Inhibition of EZH2 by chidamide exerts antileukemia activity and increases chemosensitivity through Smo/Gli-1 pathway in acute myeloid leukemia

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Abstract

Background: Dysregulation of epigenetics plays important roles in leukemogenesis and progression of acute myeloid leukemia (AML). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) reciprocally regulate acetylation and deacetylation of nuclear histone, aberrant activation of HDAC results in uncontrolled proliferation and differentiated blockage, HDAC inhibitors have been investigated as therapeutic drugs for treatment of AML.

Methods: Cell growth was assessed by CCK-8 assay, apoptosis was determined by flow cytometry in AML cell lines, CD45+ and CD34+CD38- cells from patient’s samples after stained with Annexin V-fluorescein isothiocyanate (FITC)/Propidium Iodide (PI). EZH2 expression was silenced by short hairpin RNA (shRNA). The pathway changes were detected by western blot. The effect of chidamide or EZH2 shRNA in combination with adriamycin was studied in vivo in nude mice model bearing leukemia.

Results: In this study, we investigated the antileukemia activities of HDAC inhibitor chidamide and its combinatorial effect with cytotoxic agent in AML. We demonstrated in vitro and in vivo that chidamide suppressed expression of EZH2, exerted potential antileukemia activity and increased the sensitivity AML cells and AML stem/progenitor cells to chemotherapeutic drug through Smo/Gli-1 pathway. In addition to decrease the expression of H3K27me3 and DNMT3A, inhibition of EZH2 either pharmacologically by chidamide or genetically by shEZH2 decreased the activity of Smo/Gli-1 pathway, and increased chemotherapeutic sensitivity in AML cells.

Conclusions: Inhibition of EZH2 by chidamide has antileukemia activity and increases chemosensitivity, it provides a potential strategy to improve chemotherapeutic effect in AML.

1. Background

Acute myeloid leukemia (AML) is the most common adult hematologic malignancies with poor prognosis. Nearly 80% of patients achieve initial remission after induction chemotherapy, but most of them relapse and become insensitivity to chemotherapy[1]. The treatment failure is associated with simultaneous resistance to multidrug and survival of leukemia stem cell (LSC) after chemotherapy[2]. Recent studies showed that constitutive activation of multiple pathways contributes to chemoresistance in AML[3–5]. Epigenetic modification regulates various biological functions, such as histone acetylation and deacetylation, DNA methylation and demethylation[6, 7]. Dysregulation of epigenetics plays important roles in leukemic pathogenesis and progression, hence represents a potential therapeutic target in AML[8, 9].

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) reciprocally regulate acetylation and deacetylation of nuclear histone[10]. Histone modification directly affects nucleosome structure, activities of oncogenes and transcription factors[11, 12]. Aberrant activation of HDAC deregulates the expression of multiple genes and activity of signaling pathways, resulting in uncontrolled proliferation and differentiated blockage related to leukemogenesis. HDAC inhibitors have been investigated as
therapeutic drugs for treatment of AML[13–15]. Inhibition of HDAC exerts proliferative inhibition and apoptotic induction, and has synergistic antileukemia effects in combination with cytotoxic agents in AML[16, 17]. Chidamide, a novel benzamide-type HDAC inhibitor, was demonstrated to induce cell differentiation and apoptosis by specifically inhibiting HDAC1, 2, 3 and 10[18]. It was initially developed to treat T/B cell lymphoma/leukemia, breast cancer and lung cancer[19–22]. Recent studies show that chidamide significantly inhibits growth and induces apoptosis in AML cells and LSCs, and increases the sensitivity to cytotoxic agents through disrupting multiple pathways, activating reactive oxygen species (ROS) and accumulating DNA damage[23–27]. Thus, HDAC inhibition provides a promising strategy to improve the efficacy of chemotherapy in AML patients. However, the chidamide's mechanisms of action are not fully understood.

