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*Research Paper*

# Targeting of the Antivascular Drug Combretastatin to Irradiated Tumors Results in Tumor Growth Delay

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***Purpose.*** The aim of the study is to evaluate the effects of targeting the antivascular drug combretastatin to irradiated mouse melanomas.
***Methods.*** Combretastatin was incorporated into liposomes with surfaces modified by the addition of cyclo(Arg-Gly-Asp-D-Phe-Cys) (RGD) to create an immunoliposome (IL). This addition of RGD allows the liposome to be preferentially targeted to $\alpha_v\beta_3$, an integrin up-regulated in the vasculature of irradiated tumors. C57BL mice bearing a transplanted B16-F10 melanoma were randomly assigned to one of the following treatment groups: untreated, a single dose of 5-Gy radiation (IR), IL (14.5 mg/kg of combretastatin), 5-Gy radiation plus IL, and a systemic administration of free drug (81.0 mg/kg of combretastatin).
***Results.*** In this transplanted tumor model, there was no significant increase in the volume of the IL + IR (5 Gy) treated tumors during the initial 6 days posttreatment; all other treatment groups exhibited exponential growth curves after day 3. The IL + IR (5 Gy) treatment resulted in a 5.1-day tumor growth delay compared to untreated controls.
***Conclusions.*** These findings indicate that preferential targeting of antivascular drugs to irradiated tumors results in significant tumor growth delay.

**KEY WORDS:** adhesion molecules; antivascular drugs; combretastatin; ionizing radiation; targeted drug delivery.

## INTRODUCTION

During the past decade, there has been an increasing focus on cancer therapies combining ionizing radiation with antivascular drugs. In antivascular therapy, the goal is to cause extensive tumor cell death as a result of localized vascular shutdown causing hypoxia and metabolic deprivation (1), which in tumors may cause widespread tumor cell death (2). One of the most prominent members of this group of antivascular drugs, combretastatin (CA4DP), has been successful enough to warrant clinical trials (3) and has been shown to be most effective when it has been combined with radiotherapy (3,4). Despite promising preliminary results, antivascular compounds have undesirable side effects on many normal tissues and are most effective at or above their maximum tolerated doses (3).

Increased expression of cell adhesion molecules on inflamed endothelium, which plays a key role in leukocyte recruitment, can be utilized to deliver microparticles (5), biodegradable polymers (6), or other drug delivery systems (7) to the site of inflammation. In irradiated tissue, several adhesion molecules (e.g., $\beta_3$ chain) on the luminal surface of the endothelium in the tumor and the surrounding normal tissue are up-regulated (8). The radiation-induced up-regulation of endothelial cell adhesion molecules on tumor vasculature provides a potential avenue for targeting drugs to tumors (8–10). This targeting could be especially efficacious in conjunction with modern clinical radiotherapeutic techniques, where radiation exposure is generally limited to a core of diseased tissue and the normal tissue surrounding it.

In this study, we hypothesize that $\alpha_v\beta_3$ up-regulation in irradiated tumors can be utilized to selectively deliver combretastatin to tumors. Inhibition of $\alpha_v\beta_3$ to prevent angiogenesis has been the subject of many studies (11), but the up-regulation of this integrin in response to various stimuli has been studied to a lesser extent. In the case of ionizing radiation, it has been reported that a dose of 2 Gy is sufficient to up-regulate $\alpha_v\beta_3$ between 1 and 4 h after exposure (12). We demonstrate that in transplanted tumors, significant tumor volume control can be achieved when combretastatin is preferentially targeted to irradiated (IR) tumors. This targeting is accomplished by encasing the drug in a liposome conjugated to a tripeptide sequence (RGD)

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that targets the integrin $\alpha_v\beta_3$. The long-term goal of this project is to develop a combination of radiation-antivascular therapy for treating tumors in a clinical setting.

## MATERIALS AND METHODS

Distearoyl phosphatidylcholine–PEG(2000) and DSPE–PEG–maleimide were purchased from Avanti Polar Lipids (Alabaster, AL). Cyclo(Arg-Gly-Asp-D-Phe-Cys) was custom-produced by Peptides International (Louisville, KY). Hydrogenated soy phosphatidylcholine was purchased from Northern Lipids, Inc. (Vancouver, Canada). Cholesterol was purchased from Sigma-Aldrich (St. Louis, MO).

