In Vivo Antitumour Effect of Combretastatin A-4 Phosphate Added to Fractionated Irradiation

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Abstract. *Background: The study aimed at evaluating the potential benefit from a combination of fractionated ionising radiation with the vascular-targeting compound combretastatin A-4 phosphate (CA-4-P). Materials and Methods: Syngenic rat rhabdomyosarcoma (R1), growing subcutaneously, was treated at 2 different sizes: either small (2±0.5 cm^3) or large (10.94±0.6 cm^3). Localised fractionated irradiation of the tumours (5 x 3 Gy) in 5 days was followed 1 day later by an intraperitoneal CA-4-P treatment (25 mg/kg). Results: The combined treatment of only large tumours resulted in a small additional growth delay when compared with radiotherapy only. Conclusion: The present data indicate a size-dependent increase in tumour growth delay from combining fractionated irradiation with CA-4-P.*

The vital importance of the vasculature in tumour growth and metastasis has generated a great deal of interest in strategies aimed at eliminating the existing tumour vessel network. The damage of a single tumour blood vessel will result in the death of a large number of tumour cells, because of lack of nutritional support for survival and proliferation (1, 2).

In addition to anti-angiogenesis, there is another related, but distinct, approach being investigated, which aims to preferentially damage the established neovasculature within the tumour, *i.e.* vascular targeting (1). One such vascular-targeting approach is the use of small-molecule agents that disrupt the tubulin cytoskeleton of rapidly proliferating and immature tumour endothelial cells (3-5). The lead compound in this class, combretastatin A-4 phosphate (CA-4-P), has completed phase I clinical trials (6, 7) and entered a phase II trial. Preclinical studies, involving various rodent tumour types, have demonstrated a rapid and strong reduction in blood flow and vessel occlusion when a single non-toxic dose of CA-4-P was used (8). Although severe induction of necrosis was observed, little or no retardation of tumour growth was measured (9, 10). However, a significant differential volume-response after treatment with CA-4-P was observed with the rat rhabdomyosarcoma tumour model in which a strong growth delay in large tumours, but little or no growth delay in small ones, were reported (11).

Despite the induction of profound damage in tumours, the drug is unable to kill all the tumour cells, thereby allowing re-growth from the surviving population, specifically at the periphery. The survival and proliferation of cells in the peripheral region of a solid tumour are most probably related to the presence of normal tissue vasculature surrounding the tumour mass. In general, these blood vessels are much less responsive to vascular-targeting agents. Overall, a potential clinical application of CA-4-P is its combination with a treatment that can effectively target the peripheral tumour cells, such as radiation or chemotherapy.

An improved anticancer response has been demonstrated when CA-4-P was combined with chemotherapy or hyperthermia (12-14). Studies involving the combination of single-dose irradiation with CA-4-P showed enhanced response when compared to either treatment alone (15, 16).

In the present study, whether the addition of CA-4-P to a fractionated radiation treatment could further improve the tumour response in a rat rhabdomyosarcoma was examined, in comparison with the effect obtained after radiotherapy alone.

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Materials and Methods

In vivo tumour model. Male adult WAG/Rij rats, weighing 270 -300 g, were used for all the experiments. The animals were housed 3-4 per cage and had food and water *ad libitum*. The syngeneic rhabdomyosarcoma tumour (a 1-mm^3 piece) was implanted subcutaneously in the lower flank of the animals. The tumours were selected for treatment when the size was either 2±0.5 cm^3 or 10.9±0.6 cm^3, referred to as small and large, respectively.

All experimental conditions were approved by the ethical committee of the University of Leuven, Belgium, in compliance with the national guidelines on animal research.

Radiation treatment. Local tumour irradiation, with the remainder of the body adequately shielded, was carried out with a linear accelerator (General Electric, Baden, Switzerland) using an 18-megavolt photon beam and a dose rate of 3 Gy/min.

Dosimetry was performed in treatment conditions. A perspex tissue-equivalent plate of 2.5-cm thickness was placed above the tumours. To allow for the correct positioning of the tumour, the rats were anaesthetised with sodium pentobarbital (Nembutal, max. 60 mg/kg body weight).

Drug treatment. The vascular-targeting compound, CA-4-P (OXiGENE Inc., Watertown, USA), stored at 4˚C while protected from light, was dissolved in 0.9% saline immediately before intraperitoneal (*i.p.*) injection. A dose of 25 mg/kg was used, a selection based on previous toxicity evaluations (11, 15).

