Dimethoxycurcumin, a Structural Analogue of Curcumin, Induces Apoptosis in Human Renal Carcinoma Caki Cells Through the Production of Reactive Oxygen Species, the Release of Cytochrome c, and the Activation of Caspase-3

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Purpose: Curcumin (Cur) has been reported to induce apoptosis in human renal carcinoma Caki cells. Dimethoxycurcumin (DMC), one of several synthetic Cur analogues, has been reported to have increased metabolic stability over Cur. We determined whether DMC, like Cur, induces apoptosis in Caki cells and also compared the apoptosis-inducing activity of DMC with that of Cur.

Materials and Methods: Caki cells were treated with DMC possessing four methoxy groups, Cur possessing two methoxy groups, or bis-demethoxycurcumin (BMC), which lacks a methoxy group. Cell viability was measured by using a methyltetrazolium assay. Flow cytometry and the caspase-3 activity assay were used to detect apoptosis. The release of cytochrome-c (Cyt c) was detected by Western blot analysis. The production of reactive oxygen species (ROS) was measured by flow cytometry.

Results: DMC, Cur, and BMC reduced cell viability and induced apoptosis, but the potency varied; DMC was the most potent compound, followed by Cur and BMC. ROS production, Cyt c release, and caspase-3 activity were increased, again in the order DMC > Cur > BMC. N-Acetylcysteine, a potent antioxidant, inhibited ROS production, Cyt c release, caspase-3 activation, and apoptosis induction in DMC-treated cells.

Conclusions: These results indicate that DMC, like the original form of Cur, may induce apoptosis in human renal carcinoma Caki cells through the production of ROS, the release of mitochondrial Cyt c, and the subsequent activation of caspase-3. In addition, DMC is more potent than Cur in the ability to induce apoptosis.

Key Words: Antineoplastic agents; Apoptosis; Curcumin; Dimethoxycurcumin; Renal cell carcinoma

INTRODUCTION

In recent years, a rapid increase in the costs of health care has facilitated the importance of plant-derived polyphenols for the prevention and treatment of human diseases, including cancer. Among the polyphenols, curcumin (Cur) has been most extensively investigated for its cancer chemopreventive and chemotherapeutic properties [1]. Several studies have shown that Cur is a potent inhibitor of tumor initiation in vivo [2,3] and possesses anti-proliferative activities against cancer cells in vitro [4,5]. Moreover, Cur has been reported to induce apoptosis in diverse human cancer cells [6,7]. The mechanism or mechanisms by which Cur can induce apoptosis in cancer cells remain poorly understood; however, it is most likely that Cur may induce apoptosis, at least in part, through the generation of reactive oxygen species (ROS), the release of mitochondrial cytochrome c (Cyt c), and the subsequent acti-
Apoptosis Induction by Dimethoxycurcumin

Cur, a member of the naturally occurring curcuminoid family, is a yellow-colored phenolic pigment in turmeric; the other two curcuminoids being demethoxycurcumin and bis-demethoxycurcumin (BMC). Both the inhibition of proliferation and the induction of apoptosis are associated with the cellular uptake of curcuminoids [14], which may be dependent on the number of methoxy groups at their aromatic rings. Cur is rapidly metabolized in vivo into tetrahydrocurcumin and other reduced forms in rats and mice and in vitro in human hepatic cells [15,16]. It has been demonstrated that some pharmacological activities are lost when Cur is reduced to its metabolites [15]. Thus, there is a need to develop Cur analogues with a higher metabolic stability than Cur. Dimethoxycurcumin (DMC), one of several synthetic Cur analogues, has been reported to have increased metabolic stability in comparison with Cur [17]. Interestingly, DMC can induce apoptosis in human HCT116 colon cancer cells [17], but its apoptosis-inducing effect against other cancer cells, such as renal cancer cells, has not been investigated.

