Homoharringtonine and SAHA synergistically enhance apoptosis in human acute myeloid leukemia cells through upregulation of TRAIL and death receptors

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Abstract. Single-agent histone deacetylase (HDAC) inhibitors have exhibited marked antileukemic activity in preclinical and clinical studies and have undergone trials in combination with standard chemotherapeutics. However, the mechanisms of action of combination therapies are not completely understood. In the present study, a novel strategy for treatment of acute myeloid leukemia (AML) was identified, in which the chemotherapeutic agent, homoharringtonine (HHT), was combined with suberoylanilide hydroxamic acid (SAHA), a pan-HDAC inhibitor. A synergistic effect was observed when HHT was added to SAHA to induce apoptosis in Kasumi-1 and THP-1 leukemia cells. This combination was found to significantly enhance the activation of caspase-8 and -9 compared with treatment with each drug separately. Notably, while SAHA induced upregulation of death receptor 4 (DR4) and DR5, HHT upregulated tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression in a dose-dependent manner. In addition, the synergistic effect between HHT and SAHA was blocked partially using a specific anti-TRAIL antibody. The combination therapy was also found to significantly inhibit the growth of leukemia xenografts *in vivo* with enhanced apoptosis. These results indicate that, by regulating the induction of TRAIL and activation of the TRAIL apoptotic pathway, it is possible to administer HHT at low concentrations in combination with SAHA as an effective therapeutic approach for the treatment of AML.

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Introduction

Histone deacetylase (HDAC) inhibitors represent a novel class of anticancer agents that catalyze the deacetylation of histone and non-histone proteins and modulate the expression of genes involved in multiple cellular processes, including differentiation, apoptosis and autophagy (1-2). *In vitro* studies have revealed that HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA), have a dual effect on leukemic cells, triggering apoptosis at high concentrations (3) and inducing differentiation at low concentrations (4). In addition to the modulation of gene transcription, HDAC inhibitors have pleiotropic biological effects, which may be beneficial for the destruction of acute myeloid leukemia (AML) cells. These effects include the production of reactive oxygen species, induction of oxidative damage to DNA and inactivation of HSP90 chaperone function (5,6). Hyperacetylation of HSP90 by HDAC inhibitors leads to dysfunctional chaperone activity, resulting in the degradation of leukemia-related client proteins, including BCR-ABL (7). HDAC inhibitors may also overcome the effects of leukemia fusion proteins, including AML1-ETO, PML-RARα and MLL-CBP, on leukemogenesis by targeting the HDAC complex and these fusion proteins (8,9). These observations indicate that HDAC inhibitors represent promising new agents for the treatment of AML. In a phase I clinical trial, 7/31 patients with relapsed or refractory AML exhibited a response to HDAC inhibitors, including 2 complete remissions (CRs) and 2 CRs with incomplete blood count recovery (10). However, these observations were not consistent in a phase II study where only 1 CR was observed among 37 patients (11). Based on these results, more recent studies have focused on the combination therapy of AML cells with SAHA and other antileukemia drugs (12-16).

Homoharringtonine (HHT) is a natural alkaloid, derived from various species of *Cephalotaxus*. HHT functions as a protein synthesis inhibitor and has been found to induce apoptosis in a variety of leukemic cells (17,18). Early phase I trials in the United States have confirmed its antileukemic activity; however, 4/16 patients who received daily i.v. treatment (5-6 mg/m^2/day) for 5 days exhibited severe hypotension that resulted in cardiovascular collapse (19). A phase II study

of low-dose continuous infusion HHT in AML demonstrated that there were no serious cardiovascular complications when patients were treated with an infusion of 2.5 mg/m^2/day for 15-21 days or 3.0 mg/m^2/day for 15 days (20). In addition, combination therapy of HHT and cytarabine (ara-C) in patients with late chronic-phase CML resulted in 32% cytogenetic response and significantly improved survival was reported compared with HHT alone (21). The combination regimen of HHT and ara-C has been widely used in AML patients in China. For example, elderly patients with AML were treated with HHT (2.0 mg/m^2/day for 7 days) combined with low-dose ara-C. The overall response rate was 56.5% and CR rate was 39.1% (22). Our pilot study (23) found that HHT combined with ara-C and aclarubicin results in a CR rate of 83% in *de novo* AML patients. The estimated 3-year overall survival is 53%. Collectively, these results indicate that low-dose HHT is safe and the combination therapy of HHT and other antileukemic agents is a promising approach for treating AML.

