Evaluation of the Therapeutic Potential of Orlistat on a Mouse Model of Hereditary Hemorrhagic Telangiectasia

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Abstract

Hereditary hemorrhagic telangiectasia (HHT) is a genetic vascular disorder that is a highly debilitating disorder caused by loss-of-function mutations in bone morphogenetic protein 9 (BMP9)-ALK1-Smad1/5/9 signaling. Currently, there is no cure for this disease, however, it is characterized by Arteriovenous malformations (AVMs) around the brain, gastric-intestinal tract, liver, lungs, and skin. Sirolimus, an immunosuppressant drug used in liver transplant surgery has also shown to have possible links to treat AVMs; yet it is limited by its toxicity. Orlistat and Nintendonib are a class of FDA-approved drugs that are shown to have similar effects on the liver and organs associated with HHT as Sirolimus. Through the use of western blotting and vasculature analysis in the retina, the effect of Orlistat and nintedanib on mouse with BMP9/10 inhibition was tested to study the effectiveness of Orlistat in reducing HHT pathology. Results showed that Orlistat reversed the inhibitory effect of BMP9/10 antibodies in HHT model phosphorylation of Smad ($p<0.05$). However, although transmammary transfer of BMP9 and BMP10 blocking antibodies induces AVMs in the neonatal retina, Orlistat does not improve vascular pathology in the BMP9/10-immunoblocked retina. Therefore, my data highlights a beneficial interaction and synergy between Orlistat and Nin treatments in HHT mice. Further molecular mechanism studied can be made, and results can be supported through analysis through a bleeding assay and an increased dosage of the drugs.

Evaluation of the Therapeutic Potential of Orlistat on the Liver and Retina of a Mouse Model of Hereditary Hemorrhagic Telangiectasia

1. Introduction

1.1. Rationale

Gastroenterologists are currently seeking new methods for the treatment of Hereditary Hemorrhagic Telangiectasia (HHT) [1]. HHT is a genetic vascular disorder that is characterized by vascular mutations in the liver, retina, lungs, and the skin [2]. It is an autosomal dominant disorder in which blood vessels do not properly develop. The capillaries which run between the veins and arteries are often missing, leading to frequent bleeding and spots around the body. The fragile nature of the location in between the arteries and veins often leads to bleeding and sensitivity. The primary cause is a genetic mutation that is passed on from parents. Out of the five genes that can cause HHT, three are currently known. As a result, in this study, I conducted an experiment to determine whether therapeutic targeting of pathways associated (Smad1/5/8) with HHT and vascular malformations in the lungs, liver, and retinas could rescue the signaling defects and vascular pathology of HHT.

Tacrolimus (Tac), also known as FK506, was previously found to be an effective treatment in reducing HHT pathology invitro and *in vivo* in a mouse model [3, 4]. Tac is one of the immunosuppressants mainly used for organ transplants to reduce tissue rejection [3]. Similarly, Sirolimous (Siro) has been determined to be a potential treatment of HHT, as it resulted in the restoration of retinal vasculature and restoration in the Smad1/5/8 pathway, although to a reduced degree than Tac [4]. Additionally, in a recent study, an HHT patient who underwent liver transplantation with Arteriovenous Malformations (AVMs) was treated in a series of medical treatments, including Siro, and it was found to be an effective treatment in reducing the pathological symptoms of HHT [5]. Finally, a study published within my lab demonstrated the effectiveness of Siro in combination with Nintendanib in reducing pathology in a Mouse Model [6]. Siro is an effective treatment for HHT however, it is an expensive and dangerous alternative

that often has severe side effects. There was a study conducted that demonstrated similarities in Siro and another drug called Orlistat in their impact on restoring mutated vasculature [7].

Orlistat is currently available as a weight-loss option and is a lipase inhibitor that allows the body to reduce fat intake. Because HHT is a disorder that affects the vasculature of an organism, I proposed that Orlistat would be an effective treatment for HHT that is already FDA approved, safer than alternative drugs, and is cost-effective.

My study had three aims: 1) to determine the efficiency of Orlistat in restoring vasculature in BMP 9/10 inhibited mice 2) to determine if Orlistat would be efficient in restoring Smad 1/5/8 and P-AKT pathways 3) to test combinations of Orlistat and Nintedanib and Orlistat alone to wild type mice and HHT methodology.

Therefore, my study investigated the therapeutic potential of Orlistat and Nintedanib as a possible source of treatment for HHT.

1.2. Background

HHT Gene Mutations

HHT mutations are mostly found in the ALK1 (activin receptor-like kinase 1) and ENG (endoglin) genes and define HHT1 and HHT2, which are responsible for more than 80% of HHT patients [1,2]. Mutations were also reported in SMAD4 [6] and BMP9 (bone morphogenetic protein 9), which cause rare and atypical forms of the disease called juvenile polyposis/HHT combined syndrome and HHT-like vascular anomaly syndrome [7], respectively.

