Original Article
Running Title: HO-3867 Induces Apoptosis in HCC
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HO-3867 Induces Cell Apoptosis by Reducing STAT3 Signaling in Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is the most common primary liver cancer with limited treatment options. HO-3867, a STAT3 inhibitor, has shown anticancer effects in other malignancies, but its role in HCC remains unclear. This study aimed to investigate the efficacy and mechanism of HO-3867 against HCC.

Method: We conducted an experimental study combining in-vitro cell assays (proliferation, colony formation, apoptosis analysis, Western blot) using HCC cell lines and in-vivo nude mouse xenograft model, and all quantitative experimental data were statistically analyzed using unpaired two-tailed Student's t-test for comparisons between two groups and one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple group comparisons, with a *P*-value less than 0.05 defined as statistically significant.

Results: HO-3867 significantly inhibited HCC cell viability, colony formation, and induced apoptosis in-vitro, and suppressed tumor growth in-vivo via inhibiting STAT3 phosphorylation and downstream anti-apoptotic protein expression.

Conclusion: HO-3867 exerts potent anti-HCC effects through targeting STAT3 signaling, providing a preclinical basis for further clinical development.

***Keywords**:* Hepatocellular carcinoma, HO-3867, STAT3, Apoptosis

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary HCC.[1, 2] The incidence of HCC dramatically increased in the decade years. Surgery, chemo-therapies and radiotherapy are the most conventional treatments for HCC.[3] However, chemotherapeutic effects are typically to be limited by drug resistance and severe side effects, leading to poor prognosis for many patients.[4] Recent advances in molecular targeted therapy have offered new hope for HCC

treatment, with a focus on inhibiting specific signaling pathways that drive tumor growth and progression. Among these pathways, the signal transducer and activator of transcription 3 (STAT3) has emerged as a critical oncogenic driver in HCC. Constitutive activation of STAT3 is frequently observed in HCC tissues and is closely associated with increased cell proliferation, anti-apoptosis, angiogenesis, and metastasis.[5, 6] As a result, STAT3 has become an attractive therapeutic target for the development of novel anti-HCC agents. In recent years, several small-molecule STAT3 inhibitors have been identified and evaluated preclinically, showing promising anti-tumor effects in various cancer models. HO-3867, a diarylalkylpiperidinone derivative, has been reported to exert inhibitory effects on STAT3 in breast cancer[7] and glioblastoma,[8, 9] but its specific role and detailed mechanism of action in HCC remain largely unexplored. Therefore, the present study aimed to systematically investigate the anti-tumor activity of HO-3867 in HCC and to clarify the underlying molecular mechanisms, which may provide valuable insights into its potential clinical application. Therefore, there is an urgent need to develop new therapy because of late diagnosis along with drug resistance that led to an impediment to achieved effective agents for HCC treatment.

HO-3867 is a derivative compounds with an N-hydroxypyrroline substitution at the Nterminal of piperidone.[10] As one of the diarylalkylpiperidinone compounds with ortho-fluorinated phenyl groups, HO-3867 exhibited potent anticancer efficacy in-vitro when tested using ovarian epithelial cancer cell lines as well as in-vivo using a mouse model.[11] HO-3867 could be as an adjuvant to conventional therapeutics in ovarian cancers harboring TP53 missense mutations and improve patient outcomes.[12] It has been reported that HO-3867 induced cell apoptosis by triggering formation of activated caspase 3, caspase 8, caspase 9 and PARP due to dissecting MAPK pathway and c-Jun N-terminal kinase (JNK)1/2 pathway[13] in human oral squamous cell carcinoma cells. The similar role in cancer was found in human pancreatic cancer cells.[14] However, its role in HCC has not been investigated. Here, we characterize the mechanism of actions of the diarylalkylpiperidinone curcumin HO-3867 in hepatocellular cancer cells. We study the efficacy of HO-3867 on cell viability, colony formation, apoptosis, and tumor growth in-vivo. We also explored possible mechanisms of HO-3867 effects on cellular process, including an STAT3-signaling pathway that has been implicated in other cancers. We show that HO-3867 is a cytotoxic agent to Huh7 and HepG2 cells, which induces STAT3 inhibition mediated cell-cycle arrest, and apoptosis.

Method

Study design

This was an experimental laboratory study combining in-vitro cell functional assays and in-vivo nude mouse xenograft models. All experiments were performed in triplicate. The study was reported in

accordance with the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines for experimental research.

