Anatomy & Physiology: Current Research
ISSN: 2161-0940

Trinh et al., Anat Physiol 2016, 6:3
DOI: 10.4172/2161-0940.1000213

Research Article Open Access

A Novel Anti-tumor Protein from *Calloselasma Rhodostoma* Venom in Vietnam

Kiem X Trinh[1], Pham Van Thuc[2], Chu Thi My[1], Long X Trinh[1], Thuc Thi Minh Vu[1], Bao Khanh Nguye[1]n, Francis Markland Jr[3], Stephen Sweenson[3] and Radu Minea[3]

[1]*Department of Toxicology, Vietnam Institute on Toxicology (VST), Vietnam*
[2]*Department of Toxicology, Haiphong Medical and Pharmacological University, Vietnam*
[3]*Departments of Biochemistry and Molecular Biology, Keck School of Medicine of USC, CA 90033, USA*

Abstract

Aim: Vietnam is a tropical and agricultural country. Annually, there are 30,000 cases of snake bite. Two venomous snake families cause the big medical problem. In this, *Calloselasma Rhodostoma* (CR) is the most dangerous snake. Therefore, since 2001, the scientific research collaborations between Vietnam (VN) and University of Southern California (USC) were established. Since 2014 this project has been approved by VN government. The aims of the 1st project are to establish the technological process for purification of dsintegrin from CR venom of VN (*CR*d.VN), to determine the molecular weight, structure and its biological antitumor activities.

Methods: The process of collection, lyophilisation of CR venom from VN. Protein concentration of CR venom was determined by BCA assay. High Performance Liquid Chromatography (HPLC), SDS-PAGE, Mass spectrometry (MS) analysis and sequencing by tryptic digestion were used for purification of *CR*d.VN and its molecular weight (MW) and structure. Standard cell biology methods were employed to characterize *CR*d's abilities (*in vitro*) to inhibit platelet aggregation, adhesion, migration and invasion of tumor cells. Its anti-cancer activity in a breast cancer (BC) murine model (*in vivo*) was tested.

Results: Peak 7 of HPLC (*CR*d.VN), showed a single ~10 kDa band on SDS-PAGE gel. *CR*d.VN's MW, structure and the sequence are 7.33 kDa, a monomer containing 68 amino acids with an RGD motif (position 49-51) and 6 disulfide bonds. The anticancer activities of *CR*d.VN are very strong.

Conclusion: We have shown that CRd.VN is a possible anti-tumor agent with clinical potential. However, further research is required on *CR*d recombinant production, preliminary pharmacokinetics/ toxicology properties and anti-tumor activities.

Keywords: CRd anti-cancer; CRd recombinant production; Pharmacokinetics

Introduction

Cancer is a chronic degenerative disease considered to be the second most common cause of death in economically developing countries [1].

According to a recent report by the International Agency for Research on Cancer (IARC), there are currently more than 10 million cases of cancer per year worldwide. In 2008 alone there were 12.7 million new cases of cancer worldwide and the WHO estimates that the disease will cause about 13.1 million deaths by 2030 [2].

Vietnam, there were 68.810 new cases of cancer in 2000, increasing to 126.307 in 2010 and estimates that will be 189.000 in 2020 [3].

However, cancer therapy continues involving invasive procedures, including the application of chemotherapy, surgery to remove the tumors, the use of radiation, and even no selective cytotoxic drugs [4]. Therefore, the search for new active drugs for cancer therapy is one of the goals of biotechnological research.

In the early 20th century, the idea of utilizing purified toxins as a source of therapeutics emerged. Anticancer drug developments from natural biological resources are ventured throughout the world. Snake venom toxins were also investigated as blockers of metastasis. Metastasis is one of the major causes of death in patients with cancer, being dependent on steps such as adhesion, migration, invasion of blood or lymph vessels, and finally interaction with the tissue target [5].

The ability of snake venoms to act upon tumor cells has been known for a long time. The first reported studies on using snake venom against tumor cells were related to the defibrination process. It was suggested a polypeptide from Agkistrodon rhodostoma could produce defibrination, decreasing the tumor weight and decreased spread of some tumors [6].

One of the targets investigated is integrins. They play multiple important roles in cancer pathology including tumor cell proliferation, angiogenesis, invasion, and metastasis. At least six integrin inhibitors are being evaluable in clinical trials for cancer [7].

Integrins are heterodimer receptors that evolved to mediate the complex cell-ECM interactions that regulate the ability of cells to mechanically sense their micro environment. Conversely, as epithelia transition to malignancy they evade the micro environmental constraints by both altering their integrin affinity and avidity for ECM proteins (inside-out signalling) and/or shifting their integrin expression [8,9]. The precise roles, however, played by different integrin subunits

***Corresponding author:** Kiem X Trinh, Department of Toxicology, Vietnam Institute on Toxicology, Vietnam, E-mail: kiemtrinhxuan211@yahoo.com; trinhkiem0211@gmail.com

Received March 28, 2016; **Accepted** April 12, 2016; **Published** April 18, 2016

Citation: Trinh KX, Thuc PV, My CT, Markland FJr, Swenson S, et al. (2016) A Novel Anti-tumor Protein from *Calloselasma Rhodostoma* Venom in Vietnam. Anat Physiol 6: 213. doi:10.4172/2161-0940.1000213

Copyright: © 2016 Trinh KX, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

in various aspects of tumor progression and why some integrins appear to be especially supportive of tumor progression [10] are still poorly understood. Despite these limitations, due to their pivotal roles in cancer biology, integrins represent attractive therapeutic targets.

The inhibition of angiogenesis is one of the most heavily explored treatment options for cancer, and snake venom disintegrins represent a library of molecules with different structures, potencies, and specificities and are good starting points for developing anti-angiogenesis therapeutics. Disintegrins first discovered in 1983 [11] and named in 1990 [12]. They are family of disulphide–rich, stable peptides, originally isolated from snake venom, also found in mammalian proteins (ADAMs). Disintegrins bind exclusively to activated integrins on cells, derived from multi-domain proteins by proteolytic processing, contain RGD (or alternate tri-peptide) motif at tip of a flexible 11-amino acid loop that is involved in integrin interaction. Range in size from small to medium to large and homo- and hetero-dimeric. Disintegrins hold a significant translational potential as anticancer agents based on their anti-angiogenic and anti-metastatic effects demonstrated in various experimental settings [13-15].

Along with metalloproteases, disintegrins are important compounds in most Viperid and Crotalid venoms. Disintegrins represent a family of nontoxic and nonenzymatic low molecular weight (5–10 kDa) RGD-containing peptides naturally presented in snake venoms or synthetics. Originally, these compounds are characterized by their ability to interact with integrins $\alpha IIB\beta 3$, $\alpha 5\beta 1$ and $\alpha V\beta 3$IIs expressed by a number of cells including those involved in tumor development and proliferation [16].

Based on binding experiments, $\alpha\beta$ integrins and their subtypes have been identified as major functional adhesion receptors on tumor cells. Indeed, disintegrins from several snake venoms have revealed new possibilities of uses not only in cardiovascular diseases but also as potent inhibitors of tumor cells. Thus, a number of toxins containing RGD-peptides or RGD-containing disintegrins isolated from snake venoms have also been used to elucidate target receptors in a wide variety of primary cultured tumor cells.

For almost three decades since 1983, over 100 additional disintegrins have been studied and named [17]. Despite their enormous therapeutic potential, none of these natural products or their recombinant variants has made it yet into human clinical trials. However, many of these natural polypeptides continue to be intensely investigated pre-clinically in various animal models of human diseases, most subsequent preclinical efforts have focused on the anti-angiogenic and anti-metastatic properties of these disintegrins for anti-cancer applications [18].

