Enzymatic Redox Properties of Novel Nitrotriazole Explosives Implications for their Toxicity

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The toxicity of conventional nitroaromatic explosives like 2,4,6-trinitrotoluene (TNT) is caused by their enzymatic free radical formation with the subsequent oxidative stress, the formation of alkylating nitroso and/or hydroxylamino metabolites, and oxyhemoglobin oxidation into methemoglobin. In order to get an insight into the mechanisms of toxicity of the novel explosives NTO (5-nitro-1,2,4-triazol-3-one) and ANTA (5-nitro-1,2,4-triazol-3-amine), we examined their reactions with the single-electron transferring flavoenzymes NADPH: cytochrome P-450 reductase and ferredoxin:$NADP^+$ reductase, two-electron transferring flavoenzymes mammalian NAD(P)H:quinone oxidoreductase (DT-diaphorase), and *Enterobacter cloacae* NAD(P)H:nitroreductase, and their reactions with oxyhemoglobin. The reactivity of NTO and ANTA in the above reactions was markedly lower than that of TNT. The toxicity of NTO and ANTA in bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) was partly prevented by desferrioxamine and the antioxidant *N*,*N*′-diphenyl-*p*-phenylene diamine, and potentiated by 1,3-bis-(2-chloroethyl)-1-nitrosourea. This points to the involvement of oxidative stress in their cytotoxicity, presumably to the redox cycling of free radicals. The FLK cell line cytotoxicity and the methemoglobin formation in isolated human erythrocytes of NTO and ANTA were also markedly lower than those of TNT, and similar to those of nitrobenzene. Taken together, our data demonstrate that the low toxicity of nitrotriazole explosives may be attributed to their low electron-accepting properties.

Key words: Nitroaromatic Explosives, Cytotoxicity, Oxidative Stress, Hemoglobin

Introduction

Conventional nitroaromatic explosives like 2,4,6-trinitrotoluene (TNT, Fig. 1) are toxic to mammalian species, causing damage to liver, kidney, and spleen, as well as methemoglobinemia and hemolytic crisis (Dilley *et al.*, 1982; Djerassi, 1998). The mechanisms of toxicity of TNT and other polynitroaromatics involve the oxidative stress caused by the flavoenzyme-catalyzed free-radical formation and their redox cycling (Kong *et al.*, 1989; Čėnas *et al.*, 2001), the direct oxidation of oxyhemoglobin ($Hb\text{-}Fe^{2+}O_2$) in erythrocytes (Cossum and Rickert, 1987; Marozienė *et al.*, 2001), and the formation of nitroso- and/or hydroxylamino products of two-electron reduction which may modify proteins and DNA, and induce methemoglobin ($Hb\text{-}Fe^{3+}$) formation (Facchini and Griffiths, 1981; Leung *et al.*, 1995; Homma-Takeda *et al.*, 2002).

Nitrotriazoles NTO (5-nitro-1,2,4-triazol-3-one) and ANTA (5-nitro-1,2,4-triazol-3-amine) (Fig. 1) represent explosives of a novel generation, selected for their performance analogous to classical explosives, and the concomitantly higher stability against physical constraints (Sikder *et al.*, 2001, and references cited therein). Their processing is accompanied by the release of wastewater with high compound concentration (10–15 g/l NTO), which may represent a potential threat to the envi-

Abbreviations: NTO, 5-nitro-1,2,4-triazol-3-one; ANTA, 5-nitro-1,2,4-triazol-3-amine; TNT, 2,4,6-trinitrotoluene; cL_{50}, the concentration of compound for 50% cell survival; FNR, ferredoxin:$NADP^+$ reductase; P-450R, NADPH:cytochrome P-450 reductase; NQO1, NAD(P)H: quinone oxidoreductase; NR, NAD(P)H:nitroreductase; k_{cat}, catalytic constant; k_{cat}/K_m, bimolecular rate constant; $Hb\text{-}Fe^{3+}$, methemoglobin; $Hb\text{-}Fe^{2+}O_2$, oxyhemoglobin; DPPD, *N*,*N*′-diphenyl-*p*-phenylene diamine; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; $\Delta Hf(ArNO_2^{-\cdot})$, enthalpy of the single-electron reduction of the nitroaromatic compound.

