0 h), rather longer than in mice. The value of Vd_{ext} was similar to that in mice. After oral administration peak concentrations were considerably lower and were seen later. The $AUC_{0-\infty}$ was reduced, though not greatly, and the mean oral bioavailability was 40% (range 27%–59%). The t½ values for the two routes were not significantly different ($P > 0.1$).

CB 1954 in tumour and brain. Figure 5 summarizes data from two experiments comparing the concentrations of CB 1954 in blood, whole brain and EMT6 tumours of BALB/c mice after 75 mg/kg CB 1954 injected IP in arachis oil (0.0 ml/g). Concentrations in the tumour were somewhat slow to rise but became similar to those in blood from 2 h onwards; brain levels were 2-fold lower throughout. In another experiment, blood, plasma, brain and EMT6 tumour levels were determined 45–135 min after 100 mg/kg CB 1954 injected IP in a large volume of saline (0.08 ml/g). Under approximately steady-state conditions, the mean blood: plasma ratio was 91%±3% (SE, $n=12$), the tumour: plasma ratio was 58%±2% and the brain: plasma ratio was 37%±2%. Table 2 shows data from an

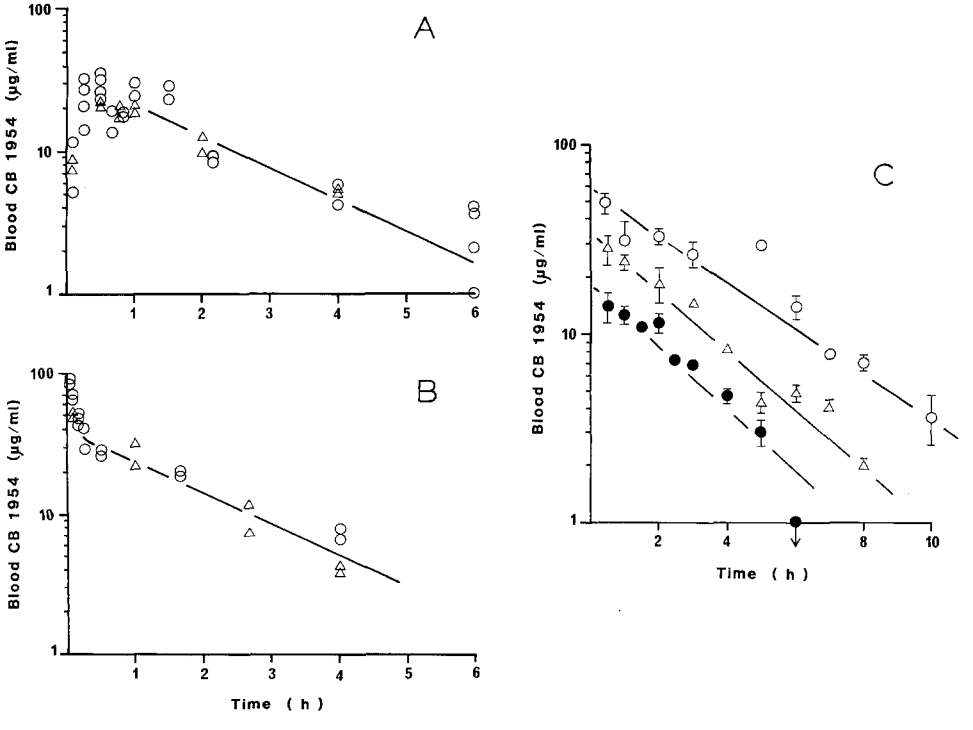

Fig. 3A–C. Kinetics of CB 1954 in BALB/c mouse blood **A** after 50 mg/kg IP in 10% acetone/arachis oil (0.01 ml/g). Each *symbol* represents one mouse; *triangles* and *circles* indicate two independent experiments; **B** after 50 mg/kg IV in 10% dimethyl acetamide/saline (0.01 ml/g). *Symbols* as in **A** (same experiments); **C** after different doses injected IP in arachis oil (0.01 ml/g): 25 mg/kg (●), 50 mg/kg (△), 100 mg/kg (○). Pooled data from four independent experiments (±SE, $n=3-11$)

