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Preclinical report

Evaluation of combretastatin A-4 prodrug in a non-Hodgkin's lymphoma xenograft model: preclinical efficacy

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Combretastatin A-4 prodrug (CA4P) is a new antitubulin agent currently in phase I/II clinical trials against solid tumors. We have previously reported on the *in vitro* activity of CA4P against a panel of malignant human B-lymphoid cell lines. In this study, we investigated the antitumor and the antiangiogenic activity of CA4P in our diffuse large cell lymphoma WSU-DLCL$_2$-SCID mouse model. WSU-DLCL$_2$ cells (10^7) were injected s.c. into 5-week-old female ICR-SCID mice. Tumor-bearing mice were treated at the CA4P maximum tolerated dose (MTD) of 800 mg/kg in different dose/schedules. CA4P showed significant antitumor activity against this lymphoma model. Best results were seen when MTD was given in two and four divided doses (400 and 200 mg/kg, respectively). CA4P given in four divided doses (4×200 mg/kg) showed a $\log_{10}$ kill of 1.01, *T/C* of 11.7% and *T–C* of 12 days. Immunohistochemical staining using anti-CD31 antibody after 6, 24, 48 and 120 h treatment revealed a significant decrease in the number of tumor blood vessels after 24 h (about 80%). Only the periphery of treated tumors revealed the presence of blood vessels. Morphological examination of the tumors after tetrachrome staining showed a necrotic center in tumors of CA4P-treated animals. New blood vessel formation was noted to emerge in tumor tissues as early as 48 h following a single dose of CA4P. The G_2/M arrest observed *in vitro* was not detected *in vivo* indicating predominance of the antiangiogenic effects with regard to antitumor efficacy *in vivo*. We conclude that CA4P has antiangiogenic activity in this lymphoma model and the use of this agent should be explored clinically in the treatment of non-Hodgkin's lymphoma. [© 2001 Lippincott Williams & Wilkins.]

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Introduction

Combretastatins are a group of cancer cell growth inhibitory compounds isolated from the South African tree *Combretum caffrum*. Combretastatin A-4 (CA-4) has been shown to have antimitotic activity by inhibiting microtubule assembly and by competing with colchicine for binding to tubulin.[1,2] CA4P is a phosphorylated form of CA-4,[3] which has been shown to be more soluble and is hydrolyzed *in vivo* to the active form (CA-4) by endogenous phosphatases. CA4P has been reported to induce vascular shut down in murine adenocarcinoma NT and human breast carcinoma xenografts.[4,5] CA4P is currently in phase I/II clinical trials against solid tumors in the UK and US.

Over the last few decades, many efforts have been made to target angiogenesis. Angiogenesis, the formation of new blood vessels from pre-existing ones, is critical for the proliferation and survival of cancer cells.[6,7] Different strategies have been employed to inhibit this phenomenon. The first approach was to target specifically the endothelial cells by angiogenesis inhibitors like endostatin[8] and angiostatin.[9] Antibodies which block growth factor receptors such as vascular endothelial growth factor (VEGF) have also been investigated and successfully shown to inhibit angiogenesis in preclinical models.[10] The second strategy was to target tumor cells as well as endothelial cells using drugs such as paclitaxel,[11] thalidomide,[12] 2-methoxyestradiol, taxol,[13] cyclophosphamide[14] and vinblastine.[15]

Over 54 000 new cases of non-Hodgkin's lymphoma (NHL) occur annually and almost half of these are considered to be aggressive NHL.[16] At the present time, the majority of patients with newly diagnosed aggressive NHL receive an anthracycline-containing induction regimen such as CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone). However, CHOP induction therapy only cures approximately 40% of aggressive NHL patients, indicating a need to develop new drugs. Diffuse large cell lymphoma (DLCL) is the most common subset of NHL and is considered the prototype of curable lymphomas. Previously, we reported on the cytotoxic effects of CA4P against a panel of malignant human B cell lines *in vitro*.[17] CA4P induced growth inhibition which was mediated by cell cycle arrest in G_2/M and mitotic catastrophe.

