Moscatilin Induces Apoptosis in Human Colorectal Cancer Cells: A Crucial Role of c-Jun NH2-Terminal Protein Kinase Activation Caused by Tubulin Depolymerization and DNA Damage

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Abstract

C-Jun NH2-terminal protein kinase (JNK) is a subfamily of the mitogen-activated protein kinase superfamily (1). Numerous lines of evidence indicate that JNK functions as a proapoptotic kinase (2, 3). Gene-targeting studies in Jun-null mice suggest that the JNK signaling pathway is important for apoptosis (4, 5). Furthermore, JNK is required for UV-induced cytochrome c release and apoptotic cell death, indicating that mitochondrial caspase activation may be responsible for JNK-induced apoptosis (6). It has been reported that constitutively active JNK2-JNK1 fusion protein can induce the caspase-dependent apoptosis pathway in Chinese hamster ovary cells (7). Overexpression of Mst1 and Ask1, which activate JNK pathways, can also trigger caspase activation and cell apoptosis (8, 9). Taken together, these studies suggest that JNK is an upstream signal for the activation of caspases. Intriguingly, several studies provide evidence that the caspase inhibitor z-VAD-fmk can inhibit JNK activation in cells responsive to various apoptotic stimuli. This indicates that JNK can serve as a downstream effector of caspase activation (10, 11).

Microtubules are composed of a backbone of α- and β-tubulin heterodimers and microtubule-associated proteins. They are involved in various cellular functions, including cell adhesion, movement, replication, and division. Microtubules are in a dynamic process of polymerization and depolymerization during cell replication and division. This makes them susceptible to numerous endogenous regulators and exogenous agents. Various antineoplastic compounds can disturb microtubule dynamics, thereby arresting cancer cell mitosis. For example, paclitaxel binds to the microtubule lattice, stabilizing microtubule bundles and impairing cell mitosis (12, 13). In contrast, vinblastine and vincristine bind to and inhibit tubulin polymerization, blocking mitosis and inducing apoptosis (14).

Moscatilin (4,4′-dihydroxy-3,3′,5-trimethoxybibenzyl) is a bibenzyl component derived from the India orchid Dendrobrium moscatum (15) and the stem of Dendrobrium loddigesii (16).
This plant was used in traditional Chinese medicine as a tonic to nourish the stomach, induce body fluid, and reduce fever. Several studies indicate that moscatilin inhibits platelet aggregation and has antimutagenic activity (17, 18). Recently, researchers have examined the antineoplastic activity of moscatilin against numerous lines of cancer cells (16). However, primary molecular targets of moscatilin have not been identified. In this study, we used several pharmacologic and biochemical assays to identify the possible molecular targets and anticancer mechanism of moscatilin in human colorectal cancer cells. We found that moscatilin induced the proapoptotic effect and cell apoptosis through the direct binding to tubulin, triggering DNA damage, and the induction of JNK activation. Furthermore, the in vivo antitumor efficacy of moscatilin was also determined to show the potential of this compound for further development.

Materials and Methods

Materials. Moscatilin was extracted and purified by one of our colleagues (C-C.C.) and the purity is more than 98% by extraction of high-performance liquid chromatography and nuclear magnetic resonance. RPMI 1640, fetal bovine serum, penicillin, streptomycin, and all other tissue culture reagents were obtained from Life Technologies. Antibodies to poly(ADP-ribose) polymerase and Bcl-2, p21 and HRP-conjugated anti-mouse, and anti-rabbit IgG were from Santa Cruz Biotechnology. Monoclonal antibody against caspase-3 was from Immgenex. Antibodies to caspase-8, caspase-9, phosphorylated JNK, JNK, phosphorylated c-Jun, and phosphorylated H2AX (Ser139), phosphorylated p53 (Ser15), and p53 were from Cell Signal Technology. Anti-α-tubulin IgG was from Serotec Product. Caspase colorimetric activity assay kits were from BioVision. Enhanced chemiluminescence detection kits were from Amersham. Antibodies to β-tubulin and FITC-conjugated anti-mouse IgG, propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at 37°C, and PI (80 μg/mL). DNA content was analyzed with the FACScan and CellQuest software (Becton Dickinson).

