Tetrandrine induces cell death in SAS human oral cancer cells through caspase activation-dependent apoptosis and LC3-I and LC3-II activation-dependent autophagy

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Abstract. Numerous studies have demonstrated that autophagy is associated with cancer development. Thus, agents to induce autophagy could be employed in some cases for the treatment of cancer. Our results showed that tetrandrine significantly decreased the viability of SAS cells in a concentration- and time-dependent manner. Tetrandrine induced nuclear condensation, demonstrated by DAPI staining. The early events in apoptosis analysed by Annexin V/PI staining indicated that the percentage of cells staining positive for Annexin V was slightly increased in SAS cells with tetrandrine treatment but was much lower following bafilomycin A1 pre-treatment. Tetrandrine caused AVO and MDC induction in SAS cells in a concentration-dependent manner by fluorescence microscopy. Tetrandrine also caused LC-3 expression in SAS cells in a time-dependent manner. Our results show that tetrandrine treatment induced the levels of cleaved caspase-3 in a concentration- and time-dependent manner. Tetrandrine treatment induced the levels of LC-3 II, Atg-5, beclin-1, p-S6, p-ULK, p-mTOR, p-Akt (S473) and raptor. Tetrandrine decreased cell viability, but bafilomycin A1, 3-MA, chloroquine and NAC protected tetrandrine-treated SAS cells against decrease of cell viability. Atg-5, beclin-1 siRNA decreased tetrandrine-induced cleaved caspase-3 and cleaved PARP in SAS cells and protected tetrandrine-treated SAS cells against decrease in cell viability. Chloroquine, NAC and bafilomycin A1 also decreased tetrandrine-induced cleaved caspase-3 and cleaved PARP in SAS cells. Our results indicate the tetrandrine induces apoptosis and autophagy of SAS human cancer cells via caspase-dependent and LC3-I and LC3-II-dependent pathways.

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Introduction

Oral cancer is one of the common cancers and is the fourth leading cause of cancer death in Taiwan male population but the 16th in females (1-3). Based on the 2008 report from the Department of Health, R.O.C. (Taiwan) indicated that 7.9 individuals per 100,000 die annually from oral cancer (1). It has been demonstrated that about 95% of these oral cavity cancers are squamous cell carcinomas and human oral cancer is largely associated with the risk factors of chronic smoking or alcohol consumption (4-6). Furthermore, the susceptibility of an individual to oral cancer is associated with the individual genetic factors and carcinogen-exposure behavior (7-9). In Taiwan, it was suggested that smoking and betel nut chewing are the two most important risk factors (1,10). Based on the above report the government indicated that approximately 85% of the oral cancer patients are regular users of betel quid (areca nut). The current treatments for oral cancer are inadequate and a significant proportion of oral cancer patients may develop local invasion and metastases (11). Numerous studies have been performed to find novel agents (especial from natural plant) which can trigger programmed cell death (apoptosis) in tumor cells and hopefully to provide a new therapeutic approach for anticancer design (12-14).

It is well documented that numerous natural compounds present anticancer effects and some of them play important roles in cancer treatment clinically. Tetrandrine ($C_{38}H_{42}O_8N_2$), a bisbenzylisoquinoline alkaloid, was isolated from the root of *Stephania tetrandra* S. Moore, has been used as an anti-inflammatory, antipyretic and analgesic herb in Chinese medicine (15-18), furthermore, in myocyte and

vascular smooth muscle cells, tetrandrine inhibits the $I_{Ca\text{-}L}$ and reduces Ca^{2+} flows into the cytosol (19,20). Tetrandrine has been found to induce cytotoxic effect such as the inhibiting proliferation and inducing apoptosis of various cancer cells such as breast cancer, lung cancer, neuroblastoma, Burkitt's lymphoma, hepatoma, glioma, leukemia and colon cancer (21-25). Tetrandrine induced G0/G1 phase arrest in Neuro 2a mouse neuroblastoma cells (26) and human Hep G2 cells (27). Tetrandrine have been demonstrated to inhibit lipid peroxidation and platelet aggregation and reduces ischemia/reperfusion injury (28-30) and tetrandrine suppressed T and B cells and inhibited the production of cytokines (29). It was reported that tetrandrine is cytotoxic to RT-2 glioma cells and it has antitumor effects on subcutaneous and intracerebral gliomas, and inhibits angiogenesis in subcutaneous gliomas (31-34). Tetradine induced apoptosis by activating reactive oxygen species and repressing Akt activity in human hepatocellular carcinoma HCC cells (35,36). Recently, it was reported that tetrandrine induced apoptosis and triggers caspase cascade in human bladder cancer cells (37).

There is no previous report on studies investigating the effects of tetrandrine on the cytotoxic on human oral cancer cells. In the present study, we investigated the antitumor effect of tetrandrine against human oral squamous carcinoma cells and the mechanism by which the association occurs. Using human oral cancer SAS cells, we demonstrated that tetrandrine exhibits antitumor activity on the cells by inhibiting the activity of the caspase pathway and inducing autophagy and apoptosis. Our study suggests that tetrandrine may be a potential agent to combat oral cancer cells.

