A Master's Thesis

Quercetin induces mitochondria mediated apoptosis and protective autophagy in human glioblastoma U373MG.

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June, 2013

퀘르세틴에 의한 뇌종양 세포주 U373MG에서 미토콘드리아 의존적인 apoptosis와 protective autophagy 유도

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Abstract

Quercetin is one of the dietary flavonoids known to have antitumor effect against several types of cancer by promoting apoptotic cell death and inducing cell cycle arrest. In this study, anticancer effect of quercetin has been evaluated against p53-mutant human glioblastoma cells U373MG. Quercetin exhibited time- and concentration-dependent cytotoxicity against U373MG. Induction of apoptotic cell death by quercetin was observed by fragmented nuclei, increased sub-G1 population and decreased mitochondrial membrane potential. Further, proteolytic activation of caspase-3, -7, and cleavage of PARP (Poly (ADP-Ribose) Polymerase) substantiated the occurrence of apoptotic cell death. Also, translocation of p53 from cytosol to mitochondria lead to release of cytochrome C from mitochondria into cytosol. Concomitantly, quercetin induced formation of acidic vesicular organelles, conversion of LC3II, indicating that quercetin also induced autophagy. Importantly, pre-treatment with chloroquine, an autophagy inhibitor, enhanced quercetin-mediated apoptotic cell death. These results show that quercetin caused protective autophagy in U373MG and the ability of quercetin in association of autophagy inhibitor may improve the ultimate outcome of glioblastoma therapy.

요약문

Flavonoid의 일종인 quercetin은 다양한 암세포 주에 대하여 항암효능이 있는 것으로 알려져 있다. 본 연구는 교모세포종인 U373MG에 대한 quercetin의 세포사멸효능과 그 기작에 대해 연구하였다. Quercetin 처리 시간과 농도 의존적으로 세포죽음이 일어났다. Hoechst 33342 염색을 통한 apoptotic body 형성, flow cytometic analysis를 통한 Sub-G1기의 축적과 mitochondrial membrane potential 감소, apoptosis 관련 단백질의 발현증가와 caspase-3, 9 의 활성 증가로 인한 apoptosis에 의한 세포죽음을 확인하였다. 또한 acridine orange 염색과 immunoblot analysis를 통하여 autophagy 현상도 일어나고 있음을 확인하였다. Autophagy inhibitor인 chloroquine 전처리를 통하여 autophagy 현상이 세포를 보호하기 위한 protective autophagy임을 확인하였으며, 이러한 autophagy를 억제하였을 때 apoptosis가 증진되어 세포죽음을 더욱 유도하는 것을 확인할 수 있었다.

1. Introduction

Apoptosis is the programmed cell death which is mediated by caspases, cystein proteases that cleave target proteins at specific aspartic acid. It is known to have two different pathways; cell surface death receptor activated extrinsic pathway and mitochondria involving intrinsic pathway. Apoptosis is responsible for cell death during development as well as in adult stage of multicellular organisms to keep balanced cellular homeostasis. On the other hand, autophagy is an evolutionary conserved and genetically programmed process degrading long-lived cellular proteins and organelles. The role of autophagy in cancer is quite complicated and controversial. It is assumed to be tumor suppressive during cancer development, but contributes to tumor cell survival during cancer progression [1]. Alternatively, during nutritional deprivation, autophagy prevents the tumor cells from dying by inhibiting apoptosis and when autophagy is prevented, the cells undergo apoptosis [2,3,4]. Regardless of promoting cell survival or cell death characteristic, autophagy is intimately linked with apoptosis, and the two processes engage in a complex and poorly understood molecular crosstalk [5]. Therefore, induction of apoptosis and inhibition of protective autophagy has become an effective means of cancer therapy [6, 7].

Quercetin is an antioxidative flavonoid ubiquitously distributed in the plant kingdom. Quercetin and related flavonoids present in fruits and vegetables have attracted much attention in recent years as potential anti cancer agents. Their anticancer effects have been attributed to anti-oxidative activity, inhibition of enzymes activating carcinogens, the modification of signal transduction pathways, and interactions with receptors and other proteins [8]. Quercetin has been reported as anticancer agent in many cancer models, such as lymphoma, ovary, endometrial, prostate, liver, and gastric cancer [9, 10, 11, 12]. Furthermore, antitumor activity of quercetin was shown by inducing apoptosis in leukemic cells, pancreatic tumor, breast cancer, hepatoma cells and prostate cancer [10, 13-17]. Several papers showed that quercetin has effect on the glioma cell line. Quercetin induced apoptosis through reduced XIAP (X-linked Inhibitor of Apoptosis Protein) and surviving protein which is related to anti-

apoptosis protein in glioblastoma cell line A172 [18]. Previous study, Michaud-Levesque et al., 2012 reported that STAT3 (Signal Transducer and Activator of Transcription 3), one of the highly expressed oncogenes in glioblastoma was blocked by IL-6 which induced by quercetin [19]. Quercetin also has anti proliferative effect on U138MG glioma cell line through arrest in the G2 checkpoint of cell cycle and decrease of the mitotic index [20]. Such effects of quercetin in glioblastoma cells seemed to be dependent on the cell type; it promote degradation of survivin and caused apoptotic cell death in U87MG, U251, A172 and LN229 but not much in U373MG [21].

Glioblastoma are the most common type of primary brain tumors in adults and the most lethal and least successfully treated tumors. This low absolute incidence combined with high morbidity, poor response rates and short survival times pose practical problems for clinical trial execution, particularly if therapy is anticipated to target a molecularly-defined subset of tumors [22]. Less than 30% patients suffered from this devastating diseases, even receiving multimodal treatments such as maximal surgical resection, combined chemotherapy and radiotherapy and adjuvant chemotheraphy, can survive only 12-15 month [23]. This scary fact underscores the need of alternative therapies for the prevention and effective treatment of glioblastoma. Previously, it has been found that methylating agent temozolomide (TMZ), the most widely used anticancer agents to treat glioblastoma, produced ROS (Reactive Oxygen Species) and activated the AMP-activated protein kinase (AMPK) which contribute to p53 activation and mTORC1 (mammalian Target Of Rapamycin Complex 1) inhibition, thereby causing apoptosis [24]. Another anticancer reagent, bevacizumab inhibits the binding of VEGF (Vascular Endothelial Growth Factor) to its receptors, Flt-1 and KDR (Kinase Domain Receptor), on the surface of endothelial cells [25]. However, glioblastoma patients survival rate still low, so urgently need to improved methods of treatment glioblastoma. Mutations of p53 are present in more than 30% of gliomas, which are the most common tumors of the brain, and constitute an early genetic event, suggesting that abnormalities of p53 are involved in development of gliomas [26]. In U373MG, p53 was mutated in R273H and it is a classic DNA contact mutant. This mutation increased malignancy and tumorigenicity from loss of the function of wild type p53 [27] although mutant and

wt p53 has conformationally same structure [28]. The mutant p53 R273H can translocate to mitochondria, then induce decrease of MOMP (Mitochondrial Outer Membrane Permeablilization), release of cytochrome C and activate caspase same as wild type of p53 [29, 30]. In this study, with the aim of exploring effective anticancer activity of quercetin, the induction of cell death mechanism by quercetin against p53-mutant human glioblastoma U373 MG cells were evaluated.

