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Quantitative structure–activity relationships in enzymatic single-electron reduction of nitroaromatic explosives: implications for their cytotoxicity

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Abstract

The mechanisms of cytotoxicity of polynitroaromatic explosives, an important group of environmental pollutants, remain insufficiently studied so far. We have found that the rate constants of single-electron enzymatic reduction, and the enthalpies of single-electron reduction of nitroaromatic compounds ($\Delta Hf(ArNO_2^{-\bullet})$), obtained by quantum mechanical calculation, may serve as useful tools for the analysis of cytotoxicity of nitroaromatic explosives with respect to the possible involvement of oxidative stress. The single-electron reduction rate constants of a number of explosives including 2,4,6-trinitrotoluene (TNT) and 2,4,6-trinitrophenyl-*N*-methylnitramine (tetryl), and model nitroaromatic compounds by ferredoxin:$NADP^+$ reductase (FNR, EC 1.18.1.2) and NADPH:cytochrome P-450 reductase (P-450R, EC 1.6.2.4) increased with a decrease in $\Delta Hf(ArNO_2^{-\bullet})$. This indicates that the reduction rates are determined by the electron transfer energetics, but not by the particular structure of the explosives. The cytotoxicity of explosives to bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) increased with a corresponding increase in their reduction rate constant by P-450R and FNR, or with a decrease in their $\Delta Hf(ArNO_2^{-\bullet})$. This points to an importance of oxidative stress in the toxicity of explosives in this cell line, which was further evidenced by the protective effects of desferrioxamine and the antioxidant *N*,*N*′-diphenyl-*p*-phenylene diamine, and an increase in lipid peroxidation. DT-diaphorase (EC 1.6.99.2) exerted a minor and equivocal role in the cytotoxicity of explosives to FLK cells.

Keywords: TNT; Tetryl; Pentryl; Redox cycling; Cytotoxicity; Ferredoxin:$NADP^+$ reductase; NADPH:cytochrome P-450 reductase; DT-diaphorase

1. Introduction

Nitroaromatic compounds are widely used in medicine, industry and agriculture. Nitroaromatic pesticides and explosive residues are considered as being toxic environmental pollutants [1]. For manifestation of their therapeutic and/or toxic properties, most nitroaromatics have to undergo a single- or two-electron enzymatic reduction in organism. Single-electron reduction of nitroaromatic compounds to their anion-radicals is catalyzed by flavoenzymes dehydrogenases-electrontransferases, e.g., NADPH:cytochrome P-450 reductase (P-450R, EC 1.6.2.4) [2–5], ferredoxin:$NADP^+$ reductase (FNR, EC 1.18.1.2) [3,6], bacterial oxygen-sensitive nitroreductases [2]. These reactions are accompanied by futile redox cycling and oxidative stress, since nitroradicals are rapidly reoxidized by oxygen with the formation of superoxide, and, subsequently, hydrogen peroxide and hydroxyl radical. Two-electron reduction of nitroaromatics to nitroso compounds and, subsequently, to alkylating hydroxylamines, is catalyzed by mammalian DT-diaphorase (NAD(P)H:quinone reductase; EC 1.6.99.2) [7–9] and bacterial oxygen-insensitive nitroreductases [10–12].

Frequently, the aerobic cytotoxicity of nitroaromatic compounds increases with an increase in their single-electron reduction potential (E_7^1) with a relationship $\Delta\log cL_{50}$/

Abbreviations: TNT, 2,4,6-trinitrotoluene; TNC, 1,3,6,8-tetranitrocarbazole; E_7^1, single-electron reduction potential; cL_{50}, compound concentration for survival of 50% cells; ΔHf, enthalpy of reaction; *P*, octanol/water partition coefficient; k_{cat}, catalytic constant; k_{cat}/K_m, bimolecular rate constant; FNR, ferredoxin:$NADP^+$ reductase; P-450R, NADPH:cytochrome P-450 reductase

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$\Delta E_7^1 \sim \sim -10\ V^{-1}$, where cL_{50} is the concentration of compound for 50% cell survival [13–17]. These relationships may reflect the relative rates of single-electron reduction of nitroaromatics initiating their redox cycling, since, as a rule, the reactivity of nitroaromatics towards single-electron transferring enzymes increases with their E_7^1 [3–6]. While such an approach represents certain simplification, it may serve as a starting point for the evaluation of cytotoxicity mechanisms, e.g., involvement of oxidative stress.

2,4,6-Trinitrotoluene (TNT), 2,4,6-trinitrophenyl-*N*-methylnitramine (tetryl) and other polynitroaromatic high explosives (Fig. 1) are contaminants of soil and ground water at their manufacturing, processing, and disposal sites [18]. TNT and related polynitroaromatics are alleged to cause cataracts [19], hemolytic crisis [20], and urinary tract tumors [21] in humans. TNT and tetryl are toxic and mutagenic to mammalian cell cultures and bacteria [22,23]. Enzymatic redox cycling of TNT [24,25], and/or the covalent binding of TNT enzymatic reduction products, e.g., 4- or 2-nitroso dinitrotoluenes, to proteins [26] appear to be the most important mechanisms of its cytotoxicity. However, little is known about the mechanisms and quantitative structure–activity relationships of cytotoxicity of other polynitroaromatic explosives (Fig. 1), as well about their mechanisms of bioreductive activation. The studies in this direction are hampered by the absence of E_7^1 values of explosives.

