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ORIGINAL ARTICLE

Antiangiogenic and antiproliferative effects of substituted-1,3,4-oxadiazole derivatives is mediated by down regulation of VEGF and inhibition of translocation of HIF-1α in Ehrlich ascites tumor cells

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Abstract
Purpose 1,3,4-Oxadiazoles are an important class of heterocyclic compounds, which play a pivotal role in various pharmaceutical applications. Here, we investigated the antiangiogenic and antiproliferative effects of the derivatives and explored its mechanism of action on EAT cells.
Methods The cytotoxic effect of the derivatives on EAT and HEK293 cells was assessed by MTT assay. Effect of the derivatives on ALP activity and proliferation was measured. Swiss albino mice transplanted with EAT cells were used as a model system to study the effect of the derivatives in vivo. Inhibition of angiogenesis in mice peritoneum, CAM and Cornea of the rat were studied. Finally, the effects on VEGF gene expression, HIF-1α translocation and cell cycle arrest were determined.
Results The IC50 range for growth inhibition of EAT cells was found to be 140–175 μM. In contrast normal HEK293 cells were resistant to the derivatives at this range. Treatment with derivatives in vivo was demonstrated by the down regulation of VEGF in EAT cells and inhibition of blood vessels formation in mice peritoneum, CAM and cornea of rat, indicating the potent angioinhibitory effect of the derivatives. VEGF promoter-luciferase reporter gene expression analysis showed suppression of VEGF gene expression in vitro. The derivatives proved to be potent antiproliferative agents as shown by FACS analysis and decreased ALP activity. Furthermore, expression of HIF-1α was also down regulated by derivatives by repressing its nuclear translocation.
Conclusions Oxadiazole derivatives are strong bioactive compounds with antiangiogenic and antiproliferative potential both in vitro and in vivo. We postulate that diminished HIF-1α nuclear presence in oxadiazole treated EAT cells could be responsible for decreased VEGF expression and antiangiogenic effects.

Keywords Oxadiazole derivatives · Antiangiogenesis · EAT cell · HEK293 cells · VEGF · Hypoxia · HIF-1α

Introduction

Angiogenesis, the formation of new blood vessels from preexisting endothelium, is a fundamental step in a variety of physiological and pathological conditions including wound healing, embryonic development, chronic inflammation, tumor progression and metastasis [1–6]. Complex and diverse cellular actions are implicated in angiogenesis, such as extracellular matrix degradation, proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes [7]. The angiogenic process is tightly controlled by a wide variety of positive or negative regulators, which are composed of growth factors, cytokines, lipid metabolites, and cryptic fragments of haemostatic proteins [8], and many of these factors are initially characterized in other biological activities. Among these molecules, vascular endothelial growth factor (VEGF), a soluble angiogenic factor produced by many tumor and normal cells, plays a key role in regulating normal and abnormal angiogenesis [9]. VEGF is found to stimulate

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endothelial cells to secrete proteases and plasminogen activator, resulting in the degradation of vessel basement membrane, which in turn allows cells to invade the surrounding matrix [10, 11]. After subsequent migration and proliferation the cells finally differentiate to form a new vessel. Enhanced expression of VEGF has been observed in many human cancers including rectal, breast, non-small cell lung and ovarian cancers [12]. High levels of VEGF have been found in a variety of effusions accompanying pathologic disorders like edema formation in the brain, human rheumatoid synovial fluid and malignant ascites [13]. Consequently inhibition of fluid accumulation and tumor growth by neutralization of VEGF has been demonstrated, underlining the importance of VEGF in malignant ascites formation [14, 15].

Tumor hypoxia is of critical importance in tumor physiology and cancer treatment and it appears to be strongly associated with malignant progression and resistance to cancer therapy. The VEGF gene and several other genes regulated by hypoxia are under the control of transcription factor Hypoxia inducible factor-1α (HIF-1α) [16]. Under hypoxic condition HIF-1α protein accumulates and translocates to the nucleus where it forms an active complex with HIF-1β and activates transcription of target genes. HIF-1α is over expressed in premalignant lesions in the colon, colorectal cancer and its metastases and is also considered to be an independent indicator of poor prognosis [17]. However, driven by the recent progress in the molecular understanding of the tumor hypoxia response, various strategies for molecular targeting of hypoxic cancer cells are emerging [18], such as blocking HIF-1α signal [19]. Therefore, pharmacological strategies designed to inhibit HIF-1α activity may represent a novel approach in cancer therapy.

Ascites tumor cells have long been known to adapt and grow under severe hypoxia, providing a large number of hypoxic cells [20–23]. The cells in ascites are potentially antiapoptotic under extreme hypoxia, under acidosis and at low-glucose levels, recapitulating, at least partly, the malignant feature of cancer cells [24]. In solid tumors, hypoxia is accompanied by a wide variety of conditions, such as low pH and low concentrations of nutrients [25]. Since it is extremely hard to reproduce such complex condition in vitro, malignant ascites provides a unique platform to evaluate hypoxia-targeting drug in vivo [26].

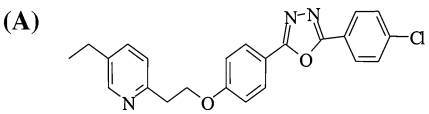
A balance between angiogenic and antiangiogenic factors has given rise to a significant interest in the use of exogenous antiangiogenic agents for the treatment of solid tumors and it has been demonstrated that antiangiogenic treatment retards tumor growth [27].Although several new chemotherapeutic drugs of both synthetic and natural origin are being discovered from time to time, disease like cancer lacks satisfactory solutions. There has been a continuous search for compounds useful in the prevention or treatment of cancer, especially for agents with reduced toxicity. 1,3,4-Oxadiazoles are an important class of heterocyclic compounds with broad spectrum of biological activities.

Compounds possessing oxadiazole moiety show anticancer and tyrosinase inhibitory activity [28]. Substituted 1,2,3-oxadiazoles have revealed antibacterial, [29, 30] antimycobacterial [31], antifungal [32], anti-inflammatory [33], analgesic [34], anticonvulsant [35] and insecticidal properties [36]. Oxadiazoles find use as fluorescent whiteners [37] and also act as muscle relaxants [38]. We have reported earlier the synthesis of new series of 2-{-[2-(5-ethypyridin-2-yl) ethoxy] phenyl}-5-substituted-1,3,4-oxadiazoles using chloramine-T by the oxidative cyclization of hydrazones derived from aromatic aldehyde and aroylhydrazines [39]. Of the many derivatives synthesized, two compounds namely 1a and 1b were used to evaluate the antiproliferative effect in the Ehrlich Ascites tumor model. We report for the first time antiproliferative and antiangiogenic effect of the oxadiazole derivatives in vitro and in vivo by propagation of ascetic transplantable tumors, such as EAT which grow as cell suspensions in the intraperitoneal cavity of mice.

