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Identification and characterization of small-molecule inhibitors of Tie2 kinase

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Abstract **Angiopoietins and Tie2 receptor were recently identified as an endothelial cell-specific ligand-receptor system that is critical for vascular development and postnatal pathologic angiogenesis by mediating vascular integrity. In this study, we identified a series of small-molecule Tie2 inhibitors, which blocked Ang1-induced Tie2 autophosphorylation and downstream signaling with an IC_{50} value at 0.3 μM. Further optimization yields improved selectivity, aqueous solubility, microsomal stability and cytochrome P450 profile for one of the compounds (compound 7). Both compound 1 and compound 7 inhibit endothelial cell tube formation. Furthermore, in a rat model of Matrigel-induced choroidal neovascularization, compound 7 significantly diminished aberrant vessel growth. Our findings demonstrate a potential clinical benefit by specifically targeting Tie2-mediated angiogenic disorders.**

Keywords: Tie2; Small-molecule inhibitor; Solubility; Stability; CYP450, selectivity; Angiogenesis; Choroidal neovascularization

1. Introduction

Abnormal growth of new blood vessels is involved in promoting different ocular pathogenesis including diabetic retinopathy and age-related macular degeneration, the two most frequent causes of severe and progressive vision loss [1]. Tie2 and angiopoietins are upregulated in the retina from patients with ischemic retinal disorders [2]. Recent studies demonstrated that Tie2 plays a key role in mediating both retinopathy and choroidal neovascularization. Immunohistochemistry with a monoclonal antibody to human Tie2 showed a prominent expression of Tie2 around and within the base of newly formed blood vessels of retinal and choroidal neovascular lesions. Soluble Tie2 receptor inhibits experimental retinal and choroidal neovascularization [1,2]. Systemic soluble Tie2 expression also inhibits and regresses corneal neovascularization [3].

Two families of receptor tyrosine kinases, VEGFR1/VEGFR2 and Tie2, have been shown to be critical factors in controlling the aberrant vascular development by interacting with their respective ligands, VEGFs and angiopoietins (Ang1 and Ang2) [4]. Interestingly, accumulating evidence suggests that Tie2 activation, through collaboration with VEGFR, regulates the pathological angiogenesis in a synergistic fashion [5], and combination of soluble Tie2 and VEGFR additively suppresses retinal angiogenesis [2]. These results indicate that the Tie2 signaling network, along with VEGF/VEGFR, is an important cascade in the induction and progression of the aberrant angiogenesis in ocular disorders. Inhibition of Tie2 signaling may provide a potentially viable therapeutic strategy in the treatment of ocular diseases due to the abnormal neovascularization.

In this study, approximately four million compounds, generated through Encoded Combinatorial Libraries on Solid Support (ECLIPS™) technology [6], were screened in an attempt to identify selective small-molecule Tie2 kinase inhibitors. Through the screening and subsequent medicinal chemistry efforts, a selective small-molecule Tie2 inhibitor was identified (compound **7**). A rat choroidal neovascularization model was used to investigate the in vivo efficacy of the compound. Our results clearly demonstrated that the Tie2 inhibitor markedly block the aberrant blood vessel formation in this animal model.

2. Materials and methods

2.1. Cell culture

Human aortic endothelial cells (HAECs) were maintained in endothelial basal media (EBM) (BioWhittaker, Baltimore, MD) supplemented with 10% fetal bovine serum and the EGM kit containing growth factors (BioWhittaker). HAECs were used in cell-based assays between passage 6 and passage 8.

2.2. High throughput screen for Tie2 kinase inhibitors using a homogeneous time-resolved fluorescence (HTRF) assay

The assay was performed in 384-well black Costar plates. Substrate mix containing final concentrations of 1.5 μg per ml of biotinyl poly-Glu, Ala, Tyr and 1 μM ATP in assay buffer (50 mM HEPES, pH 7.4, 0.2 mM Na_3VO_4, 1 mM DTT, 25 mM $MgCl_2$, 5 mM $MnCl_2$, 0.01% BSA and protease inhibitor cocktail) was added to each well followed by adding Tie2 enzyme to initiate the reaction. After incubation for 75 min at room temperature, the reaction was terminated by adding stop/detection solution containing streptavidin-APC and Europium-anti-phospho-tyrosine antibody. The plates were read by a cooled CCD imager (ViewLux, Perkin Elmer, Boston, MA) at both 615 nm and 665 nm. The screening data were analyzed using the ratio of the 665 nm to 615 nm fluorescence signal.

