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2-(2-(2-Ethoxybenzoylamino)-4-chlorophenoxy)-*N*-(2-ethoxybenzoyl)benzamine inhibits EAT cell induced angiogenesis by down regulation of VEGF secretion

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Abstract—Compounds containing amide bond play a pivotal role in various pharmaceutical applications. 2-(2-(2-Ethoxybenzoylamino)-4-chlorophenoxy)-*N*-(2-ethoxybenzoyl)benzamine **4** is shown to be a potent antiangiogenic agent. In this study, we report the microwave-assisted synthesis, single crystal X-ray structure, and antiangiogenic effect of compound **4** in EAT cell induced angiogenesis. Treatment with compound **4** in vivo demonstrated down regulation of the secretion of VEGF in EAT cells and inhibition of blood vessel formation indicating the potential angioinhibitory effect of the compound in EAT cells.

Angiogenesis, the formation of new blood vessels from preexisting vessels, is a complex process that normally occurs in adults only under specific conditions such as wound healing, inflammation, and in menstrual cycle.[1,2] Under normal conditions such as wound healing, the angiogenic process switches on and then off at the appropriate times indicating tight regulation of stimulatory and inhibitory factors.[3] Under certain pathological conditions, such as the growth of solid tumors, rheumatoid arthritis, psoriasis, and diabetic retinopathy, angiogenesis occurs in a less controlled manner.[2,4] Understanding angiogenesis and its unique characteristics in tumor growth has provided insights into a number of ways to interrupt the process. In the last decade research on antiangiogenic agents has exploded along with public interest in its potential.[5] Most studies have addressed the prognostic significance of VEGF (Vascular endothelial growth factor) expression.[6,7] VEGF expression is upregulated in a majority of human tumors including lung, breast, GI, kidney, bladder, ovary and endometrial carcinomas as well as in hematologic malignancies. Tumoral expression of VEGF leads to the formation of new blood vessels which are often tortous and leaky, unlike normal blood vessels, they stimulate the endothelial cells to secrete proteases, resulting in the degradation of vessel basement membrane, which in turn allows cells to invade the surrounding matrix.[8]

Amides are ubiquitous in life, as proteins play a crucial role in virtually all biological processes such as enzymatic catalysis, transport or storage, immune protection, and mechanical support. Amides also play a key role for medicinal chemists. An in-depth analysis of the comprehensive medicinal chemistry database revealed that the carboxamide group appears in more than 25% of known drugs.[9] Dipyridyl amides have been reported as a potent metabotropic glutamate subtype 5(mGlu5) receptor antagonist.[10] 2-Aryl-4-oxo-thiazolidin-3-yl-amides have been used to check the anti-proliferative activity for prostate cancer[11] as well as pyrazolo [1,5-a] pyrimidin-7-yl-phenyl amides as novel anti-proliferative agents.[12] Various amides are used as antidepressants, anti-inflammatory, anti-malarial drugs, anti-viral agents, steroids, anti-microbials[13] and general anesthetics.[14]

In general, amides are formed from activated carboxylic acids and amines. Carboxylic acids can either be

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Table 1. The different conditions and reagents used for the condensation reaction