In this work, we have examined the reactivity of nitroaromatic explosives (Fig. 1) towards single-electron transferring flavoenzymes ferredoxin:$NADP^+$ reductase and NADPH: cytochrome P-450 reductase, and towards two-electron transferring DT-diaphorase. We have found that the rate constants of enzymatic single-electron reduction or the enthalpies of single-electron reduction of explosives ($\Delta Hf(ArNO_2^{-\bullet})$), obtained by quantum mechanical calculation, may serve as useful tools for analysis of cytotoxicity of explosives with respect to the possible involvement of their redox cycling.

2. Materials and methods

2.1. Materials

The methods of synthesis of nitrocompounds are listed consecutively: TNT [27]; tetryl (2,4,6-trinitrophenyl-*N*-methylnitramine) and *N*-methylpicramide [28]; TNC (1,3,6,8-tetra-nitrocarbazole) [29]; pentryl (2,4,6-trinitrophenyl-*N*-nitraminoethylnitrate) [30]. Chinifur (2-(5′-nitrofuro-2′-yl)ethene-1-yl)-4-(*N*,*N*-diethylamino)-1-methyl-but-1-ylamino-carbonyl-4-quinoline) was a generous gift from Dr. N.M. Sukhova (Institute of Organic Synthesis, Riga, Latvia). The purity of nitrocompounds was determined using melting points, TLC, NMR, IR, and elemental analysis. All other compounds were obtained from Sigma or Aldrich and used as received.

2.2. Enzymatic assays and analytical procedures

Kinetic measurements were carried out spectrophotometrically using Hitachi-557 spectrophotometer, in 0.1 M K-phosphate buffer (pH 7.0) containing 1 mM EDTA at 25°C. In the experiments with DT-diaphorase, Tween 20 (0.01%) and bovine serum albumin (0.25 mg/ml) were used as activators. NADPH:cytochrome P-450 reductase from pig liver was prepared as described previously [31], the enzyme concentration was determined using $\varepsilon_{460} = 22\ mM^{-1}\,cm^{-1}$. The activity of P-450R (NADPH concentration, 100 μM) using 50 μM cytochrome *c* as the electron acceptor, was 100 s^{-1}. The reduction of cytochrome *c* was monitored using $\Delta\varepsilon_{550} = 20\ mM^{-1}\,cm^{-1}$. Ferredoxin:$NADP^+$ reductase from *Anabaena* was prepared as described previously [32] and was a generous gift of Dr. M. Martinez-Julvez and Professor C. Gomez-Moreno (Zaragoza University, Spain). The enzyme concentration was determined using $\varepsilon_{459} = 9.4\ mM^{-1}\,cm^{-1}$. The activity of FNR (concentration of NADPH, 200 μM), using 1 mM ferricyanide as electron acceptor [32], was equal to 200 s^{-1}. Rat liver DT-diaphorase was prepared as described previously [33]. The enzyme concentration was determined using $\varepsilon_{460} = 11\ mM^{-1}\,cm^{-1}$. NADPH:menadione reductase activity of DT-diaphorase was monitored according to the rate of reduction of cytochrome *c* (concentration of NADPH, 100 μM, concentration of menadione, 10 μM, concentration of cytochrome *c*, 50 μM). The rates of nitroreductase reactions of FNR, P-450R and DT-diaphorase were monitored by following the oxidation of NADPH ($\Delta\varepsilon_{340} = 6.2\ mM^{-1}\,cm^{-1}$, concentration of NADPH, 15–100 μM). Corrections were used when necessary for the formation of tetryl reduction product *N*-methylpicramide ($\Delta\varepsilon_{340} = 12.8\ mM^{-1}\,cm^{-1}$), and products of pentryl reduction with the same absorbance characteristics. The catalytic constant (k_{cat}) and the bimolecular rate constant (k_{cat}/K_m) of nitrocompound reduction 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 NADPH molecules oxidized by a single active center of the enzyme per second. The rate of oxygen consumption during enzymatic reactions was monitored using a Clark electrode. The concentrations of nitrite were determined spectrophotometrically at 540 nm, by monitoring the formation of the azo dye in the presence of sulfanilamide, naphthylethylene diamine dihydrochloride, and a fourfold diluted reaction mixture, as described [34].

2.3. 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 [17,35]. In the cytotoxicity experiments, cells (3×10^4/ml) were grown in the

presence of various amounts of nitrocompounds for 24 h, trypsinized, and counted using a hematocytometer with viability determined by exclusion of Trypan blue. The lipid peroxidation during the 24 h incubation of the cells with nitrocompounds was monitored according to the formation of malondialdehyde, using the thiobarbituric acid test [36].

