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Original Paper

Degradation of Mcl-1 through GSK-3β Activation Regulates Apoptosis Induced by Bufalin in Non-Small Cell Lung Cancer H1975 Cells

Xiao-hong Kang[a] Jing-hang Zhang[b] Qing-qin Zhang[a] Yan-hui Cui[a] Ying Wang[a] Wei-zheng Kou[a] Zhan-hui Miao[a] Ping Lu[a] Li-Fang Wang[c] Zhen-ye Xu[c] Fei Cao[d]

[a]Department of Oncology, [b]Department of pathology, The First Affiliated Hospital of Xinxiang Medical University, Xinxiang, [c]Department of Oncology, Longhua Hospital Affiliated to Shanghai University of TCM, Shanghai, [d]Department of Oncology, The First People's Hospital of Ping Ding Shan, Henan, China

Key Words
NSCLC • Bufalin • Mcl-1 • Glycogen synthase kinase-3β • Apoptosis

Abstract
Background/Aims: Mcl-1, an anti-apoptotic Bcl-2 family member, is often overexpressed in non-small cell lung cancer (NSCLC). Bufalin has been reported to induce apoptosis in various tumor cells. However, there is no report showing that bufalin could downregulate Mcl-1 expression in NSCLC. ***Methods:*** Cell proliferation was analyzed by cell counting kit-8 (CCK-8) assay in H1975 cells. Cell apoptosis was detected by flow cytometry. Mcl-1 mRNA was detected by RT-PCR. The expression of apoptosis-associated proteins in H1975 cells was detected by western blotting. The levels of Mcl-1 ubiquitination and NOXA were analyzed by Immunoprecipitation assay. ***Results:*** Cell growth was inhibited by bufalin in a time and dose-dependent manner. Bufalin induced apoptosis in NSCLC cells by activating caspase cascades and downregulating Mcl-1 expression. However, overexpression of Mcl-1 diminished bufalin-induced apoptosis. Furthermore, bufalin did not reduce Mcl-1 mRNA expression in H1975 cells, but strongly promoted Mcl-1 protein degradation. Proteasome inhibitor MG132 markedly prevented the degradation of Mcl-1 and blocked bufalin-induced Mcl-1 reduction. Bufalin did not significantly affect NOXA protein levels, but downregulated the expression of p-GSK-3β. GSK-3 inhibitor and GSK-3β siRNA resulted in increased levels of Mcl-1 and reversed the bufalin-induced Mcl-1 degradation. ***Conclusion:*** Bufalin induced cell apoptosis in H1975 cells may be through downregulation of Mcl-1. Proteasomal degradation of Mcl-1 via GSK-3β activation was involved in bufalin-induced apoptosis.

X.-h. Kang and J.-h. Zhang contributed equally to this work.

Fei Cao
Department of Oncology, The First People's Hospital of Ping Ding Shan,
117 East Youyue Road, Pingdingshang, Henan 467000 (China)
Tel. +86 0375 3399571, Fax +86 0375 2262999; E-Mail cf771225@163.com

Introduction

Lung cancer is the most prevalent cancer and one of the leading causes of cancer-related mortality worldwide [1]. It is classified into two histological subgroups: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Currently, NSCLC accounts for 80%-85% of morbidity of lung cancer. Although cisplatin-based combination chemotherapy or targeted therapy has been established as the first-line therapies against advanced or metastatic NSCLC [2, 3], the 5-year relative survival rate for NSCLC is only 17% [3]. NSCLC is generally incurable because it commonly acquires resistance to apoptosis mediated by intrinsic pathway [4, 5]. Therefore, it is urgent to explore novel therapeutic agents for NSCLC via inducing apoptosis.

