Combined inhibition of sonic Hedgehog signaling and histone deacetylase is an effective treatment for liver cancer

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Abstract. Clinical trials have revealed that inhibition of sonic Hedgehog (SHH) signaling or histone deacetylase (HDAC) holds promise as a treatment for liver cancer. Based on our previous results, it was hypothesized that dual inhibition of SHH and HDAC may contribute to more efficient targeting of this disease. The effect of SHH inhibitor vismodegib as a single-agent or in combination with HDAC inhibitor entinostat was evaluated by Cell Counting Kit-8 (CCK-8) and flow cytometric assays, as well as immunoblotting. The synergistic effect on cell viability was assessed by combination indexes. Ex vivo cultured liver cancer tissues from a patient were treated with vismodegib as a single-agent or in combination with entinostat, and analyzed by histological and immunohistochemical methods. The results revealed that the dual use of the SHH inhibitor and the HDAC inhibitor effectively synergized to inhibit proliferation, and promote apoptosis in liver cancer cells. Furthermore, the effect of the combination of these drugs was confirmed in an ex vivo culture of human liver cancer tissue. Mechanistically, combined use of SHH and HDAC inhibitors resulted in significantly greater downregulation of SHH and PI3K/mTOR signaling. In conclusion, the combined use of SHH signaling and HDAC inhibitors may be an effective therapeutic strategy for liver cancer.

Introduction

Liver cancer is commonly diagnosed, and the 5-year relative survival rate is only 18% (1,2). Current treatments applicable for liver cancer, include surgery, transcatheter arterial chemoembolization and chemotherapy (3). In recent years, the efficacy of agents that selectively target critical signaling pathways has been assessed in several clinical trials, however, no relevant improvement has been achieved to date (3,4). Therefore, it is urgent to identify therapeutic strategies for more effective therapy of liver cancer.

Recently, the activation of sonic Hedgehog (SHH) signaling has been implicated in liver cancer (5). It was revealed to play a crucial role in the initiation and maintenance of liver cancer and contribute to chemotherapeutic resistance (6). The SHH signaling pathway is initiated by the binding of the patched (Ptch) receptor, which in turn relieves smoothened (Smo) receptor from inhibition. Smo then triggers a series of intracellular events, resulting in the activation of downstream target genes including the glioma-associated oncogene homolog (Gli), which are the early transcriptional targets of SHH signaling (7,8). It has been determined that blocking Smo can inhibit the activity of the SHH signaling pathway, and Smo receptor from inhibition. Smo then triggers a series of intracellular events, resulting in the activation of downstream target genes including the glioma-associated oncogene homolog (Gli), which are the early transcriptional targets of SHH signaling (7,8). It has been determined that blocking Smo can inhibit the activity of the SHH signaling pathway, and Smo has been used as a target to develop related drugs for cancer treatment (7). It was reported that Smo antagonists including cyclopamine and vismodegib could inhibit the growth of tumors (9). Vismodegib (GDC0449) has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic or locally advanced unresectable basal cell carcinoma (10).

Several studies have revealed that SHH regulates sustained activation of histone deacetylases (HDACs) which is required for cell growth (11,12). Liver cancer patients with overexpression of HDAC1 exhibited higher incidence of cancer cell invasion, poorer histological differentiation, and a low survival rate (13,14). Aberrant regulation of HDAC2 may play a pivotal role in the development of liver cancer rendering HDAC2 a relevant target for liver cancer therapy (15). In numerous previous studies, SHH signaling regulated histone acetylation and chromatin (16) and led to carcinogenesis (17). Further studies are still required to ascertain the use of dual inhibition of SHH signaling and HDAC treat liver cancer. In the present
study, it was demonstrated that the combined use of SHH and HDAC inhibitors effectively treated liver cancer cells in vitro and ex vivo. These studies indicated that SHH inhibition may be a reasonable strategy to extend the utility of a HDAC inhibitor in liver cancer.

Materials and methods

Cell culture. The HepG2 cell line was obtained from the Cell Bank, Chinese Academy of Sciences (Shanghai, China). This is a common liver cancer cell line. The HepG2 cell line was derived from a 15-year-old white male with well-differentiated liver cancer. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The HepG2 cells were cultured in a humidified incubator containing 5% CO2 at 37°C.

