RESEARCH ARTICLE

Glaucocalyxin A Activates FasL and Induces Apoptosis Through Activation of the JNK Pathway in Human Breast Cancer Cells

Mei Li[1,2], Xiao-Gang Jiang[2], Zhen-Lun Gu[3]*, Zu-Bin Zhang[2,4]*

Abstract

This study was conducted to analyze the molecular mechanisms responsible for anti-proliferation effects of glaucocalyxin A in cultured MCF-7 and Hs578T breast cancer cells. The concentration that reduced cell viability to 50% (IC50) after 72 h treatment was derived and potential molecular mechanisms of anti-proliferation using the IC50 were investigated as changes in cell cycle arrest and apoptosis. Gene and protein expression changes related to apoptosis were investigated by semi-quantitative RT-PCR and western blotting, respectively. Involvement of phosphorylated mitogen-activated protein kinases and JNK signaling in regulation of these molecules was characterized by western blotting. Cell viability decreased in a concentration-dependent manner and the IC50 was determined as 1 μM in MCF-7 and 4 μM in Hs578T cell. Subsequently, we demonstrated that the GLA-induced MCF-7 and Hst578T cell death was due to cell cycle arrest at the G2/M transition and was associated with activation of the c-jun N-terminal kinase (JNK) pathway. We conclude that GLA has the potential to inhibit the proliferation of human breast cancer cells through the JNK pathway and suggest its application forthe effective therapy for patients with breast cancer.

Keywords: Glaucocalyxin A - cell arrest - cell death - human breast cancer - FasL - JNK pathway

Asian Pac J Cancer Prev, **14 (10)**, 5805-5810

Introduction

Breast cancer is the most common malignant tumor in women in recent years. It is estimated that more than one million new cases of breast cancer are diagnosed every year in the world, and that about half of these patients would die of this disease. Estrogen therapy is frequently used in the early-stage of breast cancer (Jemal et al., 2011). However, because of the drug resistance, breast cancer is highly resistant to estrogen as the pathology progresses (Miyoshi et al., 2010). Radiotherapy is also used after breast-conserving surgery to decrease the chance of recurrence (Veronesi et al., 2010). Nevertheless, novel therapies and chemo-therapeutic drugs are urgently needed to be developed for the treatment of breast cancer.

Apoptosis, which is characterized by cytoplasmic shrinkage, chromatin condensation and DNA fragmentation, is an active form of cell death that occurs in response to several agents, including anticancer chemotherapeutic drugs (Lawen et al., 2003). Many biomarkers and events, such as the caspase family proteins and Bcl-2 family members, could be considered as the determinants of apoptosis (Antonsson et al., 2000). The abnormal production of the molecule may trigger redox signaling pathways, such as oxidative stress, cell cycle arrest and apoptosis (Zhang et al., 2013).

Natural products have been considered as major sources of chemotherapeutic drugs in the treatment of breast cancer (Bishayee et al., 2011). Rabdosia japonica (R. japonica) is a perennial herb that is distributed widely in East Asia, and the entire R. japonica extract has been used traditionally as a folk medicine for treating gastrointestinal disorders, tumors, and inflammatory diseases (Sun et al., 2006; Hong et al., 2008; Kim et al., 2013). In this study, the anti-cancer effect of Glaucocalyxin A (GLA) and the underlying mechanisms involved were investigated in breast cancer cells.

The c-Jun N-terminal kinases (JNKs) are members of the mitogen activated protein kinase (MAPK) family that regulate multiple cellular processes, including development, differentiation, proliferation and apoptosis, in response to extracellular signals, metabolic status and environmental cues (Weston et al., 2002). The kinases are encoded by three distinct genes; JNK1 and JNK2 are ubiquitously expressed, while JNK3 is present primarily in the brain (Nakano et al., 2006). In general, the JNK and p38 cascades are activated by chemicals and environmental stress and are usually involved in cell growth and apoptosis.

