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Flaccidoxide Induces Apoptosis Through Down-regulation of PI3K/AKT/mTOR/p70S6K Signaling in Human Bladder Cancer Cells

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Abstract. *Background/Aim: Urothelial carcinoma (UC) is the most common type of genitourinary cancer with high incidence and mortality rates in men. In this study, we used the BFTC-905 and T24 bladder cancer cell lines as in vitro models to investigate the pathways involved in flaccidoxide-induced apoptosis. Materials and Methods: We utilized MTT assays, colony assays, wound-healing assays and fluorescence with TUNEL to confirm the cytotoxicity of flaccidoxide in bladder cancer cell lines. Potential proliferative and apoptotic molecular mechanisms were evaluated by western blotting. Results: The expression of anti-apoptotic proteins Bcl-2 and phosphorylated Bad (p-Bad) was attenuated with an increasing flaccidoxide concentration, while the expression of proapoptotic proteins Bax, Bad, cleaved caspase-3, cleaved caspase-9 and cleaved PARP-1 was found increased. Additionally, phosphorylation of phosphoinositide 3-kinases (PI3K), protein kinase B (AKT) and mammalian target of rapamycin (mTOR) in the PI3K/AKT/mTOR pathway was reduced, leading to a reduction in the phosphorylation of downstream 70-kDa ribosomal protein S6 kinase 1 (p70S6K), S6 ribosomal protein (S6) and eukaryotic translation initiation factor 4B (eIF4B). However, eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) protein phosphorylation was increased due to attenuation of the upstream phosphorylation of mTOR protein. Conclusion: Flaccidoxide-induced apoptosis in BFTC-905 and T24 cells is mediated by mitochondrial dysfunction and down-regulation the PI3K/AKT/mTOR/p70S6K signaling pathway.*

Urothelial carcinoma (UC) is a lesion of urinary tract epithelial cells and is also known as renal transitional cell carcinoma (TCC), as it arises from the transitional epithelium (1). UC often has a heterogeneous cell population that accounts for more than 90% of all bladder cancers, and may occur in the kidneys, ureter or bladder (2, 3). Gender differences in the prevalence of bladder cancer exist, being higher in men than in women in all countries. The mortality of bladder cancer ranks 13th of all diseases worldwide; countries undergoing rapid economic transformation have a higher mortality, while developed countries have lower rates (4, 5). As the population ages, UC is becoming a serious issue, and in particular the incidence of UC increases with age in women (6, 7). Studies have shown that risk factors for bladder cancer include smoking (8), schistosomiasis (9), occupational exposure to aromatic amines and certain chemicals (10), exposure to arsenic in drinking water (11), genetic mutations (12) and type 2 diabetes (13). Despite advances in molecular pharmacological research, bladder cancer is still a healthcare management challenge. Currently, most drugs approved for bladder cancer treatment are only effective in the early stages of cancer, and few new drugs have shown promise in clinical trials. Therefore, the

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development of new diagnostic techniques and treatments is important for the treatment of bladder cancer worldwide (14).

UC is mostly chemotherapy-sensitive and thus difficult to treat. Currently, the success of treatment depends on early detection, and treatment options include transurethral resection (15), partial or radical cystectomy of the bladder (16), immunotherapy or chemotherapy (17) and radiotherapy (18). However, post-operative follow-up is required, and the selection of treatment considers the cancer cell type, stage, and the patient's age and overall physical condition, with the goal of improving survival (19).

Surgical removal of the tumor is still the main treatment for early-stage UC, and the treatment planning may include chemotherapy or radiation therapy in order to improve the prognosis of survival and preserve bladder and urinary functions (20). However, in the case of metastasis to other organs, whereupon surgery is not suitable, systemic chemotherapy often becomes the only option for treatment (21). Commonly used anti-cancer drugs include mitomycin c, doxorubicin, epirubicin and thiotepa. Bacillus Calmette-Guerin (BCG) is a commonly-used immunomodulator administered into the bladder through a catheter in the urethra, which triggers the immune response of the host to destroy cancer cells (22, 23). Chemotherapy is currently one of the most helpful approaches to treat many cancer types, as it delivers drugs that directly inhibit tumor cell growth or destroys them. The key pathway of chemotherapy is to induce apoptosis in cancer cells under certain physiological or pathological conditions (24). During apoptosis, the expression of Bcl-2 protein on the mitochondrial membrane decreases, the expressions of pro-apoptotic proteins (Bax, Bad, Bid and Bim) increase, and the expressions of anti-apoptotic proteins (Bcl-xl, p-Bad and Mcl-1) decrease. Additionally, an increase in the mitochondrial membrane permeability leads to release of cytochrome *c* into the cytosol, resulting in activation of caspase-9 protein and caspase-3, followed by caspase-3 activating its substrate, poly (ADP-ribose) polymerase-1 (PARP-1). The process causes intracellular nuclear chromatin condensation and degradation, leading to DNA fragmentation and killing of cancer cells (25).

In recent years, many natural compounds derived from marine soft corals have been shown to have anti-tumor effects that may destroy cancer cells through apoptosis-mediated pathways (26). 11-Dehydrosinulariolide, an active metabolite isolated from soft coral *Sinularialeptoclados*, was found to induce apoptosis in oral carcinoma Ca9-22 cells through mitochondrial dysfunction and the endoplasmic reticulum (ER) stress pathway (24); it also triggers cell death in human melanoma A2058 cells *via* similar pathways (27). Neoh *et al*. (28) found that flaccidoxide-13-acetate inhibits cell migration and invasion of bladder cancer cells by modulating the expression of MMP-2 and MMP-9 through the FAK/PI3K/AKT/mTOR pathway. Wu *et al*. (29) reported that flaccidoxide-13-acetate prepared from cultured soft coral *Sinulariagibberosa* induced apoptosis in bladder cancer cells *via* mitochondrial dysfunction, ER stress, activation of p38/JNK, and inhibition of PI3K/AKT.

