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PII: S0006-2952(13)00231-1
DOI: http://dx.doi.org/doi:10.1016/j.bcp.2013.04.007
Reference: BCP 11611

To appear in: *BCP*

Received date: 1-3-2013
Revised date: 8-4-2013
Accepted date: 9-4-2013

Please cite this article as: García-Caballero M, Beffa MM, Cañedo L, Medina MÁ, Quesada AR, Toluquinol, a marine fungus metabolite, is a new angiossuppresor that interferes the AKT pathway, *Biochemical Pharmacology* (2013), http://dx.doi.org/10.1016/j.bcp.2013.04.007

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Toluquinol, a marine fungus metabolite, is a new angiossuppresor that interferes the AKT pathway

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Keywords: Toluquinol, 2-methylhydroquinone, angiogenesis, cancer, marine compounds

Short title: Angiosupressive activity of toluquinol

Conflict of interest: The authors state no conflict of interest.

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Abstract

Toluquinol, a methylhydroquinone produced by a marine fungus, was selected in the course of a blind screening for new potential inhibitors of angiogenesis. In the present study we provide the first evidence that toluquinol is a new anti-angiogenic-compound. In a variety of experimental systems, representing the sequential events of the angiogenic process, toluquinol treatment of activated endothelial cells resulted in strong inhibitory effect. Toluquinol inhibited the growth of endothelial and tumor cells in culture in the micromolar range. Our results indicate that the observed growth inhibitory effect could be due, at least in part, to an induction of apoptosis. Toluquinol induced endothelial cell death is mediated via apoptosis after a cell cycle block and caspase activation. Capillary tube formation on Matrigel and migratory, invasive and proteolytic capabilities of endothelial cells were inhibited by addition of toluquinol at subtoxic concentrations. Inhibition of the mentioned essential steps of in vitro angiogenesis agrees with the observed inhibition of the in vivo angiogenesis, substantiated by using the chick chorioallatoic membrane assay and confirmed by the murine Matrigel plug, the zebrafish embryo neovascularization and the zefrafish caudal fin regeneration assays. Data here shown altogether indicate that toluquinol has antiangiogenic effects both in vitro and in vivo that are exerted partly by suppression of the VEGF and FGF-induced AKT activation of endothelial cells. These effects are carried out at lower concentrations to those required for other inhibitors of angiogenesis, what makes toluquinol a promising drug candidate for further evaluation in the treatment of cancer and other angiogenesis-related pathologies.

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1. Introduction

Angiogenesis, the process of formation of new blood vessels from other pre-existent ones, is strictly controlled by a balance of activators and inhibitors. When angiogenic growth factors are produced in excess of angiogenic inhibitors, the balance is tipped in favor of blood vessel growth, connecting the so called "angiogenic switch". Angiogenesis is reduced in the adult to some processes related to reproductive cycles (corpus luteum formation, endometrial vascularization, placental development), wound healing and bone repair. In all these cases, angiogenesis takes places as a transient and highly regulated process. On the contrary, a persistent and deregulated angiogenesis is an essential step in the transition of tumors from a dormant state to a malignant state. Nowadays, angiogenesis is considered to be one of the hallmarks of cancer, playing a relevant role in tumor growth, invasion, and metastasis [1]. But the role of the angiogenesis switch is not limited to the neoplastic diseases pathogenesis, but it has also been related to other non-neoplastic diseases including wet macular degeneration, diabetic retinopathies, diabetes, psoriasis and rheumatoid arthritis, among others. All these facts make angiogenesis inhibition an attractive target in the field of pharmacological research. A continuously increasing number of antiangiogenic therapies are being approved for the treatment of cancer, blindness, and other angiogenesis-dependent diseases, encouraging expectations in their therapeutic potential [2].

Terrestrial fungi have been a rich source of relevant bioactive compounds, produced as secondary metabolites, and currently used for the treatment of a number of human diseases [3,4]. More recently, marine species are demonstrating to be an unexplored and prolific source of molecular diversity, yielding an increasing number of products for biotechnological applications, including the production of bioactive compounds for pharmaceutical use [5,6]. In the course of ongoing research efforts aimed at exploring the biosynthetic potential of rare marine microorganisms, the culture broth of the marine fungus *Penicillium* sp. HL-85-ALS5-R004 was selected by means of its ability to inhibit the endothelial cell differentiation in vitro. The compound responsible for the antiangiogenic activity was isolated and structurally characterized, being identified as 2-methylhydroquinone (also denominated 2,5-toluenediol or toluquinol) (Fig. 1).

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Toluquinol is a naturally occurring metabolite, previously isolated from soil-fungi, exhibiting herbicidal activity [7,8]. In this article we provide evidence of the antiangiogenic and antitumoral activities of toluquinol. Our results clearly show that this compound inhibits angiogenesis in vitro and in vivo. It inhibits the growth and induces apoptosis in tumor and endothelial cells; it inhibits endothelial tube formation on a layer of Matrigel, and decreases the endothelial proteolytic capability. In vivo, the antiangiogenic activity of this compound was shown by the chick chorioallantoic membrane (CAM), in the Matrigel plug, and in the zefrafish embrio neovascularizatuion and zebrafish caudal fin regeneration assays. Taken together, our data indicate that toluquinol inhibits several essential steps of the angiogenic process, this antiangiogenic activity being exerted at lower concentrations than those required for other previously described inhibitors of angiogenesis, including the structurally-related calcium dobesilate and gentisic acid. This work reinforces the possibility of utilizing compounds derived from marine organisms as potential new sources of angiogenesis inhibitors that could be used for the treatment of angiogenesis-related malignancies.

2. Materials and methods

2.1. Materials

Cell culture media were purchased from Biowhittaker (Walkersville, MD, USA). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, UK). Matrigel was purchased from Becton–Dickinson (Bedford, MA, USA). Recombinant human FGF basic Pro143-Ser288 (FGF2) was produced in *E.coli* at Cell Signaling Technology (Danvers, MA, USA). Recombinant human VEGF165 Ala207-Arg371 was expressed in human 293 cells at Cell Signaling Technology. Antibodies were purchased to Cell Signalling. Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals (St. Louis, MO, USA). Plastics for cell culture were supplied by NUNC (Roskilde, Denmark). Fertilised chick eggs were obtained from Granja Santa Isabel (Córdoba, Spain). Toluquinol was isolated and purified from the fermentation broth of a marine fungus by Instituto Biomar (León, Spain).

2.2. Cell culture

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Bovine aortic endothelial cells (BAECs) were isolated from bovine aortic arches as previously described [9] and maintained in Dulbecco's modified Eagle's medium (DMEM) containing glucose (1 g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (0.05 mg/mL), and amphotericin (1.25 mg/L) supplemented with 10% FBS (DMEM/10% FBS). Human fibrosarcoma HT1080 cells were maintained in DMEM containing glucose (4,5 g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (0.05 mg/mL), and amphotericin (1.25 mg/L) supplemented with 10% FBS. Human colon adenocarcinoma HT29 cells were maintained in McCoy´s 5A medium containing glutamine (2 mM), penicillin (50 IU/mL), streptomycin (0.05 mg/mL), and amphotericin (1.25 mg/L) supplemented with 10% FBS. Promyelocytic leukemia HL60 cells were maintained in RPMI1640 medium containing glutamine (2 mM), penicillin (50 IU/mL), streptomycin (0.05 mg/mL), and amphotericin (1.25 mg/L) supplemented with 20% FBS.

2.3. Extraction and Isolation of totuquinol from the marine fungus culture broth

2.5 litres of whole harvested broth of the marine fungus *Penicillium* sp. HL-85-ALS5-R004 were filtrated to separate the biomass and other solids, the antiangiogenic activity being detected in the supernatant. The broth filtrate was extracted with Amberlite XAD-1180 resin (250 ml) by agitating the resin in the broth for 1hour. The resin was collected and extracted twice with methanol (400 ml). The organic solvent was concentrated and evaporated to dryness in vacuum to yield 2.6 g of crude extract. The extract was applied to a silica gel VFC (vacuum flash chromatography) system, using a mixture of n-hexane-EtOAc and EtOAc-MeOH as eluting solvents. The fraction containing antiangiogenic activity was eluted with n-hexane-EtOAc 1:1 (39 mg). The active fraction displayed a peak by HPLC analysis, and was further purified by semipreparative HPLC, affording 17 mg of 99%-pure compound that was identified as toluquinol by NMR and HPLC-MS spectra.

2.4. Tube formation on Matrigel by endothelial cells

Matrigel (50 µL of 10.3 mg/mL) at 4 °C was used to coat each well of a 96-well plate and allowed to polymerise at 37 °C for a minimum of 30 min. 5×10^4 BAECs were added with 200 µL DMEM. Finally, the indicated concentrations of the compounds to

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be tested were added and incubated at 37 °C in a humidified chamber with 5% CO_2. After incubation for 7 h, cultures were observed (40x and 100x magnifications) and photographed with a NIKON inverted microscope DIAPHOT-TMD (Nikon, Tokyo, Japan). Each concentration was tested in duplicate, and two different observers evaluated the inhibition of tube formation. To check the viability of endothelial cells after the treatment with the compounds in this assay, BAECs were incubated in 96-well plate in the same conditions employed for the tube formation assay. After 7 h, cell viability in comparison to control untreated cells was determined by the addition of MTT essentially as below described for the cell growth assay. Aeroplysinin-1 (at a concentration of 2 μM) was used as a routine positive assay control in this assay [10].

2.5. Cell growth assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical, St. Louis, MO) dye reduction assay in 96-well microplates was used. The assay is dependent on the reduction of MTT by mitochondrial dehydrogenases of viable cell to a blue formazan product, which can be measured spectrophotometrically. BAECs (3×10^3 cells in a total volume of 100 μL of complete medium) were incubated in each well with serial dilutions of toluquinol (ten 1:1 dilutions starting at 100 μM). After 3 days of incubation (37 °C, 5% CO_2 in a humid atmosphere), 10 μL of MTT (5 mg/mL in PBS) was added to each well and the plate was incubated for a further 4 h (37 °C). The resulting formazan was dissolved in 150 μL of 0.04 N HCl–2 propanol and read at 550 nm. Four samples for every tested concentration were included in each of three independent experiments. experiment. IC_{50} values were calculated as those concentrations of toluquinol yielding a 50% of cell growth, based on a regression line in which the absorbance values at 550 nm were plotted against the logarithm of drug concentration.

2.6. Apoptosis and cell cycle assays

Apoptosis induction by toluquinol was assessed by flow cytometric analysis of propidium iodide-stained cells as follows: After treatment (14 h) with the indicated concentrations of toluquinol in complete medium, attached and unattached treated and

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control BAECs were harvested, washed (PBS), and fixed (70% ethanol, 1 h on ice). Pelleted cells were incubated (1 h protected from light) with RNase-A (0.1 mg/mL) and propidium iodide (40 μg/mL) during 1 h with shaking and protected from light. Percentages of subG1, G1, S and G2/M populations were determined using a MoFlo Dakocytomation cytometer.

For Hoechst staining experiments, cells were seeded on 8-well chamber slides and grown to sub-confluence. After treatments for 14 h with the indicated concentrations of toluquinol in complete medium, cells were washed (PBS) and fixed (formalin solution, Sigma). Chamber slides were stained with Hoechst, mounted (DAKO Cytomation Fluorescent Mounting Medium, DAKO), and observed under a fluorescence microscope (Leica, TCS-NT). The percentage of control and toluquinol-treated cells showing chromatin condensation was evaluated in ten vision fields from two independent experiments (the chromatin condensed cells were counted by fluorescence microscopy, the total cells were counted by bright field microscopy).

For DNA fragmentation studies, cells were grown to 75% confluency on 8-well Falcon humidified chamber slides and incubated for 14 h with or without the indicated concentrations of toluquinol in complete medium. The TUNEL (terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end-labeling) assay was performed with the use of the In Situ Cell Death Detection Kit (Roche Diagnostics, Barcelona, Spain), according to the manufacturer's instructions. The percentage of control and toluquinol-treated cells showing DNA fragmentation was evaluated in ten vision fields from two independent experiments (the TUNEL positive cells were counted by fluorescence microscopy, the total cells were counted by bright field microscopy).

