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Metformin sensitizes cholangiocarcinoma cell to cisplatin-induced cytotoxicity through oxidative stress mediated mitochondrial pathway

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ABSTRACT

Aims: Metformin (Met), an essential antidiabetic agent, shows antitumor activity in some cancers. A previous study showed that Met enhanced cytotoxic activity of cisplatin (Cis) in cholangiocarcinoma (CCA) in association with the activation of AMP-activated protein kinase and suppression of Akt-mTOR. However, these effects do not entirely explain the observed chemosensitizing effect. The present study investigated the interaction of Met and Cis over the enhanced antitumor effect.

Main methods: KKU-100 and KKU-M156 cells were used in the study. Cytotoxicity was assessed by acridine orange-ethidium bromide staining. Reactive oxygen species (ROS) and mitochondrial transmembrane potential ($\Delta\psi_m$) were measured by dihydroethidium and JC-1 fluorescent methods. Cellular glutathione (GSH) and redox ratio were analyzed by enzymatic coupling assay. Proteins associated with antioxidant system and cell death were evaluated by western immunoblot.

Key findings: Cytotoxicity of Cis was enhanced by Met in association with ROS formation and GSH redox stress. The antioxidants, *N*-acetylcysteine and TEMPOL, and MPTP inhibitor, cyclosporine, attenuated cytotoxicity in association with suppression of ROS formation and the losses of $\Delta\psi_m$. Met in combination with Cis suppressed expression of Nrf2 and altered the expression of Bcl2 family proteins.

Significance: The chemosensitizing effect of Met in combination with Cis is causally associated with increased oxidative stress-mediated mitochondrial cell death pathway. Met may improve the efficacy of Cis in the treatment of cancer.

1. Introduction

Cholangiocarcinoma (CCA) originates from bile duct epithelial cells and is the second most common primary liver cancer. While CCA is a rare malignancy worldwide, the incidences of intrahepatic CCA in many countries are on the rise [1]. CCA patients are usually asymptomatic in early stages and typically are diagnosed when the disease is already at advanced stage. Although complete resection of tumor is the most effective treatment, less than one-third of the patients are eligible for this surgery. Chemotherapy is usually considered to be the mainstay palliative treatment for patients with unresectable tumors [2]. However, responses to anticancer agents are generally poor probably due to the occurrence of multiple drug resistance phenotype in cancer cells [3]. Novel strategies to overcome the resistance are under active investigation. Recent works have provided evidences that suppression of transcription factor Nrf2 and its down-stream antioxidant and cytoprotective genes including HO-1 and NOQ1 could enhance the cytotoxic effect of several anticancer agents [4–6]. Furthermore, other strategies including upregulation of retinoic acid receptor-β and suppression of PI3K/mTOR show antitumor activity in cholangiocarcinoma cells [7,8].

Metformin (Met) is an eminent antidiabetic agent for the treatment of type 2 diabetes. In epidemiological studies, Met may reduce the risk for some solid cancers [9,10]. Met has demonstrated an anticancer effect and also enhanced the cytotoxic effect of other anticancer agents, such as cisplatin (Cis), 5-fluorouracil, sorafenib and etoposide [11–14]. It has been suggested that the anticancer and chemosensitizing effects of Met are mediated via activation of AMP-activated protein kinase (AMPK) and suppression of Akt-mTOR pathway. However, these effects still do not entirely explain the observed chemosensitizing effect [13–15]. Met also induces oxidative stress and increases ROS formation

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in various cells [16,17]. The increase in ROS formation in association with mitochondrial injury in breast cancer cells has been reported as a mechanism for the antitumor activity of Met [18].

It is accepted that Met mediates insulin sensitivity by specifically inhibiting mitochondrial complex I, leading to the activation of AMPK and inhibition of mTOR and its down-stream genes [19]. However, inhibition of complex I by Met, in contrast with rotenone, a potent complex I inhibitor, does not lead to increase ROS formation. This is probably due to Met suppressing the reverse electron flux-related ROS production and nature of relatively mild inhibition of complex I [20]. Hence, Met induced formation of ROS may happen through other mechanisms. In general, cellular oxidants and redox homeostasis are regulated by the cellular antioxidant system. Nrf2 is a critical transcription factor regulating expression of antioxidant and metabolic genes [21] in response to various oxidants and electrophiles [22]. Previous reports showed that Met decreased Nrf2 mRNA expression [17]. Thus, down-regulation of Nrf2 could cause an increase in oxidant formation and lead to mitochondrial dysfunction [23].

Cisplatin (Cis) is an anticancer agent that is widely used in many solid and hematologic cancers including cholangiocarcinoma [24,25]. Its mechanism of action involves induction of DNA crosslinks and oxidative stress, leading to cell death [24]. Cis activates Nrf2 and its downstream antioxidant genes resulting in a decrease in the efficacy of anticancer activity [26]. It is probable that Met enhances the cytotoxic effect of Cis because of suppression of Nrf2 and an increase in the formation of ROS. The present study investigated the chemosensitizing effect of Met when used in conjunction with Cis. The study examined whether Met enhanced Cis cytotoxicity was related to induction of oxidant formation and redox stress leading to the mitochondrial dysfunction. The study suggests a potential mechanism of chemosensitizing effect of Met in combination with Cis in the inhibition of CCA cells.

2. Materials and methods

Cell culture reagents were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA). Metformin was obtained from Abhilash Chemicals and Pharmaceuticals (Tamil Nadu, India). Cisplatin was obtained from Boryung Pharmaceutical (Seoul, South Korea). *N*-acetyl-L-cysteine (NAC), 4-hydroxy-TEMPO (TEMPOL), Acridine orange (AO), and ethidium bromide (EB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dihydroethidium (DHE) was purchased from Calbiochem (EMD Chemical, San Diego, CA, USA). JC-1 Mitochondrial Membrane Potential Assay Kit and thiol green indicator were obtained from Abcam Inc. (Cambridge, MA, USA). 1-Methyl-2-vinylpyridinium triflate (M2VP) was purchased from Fluka Chemical (Buch, Switzerland). Nuclear and cytoplasmic extraction reagents were obtained from Thermo Scientific (Rockford, IL, USA). Phosphatase inhibitor cocktail was purchased from Pierce Biotechnology (Rockford, IL, USA). Protease inhibitor cocktails and RIPA lysis buffer were obtained from VWR international, LLC (Solon, OH, USA). Luminata™ Forte Western HRP substrate was obtained from Merck Millipore Corporation (Billerica, MA, USA). Primary antibodies against Nrf2 (sc-13032), ERK1/2 (sc-135900), γ-GCSc (sc-390811), HO-1 (sc-7695), BAX (sc-493), Bcl-2 (sc-65392), cytochrome *c* (sc-13560), actin (sc-1616), goat anti-mouse IgG-horseradish peroxidase (HRP) secondary antibody (sc-2031), goat anti-rabbit IgG-HRP secondary antibody (sc-2030), and mouse anti-goat IgG-HRP secondary antibody (sc-2354) were obtained from Santa Cruz Biotechnology (San Diego, CA, USA). Primary antibodies phospho-Akt (#4056), Akt (#9272) and phospho-ERK1/2 (#9106) were purchased from Cell Signaling Technology (Danvers, MA, USA). Other chemicals were of highest purity commercially available.

2.1. Cell lines and cell cultures

The human cholangiocarcinoma (CCA) cell lines KKU-100 and KKU-M156 were used in the study. The cells were derived, established, and characterized from CCA tissues of Thai patients [27]. At the beginning of experiments, frozen cells in stocks were revived and their characteristics including cell morphology and growth kinetics were assessed before use. The CCA cells were cultured in Ham's F12 media supplemented with 50 μg/mL gentamicin, 100 U/mL penicillin, 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.3), and 10% fetal bovine serum (FBS). The cells were maintained under an atmosphere of 5% CO_2 in air at 37 °C. The CCA cells were subcultured every 2–3 days using 0.25% trypsin-EDTA, and the media was refreshed after an overnight incubation.

