Interactions of Nitroaromatic Compounds with the Mammalian Selenoprotein Thioredoxin Reductase and the Relation to Induction of Apoptosis in Human Cancer Cells*

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Here we described novel interactions of the mammalian selenoprotein thioredoxin reductase (TrxR) with nitroaromatic environmental pollutants and drugs. We found that TrxR could catalyze nitroreductase reactions with either one- or two-electron reduction, using its selenocysteine-containing active site and another redox active center, presumably the FAD. Tetryl and p-dinitrobenzene were the most efficient nitroaromatic substrates with a $k_{\text{cat}}$ of 1.8 and 2.8 s$^{-1}$, respectively, at pH 7.0 and 25 °C using 50 μM NADPH. As a nitroreductase, TrxR cycled between four- and two-electron-reduced states. The one-electron reactions led to superoxide formation as detected by cytochrome c reduction and, interestingly, reductive N-denitration of tetryl or 2,4-dinitrophenyl-N-methylnitramine, resulting in the release of nitrite. Most nitroaromatics were uncompetitive and noncompetitive inhibitors with regard to NADPH and the disulfide substrate 5,5'-dithiobis(2-nitrobenzoic acid), respectively. Tetryl and 4,6-dinitrobenzofuroxan were, however, competitive inhibitors with respect to 5,5'-dithiobis(2-nitrobenzoic acid) and were clearly substrates for the selenolthiol motif of the enzyme. Furthermore, tetryl and 4,6-dinitrobenzofuroxan efficiently inactivated TrxR, likely by alkylation of the selenolthiol motif as in the inhibition of TrxR by 1-chloro-2,4-dinitrobenzene/dinitrochlorobenzene (DNCB) or juglone. The latter compounds were the most efficient inhibitors of TrxR activity in a cellular context. DNCB, juglone, and tetryl were highly cytotoxic and induced caspase-3/7 activation in HeLa cells. Furthermore, DNCB and juglone were potent inducers of apoptosis also in Bcl2 overexpressing HeLa cells or in A549 cells. Based on these findings, we suggested that targeting of intracellular TrxR by alkylating nitroaromatic or quinone compounds may contribute to the induction of apoptosis in exposed human cancer cells.

Thioredoxin reductase (TrxR, EC 1.8.1.9) catalyzes NADPH-dependent reduction of the redox active disulfide of thioredoxin (Trx), which has a major antioxidant role and regulates many cellular functions by redox control (1, 2). Both thioredoxin and thioredoxin reductase are expressed as cytosolic (Trx1 and TrxR1) as well as mitochondrial (Trx2 and TrxR2) isoenzymes, and deletion of any of these four genes is embryonically lethal in mice (3–6). Mammalian thioredoxin reductases are homodimeric proteins of 54–58-kDa subunits, containing FAD, a redox active disulfide and a redox active selenenylsulfide (7–10). The three-dimensional structure of mammalian TrxR is similar to that of glutathione reductase, including conserved FAD- and NAPD(H)-binding domains, but TrxR has a 16-residue C-terminal extension carrying a catalytic Cys-497/Sec-498 couple that is easily accessible for substrates of the enzyme while shielding its glutathione reductase-like active site disulfide (11, 12). In the catalytic cycle, NADPH first reduces FAD, which passes redox equivalents to the redox active disulfide, located within a conserved -CVNVGC- sequence, with formation of a dithiol. Subsequently, this dithiol reduces the selenenylsulfide located at the C-terminal end of the other subunit in the dimeric enzyme (7, 9, 11, 12). This formed selenolthiol motif reduces the active site disulfide of ‘Trx, the disulfide of the synthetic model substrate, 5,5'-dithiobis(2-nitrobenzoate) (DTNB), or any of the many other substrates of the enzyme, such as lipoic acid, selenite, and several quinone compounds; the wide substrate specificity of mammalian TrxR is attributed to the easy access and reductive capacity of the C-terminal selenolthiol (1, 2, 7, 9, 11, 12). Because of the presence of three two-electron accepting redox centers, TrxR may exist in two-, four-, and six-electron-reduced states with distinct spectral properties (7, 9). It is believed that during Trx reduction, mammalian TrxR cycles between two- (EH2) and four-electron (EH4)-reduced states, with two or four electrons shared mainly between catalytic disulfide and the selenenylsulfide motif (9). This mechanism has been demonstrated for the orthologous Dro sophila melanogaster TrxR where, however, a dithiol motif substitutes for the role of the selenolthiol in the mammalian enzyme (13, 14). The same principal mechanism is seen also during quinone reduction as catalyzed by mammalian TrxR (15).

Many factors make mammalian TrxR an important target of drugs and xenobiotics, as reviewed recently (16–18). One is the wide variety of the antioxidant actions of the enzyme, because in addition to thioredoxin, TrxR reduces hydroperoxides and generates some antioxidants, e.g. lipoate, various selenium compounds, and ubiquinone (2, 18, 19); all these actions would be perturbed if TrxR should become inhibited in cells. In addition, increased Trx and TrxR levels in some tumor cell lines or in synovial fluid and tissue of patients suffering from rheumatoid arthritis (16–18, 20) may argue for the suppression of TrxR functions as a possible therapeutic approach. Moreover, selenium-compromised forms of TrxR have the capacity to induce apoptosis in cancer cell lines by a direct gain of function (21), the detailed mechanisms for which are currently being studied in our laboratory. Indeed, TrxR interacts with a variety of drugs in therapeutic use, and this may have con-
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![Figure 1. Structural formulae of selected nitroaromatic compounds studied in this work.](image)

considerable importance for the therapeutic effects. In its reduced state, TrxR is inactivated by 1,3-bis-(2-chloroethyl)-1-nitrosourea (22, 23), several anticancer platinum compounds (22, 24, 25), antiarthritic gold compounds (26), immunostimulatory dinitrohalobenzenes (2), alkylating anticancer agents such as busulfan, melphalan, and chlorambucil (22) and partially substituted quinones (15). The modification of SeCys-498 and Cys-497 by the nitroaromatic compound 1-chloro-2,4-dinitrobenzene (DNCB/CDNB) inactivates TrxR and enhances its superoxide-producing NADPH-oxidase activity, thus conferring pro-oxidant properties to the enzyme (2). Certain quinones, reduced by TrxR to easily auto-oxidizing hydroquinones, may also inactivate and at the same time confer pro-oxidant functions to TrxR (15).