It is reported that inhibition of enhancer of zeste homolog 2 (EZH2) contributes to the antitumor effect of HDAC inhibitor in neuroblastoma cells and lung cancer cells[28, 29]. EZH2 is the functional core subunit of the polycomb repressive complex 2 (PRC2), and plays a pivotal role in catalyzing the methylation of the lysine 27 of histone H3 (H3K27) [30–33]. Overexpression of EZH2 is associated with poor prognosis in lymphoma, melanoma, and breast cancer, and EZH2 is an potential therapeutic target[34, 35]. EZH2 was reported to support leukemogenesis by blocking cellular differentiation, and of EZH2 inhibitors suppressed the growth of AML cells and survival of LSCs[36, 37]. Moreover, EZH2 inhibition also suppressed the activity of Hedgehog pathway, which plays a critical role in tumorigenesis and metastasis[38–40]. Smo/Gli-1 is the key component of the signal transduction in Hedgehog pathway, its aberrant activation supports survival of LSC and induces chemoresistance in AML[41–44]. Targeted inhibition of Smo/Gli-1 pathway is demonstrated to improve chemotherapeutic effect in AML, and Smo inhibitor has been approved by the Food and Drug Administration (FDA) to treat AML patients in combination with chemotherapy[45–47]. Recent studies show that disruption of EZH2 increases the sensitivity to cytotoxic agents through Smo/Gli-1 pathway in colorectal cancer cells[48]. Thus, the strategies to improve effect by combination with HDAC inhibitor and chemotherapy are needed for treatment of AML.

Our previous study showed that EZH2 overexpression and activation of Smo/Gli-1 pathway related to poor prognosis in AML patients, and Smo inhibitor effectively decreased leukemia growth and increased chemosensitivity[49–51]. Chidamide has a promising antileukemia activity in AML. However, its mechanism of action is not clear. We here demonstrate that chidamide exerted potential activity against AML cells and AML stem/progenitor cells, and increased sensitivity to chemotherapeutic drug in vitro and in vivo by inhibition of EZH2 through Smo/Gli-1 pathway.

2. Materials And Methods

2.1 Cells

Kasumi-1 cells and HL-60/ADM cells (Institutes for Biological Sciences Cell Resource Center, Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI-1640 medium (Hyclone, USA),
supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, USA) in a humidified atmosphere of 5% CO2 at 37℃. Bone marrow samples were obtained from AML patients except M3 and healthy donors for stem cell transplantation after informed consent following the institutional ethics committee's approval of Nanfang Hospital in accordance with Declaration of Helsinki. Mononuclear cells were purified by Ficoll-Hypaque (Sigma-Aldrich, USA) density-gradient centrifugation and cultured in α-MEM supplemented with 10% fetal bovine serum. Table 1 summarizes clinical characteristics of patients.

2.2 Cell growth assay

Kasumi-1 cells and HL-60/ADM cells (2 × 10^5 cells/ml) were plated in 96-well plates and treated with chidamide (Chipscreen Bioscineces, China), Adriamycin (MedChem Express, USA), or their combination. Cell growth was assessed by CCK-8 assay kit (Dojindo, Janpan). After cells were incubated with 10 µL of CCK-8 solution for 2 hours at 37 °C, each well was measured at 450 nm using a spectrophotometer (Thermo Fisher Scientic, USA). Cell viability was determined in each treated group and compared with that of the untreated cells. Drug concentration resulting in 50% inhibition of cell growth (IC50) was calculated to evaluate the sensitivity to adriamycin or cytarabine in Kasumi-1 cells.

2.3 Flow cytometry analysis

Kasumi-1 cells, HL-60/ADM cells (2 × 10^5 cells/ml) and primary samples (5 × 10^5 cells/ml) were treated with chidamide, adriamycin, or their combination. Cell apoptosis was estimated by flow cytometry (BD Biosciences, USA) after cells were stained with Annexin V-fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) (NanJing KeyGen Biotechnology, China). Apoptosis in primary cells was assessed in CD45+ and CD34+CD38- cells after samples were incubated with CD45-APC, CD34-PC5.5 and CD38-PE Cy7 antibodies (BD Biosciences, USA) and stained with Annexin V-FITC (NanJing KeyGen Biotechnology, China).

