Original article

Chidamide in FLT3-ITD positive acute myeloid leukemia and the synergistic effect in combination with cytarabine

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ABSTRACT

Chidamide, a novel histone deacetylase inhibitor (HDACi), has been approved for treatment of T-cell lymphomas in multiple clinical trials. It has been demonstrated that chidamide can inhibit cell cycle, promote apoptosis and induce differentiation in leukemia cells, whereas its effect on acute myeloid leukemia (AML) patients with FLT3-ITD mutation has not been clarified. In this study, we found that chidamide specifically induced G0/G1 arrest and apoptosis in FLT3-ITD positive AML cells in a concentration and time-dependent manner. We also found chidamide had the cytotoxicity effect on FLT3-ITD positive and negative AML cells. Moreover, with respect to relapsed/refractory patients, chidamide showed the same effectiveness as that in de novo AML patients. Notably, chidamide synergistically enhanced apoptosis caused by cytarabine. Our results support chidamide alone or combine with cytarabine may be used as an alternative therapeutic choice for AML patients especially those with FLT3-ITD mutation or relapsed/refractory ones.

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1. Introduction

Acute myeloid leukemia (AML) is the most common form of leukemia in adults. Approximately 20–30% of AML patients harbor an internal tandem duplication mutation in the FLT3 kinase gene (FLT3-ITD) which is considered as unfavorable cytogenetics [1,2]. Histone deacetylases (HDACs) are a family of enzymes that remove an acetyl group from the e-amino groups of the lysine side-chains [3]. Previous studies have demonstrated that HDAC inhibitors (HDACIs) including TSA, sodium butyrate, suberoylanilide hydroxamic acid (SAHA) and depsipeptide are promising new agents for the treatment of different cancers especially hematological malignancies [4–7]. They potently prevented cell cycle progression in the G0/G1 or G2/M phases, leading to growth inhibition and differentiation of cancer cells [8,9]. One of the HDACIs SAHA has been approved by the USA FDA for treatment of cutaneous T-cell lymphoma (CTCL) [4,10].

Chidamide, a new histone deacetylase (HDAC) inhibitor of the benzamide class, is currently under clinical trials. It was approved for the treatment of recurrent or refractory peripheral T-cell lymphoma (PTCL) in China at 2014 [11]. Previous studies showed chidamide had efficient anti-proliferative activity on different cancer cells [12–14]. In this study, we reported the antitumor characteristics of the chidamide in FLT3-ITD positive AML or relapsed/refractory AMLs and the synergistic effect of chidamide combined with cytarabine in AML.

2. Materials and methods

2.1. Materials

Anti-phosphorylated and total RB, Acetyl-H3, Acetyl-H4, E2F1, CDK2, CDK4, CDK6, P21WAF1/CIP1, CASPASE-3, PARP, β-actin antibodies were purchased from Cell Signalling Technology (Beverly, MA, USA). Chidamide was gifted by Shenzhen Chipscreen Biosciences, Ltd (Shenzhen, China). Cytarabine was obtained from Selleck Chemicals (Houston, USA).
2.2. Methods

2.2.1. Cells and cell culture

MV4-11 and MOLM-13 cells were gifts from Professor Ravi Bhatia (City of Hope National Medical Center, Duarte, CA). All cells were cultured in IMDM medium (Gibco, Billings, MT, USA) with 10% fetal bovine serum (Gibco) and maintained in a 37°C humidified atmosphere containing 5% CO₂. Primary AML cells were isolated from the bone marrow after obtaining informed consent. Mononuclear cells were purified by standard Ficoll-Hypaque density centrifugation. The mutations in FLT3-ITD were tested by the First Affiliated Hospital of Zhejiang University. The Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China) approved this study.

2.2.2. Cell viability assay

Cells were plated in 96-well plates and treated with different doses of drugs for 24 h. The cell viability was measured using MTS proliferation assay kit. Cell line experiments were repeated three times.

2.2.3. Flow cytometry analysis

Following drug treatment, cells were harvested. After washed twice with phosphate buffered saline (PBS), cells were resuspended in 200 μl binding buffer. Then cells were incubated with 5 μl Annexin V-Fluorescein Isothiocyanate (FITC) and 10 μl Propidium Iodide (PI) (BD Pharmingen, San Diego, CA, USA). For cell cycle analysis, at the end of the drug treatment, the cells were fixed with 75% ethanol at 4°C overnight. The following day, the cells were harvested and washed, re-suspended in PBS with 100 μg/ml RNase A and 100 mg/ml PI for 30 min. The apoptosis and DNA content were analyzed by FACScan flow cytometer (Becton Dickinson, San Diego, CA, USA).

