Targeting Cancer Cells via the Reactive Oxygen Species-Mediated Unfolded Protein Response with a Novel Synthetic Polyphenol Conjugate

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Abstract

Purpose: The selective killing of tumor cells is an important strategy for cancer therapeutics. The aim of this study was to develop a novel antitumor agent that is safe for normal cells with the ability to selectively target cancer cells.

Experimental Design: On the basis of quantitative structure–activity relationship, we synthesized a novel polyphenol conjugate (E)-3-(3,5-dimethoxyphenyl)-1-(2-methoxyphenyl)prop-2-en-1-one (DPP-23). We evaluated the effect of DPP-23 on proliferation, cell cycle, and apoptosis in various tumor cells. We also assessed molecular targets of DPP-23 using genome-wide expression profiling by DNA microarray and real-time PCR array systems.

Results: DPP-23 effectively inhibited the growth of cancer cells in vitro and in vivo (xenografts in Balb/c nude mice). At a molecular level, DPP-23 targeted the unfolded protein response (UPR) in the endoplasmic reticulum (ER) through the production of reactive oxygen species (ROS) in cancer cells, but not in normal cells, resulting in selective killing of tumor cells via caspase-dependent apoptosis.

Conclusions: The selective generation of ROS in cancer cells could be an attractive strategy for the selective killing of cancer cells, while maintaining negligible cytotoxicity to normal cells. DPP-23 represents a promising novel therapeutic agent for the selective production of ROS in cancer cells. Clin Cancer Res; 20(16); 4302–13.

Introduction

Reactive oxygen species (ROS) are the major forms of free radicals and include superoxide anion radical (O₂⁻), hydrogen peroxide (H₂O₂), and the highly reactive hydroxyl radical (·OH), all of which are produced ubiquitously in most cells as byproducts of cellular aerobic metabolism. ROS are also generated by exogenous sources such as smoke, tobacco, drugs, xenobiotics, or radiation, and exogenous ROS can also affect the overall oxidative status of a cell. Under normal physiologic conditions, cellular enzymatic and nonenzymatic ROS-scavenging systems, including catalase, superoxide dismutase, glutathione peroxidase, peroxiredoxins, glutaredoxin, and thioredoxin, regulate cellular ROS levels to maintain the proper intracellular redox states. Moderate levels of ROS can regulate many signal transduction pathways that are essential for various biologic processes, such as cell proliferation and differentiation (1). Abnormally increased ROS production or decreased antioxidant capacity can damage cellular proteins, lipids, and DNA, causing irreversible oxidative damage that leads to cell death and various pathologic conditions such as cardiovascular and neurodegenerative diseases (2).

Increasing evidence has suggested beneficial effects of ROS generation on chemotherapy-induced cell death in cancer cells (3–5). This is supported by observations that several cancer chemotherapeutic agents, including vinblastin, doxorubicin, camptothecin, inostamycin, and xanthine oxidase–conjugated polymer, induce apoptosis via the increased generation of ROS (6, 7). Because some cancer cells are more sensitive to ROS than normal cells (5), ROS production could be an effective strategy for the selective killing of cancer cells without causing significant toxicity to normal cells (8, 9).

A large variety of polyphenolic compounds are enriched in vegetables, fruits, and teas, including flavonoids, tannins, curcuminoids, galloctechins, and stilbenes. These compounds possess a wide range of pharmacologic properties, including antitumor effects (10–12). Because most dietary...
Selective Targeting of Tumor Cells by DPP-23

Translational Relevance
Therapeutic strategies with limited side effects are urgently needed for use in cancer chemotherapy. The primary object of the present study was to develop a novel antitumor agent that is safe for normal cells and exerts the preferential killing of tumor cells. Polyphenols are common compounds found in dietary plants that display a broad spectrum of biologic activities. The present study synthesized a novel polyphenol conjugate (DPP-23) that produces reactive oxygen species (ROS) in a cancer-selective manner, leading to the stimulation of the unfolded protein response (UPR) and caspase-dependent apoptosis. This study supports a molecular basis for the development of agents that stimulate the UPR via the production of ROS in cancer cells and for further evaluation in early-phase clinical trials.

