OSU-03012, a non-cox inhibiting celecoxib derivative, induces apoptosis of human esophageal carcinoma cells through a p53/Bax/cytochrome *c*/caspase-9-dependent pathway

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OSU-03012 is a celecoxib derivative devoid of cyclooxygenase-2 inhibitory activity. It was previously reported to inhibit the growth of some tumor cells through the AKT-signaling pathway. In the current study, we assessed the ability of OSU-03012 to induce apoptosis in human esophageal carcinoma cells and the mechanism by which this occurs. A cell proliferation assay indicated that OSU-03012 inhibited the growth of human esophageal carcinoma cell lines with an IC_{50} below 2 μmol/l and had the most effective cytotoxicity against Eca-109 cells. Terminal deoxynucleotidyl transferase-mediated nick-end labeling assay and flow cytometry analysis showed that OSU-03012 could induce the apoptosis in Eca-109 cells. After treatment of Eca-109 cells with 2 μmol/l OSU-03012 for 24 h, the apoptosis index increased from 14.07 to 53.72%. OSU-03012 treatment resulted in a 30–40% decrease in the mitochondrial membrane potential and caused cytochrome *c* release into the cytosol. Further studies with caspase-9-specific and caspase-8-specific inhibitors (z-LEHDfmk and z-IETDfmk, respectively) pointed toward the involvement of the caspase-9 pathway, but not the caspase-8 pathway, in the execution of OSU-03012-induced apoptosis. Immunoblot analysis demonstrated that OSU-03012-induced cellular apoptosis was associated with upregulation of Bax, cleaved caspase-3, and cleaved caspase-9. Ser-15 of p53 was phosphorylated after 24 h of treatment of the cancer cells with OSU-03012. This increase in p53 was associated with the decrease in Bcl-2 and increase in Bax. An inhibitor of p53, pifithrin-α, attenuated the anticancer effects of OSU-03012 and downregulated the expression of Bax and cleaved caspase-9. Altogether, our results show that OSU-03012 could induce apoptosis in human esophageal carcinoma cells through a p53/Bax/cytochrome *c*/caspase-9-dependent pathway. *Anti-Cancer Drugs* 24:690–698 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Esophageal cancer, the eighth most commonly occurring cancer and the sixth most common cause of cancer death worldwide, occurs at a very high frequency in certain areas of China [1]. Despite advances in detection and treatment, the prognosis for esophageal cancer is still poor, with low relative survival rates [2]. To date, cytotoxic chemotherapy has not been a standard treatment for esophageal cancer. These statistics highlight the need for the development of novel and effective chemopreventive and chemotherapeutic agents for esophageal cancer.

OSU-03012, a derivative of celecoxib that lacks cox-2 inhibitory activity, has been shown to induce rapid apoptosis in primary chronic lymphocytic leukemia cells, as well as in glioblastoma, pancreatic, and breast cancer cell lines [3–7]. The mechanism of action is presumably through inhibition of the 3-phosphoinositide-dependent kinase 1 (PDK1)/AKT-signaling pathway [8]. In addition to inhibition of PDK1/AKT signaling, OSU-03012 might also have effects on other important signaling pathways. For example, OSU-03012 has been shown to induce apoptosis by activation of the intrinsic mitochondrial pathway in primary chronic lymphocytic leukemia cells [3]. OSU-03012 also inhibited c-Jun NH_2-terminal kinases/signal transducers and activators of transcription and mitogen-activated protein kinase pathways in multiple myeloma cells [9]. Further, OSU-03012 has been reported to cause PDK1/AKT-independent cell death in glioma cells [4]. These findings suggest that OSU-03012 might be a multitargeted inhibitor that exerts its functions in a cell type-dependent manner. To the best of our knowledge, no reports have been published on the effect of OSU-03012 on apoptosis in human esophageal cancer cells. In the current study, we analyze whether OSU-03012 has antiproliferative effects on human esophageal cancer cells.

Apoptosis in response to cancer therapy proceeds through activation of the core apoptotic machinery, including the extrinsic cell-death receptor and the intrinsic mitochondrial-signaling pathway [10]. The major regulators of the intrinsic pathway are the prodeath and antideath members of the Bcl-2 family [11]. The intrinsic pathway

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is characterized by mitochondrial dysfunction with release of caspase activators, including cytochrome *c* [12]. This release of cytochrome *c* into the cytosol triggers caspase-3 activation through formation of the cytochrome *c*/Apaf1/caspase-9 apoptosome complex. Upon triggering, caspases (the final executioners of apoptosis) are activated, causing degradation of cellular proteins and leading to typical morphological changes such as chromatin condensation, nuclear shrinkage, and formation of apoptotic bodies [13]. Thus, mitochondria are increasingly appreciated as a target for cancer therapy.

Because the mechanism of action of OSU-03012 in human esophageal carcinoma has not been reported, we sought to shed light on this phenomenon. In the current study, we determine whether OSU-03012-induced apoptosis in human esophageal carcinoma cell lines is related to the mitochondria and explore the molecules related to the mitochondrial pathway.