For the determination of Caspase 3/7 activity, BAECs were plated in 96-well plates (13000 cells/well) and treated for 14 h with or without different concentrations of toluquinol in complete medium. Then, Caspase-Glo® 3/7 reagent (Promega Biotech Ibérica, Madrid, Spain), was added to wells, according to the manufacturer's instructions and the luminescence was recorded at thirty min with a GLOMAX 96 microplate luminometer. The assay provides a proluminescent caspase-3/7 DEVD-aminoluciferin substrate and a proprietary thermostable luciferase in a reagent optimized for caspase-3/7 activity, luciferase activity and cell lysis.

In the apoptosis experiments, 2-methoxyestradiol (10 μM) was used as a routine positive assay control [11].

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2.7. Endothelial cell migration and invasion assays

The migratory activity of BAECs was assessed using a wounding migration assay. Confluent monolayers in 6-well plates were wounded with pipet tips following two perpendicular diameters, giving rise to two acellular 1-mm-wide lanes per well. After washing, cells were supplied with 1.5 mL complete medium in the absence (controls) or presence of different concentrations of toluquinol. Wounded areas were observed under microscope after 7 h of incubation in the dark, and photos were taken from the same areas as those recorded at zero time. The amount of migration at 7 h was determined by image analysis in both controls and treated wells and normalized with respect to their respective values at zero time, using the NIH Image 1.6 software. The regrowth of BAECs into the cell-free area was calculated as the percentage of the initial wounded area (time 0) that had been recovered by endothelial cells after 7 h. Dimethyl fumarate (at a concentration of 50 μM) was used as a routine positive assay control [12].

Invasion of fluorescence-labeled endothelial cells was assayed by using a 24-well fluorescence-opaque membrane insert, as previously described [10]. BAECs were grown to 80–90% confluence in DMEM/10% FBS and then labeled in situ with 5 μg/mL Calcein-AM in complete culture medium for 2 hr at 37°C. After washing, the cell monolayer was briefly tripsinised to lift the cells, which were washed and suspended in DMEM supplemented with 0.1% BSA (DMEM/0.1% BSA). Then cells were added to Matrigel (25 μg/filter)-coated 8 mm FALCON HTS FluoroBlok inserts at a density of $2x10^5$ cells/insert in the absence or presence of the indicated concentrations of toluquinol. DMEM/10% FBS was used as chemoattractant in the lower wells. The inserts were incubated at 37°C and the real time kinetics of cell invasion were determined by taking readings at different time points. Fluorescence of cells that had migrated through the inserts was measured on the Fluorescence Microplate Reader (FL600FA, BIOTEK Instruments, Winooski, VT) in the bottom read mode using excitation/emission wavelengths of 485/530 nm and a gain setting of 75. Number of cells that migrated through the insert was calculated by interpolation in a standard curve comparing relative fluorescence units vs. cell number. For standard curve determination calcein-AM BAECs were diluted in DMEM/BSA to give the indicated cell

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density per well. The cells were placed in 96 well assay plates and quantified in the Fluorescence Microplate Reader as described above.

2.8. Gelatinolytic activity

BAECs or HT1080 cells were grown in 6-well plates at 75% subconfluency in 6-well plates, medium was aspirated, cells were washed twice with phosphate-buffered saline (PBS) and each well received 1.5 ml of DMEM/0.1% BSA containing 200 KIU of Aprotinin/mL. Additionally, some wells received the indicated concentration of toluquinol. After 24 h of incubation, conditioned media were collected, centrifuged at 1000xg and 4 ºC for 20 min, and used for zymography. Duplicates were used to determine cell number with a Coulter counter. The gelatinolytic activity of MMP-2and MMP-9 delivered to the conditioned media was detected in gelatinograms as follows. Samples were subjected to non-reducing SDS/PAGE as above but with gelatin (1mg/mL) added to the 10% resolving gel. After electrophoresis, gels were washed twice with 50 mM Tris/HCl, pH 7.4, supplemented with 2% Triton X-100, and twice with 50 mM Tris/HCl, pH 7.4. Each wash was for 10 min and with continuous shaking. After the washes, the gels were incubated overnight at 37 °C immersed in a substrate buffer (50 mM Tris/HCl, pH 7.4, supplemented with 1% Triton X-100, 5 mM $CaCl_2$, and 0.02% Na_3N). Then, the gels were stained with Commassie blue R-250 and the bands of gelatinase activity could be detected as non-stained bands in a dark, stained background.

Quantitative analysis of zymograms and gelatinograms was performed with the NIH Image 1.6 Program.

2.9. Western blot analysis

Subconfluent BAECs cultures were incubated in DMEM medium supplemented with1% fetal calf serum for 24 h. After that the medium was replaced and cells were incubated with toluquinol (2.5 and 5 μM) or vehicle (DMEM) for 30 min, and then challenged for 20 additional min with either VEGF (100 ng/ml) or FGF2 (100 ng/ml) or the vehicle (DMEM medium). The protein lysates were obtained by scrapping the cells in a lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM sodium orthovanadate and 5 mg/mL of a

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protease inhibitors mixture). Afterwards, extracts were centrifuged at 13,000 rpm for 15 min at 4 °C, evaluated for protein concentration and stored at −80°C until the moment of analysis.These samples were denatured for 5 min at 95 °C and submitted to SDS-PAGE. After electrophoresis, samples were electro-transferred to nitrocellulose membranes, blocked with 5% dried skimmed milk in 50 mM Tris pH 8.4, 0.9% NaCl, 0.05% Tween 20 (Tris buffered saline-Tween 20, TBS-T), and incubated overnight in the presence of an anti-human phosphorylated Akt, at a dilution of 1:1000 or anti-human total Akt at a dilution of 1:1000 in TBS-T with 5% BSA. After three washing steps with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody at a dilution of 1:5000 in blocking buffer for 2 h at room temperature. After three washing steps with TBS-T, the inmunoreactive bands were detected using chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, USA) with an imaging system (Chemidoc XRS System, Bio-Rad, Hercules, California, USA) and were quantified by using ImageLab version 3.0 software. The membranes were incubated with an anti-GAPDH primary antibody at a dilution of 1:1000 to ensure equal loading. Akt activation was calculated as the p-Akt /total Akt ratio and expressed as mean $\pm$ SD of 4 independent experiments.

2.10. In vivo chorioallantoic membrane assay

Fertilised chick eggs were incubated horizontally at 38 °C in a humidified incubator, windowed by day 3 of incubation, and processed by day 8. The tested compound stock solution was added to a 1,2% solution of methylcellulose in water, and 10 µL drops of this solution were allowed to dry on a teflon-coated surface in a laminar flow hood. Then, the methylcellulose discs were implanted on the CAM, the eggs were sealed with adhesive tape and returned to the incubator for 48 h. Negative controls were always made with DMSO mixed with the methylcellulose. After the reincubation, the CAM was examined under a stereomicroscope. The assay was scored as positive when two independent observers reported a significant reduction of vessels in the treated area. In CAM experiments, aeroplysinin-1 (3 nmol/CAM) was used as a routine positive assay control [10].

2.11. Matrigel plug assay

C57BL/6 female mice were injected subcutaneously near the abdominal midline, via a 21-gauge needle with about 300 µl of Matrigel containing FGF2 (1 µg/ml) and toluquinol 10 or 100 µM (3 or 30 nmol toluquinol per plug, respectively). Control mice received the same volume of Matrigel with FGF2 mixed with a corresponding amount of DMSO without toluquinol. Blank mice were injected with Matrigel containing a corresponding dose of PBS and DMSO. After injection, the Matrigel rapidly formed a single, solid gel plug. Mice were sacrificed 4 days after injection. Plugs were then removed, photographed and the extent of neovascularization was assessed by measuring the haemoglobin content using the Drabkin's Reagent Kit (Sigma). Some of the excised Matrigel plugs were fixed with 10% formalin, embedded in paraffin, sectioned (13 µm thick), and stained with hematoxylin and eosin.

2.12. Zebrafish embryo assay

Zebrafish embryos were generated by natural pairwise mating and maintained in embryo water at 28.5 ºC. Transgenic Fli-EGFP fish (*TGfli1:EGFPy1*) had a label vasculature with the green fluorescen protein and were purchased from the Zebrafish International Resource Center (ZIRC, Eugene, OR). Embryos were manually dechorionated with forceps at 24 h post-fertilization (hpf), they were arrayed in 96-well plate, one embryo per well, and incubated with 100 µl of the indicated concentrations of the tested compounds at 28.5 ºC for 24 h. DMSO was used as both carrier of drugs and control. After incubation, fish embryos were anesthetized with tricaine (0.02%), placed on slides and examined under an epifluorescence Nikon microscope equipped with DS-L1 Nikon digital camera. Phenotypic changes were evaluated by two different observers. In these experiments, dimethyl fumarate (25 µM) was used as a routine positive assay control [12].

2.13. Zebrafish caudal fin regeneration assay

Adult transgenic Fli-EGFP fishes were anesthetized with tricaine (0.02%), and their caudal fin was partially amputated. Then, fishes were maintained for 3 days at 28.5 ºC in 100 ml water containing the indicated concentration of the compound. After 3 days, fishes were anesthetized and examined and photographed under an epifluorescence

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Nikon microscope equipped with a DS-L1 Nikon (Chiyoda-Ku, Tokyo, Japan) digital camera. SU4312 (5 μM) was used as a routine positive control in this assay [13].

2.14. Statistical analysis

All data are expressed as means ± standard deviation (SD). One-tailed Student's *t* test was used for evaluations of pairs of means, to establish which groups differed from the control group. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Toluquinol inhibits the capillary tube formation by endothelial cells

Initially, the compounds isolated during our screening program were selected for their ability to inhibit the endothelial cell morphogenesis on Matrigel, a quantitative and reliable in vitro angiogenesis assay that can be adapted for high throughput use [14]. As a result of this primary screening, toluquinol was isolated and purified from the fermentation broth of the marine fungus *Penicillium* sp. HL-85-ALS5-R004, and further characterized for its antiangiogenic activity.

In vitro, endothelial cells plated on a gelled basement matrix (Matrigel) aligned themselves to form cords, which were already evident a few hours after plating.

The minimal concentration of compound yielding a complete inhibition of BAECS alignment and cord formation was 5 μM toluquinol, with partial inhibition observed at 2.5 μM (Fig. 2). A total inhibition of endothelial tube formation could be only achieved by using 100 or 200 μM dobesilate and gentisic acid, respectively, indicating a notably lower potency of those compounds in this in vitro angiogenesis assay.

The treatment with toluquinol, at the concentrations used to inhibit the differentiation of BAECs, did not affect their viability after 7 h (results not shown).

3.2. Toluquinol inhibits the growth of endothelial and tumor cells

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Angiogenesis involves local proliferation of endothelial cells. We investigated the ability of toluquinol to inhibit the proliferation of actively growing endothelial and tumor cells. As shown in Fig. 3, toluquinol inhibited the growth of cultured BAECs with a IC_{50} value of 2.3 μM for subconfluent BAECs stimulated to grow with 10% FBS. Data obtained with HL60 leukemia, HT1080 fibrosarcoma and HT29 colon adenocarcinoma cell lines show that toluquinol is not a specific inhibitor of the endothelial cell growth, since the IC_{50} values of tumor cells were in the same range of concentrations than that of BAECs. The effect of dobesilate and gentisic acid on the growth of endothelial and tumor cells was much lower than that of toluquinol, as deduced from the higher IC_{50} values obtained for those compounds (Fig. 3B).

3.3. Toluquinol induces chromatin condensation and DNA fragmentation in endothelial and tumor cells

In an effort to elucidate the inhibition of cell growth as the result of toluquinol treatment, its effects on cell apoptosis were assessed. As a first approach to study a possible proapoptotic activity of toluquinol, nuclear morphology was investigated in BAECs, HL60, HT-29 and HT1080 cells after 14 h treatment with different concentrations of this compound. Figures 4A and 4C-left panel show that toluquinol concentrations of 5 μM or higher, induced chromatin condensation in endothelial, leukemia, colon adenocarcinoma and fibrosarcoma cells. Similar results were obtained by using TUNEL assay to detect the DNA fragmentation induced by this compound(Fig. 4B and 4C-right panel).

