MAP30 inhibits autophagy through enhancing acetyltransferase p300 and induces apoptosis in acute myeloid leukemia cells

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Abstract. Momordica anti-human immunodeficiency virus protein of 30 kDa (MAP30) has been shown to exhibit potent antitumor activities against several solid tumors. In the present investigation we demonstrated that MAP30 significantly inhibited the proliferation of acute myeloid leukemia (AML) HL-60 and THP-1 cell lines and patient AML cells through autophagy inhibition and apoptosis induction. Intriguingly, MAP30-induced cell death and apoptosis were partially rescued in combination with an autophagy activator rapamycin, and aggravated in combination with an autophagy inhibitor bafilomycin A1 in HL-60 cells, suggesting that autophagy is a pro-survival signal and its inhibition contributes to the induction of apoptosis in MAP30-induced cell death. Further mechanism analysis demonstrated that MAP30 enhanced p300, and C646, a selective inhibitor of p300, markedly promoted autophagy and partially rescued the MAP30-induced cell death in HL-60 cells and patient AML cells. Collectively, our findings suggest that apoptosis and autophagy act cooperatively to elicit MAP30-induced cell death and MAP30 may be a potential antitumor drug candidate against AML.

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Introduction

Acute myeloid leukemia (AML) is the most common type among adults, accounting for the largest number of annual deaths due to leukemia. Despite key advances in diagnosis and treatment having made the disease more treatable and curable, 5-year overall survival rate of patients with AML is still approximately 30% (1-3). The standard induction therapy for AML has changed little over the decades and includes cytarabine in combination with an anthracycline as primary therapy, which can acquire a high rate of complete remission, however, most patients eventually relapse (4). Furthermore, these chemotherapy drugs kill cancer cells as well injure normal cells. Therefore, the development of effective drugs against AML with relatively low cytotoxicity is highly desirable. Targeting metabolic processes has been revealed as a promising approach for cancer therapy (5,6).

Momordica anti-human immunodeficiency virus protein of 30 kDa (MAP30) is extracted from *Momordica charantia*, which is a vegetable widely distributed in Asia and Africa. MAP30 inactivates ribosiome through depurination of the adenine base at position 2543 of 28S ribosomal RNA and the resulting inhibition of ribosomal protein synthesis in cells (7,8). As a ribosome-inactivating protein, MAP30 has been reported to exert potent inhibitory effects and induce apoptosis on several solid tumor cells (9-12). Whether MAP30 exerts similar effects on malignant hematological cells remains unknown.

Except for apoptosis, autophagy also plays an important role in tissue homeostasis, development, and disease. Autophagy is an adaptive and protective cellular response to nutrient deprivation, growth factor withdrawal, or metabolic stress to sustain cellular homeostasis and recycle damaged cytoplasmic organelles (13). This dynamic process is characterized by the formation of double-membrane vesicles called autophagosomes, which sequester cytoplasmic organelles or long-lived proteins targeted for destruction and fuse with lysosome for succeeding degradation (14). However, the role of autophagy in cancer is complicated and depends upon tumor subtypes, stages of tumor progression, cellular context or drugs

that cause this process (6,15). Autophagy can exhibit tumor suppressive activities through the annihilation of oncogenic protein substrates and toxic unfolded proteins (16,17), and alternatively, it may exhibit tumor-promoting activities in established cancers by maintaining cellular metabolism through intracellular recycling when nutrients are limiting (15,18,19). In addition, autophagy is implicated in other important aspects of hematological malignancies as it improves immune competence and antitumor immunity, and may even help to heighten patients' tolerance to standard treatments (20). Mounting evidence has demonstrated that autophagy can be regulated by several epigenetic modifications including acetylation, phosphorylation, and ubiquitylation, and importantly, an increase of acetylated level can inhibit the autophagic flux and turnover of long-lived proteins (21-26). Therefore, a detailed investigation is needed for the role of autophagy and the underlying mechanisms according to cancer type.

Here, we present data demonstrating that MAP30 effectively inhibits and kills the AML cells HL-60 and THP-1 as well as primary AML cells. We provide compelling evidence that MAP30 induces apoptosis in a caspase-dependent manner, and attenuates cytoprotective autophagy by increasing the level of histone transacetylase p300.

Materials and methods

Reagents and antibodies. MAP30 was a gift from School of Medical Laboratory Science, Chengdu Medical College. N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), rapamycin and bafilomycin A1 (baf A1) were purchased from Selleck Chemicals (Houston, TX, USA), and C646 was purchased from Sigma-Aldrich (St. Louis, MO, USA), which were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO used did not exceed 0.1%, which had no adverse effect on the cell cultures. The antibodies against LC3, p62, beclin 1, caspase-3, caspase-8, caspase-9, PARP, survivin, Bcl-2, Bax, GAPDH, acetylated-histone H3, or histone H3 were provided by Cell Signaling Technology (Beverly, MA, USA).

