

APG-1252-12A induces mitochondria-dependent apoptosis through inhibiting the antiapoptotic proteins Bcl-2/Bcl-xl in HL-60 cells

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Abstract. Acute myeloid leukemia (AML) is the most common acute leukemia in adults. Despite improved remission rates, current treatment regimens for AML are often associated with a very poor prognosis and adverse effects, necessitating more effective and safer agents. B-cell leukemia/lymphoma 2 (Bcl-2) family proteins regulate apoptotic pathway that can be targeted with small molecule inhibitors. APG-1252-12A is a Bcl-2 homology (BH)-3 mimetic that specifically binds to Bcl-2 and Bcl-xl, which has shown efficacy in some Bcl-2 dependent hematological cancers. In this study, we investigated whether APG-1252-12A inhibits the growth of five leukemia cell lines in a concentration- or time-dependent manner by MTS assay. Following treatment of AML cell line HL-60 with this compound, cell apoptosis was detected using flow cytometry and nuclear condensation was observed after Hoechst 33258 dye. Immunoblotting for cytochrome c, cleaved caspase-3 and PARP-1 cleavage was used to demonstrate the mechanism of inducing mitochondria-dependent apoptosis by APG-1252-12A. Our findings showed that this new compound inhibited cell proliferation in five leukemia cell lines and induced apoptotic death. There was a link between the level of Bcl-2 protein and IC₅₀. APG-1252-12A targeted mitochondria

and induced caspase-dependent apoptosis by inducing the HL-60 cell cytochrome c released, PARP cleavage and caspase activation. These data suggested that APG-1252-12A is a candidate drug for the *in vivo* analysis and clinical evaluation in AML.

Introduction

Acute myeloid leukemia (AML) is a heterogenous hematological malignancy involving the clonal expansion of myeloid blasts in the bone marrow and peripheral blood with possible spread to liver and spleen. An estimated 19,950 people were newly diagnosed in 2016, 10,430 of whom will die from their disease (1). The 5-year survival rate for adult AML patients is only 26.6% based on 2006-2012 data, with a median age of 67 years at diagnosis (1). Overexpression of anti-apoptotic Bcl-2 proteins such as Bcl-2, Bcl-xl, and Mcl-1 occurs frequently in AML (2), which is widely associated with tumor initiation, progression, and drug resistance. Most AML patients become resistant to chemotherapy at some point in their course and succumb to their disease. Therefore, there is an urgent need to prevent chemoresistance or enhance chemosensitivity in a selective fashion to lead to a higher cure rate and a lower toxic burden.

Resisting cell death is a hallmark of cancer cells that contributes to tumour progression and to chemoresistance (3). Over the past three decades, over 16 members of the Bcl-2 family protein were identified and characterized (4). There are proapoptosis BH3-only proteins (such as Bim and Bad), proapoptosis multi-BH-domain proteins (such as Bak and Bax) and anti-apoptosis proteins (including Bcl-2, Bcl-xl, Mcl-1, Bfl1 and Bclw). The discovery of Bcl-2 started with a t(14;18) chromosomal translocations in human follicular lymphoma (5,6). This protein has since been shown to have a dominant role in the survival of multiple lymphoid malignancies (7,8). The pro-survival Bcl-xl protein, which was encoded by Bclx gene, was associated with drug resistance and disease progression of hematological malignancies (9). The dependence of AML

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cells on the anti-apoptotic Bcl-2 protein can be exploited for therapeutic effect using BH3 mimetics (10), a class of small molecules that mimic the inhibitory features of BH3-only proteins (11). Cancer cells have greater susceptibility to BH3 mimetic drugs than normal cells, partly because they often have higher levels of anti-apoptosis proteins and release more previously sequestered BH3-only proteins to activate Bax and Bak (12,13).

ABT-737 (14) and ABT-263 (15), both developed by Abbvie Laboratories, displace pro-apoptotic proteins from Bcl-2 and Bcl-xl and have synergistic toxicity with conventional chemotherapeutics and radiation. They require Bax for cell killing and causing MOMP in Bcl-2 dependent cancer cells (16,17), thus confirming an on-target effect. However, on-target thrombocytopenia caused by Bcl-xl inhibition limits the application of ABT-263. For the treatment of cancers that depend on Bcl-2, Bcl-2 selective inhibitor ABT-199 was created. ABT-199 does not reduce platelet lifespan and is better tolerated than ABT-263 (18). These mimetics have shown promising efficacy in various preclinical models and now in advanced clinical trials for chronic lymphocytic leukemia (CLL) and other malignancies (19-22).

Previously, our laboratory reported that small molecule Bcl-2 inhibitors ApoG2 and BM-1197 have potent antitumor effect on colorectal cancer cells (23,24). APG-1252, a new BH3 mimetic that binds to Bcl-2 and Bcl-xl with sub-nanomolar affinities ($K_i < 1$ nM) (25), was demonstrated with better *in vivo* antitumor activity than ABT-263 (26). APG-1252 achieved complete and long-term tumor regression in both H146 and H1963 SCLC xenograft models and avoid the commonly seen on-target toxicity when Bcl-xl is inhibited. APG-1252 converts into a more active metabolite APG-1252-12A (APG-1252-M1) *in vivo*. APG-1252-12A also binds with high affinity to Bcl-2 and Bcl-xl ($K_i < 1$ nM) and is over ten times more active than APG-1252 (25). Regardless, the anti-tumor effect and underlying mechanism of APG-1252-12A have not yet been evaluated in leukemia. Herein, we report our detailed investigation of APG-1252-12A in HL-60 cells. These data showed that APG-1252-12A induced mitochondria-dependent apoptosis thus warranting further investigation.

