β-Catenin and AKT are promising targets for combination therapy in acute myeloid leukemia

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In this study, we confirmed that combining HHT with ACR can result in synergistic cytotoxicity to AML cells in vitro and in vivo. Combining HHT and ACR simultaneously inhibited PI3K/AKT and WNT/β-catenin signaling in AML cells. Significant increases in growth inhibition and apoptosis were induced by an AKT inhibitor when the WNT3A gene of THP-1 cells was silenced. HHT + ACR could synergistically induce the apoptosis of CD34⁺/CD38⁺ primary AML cells. These results highlight β-catenin and AKT are promising targets for combination therapy for AML. © 2013 Elsevier Ltd. All rights reserved.

1. Background

High-dose chemotherapy regimens have been used in subjects with acute myeloid leukemia (AML) and have resulted in a high prevalence of complete remission (CR). However, favorable long-term survival has not been observed in AML patients who receive excessive chemotherapy [1]. Our previous study showed that a regimen combining homoharringtonine (HHT), cytarabine and aclarubicin (ACR), known as the HAA regimen, produced a high prevalence of CR and a favorable estimated overall survival (OS) at 3 years in patients with de novo AML [2]. This founding raised a possibility that combining HHT and ACR could bring favorable long-term survival. The natural alkaloid HHT has been confirmed to have definite antileukemic activity against several AML cell lines in vitro [3–5]. Horinaka et al. reported that once-weekly treatment with 120 nM ACR alone resulted in apoptosis in Jurkat cells [6]. However, the mechanism of action of the HAA regimen, especially the effects of HHT and ACR in combination, has not been clarified fully.

Constitutive activation of PI3K/AKT can trigger excessive cell proliferation and promote the clonogenicity of leukemic progenitors [7,8]. PI3K expression in serum and AKT phosphorylation at Ser473 has been observed in 50–80% of clinical de novo AML samples [9,10]. Compared with those with high levels of p-AKT, patients with low levels of p-AKT had significantly longer median survival times [10,11]. Our previous study showed that HHT reduced AKT activation in several AML cell lines. However, how ACR regulate the PI3K/AKT signal is less clear.

Previously, researchers focused on the WNT pathway in solid tumors (especially in colon carcinoma). Recently, there has been increasing evidence for deregulation of the WNT signal in leukemogenesis. Mutations leading to overexpression of WNT factors or abnormal methylation of WNT antagonists appear to be involved in leukemogenesis [12]. In two small scale studies, aberrant β-catenin activity of bone marrow tissue was detected in 40.7% and 22% AML patients respectively. Kaplan–Meier survival analysis showed aberrant β-catenin activity and high topoisomerase IIC activity independently predicted an adverse prognosis in AML patients [13,14]. As we known, aclarubicin can interact with topoisomerase I and II, and then inhibit DNA replication and DNA repair. However, aclarubicin alone is not enough to improve the long-term survival of AML patients. The action of HHT and ACR for WNT/β-catenin remains unknown. So we try to discuss if WNT/β-catenin signal is involved in the synergistic cytotoxicity effects of HHT and ACR to AML cells.

In the present study, we investigated the anti-leukemic effects of HHT and ACR in vivo and in vitro. Activation of the PI3K/AKT and WNT pathways in AML cells treated with HHT or/and ACR was.
evaluated by polymerase chain reaction (PCR) and Western blotting. The synergistic cytotoxic effect induced by WNT3A silencing and AKT inhibition was also assessed in AML cells. Finally, we propose the possibility of a synergistic cytotoxic effect induced by HHT and ACR in CD34+CD38− AML cells.

2. Methods

The research protocol was approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University (Permit number: 2011–14). Animal experiments were conducted in accordance with guidelines set by the National Institutes of Health (Bethesda, MD, USA).

2.1. Reagents and antibodies

HHT was obtained from the National Institutes for Food and Drug Control (Beijing, China). ACR was kindly provided by WanlePharmacia (Shenzhen, China). ACR stock solution was prepared at 0.11 mM in dimethyl sulfoxide (DMSO); Sigma−Aldrich, St. Louis, MO, USA) and kept at −20 °C. A stock solution of the AKT inhibitor triciribine (Sigma−Aldrich) was prepared at 15 mM in DMSO and kept at −20 °C. A specific inhibitor of GSK−3β, CHIR−99021, was obtained from Selleckchem company (Houston, USA). Rabbit polyclonal antibodies to WNT3A, total β−catenin, Ser33/37 and Thr41 p−β−catenin, GSK−3β, Ser9 p−GSK−3β, and AKT, Ser473 p−AKT, PI3K100x, Bcl−2, Bax, Bcl−xl, Mcl−1, caspase−3 and caspase−9 were from Cell Signaling Technology (Beverly, MA, USA). Antibodies to β−actin and Lamin B were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell lines, primary samples, and cell cultures

The AML cell line THP−1 was purchased from the Shanghai Cell Culture Institute (Shanghai, China). The Kaumi−1 cell line was kindly endowed by Professor Saijuan Chen of the Shanghai Institute of Hematology (Shanghai, China). These two cell lines were cultured in RPMI−1640 (Gibco, Billings, MT, USA) containing 2 mM l−glutamine supplemented with 10% fetal bovine serum (Gibco). Bone−marrow samples were obtained from AML patients after obtaining written informed consent. Mononuclear cells were purified by Ficol−Hyapaque (Sigma−Aldrich) density−gradient centrifugation.

