Ent-18 acetoxy-7β hydorxykaur-16en-15one from *Cronton tonkinesis* induces cell death and down- regulates anti-apoptotic gene XIAP through NF-κB signaling

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Abstract

An ent-kaurane diterpenoid ent-18acetoxy-7beta hydroxykaur-16en-15one (Cp7) was isolated from the dried leaves of Cronton tonkinesis Gagnep which is an indigenuos plant being widely used in Vietnamease Traditional Medicine. Cp7 induced cell death of 6 tested human cancer cell lines with IC50 (concentration inhibiting 50% of cell growth) values change from 15.74 ± 1.75 to 36.63 ± 1.19 μM, implying its cytotoxic potential to many kinds of cancer cells. We investigated that Cp7 induces cell death via apoptotic pathway by inhibiting p-NFκB p65 and down-regulating XIAP expression. It could become a useful compound to develop a new anti-cancer reagent.

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1. Backgrounds (Introduction)

Cronton tonkinesis belongs to *Euphobiaceae* which grows wildly in Vietnam, and is commonly known as *Kho sam cho la* in Vietnamese [1-3]. In traditional medicine, *Cronton* species are used as remedies for gastric and duodenal ulcers and many other diseases. Some ent-kauranes diterpenoids isolated from the leaves of *Cronton tonikinesis* could inhibit NF-κB activity [4, 5] but the anti-cancer effect have not well-understood.

Apoptosis is the programmed cell death with morphological characteristics including cellular shrinkage, membrane blebbing. Apoptotic signal pathways are activated through caspase cascades containing initiator and executioner caspase (caspase-3, -6, -7) activation. The activity of executioner caspase is directly blocked by IAPs (inhibitor of apoptosis proteins). Among IAPs, XIAP (X-linked inhibitor of apoptosis protein) has been shown to be highly expressed in many tumors and also induces the resistance of tumor to therapeutic agents. XIAP expression is transcriptionally regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [6], which is involved in inflammation, carcinogenesis, pro-apoptosis and anti-apoptosis [7].

In this study, we determined on the cytotoxicity of *ent-18acetoxy-7beta hydroxykaur-16en-15one* (Cp7), which isolated from *Cronton tonkinesis Gagnep*, in 6 cancer cell lines. Cp7 could induce apoptosis by downregulating XIAP via inhibition of NF-kB signaling.

2. Materials and methods

4.1 ent-18acetoxy-7beta hydroxykaur-16en-15one (Cp7)

ent-18acetoxy-7beta hydroxykaur-16en-15one (Cp7) was isolated from *Cronton tonkinesis Gagnep* in Vietnam National Institute of Medicinal Material.

4.2 Cell culture

MDA-MB-231, MDA-MB-468, HT29, PANC1, UACC257, and SK-MEL-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 medium with 10% fetal calf serum (FCS), 2mM L-Glutamine, 100U/ml penicillin and 100µg/ml Streptomycin at 37°C in a humidified 5% CO_2 and 95% air atmosphere.

4.3 WST-1 assay

Cytotoxic activity was assayed by WST-1 Cell counting kit (Wako Pure Chemical industries Ltd.) [8]. Briefly, the cells were seeded at density of $1x10^4$ cells/well to 96-well plate. After overnight incubation, 10 µl medium containing various concentrations of Cp7 was added to each well. At the indicated time points, 10µl WST-1 solution was added and the absorbance at 450 nm was measured using the micro-plate reader. Cell viability was determined by normalizing to vehicle control.

4.4 Apoptosis assays

Cells were seeded at density of $5x10^5$ cells/well to 6 wells plate and allowed to adhere overnight. The cells were treated with Cp7 at the indicated concentrations for 24 hours. The cells were washed with cold PBS and then collected. After washing the cells with cold PBS, the cell pellets were re-suspended with PBS 1x contains 1% BSA at $1x10^6$ cells/ml. 100 µl of Muse Annexin-V and Dead cell Reagent (Millipore Co.) was added to 100 µl of cells. After incubation for 20 minutes at room temperature in dark, apoptotic cells were measured by Muse system.

