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Bufothionine exerts anti-cancer activities in gastric cancer through Pim3

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ABSTRACT

Aim: Gastric cancer (GC) is the fourth most common cancer globally. Bufothionine is a major active constituent of Cinobufacini (Huachansu), which is extracted from the skin and parotid venom gland of the toad Bufo bufo gargarizans Cantor. It exhibits anti-cancer activities *in vitro*. However, whether bufothionine exerts anti-cancer activities against GC is unknown. This study was designed to evaluate the efficacy of bufothionine *in vitro* and *in vivo*.

Material and methods: MKN28 and AGS cells were chosen as cell models to study the anti-cancer effect of bufothionine. Cell viability was determined by CCK-8 assay, while the effect of bufothionine on cell membrane integrity was examined by LDH assay. Cell apoptosis was detected by Hoechst/PI staining and Annexin V-FITC/PI staining followed by flow cytometry analysis. The expression levels of proteins involved were examined using western blotting. I-Traq analysis was conducted to identify the differentially expressed genes in AGS cells following bufothionine treatment. The anti-growth effect of bufothionine was validated *in vivo* using a GC xenograft model.

Key findings: The results revealed that bufothionine prevented the growth, destroyed cell membrane and promoted apoptotic cell death of GC cells. iTRAQ analysis revealed thatPIM3 might be a molecular target responsible for the anti-cancer effects of bufothionine. It was also found that PIM3 knockdown significantly augmented the anti-growth and pro-apoptotic effects of bufothionine in GC cells. In contrast, ectopic PIM3 expression markedly dampened the anti-neoplastic activities of bufothionine. The expression of PIM3 was also suppressed by bufothionine treatment in xenograft tumor tissue.

Significance: Bufothionine exhibited anti-cancer activities *in vitro* and *in vivo* in GC *via* downregulating PIM3.

1. Introduction

Gastric cancer (GC) is the fourth most common cancer globally and the third leading cause of cancer-related deaths [1]. In China, the incidence of GC is high [2]. Currently, surgery is the mainstream therapeutic regimen for GC. Recently, a variety of chemotherapeutic agents have been introduced for the treatment of GC. However, the prognosis of GC patients remains far from being satisfactory going by the poor 5-year survival rate, which is attributed to late diagnosis and chemo-resistance [3]. Hence, it is imperative to investigate novel treatment agents for GC.

PIM3 is a member of the Provirus integrating site Moloney murine leukemia virus (Pim) family, which belongs to the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) group and exhibits serine/threonine kinase activity [4,5]. It has been evidenced that Pim3 is involved in various human malignances [6]. PIM-3 displays aberrant high expression in a variety of endoderm-derived tumors such as adenocarcinoma, hepatocellular carcinoma, and pancreatic cancer. However, the expression of PIM3 in normal organs is low [7–9]. PIM3 was found to be an oncogene given thatPIM3 overexpression correlated with enhanced tumor growth and cell survival whereas PIM3 siRNA or inhibitor delayed tumorigenesis [10]. Abnormal Pim-3 expression has also been found in the invasion and metastasis of gastric cancer [11]. These findings imply that PIM3 might function as a potential therapeutic target for GC.

Cinobufacini (Huachansu), which is extracted from the skin and parotid venom gland of the toad Bufo bufo gargarizans Cantor, has been used as a folk medicine in China for decades to treat swelling, pain, and heart failure [12]. In the past decade, cinobufacini injection has also been tested in clinical trials in combination with chemotherapeutic agents to treat lung cancer, hepatocellular carcinoma, prostate cancer and gallbladder cancer. The results of such studies have evidenced that Cinobufacini possess anti-neoplastic activity [13–15]. Bufadienolides are bioactive compounds of Cinobufacini responsible for its anti-cancer activities [16,17]. Bufothionine is a potent bufadienolide which has been found to exert anti-neoplastic activities in hepatocellular

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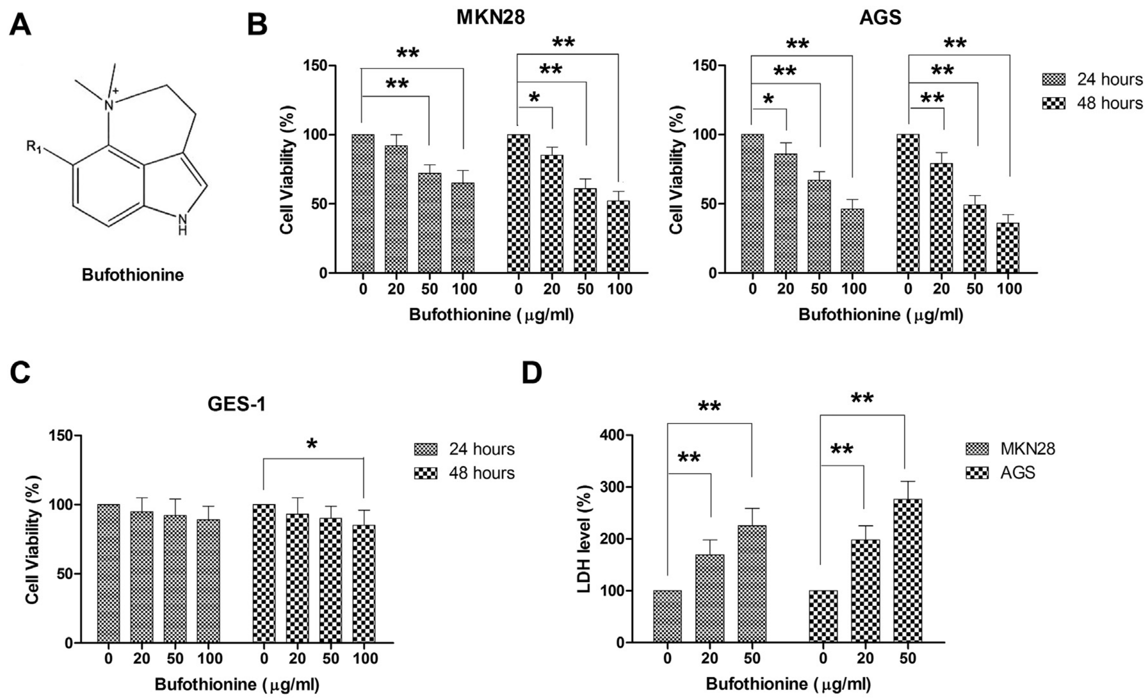

