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Original Article

Halichondrin B amide acts as tubulin binding agent to exhibit anti-tumor efficacy in hematologic cancers

Ying-Chun Li, Rong Zhang, Ying Yang, Jia Li, Ke Zhu, Kun Yao, Zhuo-Gang Liu

Department of Blood Internal Medicine, Shengjing Hospital of China Medical University, Shenyang 110004, China

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Abstract: Since microtubule dynamics play an indispensable role in cell division, cell motility, cellular transport, cell polarity and cell signalling, the microtubule appears as a highly attractive target for anticancer drug design. The present study demonstrates the role of halichondrin B amide (HCBA), an analog of halichondrin Bas an antitumor agent, its mechanism of action and pharmacokinetics. The results revealed that HCBA effectively inhibitscell growth in a variety of tumor types *in vitro.* The HCT116 DPC4 (-/-) colon cancer cell line was the most sensitive with an IC_{50} of 2.02 μM, compared to 3.78 μM in the parental HCT116. It also effectively reduced tumor growth in SCID mice human tumor xenografts of MV-4-11 acute myeloid leukemia, MM.1S multiple myeloma and DU-145 prostate cancer. HCBA caused accumulation of H69S, MM.1S, U266 and 8226/S cells in G2/M-phase after 24 h. There was a significant increase in the positive histone H3 cells from a baseline value of 4.38 to 53.45% in 8226/S cells and from 4.32 to 43.83% in MM.1S cells on treatment with HCBA. The results from pharmacokinetic studies demonstrated relatively high oral bioavailability of 83% with distribution in both plasma and bone marrow. In non-tumor bearing SCID mice injected with a single acute lethal dose of HCBA no myelosuppression was observed. Flow cytometry analysis showed cell cycle arrest in metaphase. It also caused inhibition of tubulin polymerization. Thus, HCBA appears to be a potent agent to arrest cell cyclin the metaphase and inhibit tubulin polymerization. Compared to other existing microtubule destabilizing agents HCBA has good oral bioavailability and lacks MDR cross-resistance acute myelosuppression.

Keywords: Pharmacokinetic, myelosuppression, oral bioavailability, microtubule inhibitor, tubulin polymerization

Introduction

Microtubules are the long, filamentous, tube-shaped protein polymers which constitute key components of the cytoskeleton. They are composed of two tightly linked polypeptides, α-tubulin and β-tubulin [1]. In the structure of microtubules α-tubulin are exposed at one and β-tubulin at the other end [2]. Microtubules control the position of organelles, direct intracellular trafficking of vesicles, organelles and proteins, and pull the chromosomes apart at mitosis [3]. Since microtubule dynamics play an indispensable role in cell division, cell motility, cellular transport, cell polarity and cell signalling, the microtubule appears as a highly attractive target for anticancer drug design. Themicrotubules are considered as the best cancer target identified to date and microtubule-targeted drugs are believed to be an important chemotherapeutic class of drugs [4, 5].

Microtubule-destabilizing agents destabilize microtubules by inhibiting the assembly of tubulin heterodimers into microtubule polymers or depolymerizing existing ones [6, 7]. Microtubule-binding agents, either microtubule-stabilizing or microtubule-destabilizing agents, could cause disruption of microtubule dynamics and subsequent mitotic arrest and cell apoptosis in cancer cells. The mechanism of their cytotoxic activity has been widely studied [8, 9]. The clinical success of the presently available microtubule-binding agents in cancer therapy was based mostly on their direct cytotoxic effects on tumor cells. Compared with normal endothelial cells, tumor-related endothelial cells are much more sensitive to the activity of microtubule-binding agents [10]. Microtubule-binding agents exhibit both antiangiogenic and vascular-disrupting actions and their multiple actions result a significant decrease in the blood supply to tumors compared to normal tissues [11]. Microtubule-binding agents have proved to be

useful class of drugs for vascular-targeted therapy in both preclinical and clinical studies [12, 13]. Efforts have been made to isolate microtubule-binding agents, which selectively target the tumor vasculature than exhibiting direct cytotoxic effects on cancer cells. The first microtubule-binding agent identified to have tumor vascular disrupting activity at well-tolerated doses was combretastatins [14].

