Original Article

Kaempferol Induces Cell Death in A2780 Ovarian Cancer Cells and Increases Their Sensitivity to Cisplatin by Activation of Cytotoxic Endoplasmic Reticulum-Mediated Autophagy and Inhibition of Protein Kinase B

(ovarian / cancer / kaempferol / apoptosis / cisplatin)

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Abstract. This study investigated whether kaempferol could inhibit ovarian cancer (OC) by activation of endoplasmic reticulum (ER) stress and autophagy, and tested its effect on the sensitivity of OC cells to cisplatin (cis-diamminedichloroplatinum, DPP). To study the effect of kaempferol on activation of ER stress and autophagy and find out whether its mechanism of action involves calcium (Ca^{2+}), A2780 OC cells were cultured in DMEM/F12 for 24 h with or without kaempferol (40 µmol/l) in the presence or absence of autophagy or ER stress inhibitors or a calcium chelator. To study the effect of kaempferol on the sensitivity of OC cells to DPP and the potential involvement of modulation of protein kinase B (Akt) expression, A2780 OC were incubated with kaempferol and increasing concentrations of DPP (0–20 µmol/l) and then with kaempferol at its predetermined IC_{50} (6.8 µmol/l). Compared to control cells, kaempferol increased cell apoptosis (158 %) and decreased viability (53.17 %) and proliferation (49.17 %) of A2780 OC cells. Concomitantly, it increased the protein levels of GRP78, PERK, ATF6, IRE-1, LC3II, beclin 1, and caspase 4, thus suggesting activation of cytotoxic autophagy. This was mediated by increasing intracellular Ca^{+2} levels. In addition, kaempferol increased the sensitivity of A2780 cells to DPP (IC_{50} from 6.867 ± 0.99 to 3.73 ± 0.59 µmol/l) by decreasing the protein levels of p-Akt (0.31 ± 0.09 vs 0.12 ± 0.005). In conclusion, the findings of this study encourage the use of kaempferol alone or in combination with DPP to inhibit tumorigenesis of ovarian cells.

Introduction

Ovarian cancer (OC) is one of the most lethal gynaecological cancers and the fifth leading cause of death in women worldwide (Jayson et al., 2014). In spite of the diverse therapeutic options and strategies such as surgical resection and adjuvant combination chemotherapy, the overall five-year survival rate has remained very low and less than 40 % (Ricciardelli and Oehler, 2009; Network, 2011; Siegel et al., 2011; Gasparri et al., 2017). This has been attributed to the complexity of the molecular pathways involved in the progression and development of the disease, poor screening approach, inadequate therapeutic options, high recurrence rate, and drug resistance (Jayson et al., 2014; Siegel et al., 2017). Hence, in order to improve survival among OC patients, treat the associated recurrence, and overcome the developed chemo-resistance, current advice suggests to carry out more research to understand the precise molecular basis of the disease and to develop more efficient therapeutic options.

Currently, numerous studies have shown that activation of endoplasmic reticulum (ER) stress is a golden

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Abbreviations: BiP – binding immunoglobulin protein, CHOP – C/EBP homologous protein, DMSO – dimethyl sulphoxide, DPP – cisplatin, cis-diamminedichloroplatinum, DMEM/F12 – Dulbecco's modified Eagle's medium, eIF2α – eukaryotic initiation factor 2α, ER – endoplasmic reticulum, OC – ovarian cancer, OD – optical density, PBS – phosphate-buffered saline, POD – peroxidase, ROS – reactive oxygen species, TNF – tumour necrosis factor, UPR – unfolded protein response, XBP1 – X-box binding protein 1.

approach to inhibiting tumour growth by activation of apoptosis and cytotoxic autophagy (Xu et al., 2015; Huang et al., 2018). Generally, ER is a unique cell organelle responsible for regulating protein folding, translocation and post-translation modification which aims to maintain normal cellular homeostasis (Wang et al., 2014). When biochemical, physiological and pathological stimuli perturb ER functions, unfolded or misfolded proteins accumulate in the ER lumen. This leads to activation of a phenomenon termed "ER stress", which in turns activates a number of pathways to counteract the associated damage (Wang et al., 2014). Together these pathways are called the unfolded protein response (UPR). In most cells, the UPR has a dualistic function, in which it primarily aims to reverse the damage associated with ER stress to enhance cell survival, and if unresolved, then it induces cell death through activation of apoptosis (Martinon, 2012; Yadav et al., 2014).

The UPR is composed of three different pathways that are controlled by three ER transmembrane proteins known as 1) kinase RNA-like endoplasmic reticulum kinase (PERK), 2) activating transcription factor 6 (ATF6), and 3) inositol-requiring enzyme 1α (IRE1α) (Hetz, 2012; Wang et al., 2014). However, these three proteins are maintained inactive under normal physiological conditions by forming a complex with a protein named binding immunoglobulin protein (BiP or GRP78) (Bertolotti et al., 2000). In the presence of any cellular stress, misfolded proteins bind to BiP, causing its dissociation from these proteins and so their activation. Downstream signalling of PERK includes eukaryotic initiation factor 2α (eIF2α), ATF4, and C/EBP homologous protein (CHOP), whereas the IRE1α downstream target is X-box binding protein 1 (XBP1) (Wang et al., 2014). On the other hand, autophagy is a preserved mechanism that leads to lysosomal degradation of the dysfunctional organelles and cytoplasmic contents (Dupont and Codogno, 2016).

ER stress is a major trigger of autophagy during cellular physiological and pathological stress as well as during chemotherapy (Kouroku et al., 2007; Maiuri et al., 2007; Xu et al., 2015; Huang et al., 2018). Similarly to ER stress, autophagy plays dualistic roles during chemotherapy, in which it may induce cell survival and chemotherapy resistance or apoptosis (Levine et al., 2008; Guo et al., 2017; Cheng et al., 2018). As a protective mechanism, autophagy activation degrades unfolded aggregated proteins to inhibit ER stress, thus enabling the cancerous cell to avoid ER-mediated apoptosis (Herman-Antosiewicz et al., 2006; Cheng et al., 2018).

