

## Original Research

# Therapeutic effects of an innovative BS-HH-002 drug on pancreatic cancer cells via induction of complete MCL-1 degradation

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## ABSTRACT

BS-HH-002 is a newly developed drug with excellent antitumor activity, which resulted from the modification and optimization of the side structure of the homoharringtonine (HHT). It is particularly efficient in treatment for acute myeloid leukemia and myelodysplastic syndromes. Here we tested whether BS-HH-002 also had anti-cancer effects on solid tumors, especially pancreatic cancer. The results showed that BS-HH-002 treatment resulted in the complete degradation of the anti-apoptosis protein MCL-1, thereby inhibiting proliferation and inducing apoptosis of pancreatic cancer cells. In contrast, BCL-2 and BCL-XL protein levels were still detected in apoptotic cells. Further, we compared HHT and BS-HH-002 in terms of PK and heart toxicity in animals. Compared to HHT, BS-HH-002 quickly reached high blood concentration after intravenous injection or oral administration, without causing obvious cardiac toxicity. These results indicate that BS-HH-002 is a promising new anti-cancer drug to treat pancreatic and other solid tumors.

## Introduction

Pancreatic cancer is a common malignant tumor that is characterized by unlimited proliferation, resistance to apoptosis, high occurrence of metastasis, and immune escape. In addition, pancreatic cancer is usually diagnosed at an advanced stage, which leads to poor prognosis and short survival time [1,2]. Previous studies and the WHO's 2020 Cancer Statistics Report estimated that in the United States, pancreatic cancer is the eleventh most diagnosed and the second most deadly cancer [3,4]. Currently, available treatments include surgery, radiotherapy, and chemotherapy [5–7]. The first-line clinical medication for pancreatic cancer uses mainly gemcitabine, which is a specific inhibitor of cell cycle. Combined therapies may also use oxaliplatin, cisplatin, capecitabine, paclitaxel, and 5-Fu, among other drugs, as second-line treatment [8–11]. In recent years, innovative antitumor drugs have emerged, adding new targeted treatments such as erlotinib, pembrolizumab, and trastuzumab, used in combination with immunotherapy [12–14].

The proteins of the B-cell lymphoma-2 (BCL-2) family share a common BCL-2 homologous domain (BH). The members of this family can be classified into pro-apoptotic and anti-apoptotic categories [15]. The group of pro-apoptotic proteins includes BAK, BAX, BOK, and NOXA (specifically antagonizes Myeloid Cell Leukemia-1 [MCL-1]), which

contain the four domains BH1, BH2, BH3, and BH4. In contrast BID, BIM, BIK, NOXA, and PUMA, contain only a BH3 domain. The group of anti-apoptotic proteins includes A1/BFL-1, BCL-2, BCL-XL, MCL-1, and BCL-W, which contain the four conserved domains BH1, BH2, BH3, and BH4. Among these anti-apoptotic proteins, BCL-2, BCL-XL, and MCL-1 play major roles [16,17]. BCL-2 family proteins, mainly located in the endoplasmic reticulum and around mitochondria, mediate endogenous apoptosis of cells by regulating mitochondria through an interplay between antiapoptotic and pro-apoptotic proteins [18].

MCL-1 was first identified as an element of the human myeloid leukemia pathway. It contains PEST domain, conferring it a short half-life (1–2 h). The gene encoding the MCL-1 proteins is located on chromosome 1Q21. MCL-1 protein is also expressed in some normal cells but at much lower level than in tumor cells. MCL-1 protein can be induced through different signaling pathways, among which the main stimulatory pathway uses the JAK-STAT pathway. Unlike other members of the BCL-2 family, MCL-1 transcription can be rapidly induced, allowing its expression to increase during myeloid differentiation and upon cytokine stimulation [19–21]. Amplification and overexpression of MCL-1 have been reported in a variety of human tumors, including hematological malignancies and solid tumors [22]. MCL-1 can be upregulated by such as cytokines, including IL-6 [23], IL-8 [24], and IL-10 [25], and growth

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factors, such as EGF [26], VEGF [27], and PDGF [28]. The JAK-STAT pathway is composed of four main components: a tyrosine kinase-associated receptor, JAK kinase, STAT transcription factor, and target genes. The fact that MCL-1 is rapidly upregulated through activation of the JAK-STAT signaling pathway, that acts downstream of cytokine receptors, explains why MCL-1 expression increases upon cytokine exposure [29–31].

The proteins of JAK-STAT pathway are highly expressed in pancreatic cancer cell lines and overactivated in tumor cells from clinical pancreatic cancer patients [30,32]. Combination of gemcitabine and ruzotinib (JAK1/JAK2 inhibitor) increases patient survival [33]. STAT3 plays an important role in tumorigenesis by being a convergent target activated and phosphorylated by multiple upstream oncogenic signaling pathways, including growth factor kinase and cytokine receptor pathways. Through hyperactivation and autocrine loops, STAT3 can promote the expression of key proteins such as IL-6, MCL-1, and PD-L1, of which the regulation correlates with a variety of specific cell behaviors and morphologies, and influences tumor cell resistance to apoptosis [29, 34–36].

Endogenous cell apoptosis, a complex process involving mitochondria, is key to malignant programmed cell death. Antiapoptotic proteins play an important role in tumor resistance to apoptosis. Antiapoptotic proteins favor the formation of tumor by prolonging the life of cells that would otherwise die rapidly [37]. It has been reported that the antiapoptotic protein MCL-1 plays a key role in apoptosis resistance and survival of cancer cells [38–41].

Homoharringtonine (HHT) is a lipid alkaloid with antitumor activity isolated from the Chinese herbal medicine cephalotaxus. Recent clinical studies demonstrated its efficacy for remission and post-remission treatments of acute myeloid leukemia (AML), and subsequently, for myelodysplastic syndrome (MDS)-related diseases [42]. Mechanistic studies on HHT indicated that this compound has three main effects on tumor cells, namely, it promotes tumor apoptosis, blocks cell cycle progression, and inhibits protein synthesis in eukaryotic cells [43–47]. Semisynthetic-HHT (sHHT), which was launched in 2012, ameliorates bioavailability by improving the extraction and production of sHHT, resulting in better therapy for chronic myeloid leukemia [48,49]. Although HHT shows significant inhibitory effects on tumor cells *in vitro*, its narrow tissue distribution greatly limits its clinical application. Oral and subcutaneous treatments with HHT only achieve low plasma concentration due to fast metabolic cycle in blood, whereas myelosuppression occurs at high drug concentration. For this purpose, BS-HH-002 was designed and developed by Shanghai BenSheng Pharmaceutical Corp., Ltd. by modifying the secondary structure of HHT. In the present study, BS-HH-002 was tested for the first time both *in vitro* and *in vivo* on pancreatic cancers to determine its therapeutic effect as well as its potential mechanism of action.