In the present study, human AML Kasumi-1 and THP-1 cells were used to assess the role of HHT combined with SAHA in the induction of cell death and studied the mechanisms of the synergistic effect with attention to the death receptor pathway. Low concentrations of HHT and SAHA were found to synergistically induce higher levels of apoptosis in Kasumi-1 and THP-1 cells and significantly inhibited the growth of leukemia xenografts *in vivo* compared with each agent alone. In addition, the combination upregulated expression of death receptor 4 (DR4)/DR5 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and the synergistic effect between HHT and SAHA was partially blocked by a specific anti-TRAIL antibody. Together, these observations provide a rationale for further clinical investigation of this novel combination strategy in patients with AML.

Materials and methods

Cell lines and culture. The human AML cell line, Kasumi-1, was provided by Professor SJ Chen (Shanghai Jiaotong University, Shanghai, China) and THP-1 was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (both Hyclone Laboratories, Inc., Logan, UT, USA) and 1% L-Glutamine (Life Technologies, Inc., Grand Island, NY, USA) at 37˚C in a humidified incubator containing 5% CO_2. The study was approved by the ethics committee of the First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China).

MTT assay. Effects on cell proliferation were examined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, St Louis, MO, USA).. In brief, cells were plated on 96-well plates at 1.0x10^5 cells/well, then treated with HHT (Hangzhou Minsheng Pharmacy Factory, Hangzhou, China) and/or SAHA (Binxinbio, Inc., Tianjin, China) at the indicated concentrations for 24 h. Stock MTT solution (20 μl; 2.5 mg/ml) was added to each well and cells were incubated at 37˚C for an additional 4 h. Following removal of the MTT solution in medium, DMSO (200 μl) was added to each well and absorbance at 570 nm was detected.

Apoptosis assay. Leukemic cells were treated with HHT and/or SAHA at the indicated concentrations for 24 h and then washed with cold PBS. Next, cells were co-stained with Annexin V-FITC and propidium iodide (PI) using an apoptosis detection kit (Biouniquer, Suzhou, China), according the manufacturer's instructions. Cells were analyzed with FACScan flow cytometer (Becton Dickinson, San Diego, CA, USA). To detect chromatin condensation and nuclear fragmentation, which are characteristics of apoptosis, cells were plated on glass slides for fixation by 4% paraformaldehyde for 30 min at room temperature followed by three washes with PBS. Slides were then incubated with Triton X-100 for 20 min and washed with PBS. Next, slides were stained with Hoechst 33258 for 15 min in the dark, washed 3 times with PBS and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Western blot analysis. Cells from various conditions, following the induction of apoptosis, were harvested and washed twice in PBS. Whole cell extracts were prepared using a lysis buffer (Cell Signaling Technology, Beverly, MA, USA), according to the manufacturer's instructions. Protein samples (equal protein/lane) were subjected to 12% SDS-PAGE and transferred onto nitrocellulose filters. Next, membranes were blocked in non-fat milk buffer and the following primary antibodies were applied: caspase-3, -8 and -9, poly (ADP-ribose) polymerase (PARP), histone-H3, acetylated (Ac)-H3, Ac-H4, cytochrome c, Bid (all Cell Signaling Technology), TRAIL (BD Pharmingen, San Diago, CA, USA), DR4 (Bioworld Technology, Louis Park, MN, USA), DR5 (Millipore, Billerica, MA, USA) and β-actin (housekeeping protein control; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The secondary antibody was obtained from MultiSciences Biotech (Hangzhou, China). Blots were visualized using enhanced chemiluminescence procedures according to the manufacturer's instructions.

