Withaferin A suppresses the growth of myelodysplasia and leukemia cell lines by inhibiting cell cycle progression

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Despite recent progress in various types of chemotherapy and in the wide indication of hematopoietic stem cell transplantation, treatment outcomes for acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) remain unsatisfactory from the perspective of achieving disease-free status or complete cure.1,2 There is, therefore, a need for the development of a new therapeutic strategy.

Withaferin A (WA), one of the steroidal lactones, is a bioactive constituent of the Indian herb Withania somnifera (commonly known as Ashwagandha or Indian winter cherry), a wild plant that is widely distributed across the South Asian field,3,4 and is a traditional medicine for various diseases, such as inflammatory diseases, autoimmune diseases and malignant tumors.5

The anticaner activity of WA was reported for the first time in 19676 and several investigations have since been performed to determine its potential as an anticancer agent. Previous reports have demonstrated that WA affects microtubules or vimentin intermediate filaments and, subsequently, exerts cytotoxicity or inhibition of the epithelial–mesenchymal transition.7–10 WA is reported to induce cell cycle arrest at G2/M phase, resulting in apoptosis.11–15 Nonetheless, the detailed mechanisms of action remain to be determined and may be different depending on the cells, tissues or experimental systems.

In the present study, we investigated the growth-suppressive effect of WA on human myeloid and lymphoid cell lines. We found that WA exhibits growth-suppressive effects on these cell lines and induces cell cycle arrest at G2/M phase at relatively low doses. We also found the upregulation of heme oxygenase-1 (HMOX1) and enhanced autophagy. WA has a unique growth-suppressive effect on leukemic cells and may serve as a novel therapeutic approach to the treatment of hematological malignancies.

Materials and Methods

Reagents. Withaferin A was purchased from ChromaDex (Irvine, CA, USA). It was dissolved in dimethylsulfoxide and stored at −20°C. Chloroquine (CQ) was obtained from Cell Signaling Technology (Danvers, MA, USA). It was dissolved in distilled water and stored at 4°C.

Cell lines and primary cells. A myelodysplastic cell line, MDS92, was established from the bone marrow of a patient with MDS.16 This cell line proliferated in the presence of interleukin-3 (IL-3) with a tendency to gradual maturation. Therefore, we isolated the CD34-positive fraction from MDS92 cells by the magnetic beads method with anti-CD34 monoclonal antibody for a comparative study with the other...
cell lines. MDS-L cell line, a blastic subline originated from MDS92, was mainly used in this study. MDS92 and MDS-L cells were maintained in RPMI1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol, 2.0 mM L-glutamine and 100 U/ml IL-3. HL-60 and THP-1 (both human myeloid leukemia cell lines), Jurkat (a T-acute lymphoblastic leukemia cell line) and Ramos (a Burkitt lymphoma cell line) were also used in this study.

Five human normal bone marrow CD34-positive progenitor cells were purchased from LONZA Group (Basel, Switzerland) and cultured with a serum-free medium with recombinant cytokines for myelopoiesis of hematopoietic progenitor cells, STEM ALPHA, AG (STEM ALPHA SA, Saint Genis L’Argentiére, France). Primary leukemic cells were obtained from a patient with AML at Kawasaki Medical School Hospital after informed consent. The usage of patient samples was approved by the Ethical Committee of Kawasaki Medical School. The mononuclear cell fraction was isolated from the bone marrow sample using the Ficoll–Hypaque density centrifugation method.

Cell growth assay and MTT assay. The morphological assessment was performed with May–Gruenwald–Giemsa stained cytospin slides. Cell growth was assessed by counting the number of living cells after trypan blue dye staining. Cell suspensions were plated into 96-well plates in the presence of the drug or solvent alone, incubated as above at 37°C for 1–3 days, and analyzed using the MTT assay.

Cell cycle analysis. Cells were fixed with 70% methanol for 30 min and treated with 2 mg/mL ribonuclease A (Nacalai Tesque, Kyoto, Japan) for 30 min at 37°C, then with 50 μg/mL propidium iodide (PI; Sigma, St Louis, MO, USA) for a further 20 min at room temperature. Samples were analyzed with BD FACS Aria III and Diva software (Becton Dickinson, Franklin Lakes, NJ, USA).