## Materials and methods

### Materials

BS-HH-002 was provided by BenSheng Pharmaceuticals Co., Ltd. Gemcitabine was obtained from Harbin Gloria Pharmaceuticals Co., Ltd. HHT was obtained from MinSheng Pharmaceuticals Co., Ltd. A stock solution of these compounds was prepared in DMSO and was diluted in ultra-pure water shortly before use.

### Cell viability assay

The MTT assay was used to evaluate the viability of pancreatic cancer cells. More details are provided in the Supplementary Material File.

### Measurement of apoptosis by flow cytometry

The apoptotic pancreatic cancer cells were harvested and assessed with the Annexin V-FITC/PI apoptosis assay kit (Becton, Dickinson and Company). Apoptosis was quantified using a Guava HT easyCyte instrument within 1 h.

### Western blot

All proteins were quantified by Western blot, with  $\beta$ -actin or  $\beta$ -Tubulin as the internal reference protein. More details are provided in the Supplementary Material File.

### Measurement of hERG activity

In the hERG model, cisapride was used as positive control to ensure the normal response and quality of the hERG cells compared with BS-HH-002. More details are provided in the Supplementary Material File.

### Pharmacokinetic analysis

Twelve CD1 mice in each group were used to test oral (P.O.) and subcutaneous (S.C.) treatment with BS-HH-002 and HHT. P.O. administration was conducted by gavage. More details are provided in the Supplementary Material File.

### Xenograft in BALB/c Nude mice

This study included experiments in three models of xenograft in BALB/c Nude mice. Two animal-models were induced by subcutaneous injection of Miapaca-2 (once) and KP-4 (twice) in BALB/c Nude mice. More details are provided in the Supplementary Material File.

### Hematoxylin and Eosin (HE) staining assay

The nuclei were stained first in hematoxylin solution (Solarbio) for 5–20 min and differentiated with the differentiation solution for 3 min. The cells were then stained with Eosin Y aqueous solution for 10–120 s, after which the slides were quickly washed in deionized water. Finally, the sections were dehydrated in solutions with increasing ethanol concentration, with incubations of 2–3 s each, and rinsed in 100% ethanol for 1 min. The stained sections were mounted with transparent xylene and sealed with resin.

### Immunofluorescence assay

The expression of TIP30/CC3 and KI67 proteins in KP-4 from BALB/c Nude mice model, treated with Gemcitabine and BS-HH-002 was assessed by immunofluorescence. More details are provided in Supplementary Material File.

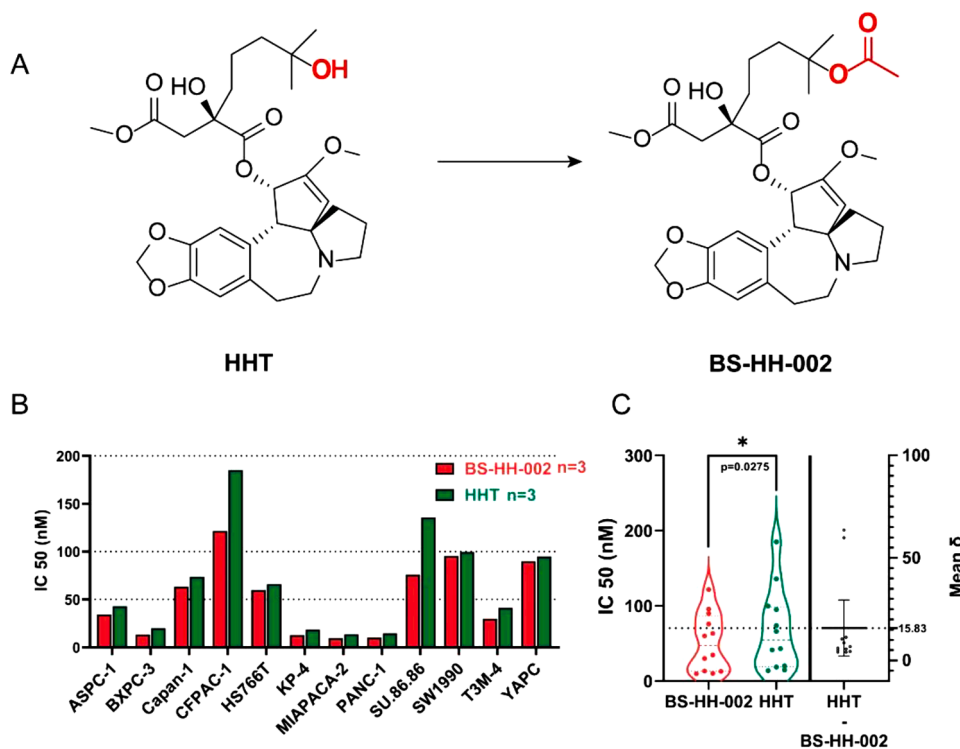
## Results

### BS-HH-002 had a strong inhibitory effect on the growth of pancreatic cancer cell lines

We first tested the cell toxicity of BS-HH-002 on a variety of pancreatic cancer cell lines. The 72-h  $IC_{50}$  of BS-HH-002 on all cells were lower than 200 nM, and the average  $IC_{50}$  was 51.35 nM. Compared with HHT, BS-HH-002 exerted a stronger inhibition on cell proliferation in some of the pancreatic cancer cells, including CFPAC-1 and SU86.86 (Fig. 1).

### Suppressive effects of BS-HH-002 on cellular oncogenic pathways

To determine the effects of BS-HH-002 on oncogenic signaling



**Fig. 1.** Comparison of the cell growth inhibition ability of BS-HH-002 and HHT on pancreatic cancer cells. (A) Molecular structures of BS-HH-002 and HHT. (B) Seventy-two-hour IC<sub>50</sub> values of BS-HH-002 and HHT in different pancreatic cancer cell lines ( $n = 3$ ). (C) Subtracting the value of BS-HH-002 from the value of HHT, significant difference analysis between the BS-HH-002 and HHT groups through paired-samples  $t$ -test, \*  $p < 0.05$ .

pathways, we measured JAK-STAT path-related protein expression in pancreatic cancer cell lines after administration. All the pancreatic cell lines had expression of STAT3, MYC, MCL-1 and BCL-XL proteins (Fig. 2A). We then treated the different pancreatic cancer cell lines with 50 nM of either BS-HH-002 or HHT for 24 h to monitor the changes in expression of the target proteins. The basal level of p-STAT3 and MCL-1 in most tested pancreatic cancer cell lines was high. After 24 h of treatment with BS-HH-002, MCL-1 decreased to varying degrees in most cell lines, but p-STAT3 and MCL-1 levels varied in different ways across the different pancreatic cancer cell lines. In contrast, except for a few cell lines, BCL-2 and BCL-XL expression did not vary significantly upon treatment (Fig. 2B). On average, BS-HH-002 used at 1  $\mu$ M degraded MCL-1 in 4 h in the different pancreatic cancer cells. In the supplementary experiment, we found that the degradation of MCL-1 protein decreased in both time and dose-dependent manners.

