

RESEARCH ARTICLE

Anticancer Effects of *Curcuma* C20-Dialdehyde against Colon and Cervical Cancer Cell Lines

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Abstract

Background: Recent attention on chemotherapeutic intervention against cancer has been focused on discovering and developing phytochemicals as anticancer agents with improved efficacy, low drug resistance and toxicity, low cost and limited adverse side effects. In this study, we investigated the effects of *Curcuma* C20-dialdehyde on growth, apoptosis and cell cycle arrest in colon and cervical cancer cell lines. **Materials and Methods:** Antiproliferative, apoptosis induction, and cell cycle arrest activities of *Curcuma* C20-dialdehyde were determined by WST cell proliferation assay, flow cytometric Alexa fluor 488-annexin V/propidium iodide (PI) staining and PI staining, respectively. **Results:** *Curcuma* C20 dialdehyde suppressed the proliferation of HCT116, HT29 and HeLa cells, with IC50 values of $65.4 \pm 1.74 \mu\text{g/ml}$, $58.4 \pm 5.20 \mu\text{g/ml}$ and $72.0 \pm 0.03 \mu\text{g/ml}$, respectively, with 72 h exposure. Flow cytometric analysis revealed that percentages of early apoptotic cells increased in a dose-dependent manner upon exposure to *Curcuma* C20-dialdehyde. Furthermore, exposure to lower concentrations of this compound significantly induced cell cycle arrest at G1 phase for both HCT116 and HT29 cells, while higher concentrations increased sub-G1 populations. However, the concentrations used in this study could not induce cell cycle arrest but rather induced apoptotic cell death in HeLa cells. **Conclusions:** Our findings suggest that the phytochemical *Curcuma* C20-dialdehyde may be a potential antineoplastic agent for colon and cervical cancer chemotherapy and/or chemoprevention. Further studies are needed to characterize the drug target or mode of action of the *Curcuma* C20-dialdehyde as an anticancer agent.

Keywords: Antiproliferative activity - apoptosis - *Curcuma* C20-dialdehyde - cell cycle arrest - cervical - colon cancer

Asian Pac J Cancer Prev, 16 (15), 6513-6519

Introduction

Cancer is the leading and second leading cause of death in developed and developing countries, respectively (WHO, 2008). The incidence and mortality of cancers are burgeoning as a result of population age and growth along with changes in lifestyles including smoking, physical inactivity, etc. in economically developing parts of the world (Jemal et al., 2011). Colorectal and cervical cancers are among the most frequently encountered cancers in humans. Colorectal cancer is the third most commonly diagnosed cancer in man and the second in woman globally. Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in females globally. More than 85% of these cases and deaths are found to occur in developing countries (Ferlay et al., 2010). A combination of late clinical presentation of symptoms and lack of adequate access to timely and standard treatment limits the cancer survival trends in developing countries (Jemal et al., 2011).

Chemotherapy is one of most practiced approaches in advanced carcinogenesis as well as metastatic condition and also an adjuvant therapy for many cancers at present. However, clinical application of this approach often features serious challenges involving toxicity, side-effects and resistance development by cancer cells which underscore the desperate need for relatively non-toxic natural products (Pan and Ho, 2008). Currently, several purified natural products and their derivatives with immense pharmacological and biological properties have been discovered as potential candidates for cancer chemotherapy. The natural products preferentially function in multiple mechanistic pathways and overcome chemoresistance in tumors with cumulative actions (Saha and Khuda-Bukhsh, 2013). The tremendous ability of natural products to act as effective scaffolds and bind bewildering types of protein domains and folding motifs makes them effective modulators of various cellular processes, contributing to immune responses, signal transduction, cell division and apoptosis (Peczuha and Hamilton, 2000).