Apoptosis assay. Apoptosis was examined using the AnnexinV Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA) and all samples were analyzed with BD FACS Aria III and Diva software (Becton Dickinson).

Autophagy assay. Autophagy assay was performed using the CYTO-ID Autophagy Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA). The cells were stained by the dedicated reagent covered from light for 30 min at 37°C and analyzed with BD FACS Aria III with Diva software (Becton Dickinson).

Immunoblotting analysis. Cell lysates were prepared in Laemmli Sample Buffer (BIO-RAD, Hercules, CA, USA) containing 2-mercaptoethanol and heated for 5 min at 95°C. Cell lysates were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk in T-PBS for 60 min at room temperature. Then the membrane was incubated with the appropriate antibodies overnight. The membrane was washed three times with T-PBS and incubated with HRP-conjugated goat antiabbit antibodies for 30 min, and specific proteins were detected using an enhanced chemiluminescence detection system.

Primary antibodies were obtained from Cell Signaling Technology Tokyo, Japan (HMOX1[HO-1], C-PARP, LC3A/B, β-actin). HRP-conjugated rabbit secondary antibody was purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA).

Gene expression profiling and gene set enrichment analysis. Gene expression profiling of MDS-L cells were analyzed in three independent experiments. Treated cells were harvested after 12 h treatment with 1 μM of WA. Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Germantown, MD, USA), converted to cDNA and amplified with a GeneChip WT Terminal Labeling and Controls Kit (Affymetrix, Santa Clara, CA, USA). The fragmentation, the labeling and the hybridization of cDNA were treated with a GeneChip Hybridization, Wash, and Stain Kit (Affymetrix). Chips were
Fig. 2. Effects of withaferin A (WA) on the morphology and cell cycle of myelodysplasia and leukemia cell lines. (a) MDS-L cells were cultured without treatment (control) or treated with 250, 500 and 1000 nM WA for 24 and 48 h. HL60, THP-1, Jurkat and Ramos cells were cultured without treatment (control) or treated with 1000 nM WA for 6 h. Cytospin preparations were stained by May-Gruenwald-Giemsa (original magnification x400). Representative images by each treatment are shown. (b) The cell cycle analyses by propidium iodide (PI) staining are shown. MDS-L, HL-60, THP-1, Jurkat and Ramos cells were treated with indicated concentrations of WA for 24 h, and the cells were stained with PI and analyzed by flow cytometry. Representative histogram patterns by each treatment are shown. (c) The cell fractions at G1, S and G2/M phase are presented by white, gray and black bars, respectively. The data represent the mean values with SD from three independent experiments.
scanned with a GeneChip Scanner 3000 7G System (Affymetrix).

The gene set enrichment analysis (GSEA Broad Institute, Cambridge, MA, USA) was performed using the gene expression profiling data and the GSEA software. Detailed information regarding these experiments can be found in Subramanian et al. and from the GSEA website (http://www.broadinstitute.org/gsea/index.jsp). (22)

Immunofluorescence staining. Cells were centrifuged onto cytospin slides and fixed for 15 min in 100% methanol at −20°C, then blocked with blocking buffer including 5% FBS and 0.3% Triton X-100 in PBS (pH 8.0) for 60 min. Immunofluorescence staining was performed using rabbit monoclonal anti-LC3A/B to detect autophagosomes, and AlexaFluor488-conjugated anti-IgG antibodies (Molecular Probes, Eugene, OR, USA) as secondary antibodies. The nucleus was stained with DAPI (Dojindo, Kumamoto, Japan). Cells were observed under an Olympus BX51 fluorescence microscope (Tokyo, Japan).

Statistical analyses. Most data were expressed as mean ± SD. Comparisons between the groups were done...
using one-way ANOVA. Differences were considered statistically significant if $P$-values were <0.05. Data analysis was performed using the Prism software (GraphPad, La Jolla, CA, USA).