2. Materials and Methods

2. 1. Reagents

Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, fetal bovine serum (FBS), penicillin and streptomycin were acquired from Gibco. Hoechst 33342 dye was purchased from Invitrogen Life Technologies, Inc. (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) and MTT were from amresco (Cleveland, OH, USA). Quercetin, Chloroquine, propidium iodide (PI), RNase A, Acridine orange and β-actin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bax, cytochrome c, caspase-3, -7 and -9 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-caspase-8 was obtained from R&D Systems (Minneapolis, MN, USA). BD^{TM} Mitoscreen (JC-1) kit was purchased from BD Biosciences (Franklin Lakes, NJ). A BCA protein assay kit was purchased from Pierce (Rockford, IL, USA), and polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA). All other reagents used were of analytical grade.

2. 2. Cell culture

Human glioblastoma U373MG cell was kindly provided by Professor Tae-Hoo Yi in the Department of Biotechnology, Kyunghee University, Korea. U373MG cell was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) heat inactivated Fetal Bovine Serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell was maintained in a humidified incubator at 37 ℃ in a 5% CO_2 atmosphere.

2. 3. Cell viability

The effect of the Quercetin on the viability of U373MG cell lines was determined using an MTT-

based assay [32]. Briefly, exponential-phase cells were collected and transferred to a microtiter plate (3×10^4 cells/ml). The cells were then incubated for 24 h, 48 h, and 72 h with various concentrations of the Quercetin and/or chloroquine. Afterwards, 0.1 mg of MTT (Amresco, Cleveland, OH, USA) was added to each well, and incubated at 37 ℃ for 4 h. The medium was carefully removed and DMSO (150 μl) was added to each well to dissolve the formazan crystals. The plates were read immediately at 570 nm on a Sunrise microplate reader (Sunrise, Tecan, Salzburg, Austria). The percentage of cell viability was calculated based on the following formula: mean value of (control group - treated group/control group) × 100%. All results were assessed in triplicate for each concentration.

2. 4. Flow cytometric analysis

To analyze the cell cycle distribution, apoptosis, autophagy, and mitochondrial membrane potentials, cells (3×10^4 cells/ml) were plated in 60 mm plates and treated with Quercetin (0-100 μM) for 48 h. For cell cycle analysis, cells were harvested, washed with PBS, fixed in 70% ethanol, rehydrated in 2 mM EDTA-PBS, treated with RNase A (25 ng/mL), and stained with PI (40 μg/ml). For the detection of autophagy, cells were stained with 10 μM AO, harvested, and kept in 2 mM EDTA-PBS containing 10% FBS. For JC-1 mitochondrial membrane detection, we followed the manufacturer's protocol. In brief, treated cells were trypsinized and washed with 1X assay buffer, stained with JC-1 for 15 min at 37 ℃ in a CO_2 incubator, and washed twice with 1X assay buffer at RT. All analyses were performed using a FACSCaliber flow cytometer (BD Biosciences). Data from 10,000 cells per sample were analyzed with CellQuest Software (BD Biosciences). Each experiment was repeated at least three times.

2. 5. Caspase activity

Caspase-3, -9 activity was measured by colormetric assay following the protocol of the commercially available kit from Sigma and Biovision respectively. Briefly, cells were lysed after 48h

quercetin treatment with or without chloroquine, aliquots (50 μl) of the supernatant were placed in a 96-well microplate containing reaction buffer. After added substrate, all mixtures were incubated overnight in a humidified environment at 37 ℃, and the concentration of the ρ-nitroanyline (ρ-NA) released from the substrate was measured with a Sunrise microplate reader at 405 nm.

2. 6. Cellular fraction and immunoblot analysis

After treatment with various concentration of quercetin, the U373MG cells were collected and washed twice with cold PBS. The cells were then lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/ml aprotinin, and 25 μg/ml leupeptin) and kept in ice for 30 min. The lysates were centrifuged for 30 min at 13,000 rpm and 4 ℃, and the supernatants were stored at -70 ℃ until use. Cytosolic and mitochondrial extracts were prepared using the fraction lysis buffer (75 mM NaCl, 8 mM Na2HPO4, 1 mM Na2H2PO4, 250 mM sucrose, 1 mM EDTA, 350 μg/ml digitonin). After lysed the cell, kept in ice for 10 min then centrifuged for 15 min at 15,000 rpm and 4 ℃. The supernants were cytosolic fraction. After pellet washed by lysis buffer, pellets were lysed in lysis buffer same as make whole lysates. The protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Aliquots of the lysates (30–60 μg of protein) were separated via 10–15% SDS-PAGE and transferred onto a polyvinylidine difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) using a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.8, and 20% methanol, v/v). After blocking with 5% nonfat dried milk, the membranes were incubated for 4 h with primary antibodies, followed by an additional 30 min of incubation with secondary antibodies in milk containing Tris-buffered saline (TBS) and 0.1% Tween 20. Human anti-caspase-3, caspase-7, cleaved PARP, cytochrome c, HSP60, LC-II, beclin-1, and DRAM antibodies were used at a 1:1,000 dilution as the primary antibodies, and horseradish peroxidase-conjugated goat anti-human IgG (Vector Laboratories, Burlingame, CA, USA) at a 1:5,000 dilution was utilized as the secondary antibody. The membrane was then exposed to X-ray film. Protein bands were detected

using the WEST-ZOL® plus western blot detection system (Intron, Gyeonggi-do, Korea).

2. 7. Statistical analysis

All results are expressed as means ± standard deviations (SDs). A one-way analysis of variance was conducted using the Statistical Package for the Social Sciences software (SPSS 17.0 for Windows, 2008, SPSS Inc., Chicago, IL, USA). A $p < 0.05$ and 0.01 were considered statistically significant. All assays were performed in triplicate.

3. Results

3.1. Effect of quercetin on cell viability in U373MG

To investigate the cytotoxic effects of quercetin on human glioblastoma U373MG, MTT assays were conducted. As shown in Fig. 1, quercetin reduced the viability of U373MG cells in a time- and dose-dependent manner. IC_{50} values of quercetin against U373MG at 48 h and 72 h were 125.70 μM and 78.04 μM, respectively. These results indicated that quercetin caused cytotoxicity in U373MG cells in long time exposure.

