Evaluation of in vitro anti-proliferative and immunomodulatory activities of compounds isolated from Curcuma longa

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ABSTRACT

The rhizome of Curcuma longa (CL) has been commonly used in Asia as a potential candidate for the treatment of different diseases, including inflammatory disorders and cancers. The present study evaluated the anti-proliferative activities of the isolated compounds (three curcuminoids and two turmerones) from CL, using human cancer cell lines HepG2, MCF-7 and MDA-MB-231. The immunomodulatory activities of turmerones (α and aromatic) isolated from CL were also examined using human peripheral blood mononuclear cells (PBMC). Our results showed that the curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) and α-turmerone significantly inhibited proliferation of cancer cells in dose-dependent manner. The IC50 values of these compounds in cancer cells ranged from 11.0 to 41.8 μg/ml. α-Turmerone induced MDA-MB-231 cells to undergo apoptosis, which was confirmed by annexin-V and propidium iodide staining, and DNA fragmentation assay. The caspase cascade was activated as shown by a significant decrease of procaspases-3, -8 and -9 in α-turmerone treated cells. Both α-turmerone and aromatic-turmerone showed stimulatory effects on PBMC proliferation and cytokine production. The anti-proliferative effect of α-turmerone and immunomodulatory activities of ar-turmerone was shown for the first time. The findings revealed the potential use of CL crude extract (containing curcuminoids and volatile oil including turmerones) as chemopreventive agent.

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1. Introduction

Various species of the perennial plant Curcuma (family Zingiberaceae) are found as common ingredients in many health supplements in Asia. Curcuma longa Linn. (CL) is a spice commonly used in Indian and Chinese cuisine and traditional medicine. In Ayurvedic medicine, the rhizome of CL was suggested to be used as a stimulant, tonic, stomachic and depurative. It was also used in combinations for sprains and bruises. According to the Ayurvedic Pharmacopoeia of India, essential oil from rhizome of CL was used as a carminative, stomachic and tonic (Ministry of Health and Family Welfare, Government of India, 2001). In ancient Chinese medicine, the use of the plant Curcuma firstly appeared in “Yao Xing Lun” during Tang dynasty (A.D.618–907) and was formally recorded in the Compendium of Materia Medica (Ben Cao Gang Mu) of Ming dynasty (A.D.1590). According to the Chinese Pharmacopoeia, CL was suggested to have the functions of eliminating blood stasis, promoting the flow of “qi”, stimulating menstrual discharge and relieving pain (Chinese Pharmacopoeia Commission, 2005).

In modern pharmacological studies, CL constituents, in particular curcumin had previously been shown to possess anti-inflammatory, anti-oxidant, and chemopreventive properties. Curcumin was known to suppress NF-κB activation (Singh and Aggarwal, 1995) and inhibited COX-2 through the arachidonic acid metabolism pathway which accounted for its anti-inflammatory activities (Plummer et al., 1999). Curcumin also suppressed tumor growth by blocking signal transduction pathways in the target cells (Mori et al., 2001). Recent reports suggested that curcumin down-regulated expression of cell proliferation and anti-apoptotic and metastatic gene products (Aggarwal et al., 2006) and potentiated antitumor activity of gemcitabine in pancreatic cancer model (Kunnunakkara et al., 2007). Furthermore, curcumin has been shown to inhibit proliferation and induce apoptosis in human leukemic cell lines (Anuchapreeda et al., 2008), human breast (Ramachandran et al., 2002, 2005; Simon et al., 1998), hepatoma (Cao et al., 2006, 2007; Chan et al., 2005), pancreatic (Kunnunakkara et al., 2007; Li et al., 2004) and colon (Cheng et al., 2007) cancer cells. On the other hand, curcumin has been suggested to...
modulate the proliferation and cellular response of various immune cell types, such as T cells, B cells, macrophages, neutrophils, NK cells and dendritic cells (Jaegia and Aggarwal, 2007; Bhauik et al., 2000).

