RESEARCH COMMUNICATION

Suppressive Effect of Maslinic Acid on PMA-induced Protein Kinase C in Human B-Lymphoblastoid Cells

Lim Yang Mooi[1]*, Wong Teck Yew[1], Yap Wei Hsum[2], Khoo Kong Soo[2], Lim Saw Hoon[3], Yeo Chew Chieng[4]

Abstract

Protein kinase C (PKC) has been implicated in carcinogenesis and displays variable expression profiles during cancer progression. Studies of dietary phytochemicals on cancer signalling pathway regulation have been conducted to search for potent signalling regulatory agents. The present study was designed to evaluate any suppressive effect of maslinic acid on PKC expression in human B-lymphoblastoid cells (Raji cells), and to identify the PKC isoforms expressed. Effects of maslinic acid on PKC activity were determined using a PepTag® assay for non-radioactive detection of PKC. The highest expression in Raji cells was obtained at 20 nM PMA induced for 6 hours. Suppressive effects of maslinic acid were compared with those of four PKC inhibitors (H-7, rottlerin, sphingosine, staurosporine) and two triterpenes (oleanolic acid and ursolic acid). The IC_{50} values achieved for maslinic acid, staurosporine, H-7, sphingosine, rottlerin, ursolic acid and oleanolic acid were 11.52, 0.011, 0.767, 2.45, 5.46, 27.93 and 39.29 μM, respectively. Four PKC isoforms, PKC βI, βII, δ, and ζ, were identified in Raji cells via western blotting. Maslinic acid suppressed the expression of PKC βI, δ, and ζ in a concentration-dependent manner. These preliminary results suggest promising suppressive effects of maslinic acid on PKC activity in Raji cells. Maslinic acid could be a potent cancer chemopreventive agent that may be involved in regulating many downstream signalling pathways that are activated through PKC receptors.

Keywords: Protein kinase C - maslinic acid - Raji cell s- PMA - cancer chemoprevention

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Introduction

The incidence and mortality rates of cancer have increased yearly and the four most frequent cancers are lung, breast, colorectal and stomach cancers (Ferlay et al., 2004). New treatments that can deliver drugs more specifically and produce less toxicity to normal cells are more desired for current cancer treatments. Herbal medicines are used when contemporary treatment fails to cure cancer. Nelson (2007) reported that more than 3,000 different plant species have been used to treat cancer worldwide.

Multiple sequential mutations acquired during carcinogenesis are required to convert a normal cell into a malignant cell (Tannock et al., 2005). Carcinogenesis is progressive, and this progression in pre-malignancy is characterised by the appearance of specific molecular and more general genotypic damage associated with increasingly severe dysplastic phenotypes (Kelloff et al., 1999a). Therefore, understanding of carcinogenesis and chemopreventive mechanisms could provide a strong base for chemoprevention studies, and for the design and development of clinical investigations. Chemoprevention aims to inhibit neoplastic development prior to or during the preneoplastic period through pharmacological, nutritional, or endocrinological interventions (Malone et al., 1992; Greenwald & Kelloff, 1996). Cancer chemopreventive agents can act as inhibitors of carcinogen formation, blocking (anti-initiation) agents, and suppressing (anti-proliferation/anti-progression) agents (Boone et al., 1990; Morse & Stoner, 1993; Kelloff, 1999b). National Cancer Institute Chemoprevention Programme has evaluated more than 1000 potential chemopreventive agents or agent combinations (Kelloff et al., 1996). These agents have been studied in Phase I, II, III and IV clinical trials for safety, pharmacokinetics and pharmacodynamics evaluations (Kelloff et al., 1997).