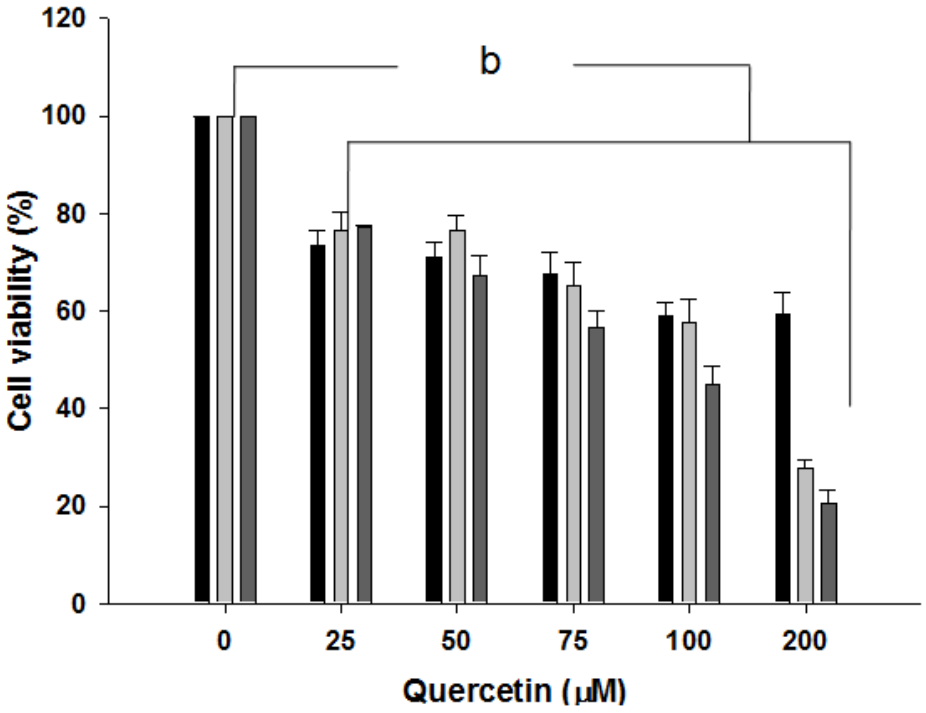


Figure 1. Quercetin inhibits cell growth and induces apoptosis. Cells were seeded, incubated for 24 h and then incubated with the indicated concentrations of quercetin for additional 48 h. Viability was determined on the basis of MTT reduction. U373MG human glioblastoma cells were treated with increasing doses of quercetin for varying lengths of time (24-72 h). The data shown represent the mean ± S.E. for one experiment performed in triplicate. ■; 24h, ■; 48h, ■ ; 72h. Values are the mean ± SD of three independent experiments. $^{a}P<0.05$, $^{b}P<0.01$, compared to the control.

3. 2. Effect of quercetin on the cell morphology

In apoptotic cell death, various morphological changes such as cell shrinkage and condensation and fragmentation of chromatin can be clearly noted. To evaluate the effect of quercetin in the induction of apoptosis, U373MG cells treated with various concentrations of quercetin were examined by fluorescence microscopy after Hoechst 33342 staining. As depicted in Fig. 2, after treatment with 25, 50, 75 and 100 μM quercetin, cells showed marked morphological changes such as condensed and fragmented chromatin and formation of apoptotic bodies (Fig. 2, white arrows).

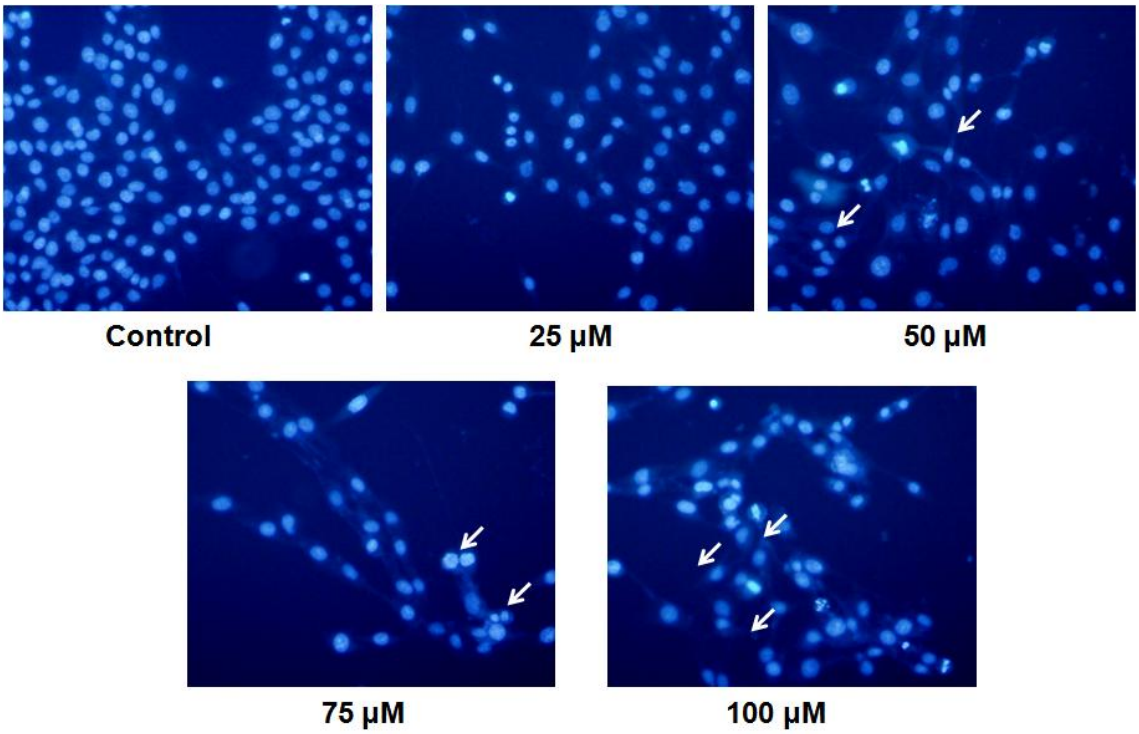


Figure 2. Effect of quercetin on the cell morphology of U373MG cells. Treated cells were stained with nuclear Hoechst 33342 and visualized under a fluorescence microscope after 48 h treatment. White arrows are indicating apoptotic bodies.

3. 3. Effect of quercetin on cell cycle distribution

Furthermore, induction of apoptosis was indicated by the accumulation of sub-G1 U373MG cells after quercetin treatment. A significant increase in sub-G1 cells were not noted in 24 h treatment; from 1.02% (0 μM) to 2.05% (100 μM). However, after 48 h of treatment, quercetin significantly increased the sub-G1 population concentration-dependently; from 0.46% (0 μM) to 31.75% (100 μM) (Table 1). Analysis of disruption of mitochondrial membrane potentials is also one of the apoptosis phenomenon.

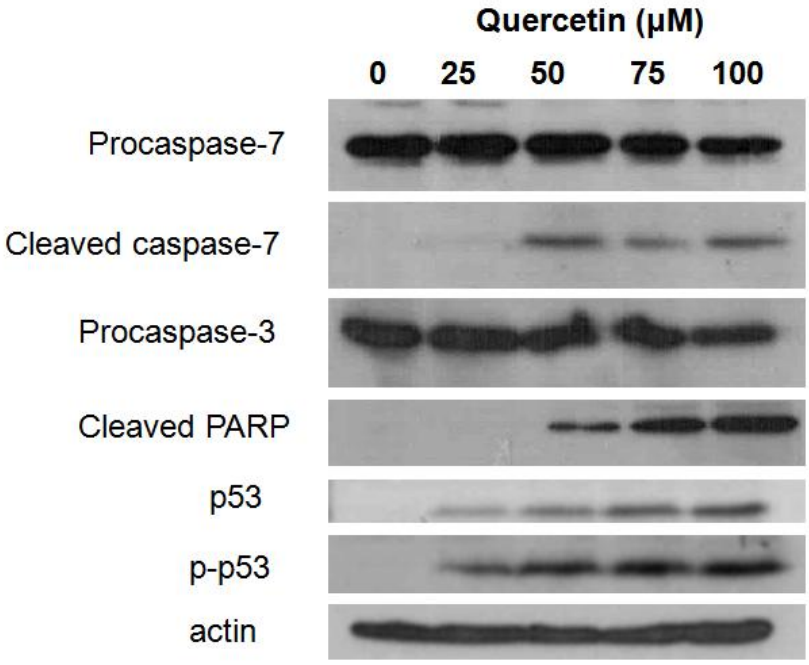
Table 1. The percentage of U373MG cells in the sub-G1 fraction after 24 h and 48 h incubation with quercetin.

