Geranyl Derivative of Phloroacetophenone Induces Cancer Cell-Specific Apoptosis through Bax-Mediated Mitochondrial Pathway in MCF-7 Human Breast Cancer Cells

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Plant-derived polyphenols inhibit cancer cell proliferation and induce apoptosis. Recently, prenylflavonoids and alkyl-phloroacetophenones have been reported for their in vitro antitumor activity. In the present study, we examined the cytotoxic activity of prenyl (3-PAP) and geranyl (3-GAP) derivatives of phloroacetophenone, and xanthohumol (XN), a prenyl-chalcone, in human breast cancer (MCF-7) and human sarcoma (HT1080) cell lines in vitro. 3-GAP showed the strongest cytotoxicity in these cell lines with IC_{50} values of less than 10 \mu M. In addition, we report that 3-GAP is a more potent anti-cancer agent for breast cancer than XN which is a well-known anticancer flavonoid. Moreover, 3-GAP did not induce cytotoxicity in the normal cell line, TCMK-1, whereas 3-PAP and XN significantly reduced TCMK-1 cell viability. In 3-GAP-treated MCF-7 cells, nuclear accumulation and transcriptional activity of p53 were increased. In addition, pro-apoptotic Bax but not B-cell lymphoma 2 (Bcl-2) expression was increased by 3-GAP. In accordance with the Bax increase, 3-GAP induced mitochondrial cytochrome c release and activated caspase-9, an initiator of the caspase cascade. In the MCF-7 cell line which does not express caspase-3, activation of caspase-7, a member of the caspase-3 subfamily, was increased by 3-GAP. The present results indicate that synthetic 3-GAP is a safe and effective anti-cancer agent, and the Bax-mediated mitochondrial pathway is the main apoptosis signaling pathway of 3-GAP in MCF-7 cells.

Key words phloroacetophenone; apoptosis; cancer selective cytotoxicity; Bax; MCF-7

Phloroacetophenone analogues which are intermediates of the flavonoid biosynthetic pathway have shown in vitro cytotoxicity against several cancer cell lines. In fact, flavonoid polyphenols from dietary plants are known to exert a variety of effects. Epigallocatechin gallate, the most intensively studied tea polyphenol, inhibits inflammation, cell growth, and tumor invasion and metastasis. It also induces apoptosis in various cancer cell types. The apoptosis-inducing action of polyphenols involves several mechanisms including p53, and B-cell lymphoma 2 (Bcl-2)/Bax proteins. Although phloroacetophenone derivatives are reported to be effective agents for reducing cancer cell viability, it has not been revealed whether they induce apoptosis in breast cancer cells.

Breast cancer is the most common cancer and one of the leading causes of death in women in America and northwestern Europe. Approximately 60% of breast cancers are hormone-dependent and require estrogen for tumor growth. In fact, breast epithelial cells undergo cyclic proliferation and apoptosis during the menstrual cycle. One of the molecules responsible for maintaining the balance between cell proliferation and apoptosis is anti-apoptotic Bcl-2 protein, the expression of which also fluctuates in a cyclic pattern in normal breast epithelium. Anti-apoptotic Bcl-2 protein localizes to mitochondrial membranes, and antagonizes the action of pro-apoptotic Bcl-2 family members such as Bax, which permeabilizes the mitochondrial membrane to induce the release of cytochrome c. Tumor suppressor protein p53, which plays a role in DNA repair and cell cycle arrest, is also involved in apoptosis regulation. Importantly, the expression of these apoptosis regulating factors, p53 and Bcl-2/Bax, is inter-related in the apoptosis of cancer cells including breast cancer.

In the present study, we (1) compared the effects of synthetic geranyl and prenyl derivatives of phloroacetophenone and xanthohumol, a prenylchalcone, on the viability of several cancer cell lines, and (2) examined the involvement of p53 and Bcl-2 family members in the mechanism of action by which geranyl phloroacetophenone induces apoptosis in MCF-7 human breast cancer cells.

