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An oral triple pill-based cocktail effectively controls acute myeloid leukemia with high translation

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Abstract

Acute myeloid leukemia (AML) is a deadly hematological malignancy characterized by oncogenic translational addiction that results in over-proliferation and apoptosis evasion of leukemia cells. Various chemo- and targeted-therapies aimed at reversing this hallmark, but most show only modest efficacy. Here we report a single oral pill containing low-dose triple small molecule-based cocktail, a highly active anti-cancer therapy (HAACT) with unique mechanisms, that can effectively control AML. The cocktail comprises of oncogenic translation inhibitor Homoharringtonine (HHT), drug efflux pump P-gpi Encequidar (ENC) and anti-apoptotic protein Bcl-2i Venetoclax (VEN). Mechanically, the cocktail potently kills both leukemia stem cells (LSC) and bulk leukemic cells via co-targeting oncogenic translation, apoptosis machinery and drug efflux pump, resulting in deep and durable remissions of AML in diverse model systems. We also identified EphB4/Bcl-xL as the cocktail response biomarkers. Collectively, we for the first time develop an oral triple pill-based cocktail with high efficacy, safety, simplicity for the AML patients, and provide proof of concept that a single pill containing triple combination cocktail is a promising avenue for AML therapy.

Introduction

Acute myeloid leukemia (AML) is a highly heterogeneous and aggressive hematologic malignancy characterized by uncontrolled proliferation and apoptosis evasion of leukemia stem/progenitor and immature leukemic cells.[1,2] Although the initial response of current chemotherapy and single-agent targeted therapy is encouraging,[3,4] drug resistance and disease relapse are often inevitable for most patients,[5,6] highlighting the urgent need for novel target and therapeutic strategy.

Given that oncogenic translational addiction is a hallmark of AML characterized by extensive activation of numerous oncoproteins and anti-apoptotic proteins, such as c-Myc, FLT-3, Bcl-

2, Mcl-1, and drug efflux pump P-gp, which plays critical roles in AML relapse and drug resistance,[7-11] we propose that targeting translational addiction, together with apoptosis machinery and P-gp, might be a potential strategy for controlling AML. HHT is a FDA approved anti-cancer drug and can selectively suppress translation of a series of short-lived oncoproteins, such as c-Myc, FLT-3 and Mcl-1,[12-14] suggesting that HHT is a pan-oncoprotein translational inhibitor. However, single-agent HHT has a low efficacy in AML, because it is a substrate of drug efflux pump P-gp and does not affect anti-apoptotic protein Bcl-2.[13,15] VEN is an oral Bcl-2 inhibitor (Bcl-2i), while efficacious for AML, but shows short-lived responses due to its inefficacy to Mcl-1-mediated resistance.[16,17] ENC is a clinically available P-gp inhibitor (P-gpi) and can greatly improve oral bioavailability of P-gp substrate paclitaxel.[18,19] Based on the above data, we hypothesized that the combination of pan-translation inhibitor HHT with Bcl-2i and P-gpi might be novel effective therapy for reversing translational addiction in AML. P-gp-related drug resistance and Bcl-2-mediated apoptosis evasion of HHT could be overcome by P-gp inhibitor ENC and Bcl-2i VEN, respectively. In addition, ENC could improve oral efficacy of HHT via inhibiting P-gp in the small intestines.[20] Therefore, we attempted to develop an oral triple pill-based cocktail by the combination of HHT, ENC and VEN and then conducted the comprehensive proof-of-concept in vitro and in vivo, and also attempted to identify its targets and mechanisms of action.

Here, we report our data on the development of the first oral triple pill-based cocktail as a novel AML therapy. We found that the triple pill-based cocktail with HHT, ENC and VEN at low doses exhibited an unprecedented therapeutic effect in various AML mouse models. In addition, the triple pill-based cocktail is well tolerable and simplicity for the AML patients, and provide proof of concept that oral cocktail is a promising avenue for AML therapy and provide proof of concept that oral cocktail is a promising avenue for AML therapy.

Results

Targeting P-gp potentiate HHT killing activity for leukemia stem/progenitor cells

Given that drug efflux pump P-gp overexpression is a marker of LSCs[21] and HHT is a substrate of P-gp[15], we hypothesized that P-gp might be a major cause of low efficacy of single agent HHT in AML. To test it, we first compared expression levels of P-gp in LSC^+ AML cell lines Kg1a and Kasumi-1, bulk AML cell lines Molm-13, THP-1, MV4-11, NB4 and HL-60 using Western blot. As shown in Fig. 1a, P-gp protein was highly expressed in $CD34^+$ AML cell lines Kg1a and Kasumi-1, but low or absent in $CD34^-$ AML cell lines Molm-13, THP-1, MV4-11, NB4 and HL-60. To further confirm these results, we examined P-gp levels in primary AML cells and normal $CD34^+$ hematopoietic stem/progenitor cells. Consistently, P-gp protein was also highly expressed in $CD34^+$ leukemia stem cells (LSCs) from primary AML samples, but low in normal hematopoietic stem cells (HSCs) (Fig. 1b, c). To determine whether there is a correlation between P-gp and CD34 levels in AML, we analyzed mRNA levels of CD34 and ABCB1 (MDR1), which encodes P-gp protein, in TCGA databases and other two datasets (AML_OSHU_2018, GSE12417), and found that the ABCB1 mRNA levels were positively correlated with CD34 levels (Fig. 1d, $R=0.6090$, $P < 0.0001$, and Supplementary Fig. 1).

We next examined the effects of the specific P-gp inhibitor ENC on killing activity of HHT for LSCs, Kg1a cells, which contains $CD34^+/CD38^-$ leukemia stem-like cells with high level of P-gp170 (Fig. 1a), were treated with HHT in the presence of ENC at non-cytotoxic concentrations for 72h and then harvested for cell viability analysis and IC50 values using MTT. As shown in Fig. 2a, single-agent HHT were resistant to $CD34^+/CD38^-$ Kg1a cells, and its IC50 value (72h) was 28.7nM (Fig. 2b). However, ENC co-treatment significantly decreased the IC50 in a dose-dependent manner. IC50 values of HHT combined with ENC at 0.01, 0.1 and 0.5μM were 10.91, 10.06, and 8.44nM, respectively (Fig. 2a, b). Similar results were observed in $P\text{-gp}^{high}$ LSCs from AML patient (Fig. 2c, d) and $CD34^+$ Kasumi-1 cells (Supplementary Fig. 2a, b). In contrast, ENC co-treatment did not affect HHT killing activity

for $CD34^-$/$P\text{-}gp^-$ Molm-13 and THP-1 cells (Fig. 2e, f, and Supplementary Fig. 1c, d). These results suggest that high expression of P-gp is associated with HHT resistance to LSCs with high P-gp level.

