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Research Paper

Inhibition and crosslinking of the selenoprotein thioredoxin reductase-1 by p-benzoquinone

Nan Shu\textsuperscript{a}, Qing Cheng\textsuperscript{b}, Elias S.J. Arnér\textsuperscript{b}, Michael J. Davies\textsuperscript{a,∗}

\textsuperscript{a} Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, 2200, Copenhagen, Denmark
\textsuperscript{b} Division of Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institute, 171 77, Stockholm, Sweden

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A B S T R A C T

Quinones are common in nature, and often cytotoxic. Their proposed toxicity mechanisms involve redox cycling with radical generation, and/or reactions with nucleophiles, such as protein cysteine (Cys) residues, forming adducts via Michael addition reactions. The selenenyl anion of selenocysteine (Sec) is a stronger nucleophile, more prevalent at physiological pH, and more reactive than the corresponding thiolate anion of Cys. We therefore hypothesized that Sec residues should be readily modified by quinones and with potential consequences for the structure and function of selenoproteins. Here, we report data on the interaction of p-benzoquinone (BQ) with the selenoprotein thioredoxin reductase-1 (TrxR1), which exposes an accessible Sec residue upon physiological reduction by NADPH. Our results reveal that BQ targets NADPH-reduced TrxR1 and inhibits its activity using 5,5′-dihydroxybenzoic acid (5-nitrobenzoic acid) or juglone as model substrates, consistent with the targeting of both the Cys and Sec residues of TrxR1. In the absence of NADPH, BQ modified the non-catalytic Cys residues, leading to subunit crosslinking, mainly through disulfides, which also resulted in some loss of activity. This crosslinking was time-dependent and independent of the Sec residue. Addition of NADPH after BQ pre-treatment could resolve the disulfide-linked crosslinking. TrxR activity loss was also observed upon incubation of J774A.1 cells or cell lysates with BQ. These data suggest that BQ readily targets TrxR1, albeit in a rather complex manner, which results in structural changes and loss of enzyme activity. We suggest that TrxR1 targeting can explain some of the cytotoxicity of BQ, and potentially also that of other quinone compounds.

1. Introduction

Quinones are ubiquitous in nature and can be found in many natural products, endogenous chemicals (e.g. dopaquinone, estrogen-derived quinones) and environmental contaminants (e.g. naphthoquinone), or generated via the metabolism of aromatic hydrocarbons (e.g. benzene, hydroquinones), drugs (e.g. acetaminophen, gefitinib) and some food constituents (e.g. phenols/polyphenols) [1]. Thus, quinone exposure can occur endogenously, pharmacologically, via the diet, and via environmental pollutants. The inherent reactivity of quinone compounds has been associated with toxicity. Specific examples include the hydroxyniline metabolite of gefitinib that further undergoes two-electron oxidation to produce a reactive quinine imine intermediate, which is associated with hepatotoxic side effects [2]. The hepatotoxicity of acetaminophen involves its liver-specific metabolite N-acetyl-p-benzoquinone imine (NAPQI) that mediates protein modifications, lipid oxidation, and calcium homeostasis changes [3]. It also targets the selenoprotein thioredoxin reductase (TrxR), which is likely to contribute to toxicity [4,5].

Two major characteristics have been demonstrated to account for quinone toxicity (reviewed in Refs. [1,6]). They readily undergo one-electron reduction to give semiquinone radical anions, with the latter rapidly undergoing electron transfer to O\textsubscript{2} to give O\textsubscript{2}⁻, and subsequently H\textsubscript{2}O\textsubscript{2} (via dismutation), and hydroxyl radicals in the presence of trace metal ions. Alternatively, quinones are Michael acceptors, and can therefore modify proteins and/or DNA at nucleophilic sites by forming covalent adducts [7,8]. Our previous research have demonstrated that cysteine (Cys) is a favoured site for quinone...
adduct, and the modification of Cys residues in protein can further lead to the protein structure changes and dysfunction [9,10].