Bcl-2 family proteins play a key role in regulating the mitochondria-mediated intrinsic pathway of apoptosis. They consist of three groups: (1) the anti-apoptotic proteins, including Bcl-2, Bcl-xL, Bcl-w and Mcl-1, which contain the Bcl-2 homology (BH) 1-4 domains; (2) the multi-domain pro-apoptotic proteins, including Bax, Bak and Bok, which contain the BH1-3 domains and are effectors of apoptosis; and (3) the BH3-only members, including Bim, Bad, Bik, Puma and NOXA, which are initiators of apoptosis [6]. During intrinsic apoptosis, the pro-apoptotic proteins Bax and Bak are stimulated by apoptotic stress and then oligomerized into proteolipid pores in the outer mitochondrial membrane. Subsequently, cytochrome *c* is released into cytosol, leading to activation of caspase cascade and ultimately to apoptosis [7]. On the other hand, the anti-apoptotic proteins maintain mitochondrial integrity through sequestration of the multi-domain pro-apoptotic proteins and prevention of their oligomerization, resulting in cell survival or resistance to apoptosis [8]. Furthermore, BH3-only proteins can bind to the anti-apoptotic Bcl-2 proteins at the BH3 groove or activate pro-apoptotic proteins and trigger apoptosis [9].

Recent studies have suggested that overexpression of the anti-apoptotic Bcl-2 family proteins or downregulation of BH3-only proteins contributed to chemotherapy resistance and poor prognosis in a variety of cancers [10]. In NSCLC, Bcl-2, Bcl-xl and Mcl-1 are often found to be upregulated, which is associated with chemotherapy resistance and metastasis of cancers [11, 12]. These findings indicate that suppression of the anti-apoptotic Bcl-2 proteins may be a promising strategy for cancer therapy and potentially overcome the resistance to conventional chemotherapy.

Bufalin is a major bioactive component derived from *Venenum Bufonis*, a traditional Chinese medicine obtained from the skin and parotid venom glands of toads [13, 14]. In recent years, increasing studies have demonstrated that bufalin could inhibit cell proliferation and induce apoptosis in a variety of tumor cells [15-22]. However, the detailed mechanisms of bufalin-induced apoptosis in NSCLC cells have not been fully elucidated. Since anti-apoptotic Bcl-2 family proteins play a major role in intrinsic apoptotic pathway and are overexpressed in NSCLC, we suspected that bufalin-induced NSCLC apoptosis might involve suppression of Bcl-2 family proteins. In the present study, we tested this hypothesis and elucidated the underlying mechanisms.

Materials and Methods

Reagents and antibodies

Bufalin, MG132 and SB216763 were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). z-VAD-fmk was purchased from Selleck Chemicals (Houston, TX, USA). Antibodies against caspase-3 , PARP, NOXA, GSK-3β, p-GSK-3β^{Ser9} were obtained from Cell Signaling (Danvers, MA, USA). Antibodies against Bcl-2, Bcl-xl, Bax and Bak were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Mcl-1 was obtained from Abcam (Cambridge, MA, USA). The chemical structure of bufalin is shown in Fig. 1A

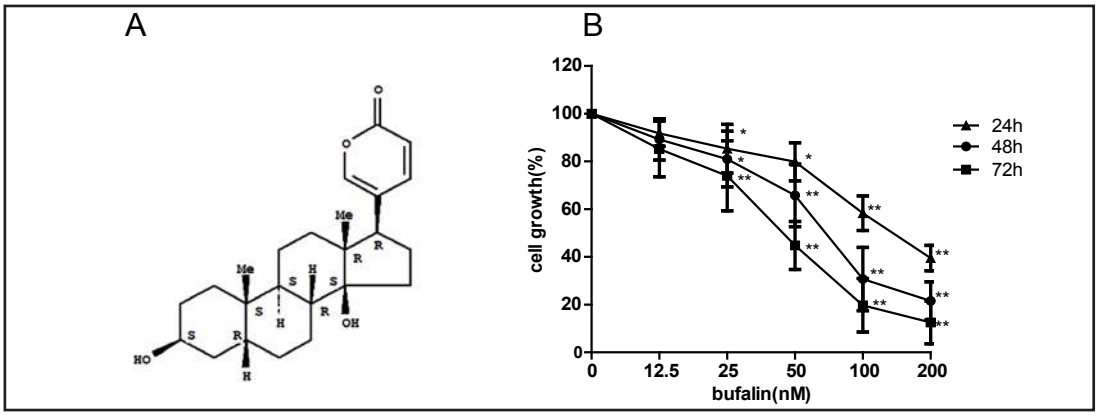

Fig. 1. Bufalin suppresses H1975 cells growth. (A), Chemical structure of bufalin. (B), H1975 cells were treated with various concentrations of bufalin for 24, 48 and 72 h, and then cell growth was determined by CCK-8 assay. The data are presented as mean±SD. (N=3, $^{*}P<0.05$, $^{**}P<0.01$ *vs* control).