In a typical cancer cell, many oncogenes are overexpressed and involved in many biochemical reactions to promote tumour development; these biochemical reactions which include protein kinase activities and protein-DNA binding activities are functionally transmit signals through multiple signalling networks for cell proliferation, differentiation and survival (Savelyeva & Schwab, 2001; Chang et al., 2009). Protein Kinase C (PKC) plays essential roles in multiple cellular

*[1]Department of Pre-clinical Science, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, Selangor, [2]Faculty of Science, Jalan Universiti, Kampar, Perak, [4]Faculty of Agriculture and Biotechnology, Universiti Sultan Zainal Abidin, Kuala Terengganu, Malaysia, [3]School of Biological Sciences, Faculty of Science, Monash University Victoria, Australia *For correspondence: ymlim@utar.edu.my*

signal transduction pathways of eukaryotic organisms. In cancer cells, PKC has been known to play its vital role in tumour development and maintenance of malignant phenotype. The distinctive function of each PKC isoform and their coordinated actions are the important factors responsible for the aggressiveness of cancers. Hence, Protein Kinase C (PKC) is regarded as the prime target for cancer chemoprevention (Kanashiro & Khalil, 1998; Dempsey et al., 2000; Barry & Kazanietz, 2001; Ventura & Maioli, 2001; Griner & Kazanietz, 2007). This study was therefore designed to evaluate the inhibitory effect of maslinic acid, a novel natural triterpene on protein kinase C activity. Four PKC inhibitors, H-7, rottlerin, sphingosine and staurosporine and two other triterpenes, oleanolic acid and ursolic acid were employed to benchmark the suppressive activity of maslinic acid. Western blot method was performed to identify the PKC isoforms suppressed by Maslinic acid in Raji cells.

Materials and Methods

Chemicals

Sphingosine, staurosporine, H-7, ursolic acid and oleanolic acid were obtained from Sigma. Rottlerin was purchased from Calbiochem. Maslinic acid was isolated from the tubers of Coleus tuberosus Benth (Lamiaceae) (Lim et al., 2010). The compound used is a chemically pure white powder (> 95 % pure as determined by HPLC) and is stable when stored at 4 ºC. A stock solution of 10 mg/mL was stored at -20 ºC. This solution was diluted in cell culture medium for use in experiments.

Cell culture

Raji cells obtained from Riken Cell Bank, Japan were maintained in commercial Roswell Park Memorial Institute Media (RPMI 1640) supplemented with 10% heat-activated fetal bovine serum (GIBCO, South America). The cells were incubated in a humidified atmosphere at 37 °C with 5% carbon dioxide (CO_2) incubator (Sanyo, Japan).

Protein Kinase C Assay

PepTag® Assay for Non-Radioactive Detection of Protein Kinase C from Promega was used to determine PKC activity. Approximately 1×10^7 cells were washed with phosphate-buffered saline (Sigma), and then suspended in 0.5 ml of cold PKC extraction buffer, and homogenized. The lysate was centrifuged for 5 minutes at 4 °C, 14,000 × g and the supernatant (extracted protein kinase C) was collected. The PKC sample was then incubated at 30 °C for 30 minutes. A 0.8 % agarose solution in 50 mM Tris-HCl (pH 8.0) was prepared. Gel was run at 100 V for 15 minutes. Once electrophoresis was completed, the gel was removed from the chamber and photographed under chemiluminescence in gel imager (AlphaInnotech FluorChem® FC2 with AlphaEase® Fc Software). The bands of interest were then cut out and heated at 95 °C until the gel slice is melted. 125 µl of the hot agarose was transferred to a tube containing 75 µl of Gel Solubilization Solution and 50 µl of glacial acetic acid. A total of 250 µl sample was quickly vortex and transferred to a well in a 96-well plate. The absorbance was read at 570 nm with microplate reader (Tecan Infinite® 200 NanoQuant with Magellan® software). PKC activity was calculated based on the Beer's law.

Western Blotting

Ten μl of each protein sample and 4 μl of Kaleidoscope Prestained Standards (Bio-Rad) were loaded on Acrylamide/Bisacrylamide gels (12% stacking gel) and the separated proteins were then transferred onto an Immobilon PVDF (poly-vinylidene-difluoride) membrane. The membrane was then probed with monoclonal or polyclonal antibodies against various isoforms of PKC (Santa Cruz Biotechnology, USA) at a dilution of 1:500 (for PKCδ), 1:1000 (for PKCα, βI, βII, η and ε) and 1:20000 (for PKC ζ). The enzyme-coupled secondary antibody (affinity purified mouse or rabbit anti-human IgG horseradish peroxidase conjugate from Cell Signalling Technology) was used at a dilution ratio of 1:10000 and detected using the Immobilon Western Chemiluminescent HRP Substrate from Millipore.