Materials and Methods

Cell lines and culture
HCT116 (human colon cancer), MIA PaCa-2 (human pancreatic cancer), U87MG (human glioblastoma), MDA-MB-231 (human breast cancer), and PrimPanc (primary pancreatic cancer), U87MG (human glioblastoma), MDA-MB-231 (human breast cancer), and PrimPanc (primary pancreatic cancer), U87MG (human glioblastoma), MDA-MB-231 (human breast cancer), and PrimPanc (primary pancreatic cancer), U87MG (human glioblastoma), MDA-MB-231 (human breast cancer), and PrimPanc (primary pancreatic cancer), U87MG (human glioblastoma), MDA-MB-231 (human breast cancer), and PrimPanc (primary pancreatic cancer), U87MG (human glioblastoma), MDA-MB-231 (human breast cancer), and PrimPanc (primary pancreatic cancer), U87MG (human glioblastoma), MDA-MB-231 (human breast cancer), and PrimPanc (primary pancreatic cancer). Cells were generous gifts of Dr. Bert Vogelstein (John Hopkins University School of Medicine, Baltimore, MD). Cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS (HyClone).

Chemical synthesis and quantitative structure–activity relationship
The detailed procedures for chemical synthesis and quantitative structure–activity relationship (QSAR) were described in Supplementary Methods.

Clonogenic survival assays
Long-term clonogenic assay was carried out as described previously (14).

In vivo tumor xenografts
Xenograft tumor experiments were performed as described previously (15, 16). The detailed procedure was described in Supplementary Methods. All animal experiments were conducted following the standards and procedures approved by the Konkuk University Institutional Animal Care and Use Committee (Seoul, Republic of Korea; no. KJ12121).

Immunofluorescence assay
Double immunofluorescence analysis was performed as described previously (17). Xenograft tumor sections were prepared and incubated with mouse anti-Ki67 antibody (cell proliferation marker) or rabbit anti-cleaved caspase-7 (Asp198) antibody (apoptosis marker) for 90 minutes, and then stained with Alexa-Fluor 488-conjugated anti-mouse or Alexa-Fluor 555-conjugated anti-rabbit antibody for 30 minutes, yielding green and red signals, respectively. Nuclear DNA was stained using 1 μg/ml Hoechst 33258 (blue signal). Labeled cells were examined under an EVOSFl fluorescence microscope.

Analysis of the cell cycle and cell death
Flow cytometry was used to assess the cell cycle and using FACScalibur (Becton Dickinson Immunocytometry Systems) or NucleoCounter NC-3000 (Chemometec), as described previously (18). Propidium iodide-stained dead cells were enumerated using a FACScalibur flow cytometer.

Annexin V staining and TUNEL assay
The presence of phosphatidylerine on the outer leaflet of the cell membrane was analyzed using a FITC-conjugated Annexin V kit (BD Pharmingen), following the manufacturer’s instructions. Positively stained cells were then analyzed by flow cytometry using a NucleoCounter NC-3000, as described previously (19). For in situ DNA fragmentation, a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was carried out using an ApoBrutU in situ DNA Fragmentation Assay Kit (BioVision), following the manufacturer’s instructions.

Immunoblot analysis
Cell lysates containing 10 to 20 μg protein were separated by SDS-PAGE, and transferred to nitrocellulose membranes. The blots were probed with respective primary antibodies then incubated with secondary antibodies conjugated to horseradish peroxidase as described previously (16). The list of primary antibodies was described in Supplementary Methods. Results were visualized using an enhanced chemiluminescence detection system (Amer sham Pharmacia Biotech Inc.).

Transmission electron microscopy
The morphologic features of autophagy were examined by transmission electron microscopy using an H-7650 transmission electron microscope (Hitachi) at an accelerating voltage of 80 kV, as described previously (20).
GFP-LC3 localization
A plasmid expressing GFP-tagged LC3 was generously provided by Dr. Seung-Jae Lee (Department of Biomedical Science and Technology, Konkuk University). MIA PaCa-2 cells were transduced with 0.5-μg GFP-LC3 plasmid, and examined under an EVOSf1 fluorescent microscope (Advance Microscopy Group) as described previously (20).