Cell culture and plasmid transfection

Human lung adenocarcinoma cell line H1975 was purchased from American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37°C in 100% humidity, 5% CO_2, and 95% air. H1975 cells were transfected with pcDNA3.1-Mcl-1, empty vector pcDNA3.1, GSK-3β siRNA or control siRNA (GenePharma, Shanghai, China), respectively with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The sense strand sequence of GSK-3β siRNA was 5'-GGGACCCAAAUGUCAAACUTT-3'. The expressions of Mcl-1 and GSK-3β were evaluated by Western blotting assays.

Cell proliferation assay

H1975 cells were seeded into 96-well plates at a density of 3×10^3 cells/100 μL/well and treated with bufalin for 24, 48 and 72 hours at indicated concentrations. Cell proliferation assays were performed using the cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) following manufacturer's instructions. Cell growth was shown as relative percentage of the untreated controls as described previously [15].

Cell apoptosis assay

Tumor cells were seeded into 6-well plate at a density of 1×10^5/well and incubated with various agents at indicated concentrations for 24 h. Cell apoptosis was detected using the Annexin V-FITC/PI Apoptosis Detection kit (Keygen, Nanjing, China) following the manufacturer's protocols. The quantitation of apoptotic cells was calculated by CellQuest software.

qRT-PCR assay

Total RNA was extracted from cells treated with Trizol reagent (Invitrogen CA) and cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. Mcl-1 mRNA was detected by RT-PCR using SYBR Premix Ex Taq (Takara Dalian, China). The primers used were as follows: Mcl-1, (forward) 5'-TGCTTCGGAAACTGGACATC-3', (reverse) 5'-TAGCCACAAAGGCACCAAAAG-3'; GAPDH, (forward) 5'-GAGTCAACGGATTTGGTCGT-3', (reverse) 5'-TTGATTTTGGAGGGATCTCG-3'. The qPCR was performed with SoFastTM EvaGreenH Supermix (Bio-Rad).

Western blotting analysis

Treated cells were harvested, washed twice with ice-cold PBS and lysed with RIPA buffer. Cell lysates were centrifuged at 12 000 rpm for 15 min at 4°C, and the protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein extracts of 50 μg were separated by SDS-PAGE gel (Biorad, France) and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk for 2 h at room temperature and incubated with primary antibodies overnight at 4°C, followed by incubation with HRP-linked secondary antibody for 1 h at room temperature.

The intensity of blot signals was quantified using Image Quant TL analysis software (General Electric, UK). Representative blots were from at least three independent experiments.

Immunoprecipitation assay

Transfection of HA-tagged ubiquitin into H1975 cells were performed using Lipofectamine 2000 according to the manufacturer's instructions. After 12 h, H1975 cells were treated with bufalin (50 nM) for 20 h, and co-treated with MG132 (10 μM) for another 4 h. Cells were harvested, lysed and incubated with Mcl-1 antibody for 4 h, followed by Protein A/G agarose (Pierce Biotechnology) overnight at 4°C. Then, the beads were washed 4 times with lysis buffer and isolated by centrifugation. The pellets were boiled in the loading buffer at 95°C for 5 min. The levels of Mcl-1 ubiquitination and NOXA were analyzed by Western blotting using anti-HA and anti-NOXA antibodies.

Statistical analysis

All experiments were performed in triplicate. The data were presented as mean ± SD. All statistical analyses were performed using SPSS Ver. 18.0 (SPSS Inc, Chicago, IL, USA), and differences were analyzed by one-way or two-way ANOVA tests. $P<0.05$ was considered to be statistically significant. All graphs in figures were created using Graph Pad Prism 5.0 (Graph Pad Software, Inc.).

Results

Bufalin inhibits the proliferation of H1975 cells

We first assessed the effects of bufalin on viability of H1975 cells by using the CCK-8 cytotoxicity assay. As shown in Fig. 1B, treatment with bufalin at various concentrations for 24, 48 and 72 hours resulted in dose-dependent reduction in proliferation of H1975 cells. These data showed that bufalin inhibited cell growth in a time and dose-dependent manner.

