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Armillaridin induces autophagy-associated cell death in human chronic myelogenous leukemia K562 cells

Wen-Han Chang[1,2] · Huey-Lan Huang[3] · Wei-Pang Huang[4] · Chien-Chih Chen[5] ·
Yu-Jen Chen[1,6,7]

Abstract Armillaridin (AM) is an aromatic ester compound isolated from *Armillaria mellea*. Treatment with AM markedly reduced the viability of human chronic myelogenous leukemia K562, chronic erythroleukemia HEL 92.1.7, and acute monoblastic leukemia U937 cells, but not normal human monocytes, in a dose- and time-dependent manner. Treatment of K562 cells with AM caused changes characteristic of autophagy. Only a small amount of AM-treated K562 cells exhibited apoptosis. By contrast, AM treatment resulted in extensive apoptotic features in U937 and HEL 92.1.7 cells without evident autophagy. The autophagy of K562 cells induced by AM involved autophagic flux, including autophagosome induction, the processing of autophagosome-lysosome fusion and downregulation of BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3). By *bcr-abl* knockdown, the growth inhibition of K562 cells caused by AM was partially blocked, suggesting that AM-induced cell death might be a *bcr-abl*-dependent mode of autophagy-associated cell death. In conclusion, AM is capable of inhibiting growth and inducing autophagy-associated cell death in K562 cells, but not in normal monocytes. It may have potential to be developed as a novel therapeutic agent against leukemia.

Abbreviations

AM	Armillaridin
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3
CML	Chronic myelogenous leukemia
CQ	Chloroquine
3-MA	3-Methyladenine
STI-571	Signal transduction inhibitor-571

✉ Chien-Chih Chen
  ccchen@sunrise.hk.edu.tw

✉ Yu-Jen Chen
  chenmdphd@gmail.com

1   Institute of Traditional Medicine, National Yang-Ming University, Taipei 11221, Taiwan

2   Department of Healthcare Center, MacKay Memorial Hospital, New Taipei City 25173, Taiwan

3   Department of Bioscience Technology, College of Health Science, Chang Jung Christian University, Tainan 71101, Taiwan

4   Department of Life Science and Institute of Zoology, National Taiwan University, Taipei 10617, Taiwan

5   Department of Biotechnology, Hungkuang University, No.1018, Sec. 6, Taiwan Boulevard, Shalu Dist., Taichung City 43302, Taiwan

6   Department of Medical Research, MacKay Memorial Hospital, No.92, Section 2, Zhongshan N. Rd., Zhongshan District, Taipei City 10449, Taiwan

7   Department of Radiation Oncology, MacKay Memorial Hospital, No.92, Section 2, Zhongshan N. Rd., Zhongshan District, Taipei City 10449, Taiwan

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Introduction

Chronic myelogenous leukemia (CML) is a disease of hematopoietic stem cells, arising from t(9;22)(q34;q11) translocation, resulting in the Philadelphia chromosome. This translocation leads to the juxtaposition of the *abl* gene from chromosome 9 and the *bcr* gene from chromosome 22, resulting in *bcr-abl* fusion genes that encode for *bcr-abl* transcripts and fusion proteins with unusual tyrosine kinase activities [1]. Signal transduction inhibitor-571 (STI-571), which can inhibit Bcr-abl tyrosine kinase activity, was first introduced in 1996 and then became the first-line therapy for CML, and it is regarded as a model for development of small-molecule targeted therapeutics. However, treatment of CML is changing rapidly because the primary curative treatment, allogeneic transplantation, has significant transplantation-related mortality; the target treatment has a better outcome, but 10–15 % of patients remain resistant to the tyrosine kinase inhibitor [2]. A number of pharmacological modalities have been tested for their ability to overcome imatinib-refractory CML, such as targeting another prosurvival pathway, autophagy [3]. Development of novel therapeutics against CML via distinct mechanisms is a legitimate strategy to overcome the current clinical dilemma.

Armillaria mellea is a medicinal fungus with a symbiotic relationship with the Chinese medicinal herb *Gastrodia elata* (tien-ma). It is commonly used to treat dizziness, headache, neurothenia, insomnia, numbness in limbs, and infantile convulsions. The extracts of *A. mellea* were reported to have antioxidant properties [4]. Armillaridin (AM), a sesquiterpenoid aromatic ester, is a compound isolated from the mycelium of *A. mellea*. It is a colorless, acicular compound with a boiling point of 132–134 °C [5]. The chemical structure is shown in Fig. 1. Although the antibacterial activities of AM have been reported, the biological properties of AM are not well understood. In our previous study, AM was reported to have therapeutic and radiosensitization effects on esophageal cancer cells [6].
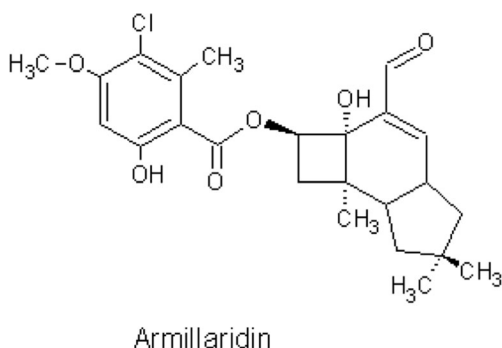

Fig. 1 Molecular structure of armillaridin (AM). AM is a sesquiterpenoid aromatic ester with antibacterial activity, $C_{24}H_{29}O_6Cl$

Autophagy is characterized by the degradation of intracellular materials within lysosomes or vacuoles [7]. The process occurs at a basal level and in response to stress. Basal level autophagy plays a role in protein and organelle turnover and is rapidly upregulated when cells need to generate intracellular nutrients and energy. Autophagy and apoptosis are important to control the turnover of organelles and proteins in cells. Autophagy blocks the induction of apoptosis in general and apoptosis-associated caspase activation turns off the autophagic process. However, in some circumstances, autophagy may help to induce apoptosis or necrosis and lead to autophagic cell death [8].