Renal carcinoma remains one of the most drug-resistant malignancies in humans and is a frequent cause of cancer mortality [18,19]. Interestingly, two studies have demonstrated that Cur can induce apoptosis in human renal carcinoma Caki cells [20,21]. This prompted us to determine whether DMC would also induce apoptosis in Caki cells. For this purpose, we compared the ability of DMC, Cur, and BMC to induce apoptosis in terms of the number of methoxy groups in their chemical structures and also investigated the possible mechanisms by which DMC could induce apoptosis.

MATERIALS AND METHODS

1. Chemicals and antibodies
Cur and BMC were isolated from the rhizomes of turmeric, as described earlier [22]. DMC was synthetically prepared, as described previously [23], at the College of Pharmacology, Wonkwang University (Iksan, Korea). The purity of each compound, as detected by HPLC, was >90%. All solvents used in this study were LC-MS grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). The culture medium used throughout these experiments was Dulbecco’s modified Eagle’s medium (Sigma-Aldrich), containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 20 mM HEPES buffer and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin). Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. Fresh medium was given every second day and on the day before the experiments were done. Compounds used in this study were dissolved in dimethyl sulfoxide (DMSO), and all cells received DMSO to a final concentration of 2% (v/v). Controls were always treated with the same amount of DMSO.

3. Cell viability assay
Cell viability was determined by a modified MTT reduction assay. MTT is a pale yellow substance that is reduced by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even fresh dead cells do not reduce significant amounts of MTT. Caki cells were cultured in a 96-well flat-bottom plate at a concentration of 5x10⁵ cells/ml. After 12 h of preconditioning, the cells were treated with various concentrations of DMC or other agents for 24 h. Thereafter, the culture medium was aspirated and 100 µl of MTT dye (1 mg/ml in phosphate-buffered saline (PBS)) was added; the cultures were incubated for 4 h at 37°C. The formazan crystals produced through dye reduction by viable cells were dissolved by using acidiﬁed isopropanol (0.1 N HCl). An index of cell viability was calculated by measuring the optical density of the color produced by MTT dye reduction at 570 nm.
4. DNA fragmentation assay
Caki cells were harvested, washed with PBS, and then lysed overnight at 56°C with a digestion buffer containing 0.5% sarkosyl, 0.5 mg/ml proteinase K, 50 mM tris(hydroxymethyl) aminomethane (pH 8.0), and 10 mM EDTA. Following lysis, the cells were then treated with RNase A (0.5 μg/ml) for 3 h at 56°C. The DNA was then extracted by using phenol/chloroform/isoamyl alcohol (25:24:1) before loading and was analyzed by 2% agarose gel electrophoresis. The agarose gels were run at 50 V for 120 minutes in Tris-borate/EDTA electrophoresis buffer. Approximately 20 μg of DNA was loaded in each well with 6x loading buffer containing 0.25% bromophenol blue, 0.25% xylene xylanol, and 40% sucrose. DNA was stained with ethidium bromide and visualized under UV light (wavelength, 260 nm), and the plates were photographed.

5. Measurement of apoptosis
Apoptosis was examined by using an FITC-labeled Annexin V/propidium iodide (PI) apoptosis detection kit (Sigma-Aldrich) according to the manufacturer’s instructions. Briefly, 1x10⁶ Caki cells were harvested and washed with PBS. The cells were resuspended in 400 μl of binding buffer. Next, 5 μl of Annexin V-FITC and 1 μl of PI were added. Flow cytometric analysis was performed immediately after staining. Data acquisition and analysis were performed by a fluorescence-activated cell scanner (FACScan) flow cytometer (Becton Dickinson, San Jose, CA, USA). Cells in the early stages of apoptosis were Annexin V positive and PI negative, whereas cells in the late stages of apoptosis were both annexin V and PI positive.

