Stronger proteasomal inhibition and higher CHOP induction are responsible for more effective induction of paraptosis by dimethoxycurcumin than curcumin

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Although curcumin suppresses the growth of a variety of cancer cells, its poor absorption and low systemic bioavailability have limited its translation into clinics as an anticancer agent. In this study, we show that dimethoxycurcumin (DMC), a methylated, more stable analog of curcumin, is significantly more potent than curcumin in inducing cell death and reducing the clonogenicity of malignant breast cancer cells. Furthermore, DMC reduces the tumor growth of xenografted MDA-MB 435S cells more strongly than curcumin. We found that DMC induces paraptosis accompanied by excessive dilation of mitochondria and the endoplasmic reticulum (ER); this is similar to curcumin, but a much lower concentration of DMC is required to induce this process. DMC inhibits the proteasomal activity more strongly than curcumin, possibly causing severe ER stress and contributing to the observed dilation. DMC treatment upregulates the protein levels of CCAAT-enhancer-binding protein homologous protein (CHOP) and Noxa, and the small interfering RNA-mediated suppression of CHOP, but not Noxa, markedly attenuates DMC-induced ER dilation and cell death. Interestingly, DMC does not affect the viability, proteasomal activity or CHOP protein levels of human mammary epithelial cells, suggesting that DMC effectively induces paraptosis selectively in breast cancer cells, while sparing normal cells. Taken together, these results suggest that DMC triggers a stronger proteasome inhibition and higher induction of CHOP compared with curcumin, giving it more potent anticancer effects on malignant breast cancer cells.

Citation: Cell Death and Disease (2014) 5, e1112; doi:10.1038/cddis.2014.85; published online 13 March 2014

Subject Category: Cancer

Breast cancer remains a major cause of morbidity and mortality worldwide. The incidence of breast cancer has been increasing over the past four decades and there are relatively few effective therapies for advanced-stage disease. Despite improvement in treatment options, the mortality of breast cancer patients has remained stable. Thus, many scientists are currently seeking new anticancer agents with better efficiency and fewer side effects.

Curcumin has demonstrated selective killing of various cancer cell types, while sparing normal cells. Despite the cancer-selective cytotoxic effects of curcumin, however, its clinical use has been limited by poor systemic bioavailability, poor absorption, rapid metabolism and conjugation in the gastrointestinal tract. In order to overcome these issues, new improved analogs of curcumin have been synthesized and tested. Dimethoxycurcumin (DMC) is a methylated analog, in which the phenolic-OH groups of curcumin have been replaced with methoxy groups. Tamvakopoulos et al. showed that DMC is more stable in cultured cells and in vivo, and has increased bioavailability compared with curcumin. In addition, DMC more potently induced apoptosis in HCT116 human colon cancer cells and Caki renal cancer cells, but was less toxic in lymphocytes, compared with curcumin. However, the mechanisms underlying the anticancer effects of DMC have not been fully explored.

Here, we show for the first time that DMC demonstrates more potent anticancer effects than curcumin on malignant breast cancer cells in vitro and in vivo, while sparing normal breast cells. We further report that DMC induces paraptosis at much lower doses than curcumin. Sperandio et al. first introduced the concept of ‘paraptosis’ as an alternative, nonapoptotic form of programmed cell death. This mode of

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Keywords: dimethoxycurcumin; curcumin; paraptosis; malignant breast cancer cells; proteasomal inhibition

Abbreviations: DMC, dimethoxycurcumin; ER, endoplasmic reticulum; CHOP, CCAAT-enhancer-binding protein homologous protein; ATF4, activating transcription factor 4; COX IV, cytochrome oxidase subunit IV; PDI, protein disulfide-isomerase; MAP kinase, mitogen-activated protein kinase; Alix, ALG-2-interacting protein X; ROS, reactive oxygen species; PGP-1-like, peptidylglutamyl peptide hydrolyzing-like; HBSS, Hank’s balanced salt solution; 3-MA, 3-methyladenine; CuDIPS, copper(II)-disopropyl salicylate; MnTBAP, Mn(III)tetrakis (4-benzoic acid) porphyrin chloride; CHX, cycloheximide; L-JNKI, L-JNK Inhibitor 1; siRNA, small interfering RNA; HMECs, human mammary epithelial cells; PI, proteasome inhibitor

