CS055 (Chidamide/HBI-8000), a novel histone deacetylase inhibitor, induces G1 arrest, ROS-dependent apoptosis and differentiation in human leukaemia cells

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INTRODUCTION

Leukaemia, also known as blood cancer, is characterized by rapid cell proliferation and abnormal cell function and is a lethal disease of the bone marrow and blood. It is usually categorized into four main types, acute myeloid leukaemia, chronic myeloid leukaemia, acute lymphocytic leukaemia and chronic lymphocytic leukaemia, according to the type of white blood cell that is affected, myelogenous or lymphocytic, and the progress of disease, either rapid or slow. Although various chemotherapeutic drugs are currently used to treat leukaemia, the development of novel drugs with minimal cytotoxicity is needed. HDACis (histone deacetylase inhibitors), cytostatic agents that induce the differentiation and apoptosis of tumour cells, have emerged as a promising new class of anticancer drugs for the treatment of leukaemia [1,2]. HDACis, such as phenylbutyrate and sodium butyrate, induce leukaemia cell apoptosis and differentiation at millimolar concentrations [3]. Newer HDACis, including benzamide derivatives such as MS-275, primarily block the activity of HDAC1 and HDAC3 [4]. Other compounds, including hydroxamic acid derivatives such as the non-selective HDACi SAHA (suberoylanilide hydroxamic acid; also known as vorinostat), have been approved by the U.S. Food and Drug Administration for the treatment of cutaneous T-cell lymphoma [5]. In addition to treating leukaemia, HDACis also induce apoptosis in many other cancers, including lung cancer [6], prostate cancer [7], breast cancer [8], multiple myeloma [9] and thyroid cancer [10]. Moreover, HDACis have demonstrated a synergistic therapeutic effect with other anti-tumour agents. For example, co-treatment of AML (acute myeloblastic leukaemia) with TRAIL (tumour-necrosis-factor-related apoptosis-inducing ligand) induces apoptosis [11]. Co-treatment of human leukaemia cells with fludarabine induces apoptosis [12]. In renal cell carcinoma, co-treatment with IL-2 (interleukin 2) induced apoptosis in vivo [13]. Finally, treatment with proteasome inhibitors induced apoptosis in hepatoma cells [14].

The molecular mechanism of HDAC inhibition to induce anti-tumour activity is primarily due to the regulation of gene transcription by the alteration of chromosome structure [15]. Gene expression profiling analysis showed that SAHA, trichostatin A and MS-275 dramatically up-regulated p21, α-tubulin, TRPM-2 (testosterone-repressed prostate message 2) and other genes associated with DNA synthesis, cell cycle and apoptosis [16]. In addition, HDACi treatment down-regulated the expression of thymidylate synthetase, TRP (tRNA proline), proteasome subunits, cytokines and other transformation-related proteins in bladder cancer cells, breast carcinoma cells and osteoblasts [17,18]. In addition, HDACis regulate essential intracellular signalling pathways that control cell survival and differentiation [19]. MS-275 blocks mTOR (mammalian target of rapamycin) signalling [20], inhibits NF-κB (nuclear factor κB) activation [21], decreases the mitochondria apoptosis-related proteins XIAP (X-linked inhibitor of apoptosis) and Mcl-1 (myeloid cell leukaemia sequence 1), and increases the level of intracellular ROS (reactive oxygen species) in leukaemia cells [11]. HDACis sensitize human malignant tumour cells to TRAIL-induced apoptosis by

Abbreviations used: ALT, alanine aminotransferase; AML, acute myeloblastic leukaemia; Bid, BH3-interacting domain death agonist; Bim, Bcl-2-interacting mediator of cell death; CML, chronic myelogenous leukaemia; DMF, dimethylformamide; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; HDACi, HDAC inhibitor; Mcl-1, myeloid cell leukaemia sequence 1; MDA, malondialdehyde; MPT, mitochondrial permeability transition; NAC, N-acetyl-L-cysteine; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PUMA, p53 up-regulated modulator of apoptosis; ROS, reactive oxygen species; SAHA, suberoylanilide hydroxamic acid; TRAIL, tumour-necrosis-factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling; WBC, white blood cell.

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up-regulating the expression of DR5 (death receptor 5) [22] and regulating EGFR (epidermal growth factor receptor) [23], TGFβ/R (transforming growth factor β receptor) [24] and cAMP-dependent signalling [25]. These efforts have led to the identification of a series of agents with HDAC inhibitory activity, such as SAHA, MS-275, trichostatin A [26], LBH589 [27] and cyclic depsipeptide (FK228 or FR901228) [28]. With the exception of SAHA and FK-228, which has been used to treat T-cell lymphoma patients, most of these compounds are currently in clinical trials for the treatment of leukaemia and other solid cancers. Nevertheless, the development of novel HDACis is needed.

