Supporting Information

The Small Molecule Chlorpyramine (C4) Targets the Binding Site of Focal Adhesion Kinase and Vascular Endothelial Growth Factor Receptor 3 and Suppresses Breast Cancer Growth *in vivo*

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Experimental Section.

RNA isolation and RT-PCR. Total RNA was isolated with RNeasy kit (Qiagen) and RT-PCR was performed using Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA). Random Hexamers (50 ng/reaction) or VEGFR-3 primer (proprietary sequence of R&D Systems, RDP-107-025, 10 pmol/reaction) was combined with 5 μg of RNA and 10 μM dNTP mix. 8 μl of master mix (cDNA synthesis buffer, 0.1 μM DTT, RNaseOUT™, DEPD-treated water, Cloned AMV reverse transcriptase) was added to the PCR tube on ice. Reaction was incubated at 50ºC for 1 hour. Reaction was terminated by incubating at 85ºC for 5 minutes. cDNA, primers, dNTP, Herculase are mixed. PCR was performed in the following manner: 10 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 70 seconds; 30 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 80 seconds. PCR products were visualized using ethidium bromide staining after agarose gel electrophoresis.

Immunocytochemistry. Cells were incubated in presence or absence of C4 and stained with anti-FAK monoclonal antibody 4.47 (Upstate) along or in combination with paxillin (BD Biosciences) or VEGFR-3 (N-terminus extracellular domain antibody from Chemicon #3757 and C-terminus intracellular domain antibody from Santa Cruz sc-321) as previously described . Detection in case of mono-staining was done with Alexa 546 secondary antibody, and for dual staining combination of Alexa 488 and Alexa 546 secondary antibody was used (Invitrogen, Carlsbad, CA). 3 X 10^4 cells were plated in 24-well plates with a cover slip placed at the bottom of the well. Then cells were fixed in 3.7% formaldehyde in PBS for 15 minutes, permeabilized with 0.1% Triton-X100 in PBS for 3 minutes, blocked with 10% normal goat serum (NGS) in 2%BSA-PBS for 1 hr and then were incubated with primary antibody at a concentration of 5 μg/ml in 2% BSA-PBS for 1 hour. Cells were incubated with the appropriate secondary antibody-florescent molecule conjugate, diluted 1:100 in 10% 2%BSA-PBS for 45 minutes in the dark and cover slips were mounted to slides using Vectashield® Hard Set™ mounting medium with DAPI (Vector Laboratories, Burlingame, CA). The slides were observed on a Leica Confocal microscope (Leica TCS SP5) running Leica LAS-AF software for instrument control and image analysis.

Figure S1. VEGFR-3 expression in breast cancer cell lines.

MCF7 breast cancer cells express undetectable level of endogenous VEGFR-3. We stably transfected MCF7 cells with a VEGFR-3 expression plasmid pcDNA3-VEGFR-3 (MCF7-VEGFR-3), and an empty expression vector, pcDNA3 (MCF7-pcDNA3).

(A) Reverse Transcription followed by polymerase Chain Reaction (RT-PCR) was performed with VEGFR-3-specific primers (RDP-107-025, R&D Systems) to demonstrate transcription of the VEGFR-3 gene in stably transfected MCF7 cells. BT474 cells also demonstrate VEGFR-3 gene transcription.

(B) Western blot analysis demonstrating expression of the VEGFR-3 (sc-321 antibody, Santa Cruz Biotechnology) in stably transfected MCF7 cells. Both BT474 and MCF7-VEGFR-3 cells expresses highly phosphorylated VEGFR-3 (Calbiochem #PC-450). Neither the parental MCF7 cell line nor MCF7-pcDNA3 demonstrates VEGFR-3 transcription or expression.

