Asian Journal of Chemistry; Vol. 24, No. 6 (2012), 2617-2621

ASIAN JOURNAL OF CHEMISTRY

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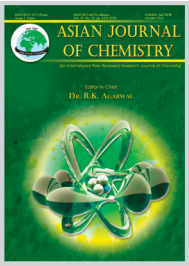

# Involvement of Reactive Oxygen Species in Sorafenib-Induced Autophagy in HepG2 Cells

HAILEI DU[1,†], LIN CHEN[2,†], WEIPING YANG[3], HAO CHEN[3], MINGMING SHI[4], VARUN SEEWOO[3] and WEIHUA QIU[3,*]

[1]Department of Thoracic Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, P.R. China
[2]Department of Respiratory Disease, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, P.R. China
[3]Department of Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, P.R. China
[4]Institute of Digestive Surgery, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, P.R. China

*Corresponding author: Tel.: +86 21 64370045, Ext: 611020; E-mail: drqwh2003@hotmail.com
†Hailei Du and Lin Chen contributed equally to this work.

(*Received*: 2 June 2011; *Accepted*: 17 January 2012) AJC-10957

Sorafenib is a newly established cancer drug found to be an effective systemic treatment for advanced hepatocellular carcinoma. However, little is known about any potential effectors that modify tumor cell sensitivity towards sorafenib. Autophagy, as a physiological cellular mechanism, is involved in both cell survival and cell death. Reactive oxygen species have been identified as signaling molecules in various pathways regulating both cell survival and cell death. In this study, it is found that the increased expression of Beclin 1 at gene and protein levels indicated that sorafenib could induce autophagy in HepG2 cells. Reactive oxygen species triggered by sorafenib may also induce autophagy. Reactive oxygen species mediated autophagy may play protective role in HepG2 cells treated with sorafenib. Reactive oxygen species inhibitors may enhance the sensitivity of molecularly targeted therapies in treatment of hepatocellular carcinoma.

**Key Words: Hepatocellular carcinoma, Autophagy, Apoptosis, Sorafenib, Beclin 1, Reactive oxygen species, NAC.**

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in many parts of the world, particularly in the Far East, South Sahara and Southern Europe[1]. Over the recent years, the occurrence of hepatocellular carcinoma has actually increased markedly in low-risk countries such as the United States[2]. Hepatocellular carcinoma is often diagnosed at an advanced stage when most potentially curative therapies such as resection, transplantation or percutaneous and transarterial interventions are of limited efficacy[3,4]. Hence, clinical development of novel therapeutic agents in hepatocellular carcinoma has begun in earnest.

Sorafenib, is a multikinase inhibitor that has shown efficacy against a wide variety of tumors in preclinical models[5]. Antitumor effects of sorafenib in renal cell carcinoma and in hepatoma have been ascribed to antiangiogenic actions of this agent through inhibition of the growth factor receptors[6,7]. It has shown modest survival benefits in advanced hepatocellular carcinoma in two randomized controlled trials, supporting the use of molecularly targeted therapies in treatment of hepatocellular carcinoma[8]. As the first anticancer drug to improve survival in advanced hepatocellular carcinoma, sorafenib has paved the way for novel therapies in the treatment of patients with hepatocellular carcinoma. However, it is still less known about any potential effectors that modify tumor cell sensitivity towards sorafenib.

Autophagy, which is a dynamic process involving the bulk degradation of cytoplasmic organelles and proteins. Based on the function of cellular recycling, autophagy plays key roles in the quality control of cellular components as well as supplying nutrients and materials for newly constructed structures in cells under metabolic stresses[9]. Recent studies suggest that autophagy may not only be important in the regulation of cancer development and progression, but also in determining the response of tumor cells to anticancer therapy[10,11].

It is widely recognized that reactive oxygen species (ROS) play an important role in cancer initiation and progression. Reactive oxygen species have been identified as signaling molecules in various pathways regulating both cell survival and cell death[12]. Many stimuli, under clinical investigation as cancer treatments, induce autophagy and reactive oxygen species generation as well. Such as hypoxia, nutrient starvation, oxidative stress and so on. Moreover, recent research has showed that autophagy is also regulated by reactive oxygen species[13].