In the present study, we present a novel benzamide-type compound CS055 (Chidamide/HBI-8000), which is a synthetic analogue of MS-275 and is currently in clinical trials in the U.S and China. Our results indicate that CS055 is a novel HDACi. CS055 induced significant cell-cycle arrest, resulting in the inhibition of cell proliferation and the induction of apoptosis and in vitro and in vivo differentiation of leukaemia cells. Moreover, CS055 induced similar apoptosis effects in peripheral blood mononuclear cells in leukaemia patients. ROS is an essential component of anti-tumour activity. The results of the present study demonstrate the potential utility of a novel HDACi.

**EXPERIMENTAL**

**Cell lines and cell culture**

The human promyelocytic leukaemia cell line HL60, K562 CML (chronic myelogenous leukaemia) cells and Jurkat T lymphocytic leukaemia cells were purchased from CCTCC (China Center for Type Culture Collection; Wuhan, China). The cells were cultured in RPMI 1640 containing 10% FBS (fetal bovine serum). The approval for patient blood collection was obtained in accordance with the institutional guidelines. Routine blood samples were obtained from a volunteer using leucocyte reduction filters. PBMCs (peripheral blood mononuclear cells) were isolated by density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare). The cells were centrifuged for 30 min at 400 g by using netural extraction reagents (Pierce) and NAC (N-acetyl-L-cysteine) and Tiron were bought from Sigma.

**Reagents**

CS055 was supplied by Chipscreen Biosciences and was dissolved in DMF (dimethylformamide). For the in vivo experiments, CS055 was suspended in 0.1% sodium carboxyl methylcellulose and stored at 4°C. Z-VAD-FMK was purchased from R&D Systems. NAC (N-acetyl-L-cysteine) and Tiron were bought from Sigma.

**Cell-cycle analysis**

The cells were washed with PBS and then resuspended in 75% ice-cold ethanol overnight. After that, the cells were harvested and resuspended in PBS with 50 μg/ml PI (propidium iodide) and 100 μg/ml RNase A for 1 h. The DNA content was analysed by flow cytometry.

**HDAC activity assay**

HDAC activity was detected as described in the Colorimetric HDAC Activity Assay kit (BioVision). Each reaction (100 μl) contained nuclear protein (50 μg) extract from leukaemia cells (isolated by Nuclear and Cytoplasmic Extraction reagents; Pierce) and HDAC substrate. To test the effect of HDACis, CS055 and MS-275 were added to the mixtures and incubated at 37°C for 1 h. The HDAC activities were measured by a microplate reader (SpectraMax M5) at 405 nm. The positive control (only nuclear extract and vehicle) was set as 100% and double-distilled water containing 10 μM Trichostatin A, a known strong HDACi, was used as a negative control and set as 0%.

**Cell viability and apoptosis assay**

Cell viability was determined by a Trypan Blue exclusion assay, which measures membrane integrity. The cell apoptosis assay included the detection of DNA fragmentation and Annexin V-FITC/PI staining assay. DNA fragmentation was detected as described in the DNA Ladder assay kit (Beyotime Biotech). Annexin V-FITC/PI staining assay was performed according to the manufacturer’s instructions (Bipiec Biopharma). The cells were analysed on a flow cytometer (Beckman Coulter).

**Measurement of ROS, Δψm and cellular surface CD11b**

The cells were washed with PBS and resuspended in serum-free RPMI 1640 medium containing 10 μmol/l carboxy-H2DCFDA (Invitrogen), 1 μM Rhodamine 123 (for Δψm measurement; Beyotime Biotech) and then incubated at 37°C for 30 min or in PBS with FITC anti-CD11b antibody (or its isotype IgG for which the cells were pre-blocked with anti-Fc antibody for 30 min) (Biolegend) for 30 min and subjected to flow cytometry analysis.

**Western blot analysis**

Following different treatments, as described in the Figure legends, the cells were harvested, washed with PBS and then lysed in RIPA buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM sodium EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM 2-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin and 1 mM PMSF] on ice for 30 min. The cell lysates were then centrifuged at 13 000 g for 30 min at 4°C and the supernatant collected. The protein in each sample was quantified by a BCA (bicinchoninic acid) assay (Pierce). Equal amounts of protein were separated by SDS/PAGE and then electrotransferred on to a PVDF membrane (Millipore). Detection was performed using chemiluminescent HRP (horseradish peroxidase) substrate (Millipore). The antibodies used were: anti-caspase-3, anti-caspase-8, anti-caspase-9, anti-PARP [poly(ADP-ribose) polymerase], anti-Mcl-1, anti-Bcl-2, pro-apoptosis Bcl-2 Family Antibody Sampler kit (catalogue number 9942), anti-c-FLIP(L), anti-[acetyl-histone H3 (Lys9)], anti-[acetyl-histone H3 (Lys18)] and anti-[acetyl-histone H4 (Lys8)] (all from Cell Signaling Technology), total anti-(histone H3), total anti-(histone H4), anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase), anti-(cyclin E1), anti-(cyclin E2), anti-Bid (BH3-interacting domain death agonist) and anti-Bcl-xL (Proteintech).

