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14. ABSTRACT Ovarian cancer (OC) is the deadliest of all gynecological cancers, with five year survival rates of <45%. One critical feature of the disease is that two-thirds of the women diagnosed have advanced disease, and the five year survival rate of this group is <30%. This project outlines the development of a recombinant version of a member of a class of proteins known as disintegrins as an innovative imaging and diagnostic agent for ovarian cancer (OC). Vicrostatin (VN) is a recombinant protein based on the venom disintegrin contortrostatin (CN), which has shown impressive antitumor and antiangiogenic activities in models of human ovarian cancer. OC cells have been shown to display integrins $\alpha v \beta 5$ and $\alpha 5 \beta 1$, and the antitumor activity of CN, and demonstrated for VN, is based on the high affinity interaction between the disintegrin and these integrins. Thus far we have developed and shown that we have a robust and viable system for the production of VN and that the protein produced displays a high affinity for integrins displayed on ovarian cancer cells. In ongoing experiments we are evaluating the imaging potential for VN to be used for both evaluation of treatment and diagnosis of OC. The high affinity of VN for the integrins found on OC cells make for an excellent candidate for improvement of OC diagnosis and therapy.					
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Annual Progress Report

Introduction

The project entitled "Development of a Multifaceted Ovarian Cancer Imaging Agent" began on April 1 2007. The project focuses on the development of a production method of a recombinant disintegrin vicrostatin (VN), which is based on the snake venom disintegrin contortrostatin (CN). The protein is delivered via intra-peritoneal injection in a liposomal formulation. PET imaging radiotracers can be covalently attached to VN and used as an imaging and diagnostic agent in ovarian cancer (OC). In the past year we have developed a robust and efficient system for the production of VN on a scale amenable to clinical translation. In addition we have developed liposomal formulations using a homogenization method that again is on a scale for clinical quantities of material. The affinity of the VN for well characterized ovarian cancer cell lines has also been evaluated. Finally, we are progressing on the development of VN as an OC imaging agent.

Summary of Specific Aims

LVN was prepared using homogenization equipment that is scaleable to commercial quantities. Our goal is to demonstrate that production of LVN by commercially relevant methods does not change its integrin binding profile and will allow for the development of an OC imaging agent. Successful completion of these initial studies has lead to the achievement of two key milestones. Our Specific Aims and Milestones for this project are:

Specific Aim 1: Prepare VN, a recombinant disintegrin with proven in vivo antiangiogenic activity (Milestone 1), and produce a liposomal formulation (LVN) with stability characteristics appropriate for clinical application (Milestone 2).

Specific Aim 2: Demonstrate imaging potential and biological efficacy of a LVN formulation in a mouse model of ovarian cancer (Milestone 3).

Specific Aim 3: Evaluate the use of VN as a novel tumor imaging agent both for diagnostic use and for evaluation of tumor suppression following treatment (Milestone 5).