On the other hand, the low intake of flavonoids is associated with an increased risk of development of different solid tumours including OC (Banks, 2001; Arts and Hollman, 2005; Xiao et al., 2011; Batra and Sharma, 2013). Kaempferol (3,4′,5,7-tetrahydroxyflavone) is a well-known flavonoid that has been reported to protect from colon, pancreatic, lung, prostate, oesophageal, hepatic and ovarian cancers (Luo et al., 2011; Chen and Chen, 2013; Kim and Choi, 2013). In females, kaemp-

ferol reduced the risk of OC (Gates et al., 2007). *In vitro*, kaempferol induces apoptosis in A2780/CP70, A2780/wt, and OVCAR-3 OC cells through activating p53 (Luo et al., 2011). Also, kaempferol sensitizes OVCAR-3 and SKOV-3 OC cells to tumour necrosis factor (TNF)-mediated apoptosis via activation of the JNK/ERK-CHOP pathway and up-regulation of death receptors 4 and 5 (Zhao et al., 2017). In addition, kaempferol induced apoptosis in hepatocellular carcinoma by activation of ER stress-induced activation of CHOP-induced autophagy (Guo et al., 2017).

In spite of these findings, the effect of kaempferol on OC cell proliferation, growth, and apoptosis with respect to its effect on ER and autophagy has not yet been investigated. In addition, the sensitivity of OC cells treated with kaempferol to cisplatin (DPP) is still unclear. Hence, in this *in vitro* study, we tested the ER and autophagy response as well as the sensitivity of human chemo-sensitive OC cells (A2780 cell line) to DPP after kaempferol treatment.

Material and Methods

Drug preparations

Human chemo-sensitive OC cells (A2780) (Cat. No. 93112519), cisplatin (DPP) (Cat. No. 15663-27-1), chloroquine (CQ) (Cat. No. C6628), kaempferol (Cat. No. K0133), sodium phenylbutyrate (4-PBA) (Cat. No. SML03090), and BAPTA-AM [bis-o-aminophenoxy) ethane-*N,N,N′,N′*-tetra-acetic acid tetrakis (acetoxymethyl ester)] (Cat. No. 1076) were purchased from Sigma Aldrich (St. Louis, MO). Cisplatin and kaempferol were prepared in dimethyl sulphoxide (DMSO) (the final concentration of DMSO was 0.1 %). Chloroquine and 4-PBA were dissolved in phosphate-buffered saline (PBS, pH = 7.4). Primary antibodies against microtubule-associated protein light chain 3 (LC3), (Cat. No. 2775), beclin 1 (Cat. NO. 3738), autophagy-related 5 protein (ATG5) (Cat. No. 2630), caspase 4 (Cat. No 4450), cleaved caspase 3 (Cat. No. 9661), PERK (Cat. No. sc-377400), CHOP (Cat. No. 2895), and p-eIF2α (Ser51) (Cat. No 9721) and β actin (Cat. No. 4970) were purchased from Cell Signaling Technology (Danvers, MA). Primary antibodies against ATF-6α (Cat. No. sc-166659), GRP78 (Cat. No. sc-13539), IRE1-α (Cat. No. sc-390960) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX).

Cell culture

A2780 cells were cultured in a humidified atmosphere (5% CO_2 at 37 °C) in Dulbecco's modified Eagle's medium (DMEM/F12) (ThermoFisher Scientific Inc, Rockford, lL) supplemented with 10% foetal bovine serum, 50 µg/ml streptomycin, 50 U/ml penicillin, 1% nonessential amino acids, and 0.625 g/ml fungizone (Fraser et al., 2008). Eighteen hours before the beginning of the treatments, A2780 cells were cultured at a density of $5×10^4$ cells in 60-mm dishes, and a cell den-

sity of < 85 % confluence was used at the time of the treatments.

Experimental procedure

Cells were grown in the presence or absence of kaempferol (40 µmol/l) for 24 h. The dose of kaempferol and the incubation period were adopted from the studies of Luo et al. (2011) and Zhao et al. (2017), who showed that proliferation of OVCAR-3 and SKOV-3, as well as A2780, was significantly inhibited at a final concentration of 40 µmol/l and higher. In some cases, cells were also co-incubated with 40 µmol kaempferol plus 10 µmol/l of CQ (an autophagy inhibitor) or 1 µmol/l of 4-PBA (an ER stress inhibitor) or 5 µmol/l of BAPTA-AM (a Ca^{2+} chelator). Control cells were incubated with diluted DMSO only (0.1%). In our preliminary studies, we have found that DMSO up to a final concentration of < 0.05 % has no effect on A2780 cell viability, proliferation, and death, as well as on the expression of markers of ER stress and autophagy, as compared to cells treated with media only.

Sensitivity to cisplatin (IC$_{50}$)

To determine the half inhibitory concentration (IC$_{50}$) of cisplatin, cells were incubated with kaempferol (40 µmol/l) with increasing concentrations of cisplatin (0, 2, 4, 6, 8, 10, 15, 20 µmol/l) in the presence or absence of CQ or 4-PBA. The IC$_{50}$ value is defined as a cisplatin concentration that inhibits cell viability by 50 %. Control cells were incubated with diluted DMSO only.

Cell viability assays

Cell viability of all treatments was measured using a cell counting kit-8 (CCK-8; Cat No. CK04-13, Dojindo, Kumamoto, Japan). In brief, at the end of the incubation periods, the medium was replaced by CCK-8 solution (10 µl) and culture media (90 µl) for 1 h. The absorbance was read in 96-well plates at an optical density (OD) of 450 nm. Cell viability was presented as the percent of control.

5-bromo-2'-deoxyuridine (BrdU) cell proliferation

A BrdU colorimetric kit (Cat. No. 11647229001; Roche Diagnostics, Indianapolis, IN) was used to measure cell proliferation in the treated groups. In brief, BrdU incorporates into newly synthesized cellular DNA and then binds to anti-BrdU-peroxidase (POD). The whole immunocomplex is then detected with the use of the 3,3',5,5'-tetramethylbenzidine substrate. In the test, each well was supplied with 10 µM BrdU and incubated at 37 °C for 2 hours. Anti-BrdU-peroxidase (POD) working solution was added to each well and incubated for 90 min at room temperature. Absorbance was measured at 370 and at 492 nm (reference range). Cell proliferation was calculated as the percent of control.

Measurements and determination of cell apoptosis

Cell apoptosis was determined in all treatments by means of western blotting (as shown later) and using quantitative measurements by a cell death determination ELISA kit (Cat. No. 11544675001, Roche Diagnostics GmbH, Mannheim Germany). In the latter procedure, the cell death determination ELISA kit measures the cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fractions. Within the test, anti-histone antibodies react with the various histones (H1, H2A, H2B, H3, and H4), and peroxidase-conjugated DNA antibody (anti-DNA POD) binds to both single- and double-stranded DNA. The developed absorbance of each reaction was read at OD of 405 nm and 490 nm (reference range). The apoptosis ratio was calculated as the percent of control.

