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Supplementary Materials for

Targeting of AML1-ETO in t(8;21) Leukemia by Oridonin Generates a Tumor Suppressor–Like Protein

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The PDF file includes:

Materials and Methods

Reagents

Oridonin (Zhao Wei Technology and Development) was dissolved in DMSO (Sigma). Oridonin formulation used in vivo treatment (provided by Jiangsu Hengrui Medicine Co., Ltd.) was dissolved in 5% glucose. Biotin-oridonin was kindly provided by F.-Y. Qiu (Guangzhou Institute of Biomedicine and Health, CAS). Cocktail of protease inhibitors (Roche Applied Science), 2′, 7′-dichlorofluorescin diacetate (DCFH-DA, Sigma), dithiobisnitrobenzoic acid (DTNB, Pierce Biotechnology), NAC (Sigma), TrxR from rat liver (sigma), recombinant human Trx expressed in *E.coli* (Sigma), GSH (Sigma), NADPH (Sigma) and insulin (Sigma) were dissolved in appropriate solutions as the instructions described.

Plasmids

Full length AML1-ETO was cloned into pSG5 (Stratagene), pcDNA3.1 (–) (Invitrogen), MigR1-GFP and pCMV4-Flag (Sigma) vectors. ΔAML1-ETO was cloned into pcDNA3.1 (+) (Invitrogen), MigR1-YFP, and pCMV4-Flag vectors. ΔAML1-ETO(ΔNHR2) was subcloned into pcDNA3.1 (–) and MigR1-YFP from pSG5-ΔAML1-ETO(ΔNHR2). The latter as well as AML1-ETO-GFP and pGL3-RUNX3 promoter luciferase reporter plasmids was a gift from S. Dong (Baylor College of Medicine). The mutations of AML1-ETO(D188V, D368A), ΔAML1-ETO(D368A), ΔAML1-ETO(C266A), ΔAML1-ETO(C347A) and

ΔAML1-ETO(C416A) were created on pFLAG-CMV4-AML1-ETO or pFLAG-CMV4- ΔAML1-ETO plasmid using QuikChange Site-Directed Mutagenesis Kit (Stratagene). To obtain His-tagged caspase-3, RHD, NHR1, NHR2, NHR3 and ZnF protein domains of AML1-ETO, their coding sequences were cloned into PET15b vector (Novagen) and schematic structures of the constructs are provided in Fig. 1B.

Cell Culture

Kasumi-1, Kasumi-1 cells with the expression of GFP or ΔAML1-ETO-GFP, NB4 and K562 cells were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics. SKNO-1 cells were cultured in RPMI 1640 medium supplemented with antibiotics, 10% FBS and 10 ng/ml recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF, Sigma). HeLa, 293T cells, Bosc23 and NIH3T3 cells were cultured in DMEM medium supplemented with 10% FBS and antibiotics.

Western blot analysis

Western blot was performed with standard protocols. AML1-ETO, ΔAML1-ETO, ΔAML1-ETO(ΔNHR2) and their derivatives were detected with anti-ETO antibody (Santa Cruz), followed by rabbit anti-goat IgG-horseradish peroxidase (HRP) antibody (Santa Cruz). Caspase-3, TrxR, Trx, AML1-ETO and AML1-ETO9a were detected by anti-caspase-3 (Cell Signaling), anti-TrxR (Santa Cruz), anti-Trx (IMCO Corporation) and anti-AML1 (Ab-2)

(Calbiochem)/anti-ETO (N-terminal) (Cell Signaling) antibodies respectively, followed by anti-rabbit IgG-HRP antibody (Amersham). β-Actin was used as internal control with anti-β-Actin (Sigma) antibody. Flag-ΔAML1-ETO-GFP was detected with anti-Flag antibody (Sigma) and anti-mouse IgG-HRP antibody (Cell Signaling). HRP activity was detected with enhanced chemiluminescence reagent (Millipore).

Immunofluorescence microscopy and image analysis

HeLa cells grown on cover glasses were transfected with AML1-ETO-EGFP or AML1-ETO9a-EGFP, ΔAML1-ETO-DsRed2, EGFP and DsRed2 using SuperFect Transfection Reagent (Qiagen). 24 hours later, the cells on cover glasses were fixed with 4% paraformaldehyde for 15 minutes. Images were captured by laser scanning confocal microscope (LSM 5, Zeiss) and analyzed by LSM Pascal software (Zeiss).

