Oridonin, a diterpenoid extracted from medicinal herbs, targets AML1-ETO fusion protein and shows potent antitumor activity with low adverse effects on t(8;21) leukemia in vitro and in vivo

Running title: Efficacies of oridonin on t(8;21) leukemia

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Oridonin, t(8;21) leukemia, AML1-ETO, apoptosis, murine model
Abstract

Studies have documented the potential antitumor activities of oridonin, a compound extracted from medicinal herbs. However, whether oridonin can be used in selected setting of hematology/oncology remains obscure. Here we reported that oridonin induced apoptosis of t(8;21) acute myeloid leukemic (AML) cells. Intriguingly, the t(8;21) product, AML1-ETO (AE) fusion protein which plays a critical role in leukemogenesis, was degraded with generation of a catabolic fragment, while the expression pattern of AE target genes investigated could be reprogrammed. The ectopic expression of AE enhanced the apoptotic effect of oridonin in U937 cells. Pre-incubation with caspases inhibitors blocked oridonin-triggered cleavage of AE, while substitution of Ala for Asp at residues 188 in ETO moiety of the fusion abrogated AE degradation. Furthermore, oridonin prolonged life-span of C57 mice bearing truncated AE-expressing leukemic cells without suppression of bone marrow or reduction of body weight of animals, and exerted synergic effects while combined with cytosine arabinoside. Oridonin also inhibited tumor growth in nude mice inoculated with t(8;21)-harboring Kasumi-1 cells. These results suggest that oridonin may be a potential anti-leukemia agent which targets AE oncoprotein at residue D188 with low adverse effect, and may be helpful for the treatment of patients with t(8; 21) AML.
Introduction

The Isodon plant, Rabdosia rubescens (RR), and its extracts, were shown in China to be able to suppress disease progress, reduce tumor burden, alleviate syndrome and prolong survival in patients with esophageal, gastric carcinoma or liver cancer.1-5 Interestingly, other Isodon plants including Isodon japonicus Hara (IJ) and I. trichocarpus (IT) were also applied as home remedies for similar disorders in Japan and Korea6. Oridonin (Fig. 1A), a bitter tetracyclin diterpenoid compound, was isolated from RR, IJ, and IT separately,7,8 suggesting oridonin should be an essential antitumor component of Isodon plants. Studies showed that oridonin induced apoptosis in a variety of cancer cells including those from prostate, breast, non-small cell lung cancers, acute leukemia (NB4, HL-60 cells), glioblastoma multiforme and human melanoma cells.9-12 Oridonin could also increase life span of mice bearing Ehrlich ascites or P388 lymphocytic leukemia.13,14 However, though studies showed that Caspase-3 (casp-3), casp-8, P53, Bcl-2/Bax, cytochrome C (cyt C)10,15,16 and nuclear factor kappa B (NFκB)17 were involved in apoptosis induced by oridonin, mechanisms underlying the antitumor activity of oridonin remain largely unknown, and whether oridonin can find clinical application still needs more investigation.

Genetic abnormalities have been shown to play a key role in leukemogenesis,18 and treatment strategies interfering oncoproteins involved in leukemia pathogenesis have been reported to have high therapeutic efficacy with low adverse effects. The BCR-ABL-targeting STI-571 in the treatment of chronic myeloid leukemia (CML),19 and the PML-RARα-targeting agents, all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) in taming acute promyelocytic leukemia (APL),20,21 can serve as paradigms. Hence molecular target-based therapies should be developed for leukemias with poor prognosis. The AML1-ETO (AE) fusion gene is the result of translocation t(8;21)(q22;q22), which is seen in 40%-80% of M2 type acute myeloid leukemia (AML M2) and 12%-20% of all cases of AML.22,23 The AE fusion protein recruits the N-CoR-mSin3-HDAC complex,24,25 inhibits transcription of AML1 target genes22,24 including interleukin-3 (IL-3),26 activates transcription of apoptotic antagonist Bcl-2,27 upregulates protein tyrosine kinase C-KIT,28 induces the
expression of granulocyte colony-stimulating factor receptor (G-CSFR) as well as myeloperoxidase (MPO), and blocks transactivation of the GM-CSF promoter. The AE oncoprotein enhances self-renewal of hematopoietic stem/progenitor cells, blocks hematopoietic differentiation, disturbs normal cell proliferation and immortalizes murine hematopoietic progenitors. Though reports suggest that additional mutations are required to cooperate with AE to cause murine full-blown leukemia, stem cells expressing AE induce a myeloproliferative disorder or distinct myeloid developmental abnormalities in mice, while deletion of a C-terminal NcoR/SMRT-interacting region strongly induces leukemia development. Recently a novel isoform of AE transcript, AML1-ETO9a (AE9a) that included an extra exon 9a of the ETO gene and encoded a C-terminally truncated AE protein, was identified from human t(8;21) AML and was shown to rapidly induce leukemia in a mouse retroviral transduction–transplantation model. These data demonstrate that AE plays a critical role in pathogenesis of t(8;21) leukemia, and AE-targeting agents might be helpful for the treatment of t(8;21) AML.

