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Redox properties and prooxidant cytotoxicity of benzofuroxans: a comparison with nitrobenzenes

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The presence of =N(→O)O- moiety in benzofuroxans may confer them the electron-accepting properties similar to those of aromatic nitrocompounds or *N*-oxides which are used as bioreductively activated antitumour and / or cytotoxic agents. However, the redox properties of benzofuroxans have not been characterized so far. We have studied the redox properties of eight benzofuroxans, determining the heats of formation of their free radicals ($\Delta Hf(e^-)$), the peak-potentials of their electrochemical reduction in the aqueous medium at pH 7.0 ($E_{p,7}$), and their reactivity towards a single-electron transferring flavoenzyme, ferredoxin : $NADP^+$ reductase (FNR). We found that the reactivity of benzofuroxans towards FNR in general is similar to that of (poly)nitrobenzenes possessing analogous values of $\Delta Hf(e^-)$, and the benzofuroxans are reduced in a single-electron way. However, the $E_{p,7}$ of the irreversible electrochemical reduction of benzofuroxans strongly deviated from the dependence of $\Delta Hf(e^-)$ of nitrobenzenes on their $E_{p,7}$. In general, the cytotoxicity of benzofuroxans in bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) is similar to that of nitroaromatic compounds possessing the analogous values of $\Delta Hf(e^-)$, whereas their cytotoxicity in mice splenocytes considerably differs. This may be caused by other parallel cytotoxicity mechanisms, e. g. reactions of benzofuroxans with –SH groups. In this work, for the first time we identified benzofuroxans as redox active compounds. Preliminarily, one may conclude that the redox properties and cytotoxicity of benzofuroxans bear certain similarities (reactions with single-electron-transferring flavoenzymes) and differences (electrochemical reduction, cytotoxicity) from nitroaromatic compounds.

Key words: benzofuroxans, nitrobenzenes, electrochemical reduction, electron-accepting properties, ferredoxin : NADP+ reductase, free radicals, oxidative stress, cytotoxicity

Abbreviations: $Ar\text{-}NO_2^-$, aromatic nitrocompound; Ar>N→O, aromatic N-oxide; $Ar\text{-}NO_2^{\cdot-}$, aromatic nitroanion-radical; $\Delta Hf(e^-)$, heat of formation of free radical; FNR, ferredoxin: $NADP^+$ reductase; k_{cat} / K_m, bimolecular rate constant in enzymatic steady-state reactions; E_7^1, single-electron reduction potential at pH 7.0; $E_{p,7}$, the reduction peak-potential in cyclic voltammetry; O_2^-, superoxide; NHE, normal hydrogen electrode; cL_{50}, compound concentration for 50% cell death.

INTRODUCTION

One of the main mammalian cell cytotoxicity mechanisms of nitroaromatic and nitrohetero-cyclic compounds ($Ar\text{-}NO_2$) is their redox cycling, initiated by the flavoenzyme-catalyzed single-electron reduction. The formation of anion-radicals of nitroaromatics ($Ar\text{-}NO_2^{\cdot-}$) is followed by their reoxidation by molecular oxygen, which yields the parent $Ar\text{-}NO_2$ and superoxide (O_2^-), the latter subsequently yielding other activated oxygen species. This results in the 'oxidative stress'-type cytotoxicity, i. e. peroxidation of lipids and the oxidative damage to proteins and DNA [1–4]. It is assumed that if the 'oxidative stress' is the main factor of cytotoxicity of $ArNO_2$, then their cL_{50} (compound concentration for 50% cell death) should decrease with an increase in their single-electron reduction potential (E_7^1) with the coefficient $\Delta \log cL_{50} / \Delta E_7^1 \sim -10\ V^{-1}$ [1–4]. The E_7^1 values of nitroaromatic compounds determined by means of pulse-radiolysis, typically range from –0.20 V to –0.50 V *vs*. normal hydrogen electrode (NHE) [1, 5, 6]. Aromatic *N*-oxides (Ar > N→O) represent another

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important group of bioreductively activated compounds, including the antitumour agent tirapazamine and its analogues [7–9]. However, they are less efficient electron acceptors than nitroaromatics, because their E_7^1 values range from –0.38 V to –0.80 V.

