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# Anti-tumor and proapoptotic effect of novel synthetic benzophenone analogues in Ehrlich ascites tumor cells

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**Abstract**—A series of substituted benzophenone analogues, (2-aroyl-4-methylphenoxy)acetamides **4a**–**e**, have been synthesized via three-step synthesis sequence beginning with the 2-hydroxybenzophenones **1a**–**e** in excellent yield. **1a**–**e** on reaction with ethyl chloroacetate afford ethyl (2-aroyl-4-methylphenoxy)acetates **2a**–**e** which on alkaline hydrolysis afforded (2-aroyl-4-methylphenoxy)ethanoic acid **3a**–**e**. Compounds **3a**–**e** on condensation with *p*-chloroaniline furnished benzophenone analogues **4a**–**e**. In the present report, we investigated the anti-tumor and proapoptotic effect of benzophenones in Ehrlich ascites tumor (EAT) cells. Treatment of benzophenones in vivo resulted in inhibition of proliferation of EAT cells and ascites formation. Further, we demonstrate that the induction of apoptosis in EAT cells is mediated through activation of caspase-3. These results suggest a further possible clinical application of these synthetic compounds as potent anti-tumor and proapoptotic compounds.


## 1. Introduction

Apoptosis or programmed cell death has been characterized as a fundamental cellular activity occurring under a wide range of physiological and pathological conditions.[1–6] It plays an essential and protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells or excess cells that have been improperly induced to divide by mitotic stimulus.[7–9] Apoptosis results from activation of preprogrammed pathway of biochemical events leading to cell death.[10,11] The p53, a tumor suppressor gene, is implicated in the induction of distinct apoptotic signaling pathways that lead to the activation of aspartate specific cystiene proteases (caspases) that mediate apoptosis.[12] The activation of apoptotic genes will culminate in morphological and biochemical changes that include skeletal disruption, membrane blebbing, decrease in adhesion and intracellular contacts, chromatin condensation, and nuclear fragments into membrane enclosed apoptotic bodies.[13] The DNA fragmentation results from activation of caspase-activated endonuclease. DNA fragmentation factor (DFF-40/CAD) is associated with the internucleosomal cleavage of DNA during apoptosis. CAD is normally sequestered in the cytoplasm via its binding to an inhibitor (ICAD/DFF-45), which suppresses its endonuclease activity and conceals its nuclear localization signal. During the induction of apoptosis, ICAD/DFF-45 is cleaved and inactivated by a caspase-3-like protease, thereby resulting in the activation of CAD, its nuclear translocation, and subsequent oligonucleosomal DNA fragmentation which is a hallmark of apoptosis.[14–16]

In this context, it is noteworthy that apoptosis inducing ability seems to have become a primary factor in considering the efficacy of chemopreventive agents. There are several natural and synthetic compounds that a induce apoptosis both in vivo and in vitro in EAT cells.[17–21]

Benzophenones are a class of compounds obtained from natural products[22] or by synthetic methods.[23] The great importance of these substances is fundamentally due to the diverse biological and chemical properties they

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possess. Benzophenones exhibit significant anti-tumor activity both in vivo and in vitro.[24–34] In addition, synthetic benzophenones, such as 2-aminobenzophenone[35] and dihydroxy-4-methoxybenzophenone[36], have proven to be antimitotic and anticancer agents, respectively. Recently, *para*-methoxy substituted benzophenones were evaluated as p38α inhibitors with high efficacy and selectivity ($IC_{50}$: 14 nM).[37] Amino- and methoxy-substituted benzophenones are reported to be potent cytotoxic agents against a panel of human cancer cell lines including multidrug resistant cell lines.[24] The tubulin binding drug combretastatin A-4 exhibits a selective toxicity for proliferating endothelial cells by induction of apoptosis.[38] Polyprenylated benzophenone derivatives will induce caspase-mediated apoptosis.[31] Encouraged by this information an attempt was made to achieve new compounds with possible anti-proliferative effect in vivo. In the present study, we designed and synthesized some novel (2-aroyl-4-methylphenoxy)acetamides **4a**–**e** and verified their anti-tumor effect and mechanism of inhibition of proliferation and ascites formation in EAT cells. We addressed the anti-tumor and proapoptotic effect of compounds **4a**–**e** and relevance of caspase-3. We suggest that these compounds could be useful for individualization of therapeutic strategies in the future.

## 2. Results

### 2.1. Compounds 4a–e inhibit proliferation of EAT cells in vitro

We examined the effect of compounds **4a**–**e** on the cellular proliferation of EAT cells by using the MTT assay. Significant dose-dependent growth inhibition was observed when cells were treated with compounds **4a**–**e** for 24 h. Compounds **4a**–**e** showed 50% growth inhibition at <50 μM (Table 1). These result suggest that compounds **4a**–**e** potently inhibit the proliferation of EAT cells.

### 2.2. Compounds 4a–e inhibit proliferation EAT cells and increases the survivality of the animals in vivo

To determine the in vivo effect on cell growth, mouse mammary carcinoma cell line EAT was treated with compounds **4a**–**e** (100 mg/kg body weight/ip), during its growth period, three doses on every other day starting from 6 days after inoculation of tumor cells. Animal weights were monitored throughout the course of treatment. Compounds were not cytotoxic to the cells as verified by the MTT assay and trypan blue dye exclusion method (data not shown). In control EAT-bearing mice, there is a gradual increase in body weight, which from day six attains an exponential phase of growth. A rise in body weight of about 7 g over 12 days period of tumor growth was observed. However, when treated with the compounds **4a**–**e**, about 49.05%, 39.18%, 30.12%, 45.76% and 61.12% decrease in the body weight was observed, respectively, indicating the anti-tumor effect of compounds **4a**–**e** (Table 1). As shown in Figure 2 compound **4a**–**e** treatment resulted in a dose-dependent inhibition of proliferation of EAT cells. Decrease in tumor growth also correlates with decrease in ascites volume of treated mice (Fig. 1). The survivality of EAT cell-bearing mice (control) halved on the 12th day after implantation and no animal survived beyond the 14th day. Compound **4a**–**e** treatment increased the survivality, where animals were alive beyond the 23rd day. Compound **4e**-treated animals survived for 23 days, whereas compound **4a**- and **4d**-treated animals survived for 20 days, and of compound **4b**- and **4c**-treated animals 17 days, correlating with the anti-tumor effect of these compounds. These results showed that compound **4e** is the most effective inhibitor of tumor growth among compounds **4a**–**d**. However, compounds **4a**–**d** did show significant inhibition of tumor growth.

All the results are listed in Table 1.

### 2.3. Compounds 4a–e induce apoptosis of EAT cells in vivo

To elucidate the possible mechanism of compound **4a**–**e** mediated tumor inhibition, we investigated the effect of compounds **4a**–**e** on EAT cells. We found that treatment of compounds **4a**–**e** to EAT cells induced a significant proportion of cells to undergo apoptosis, as determined by the flowcytometric analysis. FACS analysis of the cells indicates the formation of apoptotic bodies and condensed nuclei that show up in the sub-G1 region. As shown in Figure 3, when compared to the control cells, on receiving compound **4a**–**e** treatment some of the EAT cells undergo apoptosis and are detected in the sub-G1 area. These results suggested the breakdown of EAT cells DNA resulting in tumor killing. The obvious ramifications were the growth arrest of EAT cells.