6. Measurement of ROS production
Changes in intracellular ROS levels were evaluated by measuring the oxidative conversion of DCFH-DA to fluo-
rescent DCF. Caki cells grown in 12-well plates were loaded with 10 μM of DCFH-DA for 30 min at 37°C and were then incubated with Cur analogues under the indicated conditions. The cells were washed with PBS and harvested by gentle scraping. DCF fluorescence in 10,000 cells was detected by a FACSscan flow cytometer. The results were obtained as histogram plots of cell number to fluorescence intensity, and the mean fluorescence for each sample within an experiment was analyzed by using CellQuest Software (Becton Dickinson).

7. Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton-X 100) containing a protease inhibitor cocktail (Sigma-Aldrich). Cell extract protein amounts were quantified by using the BCA protein assay kit (Sigma-Aldrich). Protein lysates (50 μg per lane) were resolved on sodium dodecyl sulfate 10% to 14% polyacrylamide gels and transferred to nitrocellulose membranes; the membranes were then incubated with mouse polyclonal antibody against Cyt c. Peroxidase-conjugated goat anti-mouse antibody was used as secondary antibodies. Immune complexes were visualized with the use of an ECL solution (Amersham Biosciences, UK) according to the manufacturer’s instructions.

8. Measurement of caspase-3 activity

Caki cells treated with compounds were collected and washed with PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM MgCl2, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethanesulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. Cell lysates were clarified by centrifugation at 12,000 g for 20 min at 4°C. Caspase activity in the supernatant was determined by a fluorogenic assay (Promega’s CaspACE Assay System Corp., Madison, WI, USA). Briefly, 50 μg of total protein, as determined by BCA

![Image](https://example.com/image.png)

**Fig. 3.** Effects of dimethoxycurcumin (DMC), curcumin (Cur), and bis-demethoxycurcumin (BMC) on apoptosis in Caki cells. (A) Cells were treated for 12 h with solvent (control) or with 80 μM of DMC and were then stained by Annexin V-FITC/PI solution and analyzed by flow cytometry (cells in the lower right quadrant represent apoptosis). Cells were treated for 12 h with the indicated concentrations of DMC (B) or with 80 μM of DMC, Cur, or BMC (C). After Annexin V/PI staining, flow cytometric analysis was performed to determine the percentage of Annexin-V positive cells. Each bar represents the Mean±SD (n=4). *: p<0.05 compared with the control groups, †: p<0.05 compared with the DMC-treated groups.
protein assay kit (Sigma-Aldrich), was incubated with 50 μM of caspase-3-specific substrate (Ac-DEVD-AMC) at 30°C for 1 h. The release of methylcoumaryl-7-amine (AMC) was measured by excitation at 360 and emission at 460 nm with a fluorescence spectrophotometer.

9. Statistical analysis
The results were expressed as Means±SD. Statistical significance was estimated by Student’s t-test for unpaired observations between two groups or by ANOVA with Bonferroni correction for comparisons of multiple groups. At least three independent experiments were carried out for each variable. The level of significance was accepted at p < 0.05.

RESULTS
1. DMC reduces cell viability by apoptosis in Caki renal carcinoma cells
Human renal carcinoma Caki cells were exposed to increasing concentrations of DMC for 24 h. A notable loss of cell viability (approximately 40%) was observed after treatment with 80 μM of DMC (Fig. 2A). A significant reduction of cell viability was first observed after 6-h treatment with 80 μM of DMC and was found to be maximal after 24-h treatment (Fig. 2B). At 80 μM, Cur and BMC, like DMC, also reduced cell viability, but the potency varied; DMC was the most potent compound, followed by Cur and BMC (Fig. 2C). A typical ladder pattern of DNA fragmentation, which is considered a hallmark of apoptotic cell death, was observed when Caki cells were treated for 24 h with 80 μM of DMC, Cur, or BMC (Fig. 2D), which indicated that the loss of cell viability by these agents was due to the induction of apoptosis.

