A NOVEL ANTI-TUMOR PROTEIN FROM *CALLOSELASMA RHODOSTOMA* VENOM IN VIETNAM

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Abstract

Introduction: 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 Uiversity 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 disintegrin from *CR* venom of VN (*CR*d.VN), to determin the molecular weigh, structure and its biological antitumor activities. ***Methods***: The process of collection, lyophilization 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. ***The resuls***: Peak N°: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 anti-cancer activities of *CR*d.VN are very strong.

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

Keyword: *CR*d anti-cancer.

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1. Introduction

Cancer is a chronic degenerative disease considered to be the second most common cause of death in economically developing countries ("Global cancer statistics," CA: Cancer Journal for Clinicians, 2011).

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 (F.Bray et al., 2013).

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 (Statistics of Vietnam Ministry of Health, 2015).

However, cancer therapy continues involving invasive procedures, including the application of chemotherapy, surgery to remove the tumor(s), the use of radiation, and even nonselective cytotoxic drugs (L.Brannon Peppas et al., 2012).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 (Yang et al., 2005).

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 (W.D.DeWys et al.,1976).

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 (Leonardo et al., 2014).

Integrins are heterodimeric receptors that evolved to mediate the complex cell-ECM interactions that regulate the ability of cells to mechanically sense their microen- vironment. Conversely, as epithelia transition to malignancy they evade the microenvironmental constraints by both altering their integrin affinity and avidity for ECM proteins (inside-out signaling) and/or shifting their integrin expression (Hood and Cheresh, 2002; Mizejewski, 1999). The precise roles, however, played by different integrin subunits in various aspects of tumor progression and why some integrins appear to be especially supportive of tumor progression (Desgrosellier et al., 2009) 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 antiangiogenesis therapeutics. Disintegrins first discovered in 1983 (Ouyang and Huang,1983) and named in 1990 (Gould et al., 1990). They are family of disulfide –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 tripeptide) 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 antimetastatic effects demonstrated in various experimental settings (Huang et al., 2001; McLane et al., 2008; Swenson et al., 2004).

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 αIIbβ3, α5β1, andαvβ3lls expressed by a number of cells including those involved in tumor development and proliferation (R.J. Gould, S.Niewiarowski et all., 1994).

Based on binding experiments, αβ 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 (McLane et al., 2008). 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 preclinically 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 (Marcinkiewicz et al., 2003).

The integrin binding activity of disintegrins depends on the appropriate pairing of several cystein 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 (Saudek et al.,1991). Two of the most studied native disintegrins are the homodimer contortrostatin (CN) (Trikha et al.,1994b) and the monomer echistatin (MacLane et al., 2008). 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 (Zhou et al., 2000b, Zhou et al., 2000d).

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 Uiversity of Southern California (USC) was established.Since 2014 this project has been approved by VN government.

The aims of the 1st project: To establish the technological process for purification of disintegrin from *CR* venom and to determine the molecular weigh, structure and its biological antitumor activities.

2. Materials and methods

2.1. CR Venom was collected, lyophilized and supplied from Vietnam.

2.2. Cells and reagents.

The MDA-MB-435 cells were obtained from MD Anderson Cancer Center, Houston, TX and the MDA-MB-231cells 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 were 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).

2.3. 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. *Anal. Biochem*.1985.

2.4. 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 C_{18}-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 C_{18} 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 (*CR*d) starts eluting in 30% acetonitrile and is collected for lyophlyzation and further characterization

2.5. Inhibition of platelet aggreegation.

The inhibition of ADP-induced platelet aggregation by *CR*d 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 *CR*d samples are added to PRP and mixed for 30 seconds at this point ADP is added to a final concentration of 20nM to the *CR*d-PRP mixture and the level of aggregation inhibition is evaluated. These studies are presently ongoing with fractions obtained from C_{18} RP-HPLC.

2.6. Mass spectrometry (MS) analysis and sequencing by tryptic digestion.

The MS analysis and the subsequent sequencing of purified *CR*d was performed by Dr. Ebrahim Zandi (Keck School of Medicine, University of Southern California) using purified *CR*d obtained form C_{18} RP-HPLC. For sequencing, the purified disintegrin was reduced, alkylated and digested with trypsin at 37^{o}C 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 C_{18} 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.

