

An *ent*-Kaurane Diterpenoid from *Croton tonkinensis* Induces Apoptosis by Regulating AMP-Activated Protein Kinase in SK-HEP1 Human Hepatocellular Carcinoma Cells

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Hepatocellular carcinoma (HCC) is the most common type of liver cancer with high mortality worldwide. Traditional chemotherapy for HCC is not widely accepted by clinical practitioners because of its toxic side effects. Thus, there is a need to identify chemotherapeutic drugs against HCC. AMP-activated protein kinase (AMPK) is a biologic sensor for cellular energy status that acts a tumor suppressor and a potential cancer therapeutic target. The traditional Vietnamese medicinal plant *Croton tonkinensis* shows cytotoxicity in various cancer cells; however, its anticancer mechanism remains unclear. In this study, we determined whether the *ent*-kaurane diterpenoid *ent*-18-acetoxy-7 β -hydroxy kaur-15-oxo-16-ene (CrT1) isolated from this plant plays a role as a chemotherapeutic drug targeting AMPK. CrT1 blocked proliferation in dose- and time-dependent manners in human hepatocellular carcinoma SK-HEP1 cells. CrT1 induced sub-G₁ arrest and caspase-dependent apoptosis. CrT1 activated caspase-3, -7, -8, -9, and poly(ADP-ribose) polymerase, and its effect was inhibited by z-VAD-fmk suppressing caspase-3 cleavage. CrT1 induced increases in p53 and Bax levels but decreased Bcl₂ levels. In addition, CrT1 resulted in increased translocation of cytochrome *c* into the cytoplasm. We showed that CrT1-activated AMPK activation was followed by modulating the mammalian target of rapamycin/p70S6K pathway and was inactivated by treating cells with compound C. Treatment with CrT1 and aminoimidazole carboxamide ribonucleotide (AICAR) synergistically activated AMPK. CrT1-induced AMPK activation regulated cell viability and apoptosis. These results suggest that CrT1 is a novel AMPK activator and that AMPK activation in SK-HEP1 cells is responsible for CrT1-induced anticancer activity including apoptosis.

Key words hepatocellular carcinoma; AMP-activated protein kinase; *ent*-kaurane diterpenoid

Hepatocellular carcinoma (HCC) is the most common type of liver cancer with high mortality and causes 662000 deaths worldwide each year. To date, HCC has a life-threatening and unsatisfactory prognosis. Once the diagnosis is confirmed, most patients are already at the advanced stage and have lost the opportunity for surgical intervention. Further characterization of underlying molecular mechanisms in HCC would bring new insights into HCC research and clinical treatment.¹⁾ Traditional systemic chemotherapy for liver cancer has a low curative rate with toxic side effects and is not widely accepted by clinical practitioners.²⁾ Therefore, exploring alternative chemotherapy modalities and applying new chemotherapeutic drugs is necessary.

The genus *Croton* consists of about 300 species, which are widely distributed throughout tropical and subtropical regions.³⁾ *Croton tonkinensis* GAGNEP. (Euphorbiaceae), commonly known as “Kho sam cho la” in Vietnamese, is a small plant indigenous to Northern Vietnam.⁴⁾ It has been used to treat stomach ache, abscess, impetigo, gastric and duodenal ulcers, and malaria.^{5,6)} The *C. tonkinensis* crude extract shows significant cytotoxicity against breast cancer cell (MCF-7), lung cancer cell (NCI-H460), and glioblastoma (SF-268) lines.³⁾ Some constituents isolated from *C. tonkinensis* include sterols, long-chain alkyl alcohols, flavonoid glucosides, and *ent*-kaurane diterpenoids.^{4,7)} Among them, the *ent*-kaurane

diterpenoids have cytotoxic and proapoptotic activities,⁸⁾ inhibit lipopolysaccharide-induced nuclear factor kappa B activation,⁹⁾ reduce inflammation,¹⁰⁾ inhibit silent information regulator two ortholog 1 (SIRT1),¹¹⁾ and stimulate osteoblast differentiation.¹²⁾

AMP-activated protein kinase (AMPK) is a cellular energy status sensor found in all eukaryotes, which is activated under conditions of low intracellular ATP following stressors such as nutrient deprivation and hypoxia.¹³⁾ Several studies have suggested that AMPK regulates cell proliferation, cell growth, and autophagy.^{14,15)} AMPK activation by aminoimidazole carboxamide ribonucleotide (AICAR) inhibit HepG2 cells (liver cancer) growth via p53 phosphorylation.¹⁶⁾ Genetic alteration of the tumor suppressor LKB1, which activates AMPK, plays an important role in HCC tumor development.¹⁷⁾ Activating AMPK inhibits HCC by destabilizing p53 in a SIRT1-dependent manner.¹⁸⁾ These studies provide evidence that AMPK may serve as a potential target for treating HCC.