In this study, for the first time, the antitumor and antiangiogenic activity of CA4P against the diffuse large cell lymphoma cell line (WSU-$DLCL_2$) in SCID mice xenografts was studied. The administration of CA4P at the maximum tolerated dose (MTD) or at one-eighth of the MTD resulted, after 24 h, in a significant vascular shutdown in WSU-$DLCL_2$ tumors. Furthermore, the MTD used at various schedules showed different antitumor activity.

Materials and methods

WSU-$DLCL_2$ cell line

The human diffuse large cell lymphoma cell line (WSU-$DLCL_2$) was established in our laboratory at Wayne State University School of Medicine[18] and was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. All chemicals for cell culture were obtained from Sigma (St Louis, MO).

CA4P

The prodrug CA4P was dissolved in phosphate-buffered saline (PBS) and injected i.v. CA4P was isolated from the South African tree *C. caffrum*, purified and phosphorylated as previously described.[3]

WSU-$DLCL_2$ xenografts

Five-week-old female ICR-SCID mice were obtained from Taconic (Germantown, NY). Two mice received 10^7 WSU-$DLCL_2$ cells in serum-free RPMI 1640 s.c. in each flank area. When tumors reached about 2000 mg, mice were sacrificed and tumors cut into fragments of 20–30 mg, which were bilaterally transplanted s.c. via a 12-gauge trocar into groups of mice designed for trial.

Antitumor activity trial

After transplant, WSU-$DLCL_2$ fragments developed into palpable tumors (200–300 mg). Groups of six animals were removed randomly and assigned to different treatment groups. The drug and the diluent (PBS) were injected i.v. via a tail vein. A control group received PBS, another group received CA4P at the MTD, which was determined in our laboratory to be 800 mg/kg in SCID mice, in a single or divided doses as shown in Table 1.

Assessment of tumor response

The endpoints for assessing antitumor activity were in accordance with standard procedures used in our laboratory and are as follows: (i) tumor weight (mg) = $(A \times B^2)/2$, where A and B are the tumor length and width (in mm), respectively; (ii) tumor growth inhibition (T/C) is the median tumor weight in the treated group (T) when the median tumor weight in the control group reached approximately 700 mg; (iii) tumor growth delay ($T-C$), where T is the median time (in days) required for the treatment group tumors to reach 700 mg and C is the median time (in days) for the control group tumors to reach the same weight; and (iv) tumor cell kill net ($\log_{10}$) = $(T-C)$ − duration of treatment in days/3.32 (T_d), where T_d is the volume tumor doubling time (in days), estimated from the best-fit straight line from a log-linear growth plot of the control group tumors in exponential growth (100–800 mg range). In this study the antitumor activity is considered highly active (++++) when the $\log_{10}$ kill (net) is more than 2.0. Activity rating scores of (++++) or (+++) are needed for translation to clinical activity, and equate with complete and partial tumor regression, respectively. A score of either (++) or (+) is not considered active by usual clinical criteria.

Immunohistochemical staining

Three groups of animals containing bilateral s.c. tumors were used. One group was designated as a control receiving one PBS injection, i.v., while the second group received CA4P one injection (100 mg/kg) and the third group received one injection of 800 mg/kg. After 6, 24, 48 and 120 h, three animals from each group were euthanized. The tumors were excised and frozen at −80°C. Sections of 8 μm were dehydrated in cold acetone for 2 min and rinsed in

Table 1. Antitumor activity of CA4P against $DLCL_2$-bearing SCID xenografts

Agent	Dose (mg/kg/day)	Route	No. of animals	No. of injections	*T/C* (%)	*T*−*C* (days)	Log_{10} kill (net)	Mean (mg)	Range (mg)	Activity score
Diluent (control)	0.0	i.v.	6	1	100	0	0	771±281	424–1221	−−
CA4P	800	i.v.	6	1	15.6	7	0.67	110±67	46–201	+
CA4P	400	i.v.	6	2	17.7	10	1.01	140±65	90–270	+++
CA4P	200	i.v.	6	4	11.7	12	1.01	37±13	18–54	+++
CA4P	100	i.v.	6	8	20.0	9	0.22	158±46	115–238	−

Rating score of (+++, active) or (++++, highly active) is needed to effect partial or complete tumor regressions; (++, marginal activity) and (+) is not active.

