CUDC-101 overcomes arsenic trioxide resistance via caspase-dependent promyelocytic leukemia-retinoic acid receptor alpha degradation in acute promyelocytic leukemia

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Although arsenic trioxide (ATO) treatment has transformed acute promyelocytic leukemia (APL) from the most fatal to the most curable hematological cancer, many high-risk APL patients who fail to achieve a complete molecular remission or relapse become resistant to ATO. Herein, we report that 7-(4-(3-ethynylphenylamino)-7-methoxyquinazolin-6-yloxy)-N-hydroxyheptanamide (CUDC-101) exhibits specific anticancer effects on APL and ATO-resistant APL *in vitro* and *in vivo*, while showing negligible cytotoxic effect on the noncancerous cells including normal CD34$^+$ cells and bone marrow mesenchymal stem cells from APL patients. Further mechanistic studies show that CUDC-101 triggers caspase-dependent degradation of the promyelocytic leukemia-retinoic acid receptor alpha fusion protein. As a result, APL and ATO-resistant APL cells undergo apoptosis upon CUDC-101 treatment and this apoptosis-inducing effect is even stronger than that of ATO. Finally, using a xenograft mouse model, we demonstrated that CUDC-101 significantly represses leukemia development *in vivo*. In conclusion, these results suggested that CUDC-101 can serve as a potential candidate drug for APL, particularly for ATO-resistant APL. *Anti-Cancer Drugs* XXX:000–000

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Introduction

Acute promyelocytic leukemia (APL) is the M3 subtype of acute myeloid leukemia (AML), with 98% of patients harboring the t(15;17) chromosomal translocation, which involves the fusion of the genes encoding promyelocytic leukemia (PML) and retinoic acid receptor alpha (RARα) [1–4]. All-trans retinoic acid (ATRA) and arsenic trioxide (ATO) are promyelocytic leukemia-retinoic acid receptor alpha (PML-RARα) targeting drugs that bind to the RARα and PML moieties, respectively. These two drugs have transformed APL from the most fatal to the most curable hematological cancer [2,5,6]. Despite this unprecedented success, many high-risk patients fail to achieve complete molecular remission or relapse and become resistant to ATO [7–9]. Therefore, alternative agents must be developed, particularly for relapsed APL with ATO resistance.

Histone deacetylases (HDACs) are a group of 11 proteins that remove acetyl groups from lysine residues in histone tails and in other proteins [10]. A large number of studies have suggested that HDACs are overexpressed in many cancers and inhibit specific tumor suppressor genes, resulting in an aberrant epigenetic status compared with adjacent normal cells [11,12]. Thus, HDAC inhibitors (HDACi) have been targeted as promising agents for the treatment of several cancers [13]. It has been further demonstrated that in APL, fusion proteins of the RARα recruit HDACs containing corepressor complexes, which in turn deacetylate and silence genes crucial for hematopoietic differentiation [14,15]. Accordingly, HDACi may be a promising approach to treat APL.

One of the most promising agents in this category is 7-(4-(3-ethynylphenylamino)-7-methoxyquinazolin-6-yloxy)-N-hydroxyheptanamide (CUDC-101), which is a novel pan-HDACi shown to inhibit class I and II HDACs enzymatic activity *in vitro*. In most tumor cell lines tested, CUDC-101 exhibits efficient antiproliferative activity with great potency. *In vivo*, the drug promotes tumor regression or inhibition in various cancer xenograft models including nonsmall cell lung cancer (NSCLC), liver, breast, head and neck, and pancreatic cancers [16]. CUDC-101 has undergone Phase I clinical trials for some solid tumors, such as head and neck squamous cell carcinoma [17]. Up to now, the role of CUDC-101 in hematologic neoplasms has remained largely unknown.

Bone marrow mesenchymal stem cells (BMSCs) are known to provide signals and regulatory factors essential for normal cells quiescence, localization, self-renewal,

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proliferation, and differentiation [18,19]. However, it has been reported that ATO has a cytotoxic effect on BMSCs during treatment because of its own toxicity [20]. The application of ATO can also cause various adverse effects, such as leukocytosis (32–73%), differentiation syndrome (7–35%), hepatic toxicity, and even death [1,21–23]. Therefore, it is important to develop new alternative drugs that target APL cell lines without affecting the BMSCs.

In this study, CUDC-101 was demonstrated to exert specific anticancer effects in APL and ATO-resistant APL models. Mechanistically, CUDC-101 has an effect through induction of hyperacetylation at histone 3, which subsequently leads to the activation of caspase 3, causing caspase-dependent degradation of PML-RARα. As a result, APL and ATO-resistant APL cells underwent apoptosis upon CUDC-101 treatment. Notably, this apoptosis-inducing effect of CUDC-101 was found to be stronger than that of ATO. To the best of our knowledge, this is the first report that CUDC-101 functions as HDACi and has potential therapeutic value for the treatment of both APL and relapsed APL with ATO resistance.

Materials and methods

Reagents

Novel pan-HDACi CUDC-101 and caspase inhibitor Z-VAD-FMK were provided by Selleck Chemicals (Houston, Texas, USA). ATO was provided by the Affiliated Hospital of Guizhou Medical University (Guiyang, China).

Cell lines and cell culture

Human promyelocytic leukemia cell lines NB4 and HL-60 were obtained from the central laboratory of Hematopoietic Stem Cell Transplantation Center of Guizhou Province (Guiyang, China). ATO-resistant NB4 (NB4/As) cells were obtained by culturing NB4 cells with gradually increasing concentrations of ATO [24]. These cell lines were cultured at 37°C in a 5% CO_2 humidified atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Maryland, USA), penicillin (100 units/mL), and streptomycin (100 μg/mL).

Experiments involving healthy volunteers who donated blood were conducted with prior approval of Research Ethics Board of the Affiliated Hospital of Guizhou Medical University (Guiyang, China) and informed consent was obtained from all subjects. Peripheral blood mononuclear cells from healthy donors were collected and isolated by Ficoll-Hypaquedensity gradient centrifugation. Then $CD34^+$ plasma cells from healthy donors were purified as previously described [25]. The isolated normal $CD34^+$ cells were maintained in RPMI 1640 media supplemented and maintained in the same way as the APL cell lines. Normal $CD34^+$ cells were treated within 1 h of collection with various doses of CUDC-101 or ATO for 24, 48 and 72 h; the cell viability was measured with a CCK8 Kit.