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2.3. Treatment of HAECs with Ang1 and Tie2 inhibitors

Ang1 was purchased from R&D Systems. Before use, Ang1 was pre-incubated with anti-poly-histidine antibody (R&D Systems, Minneapolis, MN) to form dimers prior to the treatment of HAECs. Equal numbers of HAECs were grown to confluent monolayer and starved with serum-free EBM medium for 6 h. HAECs were pretreated with Tie2 inhibitors or the carrier DMSO (final concentration at 0.5%) for 1 h followed by the stimulation with dimerized Ang1 for 10 min at 37 °C. The same amount of protein was loaded into each well of SDS–PAGE.

2.4. Microsomal stability analysis

The compound stability in human liver microsome was evaluated as described previously [7].

2.5. CYP450 inhibition assay

Major isoforms of recombinant CYP450 (3A4, 1A2, 2C9, 2C19, and 2D6) and assay kits were obtained from the Gentest (BD biosciences, Bedford, MA). Eight different compound concentrations were used for the evaluation of inhibitory effect of the compounds on CYPs. The IC_{50} values against each CYP450 isoform were determined in duplicate for the test compounds by monitoring the production of fluorescent metabolite as described by the manufacturer.

2.6. Solubility study

Aqueous solubility analysis was performed by Absorption Systems (Exton, PA). Briefly, the test article was accurately weighed and suspended in pH 7.4 phosphate buffer. The sample was agitated for approximately 2 h and allowed to stand overnight followed by filtration through a 0.45 μm nylon syringe filter. Dilutions of the test compounds were made in 80% buffer/20% acetonitrile from a 10 mM stock solution in DMSO to achieve 1, 0.3, 0.1, 0.03, and 0.01 μM final concentrations. The standards and filtrates were assayed by LC/MS using a generic gradient method.

2.7. Endothelial cell tube formations

The tube formation assay was conducted as described [12]. HUVECs at 30 000 cells/well, were cultured on 300 μl growth factor reduced Matrigel (BD biosciences) in M199 and 0.5% fetal calf serum. The control wells included either no Ang1 or 100 ng/ml Ang-1, and the treated well contains both Ang-1 and 5 μM of the test compound. The cells were incubated for 18 h at 37 °C, 5% CO_2 and were fixed with 2.5% glutaraldehyde followed by staining with 0.02% toludine blue. The gel was dried prior to imaging. The tube length was quantified using the NIH image J software. Results are represented as total tube length (mm) for three photographic fields per experimental condition. Each treatment was performed in duplicate and the experiments were repeated three times.

2.8. Rat model of choroidal neovascularization (CNV)

Sprague–Dawley rats (female, 2 months old) were purchased from Harlan Laboratories (Indianapolis, IN). Subretinal injections of Matrigel (growth factor reduced, BD Biosciences) were performed on the temporal side of the eye under a microscope. Rats were anesthetized by intraperitoneal injection of ketamine (40 mg/kg) and xylazine (6 mg/kg). The sclera was exposed and an incision was made between the limbus and the equator with a sharp #33 needle to reach the subretinal space. The tip of a blunt #33 needle attaching to a Hamilton micro-syringe was introduced to the incision at a shallow angle toward the posterior pole and inserted 0.1–0.2 mm into the subretinal space for injection. Matrigel was diluted with phosphate buffered saline (vehicle) or compound **7** (final concentration: 10 μg/μl) in the vehicle at 3:1 (75% gel). Gel solution (1.2 μl) was slowly injected so the solution pushed its way into the subretinal space. Injected solution normally solidified within minutes and formed a bleb of 1.5–2 mm in diameter with the injection site at the anterior edge.

Blood vessels were directly labeled with a solution containing a fluorescent carbocyanine dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Sigma–Aldrich, St. Louis, MO). Animals were sacrificed by CO_2 overdose and perfused with PBS, followed by a solution containing DiI. The animal was subsequently perfused with 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4). Eyes were harvested and the anterior segments removed. The eyecups were post-fixed in the same fixative overnight and then transferred to PBS at 4 °C. For each eye, serial sections were obtained for the entire Matrigel injected area. Typically, 20–24 sections (100 μm of each) of each eye were used for analysis. Tissue was embedded in 5% agarose. Thick (100 μm) serial sections were cut on a vibratome (VT1000S, Leica Microsystems, Bannockburn, IL), mounted on glass slides with 80% glycerol, and examined by confocal microscopy. CNV index of an eye was calculated according to the following equation: $C = T\sum_{i=1}^{n} Wi$, where C is the CNV index, T is the thickness of a section, W is the measured width of CNV in a particular section, and i is the series number of the section. Each section has its own CNV index, which varies from section to section in the same eye. The index for the whole eye is the sum of all serial sections. Statistical analysis was performed and data are presented as mean ± S.D. Student's t-test was used to evaluate the difference of CNV indexes between the control and each treated group.