Cell viability assay and drug combination analysis. Cell viability was assessed by Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Vismodegib and entinostat were purchased from Shanghai Biochempartner Co., Ltd. (Shanghai, China). According to the instructions, HepG2 cells cultured in 96-well plates were treated with an indicated concentration of vismodegib (0, 1, 2, 4, 8 and 16 µM) or entinostat (0, 0.5, 1, 2, 4 and 8 µM) or a combination of the two, or DMSO for 72 h. The half-maximal inhibitory concentration (IC50) values were obtained from dose-response curves with GraphPad Prism software (version 7.00; GraphPad Software, Inc., La Jolla, CA, USA). The synergistic effect was determined by calculating the combination index (CI) using the CalcuSyn software program (version 2.1; Biosoft, Great Shelford, UK). Data from cell viability assays were expressed as the fraction of growth inhibition by the single drugs or the combination. Synergism was expressed as a CI value <1 and antagonism by a CI value >1 at 0.5 fraction affected (FA).

Cell proliferation assay. The effects of the inhibitors on cell growth were evaluated as previously described (18). Briefly, the cells were cultured in 6-well plates at 1,500 cells/well densities and then treated with vismodegib (4 µM) or entinostat (2 µM) or combination (vismodegib and entinostat at the respective concentrations). The cells were treated for ~14 days. Then the cells were fixed, stained with crystal violet, and extracted with glacial acetic acid. The optical density (OD) was assessed at 570 nm by the microplate reader (iMark™ Microplate Absorbance Reader; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Soft agar colony formation assay. The colony formation of the liver cancer cells in soft agar medium was performed as previously described (19). Briefly, the cell suspension (2x10^4 cells/well) was mixed with 0.4% soft agar (BD Biosciences, Franklin Lakes, NJ, USA) prepared with DMEM containing 10% FBS and layered onto 0.6% soft agar prepared with DMEM containing 10% FBS. In addition, liver cancer cells were treated with media supplemented with the indicated drugs. The medium was changed twice a week. The colonies were photographed and the area of these colonies was assessed by Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) at the end of the experiment.

Apoptosis analysis. HepG2 cells were grown in 6-well plates and treated with vismodegib (4 µM), entinostat (2 µM), combination or DMSO control for 48 h. Following treatment, apoptotic cells were assessed by nuclear morphology and apoptotic cell numbers were detected. Cells exposed to different treatments for 48 h were stained with acridine orange (AO) and ethidium bromide (EB) as previously described (20), and examined with a fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). Apoptosis in liver cancer cells was analyzed with an Annexin V-FITC Apoptosis Detection Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The cultured cells were trypsinized, resuspended in Annexin V binding buffer, and incubated with Annexin V and propidium iodide (PI) in the dark. Flow cytometric analysis was performed using a BD Accuri™ flow cytometer (BD Biosciences, San Jose, CA, USA) to subject the stained cells.

Cell cycle analysis. The treated cells were fixed in ice-cold 70% ethanol and stored at 4°C overnight for cell cycle analysis. After the fixed cells were washed with PBS, they were treated with 100 µg/ml RNase A at 37°C for 20 min, and stained with 50 µg/ml PI (Dojindo Molecular Technologies, Inc.). Then, the cells were subjected to fluorescence-activated cell sorting. The cell populations that were found in G0/G1, S and G2/M phases were quantified using the ModFit software (version 4.0; Verity Software House, Inc., Topsham, ME, USA).

Protein isolation and western blotting. Cells exposed to vismodegib (4 µM) and/or entinostat (2 µM) for 48 h were lysed by RIPA buffer (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China) containing protease and phosphatase inhibitors. The BCA method was used to quantify the protein of the lysate. The whole cell extracts (30 µg) was separated by 8% SDS-PAGE and the polyvinylidene fluoride (PVDF) membranes were blocking by 5% skim milk for 2 h at 4°C, then were incubated with the following primary antibodies staying overnight at 4°C: vinculin (dilution 1:2,000; cat. no. V4139; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), CDK1 (dilution 1:500; cat. no. 10762-1-AP), cyclin B1 (dilution 1:500; cat. no. 55004-1-AP), Glil (dilution 1:500; cat. no. 25733-1-AP), Glil2 (dilution 1:500; cat. no. 18989-1-AP), Gli3 (dilution 1:500; cat. no. 19949-1-AP) (all from ProteinTech Group, Inc., Chicago, IL, USA), cleaved-PARP (dilution 1:1,000; cat. no. 9541), pS6RP s255/256 (dilution 1:1,000; cat. no. 4858), pAKTs473 (dilution 1:1,000; cat. no. 20953-1-AP), and acetyl-histone H3 (dilution 1:1,000; cat. no. 9677) (all from Cell Signaling Technology, Inc., Danvers, MA, USA). Subsequently, they were incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology, Shanghai, China), which was followed by Thermoscientific™ chemiluminescence (cat. no. 32106; Thermo Fisher Scientific Co., Ltd., Shanghai, China) detection.

