1-Methoxy-Canthin-6-One Induces c-Jun NH2-Terminal Kinase–Dependent Apoptosis and Synergizes with Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Activity in Human Neoplastic Cells of Hematopoietic or Endodermal Origin

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Abstract

We investigated the effects of 1-methoxy-canthin-6-one, isolated from the medicinal plant Ailanthus altissima Swingle, on apoptosis in human leukemia (Jurkat), thyroid carcinoma (HuH7), and hepatocellular carcinoma (HepG2) cell lines. Cultures incubated with the compound showed >50% of sub-G1 (hypodiploid) elements in flow cytometry analysis; the apoptosis-inducing activity was evident at <10 μmol/L and half-maximal at about 40 μmol/L 1-methoxy-canthin-6-one. The appearance of hypodiploid elements was preceded by mitochondrial membrane depolarization, mitochondrial release of cytochrome c, and Smac/DIABLO and procaspase-3 cleavage. We subsequently investigated the effect of 1-methoxy-canthin-6-one in combination with human recombinant tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) in the four cell lines. Suboptimal concentrations (10 μmol/L 1-methoxy-canthin-6-one and 0.25 ng/mL TRAIL, respectively) of the two agents, unable to elicit apoptosis when used alone, induced mitochondrial depolarization, activation of caspase-3, and 45% to 85% of sub-G1 elements when added together to the cells. The synergism seemed to rely partly on the enhanced expression of TRAIL receptor 1 (TRAIL-R1; DR4), analyzed by immunofluorescence, by 1-methoxy-canthin-6-one. Cell incubation with 1-methoxy-canthin-6-one resulted in activating c-Jun NH2-terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family, is a key regulator of apoptosis due to cleavage of BID by caspase-8 (7, 8). The JNK-activating and proapoptotic properties of 1-methoxy-canthin-6-one were >80% prevented by the addition of the JNK inhibitor (JNKi) SP600125 (JNKI), indicating that both effects were almost completely mediated by JNK activity. On the other hand, synergism with TRAIL was reduced by about 50%, suggesting that besides up-regulating TRAIL-R1, 1-methoxy-canthin-6-one could influence other factor(s) that participated in TRAIL-induced apoptosis. These findings indicate that 1-methoxy-canthin-6-one can represent a candidate for in vivo studies of monotherapies or combined antineoplastic therapies. (Cancer Res 2006; 66(8): 4385-93)

Introduction

Species belonging to the genus Ailanthus (Simaroubaceae) have long been used in Chinese traditional medicine for their antitumoral properties (1), and isolated compounds from their extracts have been observed to induce apoptosis in human cell lines in vitro (2, 3). In particular, extracts from Ailanthus altissima Swingle display, in addition to their antigerminative properties (4), anti proliferative effects in human cell cultures (3, 5). 1-Methoxy canthin-6-one, isolated from the plant, can induce cell death in human osteosarcoma SaOS-2 cells (3). The mechanism of apoptosis induction by this compound was not previously identified.

c-Jun NH2-terminal kinase (JNK), a member of the mitogen activated protein kinase (MAPK) family, is a key regulator of apoptosis (6). Modulation of its activity can either promote or inhibit apoptotic processes, depending on cell system and contexts. Indeed, the kinase acts on a variety of targets, including, in addition to c-Jun, other transcription factors (ATF2, Elk-1, p53, and c-Myc) and proapoptotic and antiapoptotic members of the Bcl-2 family (Bcl-2, Bcl-xL, Bim, and BAD), thereby influencing levels and activities of molecules that variably participate in cell death mechanisms (6).

In the present study, we showed that 1-methoxy-canthin-6-one induced apoptosis via a JNK-dependent mechanism. Furthermore, the compound synergized with human recombinant tumor necrosis factor (TNF)–related apoptosis-inducing ligand (hTRAIL) in apoptosis induction. TRAIL, a member of the TNF gene superfamily, interacts with a complex system of receptors consisting of two proapoptotic death receptors (TRAIL-R1 and TRAIL-R2) and three decoy receptors (TRAIL-R3, TRAIL-R4, and osteoprotegerin). As a stable soluble trimer, the cytokine selectively induces apoptosis in many transformed cells but not in normal cells; differential expression levels of decoy versus proapoptotic receptors and other mechanisms seem to account for normal cell resistance to TRAIL-induced cell death. TRAIL action involves the formation of a death-inducing signaling complex (DISC) and activation of caspase-8; the apoptotic processes then follow two pathways: the mitochondrial-independent activation of caspase-3 and mitochondrial-dependent apoptosis due to cleavage of BID by caspase-8 (7, 8).