Materials and methods

Cell culture and chemicals. Tetrandrine, dimethyl sulfoxide (DMSO), propidium iodide (PI), potassium phosphates, ribonuclease-A, Triton X-100, Tris-HCl and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS), penicillin, streptomycin and trypsin-EDTA were obtained from Gibco-BRL/Invitrogen Corp (Grand Island, NY, USA). The SAS cell line (human oral squamous cell carcinoma) was obtained from Dr Pei-Jung Lu (Graduate Institute of Clinical Medicine, National Cheng Kung University, Tainan, Taiwan). Cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in 75 cm^2 tissue culture flasks at 37˚C under a humidified 5% CO_2 and 95% air atmosphere.

Assessment of cell morphology and viability. Tetrandrine was prepared and dissolved in DMSO. SAS cells ($2.5x10^5$) were plated in 24-well plates in DMEM and incubated at 37˚C for 24 h. Cells were then treated with 0, 1, 5, 10, 15, 20, 25, 30, 35 and 40 μM tetrandrine for 24 and 48 h. DMSO was used as a vehicle control. For inhibitor treatment, cells were pre-treated with autophagic inhibitor (bafilomycin A1, 3-MA, chloroquine), caspase inhibitor (pan-caspase, caspase-3, caspase-8 and caspase-9 inhibitor) or NAC (Sigma Chemical Co) and then treated with 0, 5, 10, 15, 20, 25, 30, 35 and 40 μM tetrandrine for 24 and 48 h. At the end of the incubation period, cells were photographed with a phase-contrast microscope. They were then harvested, stained with PI (5 μg/ml) and analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA) (38-40).

DAPI staining. SAS cells were treated with or without 0, 1, 10, 20, 25 and 30 μM tetrandrine for 24 h. They were then isolated, stained with DAPI and photographed using a fluorescence microscope (38-40).

Annexin V and PI staining assay. SAS cells were treated with or without 0 and 25 μM tetrandrine for 24 h then were re-suspended in Annexin V-FITC (Becton-Dickinson, San Jose, CA, USA) alone or in combination with 10 ml of PI (50 mg/ml) and were incubated at room temperature for 15 min. The staining was analysed by flow cytometry (38-40).

Electron microscopy. SAS cells were treated with or without (0 and 25 μM) tetrandrine for 24 h then were fixed with a solution containing 2.5% glutaraldehyde and 2% paraformaldehyde (in 0.1 M cacodylate buffer, pH 7.3) for 1 h. After fixation, the samples were postfixed for 30 min in the same buffer containing 1% OsO_4. Ultra-thin sections were observed under a transmission electron microscope (JEM-1200EX, JEOL Ltd., Tokyo, Japan) at 100 kV (41).

Acridine orange staining. SAS cells were treated with 25 μM tetrandrine for 0, 2, 4 and 6 h and then cells were harvested and stained with 1 mg/ml acridine orange for a period of 20 min and analysed by a fluorescence microscope (41).

Monodansyl cadaverine MDC staining. SAS cells were treated with 0, 10, 20 and 30 μM tetrandrine for 6 h and then cells were harvested and stained with 1 mg/ml MDC for a period of 20 min and analysed by a fluorescence microscope (42).

GFP-LC3 expression. SAS cells were transfected with pEGFP-LC3 for 16 h and then were treated with 0, 10, 20 and 30 μM tetrandrine for 6 h. The cells were harvested, fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were stained with LC-3-FITC for a period of 20 min and analysed by a fluorescence microscope (41).

Western blot analysis. SAS cells were pre-treated with Atg-5 or beclin-1 siRNA bafilomycin A1 then treated with 0, 10, 15, 20, 25 and 30 μM tetrandrine and then harvested by scraping into RIPA buffer and sonicated for 15 min with 30 sec pulses. The protein concentration was measured using the BCA assay (Thermo Scientific) following the instructions of the manufacturer. Incubation with primary antibodies was done overnight at 4˚C. Immunoreactive proteins were visualised with the ECL chemiluminescent detection system (Perkin Elmer Life Sciences, MA, USA) and BioMax LightFilm (Eastman Kodak, New Heaven, CT, USA) according to the manufacturer's instructions (43).

Statistical analysis. Data of control and experimental groups were expressed as mean ± standard deviation for at least three separate experiments. Statistical analyses of the data were performed using Student's t-test and one-way analysis of variance (ANOVA). Statistical significance was set at $P<0.05$ (43).