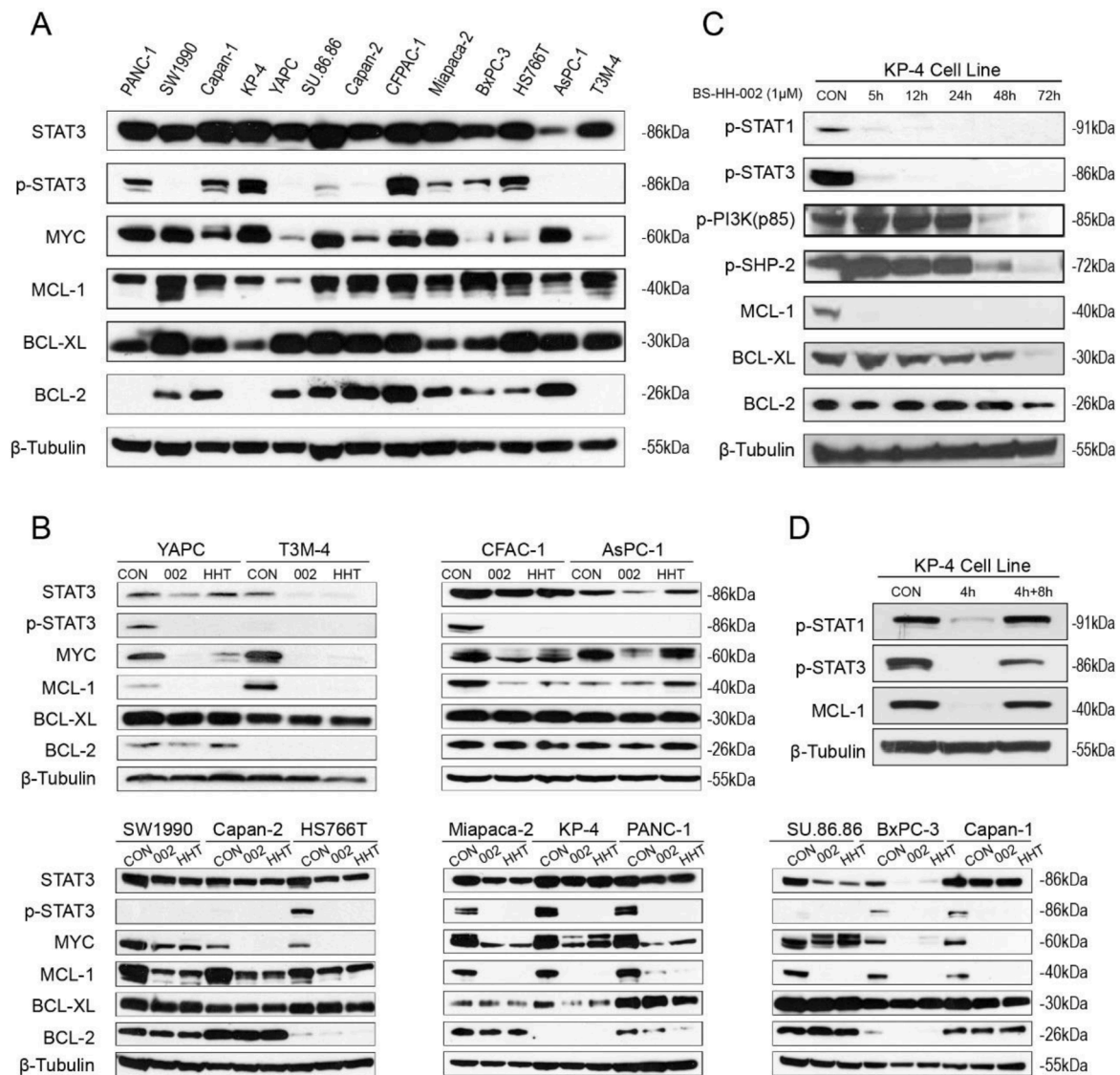
To monitor the changes in the expression of target proteins in a time-dependent manner, KP-4 cells were selected and treated with this concentration of BS-HH-002 for 5 h, 12 h, 24 h, 48 h, and 72 h. The results showed that the antiapoptotic protein MCL-1 was degraded below detection level after 5 h of treatment with BS-HH-002. The levels of p-STAT3 and p-STAT1 were also reduced after 5 h. Consistent with the changes observed for MCL-1, there was a pronounced decline of phosphorylated STAT1 and STAT3. However, the initial levels of p-STAT3 and p-STAT1 were significantly expressed. Besides, p-SHP-2 and p-PI3K-p85 were significantly decreased after 48 h of treatment (Fig. 2C). We treated KP-4 cells with 1  $\mu$ M BS-HH-002 for 4 h, and then stopped the drug for 8 h to monitor the changes in target protein levels. MCL-1 protein was largely degraded after 4 h of drug activity. The levels of p-STAT3 and p-STAT1 were also significantly reduced. After stopping the drug for 8 h, the amount of p-STAT1, p-STAT3, and MCL-1 expression increased to the same levels as those in the untreated controls (Fig. 2D).

#### BS-HH-002 induced strong apoptosis on pancreatic cancer cells

Measurement of BS-HH-002-induced apoptosis of pancreatic cancer cells showed that the apoptosis rate in Capan-1 and Miapaca-2 cells was obviously increased upon treatment for 12 h, and that Capan-1 cells initiated the post-apoptotic process more rapidly; KP-4 cells showed a large amount of apoptosis when treated for 24 h; and the apoptosis rate of three kinds of pancreatic cancer cells reached more than 90% when treated for 72 h (Fig. 3A). The results of the detection of apoptosis-related proteins in pancreatic cancer cell lines treated with BS-HH-002 showed that after 2 h of treatment in pancreatic cancer cells, the apoptotic proteolytic enzyme caspase-3 started to be activated, and PARP protein was also cleaved. The first time point of obvious increase of caspase-3 protein activation coincided with the apparition of a large number of apoptotic cells in different pancreatic cancer cell lines (Fig. 3B). The initiation of apoptosis occurred after degradation of MCL-1 protein (Supplement Fig. S1A and B). In addition, the initiation of Cyclin D1 protein degradation was correlated within 2 h of treatment with BS-HH-002. Anti-apoptotic protein detection on the apoptotic pancreatic cancer cells when treated with BS-HH-002 for 48 h showed that MCL-1 had been completely degraded in apoptotic cells. In contrast, there is no obvious change in the expression levels of BCL-2 and BCL-XL proteins (Fig. 3C).

