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The noni anthraquinone damnacanthal is a multi-kinase inhibitor with potent anti-angiogenic effects

Javier A. García-Vilas[1], Almudena Pino-Ángeles[2], Beatriz Martínez-Poveda[1], Ana R. Quesada[1,3] and Miguel Ángel Medina[1,3] *

[1]Universidad de Málaga, Andalucía Tech, Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, and IBIMA (Biomedical Research Institute of Málaga), and [3]CIBER de Enfermedades Raras (CIBERER), E-29071 Málaga, Spain

[2]Department of Chemistry, The City College of New York, New York, USA

***Corresponding author:** Dr. Miguel Ángel Medina, Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, E-29071, Málaga. Phone: +34-952137132. Fax. +34-952131674. E-Mail: medina@uma.es

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ABSTRACT

The natural bioactive compound damnacanthal inhibits several tyrosine kinases. Herein, we show that -in fact- damancanthal is a multi kinase inhibitor. A docking and molecular dynamics simulation approach allows getting further insight on the inhibitory effect of damnacanthal on three different kinases: vascular endothelial growth factor receptor-2, c-Met and focal adhesion kinase. Several of the kinases targeted and inhibited by damnacanthal are involved in angiogenesis. Ex vivo and in vivo experiments clearly demonstrate that, indeed, damnacanthal is a very potent inhibitor of angiogenesis. A number of in vitro assays contribute to determine the specific effects of damnacanthal on each of the steps of the angiogenic process, including inhibition of tubulogenesis, endothelial cell proliferation, survival, migration and production of extracellular matrix remodeling enzyme. Taken altogether, these results suggest that damancanthal could have potential interest for the treatment of cancer and other angiogenesis-dependent diseases.

1. Introduction

Angiogenesis is a hallmarks of cancer, a crucial step in the transition of tumors from a dormant state to a malignant state, and playing an essential role in tumor growth, invasion and metastasis [1]. In addition, many non-neoplastic pathologies are related to an upregulated angiogenesis [2,3]. For these reasons, there is a current genuine interest in the search for new angiogenesis inhibitors as an interesting approach for the treatment of cancer and other angiogenesis-dependent diseases. Some antiangiogenic compounds have already been approved for the treatment of cancer, blindness and other angiogenesis-dependent diseases [3-5].

Angiogenesis is a complex process that begins in response to an angiogenic stimulus with the activation of the normally quiescent endothelial cells. As a consequence, endothelial cells undergo a series of phenotypic changes, including the release of proteases that will allow them to degrade the basal membrane and subjacent extracellular matrix and migrate. Activated endothelial cells can proliferate and avoid apoptosis, and finally will differentiate forming a new capillary. In principle, any compound able to inhibit any of these steps could be a candidate for the pharmacological inhibition of angiogenesis [4]. Preclinical and clinical results seem to indicate that a multitargeted approach could produce better results in antiangiogenic therapy [6]. Our research group actively searches for, identifies and characterizes new natural bioactive compounds with multitargeted antiangiogenic effects [7-14].

Damnacanthal (3-hydroxy-1-methoxy-anthraquinone-2-aldehyde, see its chemical structure in Figure 1S in supplementary material) is a natural bioactive compound initially isolated from the phenolic phase of noni roots, although it is also present in its fruit, as well as in other Rubiaceae plants [15,16]. Damnacanthal total synthesis has already been reported [17]. Damnacanthal is characterized as the most potent known selective inhibitor of $p56^{Ick}$ tyrosine kinase [18], a protein activity with a key role in the chemotactic response of T cells to CXCL12 [19]. Additionally, damnacanthal is also able to inhibit other tyrosine kinases (PDGFR, erbB2, EGFR and insulin receptor) with IC_{50} values in the micromolar concentration range [18]. These additional effects of damnacanthal could be related to its reported antitumoral effects [20-22].

Very recently, our group has found that damnacanthal is also a potent inhibitor of c-Met and behaves as an antitumoral agent against hepatocellular carcinoma [23].

In order to get a deeper insight on the bioactivities of damanacanthal, we have screened it against a panel of 25 kinases, showing its potentital as a multi-kinase inhibitor targeting 16 of these tested kinases. Docking analysis and molecular dynamics simulations throw new light on this multi-kinase targeting by damnacanthal. Since some of the tyrosine kinases targeted by damnacanthal are involved in the regulation of angiogenesis, in the present work we analyze the potential of damnacanthal as a new antiangiogenic drug. Our results show for the first time that, indeed, damnacanthal inhibits angiogenesis *in vitro*, *ex vivo* and *in vivo* targeting several steps of the process.

2. Materials and methods

Supplementary materials and methods (including in vitro kinase inhibition assay, docking and molecular dynamics simulations, network and functional enrichment analysis, as well as the whole set of in vivo, ex vivo and in vitro assays of angiogenesis, as well as qPCR assays with the primers used in this study and listed in Table S1) are included in SI "Materials and Methods" (supplementary material).

2.1. Cell culture

Bovine aortic endothelial (BAE) cells were isolated from bovine aortic arches as previously described [24] and maintained in Dulbecco´s modified Eagle´s medium (DMEM) containing glucose (1g/L), glutamine (2mM), penicillin (50 IU/mL), streptomycin (0.05 mg/mL), and amphotericin (1.25 mg/L) supplemented with 10% FBS (DMEM/10% FBS). Human umbilical vein endothelial (HUVE) cells were isolated from umbilical cords as previously described [25] and grown on gelatin-coated dishes in Medium 199 containing 10 mM HEPES, 20% fetal calf serum, glutamine (2 mM) heparin (100 μg/mL) and endothelial cell

growth supplement (30 μg/mL). Two immortalized human endothelial cell lines kindly supplied by Dr. Arjan W. Griffioen (Maastrich Universtiy, The Netherlands) were used during this study: human umbilical vein endothelial cells (RF-24) and human microvascular endothelial cells (HMEC). These immortalized cell lines have been previously characterized [26]. Both immortalized human endothelial cell lines were grown in RPMI 1640 medium supplemented with glutamine (2mM), penicillin (50 IU/mL), streptomycin (0.05 mg/mL), and amphotericin (1.25 mg/L) supplemented with 10% fetal bovine serum, and 10% human serum. Human dermal lymphatic microvascular endothelial cells (HMVEC-dLy) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in EGM-2 MV Bullet Kit from Lonza Inc. (Walkersiville, MD, USA) in a humidified atmosphere (5%CO_2). Cells were passaged upon reaching confluence with Trypsin-EDTA solution. To maintain normal growth, hMVEC-dLy cells were used at passage from 3-7 for all experiments. All cell cultures were maintained at 37°C under a humidified 5% CO_2 atmosphere.

2.2. In vivo angiogenesis assays

In this study three different in vivo angiogenesis assays have been used: the choriallantoic membrane assay, the Matrigel plug assay and a zebrafish angigoenesis assay. Details on how these in vivo assays were performed are provided in SI Materials and Methods.

2.3.Statistical analysis

Results are expressed as mean±SD. Statistical significance was determined using the two-sided Student t-test. Values of $P< 0.05$ were considered to be statistically significant.

3. Results

3.1. Damnacanthal inhibits in vitro receptor tyrosine kinases involved in angiogenesis

We started our study by carrying out a blind in vitro screening of damnacanthal against a panel of 25 kinase activities (see Table S2 in supplementary material). Damnacanthal (10 μM)

inhibited more than 50% of the in vitro activity of 16 kinases, including 8 angiogenesis-related tyrosine-kinases: VEGFR1-3, FGFR1, 2 and 4, c-Met and EGFR

The set of 16 kinases inhibited by damanacanthal was used as a seed to perform simple network analysis by using several tools, as described in SI section "Network analysis" (supplementary material). The results of this analysis pointed to the involvement of damnacanthal in the inhibition of angiogenesis.