Establishment of subcutaneous leukemia xenografts and therapy. Severe combined immunodeficient (SCID) mice were purchased from Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China) and were housed in The School of Medicine, Zhejiang University (Hangzhou, China) under an institute-approved animal protocol. For the subcutaneous leukemia xenograft mouse model, 3 to 4-week-old female mice were inoculated subcutaneously with 1x10^7 THP-1 cells (in 0.1 ml volume) into the hind flanks. Tumor volume was measured and calculated using the following formula: Volume=(length x width^2)/2. When tumor volumes reached ~100 mm^3, mice were pooled and randomly assigned to 4 groups (n=8/group): PBS (control), intraperitoneal injection of SAHA (50 mg/kg^{-1}) for 5 days, intraperitoneal injection of HHT (1 mg/kg^{-1}) for 7 days and SAHA for 5 days combined with HHT for 7 days. One mouse from each group was selected randomly and humanely sacrificed at day 3 following treatment. Tumors were harvested and then processed for a TUNEL assay using an *In Situ* Cell Death Detection kit (Roche, Nutley, NJ, USA).

Statistical analysis. Student's t-test was used to determine statistical significance. Results of combination therapy were assessed by calculating combination index (CI) values using

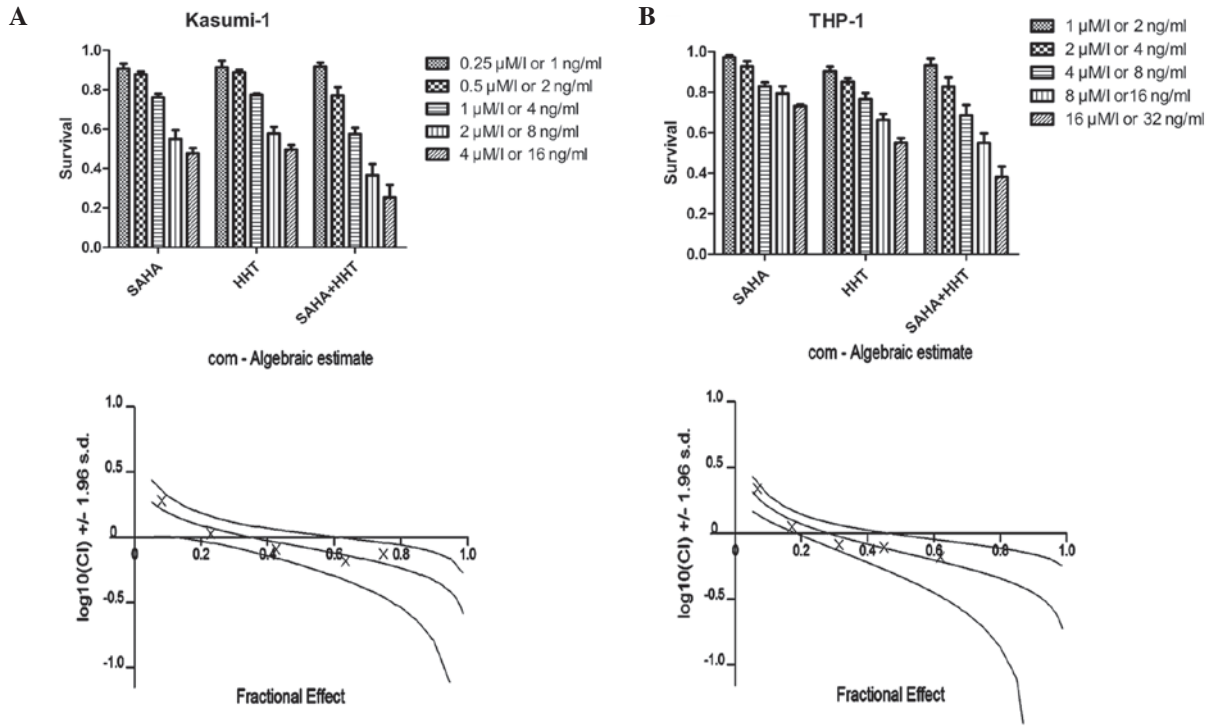

Figure 1. Co-treatment with HHT and SAHA synergistically inhibits growth of AML cell lines. (A) Kasumi-1 and (B) THP-1 cells were treated with increasing concentrations of HHT and/or SAHA for 24 h and cell viability was measured by an MTT assay. CI are presented (lower panels). Data are presented as the mean ± SEM of three independent biological samples assayed in triplicate. CI, combination index; HHT, homoharringtonine; SAHA, suberoylanilide hydroxamic acid.

CalcuSyn software (Biosoft, Cambridge, UK). To assess tumor growth curves, xenograft volumes were calculated as the mean ± SEM. *In vivo* survival curves were estimated using the Kaplan-Meier method by the log-rank test for pair-wise survival analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HHT functions synergistically with SAHA to inhibit AML cell growth. To improve the antileukemic efficacy of HHT in AML cells, we aimed to identify an effective agent that enhanced the cytotoxic effect of HHT. The effects of HHT and SAHA alone and HHT plus