Isomahanine induces endoplasmic reticulum stress and simultaneously triggers p38 MAPK-mediated apoptosis and autophagy in multidrug-resistant human oral squamous cell carcinoma cells

TANYARATH UTAIPAN1, ANAN ATHIPORNCHAI2,3, APICHART SUKSAMRARN2, SURASAK CHUNSRIVIROT4 and WARANGKANA CHUNGLOK1

1School of Allied Health Sciences, Walailak University, Nakhon Si Thammarat 80161; 2Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240; 3Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Burapha University, Chon Buri 20131; 4Structural and Computational Biology Research Group and Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

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Abstract. Advanced oral squamous cell carcinoma (OSCC) is typically aggressive and closely correlated with disease recurrence and poor survival. Multidrug resistance (MDR) is the most critical problem leading to therapeutic failure. Investigation of novel anticancer candidates targeting multidrug-resistant OSCC cells may provide a basis for developing effective strategies for OSCC treatment. In the present study, we investigated the cytotoxic mechanism of a carbazole alkaloid, namely isomahanine, in a multidrug-resistant OSCC cell line CLS-354/DX. We demonstrated that CLS-354/DX cells overexpressing multidrug resistance-associated protein 1 (MRP1) were resistant to anticancer drugs cisplatin and camptothecin. Isomahanine effectively induced cytotoxicity against CLS-354/DX cells regardless of resistance. Apoptosis as determined by FITC-Annexin V/PI staining and western blot analysis of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) was significantly induced in a time-dependent manner upon isomahanine treatment. Isomahanine-induced caspase-dependent apoptosis was determined using z-VAD-fmk. The effects on autophagy in isomahanine-treated cells were investigated via conversion of LC3B and degradation of p62/SQSTM1 (p62). Isomahanine obviously induced autophagic flux as shown by an increase in punctate GFP-LC3B and the LC3B-II/LC3B-I ratio with a concomitant decrease in p62 levels. Autophagy inhibitors 3-methyladenine (3-MA) and chloroquine (CQ) protected isomahanine-induced cell death, indicating the activation of autophagic cell death. Endoplasmic reticulum (ER) stress and MAPK activation were examined to elucidate the mechanism underlying cell death. The expression levels of PERK, CHOP and phosphorylated MAPK (p38, ERK1/2 and JNK1/2) were upregulated following isomahanine treatment. We found that p38 MAPK inhibitor (SB203580) significantly attenuated isomahanine-induced apoptosis and autophagic flux and this prevented cell death. Collectively, the present study demonstrated that isomahanine was able to induce ER stress and trigger p38 MAPK-mediated apoptosis and autophagic cell death in multidrug-resistant OSCC cells. The potential cytotoxic action of isomahanine may provide the development of anticancer candidates for treating multidrug-resistant cancer.

Introduction

Oral squamous cell carcinoma (OSCC) is the most prevalent cancer occurring in the oral cavity and is the sixth most common cancer in Asia and the eleventh worldwide (1,2). The majority of OSCC patients are typically diagnosed when the cancer develops to an advanced stage, which confers a decrease of the 5-year survival rate to 20% (1). In this advanced-stage cancer,
the phenomenon of multidrug resistance (MDR) is the most critical problem in chemotherapy, leading to therapeutic failure and disease recurrence (3). MDR can be attributed to multiple factors, such as upregulation of the ABC transporter family, aberrant apoptosis, change in DNA damage repair, miRNA regulation and cancer stem cell regulation (4). To circumvent the drug resistance of cancer cells, simultaneous activation of different cell-death pathways, such as apoptosis, autophagy and necroptosis, could be principally applicable (5-8). Hence, investigation of novel anticancer agents targeting contemporary non-apoptotic and apoptotic mechanisms may be a great step forward for multidrug-resistant OSCC treatment.

