In-Vitro and *Ex-Vivo* Investigations of the Microtubule Binding Drug Targetin on Angiogenesis

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Abstract

Background—Intervention aimed at disrupting or inhibiting newly formed vascular network is highly desired to attenuate the progression of angiogenesis-dependent diseases. In cancer, this is tightly associated with the generation of VEGF by hypoxia inducible factor-1α following its activation by hypoxia. In light of the multiple cellular roles played by microtubules and their involvement in the processing of the hypoxia inducible factor-1α transcript, modulation of microtubule dynamics is emerging as a logical approach to suppress tumor reliance on angiogenesis. Targetin is a novel noscapinoid that interferes with microtubule dynamicity and inhibits the growth of cell lines from many types of cancers.

Methods and Results—Utilizing *in-vitro* and *ex-vivo* angiogenic models, we discovered the vascular disrupting and anti-angiogenic properties of Targetin. Targetin disrupted pre-assembled capillary-like networks of human endothelial cells by severing cell-cell junctions, inhibiting endothelial cell proliferation and metabolic activity in the presence and absence of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Furthermore, we show that Targetin significantly inhibits the formation of neovasculature network sprouting from rat aortic explants stimulated with proangiogenic stimuli, namely VEGF or bFGF.

Conclusion—We conclude that Targetin is a potential clinically promising anti-angiogenic agent for the treatment of many diseases including cancers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Keywords

Drug discovery; angiogenesis; microtubule; opioid

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1. INTRODUCTION

The sprouting of new vessels from established vasculature, otherwise referred to as angiogenesis, occurs rarely during postnatal development and is regulated by a stringent balance between pro- and anti angiogenic factors [1, 2]. However, this balance is often bypassed in physiologic conditions such as wound repair and in pathological conditions such as cancer where tumor progression is fueled by angiogenesis [3, 4]. As a result, inhibition of angiogenesis has evolved as a pivotal strategy to suppress tumour growth and metastasis. This notion had prompted the evaluation of several anti-tumour agents in angiogenic models systems, with the goal of discovering compounds endowed with anti-angiogenic/vascular disrupting properties [5], including members of the noscapinoid family [6, 7].

Noscapine is an opium derived anti-tussive alkaloid which binds to tubulin and induces growth regression in a variety of cancer types [8]; but with little or no adverse toxicity to normal tissues [9]. Its excellent anti-tumour properties and ability to modulate microtubule dynamics without altering the total microtubule polymer mass have served as the rationale for us to previously synthesize several noscapine derivatives with potent anti-tumour properties and with IC_{50} concentrations much lower than that required by noscapine [10, 11]. In fact, we recently reported the strategic synthesis of a conjugated noscapine derivative, Targetin, that modulates microtubule dynamicity and inhibits the proliferation of lung, colon, prostate, breast and ovarian cancer cells more potently than parental noscapine [10]. Moreover, in pediatric gliomas, we previously discovered that Targetin modulates the expression of numerous genes implicated in cancer progression, disrupts anchorage independent growth, cell migration and proliferation, while inducing G2M cycle arrest and apoptosis [12]. Unlike noscapine and its bromo-analogue EM011, which inhibit angiogenesis by interfering with the HIF-1α angiogenic signaling [6, 7], the effect of Targetin on angiogenesis is yet to be determined. In this study, we report that Targetin suppresses endothelial cell viability, proliferation and impedes VEGF-induced formation of capillary-like structures as well as *ex-vivo* vascular outgrowths from rat aorta.

2. MATERIALS AND METHODS

2.1. Cell Lines

The Human umbilical vein endothelial cell (HUVEC-CS) was obtained from ATCC and cultured on gelatin coated plates in the presence of Dulbecco's minimum essential medium (DMEM) and 20% fetal bovine serum (FBS). A second HUVEC cell line was established as described previously [13] and propagated on a 12 day monolayer of normal human dermal fibroblasts (NHDFs) using conditions previously described [14]. All cell lines were maintained at 37°C, 5% CO_2.

2.2. Cell Viability Assay

The effect of Targetin on viability of HUVEC cells was investigated using the Cell Titer 96 Aqueous One cell proliferation kit (Promega) as previously described [15]. Briefly 2×10^3 endothelial cells were plated on 96 well gelatin-coated plates and subjected to various treatments including vehicle (PBS), Targetin, vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) for 5 days. Absorbance measurements were obtained at specific time points following addition of the MTS reagent.