3. Results and discussion

3.1. Discovery and characterization of small-molecule inhibitors of Tie2

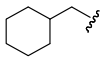
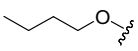
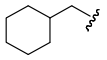
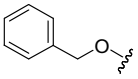
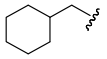
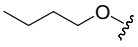
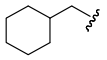
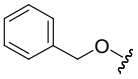
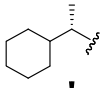
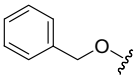
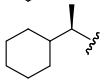
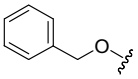
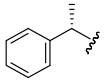
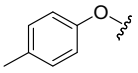
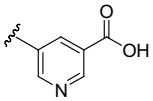
We established a Tie2 kinase assay using recombinant GST Tie2 kinase domain fusion protein as the source of the enzyme. Enzyme kinetic studies of the recombinant Tie2 kinase domain revealed that the apparent K_m for ATP was 60 μM (data not shown). To conduct high throughput screening of approximately four million compounds divided among 89 combinatorial libraries, a HTRF assay was established using biotinyl poly-Glu, Ala, Tyr as the substrate. The ATP concentration was adjusted to 1 μM in the screen. Tie2 inhibitors were identified from one specific library based on a glycinyl carbamate, and incorporating a key biaryl substituent. Several analogs were generated in this active series with examples shown in Table 1 and supplementary Fig. 1. Compounds incorporating a butyl carbamate (compound **1**) and benzyl carbamate (compound **2**) produced concentration-dependent inhibition of Tie2 kinase activity with IC_{50} values of 3.2 μM and 2.1 μM, respectively. In all active compounds, the biaryl moiety was present as a *para*-(pyridin-3-yl)benzyl (compounds **1**, **2** and **7**) or a *para*-(pyrimidin-5-yl)benzyl substituent (compound **5**) of the glycinyl nitrogen, providing a hydrogen bond acceptor at the 3-position of the terminal aromatic ring. The importance of the 3-position hydrogen-bond acceptor within the biaryl component (R^3) is demonstrated since the phenyl substituted (compound **3**) and the 4-pyridyl (compound **4**) analogs showed only weak activity (Fig. 1A and Table 1).

Compound **2** was selected from the series to investigate the inhibitory mechanism of Tie2 kinase activity. Kinetic experiments were performed by varying the ATP levels at several inhibitor concentrations. The results suggest that compounds within this series competitively interact with the Tie2 kinase domain at the ATP binding site (Supplementary Fig. 2).

3.2. Specific blockade of Ang1-triggered Tie2-mediated signaling cascade

Studies have demonstrated that activation of the Tie2 receptor by Ang1 leads to auto-phosphorylation of Tie2 [8,9]. Since Tie2 is predominantly expressed and functions on endothelial cells, HAECs, which express high levels of the Tie2 receptor, were chosen to evaluate the cellular functions of the Tie2 inhibitors. We assessed the effect of compound **1** on Tie2 auto-phosphorylation. It was demonstrated that compound **1** blocked Tie2 auto-phosphorylation in a concentration-dependent

Table 1
Blocking in vitro Tie2 kinase activity by small-molecule Tie2 inhibitors

Compound	R^1	R^2	R^3	K_i binding (μM)
1				3.2 ± 0.7
2				2.1 ± 0.4
3				>100
4				>80
5				1.2 ± 0.1
6				>100
7				1.3 ± 0.2

manner (Fig. 1B). The IC_{50} value of the compound was approximately 0.3 μM in this cellular assay. Compound **3**, an analog of compound **1**, which does not inhibit Tie2 activity in the kinase assay (IC_{50} > 100 μM), showed little inhibitory effect in the same cell-based assay (Fig. 1C). Stimulation of Tie2 receptor by Ang1 further leads to the activation of its downstream signaling pathway through p42/p44 Erk and Akt [8,9]. Ang1 was previously shown to activate the MAPK signaling cascade in human umbilical vein endothelial cells (HUVECs). The pharmacological inhibition of Erk activation with the MEK inhibitor, PD98059, suppressed Ang1-induced migration [10]. A short synthetic peptide, identified as a specific blocker of Tie2 and Ang1/Ang2 interaction, was shown to abolish the phosphorylation of p42/p44 Erk induced by Ang1 [8]. Therefore, the compound effect on these downstream cellular events was also investigated. Consistent with the previous findings, we observed a strong activation of p42/p44 Erk and Akt after Ang1 induction, and these activities were attenuated by the small-molecule Tie2 inhibitors. As shown in Fig. 1C, at 10 μM compound concentrations, compound **1** completely abolished the activation of p42/p44 ERK and Akt induced by Ang1, whereas the inactive analog, compound **3**, displayed little activity on the cellular Tie2 signal transduction pathways. Two additional analogs were also investigated for their ability to block Ang1/Tie2 signaling in HAECs. Compound **5** and compound **6** correspond to the *S*- and *R*-enantiomers of the α-methylated cyclohexylmethyl amides, respectively. Inhibition of Tie2 kinase activity was demonstrated with the *S*-isomer (compound **5**) at an IC_{50} value of 1.2 μM versus the inactive *R*-isomer (compound **6**) with an IC_5 over 100 μM (Table 1). In agreement with the kinase activities, compound **5** completely blocked Ang1-induced Tie2 receptor autophosphorylation along with activation of p42/p44 Erk and Akt when screened at 1 and 3 μM (Fig. 1D). The Tie2 inactive analog, compound **6**, did not block the Ang1 induction of Tie2 receptor phosphorylation. Thus, compounds within this series show a requirement for 3-position nitrogen at R^3 and a specific chirality at R^1 for their inhibitory effects, which are reflected both at the kinase and cell-based functional levels of the Tie2 receptor.