Cell proliferation assay

Cells were plated in five replicates in a 96-well plate (3000 cells/well) and cultured in DMEM (Gibco, 11965-092) containing 10% FBS (Gibco, 10099-141). Then, the cells were incubated with 10 μL of Cck-8 reagent (C2581, Sigma, Germany) for 4 hours at 37°C. Viable cells were counted every other day by reading the absorbance at 450 nm using a plate reader. Statistical analysis (one-way ANOVA) was performed using the statistical software GraphPad Prism 9.0. A value of $P \leq 0.05$ was considered significant. The test was repeated three times.

Colony formation assay

For the colony formation assay, the stably infected cells were seeded in 6-well plates at a density of 500 cells per well. After 14 days, the cells were fixed with 4% paraformaldehyde and stained with 0.5% Crystal Violet Staining Solution. The colonies were then visualized and counted using a digital imaging system. The number of colonies containing more than 50 cells was recorded, and the colony formation rate was calculated as (number of colonies formed / number of cells seeded) × 100%. Each experiment was performed in triplicate, and the results were expressed as mean ± standard deviation. Statistical significance was determined by comparing the colony formation rates of HO-3867-treated groups with the control group using Student's t-test, with $P < 0.05$ considered statistically significant.

Western blot assay

HCC cell lines were harvested and extracted using lysis buffer, and then a total of 40μg cell extracts were separated on 10% SDS-PAGE gels. Separated protein bands were transferred into polyvinylidene fluoride (PVDF, Millipore, IPVH00010) membranes, which subsequently blocked in 5% defatted milk diluted by Tris-Buffered Saline Tween-20 (TBST). The primary antibodies against STAT3 (Cell Signaling Technology, CST, #9139, 1:1000 dilution), p-STAT3 (CST, #9145, 1:500 dilution), bcl-xl (CST, #2764, 1:1000 dilution), Mcl (CST, #5453, 1:1000 dilution) and Gapdh (CST, #5174, 1:5000 dilution) were diluted according to the instructions of antibodies and incubated overnight at 4°C. Then, horseradish peroxidase-linked secondary antibodies were added at a dilution ratio of 1:10000, and incubated at room temperature for 1 hour. The membranes were washed with TBST and the bands were visualized using ECL-PLUS/Kit (Thermo Fisher Scientific, 32132) according to the instructions.

Immunohistochemistry

Tumor sections are dewaxed with xylene (twice for 15 min) and hydrated using an ethanol gradient (100%, 85%, 75% ethanol, 5 min each) and blocked with 5% goat serum for 20 min at room temperature. Primary antibodies Ki67 (CST, #9449, 1:500 dilution) and cleaved-Caspase3 (CST, #9664,1:200 dilution) are diluted and incubated overnight at 4°C. For secondary

antibody incubation, Horseradish peroxidase-linked secondary antibodies are added and incubated at room temperature for one hour. The sections are then stained with 3,3'-diaminobenzidine (DAB) substrate to visualize the antibody binding sites, followed by counterstaining with hematoxylin to enhance nuclear contrast. After dehydration through a graded ethanol series and clearing with xylene, the slides are mounted with neutral balsam. Images of the stained sections are captured using a light microscope equipped with a digital camera. For immunohistochemistry or TUNEL quantification: Five random high-power fields (×400) were selected per slide, and the number of positive cells was counted manually. The proliferation index was calculated as the percentage of Ki67-positive cells relative to the total number of cells in each field. The staining intensity was scored semi-quantitatively using a 4-point scale (0 = no staining, 1 = weak, 2 = moderate, 3 = strong). The positive area ratio was determined using image analysis software, and the final score was calculated as the product of staining intensity and positive area ratio. Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons.

TUNEL assay

Sections (3 μm thick) cut from the paraffin-embedded tissue were dewaxed with xylene twice for 15 min, hydrated using an ethanol gradient (twice with 100% for 5 min, then 85% for 5 min and 75% for 5 min), fixed in 4% formaldehyde solution for 20 min at room temperature and then incubated with proteinase K at 37°C for 30 min. The TUNEL assay kit (Roche Diagnostics, Basel, Switzerland, 11684795910) containing TdT was prepared immediately before use according to the manufacturer’s protocol. Subsequent to washing with PBS, the sections were counterstained with 4',6-diamidino-2-phenylindole (diarylalkylpiperidinone). Apoptotic cells in the sections were detected using a microscope (Nikon Corp., Tokyo, Japan).