Materials and methods

Cells and reagents. The leukemia cell lines HL-60, MOLM-13, U937, THP-1 and MV4-11 were donated by State Key Laboratory of Oncology in South China. Cells were cultured in RPMI-1640 (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS-22A; Carpricorn Scientific GmbH, Ebsdorfergrund, Germany) and incubated at 37°C with 5% CO₂. APG-1252-12A was kindly provided by the Ascentage Pharma Group Corp Inc. (Taizhou, China) and was dissolved in pure dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) with a stock concentration of 40 mmol/l, stored at -20°C, and diluted in the corresponding culture medium just before use.

Cell viability assay. Cell proliferation was determined by CellTiter 96Aqueous MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) assay. AML cell lines were seeded 10,000-50,000

Table I. The IC₅₀ values (μ M) of APG-1252-12A in five AML cell lines.

Cell lines	24 h	48 h	72 h
HL-60	5.35±1.04	1.52±0.30	1.40±0.43
MOLM-13	0.15±0.06	0.14±0.03	0.04±0.01
MV4-11	0.19±0.09	0.02±0.02	0.03±0.00
THP-1	1.09±0.45	1.06±0.08	0.12±0.01
U937	16.61±1.93	2.22±0.34	1.23±0.23

Data are the mean \pm SEM of three independent experiments.

onto 96-well plates containing 200 μ l of culture medium per well and treated with APG-1252-12A of serial concentrations for 24, 48 and 72 h, respectively. After that, 40 μ l of MTS (Promega, Madison, WI, USA) was added to each well and reacted for another 4 h at 37°C. Then the absorbance value was measured with a spectrophotometer at 490 nm. Cell viability was expressed as mean \pm SD of absorbance and analyzed with nonlinear regression on GraphPad Prism version 6.0. The values were performed in triplicate as a percentage relative to those obtained in untreated controls.

Apoptosis detection with nuclear staining. The morphological assessment of apoptotic HL-60 cells was detected by Hoechst 33258 (Beyotime Institute of Biotechnology, Jiangsu, China) staining. Cells (20,000) were plated in each of 6-well plate and incubated with various concentrations of APG-1252-12A and 0.1% DMSO for 24 h. The staining was performed according to the manufacturer's protocol. The morphological features of apoptosis were observed by fluorescence microscope (Olympus, Tokyo, Japan).

Flow cytometry analysis of apoptosis and cell cycle. Cell apoptosis was determined with an Annexin V-propidium iodide (PI) apoptosis detection kit (KGA108; KeyGen Biotech, Nanjing, China) by flow cytometry (Beckman Coulter, Fullerton, CA, USA). Cells (200,000) were seeded into each well of a 6-well plate and treated with indicated concentration of drug or DMSO for 24 or 48 h. After treatment, cells were harvested and washed twice with phosphate buffered saline (PBS), and resuspended in 500 μ l binding buffer containing 5 μ l Annexin V FITC and 5 μ l propidium iodide (KeyGen Biotech). Experiments were analyzed after incubating out of light in the staining solution for 10 min.

Flow cytometry was performed to analyze cell cycle position. After treatment, cells were collected, washed and fixed in 70% cold ethanol at 4°C overnight. Next, the cells were incubated with RNase for 30 min at 37°C, then stained with PI (Cell cycle Detection kit, KeyGen) in the dark at 4°C for another 30 min. Cells were analyzed with a FACS Calibur flow cytometer (Beckman Coulter), and the data were analyzed using ModFit LT 3.2 software.

Western blot analysis. Cells were lysed with 1X Cell Lysis Buffer (#9803; Cell Signaling Technology, Danvers, MA, USA), and protein concentration was measured with the Pierce BCA