3.2. Docking analysis contributes to explain the wide spectrum of damnacanthal as a multi-kinase inhibitor

We have used docking and molecular dynamics simulations to determine plausible binding modes of damnacanthal into the ATP-binding sites of VEGFR2, FAK and c-Met receptors. The selected structures of VEGFR2 and c-Met correspond to the unbound phosphorylated state, which resemble the overall conformation and structural features of activated kinases [27,28] [29]. In both structures, the active site is accessible after the activation loop is ejected upon autophosphorylation of one or more tyrosine residues in its sequence. The structure of FAK is co-crystallized with ATP and corresponds to the unphosphorylated state. Nevertheless, the activation loop is also disordered, and the binding site is therefore accessible [30]. In all cases, the binding pockets are mostly comprised by hydrophobic residues in the glycine-rich loop and hinge region. The inhibitor binds in the same location in all three receptors, but its orientation significantly varies between them (see Figure 1). A detailed analysis of the structural features of the receptor-inhibitor complexes resulting from the MD simulations is provided in SI section " Detailed description and discussion of the docking and molecular dynamic results " (supplementary material).

3.3. Damnacanthal inhibits ex vivo and in vivo angiogenesis

To analyze the global effects of damnacanthal on angiogenesis, we next made use of an *ex vivo* assay and three *in vivo* models.

In the *ex vivo* assay, the capability of new vessel formation from rat aortic ring explants was evaluated after 6 and 14 days of incubation in the absence or presence of 12.5 µM damnacanthal. Figure 2A shows that in the absence of damnacanthal (controls), aortic rings were able to generate a dense microvessel outgrowth in a collagen matrix. In contrast, the presence of 12.5 µM damnacanthal completely abrogated this microvessel outgrowth.

The chicken chorioallantoic membrane (CAM) assay was selected as our first approach to determine the *in vivo* antiangiogenic activity of damnacanthal because this is a widely used and accessible system to study angiogenesis. Figure 2B shows that in untreated CAMs (controls), blood vessels form a dense and spatially-oriented branching network of vascular structures of decreasing diameter as they branch. The inhibitory effect of damnacanthal is revealed by an inhibition of the ingrowth of new vessels in the area covered by the methylcelluose disc. In fact, doses as low as 0.5 nmol/disk strongly inhibited angiogenesis (decreasing the number of small blood vessels to a 25% of those observed in the areas of control CAMs under the control methylcellulose discs without damnacanthal) under the area occupied by the methylcellulose disks in half of the tested CAMs. Furthermore, the peripheral vessels (relative to the position of the disk) tended to grow centrifugally, avoiding the treated area, with an overall decrease in vascular density and even disruption of preformed vessels within the treated area.

The *in vivo* antiangiogenic activity of damnacanthal was confirmed by using the intradermal Matrigel plug model. Our results show that this compound caused a strong inhibition of the FGF2-mediated cell invasion in the Matrigel plug (Figure 2C). The Matrigel plugs without FGF2 were colorless showing their absence of vasculature, whereas the Matrigel plugs containing FGF2 were apparently red, due to neovascularization. Matrigels containing FGF2 with 50 µM damnacanthal were only partially red, due to a decreased blood vessel formation. The hemoglobin contents in the plugs as indicator of neovascularization confirmed the inhibitory effect of damnacanthal.

The third *in vivo* experimental approach used to evaluate the effects of damnacanthal on angiogenesis was the use of embryos from a transgenic line of zebrafish driving the GFP

expression in the endothelium. Throughout the development of the zebrafish embryos, intersegmental vessels sprout and grow upward from the aorta, and then, the tips join by anastomosis to form a dorsal vein. The lack of formation of the last intersegmental vessels in the presence of a test compound is interpreted and accepted as a sign of its antiangiogenic effect. Damnacanthal concentrations as low as 1 µM were enough to inhibit the formation of some of the last intersegmental blood vessels (Figure 2D).

3.4. Damnacanthal inhibits tubule-like structures formation by endothelial cells grown on Matrigel

In the screening programs carried out in our lab, test compounds are initially selected by their ability to inhibit the formation of "tubule-like" structures by endothelial cells grown on Matrigel. This assay simulates the final event of angiogenesis, where endothelial cells form a three-dimensional network of new tubes. This is a quantitative and reliable *in vitro* angiogenesis assay that can be adapted for high throughput use [31]. Figure 3A shows representative images of controls, as well as damnacanthal treatments at the lowest doses at which there was a complete inhibition of the formation of "tubule-like" structures on Matrigel for each endothelial cell type tested. For BAEC, we observed signficant inhibitory effects only for 40 µM damnacanthal, whereas for human endothelial cells complete inhibition was observed at 20 µM damnacanthal in the case of HMEC and at 5 µM damnacanthal in the case of RF-24 cells. At lower concentrations, damnacanthal was able to produce a partial inhibitory effect in a dose-response manner (Figure 3B).

Since one of the kinases targeted by damanacanthal is VEGFR3, which plays a key role in lymphangiogenesis, we also wanted to test whether damanacanthal is able to inhibit lymphatic vessel formation. Figure 3C shows that damnacanthal is also able to partially inhibit the formation of "tubule-like" structures by lymphatic endothelial cell immersed in collagen.

Figure 3D shows the effects of damanacanthal added to endothelial cells after the formation of "tubule-like" structures on Matrigel. These data suggest that damanacanthal can also behave as a vascular disruption agent.

3.5. Damnacanthal inhibits endothelial cell proliferation and survival

Angiogenesis involves the local proliferation of activated endothelial cells. To investigate the ability of damnacanthal to inhibit the growth of serum-activated endothelial cells, we made use of the MTT assay. Figure 4A shows that damnacanthal inhibited the growth of the three types of endothelial cells tested yielding typical survival curves with half-maximal inhibitory concentration values (IC_{50}) within the micromolar range (Table 1). Figure 4B shows that damnacanthal was also able to inhibit lymphatic endothelial cell growth (with a IC_{50} value of 31±3 µM), thus reinforcing the afore-mentioned suggestion that damnacanthal can inhibit not only angiogenesis but also lymphangiogenesis.

Flow cytometric cell cycle analysis of damnacanthal-treated endothelial cells after propidum iodide staining allowed detecting significant increases in the apoptotic sub G1 populations with increasing damnacanthal concentrations (Figure 4C). This effect was accompanied by decreases in the G2/M population. The most potent effect was observed in BAEC whereas the weakest effects were detected in HMEC (Figure 4C).

The proapoptotic effect of damanacanthal on endothelial cells was further demonstrated by using the Annexin V/7AAD assay (Figure 5A) and by in vitro determination of caspase 3/7 activity (Figure 5B). To get some additional insights on specific molecular targets of damnacanthal involved in endothelial cell proliferation and survival, we carried out Western blot analysis of proteins extracted from control and damnacanthal-treated HUVEC. ERK1/2 and Akt are two of the most relevant signaling pathways controlling endothelial cell proliferatin and survival in angiogenesis [32]. Therefore, we examined the effect of 50 µM damnacanthal on the HGF-induced phosphorylation of ERK1/2 and Akt in HUVEC. Figure 5C shows a partial inhibition of the phosphorylation of both ERK 1 and 2 and a more potent inhibitory effect of damnacanthal on the phosphorylation of Akt.

3.6. Damnacanthal inhibits endothelial cell attachment to fibronectin

During angiogenesis, actively proliferating endothelial cells must migrate into their surrounding space by a double action of extracellular matrix (ECM) reearrangement and adhesive contacts with components of this ECM. To test whether damnacanthal was able to affect endothelial cell adhesion to ECM components, we carried out an adhesion assay on fibronectin after 24-h treatment. Figure 6A shows that damnacanthal partially inhibited the adhesion of human endothelial cells to fibronectin.

Since endothelial cells attach to fibronectin through their integrin a5, we also tested whether damnacanthal was able to affect the levels of integrin a5. Figure 6B shows that damancanthal decreases the levels of integrin a5, as determined by flow cytometry.

3.7. Damnacanthal inhibits endothelial cell potential to migrate and remodel extracellular matrix

The scratch "wound healing" assay was used to study the effect of damnacanthal on the overall migratory potential of endothelial cells. Figure 7A shows that 50 µM damncanthal was able to inhibit partially endothelial cell migration as photografically recorded 7 h after the scratch "wounds" were made. The most potent effect were observed on BAEC. Incubations were extended up to 24 h after scratch "wounding", a time at which the inhibitory effects of damnacanthal were more evident even at concentrations lower than 10 µM (Figure 7B). Figures 7C and 7D shows that 50 µM damnacanthal also partially inhibited the invasive potential of human endothelial cells through a layer of Matrigel in the invasion assay.