Materials and methods

Chemicals and reagents

Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from GIBCO laboratories, Grand Island, NY, USA. Hydron™ was purchased from Sigma Co., USA. Anti-HIF-1α antibodies were purchased from Santacruz Biotechnology, CA, USA. Oxadiazole derivatives were synthesized at the laboratory of organic chemistry, Department of Studies in Chemistry, University of Mysore, Karnataka, India (Fig. 1). For the biological assays, derivatives were dissolved in DMSO and diluted with distilled water immediately before use. All other reagents were of highest analytical grade.



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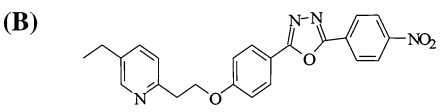

5-ethyl-2- (2-{4-[5-(4-nitrophenyl) [1,3,4] oxadiazole-2-yl] phenoxy} ethyl) pyridine

Fig. 1 Chemical structure of compound 1a and 1b

Cell culture and in vitro cytotoxicity assay

The mouse mammary carcinoma cell line, EAT and the normal human embryonic kidney cells, HEK293 were grown in DMEM supplemented with 10% heat inactivated FBS, 1% penicillin–streptomycin in a humidified incubator (5% CO_2 in air at 37°C). In vitro cytotoxic effect was determined via a modification of the MTT method [40]. Cells were plated in 96-well plates and allowed to attach for 24 h. A concentrated stock solution was prepared in DMSO and was diluted further to get the required concentration of the compounds. Cells in quadruplicate wells were exposed to the individual compounds at 0–300 μM for different time interval (24, 48 and 72 h). The medium was replaced with 100 μL of 1 mg/mL MTT solution diluted in serum free medium in each well. After 4 h of incubation, the medium was removed, and 200 μL of DMSO was added to each well to dissolve the formed formazane crystals. The absorbance was measured spectrophotometrically at 495 nm. The results represent the mean of four independent experiments and are expressed as IC50, i.e. the concentration that reduced by 50% the optical density of treated cells with respect to untreated controls.

Assessment of cell viability by trypan blue exclusion method

Cells (5×10^4 cells/well) were seeded in six well plates prior to oxadiazole derivatives addition. The cells were incubated with different doses (50, 100, 200 and 300 μM) of compounds 1a and 1b along with 1% DMSO as solvent control. Cultures were harvested and monitored for cell number by counting cell suspensions with a hemocytometer. Cell viability was checked before and after treatment with compounds using trypan blue exclusion method.

Alkaline phosphatase (ALP) assay

ALP activity was measured in control and compound treated (0–300 μM) EAT and HEK293 cells. Cells were lysed with 0.2% Triton X-100 and the lysate was centrifuged at 14,000×*g* for 5 min. The clear supernatant was used for the measurement of ALP activity and protein concentration. Enzyme activity was measured at 37°C using *p*-nitrophenyl phosphate as substrate. The amount of *p*-nitrophenol liberated was determined at 405 nm in a MediSpec ELISA reader [41].

[^{3}H] Thymidine uptake assay

To verify for the in vitro effect of compounds 1a and 1b on the proliferation of EAT and HEK293 cells, the cells (5×10^4 cells/well) were cultured in vitro in DMEM medium supplemented with 10% FBS, 1 mg/ml penicillin/streptomycin and were grown in 5% CO_2 atmosphere at 37°C for 2 days. 3[H] thymidine (0.03 MBq/ml of medium) was added prior to addition of the compounds 1a and 1b (50, 100, 200 and 300 μM). After 2 days, the cells were processed for liquid scintillation counting [42].

Animals and cell model

Swiss albino mice, New Zealand rabbit and rats were obtained from central animal facility, Department of Studies in Zoology, University of Mysore, Mysore, India. All the experiments were approved by the institutional animal care and use committee, University of Mysore, Mysore, India. Fertilized eggs were purchased from the government poultry farm, Bangalore, India. EAT cells were obtained from the American type Culture Collection (Rockville, USA). Ehrlich ascites tumor (EAT) cells are murine mammary carcinoma cells which form large volumes of ascites when grown in peritoneal cavity of mice and induce angiogenesis in the peritoneal wall.

Culture of EAT cells in vivo and oxadiazole derivatives treatment

EAT cells were grown in the peritoneal cavity of 6 to 8-week-old Swiss Albino mice by peritoneal transplantation of 0.5 ml of cell suspension (5×10^6 cells) in sterile saline. These cells grow in the 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 12–14 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 the peritoneal wall. To determine whether the compounds inhibit tumor growth and angiogenesis mediated by EAT cells in vivo, compounds [100 mg/kg body wt/ip (300 μM)] were injected into the EAT bearing mice every alternate day after the sixth day of tumor transplantation. Growth of the tumor was monitored by measuring the body weight of the animals. The animals were sacrificed on the 12th day. After harvesting the EAT cells, the abdomen was cut open and the inner lining of the peritoneal cavity of the untreated and the compound treated EAT bearing mice was examined for the extent of neovascularization.

Cell number and ascites volume

Cells along with the ascites fluid were collected from both control and treated animals. The harvested cells were resuspended in PBS and counted using a haemocytometer by trypan blue dye exclusion method. The volume of ascites obtained from control and treated animals was noted.

Mouse survivability analysis

Mouse survival analysis was performed in mice transplanted with EAT cells with and without treatment with the compounds. The animals were divided into three groups (control, compound 1a, compound 1b) with each group consisting of ten animals. All experiments were conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The weights of the mice were monitored everyday upto the life span of the animal.

VEGF–ELISA

The level of VEGF secreted by EAT cells into the peritoneal ascites was measured by ELISA as described in detail by Belakavadi and Salimath [43]. In brief, 100 µl of ascites from control or treated mice was coated in coating buffer at 4°C overnight. Subsequently, wells were washed and blocked with blocking buffer followed by incubation with anti-$VEGF_{165}$ antibodies. Bacterially expressed recombinant $VEGF_{165}$ was used to set up the standard curve. After incubation for 2 h, the wells were washed with PBST before treating with 100 µl of secondary antibodies tagged to alkaline phosphatase. Incubation was continued for 2 h at room temperature and plate was washed prior to the addition of 100 µl of substrate (pNPP). The optical density at 405 nm was measured in a MediSpec ELISA reader.

Chorioallantoic membrane (CAM) assay

Fertilized chicken eggs were incubated at 37°C in a humidified incubator. On the 11th day of development, a circular window was cut in the egg shell and glass coverslips (6-mm diameter) saturated with 25 ng/ml of vascular endothelial growth factor (VEGF) or compounds (10 µg each) were placed on the CAM and the windows were closed using sterile vegetable wrap. The windows were opened after 48 h of incubation and inspected for changes in the microvessel density in the area below the cover slip and photographed using the Nikon digital camera [44].