Apoptosis and autophagy are catalytic processes essential for maintaining cell populations and tissue homeostasis. Apoptosis can be initiated through mitochondrial-mediated and death receptor-mediated pathways. Both pathways lead to activation of a cascade of proteolytic caspases, thereby cleaving cytosolic and nuclear proteins, resulting in nuclear fragmentation and apoptotic death (9). Autophagy is well-known programmed cell death type-1, which has been utilized as a cell-death mechanism to eliminate cancer cells by a number of anticancer compounds (10). Meanwhile, autophagy is a conserved catabolic process whereby damaged cellular constituents are sequestered into double-membrane vesicles known as autophagosomes, which are subsequently degraded by lysosomal machinery (11). During autophagy, the formation of autophagosomes is processed by a conversion of microtubule-associated protein 1 light chain 3 (LC3-I to LC3-II). When the process progresses to the lysosomal degradation step, the cellular p62/SQSTM1 (p62) level is then degraded, and this whole process is referred to as autophagic flux (12). Autophagy plays a pro-death role or pro-survival role by being a ‘double-edged sword’. The pro-death role of autophagy occurs when autophagy is excessively activated, and this has shed light on its anticancer potential (13). The potential of these two cell death pathways to effectively eliminate multidrug-resistant cancer cells has been demonstrated (5,6,14).

Various stress conditions can trigger these cell-death pathways. Endoplasmic reticulum (ER) stress with the consequent unfolded protein response (UPR) is one of the stress responses that confer apoptosis and autophagy (15). Components involved in ER stress and UPR signaling, such as inositol-requiring protein-1 and transcription factor C/EBP homologous protein (CHOP), modulate the mitogen-activated protein kinase (MAPK) signaling pathway, particularly p38 MAPK and JNK, which are important mediators promoting cell death (16,17). Accumulating studies have demonstrated that activated p38 MAPK and JNK are involved in apoptosis and/or autophagy in various cancer cell lines (18-20).

Collective evidence has revealed that carbazole alkaloids exhibit a wide range of anticancer activities demonstrated both in vitro and in vivo (21-23), leading research in carbazole alkaloids to become more attractive in the pharmacological field in the last few decades (24). Isomahanine, a bioactive carbazole alkaloid abundant found in Murraya koenigii, and its isomer mahanine are capable of inducing apoptosis in human leukemia cells via a caspase-dependent pathway (22). Relevant to multidrug-resistant cancers, mahanine can enhance the chemosensitivity of colon and cervical cancer cells to the anticancer drugs cisplatin and 5-fluorouracil (25,26). Carbazole derivatives have been documented to upregulate autophagy and sensitize glioblastoma cells to anticancer drugs (27). However, the function of isomahanine in multidrug-resistant OSCC, along with the underlying mechanisms, have not yet been investigated.

In the present study, we aimed to investigate the cytotoxic mechanisms of isomahanine via apoptosis and autophagy in our established multidrug-resistant OSCC cell line (CLS-354/DX). We found that CLS-354/DX cells exhibited overexpression of multidrug resistance-associated protein 1 (MRP1) protein and a low responsiveness to conventional anticancer drugs. Isomahanine exerted an effective cytotoxic effect against CLS-354/DX cells regardless of their resistance compared to the parental cell line (CLS-354/WT). The compound induced ER stress and simultaneously triggered apoptosis and autophagic flux through the p38 MAPK signaling pathway. This finding supports the potential use of isomahanine to circumvent MDR in OSCC cells.

Materials and methods

Preparation of isomahanine. The bioactive carbazole alkaloid, i.e., isomahanine, was isolated and purified from the CHCl3 extract of M. koenigii leaves by repeated silica gel and Sephadex LH-20 column chromatography. The spectroscopic data of isomahanine were consistent with the reported values (28). The purified carbazole alkaloid was dissolved in dimethyl sulfoxide (DMSO) prior to conducting experiments.