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Medicinal plants are important sources of chemopreventive and therapeutic agents for a wide variety of solid and hematological cancers. Now-a-days, *Curcuma* species is receiving greater importance across the globe as a potential source of new drug(s) to combat a variety of ailments as the species contain a lot of compounds conferring anti-inflammatory, antioxidative, antimicrobial, antirheumatic, antivenomous, antiviral, antihepatotoxic as well as anticancerous properties. Over last few decades, researchers have devoted extensive interests in exploring the biological and pharmacological activities of *Curcuma*, especially the cultivated species (Sasikumar, 2005). Among various phytochemicals, curcuminoids which are the biologically active principles from *Curcuma* species play vital roles in the control of rheumatism, carcinogenesis and oxidative stress-related pathogenesis (Mors et al., 2000; Sasikumar, 2005).

Curcumin (diferuloylmethane), one of the curcuminoids, is a phenolic compound derived from the rhizomes of turmeric (*Curcuma longa* L.). It has been used as herbal medicine for treatment of cancers, snake bites and many other ailments for centuries (Somasundaram, 2002). Curcumin is highly popular because of its chemopreventive property against human cancers (Aggarwal et al., 2003, Guo et al., 2013). There is convincing evidence in preclinical model and in vitro assay that curcumin has growth inhibitory effect on numerous cancer cells such as breast cancer (Ramachandran et al., 2002), oesophageal cancer (O'Sullivan-Coyne et al., 2009), colon cancer (Milacic et al., 2008), bile duct cancer (Prakobwong et al., 2011) and gall bladder cancers (Liu et al., 2013).

In addition, curcumin has effects on several different targets including transcription factors, growth regulators, adhesion molecules, angiogenesis regulators and cellular signaling molecules (Aggarwal et al., 2003). It may have chemopreventive potential against cholangiocarcinogenesis through activation of multiple cell signaling pathways associated with tumor promotion and progression (Prakobwong et al., 2011). Preclinical efficacy, less toxicity as well as side effects and availability of curcumin as natural product are the compelling factors to incorporate this agent in human clinical trial for chemopreventive potential.

Curcuma zedoaroides A. Chaveerach & T. Tanee, locally known as "Wan-Phaya-Ngoo-Tua-Mia" in Thai, is a new plant in the genus *Curcuma* (Zingiberaceae). The acetone extract of *Curcuma zedoaroides* rhizomes contained a *Curcuma* C20-dialdehyde, 2-[2-(5,5,8a-trimethyl-2-methylene-decahydro-naphthalen-1-yl)-ethylidene]-succinaldehyde, as a major component with antivenomous effect (Lattmann et al., 2010). Although numerous studies documented chemopreventive potential of curcumin against a wide variety of tumors, anticancer impacts of the *Curcuma* C20-dialdehyde remain to be elucidated extensively. So the aims of the present study were to evaluate antiproliferative activity of *Curcuma* C20-dialdehyde and to unravel the underlying mechanism(s) of its tumor growth inhibition. Our results demonstrated that *Curcuma* C20-dialdehyde inhibited the growth of colon and cervical cancer cells by induction of apoptosis and

inhibition of cell cycle progression. We believe that this is the first work that shows the antiproliferative activity and underlying mechanisms of *Curcuma* C20-dialdehyde in colon and cervical cancer cell lines.

Materials and Methods

Reagents and medium

Curcuma C20-dialdehyde was prepared as previously described (Lattmann et al., 2010). Culture medium RPMI-1640 and its supplements including antibiotics and fetal bovine serum were purchased from Gibco-BRL, Invitrogen, Basle, Switzerland. WST-8 Cell Proliferation Assay Kit was purchased from Bio Vision, Mountain View, CA, USA. Vybrant Apoptosis Assay Kit #2 was purchased from Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA. Propidium Iodide (PI) and RNase A was purchased from Sigma, Dublin, Ireland. *Curcuma* C20 dialdehyde was dissolved in dimethyl sulfoxide (DMSO) (Sigma, Dublin, Ireland). The final concentration of DMSO was maintained below 0.5% (v/v) in treatment groups and also in corresponding control. For apoptosis analysis, 10 μ g/ml of camptothecin (Sigma, Dublin, Ireland) was used as positive control.