2.2. Fluorescent dye staining

Cells were stained with acridine orange and ethidium bromide (AO/EB) fluorescent dyes to evaluate cell viability, apoptosis, and necrosis, as described previously [28]. In brief, KKU-M156 and KKU-100 cells (7500 cells/well) were seeded onto 96 well plates and cultured overnight. The cells were treated with various test agents with or without the antioxidant agents NAC and TEM for the indicated period of time. Cultured cells were washed with PBS and stained with AO/EB dye solution. The viable, apoptotic, and necrotic cells were evaluated under a Nikon Eclipse TS100 inverted microscope with excitation and long-pass emission filters of 480 and 535 nm, respectively. The live cells appeared uniformly green under fluorescent microscope. The early apoptotic cell appeared in green stained cells containing with bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. The cells in the late stages of apoptosis appeared in orange stained cells containing nuclear fragmentation and chromatin condensation. Necrotic cell appeared in orange staining, but with a nuclear morphology that had no chromatin condensation. Fluorescent images were taken at predetermined areas in triplicate using Nikon Coolpix digital camera, and the numbers of cell types were counted and analyzed.

2.3. Detection of ROS

Cellular reactive oxygen species (ROS) were detected by staining with DHE, a cell-permeable florescent probe according to previously described method [28]. KKU-M156 cells at density of 2×10^4 cells/well were cultured into 96-well black plates overnight. Cells were pretreated 3 h before experiments with NAC (2 mM) and TEM (0.25 mM). Afterwards, the cells were incubated with 25 μM DHE in serum-free media at 37 °C for 30 min. The cells were then rinsed with PBS, and the fluorescent signal was measured using Gemini XPS fluorescent plate reader (Molecular Devices, San Jose, CA, USA) with excitation and emission wavelengths of 518 and 605 nm, respectively.

2.4. Assay of glutathione and glutathione disulfide

Cellular total GSH (TTGSH), reduced GSH (GSH), and glutathione disulfide (GSSG) levels were determined by a method previously described [29] with modifications. Briefly, after KKU-M156 cells were treated according to the experimental design, the cells were trypsinized and washed with cold PBS buffer. The cell pellets were resuspended and divided into three aliquots for determinations of TTGSH, GSSG, and protein concentration. An aliquot of the cell suspension was used for protein determination by the Bradford's dye binding assay. For detection of GSSG, the cell suspension was reacted with M2VP (5 mM) following the method previously described [28]. The suspensions were stored at −20 °C until analysis. For assay of GSSG and TTGSH, the cell suspensions were deproteinized by metaphosphoric acid and supernatants were collected for assay by the enzymatic method. The assay reaction was consisted of NADPH (0.2 mM), glutathione reductase (2.66 unit/mL), thiol green indicator (25 μM), and samples or a GSH standard solution (0.156–5.0 μM). The fluorescent signals were read by

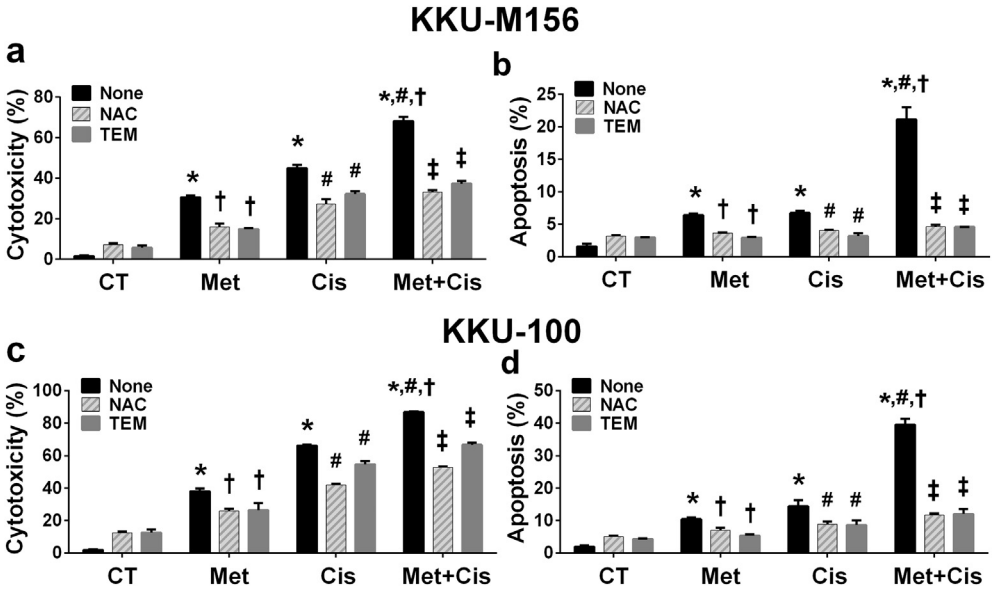

Fig. 1. Met enhances Cis-induced cytotoxicity and is associated with oxidative stress. KKU-M156 (a & b) and KKU-100 (c & d) cells were pretreated with 2 mM NAC or 0.25 mM TEMPOL (TEM) for 3 h before treatment with 3 mM Met, 3 μM Cis, or the combination of 3 mM Met and 3 μM Cis for 24 and 36 h for KKU-M156 and KKU-100 cells, respectively. Cell cytotoxicity and apoptosis were evaluated by AO/EB assay. Each bar represents the mean ± SEM from three experiments. *$p < 0.05$ vs control group, #$p < 0.05$ vs Cis alone group, †$p < 0.05$ vs Met alone group, ‡$p < 0.05$ vs combination Met-Cis group.

Gemini XPS microplate reader with excitation and emission wavelengths of 490 and 525 nm, respectively. The levels of TTGSH, GSH, and GSSG were calculated using the GSH standard curve. The ratio of GSH and GSSG was calculated as a measure of GSH redox state.

2.5. Mitochondrial membrane potential assay

The assay for mitochondrial transmembrane membrane potential ($\Delta\psi_m$) was performed by using JC-1 fluorescent dye (,5′,6,′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolyl carbocyanine iodide) staining, as previously described [28]. KKU-M156 cells (2×10^4 cells/well) were seeded onto a 96-well black plate and allowed to adhere overnight. After treatment as indicated, the cells were centrifuged at 1000 rpm at room temperature for 5 min, and 5 μM of JC-1 dye in serum-free media was added. The cells were incubated at 37 °C for 45 min, then rinsed with dilution buffer. Fluorescent signal for J-aggregates and J-monomers was read at excitation and emission wavelengths of 535 and 595 nm, and 485 and 535 nm, respectively. The ratio of J-aggregates and J-monomers was calculated. The shift down of the ratio of fluorescent intensity is indicative of depolarization of $\Delta\psi_m$.

2.6. Preparation of whole cell extract

KKU-M156 and KKU-100 cells were cultured into 6-well plates at density of 2.5×10^5 cells/well and allowed to grow for overnight. After the experiment, the cells were rinsed with cold PBS, pH 7.4, and lysed with RIPA cell lysis buffer (0.5% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, 1% NP-40, 50 mM Tris-HCl (pH 7.4), 20 mM NaF, 50 mM glycerophosphate, 20 mM EGTA, 0.5 mM PMSF, 1 mM DTT, and 1 mM Na_3VO_4) containing phosphatase- and protease inhibitors. The protein samples were scraped and transferred into a microtube. The samples were vigorously vortexed for 15 s at every 10 min for total 30 min. The protein samples were centrifuged at 12,000 rpm at 4 °C for 30 min and supernatants were collected and stored at −80 °C until used. The concentration of protein was determined using Bradford reagent.

