Tigecycline and homoharringtonine synergistically target myeloid leukemia cells by inhibiting mitochondrial translation through mTOR/4EBP1 pathway

Haiyang Yang
Zhejiang University School of Medicine First Affiliated Hospital
Chen Mei
Zhejiang University School of Medicine First Affiliated Hospital
Li Ye
Zhejiang University School of Medicine First Affiliated Hospital
Yanling Ren
Zhejiang University School of Medicine First Affiliated Hospital
Hua Zhang
Zhejiang University School of Medicine First Affiliated Hospital
Kongfei Li
Department of Hematology, Yin Zhou People Hospital
Jiansong Huang
Zhejiang University School of Medicine First Affiliated Hospital
Xin Huang
Zhejiang University School of Medicine First Affiliated Hospital
Weilai Xu
Zhejiang University School of Medicine First Affiliated Hospital
Xinping Zhou
Zhejiang University School of Medicine First Affiliated Hospital
Gaixiang Xu
Zhejiang University School of Medicine First Affiliated Hospital
Lu Wang
Zhejiang University School of Medicine First Affiliated Hospital
Chuying Shen
Zhejiang University School of Medicine First Affiliated Hospital
Hongyan Tong (tonghongyan@zju.edu.cn)
Zhejiang University School of Medicine First Affiliated Hospital https://orcid.org/0000-0001-5603-4160

Research

Keywords: Tigecycline, Homoharringtonine, AKT, mTOR, 4EBP1, myeloid leukemia

Posted Date: August 4th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-42286/v1

Tigecycline and homoharringtonine synergistically target myeloid leukemia cells by inhibiting mitochondrial translation through mTOR/4EBP1 pathway

Haiyang Yang[1,2], Chen Mei[1],Li YE[1], Yanling Ren[1], Hua Zhang[1],Kongfei Li[3],Jiansong Huang[1,2],Xin Huang[1,2],Weilai Xu[1],Xinping Zhou[1],Gaixiang Xu[1], Lu Wang[1], Chuying Shen[1],Hongyan Tong[1,2*]

Author	Email address
Haiyang Yang	haiyanglucky@163.com
Chen Mei	meichenblood@yeah.net
Li YE	yelikybs@126.com
Yanling Ren	yanlingrenxm @163.com
Hua Zhang	sapphirezh@126.com
Kongfei Li	konfeelee@126.com
Jiansong Huang	hjiansong1234@zju.edu.cn
Xin Huang	hx0628@yahoo.com
Weilai Xu	lpxwl2005@163.com
Xinping Zhou	zxp1207@163.com
Gaixiang Xu	1505136@zju.edu.cn
Chuying Shen	21818095@zju.edu.cn
Lu Wang	11818213@zju.edu.cn
Hongyan Tong	tonghongyan@zju.edu.cn

Abstract

Background: Tigecycline (TIG) is a tetracycline derivative antibiotic. Successive studies have shown that TIG is efficacious for the treatment of some solid tumors and hematological malignant diseases both *in vivo* and *in vitro*, and drug combinations appear to provide better inhibition. To explore new drug combinations for myeloid leukemia, we compared the differential combination efficacy of TIG with several anti-leukemia drugs, and explored the mechanisms of the combination of TIG and homoharringtonine (HHT) in myeloid leukemia cells both *in vitro* and *in vivo*.

Methods: Cell proliferation was assessed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyph-enyl)-2-(4-sulfophenyl)-2H-tetrazolium) and CFU-GM (colony forming unit-granulocyte and macrophage)

assays. Apoptosis was detected by flow cytometry. The combination of effects was confirmed in myeloid leukemia cells and tumor-bearing mouse model. The regulation of the AKT/mTOR (mammalian target of rapamycin)/4EBP1 (eukaryotic translation initiation factor 4E binding protein 1) pathway was assessed using a Western blot and immunohistochemistry.

Results: The combination of TIG and HHT had a strong synergistic effect in myeloid leukemia cells. The use of the drug combination *in vivo* also effectively delayed myeloid tumor development in mice. The synergistic effect of this drug combination is likely to be achieved by inhibiting mitochondrial translation and down-regulating the AKT/mTOR/4EBP1 signaling pathway.

Conclusion: The combination of TIG and HHT can synergistically enhance an anti-leukemia effect through downregulating anti-apoptotic proteins. Inhibiting mitochondrial translation through the AKT/mTOR/4EBP1 pathway might be an important mechanism.

Keywords: Tigecycline; Homoharringtonine; AKT; mTOR; 4EBP1; myeloid leukemia

Background

Tigecycline (TIG) is a new generation of tetracycline antibiotic which contains the N,N-dimethylglycylamido substitution at position 9 of minocycline [1]. The mechanism of action is similar to that of ordinary tetracyclines, and it binds to the A site of the 30 s subunit of the bacterial ribosome. Moreover, TIG also binds to the remaining part of the H34 ribosome, resulting in a stronger inhibition of bacterial translation [2]. Ribosome protection and drug efflux are the primary mechanisms by which most bacteria develop tetracycline resistance [3]. The steric hindrance of TIG is due to a large substituent at position 9 appears to surmount these resistance mechanisms [2].

It has been reported that TIG has an anti-tumor effect in many solid tumors and blood related diseases [4-7]. The inhibition of mitochondrial translation is functionally important for the selective toxicity of TIG on leukemia cells in xenograft models of human leukemia [5]. Moreover, FDA(Food and Drug Administration)-approved drug screening has determined that TIG can effectively suppress Rb/p53-deficient tumor cell proliferation (e.g., triple-negative breast cancer) by inhibiting the expression of mitochondrial protein translation (MPT) [4].

TIG has been shown to exhibit potential value in the clinical treatment of blood-related diseases (e.g., lymphoma and leukemia), especially in combination with chemotherapy drugs. The combination of TIG with daunorubicin and cytarabine has shown a strong anti-leukemic activity both *in vivo* and *in vitro* [5]. As previously reported, the mTOR/4EBP1 pathway plays an important role in regulating mRNA translation [8-10]. In our study, we screened and compared the combined effects and differences of TIG with Chidamide (CHI), Idarubicin (IDA), and HHT, respectively. It was found that TIG combined with HHT showed a strong synergistic effect. Further research revealed that this synergy is likely to be achieved by inhibiting mitochondrial translation and acting on the cellular mTOR/4EBP1 pathway.

Materials and methods

2.1 Antibodies and reagents

Antibodies specific to β-actin (#8457), Caspase3 (#9664), PARP(poly ADP-ribose polymerase) (#9532), p-Akt (#4060), p-mTOR (#5536), p-Raptor (#9111), p-4EBP1 (#13443), COX(Cyclooxygenase)-1 (#9947) including COX-4 (#4844), p-Rictor (#3806), Ki-67 (#9449) were purchased from CST (Danvers, MA). HHT was obtained from MedChemExpress (Monmouth Junction, NJ). CHI was purchased by Chipscreen Biosciences (Shenzhen, China) and IDA was purchased from the Hangzhou Minsheng Institute of

Pharmaceutical Research (Hangzhou, China). TIG was gifted by the Pfizer Biopharma (Hangzhou) Co., Ltd (Hangzhou, China).