JNKs have been proposed to function both as tumor suppressors and mediators of many malignant cell proliferation, survival and resistance to chemotherapy,

*[1]Department of Pharmacology and Laboratory of Aging and Nervous Diseases, School of Pharmaceutical Science, [2]Department of Pharmacology, Medical College, [4]Cyrus Tang Hematology Center, Soochow University, [3]Suzhou Institute of Chinese Materia Medica, Suzhou, Jiangsu, China *For correspondence: zhenlungu.2003@163.com, zubinzhang.2008@163.com*

Table 1. The List of Primers Used in RT-PCR

Genes	Primers (5'-3')	
	Sense primer	Antisense primer
Bcl-2	GGATTGTGGCCTTCTTTGAG	CCAAACTGAGCAGAGTCTTC
p53	GAAGACCCAGGTCCAGATGA	GGTAGGTTTTCTGGGAAGGG
Caspase 3	TGGAATTGATGCGTGATGTT	GGCAGGCCTGAATAATGAAA
c-fos	AAGGAGAATCCGAAGGGAAAGGAATAAGATGGCT	AGACGAAGGAAGACGTGTAAGCAGTGCAGCT
c-jun	GCATGAGGAACCGCATCGCTGCCTCCAAGT	GCGACCAAGTCCTTCCCACTCGTGCACACT
Fas	ATGCTGGGCATCTGGACCCT	TCTAGACCAAGCTTTGGATTTC
FasL	CCTCCAGGCACAGTTCTTCC	ATCTGGCTGGTAGACTCTCG
COX-2	TTCAAATGAGATTGTGGGAAAAT	AGATCATCTCTGCCTGAGTATCTT
hGAPDH	TCCTCTGACTTCAACAGCGACACC	TCTCTCTTCCTCTTGTGCTCTTGG

such as glioma (Wagner et al., 2009), breast cancer (Lee et al., 2013). However, the functional role of JNK in breast cancer after treated with GLA has not been investigated. In this study, we used pharmacologic and molecular approaches to investigate the role of JNK in the GLA-induced death of breast cancer cell.

Materials and Methods

Chemicals, reagents and instruments

A 1 mM stock solution of GLA was obtained by dissolving GLA in DMSO. RPMI-1640 medium (Gibco, CA, USA), TRIzol (Gibco, CA, USA), MTT (Sigma, St. Louis, USA), Annexin V FITC Kit (Byotime, Nantong, Jiangsu, China). The following primary antibodies were used: p-JNK, Bcl-2, Bax, p-c-jun (Cell Signaling Technology, Beverly, MA, USA), p53, p-Erk and p-c-jun (Santa Cruz Biotechnology, Inc. Dallas, Texas, USA), and β-actin (Sigma, St. Louis, USA).

Cell Culture

Human breast cancer cell line MCF-7 and Hs578T purchased from ATCC (Manassas, VA, USA), were maintained in RPMI 1640 containing 10% fetal calf serum. All cell lines were cultured at 37°C, 5% CO_2.

Cell proliferation assays

As described previously (Chiang et al., 2007), the effects of glaucocalyxin A on cell proliferation were examined by MTT method.

Briefly, 1×10^4 cells were seeded into a 96 well plate, using 100 μL culture media per well. After cells have been incubated for 12 h, the medium was replaced by 100 μL fresh cell medium containing various concentrations of GLA (0, 1, 2, 4, 8, or 16 μM). For the measurement of cell viability after being incubated for 24 h, 48 h and 72 h, MTT assay was performed as described previously to examine the impact of drugs on cells. MTT solution (5 mg/mL in PBS) was added to all the samples in the 96-well plates and the cells were allowed to incubate for 4 h at 37°C. And then, the cells and dye crystals (formazan) were dissolved by adding 100 μL DMSO and the absorbance was measured at 572 nm. The inhibition rate was calculated as follows: inhibition rate =1-(OD drug-treated/OD control) ×100%.

