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Discovery of a novel small molecule, 1-ethoxy-3-(3,4-methylenedioxyphenyl)-2-propanol, that induces apoptosis in A549 human lung cancer cells

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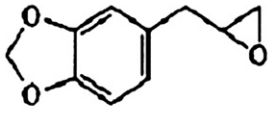
Abstract—A novel small molecule, 1-ethoxy-3-(3,4-methylenedioxyphenyl)-2-propanol (EOD), was synthesized in our laboratory. Previously, we reported pharmacological properties of EOD, triggering apoptosis in Human umbilical vein endothelial cells (HUVECs). Here, we further investigated the effects of EOD on the growth of A549 human lung cancer cells. EOD treatment induced apoptosis in A549 cells via up-regulating the expression of P53 protein, blocking cell cycle partly at G_1 phase, and ultimately activating caspase-3. In contrast, caspase-8 might be irrelevant to EOD-triggered apoptosis. This study indicated that EOD might be a potential chemopreventive agent for lung cancer. The work would encourage us to add more novel compounds to our 'library' of small molecules derived through modern synthetic organic chemistry, and would drive us to determine the proteins that the compounds target.

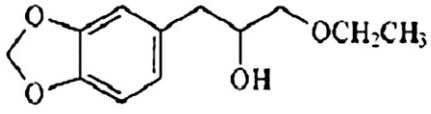
1. Introduction

Apoptosis or programmed cell death is the prevalent mechanism complementary to proliferation that is critical for the normal development and function of multicellular organisms. Rapid proliferation needs to be balanced by apoptosis to maintain a constant cell number. Retarded cell death contributes to a wide variety of human cancers.[1] As mentioned above, development of drugs that can effectively trigger apoptosis in cancer cells has been receiving considerable attention.

The use of small chemical molecules to affect biological phenomena, also known as chemical genetics, has made a significant impact in diverse areas of biology.[2–5] According to chemical genetics,[6] numerous small molecules produced by diversity oriented synthesis can be collected into a 'library' of small compounds. In addition to providing information about gene function using these small molecules, the 'library' is also a powerful tool for screening specific drugs that target specific proteins indispensable to the survival or proliferation of cancer cells. Therefore, the library would help to find effective anti-cancer drugs.



3,4-(methylenedioxy)-1-(2',3'-epoxypropyl)-benzene
(Safrole oxide)



1-ethoxy-3-(3,4-methylenedioxyphenyl)-2-propanol
(EOD)

Figure 1. The structural formulae of safrole oxide and EOD.

Keywords: 1-Ethoxy-3-(3,4-methylenedioxyphenyl)-2-propanol; A549 cells apoptosis; P53 protein; Caspase-3.
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To generate a 'library' of small chemical molecules, many series of compounds have been synthesized in our laboratory. EOD (Fig. 1) is among these molecules. Considering that vascular endothelial cells (VECs) play important roles in angiogenesis, which is also critical for promoting tumor growth and metastasis,[7] and that HUVEC has been used as one of the standards for cell-based assays in the field of anti-angiogenic drug discovery and in anti-metastasis therapy,[8] so we previously studied the effects of EOD on the growth and apoptosis in HUVEC. The results showed that EOD induced apoptosis in HUVEC by cell cycle arrest at S phase.[9] However, the effect of EOD on tumor cells is unknown. Lung cancer is the leading category of cancer death in men and women in many countries of the world. Therefore, in this study, we further investigated the effects of EOD on the growth of A549 human lung cancer cells, and revealed the mechanism by which EOD functioned, wishing for finding novel drugs for lung cancer therapy in future.

2. Results and discussion

2.1. Effects of EOD on cell growth, morphological changes, ultrastructural alteration, and chromatin condensation

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The MTT cell proliferation assay is widely accepted as a reliable way to measure the cell proliferation rate, and conversely when metabolic events lead to apoptosis or necrosis. The data obtained by MTT assay showed that exposure of cells to EOD 402–804 μM for 24 h resulted in cell viability decrease from 100% to 90.5%–77.8% (p <0.05–p <0.01) (Table 1). When the exposure continued on to 48 h, compared with the control group, cell viability reduced more significantly from 100% to 83.5%–69.8% ($p < 0.05$–$p < 0.01$) (Table 2). The data suggested that from 402 to 804 μM, EOD inhibited cell growth in a dose-dependent manner.