*2.7. Cell surface binding of CR*d *as studies by flow cytometry.*

HUVEC (human umbilical vein endothelial cells), and MDA-MB-231 or MDA-MB-435 breast cancer cells were to be grown to early confluency and starved overnight in serum-free media. The cells were harvested and resuspended in 1ml of serum-free media ($5x10^5$cells/condition) before being incubated with different amounts of *CR*d or controls for 30 min at 37^{o}C. At the end of the incubation period, the cells were pelleted, washed in ice-cold PBS containing 5% fetal bovine serum and either

analyzed in a FACSCalibur scanner (fluorescent activated cell sorter) or, depending on the assay, further incubated at 4°C 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 analyzed to determine level of *CR*d to the cells.

2.8. 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.VN) (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.

*2.9. Inhibition of cell invasion by CR*d.VN.

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, resuspended in serum-free media ($1x10^6$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 chemoattractant. The invasion plates were incubated for up to 48hr (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.

2.10. Inhibition of HUVEC tube formation.

'Endothelial Tube Formation' plates precoated with Matrigel (BD Biosciences, Bedford, MA) were used according to the manufacturer's protocol. HUVEC were seeded in triplicate ($3x10^4$cells/well) in the presence of various concentrations (0-1000 nM) of *CR*d and incubated for 16hr at 37^{o}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 MicroImaging GmbH, Munich, Germany) and averaged from at least three independent experiments.

2.11. In vivo efficacy studies.

MDA-MB-231 cells ($2x10^6$ 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 (100µg *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 400µg per injection (approx. 20µg/gr.) once a week for the duration of the MDA-MB-231 study. Tumor diameters were measured weekly with a caliper in a blind fashion and the tumor volumes calculated using the formula [length (mm) x 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.

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

3.Results

3.1. *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 indentified. Their venom was milked, centrifuged at 3,000 RPM x 15 min. The venom was taken and then kept cool (-80^{o}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^{o}C) and dark place until the time for using

Fig.1: *CR of Viet Nam.*

Fig.2: *CR venom milking.*

3.2. *The protein concentration of CR venom was determinated by BCA protein Assay.*

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

3.1. To determine the protein concentration of crude *CR* venom of VN
b) *Bicinchoninic-BCA Protein Assay to determine the protein concentration.*

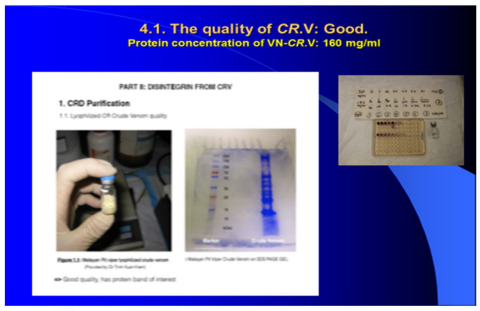

Fig.2 *The results*:

Total 30 grams of crude *CR* venom of VN were performed for 07 times with good quality.

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Fig.3:Result of protein concentration of *CR* venom.

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

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

3.2.c) *CRd*.VN was purified by HPLC & indicated on Gel

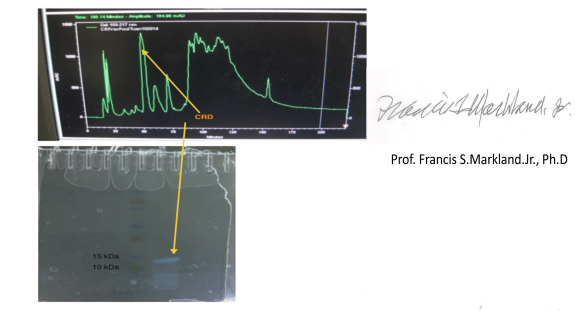

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Figure 5: HPLC analysis of fractionated *CR* venom & *CRd* was indicated on SDS gel.

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RP-HPLC analysis of HIC fractionated *CR* venom.

