Small molecule intervention of actin-binding protein profilin1 reduces tumor angiogenesis in renal cell carcinoma

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ABSTRACT

Angiogenesis plays a key role in the development and progression of renal cell carcinoma (RCC). Actin-binding protein profilin-1 (Pfn1) is overexpressed in clear cell RCC predominantly in tumor-associated vascular endothelial cells (ECs). We previously demonstrated that that EC-selective (over)expression of Pfn1 accelerates RCC progression, and conversely, genetic loss of EC-Pfn1 dramatically inhibits tumor angiogenesis impeding tumor initiation and/or progression in RCC, suggesting that Pfn1 could be an actionable therapeutic target in RCC. In this study, we demonstrate that 4,4'-((4-bromophenyl)methylene)bis(3,5-dimethyl-1*H*-pyrazole), a small molecule that we had previously identified as an inhibitor of Pfn1-actin interaction, directly binds to Pfn1 and attenuates tumor angiogenesis when directly administered into subcutaneous RCC tumors. Next, as a proof-of-concept, we demonstrate that Pfn1 inhibitor is amenable to microbubble encapsulation and release in the tumor microenvironment (TME) by ultrasound-mediated disruption of circulating microbubbles to achieve anti-angiogenic and anti-tumor benefit. Finally, we undertook a chemical optimization approach to design and synthesize 4,4'-((4-(trifluoromethyl)phenyl)methylene)bis(3,5-dimethyl-1*H*-pyrazole), a structural analog of our originally identified inhibitor, that exhibits improved anti-angiogenic efficacy. In summary, our findings suggest that tumor-localized release of Pfn1 inhibitor could be a potential therapeutic strategy in RCC.

Keywords: Profilin, actin, renal cell carcinoma, angiogenesis, microbubbles

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INTRODUCTION

Renal Cell Carcinoma (RCC) is the most common form of kidney cancer and among the different histological subtypes of RCC, clear cell RCC (ccRCC) occurs in >75% of RCC patients. Although surgical and ablative procedures successfully cure early-stage ccRCC, approximately 30% of ccRCC patients present with metastasis at the time of diagnosis [1]. Another one-third of patients, following initial treatment, develop either local recurrence and/or distant metastases with a 10% five-year survival expectation. ccRCC is characterized by near-universal loss of the entire or most of the 3p chromosome, resulting in genetic loss-of function (LOF) of the ubiquitin ligase *Von Hippel Lindau* (VHL) in >90% cases and other chromatin-remodeling and DNA-repair genes (e.g. PBRM1, BAP1, SETD2) at various frequencies in progressor tumors. A major consequence of LOF of VHL is hypoxia-inducible factor (HIF)-mediated transcriptional upregulation of various pro-angiogenic growth factors (VEGF, PDGF) leading to a highly vascularized tumor microenvironment (TME) and tumor progression [2]. Although anti-angiogenic therapies (AATs) combined with immunotherapy are front-line treatments for ccRCC [3], resistance to AATs remains a persistent clinical problem [4-6] .

Endothelial cell (EC) migration and proliferation are essential processes of angiogenesis that are highly dependent on the action of the actin cytoskeleton[7]. Actin monomer-binding protein Profilin1 (Pfn1 – the most abundant and ubiquitously expressed protein within the Pfn family) plays an important role in facilitating actin polymerization in cells. Depletion of Pfn1 generally results in reduced cellular levels of polymerized actin (F-actin), causing defects in proliferation and migration in a variety of cell types including ECs [8-12]. We previously reported that conditional knockout (KO) of the *Pfn1* gene in vascular ECs causes major defects in developmental angiogenesis[9]. Angiogenic stimulation increases Pfn1 phosphorylation in ECs in a site-specific manner (Y129), and genetic abrogation of Y129 phosphorylation of Pfn1 leads to reduced angiogenesis and tumor progression in a glioblastoma (GBM) setting [13,14]. These findings underscore Pfn1's important role in both physiological and pathological angiogenesis.

Pfn1 expression is transcriptionally elevated in both clear cell and papillary RCC which together account for >90% of RCC cases [15,16]. For ccRCC, Pfn1 is one of the top-12 differentially (over)-expressed proteins in metastatic *vs*. primary RCC[17], and Pfn1 overexpression is linked to poor

clinical outcomes in human ccRCC [16-20]. In ccRCC, we previously reported that Pfn1 expression is more commonly dysregulated in stromal cells rather than in tumor cells, most notably in tumor-associated vascular ECs [16]. Supporting these clinical correlative findings, we further showed that endothelial (over)expression of Pfn1 accelerates orthotopic tumor progression of RENCA (a commonly used murine VHL+ RCC cell line) cells in syngeneic mouse models. Conversely, triggering EC-specific loss of Pfn1 dramatically abrogates tumor formation and/or progression of RENCA tumors, and importantly, Pfn1-dependency for tumorigenicity of RENCA cells was maintained even when the VHL was genetically disrupted. As expected, RCC tumors with genetic LOF of EC Pfn1 exhibited a dramatic reduction of tumor angiogenesis[15]. Collectively, these findings suggest that Pfn1 represents a promising therapeutic target for diseases driven by excessive vascularization such as RCC.

In our previous work, we discovered two structurally distinct non-cytotoxic small molecule compounds, namely, 8-(3-hydroxyphenyl)-10-phenyl-2,4,5,6,7,11,12-heptaazatricyclo[7.4.0.0^{3},7] trideca-1(13),3,5,9,11-pentaen-13-o (denoted as compound C2) and 4,4'-((4-bromophenyl)methylene)bis(3,5-dimethyl-1*H*-pyrazole) (denoted as compound C74) that reversed Pfn1's effect on actin polymerization in biochemical assays. Treatment with either of these compounds reduced Pfn1:actin interaction in cells, inhibited cell migration and proliferation in a dose-dependent manner, and diminished abnormal neovascularization *in vivo* in ocular angiogenesis (oxygen-induced retinopathy and laser-induced choroidal neovascularization) settings[9,21-23]. Whether tumor angiogenesis is also susceptible to small molecule-mediated intervention against Pfn1-actin interaction remains unexplored. Our comparative assessment of C2 and C74 in RCC cell migration and proliferation assays previously indicated that C74 is biologically more potent than C2 [21]. Building on this foundation, in the present study, we provide the first experimental evidence for C74's ability to directly bind to Pfn1, and its utility as an anti-angiogenic agent in RCC tumors. We additionally report the design and synthesis of C74 analogs with improved *in vivo* anti-angiogenic efficacy.