**Detection of cytoplasmic cytochrome c**

The mitochondria was separated by Cell Mitochondria Isolation kit (Beyotime Biotech) and the cytoplasm lysate was subjected to Western blot analysis using anti-(cytochrome c) antibody (Proteintech).

**Lentiviral transduction**

The constructed transfer vector pHAGE.puro-Cyclin E1 was co-transfected into HEK (human embryonic kidney)-293T cells using...
FuGene HD (Roche), together with the other two plasmids, pMD.2G and psPAX2. At 48 h later, the supernatant was collected and the fresh medium was added and after a further 24 h the supernatant was collected again. The supernatant was filtered with 0.45-μm filters and then added to leukaemia cells at 8 μg/ml. At 24 h later, the virus-containing medium was replaced by fresh medium with 1 μg/ml puromycin. Stable clones were selected with puromycin.

**In vivo xenograft tumour study**

Male BALB/c nude mice (6-week-old) were obtained from the Disease Prevention Center of Hubei Province. All experiments were performed under protocols approved previously [28a]. Each mouse was inoculated in the right axilla with 2×10^7 HL60 cells suspended in 0.2 ml of PBS. At 0.5 week later, mice bearing tumours reaching 200 ± 100 mm^3 were randomized into four groups (n = 8) and received 0.1% sodium carboxyl methylcellulose as a vehicle and 12.5, 25 or 50 mg/kg of body mass CS055 daily by gavage for 20 days. Tumour volume and body mass were measured every day.

**Tumour tissue protein isolation, MDA (malondialdehyde) and TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling) assays**

The tumour tissue samples were homogenized and sonicated (2 s on and 3 s off for 3 min) in RIPA buffer on ice. Tissue lysates were then centrifuged at 12 000 g for 15 min at 4 °C to collect the supernatant. The tumour tissue samples subjected to MDA assay according to the manufacturer’s instructions (Beyotime Biotech). The MDA levels were detected by a Multi-Mode Microplate Reader (SpectraMax M5) at 532 nm. The TUNEL assay was performed to identify DNA strand breaks labelled with fluorescein according to the manufacturer’s instructions (Roche).

**Statistical analysis**

Student’s t test was used for statistical analysis, and the accepted level of significance was P < 0.05.

**RESULTS**

**CS055 is a novel HDACi**

CS055 is a novel synthetic benzamide-type HDACi which mainly acts as a class I HDACi (inhibiting HDACs 1, 2 and 3 at low nanomolar concentrations) [29]. CS055 has a chemical structure similar to the benzamide derivative MS-275 (Figure 1A). CS055 has one fluorine atom in its benzene ring and double carbon bond in its pyridine ring, whereas MS-275 does not have a fluorine atom and has a carbon–oxygen bond in its pyridine ring. Docking analysis was used to locate the CS055-binding sites within the class I HDAC catalytic core following the protocol described previously [29a]. The residues Arg^83, Tyr^91, Glu^92, Phe^141, Tyr^196, Phe^198, Gln^192 and Lys^267 of HDAC were identified as sites of CS055 recognition. Positive π-stacking was observed between the pyrrolyl ethylene chain of CS055 and the Phe^141 and Phe^198 within HDAC. The pyrrole N2 amino group forms a hydrogen bond with the hydroxy group of Glu^92 (Figure 1B).

To determine the activity and efficacy of CS055, we tested the ability of CS055 to inhibit HDAC activity. It was shown in vitro that CS055 exhibited almost the same HDAC inhibition capability as MS-275 (Figure 1C). To determine whether the compound was active, we further measured the acetylation of lysine residues on histones H3 and H4. Increased histone acetylation is expected to result from HDAC inhibition. As shown in Figure 1(D), Western blot analysis determined that the acetylation of histone H3 at Lys^9 (Ace-H3K9) and Lys^18 (Ace-H3K18), and H4 at Lys^8 (Ace-H4K8). GAPDH expression was determined as a loading control.
Figure 2  CS055 inhibits cell proliferation in leukaemia cells
CS055 treatment showed no effect on the levels of total histone H3 and H4 (Figure 1D). These results demonstrate that CS055 is a HDACi that results in increased acetylation of histone H3 and H4 at several lysine residues in HL60 and K562 cells.

CS055 inhibits cell proliferation and induces cell-cycle arrest in leukaemia cells

To determine the effects of CS055 on proliferation, HL60, K562 and Jurkat cells were treated with 0 (DMF control), 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 or 1 μM CS055 for 24, 48 or 72 h. CS055 at low concentrations dramatically inhibited cell proliferation in each cell line (Figure 2A). In comparison to SAHA, CS055 has stronger effects on inhibiting cell proliferation, whereas this compound was weaker than MS-275 (Supplementary Figure S1 at http://www.BiochemJ.org/bj/443/bj4430735add.htm). To further investigate the molecular mechanism of CS055, cell-cycle analysis was performed. After CS055 treatment, cells arrested at the G0/G1 phase in a dose-dependent manner (Figure 2B).