Cell cycle analysis

For analysis of cell cycle distribution, cells were plated into 100-mm tissue culture dishes and at approximately 30% confluence treated with different concentrations of GLA. At different time points after the treatment, floating and attached cells were collected and washed twice with PBS, fixed in ice cold 70% ethanol and stored at -20°C until use. Subsequently, cells were rinsed with PBS and incubated with PBS containing 20 mg/mL propidium iodide (Sigma) and 1 mg/mL RNase A (Sigma). Cell cycle analysis was done using FACScan flow cytometer (Becton Dickinson, San Jose, CA). The percentage of cells in different phases of cell cycle was determined by ModFit LT cell cycle analysis software (Verity Software House, Topsham, ME), considering only cells with DNA content 2 n. Apoptotic cells were identified as a sub-G1 population (DNA content, 2n) and the percentage of cells in this pre-phase was calculated considering the totality of the events.

Annexin V-PI binding assay

For the Annexin-V-PI binding assay, the Annexin-V-FITC Apoptosis Detection Kit was used according to the manufacturer's instruction. Briefly, after treatment with GLA for 24 to 72 h, cells were washed twice with cold PBS. Then, the cell suspension concentration was adjusted to approximately 1×10^6 cells/mL. The cell suspension (1.5 mL) in a microfuge tube (5×10^4 cells) was incubated with 195 μL Media Binding Reagent and 5 μL Annexin V-FITC at room temperature for 10 min in the dark. After incubation, the cells were centrifuged at 1000×g for 5 min at room temperature. The cells were gently resuspended with 190 μL cold 1×Binding Buffer, and incubated with 10 μL PI, and then analyzed by flow cytometry (Beckman-coulter) immediately. The percentage of early apoptotic cells (stained by Annexin V only) and late apoptotic and necrotic cells (stained by PI) was calculated using the Cell Quest software.

Reverse Transcription polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA). RNA (2.5 μg) was reverse transcribed using a SuperscriptTM-III kit (Invitrogen), according to the manufacturer's instruction. PCR amplification was carried out in a 50 μL PCR reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 20 pmol of each primer set, two units of Taq DNA polymerase (Transgene, Beijing, China), 0.2 mM dNTPs, and 2 mL of cDNA. The PCR conditions were described previously

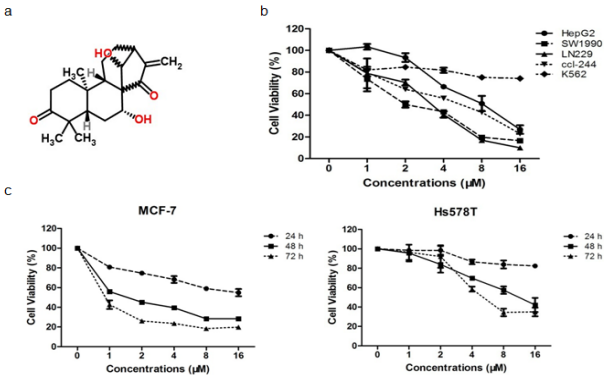

Figure 1. a. Chemical structure of Glaucocalyxin A; b. Effect of Glaucocalyxin A on the viability in different cells. Cells were treated with various concentrations (0, 1, 2, 4, 8, and 16 μM) of Glaucocalyxin A for 72 h. Cell viability was determined by the MTT assay. The cell viability was directly proportional to the production of formazan, which was measured spectrophotometrically at 572 nm; c. Effect of Glaucocalyxin A on the viability in MCF and Hs578T cells in different time

