Original Article
Melatonin increases the anti-tumor effects of sorafenib on human hepatoma cell lines via down-regulating autophagy

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Abstract: The multikinase inhibitor sorafenib was the established systemic therapy proven to increase the overall survival of patients with advanced hepatocellular carcinoma (HCC). However, benefits for patients on this therapy were modest in clinical practice. Melatonin is known to exert antitumor effects in HCC cells, this study investigated whether and how melatonin enhances the anti-tumor effects of sorafenib. We found that co-administration of melatonin and sorafenib exhibited a synergistic cytotoxic effect on HepG2 and Bel-7402 cells. In addition, melatonin and/or sorafenib increased the expression levels of Bax, and decreased the expression levels of Bcl-2, suggesting that apoptosis was enhanced by a combination of melatonin and sorafenib. We also found that sorafenib increased autophagic activity obviously compared with the untreated cells, as demonstrated by an upregulation of LC3-II and down regulation of P62. However, autophagic activity induced by sorafenib was obviously decreased after the addition of melatonin. Furthermore, chloroquine (CQ), an autophagy inhibitor, significantly increased the cytotoxicity and pro-apoptosis effects of sorafenib alone or combined with melatonin compared with those in the absence of CQ. Our present results suggest that sorafenib combined with melatonin generates synergetic effects on hepatoma cell lines. Moreover, synergism can be enhanced by autophagy inhibition. These results suggest that combination treatment of sorafenib and melatonin might be a potential strategy for HCC.

Keywords: Hepatoma, sorafenib, melatonin, autophagy, apoptosis

Introduction

Melatonin, a derivative of tryptophan, was isolated from the bovine pineal by the dermatologist Aaron B. Lerner in 1958 [1]. It is produced by the pineal gland [2], and other sources such as retina, gut, skin, platelets, lymphocytes, and bone marrow [3]. The main physiological functions of melatonin are related with regulating circadian rhythm, immunomodulation, hematopoiesis, and antioxidation [4-7]. In addition, a number of studies have reported that melatonin inhibits the growth of a variety of cancers including liver [8], lung, breast, prostate, and colon [9, 10], and decreases the growth rates of tumors *in vivo* both in transplantable animal models and in animal models induced by the administration of carcinogens [11, 12]. The reported anti-tumor mechanisms include anti-proliferation [13], induction of apoptosis [14, 15], inhibition of invasion and metastasis [16], and anti-angiogenesis [17, 18].

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the second leading cause of cancer-related mortality overall [19, 20]. It is reported that there are approximately 750,000 new cases of liver cancer per year [20]. The multikinase inhibitor sorafenib, which suppresses tumor angiogenesis and proliferation by inhibiting Raf kinase, vascular endothelial growth factor receptor (VEGFR), and platelet-derived growth factor receptor (PDGFR), was the first systemic agent to prolong the median overall survival com-

pared with placebo for patients with advanced HCC [21, 22]. However, sorafenib is beneficial in only approximately 30% of patients, and acquired resistance often develops within 6 months [22]. Considering the current situation, it is valuable to investigate how to improve the effect of sorafenib. Melatonin has been reported to be synergistic with other antitumor drugs [23]. In our preliminary experiment, anti-tumor effects were enhanced by a combination treatment of melatonin and doxorubicin [24]. Thus, it is imperative to study whether the effect of sorafenib could be enhanced by melatonin.

Macroautophagy (also referred to as autophagy) is a bulk degradation system that recycles unnecessary or dysfunctional cellular components, such as proteins or organelles, for the maintenance of cellular homeostasis [25]. However, programmed cell death instead of survival may be induced by excessively stimulation [26]. Therefore, the role of autophagy may be a double-edged sword [27]. Many studies have reported that sorafenib modulates autophagy both *in vitro* and *in vivo* experiment models [28]. It is reported that sorafenib-related cell resistance generation is correlated to autophagy, which may explain the unsatisfactory therapeutic efficacy of sorafenib. Inhibition of autophagy by the pharmacologic inhibitor-chloroquine (CQ)-could enhance antitumor effects or reverse resistance to targeted drugs [29]. Therefore, changes in the levels of autophagy induced by a combination of sorafenib and melatonin are worth exploring.

In the present study, our results revealed that sorafenib combined with melatonin have synergistic anti-tumor effects on HCC cell lines. We also found that autophagic activity induced by sorafenib was decreased after the addition of melatonin. Futhermore, inhibition of autophagy by CQ could further improve the anti-tumor effects of sorafenib alone or sorafenib combined with melatonin.

Materials and methods

Cell lines and cell culture

The human hepatoma cells HepG2 and Bel-7402 (Shanghai Cell Bank, Chinese Academy of Sciences) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum

and antibiotics, and incubated at 37°C in a humid atmosphere with 50 ml/L CO_2.