The integrin binding activity of disintegrins depends on the appropriate pairing of several cysteine residues responsible for the disintegrin fold, a mobile 11 amino acid loop from the polypeptide core displaying a tri-peptide, usually RGD (Arg-Gly-Asp) motif. Two of the most studied native disintegrins are the homo-dimer contortrostatin (CN) and the monomer echistatin. Studies of Markland have shown disintegrin contortrostatin (CN) from southern copperhead snake venom to be an effective agent in limiting tumor growth, spread and blocks breast cancer cell adhesion and migration [19-23].

In Vietnam (VN), *Calloselasma Rhodostoma* (CR) is the most dangerous snake of Viperidae. The potential toxicity of CR venom may be stronger than southern copperhead venom. Therefore, since 2001, the scientific research collaboration between VN and University of Southern California (USC) was established. Since 2014 this project has been approved by VN government [24-40].

The Aim of the 1st Project

To establish the technological process for purification of disintegrin from CR venom and to determine the molecular weight, structure and its biological antitumor activities.

Materials and Methods

CR Venom was collected, lyophilized and supplied from Vietnam.

Cells and reagents

The MDA-MB-435 cells were obtained from MD Anderson Cancer Center, Houston, TX and the MDA-MB-231 cells from Osaka University, Osaka, Japan. HUVEC were purchased from Promocell (Heidelberg, Germany) and maintained according to the manufacturer's protocol. The 'Endothelial Cell Tube Formation' plates were purchased from BD Biosciences (San Jose,CA). The tube formation inhibitor Suramin, the actin modifier Cytochalastin D was purchased from Calbiochem (San Diego, CA). The complete Matrigel was from BD Biosciences (Bedford, MA). A column-based FITC-labeling kit was purchased from Pierce (Rockford,IL). All other reagents were purchased from Sigma Chemical Co (St.Louis, MO).

Protein concentration determination of CR venom

Pierce™ BCA Protein Assay Kit, Thermo Scientific, USA (Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K. Olson,B.J., Klenk,D.C. Measurement of protein using bicinchoninic acid [24].

Purification of disintegrin from CR venom of Vietnam

Following the procedure used for purification of the disintegrin (CN) from Agkistrodon contortrix contortrix venom we utilized a multi-step HPLC purification strategy. First the CR venom was fractionated using a hydrophobic interaction chromatography (HIC) method. The CR venom is placed into a sodium phosphate buffer applied to the column and eluted with a gradient of ammonium sulfate collecting fractions with absorbance at 280 nm indicating the presence of protein. In the second step, the positive fractions were applied to a C18-reverse phase HPLC column and run using the standard elution conditions previously employed for the purification of native CN.

The collected HIC fractions were loaded onto a Vydac C18 reverse-phase (RP) column (218TP54, Temecula, CA). A ten-minute rinse of the column (at 5 ml/min) with an aqueous solution containing 0.1%TFA was followed by a linear gradient (0-100%) elution over 150 min in a mobile phase containing 80% acetonitrile and 0.1%TFA. The disintegrin (CRd) starts eluting in 30% acetonitrile and is collected for lyophilisation and further characterization

Inhibition of platelet aggregation

The inhibition of ADP-induced platelet aggregation by CRd was determined by measuring the light absorption of human platelet-rich plasma (PRP) in a specialized spectrophotometer (Chrono-log 490 optical aggregometer, Chrono-log, Havertown, PA) as previously described. Briefly, the aggregometer is blanked with platelet poor plasma (PPP) and platelet rich plasma (PRP) is used for aggregation studies. The putative CRd samples are added to PRP and mixed for 30 seconds at this point ADP is added to a final concentration of 20 nM to the CRd-PRP mixture and the level of aggregation inhibition is

evaluated. These studies are presently on going with fractions obtained from C18 RP-HPLC.

Mass Spectrometry (MS) analysis and sequencing by tryptic digestion

The MS analysis and the subsequent sequencing of purified CRd were performed by Dr. Ebrahim Zandi (Keck School of Medicine, University of Southern California) using purified CRd obtained from C18 RP-HPLC. For sequencing, the purified disintegrin was reduced, alkylated and digested with trypsin at 37ºC overnight. The resultant digestion peptide was used in the tandem LC/MS/MS for sequence analysis. The LC (liquid chromatography) consists of a reverse phase C18 column through which peptide was eluted into the mass spectrometer using the following gradients: 5-60% acetonitrile + 0.1% formic acid over 75 min and 50-90% acetonitrile + 0.1% formic acid over 10 min. Tandem MS/MS spectra was acquired with Xcalibur software on a linear ion trap LTQ instrument. Data was analyzed using Bioworks, the SEQUEST algorithm and Sage-N Sorcerer to determine cross-correlation scores between acquired spectra and NCBI protein FASTA databases or any other databases as needed

Cell surface binding of CRd as studies by flow cytometer

HUVEC (human umbilical vein endothelial cells), and MDA-MB-231 or MDA-MB-435 breast cancer cells were to be grown to early confluence and starved overnight in serum-free media. The cells were harvested and suspended in 1 ml of serum-free media (5×10^5 cells/condition) before being incubated with different amounts of CRd or controls for 30 min at 37ºC. At the end of the incubation period, the cells were pelleted, washed in ice-cold PBS containing 5% fetal bovine serum and either analysed in a FACS Cali bur scanner (fluorescent activated cell sorter) or, depending on the assay, further incubated at 4ºC for 30 min intervals with additional treatments. All cells were counterstained with propidium iodide to allow gating of necrotic cells. For each reading, 10,000 cells per sample were analysed to determine level of CRd to the cells.

Inhibition of cell migration (The colloidal gold migration assay)

Inhibition of cell migration (the colloidal gold migration assay) by disintegrins. The ability of *CR*d.VN to disrupt cell migration was assessed on gold coverslips coated with complete Matrigel on which serum-starved HUVEC, MDA-MB-231 or MDA-MB-435 cells were seeded and allowed to adhere before being incubated for up to 48 h with various concentrations of disintegrin (*CR*d) (1–1000 nM). The fungal metabolite Cytochalasin D, a potent inhibitor of actin polymerization, was used as a positive control at a concentration of 200 nM. (i) Representative images of 'phagokinetic tracks' generated on colloidal gold by migratory HUVEC exposed to different concentrations of disintegrin (*CR*d.VN) (magnification, 200; scale bar, 200 mm). (ii) The 'phagokinetic tracks' generated by migratory cells with different integrin profiles (HUVEC, MDA-MB-231 or MDA-MB-435) were quantitated digitally ('SimplePCI' imaging software) by counting the total number of pixels corresponding to every track in multiple photomicrographs for each condition. The above plotted data were averaged from multiple independent experiments for each cell line tested.

Inhibition of cell invasion by *CR*d

The ability of *CR*d to block the invasion of HUVEC, MDA-MB-231 or MDA-MB-435 cells through a reconstituted basement membrane was assessed using the fluorometric QCM™ 24-Well Cell Invasion kit (Millipore, Billerica, MA). The cells were serum-starved overnight, harvested, suspended in serum-free media (1×10^6 cell/ml) and incubated in the presence of various concentrations (0-1000 nM) of *CR*d or control peptide for 10 min at 37°C. The assay was done according to the manufacturer's protocol and used HT1080 conditioned media as a chemo attractant. The invasion plates were incubated for up to 48 hr (depending on the cell line) at 37°C in the presence of 5% CO_2. At the end of the incubation period, the invaded cells were detached, lysed and quantitated using the DNA-binding fluorescent dye CyQUANT. The relative fluorescence was measured in a SPECTRAmax GeminiEM fluorescent plate reader (Molecular Devices, Sunnyvale, CA) and the numbers averaged and plotted for each condition.