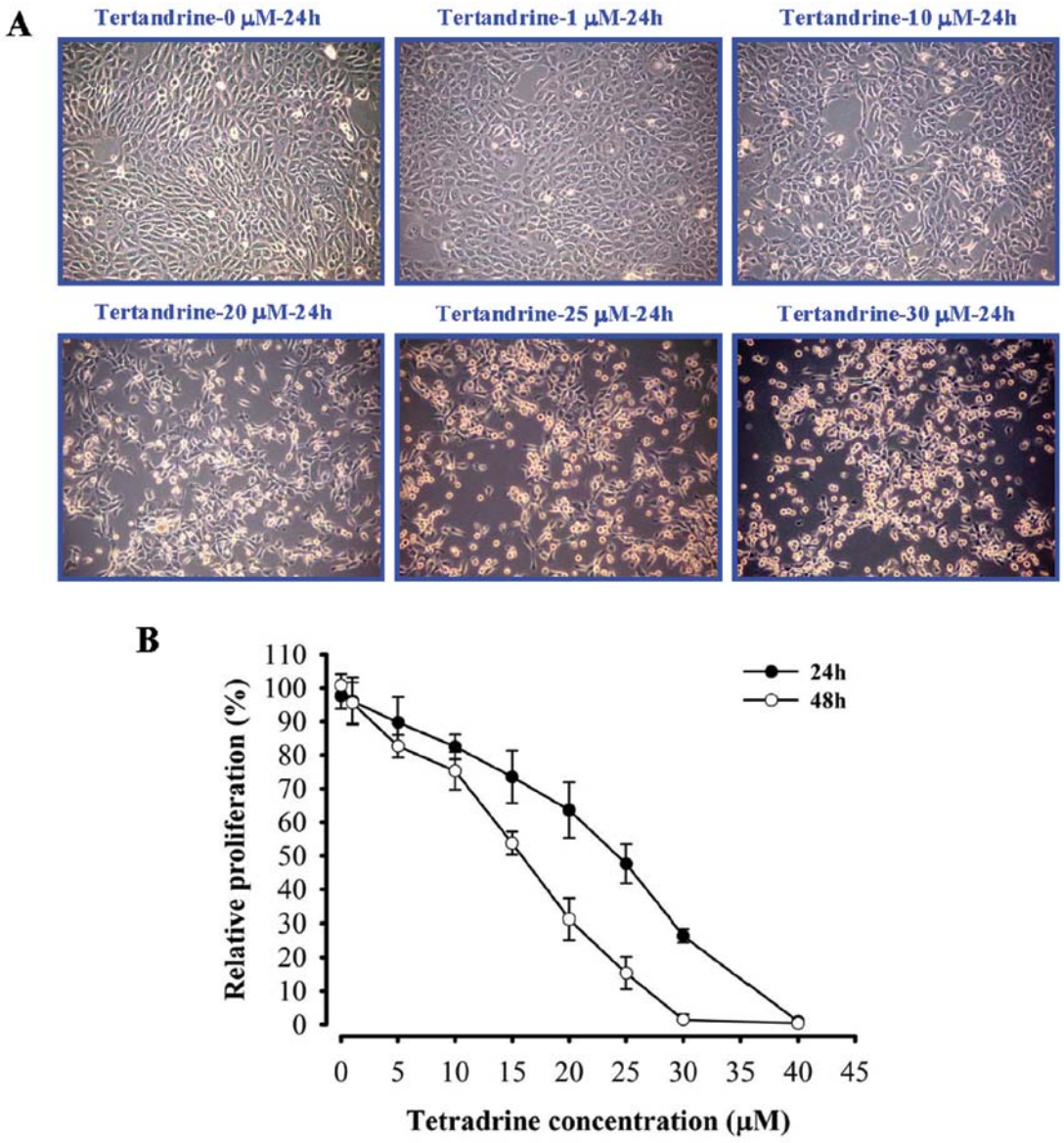

Figure 1. Tetrandrine induced cell morphological changes and decreased the percentage of viable SAS cells. Cells in 24-well plate were treated with 0, 1, 5, 10, 15, 20, 25, 30, 35 and 40 μM of tetrandrine for various time periods. (A) Cells were examined and photographed under contrast phase microscope at x200, then harvested for measuring (B) the percentage of viability by using flow cytometeric assay as described in Materials and methods. Significantly different from the control and tetrandrine treated groups at $^{*}p<0.05$.

Results

Effects of tetrandrine on cell morphology and viability in SAS cells. Cells were morphologically altered by tetrandrine treatment as shown in Fig. 1A. We tested the cell viability of tetrandrine in SAS cell lines. SAS cells were treated with 0, 5, 10, 15, 20, 25, 30, 35 and 40 μM tetrandrine for 24 and 48 h. Tetrandrine significantly decreased the growth of cells in a concentration- and time-dependent manner (Fig. 1B).

Tetrandrine-induced apoptosis in SAS cells. Induction of apoptosis by tetrandrine in SAS cells was confirmed by DAPI staining, as seen in Fig. 2B, which showed that tetrandrine induced nuclei condensation. These effects were dose-dependent as noted in Fig. 2B. Higher concentrations of tetrandrine resulted in a greater number of apoptotic cells being stained. We analysed surface exposure of phosphatidylserine, an early event in apoptosis by Annexin V/PI staining. The percentage of cells staining positive for Annexin V was slightly increased in SAS cells types after 24 h of tetrandrine treatment but was much lower following bafilomycin A1 of pre-treatment.

Tetrandrine induced autophagy in SAS cells. Autophagic vacuoles containing cellular membranous structures were increased in SAS-treated with tetrandrine for 24 h compared with untreated cells, as determined by electron microscopy (Fig. 3A). We used Acridine orange staining to AVOs, including autophagic vacuoles and lysosomes. Cells with AVOs had enhanced red fluorescence that was detected by fluorescence microscopy (Fig. 3B). Tetrandrine caused MDC induction in SAS cells in a concentration-dependent manner (Fig. 3B). Tetrandrine also caused LC-3 expression in SAS cells in a time-dependent manner.