2.2. Cell lines and primary patient cells

The myeloid leukemia cell lines included SKM-1, MOLM-13, and MDS-L cells. SKM-1 and MOLM-13 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China). MDS-L was presented by Professor Huang Gang (The Cincinnati Children's Hospital Medical Center). Genomic DNA microsatellites were used to identify the authenticity of these cell lines by Shanghai Biowing Applied Biotechnology (Shanghai, China). The SKM-1 and MDS-L cell lines were cultured in RPMI1640 (Corning) medium supplemented with 10% fetal bovine serum (FBS, Gibco) in a humidity cell incubator that was maintained at 37°C and 5% CO_2. The MOLM-13 cell lines and primary myeloid leukemia cells were cultured in Iscove's Modified Dubecco's Medium (IMDM, Corning) supplemented with 10% FBS. The bone marrow mononuclear cells of patients were isolated by Ficoll Hypaque density gradient centrifugation and treated with erythrocyte lysate. All patients signed an informed consent form.

2.3. Cell viability assay

A density of either 1×10^5 for cell lines or 2×10^5 for primary patient cells were seeded into 24-well plates. After drug treatment, the cell suspension was transferred to a 96-well plate. A cell suspension of 200 μL and 20 μL MTS solution (Promega, Madison, WI) were added to each well and incubated at 37°C or 4 h. The optical delnsity (OD) values of these wells were measured using a microplate reader (Varioskan Flash, Thermo scientific) at a wavelength of 490 nm. All cell lines and primary patient cells were tested with at least three independent replicates.

2.4. *Flow cytometry analysis*

After drug treatment, the cells were washed twice with phosphate buffer saline (PBS), and $1 - 5 \times 10^5$ cells were collected. The supernatant was centrifuged at 1500 rpm for 5 min, and 500 μL Binding Buffer was added

to prepare the cell suspension. Next, 6 mL Annexin-V FITC and 10 μL PI (propidium iodide) were added, incubated in dark for 5 min, and annexin-v FITC (radiation wavelength = 488 nm; absorption wavelength = 530 nm) was detected by the FITC signal (FL1 channel). The PE signal was detected for PI staining (FL2 channel). Both signals were analyzed using a FACScan flow cytometer (Becton Dickinson, San Diego, CA).

2.5. *Western blot analysis*

After the drug was administered, the cells were centrifugated to obtain the supernatant, and more than 1 × 10_6 cells were collected in each group. After suspending the cells in 40 μL of RIPA (Radioimmunoprecipitation assay) buffer (Thermo Fisher Scientific, Waltham, MA), they were placed on ice for 30 min at 4°C, centrifugated at 12000 × g for 20 min, and the protein supernatant was collected. The protein concentration in the supernatant was determined with BCA (Bicinchoninic acid) reagent (BBI life sciences, Shanghai, China) and then mixed with 4× loading buffer at 100°C for thermal denaturation. The protein samples were separated with SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel (Thermo Fisher Scientific, Waltham, MA) and transferred to PVDF (Polyvinylidene fluoride) membranes (Millipore, Burlington, MA). The membrane was sealed with tris-buffered saline (TBS) containing 5% skim milk for 1 h and incubated with a primary antibody on a shaker overnight at 4°C. After washing three times with tris-buffered saline tween (TBST) buffer, the membranes were incubated with the secondary antibodies (CST, Danvers, MA) for 1 h. The ECL reagent (Thermo Fisher Scientific, Waltham, MA) was catalyzed by an HRP (Horseradish peroxidase) reaction on the HRP-labeled secondary antibody to display the target protein bands, and the ChemiDoc MP imaging system (Bio-Rad, Hercules, CA) was used for imaging.

2.6. *Immunohistochemistry*

The stripped tumor tissue was cut into appropriately sized tissue pieces and fixed in 4% paraformaldehyde for two days. The tissue pieces were removed from the fixing solution, rinsed with PBS for 1 h, and proceeded. After dehydration, the tissue was completely immersed in a wax solution at 60°C overnight, cut into

continuous 5-μm thick sections, dewaxed with xylene, and then the sections were fully rehydrated with gradient alcohol and water. After the antigen was repaired, an appropriate amount of 3% H_2O_2 was added dropwise. The tissue was covered with H_2O_2 and incubated at room temperature for 10 min to block the endogenous peroxidase activity. The tissue was washed with PBS on a shaker three times for 5 min. Then, 5% goat serum was prepared with PBS added dropwise, and the tissue was blocked for 1 h . The slides were dried, the residual liquid was aspirated, placed in a wet box, the primary antibody liquid was dropped to completely cover the tissue, and incubated overnight at 4°C in a refrigerator. The tissues were washed three times with PBS on a shaker at room temperature for 10 min each time. Then 5% goat serum was diluted with biotin-labeled secondary antibody to cover the surface of the tissue and incubated at room temperature for 45 min, before washing with PBS three times for 10 min. An appropriate amount of avidin-labeled horseradish peroxidase was added to the tissue surface and incubated at room temperature for 30 min. The tissue was washed three times, 5 min each time and diaminobezidin (DAB) color solution was added to the surface of the tissue and the staining was observed under a microscope. When the color was obviously brown and the background was not visible, then placed in water to stop the color reaction. The tissues were stained with hematoxylin for 1 - 2 min and rinsed with water to stop the staining. The tissues were observed and pictures were obtained under the microscope after mounting. On sections that had been dewaxed and rehydrated, an appropriate amount of hematoxylin was added to cover the tissue surface, the nucleus was stained for 5 min, and the remaining staining solution was rinsed away with tap water. The tissues were quickly differentiated with 1% hydrochloric acid alcohol, rinsed with tap water, and an appropriate amount of eosin was added. The solution was stained on the surface of the tissue for 30 s and then rinsed with tap water. After dehydration to transparency, the tissue was observed under the microscope and photographed after sealing.

2.7 ***Mouse models***

Animal experiments in this study were performed in accordance with the "Guidelines for the Care and Use of Laboratory Animals" (NIH Publication 86-23, revised in 1985) issued by the National Institutes of Health. The animal experiment protocol was approved by the Animal Protection and Facilities Committee of Zhejiang University. Male nude mice of six-week-old were purchased from Zhejiang Academy of Medical Sciences. The SKM-1 myeloid leukemia cell line (1×10^7 cells per animal) was subcutaneously injected into the right forelimb flank of the mice. Seven days after cell injection, when the tumors were palpable, animals with approximately the same tumor volume were randomly divided into four groups consisting of five animals per group. The use of TIG (50 mg/kg /d), HHT (0.5 ng/kg/d) or a combination for two consecutive weeks. For the control group, PBS was used for the injection. Both the drugs and PBS were intraperitoneally injected. The mice were weighed every day and calipers were used to calculate the tumor volume every two days using the formula: $\pi/6$ length $\times$ width^2. Two weeks later, in accordance with ethical animal practices, each group of mice was killed, and the tumors were collected for further testing.

2.8 *Statistical analysis*

The significant differences among the groups was determined using a one-way ANOVA (Analysis of Variance) followed by a post-hoc Bonferroni's multiple comparison test. For non-parametric data, a Kruskal-Wallis followed by a Dunn's multiple comparison test was used. The minimal level of significance was $p < 0.05$. All data were presented as the mean ± standard deviation (SD). GraphPad PrismV 5.0 (GraphPad Software, San Diego, CA) was used for statistical analysis. * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Results

TIG-HHT combination can synergistically inhibit the growth of myeloid leukemia cells

To investigate the effect of the drug combination on the proliferation of myeloid leukemia cells, we used the anti-leukemia drugs IDA, HHT, and CHI in combination with TIG to treat SKM-1. The IC50 (50% of

maximal inhibitory concentration) of SKM-1 at 72 h was calculated to determine the appropriate concentration of each drug (Table S1). In the experiment, SKM-1 was exposed to 72 h in TIG, HHT, CHI, and IDA. In all combination groups of SKM-1, the effect of the drug combination on the cells was evaluated at 24, 48, and 72 h. Next, we used the median-effect method to calculate the combination index of each group to evaluate the combined effect of each group using CalcuSyn software[11]. When TIG was combined with CHI and IDA, most dose combinations had CI (Combination Index) values above 1 at 24, 48, and 72 h after the drug combination. Although the duration of action was prolonged, they did not show a better combined effect. When TIG and HHT were administered at the same time, the CI value of almost all metering combinations was less than 1, and the CI value of each metering combination of 72 h was less than 24 h and 48 h (Figure 1). This indicates that the combination of TIG-HHT shows a strong anti-leukemia response. The synergistic and cumulative effects were evaluated over time. At least three independent experiments were performed with three replicates in each group.