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ronment (Le Campion *et al.*, 1999). However, the biochemical and toxicological studies of NTO and ANTA are almost absent, except the preliminary toxicity data of NTO in mice or rats (London and Smith, 1985), and the studies of the NTO microbial degradation (Le Campion *et al.*, 1999).

In this paper, we examined the enzymatic reactions of NTO and ANTA relevant to their cytotoxicity, *i.e.*, their single- and two-electron reduction by flavoenzymes, and oxyhemoglobin oxidation. The low reactivity of NTO and ANTA in the above reactions correlated with their low observed mammalian cell cytotoxicity and methemoglobin formation activity in erythrocytes *in vitro*.

Materials and Methods

NTO and ANTA were synthesized according to the established methods (Sikder *et al.*, 2001). The purity of nitroaromatic compounds was determined using melting points, TLC, NMR, IR, and elemental analysis. All the other compounds were obtained from Sigma or Aldrich and used as received.

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, EC 1.6.4.2) from pig liver was prepared as described by Yasukochi and Masters (1976), its concentration being determined using $\varepsilon_{460} = 22\ mM^{-1}\ cm^{-1}$. The activity of P-450R using 50 μM cytochrome c as an electron acceptor (the concentration of NADPH, 100 μM) was determined using $\Delta\varepsilon_{550} = 20\ mM^{-1}\ cm^{-1}$, and was equal to 77 μmol $mg^{-1}\ min^{-1}$. Ferredoxin:$NADP^+$ reductase (FNR, EC 1.18.1.2) from *Anabaena* was prepared as described by Pueyo and Gomez-Moreno (1991) and was a generous gift of Dr. M. Martines-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 using 1 mM ferricyanide as electron acceptor (the concentration of NADPH, 200 μM) was determined using $\Delta\varepsilon_{420} = 1.0\ mM^{-1}\ cm^{-1}$, and was equal to 330 μmol $mg^{-1}\ min^{-1}$. Rat liver DT-diaphorase (NQO1, EC 1.6.99.2) was prepared as described by Prochaska (1988), its concentration being determined using $\varepsilon_{460} = 11\ mM^{-1}\ cm^{-1}$. In the experiments with NQO1, 0.01% Tween 20 and 0.25 mg/ml bovine serum albumin were added as activators (0.01%). The activity of NQO1 determined according to the rate of the menadione-mediated reduction of 50 μM cytochrome c (concentration of NADPH, 100 μM; concentration of menadione, 10 μM) was equal to 3300 μmol $mg^{-1}\ min^{-1}$. The recombinant *Enterobacter cloacae* NAD(P)H:nitroreductase (NR, EC 1.6.99.7) was prepared as described by Koder and Miller (1998), and was a generous gift of Dr. R. L. Koder and Professor A.-F. 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-HCl (pH 7.0), containing 0.5 mM desferrioxamine. The rates of enzymatic oxidation of NADPH by nitroaromatic compounds were determined according to $\Delta\varepsilon_{340} = 6.2\ mM^{-1}cm^{-1}$, at the concentration of 100 μM NADPH. The catalytic constant (k_{cat}) and the bimolecular rate constant (k_{cat}/K_m) of the reduction of nitroaromatics were calculated from the Lineweaver-Burk plots. k_{cat} is the number of NADPH molecules oxidized by the single active center of an enzyme per second. The rates obtained were corrected for the intrinsic NADPH-oxidase activity of the enzymes. The rate of oxygen consumption was monitored using a Clark electrode.

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 (Čėnas *et al.*, 2001). 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 the viability determined by the exclusion of Trypan blue. Before the count, the cells were trypsinized.

The freshly prepared suspensions of erythrocytes from healthy patients obtained from Vilnius Blood Transfusion Center were washed twice by centrifugation, resuspended in 0.01 M K-phosphate (pH 7.4) containing 0.137 M NaCl, 0.0027 M KCl, 10 mM glucose and 1 mM EDTA, and stored at 4 °C for not more than 7–10 d. For the kinetics studies, the erythrocytes were lysed in a buffer solution containing 40 μg/ml digitonin. The oxyhemoglobin (Hb-$Fe^{2+}O_2$) concentration was adjusted to 20 μM [$\varepsilon_{577} = 15\ mM^{-1}cm^{-1}$ (Winterbourn, 1985)]. The kinetics of methemoglobin (Hb-Fe^{3+}) formation were monitored according to the absorbance rise at 630 nm and the absorbance decrease