2.3. Growth inhibition assay

Cells (1 × 106 cell−line cells and 5 × 105 primary AML cells per well) were seeded in 96−well microtiter plates (Nunc, Roskilde, Denmark). After exposure to different drugs for 24 h, 20 μL 3−(4,5−dimethylthiazol−2−yl)-2,5−diphenyltetrazolium bromide (MTT; Sigma−Aldrich) solution (5 mg/mL) was added to each well and the plates were incubated for an additional 4 h at 37 °C. MTT solution in the medium was aspired before 200 μL DMSO was added to each well. The absorbance at 570 nm was measured. Each sample was assayed with six replicates per assay, and cell−line experiments were carried out in triplicate.

2.4. Evaluation of apoptosis

Cells were treated with various reagents of different concentrations or equal volumes of DMSO for 12 h, harvested, washed twice with phosphate−buffered saline (PBS), resuspended in binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM CaCl2) at 1 × 105 cells/mL. Staining of apoptotic cells was performed by Annexin V−Fluorescein Isothiocyanate/Propidium Iodide (FITC/PI) Assay kit (BD Pharmingen, San Diego, CA, USA) according to manufacturer’s instructions. A total of 5 μL Annexin V−FITC and 5 μL PI were added to the cell suspension. Apoptotic cells were detected using a FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Mononuclear cells (5 × 105/mL) obtained from primary AML cells were incubated with the drugs in 24−well plates for 12 h, collected, washed thrice with PBS and stained with CD34−PE and CD38−APC for 15 min. After washing with PBS, resuspended cells were incubated with 5 μL Annexin V−FITC for 10 min. The number of Annexin V−positive AML CD34+CD38− cells was analyzed by flow cytometry.

2.5. Hoechst staining

THP−1 cells and Kasumi cells were treated with HHT (36 nM), ACR (90 nM) and two−agent combination for 12 h. Cells were then permeabilized with 0.5% Triton X−100 for 30 min, washed with PBS, stained with 10 μg/mL Hoechst 33342, and washed with PBS. Nuclear morphology was observed immediately using a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

2.6. Gene chip analyses

RNA isolated from THP−1 cells treated with HHT, ACR, or HHT+ACR for 3 h were used for gene chip analyses. An Agilent 4 × 44K Human Genome Microarray platform (Agilent, Santa Clara, CA, USA) was used. Gene expression data were normalized using Agilent GeneSpring GX software. Differentially expressed genes were identified via a fold change (FC) of ≥2 and P<0.05. Statistical analyses of microarray data were executed using a SBC Analysis System (SAS) provided by the Shanghai Biocomp Company Limited (Shanghai, China).

2.7. Real−time reverse transcription−polymerase chain reaction (RT−PCR)

Total RNA was extracted from 1 × 105 cells using TRIzol (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse−transcribed by M−MLV reverse transcriptase (Invitrogen) with 0.25 μg random primers. RT−PCR was conducted using the one−step qSYBR Green Supermix and Cycler Real−time PCR Detection system (BioRad, Hercules, CA, USA) according to the manufacturer’s instructions. The primers used to amplify WNT3A were forward, 5′−GACCTTCGCACTGGTA−3′, and reverse, 5′−GGCAGGATTGAGACAC−3′. The primers used to amplify β−catenin were forward, 5′−TTGAGGAGCAGCCACAGATTACA−3′ and reverse, 5′−GGGGCAATATCAGTCCAA−3′. The primers used to amplify glyceraldehyde 3−phosphate dehydrogenase (GAPDH) were forward, 5′−TCAAGCCATTGTCACTGTTAC−3′ and reverse, 5′−GCTGGTGTCCAGGCGTCTT−3′. The gene expression values in control cells were set to 100%, and all other experimental samples were expressed as percentage of the untreated control cells.

2.8. Western blot analyses

Cells were washed twice in PBS and lysed in 10 mM Tris, 1 mM ethylenediamine tetra−acetic acid (EDTA), 10 mM KCI, 0.3% Triton and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The protein concentration of the samples was determined using a bicinchoninic acid (BCA) Protein Assay kit (Pierce, Rockford, IL, USA). Cell lysates containing 25 μg of total protein were separated on 8−12% SDS−PAGE and electrophoretically transferred onto immobilon−P transfer membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skimmed milk in TBS−T buffer (10 mM Tris−HCl pH 8.0, 150 mM NaCl, 0.1% Tween−20) for 2 h and incubated with primary antibodies at 1:1000 dilution overnight at 4 °C. After four washes with PBS−T, membranes were incubated with secondary antibody at 1:5000 dilution (KPL, Baltimore, MD, USA) for 2 h at room temperature. Signals were detected using an ECL kit (Amersham, Little Chalfont, UK).