Body

Preparation of LVN

Recombinant expression of a venom derived disintegrin: For a number of years the Markland laboratory has worked with contortrostatin (CN), a disintegrin isolated from *Agkistrodon contortrix contortrix* venom. A major block in the pathway to clinical development of CN was the supply of the protein itself: for purification, it only exists as a very small fraction of the total venom protein (~0.01%), and for recombinant production, its peculiar structure stabilized by numerous disulfide bonds makes its expression in commonly-employed recombinant systems a very difficult task. Nonetheless, we have successfully employed a recombinant expression system for which we developed a proprietary production method capable of generating substantially more than 20mg of purified active recombinant disintegrin from one liter of bacterial culture in small-scale laboratory conditions. To generate recombinant disintegrins, we have successfully adapted a commercially-available *E. coli* expression system consisting of the Origami B (DE3) expression host in combination with the pET32a vector (Novagen) for our production needs. A sequence-engineered form of CN, called vicrostatin (VN), has been directionally cloned into pET32a expression vector incorporating a unique TEV protease cleavage site, which facilitates the removal of the thioredoxin fusion partner from the expressed VN. Briefly for recombinant VN production, multiple colonies of transformed Origami B cells were used to establish primary cultures by inoculating 5ml LB broth batches containing carbenicillin (100µg/ml), tetracycline (12.5µg/ml), and kanamycin (15µg/ml). The primary cultures were grown overnight at 37°C and 250 rpm in a shaker-incubator and used to seed secondary cultures. Batches of fresh 500ml LB broth in the presence of the three antibiotics were then inoculated with the previously-established primary cultures and grown at 37°C and 250 rpm to an OD600 of 0.6-1.0. At this point, the cells were induced with IPTG added to a final concentration of 1mM and cultured for another 4-5 hours at either 25°C or 37°C and 250 rpm. At the end of the induction period, the cultures were centrifuged at 4000xg and bacterial pellets lysed by utilizing a scalable homogenization method for breaking open the bacteria. The cells were homogenized in a microfluidizer (Microfluidics M-110L, Microfluidics, Newton, MA) at room temperature by resuspending the cell pellets in 5 volumes of water before commencing the process. The operating conditions of the homogenizer included applied pressures of 14,000–18,000 psi, bacterial slurry flow rates of 300–400ml per minute and multiple passes of the slurry through the processor. The insoluble cellular debris was then removed by

centrifuging the bacterial lysates at 40,000xg and the soluble cell lysates collected and further analyzed by SDS-PAGE for recombinant protein expression. The expressed fusion protein (Trx-VN) was proteolysed by adding recombinant TEV to the soluble cell lysates according to the manufacturer's protocol (Invitrogen). The TEV treatment efficiently cleaved off VN from its TrxA fusion partner, the proteolysis status being monitored by SDS-PAGE. When proteolysis was complete, as assessed by SDS-PAGE, the proteolyzed lysates were passed through a 0.22µm filter, diluted 100-fold in water and ultrafiltrated through a 50kDa molecular weight cut-off cartridge (Biomax50, Millipore, MA) in a tangential flow ultrafiltration device (LabScale TFF system, Millipore, MA) that removed most of the higher molecular weight bacterial proteins. The resulting ultrafiltrates were then re-concentrated against a 5kDa molecular weight cut-off cartridge (Biomax5, Millipore MA) using the same tangential flow ultrafiltration device. VN was further purified by C₁₈-reverse phase (RP) HPLC. The recombinant disintegrin we have produced through this system is recognized by polyclonal antisera raised against native CN and inhibits ADP induced platelet aggregation in a dose dependent manner with an IC₅₀ almost identical to native CN (~60nM). Moreover, VN inhibits cell adhesion to vitronectin and fibronectin, inhibits endothelial cell and tumor cell invasion through a laminin-rich reconstituted basement membrane, and inhibits endothelial cell tube formation in a manner indistinguishable from native venom derived contortrostatin. In conclusion, due to its robustness and reproducibility, we believe that our recombinant production method will be easily translatable to bioreactors for scale up for clinical use.

Preparation of Liposomal Vicrostatin (LVN)

