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Therapeutic Discovery

Molecular Cancer Therapeutics

Synthetic Triterpenoid Cyano Enone of Methyl Boswellate Activates Intrinsic, Extrinsic, and Endoplasmic Reticulum Stress Cell Death Pathways in Tumor Cell Lines

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Abstract

We explored the effect of a novel synthetic triterpenoid compound cyano enone of methyl boswellates (CEMB) on various prostate cancer and glioma cancer cell lines. CEMB displayed concentration-dependent cytotoxic activity with submicromolar lethal dose 50% (LD_{50}) values in 10 of 10 tumor cell lines tested. CEMB-induced cytotoxicity is accompanied by activation of downstream effector caspases (caspases 3 and 7) and by upstream initiator caspases involved in both the extrinsic (caspase 8) and intrinsic (caspase 9) apoptotic pathways. By using short interfering RNAs (siRNA), we show evidence that knockdown of caspase 8, DR4, Apaf-1, and Bid impairs CEMB-induced cell death. Similar to other proapoptotic synthetic triterpenoid compounds, CEMB-induced apoptosis involved endoplasmic reticulum stress, as shown by partial rescue of tumor cells by siRNA-mediated knockdown of expression of genes involved in the unfolded protein response such as IRE1α, PERK, and ATF6. Altogether, our results suggest that CEMB stimulates several apoptotic pathways in cancer cells, suggesting that this compound should be evaluated further as a potential agent for cancer therapy. *Mol Cancer Ther; 10(9); 1635–43. ©2011 AACR.*

Introduction

Synthetic triterpenoid compounds are a class of anticancer agents possessing beneficial multifunctional activities. Thus far, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and its derivative compounds CDDO-Me and CDDO-Im are the most potent triterpenoid compounds known in terms of anti-inflammatory and anticarcinogenic activity (1). These compounds have antiproliferative activity against cancer cells, anti-inflammatory activity against mouse macrophages, and pro-apoptotic activity against various types of cancers and leukemias, as well as inducing differentiation of neuronal cell lines and preadipocytes (2). Among the many types of cancer and leukemia cells in which CDDO class triterpenoids have been reported to induce apoptosis are B- and T-cell leukemias (3–6), breast cancers [including both estrogen receptor positive and negative; refs. 7, 8], ovarian cancers (9), prostate cancers (10, 11), pancreatic carcinomas (12), various skin cancers (13), and lung cancers (14–17). In addition, CDDO, CDDO-Me, and CDDO-Im have been shown to inhibit tumor growth in xenograft models in mice and rats (8, 10, 18). Currently, CDDO-Me is in phase I clinical trials as a novel cancer therapeutic agent.

The cytotoxic mechanism of triterpenoids is multifunctional. Recent studies suggest that CDDO and its derivatives induce reactive oxygen species (12, 19), decrease mitochondrial glutathione (12, 19, 20), activate JNK and p38 mitogen-activated protein kinase stress pathways (14, 21–23), and inhibit NF-κB activity (24, 25).

The triterpenoid classes of anticancer and bioactive natural products are electrophiles (26). Recently, these electrophilic compounds were shown to trigger endoplasmic reticulum (ER) stress (27), presumably by interfering with disulfide-dependent folding of proteins in the ER. The accumulation of unfolded proteins in the ER stimulates signal transduction events that include activation of the ER transmembrane kinases IRE1α and PERK, as well as proteolytic processing and activation of the transcription factor ATF6 (reviewed in ref. 28). Downstream components of the unfolded protein response (UPR) are capable of activating the 2 major apoptotic pathways—the extrinsic cell death pathway activated by TNF family ligands and death receptors, and the intrinsic cell death pathway initiated by release of apoptogenic

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proteins from mitochondria (29–31). In several studies, it has been shown that the CDDO and its derivatives induce cell death either through intrinsic (32–34) or extrinsic pathways (14, 16, 35). Some studies indicate that both extrinsic and intrinsic pathways are involved depending on the cell type (10). CDDO-Me also induces ER stress, including *CHOP* expression, TNF-related apoptosis-inducing ligand (TRAIL) receptor expression, and caspase-dependent killing of tumor cells (27). Thus, ER, mitochondrial, and death receptor pathways are all activated by CDDO and its active analogues.