Drugs and reagents

Sorafenib (Santa Cruz, CA, USA) was re-suspended in dimethyl-sulfoxide (DMSO), and stocked at a concentration of 10 mM at -20°C. Melatonin (St. Louis, MO, USA) was diluted to 0.1 M and stocked at -20°C. Dulbecco modified Eagle medium (DMEM) was obtained from Wisent (Wisent Inc, USA). Rabbit polyclonal antibodies against human Bcl-2 and Bax were purchased from cell signaling technology (CST), and LC3 and P62 were obtained from ABclonal (USA). Anti-β-actin, anti-rabbit, anti-mouse IgG peroxidase-conjugated secondary antibodies were obtained from Bioworld (USA). BCA protein assay kit was purchased from Beyotime Biotechnology. Cell counting kit-8 (CCK-8) and Apoptosiskit were purchased from Bestbio (Bestbio, China). Cell apoptosis was measured by an Annexin V-FITC/propidium iodide (Bestbio, China) double-staining assay and flow cytometry (Cytomics™ FC500, Beckman Coulter, USA).

In vitro cytotoxicity examination

HepG2 and Bel-7402 cells in exponential growth phase were cultured at a density of 5000-10,000 cells/well in a 96-well plate. After treatment with various concentrations of drug for 48 hours, CCK-8 agent was added (10.0 μl/well), and the plates were incubated for another 2 hours at 37°C. The optical density (OD) values were read on a microplate reader (Bio-Tek, USA) at 450 nm. The proliferation inhibition rate was calculated as follows: IR=[1-(average OD value of experimental group)/(average OD value of control group)]×100%.

Analysis of in vitro drug interaction

In general, the coefficient of drug interaction (CDI) was used to assess the anti-proliferative effects of combination treatment, which were measured according to the absorbance of each group using the following equation follows: CDI=AB/(A×B). AB is the ratio of the combination groups to the control group at 450 nm; A or B is the ratio of the single agent groups to the control group at 450 nm. CDI value less than 0.95, between 0.95 and 1.05, and greater than 1.05, indicates that the two drugs are synergistic, additive, or antagonistic, respectively. CDI
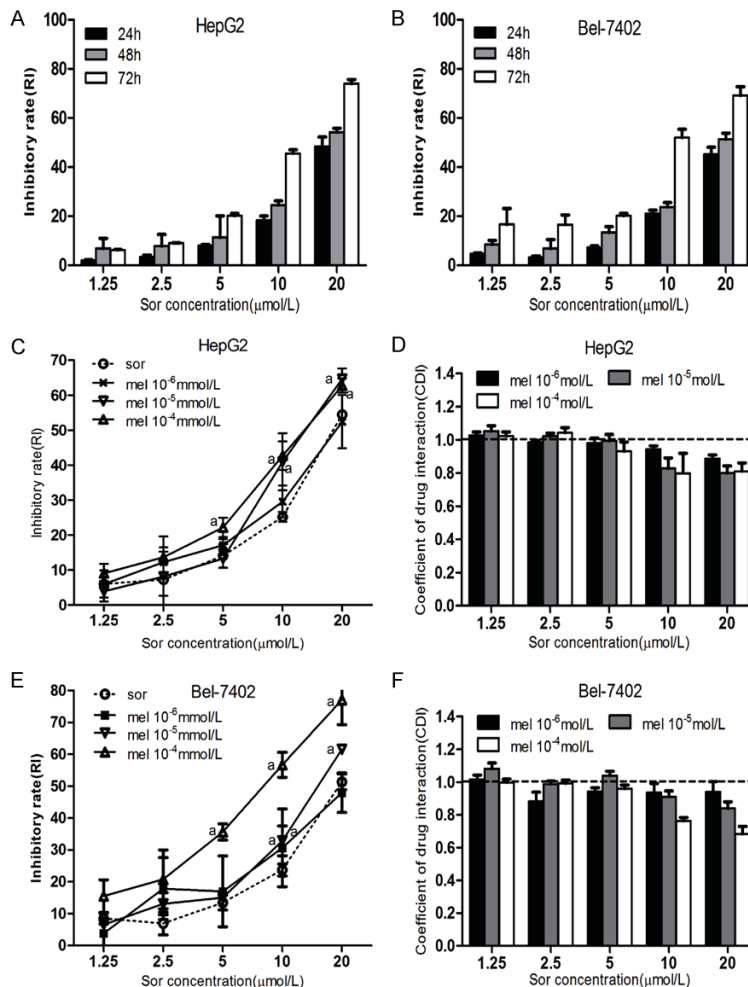
Figure 1. Effects of sorafenib, synergistic and CDI of melatonin and sorafenid on HepG2 and Bel-7402 cell. The cells were exposed to different concentrations of sorafenib for 24 h, 48 h, 72 h, The inhibitory effect of sorafenib were measured by CCk-8 assay on HepG2 (A) and Bel-7402 (B) cells. The inhibitory ratio of combination for HepG2 (C) and Bel-7402 (E) cells were determined by CCK-8 assay after cells were treated with the indicated concentrations of melatonin or sorafenib for 48 h. CDI values of the combination group were shown on HepG2 cells (D) and Bel-7402 cells (F). Date are presented as mean ± SD (error bar) of triplicate cultures. a: P<0.05 vs. sor treated alone.

less than 0.7 indicate that the drugs are significantly synergistic.