Liposomal encapsulation of VN using a homogenization method: To prepare liposomal vicrostatin (LVN), stock solutions of the phospholipids and cholesterol were prepared by dissolving each lipid in a chloroform/methanol solvent mixture. Thin lipid films were created by pipetting aliquots of the lipid solutions into round bottom glass tubes followed by solvent evaporation at 65°C under a stream of nitrogen gas. The dried lipids and cholesterol were further dried under vacuum for 48 hours. This process yielded lipid powder mixtures that were used to prepare LVN. For homogenization or sonication, VN was dissolved in a hydration buffer (10mM sodium phosphate and 262mM sucrose, pH 7.2) and added to the dried lipids. The lipid dispersion was incubated for five minutes at 50°C. The LVN was formed by either: (i) probe sonication at 10% power for 3 to 5 minutes in a Branson Probe Sonifier, or (ii) homogenization by passing the material through a microfluidizer (M110L; Microfluidics, Newton, MA). The material was processed between 10,000 and 18,000 psi while maintaining an elevated temperature (45-65°C). Samples of the liposome batch were taken during the process and the size distribution of LVN was determined with an Ultrafine Particle Analyzer (UPA150) (Microtrac, North Largo, FL). After processing, unencapsulated VN was removed by ultrafiltration using an Amicon UF membrane of 100,000 MWCO and the LVN sterilized by filtration through a 0.2µm PVDF filter. To determine the optimal processing conditions to formulate LVN in large scale, we performed a series of processing studies using the Microfluidizer and compared these preparations with the sonication method that had been used to produce lab-scale LVN for all of the *in vitro* and *in vivo* studies performed previously. The homogenized LVN was evaluated for size distribution, ease of 0.2µm filtration and percent encapsulation of VN into the liposome to determine the best formulation (Table 1). In the first experiment, 25ml of LVN (2mg/ml VN) was processed at 18,000 psi for three separate 1 minute intervals at 60-65°C and the temperature controlled using a heat exchanger set at 45°C. After each one minute homogenization cycle, a sample was evaluated for size distribution (**Figure 1**). After the third 1 minute homogenization cycle, the formation of LVN was complete based on size distribution and the relative translucence of the product. As compared to the sonicated material, the homogenized LVN was similar in size (55-85nm average range for sonicated LVN, Table 1) with an average diameter of 68nm and a standard deviation of 21.1nm. In a second study, 25ml of LVN (2mg/ml VN) was processed at 10,000 psi for five separate 30 second intervals at 50-55°C and the temperature controlled using a heat exchanger set at 45°C. The rationale for this processing condition was two-fold. First, we reduced the time/temperature and pressure of the homogenization parameters in an attempt to reduce the possibility of VN degradation and second, to produce larger vesicles that might encapsulate a greater quantity of VN. After each 30 second homogenization cycle, a sample was evaluated for size distribution (**Figure 2**), in which the average size of the LVN was 83nm and considered complete based on the size distribution and the relative translucence. Despite the larger vesicles prepared, the percent encapsulation was in the same range as the previously formulated material (**Table 1**). Under the recirculation conditions used in the first two experiments, it is possible that not all of the LVN was processed for the same amount of time due to a mixing effect during this type of processing. In a third processing experiment, 25ml of

LVN (2mg/ml VN) was processed at 18,000 psi for seven separate passes at 60-65°C and the temperature controlled using a heat exchanger set at 45°C. In this experiment, the size distribution of the sample never reached completion. The average size of the liposomes remained greater than 200nm and the standard deviation was greater than 500nm. Optically, this preparation appeared flocculent and not translucent as the other batches and was not filterable through a 0.2µM filter (data not shown). In a final experiment, 60ml of LVN (2mg/ml VN) was processed at 13,000 psi for three separate 1 minute intervals at 60-65°C and the temperature controlled using a heat exchanger set at 45°C. The final processed material showed a size distribution of 68nm (+/- 20nm). This material filtered easily through a 0.2µM filter and was optically translucent. Taken together, these data indicate LVN can be processed by homogenization and that these liposomes appear equal in size, filterability and percent encapsulation to LVN previously formed by sonication. In addition, the homogenized material has been scaled to 60ml per batch, which is 12 times that of the sonicated batch. Since homogenization has been used by others in the industry for liposome preparations, we are encouraged that this product can be scaled to volumes necessary for commercialization.

In vitro activity

Binding to integrins on cell surfaces: We have shown that the recombinant VN binds with different affinities to a panel of human ovarian cancer cell lines dependent on the integrin display status of the individual cell line (**Table 2**). Using a FACS based assay we evaluated the ability of fluorescently labeled VN to bind to Human Umbilical Vein Endothelial Cells (HUVEC) and the ovarian cancer cell lines OVCAR-3 and A2780 (**Figure 3**). This study revealed differential binding to the cells, and this appears to be dependent on their integrin profiles. The results of these studies support our hypothesis that the promiscuous nature of integrin binding by VN allows for broad targeting toward ovarian cancer.

