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Flavoenzyme-catalyzed redox cycling of hydroxylamino- and amino metabolites of 2,4,6-trinitrotoluene: implications for their cytotoxicity

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Abstract

The toxicity of 2,4,6-trinitrotoluene (TNT), a widespread environmental contaminant, is exerted through its enzymatic redox cycling and/or covalent binding of its reduction products to proteins and DNA. In this study, we examined the possibility of another cytotoxicity mechanism of the amino- and hydroxylamino metabolites of TNT, their flavoenzyme-catalyzed redox cycling. The above compounds acted as redox-cycling substrates for single-electron transferring NADPH:cytochrome P-450 reductase (P-450R) and ferredoxin:$NADP^+$ reductase (FNR), as well as substrates for the two-electron transferring flavoenzymes rat liver NAD(P)H:quinone oxidoreductase (NQO1) and *Enterobacter cloacae* NAD(P)H:nitroreductase (NR). Their reactivity in P-450R-, FNR-, and NR-catalyzed reactions increased with an increase in their single-electron reduction potential (E_7^1) or the decrease in the enthalpy of free radical formation. The cytotoxicity of the amino- and hydroxylamino metabolites of TNT towards bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) was partly prevented by the antioxidant *N*,*N*′-diphenyl-*p*-phenylene diamine and desferrioxamine, and potentiated by 1,3-bis-(2-chloroethyl)-1-nitrosourea, thus pointing to the involvement of oxidative stress. In general, their cytotoxicity increased with an increase in their electron accepting properties, or their reactivity towards the single-electron transferring FNR and P-450R. Thus, our data imply that the flavoenzyme-catalyzed redox cycling of amino and hydroxylamino metabolites of TNT may be an important factor in their cytotoxicity.
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Keywords: Trinitrotoluene; Hydroxylamino dinitrotoluene; Amino dinitrotoluene; NADPH:cytochrome P-450 reductase; Ferredoxin:$NADP^+$ reductase; NAD(P)H:quinone oxidoreductase; NAD(P)H:nitroreductase; Cytotoxicity; Oxidative stress

The toxic action of nitroaromatic compounds is most frequently caused by the enzymatic reduction of their nitro group(s) ([1] and references therein). The single-electron reduction of nitroaromatics to their anion-radicals by flavoenzymes dehydrogenases–electron-transferases, e.g., NADPH:[1]cytochrome P-450 reductase (P-450R, EC 1.6.2.4), ferredoxin: $NADP^+$ reductase (FNR, EC 1.18.1.2), and bacterial oxygen-sensitive nitroreductases [2–7] initiates their redox cycling in aerobic conditions, and, subsequently, oxidative stress. The cytotoxic alkylating hydroxylamines might be also

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[1] *Abbreviations used:* TNT, 2,4,6-trinitrotoluene; 2-NH_2-DNT, 2-amino-4,6-dinitrotoluene; 4-NH_2-DNT, 4-amino-2,6-dinitrotoluene; 2,4-$(NH_2)_2$-NT, 2,4-diamino-6-nitrotoluene; 2,4,6-$(NH_2)_3$-T, 2,4,6-triaminotoluene; 2-NHOH-DNT, 2-hydroxylamino-4,6-dinitrotoluene; 4-NHOH-DNT, 4-hydroxylamino-2,6-dinitrotoluene; DPPD, *N*,*N*′-diphenyl-*p*-phenylene diamine; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; P-450R, NADPH:cytochrome P-450 reductase; FNR, ferredoxin:$NADP^+$ reductase; NQO1, NAD(P)H:quinone oxidoreductase; NR, NAD(P)H:nitroreductase; E_7^1, potential of single-electron reduction at pH 7.0; ΔH_f, enthalpy of the reaction; k_{cat}, catalytic constant; k_{cat}/K_m, bimolecular rate constant; cL_{50}, the concentration of compound for 50% cell survival.

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formed as side-products of single-electron reduction under aerobiosis due to the competition between the reoxidation and the disproportionation of nitroradicals [8]. Under hypoxic conditions, single-electron transferring enzymes reduce nitroaromatics to amines [2] or, less frequently, to hydroxylamines [9]. The two-electron reduction of nitroaromatics by mammalian DT-diaphorase (NAD(P)H:quinone oxidoreductase; NQO1, EC 1.6.99.2) or bacterial oxygen-insensitive nitroreductases results in the formation of nitroso-, and, subsequently, hydroxylamine products ([2,10–12] and references therein).

Nitroaromatic explosives like 2,4,6-trinitrotoluene (TNT) are toxic environmental and industrial pollutants, causing hemolytic crisis, cataract, urinary tract tumors, and reproductive toxicity in humans ([9,13–17] and references therein). The TNT toxicity in mammals is exerted through the bioreductive activation, including the TNT redox cycling with the formation of reactive oxygen species [18,19], and the rapid formation of toxic hydroxylamino- and amino metabolites [9,20,21]. Hydroxylamino- and amino metabolites of TNT may be considered as possible factors of the environmental pollution as well, since they may be formed during composting and the incomplete bioremediation of TNT contaminated soil by microorganisms and fungi [22–25]. The most widely accepted mechanism of the TNT metabolite toxicity is the covalent binding of hydroxylamino-dinitrotoluenes and/or their nitroso reoxidation products to proteins [9]. In addition, hydroxylamino-dinitrotoluenes undergo the transition metal-catalyzed oxidative redox cycling causing the DNA damage [17]. The mechanisms of the toxicity of TNT amino metabolites are poorly understood, except the recently demonstrated enhanced expression of tumor suppressor *p*53 under the action of 2-amino-4,6-dinitrotoluene [26].