As CDDO and its derivatives have been recognized as promising agents for treatment of cancers, we investigated the synthesis and characterization of additional types of synthetic triterpenoid compounds. In this article, we investigated the cytotoxic mechanism of cyano enone of methyl boswellate (CEMB), a closely related analogue of CDDO (Supplementary Fig. S1). In our previous work, we synthesized triterpenoid compounds and showed that the CEMB, cyano enone of arjunolic acid and glycyrrhetinic acid, inhibit inflammation in primary mouse macrophages stimulated by IFN-γ and induce cell death of some cancer cell lines (36). Among the 3 synthetic triterpenoids that we tested, the most bioactive was CEMB, which was extracted and modified from *Boswellia serata*. The parent structure of CDDO belongs to the olean skeleton, whereas that of CEMB is a mixture of ursane and olean skeletons; the 2 isomeric skeletons differ only in the position of the methyl group on the E-ring. Both CDDO and CEMB share the cyano-enone moiety at the A-ring of the structures. The differences between the 2 structures are in the position of the carboxylate moiety and the functionality of the C-ring. Whereas CDDO has the polar carboxylate moiety on the E-ring, CEMB carries it as a pendant of the A-ring. Furthermore, CDDO has an enone functionality in the C-ring, whereas CEMB retains the unsaturated C-ring in the parent skeleton. Because the synthesis of CEMB does not involve the chemical modifications involved in the functionalization of the C-ring, the number of steps required for its synthesis is reduced in comparison with CDDO. In this study, we have investigated the *in vitro* activity of CEMB against prostate cancer and glioma cancer cells, and we have explored the mechanism by which CEMB induces apoptosis.

Materials and Methods

Reagents

The triterpenoid compound, CEMB, was synthesized from boswellic acid. For a detailed protocol describing synthesis of CEMB, refer to our earlier work (36). The CEMB compound was dissolved in dimethyl sulfoxide (DMSO) at the concentration of 10 mmol/L and aliquots were stored at −80°C. Stock compound was diluted in culture medium just before cell treatment. The ATPlite 1-step Luminescence Assay Kit was purchased from Perkin Elmer. The Caspase-Glo 8 Assay, Caspase-Glo 9, and Caspase-Glo 3/7 Assay Kits were purchased from Promega. RNAiMAX was obtained from Invitrogen. The OptiMEM was purchased from GIBCO. RPMI and Dulbecco's Modified Eagle's Medium (DMEM) media came from Mediatech Inc. Predesigned short interfering RNAs (siRNA) directed against caspase 8 (siRNA Ids: 9572, 9478, and 9663), DR4 (siRNA Ids: 202067, 4816, and 5004), DR5 (siRNA Ids: 107249, 5003), and Bid (siRNA Ids: 120775, 120774, 2556, and 120776) were purchased from Ambion. Apaf-1 siRNAs (siRNA Ids: SI02662387, SI02661876, and SI02654428) were obtained from Qiagen. TRAIL was obtained from Genentech. Primers for IRE1α 5′-3′-(F-ggcctggtcaccacaattag; R-gggtcagcactgtcctctgt), PERK (F-gctgtatccattcagcactcag; R-gcatgtcttgaaccatcacg), ATF6 (F-gcagctggatgaagttgtgt; R-ccaacatgctcataggtcca), and Ask1 (F-cctgaagcttaagtcccaacc; R-cattcactctcagccagtcg) were designed and obtained from Valuegene Inc. *CHOP*-Luc plasmid was kindly provided by Prof. Pierre Fafournoux, France.

Cell lines and culture conditions

Prostate cancer cell lines (PPC-1, Alva-31, DU145, LNCAP, and PC3) and glioma cell lines (U87, U343, U251, LN229, and C6) were obtained from the American Type Culture Collection. The cell lines have not been recently tested and authenticated. Prostate cancer cells were cultured in RPMI and glioma cells were cultured in DMEM supplemented with 10% FBS (GIBCO) and maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Cell viability assays

MTT and ATPlite assays were used for cell viability estimation. For MTT assay, cells were seeded at a density of 5×10^3 cells per well in 100 μL of culture medium in 96-well flat-bottom plates and cultured overnight. The next day, compound treatment was carried out by adding various concentrations of compounds directly to the wells followed by incubation for 72 hours. Then, 20 μL of MTT (5 mg/mL) was added to each well and plates were returned to the incubator for 3 hours. The supernatant medium was removed and the dark blue crystals that had formed were dissolved in 200 μL of DMSO, followed by reading absorbance at 550 nm. For ATP assays, in which ATP serves as a surrogate for cell viability, we used the ATPlite 1-step Luminescence Assay. Cells were plated at a density of 2×10^3 cells per well in 45 μL complete medium in 384-well solid white plates and cultured overnight. The next day, cells were cultured with various concentrations of compounds for various times (5 μL). Plates were then removed from the incubator and allowed to equilibrate to room temperature for about 10 minutes. ATPlite solution was added 25 μL per well and plates were kept in dark for 5 minutes before reading luminescence by using a luminometer (Luminoskan Ascent; Therom Electron Corporation).

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Caspase activity assays

Prostate cancer and glioma cells were plated (2×10^3 cells per well) in 384-well solid white bottom plates and cultured overnight in 45 μL of complete medium. The next day, compounds were added directly to the wells (5 μL) and cells were incubated for 6, 12, 24, or 48 hours. Caspase-Glo 8, Caspase-Glo 9, or Caspase-Glo 3/7 substrate was added (50 μl) and the plates were returned to the incubator. After 30 minutes to 1 hour of incubation, luminescence was measured.