PBS. The endogenous peroxidase activity was reduced by treating sections with peroxo-block (Zymed, San Francisco, CA) followed by one water rinse. Nonspecific binding was blocked using 5% normal goat serum. Primary rat anti-CD31 (PharMingen, San Diego, CA) at a 1:25 dilution was added to the sections for 1 h. CD31 is a transmembrane glycoprotein present on endothelial cells. Slides were then rinsed and the biotinylated secondary antibody (goat anti-rat at a 1:100 dilution) was added for 30 min. Evaluation of blood vessel density was determined by counting nine random fields in each section under ×10 magnification. Four sections from each tumor were stained. Negative controls were produced by omitting the primary antibody. For a positive control, tissue from mouse spleen was stained for CD31.

Morphological analysis

Sections of 8 μm from untreated and CA4P-treated tumors taken from three groups of animals same as above were prepared, air-dried, stained with tetrachrome for 5 min and analyzed using light microscopy. Features of apoptosis that were assessed for included cell shrinkage, nuclear chromatin condensation, formation of membrane blebs and apoptotic bodies. Features of cell necrosis included cell swelling, nuclear expansion and gross cytolysis.

Cell cycle analysis

Tumors taken from untreated and CA4P-treated SCID mice were dissociated into single-cell using a cell dissociation sieve (Sigma) and 10^6 cells were fixed in absolute alcohol for 30 min at 4°C. After treatment with RNase for 40 min at 37°C, cells were stained with propidium iodide (50 μg/ml) and analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Data on 20 000 cells was acquired and processed using Lysys II software (Becton Dickinson) in the Molecular and Cellular Imaging and Analytical Cytometry Core Facility of the Barbara Ann Karmanos Cancer Institute at Wayne State University School of Medicine.

Statistical analysis

Differences in survival curves were analyzed by Student's *t*-test. All differences were deemed significant at the 95% confidence interval.

Results

Anti-tumor efficacy of CA4P

Table 1 shows the *in vivo* preclinical trial design and activity for CA4P. CA4P was given at its MTD, which was determined to be 800 mg/kg, in one dose (800 mg/kg × 1), two doses (400 mg/kg × 2), four doses (200 mg/kg × 4) or eight doses (100 mg/kg × 8. CA4P showed significant antitumor activity against WSU-$DLCL_2$ tumors. However, CA4P did not completely abolish tumor growth in mice in any of these treatments. The maximum antitumor activity was seen when CA4P was given at 200 mg/kg every day for 4 days. The *T/C*, *T*−*C* and log_{10} kill were 11.7%, 12 days and 1.01, respectively. However, according to log_{10} kill values, only 400 and 200 mg/kg had clinically meaningful activity, while the others were not considered active by usual clinical criteria. It should be noted that the activity rating score of (+++, active) or (++++, highly active) is needed to effect partial or complete tumor regression. Furthermore, the administration of 200 and 400 mg/kg showed a visible necrosis in tumors taken from the three treated animals.

Survival curves of SCID mice

The survival curves of SCID mice bearing WSU-$DLCL_2$ tumors treated with 800 × 1, 400 × 2, 200 × 4 and 100 × 8 compared with control are presented in Figure 1. The tumor weight of all animals in the control group

reached 2000 mg within 21 days from transplantation of WSU-DLCL$_2$. Treatment with CA4P significantly increases the time for the tumors to reach 2000 mg. Survival after CA4P treatment was significantly lengthened in mice receiving 800 mg/kg × 1 ($p \leqslant 0.004$ versus control) and 100 mg/kg × 8 ($p \leqslant 0.002$ versus control) when compared to the control. Mice receiving CA4P at 200 mg/kg × 4 and 400 mg/kg × 2 also showed significantly ($p \leqslant 0.0006$ versus control) increased survival when compared with control mice. Interestingly, all treatment groups showed significantly improved survival when compared to control animals. However, no significance was observed between animal groups receiving different doses and schedules of CA4P.