Statistical analysis

The results were obtained from 3 separate experiments in 3 replicates. In all experiments, mean values ± standard deviations (SD) were calculated.

Results and Discussion

Tumour promoter phorbol 12-myristate 13-acetate (PMA) is known to induce PKC expression. PMA bind to the DAG-binding site of PKC with high affinity and promote activation of PKC. Optimum expression of PKC was determined by treating cells with different concentrations of PMA and incubation times. As shown in Figures 1 and 2, the highest PKC levels in Raji cells were induced (2.77 fold expression and 175.21 % activity) by 20 nM of PMA and the expression reached a maximum response at 6 h (2.74 fold expression and 221.86 % activity) treatment. This transient PKC activation pattern is consistent with other studies reporting that higher PMA concentrations and prolonged exposure to PMA could cause PKC depletion (Butler et al., 1991; Chang et al., 2002). Similar findings showed that highest PKC induction in B cells was obtained with 20 nM PMA and 6 hours incubation time (Kwon et al., 2006).

The suppressive effect of maslinic acid on PMA-induced PKC expression was evaluated in Raji cells. Raji cells were pre-treated with various concentrations of maslinic acid for 1 h before incubating with 20 nM PMA for another 6 h. The effect of maslinic acid was compared to four standard PKC inhibitors, including H-7, rottlerin, sphingosine, and staurosporine as well as two natural triterpenoids, oleanolic acid and ursolic acid. Staurosporine exhibited the strongest suppressive effect, followed by H-7, sphingosine, and rottlerin. Their IC_{50} were determined as 0.011, 0.77, 2.45, and 5.46 µM, respectively (Figure 3). In comparison, the effect of maslinic acid (IC_{50} = 11.52 µM) was weaker than the standard PKC inhibitors but stronger compared to ursolic acid (IC_{50} = 29.73 µM) and oleanolic acid (IC_{50}

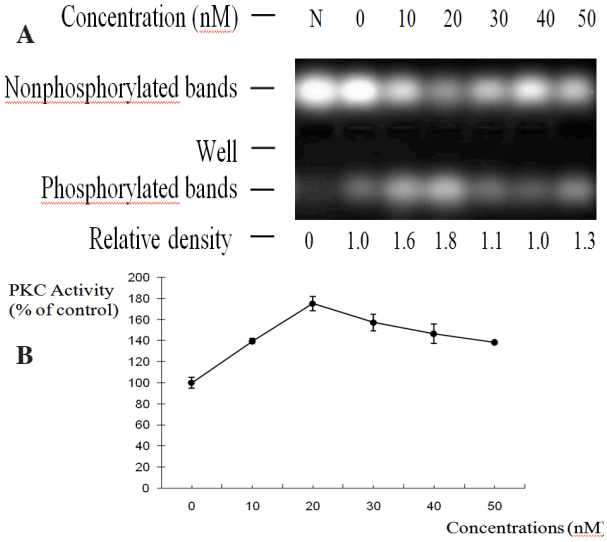

Figure 1. PKC Expression in Raji Cells Induced with Different Concentrations of PMA. Raji cells were treated with 10, 20, 30, 40, and 50 nM PMA for 6 h. PKC activities were determined using the PepTag® Assay for Non-Radioactive Detection of PKC from Promega. PKC lysates extracted from PMA-treated cells were subjected to agarose gel electrophoresis. (A) Phosphorylated PKC bands were excised, solubilized and their absorbance were read at 570 nm to determine the PKC activities. The (B) PKC activities obtained were plotted against different PMA concentrations. Each data point represents average ± SD of three independent experiments