Expression of siRNA
MIA PaCa-2 cells were transduced with MISSION shRNA lentiviral particles expressing scrambled or Beclin-1 siRNA (sc-40705, Santa Cruz Biotechnology), following the manufacturer’s instructions. Twenty-four hours after infection, 2 μg/mL puromycin was added to select for infected cells. After an additional 2 weeks, the stable silencing of Beclin-1 expression was confirmed using Western blotting. For the transient silencing of IRE1α, a pool of four target-specific 20 to 25 nucleotide IRE1α siRNAs (sc-40705, Santa Cruz Biotechnology) was used.

DNA microarray analysis
Total RNA was isolated from MIA PaCa-2 cells treated with 10 μmol/L DPP-23 for 12 or 24 hours using phenol/guanidine-based Isol-RNA Lysis Reagent (5 PRIME GmbH), following the manufacturer’s instructions. cDNA was then synthesized, and hybridized to Agilent human whole-genome 44 K v2 microarray (Agilent Technologies) that represents 34,127 annotated human genes. DNA labeling, hybridization, and data analysis were performed by the E-Biogen (http://www.e-biogen.com) with the Agilent GeneSpring software. Gene ontology was analyzed using Gene Ontology database (http://www.geneontology.org) and UPR pathway was analyzed using the KEGG Mapper tool (http://www.genome.jp/kegg/tool/map_pathway2.html). The raw data for expression profiling can be found at the website Gene Expression Omnibus (http://ncbi.nlm.nih.gov/geo) under accession number GSE 54225.

Real-time quantitative PCR for UPR gene expression profiling
MIA PaCa-2 cells were treated with 10 μmol/L DPP-23 for 24 hours, and total RNA was extracted using Isol-RNA Lysis Reagent (5 PRIME). The first-strand cDNA was synthesized from 500 ng total RNA using an iScript cDNA synthesis kit (Bio-Rad). Real-time quantitative PCR was performed using the commercially available Human Unfolded Protein Response RT²iQ Profiler PCR Array System (PAHS-0892; SABiosciences). Real-time PCR was performed using iCycler iQ (Bio-Rad). Data analysis was performed using the online tools provided by the manufacturers.

Assessing intracellular ROS levels
Cells were trypsinized and incubated with 10 μmol/L 2’,7’-Dichlorofluorescin diacetate (DCF-DA; Sigma-Aldrich) for 30 minutes. The fluorescence intensity was then analyzed using a FACSCalibur flow cytometer.

Determination of cellular GSH
Intracellular glutathione (GSH) was measured using a Glutathione Assay Kit (Cayman Chemical Company) according to the manufacturer’s instructions. Total cell lysates were prepared, deproteinization was performed, and cellular GSH contents were analyzed by measuring the yellow-colored 5-thio-2-nitrobenzoic acid (TNB), a product of glutathionylated 5,5’-dithio-bis-2-nitrobenzoic acid (DTNB) with a UV spectrometer at 405 nm.

Statistical analysis
The statistical significance in xenograft tumor growth between the vehicle-treated control and DPP-23–treated groups was analyzed using the two-sided Mann–Whitney test in GraphPad InStat version 3.0 (GraphPad Software; www.graphpad.com). P < 0.05 was considered statistically significant.

Results

Identification of a novel polyphenol conjugate that inhibits the clonogenicity of tumor cells
To identify a lead platform for the development of novel anticancer agents, we selected 35 polyphenols with known antitumor activity, including 16 flavones, eight isoflavones, 10 flavanones, and 1 chalcone (listed in Supplementary Table S1), and performed clonogenic survival assays in MIA PaCa-2 pancreatic cancer cells (Supplementary Fig. S1). The relationship between the structure of the polyphenol and their inhibitory effect on clonogenicity was analyzed using QSAR. On the basis of the interpretation of the comparative molecular field analysis (Supplementary Table S2 and Supplementary Fig. S2), we further synthesized 11 novel polyphenol conjugates (Supplementary Table S3). Of these, DPP-23 (Fig. 1A) exerted the most effective inhibition of clonogenicity (Supplementary Fig. S3A) and the cleavage of PARP (Supplementary Fig. S3B) in MIA PaCa-2 cells. It also inhibited the clonogenicity in other cancer cell lines, including human pancreatic cancer (Capan-1), breast cancer (MDA-MB231), colon cancer (HCT116), and osteosarcoma (HT1080) cells (Supplementary Fig. S3C). The remainder of this study focused on characterizing the molecular mechanism underlying the antitumor activity of DPP-23.

