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Quinacrine Induces Cytochrome c-dependent Apoptotic Signaling in Human Cervical Carcinoma Cells

Adedigbo A. Fasanmade[1,2], Edward D. Owuor[1,3], Rachel P. L. Ee[1], Dima Qato[1], Mark Heller[2], and Ah-Ng Tony Kong[1,3]

[1]Department of Pharmaceutics and Pharmacodynamics, Center for Pharmaceutical Biotechnology, College of Pharmacy, [2]Present address: Centocor Biotechnologies Inc., 200 Great Valley Parkway, Malvern, PA 19355, USA, [3]Department of Pharmaceutics, College of Pharmacy, Rutgers University, Environmental and Occupational Health Sciences Institute, 170 Frelinghuysen Road, Piscataway, NJ 08854, USA [4]Deparment of Hematology/Oncology, College of Medicine, University of Illinois at Chicago, Chicago, IL 60607, USA
1st and 2nd authors contributed equally to this paper

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Quinacrine (QU), a phospholipase-A2 (PLA-2) inhibitor has been used clinically as a chemotherapeutic adjuvant. To understand the mechanisms leading to its chemotherapeutic effect, we have investigated QU-induced apoptotic signaling pathways in human cervical squamous carcinoma HeLa cells. In this study, we found that QU induced cytochrome c-dependent apoptotic signaling. The release of pro-apoptotic cytochrome c was QU concentration- and time-dependent, and preceded activation of caspase-9 and -3. Flow cytometric FACScan analysis using fluorescence intensities of $DiOC_6$ demonstrated that QU-induced cytochrome c release was independent of mitochondrial permeability transition (MPT), since the concentrations of QU that induced cytochrome c release did not alter mitochondrial membrane potential (▲ Ψ_m). Moreover, kinetic analysis of caspase activities showed that cytochrome c release led to the activation of caspase-9 and downstream death effector, caspase-3. Caspase-3 inhibitor (Ac-DEVD-CHO) partially blocked QU-induced apoptosis, suggesting the importance of caspase-3 in this apoptotic signaling mechanism. Supplementation with arachidonic acid (AA) sustained caspase-3 activation induced by QU. Using inhibitors against cellular arachidonate metabolism of lipooxygenase (Nordihydroxyguaiaretic Acid, NDGA) and cyclooxygenase (5,8,11,14-Eicosatetraynoic Acid, ETYA) demonstrated that QU-induced apoptotic signaling may be dependent on its role as a PLA-2 inhibitor. Interestingly, NDGA attenuated QU-induced cytochrome c release, caspase activity as well as apoptotic cell death. The blockade of cytochrome c release by NDGA was much more effective than that attained with cyclosporin A (CsA), a MPT inhibitor. ETYA was not effective in blocking cytochrome c release, except under very high concentrations. Caspase inhibitor z-VAD blocked the release of cytochrome c suggesting that this signaling event is caspase dependent, and caspase-8 activation may be upstream of the mitochondrial events. In summary, we report that QU induced cytochrome c-dependent apoptotic signaling cascade, which may be dependent on its role as a PLA-2 inhibitor. This apoptotic mechanism induced by QU may contribute to its known chemotherapeutic effects.

Key words: Quinacrine, Cytochrome c, Caspase-3; Phospholipase-A-2; Cervical Carcinoma, Apoptosis

INTRODUCTION

Quinacrine (QU), also known as mepacrine, (6-chloro-9-[(4-diethylamino)-1-methyl-butyl]amino-2-methoxyacridine dihydrochloride) is a potent phospholipase A-2 (PLA-2) inhibitor that has been extensively used for clinical treatment of malaria (Wallace, 1989). QU has also been used in anticancer-related conditions, such as in intra-cavitary treatment of malignancies in the pleural and the peritoneal assumably due to its ability to inhibit interferon induced trafficking of neutrophils (Gebbia *et*

Correspondence to:An-Ng Tony Kong, Department of Pharmaceutics, College of Pharmacy, College of Pharmacy, Rutgers University, 160 Frelinghuysen Road, NJ 08854, USA
E-mail: KongT@cop.rutgers.edu

al., 1994; Banerjee, *et al.*, 1994). The mechanisms of action of QU in the treatment of malignancies has always been attributed to its role in blocking the release of arachidonic acid (AA), an intermediate generated during oxidative stress and a precursor of the prostalandins which may be involved in enhancement of cell proliferation (de Souza *et al.*, 1997). Moreover, QU has also been used clinically in combination with paclitaxel, an anticancer agent in order to enhance its potency in inducing apoptotic cell death (de Souza *et al.*, 1997).

Apoptosis or "programmed cell death" is an active physiological process (Kerr *et al.*, 1972; Au *et al.*, 1997), typically characterized by a cascade of sequential events leading to loss of membrane asymmetry and attachment, condensation of the cytoplasm and nucleus and internucleosomal cleavage of DNA. The final stages of apoptosis involve DNA fragmentation and the cell degrades into "apoptotic bodies" that are rapidly phagocytosized without eliciting a significant inflammatory damage on neighboring cells.