Treatment protocol and response evaluation. Radiation was given over 5 days, with a dose fraction of 3 Gy (3 Gy x 5) each day. CA-4-P was given as a single *i.p.* injection (25 mg/kg) 1 day after the last irradiation. The separate groups of tumour-bearing rats were treated with fractionated irradiation alone, CA-4-P alone, or received no treatment.

The response of rat rhabdomyosarcoma to the various treatments was evaluated by the classic tumour growth delay assay. Using calipers, tumours were measured 2-3 times per week, and volumes were calculated as [(a x b x c) xpi/6] where a, b, and c are orthogonal dimensions. Five to 6 rats per treatment group and a repeat experiment were used; the results of both experiments were pooled for analysis. The measurements at the different time-points are presented as means with standard error of the mean. The tumour growth delay time was derived from the growth curves: 'starting volume x5' for small tumours and 'starting volume x2' for large tumours. The differences between groups were evaluated with a multiple regression analysis, and $p<0.05$ was considered significant.

Results

Systemic toxicity evaluation of the combined treatment indicated that only in some animals was a body weight loss of, at most, 15% recorded. This reduction lasted for about 2 weeks, whereafter recovery in body weight to and above the initial value was measured.

The growth patterns of small rat rhabdomyosarcoma tumours treated with CA-4-P alone (25 mg/kg), fractionated irradiation (5x3 Gy) alone, and the combination of both agents are illustrated in Figure 1A. The figure includes the growth in the untreated condition for comparison. With the small-sized (2±0.5 cm^3) tumours at the start of the treatment, no increase in growth delay was observed (in fact, a non-significant shorter time was necessary to reach the starting volume x5) in comparison with the combination treatment or with fractionated irradiation alone.

Tumours with a large volume (10.9±0.6 cm^3) at the start of the treatment showed a longer but non-significantly different growth delay (2.8 days, at the level of the starting volume x2) after the combined fractionated irradiation and CA-4-P treatment in comparison with the result obtained after the fractionated irradiation only (Figure 1B).

Discussion

The different types of vascular-targeting agents produce a similar characteristic pattern of rapid blood flow reduction, vessel occlusion and central necrosis development in various rodent solid tumour models (5, 17). The resulting central necrosis was shown to extend to as much as 95% of the tumour, depending on the drug-sensitivity of the tumour. Despite these large intratumoral effects that have been documented for CA-4-P and related agents at doses below the maximum tolerable level, the corresponding inhibition of tumour growth has, in most cases, been quite modest or absent. Moreover, viable tumour cells at the periphery of the tumour remained, from which subsequent (re)growth occurred. The remaining peripheral rim of tumour cells is likely to have resulted from the presence of the tumour-encompassing normal tissue vessels which supply oxygen and nutrients for survival and proliferation. This suggests that, for potential improvement of the antitumour response, CA-4-P must be combined with conventional therapies, such as radiotherapy. Ionising radiation treatment is known to be effective in killing the well-oxygenated and rapidly proliferating tumour cell population, whereas CA-4-P indirectly kills hypoxic (radio-resistant) cells. There is, at present, great interest in the establishment of such potentially powerful combination treatments (18, 19).

Our investigations illustrated an advantage of the combination of fractionated irradiation and CA-4-P in rhabdomyosarcomas with a large size at treatment. Such a benefit in tumour response was, however, not observed with the smaller tumours. This might be the reflection of the relative radio-sensitivity of the rat rhabdomyosarcoma tumour type, or the result of a non-optimal combination of both agents in the treatment of small tumours. Investigations involving the vascular-targeting agent ZD6126, injected twice during a fractionated radiotherapy schedule (daily 2.5 Gy for 12 days), showed a clear-cut increase in small KHT sarcoma tumours (20). Combining irradiation as a single dose with a single CA-4-P