The quantitative measurement of apoptosis by use of the Annexin V/PI staining assay showed the percentage of cancer cells that underwent apoptosis after 12-h treatment with different concentrations of DMC (Fig. 3A, B). The induction of apoptosis was first observed after 6-h treatment

![Graphs and diagrams](https://example.com/graphs.png)

**Fig. 4.** Effects of dimethoxycurcumin (DMC), curcumin (Cur), and bis-demethoxycurcumin (BMC) on reactive oxygen species (ROS) production in Caki cells. (A) Cells stained by DCF-DA were treated for 6 h with solvent (control) or with 80 μM of DMC and were then analyzed by flow cytometry. Data are presented as log fluorescence intensity. Cells stained by DCF-DA were treated for 6 h with the indicated concentrations of DMC (B) or with 80 μM of DMC, Cur, or BMC (C). Flow cytometric analysis was performed to determine the mean fluorescence intensity of DCF-positive cells. Each bar represents the mean±SD (n=4). *: p<0.05 compared with the control groups. **: p<0.05 compared with the DMC-treated groups.
with 80 μM of DMC and gradually increased until 24 h (not shown). A comparable increase in apoptotic cells was also observed when Caki cells were exposed for 12 h to 80 μM of Cur or BMC (Fig. 3C). Again, DMC was the most effective in inducing apoptosis, followed by Cur and BMC (Fig. 3C). These data are in agreement with the ability of these compounds to reduce cell viability (Fig. 2D), which suggests the important role of the methoxy groups in DMC in the induction of apoptosis.

2. DMC induces ROS production, Cyt c release, and caspase-3 activation

The level of ROS production in cellular systems was investigated by using the specific fluorescent dye DCF-DA. This probe detects intracellular ROS and fluoresces after forming DCF inside cells. Therefore, an increase in fluorescence indicates increased production of cellular ROS. Fig. 4A and B show overlapped histograms of flow cytometric fluorescence intensity patterns of ROS produced by Caki cells after treatment with 80 μM of DMC for 6 h and a dose dependency between the concentration of DMC and ROS production, respectively. ROS production was found to be maximal after 6-h treatment with DMC, but at near basal levels after 24-h treatment (not shown). At 80 μM, Cur and BMC, like DMC, also induced significant levels of ROS, but they differed in their ability to induce ROS production (Fig. 4C). DMC was the most potent compound, followed by Cur and BMC. These results correlate well with the data on induction of apoptosis.

Cyt c is a component of the mitochondrial electron transport chain and is present in the inner membrane space. Disruption of the outer mitochondrial membrane by apoptotic stimuli, including H2O2, results in the release of Cyt c into the cytoplasm, which activates a cascade of caspases involved in apoptosis. Western blotting analysis with cytosolic fractions was carried out to examine the release of Cyt c from mitochondria in DMC-treated Caki cells. Cytosolic localization of Cyt c was clearly observed in Caki cells treated for 6 h with 40 μM or 80 μM of DMC (Fig. 5A). When examined at a given concentration (80 μM), DMC was the most

**Fig. 5.** Effects of dimethoxycurcumin (DMC), curcumin (Cur), and bis-demethoxycurcumin (BMC) on cytochrome c (Cyt c) release and caspase-3 activity in Caki cells. Cells were treated for 6 h with the indicated concentration of DMC (A) or with 80 μM of DMC, Cur, or BMC (B). Subcellular fractions were prepared and cytosolic Cyt c was detected by Western blot analysis with Cyt c antibody. The experiments were repeated three times with similar results. Cells were treated for 12 h with the indicated concentrations of DMC (C) or with 80 μM of DMC, Cur, or BMC (D). Caspase-3 activity was analyzed by a fluorogenic assay, as described in the Materials and Methods. Each bar represents the mean±SD (n=4). a: p < 0.05 compared with the control groups, b: p < 0.05 compared with the DMC-treated groups.
effective in inducing the release of Cyt c, followed by Cur and BMC (Fig. 5B). These results correlate well with the data on ROS production.