Patient samples

Bone marrow samples were collected during routine diagnostic assessment after an explanation was provided in accordance with the Declaration of Helsinki (2017–13). All participants provided written informed consent before enrolling in the study. This study was approved by the institutional review board of the Affiliated Hospital of Guizhou Medical University.

Bone marrow mesenchymal stem cells isolation and culture

BMSCs were isolated from the iliac crest bone marrow aspirate of APL patients. Bone marrow mononuclear cells were isolated with Percoll (Solarbio), Dulbecco's modified Eagle's medium (DMEM) (Euroclone) with 1000 mg/mL glucose and L-glutamine, centrifuged and plated at a density of 1000 cells/cm^2. BMSCs were then cultured in L-DMEM/F-12 (Gibco, USA) with 15% fetal bovine serum (Gibco) at 37°C with 5% CO_2. Three days later, nonadherent cells were removed carefully and the culture medium was refreshed. When primary cultures became almost confluent, the culture was treated with 0.5 mL of 0.25% trypsin containing 0.02% mmol/L ethylenediamine tetraacetic acid (Gibco) for 2 min at room temperature (25°C). A purified population of BMSCs was obtained 1 week after the initiation of culture.

Identification of Bone marrow mesenchymal stem cells derived from acute promyelocytic leukemia patients

BMSCs were obtained from the bone marrow of APL patients by plastic adhesion as previously described [26]. The cultured cells had a fibrous spindle or a triangular shape, and they were arranged in a spiral, reticular, or radial pattern, showing a high degree of homogeneity and reaching confluence in 7 days (Fig. 3a). The ability of BMSCs to differentiate into osteocytes and adipocytes was tested using alizarin red and oil red staining, respectively (Fig. 3b). Flow cytometry showed that BMSCs were positive for CD44, CD105, CD90, and negative for CD34 and CD45 (Fig. 3c). The adhesion, immunophenotype, and differentiation ability of BMSCs were characterized by referring to their identification criteria [27,28].

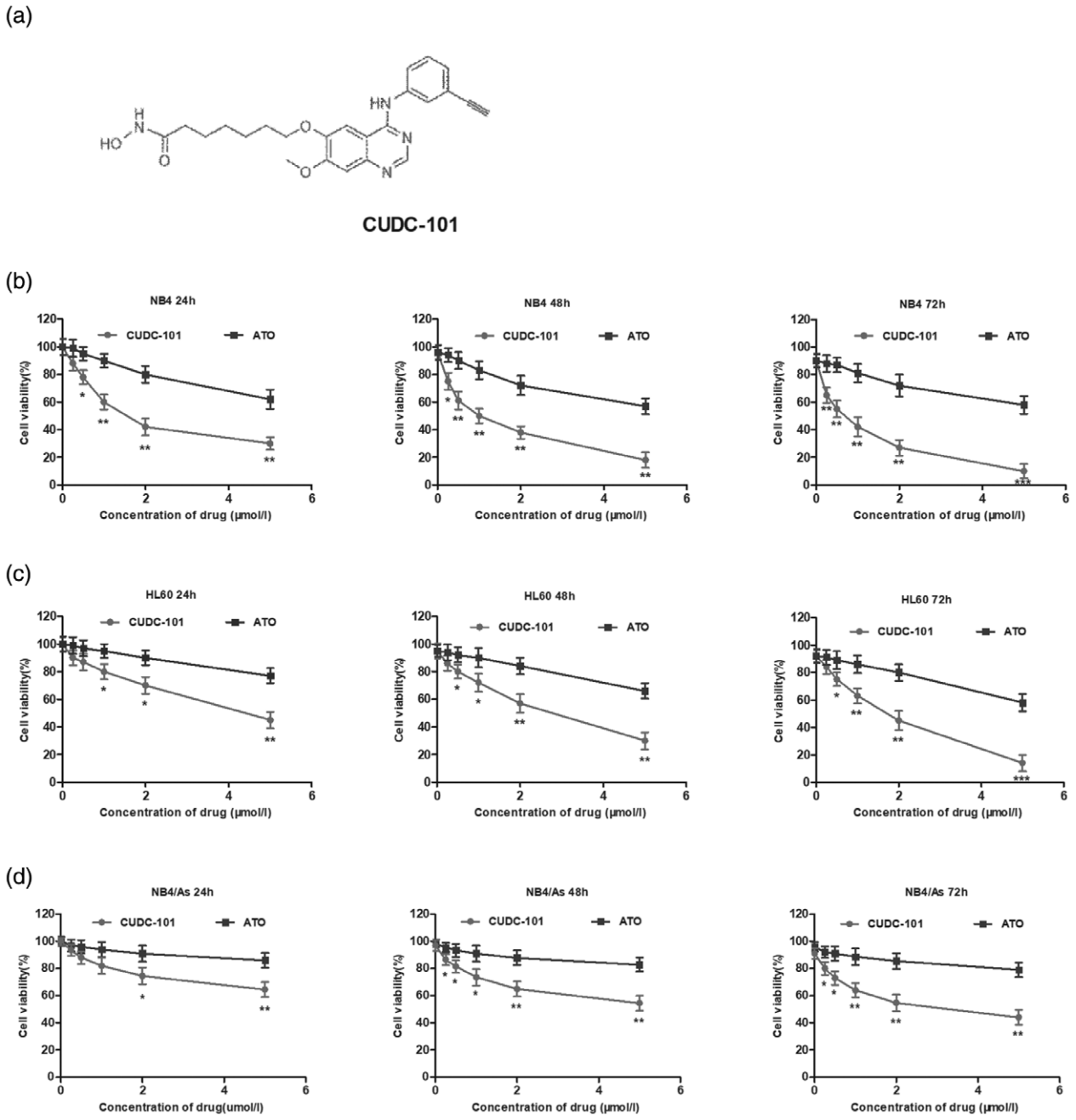
Cell viability assay

Cells were seeded at a density of 4000/well in 96-well plates. After overnight incubation, the cells were treated with different concentrations of CUDC-101 or ATO for 24, 48, and 72 h. The subsequent inhibitory effects were assessed using the Cell Counting Kit8 test (CCK-8, Dojindo, Kumamoto, Japan). The percentage inhibition was calculated and IC50 was determined using Prism 5 (GraphPad Software, USA).