MATERIALS AND METHODS

Cell Culture and Reagents. RENCA renal cell carcinoma (RCC) cells (ATCC, CRL-2947) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Human microvascular ECs (HmVECs, ATCC, CRL-3243) were cultured in MCDB-131 media with growth supplements as described previously [22].

Small molecule compounds. C74 was either purchased from commercial sources (MolPort-000-793-534) or chemically synthesized. Other analogs were synthesized as described in ***Supplementary Information*** **(SI)** section. All tested compounds were determined to be of at least 90% chemical purity. The details of chemical synthesis of various C74 analogs and structural characterization are provided in the ***SI*** section.

Molecular docking. Molecular docking was performed using GNINA (PMID: 34108002) which performs Monte Carlo search over possible ligand conformations and poses and ranks the resulting poses using a convolutional neural network [24]. Default settings were used and the protein receptor structure was kept rigid. The protein structure used for docking includes a cryptic pocket at the Pfn1:actin interface that was identified through molecular dynamics simulations, as previously described [25].

Pfn1 purification and Protein Thermal Stability Shift (PTS) assay. Recombinant wild-type (WT Pfn1 was expressed in BL21(DE3) Codon + RPIL *E. Coli* as an N-terminal fusion with a purification tag containing both αv 8X-His and the fluorescent protein, mRuby2. After lysis, the fusion protein was purified using IMAC and the His_8-mRuby2 tag liberated from the fusion protein by overnight digestion with TEV protease. The purification tag and TEV protease were then separated from Pfn1 using a second round of IMAC. The resulting Pfn1 is then subjected to ion exchange and size exclusion chromatography. The resulting purity is >99% as judged by SDS-PAGE. For PTS assays, Pfn1 was used at a concentration of 0.3 mg/ml in a buffer containing 50mM HEPES pH7.5, 50mM NaCl, 1 mM β-mercaptoethanol, and 5X SYPRO Orange. Fluorescence of SYPRO Orange was measured and plotted as a function of temperature to quantify protein unfolding. The T_m was defined as the temperature with the maximum rate of change in fluorescence.

Surface Plasmon Resonance: SPR analysis was conducted using a Reichert 4SPR instrument (Reichert Technologies) with 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween 20, and 3 mM EDTA as the running buffer. An anti-GST antibody (Sigma Aldrich, G7781) was immobilized first on carboxymethyl dextran hydrogel biosensor chips (Reichert) using standard amine coupling chemistry with 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Purified recombinant GST-Pfn1 proteins or GST alone as a negative control were then captured by the immobilized antibodies on separate channels on the chip. Analytes (purified actin and C74) were then injected over a range of concentrations at 50 μL/min with a 60 s association phase and 120 s dissociation phase. The chip surface was regenerated with 1 mM NaOH after each analyte injection. Each analyte concentration was measured in duplicate (Actin) or triplicate (C74), and the resulting sensorgrams were corrected for buffer effects. Kinetic and binding constants were calculated by fitting the sensorgrams with a 1:1 Langmuir binding model using the TraceDrawer software (Reichert).

In vitro anti-proliferation and anti-angiogenesis screening. For initial screening of compounds in a cell proliferation assay, HmVECs were seeded in the wells of a 24-well plate and treated with various compounds at a 20 μM concentration or equivalent concentration of DMSO (solvent control), and cell count was measured well-by-well by imaging and extrapolating the overall number of cells/well using a Digital Cell Imager (Millipore). For testing of anti-angiogenic capability of small molecules, HmVECs were plated on growth factor-reduced matrigel (R&D Systems, 3433-005-01) and treated with various compounds at a 20 μM concentration or equivalent concentration of DMSO as described previously [22]. Endothelial cord formation was imaged and quantified after 18 hours, with the length of branch segments used as a measure of angiogenic activity.

Lipid microbubble encapsulation of C74. Lipid microbubbles were prepared with modifications to a previously described method (PMID: 27551081). DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine; 850365C, Avanti Polar Lipids, Alabaster, AL, USA), DSPE-mPEG 2000 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly-ethylene glycol)-2000] ammonium salt; 880120C, Avanti Polar Lipids), and poly-oxyethylene (40) stearate (P3440, Millipore Sigma) dissolved in chloroform were mixed with C74 (dissolved in ethanol) in a

2:1:1:0.5 (w/w/w) ratio and dried under argon at room temperature for 25 to 30 minutes, followed by overnight vacuum drying. The dried lipid film was hydrated with saline and the resulting dispersion was sonicated with a Misonix XL2020 Sonicator Ultrasonic Processor XL (Bioventus, Farmingdale, NY, USA) at power level 5.25 in the presence of perfluorobutane (PFB) gas (FluoroMed, Round Rock, TX, USA) for 1 minute 15 seconds to form microbubbles. These were washed twice in a saline bag, incubating at room temperature for 60 minutes after each wash, then resuspended in saline. The final formulation was aliquoted into vials with PFB-filled headspace and stored at 4 °C until use. This method consistently produced microbubbles with an average concentration of ~0.5×10^9/mL and a mean diameter of 3 µm, as measured using a Coulter counter (Multisizer 4e, Beckman Coulter, Indianapolis, IN, USA) with a 30 µm aperture tube.

For in vitro testing of microbubble-encapsulated C74, HmVECs were seeded for cord formation as described above and allowed to grow for 24 h. Microbubbles loaded as indicated were added to the culture media to achieve a final ratio of 10 microbubbles per cell. The wells were covered with cell media, sealed with polycarbonate membrane and inverted. To disrupt microbubbles and release C74, Ultrasound was delivered with a single-element immersion transducer (A302S, 25.4 mm in diameter, Olympus NDT, Center Valley, PA), driven by an arbitrary function generator (AFG3252, Tektronix, Beavertona, OR) connected to a gated radio frequency power amplifier (250A250AM8, Amplifier Research, Souderton, PA). The ultrasound field was calibrated with a 200-µm capsule hydrophone (HGL-0200, Onda Corp, Sunnyvale, CA). Ultrasound pulses were delivered at 1 MHz, 0.50 MPa peak negative pressure (spatial peak temporal peak), 10 µs pulse duration, and pulse interval 1 ms (duty cycle 1%), for a total of 10 s. The media was removed after UTMD, and cells were replenished with 10% DMEM and incubated for 48 hr for cell viability assay.