Materials and methods

Reagents

OSU-03012 was obtained commercially (Selleck Chemicals, Houston, Texas, USA). It was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Solna, Sweden), and dilutions were made in RPMI-1640 to the desired in-vitro concentrations. The maximal concentration of DMSO did not exceed 0.1%, which would not affect cell growth. Pifithrin-α was purchased from Sigma Life Science (Saint Louis, Missouri, USA). The FITC-Annexin V Apoptosis Detection kit was purchased from BD Pharmingen (San Diego, California, USA).

Cell culture

Human esophageal carcinoma cell lines (Eca-109, TE-1, and TE-11) were obtained commercially (Keygen Biotech, Nanjing, China) and cultured in RPMI-1640 medium (HyClone, Logan, Utah, USA) containing 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in humidified atmosphere containing 5% CO_2.

IC_{50} determination by MTT assay

The effect of OSU-03012 on esophageal carcinoma cell viability was determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT; Sigma-Aldrich) assay, as described previously [14]. Briefly, the cancer cells were plated onto 96-well plates at a density of 5000 cells/well in RPMI-1640 medium. The cells were then treated with various concentrations of OSU-03012 (0, 2, 5, 10 μmol/l) and incubated at 37°C in a 5% CO_2 environment for 24 h. After the designated time period, 20 μl 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide was added to each well and the plates were incubated at 37°C for additional 3 h. The formazan crystals formed in the wells were dissolved in 100 μl DMSO. The absorbance was measured at 570 nm using a Spectra Max M_2 spectrophotometer (Molecular Devices, Sunnyvale, California, USA).

Apoptosis detection

To study whether the inhibition of cell proliferation by OSU-03012 was mediated by apoptosis, we used multiple methods to determine apoptosis. After treatment with 2 μmol/l OSU-03012 for 24 h, Eca-109 cells were harvested. Apoptosis was analyzed by terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL; Roche, Basel, Switzerland) performed according to the manufacturer's instructions. Hoechst 33258 (Keygen Biotech) was used to stain the nucleus. The percentage of apoptotic cells was calculated as the number of apoptotic cells compared with the total number cells on analyzing 1000 cells in randomly selected fields. We further chose the flow cytometry assay to detect apoptosis. The cells were cultured with OSU-03012 (2 μmol/l) for 24 h and then harvested for flow cytometry analysis as described previously [15]. Cells were analyzed with a FacsCalibur (Epics XL-4; Beckman Coulter, Brea, California, USA). The percentage of apoptotic cells was referred to as the apoptosis index.

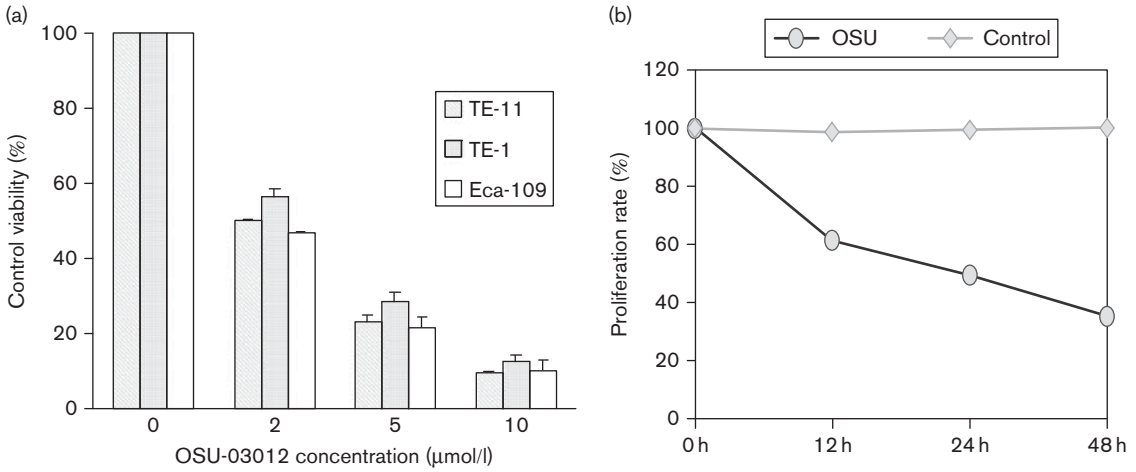
Determination of the mitochondrial transmembrane potential by flow cytometry

The mitochondrial transmembrane potential ($\Delta\psi_m$) was determined by flow cytometry using the $\Delta\psi_m$-sensitive dye, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; Calbiochem, Darmstadt, Germany) [16], as described previously [17]. Briefly, Eca-109 cells were treated with 2 μmol/l OSU-03012 for 24 h and then harvested, washed once in PBS, resuspended in 1 × PBS, and incubated with 1 μmol/l JC-1 at 37°C for 10 min. Stained cells were then washed once in PBS and analyzed by flow cytometry. JC-1 monomers emit at 527 nm (FL-1 channel) and 'J aggregates' emit at 590 nm (FL-2 channel). Valinomycin-treated cells were used for compensation (FL-1–FL-2), and a cytofluorometric profile from these cells defined the 590-nm cutoff for untreated versus treated cells.

