FIP-gts Potentiate Autophagic Cell Death Against Cisplatin-resistant Urothelial Cancer Cells

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Abstract. Background: Urothelial cancer (UC) is a common cancer among males. Once metastatic or chemoresistant diseases develop, there is little effective treatment available. A fungal immunomodulatory protein, ganoderma tsugae (FIP-gts) possesses antitumor activity against solid tumors and inhibits telomerase activity. FIP-gts induces autophagy in cancer cells and may provide an alternative pathway against chemo-resistance. Materials and Methods: Two UC cell lines were used to investigate the cytotoxicity effects and the autophagy regulation of FIP-gts using flow cytometry, acidic vesicular organelles (AVO) staining and western blotting. Results: MTT assay showed that FIP-gts and bafilomycin-A1 (Baf-A1) and or chloroquine (CQ) could enhance a significantly synergistic cytotoxicity. The treatment of UC cell lines with FIP-gts activated LC-3 II formation and AVO positive staining on western blot and flow cytometry. Interestingly, FIP-gts and Baf-A1 combined treatment was found to lead to enhancement of apoptosis along with inhibition of autophagy in parental and resistant UC cells. Conclusion: FIP-gts may have the potential to be utilized as a therapeutic adjuvant for the treatment of resistant UC cancer down-regulating Beclin-1 to activate autophagic cell death.

Cisplatin-based chemotherapy is highly effective and is becoming the standard first-line treatment for metastatic urothelial carcinoma (UC) (1, 2). However, the progression-free survival is short and the median overall survival ranges from only 8 to 14 months in metastatic UC patients. Progress of cisplatin resistance concedes treatment outcome and patient survival. In addition, there is still no useful second-line therapy except for paclitaxel following the failure of cisplatin-based regimens in the first-line setting (3). Various new chemotherapy agents have been introduced as second-line treatments with an objective response rate of up to 50%, but the accompanying toxicity is significant and the progression-free survival is quite low (4-6). Some vascular endothelial growth factor inhibitors (VEGFRi) and mammalian target of rapamycin inhibitors (mTORi) were applied in the treatment of cisplatin-resistance metastatic UC patients. Although the toxicity is acceptable, the benefits of progression-free survival is still relatively small (7).

Despite apoptosis being the major pathway leading to UC cell death in most cases of chemotherapy, a previous study of ours showed that autophagy may provide another anticancer pathway (8). In general, autophagy plays a key role in the cellular survival or autophagic cell death in response to various forms of stress. It has, thus, been considered as a novel target in cancer treatment (9). Autophagy inhibition facilitates anticancer drug resistance. At times, autophagy inhibitors facilitate to re-sensitize resistant cancer cells to anti-tumor therapy. New chemoresistant therapeutic approaches based on the balance of apoptosis and autophagy have been reported with good pre-clinical rationales (10). In addition, multiple clinical trials using novel agents targeting various signaling pathways are ongoing at this time.

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Currently, most therapeutic effects reported for Lingzhi species are attributed to triterpenoids, polysaccharides, sterols and glycoproteins (11, 12). A new glycoprotein class in Lingzhi named fungal immunomodulatory proteins (FIPs) was identified. Thus far, four FIPs, LZ-8 (Ganoderma lucidum), FIP-gts (Ganoderma tsugae), GMI (Ganoderma microsorum) and FIP-gsi (Ganoderma sinensis) exhibiting immunomodulatory activity and anti-tumor activity, have been cloned and purified from Lingzhi (13-15). GMI inhibits tumor growth and significantly induces autophagy in nude mice following A549 injection (16). FIP-gts inhibits anti-tumor activity involved in the regulation of telomerase expression (17). FIP-gts inhibits telomerase activity in lung cancer cells through nuclear export mechanisms and ER stress-induced intracellular calcium levels (18). In addition, FIP-gts inhibits the growth of A549 cancer cells, leading to cell-cycle arrest, consequently inducing premature senescence in lung cancer cells. Moreover, FIP-gts results in significant inhibition of tumor growth in a thymic nude mice implanted with A549 cells (19).