Selenocysteine (Sec, U), the sulfur-for-selenium-substituted analogue of Cys, has a relatively low pKₐ (5.2, compared to 8.3 for Cys) and is generally much more reactive with electrophiles than Cys [11]. Considering the highly polarizable nature of Sec, the exact pKₐ value may have a broader range, and is dependent on the microenvironment. A previous study has reported a pKₐ value of Sec in a peptide as low as to 3.3 [12]. Thus, under physiological condition, Sec residues will be almost completely ionized to the selenolate, unlike most Cys residues, giving Sec a higher nucleophilicity, a higher susceptibility to electrophilic attack, and a better leaving group character [13]. Therefore, we hypothesized that quinone compounds should rapidly react with Sec residues of selenoproteins.

Selenoproteins are often, but not always, oxidoreductases that have Sec as a catalytic residue. This is typically positioned close to the C-terminus, such as in TrxR, or within a Trx fold, such as in glutathione peroxidases (GPxs) [14]. The catalytic cycle of many selenoproteins involves selenolate/selenenylsulfide exchange reactions, analogous to thiolate/disulfide reactions [15]. For TrxR, the three critical motifs for its function are an enzyme-bound FAD, an N-terminal -CVNVGC- dipeptide [16,17]. The proposed mechanism of TrxR involves the electron transfer from nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) via FAD and the N-terminal motif to the C-terminal -GCUG-sequence [17,18]. Previous studies have demonstrated that certain quinone compounds can be substrates for form, as well as inhibitors of TrxR, which may trigger cellular events and Nrf2 activation, as reviewed elsewhere [19]. The enzyme can use either the N-terminal FAD/dithiol/disulfide or the C-terminal Sec-containing motif to directly catalyse mixed- or two-electron reduction reactions with quinones, depending upon the quinone structures [20]. The reduction of most of the quinones tested to date seems to require the Sec residue, whilst reduction of 5-hydroxy-1,4-naphthoquinone (juglone) also displays a Sec-dependent activity, the C-terminal motif of TrxR2 does not in influence reduction of either DTNB or juglone as much as with TrxR1 [24].

The highly-reactive and solvent-accessible Sec residue of TrxRs makes the enzyme readily modifiable by electrophilic compounds. Thus, previous studies on isolated TrxR1, or mixtures of TrxR isoforms (non-fracionated materials) have provided evidence for inhibition by cisplatin [25], dinitrobenzenes [26], curcumin [27], and some flavonoids [28], with this typically resulting in enzyme inactivation. As p-benzoquinone (BQ), the main metabolite of benzene, has been shown to react directly with Cys residues in glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and creatine kinase (CK), resulting in the enzyme inactivation and crosslink formation [10], we hypothesized that similar reactions would occur with the major cellular TrxR isoform, cytosolic TrxR1. TrxR1 predominantly exists as a non-covalently linked homodimer [17,29], though higher oligomers have also been reported [30]. Oxidation of the Trp114 residue of TrxR1 can also lead to di-Trp114 covalently linked subunits aggregating into tetramers, with this decreasing enzyme activity [31].

Here, we investigated direct covalent interactions of BQ with TrxR1, related enzyme activity changes, and the role of NADPH-mediated reduction of TrxR1 in these interactions. Furthermore, we identified the occurrence of quinone-induced and disulfide-linked TrxR1 dimers, and determined that BQ exposure inhibits TrxR activity in both intact J774A.1 cells and corresponding cell lysates. Overall, this study provides novel data on covalent interactions of BQ with TrxR1, with potential biological importance with regards to the mechanisms underlying BQ toxicity.

2. Materials and methods
2.1. Reagents

Thioredoxin reductase 1 (TrxR1) from rat and human point-mutated TrxR1 species (in which Sec is mutated to Cys or Ser) were prepared according to previously reported methods [17,32,33]. Reduced glutathione (GSH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), biotin polyethyleneoxide iodoacetamide (BPI), 5-hydroxy-1,4-naphthoquinone (juglone), 9,10-phenanthrenquinone (9,10-PQ), aurafin and p-benzoquinone (BQ) were purchased from Sigma (St. Louis, MO, USA). BQ, juglone and 9,10-PQ was prepared freshly for every experiment by dissolving these in dimethyl sulfoxide (DMSO) to give stock solutions, with these then diluted into the relevant reaction systems. All other reagents were of highest possible quality. High purity (MQ) water was obtained from a Milli-Q System (Millipore, Bedford, MA).