In this study, we investigated the effect of flaccidoxide (Figure 1) isolated from cultured soft coral *Sinulariagibberosa* in terms of inducing apoptosis and inhibiting cell proliferation in bladder cancer cells, which also involved mitochondrial dysfunction and the PI3K/AKT/mTOR/p70S6K pathway. As flaccidoxide is a new compound, very few studies have been done. The results of this study will serve as a reference for the future development of novel anti-tumor drugs.

Materials and Methods

Chemicals and antibodies. Flaccidoxide was purified from Sinulariagibberosa (30). Dimethyl sulfoxide (DMSO), 3- (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), protease inhibitor cocktail and rabbit anti-human β-actin antibodies were purchased from Sigma (St. Louis, MO, USA). Antibodies against pro-caspase-9, cleaved caspase-9, pro-caspase-3, cleaved caspase-3, Bax, Bad, p-Bad, PARP-1, Bcl-2, PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, p70S6K, p-p70S6K, p-4E-BP1, p-S6, p-eIF4B, p-eIF4E and goat anti-rabbit and horseradish peroxidase-conjugated immunoglobulin (Ig) G were obtained from Cell Signaling Technology (Danvers, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Biowest (Nuaillé, France).

Cell culture. Bladder transitional carcinoma cell lines BFTC-905 and T24 were cultured in DMEM (with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate and 4.5 g/l glucose) containing 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate. The cells were maintained in an incubator at 37˚C under 5% CO_2. For flaccidoxide treatment, cells were incubated with different concentrations (5, 10, 15, 20 and 25 μM) of flaccidoxide, and cells in the control group were incubated with DMSO only, for 24 h. All experiments were performed three times.

MTT cell viability assay. Flaccidoxide was dissolved in DMSO. For all *in vitro* experiments, the final concentration of DMSO was 0.1% v/v. MTT was used to measure the inhibition effects of flaccidoxide on the growth of BFTC-905 and T24 bladder cancer cells. Briefly, cells ($1\times10^5/cm^2$) were seeded onto each well of a 96-well plate and incubated with flaccidoxide for 24 h. A total of 50 μl MTT solution (1mg/ml in PBS) were then added into each well and allowed to react for 4 h at 37˚C. 200 μl DMSO were added to each well to dissolve the blue-violet crystals, and the plates were analyzed using an ELISA reader at an absorbance of 595 nm.

Colony assay. In a 6-well plate, BFTC-905 or T24 cells at 5×10^3 cells/ml were seeded into each well. After 24 h, the media were changed to fresh media containing different concentrations (5, 10, 15 and 20 μM) of flaccidoxide and cultured in an incubator. The cells were further cultured for 7 days in DMEM containing 10% FBS. The cells were then fixed and stained with 1% crystal violet (containing 30% ethanol) for 15 min and the cell numbers were counted after the plates had dried.

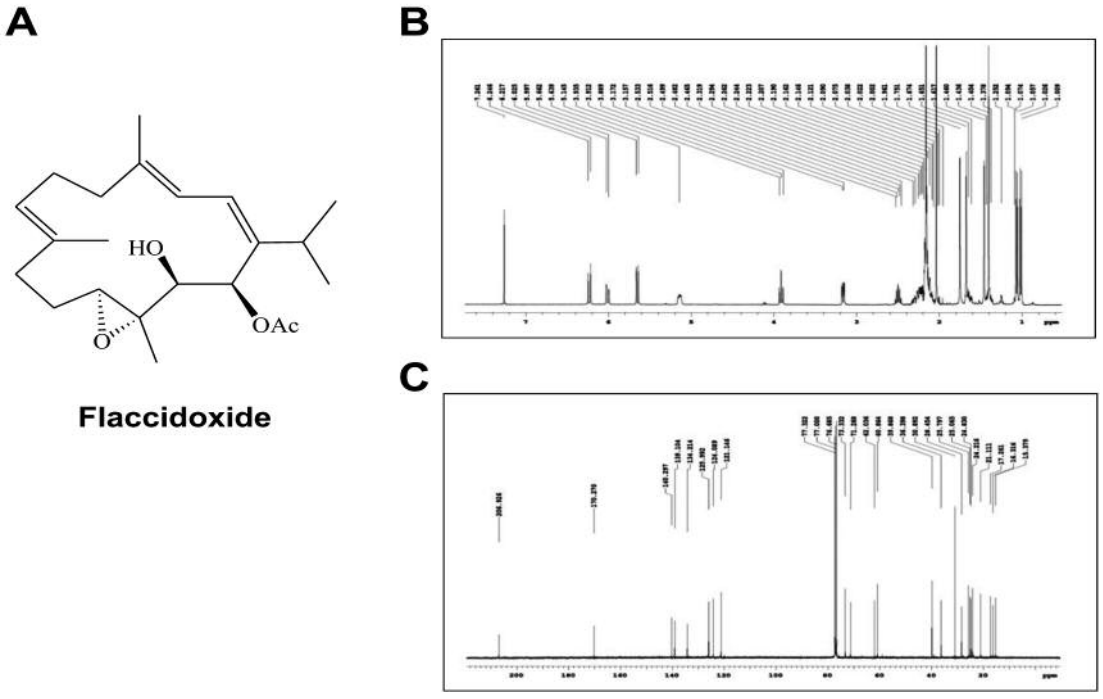

Figure 1. *(A) Chemical structure of flaccidoxide; (B) ^{1}H NMR spectrum of flaccidoxide in $CDCl_3$ at 400 MHz; (C) ^{13}C NMR spectrum of flaccidoxide in $CDCl_3$ at 100 MHz.*

Wound-healing assay. A wound-healing assay was conducted by seeding BFTC-905 and T24 cells in a 6-well plate. After the cells had reached confluence, a pipette tip was used to damage the cell layer by creating a straight line. The wound repair processes of cells treated with different concentrations of flaccidoxide (5, 10 and 20 μM) and control cells were compared.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). BFTC-905 and T24 cells at a concentration of 1×10^5 cell/ml were seeded onto coverslips placed in a 3-cm dish. After the cells had become attached, they were treated with 10 or 20 μM flaccidoxide and incubated a further 24 h. After washing with PBS, cells on the coverslips were fixed with 4% polyoxymethylene (POM) for 20 min, followed by washing 3 times with PBS. After permeabilization with 0.1% Triton-100, then washing with PBS, the cells were incubated with TUNEL for 2 h at 37˚C. Cells on the coverslips were then stained with DAPI, mounted onto slides, and the staining was analyzed using a fluorescence microscope.