In our opinion, the studies performed so far did not address another potential mechanism of the cytotoxicity of TNT metabolites, namely their flavoenzyme-catalyzed redox cycling. The recently determined single-electron reduction potentials (E_7^1) for the amino metabolites of TNT [27] point to the possibility of their action as substrates for single-electron transferring flavoenzymes, which implies the redox cycling of nitro-anion radicals, and subsequent oxidative stress. In this paper, we examined the reactivity of several amino- and hydroxylamino metabolites of TNT (Fig. 1) towards single- and two-electron transferring flavoenzymes, as well as their mammalian cell culture cytotoxicity. Taken together with the analogous data of TNT and the model nitroaromatic compounds, our results demonstrate that the flavoenzyme-catalyzed redox cycling accompanied by the oxidative stress may be an important factor in the cytotoxicity of TNT metabolites, and possibly in the cytotoxicity of TNT itself.

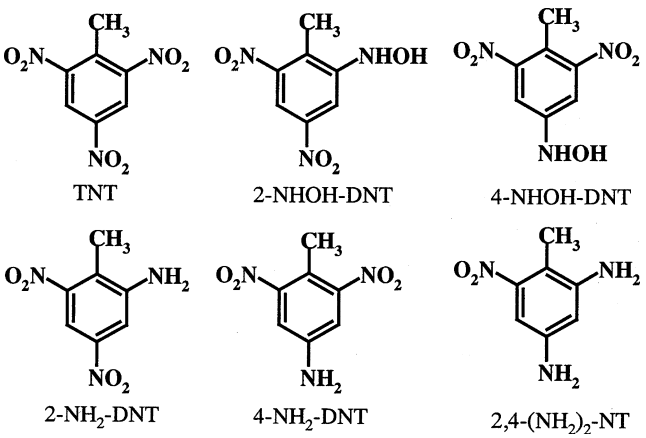

Fig. 1. The formulae of 2,4,6-trinitrotoluene (TNT) metabolites studied in this work: 2-hydroxylamino-4,6-dinitrotoluene (2-NHOH-DNT), 4-hydroxylamino-2,6-dinitrotoluene (4-NHOH-DNT), 2-amino-4,6-dinitrotoluene (2-NH_2-DNT), 4-amino-2,6-dinitrotoluene (4-NH_2-DNT), and 2,4-diamino-6-nitrotoluene (2,4-$(NH_2)_2$-NT).

Materials and methods

Materials

2-Hydroxylamino-4,6-dinitrotoluene (2-NHOH-DNT), 4-hydroxylamino-2,4-dinitrotoluene (4-NHOH-DNT), 2-amino-4,6-dinitrotoluene (2-NH_2-DNT), 4-amino-2,6-dinitrotoluene (4-NH_2-DNT), and 2,4-diamino-6-nitrotoluene (2,4-$(NH_2)_2$-NT) (Fig. 1) were synthesized according to the described methods [28,29]. All the compounds were characterized by melting points, ^{1}H NMR, UV, and IR spectroscopy. All other chemicals were obtained from Sigma and used as received.

Enzymatic assays

The kinetic measurements were carried out spectrophotometrically using a Hitachi-557 spectrophotometer in 0.1 M K-phosphate buffer (pH 7.0) containing 1 mM EDTA at 25 °C, unless specified otherwise. NADPH:cytochrome P-450 reductase (P-450R) from pig liver was prepared as described [30], the enzyme concentration was determined using $\varepsilon_{460} = 22\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$. Ferredoxin:$NADP^+$ reductase (FNR) from *Anabaena* was prepared as described previously [31], the enzyme concentration was determined using $\varepsilon_{459} = 9.4\ \mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$. Rat liver DT-diaphorase (NQO1) was prepared as described [32], the enzyme concentration was determined using $\varepsilon_{460} = 11\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$. In the experiments with NQO1, 0.01% Tween 20 and 0.25 mg/ml bovine serum albumin were added as activators (0.01%). The enzyme turnover numbers were expressed as the number of molecules of electron acceptors reduced by the active center of enzyme per second. They were equal to $100\,\mathrm{s}^{-1}$ (P-450 R, reduction of 50 μM cytochrome *c*