Effects of DPP-23 on the inhibition of tumor growth in vitro and in vivo
To assess the potential of DPP-23 as a therapeutic agent, we assessed whether DPP-23 inhibits tumor growth in vivo. HCT116 cells were inoculated subcutaneously into the flanks of athymic nude mice. At 18 days postimplantation, tumor-bearing mice were treated with DPP-23 intraperitoneally once daily. The inhibition of tumor growth in DPP-23–treated mice compared with vehicle-treated control was observed after day 24 (Fig. 1B). By day 30, the tumor sizes of the control and DPP-23–treated groups were 743 ± 135 and 453 ± 127 mm³.
respectively. Analysis of the proliferative index of tumor sections revealed decreased Ki-67 immunoreactivity, a marker of proliferation, in DPP-23–treated tumors compared with control (Fig. 1C). In contrast, treatment with DPP-23 increased active caspase-7 immunoreactivity. Importantly, there was no indication of toxicity in normal tissues (Fig. 1D). These results suggest that DPP-23 inhibited the growth of tumor xenografts in nude mice, possibly through the induction of apoptosis.

DPP-23 induces caspase-mediated apoptosis

To assess the mechanism of DPP-23–induced suppression of tumor growth, the effects of DPP-23 on the cell-cycle distribution of MIA PaCa-2 cells were assessed. Treatment with DPP-23 led to an accumulation of cells in the sub-\(G_1\) phase, with a concomitant decrease in the \(G_1\) population, in a time-dependent fashion, compared with vehicle-treated control cells (Fig. 2A). The numbers of \(G_1\)–M cells were increased at 12 hours and then decreased with an increase in
the sub-G1 cells, suggesting that DPP-23 induced cell-cycle arrest at the G2–M phase, followed by cell death. Similar results were obtained in HCT116 colon cancer cells (Supplementary Fig. S4A). Because of the increase in the population of sub-G1 cells, we next assessed whether DPP-23 induced apoptosis by costaining with FITC-Annexin V and propidium iodide. Annexin-V–positive cells (Av+) are either early apoptotic (Av+/P-; C0) or late apoptotic/necrotic (Av+/P+). Treatment of MIA PaCa-2 cells with DPP-23 resulted in an increased number of Annexin V-positive cells (Fig. 2B). Furthermore, DPP-23–treated cells exhibited increased in situ DNA fragmentation, assessed using TUNEL staining (Fig. 2C). Caspases are cysteine-dependent aspartate-directed proteases that play essential roles in apoptosis. DPP-23 increased the levels of the active forms of caspase-9, -3, and -7 and the cleavage of PARP, a substrate for caspase-3 and -7, in a time-dependent manner in both MIA PaCa-2 and HCT116 cells (Fig. 2D). In addition, inhibiting caspases using the pan-caspase inhibitor zVAD-FMK or the caspase-3 inhibitor DEVD-FMK blocked DPP-23–induced PARP cleavage (Fig. 2E). These results suggest that caspase activation plays a role in DPP-23–induced apoptosis.

DPP-23 induces autophagy

Autophagy is an adaptative mechanism that can result in either survival or cell death, depending on the stress and cell type (21). We therefore asked whether DPP-23 induces autophagy, and whether there is a relationship between autophagy and apoptosis in DPP-23–treated cells. Electron microscopy revealed that treatment with DPP-23 resulted in the appearance of numerous vacuoles in MIA PaCa-2 cells (Fig. 3A). During autophagy, microtubule-associated protein 1 light chain 3-I (LC3-I, 18 kDa) is processed to LC3-II (16 kDa), which binds to the autophagosome membrane and HCT116 cells (Fig. 2F). In addition, inhibiting caspases using the pan-caspase inhibitor zVAD-FMK or the caspase-3 inhibitor DEVD-FMK blocked DPP-23–induced PARP cleavage (Fig. 2E). These results suggest that caspase activation plays a role in DPP-23–induced apoptosis.