Rat corneal angiogenesis assay

Hydron Polymer (polyhydroxyethyl-methacrylate (poly HEMA), Sigma, St. Louis, MO, USA) was dissolved in ethanol to a final concentration of 12%. A 5-µl aliquot of this mixture was then pipetted onto Teflon pegs. Saline (group 1) or 1 µg of VEGF (group 2) or VEGF + compounds (10 µg) was added to each pellet and allowed to dry under a laminar flow hood at room temperature for 2 h. The pellets were incubated at 4°C overnight. All procedures were performed under sterile condition. Swiss albino rats weighting 300–350 g were anaesthetized with intraperitoneal injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). A drop of 0.5% proparacaine was instilled to the eye and the globe was proposed using a pair of 0.3 mm tissue forceps. Using a surgical microscope, a paracentral linear incision 1 mm from the center of the cornea, 1.5 mm in length and 50% of the corneal depth was made with a No. 11 surgical blade. To create a corneal micropocket, the incision was bluntly dissected through the stroma to the limbal area using a curved iris spatula. A single pellet was advanced into the lamellar pocket to the limbus using corneal forceps. Postoperatively, gentamicin ointment was applied to the anterior surface of the operated eye. The rats were observed for 24–72 h for the occurrence of non-specific inflammation and for localization of the pellets. On day 7, the rats were anaesthetized with ketamine and the corneas were observed under steriobinocular microscope with CCD camera and photographed [45].

Western blot analysis of HIF-1α

EAT cells (1×10^5 cells/60 mm dish) were treated with the compounds 1a and 1b in vivo. Cells were homogenized in a lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulphonyl fluoride, 10 µg/ml leupeptin and 0.5 mM dithiothritol) for 30 min on ice. The cell debris was pelleted by centrifugation at $10{,}000 \times g$, 4°C for 30 min. For the preparation of nuclear extract, the cells were extracted in a buffer containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, 0.25 M sucrose, 1 mM PMSF, 2 mg/ml leupeptin and 10 mg/ml aprotinin. About 60 µg of nuclear and/or cytosolic proteins as estimated by Lowry's method were separated on 12.5% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% non-fat skimmed milk powder in Tris-buffered saline, the membranes were incubated with antibodies against HIF-1α. The blots were washed thrice with TBST on a shaker and incubated with secondary antibody tagged to alkaline phosphatase for 2 h. After washing with TBST for 10 min, the HIF-1α proteins were detected by using the chromogenic substrate BCIP/NBT.

FACS analysis

For the determination of cell cycle phase distribution and cell proliferation, EAT cells were either untreated or treated with the compounds in vivo. Cells were collected, centrifuged and washed twice with PBS at room temperature. The cell pellets were resuspended in ice-cold 70% ethanol for overnight fixation at 4°C. The suspension was centrifuged and the cell pellet was resuspended in 0.8 ml PBS containing 1 µl of RNase (10 mg/ml) and 20 µl of PI

(1 mg/ml) and incubated for 30 mins at 37°C before analysis by flow cytometry. A total of 10,000 events were acquired and analysis of flow cytometric data was performed using ModFit software. A histogram of DNA content (*x*-axis, PI fluorescence) versus counts (*y*-axis) has been displayed.

Transient transfection and luciferase assay

To determine the effect of oxadiazole derivatives on EAT cells, cells were transfected with 2 μg of VEGF promoter-luciferase reporter constructs containing the 5′ flanking region (−2,068 bp) of the human VEGF gene promoter coupled to the promoterless luciferase reporter gene vector pCDNA3 and 2 μg of the *β* galctosidase expression vector *β*-Gal. Transient transfection assays were performed using calcium phosphate transfection kit according to the manufacturer's instructions. In brief, 2×10^5 cells were seeded in six-well plates and cultured to 60–70% confluency. The transfected cells were cultured further for 20 h followed by incubation with or without compounds (50, 100, 200 and 300 μM). Cells were washed once with phosphate buffered saline (PBS) and lysed with reporter lysis buffer. Luciferase (Luc) activity of the cell extract was determined using the luciferase assay system. Beta galactosidase (*β*-Gal) activity was determined by measuring hydrolysis of *O*-nitrophenyl *β*-D-galactopyranoside using 50 μl of cell extract at 37°C for 2 h. Absorbance was measured at A_{405}. Luciferase activity was determined using 50 μl of cell extract. The reaction was initiated by injection of 100 μl of luciferase assay substrate. Relative Luc activity (defined as VEGF reporter activity) was calculated as Luc (relative light units per 50 μl cell extract)/*β* gal activity (A_{405} per 50 μl cell extract per 2 h).

Statistical analysis

Data are expressed as mean ± SE and all statistical analysis were performed by using SPSS10.0. Comparisons among all groups were performed with the one-way analysis of variance (ANOVA) test. Statistical significance of differences between control and treated tumor cells were determined by Tuckey's post hoc test. Statistical significance was defined as $P < 0.05$ for all the tests. All experiments were performed in triplicates.

Results

Effect of compounds on cell viability in vitro

Viability of the cells was measured by both MTT and trypan blue dye exclusion method. MTT assay is a standard colorimetric assay to determine cytoxocity of potential compounds. To investigate the potential effects of compounds 1a and 1b on proliferation and survival of EAT cells and the untransformed HEK293 cells, the cells were exposed to 0–300 μM of the compounds for different time interval (24, 48 and 72 h). In this respect compounds 1a (Fig. 2a) and 1b (Fig. 2b) were totally selective for the cancer cells without any inhibitory effect on normal HEK293 cells at IC50. The IC50 value of compound 1a was found to be 170.06 μM and compound 1b was 141.6 μM. The viability of the control and treated cells was further confirmed by trypan blue dye exclusion method (Fig. 3a, b). Results show that the derivatives are potent growth inhibitors of EAT cell lines tested in this experiment. On the contrary, the untransformed normal HEK 293 cells were relatively resistant at this range.

Effect of compounds on ALP activity

Figure 4a and b depicts the effect of the compounds 1a and 1b on the ALP activity in the EAT and HEK293 cells. There was a significant decrease in the activity of ALP in EAT cells in a dose dependent manner. However, the normal HEK293 cells showed no significant effect on the ALP activity.

Antiproliferative effect of compounds

To gain insights into the antiproliferative effect of compounds on EAT and HEK 293 cells we performed a [^{3}H] thymidine incorporation assay. Significant dose dependent growth inhibition of EAT cells was observed on treatment with compounds. The results shown in Fig. 5a and b clearly indicates that compounds inhibit proliferation in a dose dependent manner when compared to 100% proliferation of EAT cells in vitro in the absence of compounds. However, the effect of the compounds on the normal HEK 293 cells was much smaller than on EAT cells.