Protein extraction and quantitation. Protein lysates were prepared from cells treated with various concentrations of flaccidoxide using Cell Extraction Buffer (BioSource International, Camarillo, CA, USA) with a protease inhibitor cocktail to lyse the cells for 30 min on ice. The lysates were cleared by centrifugation at 12,000 rpm for 30 min, and the protein concentrations of the supernatants were measured using a Bradford assay kit (Bio-Rad, Hercules, CA, USA).

Western blotting analysis. A total of 25 μg of total protein from each sample were used for western blotting analysis. After SDS-PAGE, the proteins on the gels were transferred onto PVDF membranes with Transphor TE 62 (Hoeffer) at 400 mA for 1.5 h. After blocking, the PVDF membranes were probed with primary antibodies overnight at 4˚C at the concentrations recommended by the manufacturer. After washing three times with PBST (10 mM NaH_2PO_4, 130 mMNaCl, 0.05% Tween 20), the membranes were incubated with horseradish peroxdase-conjugated secondary antibody (1:5,000 in blocking solution) for one h at room temperature. The signals on the membranes were then visualized using ECL Western Blotting Reagents (Pierce Biotechnolog; Rockford, IL, USA).

Statistical analysis. Cell viability assayand colony assay data were collected from three independent experiments and analyzed using Student's *t*-test (Sigma-Stat2.0, San Rafael, CA, USA). Results with $p<0.05$ were considered statistically significant.

Results

Cell cytotoxicity of flaccidoxide on BFTC-905 and T24 bladder cancer cells. Cell cytotoxicity of flaccidoxide was assessed by the MTT assay, and the viability of BFTC-905 and T24 cells treated with various concentrations of flaccidoxide (5, 10, 15, 20, 25 μM) decreased with an increasing flaccidoxide concentration. The results demonstrated that flaccidoxide inhibited the cell survival of these two bladder cancer cells. As shown in Figure 2, 20 μM flaccidoxide exerted an inhibition effect of ~60% at both 24 and 48 h after flaccidoxide treatment.

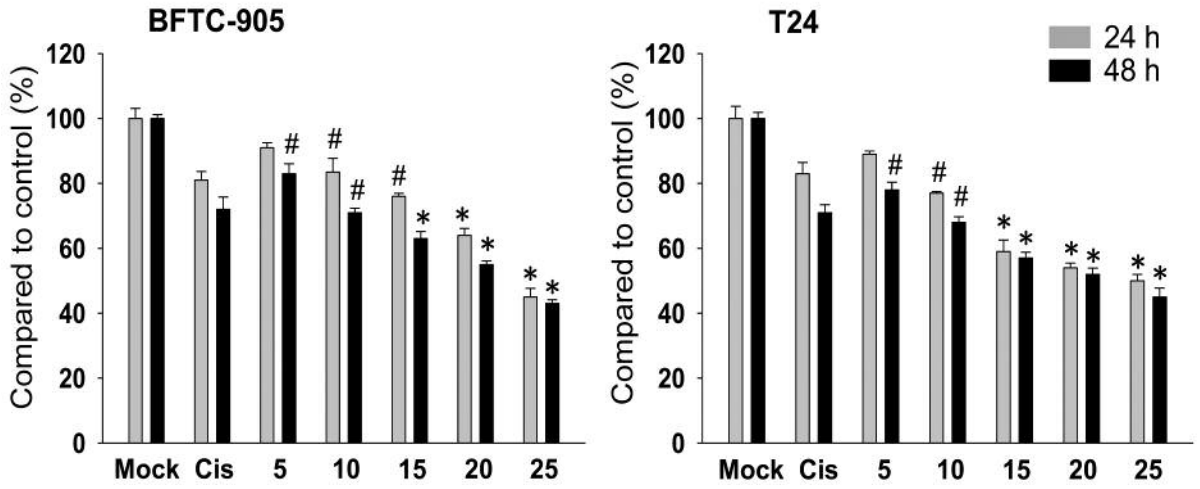

Figure 2. *Effect of flaccidoxide on cell survival of BFTC-905 and T24 bladder cancer cells. Cells were treated with different concentrations of flaccidoxide (5~25 μM) for 24 and 48 h, and cell survival was analyzed by the MTT assay (#p<0.05, *p<0.01). Mock: DMSO as the vehicle.*

