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CB 1954 revisited

II. Toxicity and antitumour activity

Paul Workman, Jane E. Morgan, Kathleen Talbot*, Karen A. Wright, Jane Donaldson, and Peter R. Twentyman

MRC Clinical Oncology and Radiotherapeutics Unit, MRC Centre, Hills Road, Cambridge, CB2 2QH, UK

Summary. We have assessed the antitumour activity of the nitrophenylaziridine CB 1954 in vitro and in vivo. For EMT6 mouse mammary tumour multicellular spheroids under hypoxic conditions in vitro, a 6-h exposure to 40 μg/ml reduced the surviving fraction to as low as 10^{-3} and the growth delay was 5.4 days. Oxic cells were twofold less sensitive. Phenyl AIC protected oxic and hypoxic cells equally. Under oxic conditions minimal cell killing was seen with HT29 cells, either in multicellular spheroids or in monolayer; a 6-h exposure to 40 μg/ml gave a spheroid growth delay of 1.5–1.7 days. No growth delay was seen with single maximum tolerated doses of CB 1954 against HT29 grown as a xenograft in immunosuppressed mice. Only minimal growth delays of 1–2 days were seen with similar doses against the EMT6 tumour and the RIF-1 and KHT sarcomas in mice. Little activity was seen with maximum tolerated doses given once a day for 5 days against EMT6 and RIF-1. No chemosensitization was measurable with CCNU, cyclophosphamide or melphalan in the KHT tumour.

Introduction

In the earlier companion paper [20] we reported our results on the pharmacokinetics and metabolism of CB 1954 (2,4-dinitro-5-aziridinyl-benzamide). Here we describe our complementary studies on the toxicity and antitumour activity of the drug.

The principal attraction of CB 1954 was originally its unique activity against the Chester Beatty Walker rat carcinoma line WS, other tumours tested being comparatively resistant [5, 6, 8–10]. Its clinical value would, of course, be dependent on the identification of human tumours which would share the peculiar biochemical property presumed to be responsible for the acute sensitivity of the UK Walker tumour. In a small unpublished study by Dr Eve Wiltshaw at the Royal Marsden Hospital there were no unequivocal tumour regressions, but it is possible that it may have been used against inappropriate tumour types. More recently, the human bladder carcinoma line EJ was found to exhibit in vitro sensitivity intermediate between that of the sensitive Walker line and the resistant TLX5 mouse lymphoma [14] and similar behaviour was observed with the human colon carcinoma HT29 (Tisdale, personal communication). No other experimental evaluation appears to have been carried out.

Having established the maximum tolerated doses for single and multiple administration, we investigated the response to CB 1954 of the HT29 tumour grown as a xenograft in immunosuppressed mice together with that of the KHT and RIF-1 sarcomas and the EMT6 mouse mammary carcinosarcoma grown in their respective syngeneic mouse hosts. Complementary experiments were carried out with HT29 and EMT6 cells grown in monolayer or multicellular spheroid culture in vitro. In view of previous reports of the preferential activity of CB 1954 towards hypoxic cells [4, 13], the response of EMT6 spheroids was compared in oxic and hypoxic conditions, and the effect of the CB 1954-protective compound phenyl AIC (4-amino-2-phenylimidazole-5-carboxamide) [6, 13] was also examined. The more recent revival of interest in CB 1954 has resulted from the demonstration of its unusually potent radiosensitizing activity both in vitro [13] and in vivo [4]. Because of this it has been used as a lead compound in the development of 'mixed-function' sensitizers such as RSU 1069, which has also been reported to exhibit potent enhancement (or chemosensitization) of the tumour response to melphalan in mice [1]. Here we also describe experiments to determine the effect of CB 1954 on response to melphalan, cyclophosphamide and CCNU in the KHT tumour in mice.

Finally, these studies are interpreted in light of the pharmacokinetic data reported in the previous paper [20].