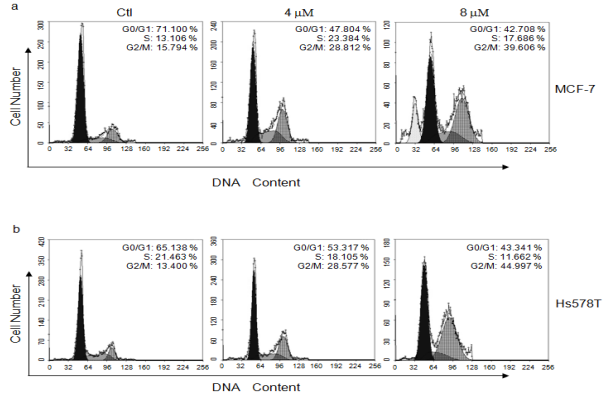

Figure 2. Cell cycle analysis of Glaucocalyxin A-treated MCF-7 cells (a) and Hs578T cells (b). Cells were treated with various concentrations (0, 4, and 8 μM) of Glaucocalyxin A for 24 h. The cells were fixed and stained with PI, and the cell cycle distribution was then analyzed by flow cytometry. The data indicate the percentage of cells in sub- G1, G0/G1, S, and G2/M phases of the cell cycle. Data was presented from three independent experiments. e. Western blot analysis of p53, p21 and G2/M transition-related proteins after WB treatment

(Kim et al., 2011).

The nucleotide sequences for the oligonucleotide primers used are shown in Table 1 (Kiaris et al., 1999; Chatzistamou et al., 2000; Hendrickx et al., 2003; Minko et al., 2005; Lu et al., 2010)

Immunoblot analysis

Proteins were extracted by lysing cells in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl [pH 6.8], 2% SDS) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/mL pepstatin, 12.5 mg/mL leupeptin, 2 mg/mL aprotinin, 1 mM sodium orthovanadate, and 1 mM sodium molybdate. Cell extracts were processed for western immunoblotting as described previously (Weston et al., 2002). The following antibodies used for immunoblotting were purchased from the indicated suppliers: p53 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); phospho-JNK (Thr183/Tyr185), JNK1/2, and Bcl-2 from Cell Signaling Biotechnology (Beverly, MA, USA), β- actin from Sigma.

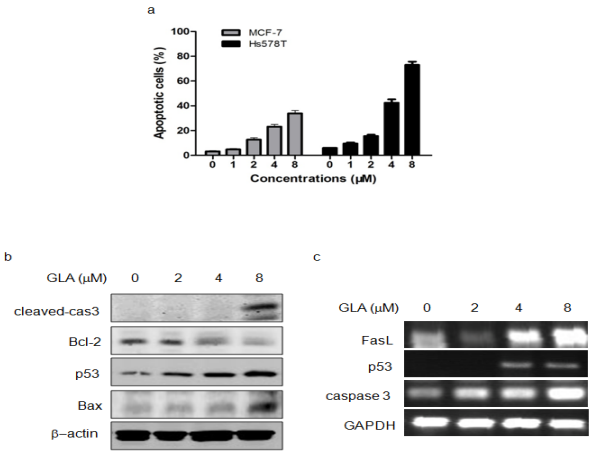

Figure 3. a. Following treatment of MCF-7 and Hs578T cells with various concentrations of GLA for 24 h, apoptotic cells were detected by Annexin V and propidium iodide double staining. Statistical analysis of the percentages of the apoptotic cells. The data shown are representatives of three experiments. *P<0.05, **P<0.01; b. the expression of cleaved caspase 3, Bcl-2, p53 and Bax were determined by Western blotting analysis; c. RT-PCR analysis of the expression of mRNA for FasL, p53, caspase 3 and β-actin in MCF-7 human breast cancers

Statistical analysis

Experiments and in vitro assays were carried out at least in triplicate. Differences between mean values were assessed by Student's t-test with two-sided P values, P < 0.05 was considered as statistically significant.

Results

GLA suppressed the viability and growth of cells through G2/M phase arrest

Many studies have demonstrated that GLA exerted a potent cytotoxic activity and had a significantly inhibitory effect on several tumor cells, such as leukemia HL60 cell (Gao et al., 2011; Yang et al., 2013). Our results revealed that a marked anti-proliferative activity was observed in MCF-7 and Hs578T cells with IC50 value of 1 μM and 4 μM after treatment of GLA for 72 h, respectively (Figure 1).