Flow cytometry

The cells in the exponential growth phase were plated in 6-well plates (1×10⁵ cells/well) and exposed to melatonin and/or sorafenib at the desired concentrations. After exposure to drugs for 48 hours, both adherent and floating cells were trypsinized and collected, then washed twice with cold phosphate-buffered saline (PBS) and centrifuged. The cell pellet was

resuspended in 400 µl binding buffer, and incubated with 5 µl/tube Annexin V/fluorescein isothiocyanate (FITC) at room temperature in the dark for 15 minutes, and then with 10 µl/tube Propidium Iodide (PI) at room temperature in the dark for 5 minutes. Suspended cells were transferred to flow cytometry test tubes and determined by flow cytometry. Data were analyzed using a flow cytometry system (Beckman Coulter, Fullerton, CA, USA).

Western blot analysis

Cells were seeded at a density of 6×10⁵ in 6-well plates for 24 hours prior to treatment. Then, cells were treated with sorafenib and/or melatonin at the desired concentrations. After treatment for 48 hours, cells were washed twice with ice-cold PBS solution and lysed. The lysates were centrifuged at 12,000 rpm for 10 minutesat 4°C, and the supernatant was resolved. The supernatant with added sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) was boiled in boiling water for 10 minutes. Protein concentrations were measured by BCA Protein Assay kit. Proteins were electrophoresed in 12.5% SDS-PAGE. After electrophoresis, the proteins were transferred onto 0.45 µm polyvinylidene fluoride (PVDF). The membranes were washed after blocking with 5% nonfat milk for 2 hours at room temperature, and proteins were detected by incubation with indicated antibody at 4°C overnight. Proteins were visualized with a horseradish peroxidase-coupled secondary antibody (1:50,000 dilution) using enhanced chemiluminescence reagents (Thermo Fisher, USA), and signals were captured using an Image QuantTM LAS-4000 Mini Imager (Fuji, Japan). β-actin was used as a loading control. The band

densities were scanned for densitometric analysis using Scion Image Software, version 4.0.3.2.

Statistical analysis

SPSS 16.0 software (SPSS Inc, Chicago, USA) was used for statistical analyses. All experiments were repeated at least three times. The measurement data was analyzed for normal distribution with the Shapiro-Wilk test. The results were reported as mean±standard deviation if normal distribution was met. Enumeration data were expressed as rates. Student's t-test was used to assess the difference between two groups. One-way ANOVOA followed by Bonferroni post hoc test was used to measure differences between mean values of the different treated groups. Non-normal distribution data were analyzed by Wilcoxon test. For apoptotic rates, we used Chi-square test to analysis differences between different treated groups. Student's t-test was used to assess the difference between two groups. One-way ANOVOA followed by Bonferroni post hoc test was used to measure differences between mean values of the different treated groups. Non-normal distribution data were analyzed by Wilcoxon test. For apoptotic rates, we used Chi-square test to analysis differences between different treated groups. A level of $P<0.05$ was accepted as statistically significant.

Results

The synergistic effects of sorafenib combined with melatonin

To study the nature of sorafenib combined with melatonin, we firstly detected the anti-proliferative effect of sorafenib on HepG2 and Bel-7402 cells by CCK-8 assay. HepG2 and Bel-7402 cells were treated with various concentrations of sorafenib (1.25-20 μM) for 24 hours, 48 hours, and 72 hours. A dose- and time-dependent anti-proliferative effect was observed on both HepG2 (**Figure 1A**) and Bel-7402 cells (**Figure 1B**). The IC50 of sorafenib were 13.21 μM and 11.83 μM for HepG2 and Bel-7402 cells at 48 hours, respectively.

Then, five doses of sorafenib (1.25 μM, 2.5 μM, 5 μM, 10 μM, and 20 μM) and three concentrations of melatonin (10^{-6} mol/L, 10^{-5} mol/L and 10^{-4} mol/L) were chosen and measured. Co-

mbined treatment with the two agents increased tumor cell growth inhibition, as compared with treatment with either sorafenib or melatonin alone on HepG2 (**Figure 1C**) and Bel-7402 (**Figure 1E**) cells. CDI values were used to evaluate the nature of the interaction. When sorafenib (10 μM) was combined with melatonin(10^{-4} mol/L), the synergistic inhibitory effect is the strongest (0.799±0.170) on HepG2 (**Figure 1D**). In this study, we chosen sorafenib (10 μM) and melatonin (10^{-5} mol/L) as the treatment concentrations, and CDI value was (0.827±0.09) for HepG2 cells at 48 hours. Because sorafenib (10 μM) is the proper concentration in human body [30], and melatonin (10^{-5} mol/L) is the pharmacological concentration.CDI of the strongest synergism was (0.683±0.065) when 10^{-4} mol/L melatonin was combined with 20 μM sorafenib on Bel-7402 cells (**Figure 1F**). And CDI value was (0.91±0.05) when the concentration was used as the same for HepG2 cells.

