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Vascular targeting of solid tumours: a major 'inverse' volume–response relationship following combretastatin A-4 phosphate treatment of rat rhabdomyosarcomas

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Abstract

Tumour-specific vascularisation may be therapeutically approached in two different ways: by antiangiogenic treatments specifically directed to dividing and migrating endothelial cells, or by agents that target principally the inadequate and ill-structured tumour vasculature. Combretastatin A-4 phosphate (combreAp), a recently synthesised prodrug (OXiGENE, Lund, Sweden), is a vascular targeting agent of the latter kind. We evaluated the effect of a single intraperitoneal (i.p.) combreAp injection on the growth of rhabdomyosarcomas syngeneic in WAG/Rij rats. Different tumour volume groups, ranging between 0.1 and 27 cm^3, were selected to assess the relationship between the size at treatment time and the response to combreAp. A double combreAp treatment (2×25 mg/kg) was investigated within the same overall aim: the relationship between growth delay and tumour size. Our results show that the systemic administration of combreAp induces a clear-cut differential growth delay in the solid rat rhabdomyosarcomas: with very large tumours ($\geqslant 14$ cm^3), a 17.6-fold stronger effect was measured than with very small tumours (<1 cm^3). This is the 'inverse' of the volume-response seen with the conventional therapeutic approaches (radiotherapy, chemotherapy or surgery). These combreAp antitumour responses were observed without treatment limiting systemic toxicity in the rats. With clinical digital subtraction angiography, using microsurgical cannulation of a major tumour draining vessel, and with histopathology, we demonstrate that growth delay is related to an early (within 3–6 h) and extensive breakdown of tumour blood vessels. The experiments involving a second injection also indicate a volume-dependent effect of combreAp in reducing the regrowth rate of small or large rhabdomyosarcomas. This significant differential volume–response obtained with 'selective' vascular targeting, stronger in larger tumours than smaller ones, suggests the potential of broadening the therapeutic window.

Keywords: Vascular targeting; Combretastatin A-4 prodrug; Tumour volume; Antitumour therapy; Tumour necrosis; Growth delay; Angiography

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1. Introduction

Maintenance and improvement of vasculature is critical for the continued growth of solid tumours. These blood vessels therefore represent a target for potential new anticancer therapies (for reviews, see e.g. [1–6]). Definitive damage will lead to an avalanche of ischaemic tumour cell death and necrosis. As a consequence, inhibition of angiogenesis and more recently, specific vascular targeting are being extensively investigated [7–10].

Angiogenesis inhibition in solid tumours is defined as the prevention of new blood vessel formation from the existing vascular bed [1,7,8,11]. The actions of these drugs thus primarily consist of inhibiting growth and migration of the endothelial cell. Subsequent to such antiangiogenic treatment, tumour growth that is critically dependent on angiogenesis will be prevented.

Vascular targeting treatments, on the contrary, are predominantly based on the fact that the already acquired tumour vasculature is selectively attacked [1,12,13]. This type of therapy takes advantage of the weaknesses of established tumour endothelial cells and their supporting structures and induces collapse, thrombosis and/or haemorrhage. Obviously, depending on the agent and the dose intensity selected for this treatment, the proliferating endothelial cells of newly growing blood vessels might serve as an additional target.

Selectively targeting the acquired tumour blood vessels may constitute a potential tool, complementing traditional anticancer therapy that is mainly directed against the malignant cells themselves. Occlusion or collapse of the tumour vessels inherently leads to ischaemic or haemorrhagic necrosis. The acute reduction of tumour vasculature will initiate an immense loss of tumour cells because of nutrient deprivation, and might thus result in stabilisation or reduction of tumour growth. This has been demonstrated with hyperthermic treatment [14,15], with flavone acetic acid injections [13,16], with tumour necrosis factor-α [13,17] and with an antitumour endothelial cell immunotoxin [18,19]. Another vascular targeting approach, involving tubulin-binding agents, recently gained increasing attention. As such, colchicine, vinblastine and dolostatin have been tested for their effect on tumour growth through antivascular activity [12,20,21]. Of particular interest is the novel compound combretastatin A-4, derived from the South African tree *Combretum caffrum* [22,23]. The more soluble phosphorylated prodrug showed the potential to induce a similar extent of vascular shutdown and necrosis, but without the severe side-effects of the parent compound [24–26]. These studies, as well as all other vascular targeting investigations have, however, been restricted to small-sized mouse tumours only. They therefore lack potentially useful information on the relationship between tumour size and the effectiveness of a selective vascular targeting compound.

The present study assessed the *in vivo* antitumour effect by vascular targeting with combretastatin A-4 phosphate prodrug (combreAp) in a broad range of tumour volumes, using the syngeneic WAG/Rij rat rhabdomyosarcoma model. This tumour grows subcutaneously (s.c.) in the flank region to much larger sizes than have been described in the literature for mouse models. Such a growth capacity up to several tens of cm^3 favours clinical relevance, as many cancer patients present with already large tumour masses. In addition, we have tested a clinical imaging technique consisting of digital subtraction angiography for its applicability to evaluate the antivasculature and antitumour effects of combreAp in our rat tumour model.

The overall goal of our research was to determine the relationship between tumour volume and the vascular/antitumour effect of combreAp. Our investigations covered: (i) the extent of the antitumour activity in terms of growth delay; (ii) histopathological examinations; and (iii) the visualisation of the effect of combreAp on the tumour vasculature with digital subtraction angiography. Using the latter two methods, we also focused on the time relationship between the combreAp injection and the sequence of the major events involved in the development of tumour damage and eventual regrowth. Finally, a double combreAp treatment was evaluated for its additional tumour growth delay capabilities and the presence of a size-dependent phenomenon.