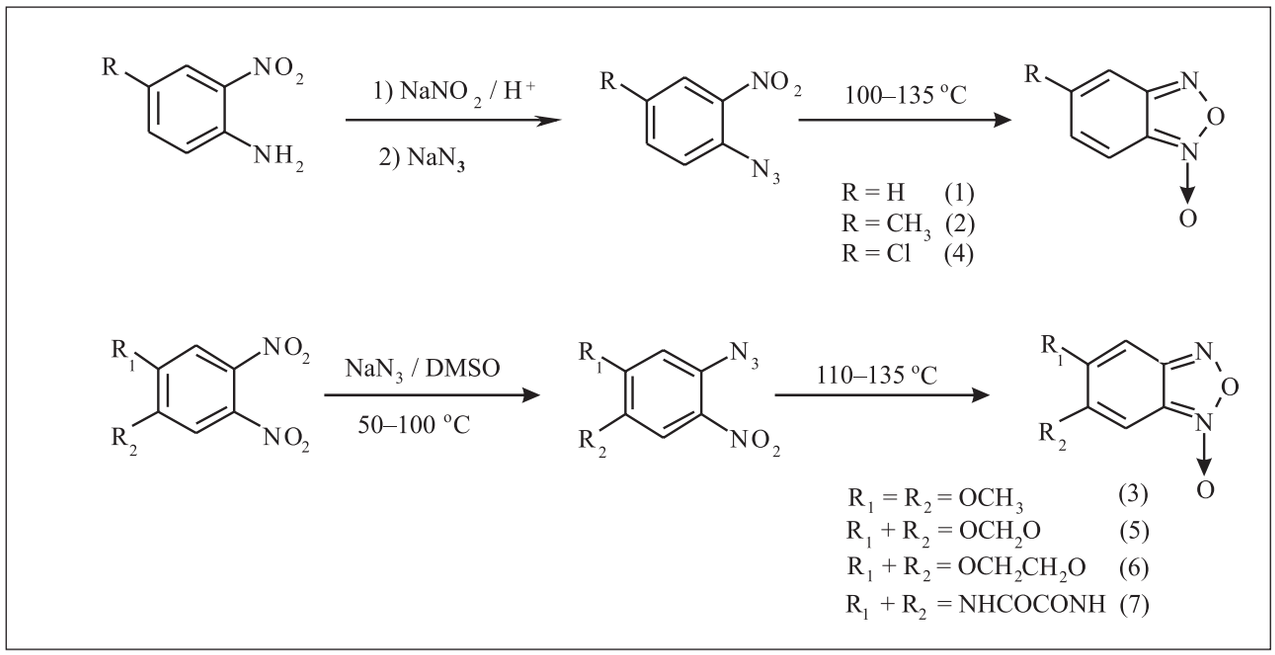
Benzofuroxans possess =N(→O)O- moiety (Fig. 1) which shares a similarity with both nitro- and *N*-oxide groups. Thus, one may expect that benzofuroxans may also undergo a single-electron enzymatic reduction, and that their electron-accepting potency may be similar to that of nitroaromatic compounds or aromatic *N*-oxides. These data may be of certain importance in medicinal chemistry and toxicology because benzofuroxans are used as activators of guanylate cyclase and inhibitors of monoamino oxidases [10, 11]. Besides, benzofuroxans comprise a new group of explosive compounds [12]. This implies the possible ecotoxicological problems due to the environmental contamination by explosive residues. However, to our best knowledge, the redox properties of benzofuroxans have not been reported so far. In this paper, we present data on the electron-accepting properties of several benzofuroxans and their mammalian cell cytotoxicity, which are compared to the analogous properties of nitrobenzenes.

EXPERIMENTAL

The synthesis of benzofuroxans was performed according to the established methods (Scheme). Mono-substituted derivatives of benzofuroxan (compounds 1, 2, 4 (Fig. 1)) were obtained by a direct one-step oxidation of corresponding *o*-nitroanilines by hypochlorite in basic medium (pH 8.0–9.5) [13]. Di-substituted benzofuroxans (compounds 3, 5, 6, 7 (Fig. 1)) were synthesized from the corresponding 1,2-dinitrobenzene derivatives by a modified two-step method [14]. 2-Nitrophenylazides were obtained by the nucleophilic substitution reaction and underwent cyclization during their boiling with acetic acid [15]. Benzotrifuroxan (compound 8 (Fig. 1)) was synthesized by the thermocyclization of 1,3,5-triazido-2,4,6-trinitrobenzene in boiled propionic acid as previously described [16]. The structure of the compounds was identified by means of elemental analysis, NMR, and IR. 2,4,6-Trinitrotoluene and 2,4,6-trinitrohenyl-*N*-methylnitramine (tetryl) were synthesized as described [4].