In this context, we believed that a possible interaction of TrxR with different classes of nitroaromatic compounds that represent a widespread group of drugs and environmental pollutants should be of significant interest. The cytotoxic, antitumor, and antiparasitic activities of nitroaromatics are generally believed to stem mainly from the redox cycling of their free radicals formed after single-electron reduction by different flavoenzymes or from the alkylation of DNA and/ or other cellular nucleophiles by the products of their two (or four)-electron reduction, the arylhydroxylamines (27–30). It has been shown previously that enzymes analogous to TrxR, such as the pyridine nucleotide-disulfide reductases trypanothione reductase and glutathione reductase, may reduce nitroaromatics and produce redox cycling radicals, although at quite low rates (31–38). Besides, nitroaromatic compounds may reversibly bind to these enzymes inhibiting the reduction of their natural disulfide substrates, glutathione and trypanothione, thus significantly impeding their natural antioxidant functions (31–35). The possible interaction of mammalian TrxR with nitroaromatics, except for the targeting of the Sec residue by dinitrohalobenzenes (2), has not been studied to date.

Here we examined the interactions of recombinant rat TrxR with a number of structurally diverse nitroaromatics, including the antitumor agents chinifur and nitracrine (36) and the explosives 2,4,6-trinitrotoluene (TNT), tetryl, and 4,6-dinitrobenzofuroxan (DNBF) (Fig. 1). We found that TrxR has a significant nitroreductase activity with some nitroaromatic compounds, that it may catalyze reductive N-denitration reactions, and that the enzyme concomitantly can be inhibited for its normal disulfide reductase activity. Considering the important cellular functions of TrxR and the direct apoptosis induction that may be provoked by selenium-compromised forms of the enzyme (21), we also studied the cytotoxic actions of some of the nitroaromatics and compared it with that of the most potent TrxR-interacting quinone, juglone (15). We found that tetryl, DNCB, and juglone may induce a pronounced cytotoxicity. Most interestingly, studying caspase-mediated apoptosis in HeLa cells, HeLa cells overexpressing Bcl2, and A549 cells, we found that tetryl had a similar profile in apoptotic induction as the classical apoptosis-inducer staurosporine, whereas DNCB or juglone provoked an apoptosis with a completely different profile that we propose may be intimately linked to their interactions with TrxR.

**MATERIALS AND METHODS**

**Enzymes and Reagents**—Recombinant mammalian TrxR was prepared as described (39). The enzyme concentration was determined according to the absorbance of FAD, $e_{563} = 11.3 \text{ mm}^{-1} \text{ cm}^{-1}$, and the specific activity in the standard DTNB assay (39) was about 20 units/mg. Double mutant (C61S/C72S) human Trx was kindly provided by Dr. Arne Holmgren (Karolinska Institutet, Stockholm, Sweden), and the recombinant Enterobacter cloacae NAD(P)H:nitroreductase, prepared as described (40), was a generous gift of Dr. Ronald L. Koder (University of Kentucky, Lexington, KY). DEVD-AMC was purchased from the Peptide Institute, Osaka, Japan. NADPH, cytochrome c, superoxide dismutase, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and gold thioglucose were obtained from Sigma and were used without additional purification. Chinifur (2′-(5′-nitrofuro-2′-yl)ethene-4-N,N′-diethyliamino)-1-methylbut-1-ylaminocarboxyl-4-quinoline), TNT, tetryl (2,4,6-trinitrophenyl-N-methylnitroamine), DNBF (4,6-dinitrobenzofuroxan) (Fig. 1), 2,4-dinitrophenyl-N-methylnitroamine, and 4-nitrophenyl-N-methylnitroamine were synthesized according to established procedures (33, 35, 41, 42). Purity was checked by the melting point, elemental analysis, IR, and NMR spectra. Nitracrine (9-(3-dimethylaminopropylamino)-1-nitroaracine) (Fig. 1) was a generous gift of Professor Igor Degterev (Institute of Chemical Physics, Moscow, Russia). Other nitroaromatic compounds were obtained from Sigma and used without additional purification.