Therefore, this study was designed to investigate autophagy and reactive oxygen species generation after sorafenib treatment in HepG2 cells and discuss whether reactive oxygen species was also in the regulation of autophagy in HepG2 cells.

## EXPERIMENTAL

The following materials were obtained from the indicated sources: Acridine orange and *N*-acetylcysteine were purchased from Sigma (St. Louis, MO). Cell counting Kit-8(CCK8) was purchased from Dojindo Japan. The antibodies: polyclonal rabbit anti-Becline-1, monoclonal mouse antiGAPDH, goat antirabbit and goat antimouse IgG secondary antibody were from Santa Cruze Biotechnology. Sorafenib was synthesized by Bayer Corporation (West Haven, CT). Compounds were dissolved in 100 % DMSO (Sigma, St. Louis, MO) and diluted with dulbecco's modified Eagle's medium (DMEM) to the desired concentration with a final DMSO concentration of 0.1 % for *in vitro* studies.

The hepatocellular carcinoma cell line HepG2 was purchased from American type culture collection (Rockville, MD) and cultured as described earlier[14]. Cells were plated 24 h before treatment and were at subconfluent density at the time of drug exposure. The logarithmically growing HepG2 was treated by one of the most active anticancer compounds. Sorafenib (S-8502, LC Laboratories, Woburn, USA ) was dissolved in 100 % DMSO (Sigma, St. Louis, MO) and diluted with dubecco's modified Eagle's medium to achieve a desired concentration of 0, 5, 10, 15 and 20 mM. DMSO was added to cultures at 0.1 % as a solvent control. To further examine whether reactive oxygen species mediated autophagy plays a role in cancer treatment, *N*-acetylcysteine was included in system. Before administrated by sorafenib, cells were pretreated with *N*-acetylcysteine at final concentration of 5 Mm for 2 h. Sorafenib were added to cell cultures to achieve the indicated concentration. In system control, *N*-acetylcysteine, without sorafenib, was solely added to HepG2 cultures at a final concentration of 5 mM. Corresponding volumes of DMSO were added as solvent controls in mock study.

### Detection method

**Colour imetric viability assay:** Cell viability was estimated using the colour imetric assay. HepG2 was plated in triplicate, at a density of 5,000 cells/well in a volume of 100 mL in 96-well microtiter plates and incubated overnight. On the following day, sorafenib and *N*-acetylcysteine were added to wells at the dosage mentioned above. In inhibition study, sorafenib was included into culture after *N*-acetylcysteine pretreatment. Plates were incubated for an additional 24 h. After incubation, 20 mL of the cell-counting solution (Dojindo Laboratories, Japan) were added to each well and incubated at 37 ºC for 3 h. The absorbance of the solution was read spectrophotometrically at 450 nm with a reference at 650 nm using a microtiter plate reader (Becton Dickinson). Cell viability was calculated according to the following formula: Cell viability (%) = [1-(A450 sample-$A_{450\ blank}$)/($A_{450\ control}$ - $A_{450\ blank}$)] × 100. The $IC_{50}$ value, at which 50 % of the cell growth inhibition compared with DMSO control, was calculated by nonlinear regression analysis using GraphPad Prism software (San Diego, CA).

**Detection of acidic vesicular organelles (AVOs) with acridine orange staining:** Cells were treated by 6 μM sorafenib for 24 h as described above, then acridine orange was added to culture medium at a final concentration of 1 mg/mL post sorafenib treated and followed by incubation in dark at room temperature for 15 min. Samples were then examined under a fluorescence microscopy. A typical acridine orange accumulation in acidic autophagic vacuoles exhibited granular distribution of bright red fluoresce in the cytoplasm, indicative of autophagosome formation. Acidic vesicular organelles fluorescence was measured with an excitation wavelength of 488 nm and an emission wavelength of 515 nm. The amount of acidic vesicular organelles was quantitatively determined according to the red-to-green fluorescence ratio, which was obtained using Photoshop software (Adobe, San Jose, USA).

