CHEMIJA. 2012. Vol. 23. No. 2. P. 141–146

Possible modes of cytotoxicity of high energy aliphatic nitroesters and *N*-nitramines for mouse splenocytes

Valė Miliukienė,

Jonas Šarlauskas,

Kastis Krikštopaitis,

Algirdas Šaikūnas,

Narimantas Čėnas*

Institute of Biochemistry of Vilnius University, Mokslininkų 12, LT-08662 Vilnius, Lithuania

In this work, we report for the first time the cytotoxicity data of a large number of structurally diverse high energy aliphatic nitrate esters and *N*-nitramines (n = 20) to mouse splenocyte cells. Their concentrations causing 50% cell death (cL_{50}) vary from 18 μM (*N*-nitro-diethanolamine dinitrate) to >250 μM (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, ethylene glycol dinitrate). The log cL_{50} of compounds does not depend on the calculated heats of their hydride adduct formation, or voltammetric reduction peak potentials, or lipophilicity. The cytotoxicity of compounds is not oxidative stress-dependent. On the other hand, their cytotoxicity is partly decreased by an inhibitor of aldehyde dehydrogenase (EC 1.2.1.10), tetraethylthiuram disulfide. It shows an involvement of this enzyme in bioreductive activation of nitrate esters and *N*-nitramines with the liberation of nitrite, which is further converted to NO.

Key words: organic nitrate ester, *N*-nitramine, cytotoxicity, aldehyde dehydrogenase

Abbreviations: cL_{50} – compound concentration causing 50% cell death, ΔHf – heat of formation, $E_{p(7)}$ – voltammetric reduction peak potential at pH 7.0, P – octanol / water partition coefficient, GTN – glycerol trinitrate, PETN – pentaerythritol tetranitrate, RDX – hexahydro-1,3,5-trinitro-1,3,5-triazine, HMX – octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, TNT – 2,4,6-trinitrotoluene, P-450R – NADPH: cytochrome P-450 reductase, ADH – aldehyde dehydrogenase.

INTRODUCTION

The aliphatic nitrate esters glycerol trinitrate (GTN) and pentaerythritol tetranitrate (PETN) (Fig. 1) are used clinically for the treatment of angina pectoris and other cardiovascular diseases [1]. It is generally accepted that these compounds undergo bioreductive denitration (Scheme) with the formation of nitrite, which is further reduced into NO [2, 3]. On the other hand, GTN, PETN, and the nitrogen analogues of nitroesters, *N*-nitramines hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and related compounds (Fig. 1) are extensively used as explosives. These compounds may be considered as important environmental pollutants because of the production of large amounts of wastewater during their manufacture, and soil pollution during military activities [4]. High concentrations of GTN were phytotoxic [5], GTN, PETN, RDX, and HMX were toxic to fish and other aquatic organisms [6]. RDX and HMX were also toxic to reptiles, birds, and mammals damaging their central nervous system, that may result in their death accompanied by neurotoxicity and convulsions ([7, 8], and references therein). Their mode(s) of toxicity differ from those of nitroaromatic explosives, e. g., 2,4,6-trinitrotoluene (TNT) in mammals, which are manifested mainly through methemoglobinemia, and damage to liver and spleen ([9], and references therein). However, in spite of their importance, the molecular

* Corresponding author. E-mail: narimantas.cenas@bchi.vu.lt

mechanisms of the toxicity of nitrate esters and related *N*-nitramines are poorly understood.

Continuing our studies on the mammalian cell cytotoxicity of explosives [9, 10], in this work we report for the first time the cytotoxicity data of a large number of structurally diverse aliphatic nitrate esters and *N*-nitramines (Fig. 1) to mouse splenocyte cells. We also attempted to clarify their cytotoxicity mechanisms.

EXPERIMENTAL

Aliphatic nitrate esters and *N*-nitramines (Fig. 1) were synthesized according to the established methods ([11], and references therein). All compounds were characterized by ^{1}H-NMR and IR spectroscopy, and by HPLC (LaChrom (Merck, Darmstadt, Germany)). All other compounds were obtained from Sigma-Aldrich, and used as received.