Reagents and antibodies. cis-diaminedichloroplatinum(II) (cisplatin), camptothecin, DMSO, 3-MA, 4-phenylbutyric acid (4-PBA), chloroquine (CQ) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Benzylxycarbonyl-ValAla-Asp (OMe) and fluoromethylketone (z-VAD-fmk) were purchased from InvivoGen (San Diego, CA, USA). Luminita™ Chemiluminescent HRP substrate was purchased from EMD Millipore (Billérica, MA, USA). All primary antibodies (rabbit), HRP-conjugated secondary antibodies (anti-rabbit) and MAPK inhibitors (U0126, SB203580 and SP600125) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell lines and culture. Human OSCC CLS-354 cells (CLS Cell Lines Service GmbH, Eppelheim, Germany) at passage no. 30-40 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Biochrom GmbH, Berlin, Germany), 1% penicillin/streptomycin and 2 mM stable L-glutamine (PAA Laboratories GmbH, Pasching, Austria). The cancer cell line was maintained in an atmosphere of 95% humidity and 5% CO2 at 37°C. CLS-354 cells, as parental cells, were oral epithelial cancer cells exhibiting epithelial-like shape, namely CLS-354/WT (Fig. 1A, left). The mesenchymal phenotype with the elongated shape was isolated by differential trypsinization as previously described (29). The cell line was named as CLS-354/DX (Fig. 1A, right).

MDR assay. Cancer cells (8x10^5) were seeded onto a 60-mm tissue culture dish and allowed to grow for 36 h. Cells (2x10^3 cells/sample) were collected, washed with
phosphate-buffered saline (PBS), and subjected to MDR assay using eFluxx-ID® Green MDR assay kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA) according to the manufacturer's instructions. The cells were analyzed immediately by flow cytometry. The mean fluorescence intensity (MFI) values were analyzed using CellQuest™ Pro (BD Biosciences, San Jose, CA, USA). The MDR activity factor (MAF) for each transporter was calculated according to the formula: 

$$ \text{MAF} = 100 \times \frac{\text{MFI}_{\text{with inhibitor}} - \text{MFI}_{\text{control}}}{\text{MFI}_{\text{with inhibitor}}} $$


Cell viability assay. Cells (1.5x10^4 cells/well) were seeded into 96-well plates and grown for 48 h. Cells were treated with various concentrations of cisplatin (10-25 µM) and camptothecin (0.3-15 µM) or isomahanine (10-25 µM) for 24 h or for various time-points. After treatment, cell viability was assessed by MTT assay. The half-maximal inhibitory concentration (IC_{50}) was calculated from concentration-response curves following a 24-h treatment. The resistance index (RI) was defined as the ratio of the IC_{50} value of the resistant cell line to the IC_{50} value of the parental cell line of each drug.

Annexin V-FITC/PI staining assay. Cells (4x10^5 cells/well seeded on a 6-well plate) were incubated with isomahanine for 0, 6, 12 and 24 h. The cells were harvested, washed with PBS, and stained with Annexin V-FITC and Annexin V-FITC/PI according to the manufacturer's instructions (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Fluorescence intensity (FL-1 and FL-2) was immediately determined by flow cytometry. For each measurement, at least 15,000 cells were counted. The percentages of Annexin V-FITC- and Annexin V-FITC/PI-positive cells were analyzed as apoptotic cells using CellQuest™ Pro (BD Biosciences).

**Analysis of GFP-LC3B puncta.** CLS-354/DX cells (2x10^4 cells/well seeded on a 24-well plate) were transduced with BacMam LC3B-GFP viral particles (MOI=30 plaque-forming units/cell), according to the manufacturer's instructions of the Premo™ Autophagy Sensor kit (Invitrogen, Carlsbad, CA, USA). After 24 h, the cells were treated with 20 µM isomahanine or 50 µM CQ. After a 16-h incubation, green fluorescent signals were monitored and imaged using a Zeiss Axio Vert.A1 inverted microscope (magnification, x40). Cells with >5 GFP-LC3B puncta were considered autophagy-positive. The percentage of cells with punctate GFP-LC3B/total GFP-LC3B-positive cells was determined.

