EPOX Inhibits Angiogenesis by Degradation of Mcl-1 through ERK Inactivation

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Abstract

Purpose: Antiangiogenic therapy is considered as an effective strategy for controlling the growth and metastasis of tumors. Among a myriad of biological activities described for xanthone derivatives, the anticancer activity is quite remarkable, but the molecular mechanism is not clearly resolved. In the present study, we investigated the antiangiogenic mechanism of 3,6-di(2,3-epoxypropoxy)xanthone (EPOX), a novel Mcl-1 targeting drug.

Experimental Design: To evaluate the antiangiogenic activity of EPOX, we did cell viability, cell cycle, tube formation assay in vitro, and Matrigel plug assay in vivo. To evaluate the effect of EPOX on the endothelial signaling pathway, we did immunoblotting, immunoprecipitation, and immunofluorescence analysis. Intracellular glutathione levels were determined with the use of monochlorobimane, a glutathione-specific probe.

Results: EPOX induced endothelial cell apoptosis in association with proteasome-dependent Mcl-1 degradation. Down-regulation of Mcl-1 resulted in an increase in Mcl-1–free Bim, activation of Bax, and then signaling of mitochondria-mediated apoptosis. Additionally, glutathione depletion and extracellular signal-regulated kinase (ERK) inactivation was observed in EPOX-treated cells. Glutathione supplementation reversed the inhibitory effects of EPOX on ERK, which increases the phosphorylation of Mcl-1 at T163. Overexpression of mitogen-activated protein/ERK kinase (MEK) partially reversed the effect of EPOX on Mcl-1 dephosphorylation, ubiquitination, and degradation, further implicating ERK in the regulation of Mcl-1 stability.

Conclusions: This study provides evidence that EPOX induces glutathione depletion, ERK inactivation, and Mcl-1 degradation on endothelial cells, which leads to inhibition of angiogenesis. Our results suggest that EPOX is a novel antiangiogenic agent, making it a promising lead compound for further development in the treatment of angiogenesis-related pathologies.

Angiogenesis, namely neovascularization from preexisting vasculature, is necessary for continued growth and metastasis of tumors (1). Angiogenesis-related molecules, such as vascular endothelial growth factor and vascular endothelial growth factor receptor, have been suggested to be ideal targets, and a large number of inhibitors have been developed. Apart from angiogenesis inhibitors, so-called vascular disrupting agents targeted at the established tumor vasculature are currently being investigated in clinical trial. Recently, we revealed that an endostatin-cytosine deaminase fusion protein possesses the tumor vessel–targeting property of endostatin to allow selective tumor-killing effect of cytosine deaminase, producing potent antitumor activity (2).

Endothelial cell apoptosis is intimately involved in the sculpting of blood vessels (3). Natural inhibitors of angiogenesis, such as endostatin, angiostatin, and thrombospondin-1, act in part through selective induction of endothelial cell apoptosis to promote active vessel regression (4). Metronomic chemotherapy, defined as the frequent administration of chemotherapeutic drugs at doses significantly below the maximally tolerated doses,
EPOX Induces Endothelial Cell Apoptosis

Translational Relevance

Although some antiangiogenic drugs have been approved in the treatment of cancer, the survival benefits of such drugs are relatively modest. Finding new drugs and understanding the mechanism governing angiogenesis are therefore essential for therapeutic improvements. We found that 3,6-di(2,3-epoxypropoxy)xanthone (EPOX) was the most effective in inhibiting endothelial cell proliferation among a series of xanthone derivatives. The antiangiogenic actions of EPOX are proposed to involve the inhibition of extracellular signal-regulated kinase in response to glutathione depletion leading to the degradation of Mcl-1, and then the stimulation of mitochondria-mediated apoptosis in response to an increase in Mcl-1–free Bim. Our study clearly shows the mechanism of EPOX and represents the most thorough investigation to date of the biological consequence of Mcl-1 inhibition in endothelial cells. Furthermore, we proposed that therapies designed to manipulate reduced glutathione content, the extracellular signal-regulated kinase/Mcl-1 pathway, and crosstalk among Bcl-2 members could be a new direction for antiangiogenic therapy.

Materials and Methods

Reagents.