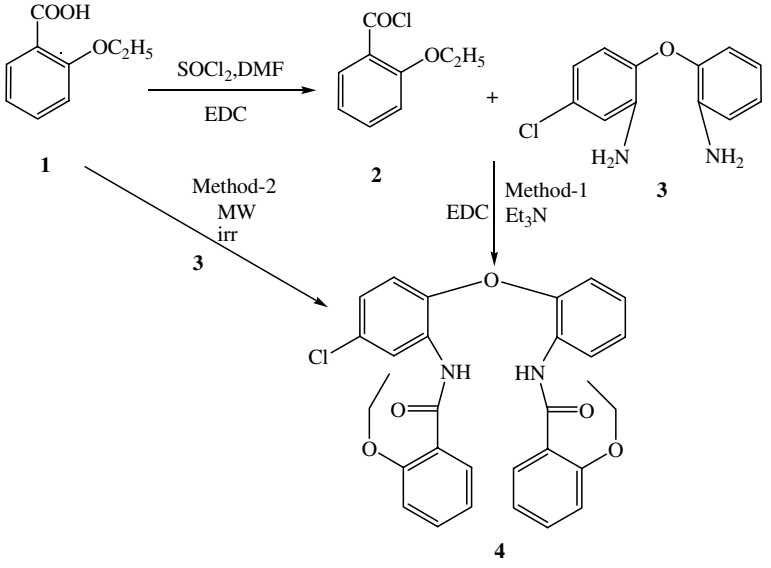
Methods	Condition	Yield%
Conventional, EDC/Et_3N	Room temperature	75
MW irradiation	80–90 s, 60 W	85

activated separately prior to the amide formation or they can be activated in situ using coupling reagents.[15] Although good results are obtained by conventional method, they are time consuming. To improve efficiency and reduce waste production to prepare amides directly from non-activated carboxylic acids and amines in the absence of coupling reagents and solvent are highly desired.[16] Microwaves (MWs) have been used to simplify and improve reaction conditions for many classic organic reactions. Reactions performed under MW-conditions proceed faster more cleanly and in much better yields than similar reactions under conventional conditions.[17,18] The MW assisted synthesis of amides has already been investigated.[19,20] However, the enormous growth in the use of microwave irradiation in the last decade in synthetic organic chemistry[21] inspired us to study this reaction. The synthesis of amides from carboxylic esters and amines under microwave irradiation is reported[22] in presence of solid potassium tertiary butoxide under solvent-free conditions that proceeds in a much shorter time. We have synthesized the compound **2** using the reported procedure[13] from our laboratory. In continuation of our work, herein, we report an efficient method for the synthesis of 2-(2-(2-ethoxybenzoylamino)-4-chlorophenoxy)-*N*-(2-ethoxybenzoyl)benzamine **4** in which 2-ethoxy benzoic acid undergoes condensation reaction with 2-(2-amino-4-chlorophenoxy)benzeneamine **3** in solvent-free medium under microwave irradiation at 60 W for 90 s.

We have reported earlier the synthesis of compound **4** by conventional method[23] using 2-ethoxy benzoylchloride **2** and triethylamine with 2-(2-amino-4-chlorophenoxy)benzeneamine **3**. Compound **4** was achieved in good yield by microwave irradiation method[24] compared to the product obtained by conventional method as shown in Table 1 and Scheme 1. IR, ^1H NMR, and CHN data provide the proof for condensed structure. For instance, in the IR spectra, compound **4** showed the bands in the region of 1675 cm^{-1} for C=O amide bond and N–H is observed in the region of 3340 cm^{-1} indicating the formation of substituted amide bond in **4**.

Crystal structure of the compound 2-(2-(2-ethoxybenzoylamino)-4-chlorophenoxy)-*N*-(2-ethoxybenzoyl)benzamine **4** was determined by X-ray diffraction method. The ORTEP[25] of the compound **4** at 50% probability is given in Figure 1. The parent phenyl rings of the molecule are planar. The dihedral angle between the planes [C4–C9] and [C13–C18] is 71.2 (3)°, while those of the planes [C13-C18] and [C20–C25] and [C20–C25] and [C29–C34] are 82.9(3)° and 83.2(3)°, respectively. The torsion angles about C9–C10–N12–C20 and C13–C18–O19–C20 are 171.9(5)° and −84.5(7)° implying that they exhibit antiperiplanar and synclinal conformations, respectively. The molecules are stacked in pairs when viewed along the *a* axis. The structure exhibits intramolecular hydrogen bonds of the type N–H…..O and C–H…..O.