2.4. Computer calculations, statistical analysis

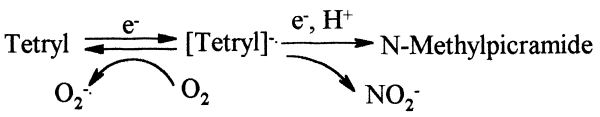
In semiempirical quantum mechanical calculations of compound heat formation (Hf) by AM1 and PM3 methods, PC Spartan Pro (version 1.0.1, Wavefunction) was used. The calculations were performed on nitrocompounds and their single-electron reduced forms. For all calculations, geometries were fully optimized. The enthalpies of reactions (ΔHf) were calculated from Eq. 1, where $ArNO_2$ denotes nitroaromatic compound, and $ArNO_2^{-\bullet}$ denotes its anion-radical:

$$\Delta Hf(ArNO_2^{-\bullet}) = Hf(ArNO_2^{-\bullet}) - Hf(ArNO_2). \quad (1)$$

The octanol/water partition coefficients (*P*) were calculated using ACD logP (version 1) software, a generous gift of Advanced Chemistry Development (Toronto, Canada). The multiparameter regression analysis was performed using Statistica (version 4.3) software (StatSoft, 1993).

3. Results

The single-electron reduction of nitroaromatic compounds by flavoenzymes ferredoxin:$NADP^+$ reductase and NADPH:cytochrome P-450 reductase is extensively documented [3–6]. Therefore, we have used FNR and P-450R as model systems for the evaluation of the redox cycling activity of nitroaromatic explosives. The reduction of TNT, TNC, and *N*-methylpicramide by FNR and P-450R was analogous to the single-electron reactions of other classes of nitroaromatics under aerobic conditions [3–6], i.e., nitrocompound oxidized excess NADPH, with consumption of a stoichiometric amount of O_2 per mole of NADPH. FNR catalyzed superoxide dismutase-sensitive reduction of added cytochrome *c* by nitrocompounds. The k_{cat} for the above compounds, tetryl, and pentryl were close to 100 s^{-1} in the FNR-catalyzed reaction, and close to 50 s^{-1} in the P-450R-catalyzed reaction. The reactions of tetryl and pentryl should be considered separately. It has been shown [37–39] that FNR performed the single-electron reductive N-denitration of tetryl, accompanied by redox cycling and nitrite formation:



(2)

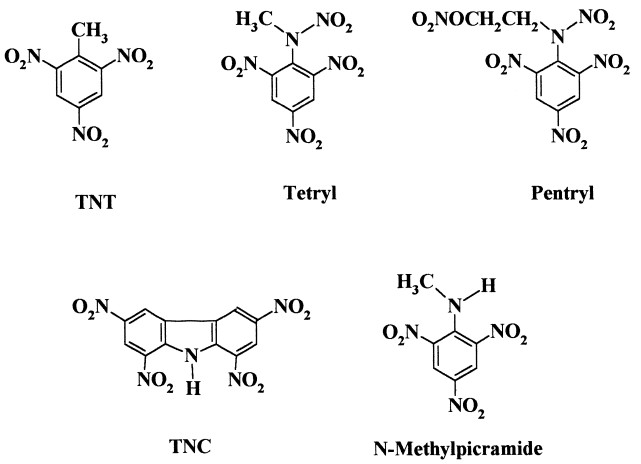


Fig. 1. The formulae of nitroaromatic explosives studied in this work: 2,4,6-trinitrotoluene (TNT), 2,4,6-trinitrophenyl-*N*-methylnitramine (tetryl), 2,4,6-trinitrophenyl-*N*-nitramino-ethylnitrate (pentryl), and 1,3,6,8-tetranitrocarbazole (TNC).

The kinetic parameters of the reaction were determined in our previous papers [38,39]. In this study, we found that the reactions of tetryl with P-450R, and the reactions of pentryl with FNR and P-450R shared the same reductive denitration mechanism (Eq. 2). Tetryl formed 0.12 ± 0.02 mol nitrite and *N*-methylpicramide per mol NADPH oxidized in a P-450R-catalyzed reaction ($n = 3$). Pentryl formed 0.45 ± 0.07 and 0.75 ± 0.09 mol NO_2^- per mol NADPH oxidized in P-450R- and FNR-catalyzed reactions, respectively. The absorbance spectra of unidentified product(s) of pentryl reduction by FNR and P-450R (λ_{max} at 340 and 420 nm) were almost the same as the spectra of *N*-methylpicramide [38,39].