($\Delta\varepsilon_{550} = 20\,mM^{-1}\,cm^{-1}$), concentration of NADPH, 100 μM), $200\,s^{-1}$ (FNR, reduction of 1 mM ferricyanide ($\Delta\varepsilon_{420} = 1.0\,mM^{-1}\,cm^{-1}$), concentration of NADPH, 200 μM), and $2000\,s^{-1}$ (NQO1, menadione-mediated reduction of 50 μM cytochrome *c* (concentration of NADPH, 100 μM, concentration of menadione, 10 μM)). The recombinant *Enterobacter cloacae* NAD(P)H:nitroreductase (NR, EC 1.6.99.7) was prepared as described [12], and was a generous gift of Dr. Ronald L. Koder and Professor Anne-Frances Miller (University of Kentucky, Lexington, USA). The enzyme concentration was determined using $\varepsilon_{454} = 14.3\ mM^{-1}\,cm^{-1}$. The kinetic studies of NR were performed in 0.1 M Tris–Cl (pH 7.0), containing 0.5 mM desferrioxamine. The rates of enzymatic oxidation of NAD(P)H by nitroaromatic compounds were determined according to the NAD(P)H oxidation rates ($\Delta\varepsilon_{340} = 6.2\ mM^{-1}\,cm^{-1}$), using 100 μM NADPH (P-450R, NQO1), 200 μM NADPH (FNR), or 150 μM NADH (NR) as an electron donor. The enzyme catalytic constant (k_{cat}) and the bimolecular rate constant (k_{cat}/K_m) of the reduction of aromatic nitrocompounds correspond to the reciprocal intercepts and slopes of plots [E]/v vs. 1/[$ArNO_2$], where [E] is the enzyme concentration and [$ArNO_2$] is the concentration of nitrocompound. k_{cat} is the number of NAD(P)H molecules oxidized by the single active center of an enzyme per second. The rates obtained were corrected for the intrinsic NADPH-oxidase rates of the enzymes.

The rates of oxygen consumption were monitored using a Clark electrode. The rate of the nonenzymatic reduction of cytochrome *c* (10 μM) was monitored at 550 nm, adding excess (50–200 μM) hydroxylamino metabolites of TNT. Horse spleen ferritin was gel filtrated on Sephadex G-25, equilibrated with 0.1 M Tris–HCl, pH 7.0. The reductive mobilization of iron from ferritin was monitored according to the rise in absorbance of Fe^{2+}-α,α′-bipyridyl complex ($\Delta\varepsilon_{522} = 8.43\ mM^{-1}\,cm^{-1}$ [33]) in 0.1 M Tris–HCl buffer, pH 7.0, containing 2 mM α,α′-bipyridyl. During the assays, the concentration of ferritin corresponded to 1.5–1.6 mM Fe^{3+}, which was determined according to $\varepsilon_{380} = 19\ ml\,(mg\ Fe)^{-1}\,cm^{-1}$ [34].

Cell culture cytotoxicity studies

The culture of bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) was grown and maintained in Eagle's medium supplemented with 10% fetal bovine serum at 37 °C as described previously [35,36]. In the cytotoxicity experiments, cells (3.0×10^4/ml) were grown in the presence of various amounts of aromatic nitrocompounds for 24 h, and counted using a hematocytometer with viability determined by the exclusion of trypan blue. Before the count, the cells were trypsinized.

Quantum mechanical calculations and statistical analysis

In the semiempirical calculations of compound heat formation (H_f) by the Austin Model 1 Hamiltonian (AM1) and Parameter Model Hamiltonian 3 (PM3) methods, PC Spartan Pro (version 1.0.1, Wavefunction) was used. For all calculations, the geometries were fully optimized. The enthalpies of nitroanion-radical formation ($\Delta H_f(ArNO_2^{-\cdot})$) were calculated from Eq. (1), where $ArNO_2$ denotes nitroaromatic compound and $ArNO_2^{-\cdot}$ denotes its anion-radical:

$$\Delta H_f(ArNO_2^{-\cdot}) = H_f(ArNO_2^{-\cdot}) - H_f(ArNO_2) \quad (1)$$

The regression analysis was performed using Statistica software (version 4.3; Statsoft, 1993).

Results

Frequently, the aerobic cytotoxicity of nitroaromatic compounds increases with an increase in their single-electron potential (E_7^1) with a relationship $\Delta\log cL_{50}/\Delta E_7^1 \approx -10\,V^{-1}$, where cL_{50} is the concentration of compound for 50% cell survival ([4,35,36] and references therein). These relationships may reflect the relative rates of the single-electron reduction of nitroaromatics initiating their redox cycling, since their reactivity towards single-electron transferring flavoenzymes, e.g., NADPH:cytochrome P-450 reductase (P-450R) or ferredoxin:$NADP^+$ reductase (FNR), increases with an increase in their E_7^1 [3,5–7]. Therefore, we have used FNR and P-450R as the model systems for the evaluation of the reactivity of TNT metabolites (Fig. 1) in their single-electron reduction reactions.. The single-electron reduction potentials of 2-amino-4,6-dinitrotoluene (2-NH_2-DNT), 4-amino-2,6-dinitrotoluene (4-NH_2-DNT) and 2,4-diamino-6-nitrotoluene (2,4-$(NH_2)_2$-NT), TNT, and other related nitrobenzenes are given in Table 1. To our knowledge, the E_7^1 values for 2-hydroxylamino-4,6-dinitrotoluene (2-NHOH-DNT) and 4-hydroxylamino-2,6-dinitrotoluene (4-NHOH-DNT) are unavailable. Thus, their enthalpies of anion-radical formation ($\Delta H_f(ArNO_2^{-\cdot})$) obtained by means of quantum mechanical calculations were used as the measure of their single-electron accepting potency (Table 1). It is known that these parameters exhibit a correlation with single-electron transfer redox potentials [37,38]. It is evident that the electron accepting potency of hydroxylamine metabolites of TNT is intermediate between that of the parent compound, TNT, and its amine metabolites (Table 1).