Cell lines and culture condition

Human colorectal carcinoma cell line (HCT116) was collected from Dr. Osamu Tetsu (University of California, San Francisco), human colon adenocarcinoma cell line (HT29 cells) and human cervical carcinoma cell line (HeLa cells) were collected from National Cancer Institute, Bangkok, Thailand. Cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, a mixture of penicillin (100 U/ml) and streptomycin (100 μ g/ml), and incubated at 37°C in a humidified atmosphere with 5% CO₂.

Antiproliferative activity assay

Antiproliferative activity was assayed by using a WST-8 Cell Proliferation Assay Kit (BioVision, Mountain View, CA, USA) in accordance with manufacturer's instructions. Briefly, cells at a density of 8×10^3 cells/well were seeded onto 96-well plates in triplicate and allowed for 24 h for attachment. After 24 h, cells were exposed to increasing concentrations of *Curcuma* C20-dialdehyde for 24, 48 and 72 h. At the end of indicated time, WST reagent was added to cultures and incubated for 90 min at 37°C. Tetrazolium salt was converted into formazan by enzyme-catalyzed reduction in viable cells. Absorbance (A) of formazan was measured at 415 nm with a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA, USA) while 655 nm was used as a reference wavelength. The number of viable cells is corresponding to the production of formazan. Cell viability was calculated and expressed as percentages by the following equation:

$$\% \text{ Cell viability} = [\text{Sample A}/\text{Control A}] \times 100$$

Apoptosis analysis by Flow cytometry

Apoptosis induction of HCT116, HT29 and HeLa cells was evaluated with flow cytometer using the Alexa

Fluor 488-Annexin V apoptosis detection kit following the manufacturer's instructions. Briefly, cells (2.5×10^5 cells/ml) were seeded in a 5.4-cm culture dish and incubated for 24 h. Cells were treated with three concentrations of *Curcuma* C20-dialdehyde (25, 50 and 100 $\mu\text{g/ml}$) for 24 h. After 24 h of exposure, cells were harvested by trypsinization, washed with cold PBS and centrifuged (3,000 rpm for 3 min). Then, pellet of cells was re-suspended and diluted in the Annexin-binding buffer to a number of 105 cells per assay, and subsequently incubated with Alexa Fluor 488-Annexin V and Propidium iodide (PI) for 15 min at room temperature. After the incubation, cells were subjected to be analyzed by a Beckman Coulter Cytomics FC500 MPL flow cytometry (Beckman Coulter, Maimi, FL, USA). The flow cytometric results on apoptosis were correlated with that of a conventional cell count technique using a fluorescence microscope.

Analysis of cell cycle profile by Flow cytometry

To analyze cell cycle profiles, HCT116, HT29 and HeLa cells were seeded at the density of 2.5×10^5 cells/ml in a 5.4-cm culture dish and allowed for 24 h. Cells were then exposed to three concentration of *Curcuma* C20-dialdehyde (25, 50 and 100 $\mu\text{g/ml}$) for 24 h. After exposure, cells were harvested, washed with PBS, centrifuged (3,000 rpm for 3 min) and fixed with 70% cold ethanol at 4°C. After 1 h-fixation, cells were washed with PBS twice and incubated with 0.5 mg/ml RNaseA (Sigma, Dublin, Ireland) for 1 h to destroy double stranded RNA. Lastly, Propidium iodide (50 $\mu\text{g/ml}$) in PBS solution was added to stain nuclear DNA in subdued light for 40 min at room temperature. The DNA histogram reflecting the percentages of cells at different cell cycle phases was determined by using a Cytomics FC500 MPL flow cytometer (Beckman Coulter, Maimi, FL, USA).

Statistical analysis

All experiments were repeated separately at least three times. Data were presented as mean \pm SD for three separate experiments. Statistical differences between sample-treated and solvent-treated cells were determined using one-way ANOVA with Duncan's post hoc test. The criterion for consideration of statistical significance was set at $p < 0.05$.

Results

Effects of *Curcuma* C20-dialdehyde on proliferation of HCT116, HT29 and HeLa cells

To evaluate the effect of *Curcuma* C20-dialdehyde on cancer cell growth, its antiproliferative activity was determined in both colon cancer cell lines (HCT116 and HT29 cells) and cervical cancer cell line (HeLa cells). *Curcuma* C20-dialdehyde exhibited antiproliferative activity against all three cancer cell lines tested. As shown in Figure 2, cell growth was significantly ($p < 0.05$) inhibited by *Curcuma* C20-dialdehyde in a dose- and time-dependent manner. Cellular sensitivities measured as IC₅₀ (concentration required to decrease cell viability 50%) are presented in Figure 2.