Received 13.9.13; revised 20.1.14; accepted 05.2.14; Edited by E Baehrecke
cell death lacks the characteristic apoptotic features (e.g., nuclear fragmentation, chromatin condensation and the formation of apoptotic bodies) and does not respond to the typical inhibitors of apoptosis (e.g., caspase inhibitors and Bcl-xL). Observations that parapaptosis can be inhibited by cycloheximide (CHX) indicate that the process requires protein synthesis, thereby distinguishing it from necrosis. Parapaptosis is also insensitive to various autophagy inhibitors. Ultrastructural observation of cells undergoing parapaptosis has identified cytoplasmic vacuolization as being characteristic of this process; the mitochondria and endoplasmic reticulum (ER) first swell, and then fuse to create large vacuoles. Parapaptosis appears to occur during the development of the nervous system, as well as in some cases of neurodegeneration. Parapaptotic cells in brain tissues were observed to be filled with small and large vacuoles. In addition, various stimuli, including paclizaxel, curcumin and ophiobolin A reportedly induce parapaptosis or parapaptosis-like cell death in resistant malignant cancer cells, suggesting that parapaptosis may provide a strategy for overcoming innate and acquired resistance to the current pro-apoptotic anticancer therapies. However, the molecular basis of parapaptosis is poorly defined, and additional evidence is required to confirm the authentic biochemical markers of this process. ALG-2-interacting protein X (Alix) has been identified as an inhibitor of parapaptosis in mitogen-activated protein (MAP) kinase activation has been associated with parapaptosis induced by insulin-like growth factor I receptor, 1-nitropyrene, paclizaxel, curcumin, celsonol and yessotoxin, although the importance of the respective MAP kinase differs depending on the stimulus. We recently showed that proteasomal dysfunction and the generation of mitochondrial superoxide are critical for the curcumin-induced dilution of mitochondria and/or the ER and subsequent parapaptotic cell death in breast cancer cells. In this study, we provide evidence suggesting that the ability of DMC to effectively induce parapaptosis via potent proteasomal inhibition and CCAAT-enhancer-binding protein homologous protein (CHOP) upregulation may be responsible for its improved anticancer effects on malignant breast cancer cells, compared with curcumin.

Results

DMC demonstrates more potent anticancer effects on breast cancer cells in vitro and in vivo than curcumin. To evaluate the anticancer activity of DMC on various breast cancer cells, we first compared its cytotoxic effects with those of curcumin (Figure 1a). We found that DMC treatment more potently induced cell death in various breast cancer cell lines (Figure 1b). Although the IC_{50} values for curcumin were 151.95, 76.27, 37.48 and 34.75 μM for T-47D, MDA-MB-435S and MDA-MB-231 cells, respectively, those of DMC were 21.75, 23.62, 20.05 and 22.44 μM, respectively. We then compared the effects of curcumin and DMC on the long-term survival and found that treatment with 10 μM DMC for 12 h completely blocked the clonogenicity of MDA-MB-435S cells, whereas at least 30 μM curcumin was required to achieve the same effect (Figure 1c). Furthermore, 10 μM DMC required a much shorter incubation time than 10 μM curcumin to inhibit the clonogenicity of these cells. These results show that the anticancer effects of DMC on long-term survival appear to be greater than that on in vitro cytotoxicity to breast cancer cells. Similar results were obtained in MDA-MB-231 cells (Supplementary Figure 1). Next, we examined the anticancer effects of curcumin and DMC in vivo. Nude mice were xenografted with MDA-MB-435S cells, injected with curcumin or DMC at two doses (25 and 50 mg/kg) at intervals of 2 days for 20 days and tumor sizes were estimated. Both curcumin and DMC dose-dependently reduced the tumor sizes, but the tumor-reducing effect of DMC at 25 mg/kg was greater than that of curcumin at 50 mg/kg (Figure 1d), suggesting that DMC demonstrates a more potent in vivo anticancer effect than curcumin. To further confirm the in vivo anticancer effects of curcumin or DMC, we utilized bioluminescence imaging, which is a more sensitive measure of tumor growth than caliper measurement. Nude mice were injected with MDA-MB-435S cells engineered to express luciferase (MDA-MB-435S/Luc). Once a palpable mass was detectable (about 2 weeks), mice were subjected to intraperitoneal injections of vehicle, 50 mg/kg curcumin or DMC every 2 days for 20 days. Bioluminescent imaging analysis showed that DMC more effectively reduced the luciferase activity in tumors compared with curcumin, indicating again that DMC inhibited tumor growth more strongly than curcumin (Figure 1e). Collectively, these results indicate that DMC demonstrates more potent anticancer effects than curcumin when tested on breast cancer cells in vitro and in vivo.

