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, angioptatin, 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,
and anticancer activities (11). The emerged properties, including tuberculostatic, antithrombotic, antiallergic, higher plants and microorganisms, have diverse biological properties, including tuberculostatic, antithrombotic, antiallergic, higher plants and microorganisms, have diverse biological properties, including tuberculostatic, antithrombotic, antiallergic, higher plants and microorganisms, have diverse biological properties, including tuberculostatic, antithrombotic, antiallergic, higher plants and microorganisms, have diverse biological properties, including tuberculostatic, antithrombotic, antiallergic, higher plants and microorganisms, have diverse biological properties, including tuberculostatic, antithrombotic, antiallergic, higher plants and microorganisms, have diverse biological properties, including tuberculostatic, antithrombotic, antiallergic, higher plants and microorganisms, have diverse biological properties, including tuberculostatic, antithrombotic, antiallergic, higher plants and microorganisms, have diverse biological properties, including tuberculostatic, antithrombotic, antiallergic, higher plants and microorganisms, have diverse biological properties, including tuberculostatic, antithrombotic, antiallergic, higher plants and microorganisms, have diverse biological properties, including tuberculosis, antithrombotic, antiallergic, and anticancer activities (11). The emerged "hit" xanthone derivatives, such as 5,6-dimethylxanthenone-4-acetic acid (DMXAA), prospermin, mangiferin, norathyriol, mangostins, and AH6809, constitute excellent starting materials to lead development in the field of cancer. DMXAA has completed phase II testing (12) and is currently undergoing phase III evaluation in non–small cell lung cancer (ATRACT-1 [Antivascular Targeted Therapy: Researching ASA404 in Cancer Treatment] trial). DMXAA induces apoptosis of tumor vascular endothelial cells and cytokine production, leading to tumor vascular collapse (13). As we previously reported, a series of oxygenated xanthones and [3-(dialkylamino)-2-hydroxypropoxy]xanthones have been continuously exposes tumor endothelial cells to these drugs. During such therapy, endothelial cell apoptosis precedes tumor cell apoptosis (5). In preclinical studies, metronomic chemotherapy was found effective for managing tumors with cancer cells that had developed resistance to the same chemotherapeutic agents (6). According to current laboratory and clinical findings, inducing apoptosis in tumor endothelial cells contributes to the antitumor efficacy of antiangiogenic and cytotoxic drugs (7).

Apoptosis is characterized by a variety of biochemical and morphologic alterations, including caspase activation, mitochondrial depolarization, chromatin condensation, cell shrinkage, and formation of apoptotic bodies (8). Intracellular redox status is recognized to play an important role in the induction of cell apoptosis. Glutathione (GSH), which accounts for >90% of cellular nonprotein thiols, is critical to maintaining cellular redox balance (9). Therefore, compounds that block the effects of GSH possess the potential to induce cancer cell apoptosis or to reverse drug resistance (10).

Xanthone compounds, secondary metabolites derived from higher plants and microorganisms, have diverse biological properties, including tuberculostatic, antithrombotic, antiallergic, and anticancer activities (11). The emerged "hit" xanthone derivatives, such as 5,6-dimethylxanthenone-4-acetic acid (DMXAA), prospermin, mangiferin, norathyriol, mangostins, and AH6809, constitute excellent starting materials to lead development in the field of cancer. DMXAA has completed phase II testing (12) and is currently undergoing phase III evaluation in non–small cell lung cancer (ATRACT-1 [Antivascular Targeted Therapy: Researching ASA404 in Cancer Treatment] trial). DMXAA induces apoptosis of tumor vascular endothelial cells and cytokine production, leading to tumor vascular collapse (13). As we previously reported, a series of oxygenated xanthones and [3-(dialkylamino)-2-hydroxypropoxy]xanthones have been prepared and tested for anticancer activity (14). 2,6-Di(2,3-epoxypropoxy)xanthone was chosen for further study, and the mechanism of action is proposed to involve a selective down-regulation of Ras (15). After examining the antiproliferative effects of xanthone derivatives on human umbilical vascular endothelial cells (HUVEC), structure-activity analysis indicated epoxidation of the hydroxyxanthone increased cytotoxicity, but ring opening of the epoxide group with dialkylamine did not enhance the antiangiogenic activity (Supplementary Fig. S1A). 3,6-Di(2,3-epoxypropoxy)xanthone (EPOX; Supplementary Fig. S1B) was found to be the most effective. The present study was therefore conducted to elucidate the mechanism whereby EPOX exerts its apoptotic effects in endothelial cells.