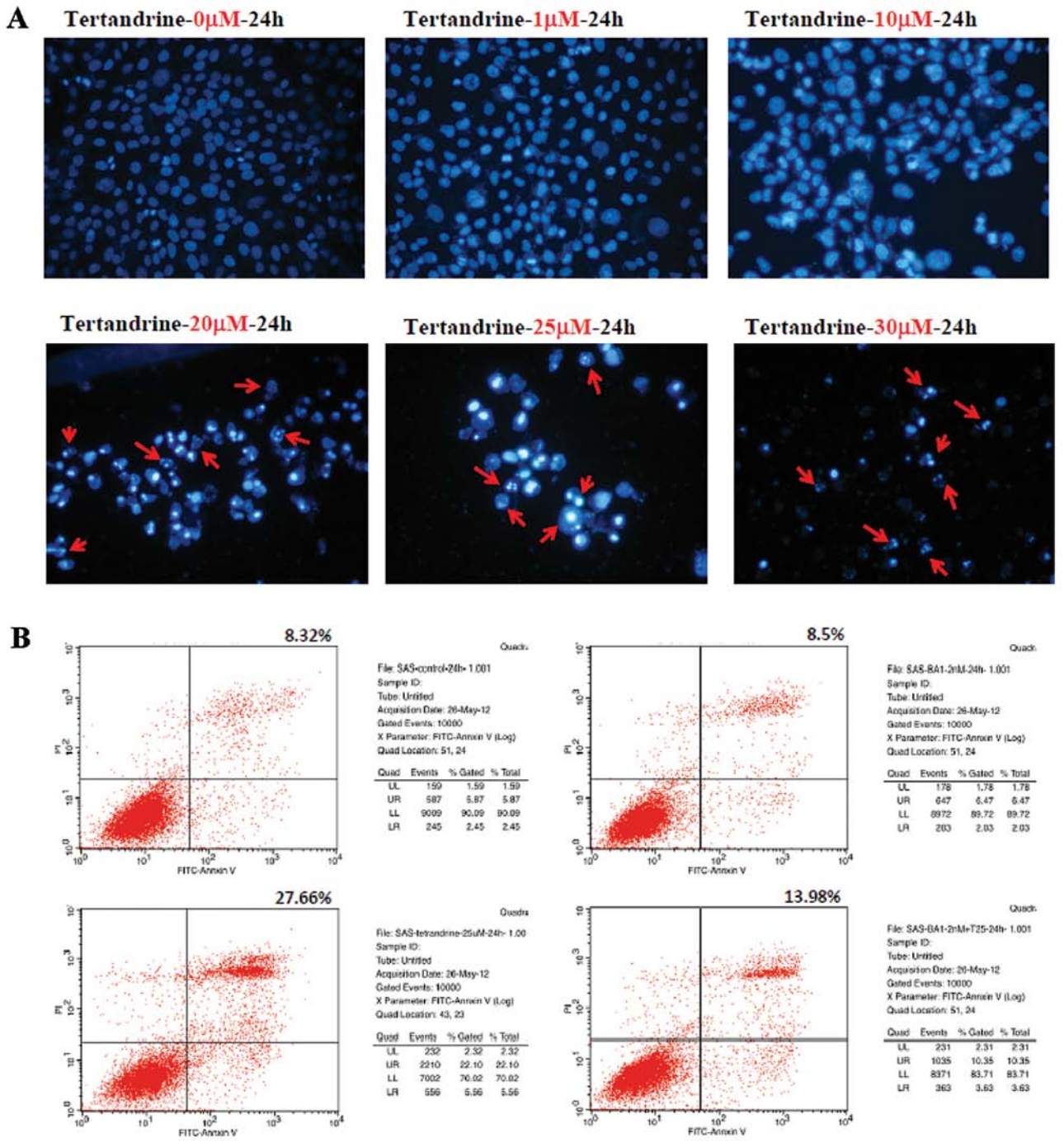

Figure 2. Tetrandrine induced DNA condension and apoptosis in SAS cells. (A) Cells in 12-well plate were treated with 0, 1, 10, 20, 25 and 30 μM of tetrandrine for 24 h. Cells were harvested and stained with DAPI and then photographed using a fluorescence microscope or (B) cells were treated with 0 and 25 μM tetrandrine for 24 h then re-suspended in Annexin V-FITC alone or in combination with 10 ml of PI (50 mg/ml) and were incubated at room temperature for 15 min. The staining was analysed by flow cytometry.

Effects of tetrandrine on levels of proteins associated with apoptosis and autophagy. Results are presented in Fig. 4A, tetrandrine treatment induced the levels of cleaved-caspase-3 in a concentration- and time-dependent manner. Tetrandrine treatment induced the levels of LC-3 II, Atg-5, beclin-1, p-S6, p-ULK, p-mTOR, p-Akt (S473), and raptor in a concentration- and time-dependent manner (Fig. 4B).

Autophagy inhibitors decreased tetrandrine-induced apoptosis in SAS cells. Results are presented in Fig. 5A, tetrandrine decreased the cell viability, but bafilomycin A1, 3-MA, chloroquine and NAC protected tetrandrine-treated SAS cells against the decrease of cell viability. On the other hand, pan-caspase, caspase-3, caspase-8 and caspase-9 inhibitor also protected tetrandrine-treated SAS cells against the decrease of cell viability (Fig. 5B).