Effect of CA4P on tumor vasculature

To study the antiangiogenic effect of CA4P, we assessed the effects of two doses of the drug (100 and 800 mg/kg)—the lowest and the highest doses used to study the antitumor activity of CA4P—6, 24, 48 and 120 h after i.v. administration of the drug. After 6 h of treatment, there was no significant difference in blood vessel number in either treatment when compared with the untreated control. However, after 24 h, at both 100 and 800 mg/kg the percentage of vascular shutdown was 83 ± 6.2 and 82 ± 3.9%, respectively, when compared to the control (Figure 2). CA4P did not completely shutdown all blood vasculature in the tumors. A small number of blood vessels were observed at the periphery of the tumor sections, while the centers of these sections showed a complete absence of blood vasculature (Figure 3). Furthermore, the blood vessels observed in tumors of treated animals seem to be different (smaller in size, less circular, less branched) from those observed in the control. Significant reduction in the number of blood vessels was also seen after 48 h (61 ± 9.5% for 100 mg/kg and 68 ± 8.5% for 800 mg/kg). After 120 h, as after 48 h, the vascularization in the tumors of CA4P-treated mice began to return but still was significantly lower compared with controls. The shutdown of blood vessels was 51 ± 6.2% for 100 mg/kg and 46 ± 1.8% for 800 mg/kg. Interestingly, this vascularization was localized in two areas situated at the periphery of the tumors.

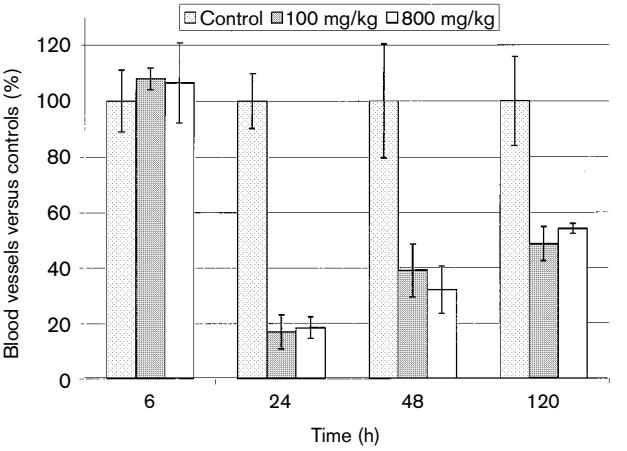

Figure 2. Effect of CA4P on the number of blood vessels of the human WSU-DLCL$_2$ growing s.c. in SCID mice. Data are expressed as percentage vascular relative to the control and are the mean of three tumors in each group. Bars = SE.