In the present study, we demonstrated that AM inhibited the viability of various human leukemia cell lines and specifically induced autophagy-associated cell death in CML K562 cells in association with *bcr-abl* dependence and the downregulation of BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) expression.

Material and methods

Cells and reagents

Human leukemic cell lines, including the CML cell line K562 and two acute myelogenous leukemia cell lines, U937 (M5) and HEL92.1.7 (M6), were obtained from the American Type Culture Collection and were provided by Dr. Sheng-Yuan Wang at Taipei Veterans General Hospital, Taipei, Taiwan. They were kept in an RPMI-1640 medium (Gibco, NY, USA) with 10 % fetal bovine serum (FBS) (Biological Industries Ltd., Kibbutz Beit Haemek, Israel) and maintained in exponential growth conditions. HEL 92.1.7 cells were cultured in an RPMI-1640 medium with 10 % FBS and 1 % sodium pyruvate (Gibco, NY, USA); other leukemic cells were cultured in an RPMI-1640 medium with 10 % FBS. With approval of the IRB (No. 09MMHIS027) at MacKay Memorial Hospital, Taipei, Taiwan, human peripheral blood mononuclear cells were acquired from healthy volunteers using the Ficoll-Paque density gradient centrifugation (GE Healthcare, Buckinghamshire, UK). CD14$^+$ cells were purified by high-gradient magnetic sorting using the miniMACS system (MiltenyiBiotec, BergischBladbach, Germany) with anti-CD14 microbeads. The purity of CD14$^+$ monocytes was >95 % as determined by flow cytometry. Cells were kept in an αMEM medium (Gibco, NY, USA) with 10 % FBS for all experiments.

Armillaridin was provided by Dr. Chien-Chih Chen (Hungkuang University, Taichung, Taiwan). It was dissolved in DMSO as stock solutions with concentrations of 1.25, 2.5, 5, and 10 mM and then stored at 4 °C until use.

Chloroquine (Sigma-Aldrich, MO, USA) was dissolved in phosphate-buffered saline and stored at 4 °C. 3-

Methyladenine (Sigma Aldrich, MO, USA) was dissolved into RPMI-1640. CQ was used at 10 μM, and 3-MA was used at 10 mM. The cells were preincubated with CQ and 3-MA for 90 min.

Cytoplasmic membrane integrity

The trypan blue exclusion assay was used to measure cell viability [9]. The numbers of viable cells were counted after 24, 48, and 72 h of exposure to AM.

Colony formation assay

Viable tumor cells (10^3) were plated into each 35-mm culture dish and allowed to grow in McCoy's 5A medium containing 20 % heat-inactivated FCS and 0.24 % agarose at 37 °C in a humidified 5 % CO_2 incubator. After 10 to 14 days, the dishes were stained with 0.4 % crystal violet and colonies containing ≥50 cells were counted.

Transmission electron microscopy

Cells were harvested, washed, and then fixed with cold 2.5 % glutaraldehyde in a 0.11 M cacodylate buffer for 30 min. Samples were then fixed in osmium tetroxide (1 %) and embedded in Epon resin (Electron Microscopy Sciences, PA, USA). Semi-thin sections were cut, stained with 5 % uranylacetate and lead citrate, and observed with a transmission electron microscope (JEM-1200EXII, JEOL, Co., Tokyo, Japan).

Cell cycle analysis by flow cytometry

After treatment, cells were washed with 10 % FBS/PBS and then fixed with ice-cold 70 % alcohol for 30 min. Cells were then incubated with Triton X-100 (Sigma-Aldrich, MO, USA), 0.2 mg/mL RNase A (Sigma-Aldrich, MO, USA), and 10 mg/mL propidium iodide (Sigma-Aldrich, MO, USA) for 10 min at 37 °C in the dark. Data were acquired using a FACSCalibur flow cytometer (Becton Dickinson, NJ, USA) and were analyzed by ModFIT software (Becton Dickinson, NJ, USA). A total of 10^4 cells were counted.

Assays for the detection of apoptotic events

Annexin V/PI staining (Trevigen Inc., MD, USA) was done according to the protocol provided by the manufacturer. Data acquisition and cell cycle analysis were performed on a FACSCalibur flow cytometer. STI-571 1 μM (Novartis Pharmaceutical Co., Switzerland) was used as a positive control for apoptosis induction in K562 cells [10]. Camptothecin 1 μM was used as a positive control for apoptosis induction in U937 cells [11] and HEL 92.1.0 cells.

Activity assay for caspases 3, 8, and 9

Caspase assays were also done according to the protocol provided by the manufacturer (Promega, WI, USA). The measurement of luminescence was done by luminometer.

GFP-LC3 assay

The green fluorescent protein (GFP) tagged-LC3 expressing vector was constructed by subcloning the LC3 coding sequence from pGST-LC3 plasmid (Provided by Dr. Tamotsu Yoshimori) into a pEGFP-C1 vector (Clontech, CA, USA). Cells were transiently transfected with GFP-LC3 by Lipofectamine™ 2000 (Invitrogen, CA, USA) based on the manufacturer's protocol. After treatments with AM, cells were observed under a fluorescence microscope.