Western blot analysis indicated that cyclin E1 and E2 protein expression was down-regulated after CS055 treatment, which is consistent with the cell-cycle analysis (Figure 2C). As the changes in cyclin E1 were much more significant than cyclin E2, cyclin E1 was up-regulated in HL60 and K562 cells by lentiviral transduction (Figure 2D). The effect on leukaemia proliferation by CS055 inhibition were largely weakened when cyclin E1 was overexpressed. It is therefore likely that cyclin E1 levels were decreased by CS055 which induces cell-cycle arrest at the G0/G1 phase.

CS055 induces caspase-dependent apoptosis in leukaemia cells

In general, anti-cancer drugs cause apoptotic cell death at elevated concentrations and thus we investigated whether CS055 causes apoptosis in leukaemia cells. As shown in Figure 3(A), inhibition of cell viability was observed in HL60, K562 and Jurkat cell lines in a concentration- and time-dependent manner after treatment with CS055. Moreover, the efficacy of drug-induced cell death was greater for CS055 than SAHA, but weaker than MS-275 in HL60 cells (Supplementary Figure S2 at http://www.BiochemJ.org/bj/443/bj4430735add.htm). To confirm the effects of CS055 on cell viability, apoptosis was analysed by Annexin V-FITC/PI double staining and a DNA ladder assay. Annexin V-positive cells were observed after CS055 treatment, indicating that these cells were apoptotic (Figure 3B). Similarly, DNA fragmentation was observed after CS055 treatment in HL60 cells (Figure 3C). These results demonstrate that CS055 induced apoptosis in a dose-dependent manner.

Two apoptotic pathways exist, the extrinsic and intrinsic, and caspase activation is essential for both pathways withBid proteins on the mitochondrial membrane being crucial regulators of these pathways [30]. To understand the mechanism of CS055-dependent apoptosis in leukaemia cells, we determined the effects of CS055 on the activation of caspases and Bid proteins as well as PARP cleavage, a marker of apoptosis. Western blot analysis demonstrated that PARP and pro-caspase 3, 8 and 9 were cleaved following CS055 treatment in HL60 and K562 cells. In addition, Bid, a protein involved in intrinsic and extrinsic apoptotic signalling [31], was cleaved (Figure 3D). Thus we concluded that both the intrinsic and extrinsic apoptotic pathways were activated after CS055 treatment. To determine whether caspases are necessary for CS055-induced apoptosis, we examined apoptotic cell death in presence of Z-VAD-FMK, a pan-caspase inhibitor. The results showed that Z-VAD-FMK significantly blocked CS055-induced cell death in HL60 and K562 cells, even at CS055 concentrations as high as 4 μM (Figure 3E). These results suggest that CS055 induces caspase-dependent intrinsic and extrinsic apoptotic cell death in leukaemia cells.

Bid 2 family proteins regulate apoptosis by effecting mitochondrial outer membrane permeabilization [32]. Anti-apoptotic Bid 2 proteins prevent cell death induced by various apoptotic stimuli by inhibiting mitochondrial cytochrome c release, preventing the activation of casapse 9 and 3 [33]. Decreased expression of these proteins often facilitates apoptosis [34, 35]. To investigate whether mitochondria are involved in CS055-induced apoptosis of leukaemia, we determined the expression of the Bid 2 family proteins. Results shown that pro-survival Bid 2 family proteins Mcl-1, Bcl-2 and Bcl-xl was decreased in a dose-dependent manner after treatment with CS055 in HL60 and K562 cells (Figure 3F). In contrast, the cytoplasmic cytochrome c gradually increased (Figure 3G).

CS055-induced cell death is dependent on intracellular ROS

A previous report has demonstrated that intracellular ROS generation is crucial for chemotherapeutic agent-induced apoptosis in various cancer cells [36]. Therefore we determined whether CS055 treatment could increase the level of ROS in leukaemia cells. Using H2DCFDA-based detection and flow cytometry, ROS accumulation was observed in both HL60 and K562 cells after treatment with CS055 (Figure 4A). In addition, co-treatment of HL60 and K562 cells with the free radical scavenger NAC (15 mM) or Tiron (15 mM) [37, 38] markedly abrogated CS055-induced ROS generation (Figure 4B and Supplementary Figure S3 at http://www.BiochemJ.org/bj/443/bj4430735add.htm).

