Blocking autophagy enhances meloxicam lethality to hepatocellular carcinoma by promotion of endoplasmic reticulum stress

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Abstract

Objectives: Meloxicam, a selective cyclooxygenase-2 (COX-2) inhibitor, has been demonstrated to exert anti-tumour effects against various malignancies. However, up to now, mechanisms involved in meloxicam anti-hepatocellular carcinoma effects have remained unclear.

Materials and methods: Cell viability and apoptosis were assessed by CCK-8 and flow cytometry. Endoplasmic reticulum (ER) stress and autophagy-associated molecules were analysed by western blotting and immunofluorescence assay. GRP78 and Atg5 knock-down by siRNA or chemical inhibition was used to investigate cytotoxic effects of meloxicam treatment on HCC cells.

Results: We found that meloxicam led to apoptosis and autophagy in HepG2 and Bel-7402 cells via a mechanism that involved ER stress. Up-regulation of GRP78 signalling pathway from meloxicam-induced ER stress was critical for activation of autophagy. Furthermore, autophagy activation attenuated ER stress-related cell death. Blocking autophagy by 3-methyladenine (3-MA) or Atg5 siRNA knock-down enhanced meloxicam lethality for HCC by activation of ER stress-related apoptosis. In addition, GRP78 seemed to lead to autophagic activation via the AMPK–mTOR signalling pathway. Blocking AMPK with a chemical inhibitor inhibited autophagy suggesting that meloxicam-regulated autophagy requires activation of AMPK.

Conclusions: Our results revealed that both ER stress and autophagy were involved in cell death evoked by meloxicam in HCC cells. This inhibition of autophagy to enhance meloxicam lethality, suggests a novel therapeutic strategy against HCC.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer in the world and the third leading cause of cancer-related death, with 748,000 new cases and 695,000 deaths worldwide in 2008 (1,2). Approximately 85% of these cases occurred in developing countries, with China accounting for more than 50% of the total (3). The main risk factors include hepatitis virus (B or C) infection, alcohol-related liver cirrhosis and non-alcoholic steatohepatitis (4). Over the last few decades, survival rates of HCC patients have significantly improved due to a series of surveillance programmes and large number of advances in medical technology for detecting and treating early stages of HCC. However, HCC patients amenable to curative therapy (surgical resection, liver transplantation or local ablation treatments) only account for 20% of the total (5). Unfortunately, development of effective therapy for the advanced-stage HCC has been rather slow. Chemotherapy is an important strategy for advanced HCC, but no systemic chemotherapy has been demonstrated to be consistently efficacious (6). Even worse, frequent acquisition of drug-resistant phenotypes is often associated with HCC chemotherapy and there are significant obstacles to achieve favourable outcomes. Sorafenib, a tyrosine kinase inhibitor with a broad inhibitory profile, is the most effective drug currently approved to treat advanced-stage disease (7,8). However, even this only prolongs survival for 2–3 months, and has not been widely adopted as it is cost-prohibitive, particularly in Asia and sub-Saharan Africa,

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which have the highest incidences of HCC. Thus, novel targeted molecular therapeutic strategies are required to greatly contribute to treatment of this condition.

Cyclooxygenase-2 (COX-2), a rate-limiting enzyme in the synthesis of prostaglandin (PG), is frequently found in inflammatory tissues and is involved in carcinogenic pathways in many organs (9). Accumulated evidence has demonstrated that COX-2 expression influences proliferation, invasion, chemoresistance and tumourigenesis in many malignant diseases (10–13). Meloxicam, a selective COX-2 inhibitor and non-steroidal anti-inflammatory drug (NSAID), is widely used to treat inflammation and can induce growth inhibition and apoptosis by suppressing COX-2 expression and PGE₂. Our previous studies have revealed that meloxicam can inhibit proliferation and induce apoptosis of HCC cells (14,15). However, up to now the mechanisms involved in meloxicam anti-hepatocellular carcinoma effects have remained unclear.

