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IN VIVO ANTITUMOR EFFECT OF VASCULAR TARGETING COMBINED WITH EITHER IONIZING RADIATION OR ANTI-ANGIOGENESIS TREATMENT

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Purpose: Interference with the tumor blood vessels through anti-angiogenesis or vascular targeting can indirectly suppress tumor growth. Vascular targeting of solid tumors, using tubulin-compromising agents, seems a promising and selective novel treatment. We aimed to evaluate the potential (hypothesis-based) benefit from combinations of vascular targeting using combretastatin A-4 phosphate (combreAp) with either ionizing radiation or anti-angiogenesis.

Methods and Materials: Rhabdomyosarcoma tumor pieces were inplanted subcutaneously (s.c.) in the lower flank region of syngeneic adult WAG/Rij rats. Tumors were grown until different sizes and stratified for the various treatment groups: small (1–3 cm^3), medium (3.1–7 cm^3), and large (7.1–14 cm^3). CombreAp was injected i.p.; injections of TNP-470 were s.c. in the neck area. Localized single-dose (8 Gy) irradiations of tumors were done under Nembutal anesthesia, always 1 day before a single combreAp (25 mg/kg) injection. The TNP-470 treatment (3 times 30 mg/kg in 1 week) started 1 day after a double (8 days interval between both) combreAp administration. Tumor responses were evaluated by the growth delay assay, and statistical significance of tumor growth change was computed.

Results: Large tumors responded better to combreAp treatment given alone than did the smaller ones, confirming our previous data with this tumor model. Combining irradiation with combreAp also resulted in a tumor size–dependent growth delay. With small and medium tumor volumes, a similar response was measured after the combination treatment when compared with irradiation only. Large tumors, however, showed a strong (at least additive) increase of the growth delay with the combined therapy; the difference in tumor growth between the two treatment groups was very significant ($p < 0.0001$).

When TNP-470 was combined with combreAp, no significant lengthening of the growth delay, irrespective of the tumor size, was present with the applied schedule.

Conclusion: The current data show a significant advantage in the combination of combreAp with irradiation in rhabdomyosarcomas having a large size (7–14 cm^3) at treatment. Such a benefit in tumor response was not observed with the smaller tumors, seemingly because irradiation as such was very effective. No significant gain in growth delay for any tumor size was observed when TNP-470, showing efficacy on its own specifically with tumors measuring <7 cm^3, was added to the combreAp treatment. This presumably reflects only little angiogenesis during the first week of rhabdomyosarcoma regrowth after the combreAp treatment. © 2001 Elsevier Science Inc.

Vascular targeting, Anti-angiogenesis, Radiotherapy, Tumor growth delay, Tumor size, Combretastatin A-4 phosphate, TNP-470.

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INTRODUCTION

Tumors require blood vessels for nutrient and oxygen supply to maintain their viability. In the first time stage of growth, this demand is anticipated by the cooptation of host vasculature. To continue tumoral expansion, the formation of additional blood supply is a prerequisite (1, 2). The presence of these morphologically and functionally abnormal tumor blood vessels makes vascular targeting an attractive approach in anticancer treatment (reviewed by, e.g., 3, 4). Tubulin-binding agents were identified to exert antivascular activity in solid tumors, though not without some morbidity (5, 6). Recent mouse tumor experiments involving combretastatin A-4 phosphate (combreAp), a compound that interferes with the tubulin polymerization, provided evidence for *in vivo* antitumor effectivity at a systemically nontoxic dose of 100 mg/kg given intraperitoneally (i.p.) (e.g., 7–10). With BD9 rats, however, a combreAp dose of 100 mg/kg induced significant though transient blood flow changes in normal tissues, but still very much less than in the tumor tissue (10). This could indicate a lesser tolerance to combreAp in these larger animals when compared with mice. Indeed, an extension of this normal tissue analysis involving doses below 30 mg/kg (including 25 mg/kg) indicated very little vascular effects, while retaining its antitumor activity (G. Tozer, personal communication and meeting report at the BIOMED II Concerted Action on 'Tissue Oxygenation', 06/2000, Pisa, Italy); a lack of clear-cut systemic toxicity at a dose of 50 mg/kg combreAp was observed with BD IX/Han Foss rats (11). Also in our previous investigations on the efficacy of combreAp toward rat rhabdomyosarcoma, we found no reflection of systemic toxicity with the WAG/Rij rats after i.p. injection of 25 mg/kg or less (12).

