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Identification and Anti-human Glioblastoma Activity of Tagitinin C from *Tithonia diversifolia* Methanolic Extract

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ABSTRACT: The *Tithonia diversifolia* methanolic extract (TDM), which showed antiproliferative activity against human glioblastoma U373 cells, with an IC_{50} value of 59.2 ± 3.7 μg mL^{-1}, was passed through silica gel chromatography and successively eluted with different percentages of EtOAc/hexane. The 10−60% EtOAc/hexane subfractions, which exhibited a comparatively higher antiproliferative activity, were isolated, and then structural identification was proceeded with ^{1}H nuclear magnetic resonance. The isolated compound was tagitinin C, a kind of sesquiterpenoid. The IC_{50} value was 6.1 ± 0.1 μg mL^{-1} in U373 treated with tagitinin C. In flow cytometric analysis and inhibition of pan-caspase, the results showed that the anti-glioblastoma effect was apoptosis-independent. In PARP, p-p38, ULK1, and LC3-II expression, the anti-glioblastoma induced by tagitinin C was likely via autophagy. In the ULK1 siRNA transfection experiment, autophagy blockade counteracted the suppression induced by tagitinin C. The result suggested that tagitinin C induces U373 cell death dependent upon autophagy under certain conditions.

KEYWORDS: *Tithonia diversifolia* methanolic extract (TDM), tagitinin C, ^{1}H NMR, apoptosis, autophagy

INTRODUCTION

Tithonia diversifolia, a bushy perennial weed, is commonly found in Nigerian fields, wastelands, and roadsides. The plant is used for ornamental purposes, fertilizer for farming, and treatment for diabetes mellitus.[1] In addition, *T. diversifolia* containing bioactive compounds has anti-inflammatory,[2] anti-diarrheal,[3] anti-amoebic and spasmolytic[4] properties. Extracts of *T. diversifolia* have been used traditionally for the treatment of diarrhea, fever, hematomas, hepatitis, hepatomas, malaria, and wounds.[5] Phytochemical investigations of *T. diversifolia* have isolated cadinane, chromene, eudesmane, flavone, germacrane, and rearranged eudesmane derivatives.[6−13] Recently, some studies showed significant antiproliferative activity of several sesquiterpenoids and flavonoids from the aerial parts of *T. diversifolia*. These compounds included tirotundin, tirotindin 3-*O*-methyl ether, tagitinin A, tagitinin C, deacetylviguiestin, tagitinin F, 1β-methoxydiversifolin, 1β-methoxydiversifolin 3-*O*-methyl ether, 4β,10α-dihydroxy-3-oxo-8β-isobutyroyloxyguaia-11(13)-en-6,12-olide, luteolin, nepetin, and hispidulin.[14,15] Among these isolates, some sesquiterpenoids and flavonoids showed anti-human promyelocytic leukemia (HL-60) and other cancer cell lines. Tagitinin C was the major sesquiterpenoid and has been identified (Figure 1).[14,15] However, there is no experimental mechanism detailing its cytotoxic activity. In the present study, we identified the active compound of anti-human glioblastoma and examined the antiproliferative mechanism.

Programmed cell death is defined as the death of a cell in any form mediated by an intracellular program. In contrast to necrosis, this is a form of cell death that results from acute tissue injury and provokes an inflammatory response. Programmed cell death is carried out in a gene-regulated process, which generally confers advantage during the life cycle of an organism. Programmed cell death serves fundamental functions during both plant and multicellular animal development. There are two types of programmed cell death involving antiproliferative activity: type-I cell death or apoptosis and type-II cell death or autophagy. Besides these two types of programmed cell death, other pathways have been discovered and are known as "non-apoptotic programmed cell death" (or "caspase-independent programmed cell death" or "necrosis-like programmed cell death").[16] These alternative routes to death are as efficient as apoptosis and can function as either backup or the major mechanism type of programmed cell death.

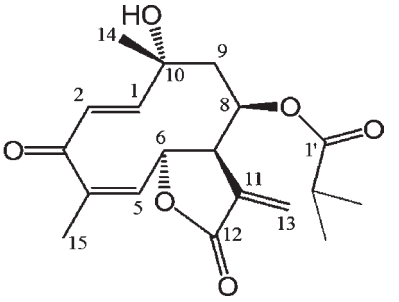

Figure 1. Structure of tagitinin C.

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Therefore, there has been a lot of activity around identification of novel pathways of cell death, which could happen either with or without the absence of efficient apoptotic machinery. In this regard, autophagy has been lighted and is activated in response to growth factor deprivation or in response to the presence of genotoxic compounds. Whereas the jury is still out on the functional relevance of this pathway in terms of its ability to serve as a stress response or a truly death effector mechanism, recent evidence seems to support that autophagy is a specialized form of cell death under certain conditions.

Malignant gliomas are resistant to many kinds of treatments, including chemotherapy, radiation, and other adjuvant therapies. In addition, glioma cells are prone to acquire drug-resistant systems. Currently, there is still need to identify chemotherapeutic agents with cytotoxic effects exclusive for malignant glioma cells. Developing novel strategies for malignant glioblastoma treatment is urgent. In our previous data,[29] we found that *T. diversifolia* methanolic extract (TDM) and tagitinin C induced *in vitro* viability inhibition, autophagosome, survivin inhibition, and G2/M arrest. These intracellular damages are consistent with autophagy, programmed cell death type II. In this study, we isolated tagitinin C from TDM and investigated the effect of tagitinin C at 2.5−10 μg mL^{-1} on human malignant glioma cells. Our data show that tagitinin C from TDM inhibited the cell growth and induced cell death. However, apoptosis was not observed in malignant glioma cells. Thus, tagitinin C seems to be a promising agent for additional investigations in the treatment of malignant gliomas.