Bufalin induces apoptosis by activating caspase cascades

We then further investigated whether bufalin could trigger apoptosis by using flow cytometry analyses with Annexin V/PI staining in H1975 cells. As depicted in Fig. 2A, different concentrations of bufalin resulted in increased proportion of apoptotic cells compared with the control, suggesting that bufalin may act as an apoptosis-inducer. To examine whether bufalin induction of apoptosis was caspase-dependent, the pan-caspase inhibitor z-VAD-fmk (zVAD) was used to pretreat H1975 cells for apoptosis analyses. As shown in Fig. 2B and C, zVAD abrogated bufalin-induced apoptosis and cleavage of Caspase-3 and PARP. These data collectively indicated that bufalin trigged caspase-dependent apoptosis in NSCLC cells.

Downregulation of Mcl-1 expression contributes to Bufalin-induced apoptosis in lung cancer cells

We subsequently tested whether bufalin-induced apoptosis involved regulation of Bcl-2 family proteins in NSCLC cells. After bufalin treatment at different concentrations for 24 h in H1975 cells, the expression of Bax and Bak was increased, whereas the expression of Mcl-1, Bcl-2 and Bcl-xl was decreased in a dose-dependent manner. Interestingly, we found that bufalin downregulated Mcl-1 expression more dramatically than that of Bcl-2 and Bcl-xl (Fig. 3A). In addition, time-course experiments showed that Mcl-1 levels were decreased after treatment with bufalin for 12 h (Fig. 3B). To confirm that inhibition of Mcl-1 crucially contributed to bufalin induction of apoptosis, we then transfected H1975 cells with pcDNA3.1-Mcl-1 to overexpress Mcl-1. As shown in Fig. 3C and D, overexpression of Mcl-1 significantly abolished bufalin-induced apoptosis. These data consistently indicated that bufalin induced apoptosis in NSCLC cells at least partially through suppression of Mcl-1.

Bufalin induces downregulation of Mcl-1 protein by activation of proteasome pathway.

To determine whether bufalin repressed Mcl-1 at transcriptional level, we detected Mcl-1 mRNA levels by RT-PCR. Compared with control, bufalin did not reduce Mcl-1 mRNA

