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In vivo peritoneal antiangiogenesis and in vitro antiproliferative properties of some bischalcone derivatives

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Abstract In this study, three, bisarylidene cyclopentanones (curcumin analogs) **3a–c** are synthesized by Claisen Schmidt condensation reaction. Antiangiogenic effects of the compounds were studied in Ehrlich ascites tumor (EAT) cells transplanted mouse in vivo. Antiangiogenic effect of **3a–c** showed reduction in ascites volume, cell number, and induced apoptotic bodies in EAT cells in tested animals. The antiproliferative effects of the **3a–c** were determined at different concentrations by MTT assay on HepG2 and HeLa cells. The compounds showed significant antiproliferative activity at 40 μM concentration. Compound **3a** (fluoro derivative) showed highest antiproliferative effect with the IC50 value 39 and 48 μm for HeLa and HepG2, respectively. Growth inhibition of the HeLa cells and antiangiogenesis was mediated by promoting apoptosis which was confirmed by DNA fragmentation study.

Keywords Bischalcone derivatives · Antiangiogenesis · Antiproliferative · MTT assay · Apoptosis

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Introduction

Angiogenesis, the formation of new blood vessels from pre-existing ones, takes place in various physiological and pathological conditions, such as embryonic development, wound healing, the menstrual cycle, chronic inflammation, and tumors development. Tumor angiogenesis is linked to a switch in the equilibrium between positive and negative regulators and mainly depend on the release by neoplastic cells of growth factors specific for endothelial cells and it is able to stimulate growth of host's blood vessels (Ribatti and Vacca, 2008). A growing tumor needs an extensive network of capillaries to provide nutrients and oxygen. The inhibition of tumor angiogenesis using novel antiangiogenic agents has gained increasing importance in cancer research and it has been demonstrated that different antiangiogenic therapies retarded experimental tumor growth (Brustmann and Naudé, 2002). In animal models, antiangiogenic dienone cyclopropoxy curcumin analogs have proven very successful in inhibiting tumor development (Chandru *et al.*, 2007). In normal cell growth, the balance between cellular proliferation and apoptosis is maintained; however, in the cancer cells, this balance gets out of control abnormal proliferation increases, thereby inducing the tumor growth. Therefore, the induction of apoptosis could be important to the cancer chemotherapy. Apoptotic inducers are screened to discover anticancer drugs (Lim *et al.*, 2007).

Curcumin, the active principle in turmeric powder, was subsequently demonstrated to be anti-inflammatory and an antioxidant (Ammon and Wahl, 1991). Curcumin inhibits several signal transduction pathways including those involving protein kinase C, nuclear factor-kappa B (NF-kB), phospholipase A2 bioactivity, arachidonic acid metabolism, and epidermal growth factor (EGF) receptor autophosphorylation (Lu *et al.*, 1994; Singh and Aggarwal, 1995; Huang

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et al., 1991: Korutla *et al.*, 1995; Rao *et al.*, 1993). Curcumin (along with two natural analogs, dimethoxycurcumin and isodemethoxycurcumin) has entered into Phase I clinical trials for chemoprevention by the National Cancer Institute (Kelloff *et al.*, 1996). It has been recently shown that curcumin induces apoptosis in Ehrlich ascites carcinoma cells by up-regulation of Bax, release of cytochrome c and activation of caspase-3 (Suman *et al.*, 2001). Synthetic chemical modifications of curcumin have been studied intensively in an attempt to find a molecule with similar but enhanced properties of curcumin. It was reported that a series of novel curcumin analogs were synthesized and screened for anticancer activity. New analogs that exhibit growth-suppressive activity 30 times that of curcumin and other commonly used anticancer drugs were identified (Ohori *et al.*, 2006). Moreover, bisarylidene cyclopentanones belong to novel class of compounds called chalcones and it was reported that chalcone derivatives exhibited good angiogenesis inhibitor activity and also antitumor/anticancer property (Bowen *et al.*, 2008). In the present study three bischalcones **3a–c** were taken to investigate the antiangiogenic effects on Ehrlich ascites tumor (EAT) in vivo and compounds were subsequently tested for their in vitro antiproliferative properties using HepG2 and HeLa cell lines.

Materials and methods

Synthesis of bischalcones

Synthesis of *bischalcones* (2*E*,5*E*)-2,5-bis(4-fluorobenzylidene)cyclopentanone (**3a**), (2*E*,5*E*)-2,5-bis(3,4-dimethoxybenzylidene) cyclopentanone (**3b**), and (2*E*,5*E*)-2,5-bis(3-methoxy-4-hydroxybenzylidene) cyclopentanone (**3c**) were carried out according to the reported method (Vogel's Text book, 1989; Butcher *et al.*, 2007).

