Meloxicam combined with sorafenib synergistically inhibits tumor growth of human hepatocellular carcinoma cells via ER stress-related apoptosis

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Abstract. Sorafenib (SOR) is a promising treatment for advanced hepatocellular carcinoma (HCC). However, the precise mechanisms of toxicity and drug resistance have not been fully explored and new strategies are urgently needed for HCC therapy. Meloxicam (MEL) is a selective cyclooxygenase-2 (COX-2) inhibitor which elicits antitumor effects in human HCC cells. In the present study, we investigated the interaction between MEL and SOR in human SMMC-7721 cells and the role endoplasmic reticulum (ER) stress exerts in the combination of SOR with MEL treatment-induced cytotoxicity. Our results revealed that the combination treatment synergistically inhibited cell proliferation and enhanced apoptosis. Furthermore, the combination treatment enhanced ER stress-related molecules which involved in SMMC-7721 cell apoptosis. GRP78 knockdown by siRNA or co-treatment with MG132 significantly increased this combination treatment-induced apoptosis. In addition, we found that the combination treatment suppressed tumor growth by way of activation of ER stress in in vivo models. We concluded that the combination of SOR with MEL treatment-induced ER stress, and eventually apoptosis in human SMMC-7721 cells. These findings provided a new potential treatment strategy against HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and the fourth primary cause of tumor-related deaths worldwide with high mortality and poor prognosis (1). Although many types of therapeutic measures including surgical resection, transarterial chemoembolization (TACE), radiation and chemotherapy have been used for the treatment of HCC, most patients progress to an advanced stage after the initial therapeutic benefit attributed to high chemoresistance, particularly due to the multidrug resistance (MDR) of HCC (2). Sorafenib (SOR), an oral multikinase inhibitor, which inhibits tumor growth and angiogenesis by way of inhibiting vascular endothelial growth factor receptor 2 and other receptor tyrosine kinases has been used as the standard treatment for advanced stages of HCC based on two large randomized phase Ⅲ trials, which led to the Food and Drug Administration (FDA) approval since it prolongs survival for 2-3 months in advanced and inoperable HCC cases (3-5). However, clinical results have been disappointing showing that a large number of advanced HCC patients are unresponsive or acquire resistance to SOR. Therefore, it is urgent to seek new effective therapy strategies to combat HCC.

Non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to reduce the risk of developing cancer (6-8). Meloxicam (MEL), a selective cyclooxygenase-2 (COX-2) inhibitor, has been demonstrated to inhibit proliferation and promote apoptosis in many malignant diseases (9-11). Our previous experimental results showed that COX-2 inhibitor exhibits antiproliferative and proapoptotic effects in HCC cell lines (12,13). However, the detailed effects and mechanisms of MEL combined with SOR for treating HCC cells have not been fully cleared. Recently, a number of studies have revealed that certain chemotherapeutics lead to cell death by the way of the ER stress-related apoptosis (14,15). Therefore, we hypothesized that ER stress promoting proapoptotic effects or inhibiting its proliferative function may be a potential target for the treatment of HCC. The endoplasmic reticulum (ER), a central cellular organelle, plays a crucial role in protein folding and maturation as well as accumulation of intracellular calcium. Small
errors in these processes could disturb normal ER processes and lead to ER stress known as the unfolded protein response (UPR). GRP78, as an ER molecular chaperone, is upregulated when ER stress is induced and functions as a sensory hub and inhibitor of three ER transmembrane receptors: eukaryotic translation initiation factor 2α kinase 3 (EIF2AK3/PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor-6 (ATF6) (16). The UPR initially targets proteins for degradation and restores the proper ER homeostasis. However, it eventually induces cell death during intense ER stress (17). Our purpose in the present study was to explore the combined effects of MEL and SOR on apoptosis and evaluate the probable mechanisms of action in HCC cell lines.

**Materials and methods**

**Cell culture and animals.** Human hepatocellular cancer (HCC) SMMC-7721 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in RPMI-1640 medium (Gibco)/ Dulbecco's modified Eagle's medium (DMEM) (HyClone) containing 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37°C in 95% air and 5% CO₂. BALB/c male athymic mice (5-6 weeks old, 18-22 g) were purchased from the Animal Supplier Center of Shandong University. All the animal studies were approved by the Ethics Committee of Shandong University. All surgical procedures were performed under anesthesia with sodium pentobarbital.