To further investigate that the inhibitory effects of compound **1** were specifically through the Tie2-associated pathway, cellular assays were performed to monitor the activation of two other receptor tyrosine kinases, EGFR and c-Met, induced by their cognate ligands, EGF and HGF, respectively, using the same cellular system as Tie2. Similar to the stimulation by Ang1, both EGF and HGF were capable of inducing their receptor autophosphorylation and the phosphorylation of p42/p44 Erk and Akt. However, although compound **1** at 10 μM showed strong inhibition on Ang1-induced Tie2 receptor, p42/p44 Erk and Akt phosphorylation, no inhibitory effects were observed on EGFR or c-Met receptor phosphorylation and downstream ERK and Akt activation. As a control, PD98059, a MAP kinase kinase (MEK) inhibitor, was included in these studies and, as anticipated, the compound displayed a significant inhibition on p42/p44 ERK phosphorylation triggered by all three receptors including Tie2, EGFR and c-Met with little effect on Akt activation (Fig. 2A–C). These results indicated that the observed effect of compound **1** was most likely through specific inhibition of Ang-1-triggered Tie2 activation pathway and not through its effect on other kinases or cytotoxicity. Consistently, no cytotoxicity was observed for compound **1** at concentration up to 50 μM using PC3 and THP1 cells (data not shown). However, subsequent studies in a kinase selectivity panel indicated that compound **1** blocked JNK1 and VEGFR2 with IC_{50} values of 3.9 μM and 6.6 μM, respectively. To selectively target Tie2 signaling pathway, further improved kinase selectivity was desired. Compound **7** was identified and possessed an IC_{50} value of 1 μM against the Tie2 receptor in the kinase assay (Table 1). Further cell-based studies also indicated that the compound inhibited Ang1-induced Tie2 autophosphorylation and p42/p44 ERK phosphorylation with IC_{50}value of approximately 0.3 μM (data not shown and Fig. 1E). No cytotoxicity was observed for compound **7** up to 50 μM using PC3 and THP1