To further verify whether P-gp is a major cause that attenuates HHT anti-leukemia activity, we examined the effects of another P-gp inhibitor tariquidar on killing activity of HHT for LSCs. As shown in Supplementary Fig. 2, similar results were observed in $CD34^+$ LSC and $CD34^-$ leukemic cells. The IC50 values of HHT monotherapy and HHT co-treated with TAR at 0.5μM were 28.17 and 9.53nM, respectively, in $P\text{-}gp^{high}$ Kg1a cells (Supplementary Fig. 2e, g). In contrast, the IC50 values of HHT monotherapy and co-treated with TAR at 0.5μM in $P\text{-}gp^{low}$ Molm-13 showed no significant differences (monotherapy versus co-treatment: 2.678 versus 1.876nM, $P > 0.05$) (Supplementary Fig. 2f).

Both ENC and TAR selectively decrease the IC50 of HHT in $P\text{-}gp^{high}$ Kg1a cells, but do not affect HHT killing activity for $P\text{-}gp^{low}$ Molm-13 cells. These results indicate that P-gp is a critical protective factor that prevents HHT from killing $P\text{-}gp^{high}$ Kg1a cells, and P-gp inhibitor enhances killing activity of HHT for $P\text{-}gp^{high}$ LSCs.

P-gp inhibitor enhances oral HHT anti-cancer efficacy in vivo

Oral single agent HHT has poor efficacy due to its poor oral bioavailability. Given that HHT is a substrate of P-gp and the small intestines express high levels of P-gp,[15,20] to determine whether P-gp inhibitor could improve efficacy of oral HHT, we next evaluated whether P-gpi ENC improve anti-leukemia efficacy of the oral HHT in AML mouse model with human AML cell line Molm-13 with low level of P-gp, which could exclude the effect of ENC on P-gp expression of tumor cells. The dose ratio of oral HHT and ENC was 1:4, because the maximum tolerable dose (MTD) of i.p HHT in NSG mice is 2.0 mg kg^{-1}, lower doses were chosen for the evaluation of anti-cancer efficacy of i.p and p.o HHT. As expected, 0.5 mg kg^{-1} doses of HHT by i.p exhibited significant tumor growth inhibition, but did not exhibit anti-

cancer effects by oral administration at the same dose or even higher dose 1.0 mg kg^{-1} (Fig. 2g). Instead, the oral combination regiments of 0.5 and 1.0 mg kg^{-1} HHT co-administered with the P-gpi ENC showed similar tumor growth inhibition compared to the i.p groups, without obvious body weight loss of mice (Fig. 2h). These results show that oral HHT combined with ENC has equivalent anti-leukemia efficacy with i.p HHT and exhibit well tolerance, suggesting that HHT could be orally administered via combination with P-gp inhibitor.

HHT and venetoclax shows synthetic lethality for AML LSC cells in vitro

In AML treatment, Bcl-2 and Mcl-1 are important obstacles of HHT and VEN, respectively.[22-24] Given that HHT can rapidly downregulate MCL-1 protein in AML cells,[12,13] and has synergistic anti-cancer activity with VEN against bulk leukemia cells,[13] we next determined whether co-treatment with HHT and VEN would exert synergistic lethality against LSC cells. $CD34^+/CD38^-$ Kg1a leukemia stem cells were treated with HHT in the presence of VEN at non-cytotoxic concentrations (≤ 5μM) for 72h and then harvested for analysis cell viability and IC50 values. As shown in Fig. 3a, b, the IC50 values of HHT combination with VEN at 0.05, 0.5 and 5μM were 26.06, 20.73 and 15.55nM, respectively. Similar results were also observed in $CD34^-$ THP-1 cells (Supplementary Fig. 3a, b). These results showed that HHT/VEN combination exhibits significantly synergistic lethality for both LSC and bulk leukemic cells.

To further verify whether Bcl-2i has synergistic lethality with HHT for LSCs, Kg1a cells were treated with HHT in the presence of another BCL-2i Navitoclax (Navi) at non-cytotoxic concentrations (≤ 0.5μM) for 72h and then harvested for cell viability analysis. The IC50 values of HHT co-treated with Navi at 0.01, 0.1 and 0.5μM were 33.25, 18.62 and 11.86nM, respectively (Supplementary Fig. 3c, d).

Potent efficacy of an oral triple pill-based cocktail of HHT/ENC/VEN

Given the marked synergy of HHT-based combination with ENC or VEN against AML cells in vitro and in vivo, we next tried to develop an oral triple pill-based cocktail using these three small molecules and then examined its efficacy and safety. The ratio of HHT, ENC and VEN in the cocktail was determined to be 1:10:100 via tolerance and anti-cancer efficacy test in mice. We first compared the efficacy of various HHT-based combinations with HHT along using $CD34^+/CD38^-$ Kg1a and Kasumi-1 cells with MTT assay. Cells were treated with different combinations at various concentrations for 72h and then analyzed IC50 values of HHT. The IC50 values of HHT in single HHT, HHT/VEN combination, HHT/ENC combination and HHT/ENC/VEN combination were 27.39, 15.89, 11.18 and 5.34nM, respectively, in Kg1a cells (Fig. 3c). The cocktail reduced the IC50 values of HHT by 5.13, 2.97, and 2.09-fold, respectively, compared with HHT, HHT/VEN and HHT/ENC combinations. Similar results were observed in Kasumi-1 cells (Fig. 3d) and primary $CD34^+$ LSCs from R/R AML patients (Fig. 3e).

To determine whether the cocktail induces more apoptosis of AML cells, we treated Kg1a with 80nM HHT, or single ENC, VEN, HHT/ENC, HHT/VEN, or cocktail at the indicated ratio HHT: ENC: VEN (1:10:100 mg mL^{-1}) for 24h and then collected cells for apoptosis analysis by FCM. We found that the cocktail treatment caused more apoptosis than that of HHT, HHT/VEN and HHT/ENC combinations in Kg1a cells, while single ENC or VEN almost performed no effect, compared with the DMSO control (Fig. 3g, h). Consistently, Western blot analysis showed that the cocktail induced more apoptosis-related molecules cleaved caspase3 and cleaved PARP than that of HHT, HHT/VEN and HHT/ENC combinations in Kg1a cells (Fig. 3f) after treatment with the cocktail for 24h. These results demonstrate that the triple molecule combination exhibits potent efficacy against AML, and its efficacy is superior to that of two molecule combination or single HHT.

To further validate above observations in vivo, we next evaluated anti-leukemia efficacy of this triple pill-based cocktail in AML NSG mouse models with AML cell line Molm-13-Luciferase. As shown in Fig. 4a, b, all the 0.25, 0.5 and 1.0mg kg^{-1} doses of HHT in the triple cocktail could result in prominent tumor growth inhibition after 5-day oral administration. Of particular note, very low dose of 0.25 mg kg^{-1} of oral HHT-based three small molecule cocktail showed significant anti-leukemia activity. These values compared favorably exceeded the effectiveness of HHT-based combination with ENC or VEN. Furthermore, 0.5 and 1.0 mg kg^{-1} doses of HHT-based cocktail induced a durable complete remission of tumor growth after receiving 20-day period of oral administration. Consistently, the cocktail treatment for 3 weeks significantly improved median and overall survival of NSG mice (Fig. 4c), the survival time in the three doses of administration groups was significantly prolonged in dose dependent manner by 40, 80 and 100% compared to the control. Here we noted that one mouse in the high-dose group died due to gavage process rather than tumor load or toxicity. Meanwhile, we developed $CD34^+$ AML mouse model with Kasumi-1-luciferase and given the same triple pill-based cocktail oral administration, a striking potent anti-leukemia activity was also observed (Supplementary Fig. 4).