Concomitant with cell growth inhibition induced by EOD, cell morphological changes were observed using phase contrast microscope. Cells gradually turned round and detached from the dishes from 24 to 48 h, accompanied with apoptotic bodies. Interestingly, much more vacuoles were present in the cytoplasm (Fig. 2).

Table 1. Effect of EOD on the proliferation of A549 cells at 24 h

Cell group		A_{570}	Viability/%
Control		0.4475 ± 0.015	100
EOD	402 μM	0.405 ± 0.005[a]	90.5 ± 3.2[a]
	804 μM	0.348 ± 0.012[b]	77.8 ± 2.7[b]

$n = 4$ in all groups. Values represent means ± SE.
[a] $P < 0.05$.
[b] $P < 0.01$ by comparison with control group.

Table 2. Effect of EOD on the proliferation of A549 cells at 48 h

Cell group		A_{570}	Viability/%
Control		1.062 ± 0.032	100
EOD	402 μM	0.887 ± 0.028[a]	83.5 ± 0.8[a]
	804 μM	0.741 ± 0.051[b]	69.8 ± 1.1[b]

$n = 4$ in all groups. Values represent means ± SE.
[a] $P < 0.05$.
[b] $P < 0.01$ by comparison with control group.

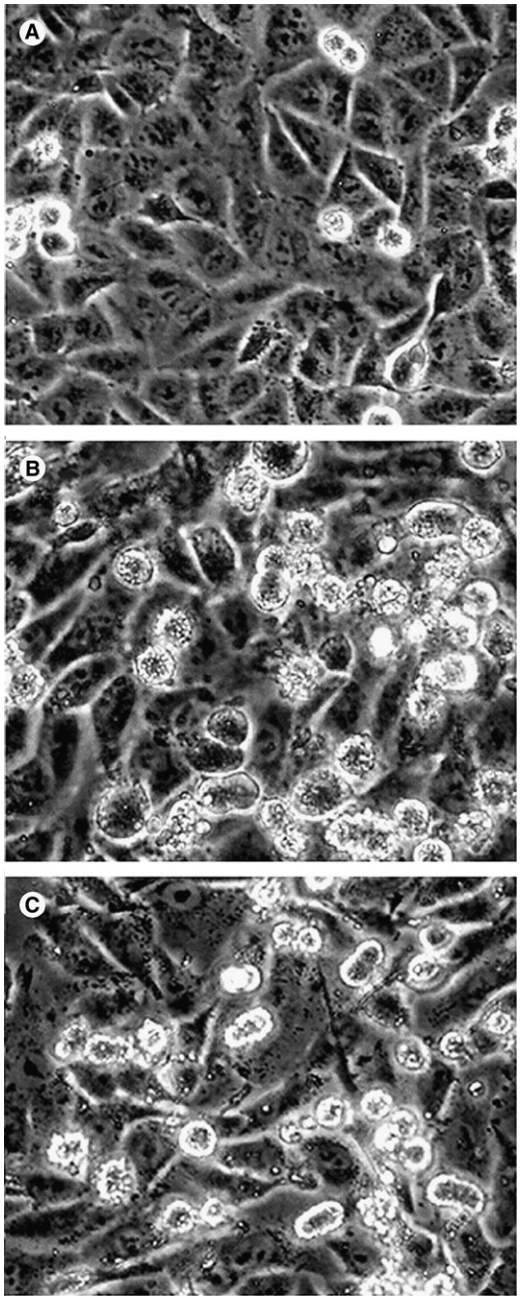

Figure 2. Effect of EOD on morphological changes of A549 cells at 48 h. (A) Cells in the control group; (B) Cells treated with EOD 402 μM; (C) Cells treated with EOD 804 μM. EOD-treated cells displayed features of apoptosis, including apoptotic bodies and many vacuoles in cytoplasm (B and C). The figures were obtained by using a Phase Contrast Microscope (×600).

Cytoplasmic vacuolization is a remarkable characteristic of apoptosis and necrosis.[10–12] Classical electron microscopy is effectively used to detect vacuoles in cells.[13] To observe the vacuoles more detailedly and to discern the mode of cell death, we observed the cells by electron microscopy. Ultrastructurally, cells incubated with EOD 402 μM for 48 h exhibited many vacuoles in the cytoplasm, and the chromatin compaction into a mass at the nuclear periphery (Fig. 3B). However, the chromatin of control cell is speckled (Fig. 3A). Chromatin condensation is widely accepted as a criterion to distinguish apoptosis from necrosis,[14] thus these results suggested that the mode of cell death triggered by EOD might be that of apoptosis.