MATERIALS AND METHODS

Materials All cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.). Dimethyl sulfoxide (DMSO), protease inhibitor cocktail, sodium dodecyl sulfate (SDS), propidium iodide (PI), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 1-chloro-2, 4-dinitrobenzen (CDNB) and sodium pyruvate were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). p53-Luc was purchased from Panomics (Fremont, CA, U.S.A.). Antibodies against p53, Bax, Bcl-2, cytochrome c, actin and lamin B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-caspase-7 antibody was from Cell Signaling Technology Inc. (Danvers, MA, U.S.A.). Prenyl- and geranyl-phloroacetophenones were synthesized in the lab of Dr. Yong Rok Lee, and designated as 3-PAP and 3-GAP, respectively (Fig. 1).

Cell Lines and Culture Conditions MCF-7 human breast carcinoma cells and HT1080 human sarcoma cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin, and 100 \mu g/mL streptomycin. Cells were main-

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tained at 37°C/5% CO₂. TCMK-1 mouse kidney epithelial cells were maintained in Minimum essential medium with Earle’s balanced salts (MEM/EBSS) supplemented with 10% FBS and 100 U/mL penicillin, and 100 µg/mL streptomycin. The culture media were replaced every other day. After reaching confluence, cells were subcultured following trypsinization with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) solution.

**Cell Viability Assay (MTT Assay)** Cell viability was assessed using the MTT staining assay. Briefly, the cells from 4- to 5-day-old cultures were seeded in 96-well microtiter plates (Nunc, Denmark) at a density of 1 × 10⁵ cells/cm². The volume of the medium in each well was 200 µL. In the control experiments, the cells were grown in media containing the drug-free vehicle. After exposure to vehicle or various concentrations of 3-GAP (as indicated in the figure legends) in a final volume of 200 µL 1% FBS media for 48 h, 20 µL of MTT (5 g MTT/L in phosphate buffered saline (PBS)) was added, and the cells were incubated for another 4 h. The medium containing MTT solution was aspirated off, and dimethyl sulfoxide (DMSO) (200 µL) was added to each culture and mixed by pipetting to solubilize the formazan salt formed. The absorbance was then measured at 540 nm using a microplate reader (Molecular Devices, Versa MAX Sunnyvale, CA, U.S.A.), and cell viability was expressed as a percent of the control culture.

**Analysis of Apoptosis by Flow Cytometry** For flow cytometry analysis, cells were plated at 5 × 10⁴ cells/cm² in 6-well plates, and incubated until they reached 50% confluency. After treatment for 6, 24, and 48 h, the cells were rinsed with PBS, harvested, fixed by the slow addition of cold 70% ethanol (to 1 mL total) while mixing, and stored at 4°C overnight. The fixed cells were pelleted, washed twice with PBS and stained in 1 mL of 20 mg/L PI, 1 g/L RNase in PBS for 20 min. Cells (n=10000) were examined by flow cytometry (Becton Dickinson FACSort) gated on an area vs. width dot...
plot to exclude cell debris and aggregates and analyzed with CellQuest (San Jose, CA, U.S.A.) software. Apoptosis was measured as the level of subdiploid DNA contained in the total gated cells. Measurements are fold-increase over matched vehicle-treated cells.

**Protein Extraction** Total protein extraction of the drug-treated pellets was achieved by addition of lysis buffer (10 mM Tris pH 7.6, 10 mM EDTA, 150 mM NaCl, 0.1% NP-40, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.7 µg/mL pepstatin A, 10 µg/mL Leupeptin, and 1 µg/mL aprotinin). The lysates were centrifuged at 12000 rpm for 30 min at 4°C. The nuclear protein extract was collected using an NE-PER (Pierce, Rockford, IL, U.S.A.) kit per the manufacturer’s instructions. The subcellular fraction was obtained using a Mitochondria Isolation Kit (Pierce, Rockford, IL, U.S.A.). The soluble protein concentrations in lysates were determined in the BCA protein assay (Pierce, Rockford, IL, U.S.A.).

**Western Blot Analysis** Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Hybond ECL nitrocellulose membranes (Amersham Life Science, Buckinghamshire, U.K.) at 200 mA for an hour. The membranes were blocked in 5% skim milk in Tris-buffered saline-Tween-20 (TBST) (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20) at room temperature for 1 h. The membrane was incubated with specific primary antibodies in skim milk-TBS at 4°C overnight. The membrane was then washed 3 times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody in skim milk-TBS for 1 h at room temperature. Following 3 washes with TBST, the immunoreactive proteins were visualized with an ECL kit (GE Healthcare). When necessary, blots were stripped of antibody in a solution of 62.5 mM Tris–HCl, 2% SDS and 100 mM β-mercaptoethanol for 30 min with constant agitation in a 65°C water bath and then reprobed. Bands were quantitated by densitometry, using image analyzing software (Multi Gauge Ver 3.2, Japan).