To determine whether ROS generation is involved in CS055-induced apoptosis, HL60 and K562 cell viability was assessed after treatment with 3 μM CS055 in the presence or absence of NAC or Tiron. The results showed that NAC and Tiron completely eliminated CS055-induced cytotoxicity (Figure 4C and Supplementary Figure S4 at http://www.BiochemJ.org/bj/443/bj4430735add.htm). Western blot analysis showed that PARP, pro-caspace 8 and pro-caspace 9 cleavage was induced by CS055 and was inhibited by NAC or Tiron (Figure 4D and Supplementary Figure S5 at http://www.BiochemJ.org/bj/443/bj4430735add.htm). In addition, Annexin V/PI analysis indicated that treatment with free radical scavengers blocked CS055-induced apoptosis in leukaemia cells, consistent with the cell viability results (Figure 4E and Supplementary Figure S6 at http://www.BiochemJ.org/bj/443/bj4430735add.htm). Thus these results suggest that CS055 induces apoptotic cell death in leukaemia cells by promoting intercellular ROS generation.

(A) Time- and dose-dependent effects of CS055 on cell proliferation in HL60, K562 and Jurkat cells. Results are means ± S.D. (B) Cell-cycle analysis. HL60 and K562 cells were treated with different concentrations of CS055 for 72 h. Cells were washed with PBS and then resuspended in 75% ice-cold ethanol overnight. The cells were then harvested and resuspended in PBS with 50 μg/ml PI and 100 μg/ml RNase A for 1 h. The DNA content was analysed by flow cytometry. (C) Western blot analysis of cell-cycle proteins cyclin E1, cyclin E2 and CDK2 (cyclin-dependent kinase 2). Cells were treated with different dose of CS055 for 72 h. (D) After cyclin E1 was up-regulated, proliferation inhibition effects of CS055 (0.8μM) were measured after 72 h by cell counting. Results are means ± S.D. **P < 0.01.
We detected the histone H3 acetylated at Lys9 or Lys18 in the presence of NAC or Tiron when cells were treated with CS055. It was found that increases in histone H3 acetylated at Lys9 or Lys18 had no significant changes in the presence of NAC and Tiron, which implied that the HDAC activity was still inhibited by CS055 when ROS was blocked (Figure 4D and Supplementary Figure S5). Therefore the anti-tumour mechanism of CS055 is related to ROS production, but not drug binding.

It was reported that induction of MPT (mitochondrial permeability transition) can lead to cell death and that it has an important role in apoptosis [39]. As shown in Figure 4(F), the results of the flow cytometry experiments demonstrated that CS055 mediated an MPT (which caused mitochondria to become depolarized, meaning ΔΨm was lost) in HL60 and K562 cells. Moreover, the loss of ΔΨm was dependent on the level of intracellular ROS (Figure 4G and Supplementary Figure S7 at http://www.BiochemJ.org/bj/443/bj4430735add.htm). These results indicate that CS055-induced apoptosis of leukaemia cells involves ROS-dependent mitochondrial dysfunction.

Figure 3   CS055 induces caspase-dependent apoptosis in leukaemia cells

(A) Time- and dose-dependent CS055-induced effects on cell viability. Results are means ± S.D. (B) HL60 and K562 cells were treated with CS055 for 72 h and apoptosis was determined by Annexin V-FITC/PI staining. Results are means ± S.D. (C) DNA ladder assay. HL60 cells were treated with CS055 for 72 h and then harvested, washed in PBS and lysed in digestion buffer [0.5% sarkosyl, 50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 0.5 mg/ml RNase A and 0.5 mg/ml proteinase K] at 55 °C for 3 h. The genomic DNA was then extracted and analysed by agarose gel electrophoresis. The molecular mass is given in kDa on the left-hand side. (D) Western blot analysis using antibodies specific against PARP, pro-caspase 3, 8, 9 and Bid. GAPDH was measured as a loading control. Cells were treated with CS055 for 72 h. (E) HL60 and K562 cells were treated with 4 μM CS055 for 72 h in the presence or absence of 50 μM Z-VAD-FMK, and cell viability was measured. Results are means ± S.D. **P < 0.01. (F and G) Western blot analysis for Mcl-1, Bcl-2, Bcl-xL, Bax and cytochrome c (Cyto-C). HL60 and K562 cells were treated with the indicated dose of CS055 for 72 h.
CS055 is a novel HDAC inhibitor that induces human leukaemia cell apoptosis

Figure 4 CS055-induced apoptosis is dependent on intracellular ROS

(A) HL60 and K562 cells were treated with the indicated concentration of CS055 for 72 h. Intracellular ROS accumulation was examined by DCFDA fluorescence intensity analysis. (B) ROS detection in presence of the free radical scavengers 15 mM NAC and/or 4 μM CS055. Cells were treated for 72 h. (C) Determination of cell viability after incubation with 15 mM NAC and/or 4 μM CS055 for 3 days. Results are means ± S.D. **P < 0.01. (D) Western blot analysis of PARP, caspase 8 and 9 cleavage, acetylation of histone H3 at Lys9 (Ace-H3K9) and Lys18 (Ace-H3K18), total histone H3 (total-H3) and GAPDH expression. Cells were treated with 4 μM CS055 and/or 15 mM NAC for 72 h. (E) Analysis showed free radical scavengers (15 mM NAC) blocked 4 μM CS055-induced apoptosis in leukaemia cells 72 h after treatment. Results are means ± S.D. **P < 0.01. (F) Mitochondrial membrane potential (∆Ψm) was examined by flow cytometry. Cells were treated with the indicated concentration of CS055 for 72 h. Results are means ± S.D. (G) NAC (15 mM) blocked the loss of ∆Ψm induced by 4 μM CS055 treatment for 3 days in leukaemia cells. Results are means ± S.D. **P < 0.01.