### 2.4. Compounds 4a–e induce nuclear condensation of EAT cells

An attempt was made to find out whether the inhibition of proliferation of EAT cells by compounds **4a**–**e** was

**Table 1.** Effect of compounds **4a**–**e** on proliferation of EAT cells in vitro, tumor growth inhibition, and survivality of the animals in vivo

| | Control | **4a** | **4b** | **4c** | **4d** | **4e** |
|---|---|---|---|---|---|---|
| $IC_{50}$ value (μM) | — | 39.67 | 38.58 | 38.22 | 37.46 | 42.50 |
| Tumor growth inhibition (%) | — | 49.05 | 39.18 | 30.12 | 45.76 | 61.12 |
| Survivality of the animals (days) | 14 | 20 | 18 | 18 | 20 | 23 |

Exponentially growing cells were treated with various concentrations (0–100 μM) of compounds **4a**–**e** for 24 h and were analyzed by the MTT assay and $IC_{50}$ values of each compound were determined. The effect of compounds **4a**–**e** on the body weight of the EAT-bearing mice was verified and percentage of inhibition after treatment was calculated taking 100% growth in the case of untreated mice. Survivality of the untreated mice was 14 days, whereas increased survivality in treated mice was observed. All the experiments contain at least five animals and each experiment was repeated thrice. Results are mean values ($n = 3$).

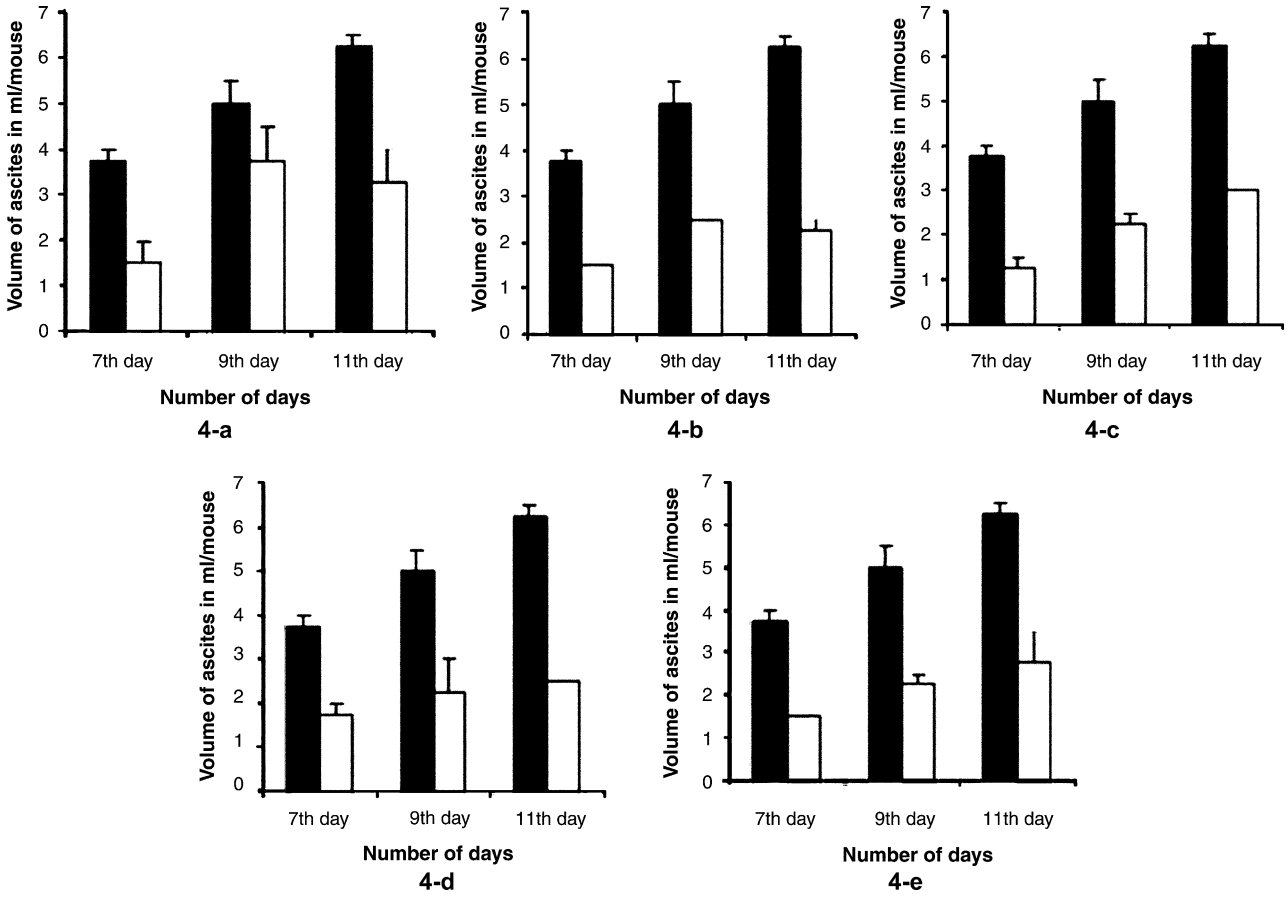



**Figure 1.** Effect of compounds **4a**–**e** on ascites volume. EAT-bearing mice treated with compounds **4a**–**e** were sacrificed after giving each dose and cells along with ascites fluid were harvested. Ascites volume was recorded. The above results are an average of three individual experiments.

due to induction of apoptosis in EAT cells. During in vivo studies, cytological analysis of acridine orange/ethidium bromide stained treated EAT cells (in vivo) showed nuclear condensation, an important feature of apoptosis as compared to the untreated EAT cells (Fig. 4).

### 2.5. Compounds 4a–e induce externalization of phosphatidylserine residues in EAT cells

During in vivo studies we verified the flipping of phosphatidylserine on the cell membrane by compounds **4a**–**e**, since phosphatidylserine flip-flops onto the cell surface in the very early stages of apoptosis. Cells undergoing apoptosis were detected using Annexin-V labeled with fluorescein isothiocyanate. EAT cells treated with compounds **4a**–**e** clearly showed early apoptotic changes like annexin binding (greenish red), while control cells did not show annexin binding (Fig. 5). These results indicate that compounds **4a**–**e** induce nuclear translocation of phosphatidylserine to the outer membrane surface in EAT cells.

### 2.6. Compounds 4a–e induce degradation of DNA in EAT cells

Biochemically, apoptosis is characterized by fragmentation of chromosomal DNA. We further verified the effect of compounds **4a**–**e** on DNA damage of EAT cells. As expected, compound **4a**–**e** treatment caused DNA fragmentation which then led to the formation of DNA ladder in EAT cells. This indicates that the apoptotic role of compounds **4a**–**e** in EAT cells ruled out the possibility of it being necrotic to EAT cells (Fig. 6).

### 2.7. Compounds 4a–e modulate p53 expression

For investigating the molecular mechanism of compounds **4a**–**e** induced apoptosis Western blot analysis of p53 protein was carried out. Result showed an increase in p53 protein levels in treated compared to untreated. Based on the above results we suggested that compound **4a**–**e**-mediated apoptosis is p53 dependent (Fig. 7).

### 2.8. Compound 4a–e treatment induces caspase-3-mediated apoptosis

Delineating the downstream signaling pathway revealed that compound **4a**–**e** treatment is activating the caspase-3 protease. In order to verify the involvement of caspase-3 upstream activator of endonuclease, EAT cells either treated with or without caspase-3 inhibitor were exposed to the compounds **4a**–**e**. The resultant lanes (3, 5, 7, 9, and 11) show the DNA fragmentation as a consequence of increased endonuclease activity and in lanes (2, 4, 6, 8, and 10) fragmentation was inhibited

