

Original Article

In vivo and *In vitro* Anti-angiogenesis Effect of Venom-derived Peptides (ICD-85)

Mandana Mombeinipour¹, Abbas Zare Mirakabadi PhD², Kamran Mansuri³, Mohsen Lotfi⁴**Abstract**

Background: Angiogenesis is the development of new blood vessels from pre-existing vasculatures. Although essential in the physiological process, it becomes pathological in various diseases including cancer. Preventing the formation of new blood vessels causes reductions in tumor size and metastasis. This study has been undertaken to elucidate the anti-angiogenesis effects of ICD-85 (derived peptides from venom).

Methods: We evaluated the ICD-85 anti-angiogenesis activity by the *in vivo* CAM assay and *in vitro* tube formation assay of human umbilical vein endothelial cells (HUVECs). The anti-proliferative activity of ICD-85 was also determined through MTT assay on HUVECs.

Results: Results of this study revealed the anti-proliferative activity of ICD-85 on the HUVEC cell line with an IC50 of 12 µg/mL. The *in vivo* CAM assay also clearly showed the prevention of new vascular formation when the chick embryos were exposed to 0.15 µg/disc of ICD-85. *In vitro* tube formation assay of HUVECs also showed the complete prevention of capillary tube formation on 18 µg/mL.

Conclusion: Based on the results obtained in this study, ICD-85 has anti-angiogenesis activity as shown by the prevention of capillary tube formation and the CAM assay.

Keywords: Anti-angiogenesis, HUVEC, ICD-85, MTT assay, venom

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Introduction

Angiogenesis is the formation of new capillaries from pre-existing microvessels, which plays a major role in several physiological and pathological events. Angiogenesis has an important role during pathological conditions such as inflammatory diseases, tumor growth and metastasis.¹ This complex is a multistep process that includes extracellular matrix degradation, endothelial cell proliferation and migration, followed by the recruitment and adhesion of pericytes or smooth muscle cells.² Angiogenesis is regulated by the balance between angiogenesis factors that include vascular endothelial cell growth factor (VEGF)^{3,4} and anti-angiogenesis factors such as endostatin.^{5,6} Preventing the formation of new blood vessels causes a reduction in tumor size and prevention of metastasis.⁷ Since the discovery of its importance, angiogenesis is considered an optimal target for anti-cancer strategies. Recently many researchers have tested different compounds to develop anti-angiogenesis agents.⁸⁻¹¹

Some of the disintegrins purified from snake venom, that bind specifically to integrins and certain tumor cells have been proposed to be anti-angiogenesis agents.¹² The antitumor activity of rhodostomin, a purified disintegrin from snake venom, has been evaluated by examining its effects on B16F10 melanoma tumor-induced angiogenesis, where it was determined to be an anti-an-

giogenesis factor.¹³

Our previous studies on ICD-85 revealed an inhibitory effect on the breast cancer cell line MDA-MB231.^{14,15} *In vivo* studies have shown that the breast tumors stop weight gain and undergo a reduction in size when ICD-85 was injected intratumorally into mice that had breast tumors. This could be an indicator of the anti-angiogenesis characteristics of ICD-85.¹⁵ Hence the present study seeks to evaluate the anti-angiogenesis effect of ICD-85 by using *in vitro* and *in vivo* models.

Materials and Methods

Materials

The cell culture medium (DMEM/F12), fetal bovine serum (FBS) were purchased from the Gibco company. Trypsin-EDTA, penicillin and streptomycin were provided by Roche company (USA). Isopropanol, 0.1 N HCl, PBS, MTT, collagen type I, cytodex-3-microcarriers were from the Sigma company (USA). Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from the National Cell Bank, Pasteur Institute of Iran and SPF fertilized eggs purchased from Venky's India Ltd.

ICD-85

The active fraction of ICD-85 is a combination of three peptides that range from 10 to 30 kDa and are derived from the venom of an Iranian snake (*Agkistrodon halys*) and a scorpion (*Hemiscorpius lepturus*).¹⁴

Cell culture

The HUVEC cell line was cultured in DMEM:F12 supplemented with 7% heat-inactivated FBS along with 100 IU/mL penicillin G and 100 µg/mL streptomycin. The culture was incubated at 37°C with humidified air that contained 5% CO₂.