### Liposome Formulation

Long circulating liposomes were composed of hydrogenated soy phosphatidylcholine (50 mol%), cholesterol (45 mol%), and distearoyl phosphatidylcholine–PEG(2000) conjugate (DSPE–PEG, 5 mol%). For preparation of long circulating liposomes with attached ligands (RGD), a part of DSPE–PEG (2 mol%) was replaced with the DSPE–PEG–maleimide functional lipid. Arginine–glycine–aspartic acid (RGD) is an ideal ligand to use because $\alpha_v\beta_3$ recognizes this sequence and is readily expressed on angiogenic endothelial cells (13,14).

Combretastatin was synthesized according to published methods (15–17). This drug was incorporated into long circulating liposomes, and the drug loading was quantified via high-performance liquid chromatography (HPLC). Briefly, a centrifugal ultrafiltration device was used to separate free (nonencapsulated) drug from the drug encapsulated in the liposomes after preparation. Free drug (nonencapsulated) concentration was determined via HPLC. An aliquot of the liposome dispersion was diluted with ethanol to extract the encapsulated drug, and total extracted drug was also measured using HPLC. These two measurements were then used to verify total drug amount encapsulated in the liposomes. This process was performed in triplicate for each batch of liposomes.

### Coupling of RGD to Liposomes

For preparation of immunoliposomes (IL) with an attached RGD recognition motif, long circulating liposomes with maleimide functional groups on the distal end of PEG chains were incubated with cyclo(Arg-Gly-Asp-D-Phe-Cys) at pH 6.5 at the molar ratio of 1:30 (RGD/maleimide). An HPLC method was developed to determine binding of the RGD to the liposome. RGD was initially dissolved in the mobile phase and injected into the HPLC to find the retention time of free RGD (approximately 14 min as shown in Fig. 1A). Following the coupling step, the liposomes were injected to determine free RGD. As shown in Fig. 1B, at 14 min, the new peak was below the quantifiable level indicating RGD had been successfully coupled to the liposome.

Based on the amount of RGD molecule used and the phospholipids concentration, a published technique (18) was used to calculate the number of RGD molecules per liposome. Based on a liposome size of 120 nm and an assumption based on the literature that there are 144,000 phospholipid molecules in every 120-nm liposome (18), we estimate that there are approximately 124 RGD molecules per liposome. This ratio was used in an effort not to affect the stability of the liposome in the circulation and to give an optimal ligand density. When ligand density was increased in a few preliminary experiments, it had severe adverse side effects on the animals. For our experiments, this ratio seemed to provide the best tumor control benefits and the least detrimental normal tissue effects.