2.2. Enzyme activity measurements

TrxR1 activity was determined using two different model substrates, DTNB and juglone [21,34]. Activity measurements were performed with DTNB (1 mM) or juglone (400 μM) in 50 mM Tris-HCl (pH 7.6), 2 mM EDTA, and 200 μM NADPH. The reduction of DTNB to form TNB− was quantified through absorbance decreases at 412 nm. Alternatively, TrxR1 activity was quantified by following the oxidation of NADPH to NADP+ as a decrease in absorbance at 340 nm, with this monitored at 10 s intervals over a 5 min period. As there are other DTNB-reactive groups in cell lysates, the TrxR inhibitor aurafin (500 nM) was added into the reaction system as a background control, with the absorbance changes in the presence of this inhibitor used to correct the experimental values from the cell lysates when using DTNB as the substrate.

2.3. Inactivation of TrxR1 by BQ in purified protein

BQ (4 and 100 μM) was incubated with TrxR1 (0.8 μM) for 5 min in 50 mM Tris-HCl (pH 7.6) containing 2 mM EDTA at 22 °C. Samples were then desalted using Zea column (Thermo, Waltham, MA, USA) and remaining enzyme activity was measured using the method described above.

2.4. Inactivation of TrxR by BQ in J774A.1 cells

Murine macrophage-like J774A.1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) containing 10% (v/v) fetal bovine serum (Invitrogen), and 2 mM L-glutamine (Thermo, Waltham, MA), at 37 °C in a humidified atmosphere of 5% CO₂.

For cell lysis preparation, cells were seeded in cell culture flask at a density of 1 × 10⁶ cell mL⁻¹. After growth to confluence, the cells were collected by centrifugation, washed twice with warm phosphate-buffered saline (PBS), and lysed in phosphate-buffered solution (50 mM, pH 7.4) containing 10 mM EDTA on ice. The lysates were then centrifuged at 14000 g for 5 min to remove debris, and the supernatants taken for experiments. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Sigma, St. Louis, MO, USA). The cell lysates were incubated with BQ (0–80 μM) for 5 min, and residual TrxR activity determined using the method described above.

For intact cell experiments, J774A.1 cells in culture were incubated with BQ (0–80 μM) for 5 min. Before harvesting, the cells were washed twice with PBS, and the cells then lysed in phosphate-buffered solution (50 mM, pH 7.4) containing 10 mM EDTA on ice. The lysates were centrifuged at 14000 g for 5 min, and the supernatants taken to determine TrxR activity. The total protein concentration was quantified using the BCA assay.
2.5. Kinetic parameter determination

TrxR1 (0.8 μM) was incubated with BQ (5–100 μM) in the presence of NADPH (200 μM) in 50 mM Tris-HCl (pH 7.6) containing 2 mM EDTA at 22 °C. Activity was monitored at 340 nm in 10 s intervals over a 5 min period, as described above, with the concentrations of NADPH consumed determined using a standard curve over the range 100–200 μM; $k_{\text{cat}}$ and $K_m$ values were calculated by Michaelis-Menten fitting using OriginPro 2016 (OriginLab, Northampton, MA, USA).

2.6. Assessment of dimer formation associated with BQ treatment

TrxR1 (0.8 μM) was incubated with BQ (0.8–100 μM) for 5 min in the presence or absence of NADPH (200 μM) at 22 °C, and 18 μL samples were removed, with these then diluted with loading buffer with or without DTT (50 mM). Samples were loaded on to 15-well SDS-PAGE gels (4–12% Bis-Tris Gel, Invitrogen) and run using NuPAGE MES SDS running buffer (Invitrogen) at 200 V for 25 min. The proteins were subsequently stained with Coomassie blue and scanned using a Gel Documentation system (Bio-Rad, Hercules, CA, USA).

2.7. Visualisation of BQ-modifications using BPI labelling

TrxR1 (0.8 μM) was incubated with BQ (0.8–100 μM) for 5 min at 22 °C. After incubation, 1 μL of sample was taken and mixed with 19 μL of BPI (100 μM) at pH 6.5 or 8.5, followed by further incubation at 37 °C in the dark for 30 min to alkylate remaining free -SH and -SeH groups in the enzyme [27]. Samples (18 μL) were then diluted with loading buffer, and subjected to SDS-PAGE (15 well, 4–12% Bis-Tris Gels, Invitrogen) using NuPAGE MES SDS running buffer (Invitrogen) at 200 V for 25 min. The separated proteins were transferred to PVDF membranes using an iBlot 2 dry blotting system (Thermo, Waltham, MA) at 200 V for 7 min. The BPI-labelled protein was then detected using horseradish peroxidase-conjugated streptavidin and enhanced chemiluminescence detection.

