Role of glutathione in augmenting the anticancer activity of pyrroloquinoline quinone (PQQ)

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Pyrroloquinoline quinone (PQQ), a bacterial redox co-factor and antioxidant, is highly reactive with nucleophilic compounds present in biological fluids. PQQ induced apoptosis in human promonocytic leukemia U937 cells and this was accompanied by depletion of the major cellular antioxidant glutathione and increase in intracellular reactive oxygen species (ROS). Treatment with glutathione (GSH) or *N*-acetyl-L-cysteine (NAC) did not spare PQQ toxicity but resulted in a 2–5-fold increase in PQQ-induced apoptosis in U937 cells. Cellular GSH levels increased following treatment by NAC alone but were severely depleted by co-treatment with NAC and PQQ. This was accompanied by an increase in intracellular ROS. Alternatively, depletion of glutathione also resulted in increased PQQ cytotoxicity. However, the cells underwent necrosis as evidenced by dual labeling with annexin V and propidium iodide. PQQ-induced cytotoxicity is thus critically regulated by the cellular redox status. An increase in GSH can augment apoptosis and its depletion can switch the mode of cell death to necrosis in the presence of PQQ. Our data suggest that modulation of intracellular GSH can be used as an effective strategy to potentiate cytotoxicity of quinones like PQQ.

Keywords: pyrroloquinoline quinone, PQQ, pro-oxidant, apoptosis, necrosis, glutathione

Introduction

Many naturally occurring flavanoids and quinones used as drugs protect against oxidative stress by scavenging free radicals. However, in the process of offering protection against free radicals, they are chemically converted into an oxidation product¹ that reacts with glutathione (GSH), thereby forming glutathionyl adducts.² This high reactivity toward thiols can result in GSH depletion and loss of protein function.³ Menadione (2-methyl-1,4-naphthoquinone, vitamin K₃), a component of multivitamin

formulations and widely used as a therapeutic agent for hypothermia and cancer, acts by thiol depletion and formation of reactive oxygen species (ROS).⁴ The molecular mechanisms of quinone cytotoxicity have been extensively reviewed.^{5,6} Two main mechanisms are: (i) covalent binding to macromolecules (protein, DNA) via Michael addition; and (ii) formation of ROS, resulting in oxidative stress that can oxidize lipids, proteins, or DNA. Covalent binding is preceded by glutathione (GSH) depletion either by conjugation, or via oxidation of GSH to GSSG.

Pyrroloquinoline quinone (PQQ) is a naturally occurring redox co-factor first discovered in bacteria in 1979⁷ and is now known to be widely distributed in mammalian cells.⁸ PQQ is a redox active essential nutrient that can generate or scavenge superoxide depending on its micro-environment. PQQ is able to

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Figure 1 PQQ-induced cytotoxicity. (A) NIH 3T3 and L929 were allowed to adhere for a minimum of 4 h. All the four different cell types were seeded at a density of 0.5×10^5 cells/ml and were incubated with different concentrations of PQQ for 48 h. The viability of the cells was assessed by MTT assay by measuring the absorbance at 550 nm and viability was calculated as percentage of untreated cells. (B) Flow cytometric profile of DNA content of cells treated with different concentrations of PQQ. The cells were labeled with PI and the samples were acquired in a FACS Vantage flow cytometer and analyzed using CELLQuest™ software. The marker M1 corresponds to the cells containing sub G₁ DNA content or apoptotic cells. (C) Apoptotic indices as determined by PI labeling of cells with sub G₁ DNA content. Values are mean $\pm$ SD of three separate experiments. *Shows significant difference as compared to the control at $P < 0.05$

oxidize the redox modulatory site of *N*-methyl-D-aspartic acid (NMDA) receptors, thus conferring protection against NMDA- or glutamate-mediated cell injury in cultured neurons.⁹ PQQ has been reported to suppress peroxynitrite formation¹⁰ and also showed antioxidant properties against lipid peroxidation.^{11,12} The most promising potential application of PQQ has been in neuroprotection,^{10,13} cardioprotection¹⁴ and as an antimelanogenic agent.^{15,16} Though PQQ has been shown to be cytotoxic to melanoma cells,¹⁵ this aspect of PQQ activity has not been explored in detail.