Tumor size has been discussed to be an important determinant in the tumor response to a specific treatment (e.g., 13, 14). Using combreAp specifically, an 'inverse' volume-response relation (with respect to radio- or chemotherapy) has been demonstrated with a single injection, at least in the rat rhabdomyosarcoma tumor model (12). Longer growth delays were measured with large tumors (>7 cm^3) as compared with those that measured less than 3 cm^3 at the start of the treatment. Notwithstanding a major necrosis induction, this vascular targeting treatment did not result in a complete tumor response (e.g., 7, 12). To find an improvement of the antitumor response, we now examined the effect from combinations of combreAp with two basically and mechanistically different treatment modalities.

The first series of experiments combined combreAp vascular targeting with ionizing radiation for treatment of rat rhabdomyosarcomas. We hypothesized we would obtain a (supra-)additive antitumor response with the combined direct (irradiation) and majorly indirect (combreAp) cytotoxic effect. It was assumed that the proliferating tumor cells, predominantly present in the peripheral host vessel–irrigated region of the tumor, should be most vulnerable to ionizing radiation. The combreAp, on the other hand, should induce, as the result of collapse of the postangiogenesis intratumoral vascularity, a cascade of indirect tumor cell death, including the killing of hypoxic, radioresistant cells. Based on this hypothesis, we further assumed to obtain a more important advantage with tumors having large sizes and inherently larger hypoxic areas at the start of the combination treatment.

In the second part of the investigations, we assumed that the tumor re-growth following the combreAp treatment would be paralleled by a renewed angiogenesis. Blocking the latter process would result in a further arrest of tumor growth. Indeed the more established anti-angiogenesis compounds, including the synthetic fumagillin analog TNP-470, were demonstrated to interfere to a variable degree with tumor growth (reviewed by, e.g., 15, 16). In the present study, TNP-470 was therefore given in conjunction with the combreAp treatment. Again we incorporated the question of tumor size–dependent effectiveness of this combination strategy. The selection of TNP-470 was related to our previous experience with this compound in the rat rhabdomyosarcoma tumor model (17).

In general, the data presented thus aimed to demonstrate an improvement of the combreAp vascular targeting treatment from the combination with either ionizing radiation or TNP-470 anti-angiogenesis and to eventually relate the results with the tumor volume.

METHODS AND MATERIALS

In vivo *tumor model*

Male adult WAG/Rij rats weighing at least 270 g were used for all the present experiments. The animals were housed 4 per cage and had food and water *ad libitum*. The syngeneic rhabdomyosarcoma, at its origin an X-ray-induced jaw muscle tumor (18), was implanted s.c. in the lower flank of lightly ether-anesthetized animals. Pieces of about 1 mm^3 were used in the sequential series for at most 10–12 passages, whereafter transplantation was restarted from the frozen cell stocks.

Treatment was initiated when tumors reached the predetermined size: either 1–3 cm^3, 3.1–7 cm^3, or 7.1–14 cm^3; these sizes are referred to respectively as small, medium, and large.

After the start of any treatment, rats were weighed regularly, and the mean body weights were compared with those for the appropriate single-agent controls, including a batch of untreated tumor-bearing rats.

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

Drug treatment

The vascular targeting compound combretastatin A-4 phosphate (combreAp) was obtained from OXiGENE, Lund, Sweden, and stored at 4°C. (The compound is presently manufactured by Bristol-Meyers Squibb, Princeton, NJ.) Immediately before i.p. injection, the necessary

amounts were dissolved in 0.9% saline. A dose of 25 mg/kg was used, a selection based on our previous toxicity evaluation (12).

The angiogenesis inhibitor TNP-470 (Takeda Chemical Industries, Japan) was kept at −20°C, and solutions were freshly prepared before use in 10% ethanol/5% arabic gum in saline. Injections of 30 mg/kg each were s.c. in the neck area of the rats; the dosage was based on previous TNP-470 experience in WAG/Rij rats (17).

Radiation treatment

Local tumor irradiation, with the remainder of the body adequately shielded, was done with a linear accelerator (Saturne 42, General Electrics) using an18-megavolt beam and a dose rate of 3 Gy/min. Dosimetry was performed in treatment condition. A perspex tissue-equivalent plate of 2.5-cm thickness was placed above the tumors. To allow a correct positioning of the tumor, rats were anesthetized with sodium pentobarbital (Nembutal, max. 0.1 mL/100 g body weight).