Concentration (μM)	Sub-G1 (%)	
	24 h	48 h
0	1.02 ± 0.35	0.5 ± 0.31
25	1.78 ± 0.89	1.71 ± 0.53
50	2.33 ± 1.17	2.26 ± 0.86
75	1.65 ± 0.65	6.66 ± 0.79[a]
100	2.05 ± 0.93	8.48 ± 0.40[a]

3. 4. Effect of quercetin on expression of apoptosis related protein

Apoptosis is form of the cell death characterized by morphological change and activation of cystein-asparate protease (caspase) and pro-apoptotic protein. In intrinsic pathway, caspase-9 was cleavage by Apaf-1 and cytochrome C then finally executioner caspase-3 and -7 were activated [33]. The apoptotic caspases can be classified as either initiator caspases or effector caspases. Initiator caspases, which include caspases-2, -8, -9 and -10, activate the caspase cascade through the removal of the inactive prodomains of the effector caspases. Once activated, effector caspases, including caspases-3, -6 and -7, cleave several dozen key substrates within the cell in order to carry out the apoptotic process [34, 35]. Caspase-3 is a key member of the effector caspases which it can be activated by apoptotic signals from both intrinsic and extrinsic pathway [36]. In the intrinsic pathway, apoptosis was mediated by release of cytochrome C from mitochondria to cytosol which interacts with monomeric APAF-1 to facilitate a conformational change in the latter, leading to its oligomerization and recruitment of caspase-9 to form the apoptosome [37, 38]. Western blot analysis showed that quercetin promoted cleavage of procaspase-3, -7 and increased cleaved form PARP, suggesting that apoptosis was cause of quercetin-induced cell death in U373MG (Fig. 3A). However, the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 were not detected in whole lysate. Previous report showed that mutant p53 could induce apoptosis by transcription independent signaling [28]. Nevertheless U373MG has no p53 transcriptional activity, our results showed that p53 expression level was does dependently increased. Under a variety cell death stress condition, p53 rapidly moves to the mitochondria and induce apoptosis [39]. In cellular fraction results showed that p53 translocated to mitochondria (Fig. 3B). In cytosolic fraction, cytochrome C was increased whereas cytochrome C was decrease in mitochondrial fraction (Fig. 3B). Collectively, these data indicate that quercetin activated the caspase cascade by translocate p53 from cytosol to mitochondria, release of cytochrome C from mitochondria, that leading to apoptotic cell death.

(A)



(B)

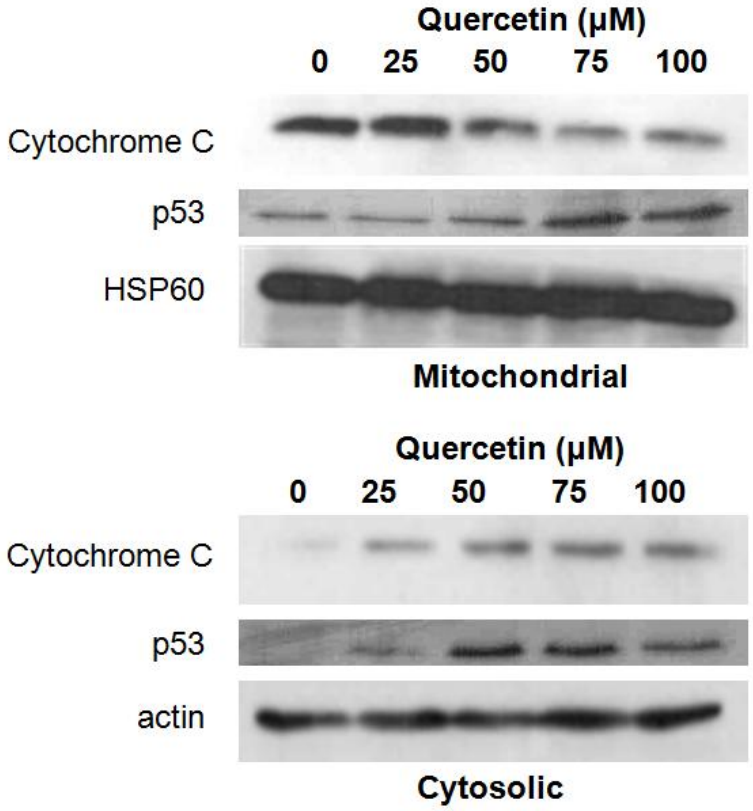
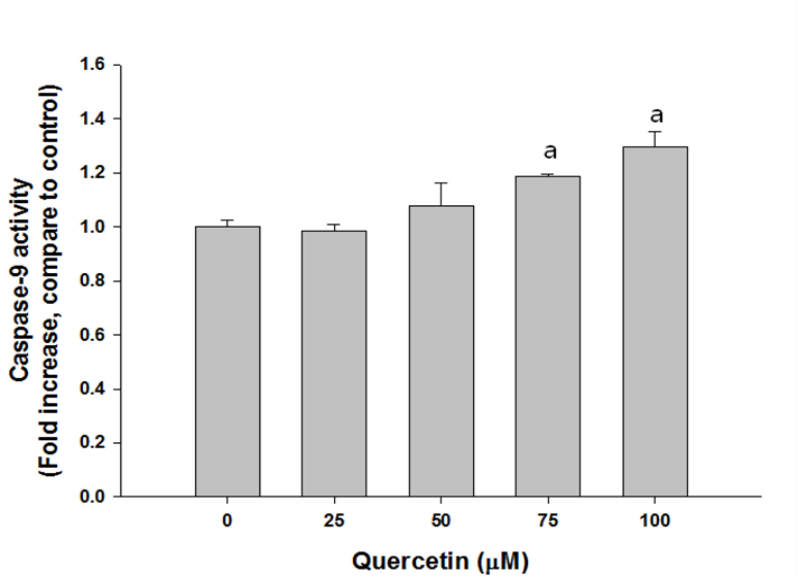


Figure 3. Expression level of apoptosis related protein in various concentrations. (A) The cellular proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were probed with the indicated primary antibodies against caspase-7, -3, and cleaved PARP, p53, phosphorylated p53 then with horseradish peroxidase conjugated goat anti-rabbit IgG. Actin was used as an internal control. (B) Cytochrome c translocation were estimated by western blotting with actin and HSP60 serving as loading controls for mitochondrial and cytosolic proteins, respectively. Mitochondrial and cytosolic fractions were isolated from cells as indicated in the methods section.

3. 5. Effect of quercetin on caspase activities

In immunoblot results showed cleaved caspase-3 and -9 signals were too weak to detect. So we quantified its enzymetic activity using commercially available kit. Quercetin treatment does-dependently increased caspase-3 activity (Fig 4B). Caspase-9 activity also increased does dependent manner in quercetin treatement (Fig 4A).