BMP9, ALK1, endoglin, and Smad4

BMP9, ALK1, endoglin, and Smad4 are members of the transforming growth factor-β signaling superfamily. They play an essential role in angiogenesis, and alterations in any of them result in a subsection of HHT. Endoglin is a gene responsible for HHT1, which differs from HHT2 that is developed from a mutation in ALK1. HHT1 is characterized by congenital AVMs, whereas HHT2 is characterized by malformations in the form of spots around the liver. Lastly, Smad4

mutations lead to juvenile polyposis, a disorder in which masses of normal tissue form within the digestive tracts. ALK1 and endoglin receptor activation leads to the formation of Smad1, Smad5, and Smad8 to trigger the formation of the Smad1/5/8 complex (**Figure 1**). Smad1/5/8 activity is observed as a difference that occurred between a mouse with HHT and one without.

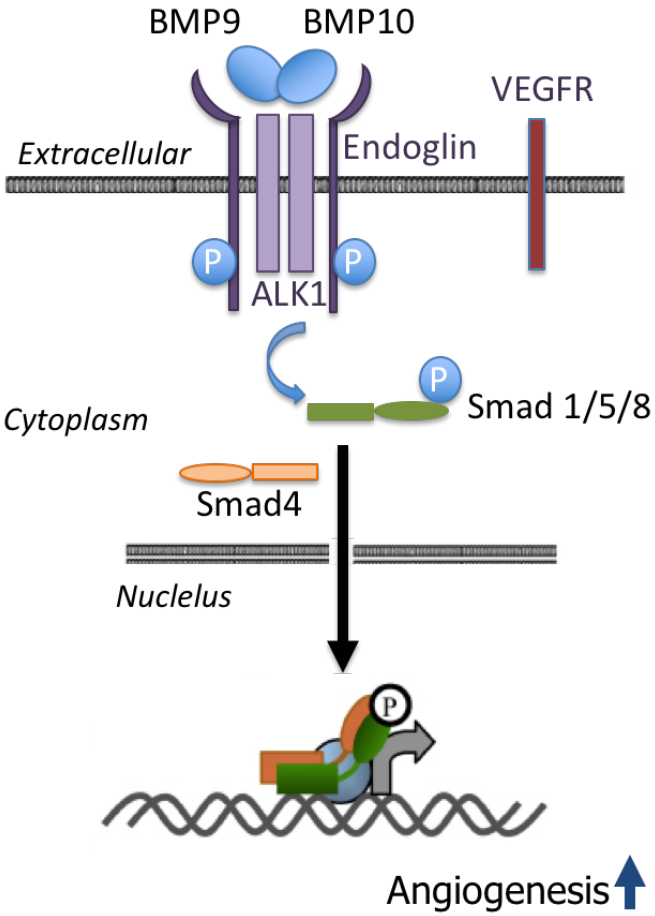

Figure 1. *BMP9 and 10 engage the ALK1-Endoglin receptor complex and activate the Smad complex. Following ligand binding, receptors are phosphorylated and induce signal through Smad1/5/8 phosphorylation. Then Smad4 binds to the Smad complex and enters into the nucleus. BMP; Bone morphogenetic protein, ALK;* activin receptor-like kinase 1, *VEGFR; Vascular Endothelial Growth Factor Receptor ds (Adopted and modified from reference 18).*

BMP 9/10 inhibition

HHT leads to a loss-of-function in the BMP9/10-ALK1-ENG signaling cascade [8,9]. BMP9/10 are produced in the liver and tissues and circulated in the body. Although the inhibition of the

BMP9/10 pathway characterizes HHT pathology, there is evidence supporting the possibility that BMP10 may not be pivotal in the development of the HHT pathology [9].

For this study, the mice were treated with anti-BMP9/10 antibodies; their inhibition leads to the formation of pathology seen in HHT patients such as abnormal hypervascularization [9,10]. Recently, it has been shown that antibody delivery and BMP9/10 immuno-neutralization in mouse pups could be achieved via injecting the lactating dams of the female mothers of the pups [10]. Through the use of antibodies against BMP9/10, such as ALK1 and endoglin, AVMs, shrinkage of vasculature, and other HHT pathology may be mimicked for testing *in vivo* models [8,9,10].

Orlistat and Nintendonib

While 1.4 million people are affected with this condition worldwide, there is no effective treatment or cure available. For this reason, in the lab, I determined whether certain FDA-approved drugs with known connections to other conditions could have disease-modifying properties in HHT mouse models and cellular models. When treated with two drugs, Sirolimus and Tacrolimus, the number of AVMs were decreased in areas throughout the body [11]. These drugs are being tested alongside others (such as Orlistat and Nintedanib) to potentially lower HHT pathology. Orlistat is a known lipase inhibitor and used for weight loss, as it reduces fat intake of the body [11,12]. Nintendanib (BIBF 1120) is a tyrosine kinase inhibitor used for the treatment of pulmonary fibrosis, and systemic interstitial lung disease [13]. Both are drugs that are already FDA approved and showed trends to be effective in restoring HHT pathology.

A recent case study reported that Nintedanib was an effective treatment for patients with epistaxis and skin telangiectasias by both HHT and idiopathic pulmonary fibrosis (IPF) [14]. Nintendanib inhibits tyrosine kinase, including vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), and platelet-derived growth factor receptor (PDGFR) [14]. These growth factors promote fibrosis, which leads to the formation of scar tissue in the lungs. Nintendanib was selected out of other receptor tyrosine kinase (RTK) inhibitors

that have similar effects because it has a known target of inhibition and is associated with minimal side effects. The drug repurposing strategy has the potential to fast-track new clinical investigations for HHT. Orlistat and Nintendanib, when administered in combination, may synergize to effectively reverse vascular malformations, and restore the Smad1/5/8 pathways in the HHT mouse model.