**Western blot analysis.** After isomahanine treatment, the cells were harvested and lysed in lysis buffer containing protease inhibitors. Protein samples (50 µg) were then prepared in Laemmli sample buffer. The proteins were separated on 8-12.5% SDS-PAGE gels and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% non-fat dry milk, washed with a mixture of Tris-buffered saline and Tween-20, and probed with primary antibodies (1:250-1:1,000) at 4°C overnight. The blot was then probed with the HRP-conjugated secondary antibody (1:5,000) at room temperature for 1 h. The proteins were visualized using the chemiluminescent detection system.

**Statistical analysis.** All results are expressed as the mean ± SEM. Data were obtained from at least 3 independent experiments. Significant differences among the different groups were considered at a p-value <0.05, using Student's t-test or one-way ANOVA. The statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).
Results

CLS-354 cells develop a multidrug-resistant phenotype. The multidrug-resistant OSCC cell line was spontaneously generated from the parental human OSCC cell line CLS-354 (passage no. 27), which is epithelial-like in shape (CLS-354/WT) (Fig. 1A, left). After being cultured for >10 passages, some cells became elongated and mesenchymal-like. These mesenchymal-like cells were isolated by differential adhesion (29) and named CLS-354/DX (Fig. 1A, right). These two cell lines had similar growth characteristics. The population doubling times of CLS-354/WT and CLS-354/DX were 31.25±0.64 and 29.17±1.20 h, respectively, which were not significantly different.

To investigate the drug-resistance profiling, the activity of a particular multidrug-resistant transporter [P-gp, MRP1/2 and breast cancer resistance protein (BCRP)] was assessed using eFluxx-ID® Green probe. The probe is a hydrophilic fluorescent dye, which is trapped within the cells unless actively pumped out by these transporters and is detectable by flow cytometry. As shown in Fig. 1B, CLS-354/WT cells exhibited an equal increase of fluorescent signals in the presence of a P-gp inhibitor (verapamil) and an MRP1/2 inhibitor (MK-571) in comparison to the control (Fig. 1B, left). These two cell lines had similar growth characteristics. The population doubling times of CLS-354/WT and CLS-354/DX were 31.25±0.64 and 29.17±1.20 h, respectively, which were not significantly different.

Isomahanine has potential cytotoxicity against CLS-354/DX cells. The responsiveness to cisplatin, a platinum-based anticancer drug and camptothecin, an anticancer alkaloid (Fig. 2A, left and middle), was investigated in the CLS-354/WT and CLS-354/DX cells following 24-h treatments using the cell viability assay. The results showed that the response of CLS-354/DX cells to cisplatin and camptothecin was significantly lower than that of CLS-354/WT cells (Fig. 2B, left and middle). The IC₅₀ values for CLS-354/DX cell line to cisplatin and camptothecin were 33.23±0.96 and >15 µM, respectively, which were 2 times and >65 times, respectively, greater than those of the CLS-354/WT cells as indicated by the RI (Fig. 2C). These data strongly support the contribution of MDR in CLS-354/DX cells. Notably, isomahanine (Fig. 2A, right) at a concentration starting from 20 µM effectively suppressed the viability of CLS-354/DX cells without relevance of resistance (Fig. 2B, right). The IC₅₀ values observed in both cell lines were not significantly different, with an RI value of 1.1 (Fig. 2C).

Isomahanine induces apoptosis in CLS-354/DX cells through a caspase-dependent pathway. The mechanism of cell death was further examined in the CLS-354/DX cells.
morphology was preliminarily observed in a time-course study following isomahanine treatment (20 µM) for 0, 6, 12 and 24 h. As shown in Fig. 3A, the cells after exposure to isomahanine for 6 h presented small cytoplasmic vacuoles. After 12 h, the cells detached and shrunk in size. Eventually, several cells became round in shape and cell death followed after 24-h treatment. Hence, apoptosis was further confirmed by flow cytometric analysis as well as western blot analysis of caspase-3 and poly(ADP-ribose) polymerase (PARP). Isomahanine time-dependently induced early apoptotic and late apoptotic cells to 5.09 and 15.55%, respectively (Fig. 3B, upper panel). Total apoptosis significantly increased in a time-dependent manner, while the maximal induction was increased to 20% following the 24-h treatment (Fig. 3B, lower panel). Isomahanine activated cleavage of caspase-3 and PARP in a time-dependent manner, and the greatest level of increase was observed upon 24-h treatment (Fig. 3C). To confirm caspase-dependent apoptotic death, the cells were treated with isomahanine along with z-VAD-fmk, a pan-caspase inhibitor. The inhibition of caspase activity significantly rescued isomahanine-induced cell death (Fig. 3D). Thus, isomahanine induced apoptotic death via a caspase-dependent pathway.