(A)



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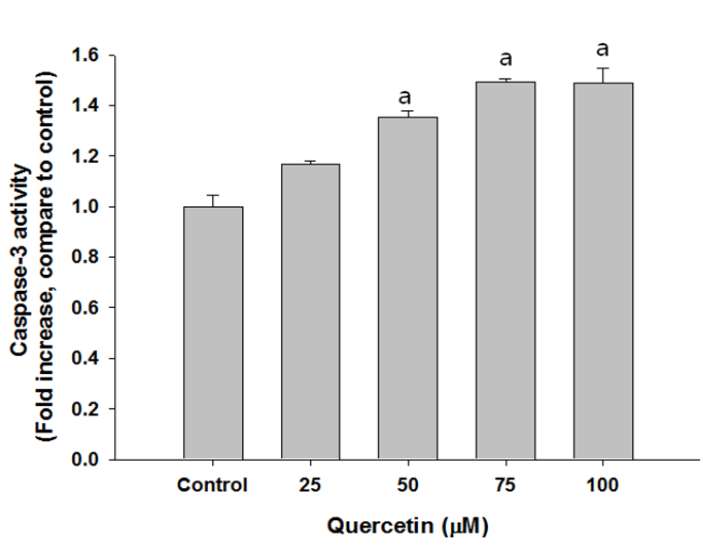
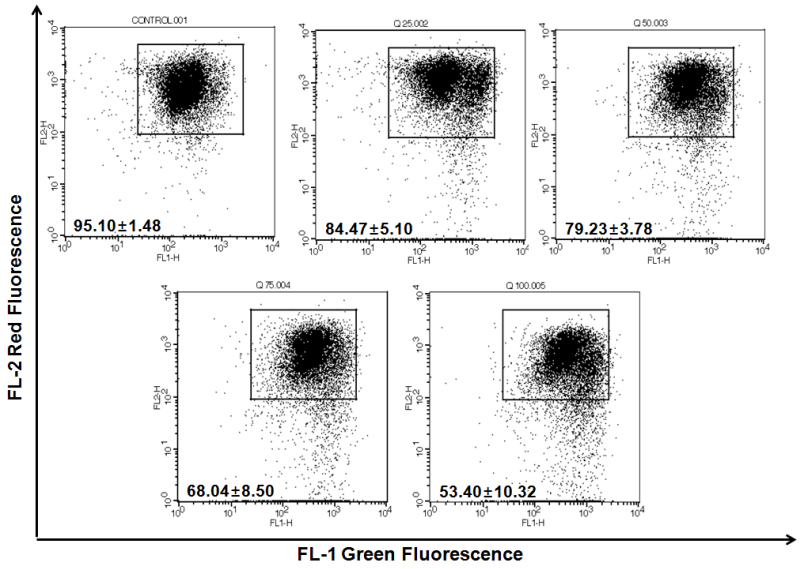


Figure 4. Activities of caspase-9 and -3 in U373MG cells. Cell lysates prepared from cells that had been treated with quercetin for 48 h were assayed for in vitro Caspase-9, -3 activity. The rate of cleavage of the caspase substrate LEHD-pNA, Ac-DEVD-pNA was determined by measuring the absorbance at 405 nm. (A) Caspase-9 activity. (B) Caspase-3 activity. All data correspond to the mean ± SD of three independent experiments. a= significantly different from the control, $p < 0.05$.

3. 6. Effect of quercetin on mitochondrial membrane potential.

Mitochondria may play a key part of apoptosis. Due to decrease of mitochondria membrane potential, certain apoptogenic factors released from intermembrane space to cytosol [40]. Since cellular fraction results showed cytochrome C released from mitochondria to cytosol, we presumed that mitochondrial membrane potential also decreased after quercetin treatment. JC-1 staining, one of the methods for measuring mitochondrial membrane potential, showed quercetin treatment disrupted membrane potential in 48 h treatment in does dependent manner (Fig. 5A, B).

(A)



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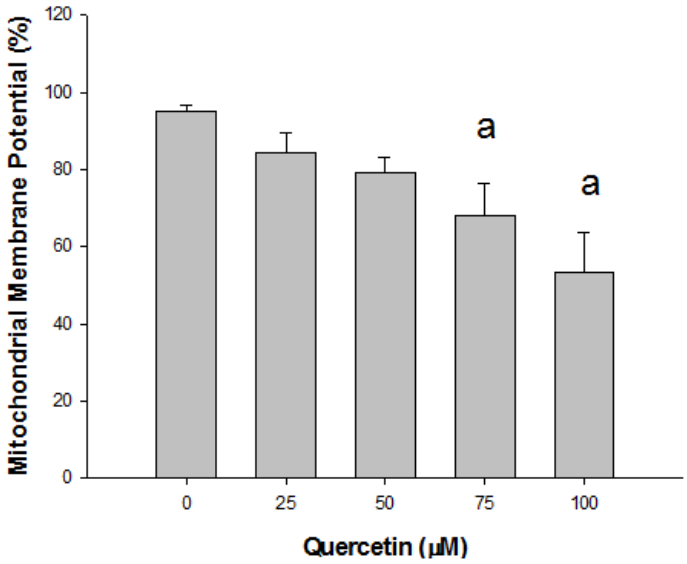
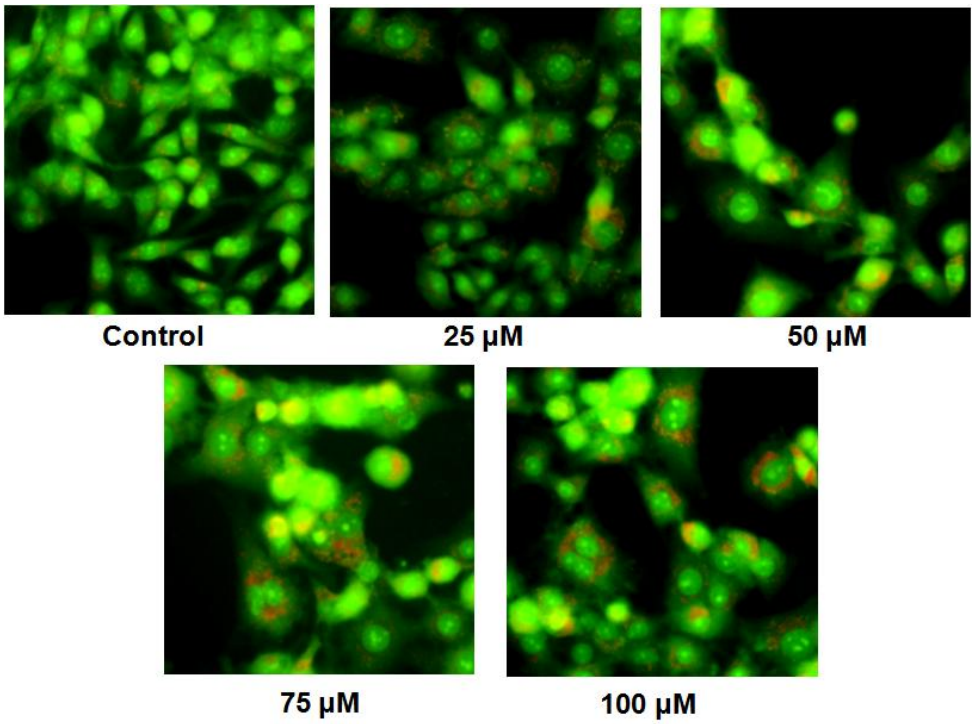


Figure 5. Mitochondrial membrane potential analysis by FACS. Flow cytometry analysis of JC-1 staining. Treated cells were trypsinized, stained with JC-1, washed, and analyzed by flow cytometric analysis. (A) Dot plot illustrating. (B) The decrease of mitochondria membrane potential % indicated that cells were undergoing mitochondrial dysfunction.

3. 7. Effect of quercetin on autophagosome formation

Quercetin was induced autophagy in U87MG, p53 wild type, but not in T98G, p53 mutant. So we investigated whether quercetin induce autophagy in one of the p53 mutant glioblastoma U373MG. We first confirmed that induction of autophagy in U373MG glioblastoma cell by quercetin through acridine orange staining. It was found that there were many red fluorescent in quercetin treatment whereas little red fluorescent were observed in control (Fig. 6A). FACS analysis also showed same phenomenon that up to 75 μM, acridine stained cell were increased. However in 100 μM, acridine orange stained cells were slightly decreased (Fig. 6B, C).