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Cell viability assay

MTT assay

Cell viability was determined using the 3-(4,5 dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay. HUVECs (12.5×10^3) were cultured in 96-well plates for 72 hours. Different concentrations of ICD-85 (24, 12, 6, 3, 1.5, 0.75, 0.37, 0.19, 0.094, 0.047, and 0.023 $\mu\text{g/mL}$) were diluted by the medium culture and added, after which, cells were incubated for an additional 48 hours. The dilution series of ICD-85 was removed; culture medium and FBS were added and plates were transferred to the incubator for an additional 48 hours. To assay cell viability, 20 μL of MTT was added to each well and plates were incubated for 6 hours. The medium was carefully removed and acidic isopropanol was added. Plates were carefully shaken until the formazan solubilized. We measured absorbance at 570 nm using an absorbance reader.¹⁶ The percentage of viable cells was calculated according to the following formula:

Percentage of viability of each concentration = (corrected mean OD of test/corrected mean OD of control) \times 100

CAM assay

Specific pathogenic-free (SPF) fertilized eggs (Venky's India Ltd.) were incubated at 37°C at a constant humidity. On day seven, two small holes were drilled into the shell, one at the base of the egg and the other on the upper surface. With the use of a gentle vacuum, an air sac was transferred to the upper surface and the CAM was dropped. Next, we created a small window (~1 cm in diameter) on the upper surface. Whatman's sterile paper disk that contained 5 μL distilled water was used as the negative control and, under sterile conditions, different concentrations (15, 30, 60, 90 $\mu\text{g/mL}$) of ICD-85 were placed on the CAM. The zones around and under the disks were observed microscopically (Olympus BX51TRF Microscope, USA) 72 hours after disc placement and blood vessels were analyzed by three independent expert observers.¹⁷

HUVEC capillary tube formation in three-dimensional collagen gel

Preparation of cytodex-3-microcarrier beads

Cytodex-3-microcarrier beads were allowed to pre-swell in phosphate buffer, then rinsed with DMEM:F12 under a sterile hood.¹⁸

Development of *in vitro* angiogenesis models

HUVECs were mixed with cytodex-3-microcarriers at an appropriate ratio in DMEM:F12 medium supplemented with 7% FBS, 100 IU/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin. Then, cell-coated beads were cultured in collagen matrix and the culture medium was added. In order to monitor anti-angiogenesis effect of ICD-85, we treated the cells with different concentrations (9, 18, 27 $\mu\text{g/mL}$) of ICD-85 against the controls. The results were analyzed microscopically after 48 hours.¹⁹

Results

Anti-proliferative effect

To investigate the anti-proliferative effect of ICD-85 on HUVECs, we performed the MTT assay using various concentrations of ICD-85. According to the results, HUVECs proliferation was inhibited by less than 20% at 0.75 $\mu\text{g/mL}$ of ICD-85. However,

more than 72% inhibition was observed when the HUVECs were exposed to 24 $\mu\text{g/mL}$ of ICD-85. The 50% proliferation inhibition (IC50) of HUVECs exposed to ICD-85 was 12 $\mu\text{g/mL}$ (Table 1).

Table 1. Inhibitory effect of various concentrations of ICD-85 on HUVECs.

Concentration of ICD85 ($\mu\text{g/mL}$)	Inhibition \pm SD (%)
24	72 \pm 3
12	50 \pm 4.12
6	42 \pm 4.36
3	33 \pm 2.29
1.5	23 \pm 2.12
0.75	19 \pm 1.78
0.37	18 \pm 4.29
0.19	15 \pm 4.57
0.093	12 \pm 1.55
0.047	5 \pm 2.5
0.023	0

Values are expressed as mean \pm SD from at least three independent experiments.

Result of inhibitory activity of ICD-85 on chick chorioallantoic membrane (CAM) model

Results of the CAM assay showed that the lowest concentration (0.075 $\mu\text{g/egg}$) of ICD-85 decreased the size and density of blood vessels compared to control group that used only distilled water. However at higher concentrations, the effect of ICD-85 was more prominent and at concentrations of 0.3 $\mu\text{g/egg}$ and higher, angiogenesis was inhibited. Also, at higher concentrations some previously formed capillary tubes and vessels were absent (Figure 1).