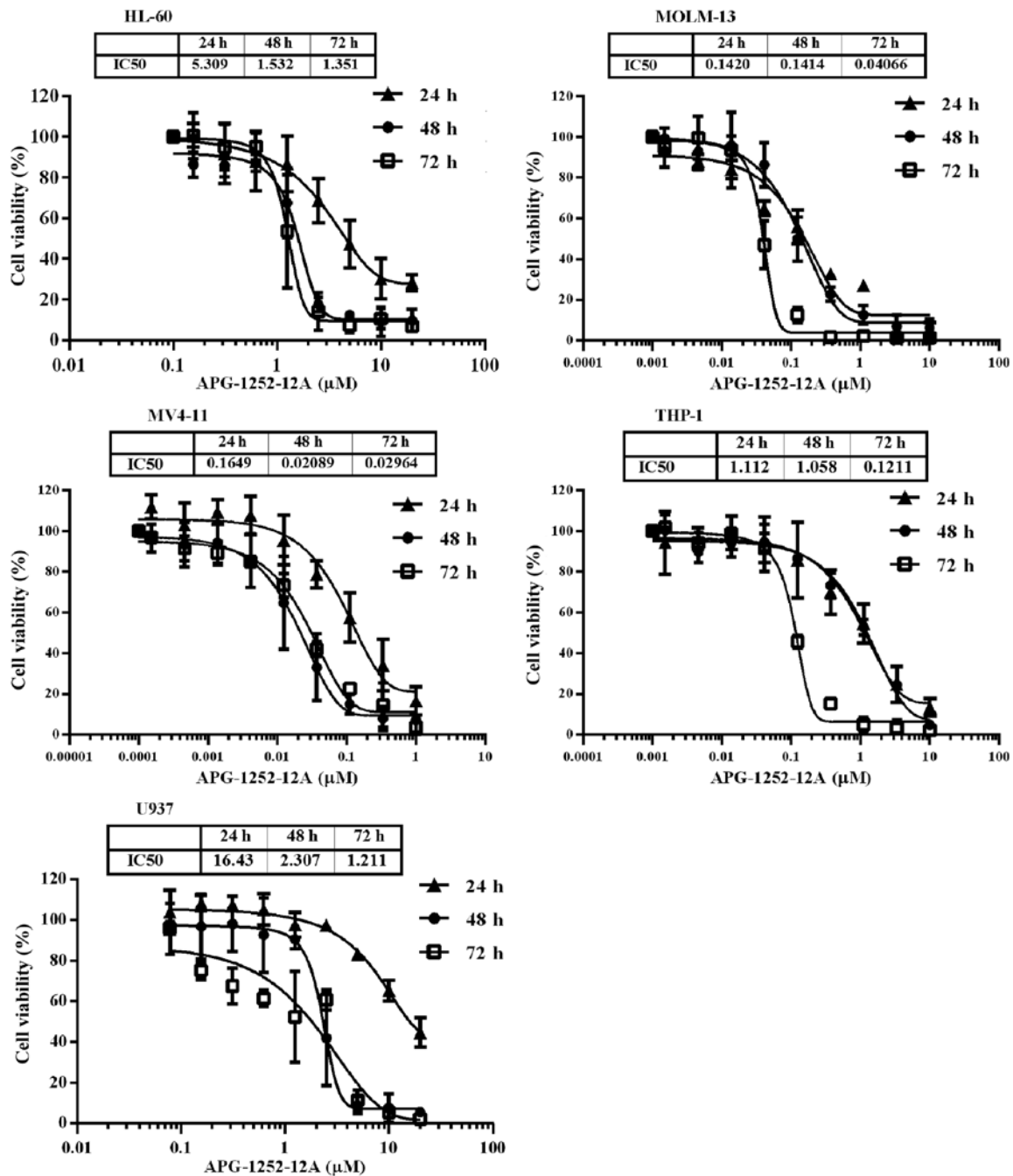


Figure 1. Anti-proliferative activity of APG-1252-12A in leukemia cell lines. MTS assays were performed using five cell lines: HL-60, MOLM-13, MV4-11, THP-1, and U937. The cells were treated with APG-1252-12A at various concentrations and 0.1% DMSO served as control group for 24, 48 and 72 h. The data are presented as the means of three independent experiments, with error bars indicating the standard deviation (SD).

protein assay kit. Total cell lysates were extracted and separated by electrophoresis in 8-15% SDS-polyacrylamide gel and transferred to PVDF membranes (Roche, Basel, Switzerland). Following blockage in 5% non-fat milk, PVDF membranes were incubated with anti-Mcl-1 (94296), Bcl-2 (4223), Bcl-x1 (2764), anti-Bax (2772), anti-Bak (6947), anti-cleaved caspase-3 (9661), anti-caspase-3 (9665), anti-cytochrome c (4272), GAPDH (2118), anti- β -actin (4970, Cell Signaling Technology) or anti-PARP-1 (sc-7150), anti-Bim antibody (sc-374358; Santa Cruz Biotechnology Santa Cruz, CA, USA). The secondary anti-mouse (sc-2005) and anti-rabbit (sc-2004) antibodies were purchased from Santa Cruz Biotechnology. Antigen-antibody

complexes were detected using Bio-Rad Clarity™ western ECL substrate and protein level were quantified by Image Lab (Bio-Rad Laboratory, Hercules, CA, USA).

Mitochondrial cytochrome c release assay. HL-60 cells were pretreated with 2.5 μ mol/l of APG-1252-12A for 6 h. Cytoplasmic fractionation was isolated using the Cytosol/Mitochondria Fractionation kit (#QIA88; Merck Millipore, Darmstadt, Germany). Following the kit recommendations, cytosolic fractions were isolated from HL-60 cells. The amount of cytochrome c in cytosol fraction was determined by western blot analysis as described above.