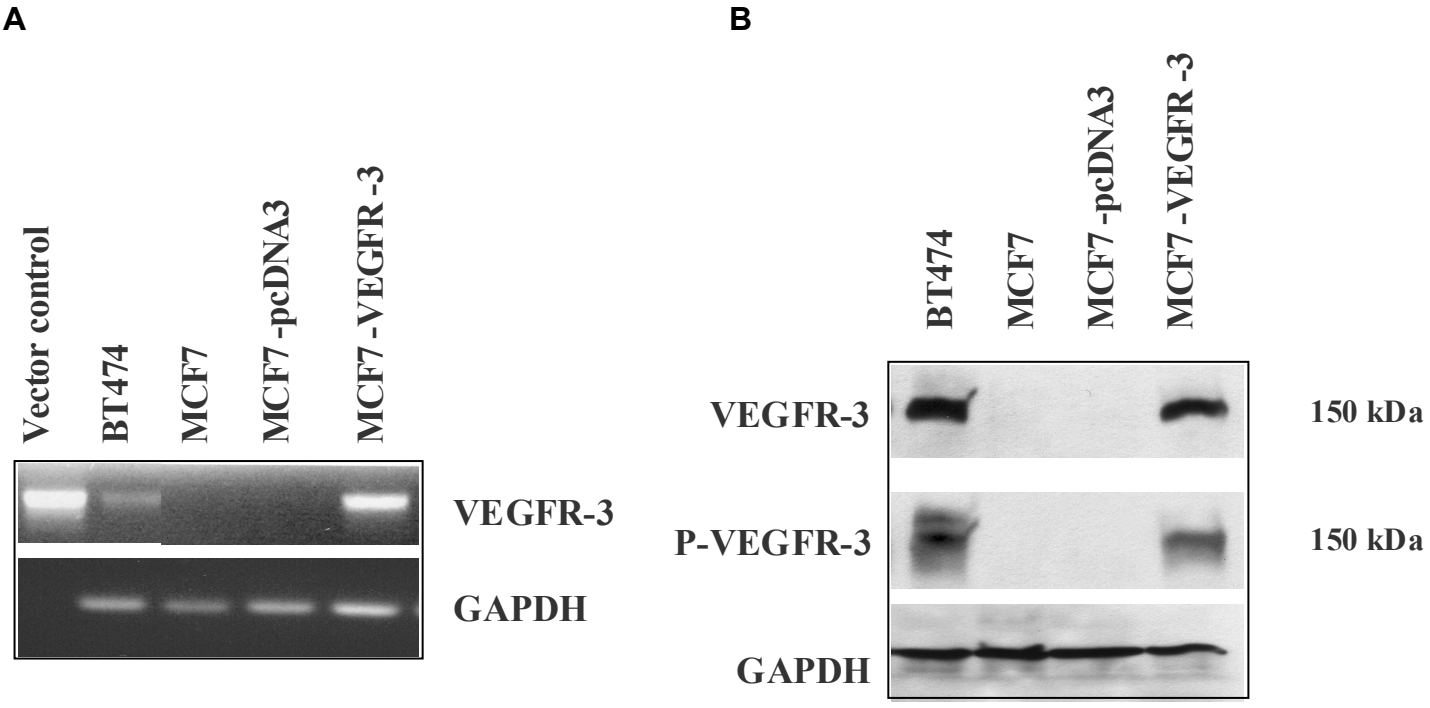


Figure S2. Specificity of the effect of small molecule 1 (C4).

Breast carcinoma cell lines BT474 and MCF7, and "normal" breast epithelial cell line MCF10A were treated with increased concentration of **1 (C4)**:

