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ORIGINAL ARTICLE

# Chidamide (CS055/HBI-8000): a new histone deacetylase inhibitor of the benzamide class with antitumor activity and the ability to enhance immune cell-mediated tumor cell cytotoxicity

**Zhi-Qiang Ning · Zhi-Bin Li · Michael J. Newman · Song Shan · Xin-Hao Wang · De-Si Pan · Jin Zhang · Mei Dong · Xin Du · Xian-Ping Lu**

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**Abstract**
*Purpose* Chidamide (CS055/HBI-8000) is a new histone deacetylase (HDAC) inhibitor of the benzamide class currently under clinical development in cancer indications. This study reports the in vitro and in vivo antitumor characteristics of the compound.
*Methods* Selectivity and potency of chidamide in inhibition of HDAC isotypes were analyzed by using a panel of human recombinant HDAC proteins. Tumor cell lines either in culture or inoculated in nude mice were used for the evaluation of the compound's antitumor activity. To investigate the immune cell-mediated antitumor effect, isolated peripheral blood mononuclear cells from healthy donors were treated with chidamide, and cytotoxicity and expression of relevant surface proteins were analyzed. Microarray gene expression studies were performed on peripheral white blood cells from two T-cell lymphoma patients treated with chidamide.
*Results* Chidamide was found to be a low nanomolar inhibitor of HDAC1, 2, 3, and 10, the HDAC isotypes well documented to be associated with the malignant phenotype. Significant and broad spectrum in vitro and in vivo antitumor activity, including a wide therapeutic index, was observed. Chidamide was also shown to enhance the cytotoxic effect of human peripheral mononuclear cells ex vivo on K562 target cells, accompanied by the upregulation of proteins involved in NK cell functions. Furthermore, the expression of a number of genes involved in immune cell-mediated antitumor activity was observed to be upregulated in peripheral white blood cells from two T-cell lymphoma patients who responded to chidamide administration.
*Conclusions* The results presented in this study provide evidence that chidamide has potential applicability for the treatment of a variety of tumor types, either as a single agent or in combination therapies.

**Keywords** Chidamide · HDAC inhibitor · Antitumor activity · Immune cell-mediated cytotoxicity

**Electronic supplementary material** The online version of this article (doi:10.1007/s00280-011-1766-x) contains supplementary material, which is available to authorized users.

Z.-Q. Ning · Z.-B. Li · S. Shan · X.-H. Wang · D.-S. Pan · X.-P. Lu (✉)
Chipscreen Biosciences Ltd., Bio-Incubator 2-601, 1st Ave. of Gaoxin Road, Hi-Tech Industrial Park, Shenzhen 518057, Guangdong, China
e-mail: xplu@chipscreen.com

M. J. Newman
HUYA Bioscience International, 12531 High Bluff Drive Suite 138, San Diego, CA 92130, USA

*Present Address:*
M. J. Newman
Aaxiion Therapeutics, Inc., 11585 Sorrento Valley Rd., Suite 105, San Diego, CA 92121, USA

J. Zhang · M. Dong
Chinese Academy of Medical Sciences, Cancer Institute and Hospital, No. 17 Panjiayuan, Chaoyang District 100021, Beijing, China

X. Du
Hematology Department, The 2nd General Hospital, No. 3002 Sungang Rd., Shenzhen 100730, Guangdong, China

## Introduction

Histone deacetylases (HDACs) are involved in the remodeling of chromatin and play a key role in the epigenetic regulation of gene expression. HDACs act as transcription

repressors by removing acetyl groups from the ε-amino-terminus of lysine residues within histones to promote tighter winding of DNA around histone proteins. Elevated expression or activity of HDACs is implicated in the development and progression of cancer [1]. Inhibition of HDAC enzymes results in increased histone acetylation, thereby inducing an open chromatin conformation and transcription of previously dormant genes. At least 18 human HDACs have been identified and are grouped into four classes. HDAC enzymes class I (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 7, and 9 as IIa, and HDAC6 and 10 as IIb), and class IV (HDAC11) utilize a zinc-catalyzed mechanism for deacetylation of histones and non-histone proteins, whereas class III (SIRT 1–7) HDACs are $NAD^+$-dependent deacetylase enzymes [2]. Although the precise biological functions of individual HDACs are still largely unknown, the importance of HDAC enzymes in the malignant phenotype has been most closely associated with Class I HDACs 1–3 [3–5]. In addition, Class IIb HDACs 6 and 10 have been found to play a role in the expression and stability of tumor angiogenesis gene products [6].

The synthesis of small-molecule HDAC inhibitors (HDACi) has been an active focus in the field of anticancer drug discovery in recent years. Several different chemical classes of HDACi have been described, including hydroxamic acids, carboxylic or short-chain fatty acids, cyclic peptides, and benzamides. Examples of each of these classes have entered clinical development as antitumor agents [7, 8]. Among them, the hydroxamic acid vorinostat (SAHA) and cyclic peptide romidepsin (FK-228) were approved in the United States for the treatment of cutaneous T-cell lymphoma [9, 10], and very recently, romidepsin for peripheral T-cell lymphoma [11].