2.7. Preparation of nuclear protein extract

Nuclear protein extract was performed as described previously [26]. KKU-100 and KKU-M156 cells were cultured with density of 1×10^6 cells/well in 100-mm plates overnight. After treatment, the cells were harvested and rinsed with cold PBS, pH 7.4. Nuclear extraction was performed according to the manufacturer's instructions (Thermo scientific, Rockford, IL, USA). Protein concentration was measured by using the Bradford's dye binding assay.

2.8. Western blot analysis

The Western blot analysis was performed according to our method previously described [5]. Briefly, the sample proteins were electrophoretically separated on a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE). The protein bands were transferred to polyvinylidene difluoride (PVDF) membranes by wet electroblotting. All blots apart from those used to detect phospho-proteins were blocked with 5% (w/v) skimmed milk powder in TBS, pH 7.6 containing 0.1% Tween-20 (TBST) at room temperature for 2 h. Blots for detection of phospho-proteins were blocked with 5% (w/v) BSA in TBST. The blots were incubated with the appropriate primary antibodies at 4 °C for overnight including rabbit monoclonal antibody against p-Akt (1:1000), rabbit monoclonal antibody against Akt (1:1000), mouse monoclonal antibody against p-ERK1/2 (1:1000), mouse monoclonal antibody against ERK1/2 (1:1000), rabbit polyclonal antibody against Nrf2 (1:2000), rabbit polyclonal antibody against BAX (1:1000), mouse monoclonal antibody against Bcl-2 (1:1000), mouse monoclonal antibody against cytochrome *c* (1:1000), mouse monoclonal antibody against γ-GCSc (1:1000), goat polyclonal antibody against HO-1 (1:1000) and goat polyclonal antibody against actin (1:5000). Afterwards, all blots were incubated with the appropriate horseradish peroxidases-conjugated secondary antibodies (1:2500) at room temperature for 2 h. The intensity of target protein bands was visualized and captured by using Luminata™ Forte Western HRP substrate (Millipore Corporation, Billerica, MO, USA) and ChemiDoc™ MP imaging System (Bio-Rad Laboratories, Hercules, CA, USA). The bands were quantified using Gel-Pro Analyzer, and the relative intensity of the targeted protein band was normalized using the intensity of β-actin as loading control.

2.9. Statistical analysis

Data were presented as mean ± SEM of three independent experiments. Comparisons between control and treatment groups were performed by ANOVA with Student-Newman-Keuls post-hoc test. The threshold for statistical significance was set at $p < 0.05$.

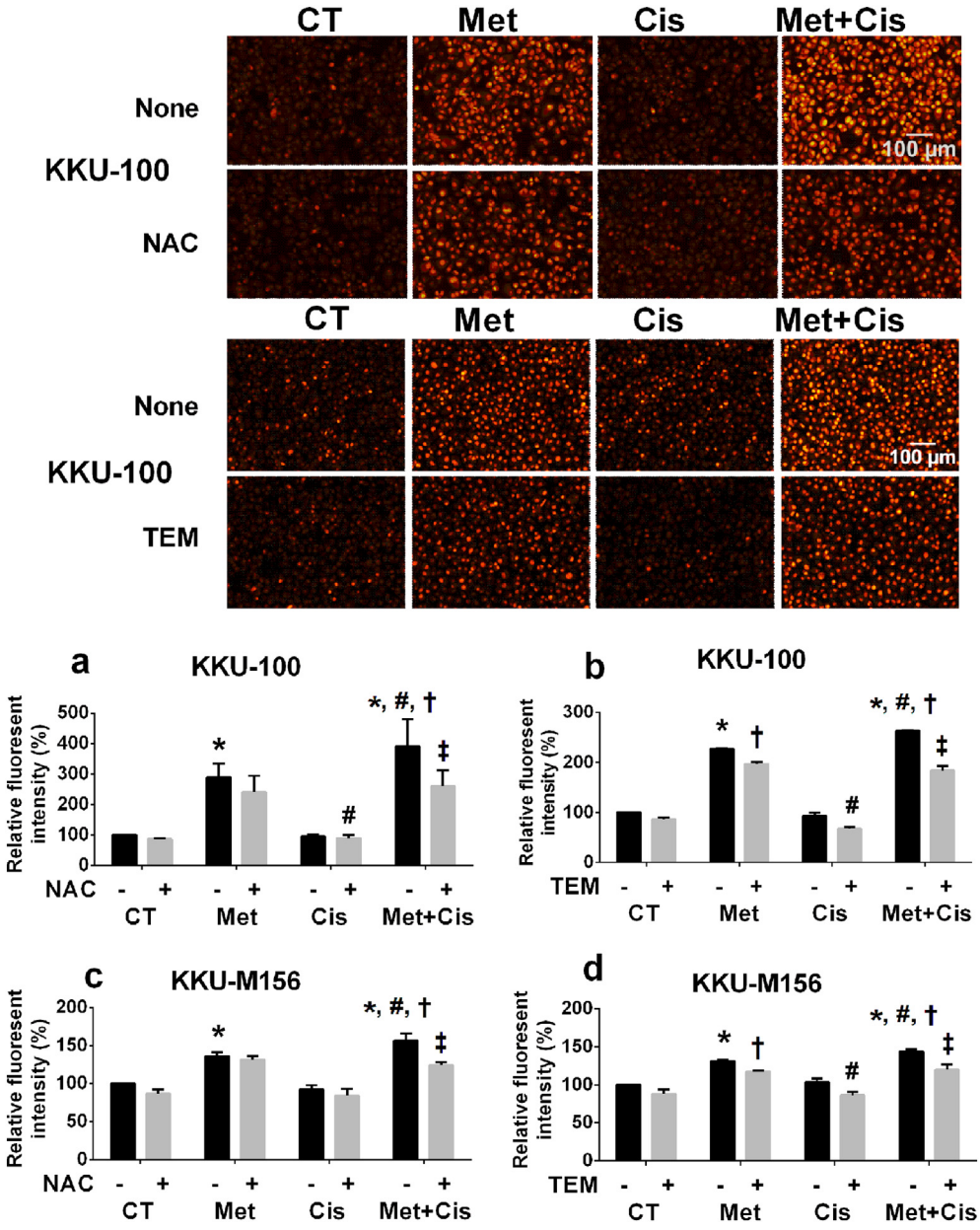

Fig. 2. Met enhances Cis-induced ROS formation in CCA cells. KKU-100 (a & b) and KKU-M156 (c & d) cells were incubated with 3 µM Cis (Cis) or 3 mM Met (Met) alone or combination Met-Cis for 6 h before ROS levels were determined by DHE assay. Cells were pretreated with antioxidant compounds, 2 mM NAC and 0.25 mM TEMPOL (TEM) for 3 h before treatment with Cis and Met. Fluorescent images of KKU-100 cells stained with DHE after various treatments are shown. Each bar represents the mean ± SEM from three experiments. $^{*}p < 0.05$ vs control group, $\#p < 0.05$ vs Cis alone group, $\dagger p < 0.05$ vs Met alone group, $\ddagger p < 0.05$ vs combination Met-Cis group.

Table 1
Effect of Met and Cis on cellular GSH level. KKU-M156 cells were incubated with Met, Cis and the combination drug for 6 h before assay for cellular TTGSH, GSH and GSSG levels, and GSH/GSSG ratio. Each value represents the mean ± SEM from three experiments. $^{*}p < 0.05$ vs control group, $^{\dagger}p < 0.05$ vs Met alone group, $^{\#}p < 0.05$ vs Cis alone group.