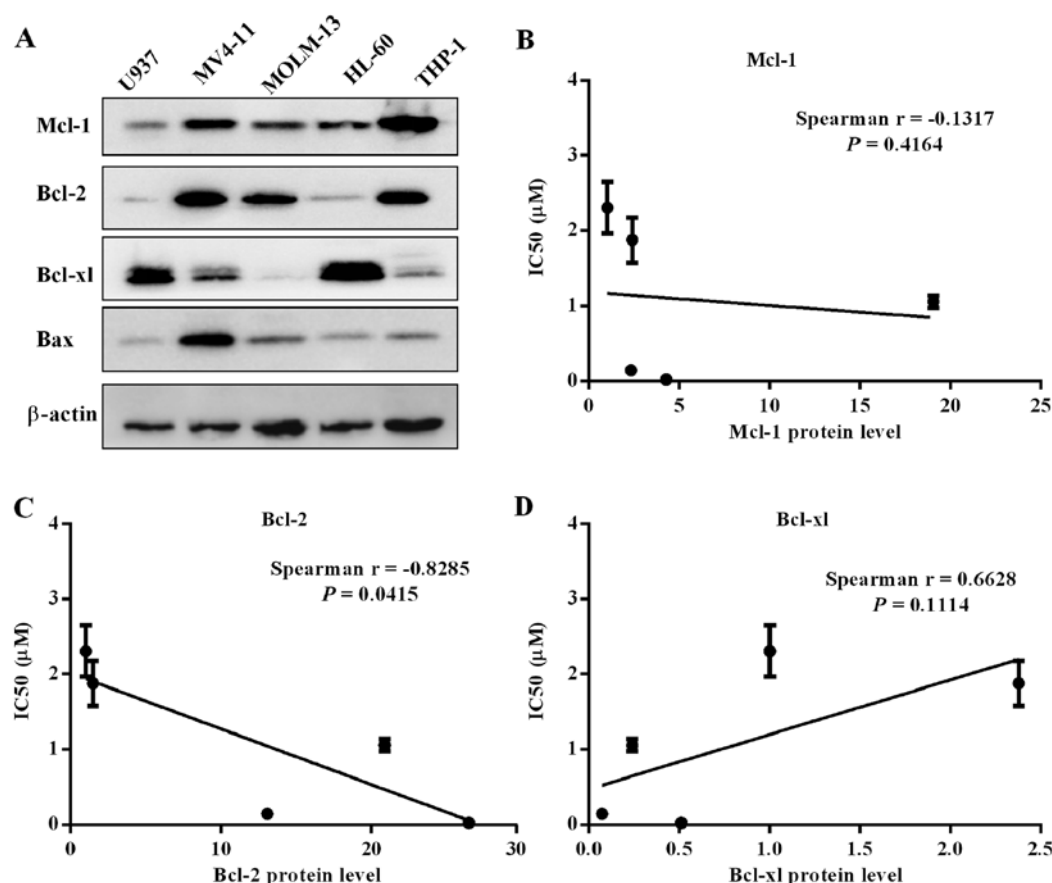


Figure 2. Endogenous Bcl-2 family protein level in five leukemia cell lines. (A) Western blot analysis of Bcl-2 family proteins in untreated leukemia cells. The band intensity was quantified with Image Lab software, and displayed numerically as a ratio of the band intensity detected in the U937 cells. (B-D) The non-parametric one-tailed Spearman's test was used to determine the correlation coefficient between IC_{50} values and protein expression. The P-values provided are nominal P-values not corrected for multiple comparisons. Significant correlations were observed between APG-1252-12A IC_{50} values and Bcl-2 protein levels.

Statistical analysis. IC_{50} values were calculated by non-linear regression analysis with GraphPad Prism software v6.0 (GraphPad Software, La Jolla, CA, USA). The results were expressed as the mean \pm standard error of mean (SEM) from at least three independent experiments. One-way analysis of variance (ANOVA) was used to compare the means between groups by SPSS 20.0 software. Differences in P-value <0.05 were considered statistically significant.

Results

APG-1252-12A inhibits growth potently in five leukemia cell lines. To test the potential utility of APG-1252-12A in leukemia, we exposed five leukemia cell lines with increasing concentrations of APG-1252-12A for 24, 48 and 72 h and then determined the IC_{50} values. The viability of these cell lines after treatment decreased significantly in a time- and dose-dependent manner (Fig. 1). The IC_{50} of APG-1252-12A ranged from <100 nM to >1000 nM and MV4-11 was the most sensitive cell line (Table I).

Bcl-2 family protein level in five leukemia cell lines. To further clarify the on-target action of cell killing via selectively binding with Bcl-1/Bcl-xl, we analyzed whether there were correlates of cell line sensitivity to APG-1252-12A. The expression of three Bcl-2 family proteins were determined by western blot

analysis (Fig. 2A). Spearman's analysis was performed to assess correlation between IC_{50} values and protein expression. The expression level of Bcl-2 correlated with sensitivity to the drug, while levels of Bcl-xl and Mcl-1 had no correlation with the drug sensitivity (Fig. 2B-D). The MV4-11 and MOLM-13 cells with high levels of Bcl-2 protein and relatively low Bcl-xl expression were more insensitive to APG-1252-12A. High expression of Bcl-xl in HL-60 and U937 cells might explain the killing mechanism of targeting Bcl-xl. THP-1 cell line had high level of Bcl-2 and Mcl-1 as well as relatively low level of Bcl-xl which supported that sensitivity to APG-1252-12A was correlated with Bcl-2 protein level.

APG-1252-12A induces apoptosis in HL-60 cells. Hoechst 33258 staining and flow cytometry were used to evaluate APG-1252-12A inducing apoptosis in HL-60 cells. Increased apoptosis was shown by analysis of nuclei changes with the electron microscopic analysis (Fig. 3A). The apoptotic bodies and nuclear fragments were stained light blue, and the normal cells were stained blue. The nuclei of the cells appeared normal, round and large with regular contours in the control groups. Cells with smaller nuclei and condensed chromatin were rare. By contrast, the treated cells showed strong morphological alterations such as nuclear shrinkage, intense fluorescence of nuclei and nuclear fragmentation. Apoptosis detection by Annexin V and PI staining showed

