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RESEARCH ARTICLE

Homoharringtonine synergy with oridonin in treatment of t(8; 21) acute myeloid leukemia

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Abstract **Collaboration of c-KIT mutations with AML1–ETO (AE) has been demonstrated to induce t(8; 21) acute myeloid leukemia (AML). Targeted therapies designed to eliminate AE and c-KIT oncoproteins may facilitate effective treatment of t(8; 21) AML. Homoharringtonine (HHT) features activity against tumor cells harboring c-KIT mutations, whereas oridonin can induce t(8; 21) AML cell apoptosis and AE cleavage. Therefore, studies should explore the efficacy of combination therapy with oridonin and HHT in t(8; 21) AML. In this study, we investigated the synergistic effects and mechanism of oridonin combined with HHT in t(8; 21) AML cell line and mouse model. The two drugs synergistically inhibited cell viability and induced significant mitochondrial membrane potential loss and apoptosis. Oridonin and HHT induced significant downregulation of c-KIT and its downstream signaling pathways and promoted AE cleavage. HHT increased intracellular oridonin concentration by modulating the expressions of MRP1 and MDR1, thus enhancing the effects of oridonin. The combination of oridonin and HHT prolonged t(8; 21) leukemia mouse survival. In conclusion, oridonin and HHT exert synergistic effects against t(8; 21) leukemia *in vivo* and *in vitro*, thereby indicating that their combination may be an effective therapy for t(8; 21) leukemia.**

Keywords AML1–ETO; c-KIT; homoharringtonine; oridonin; t(8; 21) AML; synergistic effect

Introduction

t(8; 21) acute myeloid leukemia (AML) is one of the most common types of AML; however, the efficacy of Ara-C- and anthracycline-based chemotherapies remains unsatisfactory [1]. Thus, investigational therapies should be developed to improve clinical outcomes and provide t(8; 21) AML patients with additional treatment options.

AML1–ETO (AE) is the protein product of t(8; 21) translocations and plays a leading pathogenic role in t(8; 21) leukemia by blocking hematological cell differentiation [2]. Over several years of study, oridonin, a tetracycline diterpenoid compound isolated from leaves of *Rabdosia rubescens*, has been shown to specifically participate in AE oncoprotein cleavage [3]. Oridonin induced apoptosis in AE-bearing leukemic cells by activating the intrinsic apoptotic pathway and triggering caspase-3-mediated AE degradation, thereby generating a cleaved fragment ΔAE with antitumor activity [4]. Oridonin also displayed curative properties in mice with t(8; 21) AML induced by AE9a, an AE isoform with an alternatively spliced ETO exon [4]. Therefore, oridonin may possess potential in treatment of t(8; 21) AML. The preceding results imply the importance of natural medicinal plants in drug discovery given that these organisms serve as rich resources of antitumor drugs.

AE plays an essential role in t(8; 21) AML leukemogenesis; however, additional abnormalities, such as c-KIT mutations or overexpression, are also required for full-blown leukemia development [5]. Our previous report showed that 81.3% of leukemic cells displayed c-KIT mutation or overexpression [6]. c-KIT, which is also known as CD117, is a member of the type III tyrosine kinase subclass and comprises five extracellular immunoglobulin-like domains and a split tyrosine kinase domain. c-KIT gain-of-function mutations or overexpression cause activation of downstream signaling pathways, such as the Janus kinase/signal transducers and activators of

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transcription (STAT), Ras-Raf-mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K) pathways, eventually leading to uncontrolled cell proliferation and apoptosis resistance [7]. Therefore, drugs targeting c-KIT may cause growth inhibition and/or apoptosis and thus play a role in treatment of t(8; 21) AML.

Homoharringtonine (HHT) is a natural plant alkaloid from *Cephalotaxus harringtonia*; it exhibits antitumor activity in hematological diseases [8]. Our country initially studied the usefulness of HHT as treatment for myeloid leukemia in the 1970s. HHT is currently prescribed for treatment of adult patients with chronic myeloid leukemia and who show resistance and/or intolerance to two or more tyrosine kinase inhibitors [9]. A recent multicenter, randomized, controlled clinical trial in China demonstrated the efficacy of combined HHT, cytarabine, and aclarubicin for treatment of newly diagnosed AML [10,11]. HHT has also been shown to decrease c-KIT protein expression levels by inhibiting protein translation, resulting in decreased phospho-KIT levels and abrogating the activation of several downstream signaling pathways in systemic mastocytosis and gastrointestinal stromal tumor cells with high-frequency c-KIT mutations [12,13]. Therefore, investigating the effects of HHT on c-KIT protein expression in t(8; 21) leukemia cells is valuable.

In this study, we tested the hypothesis that oridonin and HHT will exert synergistic effects to treat t(8; 21) AML. Cell Counting Kit (CCK)-8 cell viability and cytotoxicity assays showed that oridonin and HHT exerted synergistic effects on Kasumi-1 cells. Specifically, the combination of oridonin and HHT induced significant increase in cell apoptosis and facilitated AE cleavage and c-KIT oncoprotein downregulation in Kasumi-1 cells. The combination of oridonin and HHT prolonged survival in an AE- and c-KIT N822K-induced leukemia mouse model.