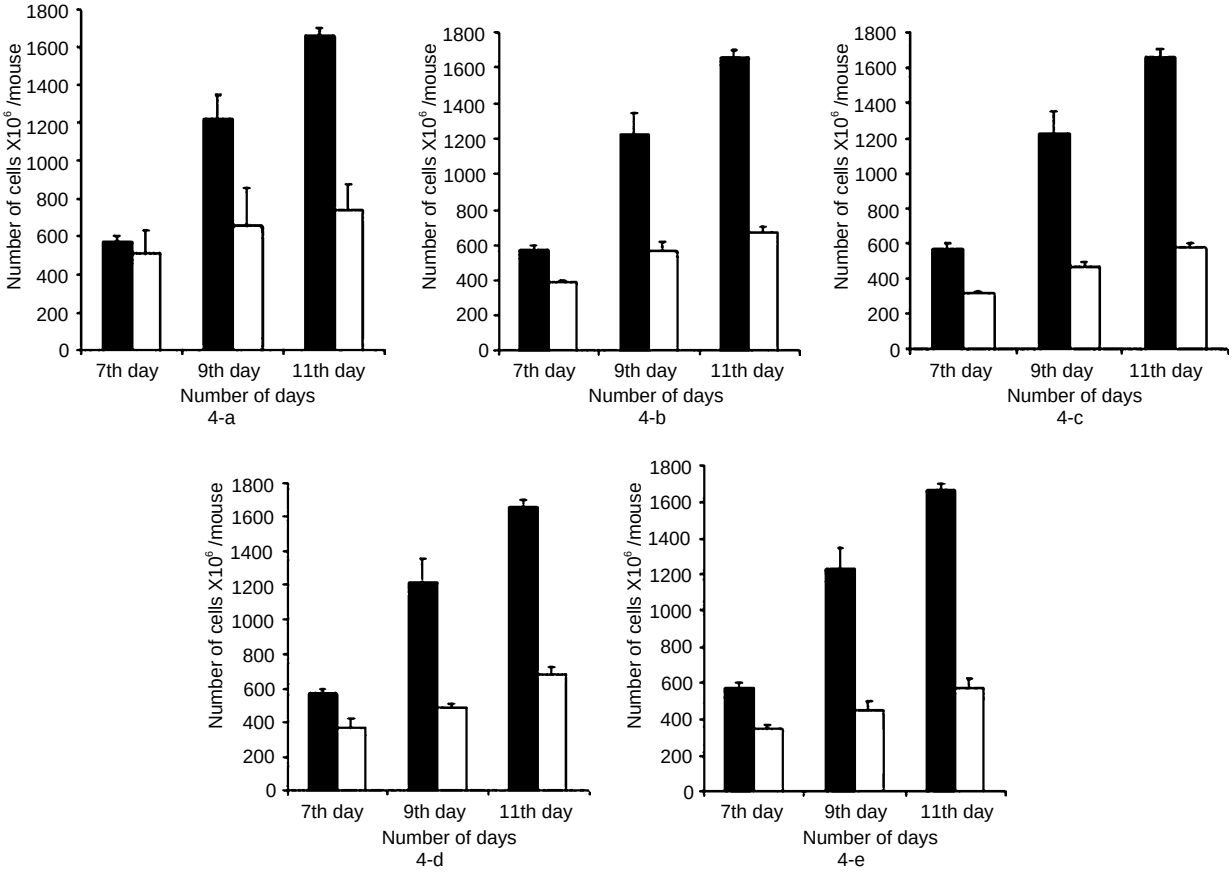



**Figure 2.** Effect of compounds **4a**–**e** on EAT cell number. EAT-bearing mice treated with compounds **4a**–**e** were sacrificed after giving each dose and cells along with ascites fluid were harvested. The number of cells per mouse was determined by counting the cells in hemocytometer. The above results are an average of three individual experiments.

by Ac-DEVD-CHO, a specific inhibitor of caspase-3 enzymes. Lane 1 is control DNA (Fig. 8).

### 2.9. Compounds 4a–e induce translocation of CAD

Since compounds **4a**–**e** activate caspase-3, a death protease responsible for apoptosis and activation of endonuclease in EAT cells, we further verified the coordinate relationship between caspase-3 and endonuclease in the cellular system of EAT cells. Western blot analysis of nuclear extract prepared from EAT cells treated with compounds **4a**–**e** in vivo showed the presence of CAD, whereas untreated cells did not show the presence of CAD (Fig. 9). In apoptotic cells, cleavage of ICAD by the caspase-3 protease allowed CAD to enter the nucleus and degrade DNA. We have demonstrated that the caspase-activated DNAse (CAD) is translocated from the cytosol to the nucleus and is responsible for the oligonucleosomal DNA fragmentation. The CAD protein was identified in the nucleus of compound **4a**–**e**-treated cell nuclear extract, while CAD was not detected in the untreated nuclear extract.

## 3. Discussion

Structurally, (2-aroyl-4-methylphenoxy)acetamides, the benzophenone analogues **4a**–**e**, exhibit activity due to the presence of amide group. The amide moiety is an important constituent of many biologically significant compounds. Amide moiety is versatile in organic compounds since all the three atoms in the O–C–N chain are potentially reactive. These results are partly due to the delocalization of the $\pi$ electrons along the O–C–N chain. This produces partial double-bond character in the C (O)–N bond. The versatility of the amide group in forming partial bonds with itself and many other functional groups is partly responsible for the structural subtleties of the biologically important proton derivatives.[39,40]

To determine whether compound **4a**–**e** treatment inhibits tumor growth, effect of compounds **4a**–**e** on EAT cell growth in vivo was checked. Earlier reports have shown that benzophenones exhibit significant anti-tumor activity both in vitro and in vivo in many of the cancer cell lines.[24–34] The synthetic morphiline and thio-morphiline benzophenones were screened for cytotoxic and anti-tumor activities against p388 murine leukemia and human lung carcinoma cells.[29] Recently, benzophenone analogues with amino and methoxy substitutes at *ortho*- and *para*-positions have been reported as potent cytotoxic agents against human cancer cell lines compared to plenstatin combretastatin A-4 analogue.[35] Our results on in vivo tumor inhibition showed that compounds **4a**–**e** have potent anti-tumor activity when compared with

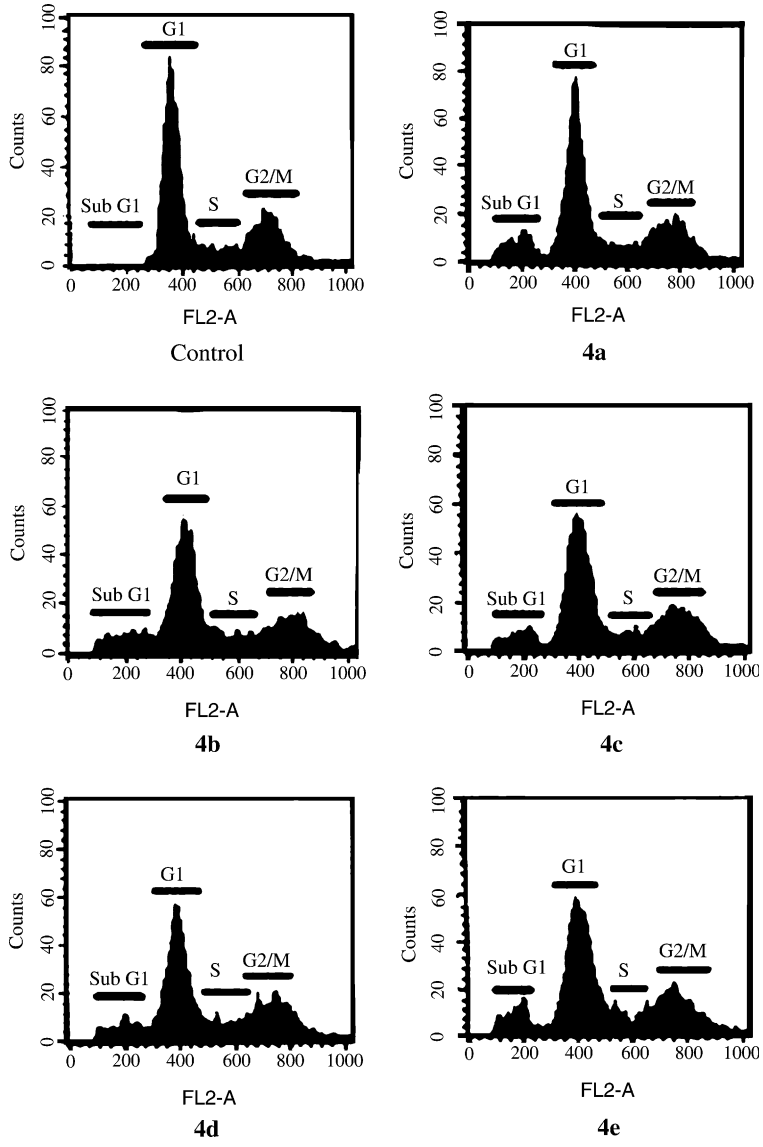



**Figure 3.** Analysis of apoptosis in EAT cells by compounds **4a**–**e**. For the determination of cell cycle-phase distribution, EAT cells from tumor-bearing mice were permeabilized and nuclear DNA was labeled with propidium iodide (PI) using Cycle TEST PLUS DNA reagent kit. Cell cycle phase distribution of nuclear DNA was determined on FACS, fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using CellQuest software (Becton–Dickinson). A total of 10,000 events were acquired and analysis of flowcytometric data was performed using ModFit software. A histogram of DNA content (*x*-axis, PI-fluorescence) versus counts (*y*-axis) has been displayed.