2.2.4. Western blot analysis

Cells were lysed in RIPA buffer (Cell Signaling Technology, Beverly, MA, USA). Whole cell lysates were separated by SDS-PAGE gel (life) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The target proteins were probed with antibodies and analyzed by the image lab software (bio-rad, california, USA).

2.2.5. Statistical analysis

Statistical significance of differences was determined using the Tukey-Kramer Comparison Test or the Student’s t test, P < 0.05 was considered statistically significant (*). Combination index (CI) was calculated using CalcuSyn software (Biosoft, Cambridge, UK). Data were expressed as mean ± SD of 3 independent experiments.

3. Results

3.1. Chidamide caused growth arrest in FLT3-ITD positive cell lines by inducing cell cycle arrest and apoptosis

To test the activity and efficacy of chidamide in FLT3-ITD positive AML cells, we treated two cell lines at concentrations of 2, 4, 8 and 16 μM. We performed MTS assay to evaluate the growth inhibition of AML cells. Following treatment with increasing doses of chidamide, viability of MV4-11 and MOLM-13 cells was respectively found to be significantly inhibited in a dose dependent manner at 24 h and 48 h (Fig. 1A).

![Fig. 1. Chidamide inhibit the growth of FLT3-ITD positive AML. The cell viability induced by chidamide in MV4-11 and MOLM-13 cells (A). Cells were treated with 2, 4, 8 and 16 μM chidamide for 24 h and 48 h. Apoptosis was measured by flow cytometry (B). Expression of PARP, Caspase-3 were analyzed by Western blotting analyses in FLT3-ITD positive cells (C). After treated with chidamide, cells were stained with propidium iodide and subjected to flowcytometry analysis to determine cell cycle distribution (D). CDK4, CDK6 and CDK2 were analyzed by Western blotting analyses at the indicated concentrations for 24 h (E).](https://example.com/fig1.png)
As a previous study showed, chidamide could cause cell cycle arrest and apoptosis in AML cells [12]. Here, we similarly tested cell cycle distribution for 24 h and apoptosis for 24 h and 48 h. As shown in Fig. 1B and C, Chidamide treatment induced cells to arrest in the G0/G1 phase and the cyclin dependent kinase (CDK)4, 6 and 2 were reduced. Generally, cell cycle arrest could lead to cell apoptosis or senescence [15]. Consistently, as shown in Fig. 1D, chidamide-treated AML cells increased Annexin-V positivity in a dose-dependent manner. In addition, we detected Caspase-3 and activation of PARP, a key event that occurs upon Caspase-3 activation during apoptosis. As a result, Chidamide was able to increase cleaved Caspase-3 and PARP (Fig. 1E) in FLT3-ITD positive AML cells.

3.2. Modulation of oncogenic signaling pathways after chidamide treatment

As previously reported, chidamide caused hyper-acetylation of histone H3, H4 [16], and increased the target gene p21 [17] that can be regulated by acetylation level of histone proteins. We treated AML cells with increasing concentrations of chidamide for 24 h. Fig. 2 shows that chidamide increased histone H3 and H4 acetylation levels, up-regulated p21 protein levels which can cause cell cycle arrest. Moreover, to determine the downstream signaling of the p21, we examined the RB/E2F1 pathway and found a significant decrease of p-RB and E2F1 (Fig. 2). These results confirmed that chidamide inhibited FLT3-ITD positive cells mainly through regulation of p21 gene.

3.3. Chidamide enhanced cytarabine induced apoptosis

We examined the effect of chidamide in combination with the front line AML chemotherapy cytarabine. Fig. 3A–C shows that the combination synergistically inhibited growth in AML cell lines. The dose-effect curves were determined by Calcsyn analyses. The CI50 at the ED50, ED75 and ED90 are presented in Fig. 3D. So we confirmed that chidamide combined with cytarabine had a strong synergistic effect in AML cell lines in vitro.