#### Improved PK/PD of BS-HH-002 compared with HHT

We then compared the PK/PD of BS-HH-002 with HHT. Oral administration or subcutaneous injection with BS-HH-002 resulted in significantly higher blood concentration than that with HHT. For both drugs, the monitoring of plasma drug concentration in individual animals was analyzed using the non-compartmental model to estimate the PK parameters of the drug plasma concentration (Fig. 4) (Tables 1 and 2; Supplement file Table 1). The results indicate an improvement of PK/PD regarding plasma concentration and rapidity of drug metabolism with



**Fig. 2.** Detection of JAK-STAT path-related protein expression in pancreatic cancer cell lines after administration and MCL-1 protein on apoptosis of pancreatic cancer cells. (A) Basic detection of important proteins in the JAK-STAT pathway of 13 pancreatic cancer cell lines. (B) 50 nM BS-HH-002 and HHT treated for 24 h. (C) 1 μM BS-HH-002 treated pancreatic cancer KP-4 cells for 5 h, 12 h, 24 h, 48 h, and 72 h. (D) The effect of STAT1 and STAT3 on the expression of MCL-1 in KP-4 cells after treatment with 1 μM BS-HH-002 for 4 h and 8 h after withdrawal was detected.

BS-HH-002 as compared with HHT. The tissue distribution in the heart was lower for BS-HH-002 than for HHT, suggesting that BS-HH-002 is superior to HHT in terms of cardiotoxicity.

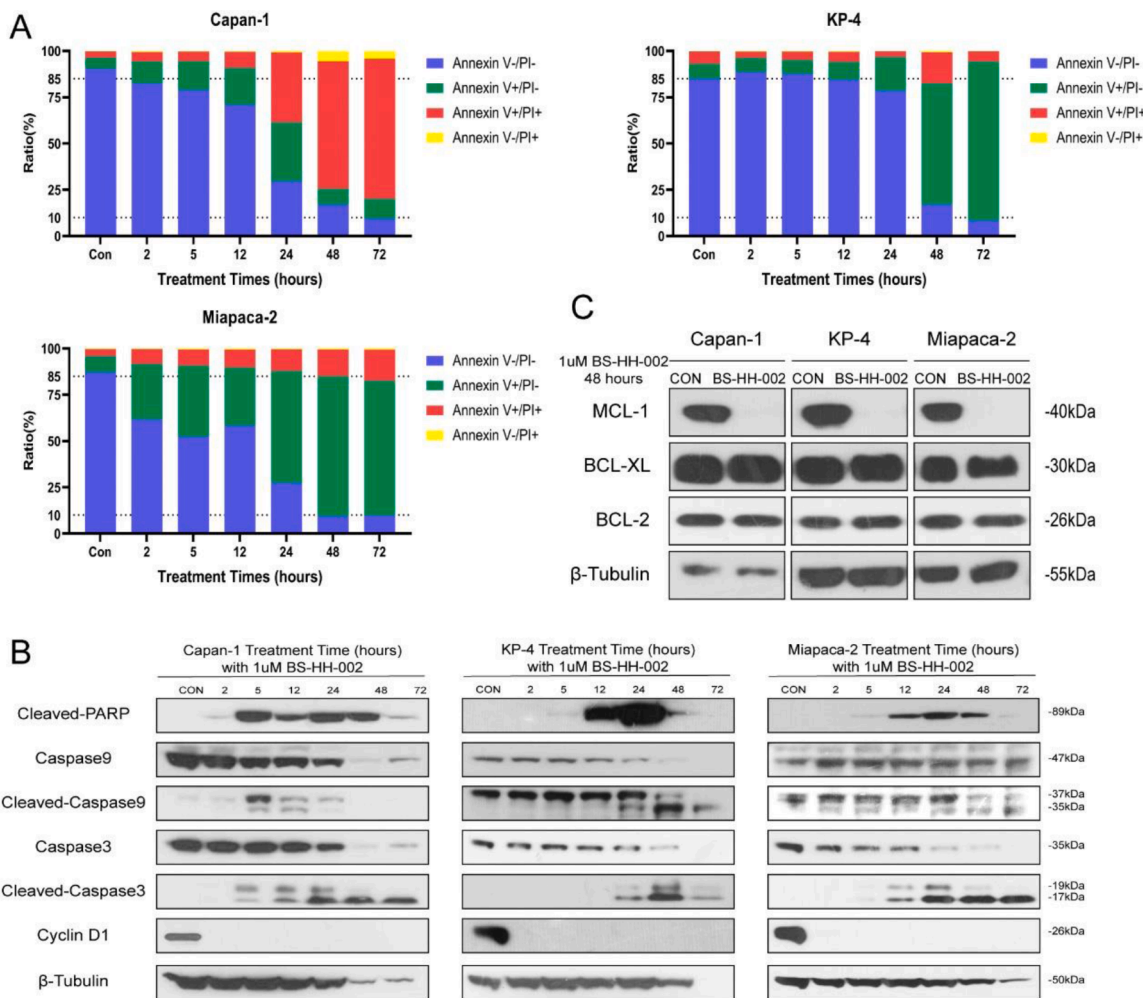
*BS-HH-002 had potent anti-tumor activity in mouse tumor models*

Depending on the treatment schedule, BS-HH-002 showed differential tumor-inhibiting effects in a mouse model of subcutaneous transplant of pancreatic cancer. In a mouse model of pancreatic cancer obtained by xenograft of KP-4 cells, BS-HH-002 was administered once a day, with cycles of 6 days of treatment followed by 3 days off-drug. The tumor volume is shown in Fig. 5A and B, and complete data are summarized in supplement file Table 2A. Compared with the vehicle group, the groups treated with BS-HH-002 or gemcitabine had fewer dark red blood vessels around the tumor mass, of which the color was lighter. The tumor volume decreased significantly upon treatment with the two drug. The tumors from five mice at least of the vehicle, the gemcitabine, and the BS-HH-002 (5.4 mg/kg) groups were analyzed to measure the protein levels of MCL-1 and BCL-XL. MCL-1 was significantly decreased in tumor cells from the BS-HH-002-treated group, and this difference