Induction of apoptosis is a very important type of cytotoxicity brought about by a number of chemotherapeutic drugs; many induce apoptosis by the mitochondrial pathway. However, recently several proteasome inhibitors have induced apoptosis through an endoplasmic reticulum (ER) stress-mediated apoptotic pathway.

Endoplasmic reticulum (ER) is the main organelle for lipid synthesis, which allows protein folding and maturation and stores high intracellular calcium. Disturbance of any of these processes induces an evolutionarily conserved cell stress response, referred to as the unfolded protein response (UPR). UPR is initially a protective mechanism that restores correct ER homeostasis, however, it eventually leads to cell death during persistent ER stress (16,17). Autophagy is a highly regulated process that allows cells to sequester cytoplasmic material by forming double-membrane vesicles (autophagosomes) and to deliver material to lysosomes for degradation (18). Previous studies have reported that some anti-neoplastic therapies lead to autophagy and apoptosis in cancer cells (19–21). Targeting autophagy enhances chemotherapy sensitivity in many cancers (22,23). Thus, we designed the present study to investigate whether targeting autophagy could enhance meloxicam lethality to hepatocellular carcinoma, by promotion of ER stress.

Materials and methods

Reagents and cell culture

Meloxicam was purchased from Merck Millipore (Darmstadt, Germany). (-)-Epigallocatechin-3-gallate (EGCG), 3-methyladenine (3-MA), Z-VAD and compound C

were obtained from Sigma-Aldrich (San Diego, CA, USA) and primary antibodies to GRP78, caspase-12, cleaved PARP and cleaved caspase-3 were obtained from Abcam (Cambridge, UK). Antibodies to IRE1, light chain 3 (LC3), phos-eIF2 α , phos-mTOR (Ser2448), phos-AMPK (Thr172) and Atg5 were bought from Cell Signaling Technologies (Danvers, MA, USA), and anti-GAPDH antibody was obtained from Abcam.

Of the five human HCC cell lines used, HepG2, Bel-7402 and Huh-7 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and SMMC-7721 and SMMC-7402 were obtained from the Type Culture Collection Cell Bank, Chinese Academy of Science (Shanghai, China). Cells were routinely cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA)/DMEM (Hyclone, Logan, UT, USA) supplemented with 10% foetal bovine serum (Gibco) and 1% antibiotics at 37 °C in 95% air and 5% CO₂.

Cell viability assay

Cell viability was determined using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, cells were seeded in culture medium in 96-well plates (5000 cells/well) and incubated at 37 °C for 24 h before drug exposure. Then, culture medium was replaced with medium containing various concentrations of meloxicam (0–100 μ M) and cells were incubated once more. Finally, they were incubated with complete medium containing 10 μ L CCK-8 solution at 37 °C for 4 h and optical density (OD) at 450 nm was measured using a Spectra Max 190 (Molecular Devices, Sunnyvale, CA, USA).

Assay of apoptosis

Cells (4 $\times$ 10⁵/well) were seeded in culture medium in six-well plates and incubated at 37 °C for 24 h and then with fresh medium with the required concentrations of meloxicam (0–80 μ M). After washing twice in cold PBS and re-suspending in binding buffer, ANXA5-FITC/PI apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) was utilized to analyse apoptotic cells by flow cytometry, according to the manufacturer's instruction.

Western blot assay

Protein concentration in supernatants was determined using Bio-Rad (Hercules, CA, USA) technology. In brief, equal amounts of protein fractions of lysate were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, Billerica, MA, USA), which were then probed

with primary antibodies overnight at 4 °C. Membranes were subsequently blotted with HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) with enhanced chemiluminescence (ECL) immunoblotting detection reagents (Millipore), for 1 h followed by visualization. Levels of protein band intensity were quantified by western blotting using Image J software (NIH, Bethesda, MD, USA).