2.9. RNA interference

WNT3A siRNA was purchased from Invitrogen. Transfection efficiency was assessed using the BLOCK iT™ Alexa Fluor™ Red Fluorescent Control (14750−100, Invitrogen). Transfection was performed using LipofectamineTM RNAiMAX (13778−075, Invitrogen) according to the manufacturer’s instructions. THP−1 cells were seeded at 3 × 105/well in 24−well plates. A total of 20 nM of each siRNA and 1 μL Lipofectamine was mixed in 100 μL Opti−MEM (Invitrogen). After incubation at room temperature for 20 min, the siRNA−lipid complex was added to THP−1 cells, incubated at 37 °C for 48 h, and then THP−1 cells were collected for RT−PCR, Western blotting, the MT assay or apoptosis analyses.

2.10. Animal xenograft tumor model

Female severe combined immunodeficient (SCID) mice aged 3–4 weeks were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). Mice were housed under specific pathogen−free conditions. THP−1 cells (1 × 106) in a final volume of 100 μL of PBS were bilaterally subcutaneously inoculated into the dorsum the SCID mice. After 10–14 days, the tumor volumes had reached 90–110 mm3, and the mice were randomly divided into four groups of control, HHT, ACR, and HHT+ACR. Mice were treated with 1 mg/kg HHT and/or 3 mg/kg ACR every day by intraperitoneal injection for 5 days. The placebo group received an equal volume of PBS.

2.11. Drugs effects and micro−posision emission tomography (PET)

After the beginning of treatment, the tumor volume was measured every 2 days. Tumor volume was calculated using the formula: tumor volume = π/6 × length × width2. Three days after the last injection, three mice from each group were killed; the tumors were excised and processed for Western blotting and histopathological analyses. To accurately evaluate the glucose metabolism of xenograft tumors, micro−PET was conducted in one mouse from each group at day 0 and day 8 from the beginning of treatment. Animals were anesthetized with pentobarbital (40 mg/kg, intraperitoneally) and injected with 0.1 μCi [18F] fluoro−D−glucose (FDG) via the tail vein. Forty−five minutes later, static acquisition in three−dimensional mode was conducted using a Micro−PET Imaging system (R4, Concorde Microsystems, Knoxville, TN, USA). For semiquantitative evaluation, the region of interest (ROI) method was used to evaluate the regional uptake of the tracer 18F−FDG. ROIs were drawn around the tumor. The tracer uptake
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Fig. 1. HHT and ACR inhibit the growth of AML cells in a synergistic manner. The rate of growth inhibition induced by HHT, ACR and HHT + ACR in THP-1 cells (A), Kasumi cells (C) and primary AML cells (E). CI values for HHT and ACR combination treatments at the molar ratio of 2.5:1 in THP-1 cells (B), Kasumi cells (D) and primary AML cells (F).

(percentage injection dose) in the ROI were recorded and calculated by ASIPro 6.0.5.0 software.

2.12. Statistical analysis

The half-maximal inhibitory concentrations (IC_{50}) were calculated using GraphPad Prism (GraphPad Software, USA). The combination index (CI) was calculated using the Chou–Talalay method (CalcuSyn software, Biosoft, San Diego, CA, USA) to ascertain if the effects of drug combinations were synergistic (CI<1), additive (CI=1), or antagonistic (CI>1) [15]. Group comparisons of parametric data were performed using the Student’s t-test. Statistical analyses were carried out with SPSS version 16.0 (SPSS, Chicago, IL, USA). P<0.05 was considered significant.

3. Results

3.1. HHT and ACR synergistically inhibit growth in AML cell lines and primary AML cells

The growth inhibition of THP-1 cells and Kasumi-1 cells by HHT and ACR were examined by exposing cells to various concentrations of HHT or ACR for 24 h and 48 h. THP-1 and Kasumi cells were plated into 96-well plates and treated with increasing concentrations of HHT (9, 18, 36 and 72 nM), ACR (22.5, 45, 90 and
180 nM) or equal volumes of DMSO. The IC_{50} values for HHT in THP-1 cells and Kasumi-1 cells at 24 h were 71.70 ± 8.77 nM and 57.37 ± 6.97 nM, respectively. The IC_{50} at 24 h for ACR in THP-1 cells was 171.10 ± 6.05 nM, and 146.80 ± 8.65 nM for Kasumi-1 cells. The IC_{50} for HHT in THP-1 cells over 48 h was 35.035 ± 7.73 nM, and 12.97 ± 3.55 nM for Kasumi-1 cells. The IC_{50} values for ACR in THP-1 cells and Kasumi-1 cells over 48 h were 161.81 ± 11.96 nM and 84.47 ± 7.11 nM, respectively. The MTT assay showed dose-dependent growth inhibition by HHT and ACR (P < 0.001). The dose–effect curves for HHT + ACR were determined by Calcusyn analyses in THP-1 cells, Kasumi-1 cells and primary AML cells. Simultaneous exposure to HHT and ACR for 24 h resulted in a strong synergistic inhibition of growth in all three cell types (Fig. 1A, C and E). The best synergistic effect was observed when the molar ratio was 1:2.5 (HHT:ACR) in THP-1 cells and Kasumi-1 cells. The CI at the median effective dose (ED_{50}) was 0.69 for THP-1 cells, 0.42 for Kasumi-1 cells and 0.34–0.55 for primary AML cells (Fig. 1B, D and F). Thus, HHT and ACR acted synergistically to inhibit the growth of AML cell lines and primary AML cells in vitro.