The single-electron reduction of amino, and hydroxylamino metabolites of TNT by FNR and P-450R was accompanied by their redox cycling, i.e., the oxidation of excess NADPH over nitrocompound, with a consumption of stoichiometric O_2 amount per NADPH

Table 1
The single-electron reduction potentials (E_7^1) [27] and enthalpies of free radical formation ($\Delta H_f(ArNO_2/ArNO_2^{-\cdot})$) of 2,4,6-trinitrotoluene (TNT) and its metabolites

No.	Compound	E_7^1 (V)	$\Delta H_f(ArNO_2/ArNO_2^{-\cdot})$ (kJ/mol)	
			(a) AM1	(b) PM3
1	TNT	−0.253	−310.70	−316.44
2	2-NHOH-DNT		−261.27	−265.27
3	4-NHOH-DNT		−255.60	−258.94
4	2-NH_2-DNT	−0.417	−249.21	−254.07
5	4-NH_2-DNT	−0.449	−242.11	−238.97
6	2,4-$(NH_2)_2$-NT	−0.502	−161.12	−163.65

oxidized. The FNR-catalyzed reduction of the amino metabolites of TNT was accompanied by the reduction of cytochrome *c*, added into the reaction mixture. The cytochrome *c* reduction rate was close to a doubled rate of NADPH oxidation and was inhibited by 30 μg/ml superoxide dismutase by 50–70%. This demonstrates the involvement of nitroanion-radicals and superoxide, formed in their reoxidation by oxygen, in the cytochrome *c* reduction. The analogous studies of hydroxylamino metabolites of TNT were not performed, since these compounds rapidly reduce cytochrome *c* directly with rate constants of $90 \pm 8.0\,M^{-1}\,s^{-1}$ (2-NHOH-DNT) and $39 \pm 5.0\,M^{-1}\,s^{-1}$ (4-NHOH-DNT). The bimolecular reduction rate constants (k_{cat}/K_m) of TNT metabolites by FNR and P-450R are given in Table 2. The k_{cat}/K_m of the amino metabolites of TNT matched the previously obtained linear log k_{cat}/K_m vs. E_7^1 relationships of other nitrobenzenes in P-450R- and FNR-catalyzed reactions [35]. The resulting linear regressions are characterized by $r^2 = 0.8276$ (P-450R) and $r^2 = 0.8126$ (FNR) ($n = 12$, data not shown). Analogously, the reactivities of TNT metabolites including hydroxylamino-DNTs with unknown E_7^1 values matched the previously obtained linear log k_{cat}/K_m vs. $\Delta H_f(ArNO_2^{-\cdot})$ relationships [35], although the resulting regressions were relatively scattered ($r^2 = 0.5273$ (FNR), and $r^2 = 0.5469$ (P-450R), AM1 method, $n = 13$) (data not shown).

Next, we examined the reactivity of TNT metabolites towards two-electron transferring flavoenzymes. As a rule, nitroaromatics are poor substrates for mammalian NAD(P)H:quinone oxidoreductase (NQO1), their k_{cat} not exceeding $5\,s^{-1}$, and their reactivities being independent of reduction potentials [11,35]. The k_{cat}/K_m and k_{cat} values of TNT and its metabolites are given in Table 2. One may note that the reactivities of 2-NHOH-DNT and 4-NHOH-DNT are similar to that of TNT, and that the reactivities of amino metabolites are much lower (Table 1). During the reduction of 2-NHOH-DNT and 4-NHOH-DNT by NQO1, we observed the oxidation of more than a double excess of NADPH over nitrocompound, the reaction being almost completely inhibited by 20 μM dicumarol, an inhibitor of NQO1 (data not shown). However, the further studies in this direction were hampered by the low reaction rates (Table 2), which were comparable to an intrinsic NADPH oxidase turnover rate of NQO1, $0.05\,s^{-1}$. For this reason, we examined the two-electron reduction of TNT by

Table 2
Bimolecular steady-state rate constants (k_{cat}/K_m) of reduction of 2,4,6-trinitrotoluene (TNT) and its metabolites by ferredoxin:$NADP^+$ reductase (FNR), NADPH:cytochrome P-450 reductase (P-450R), NAD(P)H:quinone oxidoreductase (NQO1), and by *Enterobacter cloacae* NAD(P)H:nitroreductase (NR), and their concentrations for the 50% survival of FLK cells (cL_{50})