3.4. Toluquinol treatment induces cell cycle arrest at G2/M and apoptosis in endothelial cells

In order to determine wether toluquinol could affect the cell cycle progression of endothelial cells, the cell cycle distribution of toluquinol-treated BAECs was evaluated by flow cytometric analysis. As shown in Fig. 5A, untreated BAECs showed a classic pattern of proliferating cells distributed between the G1 (59%), S (19%) and G2/M (19%) phases. Flow cytometric analysis (Figure 5A) showed that incubation with toluquinol significantly increased apoptotic sub-G1 cells in a concentration-dependent manner, confirming that the growth inhibitory effect produced by this compound on

activated endothelial cells could be due, at least in part, to an induction of apoptosis. Moreover, toluquinol, when added at 5 or 10 μM resulted in an appreciable decrease of BAECs in the G1 phase of cell cycle, accompanied by a concomitant increase in cell number in the G2/M phases. This pattern is consistent with a G2/M cell cycle arrest after incubation with toluquinol and may underlie the growth inhibitory activity observed for this compound.

Caspases activation plays a central role in the induction of apoptosis. To determine whether endothelial cells caspases were activated as a result of the treatment with toluquinol, we used a caspase-3/7 substrate DEVD-AMC, which is cleaved to a fluorescent product by caspase-3. As shown in Fig. 5B, the "effector caspase"-3 was significantly activated in 10 μM toluquinol-treated BAECs. As a positive control of caspase activation, 10 μM 2-methoxyestradiol was used.

3.5. Toluquinol inhibits the migratory, invasive and proteolytic capabilities of endothelial cell

Taking into account that endothelial cell migration and invasion are two essential steps required for angiogenesis, we decided to study the effect of toluquinol on both processes. The effect of toluquinol on BAECs migration was investigated by mean of the so called "wound healing" assay. As shown in Fig. 6A, 10 μM toluquinol produced a significant inhibition of the migratory capability of BAECs in this assay. Similar results were obtained when toluquinol was added at a concentration of 10 or 20 μM to the upper well of a Boyden Chamber invasion assay, resulting in a significant inhibition of the ability of BAECs to invade through Matrigel-coated filters (Fig. 6B).

Matrix-metalloproteinases (MMP) play a key role in angiogenesis. Gelatin zymography of conditioned media of toluquinol-treated BAECs (Fig. 6C and D, left panels) shows that toluquinol inhibited MMP-2 secretion by endothelial cells. Whereas BAECs only express MMP-2, HT1080 cells express both gelatinases: MMP-2 and MMP-9. Our results show that no effect on MMP-2 and MMP-9 levels in the HT1080 tumor cells conditioned media was observed after treatment with toluquinol up to 20 μM. (Fig. 6C and D, right panels).

3.6. Toluquinol supresses the VEGF or FGF-induced phosphorylation of Akt but is not an inhibitor of the kinase activity of their receptors

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In order to investigate the molecular mechanism of toluquinol-induced inhibition of angiogenesis, we examined the effects on the FGF2 or VEGF- activated BAECs. Previous analysis had discarded a direct effect of this compound on the kinase activity of the angiogenic factors receptors at the concentrations needed to inhibit angiogenesis in vitro (toluquinol, tested at 2 or 20 μM in an in vitro radiometric protein kinase assay, did not inhibited the kinase activity of the VEGFR1, VEGFR2, VEGFR3, FGFR1, FGFR2, FGFR3 or FGFR4 purified human recombinant proteins. Data not shown). This led us to think that the molecular target of toluquinol could be acting downstream the growth factors receptors.

Among the network of pathways governing angiogenesis, the PI3K/Akt pathway play a critical role, controlling essential cellular functions, such as migration, proliferation, differentiation, and apoptosis [15]. Phosphorylation of Akt is an important signalling component involved in both FGF2 and VEGF mediated angiogenesis. Therefore we examined the effect of toluquinol on the FGF2 and VEGF-induced phosphorylation of Akt in BAECs. Although a basal phosphorylation of Akt is observed in unstimulated cells, exposure of BAECs to VEGF (100 ng/ml) or FGF2 (100 ng/ml) resulted in an activation of this intracellular target protein, as demonstrated by the significant increase in pAkt/total Akt ratio. As shown in Fig. 7 A and B, Akt phosphorylation was abrogated in BAECs by the presence of 5 μM toluquinol.

3.7. Toluquinol inhibits in vivo angiogenesis in the chick chorioallantoic membrane and the Matrigel plug assays

Table 1 summarizes the evaluation of the *in vivo* inhibition of angiogenesis in the CAM assay by toluquinol, showing that its antiangiogenic activity is maintained as low as 5 nmol per CAM, where 70% of the eggs scored positive. No significant antiangiogenic activity in the CAM assay was exhibited at 20 nmol/CAM by calcium dobesilate or gentisic acid (results not shown). The antiangiogenic effect of toluquinol was observed as an inhibition of the ingrowth of new vessels in the area covered by the methylcellulose discs. The peripheral vessels (relative to the position of the disc) grew centrifugally, avoiding the treated area, where a decrease in the vascular density could be observed (Fig. 8A).

Toluquinol in vivo antiangiogenic activity was confirmed by using the intradermal Matrigel plug model. Our results show that this compound caused a strong inhibition of

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the FGF2-mediated cell invasion in the Matrigel plug (Fig. 8B). The Matrigel plugs without FGF2 were colorless and showed the absence of vasculature, whereas the Matrigel plugs containing FGF2 were apparently red, due to neovascularization. The formation of blood vessels was confirmed by histological analysis of the plugs (Fig. 8C). Matrigels containing FGF2 with 3 or 30 nmol toluquinol were only partially red, indicating a decreased blood vessel formation, also confirmed by histological analysis. Furthermore, we measured the haemoglobin content in the plugs as indicator of neovascularization. As shown in Fig. 8D, 30 nmol toluquinol significantly inhibited the FGF2-induced angiogenesis in the Matrigel plug in vivo assay.

3.8. Toluquinol inhibits in vivo angiogenesis in zebrafish embryo and caudal fin regeneration assays

To further evaluate the in vivo antiangiogenic activity of toluquinol, two different assays based on the transgenic (TG(fli1:EGFP)y1) zebrafish line were used. In these animals, enhanced green fluorescent protein (EGFP) expression is driven under the 15-kb promoter of the transcription factor friend leukaemia virus integration-1 (fli-1). This promoter is ubiquitously activated in endothelial cells along the complete embryo, young and adult zebrafish. This ubiquitous expression leads to green in vivo fluorescence in all endothelial cells, and permits observation of bright blood vessels at all stages of embryogenesis [16]. As shown in Fig. 9A, 20 μM toluquinol added to water reduced the number of caudal intersegmental vessels at late tail bud stages of *Fli-EGFP* transgenic zebrafish. This inhibitory effect was dose-dependent (table 1). Structurally related compounds, such as Calcium dobesilate or Gentisic acid, did not show this effect, when tested at 20 μM (0 positive results out of 8 treated embryos for each compound) (Fig. 9A).

Zebrafish caudal fin can be used as a model for regenerative angiogenesis, sensitive to chemical inhibition by antiangiogenic compounds [17]. In fin regeneration experiments, zebrafish caudal fins are amputated at mid-fin level, and then allowed to recover. Amputated blood vessels heal their ends by 1 day post amputation (dpa) and then reconnect arteries and veins via anastomosis, to resume blood flow at wound sites by 2 dpa [18]. By 3 dpa, networks of endothelial cells in the regenerated tissue formed a vascular plexus that extended to the fin tip (Fig.9B,

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left panels). As shown in Fig. 9B, when 20 μM toluquinol was added to water, a prevention of new vessel formation, accompanied to both fin regeneration arrest and vessel lumen enlargement, was observed. Fin regeneration arrest led to absence of fin blastema. Varicosities were mostly observed in inter-ray regions. In some instances, reduction of stump vasculature could be detected, suggesting distal vascular degeneration. These inhibitory effects were not observed when 20 μM calcium dobesilate or gentisic acid were used (Fig 9B right panels).

4. Discussion

The development of new blood vessels is a complex multi-step process. Endothelial cells, forming a monolayer that covers the inside surface of all the vessels, are activated by an angiogenic signal and stimulated to synthesize and release degradative enzymes allowing them to migrate, proliferate and finally differentiate to give rise to capillary tubules. Any of these steps may be a potential target for pharmacological intervention.

A remarkable number of natural compounds have been reported to inhibit angiogenesis in vitro and in vivo [19, 20]. Most of them have been isolated from plants and terrestrial microorganisms, mainly due to their higher availability and because their therapeutic effects had been previously known in folk traditional medicines. However, more recently, pharmaceutical research is also focusing on the deep sea as an unexploited source for new drugs [21]. The large diversity of marine habitats and environmental conditions cause marine organisms to produce metabolites allowing them to adapt and survive in extreme environments. The development of those biologically unique molecules, suggests the potential of marine organisms for drug discovery. In this regard, some angiogenesis inhibitors from marine origin have been described by us and others [10, 22-24].

This study shows that toluquinol, a methylhydroquinone isolated from the fermentation broth of a marine fungus is a new inhibitor of angiogenesis in vitro. It affects in a dose-dependent fashion certain functions of endothelial cells, namely, differentiation, proliferation, migration and invasion. Antiangiogenic activity of toluquinol was first detected using the in vitro differentiation assay for endothelial cells. Our results show

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that this compound inhibits capillary-like tube formation by BAECs at non-toxic concentrations which are in the same range as those required for other previously described inhibitors of angiogenesis, including the marine bromotyrosine aeroplysinin-1 [10] and the natural phenols hypericin, carnosol, carnosic acid and licochalcone A, among others [25-27]. Moreover, this activity is exerted at one or two orders of magnitude lower than those required for some other natural compounds such as dimethyl fumarate [12], the phenolic compounds hydroxytirosol, resveratrol, quercetin, aloe emodin or honokiol [28-31] and the toluquinol-structurally related calcium dobesilate and gentisic acid, which have been previously described as inhibitors of angiogenesis [32,33].

Angiogenesis involves local proliferation of endothelial cells in response to an angiogenic stimulus. In fact, several of the best characterized anti-angiogenic compounds were initially detected and selected for their capability to interfere with endothelial cell growth, although the desirable endothelial cell specificity of this effect is not a common feature [34]. Our data obtained with the MTT assay indicate a non-specific cell growth inhibitory effect in long term (3 days) treatments with micromolar concentrations of toluquinol for endothelial and tumor cells. This suggests that toluquinol could behave not only as a potential anti-angiogenic compound but also as a potential anti-tumor drug.

Typical hallmarks of apoptosis, including apoptotic cell morphology and DNA fragmentation were observed in the toluquinol-treated endothelial and tumor cells, indicating that the growth inhibitory effect produced by toluquinol on proliferative cells could be due, at least in part, by an induction of apoptosis. There is growing evidence that Reactive Oxygen Species (ROS) are important for the induction of apoptosis under both physiologic and pathologic conditions [35]. Toluquinol and some structurally related marine tripenyl toluquinones and toluhydroquinones have been reported to induce an increase of DNA damage by generation of (ROS) [36, 37] that could contribute to the observed pro apoptogenic activity of toluquinol. Moreover, given that cancer cells are often more susceptible to ROS, those compounds have been proposed to present plausible leads for new anticancer treatments [37].

The induction of endothelial apoptosis is a common mechanism exhibited by a number of angiogenesis inhibitors and has been postulated to contribute to the anti-angiogenic potential of these compounds [10, 12, 26, 38, 39]. In order to further characterize the

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induction of apoptosis in endothelial cells, cell cycle analysis was performed in toluquinol-treated BAECs after propidium iodide staining. Our results confirm that toluquinol induces endothelial cell apoptosis, as reflected by the dose-dependent sub-G1 population accumulation. Moreover, flow cytometry data show that toluquinol treatment inhibits endothelial cell growth by arresting the cell cycle at G2/M phase. Similar G2/M arrests have been described for oesophageal cancer cells after being treated with marine tripenyl toluquinones and toluhydroquinones, and they have been related to an irreversible cellular damage mediated by the ROS generation [37].

