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Preclinical Evaluations of Therapies Combining the Vascular Targeting Agent Combretastatin A-4 Disodium Phosphate and Conventional Anticancer Therapies in the Treatment of Kaposi's Sarcoma

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The antitumor efficacy of the vascular targeting agent combretastatin A-4 disodium phosphate (CA4DP) was evaluated in a xenograft model of Kaposi's sarcoma (KS) grown in athymic mice. Response to CA4DP alone or in combination with localized radiation treatment or systemic chemotherapy (cisplatin or vinblastine) was assessed using a clonogenic cell survival or tumor growth delay assay. Administering increasing doses of CA4DP to tumor-bearing mice resulted in a dose-dependent increase in tumor cell kill. CA4DP also enhanced the antitumor effects of radiation and chemotherapy ~10–100-fold. Although single doses of CA4DP as large as 300 mg/kg failed to alter tumor growth, the same total dose, administered as 3 fractions in 5 or 9 days, resulted in significant growth delay. Such repeated CA4DP exposures also significantly increased the response of KS xenografts to cisplatin. These findings suggest that CA4DP ought to be considered as a candidate agent for therapeutic evaluation in AIDS-KS patients.

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Kaposi's sarcoma (KS) is a highly vascularized neoplasm (1, 2) classified into four different types: classic, African endemic, iatrogenic or drug-associated, and AIDS-related (3). Classic KS usually follows an indolent and benign clinical course that rarely requires treatment. In contrast, AIDS-KS is a fulminant disease that requires aggressive pharmacotherapy, especially when it involves visceral organs. Presently, AIDS-KS is the fourth leading clinical manifestation of AIDS (4). Involvement of the pulmonary tract occurs in 15–50% of patients and is estimated to contribute to 25% of deaths in patients with AIDS-KS (2).

Standard treatments for KS include intralesional injection of vinblastine or α-interferon, local radiotherapy and systemic chemotherapy. These treatments are administered primarily for symptomatic relief and to prevent disease progression. Cytotoxic chemotherapy is the standard therapy for patients with extensive lesions and disease involving visceral organs and lymph nodes. A single agent such as vincristine or α-interferon is used for mild cases. More advanced cutaneous or visceral KS is usually treated with combination chemotherapy including agents such as vincristine, bleomycin, and doxorubicin. However, these treatments do not significantly prolong survival, and the clinical effectiveness is insufficient (5). Continued pursuit of more effective and less toxic agents is clearly warranted.

Because KS is a highly vasoactive neoplasm, antiangiogenic approaches and strategies directly targeting the actively growing vessels of the tumor may prove particularly suitable for KS treatment. Recently, much effort has been focused on inhibiting tumor angiogenesis since this process is such a crucial component of the pathogenesis and progression of KS (6). A large number of agents directed at interfering with the angiogenic process have been developed (7, 8) and some of these, including TNP470 and thalidomide, have been used in clinical trials in AIDS-KS patients (9–11). Another agent, combretastatin A-4 disodium phosphate (CA4DP), currently in Phase I trials, has shown significant antivascular activity in a xenograft model of AIDS-KS (12). In the light of these findings, and given its possible application in an adjuvant setting in this

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disease, the present investigations were undertaken to explore the potential therapeutic applicability of CA4DP alone or in combination with conventional anticancer therapies to treat KS xenografts.

METHODS AND MATERIAL

KS xenografts and treatments

KS xenografts were initiated by injecting the flanks of 6–8-week-old athymic NCR nu/nu mice (Frederick Laboratories, Frederick, MD) with 1×10^6 KSY-1 cells (13) and were serially passed by subcutaneous transplantation of tumor pieces. Macroscopic tumors were available for experiments 3–4 weeks later. Tumor-bearing mice were allocated to groups and received either no treatment or different treatment strategies. CA4DP (OXiGENE Inc., Lund, Sweden), cisplatin (Bristol-Myers Squibb Co., Princeton, NJ) and vinblastine (Fujisawa USA, Inc., Deerfield, IL) were injected intraperitoneally in a volume of 0.01 ml/g animal body weight. For radiation treatment, tumor-bearing mice were irradiated whole-body while unanesthetized using a ^{137}Cs source operating at a dose rate of 1.5 Gy/min. In the combination treatment studies groups, CA4DP was administered 0.5–1 h after chemotherapy or radiotherapy treatment.