2.4 EZH2 silencing by shRNA

Lentivirus-mediated short hairpin RNA (shRNA) was constructed by Genechem (Shanghai, China). The targeting sequences for EZH2 shRNA (shEZH2) were the following: shRNA-1, 5' AACAGCTGCCTAGCTTCA-3'; shRNA-2, 5'-AACAGCTCTAGACAACAAA-3'; shRNA-3, 5' GGATAGAGAATGTGGGTTT-3'. The negative control was a non-target scrambled sequence: 5'-TTCTCCGAACGTGTCACGT-3'. Kasumi-1 cells and HL-60/ADM cells were transduced by lentivirus with enhanced GreenFluorescent Protein (EGFP) and sorted by flow cytometry as described in our previous study[49]. The effect of EZH2 silencing was confirmed by real-time polymerase chain reaction (RT-PCR) and western blot. The Kasumi-1 cells and HL-60/ADM cells with the best EZH2 silencing effect were used in the subsequent experiments.

2.5 Western blot analysis

Kasumi-1 cells and HL-60/ADM cells were treated with chidamide, and cells were lysed in RIPA buffer (Sigma-Aldrich, USA). Protein levels were determined by western blot as previously described[1, 52]. Briefly, whole cell lysates were separated by SDS-PAGE gel and transferred onto polyvinylidene difluoride
(PVDF) membranes (Millipore, USA). Targeted protein was probed with primary antibody, then incubated with the secondary antibody. The immunoblots were visualized using chemiluminescence horseradish peroxidase substrate (Millipore, USA), and analyzed by the Odyssey Infrared Imaging System (LI-COR Biosciences, USA). Antibodies against Acetyl-Histone H3 (#8173), DNMT3A (#3598), H3K27me3 (#9733), EZH2 (#5246), Smo (#4940), Gli-1 (#2643), AKT (#4685), p-AKT (#9614) and GAPDH (#5174) were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GAPDH was used as a loading control.

2.6 In vivo studies

Animal experiments were performed in accordance with the Nanfang Hospital Animal Care and Use Committee approved protocols. Kasumi-1 cells or shEZH2 Kasumi-1 cells (1 × 10^7) were injected subcutaneously into the right posterior flank of BALB/c nude mice. When the tumor size was about 150–200 mm^3, the Kasumi-1 bearing mice were randomized to the following treatment groups (n = 10/group): vehicle control, adriamycin (3 mg/kg.d) by intraperitoneal injection, chidamide (12.5 mg/kg.d) by oral gavage or adriamycin plus chidamide for 7 days. Mice with shEZH2 Kasumi-1 neoplasms were randomized to the following treatment groups (n = 10/group): vehicle control, adriamycin (3 mg/kg.d) by intraperitoneal injection for 7 days. Calculation of tumor volume is V = 0.5 × longest × shortest^2. Three mice in each group were sacrificed after 7 days post treatment, tumors were weighted and fixed in 10% neutralized formalin overnight. Histopathological and immunohistochemical examination were performed to determine the protein expression of EZH2, Smo, Gli-1, p-AKT in the tumor tissues.

2.7 Statistical analysis

Cell experiments were conducted in triplicates, results were expressed as means ± SEM. Statistical analyses were performed using a two-tailed student’s t-test or one-way analysis of variance (ANOVA) for comparisons of multiple groups. *P*< 0.05 was defined as statistical significance.

3. Result

3.1 Chidamide, a novel HDAC inhibitor suppresses growth and induces apoptosis in AML cells and AML stem/progenitor cells

Kasumi-1 cells, HL-60/ADM cells and primary AML cells were treated with HDAC inhibitor chidamide. Cell viability was determined by CCK-8 assay, and apoptosis was determined by flow cytometry after Annexin-FITC/PI staining. Chidamide inhibited cell growth in Kasumi-1 cells and HL-60/ADM cells after treatment for 24, 48 and 72 hours (Fig. 1A). Chidamide also markedly induced apoptosis in Kasumi-1 cells and HL-60/ADM cells (Fig. 1B), as well as CD45 + and CD34 + CD38- stem/progenitor cells from AML patients after treatment for 48 hours (Fig. 1C). But chidamide had limited cytotoxicity in normal CD45 + and CD34 + cells from healthy donors (Supplementary Fig. S1).
3.2 Chidamide sensitizes AML cells and AML stem/progenitor cells to cytotoxic agent