Compounds inhibit growth of EAT cells and decrease the ascites secretion in vivo

In an attempt to understand the in vivo effect of compounds on proliferation of EAT cells, Swiss Albino mice were treated with compounds 1a and 1b. It was found that compounds showed significant antiproliferative effect towards EAT cells in vivo as monitored by the body weight as shown in Fig. 6a. In contrast, the weights of untreated EAT bearing mice steadily increased and they died in normal growth period of 13–14 days. Further, we tested the effect of the compounds on the survival of EAT bearing animals. Intraperitoneal transplantation of EAT cells (5×10^6 cells/mice) resulted in survival of the mice for 14 days with an increase in the number of cells and ascites volume. The

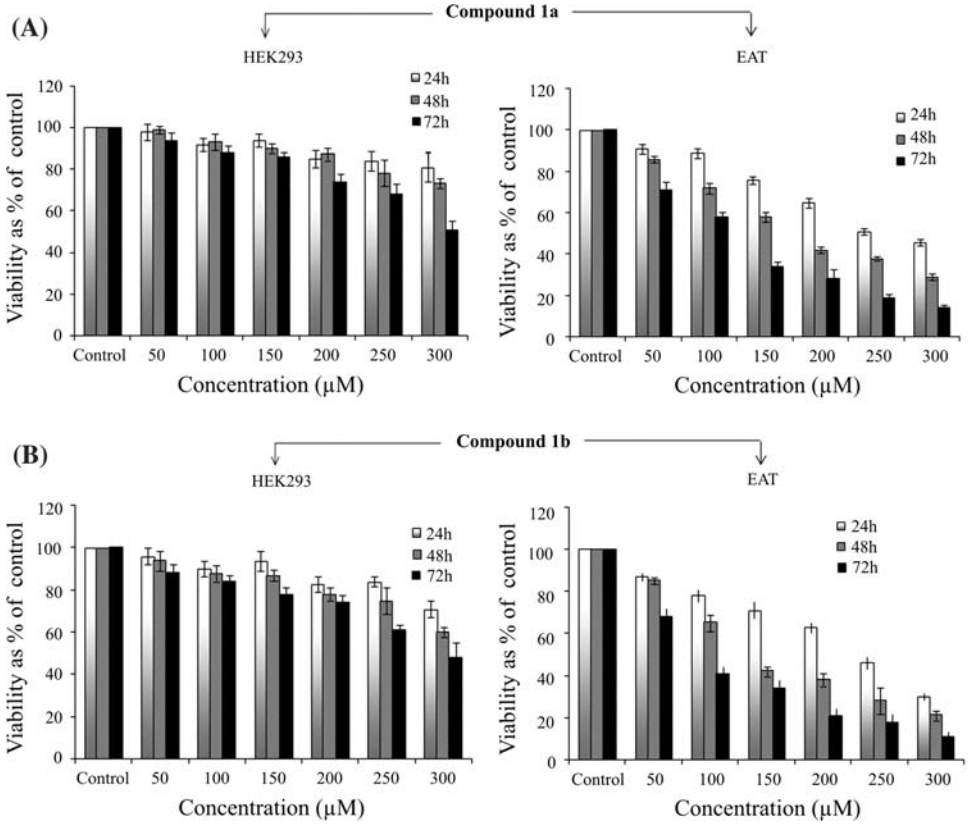

Fig. 2 Cytotoxic effects of compounds. EAT and HEK293 cells were treated with various concentrations (0–300 μM) of the **a** compound 1a and **b** compound 1b for different time intervals. Cell viability was examined with MTT assay and expressed as a percentage of the control. The results represent the mean ± SEM of four independent experiments and are expressed as IC50. Effect of compound1a and compound1b are statistically significant in comparison with control ($P < 0.001$)

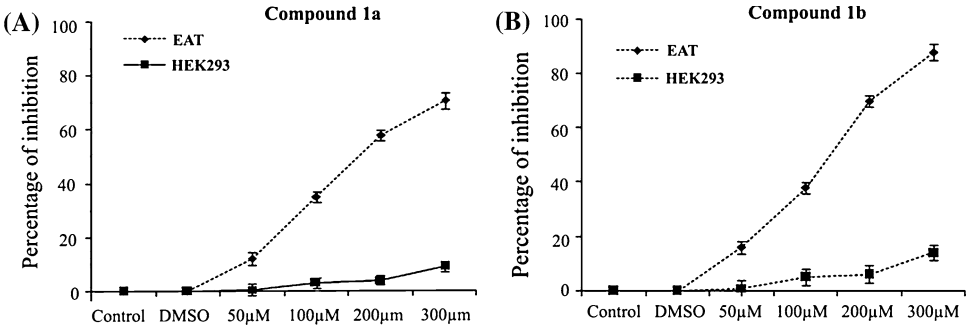

Fig. 3 Effect of compounds treatment on cell viability by trypan blue exclusion method. Cells (EAT and HEK293) were treated with 50, 100, 200 and 300 μM solution of **a** compounds 1a and **b** compound 1b along with 1% DMSO as solvent control. Results are presented as mean ± SEM of three assays. Effect of compound 1a and compound 1b are statistically significant in comparison with control ($P < 0.001$)

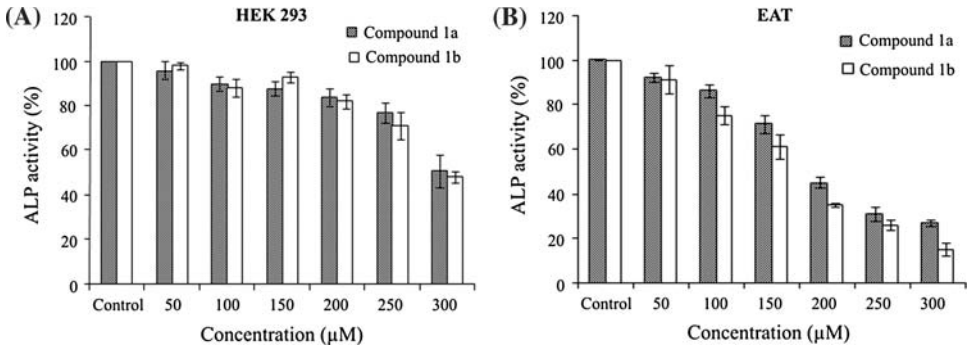

Fig. 4 Effect of the compounds on the alkaline phosphates activity. **a** HEK 293 and **b** EAT cells were treated with different concentrations of the compounds. Data shown are mean ± SEM, expressed as percentage of control. Effect of compound 1a and compound 1b are statistically significant in comparison with control ($P < 0.001$)