Results

**Withaferin A inhibits the proliferation of myelodysplasia and leukemia cell lines.** To investigate the growth-suppressive effects of WA, we treated MDS-L, HL-60, THP-1, Jurkat and Ramos cell lines with WA, and assessed their proliferation at 24, 48 and 72 h by MTT assay. As shown in Figure 1, WA exhibited a concentration-dependent growth-suppressive effect on all cell lines, with the half-maximal inhibitory concentration (IC$_{50}$) values of 171 ± 13 nM at 24 h, 90 ± 18 nM at 48 h, and 105 ± 19 nM at 72 h in MDS-L cells. The IC$_{50}$ values for the other cell lines are presented in Figure 1b. Cell counting by trypan blue dye exclusion assay displayed the same trends (data not shown).

**Withaferin A induces cell cycle arrest at G2/M phase.** The cell lines were treated with WA, and evaluation of cell morphology by May–Gruenwald–Giemsa staining demonstrated an increased number of mitotic cells (Fig. 2a). The cell cycle analysis by PI staining of the cells after WA treatment revealed an increased proportion of the cells at G2/M phase in a dose-dependent manner (Fig. 2b,c) and confirmed the induction of G2/M arrest in the five cell lines.

**Withaferin A partially induces apoptosis.** As WA ultimately led the cell lines to cell death after inducing G2/M arrest, we investigated whether WA induces apoptosis. Annexin V/PI dual staining confirmed the presence of apoptotic cells (Fig. 3a). Immunoblotting analysis also revealed that WA treatment resulted in increased expression of the apoptosis-related protein, C-PARP, confirming that WA induces apoptosis (Fig. 3b).

**Gene enrichment analysis revealed upregulation of the heme oxygenase-1 (HMOX-1) gene after treatment with WA.** Previous reports in the literature suggested that the effects of WA on cultured cells are multifaceted. Hence, we investigated the effects of WA on gene expression in MDS-L cells by microarray gene expression profiling and assessed the data by GSEA. As shown in Figure 4a, WA treatment resulted in significantly increased expression of the transcription factor gene set, V$USF_Q6_01$ (FDR $q$-value 0.036; FWER $P$-value 0.00). The expression of $HMOX1$ was most significantly increased among the genes in the set (Fig. 4b). WA-induced HMOX1 upregulation was also confirmed by immunoblotting analyses (Figs 3b,5a).

In contrast, microarray analyses did not show increased expression of $HMOX1$ after the addition of decitabine, lenalidomide and rigosertib (these are the drugs with promising anticancer effects on MDS) to MDS-L cells (data not shown).

**Chloroquine inhibits autophagy and enhances Withaferin A-induced apoptosis.** As GSEA demonstrated the upregulation of autophagy-related molecules such as $HMOX1$ and $WIP1$ during WA treatment (Fig. 4b), we investigated LC3 expression at the cellular level, and confirmed that WA treatment resulted in increased levels of LC3A/B (Figs 3b,5a). Using a flowcytometric method, WA-induced autophagy was detected on most of the cells (Fig. 5b).

Chloroquine is known to suppress autophagy by inhibiting lysosome-autophagosome fusion. (23) CQ treatment by itself
caused marked accumulation of LC3A/B-II (Fig. 5a). Hence, we assessed LC3A/B accumulation by the combined use of WA and CQ. Immunoblotting analysis revealed no apparent changes (Fig. 5a), but LC3A/B showed increased cytoplasmic accumulation on immunostaining (Fig. 5c) by co-treatment with WA and CQ. Although MDS-L cell growth was moderately suppressed by treatment with CQ alone, the combined treatment with WA and CQ increased the growth-suppressive effect (Fig. 5d). While the combined use of the two drugs did not result in any apparent changes in C-PARP (Fig. 5a), annexin V/PI staining showed an increase in the fraction of early apoptosis (annexin V-positive and PI-negative fraction shown in Fig. 5e). In regards to the cell cycle, CQ did not affect the pattern of the cell cycle with the single administration nor in combination with WA (Fig. 5f,g).

Normal bone marrow CD34-positive cells are less susceptible to Withaferin A than leukemic cell lines. To assess the effects of WA on normal hematopoietic cells, normal bone marrow CD34-positive cells were treated with WA for 48 h, and the cell growth, morphology and cell cycle were evaluated. Unlike MDS-L cells, normal CD34-positive cells revealed no apparent growth-suppressive effect (Fig. 6a), no increase in mitotic cells (Fig. 6b) and no apparent change of the cell cycle pattern in the short-term culture (Fig. 6c,d).