Kasumi-1 cells and HL-60/ADM cells were treated with chidamide, adriamycin and their combination for 24 hours. At 1.00 µmol/L, chidamide did not inhibit proliferation, but significantly increased the growth inhibition of adriamycin in AML cells (Fig. 2A), and which IC50 value was decreased from 1.39 ± 0.24 to 0.15 ± 0.05 µmol/L for Kasumi-1 cells, and 1.79 ± 0.13 to 0.25 ± 0.02 µmol/L for HL-60/ADM cells, indicating that chidamide increased the sensitivity of AML cells to chemotherapeutic drug. We then treated Kasumi-1 cells, HL-60/ADM cells and primary AML blasts with chidamide (1.00 µmol/L) in combination with adriamycin (0.13 µmol/L) for 48 hours. Chidamide alone did not have obvious cytotoxic activity, but it significantly increased apoptosis induced by adriamycin in Kasumi-1 cells and HL-60/ADM cells (Fig. 2B), as well as in CD45+ cells and CD34+CD38- stem/progenitor cells from AML patients (Fig. 2C). But chidamide did not increased apoptosis induced by adriamycin in normal CD45+ and CD34+ cells from healthy donors (Supplementary Fig. S2).

3.3 Inhibition of EZH2 by chidamide exerts antileukemia activity and increases chemotherapeutic sensitivity through Smo/Gli-1 pathway

To understand the mechanisms of action, we treated Kasumi-1 cells and HL-60/ADM cells with chidamide and determined protein levels at 48 and 72 hours by western blot. Chidamide treatment, as expected resulted in accumulation of acetylated histone 3 and decreasing expression of H3K27 trimethylation (H3K27me3) and DNMT3A (Fig. 3A). We discovered that chidamide also inhibited the expression of EZH2, activities of Smo/Gli-1 pathway and downstream signaling target p-AKT after treatment with chidamide for 48 hours, the targeted inhibition was more effective after treatment for 72 hours (Fig. 3A). It indicated that chidamide inhibited the expression of EZH2 and downstream targeted trimethylation of H3K27 and DNMT3A. Interestingly, chidamide decreased activity of Smo/Gli-1 pathway, coinciding with potential inhibition of EZH2 expression in AML cells. This would suggest that chidamide may inhibit Smo/Gli-1 pathway through disruption of EZH2 expression in AML.

To test this, we silenced EZH2 expression by shRNA in Kasumi-1 cells and HL-60/ADM cells. We found that genetic inhibition of EZH2 suppressed Smo/Gli-1 pathway and downstream signaling p-AKT, in addition to decreasing expression of H3K27me3 and DNMT3A (Fig. 3B). Smo inhibitor LED225 suppressed Smo/Gli-1 pathway, but had no effect on EZH2 expression (Fig. 3C). We also observed that shEZH2 or LED225 increased the chemotherapeutic sensitivity to adriamycin, although apoptosis was also slightly induced after EZH2 silencing in Kasumi-1 cells and HL-60/ADM cells (Fig. 3D and 3E). These data indicated that inhibition of EZH2 either pharmacologically by chidamide or genetically by shEZH2 decreased activity of Smo/Gli-1 pathway, and increased the sensitivity to chemotherapeutic drug in AML cells.
3.4 Chidamide suppresses EZH2 and Smo/Gli-1 signaling and enhances the antileukemia activity of adriamycin in an AML xenograft mouse model

