Atractylodes macrocephala Koidz (A. macrocephula, also known as Baizhu) is an important ingredient in several traditional Chinese herb complexes for the treatment of abdominal pain and gastroenterology diseases for thousands of years. We previously demonstrated the induction of ROS-mediated apoptosis by methanol extract of A. macrocephula in human leukemia cells. After purification and assessment of those active compounds from A. macrocephula ethanol extracts, in this study, we focused on the major active compound, atractylenolide I (ATL-I). Through MTT assay and morphology observation, we found cytotoxic effect of ATL-I in human K562 chronic myeloblastic leukemia (CML), U937 acute myeloblastic leukemia (AML) and Jurkat T lymphoma cells. In addition, ATL-I-induced apoptosis was demonstrated by sub G1 and fragmented chromosomal DNA detection using flow cytometry, enzyme-linked immunosorbent assay (ELISA) and agarose electrophoresis. Finally, we found ATL-I also induced caspase-3 and caspase-9 activation through the detection of procaspase-3, procaspase-9 and caspase-3 substrate poly(ADP-ribose) polymerase (PARP) by immunoblotting. Interestingly, we found that ATL-I induced not only apoptosis but also differentiation, as upregulation of CD14 and CD68 surface markers and increase of phagocytosis ability were discovered in ATL-I-treated K562 CML and U937 AML cells. Our study thus suggests the potential of developing new leukemia therapies by using ATL-I for leukemia treatment in the future.

Abbreviations: AML, acute myeloblastic leukemia; A. macrocephula, Atractylodes macrocephala; ATL-I, atractylenolide I; CML, chronic myeloblastic leukemia; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IC50, growth inhibition concentration value; MFI, mean fluorescent intensity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OD, optical density; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered saline; PI, propidium iodide; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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group, which includes butyrates, herbimycin A, guanosine triphosphate (GTP) and everolimus, induces differentiation of CML into erythroid lineages.\textsuperscript{20,22–24} Despite the studies of these drugs for decades, so far ATRA is still the only one used in clinical therapy, even though it is known to be ineffective for many patients with complicated leukemia.\textsuperscript{20} Finding more effective drugs for leukemia therapies remains therefore an important and urgent issue.

In our previous study, we found induction of apoptosis and ROS production by methanol extract of \textit{A. macrocephula} in human leukemia cells.\textsuperscript{4} This apoptosis induction can be inhibited by ROS scavengers. After isolating the main components from these extracts, we focused on the major active component, ATL-I. In this study, we isolated and examined the effects of ATL-I from baizhu crude extracts correlated with apoptosis induction in human K562 CML, U937 AML and Jurkat T lymphoma cells. Our results showed that compound ATL-I induced not only apoptosis but also differentiation of macrophage lineage in human CML and AML leukemia cells.

\textbf{Effects of ATL-I on apoptosis in human leukemia cells:} Since we found apoptosis induction by methanol extract of \textit{A. macrocephula} in human leukemia cells in our previous study, we tried to purify and analyze pure compounds from the extract. We obtained the major compound ATL-I (Fig. S1A) from \textit{A. macrocephula} ethanol extracts as described in ‘Material and Methods’ (Supporting Information). We also confirmed that it is indeed ATL-I by \textsuperscript{1}H NMR, \textsuperscript{13}C NMR and 2D NMR Spectrum analysis (data not shown).

To test growth inhibition effect of ATL-I, human K562 CML, U937 AML and Jurkat T leukemia cells were incubated with 3.13–100 \mu g/mL ATL-I for 12–72 h, and then cell viability was evaluated by MTT assay. As shown in Figure S1, ATL-I effectively induces growth inhibition of U937 AML and Jurkat T leukemia cells. According to the results of MTT assay, the 50% growth

![Figure 1](image-url)