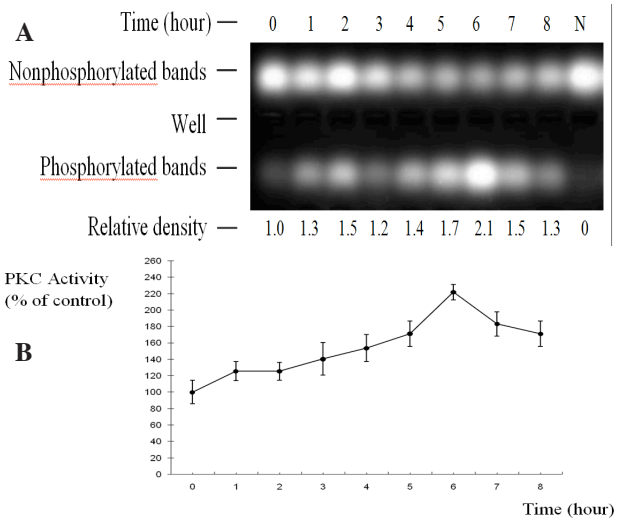

Figure 2. Time Course Study of PKC Expression in Raji Cells Induced with 20 nM PMA. Raji cells were treated with 20 nM PMA for 1, 2, 3, 4, 5, 6, 7, and 8 h. PKC activities were determined using the PepTag® Assay for Non-Radioactive Detection of PKC from Promega. PKC lysates extracted from PMA-treated cells were subjected to agarose gel electrophoresis. (A) Phosphorylated PKC bands were excised, solubilized and their absorbance were read at 570 nm to determine the PKC activities. The (B) PKC activities obtained were plotted against different incubation time points. Each data point represents average ± SD of three independent experiments

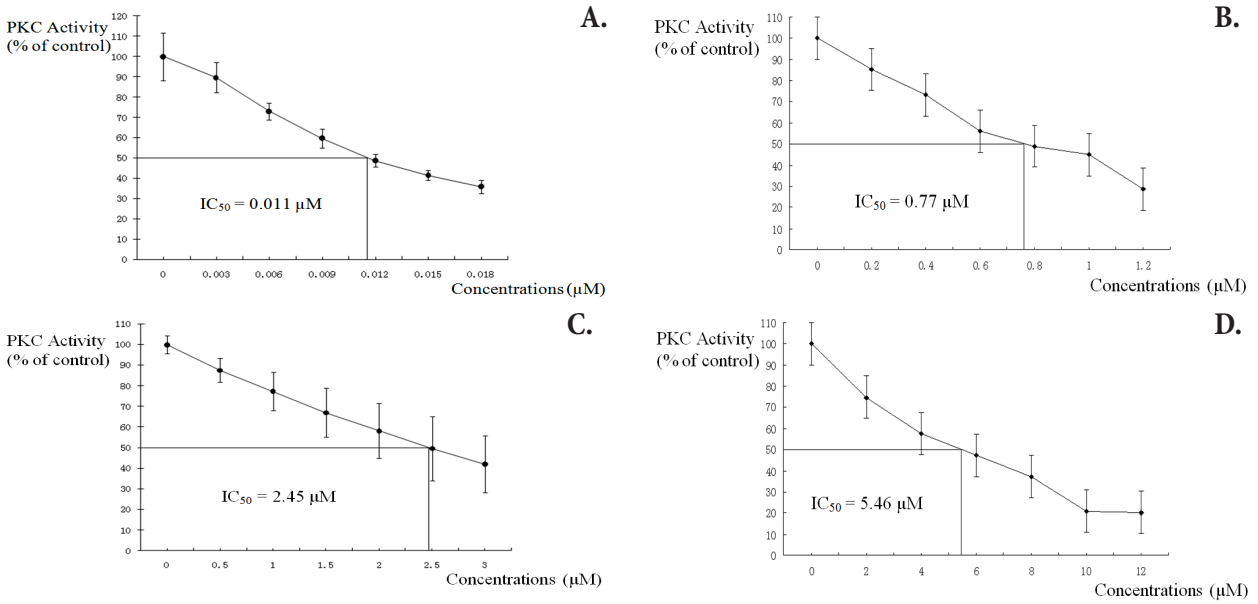

Figure 3. The Suppressive Effects of Standard PKC Inhibitors (A) Staurosporine, (B) H-7, (C) Sphingosine and (D) Rottlerin on PKC Activity in Raji Cells. PKC activities were determined using the PepTag® Assay for Non-Radioactive Detection of PKC from Promega. The PKC activities obtained were plotted against the sample concentrations. Each data point represents average ± SD of three independent experiments

= 39.29 µM) (Figure 4). Studies of plant-derived natural compounds have shown that triterpenoids such as ursolic acid, oleanolic acid and betulin are weak PKC inhibitors (Wang & Polya, 1996). This study is the first to show that maslinic acid has potential suppressive effect on PKC activity and it is more potent compared to ursolic acid and oleanolic acid.