2. Materials and methods

2.1. Tumour model

The *in vivo* tumour system used in the present project is a highly reproducible experimental syngeneic rhabdomyosarcoma in WAG/Rij rats [27]. Briefly, the tumour cell line has been derived from a radiation-induced tumour in the jaw musculature of inbred WAG-Rij rats. Alternate s.c. tumour cell inoculation and tumour piece transplantation are routinely used in our laboratory to maintain qualitatively the characteristics of the rhabdomyosarcoma. The tumour shows a regular growth pattern without the development of metastases, and has the property of s.c. growth up to 40–50 cm^3 in the flank region of rats without causing any obvious disturbance of the animals' health. Indeed, no anaemia, weight loss or change in physical behaviour was recorded during the time of this growth. This allowed us to investigate the effectiveness of combreAp in a large range of tumour sizes. It was, however, decided to sacrifice rats bearing tumours larger than 40 cm^3 (or less if adequate information is obtained earlier during growth) at any time during the follow-up period. For the present

experiments, we transplanted tumour pieces of approximately 1 mm^3 s.c. into the lower flank of adult rats (260–300 g). This technique avoids the spread of growth along the injection track, which often occured after s.c. inoculation of $1–3\times10^6$ tumour cells, and allows adequate and reproducible tumour volume measurements.

2.2. Preparation of the vascular targeting compound solution and administration

The combretastatin A-4 prodrug (OXiGENE Inc., Lund, Sweden) was dissolved in 0.9% saline immediately before use. The solution was injected intraperitoneally (i.p.) in a volume of 0.5 ml. The control rats received an i.p. injection of 0.5 ml 0.9% saline only.

2.3. Growth delay measurements

Three orthogonal diameters were measured with a caliper and used to calculate the volume of the tumour with the formula: $a\times b\times c\times\pi/6 =$ volume, expressed in our study as cm^3. Correction for skin thickness of 1 mm was applied at all the measured diameters. To evaluate the relationship between tumour size and the combreAp administration, five different tumour volume ranges were selected prior to any treatment. These ranges were <1 cm^3, 1–3 cm^3, >3–7 cm^3, >7–14 cm^3 and >14 cm^3, referred to in our study as 'very small', 'small', 'medium', 'large' and 'very large', respectively.

2.4. Histopathology examination

After removal of the tumour from the animals, at selected time intervals after the combreAp injection, transections were made of predetermined tumour size ranges. Either the whole segment (for very small and small tumours) or half of the segment (medium to very large tumours) was fixed in 10% formaldehyde solution. Care was taken to enable the evaluation of both the central and peripheral tumour area in the same slice. Following paraffin embedding, 10 μm sections were stained with the haematoxylin–eosin combination. All sections were screened independently, generally using a $\times100$ and a $\times400$ magnification, by the principal investigator and the histopathologist. The latter was blinded to the treatment protocol.

2.5. Microsurgical cannulation of the tumours for digital subtraction angiography

Spontaneously breathing anaesthetised rats (Fentanyl–Dehydroperidole (Janssen-Cilag, Belgium) mixture 0.4 ml/100 g, i.p.) were placed in dorsal decubitus and pinned to a cork operation table. Through transcutaneal and transfascia midline incision, the encapsulated tumour was exposed. The wound was retracted, and the tumour vascular pedicle was dissected under microsurgical viewing. The major feeding artery and vein were followed by proceeding proximally up to the confluence site with the iliac artery and iliac vein, respectively. Because the pedicle of the exposed tumour often appeared kinked and occasionally compressed by vascular sheet bands, the sheet and the adventitia of the vein were trimmed. Following cannulation, a flush with 0.2 cc pure heparin was made and the syringe with contrast medium was attached to allow injection during imaging. An occlusive microvascular clamp was applied proximally to prevent possible backflow of contrast medium into the blood circulation. At the end of the angiographic recording, the cannula was withdrawn and the operation wound was closed with 4/0 Prolene running suture. Only the large and very large tumours were investigated in this way since the smaller ones had a narrow vascular pedicle that made cannulation impossible.

2.6. Digital subtraction angiography technique

Following micro-dissection and cannulation of the major tumour draining blood vessel, angiography of the tumour was initiated. For this purpose, 1 ml of ioxaglate meglumine/ioxaglate sodium (Hexabrix, Codali, Belgium) 200 mg I/ml, a water soluble low osmolar ionic contrast agent, was injected. The animals remained anaesthetised to obtain recordings without movement artifacts. Pulse mode X-ray image acquisition was done at a fixed voltage of 70 kV with a Siemens Polytron apparatus, using a constant image intensifier field. Scanning of the tumour vascularity was performed at 3 frames per second during the injection. The images were stored and digital subtraction of the body-background was performed to improve the analytical quality. Selected images were printed on Agfa films. The tumour vasculature images were offered for interpretation to two independent radiologists blinded to the study plan. Number and size (numerical grading), location (peripheral; central) and gross morphology (regular or irregular) of the blood vessels were the parameters used to evaluate the antivasculature effects of combreAp.

2.7. Statistics

The results presented in the growth delay graphs are means with standard error of the mean (SEM). Differences between groups were evaluated with a Multiple Regression Analysis.

2.8. General aspects

The overall number of tumours used to assess growth delays, tissue changes and vascularity amounts to a total of approximately 320, including repeat experiments.