Endogenous LC3 and LAMP2A assay

K562 cells were fixed with paraformaldehyde and permeabilized with Triton X-100. Cells were then incubated with anti-LC3B antibody (Enzo Life Science, NY, USA) and anti-LAMP2A antibody (ABcam, MA, USA) in a press-to-seal silicone isolator. Then, cells were incubated with FITC-conjugated secondary anti-mouse antibody and Rhodamine-conjugated anti-rabbit secondary antibody. The nuclei of K562 cells were stained with DAPI. Cells were then examined under a fluorescence microscope.

shRNA for BCR knockdown

All lentivirus-based short hairpin RNA (shRNA) expression systems were obtained from the National RNAi Core Facility, Academia Sinica, Taiwan. LacO-inducible lentiviral vector pTRC-905-shBCR791 and 793 plasmids were constructed by the ligation of EcoRI- and AgeI-digested pTRC-905 vectors (a gift from the National RNAi Core Facility, Academia Sinica, Taiwan) with annealed oligonucleotides containing the same shRNA sequence against the BCR gene from pLKO.1-shBCR constructions (TCN0000000791 and TRCN0000000793). pLKO.1-shRFP was used to knockdown gene expression of was used to knock down gene expression of as non-targeting shRNA control. Lentiviruses were produced from 293T cells transfected with the vector controls pTRC-905-shBCR791, pTRC-905-shBCR793, or pLKO.1-shRFP plasmids, following the standard procedures from the National RNAi Core Facility [12]. After lentiviral infection and puromycin selection, K562 cells were incubated with or without 200 μM IPTG for 1 day and treated with DMSO, 5 or 10 μM AM for another 2 days. Flow cytometry was used to estimate the number of cells in the sub-G1 apoptotic phase, and the knockdown effects were estimated by monitoring the remaining amount of BCR protein expression.

Western blotting

Cells were cultured in 10-cm dishes in the presence or absence of AM at different concentrations. Those were lysed, and protein concentrations were determined by a bicinchoninic acid assay kit (Pierce, IL, USA). Equal amounts (50 mg in each lane) of protein extracts were electrophoresed on 10 % (w/v) SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were incubated overnight at 4 °C with primary antibodies against LC3B (#2775), Phospho-p70S6K(#9205), p70S6K (#9202), Beclin-1(#4122), BCl-2 (#2876), c-ABL (#2862) (Cell Signaling Technology Inc., MA, USA), ATG5 (# PM050) (MBL international Cooperation, Japan), and BNIP-3 antibodies (ab10433) (ABcam, MA, USA) and then conjugated with horseradish peroxidase. The antigen-antibody reaction was detected under an enhanced chemiluminescence system (Abfrontier, Seoul, Korea) and then exposed to film. Quantification of the western blot analysis was done by ImageJ [13].

Data analysis

The experiments were performed in triplets. Results are reported as mean ± standard deviation. The Student's t test and one-way analysis of variance followed by Scheffe's test were used to estimate differences in the effects of AM on the viability of cells; the necrotic and apoptotic cells were tested by annexin V-PI staining. A p value less than 0.05 considered significant.

Results

Armillaridin reduced the viability of leukemic cell lines but not normal monocytes

The viabilities of leukemic cells and CD 14$^+$ monocytes were estimated. As shown in Fig. 2, AM inhibited the viability of leukemic cell lines K562, U937, and HEL 92.1.7 in a dose- and time-dependent manner. The estimated IC$_{50}$ values at 48 h were 4.4 μM for K562, 3.0 μM for U937, and 3.7 μM for HEL 92.1.7. It was indicated that the clonogenic capacities of the cells have been changed by colony formation assay (Table 1). In contrast, AM had no significant effect on the viability of CD14$^+$ monocytes (IC$_{50}$ > 10 μM). The results indicate that AM might preferentially inhibit the viability of leukemic cells.

Morphological changes after AM treatment

As shown in Fig. 3a, the AM-treated K562 cells showed various cytoplasmic vacuoles with intact cell membranes and intact nuclei characteristic of autophagy. A small number of cells with apoptotic features, including reduced cell volume, chromatin condensation, and nuclear fragmentation, were also found. However, as demonstrated in Fig. 3b, c, AM treatment resulted in extensive apoptotic features in U937 and HEL 92.1.7 cells without evident autophagy.

Effect of AM on recruitment of LC3 to autophagosomes in K562 cells

Fluorescent microscopy of starved cells treated with AM revealed a punctate pattern of GFP-LC3 aggregation, indicating recruitment of LC3 to autophagosomes (Fig. 4, starvation). To examine whether the autophagosomes were efficiently delivered to lysosomes in AM-exposed cells, we performed immunofluorescence for both LC3B and lysosome-associated membrane protein type 2A (LAMP2A), a receptor in the lysosome membrane. The punctate of LC3B and LAMP2A merged together in both AM-exposed and starvation-triggered cells. It indicates that the process induced by AM is autophagy induction rather than autophagosome accumulation (Fig. 4). The ultrastructural morphological changes were examined by transmission electron microscopy in K562 cells as shown in Fig. 5a. There were various vesicles measuring approximately 1 μm in diameter within the cytoplasm, and the nucleus and cell membrane were intact. Fusion vesicles and the remnants of organelles in the vesicles were also noted. Annexin V staining revealed that the exposure of cells to imatinib (1 μM) alone for 48 h resulted in marked apoptotic events [14]. No obvious apoptotic or necrotic events were noted in AM-treated K562 cells (Fig. 5b). To compare the amount of apoptotic population in each cell line, we performed Annexin V/PI flow cytometry analysis and found the proportion of early apoptotic population (Annexin V positive and PI negative) in K562, U937, and HEL 92.1.7 cells by AM 10 μM treatment for 48 h was 1.86 ± 0.18, 2.69 ± 0.18, and 5.88 ± 2.32 % and the late apoptotic and necrotic population (Annexin V positive and PI positive) in K562, U937, and HEL 92.1.7 cells by AM 10 μM treatment for 48 h was 4.51 ± 2.09, 7.94 ± 1.24, and 33.03 ± 1.64 %, respectively. We also examined the enzymatic activity of caspase 3/7, caspase 8, and caspase 9. AM did not activate these caspases. By contrast, the STI-571 activated these caspases during the condition-induced apoptosis of K562 cells (Table 2).