Voltammetric studies of benzofuroxans and model (poly)nitrobenzenes (0.5–1.0 mM) were performed using a Parstat 2273 potentiostate (Princeton Applied Research) under anaerobic conditions (purging the solution with argon for 20 min) at pH 7.0 (0.05 M K-phosphate and 0.1 M KCl) and 25 °C. A rod of glassy carbon (BAS, 2.0 mm diameter) polished with a corundum abrasive served as a working electrode. A saturated Ag / AgCl electrode (+0.205 V vs. NHE) was used as a reference electrode. The voltammetric reduction peak-potentials of compounds studied in this work ($E_{p,7}$) are presented with respect to this electrode. A Pt electrode (apparent surface area 56 mm^2) was used as an auxiliary electrode.

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. Ferredoxin : NADP$^+$ reductase (FNR, EC 1.18.1.2) from *Anabaena* was prepared as described [17], and was a generous gift of Dr. Marta Martines-Julvez and Professor Carlos Gomez-Moreno (Zaragoza University, Spain). The enzyme concentration was determined spectrophotometrically using



Scheme. The synthesis of mono- and disubstituted-benzofuroxans

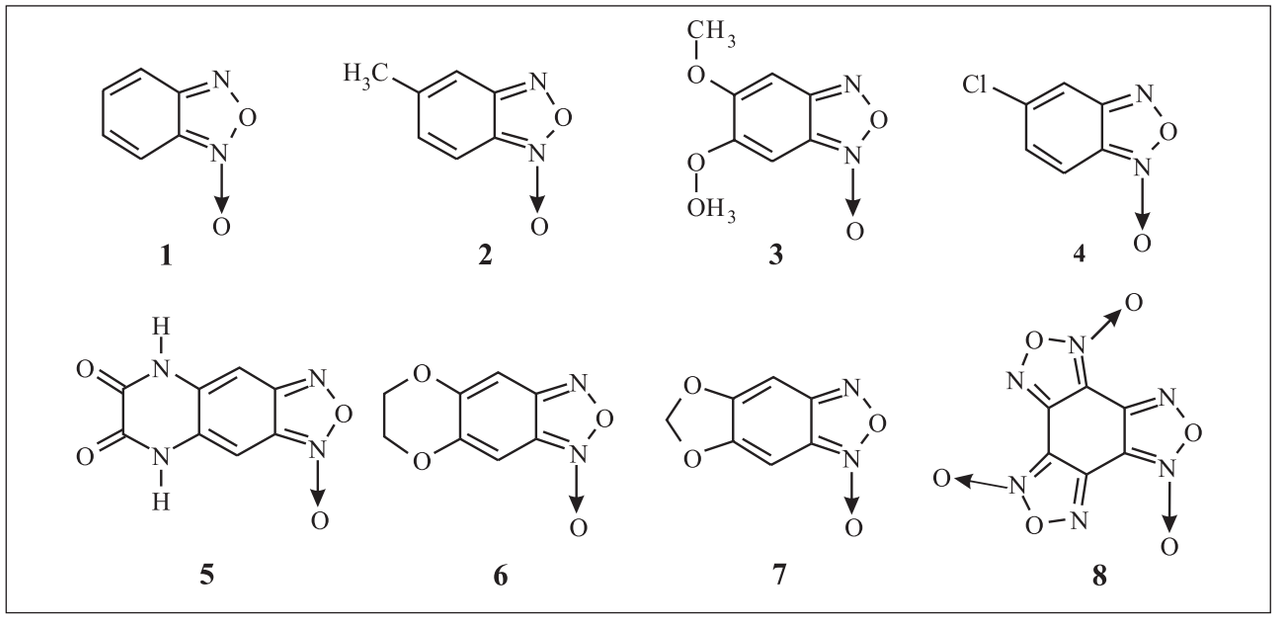


Fig. 1. The formulae of benzofuroxans studied in this work

$\varepsilon_{459} = 9.4\ mM^{-1}\ cm^{-1}$. The rates of FNR-catalyzed oxidation of 100 μM NADPH by benzofuroxans and nitroaromatic compounds, corrected for the intrinsic NADPH-oxidase activity of FNR, 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 compounds were calculated from the Lineweaver–Burk plots. k_{cat} is the number of NADPH molecules oxidized by the single active center of FNR per second. In separate experiments, cytochrome *c* (50 μM) was added to the reaction mixture. Its reduction was monitored according to $\Delta\varepsilon_{550} = 20\ mM^{-1}\ cm^{-1}$.

The heats of formation of free radicals of benzofuroxans and nitroaromatics ($\Delta Hf(e^-)$) were calculated using PC Spartan '04 Pro (Wavefunction, Inc.) according to semiempirical AM1 and PM3 methods, as described previously [18].