Isomahanine induces autophagic flux in CLS-354/DX cells. We hypothesized that autophagy may be involved in cell death upon the morphological observations. We determined autophagic induction by analysis of GFP-LC3B puncta. Cells treated with isomahanine displayed a punctate pattern of GFP-LC3B localized in the perinuclear region, indicating autophagic induction. Isomahanine-induced GFP-LC3B puncta formation by 50%, which was comparable to that of CQ (~60%), a positive control (Fig. 4A). We further performed western blotting of microtubule-associated protein 1B-light chain 3 (LC3B-II), which is an autophagosomal marker. Isomahanine induced the expression of LC3B-II in a time-dependent manner, and the expression of LC3B-II/LC3B-I ratio significantly increased after 12-h treatment (Fig. 4B). Thus, isomahanine induced autophagy in CLS-354/DX cells.

An increase in the number of autophagosomes may be due to activation or inhibition of autophagic flux. During autophagy, the cellular level of the p62 protein is typically degraded, and its decreased level serves as an indicator of autophagic flux (14). We further examined the expression of p62 with isomahanine treatment. Isomahanine markedly decreased the expression level of p62 after 24 h, indicating autophagic flux (Fig. 4C). We also excluded the effect of proteasomal degradation activity on the p62 protein using MG-132, a proteasome inhibitor. The result revealed that isomahanine decreased the level of p62 as usual, while MG-132 did not inhibit its decrease (Fig. 4D). To assess the contribution of autophagy to cell death, the effect of autophagy...
inhibitors on cell viability was determined. Results revealed that the autophagy inhibitors 3-MA and CQ significantly inhibited isomahanine-induced cell death in the CLS-354/DX cells (Fig. 4E-F). Collectively, these findings suggest that autophagic flux induced by isomahanine was involved in the cytotoxic effects of the CLS-354/DX cells.

Isomahanine induces ER stress and MAPK activation in CLS-354/DX cells. To further elucidate the cell-death mechanisms induced by isomahanine, we examined the effect of this compound on the activation of ER stress and MAPK phosphorylation. As shown in Fig. 5A, isomahanine activated the phosphorylation of protein kinase PNA (PKR)-like ER kinase (PERK), an ER stress sensor, in a time-dependent manner. Concomitantly, the compound induced the expression of the transcription factor CHOP, which is a marker of UPR (Fig. 5A). Activation of the MAPK family members, p38, ERK1/2 and JNK1/2 was also determined. The phosphorylation of p38, ERK1/2 and JNK1/2 was markedly upregulated after exposure to isomahanine for 6 h. At 12 h of treatment, the phosphorylation of p38 and ERK1/2 was strongly activated, while their activation slightly decreased after 24 h (Fig. 5A). JNK1/2 was activated to a maximal level at 6 h and decreased after 12 h (Fig. 5A).

Next, we applied 4-PBA, an inhibitor of ER stress, to demonstrate the relation between ER stress and MAPK pathway. As shown in Fig. 5B, the activated PERK and CHOP proteins were significantly decreased in the presence of 4-PBA. ER stress inhibition protected against isomahanine-activated p38 MAPK phosphorylation. Meanwhile, the activation of ERK1/2 and JNK1/2 were concomitantly increased by 4-PBA (Fig. 5B). Collectively, these results suggest that ER stress mediated the activation of p38 MAPK, but not that of ERK1/2 and JNK1/2.