Cell culture and patient samples. HL-60 and THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (both from Gibco, Grand Island, NY, USA) at 37˚C in 5% CO_2. Primary AML cells were isolated from bone marrow aspirates of five newly diagnosed and untreated patients with AML [M3, 1; M4, 4; the diagnosis and classification was established according to the world health organization criteria (27)] using Ficoll-hypaque, and cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum at 37˚C in 5% CO_2. All protocols and experiments were approved by The First Affiliated Hospital of Wenzhou Medical University Institutional Review Board for clinical experiments and use of human samples; written consents were obtained from all subjects participated in this study in accordance with the Declaration of Helsinki protocol.

Cytotoxicity assay. A Cell Counting Kit-8 (CCK-8) was used to assess the cytotoxicity of MAP30 on AML cells according to the manufacturer's protocol (Dojindo, Kumamoto, Japan). Briefly, AML cells were diluted and seeded at a density of $4x10^3$/well in 96-well plates. The cells were subsequently exposed to MAP30 at different concentrations for 48 and 72 h. Then, the CCK-8 was added, and absorbance (A) was measured at 450 nm using an ELISA reader (ELx800; Bio-Tek Instruments, Winooski, VT, USA). Cell viability rate (%) = $A_{450, MAP30}/A_{450}$, control x 100%.

Table I. The sequences of the primers used for real-time qPCR.

	Primer
p300	F:5'-AGGCTGTATCAGAGCGTAT-3'
	R:5'-TGCTTTCATTGCTGGTGT-3'
GAPDH	F:5'-ATCATCAGCAATGCCTCC-3'
	R:5'-CATCACGCCACAGTTTCC-3'

F, forward; R, reverse.

Apoptosis assay. Apoptosis was determined using the Annexin V-FITC/propidium iodide (PI) Detection kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) according to the manufacturer's instruction. Briefly, after treatment with MAP30 for 48 h, the cells were collected rapidly and washed by cold PBS and subsequently incubated with FITC-labeled Annexin V and PI for 15 min at room temperature in the dark and then analyzed with CellQuest software on a flow cytometry (FACSCalibur; BD Biosciences, Mountain View, CA, USA).

Western blot analysis. After treatment with or without MAP30 at different concentrations, the cells were collected and lysed immediately using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with PMSF and Halt protease and phosphatase inhibitor cocktail (Pierce, Rockford, IL, USA). The protein was boiled for 5 min in 1X loading buffer and subjected to western blot analysis according to our previously described method (28). Bands were visualized by enhanced chemiluminescence reagents (Thermo Fisher, Fremont, CA, USA) and the optical densities of the bands were analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Quantitative real-time PCR for gene expression analysis. Quantitative real-time PCR was used to evaluate the expression of p300 and a reference gene GAPDH according to our previously described method (29). The sequences of specific primers see Table I.

Statistical analysis. The data are presented as mean ± SEM and analyzed by one-way ANOVA followed by a post hoc Turkey's test to determine the differences between the groups. Differences were considered significant at $P<0.05$.

Results

MAP30 inhibits proliferation and induces apoptosis in AML cells. We first examined the effects of MAP30 on the proliferation of AML cell lines HL-60 and THP-1, and primary AML cells. As shown in Fig. 1A, MAP30 inhibited