Halichondrins, large polyether macrolides are isolated from the western Pacific sponge *Halichondriaokadai* and several other sponges from Axinella family. They possess an unusual 2,6,9-trioxatricyclo [3.3.2.0] decane ring system, 22-membered macrolactone ring, two exocyclic olefins, and an array of polyoxygenated pyran and furan rings [15]. The halichondrin analog, E7389 (eribulinmesylate) is currently in phase III clinical trials for the treatment of metastatic breast cancer. Phase I and phase II clinical trials have demonstrated that eribulin was active in heavily pretreated individuals while maintaining a tolerable therapeutic index. Eribulin treatment resulted in a decrease in dynamicity by suppressing the growth parameters at microtubule [16]. Binding of vinblastine to microtubules inhibited eribulin binding at low eribulin concentrations but also appeared to open additional, low-affinity binding locations for eribulin, at either one or both microtubule ends [17]. Taking into account the promising activity of halichondrins and their synthetic analogs the present study was designed to investigate the antitumor efficacy of halichondrin B amide (HCBA). In this study HCBA was evaluated in several human tumor cell lines *in vitro* and its mechanism of action in hematopoietic cell lines was determined. In addition, the study was extended to observe *in vivo* activity to several hematologic-derived human tumors in SCID mice. Pharmacokinetic studies comparing three different routes of administration suggest that oral bioavailability is quite good for the compound.

Materials and methods

Cell line and cell culture

The multiple myeloma MM.1S, RPMI 8226, NIH-H929, and U266, myelomonocyticleukemia MV-4-11, colorectal HCT-116, and prostate DU-145 and PC-3 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Drug resistant variants of parental RPMI 8226 (8226/IM10) cell line were selected for resistance to imexon [5]. The HCT-116 DPC4 (-/-) cell line, an isogenic clone of the HCT-116 colorectal line was obtained from Vogelstein laboratory at Johns Hopkins University, Baltimore, MD. The cells were cultured in RPMI 1640 (MediaTech Inc., Manassas, VA).

Drugs and chemicals

HCBA was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and caspase inhibitors from BioVision (Milpitas, CA). HCBA stock solution was prepared in DMSO and subsequently diluted into aqueous media for *in vitro* studies and for *in vivo* studies in 100% DMSO.

MTT assay

The cells at a density of 2.5×10^5 per 100 µL were seeded onto 96-well plates and incubated overnight. The cells were pre-treated with 10, 20, 30 or 50 µg/mL HCBA for 36 h followed by addition of 20 µL MTT solution to each well (5 mg/mL). Incubation of plates for 4 h at 37°C in 5% CO_2 was followed by removal of supernatants and addition of 150 µL DMSO. Plates were then placed on an orbital shaker for 5 min and the absorbance was recorded using the EnSpire™ 2300 Multilabel Plate Reader (PerkinElmer, Inc., Waltham, MA, USA) at 595 nm.

Tubulin polymerization assay

A cell-free tubulin polymerization assay was performed according to manual protocol [12, 13]. The characteristic tubulin polymerization phases (nucleation, growth, and steady state) were measured using kinetic assay in the presence of 10, 20, 30 µM or 50 µM HCBA. Paclitaxel (10 µM), vincristine (10 µM) and colchicine (5 µM) from Sigma (Sigma-Aldrich, St. Louis, MO, USA) were used as controls. After 1 min intervals the polymerization reaction was measured for 60 m at 340 nm using a Gemini XPS microplate reader (Molecular Devices; Sunnyvale, CA). The time points were plotted using SoftMax Pro® software (Molecular Devices).

Flow cytometry for cell cycle analyses

After incubation with 10, 20, 30 or 50 µM of HCBA for 24 h, the cells were washed with

Table 1. Comparison of HCBA Cytotoxicity in Human Cancer Cell Lines, *In Vitro*

Cell Type	Cell Line	IC_{50}, μM (Mean ± SEM)
Myeloma	8226/S	4.32 ± 1.21
	8226/IM10	3.45 ± 1.03
	NIH-H929	4.32 ± 1.23
	MM.1S	3.72 ± 0.82
	U266	2.43 ± 0.78
Leukemia	OCI-AML3	5.67 ± 1.76
	MV-4-11	4.75 ± 1.34
Prostate	PC-3	3.29 ± 1.08
	DU-145	4.35 ± 1.63
Colon	HCT-116	3.78 ± 1.42
	DPC4 (-/-)	2.02 ± 0.65
Lung	H69S	5.40 ± 1.78
	H69AR	2.13 ± 1.02

phosphate-buffered saline (PBS) and then fixed with 70% ethanol. Incubation with propidium iodide and RNAse A (Sigma-Aldrich, St. Louis, MO, USA) for 30 m at 37°C was followed by DNA content analysis using a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA).

