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Inhibiting Tumor Growth by Targeting Tumor Vasculature with Galectin-1 Antagonist Anginex Conjugated to the Cytotoxic Acylfulvene, 6-Hydroxylpropylacylfulvene

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Targeted delivery of therapeutic drugs promises to become the norm to treat cancer. Here, we conjugated the cytotoxic agent 6-hydroxypropylacylfulvene (HPAF) to anginex, a peptide that targets galectin-1, which is highly expressed in endothelial cells of tumor vessels. In a human ovarian cancer model in mice, the conjugate inhibited tumor growth better than equivalent doses of either compound alone. Immunofluorescence on tumor tissue demonstrated that the conjugate, like parent anginex, selectively targeted tumor vasculature and inhibited tumor angiogenesis. Increased activity from the conjugate further suggests that HPAF retains at least some of its normal cytotoxic activity when linked to anginex. More importantly perhaps is the observation that the conjugate abrogates apparent systemic toxicity from treatment with HPAF. This work contributes to the development of tumor vascular targeting agents against cancer in the clinic.

INTRODUCTION

When used at optimal doses, chemotherapeutic agents can result in considerable systemic toxicity. Recently, it was demonstrated in the clinic that chemotherapy at doses below maximally tolerated doses can be very effective when used in combination with the antiangiogenic agent, Avastin (Genentech) (*1*). In fact, a number of antiangiogenic agents have been developed and used in a similar fashion in various preclinical animal models (*2*−*4*). The most promising of these appear to be those that act directly on endothelial cells (EC) (*5*), because they are effective against a broad spectrum of tumors and less prone to induce drug resistance (*5*). Moreover, such EC-specific agents are also being used as tumor targeting moieties to increase specificity of chemotherapeutics (*6*) and to deliver contrast agents for magnetic resonance imaging (MRI) (*7*). For example, the α_v integrin-binding RGD tripeptide has been coupled to the chemotherapeutic drug doxorubicin, and shown in tumor-bearing mice to prolong survival, decrease the optimal effective dose, and reduce toxicity (*6*).

Another EC specific antiangiogenic agent, anginex (*8, 9*), targets galectin-1 (gal-1) (*10*), a cell surface glycan binding protein that is highly upregulated in tumor-activated EC (*10*−*12*). Differential stromal elevation of gal-1 over the tumor parenchyma has been reported in several cancers, including cancer of the breast (*13*), colon (*14*), prostate (*15*), and ovary (*16*). By weakly binding to "carrier protein" plasma fibronectin (*17*), anginex is transported through the cardiovascular system to the tumor vasculature where the peptide binds gal-1 (*10*). Anginex binding to gal-1 disrupts tumor EC adhesion and migration, thereby inducing EC apoptosis and resulting in the inhibition of tumor angiogenesis (*10, 18*). Because gal-1 is likely expressed in most tumor types at relatively high levels, using anginex as a tumor vasculature homing device should be widely applicable.

The acylfulvenes are a semisynthetic class of compounds derived from illudin S, a toxin from the *Omphalotus illudens* mushroom, that demonstrate broad spectrum antitumor activity in studies conducted in vitro and in vivo. Irofulven (MGI-114; 6-hydroxymethylacylfulvene) is a leading member of the acylfulvene analogues and has shown clinical activity against a variety of cancers, either as a monotherapy or in combination with other chemotherapeutic agents (*19*). Similar to other alkylating agents, dose-limiting toxicities, such as myelosuppression, neutropenia, thrombocytopenia, nausea, vomiting, and fatigue, are observed when therapeutic concentrations of irofulven are used in patients (*20, 21*). At higher doses, ocular disturbances and symptoms of renal tubular acidosis have been observed (*20, 21*). Conjugating acylfulvenes to a vascular targeting agent, like anginex, has the potential not only to reduce the chemotherapeutic's toxicity profile and maintain, or even improve, efficacy in the clinic, but also to be employed against a broader spectrum of tumors. In this report, we conjugated an irofulven analogue, 6-hydroxypropylacylfulvene (HPAF) to anginex and investigated the ability of anginex to act as a tumor vasculature homing device. In human tumor xenograft studies, we found that the conjugate indeed targeted the tumor vascu-