Preparation and administration of drug to EAT bearing mice

Inbred Swiss albino mice, 6–8 weeks old, weighing 30 ± 5 g of either sex, were used for the experiments. They were inbred and maintained in the animal house, Department of Zoology, Manasagangotri, Mysore, India. EAT cells were grown in adult Swiss albino mice intraperitoneally. Synthetic bischalcones **3a–c** were examined in this experiment. The compounds were dissolved in 0.1% dimethyl sulfoxide (DMSO) and administered to mice after 7th day of tumor transplantation to mice on the advanced stage of tumor cells when they enter into exponential growth period. The control group was administered with DMSO.

In vivo growth of EAT cells and peritoneal angiogenesis assays

EAT cell growth

EAT cells (5×10^6 cells/mouse) were injected intraperitoneally (i.p.) to 8–10 weeks old Swiss albino mice. After 6 days of tumor inoculation, 110 μg/ml body weight of compounds **3a–c** were injected intraperitoneally into EAT bearing mice every alternative day and the mice were sacrificed in batches on 13th day. Body weight was monitored daily throughout the experimental period.

Cell number and ascites volume

Either control or compounds treated EAT bearing mice were sacrificed on 13th day and a small incision was made in the abdominal region. EAT cells along with the ascites fluid were harvested into a beaker containing 2 ml saline and centrifuged at 3000 rpm for 5 min at 4°C. Subtracting the volume of saline added previously from the volume of the supernatant, gave the volume of ascites fluid. After harvesting, the EAT cells were resuspended in PBS and counted using a haemocytometer (Thippeswamy and Salimath, 2007).

Giemsa, ethidium bromide/acridine orange staining

The harvested EAT cells were smeared on clean glass slide and fixed in methanol and acetic acid and stained with Wright's Giemsa stain.

Nuclear staining was performed according to the method previously described. In brief, EAT cells of control and compounds **3a–c** treated mice were smeared on a clean glass slide, fixed with methanol/acetic acid (3:1), and air dried in a humidified chamber. The cells were hydrated with phosphate-buffered saline (PBS) and stained with a mixture of ethidium bromide/acridine orange (1:1) (10 mg/ml) solutions. The cells were immediately washed with PBS thrice and viewed under Leitz-DIAPLAN fluorescence microscope (Thippeswamy and Salimath, 2007).

Antiangiogenic effects of the compounds

Peritoneal angiogenesis The peritoneum of the mice was cut open and the inner lining of the peritoneal cavity were examined for angiogenesis in both control and compound (**3a–c**) treated tumor cells docked mice and photographed (Chandru *et al.*, 2007).

DNA fragmentation studies

The harvested EAT cells from control and compound treated mice (in vivo) and Hep G2 cells were treated with

10 μM compound for 24 h (in vitro) were lysed in a lysis buffer containing 50 mM Tris–HCl, pH 8.0 and 0.5% SDS, and incubated for 30 min at 37°C. The cell lysate was subjected to 8 M potassium acetate precipitation and left for 1 h at 4°C. The supernatant was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), followed by chloroform extraction. DNA was precipitated by adding 1:2 volumes of ice-cold ethanol. The precipitated DNA was digested with 20 μg/ml RNAse at 37°C for 30 min. The DNA was quantified and resolved on 1.5% agarose gel, viewed under UV-light and documented using UVP-Bio Doc It TM system (Thippeswamy and Salimath, 2007).

Antiproliferative studies

Cell culture and in vitro cytotoxicity assay (MTT assay)

HepG2 and HeLa cell lines were cultured and cytotoxicity assay was done, Cells were treated with compounds **3a–c** at a concentration range of 10–40 μM, respectively, to 96-well plates in antibiotic free RPMI medium containing 10% fetal calf serum. Drug treatment lasted 48 h in 5% CO_2 air atmosphere at 37°C with high humidity. After 48 h. 50 μl of 1 mg/ml solution of MTT in RPMI-1640 medium was added to each well. The culture plates were gently shaken and incubated for another 4 h. MTT was removed carefully and DMSO (100 μl) was added and shaken well. The absorbance was measured at 570 nm in an automated plate reader and the percentage of cells growth inhibition was calculated using the following formulae (Cetin and Bullerman, 2005).

$$\text{Inhibitory rate}\,\% = \text{Absorption control} - \text{Absorption test}/\text{Absorption test} \times 100$$

Determination of IC50

IC50 of compounds **3a–c** were determined by plotting of percent cytotoxicity index (Cetin and Bullerman, 2005).