Materials and methods

Cell lines and reagents

Kasumi-1 cells were cultured in Roswell Park Memorial Institute 1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

HHT sodium chloride was purchased from Hangzhou Minsheng Pharmaceutical Group Co., Ltd. (Zhejiang, China), oridonin was purchased from Zhao Wei Technology and Development Company (Shanghai, China), and biotin-oridonin (bio-ori) was kindly provided by F.Y. Qiu (Guangzhou Institute of Biomedicine and Health, CAS). MK571 and PSC833 were of analytical grade and were obtained from Sigma (USA).

Cell viability and drug combination studies

Cell viability was determined by CCK-8 cell proliferation and cytotoxicity assay kits (Dojindo, Japan). Kasumi-1 cells were seeded in 96-well plates and then incubated with increasing concentrations of HHT (0.005, 0.01, 0.02, 0.05 μmol/L). Cell viability was assessed at 48 h according to manufacturer's instructions. The concentrations at which the preceding agents mentioned inhibited cell viability by 50% (IC50) were determined using the CompuSyn software program.

For drug combination studies, Kasumi-1 cells were solely treated with each agent or with the combination of two agents at a fixed ratio for 48 h. Thereafter, growth inhibition rate of each drug or the combination of two drugs was determined by CCK-8 assay. Combination index (CI) values at the effective dose (ED) causing 75%, 90%, and 95% inhibition of cell viability, that is, ED75, ED90, and ED95, respectively, were calculated using CompuSyn software. Regarding CI data interpretation, a $CI < 0.9$ indicates synergism, a $CI = 0.9$–1.1 implies a state near synergism, and $CI > 1.1$ signifies antagonism [14].

Determination of mitochondrial membrane potential (MMP)

MMP loss was verified by flow cytometry. Kasumi-1 cells were solely treated with each drug or with the combination of two drugs for 24 h. Thereafter, the cells were incubated with 50 nmol/L $DiOC_6$ (Sigma) in the dark for 15 min at 37 °C. Loss of $DiOC_6$ fluorescence was indicative of mitochondrial inner transmembrane potential disruption. The probe was excited at 488 nm, and emission was measured through a 530 nm bandpass filter with a FACSCalibur flow cytometer (BD LSR-II, USA).

Fluorescence-activated cell sorting (FACS) analysis of apoptosis

Apoptosis was analyzed using an Annexin V/7-AAD Kit (BD Biosciences). The cells were seeded in six-well plates and then treated with oridonin and HHT at different concentrations for 24 h. Approximately 1×10^5 cells were subsequently washed with binding buffer before labeling with Annexin V/7-AAD for 15 min. Apoptotic cell counts were quantified by flow cytometry.

FACS analysis of c-KIT surface expression

After the preceding drug treatments, approximately 1×10^5 cells were collected and stained with allophycocyanin (APC) anti-human CD117 antibodies (BioLegend, USA).

CD117 expression levels on the cell surface were then determined by FACS analysis.

Analysis of intracellular bio-ori accumulation by flow cytometry

The cells were fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton for 15 min, blocked with 1% bovine serum albumin (BSA) for 30 min, and then incubated with Streptavidin APC-Cy™7 (Sav-APC-Cy7) antibodies (BioLegend) diluted in 0.1% Tween-20/phosphate-buffered saline (PBST) containing 5% BSA for 1 h at room temperature. Mean fluorescence intensity of Sav-APC-Cy7, which is an indicator of intracellular oridonin levels, was assayed by flow cytometry.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of c-KIT and ATP-binding cassette (ABC) transporter expression

Total RNA was extracted from approximately 1 million treated Kasumi-1 cells using TRIzol reagent (Invitrogen, USA). Thereafter, cDNA was synthesized from 1 μg of RNA in a 20 μL reaction mixture using random hexanucleotide primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). mRNA levels were determined by quantitative RT-PCR using gene-specific oligonucleotide primers. PCR was performed on an ABI Prism 7500 sequence detection system (Applied Biosystems, USA) using SYBR RT-PCR kit (TaKaRa, Japan). Expression levels of the target genes were determined using the $2^{-\Delta\Delta Ct}$ method and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as control.

Immunoblot assay

The treated Kasumi-1 cells were washed in cold PBS and lysed in buffer containing 50 mmol/L Tris with pH 8.0, 150 mmol/L NaCl, 1% Nonidet P 40 (NP-40), 1 mmol/L NaF, 0.1 mmol/L Na_3VO_4, 0.2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail. Protein concentrations were determined by RC DC protein assay (Bio-Rad, USA). A total of 10 μg to 50 μg of protein was then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis before being transferred onto a polyvinylidene difluoride membrane, which was probed with specific antibodies. The blots were subsequently detected with an enhanced chemiluminescence advance detection kit (Millipore, USA).

Several antibodies were used in the experiment. Anti-multidrug resistance-associated protein 1 (MRP1) rat monoclonal antibodies (MRPr1) and anti-multidrug resistance protein 1 (MDR1) rabbit polyclonal antibodies were obtained from Abcam (UK) and GeneTex Inc. (USA), respectively. Anti-RUNX1T1/ETO rabbit polyclonal antibodies (Abcam) were used to detect AE and its C-terminal truncated protein (ΔAE). Primary antibodies against c-KIT (D13A2), phospho-c-KIT (Tyr703) (D12E12), phospho-c-KIT (Tyr719), phospho-protein kinase B (AKT) (Ser473) (D9E), phospho-p44/42 MAPK (Erk) (Thr202/Tyr204) (D13.14.4E), phospho-STAT3 (Tyr705) (D3A7), phospho-STAT5 (Tyr694) (D47E7), AKT (pan) (C67E7), p44/42 MAPK (137F5), STAT3 (79D7), STAT5 (3H7), poly ADP ribose polymerase (PARP) (46D11), and caspase-3 (8G10) were purchased from Cell Signaling Technology (USA). Mcl-1 antibody (sc-12756) was purchased from Santa Cruz Biotechnology (USA). Anti-β-actin (AC-15) monoclonal mouse antibody (Sigma) was used as an internal control. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rat secondary antibodies were obtained from Cell Signaling Technology. Anti-rabbit IgG and HRP-linked antibodies were obtained from Amersham (UK).