Silencing of genes by using siRNA

PPC-1 cells were transfected with the siRNAs directed against caspase 8, DR4, DR5, Bid, Apaf-1, IRE1α, PERK, ATF6, or Ask1 for 48 hours by a reverse transfection method by using Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen). For each gene, up to 4 different siRNAs sequences were used. After 48 hours of transfection, cells were treated with various compounds, typically for 24 hours. Cell viability assays were done as described above. To confirm the silencing of genes by the siRNA, total protein or RNA was isolated after transfection and analyzed by either immunoblotting or by real time PCR (RT-PCR). The detailed protocol is given below.

Immunoblot analysis

PPC-1 cells were plated at the density of 3×10^5 cells per well in 6-well plates and cultured for overnight. The next day, cells were treated with 1 to 2 μmol/L CEMB for 24 hours. Cells were trypsinized, washed with PBS, and lysed with radioimmunoprecipitation assay buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% Na-deoxycholate, 1% Triton X100, and protease inhibitors). Protein concentrations were quantified by Bradford's assay. Samples were normalized for total protein content, loading 50 to 100 μg per lane for SDS-PAGE/immunoblot analysis.

RNA isolation and analysis

PPC-1 cells (3.5×10^5 cells per well) were plated in 6-wells plate and transfected with siRNAs for 48 hours by using RNAiMAX. Cells were trypsinized, washed with PBS, and the cell pellets were collected for RNA isolation. To determine RNA levels after compound treatments, PPC-1 cells were plated in 6-well dishes at the density of 0.5×10^6 cells per well and next day treated with compounds for 3, 6, 12, and 24 hours. After the treatment, cells were washed with PBS, trypsinized, and pelleted. QIAGEN RNAeasy Mini Kit was used to extract RNA according to the manufacturer's instructions.

Plasmid DNA transfection

PPC-1 cells were plated in 96-well plates at density of 2×10^4 cells per well in 50 μL of culture medium. After 6 hours, 3.2 μg of *CHOP*-Luc plasmid was introduced into the cells along with 0.07 μg of *Renilla*-Luc as an internal control for transfection by using Lipofectamine 2000 in 50 μL of OptiMEM. The next day, medium was removed and fresh medium was added along with various compounds for 10 hours. To terminate assays, medium was removed and cells were lysed with 1× passive lysis buffer (Promega) for 15 minutes at room temperature on a rocking platform. The Dual-Glo Luciferase Assay Kit (Promega) was used to measure the firefly/*Renilla* luciferase ratio.

Results

CEMB displays potent cytotoxic activity against prostate cancer and glioma cell lines

Because the synthetic triterpenoid CDDO is reported to have single agent apoptotic activity against prostate cancer and glioblastoma cell lines (10, 25), we tested CEMB for cytotoxic activity against several such tumor cell lines in culture ($n = 5$ each). Three days of treatment with various concentrations of CEMB, ranging from 0.1 to 2 μmol/L, resulted in dose-dependent cell death of all 10 tumor cell lines with submicromolar lethal dose 50% (LD_{50}) concentrations as measured by MTT assay (Fig. 1A and B). Results were confirmed by measuring cellular ATP levels in CEMB-treated cells, again with submicromolar LD_{50} values of all 10 tumor cell lines (Supplementary Tables S1 and S2).

CEMB-induced cytotoxicity is partially caspase dependent

We used the cell-permeable pan-caspase inhibitor z-VAD-fmk to test the caspase dependence of CEMB-induced killing of PPC-1 prostate and U251 glioblastoma cells. For these experiments, tumor cells were treated with 100 μmol/L of z-VAD-fmk and various concentrations of CEMB. CEMB-induced cell death was significantly suppressed by z-VAD-fmk in cultures of both PPC-1 and U251 cells, as measured by the surrogate indicator

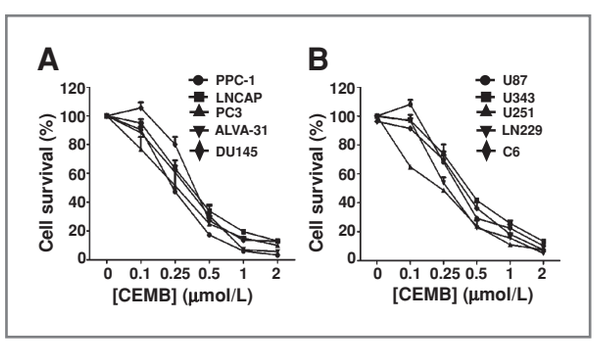

Figure 1. CEMB exhibits concentration-dependent cytotoxic activity against prostate cancer and glioma cell lines. A, prostate cancer cell lines, PPC-1, LNCAP, PC3, ALVA-31, and DU145, and B, glioma cell lines, U87, U343, U251, LN229, and C6, were treated with CEMB for 72 hours and then assayed for cell death by using MTT. The x-axis denotes the CEMB concentration (in μmol/L) and the y-axis denotes relative cell survival (% compared with control treated with DMSO) as measured by MTT assay. All the experiments were done in quadruplicate (mean ± SEM). An initial cell density of 5,000 cells per well of 96-well plates was used, adding compounds after 24 hours.