In vivo studies.

i. Matrigel plug angiogenesis assay. A single dose of 0.5 ml of growth factor reduced matrigel plugs supplemented with bFGF (500 ng) and either DMSO or compound (200 µM) was subcutaneously implanted bilaterally into the flanks of C57/B6 mice (5-6-week-old male mice). After 10 days, the plugs were excised, and infiltrating ECs into the plugs were identified by CD31 immunostaining and quantified to assess the extent of vascularization.

RCC tumor model studies**.** All animal experiments were conducted in compliance with an approved IACUC protocol, according to University of Pittsburgh Division of Laboratory Animal Resources guidelines. As previously described[21], 1 million RENCA cells were injected in a 1:1 PBS/matrigel mix subcutaneously into the flanks of 6–7-week-old male immunocompetent Balb/C mice to establish RCC tumors. For direct intratumoral injection, C74 (16 mg/kg) or equivalent amount of DMSO dissolved in saline was injected at the tumor cell inoculation site over a course of 19 days starting on day 1 before sacrificing the animals. For studies involving microbubble-based delivery of C74, subcutaneous RENCA tumors were established as described above. One hundred μl suspension of microbubbles in PBS was retro-orbitally injected into animals. Ultrasound pulses were delivered for 25 minutes with a clinical ultrasound imaging system (S3 probe, Phillips Sonos 7500). The ultrasound probe was placed on the tumor and the ultrasound was operated in ultra-harmonic mode (center frequency of 1.3 MHz) with an on-screen mechanical index of 1.6. The system was time-triggered, with 4 frames per burst and a burst interval of 2 s to allow reperfusion of the tumor with microbubbles in between bursts. The treatment was monitored by imaging the tumor using a 15L8 transducer probe on a Sequoia 512 ultrasound imaging system (Siemens Ultrasound, Issaquah, WA, USA) operated in Contrast Pulse Sequencing (CPS) mode (mechanical index = 0.20 and frame rate of 5 Hz) to confirm MB destruction by therapy pulses and subsequent reperfusion of MBs in the tumor. For tumor growth inhibition studies (N=6-8 mice per group), treatment was initiated after 5 days post inoculation and animals received a total of three UTMD treatments at two to three-day intervals. Mice were euthanized 2 days after the last treatment and tumors were harvested.

Immunohistochemistry

Tissue sections were deparaffinized and rehydrated before blocking and incubating overnight with an anti-CD31 antibody (77699, Cell Signaling Technology, 1:100). For immunodetection of the primary antibody, a secondary biotin-labeled anti-rabbit antibody (ab97049, Abcam) and streptavidin-peroxidase conjugates (11089153001, Roche) were used. The staining was detected with the Pierce DAB substrate kit (34002, ThermoFisher). For some experiments, the slides were counterstained with hematoxylin before dehydration and mounting.

Statistics

Statistical tests were performed with either one-way ANOVA followed by Tukey's post hoc test or nonparametric Mann–Whitney test when appropriate. Differences exhibiting $p < 0.05$ were considered as statistically significant.

RESULTS

C74 binds to Pfn1. While Pfn1's action enhances actin polymerization in cells, in biochemical assays without the presence of any other actin-assembly factors, Pfn1 inhibits actin polymerization through preventing actin incorporation onto the pointed ends of growing actin filaments as well as by inhibiting spontaneous actin nucleation. Our discovery of C74 (**Figure 1A**) as an inhibitor of the Pfn1-actin interaction was based on the results of a pyrene-actin polymerization screening assay where C74 reversed recombinant GST-Pfn1's inhibitory effect on actin polymerization but had no discernible effect on actin polymerization on its own [21]. In the same study, we further demonstrated that C74 treatment reduces proximity-ligand interactions of endogenous Pfn1 and actin in cells and induces other phenotypic changes (i.e. reduced cell spreading, migration, and proliferation) that are expected upon inhibition of cellular Pfn1-actin interaction. However, whether C74 directly binds to Pfn1 remained unexplored. To address this question, we performed SPR studies with C74 and immobilized GST-Pfn1 (GST served as a negative control). GST epitope-tagging enabled us to immobilize all purified proteins with an anti-GST antibody on the SPR chip thereby minimizing the masking of any binding site of Pfn1 for potential C74's interaction. As an initial test for the utility of the SPR assay to detect known ligand interactions of Pfn1, we confirmed GST-tagged wild-type (WT) Pfn1 interacts with G-actin while GST-tagged H119E-Pfn1 (a point-mutant of Pfn1 that has ~25-fold reduction in actin-binding vs WT-Pfn1[26,27]) did not exhibit any detectable interaction with G-actin as expected (**Figures 1B-C, F**). The estimated dissociation constant (K_d) of GST-Pfn1:actin interaction in the SPR assay was approximately equal to 5.3 μM (**Figure 1E**). This value is an order of magnitude higher than previously reported value (0.15 μM) for Pfn1:actin interaction measured in an isothermal calorimetric assay[26]. This discrepancy could be due to either differences between tagged- and untagged-Pfn1 in terms of their binding affinities for actin and/or assay-specific differences in the K_d measurements. We were able to detect C74's interaction with Pfn1 with an estimated K_d value approximately equal to 60 μM, consistent with its potency in previous anti-angiogenesis assays,

and H119E substitution did not impair C74's interaction with Pfn1 (**Figures 1D-E, F**). These results suggest that C74 is a low-affinity binder of Pfn1.

Intratumoral release of Pfn1 inhibitor diminishes neovascularization of RCC tumors. We previously showed that in a RENCA-based RCC mouse model, intratumoral administration of C74 leads to reduced tumor growth in a subcutaneous implant setting[21]. However, since our *in vitro* studies also showed that C74 treatment reduces RENCA cell proliferation, we could not discern the relative contributions of tumor-intrinsic vs -extrinsic effects of C74 underlying its anti-tumor action. Therefore, we performed an end-point assessment of tumor angiogenesis by performing immunohistochemistry (IHC) of tumor sections for CD31 (a marker of vascular ECs) and quantifying the relative CD31-positive areas in DMSO (vehicle control)- vs. C74-treated RENCA tumors collected from our previous *in vivo* studies. As per these analyses, C74 treatment reduced the CD31-positive area by ~30% in a statistically significant manner (**Figures 2A-B**). Encouraged by these results, we next explored whether C74 can be systemically released in a tumor-localized manner to achieve therapeutic benefit. We pursued microbubble-based delivery of C74 to overcome key limitations associated with systemic and direct injection approaches. Unlike subcutaneous tumors, access to internal organ tumors (such as RCC) is challenging for direct injection, limiting the feasibility of localized administration. Moreover, since C74 is a low-affinity binder of Pfn1, systemic delivery of C74 may require high doses to achieve therapeutic concentrations at the tumor site, potentially leading to off-target effects and toxicity. Lipid microbubbles serve as efficient carriers of small molecules and nucleic acids, and can release their payloads for localized therapeutic applications when subjected to ultrasound-targeted microbubble destruction (UTMD) [28-31]. Therefore, first, we successfully encapsulated C74 within lipid microbubbles (~3 μm in diameter), as confirmed by mass-spectrometry evaluation of microbubbles (**Figure 2C**). As a proof-of-concept *in vitro* study, we then performed cord morphogenesis (an indicator of angiogenic potential) assay with HmVECs in which we demonstrated that similar to the action of unencapsulated C74 (a positive control), UTMD of C74-encapsulated microbubbles prominently inhibits cord morphogenesis of ECs (**Figures 2D-E**). The extent of angiogenesis inhibition was more pronounced when C74 was released from microbubbles compared to the setting where C74 was directly added to the culture. We speculate that this could be due to the possible concentration differences of C74 between these two settings. To establish