Measurement of mitotic index

The mitotic index and the population of cells in metaphase (phospho-histone H3 positive cells) was analyzed by flow cytometry using a phospho-histone H3 (Ser10) antibody (Cell Signalling, Danvers, MA) and goat anti-rabbit Alexa488 secondary (Invitrogen Life Technologies, Grant island, NY). The modified Muehlbauer and Schuler [14] were used to measure the mitotic index.

Western blot analyses

HCBA-treated cells were washed twice in PBS. Then Lysis buffer (50 mM Tris-HCl pH 7.4, 137 mM NaCl, 10% glycerol, 100 mM sodium vanadate, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% NP-40, and 5 mM cocktail) 2 ml was added to the cells. BCA method was used to determine protein concentration. The protein were loaded and resolved by electrophoresis on a 10% polyacrylamide gel. The semi-dry method was used to transfer proteins onto a PVDF membrane which was then blocked with 5% non-fat dry milk overnight. After TBST washing, membrane was incubated for 2 h with primary antibodies and then washed again with TBST before incubation with secondary antibodies for 2 h. Then X-ray autoradiography was performed and the gray scale images were analysed. Relative COX-2 protein concentrations were determined by densitometry of the scanned radiography image with the Quantity One 1-D Analysis v.4.5.2 software (Bio-Rad).

Apoptosis and necrosis measurement by flow cytometry

For determination of HCBA-induced apoptosis of the cells flow cytometry using Annexin V-conjugated Alexa Fluor488 (Alexa488) and propidium iodide (PI) the cells were exposed to various HCBA concentrations for 36 h. Before exposure to HCBA, 5 μM of the caspase8 inhibitor Z-IETD-FMK (BioVision), the pan-caspase inhibitor Q VDOPh (BioVision), or a negative control caspase inhibitor (BioVision) was added to the cells. Then the cells were incubated with Alexa488 and propidium iodide in the dark. FACS Calibur instrument (BD Biosciences) equipped with the Cell Quest 3.3 software was used for analysis of stained cells.

Alkylating activity assay

The modified Friedman and Bogers' nitrobenzylpyridine assay [15-17] for alkylation activity was used to rule out direct binding to DNA.

Anti-tumor efficacy of HCBA in human tumor xenografts in SCID mice

All the animal studies were performed according to the Institutional Animal Care and Use Committee (IACUC) regulations. Myeloma (8226/S, MM.1S), leukemia (MV-4-11, OCI-AML-3) or prostate (DU-145, PC-3) tumor cells were subcutaneously inoculated in male SCID mice (8 week old). HCBA treatment was initiated was started after the mean tumor volume reached approximately 100 (**Table 2**). After every third day of tumor inoculation the parameters including tumor size, body weight, and general health of each animal was recorded. For HCBA induced delay in tumor growth, area under the curve (AUC) was determined.

Pharmacokinetic studies in normal SCID mice

Reverse phase HPLC and LCMS were used to study the pharmacokinetics of a single 50 mg/kg dose of HCBA in non-tumor bearing SCID mice. To compare bioavailability HCBA was administered in mice through intravenous (IV),