3.2. HHT and ACR synergistically induce apoptosis in AML cells

The previous experiment demonstrated that HHT and ACR both individually induced apoptosis in leukemia cell lines. In the present study, we investigated if these two drugs could synergistically induce apoptosis in AML cells using FACS analyses and Hoechst staining. Compared with single agents, a combination of HHT and ACR resulted in a significant three- to six-fold increase in apoptosis in THP-1 and Kasumi-1 cells (Fig. 2A and C). The CI at the ED_{50} was 0.32 for THP-1 cells and 0.15 for Kasumi-1 cells (Fig. 2B and D).

We also measured the level of apoptosis induced by HHT alone, ACR alone, and HHT + ACR in primary AML cells from 5 patients. The mean level of apoptosis induced by 144 nM HHT and 360 nM ACR after 1 h was 30.97 ± 6.93% and 25.08 ± 9.62%, respectively. HHT combined with ACR resulted in an apoptosis value of 63.24 ± 8.83% (Fig. 2E). The CI at the ED_{50} was 0.05–0.24 (Fig. 2F). To further confirm that HHT + ACR synergistically induced apoptosis in AML cell lines, we studied the nuclear morphology of cells after Hoechst staining. Significantly more cells with apoptotic morphology were observed in the combined group than in the single-agent groups (Fig. 2G).

3.3. HHT and ACR synergistically induce apoptosis by regulating members of Bcl-2 family and caspase family

Western blotting was used to evaluate the expression of Bcl-2, Bax, Mcl-1, caspase-3, caspase-9 and poly(adenosine diphosphate-ribose) polymerase (PARP) in AML cell lines after treatment with HHT, ACR and HHT + ACR for 3–24 h. The results showed that 36 nM HHT could decrease the expression of Mcl-1 and increase the
expression of Bax after 3 h exposure in THP-1 cells and Kasumi-1 cells. However, no change in the expression of Bcl-2 was detected in THP-1 cells and Kasumi-1 cells treated with 36 nM HHT for 3–24 h. Downregulation of Bcl-2 protein expression were observed in THP-1 cells and Kasumi-1 cells treated with 90 nM ACR for 6–24 h. Compared with HHT, a subsequent upregulation of Bax and downregulation of Mcl-1 were detected in THP-1 cells and Kasumi-1 cells exposed to 90 nM ACR (Fig. 3A and B). HHT + ACR caused a more significant increase in the expression of Bax and reduction in the expression of Bcl-2 after 6 h exposure (Fig. 3C). Furthermore, a pronounced increase in cleavage of caspase-9 and caspase-3, as well as degradation of PARP was induced by combined HHT + ACR treatment in THP-1 and Kasumi cells (Fig. 3D and E).

3.4. Genechip array and real-time RT-PCR

Using a fold change of ≥2 and P < 0.05, we identified that 28% of the WNT signaling genes were significantly differently expressed between the ACR group and untreated group; however, only 12% of the WNT signaling genes were significantly differently expressed between the HHT group and untreated group. Among these genes, WNT3A was found to be significantly downregulated in the ACR group, along with β-catenin and WNT10B. In cells treated with HHT, WNT3A and WNT10B were found to be upregulated. Another differentially expressed gene, PIK3CA, was downregulated in both the HHT group and combination group with absolute FC values of 2.74277 and 2.60000, respectively (Fig. 4). No significant difference in the expression of the PIK3CA gene was detected between the ACR group and untreated group. Next, we evaluated the gene expression of WNT3A and β-catenin in AML cells after treatment with ACR or HHT for different times (Fig. 5). A time-dependent decrease

![Fig. 3. Apoptosis detected by Western blotting analyses of caspase-3 (32 kDa, 19/17 kDa), caspase-9 (47 kDa, 37/35 kDa) PARP (116/85 kDa) and Bcl-2 family proteins in THP-1 cells and Kasumi cells. The expression of Bcl-2, Bax and Mcl-1 were regulated by HHT and ACR in THP-1 cells (A) and Kasumi cells (B). HHT + ACR simultaneously induced the up-regulation of Bax protein and the down-regulation of Bcl-2 protein after 6 h exposure (C). Caspase-9, caspase-3 and PARP were activated by combined HHT + ACR treatment in THP-1 (D) and Kasumi cells (E).](image1)

![Fig. 4. Different expression of WNT genes and PI3K between the HHT group and ACR group in THP-1 cells as detected by microarray analyses. Increased and decreased transcription levels were labeled by red color and green color respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.](image2)
in expression of WNT3A and β-catenin mRNA was confirmed in two AML cell lines treated with 90 nM ACR by real-time PCR analyses. However, the expression of WNT3A and β-catenin mRNA did not decrease in cells exposed to 36 nM HHT.

3.5. HHT and ACR cooperate to inhibit PI3K/AKT pathway signaling and WNT pathway signaling in leukemia cells

Early studies showed that HHT inhibited AKT activity in myeloma cells [11]. ACR is an inhibitor of topoisomerase II and inhibits the synthesis of DNA and RNA. Our early gene array analyses showed that ACR affected the expression of several WNT signaling genes. Therefore, we evaluated the effect of HHT and ACR on PI3K/AKT signaling and WNT signaling in AML cell lines. First, we detected the expression of PI3K, P-AKT and total AKT in AML cells after treatment with HHT or ACR. Cells were treated with 36 nM HHT for 3–24 h. Decreased protein expression levels of PI3K110 and P-AKT were observed in THP-1 and Kasumi cells treated with HHT for 3 h. In contrast, no significant differences in the expression of these two proteins were observed in cells treated with 90 nM ACR (Fig. 6A and B).