DMC effectively kills malignant breast cancer cells via induction of parapaptosis. Investigation of the cellular morphologies showed that marked vacuolation commonly preceded cell death in DMC-treated MDA-MB-435S, MDA-MB-231, MCF-7 and T-47D cells (Figure 2a). We did not observe the morphological features of apoptosis, including cellular shrinkage, cytoplasmic blebbing and apoptotic bodies, in these DMC-treated breast cancer cells.
Dimethoxycurcumin induces paraptosis

MJ Yoon et al

Cell Death and Disease
Furthermore, the DMC-induced cell death of MDA-MB 435S cells was not inhibited by various caspase-specific inhibitors, and caspase-8, -9 and -3 were not noticeably processed by DMC (Supplementary Figure 2), suggesting that apoptosis is not involved in the DMC-induced death of these breast cancer cells. In addition, hematoxylin and eoxin staining also showed severe cellular vacuolation (without noticeable nuclear condensation or fragmentation) in the sections of MDA-MB 435S xenografts treated with 25 mg/kg DMC at intervals of 2 days for 20 days, compared with those treated with vehicle (Figure 2b). When we examined whether autophagy was involved in DMC-induced cell death,
Dimethoxycurcumin induces paraptosis
MJ Yoon et al.
DMC-induced cell death was not affected by 3-methyladenine (3-MA), bafilomycin A1 and chloroquine (CQ) (Figure 2c). However, interestingly, both I and II forms of LC3, but not ATG5 and ATG6, were upregulated by DMC (Figure 2d). As LC3 was critically involved in cytoplasmic vacuolation and cell death induced by 15d-PGJ2,27 we next tested whether upregulation of LC3 has a role in DMC-induced vacuolation and subsequent cell death. We found that neither DMC-induced cell death nor vacuolation was affected by knockdown of LC3 (Figures 2e and f). Thus, our results show that autophagy and LC3 are not involved in the DMC-induced cell death of breast cancer cells.

Next, we tested whether the vacuoles induced by DMC treatment may originate from mitochondria and/or the ER. When we used MDA-MB 435S sublines expressing fluorescence selectively in mitochondria (YFP-Mito cells) or the ER (YFP-ER cells), we found that at 16 h of 20 \( \mu \)M DMC treatment, dilation of mitochondria was noted around the nuclei and a lot of small ER-derived vacuoles were also observed at the cellular periphery (Figure 3a). These vacuoles originating from both mitochondria and the ER were progressively enlarged by the prolonged exposure to DMC. When we quantitatively measured the sizes of these vacuoles, the average width of the mitochondria-derived vacuoles and that of the ER-derived vacuoles were 1.42 and 4.93 \( \mu \)m, respectively, at 24 h of DMC treatment (Figure 3b). Immunocytochemistry of cytochrome oxidase subunit IV (COX IV), a mitochondrial protein, and protein disulfide-isomerase (PDI), an ER resident protein, showed that COX IV expression in MDA-MB 435S cells treated with DMC for 16 h was observed at the boundary of vacuoles around the nuclei, whereas PDI expression was mainly detected at the boundary of enlarged vacuoles at the cellular periphery (Figure 3c). When we performed electron microscopy, most of the mitochondria had disrupted cristae and they were swollen in MDA-MB 435S cells treated with 20 \( \mu \)M DMC for 12 h (Figure 3d). Furthermore, megamitochondria arising from fusion among swollen mitochondria were frequently observed at this time. In cells treated with 20 \( \mu \)M DMC for 24 h, there were numerous fusions between swollen sections of the ER and almost the entire cellular space except the nucleus was occupied by dilated ER and a few megamitochondria. When we compared the dose-dependent effects of curcumin and DMC, we found that about fourfold higher concentration of curcumin was necessary to induce a similar extent of cellular vacuolation (Figure 3e). Accordingly, we examined whether DMC induced more effective induction of paraptosis is responsible for its stronger anticancer effects than those of curcumin. As paraptotic cell death is known to require protein synthesis,13,14 we first tested the effect of CHX on DMC-induced cell death. We found that pre-treatment of MDA-MB 435S cells with 2 \( \mu \)M CHX almost completely blocked DMC-induced cell death and cellular vacuolation (Figure 4a and b). In addition, treatment with 20 \( \mu \)M DMC reduced the protein levels of Alix, an inhibitor of paraptosis,14 more rapidly and markedly than treatment with 40 \( \mu \)M curcumin (Figure 4c). As MAP kinases have been positively associated with paraptosis,3,14,19,23–26 we examined the functional significance of various MAP kinases in DMC-induced cell death and found that inhibition of the ERK pathway (using PD98059 or U0126) or the JNK pathway (using L-JNK Inhibitor 1 (L-JNKi)), but not inhibition of p38 (using SB203580), significantly inhibited DMC-induced cell death (Figure 4d). Western blot analysis showed that the activities of ERK and JNK were markedly and similarly increased by treatment with 20 \( \mu \)M DMC or 40 \( \mu \)M curcumin (Figure 4e).