Transplantation and *in vivo* treatment studies

We established the t(8; 21) AML mouse model by transplanting 1×10^5 leukemic cells (splenic cells) harboring AE and c-KIT N822K into 8-week-old sub-lethally irradiated female recipient BALB/c mice (3.5 Gy, 24 h before transplantation). To evaluate the antileukemic activity of HHT and oridonin, we randomly assigned these tumor-bearing mice to groups in which they received saline solution, HHT (0.5 mg/kg per day), oridonin (40 mg/kg per day), or HHT combined with oridonin. Treatment was initiated on day 5 after leukemic cell transplantation and was administered for 2 weeks. Percentages of green fluorescent protein (GFP)-positive leukemic cells (GFP is a marker for c-KIT) in peripheral blood were monitored weekly by flow cytometry to assess disease progression.

Statistical analysis

All experiments were conducted at least thrice, and characteristic results were recorded. Data were exhibited as mean ± standard deviation (SD). The differences between two groups were compared by *t*-test with GraphPad Prism software. Survival time of mice was analyzed with Kaplan–Meier method. P value < 0.05 was considered statistically significant.

Results

HHT targets c-KIT protein expression and induces Kasumi-1 cell apoptosis

Kasumi-1 cells seeded in 96-well plates were treated with

increasing doses of HHT. A 48 h treatment with 5, 10, 20, and 50 nmol/L HHT inhibited 22.1% ± 2.7%, 47.5% ± 0.8%, 65.8% ± 0.9%, and 77.1% ± 3.9% of cell viability, respectively (Fig. 1A). Given that c-KIT plays a crucial role in t(8; 21) AML leukemogenesis, we assessed the effects of treatment with HHT on c-KIT expression on the surface of Kasumi-1 cells and observed that c-KIT consequently mediated constitutive survival signals. FACS analysis showed that HHT can down-regulate c-KIT expression on the cell surface in a dose-dependent manner, whereas the percentage of CD117-positive cells decreased with increasing concentrations of HHT (Fig. 1B). By contrast, no significant changes were observed in CD11b and CD34 expressions after HHT treatment. Annexin V/7-AAD labeling showed that HHT dose-dependently induced significant Kasumi-1 cell apoptosis (Fig. 1C). Therefore, we conclude that HHT may exert a potential effect on CD117-positive leukemia and is appropriate for subsequent drug combination studies.

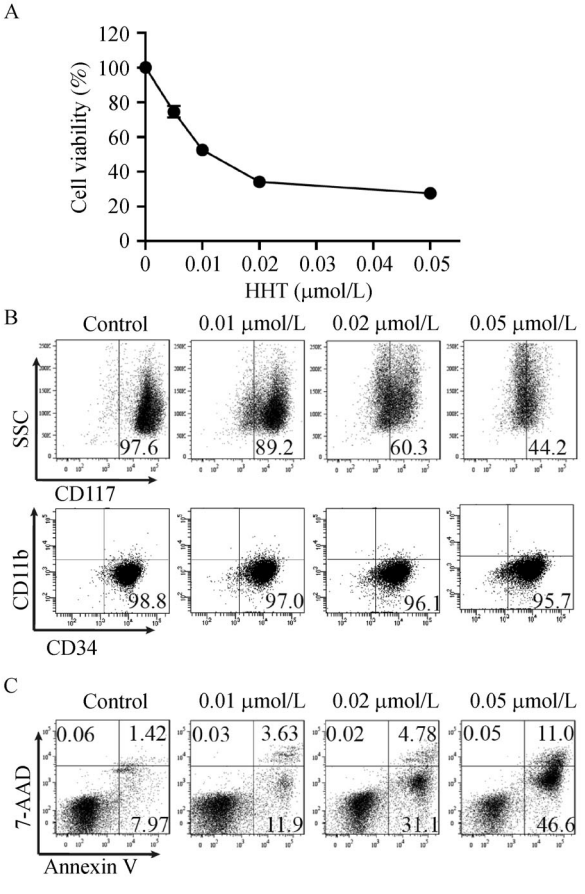


Fig. 1 HHT-induced reduction in CD117 expression and apoptosis in Kasumi-1 cells. (A) Cell viability in Kasumi-1 cells treated with different concentrations of HHT (0.005, 0.01, 0.02, and 0.05 μmol/L) for 48 h. (B) FACS analysis of CD117, CD34, and CD11b surface expression. After HHT treatment (0.01, 0.02, and 0.05 μmol/L) for 48 h, the cells were stained with monoclonal antibodies against CD117 (APC-conjugated), CD34 (PE-Cy7), and CD11b (PE) and then analyzed by flow cytometry. (C) Apoptotic effects of HHT in Kasumi-1 cells. After incubating the cells with 0.01, 0.02, and 0.05 μmol/L HHT, apoptotic cell percentages were assayed by flow cytometry after Annexin V/7-AAD double-staining.