The bimolecular reduction rate constants (k_{cat}/K_m) of nitroaromatic explosives by FNR and P-450R are given in Table 1, together with k_{cat}/K_m of model nitroaromatic compounds (partly determined in the present study, and partly taken from our previous work [38]). As observed previously [3–6], k_{cat}/K_m of nitrocompounds increased correspondingly with an increase in their E_7^1 (Table 1), the coefficient $\Delta(\log k_{cat}/K_m)/\Delta E_7^1$ being equal to 11.32 ± 1.23 V^{-1} (FNR) and 8.94 ± 0.95 V^{-1} (P-450R, data not shown). Since the values of E_7^1 of nitroaromatic explosives are not available, we have examined if the compound reactivities depended on their enthalpies of anion-radical formation ($\Delta Hf(ArNO_2^{-\bullet})$, obtained by means of quantum mechanical calculation (Table 1). It is known that these parameters exhibit a correlation with single-electron transfer redox potentials [12,41]. The linear correlations between $\Delta Hf(ArNO_2^{-\bullet})$ and $\log k_{cat}/K_m$ were characterized by $r^2 = 0.6914$ (FNR) and $r^2 = 0.7909$ (P-450R) (AM1, data not shown); however, the data were better described by a second-order polynomial regression ($r^2 = 0.7580$ (FNR), and $r^2 = 0.8255$ (P-450R) (Fig. 2)). The use of $\Delta Hf(ArNO_2^{-\bullet})$ values obtained by means of the PM3 method, gave the analogous results. This is consistent with an

Table 1
Bimolecular steady-state rate constants (k_{cat}/K_m) of reduction of nitroaromatic compounds by ferredoxin:NADP$^+$ reductase (FNR), NADPH:cytochrome P-450 reductase (P-450R), and DT-diaphorase, their single-electron reduction potentials (E_7^1), enthalpies of single-electron reduction ($\Delta Hf(ArNO_2^{-\bullet})$), their concentrations for 50% survival of FLK cells (cL_{50}), and octanol/water partition coefficients (P)

No. Compound	k_{cat}/K_m ($M^{-1}\,s^{-1}$)			E_7^1 (V) [40]	$\Delta Hf(ArNO_2^{-\bullet})$ (kcal/mol)		cL_{50} (μM)	log P
	(a) FNR	(b) P-450 R	(c) DT-diaphorase		AM1	PM3		
1. Nitrobenzene	$1.1\pm0.2\times10^{1}$[a]	$2.8\pm0.3\times10^{3}$	≤24 (≤0.06)[b,c]	−0.485	−40.07	−41.13	4370 ± 2470[b]	1.95 ± 0.4
2. *p*-Nitrobenzyl alcohol	$1.4\pm0.1\times10^{2}$	$1.2\pm0.15\times10^{4}$	≤65 (≤0.12)[c]	−0.475	−44.54	−46.04	2000 ± 400	0.75 ± 0.2
3. *p*-Nitrobenzoic acid	$2.5\pm0.2\times10^{2}$[a]	$5.6\pm0.5\times10^{3}$	$1.0\pm0.2\times10^{2}$ (≤0.05)[b,c]	−0.425	−54.54	−56.53	780 ± 390[b]	1.89 ± 0.3
4. *p*-Nitroacetophenone	$1.0\pm0.1\times10^{3}$[a]	$9.4\pm0.6\times10^{4}$	≤86 (≤0.06)[b,c]	−0.355	−51.88	−52.12	166 ± 36[b]	1.42 ± 0.3
5. 3,5-Dinitrobenzamide	$1.2\pm0.1\times10^{4}$	$3.0\pm0.3\times10^{5}$	≤60 (≤0.12)[b,c]	−0.350	−67.90	−69.11	89 ± 35[b]	0.55 ± 0.2
6. *m*-Dinitrobenzene	$2.5\pm0.2\times10^{3}$[a]	$3.3\pm0.4\times10^{5}$	$7.5\pm1.0\times10^{2}$ (0.33 ± 0.05)[c]	−0.348	−60.85	−62.65	90 ± 20	1.62 ± 0.2
7. *p*-Nitrobenzaldehyde	$8.3\pm0.7\times10^{3}$[a]	$1.3\pm0.1\times10^{4}$	≤50 (≤0.1)[b,c]	−0.325	−53.09	−53.34	25 ± 12[b]	1.56 ± 0.3
8. *o*-Dinitrobenzene	$2.2\pm0.2\times10^{3}$[a]	$5.4\pm0.4\times10^{5}$	$6.6\pm1.0\times10^{2}$ (≤0.14)[c]	−0.287	−61.50	−62.38	30 ± 5.0	1.84 ± 0.2
9. *p*-Dinitrobenzene	$3.0\pm0.2\times10^{4}$[a]	$2.3\pm0.2\times10^{6}$	$1.6\pm0.2\times10^{3}$ (1.5 ± 0.2)[c]	−0.257	−65.32	−67.17	8.0 ± 2.0	1.37 ± 0.2
10. Nitrofurantoin	$1.5\pm0.1\times10^{4}$[a]	$4.9\pm0.4\times10^{5}$	≤10[b]	−0.255	−57.86	−59.74	120 ± 36[b]	-0.54 ± 0.2
11. Nifuroxim	$7.5\pm0.5\times10^{3}$[a]	$5.0\pm0.3\times10^{5}$	≤10[b]	−0.255	−53.08	−54.48	4.9 ± 2.2[b]	0.73 ± 0.2
12. Chinifur	$6.0\pm0.4\times10^{4}$[a]	$1.5\pm0.1\times10^{6}$	≤10[b]	−0.225[d]	−62.68	−62.65	1.3 ± 0.3[b]	4.67 ± 0.2
13. 2,4,6-Trinitrotoluene	$5.3\pm0.4\times10^{3}$[a]	$1.7\pm0.1\times10^{6}$	$6.7\pm0.7\times10^{2}$ (1.0 ± 0.1)[c]	–	−74.26	−75.63	25 ± 5.0	1.68 ± 0.3
14. Tetryl	$1.1\pm0.2\times10^{5}$[e]	$2.3\pm0.1\times10^{7}$	$2.6\pm0.3\times10^{5}$ (73 ± 10)[c,e]	–	−91.21	−87.90	2.2 ± 0.3	1.49 ± 0.3
15. Pentryl	$5.5\pm0.5\times10^{5}$	$1.3\pm0.1\times10^{7}$	$5.7\pm0.7\times10^{5}$ (75 ± 10)[c]	–	−94.33	−89.98	5.0 ± 1.0	2.22 ± 0.4
16. 1,3,6,8-Tetranitrocarbazole	$7.3\pm0.5\times10^{4}$	$2.6\pm0.2\times10^{7}$	$3.6\pm0.7\times10^{4}$ (3.6 ± 0.4)[c]	–	−86.61	−87.90	8.0 ± 2.0	2.52 ± 0.4
17. *N*-methylpicramide	$2.6\pm0.2\times10^{4}$	$2.0\pm0.1\times10^{6}$	≤10	–	−71.03	−70.45	40 ± 7.0	3.02 ± 0.4