No.	Compound	k_{cat}/K_m ($M^{-1}\,s^{-1}$)				cL_{50} (μM)
		(a) FNR	(b) P-450R	(c) NQ1[a]	(d) NR[a]	
1	TNT	$1.1 \pm 0.1 \times 10^4$	$1.7 \pm 0.1 \times 10^{6}$[b]	$6.7 \pm 0.7 \times 10^2$ (1.0 ± 0.1)[b]	$9.8 \pm 1.5 \times 10^6$ (143 ± 22)[c]	25 ± 5.0[b]
2	2-NHOH-DNT	$3.1 \pm 0.2 \times 10^3$	$4.5 \pm 0.3 \times 10^4$	$6.5 \pm 1.9 \times 10^3$ (0.35 ± 0.09)	$2.14 \pm 0.19 \times 10^6$ (330 ± 28)	40 ± 7
3	4-NHOH-DNT	$2.1 \pm 0.6 \times 10^2$	$1.3 \pm 0.3 \times 10^4$	$4.1 \pm 1.4 \times 10^3$ (0.52 ± 0.10)	$2.15 \pm 0.17 \times 10^5$ (24.4 ± 3.0)	112 ± 10
4	2-NH_2-4,6-DNT	$6.2 \pm 0.4 \times 10^2$	$6.1 \pm 0.5 \times 10^3$	$1.3 \pm 0.3 \times 10^2$ ($\leqslant 0.04$)	$5.4 \pm 0.45 \times 10^5$ (72 ± 8.0)	440 ± 35
5	4-NH_2-2,6-DNT	$3.4 \pm 0.1 \times 10^2$	$2.5 \pm 0.3 \times 10^3$	$1.0 \pm 0.3 \times 10^2$ ($\leqslant 0.04$)	$1.04 \pm 0.12 \times 10^5$ (42 ± 3.0)	316 ± 20
6	2,4-$(NH_2)_2$-6-NT	$2.1 \pm 0.2 \times 10^2$	$2.0 \pm 0.5 \times 10^3$	$\leqslant 50$ ($\leqslant 0.04$)	$4.8 \pm 0.50 \times 10^3$ (0.4)	350 ± 40

[a] The values of k_{cat} (s^{-1}) given in parentheses.
[b] From [35].
[c] From [38].

E. cloacae NAD(P)H:nitroreductase (NR), which rapidly ($k_{cat} = 10$–$1000\,s^{-1}$) reduces nitroaromatic compounds to the corresponding hydroxylamines [12,38]. Earlier we have shown that the reactivity of nitroaromatic compounds in the NR-catalyzed reactions increased with an increase in their E_7^1 or decrease in their $\Delta H_f(ArNO_2^{-\cdot})$ [38]. The k_{cat}/K_mvalues of amino, and hydroxylamino metabolites of TNT towards NR (Table 2) also follow this trend, matching the previously determined k_{cat}/K_m of other nitroaromatics [38]. The resulting linear log k_{cat}/K_m vs. E_7^1 and log k_{cat}/K_m vs. $\Delta H_f(ArNO_2^{-\cdot})$ regressions are characterized by $r^2 = 0.8844$ ($n = 12$) and by $r^2 = 0.7868$ ($n = 13$, AM1 method), respectively (data not shown). Previously, we have also observed the biphasic kinetics of NR-catalyzed oxidation of excess NADH by TNT: the first phase corresponded to the rapid oxidation of a doubled to TNT amount of NADH, and the second phase corresponded to the slower oxidation of further excess NADH (Fig. 2A, curve 1) [38]. The second reaction phase was accompanied by the oxygen consumption, most probably reflecting the further reduction of hydroxylamino-DNT(s) by NR, and the reoxidation of the reduction product(s) [38]. Using 4-NHOH-DNT as NR substrate, the rate of NADH oxidation was similar to the second phase of the TNT-dependent reaction, whereas 2-NHOH-DNT caused a much higher rate (Fig. 2A). This may point to the preferential formation of 4-NHOH-DNT as the product of TNT reduction by NR. The reduction of hydroxylamino-DNT's by NR was accompanied by the oxidation of more than a double amount of NADH (Fig. 2A), and a close to stoichiometric to NADH consumption of O_2. Thus, the two-electron transferring enzymes such as NR also perform the redox cycling of hydroxylamino-DNT's. The use of superoxide dismutase-sensitive cytochrome *c* reduction assay to discriminate the redox cycling initiated by single- and two-electron transferring enzymes is problematic, since hydroxylamino-DNT's rapidly reduce cytochrome *c*. For this reason, we examined the effects of superoxide dismutase in another free-radical linked reaction, the reductive mobilization of Fe^{2+} from ferritin [39]. Although 2-NHOH-DNT reduces ferritin directly, its redox cycling in the presence of NADPH and P-450R accelerates the reaction, which is almost completely inhibited by superoxide dismutase (Fig. 2B). In contrast, the redox cycling of 2-NHOH-DNT by NR did not increase the ferritin reduction rate, and was not affected by superoxide dismutase (Fig. 2B). The same events took place during the redox cycling of 4-NHOH-DNT (data not shown). It shows that the reduction of hydroxylamino-DNT's by two-electron transferring NR does not lead to the free radical formation. Although being unidentified in the present study, the potential candidate for the final reduction product is 2,4-dihydroxylamino-6-nitrotoluene, which undergoes rapid autoxidation [40].