Inhibition of HUVEC tube formation

'Endothelial Tube Formation' plates recoated with Matrigel (BD Biosciences, Bedford, MA) were used according to the manufacturer's protocol. HUVEC were seeded in triplicate (3×10^4 cells/well) in the presence of various concentrations (0-1000 nM) of CRd and incubated for 16 hr at 37°C in the presence of 5% CO_2. The tube formation inhibitor Suramin was used as a positive control. At the end of incubation period, cells were stained with Calcein AM and imaged by confocal microscopy (LSM 510 Confocal/Titanium Sapphire Laser). The total length of tubes for each condition was quantitated in multiple fields using the Zeiss LSM Image Browser (Carl Zeiss Micro Imaging GmbH, Munich, Germany) and averaged from at least three independent experiments.

In vivo efficacy studies

MDA-MB-231 cells (2×10^6 per inoculum) were harvested and resuspended in complete Matrigel before being inoculated into the mammary fat pads of nude mice as previously described (Swenson et. al., 204). The tumors were allowed to grow for 2 weeks or until they became palpable before treatment was initiated. *CR*d was administered as an aqueous solution (100 µg *CR*d). All *CR*d administrations were made intravenously (via tail vein) twice a week for the duration of each study. Several doses were examined to determine an optimal dose, based on experience with vicrostatin we were used doses of approximately 1 mg per injection twice per week. As a control Avastin was administered intravenously (via tail vein) at the dose of 400 µg per injection (approx. 20 µg/gr.) once a week for the duration of the MDA-MB-231 study. Tumor diameters were measured weekly with a calliper in a blind fashion and the tumor volumes calculated using the formula [length (mm) × width (mm)^2] / 2, where the width and the length were the shortest and longest diameters, respectively. The average tumor volume for each study group was plotted as a function of time and type of treatment during the entire course of each study. A survival study was conducted with those treated mice who survive, and the results plotted according to Kaplan and Meier. Mice weight was measured weekly as an indication of toxicity and mice was examined weekly for signs of internal bleeding or other indications of toxic side effects [20-30].

Statistical Analysis

Statistical significance was analyzed in Prism v.3.2 (GraphPad Software, La Jolla, CA) by unpaired t-test followed by F-test to compare variances. The tumor volume distribution and immunohistochemistry data were assessed by analysis of variance (ANOVA) with a significant overall F-test followed by Dunnett's multiple comparison tests of

Citation: Trinh KX, Thuc PV, My CT, Markland FJr, Swenson S, et al. (2016) A Novel Anti-tumor Protein from *Calloselasma Rhodostoma* Venom in Vietnam. Anat Physiol 6: 213. doi:10.4172/2161-0940.1000213

treatment groups relative to control. Two-tailed $P < 0.05$ was considered significant.

Results

CR venom from VN was collected, lyophilized successfully

1,700 *CR* snakes from the wild fields in the south east of VN were collected and identified. Their venom was milked, centrifuged at 3,000 RPM × 15 min. The venom was taken and then kept cool (-80°C) immediately for 24 hrs. In the next day, *CR* venom was lyophilized in Manifold LabConco machine for 48 hrs. Lyophilized *CR* venom was stored at (-20°C) and dark place until the time for using (Figures 1 and 2).

The protein concentration of CR venom was determinate by BCA protein assay

CR venom was determinate protein concentration by Bicinchoninic (BCA) Assay (Smith, P.K.1985) for 07 times with good quality. The result was 158 ± 03 mg/ml (Figure 3).

The result of disintegrin purification from CR venom and its molecular weight

Disintegrin from CR venom of VN (*CR*d.VN) was purified by reverse phase HPLC and SDS-PAGE using a protocol originally designed for native disintegrins (Figure 4).

The structure of *CR*d.VN was determined by Mass spectrometry (MS) analysis and sequencing by tryptic digestion, performed by Dr. Ebrahim (USC) (Figures 4 and 5).

The result of *CR*d.VN inhibited platelet aggregation

Result of *CR*d.VN inhibited platelet aggregation is shown in Figure 7.

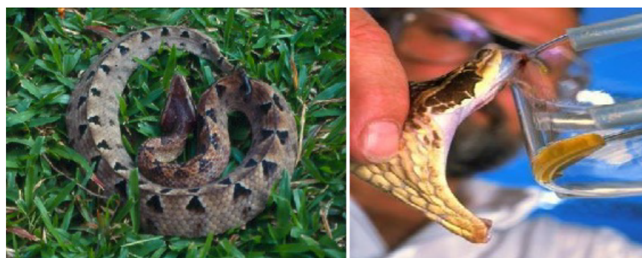
The result of cell surface binding of *CR*d.VN as studies by flow cytometry

Result of Cell Surface Binding of *CR*d.VN as Studies by Flow Cytometry is shown in Figures 7-9.

Inhibition of cell migration

*CR*d.VN significatly inhibited HUVEC, MDA-MB-231 and MDA-MB-435 cell migration in dose-dependent manner (Figures 8-10). Cell migration on complete Matrigel is an integrin-dependent mechanism. *CR*d.VN blocked this process when using different cell lines with significantly different integrin profiles. As determined by Markland et al., the MDA-MB-435 cells express significantly more copies of αvβ3 integrin than HUVEC or MDA-MB-231 cells. *CR*d.VN inhibited endothelial cell HUVEC migration.

Boyden chamber assay used: fluorescent quantitation of migrated



Figures 1and 2: CR of Vietnam and CR venom milking.

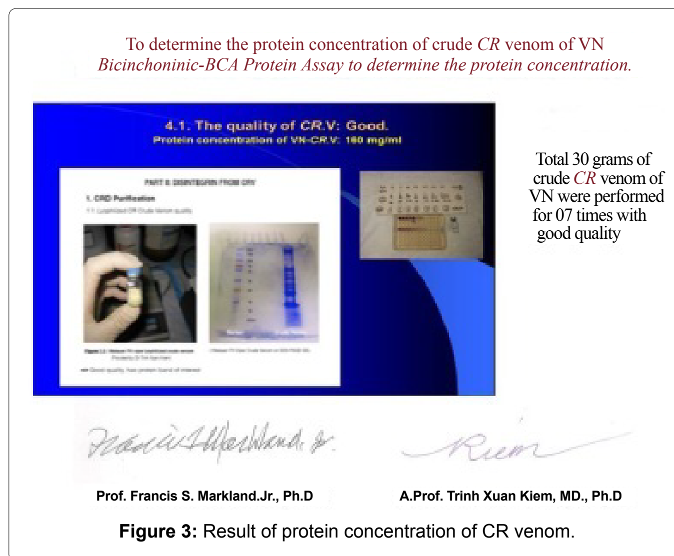


Figure 3: Result of protein concentration of CR venom.