[a]From [38].
[b]From [17].
[c]The values of k_{cat} (s^{-1}) given in parentheses.
[d]C. Houee-Levin, N. Čėnas, unpublished.
[e]From [39].

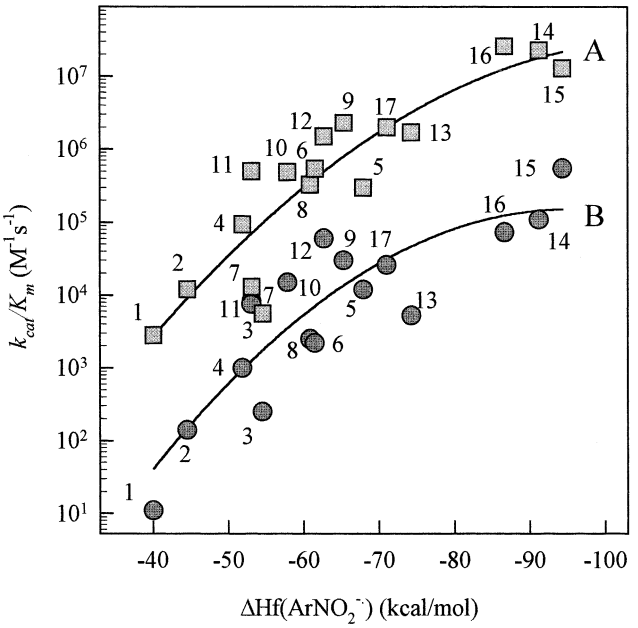


Fig. 2. The dependence of log k_{cat}/K_m of nitroaromatic compounds in NADPH:cytochrome P-450 reductase (A) and ferredoxin:NADP$^+$ reductase-catalyzed reactions (B) on their enthalpies of single-electron reduction ($\Delta Hf(ArNO_2^{\cdot-})$) calculated using the AM1 method. The numbers of compounds are taken from Table 1. The curves represent the second-order polynomial regression.

'outer-sphere' single-electron transfer mechanism [42], where the reaction rate is determined primarily by the redox potential difference of the reagents. Thus, the high reactivity of nitroaromatic explosives towards FNR and P-450R is determined not by their particular structure, e.g., the presence of benzene, furan, or carbazole ring, but by the more favourable energetics of single-electron reduction, governed by the presence of electron-accepting substituents.

As a rule, nitroaromatics are poor substrates for DT-diaphorase, their k_{cat} does not exceeds 5 s^{-1}, and their reactivity is not dependent on redox potential [7–9,17]. DT-diaphorase plays a key role in the cytotoxicity of certain nitroaromatic compounds (e.g., 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB-1954) and its derivatives), in cell lines which are extremely rich in DT-diaphorase, such as Walker cells (ca. 10^4 nmol NADPH min^{-1} (mg protein)$^{-1}$ [8], and a minor role in cells with a moderate DT-diaphorase amount [17]. We have examined the reactions of nitroaromatic explosives and several model compounds with this enzyme. The reaction rate constants, partly determined in the present study, and partly taken from our previous works [17,39], are given in Table 1. The reactions of DT-diaphorase with tetryl and pentryl also should be considered separately. We have shown that DT-diaphorase performs the single-electron *N*-denitration of tetryl accompanied by redox cycling and formation of 0.08 ± 0.01 mol nitrite per mol NADPH oxidized (Eq. 2) [39]. The reduction rate of added cytochrome *c* accounted for 132% of

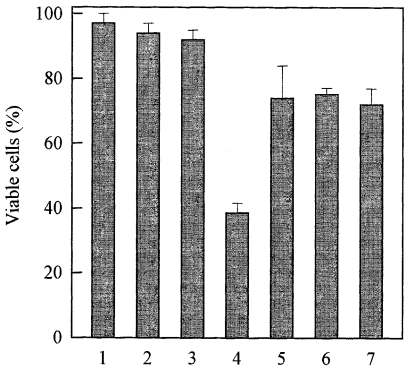


Fig. 3. The protecting effects of DPPD (2 μM) and desferrioxamine (300 μM) in the cytotoxicity of TNT (20 μM). Additions: DPPD (1), desferrioxamine (2), DPPD+desferrioxamine (3), TNT (4), TNT+DPPD (5), TNT+desferrioxamine (6), and TNT+DPPD+desferrioxamine (7). Cell viability in control experiment, $95 \pm 3\%$, $n = 3$–4.