**Reverse transcription-PCR:** Total RNA was extracted from cell lines using trizol reagent (invitrogen). Total RNA was reverse transcribed using a revert aid first-strand cDNA synthesis kit (Fermentas). PCR amplification cycles were programmed for 10 s at 95C, followed by 40 cycles of 95C for 5 s and 60C for 30 s. GAPDH was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. The following primers were used: Beclin 1:5'-AGCTGCCGTTATACTGTTCTG-3' and 5'-ACTGCCTCCTGTGTCTTCAATCTT-3';GAPDH:5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3'.

**Isolation of proteins and immunoblot analyses:** Beclin 1 is a very important and well-documented autophagy-related protein. Therefore, we used western blot to examine the changes of Beclin-1 in HepG2 and further confirm the role of autophagy following different treatment. Cells were harvested at various time points by centrifugation at 800 rpm for 5 min at 4 ºC and washed twice with cold phosphate-buffered saline (PBS). Then cells were lyzed in a lysis buffer (1 % Triton X-100 and 1 % nonidet P-40 in PBS). The supernatant was collected after high-speed centrifugation and a protease inhibitor mixture (aprotinin 30 μg/mL, leupeptin 4 μg/mL, aepstatin 2 μg/mL and PMSF 10 μg/mL) was added to the supernatants. Protein concentration was quantified using the Biorad Bradford Assay (Bio-Rad Laboratories, Hercules CA). 12.5 μL proteins were mixed with equal volume 2x electrophoresis sample buffer and boiled for 5 min. The samples were separated on 12.5 % SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad). After transfer, membranes were blocked using 5 % nonfat dry milk in phosphate-buffered saline-Tween 20 for 1 h at room temperature. Blots were probed with polyclonal rabbit anti-Beclin-1 and mouse monoclonal anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 ºC. GAPDH was used as an internal control. Goat antirabbit or goat antimouse IgG secondary antibody (1:5000; Santa Cruz biotechnology) was incubated with the polyvinylidene difluoride membranes at room temperature for 1 h and then washed with 0.5 % I-block blocking buffer. The blots were incubated with chemiluminescnet substrate and detected by enhanced chemiluminescence assay (Millipore). Band density was measured by densitometry, quantified using photoshop CS2.0 and normalized to an indicated sample in the identical membrane. Blots shown are representative of at least three independent experiments.

**Determination of intracellular reactive oxygen species:** The generation of reactive oxygen species in the cells was evaluated by a fluorometry assay using intracellular oxidation of the non-fluorescent probe 2,7-dichlorofluorescein diacetate

(DCFH-DA). 2,7-Dichlorofluorescein diacetate can passively diffuse into cells and be deacetylated by esterase to form non-fluorescent 2,7-dichlorofluorescein (DCFH). In the presence of reactive oxygen species, 2,7-dichlorofluorescein reacts with reactive oxygen species to form the fluorescent product 2,7-dichlorofluorescein, which is trapped inside the cells. When the membrane is oxidized and damaged, the fluorescence will attenuate significantly. The cells were washed with ice-cold 1x PBS and then incubated with 2,7-dichlorofluorescein-diacetate at 37 ºC for 20 min. Then DCF fluorescence intensity was detected by fluorescence spectrometry (Spectramax Gemini, Molecular Devices, USA) with an excitation wavelength of 490 nm and an emission wavelength of 530 nm. The results were expressed as relative fluorescence intensity per 104 cells. *N*-acetylcysteine (NAC, Sigma, 616-91-1), the specific inhibitor of reactive oxygen species, was further employed to confirm the specific generation of reactive oxygen species. Cells were pretreated with *N*-acetylcysteine at a final concentration of 5 mM.

**Statistical analysis:** Statistical analysis was performed using SPSS software (v.13.0). Data are expressed as mean ± SEM of at least three independent experiments. Comparisons of quantitative data were analyzed using student's t test between two groups or by one-way ANOVA for multiple groups. The statistically significant difference was defined when P value was < 0.05.