**Experimental Procedures**—All in vitro experiments were carried out in 0.1 M potassium phosphate buffer solution, pH 7.0, containing 1 mM EDTA, at 25 °C. Rapid reaction studies were performed under aerobic conditions using a DX.17MV stopped-flow spectrophotometer (Applied Photophysics). Steady-state reaction rates were monitored using a Hitachi-557 spectrophotometer. The rate of reduction of DTNB (0.20–2.00 mM) by TrxR in the presence 10–100 μM NADPH was monitored following an increase in absorbance at 412 nm ($\Delta e_{412} = 13.6 \text{ mm}^{-1} \text{ cm}^{-1}$) for the thionitrobenzoate anion) and considering that 1 mol NADPH results in the formation of 2 mol thionitrobenzoate. Reduction of nitroaromatics reflecting the nitroreductase reaction of TrxR was monitored following the rate of NADPH (50 μM) oxidation ($\Delta e_{340} = 62 \text{ mm}^{-1} \text{ cm}^{-1}$) in the presence of nitroaromatic substrates. In certain cases, the rates were corrected for the changes in absorbance of nitroaromatics at 340 nm, calculated according to their reduction data in the presence of an NADPH-regeneration system, i.e. 20 μM NADPH, 10 mM glucose 6-phosphate, and 10 units/mg glucose-6-phosphate dehydrogenase. For detection of nitroaromatic-mediated reduction of cytochrome c, 50 μM cytochrome c was added into the reaction mixture, and its reduction was monitored at 550 nm ($\Delta e_{550} = 20 \text{ mm}^{-1} \text{ cm}^{-1}$), with the direct reduction of cytochrome c by TrxR subtracted. Kinetic parameters, the catalytic constant ($k_{cat}$), and the bimolecular rate constant ($k_{cat}/K_{m}$) correspond to the reciprocal intercepts and slopes of the Lineweaver-Burk plots, $[E]v$ versus $1/[S]$, where $v$ is the initial reaction rate, and $[E]$ and $[S]$ are the enzyme and substrate concentrations, respectively. $k_{cat}$ corresponds to molecules of NADPH oxidized by enzyme active site/s. The concentrations of nitrite formed were determined spectrophotometrically at 540 nm, monitoring the formation of azo dye in the presence of sulfanilamide, naphthylethylene diamine.
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Reduction of Nitroaromatics by TrxR—Using DTNB as electron acceptor, the kinetic parameters of the current preparation of TrxR were those determined in our previous work (15), i.e. \( k_{\text{cat}} = 20.9 \pm 0.8 \, \text{s}^{-1} \), with \( k_{\text{cat}}/K_{\text{m}} = 3.50 \pm 0.2 \times 10^{-4} \, \text{m}^{-1} \text{s}^{-1} \) for NADPH and 1.52 \( \pm 0.2 \times 10^{-4} \, \text{m}^{-1} \text{s}^{-1} \) for DTNB. As summarized in Table 1, we found that nitroaromatic compounds could indeed be reduced by TrxR, thus reflecting its capacity as a nitroreductase. Among the compounds analyzed, tetryl and p-dinitrobenzene showed the highest turnover, with a \( k_{\text{cat}} \) at about 15% that seen in DTNB reduction. The reaction rates with nitroaromatics did not change by varying the NADPH concentration between 5.0 and 50 \( \mu \text{M} \), showing that the \( K_{\text{m}} \) value of NADPH was low in this reaction. The efficiency in the nitroreductase activity correlated somewhat with the single-electron reduction potential (\( E_{1/2} \)) of the nitroaromatic compounds, as demonstrated by plotting the log \( k_{\text{cat}}/K_{\text{m}} \) against the \( E_{1/2} \) which gave a weak linear correlation with \( r^2 = 0.6603 \) (not shown).

Upon the initial screen of nitroaromatics as substrates for TrxR (Table 1), we performed more thorough studies with the most efficient oxidants. In the presence of an NADPH-regeneration system, TrxR reduced p-dinitrobenzene to air-stable product(s) (Fig. 2A), whose spectra (\( A_{\text{max}} = 340 \, \text{nm} \)) were identical to those obtained using \( E. \text{cloacaee} \) nitroreductase. By using \( E. \text{cloacaee} \) nitroreductase, it was shown that the enzyme reduced nitroaromatic compounds to their corresponding hydroxylamines in two subsequent two-electron (hydride) transfers (46). In accordance with such a mechanism, we also found here that 2 mol of NADPH were oxidized per mol of p-dinitrobenzene using TrxR. Because the reaction was not accompanied by \( O_2 \) consumption, as determined using a Clark-type oxygen electrode (not shown), we excluded that nitroradicals were formed at any significant rate because they should react with oxygen and be at equilibrium with the \( O_2/O_2^- \) couple. Thus, we concluded that TrxR does not reduce p-dinitrobenzene in a single electron manner but rather reduces it directly to 1-hydroxylamino-4-nitrobenzene with a net four-electron transfer. Most interestingly, however, using enzyme that was preincubated under reducing conditions with 20 \( \mu \text{M} \) gold thioglucose for 20 min, leading to a complete loss of activity with regard to reduction of DTNB by deri-

**RESULTS**

**Enzyme Activity in Cell Lysates—**1.5 \( \times 10^6 \) cells were seeded per plate (65 \( \text{cm}^2 \)) in 5 ml of medium, treated 4 h with the indicated compounds, harvested, and washed. The resulting cell pellet was resuspended in cell extraction buffer (50 \( \text{mM} \) Tris, pH 7.6, 2 \( \text{mM} \) EDTA, 5 \( \text{mM} \) dithiothreitol, 20% (v/v) glycerol, 5 \( \text{mM} \) benzamidine, 0.5 \( \text{mM} \) phenylmethylsulfonyl fluoride, 0.5% \( \text{v/v} \) Nonidet P-40) followed by four freeze/thaw cycles. The cell extract was cleared by centrifugation at 16,000 \( \times g \) for 5 min, after which the supernatant was used for analysis of enzyme activity. For TrxR activity, a modification of the previously described end point insulin assay (44) was applied to microtiter plates. Cell extract (4 \( \mu \text{g} \) of total protein) was incubated with 12 \( \mu \text{g} \) of C61S/C72S Trx in the presence of 275 \( \mu \text{m} \) insulin, 1.3 \( \text{mM} \) NADPH, 80 \( \text{mM} \) Hepes buffer, pH 7.6, 6.7 \( \text{mM} \) Tris-Cl, pH 7.6, and 12.5 \( \text{mM} \) EDTA for 40 min at 37 °C in a total volume of 52 \( \mu \text{l} \). The reaction was stopped by addition of 200 \( \mu \text{l} \) of 7.2 \( \text{mM} \) guanidine HCl with 1 \( \text{mM} \) DTNB. The absorbance was measured at a 412 \( \text{nm} \) wavelength using the VersaMax ( Molecular Devices). For GR activity measurements, a spectrophotometric assay (45) was also adjusted to a 400-nm wavelength using the VersaMax (Molecular Devices). The efficiency in the nitroreductase activity correlated somewhat with the single-electron reduction potential (\( E_{1/2} \)) of the nitroaromatic compounds, as demonstrated by plotting the log \( k_{\text{cat}}/K_{\text{m}} \) against the \( E_{1/2} \) which gave a weak linear correlation with \( r^2 = 0.6603 \) (not shown).
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We noted that the tetryl-dependent NADPH oxidation by TrxR accelerated with time (Fig. 2B, inset). An analogous rate acceleration was observed previously in TrxR-catalyzed NADPH-dependent reduction of partially substituted quinones that covalently modified TrxR and thereby suppressed the disulfide reductase activity of the enzyme (15). Because tetryl may react with thiol groups forming the corresponding 2,4,6-trinitrophenylthiones (47), we hypothesized that the reaction acceleration could be related to TrxR modification by tetryl. Indeed, incubation of reduced TrxR with 100 μM tetryl for 1 min led to a 50–60% loss of activity with respect to DTNB reduction in the presence of 50 μM TrxR. Taken together, these data indicate that TrxR catalyzes single-electron reduction of tetryl accompanied by N-denitration (Reaction 1) (34, 41). Thus, the mechanism used in the reduction of tetryl was clearly different from the two-(four)-electron reduction of p-dinitrobenzene. The same mechanism as used with tetryl was characteristic for the reduction of the tetryl analog 2,4-dinitrophenyl-N-methylthiourea (data not shown), except that the reaction in that case was accompanied by formation of 0.25 mol of NO₂ per mol of NADPH.