Western blotting showed that ACR inhibited the expression of WNT3A and β-catenin. No changes in GSK-3β expression were observed in THP-1 and Kasumi cells treated with 90 nM ACR for 3–24 h. Interestingly, treatment of the two AML cell lines with HHT resulted in appreciable inhibition of the protein expression of β-catenin, whereas expression of WNT3A remained stable at the protein levels (Fig. 6C and D). Activated AKT inhibits the activation of GSK-3β [12], which acts as an inhibitor of β-catenin in the WNT signaling pathway. In the present study, a time-dependent increase in the expression of GSK-3β protein was observed in HHT-treated AML cells. As expected, HHT + ACR simultaneously reduced the expression of PI3 K, P-AKT, WNT3a and β-catenin at the protein level in THP-1 and Kasumi cells, with corresponding downregulation of the expression of C-MYC and cyclin D1 (downstream factors of WNT signaling) observed (Fig. 6E and F). Similar results were observed in primary AML cells (Fig. 6 G and H).

3.6. Silencing of WNT3A increases growth inhibition and apoptosis in AML cells treated with an AKT inhibitor or HHT

We used siRNAs to silence the WNT3A gene in THP-1 cells. THP-1 cells were transfected with different concentrations of positive control. Transfection efficiency was then assessed using fluorescence microscopy and flow cytometry at 12–24 h after transfection. High transfection efficiency (76.11%) was observed in THP-1 cells transfected with 20 nM red fluorescent control for 24 h (Fig. 7A). Accordingly, 20 nM of the three WNT3A siRNAs were used to silence the WNT3A gene in THP-1 cells. At 24 h and 48 h post-transfection, expression of WNT3A mRNA was determined by real-time PCR. After transfection of one siRNA (number G12) specific for WNT3A, there was a dramatic reduction in the expression of WNT3A mRNA (≈80% at 48 h) that corresponded with a decrease in the expression of WNT3A and β-catenin proteins (Fig. 7B).

To ascertain if silencing of WNT3A enhanced the effects of an AKT inhibitor or HHT on apoptosis and growth inhibition, the cytotoxicity of the AKT inhibitor triciribine or HHT was evaluated in THP-1 cells treated with specific siRNA or scrambled siRNA. Dose-dependent growth inhibition was observed in THP-1 cells transfected with WNT3A siRNA or scrambled siRNA after treatment with triciribine for 24 h. Compared with cells transfected with scrambled siRNA, a 1.47–2.02-fold increase in growth inhibition was observed in WNT3A -silenced cells after exposure to 60–120 μM triciribine for 24 h (P<0.05) (Fig. 7C). The growth
inhibition caused by HHT was also increased by WNT3A silencing in THP-1 cells. HHT led to growth inhibition of 13.56–41.74% in THP-1 cells transfected with scrambled siRNA. In THP-1 cells transfected with the WNT3A siRNA, HHT induced a 1.50–2.36-fold increase in growth inhibition (P<0.05; Fig. 7D). Furthermore, we investigated if silencing of WNT3A could increase the apoptosis induced by HHT or triciribine in THP-1 cells. Compared with cells transfected with scrambled siRNA, THP-1 cells transfected with WNT3A siRNA showed a 1.96–2.32-fold increase in the number of AV+/-P− cells (an indicator of early apoptosis) after incubation with triciribine for 24 h (Fig. 7E). After exposure to 9 nM, 18 nM and 36 nM of HHT for 24 h, the percentage of AV+/-P− cells were 20.32%, 21.21% and 20.39%, respectively, in THP-1 cells transfected with WNT3A siRNA, which was significantly higher than the respective control groups (P<0.05; Fig. 7F).

3.7. Upregulate the expression of β-catenin impaire the cytotoxicity of HHT and ACR in THP-1 cells

THP-1 cells were incubated with 50–400 nM CHIR-99021 (a GSK-3β inhibitor) for 24 h. Western blotting was used to evaluate the protein expression of β-catenin and GSK-3β. Although no change in the expression of GSK-3β was confirmed, a concentration-dependent increase in expression of β-catenin was detected in THP-1 cells treated with 100–400 nM CHIR-99021 (Fig. 8A). After treatment with 200 nM CHIR-99021 for 24 h, THP-1 cells were harvested and then were treated with HHT, ACR and HHT + ACR for 12 h. Flow cytometry analysis showed a slight decrease in apoptosis in THP-1 cells treated with HHT+ACR after exposure to CHIR-99021, but this decrease was not significant (Fig. 8B). Western blotting showed that the expression of β-catenin was only partially recovered by 200 nM CHIR-99021 (Fig. 8C).