The JNK-activating and proapoptotic properties of 1-methoxy canthin-6-one render this molecule a candidate for in vivo studies of its activity in monotherapies or combined antineoplastic therapies.

Materials and Methods

Extraction and isolation of 1-methoxy-canthin-6-one. Roots of A. altissima were collected in June 2002 at the University Campus in Fisciano...
(Salerno, Italy). A voucher specimen of the plant (DF.2002.193) is kept at the Herbarium of Pharmaceutical Botany Chair at the State University of Salerno. Air-dried roots of *A. altissima* were successively extracted at room temperature with solvents of increasing polarity [petroleum ether, chloroform, chloroform-methanol (9:1), methanol, and water]. Chloroform extract of roots, the most active in biological assays, was fractionated on a silica gel column, eluting with chloroform and mixtures of chloroform and methanol of increasing polarity. Seven hundred sixty-two fractions of 10 mL were obtained and pooled in 13 major fractions based on their TLC similarity. Fraction pool XXIII-XXVII was further purified on a silica gel column, collecting 127 fractions of 5 mL, pooled in nine major subfractions (A-I). Subfraction C, showing a strong biological activity, was shown to contain, in TLC assays, a single substance, positive to Dragendorff reagent. The identification of this active substance, recognized as 1-methoxy-canthin-6-one, was done by accurate analyses of its $^1$H-NMR, $^{13}$C-NMR, and $^{13}$C-NMR DEPT data and by comparison with literature data (ref; 9; Fig. 1).

**Cell lines and peripheral blood mononuclear cells from normal donors.** Human leukemia (Jurkat), thyroid carcinoma (ARO and NPA), and hepatocellular carcinoma (HuH7) cell lines were obtained from the American Type Culture Collection (Rockville, MD). Jurkat and ARO cells were maintained in 5% CO$_2$ at 37$^\circ$C in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) containing 10% FCS, 1% penicillin, and 1% streptomycin. HuH7 cells and NPA cells were maintained in 5% CO$_2$ at 37$^\circ$C in DMEM containing 10% FCS, 1% penicillin, and 1% streptomycin. Peripheral blood mononuclear cells (PBMC) from normal donors were isolated from heparinized blood samples through centrifugation on Ficoll density gradient (10).

**Antibodies, inhibitors, and other reagents.** Anti-human cytochrome c and Smac/DIABLO mouse monoclonal antibodies (mAb) were obtained from BD Pharmingen (San Diego, CA). Anti-human Hsp60 (H-1) and phycoerythrin-conjugated anti-integrin α4 (SG31) and anti-integrin β3 (5D2) mouse mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human α-tubulin mAB was obtained from Sigma (St. Louis, MO). Anti-human phospho-c-Jun (Ser$^63$) and phospho-JNK (G-7) rabbit polyclonal antibodies were obtained from Cell Signaling, Inc. (Beverly, MA). Anti-human caspase-3 rabbit polyclonal antibody was purchased from StressGen (Victoria, British Columbia, Canada). Etoposide and staurosporine were purchased from Sigma. Human recombinant Superkiller TRAIL and extracelluar signal-regulated kinase inhibitor U0126 were purchased from Alexis Biochemicals (San Diego, CA). JNK inhibitor SP600125 was obtained from Calbiochem (San Diego, CA).

**Immunofluorescence.** Cells ($1 \times 10^5$) were incubated with saturating amounts of phycoerythrin-conjugated mAb or, in indirect tests, primary mAb followed, after washing with PBS, by FITC-conjugated rabbit anti-mouse antibodies. After further washing with PBS, the cells were resuspended in 1% formaldehyde-PBS and analyzed with a FACScan (Becton Dickinson, San Jose, CA) flow cytometer.

**Analysis of hypodiploid (apoptotic) nuclei and caspase-3 activity.** Apoptosis was analyzed by propidium iodide incorporation in permeabilized cells and flow cytometry as described (10). Briefly, cells ($5 \times 10^5$) were washed in PBS and resuspended in 500 μL of a solution containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/mL propidium iodide (Sigma). Following incubation at 4$^\circ$C for 30 minutes in the dark, cell nuclei were analyzed with a Becton Dickinson FACScan flow cytometer. Cellular debris was excluded from analysis by raising the forward scatter threshold, and the DNA content of the nuclei was registered on a logarithmic scale. The percentage of the elements in the hypodiploid region was calculated.

Caspase-3 activity was evaluated either by Western blotting (see below) or

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**Figure 1.** Extraction procedure for 1-methoxy-canthin-6-one.
by using Caspase-3 Fluorometric Detection kit (Assay Designs, Ann Arbor, MI; ref. 11).