MMP-2 and uPA are two ECM-remodeling enzymes produced and secreted by endothelial cells that play an essential role in angiogenesis allowing the migration and invasion of endothelial cells into its surrounding space [33-35]. Gelatin and casein zymographies are simple and sensitive techniques to quantify the effects of tested compounds on MMP-2 and uPA levels, respectively. Figure 8A shows that damnacanthal is able to inhibit both the production and the secretion of BAEC, RF-24 and HMEC endothelial cell MMP-2 in a dose-response manner, as determined by the gelatin zymography assay. Quantitative data for results obtained

with this assay are provided in Figure 8B. On the other hand, damnacanthal was also able to inhibit endothelial cell uPA in a dose-response manner, as determined with both conditioned media and cell extracts from HMEC by using a casein zymography assay (see typical results in Figure 8C and quantitative data in Figure 8D). Table 2 summarizes data obtained by using qPCR to determine the relative levels of expression of mRNAs for MMPs, TIMPs, uPA and PAI. These data show that damnacanthal treatment decreased the (MMP-1/2)/TIMP and uPA/PAI ratios.

4. Discussion

In the present work, we have shown that damnacanthal is an *in vitro* inhibitor of several tyrosine kinase activities involved in angiogenesis and that damnacanthal effectively inhibits *ex vivo*, *in vivo* and *in vitro* angiogenesis.

It was known that damnacanthal can block T cell chemotaxis [19] through its extremely potent inhibition of $p56^{lck}$ tyrosine kinase activity [18]. However, at concentrations in the micromolar range, damnacanthal can also inhibit other tyrosine kinases that are more related to cancer, such as PDGFR, erbB2, EGFR and insulin receptor [18]. In fact, several antitumoral effects have been previously reported for damnacanthal [20-22]. Our group has added c-Met to the list of tyrosine kinases targeted by damnacanthal [23]. Furthermore, several effects of damnacanthal on Hep G2 hepatocellular carcinoma suggested that damnacanthal is a new inhibitor of c-Met with potential utility for hapatocellular carcinoma treatment [23]. The present report confirms the previously described inhibitory effects of damnacanthal on EGFR [20] and c-Met [23] and shows that other 6 kinases are also inhibited by damnacanthal: VEGFR1-3 and FGFR1, 2 and 4. All these tyrosine kinases are involved in angiogenesis [36,37]. Therefore, the anti-angiogenic effects of damnacanthal could be expected. Furthermore, the simple network and functional enrichment analyses herein carried out reinforce this suggestion that damancanthal could exert anti-angiogenic effects through its targeting of tyrosine kinase activities.

Tyrosine kinases inhibitors are structurally diverse, but most of them share a common mechanism of action based on the inhibition of the catalytic phosphate transfer, by occupying the ATP binding site in the protein [38]. The kinase catalytic domain has an overall conserved bilobal structure, arranged into two well-differentiated N- and C-terminal domains linked by the so-called kinase "hinge" region [27]. ATP binds into the cleft formed between the N- and C-terminal lobes and interacts with several residues in the hinge region. The docking experiments and molecular dynamics simulation performed in this work show a plausible binding mode of damnacanthal to the kinase domains of VEGFR2 and c-Met receptors, and FAK. In all three cases, the inhibitor is mainly stabilized by hydrophobic interactions with residues in the glycine-rich loop and ATP-binding site, and by specific interactions with amino acids in the hinge region (Figure 1). In summary, our results in docking and molecular dynamic studies show that damnacanthal partially occludes the ATP-binding site of the VEGFR2 and c-Met receptors, and FAK, and provide structural information of the protein-inhibitor interaction in every case.

As mentioned above, since these kinases inhibited by damnacanthal are related to angiogenesis, the anti-angiogenic effects of damnacanthal could be expected. In fact, this was consistently confirmed by four different experimental approaches, an ex vivo and three in vivo assays. The *ex vivo* aortic ring assay recapitulates the events of endothelial cell proliferation, migration and the capillary-like tube formation from aortic explants in a collagen matrix [39]. In this assay, damnacanthal concentrations ≥12.5 μM completely inhibited angiogenesis. In the CAM and zebrafish *in vivo* angiogenesis assays, damnacanthal behaves as a potent antiangiogenic compound at doses and concentrations even lower than those reported for other strong angiogenesis inhibitors [7,14,40,41]. In contrast, in the *in vivo* Matrigel plug assay 50 μM damnacanthal induced only a partial inhibition of angiogenesis, as previously reported by us in the case of aeroplysinin-1 [7].

In the present work we have also tested the specific effects of damnacanthal in each of the sequential steps of events that take part during the angiogenic process. Potential unspecific cytotoxic effects of damnacanthal were discarded in all the *in vitro* experiments with the obvious exception of the cell growth and survival assays by carrying our parallel control assays

of *in vitro* toxicity, as described in the detailed Material and Methods (supplementary material). A detailed discusion of the results obtained from *in vitro* experiments is provided in SI section "Detailed discussion of the *in vitro* results" (supplementary material). These results clearly show that damnacanthal affects both the proliferation and survival of endothelial cells, decreases the adhesiveness, as well as the migratory and invasive potential of endothelial cells and inhibits the final differentiation of endothelial cells to form new vessels. Most of these *in vitro* experiments were carried out with three diffeent types of endothelial cells: primary cultures of boviene aorta endothelial cells (BAEC), immortalized human umbilical vein endothelial cells (RF-24) and immortalized human microvascular endothelial cells (HMEC). These three types of endothelial cells correspond to great, middle and small vessels and exhibited different sensitivities to damnacanthal in different *in vitro* assays. For instance, damnacanthal-treated RF-24 cells showed the highest inhibitory effects in the tubulogenesis and the adhesion-to-fibronectin assays and in the levels of the MMP-2 extracellular remodeling enzyme. On the other hand, BAEC showed the lowest IC_{50} values in the MTT cell growth assay and the highest counting number in the subG1 subpopulation cells. These and other differences in cell type sensitivities might suggest some differential specificities for damnacanthal against the angiogenic behavior of endothelial cells derived from vessels of different sizes and origins. This posibility deserves to be further explored in the future, since its confirmation could have pharmacological interest. Some of our *in vitro* results also suggest that damancanthal could have not only anti-angiogenic effects, but could also behave as an anti-lymphangiogenic as well as an anti-vascular compound. However, these observations should be taken with caution, since more detailed investigations would be required to confirm or refuse these suggestions in a similar way to that used by us and others to confirm that toluquinol is an anti-lymphangiogenic compound [42].

In conclusion, taking the obtained results altogether the present work clearly shows that damnacanthal behaves as a multikinase inhibitor and a multitargeted antiangiogenic drug, suggesting that it could have high potential pharmacological interest for the treatment of angiogenesis-dependent diseases, including cancer. Future pre-clinical studies to test its actual

therapeutical potential seem warranted. Furthermore, the possibility of defining future synthetic derivatives with even better activities and performance deserves to be explored.

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Conflicts of interest

All authors declare they have no actual or potential competing financial interest.

Authors' contributions

JAGV carried out the *in vitro*, *ex vivo* and *in vivo* experiments and helped in the managing of bibliography. APA carried out the *in silico* experiments of docking and molecular dynamics simulations and wrote the preliminary draft of the part of the manuscript related with docking and molecular dynamics simulations. BMP carried out additional *in vitro* experiments. ARQ interpreted the results and revised the manuscript. MAM conceived the experimental work, carried out the network analysis, interpreted the results and wrote and revised the manuscript. All authors read and approved the final manuscript.

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Table 1 IC_{50} values. Half-maximal inhibitory concentration (IC_{50}) values calculated from dose-response curves as the concentration of compound yielding 50% of control cell survival. They are expressed as means ± SD of three independent experiments.

Cell line	IC_{50} (µM)
HMEC	46,01 ± 1,42
RF-24	18,75 ± 0,2
BAEC	9,88 ± 0,61

Table 2 Effects of damnacanthal on the expression levels of extracellular matrix proteases and their inhibitors in endothelial cells. Relative expression levels of messengers in damnacanthal-treated endothelial cells quantified by qPCR are given as percentages with respect to the corresponding expression levels in control, untreated cells. Data are means±S.D. of three independent experiments.

