Homoharringtonine combined with aclarubicin and cytarabine synergistically induces apoptosis in t(8;21) leukemia cells and triggers caspase-3-mediated cleavage of the AML1-ETO oncoprotein

Jiang Cao¹,a, Hao Feng¹,a, Ning-Ning Ding¹,a, Qing-yun Wu¹, Chong Chen¹, Ming-Shan Niu¹, Wei Chen¹, Ting-Ting Qiu¹, Hong-Hu Zhu² & Kai-Lin Xu¹

¹Department of Hematology, The Affiliated Hospital of Xuzhou Medical College, XuZhou 221002, China
²Department of Hematology, Peking University People’s Hospital, Beijing 100044, China

Abstract

Homoharringtonine combined with aclarubicin and cytarabine (HAA) is a highly effective treatment for acute myeloid leukemia (AML), especially for t(8;21) AML. However, the underlying mechanisms by which HAA kills t(8;21) AML cells remain unclear. In this study, SKNO-1 and Kasumi-1 cells with t(8;21) were used. Compared with individual or pairwise administration of homoharringtonine, aclarubicin, or cytarabine, HAA showed the strongest inhibition of growth and induction of apoptosis in SKNO-1 and Kasumi-1 cells. HAA caused cleavage of the AML1-ETO (AE) oncoprotein to form truncated AE (ΔAE). Pretreatment with the caspase-3 inhibitor Q-DEVD-OPh (QDO) not only suppressed HAA-induced apoptosis but also abrogated the cleavage of AE and generation of ΔAE. These results suggest that HAA synergistically induces apoptosis in t(8;21) leukemia cells and triggers caspase-3-mediated cleavage of the AML1-ETO oncoprotein, thus providing direct evidence for the strong activity of HAA toward t(8;21) AML.
supports previous results from some single-center studies [6, 7]. Moreover, as a salvage treatment, the HAA regimen has achieved a 90% CR rate in refractory/relapsed t(8;21) and inv(16)/t(16;16) AML [8]. Our data showed that one cycle of HAA treatment can yield a 93% (28/30) CR rate in de novo t(8;21) AML patients [9]. These results suggest that the HAA regimen is highly effective against t(8;21) AML.

To date, the underlying mechanisms by which HAA kills t(8;21) AML cells remain unclear. Homoharringtonine is an alkaloid derived from trees of the genus Cephalotaxus, having been used for more than 30 years to treat AML in China. The antileukemic effects of homoharringtonine are primarily based on the inhibition of protein synthesis, which induces differentiation, inhibits proliferation, and promotes apoptosis in leukemic cells [10–13]. Homoharringtonine also has a synergistic relationship with cytarabine and aclarubicin [11]. Wang et al. confirmed that combining homoharringtonine with aclarubicin can result in synergistic cytotoxicity in THP-1 and Kasumi-1 cells in vitro and in vivo [13]. Combining homoharringtonine and aclarubicin simultaneously inhibited PI3K/AKT and WNT/β-catenin signaling in AML cells [13]. However, the mechanism of action of HAA in t(8;21) AML has yet to be explored. Therefore, we investigated the mechanism of the robust antileukemia effect of HAA on t(8;21) AML in vitro.

### Methods

#### Cell lines and cell cultures

The AML cell lines SKNO-1 and Kasumi-1 were cultured in RPMI-1640 (Gibco, Billings, MT, USA) supplemented with 10% fetal bovine serum (Gibco). All cell lines were grown at 37°C in a 5% CO₂ atmosphere. Cell viability was assessed by triplicate counting of trypan blue dye-excluding cells under light microscopy.

#### Reagents and antibodies

Homoharringtonine was provided by Minsheng Pharmaceutical (Hangzhou, China). Cytarabine was provided by Pfizer Pharmaceuticals (Nerviano, Italy). Aclarubicin was obtained from Wanle Pharmaceutical (Shenzhen, China). Fludarabine was provided by Pfizer Pharmaceuticals (Fuyang, China). Caspase-3 and PARP antibodies were obtained from Abcam (Epitomics, Burlingame, CA, USA; Cambridge, UK). The antibody against ETO was obtained from Cell Signaling Technology (Danvers, USA).

#### Growth inhibition assay

The inhibition of Kasumi-1 and SKNO-1 cell growth was evaluated by CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan). Briefly, 96-well plates were seeded with 1 × 10⁵ cells per well and treated with various combinations of homoharringtonine, aclarubicin, and cytarabine. After 24 h or 48 h in culture, 10 μL of CCK-8 solution was added to each well. The samples were incubated at 37°C for 4–6 h, and absorbance was measured at 450 nm using a microplate reader (Thermo Multiskan MK3, Waltham, MA, USA). Each sample was assayed with three replicates per assay, and cell-line experiments were carried out in triplicate.