To confirm the synergistic effect of the TIG-HHT combination on different myeloid leukemia cells, we measured the IC50 of TIG and HHT for 72 h in MOLM-13, MDS-L, and the primary patient cells (Table S2). Based on the IC50 of each drug, we measured MOLM-13, MDS-L, and patient primary cells for 72 h. The effects of different concentrations of TIG in combination with HHT on the viability of the cells was measured by an MTS assay after a 72 h incubation. The data were analyzed with Calcusyn software to generate a CI versus fractional effect (cell death) plot showing the effect of the combination of TIG with HHT. After 72 h of drug treatment, the CI values of almost all metered combinations were less than 1, indicating that the TIG-HHT combination showed a strong synergistic inhibitory effect in both primary and myeloid leukemia cells. A CI < 1 indicates synergism (Figure 2).

TIG-HHT combination can inhibit significantly clone formation of myeloid leukemia

To test the effect of the TIG-HHT drug combination on the formation of myeloid leukemia cell clones in vitro, 1×10^3 SKM-1, MOLM-13, and MDS-L cells were respectively inoculated on a methylcellulose semi-solid medium. Clone formation experiments were performed and the number of clones was counted using image-J software (Figure 3A). The drug environment of myeloid leukemia cell methylcellulose semi-solid medium was: SKM-1 plus TIG 7.5 μg/mL was treated with HHT 8 ng/mL; MOLM-13 plus TIG 5 μg/mL was treated with 1 ng/mL HHT for 72 h; MDS-L plus 6 mg/mL TIG was treated with 6 ng/mL HHT; and the control group was treated with monotherapy and PBS. The use of TIG or HHT alone had a significant inhibitory effect on the colony forming ability of leukemia cells, and the addition of TIG and HHT at the same time shows a stronger blow to cell colony formation (Figure 3A and C). Combination treatment compared with single treatment and combination treatment compared to the control groups was statistically significant ($P < 0.05$ for SKM-1 and MOLM-13; $P < 0.01$ for MDS-L).

TIG-HHT combination effectively restrains tumor growth in subcutaneous myeloid leukemia mouse models

To explore the effects of drug combinations *in vivo*, we performed combination treatments, single treatments, and PBS treatments (control) on a myeloid leukemia mouse model established by a subcutaneous injection of SKM-1 in mice. After seven days, the tumor grew to a size of about 50 mm^3. We designed a treatment plan according to the report. Both the TIG and HHT group were injected once a day, and the Control group was injected with a corresponding PBS solution. Compared with the control group, TIG and HHT single-drug treatment both delayed tumor growth, and the combination showed higher efficacy ($p < 0.005$, Tukey's t-test after a one-way ANOVA) (Figure 4). Immunohistochemistry and a Western blot detection of the corresponding proteins showed that both single and combined drugs inhibited mitochondrial translation *in vivo*.

TIG-HHT combination induced the apoptosis of myeloid leukemia cells *in vitro* and *in vivo*

To test whether the inhibitory effect of TIG and HHT on cell viability was achieved by inducing and enhancing apoptosis, we performed Annexin V and PI staining on the cells treated with the drug, and analyzed the proportion of apoptotic cells by flow cytometry. The untreated cell group served as a control. The myeloid leukemia cell lines, SKM-1, MOLM-13, and patient primary cells were incubated with TIG monotherapy or combination therapy for 72 h, and apoptosis was quantified by Annexin V/PI staining. Myeloid leukemia cells were treated with different concentrations of the drugs: SKM-1 plus 7.5 mg/mL TIG were treated with 8 ng/mL HHT for 72 h; the control group was treated with monotherapy and PBS; and MOLM-13 plus 5 mg/mL TIG was performed with 1 ng/mL HHT for 72 h. As shown in Figure 3B, compared with the single-agent group and the blank group, the apoptotic rate of the cells increased significantly (Figure 3B). The mechanism of drug-induced apoptosis was studied by a Western blot analysis of the expression of cleaved caspase-3 and cleaved PARP, and the strong pro-apoptotic effect of the drug combination was revealed on a molecular level. The expression of cleaved caspase-3 and cleaved PARP changed, and the protein mass in the two drug lanes was significantly lower than that of the single drug group and the control group (Figure 3D). To further confirm that treatment with TIG and HHT alone or in combination can also inhibit cell viability by inducing and enhancing apoptosis *in vivo*, we examined the expression of cleaved caspase-3 and cleaved PARP in mouse subcutaneous tumor tissue cells by immunoblotting (Figure 5A). Compared with the single drug and the blank groups, the amounts of combined histones was significantly reduced. The immunohistochemistry of the subsequent tissue sections more intuitively confirmed a strong pro-apoptotic effect of the combined group (Figure 4E).

TIG-HHT combination inhibited mitochondrial translation by down-regulation of COX-1

In studies using FDA-approved drugs to screen leukemia cell therapies, the application of yeast whole-genome screening technology confirmed that the specific inhibition of mitochondrial translation is the mechanism of TIG's anti-leukemia effect [5]. Thus, we used a Western blot to identify the DNA and proteins

extracted from myeloid leukemia cells and tumor tissues. myeloid leukemia cells were treated with different concentrations of drugs: SKM-1 plus 7.5 mg/mL TIG was treated with 8 ng/mL HHT for 72 h, and the control group was treated with monotherapy and PBS. MOLM-13 plus 5 mg/mL TIG was treated with 1 ng/mL HHT for 72 h, and MDS-L plus 6 mg/mL TIG was treated with 6 ng/mL HHT for 72 h. Each cell line was treated with monotherapy and PBS was used as a control group. Compared with treatment of TIG or HHT alone, treatment with a combination of TIG-HHT caused a significant down-regulation of the mitochondrial gene, COX-1, expression in myeloid leukemia cells, where the nuclear gene COX-4 did not change significantly (Figure 5B). Tissue-specific protein testing revealed that the combined drug group down-regulated COX-1 gene expression in the tumor cells of subcutaneous myeloid leukemia mice without affecting the expression of the COX-4 gene (Figure 4E and 5B). SKM-1 was further exposed to a combination of drugs with different concentrations, and the inhibitory effect on the cells showed a stronger mitochondrial translation inhibitory effect as the combination drug concentration increased (Figure 5C).

TIG-HHT combination synergistically inhibited the mTOR/4EBP1 pathway

Western blot was used to identify the proteins extracted from both myeloid leukemia cells and tumor tissues. Cells were treated with different concentrations depending on the concentration of each compound used to inhibit cell proliferation: SKM-1 was exposed to 7.5 μg/mL TIG and 8 ng/mL HHT, and MOLM-13 was treated with 5 mg/mL TIG and 1 ng/mL HHT. MDS-L was exposed to 6 μg/mL TIG and 6 ng/mL HHT. Three groups of myeloid leukemia cells were treated for 72 h, and the single drug or PBS group were used as a control. The mitochondrial translation regulators, mTOR, 4EBP1, and AKT proteins were down-regulated in combined or single drug group compared to the control group. The combined TIG-HHT significantly enhanced the inhibition of protein phosphorylation. We tested the level of phosphorylation of the constituent subunit Raptor, a component of mTOR complex 1 (mTORC1) and the constituent subunit Rictor, a component of mTOR complex 2 (mTORC2). When TIG-HHT co-administration, the p-Rictor and p-Rictor were

significantly down-regulated, suggesting that the drug combination exerted a stronger inhibition on the phosphorylation of mTOR pathway proteins. The results also showed that the expression of the mitochondrial gene COX-1, and the levels of phosphorylation of AKT and 4EBP1 were reduced to a uniform level when cultured with TIG-HHT combination (Figure 5B, 5D).

