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Enzymatic redox reactions of the explosive 4,6-dinitrobenzofuroxan (DNBF): implications for its toxic action✪

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With an aim to understand the toxicity mechanisms of the explosive 4,6-dinitrobenzofuroxan (DNBF), we studied its single-electron reduction by NADPH:cytochrome P450 reductase and ferredoxin:NADP$^+$ reductase, and two- electron reduction by DT-diaphorase and *Enterobacter cloacae* nitroreductase. The enzymatic reactivities of DNBF and another explosive 2,4,6-trinitrotoluene (TNT) were similar, except for the much lower reactivity of DNBF towards nitroreductase. DNBF was less cytotoxic in FLK cells than TNT. However, their action shared the same mechanisms, oxidative stress and activation by DT-diaphorase. The lower cytotoxicity of DNBF may be explained by the negative electrostatic charge of its adduct with water which may impede cellular membrane penetration, and by the formation of its less reactive adducts with intracellular reduced glutathione.

Nitroaromatic explosives like 2,4,6-trinitrotoluene (TNT) are toxic to mammalian species, causing liver, kidney, and spleen damage, methemoglobinemia and hemolytic crisis

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Abbreviations: BCNU, 1,3-*bis*-(2-chloroethyl)-1-nitrosourea; cL_{50}, the concentration of compound for 50% cell survival; DNBF, 4,6-dinitrobenzofuroxan; DPPD, *N,N′*-diphenyl-*p*-phenylene diamine; E^1_7, single-electron reduction potential; FNR, ferredoxin:NADP$^+$ reductase; GSH, reduced glutathione; k_{cat}/K_m, bimolecular rate constant; NQO1, NAD(P)H:quinone oxidoreductase; NR, NAD(P)H:nitroreductase; P450R, NADPH:cytochrome P450 reductase; TNT, 2,4,6-trinitrotoluene.

(Dilley *et al.,* 1982; Djerassi, 1998). Their toxicity mechanisms involve the oxidative stress caused by flavoenzyme-catalyzed free-radical redox cycling (Kong *et al.,* 1989; Čėnas *et al.,* 2001), and the formation of nitroso- and/or hydroxylamino metabolites which may modify proteins and DNA, and also induce methemoglobin formation (Facchini & Griffiths, 1981; Leung *et al.,* 1995; Homma-Takeda *et al.,* 2002).

4,6-Dinitrobenzofuroxan (DNBF, Fig. 1) possesses explosive properties, and is used as a starting material for the manufacturing of thermostable explosives of new generation (Mehilal *et al.,* 2003, and references therein). DNBF and its analogues are toxic to mammalian cells (Terrier, 1982, and references therein), thus representing a threat to personnel and environment. Since DNBF is a strongly electrophilic molecule forming Meisenheimer-type adducts even with water, its cytotoxicity has been initially explained by the adduct formation with cellular –SH groups (Terrier, 1982) (Fig. 1). However, in

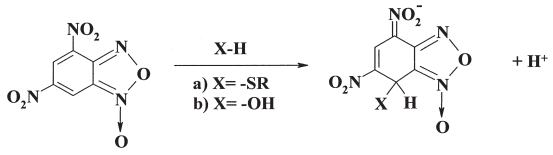

Figure 1. Formation of Meisenheimer-type adducts of DNBF with SH-groups (a) and water (b).

our opinion, the presence of reducible nitro- and N-oxide groups may confer on DNBF oxidative stress-type and/or other types of cytotoxicity related to bioreduction. Examination of flavoenzyme-catalyzed single- and two-electron reduction of DNBF, carried out in this work, shows that its mammalian cell toxicity may be consistent with the above mechanisms. Our findings enable us to hypothesize that DNBF is equally or less toxic to organisms than TNT.