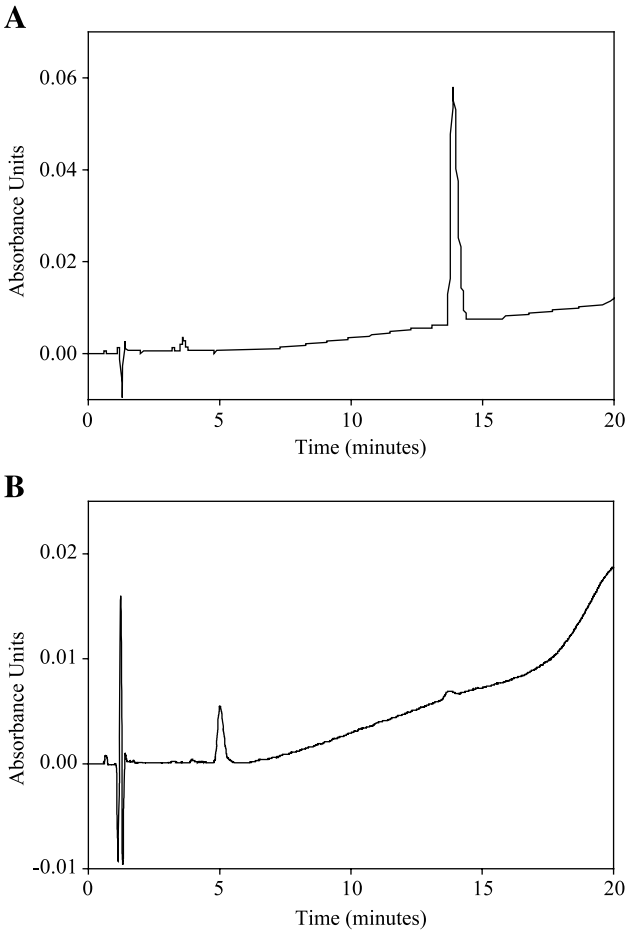


**Fig. 1.** The retention time of free arginine–glycine–aspartic acid (RGD) as determined by HPLC is indicated by a peak around 14 min (panel A). RGD coupled to liposomes shows a peak at 14 min which falls below the quantifiable level for the machine indicating no free RGD left in the system (panel B).

### Transplanted Tumor Model and Treatment

B16-F10 melanoma cells (ATCC, Manassas, VA) were injected into the rear flanks of 6- to 8-week-old C57BL male mice (Harlan, Indianapolis, IN). Tumor growth was measured daily with the aid of a metric caliper, and tumor volume was calculated by multiplying the measured depth, width, and length of a given tumor. In these tumors, which were located in the rear leg of the animals, depth, width, and length could be readily measured on a regular basis. Tumors were excised and calculated volumes were verified when the animals were euthanized at the end of the experiment. Treatments were initiated at a tumor volume of approximately 1.5 $cm^3$ at which time the animals were anesthetized using a ketamine/xylazine mixture (81:13 mg/kg) and

received a single 5-Gy dose of radiation (IR) localized to one leg from a cesium source. Immediately following irradiation, animals received a dose of ILs corresponding to a drug concentration (combretastatin) of 14.5 mg/kg via retro-orbital injection. Animals were then monitored on a daily basis for changes in tumor volume using a metric caliper as noted above.

Five different mouse treatment groups were observed: one group ($n = 6$) did not receive any treatment and served as control, another group ($n = 4$) was injected with only the free drug at a concentration of 81.0 mg/kg, another group ($n = 6$) received a single 5-Gy dose of radiation (IR) only, the next group ($n = 6$) received a single 5-Gy dose of radiation and ILs (14.5 mg/kg combretastatin), and the last group ($n = 6$) received only the ILs (14.5 mg/kg combretastatin). Tumor growth delay was defined as the time required for a treated tumor to reach a specific volume (3.5 $cm^3$) minus the time for the untreated tumor to reach that same volume. The research outlined here adhered to the *Principles of Laboratory Animal Care* (NIH publication #85-23, revised in 1985).

### Statistical Analysis

Unless otherwise noted, data are presented as mean ± SEM. Analysis of variance with planned contrasts was used to determine significant differences in tumor volume among experimental groups. All values of $P < 0.05$ were considered statistically significant.

## RESULTS

Combretastatin was preferentially targeted to irradiated B16-F10 melanoma tumors using RGD conjugated immunoliposomes (ILs), and tumor volume was measured on a daily basis. Untreated (control) tumors could only be observed for 6 days after the treatments were initiated because of the rapid growth rate of these tumors. As shown in Fig. 2, no significant difference in tumor volumes was observed between different groups until day 3 posttreatment. After day 3, all treatment groups, except for the IL + IR (5 Gy), exhibited exponential growth curves. It is important to note that there was no significant increase in the volume of the IL + IR (5 Gy) treated tumors during the initial 6 days posttreatment, and that the IL + IR (5 Gy) group exhibited significantly ($P < 0.02$) smaller tumor volumes as compared to control. It is also important to note that free drug treatment at six times the drug dose of IL + IR (5 Gy) did not result in a significant change in tumor growth as compared to control.