Treatment	TTGSH (nmole/mg protein)	GSH (nmole/mg protein)	GSSG (nmole/mg protein)	GSH/GSSG ratio
Control	102.01 ± 4.66	99.60 ± 4.52	2.40 ± 0.19	43.73 ± 3.68
3 mM Met	69.66 ± 7.46*	67.40 ± 7.32*	2.26 ± 0.14	30.64 ± 0.44*
3 µM Cis	83.74 ± 8.97*	81.27 ± 8.81*	2.47 ± 0.18	33.88 ± 1.16*
Met + Cis	79.82 ± 11.79*	76.92 ± 11.61*	2.90 ± 0.18$^{*,\dagger,\#}$	26.75 ± 1.04$^{*,\#}$

3. Results

3.1. Metformin sensitizes CCA cells to cisplatin-induced cytotoxicity involving oxidative stress

Our recent study has showed that Met enhanced Cis induced-anti-proliferation of CCA cells [13]. Moreover, it has been reported that the cytotoxic effect of Met or Cis is associated with oxidative stress [16,23]. It is probable that the chemosensitizing effect of Met when used in combination with Cis is mediated by enhanced oxidative stress. KKU-M156 and KKU-100 cells were pretreated with the antioxidant compounds NAC (2 mM) or TEMPOL (0.25 mM) before treatment with Met and Cis. Consistent with our previous study, the combination of Met and Cis showed significant increased cytotoxicity and apoptosis compared with treatment with each agent alone (Fig. 1a & b for KKU-156 cells and Fig. 1c & d for KKU-100 cells). Treatment with NAC or

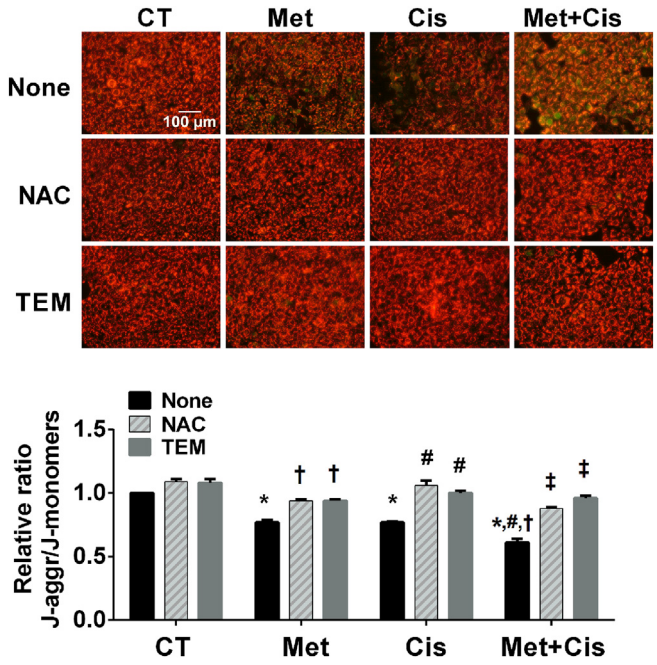

Fig. 3. Effect of Met and Cis on mitochondrial transmembrane potential. KKU-M156 cells were pretreated with 2 mM NAC (NAC) or 0.25 mM TEMPOL (TEM) for 3 h before treatment with 3 mM Met (Met), 3 μM Cis (Cis) or a combination Met-Cis for a further 6 h. The relative ratio of J-aggregates/J-monomers was determined from JC-1 assay. Fluorescent images of the cells stained with JC-1 dye are shown. Each bar represents the mean ± SEM from three experiments. $*p < 0.05$ vs control group, $\#p < 0.05$ vs Cis alone group, $\dagger p < 0.05$ vs Met alone group, $\ddagger p < 0.05$ vs combination Met-Cis group.

TEMPOL significantly suppressed cytotoxicity and greatly reduced apoptosis in cells treated with a combination of Met and Cis (Fig. 1b & d). These results suggest that the cytotoxicity and chemosensitizing effect of Met are associated with oxidant stress.

3.2. Cis and Met induce ROS generation

To demonstrate the role of oxidants in the chemosensitizing effect of Met, intracellular ROS was determined by DHE assay. It was found that Met significantly increased ROS levels (200–250% in KKU-100 cells and about 140% in KKU-M156 cells) compared to control groups. Cis induced ROS formation was not significantly different from controls in either cell type, whereas the combination of Met and Cis further increased ROS levels higher than Met alone in both KKU-100 (Fig. 2a & b) and KKU-M156 (Fig. 2c & d) cells. Treatment with TEMPOL caused a small decrease in ROS in Met or Cis groups when compared with controls in both cells (Fig. 2a–d). Moreover, NAC and TEMPOL resulted in a significant decrease in ROS levels in cells treated with a combination of Met and Cis. These findings suggest that the chemosensitizing effect of Met may be associated with induction of oxidant formation, and that ROS plays a role in the cytotoxicity observed in cells treated with Met or Cis alone.

3.3. Met and Cis induce glutathione depletion in association with oxidative stress

GSH plays an important role in cellular redox regulation, and dysregulation of GSH levels is associated with oxidative stress and cellular dysfunction. The levels of GSH and redox ratios of GSH/GSSG in KKU-M156 cells were evaluated. The KKU-M156 cells were incubated with test agents for 6 h before assay for GSH and GSSG. As shown in Table 1, total GSH and reduced GSH levels were significantly decreased by all treatments when compared with the control group. However, glutathione disulfide (GSSG) was significantly increased in the group treated with a combination of Met and Cis. Furthermore, the GSH/GSSG ratios were decreased in all treatment groups, particularly in the combination group, when compared with control or Cis treatment alone. These finding strongly suggests that GSH redox stress is an important causative agent in cytotoxicity of Met and Cis, and is important in the chemosensitizing effect of Met when used in combination with Cis.

3.4. Met and Cis induce depolarization of mitochondrial transmembrane potential

Mitochondria are organelles that are sensitive to various stressors including redox stress and ROS. Mitochondrial dysfunction initiates the sequence of mitochondrial cell death pathway [30,31]. To evaluate the underlying mechanism of chemosensitizing effect in association with mitochondrial function, KKU-M156 cells were treated with test agents with or without NAC and TEMPOL, after which mitochondrial transmembrane potential ($\Delta\Psi_m$) was measured using the JC-1 assay. Mitochondria in control cells exhibited red fluorescence, indicating accumulation of J-aggregates in the healthy mitochondrial matrix. In contrast, treatment with Met, Cis, or the combination of both agents induced the green fluorescence of J-monomers in the cytosol (Fig. 3 upper panel). A decrease in the ratio of J-aggregates/J-monomers after various treatments was indicative of depolarization of $\Delta\Psi_m$ (Fig. 3 lower panel). The combination of Met and Cis induced the loss of $\Delta\Psi_m$ significantly more than treatment with any agents alone. However, the loss of $\Delta\Psi_m$ was significantly prevented by pretreatment with 2 mM NAC and 0.25 mM TEMPOL. These findings imply that redox stress and ROS are associated with the loss of $\Delta\Psi_m$ and the chemosensitizing effect.

3.5. Cyclosporine prevents the loss of $\Delta\Psi_m$ and cell death

The opening of mitochondrial permeability transition pores (MPTP) could cause the loss of $\Delta\Psi_m$, and subsequently induce cell death. To elucidate whether the opening of MPTP was ultimately involved in the chemosensitizing effect of Met, the cells were pretreated with cyclosporine (CsA), an MPTP inhibitor, before treatment with Met or Cis. It was found that Met, Cis, or the combination of both induced the loss of $\Delta\Psi_m$, and pretreatment with CsA completely prevented the loss in every treatment groups when compared with controls (Fig. 4a). Consistent with the change in $\Delta\Psi_m$, prevention of the loss of $\Delta\Psi_m$ was associated with prevention of cytotoxicity and induction of apoptosis (Fig. 4b). It is noted that the cytotoxicity was substantially reduced (by 50–70%) but not completely eliminated. On the other hand, induction of apoptosis was almost completely suppressed to the level of controls (Fig. 4c). This suggests that the opening of MPTP is intimately involved with cell death and chemosensitizing effect caused by Met.