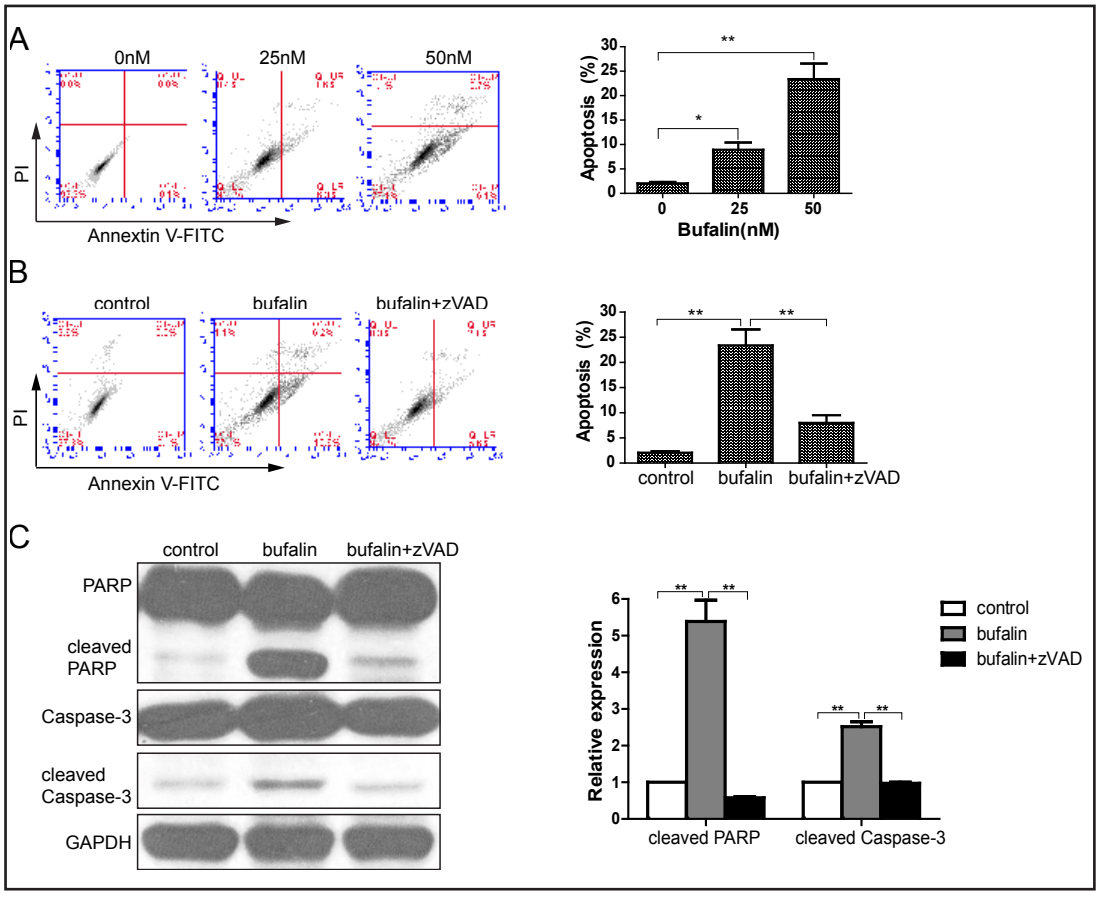

Fig. 2. Bufalin induces apoptosis in H1975 cells. (A and B), H1975 cells were treated with bufalin for 24 h or pretreated with 20 μM z-VAD-fmk for 30 min and then co-treated with 50nM bufalin for 24h. Cells were stained with Annexin V-FITC and PI and apoptosis was analyzed by flow cytometry. (C), H1975 cells were treated as B, and then whole-cell protein were extracted for western blot analysis to evaluate cleavage of caspase-3 and PARP. The data are represent as mean±S.D. of three independent experiments (N=3, $^{*}P<0.05$, $^{**}P<0.01$).

expression in H1975 cells (Fig. 4A). These results suggested that bufalin might inhibit Mcl-1 protein expression through a non-transcriptional mechanism. We then examined whether ubiquitin-proteasome system was involved in bufalin downregulation of Mcl-1. Western blotting assays showed that treatment with MG132 (proteasome inhibitor) increased the expression of Mcl-1 and counteracted the bufalin-mediated Mcl-1 repression, indicating that proteasomal degradation of Mcl-1 was involved in the effect of bufalin (Fig. 4B). To further test this hypothesis, we detected ubiquitination of Mcl-1 in H1975 cells by immunoprecipitation. Indeed, significantly enhanced ubiquitination was observed in cells treated with bufalin compared with control, indicating that ubiquitin-dependent pathway was also involved in bufalin-induced degradation of Mcl-1 proteins (Fig. 4C). Collectively, these findings provided evidence that bufalin inhibited Mcl-1 expression at least partially via activation of ubiquitin-proteasome system.

It is well-known that NOXA (the BH3-only protein) binds with Mcl-1 and mediates Mcl-1 degradation through proteasome [23]. We thus investigated whether NOXA was involved in bufalin-induced degradation of Mcl-1 in H1975 cells. Western blotting and immunoprecipitation analyses revealed that NOXA expression was not altered in bufalin-treated cells (Fig. 4C and D). Glycogen synthase kinase-3β (GSK-3β) has been demonstrated to facilitate the ubiquitination and degradation of Mcl-1 [24]. We therefore examined the effect of bufalin on GSK-3β activation in H1975 cells. Our data demonstrated that bufalin