Fig. 1. Bufothionine reduces cell viability and disrupts cell membrane integrity in GC cells. A. The chemical structure of bufothionine. B. Treatment with bufothionine at 20, 50 and 100 μg/ml for 24 or 48 h followed by cell viability assessment by CCK-8 assay. C. GES-1 cells were treated with bufothionine at 20, 50 and 100 μg/ml for 24 or 48 h. Cell viability was assessed by CCK-8 assay. D. MKN28 and AGS cells were treated with bufothionine at 20 and 50 μg/ml for 48 h. The effect of bufothionine on cell membrane integrity was assessed by LDH assay. *P < 0.05, **P < 0.01.

carcinoma cell lines [12,18]. However, the effect of bufothionine on other human malignancies has not been reported. In the present study, the effect of bufothionine on GC was explored and the underlying molecular mechanisms were investigated. The results showed that bufothionine suppresses GC growth *in vitro* and *in vivo*. Moreover, PIM3 was identified as the primary molecular target that mediates the anticancer activities of bufothionine against GC.

2. Materials and methods

2.1. Cell culture

The human GC cell line MKN28was purchased from GeneChem (Shanghai, China), AGS was purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and GES-1 (the normal gastric epithelial cells) was purchased from the American Type Culture Collection (Rockville, MD, USA). All cells were cultured in RPMI 1640, supplemented with 10% foetal bovine serum (Hyclone, Logan, UT, USA) in a humidified CO_2 (5%) incubator at 37 °C.

2.2. Viability assay

Briefly, 100 μL cells were seeded in 96-well plate at the density of 1.2×10^5 cells/ml. Following treatment with bufothionine, the cell viability was detected using the nonradioactive cell proliferation assay kit (Promega Corporation, Madison, WI) according to the manufacturer's protocol. The absorbance at 570 nm was examined by a microplate reader.

2.3. LDH activity

LDH activity assay kit (Jiancheng Biotech, Nanjing, Jiangsu, China) was utilized to evaluate LDH level. Briefly, GC cells were seeded in 6-well plates at a density of 2×10^5cells/well. Following treatment with bufothionine, NADH and pyruvate were added to the culture medium samples and incubated for 15 min at 37 °C, 0.4 M NaOH was then added to stop enzymatic reaction. A microplate reader was used to detect absorbance at 450 nm.

2.4. Hoechst 33342/PI double staining

After treatment, GC cells were rinsed twice with cold 1× PBS and incubated with Hoechst/PI staining buffer for 15 min at 25 °C in the dark. Then the level of apoptotic cell death was visualized.

2.5. Flow cytometry analysis

Cell apoptosis was detected using an Annexin V-FITC/PI apoptosis kit (BD pharmingen, NJ, USA). Briefly, after treatment, the cells were seeded at a density of 2×10^5, and then incubated with Annexin V FITC and PI staining solution for 15 min. Apoptotic cell population was then quantified using a flow cytometer.

2.6. Western blotting

Briefly, the proteins in cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride membrane. Subsequently, the membranes were incubated with the primary antibodies overnight at 4 °C. Goat anti-rabbit IgG-HRP (Beyotime, Shanghai, China) served as the second antibodies and GAPDH served as an internal control to normalize protein levels. The signals were then visualized using chemiluminescent substrate (KPL, Guildford, UK).

2.7. iTRAQ assay

Following bufothionine treatment, AGS cells were dissolved in lysis buffer to extract proteins. The protein samples were then labeled with iTRAQ labeling reagents according to the manufacturer's instructions. LC analysis and tandem mass spectrometry analysis were applied