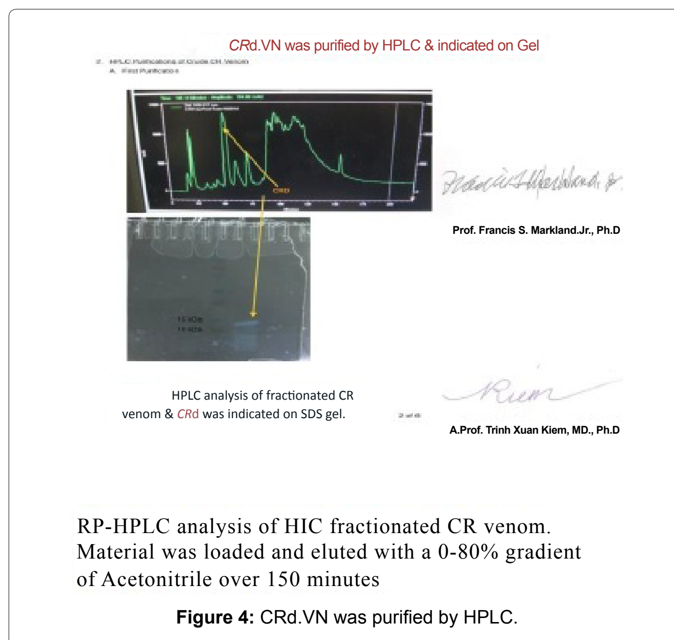


Figure 4: CRd.VN was purified by HPLC.

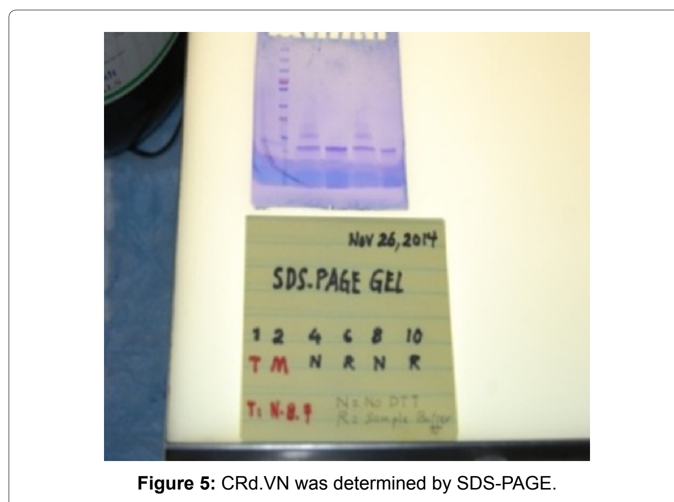


Figure 5: CRd.VN was determined by SDS-PAGE.

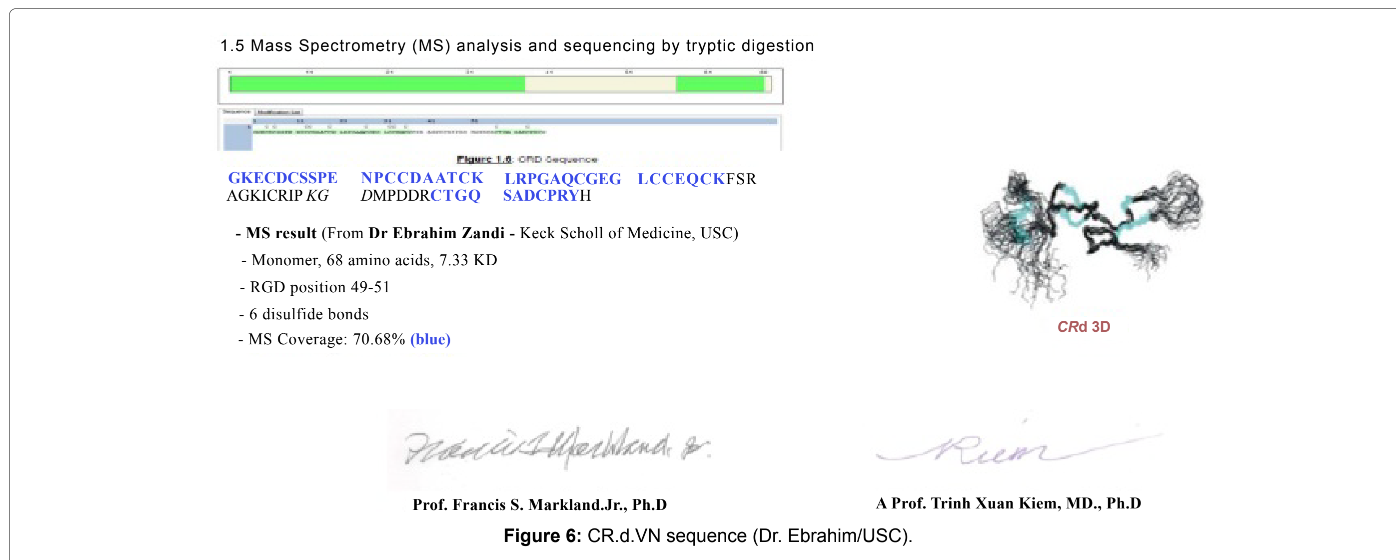


Figure 6: CR.d.VN sequence (Dr. Ebrahim/USC).

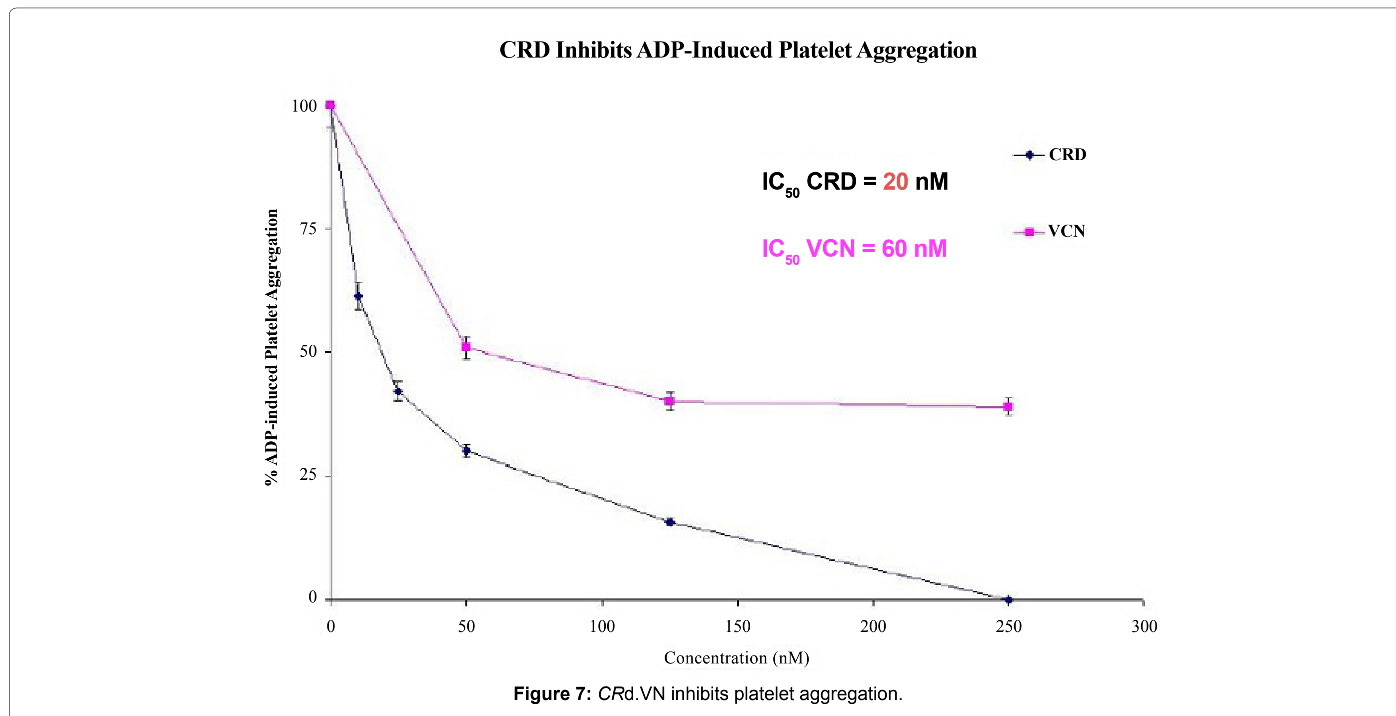


Figure 7: *CR*d.VN inhibits platelet aggregation.

cells. *CR*d.VN inhibited migration of HUVEC at low nM concentrations. Cytochalasin D (CytoD) used as a positive control (anti-migrated agent). *CR*d.VN inhibited MDA-MB-231 cell migration.