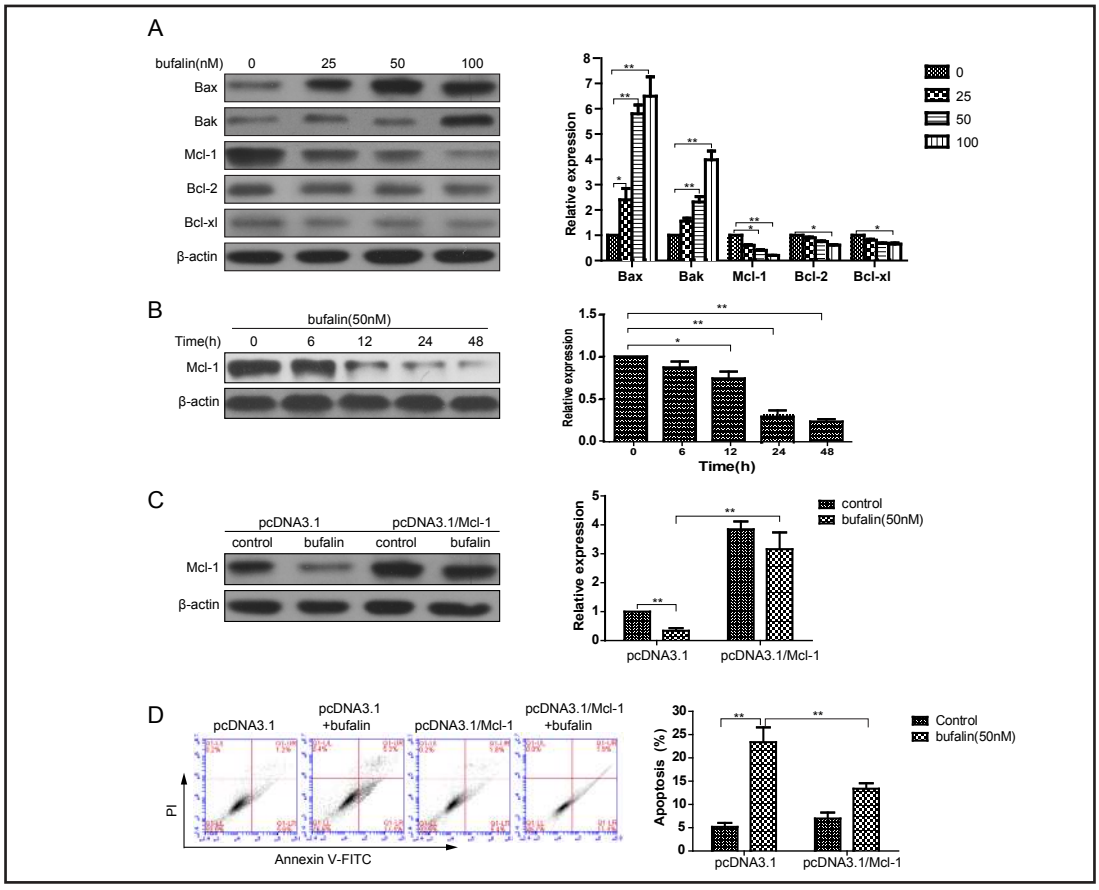

Fig. 3. Bufalin inhibits Mcl-1 expression. (A), H1975 cells were treated with indicated concentrations of bufalin for 24 h. Bcl-2 family proteins were detected by western blot. (B), H1975 cells were treated with 50 nM bufalin for 6 h, 12 h, 24 h and 48 h, Mcl-1 protein was evaluated by western blot. (C), H1975 cells were transfected with pcDNA3.1 or pcDNA3.1-Mcl-1, and then treated with 50 nM bufalin for 24h. Whole-cell protein were extracted for western blot analysis to evaluate Mcl-1. (D), H1975 cells were treated as (C), and then co-stained with Annexin V-FITC and PI. Apoptosis was analyzed by flow cytometry. The data are presented as mean±SD. (N=3, $^{*}P<0.05$, $^{**}P<0.01$).

dose-dependently decreased the abundance of p-GSK-3β (Fig. 4D). To further test the role of GSK-3β activation in bufalin-mediated Mcl-1 degradation, SB216763 (a GSK-3 inhibitor) and GSK-3β siRNA were used to manipulate GSK-3β in H1975 cells. We found that SB21673 alone increased the levels of p-GSK-3β and Mcl-1. Pretreatment with SB216763 or silencing GSK-3β by siRNA almost totally blocked the reduction of Mcl-1 in H1975 cells treated with bufalin. The apoptosis induced by bufalin was significantly attenuated by SB216763 treatment or GSK-3β siRNA as determined by cleaved PARP (Fig. 4E and F). Together, these results suggested that bufalin inhibited Mcl-1 expression via activation of GSK-3β in H1975 cells.