Activation of caspases by Cyt c is a key event during apoptosis caused by various anticancer drugs. Caspase-3 activity was significantly increased when Caki cells were exposed for 12 h to 40 μM or 80 μM of DMC (Fig. 5C). At 80 μM of DMC, an increase in caspase-3 activity was first observed after 6-h treatment with DMC, and activity gradually increased until 24 h (not shown). In comparison, the caspase-3 activity was increased in the order DMC > Cur > BMC (Fig. 5D). These results correlate well with the data on Cyt c release.

3. ROS production is involved in apoptosis by DMC

Numerous chemical and physical treatments capable of inducing apoptosis can also induce ROS production [24], suggesting a close relationship between ROS production and apoptosis. To determine whether ROS production could be responsible for the induction of apoptosis by DMC, Caki cells were treated with 80 μM of DMC in the presence or absence of the antioxidant N-acetylcysteine (NAC), and were analyzed for ROS production, Cyt c release, caspase-3 activation, and apoptosis. As shown in Fig. 6, NAC pretreatment inhibited ROS production, Cyt c release, caspase-3 activation, and apoptosis. Pretreatment of Caki cells with either 10,000 units of catalase or 50 μM of vitamin E also inhibited the induction of apoptosis by DMC (not shown).

DISCUSSION

Cur is the major component of the root of the plant Curcuma longa. A wealth of data indicates that this compound has potent antitumor effects against a variety of cancer cell lines in vitro and chemopreventive effects in carcinoma an-

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**Fig. 6.** Effects of N-acetylcysteine (NAC) on reactive oxygen species (ROS) production, cytochrome c (Cyt c) release, caspase-3 activation, and apoptosis induction in dimethoxycurcumin (DMC)-treated Caki cells. Cells were pre-incubated for 1 h with 10 mM of NAC and were then exposed for either 6 h (A, B) or 12 h (C, D) to 80 μM of DMC. ROS production (A) and apoptosis induction (D) were determined by flow cytometric analysis using DCF-DA and Annexin V plus PI, respectively. Cytosolic Cyt c (B) was detected by Western blot analysis using Cyt c antibody. Caspase-3 activity (C) was analyzed by a fluorogenic assay. Each bar represents the Mean±SD (n=4). a: p < 0.05 compared with the control groups, b: p < 0.05 compared with the groups treated with DMC alone.
imal models [1-7]. Moreover, phase I clinical trials have shown that Cur is safe even at high doses (12 g/d) in humans [25]. However, Cur exhibits poor bioavailability due to poor absorption, rapid metabolism, and rapid systemic elimination [26]. To improve the bioavailability of Cur, several approaches are being analyzed: these may include agents that block the metabolic pathway of Cur, phospholipid complexes to provide better permeability and longer circulation, and structural analogues of Cur that provide several-fold higher bioavailability than that of Cur [1]. In this study, we determined whether DMC, a structural analogue of Cur with higher metabolic stability over the original Cur, would induce apoptosis to a similar extent in human renal carcinoma Caki cells. The data presented here demonstrate that DMC is more potent than Cur in its ability to induce apoptosis.

ROS may play a crucial role in cell growth and apoptosis in cancer cells. An appropriate level of intracellular ROS promotes cellular proliferation [27], whereas excessive production of ROS leads to oxidative stress, loss of cell function, and ultimately apoptosis [24,27]. ROS production leads to the depolarization of the mitochondrial membrane and releases pro-apoptotic molecules from mitochondria into the cytosol, which may act to induce apoptosis [28]. The release of the pro-apoptotic molecule Cyt c from the mitochondrial membrane results in an increased level of Cyt c in the cytoplasm and nucleus, which may activate caspase-9, which, in turn, triggers the effector caspase-3 [28]. In this regard, a number of recent studies have suggested the possibility of using agents that promote cellular ROS accumulation to effectively kill cancer cells in vitro [28].