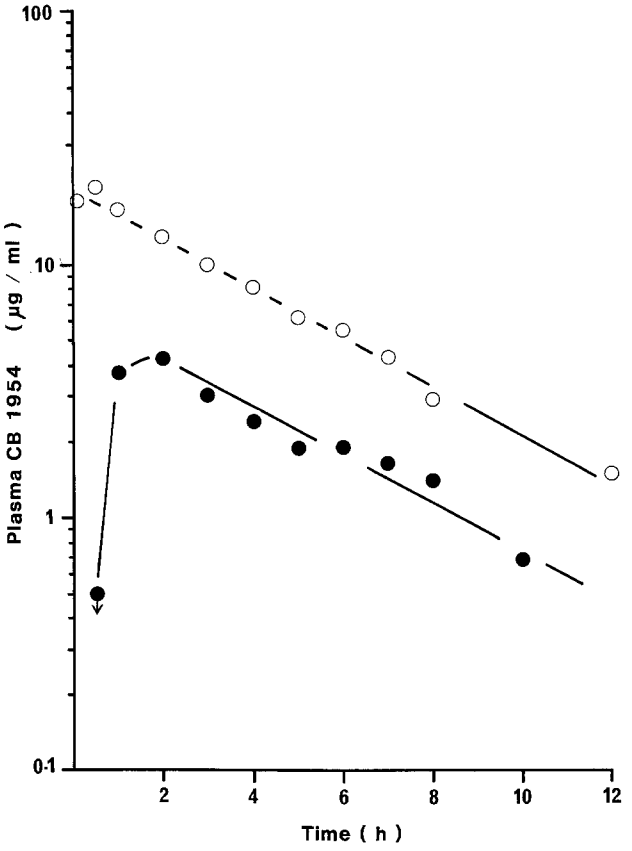

Fig. 4. Kinetics of CB 1954 in dog plasma. Data are for a single dog receiving 25 mg/kg IV in dimethyl sulphoxide (△) and orally in gelatin capsules (●)

Table 1. Pharmacokinetic parameters for CB 1954 in dogs

	IV	Oral
Peak concentration (μg/ml)	27.40 ± 3.1	8.40 ± 15
Peak time (min)	15.00	60.00
t ½ (h)	3.25 ± 0.27	2.93 ± 0.08
AUC ∞ (μg ml^{-1} h)	116.00 ± 17.00	45.00 ± 10.00
Clearance (1 kg^{-1} h)	0.23 ± 0.028	–
Oral biovailability (%)	–	40.00 ± 7.00
$V_{d_{ext}}$	1.05 ± 0.08	–

Peak times are median values; otherwise data are means ± SE of data from five dogs. Peak concentration and AUC∞ are normalized to a dose of 25 mg/kg

experiment comparing the concentrations of CB 1954 in EMT6 tumours and HT29 human colon carcinoma xenograft tumours grown contralaterally in the same immunosuppressed CBA mice. Drug penetration was similar, the tumour:blood ratios being 51%±6% and 62%±6% (SE, $n=8$) for EMT6 and HT29, respectively. Similar penetration was also observed for the RIF-1 sarcoma in C3H/He mice.

CB 1954 metabolites. The nitroreduction product CB 7060 and the aziridine ring-removed 5-amino metabolite were found in rather low concentrations (<5 μg/ml) in mouse

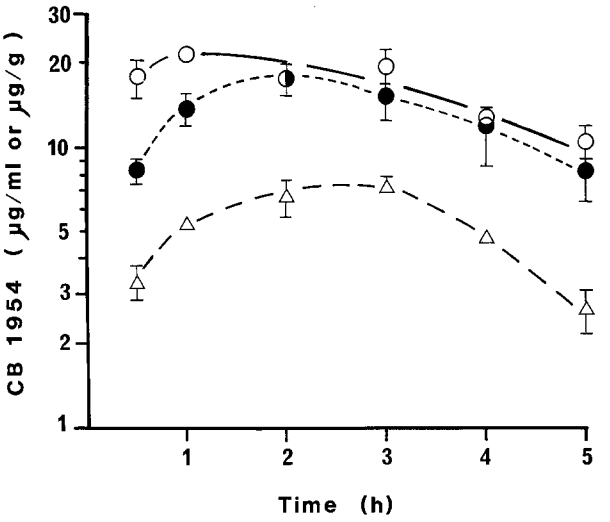

Fig. 5. Concentrations of CB 1954 in blood, brain, and EMT6 tumour of BALB/c mice. The CB 1954 dose was 75 mg/kg injected IP in arachis oil (0.04 ml/g). Values shown are means ±SE ($n=5$) of combined data from two independent experiments

blood (Fig. 6). The hydrolysis product CB 10-150 was usually absent. CB 7060 was identified in the plasma of all dogs given CB 1954, and CB 10-150 was found in most. Concentrations of these did not exceed 2 μg/ml, and the 5-amino metabolite was usually absent. Blood metabolite levels were similar in normal and tumour-bearing BALB/c and C3H/He mice and in tumour-bearing immunosuppressed CBA mice.