2.3. Cell Proliferation Assay

The colorimetric BrdU enzyme-linked immunosorbent assay (Roche) was used to assess cell proliferation. Briefly, 2×10^3 HUVEC cells were added to each well of a 96 well plate, coated with 0.01% gelatin. The cells were incubated overnight, treated daily with Targetin, vehicle, bFGF and VEGF and subjected to BrdU ELISA assessments as previously described [15].

2.4. Immunofluorescent Cytochemistry

HUVEC cells were plated on gelatin coated slides and treated with 20μM Targetin for 24 hours. Cells were subjected to immunofluorescent cytochemistry analysis as previously described [15] using the DM1A tubulin alpha antibody (Santa Cruz) and Protein-G FITC secondary antibody.

2.5. Endothelial Tube Formation Assay

The effect of Targetin on capillary-like structure formation was conducted using a previously described *in vitro* angiogenic model system [16]. Briefly, HUVEC cells were cultured on a layer of normal human dermal fibroblasts (NHDF) as described earlier [14] and treated with or without VEGF. Following the development of capillary-like structures, the cells were exposed to Targetin. Eight images per well were captured using a Nikon-TE2000 inverted microscope imaging system equipped with a photometric CoolSNAP HQ2 camera. Images of tube-like structures and capillary junctions were processed manually and graphically documented.

2.6. *Ex-Vivo* Angiogenesis Assay

The thoracic aorta of a 2 month old rat was obtained following an incision along the thoracic and abdominal cavity. The aorta was freed from fibroadipose tissues, sectioned into 1–2 mm rings and washed several times in serum free medium. A 1:1 mixture of 10X Eagle MEM (Gibco) and 23.4g/ml NaHCO$_3$ (Sigma) was prepared and added to 4 volumes rat tail collagen (Life technologies). Each aortic ring was carefully transferred into a 24 well plate containing 300 μl of diluted collagen matrix per well. Collagen gelation was induced by incubating the plates for 10 minutes at 37°C followed by the addition of vascular outgrowth induction medium containing DMEM and 20% FBS. The aortic rings were treated with or without Targetin, VEGF or bFGF and incubated at 37°C for 8 days. Images of vascular outgrowth from aortic rings were documented on days 2 and 8 using a Nikon inverted microscope imaging system.

2.7. Statiscal Analysis

Data analysis was performed using the Graphpad prism software. The mean ± standard deviation of replicate experiments is represented graphically. Comparison between treatment and control was performed with the aid of the Student's T-test and with P-values < 0.05 considered significant.

3. RESULTS

3.1. Targetin Disrupts Endothelial Cell Viability, Proliferation and Microtubule Network

Targetin is a microtubule interfering agent, with potent anti-tumorigenic properties [10]. In fact, the growth of a variety of cancer types is significantly perturbed upon exposure to 15 – 40 μM of Targetin, and especially in those that over-express the folate receptor alpha [10]. Using similar doses, we assessed the effect of Targetin on endothelial cell viability, proliferation and capillary tube formation. At sub-cytotoxic doses (5–10 μM), Targetin did not induce any profound changes in the metabolic activity of endothelial cells after 24 hour exposure. However, administration of 20 μM Targetin led to a 22% decline in metabolic activity within the same time frame. Remarkably, increasing the duration of Targetin treatment to 72 or 105 hours, resulted in a significant decrease in cell viability (P-value < 0.001) irrespective of the drug concentration used or the presence or absence of angiogenic inducers such as VEGF or bFGF (Figure 1A).

Since microtubules modulating agents often arrest proliferation by halting cell division [17], we utilized BrdU ELISA assays to further examine whether Targetin treatment could influence the proportion of cells with incorporated BrdU. As seen in Figure 1B, treatment of endothelial cells with 5 – 20μM of Targetin significantly perturbed cell proliferation after 72 hours (P-value <0.001). Moreover, the addition of angiogenic growth promoting agents such as VEGF or bFGF prior to Targetin treatment, failed to rescue HUVEC cells from the inhibitory effect of Targetin. This finding was further reinforced by immunofluorescent cytochemistry analyses of untreated endothelial cells which displayed normal microtubule arrangement and architecture. However, endothelial cells treated with 5μM, 10μM or 20μM Targetin for 24 hours, instead adopted a rounded morphology with almost complete absence of cytoplasmic microtubules (Figure 1C). In contrary, VEGF treatments were incapable of affecting the cell morphology and microtubule distribution. Indeed, the disruption of microtubule distribution by Targetin could mechanistically explain its inhibitory effect on endothelial cell proliferation considering that well organized microtubule networks are crucial for spindle formation and chromosomal segregation during mitosis.