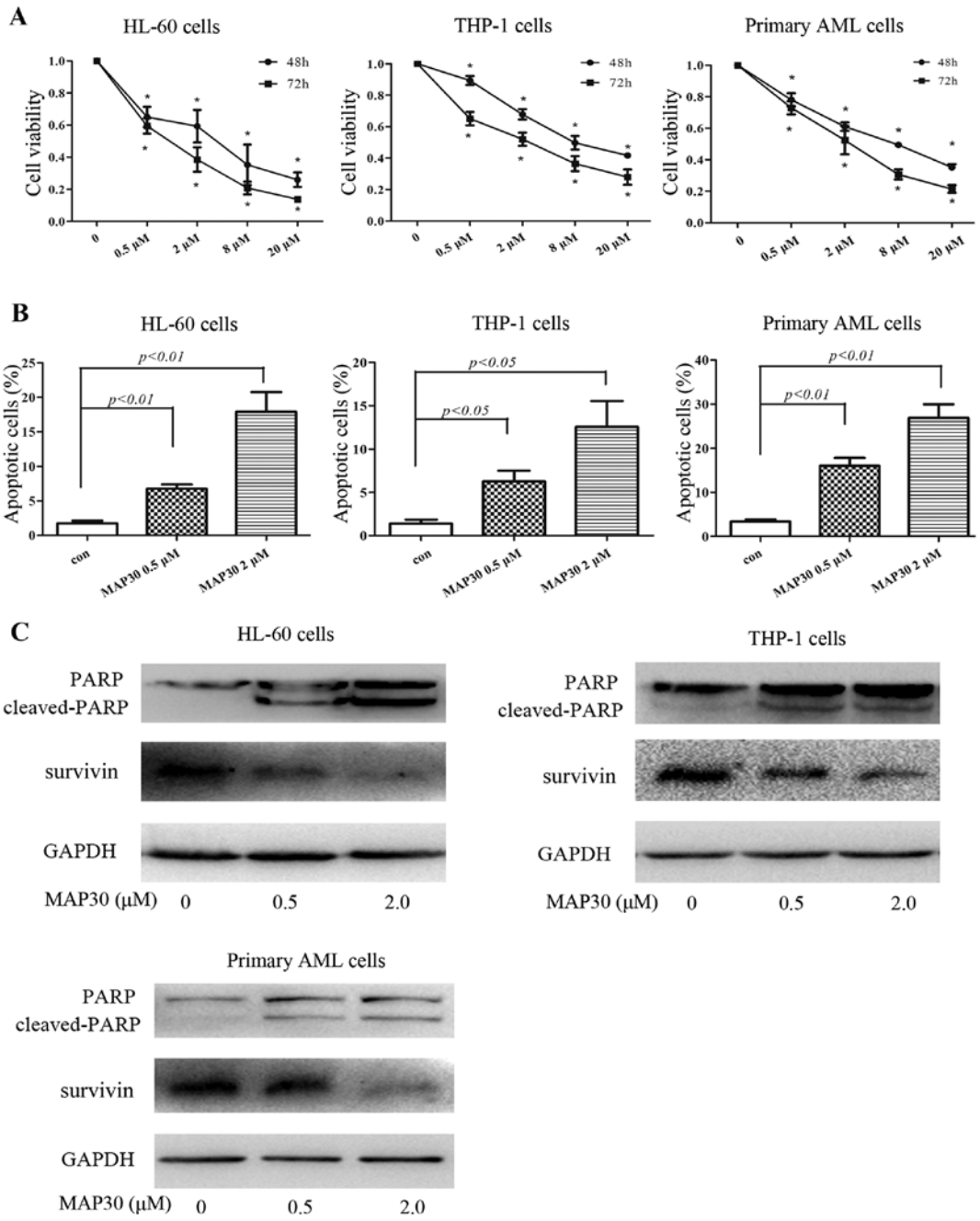

Figure 1. MAP30 induces growth inhibition and apoptosis in AML cells. (A) Two AML cell lines, HL-60 and THP-1, and primary AML cells from 5 patients with AML were treated with different concentrations of MAP30 for 48 and 72 h, and then, cell viability was measured by CCK-8 assay. (B) HL-60 and THP-1 cells and primary AML cells were pre-treated with 0.5 and 2 μM of MAP30 for 48 h, and stained with Annexin V/PI, then analyzed by flow cytometry. The percentages of Annexin V-positive/PI-negative cells are presented in bar charts. (C) HL-60 and THP-1 cells and primary AML cells were treated with 0.5 and 2 μM of MAP30 for 48 h, and then, western blot analysis was performed to assess the expression level of PARP and cleaved-PARP. Results are expressed as mean ± SEM representing at least three independent experiments. *p<0.05 vs. the respective control.

the proliferation of both HL-60 and THP-1 cells in a dose- and time-dependent manner, with IC_{50} at 48 h of 2.6 and 9.2 μM, respectively. Furthermore, MAP30 also inhibited the proliferation of primary AML cells, with IC_{50} at 48 h of 4.7 to 8.1 μM (Fig. 1A). Since the induction of apoptosis is a leading cause for MAP30-induced cytotoxicity against liver cancer cell line HepG2 (11), we next investigate whether apoptosis also occurs when AML cells are inhibited by MAP30. As shown in Fig. 1B, MAP30 (0-2 μM) dose-dependently induced apoptosis of AML HL-60 and THP-1 cells as well as primary AML cells by flow cytometric analysis using Annexin V/PI staining. As expected, high concentrations of MAP30 (8 μM, 20 μM) strongly induced apoptosis in all AML cells tested (data not shown). MAP30-induced apoptosis was further confirmed using western blot analysis of two apoptosis-related proteins PARP and survivin. After treatment with MAP30 for 48 h, cleaved-PARP was markedly increased and conversely, survivin was decreased (Fig. 1C). Taken together, these data suggested that MAP30 exhibits cytotoxicity in AML cells through inducing apoptosis.