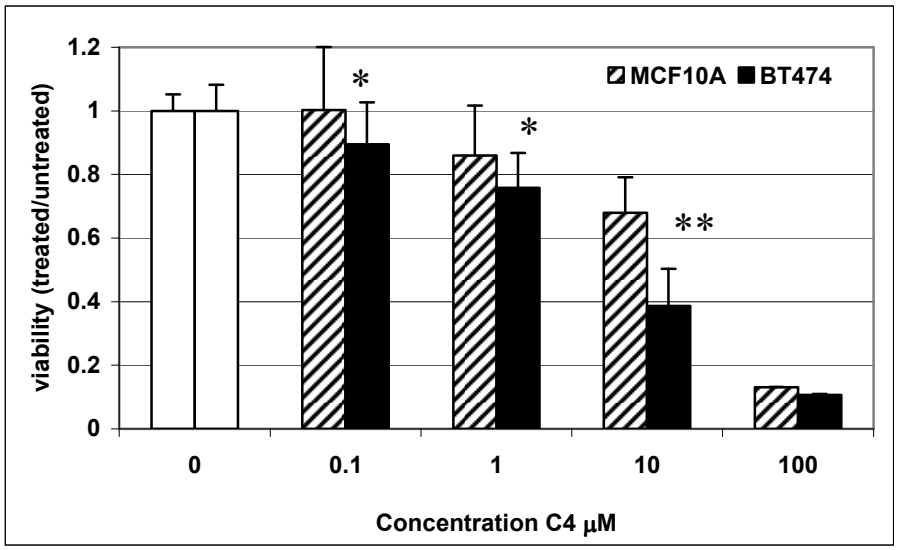
(A) Viability was measured after 24 h or 48 h of treatment in MTS assay. BT474 cells are significantly (* $P<0.05$ and ** $P<0.01$) more sensitive to **1** concentrations between 0.1 and 10 μM than normal cells, but 100 μM concentration is toxic for both cell lines; error bars represent ±SEM.

(B) Proliferation was measured after 48 h of treatment with BrdU incorporation assay. **1** has no effect on proliferation of normal MCF10 cells and cells with low /none VEGFR-3 expression. Data are represented as mean ± SEM of three independent experiments, * $P<0.05$ **$P<0.001$.

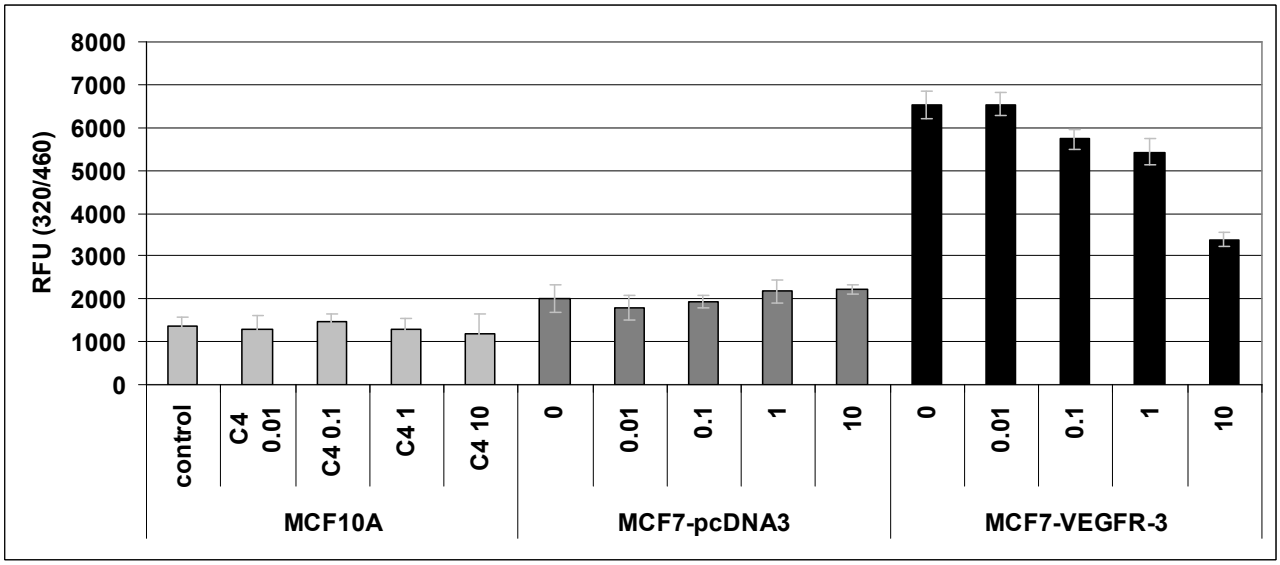
(C) BT474 cells were treated for 24 h with 10 μM of small molecules **1**, C5 and C7, selected *in silico* for FAK-VEGFR-3 binding site, and lysates were analyzed by Western blot with VEGFR-3- and FAK-specific antibody. Only small molecule **1** is capable to abrogate phosphorylation of VEGFR-3.