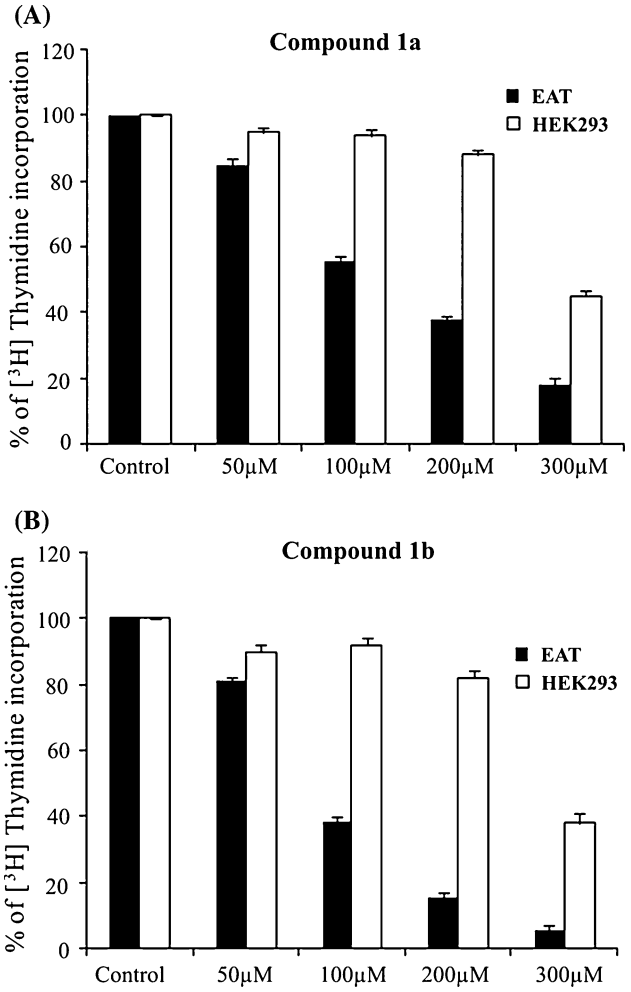

Fig. 5 Antiproliferative effect of the compounds. EAT and HEK293 (5×10^4) cells were plated in a six well plate and processed for antiproliferation activiy of compounds in a dose dependent manner (50, 100, 200 and 300 μM) using [^{3}H] thymidine (0.03 MBq/ml of medium). The data represents the mean ± SEM of three independent experiments. Effect of **a** compound 1a and **b** compound 1b is statistically significant in comparison with control ($P < 0.001$)

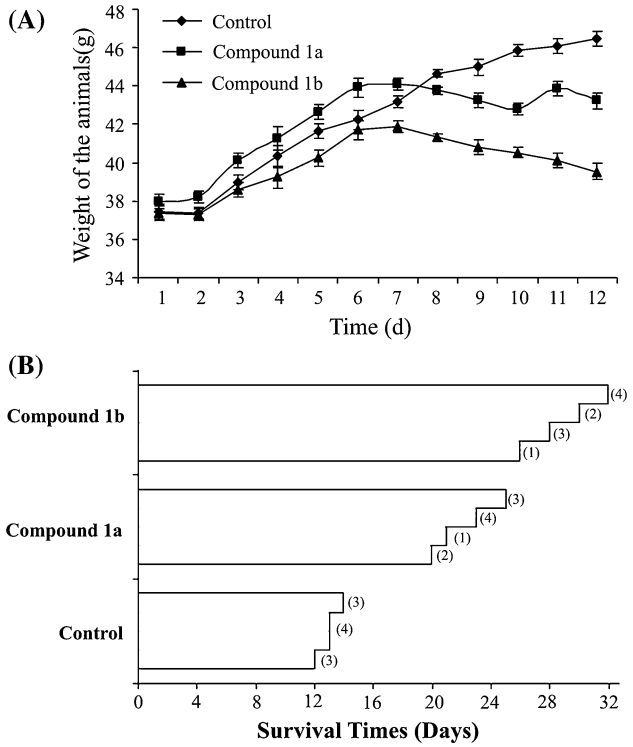

Fig. 6 Effect of the compounds on growth of EAT cells in vivo. **a** EAT cells (5×10^6 cells/mouse, i.p.) were injected into mice and weight of the mice was recorded. After sixth day of the tumor inoculation, compounds (300 μM) were injected i.p. every day till 12th day and body weights of EAT bearing mice treated with or without compounds were recorded from first day. A minimum of three mice was used for the experiment and results obtained are an average of three individual experiments. The data represent the mean ± SEM of three independent experiments. Effects of compounds are statistically significant in comparison with control ($P < 0.001$). **b** Survival studies of EAT bearing mice treated with the compounds. EAT bearing mice were treated with the compounds and continued up to 32nd day. Death of mice was checked daily and report was recorded daily. The mice were divided into three groups with ten mice in each group. The experiment was repeated thrice and the data are presented as mean from three different experiments and means of ±SEM. The number on the *bars* represent the number of mice that died

animals succumb to death upon tumor burden by 14 days after tumor transplantation. Treatment with the compounds extended the survival time of the EAT bearing mice from 14 days up to 32 days (Fig. 6b).

The total cell numbers from each group of mice either treated or untreated with the compounds as counted by the trypan blue dye exclusion method are shown in Fig. 7a, which confirmed the cell growth inhibition by compounds. Since EAT cells grow as an ascites tumor, we measured the volume of ascites secreted from the control group and the group that received compounds treatment. About 85% of ascites secretion was inhibited upon compounds treatment as shown in Fig. 7b resulting in the reduction in body weight of the animals. The result showed that compound 1b was the effective inhibitor of tumor growth in comparison to compound 1a.

Compounds inhibit VEGF production by EAT cells

Increase in peritoneal angiogenesis in tumor bearing mice is attributed to the presence of VEGF in the ascites secreted by EAT cells. Quantification of VEGF in the ascites of either treated or untreated mice indicated that the compounds interfere with the production of VEGF in vivo in EAT bearing mice. Figure 8, compares the amount of VEGF for untreated and compounds treated EAT cells. The amount of VEGF production is increased in the ascites of untreated cells as compared to the decrease in amount of VEGF production observed in the ascites of EAT bearing mice treated with compounds.

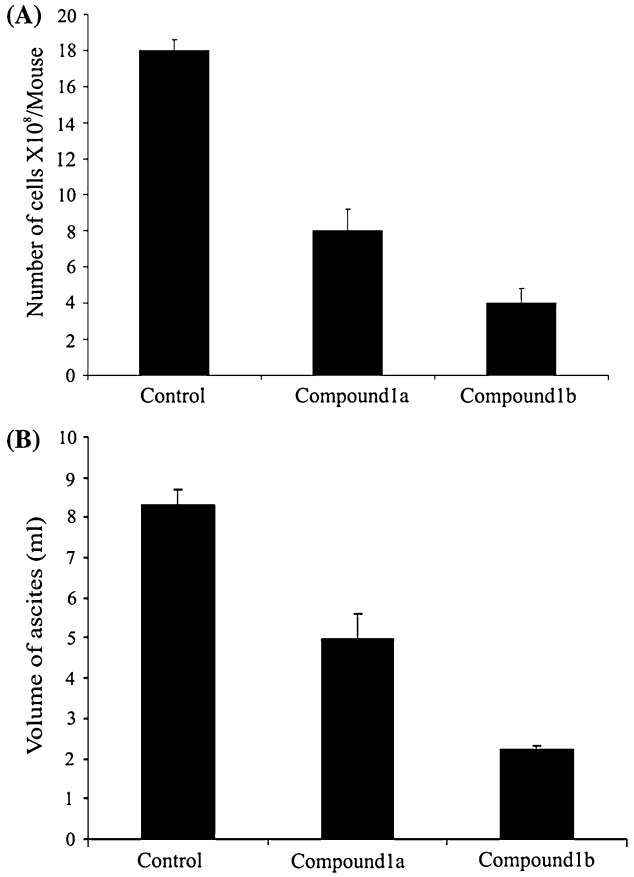

Fig. 7 Effect of the compound on EAT cell number and ascites volume. EAT bearing mice treated or untreated with compound 1a and 1b were sacrificed on the 12th day. EAT cells along with ascites fluid were harvested and ascites volume was recorded. Number of cells per mouse was determined by counting the cells in a haemocytometer. The data represent the mean ± SEM of three independent experiments. Effect of the compounds is statistically significant in comparison with control ($P < 0.001$)

When compared to control, approximately 40% inhibition is seen in the amount of protein expressed in compound 1a and 1b treated cells, respectively, treated. F test showed a significant difference between amount of VEGF and different compounds ($f = 9193.409$; $P < 0.001$) where amount of VEGF were highest in control tumor bearing mice ascites.