To explore the mechanisms leading to the loss of MCF-7 and Hs578T cells proliferation by GLA, the effects of GLA treatment on cell cycle arrest were examined. MCF-7 and Hs578T cells were incubated with various concentrations of GLA for 72 h. A dose-dependent G2/M phase arrest was observed (Figure 2).

GLA induced apoptosis and regulated the expression of apoptosis related proteins

To further understand whether GLA-induced cell death is mediated by apoptosis or necrosis, we evaluated apoptotic cell death using annexin V/PI double staining, which specifically labels apoptotic cells. As shown in Figure 3a, treatment with GLA at concentrations of 8 μM for 24 h induced apoptosis in about 38% and 75% of the MCF-7 and Hs578T cells, respectively. Additionally, treatment with GLA activated caspase-3, which the significant proteolytic cleavage of caspase-3 was detected by RT-PCR and western blot.

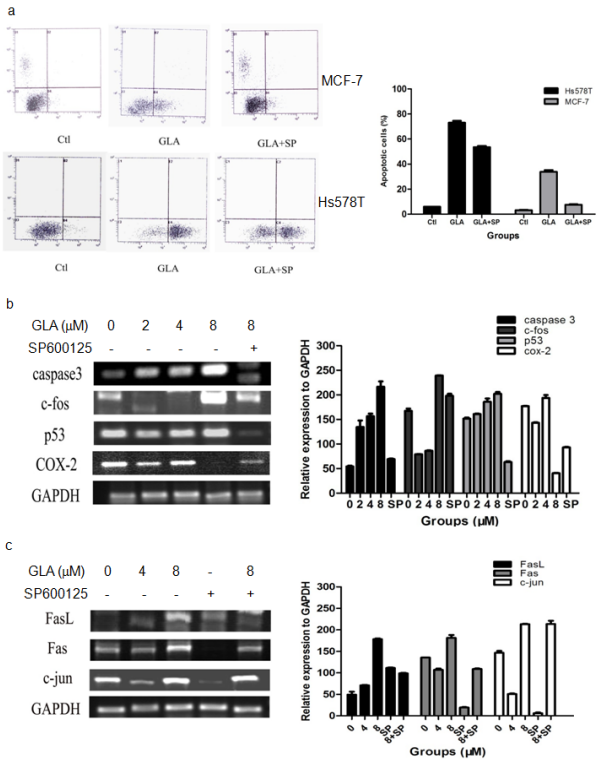

Figure 4. The Roles of JNK Signaling-related Proteins in GLA-induced Apoptosis of Human Breast Cancer Cell. a. The apoptosis of MCF-7 and Hs578T cell after treated with 8 μM with or without SP600125 for 24 h by flow cytometry; b and c. Expression of caspase 3, c-fos, p53, Fas and FasL mRNA in MCF-7 human breast cancer cell line with or without SP600125

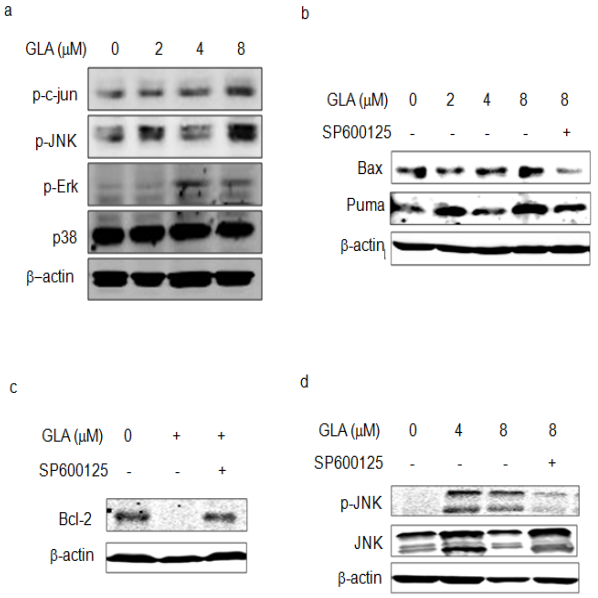

Figure 5. JNK Pathway Inhibit the Apoptosis Induced by GLA in MCF-7 Human Breast Cancer Cell. a. The JNK pathway related proteins were detected by western blot after treated with different concentrations of GLA; b, c, and d. The expression of Bax, Puma, Bcl-2 and p-JNK protein in MCF-7 cell with or without SP600125