Gene transfection and RNAi

According to the manufacturer's instructions, HepG2 and Bel-7402 cell lines were seeded on six-well plates and transfected 24 h later using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). GRP78 siRNA, Atg5 siRNA and control siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunofluorescence assay

Briefly, HCC cells seeded on coverslips in six-well plates were fixed in 4% paraformaldehyde (PFA) and 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)-labelled IgG secondary antibodies. Cells were mounted on microscope slides with DAPI mounting solution (Abcam). Fluorescent images of the cells were photographed and analysed using a light microscope (Olympus, Tokyo, Japan).

Statistical analyses

Data are presented as mean $\pm$ standard deviation (SD) and analysed by one-way ANOVA followed by Dunnett's test with SPSS software (version 17.0; SPSS China, Shanghai, China), with values of $P < 0.05$ considered statistically significant.

Results

Meloxicam induced ER stress in the HCC cell lines

As a selective COX-2 inhibitor, meloxicam has been shown to have wide anti-tumour activity in a variety of cancers (24,25). In our previous study, we have demonstrated that meloxicam inhibited HCC cell survival and its cytotoxicity increased in a concentration-dependent manner. We also found that HCC cell lines expressed different levels of COX-2 protein. HepG2 and Bel-7402 cells expressing it higher than SMMC-7402 and Huh-7 cells (15). Thus, in this study, we

chose these two cell types for the following experiments. We first used flow cytometry (FACS) with propidium iodide (PI) and annexin V-FITC staining to label apoptotic cells. As shown in Fig. 1a–d, meloxicam markedly increased apoptosis in a concentration-dependent manner. Meloxicam induced cell death in the HCC cell lines, but the underlying mechanisms had not previously been studied in detail. COX-2 inhibition has been revealed to induce ER stress in a number of cancer cells (26–28). To ascertain whether ER stress would be involved in meloxicam-induced cell death in HCC cell lines, we determined levels of ER stress markers in HepG2 and Bel-7402 cells after exposure to meloxicam for 24 h. As shown in Fig. 1e and f, meloxicam increased protein expression levels of IRE1 and GRP78/Bip as well as eIF2 α phosphorylation in a concentration-dependent manner. To further confirm observations that GRP78 was higher in HepG2 and Bel-7402 cells after treatment with meloxicam, GRP78 was visualized by immunofluorescence staining. As shown in Fig. 1g, GRP78 was notably higher after meloxicam treatment.

Meloxicam induced ER stress-related apoptosis in two HCC cell lines

Previous studies have demonstrated that caspase-12-induced apoptosis is associated with ER stress (29–31). After treatment with meloxicam for 24 h, we observed that activation of caspase-12 was significantly higher in HepG2 and Bel-7402 cell lines, consistent with cell death assays. We also observed that expression of cleaved PARP and cleaved caspase-3 significantly increased (Fig. 2a–d). Furthermore, as measured by qRT-PCR, meloxicam treatment induced elevated expression of caspase-12 mRNA (Fig. 2e). In addition, an apoptosis inhibitor (Z-VAD-FMK, Z-VAD) was used to assess cell death by meloxicam. As shown in Fig. 2f, Z-VAD notably down-regulated meloxicam-induced apoptosis. These results revealed that ER impairment by meloxicam triggered the process of apoptosis and that activation of caspases were involved in meloxicam-induced apoptosis.

GRP78 knock-down increased meloxicam-induced apoptosis

A large number of studies has demonstrated that GRP78 exerts a crucial role in ER stress-induced apoptosis and is associated with chemoresistance (27,32–34). Thus, specific siRNA to down-regulate GRP78 was introduced in both HCC cell lines. As showed in Fig. 3a and b, knock-down of GRP78 by siRNA significantly reduced