After demonstrating efficacy of HEV cocktail, we next tested its tolerability and safety in NSG mice. During oral administration, mice body weight was recorded continuously, and no weight loss was observed (Fig. 4d). Importantly, even at the higher dose of 0.5 and 1.0 mg kg^{-1} HHT in the cocktail, oral administration did not induce a weight loss. Following oral administration after 3 weeks, mice blood was collected to analyze drug safety relevance indicators, including blood counts (WBC), erythrocytes (RBC), platelets (PLT), and hepatic function (ALT, AST). We found that cocktail at high dose caused erythropenia, with no other blood routine or hepatic function changes (Fig. 4e, f). These results indicate that oral triple pill-based cocktail is more effective and tolerable as compared with single agent HHT or HHT-based combination of VEN or ENC.

The cocktail reverses oncogenic translation addiction in AML cells

Oncoproteins and anti-apoptotic proteins are known to be extensively activated in AML.[25-30] To reveal translational levels of AML, we used the OP-puro assay to measure the rate of amino acid incorporation into translating ribosomes by flow cytometry. We found that AML tumor cells exhibited significantly higher levels of baseline protein translation compared with the normal hematopoietic stem cells separated from umbilical cord blood, with a mean 10-fold increased OP-puro signals (Fig. 5a, b). Universally, primary patient AML cells also exhibited a higher protein translation level compared with normal marrow cells, but not a more significant difference compared to tumor cell lines. These results indicate that AML cells require inherently high levels of protein synthesis, namely translational addiction.

To determine whether the cocktail affects translation levels, cells with translational addiction including Kasumi-1, Molm-13, and one primary AML patient sample were incubated with cocktail at various concentrations for 2h, then analyzed translational level variance using OP-puro assay. In AML cell lines, low doses of 20nM cocktail could dramatically inhibit global protein synthesis with an exceeded effectiveness in comparison with Cycloheximide (CHX), known as eukaryote protein synthesis inhibitor, and demonstrated a dose-dependent manner (Fig. 5c, d). For the primary AML sample, higher concentrations of cocktail (containing HHT at 250 or 500nM) were used to induce a further inhibition of protein synthesis versus the equal concentration of Cycloheximide (Fig. 5e), which needed a higher cocktail concentration (IC50 of HHT cocktail was 265.9nM) in the patient sample (Supplementary Fig. 5a).

To further explore the mechanism of action for the cocktail and its influence on oncoprotein expression, AML primary and tumor cells were treated with increasing concentrations of cocktail or control DMSO, respectively, for 2h. Cellular protein was extracted, and WB was performed with a panel of 7 key oncoproteins and 4 control proteins. The results were shown in Fig. 5f - h, 7 key oncoproteins, c-Kit, c-Myc, RAS, FLT-3, β-catenin, Mcl-1, Bcl-xL,

crucial regulators of survival, proliferation and anti-apoptosis in AML, were uniformly downregulated in "high translation" cells, Kasumi-1, Molm-13, THP-1 (Supplementary Fig. 5b) or primary cell sample tested. In contrary, 4 control proteins, Xpb, Rpb1, CDK6, β-actin were not altered, signifying there is no global shutdown of translation. These results indicate that the cocktail selectively abrogates oncogenic translational addiction.

EphB4/Bcl-xL is a potential molecular biomarker of Cocktail response

Associations between biomarkers and drug response can provide insights into cellular behavior that dictates response to drug treatment and can inform clinical development of therapies. We then aimed to identify predictive molecular biomarkers for cocktail antitumor response. Previous studies reported that HHT sensitivity and resistance were associated with a panel of tumor related proteins, such as c-Myc, PLK1, Aurora kinase B, EphB4 protein, and anti-apoptotic proteins, such as Mcl-1 and Bcl-xL.[12,31,32] To determine which of these proteins would be predictive biomarkers for the cocktail response, total proteins among a panel of 10 various leukemia cell lines were extracted and the expression levels of EphB4, Bcl-xL were determined and quantified (Fig. 6a). Meanwhile, the 10 cell lines were treated with cocktail for 72h and anti-leukemia activity was compared (Fig. 6d). As shown in Fig. 6b, unexpectedly, we observed that the IC50 values of HHT in the cocktail for various leukemia cell lines were negatively correlated with EphB4 expression levels ($R = -0.7771$, $P < 0.01$), indicating that cocktail sensitivity was positively correlated with EphB4 expression levels, but not c-Myc or Mcl-1 (Supplementary Fig. 6). Interestingly, we also found that Bcl-xL expression levels were negatively associated with cocktail sensitivity ($R = 0.6323$, $P < 0.05$, Fig. 6c). Notably, we observed that the EphB4/Bcl-xL protein ratio significantly and positively correlated with cocktail sensitivity (high ratio lines exhibited lower viability, Fig. 6d, e). Our findings provide potential biomarkers for screening leukemia patients who are likely to benefit from the cocktail therapy.

Discussion

In this study, we developed a novel oral triple pill-based cocktail with high efficacy, safety and simplicity for AML. To our knowledge, this is the first report of an oral triple small molecule-based cocktail for AML.

Considering the heterogeneous and aggressive characteristics of AML, we designed a rational combination of complementary strategies without increasing toxicity based on translational addiction and apoptosis abnormality in AML patients. The cocktail is composed of low doses of three clinical available drugs HHT, ENC and VEN with different targets covering oncoprotein translation/apoptosis machinery and drug pump. Importantly, HHT, VEN and ENC are FDA approved and tolerable in humans. We demonstrate that this triple small molecule-based cocktail can induce deep and durable remissions of AML in diverse mouse models with well tolerability.

Mechanically, the cocktail selectively kills both LSCs and bulk leukemic cells via targeting translational addiction, apoptosis machinery and drug pump, which rapidly and potently down-regulates a series of oncoproteins and anti-apoptotic proteins such as c-Myc, Mcl-1 and Bcl-2 associated with refractory and relapse of AML. HHT is a potent pan-oncoprotein translation inhibitor and down-regulates a series of short-lived oncoproteins such as c-Myc, Mcl-1 and FTL3, but does not affect Bcl-2, which is frequently overexpressed and associated with apoptosis resistance in AML. VEN is a potent oral Bcl-2 inhibitor, while efficacious for AML, shows short-lived responses due to overexpression of another important anti-apoptotic protein Mcl-1. In addition, our studies demonstrated a critical role of P-gp pump in conferring resistance of HHT to P-gp^{high} LSC and low bioavailability due to high P-gp expression in the small intestines. Moreover, we showed that the small molecule ENC, a clinically available P-gp inhibitor, can greatly improve HHT-mediated killing activity for LSC and oral bioavailability of HHT. Hence, we propose that oncogenic translation addiction and apoptosis

machineries, and drug efflux pump P-gp are three logical targets for the development of the triple small-molecule-based cocktail to AML. Targeting these three targets reciprocally kill both LSCs and bulk leukemic cells, and overcome drug resistance, leading to deep and durable remissions of AML in diverse models.