Discussion

Tigecycline (TIG) has been reported to affect cellular translation and tumor proliferation [5, 12]. homoharringtonine (HHT) is an effective drug for myeloid leukemia[11]. In our study, we found the combination of TIG and HHT had a strong synergistic effect in myeloid leukemia cells. The combination *in vivo* also effectively delayed myeloid tumor development in mice. The synergistic effect of the combination might be achieved by inhibiting mitochondrial translation and downregulating the AKT/mTOR/4EBP1 signaling pathway.

Mitochondria are the source of power for various cellular activities and biochemical reactions. TIG can specifically inhibit mitochondrial gene translation by down-regulating the expression of elongation factor, Tu, thereby achieving cytotoxic effects on leukemia cells [5]. Moreover, HHT can also directly inhibit the mRNA translation in myeloid leukemia cells [13]. We explored the mitochondrial gene-encoded protein subunit COX-1 and the nuclear protein-encoded respiratory chain complex 4 protein subunit COX-4 to reveal the differential effects of the drug on nuclear translation and mitochondrial translation. In this study, we found HHT specifically inhibits mitochondrial translation. Moreover，both the single TIG and combination treatment showed levels of COX-1 protein but not COX-4 down-regulation significantly compared to the control group in each cell line. Further use of the TIG-HHT combination showed a stronger inhibition of mitochondrial translation as the concentration increased in the refractory leukemia cell line SKM-1 (Figure 5B). *In vivo* experiments also showed that the level of COX-1 gene expression in the combination group was down-

regulated in the leukemia cells of subcutaneous tumor mice without affecting the expression of the COX-4 gene (Figure 4E and 5B). Both the *in vivo* and *in vitro* experiments suggested that the combination showed a stronger inhibition of mitochondrial function, which might represent an important mechanism for the synergistic effects.

The mTOR/4EBP1 pathway plays an important role in regulating mRNA translation [8-10]. mTOR can affect translation by regulating the transcription of ribosomal RNA and tRNA [19, 20]. In addition, activated mTOR regulates translation through the phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 and an analog of the allosteric mTOR inhibitor rapamycin [21, 22].There is a mutual regulation between mTOR and AKT [15-18]. mTOR complex 1 (mTORC1) is a downstream target molecule of AKT, and mTOR complex 2 (mTORC2) can target and activate phosphorylated AKT. In this study, both TIG and HHT down-regulated Rictor phosphorylation, which is an important subunit of mTORC2 in leukemia cells. A reduction in activated mTORC2 resulted in decreased AKT phosphorylation, and mTORC1 activation downstream of AKT was also reduced. The combined use of TIG and HHT further enhanced the inhibitory effect on the level of AKT/mTOR pathway phosphorylation, which may be the mechanism of the synergistic effect of the drugs.

The inhibition of eIF4E expression or the re-expression of 4EBP1 restores the sensitivity of the cell to apoptosis[22]. HHT can effectively inhibit the growth of p-eIF4E-expressing myeloid leukemia cells and primary leukemia cells by down-regulating the level of p-eIF4E and its downstream oncoprotein, Mcl-1, as well as by increasing apoptosis [20]. Our study shown TIG may enhance the pro-apoptotic properties of HHT on myeloid leukemia cells and was molecularly validated, as previously reported [23]. We hypothesized that this phenomenon is one of the mechanisms of the synergistic effects of TIG-HHT combination treatment on myeloid leukemia cells.

It has been reported that AKT regulates mitochondrial respiratory capacity through 4EBP1[22].The re-expression of 4EBP1 can be used as a treatment for blood-related diseases that induce resistance to mTOR inhibitors due to a lack of 4EBP [22]. In our study, both the *in vivo* and *in vitro* results showed that changes in the level of intracellular 4EBP1 phosphorylation was consistent with the expression of the mitochondrial gene COX-1, which suggested that there might be a link between Akt/mTOR/4EBP1 and mitochondrial respiratory function.

Conclusion

We found that TIG combined with HHT has a synergistic anti-leukemia effect *in vitro* and significant effect on tumor volume reduction and apoptosis induction in myeloid cells and a xenograft mouse model. The down-regulating mitochondrial translation and the AKT/mTOR/4EBP1 signaling pathway might explain this effect. These findings suggest clinical potential in administration of TIG and HHT in the treatment of myeloid leukemia.

Ethics approval and consent to participate

Animal experiments in this study were performed in accordance with the "Guidelines for the Care and Use of Laboratory Animals" (NIH Publication 86-23, revised in 1985) issued by the National Institutes of Health. The animal experiment protocol was approved by the Animal Protection and Facilities Committee of Zhejiang University. All works were made to minimize the suffering of the animals.

Consent for publication

All authors of the article are happy to publish the article in the journal.

Availability of data and materials

The data and materials used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by grants from National Natural Science Foundation of China (Grant number:81970117, 81470290, and 81800121).

Authors' contributions

HYT and HYY performed most experiments, analyzed the data, wrote the manuscript; CM, LY, YLR, HZ, JSH, XH, WLX, LYM, XPZ, and GXX participated in the data analysis, provided animals, acquired and managed patients, provided facilities support; LW and CYS helped to correct the manuscript. HYT designed the overall study and supervised the experiments. All authors read and approved the final manuscript.

Acknowledgments

We would like to thank Professor Huang Gang for providing the MDS-L cell line, Chipscreen biosciences (Shenzhen Weixin Biotechnology Co., Ltd.) for providing us the agent CHI, and Zhejiang Academy of Medical Sciences providing us the nude mice and venues for animal experiments.

Authors' information

[1]Department of Hematology, the First Affiliated Hospital of Zhejiang University, Hangzhou 310003, Zhejiang Province, People's Republic of China. [2]Key Laboratory of Hematopoietic Malignancies, Diagnosis

and Treatment, Hangzhou 310003, Zhejiang Province. [3] Department of Hematology, Yin Zhou People Hospital, Ningbo 315040, Zhejiang Province, People's Republic of China.