Melatonin increase sorafenib induced apoptosis in HCC cells

To investigate whether metatonin could increase sorafenib induced tumor cells apoptosis, Annexin V/FITC-PI staining assay and Western Blot analysis were employed. HepG2 and Bel-7402 cells were treated with melatonin (10^{-5} mol/L), sorafenib (10 μM), or a combination of both for 48 hours, and apoptotic cells were measured by FCM. We found that melatonin combined with sorafenib markedly enhanced HepG2 cells apoptosis (52.5%±12.56%) compared with either melatonin (6.79%±2.34%) or sorafenib (36.8%±1.51%) alone (**Figure 2A**, **2C**). Similar results were observed in Bel-7402 cells (**Figure 2B**, **2D**). These data indicated that synergistic effects were induced by a combination of melatonin and sorafenib on HepG2 and Bel-7402 cells.

To further confirm that the synergistic effects were caused by an increase in apoptosis, the expression levels of Bcl-2 and Bax protein in HepG2 cells were determined by Western Blot assay. The protein levels of Bcl-2 were reduced, while Bax were increased after exposing HepG2 cells to combination treatment compared with those in the control cells and those treated with melatonin or sorafenib alone (**Figure 2E**). The degree of reduction in the ratio of Bcl-2/Bax
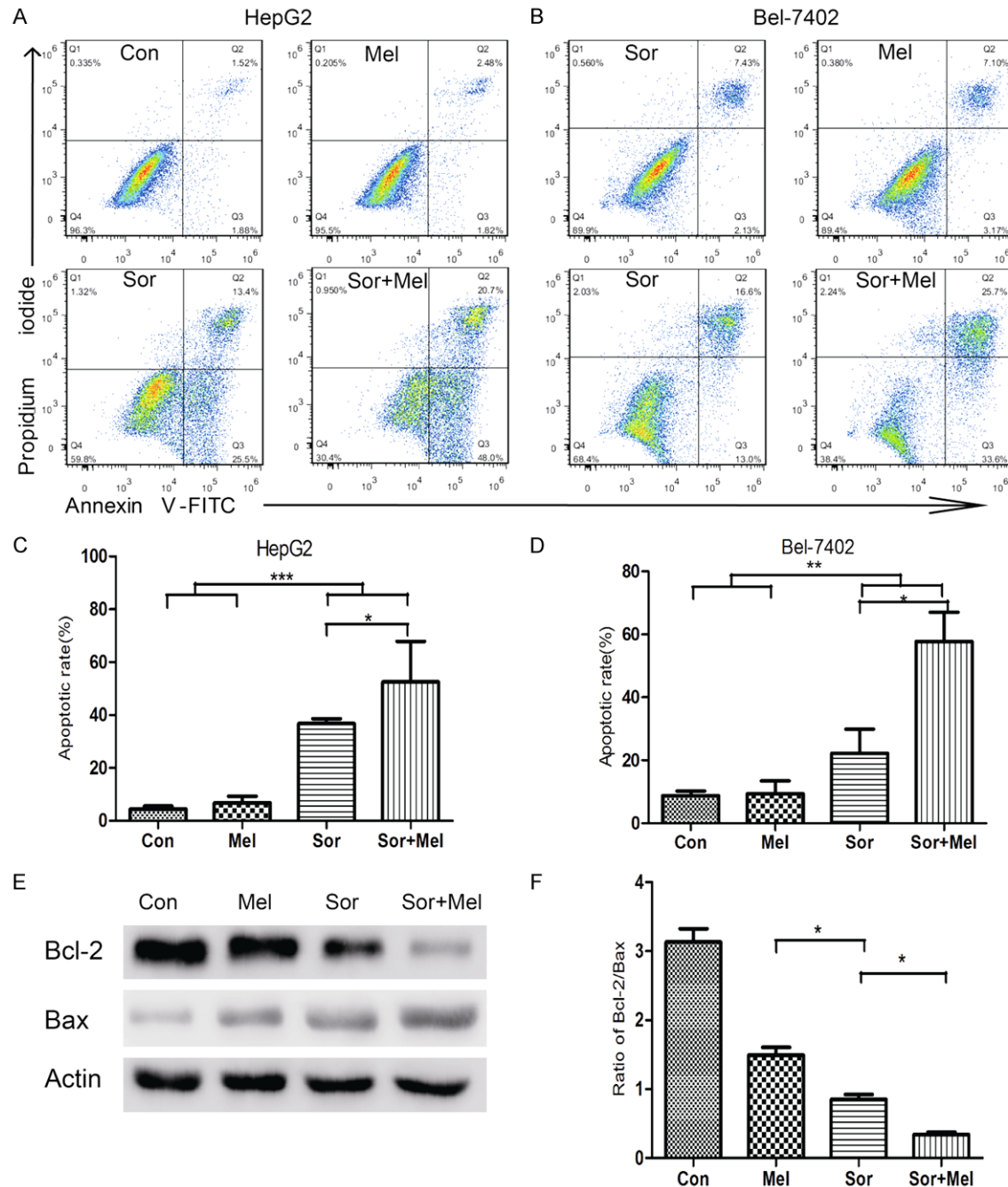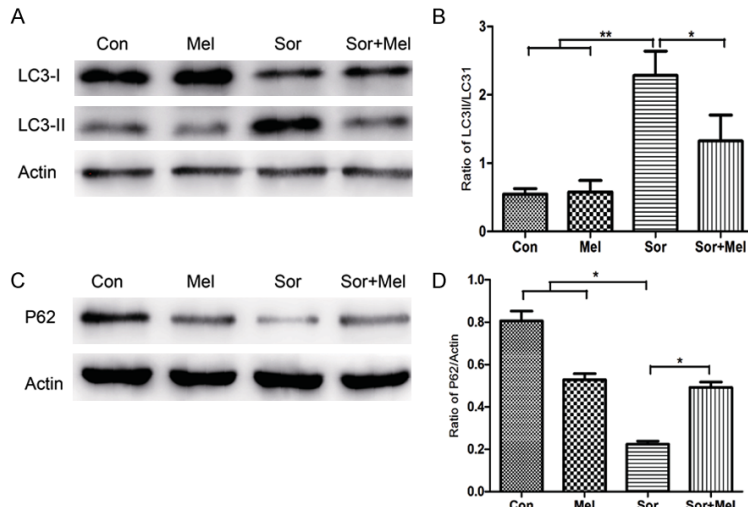
Figure 2. Effects of sorafenib and/or melatonin on apoptosis of HepG2 and Bel-7402. Cells were collected and incubated with Annexin V/FITC and propidium iodide after 48 h of treatment. The effect of each single drug and their combination on the induction of apoptosis in HepG2 (A, C) or Bel-7402 (B, D) cells were determined by FCM assay. HepG2 cells were treated with melatonin, sorafenib or a combination of both for 48 h, Westren blot analysis of expression levels of Bcl-2/Bax (E), and statistically analyzed (F). Date are presented as mean ± SD (error bar) of triplicate cultures. Con: control; Mel: melatonin; Sor: sorafenib; Sor+Mel: concurrent administration. *, ** and *** represent P<0.05, P<0.01 and P<0.001, respectively.