The primary mice splenocytes were obtained from BALB / c mice as described previously [19]. These experiments were approved by the Lithuanian Veterinary and Food Service (License No. 0171, 2007). The splenocytes (10^6/ml) were suspended in RPMI 1640 medium with 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml); their viability was determined after 24 h of incubating the splenocytes with the examined compounds according to the Trypan blue exclusion test. The compounds were dissolved in DMSO whose final concentration in the medium (0.6%) did not affect the splenocyte viability. 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 and antibiotics as described previously [20]. In the cytotoxicity experiments, cells (2.5×10^4/ml) were seeded on glass slides in 5 ml flasks in the presence or in the absence of compounds, and were grown for 24 h. Further, the slides were rinsed 3–4 times with phosphate buffer saline and stained with Trypan blue. The cells on the slides were counted under a light microscope. The statistical analysis was performed using Statistica (version 4.3, StatSoft, 1993).

All other reagents were obtained from Sigma, unless otherwise specified.

RESULTS AND DISCUSSION

Because of the similarity of =N(→O)O- moiety of benzofuroxans with the nitrogroup, their properties are further discussed in the context of redox properties of nitroaromatic compounds which are relatively well understood. According to the numerous studies ([4] and references given therein), the reactivity of nitroaromatics in flavoenzyme-catalyzed single-electron reduction reactions, which cause the 'oxidative stress'-type cytotoxicity, increases with an increase in their single-electron reduction potential (E_7^1) with the coefficient $\Delta\log(k_{cat}/K_m) / \Delta E^1_7 = 8.0–10\ V^{-1}$, where k_{cat}/K_m is the bimolecular reaction rate constant. This is in accordance with the 'outer-sphere' mechanisms of single-electron transfer [21]. If the experimentally determined E_7^1 values of compounds are absent, the heats of the formation of free radicals ($\Delta Hf(e^-)$) obtained by means of quantum mechanical calculations may be used as an approximate substitute parameter describing their reactivity [4]. The $\Delta Hf(e^-)$ values of benzofuroxans are given in Table 1, together with the $\Delta Hf(e^-)$ values of several nitrobenzenes, obtained in our previous studies [4, 18]. Our data show that the $\Delta Hf(e^-)$ values of benzofuroxans are similar to those of nitrobenzenes (12–15) and are considerably more negative than that of nitrobenzene with $E_7^1 = -0.485$ V (Table 1). It means that benzofuroxans studied in this work may be better electron acceptors than nitrobenzene.

According to quantum mechanical calculations, the *N* atom of =N(→O)O- moiety of benzofuroxans is the most probable electron-accepting site, because it possessed the highest positive Mulliken charge and the highest coefficients of the lowest unoccupied molecular orbital.

We have studied the reactions of benzofuroxans with a model single-electron-transferring flavoenzyme ferredoxin : $NADP^+$ reductase (FNR). Its reactivity is not specific towards the particular structures of nitroaromatic oxidants, and it is characterized by a linear dependence on their log k_{cat}/K_m on E_7^1 or, to a lesser extent, on their $\Delta Hf(e^-)$ ([4] and references therein). It is evident that the reactivity of benzofuroxans towards FNR is similar to the reactivity of nitroaromatics possessing analogous $\Delta Hf(e^-)$ values (Fig. 2). The reduction of the benzofuroxan derivative (5) (Fig. 1) by FNR is accompanied by a reduction of cytochrome *c* added in the separate experiments, at a rate twofold to the NADPH oxidation rate. The reduction of cytochrome *c* is inhibited by 60–65% by 100 U/ml superoxide dismutase. It shows that the reaction proceeds as a single-electron transfer with the formation of a free radical of benzofuroxan, which further is oxidized by oxygen and yields superoxide.