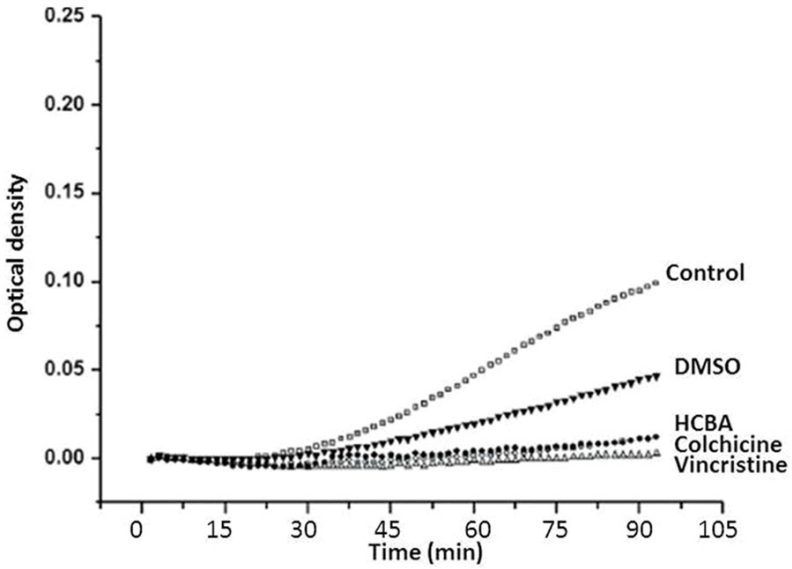

Figure 1. *In vitro* inhibition of tubulin polymerization by HCBA. The tubulin polymerization assay was performed in the presence of 10 μM vincristine, 50 μM HCBA, 5 μM colchicine, or DMSO. Tubulin polymerization phases (nucleation, growth, or steady state) were measured every minute for 105 m.

intraperitoneal (IP) or oral (PO) routes. After different intervals of HCBA administration plasma samples were collected and the DCP-046 concentration-time data analyzed using WINNONLIN Version 5.2 (Pharsight Corporation, St. Louis, MO). HCBA levels in plasma and bone marrow were measured after 15, 30, 45, and 60 m administration of a single 50 mg/kg IP dose of HCBA. For collecting bone marrow each femur was flushed with 100 μL of 0.9% NaCl and the bone marrow flushings were combined to report total HCBA (μg/mL) per mouse

Statistical methods

All the results are expressed as the mean of three experiments. The Pearson correlation coefficient was used for statistical analysis and the compounds with similar growth inhibitory response pattern would have a coefficient of 1.00 and exactly opposite agents would have a coefficient of -1.00. The differences were considered statistically significant at $P > 0.005$.

Results

Cytotoxicity effect of HCBA against human cancer cell lines

MTT dye reduction assay was used to measure growth inhibition of 13 human cell lines belonging to 5 tumor types on incubation with HCBA for 72 h (**Table 1**). The results revealed that the HCT116 DPC4 (-/-) colon cancer cell line was the most sensitive with an IC_{50} of 2.02 μM, compared to the 3.78 μM in the parental HCT116. The multidrug resistant 8226/Dox40 myeloma cell line showed a non-significant resistance compared to the parental 8226/S, whereas the MRP over-expressing H69AR cells were moresensitive to HCBA (**Table 1**). Importantly, all of the IC_{50} values are significantly lower than the peak plasma concentrations of 7.82 to 8.56 μM that are achieved in mice given tolerable HCBA doses *in vivo*.

Effect of HCBA on tubulin polymerization

The results from *in vitro* cell-free tubulin polymerization assay showed that HCBA blockedtubulin polymerization similar to that of colchicine and vincristine (**Figure 1**). Vincristine destabilizes the microtubule and colchicines binds tubulinto preventmicrotubule polymerization resulting in a flattened curve due to reduction in the amount of tubulin polymer formed.

Effect of HCBA on cell cycle

The results from flow cytometry revealed accumulation of H926, MM.1S, U266 and 8226/S cell lines in G2/M-phase on incubation with HCBA for 24 h (**Figure 2A-D**). The percentage of phosphohistone H3 positive cells in metaphase in the MM.1S and 8226/S multiple myeloma cell lines was studied using HCBA (50 μM) for 16 and 24 h. The results showeda significant increase in the positive histone H3 cells from a baseline value of 4.38 to 53.45% in 8226/S cells (**Figure 2E**) and from 4.32 to 43.83% in MM.1S cells (**Figure 2F**). However the treatment of 8226/S and MM.1S cells with colcemid increased the percentage to 23.53 and 17.84%, respectively. Thus HCBA treatment results in accumulation of the cells in the G2/M fraction and halted their cell cycle progression in the metaphase portion of mitosis.