usually represents the degree of apoptosis. The correlation between the ratio and the degree of apoptosis was negative. As indicated in **Figure 2F**, the ratio of Bcl-2/Bax in sorafenib combined with melatonin group was significantly lower than that of control or either agent group alone. These data were further verified that the combination treatment has a better ef-

the growth inhibition rates of CQ on HepG2 at the indicated concentrations were measured by CCK-8 assay (B). After exposure to melatonin (10^{-5} mol/L) or/and sorafenib (10 μmol/L) for 48 h in the presence or absence of Chloroquine (5 μg/ml), LC3 conversion and the levels of protein P62 in HepG2 cells were detected by Western Blot assay (C). The gray value for the levels of protein LC3-II/LC3-I (D) and the ration of P62 (E) were analyzed by Scion Image Software. Date are presented as mean ± SD (error bar) of at least three independent experiments. Con: control; Mel: melatonin; Sor: sorafenib; CQ: Chloroquine. #represent P<0.05 compared with other groups and *represent<0.05.

Figure 3. A combination of melatonin and sorafenib decreased the activity of actophagy. HepG2 cells were treated with melatonin, sorafenib or a combination for 48 h. The expression levels of LC3 (A) and P62 (C) were investigated by Western blot analysis. LC3-II conversion (B) and P62 increase (D) were analyzed by Scion Image software. Date are presented as mean ± SD (error bar) of triplicate cultures. Con: control; Mel: melatonin; Sor: sorafenib; Sor+Mel: concurrent administration. * and ** represent P<0.05 and P<0.01, respectively.

fect in apoptosis. These data suggested that the synergistic effects between melatonin and sorafenib were mainly attributed to melatonin increasing HCC cells apoptosis induced by sorafenib.