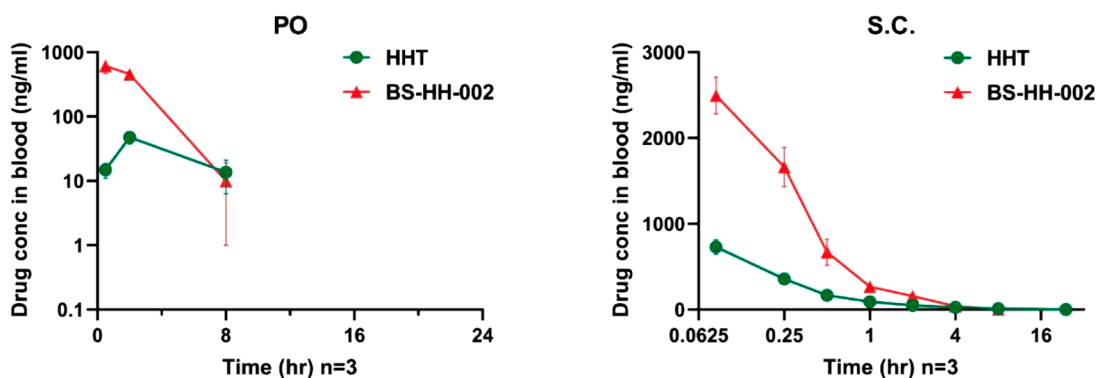
was very significant compared with the vehicle and the gemcitabine-treated groups ( $p < 0.01$ ). There was no significant difference between the gemcitabine-treated group and the vehicle group. In contrast to MCL-1 expression, there was no significant difference in BCL-XL expression between the three mouse groups. During withdrawal, the proliferation rate of KP-4 cells also increased, as well as MCL-1 expression. In fact, MCL-1 was always expressed. Compared with the BS-HH-002-treated group, the gemcitabine-treated group has a higher MCL-1 level, and the persistence of MCL-1 might inhibit tumor cell apoptosis.

*Pathological analysis and marker detection of tumor after treatment with BS-HH-002*

By measuring the changes in MCL-1 protein expression in tumor lumps before and after treatment of mice transplanted with tumor cells (Fig. 5A), and the restoration of MCL-1 protein expression (Fig. 2D), we optimized the medication regimen and implemented a protocol of continuous medication. The tumor volume in KP-4-transplanted mice was analyzed and revealed significant differences in the group receiving 3.6 mg/kg BS-HH-002 compared with the group treated with



**Fig. 3.** *BS-HH-002 induced strong apoptosis on pancreatic cancer cells.* (A) Apoptosis rate of pancreatic cancer Capan-1, KP-4, and Miapaca-2 cells treated with 1 μM BS-HH-002 at 2 h, 5 h, 12 h, 24 h, 48 h, and 72 h. (B) The expression levels of Cleaved-PARP, Caspase-9, Cleaved-Caspase-9, Caspase-3, Cleaved-Caspase-3, and Cyclin D1 proteins of Capan-1, KP-4, and Miapaca-2 cells treated with 1 μM BS-HH-002 at 2 h, 5 h, 12 h, 24 h, 48 h, and 72 h. (C) Expression of MCL-1, BCL-XL, and BCL-2 proteins were detected after treatment with 1 μM BS-HH-002 for 48 h to induce mass apoptosis in all pancreatic cancer cells.



**Fig. 4.** *Improved PK/PD of BS-HH-002 compared with HHT.* CD1 model mice were treated with 1 mg/kg of HHT and BS-HH-002 for plasma concentration of drug determination, which was administered by S.C./PO ( $n = 3$ ). In the blood concentration distribution after PO administration of HHT and BS-HH-002, the test result was BQL after 8 h. BQL: Below 80% of quantifiable limit of 1.00 ng/ml of HHT and BS-HH-002 in CD1 mouse plasma.

the vehicle ( $p = 0.0249$ ). Further, we analyzed frozen sections from tumors stained with HE and by anti-CC3/KI67 immunofluorescence. HE staining showed that the tumor cells in the vehicle group were mostly round and oval, with a little fibrosis. In comparison, in the gemcitabine and BS-HH-002 groups, the tumor cells were decreased significantly, and inflammatory cell infiltrates were slightly increased in the BS-HH-

002 group. In the BS-HH-002 group, there were significantly fewer necrotic foci, and the nuclei appeared smaller and clearer than in the vehicle group. Immunofluorescence staining of tumor masses from five mice of each experimental group was performed in triplicates. A total of 60 immunofluorescence staining results were statistically analyzed. We found that KI67 expression was significantly decreased by BS-HH-002 ( $p$

**Table 1**

Individual concentration-time data of HHT and BS-HH-002 after a PO dose of 1 mg/kg in CD1 mice.

Tissue	Compound	Sampling time (hr)				Tissue	Compound	Sampling time (hr)			
		0.5	2	8	24			0.5	2	8	24
plasma	HHT	14.8	47.7	13.7	BQL	heart	HHT	125	321	156	BQL
	BS-HH-002	615	456	10	BQL		BS-HH-002	141	171	BQL	BQL
bone marrow	HHT	BQL	669	48.6	BQL	spleen	HHT	184	742	225	BQL
	BS-HH-002	154	193	BQL	BQL		BS-HH-002	289	443	9.22	BQL
pancreas	HHT	85.8	228	211	4.86	brain	HHT	BQL	BQL	BQL	BQL
	BS-HH-002	179	228	10.1	BQL		BS-HH-002	BQL	BQL	BQL	BQL
stomach	HHT	1365	806	870	BQL	esophagus	HHT	607	BQL	BQL	BQL
	BS-HH-002	879	582	39.6	BQL		BS-HH-002	406	BQL	BQL	BQL
large intestine	HHT	105	528	689	BQL	liver	HHT	184	296	173	BQL
	BS-HH-002	79.4	100	16.8	BQL		BS-HH-002	167	275	BQL	BQL
lung	HHT	135	807	73.6	BQL	kidney	HHT	25.4	117	33.5	BQL
	BS-HH-002	641	101	BQL	BQL		BS-HH-002	138	185	BQL	BQL

\*BQL: Below 80% of lower quantifiable limit of 1.00 ng/mL of HHT and BS-HH-002 in CD1 mouse tissue.

**Table 2**

Individual concentration-time data of HHT and BS-HH-002 after a S.C. dose of 1 mg/kg in CD1 mice.