MATERIALS AND METHODS

DNBF was synthesized as described (Mehilal *et al.,* 2002), other compounds were obtained from Sigma or Aldrich and used as received. The kinetic measurements were carried out spectrophotometrically, in 0.1 M K-phosphate buffer (pH 7.0) containing 1 mM EDTA at 25°C, unless specified otherwise, using a Hitachi-557 spectrophotometer. Pig liver NADPH : cytochrome P450 reductase (P-450R, EC 1.6.4.2) and rat liver DT-diaphorase (NQO1, EC 1.6.99.2) were prepared as described (Yasukochi & Masters, 1976; Prochaska, 1988). Ferredoxin : $NADP^+$ reductase (FNR, EC 1.18.1.2) from *Anabaena* sp. was prepared as described (Pueyo & Gomez-Moreno, 1991) and was a generous gift of Dr. M. Martines-Julvez and Professor C. Gomez-Moreno (Zaragoza University, Spain). Recombinant *Enterobacter cloacae* NAD(P)H : nitroreductase (NR, EC 1.6.99.7) was prepared as described (Koder & Miller, 1998), and was a generous gift of Dr. R.L. Koder and Professor A.-F. Miller (University of Kentucky, Lexington, U.S.A.). Kinetic studies of NR were performed in 0.1 M Tris/Cl (pH 7.0), containing 0.5 mM desferrioxamine. The enzyme concentrations were determined using ε_{460} = 22 $mM^{-1}cm^{-1}$ (P450R), ε_{460} = 11 $mM^{-1}cm^{-1}$ (NQO1), ε_{459} = 9.4 $mM^{-1}cm^{-1}$ (FNR), and ε_{454} = 14.3 $mM^{-1}cm^{-1}$ (NR). The activities of P450R and FNR were determined in the presence of 200 μM NADPH using 50 μM cytochrome *c* ($\Delta\varepsilon_{550}$ = 20 $mM^{-1}cm^{-1}$) or 1 mM ferricyanide ($\Delta\varepsilon_{420}$ = 1.0 $mM^{-1}cm^{-1}$) as electron acceptors, respectively. The activity of NQO1 was determined according to the reduction of 50 μM cytochrome *c* in the presence of 10 μM menadione and 200 μM NADPH. The enzyme activities were equal to 77 μmol $mg^{-1}min^{-1}$ (P450R), 330 μmol mg^{-1} min^{-1} (FNR), and 2000 μmol $mg^{-1}min^{-1}$ (NQO1). The rates of enzymatic oxidation of NADPH (100 μM) by DNBF were determined according to $\Delta\varepsilon_{340}$ = 6.2 $mM^{-1}cm^{-1}$. The catalytic constant (k_{cat}) and the bimolecular rate

constant (k_{cat}/K_m) of the reduction of DNBF were calculated from Lineweaver-Burk plots. k_{cat} is the number of NADPH molecules oxidized by a 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 cytotoxicity experiments, cells (3.0×10^4/ml) were grown in the presence of various amounts of DNBF 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. Statistical analysis was performed using Statistica (version 4.3, StatSoft, 1993).

RESULTS AND DISCUSSION

In this work, we examined the redox reactions of DNBF which may be important in its toxicity: i) single-electron reduction by the flavoenzyme electrontransferases NADPH: cytochrome P450 reductase (P450R) and ferredoxin : $NADP^+$ reductase (FNR) which are used as model systems to study free radical formation of nitroaromatic compounds and their redox cycling (Orna & Mason, 1989; Čėnas *et al.,* 2001), and ii) reduction by rat NAD(P)H : quinone oxidoreductase (DT-diaphorase, NQO1) and *E. cloacae* NAD(P)H: nitroreductase (NR) which reduce nitroaromatics to hydroxylamines in two subsequent two-electron transfer steps (Knox *et al.,* 1993; Koder & Miller, 1998). Although the nitroreductase activity of NQO1 is low even for electron-deficient nitroaromatics, this enzyme may enhance their mammalian cell cytotoxicity (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 & Miller, 1998; Sabbioni & Jones, 2002, and references 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.

In agreement with previous observations (Terrier, 1982), an adduct of DNBF with water (Fig. 1) characterized by ε_{460} = 24.4 $mM^{-1}cm^{-1}$, was formed with $t_{1/2}$ about 30 s after introduction of a stock solution of DNBF in dimethylsulfoxide or acetonitrile into 0.1 M K-phosphate or Tris/Cl (pH 7.0, factor of dilution, 100). Thus, all the enzymatic tests were started after equilibration of DNBF solution in the buffer for 3–5 min. The kinetic parameters of DNBF are summarized in Table 1.

DNBF underwent P450R- and FNR-catalyzed redox cycling with the formation of superoxide, as evident by the following. The reactions were accompanied by consumption of a stoichiometric amount of O_2 per mole of NADPH, DNBF oxidized a significant excess NADPH, and FNR catalyzed the reduction of added cytochrome *c* (50 μM) by DNBF at 190% of the rate of NADPH oxidation (Table 1). The reduction of cytochrome *c* was inhibited by 40% by 30 μg/ml superoxide dismutase. The nature of the free radical of DNBF has not been studied so far, however, by analogy with other N-oxide-substituted nitroaromatics, the electron density should be localized mainly on the nitro, but not on the N-oxide group (Priyadarsini *et al.,* 1996). The k_{cat}/K_m values for DNBF in the FNR, P450R, and NQO1-catalyzed reactions are close or above those of the classical explosive 2,4,6-trinitrotoluene (TNT) (Table 1), showing that DNBF is an efficient electron acceptor. The low activity of DNBF in the NR-catalyzed reaction (Table 1) is unexpected, since a close