Xanthone derivatives were provided by Chun-Nan Lin (Kaohsiung Medical University, Kaohsiung, Taiwan). EPOX was synthesized by a method described previously (14). Briefly, 3,6-dihydroxanthone was reacted with one equivalent of NaOH in aqueous 2-propanol and an excess of epichlorohydrin to yield the EPOX. The melting point of EPOX is 187°C to 188°C. Anti-Bax 6A7 and all chemicals, unless specifically mentioned, were ordered from Sigma. 4,6-Diamidino-2-phenylindole, anti-HA, were purchased from Roche Diagnostics Corp. Rhodamine 123, anti–complex IV, and MitoTracker were obtained from Molecular Probes. The caspase inhibitor Z-VAD-fmk was purchased from Alexis. Caspase-3 antibody was from Imgenex. Antibodies against caspase-8, caspase-9, cleaved caspase-3, cleaved poly (ADP-ribose) polymerase, phospho and total extracellular signal-regulated kinase (ERK), phospho and total GSK-3β, and phospho and total c-Jun-NH2-kine (JNK) were purchased from Cell Signalling Technologies. Cytochrome c and Mcl-1 antibodies came from BD Pharmingen. Antibodies against Bcl-2, Bcl-xl, Bak, Bik, actin, and α-tubulin were obtained from Santa Cruz Biotechnology. Antibodies against phosphorylated Thr 163 of Mcl-1 were generated by Bethyl Laboratories. Antibodies against phosphorlated Thr 163 of Mcl-1 were kindly provided by Hsin-Fang Yan-Yen (Academia Sinica, Taipei, Taiwan). Constitutively active mitogen-activated protein (MAP)/ERK kinase (MEK) was a gift from Pier Paolo Pandolﬁ (Harvard Medical School, Boston, MA).

Cell culture and transfection.

HUVECs were obtained from human umbilical cord veins and cultured as previously described (17). The endothelial-like EAhy926 cells were maintained in DMEM supplemented with 100 μmol/L sodium hyposxanthine, 0.4 μmol/L aminopterin, and 16 μmol/L thymidine. These cells retain the properties of endothelial cells, including expression of factor VIII–related antigen and tube-forming activity (18). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Small interfering RNA (siRNA)-mediated down-regulation of Bim was done by transfecting SMARTpool specific or nonspecific control double-stranded RNA oligonucleotides (Dharmacon). For the EPOX treatment, HUVECs were seeded and then incubated with 3 μmol/L EPOX unless specifically mentioned.

Evaluation of cell viability and identification of apoptosis.

The percentage of surviving cells after treatment was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (19). Cell proliferation was determined with the use of bromodeoxyuridine labeling and detection kit (Amersham Biosciences) following manufacturer’s guidelines. Cell cycle distribution was assessed by a flow cytometry assay as described (19). For the morphologic examination, cells were examined under a phase contrast microscope (Nikon). To identify apoptotic cells, the cells were inspected by 4′,6-diamidino-2-phenylindole staining (DAPI) as previously described (19). For a quantitative assessment of oligonucleosomal DNA fragmentation, apoptosis was detected through a Cell Death ELISA PLUS kit (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Measurement of mitochondrial membrane potential was determined as detailed...
elsewhere (20). The caspase colorimetric assay kits specific for caspase-3, caspase-8, or caspase-9 (Biovision) were used to detect caspase activation.

**Tube formation assay.** Tube formation assay was done as previously described (2). In brief, 3 × 10³ endothelial cells were seeded on a layer of polymerized Matrigel (BD Biosciences) in a 96-well plate. All assays were done in triplicate. Cells were incubated for 6 h and viewed under a microscope. Tube formation was quantified by measuring the long axis of each tube in three random fields per well with the use of Image-Pro Plus software.

**In vivo Matrigel plug assay.** In vivo Matrigel plug assay was done as previously described (17). In brief, C57BL/6J mice were given s.c. injections of 0.5 mL of Matrigel (BD Biosciences) mixed with endothelial growth medium 2 (EGM2; Clonetics) growth medium. Matrigel pellets were harvested on day 7 and were processed for Masson’s trichrome staining. Hemoglobin was measured as an indication of blood vessel formation with the use of the Drabkin method (Sigma-Aldrich).