**Reagents and antibodies.** The MEL was purchased from Merck Millipore (Darmstadt, Germany), dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at 10 or 50 mM stock and diluted immediately before each experiment. SOR tosylate was obtained from bayer Health at 10 or 50 mM stock and diluted immediately before each experiment. SOR tosylate was obtained from Bayer Health Care (Berlin, Germany) and dissolved in DMSO to a 10 mM stock. MG132 was obtained from Sigma-Aldrich (San Diego, CA, USA). Primary antibodies to GRP78, caspase-12, PARP and caspase-3 were purchased from Abcam (Cambridge, UK). Antibodies to IRE1 and phos-ERF2α were obtained from Cell Signaling Technology (Danvers, MA, USA) and the anti-GAPDH antibody was obtained from Abcam.

**Measurement of cell viability.** Cell viability assays were performed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Japan). Cells (5x10⁴/well) were seeded with culture medium onto 96-well plates and incubated at 37°C for 24 h. After adaptation, cells were treated with either MEL or SOR or in combination for 48 h. Then the cells were performed by cell cycle analysis. In brief, 5x10⁵ cells were suspended in 0.5 ml of PI solution, and incubated 30 min in the dark according to the manufacturer's instructions. Cell cycle distribution was analyzed by FACS flow cytometry.

**Apoptosis assay.** Cells were stained with Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA). According to the manufacturer's instructions, the cells were incubated with 5 ml of Annexin V and 5 ml of propidium iodide (PI) for 15 min at room temperature, and then the stained cells were analyzed on a FACS flow cytometer.

**Cell migration and invasion assays.** Cells (1x10⁵) in 300 ml of RPMI-1640 medium/DMEM (with 1% FBS) containing MEL or SOR alone, or in combination were seeded into the upper chamber of a Transwell chamber (Corning, New York, NY, USA). The bottom wells of the chambers were filled with 500 ml RPMI-1640 medium/DMEM containing 10% FBS. After 48 h of incubation, the chambers were fixed with 95% ethanol and then stained with 1% crystal violet. Similarly, the cell invasion assay was performed by adding Matrigel Basement Matrix to the upper chamber.

**Western blot analysis.** Western blotting was used to evaluate apoptosis and ER stress-related signaling. After different treatments, protein concentrations in cell extracts were determined (Bio-Rad, Richmond, CA, USA), equal amounts of each sample were resolved in SDS-PAGE gels, then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and probed with specific antibodies. Blots were developed using applicable horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized by enhanced chemiluminescence with ECL (Millipore). Protein band intensities were quantified by densitometric analysis using ImageJ software (National Institutes of Health, USA).

**Gene transfection and RNAi.** For knockdown of GRP78, a small interfering RNA (si-RNA) targeting human GRP78 and a control siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SMMC-7721 cells were seeded onto 6-well plates and after 24 h were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The transfected cells were treated with MEL or SOR or in combination in complete medium for 24 h.

**Immunofluorescence assay.** Human SMMC-7721 cells seeded onto coverslips in 6-well plates, were fixed with 4% paraformaldehyde (PFA) and were permeabilized in 0.1% Triton X-100. Incubation with primary antibodies for 2 h at room temperature was followed by incubation with fluorescein isothiocyanate (FITC)-labeled IgG secondary antibodies, and then cells were mounted onto microscope slides with a DAPI mounting solution (Abcam). Fluorescent images of the HCC cells were photographed and analyzed with a light microscope (magnification, x200; Olympus, Tokyo, Japan).
**Results**

Combination of MEL and SOR significantly inhibits cell viability in SMMC-7721 cells. In order to investigate the effect of MEL, SOR and their combination on the cell viability of HCC cells in vitro, SMMC-7721 cells were treated with different concentrations of MEL (0-75 µM) or SOR (0-10 µM). As depicted in Fig. 1, treatment with MEL or SOR for 48 h significantly inhibited cell viability in SMMC-7721 cells with an IC_{50} value of 75.6±0.8 µM of MEL alone or an IC_{50} value of 10.2±1.5 µM of SOR alone. Next, we investigated whether MEL enhanced sensitivity of SMMC-7721 cells to SOR treatment. The CDI was utilized to display the effects of interaction between these two drugs. Our results showed that combination with MEL significantly enhanced SOR lethality and exhibited strong synergistic effects for SMMC-7721 cells (Table I).