SAHA on the growth of Kasumi-1 and THP-1 cells were analyzed. Following treatment with increasing doses of HHT or SAHA for 24 h, Kasumi-1 cell viability was found to be significantly inhibited in a dose-dependent manner (Fig. 1A). Consistent effects were observed in THP-1 cells that were relatively resistant to HHT and SAHA. When treated with HHT and SAHA simultaneously, these cell lines revealed significantly reduced cell viability compared with treatment alone with each agent. CI analysis revealed that the CI for Kasumi-1 and THP-1 was <1 when HHT and SAHA were used at lower concentrations (HHT, 4-16 ng/ml for Kasumi-1 and 8-32 ng/ml for THP-1; SAHA, 1-4 μM for Kasumi-1 and 4-16 μM for THP-1). These results indicate a synergistic effect between SAHA and HHT in the inhibition of AML cell growth.

Treatment with HHT combined with SAHA induces apoptosis in AML cells by activation of endogenous and exogenous apoptotic pathways. To analyze the effect of HHT and SAHA on the induction of apoptosis in AML cells, THP-1 cells were exposed for 24 h to 16 ng/ml HHT in the presence or absence of SAHA (8 μM). Fig. 2A demonstrates that an increased portion of apoptotic cells, characterized by concentrated dense fluorescence and fragmented nuclei (apoptotic body), were observed following co-treatment. Similarly, when these cells were stained with Annexin V and PI and measured by flow cytometry, the proportion of apoptotic cells (Annexin V^+ and PI^- population) among cells treated with HHT and SAHA simultaneously were found to be significantly increased compared with cells treated with each agent alone (Fig. 2B). Next, key signaling molecules in the apoptosis pathway were analyzed by western blot analysis. As revealed in Fig. 2C, combined treatment for 6 h resulted in significantly increased levels of cleaved forms of caspase-8, -9 and -3 and PARP. In addition, increased cytochrome c levels, indicative of decreased mitochondrial membrane potential, and decreased levels of Bid were observed in cells treated with HHT or SAHA alone and were further enhanced following co-treatment. These results demonstrated that SAHA enhanced the cytotoxicity of HHT against AML cells by targeting the intrinsic and extrinsic caspase pathways, which may represent one of the mechanisms by which HHT functions with SAHA to synergistically induce apoptosis.