Clinically, aggressive cytosine arabinoside (Ara-C)-based chemotherapy is the standard protocol for t(8;21) AML, and t(8;21) was shown to be a favorable prognostic factor for AML. However, others demonstrated that the median survival time of patients with t(8;21) AML was less than 2 yrs with a 5-yr survival rate of no more than 40%. To further improve the clinical outcome and to provide therapeutic options for t(8;21) AML patients, investigational therapy should be developed. Here we reported that oridonin not only induced apoptosis in AE-bearing leukemic cells by activating intrinsic apoptotic pathway, triggered a casp-3-mediated degradation of AE at D188, but also showed significant anti-leukemia efficacies without obvious side effects in two murine models for human t(8;21) AML, providing the rationale for potential clinical study to evaluate oridonin as a drug against t(8;21) leukemia.

**Materials and methods**

**Oridonin**

Oridonin with a purity of up to 99.5% was a generous gift from Mr. Wei Jiang...
(Zhao Wei Technology and Development Ltd, Shanghai) and Prof. Handong Sun (Kunming Institute of Botany, Chinese Academy of Sciences). A stock solution (10⁻² mol/L) was prepared by dissolving oridonin in DMSO (Sigma) and stored at -20°C.

Cell culture, cell viability, analysis of externalization of phosphatidylserine (PS), cell cycle and mitochondrial transmembrane potential (MTP, Δψm)

The Kasumi-1, U937, and ponasterone A (PA; Invitrogen)-inducible AE-expressing U937 (U937-A/E) cells were cultured as described previously. The diagnosis of AML M2 with t(8;21) was established according to methods described. Primary leukemic cells harvested from bone marrow samples of de novo or relapsed patients with t(8;21) AML with informed consent, were cultured as described. Approval was obtained from the institutional review board of Shanghai Jiao Tong University School of Medicine. Kasumi-1 cells at exponential growth phase were co-incubated with oridonin at the absence or presence of pan-caspases inhibitor z-VAD-fmk (BD Inc), casp-3 inhibitor z-DQMD-fmk (Sigma) or proteasome inhibitors [Lactacystin (Lact), MG-132 and Proteasome Inhibitor I (PSI); Calbiochem, CA] at indicated concentrations. The fresh cells isolated were treated with oridonin at indicated concentrations. U937-A/E cells were co-incubated with PA at the presence or absence of oridonin.

Cell proliferation was analyzed using a Cell Counting Kit-8 (CCK-8; Dojindo, Japan). Cell viability was estimated by trypan blue dye exclusion. Cell morphology was assessed by Wright’s staining of cells prepared by cytospin centrifugation. The externalization of PS was evaluated by Annexin V (AV) detection using an AV-FITC (for fluorescein isothiocyanate) Kit (Clontech, BD; USA); Cell cycle analysis and MTP assessment were performed as described.

Terminal deoxynucleotidyl transferase (TdT) in situ cell death detection (TdT-mediated dUTP nick end labeling, TUNEL) and DNA fragmentation

Apoptosis in individual cells was detected using an in situ cell death detection kit (Roche Diagnostics GmbH, Germany) according to manufacturer’s instruction and analyzed under a fluorescence microscope. For assays of DNA fragmentation, cells were collected and lysed. RNase was added to the lysate and incubated for 30 min at
37°C. Proteinase K and SDS were added, followed by incubation at 50°C for 16 h. DNA was extracted, precipitated and electrophoresed. The stained gel was visualized by transillumination with ultraviolet (UV) light and photographed.

**Analysis of the expression of AE target genes at mRNA and protein levels**

Total RNA was purified from cells using a Trizol Reagent (Gibco/BRL). First-strand cDNA was synthesized using 2 μg RNA and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega). The PCR amplification was conducted. For quantitative RT-PCR, a SYBR Green PCR Master mix (Applied Biosystems) was employed, and β-actin was co-amplified as an endogenous control to standardize the amount of the sample RNA added to the reaction. The primers used were listed in Table 1 of supporting information.

To detect the expression of G-CSFR and MPO at protein level, 1×10^6 Kasumi-1 cells were washed with PBS and incubated with FITC-conjugated anti-human G-CSFR or MPO antibody (Beckman Coulter, CA, USA) according to manufacturer’s instruction. After washed with PBS, the cells were resuspended in PBS and analyzed by flow cytometry.

**Chromatin Immunoprecipitation (ChIP)**

ChIP was conducted using the ChIP-IT Kit (Active Motif, USA) according to the manufacturer’s instruction with antibodies against the ETO (Santa Cruz), Acetyl-Histone H3, and histone deacetylase 1 (HDAC1) (Upstate). Briefly, cells treated or untreated with oridonin were fixed using formaldehyde. The DNA was sheared into small, uniform fragments using sonication, and specific protein/DNA complexes were immunoprecipitated using an antibody directed against the DNA-binding protein of interest. Following immunoprecipitation, cross-linking was reversed, the proteins were removed by treatment with Proteinase K, and the DNA was purified and analyzed by semi-quantitative RT-PCR using primers listed in Table 1 of supporting information.