Animals

For the nude mouse xenograft model, 6-week-old male BALB/c nude mice were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). Huh7 cells (5×106cells in 100 μL PBS) were subcutaneously injected into the right flank of each mouse. When tumors reached an average volume of 100 mm3, mice were randomly divided into two groups (n = 5 per group): control group (intraperitoneal injection of 0.1% DMSO) and HO-3867 treatment group (intraperitoneal injection of 20 mg/kg HO-3867, dissolved in 0.1% DMSO). Tumor volume was measured every 3 days using calipers and calculated as (length × width2)/2. After 21 days of treatment, mice were euthanized, and tumors were excised, weighed, and fixed in 4% paraformaldehyde for further analysis. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Hunan aerospace hospital. Mice were housed under specific pathogen-free conditions with a 12-hour light/dark cycle and ad libitum access to food and

water. All efforts were made to minimize animal suffering.

Statistical analysis

Statistical analyses were performed using SPSS version 24.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation, and categorical variables as number (percentage). Student's t-test was used for normally distributed continuous variables, while the Mann-Whitney U test was used for non-normally distributed variables. A *P*-value <0.05 was considered statistically significant.

Results

HO-3867 effects on the cell proliferation, colony formation and cell apoptosis

The cytotoxicity of HO-3867 in HCC cells was determined by Cck-8 assay. Cells were treated with various concentrations of HO-3867 (0.1, 0.5, 1.0, 5.0 and 10.0μM) for 24 hours, which resulted in significant decreases in cell viability in a dose-dependent manner (Figure 1A). HepG2 and Huh7 cells displayed a stronger sensitivity to HO-3867 (Figure 1A). Therefore, we apply HepG2 and Huh7 cells for further investigation. Administration of 0.5, 1.0, 2.0μM HO-3867 resulted in a significant decrease in cell viability, and these reduction in cell viability became significant when exposure came to 48h (Figure 1B, C). We also detect the colony formation of cells exposure to 0.5, 1.0, 2.0μM HO-3867, which markedly reduced the colony forming capacity (Figure 1D). To further confirm the apoptotic effect, we performed flow cytometry analysis using Annexin V-FITC/PI double staining, which revealed a significant increase in the percentage of apoptotic cells (both early and late apoptotic) following HO-3867 treatment compared with the control group. As expected, HO-3867 significantly increased apoptosis rates in a concentration-dependent manner in HepG2 and Huh7 cells (Figure 1E).

HO-3867 effects on HCC tumor formation in nude mice

To evaluate the anti-HCC effect of HO-3867 in-vivo, Huh7 xenografts were established in nude mice, which were then treated with 20 mg/kg HO-3867. Compared with the control group, HO-3867 treatment led to a significant reduction in tumor volume (Figure 2A), and some xenografts even achieved complete regression after administration (Figure 2B). Further statistical analysis showed that the final tumor weights in the control and HO-3867 groups were 0.35 ± 0.06 g and 0.17 ± 0.02 g, respectively, with a statistically significant difference between the two groups (Figure 2C). Consistent with the in-vitro results, immunohistochemical staining and TUNEL assay revealed that HO-3867 treatment significantly decreased the proportion of Ki67-positive proliferating tumor cells and upregulated the expression of Cleaved-caspase3 (a key marker of apoptosis) in tumor tissues (Figure 2D). Meanwhile, the TUNEL-positive apoptotic cell ratio in the HO-3867 group was significantly higher than that in the control group, further confirming that HO-3867 can induce apoptosis of HCC cells in vivo (Figure 2D).