Materials and methods

In vivo. Sources of drugs, formulation and methods for the growth of tumours were summarised in the previous paper [20]. Mice were treated when tumours reached 200–400 mg. The time taken by individual tumours to reach four times their initial size was determined and regrowth delay calculated for groups of six to ten mice [18, 19]. LD_{50} values were calculated by computerised probit analysis with the Generalized Linear Interactive Modelling Programme (GLIM) of the Royal Statistical Society of Great Britain.

* *Present address:* Ludwig Institute for Cancer Research, Hills Road, Cambridge, CB2 2QH, UK

Offprint requests to: P. Workman

In vitro. Techniques for the initiation and growth of multicellular spheroids were based on those of Yuhas et al. [21], as described elsewhere [11, 15]. For treatment, EMT6/Ca/VJAC mouse mammary tumour cell spheroids of appropriate size (250–300 μm or 600–800 μm) were transferred into 250 ml glass spinner vessels containing 100 ml medium (Eagle's minimum essential medium with 20% newborn calf serum) with or without test agents and continuously stirred at 37 °C. Hypoxia was produced by continuous gassing with 5% CO_2/95% N_2 ($O_2 < 10$ ppm) at a rate of 500–1000 ml/min over the surface of the medium. For oxic treatment the same spinner system was used but the vessels were gassed with 5% CO_2/95% air and then sealed during treatment. Response was assayed by cell survival following disaggregation (immediate or after 24-h delay) and by spheroid regrowth delay [11, 15].

Experiments with HT29 spheroids were carried out as described by Twentyman [15] for EMT6, except that the the medium was MEM with 10% fetal calf serum and 0.5% trypsin/0.02% versene was used for disaggregation, and the incubation period for colony formation was 16 days. Response was assayed by cell survival and regrowth delay. Experiments with HT29 monolayer cultures in logarithmic phase of cell growth also used previous protocols for EMT6 [17] with the above modifications. In growth curve experiments cells were seeded at 5×10^4 per 25 cm^2 flask and treated 3 days later. Response was assayed by cell survival after short (up to 6 h) exposures or by determining the number of cells per flask after continuous exposure to the drug.

In all in vitro experiments test agents were dissolved and added in culture medium. Concentrations of CB 1954 and metabolites in medium were determined by HPLC using the method described for plasma in the previous paper [20].

Results

Cytotoxicity against tumour cells in vitro

In vitro experiments were carried out with concentrations of 0.4, 4, or 40 μg/ml CB 1954 in the incubation medium. The last dose corresponds to the upper limit of CB 1954, which we have shown can be achieved in mouse blood and tumour with maximum tolerated doses [20]. In selected experiments concentrations of CB 1954 in the medium were determined by HPLC [20]. In no case, either oxic or hypoxic, did the concentration at 6 h fall below 95% of the original, and no metabolites could be detected. In continuous exposure experiments about 50% of the original concentration was still present after 4 days. Small amounts of the hydrolysis product CB 10-150 [20] were also seen at later times.

EMT6. Figure 1 shows the response of EMT6 mouse mammary tumour multicellular spheroids (600–800 μm diameter) incubated oxically or hypoxically with 40 μg/ml CB 1954 in spinner culture. Spheroids were disaggregated immediately after treatment and the in vitro colony-forming ability of the cells was determined. From the slopes of the survival curves, cells in hypoxic spheroids were about twice as sensitive as those in oxic ones. It can be seen that a 6-h exposure to 40 μg/ml CB 1954 reduced the surviving fraction to about 10^{-3} under hypoxic conditions, as compared with about 3×10^{-2} under oxic conditions. At the lower concentration of 4 μg/ml CB 1954 for 6 h cell survival was reduced by only 30% under oxic conditions and 60% under hypoxia (data not shown). Phenyl AIC alone had little effect on cell survival, but it provided similar protection against CB 1954 cytotoxicity under both oxic and hypoxic conditions (Fig. 1). In one experiment we compared the sensitivity of EMT6 spheroids of different sizes (600–800 μm and 250–300 μm diameter). The larger spheroids were more sensitive under both oxic and hypoxic conditions; with a 6-h oxic exposure to 40 μg/ml, for example, the surviving fraction for large spheroids was 3.3-fold lower than for small spheroids, and the difference was 5.9-fold under hypoxic conditions. Delaying the disaggregation of the spheroids allowed some recovery; for example, in one experiment 6 h at 40 μg/ml CB 1954 reduced the surviving fraction of cells in 600 to 800 μm diameter hypoxic spheroids to 5×10^{-4} with immediate disaggregation, as against 10^{-2} with a 24-h delay, giving a recovery factor of 20. In some experiments spheroid response was also measured by growth delay; for example, in one instance with spheroids 250–300 μm in diameter exposure to 40 μg/ml CB 1954 for 3 and 6 h resulted in spheroid growth delays of 1.8 and 2.4 days, respectively, with oxic treatment and of 2.5 and 5.4 days, respectively, with hypoxic treatment.