In this report, we describe the characteristic features of chidamide (CS055/HBI-8000), a new member of the benzamide class of HDACi. Chidamide inhibits HDAC1, 2, 3, and 10 in the low nanomolar concentration range with broad spectrum antitumor activity in vitro and in vivo. Mechanism studies have demonstrated that chidamide stimulates human immune cell-mediated tumor cell killing activity with increased expression of genes and proteins involved in natural killer (NK) cell functions.

## Materials and methods

### Chemicals and cell lines

Chidamide, *N*-(2-Amino-4-fluorophenyl)-4-[*N*-[(E)-3-(3-pyridyl) acryloyl]aminomethyl] benzamide ($C_{22}H_{19}FN_4O_2$; molecular weight, 390.41), was designed and synthesized by Chipscreen Biosciences Ltd. using a computer-aided, rational drug design approach [12–14]. All other comparator HDACi used were also synthesized by Chipscreen. The purity for all chemicals was over 98.0% by HPLC analysis.

All tumor cell lines were from the American Type Culture Collection (ATCC, Rockville, MD, USA). Human fetal kidney (CCC-HEK) and liver (CCC-HEL) cells were obtained from the Cell Culture Center of the Chinese Academy of Medical Sciences (Beijing, China). All cells were cultured following the vendor's instructions.

### HDAC enzyme inhibition assay

Selectivity and potency assays of chidamide inhibition of HDAC isotypes were performed and analyzed by BPS Bioscience Inc. (San Diego, CA, USA) using human recombinant HDAC proteins. All of the enzymatic reactions were incubated for 17 h at room temperature in 50 µl of reaction mixture containing HDAC assay buffer (BPS catalog number 50031), 5 µg BSA, an HDAC substrate, a purified recombinant HDAC enzyme and a test compound at a pre-defined concentration. After enzymatic reactions, 50 µl of 2× HDAC Developer (BPS catalog number 50030) was added to each well and the plate was incubated at room temperature for an additional 20 min. Fluorescence intensity was measured at an excitation of 360 nm and an emission of 460 nm using a Synergy™ 2 microplate reader from BioTek (Winooski, VT, USA). Each compound concentration was performed in duplicate. The $IC_{50}$ values were determined by analyzing concentration–response inhibition curves.

### Histone acetylation assay

Histone isolation from cells was conducted with a standard procedure. Briefly, HeLa cells were seeded into 10-cm culture dishes ($2 \times 10^6$/dish). Twenty-four hours later, cells were treated with the test compounds at different concentrations for different times. Cell pellets were obtained and resuspended in 1 ml of chilled lysis buffer (10 mM Tris–HCl, pH 6.5, 50 mM sodium bisulfite, 1% Triton X-100, 10 mM $MgCl_2$, 8.6% sucrose, 5 mM sodium butyrate) containing 1 mM PMSF and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). After centrifugation, the pellet was washed 4 times with chilled lysis buffer and gently resuspended in 1 ml of chilled nuclei wash buffer (10 mM Tris–HCl, pH 7.4, 13 mM EDTA, 5 mM sodium butyrate). After centrifugation, the pellet was gently resuspended in 318.5 µl of extraction buffer (10 mM HEPES, pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 5 mM sodium butyrate), 1.87 µl of 100 mM DTT, 5.6 µl of 100 mM PMSF, and 15 µl of 25× protease inhibitor cocktail. Then, 34.1 µl of 2.2 M $H_2SO_4$ (final concentration 0.2 M) was added to the tube drop by drop with gentle shaking. After incubation for 2 h on ice with

vigorous shaking every 20 min, the sample was centrifuged at 18,000×*g* for 10 min at 4°C; the supernatant was transferred to a pre-chilled 1.5 ml tube, and 1,125 µl of 20% trichloroacetic acid was added. The tube was incubated for 2 h on ice and inverted end-over-end every 30 min and then centrifuged at 18,000×*g* for 10 min at 4°C. The pellet was washed twice with acetone and air-dried for 30 min on ice. The extracted histones were redissolved in 40 µl of $H_2O$ and incubated at 4°C overnight. Twenty microlitres of glycerol was added to each sample and the solution was mixed gently, and then centrifuged at 12,000×*g* for 10 min at 4°C. The supernatant was transferred and stored at -80°C. Protein was quantified using a Micro BCA protein assay kit (Thermo Fisher Scientific/Pierce Biotechnology, Rockford, IL, USA).

For Western blot analysis, proteins were transferred onto Hi-Bond PVDF membranes (Thermo Scientific, Waltham, MA, USA), probed with anti-acetylated H3 antibody (Upstate Biotechnology, Inc., Waltham, MA, USA), and re-probed with anti-total histone H3 antibody (Abcam, Cambridge, MA, USA). Horseradish peroxidase-conjugated secondary antibodies (Upstate) were used with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for detection.