The research protocol was in accordance with the Ethical Committee for Animal Care and Use of the Catholic University of Leuven (K.U. Leuven, Belgium) and national guidelines.

3. Results

Systemic toxicity evaluations of a single i.p. combreAp injection clearly indicated that doses above 50 mg/kg (MTD) resulted in severe weight loss. A combreAp dose of 100 mg/kg resulted in 25% mortality in the rats. It was therefore decided to use combreAp injections of 25 mg/kg or less in all experiments, as no significant signs of general toxicity were observed in both tumour-free and tumour-bearing rats.

The s.c. growth of control tumours, evaluated between 0.1 cm^3 and less than 40 cm^3, consistently occurred at a volume doubling rate of 2.5–5 days. The effect of combreAp on the growth rate of rhabdomyosarcomas was evaluated with the five preselected tumour volume ranges. Each point of all the growth curves represents the mean volume (cm^3) of at least 20 tumours. Using a single i.p. injection of 25 mg/kg a distinct volume-dependent antitumour effect was seen (Fig. 1). With tumours referred to as very small to medium ($\leqslant 7$ cm^3), growth delays of at most 5 days were seen (Fig. 1a, b, c). Tumours with a volume at injection larger than 7 cm^3 responded well to the treatment, with growth delays between 9 and 18 days (Fig. 1d, e). The slowing of tumour growth was small, but measurable at 2 days after treatment. For the very large tumours, not only a stabilisation in size but even a small regression of tumour volume was detected (Fig. 1e). Palpation of the control and treated tumours revealed, for all the changes in volume, a consistently firm tumour mass that was later confirmed at transection. To enable a precise quantification of the growth delay relative to the treatment starting volume, it was decided to interpolate the measured growth delay at 1.5 times the increase in size. The results of these calculations are summarised in Table 1. The two extremes in the present study, i.e. no growth delay for very small tumours versus a mean of 17.6 days delay for the very large tumours, clearly demonstrate the size dependency of the combreAp effectiveness with this rhabdomyosarcoma tumour model. When the single i.p. dose of combreAp was reduced to 10 mg/kg, a smaller reduction in the growth rate was observed, but still with a more marked effect at the larger tumour volumes (data not shown). With large-sized rhabdomyosarcomas, 5 days delay in growth was measured, as compared with 9.5 days when 25 mg/kg was used with this tumour size. With medium sized tumours (>3–7 cm^3), 1.5 days growth delay was obtained after injection of 10 mg/kg combreAp instead of 4.5 days as observed with 25 mg/kg of the drug.

The digital subtraction angiographic images of control tumour-bearing rats showed the relatively rich vascularity of the large sized rhabdomyosarcomas (Fig. 2a). Vascularisation was always more intense in the periphery than in the centre of the tumour, the latter being partially necrotic (see histopathological screening). The majority of the blood vessels in these control tumours were large and showed a regular morphology. A striking effect of the treatment with 25 mg/kg combreAp on the tumour vascularity was imaged. At 3 h after drug administration, a slightly reduced number of vessels was seen in the periphery and towards the centre. These blood vessels showed an irregular shape as well as a reduction in diameter. At 6 h, a clear decrease in the number and size of blood vessels was observed and they were found to be irregular (Fig. 2b). At both time intervals, a back-pressure was present during the injection of the contrast medium. Screening the rhabdomyosarcomas at 1 day after the combreAp treatment revealed similar pictures to those at 6 h. Blood vessels were, however, now present only in the peripheral area. The images obtained at 3 days after drug injection showed that vascularity was absent in the major part of the tumour and only a few vessels remained at the

Table 1
Rat rhabdomyosarcoma and combretastatin A-4 phosphate (25 mg/kg i.p.)

Predetermined volume groups (cm^3)	Time lapse for volume change (days)[a]		Relative growth delay (days)[b]	Statistical significance for growth changes[c]
	Control	CombreAp		
Very large (>14)	3.4	21.0	17.6	$P<0.0001$
Large (>7–14)	2.6	12.1	9.5	$P<0.0001$
Medium (>3–7)	2.5	7.0	4.5	$P<0.002$
Small (1–3)	2.4	4.8	2.4	$P=0.0044$
Very small (<1)	2.9	2.9	0	–

[a] Time (days) necessary for tumours to increase 1.5× in volume.
[b] Time difference (days) between combreAp-treated and control tumours to increase 1.5× in size.
[c] Significance level of the change in tumour growth between combreAp-treated and control tumours estimated from the full growth curves (see Fig. 1) using Multiple Regression Analysis.