Many studies have shown that apoptosis is inducible by oxidative stress, increase in cytosolic calcium and changes in mitochondrial membrane potential (Farber *et al.*, 1990; Corcorana *et al.*, 1994), and in response to various cytotoxic stimuli including the activation of cell surface receptors such as Fas, tumor necrosis factor alpha 1 (TNFR1) (Sluss *et al.*, 1994), serum or growth factor withdrawal, ultraviolet (UV) irradiation, and glucocorticoids. The mitochondria are known to be a cell death integrator that plays a central role in apoptotic signaling in many cells. In the mitochondria-dependent apoptotic signaling, permeabilization of the mitochondrial membrane is a critical event that leads to the release of pro-apoptotic proteins such as pro-caspases, and caspase activators including *Smac/Diablo* and cytochrome c, apoptosis inducing factor (AIF), which subsequently activates the nucleases that will cut DNA into small fragments (Brenner and Kroimer, 2000). Most recently, Li *et al.*, (2000) have reported that a transcription factor (TR3 or Nur77 or NGFIB) translocates from the nucleus to the mitochondrial membrane where it triggers off membrane permeabilization and leads to cell death. These findings have made it essential to study the role of mitochondria in apoptotic cell death induced by various stimuli.

One of the important downstream events in the apoptotic process involves the cleavage of a series of cysteine-dependent aspartate-directed proteases called caspases (Thornberry *et al.*, 1998). These caspases are present as inactive pro-enzymes that are 30-50 kDa, and contain three domains; an amino (NH_2)-terminal domain, a large subunit (~20 kDa), and a small subunit (~10 kDa). Their activation involves proteolytic processing between domains, followed by association between large and small subunits forming heterodimers. Activated caspases then cleave pro-caspases in the ensuing caspase cascade. The first caspase to become activated is usually pro-caspase-8, and usually assembled with the death receptors (DR) such as Fas, or DR4/5 (Tewari *et al.*, 1995). It subsequently directs a cascade of downstream caspases that ultimately commit the cell to apoptotic death. To date, fourteen caspases have been identified and constitute a multi-gene family that has been classified into three subgroups according to sequence homology. Caspase-1 subfamily contains caspases 1, 4, 5, 11, 12, 13, and caspase-3 subfamily contains caspases 3, 6, 7, 8 (Thornberry *et al.*, 1998).

Conventionally, the mode of action of QU has been associated with its inhibition of PLA-2, thus blocking the release of AA from phospholipids, thereby reducing the downstream synthesis of various inflammatory eicosinoids. This is believed to account for its anti-inflammatory activities. However, the mechanism(s) by which QU-induces anti-neoplastic activity is unclear, and therefore necessitates a critical examination of the potential cell death signaling pathways. Because of the importance of caspase signaling pathways in cell death, we investigated the activation of these pathways in QU-induced apoptotic cell death. We reported, for the first time, the ability of QU to activate caspase-8, cause the release of cytochrome c, without any significant changes in mitochondrial membrane potential, and thereby activating a caspase cascade, especially caspase-3 leading to apoptotic cell death. Furthermore, this paper also reports, for the first time, that the QU-induced apoptosis may be dependent on its inhibition of PLA-2 mechanism.

MATERIALS AND METHODS

Cell and chemicals

The human cervical carcinoma HeLa cell line were purchased from American Type Culture Collection (ATCC, USA), and quinacrine (QU), nordihydroxygnaiaretic acid (NDGA), 5,8,11,14-eicosatetraynoic acid (EYTA) and arachidonic acid (AA) were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

Fluorogenic peptide substrates acetyl-L-isoleucyl-L-glutamyl-L-threonyl-L-aspartic acid α-4-methyl-coumaryl-7-amide (Ac-IETD-MCA), acetyl-L-leucyl-L-glutamyl-L-histidyl-L-aspartic acid α-4-methyl-coumaryl-7-amide (Ac-LEHD-MCA), acetyl-Tyr-Val-Ala-Asp-α-4-methylcoumaryl-7-amide (Ac-VEID-MCA), Tyr-Val-Ala-Asp-α-4-methylcoumaryl-7-amide (Ac-YVAD-MCA), and Asp-Glu-Val-Asp-α-4-methylcoumaryl-7-amide (Ac-DEVD-MCA) were purchased from Peptide Institute Inc (Minoh-shi Osaka, Japan) and caspase inhibitors including z-VAD-fmk and caspase-3-specific inhibitor (Ac-DEVD-CHO) were purchased from Calbiochem (San Diego, CA, USA).

MTS assay for qu-induced cell death

The cytotoxic cell death effect induced by QU was measured by cell viability assay using (3-(4,5-dimethyl-

thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS). Our assay method is generally as provided in the CellTiter 96^R AQ_{ueous} non-radioactive cell proliferation assay kit according to the manufacturer's instruction (Promega Corp., Madison, WI, USA), with few modifications as previously described in Yu *et al.*, 1998; Yu *et al.*, 2000b). Specifically in the current work, cells were seeded in 96-well plates containing minimum essential medium (MEM), at a density of 10^4 cells per well and incubated at 37°C under humidified 5% CO_2 for 48 h. After which, varying concentrations of QU were added. The cells were further incubated for an additional 24 h before the addition of the cell viability test substance MTS. The cells were incubated with MTS for 2 h before absorbance was measured. Viable cells were able to convert MTS tetrazolium to its formazan product with a color change measurable at 490 nm using an ELISA plate reader. In separate experiments the effects of caspase-3 specific inhibitor, AC-DEVD-CHO, on the viability of cells treated with QU was also examined.