Inhibition of cell adhesion to various ECM proteins: The effect of VN on cell adhesion was assayed using the OC cell lines OVCAR-3 and A2780, and HUVEC, incubated in serum-free DMEM (Dulbecco's Modified Eagle's Medium) with various concentrations (0-1000nM) of VN for 30 min and then seeded in wells pre-coated with 100 µl of fibronectin (Fn) or vitronectin (Vn) (15µg/ml) in a 96-well plate at 25,000 cells/well. The seeded cells were allowed to adhere to immobilized Fn or Vn for 1hr at 37C in the presence of 5% CO₂. The non-adherent cells were then washed away and the number of adherent cells for each condition was estimated colorimetrically using the MTS cell viability assay. The results were calculated in % adhesion (**Figure 4**), where the untreated control was considered as 100% adhesion. With Fn there was a dose dependent inhibition of adhesion by VN with an IC₅₀ of <100nM comparable to the value observed previously with native CN. Treatment of the cells with 1000nM VN allowed for less than 25% of the cells to adhere. Inhibition of adhesion to vitronectin by VN displayed a slightly higher IC₅₀ somewhat greater than 100nM, but again comparable to that seen with CN. With cells plated on Vn, treatment with 1000nM VN shows that less than 40% of the cells remain adherent. VN produced by the recombinant system retains biological activity equal to that of the natural protein, CN.

Inhibition of cellular invasion: To assess the ability VN to block the invasion of HUVEC, OVCAR-3 and A2780 cells through a reconstituted basement membrane, we used a cell invasion assay kit from Chemicon (Temecula, CA). This kit utilizes an invasion chamber, which consists of cell culture inserts that fit into a 24-well tissue culture plate. The inserts contain an 8 µm-pore size polycarbonate membrane, over which a thin layer of ECMatrix™ was applied. The ECMatrix™ serves as an in vitro reconstituted basement membrane and is a solid gel of ECM proteins prepared from the Engelbreth Holm-Swarm (EHS) mouse tumor. The ECM layer occludes the membranes pores, blocking non-invasive cells from migrating through. The HUVEC, OVCAR-3 and A2780 cells were starved overnight in DMEM containing 0.1% FBS and harvested and resuspended in serum free DMEM (at 1x10⁶cell/ml). The cells were incubated in the presence of various concentrations (10, 100, 1000nM) of VN for 30 min at 25C. Then 300µl of cell aliquots from each condition were added to each invasion insert, whereas the bottom well of the chamber received 500µl of chemoattractant (conditioned medium from human HT1080 fibrosarcoma cells). The invasion chamber was then incubated for 8hr at 37C in the presence of 5% CO₂ and the cells were allowed to invade through the ECM toward the chemoattractant. After 8hr, the cells that invaded through the pores into the lower chamber were detached, collected and lysed. The total DNA content from each cell lysate was determined after labeling with a DNA-binding fluorescent dye according to the manufacture's protocol. The numbers of invaded cells for each condition were approximated by further quantitating the labeled DNA using a SPECTRAMax GeminiEM fluorescent plate reader. The results were calculated in % invasion (**Figure 5**), where the untreated control

was considered as 100% invasion. VN inhibits the invasion of all of the cell lines tested. The IC₅₀ for OVCAR-3 and A2780 were <60nM (similar to that observed for CN), while the IC₅₀ for HUVEC approaches 1nM (data not shown).