Clonogenic cell survival

Clonogenic cell survival in treated or untreated tumors was assessed using an in vivo to in vitro clonogenic cell survival assay as previously described (14, 15). Briefly, 24 h after treatment, tumor-bearing mice were killed and their tumors excised and then dissociated to a single-cell suspension using an enzyme cocktail (0.025% collagenase, 0.05% pronase, and 0.04% DNase). The cells then were mixed with 10^4 lethally irradiated cells in α-MEM containing 10% fetal calf serum and plated into 60 mm Petri dishes. After 2 weeks of incubation at 37°C, colonies of 50 or more cells were counted with the aid of a dissecting microscope. Tumor-surviving fractions were determined by multiplying the calculated fraction of surviving cells by the ratio of cells recovered in treated versus untreated tumors.

Tumor growth delay

Once KS xenografts had reached 200 mm^3 in size, the mice were allocated to different groups for CA4DP or cisplatin treatment. A 100 or a 300 mg/kg dose of CA4DP was administered at this time (day 1) in the single-dose treatment studies. Mice treated with multiple dose CA4DP exposures received a 100 mg/kg dose either on days 1, 3, and 5 or on days 1, 5, and 9. When combined with cisplatin, KS-bearing mice were given a 10 mg/kg dose of cisplatin on day 1, followed by repeated injections of 100 mg/kg of CA4DP on days 1, 3, and 5. After treatment, tumors were measured daily using calipers and two perpendicular diameters were determined. Tumor volumes were estimated using the equation volume $= 4\pi r^3/3$ where $r = (a + b)/4$ and a and b represent the perpendicular tumor diameters. The time taken for each tumor to reach a size of 900 mm^3 was recorded.

Statistical analysis

Clonogenic cell survival studies consisted of 2–3 experiments each of which used 2–4 tumor-bearing mice per treatment group. Data included the mean ± SE, and an unpaired t-test was used to compare results. Regrowth delay investigations consisted of treatment groups of 7–10 mice. The median tumor responses were plotted and significance between treatment groups was determined by a Wilcoxon rank sum test ($\alpha = 0.025$).

RESULTS

Antitumor effectiveness of CA4DP was determined by measuring clonogenic cell survival in KS xenografts treated with various doses of this agent. The data demonstrated that administering increasing doses of CA4DP to tumor-bearing mice resulted in a dose-dependent increase in tumor cell kill (Fig. 1). However, doses above ~100 mg/kg yield little additional increase in tumor cell kill. These data are consistent with the histological assessments of KS xenografts treated with CA4DP; i.e. a 100 mg/kg dose of CA4DP caused both ~90% tumor cell death and necrosis 24 h after treatment (12). Cells that survive the CA4DP treatment are located at the periphery of the tumor in areas supplied by normal tissue blood vessels (12, 16). In order to eliminate these surviving tumor cells,

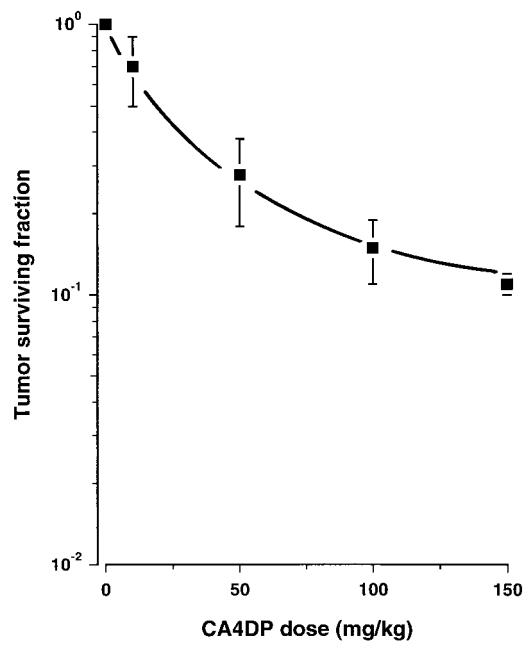

Fig. 1. Tumor cell kill in Karposi's sarcoma (KS) tumors treated with increasing doses of CA4DP. Data were determined 24 h after drug treatment and are the means ± SE of 2–3 experiments.