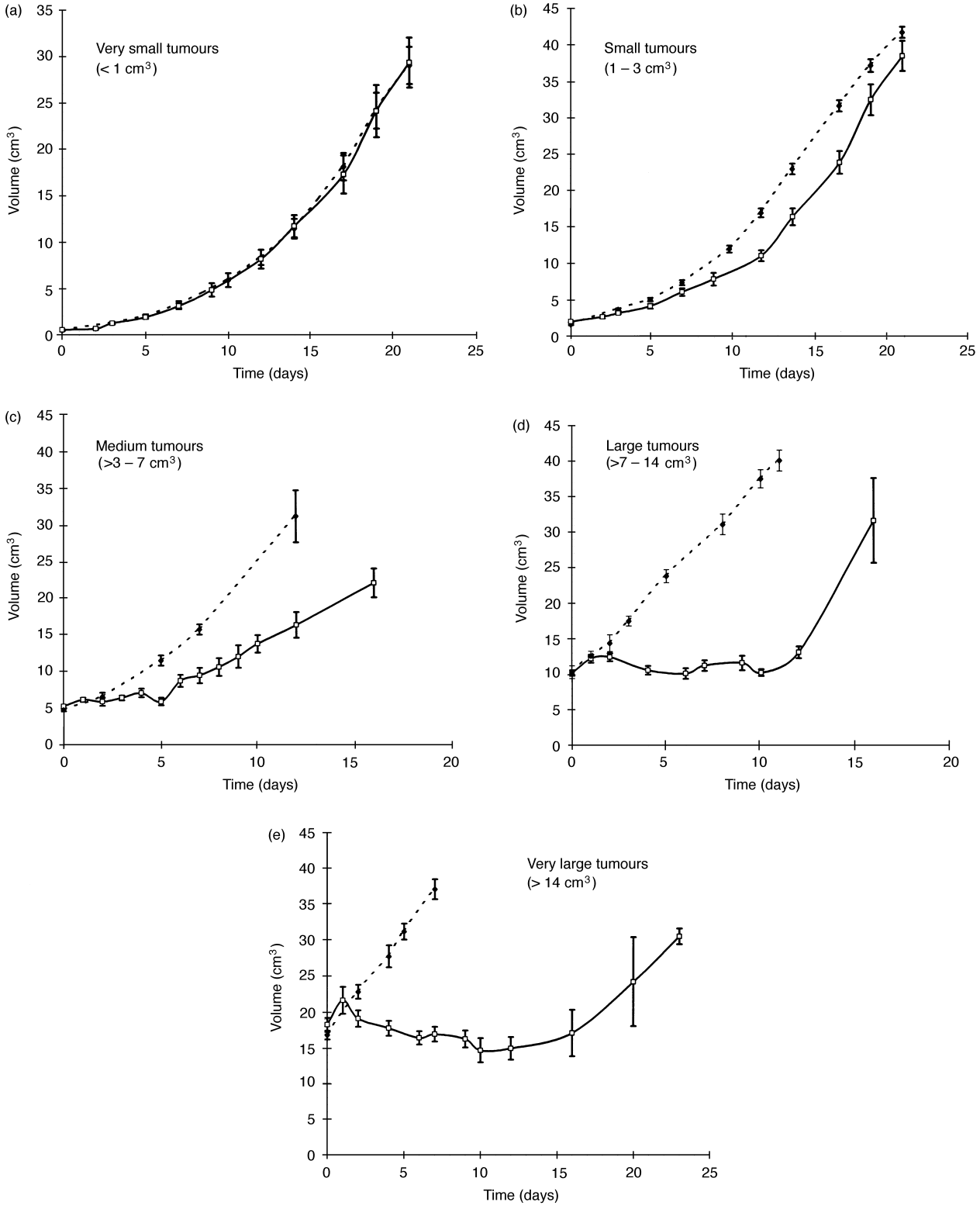

Fig. 1. Growth of rhabdomyosarcomas implanted subcutaneously (s.c.) in the flank of WAG/Rij rats: control tumours (closed symbol, broken line) and combreAp-treated tumours (open symbol, solid line). The different panels display the results with very small tumours (a); small tumours (b); medium tumours (c); large (d); and very large (e) tumours. The combreAp dosage was 25 mg/kg given as a single intraperitoneal (i.p.) injection. Vertical bars represent the standard error of the mean (SEM).

periphery (Fig. 2c). They had an irregular morphology and reduced diameter.

The histopathological examination of control rhabdomyosarcomas showed for all sizes a mainly cellular tumour with numerous actively dividing cells (Fig. 3a, b). Blood vessels were rather abundant and randomly located, showing a normal morphology. Sections of medium to large control tumours (>3–14 cm^3) revealed areas of necrosis dispersed in the centre as well as in the periphery. In very large tumours (>14 cm^3) proportionally more necrosis was seen. The blood vessels adjacent to these necrotic tumour parts were dilated. After injection of a single dose of 25 mg/kg combreAp, the picture promptly changed. Apart from the blood vessels present in the periphery of the tumour, dilatation and congestion were observable at 3 h postinjection of combreAp. Moreover, several small foci of fresh necrosis were present in the potentially viable parts of the tumours. After 6 h, the necrotic foci were larger and started to merge. After 1 day, the whole tumour showed extensive necrosis, except for a very narrow rim of potentially viable tumour cells. The blood vessels that were present in the peripheral rim showed no abnormalities. Just beneath this rim, small haemorrhagic zones were seen. Vessel wall interruption and endothelial cell damage were present in the necrotic area. At 2 and 3 days after combreAp injection, the rim of potentially viable tumour was even thinner and the haemorrhagic/necrotic area practically occupied the whole tumour volume (Fig. 3c, d): at this time interval, no bleeding was present when the tumours were transected. Analysis of the rhabdomyosarcoma tumours with volumes less than 3 cm^3 indicated that the combreAp-induced effects were quantitatively less than those