Protein extraction and western blot analysis

The effect of treatment with OSU-03012 on the expression of proteins related to the mitochondrial pathway was determined to gain insight into the mechanism of OSU-03012-induced cell death. Whole-cell extracts were prepared in lysis buffer as described previously [18]. To detect the expression of cytochrome *c*, the mitochondrial and cytosolic fractions were prepared as reported earlier [19]. Equal amounts of protein were subjected to electrophoresis on 8–15% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell BioScience GmbH, Dassel, Germany) by electroblotting. The membranes were incubated with various antibodies. The antibodies used were rabbit anti-human Bax, Bcl-2, caspase-9, caspase-8,

Fig. 1



OSU-03012 inhibits the growth of EC cell lines in a dose-dependent and time-dependent manner. Results of the MTT assay showing the percentage of viable EC cell lines (Eca-109, TE-1, and TE-11) treated with 0, 2, 5, and 10 μmol/l OSU-03012 for 24 h; OSU-03012 had the most effective cytotoxicity against Eca-109 cells (a). Eca-109 cells were treated with 2 μmol/l OSU-03012 for 12, 24, and 48 h in medium containing 10% FBS (b). The proliferation rate decreased to 35.23% in the test group, whereas it remained almost unchanged in the controls after treatment for 48 h. Columns, mean of three independent experiments; bars, SD. EC, esophageal cancer; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

and caspase-3 (1 : 1000 dilution; Immunoway, Newark, New Jersey, USA), mouse anti-human PARP (1 : 500 dilution; Abcam) and anti-cytochrome *c* (1 : 500 dilution; Abcam, Cambridge, Massachusetts, USA), and mouse anti-human phospho-p53 (1 : 1000 dilution; Cell Signaling Technology, Danvers, Massachusetts, USA). A secondary antibody to IgG conjugated to horseradish peroxidase (1 : 5000 dilution; Abcam) was used. β-Actin and Cox4 (1 : 5000 dilution; Abcam) were used as controls to demonstrate equal loading and transfer of protein. Protein bands were detected using an enhanced chemiluminescence reagent (Sigma, Ronkonkoma, New York, USA).

Viability assay after treatment with a specific caspase-9 or caspase-8 inhibitor

To determine the relative contribution of caspase-9 and caspase-8 pathways to OSU-03012-induced apoptosis, the effects of the caspase-9-specific inhibitor, z-LEHDfmk (Dublin, California, USA), and the caspase-8-specific inhibitor, z-IETDfmk (Dublin, California, USA), on OSU-03012-induced apoptosis were determined. Eca-109 cells were treated with or without a 2 h pretreatment with the caspase-9-specific inhibitor, z-LEHDfmk (40 μmol/l), or the caspase-8-specific inhibitor, z-IETDfmk (40 μmol/l). Then the cells were cultured with OSU-03012(2 μmol/l) for 24 h and cell survival was determined using MTT assays [14].

Viability assay after treatment with a specific p53 inhibitor

Eca-109 cells were treated with 2 μmol/l OSU-03012 with or without 100 μmol/l pifithrin-α for 24 h. The effect of the treatment on proliferation of the Eca-109 cells was determined by conducting the MTT assays as described above. The expression of Bax, Bcl-2, and cleaved caspase-9 was determined by western blot analysis as described above.