The electrochemical reduction of most nitroaromatic compounds proceeds as an irreversible net four-electron transfer, with the formation of hydroxylamines [22, 23]. The nitrogroups in polynitroaromatic compounds are reduced subsequently as is evidenced by the appearance of three reduction peaks of 2,4,6-trinitrotoluene (Fig. 3) and 2,4,6-trinitrophenyl-*N*-methylnitramine (Table). The subsequent reactions of the reduction products sometimes result in the appearance of electrochemically active condensation / polymerisation products which undergo electrochemical oxidation at positive potentials ([22, 23], Fig. 3). Our preliminary data show that benzofuroxans are also electrochemically irreversibly reduced at the range of the potentials of nitrobenzene reduction (Fig. 3, Table). The reduction peak currents of benzofuroxans and nitrobenzenes studied are controlled by diffusion because they are directly proportional to the square root of the potential scan rate, 10–1000 mV/s. In general, the voltammetric reduction peak-potentials ($E_{p,7}$) of nitroaromatic compounds increase with an increase in their E_7^1 values [24]. For this reason, a linear relationship has been observed between the E_7^1 values and the first $E_{p,7}$ of nitroaromatics studied in this work (Table), although it has been poorly expressed ($r^2 = 0.7845$, data not shown). On the other hand, there also exists a linear relationship between the values of E_7^1 and $\Delta Hf(e^-)$ of nitroaromatics [4]. Thus, it results in the appearance of linear although poorly expressed relationships between the first $E_{p,7}$ values of nitroaromatics and their $\Delta Hf(e^-)$ ($r^2 = 0.7193$ (AM1, Fig. 4), and $r^2 = 0.7337$ (PM3, data not shown). However, the $E_{p,7}$ values of benzofuroxans strongly deviate from this dependence (Fig. 4). For this reason, the mechanism(s) of the electrochemical reduction of benzofuroxans and their dependence on their structure are the subject of our forthcoming studies.

We have also preliminarily investigated the cytotoxicity of benzofuroxans in two mammalian cell lines, primary mice splenocytes and bovine leukemia virus-transformed lamb embryo kidney fibroblasts (line FLK). In both cell lines, the concentrations of nitroaromatic compounds causing a 50% cell death (cL_{50}) increase with a decrease in their E_7^1 with the coefficient log $cL_{50}/\Delta E_7^1 \sim -10\ V^{-1}$ [4, 19]. Analogously, cL_{50} increases with an increase in $\Delta Hf(e^-)$ of nitroaromatics [4]. Taken together with the cytotoxicity decrease by the

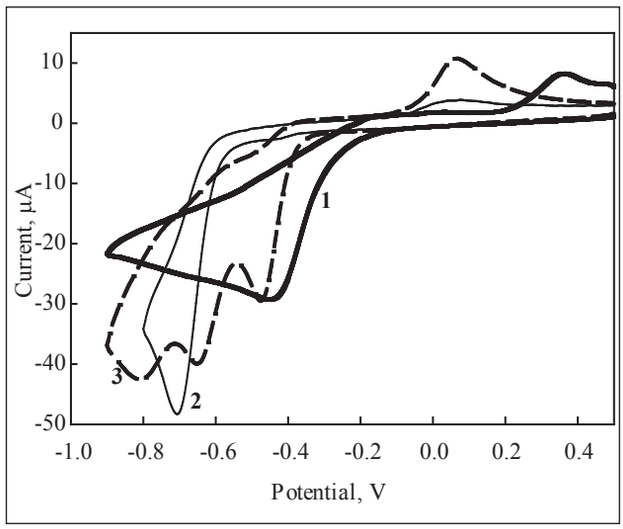


Fig. 3. Cyclic voltammograms (v = 50 mV/s) of electrochemical reduction of benzofuroxan (1), 2,4,6-trinitrotoluene (2), and nitrobenzene (3) at 0.5 mM compound concentration and pH 7.0. The starting potential is 0.5 V, the potentials are given vs. Ag / AgCl. Because of the electrode contamination, only the first scans are shown

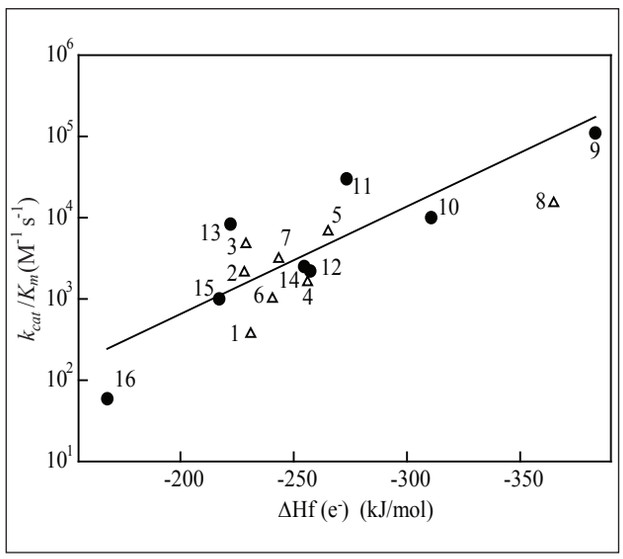


Fig. 2. Relationship between the $\Delta Hf(e^-)$ (AM1) values of nitroaromatic compounds (circles) and benzofuroxans (triangles) and their reactivity towards ferredoxin : $NADP^+$ reductase (k_{cat}/K_m). The numbers of compounds are taken from Table