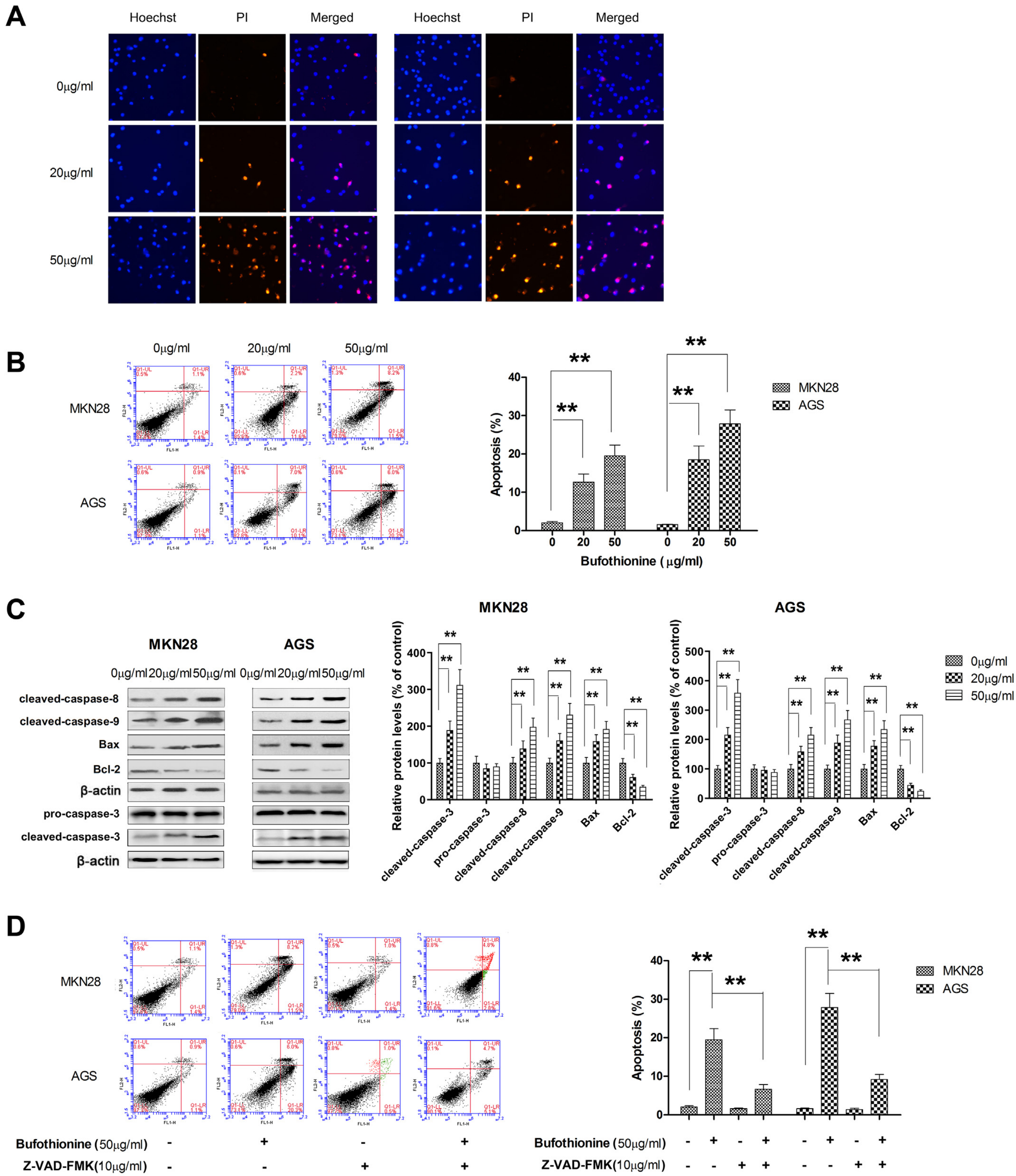

Fig. 2. Bufothionine promotes caspase-dependent apoptosis in MKN28 and AGS cells. GC cells were treated with bufothionine at 20 and 50 µg/ml for 48 h. A. Apoptotic cell population was assessed by Hoechst33342/PI double staining. B. Cell apoptosis was detected using Annexin V-FITC/PI apoptosis kit. C. The cleavage of caspase-3, caspase-8 and caspase-9 as well as expression of Bcl-2 and Bax was determined by western blotting. D. Pretreatment with specific caspase inhibitor z-VAD-FMK (10 µM, 4 h) blocked bufothionine-promoted cell apoptosis. **P < 0.01.

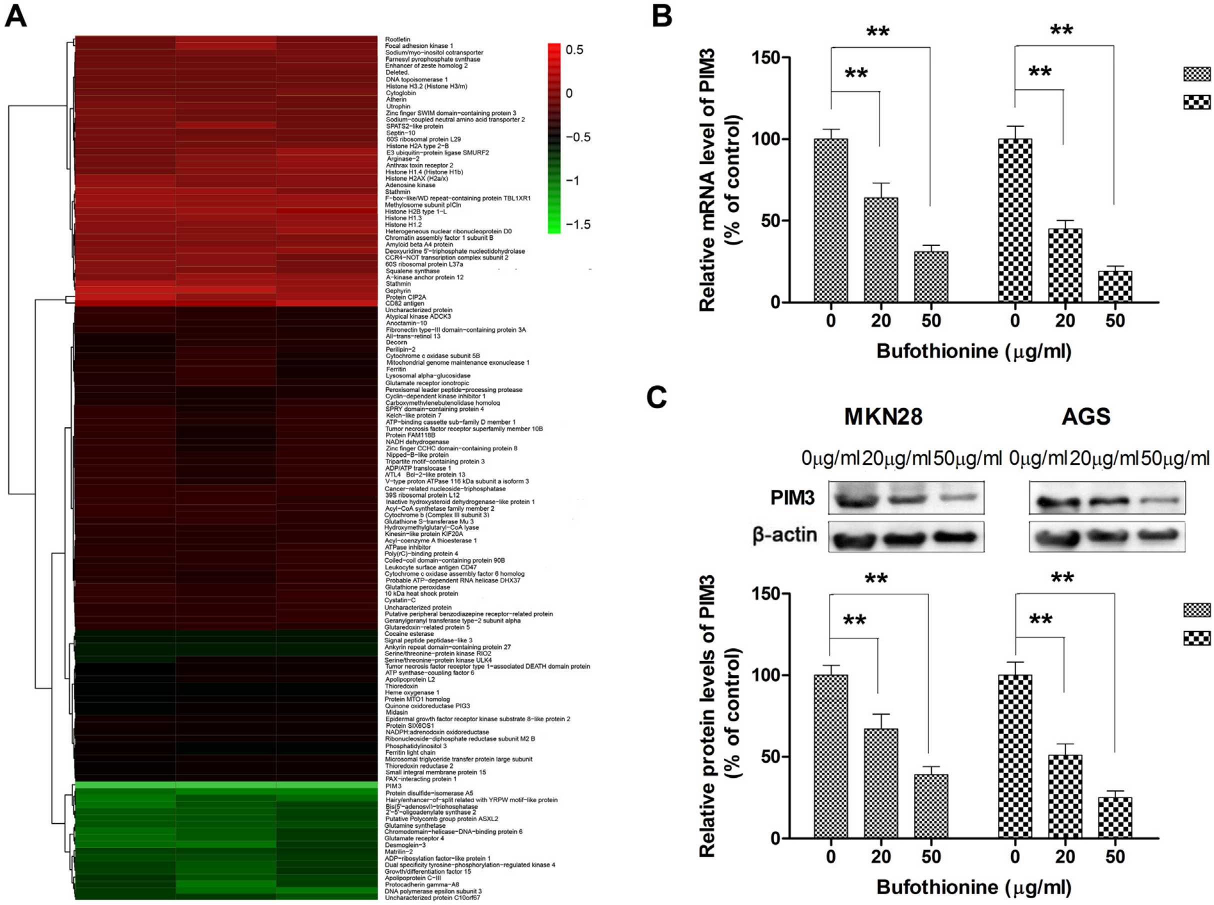

Fig. 3. Bufothionine downregulates PIM3 in GC cells. A. AGS cells were treated with bufothionine at 50 μg/ml for 48 h and differentially expressed proteins were identified by iTraq. B and C. MKN28 and AGS cells were treated with bufothionine at 20 and 50 μg/ml for 48 h and the expression levels of PIM3 mRNA and protein were examined, respectively. $^{**}P < 0.01$.