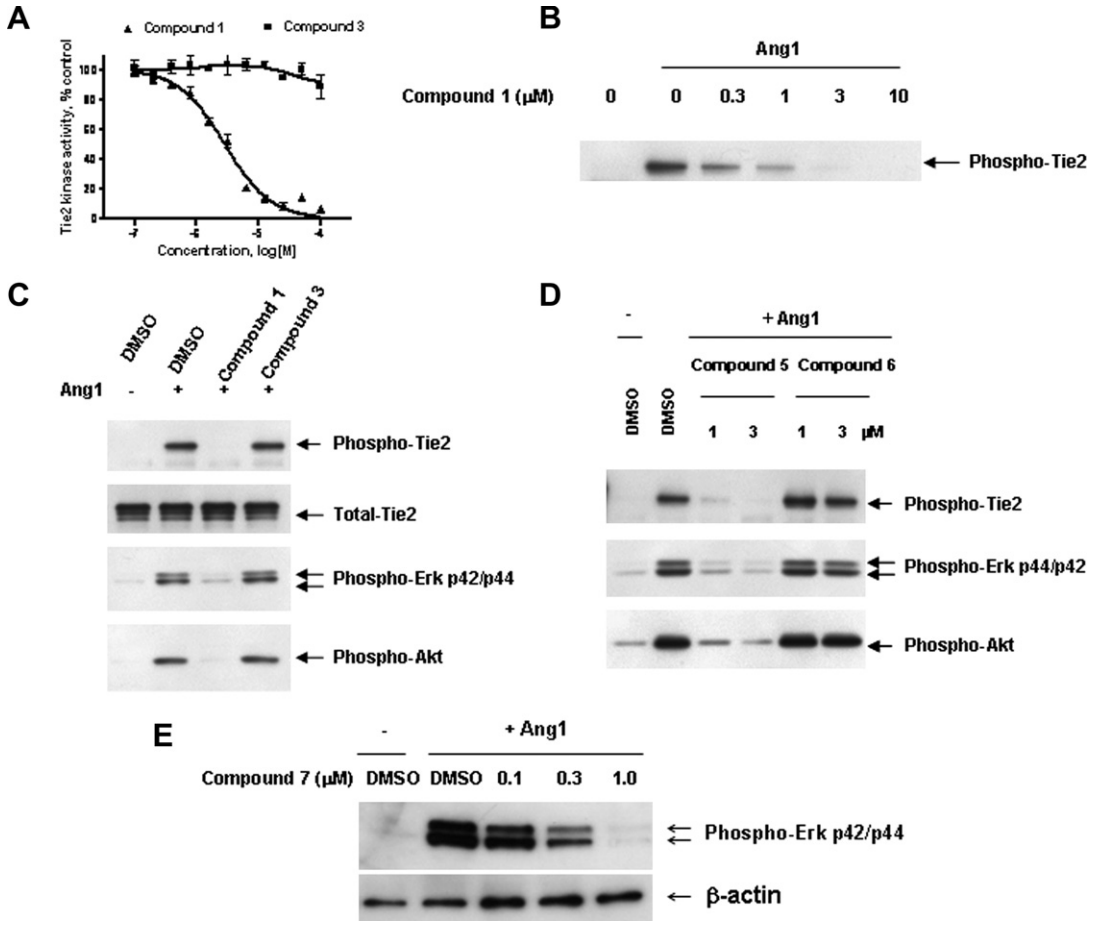


Fig. 1. Inhibitory effect on Tie2 auto-phosphorylation and its downstream signaling events in response to Ang1 stimulation. (A) Dose dependent inhibition of in vitro Tie2 kinase activity. Various concentrations of compound **1** or compound **3** were included in the Tie2 kinase assay in the presence of 1 μM ATP. (B) Inhibition of Ang1-induced Tie2 auto-phosphorylation by compound **1**. Various concentrations of the compound were incubated with HAECs for 15 min followed by incubating with Ang1 for an additional 15 min. Tie2 was immunoprecipitated, and its phosphorylation was detected by immunoblot using an anti-phospho-tyrosine antibody. The arrowhead denotes phosphorylated Tie2. The estimated IC_{50} value was approximately 0.3 μM in this cellular assay. (C) Effect of Tie2 inhibitors on the activation of Tie2, p42/p44 Erk and Akt by Ang1 in HAECs. HAECs were stimulated with 200 ng/ml Ang1 for 15 min. Phosphorylation of Tie2 and its downstream signaling proteins, p42/p44 Erk and Akt, were detected by immunoblot using ant-phosph-p42/p44 Erk and anti-phospho-Akt antibodies, respectively. The effect of compound **1** or compound **3** on Ang1-induced activation pathways were evaluated by incubating HAECs with an active Tie2 inhibitor (compound **1**) or an inactive Tie2 compound (compound **3**) at 10 μM concentration for 15 min, prior to the stimulation by Ang1 for an additional 15 min. (D) Inhibition of Ang1-induced phosphorylation of Tie2, Akt and p42/p44 Erk. HAECs were investigated in the presence of compound **5** or compound **6**, a pair of enantiomers, at 1 and 3 μM for 15 min, followed by stimulating with Ang1 for an additional 15 min. Equal amount of total cell lysates or immunoprecipitated proteins were separated by SDS–PAGE under reducing conditions. Phosphorylation of Tie2, p42/p44 Erk and Akt was measured by immunoblotting. (E) HAECs were treated with various concentrations of compound **7** for 15 min, prior to incubating with Ang1 for an additional 15 min. Phosphorylation of p42/p44 Erk was analyzed by anti-phospho-p42/p44 Erk immunoblot. The estimated IC_{50} value is 0.3 μM. The blot was also detected with anti-β-actin antibody to ensure the equal loading. The experiments were repeated three times.

cells. Importantly, compound **7** showed selectivity against all 17 tested protein kinases up to 20 μM compound concentrations (Supplementary, Table 1).

3.3. Improved physicochemical properties for compound 7

Several other key features of compound **1** were also found to be suboptimal for in vivo assessment, including poor in vitro human liver microsome (HLM) stability, a marginal cytochrome P450 inhibition profile and low aqueous solubility (Table 2). Significantly improved HLM stability was achieved with compound **7**, showing over 60% of parent compound remaining, whereas compound **1** was rapidly metabolized with 0% remaining under the same condition. In contrast to the strong inhibition on different CYP P450 isoforms by compound **1**, no significant inhibition on a CYP was observed for compound **7** up to 20 μM compound concentration. Furthermore, compound **7** demonstrated over 150-fold increase in aqueous solubility compared with compound **1**. These improvements are critical for further evaluating the compound in vivo efficacy using animal models.