Effects of *Curcuma* C20-dialdehyde on apoptosis induction in HCT116, HT29 and HeLa cells

The flow cytometry results revealed that *Curcuma* C20-dialdehyde induced apoptosis of all cancer cells tested in a dose-dependent manner (Figure 3). The early apoptosis was evidenced from Alexa Fluor 488-Annexin V positive and PI negative staining. However, late apoptosis was detected by both Alexa Fluor 488-Annexin

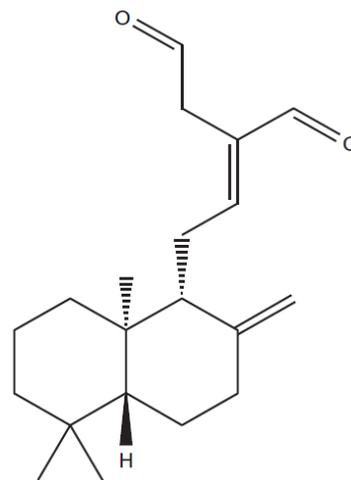


Figure 1. *trans*-Curcuma C20-dialdehyde CD; 2-[2-(5,5,8a-trimethyl-2-methylene-decahydro-naphthalen-1-yl)-ethylidene]-succinaldehyde (Lattmann et al., 2010)

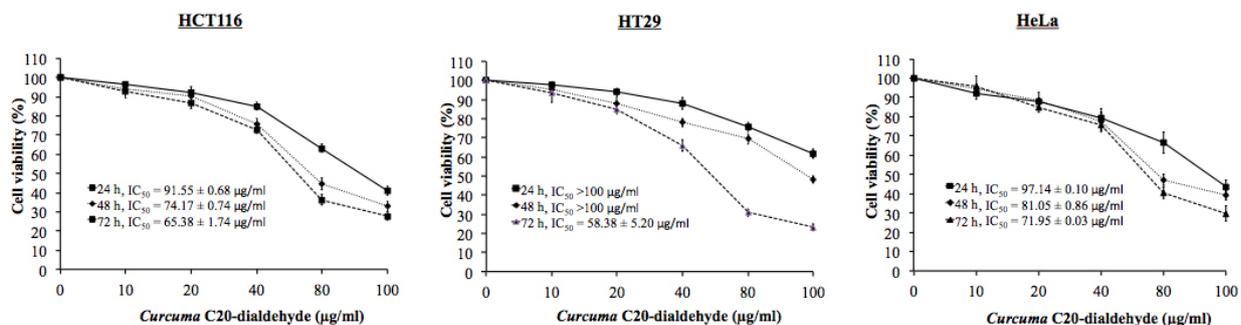


Figure 2. Effect of *Curcuma* C20-dialdehyde on Antiproliferative Activity of HCT116, HT29 and HeLa Cells. HCT116 (A), HT29 (B) and HeLa cells (C) were treated with increasing concentration of *Curcuma* C20-dialdehyde for 24, 48 and 72 h, and analyzed by WST-8 Cell Proliferation Assay. Cell viability was calculated as percentage. Data were expressed as mean \pm SD of three individual experiments