Oridonin and HHT exert synergistic effects on Kasumi-1 cells

The effects of oridonin on Kasumi-1 cells have been previously reported [3,4]. IC50 values for oridonin and HHT over 48 h in Kasumi-1 cells approximated 3.0 and 0.02 μmol/L, respectively. We tested the combined treatment effects of oridonin and HHT at a fixed ratio of 150:1. As shown in Fig. 2A and 2B, the combination of the two drugs significantly inhibited Kasumi-1 cell viability compared with either drug alone. CI values were calculated using CompuSyn software and reached 0.65 ± 0.11, 0.59 ± 0.10, and 0.52 ± 0.09 at ED75, ED90, and ED95, respectively, indicating that oridonin and HHT exerted synergistic effects on Kasumi-1 cells (Fig. 2C and 2D).

Combination treatment of oridonin and HHT induces MMP loss and apoptosis

Treatment with oridonin induced slight MMP loss, whereas treatment with HHT barely influenced MMP in Kasumi-1 cells. However, treatment with the combination of the two drugs induced a significant decrease in MMP (Fig. 3A). MMP loss is related to apoptosis. Thus, we analyzed the effects of the combination of the two drugs on apoptosis. As shown in Fig. 3B, combination treatment with oridonin and HHT caused stronger apoptosis-inducing effects than either drug alone. The apoptosis-inducing effects of the two drugs were verified by immunoblotting. Caspase-3 activation and PARP cleavage were significantly enhanced in the combination treatment group compared with those in monotherapy groups. The expression of Mcl-1, which is an anti-apoptotic protein, was nearly undetectable in the combination therapy group (Fig. 3C).

Oridonin and HHT induce significant downregulation of c-KIT oncoprotein and its downstream signaling pathway in Kasumi-1 cells

Treatment with oridonin alone cannot affect c-KIT expression on the cell surface. However, the combination of oridonin and HHT induced significant reduction in c-KIT fluorescence intensity, that is, a 55.7% reduction in c-KIT fluorescence intensity compared with the control (Fig. 4A). We then examined by Western blot analysis the total c-KIT and activated c-KIT protein expression levels post-

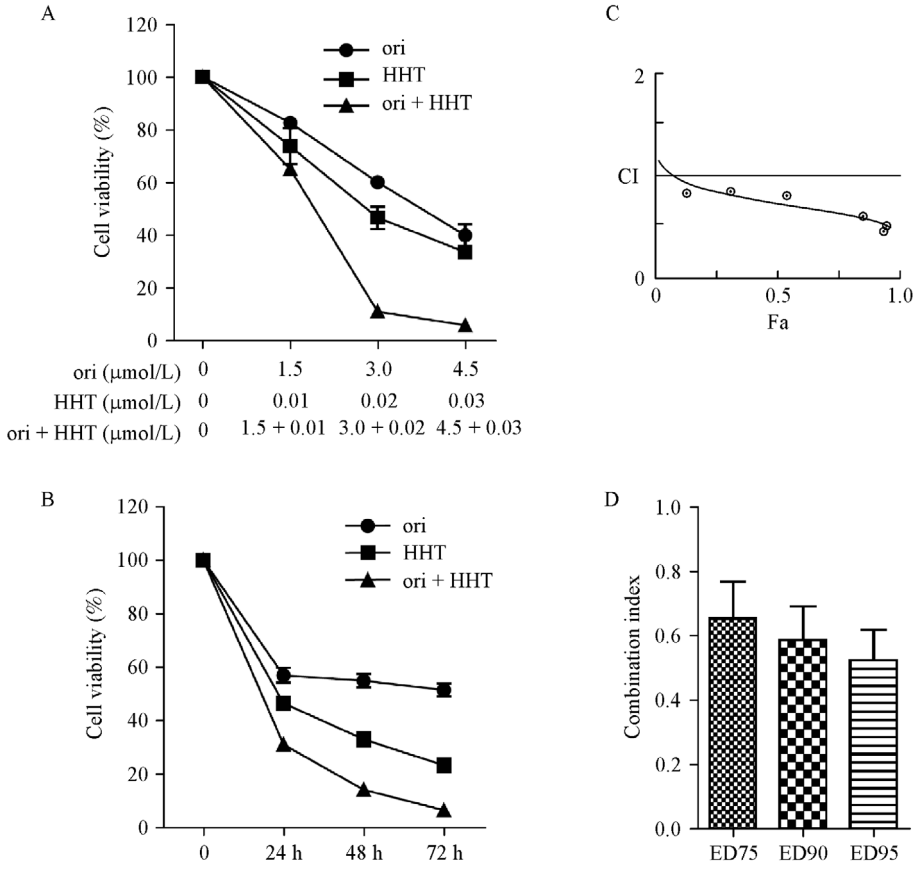


Fig. 2 Oridonin synergized with HHT to inhibit Kasumi-1 cell viability. (A) The combined cytotoxic effects of oridonin and HHT were determined by CCK-8 assay. Kasumi-1 cells were treated with different concentrations of oridonin and HHT at a fixed ratio of 150:1 for 48 h. (B) The combined cytotoxic effects of 3.0 μmol/L oridonin and 0.02 μmol/L HHT on Kasumi-1 cells at 24, 48, and 72 h. (C) Synergistic curve of HHT and oridonin. (D) CI values of oridonin and HHT after 48 h incubation at ED75, ED90, and ED95. CI values were calculated using CompuSyn software and expressed as mean ± SD from four separate experiments.