### In vitro cell growth inhibition assay

Cells were seeded into 96-well plates at 5–10 × $10^3$ cells/well (according to the growth rate of individual cell lines used). Twenty-four hours later, the test compounds were added at different concentrations, and the cells were cultured for 72 h, followed by addition of 20 µl/well of CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI, USA). After incubation of the plates for 2 h at 37°C, the absorbance at 490 nm was recorded using a 96-well plate reader. Cell viability was calculated by $A_{\text{treatment}}/A_{\text{control}} \times 100\%$ (*A* represents the absorbance recorded at 490 nm). The concentration that inhibited cell growth by 50% over the control was determined as $GI_{50}$. All of the compounds were dissolved in DMSO and were added to the culture at a 1:1,000 dilution to give a final DMSO concentration of ≤0.1%. All samples were evaluated in duplicate, and each experiment was repeated at least 3 times.

### In vivo antitumor studies

All animal experiments were performed under an Institutional Animal Care and Use Committee-approved protocol and institutional guidelines for animal welfare. Nude mice (BALB/c-nu), ages 6–8 weeks (Institute of Experimental Animal, Academy of Chinese Medical Sciences, Beijing, China), were used for the studies. HCT-8 (colorectal carcinoma), A549 (lung carcinoma), MCF-7 (breast carcinoma), and Bel-7402 (liver carcinoma) human tumor cells were passaged in vivo for at least two generations before inoculation. Tumor cells as a tumor fragment (about 1 $mm^3$) were transplanted subcutaneously into the flank of a mouse with a trocar needle. Male mice were used for inoculation with HCT-8, A549 and Bel-7402 cells, and female mice for MCF-7 cells. Treatment (8–10 mice in each experimental group) with the drugs was started 3 days after inoculation. Chidamide and MS-275, both dissolved with 0.2% carboxymethyl cellulose (CMC) and 0.1% Tween 80, were administered orally once daily for 20–28 days, depending on the tumor type. 5-fluorouracil (5-FU) (Xudong Haipu Pharmaceuticals, Shanghai, China) was administered intraperitoneally every other day. Tumor length and width were monitored every 3 days, and tumor volume was calculated. At the end of the experiment, mice were killed, and tumor burden from each animal was assessed.

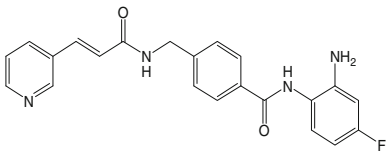
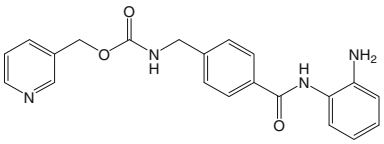
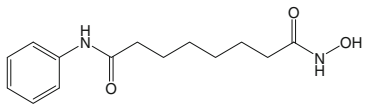
### Cellular cytotoxicity assay

Human K562 myeloid leukemia cells, classically used for the evaluation of NK cell-mediated cytotoxicity, were used as target cells. Peripheral blood mononuclear cells (PBMC) from healthy donors were obtained using lymphocyte separation medium (Amersham Pharmacia Biotech) according to the description of the manufacturer. Isolated PBMC effector cells were seeded into 6-well plates (6 × $10^6$ cells/well) and treated with chidamide at different concentrations (0–400 nM) for different times (24–72 h). In some experiments, K562 target cells were treated with 100 nM chidamide for 24 h. Both effector cells and target cells were washed and incubated together at different effector/target ratios for 4 h at 37°C, followed by cellular cytotoxicity analysis using a standard lactate dehydrogenase release assay (CytoTox 96R Non-Radioactive Cytotoxicity Assay kit; Promega). Spontaneous enzyme release by target cells alone was <15% of the maximum release as determined with target cells lysed with 1% Triton X-100. The experimental release was corrected by subtraction of the spontaneous release by effector cells at corresponding dilutions. Percentage of lysis was calculated as follows: 100 × (corrected experimental release − spontaneous release)/(maximum release − spontaneous release). Experiments were done in duplicate, and each experiment was repeated at least 3 times.

### Flow cytometric analysis

PBMC from the same healthy donors, used for cellular cytotoxicity assays, were isolated and treated with

**Table 1** Structures and $IC_{50}$s of chidamide, entinostat, and vorinostat against recombinant human HDACs

| Compound | Structure | $IC_{50}$ (μM)[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Class I | | | | Class IIa | | | | Class IIb | | Class IV |
| | | 1 | 2 | 3 | 8 | 4 | 5 | 7 | 9 | 6 | 10 | 11 |
| Chidamide[b] (HBI-8000) |  | 0.095 | 0.160 | 0.067 | 0.733 | >30 | >30 | >30 | >30 | >30 | 0.078 | 0.432 |
| Entinostat (MS-275) |  | 0.262 | 0.306 | 0.499 | 2.700 | >30 | >30 | >30 | >30 | >30 | 0.254 | 0.649 |
| Vorinostat (SAHA) |  | 0.038 | 0.144 | 0.006 | 0.038 | >30 | >30 | >30 | >30 | 0.010 | 0.021 | 0.028 |