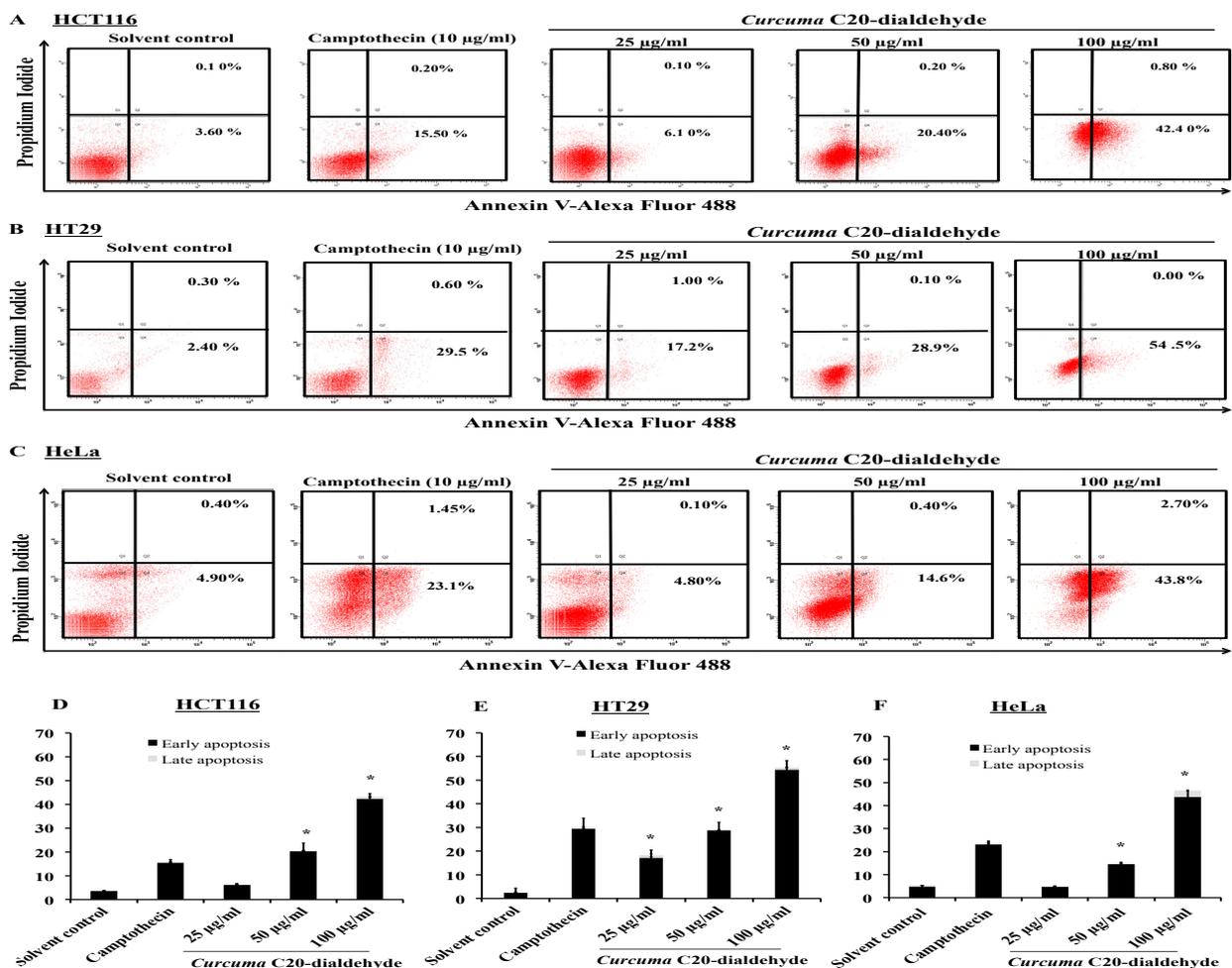


Figure 3. Effect on Induction of Apoptosis by Curcuma C20-dialdehyde in HCT116, HT29 and HeLa Cells. (A-C) Representative dot plots display the apoptotic cell death of HCT116, HT29, and HeLa cells. Cells were treated with 0, 25, 50 and 100 µg/ml of Curcuma C20-dialdehyde for 24 h, labeled with Alexa Fluor 488-Annexin V and Propidium iodide (PI) and analyzed by flow cytometer. Cells treated with solvent and Camptothecin (10 µg/ml) were used as negative and positive control, respectively. (D-F) Bar graphs show the summarized data (mean±SD) from three independent experiments performed in duplicate. *p<0.05 for comparison between drug and control treatments