the lead compound. However, we observed that compound **4e** with the methoxy group at *para*-position in ring B has shown the highest anti-tumor activity of 61.12%, whereas compounds **4a** and **4d** with the group at *meta*- and *ortho*-positions in ring B, respectively, have shown moderate decrease in body weight (49% and 45%, respectively). Compound **4b** without substituent in ring B and **4c** with the bromo group at the *ortho*-position treatment have shown about 39% and 30% decrease in body weight, respectively. The results correlated with a decrease in cell number and ascites volume to considerable amount. Treatment of compounds **4a**–**e** also increased the survivality of the animals. Compound **4e**-treated animals were alive for 23 days and the number of animals whcih survived in this group was 86%, whereas compound **4a**- and **4d**-treated animals survived for 20 days with 70% of the animals surviving. In the case of compounds **4b** and **4c**, the percentage of animals that survived was 58%, whereas the animals were alive only for 18 days.

Despite variation in the tumor inhibition activity, all the compounds were able to induce the apoptosis where the mechanism of tumor growth inhibition was involved. Our results on externalization of phosphatidylserine residues, nuclear condensation, formation of apoptotic bodies, DNA degradation, overexpression of p53, and caspase-3 inhibition assays showed that compounds **4a**–**e** were inducing caspase-3-mediated apoptosis in EAT cells. The tumor suppressor gene p53 is regarded as a key element in maintaining a balance between growth and cell death in the living system and it plays an important role in tumor growth inhibition and induction of apoptosis.[41–43] Many compounds upregulate p53

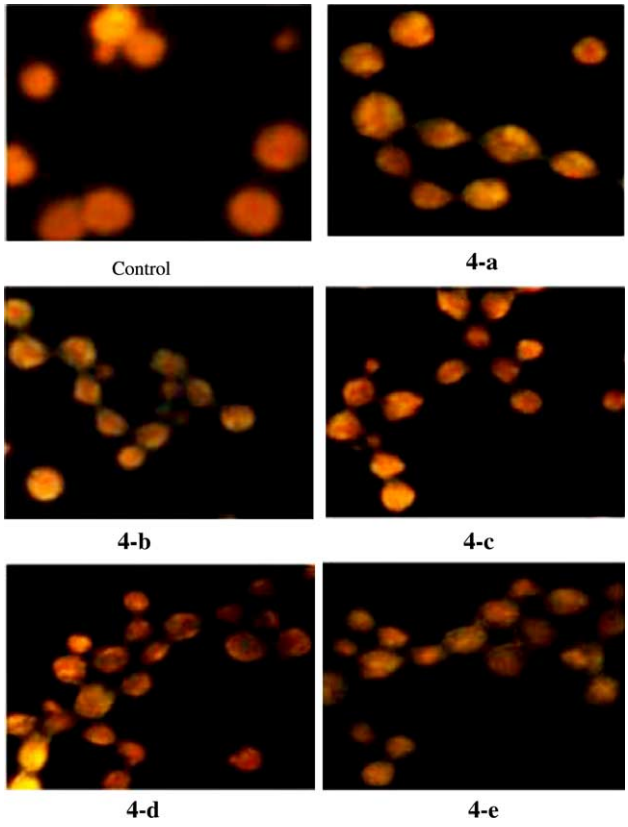



**Figure 4.** Changes in the nuclear morphology of EAT cells. Nuclear staining was performed according to the method of Srinivas et al. EAT cells either treated or untreated with compounds **4a**–**e** in vivo were smeared on a glass slide, fixed with methanol/acetic acid (3:1), and air-dried. The cells were hydrated with PBS and stained with a mixture (1:1) of acridine orange/ethidium bromide (4 μg/mL) solutions. The cells were immediately washed with PBS and viewed under a Leitz-DIAPLAN fluorescent microscope.

gene expression both in vivo and in vitro[19,44]. Our results showed that compound **4a**–**e** treatment resulted in increment of p53 protein in EAT cells and it may be an important molecular mechanism through which compounds **4a**–**e** inhibit growth of cancer cells.

The caspase family is another evolutionary conserved gene family that is involved in p53-mediated apoptosis. Caspases are cysteine proteases that play a critical role in the execution of apoptosis.[45,46] Activation of caspase-3 occurs when p53 is overexpressed in p53 null Saos-2 cells and BRK cells.[47] This suggests that the apoptotic pathways downstream of p53 may converge on caspase activation. In fact, activation of p53 induces caspase-3 activation and nuclear DNA fragmentation in many cases.[47–54] Benzophenone derivatives induce caspase-3-mediated apoptosis both in vitro and in vivo in many of the human cancer lines.[31,55] Activation of caspase-3 was observed in EAT cells treated with compounds **4a**–**e**. In addition, compounds **4a**–**e** induced activation of caspase-3, which was significantly attenuated in the presence of Ac-DEVD-CHO, suggesting a caspase-dependent mechanism of cell death by compounds **4a**–**e**. In addition, we have verified the downstream activation of caspase-activated DNase upon

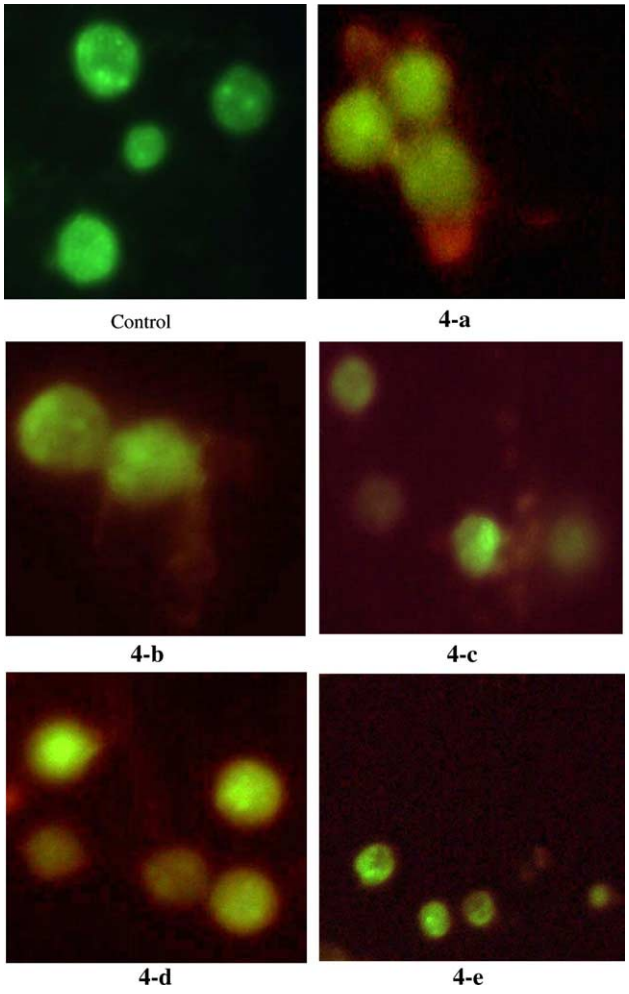



**Figure 5.** Apoptotic morphology of EAT cells upon compounds **4a**–**e** treatment. EAT cells treated with and without compounds **4a**–**e** in vivo were harvested and washed with PBS, dropped onto the slide, and air-dried. Cells were washed with binding buffer (10 mM Hepes, pH 7.5, containing 140 mM NaCl and 2.5 mM $CaCl_2$) and stained with Annexin-V detection kit as per the protocol supplied by the manufacturer. After 10 min, cells were washed thrice with binding buffer. The greenish red apoptotic cells were observed using a Leitz-DIAPLAN fluorescent microscope and photographed.