PQQ is highly reactive in solution and has been implicated in formation of adducts with other nucleophilic groups in the cells.¹⁷ We have examined the effect of PQQ in U937 human promonocytic cells and the effect of glutathione supplementation or depletion on PQQ toxicity. PQQ induced apoptosis in U937 cells which increased 2–5-fold in the presence of NAC or GSH. Depletion of cellular glutathione, on the other hand, amplified PQQ toxicity several fold and also switched the mode of PQQ-induced cell death from apoptosis to necrosis.

Materials and methods

Reagents

RPMI-1640 medium, DMEM, penicillin, streptomycin, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), *N*-acetyl-L-cysteine (NAC), propidium iodide (PI), sodium acetate, Triton X-100 and monochlorobimane (mBCl) were obtained from Sigma Chemical Company, (St Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco BRL (Paisley, UK). Annexin V-FITC, TUNEL assay kit and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation kit were from Roche Applied Science (Germany) All the cell lines were obtained from National Centre for Cell Sciences, Pune, India.

Cell culture and treatment

All cell lines were routinely maintained in media supplemented with 10% FBS (v/v), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a

humidified atmosphere of 5% CO₂ in air. U937 and EL-4 cells were grown in RPMI-1640 medium. NIH3T3 and L929 cells were grown in DMEM. For experiments, cells were seeded at a density of 5×10^5 cells/ml. Cells were pretreated with GSH (5 mM), NAC (5 mM) or BSO (5 mM) for 24 h prior to the addition of PQQ.

Estimation of cell viability by MTT assay

NIH3T3 and L929 cells were seeded and allowed to adhere in 96-well plates for a minimum of 4 h. In parallel, U937 and EL4 cells were also seeded and different concentrations of PQQ were added to all the cells at the same time. They were further cultured for 48 h. The cell viability was measured by MTT dye conversion to formazan using a MTT cell proliferation kit. Six replicates were taken for each group and the plate was read in a microplate reader at 550 nm. The viability is expressed as the percentage of the control untreated cells.

Estimation of reactive oxygen species (ROS)

Generation of intracellular ROS was measured as described previously.¹⁸ Briefly, cells were labeled with 20 μ M H₂DCFDA for 15 min at 37°C and were further incubated with 50 μ M PQQ for 2 h and analyzed in a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA, USA) using CellQuest software. Generation of ROS in cell-free system was also measured by the oxidation of DCF.¹⁹ Briefly, DCFH was prepared from DCFH-DA by mixing 0.5 ml of 1 mM DCFH-DA (in methanol) with 2 ml of 10 mM NaOH at room temperature for 30 min.¹⁹ The mixture was then neutralized to pH 7.4 with 10 ml of 25 mM NaH₂PO₄. The solution was kept in the dark on ice until used. The reactions were performed in a cuvette with RPMI-1640 medium total volume of 1 ml containing DCFH (3.3 μ M final concentration). PQQ (500 μ M) alone or along with GSH (10 mM) was incubated in RPMI-1640 medium for 3 h prior to addition of DCFH and HRP. Fluorescence of DCF generated after the reaction was measured in a spectrofluorimeter (excitation 488 nm, emission 525 nm).

Estimation of cellular glutathione (GSH)

Glutathione levels were measured using monochlorobimane (mBCl) fluorescence. mBCl is a versatile, membrane-permeable non-fluorescent probe that binds irreversibly to sulfhydryl groups yielding a fluorescent product.²⁰ Cells were labeled with 25 μ M mBCl for 10 min at room temperature and fluorescence was measured in a fluorimeter (Hidex,

Germany) with excitation and emission maxima set at 395 nm and 480 nm, respectively.²¹

Annexin V assay

Annexin V-FITC/PI dual staining was used for estimating apoptotic and necrotic cells.²² Cells were labeled with 5 μ l of annexin V-FITC and 2 μ l of propidium iodide (PI, 100 μ g/ml) and were examined using a confocal microscope (Carl Zeiss, Germany) with excitation of 488 nm and emission set at 525 nm for FITC and 585 nm for PI. Images ($\times 20$) were acquired and analyzed using an LSM image examiner. Cells labeled with PI alone were also acquired in a FACS Vantage flow cytometer and cells with more than a log cycle increase in fluorescence at FL2, 585 nm as compared to the unlabeled cells were enumerated as necrotic cells.

