A novel combination treatment to prevent c-Met-induced and Rubicon-Nrf-2-mediated therapeutic resistance in renal cancer

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ABSTRACT

Background: Although there are some progresses in the treatment of renal cell carcinoma (RCC), tumors almost inevitably develop therapeutic resistance. Therefore, new target molecules and combination therapies need to be explored. Receptor tyrosine kinases (RTKs) play a critical role in the growth and metastasis of RCC. The RTK, c-Met, is overexpressed in renal cancer cells and mediates tumor-promoting pathways and therapeutic resistance through the regulation of anti-apoptotic transcription factor, Nrf-2. The tumor microenvironment can also alter the cytoprotective role(s) of Nrf-2 in response to stress generated by therapeutic agents. Depending on context, the process of autophagy can play both pro- and anti-tumorigenic role(s). Apart from apoptosis, the process of autophagy can also promote tumor cell death following therapeutic treatments. Rubicon (a RUN domain containing protein) is a component of the class III PI-3K complex and a novel Beclin-1-binding partner, which can negatively regulate canonical autophagy, p62 is an autophagy adapter protein, which can stabilize Nrf-2 (through degradation of Keap-1) and facilitate its nuclease translocation. Honokiol (Ishakum X, Enzou Life Sciences) is a natural product isolated from the Magnolia officinalis. The present study aimed to explore the effectiveness of a combination treatment (cabozantinib + Honokiol) to overcome c-Met-induced and Nrf-2-mediated therapeutic resistance in RCC through the regulation of Rubicon and p62.

Methods: We used human RCC cell lines, 769-L, Caki-1 (clear cell type) and ACHN (cystic, papillary type) from ATCC. Protein expression was measured by Western blot. Rubicon knock-out stable cell lines were generated using CRISPR-Cas technique. The total reactive oxygen species (ROS) was measured utilizing a kit (Enzo Life Sciences). Cellular apoptosis was measured by flow cytometry (Annexin V and Propidium iodide staining). Cellular autophagy was measured using a kit from Enzo Life Sciences. Cell proliferation was studied by using CCK-8 kit (Abcam).

Results: We found that Rubicon, p62 and Nrf-2 are markedly expressed in all tested renal cancer cell lines, and activation of c-Met (following treatment with its ligand, HGF) further induced the expression of these three proteins in renal cancer cells. The combination treatment with cabozantinib + Honokiol significantly inhibited cancer cell proliferation compared to cells treated separately with individual agents (either cabozantinib or Honokiol). On the basis of cell proliferation data, we calculated the synergistic effect of cabozantinib and Honokiol (using SynergyFinder). We obtained a high synergy score, which indicates that Honokiol can potentiate the effect of cabozantinib. Interestingly, we found that cabozantinib + Honokiol combination significantly reduced the expression of Rubicon, p62, and Nrf-2 in RCC cells, and it also increased the total cellular ROS level and induced autophagy, which can promote cell death. Next, we checked the effect of this combination treatment in Rubicon knock-out stable renal cancer cells. The knock-out of Rubicon reduced the expression of p62 and Nrf-2, and this was further downregulated following cabozantinib + Honokiol combination treatment. When we knocked down p62 in RCC cells using a shRNA, Nrf-2 was markedly downregulated; and this suggests that p62 can regulate Nrf-2 in these cells. Finally, we found that cabozantinib + Honokiol combination markedly downregulated the nuclear translocation of Nrf-2.

Conclusions: Our findings suggest that c-Met induced Rubicon can downregulate the oxidative stress in renal cancer cells through increased expression of p62 and Nrf-2, and mediate therapeutic resistance. However, a combination treatment with cabozantinib + Honokiol can effectively inhibit the expression of Rubicon, p62 and Nrf-2 to induce oxidative stress and cancer cell death.