In the present study, we investigated the synergistic cytotoxic effects on UC parent and resistant cells for the FIP-gts in combination with the autophagy regulators.

Materials and Methods

Cell lines and chemicals. Two urothelial carcinoma cell lines were used: NTUB1, N/P (cisplatin-resistant sub-line), NTUB1, N/P cell lines were kindly provided by Dr. T.C. Hour (Institute of Biochemistry, Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China). All cells were maintained in RPMI-1640 medium (GIBCO, 31800-022) containing 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, 10437) and 100 ng/ml each of penicillin and streptomycin (GIBCO, 15140) at 37˚C in humidified air with 5% CO2. Bafilomycin-A1 (Baf-A1) (Sigma, St. Louis, MO, USA), 3-methyladenine (3-MA) (Sigma, St. Louis, MO, USA) and chloroquine diphosphate salt (Sigma, St. Louis, MO, USA) were purchased from Sigma.

Cell viability assay. Urothelial carcinoma cells (5×10³) were seeded onto 96-well plates containing 100 μl of culture medium. After 16 h of incubation, the medium was carefully removed and 100 μl of fresh medium containing various concentrations of gts, 3-MA, Baf-A1 and CQ were added to the wells. The cells were continuously treated for 48 h. At the end of this process, the medium was carefully removed and 100 μl of fresh medium, containing 0.5 mg/ml MTT (Thiazolyl Blue Tetrazolium Bromide) (Sigma, St. Louis, MO, USA) were added to the wells. MTT is converted from a yellowish solution to water-insoluble MTT-formazan that is dark blue in color by mitochondrial dehydrogenases of living cells. The blue crystals are solubilized with 100 μl dimethyl sulfoxide (DMSO) (Merck, Whitehouse Station, NJ, USA) and the intensity is measured colorimetrically at a wavelength of 570 nm. Absorbance values are presented as the mean±SE of three replicates for each treatment.

Assessment of apoptotic cell death. Apoptotic or necrotic cell death was evaluated using an FITC Annexin V Apoptosis Detection kit (BD Pharmingen™, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions. Cells were seeded at a density of 5×10⁵ cells onto a 6-cm plastic dish. At 16 h after seeding, the cells were incubated with gts and Baf-A1. After 48 h of drug treatment, cells were harvested and resuspended in 100 μL of binding buffer containing Annexin V-FITC and propidium iodide (PI). Cells were analyzed using a BD FACS Calibur Flow Cytometry System and the data were analyzed using CellQuest software.

Detection and quantification of acidic vesicular organelle (AVO) development. Cells were seeded at a density of 5×10⁵ cells onto 6-cm plastic dish. After 48 h of gts and Baf-A1 treatment, cells were harvested and stained with 1 μg/ml acridine orange (Sigma, St. Louis, MO, USA) in Hank’s buffered salt solution (HBSS) containing 5% FBS for 15 min. Cells were analyzed using a BD FACS Calibur Flow Cytometry System and the data were analyzed using CellQuest software.

Western blotting. Cells were lysed with RIPA buffer containing a protease inhibitor cocktail (Roche, Basel, Switzerland) and protein concentration was assayed with Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, California, USA). Equal amounts of proteins from each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride transfer membrane (PVDF) (Pall Corporation, Port Washington, NY, USA). Membrane was blocked for 1 h in PBS containing 5% nonfat milk and 0.2% Tween 20. For the detection of PARP, caspase 3, Bax, Bcl-2, Belcin-1, p62, LC3B and β-actin, anti-PARP (Cell Signaling, Danvers, MA, USA), anti-caspase 3 (Cell Signaling, Danvers, MA, USA), anti-Bax (Cell Signaling, Danvers, MA, USA), anti-Bcl-2 (Cell Signaling, Danvers, MA, USA), anti-Belcin-1 (Cell Signaling, Danvers, MA, USA), anti-p62 (GeneTex, Irvine, California, USA), anti-LC3B (Cell Signaling, Danvers, MA, USA), and anti-β-actin (Sigma, St. Louis, MO, USA) were incubated with membranes at 4°C overnight. Membranes were subsequently washed for 3-5 min in Tris-buffered saline (TBS) with 0.2% Tween-20, incubated in HRP-conjugated secondary antibody for 1 h, washed again and visualized by enhanced luminol reagent for chemiluminescence (Perkin Elmer, Waltham, MA, USA).