**Plasmid, mutagenesis and transfection**

To explore the exact cleavage site on AE, the full length AE-containing
pFLAG-CMV4-AE plasmid was used, and residues Asp (D) at 171, 188 and 192, were substituted by Ala (A) generated by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, CA, U.S.A) according to manufacturer’s instruction. After being sequenced, 20 µg of pFLAG-CMV4-AE or mutant pFLAG-CMV4-AE were mixed with U937 cells and transferred to electroporation cuvettes with a 0.4 cm gap (Bio-Rad, CA). Electroporations were performed using a Gene-Pulser II (Bio-Rad) at 280 V and 1050 µF. The samples were then transferred to complete RPMI 1640 medium and incubated at 37°C in 5% CO₂. Twelve hrs later, the cells were treated with oridonin, and the effect of oridonin on mutant AE was analyzed by Western blot. U937 cells transfected with mutant AE were also co-incubated with 1000 µg/ml G418 (Chemicon) for 30 days to select cells stably expressing mutant AE. The cells were then treated with oridonin and analyzed.

**Western blot analysis**

Cell pellets were lysed, protein extracts were quantitated and loaded on 8%-12% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose membrane. The membrane was incubated with primary antibody, washed, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz). Detection was performed by using a chemiluminescent Western detection kit (Cell Signaling). The change in cytoplasmic cyt C was detected using a Cytochrome C Releasing Apoptosis Assay Kit (BioVision, CA) according the manufacturer’s instruction.

**Murine models and oridonin treatment**

All mice used in this study were bred and maintained in a specific pathogen-free environment. Six-to eight-week-old C57 or FVB/NJ mice were irradiated by a high energy linear accelerator (6 MV, Siemens Primus) at a dose of 450 cGy. Twenty four hrs later, C57 mice were intravenously (iv) injected with 3×10⁶ leukemic cells expressing a truncated AE (AEtr),³⁷ and FVB/NJ mice were injected with 2×10⁵ cells expressing PML-RARα⁶⁶ via tail vein. When they became moribund, the mice were sacrificed, and splenic cells were isolated and inoculated into secondary recipients for passage. Xenograft model in nude mice was established by subcutaneously (sc)
inoculation of 4×10^7 Kasumi-1 cells into the right flank.

Five days after leukemic cell transplantation, the C57 mice were treated intraperitoneally (ip) with vehicle (1% PluronicF68), low dose Ara-C (25 mg/kg/d for 5 days; Ara-C-L), high dose Ara-C (25 mg/kg/d for 10 days; Ara-C-H), oridonin (2.5, 7.5, or 15 mg/kg/d, five days per week for 2 weeks), or oridonin (7.5 mg/kg/d) plus Ara-C-L. The FVB/NJ mice were treated with oridonin at 7.5 or 15 mg/kg/d, or ATRA (Sigma) at 10 mg/kg/d as a positive control. The nude mice received oridonin treatment (7.5 or 15 mg/kg/d.) when tumor was measurable. Caliper measurements of the longest perpendicular tumor diameters were performed every day to estimate the tumor volume, using the following formula: 4\pi/3\times(width/2)^2\times(length/2), representing the three-dimensional volume of an ellipse^47. Animals were sacrificed when their tumors reached 2 cm or when the mice became moribund. Survival was evaluated from the first day of treatment until death. TUNEL assay was performed to detect in situ apoptosis on tissue section using a DeadEnd™ Colorimetric TUNEL System (Promega) according to manufacturer’s instruction.

**Statistical analysis**

The life-span of mice was analyzed by Kaplan-Meier methods, while the tumor volume was analyzed with one-way ANOVA and independent-sample T test. P values <.05 were considered statistically significant and were derived from two-sided statistical tests. All statistical analysis was performed using the software SPSS 12.0 for Windows (Chicago, IL).

**RESULTS**

**Oridonin inhibits growth and induces apoptosis in t(8;21) leukemic cells**

We investigated the effects of oridonin on t(8;21)-bearing cells. We found that oridonin at 0.5~5.0 µM inhibited proliferation and growth of Kasumi-1 cells (Fig. 1, B and C). In particular, the rate of inhibition was 13%, 41%, 52%, 95%, 99% for 0.5, 1, 2, 5, 10 µM respectively. Therefore, oridonin concentrations of 0.5, 2 and 5 µM were utilized to perform the majority of the experiments. Unlike Kasumi-1 cells, primary
t(8;21) leukemic cells showed little proliferation and the cell viability was gradually decreased when no drug was added. Oridonin reduced the number of viable cells in

Fig. 1. Oridonin inhibits cell growth and proliferation of t(8;21)-bearing leukemic cells. (A), chemical structure of oridonin; (B), effects of oridonin on Kasumi-1 cell proliferation detected by using a CCK-8 Kit; (C), effects of oridonin on Kasumi-1 cell growth; (D), effects of oridonin on primary leukemic cells isolated from a relapsed patient with t(8;21) leukemia. ATO, arsenic trioxide.

samples from relapsed or refractory patients (3 cases) and de novo cases (2 cases), exemplified by cells from a patient with resistance to high dose Ara-C (Fig. 1D). This inhibition was greater at concentration of 5 µM. We also tested the effects of oridonin on NB4, HL-60, U937, and K562 cell lines, and found that the IC50 of oridonin for these cells were 8-20 µM, much higher than that for Kasumi-1 cells (1.33 µM). For fresh leukemic cells, oridonin at 5 µM induced apoptosis in a much smaller
proportion of cells in samples from patients with M3, M4, M2 types of AML without t(8;21) and CML than that in cells with t(8;21) (Table 2 in supporting information).