2.8. Statistics

Results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using SPSS 25 (IBM, Armonk, NY, USA). Data were presented as mean ± standard deviations (SD) from at least three independent experiments unless otherwise noted. Significance was set at $p < 0.05$.

3. Results

3.1. BQ-induced inhibition of TrxR1 enzyme activity

BQ was previously found to be a substrate of TrxR [20], and here we determined the enzymatic parameters for this reaction, with $k_{\text{cat}}$ determined as 7.44 ± 0.14 s$^{-1}$ and $k_{\text{cat}}/K_m$ 0.91 ± 0.22 μM$^{-1}$s$^{-1}$ (Supplementary Fig. 1), in good agreement with the earlier determinations of $k_{\text{cat}}$ 4.8 ± 0.5 s$^{-1}$ and $k_{\text{cat}}/K_m$ 0.39 ± 0.1 μM$^{-1}$s$^{-1}$ [20], when also considering the higher specific activity of the recombinant TrxR1 used in this study [33].

As shown in Fig. 1, incubation of BQ with TrxR1 induced a rapid and concentration-dependent irreversible enzyme activity loss in the presence of NADPH, as noted earlier for DTNB reduction [20], and here with both DTNB and juglone as substrates. Reduction of DTNB requires the intact, and fully reduced, Cys and Sec residues of the C-terminal tetrapeptide, while juglone reduction can proceed independently of the Sec residue in TrxR1 [21]. Therefore, the decreased reduction of both substrates, as determined here, is consistent with the targeting, by BQ, of residues in both the C-terminal and N-terminal catalytic centres of TrxR1. Moreover, in absence of NADPH, incubation with BQ also induced enzyme activity loss, especially using juglone as the substrate, though the extent of this inhibition was less pronounced.

3.2. BQ-induced modification of Cys and Sec residues in TrxR1

Previous reports have demonstrated that BPI can alkylate specific residues in proteins, with the selectively of this process determined by the pH of the reaction medium during the derivatization reaction [27,35]. At high pH values (e.g., pH 8.5), both free -SH and -SeH groups are alkylated, while at lower pH values (e.g., pH 6.5) the -SeH group is predominantly alkylated due to the lower pK$_a$ value of Sec compared to Cys.

Here, BQ-induced TrxR1 modifications were analysed by BPI labelling of free -SH and -SeH residues at these different pH values. As shown in Fig. 2A, treatment of TrxR1 with BQ for 5 min in the absence of NADPH lowered the extent of BPI labelling of the enzyme to a similar extent at both pH 6.5 and pH 8.5. In the presence of NADPH, but absence of BQ, BPI labelling of TrxR1 was clearly increased, reflecting NADPH-dependent exposure of the catalytic Cys and Sec residues, compatible with the notion that these residues are shielded by disulfide and selenenylsulfide motifs in the absence of NADPH [17]. This labelling was efficiently blocked in the presence of 100 μM BQ, suggesting that BQ competes with BPI for reaction with the catalytic Cys and Sec residues of the NADPH-reduced enzyme (Fig. 2B). These results collectively suggest efficient modification, by 100 μM BQ, of non-catalytic Cys residues in TrxR1 in the absence of NADPH, and both the catalytic Cys and Sec residues in the presence of NADPH.

3.3. BQ-induced covalent TrxR1 crosslinks

Incubation of BQ (0–100 μM) with TrxR1 (0.8 μM) also induced covalent crosslinks between TrxR1 subunits in a concentration-dependent manner. In the absence of NADPH, treatment with 100 μM BQ triggered a dramatic increase in multimeric TrxR1 bands (Fig. 3A). DTT could reduce the majority of these crosslinks, consistent with the presence of disulfide-bonded multimers (Fig. 3B). This implies that some of the BQ-dependent blockage of BPI labelling (Fig. 2) is due to formation of these species. In the presence of NADPH, no obvious disulfide-linked dimers were observed.