MATERIALS AND METHODS

Reagents Dulbecco's modified Eagle's medium (DMEM) and trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from Thermo Scientific HyClone (Logan, UT, U.S.A.). Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, U.S.A.). Antibodies to phospho (P)-AMPK, AMPK, P-mammalian target of rapamycin (mTOR), mTOR,

The authors declare no conflict of interest.

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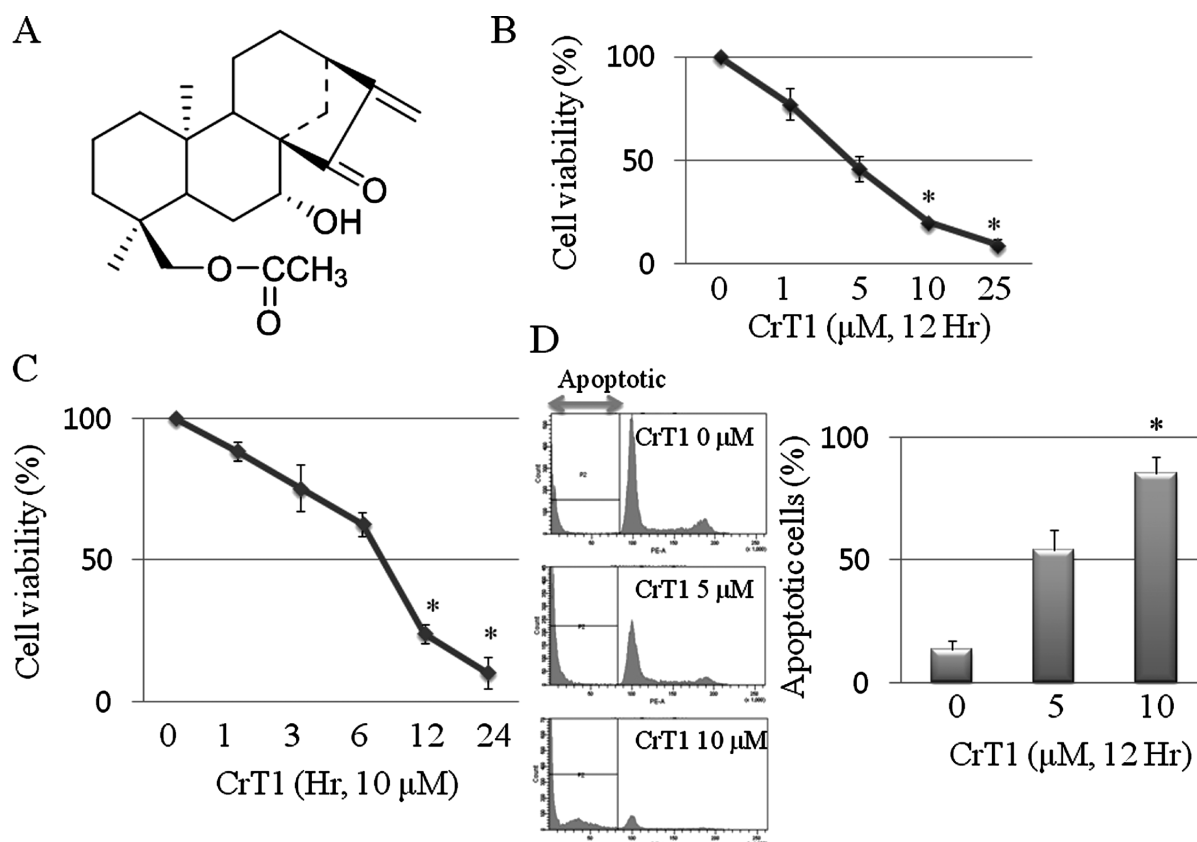