at 577 nm [$\Delta\varepsilon_{630}$ = 3.46 мм$^{-1}$см$^{-1}$, $\Delta\varepsilon_{577}$ = 10.55 мм$^{-1}$см$^{-1}$ (Winterbourn, 1985)] after the addition of excess oxidant (molar ratio 1:10–100) at 37 °C. Intact erythrocytes at 40% hematocrite (40% v/v in buffer solution) were incubated with 300 μм NTO or ANTA for 24 h. The aliquots of the reaction mixture were lysed in a digitonin solution, the Hb-Fe^{2+}O$_2$ and Hb-Fe^{3+} concentrations (μм) were calculated according to the absorbance at 577 nm and 630 nm: [Hb-Fe^{2+}O$_2$] = 66 A_{577}–80 A_{630}, and [Hb-Fe^{3+}] = 279 A_{630}–3.0 A_{577} (Winterbourn, 1985).

In the semiempirical calculations of compound heat formation *(Hf)* by the AM1) and PM3 method, PC Spartan Pro (version 1.0.1, Wavefunction) was used. For all calculations, the geometries were fully optimized. The enthalpy of nitroanion-radical formation [$\Delta Hf(ArNO_2^{-\cdot})$] was calculated from equation (1), where $ArNO_2$ denotes the nitroaromatic compound, and $ArNO_2^{-\cdot}$ denotes its anion-radical:

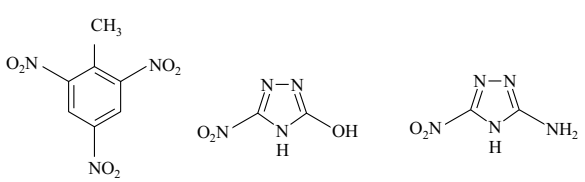
$$\Delta Hf(ArNO_2^{-\cdot}) = Hf(ArNO_2^{-\cdot}) - Hf(ArNO_2) \qquad (1)$$

The statistical analysis was performed using Statistica (version 4.3, StatSoft, 1993).

Results and Discussion

In this work, we examined the redox reactions of NTO and ANTA (Fig. 1) which may be important in their toxicity: i) single-electron reduction by flavoenzymes dehydrogenases-electrontransferases NADPH:cytochrome P-450 reductase (P-450R) and ferredoxin:NADP$^+$ reductase (FNR) which are frequently used as model systems to study the free radical formation of nitroaromatic compounds and their redox cycling (Orna and Mason, 1989; Čėnas *et al.*, 2001);

ii) their direct oxidation of oxyhemoglobin into methemoglobin



TNT NTO ANTA

Fig. 1. Structural formulae of explosives studied in this paper.

$$NTO\ (ANTA) + \text{Hb-Fe}^{2+}O_2 \rightarrow NTO^{-\cdot}\ (ANTA^{-\cdot}) + \text{Hb-Fe}^{3+} + O_2^{-\cdot} \qquad (2)$$

which may be partly responsible for the methemoglobin formation by nitroaromatic compounds in organism (Cossum and Rickert, 1987), and

iii) their two-electron reduction by rat NAD(P)H: quinone oxidoreductase (DT-diaphorase, NQO1) and *Enterobacter cloacae* NAD(P)H-:nitroreductase (NR). These enzymes reduce nitroaromatics to the corresponding hydroxylamines in two subsequent two-electron transfer steps (Knox *et al.*, 1993; Koder and Miller, 1998). Although the nitroreductase activity of NQO1 is low even for electron-deficient compounds like TNT, this enzyme may enhance the mammalian cell cytotoxicity of certain nitroaromatics (Knox *et al.*, 1993; Čėnas *et al.*, 2001). The occurrence of *Enterobacter* sp. in the gastrointestinal tract which is one of the sources of the formation of toxic aromatic hydroxylamines in mammals (Koder and Miller, 1998; Sabbioni and Jones, 2002, and references cited therein), and the high sequence homology between *E. cloacae* NR and other 'oxygen-insensitive' bacterial nitroreductases, make *E. cloacae* NR a potential target of nitroaromatics with possible relevance to their toxicity.