Previously, we have shown that reactive oxygen species (ROS), in particular mitochondrial superoxide, and proteasomal inhibition critically contribute to the paraptosis induced by curcumin.3 Thus, we first attempted to examine the functional significance of ROS in DMC-induced cell death. We found that DMC-induced cell death was very effectively blocked by pre-treatment with either Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP; the MnSOD mimetic) or copper(II)diisopropyl salicylate (CuDIPS; the CuZnSOD mimic), but not catalase (Figure 4f). FACS analysis using MitoSOX-Red showed that mitochondrial superoxide levels were time- and dose-dependently increased by DMC (Figure 4g), indicating that the increased mitochondrial superoxide has a critical role in DMC-induced cell death. Comparison of the mitochondrial superoxide levels in MDA-MB 435S cells treated with curcumin or DMC for 4 h revealed that DMC increased mitochondrial superoxide levels to a greater extent than curcumin (Figure 4h). Next, we tested whether DMC treatment could also inhibit proteasomal activity, as reflected by increased accumulation of ubiquitinated proteins. Treatment with 20 \( \mu \)M DMC progressively increased the accumulation of protein–ubiquitin conjugates (Figure 4i), whereas pre-treatment with CHX completely blocked the accumulation of ubiquitinated proteins (Figure 4j). Collectively, our results show that paraptosis is a major mode of cell death by DMC in malignant breast cancer cells.

**DMC more potently induces proteasomal inhibition and CHOP upregulation, compared with curcumin.** Next, we examined whether the more potent anticancer effects of DMC over curcumin are associated with the increased inhibition of the proteasome and we found that the accumulation of ubiquitin conjugates in DMC-treated cells far exceeded that in curcumin-treated cells at each dose (Figure 5a). Immunocytochemistry of ubiquitin also showed that the expression levels of ubiquitin were more notably elevated in MDA-MB 435S cells treated with 20 \( \mu \)M DMC than those treated with 20 \( \mu \)M curcumin (Figure 5b). Accordingly, we compared the effects of curcumin and DMC on cellular proteasome activities. The 20S proteasome is a multicatalytic protease of the 26S proteasome complex that has multiple peptidase activities, including chymotrypsin-like, trypsin-like and peptidylglutamyl peptide hydrolyzing-like (PGPH-like) activities.28 Consistent with the increased accumulation of protein–ubiquitin conjugates, treatment of MDA-MB 435S cells with DMC more effectively reduced chymotrypsin-like, trypsin-like and PGPH-like activities compared with the same doses of curcumin (Figure 5c). As synthetic fluorogenic substrates can be cleaved not only by the 20S proteasome, but also by other proteases present in cell lysates, we investigated the direct effects of curcumin or DMC on the proteolytic activities of the purified 20S proteasome. Although both curcumin and DMC dose-dependently inhibited the chymotrypsin-like, trypsin-like and PGPH-like hydrolytic activities of the purified 20S proteasome, the inhibitory effect of DMC was greater than that of...
curcumin (Figure 5d). Taken together, these results suggest that, compared with curcumin, DMC exhibits a more potent inhibitory effect against proteasomal activity in malignant breast cancer cells. As proteasomal inhibition is known to induce ER stress, we examined the expression of ER stress-associated proteins in DMC-treated cells. The protein levels...
of activating transcription factor 4 (ATF4) and CHOP were markedly increased after 4 h of DMC treatment, whereas KDEL protein levels were not noticeably affected (Figure 5e). In addition, the protein levels of Bim and Noxa, which were reportedly associated with the cytotoxic effects of proteasome inhibitor (PI), were also progressively increased by DMC treatment. Curcumin and DMC had similar effects on Bim and KDEL, slightly increasing the protein levels of Bim but not noticeably altering the expression of KDEL (Figure 5f). Interestingly, however, DMC treatment markedly increased the protein levels of CHOP, ATF4 and Noxa at much lower doses compared with curcumin (Figure 5f).