Measurement of intracellular Ca2$^+$ levels and ROS

The levels of intracellular Ca^{2+} in the cell homogenates were measured using a Flou-8 calcium flux assay kit (ab112129, Abcam, Cambridge, UK). The intracellular levels of ROS in the cell homogenates were measured using an OxiSelect™ in vitro ROS/RNS Assay Kit (Cat. NO, STA-347, Cell Biolabs, Inc. San Diego, CA). All procedures were performed in duplicate according to the manufacturer's instructions and presented as the percent of control.

Western blotting procedure

Total proteins in total cell homogenates were extracted from all cells using the M-PE mammalian protein extraction reagent (Cat. No. 78501, ThermoFisher Scientific Inc.). Protein levels were measured using a Pierce BCA Protein Assay Kit (Cat. No. 23225, ThermoFisher Scientific Inc.). Equal amounts of proteins (40 µg/well) were loaded and separated by SDS-PAGE (81–12 %). Proteins were then transferred onto nitrocellulose membranes (M-Bio-Rad, Hercules, CA) and blocked with skim milk (5%; w/v, prepared in 0.05% Tween 20 buffer [TBST]) for 1 h at room temperature. Primary antibodies were incubated with each membrane, individually, for 2 h at room temperature. Membranes were then washed with TBST buffer and incubated with the corresponding HRP-conjugated secondary antibodies for another 2 h at room temperature. Reactions were detected using a Pierce chemiluminescence reagent (Thermo Fisher Scientific Inc.), scanned and analysed using a C-Di Git blot scanner (LI-COR, Lincoln, NE) and associated software. Membranes were stripped up to four times and detection of the phosphorylated forms was done first. Protein levels of phosphorylated and total proteins were normalized to the reference protein, β-actin.

Statistical analysis

Statistical analysis for all measured parameters was done using the GraphPad Prism statistical software package (version 6). Differences among the experimental groups were assessed by one-way ANOVA, followed by Tukey's test. Data were presented as mean ± SD. Values will be considered significantly different when $P < 0.05$.
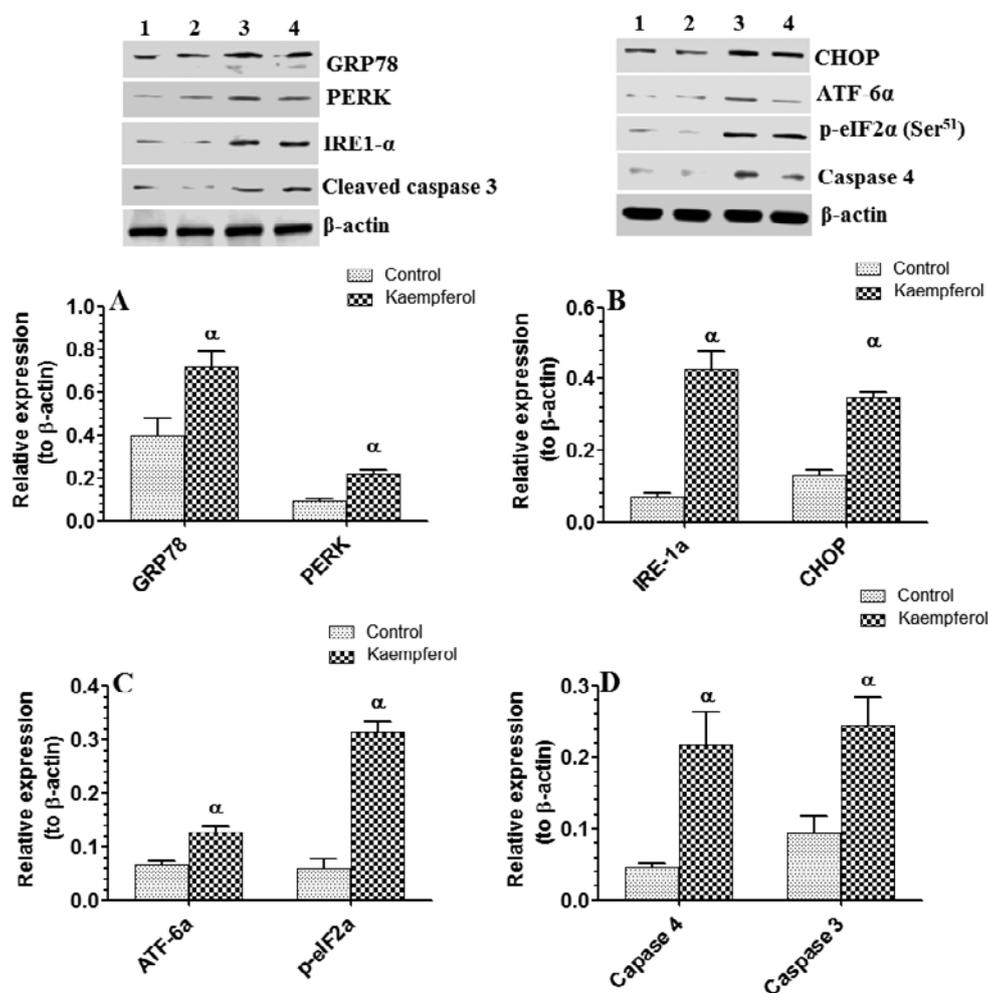
Results

Kaempferol induces ER stress and autophagy in A2780 OC cells

As shown in Fig. 1 (A-D), protein levels of the major arms of UPR including ATF6α IRE1α, PERK and their regulator, GRP78, as well as some of their downstream targets were significantly increased in A2780 OC cells that were treated with kaempferol. Similarly, protein levels of autophagy markers including LC3II, beclin 1, and ATG5 were significantly increased, whereas the protein levels of LC3I were significantly decreased in kaempferol-treated OC cells (Fig. 2, A-B). However, inhibition of ER stress by 4-PBA abolished the stimulatory effect of kaempferol on the protein levels of LC3II, beclin 1, and ATG5 (Fig. 3, A-D). Also, chelating intracellular Ca^{2+} by BAPTA-AM abolished the stimulatory effect of kaempferol on the protein levels of ATF6α, IRE1α, and PERK (Fig. 4, A-B).

