Contortrostatin, a Snake Venom Disintegrin with Anti-Angiogenic and Anti-Tumor Activity

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Key Words

Snake venom · Disintegrin · Contortrostatin · Breast cancer · Ovarian cancer · Anti-angiogenesis

Abstract

Disintegrins are soluble peptides found in snake venom. They bind to Arg-Gly-Asp (RGD)-responsive integrins with high affinity (nM range) and block integrin function. Contortrostatin (CN), the disintegrin from southern copperhead venom, is a homodimer with a molecular weight of 13,500. Each chain contains 65 amino acids with an Arg-Gly-Asp motif. CN has anti-invasive and anti-adhesive activity on tumor cells and endothelial cells in vitro, and binds to integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and/or $\alpha 5\beta 1$. In vivo studies using the human metastatic breast cancer cell line MDA-MB-435, in an orthotopic xenograft model in nude mice, revealed that CN has potent anti-tumor and anti-angiogenic activity. Recent studies have employed an intravenous liposomal delivery procedure. Liposomal delivery of CN has also been shown to provide effective in vivo anti-tumor and anti-angiogenic activity in a human ovarian cancer animal model.

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Introduction

Integrins are α/β heterodimeric glycoproteins [1]. The different α subunits combine with distinct β subunits resulting in a range of specificities toward various extracellular matrix (ECM) proteins [2]. Several classes of integrins recognize the RGD sequence present in ECM proteins [3], allowing integrins to link cytoskeletal proteins with the ECM and to be involved in bi-directional signaling that alters cellular functions. Among these interactions are the adhesion of both endothelial cells and cancer cells to ECM proteins, interactions that are integral to metastasis and tumor growth. The importance of vitronectin receptors (integrins $\alpha v\beta 3$ and $\alpha v\beta 5$) in angiogenesis is well known [4, 5]. Yet integrins are not only central to angiogenesis. Pathological examination indicates that the level of αv expression in breast cancer is directly related to migration or metastasis [6], with higher levels of integrin associated with a more metastatic phenotype.

Disintegrins are small, disulfide-rich, Arg-Gly-Asp (RGD)-containing peptides that bind to integrins on the surface of normal and malignant cells [7]. Disintegrins have been characterized from many snake venoms and were originally characterized as platelet aggregation inhibitors [8]. Nuclear magnetic resonance has been used to determine the structures of several disintegrins [9]; they display no secondary structure, but are characterized

by an RGD sequence at the tip of a flexible loop anchored by disulfide bonds and protruding from the main body of the peptide chain. Contortrostatin (CN) was purified from southern copperhead venom [10], and is a homodimer. It has a molecular mass (Mr) of 13,500; each chain has an RGD sequence and an Mr of 6,700. The CN gene is part of a precursor composed of pro-protein, metalloproteinase, and disintegrin domains [11]. The disintegrin domain encodes 65 amino acids with a molecular weight equal to that of the CN subunit. CN is truncated at its amino-terminus compared to other disintegrins; the missing sequence contains two cysteine residues. All cysteines in other disintegrins are involved in disulfide bonds; since there are two unpartnered cysteine residues in the residual CN sequence, they must be involved in its dimerization. CN binds competitively to integrins of the $\beta 1$, $\beta 3$, and $\beta 5$ subclasses, including integrin receptors for fibronectin ($\alpha 5\beta 1$), vitronectin ($\alpha v\beta 3$, $\alpha v\beta 5$), and fibrinogen ($\alpha IIb\beta 3$) [10, 12, 13]. The lack of integrin specificity by CN is an advantage in controlling cancer growth and dissemination. CN disrupts angiogenesis induced by both bFGF and VEGF, consistent with the observation that a cyclic RGD peptide had no selectivity among αv family members [14].

In the present study, we report that CN can significantly inhibit angiogenesis and tumor progression *in vivo*, using MDA-MB-435, an $\alpha v\beta 3$ -, $\alpha v\beta 5$ -, $\alpha 5\beta 1$ -positive, human metastatic breast cancer cell line, and A2780^{SEAP}, an $\alpha v\beta 5$ -, $\alpha 5\beta 1$ -positive, $\alpha v\beta 3$ -negative, human ovarian cancer cell line. Recently, a recombinant form of CN has been produced, making clinical translation of this technology a more distinct possibility (see accompanying Chapter by Minea et al., in this volume).