The PKC isoforms targeted by maslinic acid was further investigated by Western blotting. The PKC family consists of at least 12 serine-threonine kinases which are classified into three major groups: classical (α, β and γ), novel (δ, ε, η, and θ) and atypical (μ, ζ, and ι). According to Brick-Ghannam et al. (1994) and Morrow et al. (1999), B cells express the cPKC isoforms of α, βI and βII, nPKC isoforms of δ, ε and η, and aPKC of isoform ζ. Thus, seven PKC antibodies were used to determine the types of PKC isoforms expressed in Raji cells. As shown in Figure 5, PKC βI, βII, δ and ζ were expressed in PMA-induced

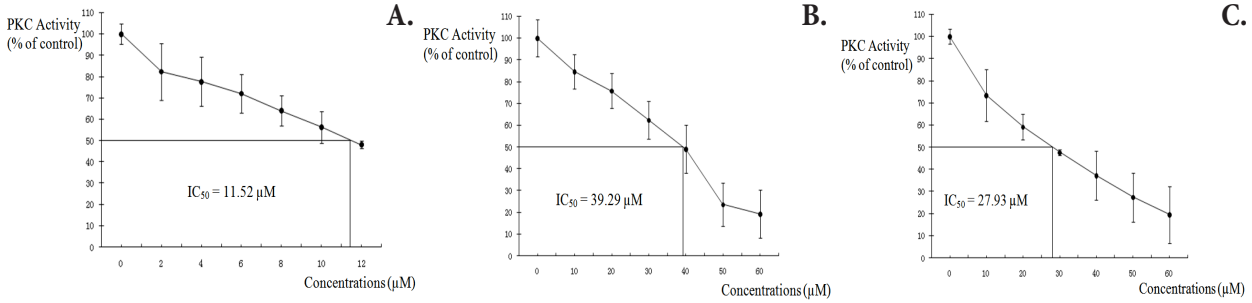

Figure 4. The Suppressive Effects of (A) Maslinic Acid, (B) Oleanolic Acid, and (C) Ursolic Acid on PKC Activity in Raji Cells. PKC activities were determined using the PepTag® Assay for Non-Radioactive Detection of PKC from Promega. The PKC activities obtained were plotted against the sample concentrations. Each data point represents average ± SD of three independent experiments

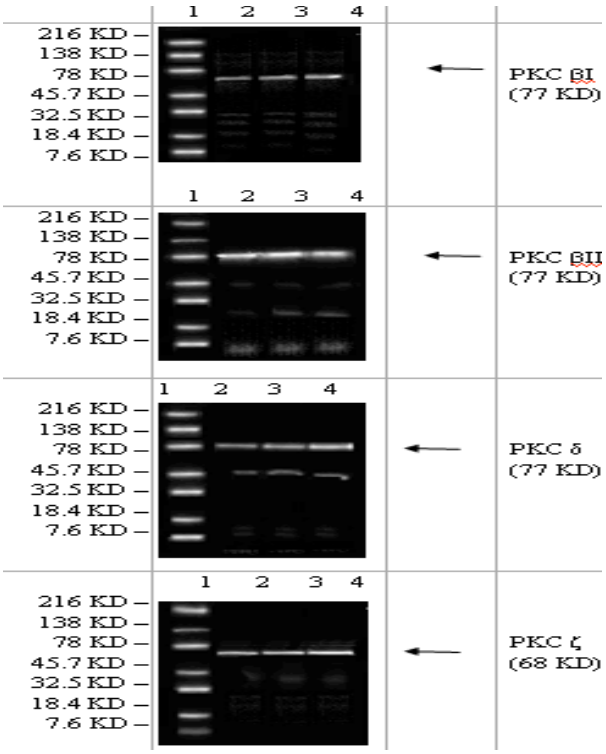

Figure 5. Expression of PKC βI, βII, δ and ζ in PMA-induced Raji cells. Raji cells were treated with 20 nM PMA for 6 h. Total cell lysates were analyzed for PKC isoform expressions by immunoblotting. Three independent experiments were carried out. Essentially identical results were obtained in three independent experiments