Melatonin decreased autophagy induced by sorafenib

It has been reported widely that autophagy and apoptosis are closely correlated [31-33]. Thus, we wonder whether melatonin increasing apoptosis induced by sorafenib was associated with the alteration of autophagic activity. As shown in **Figure 3A**, sorafenib significantly upregulated the activity of autophagy in HepG2 cells compared with the untreated cells. However, combination of melatonin significantly decreased sorafenib induced autophagic activity compared with sorafenib alone, as demonstrated by the analysis of LC3 conversion (**Figure 3B**). In addition, sorafenib significantly decreased the expression levels of P62 proteins compared with either control or sorafenib combined with melatonin (**Figure 3C**, **3D**). While, the
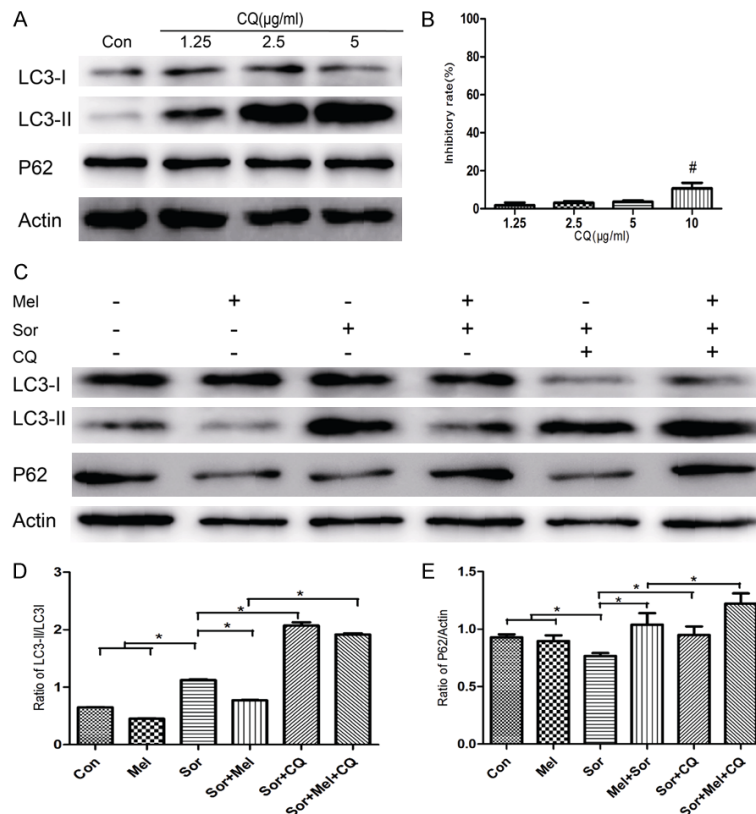
Figure 4. Effect of Chloroquine (CQ) on LC3 turn over on HepG2 cells. The cells were treated with Melatonin (10^{-5} mol/L) or/and Sorafenib (10 μmol/L) for 48 h in the presence or absence of Chloroquine (5 μg/ml). The effects of CQ on LC3 conversion were investigated by Western blot analysis (A), and

expression levels of P62 protein in the melatonin group and the control group showed no significant difference, which was consistent with the change in LC3-II/LC3-I ratio. These results indicated that autophagy might play an important role in the synergetic effects between sorafenib and melatonin.

Autophagy inhibition further enhance the synergetic effects of melatonin on the anti-tumor effects of sorafenib

To further confirm the impact of autophagy on the interaction of sorafenib and melatonin, the autophagic process was evaluated using autophagic flux analysis with a lysosomal inhibitor. HepG2 cells were pretreated with CQ 1 hour before adding melatonin and/or sorafenib. The changes of LC3 expression in the presence of CQ were compared to those in the absence of CQ. Autophagy inhibition was manifested by the accumulation of LC3-II (**Figure 4A**). We chosen CQ at the concentration of 5 μg/mL because this concentration of CQ was enough to inhibit autophagy but did not obviously cause cell death (**Figure 4B**). We observed that LC3-II accumulated significantly after lysosomal inhibition (**Figure 4C**, **4D**). And the levels of protein P62 changed correspondingly (**Figure 4E**). These results indicated that autophagy was induced by sorafenib or sorafenib combination with melatonin rather than a defect in autophagosomal degradation.

To investigate whether autophagy inhibition could influence the anti-tumor effects of sorafenib alone or combined with melatonin. HepG2 cells were treated as previously described with or without in the presence of 5 μg/mL CQ. To our expected, inhibition of autophagy by CQ increased the cytotoxicity of sorafenib alone or combined with melatonin compared with those in the absence of CQ (**Figure 5A**). Furthermore, the CDI value of sorafenib alone or combined with melatonin in the presence of CQ was 0.77±0.08 (range 0.69-0.85) and 0.70±0.08 (range 0.62-0.78), respectively (**Figure 5B**). These data confirmed that autophagy inhibition further increased the cytotoxicity of sorafenib combination with melatonin.

We also investigate whether the apoptosis was enhanced by the addition of CQ. HepG2 cells were treated with the same treatment as described previously. Flow cytometry analysis showed that apoptotic cells were increased followed by the addition of CQ (**Figure 5C**, **5D**).

These results suggested that autophagy play an important role in the synergetic effects between melatonin and sorafenib.