**Tumor xenografts.** For HCT-116 cancer cell xenograft experiments, male athymic nude mice were inoculated s.c. with 10⁷ cells per mouse. When the tumors reached the average volume of 50 mm³, the mice were randomized into two groups (n = 5 each). EPOX oral treatment at a dose of 50 mg/kg was given daily. Tumors were measured with a caliper every 3 to 4 d, and their volumes were calculated with the formula: 0.5 L × W², where L and W are the long and short diameters of the tumor, respectively. For histologic analysis, the xenograft tissue samples were stained with CD31 antibody to permit determination of the number of blood vessels. All animal experiments were done in accordance with institutional guidelines for animal welfare.

**Immunoblotting and immunoprecipitation.** Immunoblotting and immunoprecipitation were done essentially as previously described (21). For quantification of the Western analysis, the density of each band was quantified by ImageQuant software and indicated below the blots. Enriched mitochondrial fraction was isolated according to protocols from Yamaguchi et al. (22). For detection of Bax conformational change, cells were lysed with Chaps lysis buffer (10 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 1% Chaps) and subjected to immunoprecipitation with anti-Bax 6A7 as described (22).

**Detection of glutathione by monochlorobimane.** The content of glutathione was determined with the use of monochlorobimane, a permeable probe that forms blue fluorescent adducts with the reduced form of GSH. To determine the GSH level, after treatment cells in 96-well plates were incubated with 100 μmol/L monochlorobimane for 30 min in the dark. Then, plates were read on a microplate reading fluorometer with excitation and emission wavelengths of 390 and 460 nm, respectively. For imaging the intracellular GSH, cells seeded into a 4-chamber well slide were incubated with monochlorobimane as described above. Cells were fixed for detecting the fluorescence images with a fluorescence microscope (Nikon).

**Immunofluorescence analysis.** Cells were treated with EPOX for 6 h. After incubation with 25 nmol/L MitoTracker Red CMXRos (Molecular Probes) for 30 min, cells were fixed with ice-cold methanol for 15 min at 4°C and blocked for 30 min with 3% bovine serum albumin. Cells were then incubated with anti-Bax (1:100) overnight at 4°C, followed by incubation at 37°C for 1 h with FITC-conjugated secondary antibody. The slide chamber was mounted onto glass slides for microscopic observation with a Leica TCS SP2 confocal spectral microscope.

**Statistical analysis.** Each experiment was done at least thrice, and representative data are shown. Data in the bar graph are given as the means ± SE. The means were checked for statistical difference with the use of a t-test, and P values <0.05 were considered significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

**Results**

EPOX inhibits angiogenesis in vitro and in vivo. To test the hypothesis that EPOX may inhibit angiogenesis, initially we determined the effect of EPOX with the Matrigel plug assay. The Matrigel plugs in the control groups were visually bloodier than the EPOX-treated ones, suggesting a higher level of angiogenesis (Fig. 1A, top). Histologic analysis of the Matrigel pellets identified more erythrocyte-containing vessels within the control than in the EPOX-treated (Fig. 1A, middle). Quantification of angiogenesis by hemoglobin content further showed that EPOX inhibited angiogenic response in a dose-dependent manner (Fig. 1A, bottom), suggesting that EPOX attenuated angiogenesis in vivo. We proceeded to examine whether EPOX inhibits tube formation, which is a well-accepted technique to measure in vivo angiogenesis. Treatment with EPOX significantly reduced this process (Fig. 1B).

In an attempt to discover the mechanism of EPOX to inhibit angiogenesis, we first did MTT and bromodeoxyuridine incorporation assays to measure the effect of EPOX on the growth of HUVECs. EPOX was found to decrease cell viability (Fig. 1C) and DNA synthesis (Supplementary Fig. S1C) in a concentration-dependent manner. The EAHy926 cell line has been suggested to be an alternative model for HUVECs to study the control of pathologic angiogenesis by endogenous factors and pharmacologic compounds (23). Consistently, EPOX inhibited EAHy926 cell growth (Supplementary Fig. S2A) and tube formation (Supplementary Fig. S2B).

To examine whether EPOX-induced growth inhibition was associated with cell cycle regulation, the cell cycle distributions of EPOX-treated cells were analyzed by flow cytometry. As illustrated in Fig. 1D, EPOX treatment increased the percentage of cells in the sub-G1 phase. EPOX-treated HUVECs exhibited both rounding and blebbing during morphologic examination (Supplementary Fig. S2C, top). To verify the occurrence of apoptosis, cells were inspected after DAPI staining. In the absence of EPOX, cells exhibited nuclei with homogeneous chromatin distribution. However, EPOX treatment induced nuclear fragmentation and chromatin condensation (Supplementary Fig. S2C, bottom). DNA fragmentation was also increased in EPOX-treated cells in a concentration-dependent manner (Supplementary Fig. S2D). Taken together, the results suggest that EPOX treatment induces apoptosis of endothelial cells.