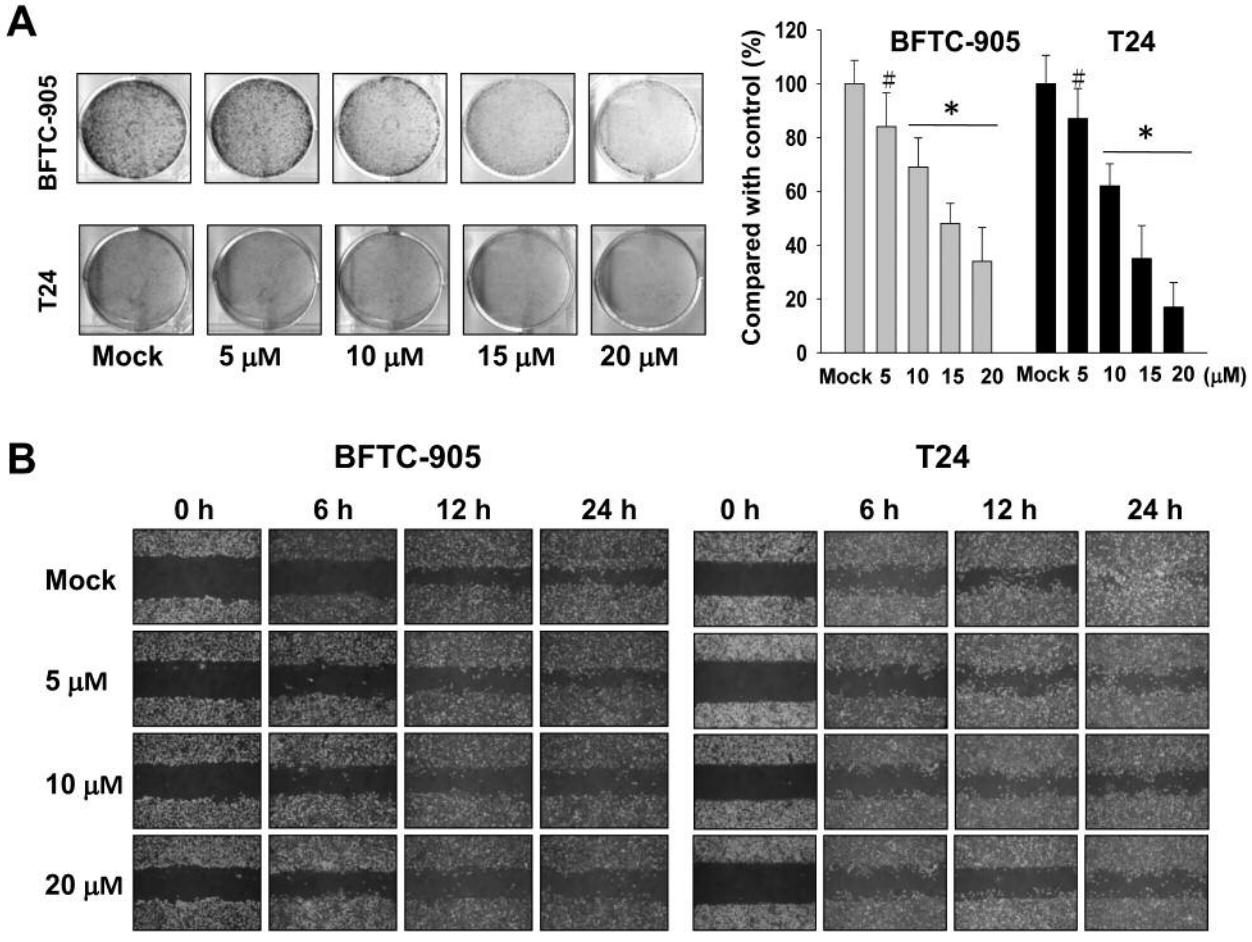

Figure 3. *Effects of flaccidoxide on the growth of BFTC-905 and T24 bladder cancer cells. (A) Cells were treated with different concentrations (5~20 μM) of flaccidoxide for 7 days and cell numbers in the cultures were counted under a microscope. (#p<0.05, *p<0.01). (B) The wound-healing assay showed that high concentrations of flaccidoxide had an inhibitory effect on the cell-healing process. Mock: DMSO as the vehicle.*

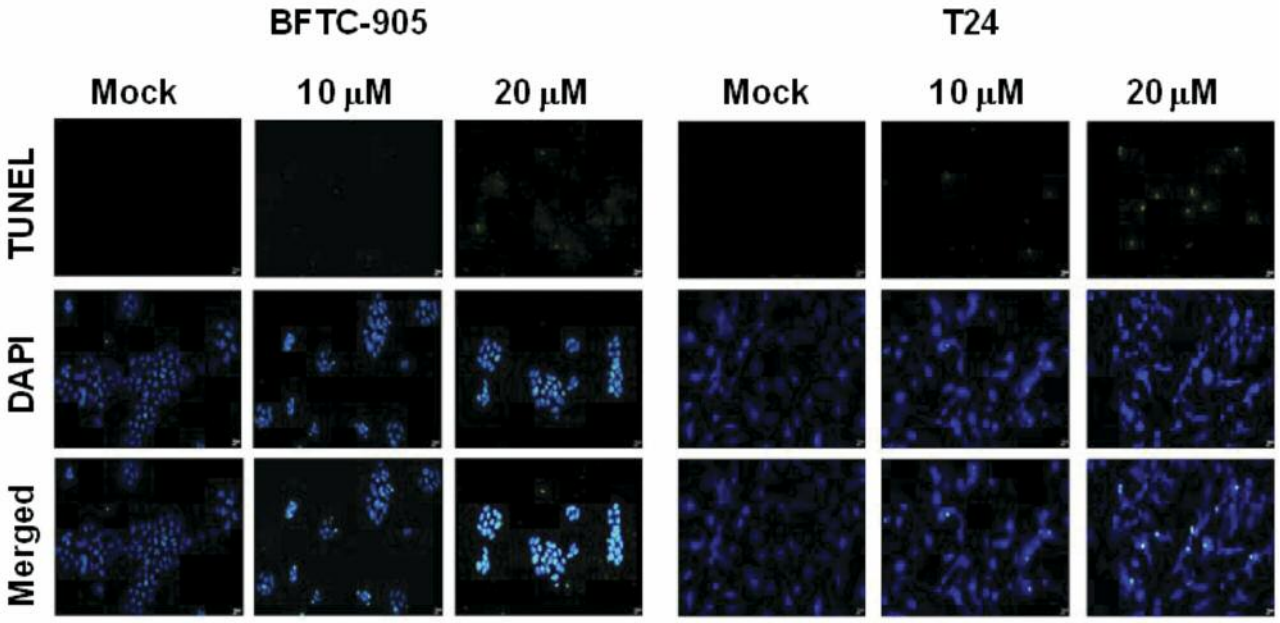

Figure 4. *Effects of flaccidoxide on the nuclear morphology of BFTC-905 and T24 bladder cancer cells. Cells were treated with flaccidoxide (10 and 20 μM) for 24 h, and nuclear morphology was assessed by TUNEL and DAPI staining and analyzed under a fluorescence microscope. Mock: DMSO as the vehicle.*