Material was loaded and eluted with a 0-80% gradient

of Acetonitrile over 150 minutes

Fig.4: *CRd.VN was purified by HPLC*

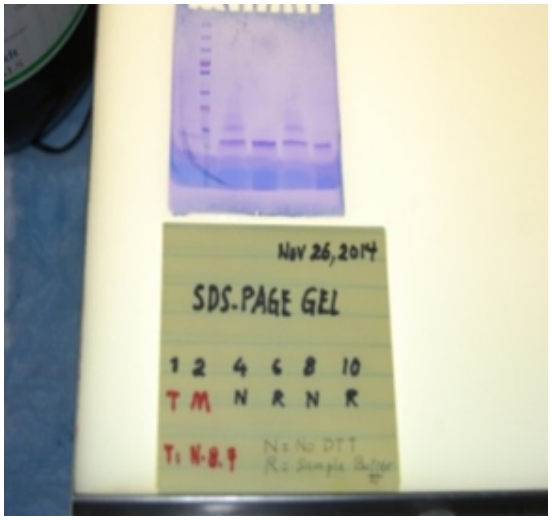


Fig.5: *CRd.VN was determined by SDS-PAGE*

3.4. The structure of CRd.VN was determined by Mass spectrometry (MS)analysis and sequencing by tryptic digestion, performed by Dr. Ebrahim (USC)

Molecular structure of *CR*d.VN

(Mass Spectrometry Analysis & Sequencing : *68 aa, 7.33 kD, RGD: position 49-51, 6 disulfide bonds)*

1.5. Mass Spectrometry (MS) analysis and sequencing by tryptic digestion

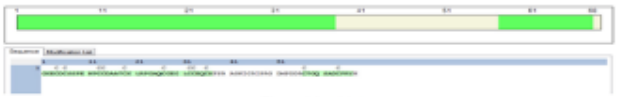
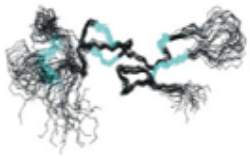


Figure 1.6: CRD Sequence

GKECDCSSPE NPCCDAATCK LRPGAQCGEG LCCEQCKFSR
AQKICRIPRG DMPDDRCTGQ SADCPRVH

- **MS result** (From **Dr Ebrahim Zandi** - Keck School of Medicine, USC)
- Monomer, 68 amino acids, 7.33 kD
- RGD: position 49-51
- 6 disulfide bonds
- MS Coverage: 70.58% (blue)



CRd 3D

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

3.5. The result of CRd.VN inhibited platelet aggregation

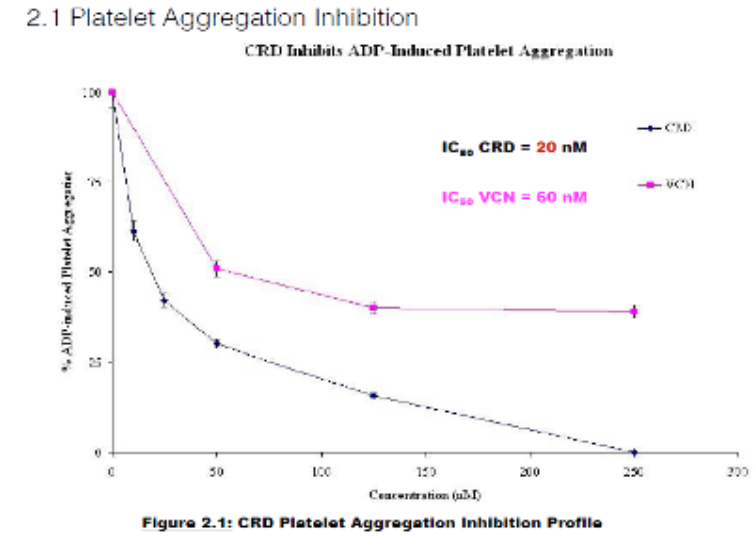

Fig. 7: *CRd.VN inhibits platelet aggregation.*

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

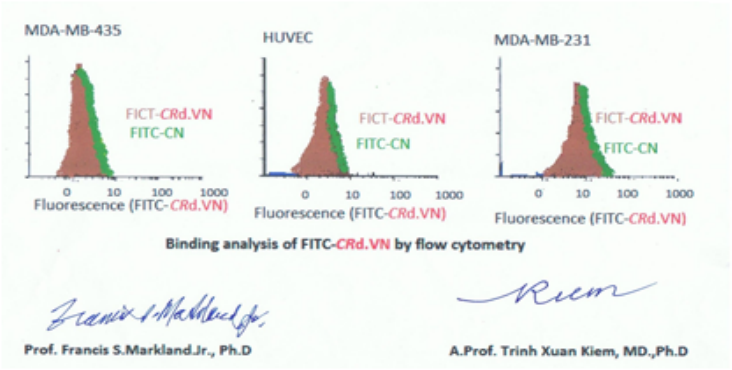

Fig.8: *The ability of cell surface binding of CRd.VN against different cell lines was tested by flow cytometry. The results show that FITC-label CRd.VN had a clearly binding profile against HUVEC, MDA-MB-435 and MDA-MB-231 cells.*