the specificity of our findings, we included several negative control groups in that same experimental setting where either C74-encapsulated microbubbles was not subjected to UTMD or C74 was transiently released by UTMD followed by immediate C74 washout and replacement with regular culture media or UTMD of microbubbles was performed without C74 as a payload (to rule out any possible detrimental effect of ultrasound cavitation on cells). In none of these negative control settings did we observe any adverse effect on cord formation by ECs when compared to the control DMSO treatment group (**Figures 2D-E**). Given these results demonstrating the feasibility of UTMD to release C74 and achieve anti-angiogenic action in cell culture settings, we next performed exploratory *in vivo* tumor model studies involving subcutaneous implantation of RENCA cells in Balb/c mice. After allowing these tumors to reach palpable size, tumor-bearing mice received retro-orbital injections of C74-loaded microbubbles three times over the course of a week, followed by ultrasound application to target the tumor site. This precision delivery approach led to a significant reduction in tumor growth and a concomitant decrease in tumor angiogenesis (**Figures 2F-J**), consistent with our in vitro findings. These results support the utility of lipid microbubble-based delivery of C74 as a promising targeted therapy to diminish neovascularization and tumor growth.

Optimization of C74 identifies analogs with improved anti-angiogenic activity. In a previous study, we established that the bromine (Br) substituent in C74 could be effectively replaced with either F or CH_3, and these analogs exhibited anti-angiogenic activity at 10 μM, an improvement over C74 [22]. Based on these promising results, we sought to identify additional analogs with improved potency, efficacy and drug-like properties. Based on our docking studies of C74 (**Figure 3A**), we focused on the aryl group as its binding pocket was not fully occupied, and our previous studies supported that modification at this position was not only tolerated but led to improvements in potency, possibly through interactions with R74 and H119. In addition, the pyrazole was predicted to engage in multiple H-bonding interactions with the target, and therefore, changes to this position were likely to lead to less active compounds.

Based on the very limited SAR available (e.g. F and CH_3 substitution led to greater efficacy than Cl or H) we designed potential new analogs based on: 1) docking studies that suggested the aryl bromine pocket was not fully occupied, 2) exploring whether lipophilicity, polarity or

electronegativity contributed to activity, and 3) optimizing the physical chemical properties such as solubility and permeability. A series of potential analogs were docked using the GNINA molecular docking software [32], and we used the docking scores and other factors (e.g. predicted interactions and physical chemical properties) to prioritize compounds for synthesis. Examples are shown in **Figure 4A** (See **SI** for synthetic protocols and characterization). The aryl halide was replaced with various pyridine isomers (UP-1, -4, and -5) to evaluate if electronegativity contributed to binding and to potentially enhance solubility; CF_3 (UP-2, -3, and -6) was introduced at various positions to probe size constraints and electronegativity, as well as enhance any metabolic instability. Several nitrogen containing bicyclic systems (UP-10 through UP-13) were introduced to probe the size of the binding site. The ketone (UP-7) and benzyl ether (UP-8) were designed to probe the size of the binding site and a potential hydrogen bond with R74, and the aniline (UP-9), which was likely more soluble, tested whether polarity or electronegativity contributed to binding.

We performed an initial testing of these compounds in EC proliferation assays at a single dose of 20 μM, and then selected those compounds (UP-2, -3, -6 and -8) that inhibited EC proliferation relative to DMSO control for qualitative evaluation of anti-angiogenic activity in EC cord formation assay. Based on this initial qualitative screening, we identified compound UP-6 4,4'-((4-(trifluoromethyl)phenyl)methylene)bis(3,5-dimethyl-1*H*-pyrazole) to be the most effective analog in terms of its ability to inhibit angiogenic activity of ECs *in vitro* (**Figure 4A).** Single-dose *in vitro* angiogenesis assay results involving compound UP-6 are shown in **Figures 4B-C**. The near-complete blockade of cord formation by 20 μM UP-6 was similar to what we had previously observed in ECs when subjected to a much higher concentration (50 μM) of C74 treatment [22]. In the same study, we also showed that C74 when applied at 10 μM is completely ineffective in reducing cord formation of EC. Encouragingly, at a 10 μM concentration, UP6 is still effective in inhibiting EC cord formation (by ~50%) while C74 is not (**Figures 4D-E**).

Based on the limited number of analogs tested and the results to date, we cannot yet formulate a basis for this difference in activity among analogs with similar electronic, size, and lipophilicity characteristics. To further characterize UP-6, we performed a Protein Thermal Shift (PTS) assay comparing the thermal stability of the Pfn1 protein with compound UP-6 and C74 vs DMSO

control. Interestingly, both C74 and UP-6 produced negative thermal shifts of Pfn1 relative to DMSO control, but the extent of the negative thermal shift was more pronounced for UP6 than C74 (**Figure 4F;** the docking model of UP6:Pfn1 interaction is shown in **Figure 3B**). This could mean that these small molecule interactions either destabilize Pfn1 to some extent or induce a conformational change that facilitates SYPRO fluorescence at lower temperatures with UP-6 having a more pronounced impact than C74.