Fluorogenic caspase activity assay

Fluorogenic caspase activity assays were performed as described previously (Yu *et al.*, 1998; Lei *et al.*, 1998). Treated cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed with lysis buffer [50 mM Tris (pH 7.4), 50 mM β-glycerophosphate, 15 mM $MgCl_2$, 15 mM EDTA, 100 µM phenylmethylsulfonyl fluoride, 1 mM DTT, and 150 µg/ml digitonin]. Ten microgram of total protein was incubated with 200 µM fluorogenic peptide substrates, in 50 µl volume, and analyzed using an excitation wavelength of 360 nm and emission wavelength of 460 nm. The effect of caspase-3 specific inhibitor (AC-DEVD-CHO) on the effect of QU-induced caspase activity was tested by pre-incubation of the cells with 50 µM of AC-DEVD-CHO for 30 min before treatment with QU. Control cells were also treated with various concentrations of the inhibitor.

Preparation of mitochondria and Western blotting of released cytochrome c

To obtain S-100 cytosolic extracts, cells were washed twice with ice-cold PBS, followed by scraping in the presence of 1 ml of buffer for cytochrome c which contained 210 mM Manitol, 70 mM Sucrose, 20 mM HEPES -KOH, pH 7.4, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM Dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and a complete cocktail of protease inhibitors (Roche Molecular Biochemicals, Indianapolis, USA). The cells were incu-bated on ice for 30 min and homogenized using a glass dounce homogenizer and a B pestle. The homo genates were ultra-centrifuged at 100,000 *g* for 30 min at 4°C. Bradford (BIO-RAD) protein assay was performed and 25 µg of total cytosolic protein resolved in a 12% SDS-poly-acrylamide gel electrophoresis and transferred to poly-vinylidene difluoride membrane using semi-dry transfer system (Fisher). The membrane was then blocked using 5% nonfat milk in TBST (20 mM Tris-HCl, pH 7.4, 8 g/l NaCl, 0.1 % Tween-20) for 1 h at room temperature. This was followed by exposure to primary monoclonal anti-cytochrome c antibody (Santa Cruz, CA, USA) in TBST contain-ing 3% nonfat milk at 4°C overnight. The membrane was then washed three times in TBST for 15 min each time after which it was exposed to secondary anti-body conju-gated horseradish peroxidase (1:10, 000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. This was followed by washing three times in TBST then the protein visualized using the ECL system (Amersham), and exposed to x-ray films.

Measurement of mitochondrial membrane potential (▲ Ψ_m)

After QU treatment, the cells were incubated with 50 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) dye for 15 minutes at 37°C. The cells were washed with ice-cold PBS and scrapped off the plates in 1 ml of PBS. The cells were then collected and centrifuged at 500 g for 5 min and resuspended in 500 µl of PBS containing 2% fetal bovine serum (FBS) and 40 nM $DiOC_6$ (Yu *et al.*, 2000a). Fluorescence intensities of $DiOC_6$ were analyzed using FACScan (Becton Dickinson, San Jose, CA), at excitation and emission wavelengths of 480 nm and 512 nm, respectively. Propidium iodide (1 µg/ml) was added to each sample to gate the dead cells before the measurements were taken. Butylated hydroxyanisole (BHA; 400 µM) treatment was used as a positive control, while untreated cells represented the negative control (Yu *et al.*, 2000a).

RESULTS

QU induces cell death in HeLa cells

Fig. 1 shows the effect of increasing concentration of QU on the viability of HeLa cells after 24 h exposure to QU. QU treatments led to a rapid decrease in cell viability between 10 and 40 µM concentrations, with an IC_{50} (50% inhibitory concentration) of about 20 µM after 24 h of exposure to the drug.

QU activates caspases

Activation of caspases is an important aspect of apoptosis, and the effect of QU on caspase activation was examined. Using 25 µM QU from the cytotoxicity curve, treating Hela cells at various time points resulted in the activation of caspases in a time- (Fig. 2A) and dose-

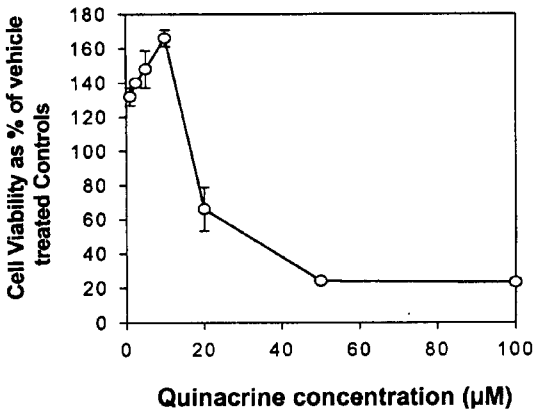

Fig. 1. The effect of quinacrine (QU) on cell growth and viability. HeLa cells were exposed to varying concentrations of QU for 24 h, and cell viability was assayed using the MTS assay. The results show the average of triplicate assays.

dependent manner (Fig. 2B). In the kinetic analysis, caspase-3-like activity increased rapidly after 1 h and peaked at 3 h, then decreased at later time points, though still remaining above the basal level (Fig. 2A).