Materials and Methods

Materials

Venom of *Agkistrodon contortrix contortrix* was purchased from the Miami Serpentarium (Punta Gorda, Fla.). CN was purified by multi-step high-performance liquid chromatography (HPLC) according to an established protocol [10, 12]. Human metastatic mammary cancer cell line MDA-MB-435 was provided by Dr. Janet E. Price, M.D. Anderson Cancer Center, Houston, Tex. Human ovarian cancer cell line A2780^{SEAP} was provided by Dr. Thomas C. Hamilton, Fox Chase Cancer Center, Philadelphia, Pa., and the SEAP detection assay kit was purchased from BD Biosciences (Palo Alto, Calif.). The monoclonal antibody (mAb) against CD31 antigen was purchased from Sigma (St. Louis, Mo.) and the immunohistochemistry detection kit was purchased from Zymed Laboratories, Inc. (South San Francisco, Calif.). All other reagents were of the best quality available.

Liposome Preparation

Liposomes were prepared using different ratios of high transition temperature lipids, cholesterol, and lipids derivatized with long-chain polymers (polyethylene glycol). The particles were formed using probe sonication according to previously described procedures [15, 16].

In vivo Efficacy of LCN in an Animal Model of Breast Cancer

For the efficacy studies, human metastatic breast cancer cells (MDA-MB-435, 5×10^5 cells) were implanted in the mammary fat pads of 5-week-old nude mice. The tumors were allowed to grow until palpable (14 days) at which time CN administration was initiated as previously described [16, 17]. In the initial breast cancer efficacy study, five mice per group were used and LCN was administered via three different modes: intravenous (IV) administration every other day (60 μ g/100 μ l of LCN per injection), IV administration twice weekly (100 μ g/100 μ l, LCN per injection) and daily intratumor (IT) administration (30 μ g/100 μ l, LCN per injection). Appropriate controls for the different delivery systems were employed. A second experiment with 10 mice per group was used to validate the conclusions from the first study and to gain a more statistically accurate evaluation of the effect of CN and LCN on tumor growth. In this trial, three groups were evaluated: LCN delivered IV twice weekly (100 μ g LCN/injection), daily native CN-IT (30 μ g CN/injection), and PBS control.

In vivo Efficacy of LCN in an Animal Model of Ovarian Cancer

For efficacy studies, human ovarian cancer cells (A2780^{SEAP}) growing as a monolayer in culture were harvested with trypsin EDTA, washed with complete medium, and resuspended in PBS. A2780^{SEAP} cells (2×10^7) were suspended in 4 ml PBS and 100 μ l of the cell suspension was injected intraperitoneally into 40 five-week-old female athymic nude mice (Balb/c/nu/nu) weighing approximately 20 g each (Simonsen Labs, Gilroy, Calif.). Each animal received 5×10^5 cells, and the tumors were allowed to implant for 4 days prior to treatment. The animals were maintained in a pathogen-free condition and received sterilized commercial food and water *ad libitum*. After 4 days, groups of 10 animals each received either: 30 μ g daily intraperitoneal (IP) injections of CN (CN-IP), 105 μ g twice weekly tail vein injections of LCN (LCN-IV Low), 210 μ g twice weekly IV injections of LCN (LCN-IV High), or daily IP PBS injections, for 28 days. Peripheral blood (40 μ l) was obtained by orbital puncture with heparinized capillary glass tubes (VWR, West Chester, Pa.). The blood was then transferred to a microfuge tube, and about 20 μ l of plasma was collected after the blood was centrifuged for 10 min at a speed of 14,000 $\times$ rpm (14.1 rcf). Plasma was stored at -20°C until SEAP analysis.

SEAP Assay

A previously described procedure was employed [18]. Two microliter of serum collected from each animal was pipetted to a 96-well microtiter plate and incubated in a 60°C oven for 30 min. Incubation at high temperatures should denature any endogenous alkaline phosphatase, but will not denature alkaline phosphatase secreted by the tumor cells. After heat treatment, the samples are allowed to cool on ice for 2 min and then 97 μ l of assay buffer (BD Biosciences, Palo Alto, Calif.) was added to the serum and allowed

to incubate at room temperature for 5 min. The assay buffer contains L-homoarginine, which inhibited any residual endogenous alkaline phosphatase, but not SEAP. The reaction mixture was then exposed to 3 μ l fluorescent 4-methylumbelliferyl phosphate (1 mM) and incubated in total darkness for 1 h at RT. Fluorescence was then determined using a CytoFluor 2300 fluorometer (Millipore, Billerica, Mass.) with excitation and emission at 360 and 460 nm, respectively.