Effects of flaccidoxide on the growth of BFTC-905 and T24 bladder cancer cells. In order to understand the effect of flaccidoxide on the growth of bladder cancer cells, BFTC-905 and T24 cells seeded at a low cell density were treated with different concentrations of flaccidoxide for 24 h and the changes in numbers of cells were analyzed. As shown in Figure 3A, BFTC-905 cells treated with 5, 10, 15 or 20 μM of flaccidoxide showed decreased cell numbers at 18, 36, 50 and 70%, respectively, while T24 cells showed decreased cell numbers at 16, 40, 70 and 85%, respectively. The results indicated that flaccidoxide inhibited the growth of BFTC-905 and T24 cells, with higher concentrations resulting in greater inhibition.

Moreover, the results of the wound-healing assay showed that the wound-healing process was delayed in cells treated with higher concentrations of flaccidoxide (Figure 3B), suggesting that flaccidoxide slowed cell proliferation of BFTC-905 and T24 bladder cancer cells.

Effects of flaccidoxide on the cell morphology of BFTC-905 and T24 bladder cancer cells. Using TUNEL and DAPI staining, the effect of flaccidoxide on the cell morphology was evaluated. Apoptotic cells have DNA fragmentation that can be detected by TUNEL and DAPI staining can reveal changes in the nuclear morphology of cells. Apoptotic cells have damaged nuclei that appear with nuclear blebbing features due to chromatin condensation, and the size of nuclear blebbing reduces in the late stage of apoptosis. As shown in Figure 4, under treatment with 20 μM flaccidoxide, some BFTC-905 and T24 cells exhibited positive staining with TUNEL. DAPI staining also indicated a nuclear blebbing pattern in cells treated with flaccidoxide. Based on the results of TUNEL and DAPI staining, 20 μM flaccidoxide induced apoptosis in BFTC-905 and T24 cells.

Association between flaccidoxide-induced apoptosis and mitochondrial dysregulation in BFTC-905 and T24 bladder cancer cells. The aforementioned results suggested that flaccidoxide induces apoptosis in both BFTC-905 and T24 bladder cancer cells. When external stress causes programmed cell death, it leads to morphological changes, such as an irregular cell shape, and swelling of mitochondria that results in its dysfunction. The process further affects the expression of proteins involved in mitochondrial function, including caspase-3, caspase-9, PARP-1, Bax, Bcl-2, Bad and p-Bad. Thus, we used western blotting to analyze the changes in Bcl-2 family proteins in flaccidoxide-induced apoptosis of BFTC-905 and T24 cells. As shown in Figure 5, the expression of Bax and Bad increased with increasing flaccidoxide concentration, whereas the expression of Bcl-2 and p-Bad were attenuated. The changes in the Bcl-2 family proteins indicated that flaccidoxide-induced apoptosis in BFTC-905 and T24 bladder cancer cells is associated with a decreased expression of Bcl-2 protein. The homeostatic balance between

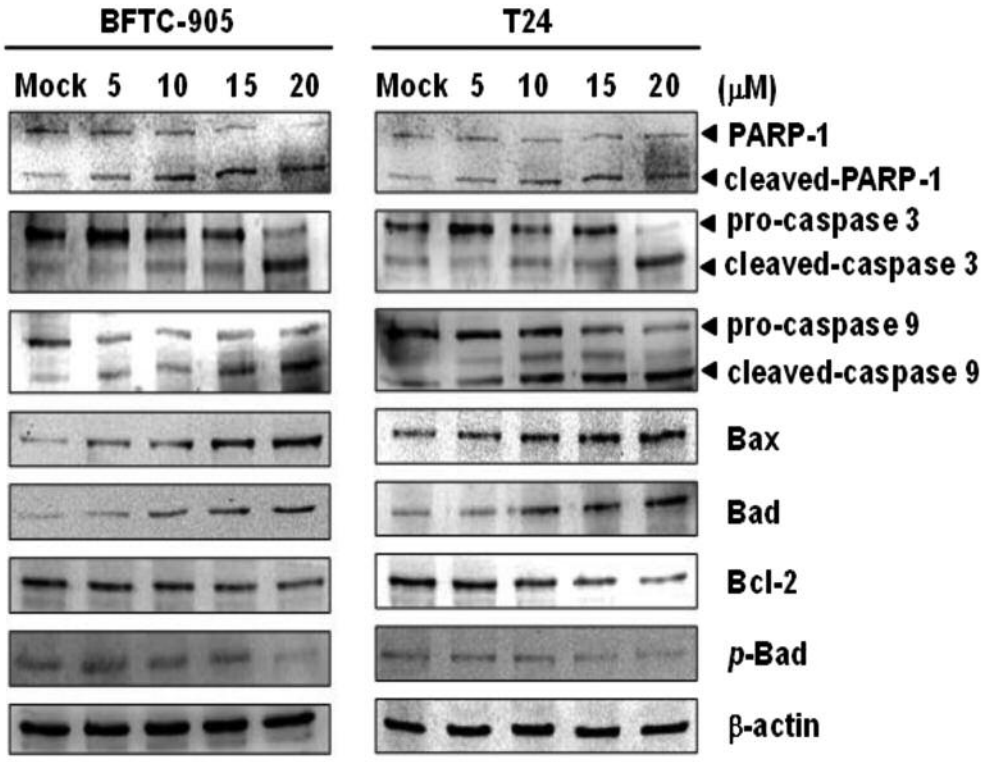

Figure 5. *Western blotting analysis of the intrinsic pathway of apoptosis mediated by mitochondrial dysfunction in BFTC-905 and T24 cells treated with flaccidoxide. Cells were treated with different concentrations of flaccidoxide (5 to 20 μM) for 24 h. Protein lysates were collected, quantified, and subjected to western blotting using antibodies against Bcl-2, p-Bad, Bax, Bad, PARP-1, cleaved-PARP-1, pro-caspase-3, pro-caspase-9, cleaved-caspase-3, and cleaved-caspase-9. β-actin served as a loading control. Mock: DMSO as the vehicle.*

Bax and Bcl-2 plays a key role in influencing the release of cytochrome c from mitochondria into the cytosol, which in turn activates activities of downstream caspases. Our results showed that the expression of Bax increased after flaccidoxide treatment, implying that flaccidoxide-induced apoptosis is mediated by mitochondrial dysfunction.