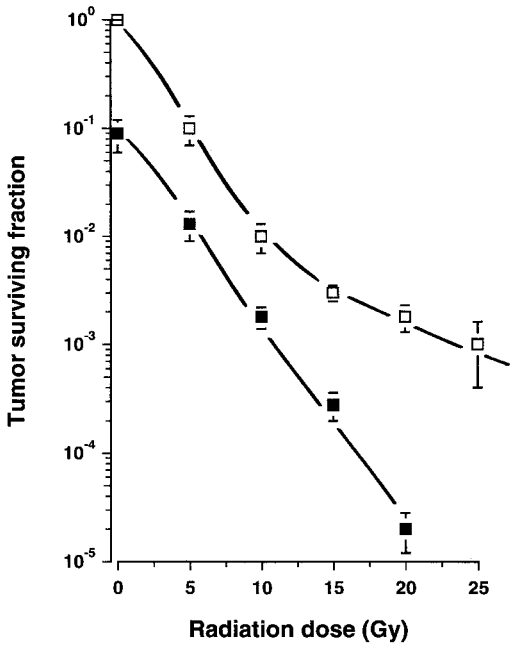

Fig. 2. Tumor cell survival in Karposi's sarcoma (KS) xenografts treated with a range of radiation doses either alone or in combination with a 100 mg/kg dose of CA4DP. In the combined treatment, CA4DP was administered 1 h after radiotherapy. Results are the means ± SE of 3 experiments. Radiation: □; radiation + CA4DP: ■.

CA4DP treatments were combined with radiotherapy or chemotherapy.

In Fig. 2 we illustrate the killing effect of various doses of radiation in KS xenografts treated with either radiation alone or radiation combined with a 100 mg/kg dose of CA4DP given 0.5–1 h after radiation. Irradiated KS tumors show a biphasic cell survival curve typically associated with the presence of a subpopulation of radiation-resistant hypoxic cells. When KS-bearing mice were treated with a combination of radiation and CA4DP, the extent of tumor cell kill was increased significantly at all doses, compared to that seen for radiation alone. The inclusion of CA4DP in the treatment protocol reduced tumor cell survival ~10–100-fold below that achieved with radiation alone. In addition, the survival curve determined from tumors treated with the combination of CA4DP plus radiation gives no indication of a 'break' down to 5 logs of cell kill, suggesting that the addition of CA4DP to the treatment influenced the proportion of radiation-resistant hypoxic cell subpopulation in KS tumors.

The possibility of producing even greater antitumor effects by adding a second agent to specifically target the rim of viable tumor cells surviving CA4DP treatment was also investigated with two chemotherapeutic agents commonly used to treat AIDS-KS patients. KS-bearing mice were treated with a range of doses of cisplatin or vinblastine either alone or in combination with 100 mg/kg CA4DP and their effect on tumor cell kill was evaluated (Fig. 3). In these studies CA4DP was administered 1 h post-chemotherapy and clonogenic cell survival was assessed 23 h later. The results demonstrated that cell kill in KS xenografts was significantly enhanced (~10-fold) when CA4DP was included in the treatment regime of either chemotherapeutic agent (Fig. 3).

In conjunction with the cell survival investigations, studies evaluating the effects of CA4DP treatment on the in situ growth of KS tumors were also performed. In the initial experiments, tumor-bearing mice were treated on reaching 200 mm^3 in size with a single dose of 100 or 300 mg/kg CA4DP. These treatments resulted in a slight, but not significant, tumor growth delay (Fig. 4, and see Table 1). Again, consistent with the histological assessments (12) and clonogenic cell survival evaluations (Fig. 1), increasing the CA4DP dose to 300 mg/kg did not lead to a greater tumor growth delay than that reached with 100 mg/kg. In an attempt to apply this agent more efficiently, studies utilizing repeated injections of CA4DP (100 mg/kg) were also performed. Treatment again commenced when the tumors reached 200 mm^3. Two different treatment schedules were used: CA4DP was administered on days 1, 3 and 5, or on days 1, 5, and 9. The results showed a superior response of the KS xenografts to the multiple CA4DP treatment schedules (Fig. 4). Both treatments induced significant growth delay compared to untreated tumors and tumors treated with either of the single doses of CA4DP (Fig. 4, and Table 1).

The combination of CA4DP treatment and systemic chemotherapy was also evaluated using tumor growth delay as the response endpoint. In these studies a single dose of cisplatin (10 mg/kg) was administered either alone or in combination with three 100 mg/kg doses of CA4DP delivered over a period of 5 days. The results (Fig. 4, and Table 1) showed that both treatments on their own could induce significant responses in KS xenografts. However, when combined, this response could be greatly enhanced such that the combination of CA4DP plus cisplatin led to significantly greater tumor growth delay than that seen for either cisplatin or CA4DP treatment alone (Fig. 4, and Table 1).