(A)



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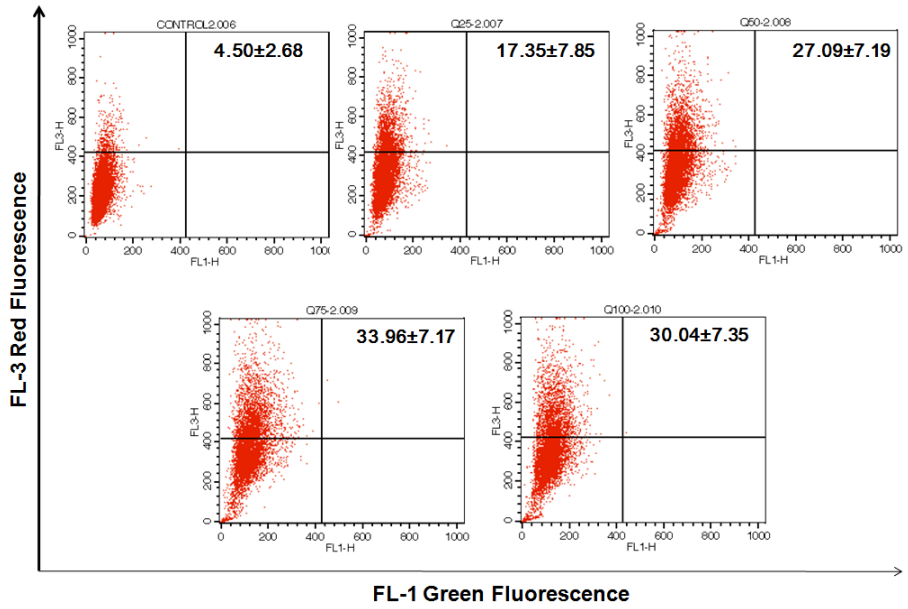


(C)

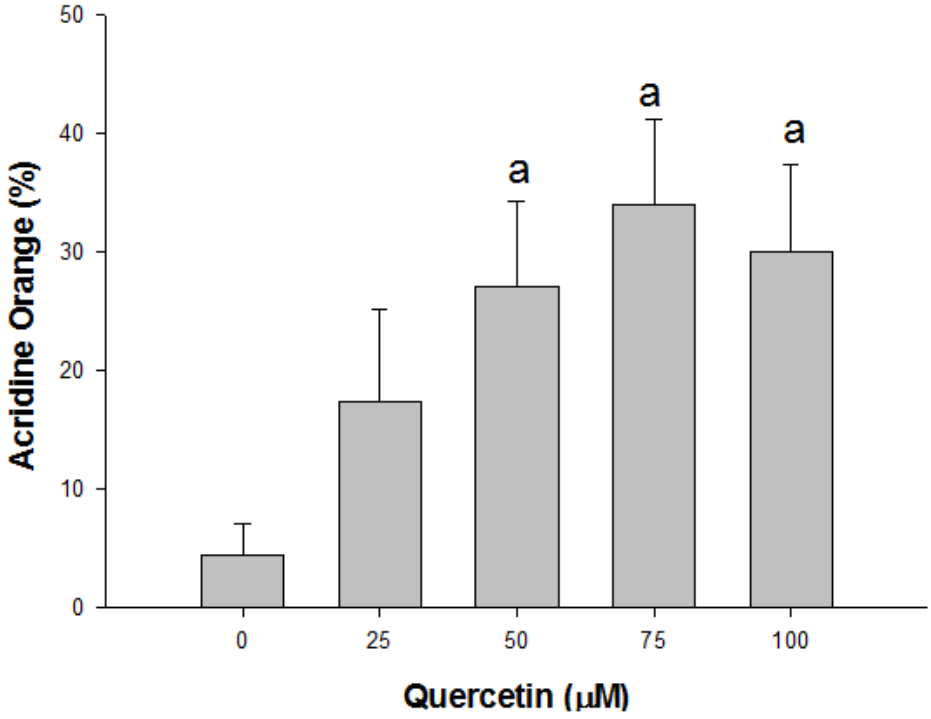
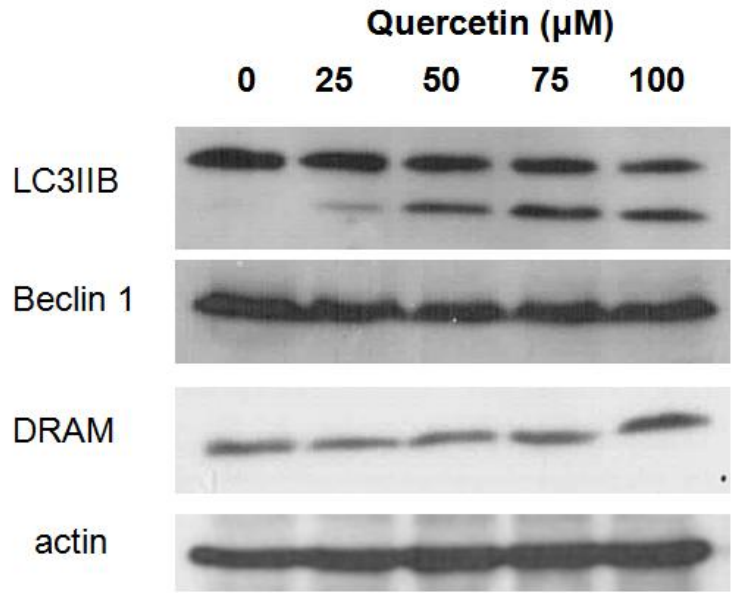


Figure 6. Formation of autophagosome by quercetin in U373MG cells. The cells were treated quercetin with various concentrations in 48h. (A) Formation of autophagosome and autolysosome. (B) Analysis of AVO-positive cells by AO staining and flow cytometry analysis. (C) Quantification of AVO-positive cell. All data correspond to the mean ± SD of three independent experiments. a= significantly different from the control, $p < 0.05$, b= significantly different from the control, $p < 0.01$,

3.8. Effect of quercetin on expression level of autophagy related protein

Similarly, it was further confirmed by up regulation of autophagy related proteins DRAM and LC3IIB. But Beclin-1 expression level was not increased. Even though Beclin-1 plays an important role in autophagy, several studies have revealed that autophagy can occur in a Beclin-1 independent manner [41]. In neurons, Beclin 1-independent autophagy might be an important contributor to caspase dependent apoptosis. [42].

(A)



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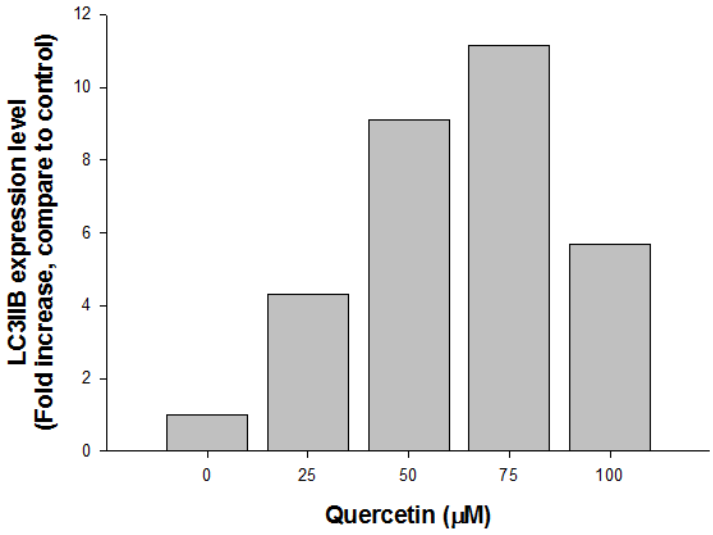


Figure 7. Expression level of autophagy related protein in various concentrations. (A) The cellular proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were probed with the indicated primary antibodies against LC3IIB, Beclin-1, DRAM, and actin and then with horseradish peroxidase conjugated goat anti-rabbit IgG. Actin was used as an internal control. (B) Densitometry analysis of LC3IIB expression level using Image J.