3.7. Inhibition of cell migration

*CR*d.VN significaly inhibited HUVEC, MDA-MB-231 and MDA-MB-435 cell migration in dose-dependent manner (Fig. 8, 9, 10). Cell migration on complete Matrigel is an integrin-dependent mechanism. *CRd.*VN blocked this process when using different cell lines with significantly different integrin profiles. As determined by Markland.F.S et al and others, the MDA-MB-435 cells express significantly more copies of αvβ3 integrin than HUVEC or MDA-MB-231 cells.

*3.7.1 CRd.VN inhibited endothelial cell HUVEC migration***.**

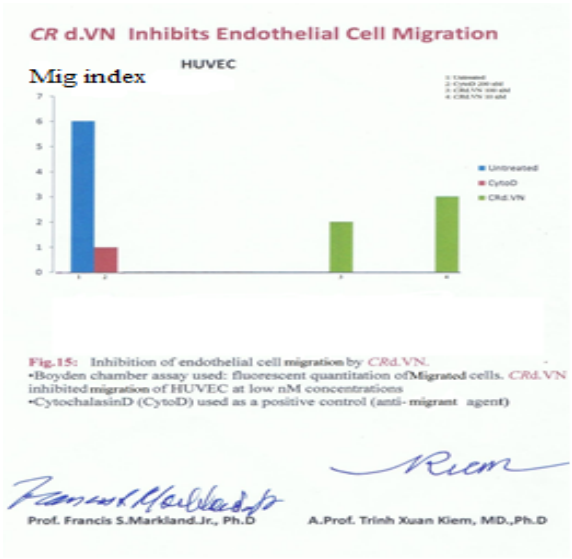

Fig.9*: Inhibition of endothelial cell migration by CRd.VN.*

•Boyden chamber assay used: fluorescent quantitation of migraed cells.

*CR*d.VN inhibited migration of HUVEC at low nM concentrations.

•Cytochalasin D (CytoD) used as a positive control (anti-migrated agen**t)**

3.7.2 CRd.VN inhibited MDA-MB-231cell migration.

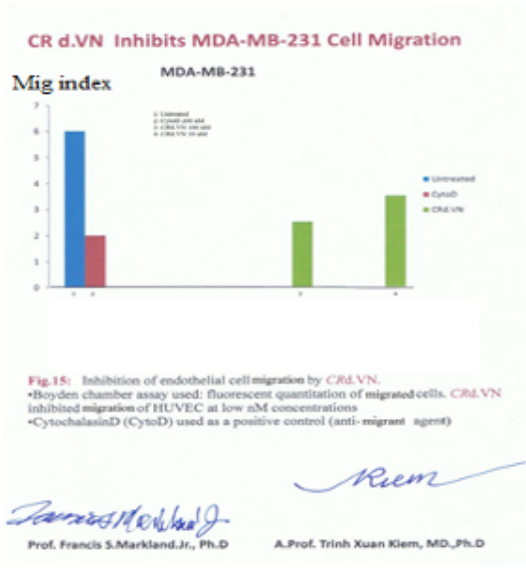

Fig.10: • *Inhibition of MDA-MB-231 cell migration by CRd.VN*

Boyden chamber assay used: fluorescent quantitation of migrant cells.

CRd.VN inhibited migration of MDA-MB-231 at low nM concentrations

•Cytochalasin D (Cyto D) used as a positive control (anti-migrant agent)