REFERENCES

1. Sum PE, Lee VJ, Testa RT, Hlavka JJ, Ellestad GA, Bloom JD, Gluzman Y, Tally FP: **Glycylcyclines. 1. A new generation of potent antibacterial agents through modification of 9-aminotetracyclines**. *Journal of medicinal chemistry* 1994, **37**(1):184-188.
2. Zhanel GG, Homenuik K, Nichol K, Noreddin A, Vercaigne L, Embil J, Gin A, Karlowsky JA, Hoban DJ: **The glycylcyclines: a comparative review with the tetracyclines**. *Drugs* 2004, **64**(1):63-88.
3. Speer BS, Shoemaker NB, Salyers AA: **Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance**. *Clinical microbiology reviews* 1992, **5**(4):387-399.
4. Jones RA, Robinson TJ, Liu JC, Shrestha M, Zacksenhaus EJJoCI: **RB1 deficiency in triple-negative breast cancer induces mitochondrial protein translation**. 2016, **126**(10):3739-3757.
5. Skrtic M, Sriskanthadevan S, Jhas B, Gebbia M, Wang X, Wang Z, Hurren R, Jitkova Y, Gronda M, Maclean N *et al*: **Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia**. *Cancer Cell* 2011, **20**(5):674-688.
6. Jia X, Gu Z, Chen W, Jiao J: **Tigecycline targets nonsmall cell lung cancer through inhibition of mitochondrial function**. *Fundamental & Clinical Pharmacology* 2016, **30**(4):297-306.
7. Yang R, Yi L, Dong Z, Ouyang Q, Zhou J, Pang Y, Wu Y, Xu L, Cui H: **Tigecycline Inhibits Glioma Growth by Regulating miRNA-199b-5p-HES1-AKT Pathway**. *Mol Cancer Ther* 2016, **15**(3):421-429.
8. Yang Q, Guan K-L: **Expanding mTOR signaling**. *Cell Research* 2007, **17**(8):666-681.
9. Mayer. C, Zhao. J, Yuan. X, Grummt. I: **mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability** *GENES & DEVELOPMENT* 2004, **18**:423-434.
10. Michels AA, Robitaille AM, Buczynski-Ruchonnet D, Hodroj W, Reina JH, Hall MN, Hernandez N: **mTORC1 Directly Phosphorylates and Regulates Human MAF1**. *Molecular and Cellular Biology* 2010, **30**(15):3749-3757.
11. Chou TC, Talalay P: **Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors**. *Advances in enzyme regulation* 1984, **22**:27-55.
12. Hu H, Dong Z, Tan P, Zhang Y, Liu L, Yang L, Liu Y, Cui H: **Antibiotic drug tigecycline inhibits melanoma progression and metastasis in a p21CIP1/Waf1-dependent manner**. *Oncotarget* 2016, **7**(3):3171-3185.
13. Pal I, Safari M, Jovanovic M, Bates SE, Deng C: **Targeting Translation of mRNA as a Therapeutic Strategy in Cancer**. *Curr Hematol Malig Rep* 2019, **14**(4):219-227.
14. Nakagawa T, Matozaki S: **The SKM-1 Leukemic Cell Line Established from a Patient with Progression to Myelomonocytic Leukemia in Myelodysplastic Syndrome (MDS)-Contribution to Better Understanding of MDS**. *Leukemia & Lymphoma* 1995, **17**(3-4):335-339.
15. Hresko RC, Mueckler M: **mTOR·RICTOR Is the Ser473Kinase for Akt/Protein Kinase B in 3T3-L1 Adipocytes**. *Journal of Biological Chemistry* 2005, **280**(49):40406-40416.
16. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM: **Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex**. *Science* 2005, **307**(5712):1098-1101.
17. Gu. Y, Zhou. H, Gan. Y, Zhang. J, Chen. J, Gan. X, Li. H, Zheng. W, Meng. Z, Ma. X *et al*: **Small-molecule induction of phospho-eIF4E sumoylation and degradation via targeting its phosphorylated serine 209 residue**. *Oncotarget* 2015, **6**:15111-15121.
18. Koritzinsky M, Goo CK, Lim HY, Ho QS, Too H-P, Clement M-V, Wong KP: **PTEN/Akt Signaling Controls**

Mitochondrial Respiratory Capacity through 4E-BP1. *PLoS ONE* 2012, **7**(9).
19. Sun Q, Li S, Li J, Fu Q, Wang Z, Li B, Liu SS, Su Z, Song J, Lu D: **Homoharringtonine regulates the alternative splicing of Bcl-x and caspase 9 through a protein phosphatase 1-dependent mechanism**. *BMC Complement Altern Med* 2018, **18**(1):164.
20. Zhou H, Xu RZ, Gu Y, Shi PF, Qian S: **Targeting of phospho-eIF4E by homoharringtonine eradicates a distinct subset of human acute myeloid leukemia**. *Leuk Lymphoma* 2018:1-13.
21. Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B: **SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity**. *Cell* 2006, **127**(1):125-137.
22. Sobol RW, Mallya S, Fitch BA, Lee JS, So L, Janes MR, Fruman DA: **Resistance to mTOR Kinase Inhibitors in Lymphoma Cells Lacking 4EBP1**. *PLoS ONE* 2014, **9**(2).
23. Cao J, Feng H, Ding NN, Wu QY, Chen C, Niu MS, Chen W, Qiu TT, Zhu HH, Xu KL: **Homoharringtonine combined with aclarubicin and cytarabine synergistically induces apoptosis in t(8;21) leukemia cells and triggers caspase-3-mediated cleavage of the MYELOID LEUKEMIA1-ETO oncoprotein**. *Cancer Med* 2016, **5**(11):3205-3213.

Figure Legends

Figure 1. TIG has in vitro antileukemia activity in combination with HHT. The combination index (CI) of TIG used in combination with IDA, HHT, or CHI in the myeloid leukemia cell line, SKM-1, was determined by MTS staining after a three day drug culture and was calculated using CalcuSyn software (three experiments each). The effect of a 72 h exposure of SKM-1 cells on different concentrations of TIG in combination with IDA, HHT, and CHI on cell viability was measured using an MTS assay after a 72 h incubation. The data were analyzed using Calcusyn software to generate a CI versus fractional effect (cell death) plot showing the effect of a combination of TIG with IDA, HHT, or CHI. CI < 1 indicates synergism.

Figure 2. Combination treatment using compounds targeting leukemic cells and patient primary cells identifies a synergistic effect. A. CI plots of DAC and IDA sequential combination in human leukemia cell lines. Cells were treated as previously described. The combinations were used at fixed molar ratios based on the IC50 values of each drug. The effects of the combinations were estimated using the CalcuSyn software, which was developed based on the median-effect method. CI < 0.8 indicates synergy; CI = 0.8 to 1.2 is additive;

and CI > 1.2 indicates antagonism. **B.** The effect of a 72 h exposure on SKM-1, MDS-L, and MOLM-13 cells and three patient primary cells to different concentrations of TIG in combination with HHT on the viability of the cells was measured by an MTS assay after a 72 h incubation. Viability was determined by MTS staining, and the results were expressed as a percentage of the results for untreated cells. ***p < 0.001; **p < 0.01, as determined by a Tukey's test after a one-way ANOVA. Error bars represent the mean ± SD.

Figure 3. Synergistic effect of the sequential combination of agents in inhibiting myeloid leukemia cell proliferation. A. Colony formation of the myeloid leukemia cell lines, SKM-1, MDS-L, and MOLM-13 cells were treated with TIG, HHT, or TIG combined with HHT, respectively. The survival of myeloid leukemia cells was evaluated by colony formation assays using Image J software. **B.** Apoptosis induced by various treatments at 72 h (**p < 0.01, one-way ANOVA, combination treatment versus control and single treatment). **C**. The survival of myeloid leukemia cells was evaluated using a colony formation assay. Mean ± SD. ***p < 0.001; **p < 0.01; *p < 0.05. **D.** Western blot of cleaved Caspase-3 and cleaved PARP-1 in myeloid leukemia cells. β-actin served as a loading control.

Figure 4.The combination of TIG and HHT has in vivo anti-leukemia activity. A. Differences in the tumor volume between combination treatments versus control and single treatments are shown after two weeks after drug treatment. **B and C.** The tumor mass and the mean volume display significant statistical differences in the tumor volume between the combination therapy and the control and single therapy. **p < 0.001 as determined by a Tukey's test after a one-way ANOVA. Error bars represent the mean ± SD. **D.** The nude mouse weight during the course of drug treatment. Error bars represent the mean ± SD. **E.** Immunohistochemical detection of the expression of cell proliferation-related molecules in the tumor tissue.