Table. **The heats of formation of free radicals of benzofuroxans and nitroaromatic compounds (ΔHf(e^-)), their reactivities towards the single-electron transferring flavoenzyme ferredoxin : $NADP^+$ reductase (k_{cat}/K_m), their single-electron reduction potentials determined by pulse-radiolysis (E^1_7), their reduction peak-potentials in cyclic voltammetry ($E_{p,7}$, 50 mV/s), and their concentrations for 50% mammalian cell death (cL_{50})**

No.	Compound	ΔHf(e^-) (kJ/mol)		k_{cat}/K_m ($M^{-1}s^{-1}$)	E^1_7 (mV)	$E_{p,7}$	cL_{50} (μM)	
		AM1	PM3	FNR	vs. NHE[a]	vs. Ag / AgCl	Splenocytes	FLK
	Benzofuroxans:							
1.	Benzofuroxan	–231.0	–225.8	3.7 ± 0.30 × 102	–	–452	60 ± 8.0	250 ± 30
2.	5-Methylbenzofuroxan	–228.2	–222.9	2.1 ± 0.10 × 103	–	–448, –0.800	94 ± 8.0	
3.	5,6-Dimethoxy-benzofuroxan	–228.9	–221.9	4.7 ± 0.20 × 103	–	–408, –0.810	>300	20 ± 4.0
4.	5-Chlorobenzofuroxan	–256.1	–245.2	1.6 ± 0.10 × 103	–	–397	47 ± 5.0	
5.	1-Oxy-5H,8H-2-oxa-1,3,5,8-tetraaza-cyclopenta[b] naphthalene-6,7-dione	–265.2	–282.7	6.7 ± 0.70 × 103	–	–634, –765	19 ± 2.0	110 ± 18
6.	6,7-Dihydro-2,5,8-trioxa-1,3-diazacyclo-penta[b] naphthalene-1-oxide	–240.6	–229.5	1.0 ± 0.10 × 103	–	–457, –760	75 ± 7.0	
7.	[1,3] Dioxolo [4′,5′ : 4,5] benzo [1,2-c]-[1,2,5]oxadiazole-1-oxide	–243.4	–231.8	3.1 ± 0.10 × 103	–	–698	22 ± 3.0	
8.	Benzotrifuroxan	–364.8	–414.5	1.5 ± 0.10 × 104	–	–759	70 ± 5.0	4.6 ± 0.7
	Nitrobenzenes:							
9.	2,4,6-Trinitrophenyl-N-methylnitramine	–383.1	–369.2	1.1 ± 0.2 × 105[b]	–156	–305, –485, –625	6.0 ± 1.5c	1.5 ± 0.3[d]
10.	2,4,6-Trinitrotoluene	–310.7	–316.4	1.0 ± 0.1 × 104[b]	–253	–470, –650, –810	10 ± 2.0[c]	25 ± 5.0[d]
11.	p-Dinitrobenzene	–273.3	–281.1	3.0 ± 0.2 × 104[b]	–257	–337, –805	2.5 ± 0.4[c]	8.0 ± 2.0[d]
12.	o-Dinitrobenzene	–257.3	–261.0	2.2 ± 0.2 × 103[b]	–287	–452,–807	60 ± 15[c]	30 ± 5.0[d]
13.	*p*-Nitrobenzaldehyde	–222.1	–223.2	8.3 ± 0.7 × 10[3b]	–315	–634	40 ± 6.0[c]	25 ± 12[d]
14.	*m*-Dinitrobenzene	–254.6	–262.1	2.5 ± 0.2 × 10[3b]	–345	–563,–805	100 ± 20[c]	90 ± 20[d]
15.	*p*-Nitroacetophenone	–217.1	–218.1	1.0 ± 0.1 × 10[3b]	–355	–532	60 ± 8.0[c]	166 ± 36[d]
16.	Nitrobenzene	–167.7	–172.1	58 ± 7.0[b]	–485	–706	1000 ± 200[c]	4370 ± 1370[d]

[a] From Refs. [5, 6]. [b, d] From Ref. [4]. [c] From Ref. [19].