Examination on t(8;21)-bearing leukemic cells upon oridonin treatment revealed the appearance of morphologic characteristics of apoptosis, such as shrinking cytoplasm, condensed chromatin, and nuclear fragmentation with intact cell membrane (Fig. 2A). Externalized PS, revealed by Annexin V staining, was significantly increased in Kasumi-1 cells treated with oridonin (Fig. 2B). After treatment with oridonin at 2 µM for 48 hrs, TdT labeling showed a proportion of Kasumi-1 cells experiencing DNA fragmentation (Fig. 2C). The sub-G1 cells also increased while Kasumi-1 cells were treated with oridonin at 2 µM for 24 hrs (Fig 2D). DNA electrophoresis using samples isolated from oridonin (2 µM) treated Kasumi-1 cells confirmed DNA fragmentation (Fig. 2E). These results demonstrated that oridonin induced apoptosis in Kasumi-1 cells. However, oridonin did not induce differentiation of Kasumi-1 cells, as determined by assessment of cell morphology, nitroblue tetrazolium (NBT) reduction assay, and detection of differentiation antigens CD11b and CD14 (data not shown).

**Collapse of Δψₘ, activation of Casp-3 and downregulation of Bcl-2**

To evaluate the effects of oridonin on Δψₘ and to determine whether cells with a low Δψₘ also lose plasma membrane integrity, we double stained the Kasumi-1 cells with propidium iodide (PI) and rhodamine 123 (Rh123), a lipophilic cation that is taken up by mitochondria in proportion to the Δψₘ. The results showed that untreated living cells were PI negative and strongly stained by Rh123 (PI-, Δψₘ high) (Fig. 2F). Upon treatment with oridonin at 0.5, 2 or 5 µM, a fraction of PI-negative and low-Rh123-staining (PI-, Δψₘ low) Kasumi-1 cells appeared in a dose- and time-dependent manner; in particular, the inhibition was greater using Oridonin at 5 µM for 48 hrs (Fig. 2F), suggesting that oridonin decreased the Δψₘ without altering plasma membrane permeability.

We tested the change of casp-3 in oridonin-induced apoptosis of Kasumi-1 cells and found that casp-3 precursor (pro-casp-3) was decreased with concentration of 5 µM of oridonin with generation of an active form, accompanied by a cleavage of its
Fig. 2. Apoptotic effects of oridonin on t(8;21) leukemic cells. (A), morphologic changes in Kasumi-1 (upper panel) and fresh leukemic (lower panel) cells untreated or treated with oridonin; (B), expression of Annexin-V on Kasumi-1 cells treated with oridonin at indicated concentrations; (C), *in situ* TdT labeling of Kasumi-1 cells.
Upper panel, control; lower panel, Kasumi-1 cells treated with 2 µM oridonin for 48 hrs; (D), cell cycle analysis showing sub-G1 content in cells treated with 2 µM oridonin. Upper panel, control; lower panel, Kasumi-1 cells treated with 2 µM oridonin for 48 hrs; (E), DNA fragmentation in Kasumi-1 cells treated with 2 µM oridonin for 48 hrs; (F), PI negative Rh123 positive [PI (-) Rh123 (+)] cells are decreased upon oridonin treatment, in particular, the inhibition was greater using oridonin treatment at 5 µM for 48 hrs; (G), effects of oridonin on Casp-3, PARP, Bcl-2, survivin, NFκB, and Fas-L. β-actin is used as a loading control.

substrate, poly ADP-ribose polymerase (PARP) (Fig. 2G and Fig. 3A). The pro-casp-9 was also decreased, while the expression of cytoplasmic cyt C was increased (Fig. 3A). Moreover, pre-treatment with pan-caspases inhibitor z-VAD.fmK and casp-3 inhibitor z-DQMD-fmk significantly reduced the percentage of AV-positive Kasumi-1 cells (Fig. 3B). These results suggested that caspases played a critical role in oridonin induced apoptosis of Kasumi-1 cells. Oridonin treatment at 5 µM also led to downregulation of Bcl-2, while the expression of Fas-ligand (Fas-L) and survivin was not modulated (Fig. 2G).