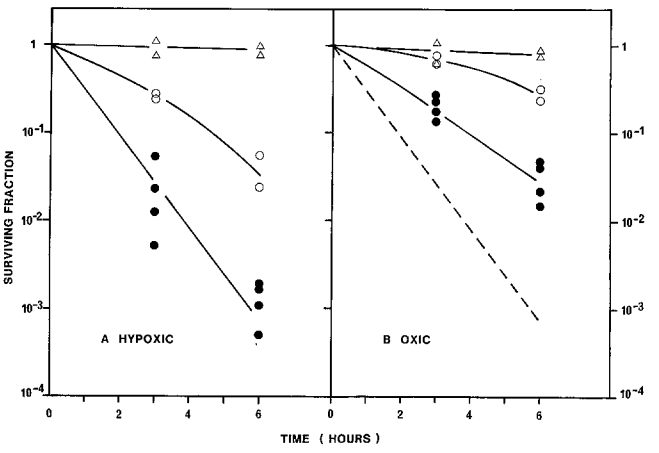

Fig. 1. Effects of CB 1954 (40 μg/ml) and phenyl AIC (50 μg/ml) alone and in combination against EMT6 mouse tumour spheroids 600–800 μm in diameter in vitro. Spheroids were incubated under **A** hypoxic or **B** oxic conditions for 3 or 6 h, then immediately disaggregated and assayed for colony formation. (△) phenyl AIC alone; (●) CB 1954 alone; (○) CB 1954 plus phenyl AIC. Each *point* represents an independent determination and data are combined from three separate experiments. The *dotted line* in **B** shows the hypoxic response to CB 1954 alone transposed from A

HT29. All experiments with HT29 were carried out under oxic conditions. In the first series of experiments monolayer cultures in logarithmic phase and spheroids 200–300 μm in diameter were exposed to concentrations of 4 or 40 μg/ml CB 1954 for 2, 4, or 6 h, followed by immediate disaggregation and assay for colony-forming ability. Very little cell kill was seen under any of these conditions, and the lowest surviving fraction of 0.2 was obtained with spheroids exposed to 40 μg/ml for 6 h. In the same experiments spheroid response was also assayed by regrowth delay, the drug being removed at the end of the ex-

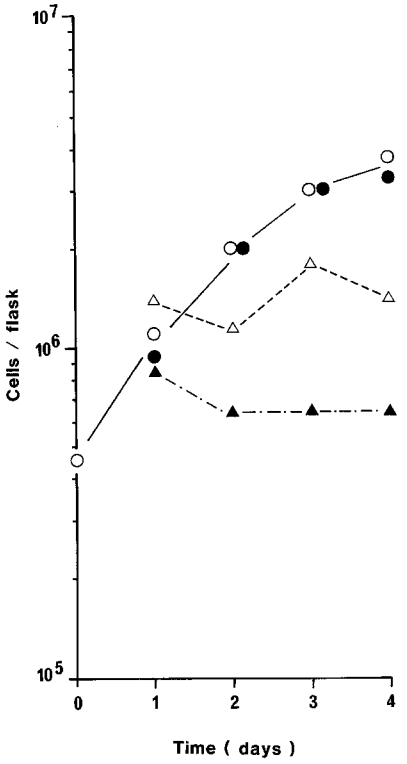

Fig. 2. Effects of continuous exposure to CB 1954 on the growth of HT29 cells in monolayer culture. (○) Control; (●) 0.4 μg/ml; (△) 4 μg/ml; (▲) 40 μg/ml in medium

posure. At a concentration of 40 μg/ml little or no effect was seen with 2- and 4-h exposures, though small growth delays of 1.7 and 1.5 days, respectively, were obtained with a 6-h exposure in replicate experiments.