treatment. As shown in Fig. 4B, total c-KIT, phospho-KIT Y703, and phospho-KIT Y719 levels decreased in the combination therapy group compared with those in the control group. We further evaluated the activity of Ras/extracellular signal-regulated kinase (ERK), PI3K, and STAT pathways, which are known signaling pathways downstream of c-KIT. We observed that phosphorylated AKT (pAKT), pSTAT3, pSTAT5, and pERK levels significantly decreased in conjunction with the abolishment of c-KIT autophosphorylation in the corresponding group compared with those in the control group (Fig. 4B). We determined how c-KIT expression was modulated on the basis of our finding, which showed that total KIT protein expression levels significantly decreased in response to combination treatment with oridonin and HHT. We investigated by RT-PCR the changes in *c-KIT* mRNA levels induced by the preceding treatments. We noted that HHT, which is a known translation inhibitor [15], caused no effect on *c-KIT* mRNA levels; high concentrations of oridonin partially decreased *c-KIT* expression levels in the corresponding groups compared with those in the control group. We also identified that the two drugs induced significant reductions in *c-KIT* mRNA expression levels when used together, thus inducing reductions in c-KIT protein expression levels (Fig. 4C).

HHT enhanced the effects of oridonin by modulating the expressions of ABC transporters facilitating oridonin extrusion

Oridonin can trigger caspase-3-dependent AE cleavage, whereas HHT regulated AE to a limited extent. However, cells treated with oridonin in the presence of HHT displayed significantly increased ΔAE levels compared with those treated with oridonin in the absence of HHT (Fig. 5A). Therefore, we postulated that addition of HHT to media containing oridonin-treated cells increases intracellular oridonin concentrations. We used bio-ori to

assess the effects of the preceding drug combination on cell apoptosis and AE cleavage. We labeled bio-ori-treated cells with Sav-APC-Cy7 and analyzed the cells by flow cytometry. As expected, the fluorescence signal of Sav, which served as an indicator of intracellular oridonin levels, significantly increased in cells treated with oridonin and HHT compared with those treated with oridonin alone (Fig. 5B).

Cancer cells often express ABC transporters at high levels to facilitate extrusion of antineoplastic drugs [16]. We determined whether HHT can regulate ABC transporter expression and reduce oridonin efflux. Based on the findings of previous reports, we assessed by qPCR the expression levels of several drug transporters known to play roles in hematological malignancy [17]. We observed that ABCC1, also known as MRP1, was expressed at a higher level than other proteins in untreated Kasumi-1 cells. We also noted that ABCB1 (also known as MDR1 or P-glycoprotein) was expressed at a relatively high level in untreated cells, and this condition facilitated the efflux of various chemotherapeutic compounds. HHT-treated Kasumi-1 cells displayed decreased MRP1 and MDR1 protein expression levels compared with untreated cells (Fig. 5C). We treated the cells with MK571 and PSC833 (also known as valspodar), which are inhibitors of MRP1 and MDR1, respectively, to determine whether MRP1 and MDR1 are efflux transporters involved in oridonin transport [18]. First, we examined the involvement of MRP1 and MDR1 in sensitivity of Kasumi-1 cells to oridonin by cytotoxicity assay [19]. As shown in Fig. 5D, the IC50 value of oridonin decreased in the presence of MK571 and PSC833. HHT, MK571, and PSC833 elevated the mean fluorescence intensity of intracellular bio-ori (Fig. 5E). The capacity of oridonin to induce apoptosis significantly increased in the presence of HHT, MK517, and PSC833 (Fig. 5F). All the preceding results support the idea that HHT increased intracellular oridonin levels and thus enhanced its effects by inhibiting ABC-mediated oridonin efflux from Kasumi-1 cells.