NADPH oxidation rate. In parallel, tetryl was reduced to unidentified product(s), presumably in a two-electron way [39]. It has been found in this study that the reduction of pentryl by DT-diaphorase proceeded similarly, and accompanied by formation of 0.18 ± 0.02 mol NO_2^- per mol NADPH oxidized. However, the reduction rate of added cytochrome *c* accounted for only 28% of NADPH oxidation rate, thus indicating a dominant two-electron reaction way. The absorbance maxima (340 nm and 420 nm) of the unidentified product(s) of pentryl reduction were similar to those obtained after reduction by FNR and P-450R (data not shown).

For the cytotoxicity experiments, we have used the bovine leukemia virus-transformed lamb kidney fibroblast line FLK used in our previous studies [9,17,35]. This line

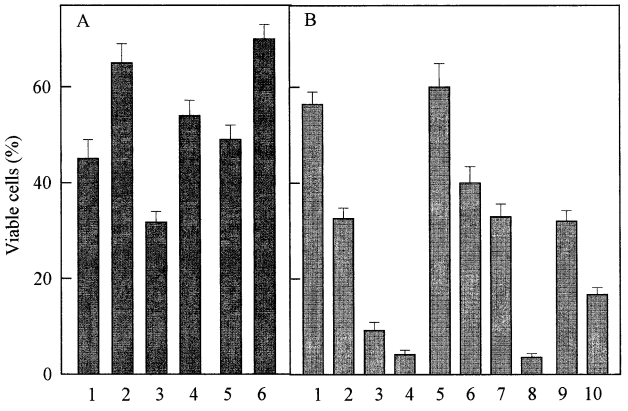


Fig. 4. The protecting (A) and potentiating effects (B) of 20 μM dicumarol in the cytotoxicity of nitroaromatic compounds. (A) Cell viability in the presence of 20 μM TNT (1), 20 μM TNT+dicumarol (2), 40 μM TNT (3), 40 μM TNT+dicumarol (4), 100 μM *m*-dinitrobenzene (5), and 100 μM *m*-dinitrobenzene+dicumarol (6). (B) Cell viability in the presence of 2 μM tetryl (1), 2 μM tetryl+dicumarol (2), 4 μM tetryl (3), 4 μM tetryl+dicumarol (4), 6 μM TNC (5), 6 μM TNC+dicumarol (6), 12 μM TNC (7), 12 μM TNC+dicumarol (8), 12 μM *p*-dinitrobenzene (9), and 12 μM *p*-dinitrobenzene+dicumarol (10). Cell viability in control experiment, $95 \pm 3\%$, $n = 3$–4. Dicumarol did not affect cell viability.

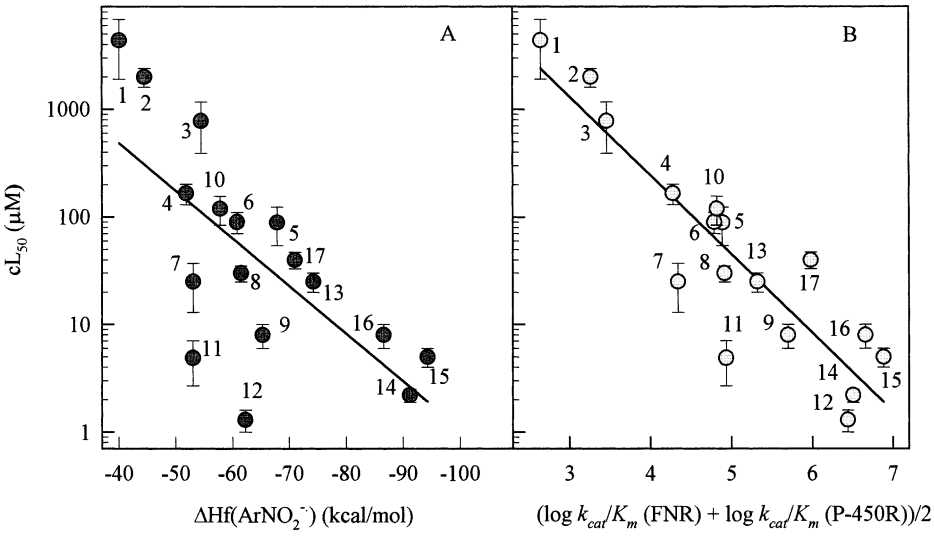


Fig. 5. (A) The dependence of cytotoxicity of nitroaromatic compounds on their enthalpies of single-electron reduction ($\Delta Hf(ArNO_2^{-\bullet})$) calculated using AM1 method,. (B) The dependence of cytotoxicity of nitroaromatic compounds on their reactivity towards ferredoxin:$NADP^+$ reductase and NADPH: cytochrome P-450 reductase (log k_{cat}/K_m (FNR)+log k_{cat}/K_m (P-450R))/2. The numbers of compounds are taken from Table 1.