3.2. Targetin Disrupts Endothelial Cell Capillary-Like Structure Formation

The anti-neovascularizing ability of tubulin binding agents relies partly on their ability to perturb endothelial cell viability, proliferation and morphogenesis into capillary-like structures [18–21]. These cellular properties could be modeled with the growth of endothelial cells on a monolayer of normal human dermal fibroblast (NHDF) as previously described by us [16]. Using Time-lapse video microscopy of this *in-vitro* angiogenesis model system, we found highly proliferating untreated endothelial cells manifesting capillary-like structures. Cells further exposed to VEGF form extensive and complex tube-

like networks became distorted after 48 hours of post-Targetin treatments (Figure 2). In fact, Targetin accelerated the retraction of endothelial cell margins and severely attenuated the formation of branches as well as the number of capillary-like structures (Figure 2). Collectively, these findings suggest that Targetin is endowed with vascular disrupting properties.

3.3. Targetin Abrogates Vascular Outgrowth in a Rat Aortic Model

The global effect of Targetin on the initiation and maintenance of neovascularization was assessed using the rat aortic ring assay, an *ex vivo* model system which robustly replicates key angiogenenic stages including sprouting, proliferation, migration and capillary tube formation by endothelial cells. Treatments of aortic explants with serum containing medium in the presence or absence of VEGF or bFGF, initiated the outgrowth of vascular structures within 48 hours (Figure 3). The sprouted endothelial cells continued to proliferate and migrate to form an intricate capillary-like meshwork, which completely covered the entire well by day 8. In contrast, the administration of 10 μM to 40 μM doses of Targetin completely abrogated the sprouting of endothelial cells from rat aortic explants. Remarkably, aortic explants treated with 20 ng/ml VEGF or bFGF plus Targetin, remained dormant and failed to show any evidence of angiogenic growth.

4. DISCUSSION

The emergence of new capillary-like vessels from pre-existing vasculature, contributes enormously in the progression of several diseases including cancer [3, 22]. Tumor growth and metastasis are accelerated by the development of new vessels within the tumor vicinity, suggesting that inhibition of angiogenesis could represent a suitable anti-neoplastic strategy [23, 24]. Drugs that target the tumor vasculature are often endowed with anti-angiogenic and/or vascular disrupting properties [22, 25, 26]. Given the multiple functions of microtubules involvement in endothelial cell morphogenesis, motility, and cell division [19, 27], it is logical that disruption of microtubule dynamics arrests sprouting and maintenance of new vasculature. In fact, several tubulin binding agents including noscapinoids were previously shown to perturb angiogenesis or disrupt vascular network through various mechanisms, including the dysregulation of HIF-1α which regulates VEGF signaling [6, 7, 28]. Among these are members of the noscapine family which modulate microtubule dynamics at doses that do not alter the monomer/polymer ratio of microtubules; hence are unassociated with adverse cytotoxic effects on normal cells which are typically induced by other tubulin binding agents [29, 30].

Targetin is a novel tubulin-binding agent that modulates microtubule dynamics and arrests the proliferation of several lung, colon, prostate, breast and ovarian cancer cell lines at doses (IC$_{50}$ between 0.3 to 30 μM) much lower than those of noscapine (IC$_{50}$ between 25 to 60 μM) [10]. Furthermore, we recently reported that Targetin perturbs the expression of several genes implicated in cancer progression including PDGFR and some members of the MAPK cascade [12]. These changes in gene expression corroborated with Targetin-mediated induction of growth arrest and apoptosis which in turn negatively affected the viability, proliferation, migration and anchorage independent growth of pediatric low and high grade glioma cell lines [12]. Since noscapinoids have been shown to attenuate HIF-1α mediated

control of VEGF pro-angiogenic signaling [6, 7], we investigated whether Targetin could abrogate the activities of exogenous VEGF or other growth promoting angiogenic stimuli such bFGF. Indeed we discovered that even though these vascular stimuli were previously reported to promote angiogenesis, their proangiogenic effects were attenuated in the presence of Targetin [31]. Mechanistically, Targetin functions by compromising endothelial cell viability and hinders cell proliferation as well as the ability to form capillary-like structures on monolayers of normal human dermal fibroblast. Targetin administration diminished endothelial cell-to-cell contact, modified microtubule distribution and accelerated the adoption of a rounded morphology by endothelial cells. Moreover, rat aortic rings exposed to Targetin remain dormant and failed to initiate neovascularization even upon proangiogenic activation by VEGF and bFGF. In conclusion, our *in-vitro* and *ex-vivo* analyses unravel for the first time, a promising role for Targetin, in the treatment of angiogenic-dependent diseases and unveils multiple avenues for future studies including an exploration of its anti-angiogenic and anti-tumour efficacy in clinical trials.