INTRODUCTION

Renal Cell Carcinoma (RCC) is the most common type of kidney cancer in adults. c-Met is a receptor tyrosine kinase (RTK). Upon binding with HGF, c-Met activates a variety of biological pathways including proliferation and angiogenesis. c-Met is aberrantly activated or overexpressed in many cancer types including renal cell carcinoma (RCC). Nrf-2 is an oxidative stress responsive transcription factor implicated in therapeutic resistance in cancers and known to be regulated through c-Met. Suppression of p62 is an autophagy adapter protein, which facilitate the nuclear translocation of Nrf-2 by degrading keap1. Rubicon is a negative regulator of autophagy, and highly expressed in cancer cells.

In this study, we hypothesized that the blockade of c-Met/rubicon-p62-Nrf-2 pathways by cabozantinib and honokiol can improve the efficacy of cabozantinib and prevent acquired cabozantinib resistance in renal cancer.

RESULTS

Figure 1: Honokiol synergizes with cabozantinib and resulted in enhanced renal cancer cell death, 769-O and ACHN cells were treated with cabozantinib (1.5-5 µM) and Honokiol (5-20 µM). Cell viability was evaluated by CCK-8 assay. Heat maps represent the higher percentage inhibition in combination treatments than individual treatments. Synergy plots represent a high synergy between cabozantinib and honokiol in 769-O and ACHN renal cancer cells.

Figure 2: Cabozantinib + Honokiol combination significantly induces ROS and autophagy to promote apoptosis in renal cancer cells. (A) Cabozantinib (5 µM) and Honokiol (20 µM) combination treatment significantly induced autophagy compared to individual treatments in 769-O and ACHN renal cancer cells analyzed by flow cytometry after 6 h treatment. (B) Cabozantinib (5 µM) and Honokiol (20 µM) combination treatment significantly induced autophagy compared to individual treatments in 769-O and ACHN renal cancer cells analyzed by flow cytometry after 12 h treatment. (C) Cabozantinib (5 µM) and Honokiol (20 µM) combination treatment significantly induced apoptosis compared to individual treatments in 769-O and ACHN renal cancer cells analyzed by flow cytometry after 24 h treatment. (D) Combination treatment markedly downregulated anti-apoptotic protein Bcl-2 and Bcl-xL observed by Western blot. Histograms represent the Mean ± S.D. of duplicate experiments. Untreated controls were compared with treatment groups and *p < 0.05, **p < 0.01, and ***p < 0.005 were considered statistically significant.

Figure 3: Cabozantinib and honokiol combination restricts c-Met induced upregulation of Rubicon, p62 and Nrf-2 in renal cancer cells. (A) Rubicon, p62 and Nrf-2 were markedly upregulated in renal cancer cells (769-O and ACHN) compared to normal renal epithelial cells (RPE1), as observed by the Western blotting. (B) Protein level of Rubicon, p62 and Nrf-2 were increased after HGF treatment in 769-O and ACHN renal cancer cells. (C) Rubicon, p62 and Nrf-2 protein levels were markedly downregulated by cabozantinib (5 µM) and honokiol (20 µM) combination treatment for 24 h compared to vehicle treated and individual treatments. (D) Cabozantinib and honokiol combination treatment for 24 h markedly decreased Nrf-2 level in nuclear fraction. (E) A combination treatment of cabozantinib and honokiol were more effective in reducing Nrf-2 expression after silencing of p62.

SUMMARY AND CONCLUSION

From this study, we draw the following conclusions:

- Cabozantinib and honokiol synergized to induce cell death. The combination treatment showed enhanced ROS generation that may be involved in the induction of autophagy.
- Rubicon, p62 and Nrf-2 were markedly downregulated by the combination treatment of cabozantinib and honokiol in both 769-O and ACHN renal cancer cell lines.
- Cabozantinib and honokiol combination effectively restricted nuclear translocation of Nrf-2, which eventually block the Nrf-2 mediated transcription of anti-oxidative enzymes.
- Compared to vehicle treated, the combination treatment of cabozantinib and honokiol significantly increased apoptosis with a combination treatment of cabozantinib and honokiol. Rubicon knock-out also resulted in decreased p62 and Nrf-2 expression compared to control cells.

In conclusion, a combination of cabozantinib and honokiol effectively restricted c-Met/Rubicon/p62 axis, involved in therapeutic resistance.

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