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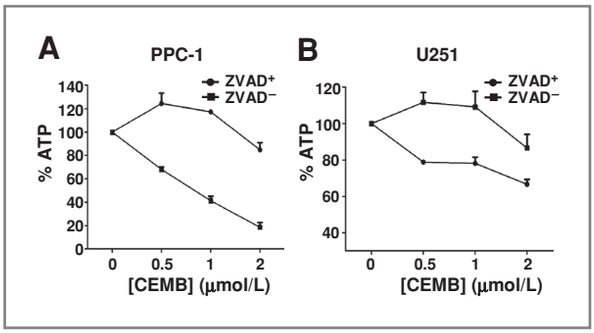

Figure 2. CEMB-induced cell death is partly caspase dependent. Prostate cancer cell line PPC-1 (A) or glioma cell line U251 (B) were plated in a 96-well plates and treated with (circle) or without (square) z-VAD-fmk (100 μmol/L) for 2 hours and then treated with CEMB at various concentrations. After 48 hours, cellular ATP levels were measured. The x-axis is the CEMB concentration (in μmol/L) and y-axis is the relative level of ATP, expressed as percentage relative to cells cultured with DMSO. Values are the average of independent measurements (bars, SE).

ATP levels (Fig. 2A and B) and by trypan blue dye exclusion (data not shown).

CEMB activates caspases

Activation of caspase 8 and caspase 9 plays key roles in the extrinsic and intrinsic apoptosis pathways, respectively. We measured the activities of the upstream initiator proteases caspase 8 (extrinsic pathway) and caspase 9 (intrinsic pathway), as well as downstream executioner proteases caspases 3 and 7 by using fluorigenic substrates that are preferentially hydrolyzed by these proteases. Staurosporine was used as a positive control for caspase 9 activation, as this broad-spectrum protein kinase inhibitor is known to cause apoptosis via the intrinsic apoptotic pathway (37). CEMB treatment resulted in increases in caspase 8, caspase 9, and caspase 3/7 activity, with maximal activity occurring typically at approximately 24 hours posttreatment (Fig. 3). Consistent with these results, CEMB also induced proteolytic processing of procaspase 8, procaspase 9, and PARP (Supplementary Fig. S2). Altogether, these findings suggest that CEMB activates both the intrinsic and extrinsic apoptosis pathways.

CEMB-induced apoptosis of PPC-1 cells requires caspase 8, TRAIL receptor DR4, Bid, and Apaf-1

We used siRNAs to investigate some of the apoptosis pathway components involved in CEMB-induced apoptosis in the PPC-1 prostate cancer cell line. Each siRNA used here was confirmed to reduce expression of the corresponding target mRNA (Supplementary Fig. S3 and S4), including extrinsic pathway components caspase 8 and DR4, and the intrinsic pathway components Bid and Apaf-1. Scrambled sequence siRNAs served as negative controls. Note that we were unable to successfully knockdown caspase 9, despite testing 4 different siRNA sequences, and the efficacy of siRNAs targeting DR5 mRNA was variable, thus precluding definitive conclusions (data not shown). In addition to CEMB, cells were treated with either TRAIL or STS as controls for extrinsic and intrinsic pathway agonists, respectively. Cell viability was assessed by ATP measurements.

CEMB-induced killing of PPC-1 cells was suppressed by caspase 8 and DR4 siRNAs (Fig. 4A and B), implying that this compound stimulates an apoptotic mechanism involving the TRAIL receptor DR4 in these prostate cancer cells. The siRNAs targeting Bid and Apaf-1 also markedly suppressed CEMB-induced cytotoxicity (Fig. 4C and D), implying involvement of the intrinsic pathway in the cytotoxic mechanism of CEMB in PPC-1 cells. Results were independently confirmed by assessing apoptosis by morphologic inspection of 4′,6-diamidino-2-phenylindole–stained cells (data not shown).

CEMB-induced cytotoxicity involves components of the ER stress machinery

Because CDDO-Me has been reported to induce apoptosis through an ER stress mechanism (27), we investigated the role of UPR signaling molecules in the cytotoxic mechanism induced by CEMB. For these experiments, siRNAs targeting IRE1α, Ask1 (an apoptotic kinase downstream of IRE1α), PERK, and ATF6, as well as corresponding scrambled sequences (as negative controls) were introduced into PPC-1 cells by lipofection. At least 2 active siRNA sequences were identified for each target, and verified by RT-PCR to reduce target mRNA levels by more than 60% (Supplementary Fig. S3 and S4). Comparisons were made of the ability of these siRNAs to reduce cell killing (measured by ATP) induced by CEMB with thapsigargin, an irreversible inhibitor of the ER Ca^{2+} ATPase (a known inducer of ER stress).

CEMB-induced cytotoxicity of PPC-1 cells was significantly reduced by all of the UPR components tested—IRE1α, Ask1, PERK, and ATF6 (Fig. 5). These siRNAs also partially rescued PPC-1 cells from thapsigargin-induced cytotoxicity, consistent with a role in ER stress. We also observed that CEMB induced modest increases (3- to 4-fold) in IRE1α and PERK mRNAs but not ATF6 or Ask1 (Supplementary Fig. S5).

