Preclinical Development of a Recombinant Anti-Integrin Therapeutic Agent with Promising Anti-cancer Translational Potential

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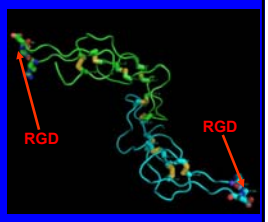
What are Disintegrins

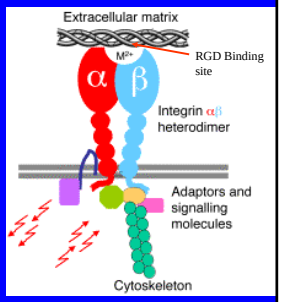
- Family of small, disulfide-rich, peptides from snake venom, but also found in mammalian species as ADAM family members
- First described in 1983 by Taiwanese investigators as platelet aggregation inhibitors
- Derived from multi-domain proteins and are (auto)proteolytically processed
- They all contain an RGD (or some alternate tripeptide) motif at the tip of a flexible 11-amino acid loop that is involved in integrin interaction
- They range in size from small to medium to large and dimeric (hetero- and homodimers)
- Present in venoms from <0.1% - >11% of the protein (venom can contain both small and dimeric disintegrins)
- Bind exclusively to integrins on cells



3-D structure of contortrostatin (CN, a homodimer from southern copperhead venom)

What are Integrins

- Family of heterodimeric transmembrane receptors involved in bidirectional signaling
- There are 24 integrins with 18 α-chains and 8 β-chains
- Mediate interactions between extra-cellular matrix (ECM) proteins and important cellular subsystems - involved in bidirectional signaling
- They form a physical link between the ECM and the actin cytoskeleton and also connect the ECM (via alternative pathways) to chromatin organization and gene expression
- Their over-expression, mislocalization and disregulated activity in cancer drives tumor progression; therefore, they are attractive targets for cancer therapy



Integrin Functions

Integrin receptors are involved in the regulation of a variety of important biological functions:

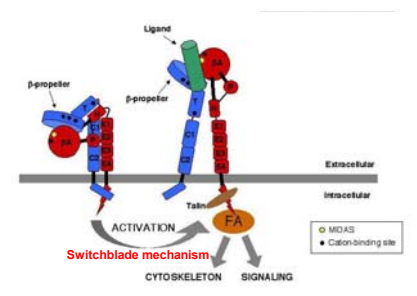
- embryonic development and proliferation
- wound repair
- hemostasis
- cell cycle involvement and prevention of programmed cell death
- motility

They are also implicated in abnormal pathological states:

- tumor directed angiogenesis
- tumor cell growth
- metastasis

Integrin Activation

- Integrins exist in two states: inactive and activated (ligand binding)
- Activation involves a conformation change and is required for ligand binding
- Disintegrins bind only to activated integrins, which are found on motile cells such as cancer cells and vascular endothelial cells during angio-genesis



Blue color represents α-chain, red color represents β-chain

Problem with Contortrostatin

CN shown to be an effective anti-cancer agent in breast, prostate, and ovarian cancer and glioma animal model studies and can be safely infused into laboratory animals with no observable adverse effects - but one major problem

AVAILABILITY - The protein exists as a small % of venom protein. Quantities of venom needed to purify clinical amounts of CN are not attainable, making translational development impractical

SOLUTION – Make a recombinant version of CN

Second Generation Agent: Recombinant Disintegrin (Vicrostatin, VCN)

- We recently developed a highly engineered *E. coli* expression system to generate recombinant version of CN - vicrostatin (VCN)
- VCN an improvement over CN (easier and cheaper to produce, retains full biological activity)

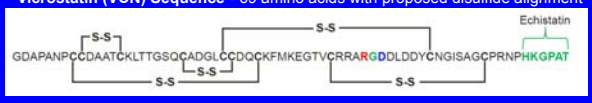
Vicrostatin (VCN): Engineered Recombinant Derivative of CN

- VCN is chimeric recombinant disintegrin designed from CN with preserved disintegrin fold displaying the same RGD motif at the tip of an 11-amino acid integrin binding loop
- It is produced as a Trx-VCN fusion protein, which is cleaved by TEV protease
- VCN has improved integrin α5β1 affinity by inserting the C-terminal tail of the viperid disintegrin (echistatin) in place of the C-terminus of CN