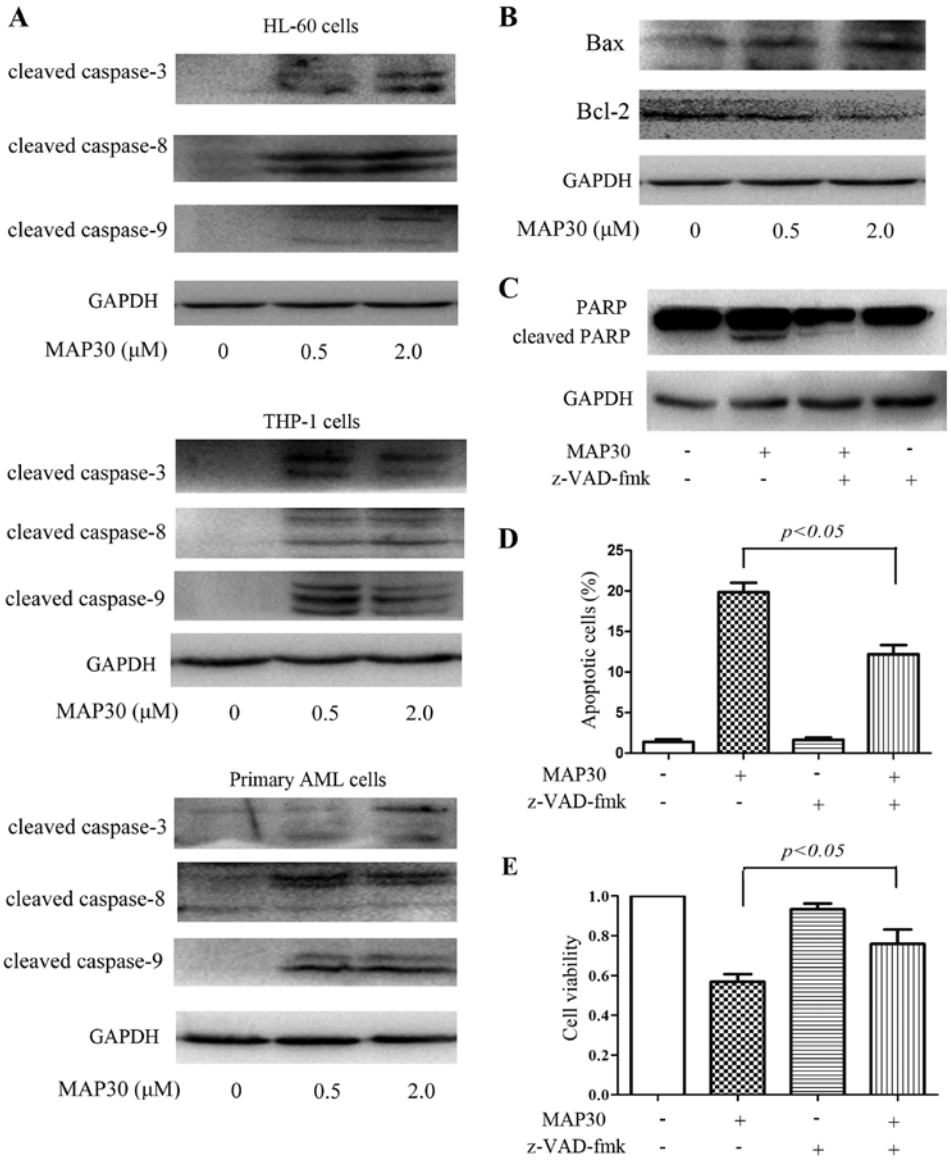

Figure 2. Both extrinsic and intrinsic caspase pathways are involved in MAP30-induced apoptosis. (A) Two AML cell lines, HL-60 and THP-1, and primary AML cells were treated with 0.5 and 2 μM of MAP30 for 48 h, and then western blot analysis was performed to assess the expression levels of caspase-3, caspase-8, and caspase-9. (B) HL-60 cells were treated with 0.5 and 2 μM of MAP30 for 48 h, and then western blot analysis was performed to assess the expression levels of Bax and Bcl-2. (C-E) HL-60 cells were incubated with 2 μM MAP30 in the presence or absence of 20 μM z-VAD-fmk for 48 h, and then, western blot analysis was performed to assess the levels of PARP and cleaved-PARP, and Annexin V/PI staining was used to determine apoptosis, and cell viability was determined by CCK-8 assay. Images shown and statistical data are expressed as mean ± SEM of at least three independent experiments.

Both extrinsic and intrinsic pathways are involved in MAP30-induced apoptosis. We further investigated the molecular mechanisms involved in MAP30-induced apoptosis. As shown in Fig. 2A, MAP30 treatment resulted in the cleavage of caspases, including the initiator caspase-8 and -9 and the effector caspase-3 in AML cell lines HL-60 and THP-1 and primary AML cells (Fig. 2A). These data suggested that MAP30 induces apoptosis of AML cells in a caspase-dependent manner, and both extrinsic and intrinsic apoptotic pathways are involved. Cleaved caspase-3 subsequently induced the cleavage of PARP, which ultimately led to apoptosis. Additionally, the expression of anti-apoptotic protein Bcl-2 was decreased and conversely, the expression of pro-apoptotic protein Bax was significantly increased in HL-60 cells treated by MAP30 (Fig. 2B), indicating that some Bcl-2 family members are also involved in caspase-dependent apoptosis induced by MAP30. Finally, a pan-caspase inhibitor z-VAD-fmk was employed to verify whether MAP30-induced apoptosis depends on caspases. As shown in Fig. 2C and D, z-VAD-fmk significantly reduced MAP30-induced apoptosis in HL-60 cells. Moreover, the growth inhibition of HL-60 cells induced by MAP30 was also partially rescued by z-VAD-fmk (Fig. 2E), further confirming MAP30 induces apoptosis in a caspase-dependent manner.