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FIGURE 2. The formation of nitroreductase reaction products of TrxR. A, reduction of 200 μM p-dinitrobenzene in the presence of an NADPH-regeneration system and 100 nM TrxR is shown. The scans are recorded in 40-min intervals, and the arrow shows the direction of absorbance changes. The dashed line shows the final spectrum of reaction product, obtained after the addition of 20 nM E. cloacae nitroreductase and 10 min of incubation. B, oxidation of 230 μM NADPH by 50 μM tetryl in the presence of 50 μM TrxR is shown. The scans are recorded in 4-min intervals, and the arrows show the direction of absorbance changes. The dashed line shows the absorbance of 50 μM tetryl. The inset depicts the kinetics of oxidation of 50 μM NADPH by 200 μM tetryl (curve 1) and by 60 μM tetryl (curve 2) in the presence of 100 nM TrxR.

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TABLE 1

| No. | Compound                      | E₁/₂(V)  | kcat (s⁻¹) | kcat/Kcat (M⁻¹·s⁻¹) |
|-----|-------------------------------|----------|------------|---------------------|
| 1   | Tetryl                        | -0.154   | 1.8 ± 0.2  | 1.4 ± 0.1 x 10⁴     |
| 2   | Chinifur                      | -0.225   | 0.08 ± 0.01| 1.8 ± 0.1 x 10³     |
| 3   | 2,4,6-Trinitrotoluene          | -0.253   | 0.05 ± 0.01| 1.8 ± 0.1 x 10³     |
| 4   | Nitrofurazic acid             | -0.255   | 0.13 ± 0.02| 2.3 ± 0.2 x 10³     |
| 5   | p-Dinitrobenzene              | -0.257   | 2.8 ± 0.3  | 3.7 ± 0.2 x 10³     |
| 6   | 2-Aminodinitrobenzene         | -0.287   | 0.05 ± 0.01| 3.5 ± 0.4 x 10³     |
| 7   | Nitrofurazic acid             | -0.303   | 0.15 ± 0.03| 1.6 ± 0.2 x 10³     |
| 8   | Nitrobenzene                  | -0.325   | 0.20 ± 0.3  | 3.3 ± 0.3 x 10³     |
| 9   | p-Nitrobenzyl alcohol         | -0.348   | 0.1 ± 0.2   | 4.5 ± 0.5 x 10³     |
| 10  | p-Nitrobenzyl alcohol         | -0.425   | <0.02       | <70                 |
| 11  | 2,4-Dinitrophenyl-N-methylthiourea | -0.485 | 0.1 ± 0.2   | 4.5 ± 0.4 x 10³     |
| 12  | Nitrobenzyl alcohol           | 1.0 ± 0.1 | 3.2 ± 0.2 x 10³ |
| 13  | Nitrobenzyl alcohol           | 0.09 ± 0.1 | 2.4 ± 0.3 x 10³ |
| 14  | Nitrobenzyl alcohol           | 0.082 ± 0.001 | 3.7 ± 0.2 x 10³ |
| 15  | Nitrobenzyl alcohol           | 0.08 ± 0.001 | 3.4 ± 0.3 x 10³ |

Data are from Ref. 65.
Data are from Ref. 66.
Data are from Ref. 35.
Data are from Ref. 67.
We next determined the $k_{cat}/K_m$ values of tetryl in the stopped-flow experiments during the 0–2-s interval (Fig. 3A), assuming 1:2 stoichiometry of NADPH oxidation to cytochrome $c$ reduction. The obtained value, 9.0 ± 1.1 × 10$^3$ M$^{-1}$ s$^{-1}$, is 30% lower than that determined under close to steady-state conditions, i.e. during the 10–30-s time interval (Table 1). Pretreatment of the enzyme by gold thioglycoside decreased its very low intrinsic NADPH oxidase activity of TrxR.

To probe further the details of the catalysis, we attempted to identify the reoxid state of TrxR responsible for reduction of nitroaromatics. Four-electron-reduced (EH$_4$) TrxR has the typical spectrum of the FAD-thiolate charge-transfer complex with $e_{440} = 2.8$ mm$^{-1}$ cm$^{-1}$ (7), whereas the two-electron-reduced (EH$_2$) TrxR has about 50% of that absorbance. Evidently, in the EH$_2$ state of TrxR, two electrons are shared between the N-terminal catalytic disulfide/dithiol and the C-terminal Cys-Sec couple. Mixing of TrxR with 6–12-fold excess of NADPH resulted in a rapid rise in absorbance at 540 nm completed in 20 ms, reaching the amplitude that thus corresponded to formation of EH$_4$ (Fig. 3B, curve 1). This signal was stable for at least 200 s because of the very low intrinsic NADPH oxidase activity of TrxR. In a separate experiment, tetryl was added to the syringe containing NADPH. Mixing then resulted in a rapid 540-nm absorbance rise to the level of EH$_4$, whereupon a fall in absorbance suggested the presence of enzyme species cycling between the EH$_4$ and EH$_2$ states (0.04 > $\Delta A_{540} > 0.02$; see Fig. 3B, curves 2 and 3) until final oxidation to the fully oxidized state occurred upon NADPH exhaustion. Thus, analogously to our previously published mechanism of quinone reduction (15), TrxR seems to cycle between the four- and two-electron-reduced states during its reduction of tetryl.