MATERIALS AND METHODS

Extraction and Isolation. To isolate, purify, and identify the active components that demonstrate inhibition of human glioblastoma cell proliferation, a large amount of methanolic extracts were prepared from leaves of *T. diversifolia* as follows. The leaves of *T. diversifolia* were collected in Hsin-Chu, Taiwan, in January 2008. A voucher specimen was deposited in the Department of Radiological Technology, Yuanpei University, Hsin-Chu, Taiwan. Collected plant materials were dried and ground into powder using a grinder and screened through a 20-mesh sieve (aperture of 0.94 mm). The leaves of *T. diversifolia* powder (200 g) were extracted 3 times with 1 L of methanol at room temperature for sonication for the duration of 5 h (100 min for each time). To minimize methanol consumption during leaves of *T. diversifolia* methanol extraction, we prolonged the extraction time to replace the use of methanol. The plant residue was filtered through a 10 μm cartridge paper, and the methanolic extracts were combined and concentrated under reduced pressure by a rotary vacuum evaporator. The methanolic extracts of the leaves of *T. diversifolia* were named TDM. The dry extract (TDM, 28.7 g) was suspended in 300 mL of H_2O, followed by an partitioning with the same volume of ethyl acetate, yielding two subfractions named TDM-EtOAc (ethyl-acetate-soluble fraction) and TDM-H_2O (water-soluble fraction). TDM-EtOAc (15.15 g) was coated with 10 g of silica gel (230−400 mesh) and then subjected to column chromatography on silica gel (230−400 mesh) with successive elution by a Hex/EtOAc and EtOAc/MeOH gradient. Subfractions with the same TLC pattern were combined into one fraction, and thus, six fractions, 0−5% EtOAc/hexane (TDM-EA-a), 10−20% EtOAc/hexane (TDM-EA-b), 20−30% EtOAc/hexane (TDM-EA-c), 30−60% EtOAc/hexane (TDM-EA-d), 60−100% EtOAc/hexane (TDM-EA-e), and 1−100% MeOH/EtOAc (TDM-EA-f), were obtained. The fractions that showed greater cytotoxicity of the human glioblastoma U373 cell were TDM-EA-b, TDM-EA-c, and TDM-EA-d. These active fractions were further purified by semi-preparative high-performance liquid chromatography (HPLC) on a normal-phase silica column at 3 mL min^{-1}, using 20, 30, or 50% EtOAc/CH_2Cl_2 as the elution to yield 7−9 compounds. The TDM-EtOAc-d fraction using a 20% EtOAc/CH_2Cl_2 system was eluted to yield the active compound tagitinin C (31.5 mg). The collected substance was then concentrated under 40 °C reduced pressure using a rotary evaporator and then freeze-dried. For the cytotoxicity-guided assay, compounds were dissolved in medium or further structure-indentified with ^{1}H nuclear magnetic resonance (NMR).

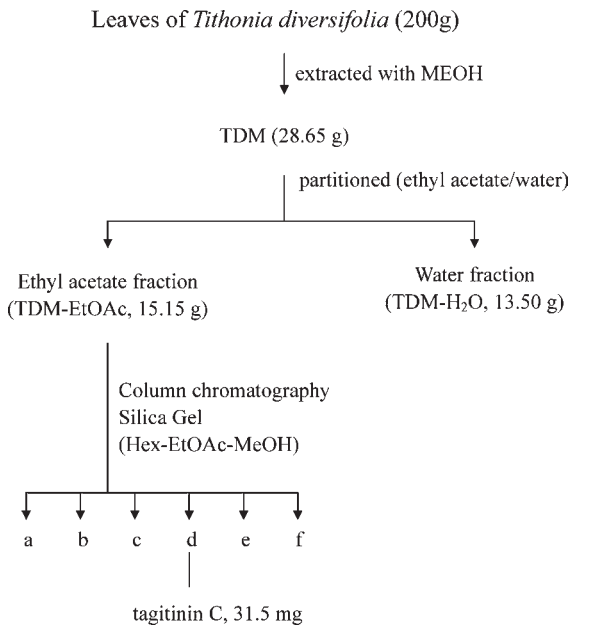

Figure 2. Schematic extraction of leaves of *T. diversifolia*.

NMR Measurements. ^{1}H NMR spectra were obtained on Varian Inova 500 NMR instruments. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} TLC plates (Merck, Darmstadt, Germany). H_2SO_4 (10%, v/v) in ethanol was aprayed on the compounds to enable visualization. Silica gel (230−400 mesh) (Macherey-Nagel, Germany) was used for column chromatography. Semi-preparative HPLC was performed with a Varian PrepStar SD-1 Varian PrepStar SD-1 (Harbour City) instrument and a Shodex RI-71 refractive index detector (Tokyo, Japan). A 10 × 250 mm inner diameter, 5 μm, normal-phase silica column (Phenomena) was used for analysis. All solvents used for chromatographic isolation were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture, Treatment, and Morphological Basement. The human malignant glioblastoma cell line U373 and Clone-9 (normal mouse liver cell) were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units mL^{-1} penicillin, 100 mg mL^{-1} streptomycin sulfate, 0.1 mM non-essential amino acid, and 1 mM sodium pyruvate then incubated at 37 °C at an atmosphere of 5% CO_2/O_2. U373 cells and Clone-9 cells (8×10^5 cells) were culture in a 6 cm dish and treated with or without tagitinin C for 24 h. The tagitinin C was predissolved in 100 mg μL^{-1} dimethyl sulfoxide (DMSO) stock solution before preparing different concentrations. To determine the morphological changes, cells were photographed with bright-field optics (Zeiss Observer Z1, Germany) after 24 h of incubation with tagitinin C.

Cell Viability Assay. Cell viability was determined by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) spectrophotometric analysis and trypan blue assay. The IC_{50} values were determined by cell reduction assay. Cells (7×10^3 cells mL^{-1}) were seeded in 96-well culture plates and allowed to adhere for 24 h. After 24 h of incubation, the extracts were added and the plates were incubated for