[a] $IC_{50}$ values were determined based on the results from 9 concentrations (range at 0.001–30 μM) tested in duplicate for chidamide or MS-275 against HDAC1, 2, 3, 6, 8, 10, and 11. For HDAC4, 5, 7, and 9, the compound concentrations at 3, 10, and 30 μM were tested in duplicate. $IC_{50}$ >30 μM means there was no significant inhibition up to the concentration tested

[b] The results are expressed as mean from three independent experiments

chidamide at 100 nM for 48 h. For flow cytometric analysis, untreated and chidamide-treated PBMC were incubated with monoclonal antibody-PE conjugates (obtained from R&D Systems Inc., Minneapolis, MN, USA) against CD16, NKG2D, GZMA (Granzyme A), or the respective isotype controls. Cells were then analyzed on a FACScan (Becton–Dickinson, Mountain View, CA, USA). Specific fluorescence indices (SFI) of staining were calculated by dividing median fluorescence values obtained with the specific mAb by median fluorescence values of the corresponding isotype control.

### Gene expression studies

Microarray gene expression studies were performed with peripheral white blood cells (WBC) from two patients with T-cell lymphoma before (0 h) and 6, 24, 48, and 72 h after administration of the first dose of chidamide. Total RNA was extracted and purified with RNeasy minikit (Qiagen). One-channel hybridization was performed with Cy3-labeled aRNA samples on microarrays produced with Operon Human Genome Array-Ready Oligo Set, V.4.0. Microarray processing was carried out as described in the supplementary *Online Resource*. Microarray signals (~35,000 probes) were normalized and compared with LOWESS regression.

### Statistical analysis

Data are expressed as mean ± SE. The significance of differences was analyzed by using a Student's *t* test. Results were considered statistically significant when $P < 0.05$.

## Results

### Profile of HDAC enzyme inhibition and induction of histone acetylation

Selectivity and potency of chidamide in inhibition of HDAC isotypes were analyzed by using a panel of human recombinant HDAC proteins and compared with that of entinostat (MS-275/SNDX-275) and vorinostat (SAHA), representatives of the benzamide and hydroxamic acid classes of HDACi, respectively [14, 15]. As shown in Table 1, chidamide inhibited class I HDACs 1–3, as well as class IIb HDAC10, at low nanomolar concentrations. Similar enzyme inhibition profiles for chidamide and MS-275 were observed, but chidamide was more potent than MS-275. Unlike SAHA, neither chidamide nor MS-275 inhibited HDAC6, and they both exhibited weaker activity against HDACs 8 and 11 (Table 1). Chidamide significantly induced histone H3 acetylation in both HeLa human

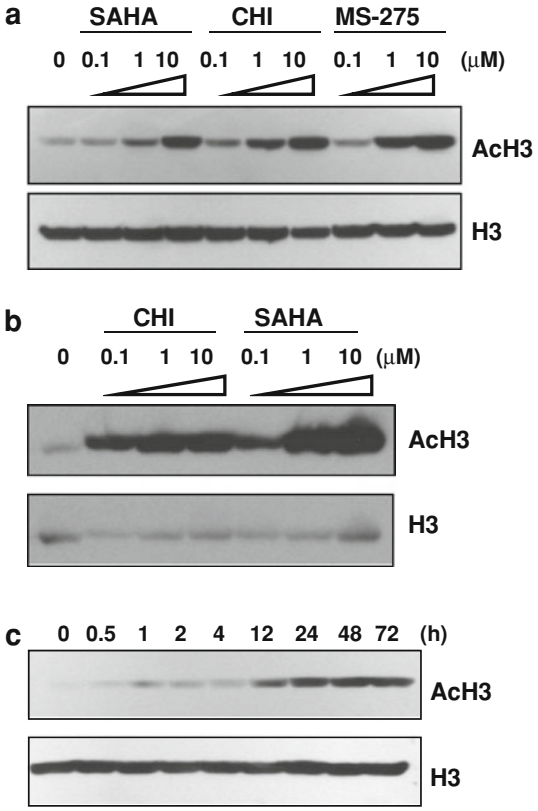



**Fig. 1** Induction of histone acetylation in cancer cells and human PBMC by chidamide. **a** HeLa cells were incubated with chidamide (CHI), SAHA, or MS-275 for 24 h at the concentrations indicated. **b** Healthy human PBMC were treated ex vivo with CHI or SAHA for 24 h at the concentrations indicated. **c** HeLa cells were incubated with CHI at 1 μM concentration for the times indicated. Histone was isolated from whole-cell lysates, and H3 acetylation status was analyzed by western immunoblot using anti-acetylated H3 antibody and re-probed with anti-H3 antibody

cervical adenocarcinoma cells and human PBMC and the extent of induction was similar to that seen with MS-275 and SAHA (Fig. 1a, b). When HeLa cells were incubated with chidamide at 1 μM concentration, H3 acetylation increased as early as 30–60 min after treatment and reached maximal levels at 24–72 h after treatment (Fig. 1c).

## Broad spectrum antitumor activity in vitro and in vivo

Cell growth inhibition studies performed with 18 human-derived tumor cell lines demonstrated that chidamide and MS-275 similarly inhibited the in vitro growth of most, but not all, tumor cells in the low micromolar concentration range (Table 2). However, chidamide, and to a lesser extent MS-275, was significantly less toxic to normal cells from human fetal kidney (CCC-HEK) and liver (CCC-HEL), indicating a differential cytotoxic response of normal cells versus cancerous cells to chidamide.