- VCN binds to the same integrins as CN (αvβ3, αvβ5, α5β1, and αIIbβ3) producing anti-angiogenic and anti-invasive activities
- VCN is an excellent drug candidate due to its bioactivity, manufacturability, solubility, and stability; it can be produced at ~200 mg/L of purified VCN
- VCN can be safely administered to mice with no discernible toxicity or immunogenicity

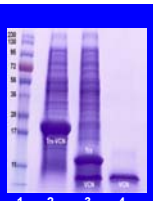
Vicrostatin: Structure & Isolation

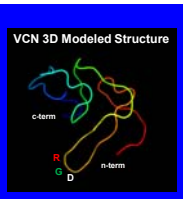
Vicrostatin (VCN) Sequence - 69 amino acids with proposed disulfide alignment



Molecule Summary:

- Thioredoxin-VCN fusion protein (lane 2) is cleaved by TEV protease (lane 3)
- Purified by IEC and RP-HPLC after cleavage (lane 4)
- Active protein is a monomer
- COOH-terminus is derived from echistatin





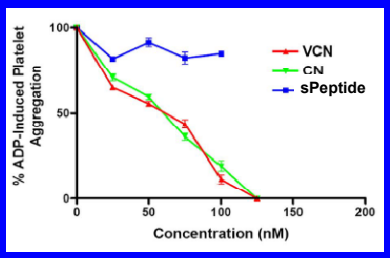
Kelley LA and Sternberg MJE. *Nature Protocols* 4, 363 - 371 (2009)

CN and VCN Affinity for Selected Integrins

Disintegrin	Integrin Kd (nM)		
	αvβ3	α5β1	αvβ5
CN	6.6	191.3	19.5
VCN	7.4	15.2	41.2

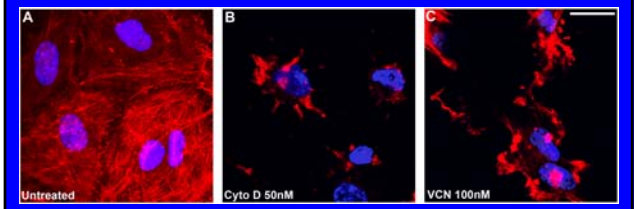
Fluorescence polarization (FP) used to determine binding constants. Differing concentrations of pure active integrins were incubated with a constant amount of FITC-VCN or FITC-CN. Kinetics of binding can be determined as with Scatchard analysis using a non-linear curve fit.

VCN & CN have Identical Effect on ADP-induced Platelet Aggregation



Different concentrations of VCN or CN were incubated with platelet rich plasma. ADP induces platelet aggregation; CN or VCN are added one minute prior to ADP. Both CN and VCN inhibit aggregation with an IC_{50} of ~60nM

VCN Mechanism of Action: Actin Cytoskeleton Reorganization

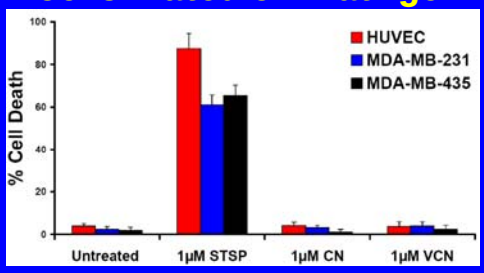


HUVEC seeded in SFM on Matrigel and treated for 3hr with FITC-VCN. Actin modifier Cytochalasin D was used as positive control. Cells were fixed, permeabilized, stained (Rhodamine-Phalloidin and Hoechst 33342) and imaged by confocal microscopy. Images shown above are x1,000. Bar is 20μm. This typifies results with motile cells

Are Disintegrins Cytotoxic

- It depends on the context of the microenvironment and the type of program executed by the targeted cells
- VCN is not cytotoxic to non-migratory cells such as HUVEC or cancer cells plated at high density in 2D
- VCN was shown to induce apoptosis in rapidly migrating and/or invading cells plated in 2.5D such as HUVEC sandwiched between two Matrigel layers, or in *in vivo* experiments