## RESULTS AND DISCUSSION

**Antiproliferation effects of sorafenib in HepG2:** To investigate the cytotoxicity of sorafenib in hepatocellular carcinoma, HepG2 cells were treated with various concentrations of sorafenib for 24, 48 and 72 h. The cell viability assay were used to evaluate the effect of sorafenib on cell proliferation on HepG2. As shown in Fig. 1, the cell viability was inhibited and sorafenib produced a dose-and time-dependent reduction in cell viability. The half maximal inhibitory concentration ($IC_{50}$) for 24h of treatment in HepG2 cells was 4.43 ± 1.50 µM. Therefore, we used 6 µM of Sorafenib for 24 h in HepG2 for further experiments.

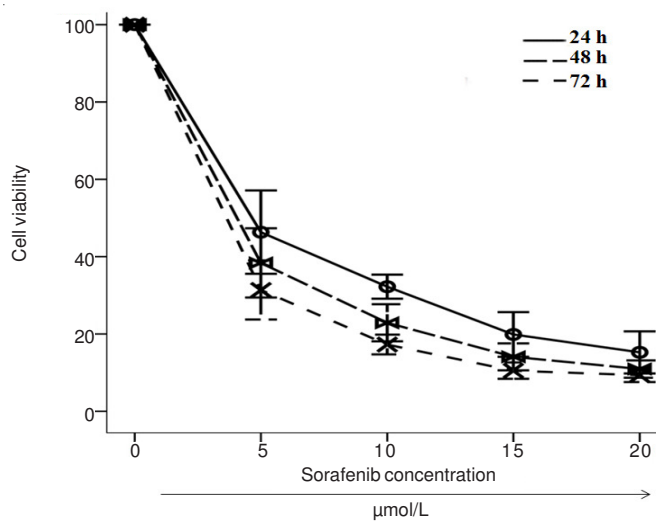


Fig. 1. Effects of sorafenib on cell viability in HepG2 cells

**Sorafenib could induce autophagy in HepG2 cells, the autophagy activity increased at both gene and protein levels:** To determine whether sorafenib could induce autophagy in HepG2 or not, we analyzed the formation of the accumulation of autophagic vacuoles (AVOs). The autophagic vacuoles can be labeled by acridine orange, which emitted bright red fluorescence in acidic vesicles but fluoresced green in cytoplasm and nucleus[15]. Vital staining of HepG2 with acridine orange revealed the appearance of acidic vesicular organelles after sorafenib treatment was employed. As is showed in Fig. 2A, through fluorescence microscope we found the number of distinct dotlike structures distributed within the cytoplasm or localized in the perinuclear regions (acridine orange stained the autophagic vacuole) was higher than in the control. Quantitatively, the red-to-green fluorescence ratio demonstrated a significant increase in HepG2 cells compared with that of the control (0.63 ± 0.01 versus 0.52 ± 0.02, p < 0.01). These results indicated that autophagic activity increased in response to sorafenib in HepG2 cells.