The BQ-induced TrxR1 crosslink formation also occurred in a time-dependent manner. Whilst disulfide bond formation occurred immediately after the reactions were initiated, non-reducible dimers were detected with reaction times greater than 5 min (Fig. 3C). This crosslink formation was independent of the presence of the catalytic Sec residue, as mutation of Sec to either Cys or Ser did not affect this BQ-induced TrxR1 dimerization (Fig. 3D and E).

3.4. NADPH-mediated protection from BQ-induced TrxR1 crosslinks

The role of NADPH in modulating the formation of BQ-induced TrxR1 subunit crosslinks was investigated by either co-incubation with NADPH, or addition of NADPH after TrxR1 pre-exposure to BQ. As shown in Fig. 4A, co-incubation with NADPH could concentration-dependently protect TrxR1 against dimerization, with NADPH concentration greater than two-fold over the BQ concentration eliminating disulfide-bonded dimer formation.

Adding NADPH after pre-exposure of TrxR1 to BQ for 5 min showed similar protective effects, as 120 μM or higher NADPH concentrations could reverse the TrxR1 crosslink formation. This phenomenon was examined further by use of columns to remove excess BQ after pre-treatment of TrxR1 with BQ, with different concentrations of NADPH then added to the samples. As shown in Fig. 4C, after desalting, concentration of NADPH as low as 20 μM NADPH could reverse crosslink formation, with both an increase in the monomer, and decreases in the oligomer bands were detected. These results suggest that BQ modification do not hinder the NADPH binding to the enzyme, and that NADPH or NADPH-reduced TrxR1 species can reduce BQ-induced inter-
molecular disulfide bonds between TrxR1 subunits.

3.5. Effect of GSH on BQ-induced TrxR1 inactivation and crosslinks

GSH is the most abundant low-molecular-mass thiol-containing molecule in cells, and consequently the potential effects of GSH on BQ-TrxR1 interactions were examined. GSH was either co-incubated with BQ and TrxR1, or added after reaction of BQ with TrxR1 for 5 min. The samples were then desalted, and residual enzyme activity determined using both DTNB and juglone as substrates. The results obtained indicate that co-incubation with GSH protects TrxR1 from inactivation by BQ, both in the presence and absence of NADPH. Adding GSH after pre-exposure of NADPH-reduced TrxR1 to BQ, did not however, have any protective effect with regards to the loss of enzyme activity. These data are consistent with a protective effect of GSH for both the N-terminal and C-terminal catalytic centres (Fig. 5A and B), and also indicate that GSH affords protection of TrxR1 activity mainly by competitive reaction with BQ.

To confirm this competitive effect of GSH, co-incubation of GSH at different concentrations (50, 100, 400 μM) with BQ (100 μM) and TrxR1 (0.8 μM) was performed, followed by analysis with SDS-PAGE as described above. The results show that co-incubation with GSH concentration-dependently decreases BQ-induced crosslinking of TrxR1, while adding GSH after the reaction initiated does not reverse the crosslinks (Fig. 5C and D). These results are again consistent with GSH protecting TrxR1 predominantly by directly scavenging BQ.

3.6. Inactivation of TrxR in J774A.1 cells by BQ

The data presented above demonstrate that BQ can trigger activity loss in purified TrxR1, whilst also being a substrate for the enzyme. The potential relevance of these reactions in more complex systems was investigated in J774A.1 cells, using both intact cells and cell lysates. Consistent with the isolated protein studies, BQ induced a concentration-dependent activity loss of TrxR after only 5 min incubation with lysates prepared from J774A.1 cells, with 40 μM BQ inducing over 90% loss of activity (Fig. 6A).

With intact cells, BQ also induced a concentration-dependent activity loss, but the extent was, as might be expected, different to that observed with the isolated protein and cell lysates. Inactivation was observed with 40 μM and 80 μM BQ, with 40 μM BQ giving rise to a 50% loss of activity in the intact cells after 5 min incubation (Fig. 6B).