Anti-angiogenic effect of ICD-85 on collagen-cytodex model

The anti-sprouting effect of ICD-85 was studied by three-dimensional culture as an *in vitro* model. While untreated control wells showed a branching pattern of tube-like vessels, capillary tube formation did not appear in the treated models. Inhibition of this model was also dose-dependent. Capillary tube formation was partially inhibited in wells treated with 9 $\mu\text{g/mL}$ of ICD-85. This concentration showed the lowest inhibitory activity. In addition sprouting was completely inhibited with 18 $\mu\text{g/mL}$ of ICD-85; eventually, at 27 $\mu\text{g/mL}$, HUVECs were destroyed (Figure 2).

Discussion

Cancer, despite the ongoing efforts from developed countries, still causes one in five deaths. Surgery, chemotherapy, and radiotherapy have their own limitations. Several studies have been undertaken during the last three decades to find the anti-cancer property of venoms and toxins. Claude Bernard, the father of physiology was the first who realized that physiologically active components of snake venom might have therapeutic potential.²⁰ Tumorigenesis is a multistep process where angiogenesis plays an important role in growth, progression and metastasis of all solid tumors. Therefore, the agents that inhibit angiogenesis could be effective in controlling primary growth and development of tumors as well as secondary metastatic tumors. Based on this hypothesis and the outcomes of preclinical studies, antiangiogenic therapy has been suggested as a most promising approach to cancer control.²¹⁻²⁴ Some disintegrins purified from snakes are proposed to be anti-angiogenesis agents.¹² For example, rhodostomin, a purified disintegrin from snake venom has been evaluated and found to have anti-angiogenesis properties.¹³

ICD-85 used in the present study is a combination of 3 peptides partially isolated from two different venoms. The molecular

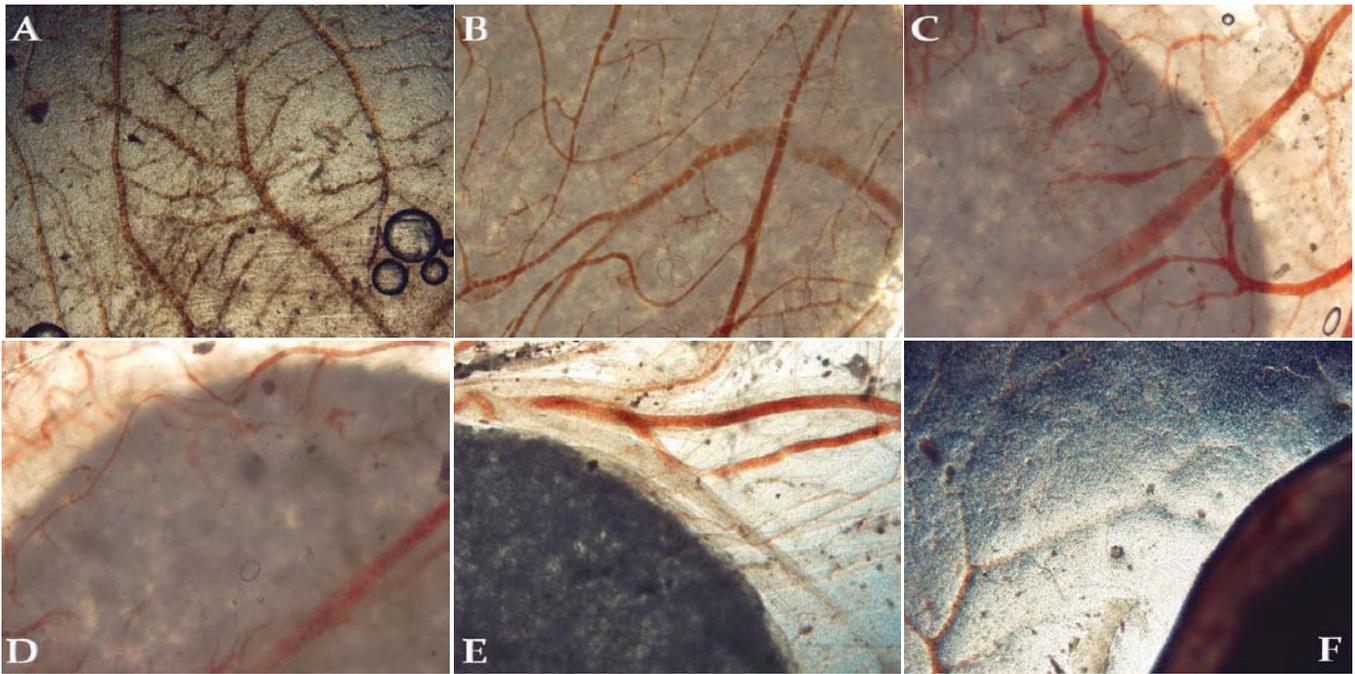


Figure 1. Anti-angiogenic effect of ICD-85 on CAM assay. Control (A). Eggs treated by 0.075 µg of ICD-85 (B). Eggs treated with 0.15 µg of ICD-85 (C, D). Eggs treated with 0.3 µg of ICD-85 (E). Eggs treated with 0.45 µg of ICD-85 (F).