EPOX treatment activates the mitochondria-mediated apoptosis pathway. The activation of selected caspases serves to identify the involvement of specific apoptotic signaling pathways. To further characterize the mechanism through which EPOX triggers apoptosis, the activation states of caspase-3, caspase-8, and caspase-9 were therefore examined. Notably, EPOX treatment resulted in the cleavage and activation of caspase-9 and caspase-3 in HUVECs (Fig. 2A).

Caspase-9 has been proposed as the predominant initiator caspase in the mitochondria-dependent intrinsic apoptosis pathway. In response to apoptotic stimuli, dissipation of the mitochondrial membrane potential results in the release of cytochrome c from the mitochondrial intermembrane space. Upon release to the cytosol, cytochrome c binds Apaf-1, and then recruits and activates procaspase-9. The mitochondrial membrane potential and release of cytochrome c from the mitochondria to the cytosol were therefore measured in untreated and EPOX-treated HUVECs. Treatment with EPOX decreased the mitochondrial membrane potential in a time-dependent manner (Fig. 2B). As shown in Fig. 2C, the efflux of cytochrome c from mitochondria was detected in EPOX-treated cells simultaneously with the accumulation of cytochrome c in the cytosol. Taken together, these findings are consistent with the
hypothesis that EPOX induces apoptosis in HUVECs through a mitochondria-mediated pathway.

**EPOX treatment induces rapid proteasome-dependent degradation of Mcl-1.** Bcl-2 proteins, a family that includes both proapoptotic and antiapoptotic members, are critical regulators of the mitochondrial pathway of apoptosis. Proapoptotic members, such as Bax and Bak, promote the release of cytochrome c from the mitochondria, whereas antiapoptotic members, such as Bcl-2, Bcl-xL, and Mcl-1, prevent release. The balance of proapoptotic and antiapoptotic Bcl-2 proteins therefore decides the fate of the cell. Because the above findings reveal that EPOX activates the mitochondrial pathway of cell apoptosis, it was of interest to identify the Bcl-2 family members involved in the apoptosis triggered by EPOX. EPOX treatment induced the rapid down-regulation of Mcl-1, whereas no significant changes in expression of Bcl-2, Bcl-xL, Bax,

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**Fig. 1.** EPOX inhibits angiogenesis. A, antiangiogenesis effect of EPOX in in vivo Matrigel plug assay. Top, macroscopic appearance of Matrigel plugs after harvest; middle, Masson's trichrome staining of cross-sections from Matrigel plugs; bottom, quantitation of active vasculature inside the Matrigel by measurement of hemoglobin content. Hemoglobin was quantified and presented compared with the control. B, tubular morphogenesis was blocked by EPOX. Tube length was quantified by measuring the length of tubes in three randomly chosen fields from each well with the use of Image-Pro Plus software and was calculated against DMSO control. C, viability measurements. Cells were treated with the indicated concentrations of EPOX for 24 or 48 h. Cell number was determined by the MTT reduction assay, and viability is expressed as the percentage of living cells present in vehicle-treated preparations. D, time-dependent changes in the sub-G1 population after EPOX treatment. Cells were incubated with 3 μmol/L EPOX and analyzed for propidium iodide–stained DNA content by flow cytometry. Values, percentage of cells with hypodiploid DNA content. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Bak, or Bim were observed (Fig. 3A). If loss of Mcl-1 after EPOX treatment was responsible for the subsequent induction of apoptosis, then maintenance of Mcl-1 should prevent apoptosis. As shown in Fig. 3B, transient transfection of Mcl-1 partially overcomes the cleavages of caspase-3 and poly(ADP-ribose) polymerase, and the loss of viability in response to EPOX treatment. These observations support the hypothesis that down-regulation of Mcl-1 contributes to EPOX-induced apoptosis in HUVECs.