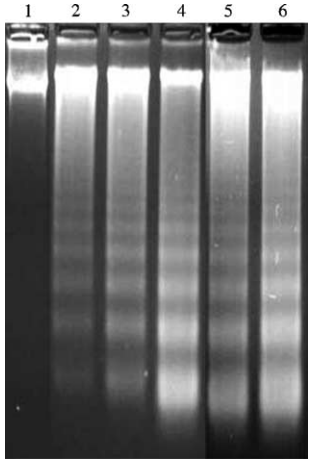



**Figure 6.** In vivo apoptotic effect of compounds **4a**–**e**. Untreated (lane 1) and compound **4a**–**e**-treated EAT cells (lanes 2–6) were used for extraction of nuclear DNA. DNA was run on 1.5% agarose gel.


**Figure 7.** Western blot analysis of p53. Nuclear extract from untreated and compound **4a**–**e**-treated EAT cells in vivo was resolved on 10% SDS–PAGE. Western blot analysis was performed for p53 using anti-p53 antibodies. Lane 1, control and lanes 2–6, compound **4a**–**e** treated.

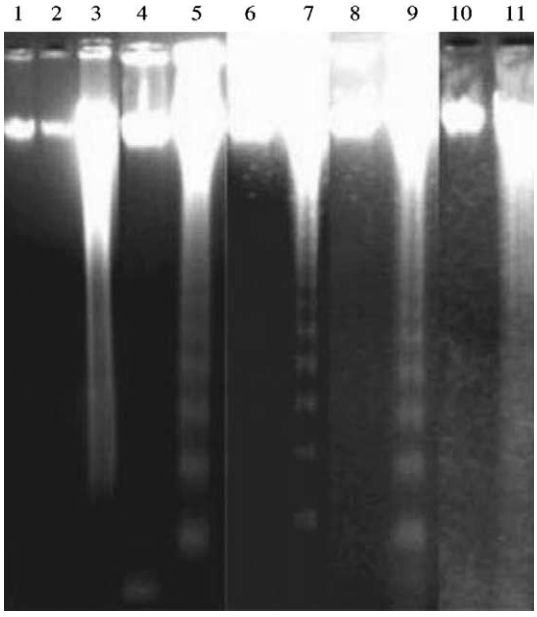



**Figure 8.** Effect of caspase-3 inhibitor on compound **4a**–**e** induced DNA degradation. EAT cells were either untreated or pre-incubated with 100 μM Ac-DEVD-CHO for 30 min followed by compound **4a**–**e** treatment for 2 h at 37 °C. Genomic DNA was isolated and verified for DNA ladder on 1.5% agarose gel, observed under UV-transillumination, and photographed. Lane 1—control DNA; lanes 3, 5, 7, 9, and 11—compound **4a**–**e**-treated DNA; lanes 2, 4, 6, 8, and 10—compound **4a**–**e** + Ac-DEVD-CHO-treated DNA.

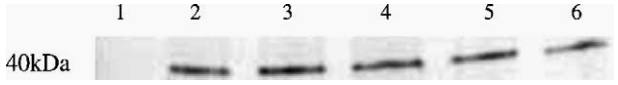



**Figure 9.** Western blot analysis of CAD. Nuclear extract from untreated and compounds **4a**–**e** treated EAT cells in vivo was resolved on 10% SDS–PAGE. Western blot analysis was performed for CAD using anti-CAD antibodies. Lane 1, control and lanes 2–6, compounds **4a**–**e** treated.

compound **4a**–**e** treatment. Caspase-activated DNase is associated with inhibitor of CAD (ICAD) in the cytosol. Upon caspase activation by external stimuli, CAD dissociates from ICAD and translocates to nucleus to disrupt the genetic material.[56] Treatment of EAT cells with compounds **4a**–**e** shows that the activation of CAD is a downstream target for caspase-3 in EAT cells. These results indicate the apoptotic signaling pathway of compounds **4a**–**e** in EAT cells.

Taking together our data compound **4e** has very potent anti-tumor activity that inhibits growth of EAT cells. However, the compounds **4a**–**d** also showed significant anti-tumor activity. All the compounds showed proapoptotic activity. These effects are likely to be induced by overexpression of p53, activation of caspase-3, and translocation of CAD into the nucleus, which is involved in the apoptosis. Such active benzophenone analogues prove to be a potential anti-tumor and apoptosis inducing factor 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.

## 4. Experimental

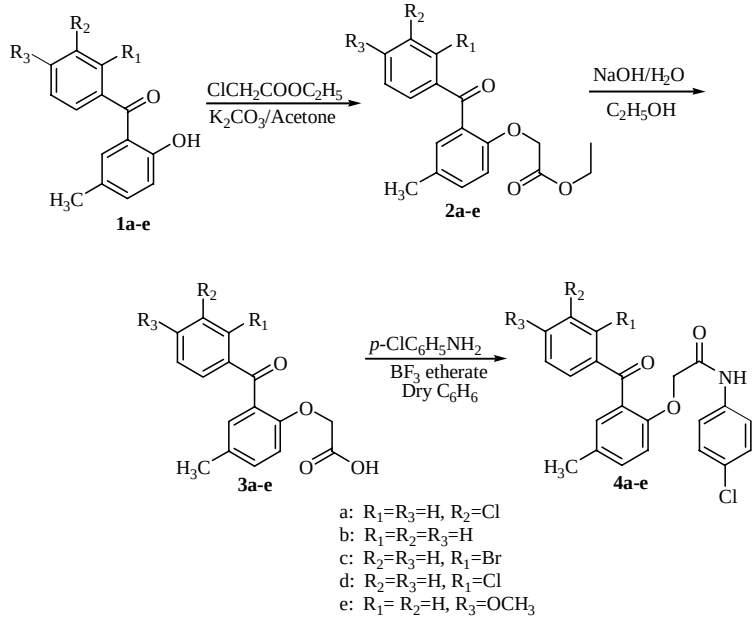
### 4.1. Chemistry

Chemicals were purchased from Aldrich Chemical Co. TLC was performed on aluminum-backed silica plates with visualization by UV light. Melting points were determined with a Thomas Hoover capillary melting point apparatus with a digital thermometer. IR spectra were recorded in Nujol on an FT-IR Shimadzu 8300 spectrometer and $^1$H NMR spectra were recorded on a Bruker 300 MHz spectrometer in $CDCl_3$. Chemical shifts were recorded in parts per million downfield from tetramethylsilane. Mass spectra were obtained with a VG70-70H mass spectrometer and elemental analysis results are within 0.4% of the calculated value.

The synthesis of the hitherto unreported title compounds **4a**–**e** is as outlined in Scheme 1. Hydroxybenzophenones **1a**–**e** on reaction with ethyl chloroacetate afford ethyl (2-aroyl-4-methylphenoxy)acetates **2a**–**e** in excellent yield, which on alkaline hydrolysis afforded (2-aroyl-4-methylphenoxy)ethanoic acid **3a**–**e**. Compounds **3a**–**e** on condensation with *p*-chloroaniline in the presence of boron trifluoride etherate and dry benzene furnished benzophenone analogues, (2-aroyl-4-methylphenoxy)acetamides **4a**–**e**.[43]

**4.1.1. Ethyl[2-(3-chlorobenzoyl)-4-methylphenoxy]acetate (2a).** A mixture of **1a** (5 g, 0.02 mol) and ethyl chloroacetate (2.4 g, 0.02 mol) in dry acetone (60 mL) and anhydrous potassium carbonate (2.8 g, 0.02 mol) was refluxed for 8 h. Subsequently, the reaction mixture was cooled and the solvent was removed under reduced pressure. The residual mass was triturated with ice water to remove potassium carbonate and extracted with ether (3 × 50 mL). The ether layer was washed with 10% sodium hydroxide solution (3 × 30 mL), followed by water (3 × 30 mL), and then dried over anhydrous sodium sulfate and evaporated to dryness to yield a crude solid. Recrystallization with ethanol gave **2a** in 80% yield. Compounds **2b**–**e** were synthesized analogously starting with **1b**–**e**, respectively.