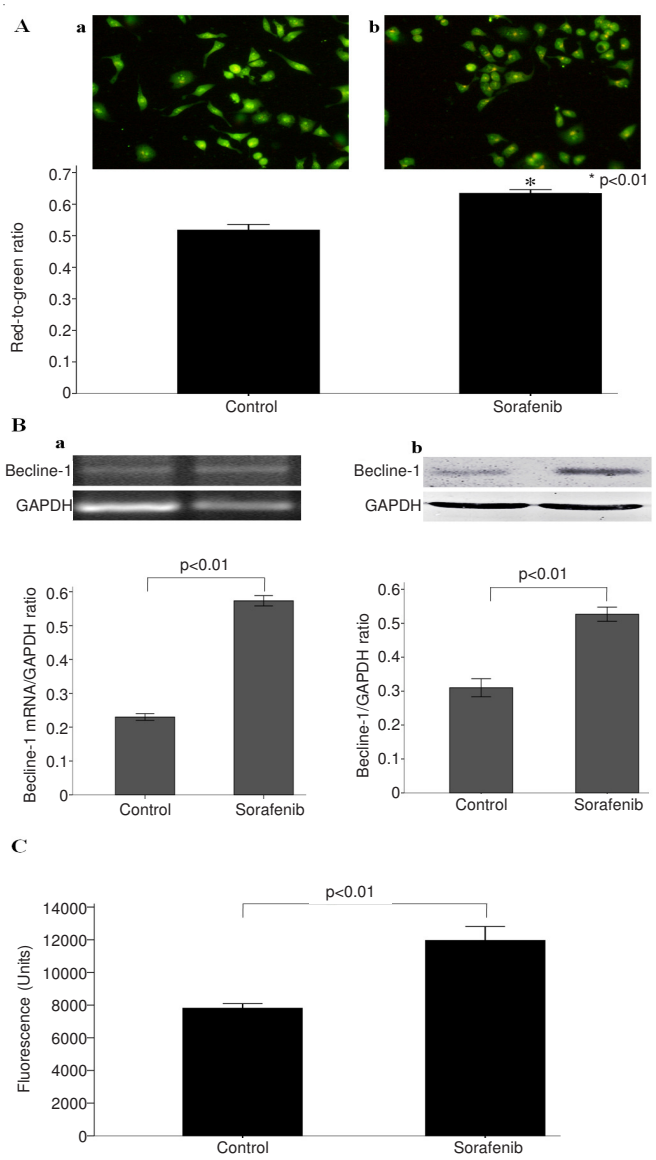


Fig. 2. Autophagy activity and reactive oxygen species generation induced by sorafenib in HepG2 cells

Beclin-1 is a haploin sufficient tumor suppressor gene and decreased expression of Beclin-1 has been reported in different tumors[16]. Then we explore the changes of Beclin-1 mRNA by reverse transcription-PCR in HepG2. Our results showed that the mRNA expression level of Beclin-1 was obviously higher in response to sorafenib compared with mocks. As is showed in Fig. 2B, we compared the bands intensities and the mean ratio of Beclin-1 mRNA to GAPDH in control was used as an endogenous control to normalize for differences. After treated with sorafenib, the mean ratio of Beclin-1 mRNA/GAPDH increased to 0.57 ± 0.02 in HepG2 compared to 0.23 ± 0.01 in negative control. Moreover, a significant difference was observed. ($p < 0.01$).

Since Beclin-1 is a very important and well-documented autophagy-related protein[17,18], to further survey the activity of autophagy, we compared the protein expression of Beclin-1 after sorafenib treated. Present data showed that the Becline-1 protein level was markedly increased after treatment of sorafenib. As is showed in Fig. 2B, the mean ratio of Beclin-1/GAPDH increased significantly compared with control (0.53 ± 0.02 vs 0.31 ± 0.03 $p < 0.01$). These results indicated that autophagy is also involved in the cell response to sorafenib treatment in HepG2 cells.

**Effect of sorafenib on intracellular reactive oxygen species production in HepG2 cells:** In order to detect the changes of intracellular reactive oxygen species, we used fluorescence spectrometry to measure the fluorescence of DCF after sorafenib treatment. As shown in Fig. 2C, elevated levels of intracellular reactive oxygen species were found that reactive oxygen species generation dramatically increased by sorafenib treatment following 6 μM sorafenib treatment for 24 h. The fluorescence intensity in HepG2 cells treated with sorafenib was approximately 1.53-fold higher compared with control. More ever, the accumulation of reactive oxygen species was significantly intreased ($p < 0.01$).

***N*-acetylcysteine reduced autophagy activity induced by sorafenib in HepG2:** To explore whether sorafenib induced autophagy is mediated by reactive oxygen species accumulation, reactive oxygen species scavenger *N*-acetylcysteine was employed to block reactive oxygen species generation. Cells were pretreated with 5 mM *N*-acetylcysteine for 2 h and then treated with 6 μM sorafenib for 24 h. By fluorescence microscopy, it is observed that with the addition of *N*-acetylcysteine, the number of autophagic vacuoles was lower than in sorafenib group. For quantitative analysis, a significant decreased in red-to-green fluorescence ratio in sorafenib treated cells compared with the sorafenib plus *N*-acetylcysteine cells (Fig. 3A).