VCN Does Not Affect Viability of Cells Plated on Matrigel



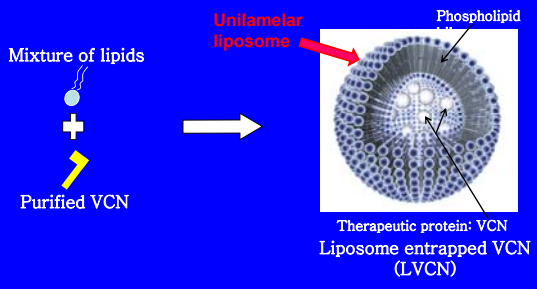
Cells were seeded in multiwell chamber slides on complete Matrigel and allowed to adhere for 1hr. Cells incubated up to 48hr with CN or VCN. Untreated cells or cells exposed to the apoptosis inducer Staurosporine were controls. Cells were TUNEL-stained and cell death plotted after digitally counting apoptosis events in images taken from multiple experiments.

We Have an Active Agent - How to Deliver it to the Tumor

- In our early animal tumor model studies CN was administered by daily intratumor injection
- This is not a clinically relevant method of administration
- There is an urgent need to develop a more effective delivery method for VCN

Liposome-Drug Formulation

Disintegrins can be easily and efficiently encapsulated into unilamelar liposomes



Lipid films created in round bottom flasks. VCN dissolved in buffer + sucrose and added to dried lipids at 50°C. LVCN particles generated by probe sonication or homogenization.

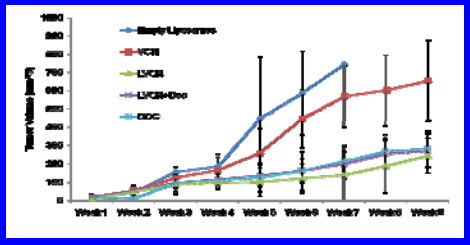
Liposomal Delivery of VCN

- The liposomal formulation of VCN is effective delivery method and clinically relevant
- Long half-life in blood allows leakage through gaps between cells lining the vessels in tumors
- Advantages of liposome-mediated drug delivery include:
  - (a) reduction of possible side effects due to "targeted" delivery
  - (b) shielding of immunogenic drugs from immune recognition
  - (c) possible decrease in dosing frequency
  - (d) facilitates delivery of drugs with short circulatory t½ such as VCN (LVCN t½ = 24.8 hr; VCN t½ ~ 0.5 to 6 hr)
  - (e) delivers VCN to the tumor (passive accumulation) due to "leaky" vessels in tumor

MDA-MB-231 Human Mammary Cancer Model

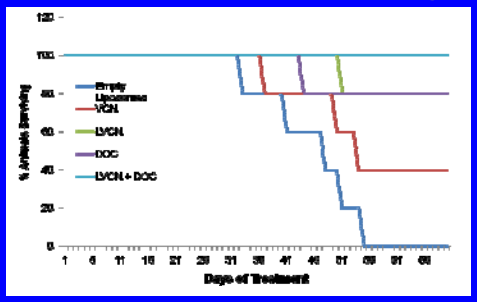
- Cells derived from pleural effusion of patient with carcinoma of the breast in 1974
- Cells are ER, PR, HER2 triple negative and are integrin $\alpha v\beta 3$ low positive, and $\alpha 5\beta 1$ and $\alpha v\beta 5$ positive
- In nude mice, tumor cells implanted orthotopically (in mammary fat pads) have ~100% take
- Tumor cells do not metastasize spontaneously to the lungs

Inhibition of MDA-MB-231 Growth by Combination Therapy



MDA-MB-231 (mammary fat pads; $2.5x10^6$ cells/mouse) in an orthotopic model. At 20 days following implantation, tumors became palpable and treatment was commenced. Groups of 5 animals were treated for 9 weeks with LVCN. LVCN was delivered by tail vein (100μg, i.v. twice weekly) alone or in combination with DOC. DOC administered i.p. at 8 mg/kg weekly for 2 weeks, then 4 mg/kg weekly