Analysis of mitochondrial transmembrane potential. Mitochondrial membrane potential ($\Delta \Psi_m$) was assessed using a cytofluorometric technique using tetramethylrhodamine ethyl ester (TMRE; ref. 12). Cells were exposed to TMRE (5 nmol/L; Molecular Probes, Eugene, OR) for 1 hour at 37°C. Changes in dye fluorescence were analyzed in a Becton Dickinson FACScan flow cytometer.

Cell lysates, subcellular fractionation, and Western blotting. Cell total protein lysates were prepared in sample buffer [2% SDS, 10% glycerol, 2% mercaptoethanol, and 60 mmol/L Tris-HCl (pH 6.8) in demineralized water] on ice. Cell cytosolic and mitochondrial fractions were generated using a digitonin-based subcellular fractionation technique as described (13). For Western blotting analysis, proteins from whole cell or fraction lysates were run on 12% SDS-PAGE gels and electrophoretically transferred to nitrocellulose. Nitrocellulose blots were blocked with 5% bovine serum albumin (BSA) in TBS/Tween 20 (TBST) buffer [20 mL/L Tris-HCl (pH 7.4), 500 mmol/L NaCl, and 0.01% Tween 20] and incubated with primary antibody in TBST/5% BSA overnight at 4°C. Immunoreactivity was detected by sequential incubation with horseradish peroxidase–conjugated secondary antibody and enhanced chemiluminescence reagents following standard protocols.

Statistical analysis. Statistical analysis was done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).1

Figure 2. Apoptosis induction by 1-methoxy-canthin-6-one. Jurkat (A), NPA (B), ARO (C), and HuH7 (D) cells were plated in RPMI supplemented with 10% of heat-inactivated FCS (10% FCS-RPMI) at a density of $1 \times 10^6$ per mL and incubated with the indicated concentrations of 1-methoxy-canthin-6-one. After 24 hours, apoptosis was measured as percentage of sub-G1 nuclei by flow cytometry (10).

Results

Induction of apoptosis in human cell lines of different origins by 1-methoxy-canthin-6-one. We have recently observed that 1-methoxy-canthin-6-one induced cell death in the human osteosarcoma cell line SaOS-2 (3). To verify and characterize the proapoptotic effect of the compound in human cells of diverse origins, we analyzed the dose-dependent induction of apoptosis in leukemia (Jurkat), thyroid carcinoma (NPA and ARO), or hepatocellular carcinoma (HuH7) cells. Following a 24-hour incubation with 1-methoxy-canthin-6-one, the four tested lines showed percentages of apoptotic (sub-G1) cells ranging from 50% to >70%, as evaluated by flow cytometry (ref. 10; see Materials and Methods). The apoptosis-inducing activity was evident at <10 µmol/L 1-methoxy-canthin-6-one and half-maximal at a compound concentration of about 40 µmol/L. The compound did not show any proapoptotic or toxic activity in PBMCs from two different donors (Fig. 2). The appearance of sub-G1 elements was evident in cells incubated with 1-methoxy-canthin-6-one for <15 hours.
(Fig. 3A). To investigate whether mitochondrial events were involved in the induction of apoptosis, we analyzed mitochondrial membrane depolarization by flow cytometry (12) and the release of cytochrome c and Smac/DIABLO (13) by Western blotting in cells incubated with the compound. The cells clearly showed a loss of mitochondrial membrane polarity 4 to 8 hours following cell incubation with 1-methoxy-canthin-6-one (Fig. 3B); in parallel, we detected a decrease in the intramitochondrial and an increase in the cytosolic amounts of cytochrome c and Smac/DIABLO proteins (Fig. 3C). These findings indicated that the mitochondrial pathway of apoptosis was triggered by this molecule. As expected, procaspase-3 was accordingly cleaved, leading to the appearance of a faint p20/19 and an intense p17 signal (13), in cells exposed to the drug (Fig. 3D).