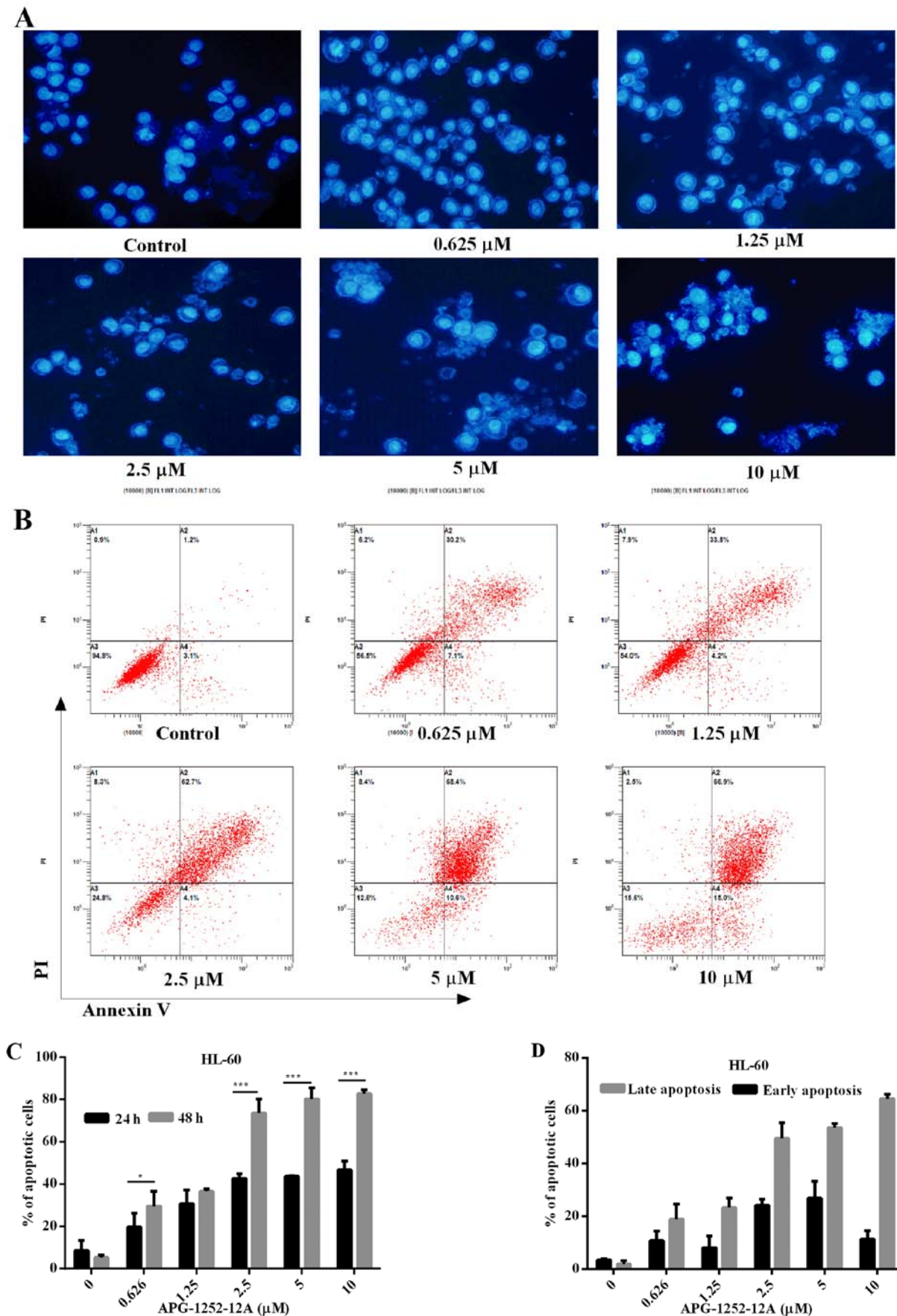


Figure 3. APG-1252-12A induces apoptosis in HL-60 cells. (A) HL-60 cells were stained with Hoechst 33258 and examined under fluorescent microscope after incubating APG-1252-12A for 24 h. (B) Representative results showing HL-60 cells in apoptosis after treatment without or with APG-1252-12A for 48 h. Flow cytometric dot plots of Annexin V/FITC and PI staining. (C) Statistical analysis of the proportion of apoptotic cells. HL-60 cells were incubated with increasing concentrations of APG-1252-12A for 24 or 48 h. (D) Distribution chart of early and late apoptotic HL-60 cells after 48 h treatment of APG-1252-12A. The data are presented as the means of three independent experiments, with error bars indicating the standard deviation (SD). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