To further confirm whether the autophagy activity changed when reactive oxygen species generation was blocked, autophagy-related protein Beclin-1 was employed. As shown in Fig. 3B, the mean ratio of Beclin-1 to GAPDH decreased to 0.11 ± 0.01 in sorafenib plus *N*-acetylcysteine group compared to 0.25 ± 0.02 in sorafenib group. Moreover, a significant difference was observed. ($p < 0.01$). Our results showed autophagy activity was reduced when reactive oxygen species generation was blocked. These results suggested that sorafenib mediated autophagy may be dependent on reactive oxygen species accumulation.

***N*-Acetylcysteine decresed the cell viability induced by sorafenib in HepG2 cell:** Furthermore, we detected whether reactive oxygen species accumulation induced by sorafenib plays an effect on cell viability. *N*-acetylcysteine, the specific inhibitor of reactive oxygen species, was added. The change of cell viability was examined using sorafenib with or without *N*-acetylcysteine. As is showed in Fig. 3C, the viability of HepG2 decreased faster in the sorafenib plus *N*-acetylcysteine group than in the sorafenib group. The combination drove almost 8 % decrease compared with the rate of cell viability in sorafenib group. The apparent decrease of cell viability could be induced statistically by sorafenib plus *N*-acetylcysteine treatment compared with sorafenib ($P = 0.024$).

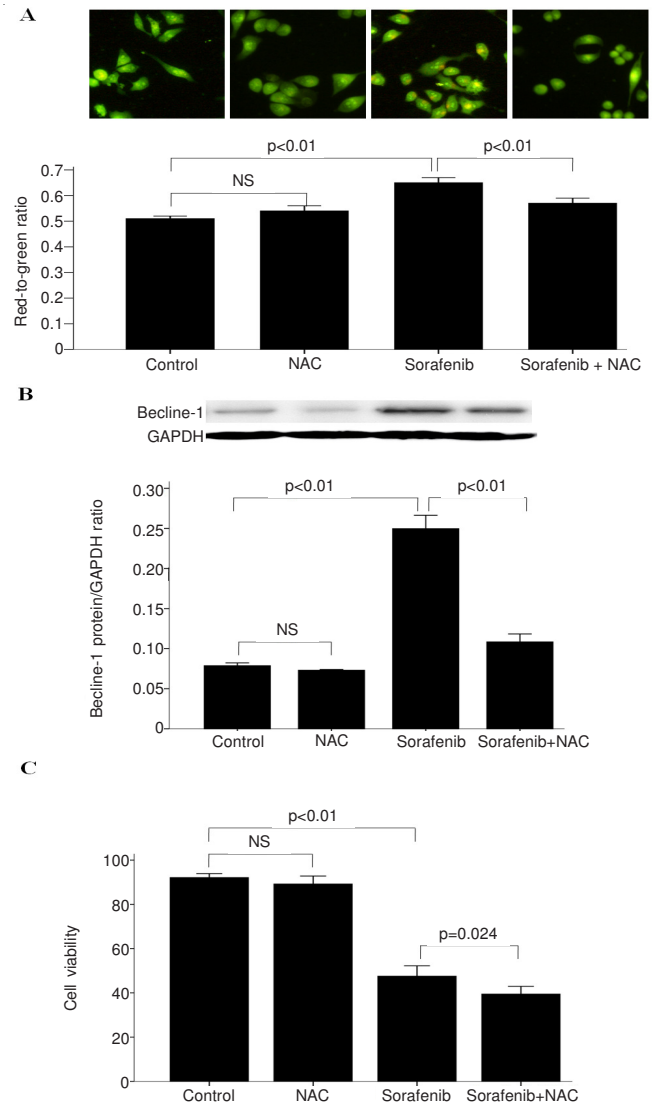


Fig. 3. Changes of autophagy activity and cell viability when reactive oxygen species generation blocked

