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Rapid dimerization of quercetin through an oxidative mechanism in the presence of serum albumin decreases its ability to induce cytotoxicity in MDA-MB-231 cells

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ABSTRACT

Quercetin is a member of the flavonoid family and has been previously shown to have a variety of anti-cancer activities. We and others have reported anti-proliferation, cell cycle arrest, and induction of apoptosis of cancer cells after treatment with quercetin. Quercetin has also been shown to undergo oxidation. However, it is unclear if quercetin or one of its oxidized forms is responsible for cell death. Here we report that quercetin rapidly oxidized in cell culture media to form a dimer. The quercetin dimer is identical to a dimer that is naturally produced by onions. The quercetin dimer and quercetin-3-O-glucopyranoside are unable to cross the cell membrane and do not kill MDA-MB-231 cells. Finally, supplementing the media with ascorbic acid increases quercetin's ability to induce cell death probably by reduction oxidative dimerization. Our results suggest that an unmodified quercetin is the compound that elicits cell death.

1. Introduction

Flavonoids are polyphenolic compounds widely distributed in human foods such as fruits and vegetables and have been shown to be beneficial for human health [1]. They are a class of secondary metabolites in plants that are responsible for pigmentation and have been shown to have a role in resistance to bacteria, fungi, viruses or insects.

Flavonoids are part of a larger group of polyphenolic compounds found in plants and over 4000 different flavonoids have been identified to date. What differentiates the flavonoids are the location of various hydroxyl groups and whether or not a double bond exists between C2 and C3 [2]. Flavonoid metabolism *in vivo* occurs primarily through modification of the hydroxyl groups through cytochrome oxidases [3–5]. However, studies on the biological effects of metabolites' bioactivities are few. Whether a flavonoid or its metabolite is primarily responsible for function varies by cell type. Olson et al. showed that quercetin (Qu; 3,3',4',5,7-pentahydroxyflavone) degrades rapidly in aqueous media, including DMEM, and the generation of ROS or reactive Qu intermediates was postulated to contribute to the proapoptotic effect in HaCat cells [6]. If ascorbic acid was used to prevent oxidation of Qu in the media, cell death was significantly decreased suggesting that the Qu intermediates may play roles in cell death. Further, Spencer et al. showed that intracellular metabolism of Qu in fibroblasts involves the formation of oxidation products although biological activity studies were not performed [7].

Qu has been shown by our lab to induce cell death in MDA-MB-231 cells through an apoptotic mechanism [8]. In our studies, we were unable to detect Qu in cell culture media to which it had been added and in cell extracts suggesting that Qu undergoes chemical modification. However, we did not investigate whether Qu or one of its products elicited apoptotic activities.

Here we report the observation of a Qu dimer formed via oxidation in DMEM. This is the first report of this specific dimer in cell culture media. We show that the Qu dimer and other oxidized products are unable to cross the cell membrane and do not induce cell death. Qu can be stabilized in the presence of ascorbic acid and this increases its apoptotic effects on MDA-MB-231 cells. Our results suggest that Qu and not its oxidation products initiate the apoptotic process.

2. Materials and methods

2.1. Materials

Qu and quercetin-3-*O*-glucopyranoside (Qu-3-O-GP, Fig. 1A) were purchased from Indofine Chemicals and stocks were prepared at 50 mM in dimethyl sulfoxide (DMSO). DMEM media, bovine growth serum (BGS), antibiotic/antimycotic, and Trypsin/EDTA were purchased from Hyclone. Ascorbic acid was purchased from Sigma. HPLC Grade water, methanol, and acetonitrile were purchased from Fisher Scientific.