Apoptosis assay

Each treated culture was washed with PBS, and stained with 5 μL of annexin V at room temperature for 15 min in dark and then with 10 μL of propidium iodide at 4°C for

Fig. 1



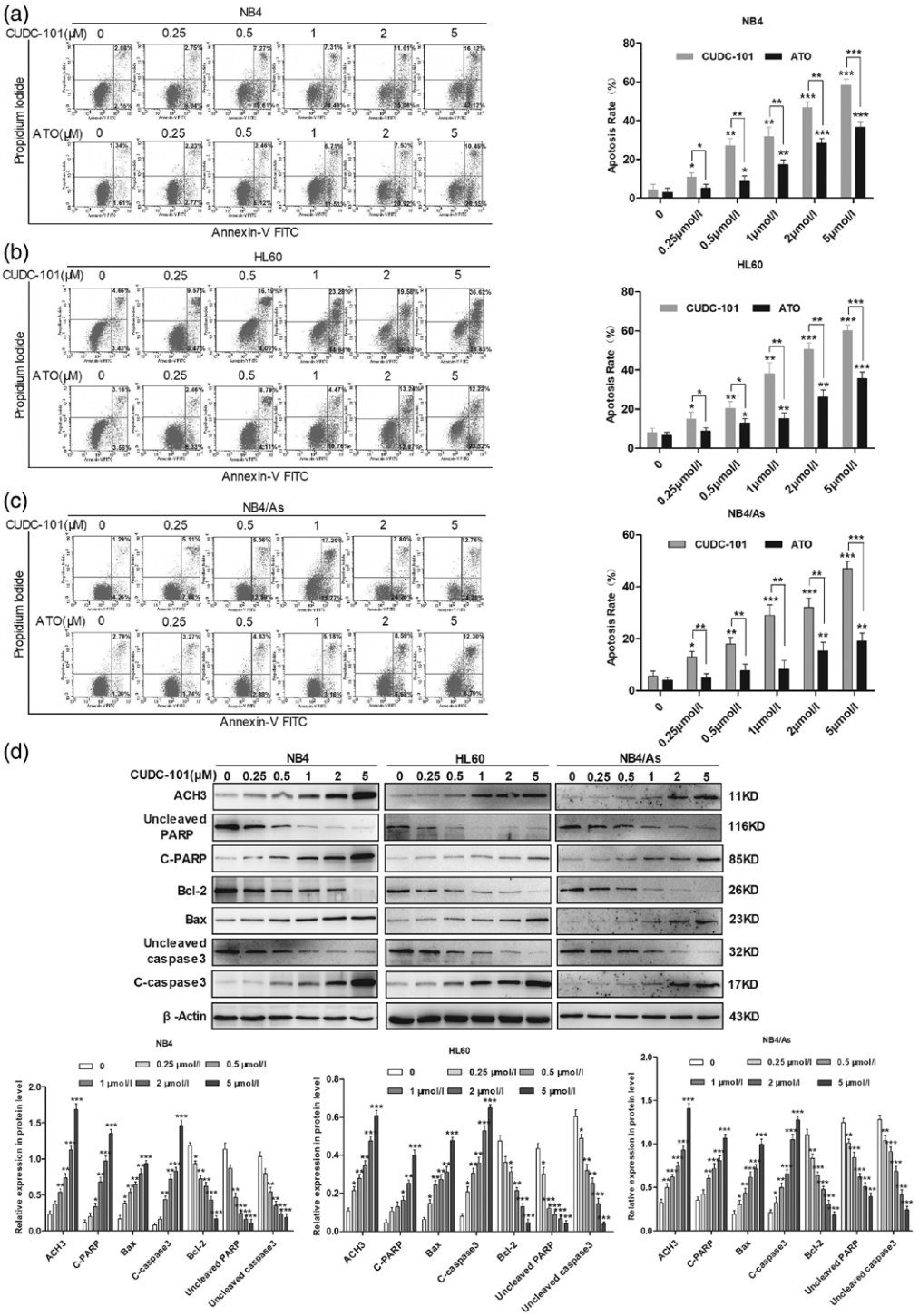
CUDC-101 exerts much stronger antiproliferative effects than ATO in APL and ATO-resistant APL cells. (a) Chemical structure of CUDC-101. (b) Effects of CUDC-101 or ATO on NB4 cell viability as detected by CCK8 assay. (c) Effects of CUDC-101 or ATO on HL60 cell viability as by CCK8 assay. (d) Effects of CUDC-101 or ATO on NB4/As cell viability as detected by CCK8 assay. Data are shown as the mean ± SD of three independent experiments; unpaired two-tailed student's test was used for statistics. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group (DMSO <0.1%). APL, acute promyelocytic leukemia; ATO, arsenic trioxide; CUDC-101, 7-(4-(3-ethynylphenylamino)-7-methoxyquinazolin-6-yloxy)-N-hydroxyheptanamide.

5 min in dark. Apoptotic cells were detected using flow cytometry (BD Biosciences, San Jose, California, USA), and data were analyzed by Cell Quest software (BD Biosciences).