To confirm whether other apoptosis related proteins, such as Bax, Bcl-2 and p53 protein was related to GLA-induced apoptosis, we treated MCF-7 cells with various concentrations of GLA (0, 2, 4, 8 μM) for 24 h and analyzed apoptosis-related proteins using Western blotting. As shown in Fig 3b, Bax, and p53 was significantly increased, however the expression of Bcl-2 was decreased.

GLA-induced apoptosis was attributed to the upregulation of Fas/FasL signaling pathway

To determine which signaling pathway was involved in cell apoptosis by GLA, expression of Fas and FasL in MCF-7 cells were examined by RT-PCR and Western blotting analysis.

In order to determine the effect of GLA on the Fas/FasL signaling pathway, we detected the gene expression of Fas and FasL at 24 h after treated with GLA, and the results showed that GLA increased the expression of FasL mRNA (Figure 3c and Figure 4c).

The roles of JNK signaling-related proteins in GLA-induced apoptosis of human breast cancer cell

Considerable evidence indicates that MAPK signaling cascades regulate not only cell growth, development and differentiation, but also apoptosis and cell growth arrest. To understand the mechanism by which GLA affects MAP kinase activation, the role of GLA in the activation of ERK, JNK and p38 MAP kinase was determined. Western blot showed that the phosphorylation of ERK and JNK was gradually, and significantly, increased after GLA treatment (Figure 4b and Figure 5). Interestingly, from Figure 4a, it can be seen that the JNK inhibitor SP600125 significantly restored cell apoptosis in response to GLA. At the protein level, the results were consistent with the results of flow cytometry and revealed that the SP600125 had a significantly opposite effect on the GLA-induced apoptosis-related proteins (Figure 5b to d). From the results obtained so far, it could be concluded that the cell apoptosis and G2 phase arrest of MCF-7 and Hs578T cells induced by GLA were mediated by activation of the JNK/MAPK signaling.

Discussion

In recent years, considerable emphasis has been given to identify new anti-cancer agents from natural sources, which could be useful for human beings.

Glaucocalyxin A (GLA), a naturally occurring Rabdosia japonica (R. japonica) var. galucocalyx (Labiatae), has a wide range of pharmacologic effects, such as inhibition of cell proliferation, inhibition of cell cycle progression, and induction of apoptosis in various cancer cell lines. Metastatic spread of cancer is responsible for 90% of human cancer related deaths and thus remains one of the important impediments on cancer curing (Steeg et al., 2006). However, whether GLA exerts an inhibitory effect on tumor metastasis in breast cancer has not been elucidated previously.

In this report, we investigated the pro-apoptotic and cell arrest effect of GLA on MCF-7 and Hs578T cells, and found that the MCF-7 and Hs578T cells were highly sensitive to GLA. All the results showed that GLA efficiently inhibited the proliferation of MCF-7 and Hs578T cells. Treatment with GLA in MCF-7 and Hs578T

cells resulted in G2 phase arrest in a dose-dependent manner. To the best of our knowledge, this study is the first to demonstrate the effect of GLA on MCF-7 and Hs578T cells.

Cell death signals from the extracellular environment or internal sensors for the cellular response are major constituents of apoptotic machinery (Liu et al., 2011). Cell surface death receptors that transmit cell death signals are activated by specific death ligands. It is demonstrated that Fas is one of the best-characterized death receptors. Upon binding of FasL onto Fas, apoptotic signals are subsequently transmitted via death adaptor molecule FADD which can mediate the activation of caspase 8, and active caspase 8 can proteolytically activate downstream effector caspases, such as caspase 3, to trigger apoptosis (Antonsson et al., 2000; Strasser et al., 2009).