Cells were exposed to 2 μM chidamide or/and 2 μM cytarabine for 24 h. Compared with single agents, the combination resulted in a significant increase in apoptosis (Fig. 3A–C). Next, we analyzed the key signaling molecules in the apoptosis pathway by western blot analysis. As presented in Fig. 3E, we observed that the combination treatment obviously increased the expression of cleaved PARP and Caspase3.

3.4. Different types of AML were sensitive to chidamide

To compare the sensitivity in different types of AML, first we identified six AML cell lines including KG-1, THP-1, HL-60, Kasumi-1, MV4-11 and MOLM-13. Then we treated 4 AML patients with FLT3-ITD mutation and 8 FLT3 wt AML patients with increasing

![Fig. 2. Chidamide inhibited of oncogenic signaling pathways. Chidamide promoted histone H3 and H4 acetylation in MV4-11, MOLM-13 and FLT3-ITD positive primary AML cells. The p21 protein levels was increased and the downstream signaling RB/E2F1 pathway was inhibited.](image-url)
concentrations of chidamide for 24 h followed by analysis of cell survival. The characteristics of the patient samples are presented in supplementary Table 1. As showed in Fig. 4A and B, we found almost no difference between FLT3-ITD mutant and wt cells.

Interestingly, we divided patients as de novo and relapsed/refractory and found that the relapsed/refractory patients showed the same effectiveness as the de novo patients (Fig. 4C). This indicates that chidamide may be a good choice for the relapsed/refractory AML patients. Primary AML cells from a de novo patient with a IDH2-172R mutation(AML#9) were treated with chidamide and cytarabine, the CI at the ED50, ED75 and ED90 were <1 (Fig. 4D). AML#11 was a MDS transformed refractory AML patient. The patient was very sensitive to chidamide and the synergistic effect with cytarabine was strong. AML#12 was a de novo patient who had a synergistic effect as well.

4. Discussion

The FLT3-ITD mutation always indicates a negative impact on outcome in clinical practice. Finding effective treatment regimens is desperately needed. The success of some HDACs such as SAHA [4,7,18], leaded to hotter research of HADC inhibitors and prompted us to search for more potent and effective HDAC inhibitors.

In previous studies, chidamide, a synthetic analogue of MS-275 [12], has showed effect in a wide variety of cancers. Li [16] reported chidamide was able to increase the levels of acetylation histone H3

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**Fig. 3. Chidamide enhanced cytarabine induced apoptosis.** Cells were treated with chidamide combined with cytarabine in MV4-11 cells (A), HL-60 cells (B) and Kasumi-1 cells (C). The CI at the ED50, ED75 and ED90 were presented (D). The combination caused significant apoptosis at 24 h (E).
and inhibit the PI3K/Akt and MAPK/Ras signaling pathways in colon cancer cells. In leukemia cells, chidamide induced G0/G1 arrest, differentiation and caspase-dependent apoptosis by cytochrome c release and ROS-mediated mitochondrial dysfunction [12]. A multicenter, open-label, pivotal phase II study showed chidamide represented significant single-agent activity and was well tolerated in relapsed or refractory PTCL [11]. In this study, we determined the effects of chidamide treatment on FLT3-ITD positive cells and AML blast samples. We found that chidamide caused cell cycle arrest at G0/G1 phase that leading to apoptosis in FLT3-ITD mutant cell lines. Our results indicated that chidamide upregulated p21 (WAF1/CIP1; CDKN1a) gene which is a universal cell-cycle inhibitor [19,20] and the downstream signaling pathway RB/E2F1 was significant decreased.

Then, we compared the sensitivity in different types of AML and found chidamide was effective in FLT3-ITD positive and relapsed/refractory AML patients (Fig. 3B and C). Nowadays, IA/DA is still the first-choice chemotherapy in AML, but resistance to cytarabine (Ara-C) is a major cause of treatment failure in this disease [21,22]. In our study, we combined chidamide with cytarabine, and the combination showed strong synergistic effect. Chidamide synergistically augmented cytarabine-induced apoptosis.

In summary, the present results suggest that chidamide is a new HDACi with potential therapeutic value in FLT3-ITD positive leukemia cells and should be considered for the treatment of relapsed/refractory AML patients. Chidamide may allow protocols to use reduced dose of cytarabine and this combination may be effective to improve tolerability for the elders who are unable to tolerate intensive induction therapy.

**Conflicts of interest**

The authors declare no competing financial interests

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopharma.2017.04.037.
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