Statistical analysis

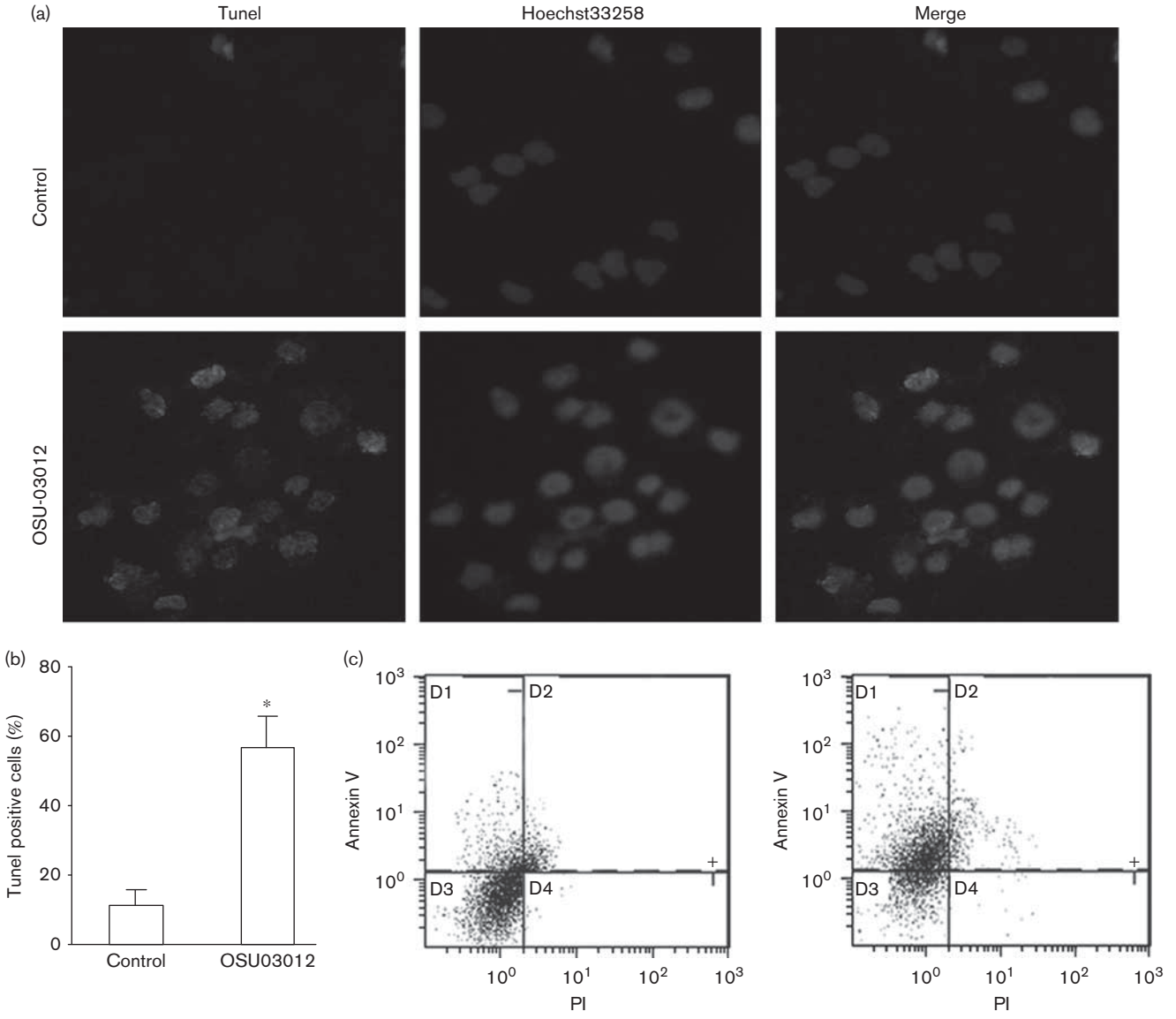
SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA) was used for all statistical analysis. Statistical significance was assessed by comparing mean values (±SD) using Student's *t*-test for independent groups. *P* less than 0.05 was considered to be significant.

Results

OSU-03012 inhibited growth of human esophageal carcinoma cells

The in-vitro activity of OSU-03012 against esophageal carcinoma cells was assessed in human esophageal carcinoma cell lines, Eca-109, TE-1 and TE-11, and evaluated after 24 h of exposure to drug. Cells were grown in the absence or the presence of different concentrations (0–10 μmol/l) of OSU-03012, and cytotoxicity was measured by the MTT assay. As shown in Fig. 1a, OSU-03012 inhibited the growth of all tested cells in a similar dose-dependent manner with IC_{50} below 2 μmol/l. Because OSU-03012 had the most effective cytotoxicity against Eca-109 cells, Eca-109 cells were chosen for the subsequent experiments. OSU-03012 treatment also resulted in a time-dependent inhibition of Eca-109 cell proliferation (Fig. 1b).

Fig. 2



Apoptosis is detectable in OSU-03012-treated Eca-109 cells. (a) After treatment with or without 2 μmol/l OSU-03012 for 24 h, Eca-109 cells were fixed for the TUNEL assay (× 200). (b) The histogram shows the rates of TUNEL-positive cells (*$P<0.05$ vs. control). Flow cytometry analysis was also used to detect apoptosis. Eca-109 cells in the logarithmic growth phase were treated with 2 μmol/l OSU-03012 for 24 h. (c) Cells were harvested, fixed, stained with Annexin V/propidium iodide, and then subjected to flow cytometric analysis. Results shown are representative of three independent experiments. PI, propidium iodide; TUNEL, transferase-mediated dUTP nick-end labeling.