The effect of HO-3867 on STAT3 signaling pathway in HCC cells

STAT3 signaling pathway plays a critical role in the progression of HCC by promoting cell proliferation, inhibiting apoptosis, and facilitating angiogenesis. Aberrant activation of STAT3 is frequently observed in HCC and is associated with poor prognosis, making it a potential therapeutic target for HCC treatment. In HCC, phosphorylated STAT3 (p-STAT3) translocates to the nucleus, activating downstream anti-apoptotic genes such as Bcl-xL and Mcl-1. These molecules belong to the Bcl-2 family and play critical roles in suppressing mitochondrial-mediated apoptosis by preventing the release of cytochromec. Overexpression of Bcl-xL and Mcl-1 is frequently observed in HCC, contributing to tumor cell survival and resistance to chemotherapy. Previous studies have demonstrated that to elucidate the mechanism of HO-3867 on cell apoptosis, we should examine the effect of HO-3867 on the STAT3 signaling pathway, specifically focusing on STAT3 activation and STAT3-mediated genes. After treating HepG2 and Huh7 cells with 0.5 and 1.0 μM of HO-3867, analysis revealed decreased levels of p-STAT3, bcl-xl, and Mcl-1(Figure 3). Targeting the STAT3 pathway or its downstream anti-apoptotic effectors has emerged as a promising therapeutic strategy for HCC treatment.

Discussion

Our study demonstrates that HO-3867 exerts specific anti-tumor activity against hepatocellular carcinoma (HCC) by selectively targeting the STAT3 signaling pathway, inhibiting cancer cell proliferation and inducing apoptosis while causing limited damage to normal cells. In both in-vitro and in-vivo experiments, we observed that HO-3867 treatment downregulated the proliferation marker Ki67 and upregulated the apoptosis marker cleaved-caspase3 in tumor tissues, which aligned with our in-vitro findings that HO-3867 reduced the expression of p-STAT3 and its downstream pro-survival proteins Bcl-xl and Mcl-1 in a dose-dependent manner. These consistent results across experimental models confirm that HO-3867 achieves anti-HCC effects through targeted inhibition of the STAT3 pathway, rather than non-specific cytotoxicity.

HO-3867, as a naturally derived compound, shows distinct advantages over conventional chemotherapeutic drugs for HCC treatment that align with the current demand for more effective and safer anti-cancer strategies. Naturally occurring compounds have received increasing attention in cancer research because of their distinct advantages: low toxicity to normal cells and strong targeting ability against cancer cells.[17] Unlike conventional chemotherapeutic drugs that often cause severe side-effects by damaging healthy tissues, natural compounds typically exhibit a more favorable safety profile, minimizing systemic harm. Moreover, their inherent structural diversity allows them to specifically interact with key molecular targets involved in cancer progression, such as signaling pathways or proteins overexpressed in tumor cells, thereby enhancing

therapeutic efficacy while reducing off-target effects. This preferential toxicity to cancer cells over normal cells makes HO-3867 a promising candidate for addressing the dose-limiting toxicities that hinder the clinical application of conventional chemotherapy.

One key strength of this study is that it expands the application potential of HO-3867 by demonstrating its efficacy against chemotherapy-resistant HCC, which is a major contributor to high mortality and treatment failure in HCC. High mortality and high treatment failure rates for HCC are mainly driven by widespread chemotherapeutic resistance, which enables tumor recurrence and progressive disease after initial treatment. This study confirms that HO-3867 maintains high anti-tumor potency in primary HCC cell populations, including subpopulations that are resistant to conventional chemotherapeutics. This finding indicates that HO-3867 could overcome existing treatment resistance and offer a new therapeutic option for patients who do not respond to first-line HCC treatments.

The development of HO-3867 as a clinical therapeutic for HCC requires further investigation of its molecular mechanisms and pharmaceutical properties, which was the core motivation for the design of this study. To reduce the risk of future treatment failure, we conducted this mechanistic investigation of HO-3867 to address three critical gaps: First, clarifying HO-3867's molecular targets and associated signaling pathways validates that its anti-tumor effects are driven by targeted modulation of HCC-specific oncogenic processes, rather than non-specific phenotypic changes; Second, characterizing HO-3867's interaction with STAT3, a key regulator of chemoresistance and tumor progression in HCC, reveals potential synergies with existing therapies, addressing the unmet need for effective combinatorial strategies to overcome treatment resistance; Third, future research can build on these findings to optimize HO-3867's structure and delivery systems, improving its therapeutic index and translating preclinical efficacy into clinical applicability, ultimately reducing resistance risk and improving patient outcomes. This study lays the foundational mechanistic foundation needed to advance the clinical development of HO-3867 for HCC.