*Compound **4** inhibits tumor induced angiogenesis.*[33] Ehrlich ascites tumor (EAT) cells. These are mouse mammary carcinoma cells. In vivo experimental studies have demonstrated that tumor growth is dependent on angiogenesis. Increased vascularity may allow not only an increase in tumor growth but also a greater enhancement of hematogenous tumor embolization. Thus, inhibiting tumor angiogenesis may halt the tumor growth and decrease metastatic potential of tumors. The in vivo treatment of compound **4** on EAT bearing mice resulted in the decrease in body weight of the mice up to 65% when compared to the untreated control animals (Fig. 2). This effect is clearly evident in the reduction of EAT cell number (Fig. 3) as well as in the ascites volume (Fig. 4) of the compound **4** treated mice.



Scheme 1.

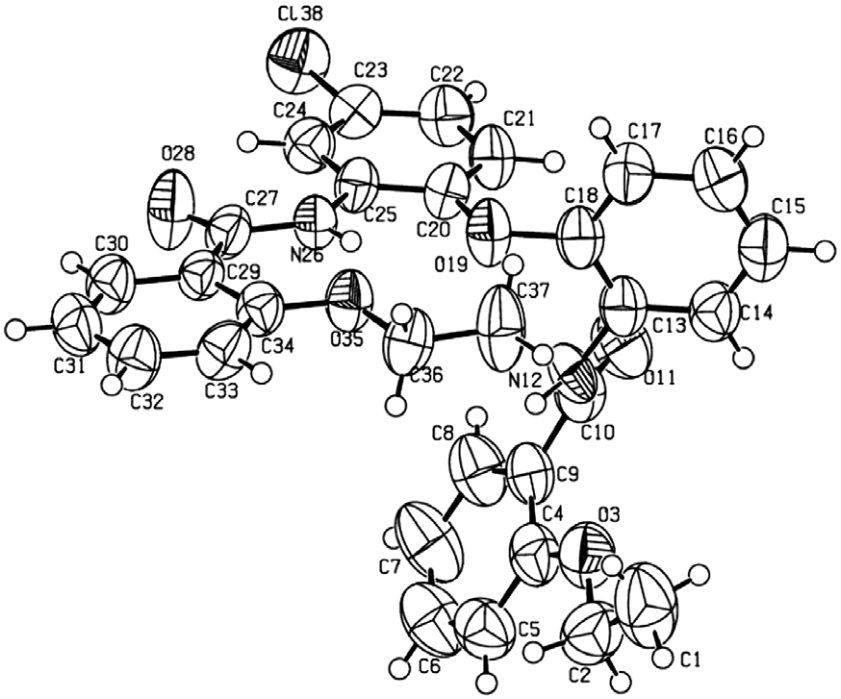


Figure 1. ORTEP diagram of the molecule 4 at 50% probability with some selected bond lengths and bond angles. Bond lengths. C2–C1:1.501(1) Å, N12–C10:1.351(6) Å, O3–C2:1.420(7) Å, N12–C13:1.387(6) Å, O3–C4:1.352(7) Å, C29–C27:1.500(7) Å, O11–C10:1.191(6) Å, Cl38–C23 1.749(5) Å. Bond angles. O3–C2–C1:105.4(6)°, C22–C23–Cl38:117.2(4)°, O11–C10–N12:122.5(6)°, C27–N26–C25:128.7(4)°, N12–C10–C9:112.4(5)°, C34–O35–C36:118.3(4)°, C18–O19–C20:117.9(4)°, O35–C36–C37:106.8(4)°.