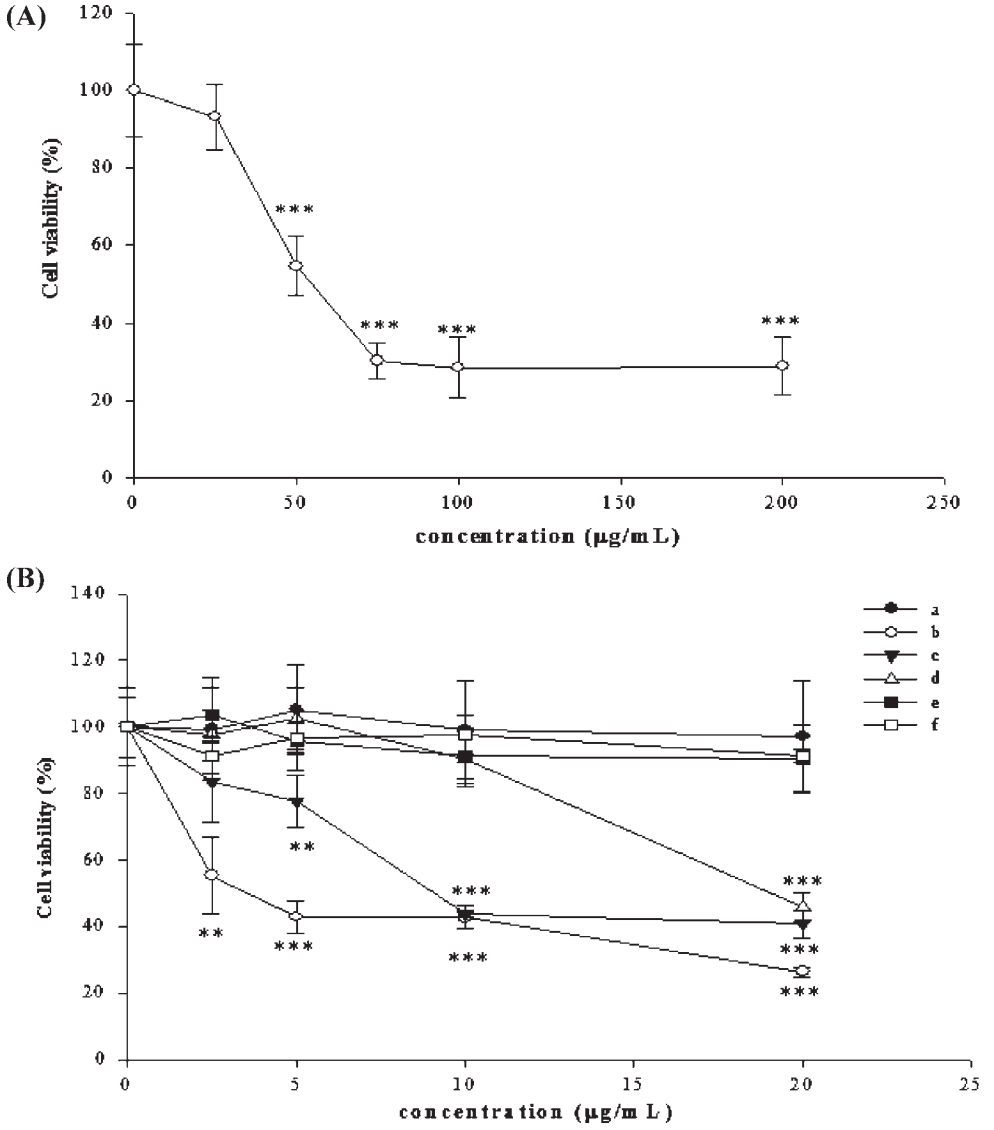

Figure 3. Effects of TDM and subfractions (a−f) of TDM on U373 cells. (A) Cytotoxic effects of TDM at various concentrations were determined using the MTT assay. (B) TDM-EA was passed through silica gel chromatography and successively eluted with (●) TDM-EA-a, (○) TDM-EA-b, (▼) TDM-EA-c, (△) TDM-EA-d, (■) TDM-EA-e, and (□) TDM-EA-f. All subfractions were dissolved in DMSO and then cultured with the cell at various concentrations. Data are represented as the mean ± SD of three independent experiments.

24 h. Cells were washed once before adding fetal bovine serum (FBS)-free medium containing MTT (1 mg mL^{-1}). After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue in the cells was dissolved in DMSO. The optical density (OD) was measured at 540 nm. The experiments were repeated 3 times.

Caspase Inhibition Assay. To determine whether caspase is involved in tagitinin-C-induced cell death, caspase inhibition assay was performed with pan-caspase inhibitor, z-VAD-fmk (BD Pharmingen) solubilized in DMSO. Cisplatin, which was used as a positive control, induces apoptosis in malignant glioma cells. Briefly, U373 cells were seeded in a 24-well plate and incubated overnight at 37 °C. At 1 h before treatment with 10 μg mL^{-1} tagitinin C, 3 μM z-VAD-fmk was added. After 1 day, cell viability was determined by the MTT assay, as described above. The cell viability of untreated U373 cells in the presence of diluted DMSO was regarded as 100%.

Assay for Apoptosis Detection. U373 cells (8×10^5 cells mL^{-1}), treated with or without tagitinin C for 12 or 24 h, were harvested and fixed with 1 mL of ice-cold 70% ethanol at 4 °C for 2 h. Total cellular DNA was stained with a propidium iodide (PI; Sigma) solution containing 250 mg mL^{-1} RNase A in PBS buffer. Annexin V is a phospholipid-binding protein, which has high affinity for phophatidylserine (PS) and is used to analyze apoptotic activity in cells. Test cells were stained with the Annexin V-FITC assay kit (Chemicon) for 15 min and then treated with PI for 30 min in the dark. Treated cells were taken as a measure of the percentage of the apoptotic cell population, using a flow cytometer (Becton Dickinson FACSCalibur).

To detect apoptosis in combination with ULK1 siRNA, U373 cells were cultured in medium containing 10 μg mL^{-1} for 24 h and then the ULK1 siRNA mixture was added in culture medium. After 24 h, tumor cells were collected and stained with PI. The percentage of the sub-G1 population was determined by flow cytometry.