Figure 5. TIG exhibits antileukemia activity with HHT by downregulating mTOR/4EBP1 pathway proteins and mitochondrial gene expression. A. Western blot of cleaved Caspase-3, cleaved PARP-1, and β-actin in the tumor tissue. **B.** Effects of TIG and HHT on mitochondrial translation protein levels of Cox-1, Cox-4, and β-actin. **C.** Effects of TIG and HHT treatment alone or in combination on the levels of mitochondrial translation proteins, Cox-1, Cox-4, and β-actin. **D.** Effects of TIG and HHT on the levels of p-Ractor, p-AKT, p-mTOR, p-Raptor, p-4EBP1, and β-actin in SKM-1,MDS-L, and MOLM-13 protein expression and three primary cells treated for 72 h. β-actin was used as a loading control.

Figures

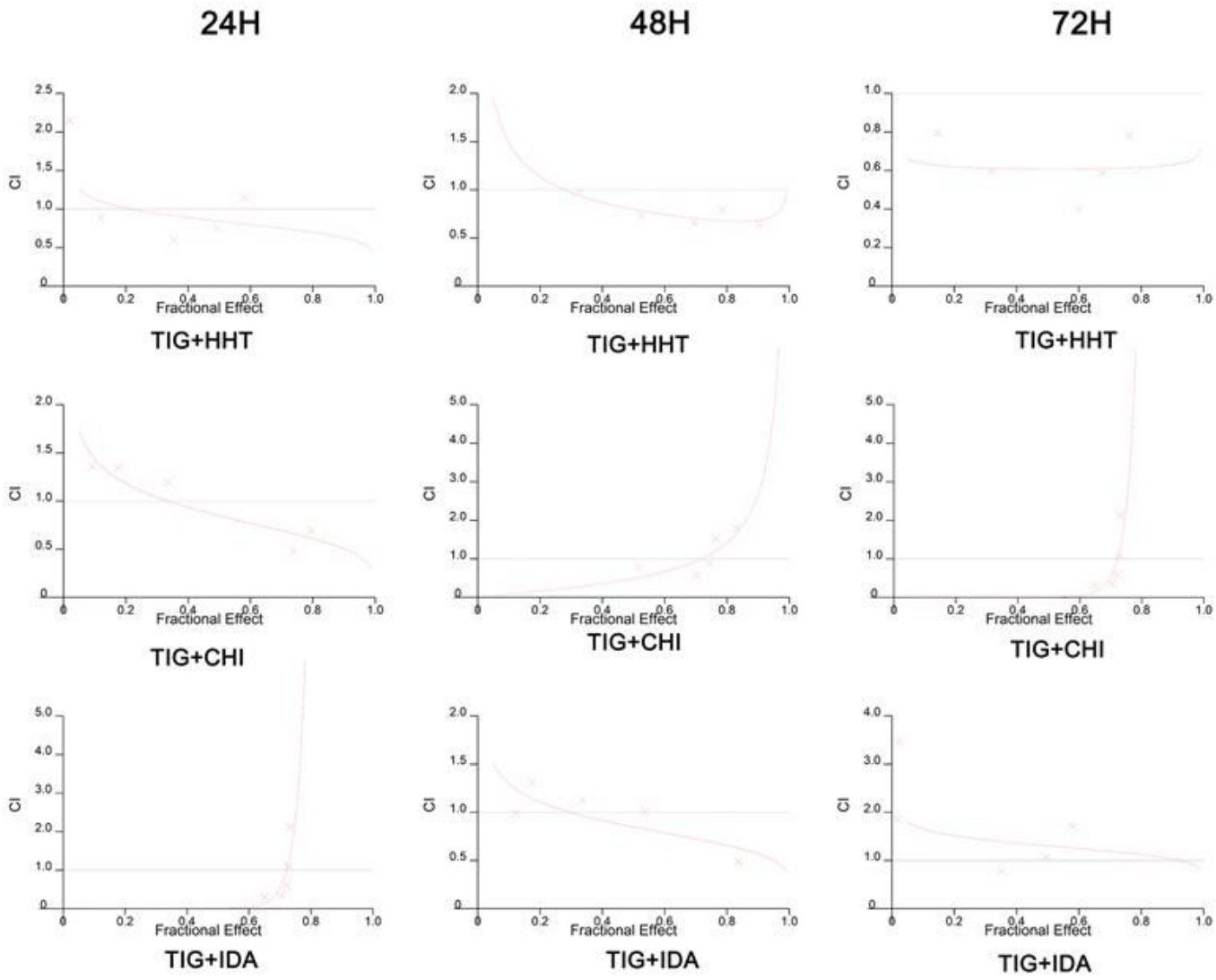

Figure 1

TIG has in vitro antileukemia activity in combination with HHT. The combination index (CI) of TIG used in combination with IDA, HHT, or CHI in the myeloid leukemia cell line, SKM-1, was determined by MTS staining after a three day drug culture and was calculated using CalcuSyn software (three experiments each). The effect of a 72 h exposure of SKM-1 cells on different concentrations of TIG in 381 combination with IDA, HHT, and CHI on cell viability was measured using an MTS assay after a 72 h incubation. The data were analyzed using Calcusyn software to generate a CI versus fractional effect (cell death) plot showing the effect of a combination of TIG with IDA, HHT, or CHI. CI < 1 indicates synergism.