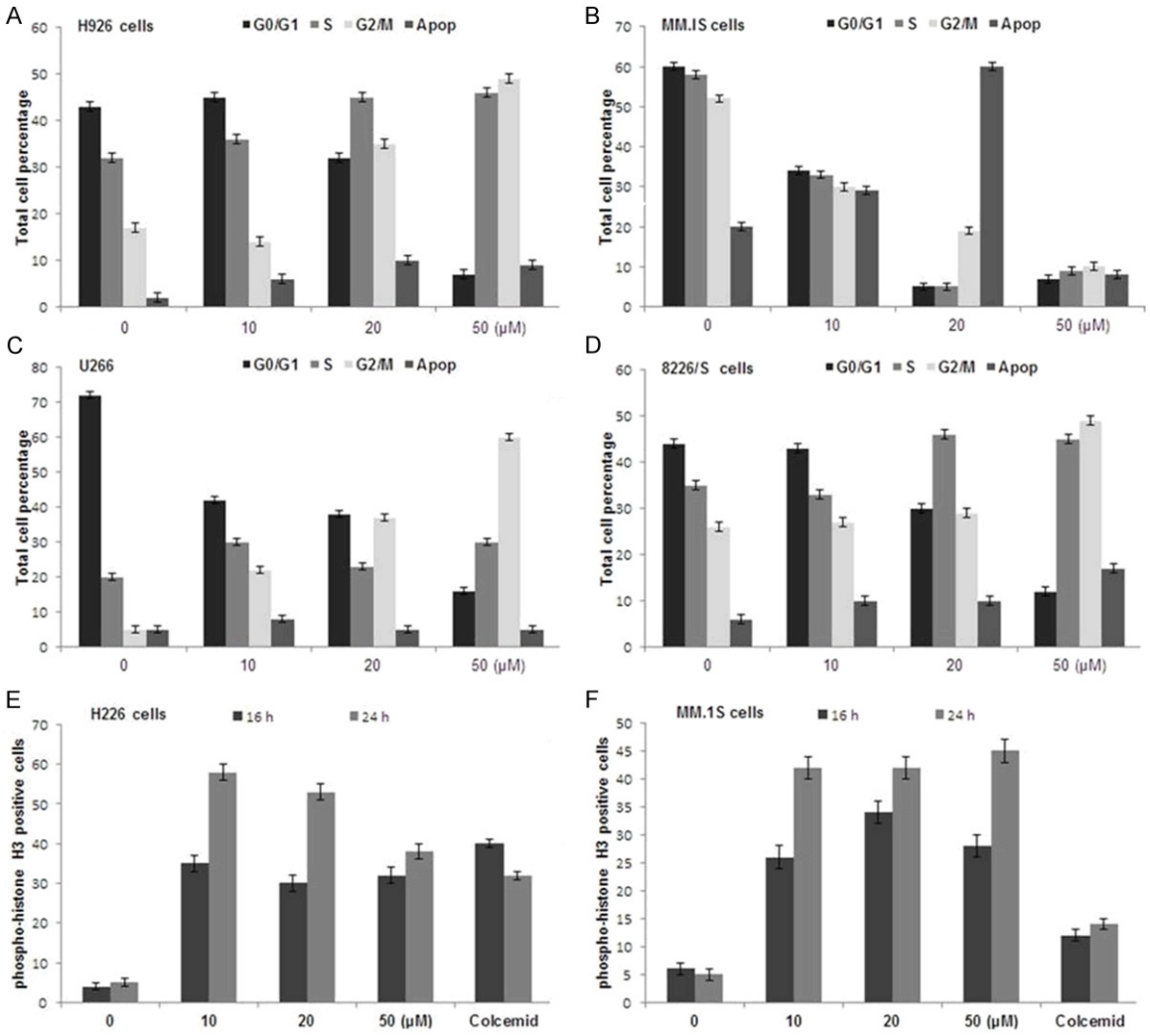

Figure 2. HCBA causes cell cycle arrest in the G2/M-phase. A-D. H929, MM.1S, U266 or 8226/S cells were incubated for 24 h with HCBA and cell cycle measured by propidium iodide staining. Cell cycle progression is halted in metaphase. E, F. 8226/S and MM.1S cells were incubated for 16 h with HCBA and stained for phospho-histone H3 positive cells.