Figure 2. Effects of DPP-23 on apoptosis. A, MIA PaCa-2 cells (1 × 105 cells/sample) were treated with 10 μmol/L DPP-23, fixed with ethanol, and stained with propidium iodide (P). The cellular DNA content was determined by flow cytometry. 2N, diploid; 4N, tetraploid. B, MIA PaCa-2 cells were incubated with FITC-labeled Annexin V and PI after treatment with vehicle (control) or 10 μmol/L DPP-23 for 24 hours. Annexin V staining was analyzed by flow cytometry using a NucleoCounter NC-3000. C, MIA PaCa-2 cells were treated with 10 μmol/L DPP-23 for 24 hours, fixed, and incubated with DNA labeling solution containing TdT enzyme and Br-dUTP for 15 minutes, followed by FITC-labeled anti-BrdUrd antibody for 30 minutes. Nuclear DNA was stained with PI. Fluorescence was observed under an EVOS® fluorescence microscope. D, HCT116 and MIA PaCa-2 cells were treated with 10 μmol/L DPP-23, and then whole-cell lysates were analyzed by immunoblotting. E, MIA PaCa-2 cells were treated with 10 μmol/L DPP-23 for 24 hours in the absence or presence of 10 μmol/L zVAD-FMK (top) or DEVD-FMK (bottom), and whole-cell lysates were analyzed by immunoblotting. An anti-GAPDH antibody was used as an internal control.
after lipid conjugation (22). Following treatment with DPP-23, the accumulation of LC3-II increased in a dose- and time-dependent manner (Fig. 3B). LC3-I exhibited a diffuse cytoplasmic staining pattern, whereas LC3-II was visualized in small puncta corresponding to autophagosomes (22). In response to DPP-23 treatment, endogenous LC3 formed a punctate fluorescence pattern (Fig. 3C), representing the association of LC3-II with autophagosomes. When we transiently transfected MIA PaCa-2 cells with a GFP-LC3 expression vector, a diffuse distribution of green fluorescence was showed in the absence of DPP-23, but formed punctate structures after treatment with DPP-23 (Fig. 3D). DPP-23 also induced the formation of LC3-II in other cancer cells, including HCT116, U-87MG, and MDA-MB-231 cells (Supplementary Fig. S4B), suggesting the induction of autophagy in various cancer cell types.

**Inhibiting autophagy facilitates DPP-23–induced apoptosis**

Beclin-1, an ortholog of yeast atg6/vps30 (23), is essential for the initiation of autophagy (24), and so silencing Beclin-1 prevents the progression of autophagy (16). In MIA PaCa-2 cells expressing Beclin-1 siRNA (siBECN1), DPP-23–induced PARP cleavage (Fig. 3E), cell death (Fig. 3F), and inhibition of clonogenicity (Supplementary Fig. S4C) were increased as compared with cells expressing scrambled siRNA (siCT). Treatment with zVAD-FMK, a pan-caspase inhibitor, completely abrogated DPP-23–induced PARP cleavage but did not affect the accumulation of LC3-II (Supplementary Fig. S4D). These data suggest that autophagy protects cells from DPP-23–induced apoptosis, whereas caspase activation had no effect on DPP-23–induced autophagy.

**p53 is not involved in DPP-23–induced autophagy and apoptosis**

p53 plays a key role in the regulation of apoptosis and autophagy (25). To determine whether p53 regulates DPP-23–induced apoptosis, we assessed the effects of DPP-23 in wild-type (p53<sup>+/−</sup>) and p53-null (p53<sup>−/−</sup>) HCT116 cells (26). DPP-23 increased the levels of p53 in wild-type HCT116 cells, but had a little effect on PARP cleavage or LC-II formation (Supplementary Fig. S5A) and the number of p53-positive cells (dead cells) were quantified by flow cytometry.
of propidium iodide-stained dead cells (Supplementary Fig. S5B), suggesting that p53 is unlikely to be associated with DPP-23–induced autophagy and apoptosis. Many human cancers contain p53 mutations, which can mediate resistance to chemotherapy-induced apoptosis. Therefore, it is possible that DPP-23 could trigger apoptosis in cancer cells irrespective of p53 status.

