Celastrol, a Triterpene, Enhances TRAIL-induced Apoptosis through the Down-regulation of Cell Survival Proteins and Up-regulation of Death Receptors*

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Whether celastrol, a triterpene from traditional Chinese medicine, can modulate the anticancer effects of TRAIL, the cytokine that is currently in clinical trial, was investigated. As indicated by assays that measure plasma membrane integrity, phosphatidylserine exposure, mitochondrial activity, and activation of caspase-8, caspase-9, and caspase-3, celastrol potentiated the TRAIL-induced apoptosis in human breast cancer cells, and converted TRAIL-resistant cells to TRAIL-sensitive cells. When examined for its mechanism, we found that the triterpene down-regulated the expression of cell survival proteins including cFLIP, IAP, Bcl-2, Bcl-xL, survivin, and XIAP and up-regulated death receptors DR4 and DR5. This increase in receptors was noted in a wide variety of cancer cells including breast, lung, colorectal, prostate, and leukemia cells. Gene silencing of the death receptor abolished the induction of DR5 expression by celastrol and associated enhancement of TRAIL-induced apoptosis. sensitization to TRAIL. Overall, our results demonstrate that celastrol can potentiate the apoptotic effects of TRAIL through down-regulation of cell survival proteins and up-regulation of death receptors via the ROS-mediated up-regulation of CHOP pathway.

Radiotherapy, surgery, and chemotherapy are considered front-line methods of cancer treatment. Radiation and chemotherapy trigger cancer cell death through several different mechanisms. These conventional treatments, however, are toxic and not effective; the tumor gradually develops resistance. In recent years, the development of more selective, tumor biology-driven agents and targets for tumor-derived molecules has become of great interest in cancer research. Furthermore, it has been suggested that a combination of conventional chemotherapy with the biological agents may result in more effective therapy.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily (1, 2) that has drawn considerable attention as an attractive molecule for treatment of various types of cancer (3). Antibodies that mimic TRAIL ability to trigger death receptors via the ROS-mediated up-regulation of CHOP and down-regulation of cell survival proteins and up-regulation of death receptors via the ROS-mediated up-regulation of CHOP pathway.

This article has been retracted by the publisher. An investigation at MD Anderson determined that images had been reused to represent different experimental conditions. Specifically, the actin immunoblot in Fig 1B, C, and the JNK1 immunoblot in Fig. 5B were reused. In Fig. 5C, the image showing MDA-MB-231 cells transfected with siRNA and treated with medium as well as the image showing MDA-MB-231 cells transfected with siRNA and treated with TRAIL were reused.

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The abbreviations used are: TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand; ROS, reactive oxygen species; CHOP, CAAT/enhancer-binding protein homologous protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; NAC, N-acetylcysteine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; XIAP, X-linked inhibitor of apoptosis; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun-N-terminal kinase; CDDO, methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate.

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9-dien-28-oate.
antitumor effects of celastrol are not limited to in vitro systems, as this triterpenoid was found to suppress the growth and metastasis of melanoma in syngeneic and xenograft mouse models (22), of human prostate tumor xenografts in mice (20), and of human glioma xenografts in nude mice (23), and it has exhibited antiangiogenic effects in zebrafish (24). Studies to define its therapeutic mechanism showed that it suppresses the nuclear factor (NF)-κB signaling pathway (21, 25) and VEGF expression (23); inhibits heat shock protein (HSP) 90 (26, 27), ERK (28), and proteasome (20, 29), and activates caspase-8 (22).