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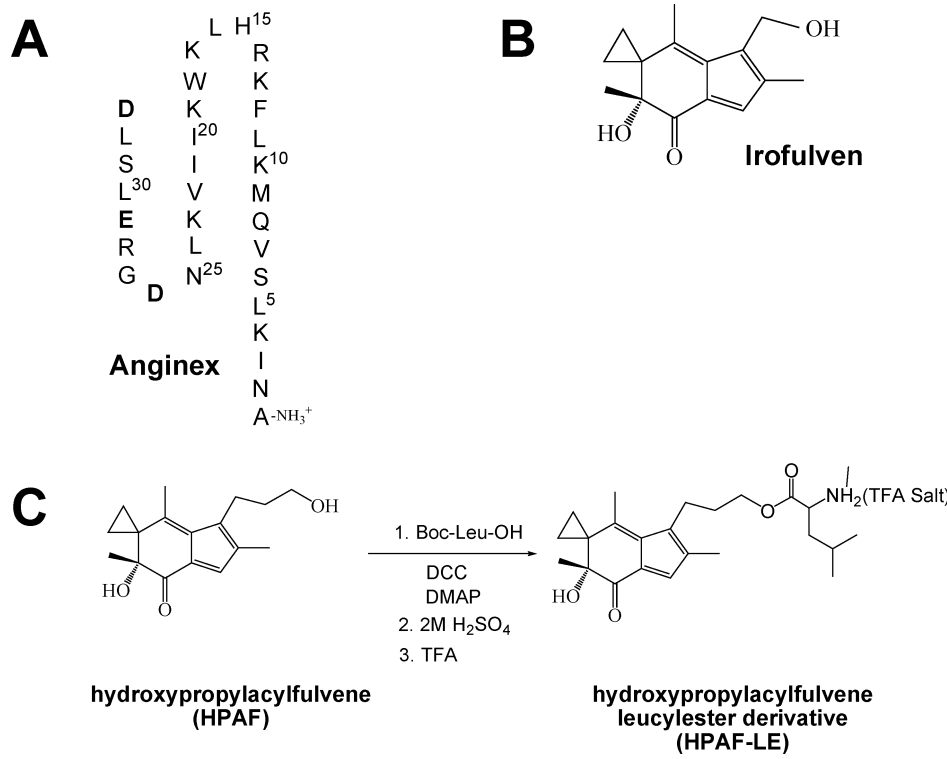

Figure 1. Synthesis of the HPAF-LE-anginex conjugate. (A) The amino acid sequence of anginex with the three possible sites for coupling of HPAF-LE, with the side-chain carboxylates D26, E29, and D33 depicted in bold. (B) The chemical structure of irofulven is shown. (C) Schematic representation of the leucylester derivative of hydoxypropylacylfulvene synthesis, starting with HPAF, is shown.

lature and was more efficacious than either agent alone, without systemic toxicity.

EXPERIMENTAL PROCEDURES

Synthesis of 6-Hydroxypropylacylfulvene Conjugated to Anginex. The irofulven analogue, 6-hydroxypropylacylfulvene, was conjugated to amino acid side-chain carboxylate groups on the peptide anginex (MW = 3941 Da) via a leucyl ester linkage as shown schematically in Figure 1. For convenience, we refer to the leucyl ester derivative of HPAF as HPAF-LE (MW = 380 Da) throughout this paper. Chemical linkage was performed manually using Fmoc chemistry. Coupling was performed by dissolving an excess (1.2 mol equiv) of HPAF in Activator 1 (HATU in DMF). This solution was cooled on ice, and then Activator 2 (DIPEA in DMF) was added. The reaction mixture remained on ice for 5 min before it was added to a solution of anginex and DMF and allowed to react overnight. The next morning, a mixture of acetonitrile and water was added to the reaction mixture, which was then lyophilized.

Because the C-terminal carboxylate of anginex is amidated, there are three possible sites for coupling of HPAF-LE to the anginex side-chain carboxylates: D26, E29, and D33 (Figure 1). To favor coupling to one site per anginex molecule, the molar ratio of HPAF-LE/anginex was adjusted to 1.2:1 during the coupling reaction. Mass spectrometry (MS) analysis on the crude conjugate indicated that the desired products were obtained, with 3941 Da for unreacted anginex and 4353 Da for the 1:1 conjugate. No 2:1 conjugate (4688 Da) was detected.

The crude conjugate was purified by HPLC (CH semiprep column) using a water/ACN gradient running from 25% to 100% ACN. For this procedure, 10 mg of crude material was dissolved in 1 mL ACN/9 mL water, sonicated for 10 min, and centrifuged. Five mL was injected per HPLC run. Although unreacted anginex (about 10% by analytical HPLC) could be separated from the reaction products by HPLC, the three 1:1 (HPAF/anginex) conjugates could not be separated, and biological studies were performed on this mixture of single adduct conjugates.

To prevent possible compound instability, we prepared fresh working solutions on the day of administration. For in vitro studies, the conjugate, e.g., was dissolved in a stock solution of 100% ethanol (30 mg/mL), aliquoted out, and kept frozen at −80 °C. On the day of use, a work solution was prepared by diluting the stock solution in media, such that the ethanol content was reduced to 3% at the highest concentration tested in vitro (20 μM). No decline in activity was observed in repeated experiments, suggesting that the compound was stable when prepared in this fashion. Similarly, in animal studies, the conjugate was made fresh on the initial day of treatment, first dissolved in pure ethanol and then diluted in PBS to reduce ethanol content (3% in the 50 mg/kg and 0.6% in the 10 mg/kg treatment group). To check for degradation, samples were routinely submitted for MS analysis, including following in vitro studies for material remaining in solution.

Antibodies. Antianginex antibody was made and used in our lab as previously described (*10*). Antigal-1 antibodies were purchased from Santa Cruz Biotechnologies, and react with both human and murine galectin-1. Galectin-1 is highly conserved intra- and interspecies, being 90% identical between human and murine amino acid sequences (*26*).

Cell Culture. Human umbilical vein derived EC (HUVEC)[1] were harvested and cultured as described earlier (*22*). Human epithelial ovarian carcinoma MA148, murine melanoma B16F10, and human alveolar epithelial carcinoma A549 cells were cultured, as described previously (*22*−*24*).