Given our findings that UP6 outperforms C74 in terms of its ability to reduce cord formation by ECs at a lower concentration than C74 *in vitro*, we wanted to further determine whether this is also true in an *in vivo* setting. Therefore, we performed matrigel plug angiogenesis assay where we co-injected bFGF (a stimulator of angiogenesis)-supplemented growth factor-reduced matrigel with either C74 or UP-6 (at 200 μM) or equivalent DMSO control bilaterally into the flanks of C57/Bl6 mice. CD31 immunostaining of plugs harvested 10 days after injection revealed that treatment of either C74 or UP-6 significantly reduced EC infiltration into the plugs, but the reduction in CD31-positivity of plugs treated with UP-6 was more prominent (>50% decrease relative to control) than that achieved by the treatment of C74 compound (~25% decrease vs control), and this difference was statistically significant (**Figures 5**). These data suggest that UP-6 has a more potent anti-angiogenic activity than C74, providing the first *in vivo* proof-of-concept that anti-angiogenic efficacy based on inhibition of the Pfn1-actin interaction can be optimized through medicinal chemistry strategies.

DISCUSSION

In this study, we report four main findings. First, our SPR studies show that our previously identified small molecule inhibitor of Pfn1-actin interaction, C74, directly binds to Pfn1. Second, our study provides the first demonstration of small molecule intervention of Pfn1 as a strategy to reduce tumor angiogenesis in RCC. Third, we provide the first proof-of-concept for the feasibility of lipid microbubble-mediated delivery of a Pfn1 inhibitor *in vivo* with intact biological activity. Fourth, we were able to design and synthesize an analog of C74 (UP-6) with improved anti-angiogenic activity *in vivo*.

In the RENCA tumor model, the degree to which tumor angiogenesis was inhibited by C74 was less than we had previously seen in the setting of genetic disruption of Pfn1 in ECs [15]. This can be due to several reasons. First, our SPR studies predicted that C74 is a low-affinity inhibitor of Pfn1 with a dissociation constant measured to be approximately 60 μM (a value that is in general agreement with our previously published data where C74 treatment in a 25 – 50 μM concentration range effectively inhibited RCC cell migration/proliferation and angiogenic activity of ECs). Second, small molecules released in the TME are expected to be taken up by multiple different types of cells, and therefore, its effect is not restricted to ECs only. In particular, fibroblasts and various immune cells also contribute to tumor angiogenesis [33,34], and at present, we do not have a clear understanding of how functional inhibition of Pfn1 impacts angiogenesis-promoting activities of those cell types in the TME. Third, since Pfn1 also interacts with many other proteins besides actin, small molecule-mediated inhibition of Pfn1-actin interaction cannot be equated to complete genetic loss-of-function of Pfn1. Fourth, we cannot rule out off-target effects of C74 that can potentially offset the anti-angiogenic effect of the inhibition on Pfn1-actin interaction. Endothelial transcriptomic studies comparing the impact of genetic loss-of-function vs small molecule inhibition may provide greater insights into the on-target specificity of C74's action.

Although our proof-of-concept tumor model studies were conducted in a subcutaneous tumor setting (a limitation of this study), our ability to attenuate angiogenesis and tumor reduction upon UTMD of systemically circulating microbubbles sets the stage for future exploration in an orthotopic RCC tumor setting to better model the disease. Our findings support the therapeutic potential of lipid microbubble-based delivery systems as a clinically translatable strategy for targeted cancer therapy. Notably, microbubbles are already FDA-approved as contrast agents for ultrasound imaging (Definity, SonoVue), which provides a strong foundation for their repurposing in therapeutic applications. In recent years, microbubble-assisted delivery has gained traction in oncology, with studies demonstrating enhanced delivery of chemotherapeutics, nucleic acids, and small molecules to solid tumors, including pancreatic [35-37], liver [38 39], and brain tumors [40], when combined with UMTD. This platform enables site-specific release of therapeutic payloads, reducing systemic toxicity while enhancing local efficacy. Our study adds to this growing body of evidence by showing that microbubble-mediated delivery of Pfn1 inhibitor can diminish tumor growth and angiogenesis in RCC. These data highlight the promise of leveraging microbubble

technology for precision delivery of anti-tumor agents, particularly for tumors located in anatomically challenging or sensitive regions. Furthermore, given our initial indication for improved anti-angiogenic activity of compound UP-6 vs C74, we will need to further explore the anti-tumor efficacy of compound UP6 in preclinical settings of RCC, either as a stand-alone agent or in combination with other anti-angiogenic drugs for examining therapeutic synergy.

At present, the underlying basis for improved biological activity of compound UP-6 over C74 is not clear. The structure activity relationship (**Figure 4A**) shows that modifications of the aryl group effect binding. This may be due to changes in the electronic structure of the ring changing the favorability of interactions with R74 and H119 (**Figure 3**). However, C74 binding is maintained in the H119E mutant (**Figure 1F**), which is unexpected if this histidine is making a cation-pi or halogen bond. This may indicate the histidine interaction is not important or that there are inaccuracies in the docking model. The negative thermal shift (**Figure 4F**) supports that there may be a significant conformational change in the protein that is not accounted for in the docking model, which used a rigid protein structure. The greater thermal shift of Pfn1 by compound UP-6 relative to C74 in the PTS assay could be indicative of an even larger structural alteration in Pfn1 elicited by its interaction with UP-6. X-ray crystallography studies in the future are expected to provide valuable structural insights into how these small molecule interactions impact the atomic arrangements in the Pfn1 molecule Finally, the therapeutic utility of targeting the Pfn1-actin interaction extends beyond cancer. Excessive or abnormal angiogenesis is also a hallmark of several other diseases, including age-related macular degeneration, diabetic retinopathy, atherosclerosis, and psoriasis. In some of these conditions, unchecked angiogenesis leads to the formation of dysfunctional blood vessels that contribute to disease progression and tissue damage. Given the clinical evidence for endothelial Pfn1 upregulation in at least atherosclerosis and diabetic retinopathy[9,41-45], small molecule inhibition of Pfn1 could be a worthwhile strategy to explore for those disease conditions in the future.

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AUTHOR CONTRIBUTIONS. DG performed experiments, conceived study design, analyzed data, wrote the manuscript, and acquired funding; PC, KX, NW, JA, CV, MW, SZ and MW performed experiments and/or generated reagents; CC generated reagents and wrote the manuscript; HS performed SPR experiments and wrote the manuscript; XC, AP, and FV assisted with and oversaw microbubble studies; TES oversaw the SPR studies and wrote the manuscript; PF performed molecular docking studies; DK, AV, DMH, and PR conceived study design, wrote the manuscript, and acquired funding.

DATA AVAILABILITY: All data are included in either main figures or supplementary information (SI).