**TrxR Inactivation and Inhibition by Nitroaromatic Compounds**

The incubation of TrxR with an NADPH-regeneration system and 100–400 μM tetryl with subsequent analysis of enzyme aliquots showed that the TrxR activity, with respect to DTNB as substrate, significantly decreased even after as little as 0.5 min of incubation. In contrast, tetryl could not inactivate oxidized TrxR, i.e. in the absence of NADPH. In an attempt to describe the inactivation of TrxR by tetryl more precisely, we examined the effects of tetryl on steady-state kinetics of DTNB reduction. We found that tetryl significantly decreased the initial rates of the reduction and subsequent decay after simultaneous addition of NADPH and tetryl (Fig. 4). In control experiments, as judged from the absorbance at 412 nm, we found that tetryl did not react directly with the DTNB reduction product, the 2-nitro-5-thiobenzoate anion. The obtained first-order apparent inactivation rate constants, $k_{i(app)}$, reached the maximal value at infinite tetryl concentration (Fig. 4B). This shows that the process may be described by the reversible binding of tetryl with the inhibition constant $K_i$ and a subsequent irreversible first-order reaction, characterized by $k_{i(max)}$. An increase in DTNB concentration slowed down the inactivation, but did not change the maximal inactivation rate (Fig. 4B), showing that DTNB and tetryl compete for the same binding center. Thus, in the presence of DTNB, the data of Fig. 4B may be analyzed according to Equation 1 (43).

$$k_{i(app)} = k_{i(max)} \times \frac{[\text{tetryl}]}{[\text{tetryl}] + K_i} \times (1 + [\text{DTNB}]/K_m(\text{DTNB}))$$

The calculated $K_m(\text{DTNB}) = 142 ± 9.0$ μM, is very close to that obtained in steady-state DTNB reduction, 138 ± 7.0 μM. The obtained values of $k_{i(max)}$ and of the apparent bimolecular rate constant of inactivation, $k_{i(max)}/K_i$, are given in Table 2. The validity of this calculation method was further analyzed by examining the inactivation of TrxR by DNCB and comparing it to the corresponding values determined earlier using the interactions with purified bovine TrxR (48). We found that DNCB inactivated TrxR much less rapidly than tetryl (Fig. 4B). We also found that increasing concentrations of DTNB slowed down the inactivation in a competitive manner (data not shown). The obtained $k_{i(max)}/K_i$ value (Table 2) was indeed well in line with the previously reported value of $\sim 200$ mm$^{-1}$ s$^{-1}$ (48). The tetryl analog, 2,4-dinitrophenyl-N-nitroamine, inactivated TrxR much less rapidly than tetryl (Fig. 4B; Table 2),

**Figure 3. Rapid reaction measurements of the nitroreductase reaction of TrxR.**

A, cytochrome $c$ reduction accompanying reduction of nitroaromatics by TrxR and NADPH was monitored in a stopped-flow spectrophotometer at 550 nm. The first syringe contained TrxR and cytochrome $c$, and the second contained NADPH and a nitroaromatic compound. Concentrations after mixing were as follows: TrxR, 4 μM; NADPH, 50 μM; cytochrome $c$, 50 μM tetryl, 100 μM (curve 1); 1,3-dinitro-4-chlorobenzene, 200 μM (curve 2). In curve 3, a control experiment without nitroaromatic compounds is shown. B, stopped-flow absorbance traces at 540 nm depict the formation of stable four-electron-reduced TrxR after mixing with NADPH (curve 1) and its initial rapid formation with subsequent decay after simultaneous addition of NADPH and tetryl (curves 2 and 3). Concentrations after mixing were as follows: TrxR, 15 μM (curves 1–3); tetryl, 100 μM (curves 2 and 3); NADPH, 80 μM (curves 1 and 3) or 160 μM (curve 2).

**Figure 4. Inactivation of TrxR by nitroaromatic compounds.** A, the time course of reduction of DTNB (1.5 mM) by 30 nM TrxR and 100 μM NADPH in the absence or presence of tetryl was followed. Additions to the reactions were as follows: control with no addition (curve 1); 100 μM tetryl added during the reaction (curve 2, time of addition indicated by arrow); 200 μM tetryl (curve 3); and 300 μM tetryl (curve 4) added before TrxR addition. B, the dependence of the first-order apparent inactivation rate constants, $k_{i(app)}$, on the concentration of nitroaromatic compounds is plotted, using tetryl (curves 1–4), 1-chloro-2,4-dinitrobenzene (curve 5), or 2,4-dinitrophenyl-N-nitroamine (curve 6). Concentrations of DTNB in the assays are as follows: 2 mM (curve 1), 1.5 mM (curve 2), 1.0 mM (curves 3, 5, and 6), and 0.5 mM (curve 4).
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**TABLE 2**

| Compound                        | Mammalian TrxR | A. thaliana TrxR[a] | GSH, k |
|---------------------------------|----------------|---------------------|--------|
|                                 | $k_{	ext{r,max}}$ | $k_{	ext{r,max}}/K_i$ |        |
|                                 | $M^{-1} s^{-1}$ | $M^{-1} s^{-1}$      | $M^{-1} s^{-1}$ |
| Tetryl                          | 732 ± 112       | 0.2                 | 0.63   |
| 2,4-Dinitrophenyl-N-methylamine | 78 ± 12         | 41.7                |        |
| 1-Chloro-2,4-dinitrobenzene     | 333 ± 65        | 4.66                | 0.03   |
| 4,6-Dinitrobenzofuroxan         | 606 ± 120       |                     | 0.02   |

[a] Data are from Ref. 34.
[b] Data are from Ref. 47.
[c] Data are from Ref. 34.
[d] Data are from Ref. 42.

although it was reduced by the enzyme at about 55% the efficiency of tetryl (Table 1). This illustrated that nitroaromatic compounds acting as substrates or inactivating agents for mammalian TrxR are two separate entities in the interactions with the enzyme.

The explosive DNBF represents another strong electrophile. It exists in a form of a Meisenheimer-type adduct with water (30), which may undergo substitution reactions with nucleophiles, e.g. thiolate (or selenolate) groups (Reaction 2).