These factors led us to investigate whether celastrol can modulate TRAIL-induced apoptosis and if so, through what mechanism. We found that celastrol can indeed enhance TRAIL-induced apoptosis through the down-regulation of various cell survival proteins and via up-regulation of TRAIL receptors. The up-regulation of death receptors by celastrol modulate TRAIL-induced apoptosis and if so, through what route remain unresolved (20, 21). Our studies show that celastrol interacts with death receptors, modulating TRAIL-induced apoptosis. We demonstrate that celastrol up-regulates death receptors, and modulates TRAIL-induced apoptosis. The results support the view that celastrol modulates TRAIL-induced apoptosis through up-regulation of death receptors and down-regulation of survival proteins. The mechanisms involved in the up-regulation of death receptors by celastrol and the role of these receptors in TRAIL-induced apoptosis are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—A 5 mmol/liter solution of celastrol (from Cayman Chemicals) was prepared in 100% dimethyl sulfoxide, stored as small aliquots at −20 °C, and then diluted as needed in cell culture medium. Soluble recombinant human TRAIL/Apo2L was purchased from PeproTech. Penicillin, streptomycin, and fetal bovine serum were purchased from Invitrogen. Anti-XIAP was purchased from BD Biosciences. Antibodies against DR4, PARP, Bcl-2, cFLIP, Bcl-xL, cIAP-1, Bid, Bax, p53, CHOP, and caspase-8 were purchased from Santa Cruz Biotechnology. Anti-DR5 was purchased from ProSci, chemokine (amino acids 1–79). The immunocomplex was incubated at 4°C for 1 hour, after which the protein was resolved on 10% SDS-PAGE, transblotted to nitrocellulose membranes, and then exposed to TRAIL for 24 h. PI staining for DNA fragmentation was performed as described previously (30).

**Live/Dead Assay**—To measure apoptosis, we used the Live/Dead Assay (Invitrogen), which assesses intracellular esterase activity and plasma membrane integrity. This assay was performed as described previously (30).

**Propidium Iodide (PI) Staining for DNA Fragmentation**—Cells were pretreated with celastrol (2 μmol/liter) for 6 h and then exposed to TRAIL (10 ng/ml) for 24 h. PI staining for DNA content analysis was performed as described elsewhere (30). A total of 10,000 events were analyzed by flow cytometry using an excitation wavelength set at 488 nm and emission set at 610 nm.

**RNA Analysis and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**—DR5 mRNA was detected using RT-PCR as follows. Total RNA was isolated from cells using TRIzol reagent (Invitrogen) as instructed by the manufacturer. One microgram of total RNA was converted to cDNA using Superscript reverse transcriptase and then amplified by platinum Taq polymerase using the Superscript One Step RT-PCR kit (Invitrogen). The total RNAs were then amplified by PCR using the following primers: DR5 sense 5′-AAGACCTTGTGTCGTGTC-3′, DR5 antisense 5′-GAACATTCGATGTCACTCCA-3′, DR4 sense 5′-GTGAGCAACGCAGAAGTGTC-3′, and DR4 antisense 5′-TCCAAGGACAAGGCCTGTGCAT-3′.

**Western Blot Analysis**—To determine the levels of protein expression, we prepared whole cell extracts as described previously (30). The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies for 1 h at room temperature, washed, and then incubated with peroxidase-conjugated secondary antibodies. After washing, immunoreactive bands were visualized by enhanced chemiluminescence (ECL). The immunoblotting was performed as described previously (31).

**JNK Assay**—To determine the effect of celastrol on the kinase activity of JNK, JNK complex from whole cell extracts was precipitated with antibody against JNK1, followed by treatment with protein A/G-agarose beads (Pierce). After 2 h of incubation, the beads were washed with lysis buffer and then assayed in kinase assay mixture containing 50 mmol/liter HEPES (pH 7.4), 20 mmol/liter MgCl2, 2 mmol/liter diethiothreitol, 20 μCi of [32P]ATP, 10 μmol/liter unlabeled ATP, and 2 μg of substrate glutathione S-transferase (GST)-c-Jun (amino acids 1–79). The immunocomplex was incubated at 30 °C for 30 min and then boiled with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized using the PhosphorImager. To determine the total amount of JNK1 in each sample, whole cell extracts were subjected to Western blot analysis using anti-JNK1 antibody.