Results

FIP-gts combine treatment with bafilomycin-A1 and chloroquine accelerates cell death in NTUB1 and N/P cells. In our previous study we identified another fungal immunomodulatory protein, GMI which significantly induces autophagy in non-small cell lung cancer cells and inhibits the tumor growth (16). 3-MA is a well-known inhibitor of autophagosome formation. Baf-A1 and CQ inhibit lysosomal acidification and degradation and are commonly used as an autophagy inhibitor (9). To assess the effects of 3-MA, Baf-A1 and CQ combined treatment with FIP-gts on urothelial cancer cell viability, NTUB1 and cisplatin resistant cell line N/P were treated with 3-MA (1 mM), Baf-A1 (2 mM), CQ (10 μM) and various concentrations of FIP-gts (0, 0.3, 0.6, 1.2 μM) for 48 h.

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Drug-induced cell death was measured by the MTT assay. After both cell lines treated with FIP-gts and 3-MA, FIP-gts alone induced cell death in a concentration-dependent manner, 52.4% and 34.8% cell viability can be detected at 48 h after high dose of drug exposure in NTUB1 and N/P cells. FIP-gts combined treatment with 3-MA also induced cell death in a concentration-dependent manner (Figure 1A and B). As shown in Figure 1C and D, approximately 9.2% and 4.8% cell viability can be detected at 48 h after drug exposure in NTUB1 and N/P cells. It revealed that FIP-gts combined treatment with Baf-A1 accelerated cell death in both cells. In Figure 1E and F, both cell lines co-treated with FIP-gts and CQ could also enhance a significantly synergistic cytotoxicity. To further verify drug-induced cell death, we treated NTUB1 and N/P cells with varied concentration of FIP-gts and Baf-A1 for 48 h and then stained the harvest cells with fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodine (PI). The amount of apoptotic cells was determined as the percentage of PI-negative/Annexin V-positive and PI-positive/Annexin V-positive cells. Initially, we treated NTUB1 and cisplatin-resistant cell line N/P with 0, 0.3, 0.6, 1.2 μM FIP-gts and varied concentrations of FIP-gts combined Baf-A1 (2 nM) for 48 h. Figure 2A shows that apoptotic cells were slightly increased from 2.59% to 24.76% after NTUB1 were treated with FIP-gts alone. However, FIP-gts cotreated with 2 nM Baf-A1 notably raised apoptotic cells from 3.13% to 52.79% on NTUB1 cells. On the other hand, 2.15% to 17.16% apoptotic cells could be detected at 48 h after FIP-gts exposure in N/P cells. At the same time, we found the amount of the apoptotic cells was obviously increased from 9.7% to 74.25% after N/P cotreated with FIP-gts and Baf-A1 (Figure 2B). These data suggested that FIP-gts combined treatment with bafilomycin-A1 accelerates cell death in NTUB1 and N/P cells.