We used a leukemia-bearing mouse model to test the *in vivo* antileukemia and chemosensitization activities of chidamide in AML. In our study, Kasumi-1 cells were subcutaneously implanted in BALB/c nude mice to establish the AML xenograft mouse model. Treatment with adriamycin or chidamide inhibited leukemia growth as measured by tumor volume and weight in the mice models, and the combination was the most effective strategy in this regard (Fig. 4A and 4C). The results support that chidamide increased the antileukemia activity of adriamycin in leukemia-bearing mice. To further demonstrate that the effect of chidamide on chemosensitivity *in vivo* was mediated at least in part through EZH2 inhibition, Kasumi-1 cells transfected with shEZH2 were subcutaneously implanted to establish the leukemia-bearing mouse model. We observed that depletion of EZH2 also increased antileukemia activity of adriamycin in the mouse model (Fig. 4B and 4C). Histopathological and immunohistochemical examinations showed that chidamide decreased the expression of EZH2, and inhibited Smo/Gli-1 pathway and its downstream signaling p-AKT. Genetic inhibition of EZH2 also suppressed Smo/Gli-1 signaling pathway in leukemic tumor tissues (Fig. 4D). These *in vivo* data suggested that EZH2 inhibition by chidamide or shEZH2 decreased leukemia growth and increased antileukemia effect of cytotoxic agent through suppression of Smo/Gli-1 pathway in the leukemia-bearing mouse model.

4. Discussion

In this study, we investigated the antileukemia activity of HDAC inhibitor chidemide and its combination with chemotherapeutic drug in AML cells *in vitro* and *in vivo*. We discovered that chidemide suppressed growth, induced apoptosis, and increased the sensitivity of AML cells and AML stem/progenitor cells to cytotoxic agent by inhibition of EZH2. We further demonstrated that in addition to decrease the expression of H3K27me3 and DNMT3A, pharmacological or genetic inhibition of EZH2 decreased the activity of Smo/Gli-1 pathway and increased chemotherapeutic sensitivity in AML.

HDAC plays an essential role in leukemic development and progression, and its inhibition increased the sensitivity to cytotoxic agents in AML cells[1]. Combination of HDAC inhibitor with chemotherapeutic drug is a potentially effective approach to improve antileukemia effect and eradicate LSC in AML through disruption of multiple signaling pathways and accumulation of DNA damage[53–56]. We showed previously that combined inhibition of HDAC and proteasome degradation had a synergistic antileukemia activity in chemoresistant AML cells[1]. In this study, we demonstrated that HDAC inhibitor chidemide induced cell apoptosis, and increased chemotherapeutic sensitivity *in vitro* in AML cells and AML stem/progenitor cells and *in vivo* in a mouse model.

In an effort to understand the mechanism of action, we found that inhibition of EZH2 by chidemide or shEZH2 decreased expression of H3K27me3 and DNMT3A, and increased cytotoxic sensitivity in AML cells and stem/progenitor cells. EZH2 is a histone methyltransferase associated with transcriptional
repression through dimethylation and trimethylation of H3K27 (H3K27me2/3), and EZH2 mediated H3K27 methylation contributes to pathological process and poor prognosis in hematological malignancies[57, 58]. Depletion of EZH2 suppresses the expression of H3K27me3, and inhibits survival of LSC in mixed lineage leukemia[59]. Our previous study also showed that EZH2 was overexpressed in AML patients with high relapse probability, and EZH2 silencing inhibited proliferation and induced apoptosis in AML cells[49]. EZH2 controls CpG methylation through directly contacting with DNA methyltransferase (DNMT) in PRC2/3, and associates with activities of DNMT1, DNMT3A, and DNMT3B[32, 57, 60]. Knockdown of EZH2 inhibits the expression of H3K27me3 and DNMT1, and epigenetic silence mediated by EZH2 and DNMT1 results in aberrant gene expression contributing to pathogenesis in gastric cancer and glioblastoma[61]. Combined inhibition of EZH2 and DNA methylation produces a remarkable synergistic activation of tumor suppressor genes and growth inhibition in leukemia cells[62]. HDAC inhibitor is also demonstrated to deplete EZH2 and DNMT1, and synergistically enhances decitabine mediated apoptosis in AML[63–65]. So targeted inhibition of EZH2 indicates a potential therapeutic strategy for treatment of AML.