3.4. Reduced tube formation upon treatment with compound 1 and compound 7

Ang1, through interaction with Tie2, has been shown to promote sprouting, cell survival and migration of endothelial cells [11–13]. Ang1 was also demonstrated to have direct effect on endothelial cell behavior during vascular remodeling. In the presence of Ang1, endothelial cells formed a well-organized network of non-fragmented, cordlike vascular structures on the Matrigel surface [14]. In this study, the effect of Tie2 inhibitors,

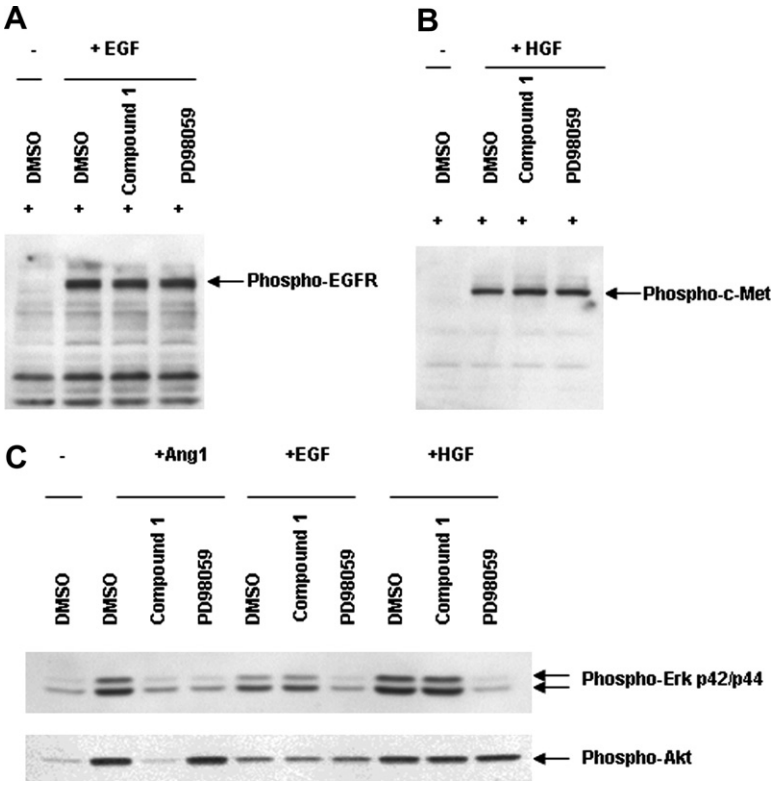


Fig. 2. Selectivity of compound **1** in cellular assays. HAECs were pretreated with compound **1** at 10 μM and PD98059, a MEK inhibitor, at 5 μM, or the vehicle, 0.5% DMSO, for 15 min. HAECs were then stimulated with Ang1(200 ng/ml), EGF (10 ng/ml), or HGF (20 ng/ml) for an additional 15 min. Phosphorylation of EGF receptor (A), c-Met (B), p42/p44 Erk and Akt (C) was detected by immunoblotting as described under Section 2. Data are representative of three experiments.

Table 2
Improved CYP profile and solubility of compound **7** versus compound **1**

Compound	Compound **1**	Compound **7**
Human liver microsomes (% of remaining)	0	66
IC_{50} for CYPs (1A2, 2C9, 2C19, 2D6, 3A4)	3A4 = 1 μM, 2C9 = 5.9 μM 2C19 = 1.6 μM 1A2 > 20 μM 2D6 = 2 μM	> 20 μM
Aqueous solubility	0.4 μM	60 μM