Analysis of microtubule polymerization in vitro. The CytoDYNAMIX ScreenTM3 (CDS-03) kits (purchased from Cytoskeleton) were used for the detection of polymerization of tubulin/microtubule. Tubulin proteins (>99% purity) were suspended (300 μg/sample) with 100 μL G-PEM buffer [80 mmol/L PIPES, 2 mmol/L MgCl₂, 0.5 mmol/L EGTA, 1.0 mmol/L GTP (pH 6.9)] plus 5% glycerol in the absence or presence of the test compound at 4°C. Then, the sample mixture was transferred to the 96-well plate, and the polymerization of tubulin was measured at 340 nm every 0.5 min for 45 min at 37°C (SpectraMAX Plus; Molecular Devices).

Immunofluorescence analysis. Cells were fixed with ice-cold methanol solution and then incubated for 1 h in a 2% bovine serum albumin/PBS blocking solution. Tubulin was detected using a mouse monoclonal anti-β-tubulin IgG followed by exposure to a goat FITC-conjugated anti-mouse secondary antibody. To visualize nuclei, cells were stained with 1 μg/mL 4,6-diamidino-2-phenylindole. All images were captured by Leica TCS SP2 confocal spectral microscope.

Western blotting. After the indicated exposure time of cells to DMSO or the agent, cells were washed twice with ice-cold PBS and reaction was terminated by the addition of 100 μL ice-cold lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1% Triton X-100]. For Western blot analysis, the amount of proteins (40 μg) was separated by electrophoresis in a 10% or 15% polyacrylamide gel and transferred to a nitrocellulose membrane. After an overnight incubation at 4°C in PBS/5% nonfat milk, the membrane was washed with PBS/0.1% Tween 20 for 1 h and immunoreacted with the indicated antibody for 2 h at room temperature. After four washes with PBS/0.1% Tween 20, the anti-mouse or anti-rabbit IgG (diluted 1:2,000) was applied to the membranes for 1 h at room temperature. The membranes were washed with PBS/0.1% Tween 20 for 1 h and the detection of signal was done with an enhanced chemiluminescence detection kit (Amersham).

Caspase activity assays. Caspase colorimetric activity assay were done as described by the manufacturer. Briefly, reaction mixtures were incubated with vehicle (0.1% DMSO) or compound for the indicated time courses, the cells were harvested by trypsinization, fixed with 70% (v/v) alcohol at 4°C for 30 min, and washed with PBS. After centrifugation, the cells were incubated in 0.1 mol/L phosphate-citric acid buffer [0.2 mol/L NaHPO₄, 0.1 mol/L citric acid (pH 7.8)] for 30 min at room temperature. Then, the cells were centrifuged and resuspended with 0.5 mL PI solution containing Triton X-100 (0.1%, v/v), RNase (100 μg/mL), and PI (80 μg/mL). DNA content was analyzed by the FACScan and CellQuest software (Becton Dickinson).

Effect of Moscatilin on Anti-Tubulin and DNA Damage

control growth (C), and cell growth in the presence of the drug (Tx), the percentage growth was calculated at each of the compound concentrations levels. Percentage growth inhibition was calculated as 100 - [(Fx - T0) / (C - T0)] × 100. Growth inhibition of 50% (IC50) is determined at the drug concentration that results in 50% reduction of total protein increase in control cells during the compound incubation.
assembled as follows: 150 to 200 μg protein (whole-cell lysates from moscatilin-treated HCT-116 cells), 50 μL of 2× reaction buffer (containing 10 mmol/L DTT), and 5 μL peptide substrate (LEHD-pNA for caspase-9 assays, IETD-pNA for caspase-8 assays, or DEVD-pNA for caspase-3 assays). Reactions were incubated at 37°C for 1 h and read at 405 nm by an ELISA reader.

