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Anti-cancer effect of cucurbitacin B on cholangiocarcinoma cells

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Abstract

The treatment of cholangiocarcinoma (CCA) is still ineffective and the search for a novel and more effective treatment is required. Cucurbitacin B (CuB) is a natural tetracyclic triterpene that possesses anti-cancer activity across a wide array of cancers. In this study, we explored the effect of CuB isolated from the Thai herb *Trichosanthes cucumerina* L on the proliferation of two CCA cell lines using MTT test. The results demonstrated that CuB exhibited potent anti-proliferation effect in a dose- and time-dependent fashion. IC_{50} values were 0.048, 0.036 and 0.032 μM for KKU-213 and 0.088, 0.053 and 0.04 μM for KKU-214 cell line at 24, 48 and 72 h of incubation, respectively. Flow cytometric analysis revealed that CuB induced G2/M phase cell cycle arrest in a dose dependent manner. Concomitantly, subG1 population was observed when the cells treated with 0.2 μM CuB in both cell lines. Western blotting demonstrated that CuB at the concentration of 0.2 μM enhanced the expression of cell cycle inhibitory proteins namely p21 and p27. On the other hand, it downregulated the expression of cell cycle driving proteins namely phosphorylated retinoblastoma protein (pRB), cyclin D1, and cyclin E. Taken together, it is suggested that CuB may inhibit CCA cell growth through suppression of cell cycle progression. The detailed molecular mechanisms underlying CuB effect on CCA cells both *in vitro* and *in vivo* should be further explored with the hope that this promising compound derived from Thai herb might be an alternative treatment for CCA.

Introduction

Although there has been progress in the development of prevention and treatment of cancer, the successful treatment of cholangiocarcinoma (CCA) remains a challenge. CCA is a cancer that is highly resistant to various anticancer drugs and, thereby, leads to poor prognosis.[1-3] Therefore, the search for new and effective anticancer agents is urgently needed.

Cucurbitacins, a class of highly oxidized tetracyclic triterpenoids, are widely distributed in the plant kingdom. To date, more than one hundred cucurbitacins and their derivatives have been identified while only a few of them have been widely investigated.[4] Naturally, cucurbitacin B (CuB) and D are the most common and have the highest content in many plants, followed by E, G, H, and I. Documented data demonstrated that cucurbitacins possess some pharmacological activities, such as anti-inflammation, and hepatoprotection.[5,6] In the past ten years, the anti-cancer effect of cucurbitacins has drawn attention of many researchers. Recent advances showed that cucurbitacins are potent anti-cancer natural products in both *in vitro* and *in vivo* models. Cucurbitacins dramatically inhibit the growth and proliferation of a series of cancer cells. They could also induce cancer cell differentiation, inhibit angiogenesis and

metastasis.[4,6-13] Accumulated data showed that cucurbitacins could induce different phases of cell cycle arrest depending on the type of cucurbitacins and the type of cell line. It has been reported that CuB induced S-phase arrest in BEL-7402, HL60, and U937 cells as well as G2/M-phase arrest in Panc-1, MiaPaCa-2, K562, SW480, and Hep-2 cells. CuE and CuI caused G2/M phase arrest in Panc-1, BEL-7402, HepG2, HL60, T24, and ES-2 cells while CuD led to S phase arrest in myeloid leukemia cells.[6]

Despite several investigations on CuB's targets and mechanism of action in various cancers, there is no evidence about the effect of CuB on CCA cells. In the present study, we aimed to investigate the effect of CuB on the liver fluke-associated CCA cell lines, KKU-213 and KKU-214, particularly the anti-proliferative properties of this compound.

Methodology

Cell lines and culture technique

The CCA cell lines, KKU-213 and KKU-214 were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan. The cell lines were maintained in DMEM medium supplemented with 10% FBS and 1% antibiotics/antimycotics, in a humidified atmosphere of 5% CO_2 at 37 °C.

Antibodies and reagents

Antibodies against total and phosphorylated retinoblastoma protein (RB and pRB), p21, p27, cyclin D1, and cyclin E were purchased from Santa Cruz Biotechnology (1:1,000; Santa Cruz, CA, USA) and the β-actin antibody was from Sigma (1:40,000). MTT reagent and propidium iodide (PI) solutions were purchased from Invitrogen (Eugene, OR, USA).