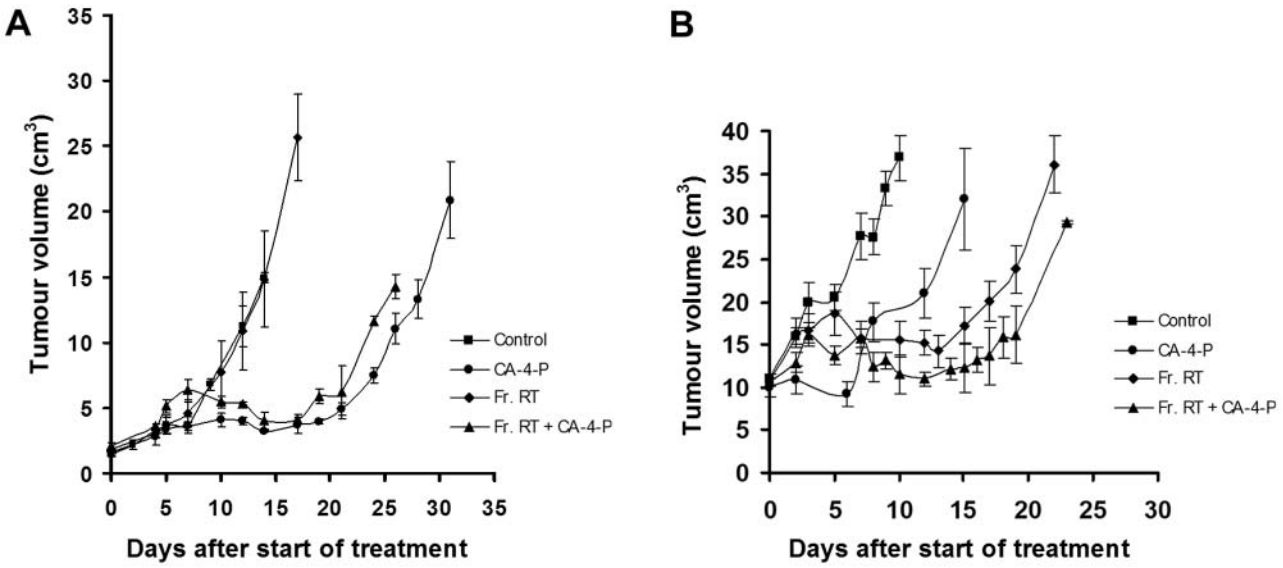

Figure 1. *Growth curves of subcutaneously growing rat rhabdomyosarcoma for two different series: (A) small-sized tumours and (B) large-sized tumours. The fractionated irradiation treatment consisted of 5 doses of 3 Gy (over 5 days) and CA-4-P given as a single dose of 25 mg/kg. For the combination treatment, CA-4-P was given 1 day after the last irradiation. The tumour volume measurements are presented as the mean, with ± SEM (when not clearly visible, they are within the symbol size).*

administration 1 day later resulted in a very similar tumour size-dependent growth delay in the same rat tumour model (15). However, the growth delay measured for large rhabdomyosarcoma tumours, after a single irradiation followed by CA-4-P, was longer than with the present combination. This is very likely the result of fewer hypoxic cells remaining after the fractionated irradiation, which are then indirectly killed by CA-4-P. Nevertheless, a possible advantage of giving irradiation before the vascular targeting relates to the recent observations of the impact of radiation-induced vascular endothelial cell damage on tumour response (21). Irradiation may, thus, sensitise endothelial cells to the subsequent vascular-targeting treatment with an improved antitumour response as the result.

We previously reported the effects of different intervals and sequences of CA-4-P and CPT-11 administration on growth delay and intratumoral uptake of CPT-11, using the syngeneic rhabdomyosarcoma tumour model. The combination treatment significantly increased tumour growth delay in comparison with both drugs alone, specifically when given simultaneously (14). The advantage of combining either the vascular-targeting agent 5,6-dimethyl-xanthenone-4 acetic acid (DMXAA) or CA-4-P with the anticancer drugs cisplatin and cyclophosphamide was evaluated preclinically *in vivo* in different tumour models (13). The greatest effects were achieved when the vascular-targeting agents were administered 1-3 h after chemotherapy.

The data on the combination of CA-4-P with radio- or chemotherapy demonstrate increases in tumour damage when compared with either treatment alone, albeit to a major extent dependent on the involved tumour type, tumour size and combination protocol. It should be noted that the improved antitumour activity seems to occur without any measurable increase in normal tissue side-effects (13, 16).

To further advance the combined treatments with vascular-targeting agents, such as CA-4-P, in general, additional experiments employing different tumour models are necessary. Our results suggest potential clinical applications, specifically when large tumours are treated.

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