HHT and SAHA synergistically induce apoptosis in AML cells via the TRAIL apoptotic pathway. To further investigate the underlying mechanism by which HHT functions synergistically with SAHA to induce apoptosis in AML cells, several signaling proteins involved in the apoptotic pathway were

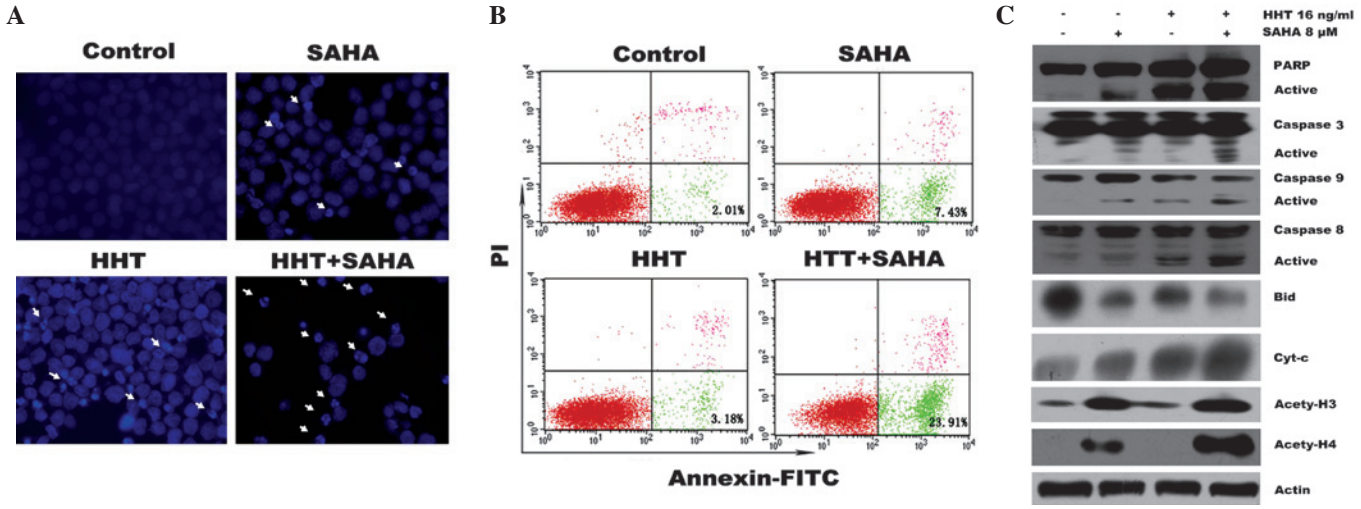

Figure 2. Synergistic induction of apoptosis by combining HHT with SAHA in AML cells. THP-1 cells were treated with 8 μM SAHA and/or 16 ng/ml HHT for 24 h. (A) Cells were stained with Hoechst 33258 and observed under a fluorescence microscope. Apoptotic cells revealed a condensed nuclear material and the formation of apoptotic bodies (arrows). (B) Cells were co-stained with Annexin V and PI and early apoptosis was measured by flow cytometry. (C) Expression of PARP, caspase-3, -8 and -9, cytochrome C and acety-H3 and -H4 were analyzed by western blot analysis in THP-1 cells treated with HHT and/or SAHA at the indicated concentrations for 6 h. HHT, homoharringtonine; SAHA, suberoylanilide hydroxamic acid; acety, acetylated; PARP, poly (ADP-ribose) polymerase; cyt-c, cytochrome c.