In addition to curcumin, other constituents of CL such as sesquiterpenoids, also possessed various biological activities. Turmeric oil was shown to offer protection against benzo(a)pyrene induced increase in micronuclei in human lymphocytes. It also decreased the number of micronucleated cells in oral submucous fibrosis patients (Hastak et al., 1997). Volatile oil isolated from CL had been reported to have antibacterial (Norajit et al., 2007) and antifungal activities (Roth et al., 1998), anti-proliferation in human leukemia (Shi et al., 2003) and lung cancer cells (Wang and Yang, 2005). Previous studies have shown that aromatic-turmerone isolated from CL could induce apoptosis in human leukemia cells (Aratanechemuge et al., 2002) and murine leukemia cells (Ji et al., 2004). The aromatic-turmerone also possessed anti-oxidant (Jayaprakasha et al., 2002) and antiplatelet activities (Lee, 2006).

There are only a few pharmacological studies regarding the extracts of CL and the individual curcuminoids of CL (other than curcumin) (Anuchapreeda et al., 2008; Funk et al., 2006; Deters et al., 2008). The present study aimed to isolate and characterize the bioactive components from CL using bioassay-guided fractionation and to evaluate the biological activities of such components using in vitro models. The anti-proliferative effects in human hepatoma cells and breast cancer cells, as well as the immunomodulatory activities in human peripheral blood mononuclear cells of the isolated compounds from CL prepared in our laboratory were evaluated.

2. Materials and methods

2.1. Materials

Dried rhizome of C. longa L. (CL) was purchased from a herbal supplier in Hong Kong with the source of origin in India. Organoletic, microscopic and chemical authentication were accomplished in accordance with the Chinese Pharmacopoea (State Pharmacopoeia Commission of People’s Republic of China, 2005). Authenti-
cated voucher specimen (No. HK 40400) was deposited in the Hong Kong Herbar-
ium of the Agriculture, Fisheries and Conservation Department of Hong Kong Special Administrative Region, China.

The organic solvents, dichloromethane, 95% ethanol, ethyl-acetate, n-hexane and methanol were purchased from Lab-Scan (Thailand) and were either HPLC-grade or AR grade.

2.2. Preparation of crude extract and sub-fractions

One kilogram of CL rhizome was cut into small pieces and soaked in 1.0 l of 95% (v/v) ethanol for 60 min. It was then extracted twice with 1.5 l of 95% (v/v) ethanol under reflux for 90 min. Following filtration, the ethanolic extracts were combined and centrifuged at 5250g for 10 min. About 2.0 l of supernatant was collected and evaporated under reduced pressure at 60 °C to dryness (with a yield of 7.7% w/w). The dried ethanolic extract was allowed to re-dissolve in 1.0 l of 10% (v/v) meth-

The remaining aequous layer was subjected to partition with firstly dichlorometh-
and then ethyl-acetate successively by following the same procedure to give dried fraction F2 (37.8% w/w) and F3 (15.1% w/w), respectively. The remaining aqueous layer was collected and concentrated under reduced pressure at 50 °C. The concentrated aqueous solution was subjected to lophophilation to give dried fraction 4 (F4, 11.8% w/w). The crude ethanolic extract and its fractions (F1–F4) have been tested for their inhibitory and stimulatory activities in cancer cells and peripheral blood mononuclear cells (PBMC), respectively. Fraction F1 showed the most potent stimulatory effect on PBMC proliferation while fractions F2 and F3 exhibited potent inhibitory activities in cancer cells (data not shown). Therefore, fractions F1–F3 were subjected to further fractionation.

2.3. Isolation of α-turmerone and α-turmerone from lipophilic fraction (F1)

The fraction F1 was subjected to silica gel column chromatography eluting with dichloromethane and methanol (98:2). The eluents were combined and evaporated to dryness to give fraction 1 (F1, 34.8% w/w). The concentrated aqueous solution was subjected to lyophilization to give dried fraction F1-A (34.8% w/w) which was 47% (w/w) active components from CL prepared in our laboratory were isolated and characterized from CL from CL by following the same procedure to give dried fraction F1-B (34.8% w/w). Table 1 summarizes the results of the fractionation and bioassays.