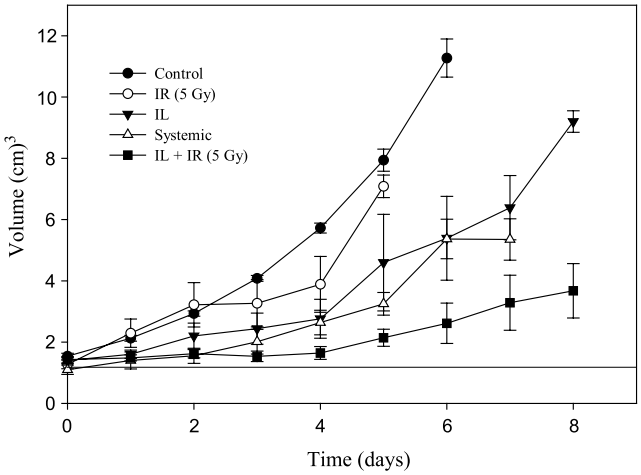


**Fig. 2.** No significant difference in tumor volumes was observed between different groups until day 3 posttreatment (Mean ± SEM, $n = 4$–6 animals per group). After day 3, all treatment groups, except for the immunoliposome (IL) + irradiated (IR) group, exhibited exponential growth curves. It is also important to note that free drug treatment at six times the drug dose of immunoliposome did not control tumor growth.

**Table I.** Tumor Growth Delay for Various Treatment Groups

| | IR (5 Gy) | IL | IL + IR (5 Gy) | Systemic |
|---|---|---|---|---|
| Growth delay (days) | 0.9 ± 0.8 | 1.9 ± 1.0 | 5.1 ± 0.9 | 2.6 ± 0.4 |

IR = Irradiated; IL = Immunoliposome; Mean ± SEM, $n = 4$–6 animals per group.

The IL + IR (5 Gy) treatment resulted in a tumor growth delay of approximately 5.1 days as compared to control (Table I). As shown in Table I, tumor growth delays for the IL + IR (5 Gy) treatment were at least twice as long when compared to any of the other treatment groups in our transplanted tumor model.

## DISCUSSION

The goal of this study was to determine if targeting antivascular drugs to irradiated tumors could cause a significant tumor growth delay as compared to treatment with systemic administration of antivascular drugs or with radiation alone. Our findings indicate that preferential targeting of antivascular drugs to tumors results in a significant tumor growth delay in transplanted and spontaneous tumors. It should be noted that a significant tumor growth delay was not observed with a free drug administration even at a drug dose of almost six times that used in the immunoliposomes.

In this study, we observed a significant tumor growth delay with only a single dose of irradiation and a single dose of immunoliposomes. In clinical settings, many tumors are treated with combination therapies consisting of multiple doses of irradiation and multiple doses of drugs (e.g., antivascular compounds). From our data, it appears that tumor regrowth begins on or after day 3. This suggests that multiple doses of radiation and immunoliposomes containing antivascular drugs should be administered as often as every 2 days. This approach may result in additional delay, or even a reversal, in growth of the tumor.

There is a large range of treatment options, including radiation therapy, available for treating smaller tumors as compared to larger tumors. On the other hand, combretastatin has been reported to be most effective with larger size tumors (19). The current study outlines our success in developing a novel therapy for treating larger tumors that are not easily treated with other approaches. The clinical applicability of this proposed therapy may depend in part on how effective this approach may be in other tumor models.

Our preliminary findings indicate that this treatment may also be effective in controlling tumor volume in spontaneous mammary ($MMTV^+$) tumors. In addition, we did not observe any normal tissue toxicity in mice bearing spontaneous tumors treated with IL + IR.

Up-regulation of adhesion molecules by ionizing radiation or other stimuli allows one to preferentially target drugs, genes, contrast agents, etc. via immunoliposomes or other drug carriers (6,8,20,21) to sites of disease. Studies from our laboratory (9,10) and others (22) indicate that ionizing radiation up-regulates many adhesion molecules with varied time courses. These up-regulated adhesion molecules may present additional targets for preferential delivery of antivascular compounds, as well as other pharmaceutical agents, to irradiated tumors.

## CONCLUSION

These findings indicate that preferential targeting of antivascular drugs to irradiated tumors results in significant tumor growth delay.

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