CS055 promotes leukaemia cell differentiation in association with intracellular ROS

Cell differentiation therapy, which is generally associated with cell death via apoptosis or senescence, is an alternative approach to leukaemia treatments. The most successful differentiation-inducing compound is all-trans-retinoic acid and is mainly effective against PML-RARA (promyelocytic leukaemia-retinoic acid receptor-α)-positive M3 [40]. We determined whether CS055 can induce leukaemia cell differentiation. CD11b expression is considered a hallmark of leukaemia cell differentiation [41]. Therefore, we examined CD11b expression by flow cytometry using an FITC anti-CD11b antibody. We demonstrated that CD11b accumulated on the surface of HL60 and K562 cells after treatment with low concentrations of CS055 in a dose-dependent manner (Figure 5B). Notably, K562 cells were more sensitive to CS055-induced differentiation than HL60 cells. DMF was used as the vehicle control and 1.3% (v/v) DMSO, which is an established
The effects of CS055 on primary leukaemia cells are unknown. Therefore we next determined the effects of CS055 on primary leukaemia cells obtained from one CML and two AML patients who did not previously receive chemotherapy. Samples from three normal adults were also examined. Peripheral WBCs (white blood cells) were isolated using Ficoll and resuspended in fresh RPMI 1640 medium supplemented with 10% FBS. CS055 treatment induced cell death in approximately 60% of the primary leukaemia cells at a concentration of 2 μM and nearly 90% at 4 μM. In contrast, WBCs from normal adults were only slightly affected by CS055 (Figure 6A). Moreover, PI staining (Figure 6B) showed increased sub-G1 cells, suggesting that CS055 treatment induced apoptotic cell death. This was confirmed by Western blot analysis of pro-caspase and PARP cleavage in leukaemia WBCs after a 72-h treatment with CS055 (Figure 6C). In addition, increased CD11b expression indicated that CS055 significantly induced human primary leukaemia cell differentiation at 2 μM after a 120-h treatment (Figure 6D). It is noteworthy that no-CS055-treated cells contained 32.8% differentiated cells. This was because these cells were isolated from human whole blood and not cell lines like HL60 or K562. Therefore CS055 exhibits considerable toxic effects in human primary leukaemia cells, in addition to cell lines.

CS055 induces apoptosis and suppresses HL60 cell xenograft growth in vivo.

To further assess the anti-tumour effects of CS055, we determined whether CS055 could inhibit tumour development in a xenograft tumour model. Inhibition of tumour growth by CS055 treatment was observed in a dose-dependent manner, demonstrating the anti-tumour activity of CS055 in vivo. Control tumours grew to an average volume of 14.51 cm³ after 20 days, and CS055-treated tumours grew to 11.68, 11.05 and 8.45 cm³, corresponding to 19.54%, 23.83% and 41.80% growth inhibition respectively. The average tumour mass in animals treated with vehicle was 9.4 ± 2.7 g and was 8.4 ± 2.4 g for animals treated with low-dose CS055. In animals treated with a moderate dose of CS055, tumour mass was 7.6 ± 1.6 g and those receiving high-dose CS055 had a tumour mass of 5.4 ± 1.5 g (P < 0.01) (Figures 7A and 7B). However, the average serum ALT (alanine aminotransferase) activity [42] (Figure 7E) and average body mass (results not shown) of the each group mice was not significantly different, indicating that CS055 has low toxicity in vivo. Additionally, CS055 treatment prolonged the survival of nude mice bearing HL60 xenografts (Figure 7C). Moreover, the level of lipid peroxidation product (MDA), which is a presumptive measure of ROS-mediated injury, was increased in tumour tissue accompanied by treatment of CS055 (Figure 7F), suggesting that CS055-induced ROS generation might play a role in vivo.

To determine whether the anti-tumour activity of CS055 in HL60 cell xenografts was attributed to the induction of apoptosis, a TUNEL assay was performed. As shown in Figure 7(F), the number of TUNEL-positive cells was significantly increased in the tumours from mice treated with CS055 compared with the corresponding vehicle-treated control. Therefore these results demonstrate that CS055 not only induced tumour cell apoptosis in vitro, but also inhibited tumour development in vivo.