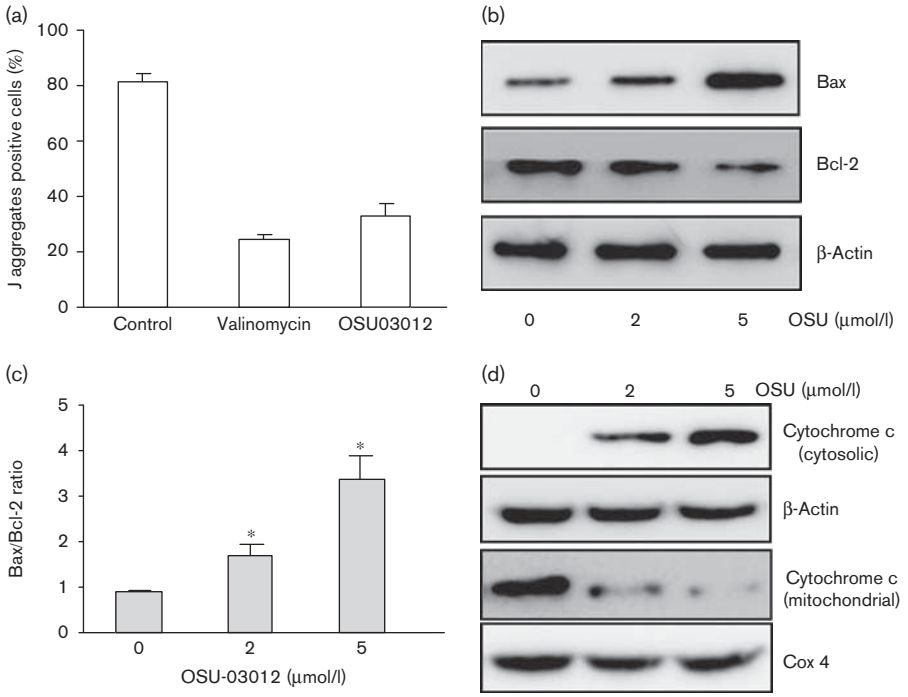
OSU-03012-induced apoptosis in human esophageal carcinoma cells

Because OSU-03012 has been reported to induce apoptosis in some cancer cells, we investigated whether it could induce apoptosis in Eca-109 cells by the TUNEL assay. As shown in Fig. 2a, apoptotic cells were detected after 24 h of treatment with OSU-03012. Control Eca-109 cells showed 11.3 ± 3.5% TUNEL-positive cells, whereas Eca-109 cells treated with 2 μmol/l OSU-03012 showed 56.9 ± 5.2% TUNEL-positive cells (Fig. 2b). This finding was further confirmed by flow cytometric analysis with annexin V/propidium iodide staining. As can be seen in Fig. 2c, after treatment of Eca-109 cells with 2 μmol/l OSU-03012 for 24 h, the apoptosis index was increased from 14.07 to 53.72%.

OSU-03012-induced apoptosis in Eca-109 cells is associated with the loss of mitochondrial membrane potential

Loss of mitochondrial membrane potential provides an early indication for the initiation of cellular apoptosis. We measured the cellular mitochondrial membrane potential

Fig. 3



Effect of OSU-03012 on $\Delta\psi_m$, the Bcl-2 family, and cytochrome *c*. Mitochondrial membrane potential was measured using the potentiometric dye, JC-1. (a) Treatment of Eca-109 cells with OSU-03012 (2 μmol/l) for 24 h resulted in a significant reduction in $\Delta\psi_m$ in comparison with control cells (*$P<0.05$). Eca-109 cells treated with valinomycin were used as a positive control. The results were expressed as the mean±SEM of three independent experiments. Western blots were detected with antibodies against Bax, Bcl-2, and β-actin. (b) A representative blot from three independent experiments is shown. The densitometric analysis of Bax and Bcl-2 bands was carried out using UN-SCAN-IT software, and the data (relative density normalized to β-actin) were plotted as the ratio of Bax to Bcl-2. (c) The data are expressed as mean±SEM of three independent experiments (*$P<0.05$ vs. control). The cytosolic and mitochondrial fractions were prepared and immunoblotted. (d) Western blots were detected with antibodies against cytochrome *c*, β-actin, and Cox4.

in Eca-109 cells after treatment with OSU-03012 (2 μmol/l). We found that treatment with OSU-03012 resulted in a 30–40% decrease in the mitochondrial membrane potential (Fig. 3a). This triggers activation of downstream events that lead to apoptosis.

OSU-03012 treatment results in an increase in the Bax/Bcl-2 ratio

Bcl-2 is an antiapoptotic protein that is associated with the mitochondrial membrane. A decrease in the mitochondrial membrane potential led us to determine the effect of OSU-03012 on Bcl-2 expression. Expression of Bax and Bcl-2 in tumor cells was analyzed using western blot analysis. As shown in Fig. 3b, treatment of Eca-109 cells with OSU-03012 was found to result in a dose-dependent increase in the level of Bax and a decrease in level of Bcl-2. Densitometry analysis of the bands revealed that treatment of Eca-109 cells with OSU-03012 resulted in a dose-dependent increase in the Bax/Bcl-2 ratio (Fig. 3c).