Kaempferol inhibits cell survival and proliferation via activation of ER stress and autophagy

Cell survival and proliferation, as well as intracellular levels of Ca^{2+}, were significantly increased, whereas the apoptosis ratio was significantly decreased in A2780

Fig. 1. Kaempferol induces endoplasmic reticulum (ER) stress in A2780 chemo-sensitive OC cells. Cells were grown in DMEM/F12 media with 0.1% diluted DMSO (Control) or 40 μmol/l kaempferol. Results as shown as the mean ± SD of N = 6 experiments. α: significantly different as compared to control. ATF6α: activating transcription factor 6α; GRP78: binding immunoglobulin protein (BiP); IRE1α: inositol-requiring enzyme 1α; PERK: kinase RNA-like endoplasmic reticulum kinase; p-eIF2α: phospho-eukaryotic initiation factor 2α; CHOP: C/EBP homologous protein. Lanes 1 & 2: control. Lanes 3 & 4: kaempferol-treated cells.
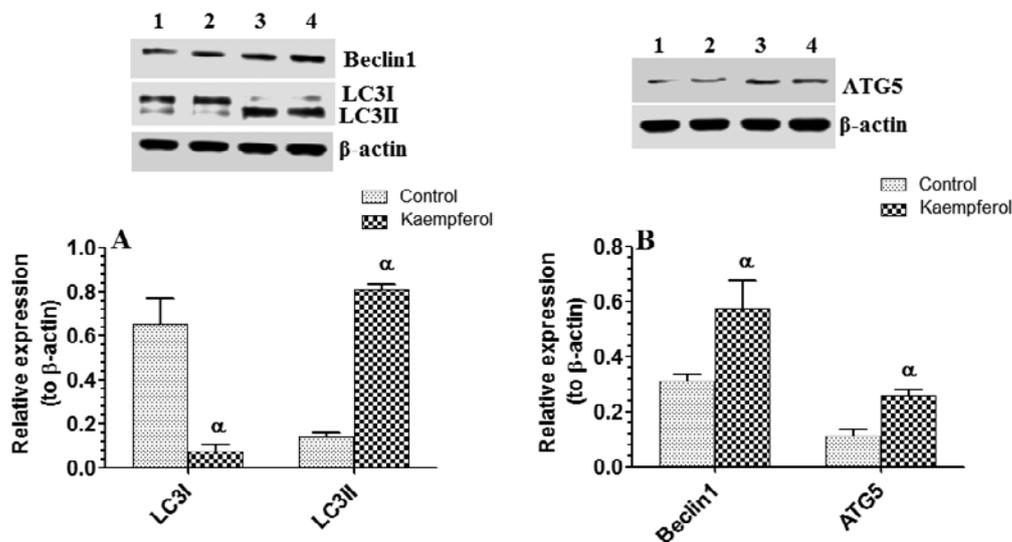
Fig. 2. Kaempferol activates autophagy in A2780 chemo-sensitive OC cells. Cells were grown in DMEM/F12 media with 0.1% diluted DMSO (Control) or 40 µmol/l kaempferol. Results as shown as the mean ± SD of N = 6 experiments. α: significantly different as compared to control. Lanes 1 & 2: control. Lanes 3 & 4: kaempferol-treated cells.
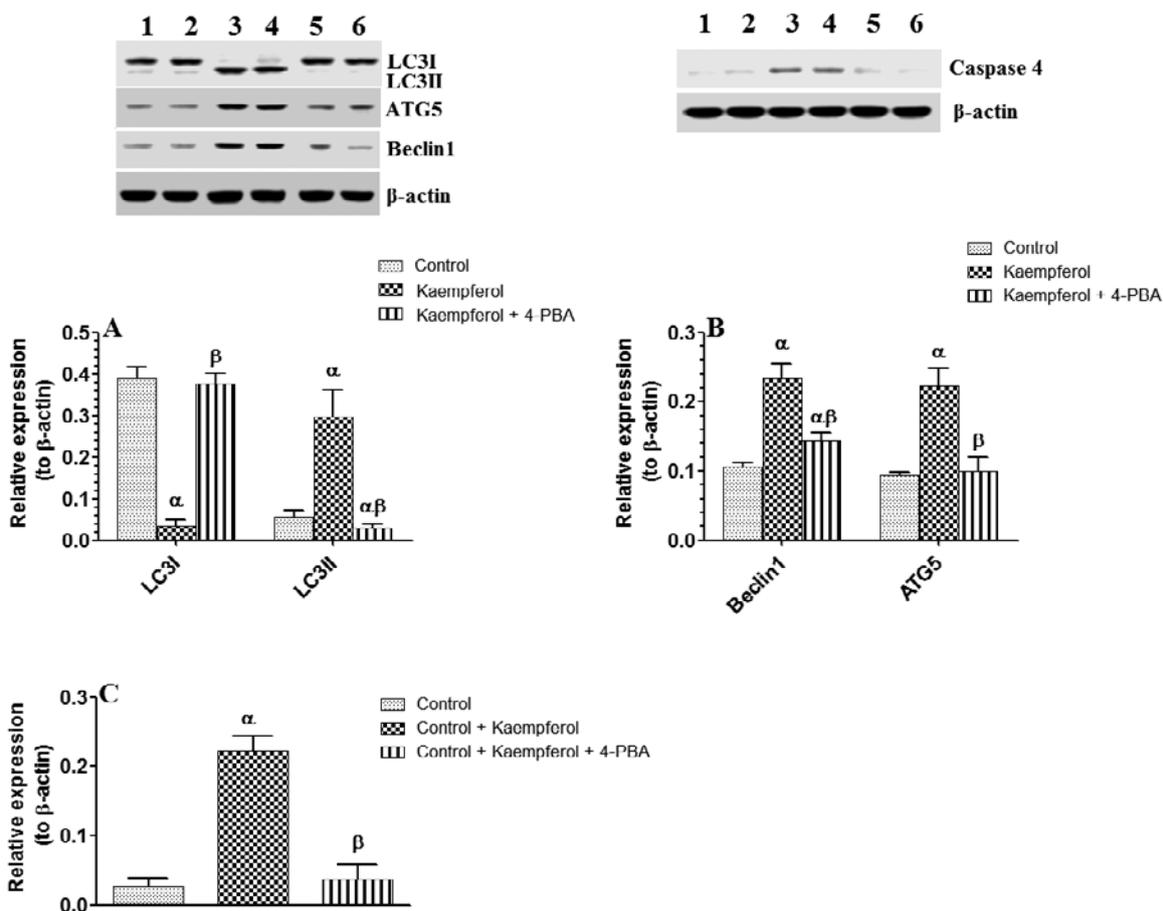
Fig. 3. Inhibition of ER stress by sodium phenylbutyrate (4-PBA) abolished the stimulatory effect of kaempferol on autophagy. Cells were grown in DMEM/F12 media and treated with 40 µmol/l kaempferol in the presence or absence of 1 µmol/l 4-PBA, an ER stress inhibitor. Control cells were treated with 0.1% diluted DMSO. Results as shown as the mean ± SD of N = 6 experiments. α: significantly different as compared to control. β: significantly different as compared to kaempferol-treated cells. Lanes 1 & 2: control. Lanes 3 & 4: kaempferol-treated cells. Lanes 5 & 6: kaempferol + 4-PBA-treated cells.
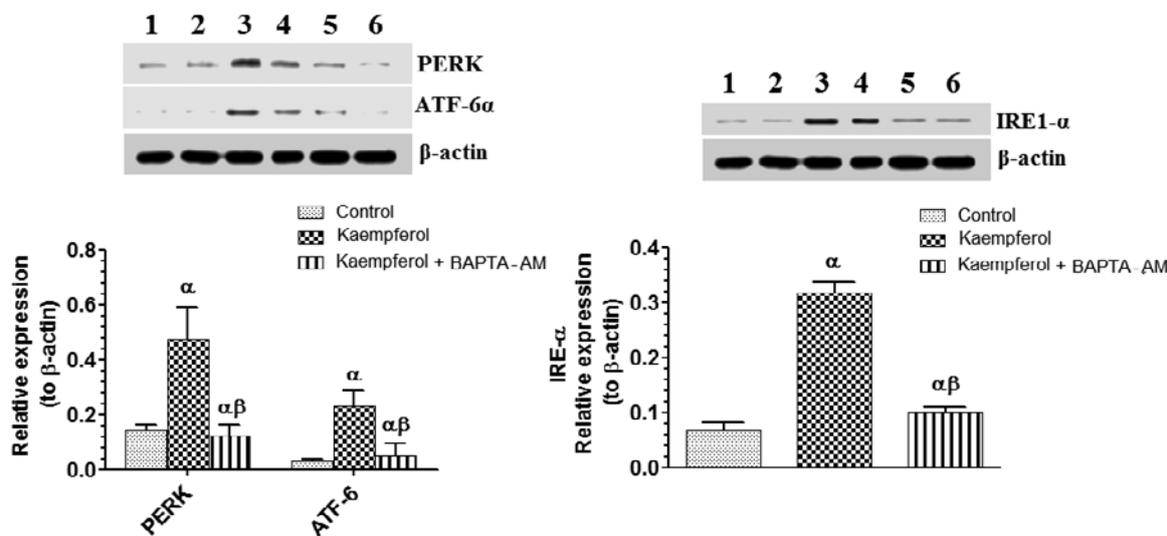
Fig. 4. Chelating intracellular calcium levels by BAPTA-AM abolished the stimulatory effect of kaempferol on ER stress. Cells were grown in DMEM/F12 media and treated with 40 μmol/l kaempferol in the presence or absence of 5 μmol/l BAPTA-AM. Control cells were treated with 0.1% diluted DMSO. Results as shown as the mean ± SD of N = 6 experiments. α: significantly different as compared to control. β: significantly different as compared to kaempferol-treated cells. BAPTA-AM: bis-(o-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester). Lanes 1 & 2: control. Lanes 3 & 4: kaempferol-treated cells. Lanes 5 & 6: kaempferol + BAPTA-AM-treated cells.