Proliferation Assay. HUVEC were seeded in a 96-well culture plate coated with 0.2% gelatin (2 h at 20 °C). MA148

[1]Abbreviations: EC, endothelial cell; HUVEC, human umbilical vein EC; PBS, phosphate buffered saline; BSA, bovine serum albumin; VTA, vascular targeting agent.

cells were seeded in noncoated 96-well plates. Both cell types were seeded at a concentration of 3000 cells per well and allowed to adhere overnight at 37 °C in 5% CO_2/95% air before treatments were initiated. Cells were then exposed to complete medium supplemented with 10 ng/mL bFGF, 0.4% Endo-Gro (VEC technologies Rensselaer, NY), 10 ng/mL VEGF, or 20% FBS, with or without various concentrations of anginex, HPAF, anginex−HPAF conjugate, or gal-1 antibody (1/1000; Santa Cruz Biotechnologies, CA) for 72 h. Cell Counting Kit (CCK-8; Dojindo, Japan) was used to assess cell proliferation rates relative to untreated cells, as described previously (*23, 25*). All measurements were done in triplicate, and each experiment was performed at least three times.

Cell Adhesion Assay. HUVEC suspended in complete medium, with or without anginex-HPAF conjugate or anginex (both 10 μM), were seeded at a concentration of 10 000 cells in a 96-well culture plate as described above and allowed to adhere for 2 h on gelatin (0.2% w/v; 50 μL) or gal-1 (1 mg/mL; 50 μL). Subsequently, the wells were aspirated and washed with PBS carefully to discard the nonadherent cells, and complete media were added to the cells. Cell Counting Kit (CCK-8) was used to calculate the number of adherent cells (*23, 25*). All measurements were done in triplicate, and each experiment was performed at least three times.

MA148 Ovarian Carcinoma Mouse Model. Female athymic nude mice (nu/nu, 5−6 weeks old) were purchased from the National Cancer Institute and allowed to acclimate to local conditions for at least 1 week. Animals were given water and standard chow ad libitum and were kept on a 12 h light/dark cycle. Experiments were approved by the University of Minnesota Research Animal Resources ethical committee. Exponentially growing human ovarian MA148 epithelial carcinoma cells were cultured, harvested, suspended in serum free RPMI (2.0×10^6 cells/mouse), and inoculated subcutaneously (s.c.) into the right flank of the mouse, as described previously (*3, 25*). Studies were carried out in a therapeutic intervention model with established tumors to test the capacity of the conjugate to inhibit or reduce tumor growth. Tumors were allowed to grow to the size of approximately 75 mm^3 prior to randomization and initiation of treatment (all treatments were administered q3d×4 IP in 3% v/v ethanol/PBS). When tumors reached the size of about 1500 mm^3, animals were euthanized as per the approved protocol. As an indirect measurement of general toxicity, body weights of mice were monitored twice weekly, using a digital balance (Ohaus Florham, NJ).

Tumor volume was determined by measuring the size of the tumors on the flanks of the mice. The diameters of tumors were measured using calipers (Scienceware, Pequannock, NJ), and the volume was calculated using the equation to determine the volume of a spheroid: $(a^2 \times b \times \pi)/6$, where a is the width of the tumor and b is the length of the tumor. A complete response was defined as tumor regression to less than 15 mm^3 (3 mm in diameter), and a partial response was defined as a smaller tumor than at the start of treatment. Stable disease was defined as tumor volume less than 100 mm^3.

Immunohistochemistry. We used immunohistochemistry on MA148 tumor tissue from mice to demonstrate that the conjugate, like parent anginex, targets gal-1. For this work, similarly sized tumors were embedded in tissue freezing medium (Miles Inc.; Elkart, IN), snap-frozen in liquid nitrogen, and subsequently cut into 5 μm sections. Preparation and procedures for the frozen tumor sections were performed as described previously, with slight modifications (*23*). Briefly, sections of tissue were fixed for 10 min in acetone, and then incubated for 30 min in PBS/5%BSA/3%FBS to saturate aspecific binding sites. After washing with PBS, the slide was incubated with 10 μg of conjugate per slide, and followed by a washing step, tissue was incubated with antianginex and antigalectin antibodies (1:50) (*10*). Following overnight incubation, slides were washed with PBS and respective secondary antibodies were added: fluorescein isothiocyanate (FITC)-conjugated for conjugate detection and phycoerythrin (PE) for gal-1 detection (Sigma Chemical; St. Louis, MO) (*10*).

Microvessel density was detected using a 1:50 dilution with phycoerythrin (PE)-conjugated antibody to mouse CD-31 (PECAM-1; Pharmingen; San Diego, CA) or FITC conjugated antibody to mouse αSMA (alpha smooth muscle actin; Pharmingen; San Diego, CA) (*24*). A minimum of 10 randomly chosen images per treatment group were acquired using an Olympus BX-60 microscope at 200× magnification and digitally analyzed and differentially quantified by morphometric analysis (*24*).