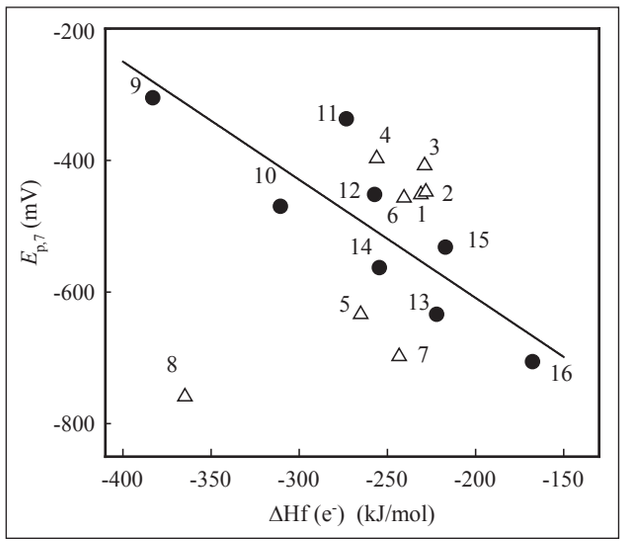


Fig. 4. Relationship between the ΔHf(e^-) (AM1) values of nitroaromatic compounds (circles) and benzofuroxans (triangles) and their electrochemical reduction peak-potentials ($E_{p,7}$). The numbers of compounds are taken from Table, the line corresponds to the first-order regression drawn through the corresponding parameters of nitroaromatic compounds

antioxidants [4, 19], these data show that the main factor of cytotoxicity of nitroaromatics in these cell lines is the 'oxidative stress' caused by their flavoenzyme-catalyzed redox cycling. We have found that the cytotoxicity of benzofuroxan in both cell lines is, at least partly, caused by the 'oxidative stress'. At the concentrations causing a 38 ± 3.0% splenocyte death (75 μM benzofuroxan) or 34 ± 2.5% FLK cell death (300 μM benzofuroxan), addition of 2.0 μM *N, N'*-diphenyl-*p*-phenylene diamine or 300 μM desferrioxamine typically increased the cell viability to ≥ 50% (data not shown). In general, the cL_{50} values of several benzofuroxans in FLK cells (Table) are similar to those of nitroaromatic compounds possessing the analogous ΔHf(e^-) values (Fig. 5A). However, the cL_{50} values of benzofuroxans show a much larger deviation from the cytotoxicity of nitroaromatics in splenocytes (Fig. 5B). These findings are unexpected and need further clarification. One of the possible explanations is that ΔHf(e^-) values may not quite well reflect the differences in the electron-accepting potency of nitroaromatic compounds and benzofuroxans in an aqueous medium. Besides, it is also possible that some other mechanism(s) of action of benzofuroxans, e. g., their reactions with intracellular –SH