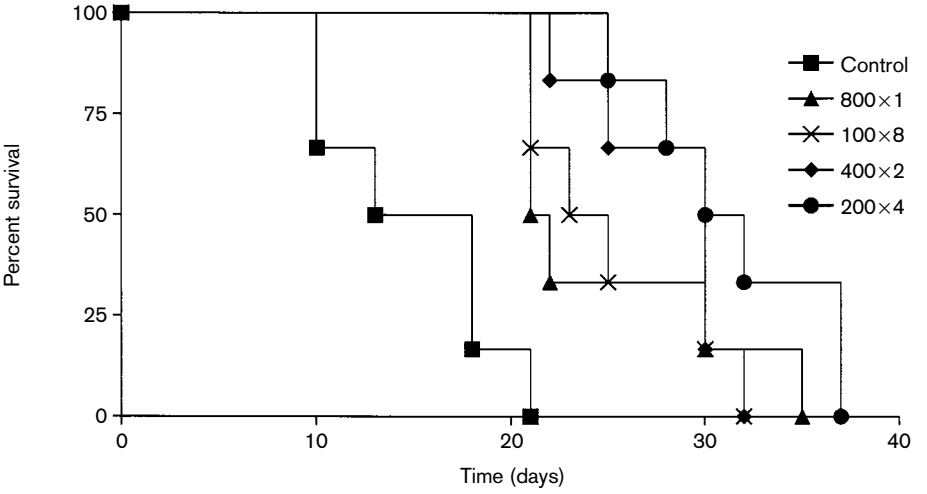

Figure 1. Tumor growth delay curves of SCID mice bearing WSU-DLCL$_2$ tumors.

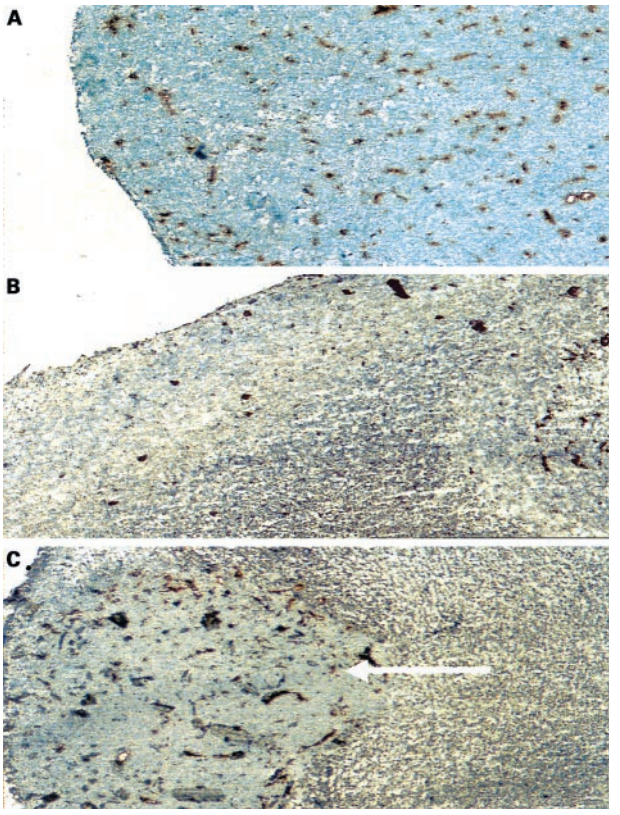

Figure 3. Immunohistochemical staining of blood vessels from WSU-DLCL$_2$-SCID mouse tumors ($\times$40). Tumor blood vessels were analyzed by staining with antibodies against CD31: (A) tumor section from untreated (Control) mouse showing the blood vessels (brown stain), (B) tumor section from 24 h CA4P-treated mouse (100 mg/kg) showing a decrease in tumor vasculature and (C) tumor section from 48 h CA4P-treated mouse (100 mg/kg) showing an area of new vasculature (arrow).

Effect of CA4P on cell cycle

We reported previously that CA4P induces G_2/M arrest in the WSU-DLCL$_2$ cell line *in vitro*.[17] To verify that CA4P exerts the same effect *in vivo*, we analyzed the effect of CA4P on cells taken from tumors. Table 2 shows that CA4P did not induce G_2/M arrest as it did *in vitro*. Animals treated with 800 mg/kg showed an increase in G_2/M from 5.9 ± 2.3 in control to 12.0 ± 7.1 after 48 h. However, statistical analysis showed no significant difference exists between the cell cycle of the control and treated tumors at any time point of the treatments.

Morphological analysis

Figure 4 represents the morphological appearance of tumor sections taken from CA4P-treated and untreated animals. We noted that after 24 h of CA4P treatment, both 100 and 800 mg/kg induced necrosis in the centers of each tumor. Viable tumor cells were seen only at the periphery. After 48 and 120 h, the formation of new healthy tissues was observed in two areas of the CA4P-treated tumors. These two areas are believed to be areas of tumor recovery, which is supported by positive findings when we stained the tissue with anti-CD31. This also showed us that these recovering areas are supported by new vasculature.