Next, we used small molecule inhibitors of ER stress–induced cell death to interrogate the role of ER stress in CEMB-mediated cytotoxicity. As controls, we used known chemical inhibitors of ER stress–induced cell death salubrinal and the benzodiazepinones compound MLS-0315763 (31) and its analogue MLS-5976228 (38). Benzodiazepinones such as MLS-0315763 inhibit ER stress–induced apoptosis downstream of IRE1α, whereas salubrinal suppresses UPR signaling downstream of PERK (39, 40). Both the benzodiazepinone compounds and salubrinal (Supplementary Fig. S1) reduced CEMB-induced cytotoxicity, as well as cell killing induced by other synthetic triterpenoids, CDDO-Me and CDDO-Im, and by ER stress inducer thapsigargin (Fig. 6). These data thus provide further evidence that CEMB kills tumor

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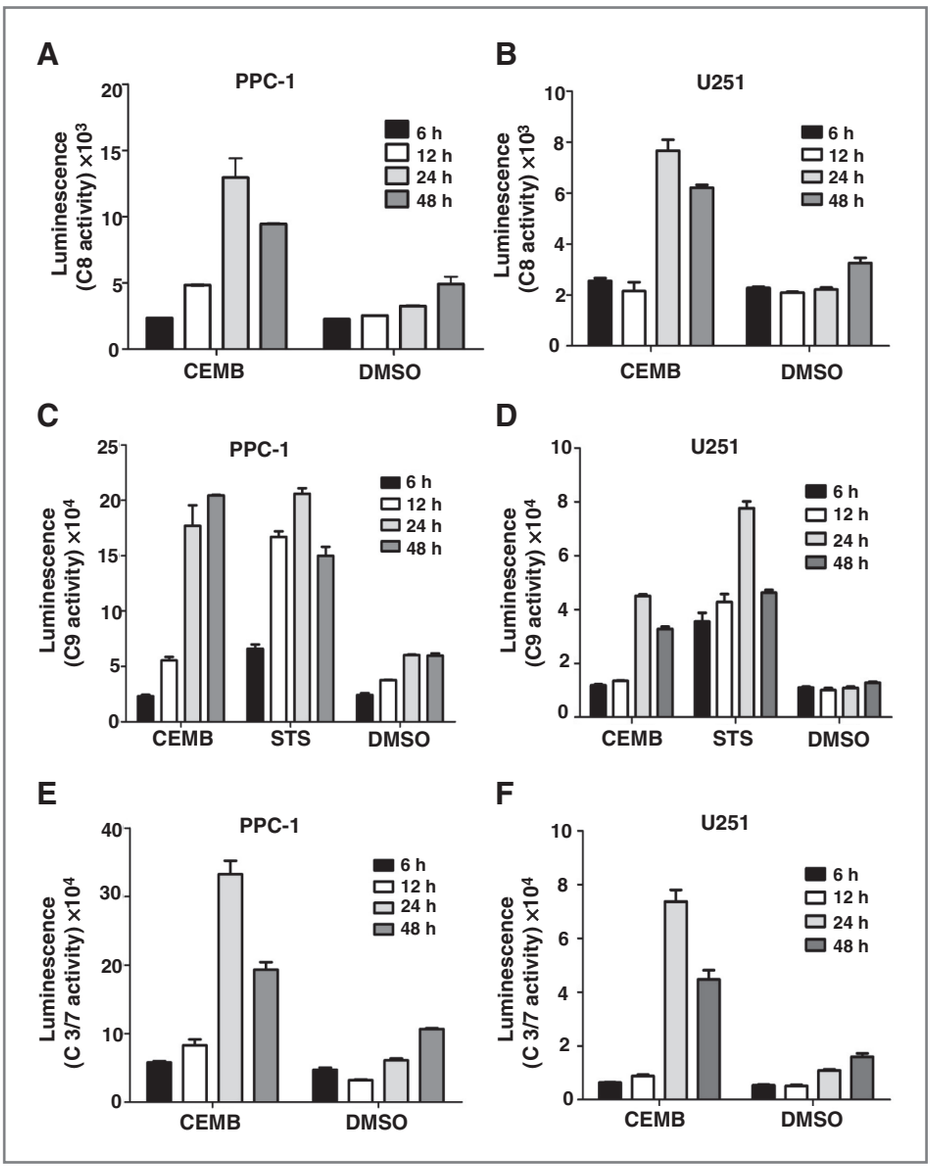

Figure 3. Activation of caspases by CEMB. PPC-1 (A, C, and E) or U251 cells (B, D, and F) were plated in 384 white plates (10^2 cells per well) and next day treated with CEMB for 6, 12, 24, and 48 hours. Relative levels of activities of caspase 8, 9, and 3/7 were measured by using fluorigenic substrates. The x-axis represents the respective compounds used, namely CEMB (2 μmol/L) and staurosporine (1 μmol/L) and DMSO (vehicle control). The y-axis denotes the luminescence of the corresponding caspases measured by caspase-Glo 8 (C8), caspase-Glo 9 (C9), and caspase-Glo 3/7 (C3/7) substrates. The experiment was repeated 2 to 3 times. Data presented here are results from one of the experiments in which assays were done in triplicate (mean ± SEM; $n = 3$).

cells, at least in part, through an ER-initiated cell death mechanism.