Current treatments of HHT

In a clinical case where an HHT patient was to undergo a liver transplant test, he was given five drugs, two of which were Siro and Tacrolimus. Both acts as immunosuppressants and are often used to lower the risk of organ rejection however, the patient following the liver transplant showed reduced to no signs of Arteriovenous Malformations (AVMs) and HHT pathology [15]. However, those drugs have been reported to be toxic even in small doses and are unaffordable for patients who need it. Tacrolimus (FK506) was previously used to test mice at the lab, and it demonstrated a significant decrease in HHT pathology [16,17]. There was a decrease in AVMs in the liver and lung, and an increase in retina density and size which signify that the mouse is getting treated.

New methods of treatment for HHT are in constant development as a result of its severity. Patients have the option to undergo treatment in the form of skin transplants, radiation therapy, or the use of experimental drugs such as bevacizumab; however, these methods are costly and are risky in terms of possible side effects [18,19]. Thus, the main challenge of HHT treatment is the limited accessibility of its treatment options resulting in an urgent need for cost-effective medication that reduces HHT pathology and AVMs.

I hypothesized that Orlistat, a drug that is FDA approved, would be efficient restoring the retina vascular density, the number of AVM and their width, and the Smad 1/5/8 and P-AKT pathways in a mouse model with BMP 9/10 inhibition.

In this study, I proposed that Orlistat (lipase inhibitor) or Nintendanib (Kinase inhibitor) would work in the trans-mammary model of BMP9/10 immunoblocked mice to restore retinal density, the number of AVMs and P-AKT and Smad1/5/8 signaling.

2. Methodology

**All methods were conducted by Author unless otherwise stated*

HHT mouse model & treatment (Performed by Mentor)

My supervisor's laboratory developed an HHT mouse model [10]. Pregnant mice (3- to 4-month-old, The Jackson Laboratory) were used in this study. DMSO, Orlistat (25mg/kg/day) or Nintendanib (0.3mg/kg/day) were treated through transmammary means. Blockage of BMP9 and BMP10 (500ug/mother) was performed to alter the retinal vasculature in mice. The lactating dams of the mother were injected once on postnatal 3 (P3) with DMSO (control), Nintendanib, or Orlistat. On P3, P4, and P5, pups were injected with Orlistat, Orlistat and Nintendanib, or vehicle (1% DMSO saline). After euthanization, the eyes were extracted and placed in 4% paraformaldehyde for 20 min on ice and retinas were isolated and analyzed by histochemistry. For Western blot, proteins were extracted from livers.

Retina Whole Mount Immunohistochemistry (IHC)

Following the preparation and preservations of the retina onto the slides, they were placed on a Nikon Eclipse T2000-S microscope, and images were taken at 4x initially to spot the AVMs and a sense of the shrinkage of the vascular density. Then a 20x lens was used to focus on three separate regions of each pedal of the retina to capture images for AVM width analysis, AVM count, and vascular density. These locations included the plexus (between artery and vein), front of the artery, and front of the vein. These images were stored, then image analysis was done using Fiji/ImageJ in a 200x200 um^2 square to calculate the density of each region. The AVM was similarly counted using Fiji/ImageJ in which irregular vessels between the artery and vein counted as an AVM. Lastly, the width was counted by dragging a scaled line from top to bottom of the AVM to determine the width for each.

Western Blotting

To determine altered Smad or AKT activation, followed by Orlistat or Nintendanib treatment, I performed Western blot using anti-phospho-Smad and anti-phospho-AKT antibodies. To determine that the same amount of protein was loaded in each well, I used anti-Actin and anti-AKT antibodies. Protein samples were mixed with SDS loading buffer 5X. The solution was then denatured at 95°C for 5 minutes. For each SDS-PAGE electrophoresis, I made a 10% acrylamide running gel and 4% stacking gel. Samples were kept in the cold, and their concentrations were measured by BCA assay as manufacturer's method. The samples were then loaded into the gels at 15ul into each well; the cap was closed, then the gel was run at 100 volts during the stacking section, then at 150 volts during the loading section of the gel. The running of the gel continued until the bands reached the bottom. The samples were transferred to a transfer buffer containing 20% Methanol, 1x transfer buffer in cold room at 200 mA for 2 hours. Following the 2 hours, the gel was transferred onto a membrane created based on a sandwich model. It consisted of a sponge on top, filter paper, the segment of the gel that has the bands, the membrane, the filter paper, then the sponge. After being rolled using a roller to remove air bubbles, it was placed in a transfer buffer then left in the cold room at 0.2A for 2 hours. In the end, the membrane was washed with a ponceau solution to determine if the run was successful then was washed twice with distilled water until all of the ponceau was removed from the membrane.