4.5 Western blot analysis

Whole cell lysates were collected as described previously [8]. Primary antibodies we used were; Caspase 3, PARP, XIAP, Phospho-NF-κB p65, and NF-κB p65 (Cell signaling Technology, Inc)

5. Results

5.1 ent-18acetoxy-7beta hydroxykaur-16en-15one (Cp7) inhibits cell viabilities.

During the search for the potent of anticancer agents from the Vietnamese natural products, we found that many compounds isolated from Cronton tonkinesis . Among them, we focused on the one new ent-Kaurane diterpenoid, *ent-18acetoxy-7beta hydroxykaur-16en-15one* (Cp7), in this study. To investigate the effect of Cp7 in cancer cells, we firstly used 6 cancer cell lines, one pancreatic carcinoma (Panc-1), one adenocarcinoma (HT-29), two melanoma (UACC-257 and SK-MEL-2), two breast cancer (MDA-MB-231 and MDA-MB468). Surprisingly, Cp7 could suppress the cell viabilities in all human cancer cell lines with IC_{50} (concentration inhibiting 50% of cell growth) values from 15.7 ± 1.8 to 36.6 ± 1.2 µM (Fig. 1 and Table 1). These suggest that Cp7 inhibits the cell viabilities in cancer cells.

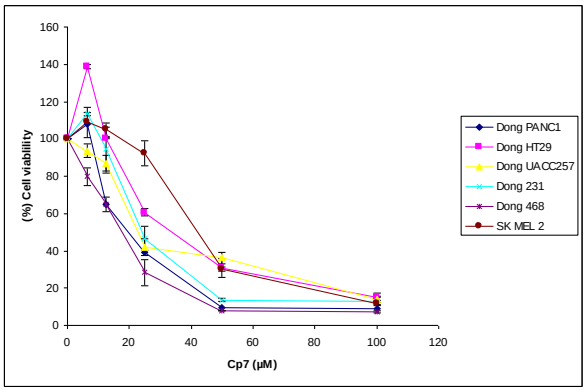


Fig. 1 6 human cancer cell lines were treated with 6.25, 12.5, 25, 50, and 100 µM of Cp7 for 24h. Absorbance was measured following incubation with WST-1 reagent and cell viability was calculated as a percentage compared with the untreated cells group.

Table.1. IC50 values of Cp7 on 6 cancer cell lines.

	PANC-1	UACC-257	HT-29	MDA-MB-231	MDA-MB-468	SK-MEL-2
IC50 (µM)	16.41±1.87	20.46± 3.59	27.47±1.18	22.74±1.75	15.74±1.75	36.63±1.19

The data were analyzed by Sigma Plot with p value was less than 0.001. Each value represents mean ± SD from 3 triplicates.

5.2 Cp7 induces apoptosis in cancer cells.

To determine the inhibition of cell viability by Cp7 is caused by apoptosis, we checked the apoptotic cell death by annexin-V staining (Fig. 2) and the expression of apoptotic markers by western blot analysis (Fig. 3) in MDA-MB-231 breast cancer cells and UACC-257 melanoma cells. As shown in Fig. 2, the induction of annexin-V-positive cells, which contain the early and late apoptotic cells, was detected in both cell lines at a dose-dependent manner. Consistent with annexin-V staining, the apoptotic markers, both cleaved caspase-3 and cleaved PARP, were induced at a dose- and time-dependent manner (Fig. 3). Collectively, these suggest that Cp7 induces apoptosis in cancer cells.