Of particular note, this triple cocktail is an oral single-pill and can be conveniently administered and provides timing and location of flexibility for patients. Oral administration of drug reportedly allows continuous exposure at low and effective concentrations during the treatment period and thereby facilitates a more flexible drug administration procedure. We also identified the EphB4/Bcl-xL as the cocktail response biomarkers.

Collectively, we for the first time develop an oral triple small molecule-based cocktail with high efficacy, safety, simplicity, and provide proof of concept that oral cocktail is a promising avenue for AML therapy. Finally, the proposed concept cocktail therapy is not limited to AML; other hematological malignancies such as other leukemia, MM, MDS and even lymphoma also have similar profiles of multiple oncoprotein and apoptosis machinery. They might also respond well to this cocktail.

Materials and methods

Cell lines

A panel of 10 human hematological tumor cell lines were used in the study, including 7 AML cell lines, Molm-13, Kasumi-1, Kg1a, THP-1, MV4-11, NB4, HL-60, and other 3 leukemia cell lines, CEM, Jurkat, Molt-4. All cell lines were cultured in RPMI-1640 medium, and supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin Solution. Cells were incubated in a humidified Incubator containing 5% CO_2 at 37 °C.

Antibodies

Primary antibodies including, GAPDH (60004-1-Ig) from Proteintech; P-gp170 (ET1611-30), Bcl-xL (ET1603-28), CDK6 (ET1612-3) from Huabio (Hangzhou, China); CD34 (ab81829),

EphB4 (ab254301), β-actin (ab8227) from Abcam; Cleaved-caspase3 (9664), PARP (9532), c-Kit (3074), FLT-3 (3462), c-Myc (13987), β-catenin (9587), Mcl-1 (94296), XPB (8746), Rpb1 (2629) from Cell Signaling Technology; RAS (sc-166691) from Santa Cruz. Secondary antibodies were obtained from Huabio (Hangzhou, China).

Reagents

HHT was obtained from TAIHUA Pharma (Shanxi, China). Encequidar mesylate (ENC) was chosen due to its better solubility compared with Encequidar, and was obtained from Bio Chem Partner (Shanghai, China). Venetoclax (VEN) was from HENGNING Medical Technology Co. LTD. Tariquidar (TAR) and Navitoclax (Navi) were from YUANYE Biotechnology (Shanghai, China). Purity of all the reagents above were more than 98% assessed by high performance liquid chromatography (HPLC).

Preparation of dosing triple pill-based cocktail solutions

The triple pill-based cocktail contains HHT at 0.25mg, ENC at 2.5mg and VEN at 25mg/pill. The dosing cocktail solutions were prepared as follows. Briefly, HHT, ENC and VEN were dissolved in dimethyl sulfoxide (DMSO) at 10, 20, 100mg mL^{-1} concentrations, respectively, and stored at -20 °C as "parent" stock solutions. The triple pill-based cocktail contains HHT, ENC and VEN at a ratio of 1:10:100 mg mL^{-1}, respectively.

Bioinformatic analysis of mRNA level

To explore the mRNA expression levels of P-gp170 and CD34 in AML, TCGA database and other two datasets, GSE12417, AML_OHSU_2018[33] were mined using R language. Duplicated genes were removed with limma packets and all data were represented in log_2(TPM).

Primary cell samples

AML primary peripheral blood samples were obtained from clinical residual biological materials with waiver of informed content from ethics approval (2022–0791). Umbilical cord blood samples were obtained from Zhejiang umbilical cord blood stem cell Bank.

Mononuclear cells were isolated using Lymphocyte Separation Medium (TBD) from samples and washed with PBS, then cultured in 20% FBS IMDM medium containing 1% Penicillin-Streptomycin. The experimental process was approved by Ethics and Scientific Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine.

Cellular protein level was detected by Western blot

Cells were cultured or treated with different concentrations of drugs, then collected and washed twice with pre-cooling PBS. M-per protein extraction reagent (Thermo scientific) containing 1% protease and phosphatase inhibitor were used to lysis cells on ice for 30min and supernatants were collected after centrifugation at 13000×g for 15 min. The protein samples were prepared after boiling supernatants with 5×loading buffer for 100 °C for 5 min and the concentrations were assessed using the Pierce BCA Protein Assay Kit (Thermo scientific). The proteins were equally sampled to 10% SDS-PAGE gels and then transferred to PVDF membranes (BIO-RAD). The membranes were blocked with 5% milk and washed 3 times with TBST buffer containing1‰ Tween-20, then incubated with commercial primary antibody overnight at 4 °C. Next day, the membranes were washed and incubated with different species of secondary antibodies (mouse or rabbit) at room temperature for 1h. Finally, the membranes were scanned using Tano 5200 Chemiluminescent Imaging System and protein levels were assessed by ImageJ software.

Cell proliferation was measured by MTT assay

To explore inhibition of cell proliferation by cocktail or its single agents, MTT assay was conducted. The specific experimental protocol was as follows. Cells were pre-seeded into 96-well plates with a suitable density, and then cultured with two-fold increasing concentration gradients of drugs for 72h in cell incubator. Thiazolyl blue tetrazolium bromide (MTT, Sangon Biotech, China) was then added to bind viable cells. After incubation for 4h, formazan crystals formed and were dissolved overnight in a triple buffer containing 10% SDS, 5% isobutanol, 0.012M HCl. The absorbance was measured with Spectramax

Absorbance Reader (Molecular devices) at 562nm. IC50 was defined as the drug concentration causing 50% cell death and was calculated by GraphPad Prism 9.0.

Cell apoptosis was detected by Flow cytometry

TUNEL experiment was performed utilizing NovoCyte flow Cytometer (ACEA, Biosciences, Inc.) and Annexin V-APC/7-AAD apoptosis kit (Multi Sciences, AP105). In summary, cells were incubated with single HHT, ENC, VEN, or combination of HHT/ENC, HHT/VEN, or triple cocktail, for 24h. HHT working concentration was 80nM, and others were added with HHT: ENC: VEN concentration ratios of 1:10:100mg mL^{-1}. DMSO was used as a vehicle control. Cells were harvested and washed twice with cold PBS, then resuspended in 200 μl Binding Buffer (1×) containing Annexin V-APC/7-AAD at room temperature away from light for 5 min. Data were detected at Annexin V-APC and 7-AAD channels, then analyzed in FlowJo 10.8.1 software.

Protein translation measurement by OP-Puro assay

Protein translational levels in cell lines and primary samples were examined employing Cayman protein Synthesis Aaasy Kit (Cayman Chemical, 601100). AML tumor Cells were treated with different concentrations of cocktail, containing HHT at 20, 50, 100nM, respectively, for 2h, meanwhile, one primary patient sample was treated with a higher concentration cocktail, containing HHT at 100, 250, 500nM, respectively. DMSO was added as a vehicle control and cycloheximide (CHX) as a positive control for protein translation inhibition. Cells were collected and processed according to the manufacturer instructions. OP-Puro MFI was detected with NovoCyte flow Cytometer (ACEA, Biosciences, Inc.) at FITC channel, with excitation/emission at 483nm/525nm. Data were analyzed in FlowJo 10.8.1 software.