A PBS-Tween wash buffer solution was created by mixing 1 Liter of 10% PBS buffer with 1mL tween. The solution was then used to wash the membrane three times for 10 minutes each. A 5% skim milk blocking buffer was created by mixing the milk powder with PBS buffer. After rinsing the milk with the tween solution once more, it was added with primary antibodies, including anti-phospho-Smad, AKT, phospho-AKT, or actin, and was incubated at 4°C overnight. After removing the membrane from the different antibodies, it is washed three times with tween for 10 minutes each. Then they were placed in a secondary antibody based on the primary. All of the antibodies were generated from rabbit, so the secondary was anti-rabbit. After being placed into

the HRP conjugated anti-rabbit antibody for an hour, it was washed in 10-minute intervals for six times.

In order to detect the HRP signal, 2 uL of ECL chemiluminescent solution were added the same amount of color reagent A and color reagent B and mixed with the membrane for exposure. The bands were detected by an x-ray film in the darkroom. Repeated trials were done for each membrane to ensure that the bands were distinct and clear for the analysis. Band intensity was measured and fold changes were determined by image analyzer and densitometer.

3. Results

Orlistat or Nintendanib do not affect vascular pathology in the BMP9/10-immunoblocked retina

From previous studies, it has been established that anti-BMP9/10 antibodies can be given to pups through transmammary means [20]. Following this, the retinal model of a mouse can be studied to determine pathological angiogenesis during stages of vascular development [20, 21]. In the same context, the in vivo potential of Orlistat and Orlistat+Nitendonib to modulate HHT vascular pathology was assessed. The mice were induced with the HHT pathology by an intraperitoneal (i.p.) injection of anti-BMP9/10 antibodies in the lactating dams of the mother on postnatal day 3 (P3). Alongside the anti-BMP9/10 antibodies given to the pups through the mother, the pups were treated with Orlistat (25 mg/kg/day, i.p.) or Nintendanib (0.3mg/kg/day, i.p.) from P3 to P6 in which they were euthanized for retinal analysis.

The retina was stained with anti-isolectin B4 antibody (green). In low magnification images (4x), the shape of vasculature and the retina vascular length were observed (**Figure 2A**). In order to quantify AVM number, AVM and vein diameter, and the vascular density, the image was captured and analyzed. All measurements and quantifications were done using Fiji software. To hasten the process of retina analysis, I developed a series of macros that allowed for a more efficient means to measure the retinal density around the front of the artery, plexus, and the front of vein regions for each pedal (**Figure 2B**).

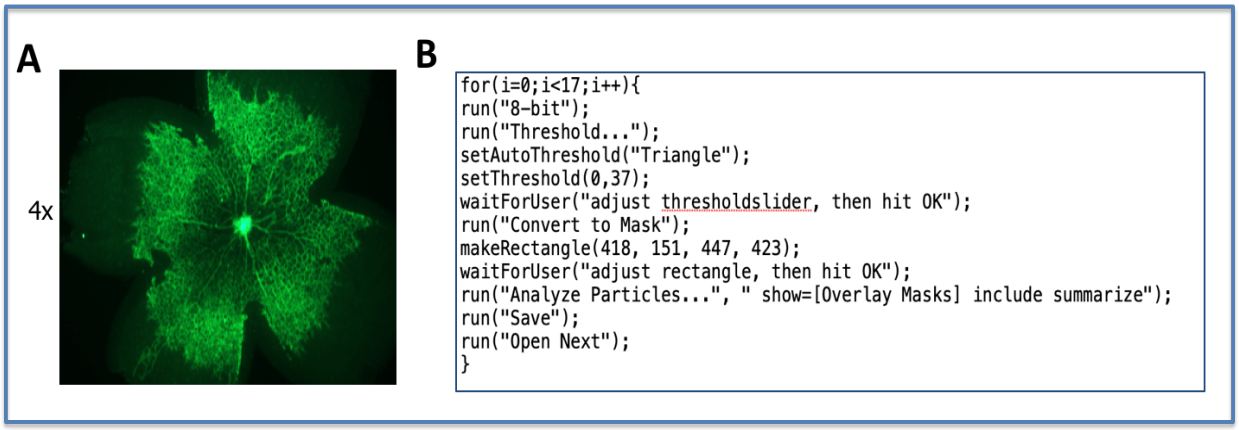

Figure 2. (A) *Representative images of retinas stained with fluorescent isolectin B4 from pups.* ***(4X)*** ***(B)*** *I developed coding sequences to analyze multiple images. A series of Fiji functions were self-programmed to prepare for density analysis. The image was automatically changed to 8-bit, and the density within a rectangle that opens is stored onto an Excel file to calculate intensity in each area. (Author)*

Results show that the delivery of anti-BMP9/10 antibodies through transmammary means was effective in demonstrating HHT pathology in the postnatal retinal vasculature (**Figure 2**). The effectiveness is included by the presence of hypervascularization and the formation of AVMs in between the veins and arteries. Representative images of the entire retina (**Figure 2A**, 4x) and particular three areas for artery, vein, and plexus (**Figure 3A-C**, 20x) are shown. In each, three distinct areas of each image were analyzed by Fiji and ImageJ as described in methods. Orlistat did not significantly reduce vein dilation and area vasculature and the density of the vascular plexus, which was still hypervascular after the treatment of Orlistat alone and Orlistat and Nintendaib (**Figure 3C**). Using my code, the surface area that the vasculature took up within the 200x200um^2 box was calculated. I then found that the Orlistat, Nintendanib+Orlistat, and Nintendanib combination treatments did not change the vascular density that was developed through the inhibition of the BMP9/10 pathways in the pups (**Figure 3, p>0.05**).