CHOP upregulation critically contributes to DMC-induced paraptosis. As ATF4 was reported to confer resistance to proteasomal inhibition, we investigated whether CHOP or Noxa may have a critical role in DMC-induced cell death. Interestingly, small interfering RNA (siRNA)-mediated suppression of CHOP, but not that of Noxa, significantly inhibited DMC-induced cell death (Figure 6a), indicating that CHOP may be critically involved in DMC-induced paraptosis. Interestingly, CHOP knockdown did not affect the DMC-induced accumulation of poly-ubiquitinated proteins (Figure 6b), suggesting that the DMC-induced upregulation of CHOP may act downstream of CHOP upregulation critically contributes to DMC-induced paraptosis. As ATF4 was reported to confer resistance to proteasomal inhibition, we investigated whether CHOP or Noxa may have a critical role in DMC-induced cell death. Interestingly, small interfering RNA (siRNA)-mediated suppression of CHOP, but not that of Noxa, significantly inhibited DMC-induced cell death (Figure 6a), indicating that CHOP may be critically involved in DMC-induced paraptosis. Interestingly, CHOP knockdown did not affect the DMC-induced accumulation of poly-ubiquitinated proteins (Figure 6b), suggesting that the DMC-induced upregulation of CHOP may act downstream of...
of proteasomal impairment. Next, we used YFP-Mito and YFP-ER cells to investigate whether CHOP knockdown affected the DMC-induced dilation of mitochondria and/or the ER. The siRNA-mediated knockdown of CHOP markedly inhibited DMC-induced dilation of the ER, compared with its effect on DMC-induced mitochondrial dilation (Figures 6c, 6d, and Supplementary Figure 3). We further confirmed the effect of CHOP knockdown on DMC-induced dilation of mitochondria and the ER by immunocytochemistry of CHOP, PDI, and COX IV. In MDA-MB 435S cells infected with the...
Dimethoxycurcumin induces paraptosis
MJ Yoon et al

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We recently showed that proteasomal dysfunction critically contributes to curcumin-induced paraptosis in malignant breast cancer cells.\textsuperscript{3} It has been reported that human cancer cells possess elevated proteasomal activity\textsuperscript{37} and are more sensitive to PIIs than normal cells,\textsuperscript{38,39} indicating that the ubiquitin–proteasome pathway is a promising target for cancer therapy. Bortezomb, the first US Food and Drug Administration-approved PI, is clinically used for the treatment of newly diagnosed multiple myeloma and mantle cell lymphoma\textsuperscript{40,41} Giant ER-derived vacuoles have been increasingly recognized as indicating perturbation of the functional link between the ER and the proteasome.\textsuperscript{3,42} Failure of the proteasomal machinery leads to the accumulation of misfolded proteins in the ER and cytoplasm; this can overwhelm cells and lead to failure of the unfolded protein response and ER-associated degradation, both of which protect cells from proteotoxicity.\textsuperscript{27} Here, we found that DMC induced a greater accumulation of ubiquitinated proteins in MDA-MB 435S cells and a mouse xenograft tumor model, compared with curcumin. In addition, DMC more potently inhibited chymotrypsin-like, trypsin-like and PGP-like activities (versus curcumin) in experiments using MDA-MB 435S cell lysates or purified 20S proteasomes. Collectively, these results indicate that DMC inhibits the proteasome more potently than curcumin, contributing to more effective induction of paraptosis. When we further examined the significance of various signals associated with PI-mediated ER stress and/or toxicity, we found that DMC upregulated CHOP more potently than curcumin, and CHOP knockdown significantly attenuated DMC-induced cell death. Interestingly, DMC-induced ER dilation was almost completely blocked by CHOP knockdown, although DMC-induced dilation of mitochondria was not greatly affected by it. We found that curcumin-induced

Discussion

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ER dilation was also effectively blocked by CHOP knockdown (Supplementary Figure 4), suggesting that CHOP may have a critical role in paraptosis, particularly in the context of ER dilation. Further work is warranted to determine whether CHOP transcriptionally controls the expression of gene products responsible for DMC-induced dilation of the ER. Collectively, our results indicate that the upregulation of CHOP via DMC-induced proteasomal inhibition has a critical role in the induction of paraptosis, contributing to the more potent anticancer effects of DMC on malignant breast cancer cells, compared with curcumin.