Statistical Analyses. Tumor volume was analyzed using general linear mixed models (*27*). During the process of model selection, the likelihood ratio test was used to determine random effects and variance−covariance structures. The final general linear mixed model of tumor volume on the original scale contained interaction terms of treatment with both time and time^2, while that of tumor volume on a natural log scale, i.e., ln(tumor volume +1), contained the interaction of treatment with time only. The variance−covariance structure of random effects was unstructured, whereas that of random errors was first-order autoregression. Using derived parameters, model-based tumor growth curves for raw data were drawn, and the integral from day 1 to day 26 for each curve was estimated from the area under the curve. This area-under-the-curve integral provided estimates of percentage inhibition in tumor growth for treatment versus control groups over the time course of the study, rather than just on a single day. We also performed nonparametric and postcomparison tests, i.e., Kruskal−Wallis and Wilcoxon Rank-Sum, as well as Student's t test to validate differences between control and treatment data sets.

RESULTS AND DISCUSSION

Initially, we assessed the ability of conjugate to inhibit proliferation of various cells in culture. Anginex inhibited endothelial cell (EC) proliferation (Figure 2A) with an IC_{50} value of 5 μM, consistent with previous results (*8, 18, 22, 23*). In comparison, both the conjugate and HPAF-LE at 5 μM exhibited greater than 90% inhibition of EC proliferation, with IC_{50} values of 1.4 μM and 0.6 μM, respectively. Results were the same whether medium was supplemented with 10 ng/mL bFGF, 10 ng/mL VEGF, 0.4% Endo-Gro, or 20% FBS. Although anginex was essentially inactive against human ovarian carcinoma MA148, murine melanoma B16F10, and human alveolar epithelial carcinoma A549 cells, HPAF-LE and conjugate were quite effective, with respective IC_{50} values of 11 μM and 14 μM against MA148 (Figure 2B), 7 μM and 23 μM against B16F10 (Figure 2C), and 0.1 μM and 0.6 μM against A549 (Figure 2D).

These results suggest that conjugation to the peptide anginex does not substantially modify the effectiveness of HPAF-LE as a chemotherapeutic agent. This is interesting because acylfulvenes are known to function intracellularly by forming DNA adducts (*28*), and for this to occur, the conjugate should be internalized by these cells. At least with EC, anginex is known to be rapidly internalized upon binding to the cell surface (*10*). Nevertheless, demonstration that the acylfulvene-based anginex conjugate can still promote formation of acylfulvene−DNA adducts requires further investigation.

To demonstrate that the conjugate still targets gal-1 like parent anginex (*10*), we employed cell adhesion and competition assays, as well as ex vivo double immunostaining (Figure 3). As illustrated in Figure 3A, conjugate and anginex significantly ($p < 0.05$) block EC adhesion on gal-1-coated surfaces, whereas

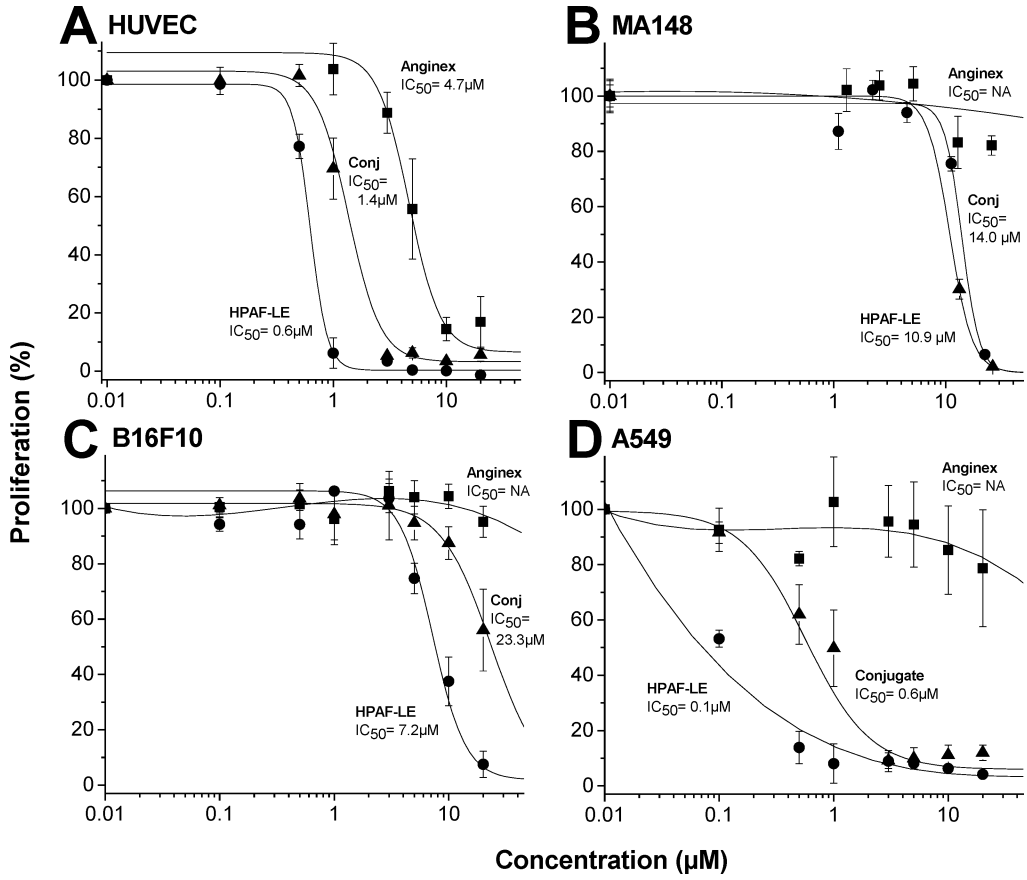

Figure 2. Differential inhibition of EC and MA148 cell proliferation by HPAF-LE, anginex, and conjugate. Concentration response curves of HPAF-LE, anginex, and conjugate on HUVEC (A), MA148 proliferation (B), B16F10 (C), and A549 (D). Average values for quantification of at least three separate experiments are shown ± SEM.