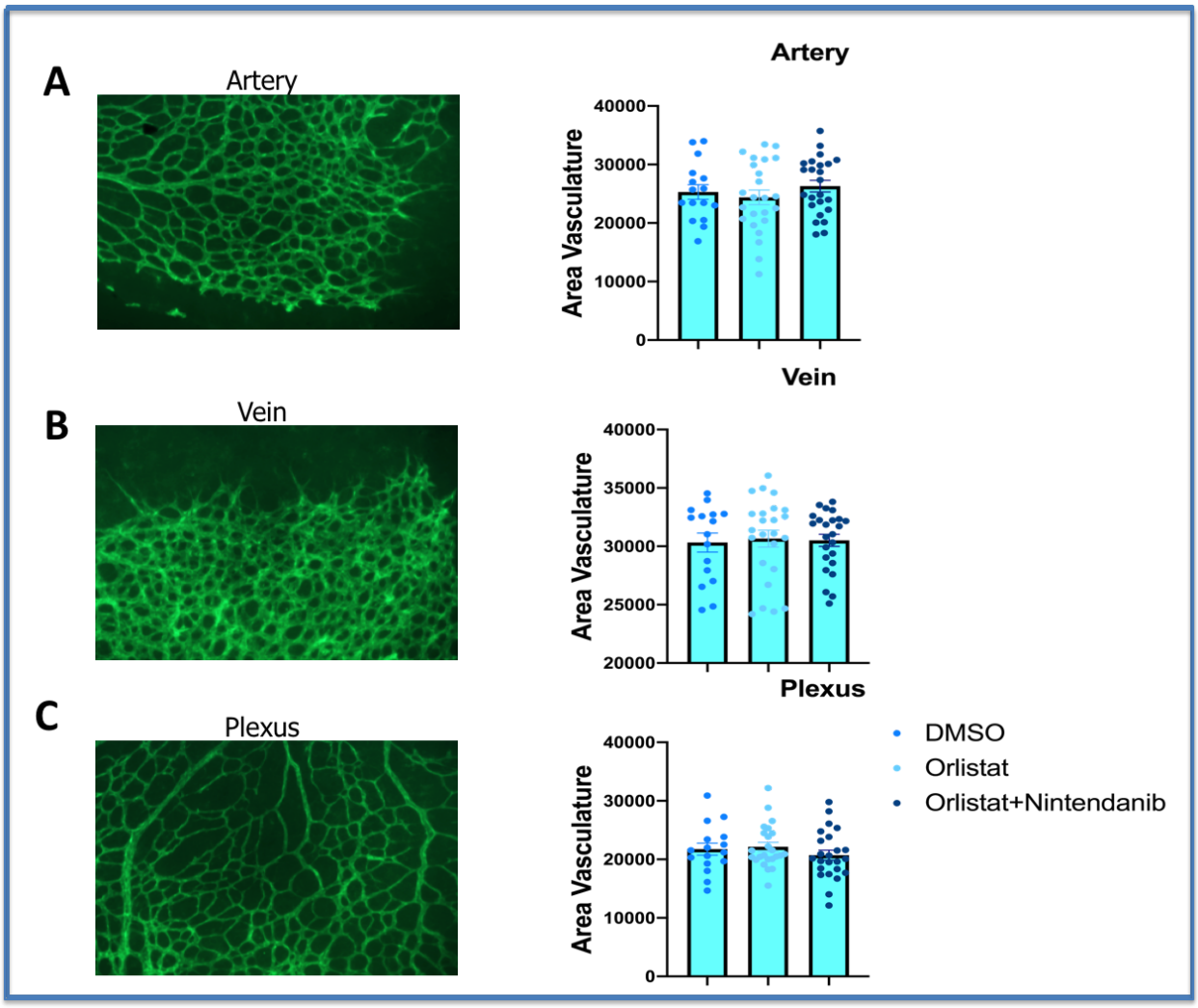

Figure 3. *Orlistat or Orlistat+Nintendanib do not change vascular pathology in the BMP9/10-immunoblocked retina. (A) Representative image of the front artery region of the retina that was calculated alongside the average vascular area of DMSO, Orlistat, and Orlistat+Nintendanib slightly goes down when treated with Orlistat, but in combination goes back up(B)Representative image of the front vein region of the retina that was calculated alongside the average vascular area of DMSO, Orlistat, and Orlistat+Nintendanib remains generally the same with no significance indicated (C) show plexus area between an artery (a) and a vein (v) (D) Image analysis by Fiji program and GraphPad Prism 8. (Author)*

Orlistat+Nintendanib reduced the number of AVMs in the BMP9/10-immunoblocked retina.

Next, I analyzed the number of AVM of BMP9/10-immunoblocked retina treated or not with Orlistat or/and Nintendanib. On average, ~4 AVMs and ~18 um AVM diameter were detected in DMSO control mice (**Figure 4A and B**). Orlistat slightly decreased both the number of AVMs

and their width (**Figure 4C and D**). Strikingly, the Orlistat and Nintendanib combination reduced the AVM number (*p=0.05*) and their diameter (*p=0.006*) by approximately 50% when compared to the DMSO treatment, suggesting that the combination drug treatment has an effect in HHT-like abnormal vascularization.