respectively to analyze the samples. Oebiotech (Shanghai, China) was used for protein identification and relative iTRAQ quantification. The cut-off values for the differentially expressed proteins were $P < 0.05$ and the fold change > 2.0 or < 0.5.

2.8. RNA extraction and qRT-PCR

Total RNA was extracted using the Trizol reagent (Life Technology, Carlsbad, CA) and then reversely transcribed to cDNA using reverse transcription kit (Promega, Madison, WI). The detection of mRNA levels of target genes was executed by qRT-PCR using Power SYBR Green PCR Master Mix (Carlsbad, CA), with GAPDH serving as the control. The primers purchased from Sangon (Shanghai, China) were as follows: forward, 5′-AAGGACGAAAATCTGCTTGTGG-3′; reverse, 5′-CGAAGTC GGTGTAGACCGTG-3′ [19]. The expression of target genes was quantified using the $2^{-\Delta\Delta Ct}$ method.

2.9. Small hair pin RNA (shRNA) silencing

shRNAs targeting PIM3 were synthesized by Sangong according to the previously described sequence [9]. shRNA (30 nM) was used to silence PIM3, and non-sense sequence served as the control. After transfection with Lipofectamine 3000 for 48 h, the cells were harvested for further analysis.

2.10. Ectopic PIM3 expression

The full-length wild-type human Pim-3 cDNA was subcloned into the pMEI-5 Neo retroviral expression vector. Retrovirus production and transduction were performed as described previously [20]. Forty-eight hours after transfection, PIM3 level was verified by western blot.

2.11. In vivo animal study

The Institutional Animal Care and Use Committee at Qingdao University approved the protocol for animal experiments. Eight-week-old male athymic BALB/c nu/nu mice were utilized in this study. AGS cells (1 × 107 cells/mL) were subcutaneously injected to the left side of mice for 21 days. Mice body weight and tumor volumes were assessed every three days until the 30th day. The cryostat sections (4 μm/section) of AGS cells xenograft tumors were stained using H&E and subjected to IHC as previously described [21]. Positive cells were scored in a semi-quantitative manner. Briefly, immunostained tumor sections were analyzed using Image J software and the proportion of Bcl-2, Bax, Pim3 and cleaved caspase3 positive cells expressing were estimated by an expert prostatic pathologist.

2.12. Biochemical parameters detection

Serum samples isolated from mice were used for the detection of routine biochemical parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urea nitrogen (BUN). The levels of ALT, AST and BUN were analyzed using all-automatic biochemical analyzer (Mindray BS-800, China).