Urinary excretion data for mice are summarized in Table 3. All three metabolites were present, but the majority of the dose (about 20%) was excreted as parent drug. In the case of BALB/c mice that received CB 10-150 (25 mg/kg) IP in 0.04 ml/g saline) 35% was recovered unchanged. The 5-amino metabolite was not seen, suggesting that removal of the aziridine ring is not via CB 10-150. However, the metabolite more polar than CB 7060 observed in CB 1954 was also seen with CB 10-150. The above metabolites were usually detectable in mouse brain and tumour, but at levels too low for accurate quantitation. However, it can be said that tissue concentrations did not exceed those in the blood.

Drug interactions. Pretreatment of C3H/He mice with the microsomal enzyme inducer phenobarbitone (80 mg/kg/day IP for 5 days) decreased the t½ of CB 1954, but only by about 10% (Fig. 6). This appeared to be due to an increase in the rate of aziridine ring-removal, whereas nitroreduction was not affected (Fig. 6 and Table 3). In the same series of experiments, other mice were given daily injections of CB 1954 (30 mg/kg/day IP in 0.01 ml/g arachis oil); this had no effect on the concentrations of CB 1954 or its metabolites in blood when the mice were given a test dose of 50 mg/kg in the same vehicle.

When administered to BALB/c mice 30 min before, the xanthine oxidase inhibitor allopurinol (32 mg/kg IP in 0.04 ml/g saline) had no effect on the pharmacokinetics of CB 1954 (50 mg/kg IP in 0.01 ml/g arachis oil). However, the concentrations of the nitroreduced metabolite CB 7060 in blood were increased by up to 50%.

Table 2. Comparative penetration of CB 1954 into the HT29 colon carcinoma xenograft and EMT6 tumour

Time (h)	CB 1954 concentration (μg/ml or μg/g)								
	Blood			HT29			EMT6		
Mouse	1	2	3	1	2	3	1	2	3
1	25.1,	25.7,	25.7	13.9,	20.5,	19.8	9.2,	22.1,	12.2
3	12.3,	8.1		5.6,	6.8		5.8,	5.0	
5	7.9,	11.0,	11.9	5.1,	5.9,	4.2	5.0,	4.3,	3.5

Tumours were grown contralaterally in the same immunosuppressed CBA mice. CB 1954 (100 mg/kg) was injected IP in arachis oil (0.01 ml/g). Values shown are for individual mice

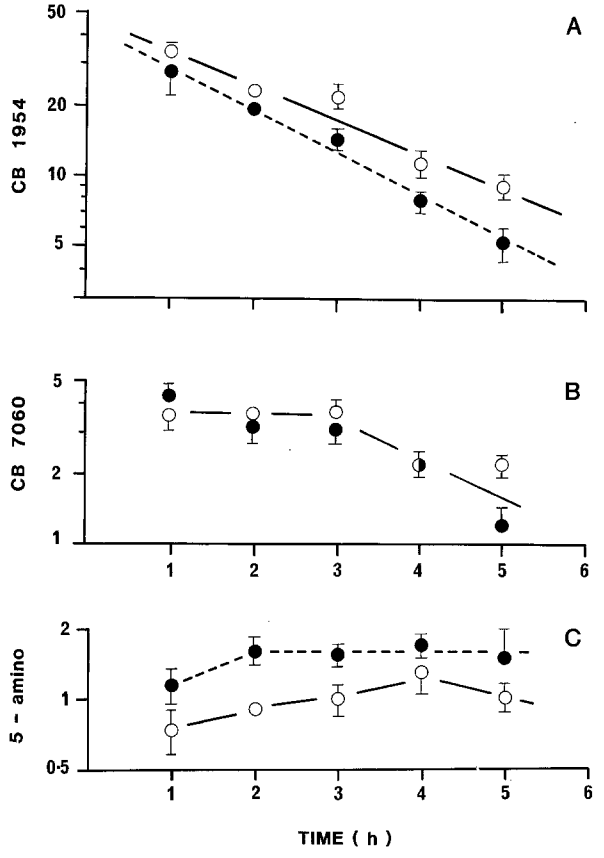

Fig. 6. Concentrations of CB 1954 and its metabolites (μg/ml) in blood of C3H/He mice. Mice were pretreated with phenobarbitone (80 mg/kg/day for 5 days) or saline before a dose of CB 1954 (50 mg/kg IP in 0.01 ml/g arachis oil). Values shown are means ±SE ($n=9$) of combined data from three independent experiments