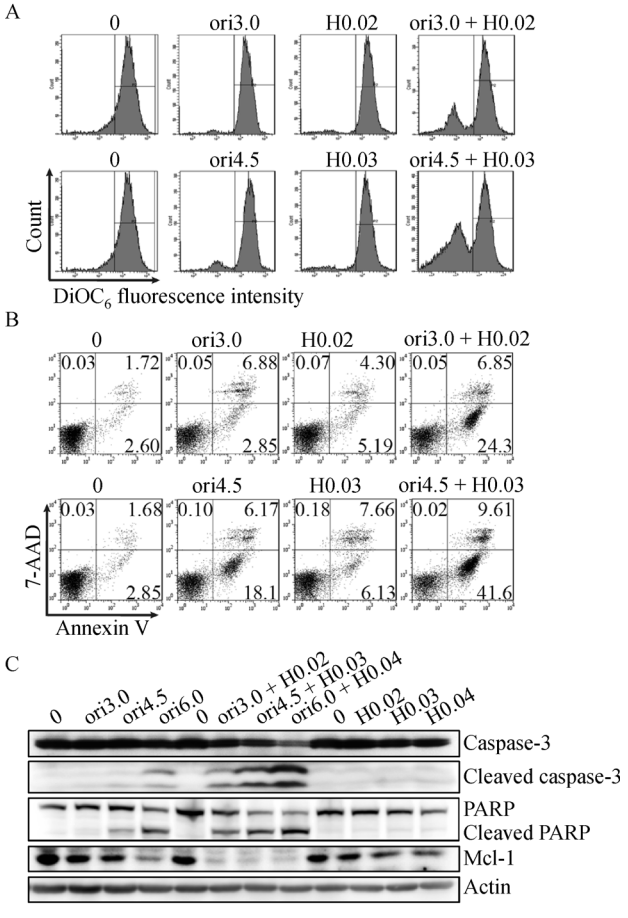


Fig. 3 Oridonin and HHT induced MMP loss and apoptosis in Kasumi-1 cells. (A) Kasumi-1 cells treated with different concentrations of oridonin and HHT were labeled with $DiOC_6$ and then assayed by flow cytometry. Mean fluorescence of $DiOC_6$, as shown in the histograms, represents MMP. (B) FACS analysis of apoptotic effects of oridonin and HHT. After treatment for 24 h, the cells were subjected to Annexin V/7-AAD double-staining and flow cytometry analysis. (C) Effects of oridonin and HHT on caspase-3, PARP, and Mcl-1 expressions. After treating the cells with the indicated concentrations of oridonin and HHT for 24 h, caspase-3 activation, PARP cleavage, and Mcl-1 down-regulations were detected by immunoblotting. The concentration unit "μmol/L" was used for oridonin and HHT treatment (e.g., ori3.0 represents 3.0 μmol/L oridonin).

Oridonin and HHT prolonged t(8; 21) leukemia mouse survival

We investigated the therapeutic effects of the combination of the two drugs using an AE- and mutated c-KIT-driven AML mouse model [5]. We randomly assigned mice with leukemia to four groups that were treated with normal saline (vehicle), 0.5 mg/kg HHT, 40 mg/kg oridonin, or oridonin and HHT. Treatment was started from day 5 after leukemia cell transplantation and continued for 14 consecutive days. We then analyzed the percentages of GFP-positive cells in peripheral blood at day 19 because these values served as indicator of tumor burden. We observed that HHT-treated mice exhibited significantly decreased GFP-positive cell percentages (mean = 9.6%) compared with those in the oridonin-treated group ($P < 0.01$) and saline-treated mice ($P < 0.001$) (Fig. 6A). Results also showed that the combination of HHT and oridonin displayed increased efficacy with respect to alleviating leukemic burden compared with that of oridonin ($P < 0.01$) or HHT ($P = 0.074$) alone (Fig. 6A). Median survival times of mice treated with oridonin or HHT reached 24 and 28 days, respectively. Notably, simultaneous administration of HHT and oridonin significantly prolonged overall survival in AML mice (median, 30 days versus 22 days for mice treated with

vehicle, $P < 0.001$) (Fig. 6B), demonstrating that the two agents exert synergistic antileukemia effects when combined.

Discussion

C-KIT mutations are closely associated with several malignant tumors, including gastrointestinal stromal tumors (GIST), AML, and mast cell leukemia [20]. Promising results have been observed in studies, in which such tumors were treated with targeted therapeutic agents designed to inhibit the c-KIT signaling network. The agents that are most commonly used to treat GISTs, in which c-KIT mutations are present in approximately 70% of cases, include tyrosine kinase inhibitors, such as imatinib [21]. Imatinib inhibits c-KIT kinase activity but cannot reduce c-KIT protein expression levels. Tumor cells may rebound after imatinib withdrawal. Therefore, improved drugs and strategies should be developed for treating patients with c-KIT mutations.

Various studies have shown that natural compounds derived from medicinal herbs elicit therapeutic responses that suppress various types of diseases while causing mild side effects. HHT, which is an inexpensive and easily acquired natural plant-derived drug, showed efficacy in treatment of patients with AML [10]. Mechanism studies revealed that HHT not only triggers rapid Mcl-1 down-regulation in myeloid leukemia cells [22,23] but also modulates c-KIT expression in mast cells harboring c-KIT D816V mutations [13].

Our previous studies showed that oridonin, a compound derived from the herb *Isodon rubescens*, selectively kills leukemic cells expressing the AE oncoprotein. Therefore, in this study, we validated the effects of combining oridonin and HHT in treatment of t(8; 21) leukemia, whose pathogenesis is associated with AE and c-KIT mutations or overexpression.

Interestingly, oridonin and HHT were shown to exert synergistic effects in cell cytotoxicity assays. HHT promoted oridonin-induced AE cleavage, whereas oridonin enhanced HHT-induced decreases in c-KIT expression on the cell surface and at the protein level. Simultaneous administration of oridonin and HHT led to significant