Figure 1. Meloxicam induced apoptosis and increased ER stress-related signalling molecules in two HCC cell lines. (a and c) Cells were treated with mock (untreated) and meloxicam (0–80 μ M) for 24 h. Apoptotic cells were analysed by FACS flow cytometry with propidium iodide (PI) and annexin V-FITC staining. (b and d) Quantitative analysis of total apoptotic (early and late) population following various concentrations of meloxicam treatment is presented. Data are presented as means $\pm$ SD of three independent experiments. * P < 0.05, ** P < 0.01 compared to mock. (e and f) Cells were treated with meloxicam (0–80 μ M). Cell lysates were harvested at 24 h and analysed by western blotting with specific antibodies to detect ER stress-related molecules IRE1, p-eIF2 α and GRP78. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments. * P < 0.05, ** P < 0.01 versus control. (g) Immunofluorescence photomicrography shows expression of GRP78 protein (magnification, $\times$ 200).

cell viability, as expected. Furthermore, down-regulation of GRP78 by siRNA notably reduced protein expression of GRP78 (Fig. 3c and d) (Fig. S1a) and significantly

enhanced increase of apoptosis (Fig. 3e and f), cleavage of caspase-3 and PARP (Fig. S1b) in meloxicam-treated HepG2 and Bel-7402 cell lines. These results suggest

Figure 2. Meloxicam induced ER stress-related apoptosis in human HCC Cells *in vitro*. (a and c) Cells were treated with meloxicam (0–80 μ M). Cell lysates were harvested at 24 h and analysed by western blotting with specific antibodies to detect ER stress-related molecule caspase-12 and pro-apoptosis molecules cleaved PARP and cleaved caspase-3. Levels of GAPDH served as a loading control. (b and d) Quantitative analysis of cleaved PARP, cleaved caspase-3 and cleaved caspase-12 following various concentrations of meloxicam treatment is presented. Data are presented as means $\pm$ SD of three independent experiments. * P < 0.05, ** P < 0.01 versus control. (e) HepG2 and Bel-7402 cells were lysed and subjected to quantitative real-time RT-PCR for measuring the levels of caspase-12. GAPDH served as an internal control. * P < 0.05, ** P < 0.01 versus control. Data are expressed as means $\pm$ SD (n = 3). (f) The effects of Z-VAD on the cell death of meloxicam in HepG2 and Bel-7402 cells. After pre-treatment with 50 μ M Z-VAD, HCC cells were treated with 80 μ M meloxicam. Apoptotic cells were analysed by FACS flow cytometry with propidium iodide (PI) and annexin V-FITC staining. The results shown are representative of at least three independent experiments. ** P < 0.01.

that GRP78 expression was associated with chemoresistance to meloxicam in human HCC cells. (-)-Epigallocatechin-3-gallate (EGCG), a flavonoid component of green tea, has been shown to block unfolded conformations of GRP78 ATPase domain and then inhibit its functions (35). In the current work, we explored properties of EGCG with regard to induction of cell death in combination with meloxicam in HepG2 and Bel-7402 cells. As shown in Fig. 3g, EGCG promoted meloxicam-induced cell death to varying degrees. Furthermore, treatment with EGCG down-regulated levels of GRP78 suggesting that it enhanced meloxicam lethality in both HCC cell types (Fig. 3h).

Involvement of autophagy in meloxicam treatment

A large number of studies has demonstrated that autophagy is activated to cope with multiple forms of cell stress (36–38). Our previous study also demonstrated, by using acridine orange staining, that meloxicam induced autophagosome formation in human HCC cells (15). To further explore the anti-tumour mechanism of meloxicam treatment here, we detected effects of meloxicam on autophagy target genes. We found that meloxicam notably increased levels of *Beclin-1*, *Atg5*, *Atg7* and activated *LC3*, whereas p62 was significantly reduced after treatment with meloxicam (Fig. 4a–d) (Fig. S2). To further confirm that LC3 was higher after treatment with meloxicam, it was visualized by immunofluorescence staining. As shown in Fig. 4e, LC3 immunofluorescence staining was notably increased after meloxicam treatment.