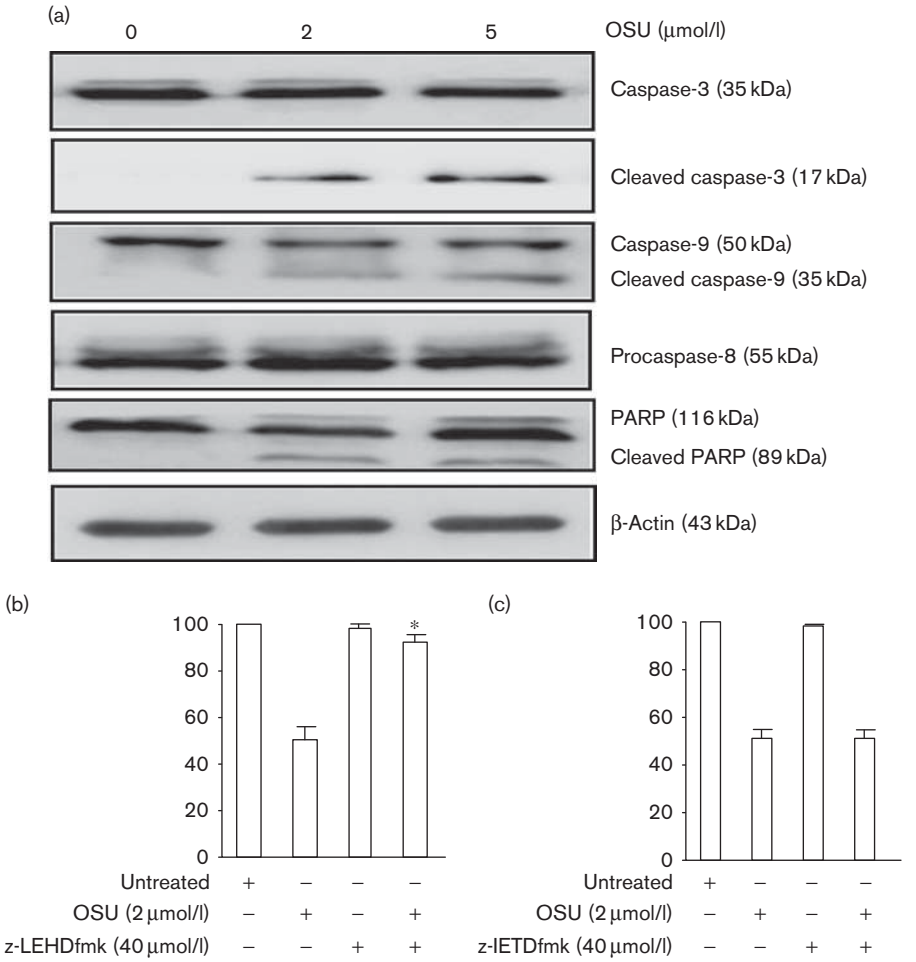
Alteration in cytochrome *c* during OSU-induced apoptosis

A change in Bax/Bcl-2 determines the commitment of cells to triggering mitochondrial release of cytochrome *c* into the cytosol [19]. We then measured the extent of mitochondrial release of cytochrome *c* using a subcellular fractionation method. As shown in Fig. 3d, treatment of Eca-109 cells with OSU-03012 induced the release of cytochrome *c* from the mitochondrial to the cytosolic compartment.

OSU-03012-induced apoptosis was mediated by a mitochondrial pathway involving activation of caspase-9

As many antineoplastic drugs induce apoptosis of cancer cells through mitochondrial apoptotic pathways [20–23], we further examined the key protein in mitochondrial pathway. As shown in Fig. 4a, treatment with OSU-03012 was found to result in a significant increase in the active form of caspase-9, caspase-3, and proteolytic cleavage of poly (ADP-ribose) polymerase (PARP). However, the active form of caspase-8 was not found after treatment of Eca-109 cells with OSU-03012 (data not shown). To address whether OSU-03012-induced apoptosis was mediated by a mitochondrial pathway involving the activation of caspase-9 or a death receptor pathway involving caspase-8, the specific caspase inhibitor of caspase-8 or caspase-9 was used during OSU-03012 treatment. As shown in Fig. 4b, the caspase-9 inhibitor significantly rescued Eca-109 cells from the

Fig. 4



OSU-03012 mediates Eca-109 cell apoptosis through a Bax/cytochrome *c*/caspase-9-dependent pathway. Total cell lysates were prepared and immunoblotted. (a) Western blots were detected with antibodies against caspase-3, caspase-8, caspase-9, PARP, and β-actin. A representative blot is shown from three independent experiments. Eca-109 cells were incubated with the caspase-9-specific inhibitor z-LEHDfmk and the caspase-8-specific inhibitors z-IETDfmk, as a single treatment or in combination with OSU-03012 for 24 h, and cell survival was measured using the MTT assay. Columns are the mean of three independent experiments and the bars represent the mean±SEM. (b) The caspase-9 inhibitor significantly rescued Eca-109 cells from the inhibitory effects of OSU-03012 (*$P<0.05$), whereas (c) the caspase-8 inhibitor had no effects. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PARP, proteolytic cleavage of poly (ADP-ribose) polymerase.

inhibitory effects of OSU-03012 (*$P < 0.05$), whereas the caspase-8 inhibitor had no effect on the inhibitory effect of OSU-03012 on Eca-109 cells (Fig. 4c).