compound **1** and compound **7**, on Ang1-induced endothelial network formation was investigated using HAECs in Matrigel system. The presence of Ang1 stimulated the capillary network formation of endothelial cells on the Matrigel, resulting in more robust tube-like structures compared with the control without Ang1. Compound treatment resulted in statistically significant inhibition on the tube formation (Fig. 3A and B). Interestingly, compound **1** treatment results in more severe disorganized vasculature in comparison with the effect of compound **7**. This is likely due to the broader activity of compound **1** with IC_{50} value around 6 μM against VEGFR2. The source of VEGF likely came through endogenous secretion from HAECs [15]. Studies have shown that induction of tube formation by Ang1 is at least partially dependent on the presence of VEGF [16]. Our current results appear to support this conclusion. Additional studies are needed to provide further clarification. It was found that the Ang1-promoted tube formation was primarily due to suppression of apoptosis of endothelial cells in a PI3-kinase-dependent manner [13]. Interaction of Ang1 and Tie2 leads to the activation of Akt, which is an important mediator of cell survival and motility. Studies have shown that Akt is required and sufficient to regulate endothelial cell survival in response to growth factor depletion. Blocking Akt diminished Ang1-mediated endothelial cell survival, a process that is essential for the tube formation mediated by Ang1/Tie2 interaction [17]. Furthermore, activating Akt rescues a Tie2 blockade-induced apoptosis of endothelial cells, and soluble Tie2 receptor has been shown to promote apoptosis of endothelial cells in culture [16]. Although stimulation of Tie2 with Ang1 leads to Akt and MAPK/ERK activation, blocking MAPK/ERK activity with a specific inhibitor PD98059 did not affect endothelial cell survival [17], indicating that Akt is the dominant mediator of Ang1-induced cell survival. In addition, it was also shown that Akt activity is required for endothelial cell sprouting in a three-dimentional collagen gel induced by Ang1 [18]. Similar to the effect of Tie2 blockade for the cellular inhibitory effects on the Ang1/Tie2 signal transduction pathway, administration of Tie2 blockers, including dominant negative Tie2 receptor or inhibitory peptide, resulted in inhibition of angiogenesis in chick chorioallantoic membrane (CAM) assay, retinal/choroidal neovascularization and tumor growth [1,19–22]. Our study demonstrated that compound **1** and compound **7**, attenuated the Ang1-induced Tie2-associated signaling network including activation of p42/p44 Erk and Akt (Fig. 1C and D) and further

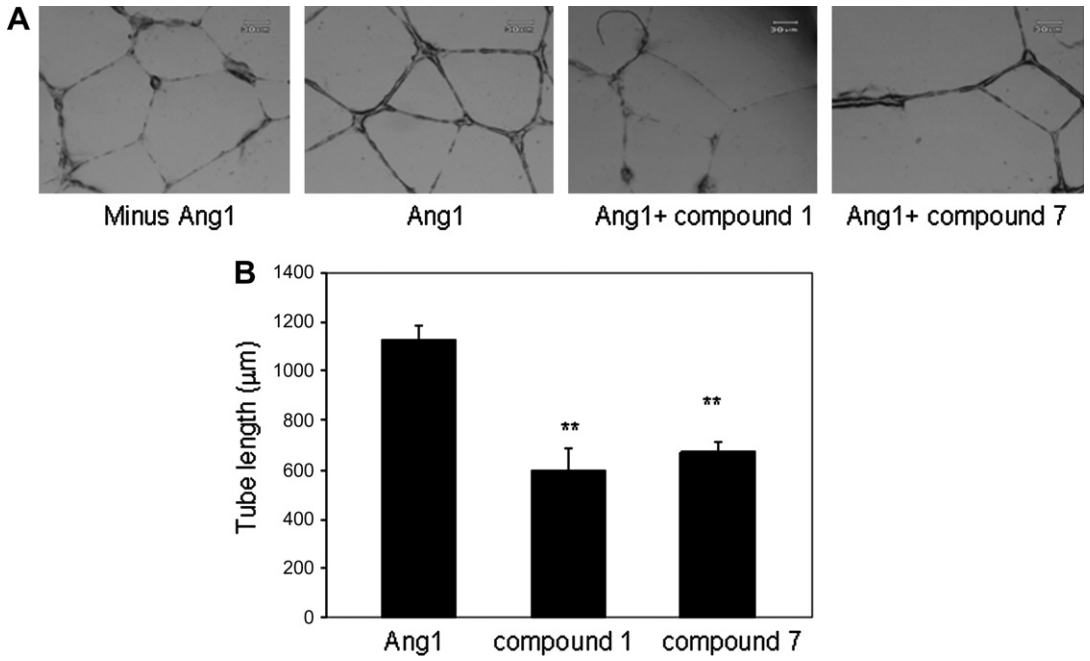


Fig. 3. Inhibition of tube formation by compound **1** and compound **7**. HAECs were seeded on Matrigel in the absence or presence of Ang1 only (control), and in the presence of Ang1 and 5 μM compound **1** or compound **7**. The cells were incubated at 37 °C and 5% CO_2 for 18 h. The morphological changes of the cells were observed and photographed under microscope. (A) Representative images of the tube formation of HAECs when treated with compound **1** or compound **7**. (B) Quantitative data on the effect of compound **1** or compound **7** on the tube formation of HAECs. $^{**}P < 0.01$ compared to control, $n = 3$.

resulted in the blockade of endothelial tube formation mediated by Tie2. These results are consistent with other studies showing a critical role of the angiopoietins/Tie2 receptor pathway in mediating vascularization [18,19,22].