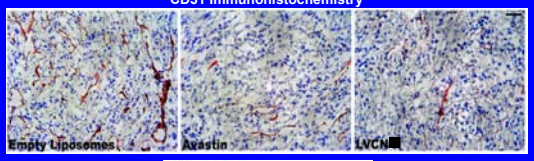
LVCN Therapy Enhances Survival of MDA-MB-231 Tumor Bearing Mice

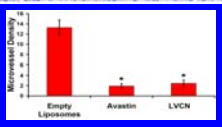


Survival of animals treated with LVCN. Survival was evaluated in the MDA-MB-231 model shown in the previous slide. In all treatment groups survival was significantly extended, while the control animals (Empty Liposomes) were all dead prior to the 8th week of treatment. Animals in the combination therapy group had 100% survival

LVCN Antiangiogenic Activity

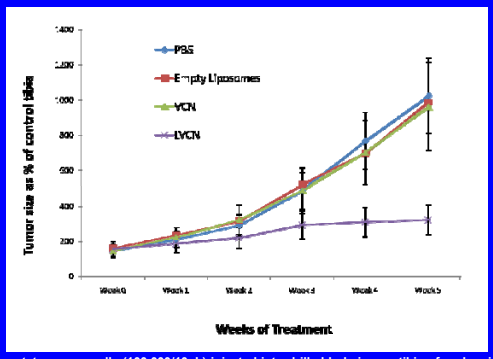
CD31 Immunohistochemistry





Comparing VCN to avastin as single agents, we calculated the anti-angiogenic activity by CD31 immunohistochemistry: LVCN has similar activity to avastin (a mAb to VEGF, which is in clinical use for colorectal cancer)

CWR22 PC Bone Metastasis Model



CWR22 prostate cancer cells (100,000/10μL) injected into drilled hole in one tibia of nude mice. Other tibia had hole drilled, but served as a control as no cells were injected. LVCN injected i.v. at 100μg twice per week. Diameters of tibia on control and injected side measured weekly and plotted as % size increase vs control

Recent Studies in Ovarian Cancer

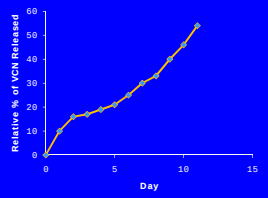
Rationale

- OC cells are carried in peritoneal fluid to 2ndary sites in the abdominal cavity and create peritoneal micrometastases in submesothelial connective tissue
- These OC cells are not accessible for surgical removal and are associated with poor therapy results and recurrence
- Hypothesis: localized intraperitoneal (i.p.) chemotherapy targets these cells and is effective in preventing recurrence
- U.S. National Cancer Institute recommends considering i.p. chemo for patients with advanced OC after surgical debulking

Vehicle for Delivery of VCN

- Use a viscoelastic gel composed of polyethylene oxide and carboxymethyl cellulose stabilized by calcium chloride
- It can be impregnated with VCN and injected i.p. and is slowly degraded in the peritoneal cavity
- This gel is approved for use in Europe and Australia for prevention of post-surgical adhesions

Gel Release Characteristics



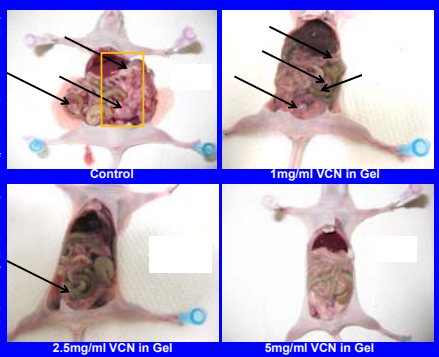
Release of VCN from gel is deletermined by measurement of released soluble ^{125}I-VCN. VCN when impregnated in gel is released with an initial rapid rate of ~16% of the loaded material over the initial 2 days. The gel remains intact for >11 days releasing nearly 55% of the loaded VCN.