3.7.3 CRd.VN inhibited MDA-MB-435 cell migration

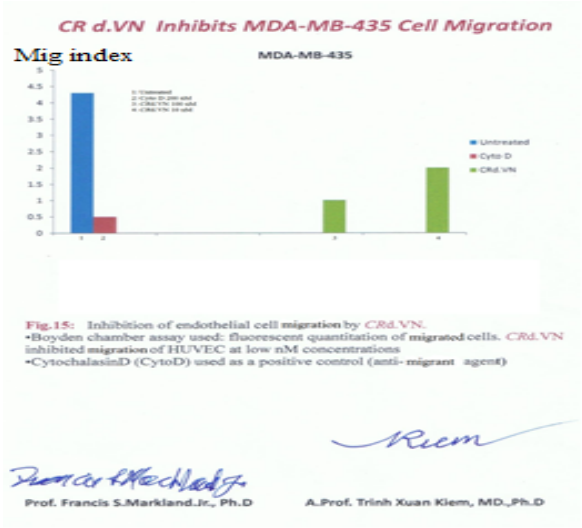

Fig. 11: *Inhibition of MDA-MB-435 cell migration by CRd.VN.*

•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 agen**t)**

3.8. The result of CRd.VN inhibited cancer cell invasion.

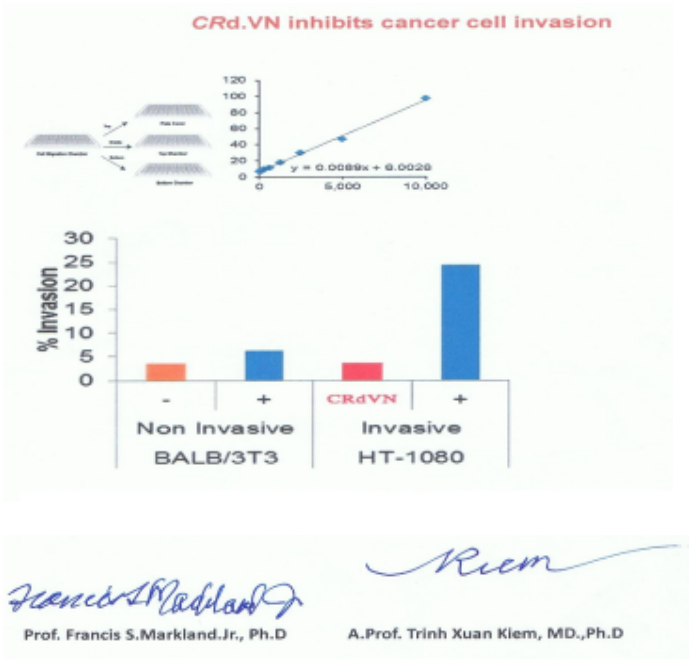

Fig.12: *Inhibition of MDA-MB-435 cell invasion by CRd.VN.*

3.9. The result of CRd.VN inhibited HUVEC tube formation by CRd.VN.

Fig.13: *Inhibition of HUVEC tube formation by CRd.VN.*

3.10 CRd.VN is not cytotoxic: Does Not Affect Viability of Cells.

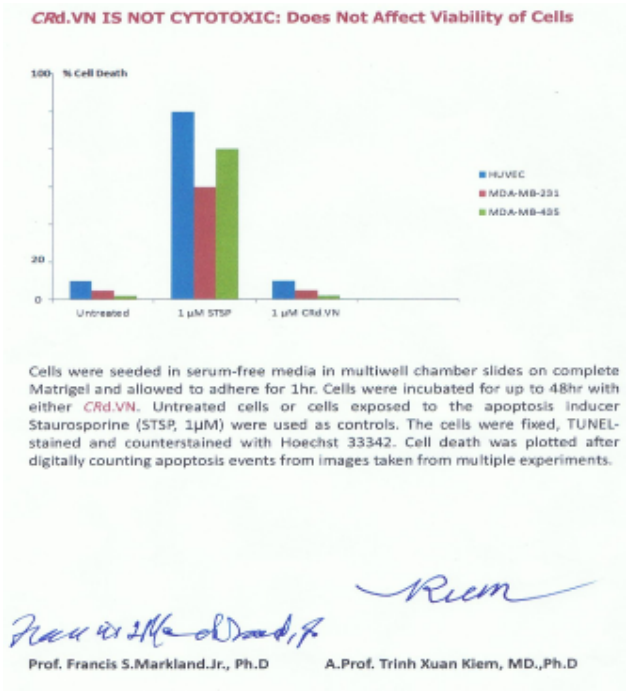

Fig.14: *CRd.VN is not cytotoxic*

Cells were seeded in serum-free media in multiwell chamber slides on complete Matrigel and allowed to adhere for 1hr. Cells were incubated for up to 48hr with either *CR*d.VN. Untreated cells or cells exposed to the apoptosis inducer Staurosporine (STSP, 1μM) 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.