Western blot analysis

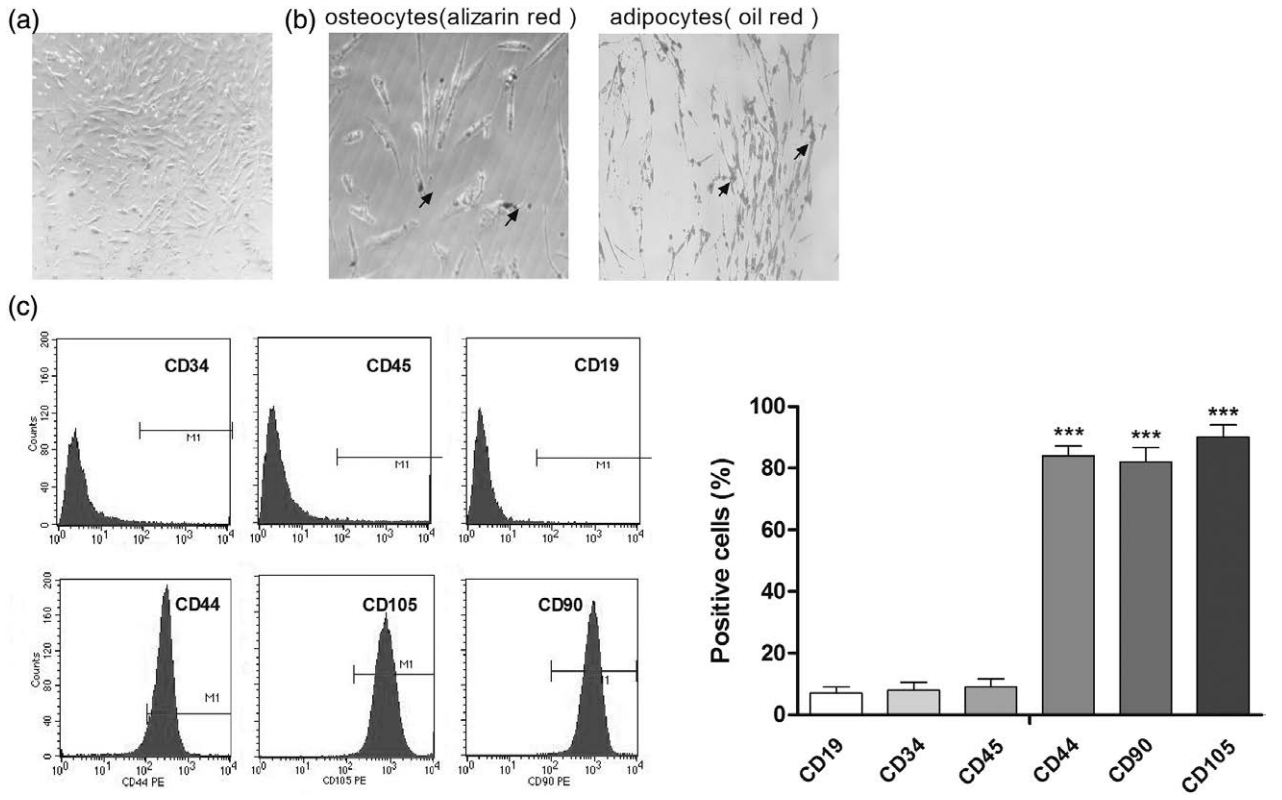
Cells were washed with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer (50 mmol/l Tris-HCl; 150 mmol/l NaCl; 0.1% SDS; 0.5% Nadeoxycholate; 1%

Fig. 2



CUDC-101 treatment induces apoptotic cell death in APL and ATO-resistant APL cells more effectively than ATO. (a) NB4 cells were treated with various concentrations of CUDC-101 or ATO for 48 h, and the apoptotic cell proportion was determined by flow cytometry. (b) HL60 cells were treated with various concentrations of CUDC-101 or ATO for 48 h, and apoptotic cells was determined by flow cytometry. (c) NB4/As cells were treated with various concentrations of CUDC-101 or ATO for 48 h, and apoptotic cells was determined by flow cytometry. (d) Apoptosis-related protein and ACH3 expression in APL and ATO-resistant APL cells were determined by western blot after 48 h of CUDC-101 treatment. An Annexin V-FITC/PI staining kit assisted in the determination of cell apoptosis. Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group. β-actin was used as a loading control in immunoblot analysis. APL, acute promyelocytic leukemia; ATO, arsenic trioxide; CUDC-101, 7-(4-(3-ethynylphenylamino)-7-methoxyquinazolin-6-yloxy)-N-hydroxyheptanamide.

Fig. 3



Characteristics of BMSCs culture and identification. (a) Morphology of BMSCs during culture. (b) Image of BMSC differentiated as osteocytes and adipocytes with alizarin red and oil red staining, respectively. (c) Surface markers of BMSCs showed that the positive rates of CD44, CD105, and CD90 were of a high proportion, whereas the expressions of CD34, CD45, and CD19 were lower than 10%. BMSCs, bone marrow mesenchymal stem cells.

NP40) with a proteinase inhibitor cocktail and phosphatase inhibitor cocktail (Roche Applied Science, Indianapolis, Indiana, USA). Cell lysates were cleared after centrifugation at 12 000 rpm at 4°C for 10 min and the supernatants were collected. Equivalent amounts of proteins (50 μg) from each lysate were resolved in 10% sodium dodecyl SDS-PAGE. Protein was transferred onto polyvinylidine difluoride membranes (EMD Millipore, Bedford, Massachusetts, USA). Membranes were blocked using 5% skimmed dry milk in TBS containing 0.2% Tween-20 at ambient temperature for 2 h and incubated them overnight at 4°C with primary antibody specific for the following proteins as appropriate at the indicated dilutions: Uncleaved caspases 3 (Santa Cruz Biotechnology, Santa Cruz, California), C-caspases 3 (Santa Cruz Biotechnology, Santa Cruz, California), Uncleaved poly(ADP-ribose)polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, California), C-PARP (Santa Cruz Biotechnology, Santa Cruz, California), Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, California), Bax (Santa Cruz Biotechnology, Santa Cruz, California), β-actin (Santa Cruz Biotechnology, Santa Cruz, California), ACH3 (Santa Cruz Biotechnology, Santa Cruz, California) and PML-RARα (C-1, sc-515796, Santa Cruz Biotechnology, Santa Cruz, California). After being washed with TBST (Tris-buffered saline and 0.1% Tween-20), the membranes were incubated with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, California) for 1 h, washed with TBST again, and detected using Tanon 4200 automatic chemiluminescence image analysis system (Tanon, Shanghai, China). Protein bands were quantified using the integration of the chemiluminescence signals on Quantity One (Bio-Rad Laboratories, Hercules, California).

Quantitative real-time PCR assay

Total RNAs were isolated from cells treated with experimental agents and detected with a real-time PCR detection system (Bio-Rad, California, USA) using the SYBR Green PCR super mix (Bio-Rad, California, USA). Human PML-RARα primers were 5′-CAGTCT CAGCCT TCTCCATCA-3′ (forward) and 5′-GCTT GTAGATGC GGG GTAGA-3′ (reverse). Human β-actin primers, 5′-CTA-CCTC ATGAAGA TCCTCA CC GA-3′ (forward) and 5′-TTC TCCTTA ATGTCACGC ACG ATT-3′ (reverse), served as internal control. Each

sample was run in triplicate and normalized to actin mRNA to assess their relative expression. A comparative CT method was adopted to analyze the gene expression level.