	MMP-1	MMP-2	TIMP-1	TIMP-4	uPA	PAI-1
HMEC	38.1 ± 12.4	47.1 ± 5.3	149.6 ± 8.1	167.3 ± 24.1	46.1 ± 10.1	61.1 ± 11.3
RF-24	2.0 ± 0.1	91.0 ± 1.9	2788.1 ± 240.8	2930.5 ± 1202.0	188.1 ± 22.1	30.4 ± 13.5

Figure legends

Figure 1. Interaction of damnacanthal with the kinase domains of three different tyrosine kinases. Final structure of the complexes generated between damnacantal and the kinase domains of VEGFR2 (purple), FAK (orange) and c-Met (teal). Receptor structures are shown in cartoon whereas damnacantal is shown as spheres. A close-up view of the binding pocket is also shown for each system, in which damnacantal is depicted as light grey sticks. Key residues in the binding site of each protein are highlighted as sticks.

Figure 2. Damnacanthal inhibits angiogenesis *ex vivo* and *in vivo*. A) Aortic ring *ex vivo* assay was performed as described in Materials and Methods. Representative photographs of aortic rings (lateral view) after 14 days of incubation in a 3D collagen gel overlaid with complete medium in the absence (control) or presence of 20 ng/mL VEGF, and with 12.5 μM damnacanthal. B) Effect of DC (0.5 nmol/disk) on *in vivo* angiogenesis, as determined by the chorioallantoic membrane (CAM) assay. Areas covered by disks are delimited by dashed circles. Arrows point to rebound of vessels outward from the treated area. Asterisks indicate disrupted vessels. Experiments were carried out as described in Materials and Methods (bar=1000μm). C) Representative Matrigel plugs that contained no FGFb, FGFb alone, or FGF plus 50 μM damnacanthal were photographed. Total hemoglobin content in the Matrigel plugs was quantified as an indicator of blood vessels formation. Data are expressed as means ± SD of at least four animals. Symbol indicates significant differences between control- untreated and treated cells (*, $p < 0.05$). D) Inhibition of zebrafish intersegmental vessels by damnacanthal**.** Transgenic TGfli1:EGFPy1 zebrafish embryos, which show green fluorescent protein (GFP) expression in endothelial cells, were incubated in the absence or presence of damnacanthal. Blood vessel morphology was recorded by fluorescence microscopy. Zebrafish intersegmental vessel assay was carried out with different doses of damnacanthal, as described in Materials and Methods (bar=50μm).

Figure 3. Damnacanthal decreases "tubule-like" structures in both endothelial and lymphatic cells, and disrupts pre-formed endothelial "tubule-like" structures. A) Representative photographs of control (untreated) and damnacanthal-treated endothelial cells on Matrigel. B) Quantitative analysis of "tubules" formed in endothelial cells treated with 2.5 and 5 μM damnacanthal. C) Representative photographs of control (untreated) and damnacanthal-treated lymphatic cells in a collagen matrix. Quantitative analysis of data is provided for the full range of tested damnacanthal concentrations in lymphatic cells. D) Representative photographs of control (untreated) and damnacanthal-treated endothelial cells in the "tubule-like" structure disruption assay. Quantitative analysis of data is provided for the full range of tested damnacanthal concentrations in endothelial cells. Quantitative data are mean ± SD for three independent experiments. Significant differences between control-untreated and treated cells: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; **** $p < 0.001$.

Figure 4. Damnacanthal decreases endothelial and lymphatic cells survival. A) Survival curve of endothelial cells in the presence of damnacanthal as determined by the MTT method. B) Survival curve of lymphatic cells in the presence of damnacanthal as determined by the MTT method. C) Effect of damnacanthal on endothelial nuclear morphology. Endothelial cells were grown on covers, treated with the indicated

concentrations of damnacanthal for 14 h, fixed with formalin, stained with Hoechst, and mounted on slides, and nuclei were observed under a fluorescence microscope. D) Representative histograms of flow cytometry results of the cell cycle distribution of subpopulations of control and damnacanthal-treated endothelial cells after 24 h of treatment. Quantitative analysis of cell cycle analysis data for the full range of tested compound concentrations. Data are mean ± SD for three independent experiments. Significant differences between control-untreated and treated cells: *, $p < 0.05$.

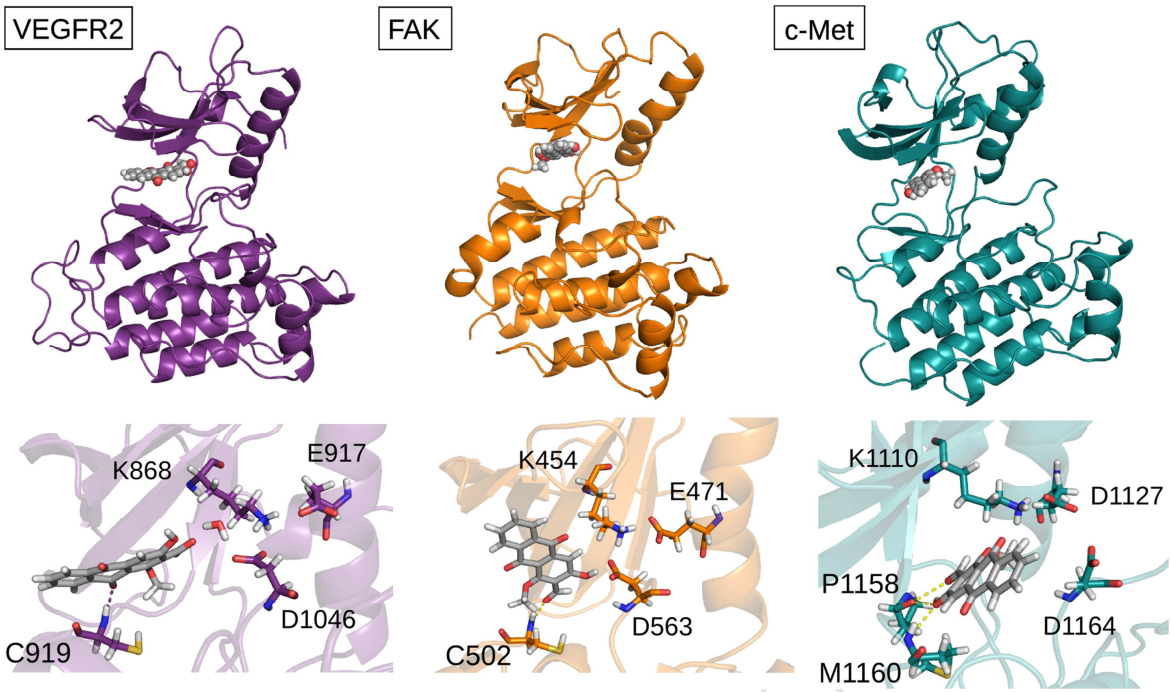
Figure 5. Damnacanthal activates apoptosis mechanism in endothelial cells. A) Quantitative analysis of data obtained with the Annexin V/7-AAD apoptosis assay. B) Quantitative caspase 3/7 activity in endothelial cells after 24 h of damnacanthal treatment. C) Western blot analysis of the effect of 50 µM damnacanthal on p-Akt/Akt and p-Erk/Erk ratios in HUVEC. GAPDH levels are used as internal controls. Strips corresponding to each of the proteins shown are cropped from different blots run under the same experimental conditions.
Quantitative data are mean ± SD for three independent experiments. Significant differences between control-untreated and treated cells: *, $p < 0.05$; ***, $p < 0.005$.