**HCT-116 xenograft models.** Male severe combined immunodeficient mice were implanted s.c. with HCT-116 cells (10⁷ per mouse). When the tumors reached the average volume of 90 mm³, the mice were divided into four groups (n = 5) and the agent treatment was initiated. Vehicle (Cremophor EL/ethanol, 1:1; 0.2 mL/mouse) or moscatilin at doses of 50 and 100 mg/kg/d were administered i.p. five times a week. The group received Taxol (20 mg/kg) once in every 4 days. The length (L) and width (W) of the tumor were measured every 3 to 4 days, and the tumor volume was calculated as L x W² / 2. The protocols of the in vivo study were approved by the Animal Care and Use Committee at National Taiwan University.

**Data analysis.** The compound was dissolved in DMSO. The final concentration of DMSO was 0.1% in cells. Data are presented as mean ± SE for the indicated number of separate experiments. Statistical analysis of data was done with one-way ANOVA followed by Bonferroni t test and P values < 0.05 were considered significant.

Fig. 1. Moscatilin-induced apoptosis in human colorectal cancer cells. A, left, cells were treated with various concentrations of moscatilin (2-fold dilutions from 100 μmol/L) for 48 h. The cell number was determined using sulforhodamine B assay as described in Materials and Methods. Right, cells were incubated with a range of moscatilin (3-100 μmol/L) for 24 h. The cell number was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods. Mean ± SE of three independent determinations, * P ≤ 0.01, compared with nontreated HCT-116 cells. B, various types of human cancer cells were treated with a range of moscatilin for 24 h. The cell number was determined using sulforhodamine B assay. The IC₅₀ of each cell line was expressed as mean ± SE of three independent determinations in logarithm scale. C, HCT-116 cells were incubated without or with moscatilin (100 μmol/L) for indicated times and subsequently analyzed by PI staining to determine their DNA proportion. Quantitative data were based on histograms. Similar results were obtained in at least three other independent experiments.
Results

Effect of moscatilin on cancer cell proliferation and cell cycle progression. Treatment of human colorectal cancer HCT-116 cells with moscatilin inhibited cell proliferation in a concentration-dependent manner with an IC_{50} of 4.5 and 48.35 μmol/L by sulforhodamine B and mitochondrial 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assays, respectively (Fig. 1A). Moscatilin also inhibited the proliferation in several types of human cancer cells, including MCF-7, N87, HT-29, A549, Hep3B, PC-3, HCT-116, and NCI/ADR-Res, with IC_{50} values of 168.87, 131.34, 34.42, 19, 13.59, 4.95, 4.48, and 1.29 μmol/L, respectively (Fig. 1B). We determined the effect of moscatilin on cell cycle progression by FACScan flow cytometry of PI staining in asynchronized HCT-116 cells. Moscatilin induced a time-dependent arrest of the cell cycle at G2-M phase. This was associated with a reduced number of cells at the G_{1} phase and followed by an increase in number of cells at the sub-G_{1} phase (Fig. 1C).

Effect of moscatilin on tubulin polymerization and Bcl-2 phosphorylation. Many anticancer agents arrest cells at the G_{2}-M phase of the cell cycle (12–14, 19). Two major mechanisms of action underlie this effect: (a) disruption of tubulin and/or microtubules and (b) activation of mitogen-activated protein kinases, particularly JNK and p38 mitogen-activated protein kinase (14, 19–21). We did the in vitro tubulin polymerization of turbidity assay to determine if tubulins were targets of the moscatilin-mediated effect. As shown in Fig. 2, tubulin normally polymerizes in a time-dependent manner in the presence of GTP at 37°C. Moscatilin inhibited tubulin polymerization in a concentration-dependent manner (Fig. 2A), suggesting that it may directly bind to tubulins and inhibit their polymerization. Taxol was used as a reference compound. We also did immunofluorescence confocal microscopy of cells stained with anti-β-tubulin antibody to examine the effect of moscatilin on the microtubule cytoskeleton. Moscatilin treatment resulted in findings similar to those for vincristine-induced microtubule depolymerization, and there was only a diffuse stain visible throughout the cytoplasm (Fig. 2B). Several lines of evidence suggest that phosphorylation of Bcl-2 is associated with the loss of its antiapoptotic function and could be a biochemical marker for cells treated with tubulin-binding agents (19, 22). Our experiments showed that moscatilin induced the phosphorylation of Bcl-2, supporting our results that moscatilin has the tubulin-binding activity (Fig. 2C).