**FIP-gts induces autophagy in urothelial carcinoma cells.** To confirm whether the FIP-gts and FIP-gts combined Baf-A1 treatment induced cell death was associated with changes in the autophagic pathway. We treated NTUB1 and N/P cells with 0, 0.3, 0.6, 1.2 μM FIP-gts and varied concentrations of FIP-gts combined Baf-A1 (2 nM) for 48 h. Autophagy is characterized by the appearance of double or multiple-membrane structures called autophagosomes. The autophagic vesicles and their contents are degraded by the cellular lysosomal system (20). The effect of FIP-gts and FIP-gts combined with Baf-A1 on the levels of acidic vesicle organelles (AVOs) was detected by staining tested cells with acridine orange solution. The percentage of AVOs in both tested cell lines following treatment was analyzed by flow cytometry. Figure 3A shows that the formation of AVOs was increased from 0.1% to 42.8% in the tested cells. We found that Baf-A1 elicited more AVOs formation in NTUB1 following treatment with FIP-gts. But, the measurement of AVOs decreased at the highest dose of FIP-gts treatment. Moreover, after FIP-gts treatment in N/P cells, the formation of AVOs also increased from 0.5% to 29.4%. Similarly, Baf-A1 induced more AVOs formation in N/P cells following treatment with FIP-gts. Also at the highest dose of FIP-gts treatment, the amount of AVOs reduced (Figure 3B). These results exhibited that treatment with FIP-gts and Baf-A1 induced enlargement of the endosomal/lysosomal system in urothelial carcinoma cells.

**The effect of FIP-gts combined with Baf-A1 treatment in NTUB1 and N/P cells.** To understand whether FIP-gts and FIP-gts combined with Baf-A1 treatment induced cell death was associated with apoptosis. We treated NTUB1 and N/P cells with 0, 0.3, 0.6, 1.2 μM FIP-gts and varied concentrations of FIP-gts combined with Baf-A1 (2 nM) for 48 h. Consistently, the cleavage of PARP is a substrate of caspase 3 and a well-known apoptotic hallmark. Immunoblot analysis revealed that NTUB1 cells treated with FIP-gts for 48 h slightly induced the formation of the active forms of PARP, however there were not obvious variation in caspase activation, Bax and Bcl-2. FIP-gts combined with Baf-A1 treatment caused induction of active forms of PARP and caspase 3. The expression of Bcl-2 was decreased and the level of Bax was slightly reduced in NTUB1 cells following treatment with FIP-gts and Baf-A1 (Figure 4A). At the beginning of autophagy, the cytosolic form of LC3 (LC3-I, 18 kDa) is converted to the phagophore and autophagosomal bound form of LC3 (LC3-II, 16kDa) (16). Using western blotting, the expression of LC3-II was obviously induced by FIP-gts and FIP-gts combined with Baf-A1 after 48 h of treatment in NTUB1 cells. Furthermore, the expression of p62 was also dose-dependently increased in the tested cells (Figure 4B). Autophagosome clearance was supported when following the degradation of p62/SQSTM1, a polyubiquitin-binding protein that is degraded by autophagy (21). As shown in Figure 4B, FIP-gts combined with Baf-A1 treatment caused a remarkable reduction in Beclin-1. In comparison with NTUB1 cells, FIP-gts and FIP-gts combined with Baf-A1 did not elicit the activation of PARP and caspase 3 in N/P cells. At the same time, Bax expression was suppressed and the level of Bcl-2 was progressively elevated for the indicated treatment in N/P cells (Figure 5A). Using immunoblot analysis we found that FIP-gts and FIP-gts combined with Baf-A1 obviously induced LC3-II expression and the amount of Beclin-1 was significantly inhibited in N/P cells. In contrast to FIP-gts treatment-alone, the expression of p62 was decreased after FIP-gts combined with Baf-A1 treatment in N/P cells (Figure 5B).

