Cancer Tumor Therapy Drug Vicrostatin Shows
Promising Inhibition of Glioma Growth and
Angiogenesis in Vivo

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May 6, 2011

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Abstract

Though glioblastoma multiforme (GBM) has been extensively researched for the last few decades, the prognosis for patients with GBM has not significantly improved. Recently, researchers were able to isolate a protein known as Contortrostatin (CN) from snake venom, which was shown to have anti-tumor properties. Researchers then made a synthetic version, known as Vicrostatin (VN), with the hope of achieving similar anti-tumor results. After obtaining promising results from in vitro studies, in vivo studies were conducted. Model tumors were injected into mice, and treatments began once the tumors became palpable. The mice were given either VN or control PBS treatments, and tumors were measured. Tumor volumes were calculated. After 28 days, the mice were sacrificed and the tumors were stained. Volume measurements showed a significant decrease in tumor volume in the VN treated group compared to the PBS control group. Staining and microvessel density analysis showed decreased vasculature and angiogenesis in VN treated tumors. These results indicate that VN may be a promising drug in extending the life expectancy of those diagnosed with GBM. We look forward to future studies involving VN to determine its exact abilities in fighting GBM and other cancer models.
INTRODUCTION

**Glioblastoma Multiforme (GBM)**

Glioblastoma multiforme (GBM) is the most aggressive form of brain tumor that can afflict an individual today. It is a form of cancer that occurs and develops in the brain, and can then metastasize to other parts of the body. It is considered to be a grade IV astrocytoma, measured on a scale of I-IV, making it the most invasive and aggressive form of brain tumors. If a brain tumor begins to develop and is left untreated, it can progress through the stages I-IV rather rapidly. Due to its rapid progression, GBM has become the most prevalent form of brain tumor, resulting in about 17,000 new cases diagnosed per year in the United States. It is currently believed that GBM is not genetic or inherited. There is also very minimal data suggesting that it is caused by any specific environmental factor (4).

Over the last few decades, the prognosis of those diagnosed with GBM has not significantly improved. Though it has been researched extensively, only minor breakthroughs have been developed to date. The prognosis for those diagnosed with GBM averages about 12-15 months of survival after diagnosis. However, this is with treatment. Without treatment, the life expectancy is much shorter.
Unfortunately, this is the case for most people around the world who do not have access to proper treatment.

Currently, the standard procedures used to treat GBM are surgery, chemotherapy, and radiation therapy. However, one of the many problems with this approach is that GBM is extremely invasive. Even with the best surgical technologies, it is nearly impossible to remove one hundred percent of the tumor. Furthermore, because of the aggressive nature of GBM, the tumor tissue that remains vigorously grows back and the GBM tumor is said to recur. Also, after substantial use of chemotherapy, patients can become resistant to the chemotherapy drugs. Because of this, there has been a great push in the last few decades to develop new strategies and drugs to improve the prognosis of those diagnosed with GBM.

**Tumor Angiogenesis**

Angiogenesis is the process by which new blood vessels are created. This occurs naturally throughout the body. It has been shown that cells need to be within approximately 0.2 mm of a blood vessel because that is the distance that oxygen can diffuse through in a living tissue (5). If a cell is any distance further than that, it can cause hypoxia and subsequently necrosis in those cells.
These blood vessels are especially important to tumors. They are critical to tumors because they bring to the tumor nutrients and biomaterials that are necessary for the tumor to grow and proliferate. In early tumor angiogenesis laboratory studies, small tumors were implanted in the ears of rabbits. Subsequently, researchers noticed over time that dense networks of capillaries and vessels began to converge on the tumor. This showed that tumors were actively recruiting blood vessels around them using growth factors (5). Important growth factors involved in this process include vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), interleukin-8 (IL-8), basic fibroblast growth factor (bFGF), angiopoietin, angiogenin, and platelet-derived growth factor (PDGF). For example, VEGF initiates the growth of capillaries, and angiopoietin 1 and 2 are responsible for recruiting pericytes and smooth muscle cells, which are important for new capillaries to mature (5). Furthermore, integrins such as αvβ3, αvβ5, and α5β1 play a critical role in pathological angiogenesis because of their involvement in the basement membrane extracellular matrix, which is greatly involved in growth (3). Because tumor cells rely so significantly on blood
vessels, angiogenesis has become a major topic of interest for cancer researchers.

The strategy behind using angiogenesis to fight cancer is to block the formation of new blood vessels by blocking any aspect of angiogenesis. By blocking angiogenesis, the tumor does not get access to its blood supply and is essentially “starved” of its nutrients that are necessary for the tumor to grow and proliferate. Angiogenesis can be blocked by interfering with any of the growth factors or integrins mentioned above.