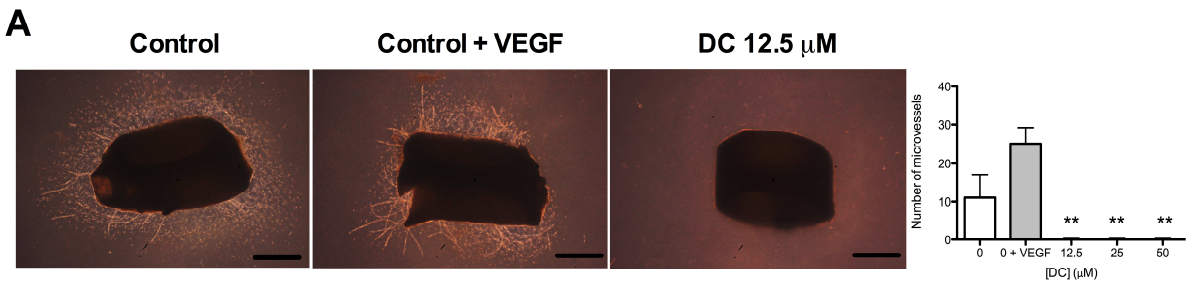
Figure 6. Damnacanthal decreases adherence ability of endothelial cells. A) Representative photographs of control (untreated) and damnacanthal-treated endothelial cells on fibronectin-coated plates. Quantitative analysis of data is provided for the full range of tested damnacanthal concentrations. B) Histograms represent quantifications of the levels of integrin alpha as determined by flow cytometry in HMEC and RF-24 after 24 h of damnacanthal treatment. Quantitative data are mean ± SD for three independent experiments. Significant differences between control-untreated and treated cells: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

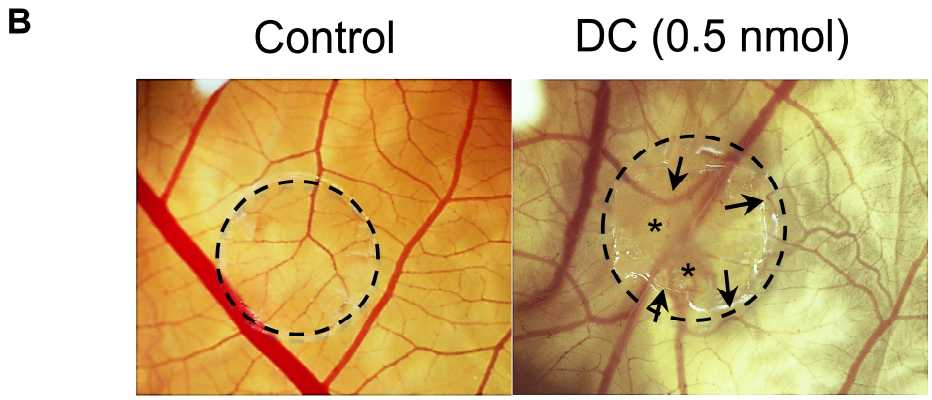
Figure 7. Damnacanthal decreases endothelial cell migration and invasion. A) Representative photographs of endothelial cell migration after 7 h of damnacanthal treatment. Quantitative analysis of data for cell-free area is shown in histograms. B) Representative photographs of endothelial cell migration after 24 h of damnacanthal treatment. Quantitative analysis of data for cell-free area. C-D) Evaluation of invading HMEC (C) and RF-24 (D) cells in the invasion assay with untreated and damnacanthal-treated cells. Quantitative data are mean ± SD for three independent experiments. Significant differences between control-untreated and treated cells: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

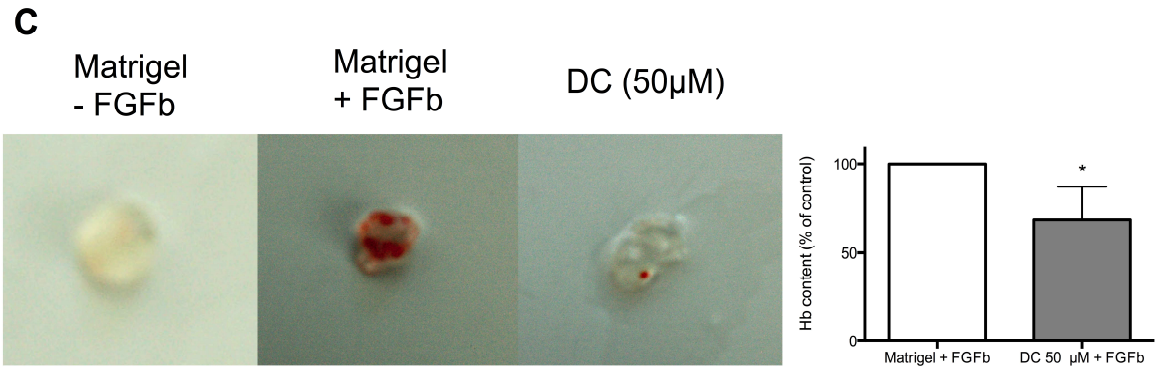
Figure 8. Damnacanthal decreased protein levels and gene expression of proteases of the extracellular matrix. A) Effect of damnacanthal on the levels of MMP-2 in both conditioned media (CM) and cell extracts (CE) from different endothelial cells. Representative results of gelatinolytic assays showing the levels of MMP-2 activity in control (untreated) and damnacanthal-treated endothelial cells are shown. B) Histograms summarize the quantitative analysis of data for the full range of tested compound concentrations. C) Effect of damnacanthal on the levels of uPA activity in

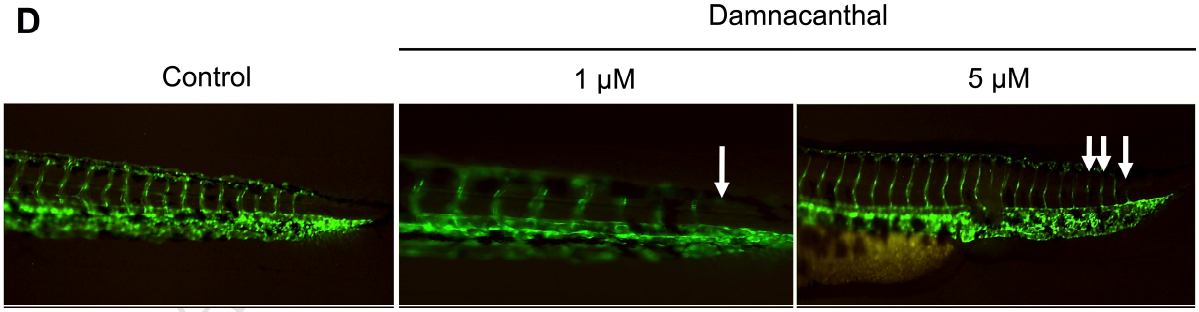
both conditioned media (CM) and cell extracts (CE) from HMEC. Representative results of plasminogen zymography assays showing the levels of uPA activity in control (untreated) and damnacanthal-treated endothelial cells are shown. D) Histograms summarize the quantitative analysis of data for the full range of tested compound concentrations. Quantitative data represent mean ± SD for three independent experiments. Symbols indicate significant differences between control-untreated and treated cells (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).
Figure 6B and 6D legend:

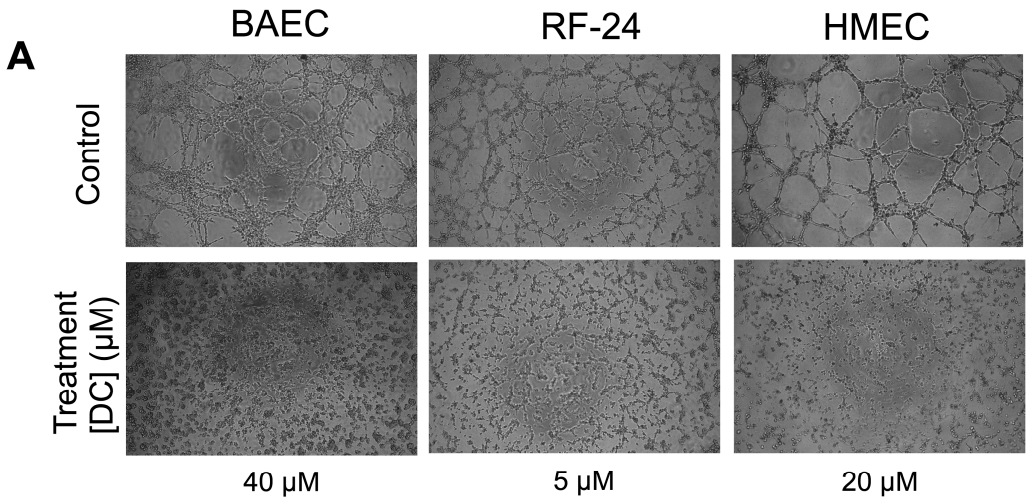


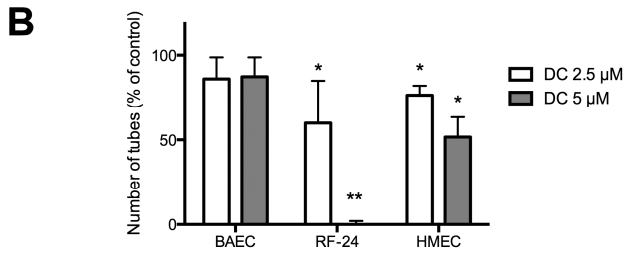

A
Control
Control + VEGF
DC 12.5 μM
Number of microvessels
40
30
20
10
0
**
**
**
0
0 + VEGF
12.5
25
50
[DC] (μM)