Caspase-8 activity increased gradually (observed first at 1 h time point) preceding caspase-3-like activity and peaked at 4 h. Caspase-9 activity was similar to that of caspase-8, though a lower level of induction was observed (Fig. 2A). Caspae-1 and 6 activities were not stimulated substantially.

Since QU activated caspase-3, a hallmark for apoptosis, we next examined whether Ac-DEVD-CHO, the specific caspase-3 inhibitor, would influence the effect of QU on cell viability. Cells treated with caspase-3 inhibitor alone at concentrations between 10 nM-100 mM did not show any significant effect on cell survival (data not shown). However, QU-induced apoptotic cell death was substantially reduced when cells were pre-treated with Ac-DEVD-CHO and the cell viability curve shifted to the right, as compared to QU alone (Fig. 3A). Analogous result was obtained in Fig. 3B, where AC-DEVD-CHO blocked QU-induced caspase-3-like activity.

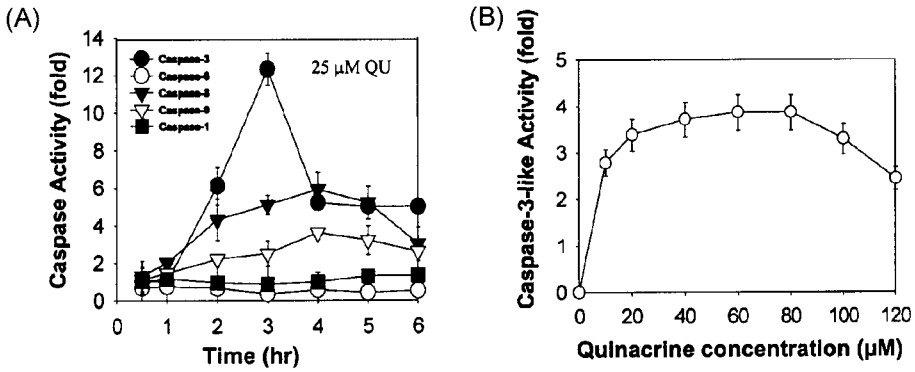

Fig. 2. The effect of QU on caspase activity in HeLa cells. The cells were treated with 25 mM QU for various times, harvested in the presence of lysis buffer. The activities of caspase-1 (Ac-YVAD-MCA), -3 (Ac-DEVD-MCA), -6 (Ac-VEID-MCA) 8 (Ac-IETD-MCA), and 9 (Ac-LEHD-MCA), were assayed using fluorogenic methods after incubation with the respective peptide substrates. The release of fluorogenic substrate AMC was monitored by a spectrofluorimeter (PerSeptive Biosystems, Inc., Framingham, MA).

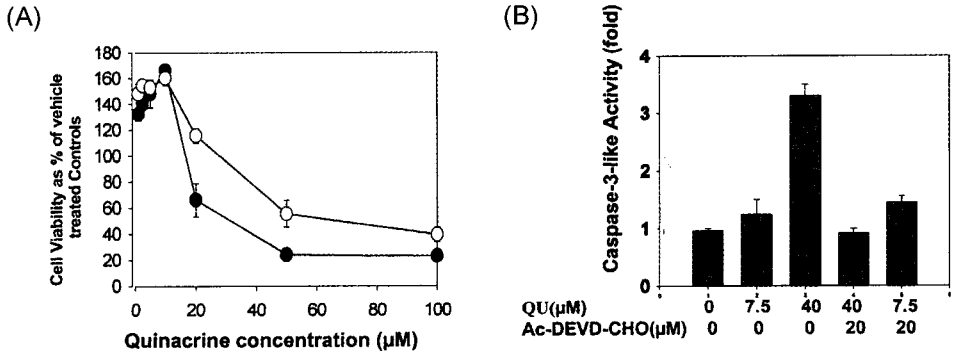

Fig. 3. A; The effect of caspase-3 specific inhibitor (Ac-DEVD-CHO) on cell viability. Hela cells were pre-treated with AC-DEVD-CHO for 1 h followed by QU treatment for 24 h then MTS assay was subsequently performed. **B**; HeLa cells were pre-treated with Ac-DEVD-CHO, followed by QU treatment as described in **A** above, for 4 h and caspase-3-like activity was assayed.

Nordihydroxyguaiaretic acid (NDGA) diminishes QU-induced caspase-3-like activity

We investigated the influence of NDGA-mediated blockade on caspases. In this study we observed that NDGA pre-treatment diminished QU-induced expression of caspase-3-like activity from about 12 fold (with QU alone) to 3 fold (Fig. 4A).

Caspase-8 and-9 activities were not significantly affected by NDGA (Fig. 4B, C). This demonstrated, for the first time, the inter-dependence between QU-induced PLA-2 inhibition and apoptotic cell death signaling.