Finally, we assessed the effects of CEMB on an UPR signaling event induced by the convergence of the IRE1α, PERK, and ATF6 pathways—namely, activation of the *CHOP* gene promoter. *CHOP* encodes a transcription factor that induces apoptosis by modulating expression of the Bcl-2, Bim, DR5, and other apoptosis genes (31). To measure *CHOP* gene activation after CEMB treatment, we transfected *CHOP*-Luc reporter gene plasmid to PPC-1 cells. As shown, *CHOP* gene promoter activity increased approximately 4-fold at 10 hours after treatment with CEMB, similar to thapsigargin (Fig. 6C). However, when PPC-1 cells were treated with salubrinal, a compound that blocks ER stress signaling in the PERK pathway (39), then *CHOP* promoter activity was not induced. We conclude that CEMB is an inducer of ER stress and UPR signaling and that CEMB mediates its cytotoxic actions, at least in part, through an ER stress mechanism.

Discussion

Triterpenoid compound boswellic acid has been used for treatment of inflammatory diseases such as arthritis and inflammatory bowel disease since ancient time in Indian medicine, particularly in ayurveda (41). In this study, we have evaluated the cytotoxic activity of CEMB on tumor cell lines, finding that CEMB as a single agent has potent *in vitro* cytotoxic activity against

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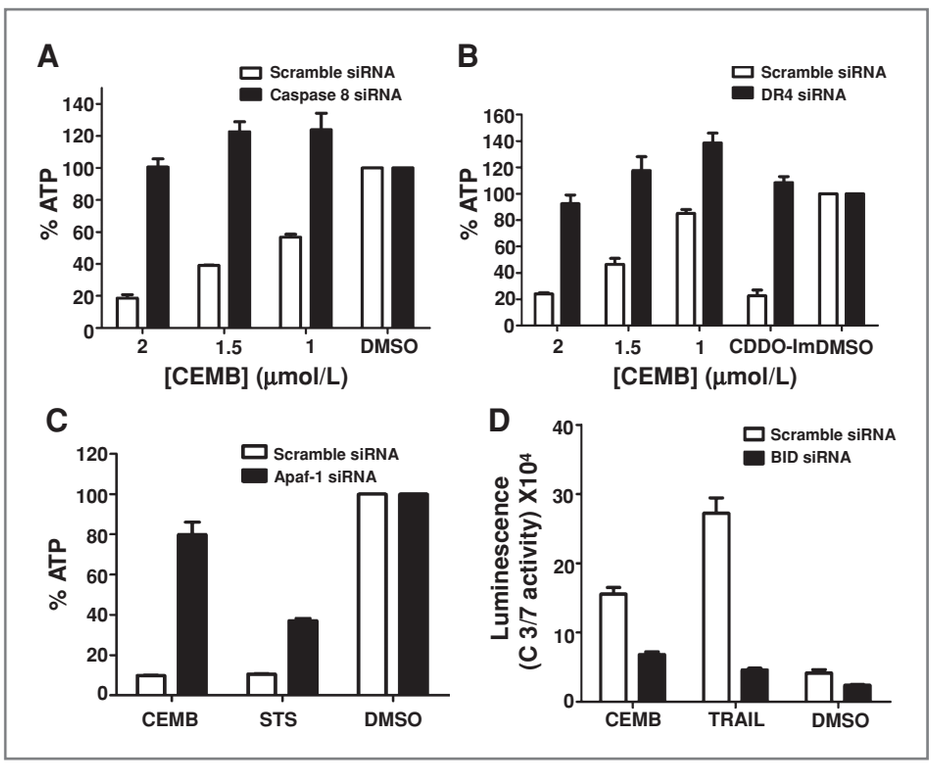

Figure 4. Caspase 8, DR4, Apaf-1, and Bid are required for CEMB-induced cell death. PPC-1 or DU145 cells were cultured in 384 well plates and transfected with caspase 8 (A), DR4 (B), or Apaf-1 (C) siRNAs. After 48 hours, cells were treated with CEMB (various concentrations), TRAIL (100 ng), or CDDO-Im (1.5 μmol/L) for 24 hours. ATP levels were measured, expressing data as percentage relative to DMSO control (mean ± SEM; $n = 3$). PPC-1 cells (D) were transfected with Bid siRNAs for 48 hours followed by treatment with CEMB (1.5 μmol/L), STS (0.25 μmol/L), or TRAIL (100 ng) for 6 hours. Caspase-Glo 3/7 activity assay was done. The x-axis denotes the compounds. The y-axis denoted the caspase-Glo 3/7 luminescence readings. [mean ± SEM ($n = 3$)].