Discussion

Bcl-2 family proteins are important in regulation of apoptosis. Overexpression of the anti-apoptotic Bcl-2 family members has been demonstrated in various tumors including lung cancer, and is associated with poor prognosis [25-27]. Among these members, Mcl-1 is a principal anti-apoptotic protein, which directly binds to the pro-apoptotic proteins Bax and Bak, and blocks their functions, leading to the evasion of apoptosis and resistance of

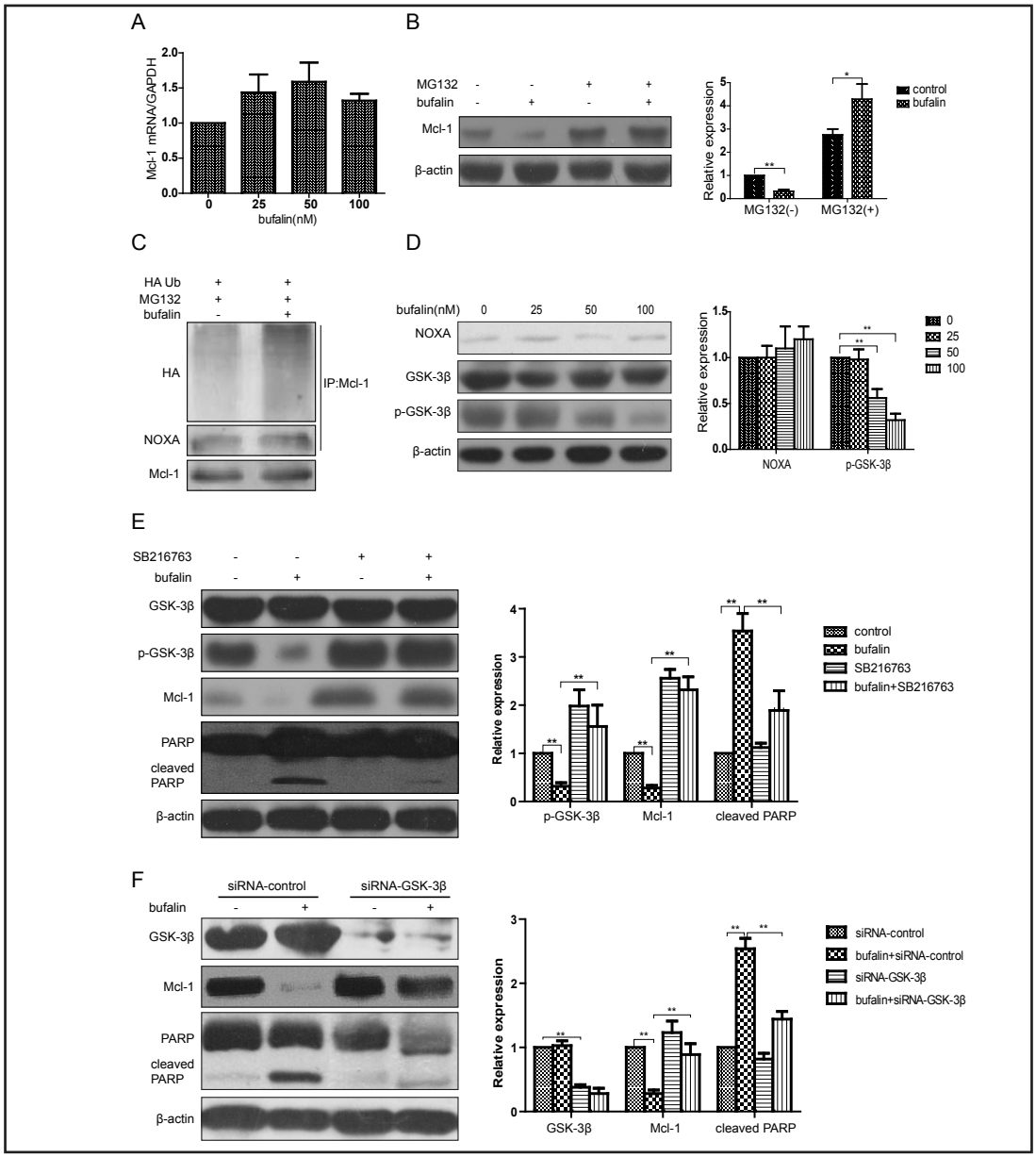

Fig. 4. Bufalin induces Mcl-1 degradation. (A), H1975 cells were treated with indicated concentrations of bufalin for 24 h. The mRNA level of Mcl-1 was detected by RT-PCR. (B), H1975 cells were pretreated with 2 μM MG132 for 2 h and then co-treated with 50 nM bufalin for 24 h. The relative level of Mcl-1 was determined by western blot analysis. (C), H1975 cells were transfected with HA-tagged ubiquitin (HA-Ub) for 12 h, then treated with 50 nM bufalin for 20 h, and co-treated with10μM MG132 for another 4 h. The levels of Mcl-1 ubiquitination and NOXA were analyzed by anti-HA and anti-NOXA western blot following anti-Mcl-1 immunoprecipitation.(D), H1975 cells were treated as (A), NOXA, GSK-3β and p-GSK-3β proteins were detected by western blot. (E), H1975 cells were pretreated with 5μM SB216763 for 2 h, and then treated with 50 nM bufalin for 24 h. The levels of GSK-3β, p-GSK-3β, Mcl-1 and PARP were determined by western blot analysis. (F), H1975 cells transfected with GSK-3β siRNA or control siRNA were treated 50 nM bufalin for 24h. The levels of GSK-3β, Mcl-1 and PARP were determined by western blot analysis. The data are presented as mean±SD. (N=3, $^{*}P<0.05$, $^{**}P<0.01$).

chemotherapies [28]. Thus, reduction of Mcl-1 could be a potential and promising therapy for cancer.

Bufalin has been shown to inhibit cell proliferation and induce cell apoptosis in several cancer cells. However, there is no report showing that bufalin could downregulate Mcl-1 expression in NSCLC. In current study, we found that bufalin downregulated Mcl-1 expression more dramatically than that of Bcl-2 and Bcl-xl. Overexpression of Mcl-1 could significantly diminish bufalin-induced apoptosis. These data collectively suggested that reduction of Mcl-1 levels was in part associated with bufalin-induced apoptosis in NSCLC cells.