Combination of MEL and SOR inhibits migration and invasion in SMMC-7721 cells. Given the association of HCC with a high degree of invasion and metastasis, we investigated whether the migratory potential of SMMC-7721 cells could be affected by exposure to MEL or SOR alone or in combination. As determined by scratch motility assay, MEL or SOR treatment alone induced a partial inhibition of migration whereas the combined treatment with MEL and SOR notably inhibited the migratory potential of SMMC-7721 cells (Fig. 2A and C). Furthermore, we applied SMMC-7721 cells to a migration and invasion assay and the results were consistent with those of the scratch assay (Fig. 2B and C).

Combination of MEL with SOR induces cell cycle arrest and apoptosis in SMMC-7721 cells. Given the superior synergistic interactions observed between MEL and SOR, we investigated the potential effects on cell apoptosis mediated by these combinations. Apoptotic cell death induction was analyzed by flow cytometry at 24 h after SMMC-7721 cells were treated with either MEL or SOR alone or in combination. As shown in Fig. 3A and B, MEL and SOR as a single agent led to apoptosis in SMMC-7721 cells. We also observed the MEL+SOR combination significantly increased apoptotic cell death compared with MEL or SOR as a single agent. Next, we used flow cytometry to evaluate the potential effects of MEL and SOR on the cell cycle distribution of SMMC-7721 cells. We
found that cell cycle arrest at the G0/G1 phase was increased with treatment of MEL or SOR compared with the control group (Fig. 3C). In addition, the MEL+SOR combination led to enhanced accumulation of cells in the G0/G1 phase compared to the single agents. These data revealed an additive mechanism of the MEL+SOR combination inducing cell death via apoptosis.

**Combination of MEL with SOR induces ER stress in SMMC-7721 cells.** Previous studies have demonstrated that COX-2 inhibitor and SOR as single agent treatments induced ER stress (19-22). To ascertain whether the MEL+SOR combination treatment enhanced ER stress in HCC cell lines, certain ER-specific signals were measured. Immunoblotting analysis results indicated that the levels of IRE1, p-eIF2α and GRP78 were upregulated in response to both MEL and SOR alone, and these ER stress marker were significantly increased by the MEL+SOR combination treatment (Fig. 4A and B). To ensure the observations that ER stress-associated markers were increased in SMMC-7721 cells after exposed to MEL or SOR alone or in combination, GRP78 were visualized by immunofluorescence staining. As shown in Fig. 4, immunofluorescence staining of GRP78 was partially increased after MEL or SOR single treatment. However, the combined treatment with MEL and SOR markedly increased GRP78 of SMMC-7721 cells. Several studies have revealed that caspase-12 is activated by continuous ER stress and plays a key role in leading to cell death not via the cytochrome c-dependent pathway (23). To investigate the involvement of ER stress in the MEL+SOR combination treatment-induced toxicity and explore potential mechanisms in the present study, western blotting assay was used to detect expression and distribution of caspase-12 proteins in SMMC-7721 cells. As shown in Fig. 4D, the activation of the caspase-12 protein was significantly increased in SMMC-7721 cells that were treated with the MEL+SOR combination compared to the single agents, in agreement with
cell death assays. These data indicated that ER impairment targeted the process of apoptosis.

**Involvement of GRP78 in combined MEL with SOR treatment-induced apoptosis.** GRP78, one of the most important protective mechanisms induced by UPR has been demonstrated to be associated with chemoresistance (24). In the present study, we explored the role of GRP78 in the MEL+SOR combination treatment-induced apoptosis. As shown in Fig. 5A, transfection of GRP78 siRNA, the MEL+SOR combination treatment significantly reduced cell viability, as expected. Additionally, transfection of GRP78 siRNA which downregulated the level of GRP78 protein (Fig. 5B), notably strengthened the increase of cell apoptosis (Fig. 5C and D) and the cleavage of PARP and caspase-3 (Fig. 5E and F) in the MEL+SOR combination-treated SMMC-7721 cells. These data revealed that GRP78 exerts a protective function in HCC cells to promote drug resistance.

**MG132 enhances the MEL and SOR combination treatment-induced apoptosis in SMMC-7721 cells.** Previous studies have revealed that the proteasome pathway exerts a crucial role in the degradation of unfolded protein (25,26). In the present study, we hypothesized that inhibition of proteasome enhances the MEL+SOR combination treatment-induced SMMC-7721 cells apoptosis attributed to the accumulation of unfolded protein. To verify our assumption, the proteasome inhibitor MG132, was used to evaluate the combination effect of the MEL+SOR on human SMMC-7721 cells. Our results showed that when exposed to low-dose (1 µM), MG132 mildly affected cell viability. However, the MEL+SOR+MG132 combination treatment significantly enhanced the cell toxicity (Fig. 6A) and apoptosis (Fig. 6B). Furthermore, MG132 significantly suppressed the MEL+SOR combination treatment-induced GRP78 expression and enhanced the cleavage of caspase-3 (Fig. 6C and D). These data revealed that proteosome inhibitor MG132 enhanced the MEL+SOR combination treatment-induced apoptosis.