Mechanistically, curcumin and DMC are both Michael acceptors ($\alpha,\beta$-unsaturated $\beta$-diketo group) that can react with sulfhydryl groups. This can induce oxidative stress by altering the cellular redox balance, potentially elevating the levels of ROS. Kunwar et al. showed that, similar to...
Dimethoxycurcumin induces paraposis
MJ Yoon et al

curcumin, DMC increased the generation of ROS in tumor cells. When we compared the mitochondrial superoxide levels in MDA-MB 435S cells treated with curcumin or DMC for 4 h, we found that DMC increased mitochondrial superoxide levels to a greater extent than curcumin (Figure 4h). The proteasome is known to be susceptible to oxidative modification and inactivation, so we presume that the severity of this DMC-induced oxidative stress may contribute to its more potent impairment of proteasomal activity. Furthermore, owing to cross-regulation, DMC-induced proteasomal inhibition may also further facilitate ROS induction by eliciting ER stress.

Identification of effective paraptotic inducers and the elucidation of critical signals involved in this alternative cell death mode may facilitate the design of novel therapeutics against malignant cancer cells with defects in their apoptotic machineries. Therefore, this study may contribute to the fields of cancer therapy and cell death research by identifying DMC as a more effective paraptotic inducer than curcumin, while also clarifying the functional significance of proteasomal modulation and CHOP induction in paraposis. To further confirm the clinical usefulness of DMC as a potential anticancer drug, future studies may be needed to examine its chemosensitizing effects for tumors and in vivo antitumor effects in a metastatic model.

Materials and Methods

Chemicals and antibodies. N-acetyl cysteine, CuDIPS and polyethylene glycol catalase, CHX, 3-methyladenine (3-MA), bafilomycin A1, CO, 4',6'-diamidino-2-phenylindole and crystal violet were purchased from Sigma-Aldrich (St. Louis, MO, USA), MitoSOX-Red, calcein acetoxymethyl ester (calcein-AM) and ethidium homodimer (EthD-1) were purchased from Molecular Probes (Carlsbad, CA, USA). MnTBAP, PD98059, U0126 and SB203580 were obtained and ethidium homodimer (EthD-1) were purchased from Molecular Probes (Eugene, OR, USA). The following antibodies were used: monoclonal anti-β-actin (Sigma-Aldrich); anti-ubiquitin, ATG6 (BENC1) and ATF4 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA); anti-KDEL (Stressgen, Ann Arbor, MI, USA); anti-Noxa and anti-Bim (BD Biosciences Pharmingen, San Jose, CA, USA); anti-phospho-ERK1/2, total ERK1/2, phospho-JNK, total JNK, ATG5, CHOP (GAD1D153), LC3B, and AIP-1/AtxA (Cell Signaling, Beverly, MA, USA); horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG (Molecular Probes).

Synthesis of DMC. DMC was synthetically prepared as described by the College of Pharmacology, Wonkwang University (Iksan, Korea) and Department of Molecular Science and Technology, Ajou University (Suwon, Korea). The purity of compound, detected by HPLC, was > 90%. All solvents used in this study were LC-MS grade and purchased from Sigma-Aldrich.

Cell culture. Human breast cancer cells lines MDA-MB 231, MDA-MB 435S, MCF-7, T-47D and MCF-10A were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (Gibco, Grand Island, NY, USA). Normal HMECs were purchased from Clonetics Corp. (San Diego, CA, USA) and maintained in mammary epithelial growth medium (MEGM; Clonetics Corp.) supplemented with bovine pituitary extract, insulin, human epidermal growth factor, hydrocortisone and antibiotics (Clonetics Corp.). The MCF-10A cells were cultured in the same MEGM additionally supplemented with 100 ng/ml cholera toxin (Calbiochem). Cell culture passage number <5 was used in this study. Curcumin (> 94% purity, Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 40 mM and stored in a dark colored bottle at −20 °C. This stock solution was diluted to the required concentration when needed.

Measurement of cellular viability. Cell viability was assessed by double labeling of cells with 2 μM calcein-AM and 4 μM EthD-1. The calcein-positive live cells and EthD-1-positive dead cells were visualized using fluorescence microscope equipped with Zeiss filter set #46 (excitation band pass, 500/20 nm; emission band pass, 535/30 nm) and #20 (excitation band pass, 546/12 nm; emission band pass, 575–640 nm) and counted (Zeiss, Oberkochen, Germany).