In this study, our results also confirmed it. The expression of FasL is the highest versus control after treated with 8 μM GLA in 24 h by RT-PCR and western blot detection. Based on the above results, it could conclude that GLA is able to induce apoptosis by upregulating Fas/ FasL signaling pathway in human MCF-7 breast cells.

JNK is a subfamily of the MAPK superfamily. MAPK pathway is involved in the development of tumor cells. JNK specifically phosphorylates the transcription factor c-Jun on its N-terminal transactivation domain, which mediates the proapoptotic function of JNK via modulating some proteins, such as Bcl-2 family protein Bim and so on. The role of JNK in tumor development remained controversial, although it was implicated in oncogenic transformation (Zhang et al., 2012). Published data implied that JNK pathway was involved in the human breast cancer cell (Sun et al., 2013), hepatoma cancer cell (Zhang et al., 2013), and so on. And also, MAPK pathway could activate its downstream Fas/ FasL signaling pathway and induce the apoptosis of cancer cells (Antonsson et al., 2000; Chen et al., 2010).

On the basis of this hypothesis, we detected the JNK pathway related proteins. And our results showed that the phosphorylation levels of Erk and JNKs were increased after treated with GLA (Figure 5a). When we treated JNK pathway inhibitor SP600125, the activation of JNKs and expression of FasL were significantly inhibited, and then cell apoptosis rate obviously declined, suggesting that GLA could activate the JNK pathway and its downstream Fas/FasL death receptor pathway to induce the apoptosis of human breast cancer MCF-7 cells (Figure 4 and 5).

These results in conclusion, we have demonstrated that GLA could activate JNK pathway and upregulate FasL protein. However, in order to fully investigate GLA-induced apoptosis, additional in vivo studies are also required. Importantly, however, the findings of this study provided a novel insight into the mechanism of GLA induced human breast cancer cell apoptosis.

Acknowledgements

This study was funded in part by a research fellowship of the Kong Health Care Association Foundation (HK20100901).

References

Antonsson BJ, Martinou C (2000). The Bcl-2 protein family. *Exp Cell Res*, **256**, 50-7.

Bishayee A, Ahmed S, Brankov M, Perloff N (2011). Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer. *Front Biosci*, **16**, 980-96.

Chatzistamou I, Schally AV, Sun B, et al (2000). Inhibition of growth of OV-1063 human epithelial ovarian cancers and c-jun and c- fos oncogene expression by bombesin antagonists. *Br J Cancer*, **83**, 906-13.

Chen KC, Chang LS (2010). Notexin upregulates Fas and FasL protein expression of human neuroblastoma SK-N-SH cells through p38 MAPK/ATF-2 and JNK/c-Jun pathways. *Toxicon*, **55**, 754-61.

Chiang CT, Way TD, Lin JK (2007). Sensitizing HER2-overexpressing cancer cells to luteolin-induced apoptosis through suppressing p21(WAF1/CIP1) expression with rapamycin. *Mol Cancer Ther*, **6**, 2127-38.

Gao LW, Zhang J, Yang WH et al (2011). Glaucocalyxin A induces apoptosis in human leukemia HL-60 cells through mitochondria-mediated death pathway. *Toxicol In Vitro*, **25**, 51-63.

Hendrickx N, Volanti C, Moens U, et al (2003). Up-regulation of cyclooxygenase-2 and apoptosis resistance by p38 MAPK in hypericin-mediated photodynamic therapy of human cancer cells. *J Biol Chem*, **278**, 52231-9.

Hong SS, Lee SA, Han XH, et al (2008). ent-Kaurane diterpenoids from Isodon japonicus. *J Nat Prod*, 71, 1055-8.

Jemal A, Bray F, Center MM, et al (2011). Global cancer statistics. *CA Cancer J Clin*, **61**, 69-90.