Cell viability assay

Cells (5×10^3 cells/100 µL/well), in a 96-well plate, were treated with various concentrations of CuB and with 0.001% dimethyl sulfoxide (DMSO) as control. After 24, 48 or 72 h of incubation, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.[14] The cytotoxicity of each compound was analyzed as relative viable cells compared with control.

Cell cycle analysis

After treatment, cells were collected, washed with PBS, fixed in 70% cold ethanol and stored at -20 °C until analysis. On the analysis day the fixed cells were washed with PBS twice and then stained with 10 µg/mL PI for 5 minutes and kept protected from light before analysis. The procedure of cell cycle analysis was previously described.[14] using BD FACSCanto II flow cytometer. The numbers of cells distributed in subG1, G0/G1, S and G2/M phases, were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Western blotting

Protein (20 µg) was subjected to a 10% SDS-polyacrylamide gel electrophoresis and transferred to a Hybond™-P PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membrane was then blocked with 3% BSA for 1 h at room temperature, probed for 1 h at room temperature with 1:1,000 of each primary antibody and 1:20,000 horseradish perosxidase (HRP)-linked secondary antibody. The immunoactive bands were detected using an enhanced chemiluminescence prime Western blotting detection reagent kit (ECL; GE Healthcare). The same membrane was stripped off and incubated with β-actin antibody as the loading control.

Results and Discussion:

CuB inhibits CCA cell growth in vitro

The anti-proliferative effects of CuB, as shown in figure 1A were screened in KKU-213 and KKU-214 cell line using the MTT assay. Cells were incubated with various concentration of CuB for 24, 48, and 72 h. The results showed that CuB strongly inhibited CCA cell growth in a dose and time dependent manners (Figure 1B). As shown in Table 1, the half maximal inhibitory concentrations (IC_{50}) values were 0.048, 0.036 and 0.032 μM in KKU-213 and IC_{50} values 0.088, 0.053 and 0.04 μM in KKU-214 cell line following 24, 48 and 72 h of incubation, respectively.

CuB induces G2/M phase cell cycle arrest of CCA cells

Cancer development is often due to perturbations in the cell cycle that leads to unlimited proliferation and confers apoptosis resistance.[15] The progression the cell cycle is exerted by cyclin, cyclin-dependent kinases (CKIs) and cell cycle inhibitors. CDK4/6-Cyclin D and CDK2-Cyclin E work in concert to relieve inhibition of RB protein-E2 factor transcription complex, whereas inhibition of the kinase activity of cyclin/CDK complex is mediated by several CKIs, including $p21^{waf1/cip1}$ and $p27^{kip1}$.[16] To examine whether the growth inhibition induced by CuB was associated with regulation of the cell cycle, the cell cycle distribution of CCA cells in the presence of CuB was analyzed by flow cytometry. As shown in Figure 2A, treatments of KKU-213 and KKU-214 cells with 0, 0.05, 0.1, and 0.2 μM for 48 h resulted in a significant accumulation of cells in the G2/M phase. This results was consistent with the study by Guo and colleagues showing that CuB induced ATM-mediated DNA damage causes G2/M cell cycle arrest in human lung cancer A549 cell line and leukemia cells.[4,17] The effects of CuB on the regulation of the cell cycle were further determined. Cell lysates of KKU-213 and KKU-214 cells treated with CuB at 0.05 and 0.2 μM for 48 h were analyzed for expression of some cell cycle regulatory proteins namely pRB, cyclin D1, cyclin E, p21 and p27 using Western blot analysis. As shown in Figure 2B, CuB markedly down-regulated the expressions of cell cycle driving proteins, pRB, cyclin D1 and cyclin E. On the other hand, CuB up-regulated the expression of cell cycle inhibitors, p21 and p27 in both cell lines.

Defective apoptosis represents a major causative factor in the development and progression of cancer. Indeed, the majority of chemotherapeutic agents act through the apoptotic pathway to induce cancer cell death. Moreover, resistance to chemotherapeutic strategies seems to be due to the alterations in the apoptotic pathway of cancer cells.[18] In the current study, the flow cytometry analysis revealed that at the concentration of 0.2 μM, the subG1 population was induced in both cell lines. Further confirmation for CuB induced cell apoptosis should be done using more specific assay such as annexin V/PI staining and also investigation of apoptosis associated protein by western blot analysis.