Fig. 1. The Cell Death Effect of *ent*-18-Acetoxy-7 β -hydroxy Kaur-15-oxo-16-ene (CrT1) on the SK-HEP1 Human Hepatocellular Carcinoma (HCC) Cell Line

CrT1 structure (A). Cells were treated with various CrT1 concentrations (B) and times (C) up to 24h, and cytotoxic effects were detected by the CCK-8 assay. The number of viable cells after CrT1 treatment is expressed as a percentage of the vehicle-only (treated with 0.1% dimethylsulfoxide) control. Data are mean $\pm$ standard deviation (S.D.) of three independent experiments. * $p < 0.005$ vs. vehicle-treated control group. Flow cytometric analysis. The sub-G₁ fraction was defined as the apoptotic cells. * $p < 0.005$ vs. vehicle-treated control group (D).

P-p70S6K, p70S6K, caspase-3, caspase-7, caspase-8, caspase-9, poly(ADP-ribose) polymerase (PARP), p53 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.), and Bax, Bcl₂, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from SantaCruz (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Transduction Lab (Lexington, KY, U.S.A.). Super Signal[®] West Pico Chemiluminescent substrate was purchased from PIERCE (Rockford, IL, U.S.A.). Cell Counting Kit-8 was from Dojindo Laboratories (Kumamoto, Japan). PromoKine Mitochondrial Apoptosis Staining Kit was purchased from PromoKine[®] (Heidelberg, Germany). z-VAD-fmk, compound C, AICAR and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Plant Material and Chemical Isolation The major *ent*-kauranoid was previously isolated from the leaves of the plant *Croton tonkinensis* and identified as *ent*-18-acetoxy-7 β -hydroxy kaur-15-oxo-16-ene (namely CrT1) (Fig. 1A). The purification and structure identification of CrT1 was demonstrated elsewhere.¹⁰ The purity of the compound was determined as 97% by HPLC.

Cell Lines and Culture Human hepatocellular carcinoma SK-HEP1 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.). Cells were grown in DMEM media supplemented with 10% (v/v) FBS, penicillin (100 U/mL)–streptomycin (100 μ g/mL) at 37°C in a humidified CO₂ (5%)-controlled incubator.

Cell Viability Assay Cells were seeded at 5×10^3 cells/mL in 96-well microplates and allowed to attach for 24h. CrT1 was added to the medium at various concentrations up to 50 μ M and different duration. After treatment, cell cytotoxicity and/or proliferation was assessed by Cell Counting Kit-8 (CCK-8). Briefly, highly water-soluble tetrazolium salt, WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], produced an orange colored water-soluble product, formazan. The amount of formazan dye generated by dehydrogenases in cells was directly proportional to the number of living cells. CCK-8 (10 μ L) was added to each well and incubated for 3h at 37°C, then cell proliferation and cytotoxicity were assessed by measuring the absorbance at 450nm using microplate reader. Three replicated wells were used for each experimental condition.

Western Blot Analysis Cells were incubated with CrT1 for 24h, and washed twice in cold phosphate buffered saline (PBS). Cells were lysed with lysis buffer (10mM Tris, pH 7.4, 150mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% NP-40, 1mM propidium iodide (PI), 1mM dithiothreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF)) and placed on ice for 1h with occasional vortexing. Centrifugation followed at 10000rpm for 10min and each cell lysates (50 μ g) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. Blots