**Combination of MEL with SOR arrests tumor growth in vivo.** Due to the superior antitumor effects of the MEL+SOR combination treatment in vitro, we explored whether the MEL+SOR combination treatment inhibited tumor growth in vivo. As shown in Fig. 7A and B, the MEL+SOR
Combination treatment exerted marked antitumor activity in SMMC-7721 xenograft tumors compared to the single agents. However, we found that the combination treatment caused only mild weight change in the in vivo models. Furthermore, we used western blotting and immunohistochemistry to analyze tumor xenografts. The results suggested that the MEL+SOR combination treatment notably activated the ER stress-related apoptosis in SMMC-7721 cell-derived tumors (Fig. 7C and D). In conclusion, our data revealed that the MEL+SOR combination treatment significantly arrests tumor growth in vivo via ER stress-associated regulatory mechanisms.

**Discussion**

Hepatocellular carcinoma (HCC), a hypervascular tumor type with characteristic of high levels of neovascularization and angiogenesis, exerts effects in the growth and progression which needs interacting approaches for effective therapy (27,28). Due to the association of single agents with treatment resistance, we considered that the combination therapy increased the lethality in HCC. Sorafenib (SOR) has been applied as the standard therapeutic strategy for advanced HCC patients. In contrast, the selective COX-2 inhibitor has been demonstrated to exert antitumor effects in various types of tumors including HCC (12,13,21,29,30). Thus, in the present study, we investigated whether the combinations of meloxicam (MEL)+SOR led to more superior antitumor effects than MEL or SOR alone in human SMMC-7721 cells. Our results revealed that either MEL or SOR alone reduce cell viability and colony formation and induce cell cycle arrest and apoptosis. However, the MEL+SOR combination exhibited more potent antitumor effects in terms of cytotoxicity and apoptotic induction via ER stress in human SMMC-7721 cells. GRP78 knockdown by siRNA or proteasome inhibitor significantly enhanced the MEL+SOR combination treatment-induced apoptosis.

ER exerts a key role in regulating protein synthesis, folding and trafficking. A large number of signal pathways have been demonstrated to disrupt the ER function and induce...
dysfunction of UPR, resulting in ER stress. The initial aim of UPR is to restore ER homeostasis, however, when pro-survival responses failed, these signaling pathways ultimately led to cell apoptosis (14,31-33). GRP78, the ER molecular chaperone, exerts a crucial role in protein folding and assembly (34). Perturbation of ER homeostasis leads to activation of ER stress which results in GRP78 dissociation (35,36). Furthermore, several studies have reported that GRP78 is associated with chemoresistance in cancer therapy (24,37,38). In the present study, the MEL+SOR combination treatment led to ER stress in human SMMC-7721 cells and is associated with the increase of IRE1, p-eIF2α, GRP78 and activation of caspase-12. Silencing GRP78 enhanced the cytotoxic and apoptotic effect of MEL+SOR combination treatment in SMMC-7721 cells. Therefore, it is concluded that GRP78 plays a protective function in HCC cells to promote drug resistance. GRP78 knockdown by siRNA notably increased the susceptibility to MEL+SOR in SMMC-7721 cells.
Certain studies have revealed that the ubiquitin proteasome pathway exerts a crucial role in intracellular protein degradation by maintaining ER homeostasis when cells encounter the UPR (25). In the present study, our results showed that combined treatment with proteasome inhibitor MG132 significantly enhanced the MEL+SOR-induced cytotoxicity and apoptosis with concomitant downregulation of GRP78 and activation of caspase-3.

In conclusion, these data demonstrated that the MEL+SOR combination treatment notably reduced cell viability and induced apoptosis in human SMMC-7721 cells. GRP78 knockdown or by proteasome inhibitor MG132 significantly enhances the MEL+SOR combination treatment-induced SMMC-7721 cell apoptosis. These findings provide a basis for and warrant future study to investigate the combination of MEL+SOR therapy for the treatment of drug resistant tumors with targeted therapy.
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