Assessment of cell viability by trypan blue exclusion method

Cells (5×10^4 cells/well) were seeded in six well plates prior to the addition of bischalcones. The cells were incubated with different doses (10, 20, 30, and 40 μM) of compounds **3a–c**, along with 1% DMSO as solvent control. Cultures were harvested and monitored for cell number by counting cell suspensions using hemocytometer. Cell viability was checked before and after treatment with compounds using trypan blue exclusion method (Paris *et al.*, 2007).

Statistical analysis

Data were expressed as mean SD and all statistical analysis was performed using Prism 3.0 software Comparisons were performed with the one-way analysis of variance (ANOVA) test. All experiments were performed in triplicates.

Results and discussion

The basic principle of cancer chemotherapy is to use drugs that have targets and preferably non overlapping toxicity. Thus a logical chemotherapeutic strategy is the combined use of apoptosis inducing compounds and cytotoxic agents (Kato *et al.*, 1994; Browder *et al.*, 2000; Ellen *et al.*, 2005; Skommer *et al.*, 2006). The bischalcones **3a–c** were synthesized as per the scheme given in Fig. 1. Tumor growth inhibition of EAT cells in vivo was determined. EAT cells grow in the mice peritoneum, forming an ascites tumor with massive abdominal swelling. The animals show a dramatic increase in body weight over the growth period and animals succumbed to the tumor burden. The weight was significantly reduced in compound treated groups compared to control indicating the effect of the compounds in preventing the tumor growth. The treated groups showed reduction in body weight due to decrease in tumor burden Fig. 2. The effect of compounds on EAT showed that the administration of bischalcones **3a–c** on day 7, 9, and 11 after tumor cell inoculation produced effective antitumor response. The compounds also exhibited corresponding reduction in mean ascites volume and cell number.

Intraperitoneal transplantation of EAT cells resulted in survival of the mice for 11 days with an increase in the number of cells. Cell growth inhibition in the compound treated mice was confirmed on counting viable cells by trypan blue dye exclusion method which is shown in Fig. 3.

Extensive peritoneal angiogenesis was observed which may be due to the secretion of the angiogenesis inducing factors in the ascites fluid. Involvement of VEGF in the

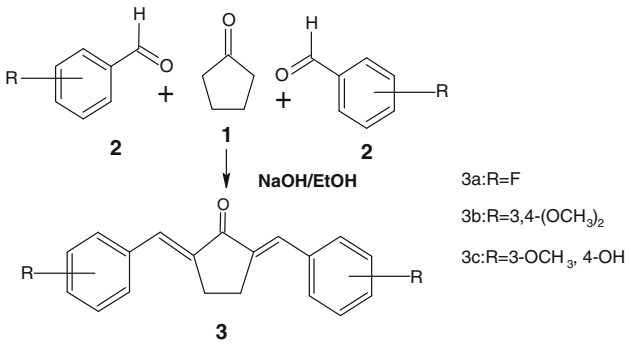

Fig. 1 Scheme for the preparation of bischalcones **3a–c**

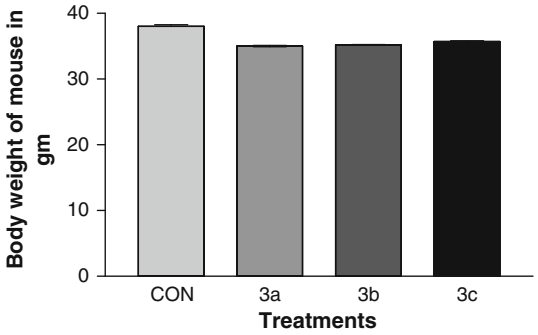

Fig. 2 Change of body weight in compounds treated mice group and control. Result expressed as body weight of mouse in grams

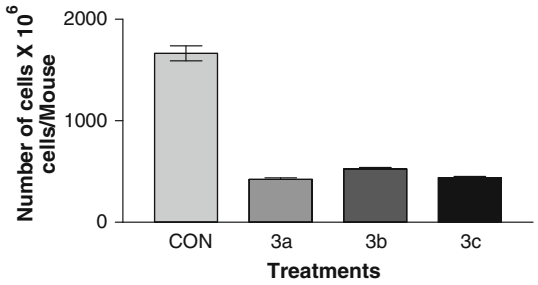

Fig. 3 Effects of **3a–c** on cell number of EAT bearing mice. The treatments showed difference in cell number from the control. The *error bars* represent standard deviation of the mean