Atg-5, beclin-1 siRNA, bafilomycin A1 and NAC decrease tetrandrine-induced cleaved caspase-3 and cleaved PARP in SAS cells. Results are presented in Fig. 6A, tetrandrine

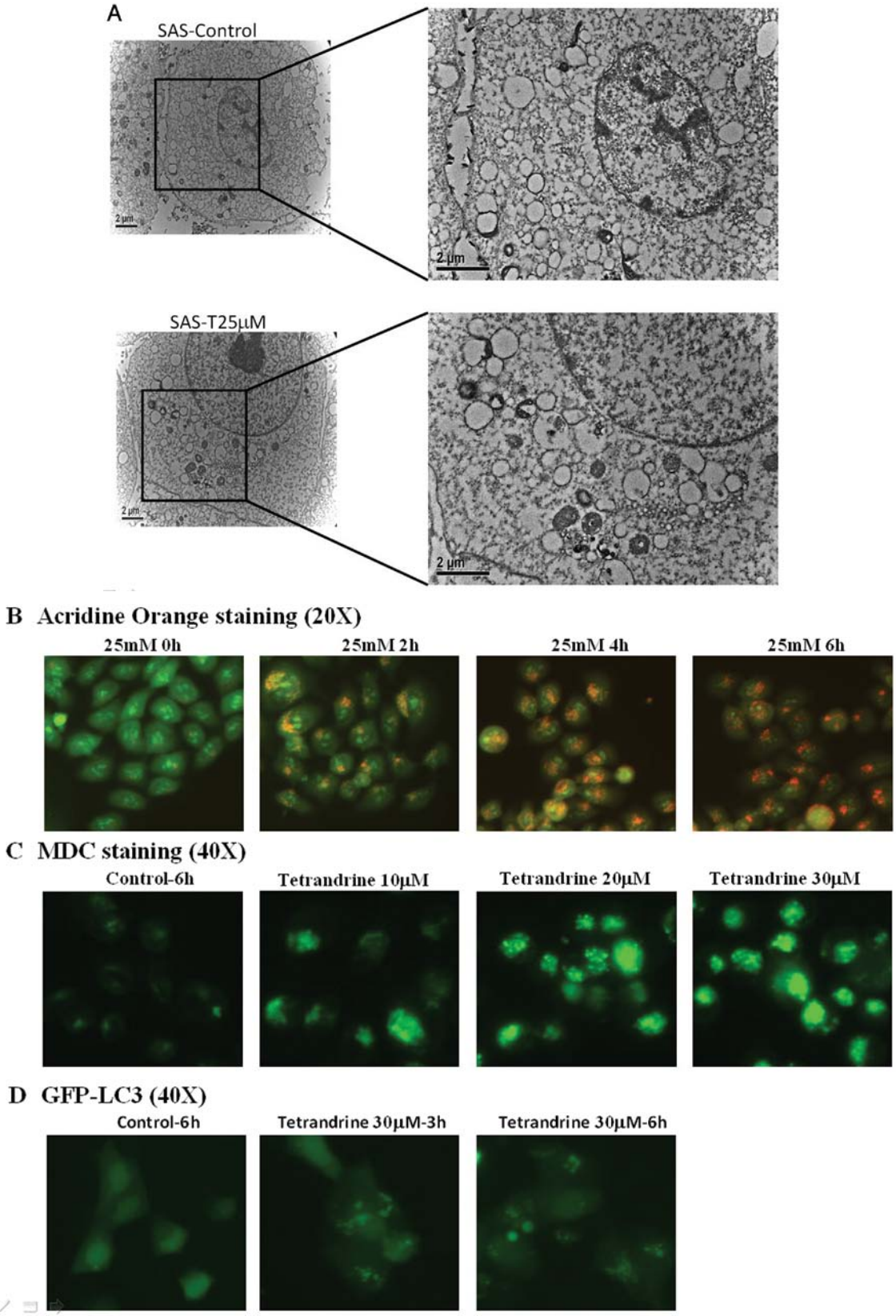

Figure 3. Tetrandrine-induced apoptosis and autophagy in SAS cells. SAS cells were treated with 0 and 25 μM tetrandrine for 24 h then all samples were postfixed for 30 min in the same buffer containing 1% OsO_4. (A) Ultra-thin sections were observed under a transmission electron microscope at 100 kV. (B) SAS cells were treated with 25 μM tetrandrine for 0, 2, 4 and 6 h then cells were harvested and stained with 1 mg/ml acridine orange for a period of 20 min and analysed by a fluorescence microscope. (C) Monodansyl cadaverine (MDC) staining. (D) GFP-LC3 expression.

treatment induced the levels of cleaved caspase-3 and cleaved PARP, however, Atg-5, beclin-1 siRNA decreased tetrandrine-induced cleaved caspase-3 and cleaved PARP in SAS cells and protected tetrandrine-treated SAS cells against

