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Anti-angiogenic effects of chemotherapeutic agents and their enhancement with OT-515 and S-NACH

Shimaa Adel Heikal
The American University in Cairo AUC, shimaa_heikal@aucegypt.edu

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Anti-angiogenic effects of chemotherapeutic agents and their enhancement with OT-515 and S-NACH

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By

Shimaa Adel Heikal
B.Sc in Pharmaceutical Sciences

Under the Supervision

of

Hassan A. N, El-Fawal, PhD
Professor of Biomedical Sciences
School of Science and Engineering
American University in Cairo
And
Shaker A. Mousa, PhD, MBA, FACC, FACB
Professor and Executive Vice President and Chairman
The Pharmaceutical Research Institute (PRI)
Albany College of Pharmacy and Health Sciences
Rensselaer, New York 12144

Biotechnology Graduate Program
School of Sciences and Engineering
American University in Cairo
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Dedication

To my parents, *Mr. Adel Heikal and Mrs. Saada Heikal*, I would have never succeeded without you by my side. A special feeling of gratitude to your love which was the source of encouragement and inspiration to me throughout my life. I owe you every single achievement in my life and will try my best to make you always proud of me.

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Abstract

Chemotherapeutic agents have been used for the treatment of numerous types of tumors with great success. Cisplatin and Doxorubicin are among the well-known chemotherapeutic drugs that showed efficacy against various types of cancers. However, cell resistance and major side effects like chemotherapy-induced peripheral neuropathy (CIPN) are limiting factors in using these compounds. Using a combination or adjuvant compounds with anti-angiogenic effects is one of the strategies suggested to decrease resistance or ameliorate chemotherapeutic toxicity. The present study investigated the anti-angiogenic effects of Cisplatin and Doxorubicin alone and combined with Sulfated non-anticoagulant heparin (S-NACH), a low molecular weight heparin LMWH that has anti-angiogenic and anti-tumor properties, or OT-515, a tempol congener-derived molecule that might act on inhibiting tumor proliferation and angiogenesis. The compounds' ability to enhance the effects of chemotherapeutic agents and decrease the doses used was tested in-vitro and in-vivo through using a chick chorioallantoic membrane (CAM) model, in the presence and absence, of tumor cells. To elucidate the mechanism of anti-angiogenic effect of the compounds, their impact on endothelial cells was studied by performing cytotoxicity assays using HUVEC and mouse endothelial cells. The results showed that combinations of Cisplatin and Doxorubicin with S-NACH or OT-515 had enhanced anti-tumor and anti-angiogenic effects than individual treatments. This suggests that OT-515 and S-NACH provide promising adjuvant therapy to reduce doses of traditional chemotherapeutic agents and ameliorate their adverse effects.

Keywords

Angiogenesis, Doxorubicin, Cisplatin, Chick Chorioallantoic Membrane (CAM), OT-515, S-NACH, CIPN

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List of abbreviations

S-NACH: Sulfated non-anticoagulant heparin

LMWH: low molecular weight heparin

CAM: Chorioallantoic membrane

HUVEC: Human umbilical vein endothelial cell

TNF: Tumor necrosis factor

TGF: Transforming growthfactor

ILs: Interleukins

MMPs: Metalloproteinases

VEGF: Vascular endothelial growth factor

FGF: Fibroblast growth factor

FGFR: Fibroblast growth factor receptor

PDGF: Platelet-derived growth factor

ECM: Extracellular matrix

EGF: Epidermal growth factor

NFκB: Nuclear factor kappa B

IκB: Inhibitor Kappa B

CIPN: Chemotherapy-induced peripheral neuropathy

ROS: Reactive oxygen species

Introduction

The use of cancer chemotherapy has expanded over the years to provide effective treatment for human cancer types. Cancer chemotherapy may, in many case, ameliorate the mortality and morbidity of cancer patients while improving their quality of life [1]. Their side effects including ototoxicity, nephrotoxicity, and neurotoxicity, are the major limiting factor in their use. To prevent toxicity and side effects, there is a need for a means of modifying the anti-cancer effects that would ameliorate adverse side effects.

Angiogenesis is an essential step for the growth and maturation of tissues. Tumor cells require angiogenesis to provide nutrients and oxygen for growth, expansion, and metastasis. Several studies have investigated angiogenesis to be well understood as an important therapeutic target for cancer treatment. Platelet-derived growth factor (PDGF) and other growth factors especially vascular endothelial growth factor (VEGF) and Fibroblast growth factor (FGF) are known as powerful angiogenic agents that mediate angiogenesis through different mechanisms [2,3] Inhibiting angiogenesis through using chemotherapeutics that affect angiogenesis as well as using an anti-angiogenic adjuvant therapy are both strategies adopted to treat cancer with decreased side effects. Sulfated non-anticoagulant heparin (S-NACH), a low molecular weight heparin LMWH that has no effect on the systemic coagulation factors but was known to release tissue factor pathway inhibitor protein that inhibits TF/VIIa complex. S-NACH has been shown to inhibit tumor proliferation, metastasis in addition to the anti-angiogenic effect that suggests different mechanisms in tumor suppression [4]. OT-515 is a tempol congener-derived molecule that is an enhanced version of OT-404. OT-404 is known to enhance the efficiency of different chemotherapeutic agents, inhibit cancer cell proliferation, angiogenesis and protect neuronal cells

through inhibiting both OS and NFκB activity [5]. These agents have been suggested as potential anti-neoplastic agents or as adjuvants to cancer chemotherapy. The study's objectives are to evaluate the direct anti-angiogenic activity of chemotherapeutics and the effect of combining S-NACH or OT-515 as adjuvant therapy to enhance the activity and minimize side effects.

In this study, the anti-angiogenic effects of Cisplatin and Doxorubicin were tested directly using the CAM model. Their ability to inhibit both tumor growth and tumor-mediated angiogenesis relied on using a CAM model implanted with the SH-SY5Y neuroblastoma cell line in Geltrex. Enhancement of this activity using OT-515 or S-NACH in combination with lower concentrations of the chemotherapeutic agents was also investigated in this model. The mechanism of action of whether this was due to direct effects on endothelium was elucidated by testing for direct cytotoxicity of the compounds on human umbilical vein endothelial cells (HUVEC).

1. Hypothesis

Anti-tumor activity of Cisplatin and Doxorubicin are mediated, in part, through the inhibition of angiogenesis, an activity enhanced by OT-515 and/ or S-NACH, while allowing dose reduction (and toxicity) of chemotherapeutic agents.

1.1. Aims

1- Establish the anti-cancer and anti-angiogenic activity of Cisplatin and Doxorubicin and test agents in the CAM model.

2- Determine the anti-angiogenic activity of OT-515 and/or S-NACH, alone, and their potential to enhance chemotherapeutic-mediated anti-angiogenic and anti-cancer activity.

3- Determine the anti-angiogenic action of OT-515 and/or S-NACH, together with chemotherapeutic, against endothelial proliferation while reducing the doses of chemotherapeutic required.

1.2. Expected outcomes

1- It is anticipated that both Cisplatin and Doxorubicin will have potent anti-angiogenic action and will inhibit the new vessels formation in CAM compared to VEGF and phosphate buffer controls. In addition, we hypothesize that OT-515 and/or S-NACH will have an additive or synergistic effect on inhibiting angiogenesis when combined with Cisplatin or Doxorubicin.

2- It is anticipated, based on earlier reports, that both OT-515 and S-NACH are likely to have some anti-angiogenic activity of their own while also enhancing Doxorubicin and Cisplatin anti-angiogenic and anti-tumor activity against that induced by neuroblastoma SH-SY5Y implanted CAM model.

3- It is anticipated that the compounds will reduce the need for high doses (concentrations) of the chemotherapeutic agent(s), often dose-limiting, thereby improving anti-tumor activity and safety.

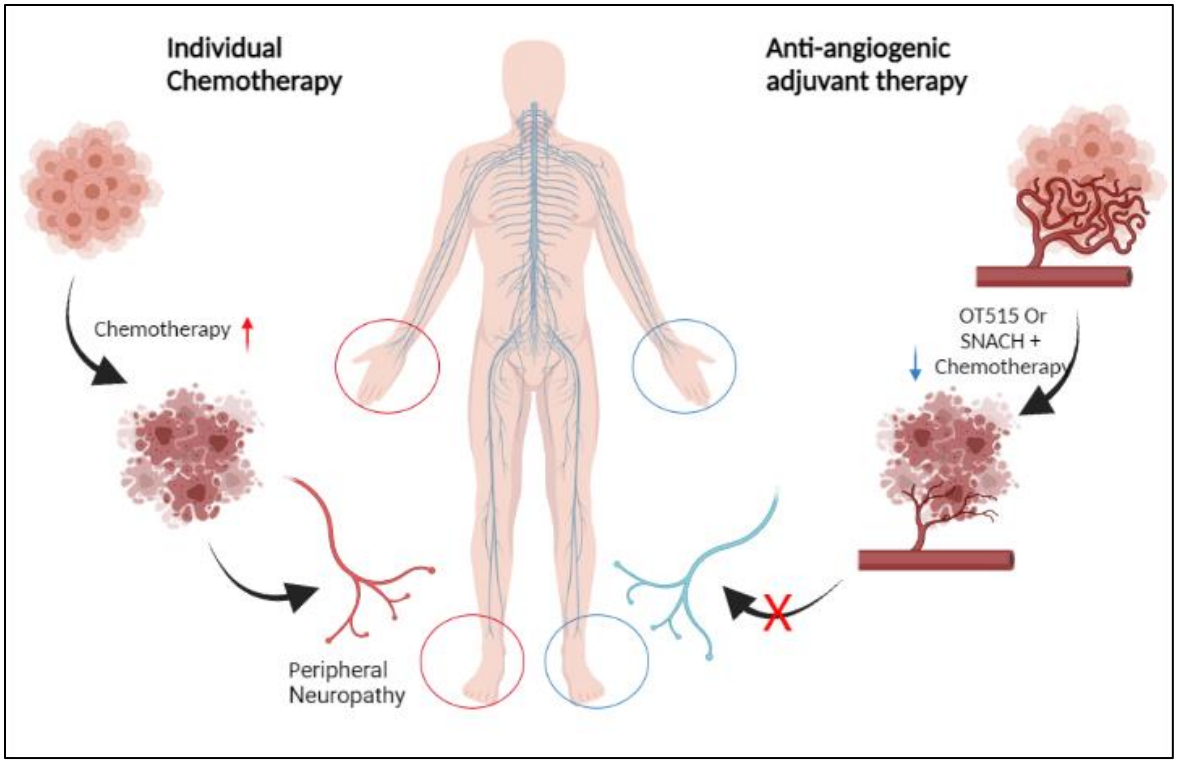

Figure 1. Anti-angiogenic adjuvant therapy as a strategy to lower the used doses of chemotherapy and avoid their side effects, mainly chemotherapy-induced peripheral neuropathy.

Chapter One: Literature Review

1. Angiogenesis in Cancer

Angiogenesis is the process of generating new capillary blood vessels. It is a pivotal mechanism for many physiological and pathological events. Physiologically, angiogenesis is required during embryonic development, wound healing, and menstruation cycle with a high degree of regulatory control. Pathological events are often associated with unregulated angiogenesis, as seen in cancer. Tumor growth and metastasis require angiogenesis to support the tumor site with an extensive network of capillaries for the proper nourishment and waste removal [6–8]. Pro-angiogenic and anti-angiogenic factors secreted by endothelia, tumor cells, and the surrounding stroma act as modulators of this process. Knowing these mediators is crucial for understanding the mechanism of the process and the appropriate methods for controlling the unregulated events. In that context, different proteins have been identified as activators of angiogenesis, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) -α and -β, tumor necrosis factor (TNF)-α, platelet-derived endothelial growth factor (PEGF), epidermal growth factor (EGF), interleukin (IL)-8, other specific growth factors and cytokines [6,9,10]. However, the blood vessel remodeling process is complex, targeting one or more pathways that might activate another mode leading to disease progression. Hence, drugs with anti-angiogenic properties targeting the common downstream signaling pathways would effectively suppress tumor progression, especially if these targets are excessively up-regulated or activated in tumor cells, compared to healthy tissue [10].

1.1. Factors involved in angiogenesis

1.1.1. Vascular Endothelial Growth Factor (VEGF)

VEGF is one of the most potent angiogenic cytokines that play a critical role in vascular formation in normal and neoplastic tissues. It is secreted as dimeric glycoprotein and has different isoforms (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E), all of which bind to specific VEGF receptors (VEGFR). The process of *de novo* formation of new blood vessels is called vasculogenesis sprouting[9,11]. Various genetic and epigenetic modifications in cancer cells enable the cells to produce high levels of VEGF. Tumor hypoxia and oncogenes are among the factors that up-regulate the expression of VEGF in tumor cells and VEGF receptors in tumor endothelial cells. Tumor cells secret VEGF into the surrounding tissue, stimulating endothelial cells on binding to its receptor on the surface of endothelial cells. This binding activates protein signaling pathways that activate the transcription of specific genes needed for new endothelial cell growth and tumor progression[12] (Fig. 2). In addition, the VEGF family has a highly conserved structure among species with high specificity for the vascular endothelium, which makes it a suitable factor to be used in various *in vitro* and *in vivo* models to stimulate angiogenesis[13].

bFGF is a member of the FGF family that binds to receptor tyrosine kinases leading to activation of the tyrosine kinase signal transduction pathways. The downstream signaling cascade leads to the differentiation of endothelial cells through a mechanism not fully understood. bFGF plays an essential role in tumor growth and angiogenesis and is crucial to converting hormone-dependent-cancers to hormone-independent entities[6].

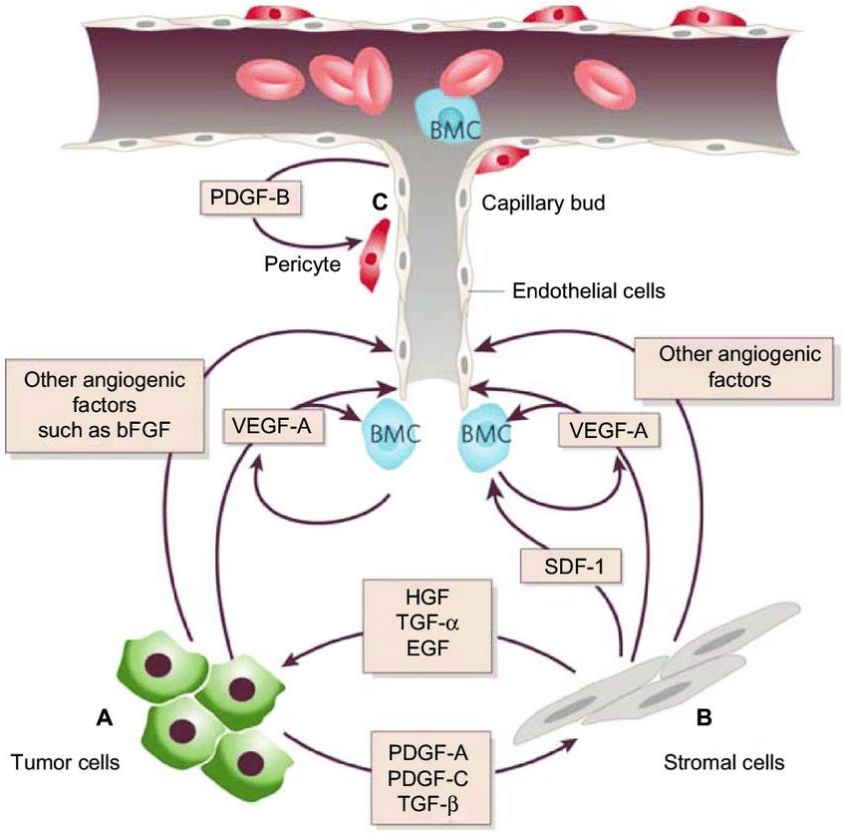

Figure 2: A schematic illustration of the factors involved in tumor angiogenesis[14]

1.1.2. Cytokines and chemokines in angiogenesis

It is now fully accepted that tumor expansion after tumorigenesis can be hampered, leading to tumor dormancy. The expansion of the tumor occurs following the angiogenesis transition in which the tumor acquires the ability to induce angiogenesis. The process is complex and tightly controlled by various pro-angiogenic cytokines and inhibitory factors, as previously mentioned. In addition to the VEGF family, non-specific and specific growth factors, interleukins also play a role in the process. IL-8

was found to be a macrophage-derived pro-angiogenic factor that helps induce the proliferation of cells and consequently induction of angiogenesis [15].

Indirect-acting factors also play a role; they affect angiogenesis by releasing direct-acting factors. For example, TNF-α is among the most studied factors as it increases VEGF expression, as well as that of its receptors, and IL-8 and bFGF in endothelial cells[7]. IL-1 is another indirect activator as it induces the expression of VEGF. However, it is not the only mechanism as IL-1β is also known to induce the expression of Angiopoietin-1. While IL-6 expression is associated with angiogenesis that is required for the development of ovarian follicles, in cancer, IL-6 and VEGF levels are correlated, indicating that IL-6 likely regulates VEGF expression as an indirect modulation effect on angiogenesis[15,16] (Fig. 2).