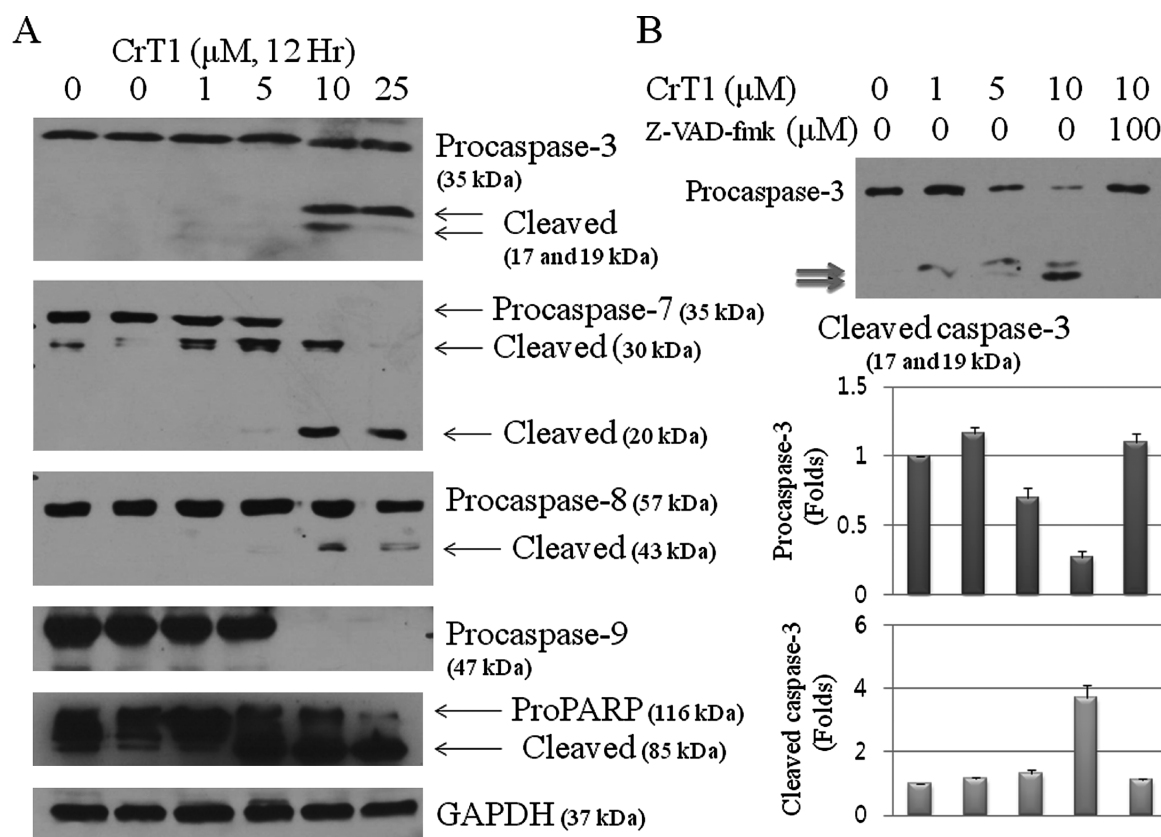


Fig. 2. Apoptosis Induced by *ent*-18-Acetoxy-7 β -hydroxy Kaur-15-oxo-16-ene (CrT1) in SK-HEP1 Cells

Cleavage of apoptosis-related proteins was measured by Western blot analysis after CrT1 treatment (0, 1, 5, 10, 25 μ M) of cells. Equal amounts of cell lysates (50 μ g) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membrane, and probed with specific antibodies for anti-caspase-3, anti-caspase-7, anti-caspase-8, anti-caspase-9, anti-poly(ADP-ribose) polymerase (PARP), and anti-GAPDH as a protein-loading control (A). The caspase inhibitor z-VAD-fmk (100 μ M) was pre-treated for 1 h before adding CrT1 (0, 1, 5, 10 μ M) to the cells, and caspase-3 cleavage was measured by Western blot analysis (B).

were blocked with 5% skim milk in PBS containing 0.05% Tween-20 for 1 h at 25°C, then incubated with primary antibodies, followed by incubation with anti-rabbit or anti-mouse horseradish peroxidase-conjugated Immunoglobulin G and visualized with enhanced chemiluminescence.

Flow Cytometric Analysis Cells with 1×10^5 cells/mL were suspended in 300 μ L PBS and 700 μ L ethanol was added. Cells were incubated at 4°C for 1 h and washed with PBS, and suspended in 250 μ L of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μ g of RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was stained by applying 250 μ L of PI (50 μ g/mL) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on the BD FACSCanto™II flow cytometer using FACSCanto™II System Software for the percentage of apoptotic cells.

RESULTS

CrT1 Inhibits SK-HEP1 Cell Growth Cells were cultured and treated with CrT1 to test the effect of CrT1 on SK-HEP1 cell growth. After cells were treated with 1, 5, 10, or 25 μ M CrT1 for 12 h, cell viability was $77.4 \pm 7.6\%$, $45.9 \pm 6.2\%$, $20.3 \pm 1.0\%$, and $9.2 \pm 2.4\%$, respectively (Fig. 1B). Cell viability was affected in a time-dependent manner and was $88.5 \pm 3.3\%$, $75.4 \pm 8.2\%$, $62.6 \pm 4.3\%$, $24.0 \pm 3.2\%$, and $10.2 \pm 5.6\%$ after cells were treated with CrT1 for 1, 3, 6, 12, and 24 h (Fig.