3. 9. Inhibition of autophagy by chloroquine promote cell death in U373MG

To further analyze whether the autophagy signal induced by quercetin was pro-survival or pro-death, we used chloroquine, as an inhibitor of autophagy. Chloroquine was treated for 2 h before quercetin treatment. Chloroquine treatment alone showed no effect on cell viability, but the treatment with quercetin showed decreased cell viability compared to that of quercetin alone treatment (Fig. 8).

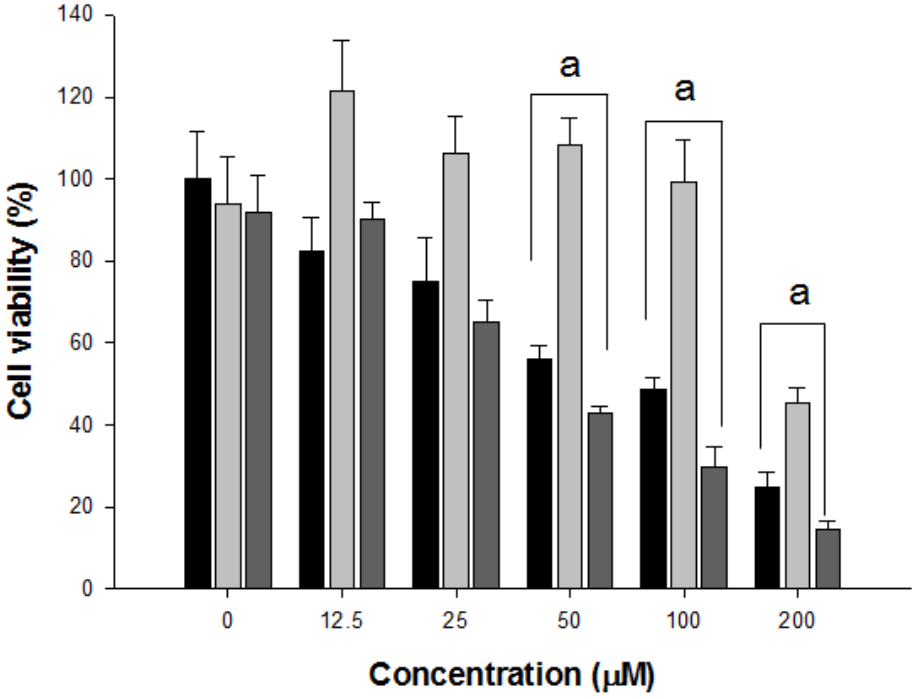


Figure 8. Inhibition of autophagy by chloroquine increase apoptotic cell death. (A) Cell viability measured by MTT Assay. Chloroquine was treated 2 h before quercetin treatment. Chloroquine concentration was 50 μM. ■; Quercetin, ■; Chloroquine, ■ ; Quercetin with Chloroquine 50 μM 2 h pre-treatment. Values are the mean ± SD of three independent experiments. a= significantly different from the control, $p < 0.05$, b= significantly different from the control, $p < 0.01$,

3. 10. Inhibition of autophagy by chloroquine promote apoptosis in U373MG

Chloroquine is the worldwide used anti-malarial drug and recently used as a potential anti-cancer agent when used in combination with anti-cancer drugs [43]. Cell cycle distribution also showed that quercetin with chloroquine more increased sub-G1 phase rather than only quercetin treatment (Table 2). Mitochondrial membrane potential was 68.0% and 54.3% in the cell treated with quercetin 75 μM alone and pretreatment of chloroquine 50 μM with quercetin 75 μM, respectively. We confirmed that inhibition of autophagy induction by quercetin was enhance apoptosis through cleavage of caspase-3, -7 and PARP.

Table 2. The percentage of U373MG cells in the sub-G1 fraction after 2 h pretreatment with chloroquine followed by incubation with quercetin for 48 h.

Concentration (μM)	Sub-G1 (%)
0	2.21 ± 1.1
CQ 50	1.45 ± 0.21
Q 75	5.18 ± 0.96
CQ 50 + Q 75	18.68 ± 3.02 [a]

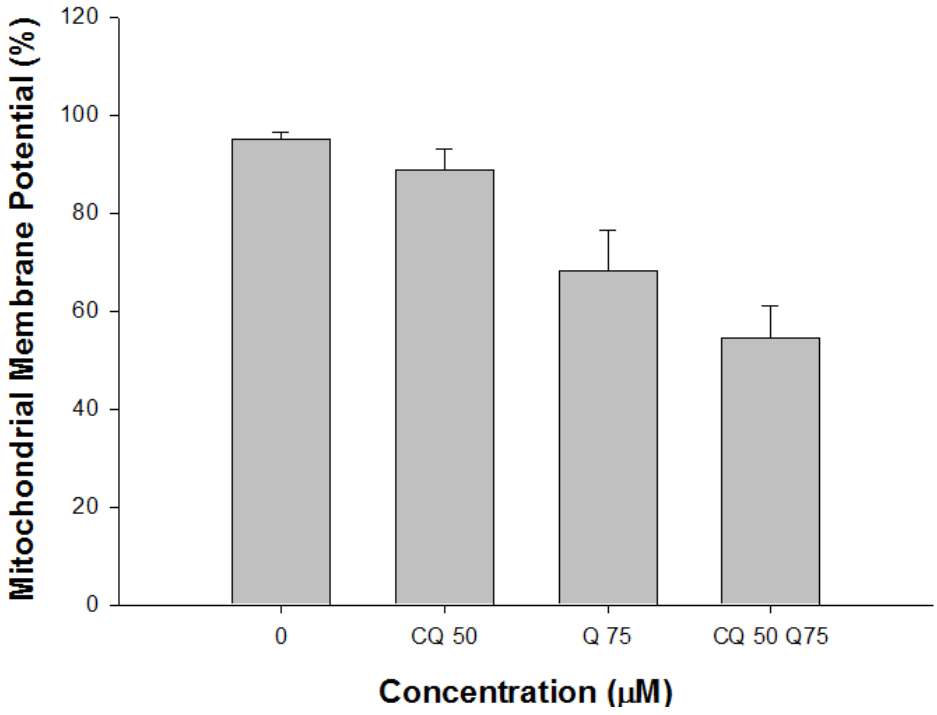
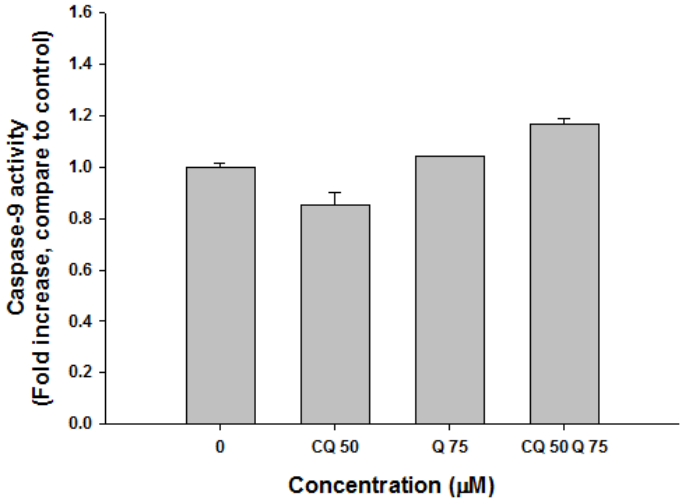