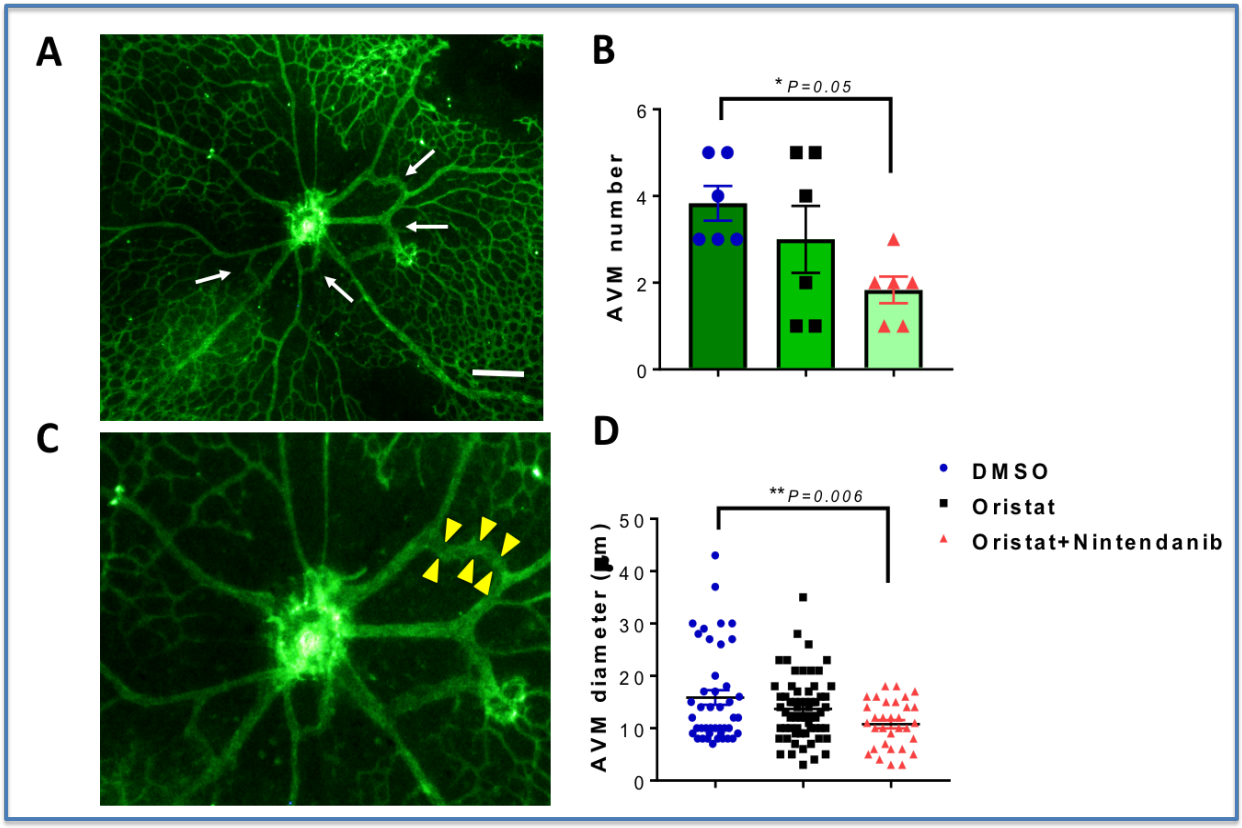

Figure 4. *Orlistat-plus-Nintendanib prevents hypervascularization, and AVMs in anti-BMP9/10 induced HHT mouse retina.* ***(A)****Representative images of retinas stained with fluorescent isolectinB4 (green) from anti-BMP9/10 antibodies treated with DMSO. Arrows in denote AVMs. Scale bar, 200 μm.* ***(B/D)*** *Scatter plots measuring AVM number and AVM diameter for treatments of Orlistat alone and in combination with Nintendanib. Data represent individual retinas and mean ± SEM (n = 18). One-way ANOVA multiple comparison test; *P<0.05, **P<0.01. (Author)*

BMP9/10 inhibition reduces phosphorylation of Smad

I next determined HHT pathology in the form of vascular malformations can be identified in the liver. The liver was selected because it is the most vascularized region; thus, HHT patients often have AVMs in the liver. The inhibition of BMP9/10 was successful by comparing the control

treated with IgG2a/2b with the anti BMP9/10 mice. In this way, I was able to confirm the success of BMP9/10 inhibition induced the phosphorylation of Smad1/5/8. This result allowed for the comparison of the effect of Orlistat and Nintendanib with that of the impact of anti-BMP9/10.

Orlistat plus Nintendanib reverses the effect of BMP9/10 antibodies

I asked whether Orlistat can reduce BMP9-specific Smad1/5/8 signaling. As shown in Figure 5, Orlistat or Orlistat+Nintendanib efficiently ameliorated phospho-Smad1/5/8, but Nitendonib did not change pSmad1/5/8 (**Figure 5A**). As a result, Orlistat by itself acts as a potential trigger for Smad1/5/8 activation. These results are similar to the effect that Tac had on Smad1/5/8 signaling in endothelial cells (15,17). Thus, Orlistat acts as a BMP9-ALK1-Smad1/5/8 signaling cascade activator that is potent.