2.4. Isolation of curcumin, demethoxycurcumin and bisdemethoxycurcumin from lipophilic fractions (F2 and F3)

The fractions F2 and F3 were combined as similar constituents were present in these fractions. The combined fraction was subjected to silica gel column chromatography eluting with dichloromethane and methanol (95:5). The eluents were monitored by thin layer chromatography (TLC) and similar fractions were combined to afford five sub-fractions (F1, F1-B1, F1-B2, F1-B3 and F1-B5). Sub-fractions F1-B3 and F1-B5 were separated by preparative TLC and identified as α-turmerone (0.03% w/w) and ar-turmerone (0.027% w/w), respectively, using 1H and 13C NMR spectral analysis and mass spectrometry.

2.5. Cell preparation

2.5.1. Cell line culture

The human hepatoma cell line HepG2, human breast cancer cell lines MCF-7 and MDA-MB-231, and human skin fibroblast cell line Hs-68 were obtained from American Type Culture Collection (MD, USA). The cancer cell lines were maintained in RPMI-1640 medium, while Hs-68 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Media and supplements were obtained from Invitrogen Gibco (NY, USA). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 and those cells in the exponential growth phase were used for experiments.

2.5.2. Preparation of human peripheral blood mononuclear cells (PBMC)

Fresh human buffy coat obtained from the Hong Kong Red Cross Blood Transfu-
sion Service was diluted with phosphate-buffered saline (PBS) at a ratio of 1:1. The diluted buffy coat sample was layered on equal volume of Ficoll-Paque™ Plus solu-
tion (GE Healthcare, UK) and then centrifuged at 800g for 20 min at 18 °C. The thin white middle PBMC layer was collected and the PBMC were washed with PBS twice and centrifuged at 100g for 10 min at 18 °C. The supernatant was discarded and the PBMC were resuspended in RPMI-1640 medium plus 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cell number was counted with a hemacy-
tometer and the viability of the cells was checked by trypan blue exclusion assay. Only the isolated cells with 95% or above viability were resuspended to the target density, which was 4 x 105 cells/ml, for experiments. The PBMC were incubated at 37 °C in a humidified atmosphere of 5% CO2.

2.6. Cell viability assay

The HepG2, MCF-7, MDA-MB-231 and Hs-68 cell lines (1 x 104/well) or PBMC (4 x 104/well) were seeded in 96-well flat-bottom culture plates (Iwaki, Japan) and incubated with various concentrations (3.125–100 μg/ml) of isolated compo-
unds from CL prepared in our laboratory (as mentioned in Sections 2.3 and 2.4) for 48 h. Plain medium was added to the control wells. The tumourones were di-
luted in absolute ethanol at 100 mg/ml and stored at –20 °C. The lyophilized pow-
der of curcuminoids was dissolved in DMSO at 100 mg/ml and stored at –20 °C. For experiments, the final concentrations of the tested compounds were prepared by diluting the stock with culture medium. Control wells and drug-treated wells re-
ceived the vehicle solvent (1% v/v ethanol or 0.5% v/v DMSO).

Following the incubation of cells with the extracts, the medium was discarded and 30 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma, USA) in PBS were added to each well and the plates were further incubated for 3 h at 37 °C. For the PBMC, the plates were centrifuged at 300g for 10 min. The supernatant was then removed and 100 μl of DMSO was added to each well to dissolve the purple formazan crystals. The absorbance at a wavelength of 540 nm was measured spectrophotometrically with a BMG FLUOstarOptima micro-
plate reader (BMG LABTECH GmbH, Germany). Results were expressed as the per-
centage of MTT absorbance with respect to vehicle-treated control cells.

2.7. Cell cycle analysis

Human breast cancer cells MDA-MB-231 (3 x 105/well) were seeded at 6-well culture plates and incubated overnight. The cells were treated with α-turmerone at various concentration for 24 h. Cells were harvested after incubation, washed and fixed in 70% v/v ethanol at 4 °C overnight. Before analysis, the cells were resuspended in PBS containing propidium iodide (PI, 20 μg/ml) and RNase A (10 μg/ml) at 37 °C in the dark for 30 min. Cell cycle distribution was analyzed by
flow cytometry (Becton Dickinson FACS Canto II, USA). Data from 10,000 cells per sample were collected and Modfit LT (Verity Software House, ME, USA) was used for cell cycle and apoptotic peak modeling.