Table 2. Effect of CA4P on the cell cycle of the WSU-DLCL$_2$ tumors growing s.c. in SCID mice after 6, 24, 48 and 120 h

Time (h)	Cell cycle	Control	100 mg/kg	800 mg/kg
6	G_0/G_1	62.7 ± 2.2	61.2 ± 0.8	64.9 ± 3.9
	S	29.5 ± 1.7	32.3 ± 2.3	28.2 ± 3.4
	G_2/M	7.8 ± 1.0	6.5 ± 3.1	6.9 ± 0.9
24	G_0/G_1	67.8 ± 0.8	66.0 ± 2.5	61.4 ± 3.0
	S	26.6 ± 1.6	28.3 ± 2.2	35 ± 2.0
	G_2/M	5.6 ± 1.4	5.7 ± 0.3	3.6 ± 5.1
48	G_0/G_1	70.7 ± 3.8	65.4 ± 2.2	64.7 ± 4.9
	S	23.4 ± 5.1	28.6 ± 1.5	23.3 ± 2.3
	G_2/M	5.9 ± 2.3	6.0 ± 2.9	12.0 ± 7.1
120	G_0/G_1	71.0 ± 10.2	66.1 ± 1.8	69.6 ± 3.0
	S	19.1 ± 11.5	25.1 ± 1.3	23.4 ± 0.1
	G_2/M	9.8 ± 1.3	8.8 ± 0.4	6.9 ± 3.1

Animals were treated with 100 and 800 mg/kg. Data are the means of three animals in each group $\pm$ SD.

Discussion

Angiogenesis is critical for tumor growth and dissemination.[6,19] Historically, antiangiogenesis research was limited to targeting the factors that stimulate new blood vessel formation, i.e. VEGF. An alternative approach has been to target the endothelial cells and the tumor cells, rather than only the endothelial cells. This necessitates the use of cancer vascular targeting drugs and currently CA4P is exceptionally promising.

In this study we showed that CA4P given to SCID mice bearing human diffuse large cell lymphoma tumors leads to a significant antitumor activity. Moreover, administering CA4P at its MTD (800 mg/kg) resulted in significant shutdown of blood vessels in the tumors.

To assess the antitumor activity in mice, CA4P was administered at the MTD (800 mg/kg) in one, two, four or eight doses (Table 1). Significant tumor growth delay was observed after every treatment. Compared with control, all doses and schedules improved survival of the animals significantly (Figure 1). At