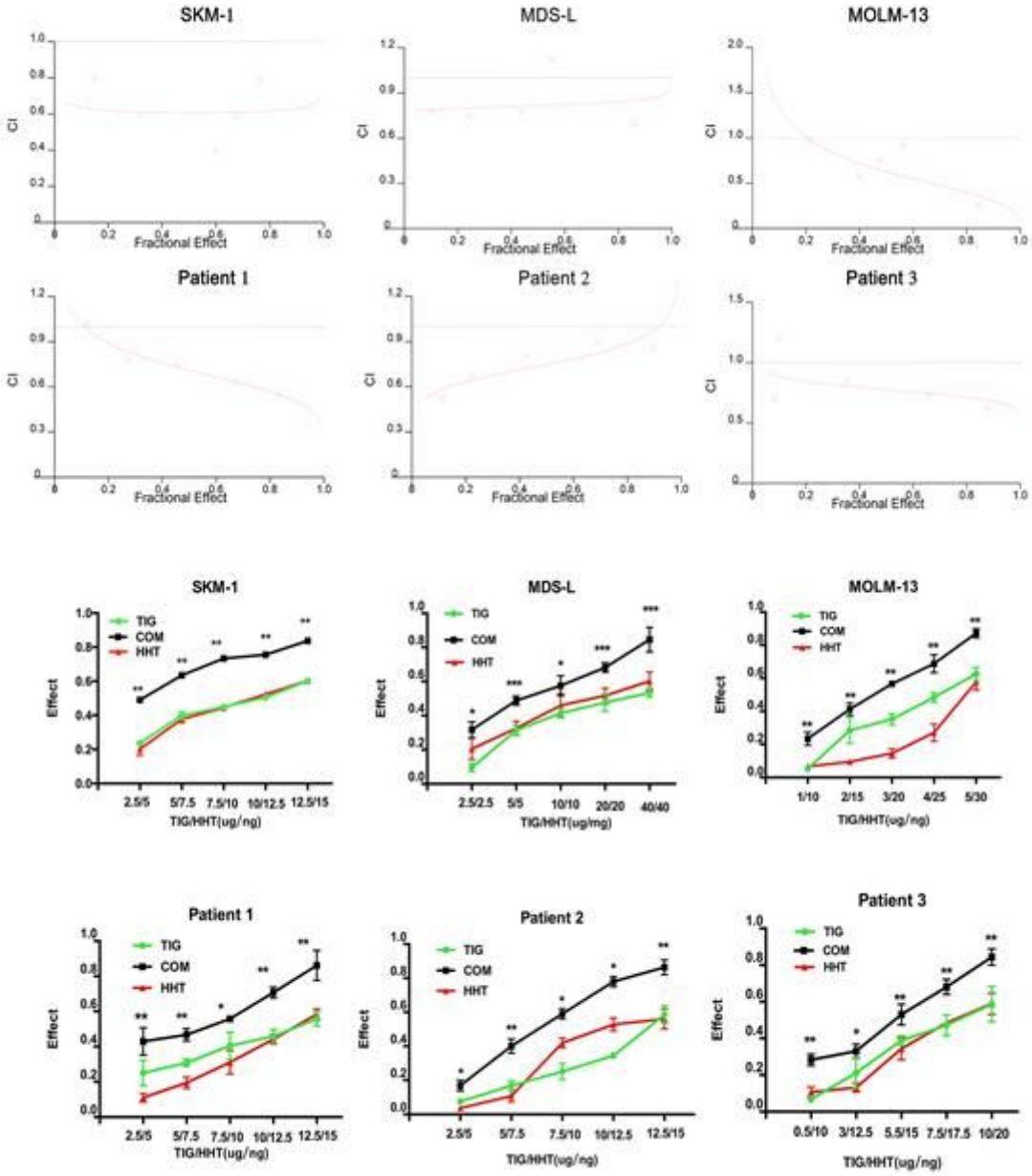

Figure 2

Combination treatment using compounds targeting leukemic cells and patient primary cells identifies a synergistic effect. A. CI plots of DAC and IDA sequential combination in human leukemia cell lines. Cells were treated as previously described. The combinations were used at fixed molar ratios based on 387 the IC50 values of each drug. The effects of the combinations were estimated using the CalcuSyn software, which was developed based on the median-effect method. CI < 0.8 indicates synergy; CI = 0.8 to 1.2 is additive; 18 and CI > 1.2 indicates antagonism. B. The effect of a 72 h exposure on SKM-1, MDS-L, and MOLM-13 cells and three patient primary cells to different concentrations of TIG in combination with HHT on the viability of the cells was measured by an MTS assay after a 72 h incubation. Viability was determined by MTS staining, and the results were expressed as a percentage of the results for untreated

cells. ***p < 0.001; **p < 0.01, as determined by a Tukey's test after a one-way ANOVA. Error bars represent the mean ± SD.

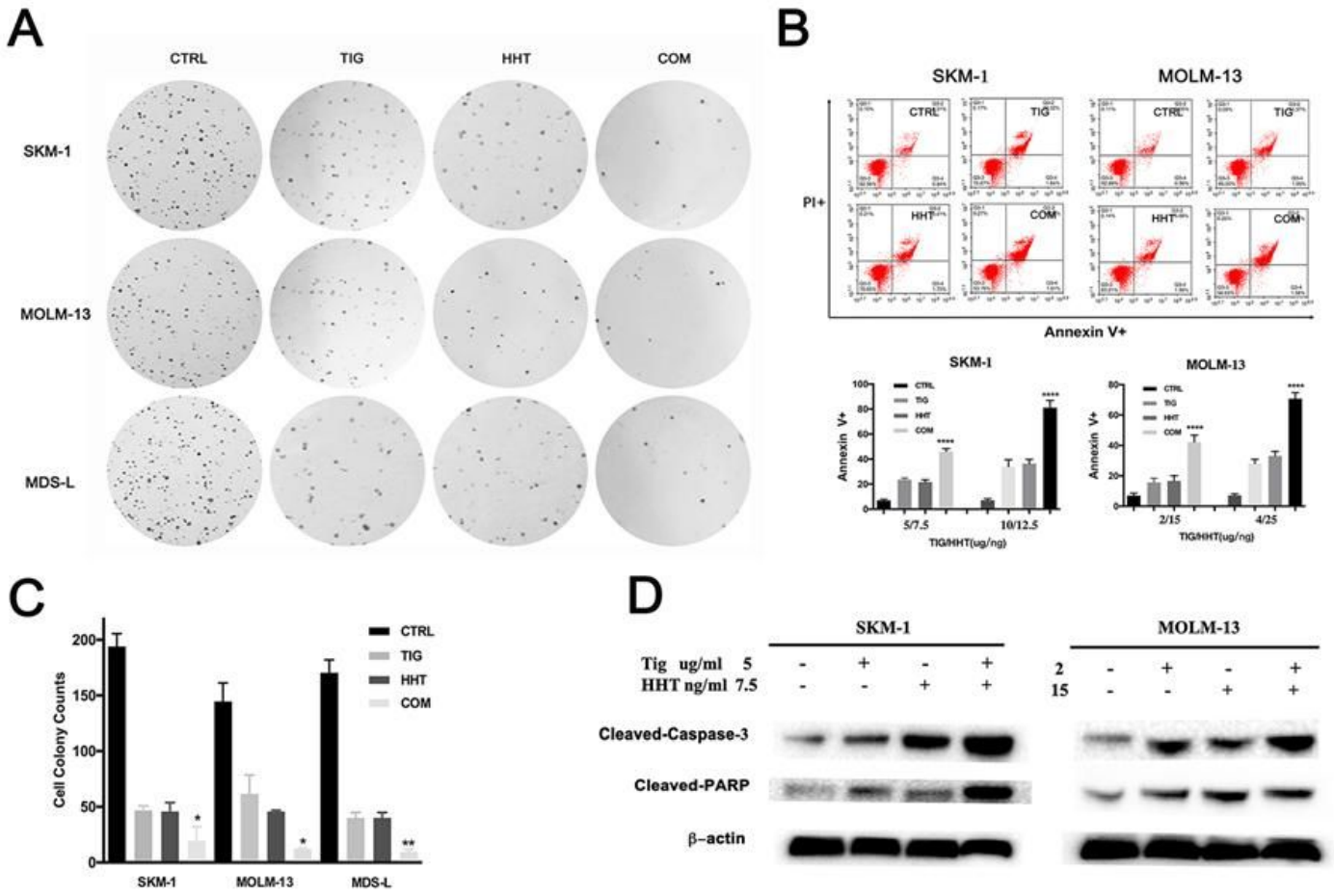

Figure 3

Synergistic effect of the sequential combination of agents in inhibiting myeloid leukemia cell proliferation. A. Colony formation of the myeloid leukemia cell lines, SKM-1, MDS-L, and MOLM-13 cells were treated with TIG, HHT, or TIG combined with HHT, respectively. The survival of myeloid leukemia cells was evaluated by colony formation assays using Image J software. B. Apoptosis induced by various treatments at 72 h (**p < 0.01, one-way ANOVA, combination treatment versus control and single treatment). C The survival of myeloid leukemia cells was evaluated using a colony formation assay. Mean ± SD. ***p < 0.001; **p < 0.01; *p < 0.05. D Western blot of cleaved Caspase-3 and cleaved PARP-1 in myeloid leukemia cells. -actin served as a loading control.