Western Blotting. Whole cell lysates were separated by 9% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and electrotransferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated in blocking solution [5% nonfat milk in phosphate-buffered saline (PBS) with 0.1% Tween 20] at room temperature for 1 h. The membrane was incubated with anti-caspase-3 (Santa Cruz Biotechnology, sc-1226), poly(ADP-ribose)polymerase (PARP),

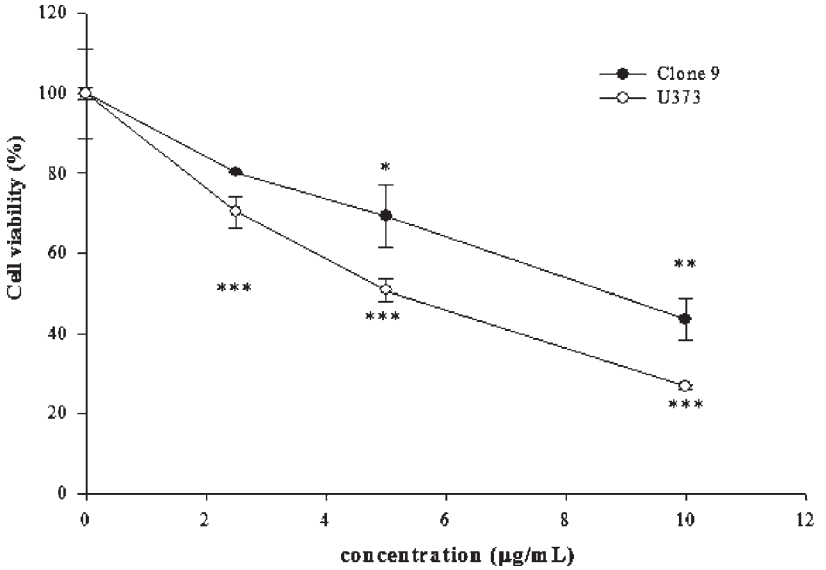

Figure 4. Effect of tagitinin C from TDM on the gioblastoma U373 cell and normal mouse liver Clone9 cell after 24 h of treatment. The cytotoxic effects of tagitinin C on the (●) Clone9 cell and (○) U373 cell at various concentrations. Data are represented as the mean ± SD of three independent experiments.

Atg1/Unc-51-like kinase 1 (ULK1), phosphorylated p38 (p-p38) (Santa Cruz Biotechnology, sc-7973), and Atg8/LC3 (microtuble-associated proteins 1A/1B light chain 3A) (Abgent, San Diego, CA) antibody at room temperature for 4 h. After washing 3 times in PBS with 0.1% Tween 20, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. The membranes were washed, incubated in a chemiluminescence reagent plus kit (Amersham), and exposed to X-ray film. The expressions of proteins were semi-quantified for a gel densitometric scanning program.

Transfection with ULK1 siRNA. For knockdown of gene expression, 10 nM ULK1 siRNA was transfected into cells in Optimem1 medium (Gibco) using the lipofectamine RNAiMAX reagent (Invitrogen) according to the instructions of the manufacturer. The mixture was added into cell culture dishes for ULK1 siRNA transfection for 24 h.

Statistical Analysis. Data were presented as the mean ± standard deviation (SD) of three independent experiments. Values were evaluated by one-way analysis of variation (ANOVA) followed by Duncan's multiple-range tests using Statistical Analysis Software (SAS Institute, Cary, NC). Control and treatment groups were compared by Student's *t* test. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Extraction and Subfractionation of the Plant Material. The leaves of TDM were extracted with methanol, as illustrated in the Materials and Methods and Figure 2. The TDM was partitioned by ethyl acetate and water. The ethyl acetate fraction (TDM-EA) was passed through silica gel chromatography and successively eluted with 0−5% EtOAc/hexane (TDM-EA-a), 10−20% EtOAc/hexane (TDM-EA-b), 20−30% EtOAc/hexane (TDM-EA-c), 30−60% EtOAc/hexane (TDM-EA-d), 60−100% EtOAc/hexane (TDM-EA-e), and 1−100% MeOH/EtOAc (TDM-EA-f). All subfractions were dissolved in DMSO, and cells were sequentially used for the experiment.

Cytotoxicities of TDM and Each Subfraction against U373 Cells. The cytotoxic effects of the TDM and subfraction at various concentrations were evaluated by the *in vitro* cytotoxicity assay against the following U373 cells. After 24 h, the viability of surviving cells in the 96-well microplates was determined quantitatively using a MTT assay in a microtiter plate reader. The subfractions were also determined at various concentrations under similar conditions. The cytotoxic effects of the TDM and subfractions against U373 cells are shown in panels A and B of Figure 3. The TDM showed antiproliferative activity against U373 cells, with an IC_{50} value of 59.2 ± 3.7 μg mL^{-1}. The subfractions exhibited relatively higher cytotoxic activity in a dose-dependent manner. The TDM-EA-b, TDM-EA-c, and TDM-EA-d subfractions exhibited a comparatively higher antiproliferative activity.

Identification of the Active Compound from Cytotoxicity-Guided Subfractions of TDM. The TDM-EtOAc-b, TDM-EtOAc-c, and TDM-EtOAc-d subfractions were collected with semi-preparative HPLC and then preceded with structure determinations. In NMR, spectra was shown in ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.89 (d, J = 17.0 Hz, 1H, H-1), 6.27 (d, J = 1.1 Hz, 1H, Hα-13), 6.19 (d, J = 17.0 Hz, 1H, H-2), 5.80 (dq, J = 8.8, 1.1 Hz, 1H, H-5), 5.74 (d, J = 1.1 Hz, 1H, H$_\beta$-13), 5.37 (br. d, J = 8.8, 1H), 5.82 (m, 1H, H-8), 3.50 (m, 1H, H-7), 3.21 (s, 1H, −OH), 2.41 (dd, J = 13.9, 6.3 Hz, 1H, Hα-9), 2.36 (seq, J = 6.9 Hz, 1H, H-2′), 1.94 (dd, J = 13.9, 10.0 Hz, 1H, H$_\beta$-9), 1.88 (br. s, 3H), 1.47 (s, 3H, H-14), 0.99 (d, J = 6.9 Hz, 3H, H-3′), 0.97 (d, J = 6.9 Hz, 3H, H-4′). The spectra data of compound tagitinin C were in agreement with the reported literature values.[2,7,13−15]

Effects of Tagitinin C from TDM on Cell Viability. The U373 cell and Clone9 were treated with tagitinin C from TDM at various doses for 24 h. The IC_{50} values were 6.1 ± 0.1 μg mL^{-1} in U373 and 8.6 ± 6.0 μg mL^{-1} in normal mouse liver Clone9. This compound from TDM showed specific killing between the cancer cell and normal cell (see Figure 4).