Co-IP and IP

293T cells were transiently transfected with Flag-tagged and/or non-Flag-tagged plasmids using SuperFect Transfection Reagent (Qiagen). 48 hours posttransfection, nuclear extracts were prepared as previously described *(S1)* and protein complexes were immunoprecipitated using monoclonal anti-Flag M2 agarose beads (Sigma) at 4°C overnight. For Co-IP, precipitated proteins were eluted in sodium dodecyl sulfate (SDS) loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with

indicated antibodies. For IP, precipitated proteins were resuspended in 100mM HEPES (pH 7.2) containing 10% glycerol for further assay.

ROS production determination

Intracellular ROS was measured using DCFH-DA. After oridonin treatment, 10 μM DCFH-DA was added into the media and incubated at 37℃ for 0.5 hour. After that, cells were washed with phosphate buffered saline (PBS) twice. The fluorescent intensity was measured by flow cytometry (LSR2，BD).

Assays for TrxR and Trx activities in cell lysates

TrxR and Trx activity were performed with an endpoint insulin assay *(S2)*. Briefly, Kasumi-1 cells were treated with 5 μM oridonin for indicated hours and lysed in the lysis buffer [20 mM Tris (pH7.5), 150 mM NaCl, 1% Triton X-100 and cocktail]. For the measurement of TrxR activity, 25 μg cell lysate from each cell extract was incubated in 50 μl buffer containing 55 mM HEPES (pH 7.6)/0.2 mM insulin/0.4 mM NADPH/2 mM EDTA/2 μM recombinant human Trx1 for 30 minutes. The reaction without Trx1 was used as the control. After incubation, 200 μl 1 mM DTNB in 6 M guanidine hydrochloride solution was added to stop the reaction, and then TrxR activity was represented by the A_{412} (Infinite M200, TECAN). For the measurement of Trx activity, 25 μg cell lysate from each cell extract was incubated in 50 μl buffer containing 55 mM HEPES (pH7.6)/0.2 mM insulin/0.4 mM NADPH/2 mM EDTA/600 nM TrxR from rat liver for 30 minutes. The reaction without TrxR was used as the control. The other procedures were the

same as the TrxR activity assay.

In vitro proteolysis of AML1-ETO protein

This experiment was performed as described *(S3)*. Briefly, Immunoprecipitated Flag-AML1-ETO, Flag-ΔAML1-ETO or their protein mutations from transfected 293T cells were preincubated with or without 0.2 mM oridonin for 1 hour, washed three times with PBS to remove oridonin, and incubated with purified recombinant caspase-3 in 100 mM HEPES (pH 7.2) containing 10% glycerol at 25 ℃ for 1 hour. The reaction was stopped by adding SDS loading buffer and then analyzed by Western blot.

SEC assay

293T cells were transiently transfected with pcDNA3.1 (–)-AML1-ETO/pSG5-AML1-ETO9a, and/or pcDNA3.1 (+)-ΔAML1-ETO using SuperFect Transfection Reagent (Qiagen), and cells were harvested 48 hours later. The nuclear proteins were extracted as described previously *(S1)* and subjected to SEC analysis using an ÄKTAFPLC chromatography system (GE Healthcare) on a Superdex 200 gel filtration column (1.0×30, GE Healthcare) preequilibrated with running buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, and 1 mM phenylmethanesulfonyl fluoride (PMSF)]

. Fractions were collected at 0.5 ml intervals and relative molecular masses were obtained by comparing elution volume to molecular mass standards. Column fractions were separated by SDS-PAGE and analyzed by Western blot with

indicated antibodies.

EMSA assay

293T cells were transiently transfected with pcDNA3.1 (–)-AML1-ETO, pcDNA3.1 (+)-ΔAML1-ETO or pcDNA3.1 (–)-ΔAML1-ETO(ΔNHR2) expression plasmid, and nuclear extracts were prepared as described previously *(S1)*. A double-stranded oligonucleotide probe corresponding to the AML1 binding site in the RUNX3 promoter (5'-GCCTGGTCCCTCAACCACAGAACCACAAGGCCAGGCCCT-3') was end-labeled with ^{32}P dATP (Perkin Elmer) using T4 Polynucleotide Kinase (Promega). Nuclear extracts as indicated were incubated with 2.5 ng double-stranded oligonucleotide probes for 0.5 hour at room temperature and analyzed by non-denaturing PAGE and the bands were detected by autoradiography.