Figure S2.

A



B



C

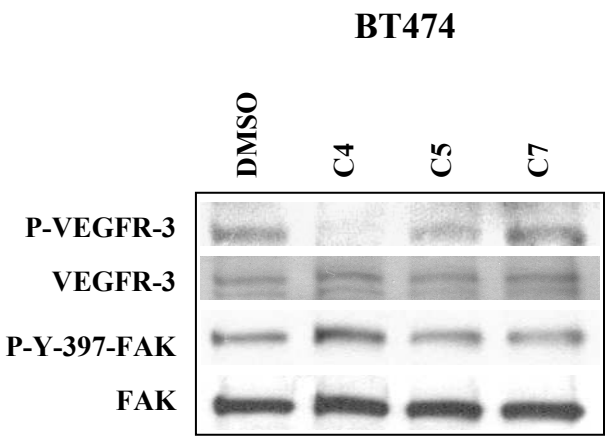


Figure S3. 1 treatment caused apoptosis in breast cancer cells expressing VEGFR-3

48 h expose to **1** leads to cell death by apoptosis, which is **1** dose-dependent and accompanied by ProCaspase 8 activation and PARP degradation. STS – staurosporine, positive control for apoptosis.

(A) BT474 breast carcinoma cells

(B) MCF7-pcDNA3 and MCF7-VEGFR-3 engineered breast cancer cells.