**Oridonin induces degradation of AE oncoprotein with D188 as a possible cleavage site for casp-3**

Western blot analysis using an anti-ETO antibody whose epitope maps at the C-terminus of ETO was performed to explore whether oridonin treatment could modulate the expression of AE oncoprotein. We found that oridonin triggered a degradation of the 94 kDa AE with generation of an approximately 70 kDa fragment (ΔAE) (Fig. 3A, upper panel). Detection using an anti-AML1 antibody (maps the Runx homology domain) confirmed the degradation of AE while the 70 kDa cleavage fragment was not detected (data not shown), indicating the ΔAE should contain the C-terminal of ETO moiety. In U937-A/E cells, the ponasterone A (PA)-induced expression of AE was also degraded by oridonin treatment with generation of ΔAE (Fig. 3A, lower panel). Indeed, treatment with PA resulted in high level expression of
Fig. 3. Effects of oridonin on AE fusion protein and the possible mechanisms. (A), in Kasumi-1 cells, oridonin causes a degradation of AE with generation of an approximately 70 kDa catabolic fragment. In this expression, Western blot assay was done using an anti-ETO antibody whose epitope maps at the C-terminal of ETO. Effects of oridonin on casp-3, PARP, casp-9 and cytoplasmic cyt-C are also shown. In U937-A/E cells, oridonin treatment also causes degradation of AE (lower panel, using the anti-ETO antibody). (B), Effects of pan-caspases inhibitor on oridonin-induced apoptosis; (C), the presence of PA which induces the expression of AE enhances apoptotic effect of oridonin on U937-A/E cells, as revealed by reduction of viable cells upon oridonin treatment; (D), pre-treatment with pan-caspase inhibitors
z-VAD.fmk (left panel) and casp-3 inhibitor z-DQMD-fmk (right panel) prevents AE from proteolysis; (E), pre-treatment with three proteasome inhibitors, PSI, lact, and MG-132, can not prevent oridonin-induced degradation of AE.

AE but not apoptosis of U937-A/E cells (Fig. 3C). However, under co-incubation with PA, oridonin treatment significantly decreased AV-negative, PI-negative proportion (viable cells) of U937-A/E cells (Fig. 3C), suggesting that the expression of AE might enhance the cellular apoptotic response to oridonin.

Pre-treatment with z-VAD.fmk not only suppressed oridonin-induced apoptosis (Fig. 3B), but also abrogated catabolism of AE and generation of $\Delta$AE (Fig. 3D, left panel). Furthermore, pre-treatment with casp-3 specific inhibitor z-DQMD-fmk almost completely blocked AE degradation and $\Delta$AE generation (Fig. 3D, right panel). However, pre-treatment with proteasome inhibitors, PSI, MG 132 and Lact, could not block oridonin-induced AE degradation (Fig. 3E). These results suggested that oridonin-induced degradation of AE was possibly mediated by casp-3.

Casp-3 prefers a DXXD-like motif-containing substrate with some variants including XXXD-X. By analyzing the amino acid sequence, we found three DXXD-like motifs, DVPD$_{99}$G, DLRD$_{473}$R, and DAED$_{549}$L in AE amino acid sequence. However, cleavage at any one of the 3 motifs could not generate a 70 kDa $\Delta$AE containing the C-terminal of ETO. In addition, only three Asp existed in the region between D133 and D287 (Fig. 4A). Hence, we hypothesized that the cleavage site(s) should be a XXXD-like, and might possibly reside on D171, D188 or D192 of AE oncoprotein. We thus performed site-directed mutagenesis to generate the substitution of A for D171, D188, and D192 (denoted as D171A, D188A and D192A, respectively; Fig. 4A). The mutant AEs were transfected into U937 cells which were then treated with oridonin. The results showed that upon oridonin treatment, the ‘wild type’, D171A and D192A-AE could be degraded with generation of $\Delta$AE. However, oridonin treatment could not cause catabolism of AE or generation of $\Delta$AE in D188A-AE mutant (Fig. 4B), suggesting D188 to be the cleavage site. Interestingly, we found that U937 cells stably expressing D188A-AE showed a less sensitivity to
oridonin as compared to U937 cells expressing wild type or D192-AE (Fig. 4C).

Fig. 4. Substitution of Ala for Asp at 188 abrogates AE catabolism and confers an extent of oridonin resistance to U937 cells. (A), schematics for mutations introduced into the AE fusion protein; (B), D188A mutant in AE abrogates AE degradation or ΔAE generation upon oridonin treatment. Wild type, D171A-, D188A- and D192A-AEs are transfected into U937 cells which are then treated without or with oridonin. Western blot is performed using an anti-ETO antibody to analyze the effects of oridonin on AE mutants; (C), oridonin induces a much lower inhibition rate of U937 cells expressing D188A-AE compared to U937 cells expressing wild type or D192-AE, suggesting D188A-AE confers oridonin resistance to U937 cells. In this experiment, U937 cells expressing AE, D188A- and D192A-AE are treated without or
with oridonin, and trypan blue dye exclusion assay is done. Inhibition rate is determined by viable cells in oridonin treatment group to that in vehicle control group.