Compound **2a**: 80%; mp 60–62 °C; IR (Nujol): 1670 (C=O), 1735 cm$^{-1}$ (ester C=O); $^1$H NMR ($CDCl_3$): $\delta$ 1.2 (t, $J$ = 7 Hz, 3H, $CH_3$ of ester), 2.3 (s, 3H, $CH_3$), 4.2 (q, $J$ = 6 Hz, 2H, $CH_2$ of ester), 4.45 (s, 2H, $OCH_2$), 7.2–7.6 (m, 7H, Ar-H); EI-MS: $m/z$ 332.5 ($M^+$, 62), 259.5 (12), 245.5 (12), 221 (100), 193 (06), 139.5 (39), 111.5 (34). Anal. Calcd for $C_{18}H_{17}ClO_4$




**Scheme 1.**

(332.5): C, 64.96; H, 5.11; Cl, 10.67. Found: C, 64.94; H, 5.07; Cl, 10.64%.

Compound **2b**: 79%; mp 61–63 °C; IR (Nujol): 1664 (C=O), 1760 $cm^{-1}$ (ester C=O); $^1H$ NMR ($CDCl_3$): $\delta$ 1.2 (t, $J = 7$ Hz, 3H, $CH_3$ of ester), 2.3 (s, 3H, $CH_3$), 4.1 (q, $J = 6$ Hz, 2H, $CH_2$ of ester), 4.5 (s, 2H, $OCH_2$), 7.1–7.7 (m, 8H, Ar-H); ms: *m/z* 298 ($M^+$, 60), 225 (10), 221(100), 211 (10), 193 (05), 105 (36), 77 (31). Anal. Calcd for $C_{18}H_{18}O_4$ (298): C, 72.48; H, 6.04. Found: C, 72.46; H, 6.02%.

Compound **2c**: 71%; mp 65–67 °C; IR (Nujol): 1665 (C=O), 1730 $cm^{-1}$ (ester C=O); $^1H$ NMR ($CDCl_3$): $\delta$ 1.21 (t, $J = 7$ Hz, 3H, $CH_3$ of ester), 2.3 (s, 3H, $CH_3$), 4.22 (q, $J = 6$ Hz, 2H, $CH_2$ of ester), 4.46 (s, 2H, $OCH_2$), 7.2–7.6 (m, 7H, Ar-H); EI-MS: *m/z* 377 ($M^+$, 61), 304 (12), 290 (12), 221 (100), 193 (06), 184 (39), 156 (33). Anal. Calcd for $C_{18}H_{17}BrO_4$ (377): C, 57.29; H, 4.50; Br, 21.22. Found: C, 57.26; H, 4.53; Br, 21.25%.

Compound **2d**: 79%; mp 52–54 °C; IR (Nujol): 1672 (C=O), 1737 $cm^{-1}$ (ester C=O); $^1H$ NMR ($CDCl_3$): $\delta$ 1.21 (t, $J = 7$ Hz, 3H, $CH_3$ of ester), 2.31 (s, 3H, $CH_3$), 4.21 (q, $J = 6$ Hz, 2H, $CH_2$ of ester), 4.46 (s, 2H, $OCH_2$), 7.25–7.7 (m, 7H, Ar-H); EI-MS: *m/z* 332.5 ($M^+$, 61), 259.5 (11), 245.5 (11), 221 (100), 193 (06), 139.5 (38), 111.5 (33). Anal. Calcd for $C_{18}H_{17}ClO_4$ (332.5): C, 64.96; H, 5.11; Cl, 10.67. Found: C, 64.99; H, 5.07; Cl, 10.63%.

Compound **2e**: 72%; mp 58–60 °C; IR (Nujol): 1660 (C=O), 1730 $cm^{-1}$ (ester C=O); $^1H$ NMR ($CDCl_3$): $\delta$ 1.2 (t, $J = 7$ Hz, 3H, $CH_3$ of ester), 2.25 (s, 3H, $CH_3$), 3.8 (s, 3H, $OCH_3$), 4.2 (q, $J = 6$ Hz, 2H, $CH_2$ of ester), 4.42 (s, 2H, $OCH_2$), 7.0 (d, $J = 8.3$ Hz, 2H, Ar-H), 7.15–7.41 (m, 3H, Ar-H), 7.49 (d, $J = 8.5$ Hz, 2H, Ar-H); EI-MS: *m/z* 328 ($M^+$, 59), 255 (09), 241 (09), 221 (100), 193 (05), 135 (35), 107 (29). Anal. Calcd for $C_{19}H_{20}O_5$ (328): C, 69.51; H, 6.09. Found: C, 69.49; H, 6.05%.

**4.1.2. [2-(3-Chlorobenzoyl)-4-methylphenoxy]ethanoic acid (3a).** Compound **2a** (2.0 g, 6.0 mmol) was dissolved in ethanol (10 mL) and treated with a solution of sodium hydroxide (0.6 g, 15 mmol) in water (10 mL). The mixture was heated under refluxed for 3 h, cooled, and acidified with 1 N hydrochloric acid. The oily precipitate was extracted with dichloromethane ($3 \times 30$ mL) and the solution was washed with water ($3 \times 25$), dried, and evaporated to give oil. Crystallization from hexane afforded **3a** as a white solid in 75% yield. Compounds **3b**–**e** were synthesized analogously starting with **2b**–**e**, respectively.

Compound **3a**: 75%; mp 120–122 °C; IR (Nujol): 1675 (C=O), 1730 (acid C=O), 3400–3500 $cm^{-1}$ (acid OH); $^1H$ NMR ($CDCl_3$): $\delta$ 2.3 (s, 3H, $CH_3$), 4.46 (s, 2H, $OCH_2$), 7.2–7.7 (m, 7H, Ar-H), 9.5 (s, 1H, COOH); MS: *m/z* 304.5 ($M^+$, 60), 260 (100), 245 (75), 193 (50), 165 (25), 139 (10), 111 (18). Anal. Calcd for $C_{16}H_{13}ClO_4$ (304.5): C, 63.06; H, 4.30; Cl, 11.63. Found: C, 63.04; H, 4.26; Cl, 11.60%.

Compound **3b**: 75%; mp 112–115 °C; IR (Nujol): 1655 (C=O), 1733 (acid C=O), 3450–3540 $cm^{-1}$ (acid OH); $^1H$ NMR ($CDCl_3$): $\delta$ 2.2 (s, 3H, $CH_3$), 4.44 (s, 2H, $OCH_2$), 6.9–7.55 (m, 8H, Ar-H), 9.2 (s, 1H, COOH); EI-MS: *m/z* 270 ($M^+$, 58), 226 (100), 211 (72), 193 (48), 165 (23), 105 (09), 77 (15). Anal. Calcd for $C_{16}H_{14}O_4$ (270): C, 71.11; H, 5.11. Found: C, 71.13; H, 5.08%.