Luciferase reporter assay

Luciferase reporter assays were performed in 293T cells transfected with a reporter corresponding to the *RUNX3* promoter and indicated plasmids using SuperFect Transfection Reagent (Qiagen). PRL-SV40 vector (Promega) was cotransfected as a control for transfection efficiency. Cells were harvested 48 hours posttransfection and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction. Total concentration of expression vectors was kept constant in all transfection experiments.

Proliferation, transformation and in vivo tumor formation assay

Bosc23 cells (2×10^6) were plated on 60 mm dish in DMEM supplemented with 10% FBS, and transfected after 24 hours with 5 μg MigR1-X [X: AML1-ETO-IRES-GFP, GFP, ΔAML1-ETO-IRES-YFP, ΔAML1-ETO(ΔNHR2)-IRES-YFP, YFP] plasmids and 2.5 μg Ecopac plasmids by Calcium Phosphate Transfection (Promega). Viral supernatants were collected 48 hours after transfection. NIH3T3 cells were infected with indicated viral supernatants and the GFP and YFP double positive cells were sorted out with MoFlo High Speed Cell Sorter (DakoCytomation).

For proliferation assay, 5×10^4 NIH3T3 cells expressing AML1-ETO, ΔAML1-ETO, ΔAML1-ETO(ΔNHR2), or coexpressing AML1-ETO and ΔAML1-ETO or ΔAML1-ETO(ΔNHR2) were seeded on six-well plates. Cells were counted by Z2 Coulter Particle Count (Beckman Coulter) after 5 days. For the transformation assay, 2×10^3 cells of each group were plated on six-well plates in DMEM with 10% FBS containing 0.3% agar on 0.5% agar base as previous described *(S4)*, and colonies were scored microscopically 14 days later. Tumor formation assay was performed by subcutaneous inoculation of 1×10^6 cells of each group into the neck of nude mouse. Tumors were detected with In Vivo Imaging System (Berthold Technologies) 16 days posttransplantation.

ChIP-qPCR and semiquantitative PCR

ChIP assay was performed as previously described *(S5)*. Real-time PCR was

performed using ABI 7000 Real-Time PCR System and the CT method ($2^{-(\text{CT of gene} - \text{CT of internal control})}$) *(S6)* was applied to quantify relative gene expression (*UBQLN1*, *CCPGI*, *IL-6R*, *BLNK*) and normalized to the expression of input. For semiquantitative PCR, total RNA was purified from cells using TRIzol Reagent (Invitrogen). β-Actin was used as an internal control. The primers were listed in table S2.

Serial replating assay

Serial replating assay was performed as described *(S7)*. Briefly, BM cells isolated from fluorouracil (25 mg/kg) treated mice were infected with indicated viral supernatants as above mentioned. Then the GFP and YFP double positive cells were sorted out and 10^3 cells were plated in M3434 complete methylcellulose media (StemCell Technologies) and cultured for 7 days (First plating round). After colonies were counted, the cultures were diluted and resuspended, and 10^4 cells were replated in M3434 media for an additional 7 days and colonies were counted (Second plating round).

Leukemia-initiating analysis

BM cells isolated from AML1-ETO9a leukemia mice were labeled with Lin-PerCP/Cy5.5, c-Kit-APC and Sca-1-PE antibodies (BD Pharmingen). The following two groups of cells were sorted out by cell sorter: $GFP^+/Lin^-/Sca\text{-}1^-/c\text{-}Kit^+$ and $GFP^+/Lin^-/Sca\text{-}1^-/c\text{-}Kit^-$. Irradiated (450 cGy) C57BL/6 mice were intravenously injected with 20, 100, 1000 or 2000 cells of

these groups. The incidence of leukemia and the survival time of the mice were followed up. The incidence of leukemia in C57BL/6 recipient mice and frequency of LICs were summarized. The frequency of LICs was calculated with Limiting Dilution Analysis Software (L-Calc).

Toxicity tests of oridonin in normal mice

C57BL/6 (6 to 8 weeks old) mice were treated with vehicle (5% glucose) or oridonin formulation (20 or 40 mg/kg per day) for 3 weeks. Body weight and blood picture of these mice were followed up.