Effect of HCBA on caspase inhibition in MM.1S cells

The myeloma cells were incubated with HCBA for 24 h followed by Western blot analysis. The results revealed cleavage of the caspases, 3, 8 and 9 suggesting the involvement of both the mitochondrial and death receptor apoptotic pathways (**Figure 3A**). HCBA-treated 8226/S and MM.1S multiple myeloma cells were co-incubated with 5 μM of the cell permeable pan-caspase inhibitor Q-VD-OPh, the cell permeable caspase 8 inhibitor Z-IETD-FMK or a caspase inhibitor negative control for 45 m prior to the addition of HCBA. After 36 h of HCBA treatment, cells were evaluated for apoptotic markers using Annexin V and propidium iodide. The results showed a decrease in HCBA-induced apoptotic cells from 53.45 to 21.26% in 8226/S and from 43.83 to 16.46% in MM.1S cells on treatment with the pan-caspase inhibitor (**Figure 3B**, **3C**). On the other hand, there was no effect of the caspase 8 inhibitor on HCBA-induced apoptotic cells, 53.45% in 8226/S and 43.83% in MM.1S cells. Therefore, caspase 8 is modestly activated by HCBA but its activation has no role for propagating the apoptotic signal.

Effect of HCBA on human hematologic and prostate cancer tumor xenografts in SCID mice

HCBA was intraperitoneally administered at concentration of 30 mg/kg for 6 days daily,

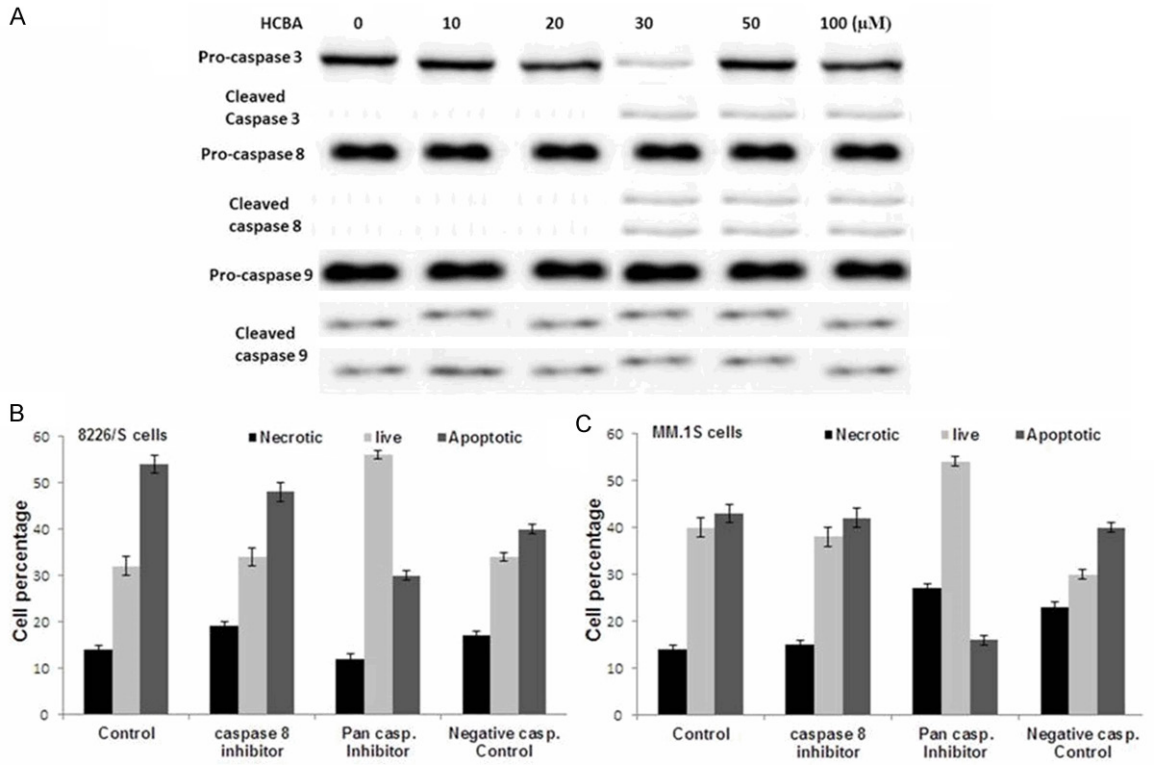

Figure 3. Activation of caspases 3, 8 and 9 by HCBA. A. HCBAtreated (24 h) MM.1S cells were examined for the activation of caspases 3, 8 and 9 by western blot. B, C. 8226/S or MM.1S cells were pre-treated for 40 m with 5 μM of the caspase 8 inhibitor Z-IETD-FMK, the pan-caspase inhibitor Q-VD-OPh, or a negative control caspase inhibitor before treatment with 0-100 μM HCBA. AnnexinV/PI staining was used to measure the cell death.