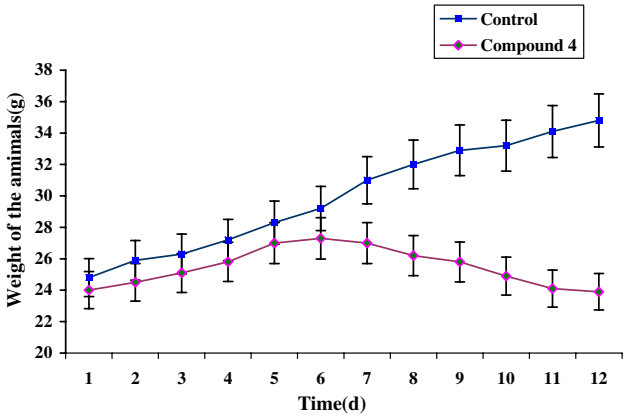


Figure 2. Effect of compound **4** against tumor growth.

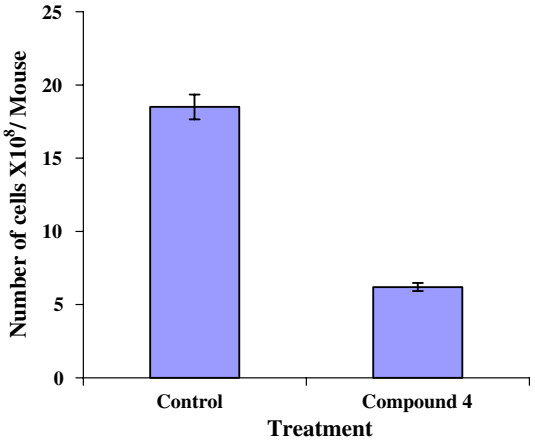


Figure 3. Effect of compound **4** on cell number.

Quantification of VEGF (Fig. 5) shows that compound **4** has dose dependent effect on secretion of VEGF under in vivo conditions compared to the untreated EAT bearing mice. The amount of VEGF increased in untreated EAT cells over the growth period, whereas the amount of VEGF in ascites of compound **4** treated EAT cells did not show any significant increase in the same growth period, suggesting a dose dependent inhibition of VEGF secretion upon compound **4** treatment in EAT cells. Angiogenesis is clearly evident in the inner peritoneal lining of EAT bearing mice and it is a reliable model for in vivo angiogenesis. Hence the peritoneal lining of compound **4** treated mice verified for its effect on microvasculature when compared to untreated EAT bearing mice (Fig. 6). Mice treated with compound **4** showed de-

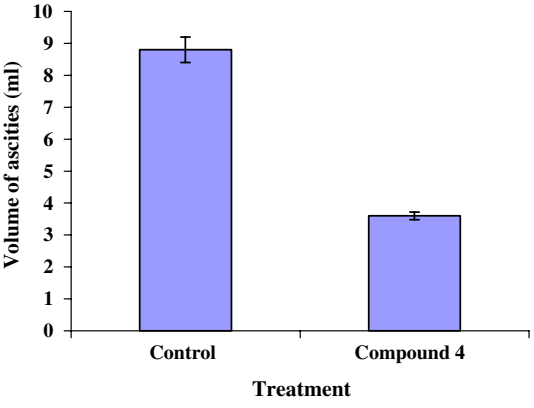


Figure 4. Effect of compound **4** on ascites volume.

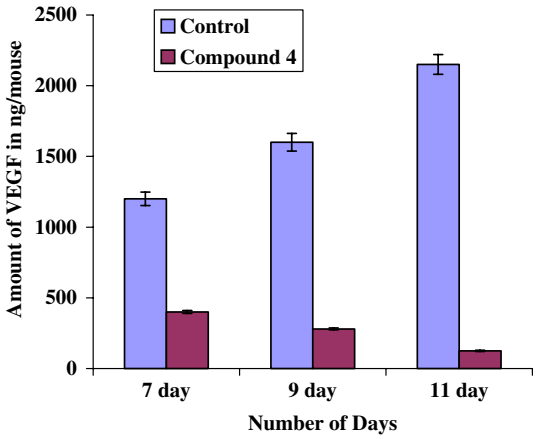


Figure 5. Effect of compound **4** on in vivo production of VEGF.

creased peritoneal angiogenesis when compared to untreated EAT bearing mice. In the CAM assay model compound **4** induced vasculature zone formation in the developing embryos. Notably, a newly formed microvessel was regressed around the compound **4** implanted disc (Fig. 7).