Xenograft mouse model

Female, 5- to 6-weeks-old nonobese, diabetic, severely compromised immunodeficient (NOD/SCID) mice were obtained from Beijing HEK Bioscience. NB4 cells (1×10^7) were implanted with matrigel (BD Biosciences) subcutaneously into the left flank of mice. All mice were randomized into two groups, a vehicle control group and a treatment group (n = 3 per group). Mice were treated with CUDC-101 (50 mg/kg; intraperitoneally) or ATO (50 mg/kg; intraperitoneally) daily for 21 days. Tumor volume was measured twice per week with calipers and was calculated as tumor volume (mm^3) = $L \times W^2/2$ (L represents the largest diameter and W is the smallest diameter of tumor). All animal experiments were approved by the Ethics Committee of Guizhou Medical University.

Statistical analysis

Every experiment was performed at least three times. Results were expressed as mean ± SEM, and analyzed with the Student's t-test. $P < 0.05$ was considered statistically significant.

Results

CUDC-101 exerts much stronger antiproliferative effects than arsenic trioxide in acute promyelocytic leukemia and arsenic trioxide-resistant acute promyelocytic leukemia cells

Using the CCK-8 assay, the antiproliferative effects of CUDC-101 on human APL and ATO-resistant APL cell lines were measured. Fifty percent growth inhibition (IC 50) was calculated in the cells treated with 0–5 μM CUDC-101 or ATO for 24, 48, and 72 h, respectively, and statistical differences are listed in Table 1. As shown in Fig. 1b and c, different concentrations of CUDC-101 considerably inhibited the growth of NB4 and HL60 cells. The IC50 values show that NB4 and HL60 cells were more sensitive to CUDC-101 than ATO. Additionally, cell growth was suppressed in a time-dependent and dose-dependent manner, corresponding to the reduced cell viability. The effects of CUDC-101 on ATO-resistant APL cell lines were also assessed. After 24 h of treatment, the viability of NB4/As cells in the ATO-treated groups was nearly consistent with that in the nontreated groups, even at an ATO concentration up to 5 μM. No significant decrease in the cell viability of NB4/As cells was observed with a treating time extended to 48 or 72 h. In contrast, CUDC-101 at the concentrations of 0–5 μM significantly inhibited the proliferation of the NB4/As cells, and cell growth was suppressed in a time-dependent manner (Fig. 1d). These data demonstrate that CUDC-101 exerts much stronger antiproliferative effects than ATO in APL and ATO-resistant APL cell lines.

Table 1 Sensitivity of acute promyelocytic leukemia cell lines to CUDC-101 or arsenic trioxide

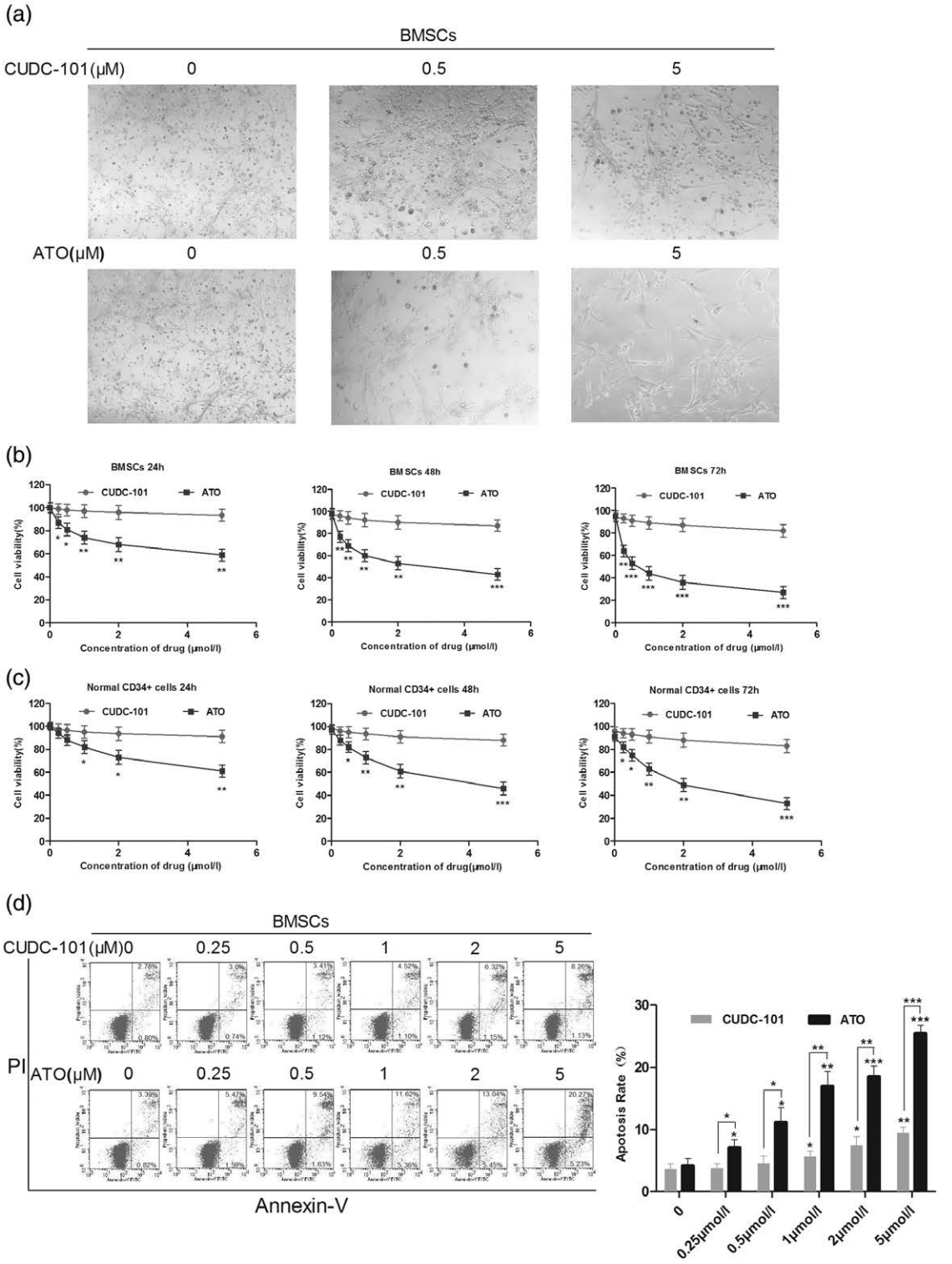
24 h	Cell lines	Drug (μM)	IC50 value (μM)
	NB4	CUDC-101	1.7390 ± 0.49
		ATO	7.9150 ± 1.40**
	HL60	CUDC-101	4.6050 ± 1.45
		ATO	15.7650 ± 2.03**
48 h	Cell lines	Drug (μM)	IC50 value (μM)
	NB4	CUDC-101	0.9735 ± 0.19
		ATO	6.9455 ± 1.05***
	HL60	CUDC-101	2.4910 ± 0.73
		ATO	13.8660 ± 7.05*
72 h	Cell lines	Drug (μM)	IC50 value (μM)
	NB4	CUDC-101	0.6218 ± 0.17
		ATO	9.4800 ± 3.87*
	HL60	CUDC-101	1.5265 ± 0.49
		ATO	10.5825 ± 5.62*

The results are expressed as the mean ± SEM; n = 3; *P <0.05, **P < 0.01, ***P < 0.01 vs. the NB4 group.
ATO, arsenic trioxide; CUDC-101, 7-(4-(3-ethynylphenylamino)-7-methoxyquinazolin-6-yloxy)-N-hydroxyheptanamide; IC50, fifty percent growth inhibition.