It should also be noted that secretion of these cytokines can alter the expression of adhesion molecules and surface markers on endothelial cells. Adhesion receptor integrin $\alpha_v\beta_3$ is among the selectively expressed markers. The interaction between integrin $\alpha_v\beta_3$ and its receptor activates specific signals that increase the survival of new endothelial cells[6].

1.1.3. NFκB and angiogenesis

Nuclear factor kappa B (NFκB) is a group of proteins that are structurally related and representing a family of inducible transcription factors that regulate a large array of genes involved in immune and inflammatory response processes. The family is composed of five members, including NFκB1 (p50), NFκB2 (p52), RelA (p65), RelB,

and c-Rel, that form various combinations of dimers. The NFκB proteins are normally sequestered in the cytoplasm due to the association with inhibitor kappa B (IκB) proteins [17,18]. When cells are stimulated, IκB is phosphorylated and degraded by IκB-kinase complex(IκK), followed by NFκB translocation to the nucleus and binding to specific promoter sequences where it regulates gene expression. The NFκB target genes are involved in different functions, including survival, proliferation, and inflammation (Figure 3)[19].

The role of NFκB signaling in angiogenesis has been investigated and connected to multiple aspects of angiogenesis. In some instances, the activation of NFκB appears to inhibit angiogenesis, but the mechanism is not fully understood. However, its regulation of extracellular matrix (ECM) might play a role in angiogenesis early events as vascular basement membrane degradation and ECM remodeling is an initial even in angiogenesis[20].

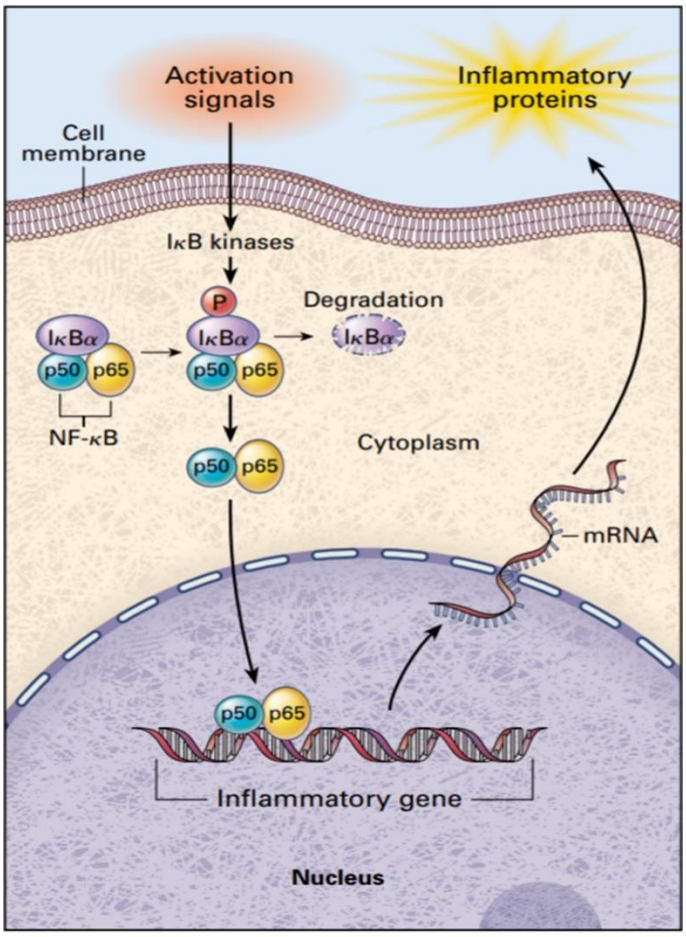

Figure 3. NFκB pathway. The stimulation of cell surface receptors activates the IκK complex and dissociate NFκB from IκB to translocate to the nucleus and turn on target genes[19].

1.2. Chemotherapeutics and angiogenesis

Conventional chemotherapeutic agents such as platinum compounds, anthracyclins, and taxanes are well established as effective agents against breast, colorectal, ovarian, lung, and testicular cancers. However, they are known to act on all dividing cells, damaging normal tissues and manifesting toxic effects[5,21]. Cisplatin is a platinum compound, a cell-cycle non-specific anti-tumor drug. It is one of the most effective chemotherapeutic drugs that can be used against various types of cancers and improves overall survival. Its anti-

cancer action is mediated through inducing DNA cross-linking, DNA damage, and cell apoptosis. The lack of cisplatin cell-specificity leads to some significant adverse effects such as nephrotoxicity, neurotoxicity, and ototoxicity [21,22]. Doxorubicin is another traditional chemotherapeutic agent, an anthracycline drug that was developed to be used in lung, gastric, ovarian, thyroid, breast, sarcoma, and pediatric cancers. Doxorubicin toxicity is the major limiting factor for its use, particularly its cardiotoxicity[23]. To prevent toxicity and side effects, there is a need for anti-cancer agents that target only tumor cells and can be used as combination therapy or a means of enhancing anti-cancer effects while ameliorating adverse side effects.

1.3. Chemotherapeutics in Neuroblastoma

Over the years, studies suggest that Cisplatin and Doxorubicin can target several cancers, including neuroblastoma. Neuroblastoma is the most frequent extracranial solid tumor in children. It accounts for more than 7% of malignancies in children with less than 40% survival[24]. Both Cisplatin and Doxorubicin were examined as commonly used in the protocols of the treatment of neuroblastoma. They were reported to induce vessel formation in different angiogenesis models when used in low doses, compared to an inhibited angiogenic effect at higher doses. This angiogenesis activation effect was also confirmed by examining samples derived from neuroblastoma patients[25]. In contrast, metronomic doses of Cisplatin were reported to affect angiogenesis through inhibiting blood vessel endothelial cells [22,26]. However, the mechanisms of action of Cisplatin and Doxorubicin

and whether their effect on angiogenesis is direct through endothelial toxicity or pharmacological through inhibition of growth factor production is unclear.

Furthermore, human-derived neuroblastoma cells are used in the *in vitro* neurotoxicity assays[27]. Neuroblastoma cell lines were also reported to be more sensitive to chemotherapy than normal endothelial cells, which require higher doses to reach IC50[25]. Taken together, more investigations of the effect of chemotherapeutics on neuroblastoma tumors and their effects on angiogenesis are required. The present study also elected to use neuroblastoma cells because of the neuropathy often associated with chemotherapy.

1.4. OT-404/ OT-515 and Angiogenesis

OT-404 is a small molecule precursor to tempol, a piperidine nitroxide that is known to have anti-cancer functions. It was reported to inhibit the proliferation of tumor cells, as well as angiogenesis and enhance the sensitivity of tumors to anti-cancer agents. A proliferation test on different cell lines showed that OT-404 has anti-proliferative activity against various cell lines. The molecule inhibited the proliferation of both chemo-sensitive and -resistant cells and enhanced the sensitivity of cancer cells to different chemotherapeutics, including Doxorubicin. Its effect on angiogenesis was studied using chorioallantoic membrane (CAM) assay stimulated with VEGF, and it showed inhibition of the pro-angiogenic response to VEGF[5].

A series of studies at the Pharmaceutical Research Institute (PRI) investigated the effect of OT-404 on NFκB and oxidative stress. The western blot analysis showed OT-404 increase cytoplasmic p65/nuclear p65 ratio with an increase in inhibitor IκB level, indicating that

OT-404 restrict NFκB to the cytoplasm and inhibits its activation translocation to the nucleus (Fig. 4A). OT-404 has also been shown to reduce the fluorescence resulted from stimulating RGC5 cells with H_2O_2 in the presence of the H_2DCFDA fluorogen that fluoresces in the presence of oxidation. This indicates that OT-404 does indeed modulate oxidative stress (Fig. 4B)[28]. In addition, OT-404 combined with Cisplatin attenuates acute hyperplasia (increased sensitivity to pain) caused by Cisplatin in rats. The study also provided evidence that OT-404 can give protection against cisplatin-induced peripheral neuropathy[28].

Although oxidative stress caused by reactive oxygen species (ROS) was believed to limit cancer initiation and progression by killing cancer cells, ROS also promote cancer initiation by promoting different aspects of tumor development and progression[29]. Oxidative stress can activate signaling pathways, including NFκB and HIF-1 transcription factors leading to the expression of growth factors, inflammatory cytokines, and chemokines that promote mutagenesis, inflammation, and cell survival. Moreover, ROS production stabilizes HIF-1α protein that induces angiogenic factors and increases VEGF level, ultimately leading to angiogenesis. Therefore, targeting oxidative stress offers an opportunity for cancer prevention and treatment [30–32].

OT-515, used in the present work, is another tempol derivative that possesses the same properties as OT-404 with higher potency, as suggested by the preliminary studies. However, investigations of the anti-tumor and anti-angiogenic effects of OT-515 are required as a potential anti-cancer adjuvant compound and is the focus of this thesis.

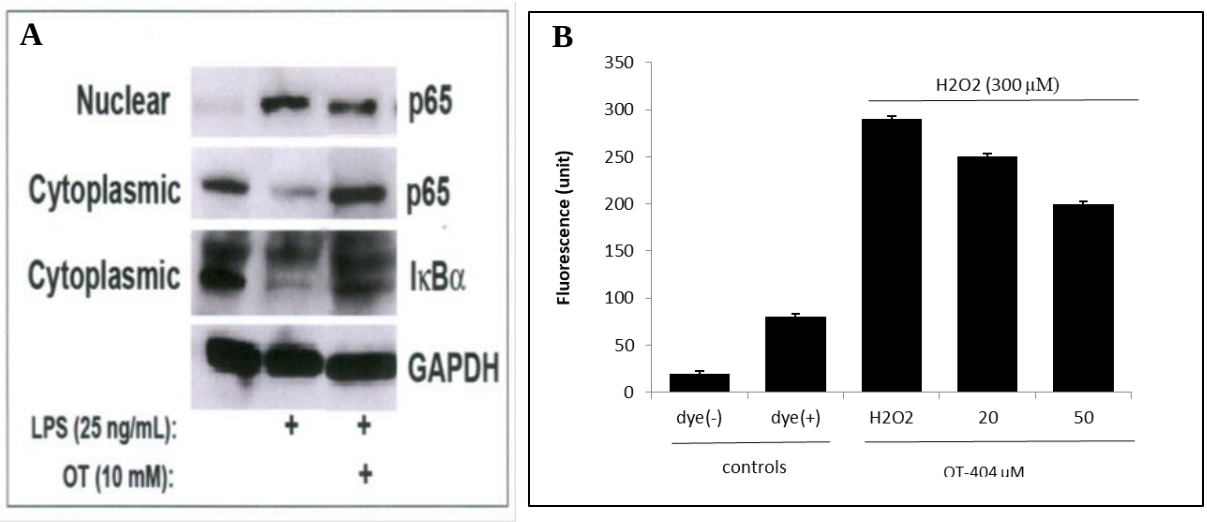

Figure 4. Prelimenary results of OT-404

A) Western blot analysis show that OT-404 retained higher level of P65 and IκB in the cytoplasm compared to their nuclear level indicating the inhibition of NFκB, B) OT-404 attenuated fluorescence intensity indicating an inhibition activity of oxidative stress in response to H2O2 (Provided by S. A. Mousa, PRI, NY).

1.5. S-NACH and angiogenesis

Heparin and its low molecular weight heparin (LMWH) derivatives -apart from their anti-coagulant effects- are increasingly shown to possess anti-inflammatory and immune-modulatory properties [33,34]. Different potential mechanisms of this anti-inflammatory effect have been discussed, such as the binding of heparin to various cytokines and chemokines and neutralizing cytokines at the inflammation site. Numerous studies demonstrate that heparin administration decreases cytokines such as IL-6, IL-8, and TNF-α. Another possible mechanism is inhibiting NFκB by preventing its translocation to the nucleus. It was also shown to induce apoptosis by modulating the TNF-α and NF-κB [34,35]. LMWH also inhibits tumor proliferation and metastasis by inhibiting Tissue Factor (TF)/VII

pathway, which is known to promote tumor metastasis and angiogenesis[36–38]. However, the anti-coagulant property of heparin and its LMWH derivatives is a major obstacle that hinders their use due to the risk of bleeding [33,39]. The concept of formulating derivatives from LMWH that are devoid of anti-coagulant properties has emerged to prompt its anti-inflammatory properties [33,39,40].

Sulfated non-anticoagulant LMWH (S-NACH) is an oxidized sulfated ultra-LMWH derivative with all the properties of LMWH with low to no systemic anti-coagulant activity. It can be administered in high doses without bleeding complications[41]. S-NACH releases tissue factor pathway inhibitor (TFPI) protein that inhibits TF/VII. Recently, S-NACH showed a protective effect on endothelial cells against thrombo-inflammation through this TFPI pathway, together with nitric oxide and other protective factors[42,43]. It also evidenced potent anti-proliferative and anti-angiogenic effects against tumor cells with minimal effects on hemostasis. S-NACH also has anti-metastatic activity, suggesting its use as a potent adjuvant therapy in cancer treatment [4,36,44]. These multimodal mechanisms of S-NACH against cancer make it another potential anti-cancer compound that needs to be studied as an adjuvant treatment in combination with chemotherapy.

2. Chemotherapy-Induced Peripheral Neuropathy (CIPN)

Chemotherapy-induced peripheral neuropathy (CIPN) is one of the common side effects that develop with the use of various traditional chemotherapeutic agents such as taxanes, platinum compounds, and vinca alkaloids. Studies reported that the incidence rates of CIPN range between 19% to more than 85% according to the type of cancer, the used chemotherapeutic, duration of therapy, and whether the patient receives an individual chemotherapeutic agent or a combination of two compounds[45,46]. CIPN is considered a major dose-limiting factor for the use of chemotherapeutics as patients must stop the treatment course or lower the chemotherapeutic doses used to avoid the pain. This eventually affects the clinical outcomes of cancer treatment and is considered a huge burden in cancer therapy.

2.1. Prognosis of CIPN

The symptoms of CIPN differ depending on the type of neurons affected (automatic, motor, or sensory), and they may be reversible or irreversible[47]. Usually, it occurs with long nerves and with a symmetric distribution. The exact mechanism of the development of CIPN is not fully understood but is generally believed to involve chemotherapeutic agents' damage to the microtubules, which affects the peripheral nerves. They also act directly on DNA or disrupt mitochondria, leading to an interference of the role of microtubules in axonal transport[21]. Although no one mechanism is known to be responsible for initiating CIPN, several factors are involved. Some studies suggested that alterations in the sodium-channel action potential leading to a prolonged open state of the channel might be a factor as it

results in hyper-excitability of sensory neurons with attendant calcium influx. The pathway involving calcium overload and downstream activation of apoptosis and oxidative stress is also believed to participate in toxic neuropathy. Activation of NFκB also has a role in the pathogenesis, and hence, interventions that act on decreasing NFκB or inhibiting oxidative stress might have a protective effect against CIPN (Fig. 5)[21,48,49].

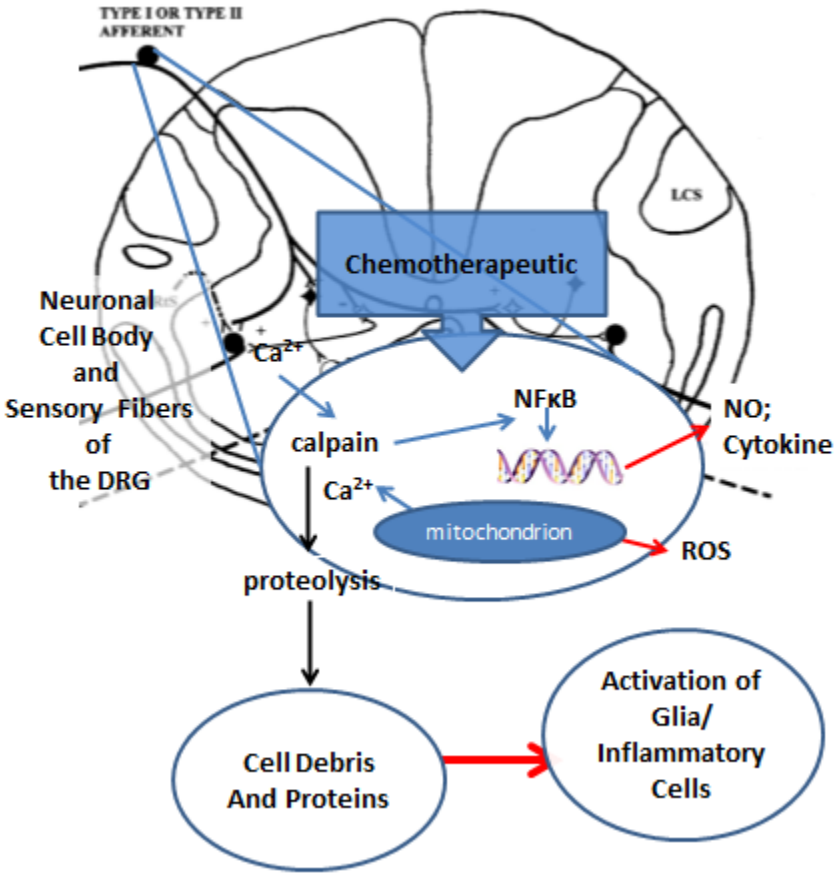

Figure 5: A schematic illustration of CIPN development[21]

3. Chorioallantoic Membrane (CAM) Model of Angiogenesis

Many *in vivo* and *in vitro* assays have been developed to study angiogenesis as a hallmark of cancer progression[50]. The chick chorioallantoic membrane assay (CAM) is one of the oldest assays that were commonly accepted and used to study angiogenesis *in situ*. The test was developed by Folkman et al. (1975), exploiting the fact that blood vessels expand and grow on the chorioallantoic membrane of fertilized eggs in the process of chick embryo development [51]. The chorioallantois is formed in the chick embryo between days 4-5 after fertilization. A network of blood vessels is formed gradually to sustain the development of organs until the CAM is fully developed by day 10-12. Therefore, the CAM displays robust angiogenic activity during this period and can respond to any pro-angiogenic stimuli by establishing new blood vessels or decrease its formation in response to anti-angiogenic factors[52]. All these features render the CAM assay a well-established test for investigating and validating pro-and anti-angiogenic treatments. CAM assay is also an easy and inexpensive method that is suitable for large-scale screening of angiogenesis. The technique depends on opening a small window in a fertilized egg to access the avascular area in the CAM. Controls and treatments are applied using a plastic or filter disc to allow the release of the compound into CAM. The effect of compounds on vascularization is determined through imaging the CAM membrane under a microscope to count the difference in blood vessel branches [53].