Supplementary Figures

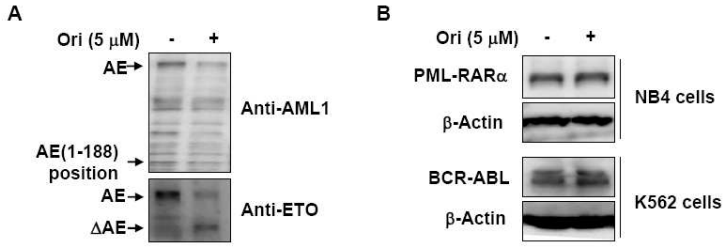

Fig. S1. Oridonin shows no effect on the generation of the N-terminal part of AML1-ETO and the protein status of PML-RARα or BCR-ABL. (A) Kasumi-1 cells were treated with oridonin and Western blot was performed to check the expression of AML1-ETO and its cleavage fragment by anti-ETO antibody or anti-AML1 antibody. **(B)** NB4 and K562 cells were treated with oridonin for 24 hours, and the protein status of PML1-RARα and BCR-ABL was detected by Western blot. β-Actin was used as internal control.

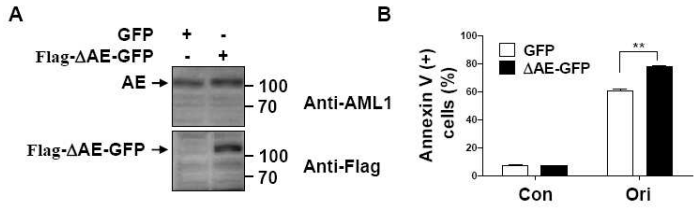

Fig. S2. Expression of ΔAML1-ETO enhances sensitivity of Kasumi-1 cells to oridonin. **(A** and **B)** Kasumi-1 cells with the expression of GFP or ΔAML1-ETO-GFP were generated by lentivirus transfection. The expression of ΔAML1-ETO in Kasumi-1 cells was detected by Western blot (A). Kasumi-1 cells with or without the expression of ΔAML1-ETO were treated with oridonin (5 μM) for 24 hours. Cells with Annexin-V^+ were counted by flow cytometry (mean ± SEM) (B). $^{**}p < 0.01$.

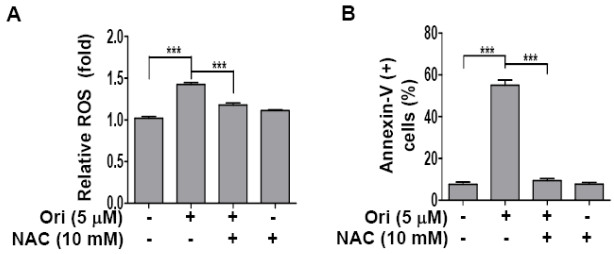

Fig. S3. NAC inhibits oridonin-induced ROS and apoptosis. (**A** and **B**) Kasumi-1 cells were preincubated with NAC for 1 hour before treatment with oridonin for 2 hours. Intracellular ROS was measured (mean ± SEM) (A), and cells with Annexin-V^+ were counted by flow cytometry (mean ± SEM) (B). ***p < 0.001.

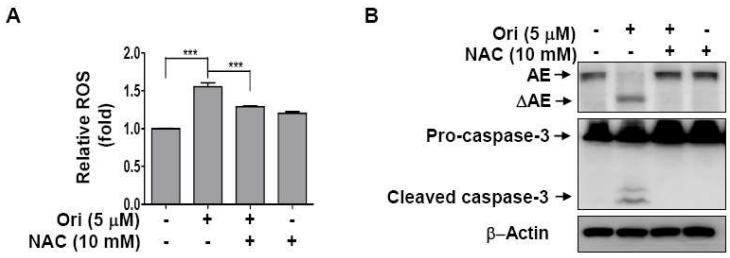

Fig. S4. NAC can block oridonin-induced ROS and ΔAML1-ETO generation in SKNO-1 cells. **(A)** SKNO-1 cells were pretreated with or without NAC for 1 hour, followed by treatment with oridonin for 2 hours and intracellular ROS was measured (mean ± SEM). ***$p < 0.001$. **(B)** SKNO-1 cells were pretreated with NAC for 1 hour, and then treated with oridonin for 12 hours. Caspase-3 activation and generation of ΔAML1-ETO were detected by Western blot.