Induction of Apoptosis-Independent Cell Death by Tagitinin C from TDM. There are two types of programmed cell death involved anticancer activity: apoptosis and autophagy. To evaluate the apoptosis-inducing effects of tagitinin C from TDM, cells were treated with 0, 2.5, 5, and 10 μg mL^{-1} tagitinin C from TDM for 12 h (shown in Figure 5A) and 24 h (shown in Figure 5B). The dot plot of Annexin V-FITC fluorescence versus PI fluorescence will show three distinct populations. In procaspase 3 and caspase 3 analyses, there was no expression in activated caspase 3

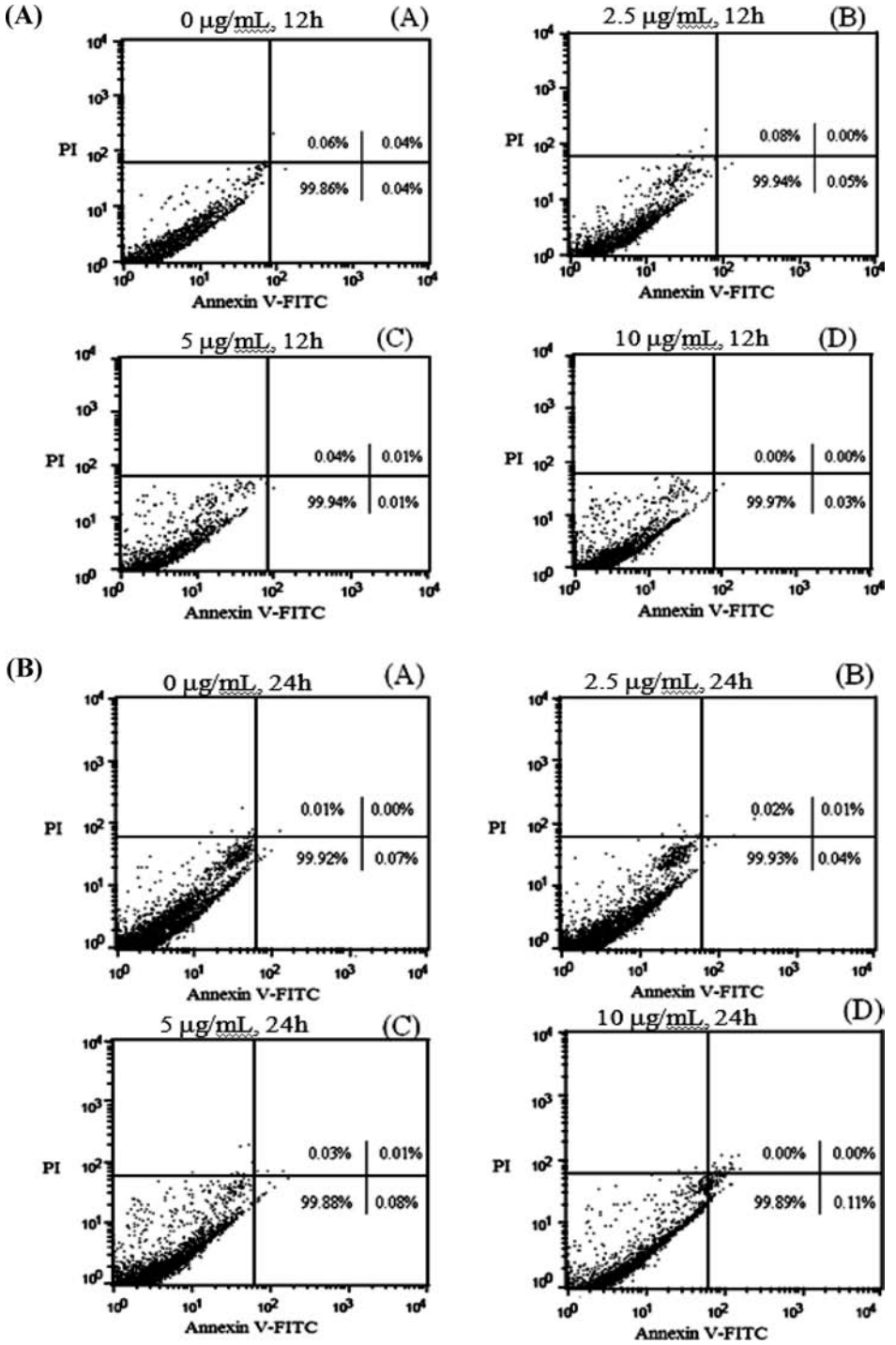

Figure 5. Flow cytometric analysis of apoptosis in the U373 cell treated with tagitinin C from TDM after 12 and 24 h of incubation. The U373 cells were treated with 0, 2.5, 5, and 10 μg mL^{-1} tagitinin C for (A) 12 h and (B) 24 h.

expression after treatment of tagitinin C from TDM for 24 h (see Figure 6A). Whereas apoptosis is mediated by caspases, the role of caspases in autophagy is debatable. Therefore, the caspase inhibition assay was performed to determine the involvement of caspase in tagitinin-C-induced cell death. Cisplatin, used as a positive control for apoptosis induction, induced apoptosis on 53% of U373-treated cells. z-VAD-fmk (3 μM) significantly increased the cell viability of U373-MG cells treated with 0.5 μg mL^{-1} cisplatin. This finding indicated that the concentration of 3 μM z-VAD-fmk was sufficient for the caspase inhibitor, z-VAD-fmk, to suppress the activation of caspases. On the other hand, 3 μM z-VAD-fmk did not significantly affect the cell viability of U373 cells treated with tagitinin C (see Figure 6B). These results indicate that tagitinin-C-induced cell death is independent of caspase activation. In flow cytometric analyses and the caspase inhibition assay, the data revealed that anti-glioblastoma activity induced by tagitinin C from TDM was apoptosis-independent.