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**Cell Lines**—Human breast adenocarcinoma MDA-MB-231, MCF7, and T47D, colon adenocarcinoma cell lines HCT116, lung adenocarcinoma H1299, embryonic kidney carcinoma A293, prostate adenocarcinoma PC3, esophageal adenocarcinoma TT, pancreatic adenocarcinoma AsPC1, chronic myelogenous leukemia K-562, and acute T cell leukemia Jurkat cells were obtained from American Type Culture Collection. Human myeloid MB-5 cells were kindly supplied by Dr. Nicholas Donato (University of Michigan Comprehensive Cancer Center, Ann Arbor, MI). HCT116 variants with deletions in p53, p21, and bax were kindly supplied by Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD). The human colon cancer cell lines HCT116 and its derivatives were cultured in McCoy’s 5A medium supplemented with 10% fetal calf serum and penicillin/streptomycin (Invitrogen). MB-5 cells were cultured in Iscove’s modified Dulbecco’s medium with 15% fetal bovine serum. A293, MDA-MB-231 and TT were cultured in Dulbecco’s modified Eagle’s medium, and other cell lines were cultured in RPMI 1640 with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

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gel electrophoresis. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes, blotted with each antibody, and detected by an ECL reagent (GE Healthcare).

**Analysis of DR4 and DR5 Surface Expression**—MDA-MB-231 cells (3 × 10⁵) were treated with celastrol and washed with 1× PBS supplemented with 0.5% bovine serum albumin (BSA) after detachment with EDTA. Cells were then stained with phycoerythrin (PE)-conjugated mouse monoclonal anti-human DR5 or DR4 (clone 71908 and 69036, respectively, R&D Systems) for 45 min at 4 °C according to manufacturer’s instructions before washing and resuspension in a fluorescence-activated cell sorting buffer (1× PBS + 0.5% BSA) for flow cytometric analysis using an excitation wavelength of 488. PE-conjugated mouse IgG2B was used as an isotype control.

**Measurement of Intercellular ROS**—Cells were pretreated with 3 μmol/liter celastrol or DMSO for 6 h and treated with 10 μmol/liter 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) dye (Invitrogen). After 30 min of incubation, the mean fluorescence intensity at 530 nm was calculated.

**RESULTS**

The goal of this study was to determine whether and how celastrol modulates the TRAIL apoptotic effects. Breast cancer MDA-MB-231 cells were used for most studies, but other cell types were also used to determine the specificity of this effect.

**Celastrol Enhances TRAIL-induced Apoptosis**—We examined first the sensitivity of various breast cancer cell lines to TRAIL. Three breast cancer cell lines, MDA-MB-231, MCF7, and T47D, were treated for 24 h with TRAIL and then assessed for cell viability using the MTT method. The MDA-MB-231 cells were found to be highly sensitive to TRAIL whereas T47D was highly resistant and MCF-7 was
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Celastrol Modulates the Expression of Death Receptors

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Celastrol Sensitizes Breast Cancer Cell to TRAIL

Celastrol Up-modulates the Expression of Bax

Celastrol Down-modulates the Expression of Antiapoptotic Proteins Linked to TRAIL Resistance

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Celastrol Induces the Expression of Death Receptor

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Celastrol Potentiates TRAIL-induced Apoptosis

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Celastrol enhances TRAIL-induced apoptosis in human breast cancer cells. As shown in Fig. 1A, left panel, cells were treated with the indicated concentration of TRAIL for 24 h. Apoptosis was determined by the Live/Dead assay. Next we determined whether celastrol could potentiate TRAIL-induced apoptosis, as indicated by plasma membrane integrity, in human breast cancer cells. To reveal celastrol enhancement of response, we used suboptimal dose of TRAIL for subsequent experiments. As shown in Fig. 1B, celastrol enhanced TRAIL-induced apoptosis from 9 to 51% in MDA-MB-231 cells and from 8 to 38% in T47D cells. We also examined apoptosis by determining the size of the sub-G1 cell pool. We found that TRAIL-induced apoptosis was increased from 10.7 to 46.8% in MDA-MB-231 cells and from 12.8 to 47.1% in T47D (Fig. 1C). Thus our results indicate that celastrol converted the TRAIL-resistant T47D cells to TRAIL-sensitive cells.