2.13. Statistical analysis

All statistical analyses were conducted using SPSS software 16.0

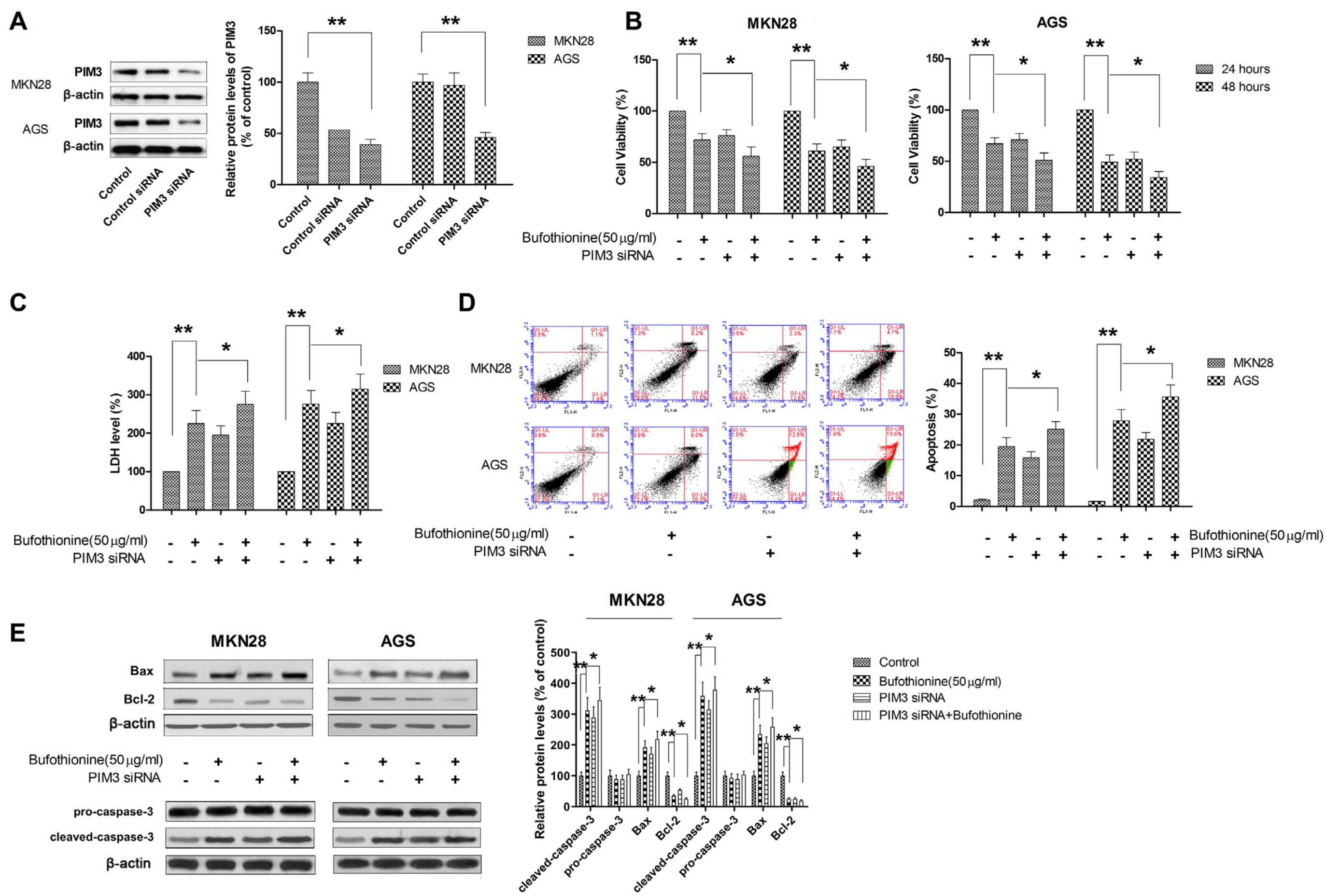

Fig. 4. Effect of bufothionine on MKN28 and AGS cells after transfection with PIM3 siRNA. GC cells were transfected with PIM3 siRNA and bufothionine treatment was started 48 h after transfection. A. 48 h post transfection, the protein expression of PIM3 was assessed by western blotting. B. Effect of the combination of bufothionine and PIM3 knockdown on cell viability was assessed by CCK-8 assay. C. Cytotoxicity of the combination of bufothionine and PIM3 knockdown on cell viability was examined by LDH assay. D. Pro-apoptotic activities of the combination of bufothionine and PIM3 knockdown were examined using Annexin V-FITC/PI apoptosis kit. E. Effect of the combination of bufothionine and PIM3 knockdown on the protein levels of cleaved caspase-3, Bax and Bcl-2. $^{**}P < 0.01$.

(SPSS Inc., Chicago, IL). Groups were compared by One-way ANOVA followed by Dunnett's *t*-test, and $P < 0.01$ was considered to be statistically significant.

3. Results

3.1. Bufothionine reduces cell viability in MKN28 and AGS cells

The chemical structure of bufothionine is displayed in Fig. 1A. Based on the preliminary experimental results, doses of 20, 50 and 100 μg/ml were chosen to test the effect of bufothionine on the cell viability of MKN28 and AGS cells. The results showed that MKN28 cells were more resistant to bufothionine-induced cell death. As shown in Fig. 1B, MKN28 cells displayed a marked loss in cell viability following bufothionine treatment at 50 and 100 μg/ml for 24 h. As for the case of 48 h treatment, bufothionine treatment at 20, 50 and 100 μg/ml caused significant reduction in cell viability. With regard to AGS cells, bufothionine treatment at 20, 50 and 100 μg/ml produced a dose-dependent cell death at both 24 and 48 h (Fig. 1B). The effect of bufothionine on the viability of normal gastric epithelial cells GES-1 was then determined. As shown in Fig. 1C, bufothionine treatment at 100 μg/ml significantly decreased the cell viability at 48 h. Similarly, bufothionine also effectively suppressed the clongenic potential of MKN28 and AGS cells in a dose-dependent manner (S2).Based on these findings, bufothionine treatment at 20 and 50 μg/ml for 48 h was chosen for the subsequent experiment. Compromised cell membrane integrity is a feature of cell death. Hence, LDH leakage assay was performed to examine whether bufothionine could destroy the cell membrane. As shown in Fig. 1D, bufothionine promoted LDH leakage in both MKN28 and AGS cells in a concentration-dependent manner. These findings supported the hypothesis that bufothionine promoted GC cell death.

3.2. Bufothionine facilitates caspase-dependent apoptosis in MKN28 and AGS cells

The role of apoptosis in bufothionine-induced cell death was first examined by Hoechst/PI staining. As shown in Fig. 2A, fragmented nuclei and condensed chromatins were observed in MKN28 and AGS cells following bufothionine treatment for 48 h. The percentage of apoptotic cell was then determined by flow cytometry. As shown in Fig. 2B, bufothionine treatment at 20 and 50 μg/ml significantly increased the apoptotic cell population in MKN28 cells to almost 10% and 20%, respectively. The results also showed that the level of cell apoptosis in AGS cells induced by bufothionine treatment was higher compared to that ofMKN28 cells (Fig. 2B). The pro-apoptotic activities of bufothionine on MKN28 and AGS cells were therefore further validated using apoptosis-related proteins. Elevated cleavage of caspase-3, caspase-8 and caspase-9 was observed in GC cells following bufothionine treatment (S3). In addition, bufothionine treatment led to a concentration-dependent upregulationofBcl-2 and downregulation of Bax, which further revealed the pro-apoptotic activities of bufothionine (Fig. 2C). Furthermore, bufothionine did cause obvious change on the