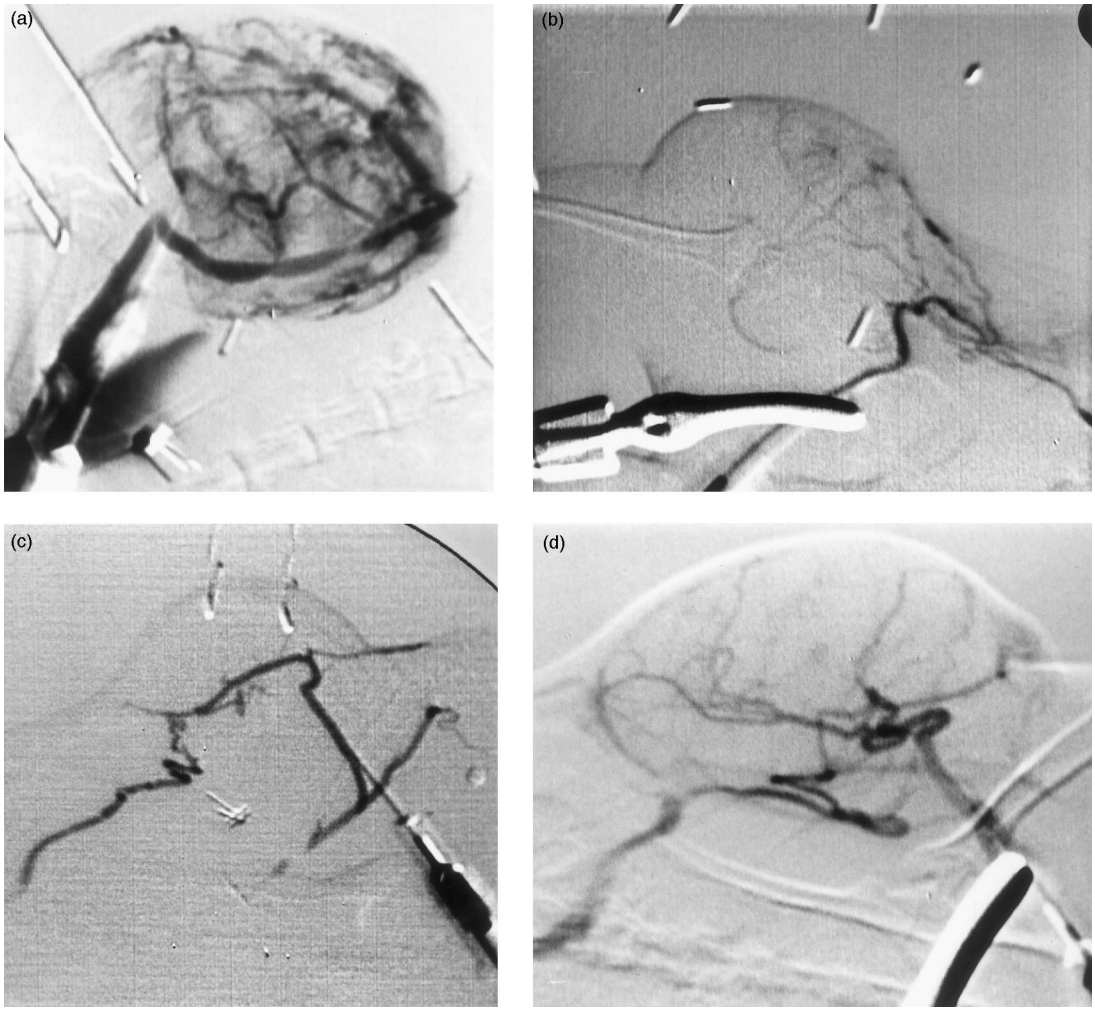

Fig. 2. Angiographic images (with digital subtraction of the body-background) of large sized rhabdomyosarcomas growing subcutaneously (s.c.) in the flank of WAG/Rij rats. They were randomly selected and representative for the imaging of tumour vascularity in control and combreAp treatment conditions. (a) Control, non-treated, tumour showing the wealth of regular blood vessels. (b) Image obtained 6 h after the combreAp treatment (25 mg/kg). A decrease in size and in number of the blood vessels was observed. (c) Angiographic screening of tumours 3 days after the combreAp injection revealed very poor vascularity, present only in the peripheral area. The remaining vessels had a reduced diameter and showed an irregular morphology. (d) At 10 days after combreAp treatment, characteristic features of newly formed vessel morphology were seen. Compared with the images obtained at the 3 day time interval, an increase in number of blood vessels was present.

described for the larger tumours: the ratio of induced necrosis to the viable looking areas was less than with the larger rhabdomyosarcomas. The timescale for presenting any vascular damage with subsequent necrosis was, however, again the same as described in detail above.