parallelism exists between the reactivities of a large number of nitroaromatics in P450R, FNR, and NR-catalyzed reactions (Nivinskas *et al.,* 2001; Čėnas *et al.,* 2001). The causes for this phenomenon are currently being studied. The formation of a Meisenheimer-type adduct of DNBF with reduced glutathione (GSH) in buffer solution is slow (k = 0.017 ± 0.002 $M^{-1}s^{-1}$, monitored according to the decrease in 460 nm absorbance (Fig. 2A)). The nitroaromatics was partly prevented by the antioxidant *N,N'*-diphenyl-*p*-phenylene diamine (DPPD) (Ollinger & Brunmark, 1991) and the iron-chelating agent desferrioxamine, whereas the NQO1 inhibitor dicumarol showed equivocal effects (Čėnas *et al.,* 2001). The cytotoxicity of DNBF was significantly decreased by DPPD and desferrioxamine, and potentiated by 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (BCNU), which inactivates the antioxi-

Table 1. Reactivity of DNBF in flavoenzyme-catalyzed reactions.

Bimolecular (k_{cat}/K_m) and catalytic (k_{cat}) steady-state reaction rate constants for DNBF reduction by: NADPH : cytochrome P450 reductase (P450R), ferredoxin : $NADP^+$ reductase (FNR), NAD(P)H : quinone oxidoreductase (NQO1), *Enterobacter cloacae* NAD(P)H : nitroreductase (NR), and redox cycling properties in FNR-catalyzed reactions. Analogous parameters of 2,4,6-trinitrotoluene (TNT) determined in our previous works (Čėnas *et al.,* 2001; Nivinskas *et al.,* 2001) are given for comparison.

Compound	DNBF		TNT	
A) Reduction rate constants:	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})
P450R	3.9±0.4 × 10^5	>1.5[a]	1.7±0.1 × 10^6	50±5.0
FNR	3.8±0.2 × 10^4	–[b]	1.0±0.1 × 10^4	–[b]
NQO1	4.5±0.3 × 10^3	–[b]	6.7±0.7 × 10^2	1.0±0.1
NR	5.9±1.0 × 10^4	8.0±1.5	9.8±1.5 × 10^6	143±22
B) FNR-catalyzed reaction rates	(μM × min^{-1})[c]:			
NADPH oxidation	11.4±0.2		3.0±0.2	
O_2 consumption	10.9±0.5		2.8±0.3	
Cytochrome *c* reduction	21.6±0.4		5.8±0.3	

[a]Enzyme inhibition above 30 μM DNBF. [b]Linear dependence of the reaction rate on substrate concentration up to its solubility limits, 100 μM DNBF or 600 μM TNT. [c] Reaction rates determined in the presence of 300 μM NADPH, 100 nM FNR, 50 μM DNBF or TNT, n = 3. In separate experiments, 50 μM cytochrome *c* was added.

enzymatic reactivity of the reaction product is 3–4 times lower as compared to the parent compound (Fig. 2B).

The cytotoxicity of nitroaromatics in FLK cells increases with an increase in their single-electron reduction potential (E_1^7) (Čėnas *et al.,* 2001). This points to oxidative stress as the main factor of their cytotoxicity, since, as a rule, the rates of single-electron reduction of nitroaromatics by flavoenzymes initiating their redox cycling increase with their E^1_7 values. The action of TNT and other dant flavoenzyme glutathione reductase (EC 1.6.4.2) and depletes intracellular GSH (Ollinger & Brunmark, 1991) (Fig. 3). This shows that DNBF cytotoxicity is due to its prooxidant character. The modest protection by dicumarol (Fig. 3) also points to a parallel potentiation of DNBF cytotoxicity by NQO1.

Since the E^1_7 of DNBF is unknown, the relationship between its cytotoxicity and enzymatic redox cycling activity may be established using a geometric mean of its reactivity with P450R and FNR, (log k_{cat}/K_m (P450R) +