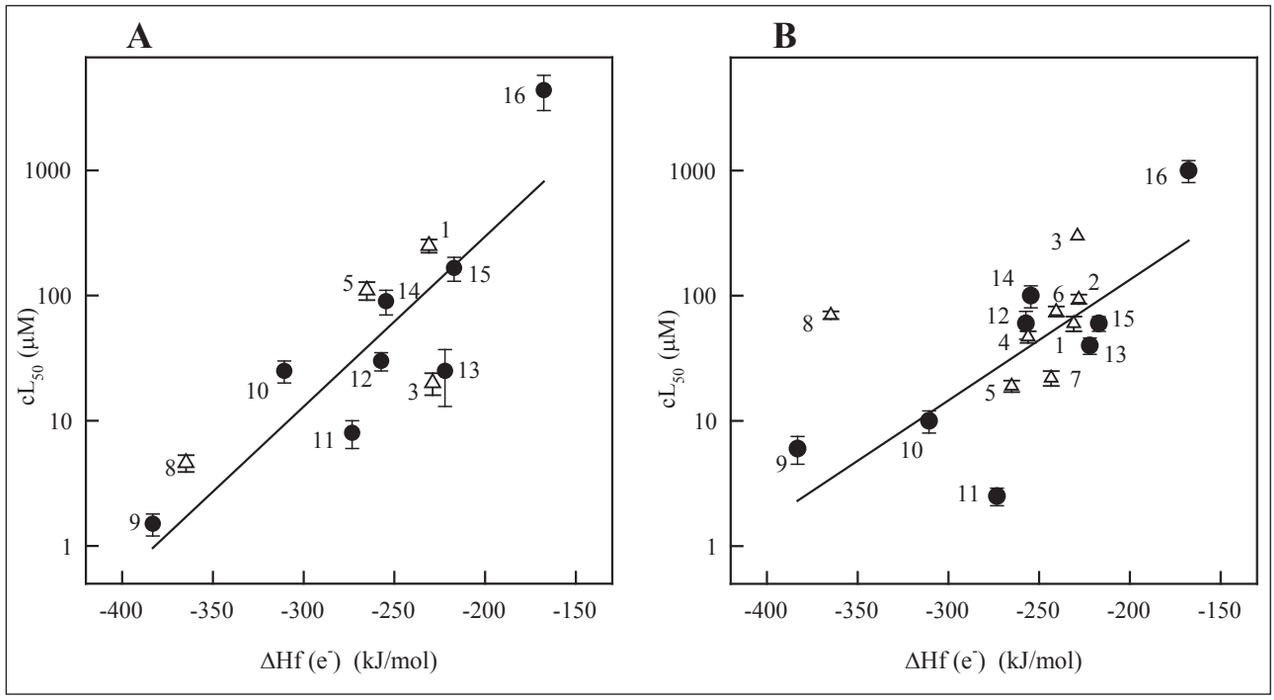


Fig. 5. Relationships between the ΔHf(e⁻) (AM1) values of nitroaromatic compounds (circles) and benzofuroxans (triangles) and their concentrations causing 50% cell death (cL_{50}) in FLK cells (A), and splenocytes (B). The numbers of compounds are taken from Table, the lines correspond to the first-order regression drawn through the corresponding parameters of nitroaromatic compounds

compounds [25] may also contribute to their cytotoxicity. A more thorough investigation of the mechanism(s) of mammalian cell cytotoxicity of benzofuroxans is the subject of our forthcoming studies.

CONCLUSIONS

In this work, for the first time we have identified benzofuroxans as the redox active compounds whose bioreductive properties may be manifested at the physiological range of redox potentials, ≥ –0.5 V vs. NHE. Although their E_7^1 values have not been determined, the quantum mechanical calculations have revealed that the electron-accepting potency of benzofuroxans may be similar to that of a number of (poly) nitroaromatic compounds, including the important explosive 2,4,6-trinitrotoluene. The benzofuroxans are reduced in a single-electron way by a model flavoenzyme, ferredoxin : $NADP^+$ reductase, which implies the possibility of their single-electron reduction by the other flavoenzymes – dehydrogenases-electrontransferases, e. g., NADPH – cytochrome P-450 reductase or NO-synthase, which may be important in their mammalian cell cytotoxicity. Although the mammalian cell cytotoxicity of benzofuroxans may not entirely depend on their electron-accepting properties due to their possible parallel reactions, our data demonstrate the role of their redox cycling in their cytotoxicity. This may serve as a guideline for the further studies of the redox properties and cytotoxicity of benzofuroxans.

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BENZOFUROKSANŲ REDOKS SAVYBĖS IR PROOKSIDANTINIS CITOTOKSIŠKUMAS PALYGINTI SU NITROBENZENAIS

S a n t r a u k a

Funkcinės =N(→O)O- grupės gali suteikti benzofuroksanams elektronoakceptorines savybes, panašias į nitroaromatinių junginių arba jų *N*-oksidų savybes. Pastarieji junginiai dažnai naudojami kaip bioredukciniu būdu aktyvuojami priešnavikiniai ir / arba citotoksiški agentai. Tačiau iki šiol benzofuroksanų redoks savybės nebuvo ištirtos. Ištyrėme 8 benzofuroksanus, nustatydami jų laisvųjų radikalų susidarymo šilumas ($\Delta Hf(e^-)$), jų ciklinės voltamperometrijos redukcijos porencialus vandeninėje terpėje ($E_{p,7}$) ir jų vienelektroninės redukcijos greičio konstantas flavininiu fermentu ferredoksin: $NADP^+$ reduktaze (FNR). Nustatėme, kad FNR redukuoja benzofuroksanus ir (poli)nitrobenzenus vienelektroniniu būdu ir panašiais greičiais, priklausančiais nuo jų $\Delta Hf(e^-)$ reikšmės. Tačiau benzofuroksanų $E_{p,7}$ ir $\Delta Hf(e^-)$ nekoreliavo su nitrobenzenų $\Delta Hf(e^-)$ ir $E_{p,7}$ tarpusavio priklausomybe. Benzofuroksanų citotoksiškumas galvijų leukemijos virusu transformuotuose ėriuko inkstų fibroblastuose (FLK linija) buvo artimas nitroaromatinių junginių su atitinkamu $\Delta Hf(e^-)$ citotoksiškumui, tačiau pelių splenocituose šių junginių citotoksiškumas smarkiai skyrėsi. Tai gali būti susieta su kitais lygiagrečiais benzofuroksanų citotoksiškumo mechanizmais, pvz., jų reakcijomis su –SH grupėmis.