2.8. Annexin-V FITC/PI staining assay

Human breast cancer cells MDA-MB-231 (3 x 10^4/well) were treated with α-turmerone at various concentrations for 24 h. Then, the cells were collected, washed and stained with annexin-V FITC kit according to the manufacturer’s protocol (Trevigen, MD, USA). Briefly, cells were washed twice with 1× binding buffer and incubated in 100 μl labeling solution containing 1 μl annexin-V FITC conjugate and 10 μl PI in the dark for 15 min at room temperature. The fluorescence of the samples was detected by flow cytometry (Becton Dickinson FACS Canto II, USA).

2.9. DNA fragmentation assay

Human breast cancer cells MDA-MB-231 (1 x 10^6) were seeded in 100 mm culture dish and incubated for 24 h to allow attachment. Various concentrations (40–80 μg/ml) of α-turmerone or 0.04% v/v ethanol (control) were added to the dishes and incubated for 48 h. After treatment, attached and floating cells were harvested and washed twice with cold PBS. The cell pellets were then lysed with whole cell lysis buffer (2% SDS, 10% glycerol, 625 mM Tris–HCl, pH6.8) for 15 min on ice. The samples were centrifuged at 6000g for 5 min to collect the DNA-containing supernatants. The supernatant was added with 50 μl of 5% SDS and 10 μl RNase A (0.4 mg/ml) at 56°C for 90 min. After incubation, 20 μl of proteinase K (1.5 mg/ml) was added into the mixture and incubated at 56°C for further 90 min. The DNA was precipitated with 30 μl of 3 M sodium acetate and 750 μl of absolute ethanol. The resulting DNA was collected by centrifugation, rinsed with cold 75% v/v ethanol and then absolute ethanol. The DNA pellet was left to air dry and the dried DNA was dissolved in 50 μl TE buffer at 37°C. The DNA was subjected to electrophoresis through a 1.5% agarose gel containing ethidium bromide. The DNA bands were visualized under ultraviolet illumination.

2.10. Western blot analysis

Human breast cancer cells MDA-MB-231 (1 x 10^6) were seeded in 100 mm culture dish and incubated for 24 h to allow attachment. Various concentrations (40–80 μg/ml) of α-turmerone or 0.04% v/v ethanol (control) were added to the dishes and incubated for 24 or 48 h. After treatment, attached and floating cells were harvested and washed twice with PBS. The cell pellets were then lysed with whole cell extraction buffer (2% SDS, 10%, glycerol, 625 mM Tris–HCl, pH8.3) for 15 min on ice. The samples were centrifuged at 6000g for 5 min to collect the cell-free supernatant. The supernatant was collected and stored at −80°C until cytokine ELISA experiments.

The effects of turmeric extracts on the proliferation of resting and mitogen (PHA)-activated PBMC were determined by (methyl-3H)-thymidine incorporation. Following incubation of cells with the extracts for 72 h, tritiated thymidine (0.5 μCi/well; GE Healthcare, UK) was added into each well and further incubated for 6 h. Then the cells were harvested on glass fiber filters by cell harvester. Radioactivity in the filters was measured by Packard TopCount NXT™ Microplate Scintillation and Luminescence Counter (PerkinElmer Inc., USA).

For in vitro cell proliferation assay, the turmerones were dissolved in absolute ethanol. During experiment, all the drug-treated or control wells were added same concentrations of vehicle (0.5% DMSO or 1% ethanol) so that the only variable was the concentrations of test compounds. In control, cell wells without vehicle have also been set in each experiment and our data showed that the presence of vehicle (DMSO or ethanol) did not significantly change in proliferation or cytokine production of PBMC cultures (n = 8, data not shown).