Reprogramming of AE targets

Whether degradation of AE could lead to reprogramming of the expression of its target genes (e.g., Bcl-2, G-CSFR, MPO, IL-3, and GM-CSF) was tested. Bcl-2 was shown to be downregulated at protein level (Fig. 2G). At mRNA level, oridonin treatment at 5 µM suppressed the expression of Bcl-2, G-CSFR and MPO in Kasumi-1 cells as detected by semi-quantitative RT-PCR, while AE was not significantly altered (Fig. 5A). The expression of these genes was further analyzed by real-time RT-PCR and the copy number of the gene transcripts at each time point (Cn) was compared to that of control (C0) (Cn/C0). We found that upon oridonin treatment, the mRNA level of AE was not perturbed significantly. While Bcl-2, G-CSFR and MPO exhibited downregulated transcript levels, IL-3 and GM-CSF were upregulated (Fig. 5B). By analysis using direct immunofluorescence assay detected by flow cytometry, we found that upon oridonin treatment, the G-CSFR and MPO were also downregulated (Fig. 5C and D).

The reversible acetylation of histones by histone deacetylases (HDACs) and acetyltransferases (HATs) plays a fundamental role in gene transcription. We tested whether oridonin might reverse epigenetic alterations. BCL-2 could bind to AML1 and AE through its DNA binding sequence for AML1 (TGTGGT).27 Using ChIP assay we found that in Kasumi-1 cells treated with oridonin, the binding activity of AE with BCL-2 promoter was decreased (Fig. 5E). This was confirmed in U937-A/E cells (Fig. 5F). However, the interaction between HDAC1 and acetyl histone H3 with BCL-2 promoter was not altered significantly (Fig. 5E). Oridonin treatment also caused dissociation of MPO promoter from AE and acetyl histone H3, IL-3 promoters from AE and HDAC1, GM-CSF promoters from HDAC1, and enhanced binding of IL-3 promoter to acetyl histone H3 (Fig. 5, E and F).
Fig. 5. Effects of oridonin on AE targets. (A), semi-quantitative RT-PCR analysis shows a downregulation of Bcl-2, G-CSFR and MPO in Kasumi-1 cells treated with oridonin; (B), quantitative real-time RT-PCR analysis of expression of AE target genes. (C and D), flow cytometry analysis shows a diminution in expression of G-CSFR (C) and MPO (D) on Kasumi-1 cells treated with oridonin; (E and F) Pharmacologic modulation of AE, HDAC1 and acetyl histone H3 on promoters of BCL-2, MPO, IL-3 and GM-CSF. ChIP assay was done in Kasumi-1 (E) and U937-A/E (F) cells treated with oridonin at indicated concentrations using anti-ETO, HDAC1 and acetyl histone H3 antibodies.
Oridonin prolongs survival and inhibits tumor growth in t(8;21) but not t(15;17) leukemia murine models

Since a truncated form of AE lacking its C-terminal 200-aa (AEtr, Fig. 4A) strongly induced leukemia development, and the D188 cleavage site was retained in the truncated form, we evaluated the efficacy of oridonin on this AEtr murine model. C57 mice receiving 3×10^6 fresh leukemic cells isolated from spleen of AEtr AML mice were treated with oridonin 5 days after leukemic cell transplantation. The results showed that oridonin prolonged survival time of mice for 21.7%-43.5% (median, 23 days for control mice versus 28, 30, 33 days for mice treated with oridonin at 2.5, 7.5, and 15 mg/kg, respectively; \( P < 0.0001 \) (Fig. 6A). In mice treated with vehicle alone, splenomegaly was observed, while histological examination revealed extensive infiltrations of immature blast cells in hematopoietic organs (peripheral blood, spleen, and bone marrow) as well as liver, which destroyed the normal architectures of these tissues. Oridonin significantly reduced disseminated disease and prevented destruction of tissue architectures (Fig. 6D). TUNEL assay revealed that oridonin induced apoptosis of leukemic cells in liver, spleen and bone marrow of mice (Fig. 6E). Ara-C at low dose (Ara-C-L, 25 mg/kg/d for 5 days) exerted therapeutic efficacy similar to that of oridonin at 2.5 mg/kg (Fig. 6B). In mice treated with 25 mg/kg/d Ara-C for 10 days (Ara-C-H), 5 mice died on day 18 due to severe bone marrow suppression (Fig. 6, B and F), while the remaining 4 mice survived for 34 to 43 days. Treatment with high dose Ara-C also led to significant reduction of body weight (Fig. 6G). However, oridonin caused neither bone marrow suppression nor weight loss (Fig. 6F and G), indicating oridonin to be a relatively safe agent. Combined use of oridonin at 7.5 mg/kg/d and Ara-C-L further prolonged survival of mice (Fig. 6B, \( P = 0.0006 \) and 0.0014 as compared to Ara-C-L and Ori 7.5 mg/kg/d treatment groups, respectively), with median survival of 34.1 days (49.6% higher than that of control mice), and 4 (33%) mice survived more than 39 days (increased 69.6% of survival time than that of control mice).