**UPR is associated with DPP-23–induced apoptosis**

To understand the mechanism of action of DPP-23, we analyzed the gene expression profiles in MIA PaCa-2 cells treated with DPP-23 using the Agilent human oligonucleotide microarray. Out of the 34,127 genes in the array, DPP-23 upregulated approximately 3% and downregulated approximately 5% (Supplementary Fig. S6). Notably, a large number of genes associated with apoptosis (such as *BAX*, *BAK*), chaperones (such as *HSPA1A/HSP70, HSPA5/GRP78/BiP*), and the UPR (such as *IRE1α, CHOP/DDIT3/GADD153, XBP1, ATF4, ATF6*) were highly upregulated by DPP-23 treatment (Fig. 4A and Supplementary Fig. S7), suggesting that ER stress is associated with DPP-23–induced apoptosis.

To confirm whether DPP-23 activates the UPR, we further analyzed expression of UPR genes using real-time PCR with a RT² Profiler PCR Array System. Consistent with the microarray data, several UPR genes were increased at least 2-fold (Fig. 4B and Supplementary Table S4). Western blotting also showed that DPP-23 increased the levels of ER resident chaperone proteins, such as glucose-regulated protein 78-kDa (GRP78)/immunoglobulin heavy chain binding protein (BiP)/HSPA5, inositol-requiring enzyme 1-α/ER-to-nucleus signaling 1 (IRE1α/ERN1), protein disulfide isomerase-1 (PDI-1), and ER oxidoreductase ERO1-Lα (Fig. 4C). In particular, the levels of the transcription factor C/EBP-homologous protein (CHOP/DDIT3/GADD153) and cleaved caspase-4, which are closely associated with ER stress-induced apoptosis (27, 28), were increased in response to DPP-23 treatment. On the basis of the known mechanism of action of caspase-4, which mediates UPR-induced apoptosis, we proposed that the UPR pathway is implicated in DPP-23–induced apoptosis. To assess this hypothesis, IRE1α, an ER transmembrane protein kinase that monitors the status of UPR, was silenced using siRNA in MIA PaCa-2 cells (Fig. 4D). Silencing IRE1α inhibited DPP-23–induced caspase-4 cleavage, suggesting that the UPR is associated with DPP-23–induced apoptosis.

**DPP-23 stimulates ER stress-mediated apoptosis through ROS generation in a cancer-selective manner**

Because oxidative stress can induce ER stress (29), we next assessed whether DPP-23 stimulated ROS production. After treatment with DPP-23, ROS accumulation was detectable within 1 hour in both MIA PaCa-2 and HCT116 cells (Fig. 5A). DPP-23 also stimulated ROS production in other cancer cell lines, including Capan-1 pancreatic cancer, MDA-MB-231 breast cancer, and HT1080 osteosarcoma cells (Supplementary Fig. S8A). We investigated whether DPP-23 stimulated ROS accumulation in normal cells. In control experiments, cisplatin, a known ROS-generating anticancer agent, induced a dose-dependent increase in ROS levels in both primary normal pancreatic epithelial cells (PrimPanc) and MIA PaCa-2 cells (Fig. 5B, top). In contrast, DPP-23 had no effect in PrimPanc cells, but stimulated the dose-dependent accumulation of ROS in MIA PaCa-2 cells (Fig. 5B, bottom). Flow-cytometric analysis of propidium iodide staining revealed that DPP-23 had little or no cytotoxicity in PrimPanc cells compared with MIA PaCa-2 (Fig. 5C), suggesting that DPP-23 selectively induces ROS formation and apoptosis in cancer cells.

GSH is an important cellular antioxidant that participates in the quenching of free radicals and ROS. Reduced GSH levels result in an accumulation of ROS, which is implicated in the progression of apoptosis (30). Thus, we analyzed whether DPP-23 depletes intracellular GSH levels. Treatment of MIA PaCa-2 cells with DPP-23 led to a decrease in the total cellular GSH level in a time-dependent manner (Supplementary Fig. S8B). N-acetyl cysteine (NAC), a thiol-containing ROS scavenger, prevented DPP-23–induced GSH depletion (Supplementary Fig. S8C). In contrast, antioxidants without thiol groups, such as α-tocopherol or Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), had no effect. These data suggest that DPP-23 reduces the cellular GSH content and that this effect is attenuated by thiol-containing antioxidants.