1C). Treatment with 10 μ M CrT1 for 12 h resulted in significant decreases in cell viability compared to that in the control group ($p < 0.001$). We evaluated apoptotic cell death using propidium iodide staining to further understand whether CrT1-induced cell death was mediated by apoptosis. CrT1 treatment at 10 μ M induced apoptosis in $85.6 \pm 6.4\%$ of the cells after 12 h (Fig. 1D). These data indicate that CrT1 inhibits proliferation and induces apoptosis in SK-HEP1 cancer cells.

CrT1 Induces Caspase-Dependent Apoptosis in SK-HEP1 Cells We assessed levels of the apoptotic markers, caspase-3, caspase-7, caspase-8, caspase-9, and poly(ADP-ribose) polymerase (PARP) using Western blot analyses. Caspase-3 mediates the proteolytic cleavage of key proteins such as the nuclear enzyme PARP, which plays an important role in chromatin condensation and degradation in apoptotic cells. CrT1 treatment (5, 10, 25 μ M) for 12 h led to procaspase-3, -7, -8, and -9 proteolysis. Full-length PARP (116 kDa) was dose-dependently cleaved into a p85 fragment after CrT1 treatment (Fig. 2A). To further confirm the involvement of caspases in apoptosis induced by CrT1, cells were pretreated with z-VAD-fmk (for 1 h), a general inhibitor of caspase, followed by CrT1 treatment. This caspase inhibitor completely rescued the cells from the caspase-3 cleavage induced by CrT1 (Fig. 2B). These results indicate that CrT1-induced apoptosis is dependent on caspase activation.

CrT1 Modulates the Bcl₂ Family and the Mitochondrial Pathway As the apoptotic pathway is the major pathway of

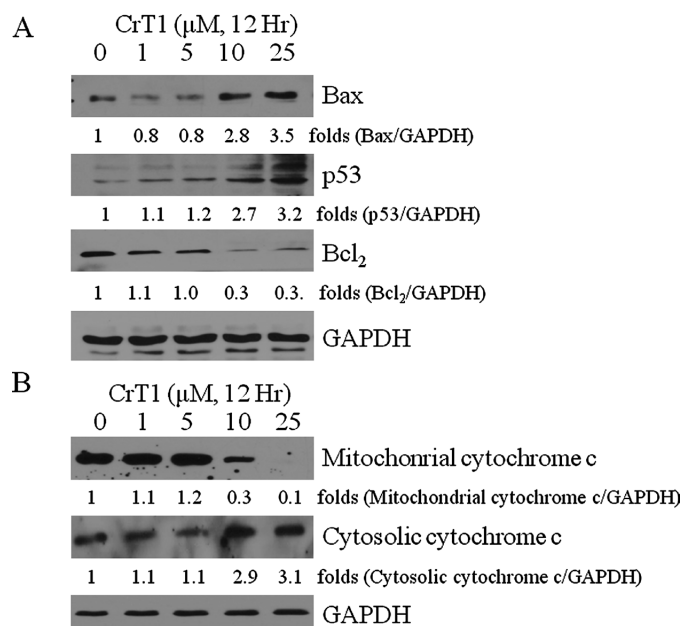


Fig. 3. *ent*-18-Acetoxy-7 β -hydroxy Kaur-15-oxo-16-ene (CrT1) Induced Mitochondrial Apoptosis

Determination of the apoptotic proteins Bcl₂, p53, and Bax in cells exposed to the indicated concentrations of CrT1 for 12h. Equal amounts of cell lysates were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blot (A). The cytosolic and mitochondrial fractions were separated after the CrT1 treatment. Cytochrome *c* (15 kDa) release into the cytosol was determined by Western blotting for cytochrome *c* in the mitochondrial and cytosolic fraction 12h after CrT1 (0, 1, 5, 10, 25 μ M) treatment (B).