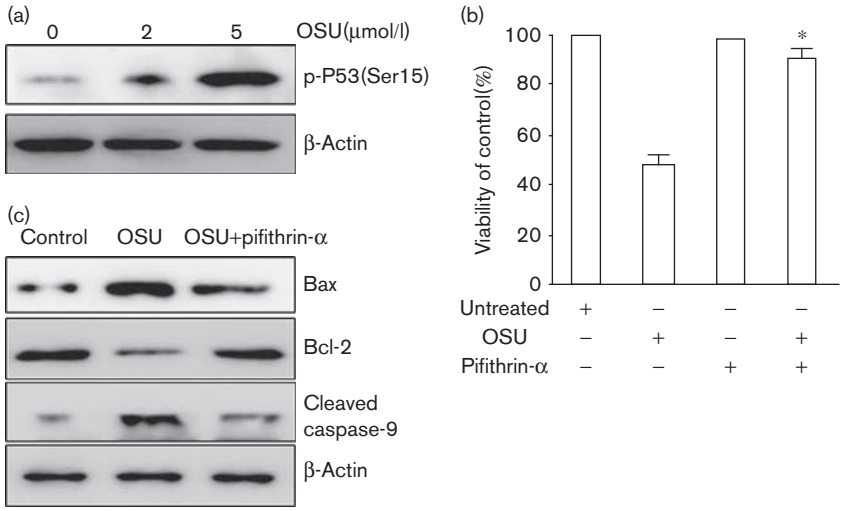
OSU-03012 activates p53

The tumor suppressor p53, a transcriptional factor, plays a key role in regulating cell death and various processes in the cell. The cellular processes regulated by p53 include cell cycle, apoptosis, DNA repair, and senescence. As OSU-03012 induced apoptosis in esophageal carcinoma cells, we decided to examine its effect on p53 activation. OSU-03012 induced a rapid increase in the phosphorylation of p53 protein on Ser-15 (Fig. 5a). These data indicate an activation of the p53 pathway. Inhibition of p53 using pifithrin-α, a specific inhibitor [24], resulted in an almost complete reversal of OSU-03012-induced apoptosis in the cancer cells (Fig. 5b), downregulating the expression of Bax and cleaved caspase-9 and up-regulating the expression of Bcl-2 (Fig. 5c).

Discussion

Recently, much work has been performed to show that OSU-03012, a novel celecoxib derivative, does not affect COX-2 activity, yet induces apoptosis through an AKT/PDK pathway. In the current study, we analyzed the molecular mechanism of esophageal carcinoma cell death induction triggered by OSU-03012. Our data suggest that OSU-03012 has potent cytotoxic activity against human esophageal carcinoma cell lines *in vitro*. It significantly inhibited proliferation and induced apoptosis in Eca-109 cells.

Fig. 5



OSU-03012 mediates Eca-109 cell apoptosis through the activation of P53. (a) After treating Eca-109 cells with OSU-03012(2 μmol/l) for 24 h, the cells were harvested and lysates were analyzed by western blotting for expression of phosphorylated p53. Eca-109 cells were incubated with OSU-03012 in the presence or absence of pifithrin-α for 24 h, and cell survival was measured using the MTT assay. (b) The P53 inhibitor significantly rescued Eca-109 cells from the inhibitory effects of OSU-03012 (*$P<0.05$), downregulating the expression of Bax and cleaved caspase-9 and (c) up-regulating the expression of Bcl-2. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

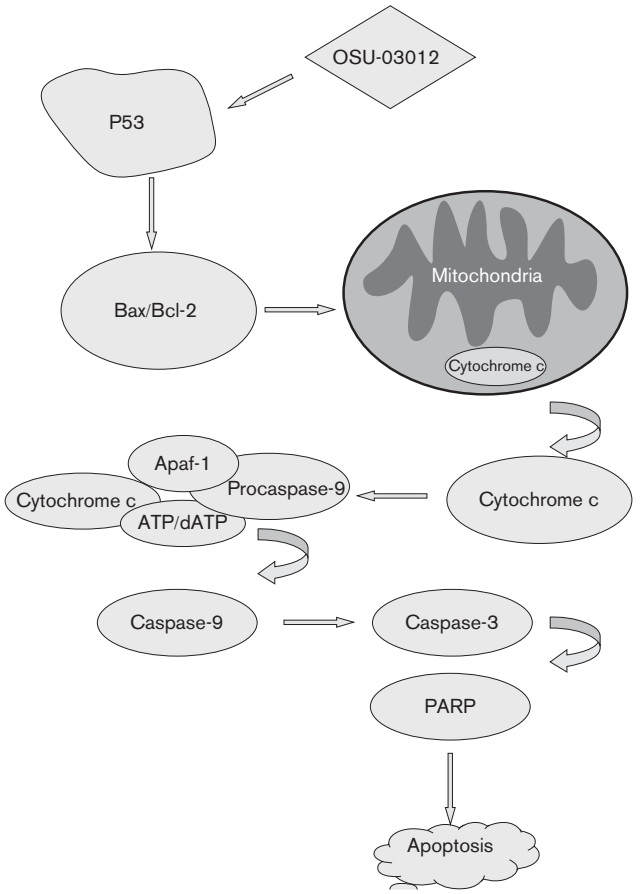
It is reported that the mitochondria plays an important role in the regulation of drug-induced esophageal carcinoma cell death [25]. Impairment of mitochondrial function may lead to apoptotic cell death [26]. Caspases are aspartate-specific cysteine proteases that play critical roles in apoptosis [27–30]. Activation of caspases results in cleavage and inactivation of key cellular proteins, including the DNA repair enzyme PARP [28]. In the mitochondrial pathway of apoptosis, caspase activation is closely linked to mitochondrial outer membrane permeabilization [31] and the mitochondrial permeability transition plays a key role in the regulation of apoptosis [32–35]. Caspase-3 is an important apoptosis protein that is activated initially in both Fas/FasL and the mitochondrial pathway. Our data showed that there was a clear increase in the 17 kDa cleaved fragment of caspase-3, indicating the activation of caspase-3 in the apoptotic process. To address whether caspase-3 is activated by a mitochondrial pathway involving the activation of caspase-9 or a death receptor pathway involving caspase-8, the specific caspase inhibitor of caspase-8 or caspase-9 was used during OSU-03012 treatment. These results pointed toward the involvement of the caspase-9 pathway, but not the caspase-8 pathway, in execution of OSU-03012-induced apoptosis.