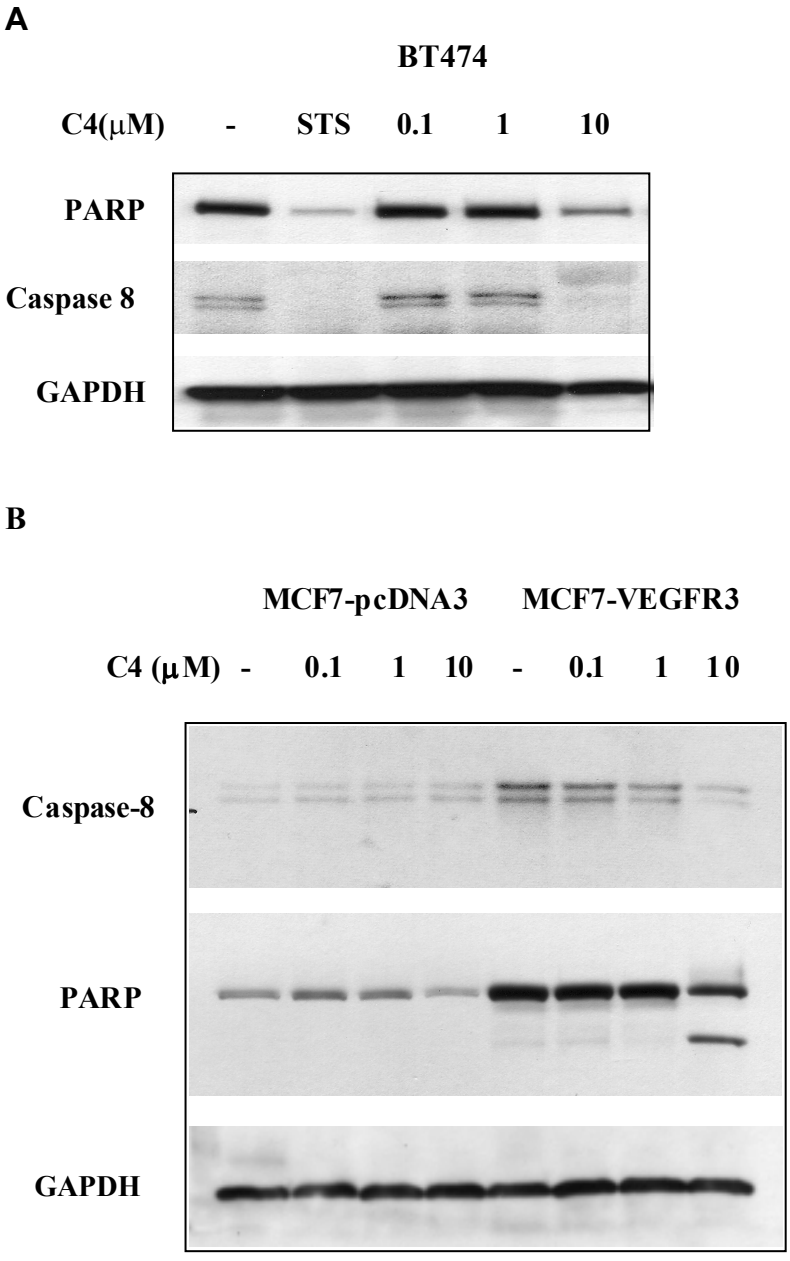


Figure S4. 1 treatment changed localization of endogenous FAK and VEGFR-3 in BT474 cells, but does not affect distribution of paxillin.

Confocal images of BT474 cells stained for FAK (left panel, antibody 4.47, Upstate), VEGFR-3 (middle panel, antibody sc-321, Santa Cruz), and paxillin (right panel, antibody # 610568 BD Biosciences) are shown with single XY, XZ, YZ cross-sections through different areas of the cells as indicated by cross-hair lines.

We used immunofluorescence confocal microscopy to analyze distribution of endogenous FAK, VEGFR-3, and paxillin in BT474 cells and how it is affected by treatment with small molecule **1**. Paxillin – focal adhesion protein, bound to FAT domain near VEGFR-3 binding site, was used as a control. BT474 breast cancer cells were grown on cover slips and were treated with 10μM **1** for 24 hr. Treated and untreated cells were individually immunostained for FAK, VEGFR-3 and paxillin, visualized with Alexa Fluor 546 secondary antibody and evaluated by confocal microscopy, followed by 3-D reconstruction. Treatment of cells with 10μM **1** for 24 hr (bottom panel) partially displaced FAK from the focal adhesions and lead to FAK relocalization closer to the cell nucleus (left panel), while paxillin cellular distribution remains unaffected by treatment (right panel). We also found that **1** treatment of BT474 cells results in VEGFR-3 relocalization from cellular cytoplasm to perinuclear compartment and to the nucleus (middle panel). Thus, we confirmed that FAK and VEGFR-3 are located in the cytoplasm, and that treatment with **1** specifically affects FAK and VEGFR-3 distribution throughout the cell and leads to relocalization of these proteins, but does not affect paxillin distribution. The same effect was revealed after staining with different VEGFR-3 antibody MAB3757 (Chemicon). It is important to note that the same redistribution of FAK and VEGFR-3 was documented when BT474 cells were treated with peptide AV3 from the VEGFR-3 binding region [1], that lead us to suggestion that compound **1** has the same effect as peptide AV3.

Figure S4.