3.8. Synergistic anti-leukemic effects of HHT and ACR in THP-1 xenograft tumors in SCID mice

To examine the interaction between HHT and ACR in vivo and further evaluate their combined therapeutic effects in AML, THP-1 xenograft tumors were allowed to grow in SCID mice before randomization in four groups: control, HHT, ACR, and HHT + ACR. Mice were treated with HHT (1 mg/kg) daily for 5 days and/or ACR (3 mg/kg) daily for 5 days. The dynamic changes in the tumor volumes are shown in Fig. 8A. On the seventh day after the end of treatment, significant delay in tumor growth was only observed in mice treated with HHT+ACR, compared with the PBS-treated controls (P<0.001). At 11 days post-treatment, significant delays in tumor growth were observed in mice treated with HHT (P<0.05) or ACR (P<0.05). The average tumor volume reached 1000 mm3 after 22 days in the HHT+ACR group, after 11 days in untreated mice, and after ≈16 days in the HHT or ACR treatment groups. Kaplan–Meier survival analyses showed favorable survival in the HHT+ACR group (Fig. 8C). Mice in the HHT+ACR group had a mean survival of 45.00±11.98 days, which was longer than that seen in control mice (27.83±7.22 days, P<0.05). However, significantly better survival was not observed in mice receiving a single agent. The mean survival time was 34.60±13.87 days in mice treated with HHT and 30.80±9.04 days in mice treated with ACR (Fig. 8B). The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay also showed increased apoptosis in the tumor tissue of the HHT+ACR group compared with a single agent (HHT or ACR) group (Fig. 8D).

Fig. 6. Western blot analyses of PI3K/AKT (A and B) and WNT/β-catenin (C and D) protein expression in THP-1 cells and Kasumi cells exposed to 36 nM HHT or 90 nM ACR for 3–24 h. THP-1 cells (E), Kasumi cells (F) and primary AML cells (G and H) were cultured in 36 nM HHT, 90 nM ACR and HHT + ACR for 6 h. Expression of WNT3a, β-catenin, PI3K110α, P-AKT, C-MYC and CyclinD1 were analyzed using Western blotting.
3.9. Glucose metabolism in tumor xenografts

The $^{18}$FDG uptake of THP-1 xenografts was assessed on day 0 and day 8 after the end of treatment in the same mice from each group. For PBS-treated mouse, the $^{18}$FDG uptake of the xenograft increased by 98.50% on day 8 compared with baseline (day 0). For HHT-treated mouse, $^{18}$FDG uptake increased by 24.41% on day 8. An increase in $^{18}$FDG uptake of 18.47% was observed in the xenografts of ACR-treated mouse on day 8. For a mouse treated with HHT + ACR, $^{18}$FDG uptake decreased by 30.69% on day 8 compared with baseline (Fig. 10).

3.10. Effects of HHT and ACR on apoptosis in CD34+/CD38− AML cells

To ascertain if HHT works in tandem with ACR to induce apoptosis in leukemia stem cell (LSC)-enriched cells, we examined the effects of a combination of HHT and ACR on the induction of apoptosis in CD34+/CD38− AML cells by flow cytometry. A single agent (HHT or ACR) could induce apoptosis in a concentration-dependent manner in CD34+/CD38− cells after 12 h exposure. Compared with single treatments, we detected more apoptotic cells in the HHT + ACR group at a molar ratio of 1:2.5. Treatment with 36 nM HHT resulted in 7–20% apoptotic cells, whereas treatment with 90 nM ACR produced 13–18% apoptotic cells. In comparison, combined agents increased apoptosis by 22–78%. CI/fractional effect analyses showed that the CI was 0.25, 0.41 and 0.62 at ED$_{50}$, ED$_{75}$ and ED$_{90}$, respectively (Fig. 11). Thus, combining HHT and ACR led to cooperative induction of apoptosis in CD34+/CD38− primary AML cells. Additionally, flow cytometry analyses showed HHT, ACR and HHT + ACR induced higher levels of apoptosis in CD34+/CD38− THP-1 cells than in CD34+/CD38− THP-1 cells; a significant difference was observed only in low-concentration groups. The percentage of AV+ cells induced by 18–36 nM HHT, 45–90 nM ACR and 18 nM HHT + 90 nM ACR were significantly higher in CD34+/CD38− group than in CD34+/CD38+ group ($\text{P} < 0.05$).

4. Discussion

In this study, we showed that HHT or ACR alone result in an inhibition of cell growth and induce apoptosis in THP-1 cells and Kasumi cells in a dose-dependent manner. These findings support the notion that HHT and ACR can induce apoptosis in AML cells [3–5]. To extend our analysis of the synergistic interactions between HHT and ACR, different ratios of HHT + ACR were used to treat two AML cell lines and primary AML cells. Cell growth assays

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**Fig. 7.** WNT3A silencing increased the cell growth inhibition and apoptosis induced by HHT or an AKT inhibitor in THP-1 cells. The transfection efficiency was 76.11% when THP-1 cells were transfected with 20 nM of red fluorescent control for 24 h (A). After the transfection of one siRNA specific for WNT3A, there was a dramatic reduction in the expression of WNT3A mRNA which led to corresponding decreases in the expression of WNT3A and β-catenin protein expression (B). Tocitirine induced significantly greater growth inhibition and apoptosis in cells transfected with WNT3A siRNA (C and E). In THP-1 cells transfected with WNT3A siRNA, HHT induced a 1.50–2.36-fold increase in growth inhibition (D). Silencing Wnt3a induced 1.5–2.0-fold increase of apoptosis in THP-1 cells exposure to different concentrations of HHT for 24 h (F).
**Fig. 8.** A GSK-3β inhibitor impaired the cytotoxicity of HHT and ACR in THP-1 cells. CHIR-99021 induced a significant increase in the protein expression of β-catenin in concentration-dependent manner (A). 200 nM CHIR-99021 slightly decreased the apoptosis induced by HHT+ACR in THP-1 cells, but this decrease was no statistically significance (B). CHIR-99021 partially recovered the decrease expression of β-catenin in THP-1 cells treated with HHT+ACR at concentration used (C).