Tissue	Compound	Sampling time (hr)				Tissue	Compound	Sampling time (hr)			
		0.5	2	8	24			0.5	2	8	24
plasma	HHT	166	48.9	11.1	0.929	heart	HHT	541	254	115	BQL
	BS-HH-002	668	155	2.57	BQL		BS-HH-002	286	98.6	BQL	BQL
bone marrow	HHT	141	412	43.5	BQL	spleen	HHT	1987	1287	282	16.5
	BS-HH-002	176	55.6	BQL	BQL		BS-HH-002	984	397	BQL	BQL
pancreas	HHT	753	512	215	5.62	brain	HHT	11	6.37	4.97	BQL
	BS-HH-002	405	180	4.2	BQL		BS-HH-002	9.67	3.37	BQL	BQL
stomach	HHT	999	587	128	9.84	esophagus	HHT	138	185	52.6	BQL
	BS-HH-002	297	97.8	8.95	BQL		BS-HH-002	68.3	28.4	BQL	BQL
large intestine	HHT	1026	807	502	35.4	liver	HHT	983	615	80.2	4.53
	BS-HH-002	485	221	19.4	BQL		BS-HH-002	435	112	BQL	BQL
lung	HHT	693	287	62.5	5.8	kidney	HHT	2010	796	95.6	5.04
	BS-HH-002	346	90.9	BQL	BQL		BS-HH-002	679	191	BQL	BQL

\*BQL: Below 80% of lower quantifiable limit of 1.00 ng/mL of HHT and BS-HH-002 in CD1 mouse tissue.

= 0.0236), while CC3 protein expression was significantly increased by both gemcitabine and BS-HH-002 used at 3.0 mg/kg ( $p = 0.004$ ;  $p = 0.461$ ).

#### BS-HH-02 had no effect to block hERG potassium channel

The dose-dependent inhibitory effect of BS-HH-002 on hERG current was compared to that of the positive vehicle cisapride by using a CHO cell line stably transfected with a hERG cDNA expressing hERG channel at normal level. Six different doses of cisapride and BS-HH-002 were tested. The graph on the left-hand side showed the monitoring of single-cell current after drug exposure. On the middle panel featuring a time-current (XY) diagram, the change of the trajectory reflected the change of current provoked by the compound. The panel on the right-hand side showed a fitting graph of the degree of inhibition of hERG by the drug. BS-HH-002 inhibited hERG with an  $IC_{50}$  value of 3.68  $\mu$ M. Cisapride, used as a positive vehicle, inhibited hERG tail current with an  $IC_{50}$  value of 0.095  $\mu$ M (Fig. 7).

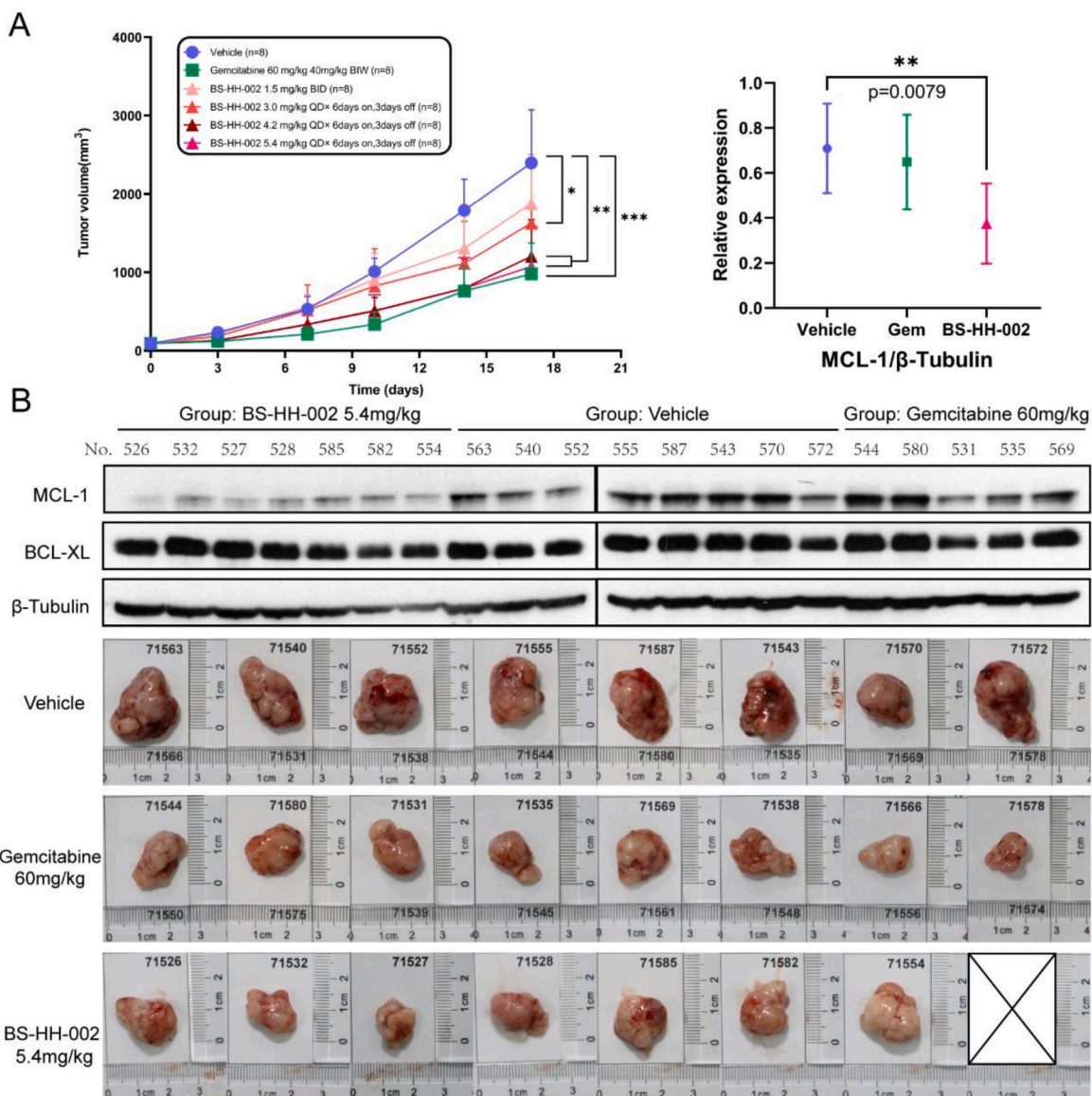
## Discussion

BS-HH-002 has been well validated for treatment of AML and MDS. The drug has been approved by the FDA to enter a phase 1 clinical study in 2018. Our study showed that BS-HH-002 was significantly effective for the treatment of both solid tumors and polycythemia vera. The mechanism and pharmacodynamics of HEL cell line and Balb/c mice model (with rhEPO induced) were verified. The discovery that polycythemia vera is mainly caused by abnormal activation of the JAK-STAT pathway due to JAK2V617F mutation led us to focus our attention on the JAK-STAT pathway. Combined with high-throughput sequencing of mRNA and protein expression after drug action, we found that BS-HH-

002 mainly treats pancreatic cancer through the JAK-STAT pathway. Another important fact is that the JAK-STAT pathway has a close crosstalk effect with the Ras, MAPK, and PI3K pathways and is crucial to the occurrence and development of malignant tumors. Indeed, we have also examined PI3K-AKT, MAPK-ERK, HIF-1 $\alpha$ , and other pathways. The phosphorylation level was significantly downregulated, thereby blocking the proliferation of pancreatic cancer cells and effectively inducing cancer cell apoptosis. In the experiment of cell apoptosis detection using flow cytometry, through the detection of three cell lines Capan-1, KP-4 and Miaapaca-2, we found that 1  $\mu$ M BS-HH-002 could achieve mass apoptosis within 72 h of treatment. We tried to extend the time of cell drug treatment to make all of the tumor cells achieve complete apoptosis under the same conditions.