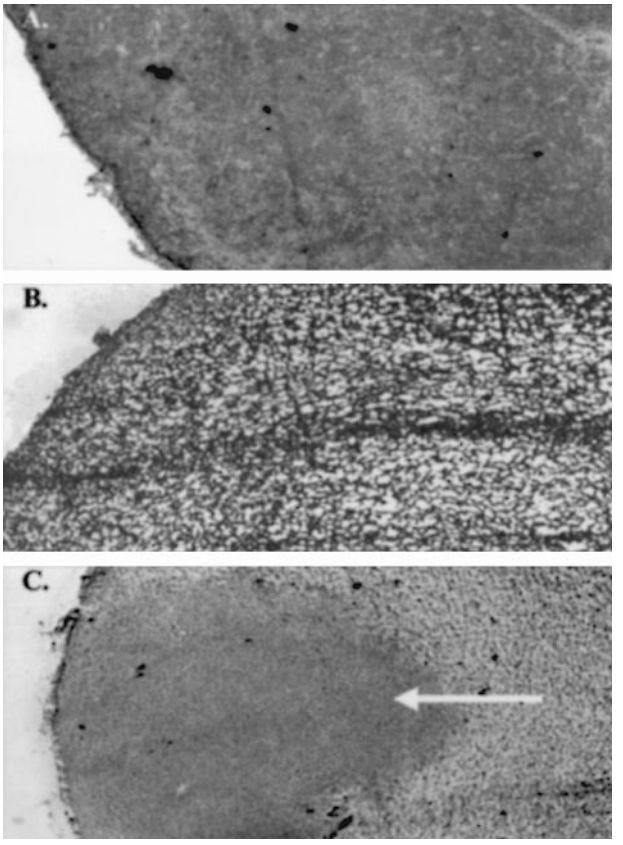

Figure 4. Morphological analysis (tetrachrome stain) of untreated and CA4P-treated WSU-DLCL$_2$ tumors ($\times$40). (A) Tumor section from untreated (control) mouse, (B) tumor section from 24 h CA4P-treated mouse (100 mg/kg) showing necrosis and (C) tumor section from 48 h CA4P-treated mouse (100 mg/kg) showing the formation of new tissue in one area of the tumor.

200 mg/kg, CA4P showed the lowest *T/C* % (11.7%) compared with 800 mg/kg (15.6%), 400 mg/kg (17.7%) and 100 mg/kg (20%). A 42% *T/C* is an indicator of antitumor effectiveness, where a value of 42% or less is considered significant antitumor activity. The same treatment had the longest tumor delay (12 days). However, if $\log_{10}$ kill value is added as a criterion, only 200 and 400 mg/kg had a clinically meaningful activity, whereas 800 and 100 mg/kg are not considered active by the usual clinical criteria (Table 1).

Results also showed that there were significant differences in mean tumor weight between the control group compared with various CA4P doses. CA4P at 200 mg/kg produced the greatest inhibition of tumor growth (37 ± 13 mg) with a range of 18–54 mg, while CA4P at 800 mg/kg produced lesser inhibition (110 ± 67 mg) and a range of 46–201 mg.

The effect of antiangiogenic agents on tumors can be measured through different methods, such as immunohistochemical staining for factors VIII and VEGF[20] specific for endothelial cells, or by measuring the blood flow.[21,22] We ascertained CA4P's antiangiogenic effects by staining immunohistochemically for CD31, a transmembrane glycoprotein present on endothelial cells. Two groups of mice were studied; one group received the MTD (800 mg/kg $\times$ 1 highest dose) while the second received one-eighth of the MTD (100 mg/kg). Blood vessels were counted after 6, 24, 48 and 120 h, showing significant shutdown after 24 h (about 80%) (Figure 2). This effect is dose independent within the dose range used because we observed the same phenomenon in mice whether treated with 800 or 100 mg/kg. Our results are similar to those reported previously,[4,5,22] except that the vascular shutdown occurred in our model 24 h following CA4P administration while others reported the same finding only 6 h after treatment with CA4P. It is important to note that the method we used to measure vascular shutdown was different from that used by other authors, which reflects the shutdown of functional blood vessels, while our method defines total vasculature. The vascular shutdown is especially pronounced in the center of the tumors. Morphological analysis showed a necrotic center and a viable periphery in the treated tumors, while control tumors showed viable centers (Figure 4), this observation was also previously reported.[22] After 48 and 120 h, two areas of the tumors, taking origin from that periphery, start to reveal the formation of new blood vessels.

Increasing doses of CA4P were not associated with improved efficacy against blood vessel formation. Tetrachome staining showed in those areas a recovery in the number of viable cells similar to those observed in the control. We have shown previously[17] that CA4P affects the tumor cells *in vitro* by inducing G_2/M arrest and mitotic catastrophe. The cell cycle analysis of tumors *in vivo* after administration of CA4P did not show the exact results. This can be explained by the fact that the plasma drug levels *in vivo* did not reach the level that was necessary to effect G_2/M cell cycle arrest *in vitro*.

CA4P's advantage as a non-specific antiangiogenic agent lies in its ability to affect the cancer cells as well as the endothelial cells. Research has effectively shown that targeting the endothelial cells only is not sufficient to suppress aggressive and advanced cancer, because cancer cells are able to induce new blood channels.[23] Dose schedule modifications may improve efficacy of CA4P. One such modification is to extend treatment with low doses to provide continuous inhibition of

blood vessel formation. This experiment is currently underway in our laboratory.

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