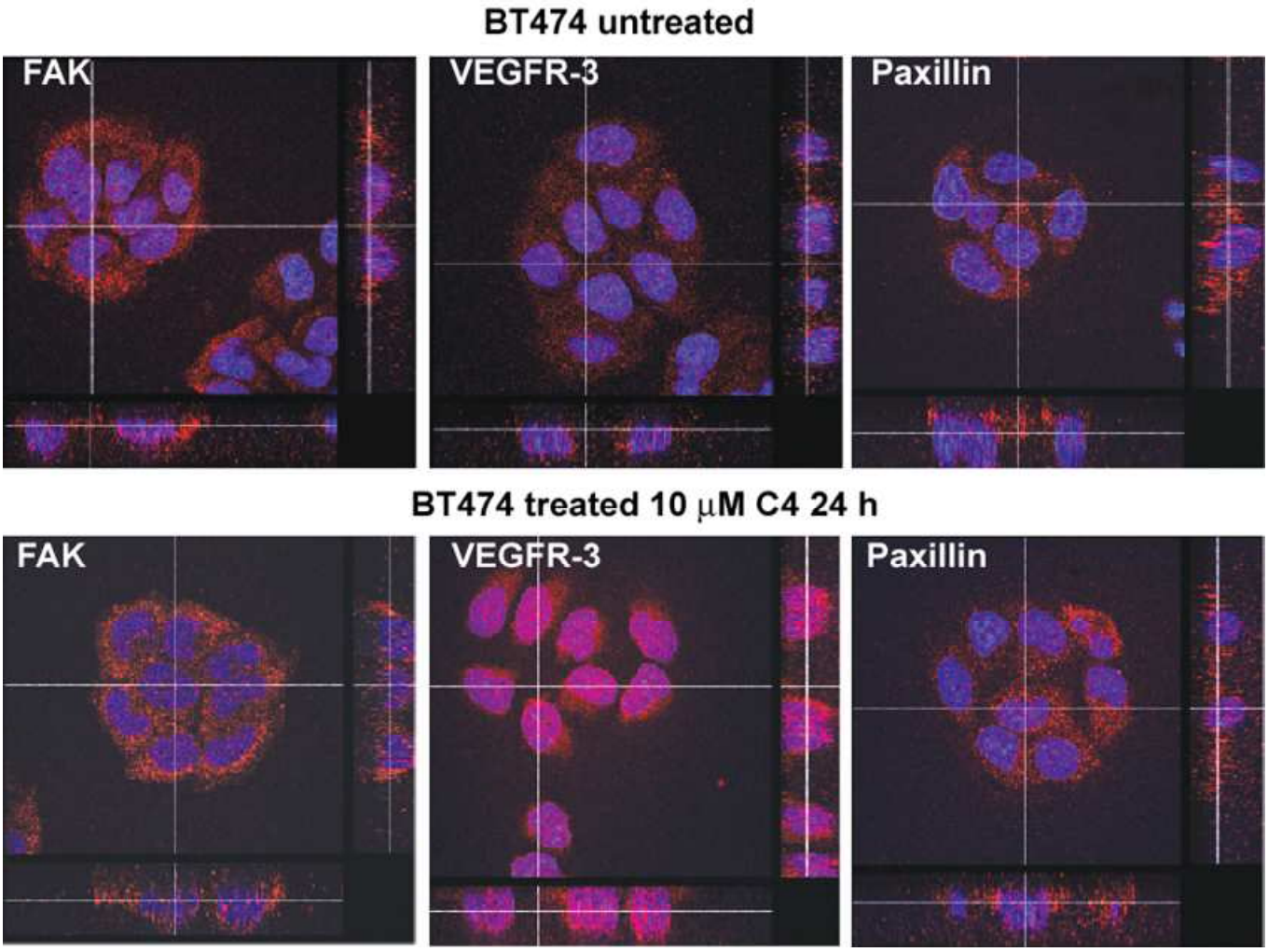

BT474 untreated
FAK
VEGFR-3
Paxillin
BT474 treated 10 μM C4 24 h
FAK
VEGFR-3
Paxillin