*CR*d.VN inhibited MDA-MB-435 cell migration

Inhibited MDA-MB-435 Cell Migration shown in Figure 11.

The result of *CR*d.VN inhibited cancer cell invasion

Result of Crd.VN Inhibited Cancer Cell Invasion shown in Figure 12.

The result of *CR*d.VN inhibited HUVEC tube formation by *CR*d.VN

Result of Crd.VN Inhibited HUVEC Tube Formation by Crd.VN shown in Figure 13.

*CR*d.VN is not cytotoxic: Does not affect viability of cells

Viability of Cells shown in Figure 14.

Cells were seeded in serum-free media in multiwall chamber slides on complete Matrigel and allowed to adhere for 1 hr. Cells were incubated for up to 48 hr with either *CR*d.VN. Untreated cells or cells exposed to the apoptosis inducer Staurosporine (STSP, 1 μM) were used as controls. The cells were fixed, TUNEL-stained and counterstained with Hoechst 33342. Cell death was plotted after digitally counting apoptosis events from images taken from multiple experiments.

Growth of MDA-MB-231 Tumors in Nude Mice and the Effect of VN.*CR*d Treatment

Tumors in Nude Mice and the Effect of VN.Crd Treatment shown in Figure 15.

Citation: Trinh KX, Thuc PV, My CT, Markland FJr, Swenson S, et al. (2016) A Novel Anti-tumor Protein from *Calloselasma Rhodostoma* Venom in Vietnam. Anat Physiol 6: 213. doi:10.4172/2161-0940.1000213

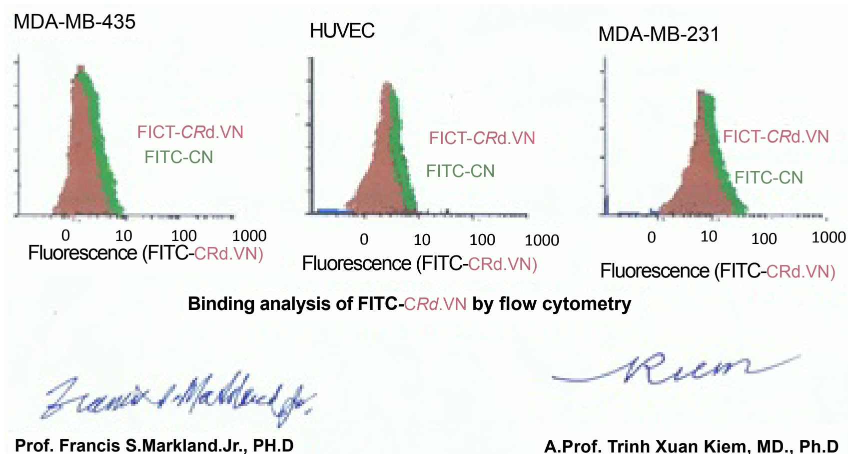


Figure 8: The ability of cell surface binding of CRd.VN against different cell lines was tested by flow cytometry. The results show that FITC-label CRd.VN had a clearly binding profile against HUVEC, MDA-MB-435 and MDA-MB-231 cells.

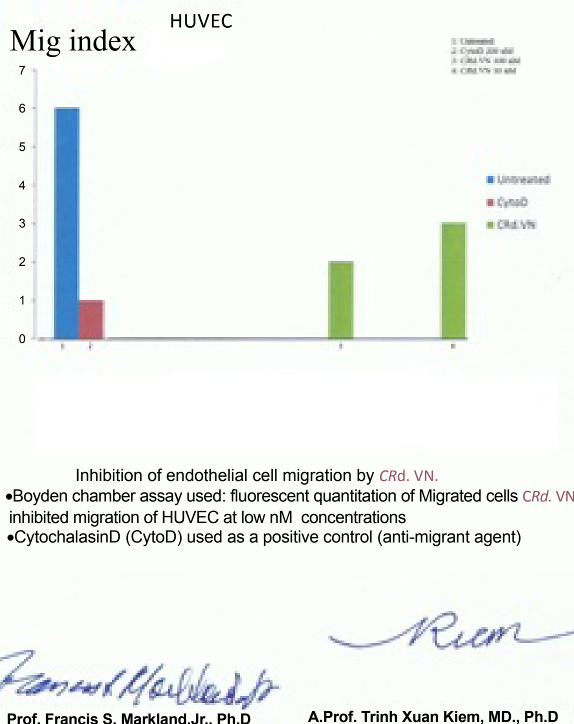


Figure 9: Inhibition of endothelial cell migration by CRd.VN.

Imaging of 231 cells in *CR*d.VN therapeutic efficacy study

231 Cells in Crd.VN Therapeutic Efficacy Study shown in Figure 16.

Discussion

From clinical experience gave idea of new drug research for cancer therapy

According to WHO Guidelines for the Production Control and Regulation of Snake Antivenom Imunoglobulins 2010, snakes of medical importance in Vietnam were identified correctly. Two venomous snake families were characterized: Elapidae and Viperidae, including *Calloselasma Rhodostoma* (CR) which is the most venomous snake of Viperidae. It was shown in many clinical studies before: Patient's platelet quantity was decreased less than 50 G/l (normal: 300 G/l). All the coagulation factors were destroyed, fibrinogen was left a trace only (normal: 3 g/l). Bleeding time was prolonged until many days (normal: 3 minutes). The patient died if not anti-venom available for treatment in time. After specific anti-venom therapy for 6 hrs, the patient recovered completely. The clinical experience was

Citation: Trinh KX, Thuc PV, My CT, Markland FJr, Swenson S, et al. (2016) A Novel Anti-tumor Protein from *Calloselasma Rhodostoma* Venom in Vietnam. Anat Physiol 6: 213. doi:10.4172/2161-0940.1000213