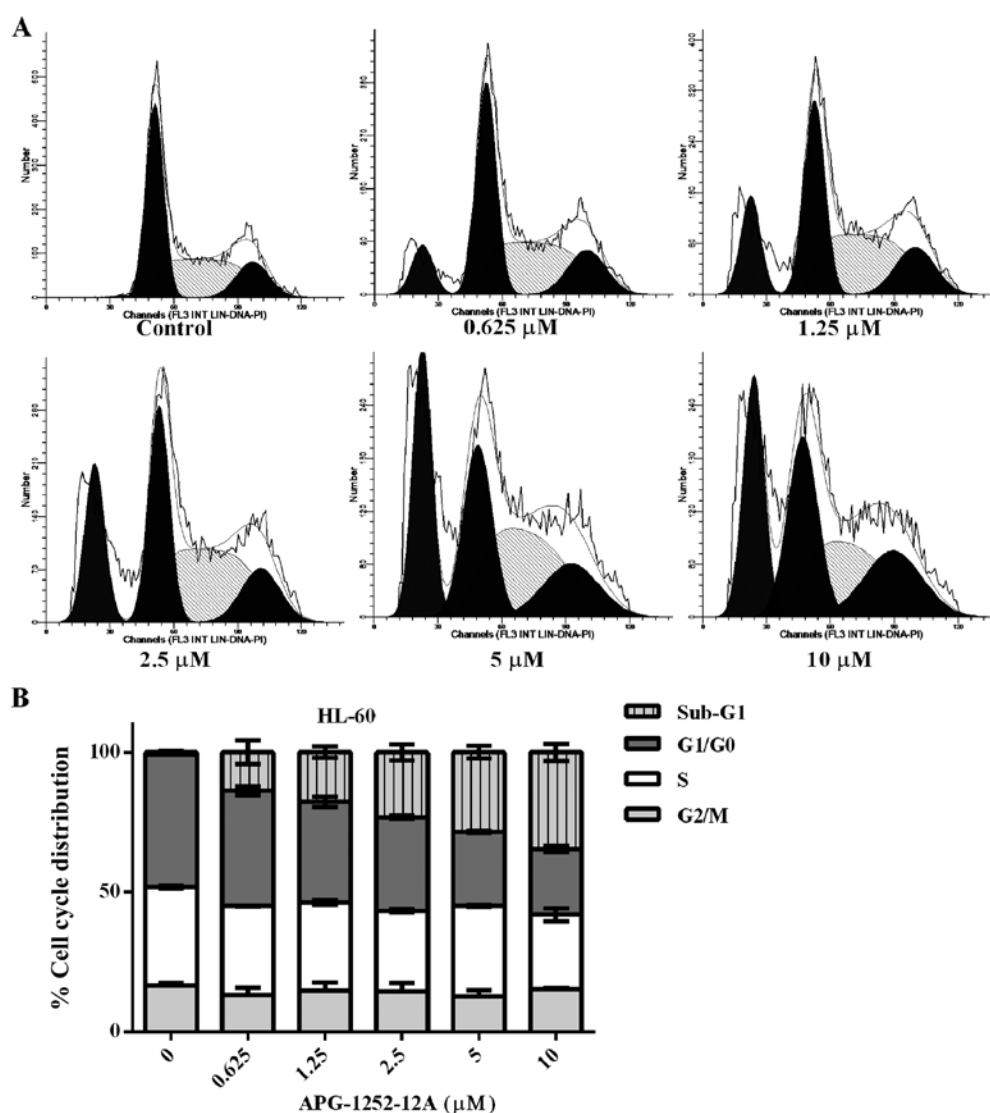


Figure 4. Cell cycle changes in HL-60 cells treated with the indicated concentrations of APG-1252-12A for 24 h. Histograms show the percentage of HL-60 cells in Sub-G1, G1, S, and G2/M phases in three independent experiments.

that when treated with APG-1252-12A alone, dramatic increase of Annexin V positive cells was seen in HL-60 cells (Fig. 3B). Flow cytometry also indicated that treatment with increasing concentrations of the drug resulted in a significant decrease of cell counts and induced apoptosis in a dose-dependent manner. Time course analysis of cells exposed to APG-1252-12A (10 μ mol/l) revealed approximately 47% cell death at 24 h, and substantially more pronounced lethality after 48 h (83%, Fig. 3C). An early and late apoptotic cell distribution chart shows more late stage HL-60 apoptotic cells than early stage after 48 h treatment of APG-1252-12A (Fig. 3D).

The sub-G1 phase increases after APG-1252-12A treatment. Cell cycle analysis of the propidium iodide stained DNA was performed in HL-60 cells. The percentage of cells in sub-G1 fraction increased significantly, pointing to APG-1252-12A-induced cell death and DNA fragmentation (Fig. 4). Treatment with increasing concentrations of APG-1252-12A resulted in a significant increase in the percentage of cells

in the sub-G1 phase. The remaining living cells showed no significant increase in the percentage of cells in the S phase of the cell cycle and the percentage of cells in the G1 and G2/M phase showed similar results. No statistically significant correlation between APG-1252-12A sensitivity and cell cycle was found.

Effect of APG-1252-12A on Bcl-2 family protein expression in HL-60 cells. To further investigate the effect of APG-1252-12A on the protein expression level of Bcl-2 family members, we chose HL-60 for analysis. After treating with serial concentrations of APG-1252-12A for 24 h, there were no significant alternations in the protein expression of Bcl-2 family except the suppression of Mcl-1 in HL-60 cells (Fig. 5A and D). A time course of Bcl-2, Bcl-xl, Bak and Bax protein levels showed that the APG-1252-12A treatment in HL-60 cells did not change their expression levels (Fig. 5B).

APG-1252-12A promotes cytochrome c release in HL-60 cells. We also observed that cell death was induced by APG-1252-12A