### Evaluation of apoptosis

Cells were treated with different combinations of reagents or equal volumes of DMSO for 12 h, harvested and washed twice with phosphate buffered saline (PBS; Gibco), resuspended in binding buffer and stained with Annexin V-APC/7-AAD according to the manufacturer’s instructions (BD Pharmingen). A total of 5 μL of Annexin V-FITC (BD Pharmingen, Torreyana Road, San Diego, CA, USA) and 5 μL of PI were added to the cell suspension. The data were expressed as the percentages of early apoptotic cells (Annexin V-APC + /7-AAD−) and late apoptotic cells (Annexin V-APC + /7-AAD+). Samples were analyzed on a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA) equipped with CellQuest Pro software (BD Bioscience). For each sample, at least 1 × 10⁵ events were acquired for downstream analysis.

#### Hoechst staining

SKNO-1 and Kasumi-1 cells were treated with different combinations of reagents or with equal volumes of DMSO 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 for 30 min, and washed with PBS. Nuclear morphology was observed immediately via fluorescence microscopy (BX31, Olympus, Tokyo, Japan).

#### Western blot assay

Cells were harvested, ground in liquid nitrogen, lysed in lysis buffer (1% Triton X-100, 0.015 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethanesulfonyl fluoride (PMSF) and 10 g/mL each of leupeptin, aprotinin, and pepstatin A) and then incubated on ice for 30 min. Lysates were centrifuged at 12,000 g for 10 min at 4°C. Supernatants were mixed with one-quarter volume of 4 × SDS sample buffer, boiled for 10 min and then separated by SDS-PAGE on 10–12% gels. After electrophoresis, proteins were transferred to nitrocellulose (NC) membranes (Millipore Corporation, Bedford, MA, USA), blocked for 1 h with 5% non-fat milk powder in TBST buffer (20 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, and 0.05% Tween-20) and then incubated with primary antibodies at 1:1000 dilution overnight at 4°C. The
membranes were washed three times with TBST and then incubated with secondary antibody at 1:5000 dilution for 1 h at RT. After extensive washing, proteins were visualized using an ECL-Plus Kit (Thermo Scientific, Rockford, IL, USA), and blots were exposed to Kodak radiographic film.

**Statistical analysis**

Data were expressed as the mean ± standard deviation (SD). The half-maximal inhibitory concentrations (IC50) were calculated using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). The combination index (CI) was calculated using the Chou–Talalay method (CalcuSyn software, Biosoft, San Diego, CA) to ascertain whether the effects of the drug combinations were synergic (CI < 1), additive (CI = 1), or antagonistic (CI > 1). Categorical variables were compared using Fisher’s exact test or the chi-square test, and continuous variables were compared using the Wilcoxon rank sum test. P < 0.05 was considered statistically significant.

---

**Figure 1.** The growth inhibition and CI in Skno-1 cells treated with different combinations. The rate of growth inhibition induced by homoharringtonine (HHT), cytarabine (Ara-C), aclacinomycin (ACR), HHT + Ara-C, HHT + ACR, Ara-C + ACR and HHT + Ara-C + ACR in SKNO-1 cells (A) for 24 h. CI values for HHT + Ara-C combination treatments at a molar ratio of 1:2.86 in SKNO-1 cells (B), HHT + ACR (1:2.5) (C), Ara-C + ACR (1:14:1) (D), and HHT + Ara-C + ACR (1:2.86:2.5) (E). We chose drug concentration gradients of HHT: 4.5, 9, 18, 36 nmol/L, Ara-C: 12.87, 25.75, 51.5, 103 nmol/L, ACR: 11.25, 22.5, 45, 90 nmol/L. CI values for HHT + Ara-C combination treatments at a molar ratio of 1:2.86 in SKNO-1 cells (B), HHT + ACR combination treatments at a molar ratio of 1:2.5 (C), Ara-C + ACR combination treatments at a molar ratio of 1:14:1 (D), and HHT + Ara-C + ACR combination treatments at a molar ratio of 1:2.86:2.5 (E).
Results

Homoharringtonine combined with cytarabine and aclacinomycin synergistically inhibited Kasumi-1 and SKNO-1 cell growth