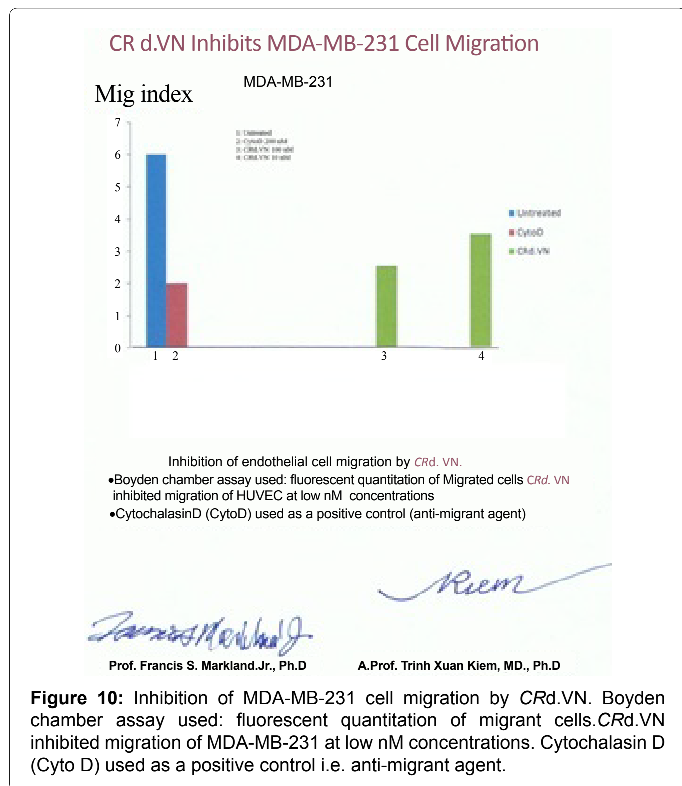


Figure 10: Inhibition of MDA-MB-231 cell migration by *CR*d.VN. Boyden chamber assay used: fluorescent quantitation of migrant cells.*CR*d.VN inhibited migration of MDA-MB-231 at low nM concentrations. Cytochalasin D (Cyto D) used as a positive control i.e. anti-migrant agent.

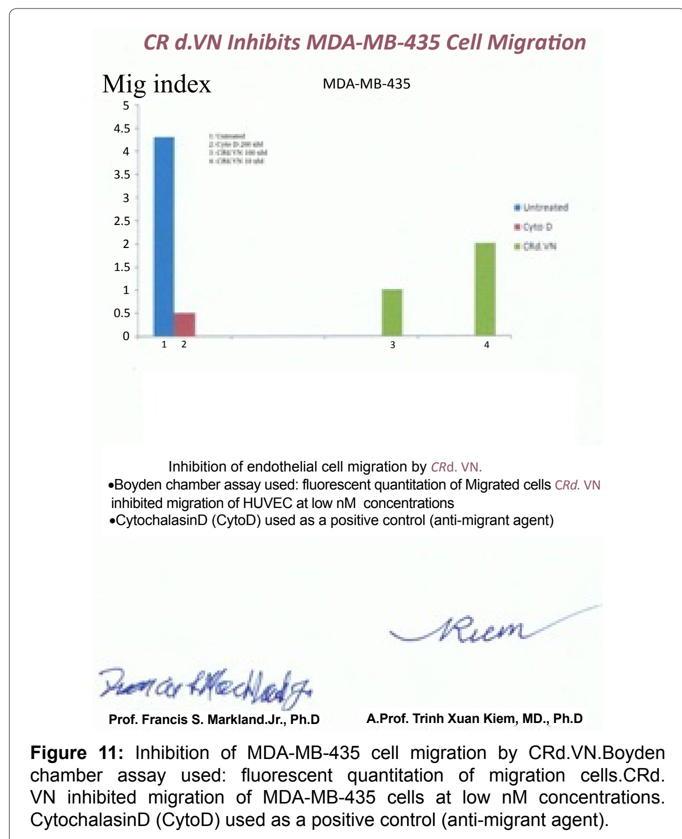


Figure 11: Inhibition of MDA-MB-435 cell migration by CRd.VN.Boyden chamber assay used: fluorescent quantitation of migration cells.CRd. VN inhibited migration of MDA-MB-435 cells at low nM concentrations. CytochalasinD (CytoD) used as a positive control (anti-migrant agent).

impressed and encouraged the toxicologists to research CR venom for new candidate drugs, especially cancer treatment, while the traditional methods for cancer therapy continue involving invasive procedures. Therefore, the research for new active drugs for cancer therapy was one of the goals of biotechnological research. This was the most promising object of the pharmaceutical industry.

In fact, as soon as ancient, a millennium after Moses, and 2,400 years ago, Hippocrates, Father of medicine held to a Greek religious belief which recognized the snake on a staff as a symbol of medicine. The snake is also connected with pharmacology. "The snake was twisted around a stick or beside a pharmapeutic cup, which also implies the use of medicines or even poison. Its poisonous properties could be parallel by similar properties of medicines".

CR venom of VN was collected, lyophilized successfully

The first, we had to find fresh and living CR snakes in the wild field

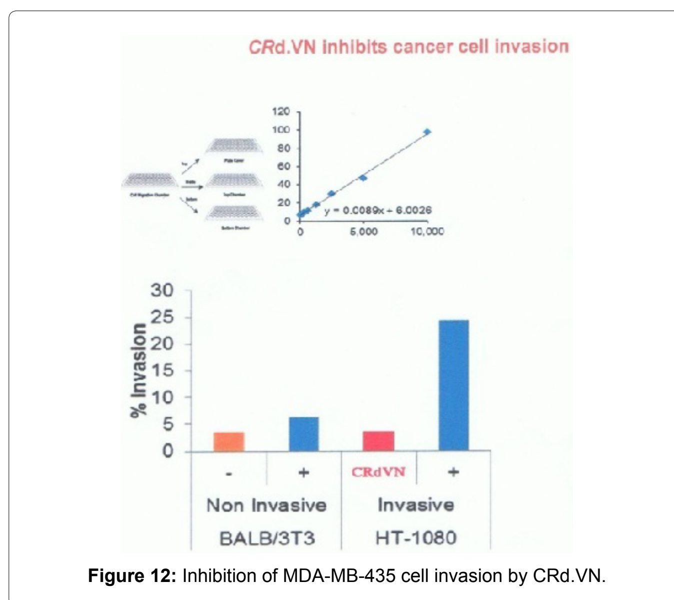


Figure 12: Inhibition of MDA-MB-435 cell invasion by CRd.VN.

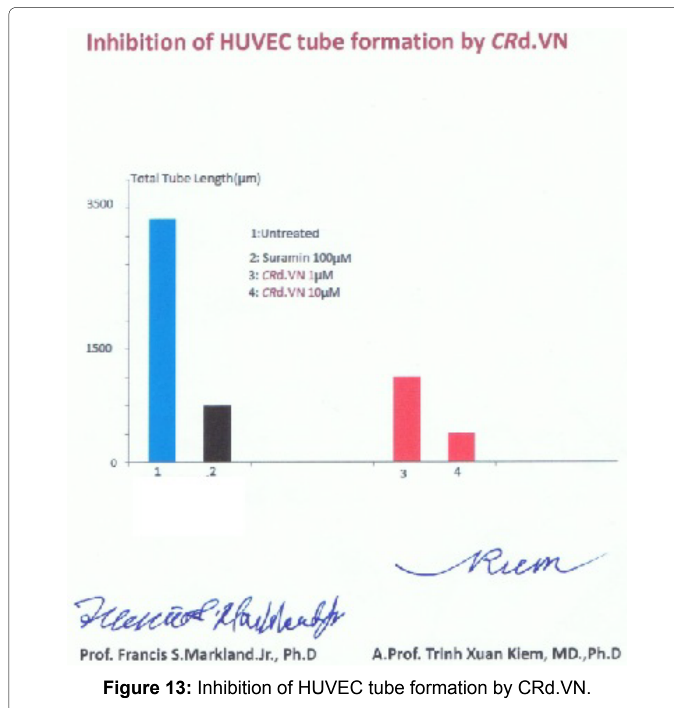


Figure 13: Inhibition of HUVEC tube formation by CRd.VN.