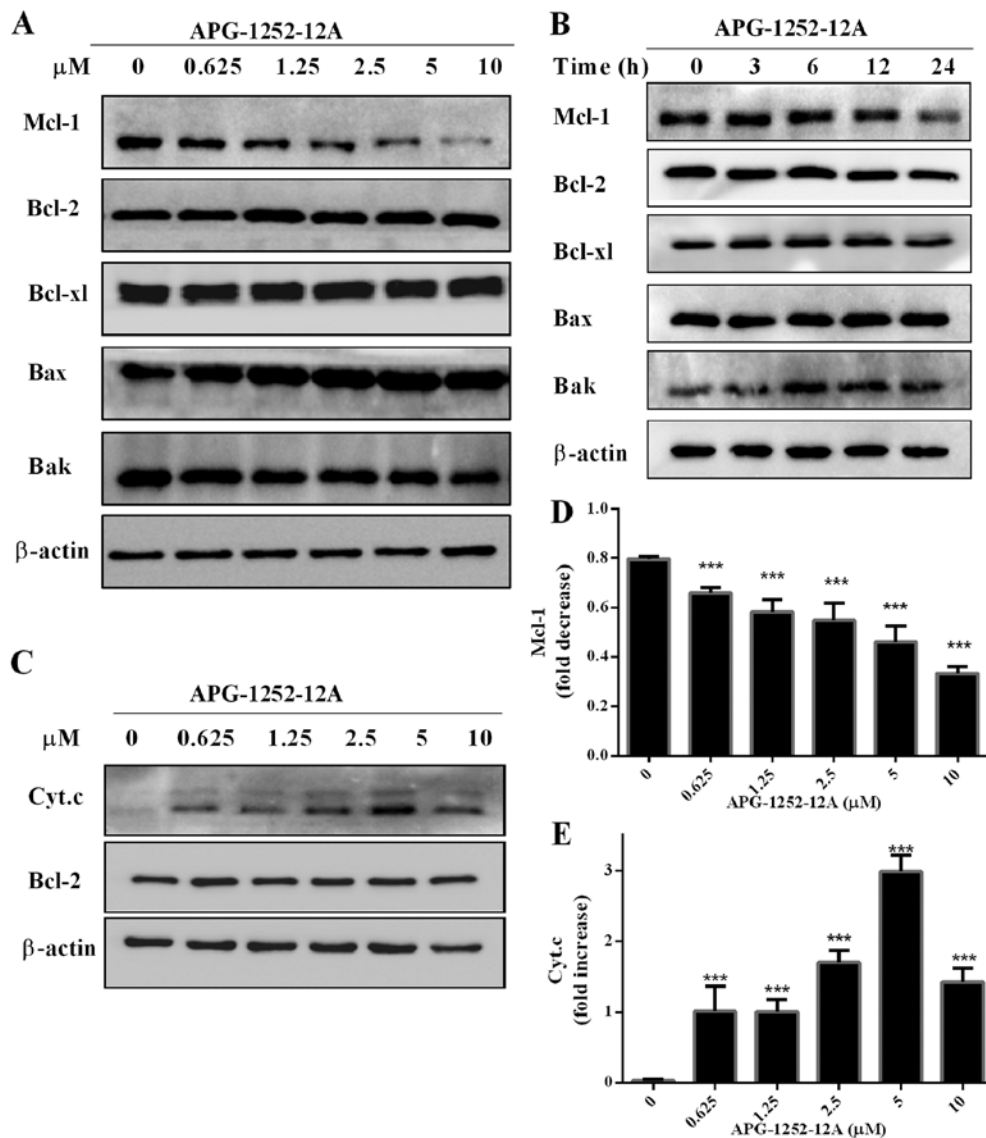


Figure 5. Effect of APG-1252-12A on HL-60 cells. (A) Bcl-2 family protein levels of Mcl-1, Bcl-2, Bcl-xL, Bax and Bak were examined by western blot analysis. HL-60 cells were treated with serial concentration of APG-1252-12A for 24 h. (B) Cells were treated with APG-1252-12A (1.25 μ M) for the indicated time points and whole cell extracts were analyzed by immunoblotting. (C) HL-60 cells were treated APG-1252-12A for 6 h and cytosolic fraction was isolated for western blot analysis. (D) Mcl-1 expression in (A) was quantified by Image Lab and normalized to β -actin. (E) The relative expression of cytochrome c in (C) was normalized to β -actin. Error bars represent the standard deviation of three independent experiments. (* P <0.05, ** P <0.01, *** P <0.001 APG-1252-12A treated group vs. control).

that underwent cytochrome c release (Fig. 5C and E). Cytoplasmic cytochrome c level was detected by western blot analysis. APG-1252-12A induced cytochrome c release at a concentration of <0.625 μ mol/l.

APG-1252-12A induces caspase-3 activation in HL-60 cells. Western blot analysis was performed with antibodies against PARP-1, caspase-3, and cleaved caspase-3. APG-1252-12A led to increase of cleaved PARP, cleaved caspase-3 and decrease of caspase-3 in HL-60 cells in a concentration-dependent manner (Fig. 6A-D). The marked cleavage/activation of caspases-3 and PARP-1 in HL-60 pronounced the loss in mitochondrial membrane potential (MOMP). These findings indicated that inhibition of anti-apoptotic Bcl-2 and Bcl-xL caused MOMP, promoting cytochrome c release followed by caspase activation.

Discussion

As multiple studies have implicated the role of Bcl-2 family proteins in AML pathogenesis and prognosis (27-29), small molecule BH3-mimetics that inhibit the anti-apoptotic functions of Bcl-2 and Bcl-xL have been developed (15,18). In clinical trials, ABT-263 significantly reduced tumour burden in most patients with CLL as a single agent or in combination with other conventional treatments (30,31). Although thrombocytopenia limited the use of ABT-263 in patients, the observed efficacy underscored the therapeutic potential of selective Bcl-2 family inhibitors. ABT-199 was also investigated as a single agent or in combination with other anti-cancer therapies for CLL (20,32). Achievement of the primary end point in the study led to the first successful US Food and Drug Administration (FDA) registration for