CUDC-101 treatment induces apoptotic cell death in acute promyelocytic leukemia and arsenic trioxide-resistant acute promyelocytic leukemia cells more effective than arsenic trioxide

Previous experiments on cell proliferation inhibition prompted investigation of whether CUDC-101 could induce apoptosis in APL and ATO-resistant APL cells. Therefore, apoptosis cells were quantified by flow cytometer. With 48-h treatment, CUDC-101 could induce the apoptosis in NB4 and HL60 cells in a dose-dependent manner (Fig. 2a and b). We compared the apoptosis-inducing activity of CUDC-101 with that of ATO in NB4 and HL60 cells. As shown in Fig. 2a and b, ATO did not exhibit enough apoptosis-inducing activity compared to CUDC-101 at the indicated concentration and time (0–5 μM; 48 h). Then, the effects of CUDC-101 on the expression of marker proteins of apoptosis were evaluated. As shown in Fig. 2d, CUDC-101 treatment altered the expression levels of Bcl-2, Bax, C-caspase 3, and C-PARP proteins (proteolytic cleavages of caspase 3 and PARP) in a dose-dependent manner. Moreover, small-molecule inhibitors of enzyme activity often have effects that occur through secondary mechanisms. To determine whether CUDC-101 increased cell apoptosis through a direct epigenetic effect, CUDC-101 was first assessed as able to affect histone acetylation by western blot. Analysis showed that CUDC-101 treatment increased expression of levels of acetylation of histone 3 (ACH3) in a dose-dependent manner in both NB4 and HL60 cells (Fig. 2d). Next, the effect of CUDC-101 was investigated on the apoptosis of ATO-resistant APL cell lines. NB4/As cells were treated with 0–5 μM CUDC-101 or ATO for 48 h. The apoptotic rates of ATO treatment groups were similar to that of the control group, but 0.5 μM CUDC-101 was able to induce significant apoptosis of NB4/As cells (Fig. 2c). Meanwhile, the immunoblot analysis showed that CUDC-101 treatment altered the expression of

Fig. 4



CUDC-101 shows negligible cytotoxic effect on noncancerous cells. (a) The morphological changes in BMSCs after CUDC-101 or ATO treatments. (b) The effects of CUDC-101 or ATO on BMSC viability were detected by CCK8 assay. (c) The effects of CUDC-101 or ATO on normal $CD34^+$ cells viability were detected by CCK8 assay. (d) BMSCs were treated with various concentrations of CUDC-101 or ATO for 48 h, and apoptotic cell was identified by flow cytometry. Data are shown as the mean ± SD of three independent experiments; unpaired two-tailed student's test was used for statistics. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group (DMSO < 0.1%). ATO, arsenic trioxide; BMSCs, bone marrow mesenchymal stem cells; CUDC-101, 7-(4-(3-ethynylphenylamino)-7-methoxyquinazolin-6-yloxy)-N-hydroxyheptanamide.

apoptosis-related proteins and ACH3 in a dose-dependent in NB4/As cells (Fig. 2d). These results strongly support CUDC-101 as an effective apoptosis inducer that induced apoptotic cell death in both APL and ATO-resistant APL cell lines, more effectively than ATO.

CUDC-101 shows negligible cytotoxic effect on noncancerous cells

Studies have shown that ATO exerts obvious cytotoxic effect on mesenchymal stem cells during treatment [20]. Our previous results show that CUDC-101 worked more effectively than ATO in killing APL cells. The next experiment investigated whether CUDC-101 shows cytotoxic effect on noncancerous cells (including normal $CD34^+$ cells and BMSCs from APL patients) like the ATO did. Then the effects of CUDC-101 on noncancerous cells were detected. As shown in Fig. 4a, BMSCs (Fig. 3 shows identification of BMSCs) from APL patients were treated with 0, 0.5, 5 μM of CUDC-101 or ATO for 48 h. The morphological changes of cell pyknosis and cell shrinkage suggest that ATO induced apoptotic cell death in BMSCs *in vitro*. However, the morphological changes in CUDC-101-treated groups were not significant. Interestingly, the viability of BMSCs in the CUDC-101-treated groups was nearly consistent with that in the nontreated groups after a 24 h of treatment, even at a CUDC-101 concentration up to 5 μM (Fig. 4b). No significant decrease in the cell viability of BMSCs was observed with the treating time extended to 48 and 72 h. By contrast, ATO at the concentrations of 0–5 μM significantly inhibited the proliferation of the mesenchymal stem cells, and cell growth was suppressed in a time-dependent manner (Fig. 4b). Furthermore, CUDC-101 at the same concentrations hardly affected normal $CD34^+$ cells, which confirms that the drug was little cytotoxic to normal donor compared to ATO (Fig. 4c). Next, we investigated the effect of CUDC-101 on the apoptosis of mesenchymal stem cells. BMSCs were treated with 0–5 μM CUDC-101 or ATO for 48 h. The percentage of apoptotic cells was determined by flow cytometry. The apoptotic rates of CUDC-101 treatment groups were similar to that of the control group, but 0.5 μM of ATO was able to induce significant apoptosis of BMSCs (Fig. 4d). These data demonstrate that CUDC-101 is a novel drug that has a negligible cytotoxic effect on noncancerous cell lines including normal $CD34^+$ cells and BMSCs from APL patients.