\textbf{Contortrostatin (CN)}

For years, researchers have looked into snake venom as a possible source for cancer drugs. Given that snake venom is dangerous to normal human tissue, researchers proposed that snake venom could similarly be harmful to cancerous tissues. Thus, if there was a way of being able to deliver snake venom to a specific target, snake venom could become a potential source of treatment.

Using this approach, Dr. Francis S. Markland Jr., at USC Keck School of Medicine, and his team isolated a protein known as Contortrostatin (CN; Fig.1). CN was found in the venom of the Southern Copperhead snake. This protein was shown to be a disintegrin, which binds to the integrins on the surface of cells and prevents their
interactions. More specifically, CN was shown to interact with $\alpha\nu\beta_3$, $\alpha\nu\beta_5$, and $\alpha\beta_1$. In doing so, CN could block the ability to signal angiogenesis.

Research studies then went on to show that CN could prevent angiogenesis around tumor cells. Studies also showed that CN interfered with the ability of VEGF and bFGF to trigger angiogenesis (2). This soon became a promising new development. However, obtaining CN from the Southern Copperhead snake was dangerous, difficult, and expensive. Because of this, researchers began to look for ways to develop a synthetic version of CN that could bypass these concerns and still have a similar effect on cancerous tumors.

**Vicrostatin (VN)**

Researchers at the University of Southern California then developed Vicrostatin (VN), a synthetic recombinant version of CN. This was shown to have similar properties and interactions as CN, while also addressing the previous concerns surrounding the method of obtaining CN. A comparison of the two drugs and their composition can be found in Fig. 1.

Initial in vitro studies showed that VN could inhibit the invasion and proliferation of tumor cells (2). However, to determine if VN could block angiogenesis, and
subsequently inhibit the growth of a tumor, in vivo studies in mice needed to be conducted.
MATERIALS AND METHODS

Materials. The drug of interest, Vicrostatin (VN), was obtained from Dr. Francis Markland’s laboratory at USC. VN was then dissolved in phosphate buffered saline (PBS).

Mice. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Southern California. Eight 4-6 week old athymic nu/nu mice were obtained (Harlan, Inc.). These mice were then kept in a pathogen-free environment to prevent any confounding variables.

Implantation. U87 glioma cells were cultured and grown in DMEM. The U87 cell line represents a common grade IV glioma cell line that has been the standard for such research for decades (1). The mice were anesthetized and U87 cells were then implanted into the subcutaneous space of each nude mouse’s dorsal right flank. Further experimental steps were put on hold until the tumors become palpable.

Tumor sizing and measurements. Once the tumors became palpable, the mice were separated into two groups: one that would be receiving VN treatments, and the other that would be receiving PBS treatment. Plastic calipers were used to measure the size of the tumors on a twice per week
schedule. Tumor volume was calculated using the ellipsoid formula, where volume = \((4/3) \times (\pi) \times (\text{length}/2) \times (\text{width}/2) \times (\text{height}/2)\). Tumor volumes were recorded and the average tumor volume in each group was calculated. Tumors were measured each time before the drug was delivered.

**Drug delivery.** The mice were given their respective drug doses on a twice per week schedule after the tumors were measured. First, the injection site was cleaned using an alcohol cleansing pad. Drugs were then delivered directly into the blood system by tail vein injections. The injection sites were then wiped with alcohol cleansing pads. If bleeding occurred, pressure was applied with the pads to stop it.

**Immunostaining.** After completing the outlined course of treatment, the mice were sacrificed. The tumors were then excised from the mice. The tumors were sliced into 8 \(\mu\)m thick slices and placed onto glass slides. The slides were then washed with PBS, followed by blocking with 5% goat serum. The primary antibody, biotinylated purified rat anti-mouse CD31 (BD Pharmingen), was prepared in 2% goat serum and applied to the slides overnight. On the next day, slides were washed with Tris buffer. After the tissues were incubated, the secondary antibody, anti-rat (Vector Labs), was prepared and applied. The ABC kit was prepared
and applied (Vector Labs). AEC Elite was then prepared and added. Finally, the slides were counterstained with hematoxylin. A positive result showed red precipitate.

**Microvessel density analysis.** The slides were imaged using the ImageJ software (National Institute of Health). The area of red precipitate in each image was measured and recorded. The microvessel density was recorded in mm$^2$ and was compared between the two groups.
RESULTS

Setup. The eight mice were injected with $5 \times 10^5$ U87 cells. The tumors became palpable after 7 days. At that point, four were randomly assigned to the VN treatment group, and the other four were randomly assigned to the PBS treatment group. Treatments were given on a twice per week schedule until the tumors began to ulcerate, at which point the treatments were stopped (Fig. 2).