In subsequent experiments the response of HT29 monolayer cultures was studied by a growth inhibition assay similar to that of Tisdale and Habberfield [14], in which cells were exposed to the drug continuously and the total number of cells per flask was determined daily. Figure 2 illustrates a representative experiment in which it can be seen that growth was inhibited almost completely at 40 μg/ml, not at all at 0.4 μg/ml, and to an intermediate extent at 4 μg/ml.

Toxicology

Toxicity studies were carried out to establish acceptable doses for single and multiple administration in our mouse strains. Data for BALB/c and C3H/He strains are summarised in Table 1. The single-dose LD_{50} values are slightly higher than the 100 mg/kg reported previously [5]. The multiple-dose schedule consisted of one dose/day for 5 consecutive days. With single doses death usually occurred within 48 h. There was very little weight loss (< 10%) at doses around the LD_{10}. With the multiple-dose schedule very marked wight loss (up to 30%) was observed at non-lethal doses. When it occurred in surviving mice the maximum weight loss was seen about 1 week after the start of treatment. At high doses, toxic symptoms developed towards the end of or just after the multiple-dose regimen. Shaking, splaying of hindlimbs, and walking on tiptoe were indicative of neurotoxicity. Insufficient data were available for estimation of an LD_{10} for the multiple-dose regimen in C3H/Km mice, but 30 mg/kg/day caused about 15% weight loss with obvious neurotoxicity, and this was regarded as the maximum tolerated dose (MTD). In terms of total dose administered the multiple-dose regimen was no more toxic than the single-dose regimen. In immunosuppressed CBA mice the single dose MTD was 75–100 mg/kg. For example, in one experiment 0/6 animals died with 8% weight loss at 75 mg/kg, and 3/9 died with 13% weight loss at 100 mg/kg.

Antineoplastic activity in mice

CB 1954 was evaluated for antitumour activity against the following solid tumours used regularly in our laboratory: the RIF-1 and KHT sarcomas grown syngeneically in C3H/He mice, the EMT6 mammary carcinosarcoma grown syngeneically in BALB/c mice, and the HT29 human colon carcinoma grown as a xenograft in immunosuppressed CBA mice. As measured by the growth delay assay, CB 1954 exhibited very little activity against any of the solid tumours tested, even at maximum tolerated doses (Table 2). Single doses of 75–100 mg/kg produced minimal growth delays of about 1 day in the KHT and RIF-1 sarcomas, and no detectable growth delay in the EMT6 tumour and HT29 xenograft. The multiple-dose regimen was used to treat the RIF-1 and EMT6 tumours. With doses of

Table 1. CB 1954 mouse toxicity data

Strain	Route	Single dose		Multiple doses	
		LD_{10}[a]	LD_{50}[a]	LD_{10}[a] or MTD[b]	LD_{50}[a]
		(mg/kg)		(mg/kg/day)	
BALB/c	IP	94 (75–119)	133 (114–156)	31[a] (25–39)	53 (41–67)
	IV	115 (79–167)	122 (109–138)	–	–
C3H/He	IP	140 (123–158)	183 (167–202)	30[b]	> 75

CB 1954 was inject in 10% dimethylacetamide/saline (0.01 ml/g) for the IV route and arachis oil or occasionally 10% arachis oil/acetone (up to 0.04 ml/g) for the IP route. Mice were observed for 1 month after injection. Values were estimated from combined data recorded in several independent experiments

[a] LD_{10} and LD_{50} values are doses causing, respectively, 10% and 50% lethality. Insufficient data were available to calculate values for multiple doses in C3H/He mice, but an MTD was defined

[b] Maximum tolerated dose

Table 2. Effects of CB 1954 against mouse tumors and a human tumour xenograft in vivo