FIGURE LEGENDS

Figure 1. **SPR-based demonstration of C74-Pfn1 interaction**. **A)** Chemical structure of C74. **B-F**) Recombinant purified wild-type and H119E mutant forms of Pfn1 were immobilized on an SPR chip as described under Materials and Methods. Purified Actin (*panels B, C*) or C74 (*panels D, E*) were then injected over the range of concentrations shown either in duplicate (Actin) or triplicate (C74) followed by a dissociation phase (*arrows*). The resulting sensorgrams are shown with the data points in color and the fitted curves in black. Response units are normalized to the amount of Pfn target protein immobilized on each channel. The reference-corrected sensorgrams were fit to a 1:1 Langmuir binding model and the resulting kinetic constants are shown in the Table (*panel F*). K_D values were calculated from the relationship $K_D = k_d/k_a$.

Figure 2: Intratumoral release of Pfn1 inhibitor diminishes neovascularization and growth of RCC tumors. (A-B) Representative images (panel A; 4× magnification) and quantification (B; based on 10× field images) of $CD31^+$ cells in subcutaneously established RENCA tumors subjected to daily intratumoral injection for 19 days with either C74 or equivalent amount of DMSO control. in Balb/c mice (**, $p < 0.01$; n = 5 mice/group; scale bar, 200 μm). **C)** Mass spectrometry confirmation of C74 encapsulation in lipid microbubbles. Only C74 characteristic mass peak was detected in microbubble samples. **D-E)** Representative cord-formation images of HmVECs for various treatment settings (*panel D*): NT: DMSO (vehicle control); C74: treatment with unencapsulated C74; MB US: empty MB with UTMD, C74 MB: C74-encapsulated MB with no UTMD; C74 MB US: C74-encapsulated MB with UTMD, and C74 US: C74-encapsulated MB with UTMD followed by immediate washout and replacement with C74-devoid culture media. The bar graph in panel E depicts the relative lengths of cords compared between the different treatment groups. **** $p < 0.0001$ from n = 3 experiments. **F-I)** Ultrasound images (*panel F*) showing microbubble (MB) infiltration into tumor vasculature (white arrows), followed by UTMD and no contrast remaining in the tumor space. Images of representative subcutaneous RENCA tumors subjected to UTMD of control vs C74-encapsulated microbubbles (*panel G*) with end-point tumor burden comparison between the two treatment groups (*panel H*). Representative H&E images (*panel I;* white arrows indicate functional tumor-associated blood vessels with presence of red blood cells) and the associated quantification (*panel J*) of the average number of tumor-

associated blood vessels per 20X field of observation between the two treatment groups (** $p < 0.01$; data pooled from 8-9 mice per group; scale bar, 50 μm).

Figure 3. Docking models of C74 and UP-6 interaction with Pfn1. The binding modes of **(A)** C74 and **(B)** UP-6 as predicted by GNINA. The unmodified hydroxypyrazoles are predicted make an identical network of hydrogen bonds in both compounds while the modified aryl ring can potentially interact with R74 and H119.

Figure 4. Summary of initial screening results of chemically synthesized C74 analogs. **A)** Structures of analogs and their impact on EC proliferation and/or follow-on cord formation assay. Only those compounds which inhibited EC proliferation relative to DMSO treatment control were subjected to cord formation assay for anti-angiogenic activity (+++ indicates highly effective in inhibiting angiogenesis at a 20 μM concentration; N/A – not applicable). **B-C)** Representative images (*panel B*; 4x magnification) and quantification (*panel B-C*; based on 4x field images) of cord formation by HmVECs on Matrigel treated with either 20 μM compound UP6 or equivalent DMSO control. Each datapoint represents quantification of cord formation in a single field of observation (****, $p < 0.0001$; n = 3 experiments; scale bar, 200 μm). **D-E)** Representative images (*panel D*) and quantification (*panel E*) of cord formation by HmVECs subjected to 10 μM of either C74 or UP6 vs DMSO (control) (* - $p<0.05$; ns – not significant). **F)** Normalized rate of change in SYPRO fluorescence as a function of temperature from the PTS assay comparing Pfn1 treated with 1mM C74, UP-6, or a DMSO control. Each data point is the average of three technical replicates, and normalized to the maximum value within the experiment.

Figure 5. Compound UP6 is more effective than C74 in inhibiting angiogenesis *in vivo*. Representative images of CD31 immunohistochemistry with hematoxylin counterstaining (panel A; 4× magnification) and quantification (panel B; based on 20X field images) of CD31+ cell infiltration in the subcutaneously implanted Matrigel plugs in Balb/C mice co-injected with a single 200 μM dosage of either C74 or 6 or DMSO control. Data are summarized from quantification of 10 bilaterally injected plugs (n=5 mice) per treatment group (**, $p < 0.01$; ****, $p < 0.0001$; scale bar, 200 μm).

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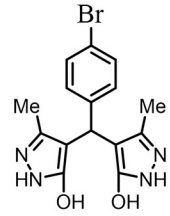
Gau et al. Fig 1

A



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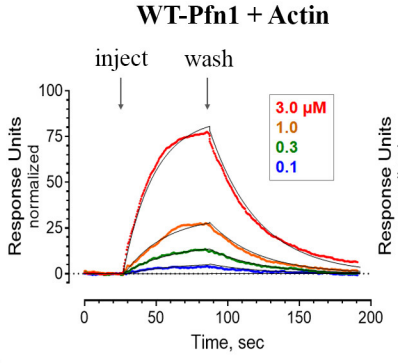

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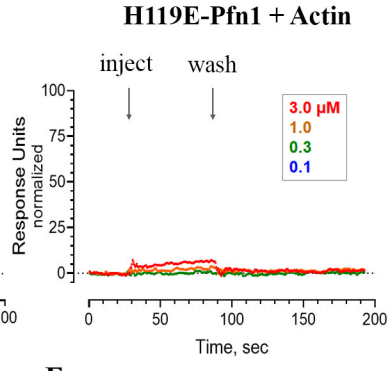

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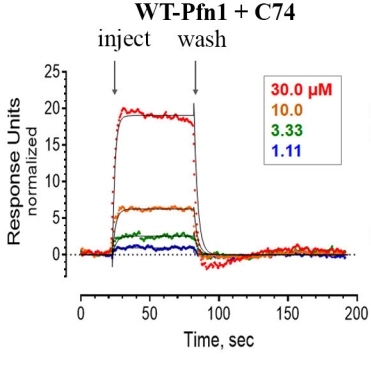