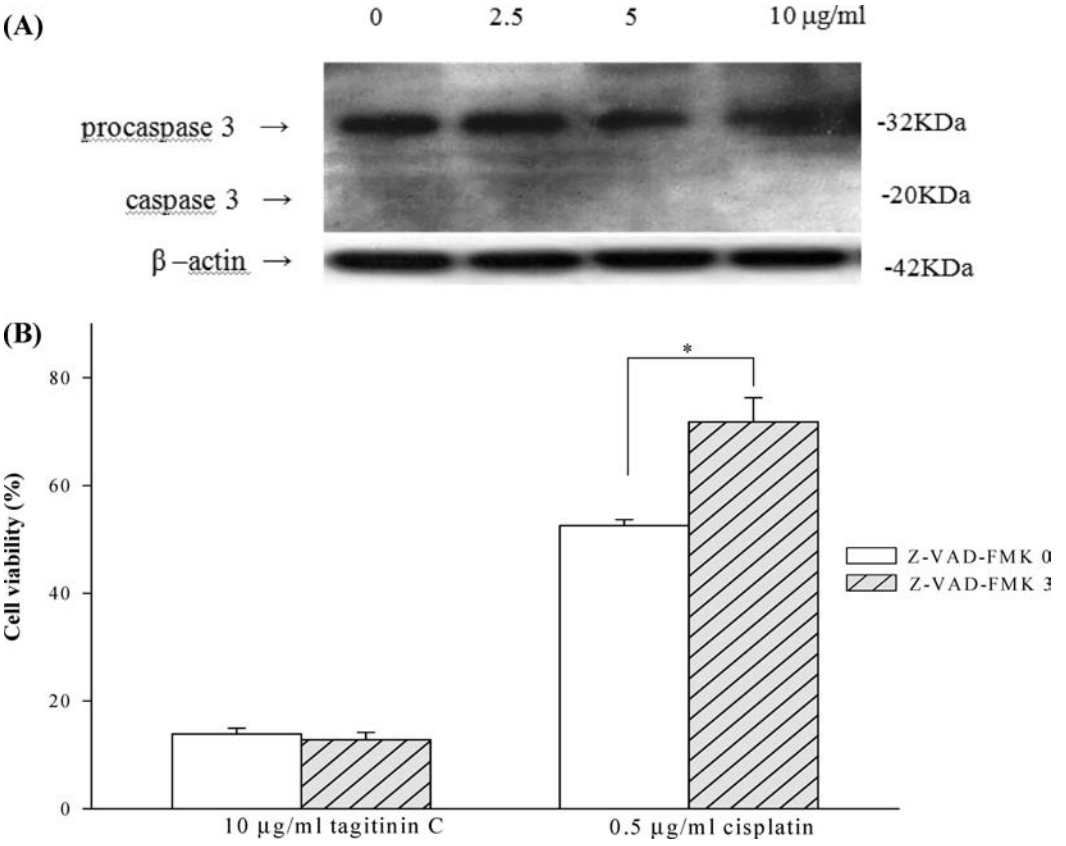

Figure 6. Effect of tagitinin C from TDM on caspases on the U373 cell after 24 h of treatment. (A) Expression of caspase 3 protein of the U373 cell treated with various concentrations was determined by western bloting. (B) Effect of the pan-caspase inhibitor, z-VAD-fmk, on the cell viability of U373 cells treated with 10 μg mL^{-1} tagitinin C. z-VAD-fmk (3 μM) was added to cells 1 h before the treatment with tagitinin C. After 1 days, the cell viability was determined. As a positive apoptosis inducer, cisplatin was used. The cell viability of untreated U373 cells in the presence of diluted DMSO was regarded as 100%. Results shown are the mean of three independent experiments. Note that the pan-caspase inhibitor, z-VAD-fmk (3 μM), did not prevent the induction of cell death by tagitinin C.

Autophagy Is Involved in U373 Cell Death Induced by Tagitinin C. To evaluate the cytotoxic effects of tagitinin C from TDM, cells were treated with 0, 2.5, 5, and 10 μg mL^{-1} tagitinin C from TDM for 24 h. Because PARP, p-p38, ULK1, and LC3 are the autophagic markers in mammalian cells, to investigate influences of tagitinin C on these proteins expression, western blotting to analyze protein alternation was carried out. With tagitinin C treatment, PARP, p-p38, ULK1, and LC3-II protein expressions were enhanced (see Figure 7). U373 cells were transfected with ULK1 siRNA for 24 h and then incubated with 10 μg mL^{-1} tagitinin C for 24 h. Autophagy blockade counteracting the suppression effect induced by tagitinin C was feeble, as evaluated by morphology, and the apoptosis ratio (sub-G1 ratio) of tagitinin-C-treated U373 cells increased significantly, as compared to the treatment of tagitinin C alone (see Figure 8). The results suggested that the tagitinin-C-induced malignant glioblastoma cell death mainly depended upon autophagy and also had potential to trigger cells that underwent apoptosis in autophagy blockade conditions.

Under oxidative stress, PARP is activated and contributes to necrotic cell death through ATP depletion. Oxidative stress is known to stimulate autophagy, and autophagy may act as either a cell death or a cell survival mechanism. PARP-1 is the founding member of the PARP family, a group of nuclear enzymes that play a critical role in DNA damage repair through poly(ADP-ribosyl)ation. Some researchers identify a novel autophagy signaling mechanism linking PARP-1 to the serine/threonine protein kinase LKB1-AMP-activated protein kinase (AMPK) mammalian target of rapamycin (mTOR) pathway, leading to stimulation of autophagy.[17]

Autophagy is an intracellular protein transport pathway common to all eukaryotic cells.[18] It is a well-known mechanism, whereby cells degrade parts of their own intracellular constituents, including cytoplasm and organelles.[19−22] The initial step of the autophagic process is the formation of a double membrane-bound vacuole (autophagosome) derived from a part of the endoplasmic reticulum[23] or from the cytoplasmic lipid pool called the phagophore.[24] The autophagosomes then receive hydrolases by fusion with lysosomes and late endosomes to form an autolysosome.