In addition, the fact that the chick embryo is not immunocompetent encouraged researchers to graft different types of cells into the CAM to establish a model for tumor angiogenesis, allowing the investigation of anti-tumor and anti-angiogenesis properties of compounds.

Tumor cells are grafted into the CAM between days 8-10, incorporated into a gel matrix, left to grow for 5-7 days until forming a developed tumor. Tumors are then harvested for the examination of cancer development, anti-tumor, and anti-angiogenesis effects of compounds[54].

Recently, various modifications have been introduced into these classic CAM assays revolutionizing their use. Recent studies have used the CAM as a model for testing the anti-metastatic capacity of cancer cells and the study of human bone regeneration[55,56].

Chapter Two: Materials and Methods

2.1. Cells and Reagents

The following cell lines, media, and reagents were used in these studies. Human Umbilical Vein Endothelial Cells HUVEC (Gibco, USA), Human Neuroblastoma SH-SY5Y (gifted by Zewail University for Science and Technology), mouse endothelial cells C 166 (ATCC, USA), Human Large Vessel Endothelial Cell Basal Medium M 200 (Gibco, USA), Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Belgium), Roswell Park Memorial Institute culture medium (RPMI 1640, Lonza, Belgium), Large Vessel Endothelial Supplement (LVES, 50X, Gibco, USA), Fetal Bovine Serum (FBS, Life Science Production, UK), Penicillin-Streptomycin (pen-strep, Lonza, Germany), Phosphate Buffer Saline PBS (Lonza, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent, Serva, Germany), dimethyl sulfoxide (DMSO, ≥99.9, molecular weight=78.13 g/mol, Serva), Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Invitrogen, USA), Vascular Endothelial Growth Factor From Mouse (Sigma Aldrich, Germany).

2.2. Detecting the possible mechanisms of action in-vitro

2.2.1. *In vitro* cell culture

All cell lines were cultured as monolayers and incubated at 37° and 5% CO_2, in a humidified atmosphere. HUVECs were cultured in M200 supplemented with 2% LVES and 0.5% Pen-Strip. C 166 and MCF7 cells were cultured in DMEM supplemented with 10% FBS and 1% Pen-Strip, while SH-SY5Y were cultured in RPMI supplemented with 10% FBS and 1% Pen-Strip. Only early passages of primary cells were used, HUVEC passages 2-5 and

C 166 passages 3-9. All cells were cultured until reaching 70% confluency then passaged for further use in cell proliferation and tumor grafting assays.

2.2.2. Cells Proliferation

To determine the cytotoxicity of compounds and their effect on cell proliferation, MTT colorimetric assay was performed as described previously[57].

HUVEC normal endothelial cells. Cells were harvested and seeded in 96-well plates, incubated for 24 hours at 37° C (5% CO2). The culture media was removed at 70-80% confluence, and cells were washed with PBS. Different treatments were added, and the mixture was incubated for 72 hours. Then 100µL of MTT (1mg/ml) was added to the cells and incubated for 4 h. DMSO was then added to solubilize the formed formazan dye. The produced violet color was measured as the absorbance of each well at 540 nm using a microplate spectrophotometer system (SPECTROstar Nano, BMG LABTECH, Germany).

2.3. Detecting anti-angiogenic properties

2.3.1. Chick chorioallantoic membrane assay

CAM model was used to elucidate the anti-angiogenic effect of the compounds using PBS as negative control and comparing them to VEGF as a positive control (Fig. 6). Ten-day-old fertilized chick embryos were incubated at 37 °C. Neovascularization was examined by CAM assay as previously described [58,59]. Using an egg candler, an avascular area of the egg was marked as the air sac. A small hole in the shell at the air sac was made using a hypodermic needle, an avascular portion of the embryonic membrane was identified, and a second hole was made on the long side of the egg over it. Mild suction was applied to the first hole to move the air sac and allow the CAM to drop. A 1 cm^2 window was sectioned

in the membrane using a drill to expose the vascular zone. A #1 sterilized filter paper disk (Millipore) was then loaded with the experimental treatments or vehicle controls and then applied to adhere to the vascular zone. Cisplatin, Doxorubicin, OT-515, and S-NACH were used and compared in the presence of standard pro-angiogenic stimuli VEGF (2µg/ml).

2.3.2. *Microscopic analysis of CAM sections*

After three days of incubation, the tissue beneath each filter disk in the CAM was resected, washed with PBS, and placed in a 35-mm petri dish for microscopic examination. Digital images of the CAM sections were taken using an Optika stereomicroscope at 10X magnification and analyzed using ImageJ software. The number of vessel branch points contained in a circular region equal to the area of each filter disk were counted.

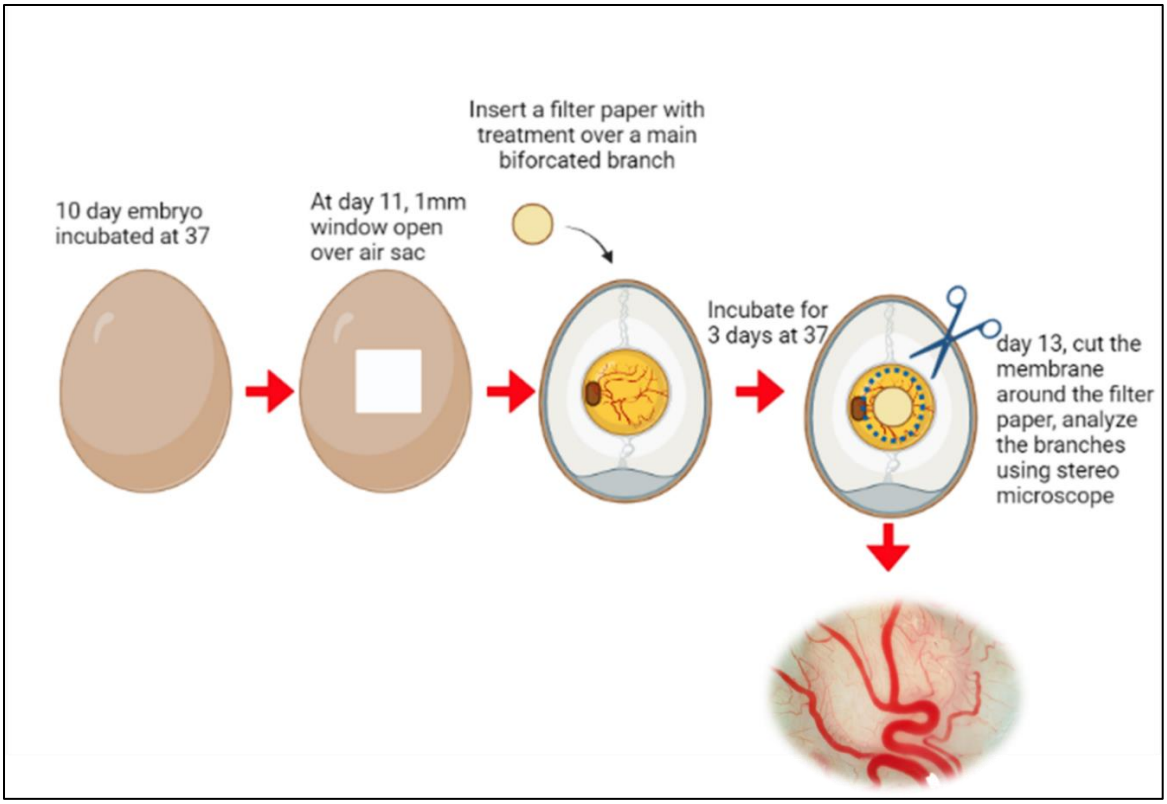

Figure 6. Chick Chorioallantoic Membrane Assay (CAM)

2.3.3. *Tumor grafting into CAM*

Neuroblastoma cells SH-SY5Y were grafted into CAM mixed with Geltrex as a protein matrix to test the effect of combining S-NACH or OT-515 with Cisplatin or Doxorubicin on tumor growth. The CAM model was prepared as mentioned above and after creating the window, tumor cells were grafted as described in previous studies[36] (Figure 7).

SH-SY5Y human neuroblastoma cells were introduced as tumor-forming cells. First, A total of 2 million cells in 20µl medium and 20µl of Geltrex were mixed and added to 10µl of treatment or PBS control. The suspension was then added to the branch point of the CAM surrounded with a 1mm silicone ring. To produce more robust tumors, a minor modification was done to the procedure. The number of cells was increased to 10 million

cells per CAM, and 30µl of Geltrex were used instead of 20. On day 7 of incubation, the tumors were harvested after treatment and examined for their weight and hemoglobin (Hb) levels. Hb level was determined as a measure of cell vascularity.

2.3.4. Determination of tumor Hb levels

The harvested tumor sections were homogenized, then centrifuged to collect the supernatant. 50µl of supernatant was then mixed with 50µl Drabkin's reagent and left at room temperature for 15-30 minutes, then transferred to a 96-well plate to measure the absorbance at 540 nm using a microplate spectrophotometer system (SPECTROstar Nano, BMG LABTECH, Germany).

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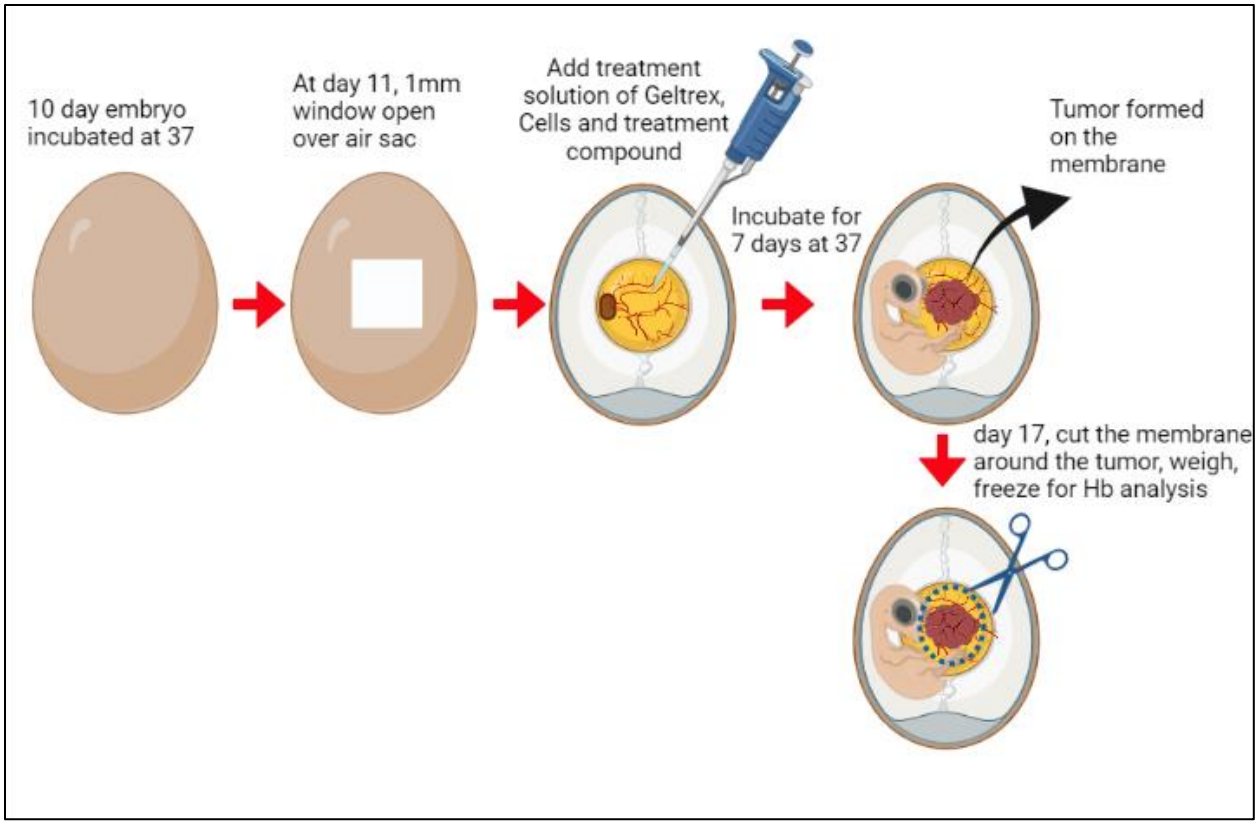


Figure 7. Tumor grafting into CAM

2.4. Statistical Analysis

Data is represented as mean ± SEM and plotted using GraphPad Prism 8.0.1 software used for statistical analysis and plot comparisons. CAM experiments were performed in triplicates with 5-8 embryos per treatment group. Data is evaluated in terms of the average number of blood vessel branch points per treatment group ± SEM while for the CAM grafts, mean tumor weight per treatment group ± SEM. One way analysis of variance ANOVA was used to compare each treatment and control, followed by a post-hoc test to evaluate the statistical significance between the untreated and treated groups. Statistical significance was defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Chapter Three: Results

3.1. Effect of combining OT-515 and S-NACH with chemotherapy on the proliferation of human neuroblastoma cells

Doxorubicin was cultured at different concentrations with human neuroblastoma cell line SH-SY5Y for 24, 48, and 72 h. Doxorubicin at a 1µg/ml concentration was particularly potent, mainly when incubated for 72 h. OT-515 and S-NACH showed an inhibitory effect on the proliferation of cells after 72 h incubation individually and in combination with Doxorubicin and Cisplatin (Supp Fig. 1). We have then combined OT-515 and S-NACH with different concentrations of Cisplatin or Doxorubicin and tested their effect on the proliferation of SH-SY5Y. Results shown in Table. 1 indicate that combining OT-515 or SNACH with Doxorubicin had a more significant anti-proliferative effect than individual Doxorubicin even when used at lower concentrations. Cisplatin combinations had the same results as shown in Table. 2. Moreover, the triple combinations of OT-515, S-NACH, and Doxorubicin or Cisplatin had more significant inhibition of proliferation compared to double combinations, particularly in the case of OT-515 and Cisplatin (Fig. 8).

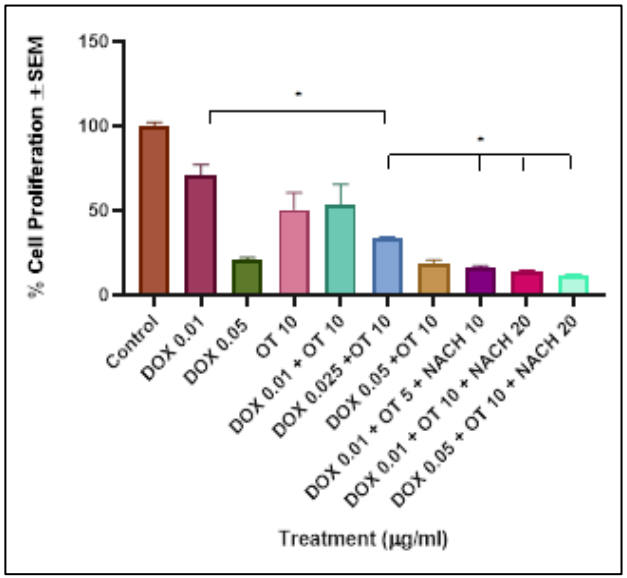
Table 1: The significance of combining OT-515 and S-NACH to Doxorubicin on treating SH-SY5Y human neuroblastoma cells.