Protective effects of autophagy during meloxicam-induced ER stress-related cell death

Previously we have demonstrated that inhibition of autophagy by 3-MA, a specific inhibitor of autophagy, enhanced pro-apoptotic activity of meloxicam *via* up-regulating expression of Bax (15). In the current work, to further investigate whether the autophagy exerted a

crucial effect on cell death, HepG2 and Bel-7402 cells (in which autophagy was inhibited by 3-MA) were treated with meloxicam. As shown in Fig. 5a–c, the 3-MA-treated cells had significantly inhibited autophagy, whereas increased levels of cell death and expression of caspase-12 cleavage after meloxicam treatment was enhanced. Furthermore, *Atg5* knock-down by siRNA notably increased ER stress-mediated apoptotic cell death (Fig. 5d–f) (Fig. S3). These data reveal that autophagy induced by meloxicam conferred protection to HCC cells against apoptosis. Meloxicam-induced autophagy alleviated ER stress, and inhibition of autophagy enhanced ER stress-related apoptosis.

GRP78 signalling pathway involved in autophagy activation induced by meloxicam in

Several studies have revealed that the GRP78 signalling pathway is required for stress-induced autophagy (32,39). Here, we investigated whether GRP78 would be required for activation of meloxicam-induced autophagy in HCC. Inhibition of GRP78 by siRNA was used to detect expression levels of GRP78 and LC3 in HepG2 and Bel-7402 cell lines after meloxicam treatment. We found that blocking GRP78 by siRNA led to down-regulation of LC3-II in cells treated with meloxicam (Fig. 6a and b) (Fig. S4). Next, we utilized EGCG to further assess that GRP78 was involved in autophagy activation induced by meloxicam. As shown in Fig. 6c and d, EGCG significantly attenuated meloxicam-induced GRP78 up-regulation, and induction of LC3-II was notably blunted by it. These data revealed that inhibition of GRP78 suppressed autophagy activation induced by meloxicam in HCC cells.

GRP78 induced autophagy activation via the AMPK–mTOR signalling pathway

Previous studies have revealed that the AMPK–mTOR signalling pathway plays a crucial role in regulating

Figure 3. Combined effects of meloxicam and GRP78 knock-down or EGCG on apoptosis of HepG2 and Bel-7402 cells. (a and b) Cells were transfected with GRP78 siRNA (20 nM) or control siRNA (20 nM), then exposed to meloxicam (80 μ M) or not for 24 h and then cell viability was determined by CCK-8 assay. Data are representative of three independent experiments and are expressed as mean $\pm$ SD. * P < 0.01 versus control; # P < 0.05 versus meloxicam. (c and d) Cells were transfected with GRP78 siRNA and negative siRNA as a control, and then treated with meloxicam (80 μ M). Cell lysates were harvested and analysed by western blotting with specific antibodies against GRP78. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments. * P < 0.01, # P < 0.05. (e and f) Cells were transfected with GRP78 siRNA (10 nM) or negative siRNA (10 nM) (as a control), and then exposed to 80 μ M meloxicam. The combined effect of meloxicam and GRP78 knock-down on apoptosis was determined by flow cytometry (FACS) with annexin V-FITC and PI labelling. Data are presented as means $\pm$ SD of three independent experiments. * P < 0.01. (g) Cells were incubated in the presence of 80 μ M meloxicam and EGCG (10 and 30 μ M) individually or in combination. Cells were stained with annexin V-FITC and PI for apoptotic analysis by FACS flow cytometry. Data are presented as means $\pm$ SD of three independent experiments. * P < 0.05 compared to meloxicam alone. (h) Cell lysates were harvested and analysed by western blotting with specific antibodies against GRP78. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments.