Anti-Angiogenic Activity of LCN in Breast and Ovarian Cancer

Since previous *in vivo* studies with CN indicated that there was a dramatic inhibitory effect on angiogenesis in growing tumors [19–21], we examined the effect of LCN on tumor angiogenesis in the MDA-MB-435 and A2780^{SEAP} models. Tumors from treated and untreated mice in the LCN efficacy studies were fixed in 4% normal buffered formalin and embedded in paraffin blocks as described [16, 22]. Anti-CD31 (PECAM-1) monoclonal antibody was used for immunohistochemistry. A number of properties of CD31 make it an optimal reporter molecule for determination of tumor angiogenesis [23].

Results

Contortrostatin Inhibits MDA-MB-435 Mammary Cancer Growth

MDA-MB-435 cells were implanted in the mammary fat pads of nude mice and the tumors were allowed to grow until palpable (14 days) at which time drug administration was initiated. In the initial study, five mice per group were used and LCN was administered via three different methods (fig. 1a, left). A second experiment with 10 mice per group employed three groups: LCN delivered IV twice weekly (100 μ g LCN/injection), daily native CN-IT (30 μ g CN/injection), and PBS control (fig. 1a, right). The results of both studies reveal statistical significance ($p < 0.0001$) between both treatment groups and control, and the data correlates well with previous observations about tumor growth rates and the effect of CN on tumor size [17]. Intravenous LCN showed equivalent anti-tumor activity to direct intratumor administration of native CN, regardless of whether LCN is delivered IV every other day or twice weekly. However, intratumor administration of LCN shows no effect on tumor growth. The reason for this is under study, but we suspect it is related to the site of delivery. Intratumor injection delivers LCN to the necrotic core of the tumor where intratumoral pressure may inhibit release of CN. With LCN-IV, we hypothesize that CN is slowly released from the deposits of liposomes that accumulate as they leak from the vasculature throughout the tumor. Not only do these experiments reveal a statistically significant difference between intravenous LCN and untreated PBS control, but they also indi-

cate that twice weekly intravenous administration of LCN is as effective as daily intratumor administration of CN, and compared to intratumor delivery, the intravenous method of administration of LCN is directly translatable to the clinic for metastatic breast cancer.

As expected IV delivery of native CN showed no anti-tumor efficacy; this was in large part due to the very short circulatory half-life of CN. Thus, these experiments indicate the significant advantage of intravenous delivery of LCN as compared to IV delivery of unencapsulated CN. LCN is available in the circulation for a substantially longer period of time and passively accumulates at the site of the mammary tumor, presumably due to the leakiness of the angiogenic vasculature of the tumor [16].

Anti-Angiogenic Activity of LCN in Breast Cancer

We examined the effect of LCN on tumor angiogenesis in the MDA-MB-435 model. Tumors from treated and untreated mice in the LrCN and LCN efficacy studies were fixed as described in Materials and Methods. Quantitation of the stained vessels was performed using 'hot spot' analysis [17, 20, 24, 25]. Anti-CD31 (PECAM-1) monoclonal antibody was used for immunohistochemistry because it has been reported to be highly expressed on angiogenic vasculature [23]. Figure 1b presents a graph of the quantitation of CD31 immunostaining in the different treatment groups: PBS, LCN-IT (30 μ g twice weekly), and LCN-IV (105 μ g twice weekly). There is a statistically significant ($p < 0.0001$) reduction of microvascular density in the LCN group as compared to the PBS control, which corresponds to ~95% reduction of angiogenesis.

Contortrostatin Inhibits A2780^{SEAP} Tumor Formation

Six-week-old female athymic nude mice were injected intraperitoneally with 5×10^5 A2780^{SEAP} cells and the cells were allowed to implant for 4 days. After 4 days the animals (10 animals per group) were treated with either: PBS, IP native CN, or LCN-IV (at two doses) for 28 days (see Materials and Methods). Thirty-two days after intraperitoneal injection of A2780^{SEAP} cells in nude mice, all animals were euthanized and necropsy was performed. The peritoneal cavity of each animal was photographed and the occurrence of tumors was noted in the following organs: liver, spleen, kidney, bladder, ovary, stomach, and intestine. Gross examination of the peritoneal cavity shows a dramatic decrease in the numbers and size of tumors formed in the treated animals as compared to the control group (data not shown). Tumors were then col-