**Table 2** $GI_{50}$s of chidamide and MS-275 against transformed and normal human cultured cells

| Cell line | Cell type | $GI_{50}$ (μM) | |
|---|---|---|---|
| | | Chidamide | MS-275 |
| Transformed cell lines | | | |
| A549 | Lung | 8.2 ± 2.9 | 10.8 ± 3.4 |
| DU-145 | Prostrate | 25 ± 6.7 | 13 ± 4.3 |
| LNCaP | Prostrate | 4.0 ± 1.2 | 2.5 ± 0.8 |
| MCF-7 | Breast | 5.0 ± 1.3 | 6.3 ± 1.7 |
| MB-231 | Breast | 7.9 ± 2.1 | 5.0 ± 2.0 |
| SK-OV-3 | Ovarian | >50 | >50 |
| HeLa | Cervical | 40 ± 8.3 | 25 ± 4.2 |
| SK-N-SH | Neuroblastoma | >50 | >50 |
| HCT-8 | Colon | 7.2 ± 1.7 | 1.2 ± 0.3 |
| SMMC | Liver | 16 ± 3.2 | 20 ± 2.8 |
| HepG2 | Liver | 4.0 ± 1.5 | 3.2 ± 1.1 |
| PANC-1 | Pancreas | 6.3 ± 2.1 | 5.0 ± 1.7 |
| SGC-7901 | Gastric | >50 | >50 |
| U2OS | Osteosarcoma | 2.0 ± 0.6 | 1.0 ± 0.3 |
| Raji | B-cell lymphoma | 4.0 ± 0.9 | 6.3 ± 1.4 |
| HL-60 | Myelogenous leukemia | 0.4 ± 0.1 | 0.32 ± 0.1 |
| 28SC | Myelomonocytic leukemia | 5.8 ± 1.2 | 4.0 ± 0.8 |
| Jurkat | T-cell lymphoma | 6.3 ± 0.9 | 6.3 ± 1.1 |
| Normal cells | | | |
| CCC-HEK | Human fetal kidney cells | >100 | 30 ± 6.9 |
| CCC-HEL | Human liver cells | >100 | 40 ± 5.7 |

The results are expressed as mean ± SE from at least three independent experiments

The in vivo antitumor activity of chidamide was evaluated by using athymic nude mice subcutaneously inoculated with human tumor cells, starting with HCT-8 colorectal carcinoma. As shown in Fig. 2a and b, chidamide in the dose range of 12.5–50 mg/kg dose-dependently reduced tumor size and tumor weight, and the dose of 50 mg/kg produced similar or greater efficacy compared with the control drugs 5-fluorouracil (5-FU, 20 mg/kg) and MS-275 (25 mg/kg, which was reported as the maximum tolerated dose in xenograft models [15]). However, chidamide was well-tolerated at the above doses in the tumor-bearing animals, whereas the control drugs caused significant weight loss (Fig. 2b). Similar significant and dose-dependent effects of chidamide were also observed with nude mice inoculated with other tumor cells, including A549 lung carcinoma, BEL-7402 liver carcinoma, and MCF-7 breast carcinoma (Fig. 2c). No gross body weight loss was observed during or at the end of experiments in animals treated with the indicated doses of chidamide (data not shown).