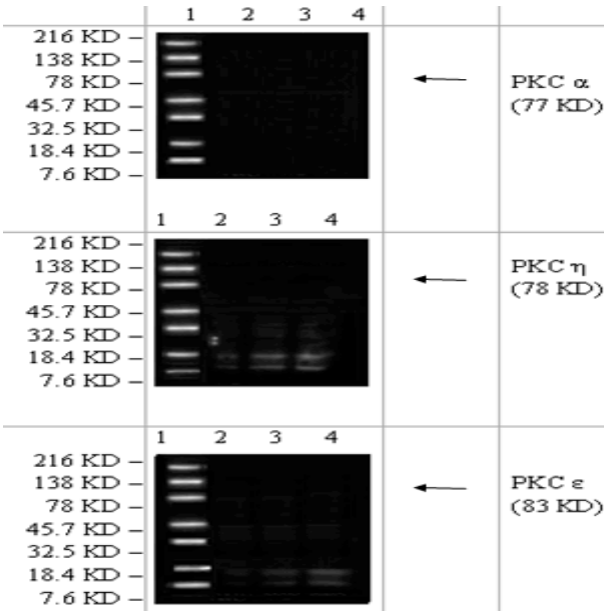

Figure 6. Immunoblot showing the effect of PMA (20 nM) on the expression of PKC α, η and ε in Raji cells. There are no corresponding PKC isoforms bands were detected. Three independent experiments were carried out. Essentially identical results were obtained in three independent experiments

Raji cells. Meanwhile, expression of PKC α, η, and ε were not detected in PMA-induced Raji cells (Figure 6). The suppressive effect of maslinic acid on PKC βI, βII, δ and ζ was determined. The results showed that maslinic acid suppressed PKC βI, PKC δ, and PKC ζ expression in a concentration-dependent manner but did not affect PKC βII (Figure 7). At 20 μM concentration, PKC βI expression was almost undetectable.

Maslinic acid is a potential anti-tumour agent which has been shown to inhibit growth and induce apoptosis in several tumour cell lines (Kim et al., 2000; Reyes-Zurita et al., 2006). There are also studies reporting that maslinic acid suppress pro-inflammatory cytokines production (Martin et al., 2006) and regulate inflammatory gene expression (Guillen et al., 2009). Focusing on the distinct targets of maslinic acid upstream in the protein kinase signaling may help to further elucidate its molecular mechanism. Our study investigates the suppressive effect of maslinic acid on PKC expression in Raji cells.

In this study, PMA was used to induce the activation of PKC in Raji cells. Highest PKC activation was induced by 20 nM of PMA for 6 h. Pre-treatment of Raji cells with maslinic acid suppressed PMA-induced PKC activity in a concentration-dependent manner, with IC_{50} value of 11.52 μM. Western blotting study showed that maslinic acid inhibited activities of PKC βI, δ, and ζ isoforms. Since PMA is a tumour promoter which stimulates cell proliferation, alteration of cell morphology and enhancing cell transformation (Ruddon, 2007), the suppressive effect of maslinic acid on PMA-induced PKC activation is likely to contribute to its anti-tumour-promoting effects in Raji cells.

One of the possible molecular mechanisms for inhibition of PKC activities may be due to the interference of PKC membrane translocation. Studies have shown that PMA substitute its binding to PKC by increasing the affinity of PKC to Ca^{2+} and phosphatidylserine, thereby causing the translocation of PKC from cytosol to plasma membrane and its activation (Nishizuka, 1984; Sharkey et al., 1984). Blocking PMA from intercalating into