Figure 9. Flow cytometry analysis of mitochondrial membrane potential. Quercetin treated cells were trypsinized, stained with JC-1, washed, and analyzed by flow cytometric analysis. (A) Dot plot illustrating. (B) The decrease of mitochondria membrane potential % indicated that cells were undergoing mitochondrial dysfunction. CQ ; Chloroquine, Q ; Quercetin

(A)



(B)

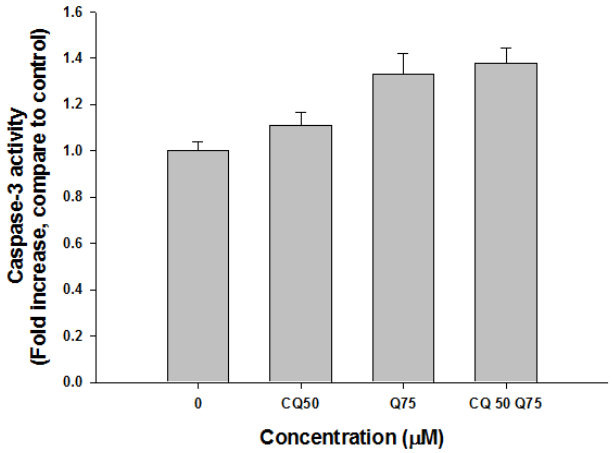


Figure 10. Enhancement of caspase-9 and -3 activity with pre-treatment of chloroquine. Cell lysates prepared from cells that had been treated with quercetin with or without chloroquine for 48 h were assayed for in vitro Caspase-9, -3 activity. The rate of cleavage of the caspase substrate LEHE-pNA, DEVD-pNA was determined by measuring the absorbance at 405 nm. (A) Caspase-9 activity. (B) Caspase-3 activity. All data correspond to the mean ± SD of three independent experiments. a= significantly different from the control, $p < 0.05$. CQ ; Chloroquine, Q ; Quercetin

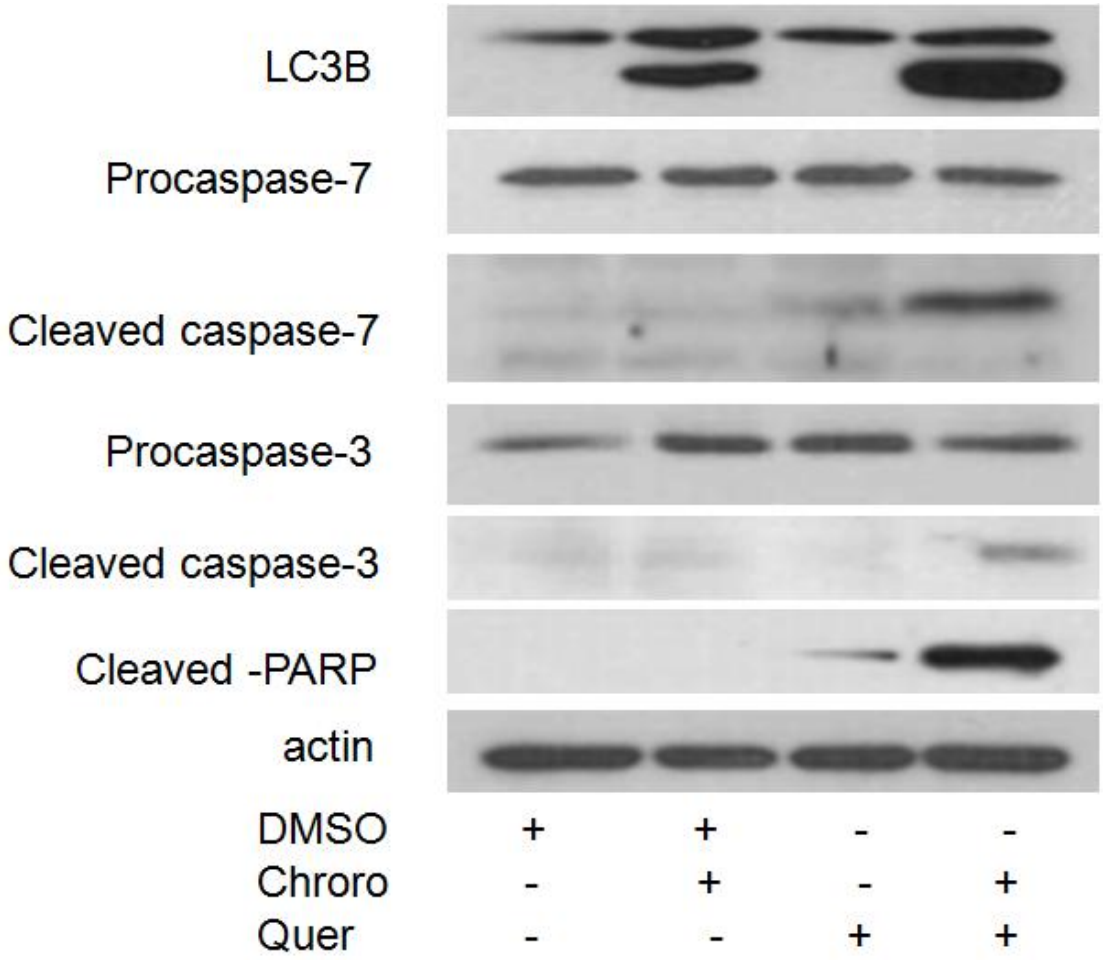


Figure 11. Western blot analysis of apoptosis related protein expression. Cells were lysed after incubation with quercetin in pretreatment of chloroquine 50 μM. After 48 h, cell lysates were subjected to western blotting with antibodies against caspase-7, -3, cleaved PARP and actin. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were probed with the indicated primary antibodies. actin was used as an loading control. CQ ; Chloroquine, Q ; Quercetin.

4. Conclusion

Our results indicated that quercetin exhibited cytotoxicity against U373MG time- and concentration-dependently. Quercetin treatment showed increase of sub-G1 cell cycle distribution, apoptotic body, cleavage of caspase-7 and PARP, and decrease of mitochondrial membrane potential which were characteristic of apoptosis. The results showed that translocation of p53 from cytosol to mitochondria and release of cytochrome C from mitochondria into cytosol indicating that quercetin induced mitochondria mediated apoptosis. Concomitantly, quercetin induced formation of acidic vesicular organelles, conversion of LC3II, elevation of DRAM, indicating that quercetin also induced autophagy. Importantly, pre-treatment with chloroquine, an autophagy inhibitor, enhanced quercetin-mediated apoptotic cell death.

In summary, we demonstrated that one of the famous flavonoid, quercetin induced cell death in human glioblastoma U373MG through mitochondria mediated apoptosis. Also quercetin induced protective autophagy in U373MG. Inhibition of autophagy induced by quercetin was promoted apoptosis in U373MG.

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