Cur, like most polyphenols, has been reported to induce ROS production when exposed to cancer cells [8-12]. Moreover, two studies have demonstrated that Cur can induce apoptosis through ROS production in human renal carcinoma Caki cells [20,21]. In light of the structural similarity between Cur and DMC, DMC can be recognized to have a chemical property resembling that of Cur. Indeed, induction of apoptosis by DMC in Caki cells was accompanied by an increase in the intracellular ROS level, the release of mitochondrial Cyt c into the cytosol, and the activation of caspase-9, which, in turn, triggers the effector caspase-3 [28]. In this regard, a number of recent studies have suggested the possibility of using agents that promote cellular ROS accumulation to effectively kill cancer cells in vitro [28].

Cur, like most polyphenols, has been reported to induce apoptosis through ROS production in human renal carcinoma Caki cells [20,21]. In light of the structural similarity between Cur and DMC, DMC can be recognized to have a chemical property resembling that of Cur. Indeed, induction of apoptosis by DMC in Caki cells was accompanied by an increase in the intracellular ROS level, the release of mitochondrial Cyt c into the cytosol, and the activation of caspase-9. NAC, an antioxidant agent, blocked the DMC-induced ROS production, blocked Cyt c release, and rescued cells from DMC-induced apoptosis. These results indicate that DMC, like Cur, induces apoptosis in Caki cells through a ROS-dependent signaling pathway, and this raises the question of how the three Cur analogues (i.e., DMC, Cur, and BMC) vary in their ability to induce apoptosis in renal carcinoma cells. DMC contains four methoxy groups, Cur contains two, and BMC contains none (Fig. 1). Our finding that DMC is maximally active, Cur is intermediate, and BMC is least active suggests that the methoxy groups do contribute to the enhancement of apoptosis. How the methoxy groups enhance apoptotic activity, however, is currently unclear. Recently, Tamvakopoulos et al showed that in comparison with Cur, the metabolism of DMC is less extensive in a cellular system [17]. Thus, it is possible that the differential efficiency of the Cur analogues may be associated with their different metabolic stabilities. Also, the differential efficiency of the Cur analogues may be caused by a different permeability of the compounds across the cell membrane, probably due to the number of hydrophobic moieties. Hsu and Cheng previously showed that increased apoptosis in colon cancer cells appears to be associated with the cellular uptake of Cur analogues [25].

Accumulating evidence suggests that the mode of Cur-induced cell death is mediated both by the activation of cell death pathways and by the inhibition of cell survival pathways. The role of mitochondria, among others, is well established in the induction of cell death by Cur [29], implying that the mitochondrial pathway might be the major pathway mediating the Cur-induced cell death. As evidenced by numerous studies, Cur-induced ROS formation causes the release of Cyt c from the mitochondria to the cytosol, hence leading to activation of caspase-9 and caspase-3 [29]. Additionally, Cur has been shown to inhibit the expression of anti-apoptotic proteins, including Bcl-2, Bcl-xL, and inhibitors of apoptosis (IAP) [29]. Indeed, Cur causes apoptosis of Caki cells, which is preceded by the sequential dephosphorylation of Akt, the down-regulation of the anti-apoptotic proteins (e.g., Bcl-2, Bcl-xL, and IAP), the formation of ROS, the release of Cyt c, and the activation of caspase-3 [20]. In our study, we have shown that DMC can induce apoptosis of Caki cells by the formation of ROS, the release of Cyt c, and the activation of caspase-3; however, other pathways could also be involved in DMC-induced cell death. Further studies are needed to investigate whether DMC, like the original form Cur, would down-regulate the expression of the anti-apoptotic proteins in Caki cells.

**CONCLUSIONS**

Our results have provided experimental evidence supporting that DMC, like the original form Cur, can induce apoptosis in Caki cells through the production of ROS, the release of mitochondrial Cyt c, and the subsequent activation of caspase-3. DMC, however, is more potent than Cur in its ability to induce apoptosis. Similar to Cur, DMC also seems to have multiple molecular targets, and its enhanced potency in cancer cell lines renders it a strong candidate for therapeutic applications for renal cancer as well as other cancers.

**Conflicts of Interest**

The authors have nothing to disclose.

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