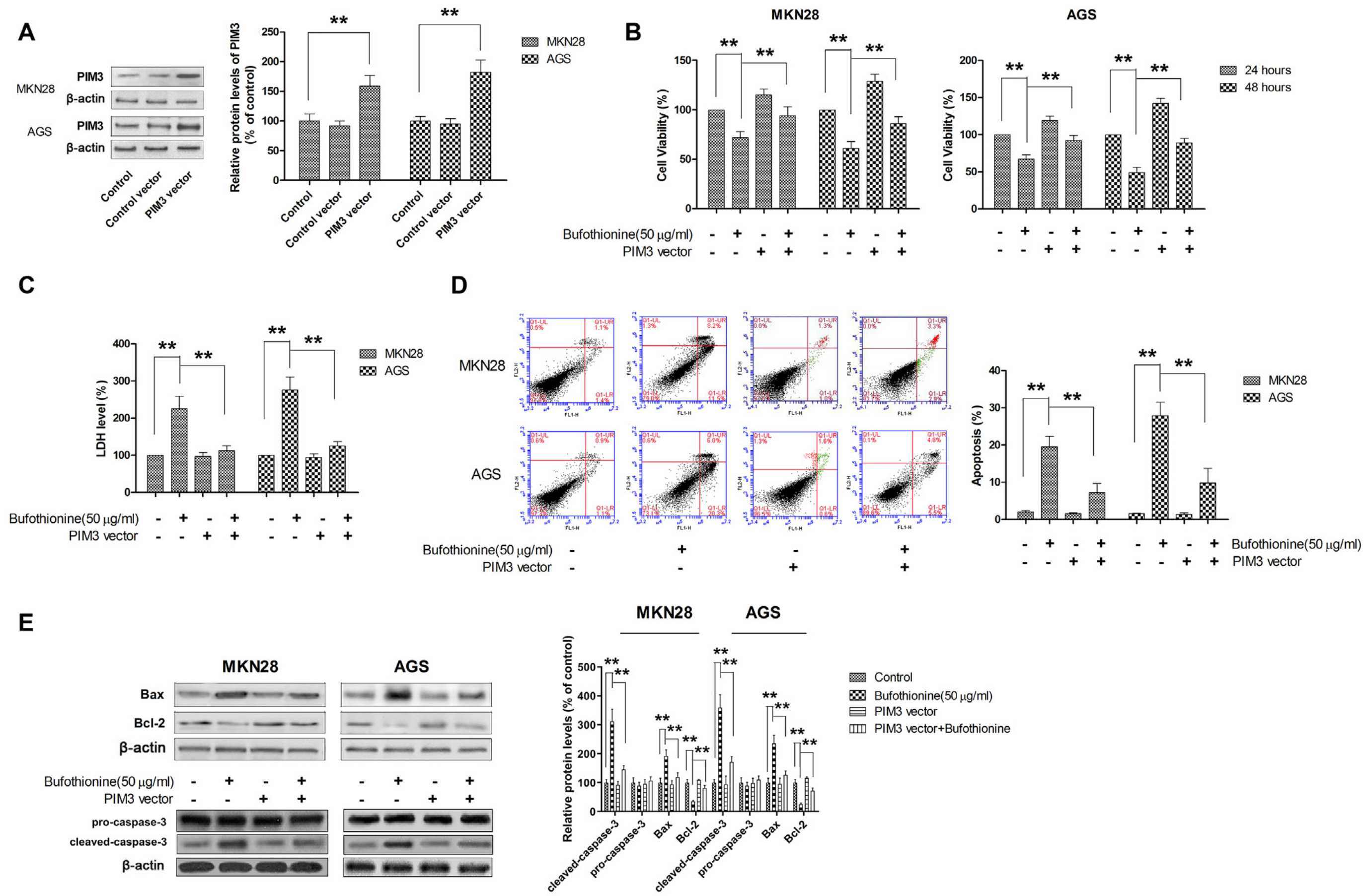

Fig. 5. Effect of bufothionine on MKN28 and AGS cells after transfection with PIM3 overexpressing vector. GC cells were transfected with PIM3 overexpressing vector and bufothionine treatment was started 48 h after transfection. A. 48 h post transfection, the protein level of PIM3 was assessed by western blotting. B. Effect of the combination of bufothionine and ectopic PIM3 expression on cell viability was assessed by CCK-8 assay. C. Cytotoxicity of the combination of bufothionine and ectopic PIM3 expression was assessed by LDH assay of cell viability. D. Pro-apoptotic activity of the combination of bufothionine and ectopic PIM3 expression was examined using Annexin V-FITC/PI apoptosis kit. E. Effect of the combination of bufothionine and ectopic PIM3 expression on the protein levels of cleaved caspase-3, Bax and Bcl-2 was examined by western blotting. **$P < 0.01$.

expression levels of apoptosis inducing factor (AIF) and endonuclease G (Endo G) (S3), which are two key genes that involved in caspase-independent manner. MKN28 and AGS cells were pretreated with specific caspase inhibitor z-VAD-FMK at 10 μM for 2 h before incubation with bufothionine at 50 μg/ml for 48 h. As shown in Fig. 2D, z-VAD-FMK did not cause any significant change on the number of apoptotic cells. However, pretreatment with z-VAD-FMK significantly blocked the pro-apoptotic activities of bufothionine in both MKN28 and AGS cells, which indicated that bufothionine promoted apoptotic cell death in a caspase-dependent fashion.

3.3. Bufothionine downregulates PIM3 in MKN28 and AGS cells

ITraq proteomic analysis was conducted to identify the potential target of bufothionine. After incubation with 50 μg/ml bufothionine for 48 h, the differentially expressed proteins in AGS cells are listed in Fig. 4A. The results from iTraq proteomic analysis showed that PIM3 expression was remarkably suppressed by bufothionine. Hence, PIM3 mRNA level was determined by qRT-PCR in both MKN28 and AGS cells. After bufothionine treatment, the mRNA level of PIM3 in GC cells was downregulated in a concentration dependent manner (Fig. 3B). Correspondingly, the PIM3 protein level was also repressed by bufothionine treatment (Fig. 3C).