iv. Inhibition of HUVEC tube formation: To assess the ability of VN to interfere with tube formation, HUVEC cells were maintained in EGM-2 complete media and grown to confluency. The HUVEC cells were then harvested by brief trypsinization, washed in the presence of soybean trypsin inhibitor (1mg/ml), and resuspended in basal media. After being maintained in suspension for 15-30 min, cells were seeded on to Endothelial Cell Tube Formation plates (BD BioCoat™ Angiogenesis System), an *in vitro* endothelial tubulogenesis system, at a concentration of 25,000 cells per well and immediately treated with various concentrations of VN, CN (control), or Suramin salt (supplier provided positive control) and incubated for 18 hours at 37C. At the end of the incubation period, cells were washed twice with PBS and then stained with 8 µg/ml Calcein AM in PBS at 37C. After 30 min the cells were washed again two times with PBS and then imaged (**Figure 6**) using confocal microscopy at 2.5X and 10X magnifications. On the captured images, the total length of tubes was quantitated with Zeiss LSM image software and data plotted (**Figure 7**) against the total length of tubes (in µm) generated by untreated cells. Representative tubes from 6 different wells were measured by 3 separate individuals and averaged to form each data point (**Figure 7**). VN inhibits tube formation as effectively as CN (data not shown).

Key Research Accomplishments

- Developed and implemented an expression procedure for the production of VN on a scale that can be used for translation into the clinic
- Developed a homogenization method for liposomal encapsulation of VN amenable for scale –up
- Evaluated the ability of VN to bind to integrins present of tumor cell surfaces
- Through integrin ligation evaluated the ability of VN to inhibit processes critical to tumor progression including cell adhesion and tumor cell invasion
- Observed and evaluated the ability of VN to limit tube formation, an in vitro assay of anti-angiogenesis

Reportable Outcomes

A manuscript is in preparation describing the production of VN and assays of VN's activity

Conclusion

LVN is a novel liposomal formulation of a disintegrin engineered using standard recombinant techniques. The results from the Phase I studies clearly show that LVN prepared by a commercially viable technique retains integrin binding and antiangiogenic activity equivalent to the laboratory prepared material. Based on these characteristics, in the next phase of the studies we will evaluate *in vivo* OC cell binding and subsequent use of VN as a pet imaging agent.

References

None

Appendices

None

Table 1 Properties of Homogenized Liposomes

Condition	Size Distribution +/- StDev	Filterability	Percent Encapsulation
Sonicated LVN	62nm +/- 12.3nm	Good	74
Homogenized LVN 1	68nm +/- 21.1nm	Good	72
Homogenized LVN 2	83nm +/- 47.1nm	Good	75
Homogenized LVN 3	225nm +/- 750nm	Poor	Not Determined
Homogenized LVN 4	68nm +/- 12.6nm	Good	78

LVN1-4 were prepared under slightly different homogenization conditions

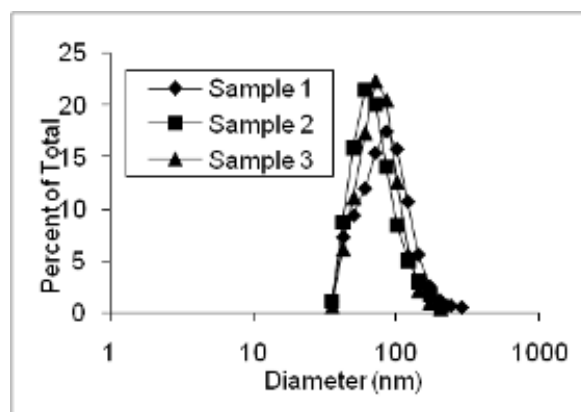


Figure 1 Size distribution of LVN processed by homogenization at 18,000psi for 3 minutes. Samples collected after 1 minute intervals.

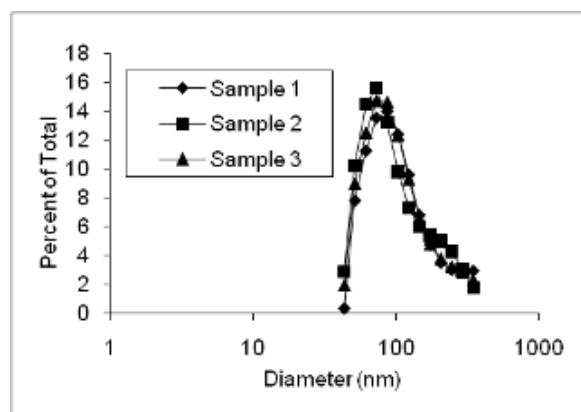


Figure 2. Size distribution of LVN processed by homogenization at 10,000psi and sampled at 1.5 (sample 1), 2 (sample 2) and 2.5 (sample 3) minutes of processing.