Therefore, it is essential to identify protein components of autophagic membrane to unravel the mechanism of the phenomenon. At least 12 Atg (autophagy-related) and 4 other proteins are known to be involved in mammalian macroautophagy initiation and execution.[25,26] The formation of autophagosomes appears to follow a pathway conserved across species, and most findings made in yeast or other organisms also apply to mammalian autophagy. Macroautophagy components are expressed in neurons and neuronal cell lines. During the first step, induction, autophagy can be induced via mTOR-dependent or-independent pathways, which stimulate the nucleation and expansion of the phagophore/isolation membrane. Previous

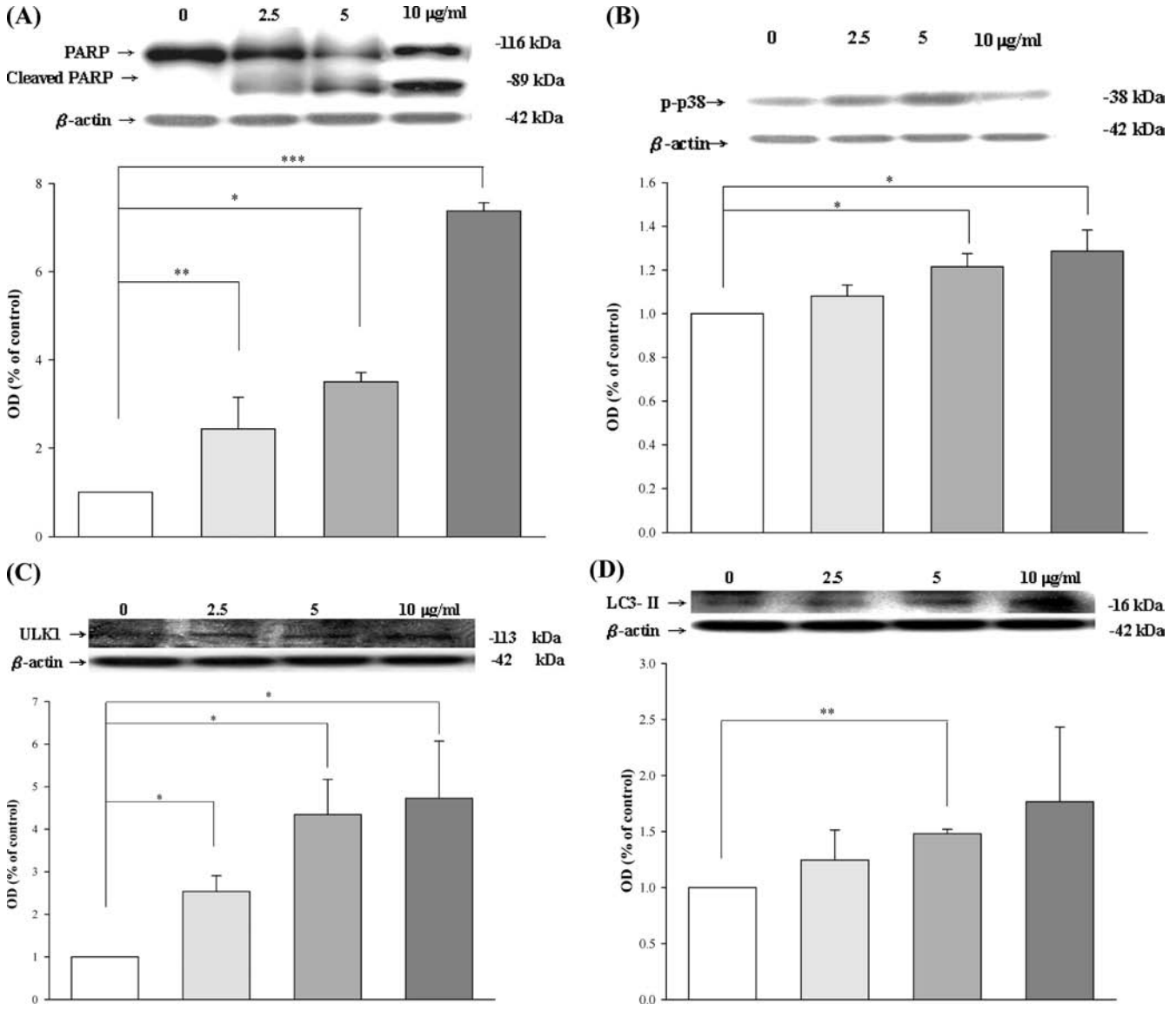

Figure 7. Effect of tagitinin C from TDM on autophagic markers on the U373 cell after 24 h of treatment. The expressions of (A) PARP, (B) p-p38, (C) ULK1, and (D) LC3-II of the U373 cell treated with various concentrations were determined by western bloting, and the OD values of the protein assay were scanned by the densitometer.

research showed conflicting data about the function of p38 MAPK in autophagy. This could be an analogy to the p38 MAPK pathway in mammalian cells, where osmosensing toward p38 MAPK is required for autophagy regulation by hypo-osmotic or amino-acid-induced cell swelling.[27] It appears that Atg1/ULK1 can act as a convergence point for multiple signals that control autophagy and can bind to several Atg proteins, regulating phosphorylation states and protein trafficking in mTOR-dependent pathways. Here, we demonstrated that p38 and Atg1/ULK1 were a contributive factor to tagitinin-C-induced autophagy. In the second step, vesicle nucleation, a multiprotein complex surrounding Atg6/Beclin 1 with PI3K activity (mediated by PIK3C3) is important for the formation of the autophagosomal membrane. In the third step, sequestration, two ubiquitin-like modification systems are essential for mammalian autophagy; Atg12, Atg7, Atg10, Atg5, and Atg16 are necessary early in autophagy for the formation of the phagophore or isolation membrane. Atg8/MAP-LC3 is cleaved by Atg4, activated by Atg7, transferred to Atg3, and conjugated to the phospholipid phosphoethanolamine in the phagophore. Atg8/MAP-LC3 makes it a general marker for autophagic membranes, which is essential for the dynamic process of autophagosome formation. Pro-LC3 is processed to its cytosolic form, 18 kDa LC3-I, which is modified to a membrane-bound form, 16 kDa LC3-II. LC3-II is localized to autophagosomes and preautophagosomes, making this protein an autophagosomal marker. After the fusion of autophagosomes with lysosomes, intra-autophagosomal LC3-II is degraded by lysosomal hydrolytic enzymes.[28] We described here that, under the conditions of tagitinin-C-induced autophagy, LC3-II was abundantly expressed through LC3-I conversion.