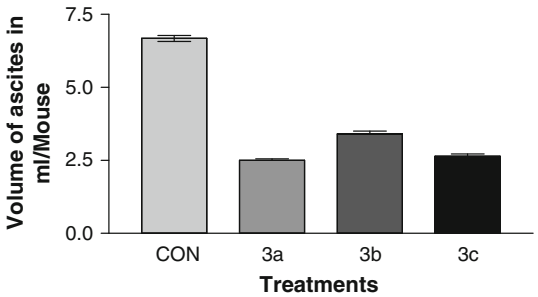

Fig. 4 Effect (in vivo) of compounds **3a–c** on proliferation of EAT cells and formation ascites cells

formation of malignant ascites has been well documented (Kumar *et al.*, 2009). The increase in the volume of ascities in the control group was due to the secretion of ascites by EAT cell whereas in the case of compound treated groups there was reduction in the ascites volume about 70–80% as shown in Fig. 4. Among the compounds tested, **3a** showed significant reduction in ascities volume. The result supports that compound **3a** was the effective inhibitor of tumor growth in comparison to compounds **3b** and **3c**.

The effect of the compounds on peritoneal angiogenesis was evaluated. Figure 5 shows reduction in vascularization in the compounds treated mice groups. Data clearly indicates that extensive angiogenesis in the peritoneum of EAT bearing control mice than in **3a–c** treated groups. The inhibition of the formation of new blood vessels may be due to the prevention of secretion of such factors in compound treated mice.

The compounds showed good antiproliferative property in vitro on HepG2 and HeLa cells. The findings demonstrated the potent anticancer activity of the curcumin analogs against EATS in vivo and HepG2 and HeLa cells in vitro.

MTT assay is a standard colorimetric assay can be used to determine the cytotoxicity of compounds. To investigate the potential antiproliferative effect of compounds **3a–c** on proliferation and survival of HepG2 and HeLa cells. Result shows that the compounds **3a–c** was selective against proliferative cells without any inhibitory effect on normal HepG2 cells Fig. 6. The IC50 values for **3a–c** were found to be 39, 110, 42 μM against HeLa cells and 48, 106, 44 μM against HepG2 cells, respectively.

Induction of apoptosis by other bischalcones both in vitro and in vivo studies has been reported (Skommer *et al.*, 2007; Girdhani *et al.*, 2005). An attempt was made to identify whether the compounds **3a–c** induced inhibition of EAT cell growth was due to induction of apoptosis. Giemsa, ethidium bromide/acridine orange staining of EAT cells indicated typical apoptotic morphology condensed nuclei, membrane blebbing and formation of apoptotic bodies. On treatment with compounds **3a–c**, most of the cells undergo apoptosis and showed all sorts of apoptotic morphology compared to control cells which are shown in Fig. 7.

DNA fragmentation is the biochemical hallmark of apoptosis. Cell genomic DNA showed the typical formation of DNA fragments as ladder in compounds **3a–c** treated EAT cells in vivo and as well as in HepG2 at 10 μM concentration which is illustrated in Fig. 8. In vivo treatment with compounds **3a–c** on EAT cells showed change in the morphology of the cells which is witnessed by the presence

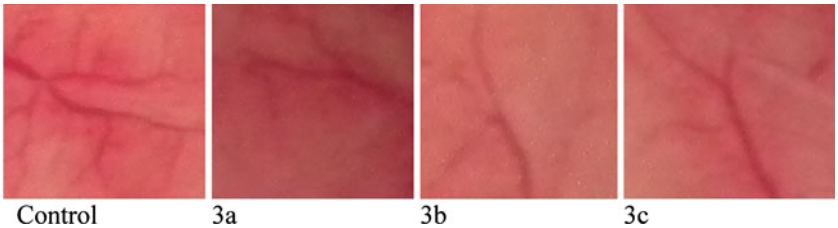

Fig. 5 Suppression of angiogenesis in vivo by **3a–c**. Decreased vasculature was observed in treated groups compared to control