3.4. PIM3 downregulation exacerbates anti-neoplastic activities of bufothionine

Based on the aforementioned results, experiments were designed to test the effect of PIM3 downregulation in the anti-neoplastic activities against GC cells. At first, the expression of PIM3 was decreased by targeted siRNA treatment (Fig. 4A). Fig. 4B shows that bufothionine reduced cell viability to a larger proportion in both MKN28 and AGS cells afterPIM3 knockdown. The results from LDH leakage assay also supported the finding that the cytotoxicity of bufothionine on MKN28 and AGS cells was more profound followingPIM3 knockdown (Fig. 4C). Flow cytometry results showed that PIM3 knockdown enhanced the apoptosis effect of bufothionine in GC cells (Fig. 4D). Further, PIM3 knockdown exacerbated the bufothionine-induced expression of cleavage of caspase-3 (Fig. 4E). At the same time, PIM3 enhanced bufothionine-induced upregulation of Bax and downregulation of Bcl-2 in GC cells (Fig. 4E). In the subsequent experiments, GC cells were transfected with PIM3 overexpressing vector to elevate PIM3 expression by > 150% compared with parental cells (Fig. 5A). The results from CCK-8 assay showed that ectopic PIM3 expression markedly compromised bufothionine-induced cell death in both MNK28 and AGS cells (Fig. 5B). The destructive effect of bufothionine in both GC cells lines was also decreased by ectopic PIM3 expression (Fig. 5C). Ectopic PIM3 expression significantly dampened the apoptosis-inducing effect of bufothionine on MKN28 and AGS cells, which was also validated by

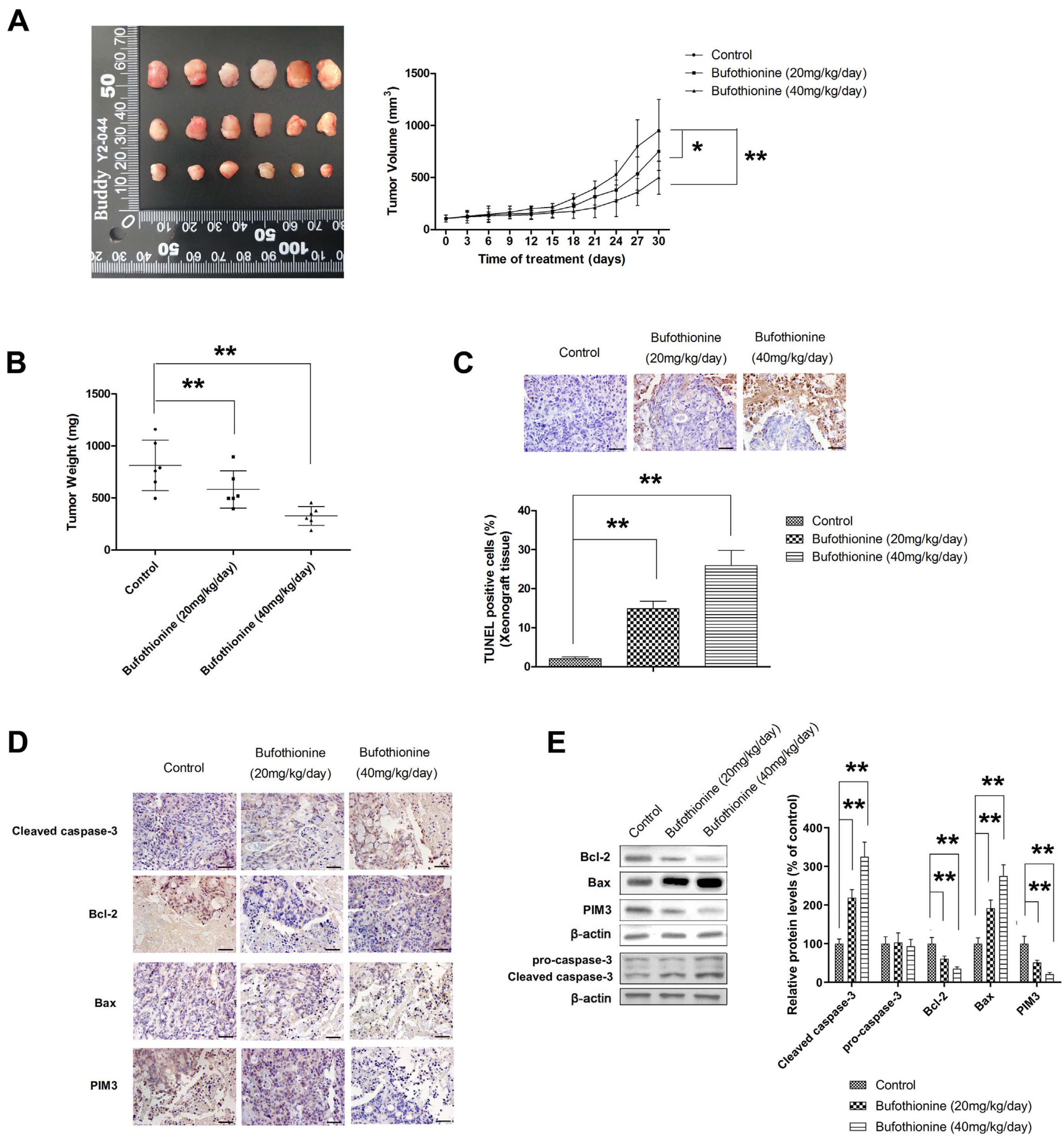

Fig. 6. Bufothionine suppresses xenograft tumor growth. The treatment started when the tumor reached 100 mm^3 and continued for 30 days before the mice were sacrificed. A. Bufothionine significantly suppressed tumor growth *in vivo*. B. The tumor was harvested at the end of the experiment and its weight was documented. C. The TUNEL assay was performed to quantitatively analyze cell apoptosis in the tumor tissue. D. The expression of PIM3, cleaved caspase-3, Bax and Bcl-2 in tumor tissues was visualized by IHC assay. E. The protein levels of PIM3, cleaved caspase-3, Bax and Bcl-2 in tumor tissues were quantitatively analyzed by western blotting. $^{**}P < 0.01$.

western blotting assay on cleaved caspase-3, Bcl-2 and Bax (Fig. 5E). These results indicated that PIM3 downregulation exacerbated the anti-cancer activities of bufothionine against GC cells.