The following concentrations were used: homoharringtonine (4.5, 9, 18 and 36 nmol/L), cytarabine (12.87, 25.75, 51.5, and 103 nmol/L) and aclacinomycin (11.25, 22.5, 45, and 90 nmol/L). DMSO was used as the control. The 24 h IC₅₀ values of homoharringtonine, cytarabine, and aclacinomycin for Kasumi-1 and SKNO-1 cells were (34.21 ± 3.51 nmol/L) and (28 ± 2.65 nmol/L), (195.07 ± 13.21 nmol/L) and (275.03 ± 8.57 nmol/L), and (148.9 ± 6.07 nmol/L) and (102.78 ± 13.78 nmol/L), respectively (n = 3). The 48 h IC₅₀ values of homoharringtonine, cytarabine, and aclacinomycin for Kasumi-1 and SKNO-1 cells were (16.90 ± 2.21 nmol/L) and (13.91 ± 1.72 nmol/L), (87.91 ± 14.84 nmol/L) and (144.27 ± 9.66 nmol/L), and (53.57 ± 7.21 nmol/L) and...
(51.37 ± 7.87 nmol/L) respectively (n = 3). The inhibition of Kasumi-1 and SKNO-1 cell growth was concentration-dependent. The CCK-8 assay showed dose-dependent growth inhibition by homoharringtonine (HHT), cytarabine (Ara-C) and aclarubicin (ACR) (P < 0.05). The growth-inhibition effect of HAA on SKNO-1 and Kasumi-1 cells was stronger than that of any two-drug combination at 24 h (Fig. 1A, 2B) (P < 0.001). Using CalcuSyn 2.0, we found that HAA (1:2.86:2.5) also showed the strongest synergistic effect with CI values of (X and Y) for SKNO-1 and Kasumi-1 cells, respectively (Fig. 1B–E and 2B–E).
Homoharringtonine combined with cytarabine and aclarubicin synergistically induced apoptosis in Kasumi-1 and SKNO-1 cells

The concentrations of homoharringtonine, cytarabine, and aclarubicin were 36, 103 and 90 nmol/L, respectively. SKNO-1 and Kasumi-1 cells treated with HAA had the highest percentage of apoptotic cells compared with any two-drug combination or with the individual drugs (Fig. 3, n = 3). Moreover, pretreatment with 50 μmol/L of the caspase-3 inhibitor Q-DEVD-OPh (QDO) for 12 h
significantly suppressed HAA-induced apoptosis. Hoechst staining also showed a higher number of condensed and/or fragmented nuclei in Kasumi-1 cells treated with HAA compared with any individual drug or pairwise combination of drugs (Fig. 4, n = 3).

**Homoharringtonine combined with cytarabine and aclarubicin synergistically induced cleavage of the AML1-ETO (AE) oncoprotein in Kasumi-1 and SKNO-1 cells**

The concentrations of homoharringtonine, cytarabine, and aclarubicin were 36, 103, and 90 nmol/L respectively. Cleavage of the AML1-ETO (AE) oncoprotein in Kasumi-1 and SKNO-1 cells treated with HAA was higher than that observed for any individual drug or pairwise combination of drugs (Fig. 5, n = 3). Western blotting revealed six truncated AEs (ΔAEs) following treatment with the individual drugs or the various drug combinations, which was not markedly in the combination of fludarabine (95 nmol/L) adding cytarabine (103 nmol/L) (Fig. 5F).

Pretreatment with 50 μmol/L of the caspase-3 inhibitor Q-DEVD-OPh (QDO) for 12 h abrogated AE cleavage and the generation of ΔAE (Fig. 5G, H, n = 3). We further analyzed the expression of caspase-3, cleaved caspase-3 (Δcaspase-3), PARP, and cleaved PARP (ΔPARP). The activation of caspase-3 and the degradation of PARP were significantly inhibited in each group except for that treated with the combination of homoharringtonine and aclarubicin (Fig. 6, n = 3).

**Discussion**

Our study showed that, compared with single drugs or two-drug combinations, HAA synergistically induced apoptosis in t(8;21) leukemia cells and triggered the caspase-3-mediated cleavage of the AML1-ETO oncoprotein. This study provides in vitro evidence to explain the high efficacy of HAA treatment in t(8;21) AML patients.
HAA Synergistically Induces Apoptosis in t(8;21) AML

J. Cao et al.

The CI of HAA at the median effective dose (ED50) was 0.52 for SKNO-1 cells, 0.52 for Kasumi-1 cells. The high efficacy of HAA against t(8;21) AML may be due to the different mechanisms by which homoharringtonine, cytarabine, and aclacinovic kill leukemia cells. Homoharringtonine is an alkaloid derived from the genus Cephalotaxus. The antileukemic effects of homoharringtonine are primarily based on the inhibition of protein synthesis, which induces differentiation, inhibits proliferation, and promotes apoptosis in leukemic cells [14, 15]. The cytotoxicity of homoharringtonine is cell-cycle specific, primarily affecting cells in the G1 and G2 phases [16]. Moreover, homoharringtonine has been demonstrated to have synergistic effects with cytarabine [13, 17].