log k_{cat}/K_m (FNR))/2 (Čėnas *et al.,* 2001). This reactivity parameter for DNBF, 5.08, is almost identical with that for TNT, 5.12 (Table 1). However, the concentration of DNBF for 50% FLK cell survival (cL_{50}), 70 ± 8 μM, is higher than cL_{50} of TNT, 25 ± 5 μM (Čėnas *et al.,* 2001). Other compounds with similar redox properties are also more toxic in FLK cells than DNBF, e.g., *p*-dinitrobenzene (cL_{50} = 8 ± 2 μM), and *o*-dinitrobenzene (cL_{50} = 30 ± 5 μM) (Čėnas *et al.,* 2001). Thus, the FLK cell cytotoxicity of DNBF is in line with its redox cycling properties, being even lower than may be expected. It seems that, contrary to previous suggestions (Terrier, 1982), the possibility of DNBF reacting with cellular –SH groups and other nucleophiles (Fig. 1) does not enhance its cytotoxicity. Possible reasons for the decreased DNBF cytotoxicity may be the negative electrostatic charge of the adduct with water (Fig. 1) which may slow down its cellular membrane penetration, and the formation of an adduct with cellular GSH, which is a less active flavoenzyme substrate (Fig. 2).

Another important toxicity mechanism is the reduction of polynitroaromatic compounds by gastrointestinal tract nitroreductases, since the hydroxylamines formed exert their toxic action in the whole organism

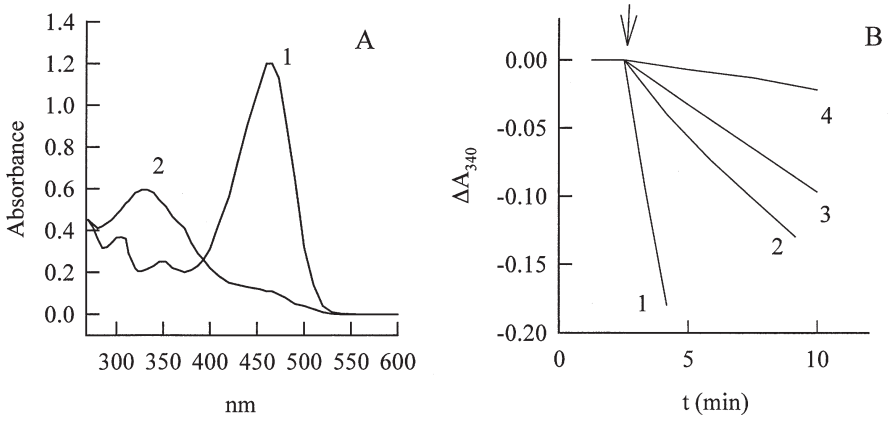

Figure 2. (A) Spectra of 50 μM DNBF before (1) and after (2) 6 h incubation with 10 mM GSH in 0.1 M K-phosphate buffer, pH 7.0. (B) Kinetics of oxidation of 200 μM NADPH by 50 μM DNBF in the presence of 100 nM ferredoxin:$NADP^+$ reductase (1, 2) and 100 nM NAD(P)H:quinone oxidoreductase (3, 4) before (1, 3) and after (2, 4) DNBF incubation with 10 mM GSH for 6 h.

Arrow indicates the time of enzyme addition. The presence of 10 mM GSH in the solution without incubation did not affect the reaction rates.

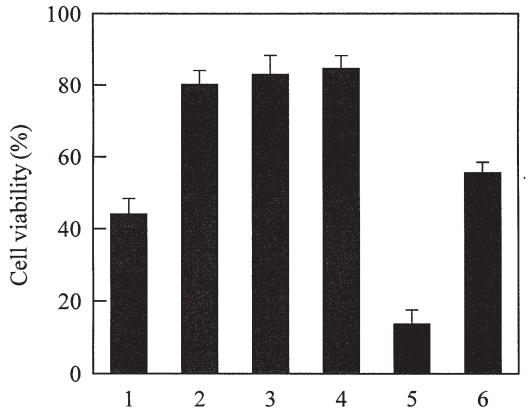

Figure 3. The effects of DPPD (2 μM), desferrioxamine (300 μM), BCNU (20 μM), and dicumarol (20 μM) on the cytotoxicity of 75 μM DNBF in FLK cells.

Additions: DNBF (1), DNBF + DPPD (2), DNBF+ desferrioxamine (3), DNBF + DPPD + desferrioxamine (4), DNBF + BCNU (5), and DNBF + dicumarol (6), n = 5, $P < 0.005$ for 2–5 against 1, and $P < 0.05$ for 6 against 1. Cell viability in control experiments, 97±2%; DPPD, desferrioxamine, BCNU, and dicumarol decreased cell viability by 1–3%.

(Sabbioni & Jones, 2002, and references therein). DNBF may be considered safer in this aspect as well, since it is a less active *E. cloacae* nitroreductase substrate than TNT. Although the data of our work do not cover the DNBF cytotoxicity mechanisms in other cell lines with a different capacity of xenobiotic metabolism and the other possible aspects of DNBF toxicity, they enable us to hypothesize that DNBF is in general equally or less toxic than TNT.

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