Individual Treatment (µg/ml)	Avr. Proliferation %	P value	Double Combination (µg/ml)	Avr. Proliferation %	P value	Triple Combination (µg/ml)	Avr. Proliferation %	P value
DOX 0.005	81.02808175	0.5372	**DOX 0.0025 + NACH 20**	67.56134	0.0878			
			DOX 0.005 + NACH 20	73.34932	0.4603			
DOX 0.01	70.72780191	0.0035	**DOX 0.01 + OT 10**	53.36801	<0.0001	**DOX 0.01 + OT 5 + NACH 10**	16.87323	<0.0001
			DOX 0.01 + NACH 10	38.45634	<0.0001			
			DOX 0.01 + NACH 20	62.23162	<0.0001	**DOX 0.01 + OT 10 + NACH 20**	14.04943	<0.0001
			DOX 0.025 +OT 10	34.27018	<0.0001	**DOX 0.017 +OT6.6+NACH 6.6**	40.74346	<0.0001
			DOX 0.025 + NACH 10	25.72033	<0.0001			
DOX 0.05	21.1749927	<0.0001	**DOX 0.05 +OT 10**	18.4255	<0.0001	**DOX 0.03 +OT 6.6+NACH 6.6**	28.289	<0.0001
			DOX 0.05 +NACH 10	63.02427	<0.0001			
			DOX 0.05 + NACH 20	17.58331	<0.0001	**DOX 0.05 + OT 10 + NACH 20**	12.21643	<0.0001

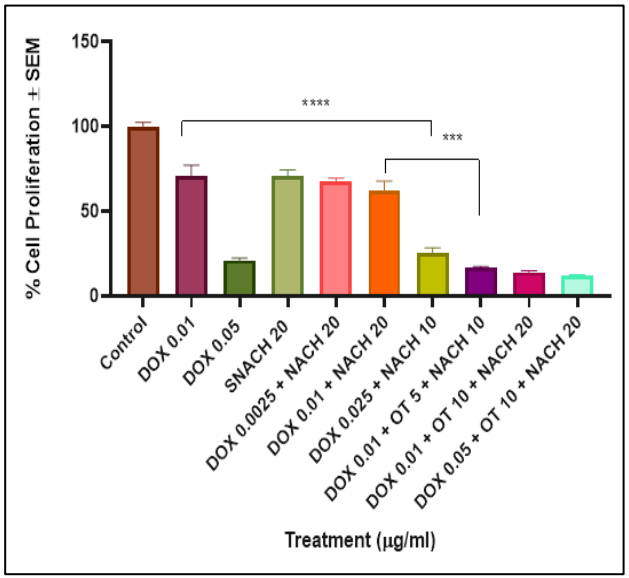
Table 2: The significance of combining OT-515 and S-NACH to Cisplatin on treating SH-SY5Y human neuroblastoma cells.

Individual Treatment (µg/ml)	Avr. Proliferation %	P value	Double Combination (µg/ml)	Avr. Proliferation %	P value	Triple Combination (µg/ml)	Avr. Proliferation %	P value
CIS 5	81.67211	0.6228	CIS 2.5 + OT 10	88.62015	0.9995	CIS 1.6 +OT6.6 +NACH 6.6	111.5037	>0.9999
			CIS 2.5 + NACH 10	115.4339	0.9995	CIS 3.3 +OT 6.6+ NACH 6.6	105.6414	>0.9999
			CIS 2.5 + NACH 20	87.20412	>0.9999			
			CIS 5+OT 10	56.98033	<0.0001	CIS 5 + OT 5 + NACH 10	24.32081	<0.0001
			CIS 5 +NACH 10	79.53361	0.3485			
			CIS 5 + NACH 20	70.8145	0.0037	CIS 5 + OT 10 + NACH 20	17.509	<0.0001
CIS 10	76.28871	0.0918	CIS 10 + OT 10	50.61439	<0.0001	CIS 10 + OT 10 + NACH 20	13.45494	<0.0001
			CIS 10 + NACH 10	63.33389	0.016			
			CIS 10 + NACH 20	66.49547	0.0004			
CIS 15	64.37424	0.0251						

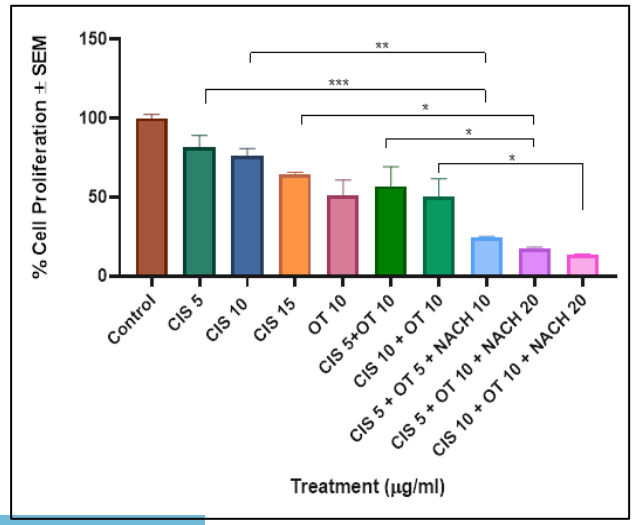
Doxorubicin- OT515



Doxorubicin- SNACH



Cisplatin - OT515



Cisplatin - SNACH

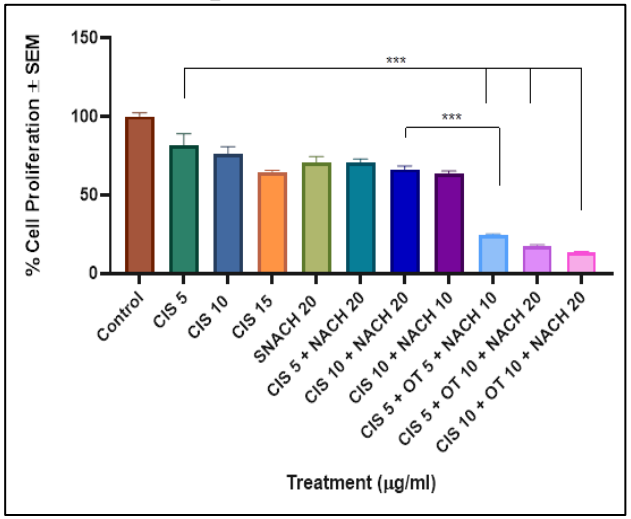


Figure 8. Testing the anti-proliferative effect of OT-515 and S-NACH on SH-SY5Y neuroblastoma cells and their enhancing effect when combined to chemotherapy.

A) Combining OT-515 to Doxorubicin at different concentrations enhanced the anti-proliferation of cancer cells. B) S-NACH at different concentrations with Doxorubicin. C) OT-515 combinations with Cisplatin. D) S-NACH combinations with Cisplatin. Data represent mean ± SEM, n = 6-18, One-way ANOVA; **p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

3.2. Anti-angiogenic effects of combining OT-515 and S-NACH with chemotherapy

To investigate the effect of OT-515 and S-NACH and their combinations with chemotherapy on angiogenesis, we used the CAM assay. We tested the effect of compounds on normative angiogenesis by applying compounds directly to the CAM membrane using PBS as a negative control. The pictures showed a slight inhibition of the formation of blood vessels after applying the filter discs containing the treatments (Fig. 9). However, counting the branch numbers indicated no significant difference between the treatments and the control (Fig. 10), which suggested that treatments do not affect normative angiogenesis in the absence of a pro-angiogenic factor or stimuli. Moreover, when comparing the effects of Doxorubicin and Cisplatin, Cisplatin showed to have a more potent anti-angiogenic effect on normative angiogenesis as only 1µg/ml of Cisplatin caused inhibition of approximately 50%. In comparison, we used up to 20µg/ml of Doxorubicin to achieve the same result.

To induce angiogenesis on CAM, different concentrations of VEGF were applied (Supp Fig. 2). The results indicated that using a filter disc with VEGF (2µg/ml) on CAM induced blood vessel formation 2.3 folds (p-value < 0.0001) compared with PBS negative control. The addition of OT-515 and S-NACH inhibited the VEGF-stimulated angiogenic effect individually and when combined with Doxorubicin or Cisplatin (Fig. 11). Counting the blood vessel branches indicated that both Cisplatin and Doxorubicin also have an anti-angiogenic effect, which significantly inhibited the pro-angiogenic response caused by VEGF (p-value < 0.0001). Double and triple combinations of OT-515, S-NACH, and Doxorubicin or Cisplatin significantly inhibited the pro-angiogenic response as well (Fig. 12). Finally, although we used half the doses of chemotherapy in the combinations, the inhibitory effect was consistent, indicating that both OT-515 and S-NACH

enhance the anti-angiogenic effect of chemotherapy, opening a room for reducing the used therapeutic doses.

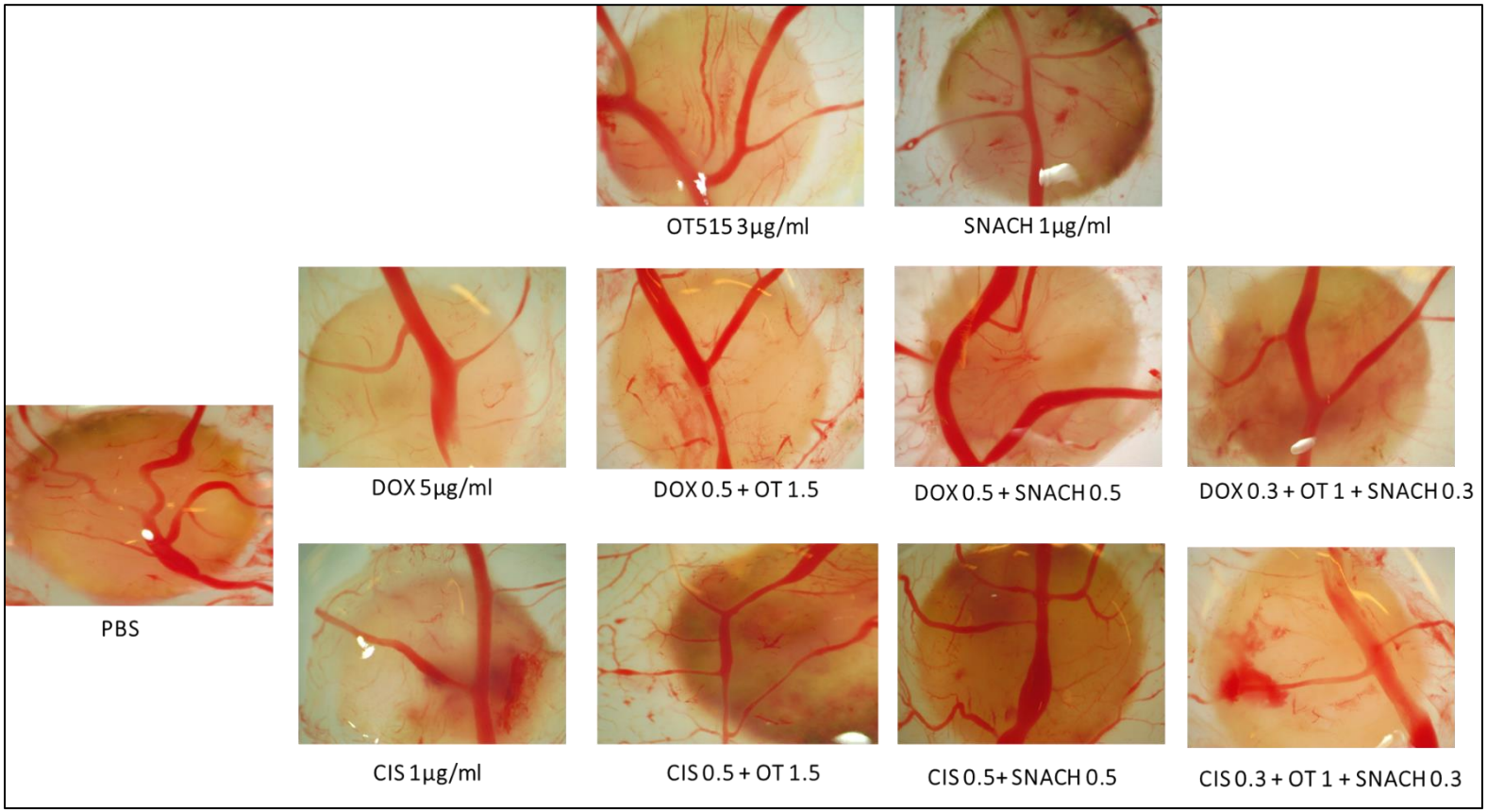


Figure 9. Effect of OT-515 and SNACH combined to chemotherapy on normative angiogenesis using check CAM assay.

Pictures were taken under and Optika stereomicroscope at 10x magnification.

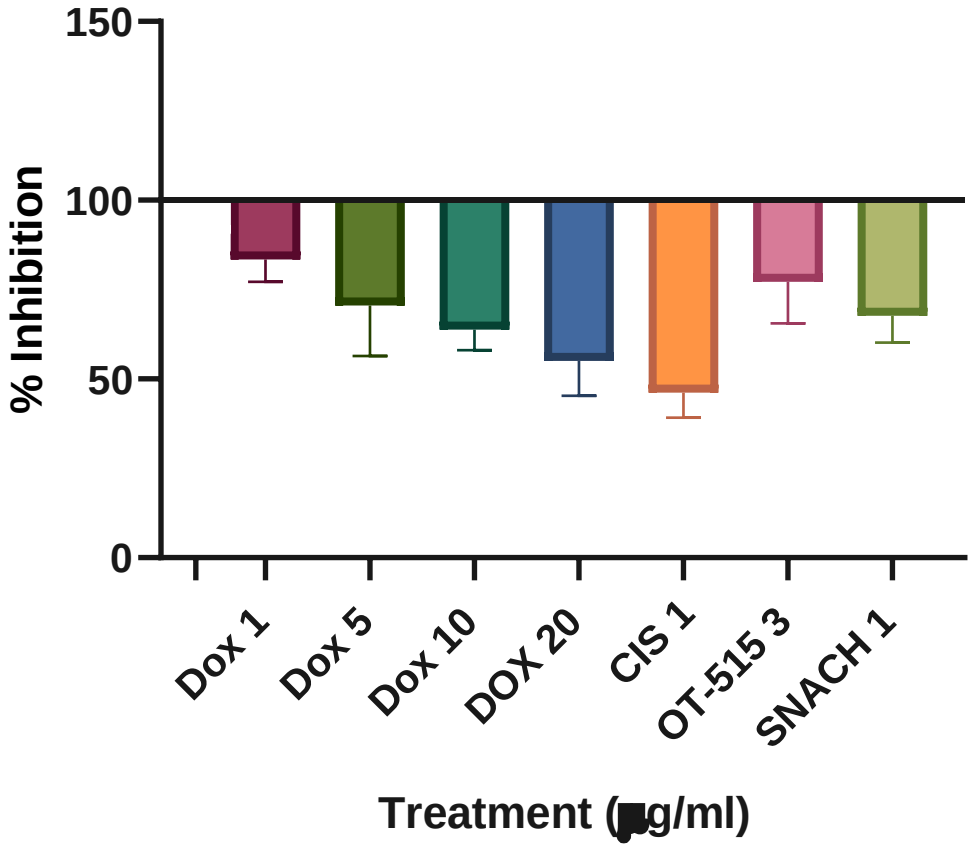


Figure 10. Normative angiogenesis on chick CAM assay and its inhibition by OT-515 and SNACH combined to chemotherapy.

Treatments were added, and vessel branch number was counted using Imagej. Data normalized to PBS negative control and presented as % inhibition of PBS (100%). n= 5 CAMs per group.