Table 2. Antitumor efficacy of HCBA against human tumor cell lines

HCBA (60 mg/kg)	Human Tumor Cell Line	Mean (SD), mm^3		Tumor AUC	
Schedule	Tumor Type	Control	DPC046	% Change	*P*-value
Dailyx6, off 6 d, repeat 1×	OCI-AM-3 (Acute Myeloid Leukemia)	19234.4 (6534.7)	10654.7 (3543.7)	-32.82%	NS
Dailyx6, off 6 d, repeat 2×	MV-4-11 (Acute Myeloid Leukemia)	23234.8 (8335.6)	9675.8 (4345.7)	-42.48%	0.1453
Dailyx6, off 6 d, repeat 1.5×	MM.1S*1* (Multiple Myeloma)	24423.8 (8654.5)	7654.3 (3223.2)	-64.23%	0.2549
Every third day, repeat 10×	8226/S (Multiple Myeloma)	33678.8 (10567.6)	30543.9 (9876.9)	-6.23%	NS
Dailyx6, off 7 d, repeat 2×	DU-145 (Prostate Cancer)	73544.8 (13446.5)	35765.7 (12867.1)	-53.46%	0.1655
Dailyx6, off 6 d, repeat 1×	PC-3 (Prostate Cancer)	12256.8 (3498.9)	6898.7 (1856.4)	-27.45%	NS

stopped for 7 days and the course was repeated in three cycles. All the mice survived with a significant loss of body weight over the repeated courses. The *SCID* mice transfected with human tumor cell lines showed a significant decrease in tumor growth on treatment with HCBA. In 3 of the 6 human tumor xenografts a significant decrease in tumor growth was observed (**Table 2**). These include 1 of 2 acute myeloid leukemia cell lines (MV-4-11 cells), 1 of 2 multiple myeloma cell lines (MM.1S cells), and 1 of 2 prostate cancer cell lines (DU-145 cells). There was significant about 48% growth inhibition in 5 of the xenograft experiments but no growth inhibition was observed in 8226/S myeloma cell line.

Effect of HCBA on hematologic toxicity in SCID mice

The blood samples from non-tumor bearing mice treated with a single 50 mg/kg or 75 mg/kg dose of HCBA were collected after 1, 3 and 6 days of administration. The samples were analyzed for the hematologic toxicity of HCBA. The erythrocyte levels and indices, white blood cells (total WBC, lymphocytes and leukocytes) and the platelet count was determined. The analysis of blood samples showed no signifi-

Table 3. Pharmacokinetic parameters in mice given 50 mg/kg of HCBA by 3 routes of administration

Pharamacokinetic parameter (units)	Mean values by route of administration		
	IV	IP	PO
Cmax (μg/mL)	2.18	5.23	2.09
Tmax (h)	0.23	0.19	0.18
T ½ (h)	9.39	6.43	4.43
AUC (hμg/mL)	6.43 (0.38)	6.68 (0.84)	5.32 (0.97)
Clearance (L/h/kg)	23.5	-	-
Vol. Dist. (L/kg)	310.4	-	-
F (bioavailabilty)	-	1.56	1.43

cant changes in the levels of these blood parameters at any of the time points. The drug dose was selected to include an acutely tolerated dose of 50 mg/kg and a lethal acute dose of 75 mg/kg. These results demonstrate that the dose-limiting toxicity of HCBA does not involve acute myelosuppression.