In this study, we found that EZH2 inhibition by chidemide disrupted Smo/Gli-1 pathway and downstream signaling molecule p-AKT, and increased the sensitivity to cytotoxic agent in AML cells and stem/progenitor cells. Mechanistic studies showed that depletion of EZH2 by shRNA decreased expression of H3K27me3 and DNMT3A, and inhibited activity of Smo/Gli-1 pathway, while disruption of Smo/Gli-1 pathway didn't affect EZH2 expression. Moreover, targeted inhibition of EZH2 or Smo increased the susceptibility to chemotherapeutic drug in AML cells. Smo/Gli-1 pathway play a critical role in embryogenesis and developmental processes including proliferation and differentiation[66], its activation induces resistance to cytotoxic agents in AML cells and LSCs[41, 67]. Our previous study demonstrated that inhibiting Smo/Gli-1 pathway increased the sensitivity to cytotoxic agents in chemoresistant AML cells[50, 51]. It is reported that Smo inhibitor in combination with chemotherapy applies to treat relapsed/refractory AML with good tolerability and efficacy[45, 68]. Both EZH2 and HDAC inhibitors as epigenetic modulators exhibit multiple-targeted approach in modulating Smo/Gli-1 pathway, their therapeutic potentialities are further investigated for treatment of cancers[38]. Our study indicated that inhibition of EZH2 decreased the activity of Smo/Gli-1 pathway, and contributed to the increasing chemosensitivity in AML.

**Conclusions**

We demonstrated that disruption of EZH2 by chidemide exerted antileukemia activity and increased sensitivity to cytotoxic agent in AML cells and stem/progenitor cells through Smo/Gli-1 pathway, it indicated that targeted inhibition of HDAC is a promising therapeutic strategy for treatment of AML. These findings provide a mechanistic basis for clinic development of HDAC inhibitor to overcome drug resistance and improve chemotherapeutic effect in AML patients.

**Abbreviations**
AML
Acute myeloid leukemia
LSC
Leukemia stem cell
HAT
Histone acetyltransferase
HDAC
Histone deacetylase
EZH2
Enhancer of zeste homolog 2
FDA
Food and Drug Administration
RT-PCR
Real-time polymerase chain reaction

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki, and the protocols approved by the ethics committee at Nanfang Hospital, Guangzhou China. Written consent for use of the samples for research was obtained from patients.

Consent for publication

Not applicable.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article.

Competing interests

No potential conflicts of interest were disclosed by authors.

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Tables

Table 1. Characteristics of AML patients and experiments

| Pt No | Source | % Blasts | Disease status       | Molecular mutation | Cytogenetic | Experiments |
|------|--------|----------|----------------------|--------------------|-------------|-------------|
| 1    | BM     | 86       | New diagnosis        | IDH1+TET2          | 46,XX       | Chi         |
| 2    | BM     | 71       | New diagnosis        | Negative           | 46,XY       | Chi         |
| 3    | BM     | 92       | Relapse/refractory   | FLT3-ITD           | Complex     | Chi         |
| 4    | BM     | 65       | New diagnosis        | Negative           | t(8;21)     | Chi         |
| 5    | BM     | 46       | New diagnosis        | Kit D816           | t(8;21)     | Chi         |
| 6    | BM     | 77       | Relapse              | Negative           | Complex     | Chi+ADM     |
| 7    | BM     | 63       | New diagnosis        | NPM1+DNMT3a        | 46,XY       | Chi+ADM     |
| 8    | BM     | 90       | New diagnosis        | FLT3-ITD+NPM1      | Complex     | Chi+ADM     |
| 9    | BM     | 74       | New diagnosis        | Negative           | Inv(16)     | Chi+ADM     |
| 10   | BM     | 59       | Relapse/refractory   | FLT3-ITD+DNMT3a    | 46,XY       | Chi+ADM     |
| 11   | BM     | 81       | Relapse              | Negative           | 47,XX,+8    | Chi+ADM     |

Abbreviation: Pt No, Patient number; BM, Bone marrow; Chi, Chidamide; ADM, Adriamycin.