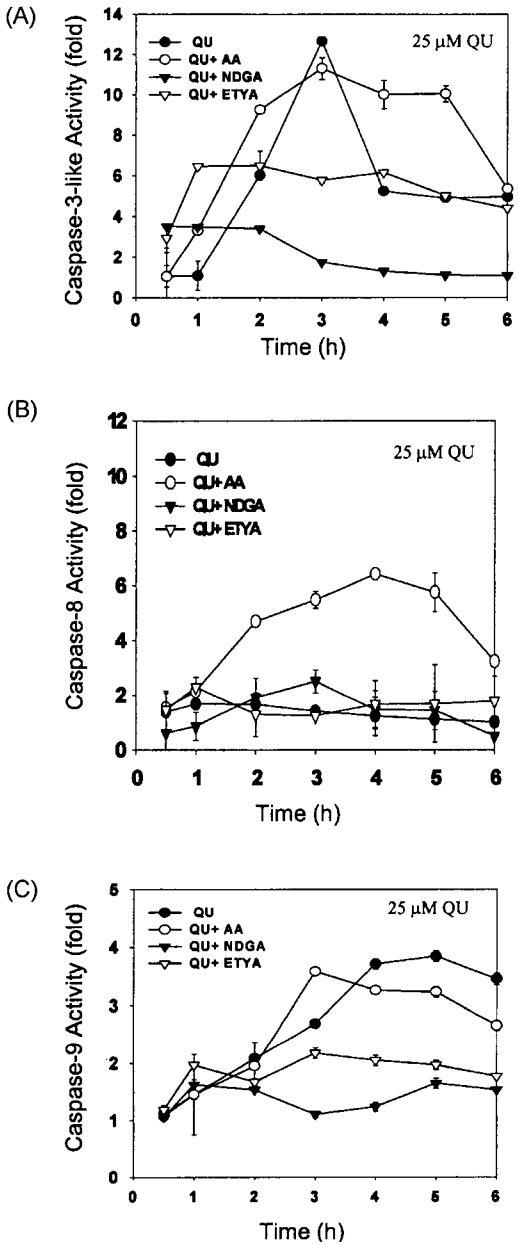

Fig. 4. The effect of pre-treatment with phospholipase-A-2 (PLA-2) inhibitors nordihydroxyguaiaretic acid (NDGA), 5, 8, 11, 14-eicosatetraynoic acid (ETYA), arachidonic acid (AA), respectively, followed by Quinacrine (QU) treatment, on caspase activity. **A**; The effects of QU alone, AA, QU+AA, QU+NDGA, QU+ETYA on caspase-3-like activity, **B**; The effects of QU alone, AA, QU+AA, QU+NDGA, QU+ETYA on caspase-8 activity, **C**; The effects of QU alone, AA, QU+AA, QU+NDGA, QU +ETYA on caspase-9 activity.

5,8,11,14-Eicosatetraynoic acid (ETYA) partially diminishes caspase-3-like activity

The inhibition of caspase-3-like activity following ETYA pre-treatment was generally much lower than that due to NDGA, resulting in relatively higher caspase-3-like activity than with NDGA (Fig. 4A). This strongly suggests that ETYA only partially blocked the cell death signal to caspase-3.

Arachidonic acid (AA) sustains QU-induced caspase-3-like activation

Supplementation of HeLa cells with AA shifted the QU-induced caspase-3-like activity curve to the left implying an increased sensitivity of caspase activation by QU (Fig. 4A). Further, AA pre-treatment also sustained caspase-3-like activity for up to 5 hr (Fig. 4A). AA supplementation also augmented QU-induced caspase-9 activity, but not as much as that seen with caspase-3 (Fig. 4C).

QU-induced time- and concentration-dependent release of cytochrome c

In order to understand the mechanism through which QU stimulated caspase activity, the role of mitochondrial events was investigated. QU treated HeLa cells showed a concentration- (Fig. 5A) and time-dependent release of cytochrome c (Fig. 5B). In the dose-response assay, the maximum release of cytochrome c occurred about 40 μM of QU and diminished at 60 μM (Fig. 5A). The kinetic study showed that, at 25 μM QU, the peak of cytochrome c release occurred after 3 h treatment, and faded off after 4 h (Fig. 5B).

NDGA abolishes QU-induced cytochrome c release

In order to examine the relationship between QU inhibition of PLA-2 pathway and apoptotic signaling, we employed two inhibitors of AA metabolism (NDGA and ETYA) to block the downstream of PLA-2 product metabolism, while examining apoptotic signaling. NDGA inhibits the AA metabolic pathway through competition with AA for the lipooxygenases. Cells pre-treated with NDGA attenuated QU-induced cytochrome c release in a dose-dependent manner (Fig. 6). This NDGA blockade was much more effective than that observed from pre-treatment with cyclosporin A (CsA), a mitochondrial permeability transition (MPT) inhibitor. This strongly suggests

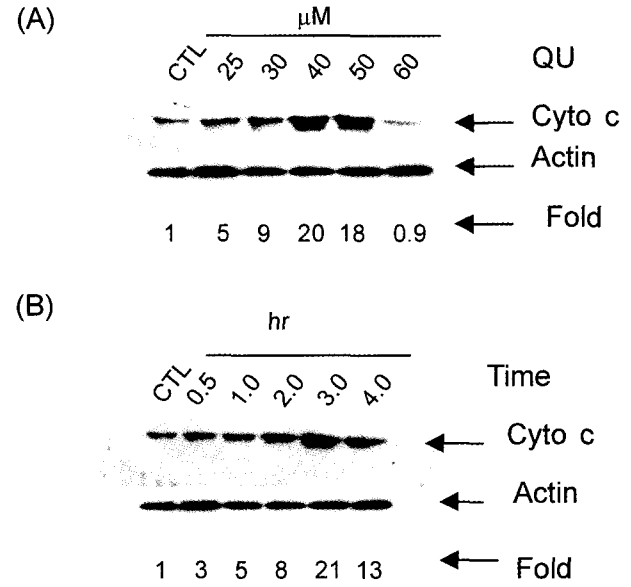

Fig. 5. Quinacrine-induced release of cytochrome c from the mitochondria. **A**; Dose-dependent cytochrome c release caused by QU. Cells were treated with varying concentrations of QU, then harvested in cytochrome c specific lysis buffer, and 10 μg of protein from the cell lysates analyzed by Western blotting using anti-cytochrome c primary antibody as described in the materials and methods section above. **B**; Kinetics of QU-induced cytochrome c release. Cells were treated with 25 μM QU for at different time points, then harvested as described in **A**; followed by Western blotting for cytochrome c release.