Assay for apoptosis: propidium iodide labeling

Forty-eight hours following treatment, cells were harvested, washed with PBS and labeled with hypotonic propidium iodide solution (50 μ g/ml PI, 0.1% sodium citrate and 0.1% Triton X-100) and analyzed by flow cytometry. Cells with less than G₁ DNA content (FL2, 585 nm) were estimated as apoptotic cells.

TUNEL (terminal deoxynucleotidyl transferase [TdT] mediated nick end-labeling) assay

Briefly, cells were labeled with the enzyme TdT and dUTP-FITC at 37°C for 1 h and analyzed by flow

Figure 2 Effect of antioxidant enzymes on PQQ-induced cytotoxicity. PQQ-induced apoptosis in cells pretreated with catalase and superoxide dismutase

cytometry.²² Cells treated with dUTP-FITC alone served as the negative controls.

Figure 3 Effect of GSH on PQQ-induced cytotoxicity (A) U937 cells were cultured with 50 μ M PQQ alone or in the presence of 10 mM NAC. Three hours later, cells were harvested and labeled with annexin-FITC/PI and the number of single positive or double positive cells enumerated by confocal microscopy. Representative images from each of the treatments are shown. Three such experiments were carried out. (B) Flow cytometric profile of DNA content of cells treated with different concentrations of PQQ in presence of GSH or NAC. The cells were labeled with PI and the samples were acquired and analyzed using CELLQuest™ software in a FACS Vantage flow cytometer. The marker M1 corresponds to the cells containing sub G₁ DNA content or apoptotic cells. (C) Percentage of apoptotic cells as determined by PI labeling of cells (sub G₁ DNA content). Values are mean $\pm$ SD of three separate experiments. *Shows significant difference as compared to the control at $P < 0.05$. (D) Flow cytometric profile of DNA content of cells treated with different concentrations of PQQ in presence of GSH. The cells were labeled with TdT and dUTP-FITC and the samples were acquired in a FACS Vantage flow cytometer and analyzed using CELLQuest™ software. The marker M1 corresponds to the cells positive for FITC or apoptotic cells. Open histograms represent the negative control with only dUTP-FITC and the filled histograms represent the treatment with TdT and dUTP-FITC

Statistical analysis

All experiments were repeated at least three times and the results were found to be reproducible. Student's *t*-test was used to calculate the significance of differences between the groups. The level of statistical significance was set at $P < 0.05$.

Results

PQQ-induced apoptosis in U937 cells

PQQ induced significant cell death in EL-4 cells as well as in U937 cells as shown by MTT assay (Fig. 1A). There was no significant loss of viability of NIH3T3 as well as L929 cells (used as non-transformed controls) due to PQQ treatment (Fig. 1A). PQQ induced apoptosis in U937 cells (increase in sub G₁ DNA containing cells) and this effect was dose dependent (Fig. 1B,C).

Modulation of PQQ-induced apoptosis by antioxidants

PQQ has been reported to generate hydrogen peroxide¹² and, hence, the sparing effect of antioxidant enzymes and glutathione was assessed. Catalase

treatment completely neutralized the toxicity of PQQ, whereas superoxide dismutase did not have any effect (Fig. 2). Pretreatment of the cells with precursor of GSH, *N*-acetylcysteine (NAC) or GSH itself augmented the toxicity of PQQ 2–5-fold. This was due to increased apoptosis as seen from annexin V staining and corresponding absence of PI positive cells (Fig. 3A). Figure 3B,C shows the increase in percentage of apoptotic cells 48 h following treatment with PQQ and a further 2–2.5-fold increase in apoptosis when cells were pretreated with NAC. This effect of NAC or GSH was also dose dependent (Fig. 3C). Cells treated with NAC and GSH alone served as the controls (data not shown). This was further confirmed with TUNEL assay (Fig. 3D).