Discussion

Our results indicated that sorafenib combined with melatonin inhibited cell proliferation and induced apoptosis in HepG2 and Bel-7402 cells. CDI analysis showed a synergetic effect compared with a single agent. Apoptosis enhanced by combination treatment could be further confirmed using the proteins Bcl-2 degradation and Bax elevation. We also found that combination treatment down-regulated the levels of autophagy induced by sorafenib. Moreover, the results from our study also indicated that addition of CQ could increase the anti-tumor effects of sorafenib alone or combined with melatonin. Our results also indicated that melatonin enhanced the anti-tumor effects of sorafenib. Additionally, combination treatment increased the apoptosis and decreased autophagic activity compared with sorafenib alone, as demonstrated by a downregulation of LC3-II and Bcl-2 protein levels and upregulation of P62 and Bax protein levels. Furthermore, inhibition of autophagy by CQ resulted in an enhanced synergistic effect when sorafenib and melatonin were co-administered.

Sorafenib was the first systemic agent for patients with HCC. However, the therapeutic effect is unsatisfactory. A phase III randomized study reported that median overall survival in the sorafenib group was 10.7 months (95% CI 9.4-13.3) versus 7.9 months (6.8-9.1) in the placebo group (hazard ratio 0.69, 95% CI 0.55-0.87; p=0.0001) [21]. A subsequent Asia-Pacific trial by Cheng et al. showed survival benefits of sorafenib (6.5 months vs. 4.2 months) [22]. These studies indicate that benefits acquired from this agent are limited for patients with HCC. A number of studies investigated how to improve the treatment efficacy of sorafenib, such as using sorafenib combined with 5-fluorouracil, doxorubicin or erlotinib. Gao Y et al. recently reported that melatonin synergizes the chemotherapeutic effect of 5-fluorouracil in colon cancer [23]. Our preliminary experiment indicated that melatonin combined with doxorubicin could enhance the antitumor effect [24]. The synergetic anti-tumor effects of melatonin have attracted attention in many tumor cells, and related research studies are increas-
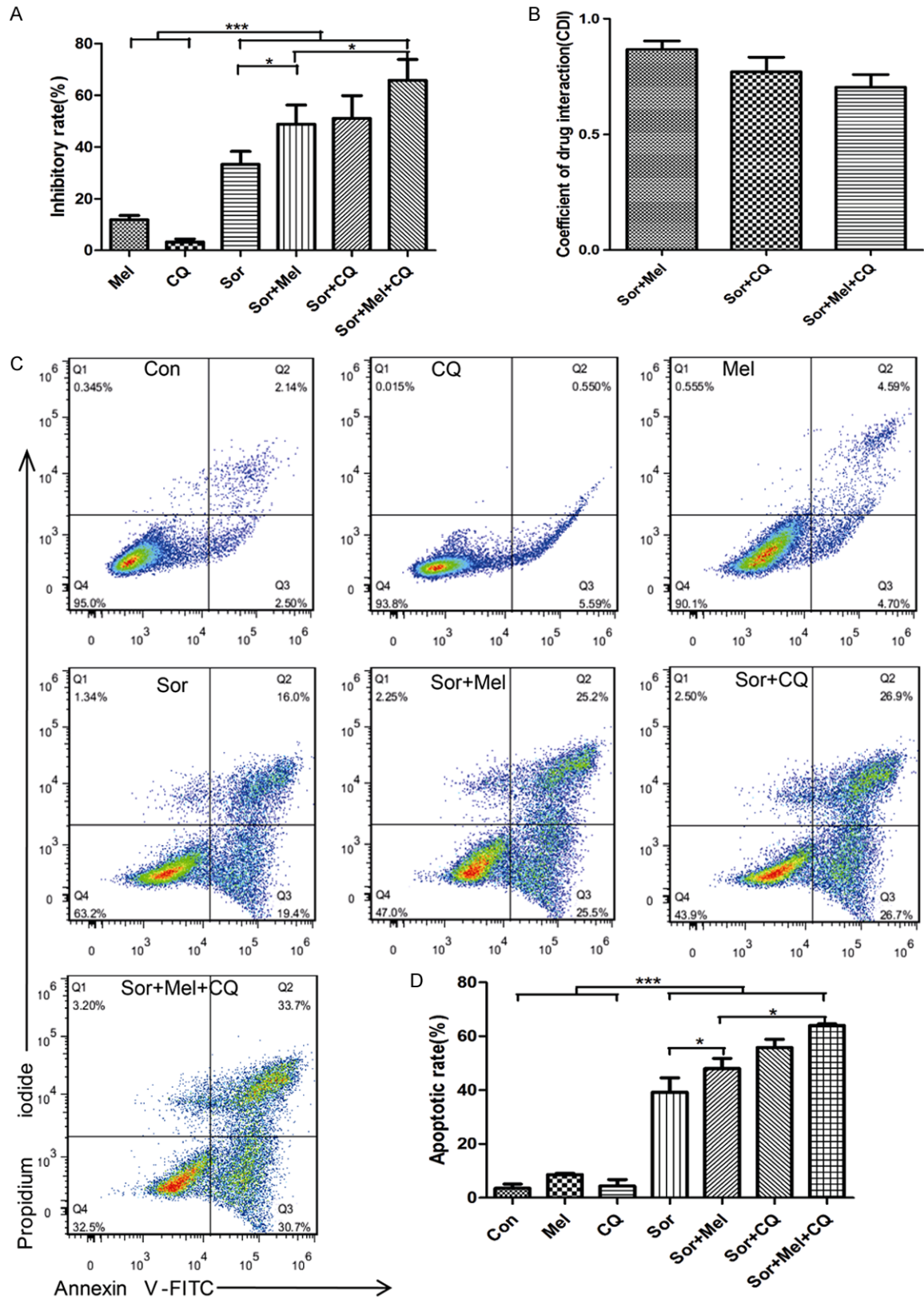
Figure 5. Inhibition autophagy by chloroquine (CQ) increased the antitumor effects of combination treatment of melatonin and sorafenib. The cells were treated with melatonin (10^{-5} mol/L) or/and sorafenib (10 µmol/L) for 48 h in the presence or absence of chloroquine (5 µg/ml). The inhibitory effect was measured by CCK-8 assay (A) and FCM (C, D). CDI values were shown (B). Date were determined as mean ± SD (error bar) of at least three independent experiments. * and *** represent P<0.05 and P<0.001, respectively.