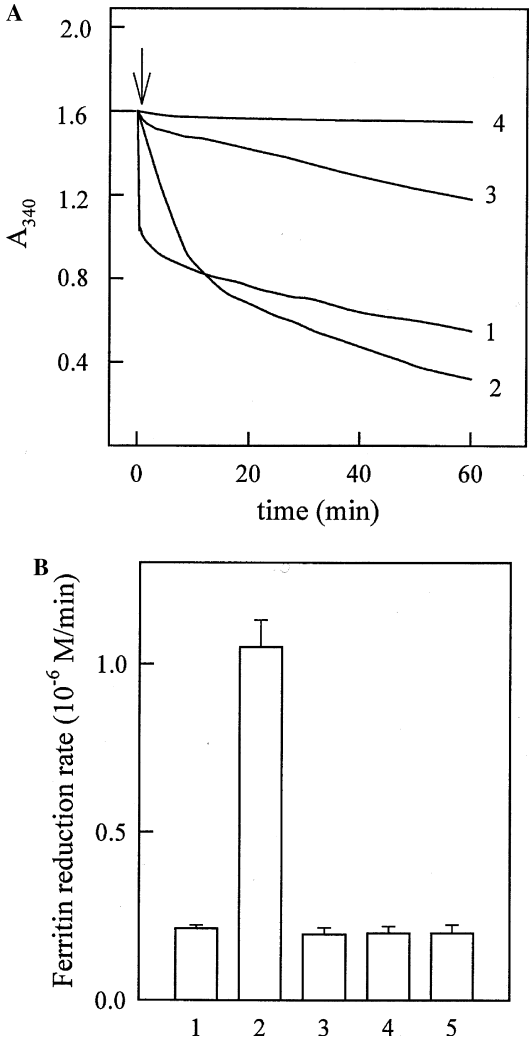

Fig. 2. Redox cycling events during the enzymatic two-electron reduction of hydroxylamino dinitrotoluenes. (A) Oxidation of 250 μM NADH by 20 nM *Enterobacter cloacae* nitroreductase using TNT or its hydroxylamino metabolites as the electron acceptors: 50 M TNT (1), 50 μM 2-NHOH-DNT (2), 50 μM 4-NHOH-DNT (3), and 50 μM 2-NHOH-DNT in the presence of 20 μM dicumarol (4). (B) Reductive Fe^{2+} mobilization from ferritin by the direct action of 50 μM 2-NHOH-DNT (1), and during its enzymatic reduction by NADPH:cytochrome P-450 reductase (2,3) or by *Enterobacter cloacae* nitroreductase (4,5) in the presence of 100 μM NADPH. The enzyme amount was adjusted to give the NADPH oxidation rate of 10 μM/min, the measurements were performed in the absence (1,2,4) and in the presence of 30 μg/ml superoxide dismutase (3,5).

For the cytotoxicity experiments, we used the bovine leukemia virus-transformed lamb kidney fibroblast line FLK, used in our previous studies [35,36]. This line is characterized by the activity of NADPH:cytochrome *c* reductase, 43 ± 1 nmol cytochrome *c* min^{-1} (mg protein)$^{-1}$; and NQO1, 260 ± 30 nmol NADPH min^{-1} (mg protein)$^{-1}$. Table 2 shows the concentrations of TNT metabolites for the 50% cell survival (cL_{50}). The cytotoxicity of 2-NHOH-DNT and 2-NH_2-DNT almost did not change when the serum amount was decreased from

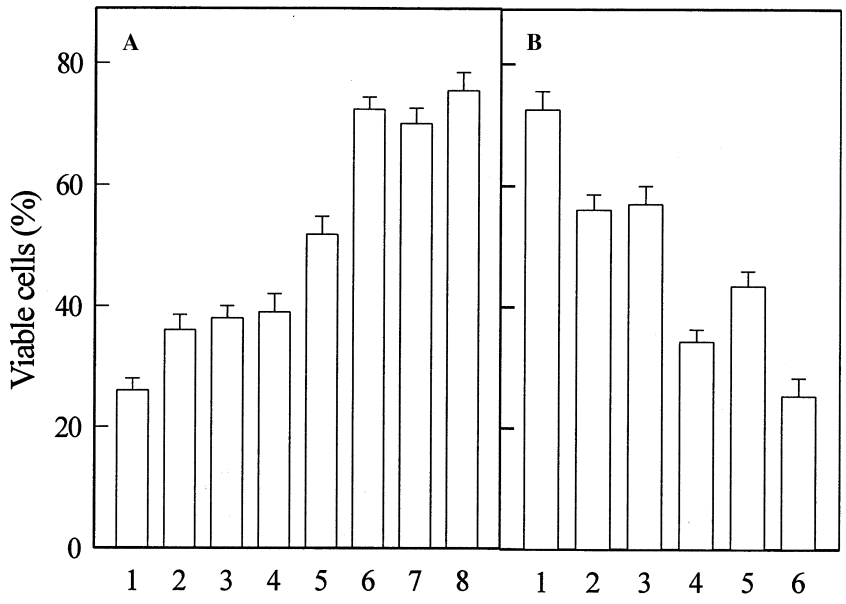

Fig. 3. (A) The protecting effects of DPPD (2 μM) and desferrioxamine (300 M) on the cytotoxicity of 60 μM 2-NHOH-DNT and 500 μM 2-NH_2-DNT. Additions: 2-NHOH-DNT (1), 2-NHOH-DNT + DPPD (2), 2-NHOH-DNT + desferrioxamine (3), 2-NHOH-DNT + DPPD + desferrioxamine (4), 2-NH_2-DNT (5), 2-NH_2-DNT + DPPD (6), 2-NH_2-DNT + desferrioxamine (7), and 2-NH_2-DNT + DPPD + desferrioxamine (8). (B) The potentiating effects of BCNU (15 μM) on the cytotoxicity of 2-NH_2-DNT and 2-NHOH-DNT. Additions: 250 μM 2-NH_2-DNT (1), 250 μM 2-NH_2-DNT + BCNU (2), 20 μM 2-NHOH-DNT (3), 20 μM 2-NHOH-DNT + BCNU (4), 40 μM 2-NHOH-DNT (5), and 40 μM 2-NHOH-DNT + BCNU (6). Cell viability in control experiments, 97 ± 2%, the addition of DPPD, desferrioxamine, or BCNU did not affect cell viability by more than 2%, $n = 3$–4.