We further investigated the effects of WA on a pathological but less aggressive cell line MDS92, the parental cell line of MDS-L. The CD34-positive fraction of MDS92 (comparable to blastic MDS-L cells) proliferated more slowly than MDS-L (the doubling time of MDS92 and MDS-L was over 60 h and <24 h, respectively). As compared with MDS-L cells, the CD34-positive fraction of MDS92 cells revealed weaker...
growth suppression (Fig. 6a), no increase in mitotic cells (Fig. 6b) and no apparent change of the cell cycle pattern (Fig. 6c,f) than MDS-L cells.

Aiming at clinical application of WA to AML, we examined the effects of WA on the bone marrow leukemic cells of an untreated AML patient and found that WA exerted a subtle toxicity on primary leukemic cells with no apparent effects on morphology and cell cycle pattern (Figs S1–S4).

Discussion

Despite several reports of the potential of WA as an anticancer agent, no clinical applications have been reported, and the precise mechanisms of action are largely unknown. In this study, we investigated the in vitro effects of WA on myelodysplasia and leukemia cell lines to ascertain the potential utility of WA for the treatment of leukemia and MDS. Concentration-dependent growth-suppressive effects were confirmed in all cell lines studied, and the IC_{50} values ranged from 60 to 200 nM at 72 h (Fig. 1), which are lower than the range of IC_{50} values reported for other cancer cell lines (300 to 1000 nM at 72 h), suggesting that human leukemia cell lines are comparatively highly susceptible to WA. WA treatment also resulted in an increased number of mitotic cells, and the cell cycle analysis showed that WA induces G2/M arrest (Fig. 2).

Fig. 4. Expression of the gene set “V$USF_Q6_01” in withaferin A (WA)-treated MDS-L cells by the gene enrichment analysis (GSEA). (a) WA (1000 nM)-treated (WFA) or untreated MDS-L cells were harvested at 12 h. Gene expression profiling of MDS-L cells was examined in triplicate experiments and obtained data were used for GSEA by handling the GSEA software and the Molecular Signatures Database according to the references. The gene set “V$USF_Q6_01” was strongly upregulated by WA treatment and several statistical values are also presented. (b) The heat map presentation of the top 31 activated genes out of 218 genes included in the gene set is shown in the triplicate experiments (WFA, WA-treated; non-WFA, untreated). In (b), this gene set contains HMOX1 as the most activated gene and further indicates the upregulation of an autophagy-related molecule WIPI1 by WA treatment.
As WA presumably has multifaceted mechanisms of action, the effects on gene expression were explored by microarray gene expression profiling and GSEA. After 12 h of WA treatment, the activation of the V$USF_Q6_01$ gene set was found to be the most significant (Fig. 4a). Although this set is composed of genes with promoter regions (−2 kb–+2 kb) around the transcription start site containing the motif (NRCCACGT-GASN), the implication of this motif is not clear at present (see http://www.broadinstitute.org/gsea/index.jsp). Detailed analysis of the genes in the set confirmed that HMOX1 was rated the top of the genes by GSEA (Fig. 4b). Increased HMOX1 expression was also confirmed at the protein level (Figs 3b, 5a).

HMOX1 is the rate-limiting enzyme during the degradation of heme into carbon monoxide (CO), biliverdin and ferrous iron, and its activity is crucial for tissue homeostasis such as mediation of oxidative stress, attenuation of inflammatory response, regulation of apoptosis, and acceleration of autophagy. Previous reports indicated that antitumor drug administration results in an increase in HMOX1 expression in tumor cells, which might be a pro-survival mechanism linked to the induction of autophagy. This suggests that autophagy is induced in tumor cells treated with antitumor drugs and is, thereby, involved in acquired resistance to chemotherapy.