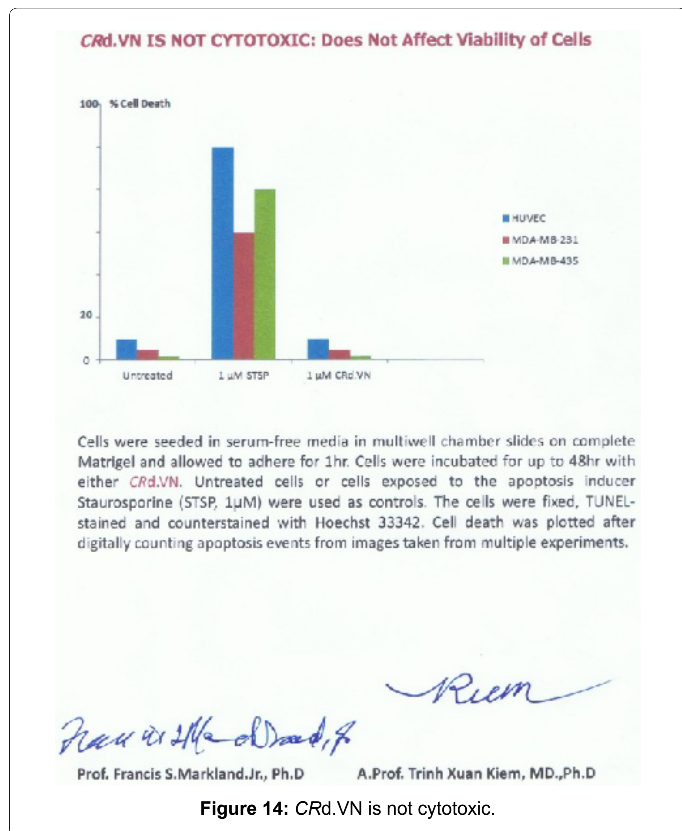


Figure 14: *CR*d.VN is not cytotoxic.

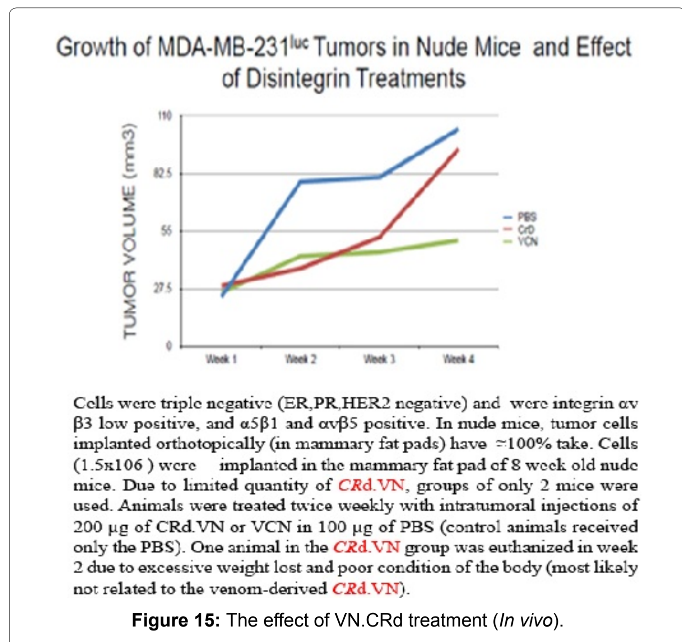


Figure 15: The effect of VN.CRd treatment (*In vivo*).

to collect them for identification of family and species. The healthy snakes were milked to collect venom for centrifuge and lyophilization (WHO Guidelines for the Production Control and Regulation of Snake Antivenom Imunoglobulins 2010). The result, we got the best quality of CR venom with protein concentration was 158 ± 03 mg/ml (Smith, P.K BCA Assay.1985). Especially we found out disintegrin (CRd) on gel by quality control assay SDS-PAGE (Figures 2-4).

*CR*d.VN was purified, characterized the molecular weight, structure and the biological anti-tumor activities successfully

One of the targets investigated is integrins. Because integrins play multiple important roles in cancer pathology including tumor cell proliferation, angiogenesis, invasion and metastasis; disintegrins purified from snake venom have an excellent activity to bind specifically on activated integrins. Their inhibition of angiogenesis is especially for cancer treatment, which stimulates the development of most bio-drugs based on toxins. *CR*d.VN purified and characterized the biological activity is the most important event of toxicological field of Vietnam.

The efficient disruption of integrin-mediated interactions between tumorigenic ECM and angiogenic ECM in the tumor microenvironment seems to be critical from the therapeutic standpoint since, as recently reported.

The critical involvement of integrins in both angiogenesis and tumor invasion provides the rationale for developing therapeutic antagonists aimed at disrupting this molecularly intertwined process. Most efforts of the pass were focused on anti-intergrin agents targeting the RGD-binding alphav member, a subclass of integrins thought to play pivotal roles in the regulation of pathological angiogenesis, which prompted the development of a number of small RGD-mimetics.

In this study, we showed that a disintergrin purified from *CR* venom of VN (*CR*d.VN) has the excellent biological activities on inhibition of cancer development such as the ability of cell surface binding of *CR*d.VN against different cell lines, the anti-migration, anti-invasion, inhibited platelet aggregation properties. *CR*d.VN also targets integrins αvβ3, αvβ5 and α5β1, while displaying a higher affinity than CN for integrin α5β1. For instance, recent studies have demonstrated that the usage of various anti-VEGF/PDGF strategies is linked to an increased risk of early metastasis in animal cancer models. Although the clinical relevance of these preclinical studies is not yet clear, these data support the idea that not only is there an imperative need to design novel anti-angiogenic drugs with better anti-invasive properties, also to test the impact of standard of care anti-angiogenics such as Avastin on metastasis [30-40].

Conclusion

We developed a novel disintegrin (*CR*d.VN) and showed the excellent biological activities on anti-tumor efficacy (*in vitro)* and *(in vivo)*. It is a possible anti-tumor agent with clinical potential.

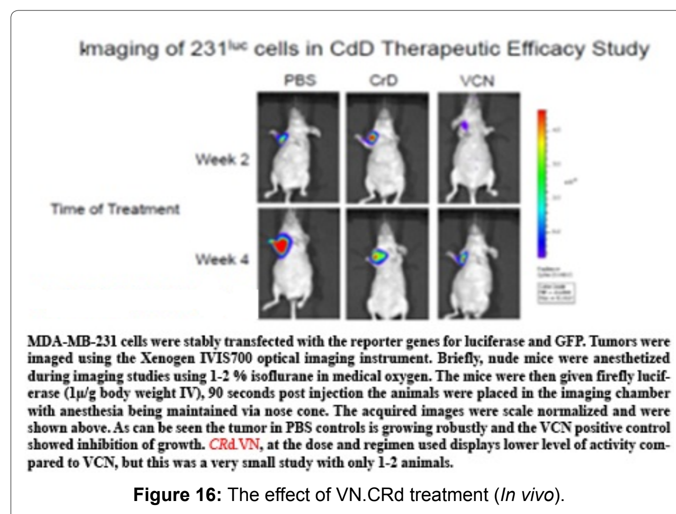


Figure 16: The effect of VN.CRd treatment (*In vivo*).