Compound **3c**: 74%; mp 125–127 °C; IR (Nujol): 1670 (C=O), 1735 (acid C=O), 3410–3510 $cm^{-1}$ (acid OH); $^1H$ NMR ($CDCl_3$): $\delta$ 2.3 (s, 3H, $CH_3$), 4.45 (s, 2H, $OCH_2$), 7.1–7.6 (m, 7H, Ar-H), 9.4 (s, 1H, COOH); EI-MS: *m/z* 349 ($M^+$, 58), 305 (100), 290 (71), 193

(45), 184 (09), 165 (25), 156 (18). Anal. Calcd for $C_{16}H_{13}BrO_4$ (349): C, 55.01; H, 3.72; Br, 22.92. Found: C, 55.04; H, 3.75; Br, 22.94%.

Compound **3d**: 69%; mp 125–127 °C; IR (Nujol): 1655 (C=O), 1730 (acid C=O), 3410–3555 $cm^{-1}$ (acid OH); $^1H$ NMR ($CDCl_3$): $\delta$ 2.2 (s, 3H, $CH_3$), 4.45 (s, 2H, $OCH_2$), 6.9–7.6 (m, 7H, Ar-H), 9.3 (s, 1H, COOH); EI-MS: *m/z* 304.5 ($M^+$, 58), 260 (100), 245 (76), 193 (51), 165 (26), 139 (11), 111 (19). Anal. Calcd for $C_{16}H_{13}ClO_4$ (304.5): C, 63.06; H, 4.30; Cl, 11.63. Found: C, 63.09; H, 4.27; Cl, 11.65%.

Compound **3e**: 70%; mp 130–132 °C; IR (Nujol): 1660 (C=O), 1738 (acid C=O), 3470–3575 $cm^{-1}$ (acid OH); $^1H$ NMR ($CDCl_3$): $\delta$ 2.25 (s, 3H, $CH_3$), 3.8 (s, 3H, $OCH_3$), 4.42 (s, 2H, $OCH_2$), 7.0 (d, $J$ = 8.3 Hz, 2H, Ar-H), 7.15–7.45 (m, 3H, Ar-H), 7.5 (d, $J$ = 8.5 Hz, 2H, Ar-H), 9.1 (s, 1H, COOH); EI-MS: *m/z* 300 ($M^+$, 55), 256 (100), 241 (70), 193 (45), 165 (21), 135 (08), 107 (14). Anal. Calcd for $C_{17}H_{16}O_5$ (300): C, 68.0; H, 5.33. Found: C, 68.03; H, 5.35%.

**4.1.3. [2-(3-Chlorobenzoyl)-4-methylphenoxy]-*N*-(4-chlorophenyl)-acetamide (4a).** A mixture of **3a** (1.0 g, 3.2 mmol), *p*-chloroaniline (0.51 g, 4.0 mmol), and boron trifluoride etherate (0.83 g, 6.0 mmol) in dry benzene (15 mL) was refluxed for 6 h. The refluxing solvent was dried by circulation over anhydrous sodium sulfate in a Soxhlet's apparatus. The reaction mixture was washed with 10% aqueous sodium hydroxide (3 × 20 mL) and 10% hydrochloric acid (3 × 20 mL), and then with water (3 × 25 mL). After being dried with anhydrous sodium sulfate, the organic layer was concentrated under reduced pressure. The amide was purified by crystallization from ethanol. Further purification was carried out by chromatography on silica gel using hexane and ethanol (7:2) as eluent to give **4a** as a white solid in 70% yield. Compounds **4b**–**e** were synthesized by analogous procedures using **3b**–**e**, respectively.

Compound **4a**: 72%; mp 127–129 °C; IR (Nujol): 1670 (C=O), 1705 (amide C=O), 3405 $cm^{-1}$ (NH); $^1H$ NMR ($CDCl_3$): $\delta$ 2.23 (s, 3H, $CH_3$), 4.45 (s, 2H, $OCH_2$), 4.82 (br s, 1H, CONH), 6.9–7.26 (m, 7H, Ar-H), 7.3 (d, $J$ = 8.55 Hz, 2H, Ar-H), 7.61 (d, $J$ = 8.6 Hz, 2H, Ar-H); EI-MS: *m/z* 415 ($M^+$+1, 10), 397 (11), 302.5 (06), 245.5 (15), 167.5 (100), 139.5 (22), 111.5 (24). Anal. Calcd for $C_{22}H_{17}Cl_2NO_3$ (414): C, 63.76; H, 4.10; Cl, 17.14; N, 3.38. Found: C, 63.73; H, 4.14; Cl, 17.11; N, 3.40%.

Compound **4b**: 71%; mp 105–107 °C; IR (Nujol): 1665 (C=O), 1701 (C=O of amide), 3402 $cm^{-1}$ (NH); $^1H$ NMR ($CDCl_3$): $\delta$ 2.2 (s, 3H, $CH_3$), 4.4 (s, 2H, $OCH_2$), 4.72 (br s, 1H, CONH), 6.8–7.2 (m, 8H, Ar-H), 7.22 (d, $J$ = 8.55 Hz, 2H, Ar-H), 7.52 (d, $J$ = 8.6 Hz, 2H, Ar-H); EI-MS: *m/z* 379.5 ($M^+$+1, 10), 361.5 (11), 302.5 (06), 210.5 (15), 167.5 (100), 105 (22), 77 (25). Anal. Calcd for $C_{22}H_{18}ClNO_3$ (379.5): C, 69.57; H, 4.78; Cl, 9.33; N, 3.69. Found: C, 69.54; H, 4.75; Cl, 9.36; N, 3.64%.

Compound **4c**: 72%; mp 112–114 °C; IR (Nujol): 1668 (C=O), 1700 (C=O of amide), 3401 $cm^{-1}$ (NH); $^1H$ NMR ($CDCl_3$): $\delta$ 2.21 (s, 3H, $CH_3$), 4.41 (s, 2H, $OCH_2$), 4.75 (br s, 1H, CONH), 6.88–7.25 (m, 7H, Ar-H), 7.25 (d, $J$ = 8.55 Hz, 2H, Ar-H), 7.59 (d, $J$ = 8.6 Hz, 2H, Ar-H); EI-MS: *m/z* 459.5 ($M^+$+1, 10), 407 (11), 302.5 (06), 290 (15), 184 (22), 167.5 (100), 156 (24), 111.5 (25). Anal. Calcd for $C_{22}H_{17}BrClNO_3$ (458.5): C, 57.57; H, 3.92; Br, 17.44; Cl, 7.74; N, 3.05. Found: C, 57.54; H, 3.95; Br, 17.41; Cl, 7.77; N, 3.09%.

Compound **4d**: 72%; mp 96–98 °C; IR (Nujol): 1675 (C=O), 1710 (amide C=O), 3410 $cm^{-1}$ (NH); $^1H$ NMR ($CDCl_3$): $\delta$ 2.25 (s, 3H, $CH_3$), 4.46 (s, 2H, $OCH_2$), 4.85 (br s, 1H, CONH), 6.92–7.28 (m, 7H, Ar-H), 7.32 (d, $J$ = 8.55 Hz, 2H, Ar-H), 7.63 (d, $J$ = 8.6 Hz, 2H, Ar-H); EI-MS: *m/z* 415 ($M^+$+1, 09), 397 (10), 302.5 (05), 245.5 (14), 167.5 (100), 139.5 (21), 111.5 (23). Anal. Calcd for $C_{22}H_{17}Cl_2NO_3$ (414): C, 63.76; H, 4.10; Cl, 17.14; N, 3.38. Found: C, 63.80; H, 4.15; Cl, 17.01; N, 3.40%.

Compound **4e**: 72%; mp 119–121 °C; IR (Nujol): 1665 (C=O), 1710 (C=O of amide), 3410 $cm^{-1}$ (NH); $^1H$ NMR ($CDCl_3$): $\delta$ 2.32 (s, 3H, $CH_3$), 3.78 (s, 3H, $OCH_3$), 4.39 (s, 2H, $OCH_2$), 4.79 (br s, 1H, CONH), 6.87 (d, $J$ = 8.3 Hz, 2H, Ar-H), 6.9–7.21 (m, 3H, Ar-H), 7.25 (d, $J$ = 8.55 Hz, 2H, Ar-H), 7.58 (d, $J$ = 8.6 Hz, 2H, Ar-H), 7.59 (d, $J$ = 8.5 Hz, 2H, Ar-H); EI-MS: *m/z* 410.5 ($M^+$+1, 09), 392.5 (10), 302.5 (06), 241 (05), 167.5 (100), 135 (22), 111.5 (25), 107 (17). Anal. Calcd for $C_{23}H_{20}ClNO_4$ (409.5): C, 67.39; H, 4.88; Cl, 8.66; N, 3.41. Found: C, 67.43; H, 4.91; Cl, 8.63; N, 3.44%.