2.13. Determination of human IL-2, IFN-γ and TNF-α production by PBMC

The PBMC culture supernatants were subjected to test for the concentrations of cytokines such as IL-2 and IFN-γ by enzyme-linked immunosorbent assay (ELISA; BD Pharmingen, USA). The assay was carried out according to the procedures recommended in the ELISA kit manual.

2.14. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analysis and significance, as measured by the Student’s t-test for paired samples or one way analysis of variance (ANOVA) followed by Bonferroni post-test, as appropriate, were performed using GraphPad Prism software version 3.0 (GraphPad Software, USA). In all comparisons, P < 0.05 was considered statistically significant.

3. Results

3.1. Isolation and characterization of α-turmerone, ar-turmerone, curcumin, demethoxycurcumin and bisdemethoxycurcumin from lipophilic fractions

Compounds F1-B3 and F1-B5 were characterized and identified as α-turmerone and ar-turmerone, respectively, using 1H and 13C NMR spectral analysis and mass spectrometry (Table 1a). Chemical shifts of the turmerones were in accordance with the reported values (Golding and Pombovillar, 1992; Dhingra et al., 2007). The compounds F2&3-A, -C and -E were characterized and identified as curcumin, demethoxycurcumin and bisdemethoxycurcumin, respectively, using 1H NMR spectral analysis and mass spectrometry (Table 1b). Chemical shifts of the curcuminoïds were in accordance with the reported values (Jayaprakasha et al., 2002; Park

Table 1a

<table>
<thead>
<tr>
<th>Carbon no.</th>
<th>1H NMR (DMSO)</th>
<th>13C NMR (CDCl3)</th>
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<td>α-Turmerone</td>
<td>ar-Turmerone</td>
<td>α-Turmerone</td>
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<td>1</td>
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<td>5.91 (1H, br)</td>
<td>7.28 (1H, s)</td>
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<td>3.45 (1H, m)</td>
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<td>13</td>
<td>2.03 (3H, s)</td>
<td>2.46 (3H, s)</td>
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The effects of turmeric extracts on the proliferation of resting and mitogen (PHA)-activated PBMC were determined by (methyl-3H)-thymidine incorporation. Following incubation of cells with the extracts for 72 h, tritiated thymidine (0.5 μCi/well; GE Healthcare, UK) was added into each well and further incubated for 6 h. Then the cells were harvested on glass fiber filters by cell harvester. Radioactivity in the filters was measured by Packard TopCount NXT™ Microplate Scintillation and Luminescence Counter (PerkinElmer Inc., USA).

For in vitro cell proliferation assay, the turmerones were dissolved in absolute ethanol. During experiment, all the drug-treated or control wells were added same concentrations of vehicle (0.5% DMSO or 1% ethanol) so that the only variable was the concentrations of test compounds. In control, cell wells without vehicle have also been set in each experiment and our data showed that the presence of vehicle (DMSO or ethanol) did not significantly change in proliferation or cytokine production of PBMC cultures (n = 8, data not shown).
et al., 2005; Uehara et al., 1992). Their chemical structures are shown in Fig. 1.

3.2. Comparison of inhibitory activities on cell proliferation of HepG2, MCF-7 and MDA-MB-231 cells among different curcuminoids and turmerones

Assays for proliferative response in human hepatoma cells (HepG2) and human breast cancer cells (MCF-7 and MDA-MB-231) were performed to evaluate the anti-proliferative effects of three curcuminoids isolated from CL ethanolic extracts (Fig. 2). The cells were treated with a series of concentrations of curcuminoids from 1.6 to 50.0 μg/ml for 48 h. As shown in Fig. 2, all three curcuminoids showed significant anti-proliferative activities in HepG2, MCF-7 cells and MDA-MB-231 cells in a concentration-dependent manner (Fig. 2A–C). The concentrations producing 50% growth inhibition (IC50) of the curcuminoids on the three cell lines are listed in Table 2. Among the species tested, curcumin exhibited the most potent inhibitory effect on MDA-MB-231 cells proliferation (IC50 = 11.0 μg/ml). Demethoxycurcumin and bisdemethoxycurcumin could achieve 50% proliferation inhibition with a concentration of 11.4 and 12.1 μg/ml, respectively (Table 2).