Autophagic flux in AM-treated K562 cells

Concentration-dependent LC3-II blotting revealed that LC3-II expression increased in a concentration-dependent manner by treatment with AM in K562 cells (Fig. 6a). The cells were incubated for a longer time, to 72 h. It showed that LC3-II accumulated at 48 h and disappeared at 72 h after treatment, indicating an active autophagic flux rather than a persistent

Fig. 2 AM reduced the viability of human leukemic cell lines and the normal counterpart, CD 14[+] monocytes. All cells were treated with various concentrations of AM for 24, 48, or 72 h. Trypan blue exclusion tests were performed to examine cell viability. Data from three separate experiments are expressed as mean ± standard deviation. *$p < 0.05$. **a** K562 cells, **b** U937 cells, **c** HEL 92.1.7 cells, **d** CD14^{+} monocytes
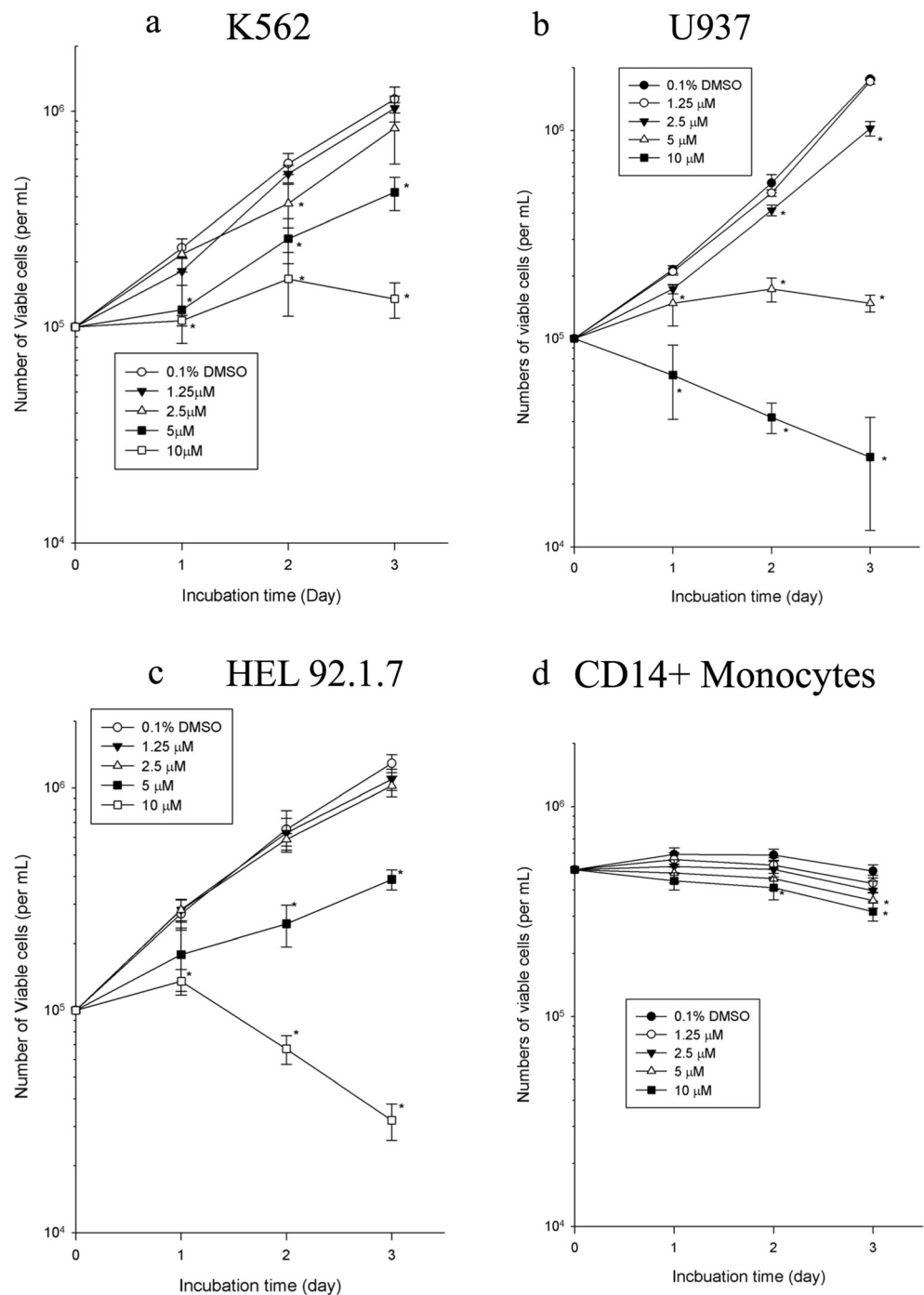

Table 1 Colony formation assay of K562 cells on treatment with AM, AM with CQ, and AM with 3-MA
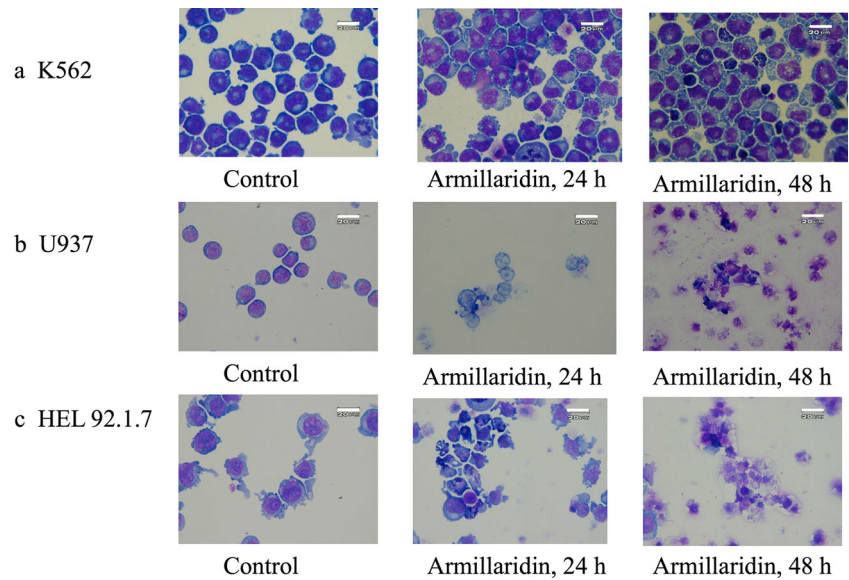
	Survival (%)
Control	100.00
AM 10 μM	50.85 ± 2.68
AM 10 μM, pretreated CQ	65.17 ± 3.63
AM 10 μM, pretreated with 3-MA	35.15 ± 3.54

Three independent tests were done. Data was shown as mean ± standard deviation. It showed significant differences between each of the groups

accumulation of autophagosomes (Fig. 6b). The accumulation of LC3-II was also found in AM-treated U937 and HEL 92.1.0 cells (Fig. 6c).