10 to 2.5%. The antioxidant *N*,*N*′-diphenyl-*p*-phenylene diamine (DPPD) [41], and the iron chelator desferrioxamine, the latter preventing the Fenton reaction, gave partial protection against the cytotoxicity of 2-NHOH-DNT and 2-NH_2-DNT (Fig. 3A), and 4-NHOH-DNT and 4-NH_2-DNT (data not shown). On the other hand, the alkylating agent 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU), which inactivates glutathione reductase and decreases the amount of intracellular reduced glutathione [42], potentiated the cytotoxicity of 2-NHOH-DNT and 2-NH_2-DNT (Fig. 3B). It points to the prooxidant character of cytotoxicity of TNT metabolites. We failed to observe the effect of an inhibitor of NQO1, dicumarol (20 μM), on the cytotoxicity of hydroxylamino-DNT's. Finally, we analyzed the dependence of the cytotoxicity of TNT metabolites on their electron accepting properties. For this purpose, their cL_{50} were compared with the previously published data on the cytotoxicity of a series of nitrobenzenes with available E_7^1 values in FLK cells [35] (Fig. 4A). For all the compounds, the linear log cL_{50} vs E_7^1 dependence is characterized by $r^2 = 0.8230$ (Fig. 4A). However, the cL_{50} of the amino metabolites (compounds 4–6) seems to be lower than may follow from their E_7^1 values (Fig. 4A), their omission even improved the correlation ($r^2 = 0.9481$, data not shown). In our previous studies of the cytotoxicity of nitroaromatic explosives and natural hydroxyanthraquinones [35,36], we have found that the geometrical mean of the compound reactivity in FNR- and P-450R-catalyzed reactions (log k_{cat}/K_m (FNR) + log k_{cat}/K_m (P-450R))/2 may serve as the parameter substituting the unavailable E_7^1 value. Thus, we extended this approach for the description of 2-NHOH- and 4-NHOH-DNT's with unavailable E_7^1. The log cL_{50} vs. (log k_{cat}/K_m (FNR) + log k_{cat}/K_m (P-450R))/2 dependence is best described by two separate first order regressions, the first one for the nitroaromatics not containing amino- or hydroxylamino groups ($r^2 = 0.8855$), and the second one for the hydroxylamino- and amino metabolites of TNT (compounds 2–6) with $r^2 = 0.7324$ (Fig. 4B). For all the compounds, the linear log cL_{50} vs. (log k_{cat}/K_m (FNR) + log k_{cat}/K_m (P-450R))/2 correlation is characterized by $r^2 = 0.7877$ (data not shown). Thus, the hydroxylamino- and amino metabolites of TNT may indeed be considered as being slightly more cytotoxic than the model nitroaromatics with equal electron accepting potency (Fig. 4B).

Discussion

It has been noted that although the bioremediation may reduce the levels of TNT in the environment, there remains a potential hazard associated with the cytotoxic and mutagenic action of TNT metabolites [21–25]. The available mammalian cell cytotoxicity data of TNT and its metabolites are summarized in Table 3. The data of our work, showing a decreased cytotoxicity of

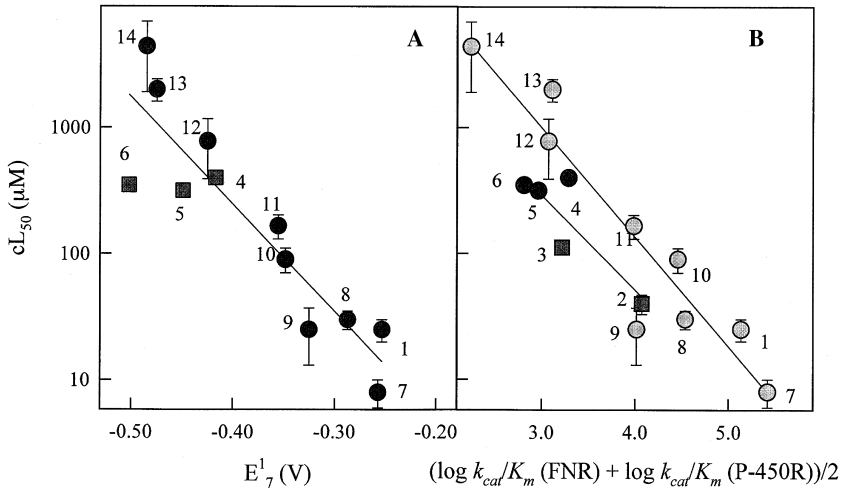

Fig. 4. (A) The dependence of cytotoxicity of TNT, its metabolites, and model nitroaromatic compounds on their single-electron reduction potentials (E_7^1), (B) or on their reactivity towards ferredoxin:NADP$^+$ reductase and NADPH:cytochrome P-450 reductase (log k_{cat}/K_m (FNR) + log k_{cat}/K_m (P-450R))/2. The numbers for TNT and its metabolites (compounds 1–6) are taken from Table 2.. The data for other nitroaromatic compounds are taken from our previous work [35]: *p*-dinitrobenzene (7), *o*-dinitrobenzene (8), *p*-nitrobenzaldehyde (9), *m*-dinitrobenzene (10), *p*-nitroacetophenone (11), *p*-nitrobenzoic acid (12), *p*-nitrobenzyl alcohol (13), and nitrobenzene (14). The hydroxylamino- and amino- metabolites of TNT (compounds 2–6) comprise a separate series in (B).