To evaluate the effects of turmerones isolated from CL on cell proliferation of cancer cells, cells were treated with a series of concentrations of turmerones from 3.125 to 100 μg/ml for 48 h. In Fig. 2, α-turmerone at concentration of 50 μg/ml caused nearly 70% inhibition of cell proliferation compared to the control in all three cell lines. The inhibitory activities of α-turmerone (12.5–100 μg/ml) were significantly lowered than those of ar-turmerone. To inhibit cell proliferation of HepG2 and MDA-MB-231 cells at 50%, the concentrations of α-turmerone were lowered than that required for MCF-7 cells (Table 2).

Table 1b

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<td>160.4</td>
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<td>10, 10'</td>
<td>7.06 (2H, dd, J = 1.8, 8.4)</td>
<td>160.4</td>
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</tbody>
</table>

Fig. 1. Chemical structures of α-turmerone, ar-turmerone, curcumin, demethoxycurcumin and bisdemethoxycurcumin.

Fig. 2. α-turmerone and ar-turmerone chemical structures.
3.3. Effects of curcuminoids and turmerones on cell viability of normal skin cells

To determine whether the inhibition of cell proliferation on these cancer cell lines was selective, human normal fibroblast (Hs-68) cells were used as a negative control. Hs-68 cells were incubated with the curcuminoids and turmerones under the same conditions as the cell viability assay of the cancer cells. The tested curcuminoids (3.125–50 \( \mu \)g/ml) induced no significant suppression on the proliferation of Hs-68 (Table 2). The maximum percentage of inhibition of cell proliferation of each tested curcuminoids (50 \( \mu \)g/ml) was ranged from 23.5% to 63.4% (\( n = 4 \)). Besides, the tested turmerones (3.125–100 \( \mu \)g/ml) did not show significant suppression on the proliferation of Hs-68 (Table 2). When compared with the cancer cells, Hs-68 cells were much less susceptible to the anti-proliferative effects of the curcuminoids and turmerones at the concentration of 50 \( \mu \)g/ml, respectively.

3.4. Presence of apoptotic cell fraction in \( \alpha \)-turmerone-treated MDA-MB-231 cells

Since the IC\(_{50}\) value of \( \alpha \)-turmerone was found to be the smallest in MDA-MB-231 cells, further experiments were performed in MDA-MB-231 cells to elucidate the mode of cell death induced by \( \alpha \)-turmerone. Cell cycle phases were detected by propidium iodide (PI) staining. As shown in Fig. 3A and B, the population of sub-G\(_1\) phase increased from 2.80% to 38.86% after 48 h of \( \alpha \)-turmerone exposure (30–45 \( \mu \)g/ml). Apoptotic cells, as judged from the appearance of a sub-G\(_1\) peak, were observed in \( \alpha \)-turmerone-treated cells. However, there was no arrest at any phase of the cell cycle after treatment. In addition, DNA agarose gel electrophoresis experiment showed that DNA fragmentation, a marker of apoptosis, was detected in \( \alpha \)-turmerone-treated MDA-MB-231 cells after 24 and 48 h (Fig. 3C). However, the pattern was not detected in the solvent control (0 \( \mu \)g/ml).

3.5. \( \alpha \)-Turmerone treatment induced phosphatidylserine externalization in MDA-MB-231 cells

To further confirm whether \( \alpha \)-turmerone caused apoptosis in MDA-MB-231 cells, annexin-V FITC/PI staining experiment was performed. The occurrence of phosphatidylserine (PS) externalization onto the cell surface is a characteristic of apoptotic cells. The results showed that the proportion of early apoptosis cells (annexin-V FITC positive and PI negative) increased with the concentrations of \( \alpha \)-turmerone applied (Fig. 4).