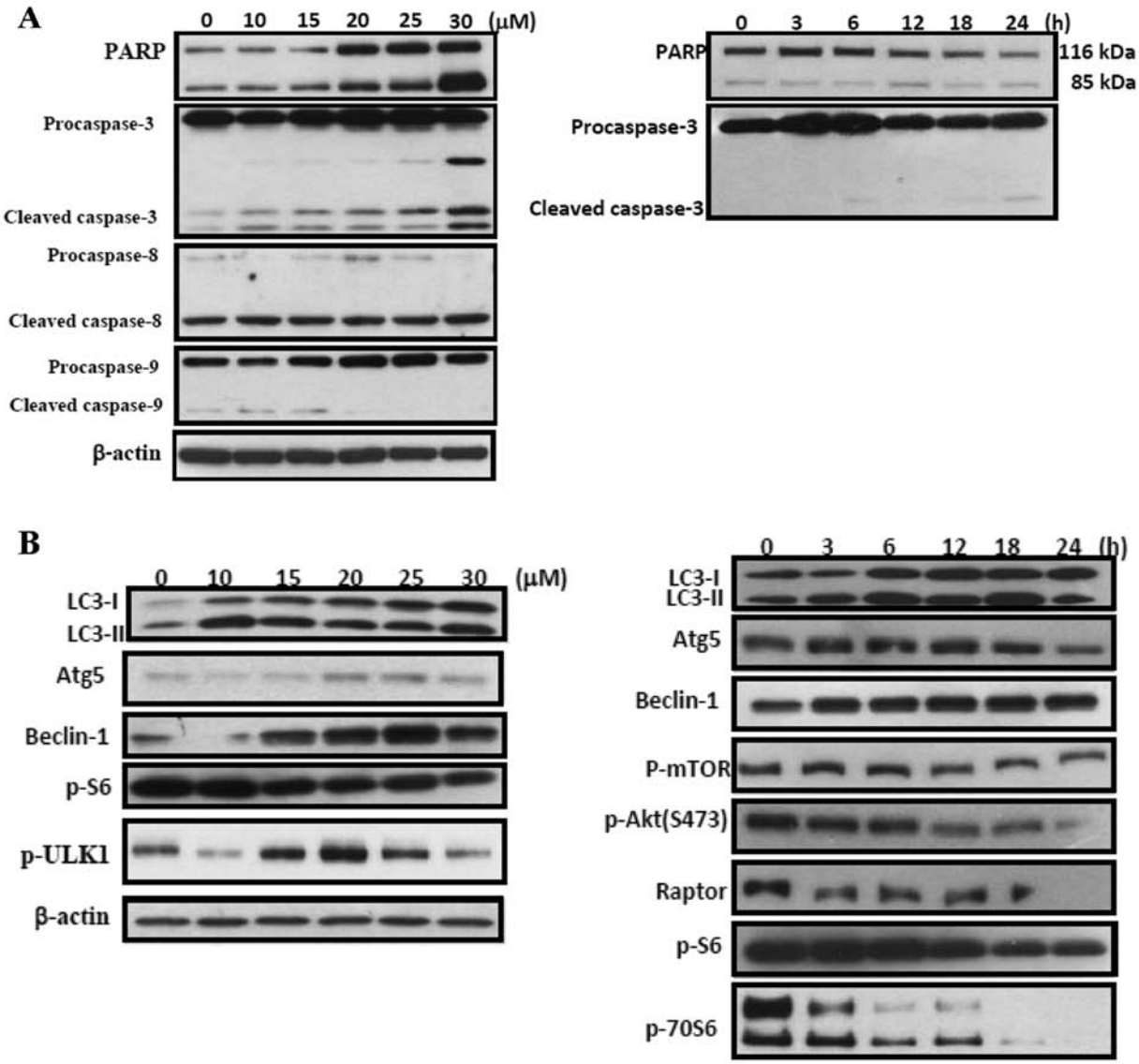

Figure 4. Tetrandrine affects the protein levels of apoptosis and autophagy in SAS cells. SAS cells were treated with 0, 10, 15, 20, 25 and 30 μM tetrandrine and then harvested by scraping into RIPA buffer and sonicated for 15 min with 30-sec pulses. The protein concentration was measured using the BCA assay then incubated with primary antibodies overnight at 4˚C. Immunoreactive proteins were visualised with the ECL chemiluminescent detection system and BioMax LightFilm (A and B) as described in Materials and methods.

decrease of cell viability. In Fig. 6B, chloroquine, NAC and bafilomycin A1 also decreased tetrandrine-induced cleaved caspase-3 and cleaved PARP in SAS cells. In Fig. 6C, bafilomycin A1 decreased tetrandrine-induced LC3 expression in SAS cells.

Discussion

Oral cancer is most frequent in men between 55 and 65 and in women between 50 and 75, and in men this disease is the fourth most common cause of cancer death (44,45). The treatment for oral cancer is still unsatisfactory. Thus, it is necessary to identify and develop new anticancer agents that will selectively target the tumor. It was reported that the induction of apoptosis through the caspase cascade triggered by tetrandrine has inhibitory effects to various tumor cells (20,21,27,37). Many studies have demonstrated that tetrandrine has pharmacologic potential in cancer therapy but to date the effects of tetrandrine on human oral cancer is little known and have not been identified and reported in oral cancer cells. In the present study, we demonstrated the anticancer effect of tetrandrine on oral SAS cancer and elucidate the underlying mechanisms of action. Tetrandrine treatment showed growth inhibitory effect and cell apoptosis and autophagy induction on oral cancer SAS cells, which was associated with caspase activation (Fig. 4) and autophagic vesicle formation (Fig. 3). To the best of our knowledge, this is the first report of the effects of tetrandrine on human oral cancer.

The current studies indicate that SAS cells are sensitive to the cytotoxic actions of the tetrandrine and the sensitivity was largely similar to that observed previously in the liver and bladder tumor cell lines (27,31,35,37). Based on the