Treatment protocols and response evaluation

CombreAp *plus* ionizing radiation: CombreAp was given as a single i.p. injection (25 mg/kg) and combined with a single radiation dose of 8 Gy, given 1 day before the combreAp.

CombreAp *plus* TNP-470: For the different preselected tumor sizes, combreAp was injected i.p. (25 mg/kg) 2 times (days 0 and 8). The TNP-470 (30 mg/kg) was given s.c. 3 times during 7 days, starting 1 day after the second combreAp injection (days 9, 12, and 15).

The response of rat rhabdomyosarcoma to the various treatments was evaluated by the classical tumor growth assay. Using calipers, tumors were measured 2–3 times per week, and volumes were calculated as $[(a \times b \times c) \times \pi/6]$ where a, b, and c are orthogonal dimensions. Tumor growth delay time was derived from the mean growth curves.

Computed analysis to differentiate between the treatment groups was performed using generalized linear models based on the asymptotic chi-squared distribution of the likelihood ratio statistics. Values of $p < 0.05$ were considered significant.

RESULTS

Most of the combination experiments indicated the absence of a straight body weight reduction when compared with the use of either agent alone. Only in the series combining the double combreAp (2×25 mg/kg i.p.) and the triple TNP-470 (3×30 mg/kg s.c.) a body weight loss of at most 10–15% was recorded. This reduction lasted for about 2–3 weeks in some animals, before recovery in body weight was measured.

CombreAp *plus* ionizing radiation effect

Figure 1 (A, B, and C) illustrates the growth pattern of rat rhabdomyosarcoma treated with combreAp alone (25 mg/kg), single-dose irradiation (8 Gy) alone, and combreAp combined with irradiation and includes the growth in untreated condition for comparison. The different treatments were evaluated in tumors with the various preselected volumes ranging between 1 and 14 cm^3, and these experiments were carried out two times independently. These data demonstrate a clear-cut size-dependent strengthening of the growth delay as compared with radiotherapy alone. With small tumors (1–3 cm^3) at the start of the treatment, no increase in growth delay was seen with this combination (Fig. 1A). The medium and large tumors, on the contrary, showed respectively a weak (3 days) or strong (10 days) increase in the growth delay (Fig. 1B and 1C, respectively). The resulting growth delays and related significance levels are summarized in Table 1A for each tumor size and treatment.

CombreAp *plus* TNP-470 effect: Rats bearing s.c. rhabdomyosarcoma tumors were treated twice with a combreAp dose of 25 mg/kg. The time interval between the two i.p. injections was 8 days, a selection based on our previous studies with rat rhabdomyosarcoma. The TNP-470 was injected s.c. three times in 7 days, with the first injection of 30 mg/kg at 1 day after the second combreAp administration. Experiments were carried out in duplicate. The results of these tumor size–related experiments are displayed in Fig. 2 (A: small, B: medium, and C: large tumors). The application of the TNP-470 after the combreAp introduced only a minor, nonsignificant additional growth delay of 2 days, compared with that seen for combreAp alone. This additional growth delay was independent of the tumor size at the start of the treatment (See Table 1B for details).

DISCUSSION

The introduction of anti-angiogenesis in the 1980s, and more recently of vascular targeting, has provided new ways to attack solid tumor growth and its spread in the body. The mode of action involves a selective interference with blood vessel constituents and subsequently a cascade of tumor cell death.

In the broad perspectives of anticancer vascular targeting, modalities such as targeting tissue factors, necrosis induction through selective damage of the tumor vascular network, blocking antibodies to angiogenic factors, or endothelium-related gene therapy are being tested (e.g., 19–23). Separately, the use of vascular targeting with tubulin-compromising compounds carries a substantial promise (5, 7, 24, 25). Specifically with combreAp, a single injection of a nontoxic dose resulted in an extremely rapid and extensive collapse of tumor vasculature. This effect and the resulting indirect antitumor response have been demonstrated with several rodent tumor models (7–10). Whereas these detailed studies involved only small tumors ($<<3$ cm^3), we demonstrated at least with a rat rhabdomyosarcoma tumor model that the extent of growth delay depended on the size