weight of active fractions of ICD-85 range from 10 to 30 kDa and have been derived from the venoms of an Iranian snake (*Agkistrodon halys*) and a scorpion (*Hemiscorpius lepturus*).¹⁴ The combination of these peptides are used because they work together synergistically having anti-proliferative activity on cancer cells and *in vivo* suppression of tumors in mice.¹⁵

The results of the present study demonstrated that ICD-85 strongly inhibited growth and cell proliferation as well as reduced survival, causing HUVE cell death. Anti-proliferation activity of

ICD-85 was carried out on the tube-forming endothelial cells using the MTT assay.²⁵ ICD-85 inhibited endothelial cell viability in a concentration-dependent manner. Our previous studies on MDA-MB231^{14,15} and HL60 cells²⁶ showed that ICD-85 effectively inhibited the growth of cancer cells. We determined the 50% inhibitory concentration of ICD-85 on HUVECs to be 12 µg/mL. However, in the present study the maximum concentration of ICD-85 which showed consistency and reproducibility of results with 72% inhibition of HUVEC cells was 24 µg/mL; when

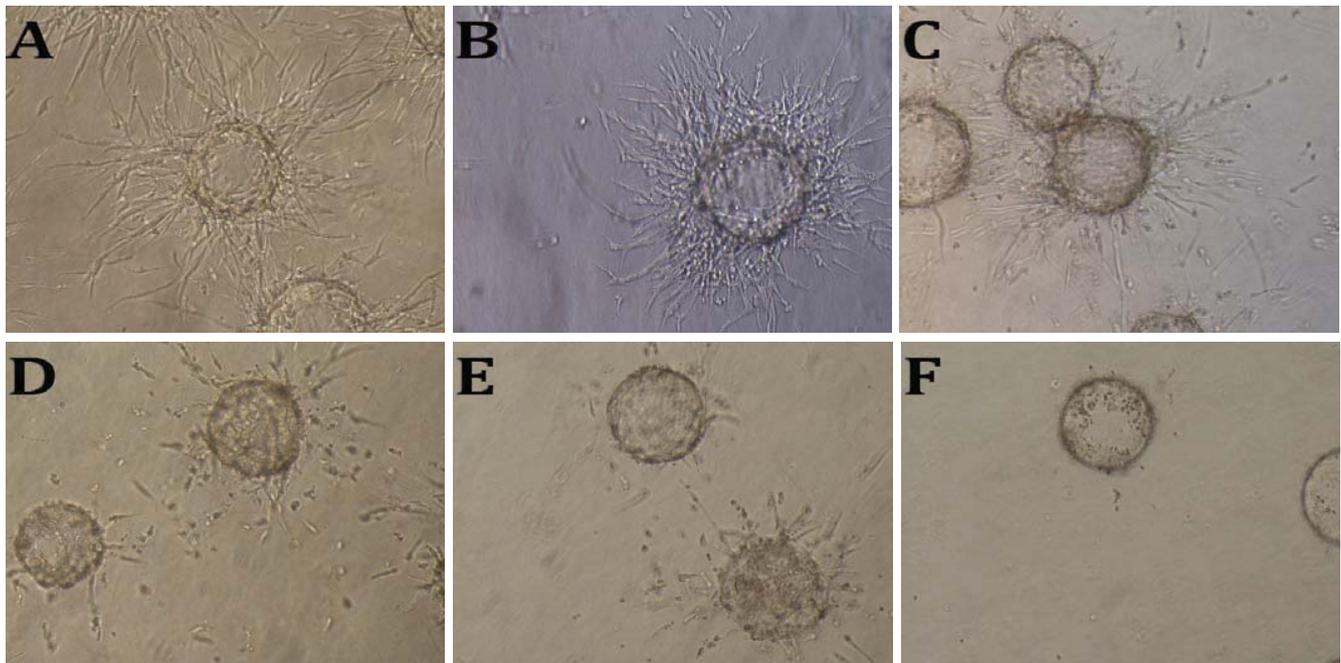


Figure 2. Effect of ICD-85 on *in vitro* angiogenesis. HUVECs were cultured on three microcarrier beads and seeded on a three-dimensional cytodex-3 microcarrier model. Sprouting at control was induced by adding medium that contained growth supplements (A, B). Angiogenesis of the endothelial cells treated by ICD-85 at 9 µg/mL (C). 100% of inhibition of sprouting observed at 18 µg/mL of ICD-85 (D, E). Cell destruction observed at 27 µg/mL of ICD-85 (F).