The reaction rates of NTO and ANTA with P-450R and FNR followed the linear dependence on compound concentrations up to the limit of their solubility (3.5 мм). The reactions were accompanied by the consumption of a stoichiometric amount of O_2 per mole of NADPH. FNR catalyzed the reduction of added cytochrome c (50 μM) by NTO and ANTA, at a rate of 175–185% NADPH oxidation. The reduction of cytochrome c was inhibited by 70–80% by 30 μg/ml superoxide dismutase. Taken together, the data indicate that NTO and ANTA undergo P-450R- and FNR-catalyzed redox cycling with the formation of superoxide:

$$2\ NTO\ (ANTA) + NADPH \xrightarrow{\text{P-450R, FNR}} 2\ NTO^{-\cdot}\ (ANTA^{-\cdot}) + NADP^+ + H^+ \qquad (3)$$

$$NTO^{-\cdot}\ (ANTA^{-\cdot}) + O_2 \longrightarrow NTO\ (ANTA) + O_2^{-\cdot} \qquad (4)$$

The kinetic parameters of redox reactions of NTO and ANTA are presented in Table I. Typically, the reactivity of P-450R, FNR, *E. cloacae* NR, and Hb-Fe^{2+}O$_2$ increases with an increase in the single-electron reduction potential (E^1_7) of nitroaromatics (Orna and Mason, 1989; Nivinskas *et al.*, 2001; Marozienė *et al.*, 2001). The E^1_7 values of

Table I. The enthalpies of single-electron reduction [$\Delta Hf(ArNO_2^{-\cdot})$] of 5-nitro-1,2,4-triazol-3-one (NTO) and 5-nitro-1,2,4-triazol-3-amine (ANTA), and their bimolecular steady-state reduction rate constants ($k_{cat/Km}$) by NADPH:cytochrome P-450 reductase (P-450R), ferredoxin:$NADP^+$ reductase (FNR), NAD(P)H:quinone oxidoreductase (NQO1), and *Enterobacter cloacae* NAD(P)H:nitroreductase (NR), and their rate constants of oxyhemoglobin (Hb-$Fe^{2+}O_2$) oxidation (k). The values of catalytic constants (k_{cat} [s^{-1}]) are given in parentheses when available. The analogous parameters of 2,4,6-trinitrotoluene (TNT) and nitrobenzene determined in our previous works (Čėnas *et al.*, 2001; Nivinskas *et al.*, 2001; Marozienė *et al.*, 2001) are given for comparison.

No. Compound	$\Delta Hf(ArNO_2^{-\cdot})$ [kJ/mol]		k_{cat}/K_m or k [$M^{-1}s^{-1}$]						
	a) AM1	b) PM3	a) P-450R	b) FNR	c) NQO1		d) NR		e) Hb-$Fe^{2+}O_2$
NTO	– 241.58	– 236.35	$9.0 \pm 0.2 \times 10^2$	55 ± 5.0	(≥ 0.09)	27 ± 3.0	($\geq$3.0)	$1.7 \pm 0.2 \times 10^4$	0.08 ± 0.02
ANTA	– 148.29	– 159.49	$1.7 \pm 0.2 \times 10^3$	$2.6 \pm 0.2 \times 10^2$	(≥ 0.09)	29 ± 3.0	(≥ 5.0)	$7.9 \pm 0.6 \times 10^3$	0.12 ± 0.02
TNT	– 308.44	– 316.43	$1.7 \pm 0.1 \times 10^6$	$1.0 \pm 0.1 \times 10^5$	(1.0 ± 0.1)	$6.7 \pm 0.7 \times 10^2$	(143 ± 22)	$9.8 \pm 1.5 \times 10^6$	3.30 ± 0.25
Nitrobenzene	– 167.76	– 172.08	$2.8 \pm 0.3 \times 10^3$	11 ± 2.0	(≤ 0.06)	≤ 24	(10 ± 1.3)	$3.0 \pm 0.3 \times 10^4$	0.10 ± 0.02

NTO and ANTA are still unavailable, however, the enthalpies of their free radical formation [$\Delta Hf(ArNO2^{-\cdot})$] calculated by quantum mechanical methods show that these compounds may be weaker electron acceptors than TNT with E^1_7 = – 0.253 V (Riefler and Smets, 2000) (Table I). It is evident that in both single- and two-electron reduction by flavoenzymes and in Hb-$Fe^{2+}O_2$ oxidation, the reactivity of NTO and ANTA is much lower than that of TNT, and similar to that of nitrobenzene [E^1_7 = –0.485 V (Wardman, 1989)] (Table I).