Because TRAIL mediates apoptosis through the activation of caspase-8, caspase-9, and caspase-3, we next examined the effect of celastrol on these caspases and poly (ADP-ribose) polymerase (PARP) cleavage as induced by TRAIL in MDA-MB-231 and T47D breast cancer cells. As shown in Fig. 1D, although celastrol and TRAIL alone had a minimal effect on the activation of these caspases and on cleavage of PARP, the combination of the two was highly effective in activation of all caspases and consequent PARP cleavage. These results indicate that celastrol can enhance TRAIL-induced apoptosis. In case of T47D, the resistance to TRAIL was reversed by celastrol.

Celastrol Down-modulates the Expression of Antiapoptotic Proteins Linked to TRAIL Resistance

Celastrol down-regulated the expression of cFLIP (both long and short forms), Bcl-2, Bcl-xl, and cIAP-1 and survivin (Fig. 2A). Whereas the down-regulation of survivin and XIAP was less pronounced, the down-regulation of cFLIPL was quite dramatic and was dose-dependent. Whether this down-modulation of antiapoptotic proteins was time-dependent, was also examined. Celastrol down-regulated cFLIP, Bcl-2, Bcl-xl, and cIAP-1, XIAP, and survivin in a time-dependent manner (Fig. 2B).

Celastrol Up-modulates the Expression of Bax—Whether celastrol can modulate the expression of pro-apoptotic proteins was also examined. We found that celastrol up-regulated the expression of Bax in a dose- and time-dependent manner but had little effect on Bid at the dose and time employed (Fig. 2, C and D).

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Celastrol Modulates the Expression of Death Receptors—To further elucidate the underlying mechanism that may be responsible for the potentiation of TRAIL-induced apoptosis by celastrol, we began by examining the effect of celastrol on the expression of death receptors. Celastrol significantly increased the expression of DR5 proteins in a dose-dependent manner, with optimum induction occurring at around 2–3 μmol/liter in human breast cancer MDA-MB-231, MCF-7, and T47D cells (Fig. 3A, upper panel). Celastrol also induced the expression of DR4 in MDA-MB-231 and MCF-7 cells. No induction of DR4 in TRAIL-resistant breast cancer T47D cells was observed (Fig. 3A, middle panel).

Whether this induction of the DRs was time-dependent was also examined. In brief, MDA-MB-231 cells were treated with 3 μmol/liters of celastrol for indicated times, and then analyzed the levels of DRs by Western blotting analysis. Celastrol induced both DR5 and DR4 in a time-dependent manner, with optimum induction occurring after 12 h (Fig. 3A, lower panel).

Whether TRAIL receptors are induced by celastrol at the transcriptional level was investigated by reverse-transcriptase PCR. Celastrol substantially up-regulated both DR4 and DR5 mRNA expression in a time-dependent manner (Fig. 3B). Whether celastrol induces cell surface expression of TRAIL receptors, was also examined. As shown in Fig. 3C, celastrol also

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**FIGURE 1.** Celastrol sensitizes breast cancer cell to TRAIL. A, left, cells (3000 cells/well) were incubated various concentrations of TRAIL. After 24 h, cell viability was determined by the MTT assay. Right, cells were pretreated with celastrol for 6 h, washed with PBS to remove celastrol, and then were exposed to the indicated concentrations of soluble TRAIL for 24 h. The cell viability was determined by the MTT assay. Points, mean percentage relative to control-treated cells (n = 5); bars, standard deviation. B, MDA-MB-231 and T47D cells were treated with 2 μmol/liter celastrol for 6 h and washed with PBS to remove celastrol. Then cells were treated with the indicated concentration of TRAIL for 24 h. Apoptosis was determined by the Live/Dead assay. C, cells were exposed to 2 μmol/liter celastrol for 6 h, and then the celastrol was removed. Then cells were treated with TRAIL (10 ng/ml) for 24 h. Cells were stained with PI, and the sub-G1 fraction was analyzed using flow cytometry. D, cells were pretreated with celastrol for 6 h and washed out. Then cells were treated with TRAIL for 24 h. Whole cell extracts were prepared and analyzed by Western blotting using antibodies against caspase-3, caspase-8, caspase-9, and PARP.
increased the cell surface expression of both DR5 and DR4 in breast cancer MDA-MB-231 cells. However, the level of DR4 cell surface expression induced by celastrol was lower than that of DR5.