Currently, a large variety of chemotherapeutic drugs are being used to treat cancer. Unfortunately, many compounds hold limited efficacy, due to problems of delivery and penetration, and a moderate degree of selectivity for the tumor cells, thereby causing severe damage to healthy tissues. From our studies, it is clear that compound **4** has antiangiogenic effect as shown by peritoneal angiogenesis assay, chorioallantoic membrane (CAM) assay, and also from the reduction in the EAT cell number, ascites volume, and body weight of the animals in vivo. It is found to be a consequence of an anti-angiogenic effect, and it would provide major impetus to a large segment of the medical oncology community to become much more actively engaged in angiogenesis research and antiangiogenic therapies to treat these types of cancer. The above study shed light toward the identification of new antiangiogenic molecules to the cancer therapy. Further research to know the mechanism of inhibition and the modifications of

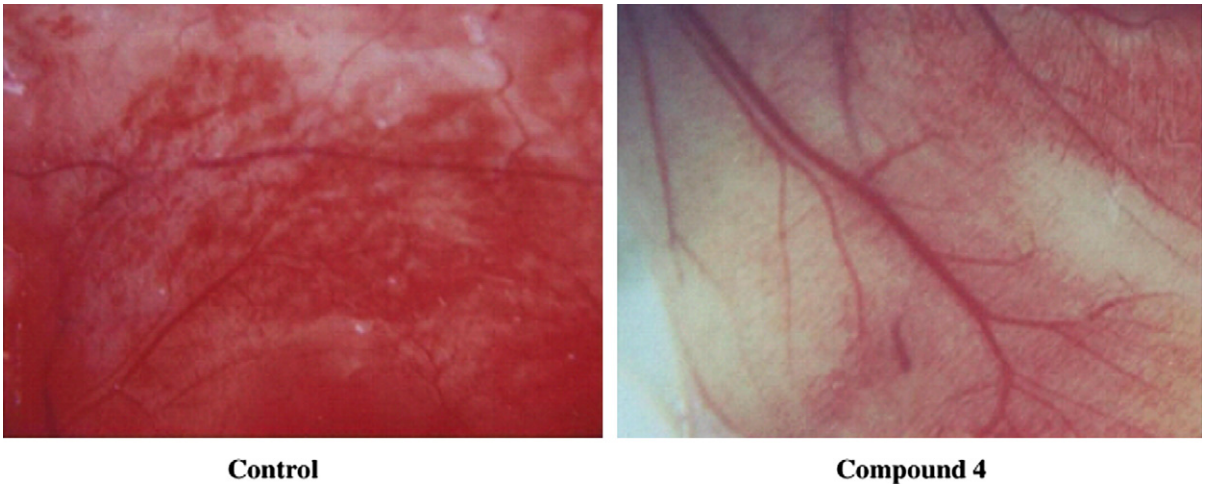


Figure 6. Peritoneal angiogenesis.