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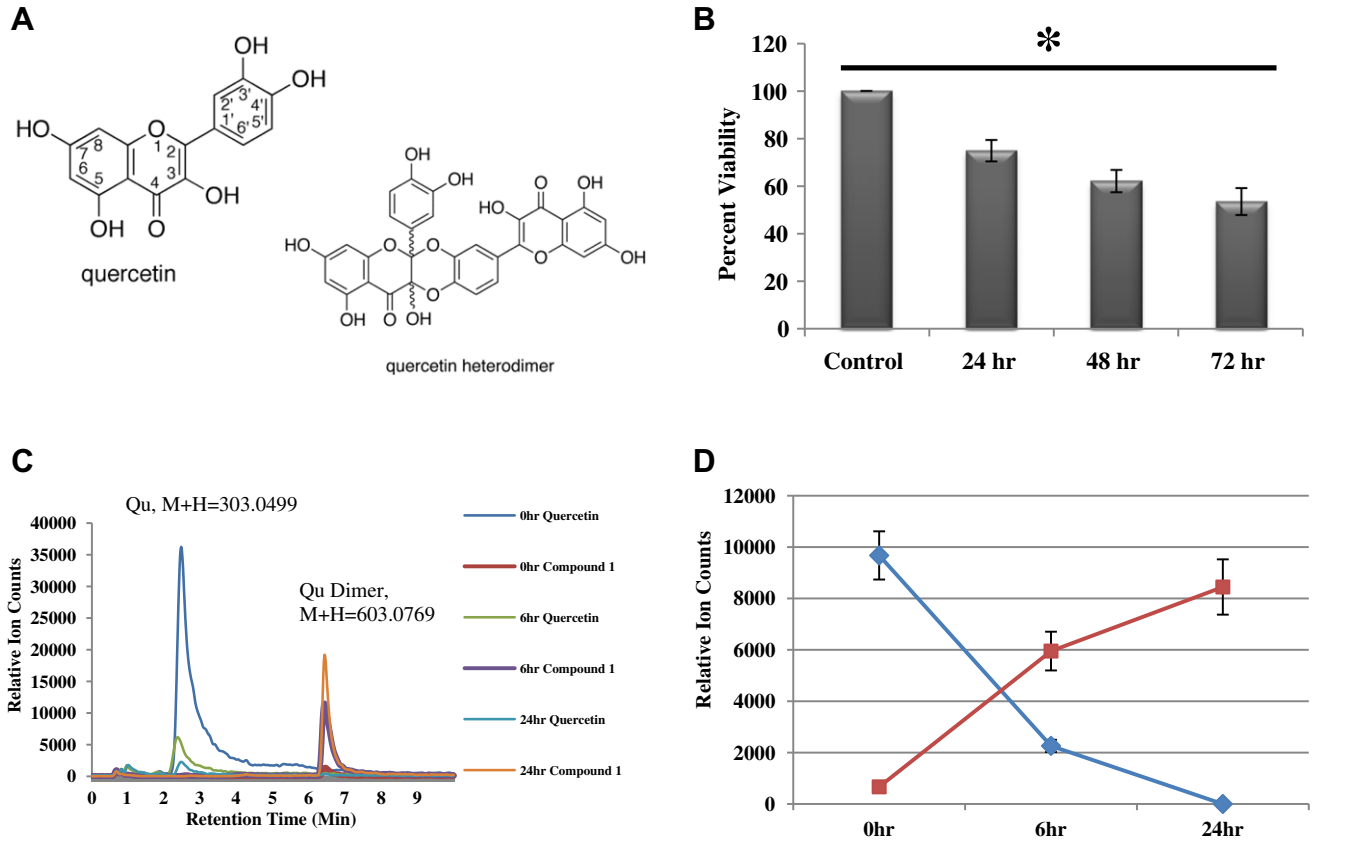

Fig. 1. Qu kills MDA-MB-231 cells but cannot be detected in culture media. (A) Structure of Qu and Qu dimer in this study. Numbering is indicated on Qu. (B) Cell viability of MDA-MB-231 cells decrease over time after 100 μM Qu treatment. One-way ANOVA was performed for comparison, $^{*}p < 0.05$. (C) HPLC chromatogram showing Qu (M + H = 303.0499) levels decrease while Qu dimer (M + H = 603.0769) increases in complete media over time. HPLC chromatogram showing detection of masses (M + H) 303.0499 (Qu) and 603.0769 (Qu dimer) in cell culture media at 6 and 24 h after treatment with Qu at 100 μM. (D) Graphical representation of the relative amounts of Qu and Qu dimer detected in media over time.