Suppression of peritoneal angiogenesis by compounds

To evaluate the effect of the compounds on peritoneal angiogenesis, the animals were sacrificed on the 13th day. The animals were dissected to observe the effect of compounds on the extensive angiogenesis. Results in Fig. 9a clearly indicate extensive angiogenesis in the peritoneum of control EAT bearing mice. Mice treated with different compounds (1a and 1b), however, showed significant inhibition of in vivo angiogenesis in the peritoneum of tumor bearing mice.

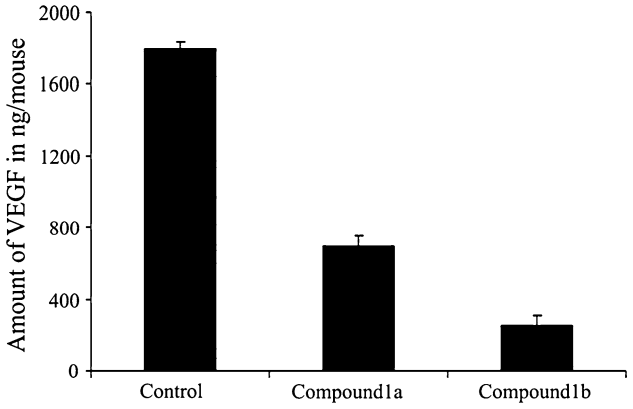

Fig. 8 Effect of compounds on in vivo production of VEGF. EAT bearing mice either untreated or treated with compound 1a and 1b were sacrificed and the ascites was collected. Sandwich ELISA was carried out to quantitate VEGF in ascites fluid using anti-$VEGF_{165}$ antibodies. The data represent the mean ± SEM of three independent experiments. Effects of compounds are statistically significant in comparison with control ($P < 0.001$)

Antiangiogenic effect of compounds in vivo

Chorioallantoic membrane assay and rat cornea assay are the standard angiogenesis assays used for validation of angioinhibitory activity of any compound. In vivo angioinhibitory effect of compounds was clearly evident from results obtained in CAM assay. Results as shown in Fig. 9b exhibit reduction of angiogenesis in the CAM at the site of application of compounds as compared to the extensive angiogenesis seen in the normal CAM. The data shown represents the results using a minimum of five eggs in each group. In order to evaluate the efficacy of the compounds on corneal angiogenesis, we performed a rat cornea assay. Results in Fig. 9c, indicate that hydron pellets containing saline failed to stimulate an angiogenic response in rat corneas 5–7 days after implantation. A strong angiogenic response was seen with the pellet containing recombinant VEGF (1 μg/μl). This angiogenic response was completely blocked by addition of compounds to the pellet containing VEGF.

Compounds inhibits the nuclear translocation of HIF-1α

In order to investigate the underlying mechanism of antiangiogenesis of compounds we studied the expression of transcription factor HIF-1α known to be responsible for VEGF regulation. We observed that in vivo treatment of compounds expressed HIF-1α protein levels in the cytosolic fraction than in the nuclear extract where as in the untreated, increased expression of HIF-1α in nuclear extracts than in the cytosol indicating inhibition of nuclear translocation of HIF-1α (Fig. 10).

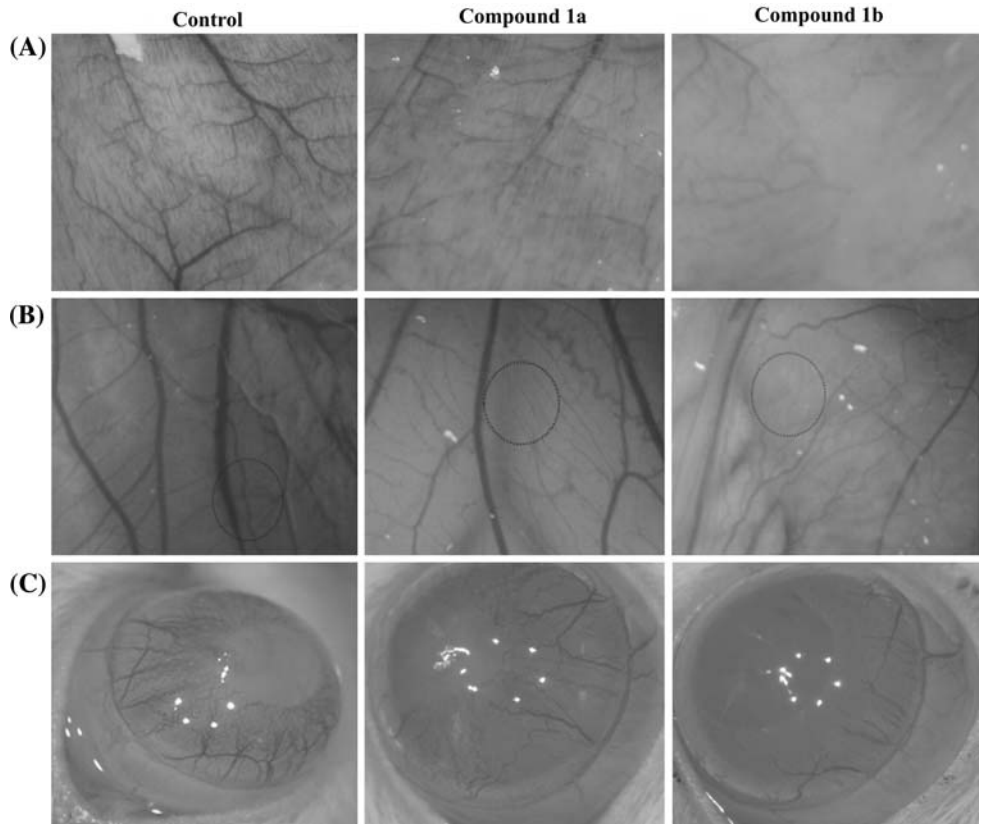

Fig. 9 Inhibition of angiogenesis in vivo. **a** Mice either treated or untreated with compound 1a and 1b were sacrificed on the 12th day and dissected to observe the effect of compounds on peritoneal angiogenesis. Inhibitions of growth of new blood vessels by the compounds are evident. Typical result from three independent experiments is shown. **b** Inhibition of angiogenesis in Chorio Allantoic Membrane. PBS, VEGF or Compounds (200 μM) were applied on the CAM of the 11th day old chick embryo and the window was sealed. The window was reopened on the 13th day and the applied area was inspected for changes in vessel density. **c** Inhibition of angiogenesis in cornea of the rat. Hydron polymer, VEGF or VEGF with compounds were implanted into the cornea of the rat. On the seventh day the cornea were inspected for the inhibition of VEGF induced corneal angiogenesis by incorporation of compounds in the hydron polymer. The values are shown as mean ± SEM of three independent experiments. Effect of compounds are statistically significant in comparison with control ($P < 0.001$). Typical results from three independent experiments are shown