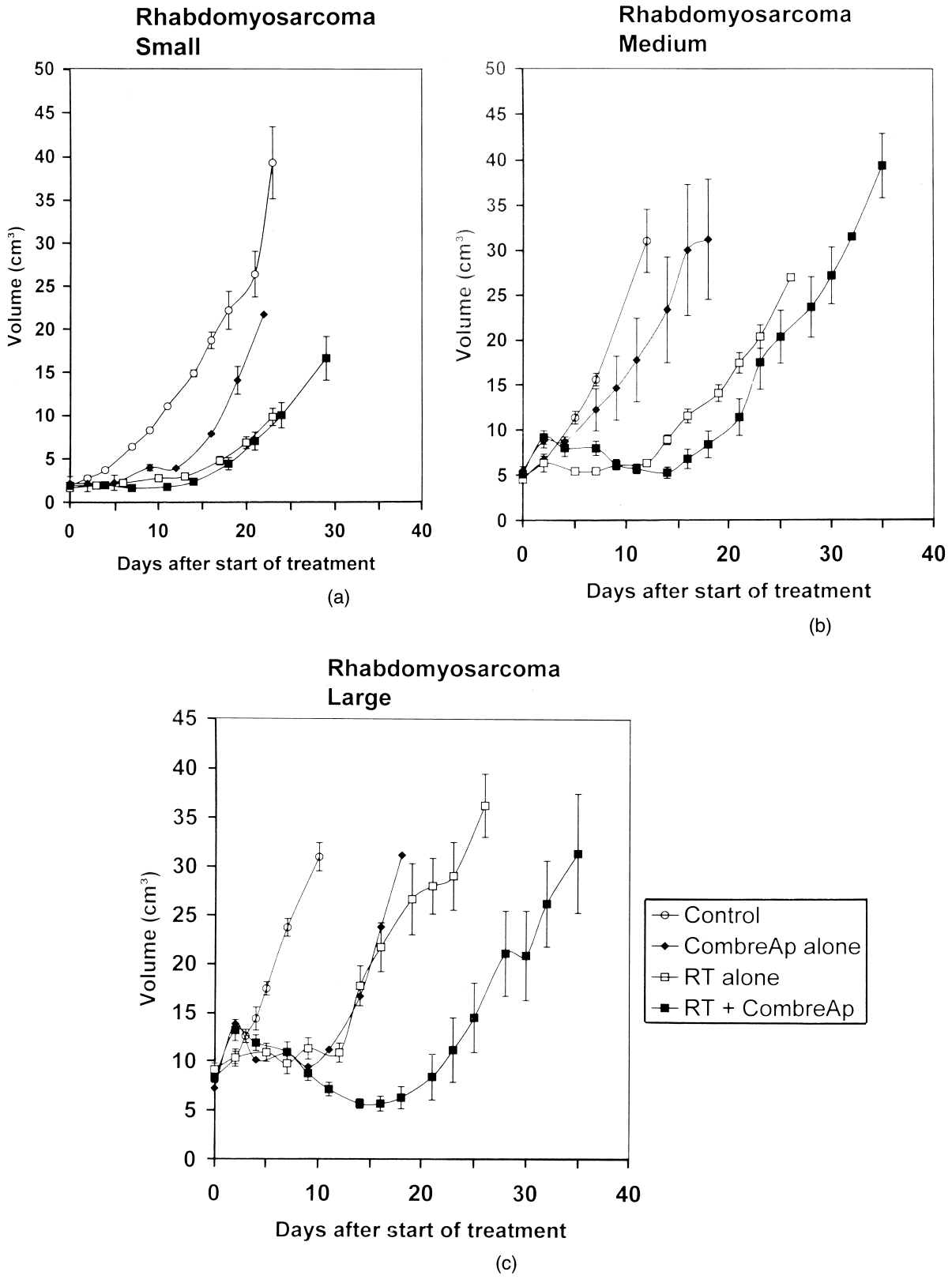

Fig. 1. CombreAp *plus* ionizing radiation; growth curves for rat rhabdomyosarcoma in the three different treatment series: (a) small, (b) medium, or (c) large tumors at the start of treatments. combreAp = 1 × 25 mg/kg, given i.p.; RT = 8 Gy single dose; combination = irradiation at 1 day prior combreAp. All values represent the mean ± SEM (when not detectable, they are within the symbol size). On average, 10–15 tumors were used for each of the treatment groups.