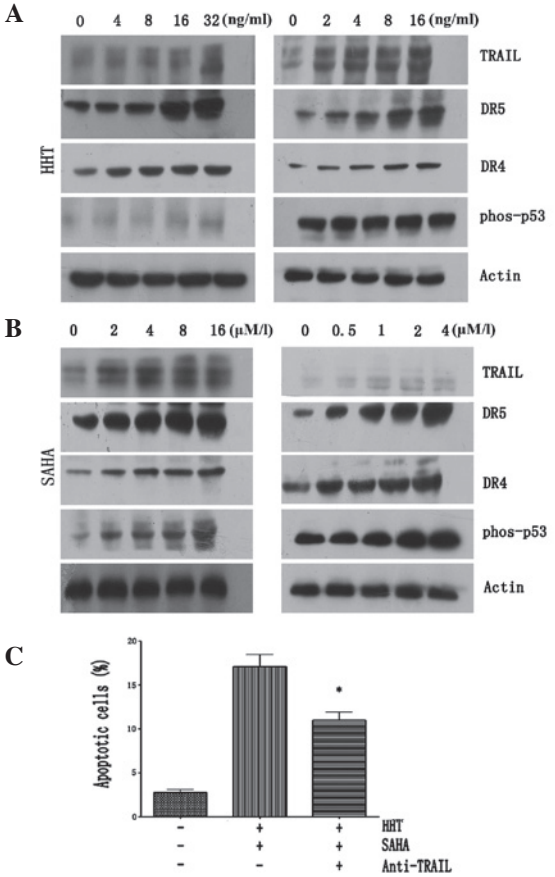

Figure 3. HHT and SAHA regulate the expression of TRAIL and DR4/DR5. Kasumi-1 and THP-1 cells were treated with increasing concentrations of (A) HHT or (B) SAHA for 24 h and the expression of TRAIL, DR4/DR5 and phosphorylated p53 was analyzed by western blot analysis. (C) Percentage of apoptotic cells was analyzed by flow cytometry in Kasumi-1 cells treated with HHT and SAHA simultaneously at the indicated doses for 23 h, prior to which cells were cultured in the presence or absence of anti-TRAIL antibody for 1 h. *P<0.01 Group treated with HHT + SAHA in the presence of anti-TRAIL antibody vs. absence of the antibody. HHT, homoharringtonine; SAHA, suberoylanilide hydroxamic acid; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR, death receptor; phos, phosphorylated.

analyzed. As demonstrated in Fig. 3A, treatment with HHT for 6 h upregulated the expression of TRAIL and DR5 in both cell lines in a dose-dependent manner, while levels of DR4 and phos-p53 protein expression were not altered. Consistent with a previous study (24), significant upregulation of DR5 and phos-p53 was identified to be induced by SAHA treatment. However, marked upregulation of DR4 upon SAHA treatment was observed in Kasumi-1 cells, but not in THP-1 cells (Fig. 3B), which may explain resistance to SAHA. To confirm the role of the interaction between TRAIL and DR4/DR5 in apoptosis induced by HHT plus SAHA, THP-1 cells were incubated with a specific anti-TRAIL antibody for 1 h, followed by co-treatment with HHT (8 ng/ml) and SAHA (4 μM) for 23 h. Next, cells were co-stained with Annexin V/PI and analyzed by flow cytometry. As revealed in Fig. 3C, the proportion of apoptotic cells induced by HHT plus SAHA was found to be significantly decreased (P<0.01) when cells were co-cultured with the anti-TRAIL antibody, confirming that HHT functions synergistically with SAHA to induce apoptosis in AML cells in a TRAIL-dependent manner.

HHT and SAHA abrogate growth of xenografted AML cells in SCID mice. Human leukemia xenografts were established by subcutaneously injecting THP-1 cells ($1x10^7$) into the right flank of female SCID mice. Tumor size was measured regularly following inoculation and mice (each group, n=8) bearing a tumor of 100-130 mm^3 were randomized to receive treatment with intraperitoneal injection of PBS, HHT, SAHA or two drugs. The treatment regimen was as follows: monotherapy groups, injections of 50 mg/kg SAHA for 5 consecutive days or 1 mg/kg HHT for 7 consecutive days; and combination therapy group, injections of 50 mg/kg SAHA for 5 days and 1 mg/kg HHT for 7 days. When tumor xenografts were established, tumor volume in the control group markedly increased in a time-dependent manner and reached 3,102±238 mm^3 on day 27. However, the volume of