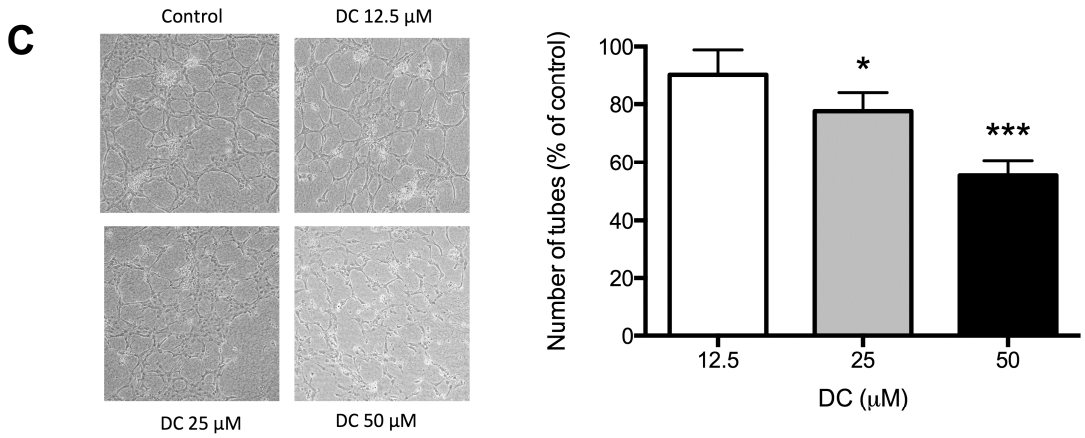

B
Control
DC (0.5 nmol)
*
*

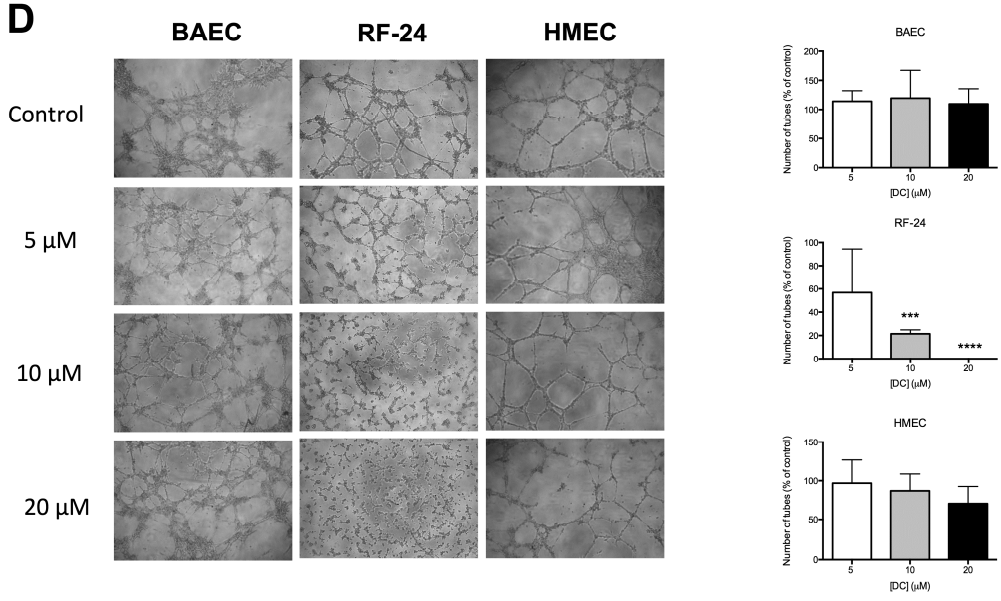

C
Matrigel
- FGFb
Matrigel
+ FGFb
DC (50μM)
Hb content (% of control)
100
50
0
*
Matrigel + FGFb
DC 50 μM + FGFb