both conjugate and anginex could not block adhesion of EC to gelatin-coated surfaces. In addition, the inhibitory proliferation by both compounds was diminished by the presence of anti-gal-1 antibody (Figure 3B).

Using double immunostaining of cross sections from untreated MA148 xenograft tumors, we first showed that the antianginex antibody cross-reacted with the conjugate, indicating that the epitopes on anginex peptide were not blocked by the presence of the conjugated irofulven analogue (Figure 3D, middle panel). Then, we showed that gal-1 is present in the stroma of MA148 tumor tissue (Figure 3C,D upper panel), confirming earlier reports (*27*), and that gal-1 and conjugate could be identified by immunofluorescence (Figure 3D). The merged images, which illustrate the morphology of tumor blood vessels, demonstrate colocalization of gal-1 and conjugate (Figure 3D, lower panel). This in turn allows us to conclude that the conjugate indeed targets gal-1, and that conjugation of HPAF-LE to the peptide does not interfere with its ability to target gal-1.

Next, we compared the in vivo activity of HPAF-LE (5 and 10 mg/kg) to that of irofulven (1.5 and 3 mg/kg) and carboplatin (32.5 mg/kg) in the MA148 human ovarian carcinoma model (IP administration on a q3d×4 schedule, Figure 4A). Carboplatin was used as the standard comparator in this model because platinum agents are the most widely used drugs in first-line defense against ovarian cancer (*3, 29*). Our study demonstrated a dose response in both irofulven and HPAF-LE treated mice. Although HPAF-LE at 5 mg/kg or 10 mg/kg inhibited MA148 tumor growth to a similar degree to irofulven at 1.5 mg/kg or 3 mg/kg, respectively, irofulven was about 3-fold more effective than HPAF-LE at the same dose (Figure 4B). On the other hand, HPAF-LE at 10 mg/kg had essentially the same effect as carboplatin at 32 mg/kg, which amounts to a 3-fold improvement in dose over this platinum agent. The toxicity profiles of all compounds tested appeared to be very similar, with body weight gains being somewhat suppressed compared to those in vehicle-treated mice (Figure 4C).

To assess the effect of the conjugate on tumor growth, we treated MA148 tumor-bearing mice with the conjugate (10 mg/kg and 50 mg/kg), along with several control groups (vehicle treatment, and stand-alone treatments with anginex (10 mg/kg and 50 mg/kg) and HPAF-LE (5 mg/kg)). Figure 5A shows tumor growth curves for all groups. Even though the time course for the study is shown out to day 28, mice received treatment only during the first 10 days and were euthanized when tumors reached the size of about 1500 mm^3. Overall, tumor growth was inhibited in all treated groups compared to that in the vehicle-treated control group, and both anginex and the conjugate showed a dose response. On the day when control mice had to be sacrificed (day 23), Student's *t*-test indicated that the conjugate at 50 mg/kg inhibited tumor growth by 94% ± 1.6% compared to vehicle-treated control mice ($p = 0.02$), whereas equimolar doses of anginex (50 mg/kg) and HPAF-LE (5 mg/kg) inhibited tumor growth by 76% ± 7.3% ($p = 0.03$) and 82% ± 5.0% ($p = 0.03$), respectively (Figure 5B). Analysis of data on day 23 using Kruskal−Wallis and Wilcoxon Rank-Sum nonparametric and post-comparison tests showed that, while there were no significant differences between anginex (10 and 50 mg/kg), HPAF-LE (5 mg/kg), and conjugate (10 mg/kg) groups, there was a significant ($p = 0.003$) difference between conjugate (50 mg/kg) and HPAF-LE treated groups. This was supported by statistical analysis over the time course of the study using general linear mixed models, which showed significant differences between the control group and all other groups (anginex 10 mg/kg, $p < 0.01$; anginex 50 mg/kg and conjugate