Activation of caspases is considered to be the prerequisite to define apoptotic cell death [40]. The induction of endothelial apoptosis by toluquinol was confirmed using a caspase3/7 fluorogenic substrate. Measurement of the activity of the effector caspase 3, showed an activation of the caspase proteolytic cascade after treatment of BAECs with toluquinol, what suggests that caspase-dependent mechanisms could play a role in the apoptosis induction within those cells.

Angiogenesis involves the acquisition by endothelial cells of the capability to migrate and invade through extracellular matrix, degrade the basement membrane and, in general, to remodel the extracellular matrix. Our results indicate that toluquinol produced a significant inhibition of the migratory and invasive capabilities of BAECs, what could contribute to its antiangiogenic activity. Matrix-metalloproteinases (MMP) play a key role in angiogenesis and lymphangiogenesis [41]. Among the MMPs, MMP-2 is the predominantly expressed protease in endothelial cells capable to degrade type IV collagen. Endothelial MMP-2 is needed to trigger tumor angiogenesis in vitro e and in vivo [42,43]. In this study we demonstrate that incubation with toluquinol inhibits MMP-2 secretion by BAECs. Given that proteolytic degradation of extracellular matrix components by MMP-2 is critical for angiogenesis, the inhibition of MMP-2 secretion could contribute to the anti-angiogenic effect of toluquinol. In contrast, no effect on MMP-2 and MMP-9 levels in the HT1080 tumor cells conditioned media was observed after treatment with this compound, suggesting an endothelial-specificity of this effect. Further investigation is warranted to clarify this suggestive potential specificity.

The PI3K/Akt pathway is a central signaling molecule in regulating crucial cellular functions including cell survival, proliferation, migration and tumor growth, making this pathway an exciting target for molecular therapeutics [44]. Akt plays an important role in regulating normal vascularization and pathological angiogenesis [45]. The kinase

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domain phosphorylation of Akt is stimulated by a wide variety of growth factors and mitogens, including FGF2 and VEGF [15]. Mechanistically, we found that toluquinol caused the repression of Akt phosphorylation in endothelial cells not only in response to VEGF but also in response to FGF2, what indicates that components of both growth factors pathways could be major targets in the molecular mechanism of this compound. The finding that toluquinol was not a direct inhibitor of the kinase activity of the VEGF or FGF2 receptors indicates that this compound exerts its antiangiogenic effect by targeting signaling events downstream of the growth factors receptors. Data presented here suggest that toluquinol could restrain vital signalling pathways governing the succession of events during angiogenesis and propose a role of this compound as a modulator of the Akt survival pathway.

Inhibition by toluquinol of essential steps of in vitro angiogenesis agrees well with the effect on the in vivo angiogenesis that has been observed by us. On the one hand, our results show that toluquinol is active in the CAM and the matrigel plug assays at concentrations that are lower than those required for other previously described inhibitors of angiogenesis, including the natural compounds dimethyl fumarate, carnosol, hydroxytyrosol, aloe emodin, or hyperforin, among others [12, 26, 28, 30, 46]. On the other hand, the present research work shows a confirmatory evidence of the potential of toluquinol to inhibit in vivo angiogenesis, by using other independent model systems, namely, that of embryo neovascularization [16] and caudal fin regeneration [17] of genetically modified zebrafish. All these results suggest the potential of toluquinol as a new antiangiogenic drug. Some other hydroquinone-related molecules have been described as possible inhibitors of angiogenesis. 2,5-dihydroxybenzene sulfonate (dobesilate) and 2,5-dihydroxybenzoic acid (gentisic acid) have been reported to inhibit FGF-related angiogenesis by direct interaction with the heparin-binding domain of fibroblast growth factor-1 (FGF1) [33]. Recently reported data, showing that calcium dobesilate inhibits VEGF-induced angiogenesis [47], suggest a possible role of this compound in the control of pathological angiogenesis, mainly driven by this factor, and could help to explain its reported efficacy in the treatment of rosacea or diabetic retinopathies [48,49]. More recently, dobesilate has been described to inhibit glioblastoma invasion, reducing its associated angiogenesis [50]. In the present study, calcium dobesilate and gentisic acid have been used in order to compare their effect on in vitro and in vivo angiogenesis with that of toluquinol.

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Our results indicate that both compounds present a notably lower potency in all the assays here used to characterize the in vitro and in vivo antiangiogenic activity of the compounds. Whether the three compounds have a similar molecular mechanism, or they have different pharmacological targets is an interesting question that deserves further investigation.

In summary, data presented here suggest a potential application of the marine metabolite toluquinol, in the treatment of cancer. On the one hand, toluquinol exhibits anticancer properties, derived from the direct inhibition of tumor cell growth. On the other hand, it inhibits angiogenesis in vitro and in vivo, affecting several steps of the angiogenesis process, namely proliferation, migration, invasion, differentiation and proteolytic capability. A critical overview of the results obtained at the clinical setting with angiogenesis inhibitors indicates that agents targeting more than one pathway may be beneficial in the treatment of cancer angiogenesis. The inhibitions of tumor growth and FGF2- or VEGF-induced angiogenesis by toluquinol indicate that this compound could behave as a multi-target inhibitor and suggest its potential as a lead compound for the treatment of cancer-related angiogenesis. Although additional studies will be needed to elucidate the molecular mechanisms underlying the anti-angiogenic activity of toluquinol and the pharmaceutical and toxicological profiles of this compound, our results suggest that toluquinol could serve as a potential candidate for antiangiogenic therapies. These data indicate the great value of marine products as candidates for further pharmaceutical studies for feeding the antiangiogenesis drug pipeline. Further efforts to determine the pharmacological potential of toluquinol are warranted.

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Acknowledgment

Melissa García is recipient of a predoctoral FPU grant from the Spanish Ministry of Science and Innovation. This work was supported by grants PS09/02216 and TRACE PT2008-0145 (Spanish Ministry of Science and Innovation, ISCIII and FEDER), Fundación Ramón Areces and PIE CTS-3759 (Andalusian Government and FEDER). The "CIBER de Enfermedades Raras" is an initiative from the ISCIII (Spain). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Authors are indebted to Auxiliadora López Jiménez for

her excellent technical assistance and to Juan Antonio Guadix for his collaboration during the realization of the matrigel plug experiments.

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Fig. 1. Chemical structure of toluquinol

Fig. 2. Toluquinol inhibits endothelial cell tube formation in vitro in a dose-dependent manner at non-toxic doses.
BAECs seeded on Matrigel formed tubes (left panels). 5 μM toluquinol completely inhibited BAECs alignment and cord formation, with partial inhibition observed at 2,5 μM. A total inhibition of endothelial tube formation could only be achieved by using 100 or 200 μM dobesilate and gentisic acid, respectively. 2 μM aeroplysinin-1 was used as a positive control of inhibition. Cells were photographed 7 h after seeding under an inverted microscope (bar=100 μM).

Fig. 3. Toluquinol inhibits endothelial and tumor cell growth
(A) Dose-dependent effect of the incubation for 3 days with toluquinol on the in vitro growth of BAECs (▲),HL60 (x), HT29 (♦), and HT1080(■). Data are expressed in terms of percentage of growth of toluquinol-treated cells with respect to control cells. The figure illustrates a representative experiment performed with quadruplicate samples. Each point represents the mean of quadruplicates; SD values were typically lower than 10% of the mean values and are omitted for clarity.
(B) Half-maximal inhibitory concentration (IC50) 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 with quadruplicate samples each.

Fig. 4. Toluquinol induces chromatin condensation and DNA fragmentation in endothelial and tumor cells
(A) Effect of toluquinol on cell morphology (Hoechst staining). Cells with condensed chromatin are highlighted (bar=50 μm).
(B) Effect of toluquinol on and DNA fragmentation (TUNEL assay) (bar=50 μm).
(C) Percentage of control and toluquinol-treated cells showing chromatin condensation (left panel) or DNA fragmentation (right panel). Values are expressed as mean ± SD of the counts evaluated in ten vision fields from two independent experiments (chromatin condensed or TUNEL positive cells were counted by fluorescence microscopy, total cells were counted by bright field microscopy).* $P<0.05$, #$P<0.005$ versus untreated control
In all experiments, cells were incubated for 14 h with or without the indicated concentrations of toluquinol in complete medium.

Fig. 5. Effect of toluquinol on endothelial cell cycle distribution and caspase 3 activity
(A) BAECs were exposed for 14 h to toluquinol at the indicated concentrations, stained with propidium iodide and percentage of subG1, G1, S and G2/M cells were determined using a MoFlo DakoCytomation cytometer. A representative result and the calculated values for cell subpopulations, expressed as means ± SD of three independent experiments are shown.
(B) Effect of toluquinol (14 h incubation) on the endothelial cells caspase-3-like activity. 10 μM 2- methoxyestradiol (2ME) was used as a positive control of caspase induction. Results are mean ± SD of three independent experiments.

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*P<0.05, #P<0.005 versus untreated control.

Fig. 6. Effect of toluquinol on the migratory, invasive and proteolytic capabilities of endothelial cell
(A) Left: Confluent BAECs monolayers were wounded and fresh culture medium was added either in the absence or presence of the indicated concentrations of toluquinol. Photographs were taken at the beginning of the assay and after 7 h of incubation (shown in the pictures). Broken lines indicate the initial (time 0) wound edges (bar=100 μm). Right: The regrowth of BAECs into the cell-free area was calculated as the percentage of the initial wounded area recovered by endothelial cells after 7 h. They are expressed as mean ± SD, ** P<0.01 versus control (n=3). 50 μM dimethyl fumarate (DMF) was used as a positive control of inhibition.
(B) Invasion assay was carried out in the presence or absence of the indicated concentrations of toluquinol, as described in Material and Methods. Experiments were conducted in 3 replicates, and similar results were repeated 2 times. Data represent the number of invading cells per hour and are given as means ± SD of 3 replicates of each sample. *P < 0.02 and ** P<0.01 versus untreated control.
(C) Conditioned media from BAECs (left panel) or HT1080 cells (right panel) were treated during 24 h with the indicated concentrations of toluquinol, normalized for equal cell density and used for gelatin zymography as indicated in Material and Methods.
(D) Quantification of the normalized relative inhibitory effect on BAECs MMP-2 activity (left panel) or HT1080 MMP-2 and MMP-9 activities (right panel). Data are given as percentage of the untreated control, and they are means + SD of three experimental values. ** P<0.01 versus untreated control.

Fig. 7. Toluquinol inhibits VEGF- and FGF2-induced Akt phosphorylation in BAECs.
(A) Representative western blot showing the effects of VEGF, FGF2 and toluquinol on the content of phosphorylated Akt and total Akt in protein extracts from BAECs. Cells were incubated with toluquinol (2.5 and 5 μM) or vehicle (DMEM) for 30 min, and then challenged for 20 additional min with either VEGF (100 ng/ml) or FGF2 (100 ng/ml) or the vehicle (DMEM medium).
(B) Western blots were quantified by densitometry and pAkt/total Akt ratio are expressed as the percentage of the P-Akt/total Akt ratio in unstimulated BAECs (Control) (means + SD of four independent experiments)
* P < 0.05 versus unstimulated control (in absence of VEGF, FGF2 or toluquinol),
# P < 0.05 versus the corresponding VEGF- or FGF2-stimulated cells (in the presence of the growth factor, in absence of toluquinol).

Fig. 8. Toluquinol inhibits angiogenesis in vivo.
A) Chorioallantoic membrane assay of toluquinol. Left panel: methylcellulose disc containing the substance vehicle alone. Central panel: methylcellulose disc containing 10 nmol of toluquinol. Right panel: methylcellulose disc containing 3 nmol of aeroplysinin-1 (positive control of inhibition). Circles show the locations of the methyl cellulose discs (bar=1 mm).
B) Representative Matrigel plugs that contained no FGF2 (Control), FGF2 alone, or FGF2 plus 3 or 30 nmol toluquinol were photographed.
C) Representative sections of the plugs stained by hematoxylin and eosin (bar=100 μm).

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D) The total haemoglobin content in the Matrigel plugs was quantified as an indicator of blood vessel formation. Data are expressed as means ± SD of at least 5 animals. (* $P<0,05$ versus control of Matrigel + FGF2).