Two experiments were done to investigate the effects of the CB 1954-protective agent, phenyl AIC, on the pharmacokinetics of CB 1954 in BALB/c mice. CB 1954 (75 mg/kg IP 0.02 ml/g in arachis oil) was given 30 min after 100 mg/kg phenyl AIC (IP in 0.02 ml/g in arachis oil) or arachis oil alone. Phenyl AIC did not alter the blood concentrations of CB 1954 or its metabolites. Neither did it have any effect on brain or EMT6 tumour concentrations. the HPLC assay used allowed the simultaneous determination of phenyl AIC itself in these samples,

Table 3. Urinary excretion of CB 1954 and its metabolites in mice

	Percentage of dose[a] excreted in urine		
	BALB/c	C3H/He	C3H/He after phenobarbitone[b]
CB 7060	3.2 ± 1.4	3.7 ± 2.0	4.8 ± 1.0
CB 10–150	2.5 ± 0.4	2.6 ± 0.6	3.7 ± 0.2
5-amino	1.6 ± 0.4	1.6 ± 0.4	4.7 ± 1.0
CB 1954	16.4 ± 3.6	20.8 ± 5.8	24.8 ± 2.6
Total	23.7 ± 5.3	28.8 ± 8.9	38.1 ± 2.0

[a] The CB 1954 dose was 50 mg/kg injected IP in 0.01 ml/g arachis oil. Results are means ± SE of three independent determinations

[b] Pretreatment regimen: 80 mg/kg/day for 5 days

the Hichrom column being more suitable than the cartridge column. Blood concentrations reached 30–40 μg/ml 30–60 min after injection and declined monophasically with a t½ of about 30 min. Tumour concentrations were similar to those in blood throughout, but brain concentrations were much lower and did not exceed 5 μg/g.

Discussion

The HPLC method described here has allowed the rapid identification and quantitation of CB 1954 and several metabolites in biological fluids and tissues. Previous methods involving labelled drug [8], thin-layer chromatography with mass spectrometry [11] and spectrophotometric assay [16] were, for differing reasons, of limited value for detailed pharmacokinetic studies.

A brief review of the physicochemical properties of CB 1954 may provide a useful basis for interpretation of the pharmacokinetic data. We found the drug to be surprisingly stable to hydrolysis in aqueous solution at physiological pH, with no more than 20% degradation at 2 weeks. In contrast, complete hydrolysis occurred instanteously in dilute acid. Thus, as the pH is lowered the more rapid acid-catalysed reaction predominates over the slower uncatalysed hydrolysis [13]. The reaction at physiological pH will proceed more rapidly in the presence of powerful nucleophiles, and thus alkylation may be swifter in vivo than in aqueous solution.

The aqueous solubility of CB 1954 is useful for pharmaceutical formulation. Particularly in the presence of 10% dimethyl acetamide, aqueous solubility was sufficient for IP and IV administration in mice. Arachis oil was also used for IP injection and dimethyl sulphoxide was used for IV administration in dogs.

The octanol-water partition coefficient of the compound (1.4–1.6) is such that, on the basis of the structure-pharmacokinetic relationships of neutral 2-nitroimidazoles [4, 24, 25], we would anticipate good tissue penetration together with elimination by both metabolic and renal pathways. The extent of binding to plasma protein (53–60%) was found to be similar to that of benznidazole, for which tissue penetration was slower but otherwise similar to the behaviour of unbound analogues [4, 25, 29]. For both drugs, binding may involve a combination of hydrogen bonding and hydrophobic interactions with protein.

The one-electron reduction potential of CB 1954 (−385 mV) is almost identical to that of the 2-nitroimidazole misonidazole (−389 mV) [17]; thus, other factors being equal, participation in redox processes might be expected to be similar.

Although there was a slight suggestion of lengthening of the elimination half-life with increasing dose in mice, pharmacokinetic behaviour was essentially linear in mice and dogs. Peak concentrations of up to 100 μg/ml were obtained with an IV dose of 50 mg/kg in mice, whereas half the dose produced a mean peak of 27 μg/ml in dogs. Rapid absorption was seen after IP dosing in mice, but peak concentrations were 3 to 5-fold lower than with the IV route; nevertheless, bioavailabity was high at 85%. A similar reduction in peak concentration was seen with oral administration in the dog, although once again absorption was rapid and the bioavailabity of 40% was unexpectedly high considering the lability of the drug at the low pH prevailing in the stomach.