of the tumor at the time of combreAp injections; i.e., the larger the tumor (>>3 cm^3), the firmer the response (12). In comparison with the standard radio- or chemotherapy, this is clearly an inverse size-dependent responsiveness. It is therefore in our opinion important to introduce when possible the volume-response relation into studies, especially

Table 1. Growth delays with rat rhabdomyosarcomas for the various preselected tumor volumes and the different treatments

A. Single dose irradiation (8 Gy, at d0) and combreAp (1 × 25 mg/kg, at d1)

Volume (cm^3)	SD irrad. + combreAp	SD irrad. alone	CombreAp alone
Small (1–3)	13.5 days (n.s.)*	13 days	5 days
Medium (3.1–7)	18 days (n.s.)*	15 days	6 days
Large (7.1–14)	22 days (<0.0001)*	12 days	8 days

B. combreAp (2 × 25 mg/kg, on days 0 and 8) and TNP-470 (3 × 30 mg/kg, on d9, d12, and d15)

Volume (cm^3)	combreAp + TNP-470	combreAp alone	TNP-470 alone
Small (1–3)	8 days (n.s.)†	5 days	6 days
Medium (3.1–7)	9 days (0.047)†	7 days	6 days
Large (7.1–14)	15 days days (n.s.)†	13 days	8 days

Growth delays were derived at the level of the time necessary to reach 8× the starting volume (small tumors), 4× the starting volume (medium tumors), and 3× the starting volume (large tumors) for the treated tumors *minus* the time to reach the same volume in control condition.

Significance of difference in growth delay for the combined treatment:

* *versus* SD irrad. alone (A), or

† *versus* combreAp alone (B).

n.s. = not significant.

so since patients often present with tumors much bigger than 1–3 cm^3.

On the other hand, and for two decades already, anti-angiogenesis has also been the topic of extensive anti-cancer research. The effectiveness within the wide range of angiogenesis inhibitors under investigation may be categorized between "some growth retardation" and tumor "dormancy." With TNP-470, a fumagillin analog that inhibits proliferation and migration of endothelial cells and consequently disables new vessel formation, *in vivo* activity has been demonstrated with a battery of tumor models. Our earlier investigations in rat rhabdomyosarcoma with TNP-470 also showed tumor growth retardation, with the effectiveness depending on the schedule/dosing used and on the tumor volume at the start of the treatment as well (17).

Both these two different modes of vasculature-directed treatments, since not curative on their own, have been combined in a few studies with tumor cell cytotoxic agents, e.g., cis-Platinum, Cyclophosphamide, Taxol, or ionizing radiation, and this with variable success (8, 26–28).

The present *in vivo* investigations, using a rat rhabdomyosarcoma tumor model, examined the effect from combining combreAp either with ionizing radiation or with TNP-470 anti-angiogenic treatment.

Ionizing radiation *plus* combreAp

Theidea of applying irradiation before the combreAp treatment was to avoid a reduced radiation efficacy due to radioresistance from an enlarged hypoxic tumor cell population. CombreAp introduces necrosis, a process that indeed is preceded as well as paralleled by hypoxia. The selection of 8 Gy was for the most part based on the clinical use of such a fraction size, as demonstrated for the relief of pain from skeletal metastases (e.g., 29, 30). Our results with combreAp (25 mg/kg) given 1 day after the single irradiation demonstrate a tumor size–dependent additional growth delay when compared with radiotherapy given alone. The effectiveness of the combined treatment was much more beneficial—or at least additive—in tumors larger than 7 cm^3 vs. those that were smaller at treatment time, thus confirming our hypothesis. This differential overall response seen between large and small tumors seems related to the stronger impact of the combreAp treatment, and conversely, related to a lesser radiation effect because of the presence of many more hypoxic cells in large tumors. The existence of severe hypoxia in larger tumors has been indicated previously in the rat rhabdomyosarcoma, in a direct way using the hypoxic marker NITP and indirectly with the degree of anaerobic bacterial colonization with apathogenic *Clostridium* (31). On the other hand, the lack of a positive change in growth delay in small tumors may be explained as the result of the very efficient tumor cell killing from the irradiation as such (most cells sufficiently oxygenated). The induction of combreAp-related vessel damage and the subsequent tumor cell death would thus not substantially add (measurable in a growth delay assay) to the tumor cell killing from the preceding irradiation. Our data differ from the results obtained with the mouse adenocarcinoma CaNT tumor model by Chaplin *et al.*, demonstrating a clear-cut gain in growth delay from combining combreAp with irradiation (8). In contrast with our study, however, a fractionated irradiation schedule was used in combination with a double combreAp injection. Besides this observation, the