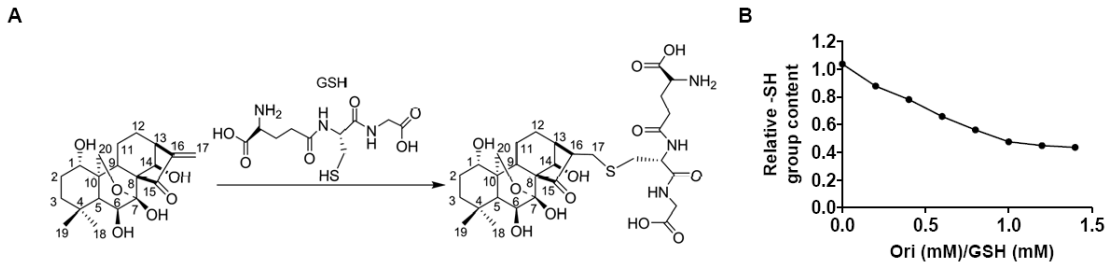

Fig. S5. Oridonin binds to GSH in vitro. (A) Chemical structure of oridonin and the reaction equation of oridonin and GSH. **(B)** DTNB assay was performed to measure -SH group content of GSH after reaction with oridonin (mean ± SEM).

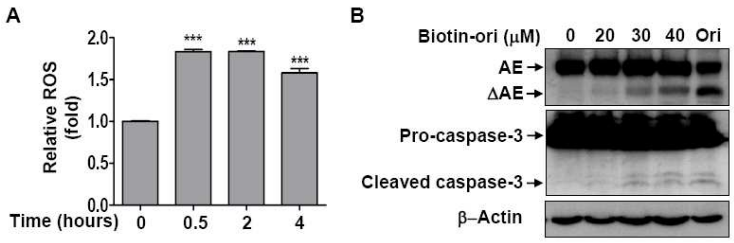

Fig. S6. Biotin-oridonin induces ROS and ΔAML1-ETO generation in Kasumi-1 cells. (A) Kasumi-1 cells were treated with biotin-oridonin (40 μM) for indicated hours and intracellular ROS was measured (mean ± SEM). ***$p < 0.001$. **(B)** Kasumi-1 cells were treated with biotin-oridonin. Caspase-3 activation and generation of ΔAML1-ETO were detected by Western blot. 5 μM oridonin was used as positive control.

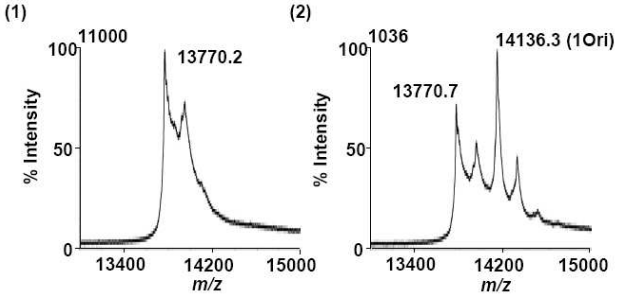

Fig. S7. Oridonin directly binds to Trx. Mass spectrum analysis of Trx in the absence (1) and in the presence (2) of oridonin.

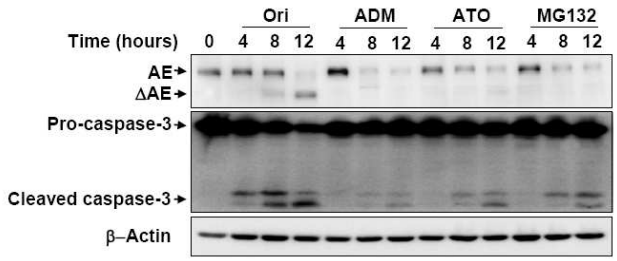

Fig. S8. Oridonin selectively leads to the generation of ΔAML1-ETO. Kasumi-1 cells were treated with oridonin (5 μM), doxorubicin (ADM, 1.3 μg/ml), MG132 (0.24 μM) or arsenic trioxide (ATO, 10 μM) for indicated hours. Western blot was performed to check the generation of ΔAML1-ETO and caspase-3 activation.