Aclarubicin, an alternative to daunorubicin, showed promising antileukemic efficacy in combination with cytarabine [18]. Homoharringtonine, aclarubicin and cytarabine may also have no cross-resistance, which may partially explain their potent antileukemia activity.

We wanted to elucidate the detailed events that occur during HAA treatment in leukemia cells with t(8;21). We found that HAA showed strongly inhibited growth and induced apoptosis in SKNO-1 and Kasumi-1 cells compared with individual drugs or two-drug combinations. Our results support those of previous reports in which homoharringtonine, either alone or combined with aclarubicin, promoted apoptosis in Kasumi-1, or primary AML cells.

The caspase-3-mediated cleavage of the AML1-ETO oncprotein was shown to be a major molecular mechanism of HAA activity in leukemia cells with t(8;21). When caspase-3 action was blocked by an inhibitor, these cleaved fragments disappeared, strongly suggesting that caspase-3 either directly or indirectly contributes to apoptosis-related cleavage. Chen and coworkers also reported that the AML1-ETO oncprotein was degraded in parallel with caspase-3 cleavage. Chen and coworkers also reported that the AML1-ETO and interferes with the trans-regulatory functions of the remaining AML1-ETO oncprotein [22]. These results may partially explain why t(8;21) leukemia cells were more sensitive to HAA.

Our study has certain limitations. Apart from t(8;21) AML or primary cells from t(8;21) AML patients, no other types of AML cell lines were used in this study. This deficit may be improved in the future. We also have not included our clinical data on the efficacy of HAA treatment on t(8;21) AML patients, which are described separately.

In summary, our study suggests that HAA synergistically induces apoptosis in t(8;21) leukemia cells and triggers the caspase-3-mediated cleavage of the AML1-ETO oncprotein, thereby providing new insights into the biology and treatment of t(8;21) AML.

Acknowledgments

We gratefully acknowledge the valuable cooperation of Dr Honghu Zhu from Department of Hematology, Peking University People’s Hospital who made a great contribution to our study.