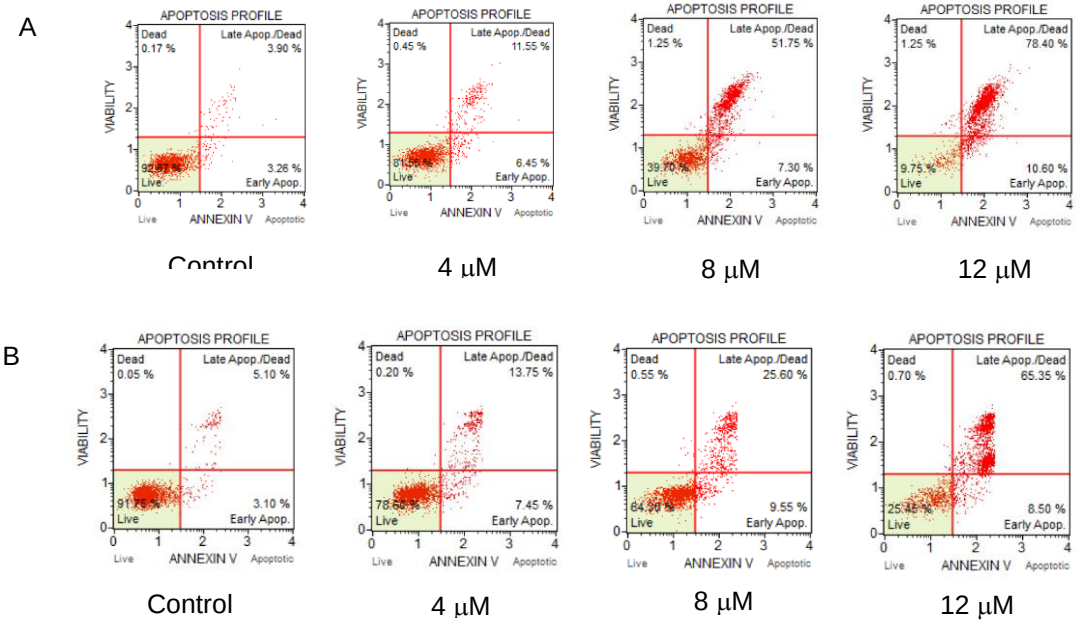
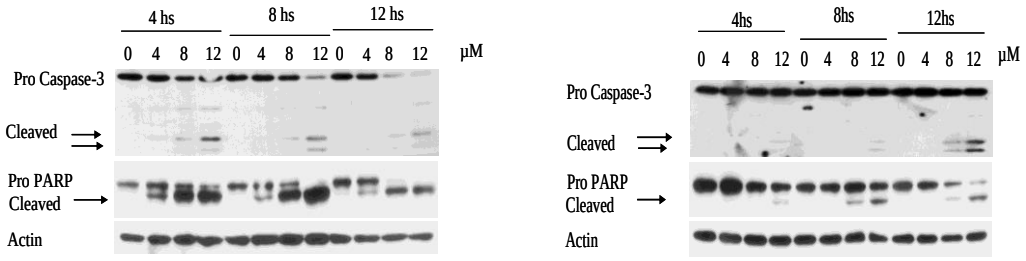


Fig. 2. MDA-MB-231 (A) or UACC-257 (B) were treated with Cp7 for 24h at the indicated dose. The cells were labeled with Annexin-V and Dead cell kit solution and analyzed by Muse system. The values indicated the percentage of living, early apoptotic, late apoptotic/dead, and dead cells. At the dose 12 μM the percentage of total apoptotic cells were 89% and 73.95% (data not shown) in MDA-MB-231 and UACC-257, respectively.



A. MDA-MB-231 cell line B. UACC-257 cell line

Fig 3. Cp7 induces apoptosis in MDA-MB-231 (A) and UACC-257 (B). Apoptotic markers (arrow), Caspase-3 and PARP cleavage, were detected by western blot analysis. β-Actin was used as a loading control.

5.3 Cp7 inhibits XIAP expression through NF-κB signaling.

Next to investigate whether Cp7 affects the expression of anti-apoptotic protein, we checked some anti-apoptotic protein such as BCL-xL, MCL1, or XIAP in MDA-MB-231 and UACC-257 cells. Shown in Fig. 4, XIAP was only suppressed by Cp7 in both cell lines at a dose- and time-dependent manner, whereas other proteins we checked such as Bcl-XL and Mcl-1 were not affected. In addition, the expression of NF-κB p65, one of the upstream

transcription factors of XIAP, was inhibited, following to the phosphorylation of p65. These suggested that Cp7 might inhibit XIAP expression via NF-κB signaling.