Fig. 9. Inhibition of the zebrafish neovascularization by toluquinol.
(A) Live fluorescent zebrafish embryo assay.Transgenic *TGfli1:EGFPy1* zebrafish embryos, which show green fluorescent protein (GFP) expression in endothelial cells, were incubated for 24 h without or with 10 or 20 μM toluquinol, or 20 μM dobesilate or gentisic acid. 25 μM dimethyl fumarate was used as positive control of inhibition.
(B) Zebrafish caudal fin regeneration at 3 days post amputation(dpa) in the presence or absence of the toluquinol, calcium dobesilate or gentisic acid, at a concentration of 20 μM. Discontinuous white lines show the amputation planes. SU4312 (5 μM) was used as positive control of inhibition. Bars represent 50 μm.

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Table 1. Inhibition of *in vivo* angiogenesis by toluquinol

	Dose (nmol/CAM)	Positive/Total	% inhibition
CAM assay	0	0/12	0
	2	1/9	11
	5	7/10	70
	10	9/10	90
	20	9/10	90
	[toluquinol] (μM)	Positive/Total	% inhibition
Zebrafish embryo assay	0	0/12	0
	5	1/12	8
	10	9/12	75
	20	12/12	100

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Figure 1

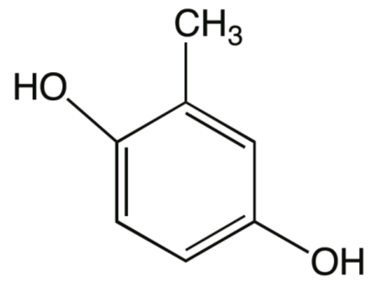

Figure 2

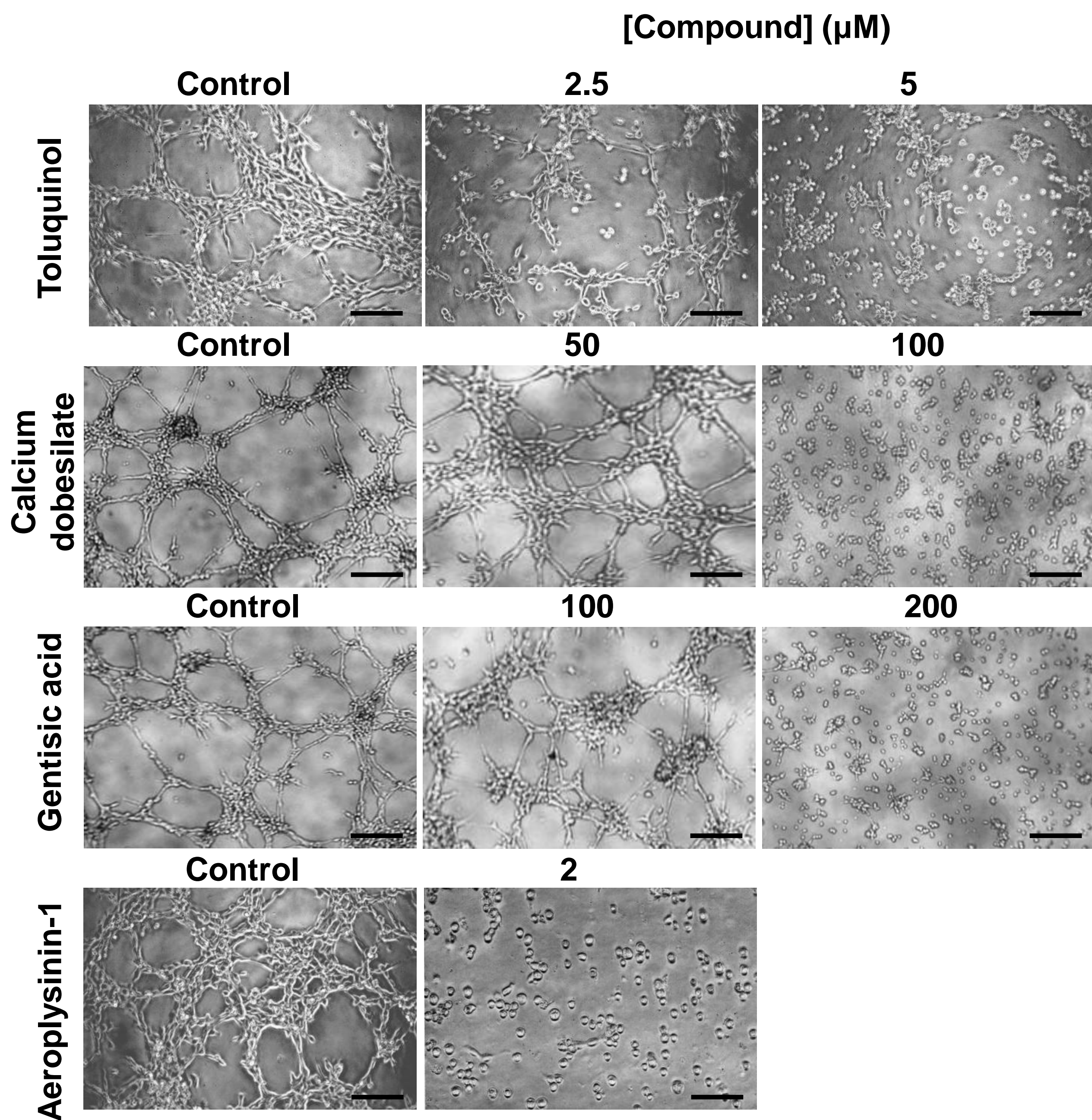

Figure 3

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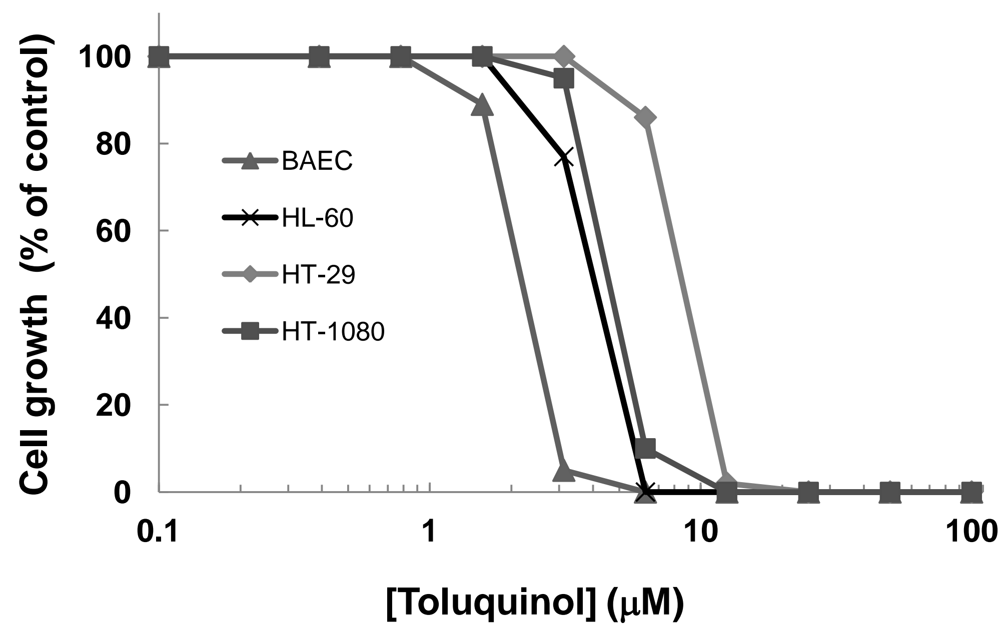
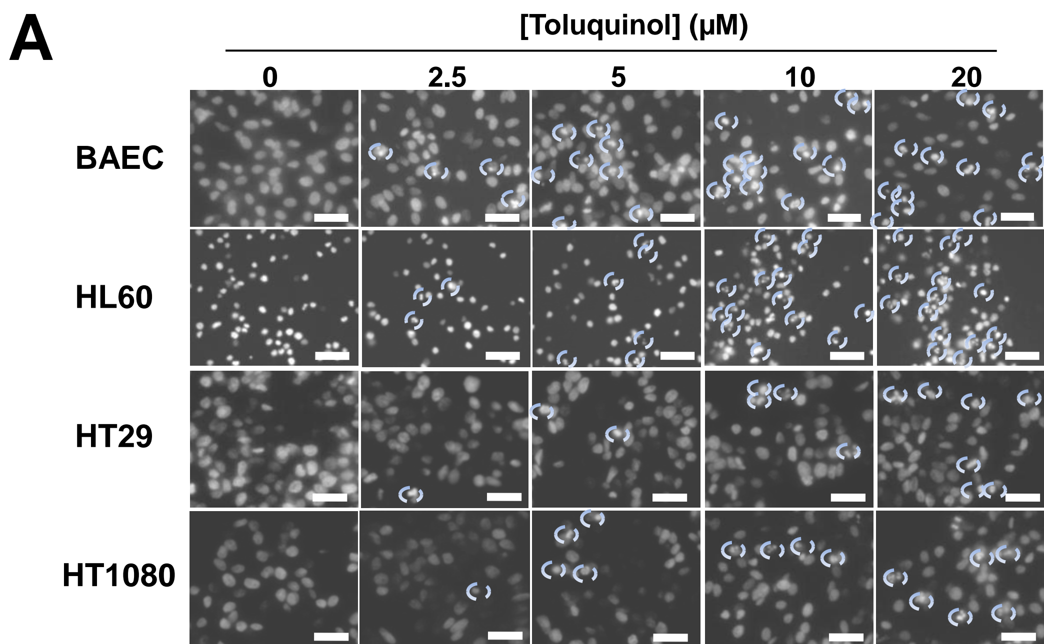
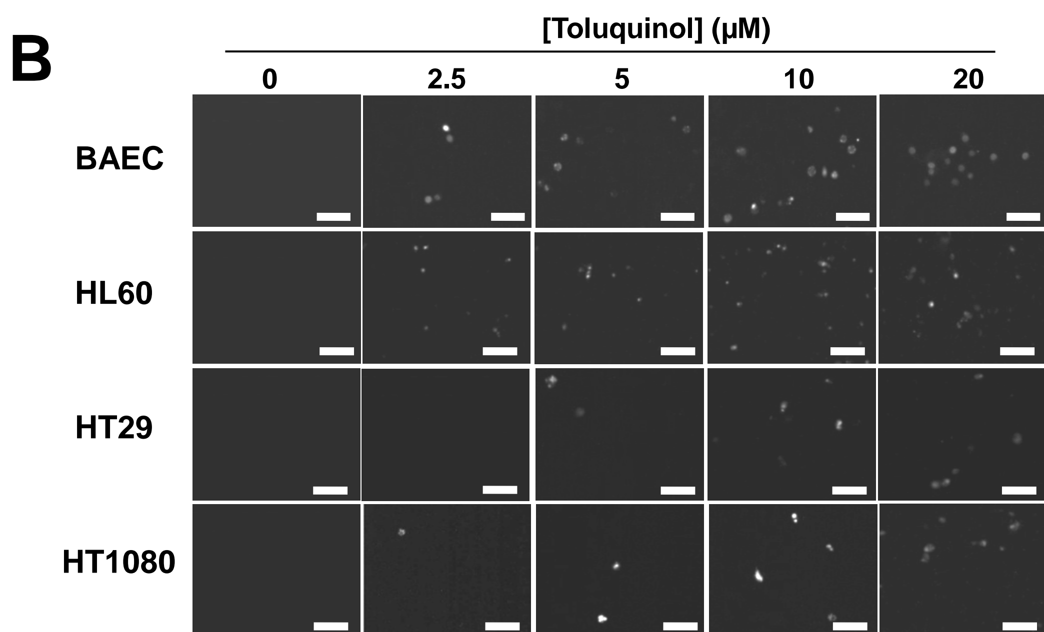


B

IC50 (μM)	BAEC	HL-60	HT-29	HT-1080
Toluquinol	2.3 ± 0.7	3.2 ± 0.9	8.6 ± 0.3	5.8 ± 2.3
Calcium dobesilate	126 ± 27	98 ± 18	25 ± 6	87 ± 5
Gentisic acid	>100	>100	>100	>100