4. Discussion

The data obtained in this study indicate that TrxR1 is readily targeted by BQ in the presence of NADPH, resulting in a rapid, concentration-dependent, loss of enzyme activity (Figs. 1 and 6). Identical concentrations of BQ induced a lower extent of loss of activity in intact J774A.1 cells, when compared to cell lysates, suggesting the cellular uptake, metabolism and efflux processes may be critical in determining the extent of BQ-TrxR interaction in cells (including GSH scavenging, as here also found in vitro). The high percentage loss of cellular TrxR activity (~90%) induced by BQ may indicate targeting of both TrxR1 and
TrxR2 (the mitochondrial isoform), though no definitive information has been obtained on this point. It should, however, be noted that because TrxR1 is responsible for the majority of the TrxR activity in cells (as it is present at much higher levels and has a higher catalytic efficiency [24]), it is possible that BQ only targets the TrxR1 isoform. Further studies are required to clarify this point. The results obtained demonstrate that the observed BQ-induced inactivation is likely to be due to direct modification of both Sec and Cys residues in isolated TrxR1 (Fig. 7), as BPI labelling of these residues was decreased on incubation with BQ at both pH 6.5 and 8.5. Similar inhibition effects on TrxR have been observed with other electrophiles, including 4-hydroxy-2-nonenal [35] and curcumin [27], two different α,β-unsaturated ketone compounds that inhibit both purified TrxR, and this enzyme in HeLa cells.

As reported previously, BQ is a substrate of TrxR1, with the enzymatic parameters determined in this study ($k_{cat} 7.44 \pm 0.14 \text{ s}^{-1}, k_{cat}/K_{m} 0.91 \pm 0.22 \text{M}^{-1} \text{s}^{-1}$) similar to those reported earlier ($k_{cat} 4.8 \pm 0.5 \text{ s}^{-1}, k_{cat}/K_{m} 0.39 \pm 0.1 \text{M}^{-1} \text{s}^{-1}$) [20]. However, we have previously reported that BQ can react rapidly with Cys residues on proteins, with the rate constant, $k$, for addition of BQ to the Cys34 residue in BSA being $\sim 10^5 \text{M}^{-1} \text{s}^{-1}$ [10]. On the basis of previous data indicating that the rate constants for reaction of Sec, when compared to Cys, with electrophiles is typically 2-3 orders of magnitude higher at pH 7.0 [36], and that a similar rate enhancement is seen in oxidant reactions (see, e.g., Refs. [37–39]), it is expected that the rate constant for adduction of BQ with TrxR1 would also be very fast and significantly greater than $10^5 \text{M}^{-1} \text{s}^{-1}$, though this needs to be experimentally verified. Therefore, in the presence of NADPH, the reactions of BQ with TrxR1 are complex, with both redox cycling and covalent adduction reactions occurring, and both processes may contribute to the loss of enzyme activity.

In the presence of NADPH, BQ did not induce a high level of crosslinking of TrxR1, with only low levels of (uncharacterized) non-reducible dimer bands observed. These linkages may be related to the BQ-induced non-reducible crosslinks observed with other proteins (e.g., GAPDH and CK), which have been proposed to contain quinone-mediated linkages arising from two successive Michael addition reactions [10]. Yu et al. [40] have demonstrated the formation of similar quinone bridge species as a result of BQ-induced modification of annexin V. A previous study has also reported that TrxR1 can form tetramers involving di-Trp (Trp114-Trp114) linkages when subjected to oxidation [31]. A similar process may also occur here as TrxR1-induced redox cycling of BQ is likely to generate $\mathrm{O}_2^-$ and downstream species. Further characterization of these dimers is therefore required to determine whether these contain quinone-bridge structures, di-Trp linkages, or both.

In contrast to our expectations, BQ could also induce TrxR1 enzyme activity loss in the absence of NADPH, although the extent was less marked. BPI labelling experiments indicate that BQ can also modify the non-catalytic Cys residues in the protein (Fig. 7), and these alterations