The generation of large condensed chromatin bodies is a characteristic of apoptotic cells, which is routinely used as a definite marker for cell apoptosis.[15,16] To assay the chromatin condensation of the cell population in response to EOD, A549 cells were stained by Hoechst 33258, a classical way of identifying chromatin condensation. The results indicated that nuclei of the cells treated with EOD 402 μM were stained into brightly blue, while the nuclei of most control cells into average slightly blue (Fig. 4). The results demonstrated that in early apoptosis, condensation of chromatin appeared to commence in several distinct regions within nucleus.

Apoptosis is a form of programmed cell death that is characterized by specific morphologic and biochemical properties.[10] Morphologically, apoptosis is characterized by a series of structural changes in dying cells: blebbing of the plasma membrane, cytoplasmic vacuolization, condensation of chromatin, and cellular fragmentation into membrane apoptotic bodies. Based on the data mentioned above, the cell death induced by EOD fitted the criteria for apoptosis, which indicated that EOD initiated classical apoptosis in A549 cells.

Apoptosis and necrosis need to be differentiated. The ability to differentiate between these two modes of cell death could have a significant impact on accessing the outcome of anti-cancer drug therapy in the clinic.[17] To examine the drug toxicity and to further substantiate the mode of cell death induced by EOD, LDH assay was performed. Considering that the higher the drug

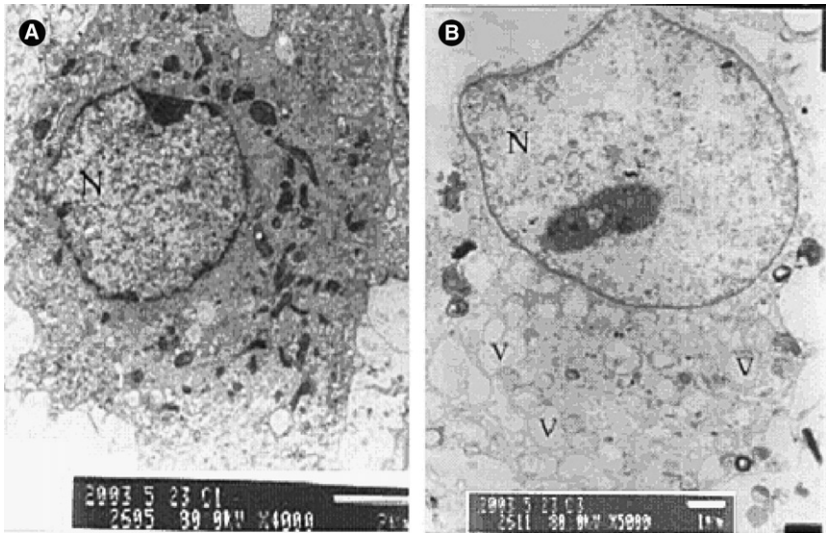

Figure 3. Effect of EOD on the ultrastructure of A549 cells at 48 h. (A) Cells in the control group (×4000); (B) Cells treated with EOD 402 μM for 48 h (×5000). Cells treated with EOD showed the characteristics of apoptosis, including cytoplasmic vacuolization and chromatin compaction into a mass at the nuclear periphery. N: nucleus; V: vacuole.

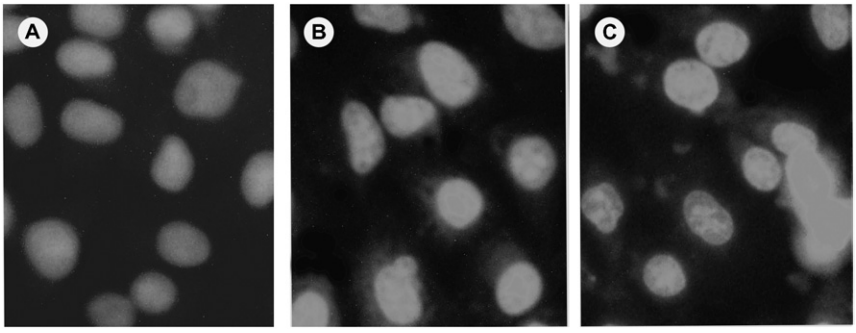

Figure 4. Effect of EOD on chromatin condensation of A549 cells at 48 h. (A) Cells in the control group; (B) Cells treated with EOD 402 μM; (C) Cells treated with EOD 804 μM. After treated for 48 h, cells were stained with Hoechst 33258. Apoptotic cells were stained into highly condensed, brightly staining nucleus (B and C). While normal cells were stained into average slightly blue. (×600).