We assessed whether ROS accumulation was linked directly to DPP-23–induced apoptosis. Pretreatment of MIA PaCa-2 cells with 2 mmol/L NAC (a ROS scavenger) efficiently blocked DPP-23–induced ROS production (Fig. 5D, top graph). Pretreatment with NAC also reduced the DPP-23–induced accumulation of UPR proteins (GRP78/BiP, IRE1α, CHOP), as well as caspase activation (caspase-4, caspase-7, and PARP cleavages; Fig. 5D, bottom). In addition, the DPP-23–induced increased sub-G1 population was reduced by pretreatment with NAC (Fig. 5E). These results suggest that DPP-23 selectively induces ROS production in cancer, but not normal cells, and that ROS production is upstream of ER stress-mediated apoptosis.

**DPP-23–induced ROS stimulates the MAPK signaling pathways that differentially regulate autophagy and apoptosis**

The activation of MAPKs is important for many aspects of survival and apoptosis (31, 32). Therefore, we examined the role of MAPKs on DPP-23–induced apoptosis. Treatment of serum-starved MIA PaCa-2 cells with DPP-23 induced the phosphorylation of ERK1/2, JNK1/2, and p38 MAPK in a time-dependent manner (Fig. 6A), which was prevented by pretreatment with NAC (Fig. 6B), suggesting that DPP-23–induced ROS production leads to the stimulation of all three MAPKs.

Specific chemical inhibitors were utilized to investigate the potential involvement of MAPK family members in mediating DPP-23–induced apoptosis or protective autophagy. Pretreatment of MIA PaCa-2 cells with the MEK inhibitor U0126 strongly reduced DPP-23–induced LC3-II formation, but facilitated caspase-7 and PARP cleavage (Fig. 6C). In contrast, pretreatment with the p38 MAPK inhibitor PD98059 reduced DPP-23–induced LC3-II and PARP cleavage (Fig. 6D). These results suggest that MAPKs differentially regulate apoptosis and autophagy in response to DPP-23.

**Pharmacological agents**

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inhibitor SB203580 or the JNK inhibitor SP600125 had little effect on the formation of LC3-II, but decreased the cleavage of both caspase-7 and PARP significantly (Fig. 6C). These data suggest that ERK1/2 mediates the induction of autophagy, facilitating cell survival against ROS-induced cellular stress, whereas p38 and JNK play roles in DPP-23–induced apoptosis, but have little effect on the induction of autophagy.

**Discussion**

The identification of novel therapeutic strategies with limited side effects is a major goal for cancer chemotherapy. The aim of the present study was to develop less toxic anticancer agents. On the basis of QSAR analysis, we finally selected a novel polyphenol conjugate, DPP-23, that induced apoptosis in cancer cells but not in normal cells.
Mechanistically, DPP-23 induced caspase-dependent apoptosis, supported by observations including decreased Bcl-2 levels, and increased active caspase-4, -9, and -3 following DPP-23 treatment. DPP-23 also activated the UPR pathway in the ER, suggesting that DPP-23 could favor protein misfolding, resulting in ER stress and the activation of the UPR. The UPR is an adaptive mechanism aimed at repairing unfolded or misfolded proteins that have accumulated inappropriately in the ER. Three ER transmembrane proteins that serve as UPR sensors (PERK, IRE1, and ATF6) are inactivated via the association with the molecular chaperone GRP78. Upon the perturbation of protein folding, GRP78 dissociates from these sensors, and subsequently activates the UPR (33). Activated UPR proteins transmit stress signals to restore homeostasis and normal ER function by inhibiting the synthesis of new proteins and increasing the production of molecular chaperones involved in protein folding (34). However, when the activation of UPR is prolonged or severe, cell death programs can become activated (35, 36). IRE1 can recruit TRAF2 and ASK1, which leads to the activation of JNK. Activated JNK facilitates apoptosis through inhibition of the antiapoptotic protein Bcl-2. In addition, TRAF2 stimulates the activation of XBP1 and PERK, which in turn activate ATF4. The activation of ATF4 leads to the increased expression of CHOP/GADD153, a transcription factor that suppresses the antiapoptotic Bcl-2 and induces proapoptotic Bim (37). Accumulating evidence has demonstrated that CHOP is one of the most important mediators of ER-stress–induced apoptosis (38–40). In this study, we found that DPP-23 increased CHOP expression at the mRNA and protein levels (Fig. 4), and activated JNK and p38 MAPK (Fig. 6). In addition, we observed that inhibition of either JNK or p38 MAPK abrogated DPP-23–induced caspase-7 and PARP cleavage (Fig. 6). Because p38 MAPK stimulates CHOP, it is possible that UPR-induced CHOP activation could contribute to DPP-23–induced apoptosis.