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**Fig. 3.** BQ-induced TrxR1 crosslinking formation. TrxR1 (0.8 μM) was incubated with BQ (0–100 μM) for 5 min with or without NADPH (200 μM) in Tris-EDTA buffer (pH 7.6) at 22 °C. Samples were subjected to SDS-PAGE under (A) non-reducing and (B) reducing conditions, followed by Coomassie blue staining. (C) TrxR1 (0.8 μM) was incubated with BQ (0–100 μM), and aliquots were taken immediately and after 5, 10, 20, 40, and 60 min after the initiation of the reaction, and subjected to SDS-PAGE under non-reducing and reducing conditions followed by Coomassie blue staining. (D, E) Native TrxR1 (U, 0.8 μM) and mutated TrxR1 (Sec to Cys, C; Sec to Ser, S, 0.8 μM) was incubated with BQ (100 μM) in Tris-EDTA buffer (pH 7.6) at 22 °C for 5 min with or without NADPH. Samples were subjected to SDS-PAGE under (D) non-reducing and (E) reducing conditions followed by Coomassie blue staining.
may be responsible for the detection of high levels of protein crosslinks under these conditions. The majority of these linkages appear to be due to the rapid formation of disulfide bonds, with oligomer formation detected immediately after mixing of BQ with TrxR1. In contrast, non-reducible crosslink formation was slow and could only be detected at longer incubation times, with this increasing in a time-dependent manner (Fig. 3C).

In the presence of NADPH, a significant loss of the parent TrxR1 monomer band was identified on exposure to 100 μM BQ (Fig. 3A). This loss could be reversed by DTT, consistent with the presence of disulfide bonds (Fig. 3B). This may occur via direct thiol-disulfide exchange, a known pathway to disulfide-bonded crosslinks [41,42], or via a quinone-triggered reaction [43,44]. The latter may be the pathway operating here, with the presence of NADPH switching disulfide bond formation from intermolecular to intramolecular. BQ has been reported previously to modify Cys residues on proteins in erythrocyte ghost membranes, with this inducing the formation of high molecular mass aggregates, and oxidation of epigallocatechin-3-gallate has been shown to form quinone intermediates that can induce aggregation of membrane proteins [45].

Neither the loss of the parent protein band, nor this aggregation, was reversed by mutation of the C-terminal Sec (to Cys or Ser; Fig. 3D and E), indicating that these crosslinks are not related to reaction of BQ with the C-terminal Sec residue, or the native enzymatic activity of TrxR1. The observation that the disulfide-bonded crosslinks can be reduced by NADPH (Fig. 4), also implies that BQ addition does not damage the NADPH binding site, or modulates the intra-protein electron transfer reactions that occur during the catalytic cycle of TrxR1.

As quinones can serve as both redox-cycling substrates and electrophiles with TrxR1, the role of these two pathways in crosslink formation was examined using additional substrates with different specificities. In the absence of NADPH, the alternative quinone, juglone (which reacts with the N-terminal Cys residues in addition to the C-terminal Sec-containing active site) induced limited crosslink formation, which could be repaired by DTT consistent with disulfide generation. In contrast, 9,10-PQ did not induce crosslink formation (Supplementary Fig. 2). These data are consistent with the electrophilic properties of quinones being critical to TrxR1 crosslink formation. Furthermore, aurano, a well-established TrxR1 inhibitor that binds irreversibly with Cys and Sec residues [46] did not induce crosslink formation, suggesting that this is a specific property of electrophilic quinones (Supplementary Fig. 2).

GSH has been reported to protect GAPDH against BQ-induced loss of activity by acting as both a competitive target for BQ (with this resulting in the formation of GSH-BQ adducts), or by acting as a secondary nucleophile that can react with the initial quinoprotein adduct via a S-transarylation pathway to repair the protein with consequent formation of a GSH-BQ adduct [10,47]. In the light of this data, the protective effect of GSH against loss of TrxR1 activity was also investigated. Co-incubation with GSH could decrease the extent of BQ-induced TrxR1 activity loss, while adding GSH after pre-treatment of the TrxR1 with BQ had no protective effect (Fig. 5A and B). Co-incubation with GSH, but not addition after pre-exposure, also decreased BQ-induced TrxR1 crosslink formation (Fig. 5). These data suggest that BQ-induced TrxR1 inactivation is irreversible, and GSH limits BQ-induced damage primarily by acting as a competitive target for BQ.