Tumour	Dosage regimen	Vehicle	Growth delay (days)	Weight loss (%)	Survivors/total
RIF-1 sarcoma	150 mg/kg × 1	A	2.3	12	10/10
	100 mg/kg × 1	A	0.8	4	10/10
	30 mg/kg/day × 5	B	1.5	15	11/12
	20 mg/kg/day × 5	B	3.5	13	11/12
KHT sarcoma	75 mg/kg × 1	B	0.7	9	8/9
EMT6/Ca/VJAC carcinosarcoma	75 mg/kg × 1	A	0	10	10/10
	50 mg/kg × 1	A	0	9	10/10
	30 mg/kg/day × 5	B	1.2	16	10/11
	20 mg/kg/day × 5	B	0.7	10	10/11
HT29 colon carcinoma xenograft	100 mg/kg × 1	A	0	13	6/9
	75 mg/kg × 1	A	0	8	6/6

Vehicles: A, 10% acetone/arachis oil; B, saline

Table 3. Effects of CB 1954 on KHT tumour response to cyclophosphamide, CCNU and melphalan in vivo

Treatment		Growth delay (2 SE)
CB 1954 alone	(75 mg/kg)	0.75 (0.21–1.4)
Cyclophosphamide alone	(75 mg/kg)	13.2 (11.7–14.9)
Cyclophosphamide alone	(150 mg/kg)	24.1 (20.4–28.4)
Cyclophosphamide alone plus CB 1954	(75 mg/kg) (75 mg/kg)	13.7 (12.7–14.7)
CCNU alone	(10 mg/kg)	7.1 (6.4–7.8)
CCNU alone	(20 mg/kg)	16.8 (14.2–19.5)
CCNU plus CB 1954	(10 mg/kg) (75 mg/kg)	8.8 (7.5–10.4)
Melphalan alone	(10 mg/kg)	5.8[a] (4.9–6.7)
Melphalan alone	(15 mg/kg)	8.0[b] (6.6–9.5)
Melphalan plus CB 1954	(10 mg/kg) (75 mg/kg)	6.8[c] (5.1–8.9)

Lethalities: [a] 1/8; [b] 4/9; [c] 1/9

20–30 mg/kg/day growth delays of 2–3 days were obtained with RIF-1 and of 1 day with EMT6. These minimal effects were accompanied by 10%–15% weight loss and about 10% lethality.

Chemosensitization in mice

Table 3 shows the results of a representative experiment comparing the activities of cyclophosphamide, CCNU, and melphalan, alone or in combination with 75 mg/kg CB 1954, against the KHT sarcoma in C3H mice. The nitrogen mustards and the nitrosourea were given at two doses alone, and for all three drugs significantly longer growth delays were seen at the higher dose. Similarly for all three the combination of the low drug dose with CB 1954 produced very little increase in response over that of the low dose alone, and in general the increase could be accounted for by the growth delay of about 1 day seen with CB 1954 alone.

Discussion

In the in vitro experiments described here the upper concentration of CB 1954 was equal to the maximum obtainable in mouse blood and tumour [20]. HPLC measurements showed that the drug was very stable under the tissue culture conditions used. Despite a 3- to 6-h exposure to 40 μg/ml CB 1954 under oxic conditions very little growth delay was observed with HT29 human colon carcinoma spheroids, and minimal cell killing was seen for those cells treated as spheroids or in monolayer culture. With HT29 monolayer cultures under continuous exposure to CB 1954, cell growth was inhibited almost completely at 40 μg/ml and partially at 4 μg/ml. For comparison, in previous studies using a similar assay 50% inhibition of cell growth was observed at 0.0002 μg/ml and 40 μg/ml for the highly sensitive UK Walker tumour and the comparatively resistant TLX5 lymphoma, respectively, while the value for the EJ human bladder carcinoma was 0.7 μg/ml [14], with HT29 also exhibiting intermediate sensitivity (Tisdale, personal communication).