3.6. Effects of Met and Cis on the expression of Nrf2 and its associated proteins

The results above suggested that the chemosensitizing effect was caused by an increase in oxidative stress. Treatment with Met and Cis may repress cellular antioxidant system resulting in an emergence of oxidants and redox stress. We investigated Nrf2 (nuclear factor-erythroid 2 like 2, or NFE2L2), a critical transcription factor regulating antioxidant and cytoprotective genes in response to various oxidative stressors, along with its associated proteins. It was found that nuclear Nrf2 protein was strongly suppressed by Met and the combination of Met and Cis in KKU-M156 (Fig. 5a) and in KKU-100 (Fig. 5b) cells within 6 h of treatment. Cis treatment alone in KKU-M156 cells had a tendency to increase Nrf2 expression where the expression of Nrf2 in KKU-100 cells was markedly increased compared with controls. Changes in proteins associated with Nrf2, including ERK, Akt, γ-GCSc,

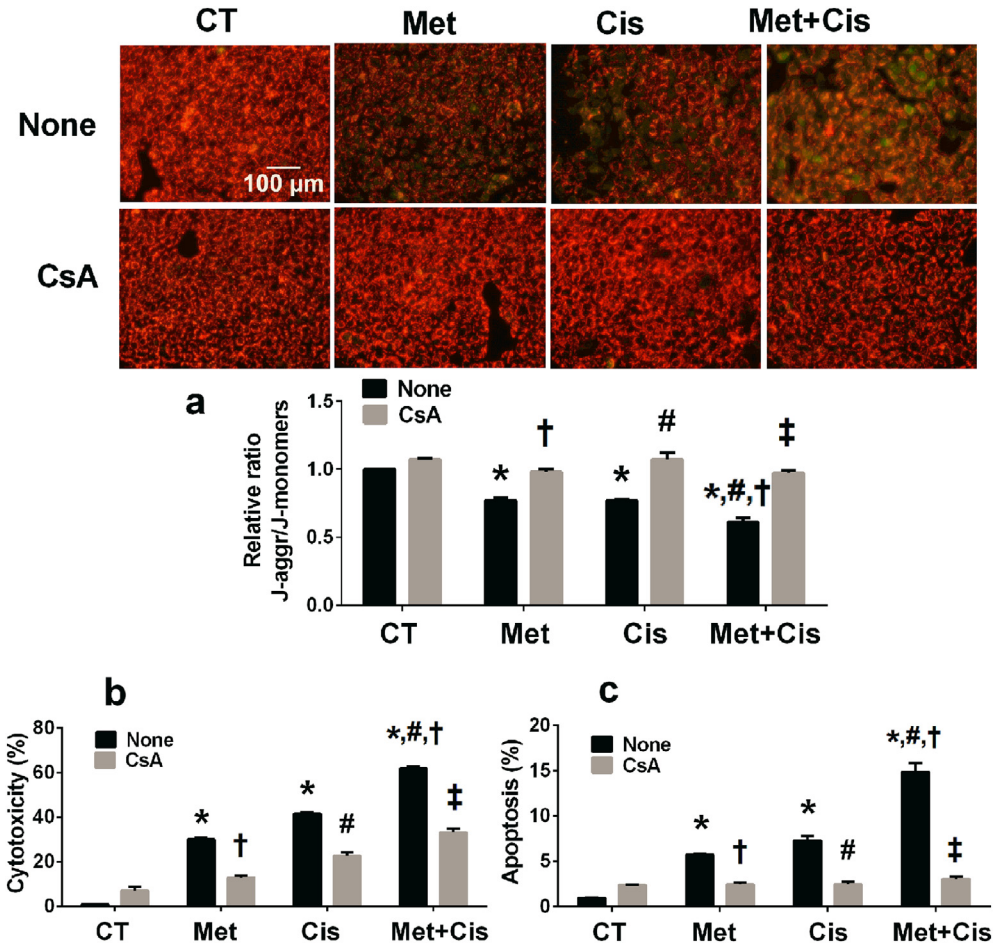

Fig. 4. CsA prevents the loss of $\Delta\Psi_m$ and cell death. KKU-M156 cells were preincubated for 3 h with 50 nM cyclosporine (CsA) before treatment with 3 mM Met (Met), 3 μM Cis (Cis) or the combination Met-Cis for 24 h. $\Delta\Psi_m$ was evaluated by JC-1 assay and cytotoxicity of the cells was estimated by AO/EB assay. Fluorescent images of treated cells stained with JC-1 are shown. Each bar represents the mean ± SEM from three experiments. *$p < 0.05$ vs control group, #$p < 0.05$ vs Cis alone group, †$p < 0.05$ vs Met alone group, ‡$p < 0.05$ vs combination of Met-Cis group.

and HO-1, were determined by western blot analysis. Expression of p-ERK (Fig. 5c) and p-Akt (Fig. 5d) was decreased by treatments with Met, Cis and the combination of both. The levels of γ-GCSc protein were unchanged in all treatment groups after 6 h and 24 h (Fig. 5e & f). On the other hand, HO-1 protein was significantly repressed by the combination of Met and Cis at 6 h and 24 h (Fig. 5g & h).

3.7. Met and Cis induce expression of proteins associated with cell cytotoxicity

As treatment of Met and Cis induced cell death in association with mitochondrial cell death pathway, we investigated proteins involved in the pathway, including Bax, Bcl-2 and cytochrome *c*, by western immunoblotting in KKU-M156 cells. Bax expression had a trend to increase at 6 h (Fig. 6a) and was significantly increased at 24 h (Fig. 6b). On the other hand, Bcl-2 expression was unchanged during 6 h (Fig. 6c) and significantly decreased in cells treated with Met or the combination of Met and Cis at 24 h (Fig. 6d). All drug treatments caused markedly increase in cytochrome *c* expressions at 24 h (Fig. 6f), but not at 6 h (Fig. 6e).

4. Discussion

Chemotherapy resistance is an important cause of failure in the treatment of CCA. Several strategies have been attempted to overcome this resistance and to increase the efficacy of chemotherapy. Metformin is well-known agent for treatment of type 2 diabetes and shown to reduce risk of some cancers [9,10]. It has been suggested that the antiproliferation and chemosensitizing effects of Met are mediated through the activation of AMPK and inhibition of Akt-mTOR [11,13,14]. In the present study we demonstrated that oxidative stress plays important role in the chemosensitizing effect of Met. Furthermore, this chemosensitizing effect is causally associated with suppression of Nrf2 expression and increased oxidative stress, leading to the loss of $\Delta\Psi_m$ and induction of cell death via the mitochondrial pathway.

In present study we showed that cytotoxicity is attenuated by antioxidant agents. Two antioxidant agents with different mechanisms were used. NAC acts as a redox modifier and also is a precursor for synthesis of GSH [32]. TEMPOL is a potent nitroxide antioxidant and mimics the activities of superoxide dismutase (SOD) and catalase [33]. The cytoprotective effect conferred by treatment with antioxidants implies that ROS and GSH redox stress play a role in the antiproliferative and chemosensitizing effects. It is noted that NAC or TEMPOL only partially prevented cell death. Hence, other mechanisms of tumor suppression independent of oxidative stress such as Cis-induced crosslink of DNA [24], Met-mediated AMPK-Akt-mTOR pathway, and other mechanisms may play a role in the induction of cell death. Nevertheless, the chemosensitizing effect by Met is mainly mediated via oxidative stress, since the enhanced cytotoxic effect by the combination of agents is largely abolished by NAC and TEMPOL.