**Up-regulation of DR5 by Celastrol Is Not Cell Type-specific**—Celastrol induced the expression of both DR5 and DR4 in a dose-dependent manner in lung cancer cells (H1299), colon cancer cells (HCT116), prostate cancer cells (PC3), esophageal cancer cells (TT), pancreatic cancer cells (AsPC-1), myeloid cells (KBM-5), T leukemia cells (Jurkat), and chronic myeloid leukemia cells (K-562) (Fig. 3D). Together, these findings suggest that the up-regulation of DR5 and DR4 by celastrol is not cell type-specific.

**Gene Silencing of TRAIL Receptor Abolished the Effect of Celastrol on TRAIL**—To verify that the up-regulation of DR5 by celastrol is needed for sensitization to TRAIL, we used siRNA targeting DR5 and DR4 and thus abolished celastrol-induced expression of these receptors. As shown in Fig. 4A, transfection of cells with DR5 siRNA but not with control siRNA (scrambled RNA; scRNA) reduced the celastrol-induced DR5 up-regulation. Similarly, transfection of cells with siRNA for DR4 reduced the celastrol-induced DR4 expression (Fig. 4A). However, DR4 siRNA had minimal effect on the expression of celastrol-induced DR5 expression.

We next examined whether the suppression of DR5 or DR4 by siRNA could abolish the sensitizing effects of celastrol on TRAIL-induced apoptosis using an esterase staining assay (the Live/Dead Assay; Fig. 4B) and the size of the sub-G1 cell pool (Fig. 4C). The results indicated that the effect of celastrol on TRAIL-mediated apoptosis was effectively blocked in cells transfected with either DR5 or DR4 siRNA (Fig. 4B). On the other hand, treatment with control siRNA had no effect (Fig. 4B). Silencing of DR5 had more dramatic effect on TRAIL-induced apoptosis than that of DR4. The silencing of both receptors abolished the apoptosis as much as silencing of DR5 alone, thus suggesting that DR5 is a major player in TRAIL-induced apoptosis.

**Celastrol-induced Up-regulation of DR5 Up-regulation Is p53-independent**—Because p53 has been reported to regulate DR5 expression (33), we next examined the role of p53 in celastrol-induced DR5 up-regulation. Because celastrol induced DR4 and DR5 in MDA-MB-231 cells, which are known to express mutant p53 protein (Fig. 5A), p53 may not be needed for celastrol to induce TRAIL receptors. Furthermore, when we treated HCT116 colon cancer cells, which do not express p53 with celastrol, we again found that celastrol
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expression of CHOP, with optimum induction occurring at around 12 h (Fig. 5A).

Activation of JNK by Celastrol Is Not Needed for DR5 Up-regulation—Other triterpenoids, such as methyl-2-cyano-3,12-dioxooleana-1, 9-dien-28-oate (CDDO-Me), and acetylketo-β-boswellic acid, have been reported to induce DR5 expression through JNK activation (36, 37). We further examined the possibility that celastrol activates the JNK pathway in breast cancer cells. An immunoprecipitated kinase complex assay indicated that celastrol could induce JNK activation in a time-dependent manner (Fig. 5B, upper panel), but under the same conditions, it has no effect on total JNK protein level. These results are similar to previous results in melanoma cells (22).

Next, we examined whether inhibition of JNK could block DRs expression using specific inhibitor for JNK. As shown in Fig. 5B (lower panel), pretreatment of cells with a JNK inhibitor (SP600125) suppressed celastrol-induced up-regulation of DR5 partially, but had a greater effect on DR4 expression. These results suggested that JNK was involved in celastrol-induced DRs up-regulation.