concentration, the more likely necrosis is to occur, we used the highest concentration (804 μM) in this assay. There was no significant difference ($P > 0.05$, $n = 3$) in LDH release between the cells in the control group and in the test group (Fig. 5). The data evidenced that EOD, 804 μM, did not result in necrosis of A549 cells within 48 h.

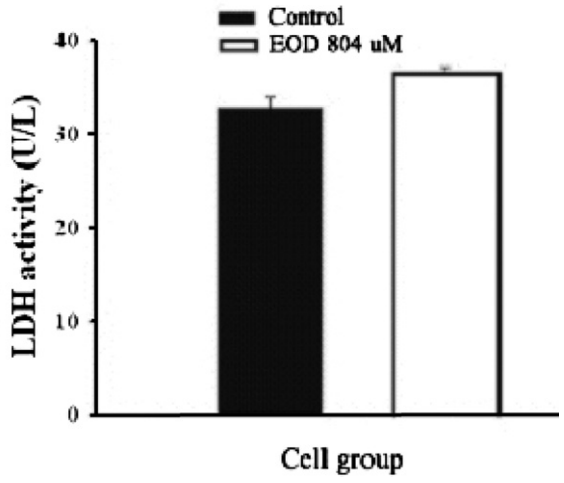

Figure 5. Effect of EOD on the release of LDH from A549 cells. Cell culture medium from the test group and the control group was collected after treatment with or without EOD 804 μM for 48 h. Light absorption was analyzed at 340 nm using a model Cintra 5 UV–vis spectrometer. LDH activity was calculated by the formula described in 'Materials and Methods'. There was no significant difference in LDH release between the two groups ($p > 0.05$, $n = 3$).

Based on these findings, we concluded that EOD, 402–804 μM, inhibited cell growth by inducing apoptosis instead of necrosis. Unbridled proliferation is one typical characteristic of potentially immortal tumor cells. If the growth of cancer cells can be inhibited, the tumor would be under control. We hypothesized that EOD might prevent lung carcinoma growth by induction of cell apoptosis.

2.2. Effects of EOD on cell cycle distribution

Mammalian cell growth and proliferation are mediated via cell cycle progression. Loss of cell cycle control can initiate the apoptotic program.[18,19] For example, tetrandrine induces caspase-mediated apoptosis and cell cycle arrest at the G1 phase in A549 human lung carcinoma cells.[20] Previously, we found that EOD blocked HUVEC at S phase and led the cells to apoptosis.[9] In this work, exposure of A549 cells to EOD 402 μM for 48 h induced G_1 phase fraction increase from 77.37% to 84.45% and S phase fraction decrease from 16.30% to 10.47% (Fig. 6A). Similarly, when A549 cells were incubated with EOD 804 μM for 48 h, the cell number in G_1 phase increased from 64.51% to 74.69% and in S phase decreased from 30.59% to 17.36% (Fig. 6B). Obviously, EOD blocked A549 cell cycle partly at G_1 phase within 48 h.

EOD blocked A549 cells partly at G_1 phase, restraining them from progress through the checkpoint. Therefore, the cells were triggered alarm signal leading to apoptosis. In addition, cell cycle arrest at G_1 phase caused the great decrease in the cell number of S phase. A