The extent of BQ-induced damage in vivo is therefore likely to be dependent on the relative concentrations of GSH versus TrxR (which will be heavily weighted in favour of GSH), and also the relative rate constants for reaction of BQ with these two species. We have reported kinetic data for the GSH reaction \( k_2 \approx 6 \times 10^5 \text{M}^{-1} \text{s}^{-1} \) at 10°C and pH 7.4 [10], but the corresponding data for TrxR1 are not available. However as indicated above, it would be expected that the rate constant for reaction with the protein would have a higher value due to the greater nucleophilicity of the selenite anion (\( \text{RSe}^- \)) compared to the (neutral) thiol of GSH. Whether this greater reactivity outweighs the concentration difference remains to be established, but it is clear that TrxR1 can still be inhibited in a cellular context. Hence, we propose
that GSH is likely to be the major target, but that due to the high reactivity of TrxR1 (and possibly TrxR2), enzyme inactivation will still occur. The non-reversibility of BQ adduction and hence loss of enzyme activity may have a cumulative effect in cells, especially if exposure to BQ (or other quinones) is at a significant level and chronic (e.g. via ongoing environmental exposure, or as a result of long-term drug treatment).

TrxR is critical to the cellular Trx antioxidant system, and together

![Graph A](image1)

![Graph B](image2)

**Fig. 5.** Effect of GSH on BQ-induced TrxR1 activity loss and crosslinking formation. (A, B) TrxR1 (0.8 μM) was incubated with BQ (100 μM) with or without NADPH (200 μM) in Tris-EDTA buffer (pH 7.6) at 22 °C. GSH (400 μM) was added at the same time (CG) or 5 min after the initiation and incubated for a further 5 min (AG). Samples were desalted by column chromatography followed by assessment of activity using (A) DTNB, or (B) juglone. (C, D) TrxR1 (0.8 μM) was incubated with BQ (100 μM) without (C) or with (D) NADPH (200 μM) for 5 min in Tris-EDTA buffer (pH 7.6) at 22 °C. GSH (0–400 μM) was added at the same time or 5 min after the initiation of the reaction, and incubated for a further 5 min. Samples were subjected to SDS-PAGE under non-reducing conditions followed by Coomassie blue staining. #p < 0.05, ##p < 0.01 vs. control group, *p < 0.05, **p < 0.01 vs. BQ treatment group.

![Graph C](image3)

![Graph D](image4)

**Fig. 6.** Inhibition of TrxR by BQ in J774A.1 cell lysate (A) and intact cells (B). J774A.1 cell lysates from 1 × 10⁶ cells, or intact cells (1 × 10⁶) were incubated with the indicated concentrations of BQ for 5 min. Residual TrxR activity was then analysed using DTNB as the substrate. For further details see Materials and methods. *p < 0.05, **p < 0.01 vs. control (CON) samples with no added BQ.
Abbreviation: nCys: non-catalytic Cys residues.

including cell death pathways [48], cancer [49], inflammation [50], and neurodegenerative disease [51]. Moreover, inhibition of this system, and especially TrxR activity, has been strongly associated with the cytotoxic effects of multiple compounds including 4-hydroxy-2-nonenal [35], flavonoids [28], and arsenic trioxide [52]. Trx, the substrate of TrxR, can also serve as the negative regulator to suppress the activation of apoptosis signal-regulating kinase (ASK1) [53]. Therefore, inhibition of TrxR may lead to the accumulation of oxidised Trx, and result in the activation of ASK1 and cell apoptosis. Previous research has also demonstrated thatildeoquinone can inactivate TrxR in Mia PaCa-2 cells, which results in increased levels of oxidised Trx, activation of the ASK1-p38/c-Jun NH2-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) signalling pathway and cell apoptosis [54]. The inhibition of TrxR activity by BQ in the J774A.1 cells studied here suggests that the activation of the ASK1-MAPK signalling pathway and cell apoptosis may also be triggered in these cells. Preliminary studies suggest that this is correct (Shu, N. et al., unpublished data). This study therefore provides a new insight into the potential biological targets of BQ, and provides a potential new mechanism for BQ-induced cell apoptosis.

As summarized in Fig. 7, this study demonstrates that BQ can irreversibly modify the Sec residue of TrxR1 resulting in a loss of enzyme activity in the presence of NADPH. GSH can protect against this activity loss when present concurrently with TrxR1, but not after damage has already been induced by BQ. This occurs primarily through competitive reaction with BQ. In the absence of NADPH, BQ can modify the non-catalytic Cys residues of TrxR1, leading to crosslink formation, with these modifications being reversible and also modulated by GSH. These results provide new insights into cytotoxicity mechanisms of BQ.

Declaration of competing interest

The authors declare no conflicts of interest with regard to the data presented.

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