2.2. Cell culture

MDA-MB-231 cells were purchased from American Type Culture Collection (ATCC) and maintained in DMEM with high-glucose, L-glutamine, and sodium pyruvate, supplemented with 10% BGS and 1× antibiotic/antimycotic (Hyclone) in a 37 °C humidified atmosphere containing 5% CO_2. This media is referred to as complete media in the text. Cells were passaged based on dilutions and confluency as recommended by ATCC.

2.3. Cell viability assays

Cell viability was determined using trypan blue exclusion assay (TBE). Cells were plated at a density of 0.5×10^5 cells/well in 6-well culture dishes. 24 h later, media was changed and cells were treated with 100 μM Qu or DMSO (<0.2%) as a control. Cells were harvested by trypsinization at 24, 48, and 72 h post treatment and resuspended in 1 ml of PBS. Cells were briefly vortexed and an aliquot was combined with an equal volume of 0.4% trypan blue in PBS. Live cells (lacking trypan blue) were counted using a hemocytometer. Cell viability is reported as the number of live cells present in the drug treatment divided by the amount of cells present in the DMSO treatment multiplied by 100 to give a percentage.

2.4. Extraction of Qu from cells

After living cells were counted, cells were pelleted a second time at 100*g* and PBS was removed. Cells were resuspended in 5 ml of 40% methanol (HPLC grade), vortexed, placed on ice for 15 min and vortexed a second time. Cell debris was pelleted at 500*g* for 10 min and supernatant was transferred to a new vial for LC–MS analysis. The pellet was resuspended in another 1 ml of 40% methanol, vortexed, placed on ice for 15 min and vortexed a second time. The debris was pelleted at 20,000*g* for 10 min and supernatant was transferred to a vial for LC–MS analysis.

2.5. Determination of Qu, Qu dimer, and Qu-3-O-GP stability

Stability of compounds in culture media was determined by preparing 100 μM of each compound in complete media, incubating at 37 °C, and after various time points, extracting in 40% methanol. Precipitated salt and protein was pelleted at 20,000*g* for 15 min and supernatant was analyzed using LC–MS as described below.

Where indicated, Qu levels were also monitored in phosphate buffered saline (PBS) with either 10% FBS, 2.5 mg/ml bovine serum albumin or 2.5 mg/ml actin. Where indicated, FBS was boiled for 5 min at 95 °C to inactive proteins. 100 μM Qu was then added to the individual solutions. 1 ml aliquots were then removed and incubated either 4 °C, 23 °C, and 37 °C for 0, 6, and 24 h. Samples were then extracted with 40% methanol and centrifuged at 20,000*g* for 15 min. Supernatants were transferred to new vials and analyzed using HPLC–MS as described below.

2.6. Purification, separation and analysis of the Qu dimer

The Qu dimer was isolated by incubation of Qu in complete media at 37 °C for 24 h. The media was then lyophilized and the

powder was collected and resuspended in methanol. The dimer was purified by thin layer chromatography (TLC) eluting with methanol:dichloromethane (1:9 v/v) on glass-backed plates. The TLC's zone containing the Qu dimer product was scraped and the silica was eluted with 5 ml of methanol:dichloromethane (2:8 v/v). The eluate was concentrated under vacuum. The crude dimer was found to be identical to the synthetic dimer (see below) by ^{1}H-NMR and high resolution HPLC coupled mass spectrometry (HPLC–MS).

A synthetic sample of the Qu dimer was prepared by a previously published protocol [9]. Briefly, 340 mg of Qu were mixed with 80 ml of acetonitrile. While stirring this solution, a 20 ml solution of 50 mM potassium ferricyanide and 50 mM sodium carbonate was added drop wise over 30 min. The solution was stirred for 3 h in the dark and concentrated hydrochloric acid was added to adjust the pH to 2. The solution was then concentrated under vacuum to remove the acetonitrile and was extracted four times with 40 ml of ethyl acetate:diethyl ether (8:2 v/v). The extract was dried ($MgSO_4$) and solvents were removed by rotary evaporator *in vacuo*. The final product was purified by preparative TLC eluting with methanol:dichloromethane (1:9 v/v). The product had TLC, 1H-NMR, and LC–MS characteristics identical with those previously reported [9].