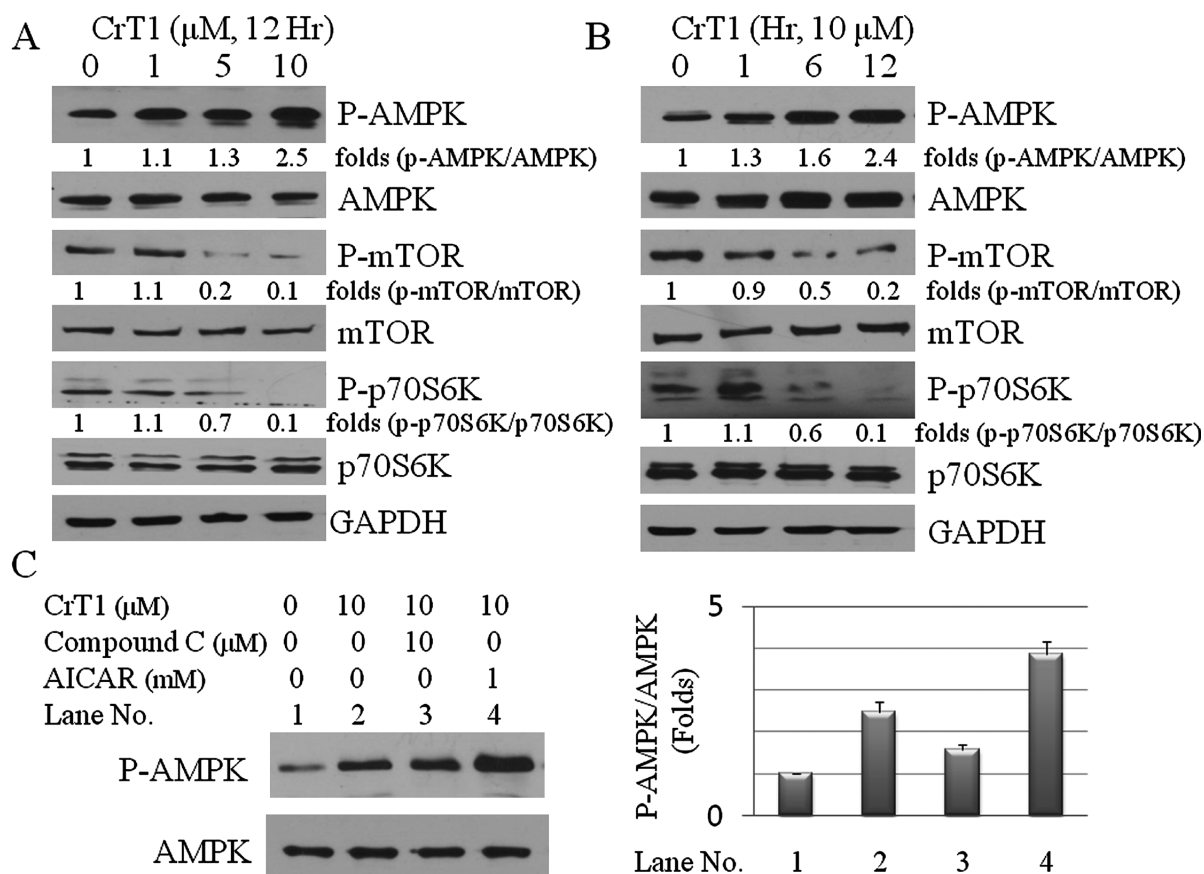


Fig. 4. *ent*-18-Acetoxy-7 β -hydroxy Kaur-15-oxo-16-ene (CrT1) Activates AMP-Activated Protein Kinase (AMPK) in SK-HEP1 Cells

After treatment with various CrT1 concentrations (A) and times (B), cells were lysed and the levels of phosphorylated AMPK (Thr172), AMPK, phosphorylated mammalian target of rapamycin (mTOR) (Ser2448), mTOR, phospho-p70S6K (Thr389), and p70S6K were determined by Western blot analysis. Cells were treated with CrT1 (0, 1, 5, 10 μ M) for 0, 1, 6, and 12h and with compound C (10 μ M, AMPK inhibitor) before being stimulated with CrT1, and CrT1 or/and 1 mM AICAR (AMPK activator) (C).

CrT1-induced cell death in SK-HEP1 cells, we observed expression of the proapoptotic proteins p53 and Bax, and the antiapoptotic protein Bcl₂.¹⁹ As a result, CrT1 induced increases in p53 and Bax levels but decreased Bcl₂ levels (Fig. 3A). To investigate the release of mitochondrial protein into cytosol, the cytosolic and mitochondrial fractions were separated, and translocation of cytochrome *c* into the cytosol was monitored. Treatment of cells with CrT1 resulted in increased translocation of cytochrome *c* into cytoplasm (Fig. 3B). These findings indicate that Bcl₂ is involved in CrT1-induced apoptosis, which may be mediated by increasing cleaved caspase protein levels.