Next, we investigated the relationship between flaccidoxide-induced apoptosis and caspase activation. Caspases are a group of kinases that regulate cell survival and apoptosis. Mitochondrial dysfunction causes cytochrome *c* release into cytosol, leading to activation of caspase-3 and caspase-9, and resulting in apoptosis. Using western blotting, we found that the expression of pro-caspase-3 and pro-caspase-9 were decreased, while the expression of activated forms of caspases (cleaved-caspase-3 and cleaved-caspase-9) were increased in BFTC-905 and T24 cells treated with flaccidoxide. In addition, overexpression of cleaved PARP-1 was observed in the nucleus, which is linked to mitochondrial dysfunction. Our results indicated that flaccidoxide is involved in the regulation of apoptosis-related proteins. It could be inferred that flaccidoxide induced the pathway of mitochondrial dysfunction, which further activated caspase-dependent apoptosis.

Effects of flaccidoxide on the PI3K/AKT/mTOR/p70S6K pathway in BFTC-905 and T24 bladder cancer cells. Tian *et al*. (31) showed that the PI3K/AKT/mTOR pathway is involved in cell proliferation, differentiation, survival and apoptosis. Therefore, we next investigated whether inhibition of the PI3K/AKT/mTOR pathway occurs in cells treated with flaccidoxide, which then prevents bladder cancer cell growth by triggering apoptosis. As presented in Figure 6, western blotting showed that the expression of phosphorylated PI3K, AKT and mTOR were reduced, while the expression of PI3K, AKT and mTOR were unchanged in the bladder cancer cells treated with flaccidoxide. The results indicated that flaccidoxide inhibits activation of the PI3K/AKT/mTOR pathway, suggesting that flaccidoxide may reduce bladder cancer cell proliferation by promoting apoptosis through down-regulation of the PI3K/AKT/mTOR pathway.

p70S6K is a downstream target of mTOR kinase (32), and studies have demonstrated that p-mTOR activates p-p706S kinase, leading to p-S6 activation (33, 34). Our results showed that flaccidoxide-treated cells had lower expression levels of p-mTOR, as well as reduced expression of its downstream targets, p-p706SK, p-S6 and p-eIF4B. While it

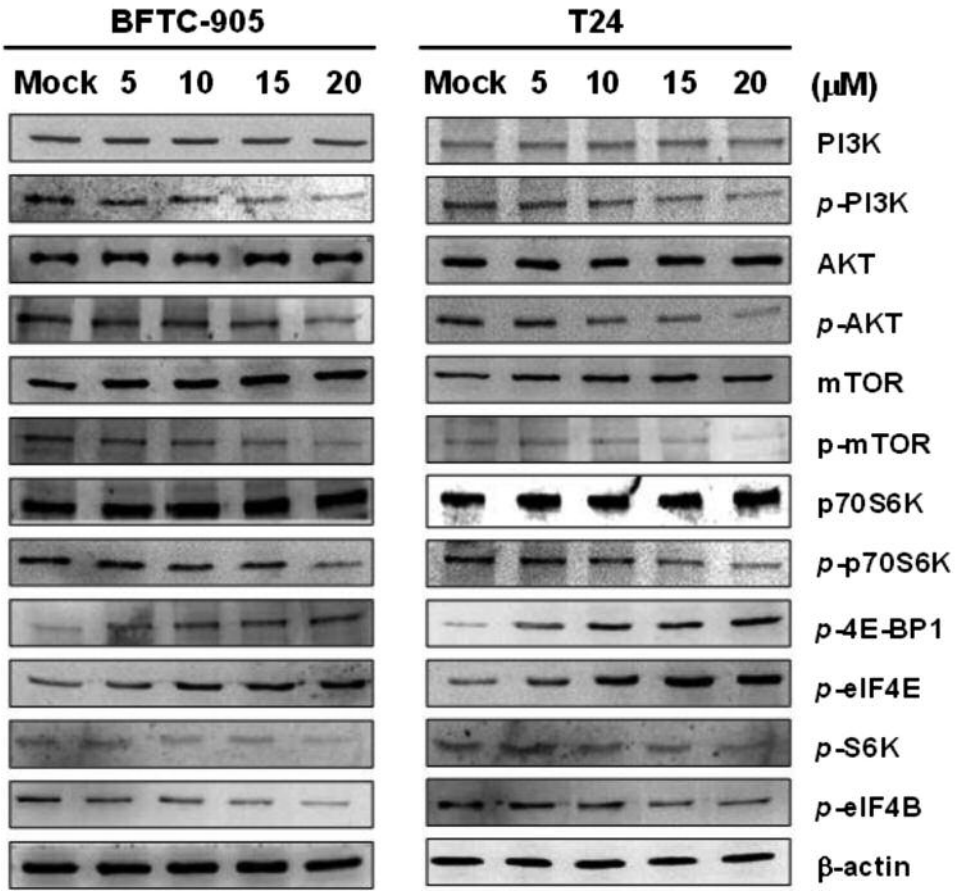

Figure 6. *Western blotting of the effects of flaccidoxide treatment on the PI3K/AKT/mTOR/p70S6K pathway in BFTC-905 and T24 bladder cancer cells. Cells were treated with different concentrations (5-20 μM) of flaccidoxide for 24 h. Protein lysates were collected, quantified, and subjected to western blotting with antibodies against AKT, p-AKT, PI3K, p-PI3K, mTOR, p-mTOR, p70S6K, p-p70S6K, p-4E-BP1, p-S6, p-eIF4E and p-eIF4B. β-actin served as a loading control. Mock: DMSO as the vehicle.*

is known that p-mTOR can inhibit 4E-BP1expression, which leads to an increase in the eIF4E expression, our study results also revealed that flaccidoxide treatment induced the expression of p-4E-BP1, but repressed the expression of p-eIF4E, suggesting that flaccidoxide-induced apoptosis in bladder cancer cells is mediated by a reduction of the PI3K/AKT/mTOR/p706SK pathway.