To the best of our knowledge, this is the first systematic study demonstrating that HO-3867, an orally deliverable STAT3 inhibitor, induces apoptosis in HCC cells with greater potency than in normal cells, as evidenced by Annexin V staining and TUNEL assay results in Figures 1E and 2D. Most importantly, we show that HO-3867 is highly effective in primary cancer cell populations, including cells known to be resistant to conventional chemotherapeutics. The specific anti-tumor activity of HO-3867 against HCC provides new evidence supporting the development of natural STAT3 inhibitors as targeted cancer therapies.[18] While additional in-vivo studies are needed to confirm that HO-3867's selective cytotoxicity is mediated exclusively through STAT3 signaling, the consistent results

from our in-vitro and ex-vivo experiments strongly support its therapeutic potential. As safe and targeted inhibitors are an essential tool for future therapeutic intervention, particularly in cancer treatment, the present results provide a convincing argument for the pre-clinical evaluation of HO-3867 in the treatment of HCC, including cancers that have developed resistance to conventional chemotherapeutic intervention.[19,20]

The present study has three key limitations that require further investigation to validate and expand our findings. First, the in-vivo experiments were conducted using only Huh7 xenografts, and further validation in other HCC cell lines and immunocompetent mouse models is needed to confirm the generalizability of our results across different HCC subtypes and in the context of an intact immune system. Second, the long-term toxicity and pharmacokinetic properties of HO-3867 were not evaluated in this study, which are critical pieces of data needed to assess its clinical safety and translate it to human trials. Third, the specific molecular interactions between HO-3867 and STAT3 require further biochemical characterization to confirm the direct binding of the compound to its target and clarify the allosteric or competitive mechanisms underlying STAT3 inhibition. Each of these limitations defines clear directions for future research to advance the development of HO-3867 as a clinical HCC therapeutic.

Beyond its limitations, this study offers important strengths that advance the field of targeted HCC therapy. It is the first systematic investigation to report the anti-tumor activity of HO-3867 across multiple experimental models, including clinically relevant chemotherapy-resistant primary HCC cell populations, which fills a key gap in knowledge about natural STAT3 inhibitors for HCC treatment. Our results consistently confirm the anti-proliferative and pro-apoptotic effects of HO-3867 are linked to STAT3 pathway inhibition across in-vitro and in-vivo experiments, providing robust preliminary evidence for its targeted mechanism of action. Furthermore, HO-3867 is orally deliverable, a favorable pharmaceutical property that improves its potential for clinical translation compared with intravenously administered inhibitors. Our study therefore provides a strong foundational basis for further preclinical development of HO-3867 as a novel therapeutic for HCC.

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Authors' Contributions

Zhiya Xiao was responsible for the conception and design of the study, data acquisition, and analysis. Jun Hu contributed to the interpretation of data,

drafting the manuscript, and critical revision for important intellectual content. Both authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of Interest

None declared.

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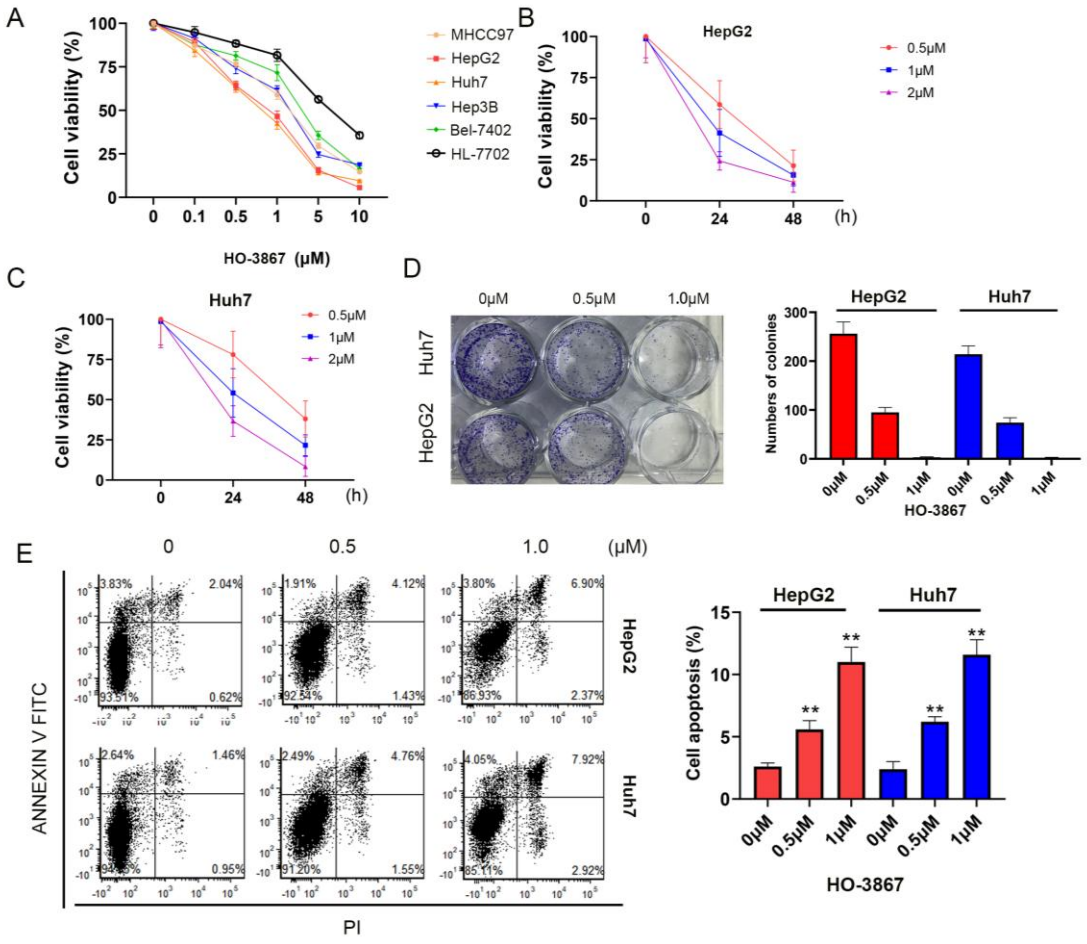