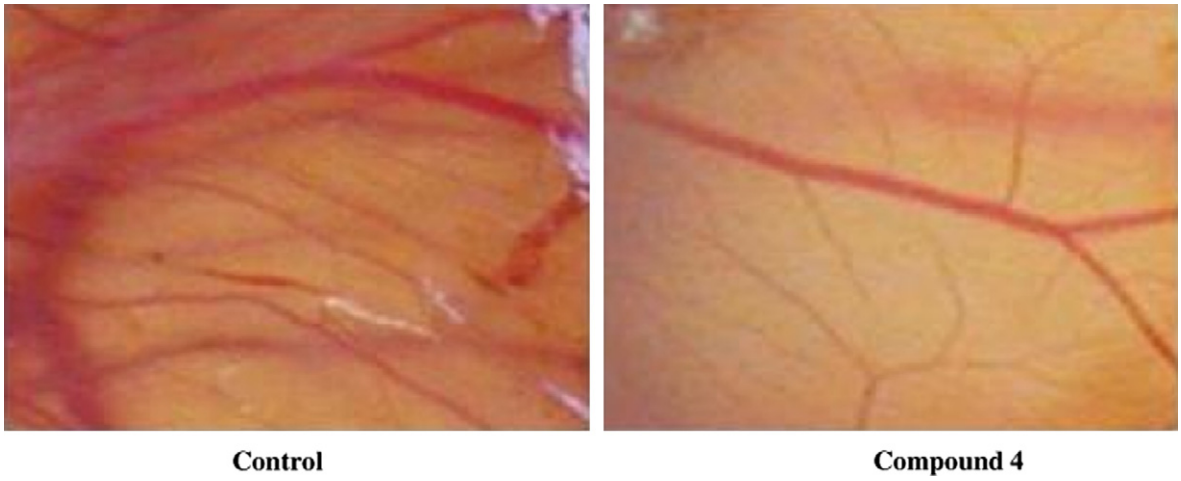


Figure 7. Chorioallantoic membrane assay.

the compound **4** to improve the potency is currently under progress in our laboratory.

Supplementary data

The full crystallographic details have been deposited at Cambridge Crystallography Data Center (CCDC No. 283578).