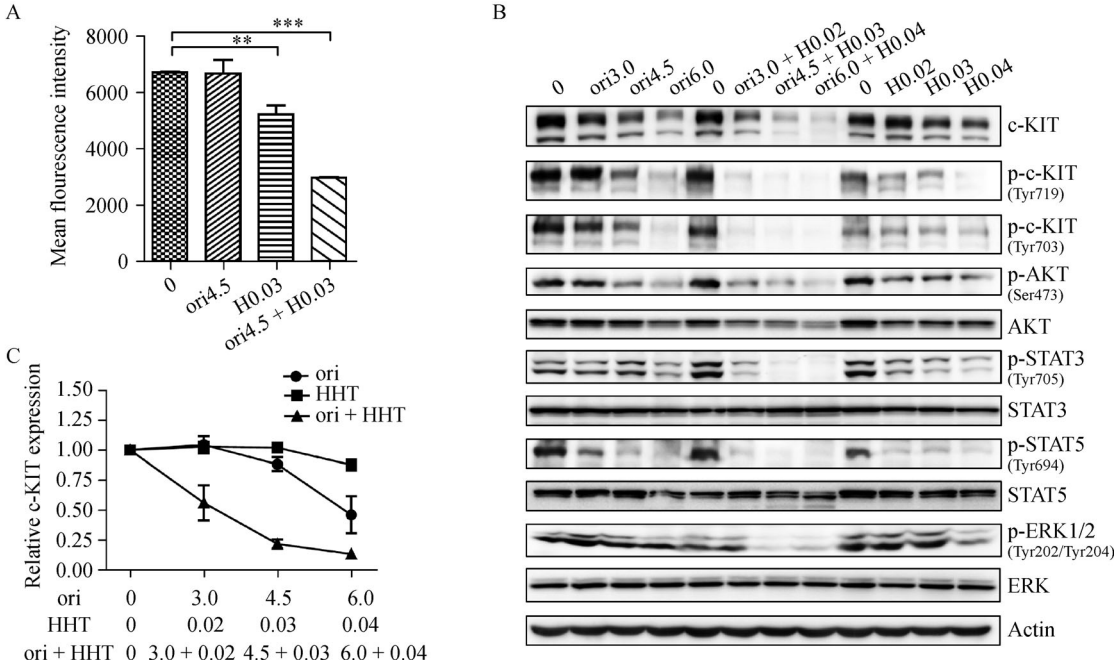
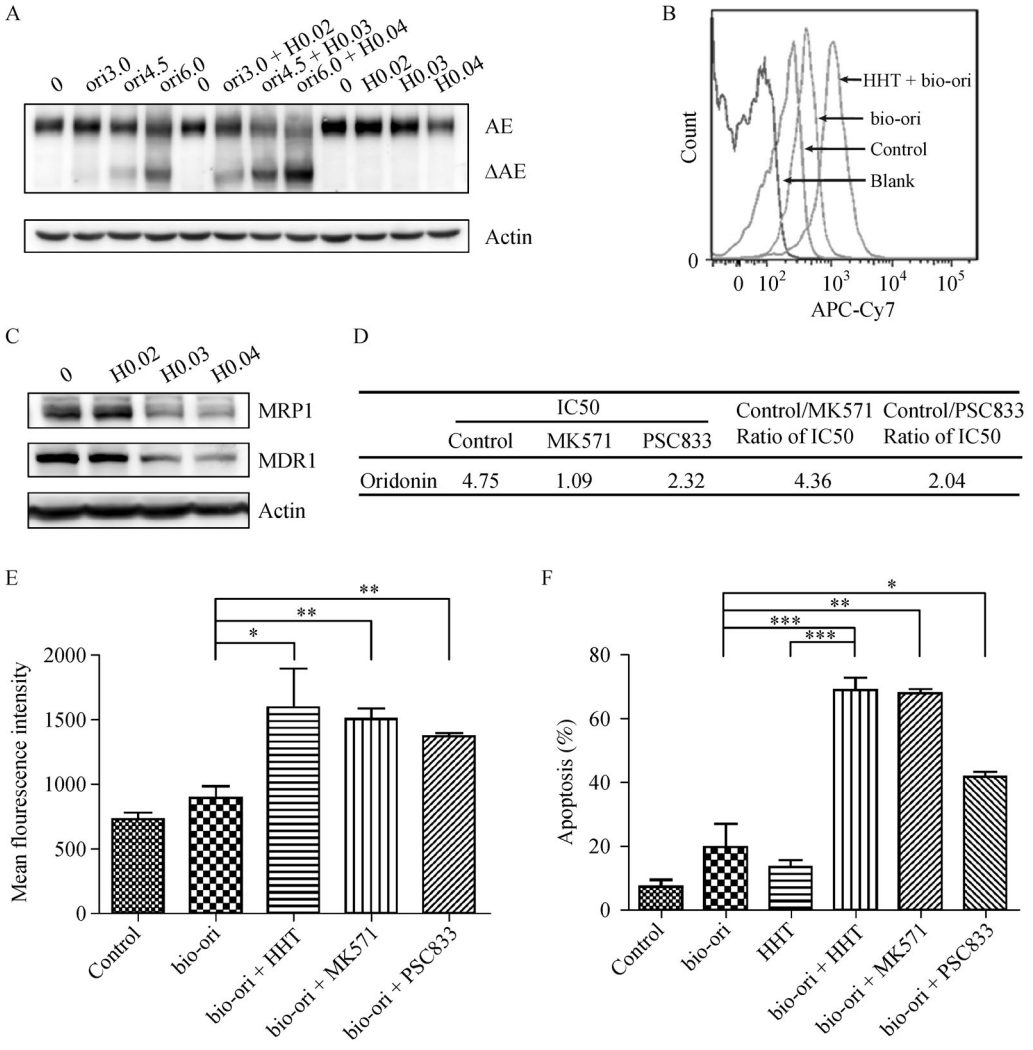


Fig. 4 Expressions of c-KIT oncoprotein and its downstream signaling pathway were downregulated in oridonin + HHT-treated Kasumi-1 cells. (A) c-KIT expression on cell surface. Mean fluorescence of c-KIT in Kasumi-1 cells exposed to 4.5 μmol/L oridonin, 0.03 μmol/L HHT, or both was analyzed by FACS and was compared with that of untreated control cells. **, $P < 0.01$; ***, $P < 0.001$. (B) Significant c-KIT pathway inhibition was induced by the combination of oridonin and HHT. Phosphorylated and total c-KIT, STAT3, STAT5, ERK, and AKT protein expression levels were evaluated by Western blot analysis using specific antibodies. Equal loading was confirmed with β-actin. These blots are representative of multiple independent experiments. (C) Changes in c-KIT expression after treatment with oridonin and HHT. The chart shows changes in c-KIT mRNA levels, which were normalized to GAPDH levels. Data are mean and SD from three independent experiments performed in triplicate. The concentration unit "μmol/L" was used for oridonin and HHT treatment (e.g., ori3.0 represents 3.0 μmol/L oridonin).