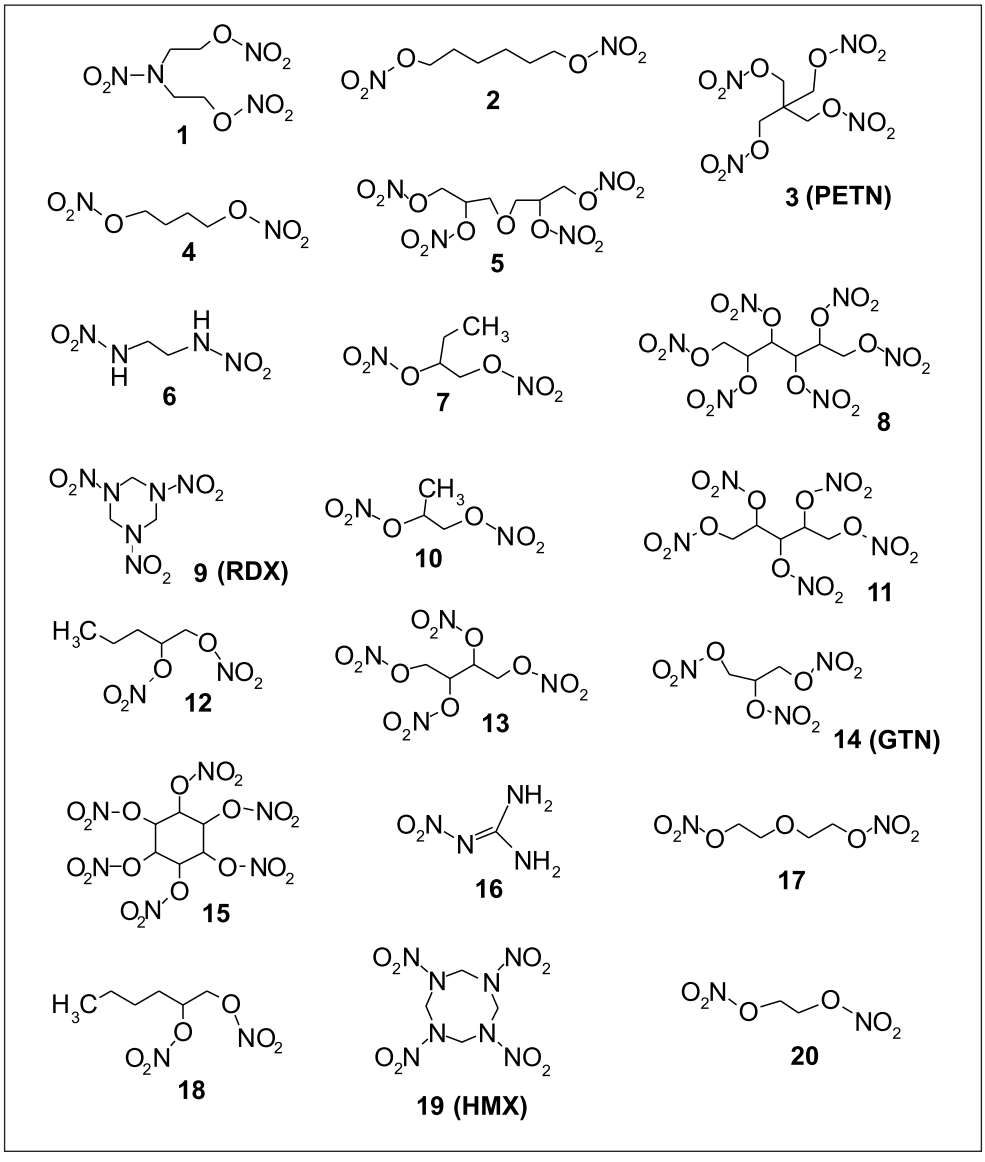
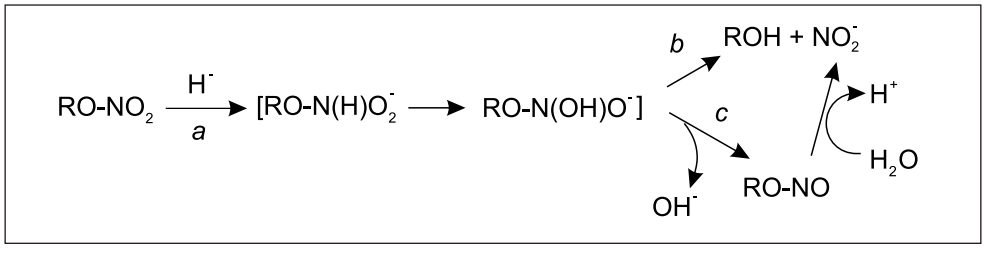