Isomahanine induces p38 MAPK-mediated apoptosis and autophagy in CLS-354/DX cells. Activation of MAPK in response to ER stress can promote cell death by apoptosis and autophagy. To study the role of MAPK in apoptosis- and autophagy-induced cell death, we assessed the cell viability with isomahanine treatment in the presence of inhibitors of
As shown in Fig. 6A, SB203580 and U0126 significantly suppressed isomahanine-induced cell death, implying that p38 MAPK and ERK participate in cell death. The function of p38 MAPK and ERK in the induction of apoptosis and autophagy was further examined using inhibitors SB203580 and U0126. Isomahanine activated caspase-3, LC3B-II and CHOP and concomitantly decreased the expression level of p62 as usual. SB203580 inhibited MAPKAPK-2, a downstream effector of p38 MAPK (Fig. 6B). The blockade of p38 MAPK completely inhibited isomahanine-activated caspase-3, LC3B-II and p62, but SB203580 did not affect CHOP expression (Fig. 6C). U0126 markedly inhibited ERK phosphorylation (Fig. 6B), and this attenuated only isomahanine-activated caspase-3 (Fig. 6C). These data indicated that p38 MAPK was an important cytotoxic mediator mediating both apoptosis and autophagic flux in response to isomahanine. Meanwhile, the activation of ERK, in part, regulated apoptosis under the tested conditions. As CHOP was not affected by MAPK inhibition, ER stress may be an upstream regulator involved in this activation. The overall cytotoxic mechanism of isomahanine against CLS-354/DX cells is illustrated in Fig. 6D.
Discussion

MDR in advanced OSCC is associated with disease recurrence and poor patient survival (30,31). To overcome multidrug-resistant cancer, identification of effective therapeutic regimens suitable for drug resistance in different tumors by targeting different cell-death pathways, particularly apoptosis and autophagy, has recently received attention (5-8,32). Herein, we isolated a carbazole alkaloid, namely, isomahanine from M. koenigii leaves. The cytotoxic activity of this compound against the multidrug-resistant OSCC cell line CLS-354/DX was examined. We discovered that isomahanine (20 µM) exerted cytotoxicity against CLS-354/DX cells regardless of resistance after stopping treatment. However, some cells were able to recover their proliferation following treatment with low concentrations of isomahanine (10 µM). These cells may acquire resistant phenotypes due to a pro-survival autophagy; hence, the confirmation process may take a period of time from several months to a year (33). Isomahanine cytotoxicity was due to the induction of apoptosis and autophagic cell death in CLS-354/DX cells. This provided the notion that isomahanine may be useful for eliminating multidrug-resistant cancer cells.

CLS-354/DX cells spontaneously developed MDR through increased efflux activities of MRP and P-gp, increased expression of the MRP1 protein and elevated IC50 and RI values to cisplatin and camptothecin in comparison to the parental CLS-354/WT cells (4). Overexpression of MRP1 indicates intrinsic drug resistance, which confers resistance to a wide

![Figure 6. p38 MAPK mediates autophagy- and apoptosis-induced cell death in isomahanine-treated CLS-354/DX cells. (A) Cell viability of CLS-354/DX cells was assessed by MTT assay after treatment with isomahanine (20 µM) in the absence or presence of inhibitors against p38 (20 µM SB203580) (left), ERK (10 µM U0126), and JNK (25 µM SP600125) for 24 h. Data are expressed as mean ± SEM of 4 independent experiments; ***p<0.001 vs. the control without any treatment; †p<0.05, ‡p<0.001 vs. the isomahanine treatment alone. Cells were treated with isomahanine (ISM) (20 µM) for 24 h in the absence or presence of SB203580 (SB) (20 µM) and U0126 (U0) (10 µM). After treatment, (B) expression of MAPKs (MAPKAPK2, p38 and ERK1/2) was determined by western blotting, and (C) expression of apoptosis, autophagy, and ER stress markers was determined by western blot (left) and densitometric analyses of relative expression level (right). Data are expressed as mean ± SEM of 3 independent experiments; *p<0.05, **p<0.01, ***p<0.001 vs. the control without any treatment; †p<0.05 vs. isomahanine treatment alone. (D) The proposed cytotoxic mechanism of isomahanine in CLS-354/DX cells. 3-MA, 3-methyladenine; 4-PBA, 4-phenylbutyric acid; CQ, chloroquine; UPR, unfolded protein response.]