Gene Silencing of CHOP Abolishes Celastrol-induced Up-regulation of DR5 Expression and Its Effect on TRAIL-induced Apoptosis—To clarify the functional role of CHOP in celastrol-induced up-regulation of death receptors, CHOP siRNA was used. Whereas DR5 was up-regulated by celastrol in non-transfected and control-transfected (scRNA) cells, transfection with CHOP siRNA significantly abrogated the up-regulation of DR5 (Fig. 5C, top panel). CHOP siRNA had no effect on celastrol-induced DR4 expression (Fig. 5C, middle panel).

We next examined whether the suppression of CHOP by siRNA could abrogate the effects of celastrol on TRAIL-induced apoptosis using an esterase-staining assay (the Live/Dead Assay). Celastrol had significantly reduced effects (from 54 to 24%) on TRAIL-induced apoptosis in cells transfected with CHOP siRNA (Fig. 5D, middle panel), whereas treatment with control siRNA had no effect (Fig. 5D, bottom panel). These results indicate that CHOP-dependent DR5 up-regulation contributes to the sensitizing effect of celastrol on TRAIL-induced apoptosis.

Celastrol-induced Up-regulation of CHOP and DR5 Requires ROS—How celastrol induces CHOP-DR5 was further investigated. That ROS plays a critical role in TRAIL-induced apoptosis and in induction of DR5 by various agents has been demonstrated (38, 39). To understand the roles of ROS in celastrol-induced DR5 expression, we examined whether celastrol can induce ROS production. As shown in Fig. 6A (left panel), treatment with celastrol increased the production of ROS. This induction of ROS generation was markedly reversed by pretreatment with ROS scavenger NAC (Fig. 6A, right).

Whether ROS production is needed for expression of DR5 by celastrol, was determined next. For this, we pretreated cells with various concentrations of NAC and a thiol antioxidant GSH for 1 h and then exposed them to celastrol for 24 h. We found that celastrol significantly up-regulated DR5 expression, whereas pretreatment of NAC blocked celastrol-induced DR5 protein expressions completely (Fig. 6B). Glutathione also abrogated the effect of celastrol on DR5 expression (Fig. 6B).
We also investigated whether ROS production mediates celastrol-induced CHOP induction. For this, cells were exposed to various concentration of NAC or GSH for 1 h, and then treated with celastrol for a further 12 h. As shown in Fig. 6C, celastrol markedly up-regulated CHOP expression, whereas pretreatment with NAC or GSH blocked the celastrol-induced expression of CHOP, thus indicating that ROS generation is critical for celastrol-induced up-regulation of DR5.

**Blockage of ROS Production Reversed Effect of Celastrol on TRAIL-induced Apoptosis**—We next examined whether scavenging of ROS could attenuate the TRAIL-induced cell death potentiated by celastrol. As shown in Fig. 6D, celastrol significantly enhanced TRAIL-induced apoptosis in breast cancer cells, and pretreatment of cells with NAC markedly reduced this celastrol-induced enhancement from 49 to 18%. These results suggest that ROS is needed for the sensitization of cells to TRAIL by celastrol.

### DISCUSSION

Among all the apoptosis-inducing cytokines, TRAIL is the only one still being actively pursued for its anticancer properties in the clinic. Many human cancer cell types, however, are resistant to TRAIL-induced apoptosis including chronic lymphocytic leukemia, astrocytoma, meningioma, and medulloblastoma (40, 41). Agents that can sensitize tumor cells to TRAIL have a great potential for making cancer therapy more effective.

In the present report we explored the role of celastrol, derived from the traditional Chinese medicine “Thunder of God vine,” for its role in sensitizing tumor cells to TRAIL. Using human breast cancer cells, we demonstrate that celastrol significantly enhanced the TRAIL-induced apoptosis. When examined for the mechanism, we found celastrol down-regulated the expression of cFLIP, an inhibitor of caspase-8 (9). Both the short and long forms of cFLIP were equally modulated. Similarly, the down-regulation of cFLIP by the peroxisome proliferator-activated receptor (PPAR)-γ agonist rosiglitazone has been shown to sensitize cells to TRAIL (42). We found that celastrol also significantly down-regulated the expression of Bcl-2. The latter
has been linked with suppression of apoptosis by TRAIL (43). In addition we found down-regulation of Bcl-xL, IAP-1, survivin, and XIAP, though to a lesser degree. All these proteins have been linked to TRAIL resistance (10–12, 15). How these survival proteins are down-regulated by celastrol is less clear. Most of the antiapoptotic proteins described above are regulated by NF-κB. Because celastrol has been shown to suppress NF-κB activation (21), it is possible that down-regulation of expression of these proteins is through the down-regulation of NF-κB.