OC cells that were treated with kaempferol and completely reversed in kaempferol-treated cells that were co-incubated with either CQ (autophagy inhibitor) or 4-PBA (ER stress inhibitor) (Fig. 5, A-C). However, the levels of ROS remained unchanged when kaempferol-treated cells were compared to control cells (Fig. 5, D). These data suggest that apoptosis afforded by kaempferol is mediated by activation of ER stress and autophagy and requires Ca^{+2} but not ROS.

Kaempferol increases sensitivity of A2780 chemosensitive OC cells to cisplatin (DPP)-induced cell death independent of ER and autophagy

To determine the sensitivity of A2780 to DPP, cells were incubated with increasing concentrations of DPP (0, 2, 4, 6, 8, 10, 15, 20 μmol/l) and kaempferol (40 μmol/l) in the presence or absence of 4-PBA or CQ and IC_{50} values were determined. The percentages of inhibition of cell survival versus log concentration of all treatments are shown in Fig. 6, A and the calculated IC_{50} (μM/l) for DPP with each treatment are shown in Fig. 6, B. IC_{50} of DPP in control cells was 6.867 ± 0.99 μmol/l (Fig. 6, B). However, the sensitivity of DPP was significantly increased to 3.73 ± 0.59 μmol/l in kaempferol-treated cells and did not significantly differ in kaempferol-treated cells and cells incubated with either 4-PBA or CQ (3.65 ± 0.92 and 4.43 ± 0.66 μmol/l, respectively) (Fig. 6, B). On the other hand, a significant decrease in survival rate and proliferation with a significant increase in apoptosis ratio were noticed in A2780 cells that were treated with DPP or kaempferol alone, or received the

combined treatment (Fig. 6, C-E). On the other hand, the alteration in the levels of all these parameters was more significantly profound in the cells co-treated with a combination of DPP and kaempferol (Fig. 6, C-E).

Kaempferol sensitizes the A2780 OC cells to cisplatin (DPP)-induced cell death by inhibition of Akt

Abundant protein levels of Akt were seen in all treated groups. However, treatment of A2780 OC cells with kaempferol or DPP alone or in combination did not alter the total protein levels of Akt (Fig. 7, A). Still, decreased levels of p-Akt (Ser[473]) with a concurrent increase in p53 and caspase 3 were noticed in cells treated with kaempferol or DPP alone or in combination (Fig. 7, A-B). Interestingly, the maximum decrease in p-Akt (Ser[473]) and the maximum increase in p53 and cleaved caspase 3 were noticed when the cells received concomitant administration of DPP and kaempferol (Fig. 7, A-B).

Discussion

The findings of the current study show that kaempferol is able to inhibit cell proliferation and induces apoptosis in A2780 OC cells by triggering ER stress-mediated cytotoxic autophagy. In addition, and independent of ER stress and autophagy, it shows that kaempferol sensitizes OC cells to DPP-induced cell death by inhibition of the Akt signalling pathway.