Clonogenic cell survival assay. MDA-MB 435S cells were plated in six-well plates at a density of 1 × 10^3 cells per well in triplicate and treated with curcumin or DMC of different dose for various time; and then, cells were treated with fresh drug-free medium and incubated for additional 9 days. After 9 days, cells were fixed in cold-methanol and stained with 0.5% crystal violet. Image acquisition was conducted using a digital camera.

Knockdown experiments using siRNAs and shRNAs. The siRNA duplexes used in this study were purchased from Invitrogen (Carlsbad, CA, USA) and have the following sequences: CHOP (NCBI accession no. NM_004083, 5'-GACGUCUGAUUGCAAGUGGU-3'); Noxa (Santa Cruz Biotechnologies, cat. no. sc-37335); LC3B (Santa Cruz Biotechnologies, cat. no. sc-43990). BLOCK-iT Fluorescent Oligo (Invitrogen) or Negative Universal Control (Invitrogen) was used as the control. After annealing of the pairs of siRNA oligos, cells in 24-well plates were transfected with 40 nM siRNA oligonucleotides using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions. For the knockdown experiments using CHOP-targeting shRNA, HEK293TN cells were transfected with the plasmid containing the non-targeting shRNA (SHC002V, Sigma-Aldrich) or the plasmid containing CHOP-targeting shRNA (TRCN0000364328, Sigma-Aldrich), together with pMD2.G (the envelope plasmid) and pSAX2.0 plasmid expressing packaging plasmid, using TransIT®-2020 transfection reagents (Mirus Bio, LLC, Madison, WI, USA) according to the manufacturer’s instructions. After 48 h of transfection, production of DMC, MDA-MB 435 cells were infected with the filtered lentiviral medium (derived from HEK293TN cultures) supplemented with 4 μg/ml polybreny.

Establishment of the stable cell lines expressing the fluorescence specifically in mitochondria or ER. To establish the stable cell lines expressing the fluorescence specifically in mitochondria or ER, MDA-MB 435S cells were transfected with the pEYFP-Mito or pEYFP-ER vector (Clontech, Mountain, CA, USA). Stable cell lines expressing pEYFP-Mito or pEYFP-ER (YFP-Mito or YFP-ER) were selected with fresh medium containing 500 μg/ml G418 (Calbiochem). To quantitatively measure the dilation of mitochondria and the ER induced by DMC, we analyzed the average width of the vacuoles originated from mitochondria and the ER in YFP-Mito and YFP-ER cells using AxioVision Rel. 4.8 software (Zeiss). More than 200 clearly identifiable vacuoles derived from mitochondria in 50 YFP-Mito cells and > 200 clearly identifiable vacuoles derived from the ER in 50 YFP-ER cells per experiment, randomly selected, were measured in three independent experiments.

Measurement of mitochondrial superoxide production. To measure mitochondrial superoxide production, cells were loaded with 2.5 μM MitoSOX-Red for 20 min in the dark, washed with Hank’s balanced salt solution (HBSS) with Ca^2+ and Mg^2+ and further processed for flow cytometry.

Western blotting. Cells were washed in PBS and lysed in boiling sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris (pH6.8), 1% SDS, 10% glycerol and 5% β-mercaptoethanol). The lysates were boiled for 5 min, separated by SDS-PAGE and transferred to an Immobilon membrane (Millipore, Bedford, MA, USA). After blocking nonspecific binding sites for 1 h by 5% skim milk, membranes were incubated for 2 h with specific Abs. Membranes were then washed three times with TBST and incubated further for 1 h with HRP-conjugated anti-rabbit, mouse or goat antibody. Visualization of protein bands was accomplished using ECL (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The protein band intensity was quantified by densitometric analysis using the NIH ImageJ program (National Institutes of Health, Bethesda, MD, USA). The representative results from at least three independent experiments are shown.