In nude mice inoculated with 4×10^7 Kasumi-1 cells sc, all animals developed a
Fig. 6. *In vivo* efficacy of oridonin on t(8;21) leukemia. (A), oridonin prolongs life-span of C57 mice bearing AEtr-expressing leukemic cells; (B), oridonin (7.5 mg/kg/d) plus Ara-C (25 mg/kg/d for 5 days, Ara-C-L) further improves survival of the mice; (C), however, oridonin treatment can not prolong life span of FVB/NJ mice bearing PML-RARa-expressing leukemic cells; (D), oridonin reduces leukemic cell
infiltration in C57 mice; (E), TUNEL assay is performed on specimen from C57 mice. The results show that oridonin induces apoptosis of leukemic cells in vivo; (F), marrow pathological section stained with hematoxylin-eosin (HE) of mice treated with Ara-C and oridonin; (G) Changes in body weight of mice treated with oridonin and Ara-C; (H) oridonin inhibits tumor growth in nude mice inoculated with Kasumi-1 cells. The numbers in parentheses represent the number of animals used in each group of this study.

measurable tumor after a mean of 5 (3 to 7) days. Oridonin at 7.5 and 15 mg/kg significantly inhibited tumor growth compared to controls mice (P=0.0004, respectively; Fig. 6H). However, oridonin at 7.5 and 15 mg/kg/d did not prolong survival of FVB/NJ mice injected with PML-RARα-expressing cells (P=0.11, Fig. 6C), while ATRA at 10 mg/kg/d did show significant therapeutic efficacy (P<0.0001). Taken together, these results demonstrated that oridonin has significant in vivo anti-leukemia efficacy for t(8;21) AML.

**DISCUSSION**

Which types of hematological malignancies could represent the most sensitive ones to oridonin treatment, and whether oridonin could be used in clinic setting remain unclear. Here we showed that oridonin triggered apoptosis in more than 50% of t(8;21) leukemic cells in vitro at concentration ≥2 µM accompanied by degradation of AE oncoprotein, and showed significant anti-leukemia efficacies with low adverse effects in vivo. These data suggest possible beneficial effects for patients with t(8;21) AML.

The extrinsic and intrinsic apoptotic pathways that ultimately lead to activation of effector caspases (Casp-3, -2 and -7) have been characterized. The extrinsic pathway is initiated by ligation of death receptors (CD95/Fas, TNF receptor, and TRAIL receptor) to stimulate activator caspases (casp-8 and -10), which in turn cleave and activate effector caspases. The intrinsic pathway requires disruption of the mitochondrial membrane and release of proteins, and disruption of this pathway is
extremely common in cancer cells. We found that oridonin disrupted the Δψm of Kasumi-1 cells without altering plasma membrane permeability (Fig. 2F), downregulated Bcl-2, (Fig 2G), activated casp-9 and caused the release of cyt C to cytoplasm (Fig. 3A), suggesting the mitochondrial permeability transition transition pore is opened and the functions of mitochondria are impaired and machineries of death are unleashed. However, oridonin did not modulate the expression of Fas-L, or survivin which is a member of inhibitor of apoptosis protein (IAP) family (Fig 2G). At protein level, oridonin did not regulate the NFκB expression (Fig. 2G), yet modulation of NFκB might also play a role in oridonin-induced apoptosis, because oridonin was shown to interfere with the DNA-binding activity of NFκB to its response DNA sequence, and affect the translocation of NF-kB from the cytoplasm to nuclei without altering IκB-α phosphorylation and degradation. Though changes of TNF, TRAIL and casp-8 and -10 were not determined, it was unlikely that the extrinsic apoptotic pathway played a major role in oridonin induced apoptosis. The decrease of the pro-casp-3 and increase of the active p17 subunit, as well as the proteolysis of PARP (Fig. 2G and Fig. 3A) indicated that casp-3 was activated. Thus oridonin may trigger apoptosis by insulting mitochondria and activating the intrinsic apoptosis pathway which results in activation of effector caspases.