Figure 4 Effect of PQQ and NAC on intracellular GSH and ROS. U937 cells were pretreated with 10 mM NAC followed by 50 μM PQQ. (A) Glutathione levels as estimated by changes in fluorescence of mBCl. (B) Intracellular ROS levels as estimated by changes in fluorescence of DCF. (C) Oxidation of DCFH in a cell-free system. Values are mean ± SD of three separate experiments. *Shows significant difference as compared to the control at $P < 0.05$

Figure 5 Effect of PQQ in glutathione-depleted cells. (A) U937 cells were cultured with 50 μ M PQQ alone or in presence of 10 mM NAC. Three hours later, cells were harvested and labeled with annexin-FITC/PI and the number of single positive or double positive cells enumerated by confocal microscopy. Representative images from each of the treatments are shown. The experiment was repeated for a minimum of three times. (B) Loss of membrane integrity was monitored by flow cytometric analysis of propidium iodide uptake by the cells. Cells with increased fluorescence indicate necrosis. (C) Percentage of necrotic cells induced by different concentrations of PQQ in BSO-treated cells. The data-points indicate the mean percentage of cells positive for PI uptake $\pm$ SEM. Representative data from one of the three experiments are shown. (D) Histogram of percentage necrotic cells induced by PQQ and BSO in presence of GSH and catalase

Mechanism of action of PQQ

To understand the mechanisms of this increased cell death in the presence of NAC and PQQ, we estimated the cellular glutathione concentration. Though there was an increase in GSH level following treatment with

NAC, it was completely depleted in the presence of PQQ (Fig. 4A). As a result of depletion of cellular thiol pools, there was a concomitant increase in intracellular ROS (Fig. 4B). PQQ alone caused a significant increase in intracellular ROS. However, in the presence of NAC or

GSH, there was 1.5-fold increase in the ROS. A similar increase of 1.5–2-fold in the generation of ROS was also observed when PQQ was incubated along with GSH in a cell-free system (Fig. 4C).

Depletion of glutathione switched PQQ-induced cell death from apoptosis to necrosis

To examine the effect of depletion of cellular glutathione on PQQ-induced cell death, U937 cells were pretreated with BSO, an inhibitor of γ -glutamyl-cysteine synthetase and a known agent for glutathione depletion.²³ PQQ caused massive necrosis in glutathione-depleted cells (Fig. 5A). There was increase in annexin-FITC positive cells following PQQ treatment, whereas BSO- and PQQ-treated cells showed dual labeling of annexin FITC and PI. Flow cytometric analysis of PI uptake by these cells also showed similar results with more than 80% of cells dying of necrosis (Fig. 5B,C).

Increased ROS accompany necrosis induced by PQQ in glutathione depleted cells

Since catalase could give complete protection against PQQ-induced apoptosis, we wanted to study if the necrosis induced by PQQ in glutathione-depleted cells could be spared by catalase or glutathione. Cells were depleted of GSH by pretreatment with BSO. They were incubated with catalase and/or GSH before addition of PQQ. Presence of catalase or GSH protected against PQQ necrosis in glutathione-depleted cells. In combination, they suppressed necrosis synergistically (Fig. 5D). Depletion of GSH by BSO was accompanied by an increase in intracellular ROS (Fig. 6B). Depletion of glutathione by PQQ was to the same extent as that by BSO (Fig. 6A).

Discussion

Medicinal research is in a constant quest to identify new anti-tumor drugs. Though PQQ has been shown to cause cell death in Jurkat cells¹² and melanoma cells¹⁵ at $> 20 \mu\text{M}$, this aspect has not been explored in detail. Interestingly, Kumazawa *et al.*²⁴ reported PQQ-induced increase in proliferation of Ras transformed NIH3T3 cells in 0.5% serum containing medium. We did not observe any toxicity in non-transformed 3T3 or L929 cells with PQQ treatment nor an increase in proliferation in 10% FBS supplemented growth medium (Fig. 1A). The fibrosarcoma cell line showed a marginal decrease in viability at the highest concentration of PQQ used (Fig. 1A). In contrast, the lymphoma cell line EL-4 and monocytic leukemia cell line U937 showed significant loss in viability (Fig.