References

1. Zhu, H. H., X. H. Zhang, Y. Z. Qin, D. H. Liu, H. Jiang, and H. Chen. 2013. MRD-directed risk-stratification treatment may improve outcome of t(8;21) AML in the first complete remission: results from AML05 multicenter trial. Blood 121:4056–4062.
2. Liu Yin, J. A., M. A. O’Brien, R. K. Hills, S. B. Daly, K. Wheatley, and A. K. Burnett. 2012. Minimal residual disease monitoring by RT-qPCR in core-binding factor AML allows risk stratification and predicts relapse: results of the United Kingdom MRC AML-15 trial. Blood 120:2826–2835.
3. Jourdan, E., N. Boissel, S. Chevret, E. Delabesse, A. Renneville, P. Cornillet, et al. 2013. Prospective evaluation of gene mutations and minimal residual disease in patients with core binding factor acute myeloid leukemia. Blood 121:2213–2223.
4. Jin, J., J. X. Wang, F. F. Chen, D. P. Wu, J. Hu, J. F. Zhou, et al. 2013. Homoharringtonine-based induction regimens for patients with de-novo acute myeloid leukemia: a multicentre, open-label, randomised, controlled phase 3 trial. Lancet Oncol. 14:599–608.
5. Wang, J. M., S. Q. Lü, J. M. Yang, X. M. Song, C. Li, C. M. Huang, et al. 2009. A homoharringtonine-based induction regimen for the treatment of elderly patients with acute myeloid leukemia: a single center experience from China. J. Hematol. Oncol. 2:32.
6. Jin, J., D. Z. Jiang, W. Y. Mai, H. T. Meng, W. B. Qian, H. Y. Tong, et al. 2006. Homoharringtonine in combination with cytarabine and aclarubicin resulted in high complete remission rate after the first induction therapy in patients with de novo acute myeloid leukemia. Leukemia 20:1361–1367.
7. Ye, P. P., Q. T. Mu, F. F. Chen, W. Y. Mai, H. T. Meng, W. B. Qian, et al. 2013. Efficacy and safety of the HAA regimen as induction chemotherapy in 236 de novo acute myeloid leukemia. Zhonghua Xue Ye Xue Za Zhi 34:825–829.
8. Yu, W., L. Mao, J. Qian, W. Qian, H. Meng, W. Mai, et al. 2013. Homoharringtonine in combination with cytarabine and aclacinomycin in the treatment of refractory/relapsed acute myeloid leukemia: a single-center experience. Ann. Hematol. 92:1091–1100.
9. Zhu, H. H., H. Jiang, Q. Jiang, J. S. Jia, Y. Z. Qin, X. J. Huang, et al. 2016. Homoharringtonine, aclacinomycin and cytarabine (HAA) regimen as the first course of induction therapy is highly effective for acute myeloid leukemia with t (8;21). Leuk. Res. 44:40–44. doi:10.1016/j.leukres.2016.02.012.
10. Gurel, G., G. Blaha, P. B. Moore, and T. A. Steitz. 2009. U2504 determines the species specificity of the A-site cleft antibiotics: the structures of tiamulin, homoharringtonine, and bruceantin bound to the ribosome. J. Mol. Biol. 389:146–156.
11. Lü, S., and J. Wang. 2014. Homoharringtonine and omacetaxine for myeloid hematological malignancies. J. Hematol. Oncol. 7:2.
12. Zhang, T., S. Shen, Z. Zhu, S. Lu, X. Yin, J. Zheng, et al. 2016. Homoharringtonine binds to and increases myosin-9 in myeloid leukemia. Br. J. Pharmacol. 173:212–221.
13. Wang, L., L. S. You, W. M. Ni, Q. L. Ma, Y. Tong, L. P. Mao, et al. 2013. β-Catenin and AKT are promising targets for combination therapy in acute myeloid leukemia. Leuk. Res. 37:1329–1340.
14. Shen, J. P., H. Yang, W. M. Ni, and W. B. Qian. 2012. Cytotoxicity of homoharringtonine on leukemic stem-like cells in AML cell line KG-1. Zhe jiang Da Xue Xue Bao Yi Xue Ban 41:485–490.
15. Kantarjian, H., S. O’Brien, E. Jabbour, G. Barnes, A. Pathak, and J. Cortes. 2015. Effectiveness of homoharringtonine (omacetaxine mesylate) for treatment of acute myeloid leukemia: a meta-analysis of Chinese studies. Clin. Lymphoma Myeloma Leuk. 15:13–21.
16. Baaske, D. M., and P. Heinze. 1977. Cytotoxicity and cell cycle specificity of homoharringtonine. Antimicrob. Agents Chemother. 12:298–300.
17. Fanucchi, M. P., X. R. Kong, and T. C. Chou. 1986. Hexamethylene bisacetamide (HMBA) does not enhance the cytotoxic effects of adriamycin (ADR), 1, beta-D-arabinofuranosylcytosine (ARA-C) and harringtonine (HT) in HL-60 cells [abstract 1492]. Proc. Am. Assoc. Cancer Res. 27:376.
18. Takahashi, I., M. Hara, K. Uchida, K. Takaoka, S. Watanabe, M. Lai, et al. 1982. Treatment of relapsed acute myelocytic leukemia with a combination of aclacinomycin and cytosine arabinoside. Acta Med. Okayama 36:77–80.
19. Zhou, G. B., H. Kang, L. Wang, L. Gao, P. Liu, J. Xie, et al. 2006. Oridonin, a diterpenoid extracted from medicinal herbs, targets AML1-ETO fusion protein and shows potent antitumor activity with low adverse effects on t(8;21) leukemia in vitro and in vivo. Blood 109:3441–3450.
20. Wang, L., W. L. Zhao, J. S. Yan, P. Liu, H. P. Sun, G. B. Zhou, et al. 2007. Eriocalyxin B induces apoptosis of t(8;21) leukemia cells through NF-kappaB and MAPK signaling pathways and triggers degradation of AML1-ETO oncoprotein in a caspase-3-dependent manner. Cell Death Differ. 14:306–317.
21. Zhen, T., C. F. Wu, P. Liu, H. Y. Wu, G. B. Zhou, Y. Lu, et al. 2012. Targeting of AML1-ETO in t(8;21) Leukemia by Oridonin. Sci. Transl. Med. 4:127ra38. doi:10.1126/scitranslmed.3003562.
22. Lu, Y., Z. G. Peng, T. T. Yuan, Q. Q. Yin, L. Xia, and G. Q. Chen. 2008. Multi-sites cleavage of leukemogenic AML1-ETO fusion protein by caspase-3 and its contribution to increased apoptotic sensitivity. Leukemia 22:378–386.