We assessed autophagic flux by combining chloroquine (CQ) or 3-methyladenine (3-MA) with AM and found that pretreatment with 10 μM of CQ enhanced the accumulation of LC3-II in K562 cells, as shown in Fig. 7. In contrast, pretreatment with 10 mM of 3-MA resulted in a decreased accumulation, indicating that AM-induced autophagy affected autophagic flux through the induction to autophagosome-

Fig. 3 Morphological changes of leukemic cells under AM treatment. Cells were treated with 10 μM AM for 24 or 48 h. Light microscopy at magnification ×1000 was used for morphological observation. **a** K562 cells, **b** U937 cells, **c** HEL 92.1.7 cells

a K562

Control   Armillaridin, 24 h   Armillaridin, 48 h

b U937

Control   Armillaridin, 24 h   Armillaridin, 48 h

c HEL 92.1.7

Control   Armillaridin, 24 h   Armillaridin, 48 h

lysosome fusion. There was an increased survival with pretreatment of CQ (50.85 vs. 65.17 %) and a decreased survival with pretreatment of 3-MA (50.85 vs. 35.15 %), according to colony formation assay (Table 1).

Mechanisms of AM-induced autophagy

Key molecules related to autophagy were expressed in a time-dependent manner (Fig. 8). Exposure to AM resulted in a time-dependent decrease in BNIP3 expression in K562 cells. The expression of phosphorylated p70s6k showed an initial increase followed by a decline during 24-h treatment. The

Control   Armillaridin   Starvation 6 h

LC3B

LAMP2A

Merged

Fig. 4 AM induced autophagy in leukemia cells. Plasmid carrying GFP-LC3 was transfected into K562 cells prior to treatment. Punctate pattern aggregation of GFP-LC3, indicating recruitment of LC3 to autophagosome, was observed by fluorescent microscopy in starved cells treated with AM. Three independent tests were done. Endogenous LC3B and LAMP2A were observed by fluorescent microscopy in cells treated with AM and starvation. Magnification ×1000. *Blue*, DAPI. *Green*, FITC LC3B. *Red*, Rhodamine-LAMP2A

expression of p70s6k, Atg5, and beclin-1 showed no evident changes.

Blockage of AM-induced cytotoxicity in BCR-ABL knockdown K562 cells

The main difference between K562 cells and acute myelogenous leukemic cells are the *bcr-abl* fusion gene. Knockdown of *bcr-abl* was performed with an isopropyl-β-D-thio-galactoside (IPTG) induction system and was validated by western blotting analysis (Fig. 9a). The *bcr-abl* knockdown partially blocked AM-induced cytotoxicity and apoptosis in K562 cells (Figs. 9b, c), indicating that AM-induced autophagy might be mediated by a BCR-ABL-dependent mode of autophagy-associated cell death.
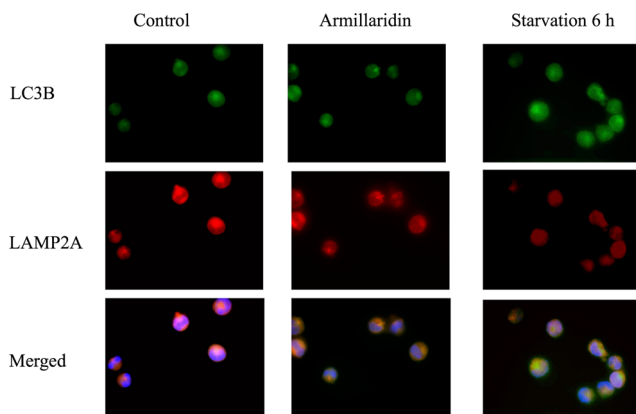
Discussion

The results of this study showed that AM reduced cell viability and induced extensive autophagy in human leukemic cells. Current data demonstrate that AM affects autophagy flux in LC3 turnover assays. This autophagic event involved downregulation of BNIP3 and was independent of the early externalization of phosphatidylserine, an early event of apoptosis. It also showed that AM preferentially inhibited the viability of myeloid leukemic cells without marked toxicity to the normal counterpart monocytes.