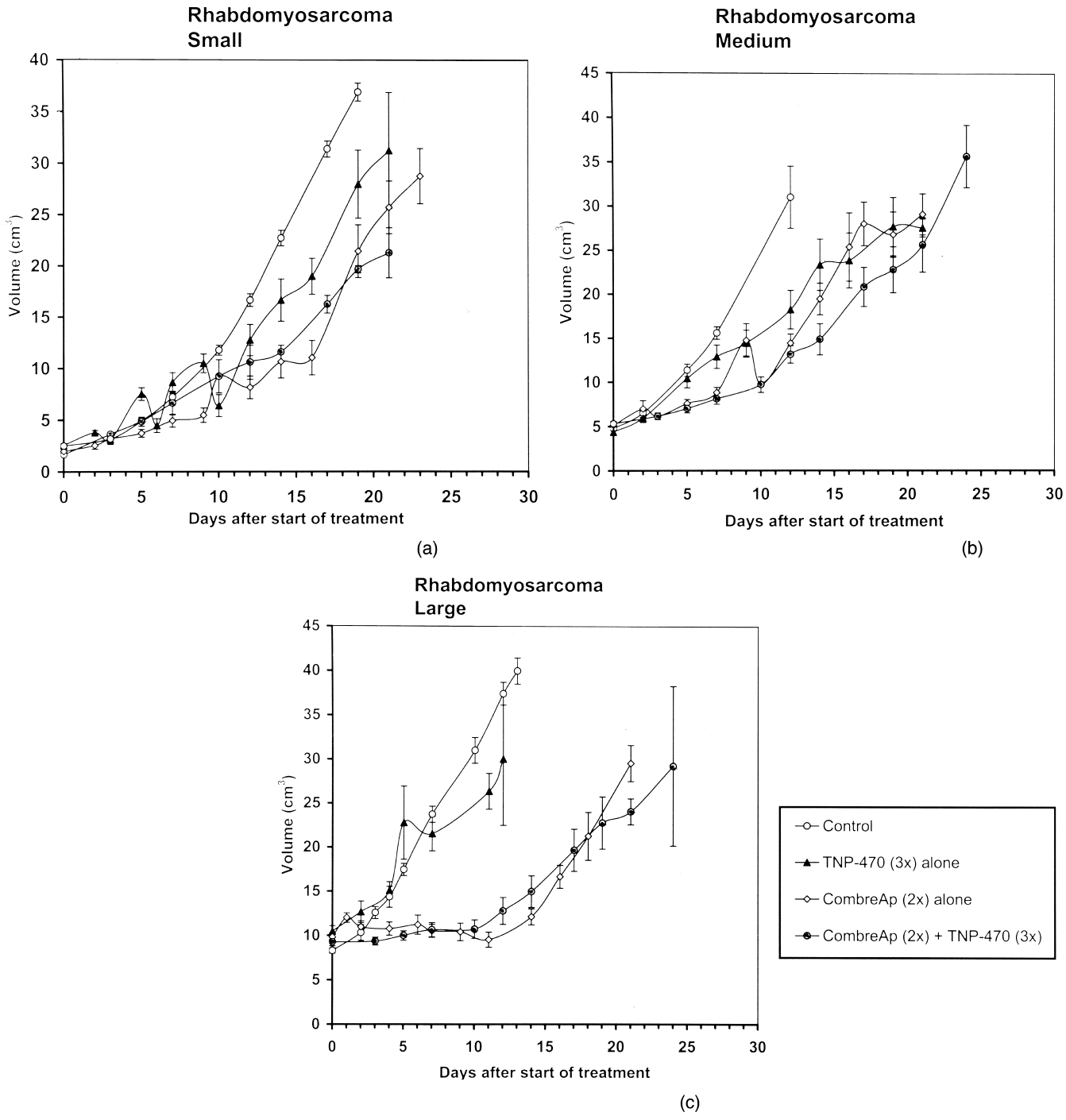

Fig. 2. CombreAp plus TNP-470; growth pattern of rat rhabdomyosarcoma for either control condition and for the various treatment series. Treatments: combreAp alone (2 × 25 mg/kg i.p. in 8 days) and TNP-470 alone (3 × 30 mg/kg s.c. in 1 week); and the combination of combreAp (25 mg/kg) injected on day 0 and day 8, followed by the TNP-470 treatments (30 mg/kg) 1 day later (days 9, 12, and 15). Results are presented for (A) small, (B) medium, and (C) large tumors at the start of treatments. All values represent the mean ± SEM. (If not detectable, they are within the symbol size). On average, 10–15 tumors were used for each of the treatment groups.