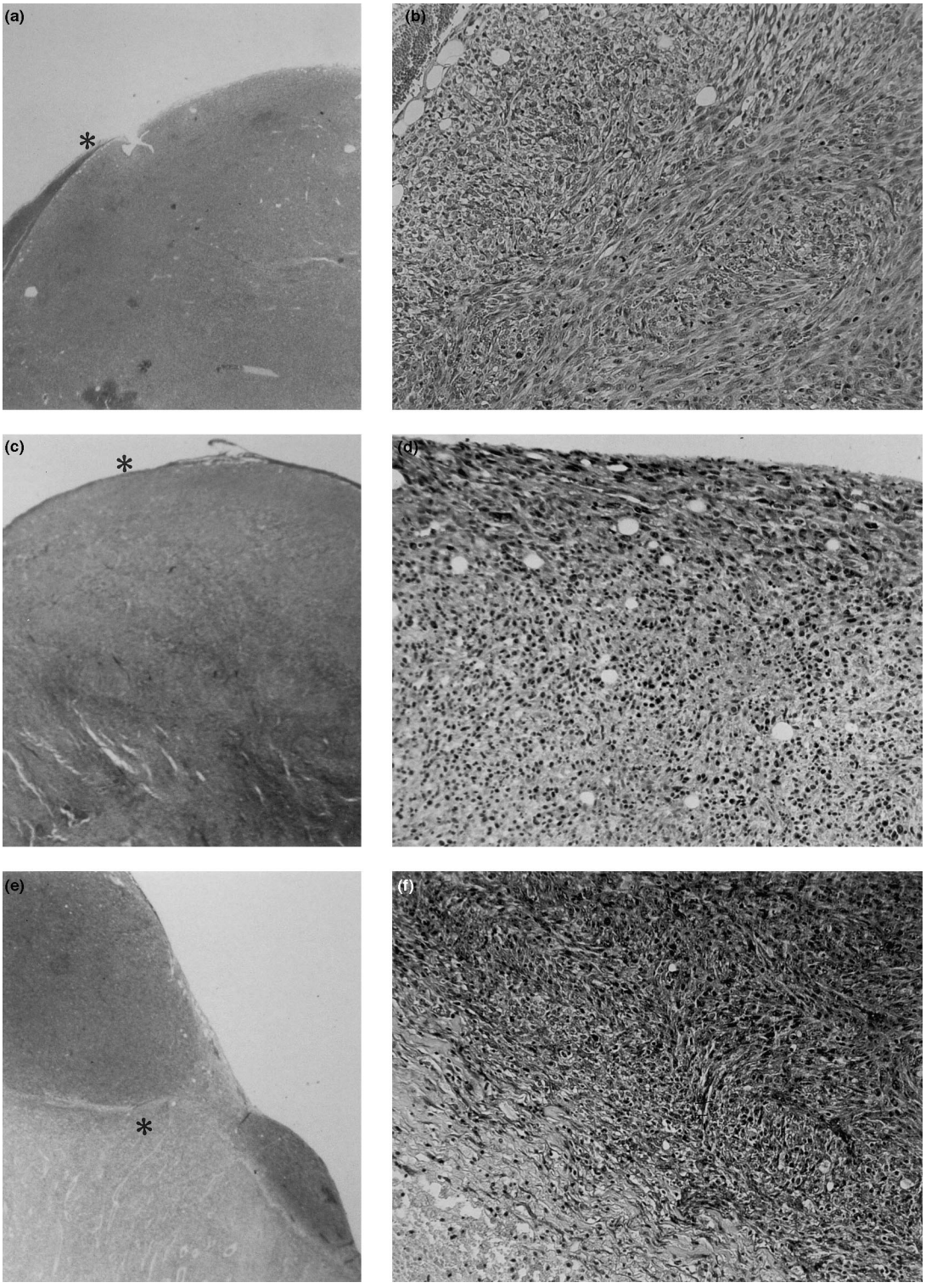

Fig. 3. Vascular targeting effect with combreAp in rat rhabdomyosarcomas. Photographs are representative of the tissue changes in large tumours, as observed at day 2–3 (c and d) and at day 10–11 (e and f) following a single intraperitoneal (i.p.) injection of 25 mg/kg combreAp. The (a) and (b) are microscopic images of control (no combreAp) tumours. The paraffin-embedded tumour tissue slices are stained with haematoxylin/eosin. Magnifications are ×10 (a, c and e, with ∗ indicating the magnified area) or ×100 (b, d and f).

The strong antitumour effects observed in the large and very large tumours, after the single combreAp injections and with three different techniques, were, however, not permanent. Subsequent to the period of reduced growth rate, a renewed increase in size was measured in these tumours, at a rate similar to the tumour growth observed in the control groups (Fig. 1d, e). This regrowth is also clearly documented with the histopathological and the angiographical approach. Indeed, when screening the slices of the large and very large tumours, removed and prepared at 10–11 days after the 25 mg/kg combreAp injection, several peripheral thickenings and outgrowths were present (for example in Fig. 3e, f). In the intermediate areas, a 'capsule' had been formed that consisted of fibrous tissue in which some dispersed tumour cells were present. In most tumours, this fibrous capsule surrounded the completely necrotic original tumour mass. The blood vessels were, for the same time interval, angiographically imaged only at the periphery (Fig. 2d): they were relatively large in size and number, having a regular morphology comparable with the peripheral vessels of control untreated tumours. In contrast, the histopathological analysis of medium and even more so of the small tumours showed that growth of the combreAp-treated tumours rather occurred as a broad expansion of the potentially viable peripheral area.

With the strategy to introduce a second combreAp injection at a specific stage of tumour regrowth, the same message was obtained: a slightly stronger effect was observed with larger tumours compared with small-sized tumours. As described above, the regrowth of the large tumours from the remaining potentially viable rim often showed a nodular pattern. These outgrowths shared the size of very small or small tumours prior to any treatment ($\leqslant 3$ cm^3). Although they were responsive, it was to a lesser extent than could be expected if the tumours were still considered large, based on their total volume (Fig. 4a). The interpolated additional growth delay at 1.5 times the increase in size following the second injection is approximately 3–4 days ($P < 0.001$). Regrowth of small tumours occurred as an overall expansion at the tumour edge. Following the second combreAp treatment, given when these tumours reached 4–7 cm^3, they showed a growth delay of approximately 5 days (Fig. 4b). In fact, this growth delay corresponds with the one measured for medium tumours when only a single combreAp injection was used (see Table 1).