Fig. 1. Inhibition of MDA-MB-435 tumor growth and angiogenesis by LCN. Human mammary tumors (MDA-MB-435) were established by implantation of 5×10^5 cells into the mammary fat pads of 5-week-old female nude mice. **a** Groups containing 5 mice were treated as described in Materials and Methods (left panel). In the right panel there are 10 mice per group. Tumor mass was determined as described in Materials and Methods following weekly measurement with a caliper. LCN was statistically as effective as daily intratumor CN delivery when both were compared to control ($p < 0.0001$). Thus, intravenous administration of LCN is an effective

means of limiting tumor growth in the orthotopic, xenograft model of human breast cancer. Data shown represent the geometric mean of the tumor sizes. Error bars are 95% confidence intervals. **b** Positive staining areas on CD31-immunostained slides were quantitated in terms of pixels with SimplePCI advanced imaging software. Ten animals were used per group and the data represents 10 areas from three sections from each mouse, the average area in pixels of CD31-stained vessels in each treatment group is presented ($\pm$ SD). CN-IT group shows 82% inhibition, while LCN-IV shows 94% inhibition of angiogenesis, compared to PBS control.

lected from each organ in all animals and immediately fixed in formalin solution and processed for immunohistochemistry analysis using the neovessel marker CD31 to determine the amount of angiogenesis.

Contortrostatin Inhibits Tumor Burden as Determined by SEAP Activity

Each of the 40 A2780^{SEAP} tumor-bearing animals were bled twice weekly; plasma was prepared from whole blood and stored at -20°C until SEAP assay was performed. To

Fig. 2. Effect of CN on blood SEAP levels and angiogenesis in nude mice harboring A2780^{SEAP} human ovarian cancer. **a** Blood SEAP levels (geometric mean $\pm$ standard deviation) in the blood of control, and CN-treated groups are shown. There were no statistically significant differences in the SEAP levels (at days 5 and 8) between the four groups, but there were statistically significant differences in SEAP levels between control and the LCN-treated groups after day 12. In addition, there is a statistically significant difference between control and CN-IP-treated group at days 19 and 22 (overall $p = 0.0002$). CN-IP ($\blacktriangle$), LCN-IV Low ($\circ$), LCN-IV High ($\blacksquare$)-treated animals inhibit tumor burden by 14, 44, and 43%, respectively, as compared to the PBS control group ($\square$). See Materials and Methods for details. **b** CN was delivered intravenously at two doses, twice weekly (105 μ g CN, LCN-IV, and 210 μ g LCN-IV High). All methods of CN administrations showed potent inhibition of angiogenesis in the tumor sections as compared to PBS control tumors. Positive staining areas on CD31-stained slides were quantitated in terms of pixels with SimplePCI advanced imaging software. The CN-IP and LCN-IV-Low groups show $\sim 63\%$ inhibition, while the LCN-IV-High group shows $\sim 87\%$ inhibition of tumor-induced angiogenesis as compared to PBS-treated animals. The average area in pixels of CD31-immunostained vessels in each treatment group is presented ($\pm$ SD).

approximate the relative tumor burden for each treatment group a graph of SEAP activity (fluorescence intensity) as a function of collection day was prepared (fig. 2a). A difference in relative SEAP activity cannot be seen in control and treatment groups until day 12, similar to previous reports [18]. As expected, all the treated groups had lower SEAP values as compared to the control. However, the pattern was different than expected. We anticipated a dose-dependent decrease in tumor burden with increased dose of CN; however, this was not what we observed. Daily IP injection of CN showed only 14% decrease in SEAP activity as compared to control, while LCN-IV Low and High showed 44 and 43% decrease, respectively. Over a 3-fold decrease in SEAP values was observed when comparing IP and IV delivery routes for

CN. A dose-dependent response was expected because it had been previously established that circulatory SEAP levels are directly proportional to tumor size [18].

Quantitation of Positive CD31 Staining in A2780^{SEAP} Tumor Sections

To quantitate the anti-angiogenic effect of CN, three 'hot spots' from each CD31 stained tumor were digitally captured as previously described [16]. The software assigns a pixel value for positive CD31 staining, and the average of all the pixels in all the tumors found in each treatment group is shown as a bar graph (fig. 2b). CN-IP and the low dose of LCN-IV inhibited angiogenesis by 62 and 63%, respectively. This is not surprising since the weekly dosage for both groups is

equivalent (210 μg CN/week), although the modes of delivery are different. On the other hand, the high dose of LCN-IV (420 μg CN/week) inhibited angiogenesis by 87%. Our findings indicate that twice weekly intravenous delivery of liposomal CN appears to be as effective as daily intraperitoneal injections at equivalent dosages. In addition, there is a significant increase in inhibition of angiogenesis when the weekly dose is doubled, thus implying a dose-dependent inhibition. Finally, twice weekly IV injections are a more clinically relevant mode of delivery than daily IP injections, and they are equally as effective.