To determine how EPOX reduces Mcl-1 expression, we first measured Mcl-1 mRNA content by reverse transcription-PCR and found that Mcl-1 mRNA values were unaffected by EPOX (Supplementary Fig. S3A). We then investigated the degradation of Mcl-1. As shown in Fig. 3C, Mcl-1 degradation in EPOX-treated HUVECs was almost fully blocked by exposure to the proteasomal inhibitor MG132. An increase in ubiquitination of Mcl-1 was also observed for EPOX-treated compared with nontreated preparations. Consistently, in the presence of cycloheximide, which inhibits de novo protein synthesis, the rate of degradation of Mcl-1 was significantly greater in EPOX-treated compared with vehicle-treated cells (Supplementary Fig. S3B). These findings support the proposal that EPOX decreases Mcl-1 level through activation of the ubiquitin-dependent and proteasome-dependent pathways.

Mcl-1 is also described as a substrate for caspase-mediated degradation. In previous data, cleavage of caspase-3 was observed in EPOX-treated HUVECs. To ascertain whether caspase activation contributes to Mcl-1 down-regulation in these cells, experiments were also conducted in the presence of ZVAD-fmk, an established caspase inhibitor. In EPOX-treated HUVECs exposed to ZVAD-fmk, caspase-3 and poly(ADP-ribose) polymerase processing were significantly reduced, and viability was...
increased compared with preparations not treated with ZVAD-fmk (Fig. 3D). However, exposure to ZVAD-fmk did not notably influence Mcl-1 down-regulation in these cells, suggesting that this down-regulation occurred upstream of caspase activation.

**Mcl-1-free Bim generated in EPOX-treated HUVECs activates Bax.** Mcl-1 is known to sequester Bim in an inactive state. Experiments were therefore done to ascertain whether Mcl-1/Bim complexes are affected in EPOX-treated HUVECs. Endogenous Mcl-1/Bim complexes were detectable in extracts of non-treated HUVECs. After 6 hours of EPOX treatment, however, an increase in Mcl-1-free Bim was observed (Fig. 4A). To examine the effects of Bim on the apoptotic response to EPOX, Bim was knocked down by RNA interference (RNAi). Knockdown of Bim conferred partial resistance to EPOX-induced caspase-3 and poly(ADP-ribose) polymerase processing, and the subsequent apoptosis (Fig. 4B).

Once liberated from Mcl-1, Bim is able to activate Bax directly (24). A change in Bax conformation to expose its N-terminal and C-terminal regions is intimately associated with the mitochondrial targeting and proapoptotic activity of the protein (25). To ascertain whether Bax is activated in EPOX-treated HUVECs, the protein was immunoprecipitated from cell extracts with the use of an antibody (anti-Bax 6A7) that recognizes only the conformationally altered protein. As shown in Fig. 4B, Bax underwent a conformational change in EPOX-treated cells. However, knockdown of Bim attenuated Bax activation in EPOX-treated cells, implying that Bim is involved in the activation. Subcellular fractionation (Fig. 4C) and confocal microscopy (Fig. 4D) confirmed Bax translocation...
to mitochondria during EPOX treatment. In untreated cells, the cytoplasm displayed diffuse immunostaining for Bax, whereas a punctate distribution, colocalizing with MitoTracker staining, was observed after EPOX treatment.

Glutathione depletion is associated with mitochondrial alterations in response to EPOX. To further characterize the upstream signals involved in the induction of apoptosis in EPOX-treated HUVECs, specific pharmacologic agents were tested for their capacity to ameliorate the apoptosis. Modulators of the “oxidant,” “calcium,” and “arachidonic acid” pathways associated with the induction of endothelial cell apoptosis in response to stimuli, including lipopolysaccharide, hypoxia, hydrogen peroxide, tumor necrosis factor α, IFN γ, activated neutrophils, or oxidized low-density lipoprotein (26), were tested. As shown in Fig. 5A, only glutathione ethyl ester, a cell-permeable analogue of GSH, significantly reduced EPOX-induced cell death.

Supplementation with glutathione ethyl ester was done for 6 hours followed by replacement with nonsupplemented medium to avoid nonspecific reactions between glutathione ethyl ester and EPOX. Supplementation was found to reverse the effect of EPOX on cell growth (Fig. 5B) and tube formation (Supplementary Fig. S3C). With regard to the importance of glutathione for the susceptibility of endothelial cells to EPOX, it was of interest to observe the changes in intracellular GSH levels after EPOX treatment. Cells were incubated with monochlorobimane, a well-established GSH-specific probe, and then analyzed for GSH content with a fluorescence microplate reader. EPOX treatment induced the depletion of GSH in a time-dependent manner (Fig. 5C). When fluorescence microscopy was used, consistently EPOX treatment was found to cause a striking decrease in monochlorobimane fluorescence (Supplementary Fig. S3D). These findings implicate GSH depletion in the induction of apoptosis by EPOX.