autophagy (39,40). Thus, we investigated whether phosphorylation of AMPK and mTOR was involved in GRP78-mediated autophagy during meloxicam treatment. As expected, meloxicam significantly activated AMPK but inhibited phosphorylation of mTOR in HepG2 and Bel-7402 cells. However, GRP78 siRNA or EGCG suppressed changes of p-AMPK and p-mTOR induced by meloxicam (Fig. 7a and b) (Fig. S5a). To further investigate the mechanism of the AMPK–mTOR signalling pathway in meloxicam-induced autophagy, compound C, a chemical inhibitor of AMPK, was employed before treating with meloxicam. Our results revealed that treating HepG2 and Bel-7402 cells with compound C reduced meloxicam-induced activation of AMPK. In addition, we also

found that inhibition of activation of AMPK notably reduced expression of LC3 (Fig. 7c and d). However, rapamycin, an inhibitor of mTOR, attenuated the effect of compound C on expression of LC3 during meloxicam treatment (Fig. S5b). To further support the importance of the AMPK–mTOR pathway in HCC cell treatment with meloxicam, we evaluated the effect of compound C on meloxicam-induced apoptosis. Results indicated that compound C significantly enhanced meloxicam lethality in HepG2 and Bel-7402 cells (Fig. S5c). These data suggest that AMPK–mTOR is involved in autophagy activation during meloxicam treatment. GRP78 induced autophagy activation *via* the AMPK–mTOR signalling pathway.

Figure 4. Involvement of autophagy in meloxicam treatment in HCC cells. (a–d) Cells were treated with meloxicam (0–80 μM). Cell lysates were harvested at 24 h and analysed by western blotting with specific antibodies to detect autophagy-related molecules Atg5 and LC3. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments. * $P < 0.05$ versus control, ** $P < 0.01$ versus control. (e) Immunofluorescence photomicrography shows expression of LC3 (magnification, $\times 200$).

Figure 5. Inhibition of autophagy enhanced ER stress-mediated apoptotic cell death. (a) HepG2 and Bel-7402 cells were treated with meloxicam for 24 h in either the presence or absence of 3-MA (2 mM). Apoptotic cells were analysed by FACS flow cytometry with propidium iodide (PI) and annexin V-FITC staining. $**P < 0.01$ versus control, $***P < 0.01$ versus meloxicam. (b and c) Cell lysates were harvested and analysed by western blotting with specific antibodies against LC3 and caspase-12. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments. $**P < 0.01$. (d) HepG2 and Bel-7402 cells were transfected with either negative or Atg5 siRNA, then exposed to meloxicam for 24 h. Apoptotic cells were analysed by FACS flow cytometry with propidium iodide (PI) and annexin V-FITC staining. $**P < 0.01$ versus control, $***P < 0.01$ versus meloxicam. (e and f) Cell lysates were harvested and analysed by western blotting with specific antibodies against Atg5, LC3 and Caspase-12. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments. $**P < 0.01$.

Discussion

Selective COX-2 inhibition has been demonstrated to have anti-tumour effects on various malignant diseases (12,15,41). However, the exact mechanisms of meloxicam on HCC had not been fully explored. Our previous

studies demonstrated that meloxicam executed its anti-tumour effects by inhibiting cell migration, invasion, adhesion and colony formation, and inducing autophagy and apoptosis, in both COX-2-dependent and -independent pathways (15). In the current study, our data revealed that meloxicam lead to apoptosis in HCC cells by induc-

Figure 6. Autophagy after ER stress activated by the GRP78 signalling pathway. (a and b) Cell lysates were harvested and analysed by western blotting with specific antibodies against GRP78 and LC3. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments. * $P < 0.05$ versus control, ** $P < 0.01$ versus control. # $P < 0.01$ versus GRP78 siRNA(10 nM). (c and d) Protein levels of GRP78 were validated after EGCG treatment. Baseline for GRP78 and LC3 protein expression was also detected using western blotting analysis. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments. * $P < 0.05$ versus control, ** $P < 0.01$ versus control. # $P < 0.05$ versus EGCG (10 μ M), ## $P < 0.01$ versus EGCG (10 μ M).

tion of ER stress and concomitant autophagy activation. Blocking autophagy enhanced meloxicam lethality *via* increase of ER stress-mediated apoptotic cell death.