Acknowledgments

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SUMMARY POINTS

- Targetin is a new microtubule binding agent which is conjugated with a folate ligand (folate group) at the C9 position of Noscapine.

- Targetin disrupts the pre-assembled capillary-like network of human endothelial cells by severing cell-cell junctions, inhibiting cell proliferation and metabolic activity in the presence and absence of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).

- Targetin significantly inhibits the formation of neovasculature network sprouting from rat aortic explants stimulated with the proangiogenic stimuli, namely VEGF and bFGF.

- Our study collectively suggests that Targetin has excellent anti-angiogenic properties which can be useful to treat a wide variety of disorders, including cancers, and therefore warrants further investigations in future clinical trials.
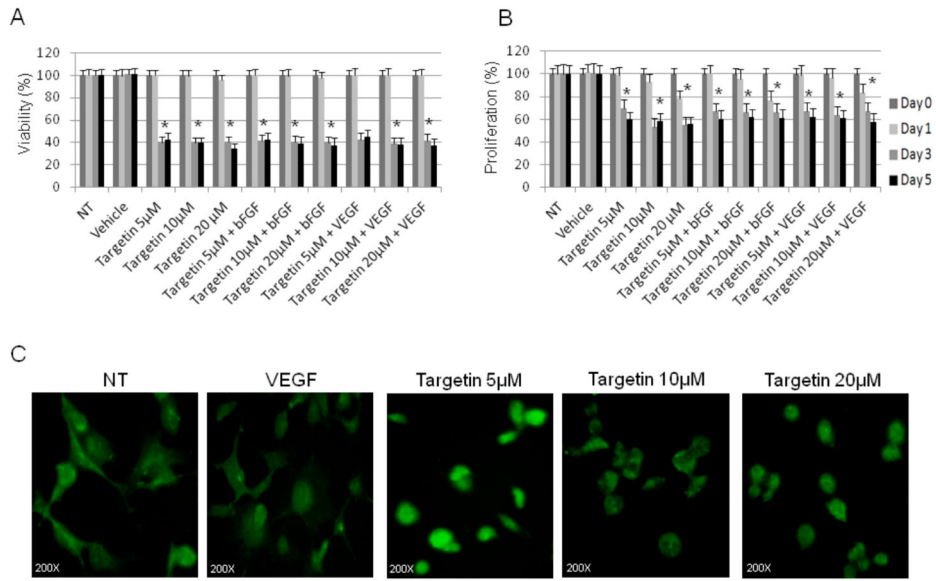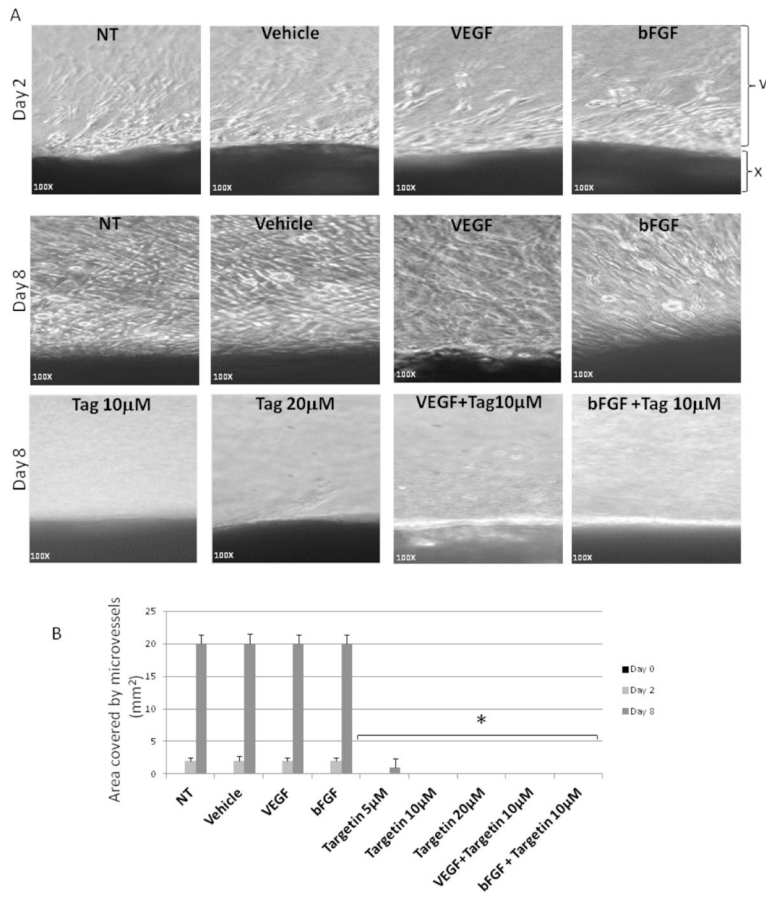
Figure 1.
Targetin perturbs metabolic activity and hinders proliferation of endothelial cells. Endothelial cells were exposed to Targetin within the indicated time frame. (**A**) Metabolic activity and (**B**) proliferation, were evaluated using the MTS and BrdU ELISA assays respectively. (**A**) Targetin treatments of 5μM, 10μM and 20 μM diminish endothelial cell viability and (**B**) proliferation. Pre-treatment of endothelial cells with 20 ng/ml VEGF or bFGF prior to Targetin exposure failed to augment endothelial viability and proliferation. (**C**) Alpha tubulin antibody (DM1A) immuno-staining revealed alterations in the endothelial cell morphology and distribution of microtubules following exposure to Targetin. Asterisk denotes a P-value <0.05, which is a significant difference between untreated and Targetin-treated cells. Error bars are Mean ± Standard Deviation of replicate experiments.
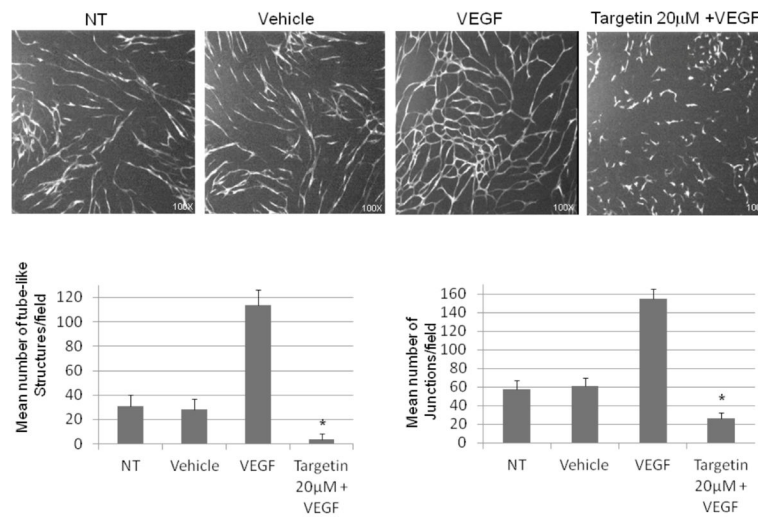
Figure 2.