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range of anticancer drugs, including camptothecin (34) and cisplatin (35). High expression of MRP1 was observed in tongue carcinoma, which was consequently associated with cisplatin resistance, suggesting the existence of this protein in head and neck cancer samples (36). A slightly increased MDR1 activity observed in CLS-354/DX cells may be due to MRP1, which has a significantly overlapping resistance profile with P-gp (37).

Isomahanine triggered CLS-354/DX cells to undergo apoptosis via the caspase-dependent pathway. Our observation was consistent with an earlier study that reported ROS-mediated caspase-dependent apoptosis in leukemia cells (21). Disruption of mitochondrial function may be a contributing effect to apoptosis. The release of cytochrome c into the cytosol can lead to formation of apoptosomes and activation of caspase-3 through a mitochondrial-dependent pathway (38,39). Next, we clearly demonstrated that isomahanine activated autophagic flux, which contributed to the cytotoxicity against CLS-354/DX cells. Accumulating data have shown that plant alkaloids selectively targeted autophagic cell death against multidrug-resistant cancer cells via different mechanisms, such as activation of AMP-activated protein kinase (40), ER stress, inhibition of the Akt/mTOR pathway (41) and induction of autophagic flux (42). Although the role of autophagy in cancer is still controversial as autophagy can either drive cell survival or cell death, pro-death is likely effective in particular multidrug-resistant cancer cells (40,42). Hence, the isomahanine-induced autophagic pathway may circumvent conventional drug resistance in cancer.

In our experimental setting, ER stress was activated following isomahanine treatment as shown by the increased levels of p-PERK and CHOP. ER stress is a crucial stress signal caused by increased accumulation of unfolded proteins within the ER. From a structural point of view, isomahanine may disrupt protein folding in the ER, similar to the effect of plant alkaloid ellipticine-induced ER stress (43). Planar structure of the carbazole moiety and lipophilic property of isomahanine may contribute to ER disruption. Excessive or prolonged ER stress response can further initiate cell death pathways (15). Three canonical MAPKs (ERK1/2, JNK and p38) are recognized to be activated in response to ER stress-induced cell death (16). We found that p38 MAPK and ERK1/2 are involved in isomahanine-induced apoptosis. During ER stress, inositol-requiring protein-1 activates apoptosis signal-regulating kinase 1, which is essential for the activation of p38 MAPK and JNK (16,44). Isomahanine-induced p38 MAPK not only triggered apoptosis but also regulated autophagic flux. This may be due to the fact that autophagy requires p38 MAPK at the sequestration step during autophagosome formation induced by nutrient deprivation (45). This mechanism is consistent with previous studies on human gingival fibroblasts and human tongue squamous cell carcinoma cells (45,46). p38 MAPK can act as an important mediator of apoptotic and autophagic cell death induced by isomahanine. ER stress induced or decreased ERK1/2 activation with different kinetics. Short-term induction of ER stress (<6 h) resulted in a decrease in ERK phosphorylation, while long-lasting activation (>10 h) induced ERK phosphorylation in hepatocellular carcinoma cells (47). In addition, we demonstrated that the MEK inhibitor rescued isomahanine-induced cell death and decreased cleavage of caspase-3. ERK-dependent apoptosis can be activated by mitochondrial cytochrome c release or caspase-8 activation, permanent cell cycle arrest, or autophagic vacuolization, depending on the different cellular contexts (48).

In conclusion, the present study elucidated the cytotoxic mechanism involved in the apoptosis and autophagy induced by isomahanine in multidrug-resistant OSCC cells. Isomahanine was capable of inducing ER stress, which may regulate apoptosis as well as autophagic cell death via p38 MAPK. The simultaneous autophagic cell death with apoptosis induced by isomahanine may provide a novel anticancer strategy to circumvent MDR in cancer.

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