ER of eukaryotic cells, is an important organelle involved in protein biosynthesis and folding, post-translational modifications, calcium homeostasis, cell

Figure 7. GRP78 induced autophagy activation *via* AMPK-mTOR signalling pathway. (a and b) Cell lysates were harvested and analysed by western blotting with specific antibodies against p-AMPK and p-mTOR. Levels of GAPDH served as a loading control. (c and d) Representative blots of p-AMPK and LC3 treated with 80 μ M meloxicam in presence or absence of 20 μ M compound C. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments. ** $P < 0.01$ versus control, ## $P < 0.01$ versus meloxicam.

homoeostasis and apoptosis. Accumulation of unfolded/misfolded proteins induces the ER stress response, known as the unfolded protein response (UPR), when homoeostasis is disrupted. UPR consists of three signalling branches: protein kinase and site-specific endoribonuclease (IRE1), protein kinase R-like ER kinase/

pancreatic eIF2 kinase (PERK/PEK) and activating transcription factor 6 (ATF6). UPR is considered to be a cytoprotective response in the biological microenvironment. However, incessantly prolonged UPR signalling induces cancer cell apoptosis. GRP78 is a prominent ER-resident chaperone of the pro-survival pathway in

the UPR and exerts a crucial role in protein folding and assembly. Many studies have revealed that GRP78 exerts an important role in tumour formation and progression. Over-expression of GRP78 confers drug resistance, whereas inhibition GRP78 enhances chemosensitivity in anti-cancer therapy (42). However, the extent of GRP78 expression and its contribution to chemoresistance in HCC remains unclear. Our results revealed that meloxicam treatment induced activation of UPR target genes. In addition, GRP78 knock-down by siRNA or EGCG increases apoptosis suggesting that GRP78 is involved in the process of action of meloxicam.

Autophagy is a catabolic process for autophagosomal-lysosomal degradation of bulk cytoplasmic contents (43). It exerts a crucial role in mediating turnover of intracellular proteins and other macromolecules; it can be induced under conditions such as ER stress (44). Incessantly prolonged UPR signalling leads to expression of chaperones and proteins involved in the recovery process. The pre-autophagosomal structure is assembled, and subsequently transport of autophagosomes to the vacuole is stimulated in an Atg protein-dependent manner (41). Furthermore, autophagy plays a key role in contributing to drug resistance and protecting tumours from toxicity (45). In the present study, we found that meloxicam treatment induced LC3 redistribution and LC3-II accumulation indicating formation of autophagosomes. Autophagy is a complex process and various molecules are involved in autolysosome formation, the major key ones being, beclin-1, p62, Atg5 and also Atg7, important in the vesicle formation. Our data revealed that meloxicam not only enhanced extent of beclin-1, Atg5 and Atg7 but also inhibited expression of p62 suggesting that meloxicam lead to autophagosome formation in the human HCC cells. Because GRP78 enhances aggresome delivery to autophagosomes to promote drug resistance, we assumed that the GRP78 signalling pathway was required for activation of meloxicam-induced autophagy. Our results suggested that both GRP78 siRNA and EGCG attenuated meloxicam-elicited GRP78 up-regulation. Furthermore, we also found that LC3 conversion was inhibited by GRP78 siRNA and EGCG. These results demonstrate that meloxicam treatment induced ER stress signals of GRP78 which have a crucial role in induction of autophagy.