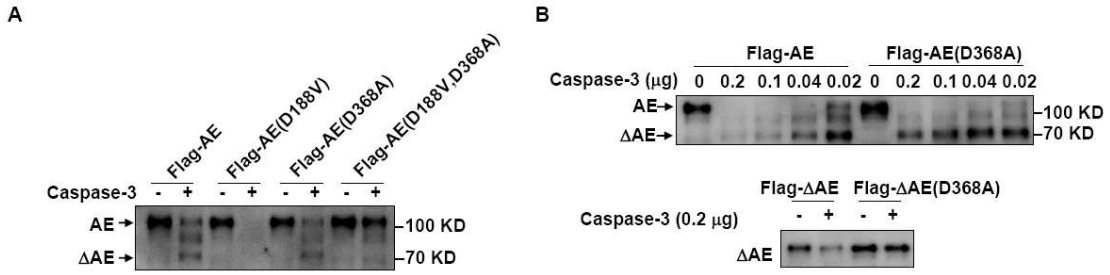

Fig. S9. D368A mutation of AML1-ETO or ΔAML1-ETO prevents it from cleavage by caspase-3. **(A)** Immunoprecipitated Flag-AML1-ETO, Flag-AML1-ETO(D188V), Flag-AML1-ETO(D368A) or Flag-AML1-ETO(D188V, D368A) was incubated with recombinant activated caspase-3 (0.2 μg) for 1 hour and the mixtures were detected by Western blot. **(B)** Flag-AML1-ETO, Flag-AML1-ETO(D368A), Flag-ΔAML1-ETO or Flag-ΔAML1-ETO(D368A) was incubated with indicated amount of recombinant activated caspase-3 for 1 hour. Western blot was performed to detect the proteolysis of AML1-ETO or ΔAML1-ETO.

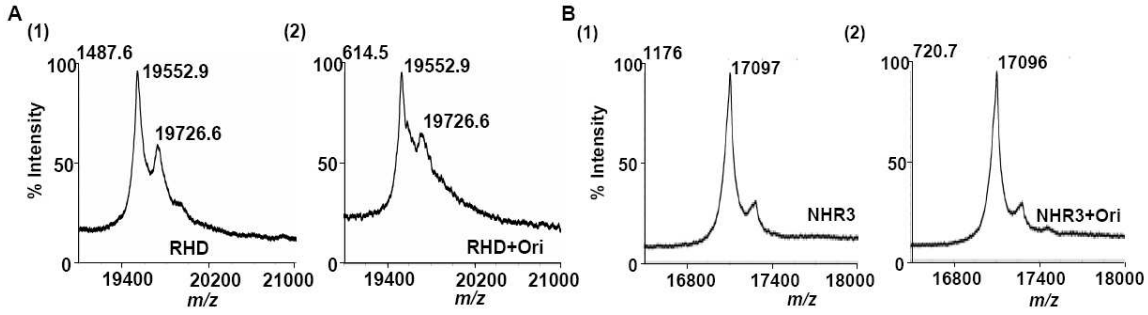

Fig. S10. Oridonin is unable to bind to RHD and NHR3 domains. (A and **B)** Mass spectrum analysis of RHD (A) or NHR3 (B). (1) Protein domain profiles in the absence of oridonin. (2) Protein domain profiles in the presence of oridonin.