Efficacy comparisons of i.p HHT, p.o HHT and p.o HHT/ENC COM in vivo

All the animal experiments involved in this paper had been approved by the Institution's Ethics Committee and conformed to the principles of animal welfare. For AML orthotopic

mouse modeling, 8-week NSG (NOD/SCID/IL2Rγ−/−) mice (weight more than 20g) were purchased from Biocytogen (Beijing, China), Molm-13 and Kasumi-1 cells were stably transduced with a lentiviral luciferase plasmid (Addgene). Molm13-luci Cells were injected into NSG mice through the tail vein with a density of 5×10^5 per mouse. On day 6 after injection, a visible tumor fluorescent signal (average intensity at 10^5 photons/second) could be detected using IVIS lumina LT series III in vivo imaging system (caliper lifesciences, USA). Tumor-bearing mice were assigned randomly into seven groups, vehicle (sterilized deionized water), HHT (0.5 or 1.0mg kg^{-1} dose) or HHT/ENC COM (1:4 dose ratio, containing HHT 0.5 or 1.0mg kg^{-1}) applied to oral administration, and the other two groups with HHT dose 0.5 mg kg^{-1} or 1.0 mg kg^{-1} by intraperitoneal injection, once a day after the modeling for 21 continuous days. Tumor burden was monitored by in vivo fluorescence imaging, mice weight and activity were recorded, every 7 days, separately.

Efficacy evaluation of the oral triple pill-based cocktail of HHT/ ENC/VEN in vivo

AML Molm13-luci or Kasumi-1-luci Cells were injected into NSG mice through the tail vein with 5×10^5 per mouse to establish AML orthotopic model as described above. Once fluorescence signals detected, mice were evenly divided into four treatment groups (n=3 per group): vehicle and various doses of cocktail (containing HHT at 0.25, 0.5, 1.0 mg kg^{-1}, respectively), to receive oral administration once a day for 3 weeks consecutively. Tumor fluorescence signals were detected every 5 days using the in vivo imaging system, in the period, mice body weight and activity were monitored synchronously. After 3 weeks of oral administration, blood samples were collected from mice orbital sinus to detect blood routine and biochemical liver enzyme. The survival of the mice was reasonably recorded until the end of the experiment.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.0 and $P < 0.05$ was considered statistically significant. For analysis of statistical differences between treatment

groups and control, Student's t-test (two-tailed) and Two-way ANOVA were applied. Mann-Whitney test was chosen if the data were not in line with Gaussian distribution. Kaplan–Meier curves were generated to represent the survival of mice, and variation was compared using log-rank test. Pearson correlation was used for correlation analysis, and spearman correlation was used when data did not conform to Gaussian distribution. In all figures, data bars were shown as mean ± sd with three duplicable tests. Difference was shown as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and ns $P > 0.05$.

Data availability statement

All data are contained within the manuscript and supplementary materials.

Acknowledgements

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Conflict of interests

The authors declare no competing interests.

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Figures

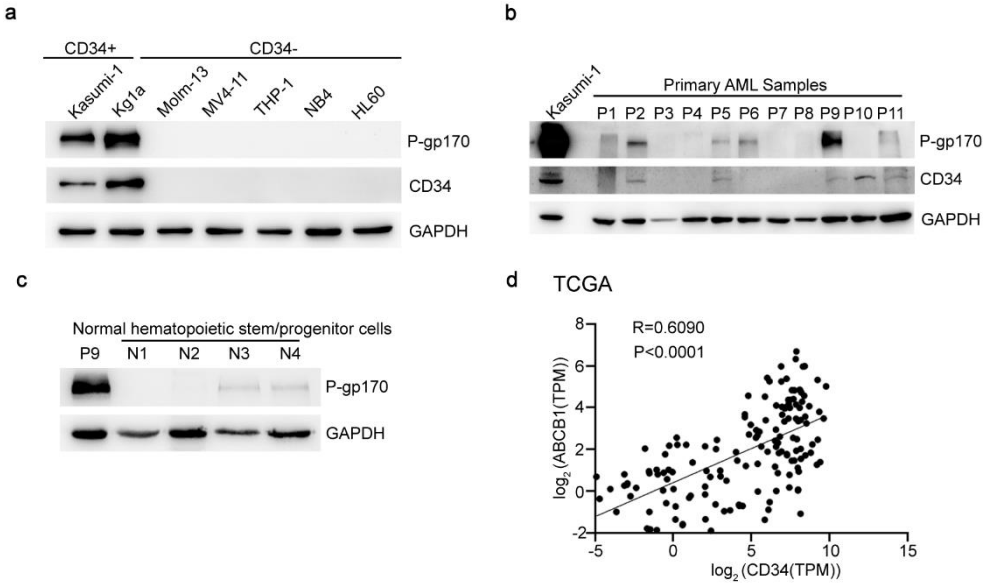

Fig.1 Expression levels of P-gp and CD34 in AML and normal hematopoietic cells.

a Western blot analyses of P-gp170 and CD34 protein levels in 7 various AML cell lines.

b-c Protein expression levels of P-gp170 and CD34 in AML peripheral blood mononuclear cells (Kasumi-1 as a positive control) and normal hematopoietic stem cells from umbilical cord blood samples (The 9th primary AML sample as a positive control).

d mRNA level analysis of CD34 and ABCB1 (encoding p-gp170) from TCGA database. Data were shown in $\log_2$(TPM). Spearman's correlation represented the correlation intensity (n=151, R=0.6090, **** P < 0.0001).