	IC50			Control/MK571 Ratio of IC50	Control/PSC833 Ratio of IC50
	Control	MK571	PSC833		
Oridonin	4.75	1.09	2.32	4.36	2.04

Fig. 5 By downregulating MDR1 and MRP1 expressions, HHT increased intracellular oridonin concentrations. (A) Immunoblot analysis of AE status. Changes in AE and AE expression in Kasumi-1 cells treated with increasing doses of oridonin, HHT, or both are presented. β-actin was used as internal control. (B) Histograms of FACS analysis of bio-ori. Kasumi-1 cells treated with 50 μmol/L bio-ori with or without 30 nmol/L HHT were fixed, permeabilized, and stained with Sav-APC-Cy7. Fluorescence intensity of Sav was indicative of the bio-ori amount detected by flow cytometry. These results are representative of three independent experiments. (C) MRP1 and MDR1 protein expression levels decreased with increasing dose of HHT. Rat monoclonal antibody to MRP1 and polyclonal MDR1 antibody were used, and β-actin was used as loading control. (D) Cytotoxic effects of oridonin in the presence of specific inhibitors. Oridonin was added to Kasumi-1 cells treated with 50 μmol/L MRP1 inhibitor MK571 or 5 μmol/L MDR1 inhibitor PSC833. After 24 h exposure, the cytotoxic effects of oridonin were measured by CCK-8 assay. FACS analysis of the increases in bio-ori levels (E) and apoptosis (F) facilitated by MK571, PSC833, and HHT. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with bio-ori alone. The concentration unit "μmol/L" was used for oridonin and HHT treatment (e.g., ori3.0 represents 3.0 μmol/L oridonin).

decreases in total and phosphorylated c-KIT protein levels. The combination of oridonin and HHT synergistically induced apoptosis and triggered MMP loss, Mcl-1 down-regulation, and consequently, caspase-3 activation. HHT also remarkably reduced protein expression levels of ABC transporters MRP1 and MDR1, which are associated with the transport of oridonin, thereby enhancing the efficacy of oridonin in t(8; 21) leukemia treatment.

We further evaluated the antitumor capacity of oridonin and HHT in an AE- and mutated c-KIT-driven leukemia mouse model. As expected, the two drugs exerted synergistic effects on leukemic mice. Our data necessitate further clinical research regarding the effects of combination therapy with oridonin and HHT on t(8; 21) leukemia.

Conclusions

Overall, this study showed that oridonin and HHT exerted synergistic effects on the leukemia cell line Kasumi-1 and

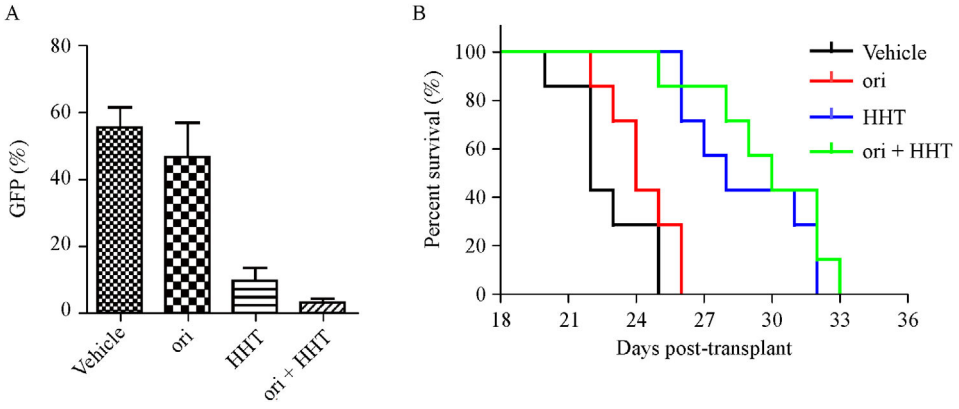


Fig. 6 Oridonin in combination with HHT exerted distinct antileukemia efficacy in treatment of t(8; 21) AML mouse model. A total of 1×10^5 leukemic cells harboring AE and c-KIT N822K were injected into 8-week-old sublethally irradiated female BALB/c mice. On day 5, the tumor-bearing mice were randomly assigned to groups receiving saline solution (vehicle, $n = 7$), HHT (0.5 mg/kg per day, $n = 7$), oridonin (40 mg/kg per day, $n = 7$), or HHT and oridonin ($n = 7$). (A) Percentages of GFP-positive cells in the peripheral blood at day 19 were analyzed by FACS. (B) Kaplan–Meier survival curves of leukemic mice treated with vehicle, oridonin, and/or HHT.

the mouse model and thus posed synergistic effects on t(8; 21) leukemia. This study provides us with better understanding of the mechanism underlying the synergistic effects of oridonin and HHT in t(8; 21) leukemia. The findings suggest that the combination of oridonin and HHT bears potential as treatment for t(8; 21) leukemia.

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Compliance with ethics guidelines

Weina Zhang, Ying Lu, Tao Zhen, Xinjie Chen, Ming Zhang, Ping Liu, Xiangqin Weng, Bing Chen, and Yueying Wang declare no competing interests. All animal experiments were conducted in accordance with the ethical guidelines on animal care of our institution and were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine.

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