that NDGA has the ability to block specific factors that are important for the release of cytochrome c induced by QU. This observation may also suggest that by blocking the activity of lipooxygenases, NDGA also interferes with QU-induced cytochrome c dependent apoptotic signaling.

partially blocks cytochrome c release

EYTA is an analogue of AA in which the double bonds in AA are replaced with triple bonds, making it a false substrate for the cyclooxygenases that metabolize AA to the downstream components of the arachidonate pathway such as prostaglandins and eicosinoids. Unlike NDGA, ETYA pre-treatment of HeLa cells only partially blocked QU-induced release of cytochrome c at very high concentrations above 30 μM (Fig. 6).

QU causes cytochrome c release independent of mitochondrial membrane permeability transition (MPT)

FACScan analysis was conducted in a flow cytometer to investigate the mechanism by which QU induces the release of pro-apoptotic cytochrome c from the inter-mitochondrial space. Because mitochondrial permeability transition (MPT; $\blacktriangle\Psi_m$) has been implicated in the release of cytochrome c induced by several stimuli, we analyzed

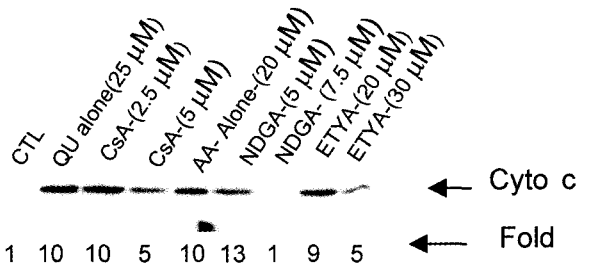

Fig. 6. The effect of NDGA, ETYA, AA, and cyclosporin A (CsA), AA on cytochrome c release from mitochondria. Cells were pre-treated with two different concentrations of the agents followed by QU (25 μM) treatment, then cytochrome c release assayed.

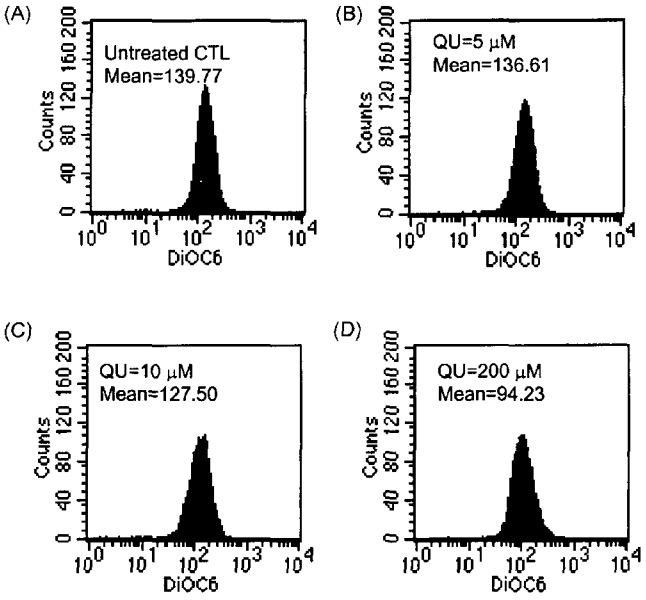

Fig. 7. Insignificant effect of QU on mitochondrial transmembrane potential assayed by FACScan in a flow cytometer, following QU treatment. **A**; membrane potential of control untreated cells, **B**; membrane potential of cells treated with 5 μM QU. **C**; membrane potential of cell treated with 10 μM QU, **D**; membrane potential of cells treated with 200 μM QU.

the changes in $\blacktriangle\Psi_m$ following QU treatment. This analysis revealed that QU treatment did not lead to any significant dose-dependent reduction in the mitochondrial membrane potential ($\blacktriangle\Psi_m$), measured as differences in $DiOC_6$ fluorescence intensities (Fig. 7A-D). However, since the concentrations (25-50 μM QU) that led to cytochrome c release and caspase activity, does not influence the MPT of $DiOC_6$, then these results strongly suggested that $\blacktriangle\Psi_m$ may not be a critical step in this apoptotic signaling pathway.