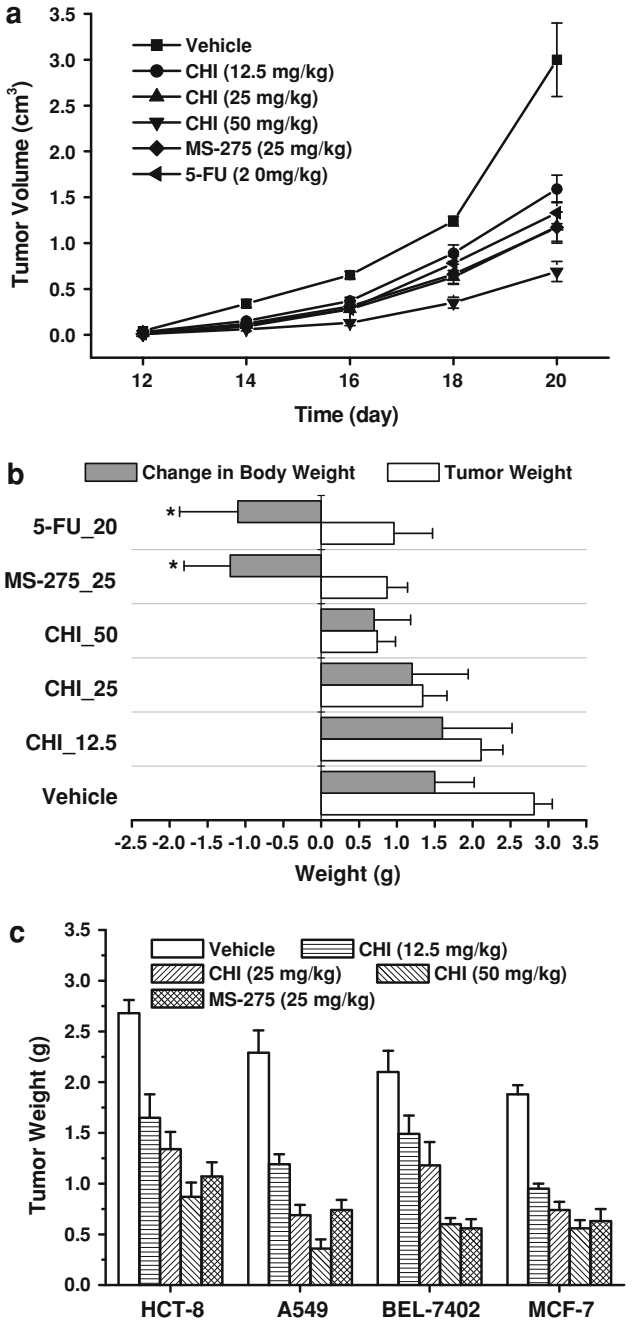



**Fig. 2** In vivo antitumor effect of CHI against human tumor xenografts. **a** Nude mice implanted with HCT-8 colorectal carcinoma cells were treated with CHI, MS-275, or 5-FU as described in "Materials and methods", and tumor volumes were measured on the indicated days. **b** At the end of the experiment, body weight from each animal was determined before killing, followed by tumor weight evaluation. Change in body weight refers to the difference in body weight between day 21 and 0. *$P < 0.05$ between the group treated with MS-275, or 5-FU and the group treated with CHI at a dose of 50 mg/kg. **c** Nude mice implanted with HCT-8 colorectal carcinoma, A549 lung carcinoma, BEL-7402 liver carcinoma, and MCF-7 breast carcinoma cells were treated with CHI or MS-275 as described in "Materials and methods", and tumor burden was determined for each animal at the end of experiments after killing. Eight–ten animals were included in each group

### Stimulation of immune cell-mediated cytotoxicity with enhanced expression of associated proteins and genes

Potential immune system-mediated antitumor effects of chidamide were evaluated using PBMC from healthy donors. The PBMC were incubated with chidamide and then co-incubated with K562 human myeloid leukemia cells in order to determine the potential for immune cell-mediated tumor cell lysis. Figure 3a demonstrates that treatment of healthy donor PBMC with chidamide significantly enhanced lysis of K562 cells, classically used as target cells for the evaluation of NK cell-mediated cytotoxicity. Maximal target cell lysis activity was observed with 100 nM chidamide (Fig. 3b), and 48 h was the optimal incubation time with this concentration (Fig. 3c). Chidamide did not produce any cytotoxic effect on PBMC at up to 72 h treatment with 200 nM concentration compared with solvent-treated cells (data not shown). Incubation of target (K562) cells alone with chidamide did not enhance lysis in the presence of untreated PBMC, but lysis was significantly enhanced when both target and effector cells were treated with chidamide, compared with effector cells treated alone (Fig. 3d). The expression levels of a panel of proteins known to be involved in immune cell-mediated cell lysis were analyzed by flow cytometry. As shown in Fig. 4a, significant upregulation of proteins which function as the NK-activating receptors, CD16 and NKG2D, and the cytotoxic enzyme, GZMA, were observed in PBMC from two different donors treated ex vivo with 100 nM chidamide for 48 h.

Microarray gene expression studies were performed on peripheral WBC from two patients with T-cell lymphoma before (0 h) and 6, 24, 48, and 72 h after administration of the first dose of chidamide. These two patients exhibited responses to chidamide in completed phase I and ongoing phase II clinical trials. As shown in Fig. 4b, the expression of genes involved in immune cell-mediated antitumor functions was significantly upregulated by the first dose of chidamide, including NK-activating surface receptors (e.g., CD16, NKG2D, and KLRG1), cytotoxic enzymes (e.g. GZMH, GZMA and Perforin) and an important apoptosis mediator, FASLG.

## Discussion

Chidamide (CS055/HBI-8000) is a new member of the benzamide class of HDACi, discovered using a computer-aided rational drug design approach. In order to design compounds with the potential to block the catalytic pocket of Class I HDACs, the crystal structure of archaebacterial histone deacetylase analog histone deacetylase-like protein