prostate cancer and glioblastoma cell lines. Most chemotherapeutic drugs induce apoptosis by the intrinsic apoptosis pathway involving mitochondria. Our present study showed that early apoptotic events such as caspase 8 and caspase 9 activation seem to be induced by CEMB treatment, suggesting that both extrinsic and intrinsic pathways are involved. Rescue of PPC-1 cells from CEMB by caspase 8 siRNA indicates a functionally

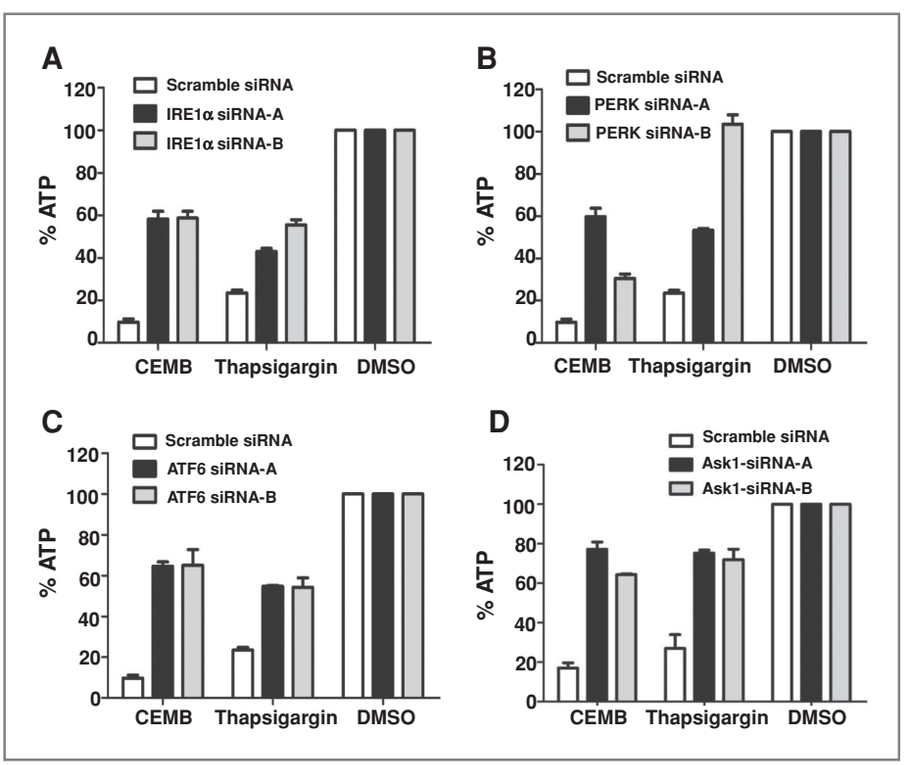

Figure 5. siRNAs targeting ER stress pathway protect against CEMB-induced cytotoxicity. PPC-1 cells were transfected with IRE1α (A), PERK (B), ATF6 (C), or Ask1 (D) siRNAs for 48 hours and then treated with CEMB (2 μmol/L) or thapsigargin (5 μmol/L) for 24 hours. ATP levels were measured and data expressed as percentage relative to DMSO treated cells transfected with scrambled control siRNAs (mean ± SEM; $n = 3$).

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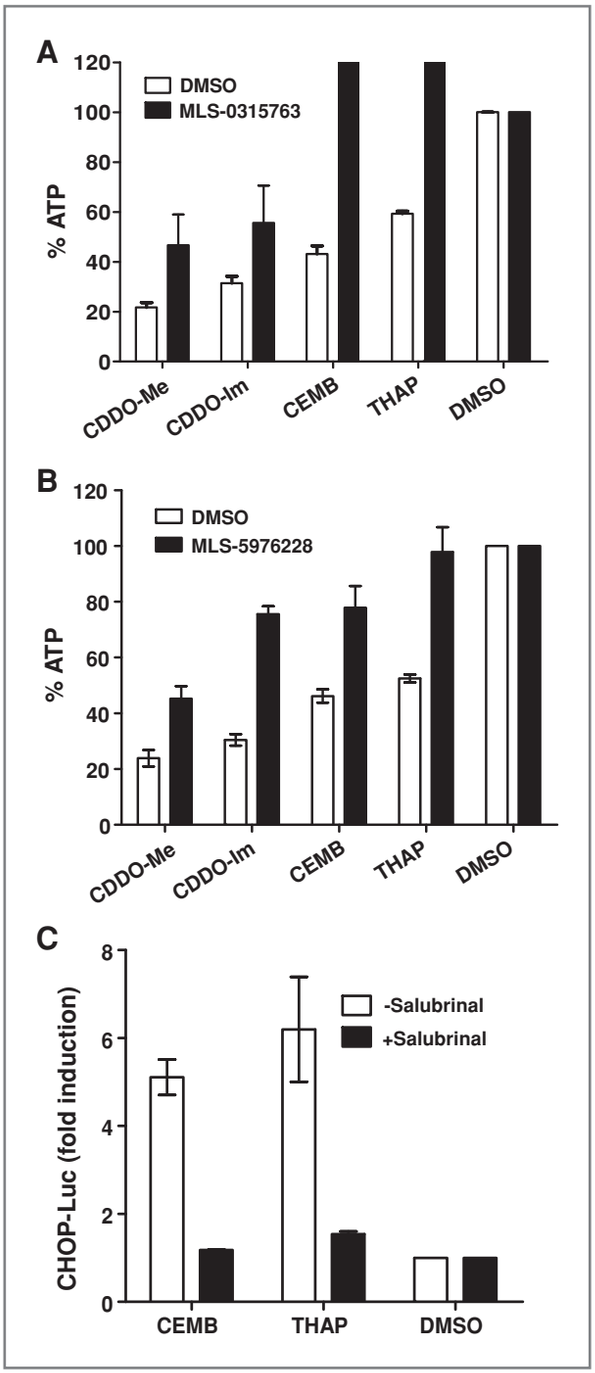