Caspase inhibitor (z-VAD) blocks cytochrome c release

The lipooxygenase and cyclooxygenase inhibitors

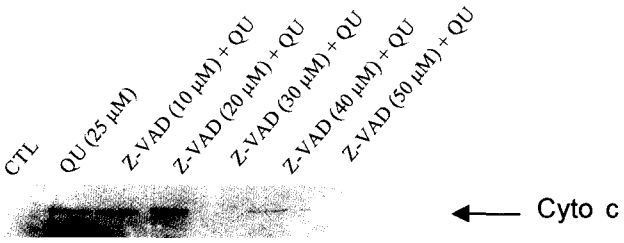

Fig. 8. The effect of pre-treatment with caspase inhibitor (z-VAD-fmk) on cytochrome c release. A concentration range from 10 μM z-VAD-fmk to 50 μM was employed followed by cytochrome c assay. QU alone was used as a positive control for cytochrome c release.

(NDGA, ETYA) diminished cytochrome c and the activities of caspase-9 and 3, strongly suggesting that these caspases are downstream of cytochrome c release. Therefore, in order to understand the position of caspase-8 relative to QU-induced mitochondrial events, caspase inhibitor z-VAD was used to block out all caspases including caspase-8, and examine cytochrome c release. Results show that blocking out of all caspases had an effect of inhibiting cytochrome c release thereby implying its upstream position relative to the mitochondrial events (Fig. 8). The dose-dependent decrease in cytochrome c release shows that concentrations higher than a threshold level of 20 μM z-VAD blocked cytochrome c release (Fig. 8).

DISCUSSION

Many studies have reported the use of QU as a potent prototype of PLA-2 inhibitor (Flynn, 1987). In this study, our results show that QU-induced apoptosis in human cervical carcinoma cells may be dependent of its PLA-2 inhibitory function. This PLA-2 dependent signaling pathway may contribute pharmacologically to the known anti-neoplastic effect exerted by QU. To elucidate the potential biological mechanism(s) elicited by QU leading to apoptotic cell death, we studied the activation of caspases, as critical proteases in apoptosis (Thornberry and Lazenbnik, 1998). QU-induced activation of caspases, and especially the death effector caspase-3-like activity which represents a major hallmark that commits the cell to suicide.

In the mitochondria-dependent apoptotic signaling model, cytochrome c is complexed with Apaf-1 forming an apoptosome (Green and Reed, 1998; Yu *et al.*, 2000a), leading to the activation of caspase-9, which in turn activates caspase-3 and apoptosis. The fact that Ac-DEVD-CHO blocked QU-induced cell death emphasized the critical role of caspase-3 in this apoptotic pathway. However, despite releasing cytochrome c from the mitochondria, QU was not able to cause any significant changes in $\blacktriangle\Psi_m$. This was interesting and strongly implied that changes in $\blacktriangle\Psi_m$ may not be necessary for the QU-induced release of cytochrome c and caspase activation.

PLA-2 belongs to a family of esterases that hydrolyze the sn-2 ester bonds in phospholipids thereby releasing products that are important in several cellular signaling processes (Cummings *et al.*, 2000). Because QU is conventionally known to act as a potent PLA-2 inhibitor, in this study we examined the influence of two of the down stream products of PLA-2, AA metabolizing enzyme, NDGA (lipooxygenase inhibitor) and ETYA (cyclooxygenase inhibitor). The NDGA-induced inhibition of cytochrome c release and reduction in caspase-3-like activity observed in this study suggests that this inhibitor may be targeting certain factors on the mitochondrial membrane, thereby blocking the release of cytochrome c, which in turn diminishes downstream apoptotic signaling events like caspase activity and apoptosis.

Interestingly, AA supplementation did enhance QU-induced caspase-3-like activity. This agrees with findings from several studies that have examined the role of PLA-2 and reports that cyclooxygenase inhibitors such as aspirin that enhance the accumulation of AA by blocking its catabolic pathways, lead to apoptosis (Jan *et al.*, 2000). Furthermore, recent studies involving bone marrow-derived murine mast cells have shown that the accumulation of AA and not other unsaturated fatty acids or cytosolic PLA-2 expression, was correlated to the number of apoptotic cells (Fonteh *et al.*, 2000) Therefore our results show a potential link between the AA metabolism and apoptotic signaling. These studies have also confirmed that free AA accumulation was not related to the decreased activities of PLA-2-dependent lipooxygenases and cyclooxygenases, there by suggesting that the QU-induced apoptotic signaling observed in this study was indeed as a result of a pathway independent of its role as a PLA-2 inhibitor (Fonteh *et al.*, 2000).

In view of the above observations we developed, for the first time, a simplistic signaling model, with respect to apoptosis in human cervical carcinoma cells (see Fig. 9 above). In this model QU induces a signaling cascade that starts from yet unidentified receptors on the cell surface, followed by the activation of pro-caspase-8, caspase-8 and then a two-way signaling develops. The first signal targets caspase-3 and leads to apoptosis, while the next pathway involves the induction of cytochrome c release, including other pro-apoptotic proteins like Apaf-1, AIF which complex with caspase-9. This activated complex then delivers a downstream signal that activates pro-caspase-3 and caspase-3, and eventually leading to apoptosis. In this simplistic model, caspase-8 induces the release of cytochrome c through BID cleavage (Gross *et al.* 1999), while NDGA abolishes the release of cytochrome c by, possibly, targeting certain proteins on the mitochondrial membrane. This role of NDGA in blocking cytochrome c release and caspase activation is very similar to that observed by Bcl-2, which resides on the outer mito-