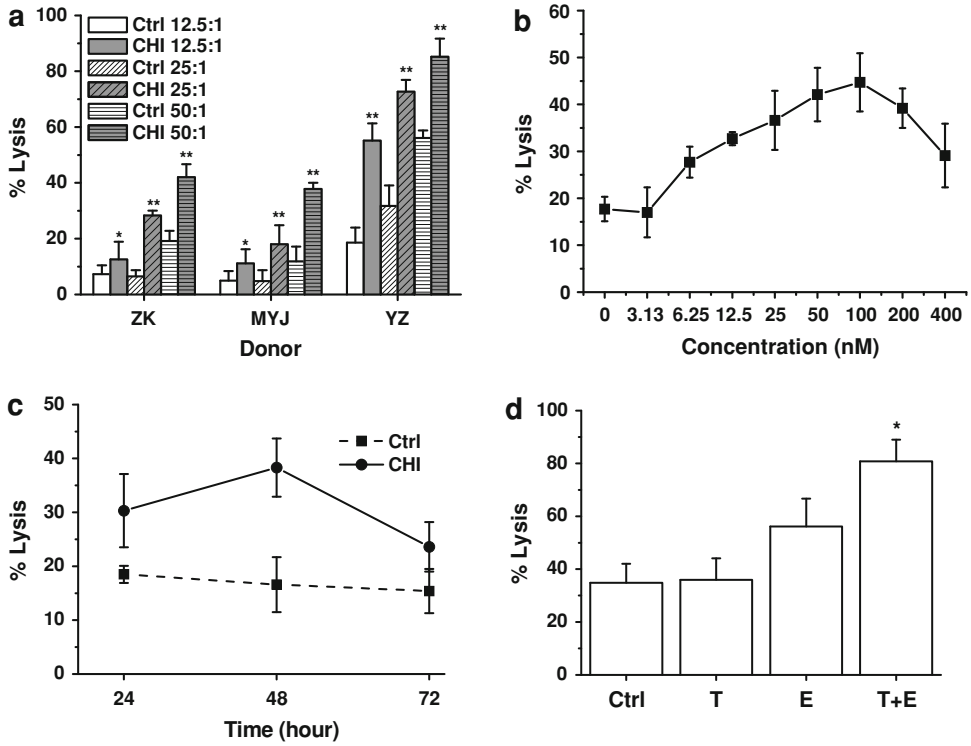



**Fig. 3** CHI enhances immune cell-mediated lysis of K562 human myeloid leukemia cells. **a** PBMC isolated from healthy donors were treated with 100 nM CHI for 48 h, and then co-incubated with K562 cells at different ratios, followed by cellular cytotoxicity analysis. $*P < 0.05$ and $**P < 0.01$ compared with the effector cells without CHI treatment, respectively. **b** PBMC were treated with different concentrations of CHI as indicated for 48 h, followed by cellular cytotoxicity analysis using *E/T* ratio of 25:1. **c** PBMC were treated with 100 nM CHI for the indicated times, followed by cellular cytotoxicity analysis using *E/T* ratio of 25:1. **d** Cellular cytotoxicity analysis using *E/T* ratio of 25:1 in the absence or presence of 100 nM CHI treatment for 48 h: Ctrl, no CHI treatment for both PBMC and K562 cells; T, K562 cells treated with CHI only; E, PBMCs treated with CHI only; T + E, both PBMC and K562 cells treated with CHI. $*P < 0.05$ compared with the effector cells treated only. The results are expressed as mean ± SE from three independent experiments

(HDLP, 1C3R) was employed for molecular docking and quantitative structure–activity relationship (QSAR) studies with hydroxamate and benzamide HDACi. QSAR studies with 124 HDACi identified key structural components for $Zn^{++}$-independent inhibition by benzamides and led to the discovery of chidamide [12–14].

Class I HDACs 1–3 have been well documented to be associated with the malignant phenotype [3–5]. Our side-by-side HDAC enzyme inhibition profiling of chidamide, MS-275 and SAHA demonstrate that chidamide is a low nanomolar inhibitor of HDAC1–3. Chidamide is more potent than MS-275 against these enzymes and also compares favorably to the published data for another HDACi of the benzamide class, MGCD0103 [16, 17]. We were surprised to see low nanomolar inhibition of HDAC10 by chidamide and to a lesser extent by MS-275, as inhibition of this HDAC by benzamides has not been reported previously. This observation may be relevant to the antitumor potential of chidamide, as HDAC10 promoter polymorphisms and increased HDAC10 activity have been reported to be associated with certain carcinomas [18]. Consistent with its HDAC inhibitory activity, chidamide significantly enhances histone acetylation in both tumor-derived cells and human PBMC. Given the known importance of histone acetylation for regulating transcription, gene expression changes resulted from HDAC inhibition by HDACi play a key role in HDACi-mediated antitumor effects [19]. However, apart from HDACs, the accumulating evidence has shown that HDACi may also have actions on other targets, such as those involved in DNA replication and repair [20] and many non-histone proteins [21], which make contributions to HDACi-mediated antitumor effects [19].