MAP30 inhibits autophagy in AML cells. Low concentration of MAP30 (2 μM) exhibited strong cytotoxicities against AML cells (Fig. 1A), whereas only modest apoptosis was observed in AML cells induced by this concentration of MAP30 (Fig. 1B), suggesting that there are other mechanisms involved in MAP30-induced cytotoxicity besides the induction of apoptosis. Histone deacetylase inhibitors (HDACi) have

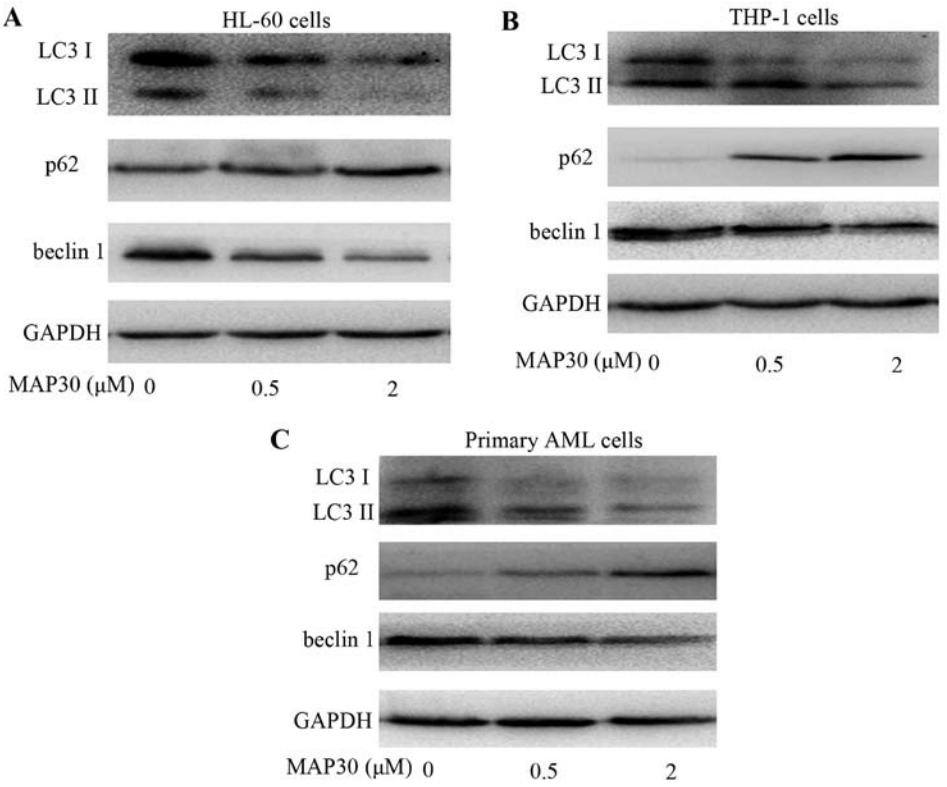

Figure 3. MAP30 inhibits autophagy in AML cells. The AML cell lines, HL-60 (A) and THP-1 (B), and primary AML cells (C) were treated with 0.5 and 2 μM of MAP30 for 48 h, and then, western blot analysis was used to detect autophagy-associated protein LC3I/II, p62 and beclin 1. GAPDH was used as a loading control. Images representing at least three independent experiments are shown.

been shown to kill leukemia cells while sparing normal cells by inhibiting autophagy (30,31). Accumulating evidence shows that one key aspect of the pro-survival function of autophagy is achieved through its ability to block necrotic cell death (32). To investigate whether MAP30 influences the autophagic flux of AML cells, the expression of autophage-related proteins, including LC3, p62, and beclin 1, were determined. As shown in Fig. 3, the expression of LC3II and beclin 1 were significantly decreased, whereas, p62 was increased in the two AML cell lines HL-60 and THP-1 as well as primary AML cells exposed to MAP30. These findings further supported the notion that MAP30 treatment inhibits the autophagic flux in AML cells.

Inhibition of autophagy is implicated in the cytotoxicity and induction of apoptosis of MAP30 in AML cells. Undoubtedly, MAP30 can inhibit autophagy, which prompted us to clarify whether the inhibition of autophagy plays an important role in antileukemic effects of MAP30. We used autophagy activator rapamycin, and another classical autophagy inhibitor baf A1, which inhibits the vacuolar ATPase required for the fusion between autophagosomes and lysosomes. Both rapamycin and baf A1 almost did not influence the cell viability and induce apoptosis of HL-60 cells and primary AML cells (Fig. 4), but at these same concentrations of rapamycin and baf A1 can activate and inhibit the autophagic flux of HL-60 cells, respectively (data not shown). As expected, rapamycin reduced the cytotoxicity and apoptosis in HL-60 cells induced by MAP30 (Fig. 4A, C and E), and conversely, baf A1 exacerbated the cytotoxicity and apoptosis in HL-60 cells induced by MAP30 (Fig. 4B, D and E). Similarly, rapamycin attenuated the cleavage of PARP and the inhibition of survivin, and baf A1 had a synergistic effect on the cleavage of PARP and the inhibition of survivin in primary AML cells induced by MAP30 (Fig. 4F). Taken together, these data further indicated that the inhibition of autophagy is implicated in MAP30-induced cytotoxicity and induction of apoptosis in AML cells.