DISCUSSION

Kaposi's sarcoma is the most common tumor seen in HIV-infected patients. Several chemotherapeutic agents including vinca alkaloids, bleomycin, doxorubicin, and cisplatin are commonly used to treat AIDS-KS patients (3). More recently, other agents, most notably, anthracyclines, paclitaxel, and antiangiogenesis agents TNP-470 and thalidomide, have led to some advances being made in the management of this disease (3, 17, 18).

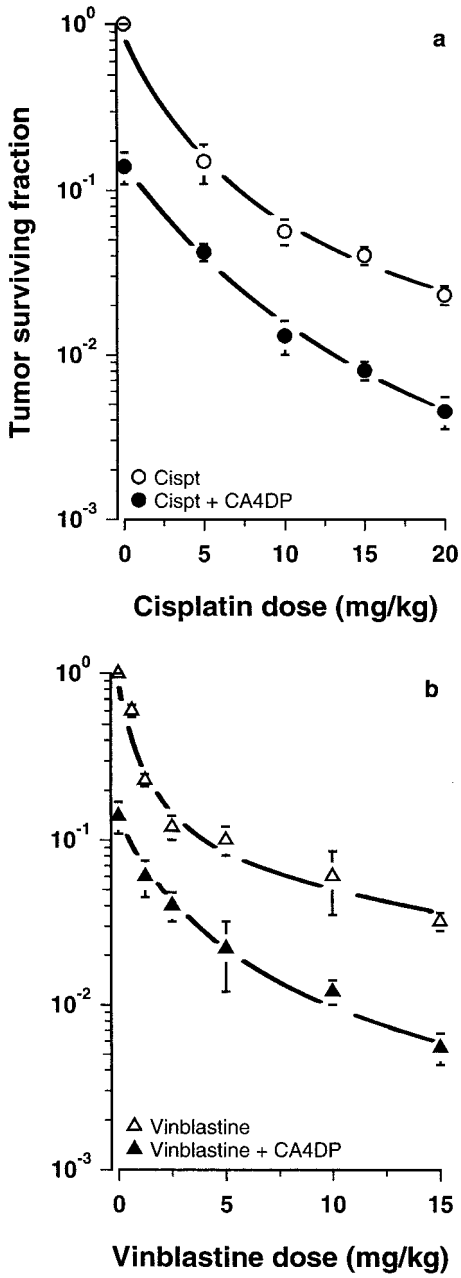

Fig. 3. Tumor cell survival in Karposi's sarcoma (KS) xenografts treated with a 100 mg/kg CA4DP 1 h after a range of doses of cisplatin (a) or vinblastine (b). Data are the means ± SE of 2–3 experiments.

Vinca alkaloids (either vincrinstine, vinblastine, or an alternating regimen of the two) exert their anticancer effect by binding to tubulin and preventing its polymerization to form microtubules, thus inhibiting a number of cellular processes, including mitosis. Like vinca alkaloids, CA4DP binds to tubulin and inhibits tubulin polymerization (19). Paclitaxel also interferes with microtubule dynamics but does so by promoting the formation of highly stable microtubules that resist depolymerization (20).

In vitro paclitaxel was found to inhibit the growth of KS-derived spindle cells and to be a potent inhibitor of endothelial cell proliferation (20). Similarly, CA4DP inhibits KSY-1 cell growth and endothelial cell migration and proliferation (21, 22). In vivo vinca alkaloids may express some antivascular action, but these effects typically are seen only at high doses approaching the maximum tolerated dose (MTD) and often only in the presence of significant morbidity (23). In contrast, CA4DP demonstrates antivascular effects at very low non-toxic (< 1/10 MTD) doses (16, 21, 24). Results from our laboratories and those of others have shown that not only CA4DP has specific effects on actively dividing endothelial cells but also that this agent can cause rapid vascular shutdown in a variety of preclinical tumor models (12, 16, 21, 25, 26). Because KS is a highly vascularized neoplasm with a cellular origin suggested to be endothelial cell derived, and because tubulin-binding agents have previously been found to be active in KS, the present study was undertaken to examine the efficacy of CA4DP in an in situ model of this disease.