In our recent published data,[29] we found that human glioblastoma U373 cell death induced by tagitinin C or TDM was apoptosis-independent and induced autophagosome, survivin inhibition, and G2/M arrest. Here, we report a mechanism of tagitinin-C-induced cell death with hallmarks of both autophagy and apoptosis in human glioblastoma cell lines. Despite blocking

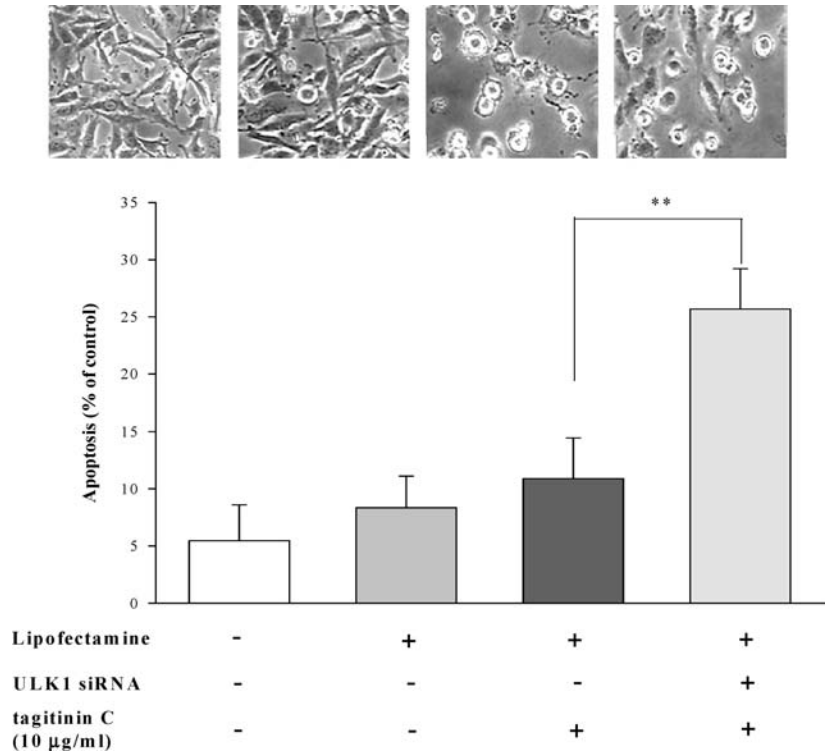

Figure 8. Effect of autophagy blockade on cell death induced by tagitinin C. U373 cells were transfected with ULK1 siRNA for 24 h and then incubated with tagitinin C for 24 h.

apoptotic features, inhibition of caspase 3 activity failed to rescue cell viability. Interestingly, cleaved PARP, p-p38, ULK1, and LC3-II protein expressions in U373 after treatment of tagitinin C revealed serial signal processes consistent with autophagy. In addition, when autophagy was prevented at an early stage by ULK1 siRNA, the apoptosis ratio (sub-G1 ratio) significantly increased. Of note, neither apoptosis nor autophagy was able to salvage cells from the effect of tagitinin C inducing cell death, suggesting that both pathways were essential in death execution. This result was similar to the antitumor effect of As_2O_3 in glioblastoma cells.[30] In several settings, autophagic signaling sets the stage for apoptosis to occur, while in others, inhibition of autophagy triggers an apoptotic cell death program.[31,32] In our study, we demonstrated that after treatment with less than 10 μg mL^{-1} tangitinin C, the autophagosomes of U373 cells were stained as described by ref 29. Additional supports, such as p-p38, ULK1, and LC3-II protein expressions, to the fact that autophagy caused cell damage after exposure to tangitinin C were found when we demonstrated that inhibition of caspase did not alter tangitinin-C-induced cell death. Finally, we corroborated the mechanism of tangitinin-C-induced cell death using ULK1 siRNA, an autophagy inhibitor. It has been suggested that, after exposure to low doses of tagitinin C (<10 g mL^{-1}), autophagy occurred. Thus, after exposure to low doses of tagitinin C and ULK1 siRNA, autophagy was inhibited and apoptosis occurred alternatively.

Classically, autophagy is described as a cellular clearance mechanism to remove damaged organelles and protein aggregates and, thus, serves as a cytoprotective mechanism to counteract some stress, such as oxidative stress in cells. On the other hand, autophagy is characterized by the presence of acid vesicular organelle formation in the cell cytoplasm.[29] This leads to the disruption of cytoplasm organelles before nuclear collapse. Autophagy has been documented in some human carcinoma cells after treatment with ionizing radiation or chemotherapeutic drugs. We have already demonstrated that, after treatment with 1 μg mL^{-1} tagitinin C, the acidic vesicular formation occurred before nuclear changes. It has been suggested that acidic vesicular formation and digestion of material in the acidic vesicular formation are dependent upon acidification of cellular organelles. Autophagy may preserve cellular homeostasis while suppressing the latent apoptotic program, meaning that, under conditions of nutrient deprivation, autophagy can actually suppress cell death, presumably by providing endogenous metabolites when exogenous nutrients are missing. While it is not a new finding that autophagy can rescue cells under conditions of starvation, it is novel that autophagy actually prevents the apoptotic default pathway to be activated. This finding may be incorporated into a more general hypothesis, suggesting the existence of a double switch between the two principal lethal signaling pathways. Prevention of autophagy can precipitate apoptotic cell death, as shown here. Thus, the presumed mechanistic contraposition of the type-I and type-II forms of cell death deserves close scrutiny and critical re-evaluation. Some reports have also shown that reactive oxygen species (ROS) could serve as signaling molecules that directly or indirectly activate autophagy.[33] Of course, we will study the relationships between apoptotic cell death and autophagic cell death in the future.

In conclusion, we showed that tagitinin C produces *in vitro* growth inhibition in the human U373 glioblastoma cell at 2.5−10 μg mL^{-1} tagitinin C. We have demonstrated that the cytotoxic effects after exposure to a certain concentration of tagitinin C is caused by autophagy. However, when autophagy is inhibited, apoptosis occurs. These findings suggest that tagitinin

C should be further investigated as a potential adjuvant chemotherapeutic agent.

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