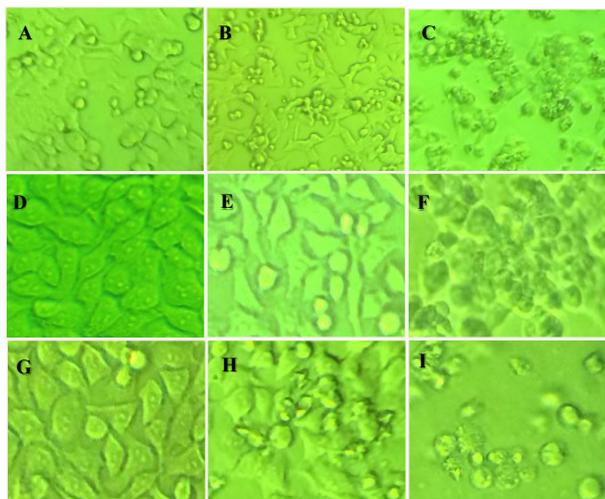


Figure 4. Morphological Changes Leading to Apoptosis by Curcuma C20-dialdehyde in HCT116, HT29 and HeLa Cells. HCT116 (A-C), HT29 (D-F) and HeLa (G-I) cells were treated with 0, 50, 100 µg/ml of Curcuma C20-dialdehyde, respectively, for 24 h. Morphological changes including shrinkage of cell, membrane blebbing, fragmentation of cells etc. upon exposure to Curcuma C20-dialdehyde were observed under microscope (Olympus 20X) and photographed

V and PI positive staining. The percentages of apoptosis increased from 3.60±0.21% to 42.40±2.14% in HCT116, from 2.40±1.98% to 54.50±3.80% in HT29 and from 4.90±0.45% to 43.80±2.76% in HeLa cells upon 24 h-exposure to Curcuma C20-dialdehyde at the concentration of 100 µg/ml. The observed morphological changes due to apoptosis induction after treatment with different concentrations are presented in Figure 4.

Effects of Curcuma C20-dialdehyde on cell cycle progression in HCT116, HT29 and HeLa cells

As presented in Figure 5A and 5D, low concentration (25 µg/ml) of Curcuma C20-dialdehyde increased the percentages of G1 phase in HCT116 cells from 75.3±0.71% to 87.00±1.84% and moderate concentration (50 µg/ml) induced the cell cycle arrest at G2/M phase with more sub-G1 fraction (20.75±1.32%). However, the highest concentration treatment in HCT116 cells caused more cell death evidenced by the presence of more sub-G1 fraction (52.55±2.3%). The treatment of HT29 cells with 50 µg/ml of Curcuma C20-dialdehyde resulted in cell cycle arrest at G1 phases (82.45±1.41%) (Figure 5B and 5E). In addition, the sub-G1 fraction gradually