In vivo model for Gel delivery of VCN

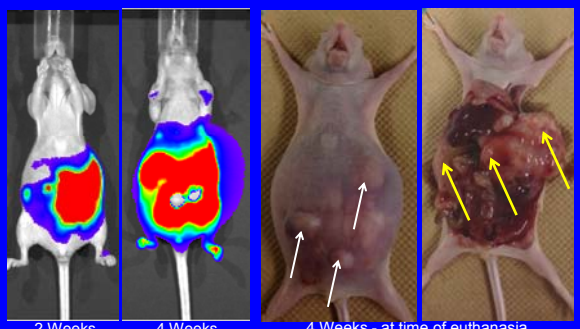
- 2x10^6 SKOV-3^{Luc} cells (human ovarian cancer cell line) delivered via i.p. injection (integrin αvβ3, αvβ5 and α5β1 positive)
- 3 animals per group (repeating study now with 10 mice/group) - cells stably infected with luciferase gene to monitor tumor growth
- OC cells were allowed to implant and grow for 2 weeks, mice displayed slightly enlarged abdomens
- Treatment with gel initiated, and gel delivered via i.p. injection weekly
- Mice were sacrificed as abdominal distension became significant
- Necropsy of animals plus luminescent imaging allowed observation of extent of tumor growth and dissemination

SKOV-3: Dose Response

- 2.0x10^6 cells implanted i.p. and allowed to grow 2 weeks
- Treatment then is begun, with one injection of gel per week (1ml) impregnated with varying doses of VCN
- Treat for 4 weeks, animals are then euthanized and necropsied, and extent of tumor spread is shown
- Arrows + orange box point out the tumors



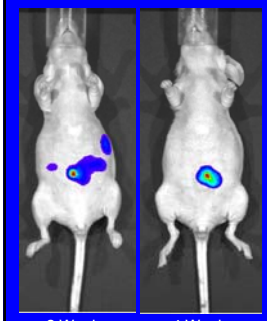
SKOV-3^{Luc} Tumor Bearing Mice Treated with Gel Alone



2 Weeks 4 Weeks
Control: Optical imaging results
Red indicates highest luminescense

4 Weeks - at time of euthanasia
(White arrows are gel injection sites; yellow arrows indicate tumor sites)

SKOV-3^{Luc} Tumor Bearing Mice Treated with VCN-Gel



2 Weeks 4 Weeks

Treatment: 5mg/ml VCN in Gel

4 Weeks - at time of euthanasia
(No visually observable tumor)

Conclusions: VCN Potential

- Easily produced
- Unique structure – RGD motif, amino acids in RGD loop, COOH-tail
- Binds integrins and blocks tumor dissemination and angiogenesis
- Non-toxic - VCN induces apoptosis in tumor and endothelial cells
- Excellent anti-tumor and anti-angiogenic activity in animal models
- Has significant anti-invasive activity
- In ovarian cancer VCN-Gel results are striking - i.p. delivery is effective
- Advantages for OC: intraperitoneal dispersion, higher patient compliance, less adhesions

Competing Interests

Dr. Markland, is a co-founder of Applied Integrin Sciences, Inc., a start-up company aimed at developing disintegrin-based therapeutic and diagnostic solutions. He has no financial interest in the Company or involvement on any Company boards.

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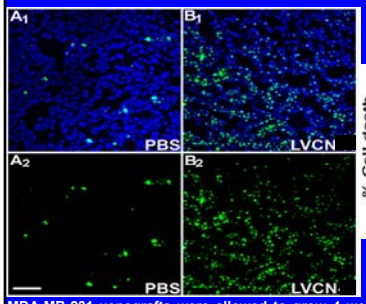
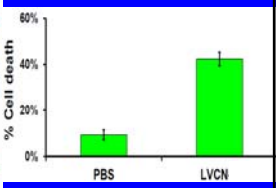
RESERVE SLIDE

LVCN Induces Apoptosis in the MDA-MB-231 In Vivo Model





MDA-MB-231 xenografts were allowed to grow 4 weeks before starting treatment. Animals received LVCN (100µg VCN per injection) i.v. every other day; control group saline only. Animals sacrificed after 6 doses of treatment. To assess the impact of VCN on cell death, tumor cryostat sections from each group were stained with FITC-TUNEL, counterstained with Hoechst. Confocal images x250 are shown. Panels A1-B1 – TUNEL-Hoechst; Panels A2-B2 - TUNEL only. Cell death was quantitated using 'SimplePCI'