The KSY-1 cell line originated from cells isolated from the pleural effusion of an AIDS-associated KS patient (13). KSY-1 cells promote tumorigenesis, angiogenesis, and metastasis in immunodeficient mice. The model's similar biological, morphological and immunophenotype make it a valuable adjunct for studies related to pathogenesis and therapy of AIDS-KS (12). In the present study, KSY-1 cells were used to initiate KS xenografts in athymic mice

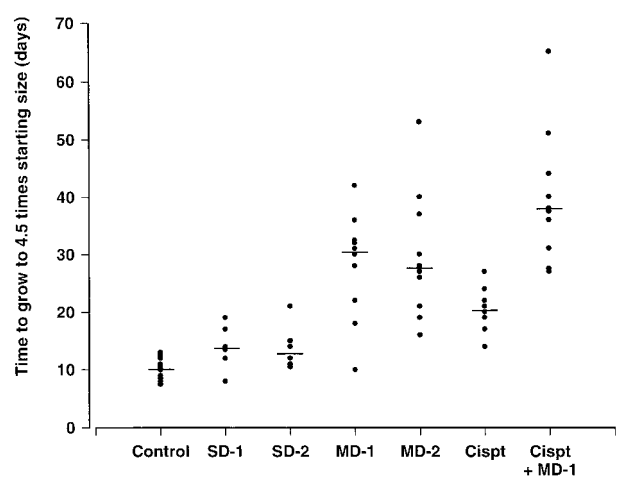

Fig. 4. Growth delays in Karposi's sarcoma (KS) tumors treated with single or multiple doses of CA4DP, a single dose of cisplatin, or a single dose of cisplatin plus multiple doses of CA4DP. Single doses (SD-1 or SD-2) of CA4DP (100 or 300 mg/kg) or cisplatin (10 mg/kg) were administered on day 1 at a tumor size of ~200 mm^3. Multiple 100 mg/kg doses of CA4DP were administered on days 1, 3, and 5 (MD-1) or on days 1, 5, and 9 (MD-2). In the combination treatment, a single dose of cisplatin (10 mg/kg) was given 1 h prior to the first 100 mg/kg dose of CA4DP in the MD-1 treatment schedule. Each datum point represents the response of an individual tumor; median response: –.

Table 1

Response of Karposi's sarcoma (KS) xenografts to single and multiple doses of CA4DP administered either alone or in combination with a single dose of cisplatin

Treatment	Median time to 900 mm^3 (days)	Growth delay (days)	Significance[4] vs. Group 1	Significance[4]
1 Control	10.0	–	–	
2 SD (100)[1]	13.5	3.5	NS	
3 SD (300)[1]	12.5	2.5	NS	
4 MTD (1, 3, 5)[2]	30.0	20.0	$p<0.025$	4 vs. 5 NS
5 MTD (1, 5, 9)[2]	27.0	17.0	$p<0.025$	
6 Cispt[3]	20.5	10.5	$p<0.025$	6 vs. 7 $p<0.025$
7 Cispt + CA4DP[3]	38.0	28.0	$p<0.025$	7 vs. 4 $p<0.025$

[1]Single doses of 100 or 300 mg/kg CA4DP given on day 1 (tumor size $\sim$200 mm^3).
[2]100 mg/kg doses of CA4DP given on days 1, 3, and 5, or days 1, 5, and 9.
[3]10 mg/kg cisplatin given on day 1 either alone or in combination with three 100 mg/kg doses of CA4DP given on days 1, 3, and 5.
[4]Significance determined by the Wilcoxon rank sum test ($\alpha = 0.025$), which compares the probability distributions of growth times of control versus treated tumors.

in order to assess their antitumor response to the vascular targeting agent CA4DP used alone or in combination with radiation or conventional anticancer drugs.

Results from the clonogenic cell survival assay established that CA4DP treatments led to a concentration-dependent killing of KS tumor cells (Fig. 1). This killing manifested itself primarily as a rapid loss of viable cells from the cell population within the 24-h period after CA4DP treatment. The clonogenic cell survival evaluations were consistent with histological observations (12). For example, a 100 mg/kg dose of CA4DP which induced $\sim$90% necrosis in KS xenografts also reduced the viable tumor cell population to $\sim$10% of the pretreatment value. Interestingly, despite the extensive tumor cell kill and induction of necrosis, a single 100 mg/kg dose of CA4DP had little effect on the growth of KS xenografts (Fig. 4). This lack of change in tumor growth following the 100 mg/kg CA4DP treatment is probably a consequence of a balance between the growth of new cells from the surviving rim and the removal of necrotic material from the tumor's core. Increasing the single dose to 300 mg/kg did not result in a greater growth delay in KS xenografts (Fig. 4). But this result is not surprising given the extent of cell death and necrosis caused by a 100 mg/kg CA4DP dose. Increasing the dose further would have little additional effect on the tumor cells that survive, owing to their location near normal tissue blood vessels.