Figure 1. HO-3867 attenuates the cell proliferation, colony formation and induces cell apoptosis in hepatocellular carcinoma cells. (A). Cck-8 assay detects various liver cancer cells and normal liver cells with 0-10μM HO-3867. (B-C). Cell proliferation data obtained from HepG2 and Huh7 cells treated with the compounds in the concentration range 0, 0.5, 1.0, 2.0μM for 24 h. Statistical significance is shown in comparison with the corresponding HO-3867 values. (D). Colony formation assay was performed on HepG2 and Huh7 cells treated with HO-3867. One hundred cells were treated with increasing concentrations of HO-3867 for 24 h. Colonies were counted 14 days after treatment. (E). Annexin V-FITC/PI double staining on cells, The Annexin V-positive cells were subsequently quantified ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

HO-3867: A synthetic selective cyclooxygenase-2 inhibitor with pro-apoptotic and anti-proliferative activity against multiple cancer types; CCK-8: Cell Counting Kit-8, a colorimetric assay used to assess cell viability and proliferation; HepG2: A widely used human hepatocellular carcinoma cell line; Huh7: A well-characterized human hepatocellular carcinoma cell line; Annexin V-FITC/PI: Annexin V conjugated with fluorescein isothiocyanate/propidium iodide, a double staining combination used to detect and quantify apoptotic cells by flow cytometry; a statistical measure used to assess the significance of observed differences between experimental groups.