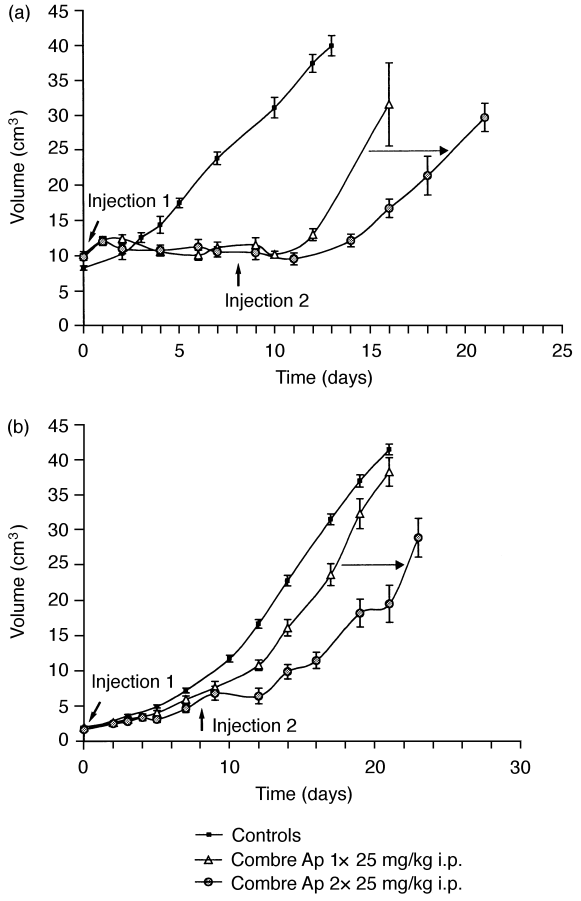

Fig. 4. Growth of subcutaneous (s.c.) implanted rhabdomyosarcomas: changes in growth delay after the use of 1×25 mg/kg (△) or 2×25 mg/kg (○) combreAp given intraperitoneally (i.p.) (time of injections is indicated). The figure shows the results for large (a) and for small (b) tumours. The additional gain in the growth delay is identified with an arrow. Vertical bars represent the standard error of the mean (SEM).

4. Discussion

Cancer growth requires the expansion of the vascular network to balance the cellular needs for oxygen and other nutrients. This vessel sprouting occurs initially from the host and secondarily from both the host and the acquired tumour blood vessels. Eventually, the tumour expands between the surrounding newly formed capillary sprouts. It has been well described that this tumour neovasculature shows an irregular pattern of convoluted and dilated vessels with less differentiated and more rapidly proliferating endothelial cells. It is therefore attractive to introduce compounds that directly attack the vulnerable tumour blood vessels with the subsequent initiation of a cascade of tumour cell death. This should at its best occur without treatment limiting normal tissue damage.

The combretastatin A4 phosphate prodrug (combreAp) belongs to a family of tubulin inhibitors for which isolation, structure and activity have been described [12,22,23,28]. The major target of the drug activity seems to be the ill-structured tumour blood vessels and their endothelial cells, with more specifically the inhibition of tubulin polymerisation. Apoptotic death has been demonstrated *in vitro* with normal human umbili-

cal vein endothelium [29]. More details about the mechanisms of action of combreAp, specifically in tumour blood vessels, remain to be unravelled, and further discussion of this aspect is beyond the scope of the present paper.

Recently, using transplanted tumours in mice, several laboratories have shown that a single administration of combreAp induced a vascular shutdown as early as 6–12 h after injection, with the subsequent formation of haemorrhagic necrosis [24–26,30]. To our knowledge, all of these reported *in vivo* data involved the treatment of very small rodent tumour models. It is obviously important to screen, when possible, the activity of such a compound in larger tumours of any type. In radio- and chemotherapy it is firmly documented that a differential antitumour effectivity exists, for different reasons, at least partly related to tumour sizes at treatment times.

Our investigations, using the established rat rhabdomyosarcoma cancer model, are intriguing in that they illustrate the dramatic change in growth pattern from combreAp relative to the tumour volume at the time of treatment. With the very small and small rhabdomyosarcomas, no or a borderline significant growth delay was measured respectively. This result is similar to data published on very small murine tumours, where no growth delay was seen at a systemically non-toxic combreAp dose [25,26,30]. However, as the rhabdomyosarcoma tumour volume at the time of the combreAp injection increased (>3 cm^3), the growth delay became more important. For instance, the difference in delay with the rhabdomyosarcoma between the small (1–3 cm^3) or very small (<1 cm^3) and the very large (>14 cm^3) tumours was approximately 7- and 17-fold, respectively. Moreover, a significant shrinkage was also observed with the larger tumour sizes rapidly after the combreAp injection (see Fig. 1b). This 'inverse' (compared with e.g. radio- or chemotherapy) volume–response relationship (compared with e.g. radio- or chemotherapy) could be expected, if one accepts that the corollary requirement for newly acquired blood vessels increases with the size of the tumour. This implies at first glance that larger tumours may have proportionately more ill-formed and eventually combreAp sensitive vasculature than small tumours, that initially have their nutrient supply through the peripheral and the co-opted host vasculature [6,31]. A further and strong impairment of an already inadequate blood supply due to combreAp activity will consequently stop tumour growth and may lead to tumour growth retardation, as is clearly demonstrated in the present study specifically with the tumours larger than 7 cm^3.