Figure 6 Effect of PQQ and BSO on intracellular GSH and ROS. (A) Glutathione levels were estimated as change in fluorescence of mBCl. (B) Intracellular ROS levels were estimated as changes in fluorescence of DCFDA. Values are mean $\pm$ SD of three separate experiments. Significant difference as compared to the control at $*P < 0.05$ or $**P < 0.01$

1A). Hence the U937 cell line was used for all further investigations. PQQ induced apoptosis at $20 \mu\text{M}$ (Fig. 1B,C) which is in agreement with other studies reported in the literature.^{12,15}

Enhancement of ROS production has long been associated with the apoptotic response induced by several anticancer agents.^{25–27} The status of intracellular redox is regulated by antioxidant enzymes (SOD, catalase, glutathione peroxidase) and non-enzymatic antioxidants (GSH, vitamin C). Catalase could completely spare the cells from PQQ cytotoxicity whereas superoxide dismutase did not have any effect (Fig. 2). This was in agreement with an earlier report.¹² Conversely, pretreatment of cells with NAC or glutathione resulted in a completely different

outcome. PQQ-induced cytotoxicity was augmented in NAC- or GSH-treated cells with a 2–2.5-fold increase in apoptosis (Fig. 3). Treatment with PQQ resulted in depletion of intracellular glutathione (Fig. 4A) along with an increase in ROS (Fig. 4B) suggesting a probable mechanism of PQQ cytotoxicity. Similar findings have been reported for menadione-induced cytotoxicity.²⁸

This increase in generation of ROS when PQQ was incubated with GSH was observed even in cell-free systems. It can be speculated that formation of PQQ–thiol conjugate may be responsible for this increased cytotoxicity due to increased generation of ROS. Oxidative stress arises when the quinone is reduced to a semiquinone radical which reduces oxygen to form superoxide radical and regenerate the quinone. This futile redox cycling and oxygen activation forms cytotoxic levels of hydrogen peroxide. Many quinones have been reported to form GSH conjugates which also undergo futile redox cycling and oxygen activation.²⁹ This futile redox cycling of PQQ in medium could be triggered by many components of the medium such as ascorbic acid, which has been reported to increase redox cycling of many quinones.³⁰ It has been reported that when intracellular thiols are increased with *N*-acetylcysteine, it spared the intracellular glutathione and diminished cytotoxicity and apoptosis.³¹ Though cellular toxicity of menadione is due to formation of menadione–thiol conjugates,⁴ pretreatment with cysteine completely protected against menadione-induced cell death.²⁸ Similar results have been reported for γ -tocopheroyl quinone–GSH conjugates.^{32,33} Increased toxicity due to pretreatment with NAC or GSH similar to our findings has been reported for some compounds like pyrogallol³⁴ and melanin.³⁵

PQQ-induced apoptosis was enhanced by treatment with NAC and the mode of cell death was completely switched to necrosis upon glutathione depletion (Fig. 5). This necrosis was completely abrogated by GSH and catalase (Fig. 5D) and was mediated by increase in cellular ROS (Fig. 6B). Depletion of intracellular GSH by BSO treatment has been used as a strategy to sensitize tumor cells to apoptosis induced by chemotherapeutic agents.³⁶

Conclusions

PQQ exhibits profound cytotoxicity and it is dependent on the intracellular thiol status. Conjugation with thiol and generation of oxidative stress seemed to be the major mechanism of action of

PQQ. Increase in glutathione levels increased PQQ toxicity and decrease in glutathione increased it several fold more and also switched the mode of cell death from apoptosis to necrosis. Our study describes the induction of cell death at both ends of the spectrum, apoptosis as well as necrosis, by modifying the thiol status of the cell. The parallel pattern of changes of the thiol content and intracellular ROS and the level of apoptosis and necrosis versus thiol status suggest that the effects of PQQ on the redox equilibrium of the cells may play a decisive role in determining its effects on cell proliferation and viability.

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