2.7. Quantitation of Qu, Qu dimer, and Qu-3-O-GP using HPLC–MS

An Agilent 1200 Series HPLC with a 150 mm × 2.1 mm Agilent Cogent Bidentate C18 column was coupled to an Agilent QTOF6520. A binary mobile phase system of solvent A (0.1% formic acid) and solvent B (100% acetonitrile) was used to elute material from the column. A flow rate of 0.5 ml/min was used. The column was first equilibrated into 30% B before 10 μl of sample was injected onto the column. A gradient was used to separate compounds from the column. The gradient began with 30% B for 2 min and increased linearly to 95% B at 10 min, at which time the gradient decreased linearly to 30% B at 15 min to return to the initial starting conditions. MS data were collected in full scan positive mode over the mass range of 100–1500*m/z*. Ion voltages and gas settings were as follows: fragmentor, 150 V; skimmer, 65 V; drying gas, 8 L/min; gas temperature, 350 °C. Agilent MassHunter Acquistion software version B.0.4 was used for data acquisition and analysis. We used Agilent's Mass Profiler Software to determine mass differences between control media and media containing Qu which helped us to identify the Qu dimer (Fig. 1).

2.8. Apoptosis assays

Apoptosis in MDA-MB-231 cells was assayed by annexin V and propidium iodide (PI) co-staining using an Annexin-V-AlexaFluor 488 staining kit (Invitrogen) following a standard protocol. 1×10^6 Cells were plated in a 10 cm dish and 24 h later 100 μM Qu, 100 μM Qu with 0.5 mM ascorbic acid, 0.5 mM ascorbic acid, or DMSO was added to the cells. Cells were analyzed 24 h after treatment for apoptosis. Prior to trypsinization, cells were observed through a 10× objective and a 10× eye piece (100× total magnification) on an Olympus CK2 inverted microscope with phase-contrast. Phase-contrast images were captured with a CCD camera attached to the microscope using AmScope software (version 86×). For the apoptosis assay, cells were harvested by addition of 0.25% trypsin, 5.3 mM EDTA for 2 min at 37 °C. Trypsin and EDTA were inactivated by addition of complete media. Cells were collected by centrifugation at 100*g* and resuspended in 1 ml of room temperature annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). 100 μl of cell suspension was transferred to a 1.5 ml eppendorf tube containing 5 μl of AlexaFluor 488-conjugated annexin V. The cells were incubated at room temperature for 15 min and then 400 μl of annexin-binding buffer plus 10 μl of PI (50 μg/ml) was added to the cells. The stained cells were then analyzed by flow cytometry using a BD FACSCaliber instrument. The instrument was set for FL 1 (annexin V) vs FL3 (PI) bivariant analysis. Data from 10,000 cells/sample was collected and dot plots of FL1 vs FL3 were generated. The quadrants were set based on the population of healthy, unstained cells in untreated samples compared to cells treated with 1 μM staurosporine as a control (data not shown). CellQuest Pro was used to calculate the percentage of the cells in the respective quadrants. A minimum of three different experiments was performed.

2.9. Statistical analysis

A one-way ANOVA was conducted for cell death over three different time points at a fixed concentration of Qu (100 μM) compared to DMSO treatment at 72 h. For comparison between two groups in all other assays, the data was analyzed using the two-sided, two independent sample Student *t*-test with 95% confidence intervals reported. A *p*-value of $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Detection of Qu and Qu dimer in complete media

Qu is a member of the flavonoid family and belongs to the subclass of flavonols (Fig. 1A). We previously showed that cell death and apoptosis varied depending on the type of flavonoid used. Qu (100 μM) was able to induce cell death in MDA-MB-231 cells over time with maximum death seen after 72 h (Fig. 1B). However, we could not detect Qu present in cell extracts, in contrast to other flavonoids [8]. Therefore, we monitored Qu levels in cell culture media over time by following a standard extraction protocol as described in Section 2. As shown in Fig. 1C and D, there was a noticeable decrease in the amount of Qu (M + H of 303.0499) in the media over time. Using Agilent's Mass Profiler software, we were able to detect a compound that increased over time with an M + H mass of 603.0769 suggesting that Qu was being modified to become this compound.