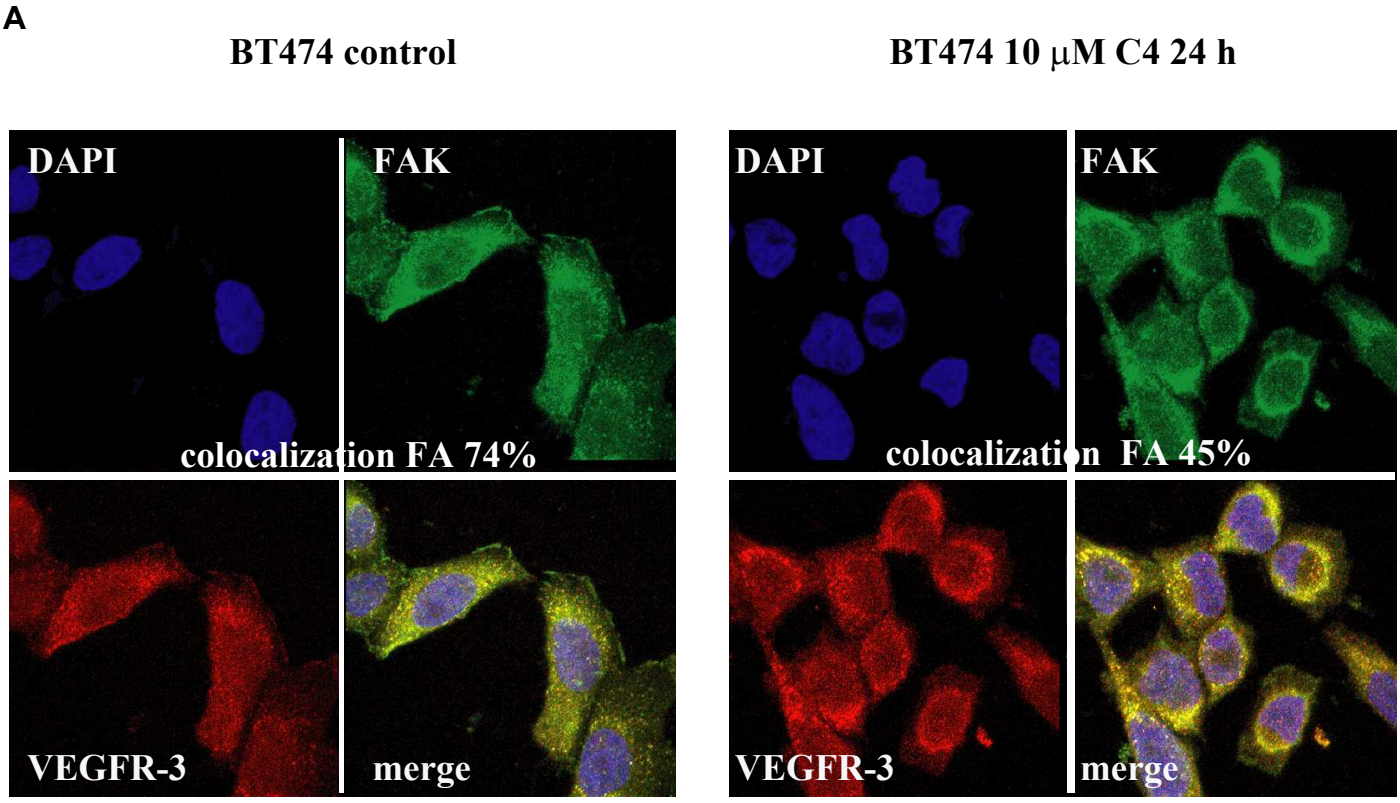
Figure S5. Analysis of FAK and VEGFR-3 colocalization.

(A) Confocal images of BT474 cells DMSO treated (left panel) and treated with 10 μM of **1** for 24 h (right panel) stained for FAK (antibody 4.47 Upstate and Alexa Fluor 488, Molecular Probes) and VEGFR-3 (antibody MAB3757, Chemicon and Alexa Fluor 546), demonstrating relocalization of both proteins after treatment with small molecule **1** and decrease of proteins colocalization in multiple cells.

(B) MCF7-VEGFR-3 engineered breast cancer cells with overexpression of VEGFR-3 protein were DMSO treated (top panel) and treated with 10 μM of **1** for 24 h (bottom panel) stained for FAK (antibody 4.47 Upstate and Alexa Fluor 488, Molecular Probes) and VEGFR-3 (antibody sc-321, Santa Cruz Biotechnology, and Alexa Fluor 546). Pattern of VEGFR-3 staining with sc-321 antibody is the same as with MAB3757, Chemicon antibody (Figure 3C) and both stainings revealed that treatment with **1** lead to redistribution of the VEGFR-3 protein and to decrease in colocalization of VEGFR-3 and FAK. Both methods revealed 40-60% drop in colocalization of FAK and VEGFR-3.

Figure S5.