Ex vivo culture of patient tumor tissue. The case of a 59-year-old patient with diagnosed primary moderately differentiated liver cancer without treatment history was employed. The acquisition of tumor tissue was executed with
an Institutional Review Board protocol approved by Binzhou Medical University (Yantai, China) and written informed consent was provided by this patient. The primary liver cancer specimens from this patient who underwent a hepatic segmentectomy were obtained and subjected to ex vivo culture experiments. For the ex vivo culture, 1 cm² hemostatic gelatin dental sponges were soaked in the medium, in which DMEM base was supplemented with 10% FBS, hydrocortisone (1 mg/100 ml; Sigma-Aldrich; Merck KGaA), antibiotic/antimycotic solution (Gibco; Thermo Fisher Scientific, Inc.), and insulin (1 mg/100 ml) for 1 h. Then, liver cancer tissues were cut into 1 mm³ blocks, and transferred to the top surface of hemostatic gelatin dental sponges. Tissue blocks were treated with SHH pathway inhibitor vismodegib and HDAC inhibitor entinostat as single-agents or in combination for 48 h, and then fixed with paraformaldehyde. The tissue blocks were embedded in paraffin, sectioned with microtome, and stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) was implemented using antibodies Bcl-2 (dilution 1:200; cat. no. 12789-1-AP), Bax (dilution 1:200; cat. no. 50599-2-Ig) and Ki-67 (dilution 1:400; cat. no. 27309-1-AP) (all from ProteinTech Group, Inc.). The results of the staining were quantified by Image-Pro Plus software. Statistical analysis. All numerical data were presented as averages and the standard deviation (SD) of the mean. The data was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Statistical significance was regarded at P<0.05. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Combined use of vismodegib and entinostat inhibits the growth of liver cancer cells. Given that the SHH signaling pathway regulated histone acetylation and led to cancinogenesis (20), the liver cancer cell line, HepG2, was chosen for examination. Cell proliferation assay using CCK-8 revealed that the IC₅₀ value of vismodegib for HepG2 cells was 4.012 µM (Fig. 1A), and the IC₅₀ value of entinostat for these cells was 1.992 µM (Fig. 1A). Then, the HepG2 cells were treated with different concentrations of vismodegib and entinostat, each alone and in combination for 72 h. The median-effect analysis was used to assess the effect of the drug combination on proliferation inhibition. Combined treatment with vismodegib and entinostat resulted in a synergistic increase in proliferation inhibition and synergistic CI value of <1 at 0.5 FA in HepG2 cells (Fig. 1B).

We also observed that SHH signaling inhibitor vismodegib as single-agent slightly reduced the viability of HepG2 cells (Fig. 1C). Conversely, the HDAC inhibitor entinostat revealed a moderate inhibitory effect on the viability of these liver cancer cells (Fig. 1C). However, the combined use of vismodegib and entinostat almost completely suppressed the growth of HepG2 cells (Fig. 1C).

The observation that the combination treatment inhibited cell proliferation in two-dimensional culture conditions led us to further assess the effect of the drug combination in three-dimensional conditions. Inhibition of the SHH pathway by the use of vismodegib alone slightly inhibited colony formation efficiently and inhibition of entinostat exhibited a moderate inhibitory effect. As anticipated, the combined use of vismodegib and entinostat inhibited the formation of colonies in HepG2 cells almost completely (Fig. 1D). These results indicated that dual use of SHH signaling inhibitor vismodegib and HDAC inhibitor entinostat provided a synergistic inhibitory effect on the proliferation of liver cancer cells.