3.2. Purification and analysis of the Qu compound

The Qu compound was purified from cell culture media as described in Section 2. The purified compound was analyzed by HPLC–MS. By comparing with a synthetic standard, we were able to establish that the purified compound was identical to a Qu heterodimer previously reported in onions and produced synthetically [9,10]. Fig. 1A shows the structure of the Qu heterodimer that we will refer to as Qu dimer throughout the rest of the article.

3.3. Kinetics of formation of the Qu dimer

The Qu dimer was detected in incubations of Qu with complete media. We postulated that the Qu dimer was being generated through an interaction with a protein present in the serum. Therefore we prepared phosphate buffered saline (PBS) with 10% BGS or 10% BGS that was boiled for 20 min to inactivate and denature proteins. We then added 100 μM Qu and monitored the levels of Qu and Qu dimer while incubating at 37 °C (Supplemental Fig. S1). The levels of Qu decreased over time regardless of whether the serum was boiled or not. However, the levels of the Qu dimer only increased significantly in the presence of the non-boiled serum suggesting that dimer formation depended on a protein that was not denatured. We were unable to determine what Qu was being

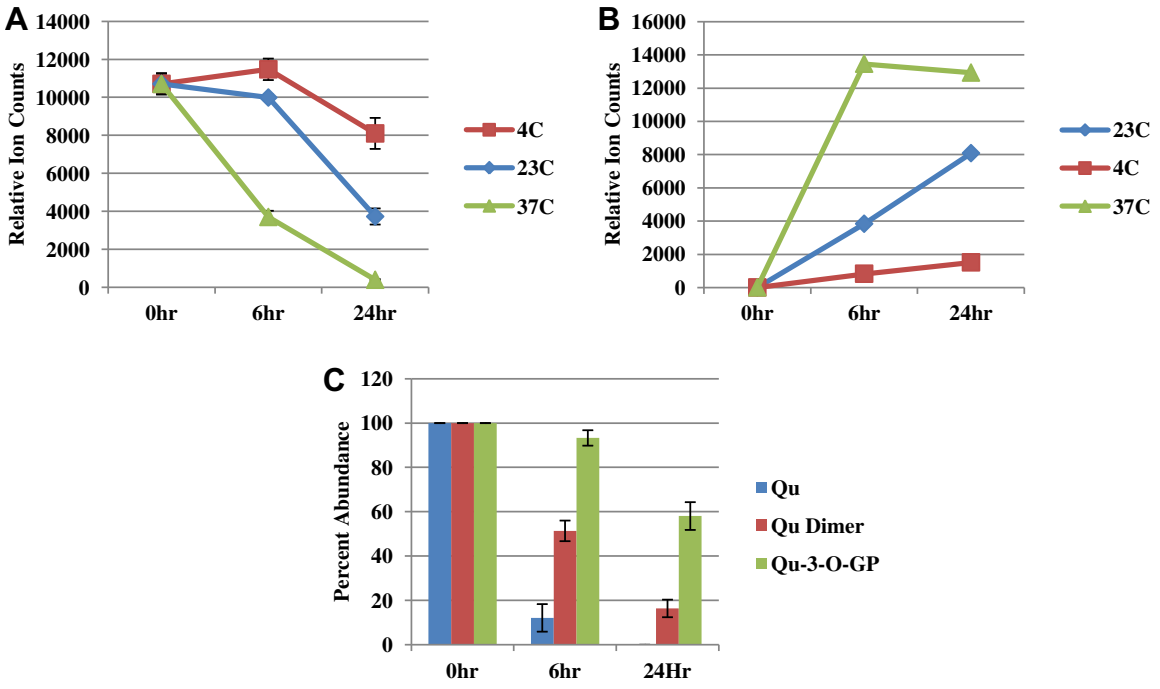

Fig. 2. Kinetics of Qu degradation and Qu dimer formation in the presence of serum albumin. (A) Qu levels decrease over time with maximal decrease seen with 37 °C. (B) Qu dimer levels increase over time with maximal increase seen with 37 °C.