Synergy of 1-methoxy-canthin-6-one and hrTRAIL. To further explore the potential use of 1-methoxy-canthin-6-one as antineoplastic agent, we investigated its proapoptotic effect in combination with TRAIL, a molecule that induces apoptosis in a vast series of different neoplasias (7, 8). To this purpose, we analyzed apoptosis by flow cytometry in cells treated with suboptimal doses of both compounds. When different cell lines were incubated with 10 μmol/L 1-methoxy-canthin-6-one and/or suboptimal TRAIL, the single agents induced <10% of apoptotic elements, whereas their combination elicited apoptotic responses ranging from 45% to >80%. Such effect was not detectable in normal PBMCs (Fig. 4A). The synergistic effect in neoplastic cells was evident at the levels of caspase-3 activation (Fig. 4C) and mitochondrial depolarization (Fig. 4B), indicating that its mechanism was to be investigated in

**Figure 3.** Effect of 1-methoxy-canthin-6-one on mitochondrial membrane depolarization, cytochrome c, and Smac/DIABLO release and caspase-3 cleavage. A, Jurkat cells were plated at a density of $1 \times 10^6$ per mL in 10% FCS-RPMI, in the absence or presence of 1-methoxy-canthin-6-one (40 μmol/L). Apoptosis was measured at the indicated time intervals as percentage of sub-G1 nuclei by flow cytometry. B, Jurkat cells were incubated at 5 × 10^5 per mL in 10% FCS-RPMI, in the absence or presence of 1-methoxy-canthin-6-one (40 μmol/L). At the indicated time intervals, mitochondrial membrane depolarization was evaluated by cell staining with TMRE (5 nmol/l) for 1 hour at 37 °C followed by flow cytometric analysis (12). Experiments were done at least twice. C, Jurkat cells were incubated with staurosporine (250 nmol/L) was used as a positive control for cytochrome c and Smac/DIABLO release (47). At the indicated time intervals, cells were harvested, cytosolic and mitochondrial fractions were generated (13), and subcellular fractions were analyzed with anti-cytochrome c, anti-Smac/DIABLO, and anti-Hsp60 (as intramitochondrial marker protein) antibodies by Western blotting. Experiments were done at least twice. D, Jurkat cells were incubated at 5 × 10^5 per mL in 10% FCS-RPMI, in the absence or presence of 1-methoxy-canthin-6-one (40 μmol/L). Cell incubation with etoposide (50 μmol/L) was used as a positive control for caspase-3 activation and appearance of the caspase-3 active cleaved forms p20/19 and/or p17 (48, 49). At the indicated time intervals, cell lysates were obtained and analyzed with an anti-caspase-3 antibody by Western blotting. Experiments were done at least twice.
earlier step(s) of the apoptotic program. Therefore, we verified whether incubation with 1-methoxy-canthin-6-one modulated the expression of TRAIL receptors. As shown in Fig. 5, Jurkat cells incubated with the molecule (10 μmol/L) showed an enhanced binding to an anti-TRAIL-R1 antibody, as detected by immunofluorescence: indeed, the mean intensity of fluorescence increased from 24.1 (Fig. 5, TRAIL-R1, green) to 44.8 after 15 hours of cell incubation with the drug (Fig. 5, TRAIL-R1, orange). This effect is specific...
because we could not detect any increase in the expression of other TRAIL receptors or unrelated proteins (i.e., integrins; Fig. 5).

Role of JNK in 1-methoxy-canthin-6-one-induced apoptosis. The activity of JNK influences cell apoptosis (6, 14–18) and mediates the proapoptotic effects of some antineoplastic compounds (19–27). Furthermore, it regulates the expression of TRAIL-R2 (DR5; refs. 28–31). We therefore investigated the role of the kinase in 1-methoxy-canthin-6-one–induced apoptosis and synergism with hrTRAIL. Incubation of Jurkat cells with 1-methoxy-canthin-6-one induced JNK activation, as revealed by the appearance of the phosphorylated forms of JNK and its substrate c-Jun, analyzed by Western blot (Fig. 6A). To verify whether JNK activation was required for apoptosis induction, we used the JNK inhibitor (JNKI) SP600125 (32). The addition of this molecule to cell cultures inhibited apoptosis by >80% (Fig. 6C), indicating that JNK activity mediated the effect of the drug. Furthermore, the inhibitor reduced TRAIL-R1 up-regulation (Fig. 7A) and the synergistic activity of 1-methoxy-canthin-6-one with hrTRAIL (Fig. 7B). Indeed, whereas apoptosis induced by the two agents together in cell culture was >45%, it was reduced to 21% in the presence of JNKI. Because the synergism was not completely abrogated by the inhibitor, other element(s), in addition to JNK activation, apparently contributed to the enhancement of TRAIL–induced apoptosis by 1-methoxy-canthin-6-one.