To evaluate the overall blood vessel changes, standard neuroradiological and interventional radiological digital subtraction angiography was performed with an iodinated contrast-medium and image analysis. The applied intra-operative technique avoids the dilution of the contrast medium in the total body circulation, which occurred when we used injections in the tail vein. The angiographic images obtained at 3 h after the combreAp injections already showed effects on the tumour vascularity. At later time intervals (up to 3 days) reduction in vessel diameter, changes in morphology and extensive loss of blood vessels within the tumour were obvious. The histopathological microscopy observations of tumour slices were in agreement with these findings, with the first signs of vascular damage (dilation and congestion) being clearly detectable at 3 h postinjection. At 6 h after the combreAp treatment, evidence of damage of blood vessels and endothelium was detectable with the microscopic analysis. An obvious deduction from the combination of the two clinico-analytical approaches is the relationship between the 'disappearance' of blood vessels and the increase in necrosis, both phenomena clearly present 1 day after the combreAp treatment. The early presence of tissue changes was also observed histopathologically in the smaller rhabdomyosarcomas, similar to the reports on small mouse tumour models which documented acute vascularity changes and blood flow reductions [12,24,26]. Such an acute manifestation of necrosis has been reported separately with experiments involving mouse tumours treated with flavone acetic acid (FAA) [20]. A strong reduction in tumour blood flow, indicative of vessel damage, was measured within 4–6 h after FAA administration [16,21,32]. The overall pattern of the combreAp-induced necrosis, being preceded by vascular defects, also resembles the antitumour effects observed with tumour necrosis factor-α. Both Baguley and colleagues [20] and Mahadevany and coworkers [32] discussed the involvement of the tumour necrosis factor-α when analysing the antitumour effects of FAA in their biological systems. Anyhow, the straightforward relationship between the severity of vascular shutdown and the antitumour effect seems obvious. Indeed, a significant growth delay was only obtained following a temporary clamping of tumour blood supply for 12–14 h, whereas 1–2 h occlusion induced little changes in tumour growth [33]. It seems clear that the suppression of rhabdomyosarcoma tumour growth by combreAp is mainly the result of selective targeting of the acquired tumour neovasculature. This is consistent with the fact that the absolute tumour volume, and inherently the absolute number of tumour cells depending on these established ill-formed blood vessels for their survival, seems to be an important parameter determining the growth delay after treatment. In addition, larger tumours inherently may have more newly formed blood vessels which can be aberant in structure and function and thus more vulnerable to the action of such agents. A correlation between small and large rhabdomyosarcomas and their vasculature may be indirectly deducible from a comparison of our results obtained with

antiangiogenesis treatment in the same tumour model [34] and the present combreAp data. With the use of TNP-470, a fumagillin analogue and specific angiogenesis inhibitor, growth delays were more pronounced in tumours smaller than 7 cm^3. This contrasts, with regard to tumour volume, to the effect seen with combreAp administration. Both these observations obtained with vascular targeting and anti-angiogenesis, taken together may indicate differences in tumour size-related vascular quality (and quantity) at least in rat and rhabdomyosarcoma.

It could also be hypothesised that combreAp finds activation through hypoxia, present to a much greater extent in large tumours compared with those smaller than 3 cm^3. It may be worthy to think about this possibility, since we earlier showed with the same tumour model that only tumours larger than 4–5 cm^3, having a sufficient amount of severe hypoxia/necrosis, could be colonised with anaerobic bacteria [35]. The possibility for such an inter-related activation will be investigated.

Finally, some direct cytotoxic effects of combreAp towards the tumour cell population, as indicated in the recent literature for some tumour cell types [25,30,36], may have to be taken into account in our *in vivo* rhabdomyosarcoma studies.

The present data describing the influence of tumour size on the outcome of vascular targeting treatment, may be compared with only a few other studies. Indications for a similar size–response relationship have to our knowledge only been suggested for hyperthermia and for FAA treatments [15,16]. In some of these experiments, the effect of treatment was also less with very small tumours compared with relatively larger ones. However, the use of these agents at their respective anticancer effectivity was hampered by severe side-effects. The absence of obvious systemic toxicity related to the combreAp dosage and injection site, used in our investigations, are therefore an additional advantage. The rats maintained normal activity, with no evidence of bleeding, diarrhoea or skin lesions at the tumour transplantation or injection site. The absence of normal tissue injury in parallel with strong tumour cell kill has been quoted by other research groups using various mouse tumour models [24–26,30].

In view of the absence of systemic side-effects, the overall research with combreAp encourages the use of such tumour vessel targeting drugs as a novel cancer treatment modality, specifically for large tumours. With the present rhadomyosarcoma tumour model, a clear-cut differential tumour volume versus response relationship with combreAp injection is demonstrated. In addition, the double combreAp treatment indicates this positive volume-dependent growth delay effect. Furthermore, the data demonstrate that even tumours smaller than 3 cm^3 can be significantly inhibited in growth with an appropriately scheduled repeat injection of combreAp. This 'inverse' response is at first surprising, as well as promising, since traditional anticancer therapies, such as radiotherapy and chemotherapy, are less effective on large solid tumours than on small ones [37,38].

It is obvious that, since a single combreAp treatment is not curative on its own, a combined strategy with e.g. radiotherapy and/or chemotherapeutic drugs will be necessary to improve tumour control. Pilot experiments involving the mouse CaNT tumour model, with either irradiation or with cisplatin in combination with combreAp, provide a positive indication [30].

Corroborating the results described herein with the rhabdomyosarcoma tumour model, it is conceivable that patients who present with large inoperable solid tumours or who relapse after other treatments, may benefit from a vasculature targeting treatment. At present, phase I trials with combreAp treatments are ongoing in Europe and the USA, of which the preliminary results are promising.

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