We further found that celastrol significantly up-regulated the expression of both the TRAIL receptors, DR4 and DR5. The increase in expression occurred at the transcriptional level and was noted in cancer cells of the breast, lung, prostate, esophageal, and colon and in myeloid and leukemic cells. Celastrol not only induced expression of protein but also mRNA for the death receptor. We also demonstrated the up-regulation of cell surface receptors by celastrol. A large number of agents have been shown to induce death receptors including triterpenoid such as boswellic acid (37) and CDDO (36). We further found that silencing of death receptor induction abolished the effect of celastrol on TRAIL-induced apoptosis, thus suggesting that these receptors play a critical role. A recent report indicated that repeated treatment with subtoxic doses of TRAIL induces resistance to apoptosis in MDA-MB-231 cells (44). This resistance to TRAIL was reported to be mediated through the down-regulation of expression of cell surface death receptors and up-regulation of cFLIP. Because celastrol up-regulates death receptors and down-regulates cFLIP as described here, it should reverse the resistance to subtoxic doses of TRAIL.

Our studies further revealed that induction of TRAIL receptors is mediated through the induction of CHOP. Celastrol induces CHOP and the silencing of CHOP gene abolished the effect of celastrol on the induction of death receptors and on the TRAIL-induced apoptosis. Other terpenoids, such as CDDO-Me and acetyl-keto-β-boswellic acid, were also reported to induce expression of DR5 through CHOP induction (37, 45).
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Similarly celastrol induced the activation of JNK, an effect that was needed for induction of death receptors. The expression of DR5 by CDDO and boswellic acid also require JNK activation (37, 45). Numerous reports indicate that CHOP binds to the DR5 promoter and up-regulates the receptor expression (35, 37).

We found that perhaps the most important upstream signal linked to modulation of TRAIL signaling is ROS. We found to induce ROS, and quenching of ROS abolished the effect of celastrol on induction of CHOP and thus abolished the potentiation of apoptosis by celastrol. These results suggest that the sulforaphane for inducing ROS can show that celastrol induces apoptosis through the induction of CHOP through the activation of JNK and CHOP (see Fig. 7). Although p53 induction has been linked to modulation of TRAIL signaling is ROS. Celastrol was found to induce ROS, and quenching of ROS abolished the effect of celastrol on induction of CHOP and of death receptors (33), we found that perhaps the most important upstream signal linked to modulation of TRAIL signaling is ROS.

Celastrol has been shown to be a potent inhibitor of the proteasome activity (20). Several proteasome inhibitors such as MG-132 (46), PS-341 (47), and NPI-0052 (48) have been shown to induce TRAIL receptors. Like celastrol, MG132 also induces DR5 through induction of CHOP (35). Unlike celastrol, however, PS-341 sensitized the cells to TRAIL despite the up-regulation of the expression of cFLIP in lung cancer cells (47). These results suggest that different proteasome inhibitors may mediate their effects through different mechanisms. Celastrol is also a potent activator of heat shock protein (HSP)-90 (26, 29, 49). Inhibitors of the HSP-90 such as geldanamycin have been shown to sensitize cells to TRAIL (50). This effect of HSP90, however, was mediated through the inhibition of NF-κB. Celastrol has been shown to suppress NF-κB activation, which may also contribute to TRAIL sensitivity.

Overall our studies provide strong evidence that celastrol could potentiate TRAIL-induced apoptosis through down-regulation of cell survival gene products and up-regulation of death receptors. Among all the triterpenoids tested till now, celastrol was found to be most active. Further studies in animals are needed to investigate the anticancer potential of celastrol in combination with TRAIL.

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