Compatible with Reaction 2, we found that DNBF inactivated reduced TrxR at a significant rate (Table 2). For comparison, it is important to note that in terms of bimolecular rate constants, the reactivity of TrxR with the hereby examined compounds is several orders of magnitude higher than that with reduced GSH and several times higher than the reactivity of the low molecular-weight-type non-selenoprotein TrxR of *Arabidopsis thaliana* (Table 2). Moreover, under identical conditions, 400 μM of o-, m-, and p-dinitrobenzenes or 4-nitrophenyl-N'-methylisatinamne, in contrast, could not inactivate mammalian TrxR.

Monitoring the initial rates of TrxR-catalyzed DTNB reduction, we found that at a fixed concentration of DTNB and varied NADPH concentrations, dinitrobenzenes, nitrofurans, and tetryl all acted as noncompetitive inhibitors with respect to NADPH (Fig. 5A). At a fixed NADPH concentration (100 μM) and varied DTNB concentration, we further found that most of the nitroaromatic compounds acted as noncompetitive inhibitors with respect to DTNB (Fig. 5B), with $K_i$ values of 17 μM for p-dinitrobenzene, 60 μM for chinifur, 80 μM for nifuroxime, 100 μM for nitrofurantoin, 300 μM for m-dinitrobenzene, and 400 μM for nitrocrine or TNT. Tetryl and DNBF, in contrast, acted as competitive inhibitors with respect to DTNB with $K_i$ values of 12.5 and 5.0 μM, respectively. However, the rapid inactivation of TrxR by these two compounds somewhat complicated the analysis and may have lead to a slight overestimation of their efficiency as competitive inhibitors.

**Inhibition of Cellular TrxR or GR Activity—**We next wished to study whether the nitroaromatic compounds could irreversibly inhibit TrxR activity also in cultured human cancer cells. For this purpose, we examined the inhibitory effects in A549, HeLa, or HeLa cells overexpressing Bc12, using the nitroaromatic compounds acting as irreversible TrxR inhibitors, i.e. tetryl, DNBC, and DNBF (Table 2), or the quinone compound juglone (5-hydroxy-1,4-naphthoquinone) identified previously as an efficient irreversible inhibitor of TrxR (15). In addition, we studied the effects of TNT, a weak reversible inhibitor of TrxR (see above). As a control for the cellular TrxR inhibition, we treated the cells with staurosporine, a well studied inducer of apoptosis that presumably should not lead to direct TrxR inactivation. We also measured the activity of the closely related flavoenzyme GR in the same cell extracts as those analyzed for TrxR activity. We found that after incubating the cells for 4 h with 50 μM compound, DNBC had clearly inhibited cellular TrxR but not GR activity, whereas juglone inhibited both enzymes but TrxR significantly more so than GR. There were no major effects on the cellular activity of either of these two enzymes by using the other substances (Fig. 6).

**Cytotoxic Effects—**Performing the experiments shown in Fig. 6, we noted a considerable cytotoxic effect after only 4 h of incubation with tetryl, juglone, or DNBC, as displayed by significant cell detachment. Production of necrotic or late apoptotic cells, as detected using trypan blue uptake, was most pronounced with juglone in all of the three cell types. It occurred also using DNBC and tetryl, however, with A549 cells being rather resistant to DNBC and with tetryl generally provoking less trypan blue uptake than the other two compounds (Fig. 7). As juglone was the most cytotoxic compound, we analyzed its dose- and time-dependent cytotoxicity, showing that ~50% detached cells was seen after incubation with 25 μM juglone for 4 h in all three cell types (Fig. 8). Analyzing caspase-3/7 activation (i.e. induction of DEVDase activity) as a measure of apoptosis, we found striking differences between the cytotoxic compounds and between the cell types analyzed. Caspase activation triggered by juglone and DNBC was pronounced in all of the three
cell types, and overexpression of Bcl2 had no protective effect. In contrast, staurosporine treatment was clearly the most potent apoptotic trigger in HeLa cells, although it had no effect in the other two cell types under the same conditions. Most interestingly, tetryl showed a similar profile in its activation of caspase activity as staurosporine, which was thereby in striking contrast to the caspase activation profile displayed by juglone and DNCB. These results are summarized in Fig. 9.

**DISCUSSION**

Many nitroaromatic compounds are environmental pollutants or drugs, causing a number of toxic effects on the hematopoietic, cardiovascular, nervous, or reproductive systems (27, 29, 30, 49, 50). A major mechanism for the cytotoxicity is believed to involve reductive activation by cellular enzymes with subsequent redox cycling, provoking oxidative stress, a feature shared with various cytotoxic quinones. The exact molecular pathways leading to the cytotoxicity, however, need more thorough understanding. The data presented here suggest that interactions with TrxR may be profoundly connected with the cytotoxicity of selected nitroaromatic or quinone compounds. The type of interactions with TrxR, found here to involve reductive N-denitration reactions and redox cycling in combination with irreversible alkylation of the enzyme, suggests that TrxR could be a conceivable intracellular target through several different mechanisms. The clear correlation between cellular inhibition of TrxR activity and the cytotoxicity of the nitroaromatic compound DNCB and the quinone juglone suggests that these compounds may indeed target TrxR as a major mechanism for their cytotoxicity, while the cytotoxicity of tetryl should likely act through other pathways as it was a less potent inhibitor of cellular TrxR activity and showed another profile of toxicity than DNCB or juglone, although we found that tetryl was a potent substrate and inhibitor of TrxR in vitro.
DNCB and other dinitrohalobenzenes are the only nitroaromatic compounds that were analyzed previously for interactions with purified mammalian TrxR, shown to be efficient inhibitors of the enzyme by derivatizing the selenolthiol motif while concomitantly inducing a significant NADPH oxidase activity in the derivatized enzyme (48). Here we found that a number of nitroaromatic substrates were better substrates for TrxR than DNCB (Table 1). Interestingly, the nitroreductase reactions of TrxR could either proceed through single electron-reductive N-denitration of nitroaromatic N-nitramines concomitant with their redox cycling or through two-electron reduction with other types of nitrocompounds. Before considering the cytotoxic consequences of these reactions or of the fact that some nitroaromatics inhibited TrxR, we first need to discuss the molecular mechanisms of the interactions between pure TrxR and nitroaromatics.