Interestingly, I found that phosphorylated AKT (P-AKT) was decreased in conditions of specific challenges with BMP9/10, and it is further decreased by Orlistat plus Nitendonib (**Figure 5B**). These results suggest that Orlistat may be a potent Smad1/5/8 signaling regulator, and the combination of Orlistat and Nitendanib may have an additional effect on hypervascularization.

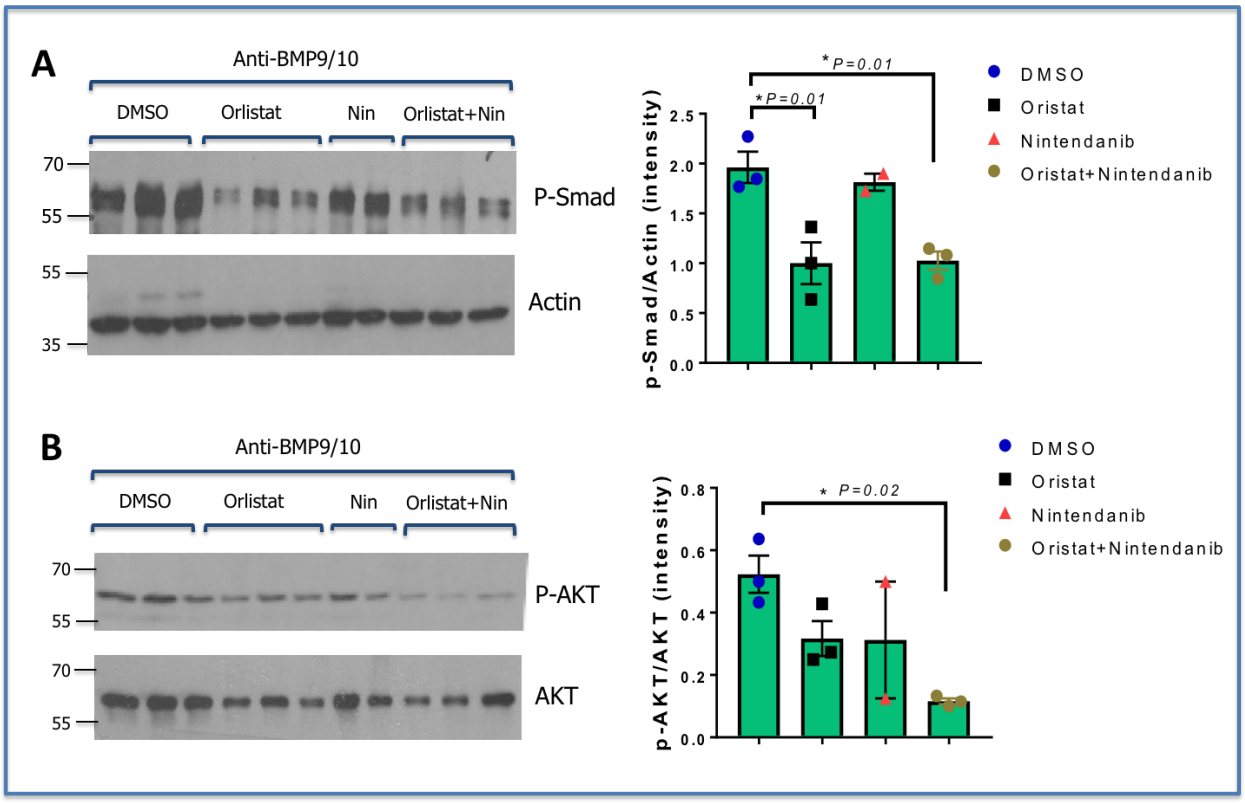

Figure 5. (A and B) *Orlistat + Nintedanib results in decreased Smad and AKT activation. Western blot from liver lysates. Antibodies for Phospho-Smad1 (Ser463/365)/Smad5 (Ser463/465)/Smad8 (Ser465/467), Phospho-AKT (Ser473), AKT and Actin were used. One-way ANOVA multiple comparison test; *P<0.05 (Author).*

4. Discussion & Limitations

My study shows that Orlistat is a potent activator of Smad1/5/8 signaling in mouse models of HHT (**Figure 2**). The density analysis demonstrates that an Orlistat treatment results in the down-regulation of Smad1/5/8 in which the bands resemble those of wild type mice without anti-BMP9/10. When compared to the control-treated with DMSO, there is a significant change (p=0.01), indicating that Orlistat by itself has a positive effect on reducing HHT pathology. Additionally, Nintedanib, by itself, did not result in a significant change in Smad1/5/8 signaling, however, when treated alongside Orlistat (**Figure 5A**), it did result in the Smad1/5/8 signaling going down (*p=0.01*). Not only did the Orlistat treatment greatly improve HHT vascularity, it also readjusted the gene expression and signaling defects that were caused by ALK1 inhibition

(**Figure 4**). These findings may suggest that Orlistat has therapeutic potential in HHT. The down signaling of AKT, in which Orlistat and Nintentanib, by itself, significantly decreased density and when treated alongside each other the effect was even stronger (*p=0.02*). This suggests that for AKT signaling, Orlistat and Nintendanib both have a role in reducing HHT pathology, however when both are used, the impact is much greater. (**Figure 5B**, *p=0.02*). Therefore, the data suggests beneficial interaction and synergy between Orlistat and Nintendanib treatments in HHT mice.