Reduced GSH levels are frequently associated with activation of apoptosis through mitochondrial pathways (27). Studies were therefore done to explore the role of GSH in the mitochondrial alterations observed in response to EPOX treatment. As shown in Supplementary Fig. S4A, glutathione ethyl ester supplementation significantly restored the collapse of
mitochondria membrane potential in response to EPOX treatment. Additionally, pretreatment with glutathione ethyl ester reduced the down-regulation of Mcl-1, the conformational change in Bax, and the cleavages of caspase-3 and poly(ADP-ribose) polymerase after EPOX treatment (Fig. 5D). Depletion of GSH therefore seems to serve as an upstream event for induction of Mcl-1 degradation by EPOX.

Down-regulation of Mcl-1 by EPOX involves inhibition of ERK activation. The stability of Mcl-1 changes in response to phosphorylation by ERK, JNK, and GSK-3β (16, 28, 29). Among these three kinases, ERK was found to be the most significantly inactivated by EPOX treatment (Fig. 6A). When a constitutively active MEK that activated ERK was transiently transfected into HUVECs, the ERK inactivation by EPOX was partially restored. Interestingly, Mcl-1 down-regulation, downstream apoptotic pathway activation (Fig. 6B), and the loss of viability (Supplementary Fig. S4B) in response to EPOX were also attenuated, consistent with a role for ERK inactivation in EPOX-induced Mcl-1 degradation.

Phosphorylation of Mcl-1 at T163, the conserved ERK site located within the proline, glutamic acid, serine, and threonine (PEST) region (16), was abolished by treatments with the MEK inhibitors PD98059 and U0126 (Supplementary Fig. S4C). Domina et al. (28) showed that phosphorylation of Mcl-1 at T163 by ERK serves to prolong the half-life of Mcl-1. Half-life prolongation is attributable to the binding of Pin1 at the phosphorylated T163 Site, which serves to protect Mcl-1 from degradation by interfering with its ubiquitination (29, 30). Accordingly, treatments with MEK inhibitors promoted Mcl-1 ubiquitination (Supplementary Fig. S4C).

Experiments were done to examine T163 phosphorylation of Mcl-1 after EPOX treatment and to ascertain whether ERK
activation attenuates the effect of EPOX. As shown in Fig. 6C, overexpression of constitutively active MEK, which served to activate ERK, reduced the T163 dephosphorylation and ubiquitination of Mcl-1 in response to EPOX treatment. Interestingly, glutathione ethyl ester supplementation was observed to reverse the effects of EPOX on ERK inactivation, on Mcl-1 dephosphorylation, and on Mcl-1 ubiquitination and degradation (Figs. 5D, and 6A and D). These observations, which are consistent with those of others (28, 31), highlight the involvement of the ERK in the regulation of Mcl-1 stability. These findings also reveal that ERK is involved in the down-regulation of Mcl-1 in EPOX-treated HUVECs.

Discussion
The increasing use of antiangiogenic drugs for the treatment of cancer is the consequence of decades of extensive basic and clinical research. However, the clinical benefits of antiangiogenic therapy are relatively modest, with survival measured in months (32). Better understanding of the molecular mechanisms governing angiogenesis and of the molecular responses to antiangiogenic therapies are required for therapeutic improvements. The present study reveals that the xanthone derivative EPOX induces apoptosis in endothelial cells through pathways initiated by GSH depletion and Mcl-1 degradation. In this regard, EPOX was also examined for apoptotic effects with the use of a panel of normal cells and cancer cell lines (Supplementary Table S1). This agent produced prominent apoptotic effects in several types of cancer cells, and modest apoptotic effects in normal prostatic cells and fibroblasts. In a colon cancer xenograft model, treatment with EPOX caused a significant inhibition of tumor growth (Supplementary Fig. S5A) without overt toxicity (Supplementary Fig. S5B). We also observed that the EPOX-treated tumor had less CD31+ vessel structures than the control group (Supplementary Fig. S5C), supporting the potential of EPOX as an angiogenesis inhibitor for cancer therapy.