While it was apparent that maximum antitumor efficacy occurred with doses of $\sim$100 mg/kg and that little could be gained by increasing the exposure dose further, we reasoned that administering multiple doses of CA4DP at times when the tumor is regrowing and the tumor vasculature is recovering and/or re-establishing itself might prove to be a much more efficient application of this agent. Two different treatment schedules were examined in KS-bearing nude mice. The results showed that unlike the single-dose treatments, both of the multiple-dose schedules caused significant growth delay in the xenografts (Fig. 4 and Table 1). Multiple doses of CA4DP were clearly far more effective at inhibiting KS growth than single-dose treatments. For example, administering three 100 mg/kg dose fractions of CA4DP, as opposed to a single 300 mg/kg treatment, increased the growth delay by $\sim$14–17 days (Table 1). None of these treatment schedules resulted in any overt animal toxicities (death, weight loss), consistent with previous observations made with this agent (21, 26).

The present studies also investigated the efficacy of combining CA4DP with conventional anticancer therapies. The rationale for such combinations was based on two factors. First, it is logical to combine drugs or treatment modalities with different mechanisms of action or different dose-limiting toxicities to obtain a therapeutic benefit. Second, as discussed above, our CA4DP studies showed that this agent, though effective at inducing large-scale tumor necrosis, failed to eliminate a viable rim of tumor cells surviving at the tumor's periphery (12). Consequently, CA4DP treatment was combined with radiation therapy. Results from the clonogenic cell survival investigations demonstrated that combining CA4DP with radiation treatment could significantly enhance the tumor cell killing effect compared to radiation alone. Results also showed that when CA4DP was used in conjunction with radiotherapy, the tumor's hypoxic cell population appeared to be significantly impacted. For radiation treatment alone, the 'break' in the KS cell survival curve reflects the presence of the hypoxic cell population in this tumor (Fig. 2). It was observed that the inclusion of CA4DP in the treatment strategy altered the radiation dose-response curve: the 'break' seen with radiation treatment alone was eliminated with the combination treatment. This demonstrates that CA4DP could improve the radiation response of tumors by impacting the radiation refractory hypoxic cell subpopulation of tumors. Studies examining the effects of CA4DP and radiotherapy on tumor growth were not conducted as

part of the present series of experiments. However, we (26) and others (24) have shown previously that the combination of CA4DP and radiation can lead not only to enhancements in tumor cell kill but also to increased inhibition of tumor growth and improved tumor control.

The present studies also investigated the efficacy of combining CA4DP with conventional chemotherapeutic agents. Owing to the rapid action of CA4DP against the tumor vasculature, in the combination studies, cisplatin and vinblastine treatment preceded CA4DP in order not to interfere with the tumor uptake of the chemotherapeutic agents. The results showed that the CA4DP–chemotherapy combination led to significantly enhanced tumor cell kill (Fig. 3). Most critically this enhancement could still be achieved at the maximum tolerated single doses of cisplatin (20 mg/kg) and vinblastine (15 mg/kg). Alternatively, the same antitumor effects could be achieved at much lower doses of cisplatin and vinblastine when CA4DP was included in the treatment.

Combining cisplatin with CA4DP also proved more effective at inhibiting KS growth than either treatment alone (Fig. 4, Table 1). The results showed that the combination of cisplatin and CA4DP led to a superior growth delay and an additive antitumor effect in KS xenografts. While direct assessments of normal tissue toxicities were not made, overt responses of animals treated with cisplatin or cisplatin plus CA4DP were identical. In addition, CA4DP treatment failed to enhance the bone marrow toxicity of cisplatin (data unpublished). Taken together, these findings demonstrate that combining the antivascular agent CA4DP with another agent that kills the tumor cells directly is an effective way of inhibiting KS growth.

In conclusion, the vascular targeting agent CA4DP alone can cause extensive tumor cell killing and growth delay in KS xenografts. This agent also can significantly enhance the antitumor effects of radiation therapy and conventional anticancer agents. These findings suggest a possible application for CA4DP in the clinical management of KS. The Phase I clinical trials with CA4DP are almost complete. The present results suggest that the evaluation of CA4DP in patients with AIDS-KS be considered in future Phase II studies.

ACKNOWLEDGEMENT

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