Figure 4 grayscale for printed version





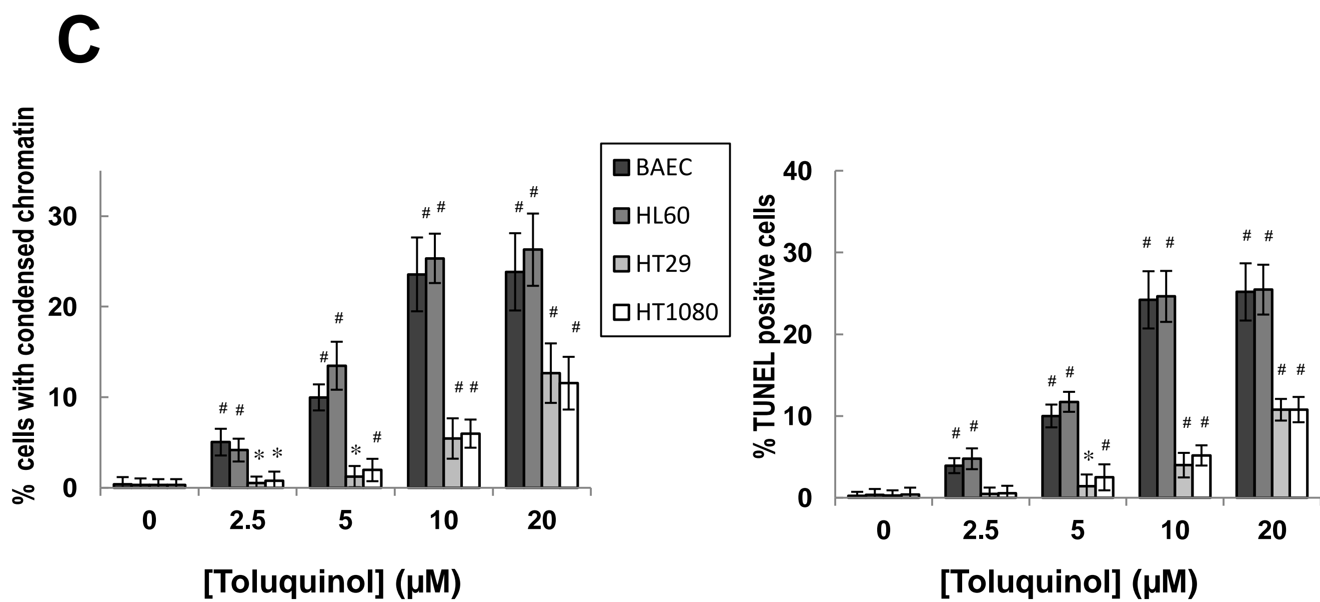
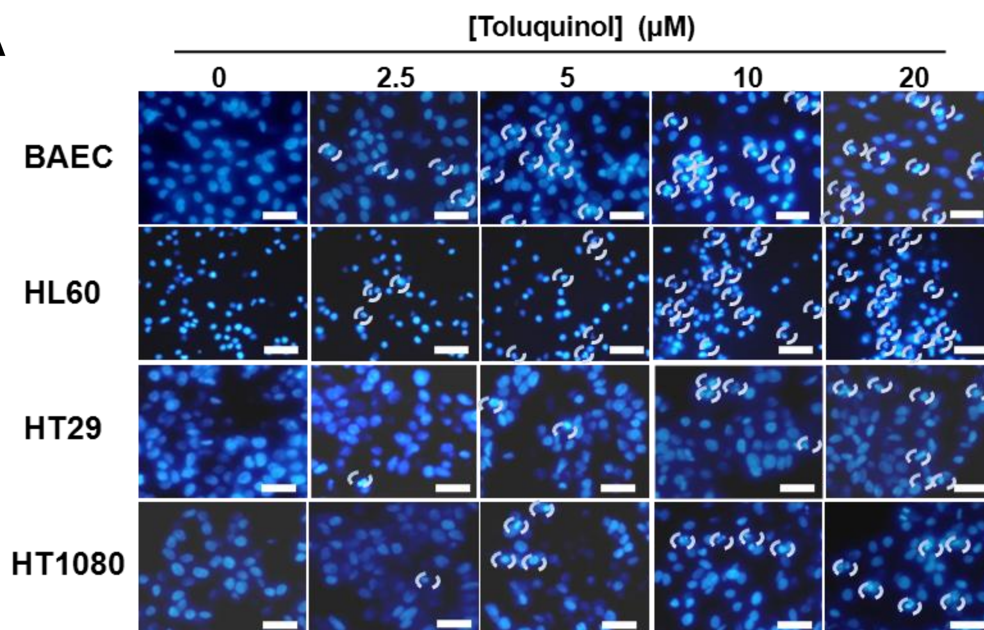
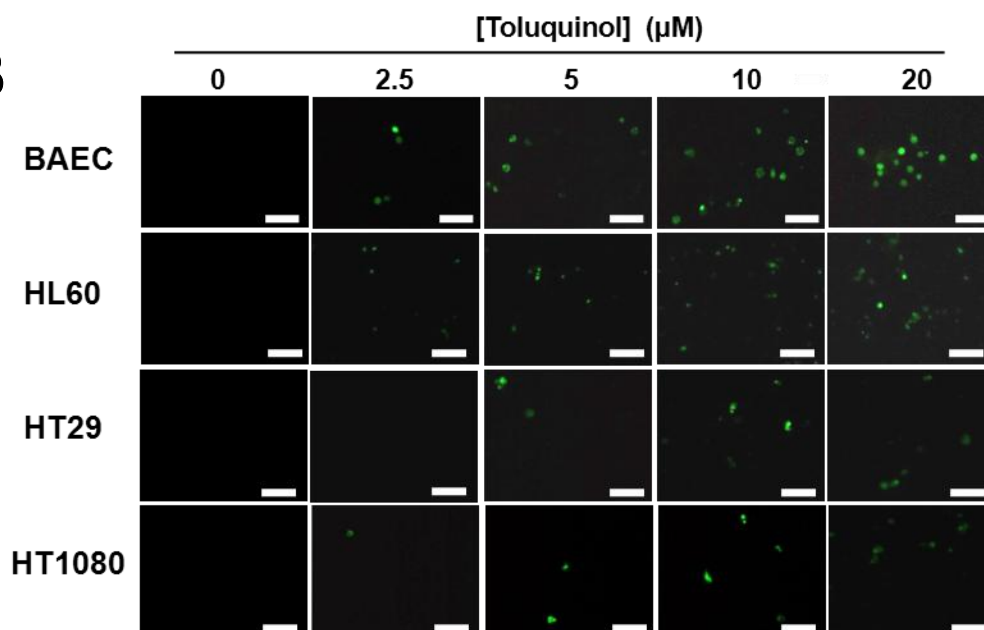


Figure 4 color (for web-based version)





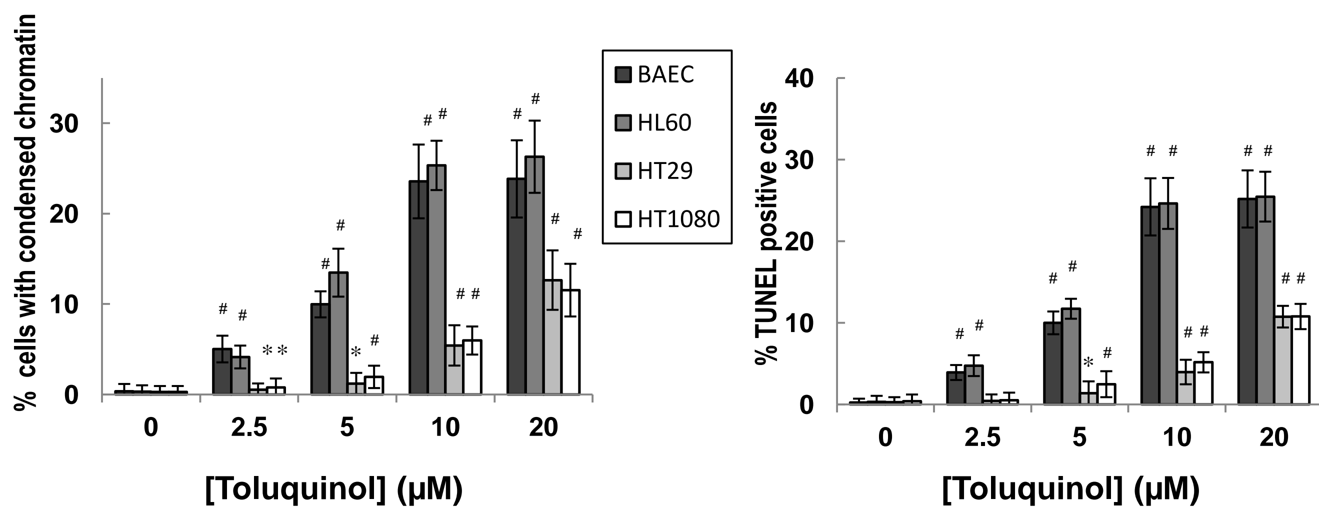
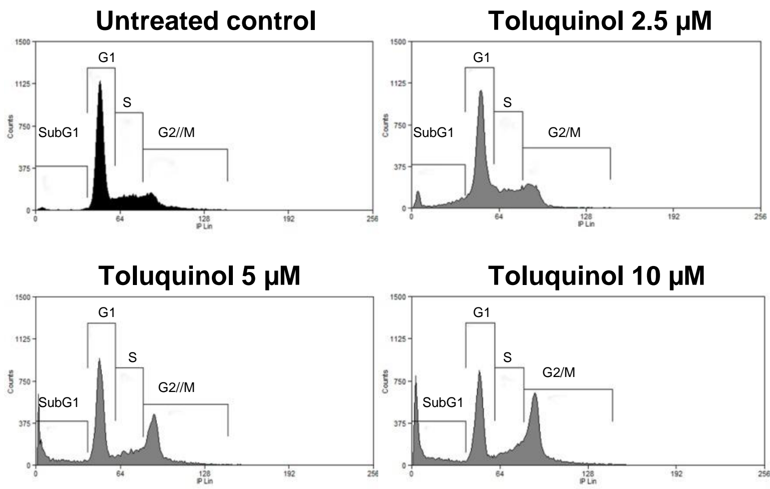


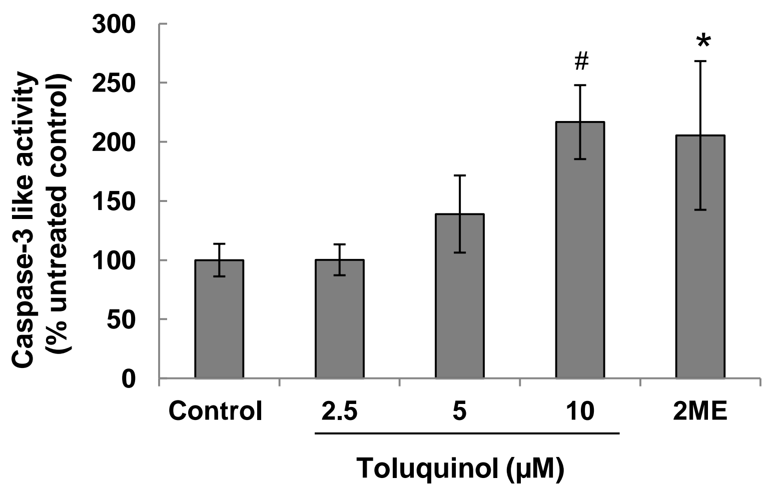
Figure 5

A



	Untreated control	Toluquinol 2.5 µM	Toluquinol 5 µM	Toluquinol 10 µM
SubG1	4 + 2	12 + 4*	18 + 5*	24 + 6#
G1	59 + 6	48 + 2*	39 + 4*	31 + 3#
S	19 + 2	21 + 6	14 + 5	15 + 5
G2/M	19 + 4	18 + 5	30 + 5*	35 + 4*