degraded to in the presence of the boiled serum. However, there are numerous reports that indicate that Qu can be oxidized to various compounds [11,12] suggesting that the compounds that are generated may not be detected by our HPLC–MS system. Taken together these results suggest to us that Qu in the absence of serum is transformed to a compound that we cannot detect and in the presence of serum is predominantly converted to the Qu dimer. Because boiled serum could not produce the Qu dimer, it suggests the Qu dimer is being generated in the presence of a protein that can be heat inactivated.

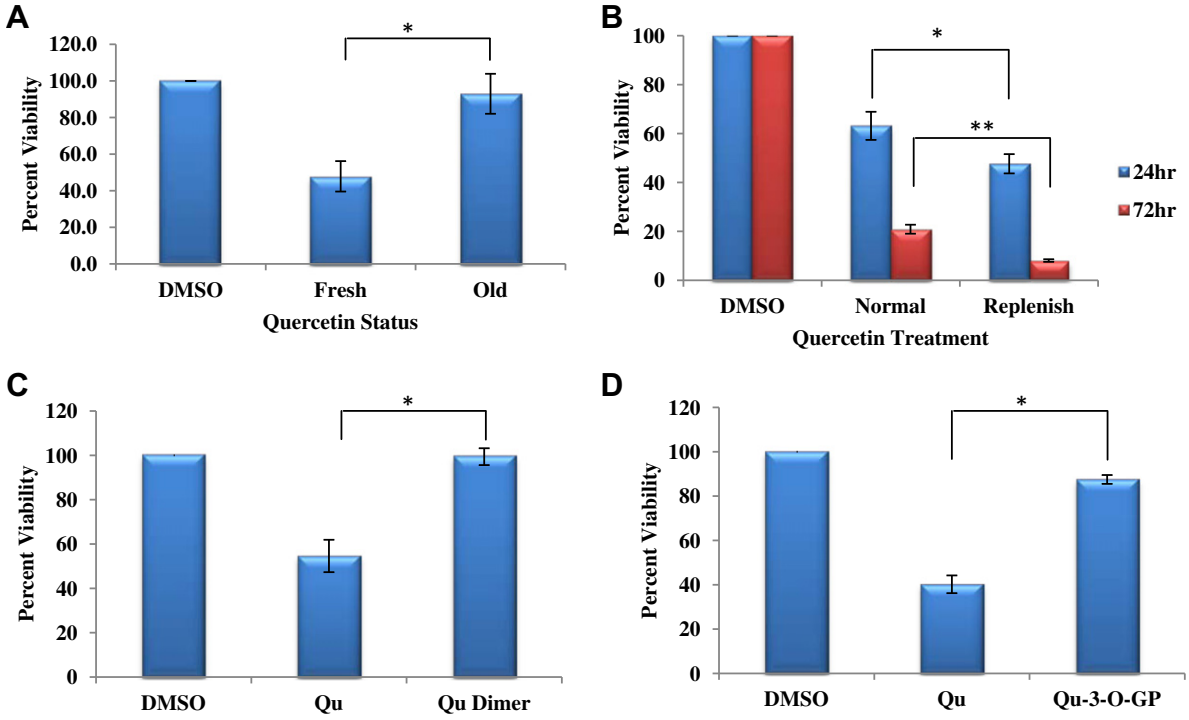

Fig. 3. Cell viability studies with Qu, Qu dimer, and Qu-3-O-GP. (A) Qu was either prepared fresh or left in media (old) for 24 h prior to adding to MDA-MB-231 cells. The old treatment does not induce cell death after 72 h compared to the fresh treatment. (B). Adding fresh Qu every 6 h for 12 h increases the amount of cell death in MDA-MB-231 cells at 24 and 72 h. (C) Purified Qu dimer does not induce cell death compared to Qu after 100 μM treatment for 72 h. (D) Qu-3-O-GP does not induce cell death compared to Qu after 100 μM treatment for 72 h. For all Figures, $^{*}p < 0.5$ and ** corresponds to $p < 0.0005$.