Importantly, DPP-23 stimulated ROS production in various cancer cells, and pretreatment with the ROS scavenger NAC abrogated DPP-23–induced UPR and apoptosis. These
results suggest that ROS production is coupled to DPP-23–induced UPR. However, the mechanism by which DPP-23 promotes ROS production remains unclear. Given that GSH plays a critical role in scavenging intracellular ROS, one possible explanation is that depletion of GSH by DPP-23 is associated with ROS accumulation. Piperlongumine (PL), a small, naturally occurring, electrophilic molecule, increases ROS in both cancer and normal cells; however, it induces apoptotic cell death only in cancer cells (41). Further analysis of the structure–activity relationship using PL analogues revealed that the electrophilicity of the C2-C3 olefin is necessary for GSH depletion and ROS elevation (42). Because DPP-23 contains a Michael acceptor moiety like PL, it is plausible that DPP-23 could react with a thiol unit to deplete cellular GSH.

In this study, we revealed that DPP-23 did not induce the accumulation of ROS in PrimPanc normal pancreatic cells (Fig. 5A). However, the reason that DPP-23 accumulates ROS only in cancer cells is yet to be elucidated. In our preliminary studies, DPP-23 upregulated the expression of genes related to the redox detoxification system in PrimPanc cells (Supplementary Fig. S8B). This suggests that DPP-23 activates the normal antioxidant system in noncancer cells. However, most of these genes are constitutively expressed in MIA PaCa-2 pancreatic cancer cells, but are downregulated after treatment with DPP-23 (Supplementary Fig. S8D). Therefore, it is tempting to speculate that DPP-23–stimulated ROS in normal cells could be effectively detoxified by the upregulation of antioxidant enzymes. In most cancer cells, ROS are produced constitutively due to the continuous oncogenic signals and uncontrolled cell proliferation (43). As a consequence, cancer cells progressively acquire adaptive mechanisms against the excessively oxidative environment. However, this may also render cancer cells
dependent on their antioxidant system to maintain the redox balance, making them more vulnerable to agents that impair the antioxidant capacity than normal cells (9). Normal cells can therefore have a reduced susceptibility to oxidative insults than cancer cells. Our data suggest that DPP-23 can selectively kill cancer cells through the accumulation of ROS only in cancer cells. However, further studies, including identifying the mechanism underlying the selective downregulation of redox detoxification enzymes by DPP-23 in cancer cells, are needed to support our hypothesis.

Notably, the increase in the population of G2–M cells was accompanied by a decrease in the fraction of G1 cells. These data suggest that DPP-23 induces G2–M arrest. It is well known that the dynamic processes of tubulin polymerization and depolymerization play crucial roles in cell division. Dysregulation of tubulin dynamics may inhibit mitotic progression, resulting in mitotic arrest and apoptosis (44). Previous studies have demonstrated that some polyphenol compounds, such as chalcone-amido-benzothiazole conjugates (45) and 2-hydroxy-4-methoxy-2,3′-benzochalcone (46), exhibit mitotic arrest by disrupting microtubule polymerization. Thus, it is likely that DPP-23 binds to tubulin and arrests the cell cycle at the G2–M phase in a manner similar to that of antimitotic chalcones, suggesting that DPP-23 might also possess antimitotic activity.

In summary, we synthesized a novel polyphenol conjugate DPP-23, which increased ROS production. DPP-23 also triggered protective autophagy and ER stress-mediated apoptosis selectively in cancer cells. ERK1/2 was essential for DPP-23–induced prosurvival autophagy, whereas p38 and JNK played important roles in DPP-23–induced apoptosis. We propose that DPP-23 has the potential to be developed into a novel chemotherapeutic agent that targets the UPR through the production of ROS for the selective killing of cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: Y.H. Lee
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.H. Lee
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.Y. Shin, J.M. Lee, M.S. Lee, H. Jung, Y.H. Lee
Writing, review, and or revision of the manuscript: S.Y. Shin, Y.H. Lee
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.Y. Shin, J.M. Lee, M.S. Lee, Y. Lim
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References
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