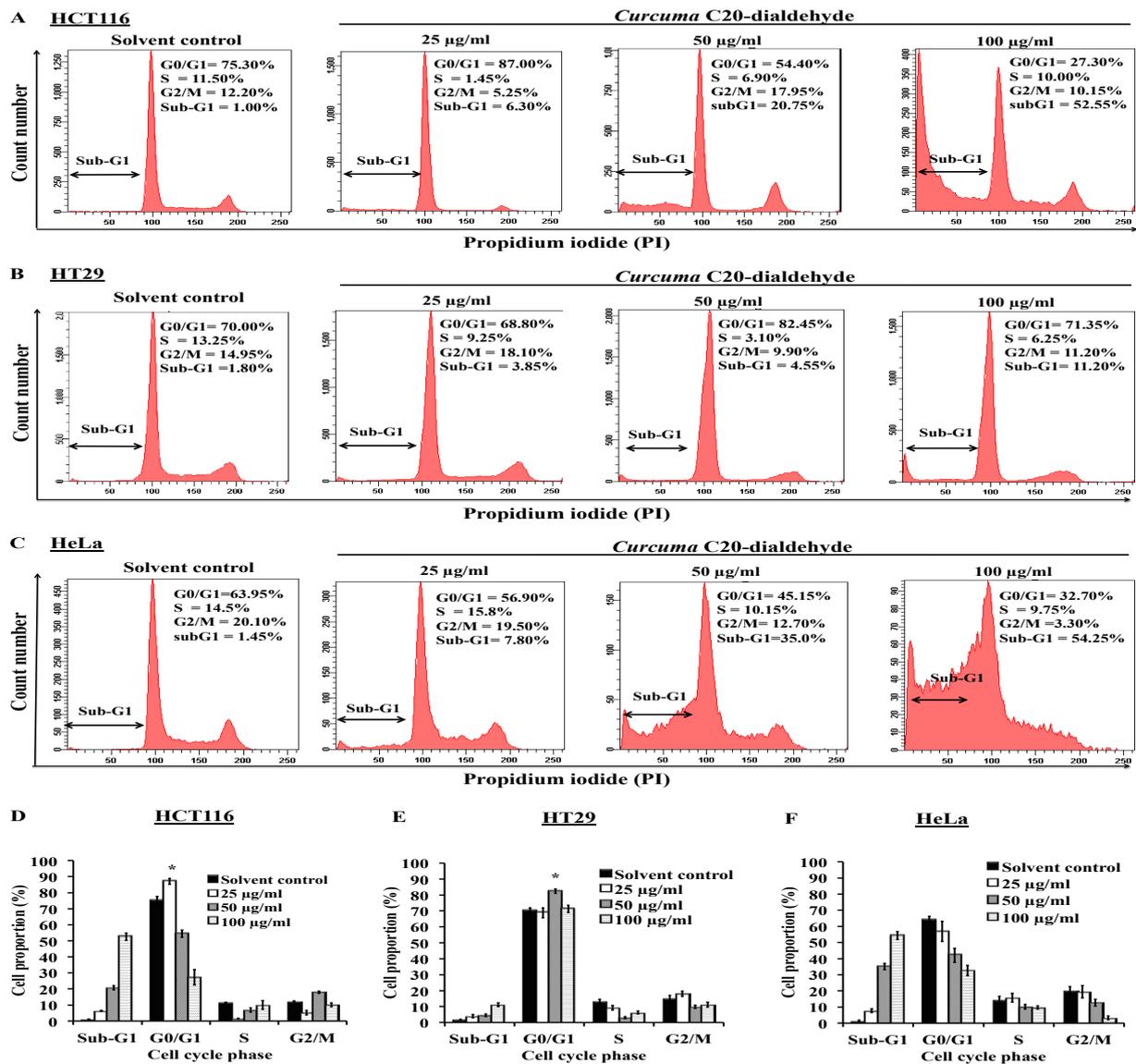


Figure 5. Effect on Induction of Cell Cycle Arrest by *Curcuma* C20-dialdehyde in HCT116, HT29 and HeLa Cells. (A-C) Representative DNA histograms on cell cycle profile of HCT116, HT29 and HeLa cells. Histograms showed a number of cells per channel (vertical axis) vs DNA content (horizontal axis). Cells were treated with 0, 25, 50 and 100 µg/ml of *Curcuma* C20-dialdehyde for 24 h, stained with Propidium iodide and analyzed using flow cytometry. (D-F) Bar graphs show summarized data (mean±SD) for three independent experiments on proportion of cells in different phases of cell cycle for HCT116, HT29 and HeLa cells, respectively. *p<0.05 for comparison between drug and control treatments.

increased with increasing concentrations of *Curcuma* C20-dialdehyde in HT29 cells. The treatments with *Curcuma* C20-dialdehyde at the concentrations used in this study did not block the cell cycle progression in HeLa cells (Figure 5C and 5F). However, the increased sub-G1 fractions were observed in HeLa cells treated with the increasing concentrations of *Curcuma* C20-dialdehyde. Our results indicated that the ability of *Curcuma* C20-dialdehyde to induce cell cycle arrest is depending on the cellular model and concentrations.