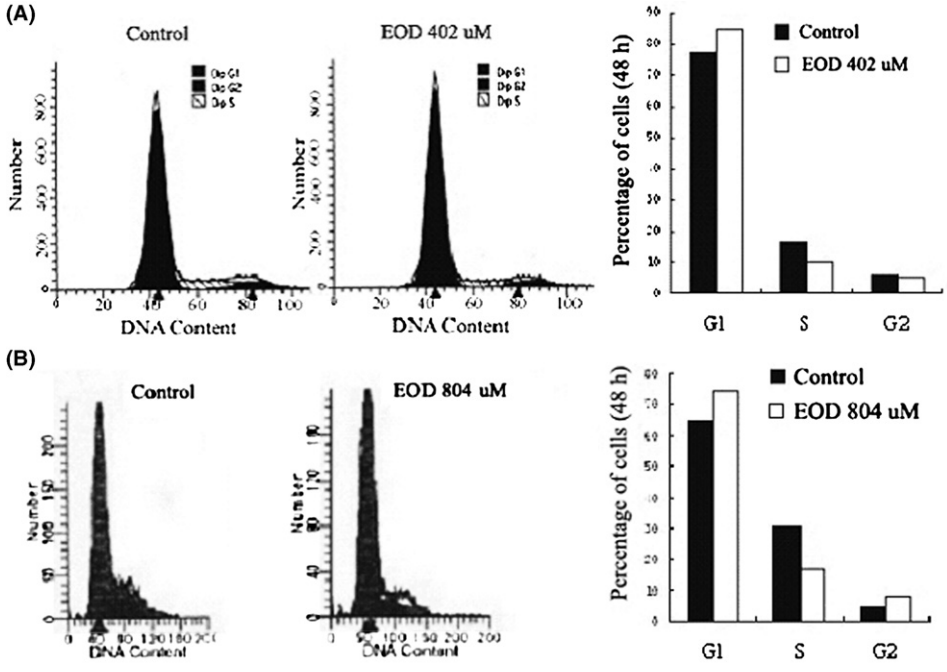

Figure 6. Effect of EOD on cell cycle distribution in A549 cells at 48 h. (A) Cells in the control group and EOD 402 μM treated group; (B) Cells in the control group and EOD 804 μM treated group. Cells were harvested after treatment with or without EOD, 402 μM or 804 μM, for 48 h. After cell fixation, RNA hydrolysis, and DNA staining with PI, DNA content of cells was analyzed using a FACSCalibur flow cytometry. Cell cycle distribution was shown on the left, while the percentage of the cells in each phase was depicted as a bar chart on the right. EOD blocked A549 cells partly at G_1 phase. Data were representative of two independent experiments.

low S-phase fraction indicates a slow cell division and a slow-growing tumor. Drugs that promote cell apoptosis, stop the process of cancer cell division, or even slow it down, are likely to be good anti-tumor agents.[21] Our findings suggested that EOD might be an effective agent against lung cancer. The evidence led us to find the putative proteins that participated in the apoptosis signal transduction pathway induced by this drug.

2.3. Effect of EOD on the expression of P53 protein

The P53 tumor suppressor protein is a sequence-specific DNA binding transcription factor that induces cell cycle arrest or apoptosis in response to genotoxic stress.[22] It has been reported that P53 protein induced G1 arrest and apoptosis in mutant p53 transfected Saos-cells in response to 9-hydroxyellipticine.[23] In this study, to understand the mechanism by which EOD blocked cell cycle partly at G_1 phase, we examined the expression of P53 protein by immunoblotting. Considering that the drug could initiate cell apoptosis at the concentrations from 402 to 804 μM, we selected the lowest concentration (402 μM) as a representative to study in this assay as well as in ultrastructural observation mentioned above and in the experiments below. Compared with the control group, the expression of P53 protein was up-regulated to three folds (Fig. 7). Hsu revealed that acacetin significantly increased the expression of p53 and caused cell cycle arrest and apoptosis in human non-small cell lung cancer A549 cells.[24] Consistent with this report, our finding demonstrated that P53 protein might be involved in EOD-evoked cell apoptosis by activation of cell cycle arrest, which implicated the molecular connection between the cell cycle and apoptosis.

2.4. Effect of EOD on the activity of caspases

It has been established that caspases initiate the commitment of cells to apoptosis caused by several stimuli.[25] Caspases are synthesized as inactive proenzymes that require proteolytic cleavage for activation. Caspase-3, the active form of procaspase-3, is involved in the execution phase of apoptosis, where cells undergo morphological changes such as DNA fragmentation, chromatin condensation, and apoptotic body formation.[26–28] Since caspase-3 levels correlate with the apoptotic index, it may be a measure of ongoing apoptosis and thus a potential marker for predicting response to cytotoxic drugs.[29]