higher concentrations of ICD-85 were used the results inconsistent. Hence, in the present study we reported the effect of ICD-85 on HUVECs only up to 24 $\mu\text{g/mL}$. HUVECs are not a cancer cell line, however since these cells are involved in new tube formation, their inhibition by ICD-85 can be an indicator of the anti-angiogenesis effect of these peptides.²⁷ In another study by our group, when HeLa as cancer cell line and MRC-5 as normal cells were treated with ICD-85, we determined that ICD-85 inhibited the growth of HeLa cells at a concentration less than 10 $\mu\text{g/mL}$ in a dose-dependent manner. It had a mild effect on normal MRC-5 cell growth and viability at a concentration of 60 $\mu\text{g/mL}$ which was six-times more than the cytotoxic dose against HeLa cancer cells.²⁸ In 2009, Chen et al. reported that the IC50 of L-amino acid oxidase derived from *Naja atra* venom on HUVECs was 21.42 mg/L.²⁹ In order to block tumor growth and metastasis formation, a number of inhibitors that target tumor vasculature have been identified by *in vitro* and *in vivo* anti-angiogenesis studies.³⁰

Inhibition of branching and capillary tube formation on HUVECs was evaluated in a dose-dependent manner. The anti-angiogenic potential of ICD-85 on a cytodex-3-microcarrier bead model showed that these peptides at a concentration of 18 $\mu\text{g/mL}$ could completely inhibit angiogenesis. In a similar study, Seyfi et al. have reported complete inhibition of capillary tube formation by 100 $\mu\text{g/mL}$ of an aqueous fraction of shallot.³¹ Since *in vitro* assays are relatively inexpensive with more rapid results, they are often used by numerous researchers. We have attempted to confirm our *in vitro* results by *in vivo* studies. Results of the CAM assay showed that the lowest used concentration (0.075 $\mu\text{g/egg}$) of ICD-85 decreased the size and density of blood vessels compared to the control group. However, at higher concentrations the effect of ICD-85 was more prominent and with complete inhibition of angiogenesis as well as some previously formed capillary tubes and vessels. The CAM assay is probably the most widely used *in vivo* assay for studying angiogenesis.^{32,33} The lack of a mature immune system in 7–8-day-old chick embryos allows for the study of anti-angiogenesis.³⁴ Jeon et al. used the CAM assay and showed that the plasma hyaluronan binding protein (a novel serine protease) at 10 $\mu\text{g/disc}$ could partially inhibit angiogenesis.³⁵ Other researchers have also identified anti-angiogenesis peptides, such as disintegrins, from snake venom. Disintegrins that have been isolated from snake venom are a family of low-molecular-weight, peptides that bind specifically to integrins on platelets and other cells such as vascular endothelial cells and some tumor cells.¹² Triflavin from *Trimeresurus flavoviridis* venom is another peptide with disintegrin properties that has been shown to inhibit angiogenesis both *in vitro* and *in vivo*.³⁶

The anti-angiogenesis effect of ICD-85 could be through its binding to VEGFR and other VEGF receptors in endothelial cells. VEGF can trigger multiple cellular responses such as promoting cell survival, preventing apoptosis, and cytoskeletal remodeling, all of which promote angiogenesis. Several lines of evidence indicate that endothelial cell apoptosis plays a critical regulatory role in angiogenesis.³⁷ Our previous studies have shown that ICD-85 induces apoptosis in the HL60 cell line.²⁶ We have demonstrated that ICD-85 increases caspase-8 activity.²⁷ Hence, there is a possibility that the antiangiogenic activity of ICD-85 is at least partially related to induction of apoptosis in endothelial cells. In conclusion, based on the results obtained in the present study as well as our previous studies, we hope that ICD-85 can be considered as an anti-angiogenic agent.

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