Next, we examined the FLK cell cytotoxicity of NTO and ANTA, and their methemoglobin formation in erythrocytes (Table II). It is evident that NTO and ANTA are markedly less cytotoxic and Hb-Fe^{3+}-forming agents than TNT. Previously, we have shown that the oxidative stress was the main factor of cytotoxicity of TNT and other nitroaromatics in FLK cells, and that the compound cytotoxicity increased with an increase in their E^1_7 value and/or reactivity in P-450R- and FNR-catalyzed reactions (Čėnas *et al.*, 2001). The cytotoxicity of NTO and ANTA is similar to nitrobenzene (Table II) which possesses similar reactivity towards P-450R and FNR (Table I). As in our previous studies of TNT and other nitroaromatics (Čėnas *et al.*, 2001), the prooxidant character of NTO cytotoxicity is evidenced by the partial protection by the antioxidant *N,N′*-diphenyl-*p*-phenylene diamine (DPPD) (Ollinger and Brunmark, 1991) and the iron-chelating agent desferrioxamine, the latter preventing the Fenton reaction (Fig. 2). In contrast, 1,3-bis-(2-chloroethyl)-1-nit-

Table II. The concentrations of 5-nitro-1,2,4-triazol-3-one (NTO), 5-nitro-1,2,4-triazol-3-amine (ANTA), 2,4,6-trinitrotoluene (TNT), and nitrobenzene for 50% survival of FLK cells (cL_{50}), and the content of methemoglobin (Hb-Fe^{3+}) formed during 24 h incubation of 300 μM compounds with erythrocytes at 40% hematocrite (n = 3). The content of Hb-Fe^{3+} in control erythrocytes was 0.6±0.2%.

No. Compound	cL_{50} [μM]	Hb-Fe^{3+} (%)
1. NTO	≥ 3500	1.2 ± 0.3
2. ANTA	3000 ± 400	2.2 ± 0.5
3. TNT	25 ± 5.0[a]	48.1 ± 6.3
4. Nitrobenzene	4370 ± 1470[a]	1.1 ± 0.3

[a] From Čėnas *et al.* (2001).

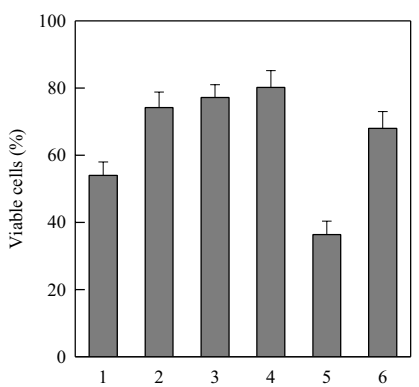


Fig. 2. The effects of DPPD (2 μM), desferrioxamine (300 μM), BCNU (20 μM) and dicumarol (20 μM) in the cytotoxicity of 3 mM NTO in FLK cells. Additions: NTO (1), NTO + DPPD (2), NTO + desferrioxamine (3), NTO + DPPD + desferrioxamine (4), NTO + BCNU (5), and NTO + dicumarol (6), n = 5, p = 0.02–0.04 for 2–5 against 1, and p = 0.073 for 6 against 1. Cell viability in control experiments, 97±2%; DPPD, desferrioxamine, BCNU, and dicumarol decreased cell viability by 1–3%.

rosourea, which inactivates the antioxidant flavoenzyme glutathione reductase (EC 1.6.4.2) and depletes intracellular reduced glutathione (Ollinger and Brunmark, 1991), potentiated the cytotoxicity of NTO (Fig. 2). The analogous effects were observed in the cytotoxicity of ANTA (data not shown). In contrast, the protecting effect of dicumarol, an inhibitor of NQO1 in the NTO cytotoxicity was less evident and statistically insignificant (Fig. 2), most probably, due to the low reactivity of NTO towards NQO1 (Table I).

In contrast to the toxicity of TNT in rats and in mice with LD_{50} = 0.6–1.3 g/kg when given perorally (Dilley *et al.*, 1982), NTO was reported to be nontoxic [LD_{50} > 5 g/kg (London and Smith, 1985)]. However, these summary investigations did not account for the risk that NTO and ANTA may possess, and did not explain the mechanism of their activation/detoxification in the organism. The data on the enzymatic reactivity, mammalian cell cytotoxicity of NTO and ANTA, and their methemoglobin formation *in vitro* obtained in our work, imply that their low toxicity may be at least partly attributed to their low bioreductive activation rates, which are in turn related to their weak electron accepting potency.

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