A

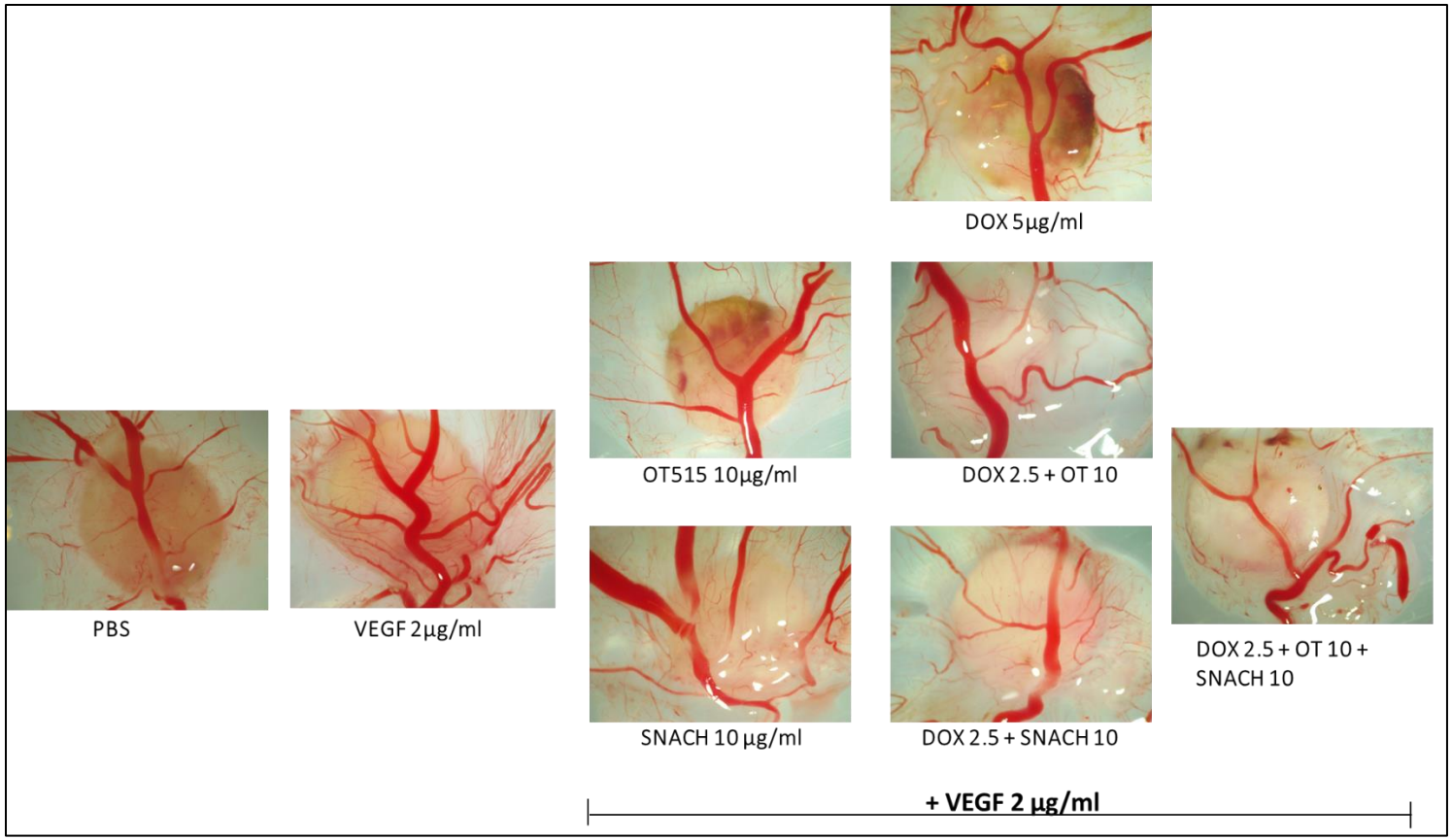


Figure 11A and B. Effect of OT-515 and SNACH combined with doxorubicin on VEGF-induced angiogenesis using check CAM assay.

Pictures were taken under and Optika stereomicroscope at 10x magnification. PBS is negative control, all other groups were first treated with 2µg/ml VEGF to induce angiogenesis A) OT-515 and SNACH were combined to Doxorubicin, B) combinations with Cisplatin. n= 3-8 CAMs per group.

B

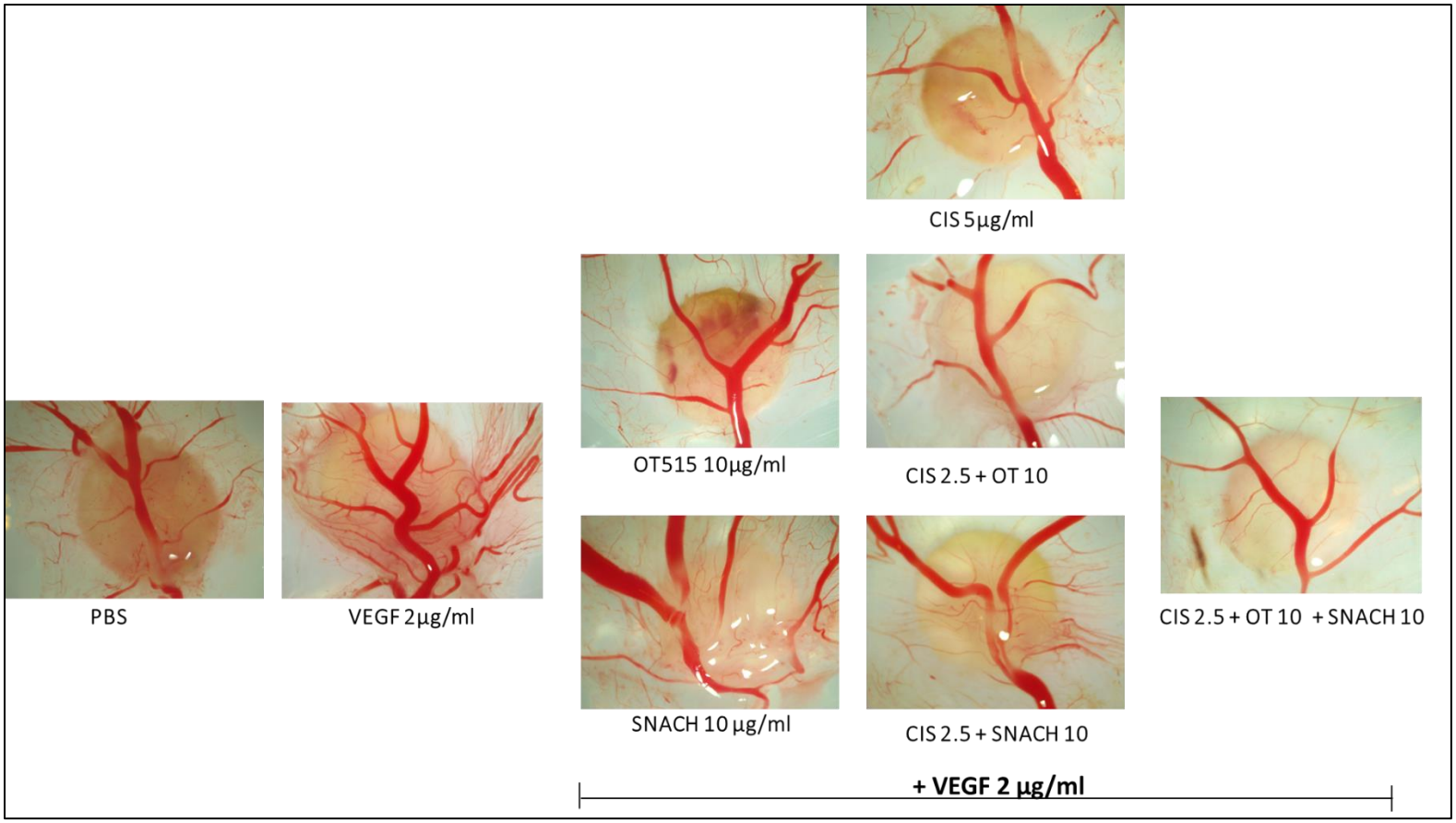


Figure 12. Effect of OT-515 and SNACH combined to chemotherapy on VEGF-induced angiogenesis using check CAM assay.

Pictures were taken under and Optika stereomicroscope at 10x magnification. PBS is negative control, all other groups were first treated with 2µg/ml VEGF to induce angiogenesis A) OT-515 and SNACH were combined to Doxorubicin, B) combinations with Cisplatin. n= 3-8 CAMs per group.

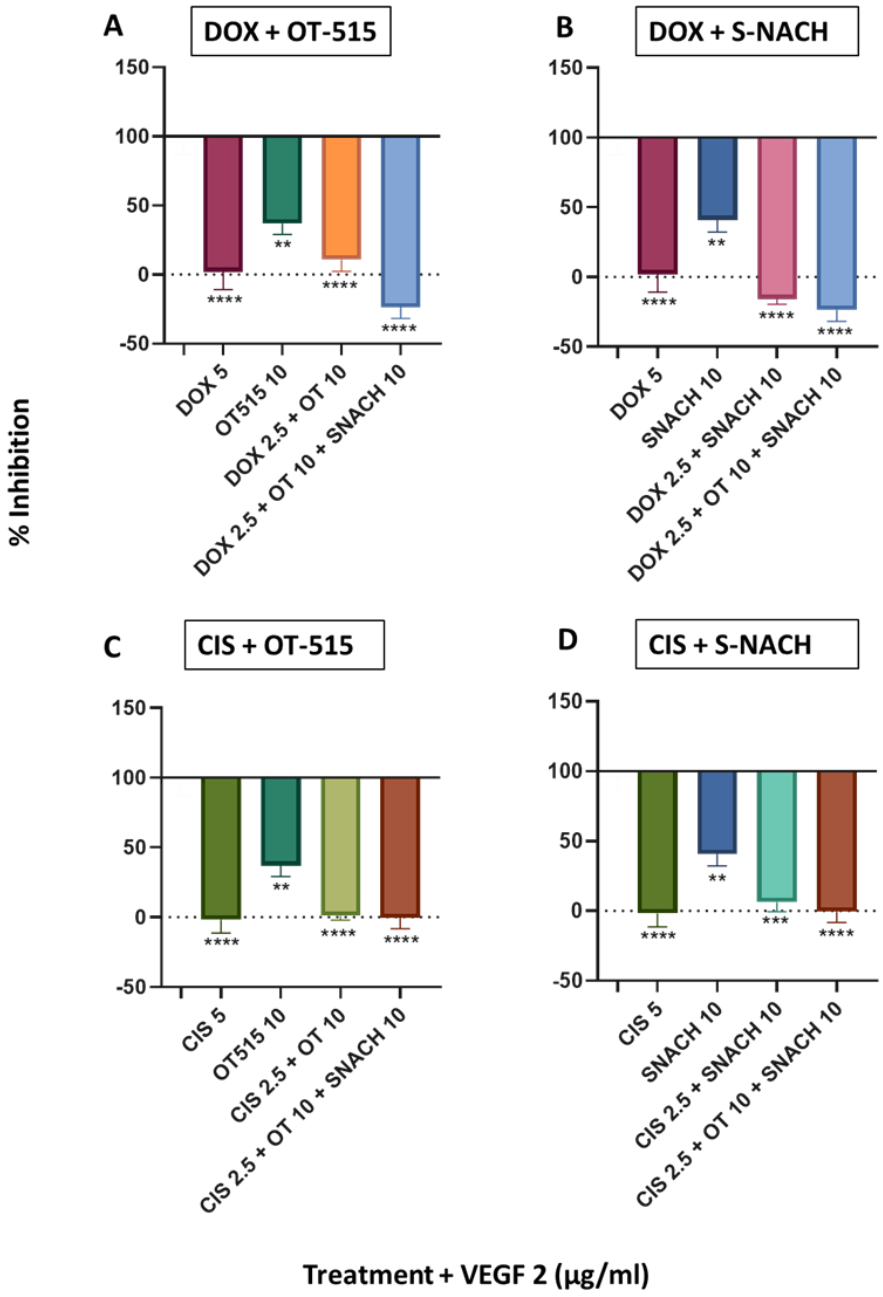


Figure 13. Inhibition by OT-515 and SNACH combined to chemotherapy of VEGF-induced angiogenesis using check CAM assay.

All treatments are normalized to PBS negative control; all groups were first treated with 2µg/ml VEGF to induce angiogenesis then treatments were added, and vessel branch number was counted using Imagej. A) OT-515 combined to Doxorubicin, B) S-NACH combined to Doxorubicin, C) OT-515 combined to Cisplatin, D) S-NACH combined to Cisplatin. Data represented as % inhibition of branch number (PBS = 100%), Doses represented as (µg/ml) which is equivalent to (0.02 µg/CAM), n= 3-8 CAMs per group. Significant reductions analyzed with One-way ANOVA; **p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001

3.3. Inhibition of tumor growth and tumor angiogenesis in the CAM model

We used the neuroblastoma grafted CAM model to investigate the in-vivo effect of OT-515, SNACH, and their combinations on tumor angiogenesis and growth. We first introduced $2*10^6$ neuroblastoma cells SH-SY5Y into CAM using Geltrex as a protein matrix to allow the growth of tumors. OT-515, S-NACH were applied individually and combined with Doxorubicin and Cisplatin using Geltrex as negative control and SH-SY5Y cells in media as a positive control. Pictures showed a slight inhibition of the formed tumors when treated with the compounds (Fig. 13). However, the results of tumor weights did not show a significant inhibitory effect of the compounds except for some concentrations of Doxorubicin (Fig. 14). Some modifications were applied to the protocol to allow the growth of larger tumors to spot the difference.

$10*10^6$ cells were introduced into CAM with an increased Geltrex of 50% of the total solution. Pictures showed tumor size and angiogenesis inhibition when adding the treatments and their combinations (Fig. 15). Harvesting the tumors also showed the difference in tumor size between the control, the individual treatments, and the combinations (Fig. 16). The tumor weight results indicated that the administration of OT-515 and S-NACH, separately or in combination with Doxorubicin or Cisplatin, significantly inhibited tumor growth ($p\text{-value} < 0.0001$). Using half the doses of chemotherapy combined with OT-515 or S-NACH had comparable significant inhibitory effects compared to individual chemotherapy, which confirms previous results (Fig. 17). The triple combinations showed a slightly enhanced effect than the double combinations.

To investigate the in-vivo effect of treatments on tumor angiogenesis, we used Drabkin's method to measure the hemoglobin content of the neuroblastoma SH-SY5Y CAM-harvested tumors with and without the addition of treatments and compared to control hemoglobin (Supp Fig. 3). All individual treatments showed statistically significant inhibition compared to the control (p-value

< 0.05). The combinations also showed significant inhibition that exceeded the individual results (p-value < 0.001) although not reaching significance compared to individual treatments. Triple combinations also showed an enhanced effect than the double combinations but with no significance (Fig. 18). However, the doses of chemotherapy used in combinations were half the doses of individual treatments, which confirms that the addition of OT-515 and S-NACH gives the major anti-tumor and anti-angiogenesis effect while avoiding the side effects caused by chemotherapy reducing their doses.

For further verification of the results of CAM tumor growth, we tried the same tumor CAM model using two different types of human-derived cancer cells, neuroblastoma cells (SH-SY5Y) and breast cancer cells (MCF-7) with and without Doxorubicin. The results showed an inhibition of the growth of both tumors, although the growth of SH-SY5Y cells and their inhibition by Doxorubicin was more significant (Fig. 19).

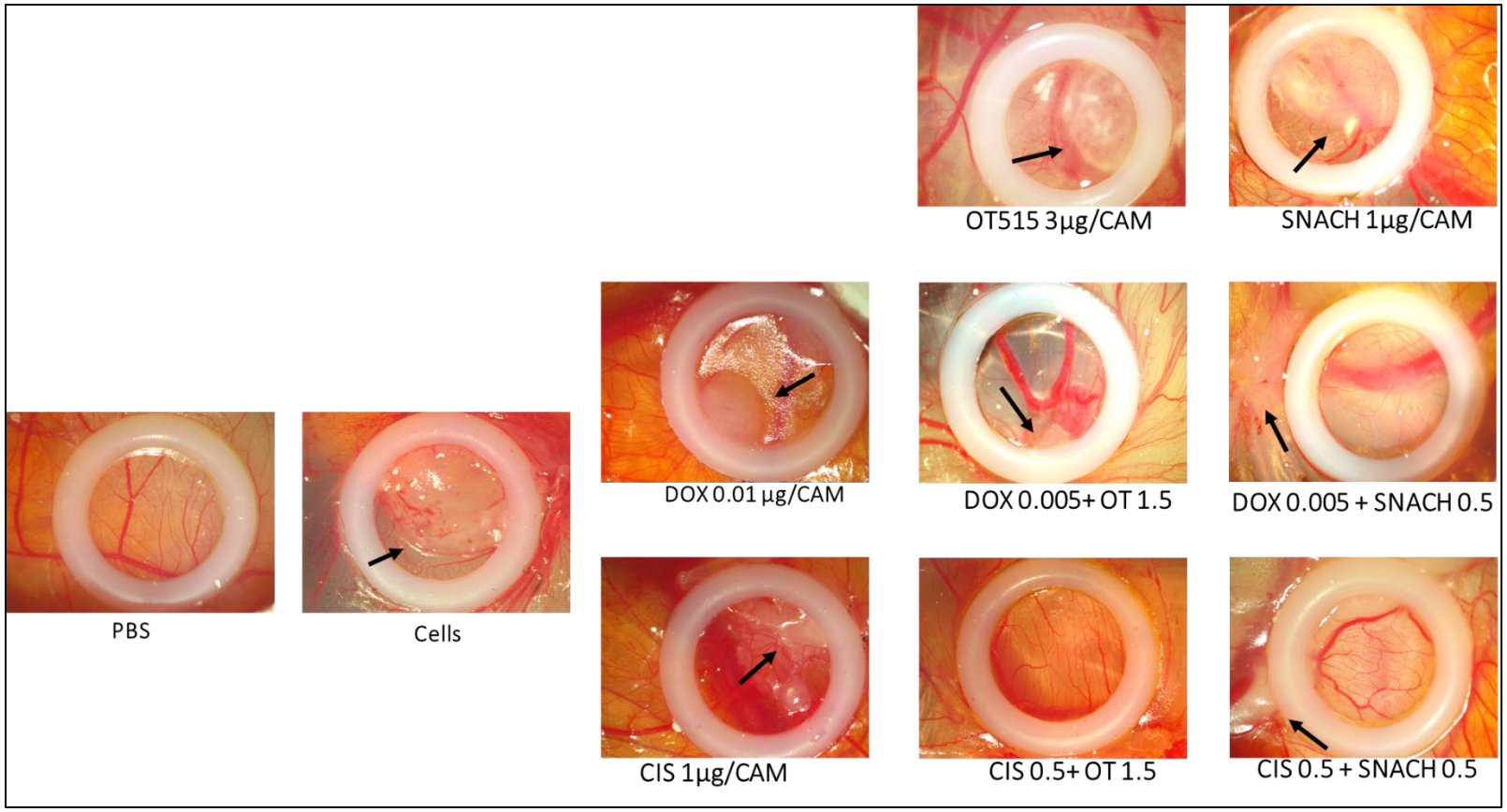


Figure 14. Typical Stereomicroscope images (7x) showing the growth of human neuroblastoma tumors on top of the CAM membranes and the inhibition caused by treatments.