AM treatment caused the downregulation of BNIP3 expression in CML K562 cells. The upregulation of BNIP3, a proapoptotic protein that binds to antiapoptotic proteins of the Bcl-2 family, may cause autophagy in hypoxia-induced autophagy [15]. BNIP3 is also a proautophagic protein and
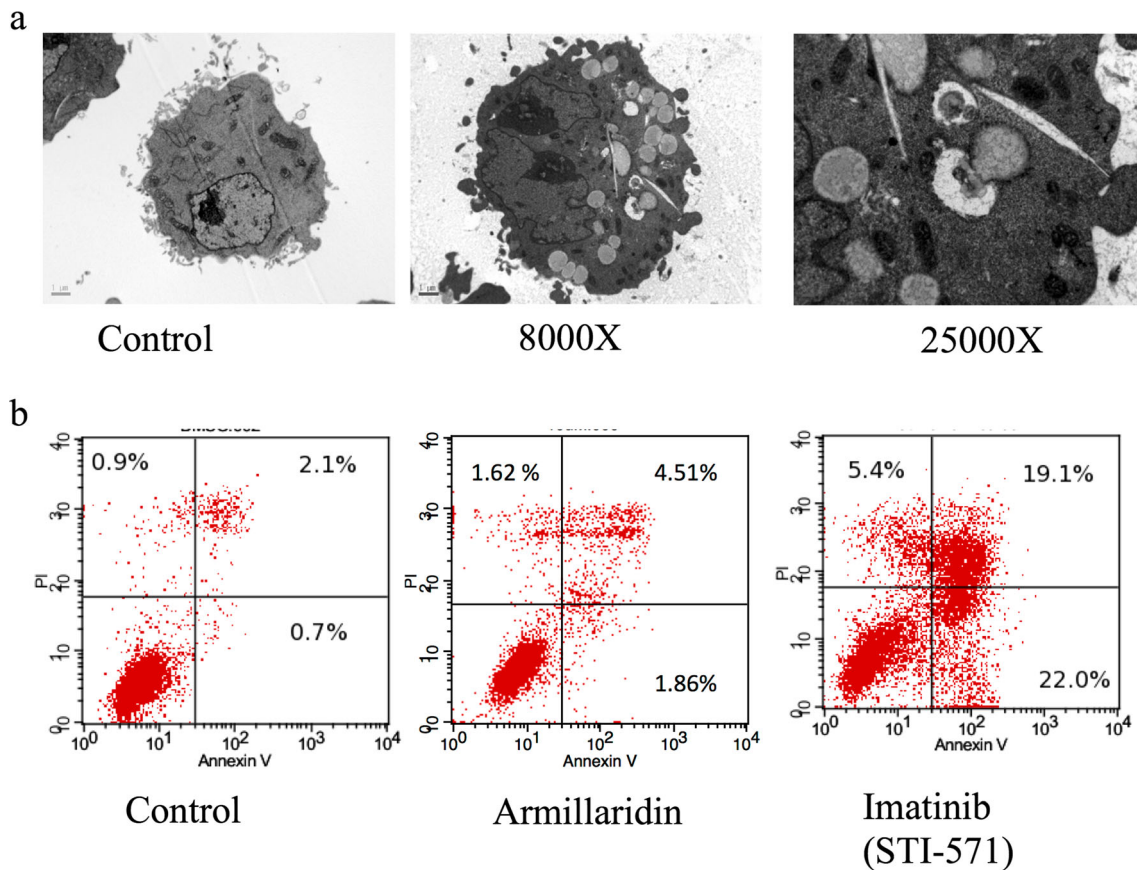
Fig. 5 AM induced autophagy without evident apoptosis in chronic myelogenous leukemia cells. **a** Transmission electron microscopy at magnification ×8000–25,000 was used for the morphological observation of AM-treated K562 cells. **b** Cells were treated with 0.1 % DMSO, 5 μM AM, or 1 μM STI-571 (as a positive control of apoptosis) for 48 h. Harvested cells were stained with propidium iodide for necrotic cells and Annexin V for externalization of phosphatidylserine. Flow cytometry was used for analysis. Data from three separate experiments are expressed as mean ± standard deviation. *$p < 0.05$

induces autophagy by disrupting the Bcl-2-beclin-1 interaction and activating beclin-1 [16]. The upregulation of BNIP3 in autophagy was revealed in ceramide-induced and arsenic-trioxide-induced autophagic cell death in malignant glioma cells and in platonin-induced autophagic cell death in leukemia cells in prior studies [17–19].

Table 2 Effect of AM on caspase activities in K562 cells

	Control	AM 5 μM	AM 10 μM	STI-571 1 μM
Caspase 3/7	1	0.87 ± 0.07	0.80 ± 0.12	11.25 ± 1.34
Caspase 8	1	1.03 ± 0.15	0.96 ± 0.18	43.05 ± 3.05
Caspase 9	1	0.93 ± 0.13	0.95 ± 0.21	29.99 ± 1.52

Cells were treated with AM 5 μM, AM 10 μM, and STI-571 1 μM for 48 h. Three independent tests were done. Data was shown as mean ± standard deviation. Activity assays for caspases 3/7, 8, and 9. AM did not activate the activity of these caspases. By contrast, the STI-571 activated these caspases during condition-induced apoptosis of K562 cells

AM armillaridin, *CQ* chloroquine, *3-MA* 3-methyladenine

There were no obvious changes in bcl-2 and beclin-1 in K562 cells treated with AM. It must also be mentioned that loss of BNIP3 was protective against hypoxia-induced autophagic cell death [15]. Conversely, in our study, the decreased expression of BNIP3 by AM was evident in a time-dependent manner. Given that BNIP3 is known as tumor suppressor and contributor to hypoxia-induced autophagy, further elucidation of the role of BNIP3 in AM-induced autophagy in K562 cells is needed.