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-dihydroxysteranthone 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 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 kinase (JNK) were purchased from Cell Signalining Technologies. Cytochrome c and Mcl-1 antibodies came from BD Pharmingen. Antibodies against Bcl-2, Bcl-xL, Bak, Bim, 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 generated by Bethyl Laboratories. Antibodies against phosphorlated Thr 163 of Mcl-1 were generated by Bethyl Laboratories. Antibodies against phosphorlated Thr 163 of Mcl-1 were generated by Bethyl Laboratories. Antibodies against phosphorlated Thr 163 of Mcl-1 were generated by Bethyl Laboratories. Antibodies against phosphorlated Thr 163 of Mcl-1 were generated by Bethyl Laboratories. Antibodies against phosphorlated Thr 163 of Mcl-1 were generated by Bethyl Laboratories. Antibodies against phosphorlated Thr 163 of Mcl-1 were generated by Bethyl Laboratories. Antibodies against phosphorlated Thr 163 of Mcl-1 were generated by Bethyl Laboratories. Antibodies against phosphorlated Thr 163 of Mcl-1 were generated by Bethyl Laboratories.
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 MIT 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 homogenous 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,
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 maintained.

![Fig. 2. EPOX promotes activation of the mitochondrial pathway of apoptosis. A, caspase activation. Cells were treated with EPOX (3 μmol/L) for the indicated times before immunoblotting and detection of caspase activity. Arrowhead, cleaved forms of caspases. Means ± SE of three independent determination of caspase activity were checked for statistical difference. *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with nontreated cells. B, mitochondrial membrane potential. Cells were treated with EPOX (3 μmol/L) for the indicated times and then harvested for the determination of mitochondrial membrane potential through measuring rhodamine 123 fluorescence intensity by flow cytometry. Results are compared with the DMSO control. C, translocation of cytochrome c. Translocation of cytochrome c from the mitochondria to the cytosol after 4 or 6 h of treatment without or with EPOX (3 μmol/L) was measured by subcellular fractionation and immunoblotting.](image-url)
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

![Fig. 4.](image_url)

Mcl-1–free Bim generated by EPOX treatment activates Bax in endothelial cells. A, dissociation of Bim from Mcl-1 in EPOX-treated cells. Cells were incubated without or with EPOX (3 μmol/L) for 6 h. Lysates were treated with anti–Mcl-1 antibody. Bound (IP pellet), unbound fractions (deplete supernatant, 5% of treated lysates), and total cell lysate were subjected to western blotting. B, knockdown of Bim by Bim siRNA attenuates EPOX-induced apoptosis. Cells were pretreated with scramble or Bim siRNA for 72 h before incubations without or with EPOX (3 μmol/L). Top, results of immunoblotting after 6 h of incubation; bottom, viabilities after 24 h of incubation. C, Bax translocates from the cytosol to the mitochondria after treatment with EPOX. Cells were incubated without or with EPOX (3 μmol/L) for 4 or 6 h. Cytosolic and mitochondrial fractions were prepared and examined by western blotting. D, confocal microscopy revealing the translocation of Bax from cytosol to mitochondria in response to EPOX treatment. Cells were treated without or with EPOX (3 μmol/L) for 6 h and stained with anti-Bax antibodies followed by FITC-conjugated secondary antibody. Mitochondria were stained with MitoTracker.
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.
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′-dichlorodihydrofluorecein diacetate or hydroxyethidine (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 therapeutic 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 anticancer 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 antiapoptotic 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 Pin1.5

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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