AMP-activated protein kinase (AMPK) has been considered to be an energy sensor regulating energy balance; it is activated when there is increase in AMP/ATP ratio under cell stress. Mammalian target of rapamycin (mTOR) plays a crucial role in maintaining cell population growth. Several studies have demonstrated that AMPK activates autophagy by way of blocking the target of mTOR (46,47). In our present investigation, meloxicam treatment activated phosphorylation of AMPK but

inhibited phosphorylation of mTOR in HepG2 and Bel-7402 cells. GRP78 siRNA or EGCG inhibited changes of p-AMPK and p-mTOR induced by meloxicam. Furthermore, blocking AMPK by chemical inhibitor compound C attenuated expression of LC3 and enhanced apoptotic cell death during meloxicam treatment. To further ascertain whether this process was actually through mTOR signalling, we blocked mTOR with rapamycin, an inhibitor of mTOR, under conditions of meloxicam treatment with compound C and assessed induction of autophagy. Our results showed that suppression of mTOR by rapamycin attenuated effects of compound C. These data revealed that the AMPK–mTOR pathway involved in GRP78 led to autophagy during meloxicam treatment, in agreement with recent studies suggesting that activation of AMPK leads to autophagy (48,49).

Autophagy has been considered to be paradoxical in character, being involved in both promotion or inhibition of cancer cell survival (50). In this study, our results revealed that combined treatment with specific inhibition of autophagy by 3-methyladenine (3-MA) or Atg5 siRNA knock-down notably blocked autophagy activation and increased pro-apoptotic effects of meloxicam in HCC cells. In addition, we found that caspase-12 activation, increased after autophagy, was blocked in HepG2 and Bel-7402 cell lines. As a consequence, autophagy exerted a protective effect during meloxicam.

In summary, the present study indicates that meloxicam induced ER stress and lead to autophagy in human HCC cells. Blocking autophagy enhanced meloxicam lethality for HCC, by activation of ER stress-related apoptosis. This might provide a novel therapeutic strategy for treatment of HCC.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 The combined effects of meloxicam and GRP78 knockdown on apoptosis of HepG2 and Bel-7402 cells. (a) Cells were transfected with #2 GRP78 siRNA and negative siRNA as a control, and then treated with meloxicam (80 μ M) or not. Cell lysates were harvested and analyzed by Western blotting with specific antibodies against GRP78. Levels of GAPDH served

as a loading control. $*P < 0.05$, $**P < 0.01$. (b) Cells were transfected with GRP78 siRNA and scramble siRNA as control; then treated with 80 μ M meloxicam. Cell lysates were harvested and analyzed by Western blotting with specific antibodies against cleaved caspase-3 and cleaved PARP. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments.

Fig. S2 Involvement of autophagy in meloxicam treatment in HCC cells. Cell lysates were harvested and analyzed by western blotting with specific antibodies against Beclin-1, Atg7 and p62. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments. $*P < 0.05$ vs. control, $**P < 0.01$ vs. control.

Fig. S3 Inhibition of autophagy enhances ER stress-mediated apoptotic cell death. Cells were transfected with #2 Atg5 siRNA and negative siRNA as a control, and then treated with meloxicam (80 μ M). Cell lysates were harvested and analyzed by Western blotting with specific antibodies against Atg5, LC3 and Caspase-12. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments. $*P < 0.05$, $**P < 0.01$.

Fig. S4 Autophagy after ER stress is activated by the GRP78 signaling pathway. Cell lysates were harvested and analyzed by Western blotting with specific antibodies against GRP78 and LC3. Levels of GAPDH served as a loading control. Results shown are representative of at least three independent experiments. $**P < 0.01$ vs. control.

Fig. S5 GRP78 induces autophagy activation via AMPK-mTOR signaling pathway. (a) Cell lysates were harvested and analyzed by Western blotting with specific antibodies against p-AMPK and p-mTOR. Levels of GAPDH served as a loading control. $**P < 0.01$. (b) Cells were pretreated with compound C (20 μ M) and/or Rapamycin (100 nM) and then followed with or without meloxicam (80 μ M) for 24 h. Cell lysates were harvested and analyzed by Western blotting with specific antibodies against LC3. $**P < 0.01$. (c) Annexin V/PI double-staining assay of HepG2 and Bel-7402 were analyzed by flow cytometry. Results shown are representative of at least three independent experiments. $**P < 0.01$.