It has been reported that ER stress is usually associated with the activation of autophagy, and prolonged ER
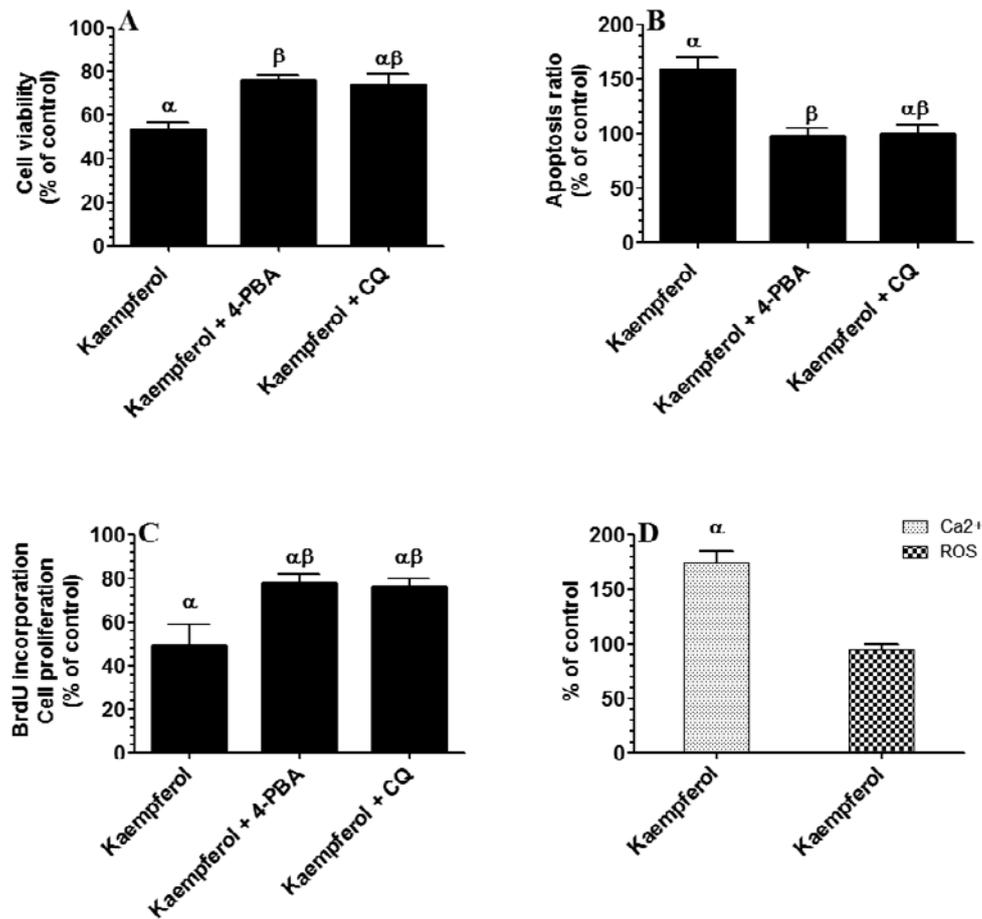
Fig. 5. Kaempferol inhibits cell survival and proliferation, induces apoptosis, and increases intracellular Ca^{2+} levels in A2780 chemo-sensitive OC cells via activation of ER stress and autophagy. Cells were grown in DMEM/F12 media and treated with 40 μmol/l kaempferol in the presence or absence of 10 μmol/l of CQ or 1 μmol/l 4-PBA. Control cells were treated with 0.1% diluted DMSO. Results as shown as the mean ± SD of N = 6 experiments. α: significantly different as compared to control. β: significantly different as compared to kaempferol-treated cells. γ: significantly different as compared to kaempferol + 4-PBA-treated cells. CQ: chloroquine (an autophagy inhibitor); 4-PBA: sodium phenylbutyrate (an ER stress inhibitor).

stress leads to the activation of cell apoptosis (Yadav et al., 2014). Reactive oxygen species (ROS) and intracellular Ca^{2+} overload are two important inducers of ER stress in the cell (Kim et al., 2008). In this study, we have found that all arms of UPR including PERK, ATF6, IRE1α, and their downstream targets p-eIF2α and CHOP were significantly increased in A2780 OC cells after the kaempferol treatment. This response was associated with increased intracellular Ca^{2+} levels with no alterations in the levels of ROS, thus suggesting that the disturbance of Ca^{2+} is one mechanism by which kaempferol induces ER stress in OC cells. To confirm this hypothesis, incubation of cells with kaempferol plus an intracellular Ca^{2+} chelator, BAPTA-AM, completely abolished the ER stress response to kaempferol-induced activation of UPR, thus confirming that a high intracellular Ca^{+2} level is an indispensable factor by which kaempferol induces ER stress.

However, it has been previously shown that ER stress in tumour cells has a dualistic action, to promote cell survival or induce cell death. Indeed, it was shown that a mild UPR and ER stress serve as an adaptive mechanism to enable cancerous cells to create multiple anti-apoptotic mechanisms for preventing cell death and chemotherapy-induced cell death, thus acting as a major factor in inducing chemotherapy drug resistance (Brown and Giaccia, 1998; Moenner et al., 2007; Wang et al., 2014). Also, and as mentioned before, when ER stress is so excessive or severe that it exceeds the protective ability of UPR, the cancerous cells undergo cell apoptosis (Martinon, 2012; Wang et al., 2014; Yadav et al., 2014). Human caspase 4 is the closest paralogue of caspase 12 found in rodents and is usually used as a downstream marker of ER stress-induced apoptosis (Cheng et al., 2018).