concomitant application of carbogen breathing may offer some explanation for the discrepancy. In addition, and of at least equal importance, the overall response to such a combined treatment will be related to the different characteristics of each type of tumor cell—difference in sensitivity to irradiation, along with difference between radiation-damaged cells' capacity to tolerate prolonged hypoxic condition as likely introduced by the combreAp treatment. The differential effectiveness will obviously also depend on the amount and inherent quality of the acquired tumor vessels in the various *in vivo* models. The *in vivo*/*in vitro* mouse KHT sarcoma experiments from Li *et al.* also show evidence of enhanced tumor cell kill when combreAp is combined with single-dose irradiation, but the results are not translated into growth delays (32). Very small tumors were used in this study. This perhaps explains the lack of a clear-cut growth

delay, as also observed in our studies with the very small tumors (12). Notwithstanding the differences in experimental design, it remains a question whether these tumor-type ranges of improved response incorporate a broadening of the therapeutic window, and therefore whether this aspect deserves an answer with appropriate *in vivo* normal tissue models. Based on the presently available information in the literature about the combreAp selectivity, combined treatment limiting normal tissue reactions should not be expected.

CombreAp *plus* TNP-470

We also aimed to identify with rat rhabdomyosarcoma the possibility of an improved response from combining combreAp with TNP-470, based on their different vessel-related action mechanisms. We hypothesized that the use of TNP-470 after the combreAp injections, specifically during the time of tumor regrowth, may result in increased tumor cell kill through the inhibition of vascularity renewal. Proof of the principle of anti-angiogenic activity being the major mechanism of TNP-470 antitumor activity has been discussed by several research groups (e.g., 33, 34). Underlying our reasoning is that rat rhabdomyosarcoma regrowth was clearly paralleled by angiogenesis, as evidenced with digital subtraction angiography of large tumors (12).

The present experiment results obtained with the combreAp *plus* TNP-470 combination show no statistically significant strengthening of the growth delay in rat rhabdomyosarcoma as compared with the effect from combreAp alone. Independent of tumor size at the start of the treatments, about 2 days of absolute extra growth delay were measured (see Table 1B). The easy explanation for the very small effect from the TNP-470 for all tumor sizes may be found in the possibility that not many new blood vessels were being formed during the time of the TNP-470 application. This would obviously result in less vascular-related antitumor effects reflected in a larger growth delay. Should we, on the other hand, think of a reduced accessibility of the TNP-470 that was injected after the combreAp? Possibly, but we hypothesized that for the major part the acquired intratumor vasculature will be severely damaged by combreAp, leaving host vessels at the periphery of the tumor intact. This has been indicated in the rat rhabdomyosarcoma using digital subtraction angiography and standard histopathology techniques (12). Also in publications from other teams, it has been reported that vasculature remains intact at the viable rim of combreAp-treated tumors. Since vessel sprouting can occur only from these intact vessels in the tumor periphery, TNP-470 should not find any obstruction to reaching its target and should thus do the job.

In conclusion, the present data and those from other groups demonstrate a potential benefit from combining ionizing radiation with combreAp treatments, and at the same time illustrate a tumor size and type dependent efficacy. Our results also indicate no significant improvement from the combination of TNP-470 treatment with combreAp injection, at least not in the present combination.

The mechanisms of combreAp activity are not yet fully understood, necessitating more experimental data involving different sequences and treatment duration/fractionation, as well as immunohistochemical measurements related to endothelial cell growth factors and hypoxia.

The limited preclinical studies are only the beginning of a journey involving vascular targeting, but at least support the potential use of combreAp in combination with classical anticancer therapies. Phase I clinical trials using combreAp alone have been conducted in several centers, and meeting reports indicated promising results.

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