Combined use of vismodegib and entinostat synergistically induces apoptosis in liver cancer cells. To investigate whether apoptosis was the pathway of cell death after treatment, cell morphological changes were observed and the proportion of apoptotic cells was evaluated by AO/EB staining and flow cytometric assays, respectively. The results indicated that only sporadic apoptotic cells were observed in the vehicle group, however, more apoptotic cells were observed in the vismodegib and entinostat single-agent treated groups. The number of apoptotic cells significantly increased in the combined treatment group of HepG2 cells (Fig. 2A). While vismodegib or entinostat alone produced a mild increase in Annexin V-positive cells, combined treatment resulted in significantly increased Annexin V-positive cells in HepG2 cells (Fig. 2B). Consistent with this result, the combination treatment also enhanced the abundance of cleaved-PARP, a marker for active apoptosis, in HepG2 cells. (Fig. 2C).

Combination treatment using vismodegib and entinostat has potent inhibition activity in liver cancer specimens. An ex vivo culture model of liver cancer was employed to assess the therapeutic effect of vismodegib combined with entinostat. Briefly, we dissected fresh surgical specimens of primary liver cancer into ~1 mm³ blocks. These blocks were exposed to vismodegib and entinostat as single-agents or in combination for 48 h on the absorbable gelatin sponge. The results of histological examination revealed that the tissue structure of vehicle-treated explants was similar to that of the primary tumor tissue (Fig. 3A). The blocks treated with vismodegib and entinostat displayed marked disrupted cellular integrity compared to that in the single-agent treatment groups (Fig. 3A). Consistently, the combination treatment significantly reduced proliferation as determined by Ki-67, and significantly enhanced apoptosis as determined by downregulated Bcl-2 and upregulated Bax (Fig. 3B). Collectively, these data ascertained the potential use of vismodegib and entinostat to treat liver cancer.

Combined use of vismodegib and entinostat synergistically induces G2/M arrest of liver cancer cells. To assess whether the effects of combination treatment on liver cancer cells involved changes in the cell cycle, HepG2 cells were treated with vismodegib and entinostat alone or combined treatment for 48 h. The results revealed that combination treatment significantly increased the number of cells at the G2/M phase and significantly decreased the number of cells at the S phase (Fig. 4A). However, an increase of sub-G1 populations was also observed. While vismodegib and entinostat as single-agents led to a slight increase of sub-G1 populations, dual treatment with vismodegib and entinostat resulted in a marked increase of sub-G1 in HepG2 cells (Fig. 4A). The present
study determined that combination therapy induced G2/M arrest and triggered apoptosis of HepG2 cells. With regards to the biomarkers of the cell cycle, vismodegib combined with entinostat reduced the expression of cyclin B1 and cyclin dependent kinase 1 (CDK1) (Fig. 4B), which further indicated that the combination treatment resulted in G2/M phase arrest.

Vismodegib as a single agent or in combination with entinostat attenuates PI3K/mTOR signaling. Western blot analysis revealed that vismodegib as a single agent or in combination with entinostat resulted in a marked reduction of the expression of Gli1, Gli2 and Gli3 effectors of SHH signaling in HepG2 cells (Fig. 5A). Notably, a slight effect was observed with...
SHH signaling in liver cancer cells treated with entinostat as a single-agent. In addition, the results revealed that entinostat as a single-agent.

Figure 3. Reaction of primary tumor explants to vismodegib and entinostat as single agents or in combination. (A) Representative H&E staining images of surgical specimens and tumor explants by ex vivo culture. (B) Representative images of immunohistochemical staining analyses of Ki-67, Bcl-2 and Bax on tumor explants treated with vismodegib (4 µM) and entinostat (2 µM) as single agents or in combination. Scale bars, 50 µm. The percentage of positive cell expression of Ki-67, Bcl-2 and Bax in each group was examined, and the results were expressed as the number of positive cells expressed over the total number of cells counted. The data are presented as the mean ± SD of 3 different tumor explants. *P<0.05; **P<0.01; ***P<0.001. H&E, hematoxylin and eosin.

Figure 4. The combined use of vismodegib and entinostat synergistically induces G2/M arrest of liver cancer cells. (A) HepG2 cells were cultured with vismodegib (4 µM) for 24 h, entinostat (2 µM) or a combination of these drugs, and analyzed by flow cytometry. The percentages of apoptotic cells in sub-G1 peaks and those of cells in the G1, S and G2/M phases are indicated. (B) Western blot analysis of proteins (cyclin B1 and CDK1) as revealed in liver cancer cells treated with vismodegib (4 µM) and entinostat (2 µM) as single agents or in combination. Vinculin was used as a loading control.