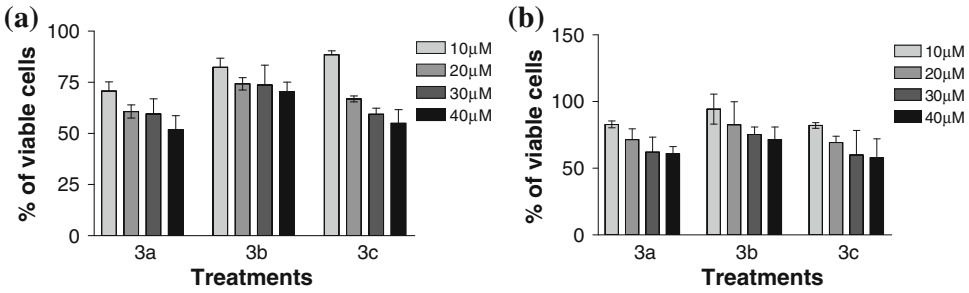

Fig. 6 MTT assay: **a** MTT assay using HeLa cells. **b** MTT assay using Hep G2 cells

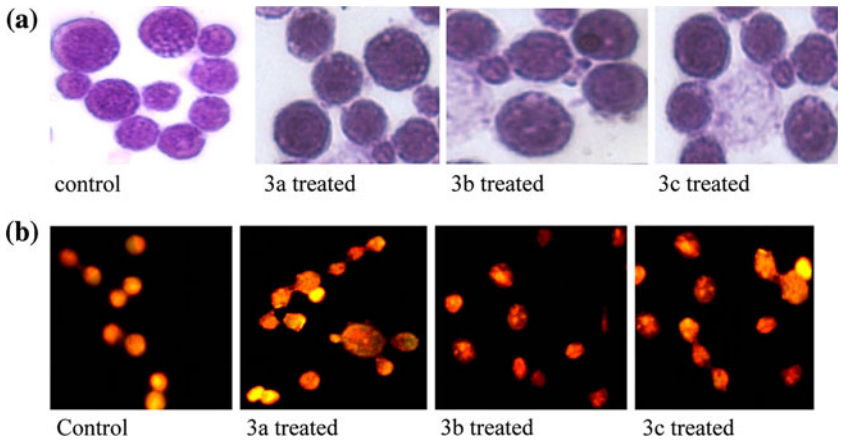

Fig. 7 Changes in the morphology of EAT cells after treatment with synthetic compounds **3a–c**. **a** Giemsa staining, **b** acridine orange:ethidium bromide staining. The apoptotic bodies and condensed nuclei are evident in the compound treated groups

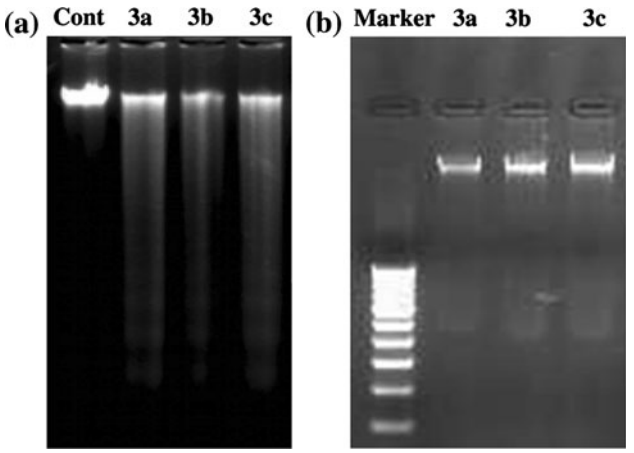

Fig. 8 DNA fragmentation study **a** DNA fragmentation found in EAT cells isolated from **3a–c** treated mouse. *Lane a*: control, *lane b*: **3a**, *lane c*: **3c**. **b** DNA fragmentation found in compounds **3a–c** treated HepG2 cells. *Lane a*: marker, *lane b*: **3a**, *lane c*: **3b**, *lane d*: **3c**

of apoptotic bodies, nuclear condensation, intra nucleosomal fragmentation, and DNA fragmentation. The effect would be immediate and direct on the tumor cells (Prabhakar *et al.*, 2006). This explains the tumor growth inhibitory and apoptotic effects of the compounds **3a–c**.

In the present findings, the compound **3a** showed good in vivo antitumor and antiangiogenic activities against EAT cells. This activity might be due to with the bioactive fluoro group (Holla *et al.*, 2006) at para-position and in rings A and B. The decrease in the activities of **3b** and **3c** compared to **3a** could be attributed to the presence of methoxy and hydroxyl group at 3,4-position. Thus the structural modifications have profound influence on antitumor, antiproliferative, and angioinhibitory activities of bischalcones. From these findings it can be concluded that all the three bischalcones **3a–c** can be considered as promising antitumor, antiproliferative, and angioinhibitory compounds. However, further investigations are needed to understand the mechanism of action of the compounds and to examine the possible utility of the compounds in cancer therapy.

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Conflict of interest None.

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