MAP30 inhibits autophagy by enhancing acetyltransferase p300. Since MCP30, a mixture of MAP30 and α-MMC, was shown to promote histone H3 and H4 protein acetylation in our previous report (33), we next investigate whether the promotion of acetylation of MCP30 are attributed to its main ingredient MAP30. As shown in Fig. 5A and B, MAP30 treatment increased the mRNA and protein expression levels of p300 in HL-60 cells, which consequently promoted the acetylation of histone H3 (Fig. 5C). Similar results have been observed in primary AML cells treated by MAP30 (Fig. 5B and C). Accumulating evidence has shown that p300 is a major endogenous repressor of autophagy (23). To investigate whether the inhibition of autophagy of MAP30 is attributed to p300, C646, a pharmacological inhibitor of p300, was used. As shown in Fig. 5D and E, C646 almost completely reversed the acetylation of histone H3 and promoted the autophagic flux in the presence of MAP30 in HL-60 cells. Therefore, C646 partially rescued the cytotoxicity and apoptosis caused by MAP30 in HL-60 cells and

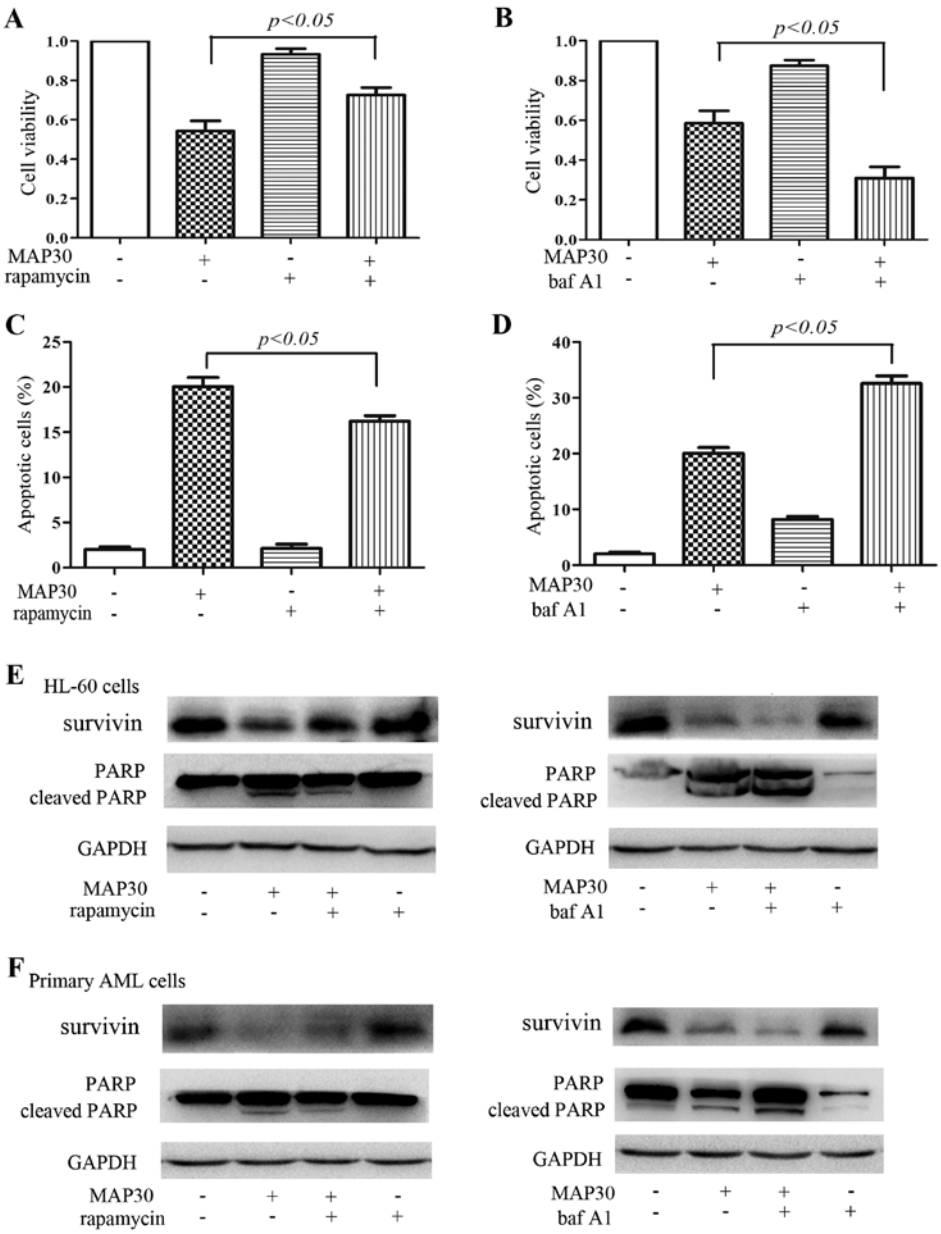

Figure 4. Autophagy inhibition is involved in MAP30-induced cell death and apoptosis in AML cells. (A-D) HL-60 cells were treated with 2 μM MAP30 for 48 h in the absence or presence of 50 nM rapamycin or 10 nM baf A1, and then, the cell viability and apoptosis were determined by CCK-8 assay and Annexin V/PI staining, respectively. (E and F) HL-60 cells and primary AML cells were treated with 2 μM MAP30 for 48 h in the absence or presence of 50 nM rapamycin or 10 nM baf A1, then western blot analysis was performed to assess the levels of survivin, PARP and cleaved-PARP. Statistical data and images representing at least three independent experiments are shown.

primary AML cells (Fig. 5F and G). Taken together, these findings suggested that MAP30 inhibits the autophagic flux of AML cells by enhancing the acetyltransferase activity of p300.

Discussion

Increasing evidence has shown that MAP30 induces cells death in several solid tumor cells, mostly resulting from apoptosis. Here we have identified MAP30 as a potent antileukemic agent, in which autophagy inhibition is a critical step in mediating the antileukemic effects of MAP30 by increasing the p300, an endogenous repressor of autophagy (23,34,35), and subsequently potentiates the induction of apoptosis. To our knowledge, this is the first report on MAP30 against hematological malignancies.

Apoptosis has been deeply studied in the past two decades and is widely accepted as a major mechanism of regulated cell death. Therefore, measurement of apoptosis is frequently used to evaluate the antitumor effects of cytotoxic agents besides cell viability assessment. Our results demonstrated that MAP30 potently inhibited the proliferation of HL-60 and THP-1 cells as well as primary AML cells isolated from AML patients, and the induction of apoptosis might be a major mechanism, similar to two previous studies (9,11). Further molecular studies unraveled the contribution of both the extrinsic pathway regu-