Discussion

Natural compounds have been increasingly examined as candidates for new drug development, and this trend has shifted to focus on marine natural compounds in recent years. A variety of marine soft corals have been demonstrated to contain a diverse range of novel compounds that possess biological activities, which have considerable potential for development as new medicines (26). Natural products from soft corals have successively been shown to possess various bioactivities, including anti-cancer (35), antiviral (36), antibacterial (37), anti-diabetic (38) and anti-inflammatory effects (39). Flaccidoxide-13-acetate derived from soft corals *Cladiellakashmani* (28) and *Sinulariagibberosa* (29) has been shown to induce apoptosis in bladder cancer cells. Many of the natural compounds obtained from soft corals have bioactivities against foreign substances, and whether or not this feature indicates an association with the special survival mode of corals as a defense against predators requires further investigation.

Our results demonstrated that 20 μM flaccidoxide has an inhibition effect against BFTC-905 and T24 cell proliferation, with cytotoxicities greater than 40% (Figure 2). The outcomes of a cell colony assay and wound-healing study (Figure 3) further indicated that flaccidoxide suppresses cell growth of these bladder cancer cell lines.

Apoptosis is a process in which cells undergo a series of cell death morphological changes, including cell membrane contraction, chromatin condensation, DNA fragmentation,

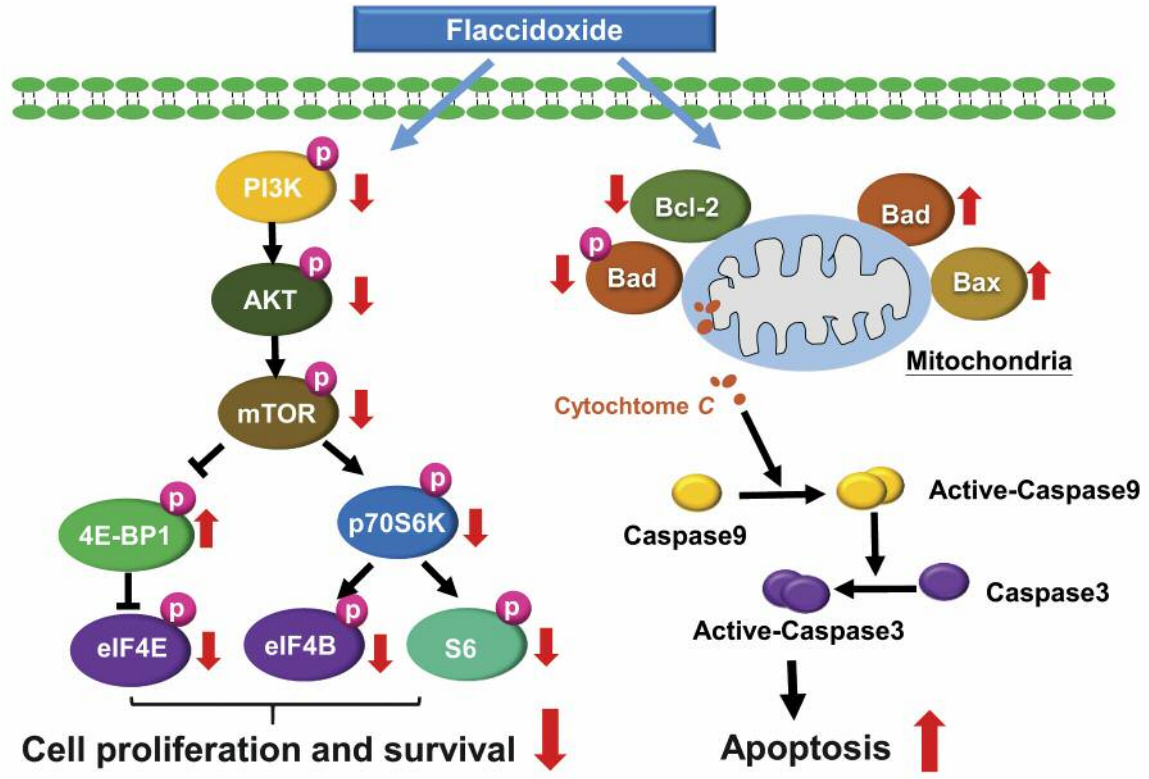

Figure 7. *Hypothetical illustration of the flaccidoxide-associated apoptosis pathway in BFTC-905 and T24 cells.*

and swelling of bubbles on the cell surface, resulting in fragmentation of the cell and breakdown of the cytoskeleton. Therefore, using TUNEL and DAPI staining, flaccidoxide-induced cell morphological damage in BFTC-905 and T24 cells can be observed. We demonstrated that DNA fragmentation was detected by TUNEL in BFTC-905 and T24 cells treated with flaccidoxide, and DAPI staining also revealed damage in well-defined nuclear envelopes due to nuclear fragmentation in BFTC-905 and T24 cells (Figure 4). The results indicated that flaccidoxide inhibited cell proliferation and triggered apoptosis in these bladder cancer cell lines.