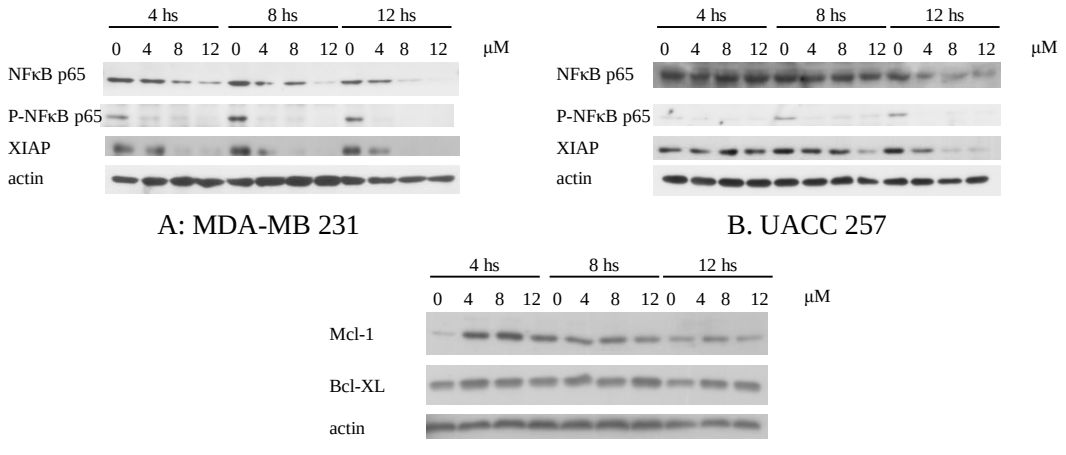


Fig. 4 Cp7 inhibits expressions of XIAP and NFκB p65 in apoptotic cells. NFκB p65, XIAP and p-NFκB p65 were detected in total cell extracts by goat-antibodies and rabbit antibody respectively. **A, B.** Cp7 mediated reduction of NFκB p65 and active form phosphorylated NFκB p65. This reduction depends on time and dose manner. XIAP was down-regulated by treated Cp7. **C.** Cp7 treatment did not result in a reduction in expression of anti-apoptotic protein such as Bcl-XL, Mcl-1 in MDA MB 231 cell line.

6. Discussion

In this study, we showed the inhibition of cell viabilities by *ent-18acetoxy-7beta hydroxykaur-16en-15one* (Cp7) isolated from the dried leaves of *Cronton tonkinesis Gagnep*, in various human cancer cells. The Cp7-induced apoptosis is mediated through the inhibition of XIAP and NF-κB signaling.

Ent-Kauranes are diterpenoid compounds commonly found in many plants. These compounds exhibit significant anti-tumor, antibacterial and anti-inflammatory activities [3]. Especially, other ent-kauranes isolated from *Cronton tonkinesis* have also been reported to have anti-cancer effects through apoptosis in hepatocelluar carcinoma cells, breast cancer cells, and Raw 264.7 by unknown mechanisms [3]. In this study, we firstly reported that an ent-kaurane *ent-18acetoxy-7β hydroxykaur-16en-15 one* (Cp7) isolated from *Cronton tonkinesis* has anti-tumor effects in many types of cancer cells (Fig. 1 and Table 1). The cytotoxicity could enhance until 90% in the case of 100 □M concentration for 24h, implying that almost all cancer cells could be killed regardless of their genetic or phenotypic backgrounds.

We newly identified the molecular mechanisms how one of Ent-Kauranes, Cp7, could show anti-tumor effects through inhibiting NF-κB signaling. It also has been reported that NFκB is activated in cancer cell by several chemotherapies and this response inhibits the ability of cancer therapy to induce cell death [7]. Because some chemo-resistances are caused by activated NF-κB signaling, Cp7 could increase the sensitivities to chemotherapy even in the case of acquired drug-resistant tumors. Far from anti-tumor effects, NF-κB signaling is also involved in the inflammatory or angiogenesis. It suggests that Cp7 could be a good candidate not only for anti-tumor reagents but also anti-inflammatory reagents for the patients with allergy.

NF-κB signaling we identified is known to regulate XIAP expression besides BCL-xL and MCL1 [9]. Even though Cp7 inhibits NF-kB p65 in our results, BCL-xL and MCL1 expression were not affected. It could be possible that Cp7 only inhibit the p65-XIAP axis. Nevertheless, Cp7 could have multiple functions, which suppresses XIAP expression independent on NF-κB. Collectively, these results strongly suggest that Cp7 could become a good lead compound for anti-tumor therapy regardless of their tumor types.

7. Reference

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