In accordance with that, along with the high levels of ER stress, we also found a significant increase in protein levels of both caspase 4 and cleaved caspase 3 in the kaempferol-treated OC cells. In addition, inhibition of ER stress by 4-PBA, a selective ER stress inhibitor,
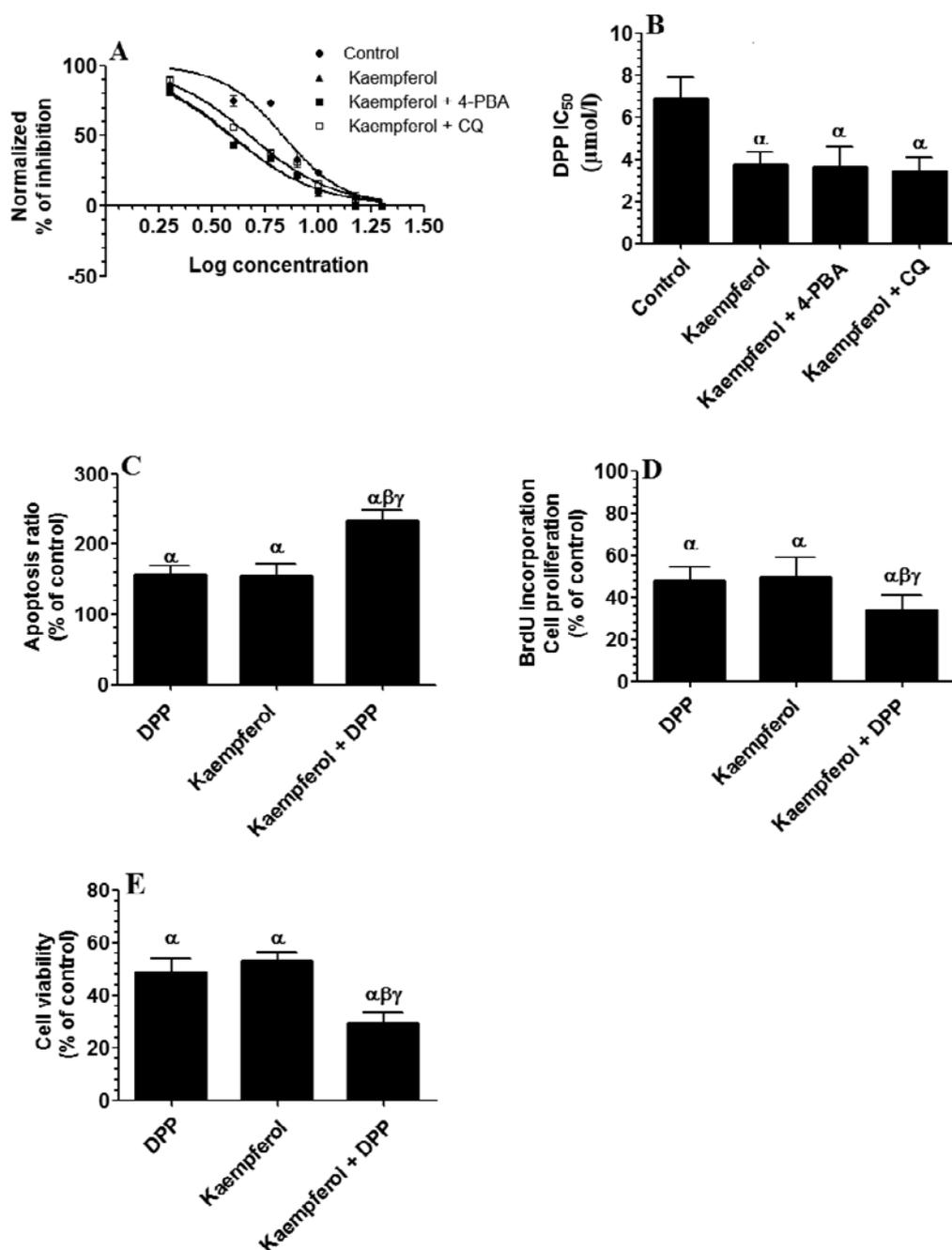
Fig. 6. Kaempferol increases sensitivity of A2780 chemo-sensitive OC cells to cisplatin (DPP)-induced cell death independent of ER and autophagy. Cells were grown in DMEM/F12 media and treated with 40 µmol/l kaempferol (in the presence or absence of 10 µmol/l CQ or 1 µmol/l 4-PBA with increasing concentrations of DPP (0, 2, 4, 6, 8, 10, 15, 20 µM). For DPP treatments, cells were incubated individually with 40 µM kaempferol or DPP (6.8 µM). Control cells were treated with 0.1% diluted DMSO. Results as shown as the mean ± SD of N = 6 experiments. α: significantly different as compared to control. β: significantly different as compared to DPP-treated cells. γ: significantly different as compared to kaempferol-treated cells. CQ: chloroquine (an autophagy inhibitor); 4-PBA: sodium phenylbutyrate (an ER stress inhibitor).

completely abolished the activation of kaempferol-induced activation of caspase 4, indicating the direct role of ER stress in the kaempferol-mediated apoptotic pathway. These findings support the findings of Guo et al. (2017), who showed that kaempferol is able to induce cell apoptosis in hepatocellular carcinoma by activation of ER stress and CHOP.

On the other hand, ER stress is closely associated with subsequent activation of autophagy. Autophagy is an important dynamic and evolutionarily conserved
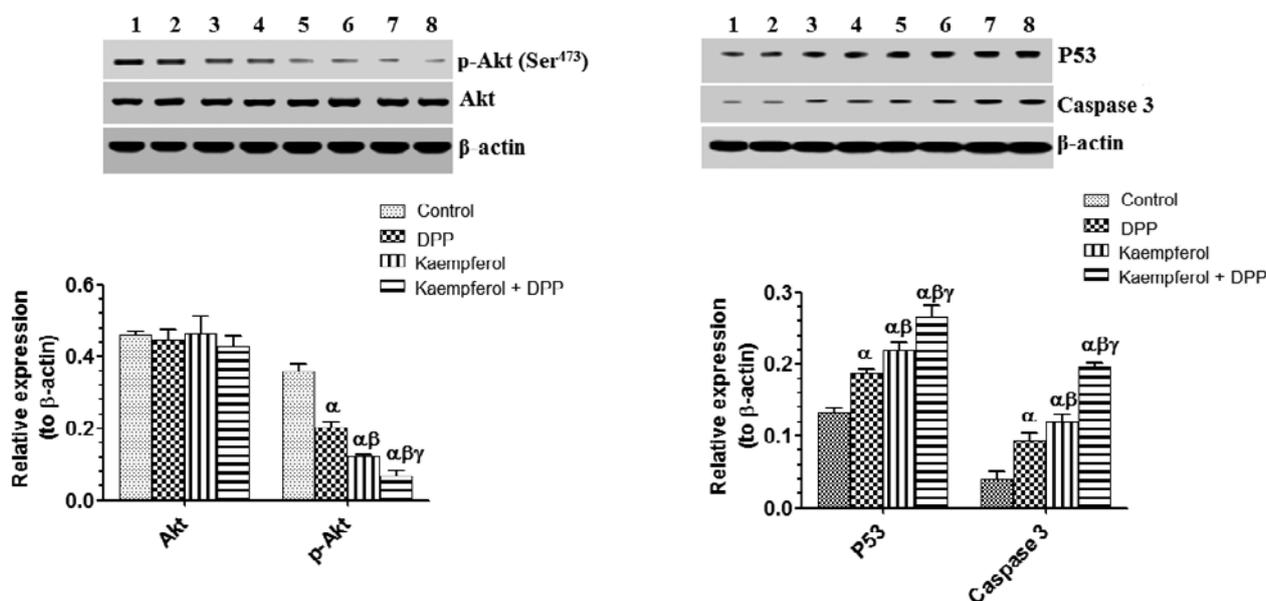
Fig. 7. Kaempferol sensitizes A2780 OC cells to cisplatin (DPP)-induced cell death by inhibition of Akt. Cells were grown in DMEM/F12 media and treated with 40 μmol/l kaempferol and/or 6.8 μmol/l DPP. Control cells were treated with 0.1% diluted DMSO. Results as shown as the mean ± SD of N = 6 experiments. α: significantly different as compared to control. β: significantly different as compared to DPP-treated cells. γ: significantly different as compared to kaempferol-treated cells. CQ: chloroquine (an autophagy inhibitor); 4-PBA: sodium phenylbutyrate (an ER stress inhibitor). Lanes 1 & 2: control. Lanes 3 & 4: DPP-treated cells. Lanes 5 & 6: kaempferol-treated cells. Lanes 7 & 8: kaempferol + DPP-treated cells.