Acknowledgments

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References and notes

1. Varner, J. A.; Cheresh, D. A. In *DeVita VT*; Hellman, S., Rosenberg, S. A., Eds.; Important Adv. Oncol.; Lippincott-Raven: Philadelphia, 1996; Vol. 69.
2. Van Hinsbergh, V. W. M.; Collen, A.; Koolwijk, P. *Ann. Oncol.* **1999**, *10*, 560.
3. Hanahan, D.; Folkman, J. *Cell* **1996**, *86*, 353.
4. O'Reilly, M. S. *Invest. New Drugs* **1997**, *15*, 5.
5. Kerbel, R. S. *Carcinogenesis* **2000**, *21*, 505.
6. Takayashi, Y.; Kitadai, Y.; Bucana, C. D. *Cancer Res.* **1995**, *72*, 319.
7. Or, R.; Feferman, R.; Shoshan, S. *Exp. Hematol.* **1998**, *26*, 217.
8. Houck, K. A.; Ferrara, N.; Winer, J.; Cachianes, G.; Philips, H. S.; Ferrara, N. *Nature* **1993**, *362*, 841.
9. Ghose, A. K.; Viswanadhan, V. N.; Wendoloski, J. J. J. *Comb. Chem.* **1999**, *1*, 55.
10. Bonnefous, C.; Vernier, J.-M.; Hutchinson, J. H.; Chung, J.; Reyes-Manalo, G.; Kamenecka, T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1197.
11. Gududuru, V.; Hurh, E.; Dalton, J. T.; Miller, D. D. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5289.
12. Gopalsamy, A.; Yang, H.; Ellingboe, J. W.; Tsou, H.-R.; Zhang, N.; Honores, E.; Powell, D.; Miranda, M.; McGinnis, J. P.; Rabindran, S. K. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1591.
13. Priya, B. S.; Basappa; Nanjunda Swamy, S.; Rangappa, K. S. *Bioorg. Med. Chem.* **2005**, *13*, 2623.
14. Kirk, K. L.; Filler, R. In *Biomedical Frontiers of Fluorine Chemistry,* Symp. Ser.; American Chemical Society: Washington, DC, 1996; Vol. 3, p 1.
15. Sheehan, J. C.; Hess, G. D. *J. Am. Chem. Soc.* **1955**, *77*, 1067.
16. Gelens, E.; Smeets, L.; Sliedregt, L. A. J. M.; van Steen, B. J.; Kruse, C. G.; Leurs, R.; Orru, R. V. A. *Tetrahedron Lett.* **2005**, *46*, 3751.
17. Lidstorm, P.; Tierney, J.; Wathey, B.; Westman, J. *Tetrahedron* **2001**, *57*, 9225.
18. Kuhnert, N. *Angew. Chem., Int. Ed.* **2002**, *41*, 1863.
19. Vasquez-Tato, M. P. *Synlett* **1993**, 506.
20. Perreux, L.; Loupy, A.; Volatron, F. *Tetrahedron* **2002**, *58*, 2155.
21. (a) Abramovitch, R. A. *Org. Prep. Proc. Int.* **1991**, *23*, 683; (b) Caddick, S. *Tetrahedron* **1995**, *51*, 10403; (c) Galema, S. A. *Chem. Soc. Rev.* **1997**, *26*, 233; (d) Loupy, A.; Petit, A.; Hamelin, J.; Texier-Boullet, F.; Jacquault, P.; Mathe, D. *Synthesis* **1998**, 1213; (e) Varma, R. S. *Green Chem.* **1999**, 43.
22. Zradni, F.-Z.; Texier-Boullet, F.; Hamelin, J. *Fifth Int. Electr. Conf. Synth. Org. Chem.* **2001**, 1.
23. Priya, B. S.; Nanjunda Swamy, S.; Tejesvi, M. V.; Basappa; Sarala, G.; Gaonkar, S. L.; Naveen, S.; Shashidhara Prasad, J.; Rangappa, K. S. *Eur. J. Med. Chem.* **2006**, *41*(11), 1262.
24. Experimental: *Microwave irradiation method*. The compounds 2-(2-amino-4-chlorophenoxy)benzeneamine **3** (0.25 g, 1.21 mmol) and 2-ethoxy benzoic acid **1** (0.281 g, 1.691 mmol) were mixed and the slurry mass was kept for 80–90 s in a microwave oven at 60% power in solvent free condition. After completion of the reaction, the reaction mass was worked up as described in the conventional method.[23]
25. Johnson, C. K. *ORTEP-II, A Fortran Thermal-Ellipsoid Plot Program, Report ORNL-5138*; Oak Ridge National Laboratory: Oak Ridge, TN, USA, 1976.
26. Otwinowski, G.; Minor, W. *Macromolecular Crystallography* **1997**, *276*: part A, 307, Carter, C. M., Jr.; Sweet, R. M., (Eds.), Academic Press.
27. Mackay, S.; Gilmore, C. J.; Edwards, C.; Stewart, N.; Shankland, N. maXus Computer Program for the Solution and Refinement of Crystal Structures, Bruker Nonius, The Netherlands, MacScience, Japan and The University of Glasgow, **1999**.
28. Sheldrick, G. M., (SHELXS{97}, SHELXL{97}), University of Gottingen, Germany, **1997**.
29. A good single crystal of the title compound with dimensions $0.3 \times 0.25 \times 0.25$ mm was chosen for X-ray diffraction studies. The measurements were made on a DIPLabo Imaging plate diffractometer with graphite monochromated Mo Kα radiation from a sealed anode generator operated at 50 kV and 36 mA. The crystal to detector distance is fixed at 120 mm with a detector area of 440×291 mm^2. Thirty-six frames of data were collected by the oscillation method with each frame being exposed for 400 s. Successive frames were scanned in steps of 5°/min with an oscillation range of 5°. Image processing and data reduction were done by Denzo.[26] The reflections were merged with Scalepack.[27] All the frames could be indexed using primitive triclinic lattice. The structure was solved by direct methods using SHELXS-97[29] and refined by least squares methods using SHELXL97.[28] The final cycle of full matrix least squares refinement was based on 5344 independent reflections and 346 parameters and converged with $R_1 = 0.0905$ and $\omega R_2 = 0.2136$. The hydrogen atoms were placed at chemically acceptable positions and were not included in the refinement. Full crystallographic details have been deposited at the Cambridge Crystallographic Centre.
30. Prabhakar, B. T.; Khanum, S. A.; Jayashree, K.; Salimath, B. P.; Shashikanth, S. *Bioorg. Med. Chem.* **2006**, *14*(2), 435.
31. Mahadesh, B.; Salimath, B. P. *Mol. Cell. Biochem.* **2005**, *57*, 273.
32. Gururaj, A. E.; Belakavadi, M.; Venkatesh, D. A.; Marmé, D.; Salimath, B. P. *Biochem. Biophys. Res. Comm.* **2002**, *297*, 934.
33. *Preparation of compound* **4** *for in vivo treatment*. Compound 2-(2-(2-ethoxy benzoyl-amino)-4-chlorophenoxy)-*N*-(2-ethoxybenzoyl)benzamine was dissolved in DMSO. Compound **4** 100 mg/kg body weight/ip, 300 μl/mouse was injected into the Ehrlich's ascites tumor cells (EAT cells) bearing mice every alternate day after 6 days of cell growth and the mice were sacrificed on the 12th day.
In vivo culture of Ehrlich ascites tumor cells and effect of compound **4** *against tumor growth*.[30] Six- to eight-week-old female mice were acclimated for 1 week while caged in group of five. Mice were housed and fed on a diet of animal chow and water ad libitum throughout the experiment. All experiments were approved by the Insti-