Geltrex is negative control, all other groups Have SH-SY5Y cells at $2*10^6$ cells/CAM.

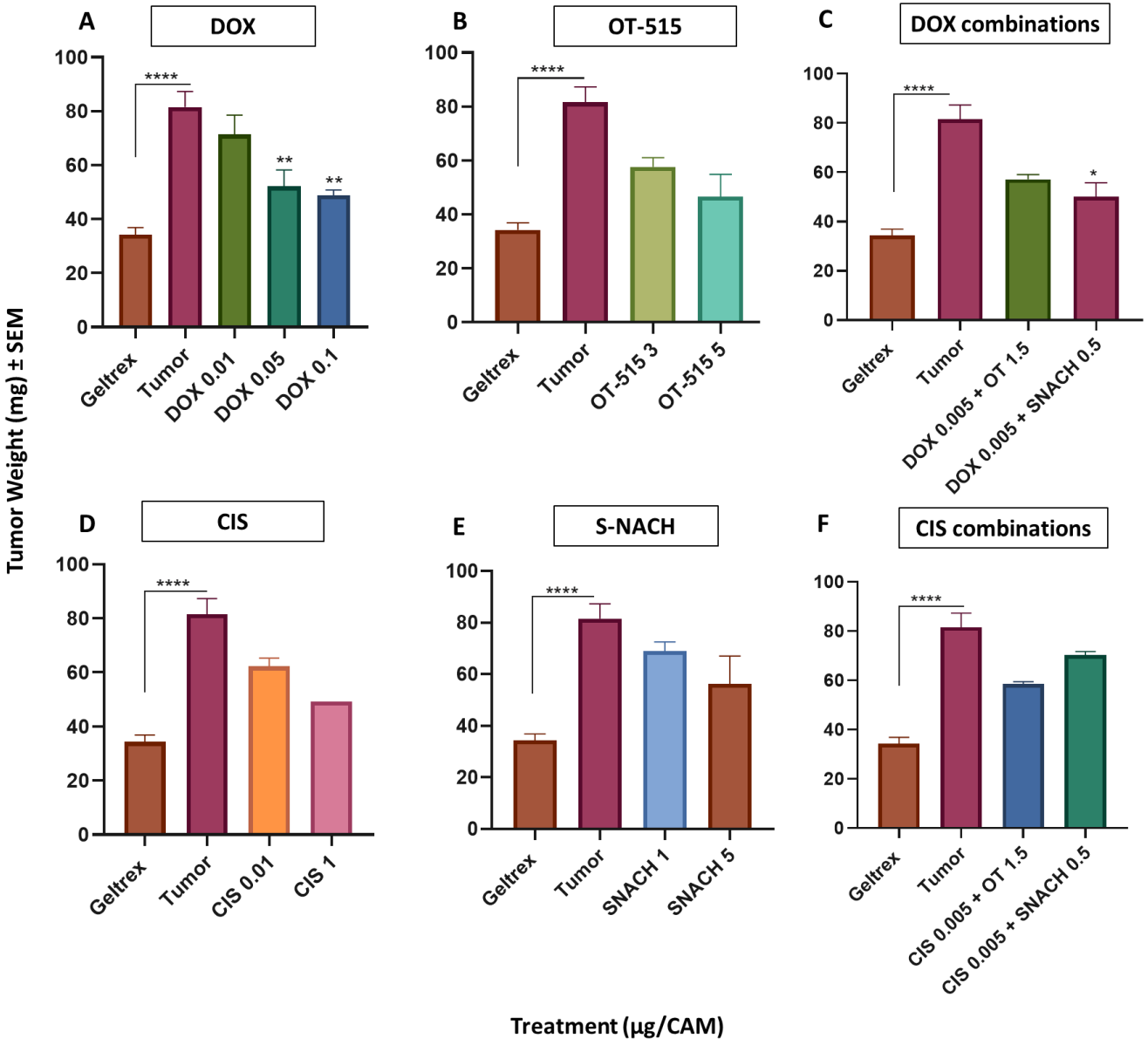


Figure 15. Human neuroblastoma tumor growth (SH-SY5Y) in the chick CAM model and the reduction associated with adding treatment compounds.

Geltrex is a negative control, and all other groups have SH-SY5Y cells at $2*10^6$ Cells/CAM with or without treatments. Data represent mean ± SEM, n = 3-8 per group. Significant reductions in tumor angiogenesis when adding treatments compared to control analyzed with One-way ANOVA; ***p < 0.0005, **p< 0.001

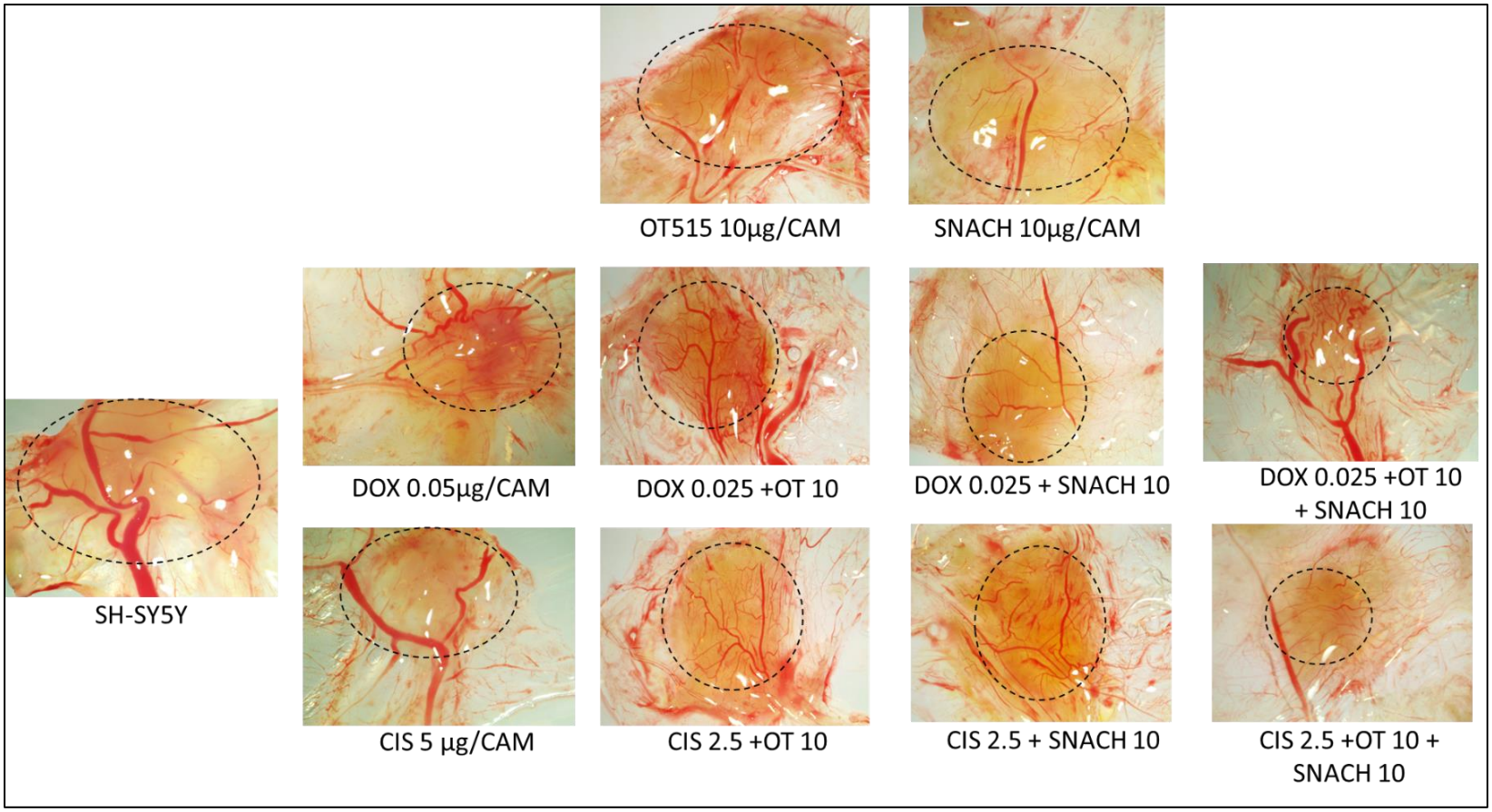


Figure 16. Effect of OT-515 and SNACH combined to chemotherapy on inhibiting the angiogenesis and growth of human neuroblastoma tumors grafted into CAM membranes.

Images taken with a stereomicroscope at 7x magnification. All groups have SH-SY5Y cells at $10*10^6$ cells/CAM.

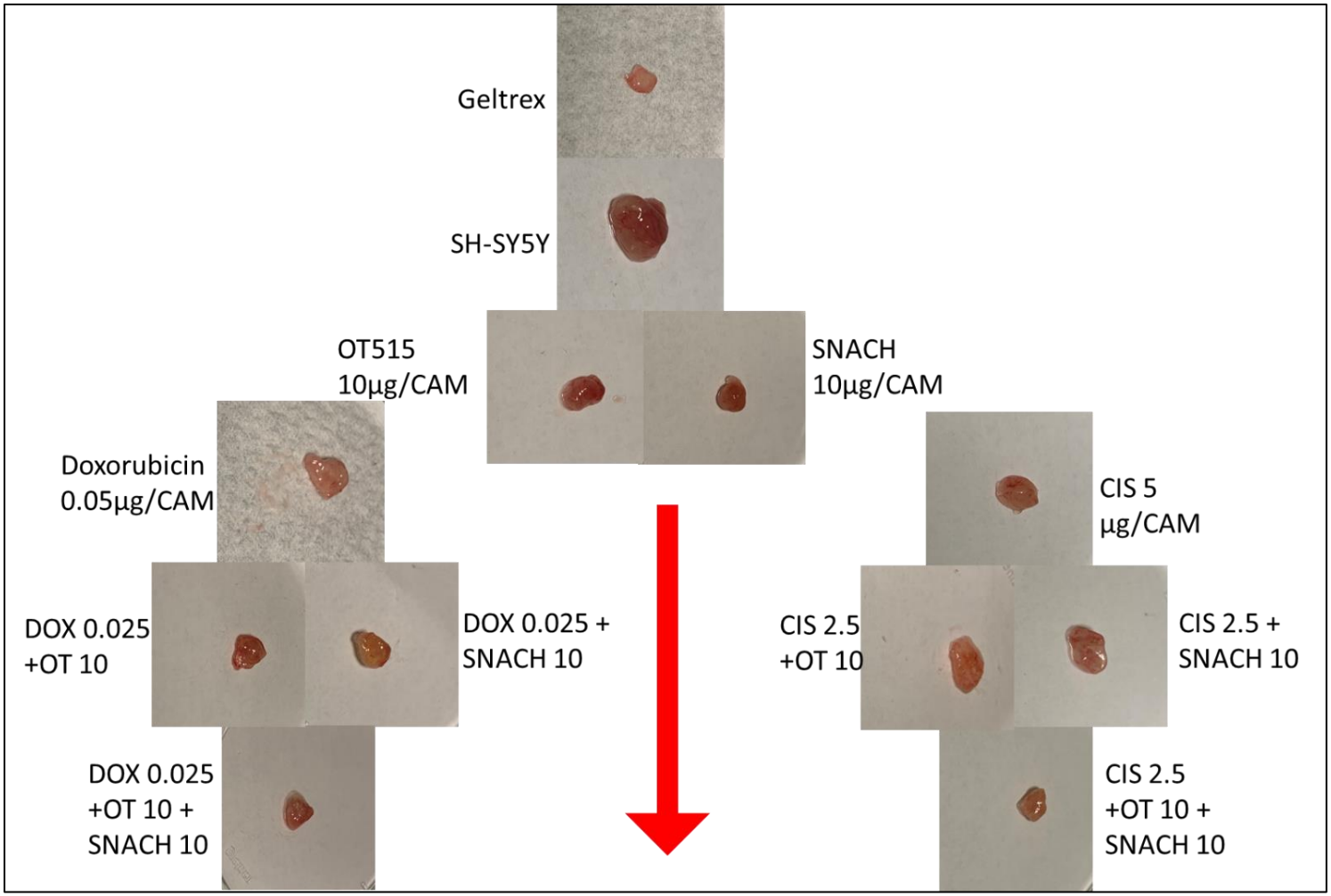


Figure 17. Inhibition of tumor growth of human neuroblastoma tumors grafted into CAM by OT-515 and SNACH combined to chemotherapy.

Geltrex is negative control, and all other groups have SH-SY5Y cells at $10*10^6$ cells/CAM. Treatment's concentrations represented as µg/CAM.

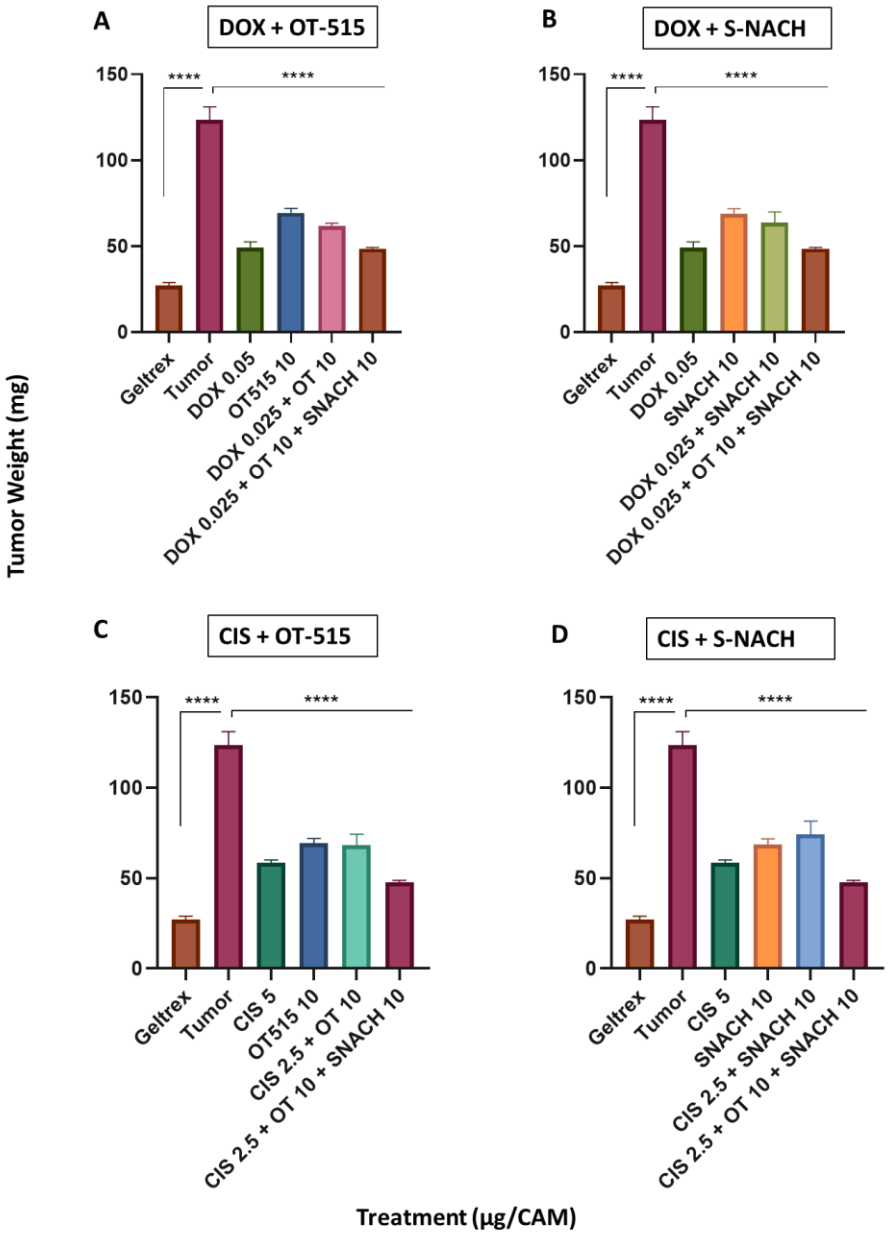


Figure 18. Effect of OT515 and SNACH combined to chemotherapy on human neuroblastoma tumor growth (SH-SY5Y) in the chick CAM model.