CrT1 Activates AMPK Because identifying new AMPK activators is essential for developing anticancer agents,²⁰ we investigated the effects of CrT1 on AMPK phosphorylation at Thr-172. CrT1 activated AMPK in dose- and time-dependent manners (Figs. 4A,B). AMPK activation was associated with decreased mammalian target of rapamycin and 70kDa ribosomal protein S6 kinase phosphorylation (Figs. 4A,B). We used a synthetic AMPK inhibitor (compound C) on SK-HEP1 cells to examine CrT1 action on AMPK activation. As expected, the elevated AMPK activity induced by CrT1 was downregulated (Fig. 4C). To confirm this result, we treated cells with both an AMPK activator (AICAR) and CrT1. Co-treatment with AICAR and CrT1 increased AMPK even more dramatically (Fig. 4C). These results indicate that CrT1 activates AMPK in SK-HEP1 cells.

CrT1-Induced AMPK Regulates Apoptosis and Cell Growth To test whether AMPK phosphorylation was responsible for CrT1-induced cell death, we treated cells with CrT1 (10 μ M) for 12h and analyzed apoptosis-related proteins by Western blotting. The selective AMPK inhibitor compound C reversed CrT1-induced PARP and caspase-3 cleavage (Fig. 5A), and cell growth inhibition ($p < 0.05$) (Fig. 5B). These results suggest that CrT1-induced anti-proliferation and apoptosis are mediated by activating AMPK.

DISCUSSION

ent-Kauranes are diterpene-type compounds commonly found in most plant species, particularly those of the Euphorbiaceae family. Previous studies have indicated that *ent*-kaurane diterpenoids have anticancer activity.^{7,8,21} In this study, we found that a major *ent*-kaurane diterpenoid, CrT1, isolated from Vietnamese plant *Croton tonkinensis*, inhibited proliferation and enhanced apoptosis in human hepatocellular carcinoma SK-HEP1 cells. Our results showed that CrT1 induced cell death in SK-HEP1 cancer cells, as determined by the CCK-8 assay (Figs. 1B,C) and a flow cytometric analysis (Fig. 1D). We also examined the cytotoxic effect of CrT1 using other cancer cell lines such as gastric cancer (NCI-N87, SNU-620, and SNU-484), colon cancer (HT-29, HM-7, and SNU-C5), and breast cancer (MCF-7 and SKBR3), which resulted in broad cytotoxic activity in all of these cell lines with IC₅₀ values ranging from 8.4 to 31.2 μ M (data not shown). However, we observed that CrT1 showed no toxicity to fibroblasts (NIH-3T3) (data not shown). This means that normal cells were less affected than tumor cells, and that the anticancer activity induced by CrT1 was focused on tumor cells. These results suggest that CrT1 is probably a safe compound for clinical use to diminish the side effects to normal cells receiving cancer therapy.

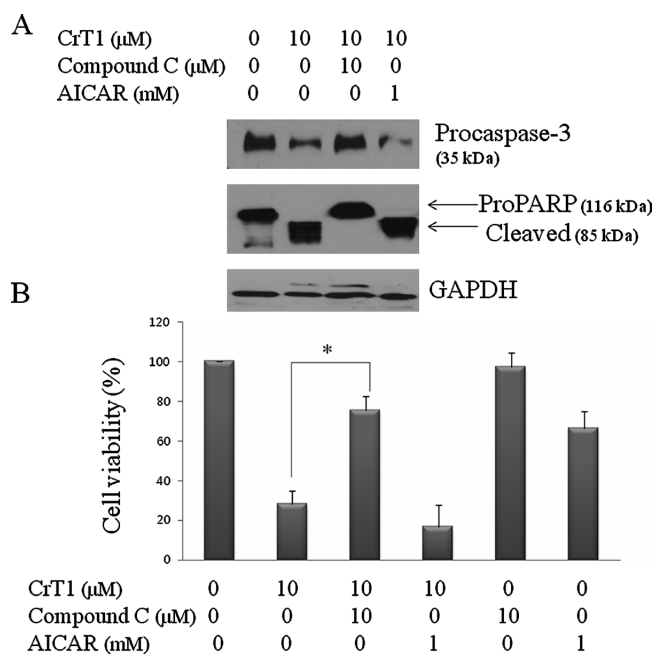


Fig. 5. *ent*-18-Acetoxy-7 β -hydroxy Kaur-15-oxo-16-ene (CrT1)-Induced AMP-Activated Protein Kinase (AMPK) Activation Regulates Apoptosis and Proliferation in SK-HEP1 Cells