is characterized by the activity of NADPH:cytochrome *c* reductase, 43 ± 1 nmol cytochrome *c* min^{-1} (mg protein)$^{-1}$; NADH:cytochrome *c* reductase, 141 ± 8 nmol cytochrome *c* min^{-1} (mg protein)$^{-1}$; and DT-diaphorase, 260 ± 30 nmol NADPH min^{-1} (mg protein)$^{-1}$ [35]. Table 1 shows the concentrations of nitroaromatic explosives for 50% cell survival (cL_{50}), and the cL_{50} values for model compounds (partly determined in the present study, and partly taken from our previous work [30]). The toxicity of several randomly selected compounds almost did not change when the serum amount was decreased from 10% to 2.5%, e.g., cL_{50} of TNC decreased from 8.0 ± 2.0 μM (Table 1) to 6.5 ± 1.5 μM, cL_{50} of TNT increased from 25 ± 5.0 μM to 30 ± 5.0 μM, and cL_{50} of *p*-nitrobenzyl alcohol increased from 2000 ± 400 μM to 2400 ± 300 μM. Thus, the nonspecific binding of nitrocompounds with serum exerts minor influence on their toxicity. It is evident that tetryl, pentryl and TNC are more toxic than TNT and are among the most cytotoxic compounds in this study (Table 1). As in our previous studies of toxicity of other nitroaromatic compounds to FLK cells [17], the antioxidant *N*,*N*′-diphenyl-*p*-phenylene diamine (DPPD) [43] and the iron-chelating agent desferrioxamine, the latter preventing the Fenton reaction, gave the partial protection from cytotoxicity of TNT (Fig. 3), and tetryl (data not shown). Other evidence of oxidative stress was an increase in the intracellular content of the lipid peroxidation product malondialdehyde. After 24 h incubation with 60 μM TNT or 6 μM tetryl that resulted in 85–90% cell death, the content of malondialdehyde was equal to 2.2 ± 0.4 nmol/10^6 cells, whereas in untreated cells it was equal to 0.6 ± 0.2 nmol/10^6 cells.

We have also endeavoured to assess the possible role of DT-diaphorase in the cytotoxicity of nitrocompounds. Dicumarol, an inhibitor of DT-diaphorase, gave partial protection against the cytotoxicity of TNT and *m*-dinitrobenzene, but potentiated the cytotoxicity of *p*-dinitrobenzene, TNC and tetryl (Fig. 4A,B). However, studies at several fixed concentrations of the above compounds revealed that dicumarol affected cL_{50} values not significantly, i.e., decreased or increased them approximately by 1.5 times (data not shown).

4. Discussion

The data of this work show that polynitroaromatic explosives like TNT, tetryl, pentryl and TNC (Fig. 1) were among the most active substrates in redox cycling reactions catalyzed by ferredoxin:$NADP^+$ reductase and NADPH:cytochrome P-450 reductase (Table 1 and Fig. 2). Although the values of E_7^1 for these compounds are not currently available, the use the of enthalpies of free radical formation as the correlation parameter (Fig. 2) provides good evidence that their reactivity is related not to their particular structure, but to the favourable energetics of single-electron reduction. The reasons for the high reactivity of tetryl and pentryl in DT-diaphorase-catalyzed reactions, and the poor reactivity of TNT and TNC (Table 1) remain unclear, as well as the general criteria for substrate specificity in DT-diaphorase catalysis [44–46].

We have shown previously [17], that the toxicity of a number of aromatic nitrocompounds to FLK cells increased with an increase in their E_7^1 and their octanol/water partition coefficient (*P*, Table 1), and that DT-diaphorase played a minor and equivocal role. The cytotoxicity was accompanied by lipid peroxidation, and was partly prevented by DPPD and desferrioxamine. These data pointed to oxidative stress as the most important cytotoxicity factor. In this work, we have extended the

number of model nitrocompounds with available E_7^1 values that have been used for cytotoxicity studies (Table 1). Again, the cytotoxicity of compounds 1–12 (Table 1) is described by a multiparameter Eq. 3, which confirms the importance of compound redox cycling activity and lipophilicity:

$$\log cL_{50}\ (\mu M) = -(1.4216 \pm 0.4472) - (10.7381 \pm 1.1942)$$
$$E_7^1\ (V) - (0.2094 \pm 0.0847) \log P,\ r^2 = 0.9125 \quad (3)$$

Interestingly, if compound reactivity towards DT-diaphorase (k_{cat}/K_m (DT), Table 1) is introduced as a third parameter, it does not improve the correlation, and points to an equivocal role of DT-diaphorase in cytotoxicity:

$$\log cL_{50}\ (\mu M) = -(1.3436 \pm 0.5361) - (10.7381 \pm 1.1942)$$
$$E_7^1\ (V) - (0.2095 \pm 0.0893) \log P - (0.0444 \pm 0.1452)$$
$$\log k_{cat}/K_m\ (DT)\ (M^{-1}\ s^{-1}),\ r^2 = 0.9135 \quad (4)$$

This agrees with the data in Fig. 4, which shows that an inhibitor of DT-diaphorase, dicumarol, may either decrease or increase the cytotoxicity of nitroaromatics.