Discussion

It has long been established that angiogenesis is necessary for tumor growth and dissemination [26, 27]. Kim et al. showed that a monoclonal antibody against vascular endothelial growth factor blocked the growth of human tumors transplanted into mice [29]; this was the first conclusive *in vivo* evidence that anti-angiogenic therapy inhibited tumor growth [9, 30]. Angiogenesis appears to be an early event in epithelial ovarian cancer [31] and pro-angiogenic molecules are secreted by ovarian cancer cells to promote peritoneal dissemination [32]. There are a number of anti-angiogenic drugs currently in clinical trials [30], and a combination of anti-angiogenic treatments seems to have an additive effect in the inhibition of tumor growth [33]. Recently, the FDA has approved the first anti-angiogenic drug Avastin, a monoclonal antibody against VEGF, for cancer treatment. Ultimately, anti-angiogenic therapy should provide an additional cancer treatment suitable for combination with standard therapies [34].

Previous animal studies have shown that native ^{125}I -CN administered intravenously has a circulatory half-life of only 0.5 h [16]. To overcome this problem, CN was encapsulated in liposomes. Intravenously injected L- ^{125}I -CN has a circulatory half-life of 19 h [16]. Intravenous delivery of LCN is a more clinically relevant method of administration than intratumor delivery and twice weekly IV delivery of LCN displays anti-tumor and anti-angiogenic activity as effective as daily IT administration.

To assess *in vivo* biological activity of CN, the MDA-MB-435 breast cancer nude mouse model was initially employed. Breast cancer grows at the primary site as a solid tumor; this provides a distinct advantage over the ovarian cancer mouse model in which the tumors dis-

seminate throughout the peritoneal cavity, making it difficult to quantitatively assess tumor burden. In the mammary tumor model, when the tumors are palpable (at 2 weeks) IV injection of LCN was initiated and continued for 6 weeks; weekly measurements of tumor mass were recorded and plotted. In the ovarian cancer model, LCN was administered intravenously for 4 weeks. A significant decrease of tumor mass in both CN- and LCN-treated groups in breast and ovarian cancer models were observed, although quantitation in the ovarian cancer model was not satisfactory.

Immunogenicity of intravenously administered LCN is of concern. However, in long-term (10 weeks) studies, we have seen no immune responses. Immunocompetent mice were intravenously injected with CN or LCN twice weekly, in the presence or absence of Freund's adjuvant (administered subcutaneously), and bled at the beginning of the experiment and then several times during it to analyze the presence of specific neutralizing anti-CN antibodies by ELISA. Only native CN in the presence of Freund's adjuvant (forced immune response) was found to be weakly immunogenic, but the LCN group, even in the presence of adjuvant, was not immunogenic [16]. These results, plus findings from previous *in vitro* and *in vivo* studies, suggest that IV administration of LCN will be suitable for long-term use in a clinical setting.

The dramatically increased circulatory half-life of LCN over native CN accounts for the accumulation of LCN at the tumor site due to the increased number of passes of LCN through the tumor. This leads to passive accumulation of LCN at the tumor due to the 'leakiness' of the angiogenic vasculature of the tumor [35]. Previous reports have shown that tumor vasculature lacks hierarchy, is structurally heterogeneous, and hyper-permeable to plasma proteins [36]. The hyper-permeability or 'leakiness' may be caused by the lack of pericytes in some tumor vasculature [37, 38]. Previous reports have shown that newly growing vasculature at the site of tumors is not intact [9, 28]. The size of liposomes with encapsulated CN is approximately 140–170 nm, and the liposomes are able to leak out of the newly growing angiogenic vessels, which supply blood to the tumor. Once the liposomes exit the 'leaky' vessels and form depots, lipases and other extracellular enzymes presumably aid in the slow degradation of liposomes releasing the entrapped protein [40].

The current study with the ovarian cancer cell line A2780^{SEAP} indicates that, as with the MDA-MD-435 breast tumor model, we have developed an effective and more clinically relevant method of delivery, liposomal encapsulation of CN (LCN). LCN shows significant effi-

cacy in inhibiting tumor dissemination and angiogenesis in an $\alpha v\beta 3$ -negative, $\alpha v\beta 5$ - and $\alpha 5\beta 1$ -positive human ovarian cancer cell line following intravenous administration. In the study, LCN was delivered IV twice weekly while native CN was delivered IP daily. Both forms of treatment (at equivalent doses) showed a significant decrease in tumor growth and angiogenesis. However, IV administration of LCN allowed for a high level of tumor uptake and dose-dependent efficacy was observed. Therefore, as in the breast cancer model, twice weekly IV administration of LCN was a more effective method of de-

livery than daily IP introduction of native CN, both in limiting ovarian cancer dissemination and in inhibiting tumor angiogenesis. Thus, liposomal delivery of CN represents a clinically relevant method of administration.

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