Hepatocellular carcinomas with poor prognosis are characterized by rapid cell proliferation and strong expression of antiapoptotic genes[19]. As a multikinase inhibitor, sorafenib has shown efficacy to block tumor cell proliferation and angiogenesis by inhibiting serine/threonine kinases (c-RAF) as well as the receptor tyrosine kinases vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR). Sorafenib has shown the optimal

results in improvement of survival and the time to radiological progression in patients with advanced hepatocellular carcinoma[20-22]. The proliferative inhibitory abilities of sorafenib have been shown to be due to the targeting of several kinases, such as serine/threonine kinases (c-RAF). However, the multitude of targets attributed to sorafenib may produce variable effects on different cells. To determine the possible clinical application of sorafenib in hepatocellular carcinoma therapy, we examined the antiproliferation effects of sorafenib on HepG2 cells *in vitro*. In present study, it is showed that sorafenib is effective in reducing the growth of human hepatocellular carcinoma *in vitro*. Notably, sorafenib inhibit HepG2 cells proliferation in a dose and time-dependent manner.

Autophagy is a major mechanism for degrading long-lived cytosolic proteins and the only known pathway for turnover of large cellular structures such as organelles and protein aggregates. The stimulation of autophagy in cancer cells was often observed in response to anticancer treatments[23]. However, the role of autophagy in cancer is both complex and controversial. It remains questionable whether the observed autophagic response is a survival attempt by tumor cells or a killing mechanism of anticancer agents. In present experiment, it is found that the number of autophagic vacuoles increased after sorafenib treatment. Meanwhile, significant increases were also observed in Beclin-1 expression occurred both at mRNA and protein levels in HepG2 cells after sorafenib treatment compared with control. The autophagy activity results demonstrated that autophagy could induce by sorafenib in HepG2 cells.

Reactive oxygen species generation is an established anticancer mechanism for many existing drugs. It has been identified as signaling molecules in various pathways[24]. Similar to autophagy, reactive oxygen species is also involved in both cell-survival and cell-death[25]. It is widely recognized that reactive oxygen species play an important role in cancer initiation and progression[12]. Present experiment showed that the fluorescence of 2,7-dichlorofluorescein was increased after sorafenib treatment compared with mock. The results suggested reactive oxygen species was also triggered by sorafenib.

Because reactive oxygen species and autophagy are similarly involved in cell-survival and cell-death pathways, as well as cancer progression and treatment, to date, the precise relationship between autophagy and reactive oxygen species remains unclear. Recently study indicated that the reactive oxygen species (ROS), originated from mitochondrial oxidative stress, seems to play a central role in autophagy[13]. Although reactive oxygen species play an important role in regulating autophagy, the reverse also occurs. In our study, we observed that the fluorescence of 2,7-dichlorofluorescein was increased after sorafenib treatment compared with mock. More importantly, the autophagy activity was significantly blocked when the reactive oxygen species scavenge *N*-acetylcysteine was added. Present data demonstrated that sorafenib stimulated the reactive oxygen species generation, which shows that these oxygen radicals were partly source for autophagy, which was induced by sorafenib. Furthermore, *N*-acetylcysteine showed significantly effect on HepG2 cells viability inhabited by sorafenib. Since many cancers are intrinsically resistant to apoptosis or develop resistance during treatment, causing cell death through autophagy and/or reactive oxygen species are attractive strategies. Thus, we hypothesized that ROS inhibitors may enhance the sensitivity of molecularly targeted therapies in treatment of hepatocellular carcinoma. However, the effects of sorafenib on mitochondria function and apoptotic process needed to be further investigation.

In summary, present results demonstrated that sorafenib could inhibit proliferation of HepG2 cells and increase intracellular reactive oxygen species levels. Reactive oxygen species triggered by sorafenib may also induced autophagy. Moreover, reactive oxygen species mediated autophagy may play protective role in HepG2 cells treated with sorafenib.

## ACKNOWLEDGEMENTS

This study was supported by Nature Science Foundation of China (81172326, 30872511), Shanghai Science and Technology Commission Grant (10ZR1419400) and Shanghai Charity Foundation for Cancer Research.

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