Figure 6. Chemical inhibitors of ER stress protect against CEMB-mediated cytotoxicity. Tumor cells were cultured with 10 μmol/L of benzodiazepinone MLS-0315763 (A), MLS-5976228 (B), or DMSO, then cultured with CEMB, CDDO analogues, or thapsigargin (THAP) for 48 hours. Relative cell survival was measured by ATP assay as described above (mean ± SD; $n = 3$). To measure *CHOP* gene promotion activation (C), PPC-1 cells were transfected with *CHOP*-Luc reporter gene plasmid and *Renilla*-Luc normalization plasmid. The next day, cells were treated with (black bars) or without (white bars) salubrinal for 1 hour, then treated with DMSO, CEMB, or thapsigargin for 6 hours. The ratio of firefly:*Renilla* luciferase activity was measured, expressing results as fold induction relative to cells treated with DMSO alone (mean ± SEM; $n = 3$).

important role for the extrinsic pathway, which is further supported by results of DR4 knockdown as well as Bid knockdown (a connector of the extrinsic pathway to the intrinsic pathway via caspase 8–mediated cleavage and activation of Bid; ref. 42). We also observed a role for the intrinsic apoptosis pathway, in that Apaf-1 knockdown rescued PPC-1 cells from CEMB. Unfortunately, we were unable to achieve caspase 9 mRNA knockdown to further confirm the involvement of this pathway. Thus, our data suggest that at least in the tumor cell lines evaluated here, CEMB causes cell death by activating extrinsic and intrinsic pathways of apoptosis, possibly by first activating caspase 8 to result in Bid activation and then causing mitochondrial involvement. It should be noted that activation of proapoptotic Bcl-2 family members such as Bid is well known to cause mitochondrial outer membrane permeabilization, which induces in parallel rapidly acting caspase-dependent and slower acting caspase-independent cytotoxic mechanisms (reviewed in ref. 29). Thus, killing by CEMB is likely to be only partly caspase dependent. Because chemoresistant cancers tend to have defects in the intrinsic mitochondrial pathway, synthetic triterpenoids such as CEMB may provide an alternative route to successfully inducing apoptosis of refractory cancer cells.

On the basis of experiments using siRNAs targeting proximal components of the ER stress/UPR machinery, as well as chemical inhibitors of ER stress signaling, we determined that the cytotoxic mechanism of CEMB involves, at least in part, the ER stress pathway. Previous studies have documented an important role for ER stress in the cytotoxic mechanism of other triterpenoids, such as CDDO analogues (27). The electrophilic nature of CEMB and other triterpenoid compounds presumably explains why they induce ER stress. The mechanisms linking ER stress to apoptosis are diverse (31). Included among the ER stress inducers of apoptosis is induction of *CHOP*, a transcription factor that stimulates expression of TRAIL receptors and that modulates expression of Bcl-2 family genes (31). We observed stimulation of *CHOP* gene promoter activity following treatment with CEMB. Because the ER stress response is capable of stimulating both intrinsic and extrinsic apoptosis pathways, the dominant pathway will likely depend on the specific tumor cell line or cancer type. Also, ER stress–initiated cell death mechanisms typically are only partly caspase dependent (reviewed in ref. 31), presumably explaining why siRNA-mediated knockdown of Apaf-1 only partially rescued cells from CEMB-mediated cytotoxicity.

In summary, compared with CDDO and its analogues, CEMB is a more easily synthesized triterpenoid compound, showing potent cytotoxic activity against tumor cell lines in culture and displaying a cytotoxic mechanism of action similar to CDDO family compounds, including inducing ER stress and evidence of activation of both intrinsic and extrinsic apoptosis pathways. The relative

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merits of CEMB compared with CDDO analogues currently in clinical testing with respect to pharmacology and toxicology remain to be determined. Cytotoxic activity of CEMB against cultured hepatocytes was similar to CDDO-Im tested at concentration of 1 μmol/L or less (data not shown). Further mechanistic studies and evaluations of CEMB in preclinical models will help to define the overall potential of CEMB as a novel therapeutic agent for cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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