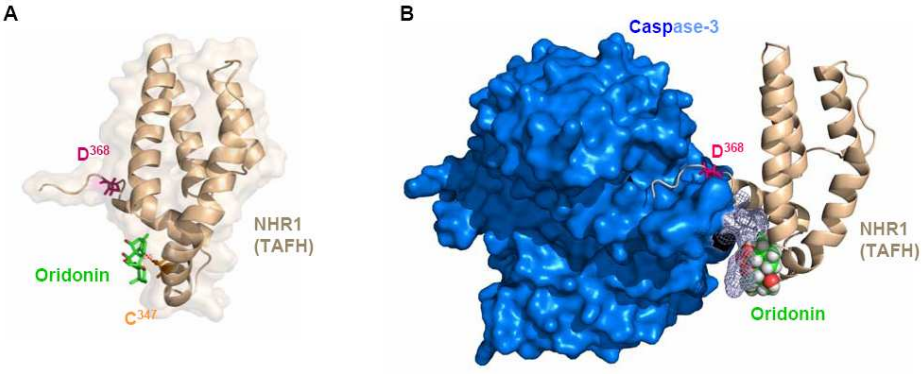

Fig. S11. The mode of oridonin binding to C^{347} of NHR1 hinders the interaction between NHR1 and caspase-3. (A) Oridonin binding to ETO NHR1 (TAFH) domain as simulated by DOCK 4.0 program. Oridonin binds to the surface of NHR1 domain and forms covalent interaction with -SH of C^{347} side chain. **(B)** Binding of oridonin at NHR1 domain (PDB code: 2PP4) in the context of interaction with caspase-3 (PDB code: 1I3O) as simulated by ZDOCK. The oridonin binding to NHR1 domain is obviously not favorable to the caspase-3 cutting its substrate ETO on D^{368}, because oridonin binding results in an inevitable collision between caspase-3 and oridonin. The collisional region was shown as gray colored mesh. NHR1 domain and caspase-3 were shown as wheat colored cartoon and blue colored surface, respectively. The oridonin was presented as sticks (A) or sphere (B) in green. C^{347} was highlighted with orange sticks and D^{368} of ETO was highlighted with pink sticks.

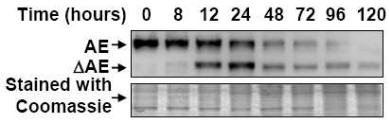

Fig. S12. Oridonin-induced ΔAML1-ETO is present in the treated t(8;21) cells for a long time. Western blot was performed to detect expression of AML1-ETO and generation of ΔAML1-ETO upon oridonin treatment (5 μM) in Kasumi-1 cells during a long period of time.

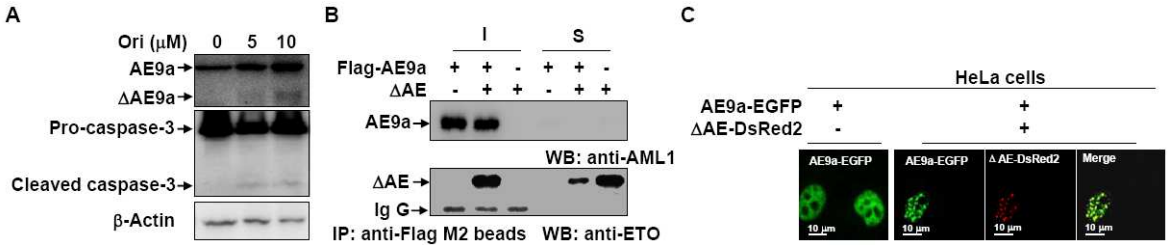

Fig. S13. ΔAML1-ETO interacts with AML1-ETO9a. (A) AML1-ETO9a (AE9a) cells isolated from AML1-ETO9a leukemia mice were treated with oridonin for 24 hours. Western blot was performed to detect the generation of ΔAML1-ETO9a [anti-ETO (N-terminal) antibody] and caspase-3 activation. **(B)** Co-IP analysis of nuclear extracts of transfected 293T cells to check interaction between AML1-ETO9a and ΔAML1-ETO. **(C)** Direct immunofluorescence showed nuclear localization of AML1-ETO9a, as well as colocalization of AML1-ETO9a and ΔAML1-ETO.

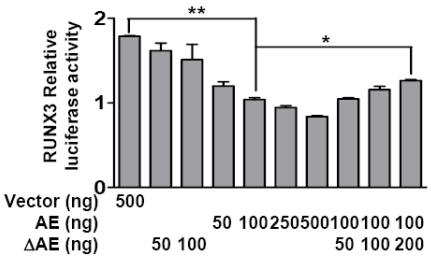

Fig. S14. ΔAML1-ETO relieves the transcriptional repression effect of AML1-ETO. Luciferase reporter assay in 293T cells transfected with pGL3-RUNX3 promoter luciferase reporter plasmid and plasmids encoding AML1-ETO and/or ΔAML1-ETO. Relative activities (mean ± SEM) were determined based on three independent experiments. *$p < 0.05$; **$p < 0.01$.