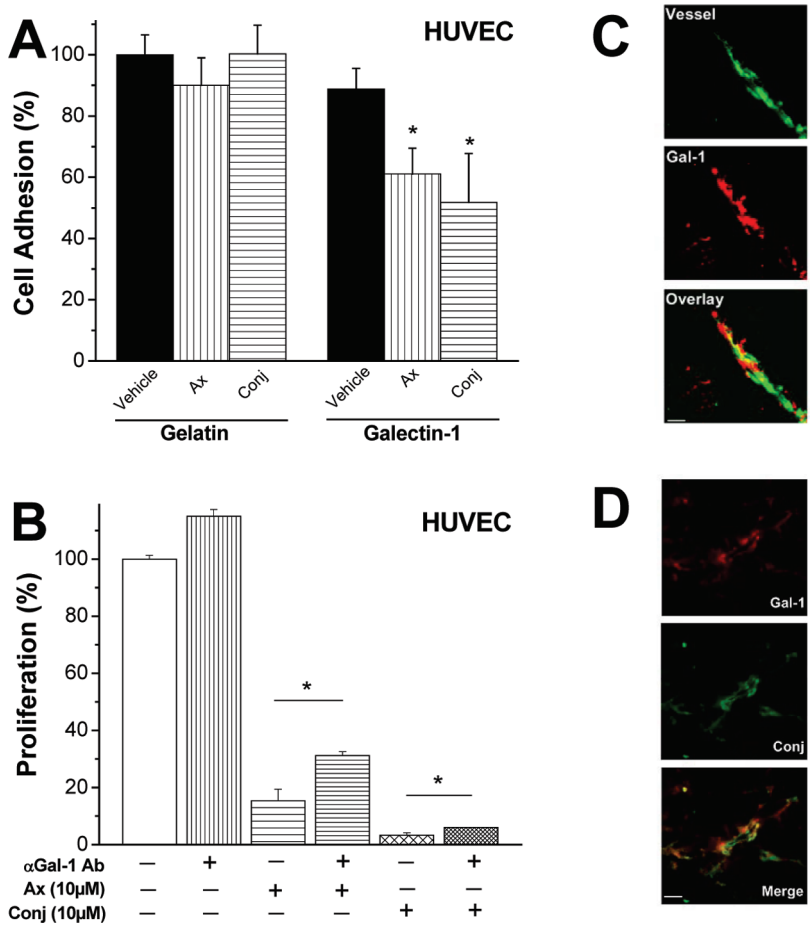

Figure 3. (A) EC adhesion to gal-1 coated surface, but not to gelatin-coated surface, is inhibited by conjugate (10 μM) or anginex (10 μM). (B) Conjugate (10 μM) or anginex (10 μM) EC proliferation inhibition is significantly reduced by anti-gal-1 antibody (1/1000). (C) Immunohistochemistry staining of mouse vessels, pericytes (green; αSMA), gal-1 expression (red); co-localization of gal-1 and mouse vessels (yellow; CD31). (D) Immunohistochemistry staining of mouse vessels, showing that HPAF-LE anginex conjugate targets gal-1 in MA148 tumors: gal-1 expression (red); conjugate binding (green); co-localization of gal-1 and conjugate (yellow). Original magnification 200×; scale bar = 50 μm.

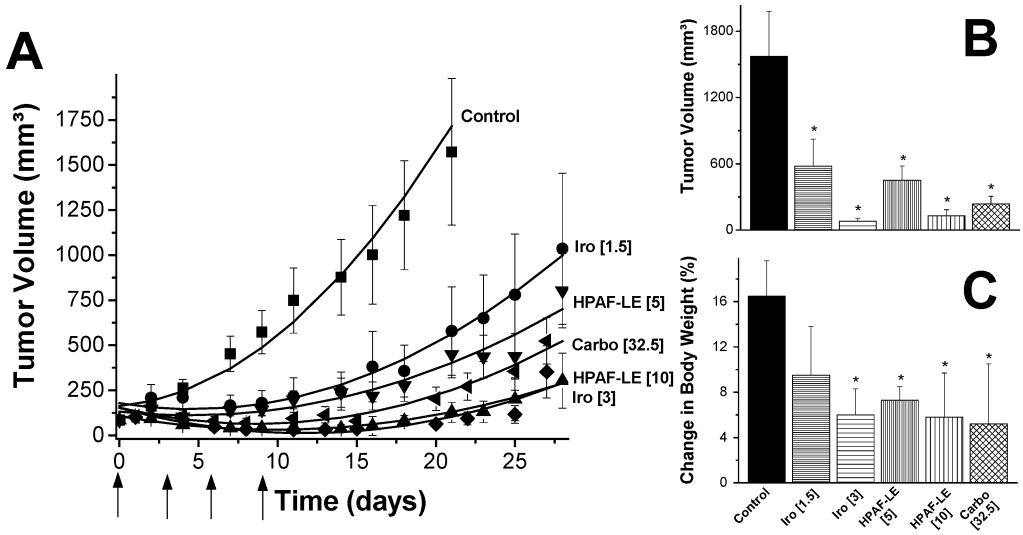

Figure 4. MA148 tumor growth inhibition by HPAF-LE, irofulven, and carboplatin. (A) Six groups of mice were studied (treatments were all administered IP q3d×4): two HPAFLE groups (5 mg/kg, $n = 7$, and 10 mg/kg, $n = 7$), two irofulven groups (1.5 mg/kg and 3 mg/kg, each $n = 8$), one carboplatin group (32.5 mg/kg, $n = 8$), and one control group (vehicle, $n = 7$), as indicated. Tumors were allowed to grow to the size of approximately 75 mm^3 prior to initiation of treatment. Tumor volumes are plotted as mm^3 (± SEM). (B) MA148 tumor growth inhibition by the individual treatments derived from the growth curves in panel A. Data represent means ± SEM on day 21 after the initiation of treatment. (C) The average gain in body weight (in %) for the experimental groups indicated. Data represent means ± SEM on day 21 after the initiation of treatment.