tutional Animal Care and Use Committee of the University of Mysore, Mysore, India. Ehrlich ascites tumor (EAT) cells were grown in the peritoneum cavity of six- to eight-week-old Swiss albino mice by peritoneal transplantation of 0.5 ml of cell suspension (5×10^8) in sterile saline. These cells grow in mice peritoneum forming an ascites tumor, with massive abdominal swelling. The animals showed a dramatic increase in body weight over the growth period and the animals succumbed to the tumor burden 10–12 days after implantation. The number of cells increased over the 10 days of growth with formation of 6–7 ml of ascites fluid with extensive neovascularization in the inner lining of peritoneal wall. To determine whether the compound inhibits tumor growth and angiogenesis mediated by EAT cells in vivo, compounds (100 mg/kg body wt/ip 300 μl/mouse) were injected into the EAT bearing mice every alternate day after 6th day of tumor transplantation and growth of the tumor was monitored by taking the body weight of the animals.

Effect of compound **4** *on cell number and ascites volume*. After monitoring the body weight, animals were sacrificed on the 12th day and the EAT cells along with ascites fluid were harvested and centrifuged at 3000 rpm for 10 min at 4 °C. The pelleted cells were counted by Trypan blue dye exclusion method using a hemocytometer. Supernatant gave the volume of ascites fluid.

Effect of compound **4** *on in vivo production of vascular endothelial growth factor (VEGF)*. Ascites fluid was collected (each dose after treatment) after sacrificing the animal every alternate day after six days. VEGF ELISA was carried out to quantitate VEGF in the Ascites fluid using anti-VEGF165 antibodies as reported earlier.[31]

Peritoneal angiogenesis assay. After harvesting the EAT cells, the abdomen was cut open and the inner lining of the peritoneal cavity of untreated and compound **4** treated EAT bearing mice was examined for the extent of vascularization. The peritoneal lining was photographed using Nikon digital camera.[31]

Chorioallantoic membrane (CAM) assay. The fertilized eggs were incubated at 37 °C in a humidified and sterile atmosphere for 10 days. A window was made under aseptic conditions on the eggshell to check for proper development of the embryo. The window was resealed and allowed to develop further. On the 12th day, saline, compound **4** (10 μg per egg) were air-dried on sterile glass coverslips. The window was reopened and the coverslip was inverted over the CAM. The window was closed again, and the eggs were returned to incubator for another 2 days. The windows were opened on the 14th day and inspected for changes in the microvessel density in the area below the coverslip and photographed using in Nikon digital camera.[32]