mechanism aimed to maintain the cellular homeostasis; meanwhile, autophagy has a double-faced effect on cancerous cells as it can either promote or suppress survival (Cheng et al., 2018; Bel and Hooper, 2018; Besio et al., 2018). ATG5, LC3 (I/II), and beclin 1 are considered major markers of active autophagy (Mizushima and Komatsu, 2011). In addition, the conversion of LC3-I to LC3-II is a critical event in autophagy initiation in the presence or absence of 4-PBA (Mizushima and Komatsu, 2011).

As expected, we found a significant decrease in LC3I protein levels and a significant increase in protein levels of LC3II, ATG5 and beclin 1 in kaempferol-treated A2780 OC cells. To clarify whether kaempferol-induced ER stress is behind this autophagy response, we re-measured the protein levels of all these markers in kaempferol-treated cells co-incubated with 4-PBA. Interestingly, the protein levels of all autophagy markers were significantly inhibited with an increased protein level of LC3I, confirming that kaempferol-induced autophagy in OC cells is mainly due to the induced ER stress. In addition, to further investigate whether this autophagy response is cytotoxic or protective, we measured the survival ratio, apoptosis rate and cell proliferation in kaempferol-treated A2780 OC cells in the presence or absence of CQ. CQ is a lysosomotropic drug that raises the intralysosomal pH level and inhibits the latest step in autophagy (Amaravadi et al., 2007). As expected, CQ inhibited cell apoptosis and increased sur-

vival and proliferation ratios, suggesting that kaempferol-induced autophagy is apoptotic. Similarly to these data but in hepatocarcinoma cells, kaempferol induced apoptosis by activation of ER stress-mediated activation of CHOP-induced autophagy (Guo et al., 2017).

On the other hand, chemotherapy drug resistance is a universal but important phenomenon in cancer treatment. Both ER stress and autophagy can induce drug resistance in cancerous cells (Carew et al., 2007; Wang et al., 2014). Hence, we next extended our study to investigate the effect of kaempferol on the sensitivity of OC cells to DPP and find out the minimum possible mechanisms behind this. Accordingly, we found increased sensitivity to DPP with kaempferol treatment, as evident by the lower IC_{50} of DPP. These data may suggest the possibility to use kaempferol for advanced clinical studies to increase the effectiveness of DPP therapy against OC. However, this effect was independent of ER stress or autophagy activation, as incubation of OC cells with 4-PBA and CQ failed to alter their sensitivity to DPP.

To investigate the precise mechanism by which kaempferol enhances the sensitivity of A2780 OC cells to DPP, we targeted the PI3K/Akt signalling pathway, given its essential role in cell survival, the well-reported higher levels of its members in OC and their roles in DPP resistance (Kolasa et al., 2009; Mabuchi et al., 2015; Janku et al., 2018). Indeed, it was shown that pharmacological inhibition of the PI3K/Akt signal transduction pathway enhances vulnerability of the can-

cerous cells to chemotherapy, radiotherapy, and hormonal treatment (Engelman, 2009; Nikolopoulos et al., 2010). Also, DPP induces cell apoptosis in chemo-sensitive cancerous cells, including OC, by up-regulation and activation of p53 and higher levels of functional p53, higher chemo-sensitivity and improved clinical outcome in OC patients (Song et al., 1999; Fraser et al., 2008; Geretto et al., 2017). In addition, Akt inhibits p53 nuclear accumulation and activity by inhibiting the p53 content and activity (phosphorylation), disturbing its subcellular localization, and promoting its ubiquitin-dependent proteolysis (Gottlieb et al., 2002; Ogawara et al., 2002)

In this study, both DPP and kaempferol, administered individually, lowered the protein levels of p-Akt (Ser^{473}) and enhanced that of p53, with a more profound effect to be seen with kaempferol treatments. The maximum decrease in Akt phosphorylation and the maximum increase in p53 levels were noticed with the combined treatments. Hence, it could be concluded that kaempferol enhances the sensitivity of OC cells to chemotherapy at least by inhibition of the PI3K/Akt signalling pathway. Thus, in addition to the induction of ER stress and cytotoxic autophagy, these data could suggest that the apoptotic effect afforded by kaempferol is also mediated, in part, by activation of p53. This could explain why we observed a partial increase in cell survival and a partial decrease in cell apoptosis when kaempferol-treated cells were co-incubated with 4-ABP and CQ. In support, Luo et al. (2011) have shown that kaempferol induces cell death in OVCAR-3 and A2780 OC cells by upregulating p53.

Overall, the findings of this study are unique and show that kaempferol could be used as a novel chemotherapeutic drug to induce cell death in OC cells and to enhance the action of DPP. The mechanisms of action could be multiple and include, at least, activation of cytotoxic ER/autophagy and inhibition of cell survival by the inhibition of Akt.

Acknowledgement

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and approved by the ethical committee at the College of Science at King Khalid University.

Authors' contributions

A. E. and A. S. designed the experimental procedure. A. E. received the funding for this study. A. E. and M. A. A. performed cell culture and treatments and supervised the technical staff during the measurements. A. A. and S. A. analysed the collected data and prepared their graphical representation. A. E., A. A. and S. A. generated the first draft of this manuscript. A. S. finalized the manuscript.

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