CUDC-101 treatment induces caspase-dependent promyelocytic leukemia-retinoic acid receptor alpha degradation

The PML-RARα fusion protein dictates not only the phenotype of APL but also the response of APL to treatment [2], and several studies have shown that PML-RARα expression induces strong resistance to apoptosis [29]. Therefore, we next analyzed the effects of CUDC-101 on PML-RARα. As shown in Fig. 5a and b, the PML-RARα levels were significantly decreased in NB4 and NB4/As cells after CUDC-101 treatment for 48 h. Caspase family members have been recognized as key participants in apoptosis and play important roles in PML-RARα degradation [30,31]. Therefore, we treated cells with CUDC-101 in the presence or absence of the caspase inhibitor Z-VAD-FMK. As exhibited in Fig. 5c, in both APL and ATO-resistant APL cells, the CUDC-101-induced apoptosis was significantly attenuated by the addition of Z-VAD-FMK. Additionally, the immunoblot results showed that Z-VAD-FMK blocked CUDC-101-induced PML-RARα degradation and alterations of apoptosis-related proteins such as C-PARP, Bcl-2, Bax, and C-caspase 3. However, histone 3 hyperacetylation induced by CUDC-101 could not be blocked by Z-VAD-FMK (Fig. 5d), indicating that CUDC-101-induced histone 3 hyperacetylation is an event that occurs earlier than PML-RARα degradation and apoptosis. These results suggest that CUDC-101-induced hyper ACH3 activates caspase 3 and subsequently induces caspase-dependent PML-RARα degradation and apoptosis.

CUDC-101 inhibits the proliferation of acute promyelocytic leukemia *in vivo*

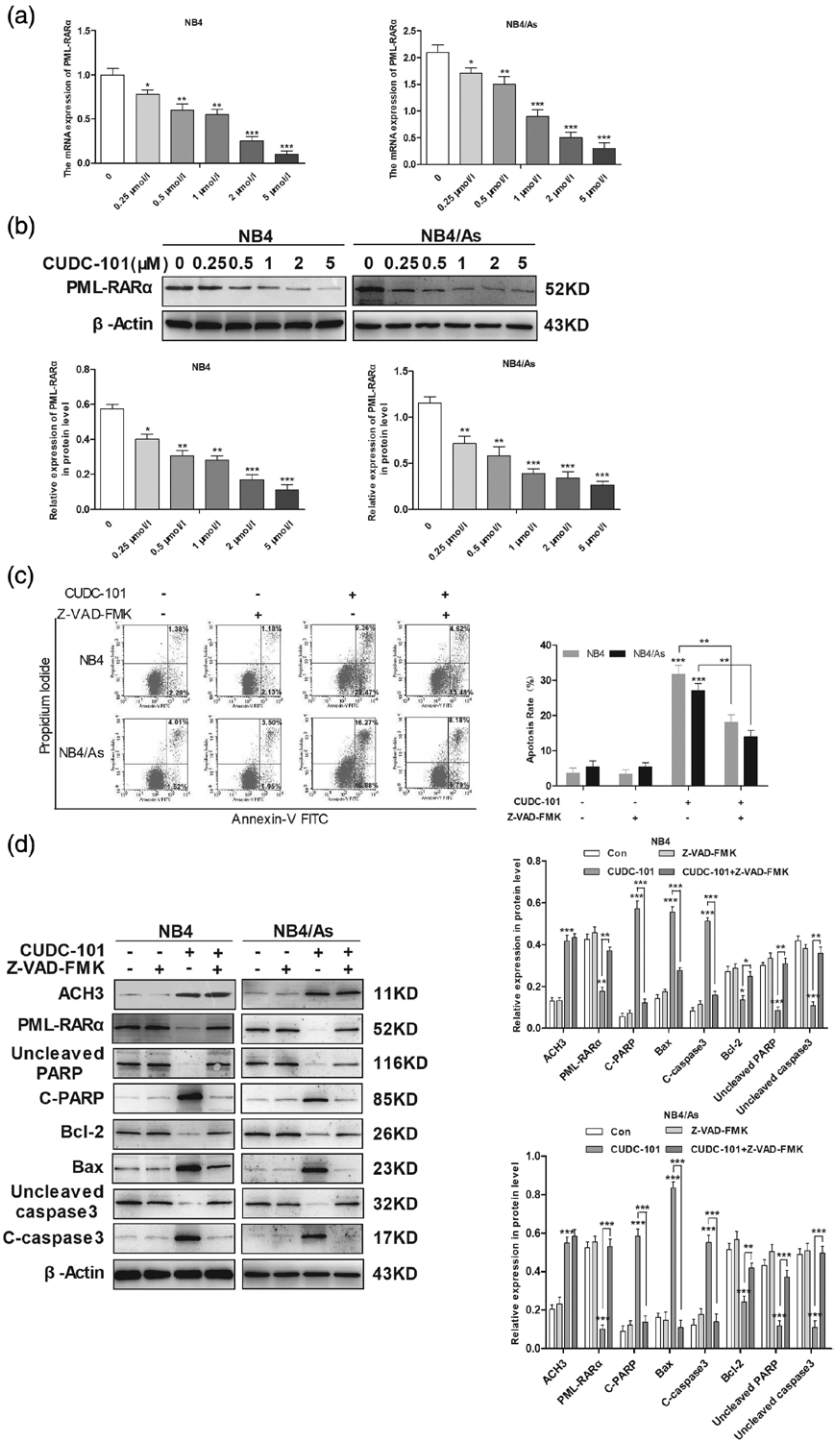
Given CUDC-101 effectively induces APL cells death *in vitro*, in-vivo effects were investigated by employing a xenograft mice model. For this purpose, NB4 cells were injected into the right flanks of the (SCID. All mice were randomized into a treatment group and a vehicle control group (n = 3 per group). Mice were treated with CUDC-101 (50 mg/kg, intraperitoneally) or ATO (50 mg/kg, intraperitoneally) daily for 21 days. The result shows that CUDC-101 exhibited significant antitumor activity in the NB4 xenograft mode compared with ATO (Fig. 6a and b). These findings suggest that the CUDC-101 is efficient in inhibiting tumor growth *in vivo* and performs better than ATO.

Discussion

There is a need for a new chemotherapeutic agent to improve prognosis with ATO and minimize treatment toxicity. Patients with APL have been successfully treated with ATO [2,5,6]. However, resistance to this drug and its toxicity are major with this treatment modality [8,9,20]. Additionally, early death and relapse remain as obstacles to further improvement of remission rates of remission and long-term survival. The acute and chronic adverse effects of ATO should be considered for more appropriate management [1].