Pharmacokinetics of HCBA administration

Non-tumor bearing SCID mice were administered 50 mg/kg HCBA by IV, IP and oral gavage routes. The mean AUC's for the IV and IP route were 6.13 and 4.98 μg/mL, respectively. For the oral route of administration the AUC was 2.87 h μg/mL with a significantly better oral bioavailability of 84% compared to the other two routes. A bone marrow drug distribution study was performed in the femurs removed at 45 and 90 minutes following 50 mg/kg IP injection. The mean HCBA concentrations in the femurs at 45 and 90 minutes were approximately 0.82 μg/mL. This was comparable to mean 30, 45, 60 and 90 minute plasma concentrations of 7.08, 1.87, 1.64 and 0.893 μg/mL, indicating very poor penetration of the drug into the marrow. At 45 and 90 minutes the femur concentrations (0.82 μg/mL) are comparable to the IC_{50} values for all the *in vitro* tested cell lines (43 to 267 nM) for a 72 h exposure (**Table 3**). Therefore, cytotoxic HCBA concentrations did not induce any significant hematopoietic toxicity in the bone marrow after the administration.

Discussion

Microtubule inhibitors are routinely used for the treatment of solid tumors and hematological malignancies. The commercially available ones include vinca alkaloids (vincristine, vinblastine, vinorelbine), taxanes (paclitaxel, nab-paclitaxel, docetaxel and cabazitaxel) and a Halichondrin B analog (eribulinmesylate) and epothilone (ixabepilone) [18]. These microtubule inhibitors either inhibit tubulin assembly (microtubule destabilizing agents) or prevent microtubule disassembly (microtubule stabilizers) [18]. However, both the ways are associated with dose-limiting clinical toxicities like myelosuppression and cumulative-dose related peripheral neuropathy. The limitations of the presently available microtubule inhibitors include intrinsic or acquired resistance and non-reliable oral route administration. In addition lack of the efficient synthetic methods for the microtubule inhibitorsfrom the complex natural product structures also hinders their use. The present study demonstrates the antitumor efficacy, mechanism of action and pharmacokinetics of HCBA, a derivative of halichondrin B. One of the best advantage of HCBA is that it does not appear to be a substrate for the classic multidrug resistance protein ABCB1 (Pgp/MDR1). Being substrate for ABCB1 (Pgp/MDR1) confers about 200 fold resistance to vincristine in the 8226/Dox40 cell line. HCBA was also unaffected by overexpression of the alternate multidrug resistance protein (MRP, ABCC-2 or c-MOAT).

In the present study the most responsive human tumor cell line to HCBA was HCT-116 colon cancer cell line with DPC4 (*SMAD4*) homozygous (-/-) deletion. The results revealed that HCBA acts as a microtubule destabilizing agent. The microtubule destabilizing property ofHCBA was proved by flow cytometry data showing that HCBA stops cell cycle progression in the metaphase portion of mitosis and by the observation that the mechanism of cell death with HCBA is apoptosis mediated through the intrinsic or mitochondrial pathway. HCBA activated both the mitochondrial apoptosis pathway and the death receptor pathway mediated by caspase 8. However, caspase 8 inhibition has not effect on HCBA induced apoptosis, whereas the pan-caspase inhibitor addition caused a significant reduction in HCBA induced apoptosis. This confirmed that caspase 8 is

not directly activated by the HCBA treatment. Treatment of *SCID* mice with HCBA causeda significant tumor growth inhibition in human hematologic cancer cell lines and in prostate cancer cell lines. Thus HCBA significantly reduced tumor growth. Moreover, the activity seen in the *SCID* mice tumors was in agreement with the type known to be sensitive to microtubule inhibiting drugs in human patients. Another interesting observation with HCBA was the surprisingly good oral bioavailability (83%) with a simple oral formulation, and the fact that myelosuppression was not seen after the administration of acute high doses of the drug. The PK studies of HCBA distribution into mouse bone marrow clearly showed that the marrow isexposed to active drug levels. Therefore, the lack of acute bone marrow toxicity with HCBA marks a significant improvement over other microtubule inhibitors such as vinblastine and the taxanes.

In conclusion, HCBA causes cell cycle arrest in metaphase and inhibition of tubulin polymerization. Compared to other existing microtubule destabilizing agents HCBA has good oral bioavailability and lacks MDR cross-resistance acute myelosuppression.

Disclosure of conflict of interest

None.

Address correspondence to: Zhuo-Gang Liu, Department of Blood Internal Medicine, Shengjing Hospital of China Medical University, No. 36 Miyoshi Street, Heping District, Shenyang 110004, Liaoning, China. Tel: 0086-024-83955555; Fax: 0086-024-83955555; E-mail: liuzhuogang401@gmail.com

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