The action of polynitroaromatic explosives on FLK cells was partially inhibited by DPPD and desferrioxamine (Fig. 3), and was also accompanied by lipid peroxidation. This indicates that oxidative stress plays an important role in cytotoxicity. Since the E_7^1 values for explosives are not available, the use $\Delta Hf(ArNO_2^{-\bullet})$ as a parameter for a description of cytotoxicity may be considered. However, the correlations between log cL_{50} and $\Delta Hf(ArNO_2^{-\bullet})$ were poor ($r^2 = 0.4717$ (AM1) (Fig. 5A), $r^2 = 0.4645$ (PM3), data not shown), although they were slightly improved using log P as a second variable, giving $r^2 = 0.5130$ (AM1):

$$\log cL_{50}\ (\mu M) = -(4.5709 \pm 0.7998) + (0.0414 \pm 0.0124)$$
$$\Delta Hf(ArNO_2^{-\bullet})\ (kcal/mol) - (0.1877 \pm 0.1723) \log P \quad (5)$$

On the other hand, the use of nitroaromatics reactivity in single-electron enzymatic reduction reactions as the parameter of correlation, gave more straightforward results. The linear dependence of log cL_{50} on the geometrical mean of reactivity of nitroaromatic explosives and model compounds in FNR- and P-450R-catalyzed reactions (log k_{cat}/K_m (FNR)+log k_{cat}/K_m (P-450R))/2 was characterized by $r^2 = 0.7755$ (Fig. 5B). The correlation was slightly improved by using log P as a second variable:

$$\log cL_{50}\ (\mu M) = (5.3139 \pm 0.5036) - (0.7311 \pm 0.1063)$$
$$(\log k_{cat}/k_m\ (FNR)\ + \log k_{cat}/K_m\ (P{-}450R))/2$$
$$(M^{-1}\ s^{-1}) - (0.1510 \pm 0.1094) \log P,\ r^2 = 0.8025 \quad (6)$$

This correlation, supported by the data on antioxidant protection (Fig. 3), shows that the toxicity of nitroaromatic explosives to FLK cells is mainly determined by their redox cycling reactions. It is interesting to note that tetryl and pentryl also follow the same cytotoxicity vs. redox cycling rate relationship (Fig. 5B) as other nitroaromatic compounds, although their redox cycling reactions are more complex, being accompanied by nitrite formation (Eq. 2).

The effects of dicumarol (Fig. 4A,B) and Eq. 4 point to a minor and equivocal role of reactions of explosives and model compounds with DT-diaphorase in their cytotoxicity to FLK cells. Indeed, the formation of alkylating hydroxylamine products of nitroreduction may be prevented by the inhibition of DT-diaphorase. However, it is difficult to explain the potentiation of cytotoxicity of several nitroaromatic compounds by dicumarol (Fig. 4B). In this respect one should note, that our understanding of the cytotoxic consequences and metabolic fate of products of two- or four-electron reduction of nitroaromatics by DT-diaphorase is far from complete. The mono- and dihydroxylamine products of TNT reduction by bacterial nitroreductases are unstable, apart from being reoxidized by oxygen, they may react with nitroso compounds forming azoxy derivatives, or isomerize to aminophenols [47,48]. The products of the reduction of TNT and other polynitroaromatics by DT-diaphorase were not characterized in this study. However, our preliminary data indicate, that the products of DT-diaphorase-catalyzed reduction of TNT and *o*-, *m*-, and *p*-dinitrobenzenes are unstable, being converted to secondary products, which acted as more efficient substrates for DT-diaphorase. Evidently, the DT-diaphorase-related cytotoxicity is a complex function of the activity of the compound as a nitroreductase substrate, the alkylating activity of nitroso- or hydroxylamine products, and the rates of side-reactions or rearrangement of products, which may cause loss of their alkylating ability.

The cytotoxic, mutagenic and carcinogenic properties of nitroaromatic environmental pollutants are frequently related to their electron-accepting properties, which may be linked to their bioreductive enzymatic activation [1]. Interestingly, tetryl has been reported to be three times more potent mutagen than TNT for *Salmonella typhimurium* [22], which is in line with its better redox cycling properties and higher mammalian cell culture cytotoxicity (Table 1). The data of this work indicate that the reactivity of nitroaromatic explosives in single-electron enzymatic reduction reactions may serve as a useful tool for a quantitative description of their cytotoxicity with the possible involvement of oxidative stress. This approach may be extended for nitroaromatic and other compounds with unknown single-electron reduction potential values. The more thorough studies in this direction with emphasis on structure–activity relationships causing apoptotic cell response are under way.

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