10 mg/kg, $p = 0.001$; HPAF-LE and conjugate 50 mg/kg, $p < 0.001$), as well as, e.g., between conjugate (50 mg/kg) and HPAF-LE treated groups ($p = 0.04$). Furthermore, at the end of the study on day 37 (absent control group due to sacrifice),

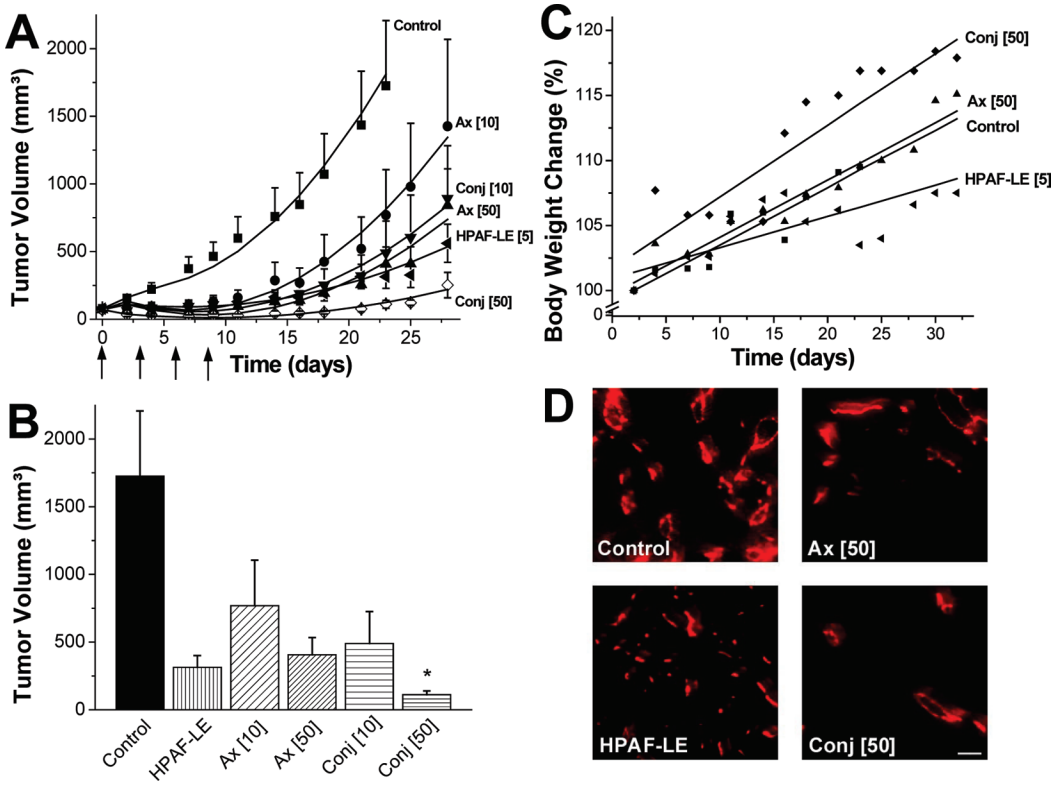

Figure 5. MA148 tumor growth inhibition by HPAF-LE-anginex conjugate. (A) Six groups of mice were studied (treatments were all administered IP q3d×4): two conjugate groups (10 mg/kg and 50 mg/kg), two anginex groups (10 mg/kg and 50 mg/kg), one HPAF-LE group (5 mg/kg), and one control group (vehicle), as indicated. Tumors were allowed to grow to the size of approximately 75 mm^3 prior to initiation of treatment (n = 10−12 animals per treatment). (B) MA148 tumor growth inhibition by the individual treatments derived from the growth curves in panel A. Data represent means ± SEM on day 23 after the initiation of treatment. *$p < 0.003$ for conjugate compared to other treatment groups. (C) The average change in body weight, normalized to individual weights at the start of treatment (in %) for the experimental groups indicated, are plotted over time with linear line fitting (no significant differences $p < 0.9$). (D) Representative images illustrate the average amount of vessel density staining on tumor tissue from control, anginex (50 mg/kg), HPAF (5 mg/kg), and conjugate (50 mg/kg) treated groups. Original magnification 200×; scale bar = 50 μm.

conjugate treated mice had an average tumor size of 600 mm^3, whereas the average tumor size of HPAF-LE treated mice reached this value on day 32, amounting to a tumor growth delay time of about 5 days in mice treated with conjugate compared to HPAF-LE.

This improvement in efficacy from the conjugate was also reflected in the level of individual tumor responses (Table 1). On day 11, analysis of individual mice showed that administration of conjugate at 50 mg/kg resulted in 20% complete remissions, 60% partial responders, and 20% stable diseases, whereas the HPAF-LE treated group (5 mg/kg) had 8% complete and partial responders and 17% stable diseases. At the lower dose of conjugate (10 mg/kg), 17% of the mice had either a complete or partial remission, or stable disease. In the anginex-treated groups, administration of 50 mg/kg caused 17% partial responders and 67% stable diseases, whereas at 10 mg/kg, there was 50% stable diseases. At the end of the study, more than 2 weeks after the last treatment, tumors relapsed in conjugate-

Table 1. Tumor Responses after Different Treatment Regimens[a]

treatment	stable (%)	response partial (%)	complete (%)
Control	0	0	0
HPAF-LE [5]	16.6	8.3	8.3
Anginex [10]	50	0	0
Anginex [50]	66.7	16.7	0
Conjugate [10]	16.7	16.7	16.7
Conjugate [50]	20	60	20

[a] Tumor response rates were evaluated on day 11, two days after the last treatment. n = 10−12 animals per treatment.