**Down-regulation of Beclin-1 expression is not associated with ubiquitin-proteasome pathway.** As shown in the western blot analysis, it is illustrated that the expression of Beclin-1 was down-regulated following treatment in NTUB1 and N/P cells.
Figure 1. Cytotoxic effects of 3-methyladenine (3-MA), bafilomycin-A1 (Baf-A1) and chloroquine (CQ) combined treatment with gts in NTUB1 and N/P cells. (A), (B) NTUB1 and N/P cells were co-treated with 3-MA (1 mM) and various concentrations of gts (0, 0.3, 0.6, 1.2 μM) for 48 h. (C), (D) Cells were co-treated with Baf-A1 (2 nM) and various concentrations of gts (0, 0.3, 0.6, 1.2 μM) for 48 h. (E), (F) Cells were co-treated with CQ (10 μM) and various concentrations of gts (0, 0.3, 0.6, 1.2 μM) for 48 h. Cell viability was measured by the MTT assay and the results are presented as the calculated cell growth inhibitory ratio. Experiments were repeated three times.
Figure 2. The detection of apoptosis in NTUB1 and N/P cells exposed to gts and Baf-A1. Cells were treated with gts (0, 0.3, 0.6, 1.2 μM) and combine treatment with bafilomycin-A1 (2 nM) for 48 h. After treatment, the cells were harvested and stained with annexin V-FITC/PI and the percentage of the apoptotic cells was analyzed by flow cytometry.
Figure 3. The detection of autophagy in NTUB1 and N/P cells exposed to gts and Baf-A1. Acridine orange was used to stain acidic vesicular organelles (AVOs) in gts (0, 0.3, 0.6, 1.2 μM) and Baf-A1 (2 nM)-treated NTUB1 (A) and N/P (B) cells for 48 h. The percentage of AVOs was measured by flow cytometry.
In particular, the levels of Beclin-1 were remarkably decreased under FIP-gts combined with Baf-A1 treatment in both cells. Beclin 1 is a coiled-coil protein involved in the regulation of autophagy in mammalian cells (22). To confirm the contribution of the decrease of Beclin-1 in NTUB1 and N/P cells, we analyzed the effect of MG-132, a well-known proteasome inhibitor. Proteasome inhibitors are small molecules or drugs that block the function of the proteasome and thus stop protein degradation. When the proteasome function was inhibited, it could lead to accumulation of protein load.

Figure 4. Effect of gts combined treatment with Baf-A1 on protein expressions in NTUB1 cells. A, Cells (5x10^5 cells/6-cm dish) were co-treated with Baf-A1 (2 nM) and various concentrations of gts (0, 0.3, 0.6, 1.2 μM) for 48 h. Following treatment, cells were harvested and lysed for detection of protein expression related to apoptosis by western blotting. B, Cells were examined for protein expression related to autophagy by western blot. β-Actin was used as a loading control.
First, we cotreated NTUB1 and N/P cells with 1.2μM FIP-gts, 2 nM Baf-A1 and 20 μM MG-132 for 48 h, and then harvested cell pellets to determine the level of Beclin-1 by western blot. As shown in Figure 6A and B, the expression of Beclin-1 was still significantly reduced but did not cause the accumulation of protein load after cotreatment with 20 μM MG-132 in both cells. These data suggest that the degradation of Beclin-1 in both cells is not associated with the ubiquitin-proteasome pathway.

**Discussion**

Cross-talk between apoptosis and autophagic cell death has been regularly studied in recent years. Boya *et al.* found that the cytoprotective potential of autophagy and inhibition of autophagy may trigger apoptosis (23). Kim *et al.* reported that oxaliplatin-based combination therapy can enhance colorectal cancer cell apoptosis through inhibition of Beclin-1-induced autophagy (24). Both Boya and Kim declared the
opposite and interacting reaction between apoptosis and autophagy. Kung et al. demonstrated that autophagy blockade sensitizes prostate cancer cells through Src tyrosine kinase inhibition (25). They concluded that Src tyrosine kinase inhibitor and autophagy modulator served as a potential treatment for relapsed prostate cancer. Our study showed the cytotoxic effect of FIP-gts on both cisplatin-sensitive and -resistant UC cells through autophagic flux. FIP-gts and BAF-A1 significantly increased large amounts of autophagosomes and induced apoptosis (Figure 2 and 3). We hypothesized that the accumulated autophagosomes result in autophagic cell death and apoptosis.