**Fig. 9.** Dynamic changes in tumor volume and overall survival time in xenograft mice after the administration of agents. A significant delay in tumor increase was observed only in the HHT+ACR group (A). Exposure to HHT+ACR brought a longer survival time and better long-term survival (B and C). TUNEL staining: more TUNEL-positive cells were observed in the HHT+ACR group (D).
HHT with ACR elicited slower tumor growth and improved survival compared with the single-drug treatments. \(^{18}\)FDG-PET images revealed that the FDG accumulation of xenografts decreased in the HHT + ACR group on day 8 by 30.69% compared with the baseline day 0, although the tumor volume continued to increase. TUNEL assay also showed increased apoptotic cells in the tumor tissue of the HHT + ACR group compared with control group and a single agent (HHT or ACR) group. These data suggested that HHT + ACR could inhibit the metabolic activity of THP-1 xenografts by inducing cell apoptosis, and then delay the growth of xenografts. Therefore, our studies suggest that combining HHT with ACR can result in synergistic cytotoxicity to AML cells in vitro and in vivo.

To elucidate the molecular mechanisms involved in the synergistic effects of HHT and ACR, gene chip analyses were used to identify the genes that were differentially expressed in cells treated with HHT and ACR. ACR was found to significantly downregulate the expression of several WNT signaling genes, but not PI3K genes, in AML cells. HHT significantly downregulated the expression of the PI3K gene. Quantitative PCR for WNT3A and β-catenin confirmed the findings of the microarray analyses. It has been established that many bcl-2 family members \([3,4,16,17]\), and AKT signaling and JAK/STAT [4] signaling are involved in HHT-mediated killing in leukemia cells. Our results also showed obvious suppression of PI3K/AKT signaling by HHT in AML cell lines and primary AML cells. These findings suggest that HHT inhibits cell proliferation and induces apoptosis via suppression of PI3K/AKT signaling. The identity of the signal by which ACR induces apoptosis in AML is not known. Recently, ACR was confirmed to enhance the induction of TRAIL-mediated apoptosis via upregulating DR5 expression in acute lymphoblastic leukemia Jurkat cells [6]. Our findings showed that ACR failed to downregulate PI3k 1105 and P-AKT protein expression in AML cells.

The WNT signaling pathway is known to be a key regulator of many embryonic development events, including hemopoiesis. Several members of the WNT/β-catenin pathway are involved in the self-renewal and differentiation of hematopoietic stem cells (HSCs) \([18,19]\). In murine hematopoietic cells, WNT3a was shown to induce higher levels of β-catenin in the presence of Frizzled-4 receptor [20]. WNT3a has been reported to promote the proliferation of leukemia stem/progenitor cells in some AML cell lines by reducing the level of phosphorylated β-catenin [21]. Simon and colleagues showed that CD34+ progenitor cells expressing mutant β-catenin failed to differentiate toward myelomonocytes [22]. These results suggest that constitutive activation of WNT3a and β-catenin is implicated in the excessive proliferation and differentiation arrest of progenitor cells. In the present study, we showed that the activity of wnt3a and β-catenin were significantly reduced.

**Fig. 10.** Micro-PET. On day 8 after the end of treatment, the \(^{18}\)FDG uptake of THP-1 xenografts was about one-fold increase in a PBS-treated mouse compared with baseline (a). For HHT-treated mice and ACR-treated mice, \(^{18}\)FDG uptake increased by about 20% on day 8 (b and c). Only for a HHT + ACR-treated mouse, 30.69% decrease of \(^{18}\)FDG uptake was detected (d). The xenografts are labeled by red arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

showed a synergistic anti-proliferative effect, as indicated by the CIs at four concentration ratios of HHT + ACR: 1:2, 1:3, 1:4 and 1:5 (complete data not shown). Similarly, combining HHT with ACR strongly and synergistically induced apoptosis in AML cells. We also report the cooperative anti-tumor activity of HHT in combination with ACR in SCID mice carrying THP-1 cell xenografts.