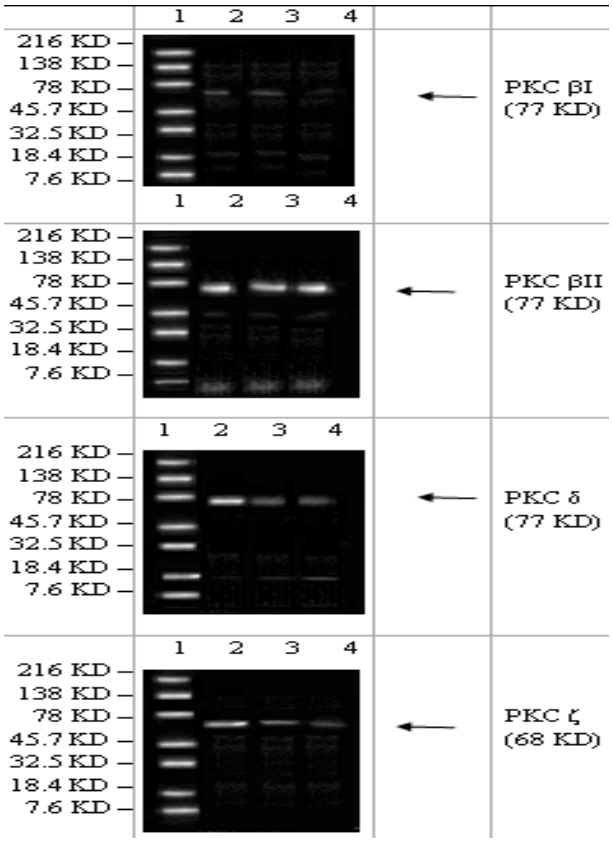

Figure 7. The Suppressive Effect of Maslinic Acid on PKC βI, βII, δ and ζ in Raji cells. Raji cells were Treated with 5 μM, (lane 2), 10 μM (lane 3) and 20 μM (lane 4) of maslinic acid for 1 h before incubating with 20 nM PMA for another 6 h. Total cell lysates were analyzed for PKC isoform expressions by immunoblotting. Three independent experiments were carried out. Essentially identical results were obtained in three independent experiments

the membrane to activate PKC may inhibit its activity. Pentacyclic triterpenes are structurally similar to sterols and interaction with the cell membrane is thought to be the molecular mechanism underlying their biological effects. It has been suggested that maslinic acid binds to transmembrane domains and competes with cholesterol (Cho) for the hydrogen-bonded ester carbonyl groups, thereby disturbing the localization and the physiological function of membrane-related proteins (Prades et al., 2011).

Inhibition of PKC activity by maslinic acid may explain the regulation of downstream targets in the signaling cascade. For instance, ursolic acid from the ursane group suppresses COX-2 protein via inhibition of PKC, ERK1/2, JNK and p38 MAPKs activation in PMA-treated human mammary epithelial cells (Subbaramaiah et al., 2000). Lupeol, a lupane-type triterpenoid, has been found to significantly reduce the expression of (PKCα)/ODC, PI3K/Akt and MAPKs pathways along with a significant reduction in the activation of IKKα, and degradation of IκBα, thereby inhibiting NF-κB activity (Chaturvedi et al., 2008). Since maslinic acid suppresses NF-κB and downstream gene expression such as COX-2, VEGF, cyclin D1 and MMP-9 (Li et al., 2010; Hsum et al., 2011), it is possible that the suppression might be achieved, at least in part, via inhibition of the PKC signaling pathway.

Maslinic acid showed marked inhibition activities on PKC βI, δ, and ζ isoforms. PKC βI expression was almost completely suppressed by 20 μM maslinic acid. The result suggests that maslinic acid have remarkable selectivity towards PKC βI suppression. Other natural triterpenoids such as α-amyrin have been reported to inhibit PMA-induced mouse skin inflammation through suppressing PKCα (Medeiros et al., 2007). Celastrol, a quinine methide triterpene, inhibited epithelial mesenchymal transition through inhibition of PKCα, PKCδ, and Rac1 in antigen-stimulated RBL2H3 cells (Kim et al., 2009). The study of isoform-selective PKC inhibitors can be used to elucidate the physiological and pathophysiological roles of individual PKC isoforms.

Since PKC is related to the tumour development, inhibition of PKC may lead to inhibition of cells growth and spreading of cancer cells. This study suggests that maslinic acid could be a potent chemopreventive agent, which acts as PKC inhibitor. However, underlying mechanism of action of maslinic acid on PKC inhibition should be further explored. Investigation on the binding interaction between PKC and maslinic acid should be carried out to study its mechanism of action on cell membranes. In addition, the effects of maslinic acid on PKC isoform localization should be investigated.

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