Table 2. Immunofluorescent Analysis of Microvessel Density

	vessel density[a]	end points[b]	branch points[c]	vessel length[d]
Control	17398 ± 1068	58.8 ± 7.5	9.8 ± 1.1	14.8 ± 0.9
HPAF-LE [5]	14099 ± 1009[e]	76.9 ± 6.1[e]	5.6 ± 0.9[e]	11.0 ± 0.9[e]
Anginex [10]	11221 ± 1124[e]	30.0 ± 7.1[e]	6.0 ± 1.3[e]	7.1 ± 1.1[e]
Anginex [50]	9819 ± 874[e]	41.1 ± 7.5[e]	3.6 ± 0.8[e]	7.2 ± 0.9[e]
Conjugate [10]	10302 ± 1330[e]	22.2 ± 3.4[e]	3.8 ± 1.0[e]	6.0 ± 0.6[e]
Conjugate [50]	8471 ± 612[e,g]	11.0 ± 2.2[e,f,g]	2.3 ± 0.7[e,g]	4.8 ± 0.8[e,g]

[a] Following binarization of images (magnification 200×), microvessel density was estimated by scoring the total number of white pixels per field. Results show the mean white pixel count per image ± standard error. At least 3 different tumors per treatment group were analyzed with a total of 10 or more images. [b] Mean number of vessel end points ± standard error as determined after skeletonization of the images. [c] Mean number of vessel branch points/nodes per image in pixels ± standard error as determined after skeletonization of the images. [d] Mean total vessel length per image in pixels ± standard error as determined after skeletonization of the images. [e] $p < 0.001$. Experimental group compared to vehicle (Kruskal−Wallis Test). [f] $p < 0.01$. Conjugate group (50 mg/kg) compared to molar equivalent Anginex (50 mg/kg) Wilcoxon Rank-Sum Test. [g] $p < 0.03$. Conjugate group (50 mg/kg) compared to molar equivalent HPAF-LE group (5 mg/kg) Wilcoxon Rank-Sum Test.

treated animals. The control group showed no responders and no stable diseases.

Although we found that the effective concentration of conjugate in vitro is lower than its effective dose in vivo, we realize that such differences are not always readily explainable, since in vivo exposure is multifaceted and would require complete pharmacokinetic and dynamic studies. Nevertheless, a dose of 50 mg/kg is not that unusual for a protein/peptide-based therapeutic in mouse studies (*30*), and we did observe the absence of apparent toxicity from the conjugate or anginex at either dose (10 mg/kg or 50 mg/kg) in any of the mice. Apparent lack of toxicity is based on normal weight gain (Figure 5C) and normal behavior in mice during the study. Trend line fitting ($R^2 > 0.8$) revealed that the rate of weight gain in the HPAF-LE (5 mg/kg) treated animals was reduced 3-fold as compared to the conjugate (50 mg/kg) treated animals, i.e., 0.19% vs 0.58% weight gain per day (Figure 5C). This is consistent with dose limiting toxicities usually observed from agents of this class of alkylating chemotherapeutics (*20*).

To demonstrate changes in microvessel density, we used immunohistochemistry (CD31 staining) on cryosections of MA148 tumor tissue from treated and untreated mice (Table 2 and Figure 5D). Morphometric analysis of viable tissue showed that microvessel density in treated groups was reduced in comparison to that in control tumor tissue. General morphology of tumor tissue showed that mice treated with higher doses of anginex or conjugate had regional antivascular effects, resulting in necrotic centers and surviving rims. At either dose, effects from conjugate and anginex are comparable, whereas there is a significantly ($p < 0.03$) smaller effect from HPAF-LE at a molar equivalent dose. These data indicate that conjugation of HPAF-LE to anginex does not affect the ability of anginex to function as an antiangiogenic agent. On the other hand, MA148 tumor growth inhibition (Figure 5A) is significantly ($p = 0.01$) greater from treatment of mice with the conjugate (50 mg/kg) compared to that from anginex (50 mg/kg), and this enhancement should result from the presence of HPAF-LE on targeting peptide anginex.

Although the precise mechanism by which anginex-conjugated HPAF-LE functions in situ remains unknown, it may be that improved efficacy is attributable to increased in vivo exposure of the acylfulvene moiety at the target site, i.e., targeting and sequestering within the tumor, and/or greater serum half-life. Moreover, although it is indirect evidence, it appears that conjugation of HPAF-LE to anginex reduces the inherent toxicity from the acylfulvene moiety. Body weight gain was normal during the course of the treatment with the conjugate (5.5% weight gain compared to 5.6% weight gain for control animals, Figure 5C), whereas it was significantly ($p = 0.03$) lower (3.2%) in HPAF-LE treated mice. Taken together, this study suggests that HPAF-LE can be made more effective when conjugated to anginex as a tumor targeting agent.

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