Cell growth inhibition studies performed with various human tumor-derived cell lines demonstrate that chidamide inhibits the in vitro growth of most tumor cells in the low micromolar concentration range. Similar results have been reported for most HDACi, regardless of chemical class [22, 23]. Our results also demonstrate that chidamide is significantly less toxic to normal cells, compared with tumor cells (Table 2). Consistent with its broad spectrum antitumor activity and significant selectivity for malignant versus

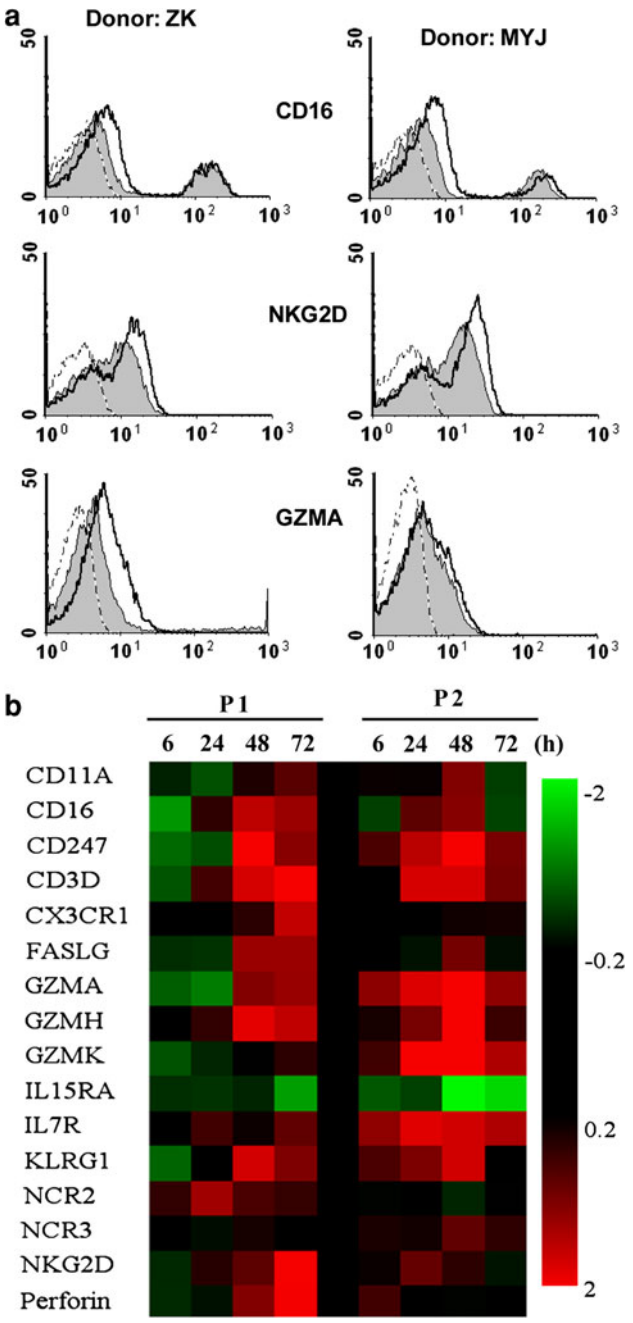



**Fig. 4** CHI enhances the expression of proteins and genes in NK functions. **a** Isolated PBMC from two different healthy donors were treated with 100 nM CHI for 48 h, and the expression of the indicated proteins before and after treatment was analyzed by flow cytometry. *Shaded histograms*, staining of untreated cells; open histograms in *solid line*, staining of treated cells; *open histograms* in *dot*, staining of treated cells with isotype control. **b** Gene expression analysis in peripheral WBC from two lymphoma patients by microarray gene-profiling studies. The numbers at *right* represent the fold changes of expression of the indicated genes at various times after the first dose of CHI at 32.5 mg (P1) and 30 mg (P2) over the expression of the genes before dosing. Procedures are referred to the supplementary *Online Resource*

normal cells, chidamide demonstrated dose-dependent antitumor activity in vivo in a panel of mouse (data not shown) and human xenograft models, in the absence of apparent toxic effects.

Several potential antitumor mechanisms of HDACi have been established, including inhibition of cell proliferation, induction of apoptosis and cellular differentiation, and suppression of angiogenesis [19]. In addition, HDACi have also been shown to sensitize tumor cells to NK cell killing, for example, by inducing the expression of MHC class I-related proteins [24] and NKG2D ligand [25] on tumor cells. However, the ability of HDACi to directly affect the antitumor functions of immune cells has not previously been demonstrated. In the current report, we provide evidence that ex vivo treatment with nanomolar concentrations of chidamide enhances immune cell-mediated cytotoxicity by human PBMC, accompanied with the increased expression of proteins involved in NK activities. Furthermore, gene expression profiling studies on peripheral WBC from T-cell lymphoma patients who responded to chidamide treatment demonstrate increased expression of a panel of genes involved in tumor immune activities. These data indicate that chidamide, and probably other HDACi as well, may produce indirect immune system-mediated antitumor effects, in addition to direct antitumor and antiangiogenic activities. If verified in the clinic, this mechanism has implications for the design of future HDACi clinical trials, particularly with respect to the development of biomarkers and the potential for activity in the minimal residual disease setting [2].

In summary, chidamide is an orally bioavailable, low nanomolar inhibitor of cancer-associated HDAC enzymes with favorable pharmacology and tolerability profiles relative to existing benzamide and non-benzamide HDACi. Immune cell-mediated antitumor activity has been observed with the compound, which has not been previously reported for other HDACi. The results presented in this study provide evidence that chidamide has potential applicability for the treatment of a variety of tumor types, either as a single agent or in combination therapies. Chidamide is currently being evaluated in Phase II studies in China, and Phase I studies in the US, for lymphomas and solid tumors.

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