To finally confirm that the cells exposed to EOD were actually undergoing apoptosis, and to determine whether caspase-3 was involved in the cell death pathway, colorimetric assay for the activity of caspase-3 was performed. Compared with the cells in the control or the solvent control group, light absorption at 405 nm in the cells incubated with EOD 402 μM for 48 h increased markedly from 0.140 to 0.254 ($P < 0.01$, $n = 3$) or significantly from 0.134 to 0.254 ($P < 0.01$, $n = 3$), respectively (Fig. 8, A). However, the activity of caspase-3 was hardly affected by ethanol at the concentration of 0.045% (v/v) ($P > 0.05$, $n = 3$). Obviously, the activity of caspase-3 was significantly up-regulated by the drug, which verified that caspase-3 was activated and participated in the cell death pathway induced by EOD. Since procaspase-3 fails to be activated during necrosis,[30] so we concluded that the cell death evoked by EOD was the kind of apoptosis.

In general, caspase-8 acts as an upstream caspase and there are two pathways downstream of caspase 8. In some cells, such as thymocytes and fibroblasts, caspase 8 directly activates caspase 3. In other cells such as hepatocytes, caspase 8 uses the mitochondrial pathway through the cleavage of Bid and subsequent release of cytochrome *c* to amplify the apoptotic signal.[31] Recently, caspase-8 as an executioner caspase in the anticancer drug-induced apoptosis was reported.[32–34] To examine whether caspase-8 is involved in the apoptosis pathway, we subsequently detected the activity of caspase-8. Different from caspase-3, light absorption at 405 nm had no obvious alteration after the treatment with EOD, 402 μM (from 0.145 to 0.144 vs the control group, $P > 0.05$; $n = 3$; from 0.122 to 0.144 vs the

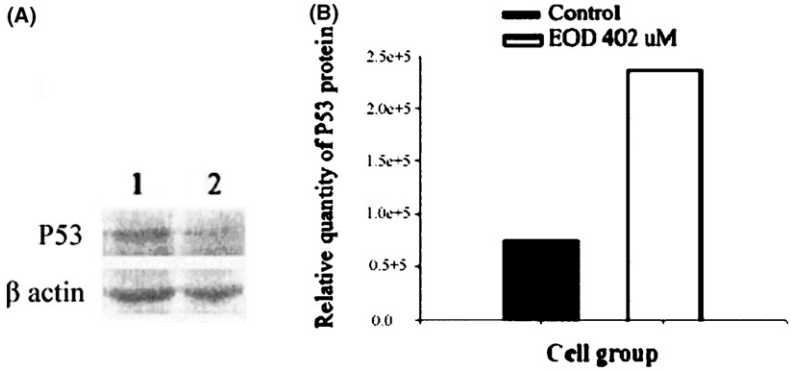

Figure 7. Effect of EOD on the expression of P53 protein in A549 cells at 48 h. Extracts were prepared from the cells in the control group and the group incubated with EOD 402 μM. The proteins were separated by a 7.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was then sequentially probed with antibodies against P53 protein and β-Actin, incubated with HRP-linked secondary antibodies, followed by color development with DAB. The bands of proteins were shown on the left (A) (Lane 1, the group treated with EOD 402 μM; Lane 2, the control group). The relative quantity of P53 protein, depicted as a bar chart on the right (B), was analyzed by JEDA software. The expression of P53 protein was up-regulated to 3 folds by EOD. Data were representative of two independent experiments.

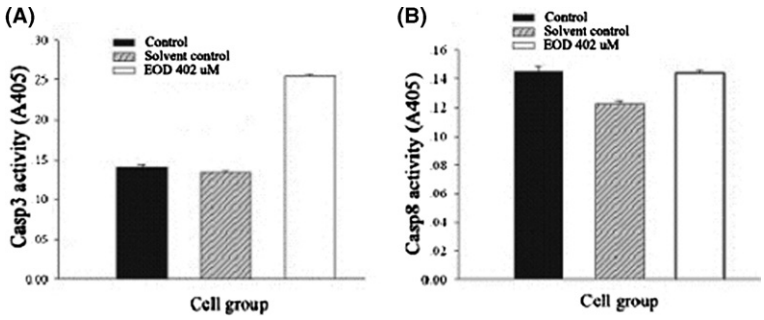

Figure 8. Colorimetric assays of caspase-3 and caspase-8 activity at 48 h. Extracts were prepared from the cells in the control group, in the test group incubated with EOD 402 μM, and also in the solvent control group, in which A549 cells were cultivated in the medium containing ethanol 0.045% (v/v). Caspase-3 activity (A) and casapse-8 activity (B) was determined by measuring the cleavage of DEVD-pNA. Data are means ± S.D. from three independent experiments. The activity of caspase-3 was significantly up-regulated by EOD ($P < 0.01$ vs the control or the solvent groups, $n = 3$). In contrast, there were almost no changes in the activity of caspase-8 ($P > 0.05$ vs the control or the solvent control group, $n = 3$). Ethanol at the concentration hardly affected the activity of caspase-3 or caspase-8.