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, et al. (2011) Global cancer statistics. CA Cancer J Clin 61: 69-90.
2. Bond A (2006) Exenatide (Byetta) as a novel treatment option for type 2 diabetes mellitus. Proceedings 19: 281-284.
3. Kamiguti AS, Zuzel M, Theakston RDG (1998) Snake venom metalloproteinases and disintegrins: interactions with cells. Brazilian Journal of Medical and Biological Research 3: 853-862.
4. Brooks PC (1996) Role of integrins in angiogenesis. Eur J Cancer 32A: 2423-2429.
5. Yeh CH, Peng HC, Yang RS, Huang TF (2001) Rhodostomin, A Snake Venom Disintegrin, Inhibits Angiogenesis Elicited by Basic Fibroblast Growth Factor and Suppresses Tumor Growth by A Selective avß3 Blockade of Endothelial Cells. Molecular Pharmacology 59: 1333-1342.
6. Contois L, Akalu A, Brooks PC (2009) Integrins as "functional hubs" in the regulation of pathological angiogenesis. Semin Cancer Biol 19: 318-328.
7. Tang C, Yang R, Liu C, Huang T, Fu W (2004) Differential susceptibility of osteosarcoma cells and primary osteoblasts to cell detachment caused by snake venom metalloproteinase protein. Toxicon 43: 11-20.
8. Ellis LM, Reardon DA (2009) Cancer: The nuances of therapy. Nature 458: 290-292.
9. Golubkov V, Hawes D, Markland FS (2003) Anti-angiogenic activity of contortrostatin, a disintegrin from Agkistrodon contortrix contortrix snake venom. Angiogenesis 6: 213-224.
10. Goodman SL, Hölzemann G, Sulyok GA, Kessler H (2002) Nanomolar small molecule inhibitors for alphav(beta)6, alphav(beta)5, and alphav(beta)3 integrins. J Med Chem 45: 1045-1051.
11. Hood JD, Cheresh DA (2002) Role of integrins in cell invasion and migration. Nat Rev Cancer 2: 91-100.
12. Schiang H, Yang RS, Huang TF (1995) The Arg-Gly-Asp- containing peptide, rhodostomin, inhibits in vitro cell adhesion to extracellular matrices and platelet aggregation caused by Saos-2 human osteosarcoma cells. British Journal of Cancer.
13. Calvete JJ, Moreno-Murciano MP, Theakston RD, Kisiel DG, Marcinkiewicz C (2003) Snake venom disintegrins: novel dimeric disintegrins and structural diversification by disulphide bond engineering. Biochem J 372: 725-734.
14. Jurasz P, Alonso-Escolano D, Radomski MW (2004) Platelet--cancer interactions: mechanisms and pharmacology of tumour cell-induced platelet aggregation. Br J Pharmacol 143: 819-826.
15. Kang IC, Lee YD, Kim DS (1999) A novel disintegrin salmosin inhibits tumor angiogenesis. Cancer Res 59: 3754-3760.
16. Kim S, Bell K, Mousa SA, Varner JA (2000) Regulation of angiogenesis in vivo by ligation of integrin alpha5beta1 with the central cell-binding domain of fibronectin. Am J Pathol 156: 1345-1362.
17. Kim S, Harris M, Varner JA (2000) Regulation of integrin alpha vbeta 3-mediated endothelial cell migration and angiogenesis by integrin alpha5beta1 and protein kinase A. J Biol Chem 275: 33920-33928.
18. Brannon-Peppas L, Blanchette JO (2012) Nanoparticles and systems, target for cancer therapy. Advanced Drug Delivery Reviews 64: 206-212.
19. Calderon LA, Soares AM (2014) Antitumoral Activity of Snake Venom Proteins: New Trends in Cancer Therapy. BioMed Research International.
20. Au LC, Choo KB (1991) A common precursor for a putative hemorrhagic protein and rhodostomin, a platelet aggregation inhibitor of the venom of Calloselasma rhodostoma: Molecular cloning and sequence analysis. Biochemical and Biophysical Research Communications.
21. Trikha M, Clerck YA (1994) Contortrostatin, a snake venom disintegrin, inhibits ß1 integrin- mediated human metastatic melanoma cell adhesion and blocks experimental metastasis, Cancer Research pp: 4993-4998.
22. Mohit T, Francis S (1994) Contortrostatin, a Snake Venom Disintegrin, Inhibits ß1 Integrin -mediated Human Metastatic Melanoma Cell Adhesion and Blocks Experimental Metastasis.
23. Hidetomo N, David JR (2013) Can anesthetic techniques or drugs affect cancer recurrence in patients undergoing cancer surgery? Journal of Anesthesia 27: 731-741.
24. Radu M, Corey H, Barbara R, Kyle B (2012) Development of a chimeric recombinant disintegrin as a cost-effective anti-cancer agent with promising translational potential, Toxicon 59: 472-486.
25. Reardon A, Nabors LB, Stupp R, Mikkelsen T (2008) Cilengitide: an integrin-targeting arginine-glycine-aspartic acid peptide with promising activity for glioblastoma multiforme. Expert Opin Investig Drugs 17: 1225-1235.
26. Gould RJ, Polokoff MA, Friedman PA (1990) Disintegrins: a family of integrin inhibitory proteins from viper venoms. Proceedings of the Society for Experimental Biology and Medicine pp: 168-171.
27. Kamath S, Buolamwini JK (2006) Targeting EGFR and HER-2 receptor tyrosine kinases for cancer drug discovery and development. Med Res Rev 26: 569-594.
28. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, et al. (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76-85.
29. Brooks SA, Lomax-Browne HJ, Carter TM, Kinch CE, Hall DM (2010) Molecular interactions in cancer cell metastasis. Acta Histochem 112: 3-25.
30. Niewiarowski S, McLane MA, Kloczewiak M, Stewart G (1994) Disintegrins and other naturally occurring antagonists of platelet fibrinogen receptors Seminars in Hematology, 31: 289-300.
31. Yang SH, Lu MC, Chien CM, Tsai CH, Lu YJ, et al. (2005) Induction of apoptosis in human leukemia K562 cells by cardiotoxin III. Life Sci 76: 2513-2522.
32. Trikha M, Rote WE, Manley PJ, Lucchesi BR, Markland FS (1994) Purification and characterization of platelet aggregation inhibitors from snake venoms. Thromb Res 73: 39-52.
33. Trikha M, Flores M, Markland FS (1994) Disintegrns inhibit mammary carcinoma binding to extracellular matrix protein. FASEB J 8: A873.
34. Trikha M, De Clerck YA, Markland FS (1994) Contortrostatin a snake venom disintegrin, inhibits beta 1 integrin-mediated human metastatic melanoma cell adhesion and blocks experimental metastasis. Cancer Res. 54: 4993-4998.
35. Vyas VK, Brahmbhatt K, Bhatt H, Parmar U (2013) Therapeutic potential of snake venom in cancer therapy: current perspectives. Asian Pac J Trop Biomed 3: 156-162.
36. DeWys WD, Kwaan HC, Bathina S (1976) Effect of defibrination on tumor growth and response to chemotherapy. Cancer Res 36: 3584-3587.
37. Yeh CH, Peng HC, Huang TF (1998) Accutin, a new disintegrin, inhibits angiogenesis in vitro and in vivo by acting as integrin alphavbeta3 antagonist and inducing apoptosis. Blood 92: 3268-3276.
38. Zhang Y (2015) Why do we study animal toxins? Dongwuxue Yanjiu 36: 183-222.
39. Zhou Q, Ritter M, Markland FS (1996) Contortrostatin, a snake venom protein, which is an inhibitor of breast cancer progression. Mol Biol Cell 7: 425a.
40. Zhou Q, Nakada MT, Arnold C, Shieh KY, Markland FS Jr (1999) Contortrostatin, a dimeric disintegrin from Agkistrodon contortrix contortrix, inhibits angiogenesis. Angiogenesis 3: 259-269.