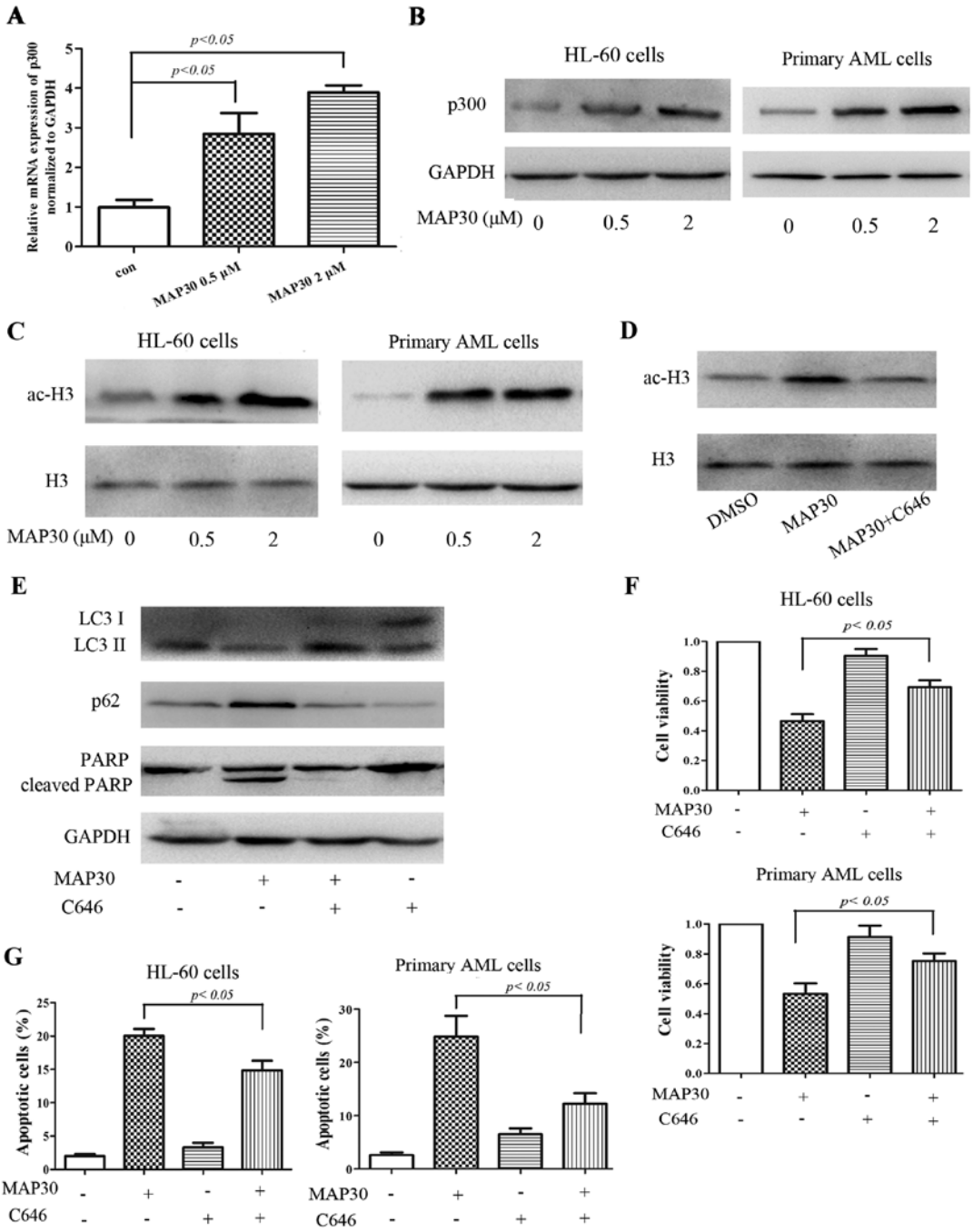

Figure 5. MAP30 inhibits autophagy by enhancing acetyltransferase p300. (A-C) HL-60 cells and primary AML cells were treated with 0.5 and 2 μM of MAP30 for 48 h, and then, real-time PCR was performed to evaluate the mRNA level of p300, and western blot analysis was performed to assess the levels of p300, GAPDH, ac-histone H3 (ac-H3), and histone H3 (H3). (D and E) HL-60 cells were treated with 2 μM MAP30 for 48 h in the absence or presence of C646, then western blot analysis was performed to assess the levels of ac-H3, LC3I/II, p62 and PARP. (F and G) HL-60 cells and primary AML cells were treated with 2 μM MAP30 for 48 h in the absence or presence of C646, and then, cell viability and apoptosis were determined by CCK-8 assay and Annexin V/PI staining, respectively. Statistical data and images representing at least three independent experiments are shown.

lated by caspase-8 cleavage and intrinsic pathway regulated by caspase-9 cleavage in MAP30-induced cell apoptosis of AML cells. Consistent with our results, MAP30 also induces apoptosis in hepatocellular carcinoma HepG2 cells through both extrinsic and intrinsic pathways (11).

The precise role of autophagy in cancer development and treatment is still controversial. In AML, recent evidence suggests that autophagy plays a pro-survival role in t(8;21) AML cells (36), but the exact role of this process in different subtypes of this hematologic tumor is still undefined. As autophagy is an important catabolic process for degrading bulky cytosolic contents, its inhibition is associated with the production of reactive oxygen species (ROS), metabolic insufficiency and increased proteotoxicity, and thus, promotes cellular damage, reduces stress tolerance and compromises survival (37). Many anticancer agents or radiotherapy can trigger cell autophagy as a pro-survival activity, which protects cancer cells against their killing action (38). In addi-