B



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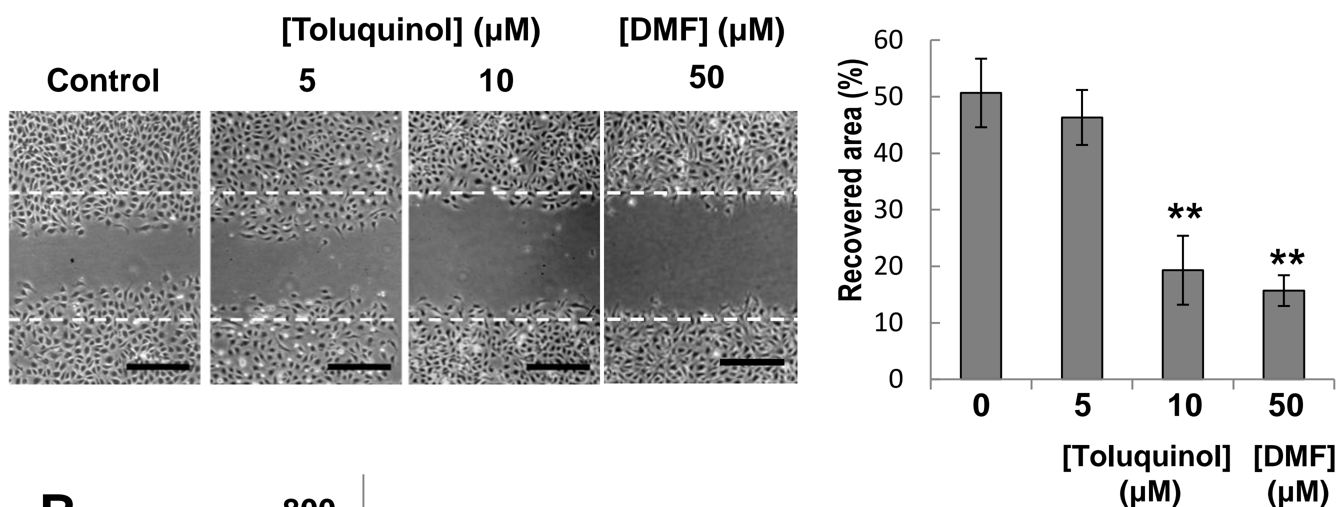
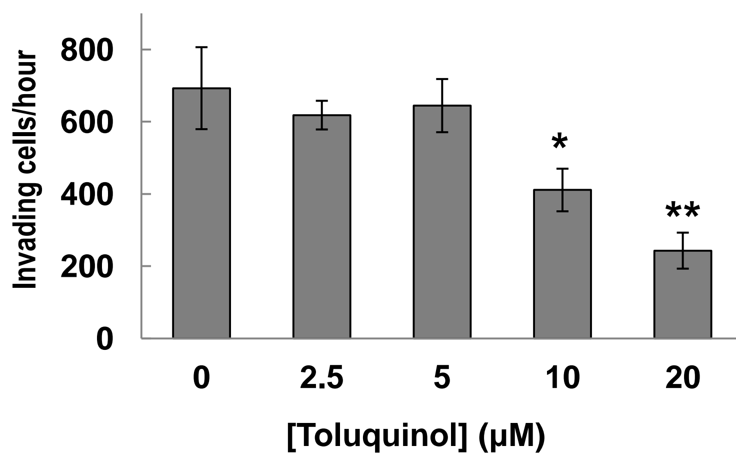
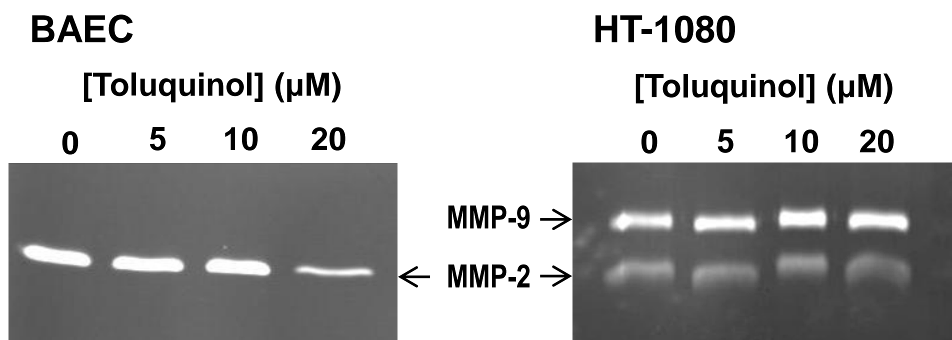






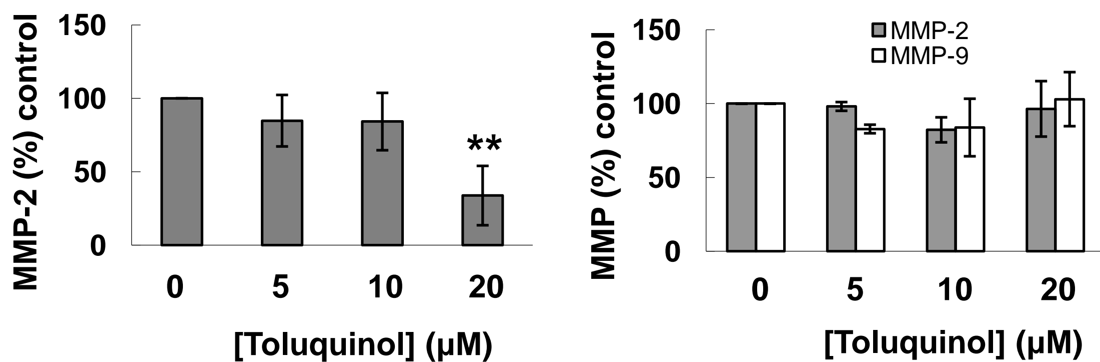


Figure 7

A

+ FGF2 | + VEGF

Toluquinol (µM) | Toluquinol (µM)

Control 0 2.5 5 0 2.5 5

P-Akt

Akt

GADPH

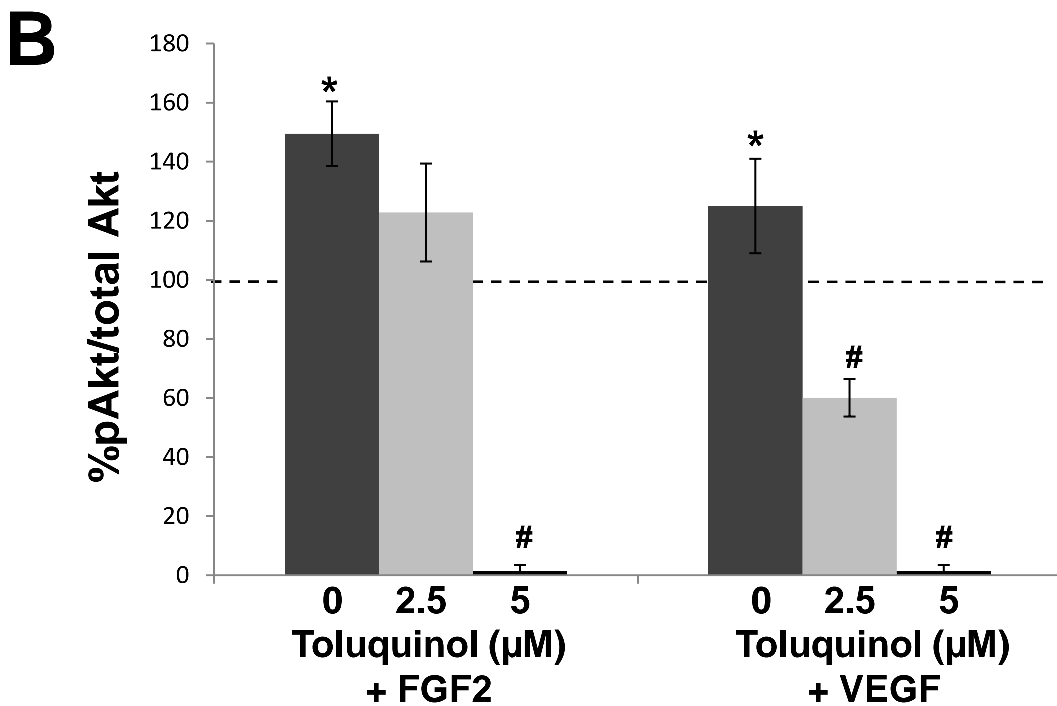
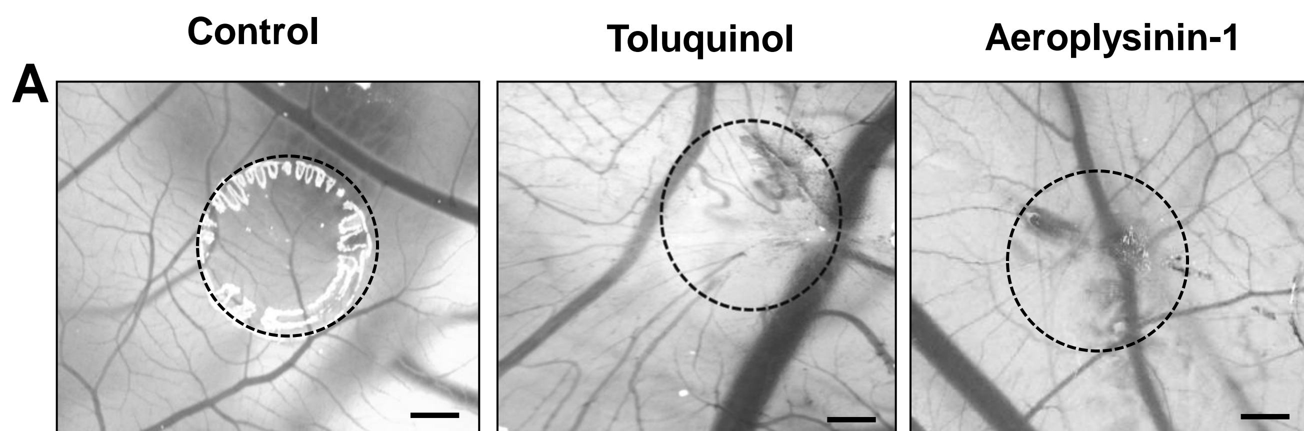
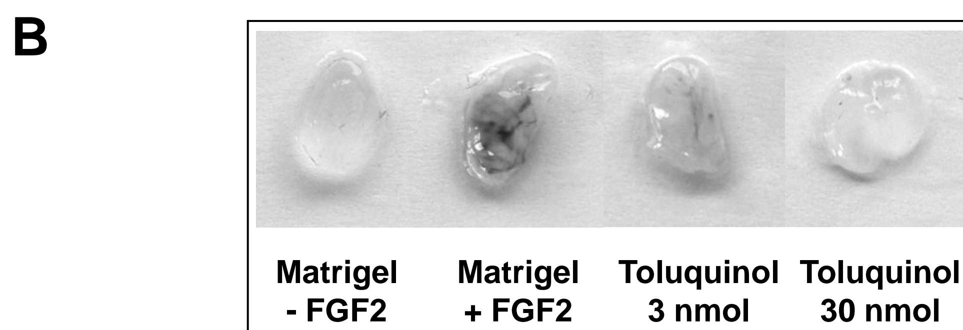
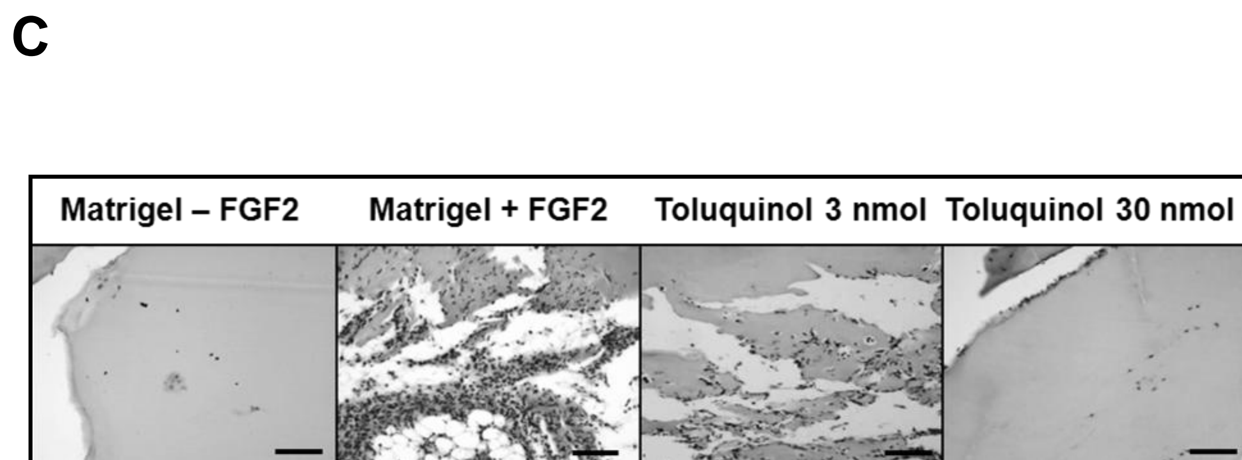