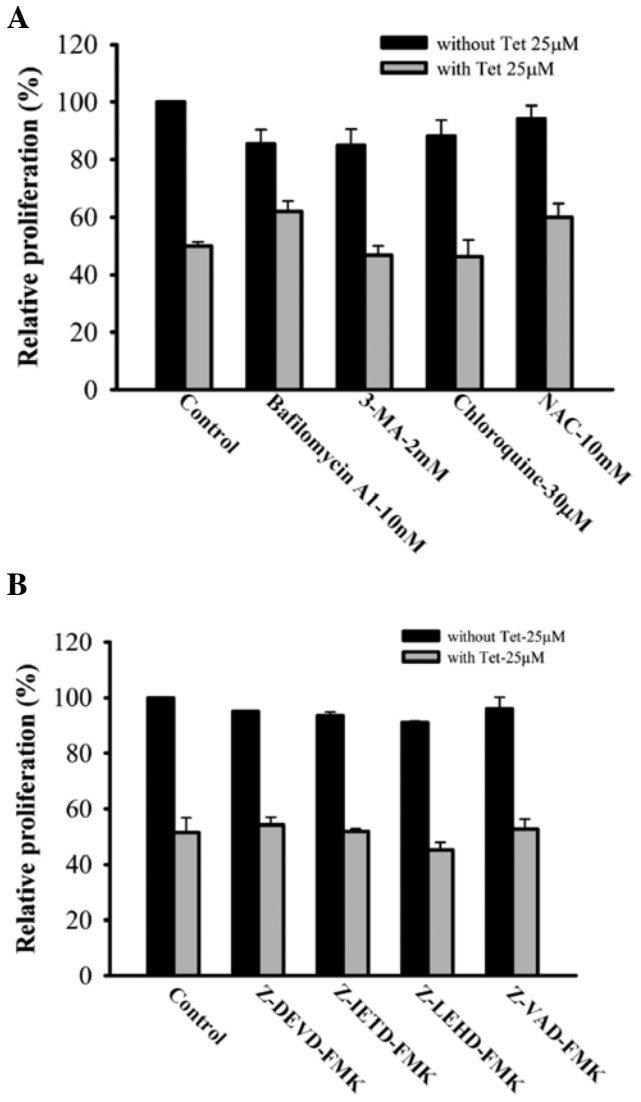

Figure 5. Bafilomycin A1, 3-MA, chloroquine, NAC and caspase inhibitor protected tetrandrine-treated SAS cells against decrease of cell viability. (A) Cells in 12-well plate were pre-treated with bafilomycin A1, 3-MA, chloroquine, NAC and (B) caspase inhibitor for 1 h then treated with 25 μM of tetrandrine for various time periods. Cells were examined and photographed under contrast phase microscope at x200 then harvested for measuring the percentage of viability by using flow cytometeric assay as described in Materials and methods. Significantly different from the control and tetrandrine treated groups at $^{*}p<0.05$.

observation and results of Annexin V and electro-microscope examination indicated that tetrandrine induced cell death may be through the formation of autophagy and the induction of apoptosis in SAS cells (Fig. 2). Autophagy from several complementary assays provided evidence for the formation of autophagic vesicles in SAS cells and LC3-I and II expression which confirmed the occurrence of autophagic flux. This is in agreement with a report on human hepatocellular carcinoma (HCC) cells after exposure to tetrandrine inducing autophagy (17). Tetrandrine also promoted apoptosis in SAS cells, although at low levels, however, both may lead to cell death. In contrast, tetrandrine appears to preferentially promote autophagy in SAS cells.

Based on other reports autophagy is quite controversial, for example, substantial evidence shows autophagy may occur through cytoprotective or through cytotoxic functions in different cancer cells (46,47). Herein, our results confirm that autophagy mediates the cell-killing effects of tetrandrine on SAS cells. The main reason is that the observations of autophagy from tetrandrine treated SAS cells was blocked by using preatment of 3-MA, NAC, chloroquine or bafilomycin that demonstrated only a modest degree of protection from the tetrandrine (Fig. 5). It may be due to autophagy being blocked thus leading to the tetrandrine-treated cells to cause apoptosis. These reasons are also seen from other studies (48,49). These observations indicate that SAS cells exposed to tetrandrine may be lethal to the tumor cells and that the cells may die by an alternative pathway if the primary pathway is blocked. Thus, further investigation is needed by *in vivo* experiments to investigate whether or not tetrandrine can be used as a potential anti-oral cancer agent. Other reports have demonstrated that if an agent is capable of promoting autophagy it is likely to have clinical utility (50-54).

We demonstrated that tetrandrine induced apoptosis in SAS cells based on the results from flow cytometric assay (Fig. 2) and this may involve the activations of caspase-8, -9 and -3 (Fig. 4). Therefore, the possibility of mitophagy exists, at the low dose of tetrandrine and then selective removal of damaged mitochondria. However, other studies demonstrated that tetrandrine induced the release of cytochrome *c* but did not cause the activation of the caspases or apoptosis in human hepatocellular carcinoma cells (27). It was reported that during mitophagy (55), cell did not show caspase activation in apoptotic cell death. Our results show that tetrandrine promoted the expressions of LC3-I and -II (Fig. 4) and it is well documented that LC3-I and -II are involved in the development of autophagy and both proteins play an important role during autophagy (56-58). Furthermore, other proteins such as Atg-5, Atg-7 and beclin-1 are also involved in the development, especial Atg-5 is essential for autophagy (59-62). In this study, we found that protein levels of Atg-5 and beclin-1 (Fig. 4) were increased after tetrandrine treatment in SAS cells. As shown in Fig. 6, tetrandrine-induced autophagy in SAS cells is dependent on the upregulation of Atg-5 and beclin-1. However, tetrandrine did not affect the levels of Atg-7 in SAS cells, but other reports show that tetrandrine promoted the expression of Atg-5 and Atg-7 in HCC cells (17). The detailed mechanism of Atg-5 and Atg-7 in tetrandrine treated SAS cells will also require further investigation as other factors may be involved. We also used siRNA to knock down Atg-5, beclin-1, and LC3-1 and -II. The results show affect on Atg-5 and beclin-1 but not on LC3-I and LC3-II in SAS cells. After the knockdown of Atg-5 and beclin-1 by siRNA the cell proliferation was decreased; however, no significant reduction was observed in LC3-1 and LC3-II siRNA treatment (Fig. 6).