In the mitochondria-dependent pathway of apoptosis, both cytosolic protein Apaf1 and cytochrome *c* participate in the activation of caspase-9, which in turn processes procaspase-3 to generate active caspase-3 [36]. As an apoprotein, cytochrome *c* is nearly undetectable in the cytosol of normal cells [37]. However, the release of cytochrome *c* from the mitochondria to the cytosol can be induced during apoptosis when the mitochondrial pathway is involved. To further assess whether cytochrome *c* was released from the mitochondria to the cytosol, we examined the level of cytochrome *c* in the cytosol and the data revealed that cytochrome *c* was markedly upregulated on treatment with OSU-03012.

Bax has a proapoptotic effect and causes release of cytochrome *c* [38–40] and an increase in outer membrane permeability [41]. During the apoptotic process, the increased expression of Bax can induce apoptosis by suppressing the activity of Bcl-2 [42], and the ratio of Bcl-2 to Bax, rather than Bcl-2 alone, was more important for survival during drug-induced apoptosis [43]. Our data showed that there was an increase in Bax expression, a decrease in Bcl-2 expression, and a reduction in mitochondrial membrane potential, which is suggestive of permeabilization of the mitochondrial outer membrane in Eca-109 cells treated with OSU-03012.

Our data also demonstrate that OSU-03012 treatment results in the activation of p53. Further, inhibition of p53 attenuates the ability of OSU-03012 to induce apoptosis in the esophageal carcinoma cells. These observations support the inference that activation of p53 is the major mechanism by which OSU-03012 inhibits the proliferation of esophageal carcinoma cells. Phosphorylation of Ser-15 of p53 interferes with the interaction between this protein and its negative regulator, MDM2 [44–46]. As a result, ubiquitination and subsequent degradation of p53 is inhibited [47]. Activation of p53 decreased the

Fig. 6



Model of the molecular mechanisms of OSU-03012-induced apoptosis. OSU-03012 activates P53, and this in turn decreases the expression of Bcl-2 and activates Bax to form Bax multimers. Bax induces mitochondrial damage and release of cytochrome *c*. The apoptosome recruits and processes caspase-9 to form a holoenzyme complex. This cascade activates the downstream executioner caspase-3, resulting in apoptosis. PARP, proteolytic cleavage of poly(ADP-ribose) polymerase.

expression of Bcl-2 [48], a result we have also observed in OSU-03012-treated Eca-109 cells. Thus, activation of p53 is likely a key molecular event that triggers the apoptotic pathway in the esophageal carcinoma cells.

Apparently, OSU-03012 induces cancer cell death through the mitochondrial pathway, as evidenced by the loss of mitochondrial membrane potential and the release of mitochondrial cytochrome *c*. The association of the cytosolic cytochrome *c* with Apaf1 and procaspase-9 contributes to the formation of an apoptosome, leading to the activation of caspase-9, as observed by others [49], which then activates effector caspases such as caspase-3, thus resulting in apoptosis. On the basis of the results of our investigation, we proposed a schematic model (Fig. 6) to show how OSU-03012 changed different molecular events, leading to apoptosis in human esophageal cancer cells: OSU-03012 activates p53, and this in turn decreases the expression of Bcl-2 and activates Bax to form Bax multimers. Bax induces mitochondrial damage and release of cytochrome *c*, which is followed by subsequent apoptosis through caspase-3.

Taken together, our present study demonstrated that OSU-03012 is likely to mediate cell death by acting on a p53/Bax/cytochrome *c*/caspase-9-dependent pathway. These results suggested that the mitochondrial apoptotic pathway at least partially promoted the beneficial effect of OSU-03012 therapy against esophageal carcinoma. The observations outlined in our studies demonstrate that OSU-03012 has the potential to be developed as a chemopreventive and chemotherapeutic agent for human esophageal carcinoma. In the future, studies focusing on cell signaling and the biological significance of OSU-03012-induced apoptosis and cell cycle arrest would lead to the exploration of the mechanisms of chemotherapeutic potency of OSU-03012 in human cancer.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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