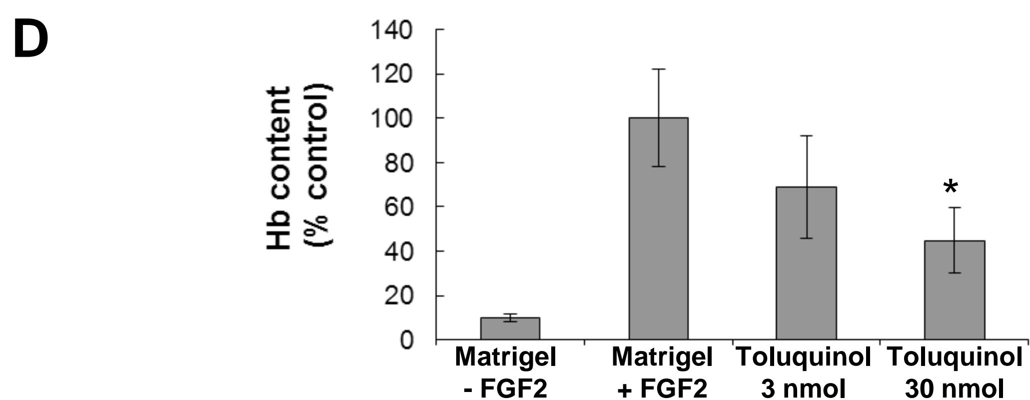
Figure 8 grayscale for printed version





C





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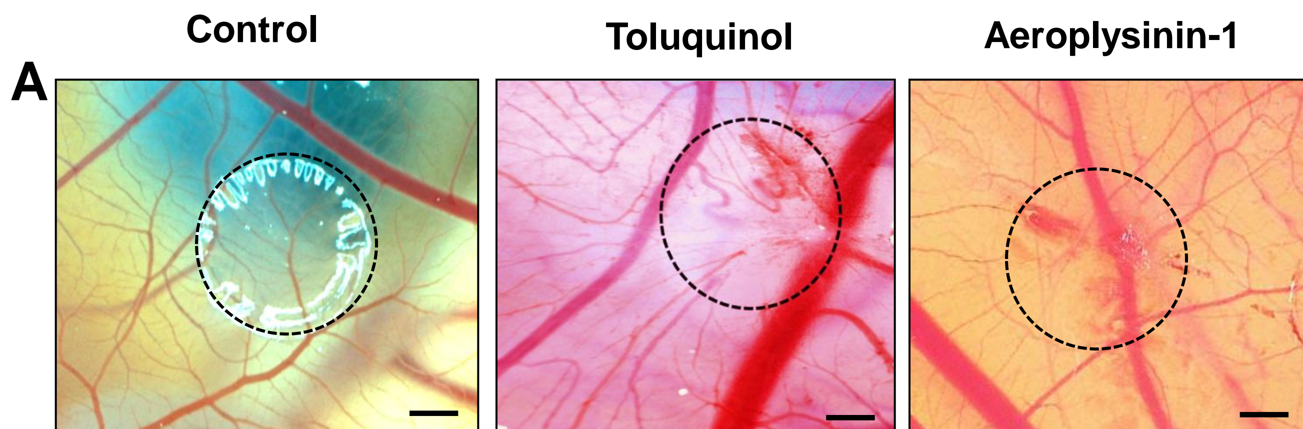

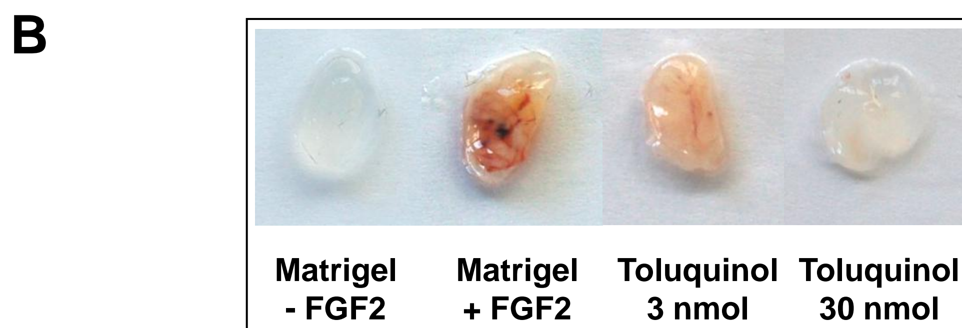

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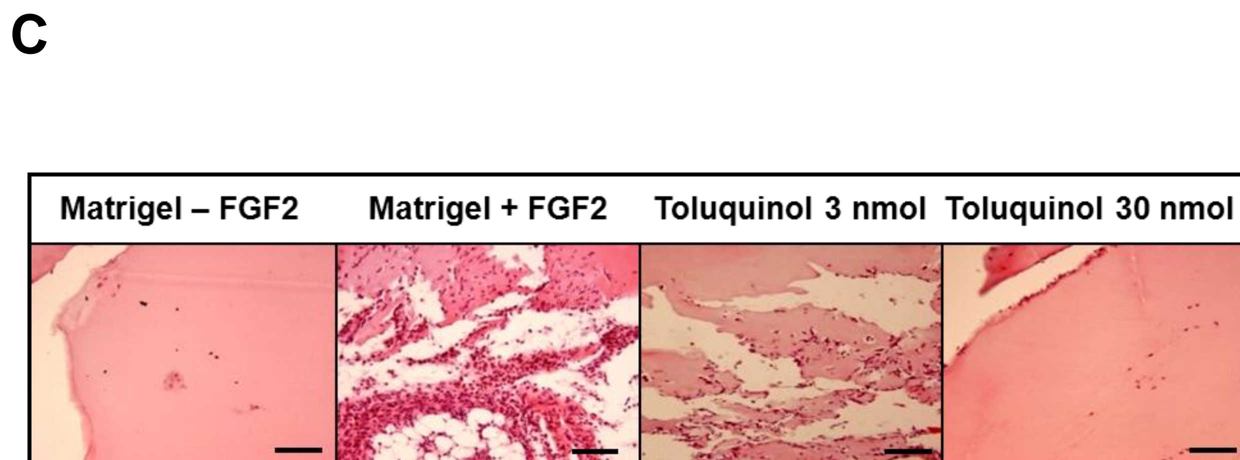


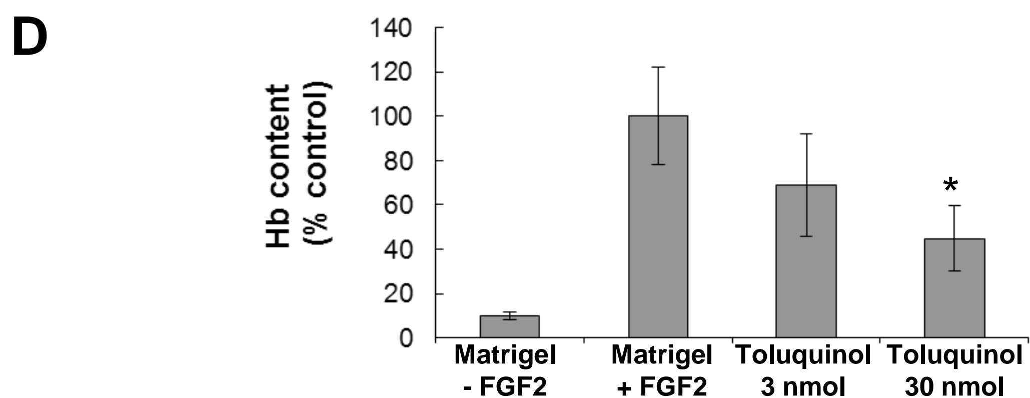
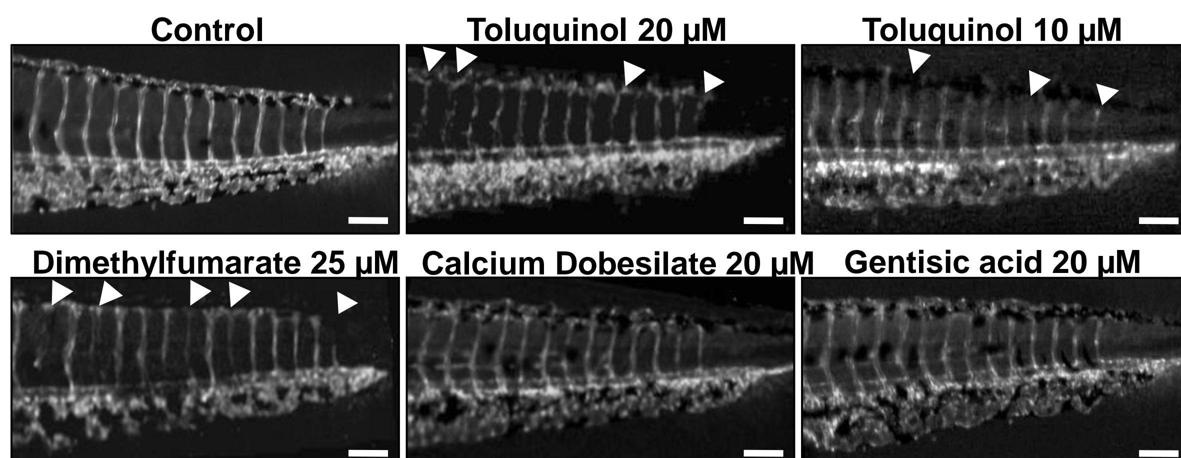


Figure 9 grayscale for printed version

A



B

Control

Toluquinol 20 µM

SU4312 5 µM

0 dpa

3 dpa

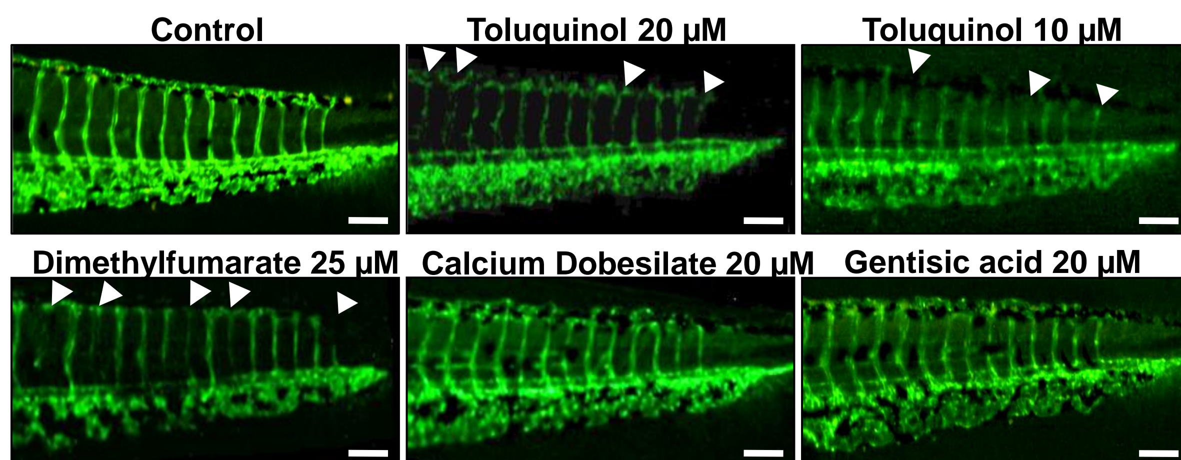
Calcium Dobesilate 20 µM

Gentisic acid 20 µM

0 dpa

3 dpa

Figure 9 color (for web-based version)



B

Control

Toluquinol 20 μM

SU4312 5 μM

0 dpa

3 dpa

Calcium Dobesilate 20 μM

Gentisic acid 20 μM

0 dpa

3 dpa

Toluquinol inhibits angiogenesis *in vitro* and *in vivo*