GSH effectively scavenges free radicals and other reactive oxygen species, both directly and indirectly through enzymatic reactions. Depletion of intracellular GSH content is frequently accompanied by a concomitant increase in reactive oxygen...
species. However, GSH depletion could lower the reducing capacity of the cells, thereby promoting oxidative stress independently of reactive oxygen species (33). In the present study, supplementation with glutathione ethyl ester, but not with other antioxidants, ameliorated EPOX-induced apoptosis. Furthermore, EPOX treatment did not promote accumulation of reactive oxygen species as monitored through use of 2′,7′-dichlorodihydrofluorescein diacetate or hydroxyethidium (data not shown). Reactive oxygen species have been shown to activate ERK in a variety of cell types (34), although the mechanisms responsible for the activation have not been identified clearly. In the current study, EPOX treatment was observed to suppress activation of ERK in endothelial cells. Taken together, these findings support the hypothesis that EPOX induces endothelial cell apoptosis independently of reactive oxygen species. Further studies are clearly required to determine the mechanism whereby GSH depletion leads to ERK inactivation after EPOX treatment.

Because most tumors are recognized to have defects in the p53 pathway or to overexpress a Bcl-2 homologue, the thera
tapeutic potential of anticancer drugs that, like the BH3 domain, bind one or more Bcl-2 homologues and trigger apoptosis are of significant current interest. Such “BH3 mimetics” should prove more effective than antiapoptosis agents acting far upstream (35). A structure-based approach recently resulted in the development of ABT-737, an agent that binds strongly to Bcl-2, Bcl-xL, and Bcl-w, but not to Mcl-1 (36). Because inactivation of Mcl-1 is critical for cell death to proceed (37), knockdown of Mcl-1 with the use of siRNA was found to sensitize cells to the effects of ABT-737 (38). Small molecules that target Mcl-1, such as obatoclax (39), should serve to complement the antiapoptosis actions of ABT-737. However, it should be noted that the rapid and marked down-regulation of Mcl-1 through proteasome-dependent degradation, as observed in the present study, was found to contribute to EPOX-induced apoptosis. EPOX in combination with ABT-737 should therefore represent an effective therapy for induction of tumor cell apoptosis (40).

Current evidence supports the importance of Raf/MEK/ERK signaling in stimulation of angiogenesis. Raf-1 knockout mice die from vascular defects in the yolk sac in association with an increased propensity for apoptosis (40). In response to intratumor hypoxia, the proangiogenic factors vascular endothelial growth factor, basic fibroblast growth factor, and platelet-derived growth factor are up-regulated and secreted. Interestingly, all of these growth factors exert their signaling effects through the Raf/MEK/ERK pathway. Furthermore, sorafenib, a Raf kinase inhibitor, strongly inhibits angiogenesis in tumor xenograft models (41). Although sorafenib was recently approved by the Food and Drug Administration for treating advanced renal cell carcinoma (42), the molecular mechanism through which this agent promotes apoptosis is not completely characterized. However, sorafenib was recently reported to enhance proteasome-mediated Mcl-1 degradation (31) and to increase apoptosis induced by tumor necrosis factor–related apoptosis-inducing ligand through down-regulation of Mcl-1 (43).

The findings of the present study highlight the involvement of the ERK in the regulation of Mcl-1 stability. Recent findings in our laboratory revealed that ERK could phosphorylate Mcl-1 at Thr 92 and Thr 163, which is required for the association of Mcl-1 and Pin1 (30). A novel mechanism was identified linking the ERK-Pin1 pathway with Mcl-1–mediated chemoresistance. Consistent with these findings, the inhibition of ERK activation in response to EPOX treatment was also found to promote the dissociation of Mcl-1 from Pin1.

Supplementary Fig. S6 presents a model, based on the findings of the present report, for EPOX-induced apoptosis in endothelial cells. In response to EPOX treatment, the depletion of GSH signals ERK inactivation, which results in the dephosphorylation and down-regulation of Mcl-1 through the proteasome-mediated degradation. The liberation of Bim from Mcl-1 serves to induce the conformational change in Bax required for its activation. Upon translocation to the mitochondria, the activated Bax triggers apoptosis through the release of cytochrome c, and activations of caspase-9 and caspase-3. It is therefore proposed that therapies designed to manipulate GSH content, the ERK/Mcl-1 pathway, and crosstalk among Bcl-2 members could prove effective for suppression of angiogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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