Geltrex is a negative control, and all other groups have SH-SY5Y cells at $10*10^6$ Cells/CAM with or without treatments. A) OT-515 combined to Doxorubicin, B) SNACH combined to Doxorubicin, C) OT-515 combined to Cisplatin, D) SNACH combined to Cisplatin. Data represent mean tumor weight ± SEM, n = 3-8 per group. Significant reductions in tumor angiogenesis when adding treatments compared to control analyzed with One-way ANOVA; ****$p < 0.0001$

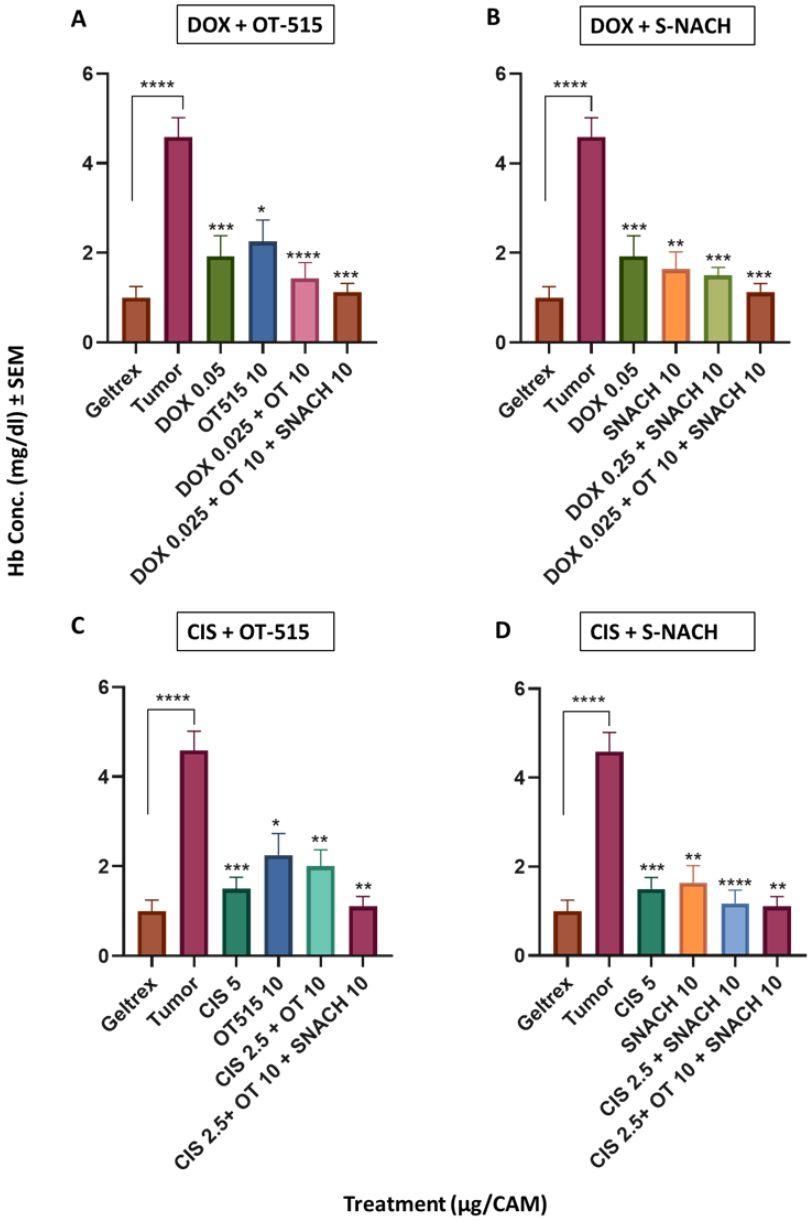


Figure 19. Hemoglobin content of the harvested tumors from CAM Neuroblastoma implant model, determined by Drabkin's method.

Geltrex is a negative control, and all other groups have SH-SY5Y cells at $10*10^6$ Cells/CAM with or without treatments. A) OT-515 combined to Doxorubicin, B) SNACH were combined to Doxorubicin, C) OT-515 combined to Cisplatin, D) SNACH combined to Cisplatin. Data represented as mean ± SEM, n = 3-8 per group. Significant reductions in tumor angiogenesis when adding treatments compared to control analyzed with One-way ANOVA; **p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001

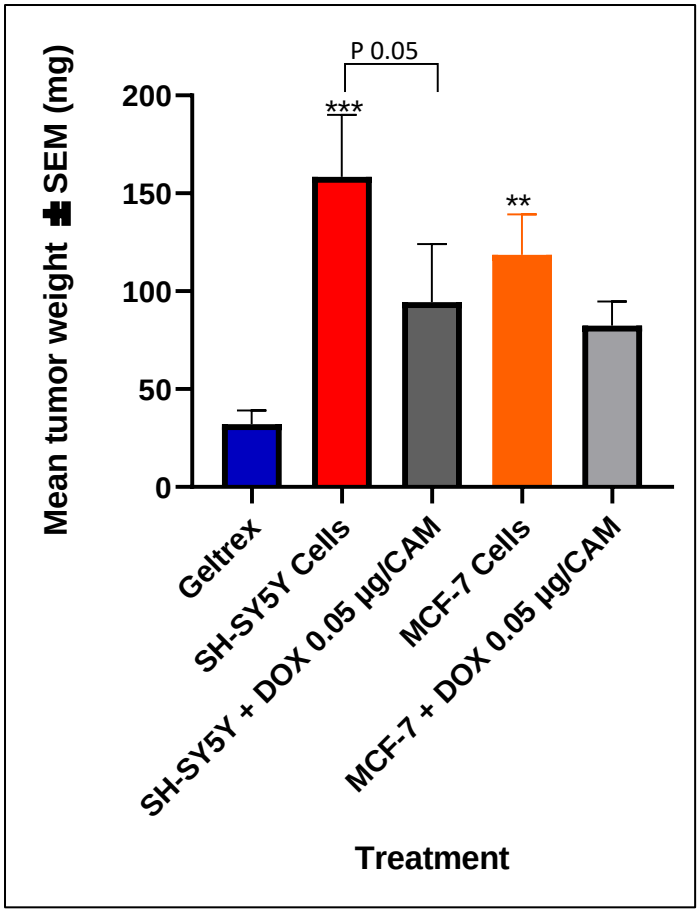


Figure 20. Human breast cancer (MCF-7) and human neuroblastoma tumor growth (SH-SY5Y) in the chick CAM model.

Geltrex is a negative control, and all other groups have cells at $2*10^6$ Cells/CAM with or without treatments. Data represent mean ± SEM, n = 3-5 per group.

3.4. Anti-proliferative effect of OT-515 and S-NACH combined with chemotherapy on normal endothelial cells

To elucidate a possible mechanism of the anti-tumor effect of the various treatments, we performed additional anti-proliferation tests to investigate their activity on the proliferation of normal endothelial cells. OT-515 and S-NACH significantly inhibited the proliferation of human endothelial vascular cells (HUVEC) p-value < 0.001 (Fig. 20). Combining OT-515 and S-NACH with Doxorubicin and Cisplatin showed significant inhibition of proliferation compared to individual chemotherapy (p-value < 0.0001). These findings suggest that the anti-tumor effect of OT-515 and S-NACH is due to possible impact on mediators, or responses to mediators, involved in the formation of blood vessels. The exact mechanism still needs further investigation.

To verify the results, we performed the same assay on mouse endothelial cells (C-166) while also comparing the inhibitory effect of the treatments to VEGF. We first applied different concentrations of VEGF on C-166 to determine the physiological dose that induces the proliferation of endothelial cells in-vitro (Supp Fig. 4). The application of 10ng VEGF significantly increased the proliferation of C-166 mouse endothelial cells (p-value < 0.05). All the treatments had a significant inhibition compared to VEGF (p-value < 0.0001). The double combinations of OT-515 or S-NACH with chemotherapy showed an anti-proliferative effect significantly more than individual chemotherapy even when using half the dose (Fig. 21). In addition, using a triple combination of OT-515, S-NACH and Cisplatin had the most significant inhibition compared to the Cisplatin double combinations (p-value < 0.0001), which was also more significant than the triple combination with Doxorubicin compared to Doxorubicin double combinations (p-value < 0.05).

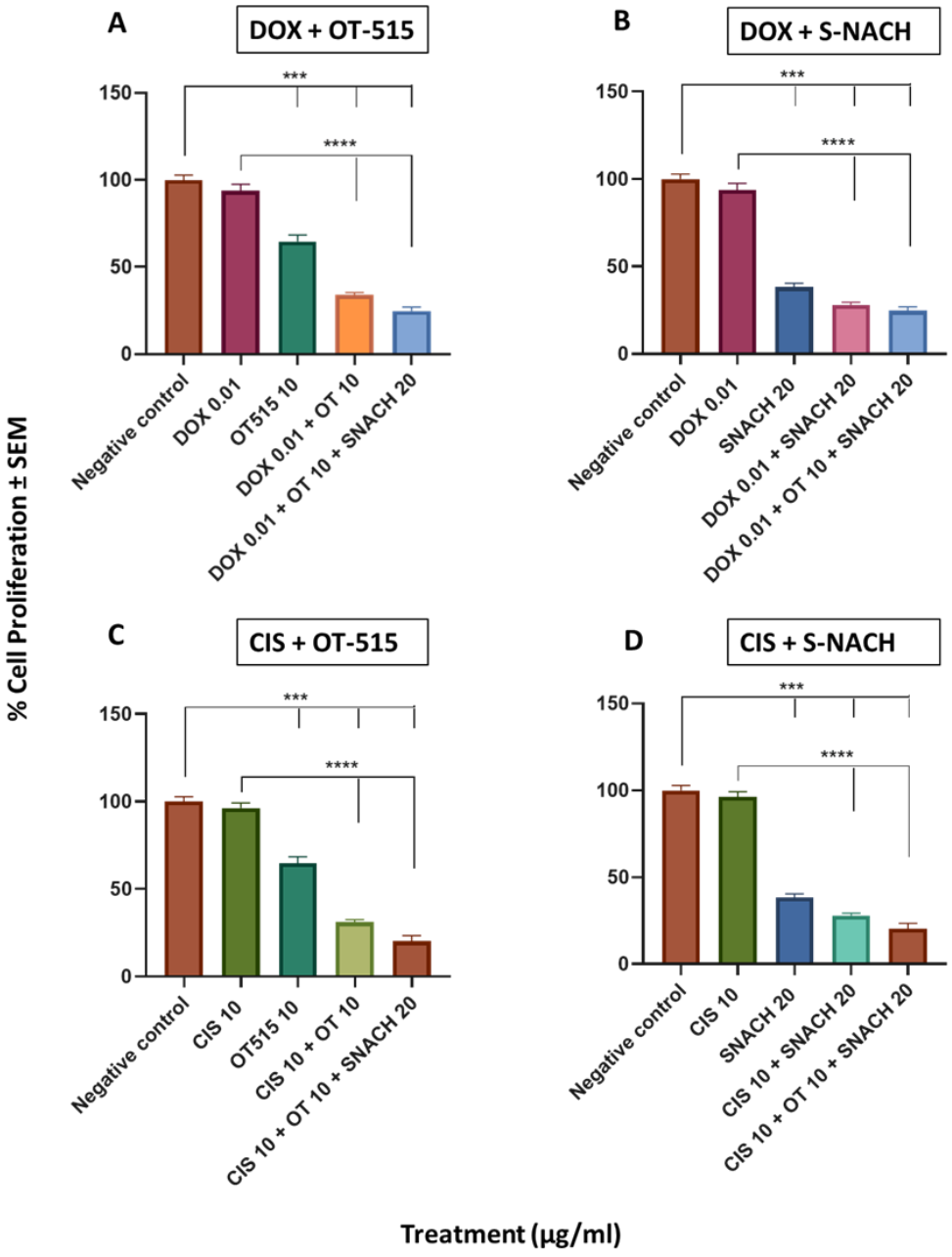


Figure 21. Proliferation inhibition with OT-515 and S-NACH combined to chemotherapy on human umbilical vascular endothelial cells (HUVEC).

Data represent mean cell proliferation ± SEM, n = 4-6, One-way ANOVA; ***p < 0.001, and ****p < 0.0001.

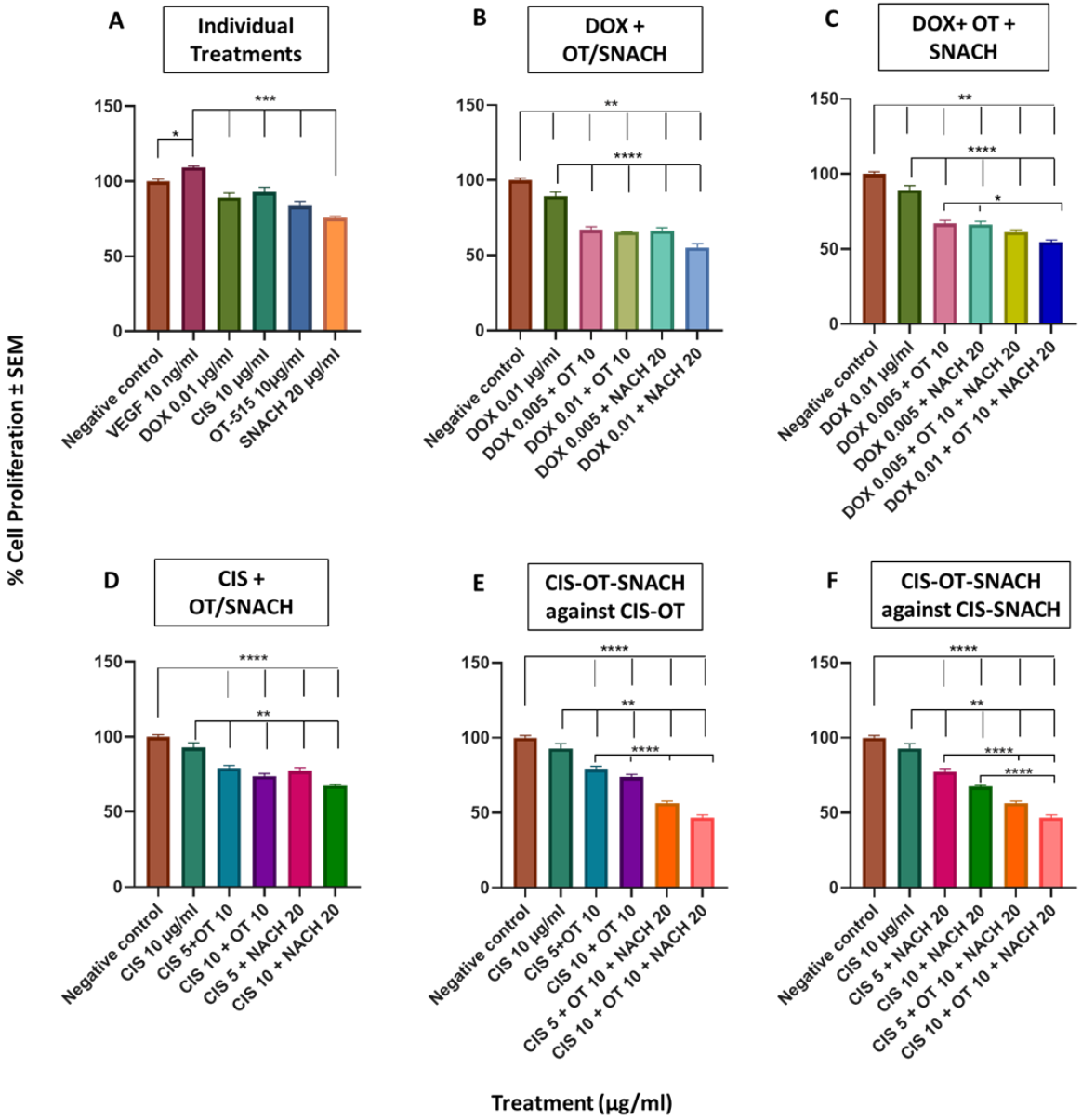


Figure 22. Comparing the anti-proliferative effect of OT-515 and S-NACH combined to chemotherapy with VEGF-stimulated proliferation on mouse endothelial cells C-166.

A)The anti-proliferation effect of individual treatments compared to negative control as well as to VEGF, B) Combining OT-515 and S-NACH to Doxorubicin at different concentrations enhanced the anti-proliferation of cancer cells, C) Triple combinations of Doxorubicin, OT-515 and SNACH, D) OT-515 or S-NACH double combinations with Cisplatin, E) Triple combinations of Cisplatin compared to CIS-OT, F) Triple combinations of Cisplatin compared to CIS-SNACH. Data represent mean % cell proliferation ± SEM, n = 6-12, One-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Chapter Four: Discussion

This study provides evidence that both the tempol derivative OT-515 and the sulfated non-anticoagulant low molecular weight heparin (S-NACH) have anti-tumor proliferation and anti-angiogenesis properties. These properties were shown when using the compounds separately or combined with classical anti-cancer chemotherapy such as Doxorubicin and Cisplatin.