Our results showed relatively higher survival rates by pretreating with the lysosomotropic reagent CQ before administering AM, which indicated that AM induced autophagy-associated cell death through the autophagy process. Pretreatment of 3-MA at the condition mitigating the LC3-II conversion (as shown in Fig. 5c) further reduced the survival of AM-treated K562 cells. The role of 3-MA may counteract the induction of autophagy with accompany by promoting apoptosis. It implicates that the development of autophagy and apoptosis seems an interchangeable or interactive process. For example, apoptosis induced by calpain inhibitor decreased oridonin-induced
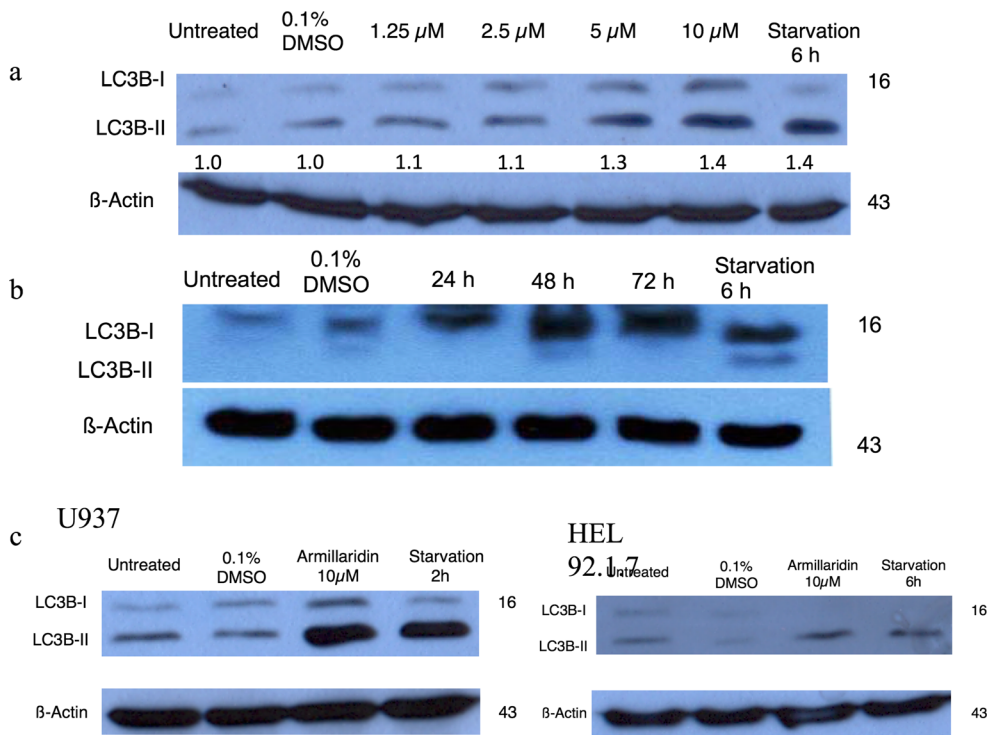
Fig. 6 Effect of AM on expression of LC3B in K562 cells. **a** K562 cells were treated with serial concentrations of AM for 24 h. Protein extracts were subjected to western blotting. Data from three separate experiments showed a similar trend. *Lane 1*, untreated; *lane 2*, 0.1 % DMSO; *lane 3*, 1.25 μM AM; *lane 4*, 2.5 μM AM; *lane 5*, 5 μM AM; *lane 6*, 10 μM AM; *lane 7*, starvation for 6 h. **b** K562 cells were treated with 10 μM of AM for 24, 48, and 72 h. Protein extracts were subjected to western blotting. Data from three separate experiments showed a similar trend. *Lane 1*, untreated; *lane 2*, 0.1 % DMSO; *lane 3*, AM 24 h; *lane 4*, AM 48 h; *lane 5*, AM 72 h; *lane 6*, starvation 6 h. **c** U937 and HEL 92.1.7 cells were treated with 10 μM AM for 24 h. Protein extracts were subjected to western blotting. *Lane 1*, untreated; *lane 2*, 0.1 % DMSO; *lane 3*, 10 μM AM; *lane 4*, post 6-h starvation

autophagy. By contrast, the inhibition of autophagy by 3-MA increased oridonin-induced apoptosis in L929 cells [20].

We showed that AM induced autophagy-associated cell death in K562 cells and apoptosis in a small population of K562 cells, whereas AM induced extensive apoptosis and autophagy in U937 and HEL 92.1.7 cells. The opposite roles played by autophagy are not only dependent on the cell type but, more importantly, on the entity or duration of stress conditions. We also found that *bcr-abl* knockdown partially blocked AM-induced cytotoxicity and apoptosis in K562 cells. It is known that the *bcr-abl* fusion gene exists in K562 cells but not in other leukemic cell lines we used, suggesting that the *bcr-abl* fusion gene might play a role in triggering autophagy-associated cell death rather than apoptotic cell death.
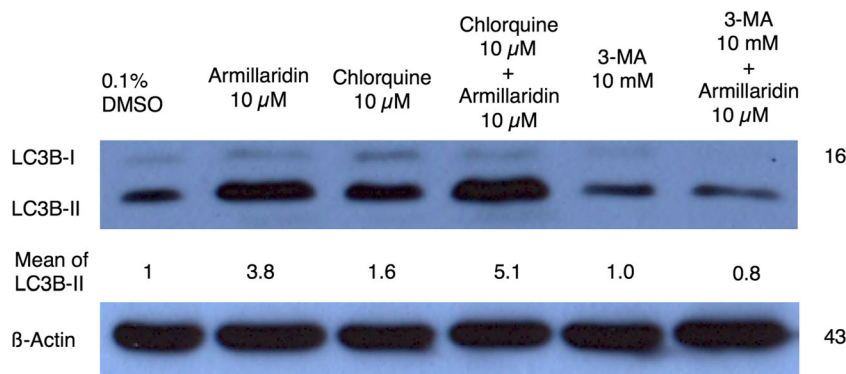

Fig. 7 Effect of AM on expression of LC3B under autophagy inhibitor in K562 cells. Cells were treated with indicated conditions of AM for 24 h. Protein extracts from whole cells were subjected to western blotting. *Lane 1*, 0.1 % DMSO; *lane 2*, 10 μM AM; *lane 3*, pretreated with 10 μM chlorquine (CQ); *lane 4*, pretreated with 10 μM CQ and then treated with 10 μM AM; *lane 5*, pretreatment of 10 mM 3-methyladenine (3-MA); *lane 6*, pretreatment of 10 mM 3-MA and then treated with 10 μM AM. Mean band intensities of LC3B-II are shown in **a** and **b**