Moreover, the vein and plexus areas of the retina suggests that Orlistat + Nintendanib does reduce HHT pathology but not significantly **(Figure 2).** The study indicates that Orlistat + Nintendanib may reduce HHT pathology; a higher concentration may be used. Further studies must be conducted. The maintenance of the BMP9/10 and ALK1 signaling is required for the development of normal vasculature. In line with these studies, I show that Smad1/5/8 signaling activation with Orlistat or BMP9 had the opposite effect. Thus these results suggest that ALK1 plays a central role in the transcriptional regulation of angiogenesis and highlights how the loss of function of ALK1 is associated with the deregulation of angiogenesis [22]. Therefore, it appears that AKT may control Smad signaling via several independent pathways.

In a mouse model of HHT, it has been demonstrated that HHT vascular pathology is prevented through P13K/Akt inhibition, where a BMP9/ALK1 blockade results in the overactivation of P13K/Akt [23]. Although I measured Smad and AKT activation to determine the inhibition of Orlistat/Nintendonib under BMP signaling (**Figure 5**), the downstream pathway of Smad and AKT signaling is unclear. The data suggests that Orlistat treatment alone might similarly reduce the Smad and Akt activation, but Nintendanib alone did not change their activation (**Figure 5**). The combination of Orlistat and Nintendanib dramatically alter the activation of AKT activation and AVM (**Figure 4 and 5**). It is not clear whether or how VEGFR control Smad1/5/8 and mTOR remains unclear (**Figure 1**). HHT pathogenesis requires the overactivation of the mTOR and VEGFR2 pathways [24]. Another aspect that I can test to determine the effect of Orlistat+Nintendanib is through the use of a bleeding assay. By detecting the amount of blood

within the tissues of the treated and untreated anti-BMP9/10 mice, the effectiveness of the drug can be analyzed through measuring the amount of blood that is caused by vascular malformations.

ID1 is induced by BMP-Smad signaling [21]. Thus, ID1 may be the other possible target of BMP signaling. I would like to try to see the expression level of ID1 when Smad was phosphorylated. If I could measure ID1 directly, it would be a great strategy to detect BMP signaling in this HHT system and a good screening gene to test other candidates. To understand complex signaling pathways, I could not use entire liver tissue or retina; thus, Human Umbilical Vein Cells (HUVEC) cell line may be helpful for Western blot. I may manipulate the specific receptor signaling using siRNA for VEGFR or BMP receptors to distinguish specific signal transduction.

Given more time, the manipulation of the dosage of both Orlistat and Nintendanib would provide valuable information to determine the optimal treatment that maintains a low mortality rate yet also results in the highest rate of restoration of HHT pathology.

5. Conclusion and Future Studies

I used a reliable model to study HHT pathogenesis and provides pre-clinical therapeutic investigations for HHT. Although the vascular density was not significantly altered, the combination of Orlistat+Nintendanib resulted in a significant reduction of AVMs and a decrease in the width of the AVMs (**Figures 3 and 4**). Moreover, the data indicates that Orlistat+Nintendanib is able to normalize Smad1/5/8 signaling in comparison to the BMP9/10 inhibited mice (**Figure 5**). I propose that the Orlistat+Nintendanib combination has therapeutic potential in HHT. My contributions in this study will be expanded to test many other FDA-approved drugs measuring retina vasculature formation.

From continuous research around the world, significant strides have been made in surgical procedures such as cauterization and embolization to assist in reducing the effect of HHT in patients [23]. Despite this, there still is no cure for the disease, and as a result, it remains to be

potentially life-threatening if not continuously treated for. Several therapies have been proposed in preclinical models and some of them are currently being investigated in clinical trials [23]. Notably, Tac showed some efficacy in reducing pathology in HHT model and patients [24,25].

The primary reasoning behind the usage of Orlistat and Nintendanib in this study is their availability and safety when compared to the alternative drug treatment options available to patients. However, Nintendanib is still known to have side effects in large doses (>300mg/day) [26]. The side effects include abdominal pain, diarrhea, nausea, and vomiting. While the results are not as severe, they're details that need to be further elaborated and studied when used alongside Orlistat if this treatment were to be publicly used. Furthermore, because a mouse model was used, human testing will ultimately be a necessary step for HHT treatment.

In terms of therapeutic approaches, the drug dosing and circulating concentration needs to be considered carefully. In line with the observed effects on these targets in BMP9/10-immunoblocked mice, optimal concentrations of Orlistat and Nintendanib are therefore expected to inhibit *in vivo* mTOR and VEGFR2. Moreover, drug delivery, such as cellular uptake and accumulation *in vivo* is also essential. These parameters can significantly reduce serum concentration while increasing local drug delivery. Altogether the administered drugs reach concentrations within the expected therapeutic range are need to be considered.

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