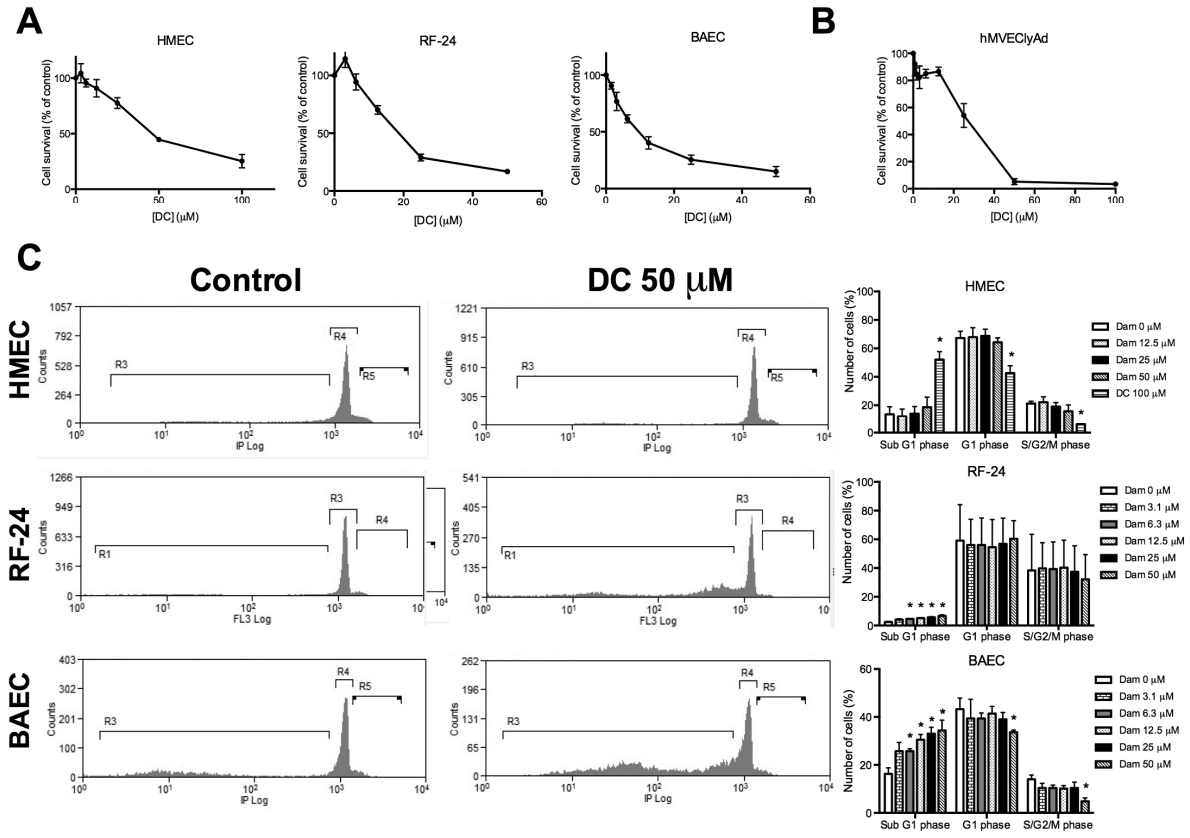

D
Damnacanthal
Control
1 μM
5 μM

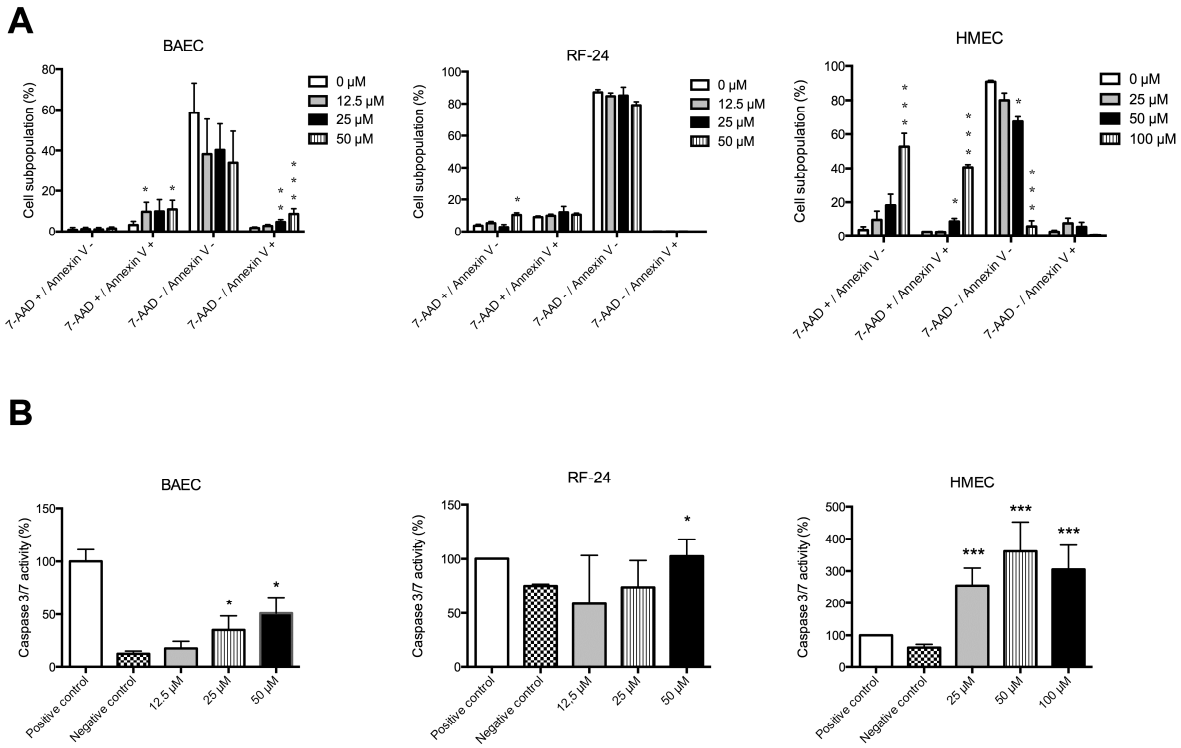

A
BAEC
RF-24
HMEC
Control
Treatment [DC] (μM)
40 μM
5 μM
20 μM

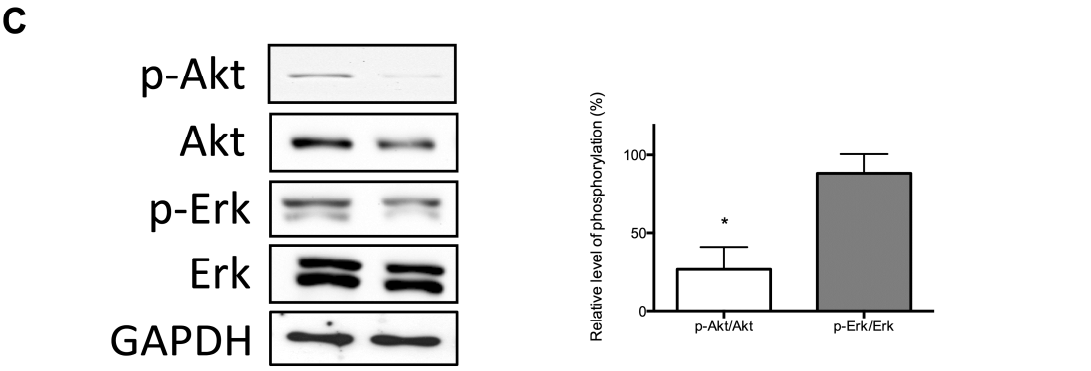

B
Number of tubes (% of control)
100
50
0
*
**
*
*
BAEC
RF-24
HMEC
DC 2.5 μM
DC 5 μM

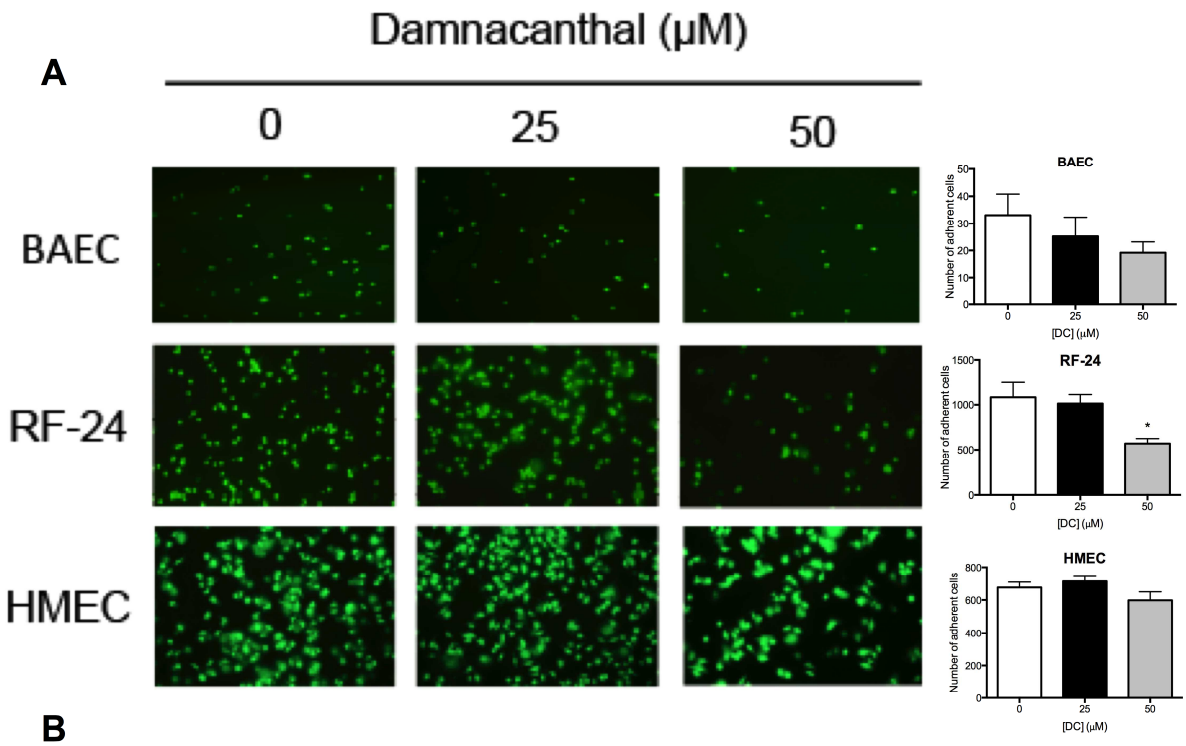

C
Control
DC 12.5 μM
DC 25 μM
DC 50 μM
Number of tubes (% of control)
100
80
60
40
20
0
*
***
12.5
25
50
DC (μM)

