RE: Inhibition of autophagy significantly enhances combination therapy of sorafenib with HDAC inhibitors in human hepatoma cells

Responses to Comments by Reviewer 1:

(1) The title is Inhibition of autophagy significantly enhances combination therapy of sorafenib with HDAC inhibitors in human hepatoma cells. The authors should present more details to enhance anti-tumor effect or tumor growth by inhibition of autophagy.

Thanks to the reviewer's constructive comment. When autophagy was inhibited by silencing Beclin-1, we detected the growth inhibitory effects and apoptosis. As shown in Figure 4 and Supplemental Figure 1, the knockdown of Beclin-1 increased the growth inhibitory effects of the combination of vorinostat and sorafenib, as well as the cell cycle alterations and induction of apoptosis. This issue has been addressed on page 11 in the revised manuscript and in supplemental materials.

(2) In figure 3, the authors should provide more legends. Moreover, Sorafenib, vorinostat or combination-induced modulation of apoptosis- and cell cycle- and autophagy-relevant proteins. The authors should provide more data for autophagy-relevant proteins (LC3-II, atg7).

We have changed the figure legend of figure 3. This issue has been addressed on page 18 and 19 in the revised manuscript.

Meanwhile, we detected the expression of other autophagy-relevant proteins (LC3-II, atg7) in the cells treated with vorinostat or sorafenib (figure 3). This issue has been addressed on page 10 in the revised manuscript.

.

(3) Previous studies showed that both vorinostat and sorafenib induced autophagy and apoptosis for anti-tumor effects. The authors showed that knockdown of Beclin-1 enhanced synergistic effect of combination of vorinostat with sorafenib to stimulated cell cycle alterations and apoptosis. How about the autophagy in this situation. The authors should show that LC3-II data for autophagy after SiBeclin-1. Do you have data about pharmacological modulation of autophagy in synergistic effect of combination of vorinostat with sorafenib?

Previous studies showed chemotherapy induced activation of autophagy, which may contribute to drug resistence. (Carew JS, Medina EC et. al. Autophagy inhibition enhances vorinostat-induced apoptosis via ubiquitinated protein accumulation. J Cell Mol Med. 2010; Modulating autophagy for therapeutic benefit.Carew JS, Nawrocki ST, Cleveland JL.Autophagy. 2007).

The expressions of autophagy-relevant proteins were detected in the vorinostat and sorafenib combination treatment groups. As shown in Figure 3, the vorinostat and sorafenib combination treatment increased the expression of Beclin1, ATG5, and ATG7.

In addition, knockdown of Beclin-1 resulted in a decrease in levels of LC3-II (Figure 3).

We also investigated if the autophagy inhibitor can also enhance synergistic effect of the combination of vorinostat and sorafenib. As shown in Supplemental Figure 1, the autophagy inhibitor 3-MA also enhanced the synergistic effect of the combination of vorinostat and sorafenib.

These issues have been addressed on page 11 in the revised manuscript.

(4) There is minor language polishing.

We have used the services of AJE to improve the quality of this manuscript.

Responses to Comments by Reviewer 2:

Comments: The study is interesting and relevance but the results need more clarification, especially on the mechanism of treatment that has not sound leading to their conclusion.

The anti-proliferation effects were based on percent cell viability assay (Fig 1 & 2). It was stated in the Methods that the first inhibitor was incubated for 24h and the second inhibitor was added for 48h. What about the single treatment time?

The single treatment time was consistent with the combined treatment group. For example, in single treatment group in Figure 1, cells were treated with Vorinostat for 72h, or with sorafenib for 48h.

The cell survivals were less than 10% in all of the combined treatment (2.5 uM/L of each). Whether the minimal amount of survival cells presented reliable results of the cell activities?

Thanks to the reviewer's constructive comment. To evaluate the viability of cells after the combined treatment, the cells were also labeled with 0.4% Trypan blue for determination of viability. And there were still a small number of live cells not colored.

The results obtained in Fig 2 were derived from the same experimental periods and the inhibitor concentration as in Fig 1, please clarify whether variation of time affected the interpretation.

In Fig.1, the growth inhibitory effects were observed after incubating the HCC cells with the combination of vorinostat and sorafenib for 48 h.

We also determined the growth inhibitory effects after treatment by the combination of vorinostat and sorafenib for 24 h. The combination of vorinostat and sorafenib also synergistically reduces cell proliferation, and survival rates of cells were higher 24h after treatment than they were after 48h.

Since the number of live cells was too small after 48h treatment, it was hard to obtain enough cells to analyze the cell cycle and caspase activity. Therefore, the incubation time was changed to 24h in Fig.2. This issue has been addressed on page 9 in the revised manuscript.

Fig 3 & 4 should contained more detail explanation (text & legend)

We have changed the figure legends of Fig.3 and Fig.4. This issue has been addressed on page 18 and 19 in the revised manuscript.

After treatment with sorafenib or HDAC inhibitor, the results did not show significant difference of beclin-1 and ATG5 bands density. However, with the combination, both bands density seemed to increase. These data did not show statistic difference.

We re-analyzed the beclin-1 and ATG5 expression in this experiment. As shown in Figure 3A, a significant increase in Beclin-1, ATG5, ATG7, and LC3 were observed in the cells treated with vorinostat or sorafenib.

The other autophagy associated proteins should be included and analyzed. According to the title of

this experiment, the inhibition of autophagy to enhance anti-tumor effect was expected.

The expressions of other autophagy-relevant proteins were detected in the vorinostat and sorafenib combination treatment groups. As shown in Figure 3, the vorinostat and sorafenib combination treatment increased the increased expression of Beclin-1, ATG5, ATG7, and LC3.

We also investigated whether the inhibition of autophagy can enhance the synergistic effect of the combination of vorinostat and sorafenib. As shown in Figure 4, knockdown of Beclin-1 or the autophagy inhibitor 3-MA enhanced the synergistic effect of the combination of vorinostat and sorafenib.

These issues have been addressed on page 10 and 11 in the revised manuscript.

What was the purpose of using SiSCR? Variation in protein levels after each treatment should be counted in this experiment? Why beclin-1 and ATG5 were also suppressed by SiSCR? What about the other presented parameters?

The SiSCR (siRNA scramble) was used to evaluate the nonspecific effects on transfection on gene expression. We optimized the experimental conditions and re-analyzed the expressions of autophagy-relevant proteins in this experiment. As shown in revised Fig.3, no obvious change was observed in SiSCR group. This issue has been addressed on page 10 in the revised manuscript.

In Fig.3, the experiments were performed independently in triplicate and representative images were shown. The corresponding densitometric analysis of three independent experiments was also shown.

From these results, what actually potentiated anti-tumor effect?

Previous study shows chemotherapy-induced autophagy contributed to drug resistance. In our experiments, we also confirmed that inhibition of autophagy enhanced the synergistic effect of the combination of vorinostat and sorafenib.