ing rapidly [34, 35]. In the present study, our results show that sorafenib inhibits proliferation of HepG2 and Bel-7402 in a dose- and time-dependent manner. Moreover, combination treatment using sorafenib with melatonin for 48 hours resulted in a marked increase in the inhibition of proliferation, compared with treatment with sorafenib or melatonin alone. In addition, cell apoptosis could be enhanced by combination treatment, as demonstrated by the apoptosis detection. These results indicated that the therapeutic effect may be enhanced by the combination treatment of sorafenib and melatonin.

Recently, the pro-apoptotic effects of melatonin in many tumor cells have attracted attention, and related studies are increasing rapidly [36, 37]. Evidence of melatonin-driven death pathways is currently being gathered [38]. It was reported that the growth inhibition induced by melatonin was related to downregulation of the antiapoptotic protein Bcl-2 and upregulation of the pro-apoptotic protein Bax [39]. In order to explore the apoptotic effects induced by melatonin and/or sorafenib, the expression levels of proteins Bcl-2 and Bax were detected. They have contrary functions: Bcl-2 acts to inhibit apoptosis, whereas Bax reverses this effect. In our present study, melatonin and/or sorafenib increased the expression of Bax, and decreased the expression of Bcl-2. The ratio of Bcl-2/Bax was decreased obviously when hepatoma cells were treated with a combination of melatonin and sorafenib.The results of our present study indicated that the expression levels of Bcl-2 and Bax protein may be involved in the synergetic effect induced by combination treatment.

Some studies have been reported that the increase of apoptosis was related to the elevation of autophagy [32, 33]. Autophagic process involves a highly conserved group of macromolecules which were discovered in yeasts [40], and autophagosome formation is conducted by five groups of proteins which include ULK1 (unc-51-like kinase) complex [41], Beclin 1-Vp-34 (vacuolar protein sorting 34) complex [42], Atg9 (autophagy-related gene 9), and the WIPI-1 (WD-repeat protein interacting with phosphoinositides) system [43], as well as LC3 (microtubule-associated protein 1 light chain 3) [44]. It has been reported that autophagic activity by sorafenib is dose- and time-dependent in HepG2, MHCC97-L, Huh7, and PLC/PRF/5 HCC cells [45]. The report claimed that sorafenib induced autophagosome formation and enhanced autophagic activity, which conferred a survival advantage to hepatoma cell. Our present study observed that LC3 conversion induced by sorafenib is clear. And the levels of protein P62 had the corresponding inverse changes. However, it is surprisingly that autophagic activity was decreased when the cells were treated with a combination of sorafenib and melatonin compared with sorfenib alone. The expression levels of P62 protein increased correspondingly. Some studies reported autophagic activity could be suppressed by melatonin [46, 47]. In general, autophagy is seen as a protective mechanism in cell survival. Thus, our results indicated that down-regulating autophagy induced by combination treatment may be involved in the synergistic anti-tumor effects. CQ, as a pharmacologic inhibitor of autophagy, has been proved to increase tumor cell death. Our preliminary research reported that CQ could significantly reverse the antagonistic effects of gefitinib combined with cisplatin in epidermal growth factor receptor (EGFR) mutant non-small-cell lung cancer cells [29]. We inhibited autophagy by CQ and found that the synergetic effect of combination treatment was significantly increased after the addition of CQ. These findings could further confirm that autophagy degradation plays a important role in the synergetic effects of combination treatment.

In conclusion, our present studie indicated that sorafenib combined with melatonin have synergistic anti-tumor effects on HCC cell lines. And the synergistic effects may be attributed to melatonin which could reduce the activity of sorafenib induced autophagy. Furthermore, inhibition of autophagy by CQ could further improve the antitumor effects of sorafenib alone or combined with melatonin. All of these results suggest that combination treatment of targeted agents and melatonin may be a potential therapy in HCC treatment in the future.

Acknowledgements

Disclosure of conflict of interest

None.

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