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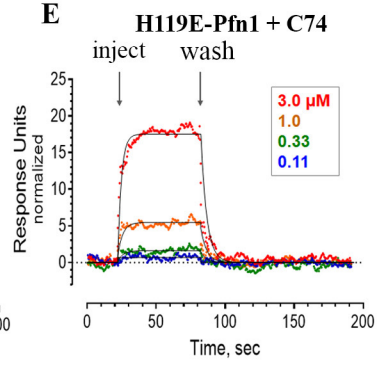
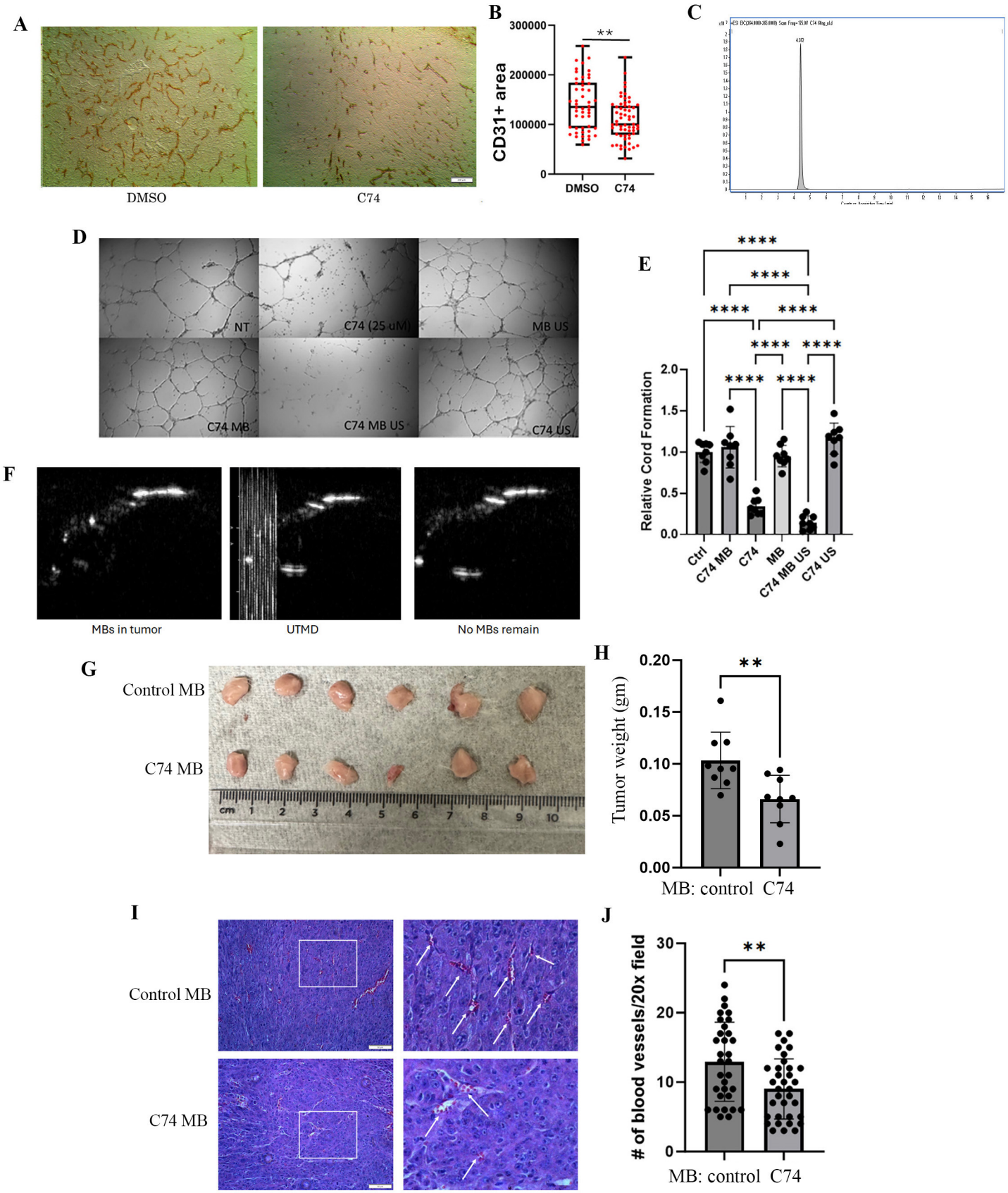


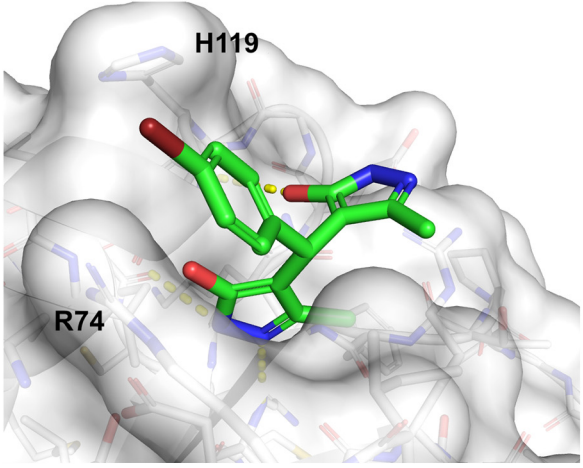
F

Ligand	Analyte	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	Chi^2 (RU^2)
WT-Pfn1	Actin	5.58×10^{3}	2.95×10^{-2}	5.29×10^{-6}	0.67
H119E-Pfn1	Actin	No Interaction			
WT-Pfn1	C74	6.29×10^{3}	3.72×10^{-1}	5.91×10^{-5}	0.29
H119E-Pfn1	C74	4.93×10^{3}	2.36×10^{-1}	4.79×10^{-5}	0.14