solvent control group, $P > 0.05$, $n = 3$) (Fig. 8, B). The result substantiated that caspase-8 was not involved in the apoptosis pathway induced by EOD, and caspase-3 activation was independent of caspase-8 within 48 h.

Apoptosis can be triggered through a biochemical chain of events known as the Fas/CD95 pathway. When activated, the Fas/CD95 pathway initiates a cascade of signals within the cell that ultimately turns on caspase. In a genetic screen for essential components of the Fas-mediated apoptotic cascade, Juo et al. evidence that caspase-8 occupies an essential and apical position in the Fas signaling pathway.[35] A Jurkat T lymphocyte cell line deficient in caspase-8 was completely resistant to Fas-induced apoptosis. Complementation of this cell line with wild-type caspase-8 restored Fas-mediated apoptosis.[35–37] Based on the data that caspase-8 was not activated in this study, we conjectured that A549 cell apoptosis triggered by EOD might be irrelative to the Fas pathway.

In summary, EOD, 402–804 μM, has a marked inhibition effect on cell proliferation of human lung cancer cell line A549. This function of EOD might be performed by up-regulation of P53 protein, blocking cell cycle partly at G_1 phase and activation of caspase-3. EOD-induced apoptosis in A549 cells was independent of activation of caspase-8. EOD might be a potential chemopreventive agent for lung cancer cells. This study would drive us to investigate the protein that EOD targets and would encourage us to prepare more effective compounds promoting apoptosis in lung cancer cells by chemical modification.

3. Experimental

3.1. Materials

Safrole [98% (GC)] was purchased from Fluka AG and 3-chloroperoxybenzoic acid from ACROS. Safrole oxide [3,4-(methylenedioxy)-1-(2′,3′-epoxypropyl)-benzene] was synthesized by the reaction of safrole with 3-chloroperoxybenzoic acid and purified by silica gel column chromatography. 1-ethoxy-3-(3,4-methylenedioxyphenyl)-2-propanol (EOD) was synthesized by the reactions of safrole oxide with sodium ethoxide in alcohol at refluxing temperature for 8 h and purified by silica gel column chromatography.[38] RPMI 1640 was obtained from Gibco BRL Co. (Grand Island, USA), newborn calf serum from Hyclone (USA). Monoclonal P53 protein antibody (mouse anti-human), ApoAlert caspase-3 and caspase-8 colorimetric assay kits were all purchased from BD Biosciences Clontech. Monoclonal anti-β-actin antibody (mouse anti-human) and HRP-linked secondary antibodies (goat anti-mouse) were purchased from Santa Cruz Biotech, Santa Cruz, CA. EOD was dissolved in ethanol and applied to cells such that the final concentration of ethanol in the culture medium was below 0.1% (v/v). Ethanol at a concentration of 0.1% (v/v) did not affect the viability of the cells.

3.2. Cell cultures

A549 lung cancer cells were cultured in RPMI 1640 medium, supplemented with 10% (v/v) newborn calf serum at 37 °C in 5% CO_2 and 95% air. The cells were routinely seeded at the density of 100,000/cm^2 into 96-well plates or other appropriate dishes in the medium. Twenty four hours after planting, the experiments below were performed.

3.3. Cell growth assay

Cells were seeded into 96-well plates and treated with or without EOD, 402–804 μM, for 24–48 h. Cell growth was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) (Sigma Chemical Co., USA) assay as described by Price et al.[39] The light absorption was measured at 570 nm using SpectraMAX 190 microplate spectrophotometer (GMI Co., USA).

3.4. Observation of cell morphological changes

Cells were seeded into 24-well plates and incubated with or without EOD, 402–804 μM. After 24 or 48 h, cell morphological changes were observed, respectively, with Phase Contrast Microscope. (Nikon, Japan).