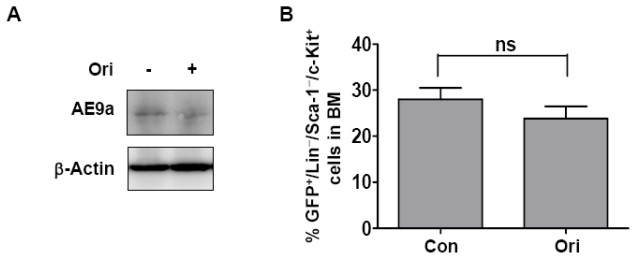

Fig. S15. Oridonin slightly decreases AML1-ETO9a expression and LIC population in vivo. (A and **B)** AML1-ETO9a mice were treated with oridonin formulation 20 mg/kg per day for 3 weeks. After that, spleen and BM cells were isolated. The expression of AML1-ETO9a in spleen cells was detected with anti-ETO (N-terminal) antibody by Western blot (A). The amount of GFP^+ cells was well controlled and β-Actin was used as internal control. Comparison of the percentages of $GFP^+/Lin^-/Sca\text{-}1^-/c\text{-}Kit^+$ cells within total BM cells of vehicle- (Con) and oridonin- (Ori) treated mice (mean ± SEM) (B). ns, no significance.

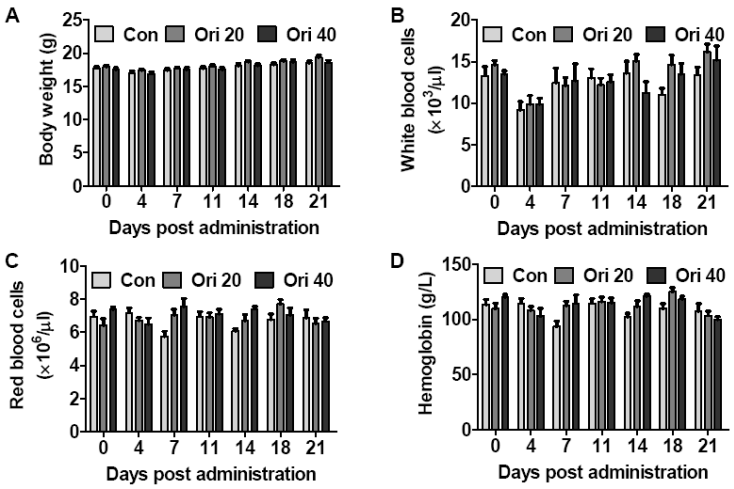

Fig. S16. Oridonin treatment does not affect blood picture of normal mice. (A to D) Normal C57BL/6 mice were treated with oridonin formulation (20 or 40 mg/kg per day) for 3 weeks. Their body weight and blood picture were followed up: (A) body weight, (B) white blood cells, (C) red blood cells, and (D) hemoglobin.

Table S1. The LIC frequency in $GFP^+/Lin^-/Sca\text{-}1^-/c\text{-}Kit^+$ or $GFP^+/Lin^-/Sca\text{-}1^-/c\text{-}Kit^-$ cells.

Group	Number of cells injected	Number of recipient mice	Number of leukemic mice	LIC frequency
$GFP^+/Lin^-/Sca\text{-}1^-/c\text{-}Kit^+$	2000	9	9	1/167
	1000	13	13	
	100	10	5	
	20	7	0	
$GFP^+/Lin^-/Sca\text{-}1^-/c\text{-}Kit^-$	2000	9	3	1/6159
	1000	3	0	
	100	5	0	
	20	7	0	

Table S2. List of primers used for ChIP-qPCR or semiquantitative PCR

	Primer used for ChIP-qPCR	Primer used for semiquantitative PCR
Primer name	Sequence (5'-3')	Sequence (5'-3')
UBQLN1-forward	GCTAATATCAACATCGCTATCGCC	ACAAGAGCAGTTTGGTGGTA
UBQLN1-reverse	CTTTCCCACCTTGCAACCTTTA	GCTGATGAACTCTGGGAAGT
CCPG1-forward	CTGATCCACCCAGCTATTCATC	CTTGTCGGTCTGGACTGATG
CCPG1-reverse	TGGTAGGAGTAGGTGGTGCCTC	AATGCTTGAAGCTCCTCTTG
IL-6R-forward	GATCTGGGTTAGGATTGGCCTG	CGGTCAAAGACATTCACAACATG
IL-6R-reverse	GCAACCACAAGGACCTTGAAGG	ACTCGCCTTGCCCGAACTCC
BLNK-forward	GGCCCTGACTGATGGAAATTAC	
BLNK-reverse	CAGCAGGTGACCATCCCTTTAG	
GAPDH-forward		CTCCTCCTGAGCGCAAGTACTC
GAPDH-reverse		TCCTGCTTGCTGATCCACATC

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