Oxidative stress is apparent in the increased formation of ROS and decrease in GSH redox ratio, where the latter is an indicative of

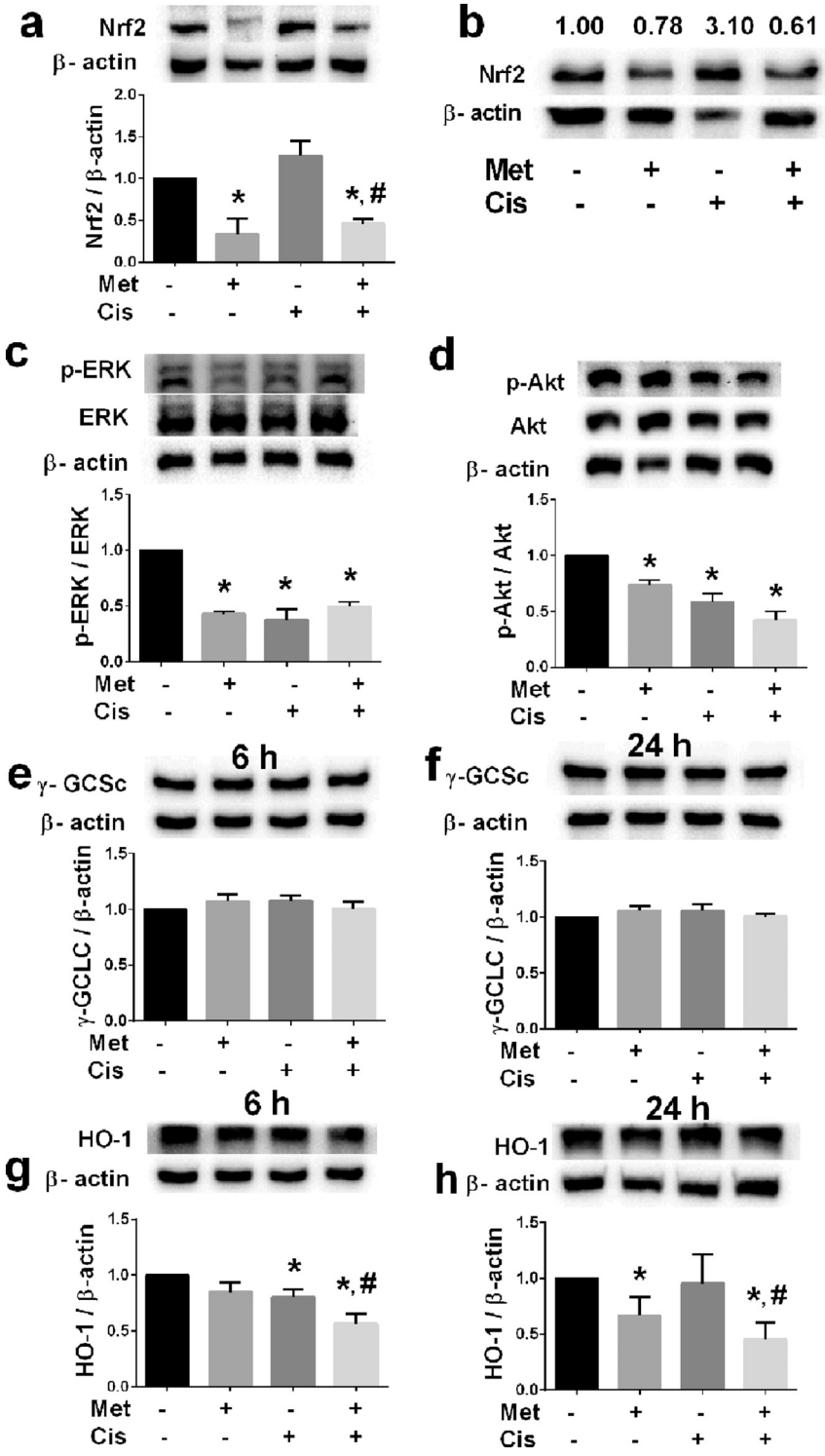

Fig. 5. Effect of Met and Cis on expression of Nrf2 and its associated proteins. CCA cells were treated with 3 mM Met (Met) and 3 μM Cis (Cis) before the expression of proteins was evaluated by western immuno blotting. The expression of nuclear Nrf2 in KKU-M156 and KKU-100 cells is shown in (a) & (b), respectively. A representative image of Nrf2 from KKU-100 cells is shown. Expression of proteins from KKU-M156 cells including (c) p-ERK, (d) p-Akt proteins at 6 h and γ-GCSc at (e) 6 h and (f) 24 h, and HO-1 at (g) 6 h and (h) 24 h and representative protein bands are shown. The protein intensity of p-ERK and p-Akt was calculated as a ratio to the total ERK and Akt. The relative intensity of Nrf2, γ-GCSc, and HO-1 proteins, normalized with β-actin as a loading control. Protein expression was calculated as ratio to the control group. Each bar represents the mean ± SEM from three experiments. $*p < 0.05$ vs control group, $\#p < 0.05$ vs Cis alone group.

dysregulation of cellular GSH redox potential. Cellular redox potential regulates vast number of biological processes, from anabolism, catabolism to cell death [34]. Disruption of redox state, as indicated by a decrease in GSH/GSSG ratio, affects the function of redox-sensitive proteins, such as enzymes and transcription factors, and eventually disrupts cell function. In the present study, Met, Cis and the combination of Met and Cis cause dysregulation of GSH redox status. The association of changes in redox status with cytotoxicity supports the notion that oxidative stress causes the chemosensitizing effect.

GSH redox status plays an important role in the process of cell death mediated by proteins in the Bcl2 family. Bcl2 modulates the MPTP and prevents Bax or Bak, a multidomain proapoptotic Bcl2 family proteins, from forming mitochondrial outer membrane permeabilization (MOMP), which in turn leads to the release of cytochrome *c* and initiation of cell death cascade [30,31]. Mitochondria function as cellular powerhouses and also play a central role in cell death. ROS and redox stress trigger mitochondrial dysfunction which is involved with the opening of MPTP [30,31]. The opening of MPTP can be activated by oxidative stress, Ca^{2+} overload, high phosphate concentration, and depletion of adenine nucleotides in the mitochondrial matrix [30].