The $k_{cat}$ value in the reduction of the best nitroaromatic substrates for TrxR was about 15% of maximal turnover, as illustrated using the model substrate DTNB (Table 1), i.e. in a similar range as for several related enzymes. It was higher than the corresponding parameter for glutathione reductase ($\approx 2\%$ (33, 35)) and trypanothione reductase ($\approx 4\%$ (31)), and it was similar to that of the low molecular weight-type non-seleno-

protein TrxR from A. thaliana ($\approx 20\%$ (34)). The findings that tetryl was a competitive inhibitor of TrxR with respect to DTNB and that preincubation with gold thioglucose inhibited the tetryl reduction by 80% strongly suggest to us that tetryl interacts with and is reduced by the selenolthiol motif of the reduced enzyme. This is a rather unique reaction because it constitutes an uncommon nitroreductase reaction involving a Sec residue, and because other flavoenzymes such as glutathione reductase or trypanothione reductase reduce artificial quinones or nitroaromatic substrates solely via their enzyme-bound FAD and not through their proper active sites (51, 52). In this context it should be noted that we detected production of superoxide in the TrxR-catalyzed tetryl reduction, which together with the residual 20% activity upon gold thioglucose preincubation suggests to us that tetryl, in addition, may also be reduced by a second redox active moiety of the enzyme. This should presumably be the enzyme-bound FAD, reminiscent of the reactions proposed to occur with quinone substrates (15, 48). For several of the other nitroaromatic substrates, e.g. p-dinitrobenzene and chinifur, the pattern of reduction and the noncompetitive inhibition with regard to DTNB indicate that those compounds are not at all reduced by the selenolthiol motif of the enzyme but may solely be reduced by the FAD moiety.

In contrast to the generally predominant two-electron reduction of
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quiones (15), we found here that TrxR could reduce nitroaromatics by one or two-electron reactions (e.g., with p-dinitrobenzene) or with mainly one-electron transfers (e.g., with tetryl). The latter type of reaction is reminiscent of the homolytic reductive cleavage of S-nitrosoglutathione by TrxR purified from calf thymus or human placenta (53). In that case, GSNO was reduced by TrxR with a $k_{\text{cat}} = 0.6 \text{ s}^{-1}$ and $k_{\text{cat}}/K_m = 105 \text{ M}^{-1} \text{s}^{-1}$ thus liberating free GSH and nitric oxide (53). Those kinetic parameters may be compared with the reduction of nitroaromatic compounds studied here (as listed in Table 1), displaying a similar range of $k_{\text{cat}}$ values but generally higher $k_{\text{cat}}/K_m$ values than for GSNO, suggesting that the homolytic cleavage of GSNO and the reduction of nitroaromatic compounds by mammalian TrxR can occur at about the same rate but that the affinity for nitroaromatic compounds appears higher than for GSNO.

The two different reduction mechanisms for tetryl should not be due to the subsequent TrxR modification upon tetryl reduction because the tetryl-mediated cytochrome c reduction was also detected at a significant rate before the enzyme modification had occurred. This peculiar mechanism of two concomitant modes of reduction of one substrate may in part be explained by the intrinsic properties of the tetryl molecule, because DT-diaphorase (EC 1.6.4.3) that generally catalyzes two-electron reduction of nitroaromatics, may also perform reductive N-denitration of tetryl in a one-electron manner (Reaction 1) (41). In the case of TrxR, the different modes of reduction of tetryl or other nitroaromatics may of course also be attributed to the combined involvement of a FAD moiety and the selenolthiol motif, the different ratios of which must depend upon the properties of the individual nitroaromatic substrate. This can be reflected in the fact that pretreatment with gold thioglucose suppressed the tetryl reduction by 80%, although p-dinitrobenzene reduction was only suppressed by 30% (thus presumably having a greater extent of reduction by the FAD moiety directly).

Considering the possible mechanisms with which the nitroreductase reactions of TrxR could have an impact on cell viability, one should note that both one- and two-electron reduction of nitroaromatics may theoretically contribute to cytotoxic effects because both types of reaction may yield potentially toxic products, i.e., either redox cycling radicals or alkylating hydroxyamines (27, 29). Substrates competing for catalysis by TrxR may obviously also interfere with the normal functions of the cellular thioredoxin system. As discussed above, the nitroreductase activity of TrxR was higher than that of the structurally related glutathione reductase (32, 33) and similar to that of DT-diaphorase, which is one of the enzymes previously believed to be most important as a cytotoxic nitroreductase (54). However, in terms of efficiency ($k_{\text{cat}}/K_m$) in reduction of nitroaromatics, TrxR was by 2–3 orders of magnitude less active than the mitochondrial NADH:ubiquinone reductase (EC 1.6.99.3) (55) or the microsomal NADPH:cytochrome P-450 reductase (EC 1.6.2.4) (41); those enzymes are probably the most important for the total nitroreductase activity in a majority of mammalian cells.

Inhibition of the cellular activity of TrxR may certainly be an important factor contributing to cytotoxicity of a xenobiotic compound. Our data clearly show that substantial irreversible inhibition occurred within a few hours of treatment of cells using a low concentration (50 μM) of the nitroaromatic compound DNCB and the quinone juglone. For the other nitroaromatics studied in our cell experiments, however, we could not detect any irreversible inhibition of the cellular TrxR activity under identical conditions. It could always be argued that the potential for inhibition would still be possible by reversible competition, which would not be detected using activity measurements with cell extracts. Inhibition could likely also occur using higher concentrations, because the enzyme could be inhibited by several of the compounds in vitro. The reversible modes of noncompetitive or uncompetitive inhibition of pure TrxR toward both NADPH and DTNB by some of the nitroaromatic compounds, however, had $K_i$ values well above the micromolar range, and it therefore seems less likely that such inhibition should be of major significance for the possible manifestation of the cytotoxicity of nonalkylating nitroaromatic compounds. In significant contrast, we propose that the irreversible inactivation of TrxR by the alkylating nitroaromatic compounds may be a significant mechanism contributing to their cytotoxicity.