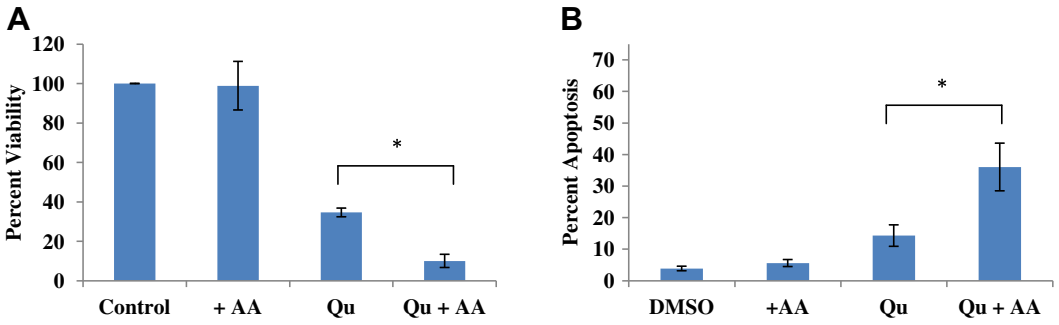

Fig. 4. Ascorbic acid increases the killing potential of Qu. Cells were treated with DMSO, 0.5 mM ascorbic acid, 100 μM Qu, or 100 μM Qu and 0.5 mM ascorbic acid. (A) Trypan blue exclusion assay to measure percent viability after 72 h treatment. (B) Annexin V assay to monitor apoptosis after 24 h treatment. $^{*}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The predominate protein that is found in serum is albumin. Oxidized Qu has previously been shown to covalently bind to human serum albumin [13]. Therefore we speculated that Qu may also be able to bind to bovine serum albumin. We tested the ability of pure albumin in PBS to generate the Qu dimer. We also tested these reactions at 4 °C, 23 °C, and 37 °C (Fig. 2). In each case, Qu levels decreased over time with maximum decreases seen with increasing temperature (Fig. 2A). Qu dimer formation was dependent on time and temperature with maximum dimer formation occurring at 37 °C after 24 h (Fig. 2B). The Qu dimer was not formed in the presence of actin (Supplemental Fig. S2) suggesting that the dimer formation was specific to the protein albumin. Interestingly, actin appears to stabilize Qu in PBS compared to PBS alone after 6 or 24 h incubations at 37 °C.

We monitored the stability of the Qu, purified Qu dimer, and a naturally occurring Qu analog, Qu-3-O-GP, which contains a glucopyranoside on C3. Since C3 is necessary for formation of the Qu dimer, we suspected that the glycosylated compound would be more stable. We prepared 100 μM of the compounds in media supplemented with 10% BGS and monitored levels over time (Fig. 2C). The stability of the compounds was Qu-3-O-GP > Qu dimer > Qu. Qu-3-O-GP had a half-life of greater than 24 h, Qu dimer had a half-life of 6 h, and Qu had a half-life of 1 h.

3.4. Identification of the Qu derivative(s) responsible for cell death

We next conducted a series of studies to ascertain whether Qu, Qu dimer, and/or Qu-3-O-GP can induce cell death in MDA-MB-231 (Fig. 3). Fig. 3A shows that the addition of fresh Qu to MDA-MB-231 cells results in cell death. However, allowing the Qu to sit in the media with serum for 24 h at 37 °C, thereby forming the Qu dimer, prior to exposure to the MDA-MB-231 cells does not induce cell death. Fig. 3B shows that if we repeatedly add fresh Qu (0 h, 6 h, and 12 h) to the media to make up for the Qu that is lost to dimerization, cytotoxicity at 24 and 72 h increased significantly compared to the one time treatment of Qu ($p < 0.05$). Fig. 3C shows that the purified synthetic Qu dimer is unable to induce cytotoxicity in MDA-MB-231 cells. Finally, Fig. 3D shows that Qu-3-O-GP is also unable to induce cytotoxicity in MDA-MB-231 cells. Neither the Qu dimer nor Qu-3-O-GP were detected in MDA-MB-231 cell extracts (data not shown) suggesting that they were unable to cross the cell membrane.