Effect of moscatilin on JNK phosphorylation and apoptosis signaling. We used pharmacologic inhibitors to examine the roles of p38 and JNK. FACScan flow cytometry of PI staining showed that SP600125 (a specific JNK inhibitor), other than SB203580 (a specific p38 inhibitor), significantly reduced moscatilin-induced apoptosis in HCT-116 cells (Fig. 3A). Moscatilin induced phosphorylation of JNK1/2 in a concentration-dependent manner (Fig. 3B). We also observed JNK-mediated...
phosphorylation of c-Jun in cells treated with moscatilin; this effect was completely abolished by SP600125 (Fig. 3B). These data suggest that activation of the JNK/c-Jun pathway may contribute to moscatilin-induced apoptotic cell death.

Following these results, we investigated the effect of moscatilin on activation of caspases, proteases that have important roles in apoptosis. Our data show that moscatilin induced the activation of caspase-9 and caspase-3 but not caspase-8 (Fig. 4A). Poly(ADP-ribose) polymerase, the downstream substrate of caspase-3, was also cleaved in moscatilin-treated cells (Fig. 4A). Notably, SP600125 significantly inhibited moscatilin-mediated caspase activation (Fig. 4B), indicating a critical role for JNK on apoptosis signaling.

**Determination of the effector responsible for JNK activation.** Our previous data show that moscatilin is a tubulin-binding agent. It is important to show if the activation of JNK results from the binding of moscatilin to tubulin. However, no known agent can block the binding of moscatilin to tubulin. Accordingly, a parallel experiment was done and the data showed that SP600125 significantly inhibits HCT-116 cell apoptosis (sub-G1 phase) caused by Taxol and vincristine (Fig. 5A). This suggests that these two tubulin-binding agents may also induce apoptosis through JNK activation in HCT-116 cells. Agents that cause DNA damage may also induce apoptotic cell death through the activation of JNK (23). Thus, we used the Comet assay to study the effect of moscatilin on DNA damage. Our results show that a 1-hour exposure of cells to moscatilin induces DNA damage in a concentration-dependent manner (Fig. 5B). Phosphorylation of H2AX (γ-H2AX) is one of the early chromatin modifications induced by camptothecin and an extremely sensitive marker for double-strand breaks (24).

Our result shows that moscatilin also caused phosphorylation of H2AX in a concentration-dependent manner (Fig. 5C). Following DNA damage, p53 may play a central role by induction of numerous downstream events, including DNA repair or apoptosis. To investigate the role of p53 in moscatilin-induced apoptosis, we examined downstream effectors and the phosphorylation state of p53 in HCT-116 cells. Our results show that moscatilin induced an up-regulation and phosphorylation of p53 at Ser^15_, with a concomitant increase in the protein levels of p21^WAF1/CIP1_ (Fig. 5C). Taken together, our data suggest that moscatilin-induced apoptosis may be partly attributed to its damage of DNA.

**Examination of in vivo efficacy by cancer xenografts in severe combined immunodeficient mice.** We subsequently carried out an initial in vivo study using HCT-116-derived cancer xenografts in severe combined immunodeficient mice. As shown in Fig. 6, the i.p. treatment with moscatilin (50 and 100 mg/kg) caused a significant inhibition of tumor growth without the loss of body weight. This initial in vivo experiment suggests that moscatilin may be an effective anticancer agent at dosages that induce negligible toxic effects (Fig. 6).

**Discussion**

Tubulin-binding agents, including polymerizing and depolymerizing drugs, are widely used in cancer chemotherapy. Currently, new tubulin-binding agents are of continued interest in the drug development. Two significant issues must be addressed before developing a new tubulin-binding agent for clinical studies. First, most tubulin-binding agents are derived from natural products that have complex chemical structures and are difficult to synthesize. Tubulin-binding agents with simple chemical structures, such as moscatilin, may be easier to synthesize and develop as clinical drugs. Second, cancer cells can develop resistance to many tubulin-binding agents, such as Taxol and vincristine, because these drugs are substrates of P-glycoprotein (25, 26). Moscatilin has effective antiproliferative activity in P-glycoprotein-rich NCI/ADR-Res cells indicating that it is not a P-glycoprotein substrate (Fig. 1B).