The anti-apoptotic BCL-2 family proteins inhibit both apoptosis and autophagy, therefore, down-regulation of anti-apoptotic BCL-2 family proteins and enhanced autophagy must co-exist in cells dying in response to an apoptosis in parental UC cells (Figure 4). However, there is an increased dependence on anti-apoptotic proteins, BCL-2, autophagic flux is also happened in the drug-resistant cells (Figure 5). The decrease of Beclin-1 and induction of apoptosis occurred in response to the combination of Baf-A1 and FIP-gts in parental and resistant cells (Figures 2 and 5). This suggests that autophagy specific inhibitors (3-MA, CQ and Baf-A1) or down-regulation of Beclin-1 enhanced FIP-gts-induced apoptosis.

![Figure 6. Effect of gts and Baf-A1 on degradation of Beclin-1](image-url)
apoptosis and FIP-gts-mediated autophagy may protect UC cells from undergoing apoptotic cell death. FIP-gts induces the cancer cell G1 arrest (19).

There is dual role of autophagy’s function in cancer. Autophagy inhibits tumorigenesis and promotes cancer cells survival in the other hand (26, 27). The cross-talk between apoptosis and autophagy is determined by the expression of bcl-2 and Beclin-1. Lima et al. reported that down-regulation of bcl-2 induced significant autophagic cell death (28). In NTUB1 cell line, we can see that expression of bcl-2 decreased with the increase in autophagy (Figure 4). Interestingly, N/P cells did not correspond to this phenomenon. The bcl-2 level increased and autophagy also increased.

Beclin-1 (also called Atg6) is essential for the double-membrane autophagosome formation, which is required during the initial steps of autophagy (29). It is very interesting and puzzling that the combination of FIP-gts and BAF-A1 decreased Beclin-1 significantly (Figure 5). Beclin-1 has been considered as a cancer suppressor. Reduced expression of Beclin-1 was associated with cancer cell transformation and poor outcome. In our study, autophagy flux still increased with significant cell death even when expression of Beclin-1 decreased.

Qu et al. demonstrated that Beclin-1 is a haplo-insufficient tumor-suppressor gene and provides genetic evidence that autophagy is a novel mechanism of cell-growth control and tumor suppression (30). Although autophagy confers chemoresistance in some cancers, its role in the development of resistant urothelial and prostate cancers remains unknown. Recently, Melanoma differentiation-associated gene 7 (mda-7)/interleukin-24 (IL-24) physically interacted with Beclin-1 in a manner that could inhibit Beclin-1 function reach apoptosis (31). Autophagy was dramatically reduced after depletion of Atg5 and Beclin-1, two canonical autophagy genes, and was associated with an inhibition of the androgen-induced mTOR pathway. The depletion of Atg5 and Beclin-1 significantly increased apoptosis induced by androgen deprivation or bicalutamide castration resistance prostate cancer cells (32). Arsenic induced Beclin 1 expression and decreased the DAPK promoter hypermethylation that provided the mechanism of arsenic-induced carcinogenesis in urothelial cells (33). Baf-A1 is a specific autophagy inhibitor targeting the vacuolar typeH+-ATPase (V-ATPase) and blocks the fusion of autophagosomes with lysosomes. Chen et al. summarized that the autophagy changed cancer cells to anticancer therapy. Bafilomycin A1 can effectively sensitize the cancer cells to therapeutic agents (34). In our previous study, GMI, another recombinant fungal immunomodulatory protein cloned from Ganoderma microsorum, induced autophagic cell death in lung cancer cells (16). Lysosome inhibitors Baf-A1 and CQ increased GMI-mediated autophagic cell death. GMI and bafilomycin-A1 co-treatment induced accumulation of large amounts of autophagosomes, but did not significantly induce apoptosis. GMI induced autophagy through Akt/mTOR signaling pathway (35). In this study, we found that combination of FIP-gts and BAF-A1 could induce apoptosis in parental and resistant UC cells (Figures 2 and 4).

Based on these findings and the related literature, it would appear that FIP-gtsmediates an autophagy-promoting cell death mechanism and that changing the autophagy status might be a potential therapeutic target in cisplatin-resistant UC cells in the future.

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