Fig. 8 Autophagy-related
signaling in AM-treated K562
cells. Cells were treated with
10 μM of AM for indicated times.
Protein extracts from whole cells
were subjected to western
blotting. Data from three separate
experiments showed a similar
trend. *Lane 1*, 0 h; *lane 2*, 2 h;
lane 3, 4 h; *lane 4*, 8 h; *lane 5*,
16 h; *lane 6*, 24 h; *lane 7*
starvation for 6 h. Mean band
intensity of LC3B-II compared
with starvation group is shown
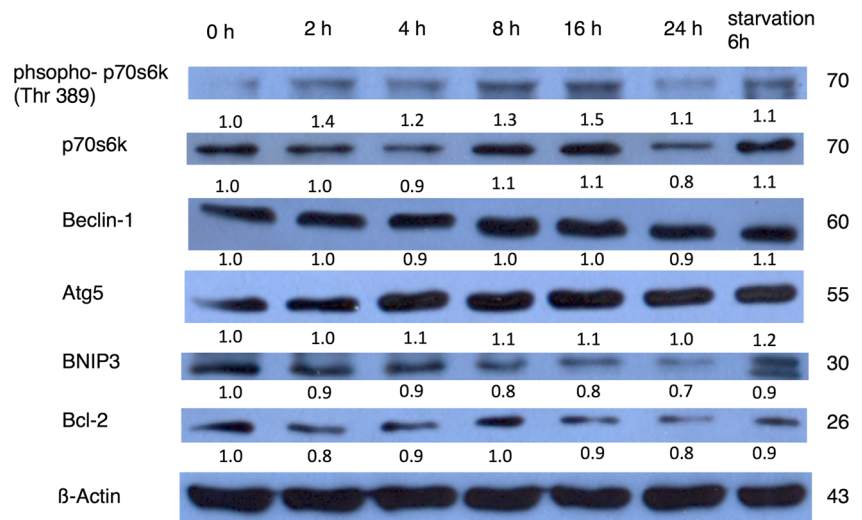
Gousseties et al. [21] demonstrated "that decreasing BCR-ABL expression by arsenic trioxide lead autophagy in K562 cells involving p62/SQSTM1-medicated localization of the oncoprotein to the autolysosomes and subsequent degradation medicated by the protease cathepsin B." This event could be reversible by inhibition of autophagy. Bellodi et al. [3] showed that the inhibition of BCR-ABL with STI-571 induced apoptosis and autophagy, and autophagy inhibits apoptosis in CML cells. Sheng et al. [22] demonstrated that BCR-ABL suppressed autophagy through the regulation of mTOR transcription. The interplay of BCR-ABL and autophagy varies depending on the different circumstances. In our study, AM induced autophagy-associated cell death without involving BCR-ABL expression (Supplemental Fig. 1). Knockdown of

bcr-abl resulted in less growth inhibition and a greater hypoploidy population in K562 cells treated with AM. It suggests that the development of autophagy in AM-treated K562 cells might be counteracted in a *bcr-abl*-dependent manner.

Conclusion

In summary, armillaridin is capable of inhibiting growth in human leukemia cells but not in normal monocytes. Armillaridin-induced cell death might be a *bcr-abl*-dependent mode of autophagy-associated cell death in CML K562 cells. It could be considered a novel therapeutic drug against leukemia.

Fig. 9 Effect of shBCR on cell growth inhibition in K562 cells. K562 cells were infected with lentivirus produced from pLKO.1-shRFP (shRFP), pTRC-905-shBCR791 (shBCR791), or pTRC-905-shBCR793 (shBCR793). After being selected with puronycin, cells were incubated with or without 200 μM of IPTG for the induction of shRNA expression for 1 day, and then treated with DMSO, 5 or 10 μM of AM for 2 days. The knockdown effect of the *bcr-abl* gene was detected by western blot analysis using antibodies against *bcr* and actin (**a**). Data from three separate experiments showed a similar trend. Growth inhibition effects (**b**) and percent of apoptotic cells (**c**) under AM treatment were detected by MTT assay and flow cytometry
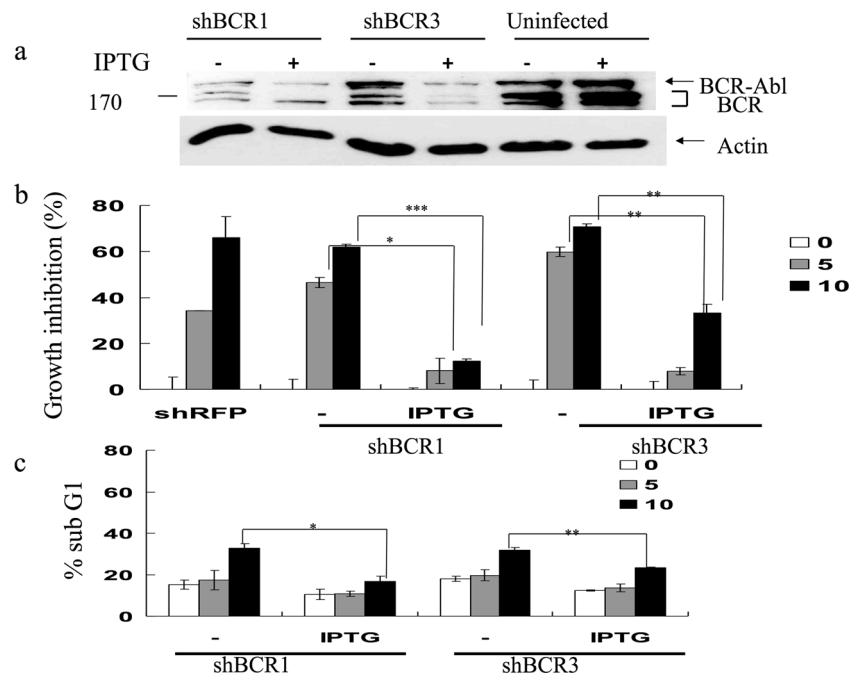
Compliance with ethical standards

Conflicts of interest None.

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