**DISCUSSION**

Recently, HDACIs have gained considerable interest as anticancer agents due to their ability to induce differentiation and apoptosis, particularly in haematopoietic malignancies, including leukaemia, T-cell lymphoma and multiple myeloma. Although
multiple HDACis are currently in clinical trials, systemic side effects are important considerations for these therapeutic agents. Some potent HDACis, such as Trichostatin A and trapoxin B, have not been used clinically due to toxicity [43].

CS055, a novel HDACi, is currently in Phase I clinical trials in the U.S. and Phase II/III for T-cell lymphoma and several other solid tumours in China. The Phase I clinical study in China demonstrated that CS055 shows good safety and tolerability in patients with advanced lymphomas and some types of solid tumours (G. Cao, personal communication). The preliminary results also showed initial efficacy of CS055 in lymphoma patients [44]. In the present study, we provide the first demonstration of the potential capability of CS055 to treat leukaemia by inducing differentiation and apoptosis. Our in vivo, ex vivo and in vitro data determined that CS055 is a HDACi and a promising drug for the treatment of leukaemia.

As shown in Figure 1(A), CS055 is a synthetic benzamide-type HDACi that has a similar chemical structure to MS-275. CS055 has a stable structure moiety of C—C—C = O, while MS-275 at the same position shows a potentially unstable moiety of C—O—C = O, which might be responsible for the eight major metabolites reported when MS-275 was administered to rats. Therefore, compared with MS-275, CS055 displays lower toxicity, better tolerance and more stability during administration to animals. Moreover, a relatively long T1/2 of 18 h, rapid biomarker response in 24–48 h, lack of dose-limiting fatigue, body mass suppression or QTc prolongation (G. Cao, personal communication) give CS055 more competitive advantages than other HDACis in single agent and combination approaches.

CS055-induced growth arrest, apoptosis and differentiation of leukaemia cells appeared to be dose-dependent. CS055 treatment induced 60–75% inhibition of leukaemia cell proliferation at 1 μM and 90% cell differentiation at 2 μM. Furthermore, a relatively higher dose of CS055 (4 μM) evoked intracellular ROS generation followed by the loss of mitochondrial membrane potential and cytosolic release of cytochrome c, resulting in activation of the caspase cascade and apoptosis. CS055 showed some functions on cell cycle and differentiation at concentrations below 2 μM. However, no significant effects on HDAC activity were observed at these low concentrations. A probable interpretation is that many anti-tumour drugs have multi-functions and multi-targets. CS055 might have some other pathways leading to cell-cycle arrest like decreasing cyclin E expression level (Figure 2D) and differentiation as well at concentrations lower than 2 μM. The second reason was that CS055 only inhibited some parts of HDAC activity at low concentration [29], and it would inhibit more HDAC activity at high concentration. So CS055 showed a weak activity to the total HDAC below 2 μM.

In previous studies, it was found that MS-275 exerted similar dose-dependent effects on leukaemia cells. MS-275-mediated growth arrest and increased expression of differentiation markers (CD11b) in U937 cells were accompanied by down-regulation of cyclin D1 [11]. In the present study, expression levels of cyclin D1, D2 and D3 have not shown any significant alterations after CS055 treatment; however, cyclin E1 and E2 expression were largely blocked. Although the G1/S-phase transition is regulated by both cyclin D and E, these findings showed G1 arrest induced by HDACis had a much more complicated regulation mechanism than previous conjecture.

Bcl-2 family proteins which regulate apoptosis include two groups: anti-apoptotic and pro-apoptotic [45]. In the present study, we have found that anti-apoptotic Bcl-2 family proteins (Mcl-1, Bcl-2 and Bcl-xL) levels were decreased by CS055 treatment to facilitate apoptosis (Figure 3F). However, the levels

Figure 6  Ex vivo CS055-induced differentiation and apoptotic cell death in peripheral WBCs from leukaemia patients

(A) CS055 caused cell death of the peripheral WBCs from leukaemia patients, but not from healthy volunteers. Peripheral WBCs of three leukaemia patients and three normal adults were treated with CS055 at the indicated dose for 3 days and viability was determined. (B) Leukaemic peripheral WBC death by apoptosis. The apoptotic sub-G1 cells were determined by PI staining, followed by flow cytometric analysis. (C) Cell lysates from WBCs treated with different concentrations of CS055 for 3 days were subjected to Western blot analysis of caspase and PARP cleavage. (D) Ex vivo induction of CD11b expression. CS055 (0, 1, 2 and 4 μM) was added to patient WBCs. After CS055 treatment, CD11b expression was determined by FACS analysis using FITC anti-human CD11b antibody as described in Figure 5.
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Figure 7 CS055 suppresses the growth of HL60 xenografts and induces apoptosis in vivo