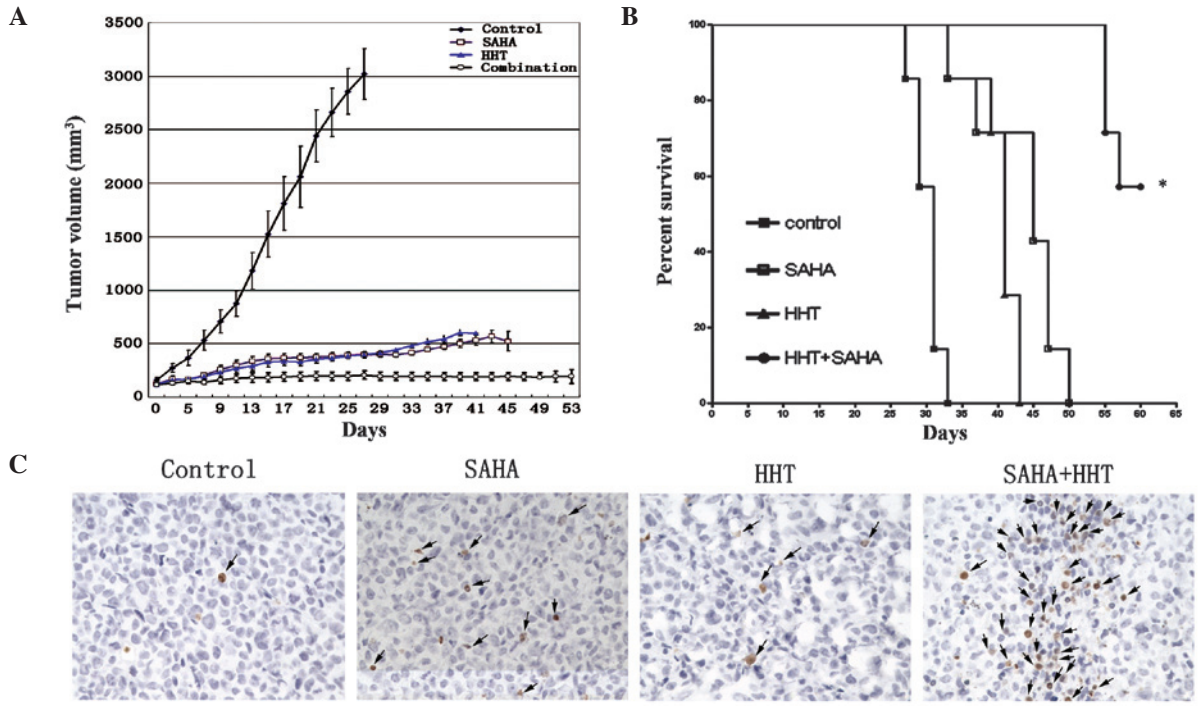

Figure 4. Antileukemic activity of HHT combined with SAHA *in vivo*. (A) THP-1 xenografts were established in mice (n=8) and treated with HHT and/or SAHA. Tumor size and volume were measured and are presented as the mean ± SE. (B) Kaplan-Meier survival curves for mice with THP-1 xenografts treated with HHT and/or SAHA at various times. *P<0.0001 Group treated with HHT + SAHA vs. HHT group, or SAHA group, respectively. (C) Tumor xenografts were harvested and subjected to immunohistochemical analysis using a TUNEL assay. Arrows indicate apoptotic cells. HHT, homoharringtonine; SAHA, suberoylanilide hydroxamic acid.

tumors in the monotherapy group was only increased slightly (HHT, 602±28.8 mm^3; SAHA, 521.1±91.6 mm^3) and tumor growth was inhibited further in mice receiving HHT and SAHA simultaneously. In one case, a tumor xenograft in the combination therapy group disappeared completely (Fig. 4A). In addition, mice in the combination treatment group were found to survive for significantly longer periods than mice in the other groups (Fig. 4B; P<0.0001). When tumor xenografts were subjected to a TUNEL assay, tumors from the combination therapy group revealed higher numbers of apoptotic cells than the monotherapy group (Fig. 4C). These results further confirmed that the combination of HHT and SAHA is effective in inhibiting the growth of AML cell-derived tumors grafted in SCID mice via enhanced apoptosis.