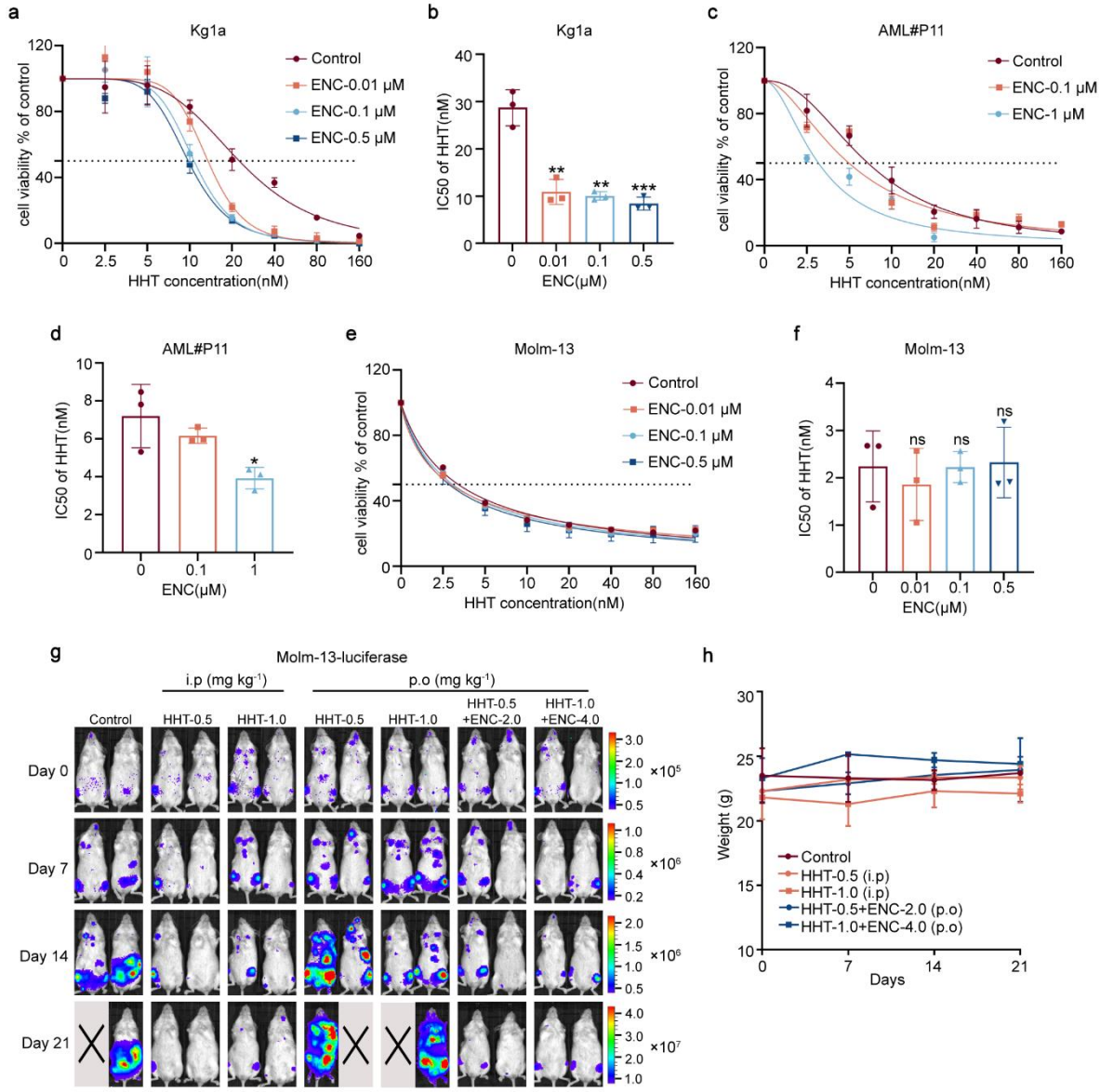

Fig.2 Effects of the specific P-gp inhibitor ENC on killing activity of HHT in vitro and in vivo.

a Cell viability of single HHT or co-treatment with p-gp inhibitor ENC in $CD34^{+}$/$P\text{-}gp^{+}$ Kg1a cells for 72h.

b HHT IC50 of single agent or combined with ENC in Kg1a cells were measured by GraphPad Prism 9.

c Primary AML patient sample was treated with increasing concentrations of HHT or with the presence of 0.1, 1μM noncytotoxic ENC for 72h.

d IC50 were calculated and data represented with histograms.

e-f Drug dose-inhibition assays of HHT or HHT/ENC COM in $CD34^-$/$P\text{-}gp^-$ Molm-13 cells. Curve charts were shown as representative of at least three independent experiments, and bar charts were results of HHT IC50 at three independent tests. Statistical analyses between COM groups and HHT single group were performed by using two-tailed t-test and error bars were shown with mean ± sd, (n=3, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns $P > 0.05$).

g In vivo bioluminescence imaging of Molm-13-luci tumor-bearing mice every 7 days.

h Mice body weight of each group.

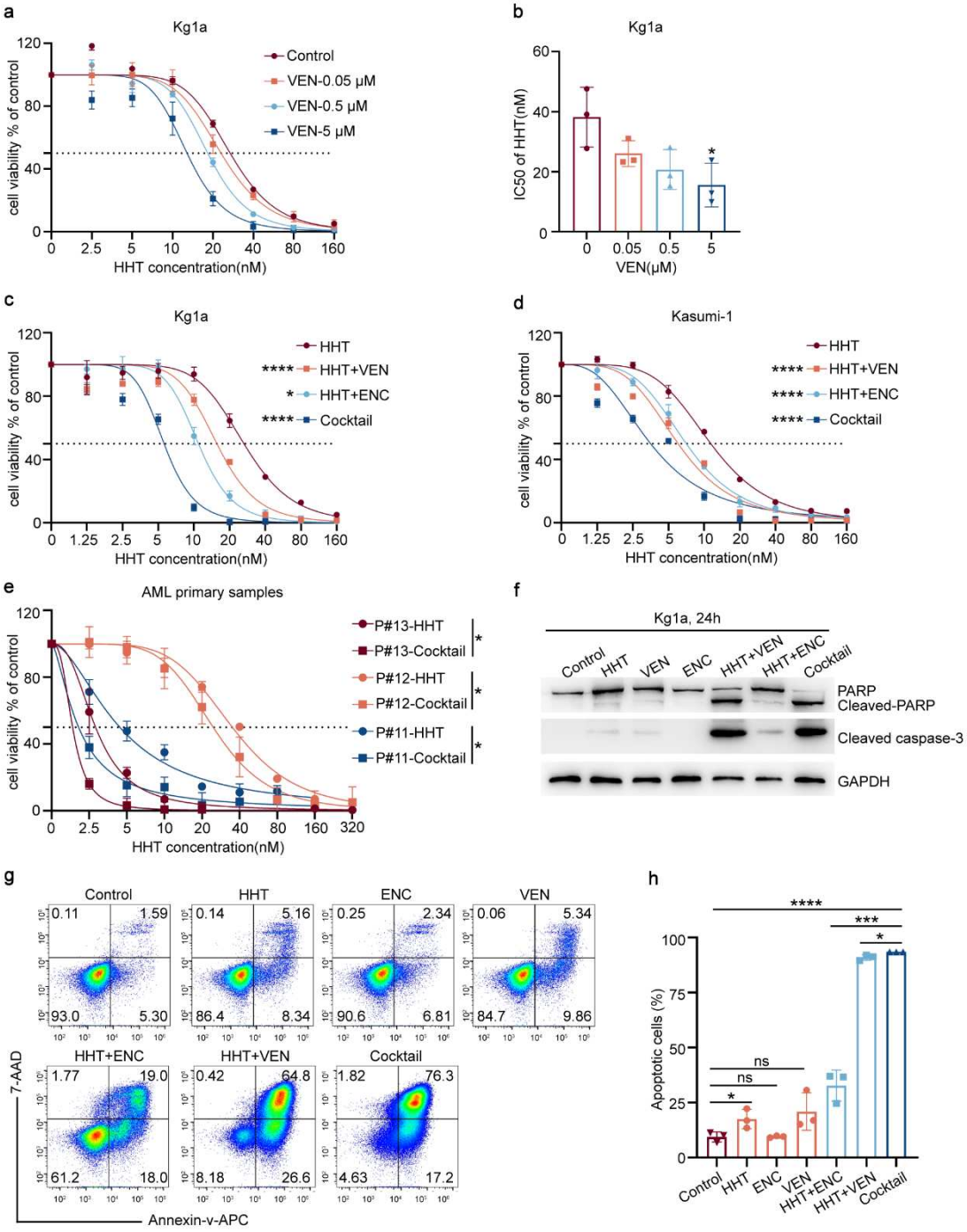

Fig.3 HHT, ENC and VEN show synthetic lethality for AML LSC cells in vitro.