3.5. The anti-growth activities of bufothionine against GC in vivo correlates with downregulation of PIM3

The anti-cancer activities of bufothionine against GC were also evaluated in a xenograft mouse model. Treatment with bufothionine for 30 days remarkably suppressed the tumor growth and tumor weight in a dosage-dependent fashion (Fig. 6A and B). Moreover, bufothionine treatment promoted apoptosis *in vivo* in a dosage-dependent manner (Fig. 6C). The results from IHC assay also showed that bufothionine treatment promoted caspase-3 cleavage expression, downregulatedBcl-2 and upregulated Bax, which correlated with PIM3 downregulation (Fig. 6D). The results from western blotting assay also supported the findings that, bufothionine treatment activated casapsae-3, upregulated Bax, and downregulated Bcl-2 and PIM3 in a dosage-dependent manner (Fig. 6E). The general toxicity of bufothionine was assessed by monitoring the changes of mice weight, levels of hepatic enzymes and urea nitrogen level. As shown in Supplementary Fig. 1A, bufothionine treatment at the indicated dosage did not significantly alter the body weight of xenograft mice model. Meanwhile, there was no significant change in hepatic and renal functions (Supplementary Fig. 1B and C). Moreover, no histological change was found in the liver, lung, kidney, heart and spleen tissues (Supplementary Fig. 1D). These results indicated that bufothionine was well-tolerated by murine models.

4. Discussion

Cinobufacini (termed as Huachansu in Chinese), is an animal-derived drug which has been employed in the treatment of human malignancies [22]. It has been found that cinobufacin suppresses proliferation and promotes apoptosis of GC cells [22,23]. In addition, Cinobufacin has been tried as an adjuvant treatment in the conventional chemotherapy in patients with GC, which showed that Cinobufacin could increase the response rate and disease the control rate of chemotherapy in advanced GC [24,25].Despite several reports have shown that bufadienolides was the main bioactive components of Cinobufacin. Little is known about which compound mainly contributes its suppressive role in GC. The results of the present study revealed that one of the bufadienolide compounds in Cinobufacin, bufothionine, promotes cell death and apoptosis of GC cells. In addition, it was found that PIM3 is the primary target responsible for the anti-cancer activities of bufothionine in GC. To sum up, bufothionine affected GC progression through down-regulating PIM3 level.

Apoptosis is a programmed cell death which can be triggered by external or internal stimuli. In fact, majority of chemotherapeutic agents eradicate malignant tumor cells by inducing apoptosis [26]. In the present study, it was found that bufothionine increased nuclear chromatin density, which reflected the condensation, coagulation, and fragmentation of the nuclear chromatin. Flow cytometric assay also showed that bufothionine dramatically promoted GC cell apoptosis. Activation of caspase cascade is the hallmark for apoptotic cell death [27]. Among the caspases, activation of casapse-8 and casapse-9 leads to apoptosis through extrinsic and intrinsic pathways, respectively [28]. The results indicated that bufothionine markedly elevated the intracellular level of cleaved caspase-3, cleaved caspase-8 and cleaved caspase9, suggesting the involvement of both intrinsic and extrinsic apoptotic pathways. Moreover, caspase inhibitor blocked the bufothionine-induced apoptosis, suggesting that bufothionine triggered caspase-dependent apoptosis. The apoptotic process is also tightly regulated by a series of pro-apoptotic and anti-apoptotic proteins, including Bax, Bak, Bid, Bcl-2, and Bcl-xL. Particularly, the interaction between Bax and Bcl-2 triggers mitochondrial membrane disruption and subsequent release of mitochondrial cytochrome *c* into the cytoplasm, which lead to the activation of caspase-cascade [29]. Here, it was observed that bufothionine upregulated pro-apoptotic protein Bax and downregulated anti-apoptotic protein Bcl-2, implying that mitochondrial dysfunction was involved in the bufothionine-induced apoptosis. These findings are in agreement with the report by Zhou which showed that bufothionine promoted cell apoptosis in mouse liver tumor cells by activating mitochondrial apoptosis signaling [18].

The pim family members possess serine/threonine kinase activity [30]. Recently, three kinases have been added to this family, termed as PIM1, PIM2, and PIM3 [31]. PIM1, as a proviral insertion site in Moloney murine leukemia virus T cell lymphomas, positively contributes to lymphomagenesis. On the other hand, PIM2 was initially identified as a proviral integration site [32,33] whereas PIM3 was initially identified as an oncogenic gene [34]. It was later found that PIM3 is expressed in normal brain and heart tissue [4,7,8]. Interestingly, PIM3 is unregulated in cancerous tissues, especially endoderm-derived tumors including the colon, liver, pancreas, and stomach [4,7,8]. Since then, small molecule Pim kinase inhibitors were developed [35,36]. An earlier study showed that a stemonamide synthetic intermediate, named T-18, exhibited anticancer activities against GC by functioning as a PIM3 inhibitor [37]. Our results showed that PIM3 is a potential therapeutic target for GC treatment. The experimental data in this study revealed that the anti-growth and pro-apoptotic activities of bufothionine were attributed to downregulation of PIM3. Moreover, the results provided compelling evidence that PIM3 is a key mediator of bufothionine-induced apoptosis of GC cells, which is consistent with studies in colorectal cancer, glioma and pancreatic cancer [6,38,39]. Several studies have shown that PIM3 positively contributes to metastasis [6], chemoresistance [40,41], and radio resistance [42]. Therefore, it is reasonable to believe that bufothionine might exhibit anti-metastatic activities or enhance the sensitivity to chemotherapy and radiotherapy by downregulating PIM3.

In summary, the findings in this study show that bufothionine suppresses GC growth *in vitro* and *in vivo*. Moreover, PIM3 may act as the primary molecular target that mediates the anti-cancer activities of bufothionine against GC. These findings provide novel insights into the chemotherapeutic potential of bufothionine for GC treatment.

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Author contribution

Guojun Wang designed the research, provided the fund and wrote the paper; Guanghui Liu, Yanwei Ye and Yang Fu performed experiments; Xiefu Zhang analyzed the data.

Declaration of Competing Interest

None.

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