Apoptosis can be triggered through intrinsic and extrinsic pathways (40). The intrinsic pathway is regulated by members of the B cell lymphoma-2 (Bcl-2) family of proteins. Overexpression of Bcl-2 is known to inhibit lymphocyte apoptosis. Bcl-2 protein is located on the mitochondrial inner membrane, suggesting that mitochondria play important roles in apoptosis, as has been reported in many recent studies (41). In the Bcl-2 protein family, Bcl-2 has an anti-apoptotic effect, while Bax has a pro-apoptotic effect. Disruption in the homeostatic balance between Bcl-2 and Bax may trigger the intrinsic pathway if the expressions of pro-apoptotic proteins Bak, Bad, Bid, Bim and PUMA are increased, while down-regulation of the expressions of anti-apoptotic proteins Bcl-xl, p-Bad and Mcl-1 will prevent apoptosis (25).

As shown in Figure 5, flaccidoxide-treated BFTC-905 and T24 cells presented decreased Bcl-2 and *p*-Bad expression and increased Bax and Bad expression, indicating that bladder cancer cells were moving towards apoptosis induced by flaccidoxide. Additionally, under stress conditions, the mitochondrial membrane permeability is altered, and cytochrome *c* is released into the cytosol, binding to ARAF-1 and subsequently activating caspase-3 and caspase-9, and inducing PARP-1 expression in the nuclei. The process ultimately induces programmed cell death in cancer cells (42). Taken together, our results suggested that flaccidoxide inhibits cell proliferation in BFTC-905 and T24 bladder cancer cells though apoptosis, and the pathway is mediated by activation of caspase-3, caspase-9 and PARP-1 protein expression.

Intracellular signaling pathways regulate important cell functions, such as the cell cycle, and the PI3K/AKT pathway is thought to link extracellular signaling pathways to regulate cell metabolism, proliferation, differentiation, survival, growth, and tumor metastasis (43, 44). PI3Ks are heterodimeric molecules that possess oncogene catalytic activity, and are composed of two subunits, a p110 catalytic

subunit and a p85 regulatory subunit. By using cancer-specific gain-of-function mutations, Vogt *et al*. (45) reported that phosphoinositide 3,4,5 trisphosphate (PIP_3) is essential for PI3K-mediated oncogenicity, and it is also critical for the enzymatic activity and ability to signal constitutively for PI3K. Dysregulation of the PI3K/AKT signaling pathway has been shown to be associated with many human diseases, including cancer, diabetes, cardiovascular diseases and neurological disorders.

Nobiletin, a polymethoxylated flavonoid, isolated from citrus fruit peel, has been reported to induce apoptosis in BFTC human bladder cancer cells (46). The author also demonstrated that the PI3K/AKT/mTOR signaling pathway is involved in the event. A study by Reddy *et al*. (47) indicated that peruvoside, a compound derived from a plant, induced apoptosis in several different types of human cancer cell though PI3K/AKT/mTOR signaling. Using western blotting, we found that phosphorylation of PI3K, AKT and mTOR was reduced with increasing concentrations of flaccidoxide, suggesting that flaccidoxide may trigger dysregulation of the PI3K/AKT/mTOR pathway, leading cells to apoptosis. The findings supported that flaccidoxide-induced apoptosis in BFTC-905 and T24 cells is associated with the PI3K/AKT/mTOR pathway.

In the PI3K/AKT/mTOR pathway, mTOR further affects two downstream transport pathways: one is activation of phosphorylation of the p70S6K/S6 pathway, while the other is inhibition of 4E-BP-1, which promotes transcription of eIF4E (48). Recently, Kim *et al*. (49) found that the use of Tamoxifen, a selective estrogen receptor modulator, attenuated renal tubular interstitial fibrosis and significantly reduced the phosphorylation of PI3K, AKT, mTOR and p70S6K target proteins. This approach has become a novel therapeutic option for the prevention and treatment of renal fibrosis. Lin *et al*. (50) studied thrombin-induced interleukin-8 (IL-8) secretion in lung epithelial cells, and found that transfection of a dominant negative mutant of AKT to cells decreased the thrombin-induced increase in the phosphorylation of mTOR and p70S6K. Their results further indicated that thrombin triggers the AKT/mTOR/p70S6K signaling pathway to elevate NF-κB activation and subsequently stimulates IL-8 secretion from human lung epithelial cells. In our current study, the results of western blotting analysis showed that flaccidoxide treatment of BFTC-905 and T24 cells diminished the phosphorylation of AKT, mTOR and p70S6K, and finally affected downstream S6 and eIF4B protein expressions, while phosphorylation of 4E-BP-1 protein was increased due to reduction in the phosphorylation of upstream mTOR.Taken together, our findings suggested that flaccidoxide-induced apoptosis in BFTC-905 and T24 bladder cancer cells is dependent on the down-regulation of PI3K/AKT/mTOR/p70S6K signaling pathway.

Conclusion

Flaccidoxide, a natural compound derived from marine soft corals, induces BFTC-905 and T24 cells towards apoptosis in a dose-dependent manner. Based on evidence from our current study and the signaling pathway mechanisms revealed in previous studies, apoptosis induced by flaccidoxide in bladder cancer cells is mediated by mitochondrial dysfunction and down-regulation of the PI3K/AKT/mTOR/p70S6K pathway. The hypothetical mechanism of flaccidoxide in BFTC-905 and T24 cells is illustrated in Figure 7. Coral reef organisms are rich resources that contain many special compounds possessing bioactivity. Many marine natural compounds have anti-cancer effects, and have great potential to be developed into new medicines for cancer therapy. In order to identify new drugs for the treatment of cancers that are difficult to treat, more studies are required to reveal the underlying anti-cancer mechanisms of new marine-derived compounds.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Yu-Jen Wuand Yih-Gang Goan conceived, designed, and performed the experiments. Jui-Hsin Su isolated and identified the compound.Bing-Sang Wong and Wen-Tung Wu performed the experiments and analyzed the data. Yu-Jen Wuand Yih-Gang Goan wrote the paper. All Authors read and approved the final manuscript.

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