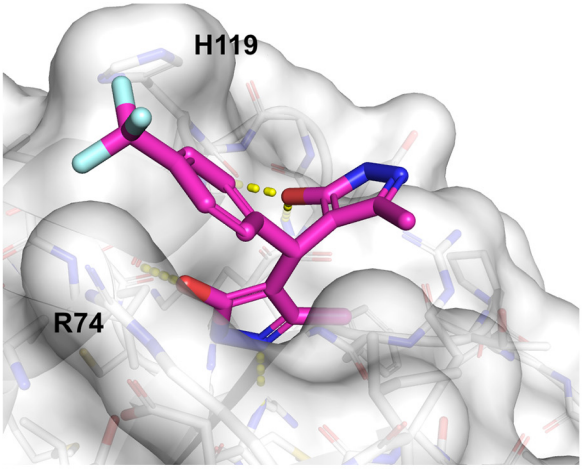
Gau et al. Fig 2



Gau et al. Fig 3



C74



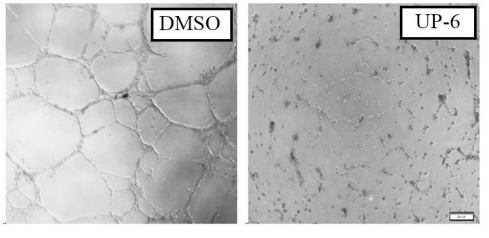
UP-6

Gau et al. Fig. 4

A

Compound/structure	Inhibits EC proliferation	Inhibits EC cord formation	Compound/structure	Inhibits EC proliferation	Inhibits EC cord formation
UP-1	No	N/A	UP-8	Yes	++
UP-2	Yes	++	UP-9	No	N/A
UP-3	Yes	+	UP-10	No	N/A
UP-4	No	N/A	UP-11	No	N/A
UP-5	No	N/A	UP-12	No	N/A
UP-6	Yes	+++	UP-13	No	N/A
UP-7	No	N/A			

B



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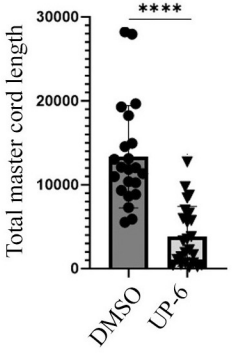

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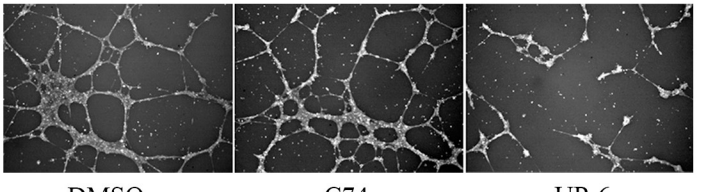
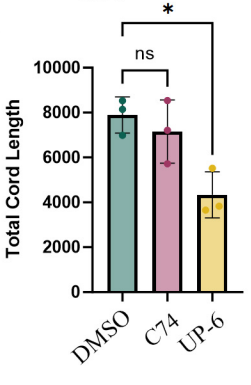


DMSO C74 UP-6

E



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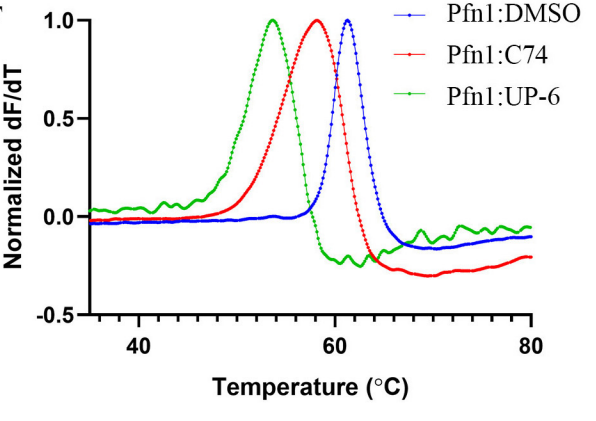
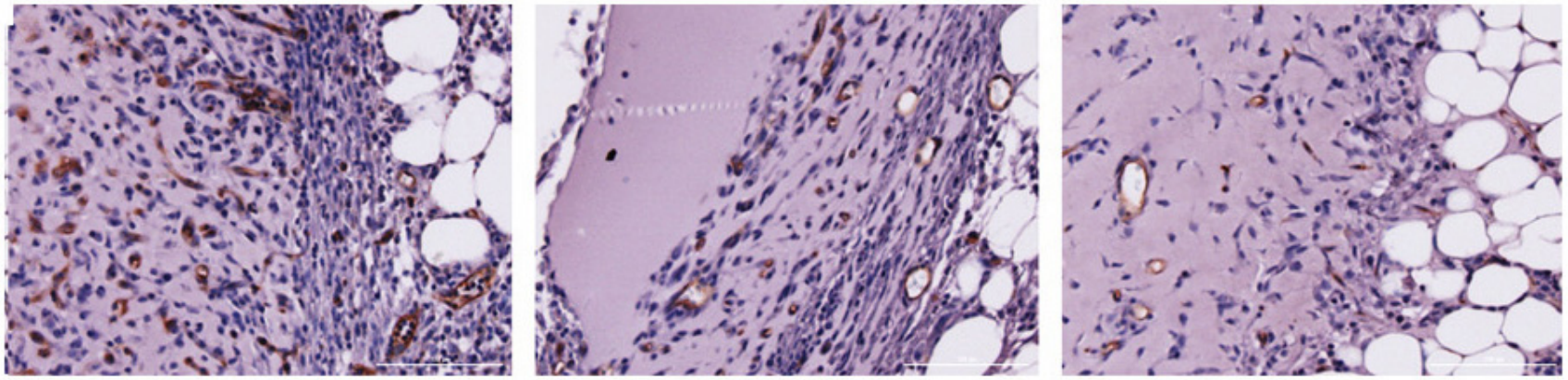


Gau et al. Fig. 5

A



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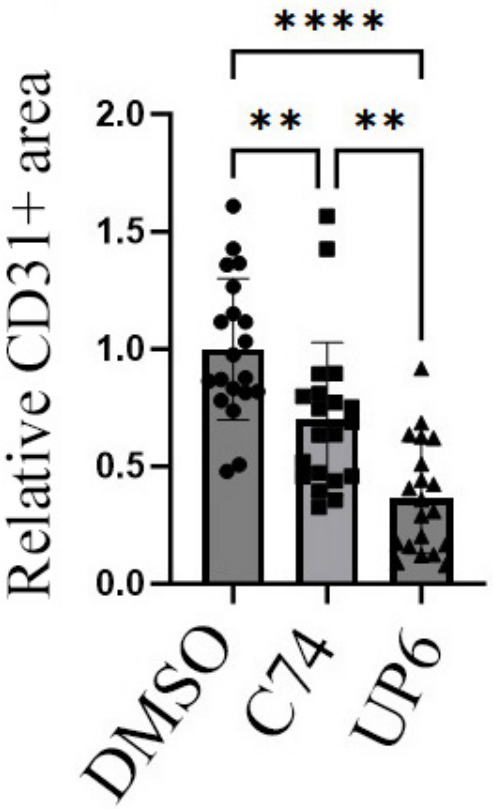
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