3.6. \( \alpha \)-Turmerone induced apoptosis via activation of caspase-dependent pathway

For elucidating the molecular mechanisms of action of \( \alpha \)-turmerone in MDA-MB-231 cells, the apoptosis signal transduction was investigated. Western blotting (Fig. 5) showed that \( \alpha \)-turmerone significantly decreased the expression of cyclin D1, which is involved in cancer cell proliferation. Caspases play a crucial role in initiation and execution of apoptosis (Nunez et al., 1998). Caspases-8 and -9 are known as initiator caspases, whereas caspase-3 is considered as executioner caspase (Budihardjo et al., 1999). Two
distinct and well-known initiator caspases, caspase-8 for the death receptor-mediated and caspase-9 for the mitochondria-mediated pathways, have been shown to initiate apoptosis. Therefore, the expression levels of caspases-3, -8 and -9 as well as PARP, the substrate of caspase-3, were detected by Western blot analysis. As shown in Fig. 5, α-turmerone treatment significantly decreased the expression levels of procaspases-3 and -8. Proteolytic cleavage of mitochondria-mediated apoptotic cell death initiator procas-
Pase-9 was observed in α-turmerone-treated MDA-MB-231 cells and the smaller fragments p35 and p37 were shown. Cleaved PARP was also observed in α-turmerone-treated MDA-MB-231 cells.

3.7. α-Turmerone caused mitochondrial transmembrane depolarization in MDA-MB-231 cells and altered expression of Bcl-2 protein

The mitochondria membrane potential change (ΔΨm) was measured by JC-1 staining. Mitochondria with normal ΔΨm concentrated JC-1 into aggregates (red fluorescence), while in depolarized mitochondria, JC-1 formed monomer (green fluorescence). The ΔΨm in cells treated with α-turmerone (25–45 μg/ml) for 24 h was decreased in a concentration-dependent manner. The percentage of depolarization cells was increased from 22.6% to 92.2% (Fig. 6). On the other hand, the Bcl-2 protein expression level in α-turmerone-treated cells was investigated. As shown in Fig. 5, treatment of MDA-MB-231 cells with α-turmerone slightly down-regulated Bcl-2 expression.

3.8. Effects of α-turmerone and ar-turmerone on cell proliferation and cytokine production of PBMC

Different concentrations of α-turmerone (AL) and ar-turmerone (AR) were tested in cultured PBMC to examine their effects on the proliferation of PBMC. The turmerones were added to PBMC with polymyxin B in order to rule out the possible contamination by the endotoxin. As shown in Fig. 7, α-turmerone (AL, 10 or 15 μg/ml) and ar-turmerone (AR, 5–15 μg/ml) were found to significantly stimulate the proliferation of PHA-activated PBMC after 72 h treatment. However, turmerones added at 5–15 μg/ml did not alter the proliferative response of resting PBMC (without mitogen).

Fig. 5. Effects of α-turmerone on cyclin D1, Bcl-2, PARP, procaspase-3, -8 and -9 expression levels in MDA-MB-231 cells. Total proteins of α-turmerone-treated (40 or 60 μg/ml) cells was isolated for testing the expression by Western blotting. All results are representative of three independent experiments.

Fig. 6. α-Turmerone caused mitochondrial transmembrane depolarization in MDA-MB-231 cells. Cells were stained with JC-1 fluorescence dye and changes in ΔΨm were determined by flow cytometry using green (FITC) and red (PI) channels. All results are representative of three independent experiments.
not shown). When ar-turmerone was added to PHA-activated PBMC at 10 μg/ml, the resulting proliferation responses were slightly higher than those stimulated by α-turmerone (Fig. 7A). After incubating the PBMC with α-turmerone (AL) and ar-turmerone (AR), the culture supernatants were collected. The concentrations of TNF-α, IL-2 and IFN-γ were determined by ELISA. After 24 h stimulation in PHA-activated PBMC, the TNF-α production was significantly increased by α-turmerone (AL) and ar-turmerone (AR) at 10 and 15 μg/ml (Fig. 7C). The productions of IL-2 and IFN-γ in PHA-activated PBMC were higher than those in resting PBMC (IL-2: 5867.2 ± 3185.4 vs 103.4 ± 81.2 pg/ml; IFN-γ: 6158.2 ± 1954.1 vs 325.7 ± 135.1 pg/ml). However, neither α-turmerone (AL) nor ar-turmerone (AR) further altered the productions of IL-2 and IFN-γ in PHA-activated PBMC (data not shown). On the other hand, in resting PBMC, ar-turmerone (AR) significantly increased TNF-α, IL-2 and IFN-γ productions (Fig. 7B, D and E).