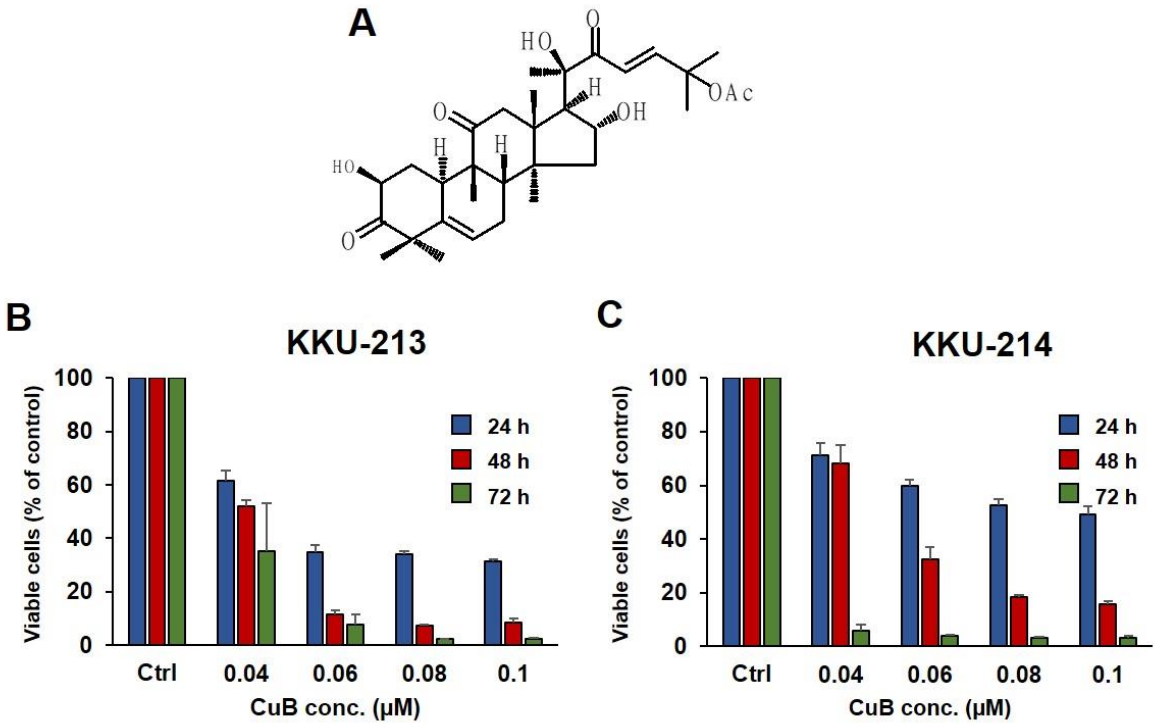

Figure 1. Anti-proliferative effect of CuB on CCA cells. (A) Chemical structure of CuB. (B) KKU-213 and (C) KKU-214 cells were treated with various concentration of CuB as indicated for 24, 48 and 72 h. The MTT assay was performed and the cell viability was calculated given the controls as 100%. Data are expressed as the mean ± SEM of triplicate assay.