tion, autophagy is also needed for leukemia cells due to high metabolic demand. In our present study, except the induction of apoptosis, MAP30 also significantly inhibited autophagy in two AML cell lines tested and all primary AML cells. Based on the above, we speculate that MAP30 can break the equilibrium of metabolism by suppressing basal autophagy, resulting in accumulation of dysfunctional mitochondria, subsequently triggering ROS production and DNA damage, and ultimately inducing apoptosis and necrotic cell death. To confirm our speculation, accompanied with MAP30 treatment, the autophagy activator rapamycin was adopted to rescue and further induce autophagy, and the autophagy inhibitor baf A1 was used to aggravate the inhibition of autophagy in HL-60 cells and primary AML cells. We found that MAP30-induced cell death significantly aggravated due to autophagy inhibition when MAP30 combined with baf A1. Moreover, the MAP30-induced cell death was partially rescued by rapamycin. Thus, based on these findings, we think that MAP30-induced autophagy inhibition contributes to the induction of apoptosis and the resulting cell death in AML cells. Further investigation is needed to clarify the relationship between autophagy and necrotic cell death in MAP30-induced AML cell death.

MAP30 has been reported to increase the acetylation level, while the underlying mechanisms remain unclear. We found that p300 is a target of MAP30 action, and it increased the expression of p300 at both the mRNA and protein levels in AML cells. Moreover, the acetylation level of histone H3, an indicator of p300 activity, was also increased by MAP30 treatment. It has been well identified that p300 is a potent autophagy inhibitor (35). To further identify the role of p300 in MAP30-induced cell death, C646, a selective pharmacological inhibitor of p300 activity, was used and partially rescued the MAP30-induced cell death. Further research is needed to investigate how MAP30 increased the p300 in AML cells.

In conclusion, although autophagy and apoptosis are two independent cell death pathways, our findings offer specific insight by which the pathways acted collaboratively to elicit MAP30-induced cell death in AML cells. Our findings identify MAP30 as a potent anti-AML agent and provide molecular insight into the anticancer potential of MAP30 by showing that both autophagic and apoptotic signaling can work together in the induction of cell death.

Acknowledgements

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