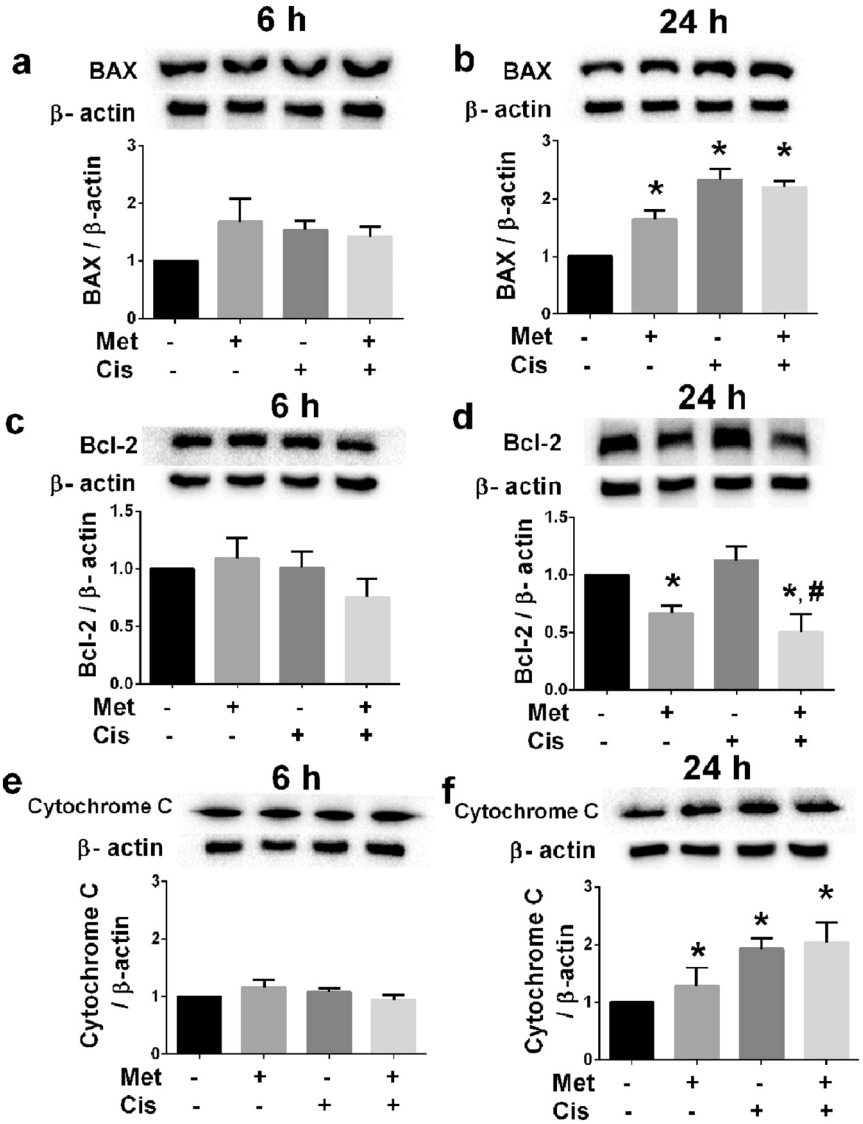

Fig. 6. Effect Met and Cis on the expression of proteins associated with cytotoxicity. KKU-M156 cells were incubated with 3 mM Met (Met), 3 μM Cis (Cis), or a combination of 3 mM Met and 3 μM Cis for 6 and 24 h. Immunoblotting was performed to detect expression of Bax (a & b), Bcl-2 (c & d), and cytochrome *c* (e & f). Representative bands from one experiment are shown. The relative intensity of target proteins normalized with β-actin as a loading control was calculated as ratio to the control group. Each bar represents the mean ± SEM from three experiments. *$p < 0.05$ vs control group, #$p < 0.05$ vs Cis alone group.

Prolonged opening of MPTP leads to the depolarization of $\Delta\Psi_m$, uncoupling of oxidative phosphorylation, ATP depletion, formation of MOMP, mitochondrial swelling, and resulting in cell death [30,31]. The present study showed that ROS and redox stress lead to increased Bax and decreased Bcl2 expression in association with the loss of $\Delta\Psi_m$, and eventually the release of cytochrome *c*.

The loss of $\Delta\Psi_m$ in the cells treated with Met and/or Cis is causally associated with cell death. This was validated by using cyclosporine, an inhibitor of MPTP, since cyclosporine prevented the loss of $\Delta\Psi_m$ and prevent cell death (Fig. 4). It should be noted that prevention of the loss of $\Delta\Psi_m$ may not necessarily prevent cell death, if the loss of $\Delta\Psi_m$ is collateral damage from primary insult that causes cell death, and incidentally recruits Bcl2 family proteins to open the MPTP. This has been demonstrated in phenethyl isothiocyanate-induced cell death in KKU-214 cells, in which prevention of the loss of $\Delta\Psi_m$ could not prevent cell death [35]. The present study indicates that mitochondria play critical role in cell death caused by Met, Cis and the combination of both agents.

The present study shows that Met and Cis increase oxidative stress. This is consistent with a previous study that demonstrated Cis inducing DNA damage and oxidative stress [24]. However, Met induced oxidative stress may not involve with suppression of mitochondrial complex I [20]. Our study suggests that Met causes oxidative stress by suppression of Nrf2 expression in CCA cells. In contrast, Cis has a tendency to increase expression of Nrf2. It has been suggested that over-expression of Nrf2-regulated antioxidant genes may cause resistance to anticancer drugs in pancreatic and CCA cells [5,36]. The suppression of Nrf2 induces oxidative stress by down-regulation of antioxidant, cytoprotective, and metabolic genes [21–23]. It is therefore possible that the chemosensitizing effect of Met may be caused by the suppression of Cis-induced Nrf2-antioxidant gene expression. The Nrf2 regulated genes including the potent cytoprotective enzyme HO-1 contribute to cancer resistance, and these genes are down-regulated by Met. Although γ-GCLC, a rate limiting enzyme in GSH synthesis, is unaltered by any treatments, the changes in cellular GSH levels and GSH redox ratio clearly indicate the presence of oxidative stress. Moreover, up-stream enzymes that regulate Nrf2, including Erk and Akt [37,38], were down-regulated by Met and Cis. This is consistent with previous studies in which Met suppressed activation of Akt [13,14,16].

In conclusion, Met enhances Cis-induced cytotoxicity and apoptosis

in CCA cells. The chemosensitizing effect of Met is associated with induction of ROS and GSH redox stress and down-regulation of Nrf2-antioxidant enzyme expression. The increase in oxidative stress is causally associated with mitochondrial dysfunction and initiation of cell death. The use of Met to enhance anticancer activity of Cis on CCA cells may be a novel strategy for CCA treatment.