*3.11. Growth of MDA-MB-231 tumors in nude mice and the effect of VN.CR*d *treatment.*

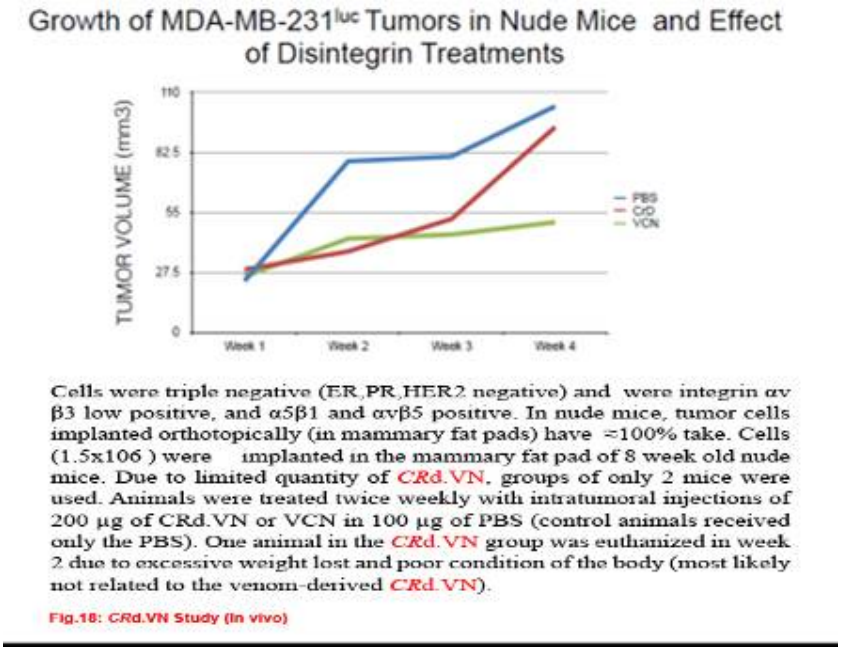

Fig.15: *The effect of VN.CRd treatment(In vivo)*

3.12. Imaging of 231 cells in CRd.VN Therapeutic Efficacy Study

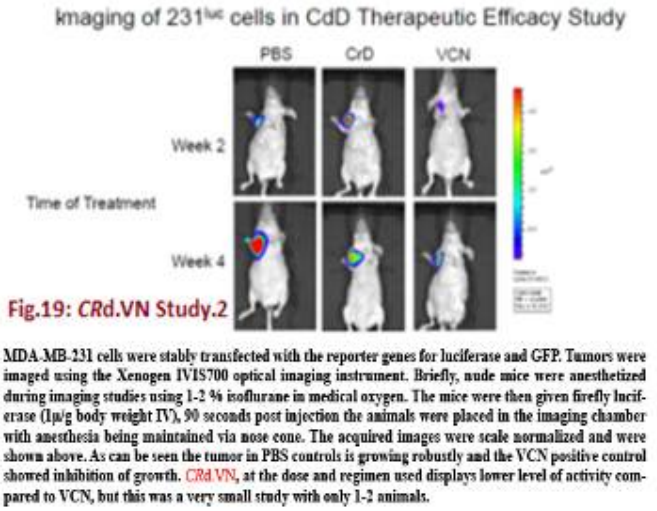

Fig.16: *The effect of VN.CRd treatment(In vivo)*

4. Discussion

4.1. 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: 3g/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 impressed and encouraged the toxicologists to research *CR* venom for new candidate drugs, specially 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 paralled by similar properties of medicines".

4.2. CR venom of VN was collected, lyophilized successfully.

The first, we had to find fresh and living *CR* snakes in the wild field 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 (*CR*d) on gel by quality control assay SDS-PAGE (Fig.3,4,5).

4.3. CRd.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 EC in the tumor microenviroment seems to be critical from the therapeutic standpoint since, as recently reported (Baluk et al.,2015).

The critical involvement of integrins in both angiogenesis (Contois et al., 2009) and tumor invasion (Hood and Cheresh, 2002) provides the rationale for developing therapeutic antagonists aimed at disrupting these molecularly intertwined process (Folkman, 2007). 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 (Nemeth et al.,2007)

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 diplaying 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 (Ebos et al.,2009). Although the clinical relevance of these preclinical studies is not yet clear (Ellis, 2009), 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.

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.

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