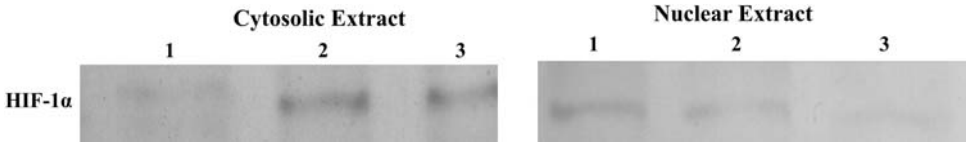

Fig. 10 Effect of the compounds on nuclear translocation of HIF-1α. Western blot analysis of HIF-1α expression in cytosolic extracts and nuclear extracts of EAT cells was performed using anti HIF-1α antibodies. Nuclear extracts were prepared from both control and compounds treated cells. 50 μg of protein sample was resolved on 12% SDS-PAGE and transferred to nitrocellulose membrane. HIF-1α protein was detected using anti-HIF-1α rabbit polyclonal antibody. Data were represented as mean ± SEM of three independent experiments

Effect of compounds on cell cycle progression in EAT cells

To elucidate the possible mechanism of compounds mediated tumor inhibition, we investigated the effect of compounds on EAT cells in vivo. We found that treatment of compounds on EAT cells induced a significant proportion of cells to undergo apoptosis, as determined by the flow-cytometric analysis. Cell cycle analysis by flow cytometry was used to quantitatively estimate the number of cells in each phase of cell cycle. The untreated cells (control) showed a typical distribution in G1, S, and G2 phases on flow cytometry. After treatment, number of cells in sub G0 area increased by 60% (Fig. 11). These results suggested the antiproliferative and antitumor effects of compounds on EAT cells. The obvious ramifications were the growth arrest of EAT cells.

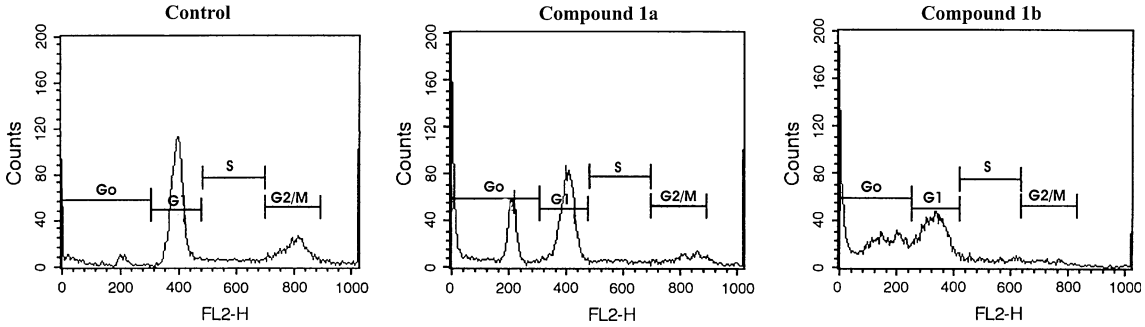

Fig. 11 Analysis of cell cycle phase distribution in EAT cells by compounds. EAT cells from tumor bearing mice, either untreated or treated with compounds 1a and 1b were fixed in 70% ethanol and the nuclear DNA was labeled with Propidium Iodide (PI). Cell cycle phase distribution of EAT nuclear DNA was determined by single label flow cytometry. Histogram display of DNA content (*x*-axis, PI-fluorescence) versus counts (*y*-axis) has been shown. Each plot is representative of three similar experiments

Down regulation of VEGF gene expression by oxadiazole derivatives

To determine whether compounds modulate VEGF gene expression, we tested the effect of compounds on VEGF promoter luciferase reporter gene analysis. The results indicated that when compared to normal untransformed HEK 293 cells, the activity of VEGF gene expression is higher in EAT cells. Compared to the basal expression of VEGF in untreated EAT cells, a repression of upto 80% was observed in compounds treated cells in a dose dependent manner (Fig. 12a, b), while a maximum of 25% inhibition of VEGF gene expression was seen in HEK 293 cells.

Discussion

In vivo experimental studies have demonstrated that tumor growth and metastasis are dependent on angiogenesis [46, 47]. It is well known that ascites tumor growth including EAT cells are angiogenesis dependent [48]. Ehrlich ascites tumor (EAT) cells are murine mammary carcinoma cells which form large volumes of ascites when grown in peritoneal cavity of mice and induce angiogenesis in the peritoneal wall. In the present study, we studied the antiangiogenic and antiproliferative effects of oxadiazole derivatives on EAT cells. The results reported herein reveal that the compounds exert antiangiogenic and antiproliferative properties and inhibit growth of cultured EAT cells. In this report we have focused our attention on delineating the antiangiogenic activity of two new substituted oxadiazole derivatives.

Chemotherapy very often induces severe side effects, which are in part a consequence of destruction on normal cells. As a complementary part for this study, it was thought worthwhile to evaluate the differential cytotoxic effect of the oxadiazole derivatives on both cancerous and normal cells. MTT assay is a standard colorimetric assay for measuring cell growth. It is used to determine cytotoxicity of potential medicinal agents and other toxic materials. The antiproliferative activity of the two compounds under study may be explained by its inhibitory action on proliferation of tumor cells in vitro in a dose and time dependent manner (IC50 = 140–175 μM) as compared to the little effect on the untransformed HEK293 cell line. In our study, the addition of the compounds to EAT cells resulted in decline in the activity of ALP. The compounds under study did not exhibit any toxic effect as evidenced by the prolonged life span of the animals transplanted with the EAT cells receiving treatment. The animals receiving treatment with the compounds showed an increased life span of 32 days as compared to the control group that succumbed to the tumor burden within 14 days. Of the two derivatives compound 1b proved to be more potent antiangiogenic and antiproliferative when compare to compound 1a. This may be attributed to the presence of NO_2 group at para position of compound 1b.