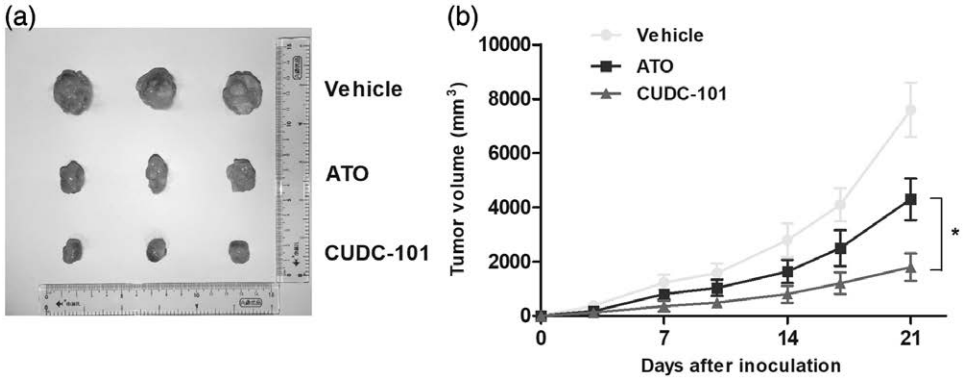
HDACs regulate the activities of tumor-suppressor genes and oncogenes, playing key roles in tumorigenesis [32]. They have been investigated in preclinical studies for solid tumors and hematological malignancies including APL [32,33]. In this study, a novel pan-HDACi CUDC-101 was used tentatively to treat hematologic disease for the first time. Notably, CUDC-101 possesses some

Fig. 5



CUDC-101 treatment induces caspase-dependent PML-RARα degradation. (a) Total RNA was extracted from NB4 and NB4/As cells treated with CUDC-101 for 48 h at the indicated concentrations (0–5 μM) and were subjected to real-time PCR analysis to detect PML-RARα mRNA levels. (b) Immunoblot analysis of PML-RARα expression in NB4 and NB4/As cells treated with CUDC-101 for 48 h at the indicated concentrations (0–5 μM). (c and d) NB4 and NB4/As cell lines were treated with 80 μM Z-VAD-FMK for 1 h, and then 1 μM CUDC-101 was added; 48 h later, cell apoptosis was measured by an Annexin V-FITC and PI staining kit (c). The expression levels of related proteins were evaluated by immunoblot analysis (d). Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, unpaired two-tailed Student's *t*-test. β-actin was used as a loading control. CUDC-101, 7-(4-(3-ethynylphenylamino)-7-methoxyquinazolin-6-yloxy)-N-hydroxyheptanamide; PML-RARα, promyelocytic leukemia-retinoic acid receptor alpha.

Fig. 6



CUDC-101 inhibits the proliferation of APL *in vivo*. (a) Images of tumors obtained from NOD/SCID mice of the respective groups. (b) NOD/SCID mice implanted subcutaneously with NB4 cells were treated with CUDC-101 or ATO daily by intraperitoneally. Tumor size was measured with calipers on days 0, 3, 7, 10, 14, 17, and 21. Calculations are described in the materials and methods section. Data presented as mean ±SD; *, $P < 0.05$, vs. vehicle group. CUDC-101, 7-(4-(3-ethynylphenylamino)-7-methoxyquinazolin-6-yloxy)-N-hydroxyheptanamide; APL, acute promyelocytic leukemia; ATO, arsenic trioxide; SCID, severe combined immunodeficiency mice.

advantages over the ATO. We showed that CUDC-101 decreased proliferation and induced apoptosis in both APL and ATO-resistant APL cell lines. This finding is consistent with previous reports on the growth inhibitory effect of CUDC-101 in various cancer cells [16]. The calculated IC50 value for the antiproliferative effect of CUDC-101 on APL and ATO-resistant APL cells was lower than that of ATO. The antiproliferative effect was observed at concentrations with no obvious toxic effect on noncancerous cell lines including normal $CD34^+$ cells and BMSCs from APL patients, suggesting specificity of CUDC-101 action in APL and ATO-resistant APL cells. We further demonstrated that CUDC-101 exerts its effects by induced hyper ACH3, caspase-dependent degradation of PML-RARα, and apoptosis. These findings highlight the potential of CUDC-101 for APL therapy and for overcoming ATO resistance.

Several therapeutics have been tested to overcome APL, such as ATO, BKM120 [34], EGCG [35], and lithium chloride [36], among these, ATO is considered the most active agent. ATO has been used for a longtime in traditional Chinese and Western medicine [37]. As shown by previous in vivo studies, a low concentration of ATO is able to induce cell differentiation and high concentrations elicit APL cell apoptotic death [38,39]. The PML-RARα fusion protein is clearly the central player driving APL, which also induces strong resistance to apoptosis in APL cells [29]. ATO could directly target the PML component of PML-RARα and degrade it, which leads to apoptosis of APL cells. Similarly, CUDC-101 treatment induces PML-RARα degradation in APL and ATO-resistant APL cells. The difference is ATO degrade PML-RARα mainly through ubiquitin proteasome system [40,41], while CUDC-101 induces caspase-dependent PML-RARα degradation. Moreover, CUDC-101 induces apoptosis in both APL and ATO-resistant APL cells, and its apoptosis-inducing effect is more potent than ATO, which is the first-line therapy for APL. On the contrary, the caspase inhibitor Z-VAD-FMK blocked CUDC-101-induced PML-RARα degradation and apoptosis, but proteins related to the histone acetylation, such as ACH3, could not be blocked by Z-VAD-FMK. Therefore, we conclude that CUDC-101 directly induces hyperacetylation of histone 3, which subsequently leads to the activation of caspase 3, and the activated-caspase 3 cleaves the PML-RARα fusion protein and subsequently facilitates APL and ATO-resistant APL cells undergoing apoptosis. The precise mechanism of CUDC-101-induced degradation of PML-RARα remains unclear. Yet, the apoptosis inducing effect of CUDC-101 offers a potent therapeutic advantage of CUDC-101 and its role in overcoming APL warrants further validation in patient-derived samples in future studies.

Taken together, these findings indicate that the novel pan-HDAC inhibitor CUDC-101 may be a promising candidate drug for newly diagnosed APL and relapsed APL with ATO resistance. The potential therapeutic value of CUDC-101 in APL and other cancers warrants further study.

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Conflicts of interest

There are no conflicts of interest.

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