Table 2 Integrin Profiles of Selected Ovarian Cancer Cell Lines

Ovarian Cancer Cell Lines	Relative Integrin Surface Expression				
	α_v	$\alpha_v\beta_3$	$\alpha_5\beta_1$	β_1	$\alpha_2\beta_1$
OVCAR-3	+	-	+++	+++	+++
A2780	++	-	++	+++	+++
Reference Cell Line					
MDA-MB-435	+++	+++	++	++	+

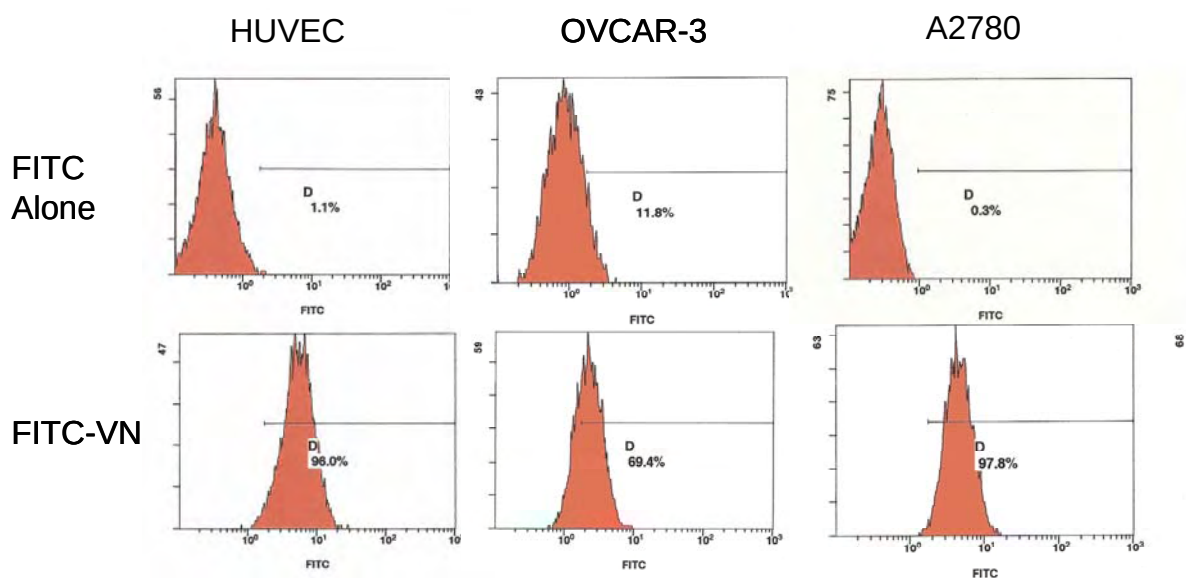


Figure 3: Binding analysis of FITC-VN by FACS. HUVEC OVCAR-3 and A2780 were incubated with FITC alone and FITC-VN. The direct binding of labeled protein was assessed by flow cytometry. As shown in the above representative flow-cytometric plots, FITC labeled VN binds avidly to both cell lines as well as the primary vascular endothelial cells.