A


BT474 control
BT474 10 μM C4 24 h
DAPI
FAK
colocalization FA 74%
VEGFR-3
merge
DAPI
FAK
colocalization FA 45%
VEGFR-3
merge

B

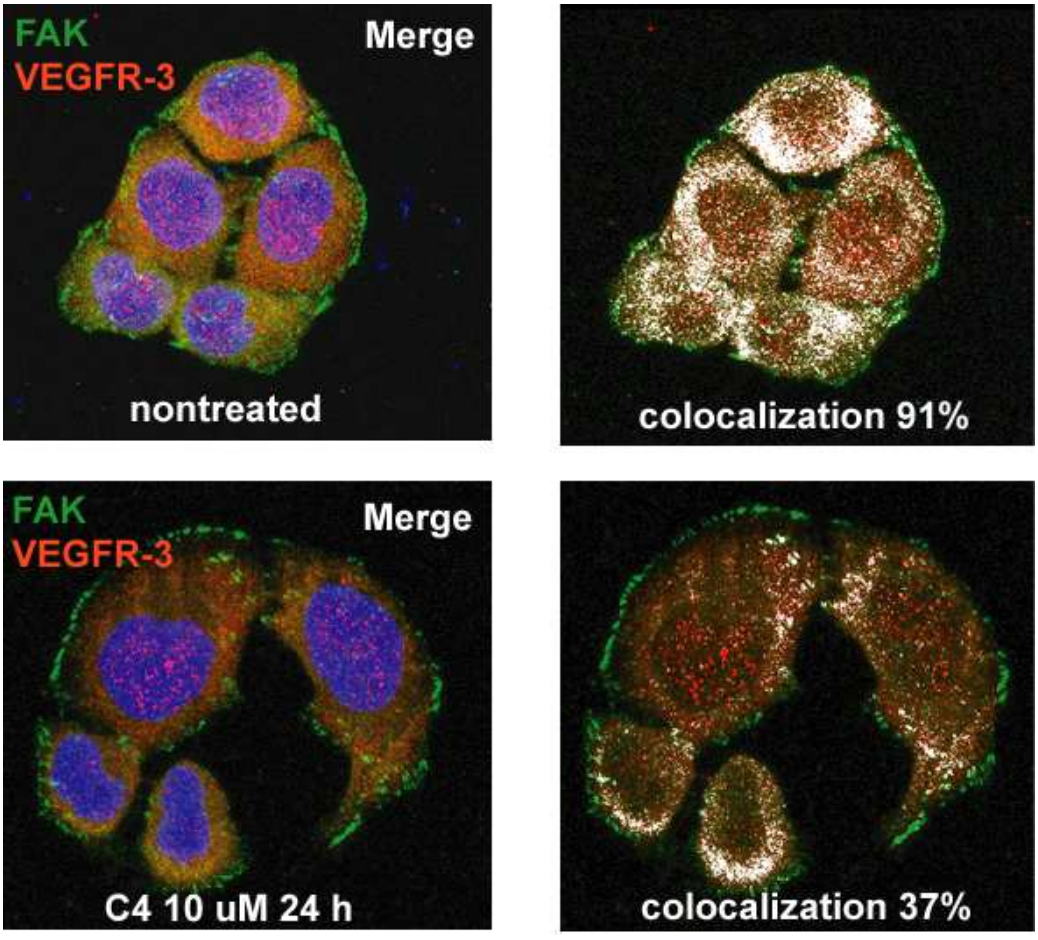

FAK
VEGFR-3
Merge
nontreated
colocalization 91%
FAK
VEGFR-3
Merge
C4 10 uM 24 h
colocalization 37%

Figure S6. 1 reduced tumor growth in xenotransplant mouse model.

(A and B) Photographs of the average tumors in mice 22 days after subcutaneous right flank inoculation of 2 X 10^6 BT474 (A) or 5 X 10^6 MCF7-VEGFR-3 (B) cells. Treatment with 60 mg/kg **1**, IP, once a day started next day after inoculation. Mice were sacrificed 21 days later and tumors were measured for size and weight.

(C) BT474 and MCF7-VEGFR-3 **1**-treated tumors weight in comparison with vehicle treated tumors, error bars represent ±SEM. * P<0.05