**Fig. 11.** The synergistically effects of HHT and ACR on apoptosis in CD34+/CD38− AML cells. Primary AML cells were incubated with antibodies against CD34 and CD38 for 15 min and then stained with Annexin V. The percentage of AV+ cells in the CD34+/CD38− group and CD34+/CD38− group was identified by flow cytometry (A). CI/fractional effect curves indicating the synergistic induction of apoptosis by HHT + ACR in CD34+/CD38− AML cells (B).
by ACR in AML cell lines, and that this was followed by growth inhibition and apoptosis. Therefore, one of the possible mechanisms of cytotoxicity due to ACR in AML cells is downregulation of the WNT3a signaling, instead of PI3K/AKT signaling. Inconsistent with the findings from RT-PCR, an apparent downregulation of β-catenin protein expression was observed in AML cells exposed to HHT for 3 h (even though significant changes in WNT3a expression were not detected). These findings prompted that HHT may regulate the expression of β-catenin at protein level rather than mRNA level. Studies have suggested that AKT activation due to phosphorylation at Ser473 results in inactivation of GSK3β in many carcinoma cells (including leukemia cells), followed by upregulation of β-catenin [12,23–25]. Activated AKT can even promote the activation of β-catenin directly by phosphorylating AKT at Ser552 [26]. In the present study, after downregulation of P-AKT protein expression, a time-dependent increase in the expression of GSK-3β protein was observed in HHT-treated AML cells. Therefore, we conjecture that the downregulation of β-catenin activity induced by HHT was due to inhibition of AKT activity and an increase in GSK-3β expression in AML cells.

Our results showed that HHT+ACR induced simultaneous downregulation of PI3K, P-AKT, WNT3a and β-catenin at the protein level in AML cells. Using WNT3a siRNA interference, we showed that WNT3a silencing could significantly increase the cell growth inhibition and apoptosis induced by HHT or an AKT-specific inhibitor in THP-1 cells. Combination of an AKT-specific inhibitor and ACR also elicited synergistic effects upon the induction of cell growth inhibition and apoptosis in THP-1 cells (data not shown). All these findings provide evidence that one of the possible mechanisms of synergistic cytotoxicity induced by HHT and ACR is the simultaneous impairment of PI3K/AKT signaling and WNT/β-catenin signaling in AML cells. Among these two pathways, β-catenin was highlighted as the common target molecule of HHT and ACR. Using a GSK-3β inhibitor, we tried to ascertain if the cytotoxicity of HHT+ACR could be impaired through upregulation of β-catenin activity. A slight decrease in apoptosis was observed in THP-1 cells treated with HHT+ACR after exposure to a GSK-3β inhibitor, but this decrease was not significant. Western blotting showed that the expression of β-catenin was only slightly recovered by the GSK-3β inhibitor (at the concentration used). These findings provide a possible explanation for the non-significant decrease in apoptosis induced by the GSK-3β inhibitor. Further studies focusing on the induction of β-catenin overexpression should be carried out. In addition, other pathways may be involved in the synergistic cytotoxicity induced by HHT+ACR in AML cells.

Recently, the role of WNT/β-catenin signaling in LSCs has received increasing attention. Using a microarray assay, Majeti and colleagues reported a distinct upregulation of WNT pathway genes in LSCs [27]. A recent study showed that AML could be induced in C57BL/6 mice by transplantation of granulocyte/macrophage progenitor (GMP)-Hoxa9/M cells transduced with a retrovirus carrying β-catenin, but not by GMP-Hoxa9/M cells without a retrovirus transducer [28]. Knockdown of the expression of β-catenin in DLL LSCs resulted in a further reduction of colony formation. Silencing of β-catenin at an early stage of transformation can completely abolish the potential leukemogenic ability of DLL-transduced cells [29]. Hence, we conclude that the β-catenin signal is required for the transformation and self-renewal of LSCs. Furthermore, AKT phosphorylation on Ser473 has been detected in CD34+, CD34low/neg and CD123+ populations containing immature leukemia cells [30]. Kharas et al. found that 10% of recipient mice transduced by excessive AKT HSCs developed AML. Short-term elevation of AKT levels causes expansion of lin−/c-kit/sca− cells (LSK) [31]. In the present study, HHT+ACR simultaneously impaired the activity of WNT/β-catenin signaling and PI3K/AKT signaling in AML. These findings raise the interesting possibility that HHT+ACR synergistically target AML LSCs. Using flow cytometry, we found that HHT+ACR could synergistically induce the apoptosis of CD34+/CD38− primary AML cells. Despite popular opinion, HHT+ACR induced appreciably more apoptosis in CD34+/CD38− cells than in CD34+/CD38+ cells (even though a significant difference was detected only in the low-concentration groups). Some authors have found that CD34+ progenitors in peripheral blood express intermediate levels of β-catenin activation, but this β-catenin activation is rapidly lost upon myeloid differentiation [22,32]. Different levels of WNT activation have different roles in the in vivo homing properties of HSCs, myeloid progenitors and LSCs [33]. High levels of β-catenin activation result in impaired differentiation and the development of leukemia [34]. Taken together, these results suggest that the β-catenin signal regulates hemopoiesis and the development of leukemia in a dose-dependent manner. Hence, different levels of β-catenin activation may have led to different sensitivities to our agents in the two sub-groups of AML cells.

5. Conclusions

In conclusion, we have provided evidence that the combination of HHT and ACR induces a cooperative anti-cancer effect in AML cells in vitro and in vivo. PI3K/AKT signaling and WNT/β-catenin signaling are all involved in the mechanism of synergistic cytotoxicity induced by HHT+ACR in AML cells. Additionally, this synergistic induction of apoptosis was also observed in CD34+/CD38− AML cells. Taken together, these data suggest that β-catenin and AKT in combination are promising therapeutic targets for AML cells, including LSC-enriched cells.

Conflict of interest

All authors have no conflict of interest to report.

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