In the experiments with large EMT6 mouse mammary tumour spheroids, where cells were plated for cell survival determination immediately after treatment a 6-h exposure to 40 μg/ml CB 1954 produced a 1.5- to 2-log cell kill under oxic conditions and a 3-log cell kill under hypoxic conditions; under both exposure conditions protection was afforded by phenyl AIC. These data are in accordance with those of Stratford et al. [13] for V79 Chinese hamster cells in single-cell spinner culture. Delaying disaggregation by 24 h resulted in a considerable reduction in the amount of cell kill, presumably reflecting the repair of potentially lethal damage [15]. With small EMT6 spheroids the growth delay resulting from 6 h at 40 μg/ml CB 1954 was about 5.7 days for hypoxic exposure and only 2.5 days for oxic exposure. The relationship between growth delay and cell survival for CB 1954 was similar to that found previously for nitrosoureas, nitrogen mustards and *cis*-platinum [15].

Administration of well-defined, maximum tolerated doses of CB 1954 produced essentially no antitumour activity in the RIF-I or KHT mouse sarcomas, the EMT6 mouse mammary carcinosarcoma or the HT29 colon car-

cinoma xenograft. In the original in vivo screening studies, the drug exhibited unique selective potency towards the rat UK Walker carcinosarcoma, the other tumours tested proving comparatively resistant [5, 6, 8–10].

The lack of activity of CB 1954 against our tumours is perhaps not surprising in view of the weak in vitro activity described above together with the drug exposures used in vitro and those attainable in vivo [20]. Thus, a close to maximum tolerated dose of 75 mg/kg produces a plasma exposure of only about 100 μg ml^{-1} h, whereas an exposure of 120 μg/ml^{-1} h (40 μg/ml for 3 h) gives minimal growth delay in vitro.

The present work also shows that at close to maximum tolerated doses CB 1954 exhibits minimal chemosensitizing activity with CCNU, cyclophosphamide or melphalan in the KHT tumour. This is in contrast to the nitroimidazole misonidazole, which at a high dose (1 g/kg) enhances the activity of all three agents in this tumour [12, 16]. In addition, the nitroimidazole aziridine analogue RSU 1069 has been shown to enhance the antitumour activity of melphalan [1]. RSU 1069 also exhibits potent radiosensitization in vivo [1], and further work on this interesting series of mixed-function sensitizers is in progress.

The precise mechanism of action of CB 1954 is still uncertain. It possesses monofunctional alkylating potential because of the aziridine group [8, 9], and it is interesting that the relationship between growth delay and cell kill in EMT6 spheroids is most similar to that for alkylating agents than for other types of drug, including antimetabolites [15]. However, the murine tumours used here respond quite well to difunctional alkylating agents and nitrosoureas (e.g., see ref. [16]), and the lack of CB 1954 response confirms earlier reports of lack of collateral sensitivity with the above agents [5]. CB 1954 also exhibits characteristics of a purine antimetabolite [4, 6] and Tisdale and Habberfield [14] have shown drug sensitivity to correlate with susceptibility to inhibition of ribonucleotide reductase. It may be that both alkylation of DNA and enzyme inhibition are involved in the mechanism of action, to an extent depending on the cell line. The preferential cytotoxicity of CB 1954 towards hypoxic cells, reported previously [4, 10] and confirmed here, indicates a likely third component in the mechanism. Nitroreduction is almost certainly involved, and an amine nitroreduction product is identifiable in plasma, urine, and tumour [7, 17]. The terminal nitroreduction products possess reduced antitumour activity [3, 7], and it is likely that the selective activity of CB 1954 against hypoxic cells is due to reactive intermediates, as with other nitroaryl compounds [2].

No other tumours have been identified which approach the unique susceptibility of the UK Walker tumour to CB 1954. We have confirmed that the drug has little activity against other rodent or human tumours in vivo, and in this case the drug exposures were precisely defined. It may be possible to identify human tumours with similar biochemistry and sensitivity to the Walker tumour, and the demonstration of ribonucleotide reductase as a target enzyme [14] may be a step in this direction. At present CB 1954 remains an interesting experimental drug in search of a human tumour to treat.

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