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References

[1] J.M. Banales, V. Cardinale, G. Carpino, M. Marzioni, J.B. Andersen, P. Invernizzi, et al., Expert consensus document: Cholangiocarcinoma: current knowledge and future perspectives consensus statement from the European Network for the Study of Cholangiocarcinoma (ENS-CCA), Nat. Rev. Gastroenterol. Hepatol. 13 (5) (2016) 261–280.
[2] S.A. Khan, B.R. Davidson, R.D. Goldin, N. Heaton, J. Karani, S.P. Pereira, et al., Guidelines for the diagnosis and treatment of cholangiocarcinoma: an update, Gut 61 (12) (2012) 1657–1669.
[3] J.J. Marin, E. Lozano, O. Briz, R. Al-Abdulla, M.A. Serrano, R.I. Macias, Molecular bases of chemoresistance in cholangiocarcinoma, Curr. Drug Targets 18 (8) (2017) 889–900.
[4] S. Kongpetch, A. Puapairoj, C.K. Ong, L. Senggunprai, A. Prawan, U. Kukongviriyapan, et al., Haem oxygenase 1 expression is associated with prognosis in cholangiocarcinoma patients and with drug sensitivity in xenografted mice, Cell Prolif. 49 (1) (2016) 90–101.
[5] P. Samatiwat, A. Prawan, L. Senggunprai, V. Kukongviriyapan, Repression of Nrf2 enhances antitumor effect of 5-fluorouracil and gemcitabine on cholangiocarcinoma cells, Naunyn Schmiedeberg's Arch. Pharmacol. 388 (6) (2015) 601–612.
[6] P. Zeekpudsa, V. Kukongviriyapan, L. Senggunprai, B. Sripa, A. Prawan, Suppression of NAD(P)H-quinone oxidoreductase 1 enhanced the susceptibility of cholangiocarcinoma cells to chemotherapeutic agents, J. Exp. Clin. Cancer Res. 33 (2014) 11.
[7] H.Y. Ren, B. Chen, G.L. Huang, Y. Liu, D.Y. Shen, Upregulation of retinoic acid receptor-beta reverses drug resistance in cholangiocarcinoma cells by enhancing susceptibility to apoptosis, Mol. Med. Rep. 14 (4) (2016) 3602–3608.
[8] S. Yothaisong, H. Dokduang, A. Techasen, N. Namwat, P. Yongvanit, V. Bhudhisawasdi, et al., Increased activation of PI3K/AKT signaling pathway is associated with cholangiocarcinoma metastasis and PI3K/mTOR inhibition presents a possible therapeutic strategy, Tumour Biol. 34 (6) (2013) 3637–3648.
[9] C.J. Currie, C.D. Poole, E.A. Gale, The influence of glucose-lowering therapies on cancer risk in type 2 diabetes, Diabetologia 52 (9) (2009) 1766–1777.
[10] G. Libby, L.A. Donnelly, P.T. Donnan, D.R. Alessi, A.D. Morris, J.M. Evans, New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes, Diabetes Care 32 (9) (2009) 1620–1625.
[11] S. Ling, T. Feng, Q. Ke, N. Fan, L. Li, Z. Li, et al., Metformin inhibits proliferation and enhances chemosensitivity of intrahepatic cholangiocarcinoma cell lines, Oncol. Rep. 31 (6) (2014) 2611–2618.
[12] Y. Liu, C. He, X. Huang, Metformin partially reverses the carboplatin-resistance in NSCLC by inhibiting glucose metabolism, Oncotarget 8 (43) (2017) 75206–75216.
[13] J. Wandee, A. Prawan, L. Senggunprai, S. Kongpetch, O. Tusskorn, V. Kukongviriyapan, Metformin enhances cisplatin induced inhibition of cholangiocarcinoma cells via AMPK-mTOR pathway, Life Sci. 207 (2018) 172–183.
[14] D. Wang, X. Wu, In vitro and in vivo targeting of bladder carcinoma with metformin in combination with cisplatin, Oncol. Lett. 10 (2) (2015) 975–981.
[15] G. Yu, W. Fang, T. Xia, Y. Chen, Y. Gao, X. Jiao, et al., Metformin potentiates rapamycin and cisplatin in gastric cancer in mice, Oncotarget 6 (14) (2015) 12748–12762.
[16] E.A. Queiroz, S. Puukila, R. Eichler, S.C. Sampaio, H.L. Forsyth, S.J. Lees, et al., Metformin induces apoptosis and cell cycle arrest mediated by oxidative stress, AMPK and FOXO3a in MCF-7 breast cancer cells, PLoS One 9 (5) (2014) e98207.
[17] M.T. Do, H.G. Kim, T. Khanal, J.H. Choi, D.H. Kim, T.C. Jeong, et al., Metformin inhibits heme oxygenase-1 expression in cancer cells through inactivation of Raf-ERK-Nrf2 signaling and AMPK-independent pathways, Toxicol. Appl. Pharmacol. 271 (2) (2013) 229–238.
[18] Z.Y. Gao, Z. Liu, M.H. Bi, J.J. Zhang, Z.Q. Han, X. Han, et al., Metformin induces apoptosis via a mitochondria-mediated pathway in human breast cancer cells in vitro, Exp. Ther. Med. 11 (5) (2016) 1700–1706.
[19] B. Viollet, B. Guigas, N. Sanz Garcia, J. Leclerc, M. Foretz, F. Andreelli, Cellular and molecular mechanisms of metformin: an overview, Clin. Sci. (Lond.) 122 (6) (2012) 253–270.
[20] C. Batandier, B. Guigas, D. Detaille, M.Y. El-Mir, E. Fontaine, M. Rigoulet, et al., The ROS production induced by a reverse-electron flux at respiratory-chain complex 1 is hampered by metformin, J. Bioenerg. Biomembr. 38 (1) (2006) 33–42.
[21] J.D. Hayes, A.T. Dinkova-Kostova, The Nrf2 regulatory network provides an interface between redox and intermediary metabolism, Trends Biochem. Sci. 39 (4) (2014) 199–218.
[22] M. Kobayashi, M. Yamamoto, Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation, Antioxid. Redox Signal. 7 (3–4) (2005) 385–394.
[23] V. Sompakdee, A. Prawan, L. Senggunprai, U. Kukongviriyapan, P. Samathiwat, J. Wandee, et al., Suppression of Nrf2 confers chemosensitizing effect through enhanced oxidant-mediated mitochondrial dysfunction, Biomed. Pharmacother. 101 (2018) 627–634.
[24] S. Dasari, P.B. Tchounwou, Cisplatin in cancer therapy: molecular mechanisms of action, Eur. J. Pharmacol. 740 (2014) 364–378.
[25] J. Valle, H. Wasan, D.H. Palmer, D. Cunningham, A. Anthoney, A. Maraveyas, et al., Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer, N. Engl. J. Med. 362 (14) (2010) 1273–1281.
[26] P. Samatiwat, A. Prawan, L. Senggunprai, U. Kukongviriyapan, V. Kukongviriyapan, Nrf2 inhibition sensitizes cholangiocarcinoma cells to cytotoxic and anti-proliferative activities of chemotherapeutic agents, Tumour Biol. 37 (98) (2016) 11495–11507.
[27] P. Yonglitthipagon, C. Pairojkul, Y. Chamgramol, J. Mulvenna, B. Sripa, Up-regulation of annexin A2 in cholangiocarcinoma caused by *Opisthorchis viverrini* and its implication as a prognostic marker, Int. J. Parasitol. 40 (10) (2010) 1203–1212.
[28] B. Buranrat, A. Prawan, U. Kukongviriyapan, S. Kongpetch, V. Kukongviriyapan, Dicoumarol enhances gemcitabine-induced cytotoxicity in high NQO1-expressing cholangiocarcinoma cells, World J. Gastroenterol. 16 (19) (2010) 2362–2370.
[29] N. Kittiratphatthana, V. Kukongviriyapan, A. Prawan, L. Senggunprai, Luteolin induces cholangiocarcinoma cell apoptosis through the mitochondrial-dependent pathway mediated by reactive oxygen species, J. Pharm. Pharmacol. 68 (9) (2016) 1184–1192.
[30] A.P. Halestrap, A.P. Richardson, The mitochondrial permeability transition: a current perspective on its identity and role in ischaemia/reperfusion injury, J. Mol. Cell. Cardiol. 78 (2015) 129–141.
[31] J.S. Kim, L. He, J.J. Lemasters, Mitochondrial permeability transition: a common pathway to necrosis and apoptosis, Biochem. Biophys. Res. Commun. 304 (3) (2003) 463–470.
[32] T. Parasassi, R. Brunelli, G. Costa, M. De Spirito, E. Krasnowska, T. Lundeberg, et al., Thiol redox transitions in cell signaling: a lesson from N-acetylcysteine, ScientificWorldJournal 10 (2010) 1192–1202.
[33] C.S. Wilcox, A. Pearlman, Chemistry and antihypertensive effects of tempol and other nitroxides, Pharmacol. Rev. 60 (4) (2008) 418–469.
[34] V. Mallikarjun, D.J. Clarke, C.J. Campbell, Cellular redox potential and the biomolecular electrochemical series: a systems hypothesis, Free Radic. Biol. Med. 53 (2) (2012) 280–288.
[35] O. Tusskorn, L. Senggunprai, A. Prawan, U. Kukongviriyapan, V. Kukongviriyapan, Phenethyl isothiocyanate induces calcium mobilization and mitochondrial cell death pathway in cholangiocarcinoma KKU-M214 cells, BMC Cancer 13 (2013) 571.
[36] A. Lister, T. Nedjadi, N.R. Kitteringham, F. Campbell, E. Costello, B. Lloyd, et al., Nrf2 is overexpressed in pancreatic cancer: implications for cell proliferation and therapy, Mol. Cancer 10 (2011) 37.
[37] H.J. Jang, E.M. Hong, M. Kim, J.H. Kim, J. Jang, S.W. Park, et al., Simvastatin induces heme oxygenase-1 via NF-E2-related factor 2 (Nrf2) activation through ERK and PI3K/Akt pathway in colon cancer, Oncotarget 7 (29) (2016) 46219–46229.
[38] Z. Qi, X. Ci, J. Huang, Q. Liu, Q. Yu, J. Zhou, et al., Asiatic acid enhances Nrf2 signaling to protect HepG2 cells from oxidative damage through Akt and ERK activation, Biomed. Pharmacother. 88 (2017) 252–259.