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 In a small unpublished study by Dr Eve Wiltshaw at the Royal Marsden Hospital there were no unequivocal tumour regressions, but it is possible that it may have been used against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 carcinomas 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 growth delay calculated for groups of six to ten mice [18, 19]. LD₅₀ values were calculated by computerised probit analysis with the Generalized Linear Interactive Modelling Programme (GLIM) of the Royal Statistical Society of Great Britain.
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% CO2/95% N2 (O2 < 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% CO2/95% 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 cm² 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 oxicly 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^{-4} 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^{-7} 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.

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-