### 4.2. Biology

Swiss albino mice were obtained from Central Animal Facility, Department of Zoology, University of Mysore, Mysore, India. MTT assay kit was procured from Merck. Annexin-V apoptosis detection kit was procured from Sigma, USA. ACDEVD-CHO caspase-3 inhibitor, anti-p53, and anti-caspase DNase antibodies were procured from Santa Cruz Biotechnology, USA. All other reagents were of highest analytical grade.

**4.2.1. Cell culture and in vitro compound 4a–e treatment.** EAT cells were grown in NCTC-135 medium with 10% FCS and penicillin/streptomycin (1 mg/mL). After the cells were 80% confluent, they were treated with various concentrations of compounds **4a**–**e** (0–100 μM) for various time periods (0–24 h). Then, the cells were trypsinized and collected before being used for further experiments.

**4.2.2. Cytotoxicity assay.** In vitro growth inhibitory effect of compounds **4a**–**e** on EAT cells was determined by measuring the absorbance of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, MERCK) dye for living cells as per the manufacturer's instructions. Briefly, EAT cells were seeded in 96-well microtiter plates containing NCTC medium. After exposure to compounds **4a**–**e** for 24 h in a dose-dependent way for

various time periods (0–24 h), 50 μL of MTT solution (2 mg/mL in PBS) was added to each well, and plates were incubated for an additional 4 h at 37 °C. The MTT solution in medium was then aspirated off. To dissolve the formazan crystals formed in viable cells, 200 μL DMSO was added to each well before measuring the absorbance at 570 nm.

**4.2.3. Animals and in vivo tumor growth.** Six- to eight-week-old female mice were acclimated for 1 week while caged in groups of five. Mice were housed and fed a diet of animal chow and water ad libitum throughout the experiment. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Mysore, Mysore, India. The EAT cell lines were maintained by in vivo passage. Swiss albino mice bearing EAT were sacrificed and the tumors were resected under aseptic conditions. A suspension of the resected tumor cell was made in 0.9% normal saline. Approximately $5 \times 10^6$ cells/mouse (300 μL of suspension) were injected into dorsa of the mice (weighing ±30 g) intraperitoneally. These cells grow in mice peritoneum forming an ascites tumor with massive abdominal swelling. The animals showed a dramatic increase in the body weight over the growth period and the animals succumbed to the tumor burden 14–16 days after implantation. The number of cells increased over the 10 days of growth with formation of 6–7 mL of ascites fluid.

**4.2.4. In vivo compound 4a–e treatment.** To determine whether compounds **4a**–**e** inhibits tumor growth in vivo, compounds **4a**–**e** (100 mg/kg body weight/ip) were injected into the EAT-bearing mice intraperitoneally using a 26 gauge needle on every alternate day starting 6 days of tumor implantation and growth of the tumor was monitored by taking the body weight of the animals every day. Control mice were injected with 0.2 mL saline (ip) on every alternate day. Each treatment consisted of at least five mice and each experiment was repeated thrice. After giving each dose (compounds + 200 μL of 0.9% saline), animals were sacrificed and the EAT cells along with ascites fluid were harvested. The set of animals was used to study the survivality after treatment until their death.

**4.2.5. Isolation of EAT cells from mice peritoneal cavity.** The EAT cells were isolated from the peritoneal cavity of tumor-bearing mice (control or treated). Two to three milliliters of sterile PBS was injected into the peritoneal cavity of the mice and the peritoneal fluid containing the tumor cells was withdrawn, collected in sterile petri dishes, and incubated at 37 °C for 2 h. The cells of macrophage lineage adhered to the bottom of the petri dishes. The non-adherent population was aspirated out gently and washed repeatedly with PBS. EAT cells were then separated from other non-adherent contaminating cells by fluorescence-activated cell sorter (FACS). Moreover, viability of these cells was assessed and was found to be >95% by trypan blue dye exclusion. The viable EAT cells were processed for further experiments.

**4.2.6. Detection of apoptosis by flowcytometry.** For the determination of cell cycle-phase distribution, EAT cells from tumor-bearing mice were permeabilized and nuclear DNA was labeled with propidium iodide (PI) using Cycle TEST PLUS DNA reagent kit. Cell cycle-phase distribution of nuclear DNA was determined on FACS, fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using CellQuest software (Becton–Dickinson). A total of 10,000 events were acquired and analysis of flowcytometric data was performed using ModFit software. A histogram of DNA content (*x*-axis, PI-fluorescence) versus counts (*y*-axis) has been displayed.

**4.2.7. Trypan blue assay.** EAT cells were treated with different concentrations of compounds **4a**–**e** for various time periods (0–8 h). Cell viability was assessed by mixing aliquots of cell suspension with 0.4% trypan blue. Cells that picked up the dye were considered to be dead.

**4.2.8. Acridine orange/ethidium bromide staining.** Nuclear staining was performed according to the method of Srinivas et al.[57] EAT cells either treated or untreated with compounds **4a**–**e** in vivo were smeared on a glass slide and fixed with methanol/acetic acid (3:1), and air-dried. The cells were hydrated with PBS and stained with a mixture (1:1) of acridine orange/ethidium bromide (4 μg/mL) solutions. The cells were immediately washed with PBS and viewed under a Leitz-DIAPLAN fluorescent microscope.

**4.2.9. Annexin-V staining.** EAT cells treated with and without compounds **4a**–**e** in vivo were harvested and washed with PBS, dropped onto the slide, and air-dried. Cells were washed with binding buffer (10 mM Hepes, pH 7.5, containing 140 mM NaCl and 2.5 mM $CaCl_2$) and stained with Annexin-V detection kit as per the protocol supplied by the manufacturer. After 10 min cells were washed thrice with binding buffer. The greenish red apoptotic cells were observed using a Leitz-DIAPLAN fluorescent microscope and photographed.

**4.2.10. DNA isolation.** EAT cells were collected from mice treated with or without compounds **4a**–**e** in vivo and DNA was isolated using the phenol–chloroform method. In brief, cells were lysed in a 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 subjected to phenol/chloroform/isoamyl alcohol (25:24:1) extraction and once to chloroform extraction. DNA was precipitated by adding 1:2 volumes of ice-cold ethanol. The precipitated DNA was dissolved in 50 μL TE buffer (pH 8.0). The DNA was digested with 20 μg/mL RNase at 37 °C for 1 h. The DNA was quantitated and equal concentration of DNA (50 μg) was resolved on 1.5% agarose gel, viewed under UV light, and documented using Uvp-BioDoc-ItTM system.

**4.2.11. Caspase-3 inhibition assay.** EAT cells were harvested and washed with PBS and suspended in HBSS buffer, pH 7.4. The cells were preincubated with or without caspase-3 inhibitor AC-DEVD CHO (100 μM) at 37 °C for 1 h. Subsequently, the cells were treated with compounds **4a**–**e** and incubated for 2 h at 37 °C. DNA

was isolated and fragmentation was visualized by 1.5% agarose gel electrophoresis.

**4.2.12. SDS–PAGE and immunoblot analysis.** EAT cells treated and untreated with compounds **4a–e** in vivo were harvested and nuclear protein extracts were prepared. For Western blots, 20 μg of the protein per lane was separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels, transferred to nitrocellulose membranes, and reacted with the desired antibody. The proteins were detected by the secondary antibody conjugated to alkaline phosphatase and developed with substrate BCIP-NBT.

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