Recent studies have examined the role of proteins in the Bcl-2 family on the regulation of cell survival. In particular, many studies have examined antiapoptotic Bcl-2 proteins, which are capable of antagonizing proapoptotic proteins and preventing the loss of mitochondrial membrane potential. In our study, we showed that moscatilin induced the band shift of the Bcl-2 expression and the subsequent down-regulation in HCT-116
Fig. 4. Analysis of the contribution of the caspases pathways to moscatilin-mediated apoptosis. **A**, cells were treated with moscatilin (3-30 μmol/L) and then harvested for detection of caspase-9 and caspase-3 activations as well as poly(ADP-ribose) polymerase cleavages. Whole-cell lysates were separated and detected using Western blot analysis. **Right**, moscatilin induces caspases activities. Cells were treated with moscatilin (3-30 μmol/L) for 24 h and then harvested for detection of caspase-8, caspase-9, and caspase-3 activities. Mean ± SE of three independent determinations. *, $P < 0.05$; **, $P < 0.01$, compared with nontreated cells. **B**, SP600125 attenuated the cleavage of caspase-9, caspase-3, and poly(ADP-ribose) polymerase. Cells were treated with moscatilin 10 μmol/L in the absence or presence of SP600125 10 μmol/L for 24 h and then harvested for detection of caspase-9 and caspase-3 activations as well as poly(ADP-ribose) polymerase cleavages. Whole-cell lysates were separated and detected using Western blot analysis. **Right**, SP600125 reversed moscatilin-triggered caspase-9 and caspase-3 activities. Cells were treated with moscatilin 10 μmol/L in the absence or presence of SP600125 20 μmol/L for 24 h and then harvested for detection of caspase-9 and caspase-3 activities. Mean ± SE of three independent determinations.
cells, indicating the phosphorylation and degradation of Bcl-2 (Fig. 2C). Several lines of evidence suggest that Bcl-2 phosphorylation is associated with the loss of its antiapoptotic function (27), whereas other studies suggest that Bcl-2 phosphorylation is only a biochemical marker in cells halted in the M phase by tubulin-binding agents (28). Although we did not identify the function of phosphorylated Bcl-2, our data on Bcl-2 phosphorylation confirms the tubulin binding of moscatilin (Fig. 2A).

In recent decades, the molecular basis of the DNA damage pathways has been unraveled and the DNA damage caused by anticancer agents has been understood. This understanding facilitates the use of DNA damage-inducing agents and/or DNA repair inhibitors in the treatment of tumors (29–31). Numerous lines of evidence suggest that DNA damage activates the p53 signaling pathway and that this induces apoptotic cell death in various types of tumors (32, 33). Thus, triggering this pathway is a potential approach to anticancer therapy. Moscatilin induced an early DNA double-strand break (1 hour) and subsequently caused H2AX phosphorylation as well as up-regulated and activated p53 by phosphorylation at Ser^{15}, which caused induction of p21^{WAF1/CIP1}. This suggests that the

Fig. 5. Moscatilin induces DNA damage, p53 phosphorylation, and p21^{WAF1/CIP1} up-regulation. A, cells were incubated with indicated reagents for 24 h and subsequently analyzed by PI staining to determine their DNA proportion. Data acquisition and analysis were done on a FACScan flow cytometry as described in Materials and Methods. Quantitative data were based on histograms. Similar results were obtained in at least three other independent experiments. B, cells were treated with indicated reagents (CPT, camptothecin; MCT, moscatilin) for 1 h and then detected by Comet assay as described in Materials and Methods. Nuclei with damaged DNA have the appearance of a Comet with a bright head and a tail, whereas nuclei with undamaged DNA appear round with no tail. The percentage of cells with Comet tail was analyzed in 50 cells for one slide. Bar, 20 μm. Mean ± SE of three independent determinations.