Discussion

HHT has demonstrated a marked efficacy in AML. In a phase II trial, continuous infusion of HHT administered at a daily dose of 5 mg/m^2 led to CR in 7/43 patients with relapsed AML (25). However, HHT is associated with serious cardiovascular complications, including hypotension at high doses and/or in short infusion schedules (25). Previously, we demonstrated that HHT (4 mg daily) combined with ara-C and aclarubicin resulted in a high CR rate and longer survival in newly diagnosed non-M3 AML patients (23). Additionally, a Chinese study revealed that combination treatment with HHT (2 mg/m^2/day) plus ara-C was an effective induction regimen in elderly patients with *de novo* AML (22). There were no severe cardiovascular complications observed in these clinical trials, indicating that reducing drug dosage abrogates the occurrence of cardiotoxicity and that HHT-based combination therapy is a promising therapeutic approach to improve patient outcome.

In the present study, the effects of HHT combined with SAHA on AML cells were investigated and a synergistic effect between HHT and SAHA on Kasumi-1 and THP-1 cells was identified by calculation of CI. The combination therapy also enhanced apoptosis in THP-1 cells and was found to significantly inhibit the tumor growth of leukemia xenografts *in vivo* compared with each agent alone. These results demonstrate that synergistic interactions occurred in both AML cell lines treated with low concentrations (2-16 ng/ml) of HHT and clinically achievable doses of SAHA (26), namely, 0.5-4 μM. Previously, a pharmacokinetic study of semisynthetic HHT demonstrated that the mean peak plasma and minimum concentrations were 78 and 27.4 ng/ml, respectively, at day 5 following the final subcutaneous injection of 3 mg/m^2/day (27). These concentrations exceeded those required to inhibit 50% of the growth of AML HL-60 cells *in vitro* (20 ng/ml) (28). Consistent pharmacokinetic parameters were observed in AML patients treated with natural HHT (29). Collectively, these observations indicate that co-administration of HHT and SAHA results in a marked increase in antileukemic activity and represents an attractive combination therapy for AML.

In the current study, mechanistic analyses in AML cell lines indicated that the induction of apoptosis via activation of intrinsic and extrinsic apoptotic pathways may contribute to the potent synergism between HHT and SAHA. Notably, induction of apoptosis was accompanied by upregulation of TRAIL expression induced by HHT and increased expression of DR4/DR5 induced by SAHA. SAHA has been previously demonstrated to upregulate DR4/DR5 in AML HL-60 and

U937 cells (24). HHT also slightly enhanced expression of DR4/DR5. To the best of our knowledge, this is the first study demonstrating that HHT treatment leads to increased expression of TRAIL and DR5 in AML cells. Previously, HHT has been revealed to induce apoptosis in various types of leukemic cells, which was characterized by cytochrome c release and activation of caspase (30). In AML cells, HHT leads to a decrease in mitochondrial membrane potential (31), which is regulated by activation of caspase-9 via cleaved Bid as a result of caspase-8 activation. In the present study, enhanced activation of caspase-9 and increased release of cytochrome c were observed, and may have been the result of enhanced activation of the extrinsic apoptosis pathway induced by upregulation of TRAIL and DR4/DR5.

It is well known that TRAIL has a marked apoptotic effect in tumor cells but not in normal cells, thereby representing a promising cancer therapeutic agent. By interacting with its cognate cell receptors, DR4 and DR5, TRAIL activates the death receptor-mediated apoptotic signaling (extrinsic) pathway, resulting in activation of caspase-8 and additional downstream caspases (32). In specific cells, activation of caspase-8 leads to induction of the intrinsic apoptotic pathway by Bid cleavage (32). Previous studies have confirmed that primary AML cells are generally resistant to apoptosis induction by TRAIL, which may be due to the expression of TRAIL decoy receptors and down-regulation of DR4 (33,34). Min *et al* (35) investigated DR4/DR5 expression in primary AML cells and found that 9/29 (31%) patients were $DR4^-$, whereas all patients were $DR5^+$. These studies and current observations demonstrate that co-treatment with clinically relevant doses of HHT and SAHA results in upregulation of the TRAIL pathway and enhanced cell death, which was reduced by an anti-TRAIL antibody. Therefore, we concluded that co-treatment with HHT and SAHA may be effective for the treatment of AML by targeting the TRAIL pathway; however, further clinical studies are required.

Acknowledgements

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