**p53 Luciferase Reporter Gene Assay** Transient transfections were performed with Lipofectamine according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, U.S.A.). Briefly, cells were plated in 24-well plates at a density of 1 × 10⁵ cells/well. After 24 h, cells were transfected with plasmid mixtures of 0.5 µg of promoter-linked luciferase vector and 0.05 µg of pRL-TK vector for 5 h in OptiMEM media (Invitrogen). The transfected cells were treated with 3-GAP for 24 h. The cell lysates were used in a luciferase assay using a dual luciferase reporter assay kit (Promega, Madison, WI, U.S.A.), and the emitted light was measured with a luminometer (Turner BioSystems, Sunnyvale, CA, U.S.A.).

**Statistical Analysis** The data are expressed as mean ± S.E.M. of 3 independent experiments. Statistical analysis was performed with the Student’s t-test or analysis of variance (ANOVA) followed by the Student–Newman–Keuls comparison method to calculate differences between groups.
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(GraphPad Prism 4.0, San Diego, CA, U.S.A.). \( p \) values less than 0.05 were considered statistically significant.

RESULTS

Cancer Cell-Selective Cytotoxicity and Apoptosis-Inducing Activity of 3-GAP To compare the cytotoxic effects of the phloroacetophenone derivatives, 3-PAP and 3-GAP, we performed the MTT assay in cancer cells. Treatment with 3-GAP for 48 h decreased the viability of MCF-7 and HT1080 cells in a concentration-dependent manner. In the case of 3-PAP, although the inhibitory effect of 3-PAP on MCF-7 cell viability was concentration-dependent, the cytotoxic effect of 3-PAP was much less than that of 3-GAP (Fig. 2A). Since the prenylchalcone xanthohumol is known to have anticancer activity,\(^{23}\) we also compared the effect of 3-GAP with that of XN. 3-GAP was more effective than XN in inducing cytotoxicity in MCF-7 cells; similar cytotoxic effect of 3-GAP was observed in HT1080 cells (Fig. 2B). As summarized in Table 1, the concentration of 3-GAP at which the growth of cancer cells was inhibited by 50% (IC\(_{50}\)) was 5.3 and 4.5 \( \mu M \) in MCF-7 and HT1080 cells, respectively. When the cytotoxicity of the compound against a normal cell line was examined, 3-GAP did not reduce the viability of TCMK-1 normal mouse kidney epithelial cells (Fig. 2C) whereas 3-PAP significantly decreased the viability of TCMK-1 cells, which was less effective in the treatment with XN.

We then examined whether 3-GAP-induced cancer cell death was due to apoptosis in MCF-7 cells. In the analysis of hypodiploid DNA content detected by PI staining, 3-GAP induced time-dependent apoptosis detected at 6, 18, 36 h in MCF-7 cells (Fig. 3).

3-GAP Increased Nuclear Accumulation and Transcriptional Activity of p53 in MCF-7 Cells We further explored the signaling molecules involved in 3-GAP-induced apoptosis in MCF-7 cells. p53 is known to be involved in cancer cell apoptosis induced by polyphenols. Likewise, 3-GAP induced nuclear accumulation of p53 in a concentration-dependent manner based on Western blot analysis (Fig. 4A). Next, using a transient transfection assay with a p53-luciferase reporter gene, we examined the relative transcriptional activity of p53 binding to its promoter. In 3-GAP-treated MCF-7 cells, p53-Luc reporter activity was significantly increased (Fig. 4B).

Involvement of Bax and Mitochondrial Pathway in 3-GAP-Induced Cell Death of MCF-7 Cells Since p53-dependent Bax increase is reported as an apoptosis-signaling pathway in breast cancer cells,\(^{24}\) and Bcl-2 family of proteins are important factors in apoptosis regulation,\(^{25}\) we then examined the level of Bax and Bcl-2 in 3-GAP-treated MCF-7 cells. In our study, the level of Bax protein was increased by 3-GAP in a time-dependent manner while the Bcl-2 level was not significantly altered; thus, the Bax/Bcl-2 ratio was increased by 3-GAP treatment (Fig. 5A).