\textbf{Figure 1.} Induction of fragmented chromosomal DNA by ATL-I in human leukemia cells. (A and B) ATL-I-induced sub G1 chromosomal DNA formation in human leukemia cells. Jurkat T lymphoma (A), U937 AML and K562 CML cells (B) were left untreated, treated with DMSO, various concentrations of ATL-I (\mu g/mL) or 10 \mu M camptothecin (positive control) for 24 h (Jurkat and U937) or for 48 h (K562). Percentages of sub G1 cells were analyzed by flow cytometry. (C) Apoptotic cells with fragmented chromosomal DNA in cytosol as detected by the Cell Death Detection ELISAPLUS Kit. * \textit{p} < 0.05 (\textit{t}-test) as compared to untreated control.
Figure 2. Activation of apoptosis-related enzymes by ATL-I in human leukemia cells. (A) U937 and (B) Jurkat. Cells were left untreated, treated with DMSO, 6.25–50 µg/mL ATL-I for 24 h. Caspase 3, caspase 9, caspase 3 substrate PARP and Actin (as loading control) were assessed by Western blot analysis.

Figure 3. Induction of differentiation markers by ATL-I in human leukemia cells. (A) U937 and (B) K562. Cells were left untreated, treated with DMSO, different concentrations of ATL-I (µg/mL) as indicated, or 1 µM TPA (positive control) or 10 µM camptothecin (Cam, negative control) for 72 h. Cell surface markers CD14 and CD68 were detected and analyzed by flow cytometry. *P < 0.05 (t-test) as compared to untreated control.
Inhibition concentration (IC_{50}) values of ATL-I are 44.67, 37.63, 24.44 μg/mL for 12, 24, 48 h-treatment in Jurkat cells, and 24.11, 14.91, 4.23 μg/mL for 12, 24, 48 h-treatment in U937 cells, respectively. High doses of ATL-I also induce growth inhibition of K562 CML cells (Fig. S1B). To test whether growth inhibition of ATL-I causes cell death or growth arrest, we observed the morphology of these treated cells. As shown in Figure S2, ATL-I induces severe cell death. These data suggest growth inhibition effect of ATL-I is due to cell death induction in human K562 CML, U937 AML and Jurkat T lymphoma cells.

Since apoptosis induction by methanol extract of _A. macrocephala_ was found in our previous study, we wondered whether the major compound ATL-I is the ingredient responsible for inducing apoptosis. We first evaluated induction of fragmented chromosomal DNA by ATL-I using cell cycle analysis. As shown in Figure 1A–B, more than 50% of U937 and K562 cells show fragmented chromosomal DNA (sub G1 cell population) when cells were exposed to 50 or 100 μg/mL ATL-I. We also confirmed ATL-I induces apoptosis using detection of cytosolic DNA fragments (apoptosis) by enzyme-linked immunosorbent assay (ELISA) as described in ‘Materials and Methods’. As compared to untreated or dimethyl sulfoxide (DMSO, vehicle control) treatment cells, OD values are increased about two-fold in 6.25 μg/mL ATL-I treatment cells (Fig. 1C). To further confirm ATL-I-induced apoptosis, we directly detected fragmented chromosomal DNA from cells by DNA agarose electrophoresis, as shown in Figure S3. These data suggest ATL-I-induced apoptosis in human AML U937, Jurkat T and CML K562 cells.

Since apoptosis induction involves procaspase degradation and caspase activation inside cells, we next checked caspase status in ATL-I-treated cells by immunoblotting. As shown in Figure 2, we found obvious procaspase-9 and procaspase-3 degradation in ATL-I-treated AML U937 and Jurkat T cells. We also found obvious degradation of poly(ADP-ribose) polymerase (PARP), a well-known caspase-3 substrate, in 50 μg/mL ATL-I-treated cells. These data suggest at least the involvement of intracellular caspase-3 and 9 in ATL-I-induced apoptosis in human leukemia cells.