Targetin disrupted endothelial cell pre-established capillary-like structures. Endothelial cells were cultured on a monolayer of normal human derived skin fibroblast. An intricate network of capillary-like structures formed 4 days later, in the presence or absence of VEGF. Treatment with Targetin abrogated the established capillary-like structures leading to a reduction in the average number of junctions and cells with tube-like structures. Eight images per well were obtained and the Mean ± Standard Deviation is represented graphically. P-value < 0.05 is denoted by an asterisk, representing a significant difference between Targetin treated and untreated cells.

Figure 3.
Targetin abrogated the formation of new vessels from pre-existing vasculature. Aortic explants were implanted in a matrix containing rat tail collagen, EMEM and $NaHCO_3$. (**A**) Exposure of rat aorta to serum containing medium in the presence or absence of VEGF or bFGF, spontaneously induced the sprouting of endothelial structures, which later developed into an extensive network of capillary-like vessels. However, the exposure of explants to the same condition and with Targetin, induced a deficiency in vascular outgrowth. (**B**) Differences in treatment can be observed from the quantitative analyses of the area covered by microvessels. P-value < 0.05 is denoted by an asterisk, representing a significant difference between Targetin treated and untreated cells. Error bars are Mean ± Standard Deviation of replicate experiments. NT = untreated; Tag = Targetin; x = aortic explant; v = vascular outgrowth.