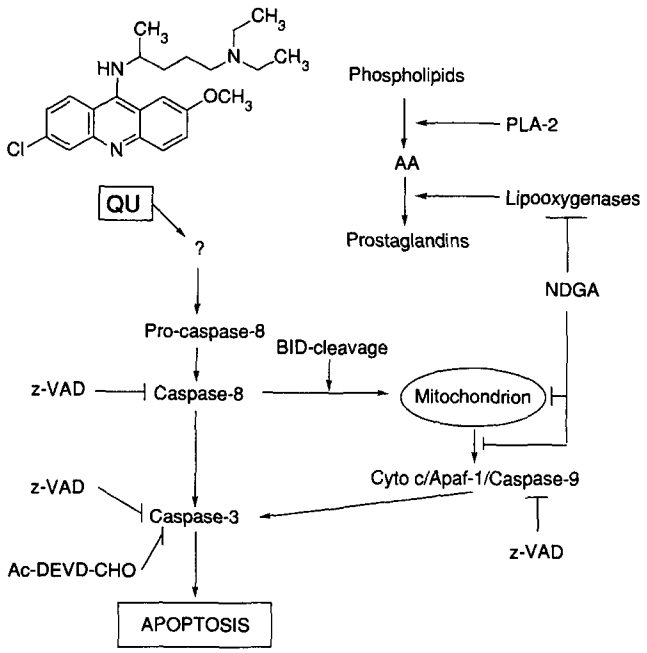

Fig. 9. A signaling model for QU-induced apoptosis is proposed, showing the role of mitochondrial cytochrome c release in enhancing a caspase cascade that leads to apoptosis.

chondrial membrane (Kluck *et al.*, 1997). Also, in this model we assume that the observed enhancement of caspase activity by AA may be occurring through its role in targeting generating a gene induction as a feedback mechanisms due to its (AA) accumulation in the cell. Increased AA levels in the cell leads to gene induction for higher lipooxygenase levels thereby reducing the amount of NDGA that is available to bind on the mitochondrial apparatus. It was, though, interesting that ETYA, a false substrate for cyclooxygenase neither activated nor completely blocked caspase-3-like activity induced by QU.

While several apoptotic stimuli have signaling models that involve the depolarization of the mitochondrial membranes, many com-pounds do show no effect on the membrane potential, yet still leading to cytochrome c release (Kim *et al.*, 2000). QU falls in this category and hence imply that cyto-chrome c release may occur in several ways apart from reduction in the membrane potential, ways that are yet to be elucidated. The recent observation by Srinivasula *et al.* (2000) demonstrating that Smac/DIABLO acts to regu-lated cytochrome c-dependent apoptosis by neutralizing the inhibitors of apoptosis proteins (IAP) and leading to caspase activation using its NH_2-terminus, also generates another possibility that NDGA may possibly be targeting and binding some other pro-apoptotic proteins and ren-dering them ineffective in initiating the mitochondrial-dependent release of cytochrome c as a result of QU treatment. The role of gene induction in modulating the mitochondria-dependent events observed above becomes even more important to examine in the light of the latest findings relating to the translocation of certain transcrip-tion factors e.g., TR3, from the cell nucleus to the mitochondrial membrane thereafter affecting the mitochondrial membrane potential (Li *et al.*, 2000). However, other studies have reported that the inhibition of PLA-2 pathway using a combination of CsA and aristolochic acid (ArA) inhibited cell death (Tifani *et al.* 2000).

The importance of cytochrome c in apoptosis cannot be over-emphasized. Several studies have shown that cytochrome c is a basic requirement for the initiation of the apoptotic program through its action in activating caspase-3 (Yang *et al.*, 1997). Therefore the regulation of QU-induced cytochrome c by NDGA which directly regulates apoptosis in Hela cells, however, the mechanism through which this inhibitor acts remain to be elucidated.

By comparing QU to other apoptosis-inducing agents, the activation of caspase-3 by QU appeared after 1 h and peaked at 3 h which contrasts from other compounds such as chelerythrine, i.e., natural alkaloids which have been shown to induce apoptotic cell death that exhibit delayed kinetics, caspases was stimulated after 6 h. The kinetics of caspase activation by QU is very similar to that induced by phenyl isothiocyanates (PEITC) (Yu *et al.*, 1998), or *tert*-butyl-4-hydroxyanisole (BHA) (Yu *et al.*, 2000a).

In summary, this study has demonstrated that QU activates upstream caspase-8, which activates caspase-3, and that the release of cytochrome c from the mitochondria, which is independent of changes $\blacktriangle\Psi_m$, help to enhance the caspase-driven cell death mechanism. Though QU has been shown to induce anti-proliferative/anti-neoplastic action in several studies previously (Gebbia *et al.*, 1994; Banerjee *et al.*, 1994), this report presents the first evidence for the activation of an important mitochondria-dependent cellular signaling pathway, directly contributing to the pharmacological anti-cancer effect of QU. Future studies should examine the role of gene induction and transcriptional activation, and whether they are responsible for the patterns of cytochrome c release, caspase activity, and that of other cell death factors (e.g., death receptors) that may be related to the caspase-8 activity observed above. Other mechanisms such as p53 (Huang *et al.*, 1998) should also be examined to provide a comprehensive understanding of QU action. The possibility of receptors being involved in this QU-induced apoptotic mechanism is justified by the fact that upstream caspase-8 was activated and sustained for about 3 h.

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