C, cells were treated with moscatilin (3–30 μmol/L) and then harvested for the detection of phosphorylated pS{sup}\text{3′-3′}pS{sub}3, pS{sub}3, p21^{WAF1/CIP1}, and γ-H2AX protein expression. Whole-cell lysates were separated and detected using Western blot analysis.

Fig. 6. Antitumor effect of moscatilin in the HCT-116 xenograft model. A, groups of control, 50 and 100 mg/kg moscatilin, and Taxol. HCT-116 cells were injected s.c. in the flanks of severe combined immunodeficient mice. The mice were sacrificed when the tumor size of control group reached 2 g, which was 15 d after the first administration. The growth curves are the mean of the tumor sizes measured within each group, and the tumor sizes were measured at the days of administration. The differences of tumor sizes between control and treated mice were statistically significant. Mean ± SE of each mouse in the four groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001. B, body weights measured every day between the first and the second administrations and then at the days of administration. There are no significant differences after the treatments in each group. Mean ± SE from each mouse in the four groups.
moscatilin-mediated induction of apoptosis may be caused by its damage of DNA. Mitochondria-mediated caspase-9 activation (intrinsic pathway) and death receptor-induced caspase-8 activation (extrinsic pathway) are two major apoptosis pathways. In the intrinsic pathway, mitochondria play an important role by releasing apoptogenic factors following the loss of mitochondrial membrane potential. This effect is regulated by the Bcl-2 family proteins (34). Recent evidence indicates that tubulin-binding agents and DNA damage-inducing agents mediate their effects by stimulating the intrinsic pathway (19, 22, 27, 35). In support of this, our results showed that moscatilin induced the phosphorylation and degradation of Bcl-2 and lead to activation of the caspase-9/3 cascade (Figs. 2C and 4A), suggesting that moscatilin-induced apoptosis mediated by induction of the intrinsic pathway.

JNK and p38 regulate cell proliferation, differentiation, and apoptosis. These well-known mitogen-activated protein kinases are activated following numerous stressors, including oxidant stress, ionizing radiation, cytokines, and DNA damage. The activation of JNK exhibits apparently opposing consequences, which lead to cell survival or execution of apoptosis. Some studies show that various cancer cell lines have high levels of JNK activity (36). The inhibition of JNK by antisense DNA suppresses growth of certain tumor cells by triggering apoptosis (37, 38). In contrast, other studies show that JNK functions as a proapoptotic kinase and that JNK activation is required for triggering the mitochondrial-dependent apoptotic pathway in UV-induced apoptosis (6). JNK activation is also responsible for cisplatin-induced apoptosis in cancer cells (39). A recent study showed that JNK and p38, but not ERK, contribute to the cell apoptosis observed after treatment with tubulin-binding agents (40). In our study, we investigated the role of JNK and p38 on moscatilin-mediated apoptotic pathway by using specific mitogen-activated protein kinase inhibitors. Our results showed that a JNK inhibitor (SP600125), but not a p38 inhibitor (SB203580), significantly inhibited moscatilin-induced apoptosis. SP600125 blocked moscatilin-induced activation of caspase-9 and caspase-3. Moreover, JNK can phosphorylate transcription factors, such as c-Jun and p53. Phosphorylation of c-Jun regulates the expression of specific genes that mediate cell apoptosis (36). c-Jun phosphorylation is a critical downstream signal of JNK signaling for apoptosis of mature neurons (41) and for UV-induced apoptosis of cancer cells (42). Our results show that moscatilin induces c-Jun phosphorylation and p53 activation. This suggests that JNK plays a central role in the proapoptotic effect of moscatilin.

In conclusion, our study suggests that moscatilin induces apoptosis of colorectal HCT-116 cells through tubulin depolymerization and DNA damage stress and that this leads to activation of JNK and the mitochondria-involved intrinsic apoptosis pathway. The HCT-116 xenograft models in severe combined immunodeficient mice show the in vivo efficacy of moscatilin. Taken together, our study suggests that moscatilin has great potential as a clinical anticancer agent.

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