Discussion

This study identifies and characterizes the apoptosis-inducing activity of 1-methoxy-canthin-6-one, isolated from A. altissima, in human neoplastic cell lines of hematopoietic or endodermal origin. Understanding of such an effect of this molecule supports its potential therapeutic application. The mechanism of 1-methoxy-canthin-6-one activity seemed to entirely rely on JNK activation; indeed, the inhibition of the kinase was able to completely prevent apoptosis induced by the compound. In this respect, these findings provide one of the clearest evidence attesting that JNK activation can exert a major role in the activity of antineoplastic molecules (19–27).

The MAPK subfamily JNK has three isoforms ([JNK1, JNK2, and JNK3]; JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is mainly expressed in neuronal and heart tissues (6, 15, 33). Among MAPKs, JNKs are preferentially activated by cytotoxic stresses (oxidative/nitrosative stress, heat and osmotic shock, X-ray, and UV irradiation) and proinflammatory cytokines, including TNF-α, interleukin-1 (IL-1), and IL-18 (34–38). Although initially identified by its ability to phosphorylate the transcription factor c-Jun, JNK was subsequently found to act also on other substrates, such as the transcription factors ATF2, Elk-1, p53 (33), c-Myc and proapoptotic (Bim and BAD) or antiapoptotic (Bcl-2 and Bcl-xL) proteins belonging to the Bcl-2 family. This justifies the opposite effects (proapoptotic or antiapoptotic) that JNK has been shown to exert in diverse systems (39–42). Genetic disruption of jnk1 and jnk2 alleles in mice resulted in increasing apoptosis in hindbrain and forebrain regions (39, 40), suggesting that JNK may be required for cell survival. Furthermore, some reports showed that inhibition of JNK2 activity suppressed tumorigenesis through promoting apoptosis (41). One of the molecular mechanisms by which JNK can suppress apoptosis is inhibition of BAD proapoptotic activity through phosphorylation at Thr201 (18). On the other hand, numerous evidence indicate that JNK is involved in proapoptotic pathways (14, 16, 17, 42, 43). JNK activation is required for...
UV-induced apoptosis, and its absence caused cell failure to release cytochrome c (14). In TNF-α-induced apoptosis, in the absence of nuclear factor-κB activation, prolonged JNK activation promotes cell death (16, 42), probably via its contribution to the proteolysis of the proapoptotic molecule BID (17). In the DNA damage response, JNK activation is responsible for 14-3-3 protein phosphorylation followed by release of sequestered c-Abl and its nuclear targeting, leading to apoptosis (43). Therefore, it seems that the proapoptotic activity of JNK can be exerted through more than one mechanism.

Various antineoplastic agents, including topoisomerase inhibitors (25), histone deacetylase inhibitors (23), rapamycin (27), and others (19–22, 24, 26), have been shown to activate JNK. Inhibition of the kinase mostly reduced the proapoptotic activity of these agents (19–27), whereas completely abrogated that displayed by 1-methoxy-canthin-6-one. In this case, therefore, JNK activation did not simply participate in promoting or amplifying the proapoptotic signal but was decisive for subverting the cell survival/death balance. These findings strongly contribute in indicating that levels of JNK activity can determine cell fate in some systems (6, 44). They introduce an element of caution in evaluating antineoplastic approaches based on the use of JNK inhibitors (45) and might instead support the potential utility of strategies aimed at specifically activating the kinase, to trigger apoptosis in neoplastic cells (46).

JNK activity has been shown to influence the levels of TRAIL-R2 (28–31). We found that it can also up-regulate TRAIL-R1 because the levels of this receptor were enhanced by 1-methoxy-canthin-6-one in a JNK-dependent manner: indeed, JNK inhibition completely prevented the effect. However, the inhibition of the kinase only partially reduced the synergism of 1-methoxy-canthin-6-one with TRAIL. A more extensive analysis of such factors (DISC components, IAPs, etc.) could contribute to deeply evaluating the proapoptotic activity of 1-methoxy-canthin-6-one and its synergism with agents that induce tumor cell death.

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References


Figure 7. Effect of JNK inhibition on the enhancing of TRAIL-induced apoptosis by 1-methoxycanthin-6-one. A, Jurkat cells were incubated overnight at $5 \times 10^5$ per mL in 10% FCS-RPMI, in the absence or presence of JNKI or ERK1 (20 μmol/L), and treated with 1-methoxycanthin-6-one (10 μmol/L) for additional 2 hours. Then cell expression of TRAIL-R1 was analyzed by flow cytometry. B, Jurkat cells were treated with JNKI (20 μmol/L) overnight and then incubated with 1-methoxycanthin-6-one (10 μmol/L) with or without TRAIL (0.25 ng/mL). After 24 hours, apoptosis was measured as percentage of sub-G1 nuclei. Representative results. Columns, means of triplicate determinations; bars, SD.


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