In our study, we found that pancreatic cancer cells with high expression of p-STAT3 had a significant decrease in STAT3 phosphorylation level, but not total STAT3 protein expression level, after BS-HH-002 treatment, which was consistent with the changes in MCL-1 expression. BS-HH-002 can simultaneously reduce MCL-1 protein at the transcriptional and translational levels. Thus, MCL-1, a protein with a short life span, can be rapidly degraded in cancer cells. We have carried out the binding experiment of BS-HH-002 with ribosome and found that BS-HH-002 has a certain binding force with ribosome, but the binding force is slightly lower than that of HHT. BS-HH-002 can also inhibit the proliferation of tumor cells at the translational level, but BS-HH-002 significantly inhibits the phosphorylation of STAT3, the transcription factor of MCL-1, which was consistent with the changes in MCL-1 mRNA expression, reducing MCL-1 transcription and leading to the rapid degradation of the PEST structure of MCL-1.

HHT was involved in cell cycle inhibition and could clearly target tumor cell in the G1 phase, preventing their entry into in the G2/M phase. In our study, we found that BS-HH-002 can degrade C-MYC and



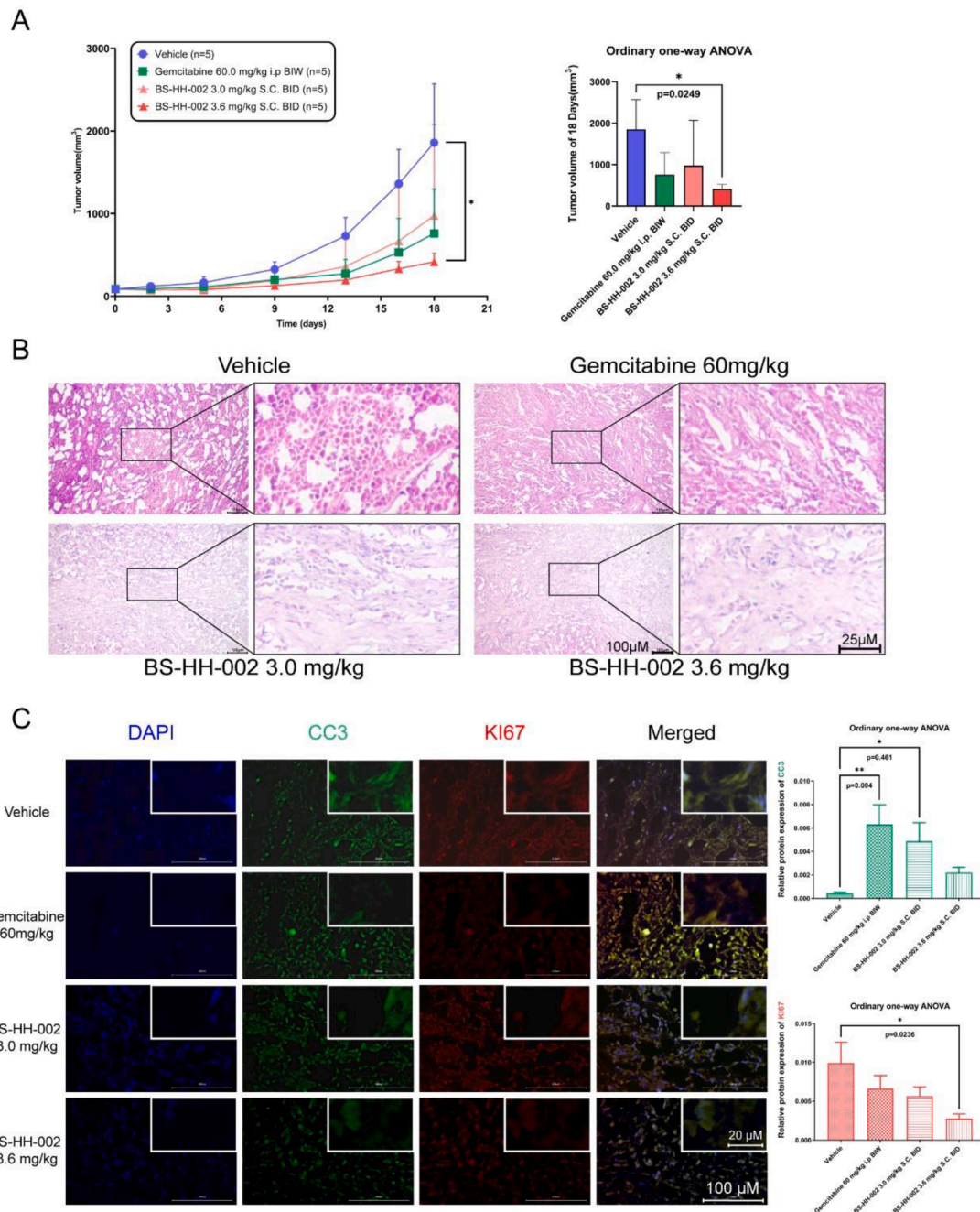
**Fig. 5.** The tumor inhibition effect of BS-HH-002 in vivo in mouse model. (A) In vivo efficacy test using gemcitabine and BS-HH-002 in BALB/c mouse model of KP-4 transplanted tumor ( $n = 8$ ). (B) After the KP-4 transplantation experiment, the transplanted tumor homogenate was taken out to extract protein and the protein expression of MCL-1 was detected. BS-HH-002 has different tumor-inhibiting effects in different treatment schedule in pancreatic cancer mice subcutaneously transplanted tumor models. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

Cyclin D1 proteins, suggesting that BS-HH-002 can inhibit cell cycle progression. However, through cell cycle detection, we found that BS-HH-002 could increase the proportion of the G1 phase in a short time, but with the advancement of cell apoptosis, the proportion of the G1 phase decreased, and cycle-specific cell inhibition was not obvious.