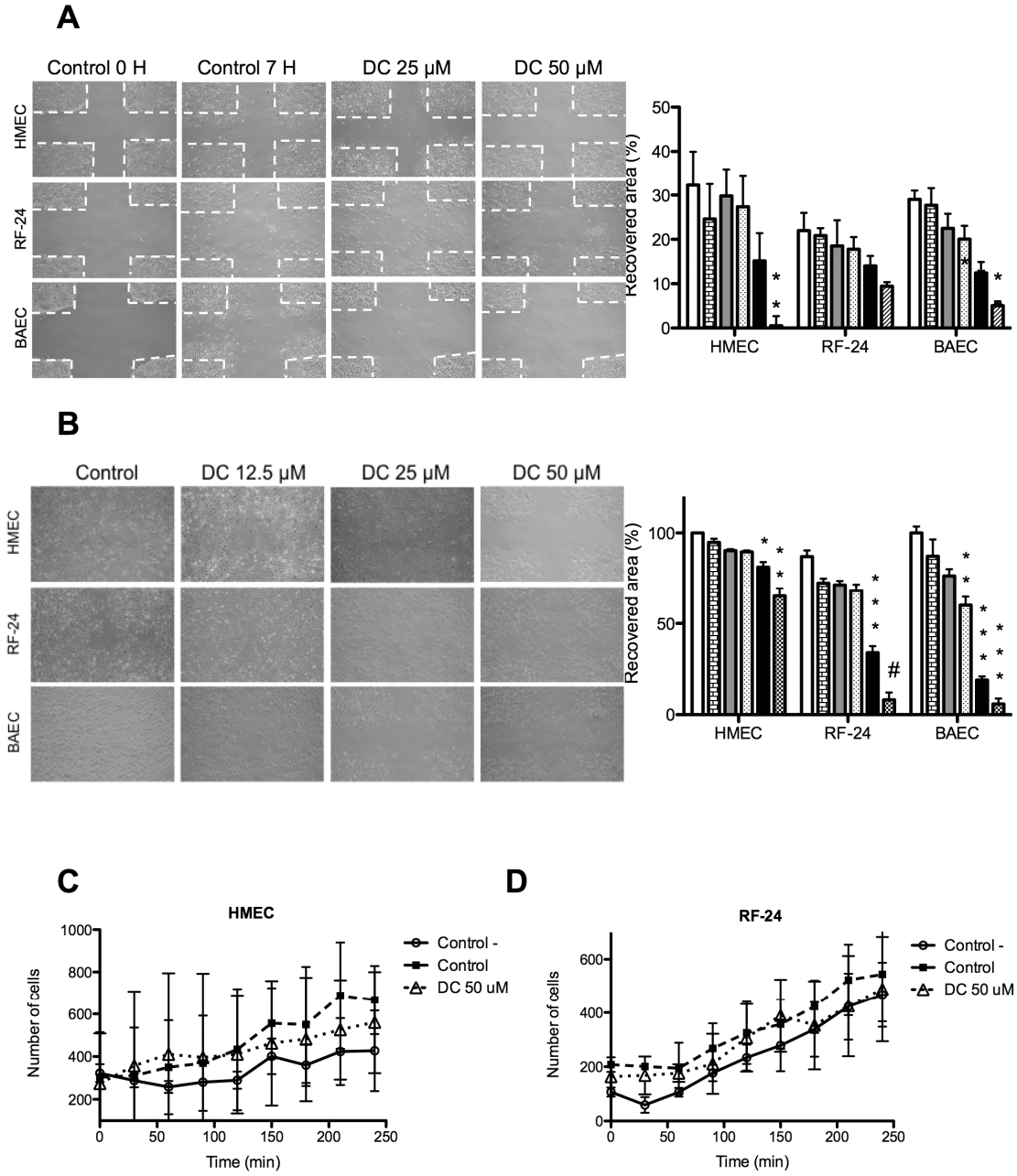

D
BAEC
RF-24
HMEC
Control
5 μM
10 μM
20 μM
BAEC
Number of tubes (% of control)
200
150
100
50
0
5
10
20
[DC] (μM)
RF-24
Number of tubes (% of control)
100
80
60
40
20
0
***
****
5
10
20
[DC] (μM)
HMEC
Number of tubes (% of control)
150
100
50
0
5
10
20
[DC] (μM)

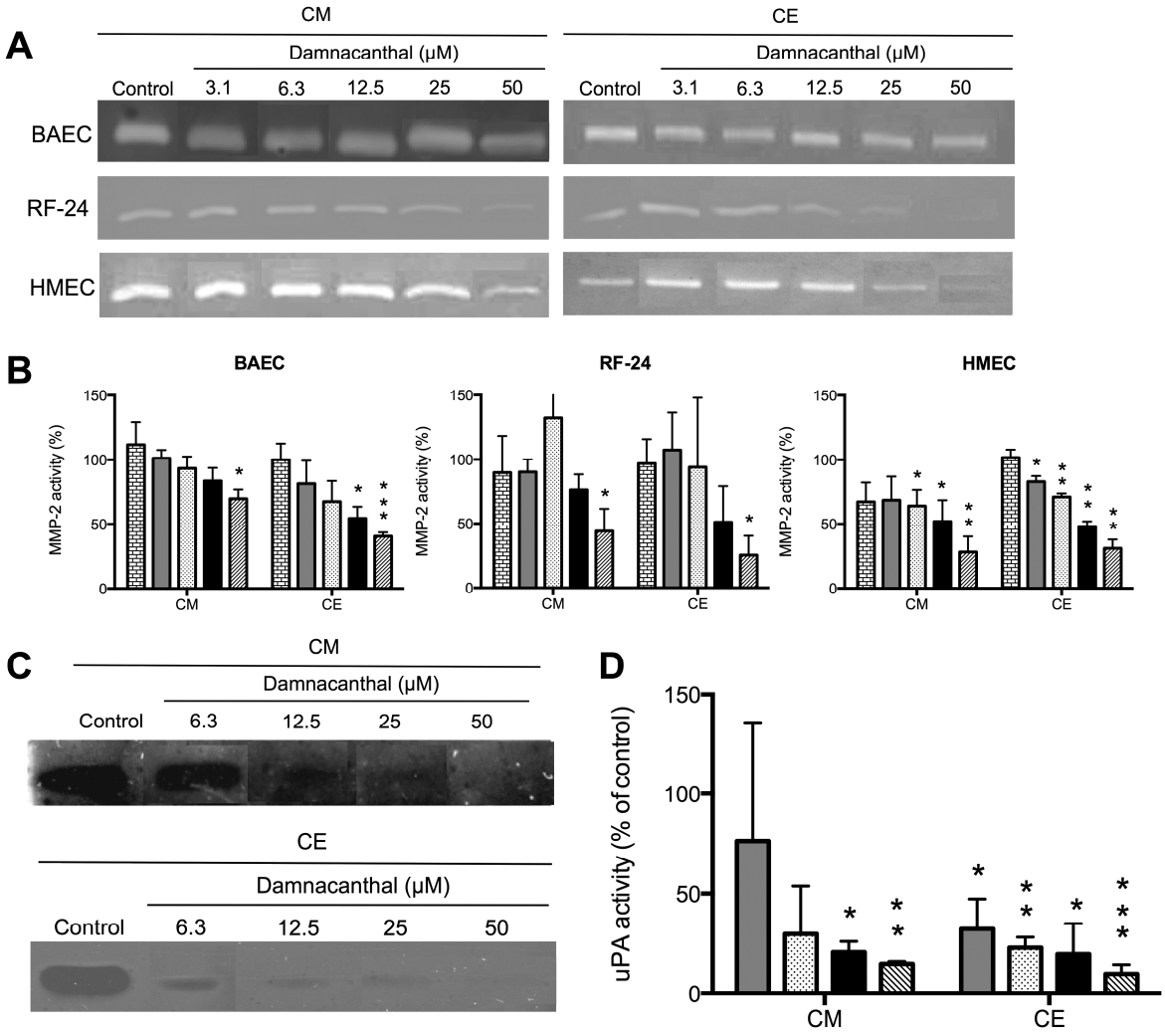



A
BAEC
RF-24
HMEC
Cell subpopulation (%)
0 µM
12.5 µM
25 µM
50 µM
100 µM
7-AAD + / Annexin V -
7-AAD + / Annexin V +
7-AAD - / Annexin V -
7-AAD - / Annexin V +
B
BAEC
RF-24
HMEC
Caspase 3/7 activity (%)
Positive control
Negative control
12.5 µM
25 µM
50 µM
100 µM


C
p-Akt
Akt
p-Erk
Erk
GAPDH
Relative level of phosphorylation (%)
p-Akt/Akt
p-Erk/Erk




A
Control 0 H
Control 7 H
DC 25 µM
DC 50 µM
HMEC
RF-24
BAEC
Recovered area (%)
50
40
30
20
10
0
HMEC
RF-24
BAEC
B
Control
DC 12.5 µM
DC 25 µM
DC 50 µM
HMEC
RF-24
BAEC
Recovered area (%)
100
50
0
HMEC
RF-24
BAEC
C
HMEC
Number of cells
1000
800
600
400
200
0
50
100
150
200
250
Time (min)
Control -
Control
DC 50 uM
D
RF-24
Number of cells
600
400
200
0
0
50
100
150
200
250
Time (min)
Control -
Control
DC 50 uM



HIGHLIGHTS

- Damnacanthal inhibits several angiogenesis-related tyrosine kinases.
- Docking and molecular dynamics provide insights on these inhibitory effects.
- Damnacanthal is a very potent inhibitor of ex vivo and in vivo angiogenesis.
- Specific effects of damnacanthal on different angiogenic process steps are shown.