3.5. Observation of cell ultrastructure changes

Cells were seeded into 100 mm Falcon plastic dishes (Becton Dickinson, USA). After incubated with or without EOD 402 μM for 48 h, cells were harvested by 0.25% trypsin-0.02M EDTA treatment, and then centrifuged (300 g, 10 min, room temperature). The cell pellets were primarily fixed in 3% glutaraldehyde overnight at 4 °C, post-fixed in 1% OsO4 for 1 h at 4 °C, pre-stained in 0.5% uranyl acetate overnight on a rotator at room temperature, dehydrated in a graded series of alcohols, infiltrated with spur resin, polymerized at 60 °C overnight (12–16 h), sectioned on ultramicrotome, and then post-stained using lead citrate for 10 min. The samples were examined with a JEM-1200EX electron microscope (JEDL Japan).

3.6. Detection of chromatin condensation

Chromatin condensation was detected by nucleus staining with Hoechst 33258. Briefly, after treatment, cells were fixed with 2% formaldehyde for 10 min, washed with PBS (phosphate-buffered saline), stained with PBS/0.1% TritonX-100/10 uM Hoechst 33258 for 5 min at room temperature, and then were visualized by fluorescence microscopy (Nikon, Japan). In this way, apoptotic cells would be stained into brightly blue because of their chromatin condensation.[40]

3.7. LDH assay for drug toxicity

The detection was performed on the control cells and the cells treated with EOD 804 μM for 48 h using Lactate Dehydrogenase (LDH) kit (ZhongSheng Co., BeiJing, China) according to the manufacturer's instructions. Light absorption was analyzed at 340 nm using a model Cintra 5 UV–vis spectrometer (GBC Co., Australia). LDH activity was calculated by the formula:

$$\mathrm{LDH(U/L)} = (\Delta A_{\mathrm{sample/min}} - \Delta A_{\mathrm{blank/min}}) \times F$$

$$F = 1000 \times V_{\mathrm{total}}/(V_{\mathrm{sample}} \times \text{extinction coefficient})$$

The extinction coefficient of mmol NADH at 340 nm is 6.3.

3.8. Determination of cell cycle distribution

The cells were cultured in the medium with or without EOD 402–804 μM for 48 h. Cell cycle distribution was determined by flow cytometry (Becton Dickinson, USA) as described by Sugiyama et al.[41]

3.9. Western blotting

Cells were cultured in the absence or presence of EOD 402 μM for 48 h. The total protein of the cells was prepared as described by Lipscomb et al.[42] Protein concentration of each lysate was determined by the Bradford protein assay.[43] Equal amount of total protein, 80 μg, was loaded on 7.5% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose transfer membrane (Amersham Pharmacia Biotech., USA). After blocking with 5% skimmed milk in PBS, 0.5% (v/v) Tween 20 for 1 h, the membrane was incubated with monoclonal P53 protein antibody (mouse anti-human) overnight at 4 °C, then incubated with HRP-linked secondary antibodies (goat anti-mouse) for 1 h at room temperature (RT), followed by color development with 0.06% DAB (diaminobenzidine) and 0.03% H_2O_2 in PBS for 3–5 min, RT. Distilled water was used to cease the reaction. Monoclonal anti-β-actin antibody (mouse anti-human) was used to ascertain that equal amount of protein was loaded. The relative quantity of P53 protein was analyzed by JEDA software (JEDA com. China).

3.10. Caspase activity assay

Caspase-3 and caspase-8 activity in A549 cells was assessed according to the instructions provided by BD Bioscience. Com. Briefly, 2×10^6 cells incubated with or without EOD 402 μM were lysed with lysis buffer and centrifuged at 10,000*g* for 10 min at 4 °C. The supernatants were mixed with reaction buffer containing DTT (10 mM) and caspase-specific substrate, and incubated for 1 h at 37 °C. Samples were read at 405 nm using a microplate reader. Comparing the reading of the test group and the control group allows determination of the fold increase in protease activity. In this assay, cells cultivated in the medium containing ethanol 0.045% (v/v) were used as a solvent control group.

3.11. Statistical analyses

Data were expressed as means ± SE and analyzed by *t*-test. Differences at $p < 0.05$ were considered statistically significant.

Acknowledgments

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