Mcl-1 protein has short half-life and contains proline-, glutamic acid-, serine- and threonine-rich (PEST) sequences, which play a primordial role in protein degradation [29]. Therefore, Mcl-1 expression is tightly regulated by proteasome-mediated degradation [30, 31]. In the present study, we found that bufalin had no apparent effect on Mcl-1 mRNA expression, and that MG132 markedly blocked bufalin-induced Mcl-1 reduction. Moreover, bufalin significantly increased the levels of Mcl-1 ubiquitination. These results suggested that the potential mechanism by which bufalin downregulated Mcl-1 was related to proteasomal degradation.

NOXA and activated GSK-3β were both well-known modulators of Mcl-1 protein degradation via proteasome. NOXA binds strongly with Mcl-1 and modulate Mcl-1 protein sequestration and degradation [23]. However, bufalin did not significantly affect NOXA protein levels in the present study. GSK-3β activation can phosphorylate Mcl-1 at Ser159 [24]. The present data showed that bufalin downregulated the expression of Mcl-1 accompanied with the reduction of p-GSK-3β. Therefore, we thought that GSK-3β activation mediated Mcl-1 protein degradation contributes to bufalin-induced apoptosis in H1975 cells.

Besides NOXA and activated GSK-3β, there are several factors modulate Mcl-1 protein degradation. For example, deubiquitinase USP9X could interact with Mcl-1 and remove the Lys48-linked polyubiquitin chains, resulting in stabilization of Mcl-1 [32]. Ku70 directly binds to Mcl-1 leading to deubiquitination and stabilization of Mcl-1 [33]. Therefore, further work may be needed to determine whether proteasomal degradation of Mcl-1 upon bufalin treatment was associated with USP9X and Ku70.

In conclusion, our current findings demonstrated that bufalin induced apoptosis at least in part, through proteasomal degradation of Mcl-1. Therefore we suggest that bufalin may be a effective inhibitor of Mcl-1 in the treatment of NSCLC.

Acknowledgments

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Disclosure Statement

The authors have declared no conflict of interest.

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