Figure 5. Vismodegib as a single agent or in combination with entinostat sufficiently weakens activation of PI3K/mTOR signaling. (A-C) Western blot analysis of proteins (acetyl-histone 3, Gli1, Gli2, Gli3, pAKT and pS6RP) as indicated in liver cancer cells treated with vismodegib (4 µM) and entinostat (2 µM) as single agents or in combination for 48 h. Vinculin was used as a loading control.
Hedgehog-dependent growth of neural progenitors and tumor
activated SHH signaling in neural progenitors and medul-
possibilities for the selection of drug treatment options for
enhance the efficacy of the SHH inhibitor and provide more
inhibitor was used in combination with an SHH inhibitor to
sensitivity to tumors. Therefore, in the present study a HDAC
is used to increase the therapeutic effect of the inhibitor and
vismodegib is challenging, due to the possible presence of
tumors using SHH signaling inhibitors, a combination therapy
non-canonical Gli activation mechanisms. For the treatment of
vismodegib is sensitive to liver cancer: radiation (26). However, SHH inhibition in liver cancer with
sion of cancer. Several HDACs have been revealed to have
aberrant expression in liver cancer (15). In the present study
concerning gene expression characteristics as a predictor of
survival in liver cancer patients, it was determined that HDAC
overexpression was associated with lower survival rates in liver
cancer patients (4,29). It has been reported that Gli1 and Gli2
are acetylated proteins, and their HDAC-mediated deacety-
lation was revealed to promote transcriptional activation and
positive autoregulation of the loop by Hedgehog-induced
upregulation of HDAC1 (28,30). Consistent with this, our
present study revealed that the dual use of SHH and HDAC
inhibitors could effectively inhibit Gli expression and exert
effective antitumor activity in liver cancer cells. In the present
study, an ex vivo culture model of surgically resected fresh
live cancer specimens was used to evaluate drug efficacy.
Since the cultured tissue explants retained tissue structure
and cellularity, as observed in primary tumors, this approach
provided a suitable platform to assess acute treatment response
to liver cancer. Our results indicated that the combination of
vismodegib and entinostat was effective in promoting the
death of tumor explant cells.

The present results revealed that inhibition of SHH signaling
not only inhibited the growth of liver cancer cells and promoted
apoptosis, but also induced G2/M cell cycle arrest. In addition,
SHH signaling pro-apoptotic factors (cleaved PARP and Bax)
were upregulated, and the activity of anti-apoptotic factors
(including Bcl-2) were inhibited. SHH-related pathways do
not always work directly, but rather induce complex cascades
of networks that intersect with other pathways to function in
different biological processes. The PI3K/mTOR signaling
pathway is closely related to SHH (31). Studies have indicated
that the PI3K/mTOR signaling has a synergistic effect on SHH
signaling in embryonic development and cancer (21,31). To
further investigate the relationship between the PI3K/mTOR
and SHH pathways in liver cancer, the results in the present
study clearly revealed that combined use of SHH and HDAC
inhibitors blocked the activation of the PI3K/mTOR pathway
and played an antitumor role. Therefore, it is suggested that
the treatment of SHH signaling should consider the effect of
PI3K/mTOR pathway.

In summary, our data indicated that HDAC inhibition
contributed to the sensitivity of SHH inhibition, and inhibition
of PI3K/mTOR by inhibition of SHH and HDAC may play a
key role in this synergy. Although the number of liver cancer
cell line models and ex vivo models studied in the present study
was limited, our data indicated that the combined use of SHH
and HDAC inhibitors may benefit patients with liver cancer.

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Availability of data and materials
The datasets used during the present study are available from
the corresponding author upon reasonable request.
Authors’ contributions

DW, JL and HC conceived the project and designed the experiments. JL and HL performed the cell viability assay and the drug combination analysis. HC contributed to the western blotting tests and data analysis. HC and YSh were responsible for the cell proliferation assay and the soft agar colony formation assay. HS contributed to the apoptosis and cell cycle analysis experiments.YL, YSu and YW contributed to the experiments about the clinical specimen. HC and DW wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The acquisition of tumor tissue was executed with an Institutional Review Board protocol approved by Binzhou Medical University (Yantai, China) and written informed consent was provided by this patient.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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