Some studies have found that, unlike BCL-2 and BCL-XL, MCL-1 has a strong affinity for the pro-apoptotic proteins BAK and BH3-only proteins including BIM, NOXA, and PUMA [50–52]. For all these reasons, the development of MCL-1 target-related drugs holds a high potential for new anticancer treatments. In recent years, the development of selective small-molecule inhibitors mimicking BH3, and antagonizing MCL-1 have brought significant progress in the field of oncology. These include maritoclax [51], UMI-77 [53], AMG-176 [54], AZD5991 [55], and the MIK665/S64315 and S63845 series [56]. Multiple drug candidates have entered clinical trials, including AMG-176, AMG-397, S64315/MIK665, and AZD5991. In addition, a large number of studies using gene knockouts have confirmed the importance of MCL-1 and its antiapoptotic function for normal cell development, such as that of

hematopoietic stem cells, hepatocytes, neutrophils, cardiomyocytes, and neurons [57]. Therefore, when inhibiting the function of MCL-1, the possible adverse effects need careful consideration. The treatment of tumors with MCL-1 inhibitors often has obvious cardiotoxicity.

In our previous studies, the prodrug HHT showed some cardiotoxicity and could induce heart anomalies such as sinus tachycardia and premature atrial beats, which led to a limitation on the dose window for its administration. Here, we measured the activity of BS-HH-002 on the potassium channel hERG to predict its cardiovascular toxicity. In fact, BS-HH-002 at high concentration had a micro molar inhibition of hERG, but the actual concentration of the drug was not very high. Regarding its tissue distribution and hERG level, BS-HH-002 showed a lower accumulation and faster clearance rate in the heart than HHT, which constitutes a significant improvement achieved by BS-HH-002 for cardiotoxicity compared with HHT. Since drugs flexibility regarding their mode of administration and safety depends on their metabolic efficiency and BS-HH-002 was metabolized faster than HHT, BS-HH-002 may allow the design of safer and patient-tailored therapies. We have



**Fig. 6.** Pathological analysis and marker detection of tumor after treatment with BS-HH-002. (A) *In vivo* efficacy of BS-HH-002 used at increasing concentrations in BALB/c mouse models of KP-4 xenografts, after optimizing the form of medication. \* $p < 0.05$ , \*\* $p < 0.01$  ( $n = 5$ ). (B) At the end point of the KP-4 transplantation experiments, the transplanted tumors were analyzed by to histology with HE staining. (C) The expression of TIP30/CC3 and KI67 proteins in KP-4 transplanted BALB/c Nude mice was analyzed by immunofluorescence. \* $p < 0.05$ , \*\* $p < 0.01$ .

tried surface plasmon resonance (SPR) analysis and co-crystallization experiment of MCL-1 protein and BS-HH-002, and we found that the two structures could not be directly combined and had low affinity. Therefore, BS-HH-002 has no obvious cardiovascular toxicity in contrast to other MCL-1 inhibitors. Importantly, BS-HH-002 is not an antagonist of MCL-1. It achieves its treatment effects mainly by reducing MCL-1 through the regulation of JAK-STAT and other relevant pathways to restore apoptosis in cancer cells.

We tested the ability of BS-HH-002 to induce apoptosis in pancreatic cancer cells and found that apoptosis rate accelerated significantly after complete degradation of MCL-1 protein. Several major antiapoptotic proteins, including MCL-1, BCL-XL and BCL-2, were detected in

apoptotic pancreatic cancer cells. However, only MCL-1 protein was completely degraded treated with BS-HH-002. These results were similar to those obtained with other types of solid tumors, including colorectal cancer, lung cancer, liver cancer, and prostate cancer, in which the antiapoptotic protein MCL-1 was not detected during apoptosis. In apoptotic cancer cells, BCL-XL and BCL-2 expression did not change significantly. Therefore, MCL-1 may play a decisive role in the spontaneous initiation of apoptosis in cancer cells. As long as the MCL-1 protein is expressed, apoptosis in cancer cells is inhibited.

In conclusion, BS-HH-002, which is derived from the natural small molecule drug HHT, can effectively induce complete degradation of overexpressed MCL-1 in pancreatic cancer cells, and restore apoptosis



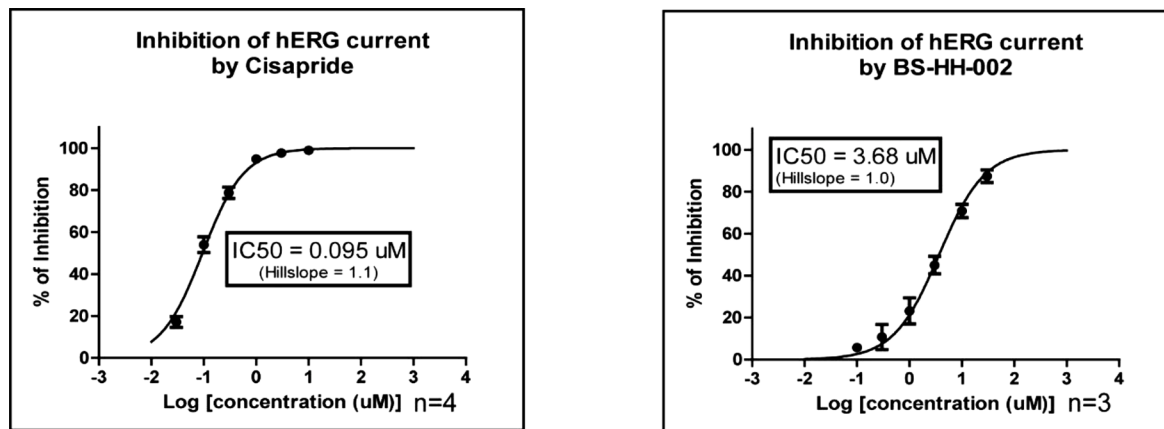


Fig. 7. BS-HH-002 had no effect to block hERG potassium channel. Model hERG was stimulated by cisapride, used as a positive vehicle, and BS-HH-002 to obtain the inhibitory effect of tail currents with value of  $IC_{50}$  ( $n=3$ ).

without obvious cardiotoxicity or other side effects. Moreover, BS-HH-002 has a higher blood drug concentration that can be achieved within a short period of time, compared with HHT. BS-HH-002 has the advantage to affect multitargets, which leads to inhibition of several oncogenic pathways crucial for tumor occurrence and development, compared to many single-target drugs. Therefore, we conclude that BS-HH-002 is an innovative anticancer drug that may break through drug-resistance barriers and offer long-term efficacy. BS-HH-002 may provide a new hope for effective anti-cancer treatment.

#### CRedit authorship contribution statement

**A-min Wang:** Validation, Resources, Writing – original draft. **Ru Qiu:** Methodology, Writing – original draft, Formal analysis. **Duo Zhang:** Validation, Resources. **Xiao-yan Zhao:** Conceptualization, Methodology, Supervision, Project administration.

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#### Declaration of Competing Interest

I am authorized on behalf of all the authors of this article to confirm that no author has any conflict of interest to disclose, and all of the authors have approved the version submitted for publication. The work in this article is original and has not been published previously, and the article is not under consideration for publication by any other journal.

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