a-b Cell viability of single HHT or co-treatment with Bcl-2 inhibitor VEN in $CD34^+$/$P\text{-}gp^+$ Kg1a cells (a) for 72 h. IC50 of HHT in single agent or combined with VEN were evaluated and shown with histograms (b) (n = 3, two-tailed t-test, * $P < 0.05$ versus HHT single group).

c-d Drug dose-inhibition assays of various HHT-based combinations in $CD34^+$ Kg1a (c) and Kasumi-1 (d) cells. Ratio of HHT/ENC/VEN was 1:10:100mg mL^{-1}.

e Comparison of single and triple cocktail inhibition effects in AML PBMCs. Statistical analyses for drug inhibition curves between COM groups and HHT single group were performed using two-way ANOVA and error bars were shown with mean ± sd, (n=3 independent replicates, * P < 0.05, **** P < 0.0001).

f-g Representative TUNEL assay of cell apoptosis (g) and western blot analysis of apoptosis-related molecules (f) (PARP, Cleaved-PARP, Cleaved caspase-3) after treatment with single or various HHT-based combinations in Kg1a cells for 24h. DMSO as negative control.

h Quantification of apoptosis cells in TUNEL assay (n = 3, two-tailed t-test, * P < 0.05, *** P < 0.001, **** P < 0.0001, ns P > 0.05).

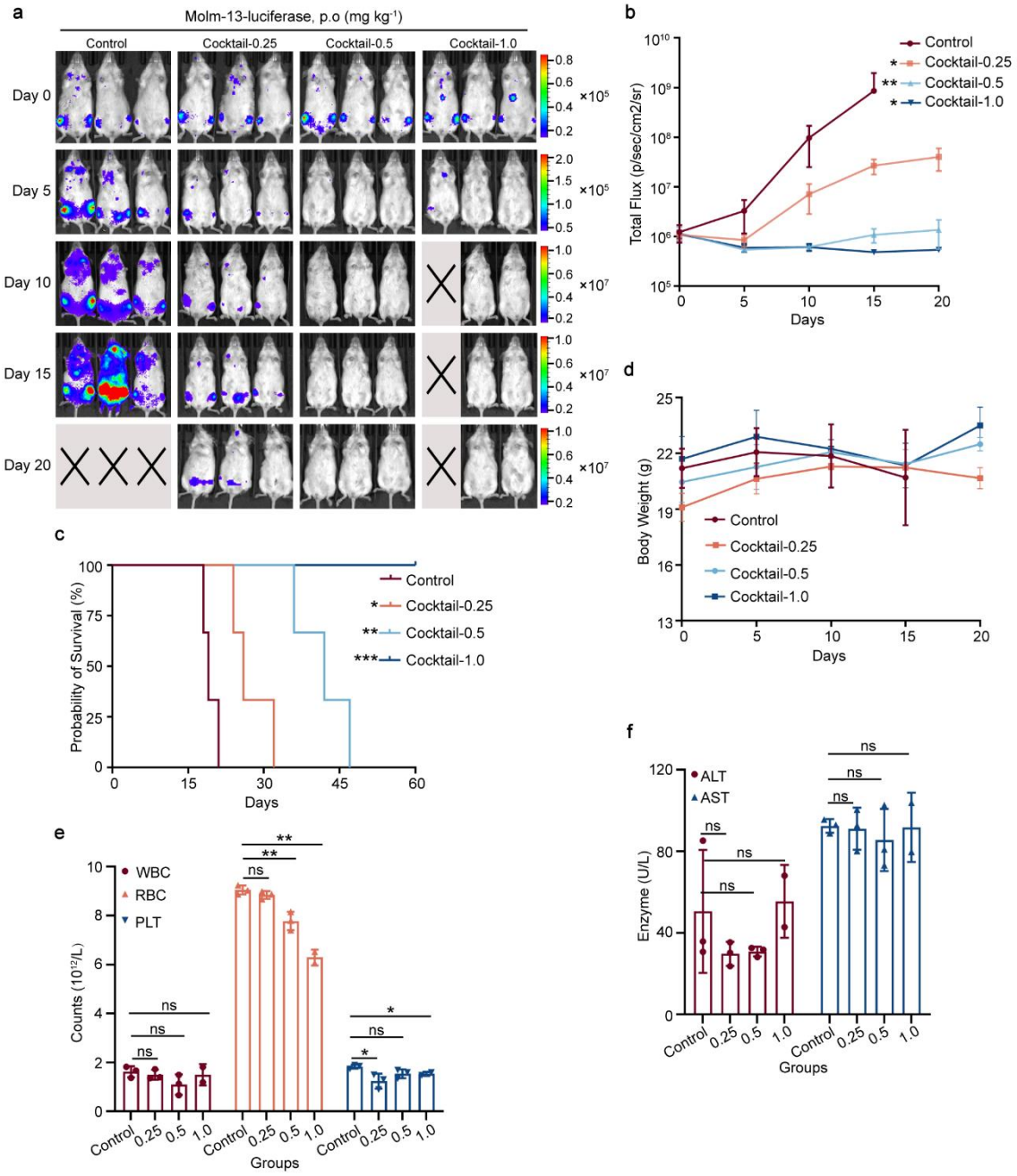

Fig.4 Potent efficacy of an oral triple pill-based cocktail containing HHT, ENC and VEN against resistant AML in vivo.

a-d Mice were evenly grouped on day 0 (6 days after modeling) and received oral administration of sterilized deionized water, different doses of cocktail, containing HHT at 0.25, 0.5, 1.0mg kg-1 respectively, once a day lasting for 3 weeks. Shown are In vivo bioluminescent images (a), quantitative fluorescence signals (b), survival curves (c) and mice body weight (d) of each group over time.

e-f Blood was collected after the drug withdrawal, hemogram index (e) including WBC, RBC, PLT, and liver enzymes (f) were evaluated.

Data were shown as histograms and error bars with mean ± sd. Statistical analyses were performed using two-tailed t-test for difference among treatment groups and control, while log-rank test for survival curves, (n=3, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns $P > 0.05$).