**Differentiation induction of human leukemia cells by ATL-I:** Since some anti-cancer drugs (e.g., lapatinib, everolimus and TPA) induce not only cell death but also differentiation in leukemia cells, we wondered the potential of differentiation induction by ATL-I. We first checked the monocyte/macrophage markers CD68 and CD14 by flow cytometry. As shown in Figure 3, ATL-I treatment effectively induces upregulation of CD14 or CD14/CD68 in K562 CML or U937 AML cells, similar to positive control TPA treatment. This is in drastic contrast to another anti-cancer drug, camptothecin, which did not induce upregulation of CD14 in both cells (Fig. 3). To further confirm induction of macrophage differentiation by ATL-I, we checked phagocytosis ability of cells after cells were incubated with fluorescent latex beads. As shown in Figure 4, mean fluorescent intensity (MFI) in ATL-I-treated cells shows about 50% increase (Fig. 4A, U937) or two-fold increase (Fig. 4B, K562) as compared to MFI in DMSO-treated cells. Similar to Figure 3, positive control TPA treatment, but without camptothecin treatment, also induces phagocytosis ability of U937 cells (Fig. 4A). This indicates induction of macrophage differentiation markers and phagocytosis ability in ATL-I-treated K562 CML and U937 AML cells.

_A. macrocephala_ (also called Baizhu) is traditionally used in treating gastroenterology diseases in Chinese herbal medicine. Our previous study indeed showed ROS-mediated apoptosis induction by _A. macrocephala_ methanol extracts. In this study, we focus on identifying the active components correlated with cytotoxicity and antitumor ability in our _A. macrocephala_ extracts. In line with this, we first purified the components of the extract, and found that the major component ATL-I from the extract induced both apoptosis and differentiation of macrophage lineage in K562 CML and U937 AML cells. According to our results about sub G1 DNA detection (Fig. 1A–B) and fragmented chromosomal DNA detection (Figs. 1C and S3), we found that the cytotoxicity of ATL-I is due to apoptosis induction in K562 CML, U937 AML and Jurkat T lymphoma cells. We also found the involvement of caspase-3 and caspase-9 activation during ATL-I-induced apoptosis, through the detection of procaspase-3, procaspase-9 and caspase-3 substrate...

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**Figure 4.** Induction of differentiation (phagocytosis ability) by ATL-I in human leukemia cells. (A) U937 and (B) K562. Cells were left untreated, treated with DMSO, different concentrations of ATL-I (μg/mL), or 1 μM TPA (positive control) or 10 μM camptothecin (Cam, negative control) for 4 d or 6 d (right panel of B). Cells were then incubated with fluorescent latex beads for 2 h and phagocytosis ability of cells was detected by flow cytometry. (Right panel) Mean fluorescent intensity (MFI) of cells was analyzed. *P < 0.05* (t-test) as compared to untreated control.
PARP degradation by immunoblotting after leukemia cells were treated with ATL-I (Fig. 2). Moreover, we confirmed ATL-I-induced differentiation of macrophage lineages according to the increase of CD14 and CD68 markers (Fig. 3) and increase phagocytosis (Fig. 4) after ATL-I treatment in K562 CML and U937 AML cells.

ATL-I-induced apoptosis was demonstrated at least in lung cancer, ovarian cancer and leukemia cells, similar to our study. However, to the best of our knowledge, this is the first report about ATL-I-induced apoptosis in accompany with differentiation in leukemia cells, like some anti-leukemia drugs induce both cell death and differentiation, as well as our positive control TPA. In addition, we also found induction of ROS production by methanol extract of A. macrocephula in our previous study. This phenomena correlates with upregulation of ROS production when U937 cells differentiating into phagocytic lineage cells. Interestingly, K562 CML cells can be differentiated toward either megakaryocytic or erythroid lineage. Our findings indicate that the ATL-I K562 cells differentiate toward megakaryocytic lineage.

In conclusion, our findings confirm induction of cytotoxicity, apoptosis and differentiation by ATL-I in human AML and CML leukemia cells. Further investigation of the detailed mechanisms about ATL-I-involvements could potentially lead to a new approach of using ATL-I in anti-leukemia treatment in the future.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.03.021.

References and notes