As discussed above, many alkylating agents, among them several used for anticancer therapy, can rapidly inactivate mammalian TrxR. In the case of dinitrohalobenzenes, the direct modification of the selenolthiol motif of TrxR was demonstrated by mass spectrometric analyses (48). It is likely that most electrophilic compounds reacting with TrxR target the selenolthiol motif of the enzyme, although this has not been directly shown for all compounds inactivating the enzyme. The selenolate group with its higher nucleophilicity and lower $pK_a$ makes selenocysteine a potent target for electrophilic compounds (56), especially when situated at the easily accessible C terminus as it does in TrxR (57). The protective effect of the DTNB disulfide substrate for inactivation by tetryl and DNBF is furthermore clearly compatible with the notion that an alkylation takes place at the Sec-containing active site motif.

It is evident that the cytotoxicity and induction of apoptosis triggered by DNCB and juglone as studied here were events that correlated closely with inactivation of cellular TrxR activity, which also notably was much more inhibited than the related flavoenzyme glutathione reductase. The effect of tetryl on the cellular activity of TrxR was apparently less significant than using DNCB or juglone, although tetryl was a more efficient TrxR inhibitor than DNCB in vitro. This incongruence between inhibition by tetryl of the pure enzyme and that in cells may possibly be explained by the subcellular dynamics, i.e., tetryl may perhaps not reach TrxR intracellularly so that inhibition can occur. Moreover, tetryl may be catabolized and thereby removed by denitration reactions (see Reaction 1) that are catalyzed also by other NADPH-oxidizing flavoenzymes such as cytochrome P-450 reductase and DT-diaphorase (41). Similarly, the lack of inhibition of cellular TrxR activity using DNBF and the total lack of cytotoxicity by this compound under the conditions utilized here may also be explained by its less efficient entry into cells, in this case probably because it exists as a negatively charged Meisenheimer-type adduct with water.

We find the diverse patterns of cytotoxicity provoked by DNCB and juglone on one hand and tetryl on the other to be very interesting. As common in studies of apoptotic cell death, we used staurosporine as a positive control for triggering apoptosis that is blocked by overexpression of Bcl2 (58). The Bcl2 protein blocks, among other effects, cytochrome c release from mitochondria and thereby inhibits formation of the apoptosome that triggers caspase activation (59, 60). Although the A549 cell line is rather heterogeneous, different subclones seem to express TrxR at similar levels and the parental cell line as such is quite resistant to apoptosis induction (61). Therefore, the predominant caspase activation triggered by staurosporine in HeLa cells but not in A549 cells or in HeLa cells overexpressing Bcl2, as found here, was expected. We find it very interesting, however, that under the conditions utilized here tetryl had the very same profile of cytotoxicity between the three cell lines studied as did staurosporine. This finding together with the lack of cellular TrxR inhibition by tetryl show that tetryl may indeed provoke significant apoptotic cell death, but through a pathway that we find unlikely to involve TrxR as one of its major intracellular targets. Our findings suggest that the apoptotis provoked
by tetryl in HeLa cells should involve a signaling cascade that converges at the mitochondrial pathway for caspase activation, which can be blocked by Bcl2. The identities of cellular targets for tetryl remain to be elucidated. In sharp contrast, we judge that the cell death provoked by DNBC or juglone is highly likely to involve TrxR as a major intracellular target molecule, the interactions with which may contribute to triggering of apoptotic, and possibly also necrotic cell death. The trypan blue uptake in A549 cells provoked by DNBC (i.e. predominantly necrotic or late apoptotic cell death) was less than that provoked by juglone, which may be due to the high glutathione S-transferase activity in A549 cells (61) that may rapidly detoxify a major fraction of the added DNBC. The caspase activation, however, was provoked to a similar extent by DNBC and juglone in all three cell types. That effect may be signaled through targeting of TrxR.

We have earlier shown that selenium-compromised TrxR, such as that formed after derivatization with electrophilic compounds, may rapidly and directly provoke apoptosis by a gain of function, as illustrated upon the introduction of minute amounts of cisplatin-derivatized TrxR into cells (21). The apoptotic signaling thus triggered seemed not to be related to either an induced NADPH oxidase activity or an inhibition of the intracellular thioredoxin system but rather to involve a direct induction of apoptosis through yet unknown pathways (21). Based upon the results presented here, we would argue that the apoptotic effects of DNBC and juglone may involve the intracellular formation of selenium-compromised forms of TrxR for the following reasons. 1) It is clear from earlier studies (15, 48) as well as from the present analysis that both DNBC and juglone have the capacity to rapidly and irreversibly inactivate TrxR in vitro. 2) We found herein that the cellular TrxR activity was significantly inhibited in an irreversible manner after only 4 h of incubation with either DNBC or juglone. 3) The cell death provoked by DNBC or juglone was significant in all the three cell types studied and was not blocked by Bcl2, in major contrast to the effects of staurosporine or tetryl, thus suggesting another mode of cytotoxicity. Both DNBC and juglone triggered caspase activation, and this effect was also similar in all three cell types studied, which coincides with the TrxR inhibition in all three cell types, but not with the corresponding effects of staurosporine or tetryl. All of these effects would be compatible with a notion that selenium-compromised forms of the TrxR protein were produced in the cells upon DNBC or juglone treatment, and that triggering of apoptosis by those TrxR-derived proteins involves induction of caspase activation that cannot be prevented by Bcl2. This pattern indeed seems to be a property of the apoptosis signaling pathway triggered by selenium-compromised TrxR.5 The findings of others are also compatible with this proposed model for apoptosis signaling through selenium-compromised TrxR, such as the fact that arsenic oxide induces cell death that cannot be prevented by Bcl2 (62, 63) and knowing that arsenic oxide is a highly potent TrxR-targeting agent that derivatizes its seleno-thiol motif (57). Moreover, apoptosis induction provoked by tetryl treatment seems to be able to occur through targeting of the Sec residue in TrxR as one of its mechanisms, in addition to the cisplatin reaction with nuclear DNA (21, 25, 64). Thus, we propose that one of the molecular mechanisms leading to the apoptosis detected in cells exposed to DNBC or juglone, as found here, could be directly linked to the derativization of cellular TrxR.

To conclude, in this study we have discovered novel interactions of TrxR with several nitroaromatic compounds, and we analyzed these interactions in detail, demonstrating a novel type of catalysis with N-denitration reduction, redox cycling involving unique concomitant 

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