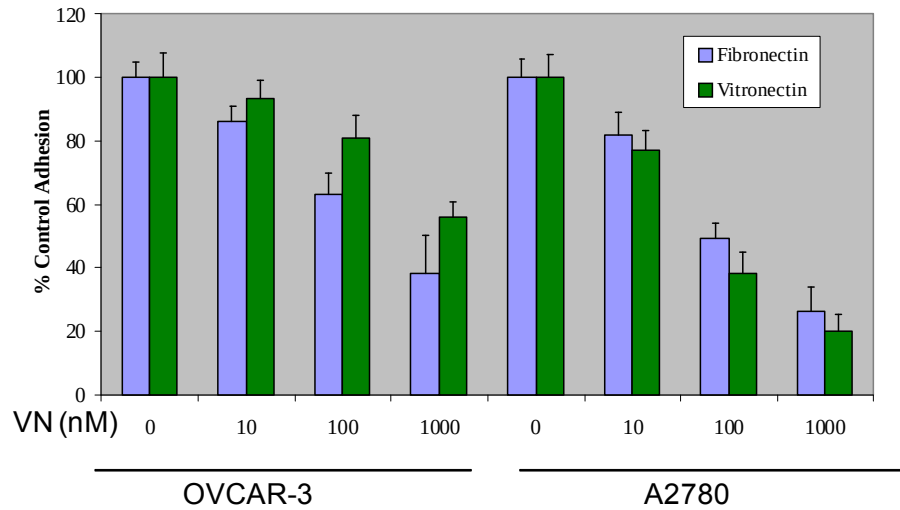


Figure 4: Inhibition of human ovarian carcinoma cells adhesion to extracellular matrices. OVCAR-3 and A2780 cells were pre-incubated with various concentrations of VN (0-1000nM) and then allowed to adhere onto either purified human fibronectin (blue bars) or vitronectin (green bars). The number of adherent cells for each condition was quantitated using an MTS-based assay. The adhesion of both cell lines was inhibited by VN in a dose dependent manner.

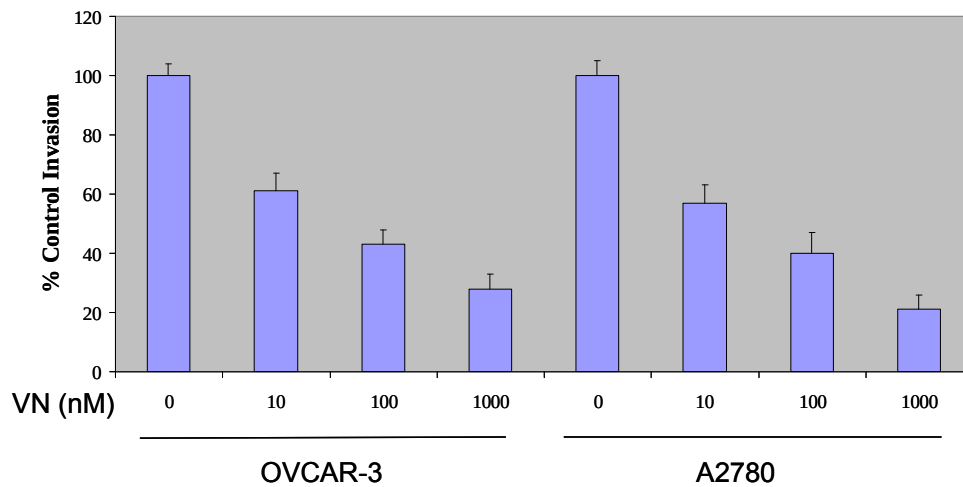


Figure 5: Inhibition of tumor cell invasion through a reconstituted basement membrane. OVCAR-3 and A2780 were preincubated with various concentrations of VN (0-1000nM) for 10 minutes before being seeded on porous inserts coated with ECMatrix and allowed to migrate against a chemoattractant gradient for 18hrs. The invaded cells were detached, lysed, stained with CyQuant, a DNA-binding fluorescent dye, and quantitated in a fluorescent plate reader. The invasion of both the OVCAR-3 and A2780 was inhibited in a dose dependent manner by VN. The IC_{50} in both cell lines is <20nM, comparable to that observed with venom purified CN.