Kiaris H, Schally AV, Sun B, et al (1999). Inhibition of growth of human malignant glioblastoma in nude mice by antagonists of bombesin/gastrin-releasing peptide. *Oncogene*, **18**, 7168-73.

Kim BW, Koppula S, Hong SS, et al (2013). Regulation of microglia activity by glaucocalyxin-A: attenuation of lipopolysaccharide-stimulated neuroinflammation through NF-kappaB and p38 MAPK signaling pathways. *PLoS One*, **8**, e55792.

Kim BW, Koppula S, Kim IS, et al (2011). Anti-neuroinflammatory activity of Kamebakaurin from Isodon japonicus via inhibition of c-Jun NH(2)-terminal kinase and p38 mitogen-activated protein kinase pathway in activated microglial cells. *J Pharmacol Sci*, **116**, 296-308.

Lawen A (2003). Apoptosis-an introduction. *Bioessays*, **25**, 888-96.

Lee HT, Kim SK, Cho MR I, et al (2013). Effects of the activated mitogen-activated protein kinase pathway via the c-ros receptor tyrosine kinase on the T47D breast cancer cell line following alcohol exposure. *Oncol Rep*, **29**, 868-74.

Liu WH, Chang LS (2011). Fas/FasL-dependent and -independent activation of caspase-8 in doxorubicin-treated human breast cancer MCF-7 cells: ADAM10 down-regulation activates Fas/FasL signaling pathway. *Int J Biochem Cell Biol*, **43**, 1708-19.

Lu B, Hu M, Liu K, Peng J (2010). Cytotoxicity of berberine on human cervical carcinoma HeLa cells through mitochondria, death receptor and MAPK pathways, and in-silico drug-target prediction. *Toxicol In Vitro*, **24**, 1482-90.

Minko T, Batrakova EV, Li S, et al (2005). Pluronic block copolymers alter apoptotic signal transduction of doxorubicin in drug-resistant cancer cells. *J Control Release*, **105**, 269-78.

Miyoshi Y, Murase K, SaitoK Oh M (2010). Prediction of hormone sensitivity for breast cancers. *Breast Cancer*, **17**, 86-91.

Nakano IH, Kornblum I (2006). Brain tumor stem cells. *Pediatr Res*, **59**, R54-8.

Steeg PS (2006). Tumor metastasis: mechanistic insights and clinical challenges. *Nat Med*, **12**, 895-904.

Strasser A, JostS Nagata PJ (2009). The many roles of FAS receptor signaling in the immune system. *Immunity*, **30**, 180-92.

Sun HD, Huang SX, Han QB (2006). Diterpenoids from Isodon species and their biological activities. *Nat Prod Rep*, **23**, 673-98.

Sun Y, Zou M, Hu C, et al (2013). Wogonoside induces autophagy in MDA-MB-231 cells by regulating MAPK-mTOR pathway. *Food Chem Toxicol*, **51**, 53-60.

Veronesi U, Orecchia R, Luini A, et al (2010). Intraoperative radiotherapy during breast conserving surgery: a study on 1,822 cases treated with electrons. *Breast Cancer Res Treat*, **124**, 141-51.

Wagner EF, Nebreda AR (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer*, **9**, 537-49.

Weston CR, Davis RJ (2002). The JNK signal transduction pathway. *Curr Opin Genet Dev*, **12**, 14-21.

Yang WH, Zhang Z, Sima YH, et al (2013). Glaucocalyxin A and B-induced cell death is related to GSH perturbation in human leukemia HL-60 cells. *Anticancer Agents Med Chem*, **13**, 1280-90.

Zhang Z, Miao L, Lv C, et al (2013). Wentilactone B induces G2/M phase arrest and apoptosis via the Ras/Raf/MAPK signaling pathway in human hepatoma SMMC-7721 cells. *Cell Death Dis*, **4**, e657.

Zhang ZB, Jiang XG, Liang ZQ, Gu ZL (2012). Arsenic trioxide inhibits the growth of human glioma stem cells through activating the JNK pathway. *Mol Cell Toxicol*, 187-93.