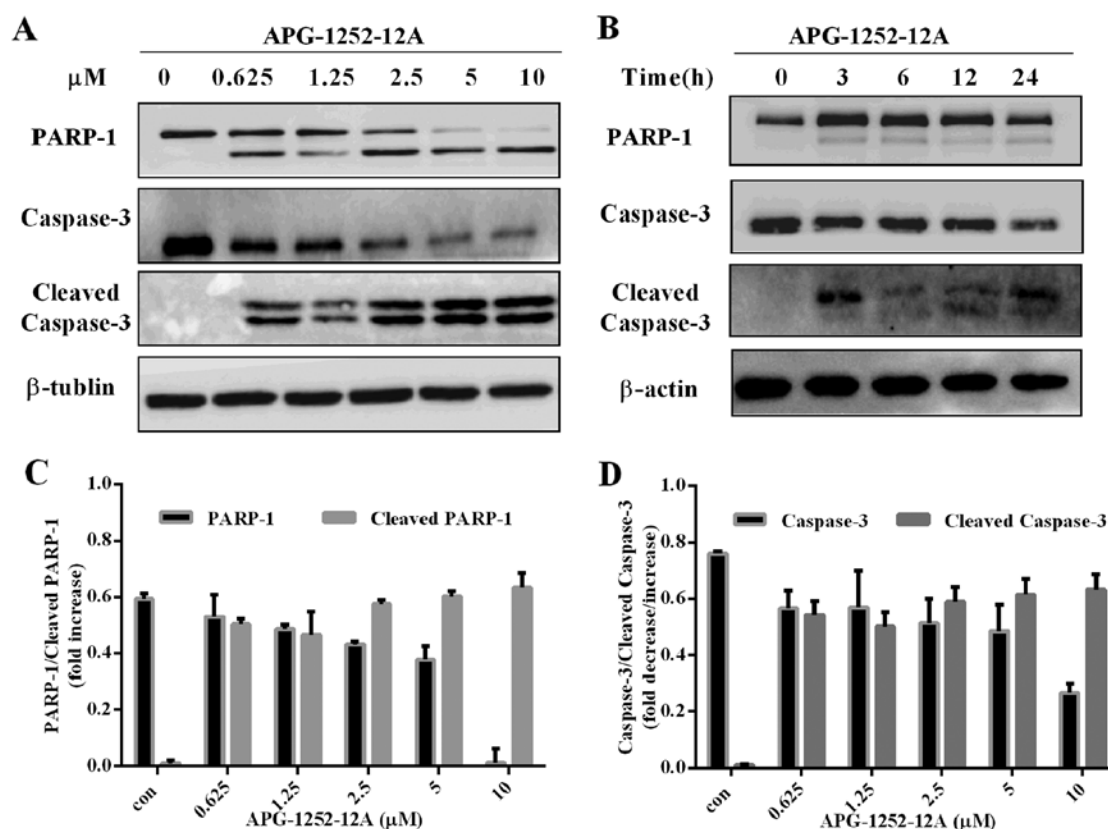


Figure 6. APG-1252-12A induces apoptosis associated with mitochondria-dependent pathway. (A) Western blot analysis of PARP-1, caspase-3 and cleaved caspase-3 in APG-1252-12A-treated HL-60 cells. (B) HL-60 cells were treated with APG-1252-12A (1.25 μ M) for 0, 3, 6, 12 and 24 h and were analyzed by immunoblotting. (C) Relative changes of PARP and cleaved PARP were examined in HL-60 cells. (D) The relative expression of caspase-3 and cleaved caspase-3 in APG-1252-12A-treated HL-60 cells were normalized to β -actin.

ABT-199. Bai and colleagues (25) identified APG-1252-12A as an active metabolic product of APG-1252 *in vivo*, and bound to Bcl-2 and Bcl-x1 with sub-nanomolar affinities. In this study, we tested the impact of Bcl-2/Bcl-x1 dual inhibitor APG-1252-12A in five leukemia cell lines. The results of MTS assay showed that application of APG-1252-12A to leukemia cell lines significantly inhibited cell proliferation. The IC_{50} was in low nanomolar range, a range might be achievable in clinical trials. In light of this observation, it is possible that the level of Bcl-2 family members might be related to sensitivity to APG-1252-12A.

We investigated whether the Bcl-2 protein level in five leukemia cell lines was associated with the sensitivity to APG-1252-12A. Leukemia cell lines express Bcl-2 with varied expression of Bcl-x1. Though ABT-737 bound to Bcl-2 and Bcl-x1 proteins with similar affinities (14), it was surprising that the level of Bcl-x1 expression did not correlate with the sensitivity of AML cells to APG-1252-12A. The levels of Bcl-x1 and Mcl-1 had no correlation with the drug sensitivity. It was found that increased expression of Bcl-2 was associated with increased sensitivity to Bcl-2 inhibitor which was similar to those previously reported (10). The Bcl-2 protein level correlated with cell line sensitivity to APG-1252-12A suggested an on-target action of killing. The role of Bcl-2 in the survival of tumor cells is well established, so the drugs that inhibited these proteins might be useful therapeutically.

Herein, we demonstrated that HL-60 cells treated with APG-1252-12A developed an accumulation of apoptotic

cells. The drug acted in a concentration- and time-dependent manner. Hoechst staining and flow cytometry analysis of APG-1252-12A-treated HL-60 cells suggested the occurrence of apoptosis. Furthermore, the effect of varying concentrations of inhibitors on cell cycle distribution was determined by flow cytometric analysis. The increase of the sub-G0/G1 phase of cells indicated typical late stages of apoptosis.

Studies in cell lines and primary cells have revealed that high expression of all anti-apoptotic Bcl-2 family members, Bcl-2, Bcl-x1, Bcl-w, A1 and Mcl-1, were capable of inhibiting the mitochondrial apoptotic pathway (33). We also investigated the mechanism of antitumor activity of APG-1252-12A. Our findings are consistent with the above studies that administration of APG-1252-12A to HL-60 cells rapidly induced hallmarks of apoptosis, including cytochrome c release, caspase-3 and PARP-1 activation. Normally, releasing cytochrome c from mitochondria to the cytoplasm is a critical signal of caspase activation (34-36). The occurrence of cytochrome c release suggested that APG-1252-12A might induce in AML a form of apoptotic cell death that can include caspase activation as an essential pathway. As MOMP and cytochrome c release are usually viewed as characteristics of no return in apoptosis, APG-1252-12A achieves a potent cell killing effect in AML cell line. The Bcl-2 anti-apoptotic members are helical proteins with an open groove that binds to the BH3 domain on the proapoptotic partner. The anti-apoptotic Bcl-2-like proteins provide a barrier against MOMP by binding proapoptotic BH3-only protein (such as Bim and Bid) and keeping multidomain target (Bax

and Bak) in an inhibited state (37,38). BH3 mimetic compounds, such as ABT-737 and ABT-263, binds to anti-apoptotic Bcl-2 family proteins and liberates proapoptotic BH3-only proteins. The proapoptotic BH3-only protein stimulate apoptosis not only by binding anti-apoptotic Bcl-2-like proteins to release Bax and Bak but also by directly activating Bax and Bak. Previous discoveries revealed that Bax and Bak have important roles in unleashing the effector phase of mitochondrial apoptosis and must change shape to cause MOMP and apoptosis (39-41).

The ability of APG-1252-12A to induce HL-60 mitochondrial apoptosis was confirmed *in vitro*. These findings have some implications for the investigation of APG-1252-12A, suggesting that inhibiting Bcl-2 and Bcl-xL protein could activate the intrinsic apoptotic pathway. Our work might provide a foundation for studies in APG-1252-12A as a single agent *in vivo* which can be exploited as a potential therapeutic drug in AML.

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