4. Discussion

The use of traditional medicine has expanded and health supplement consist of different types of herbal medicines have become very popular in Asia in recent years. C. longa (CL) and its extracts, curcumin, are widely consumed as food additive and medicine, which are believed to possess anti-inflammatory, antioxidant, anti-cancer and immunomodulatory properties. However, scientific investigations to compare their pharmacological effects are seldom reported. In the present study, the anti-proliferative activities on human cancer cells and immunomodulating activities in human PBMC of the CL extracts were evaluated in vitro. All the extract fractions and isolated compounds were prepared by bioassay-guided fractionation process.

The present study demonstrated that the curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) and
α-turmerone isolated from CL lipophilic fraction significantly inhibited proliferation of cancer cells in a dose-dependent manner, with curcumin being the most potent. Dose–response curves were obtained and demonstrated IC_{50} values of the curcuminoids ranged from 11.0 to 28.2 μg/ml in three cancer cell lines. On the contrary, the tested compounds (demethoxycurcumin, bisdemethoxycurcumin, α-turmerone and ar-turmerone, 3.125–25 μg/ml) did not induce any significant inhibition of cell proliferation in human normal skin fibroblasts, illustrating their selective cytotoxic effects on cancer cell lines (Table 2).

The mechanisms of action of the cytotoxic effects of curcumin in hepatoma cells (Cao et al., 2006, 2007) and breast cancer cells (Ramachandran et al., 2002, 2005; Mehta et al., 1997; Shao et al., 2002) have been demonstrated. They suggested that curcumin induced mitochondrial and nuclear DNA damage in HepG2 cells and apoptosis in MCF-7 and MDA-MB-231 cells. These results were consistent with our present findings that all three tested curcuminoids significantly inhibited proliferation of HepG2, MCF-7 and MDA-MB-231 cells in a dose-dependent manner (Fig. 2). The anti-inflammatory activities (Funk et al., 2006; Lantz et al., 2005) and inhibitory activities on WT1 gene expression in leukemic cells (Anuchapreeda et al., 2008) of demethoxycurcumin and bisdemethoxycurcumin have been reported and compared with the activities of curcumin. Their results suggested that curcumin showed higher efficacy. However, a recent study demonstrated that demethoxycurcumin and bisdemethoxycurcumin showed higher antimitastasis potency than curcumin by the differentially regulated mitochondrial apoptosis (Ramachandran et al., 2002, 2005; Mehta et al., 1997; Shao et al., 2002). Along with these results, α-turmerone and ar-turmerone showed stimulating activities in PBMC. Therefore, the response of PBMC may be specific to such turmerones instead of other contaminants. On the other hand, the compounds curcumin, demethoxycurcumin and bisdemethoxycurcumin, which were isolated from F2 and F3, have not been tested in PBMC because fraction F2 and F3 showed neither stimulatory nor inhibitory effect on PBMC proliferation (data not shown). Moreover, a previous study has demonstrated that in fact curcumin inhibited PHA-induced proliferation and IL-2 production in PBMC (Yadav et al., 2005).

In conclusion, bioassay-guided fractionation of the rhizome of C. longa led to the isolation of the curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin), α-turmerone and ar-turmerone. Curcuminoids and α-turmerone significantly inhibited proliferation of cancer cells in a dose-dependent manner. Both α-turmerone and ar-turmerone stimulated PBMC proliferation and cytokine production in vitro. This is possibly the first report on the anti-proliferative effect of α-turmerone and immunomodulatory activity exerted by ar-turmerone. These findings revealed the potential use of C. longa extracts (including curcuminoids and turmerones) could be illustrated in the present study.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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