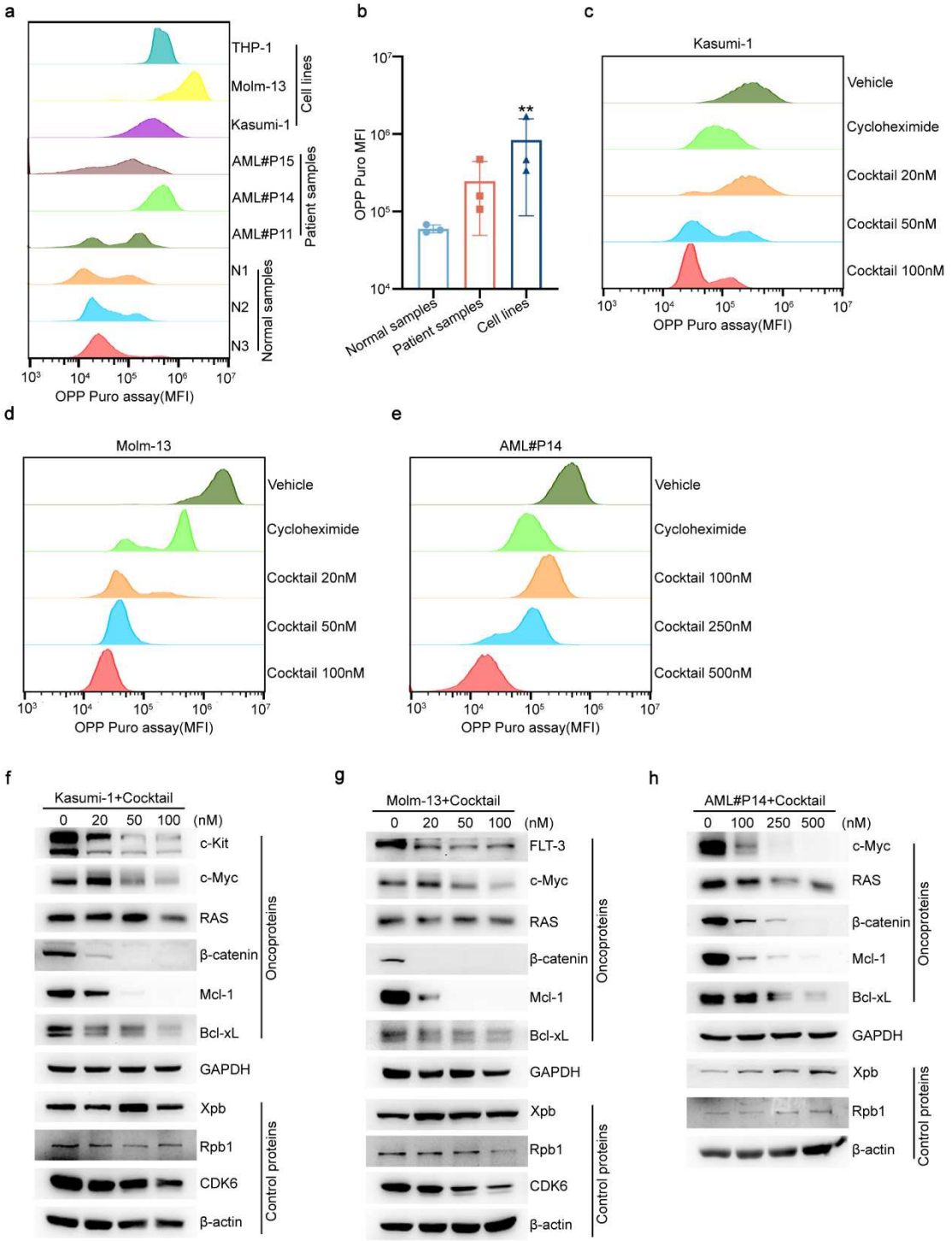

Fig.5 The triple pill-based cocktail acts through inhibition of oncoprotein synthesis.

a-b Translation levels were compared between AML tumor cell lines, primary AML patient sample and normal samples (n=3) using OP-puro and flow cytometry (a). Median fluorescence intensity (MFI) was quantified and represented with bar graphs (b).

Two-tailed t-test was used for counting difference among normal samples versus the other two groups (mean ± sd, ** $P < 0.01$).

c-e Histogram demonstrates the effects of cocktail on global protein synthesis in translational addiction cells, Kasumi-1 (c), Molm-13 (d), and primary AML sample cells (e). Cells were treated with various concentrations of cocktail for 2h, Vehicle is the negative control, CHX is the positive control. Increasing concentrations of cocktail induced decreasing OPP Puro MFI.

f-h Critical oncoproteins, including c-Kit, c-Myc, RAS, FLT-3, β-catenin, Mcl-1, Bcl-xL levels after treatment of cocktail among Kasumi-1 (f), Molm-13 (g), and primary AML sample (h), while Xpb, Rpb1, CDK6, β-actin as control proteins. Representative images by western blot were shown.

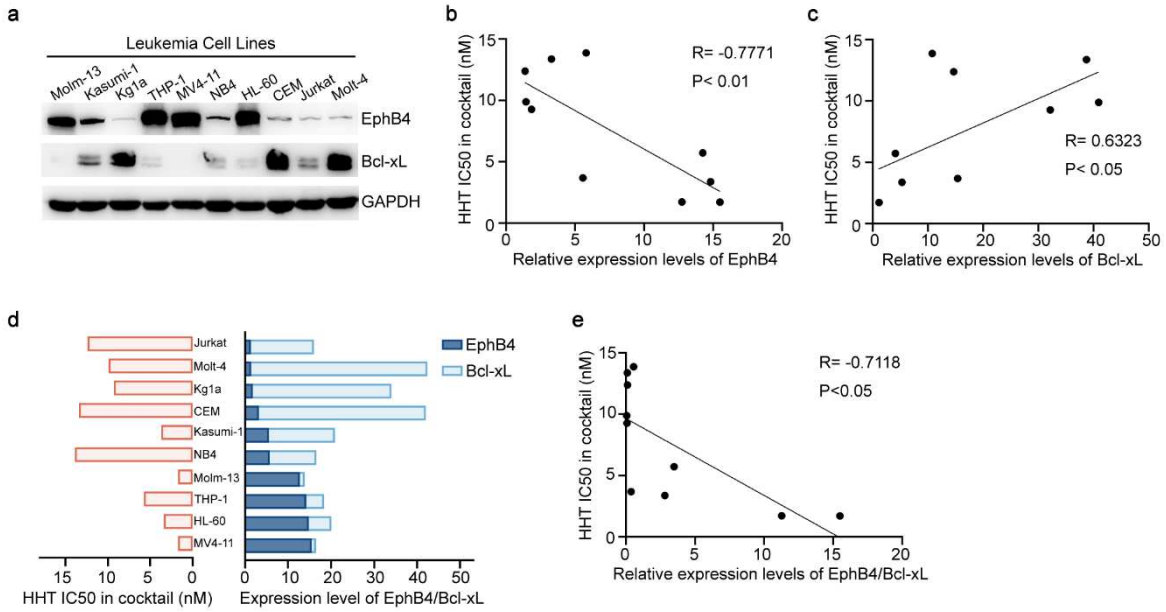

Fig.6 EphB4/Bcl-xL could be a potential molecular biomarker of cocktail response.

a Western blot of EphB4 and Bcl-xL expression levels in 10 various hematological malignant cell lines, including 7 AML and other 3 leukemia cell lines.

b-c Correlation between cocktail antitumor activity and EphB4 (b), Bcl-xL (c) protein levels in cell lines.

d Protein levels were quantified by Image J and shown as EphB4 (dark blue), Bcl-xL (cambridge blue) and MTT assays of HHT IC50 in cocktail (red). Data were shown with bar charts.

e Correlation between cocktail antitumor activity and relative expression levels of EphB4/Bcl-xL. Pearson correlation was applied to analyze the relevance (n=10, * $P < 0.05$, ** $P < 0.01$).

Supplementary Files

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