Table 3
Comparison of the cytotoxicity of 2,4,6-trinitrotoluene (TNT) and its metabolites in different mammalian cell cultures

Compound	cL_{50} (μM)				
	(a) Chinese hamster ovary K1, 24 h [21]	(b) Chinese hamster Lung V79, 24 h [43]	(c) Rat hepatoma H4IIE, 24 h [21]	(d) Human limfoblast TK-6, 48 h [43]	(e) FLK, 24 h, this work
TNT	106	197 ± 36	17.6	22.0 ± 5.0	25.0 ± 5.0
2-NHOH-DNT	—	—	—	—	40.0 ± 7.0
4-NHOH-DNT	18.8	—	28.2	—	112 ± 10
2-NH_2-DNT	>1270	222 ± 76	91.3	168 ± 14	440 ± 35
4-NH_2-DNT	>1270	>328	335	248 ± 51	316 ± 20
2,4-$(NH_2)_2$-NT	>1500	>600	>1500	>600	350 ± 40
2,4,6-$(NH_2)_3$-T	—	5.2 ± 0.8	—	3.3 ± 0.5	—

mono- and diamino metabolites as compared to TNT and its hydroxylamino metabolites (Table 3), are in line with the previous findings. It is interesting to note that 2,4,6-triaminotoluene (2,4,6-$(NH_2)_3$-T) possessed unexpectedly high cytotoxicity (Table 3). However, this compound has not been studied in our work, since it is not formed during the TNT metabolism in mammals, and may be formed in microorganisms only under enforced conditions [21,25]. Except some mechanistic studies of hydroxylamino-DNT's [9,17], the toxicity mechanisms of TNT metabolites are poorly understood at least partly due to the insufficient relevant enzymatic studies. The comparative enzymatic and cytotoxicity studies of a large number of nitroaromatics performed in this work point to the flavoenzyme-catalyzed redox cycling of the TNT metabolites as an important factor in their toxic action.

Our data show that the TNT metabolites (Fig. 1) were less active substrates than TNT in the redox cycling reactions catalyzed by ferredoxin:NADP$^+$ reductase and NADPH:cytochrome P-450 reductase (Table 2). This is consistent with the less favorable energetics of free radical formation (Table 1), and reflect the general trend in P-450R- and FNR-catalyzed nitroreductase reactions (3,6,7). The reasons for the low albeit comparable reactivity of TNT and its hydroxylamino metabolites in NQO1-catalyzed reactions (Table 2) remain unclear, as well as the general criteria for the nitroaromatic substrate specificity in NQO1 catalysis [11,44]. However, the poor reactivity of nitroaromatics contrasting with the high quinone reductase activity of NQO1, may be partly explained by the inefficient stacking of nitroaromatics with the izoalloxazine ring of FAD [45]. In contrast, *E. cloacae* nitroreductase catalyzes relatively fast reduction of hydroxylamino- and monoamino-DNT's (Table 2). As it has been noted before [38], the reactivity of nitroaromatics increases with an increase in their single-electron accepting potency, which probably reflects the possibility of a three-step (e^-, H^+, e^-) hydride transfer during their reduction, with a partly

rate-limiting first electron transfer. Although the data on the NR reactivity are more relevant to the TNT biodegradation mechanisms [22–25] than to its mammalian cell cytotoxicity, they enabled us to characterize the autoxidation of products of the two (four)-electron reduction of hydroxylamino-DNT's (Figs. 2A and B), which may take place under the action of NQO1 in mammalian cells, although with much lower rates (Table 2).

The protective effects of DPPD and desferrioxamine, and the potentiating effect of BCNU towards the cytotoxicity of hydroxylamino- and amino metabolites of TNT (Figs. 3A and B) are analogous to those observed in the cytotoxicity of TNT and model nitroaromatics in FLK cells [35], and point to the prooxidant character of their action. The data of Figs. 4A and B show that log cL_{50} of TNT metabolites follow similar dependence on their E_7^1 and enzymatic reactivity as do the model nitroaromatic compounds. Taken together, these data indicate that the enzymatic redox cycling of TNT metabolites, presumably initiated by their single-electron reduction, is an important factor of their cytotoxicity in FLK cells. Since the cytotoxicity of hydroxylamino-DNT's is not affected by dicumarol, it is unlikely that their reactions with NQO1 contribute to their cytotoxicity. Evidently, other modes of action of hydroxylamino-DNTs, e.g., their covalent binding to proteins [9], or the transition metal-catalyzed redox cycling [17] slightly enhance their cytotoxicity as compared to the model compounds (Figs. 4A and B). The enhanced cytotoxicity of the amino metabolites of TNT (Figs. 4A and B) is most probably not related to their bioreductive activation, since 2,4,6-triaminotoluene, which does not possess nitrogroups, may be even more toxic than TNT (Table 3). Among the other potential mechanisms, one may suggest the possibility of aromatic ring or N-hydroxylation of the amino metabolites of TNT by cytochrome P-450 1B1, which may be present in FLK cells as in other extrahepatic fibroblasts [46,47]. The studies in this direction are currently under way.

Acknowledgments

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