As in normal angiogenesis tumor angiogenesis appears to rely heavily on VEGF. VEGF plays fundamental role in the fluid accumulation and tumor growth in ascites tumor. By secreting VEGF, ascites tumor enhances the permeability of preexisting microvessel lining of peritoneal cavity to stimulate ascites formation thereby tumor progression [49]. Inhibition of fluid accumulation and tumor growth by neutralization of VEGF has been demonstrated, underlining the importance of VEGF in malignant ascites formation [14, 50]. According to this view the elimination of VEGF may inhibit the ascites tumor. Quantification of growth factor VEGF by ELISA in our studies indicated a decreased amount of VEGF in ascites of the compounds treated animals. The in vivo effect of compounds on proliferation of endothelial cells is especially visible when observing blood vessel formation in CAM and peritoneum of mice. Our results show that there was peritoneal angiogenesis in mice bearing ascites tumor cells, which was due to the secretion of VEGF there by accumulation of ascites fluid. Since there

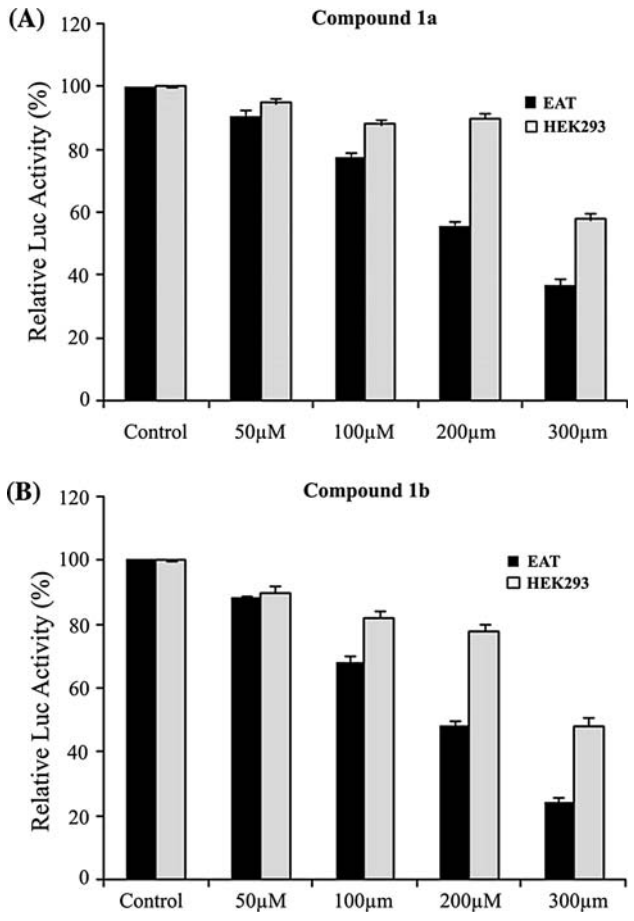

Fig. 12 Effect of compounds on VEGF Promoter Activity. EAT and HEK 293 cells were transiently transfected with 2 μg of VEGF promoter-Luciferase reporter construct. Cells were treated with different concentrations of the compounds. Forty-eight hours later, cells were assayed for luciferase activity. **a** Compound 1a and **b** compound 1b repressed VEGF promoter activity in a dose dependent manner in EAT cells. The data represent the mean ± SEM of three independent experiments. Effects of compounds are statistically significant in comparison with control ($P < 0.001$)

is inhibition of angiogenesis by compounds, it supports the view that compounds may repress the expression of VEGF, thereby inhibiting the accumulation of ascites fluid, formation of new blood vessel and tumor growth which are angiogenesis dependent. Further, the role of the oxadiazole derivatives on the regulation of VEGF expression was investigated at the gene level. This finding is in agreement with the repressing effect of the oxadiazole derivatives on endogenous VEGF expression. In transient transfection assays, we could see that the oxadiazole derivatives downregulated the VEGF promoter activity in EAT cells in a dose dependent manner. This result suggests that transcriptional repression of the VEGF gene represents the mechanism by which the oxadiazole derivatives down regulates VEGF expression.

Inhibition of VEGF secretion could be due to inhibition of activity of transcription factors, which are involved in the regulation of VEGF gene expression. Angiogenesis is often stimulated by hypoxic conditions such as those that can occur during tumor growth. It is well known that the microenvironment of ascites tumor models including EAT cells in vivo is hypoxic [20–23] and increased expression of HIF-1 α and VEGF secretion in these model systems is mainly due to hypoxic environment [23, 48]. HIF-1α is a hypoxia-activated transcription factor that can regulate VEGF synthesis. HIF-1α activity is dependent upon the level of available inhibitor of HIF-1α that halts tumor growth by blocking tumor angiogenesis and tumor adaptation to hypoxia, making this component another good antiangiogenic target [51]. A solid tumor is highly heterogeneous in terms of its microenvironment, including properties such as oxygen level, pH and concentration of nutrients [52]. Efficacy and drug delivery are the major obstacles to be overcome in developing a tumor hypoxia therapy for solid tumors [53]. In comparison with this complexity, cultured hypoxic conditions might be too simplified to test hypoxia-targeting drugs appropriately. In contrast malignant ascites are characterized by conditions that include hypoxia, low-pH and low-glucose concentrations and it provides large number of homogeneous hypoxic cells that are adapted to hypoxia. Moreover, host response to hypoxia-targeting therapy can be observed [23]. However, to date, only few antitumor agents targeting HIF-1α has been reported in ascites tumor cells in vivo [23, 26]. Here, we show that the oxadizole derivatives have the potential to become the antiangiogenic agent to target HIF-1α. The present study showed that the oxadizole derivatives may influence angiogenesis by down regulating the expression of nuclear HIF-1α while increasing its abundance in the cytosolic fraction of EAT cells. We also demonstrated that oxadiazole derivatives inhibits the expression of VEGF in EAT cells. This was due to the consequence of inhibitory activity of HIF-1α. Angiogenic processes controlled by VEGF like peritoneal angiogenesis, ascites formation and tumor growth were also subsequently impaired. Our results clearly demonstrate increased expression of HIF-1α in cytosolic fraction in oxadiazole derivatives treated cells as compared to the decreased expression of HIF-1α in nuclear extract.

Taken together our data clearly indicates that the oxadiazole derivatives are potent antiangiogenic drugs that inhibit not only tumor growth but also angiogenic properties of Ehrlich ascites tumor cells. These effects are likely to be mediated by inhibition of activity of transcription factor like HIF-1α, by inhibiting its nuclear translocation. Consequently reduced nuclear presence and activity of HIF-1α could be responsible for inhibition of hypoxic up regulation of VEGF gene expression, resulting in the decreased ascites and decreased microvessel density and there by tumor growth which is angiogenesis dependent. Such oxadiazole derivatives may prove to be potential antiangiogenic drugs

which could be further developed and translated into a therapeutic regime for treatment of human cancer where formation of peritoneal malignant ascites is a major cause of morbidity and mortality. The above study sheds light on the identification of new antiangiogenic molecules to cancer therapy. Further research to know the mechanism of inhibition and the modification of the oxadiazole derivatives to improve the potency is currently under progress in our laboratory.

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