After pretreatment with compound C (10 μ M) or AICAR (1 mM), cells were treated for 12h with CrT1 (10 μ M), and caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage was determined by Western blot analysis (A). After pre-exposure to compound C or AICAR, cells were treated for 12h with CrT1 (10 μ M), and cell viability was determined by the CCK-8 assay. Data are expressed as percent relative to control. * $p < 0.05$ (B).

Several researchers have focused on the effect of *ent*-kaurane diterpenoids during apoptosis. For example, erioalexin B induces apoptosis against four pancreatic adenocarcinoma cell lines (PANC-1, SW1990, CAPAN-1, and CAPAN-2),²² 13-hydroxy-15-oxo-zoapatanin induces apoptosis in human leukemia (U937 and Molt4),²³ weisiensin B induces apoptosis in human hepatoma (BEL-7402),²⁴ ponidicin induces apoptosis in lung cancer (A549 and GLC-82),²⁵ and *ent*-16 β -17 α -dihydroxykaurane induces apoptosis in breast cancer (MCF-7).⁸ In this study, we found that CrT1 treatment caused apoptosis in SK-HEP1 cells (Figs. 2A,B).

The apoptotic cell death pathway is mediated by Bcl₂ family proteins, a group of antiapoptotic proteins that regulate the passage of small molecules such as cytochrome *c*, which activates the caspase cascade through the mitochondrial transition pore.²⁶ *ent*-Kaurane diterpenoid ponidicin treatment causes downregulation of the anti-apoptotic proteins Bcl₂ and survivin and upregulates the Bax pro-apoptotic protein during apoptosis²⁵; however, the apoptotic mechanism of *ent*-kaurane diterpenoids remains unclear. Similarly, CrT1 downregulated the anti-apoptotic protein Bcl₂ and upregulated pro-apoptotic proteins (Bax and p53) (Fig. 3A), which resulted in a reduction in mitochondrial membrane potential and increased cytochrome *c* release into the cytosol (Fig. 3B).

Primary liver cancer is the fifth most common cancer and has the third highest mortality rate worldwide. HCC represents approximately 85% of all primary liver cancers.²⁷ Overall, 5-year survival is <15%, and is <2% in patients with advanced stage disease.²⁸ Therefore, preventing HCC is a good strategy to reduce mortality and morbidity. A number of significant risk factors, such as hepatitis B and C viruses,

have been identified to increase the risk for HCC.²⁹⁾ Some epidemiological studies have shown an increasing role for diabetes in the development of HCC. Patients with type II diabetes have a 2- to 3-fold increased relative risk for HCC,^{30–32)} and rodents diabetic models have an increased risk for HCC, illustrating a direct link between diabetes and HCC.^{33,34)} Epidemiological evidence shows a significant reduction in HCC in patients with diabetes who are taking the AMPK activator metformin.^{35,36)} AMPK is the primary regulator of the cellular response to reduce ATP levels in eukaryotic cells.³⁷⁾ Literature on the activation of AMPK in various cancers including HCC has grown rapidly in the past few years.³⁸⁾ One of the pharmacological AMPK activators, AICAR, induces hepatocellular cancer cell death,³⁹⁾ suggesting that activating AMPK could be a potential therapeutic target for HCC. In this study, we demonstrated that CrT1 increases AMPK activation (Figs. 4A,B), and that the AMPK phosphorylation induced by CrT1 was inhibited by compound C, an AMPK inhibitor, and was synergistically activated by AICAR (Fig. 4C). In addition, CrT1-induced apoptosis and anti-proliferation were regulated by AMPK (Figs. 5A,B). No reports are available on diterpenoids including *ent*-kauranes as AMPK activators. These results suggest that CrT1 is a novel AMPK activator, and that activating AMPK is responsible for CrT1-induced anti-proliferation and apoptosis in SK-HEP1 cells.

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