The Bcl-2 family is localized to the mitochondrial membrane and modulates apoptosis by permeabilizing the mitochondrial membrane, leading to release of cytochrome c. 3-GAP, like most anticancer drugs that induce the release of cytochrome c into cytosol,\(^{26}\) induced cytochrome c release from mitochondria into cytosol in a time-dependent manner (Fig. 5B).

The release of cytochrome c into the cytosol induces activation of the caspase cascade.\(^{27}\) 3-GAP also activated caspase-9 in a time-dependent manner (Fig. 5C). Since the MCF-7 cell line does not express caspase-3, a critical component of the execution of apoptosis, due to a deletion on the caspase-3 gene, the cell line undergoes caspase-3-independent apoptotic pathways.\(^{28}\) Caspase-7, a member of the caspase-3 subfamily,
has been shown to be involved in apoptosis in MCF-7 cells.\textsuperscript{29)}
3-GAP significantly increased caspase-7 activation in MCF-7 cells (Fig. 5C).

**DISCUSSION**

Various plant-derived polyphenols inhibit cancer cell proliferation and induce apoptosis. Phloroacetophenone derivatives such as thouvenol A and thouvenol B are reported to have cytotoxic activity against A2780 ovarian cancer cells.\textsuperscript{30)} In a recent report by Basabe et al.,\textsuperscript{2)} the cytotoxic activities of various derivatives of phloroacetophenone in several cancer cell lines, HT-29, A549, HeLa, and HL-60 have been compared. Geranyl-phloroacetophenone was the most effective in inhibiting cell viability than farnesyl, oleyl, or stearyl derivatives. Despite this report of anti-cancer activity, cytotoxicity of phloroacetophenone derivatives against normal cells has not been reported. In the present study, we demonstrate that 3-GAP exerts strong cytotoxicity in cancer cell lines, and is more effective than 3-PAP. The IC\textsubscript{50} of 3-GAP was 5 µM or less in MCF-7 and HT1080 cancer cell lines. Previously, when the potential of the flavonoids, XN and genistein, to serve as anti-cancer agents for breast cancer has been evaluated,\textsuperscript{23,31)} XN was several fold more potent than genistein, a flavonoid currently being used in clinical trials as a chemopreventive agent for breast cancer in humans. Now, in our present study, we report that 3-GAP is a more potent anti-cancer agent than XN. Furthermore, 3-GAP did not induce reduction in the viability of the normal cell line, TCMK-1. When 3-PAP was compared to the prenylchalcone XN, 3-PAP was less cytotoxic to decrease cell viability in both cancer and normal cell lines.

In the present study, we found that the expression levels of
p53 and Bax were increased in 3-GAP-treated MCF-7 cells. Wild-type p53 not only down-regulate ant-apoptotic Bcl-2 but also up-regulate pro-apoptotic Bax. However, in our study with MCF-7 cells which express wild-type p53, the Bcl-2 level was not changed by 3-GAP treatment despite p53 nuclear translocation and transcriptional activity increase. The increase in the Bax/Bcl-2 ratio induces Bax translocation and disruption of mitochondrial membrane. In addition, the action of p53 on Bcl-2 level in apoptosis regulation is mediated in 2 ways: by direct trans-repression of Bcl-2 transcription and by transcription-independent, direct binding with Bcl-2. p53 released from p53-Bcl-2 complex can directly induce mitochondrial permeabilization and subsequent apoptosis. In the present study, although 3-GAP-increased p53 transcriptional activity did not accompany a change in the Bcl-2 level, the cytosolic level of the mitochondrial protein, cytochrome c was increased. These results imply that mitochondrial disruption and cytochrome c release may be important events in 3-GAP-induced apoptosis in MCF-7 cells, regardless of p53 increase. Furthermore, 3-GAP activated caspase-9, which is an initiator of caspase cascade, and caspase-7, an executioner protease in apoptosis signaling.

In conclusion, 3-GAP is a safer and more effective anti-cancer agent than 3-PAP or XN, and the Bax-mediated mitochondrial pathway is the main apoptosis signaling pathway of 3-GAP in MCF-7 cells.

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