Taken together, we found that tetrandrine may be a promising chemotherapeutic agent with a variety of anticancer effects such as induction of autophagy and apoptosis in human oral cancer SAS cells *in vitro*. Tetrandrine treatment induced cancer cells to undergo apoptosis and autophagy. The upregulation of Atg-5, and beclin-1 was essential to the induction of tetrandrine-induced autophagy in SAS cells (Fig. 4). These findings suggest that tetrandrine may be a potential clinical candidate for the treatment of SAS.

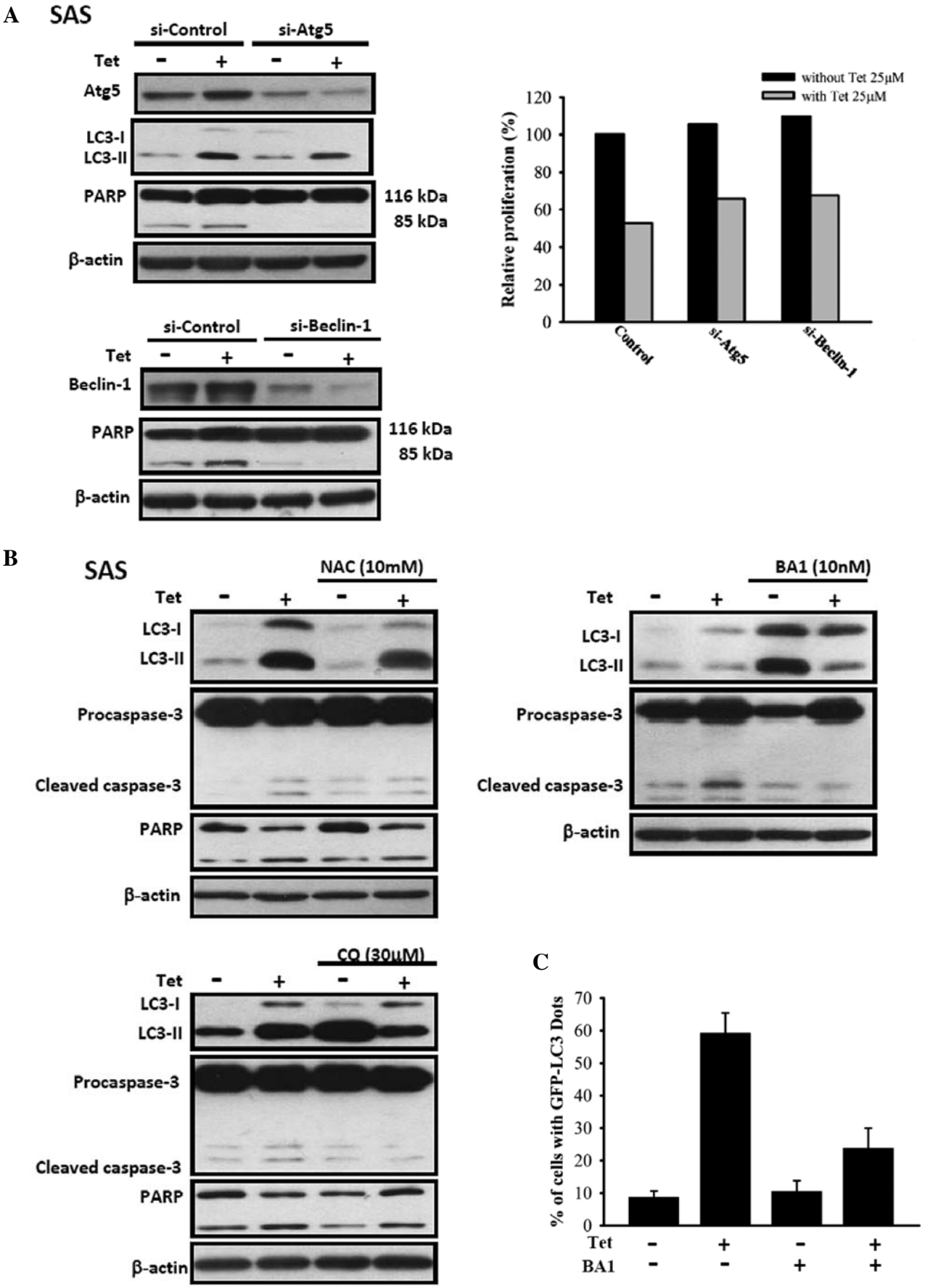

Figure 6. Atg-5, beclin-1 siRNA, bafilomycin A1 and NAC decreased tetrandrine-induced cleaved caspase-3 and cleaved PARP in SAS cells. Cells in 75-T plate were pre-treated with Atg-5, beclin-1 siRNA, bafilomycin A1 and NAC for 12 h then treated with 25 μM of tetrandrine for various time periods. The protein concentration was measured using the BCA assay then incubation with primary antibodies was done overnight at 4˚C. Immunoreactive proteins were visualised with the ECL chemiluminescent detection system and BioMax LightFilm (A and B) as described in Materials and methods.

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