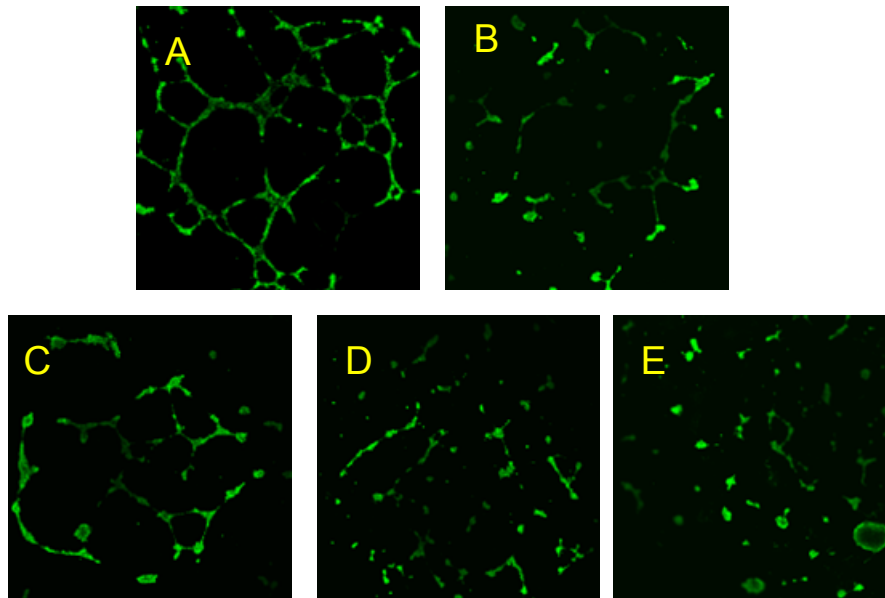


Figure 6: Inhibition of HUVEC tube formation by VN (representative images from multiple experiments). HUVEC cells were plated on 'Endothelial Cell Tube Formation' plates (BD Biosciences) in the presence of various concentrations of VN (0-1000nM), or a known tube formation inhibitor Suramin (used as a positive control). Representative figures from independent experiments were shown above: panel A - untreated control; panel B- 100uM suramin; panel C - 1nM VN; panel D -10nM VN; and panel E 1000nM VN. Cells were stained with Calcein AM and imaged using confocal microscopy. VN is an effective inhibitor of tube formation.

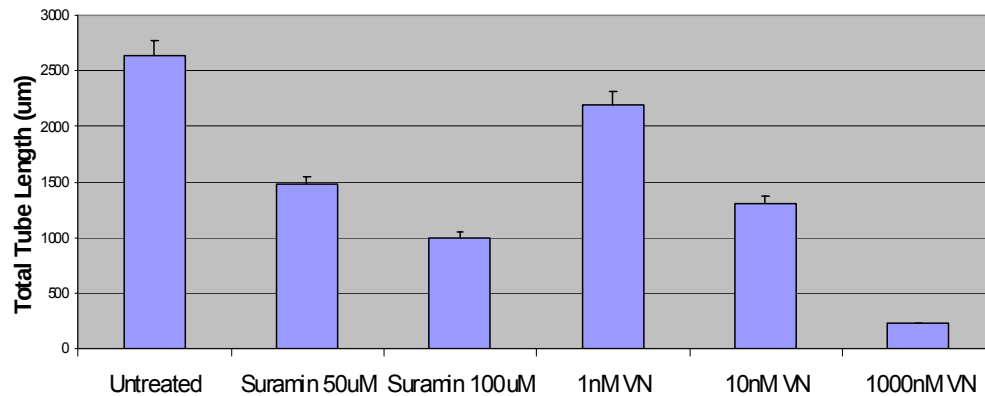


Figure 7: Quantitation of tube formation inhibition by varying concentrations of VN. The tubes formed by HUVECs were quantitated in multiple fields collected from three repeated experiments by computing the total tube length with Zeiss LSM image software and averaged to form each data point. The data shown above was assembled from multiple independent experiments.