Discussion

We, to the best of our knowledge, are the first who studied the anticancer effects of *Curcuma* C20-dialdehyde on cancer cell lines. Our study demonstrated that *Curcuma* C20-dialdehyde had the potential for inhibition of cancer

cell growth in vitro. The antiproliferative effect posed by *Curcuma* dialdehyde was time- and dose-dependent for both colon cancer (HCT116, HT29) and cervical cancer (HeLa) cell lines. In line with our findings, some researchers also reported antiproliferative activity of some natural compound from *Curcuma* sp. and other plants. It was reported that curcumin had potent dose-dependent antiproliferative effects in cholangiocarcinoma cells (Prakobwong et al., 2011) and colorectal carcinoma LoVo cells (Guo et al., 2013), human osteosarcoma (HOS) cells (Lee et al., 2009). Some oils of *Curcuma* species were known to confer growth inhibitory impacts on several cancer cell lines. *Curcuma* zedoaria oil had a significant inhibitory effect on the proliferation of SGC-7901 cells and could induce apoptosis (Guo SB et al., 2013). Essential oil of *Curcuma* wenyujin (CWO) was found to inhibit the growth of HepG2 cells in a dose-dependent fashion by

inducing a cell cycle arrest at S/G2 (Xiao et al., 2008). To investigate the mechanisms by which *Curcuma* C20-dialdehyde confers growth inhibitory effect in cancer cells, we assessed cell cycle phase distribution and apoptosis induction in vitro. Our results demonstrated that *Curcuma* C20-dialdehyde could induce apoptosis of all cancer cells tested in this study in a dose-dependent manner, indicating possibility of curative and preventive windows. Among the cell lines studied, HT29 was found to be more responsive in apoptosis induction to *Curcuma* C20-dialdehyde where more than 50% cells underwent apoptosis with 100 μ g/ml of the drug. Furthermore, this compound also induced the cell cycle arrest at G1 phase of cell cycle for HCT116 cells and HT29 cells while there had not been any evidence of cell cycle arrest in HeLa cells. Exposure to low level of *Curcuma* C20-dialdehyde showed a substantial delayed at G0/G1 phase in HCT116 cells where moderate concentration arrested cell cycle at the same phase in HT29 cells. Increasing percentages of sub-G1 for all three cell lines with the higher concentrations indicated more cell death, which coincided with apoptosis induction. So, it was evident from our study that concentrations and types of cell lines had played the decisive role in underlying mechanisms of growth inhibition. Variations in the extent of DNA damage and ability of damage repair mechanisms are the controlling factors for determining the fates of neoplastic cells. Whether or not *Curcuma* C20-dialdehyde will be effective in vivo, it needs further investigation. Our results for mechanism of growth inhibition were corroborated by several previous research findings. Live/Dead assay showed that curcumin significantly increased apoptotic cell death in CCA cells in a dose-dependent manner (Prakobwong et al., 2011). Guo et al. (2013) reported that curcumin induced the cell cycle arrest of LoVo cells at the S phase and apoptosis of colorectal carcinoma LoVo cells in a dose-dependent manner through a mitochondria-mediated pathway. It has also been reported that curcumin caused death of human osteosarcoma (HOS) cells by blocking cells successively in G1/S and G2/M phases and inducing apoptosis by activating the caspase-3 pathway (Lee et al., 2009). *Curcuma zedoaria* oil induced inhibition of SGC-7901 cell proliferation by retardation of G0/G1 phase (Guo et al., 2013). Extensive research over a few decades has contributed in identification of various molecular targets that can potentially be used both for the prevention and treatment of cancer. The active principles identified in fruit and vegetables modulate various cell signaling pathways pertaining to carcinogenesis (Aggarwal and Shishodia, 2006). Epidemiological studies have consistently shown that dietary intakes of phytochemicals with fruits and vegetables are strongly associated with reduced risk of developing cancers (Liu, 2004). Considering the findings of the present study and related discussions, we can envisage *Curcuma* C20-dialdehyde as chemopreventive agent and suggest the possibility of developing it as an anticancer compound for cancer prevention and treatment in the future.

Acknowledgements

This research was supported by the National Research Council of Thailand (through Khon Kaen University, Fiscal year 2014-2015) and the Faculty of Science Research Grant, Khon Kaen University, Thailand. Supattra Chaithongyot is a scholarship student under the Development and Promotion of Science and Technology Talents (DPST).

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