The AE oncoprotein is critical for leukemogenesis of t(8;21) AML. Intriguingly, oridonin treatment caused degradation of AE with generation of an approximately 70 kDa fragment (Fig. 3A). In U937-A/E cells, the PA-induced expression of AE enhanced the apoptotic effects of oridonin, whereas oridonin also induced degradation of AE in an identical manner to that in Kasumi-1 cells (Fig. 3A, lower panel). On the other hand, the transcription of AE was not significantly altered (Fig. 5A and B). These results demonstrated a cleavage of AE induced by oridonin. Consequently, degradation of AE might cause reprogramming of its target genes. Our results seem to confirm this notion. As shown in Fig. 2G and Fig. 5A and B, BCL-2 was downregulated; this might result from cleavage of AE which led to transcriptional inactivation (Fig. 5A and B), and activation of intrinsic apoptotic pathway might also play a role. Previous studies showed that murine homolog of human AML1,
PEBP2/CBF, regulated murine MPO,\textsuperscript{51,52} and AML1 binding site was found in human MPO\textsuperscript{53}, suggesting that AE might suppress MPO transcription and AE catabolism might lead to upregulation of MPO. However, oridonin treatment caused downregulation of MPO (Fig. 5A and B), suggesting that other molecules as well as chromatin confirmation could involve the regulation of MPO gene expression, and this could be a complex scenario. Using ChIP assay, we found that in Kasumi-1 and U937-A/E cells treated with oridonin, MPO promoter was dissociated from AE, and slightly from HDAC1 (Fig. 5, E and F). Theoretically these phenomena seemed to be able to facilitating MPO transcription, but the binding of MPO promoter to acetyl histone H3 decreased markedly at 48 h of treatment time course (Fig. 5E, 2\textsuperscript{nd} panel). This event might silence MPO, corroborating a report showing the upregulation of MPO by AE mediated indirectly through upregulation of C/EBP\textsubscript{ε}.\textsuperscript{29} The mechanisms for G-CSFR downregulation by oridonin treatment (Fig. 5, A through C) might be similar to these. On the other hand, IL-3 has a consensus TGTGGT and a similar TGTGGG sequence in its promoter, thus can be activated by AML1 and repressed by AE.\textsuperscript{26} As shown in Fig. 5E and F, oridonin dissociated IL-3 promoter from AE and HDAC1, and slightly enhanced binding to acetyl histone H3. These can explain the upregulation of IL-3 (Fig. 5B). GM-CSF is another TGTGGT-containing AML1 target gene suppresses by AE.\textsuperscript{30} Upon oridonin treatment of Kasumi-1 cells, the expression of GM-CSF was increased (Fig. 5B). Unlike IL-3, GM-CSF promoter was dissociated from AE slightly; however, when Kasumi-1 cells were treated with oridonin at 5 \(\mu\)M for 48 h, GM-CSF promoter was completely dissociated from HDAC1, suggesting that release of transcription suppressor may mainly contribute to upregulation of GM-CSF, and recruitment of HDAC1 suppressor complex might also involve in GM-CSF inactivation by AE. Since the regulation network of gene expression is complex where regulators as well as status of DNA methylation, histone acetylation, methylation and other modifications play critical roles, these results only provide a first step to understand the chemical biology of oridonin on t(8;21) leukemic cells.

Degradation of protein through hydrolysis of peptide bonds is a complex process. In APL, degradation of the PML-RAR\textsubscript{α} fusion protein by ATRA and ATO has been
shown to be mediated by proteasome and/or caspases.\textsuperscript{54,55} We tested the roles for caspases and proteasome to play in AE degradation. We found that pan-caspase inhibitor and casp-3 inhibitor not only blocked oridonin-induced apoptosis in Kasumi-1 cells, but also abrogated AE degradation (Fig. 3B and D). However, proteasome inhibitors used in this study could not obstructed AE catabolism (Fig. 3E). Indeed, the proline-rich AML1 protein was shown to be degraded through the ubiquitin-proteasome system, while proteasome inhibitors were able to protect AML1 from proteolytic degradation.\textsuperscript{56} Since casp-3 is the executioner of apoptosis induced by oridonin, it is likely that AE degradation was mediated by casp-3. Because AE was cleaved with generation of a 70 kDa fragment containing the carboxyl terminal of ETO, we speculated the cleavage site(s) should be localized in D171, 188 or 192 at AML1 moiety or N-terminal of ETO moiety (Fig. 4A). To test this hypothesis, D171A, D188A and D192A mutants were generated and mutant AE was transfected into U937 cells which were then treated with oridonin. We found that the ‘wild type’, D171A and D192A-AE could be cleaved by oridonin-treatment with generation of ΔAE. However, oridonin could neither cause catabolism of AE nor generation of ΔAE in D188A-AE (Fig. 4B), suggesting the D188 was the cleavage site. Consequently, at cellular level D188A-AE conferred oridonin resistance to U937 cells (Fig. 4C). Hence the PD\textsuperscript{188}S in AE represents a novel cleavage site for casp-3. An intriguing question is raised here: can mutation in D188 perturb the leukemogenic activity of AE? Additionally, Yan et al\textsuperscript{37} reported that deletion of an AE C-terminal NcoR/SMRT-interacting region strongly induced leukemia development. Whether ΔAE acts as a dominant negative form for the full-length AE and thereby facilitates oridonin-induced apoptosis could be interesting questions for future work.

The in vivo efficacy of oridonin on t(8;21) leukemia had been demonstrated in two different murine models. In C57 mice bearing AEtr-expressing leukemic cells, oridonin prolonged survival and reduced infiltration of leukemia (Fig. 6, A and D). TUNEL assay showed that oridonin also induced apoptosis of leukemic cells in vivo (Fig. 6E). Moreover, severe bone marrow suppression and body weight loss were not observed in mice treated with oridonin as compared to high dose Ara-C treatment (Fig. 7).
6, F and G), suggesting oridonin to be a relatively safe agent. Combined use of oridonin at 7.5 mg/kg/d and Ara-C-L further prolonged survival of mice (Fig. 6B). In nude mice inoculated with Kasumi-1 cells, oridonin significantly inhibited tumor growth (Fig. 6H). These data suggest that oridonin could be effective in treating human t(8;21) leukemia.

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Oridonin, a diterpenoid extracted from medicinal herbs, targets AML1-ETO fusion protein and shows potent antitumor activity with low adverse effects on t(8;21) leukemia \textit{in vitro} and \textit{in vivo}

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