Our data suggest that both apoptosis and autophagy occurred coincidentally after WA treatment in MDS-L cells (Figs 3a, b, 5a,b). It is likely that WA triggered multiple and apparently opposite signals related to cell survival or death. The viable
Fig. 5. continued.
Fig. 6. Withaferin A (WA) has no apparent suppressive effect on the growth of normal bone marrow CD34-positive cells in the short-term culture. (a) Normal human bone marrow CD34-positive cells \( n = 3 \), MDS-L and MDS92 (CD34-positive fraction) cells were treated with the indicated concentrations of WA for 24 and 48 h, and cell count was assessed by trypan blue dye exclusion assay. The data represent the mean values with SD from three independent experiments. (b) Normal human bone marrow CD34-positive cells \( n = 3 \) and MDS92 (CD34-positive fraction) cells were treated with the indicated concentrations of WA for 24 and 48 h, and cytospin preparations were stained by May–Gruenwald–Giemsa (original magnification ×400). Representative images for each treatment are shown. (c, e) The cell cycle analyses by PI staining are shown. Human normal bone marrow CD34-positive cells (sample 1 and sample 2) and MDS92 (CD34-positive fraction) cells were treated with indicated concentrations of WA for 24 h, and the cells were stained with PI and analyzed by flow cytometry. Representative histogram patterns for each treatment are shown. (d) From the data of two normal CD34-positive cells shown in (c), the cell fractions at G1, S and G2/M phase are presented by white, gray and black bars, respectively. (f) From the data of MDS92 cells shown in (e), the cell fractions at G1, S and G2/M phase are presented by white, gray and black bars, respectively. The data represent the mean values with SD from three independent experiments.
cell count might express the result of net balance between apoptosis and autophagy (anti-apoptosis). Our hypothesis is that WA-induced autophagy renders the cells resistant to some degree to apoptosis induced simultaneously by WA, and that CQ releases suppression of apoptosis by unlocking autophagy and, consequently, accelerates apoptosis. In fact, combined treatment with WA and an autophagy inhibitor CQ resulted in enhanced apoptosis (Fig. 5d,e).

It has been reported that therapeutic efficacy is enhanced by the inhibition of autophagy activated during anticancer chemotherapy. On this basis, a phase 1 clinical study was conducted on bortezomib in combination with an autophagy inhibitor, hydroxychloroquine, for the treatment of multiple myeloma. CQ and hydroxychloroquine have been used as autophagy inhibitors in several studies.

Our data seem to be inconsistent with a previous report using breast cancer cells that autophagy did not affect WA-mediated cell death. Valid explanation is difficult at present, but it might depend on the difference of cell types used in the experiments, and particularly high sensitivity of leukemia/lymphoma cells to WA might be related to cell death accelerated by autophagy inhibition. Although there is no informative literature on whether autophagy induction by WA is related to its cytoidal effect, our study suggests that the combination of WA and autophagy inhibitors is a potential treatment option.

To the best of our knowledge, only a few reports indicate that WA has no significant effect on normal tissue, and there are no reports describing its effects on normal hematopoietic cells. We therefore compared the growth-suppressive effects of WA among human normal bone marrow CD34-positive cells, MDS-L cells and MDS92 cells. Unlike MDS-L cells, normal CD34-positive cells revealed neither an apparent growth-suppressive effect nor an apparent change of the cell cycle pattern (Fig. 6). In contrast, MDS92 cells (CD34-positive fraction) revealed a slight but less growth-suppressive effect than MDS-L cells. Taken together, the results suggest that more aggressive or malignant cell populations...
tend to show more damage with short-term exposure to WA. However, longer treatment with WA should be attempted to assess the cytotoxic effects on the normal hematopoietic system more precisely.

In addition to studies using normal hematopoietic cells, the in vitro effects of WA on primary leukemic cells should be checked before clinical trials are planned. In the present study, we examined only one AML case and found a subtle toxicity in human breast cancer cells. Curr Cancer Drug Targets 2013; 13: 640–50.

This study showed that WA has a unique growth-suppressive effect on leukemia/lymphoma cell lines but also suggested that the antitumor effect of WA itself is limited. Its use in combination with autophagy inhibitors or the other agents with different mechanisms of action may provide a basis for a novel therapeutic approach to the treatment of leukemia and MDS.

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Disclosure Statement
The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** The evaluation of primary leukemia cells treated with withaferin A (WA) by trypan blue.

**Fig. S2.** The morphological assessment of primary leukemia cells treated with withaferin A (WA) by May–Gruenwald–Giemsa staining.

**Fig. S3.** The cell cycle analyses by PI staining in primary leukemic cells treated with withaferin A (WA).

**Fig. S4.** Effect of withaferin A (WA) on apoptosis in primary leukemic cells.