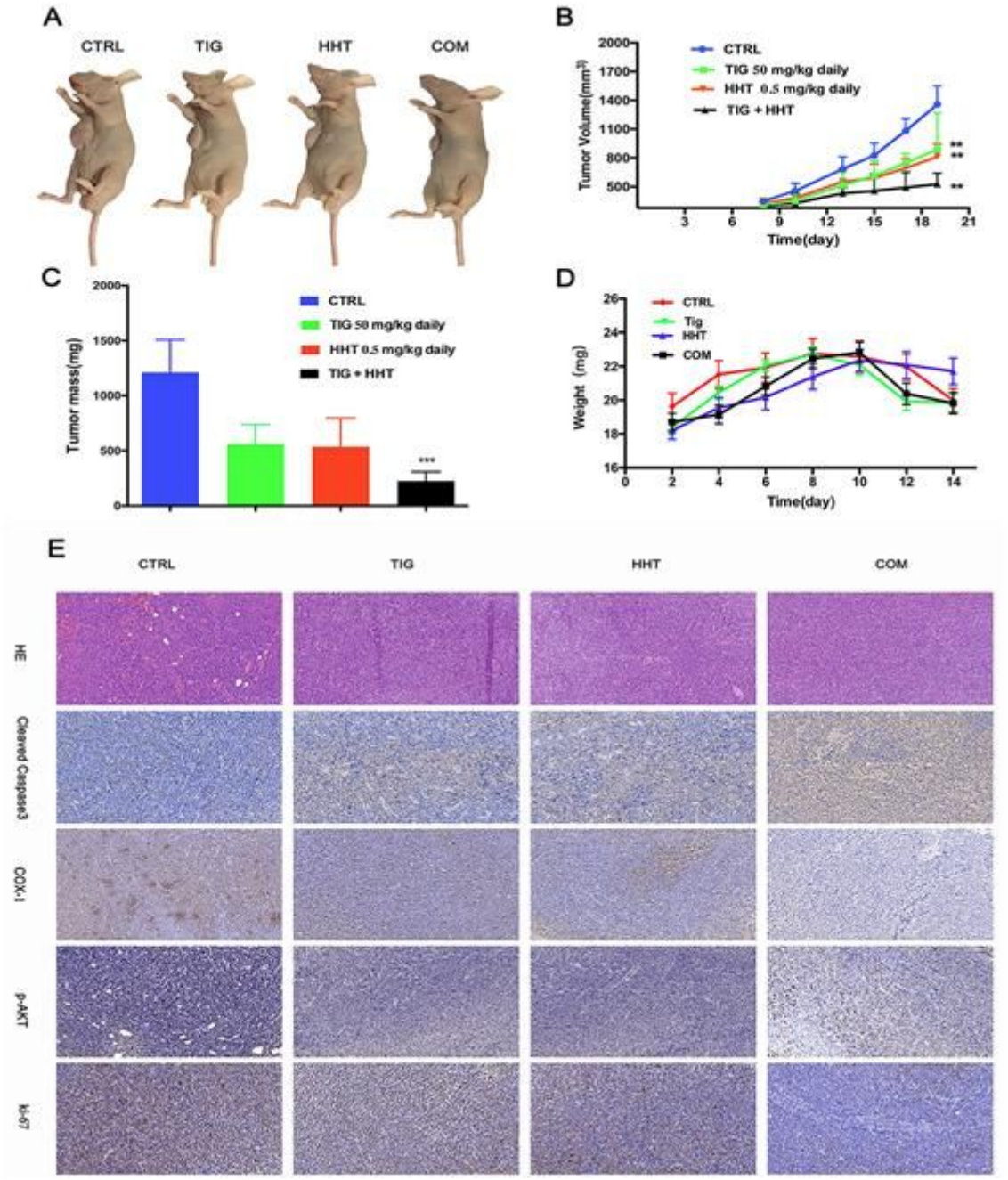

Figure 4

The combination of TIG and HHT has in vivo anti-leukemia activity. A. Differences in the tumor volume between combination treatments versus control and single treatments are shown after two weeks after drug treatment. B and C. The tumor mass and the mean volume display significant statistical differences in the tumor volume between the combination therapy and the control and single therapy. **p < 0.001 as determined by a Tukey's test after a one-way ANOVA. Error bars represent the mean ± SD. D. The nude mouse weight during the course of drug treatment. Error bars represent the mean ± SD. E. Immunohistochemical detection of the expression of cell proliferation-related molecules in the tumor tissue.

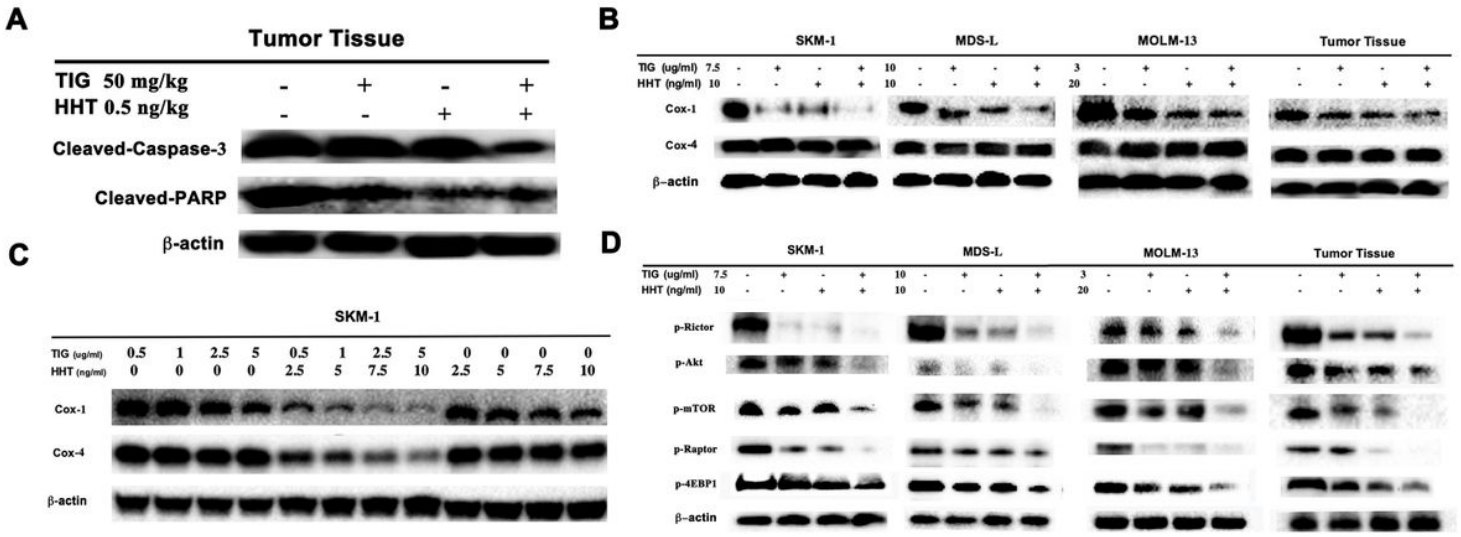

Figure 5

TIG exhibits antileukemia activity with HHT by downregulating mTOR/4EBP1 pathway proteins and mitochondrial gene expression. A. Western blot of cleaved Caspase-3, cleaved PARP-1, and β-actin in the tumor tissue. B. Effects of TIG and HHT on mitochondrial translation protein levels of Cox-1, Cox-4, and β-actin. C. Effects of TIG and HHT treatment alone or in combination on the levels of mitochondrial translation proteins, Cox-1, Cox-4, and β-actin. D. Effects of TIG and HHT on the levels of p- Ractor, p-AKT, p-mTOR, p-Raptor, p-4EBP1, and β-actin in SKM-1,MDS-L, and MOLM-13 protein expression and three primary cells treated for 72 h. β-actin was used as a loading control.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.pdf
- TableS2.pdf
- TableS3.pdf
- TableS4.pdf