Several classical chemotherapeutics, including Doxorubicin and Cisplatin, are well-known to be neurotoxic agents and cause chemotherapy-induced peripheral neuropathy (CIPN), which, in many cases, is a dose-limiting event that and requires discontinuing the treatment[21,47,60]. Although several mechanisms of CIPN have been identified, reducing the doses of chemotherapeutics used in treatment is one of the strategies implicated in CIPN prevention[61,62]. Moreover, combining chemotherapy with other adjuvant therapy to treat aggressive tumors is also among the commonly used strategies to target the tumor with multiple mechanisms and achieve remarkable anti-tumor response with the lowest side effects. The findings that OT-515 and S-NACH have anti-proliferative effects (Supp Fig 1) prompted the investigation of their effect when combined with lower doses of chemotherapeutics. The data presented in (Fig 8) showed that using a combination of OT-515 or S-NACH with chemotherapy allowed the use of lower doses of chemotherapy while getting the same or even enhanced anti-tumor effect. Taken together, these findings suggest that OT-515 and S-NACH could be used as adjuvant therapy with reduced chemotherapeutic doses to avoid CIPN.

We performed more investigation of the mechanisms of action of agents and their *in situ* anti-tumor effects. The data presented in Figs 12, 17, and 18 indicated that OT-515 and S-NACH enhance anti-tumor and anti-angiogenesis effects when combined with Doxorubicin or Cisplatin

in situ. However, further optimization to elicit the optimal dosages are required. Previous studies have tested the impact of combining OT-404 and S-NACH with chemotherapy with promising results on the increased uptake and enhanced effect of chemotherapeutics, as well as the role of tempol derivatives and their ability to reverse the resistance to Doxorubicin in different cell lines. Resistance is yet another limiting factor of chemotherapy use [36,63] but was not investigated in the present study. Taken together, OT-515, based on what has been demonstrated with OT-404, and S-NACH act on tumors through multiple targets, which offer an advantageous technique in possible treating aggressive tumors and reducing adverse side effects.

This study also demonstrated that OT-515 and S-NACH inhibit the proliferation of VEGF-stimulated CAM vasculature and VEGF-stimulated normal endothelial cells such as human-derived HUVECs and mouse-derived C-166 cells, with an enhanced inhibitory effect when combined with chemotherapy. Therefore, it can be inferred that this significant anti-tumor effect of the compounds is achieved by inhibiting the proliferation of endothelial cells, arresting vessel growth, and inhibiting angiogenesis required for tumor progression.

Angiogenesis is a well-known hallmark of cancer progression as it is the process by which tumor cells are supplied with nutrients. It is a complex process that comprises various factors, enzymes, and cytokines. VEGF is the main factor inducing angiogenesis by endothelial cells through the induction of proliferative pathways, chemokines, and cytokines[64,65]. Anti-angiogenic drugs have been of great interest for researchers to be translated into the clinic either individually or in combination with chemotherapy as they appear to have a limited toxic profile and, in many cases, can target, directly or indirectly, multiple anti-tumor mechanisms[66,67]. Our findings that OT-515 and S-NACH can inhibit the VEGF-induced angiogenesis are of great importance as they

demonstrate a new strategy to combine with chemotherapy for superior anti-tumor activity, fewer toxic effects, and drug resistance.

Several studies reported that anti-angiogenic agents could mediate the CIPN side effects through unclear mechanism[68]. In contrast, compounds with anti-angiogenic effects have been shown to work as neuroprotectors and ameliorate CIPN when used in combination therapy[69]. Previous work from our laboratory has indicated that OT-404 can ameliorate CIPN caused by Cisplatin through a dual anti-oxidant and NFκB inhibitory activity, in addition to its effect on inhibiting TNF-α, which is involved in the cycle that induces NFκB activation[28,70]. Taken together, in addition to having anti-angiogenesis and anti-tumor effects, OT-515 might have a protective effect against CIPN through ROS and anti-inflammatory pathways. Further studies are required to elucidate the exact mechanism of action.

Conclusion and future prospects

This study used a combination of well-established in-vitro and in-vivo models to provide evidence that OT-515, the tempol derivative, and S-NACH have anti-tumor and anti-angiogenesis effects and can inhibit multiple pathways of cancer progression. They have a remarkable enhancing (synergistic?) effect when combined with traditional chemotherapy such as Doxorubicin and Cisplatin, representing a unique strategy of targeting tumors while reducing doses of chemotherapeutic required, thereby suggesting a role in reducing toxicity while augmenting anti-cancer activity. Further investigations are required to define the exact mechanism of action, the pathways involved, and the impact of the compounds on other tumor types, as well as their *in vivo* utility.

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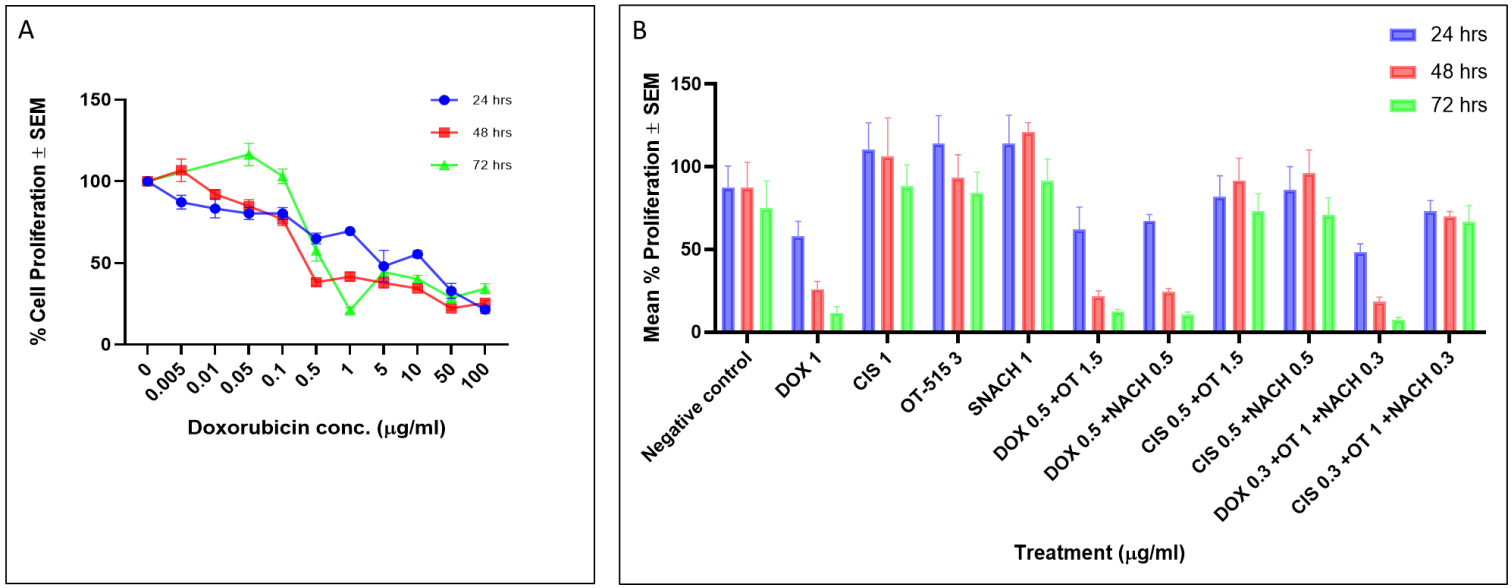
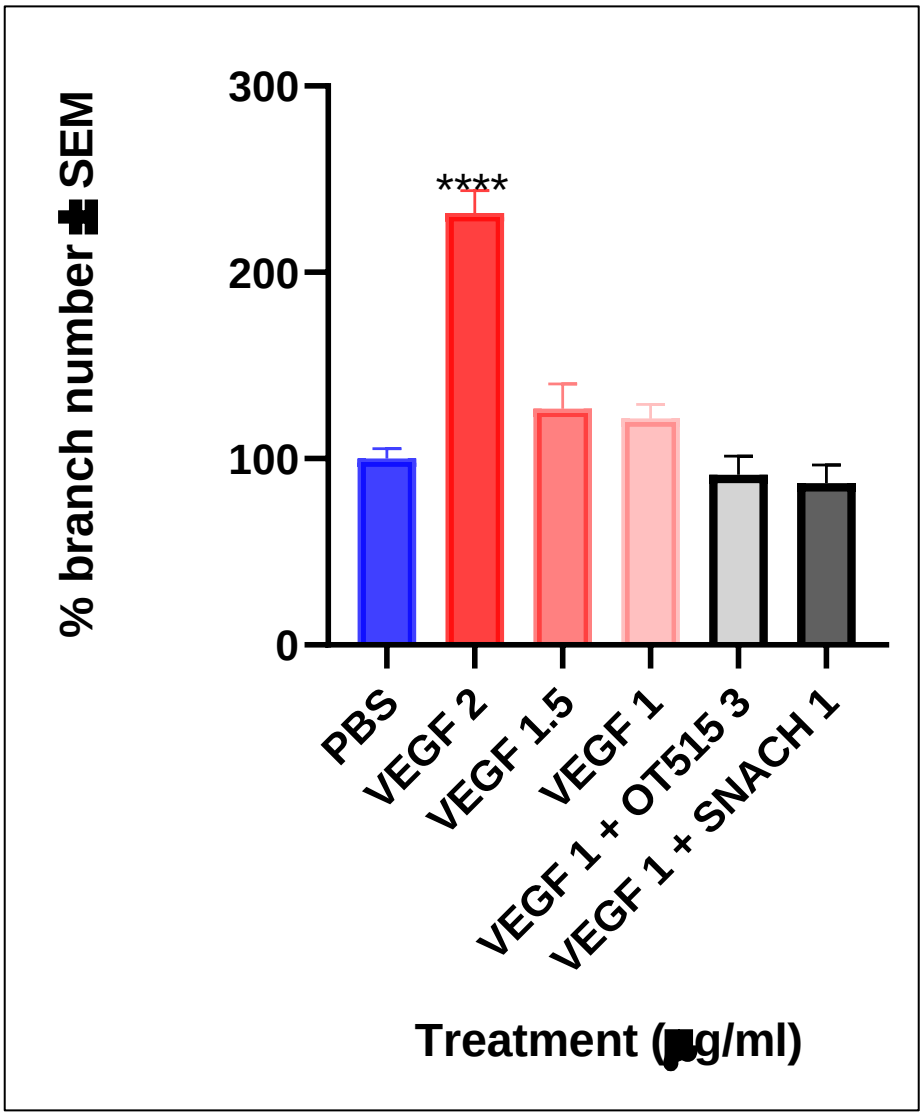
Appendix



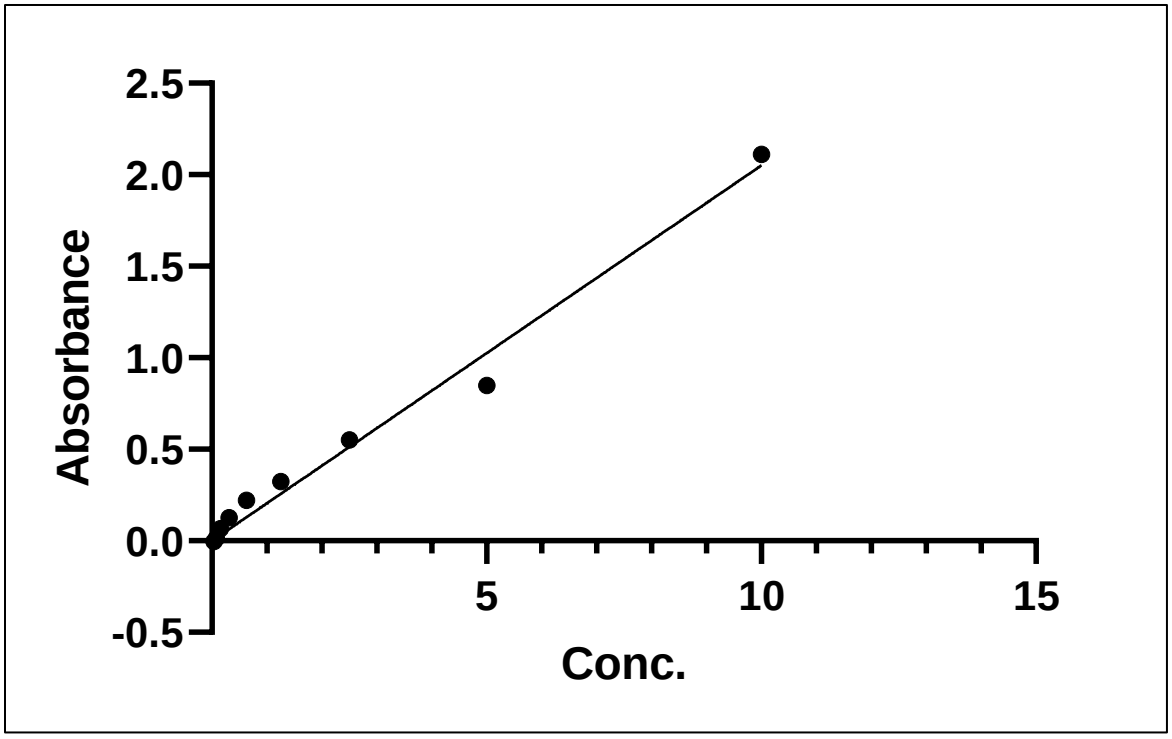
Supp Figure 1. Effect of compounds incubation for 24, 48 and 72 h on the proliferation of human neuroblastoma SH-SY5Y cells.

A) Doxorubicin at different concentrations ranging from 0.005 to 100 µg/ml inhibited cancer cell proliferation to different degrees. B) OT-515 and S-NACH were combined to Doxorubicin and Cisplatin and incubated with cells, cell proliferation was measured after 24, 48, and 72 h to determine the degree of inhibition at each time point. Data represent mean ± SEM, n = 6.

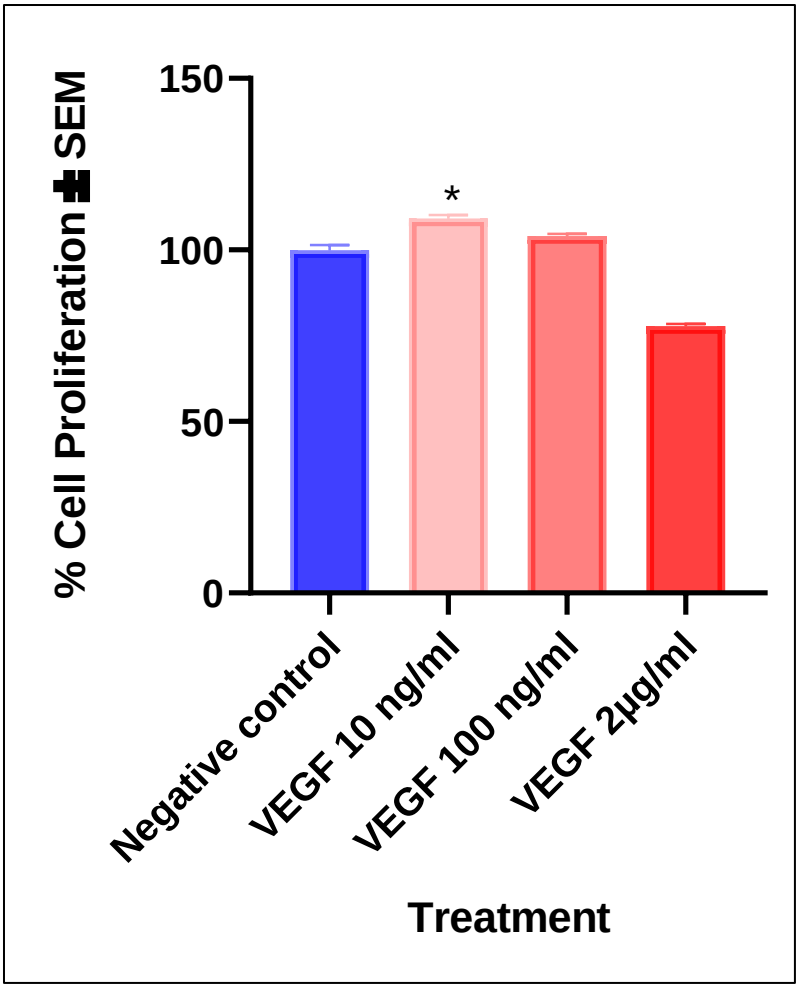


Supp Figure 2. Effect of different concentrations of VEGF on inducing angiogenesis in the CAM model.

Data represent mean ± SEM, n = 5 per group, One-way ANOVA; ****p < 0.0001.



Supp Figure 3. Hemoglobin standard curve with control blood solution for interpolation of CAM harvested tumor samples



Supp Figure 4. Effect of VEGF different concentrations on the proliferation of mouse endothelial cells C-166

Data represent mean ± SEM, n = 4-6, One-way ANOVA; *p < 0.05.