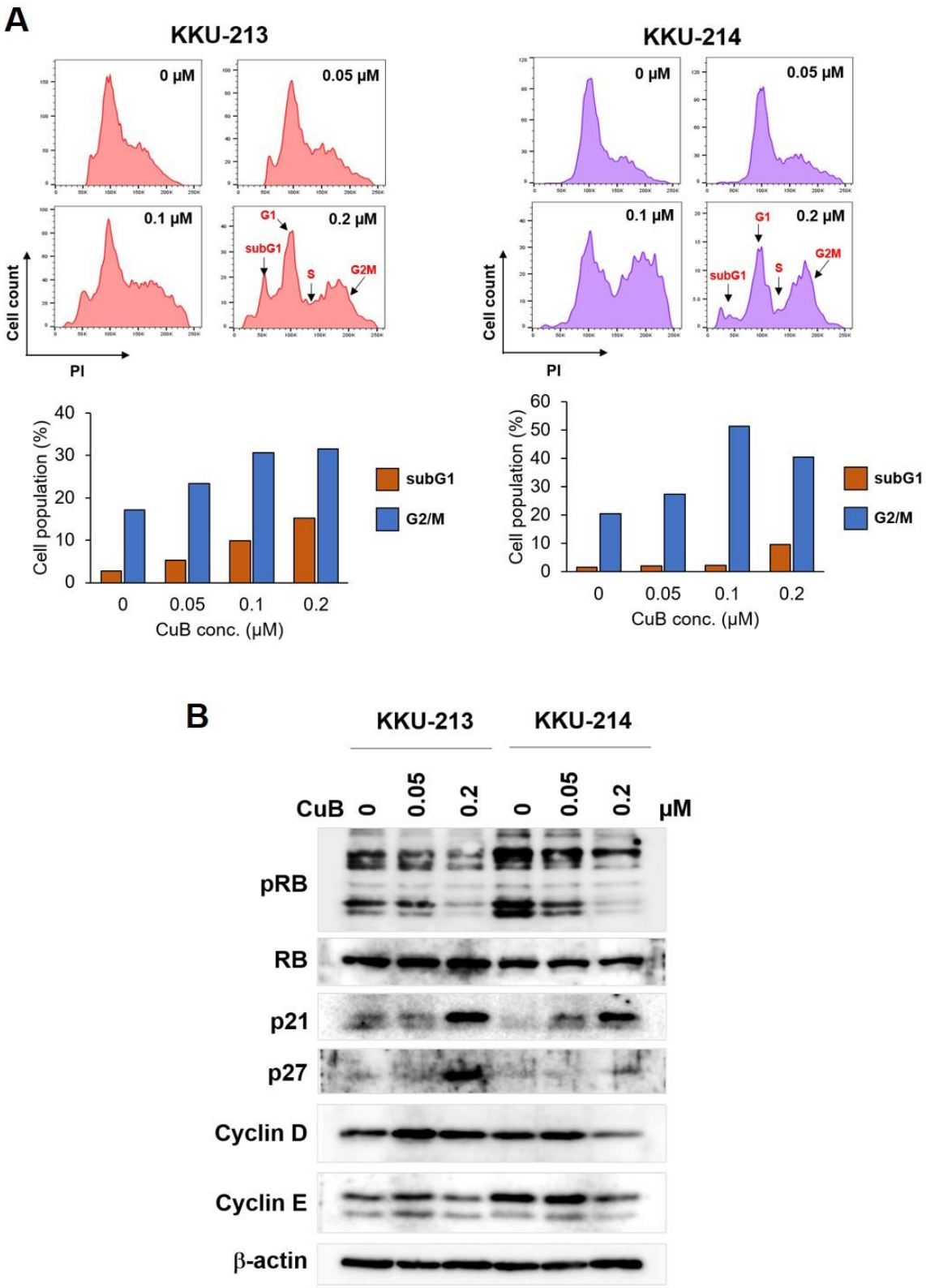

Figure 2. Effect of CuB on cell cycle distribution and cell cycle regulatory proteins. (A) CCA cells, KKU-213 and KKU-214 were treated with 0, 0.05, 0.1 and 0.2 μM of CuB for 48 h. The cell cycle distribution was analyzed using flow cytometry. (B) CCA cells were treated with 0, 0.05, and 0.2 μM of CuB for 48 and the expression of cell cycle regulatory proteins were determined using Western blotting.

Table 1. The half-maximal inhibitory concentrations of CuB at 24, 48, and 72 h of incubation.

Cell line	IC_{50} (μM, MTT assay)		
	24 h	48 h	72 h
KKU-213	0.048	0.036	0.032
KKU-214	0.088	0.053	0.040

Conclusion

CuB was evaluated for its anti-cancer activity against CCA cell lines. CuB exhibited potent anti-proliferative activity in a dose and time dependent manner. It was further shown to inhibit the growth of CCA cells by arresting the cells at G2/M phase of the cell cycle and probably induced cell death. Further studies on the efficacy and safety in normal cells an animal model are needed to propose CuB as a candidate for a supplemental or alternative therapeutic approach of CCA.

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