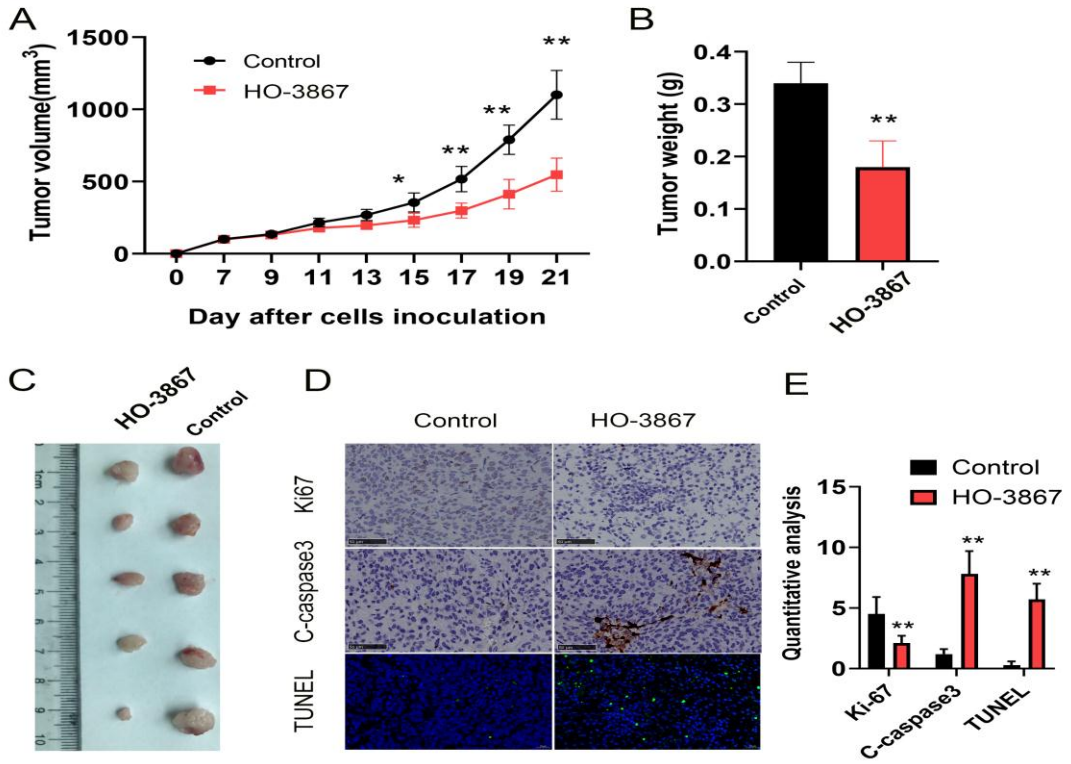

Figure 2. (A). Growth curves for the HO-3867 effects on tumor growth. (B-C) tumor weight and size between control and HO-3867 treatment. n = 5 mice per group for nu de mouse xenograft models (D). Up panel, Tumor section are detected by immunohist ochemistry (IHC) for Ki67 and cleaved-caspase3(C-caspase3), Down panel, TUNEL assay to examine the cell apoptosis in tumor. (E). For IHC Quantification, 5 random × 400 fields/slide,
count % of Ki67 or C-caspase3-positive cells relative to total cells. TUNEL Quantific ation, 5 random × 400 fields/slide,

calculate % of TUNEL-positive apoptotic cells using ImageJ software (version 1.8.0). The scale bar is 20m (n = 5, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

HO-3867: A selective inhibitor of signal transducer and activator of transcription 3 (STAT3), a synthetic compound with anticancer activity; n: Number of biological replicates; IHC: Immunohistochemistry; Ki67: Proliferation marker protein Ki-67, a nuclear marker for cell proliferation; C-caspase3: Cleaved caspase-3, an activated marker of the apoptotic cascade; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling, an assay for detecting DNA fragmentation during apoptosis; ImageJ: Open-source Java-based image processing software for biological image analysis version 1.8.0: Release version of the ImageJ software used for image quantification; μm: Micrometer, unit of measurement for the scale bar

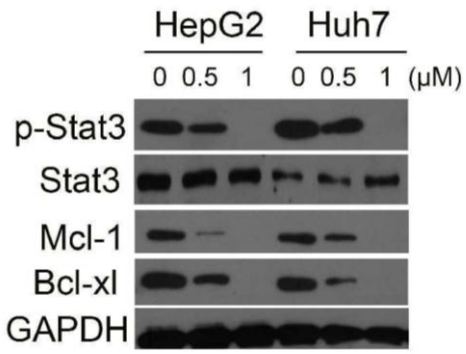

Figure 3. Effect of HO-3867 on key proteins involved in cell apoptosis and STAT3 signaling. proliferation. HepG2 and Huh7 cells were incubated with increasing concentrations (0, 0.5, 1.0μM) of HO-3867 for 24 h. The protein level was detected by using western blotting.

HO-3867: A selective inhibitor of signal transducer and activator of transcription 3 (STAT3), a synthetic compound with anticancer activity; HepG2: A human hepatocellular carcinoma cell line derived from a well-differentiated hepatocellular carcinoma; Huh7: A human hepatocellular carcinoma cell line derived from a well-differentiated hepatocellular carcinoma; STAT3: Signal transducer and activator of transcription 3, a transcription factor involved in multiple cellular processes including cell proliferation, apoptosis, and tumorigenesis; μM: micromolar, a unit of concentration equal to 10^{-6} moles per liter; Western blotting: A widely used analytical technique to detect specific proteins in a sample of tissue homogenate or cell lysate