The volume of distribution (1.2–1.3 l/kg) suggested the likelihood of good tissue penetration by CB 1954. Tumour concentrations rose slowly, possibly due to protein binding as predicted earlier, but tumour: plasma ratios equilibrated at 50%–90% later. Interestingly, brain penetration was considerably less efficient, maximum brain : plasma ratios being in the range 37%–50%.

Elimination was biphasic in the mouse but apparently monophasic in the dog. Elimination half-lives were 1.4–2 h and 2.5–4 h, respectively, and were independent of route of administration. Also, as predicted earlier, drug elimination occurred by both metabolic and renal pathways. We found 16%–25% of the drug dose was excreted unchanged in mouse urine, which compares with the figure of 34% reported by Jarman et al. for the rat [11]. Of the remaining urinary radioactivity in the rat 8% was associated with the 4-amino nitroreduction product CB 7060, 1% with the aziridine ring-opened hydrolysis product CB 10-150, and 1% with the aziridine ring-removed 5-amino compound; 3% was unidentified. We have identified the same metabolites in mouse urine, the recoveries being 3%–4% for CB 7060, 2%–3% for CB 10-150, and 1%–2% for the 5-amino compound. We also observed an additional peak on HPLC due to an unidentified metabolite of greater polarity which was present in similar amounts to those mentioned above. Low levels of the various metabolites were also observed in the circulation of mice and dogs as well as in mouse tumour and brain. As in the rat study [11] the putative 2-amino nitroreduction product CB 10-236 was not found as a metabolite in mice or dogs. After administration of CB 10-150 to mice the 5-amino metabolite was not seen, apparently indicating that hydrolysis is not the first step in ring removal. The unidentified metabolite seen with CB 1954 was also seen after CB 10-150.

We found no alterations in CB 1954 pharmacokinetics after repeated dosing in mice. Nor were there any changes after administration of the protective agent phenyl AIC. Phenobarbitone pretreatment resulted in a small reduction in elimination half-life, apparently via an increase in aziridine ring-removal with no effect on nitroreduction. Because xanthine oxidase has been implicated in the reduction and activation of nitroaryl compounds [3], we also investigated the effect of the enzyme inhibitor allopurinol. We in fact found that concentrations of the nitroreduced metabolite CB 7060 in mouse blood were increased rather than decreased, but it should be noted that we have previously demonstrated the ability of allopurinol to delay the elimination of hydrophilic drugs and metabolites by inhibiting their renal clearance [27].

The antitumour activity of CB 1954 has been attributed both to its monofunctional alkylating potential [12] and to its ability to act as an antimetabolite [8–10], in particular by inhibition of ribonucleotide reductase [18]. Metabolic activation of the drug has been considered [5, 11, 15], but the known metabolites described previously and in this paper possess considerably reduced antitumour activity [5, 11]. Activation at the target cell level cannot be ruled out, however, and the preferential activity of CB 1954 against hypoxic cells [6, 17, 30] may be due to reactive intermediates produced by nitroreduction, as is the case for other nitroaryl compounds (e.g. ref. [3]). In support of this possibility we have identified significant levels of the 4-amino nitroreduction product CB 7060 in the circulation and in tumour tissue.

The mechanism of greater hypoxic cell radiosensitization than with misonidazole [1, 2] is also unknown, but DNA-targeting by the aziridine group is a plausible hypothesis.

To give an idea of the concentrations of CB 1954 required for antitumour activity, Tisdale and Habberfield [18] reported that under oxic conditions 50% inhibition of cell growth required as little as 0.002 μg/ml for the sensitive Walker tumour line, 40 μg/ml for the highly resistant TLX5 mouse lymphoma and 0.7 μg/ml for the human bladder carcinoma line EJ. As far as radiosensitization is concerned, in hypoxic V79 cells 50 μg/ml CB 1954 produced an enhancement ratio of 2.2, and significant effects were seen at lower concentrations [17]. The present study has shown that concentrations in this active range can readily be achieved in the blood and tumour tissue of experimental animals. The companion paper to this [30] describes the activity of CB 1954 against various of our laboratory tumours, for which pharmacokinetic data are given here.

The unusual radiosensitizing properties of CB 1954 have led to the development of other mixed-function sensitizers such as RSU 1069 [2]. The pharmacokinetic data reported here should provide a basis for interpreting the antitumour activity of this intriguing drug, and may facilitate the future design of new analogues.

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