HL60 cell tumour xenografts were established in Balb/c nude mice. Animals were randomly divided into four groups (n = 8) and were injected daily with CS055 at 12.5, 25 and 50 mg/kg body mass per day i.g. for 20 days. Control animals received vehicle consisting of 0.1 % sodium carboxyl methylcellulose in sterile water. Animal mass and tumour volume was measured daily. (A) Tumour volume after treatment of CS055. (B) Tumour weight after 20 days of treatment. (C) Survival rate of nude mice with xenografts. (D) MDA level of tumour tissue proteins exacted from HL60 cell xenografts. (E) Average serum ALT activity of nude mice bearing HL60 cell tumour xenografts. In (A-E) results are means ± S.D. *P < 0.05. (F) Apoptosis analysis of the tumour tissue by TUNEL staining.

of two pro-apoptotic Bcl-2 family proteins Bim (Bcl-2-interacting mediator of cell death) and PUMA (p53 up-regulated modulator of apoptosis) were reduced as well after treatment with 4 μM CS055 (results not shown), which differs from studies published previously [46,47] showing that HDACis often activate Bim to induce apoptosis. We hypothesize that it is probable that CS055 decreased Bim or PUMA levels through other functions.

Interestingly, differentiation may be associated with apoptosis, and differentiation therapy may be useful in combination with intensive chemotherapy to increase the susceptibility of leukaemia to drug-induced apoptosis, although differentiation and apoptosis were independently regulated in leukaemia cells [48]. Previously, ROS was found to be essential for activation of differentiation in HL60 cells [49,50]. In the present study, we have demonstrated that ROS was not only involved in CS055-induced apoptosis, but also CS055-induced differentiation, since the ROS scavenger NAC could also inhibit CS055-induced leukaemia cell differentiation (Figure 5C). We found that ROS modulation by HDACis affected differentiation as well as that by PMA or DPI (diphenyleneiodonium) [49,50].

Even though CS055 is currently in clinical trials and has shown excellent anti-cancer effects, the more detailed mechanisms are still to be determined. The results of the present study suggest that CS055 is a HDACi with potential therapeutic value in
several haematological malignancies via the inhibition of cell proliferation, inducing differentiation and apoptosis in human leukaemia cells (Figure 8).

In summary, the present results suggest that CS055 is a new HDACi with potential therapeutic values in several haematological malignancies via the inhibition of cell proliferation, inducing differentiation and apoptosis in human leukaemia cells. Currently, CS055 is in clinical trials for the treatment of various forms of cancer, and the present study indicates that this compound should be considered for the treatment of leukaemia. The development of novel HDACis will significantly accelerate the discovery of anti-tumour agents and improve cancer therapy.

AUTHOR CONTRIBUTION

Wenhua Li and Ke Gong designed the project and wrote the paper. Ke Gong and Jia Xie designed and performed the experiments, analysed the data and interpreted the results. Hong Yi designed and performed the docking analysis, and worked on associated data interpretation. Wenhua Li obtained the funding. All authors reviewed and approved the paper prior to submission.

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SUPPLEMENTARY ONLINE DATA

CS055 (Chidamide/HBI-8000), a novel histone deacetylase inhibitor, induces G₁ arrest, ROS-dependent apoptosis and differentiation in human leukaemia cells

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Figure S1 Anti-proliferative effects of the HDACis CS055, MS-275 and SAHA

HL60 cells were treated with CS055, MS-275 and SAHA for 72 h at the indicated concentration. The cells were then counted. Results are means ± S.D.

Figure S2 HL60 cells were treated with three HDAC inhibitors, CS055, MS-275 and SAHA for 72 h

Cell viability was then determined.

Figure S3 ROS detection in HL60 and K562 cells in the presence of the free radical scavenger Tiron (15 mM)

Figure S4 Determination of HL60 and K562 cell viability after incubation for 3 days

**P < 0.01.

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Figure S5 Western blot analysis of PARP, caspase 8 and 9 cleavage, acetylation of histone H3 at Lys9 (Ace-H3K9) and Lys18 (Ace-H3K18), total histone H3 (total-H3) and GAPDH expression

Figure S6 The free radical scavenger Tiron blocked CS055-induced apoptosis in leukaemia cells

Results are means ± S.D. **P < 0.01.

Figure S7 Tiron blocked the loss of ∆Ψm in leukaemia cells

Results are means ± S.D. **P < 0.01.

Figure S8 Oxidative burst by PMA stimulation occurred in CS055-treated leukaemia cells

After treatment with 2 μM CS055 for 72 h, HL60 and K562 cells were collected by centrifugation and then resuspended and rescultured in fresh medium overnight. Leukaemia cells were incubated 0.5 μM DHR, with or without 10 nM PMA, at 37 °C for 30 min. Oxidative burst was determined by measuring the conversion of non-fluorescent DHR into fluorescent rhodamine 123. The ability to oxidize DHR was defined as the increase in cells with relative high fluorescence intensity (F.I.) after PMA stimulation.