A previous report has shown that Qu can be stabilized in media in the presence of ascorbic acid leading to a decrease in the apoptotic effect seen on HaCat cells [6]. We wanted to determine how ascorbic acid would affect Qu cytotoxicity in our system. Fig. 4A shows that the viability of MDA-MB-231 was significantly less after treatment with Qu plus ascorbic acid (Qu + AA) than Qu or AA treatment alone after 72 h ($p < 0.05$). We also evaluated phosphatidyl serine flipping, an early marker for apoptosis, with Annexin V staining after 24 h of treatment. Treatment of cells with Qu + AA showed significantly higher amounts of phosphatidyl serine flipping than with Qu or AA alone ($p < 0.05$). Supplemental Fig. S3 shows images acquired after treatment with Qu and AA. There is a decrease in the cell population with Qu + AA treatment compared to Qu or AA alone.

4. Discussion

Modification of Qu via an oxidative pathway is not novel. A Qu dimer has previously been shown to be present in onions and has been prepared synthetically through oxidation mechanisms [9,10]. However, this is the first report identifying a Qu dimer in cell culture media in the presence of albumin. Although a previous report showed that Qu was not stable in media over time and suggested that Qu was being oxidized, Olson et al. never identified any of the oxidized compounds [6]. We have identified the Qu dimer as one of the compounds found in media. We were unable to identify any other compounds using our system. Albumin greatly increased the amount of the Qu dimer that was formed over time. In the absence of albumin or serum, Qu appeared to degrade to an undetectable form.

The dimerization and degradation appear unique to Qu. We were unable to detect dimer or degradation for numerous other flavonoids using LC–MS analysis (data not shown). We suggest that this is due to the location of hydroxyl groups unique to Qu which are involved in the oxidation. Both dimerization and degradation appear to require the 3 hydroxyl group as a derivative of Qu, Qu-3-O-GP, which contains a glucopyranoside group attached to the oxygen off of the 3-carbon was unable to form a dimer or be degraded over time.

Although there have been numerous reports in the literature on actions of flavonoids on cancer cells, there has been little data shown to determine if the biological response seen is due to the flavonoid or an oxidized product. Our results suggest that the oxidative products of Qu are not sufficient to induce cell death as we are not able to see cell death after Qu has been oxidized in the media. Further, Qu-3-O-GP is also unable to induce cell death although we believe this to be because it cannot enter the cells based on HPLC-coupled mass spectrometry.

Although we could not detect Qu within the cell by LC–MS, we believe that Qu is getting into the cell and is responsible for the cell death. However, we cannot rule out the possibility that Qu is being modified once it enters the cell and the modified version is responsible for cell death that we are seeing. For example, it has previously been shown that Qu is able to form conjugates with free thiol groups such as glutathione [14]. However, in a subsequent study, Boots et al. went on to

show that the glutathionyl-Qu adducts were not stable, intracellularly disassociating quickly to a Qu dimer similar to what we detect [15]. Although we were unable to detect glutathione conjugated Qu in either media or cell extracts by LC–MS (data not shown), it is possible that these modifications or other modified conjugates do exist. We suggest that if they do exist, they are either occurring within the culture media and do not cross the cell membrane or if they are able to cross the cell membrane, do not have an effect on apoptosis (see Fig. 3A).

Finally, we suggest Qu potentially is a more potent killer of cancer cells than previous reported due to the instability of Qu. Using ascorbic acid to increase the levels of unmodified Qu in the media, we show an increase in the overall amount of cell death. If Qu levels could be stabilized in the cell without affecting its ability to induce cell death, we would predict a much lower IC50 than we have previously reported [8]. Current efforts are underway in the lab to make derivatives that can get into the cell but have the derivation removed upon entering the cell to determine if this will increase the ability to induce cell death.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.080.

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