Measurements and drug delivery. Measurements were taken on the same twice per week schedule using plastic calipers. The average tumor size of each group was calculated. After the tumors were measured, a new dosage of either VN or PBS was delivered via tail vein injection. These measurements and treatments were continued for 28 days before the tumors began to ulcerate.

From the data, we calculated that there was a significant size difference between the VN treated group and the PBS treated group (Fig. 3). By day 28, the PBS treated tumors were on average 46.2% larger than VN treated tumors.

CD31 immunohistochemical staining. After slicing the tumors and staining them for CD31, there was clearly more red precipitate on the PBS treated group tissues than on the VN treated group tissues (Fig. 4).
Microvessel density analysis. The images in Fig. 4 were then quantitatively analyzed to determine the amount of red in each image. This indicated the amount of positive staining for CD31.

Quantitative analysis of the two groups show that PBS treated tumors had approximately 5.6 times more microvessel density than the tumors in the VN treated group (Fig. 5).
Discussion

In this study, we did not see any blatant adverse side effects. VN was well tolerated by the mice for the entire course of 28 days. This likely shows that VN is not toxic to the mice, and can perhaps be used in other model systems. Also, as seen by the tumor volume growth, the average PBS control tumor was 46.2% larger than the average VN treated tumor by the end of the study (Fig.3). This indicates that VN successfully inhibited subcutaneous tumor growth. From the tumor pictures and the microvessel density analysis, we found a 5.6 fold increase when comparing the average PBS microvessel density to the average VN microvessel density (Fig.4 and Fig. 5). This shows that VN successfully inhibited new vasculature in the treated groups. From this, we can conclude that VN has potent anti-tumor and anti-angiogenic properties in our preliminary in vivo studies, and we believe it is worthy of further investigation to determine its potential in GBM treatment.

Based on our results, we believe that there are many roles that VN can play in GBM treatment, as well as cancer treatment as a whole. One of the most difficult issues to deal with in GBM treatment is tumor recurrence. Currently,
the treatment for GBM is surgical resection of the tumor, then chemotherapy, and finally radiation therapy. However, due to the highly invasive nature of GBM, it is almost impossible to completely resect the tumor. After a while, the little bits of GBM that are left behind end up re-growing into full tumors. We believe that VN could be used to prevent the little bits of tumors from re-growing. This would ideally slow down, or even prevent, tumor recurrence.

Also it is important to see how the effects of VN compare with the effects of other drugs that are currently being used in treatment. Such drugs currently include temozolomide and bevacizumab (Avastin), the latter also being an anti-angiogenic drug. Not only is it important to see how VN compares to these drugs, it is also necessary to see how VN interacts with these drugs if they are used together. Perhaps, using a few or all the drugs together would cause a synergistic effect that could be a powerful new approach to GBM therapy.

Finally, we believe that there is a lot of potential for VN, not just in GBM treatment but also perhaps in other types of cancer lines. We believe that VN is worth investigating in other cancers, such as breast cancer, lung cancer, melanoma, etc. To explore this further, we would eventually like to also determine if VN could be delivered
in any other form other than through injection. Perhaps VN could be delivered as a topical ointment or in a spray aerosol form.

Given the promising results from VN treatment, we believe VN has great potential to open a new door in GBM and cancer therapy. We look forward to seeing what else VN can do in the future.
References


FIG. 1. Comparison of CN and VN. VN is shown to be a recombinant monomeric version of CN (2). VN is monomeric, but has a similar composition to CN. The G in the N terminus is the result of the cut at the protease site. The HKGPAT is used in hope of better binding to the integrin to inhibit angiogenesis.
FIG. 2. Treatment schedule. Implanted tumors became palpable after 7 days. VN and PBS treatments then began on day 7 and continued for another 28 days, making the experiment last a total of 35 days.
FIG. 3. Average tumor size over time. Tumors were measured on a twice per week schedule. Measurements were taken right before a new dosage was given.
FIG 4. CD31 staining results. Tumors were sectioned and immunostained to detect CD31 in PBS control mice (A-C) and VN-treated mice (D-F). There is significantly less red precipitate in images D-F, which represent VN treated tumors, than in images A-C, which represent PBS treated tumors.
FIG. 5. Microvessel density analysis. The amount of red precipitate, indicating presence of CD31, was measured in mm² from the pictures. There is significantly less red staining in the VN-treated group than in the control PBS-treated group.