Immunocytochemistry. After treatments, cell were fixed with acetone/ methanol (1:1) for 5 min at −20 °C and blocking in 5% BSA in PBS for 30 min. Fixed cells were incubated overnight at 4 °C with primary antibody (anti-ubiquitin (1:500, mouse; Santa Cruz Biotechnologies), anti-CHOP (1:500, mouse; Cell Signaling), anti-COX IV (1:500, rabbit; GeneTex, Irvine, CA, USA) and anti-PDI (1:500, rabbit; Stressgen)) diluted in PBS and then washed three times in PBS.
Transmission electron microscopy. Cells were prefixed in Karnovsky’s solution (1% paraformaldehyde, 2% glutaraldehyde, 2 mM calcium chloride, 0.1 M cacodylate buffer, pH 7.4) for 2 h and washed with cacodylate buffer. Post-fixing was carried out in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 h. After dehydration with 50–100% alcohol, the cells were embedded in Poly/Bed 812 resin (Pelco, Redding, CA, USA) and polymerized, and observed under electron microscopy (EM 902A, Zeiss).

20S proteasome activity assay in intact cells. MDA-MB 435S cells cultured in 96-well plates (1 × 10^4 cells per well) were treated for various time points with different concentrations of curcumin or DMC. After the additional 2 h incubation with the specific fluorogenic peptide substrates, production of hydrolyzed AMC groups was measured using a fluorometer (SPECTRAmax Gemini EM, Molecular Devices, Union City, CA, USA) with 355-nm excitation and 465-nm emission wavelengths. The fluorogenic substrates Bz-Val-Methyl-acyl-AMC, Z-Leu-Leu-Val-Tyr-AMC (Cayman Chemical, Ann Arbor, MI, USA) were used to determine the 20S proteasome trypsin-like, chymotrypsin-like and PGPH-like activities, respectively.

Inhibition of purified 20S proteasome activity. Purified rabbit 20S proteasome (70 ng) was incubated with DMC or curcumin at different concentrations or the solvent DMSO for 2 h at 37°C. After proteinase K digestion, the assay was performed with D-luciferin, the mice were anesthetized with isoflurane and imaged using IVISTM 100 (Caliper Life Sciences, Hopkinton, MA, USA) and various exposure times (1 to 2 min) for image optimization. The results were analyzed with the Live Image 2.20 software package (Xenogen Corp, Alameda, CA, USA). The signal intensity was quantified as the sum of all detected photon counts within the region of interest (ROI) after subtraction of background luminescence measured from the corresponding ROI of control mice.

Statistical analysis. All data were presented as mean ± S.D. from at least three separate experiments. Student’s t-Test was applied to evaluate the differences between treated and control groups with cell viability. Data from multiple groups were analyzed by one-way ANOVA, followed by Bonferroni multiple comparison test. For all the tests, the level of significance was values of P < 0.05.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements. This research was supported by the National Research Foundation of Korea (NRF) funded by the Korean government (MSIP) (No. 2011-0018141 (Mid-Career Researcher Program)).

Author contributions
MJY and KSC designed research; MJY, JYK, JAL, YK, YL, JHP, BYL, HSK, S-AK, A-RY, E-YK, and IAK performed all experiments; MJY and KSC wrote the paper; MAK, C-OY, KL and KSC analyzed data.

and incubated for 1 h at room temperature with anti-rabbit or anti-mouse Alexa Fluor 488 or 594 (1: 500, Molecular Probes). Slides were mounted with ProLong Gold antifade mounting reagent (Molecular Probes) and cell staining was visualized with a fluorescence microscope using Zeiss filter sets #46 and #46HE (excitation band pass, 598/25 nm; emission band pass, 647/70 nm).

In vivo tumor imaging. Following the establishment of MDA-MB 435S cells that stably expressed luciferase (MDA-MB 435S/Luc), 2 × 10^6 MDA-MB 435S/Luc cells were injected into the left thighs of 6–8 weeks old male nude mice (Oriente Bio.). Two weeks after cell injection, mice were randomized into groups (n = 5 animals per group) and received 100 µl of vehicle, 50 mg/kg curcumin or DMC by intraperitoneal injections at intervals of 2 days for 20 days. For bioluminescence imaging, mice were injected intraperitoneally with α-luciferin (100 mg/kg body weight in 0.1 ml of sterile PBS). Thirteen minutes after injection with α-luciferin, the mice were anesthetized with isoflurane and imaged using IVISTM 100 (Caliper Life Sciences, Hopkinton, MA, USA) and various exposure times (1 to 2 min) for image optimization. The results were analyzed with the Live Image 2.20 software package (Xenogen Corp, Alameda, CA, USA). The signal intensity was quantified as the sum of all detected photon counts within the region of interest (ROI) after subtraction of background luminescence measured from the corresponding ROI of control mice.

Dimethoxycurcumin induces paraptosis
MJ Yoon et al

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