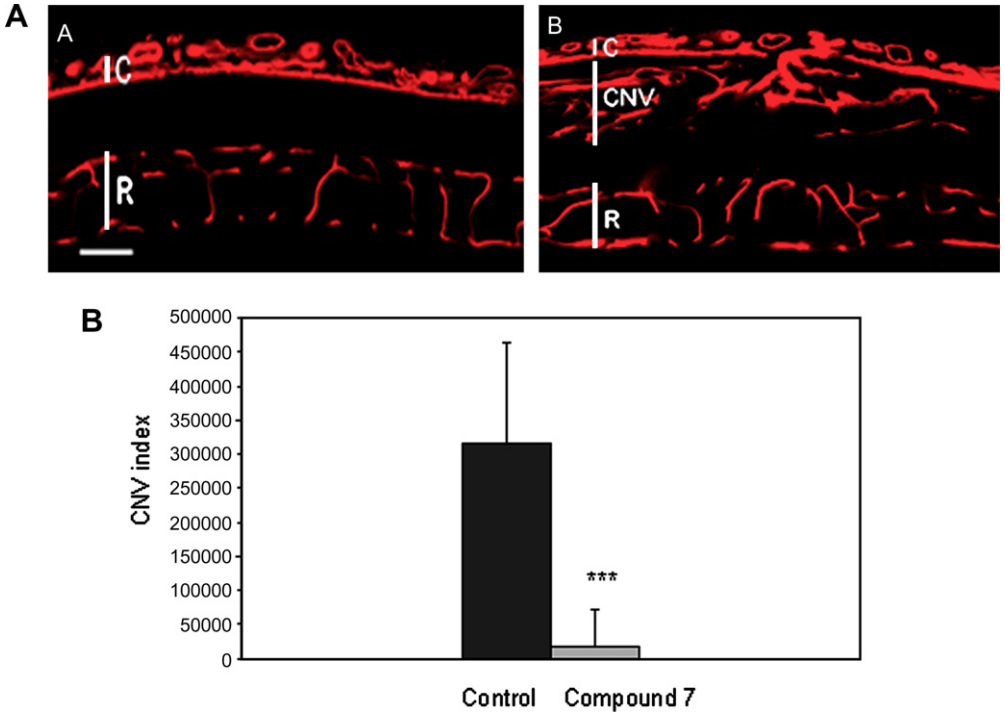


Fig. 4. Attenuated Matrigel-induced rat choroidal neovascularization by compound **7**. (A) The network of choroidal (C) and retinal (R) blood vessels in a normal retina are shown in panel A. A dense network of new blood vessels originated from choriocapillaris (CNV) invaded the Matrigel layer 10 days after subretinal injection of Matrigel (panel B). Scale bar: 100 μm. (B) Efficacy of compound VII on the rat CNV. Each group contains 10 animals. Animals were killed at 10 days. Each bar represents the mean ± S.D. from 10 animals. $^{***}P < 0.001$ versus PBS-treated group, Student's *t*-test.

3.5. Compound 7 reduces Matrigel-induced choroidal neovascularization in rats

Tie2 expression is prominent around and within the base of newly formed blood vessels of retinal and choroidal neovascular lesions. Soluble Tie2 receptor that prevents binding of angiopoietins to the Tie2 receptor on the cell surface has displayed efficacy in experimental retinal and choroidal neovascularization [1]. In this study, we investigated whether the Tie2 inhibitor, compound **7**, is efficacious in a rat model of Matrigel-induced choroidal neovascularization. When injected into the subretinal space, Matrigel formed a layer between the choroids/retinal pigment epithelium and the retina. Many new blood vessels originating from the choriocapillaris invaded the Matrigel layer to form a dense network (Fig. 4A). Matrigel alone (diluted with PBS) was injected into one eye of an animal as control, and the contralateral eye was injected with Matrigel containing the Tie2 inhibitor. Animals were sacrificed at day 10 after injection and the eyes were collected. Compound **7** markedly attenuated CNV in this animal model and the CNV index in the compound treated eyes was reduced by 80% ($^{***}P < 0.001$) (Fig. 4B). The compound showed striking in vivo efficacy in the rat model of choroidal neovascularization, suggesting an essential and independent role of Tie2-mediated angiogenesis versus that of VEGFR2 in controlling the progression of the disease, since compound **7** has no effect on VEGFR2 activity at the concentration administered. Interestingly, recent observations showed that angiopoietins can also bind to Tie1, another important kinase in regulating angiogenesis [23]. The activity of compound **7** against Tie1 kinase remains to be determined. Nevertheless, our data provide further evidence that the Tie2 receptor is an important target responsible for mediating aberrant vascular growth, and blocking Tie2 activation with selective inhibitors may provide a new approach for treating pathological neovascularization.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.02.003.

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