Fig. 1. Formulae of aliphatic nitroesters and *N*-nitramines studied



Scheme

4- to 8-week old male and female BALB/c mice (24 ± 2.0 g) were kept under standard conditions, and were given food and water *ad libitum*. The mice were sacrificed by decapitation, their spleens were removed according as described [10]. These experiments were approved by the Lithuanian Veterinary and Food Service (License No. 0171, 2007). Spleens of three to five mice for each experiment were used as a source of splenic lymphocytes (splenocytes). Erythrocytes were lysed by 5 min exposure in 3 ml ACK lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). After washing the cells twice with RPMI 1640 medium, they were resuspended at the concentration of 1.0×10^6 cells/ml in RPMI 1640 medium with 5% heat inactivated fetal bovine serum (Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml), and were used for further experiments. Cell viability was determined after 24 h incubation of splenocytes with the examined compounds in 96-well cell culture plates (200 μl cell suspension per well) at 37 °C in a humidified atmosphere containing 5% CO_2, 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 cyclic voltammetry experiments were performed using a Parstat 2273 (Princeton Applied Research) potentiostat controlled by Power Suite electrochemical software. A glassy carbon (Princeton Applied Research, diameter 2 mm) working electrode, a saturated Ag/AgCl (+205 mV vs. NHE) reference electrode, and a Pt wire (56 mm^2) as an auxiliary electrode were used in a standard three-electrode scheme. The glassy carbon electrode was polished with a suspension of alumina powder (1 μm), and then rinsed thoroughly with deionized water. The anaerobic conditions were obtained by purging the solutions (0.05 M K-phosphate + 0.1 M KCl, pH 7.0, 25 °C, compound concentration, 0.4–1.0 mM) with argon for 20 min. Stock solutions of compounds (0.1 M) were prepared in DMSO. Because of the electrode fouling during the repetitive scans in the presence of nitrate esters and *N*-nitramines, the electrochemical parameters referring only to the first scan are presented in this work.

The quantum mechanical calculations were perfomed using PC SPARTAN'04 PRO (Wavefunction, Inc., Irvine, CA, USA, 2003), as it has been described before [11]. The octanol / water partition coefficients of compounds (log *P*) were calculated using ACDLabs (Advanced Chemical Development, Toronto, Canada).

RESULTS AND DISCUSSION

We examined the cytotoxicity of GTN, PETN, RDX, HMX, and a number of their analogues (Fig. 1) in primary mouse splenocytes, determining their concentrations for 50% cell survival (cL_{50}, Table). The obtained cL_{50} values vary from 18 μM to >250 μM (Table), thus in several cases being close to the cytotoxicity of nitroaromatic explosives TNT (cL_{50} = 10 μM), and tetryl (cL_{50} = 6.0 μM) in the same cell line [10].

Next, we attempted to assess the mechanisms of cytotoxicity of aliphatic nitrate esters and *N*-nitramines. For comparison, the cytotoxicity of nitroaromatic explosives in splenocytes and other cell lines increases with an increase in their electron accepting potency [9, 10], which, taken together with the protection by the antioxidants, points to the main role of the oxidative stress, i. e., the single-electron enzymatic reduction of nitroaromatics into anion-radicals, and their subsequent redox cycling with the formation of superoxide and other reactive oxygen species. In mammalian cells, these reactions are performed mainly by flavoenzyme NADPH: cytochrome P-450 reductase (P-450R, EC 1.6.2.4) [9]. In this context, some data point to the slow single-electron enzymatic reduction of GTN by P-450R, and by other single-electron transferring metalloflavoenzyme, xanthine oxidase (EC 1.17.3.2) [12, 13], which probably may contribute to the oxidative stress-type cytotoxicity. Besides, there is also a posibility of a multistep (e^-, H^+, e^-) hydride transfer in the initial stage of the reductive denitration of nitroesters by reduced flavins (Scheme, step *a*) [3], i. e., the transient formation of free radicals that may react with oxygen . In order to assess the role of the oxidative stress, we examined the effects of antioxidant *N,N'*-diphenyl-*p*-phenylene diamine (3.0 μM) and the iron-chelating agent desferrioxamine (300 μM) on the cytotoxicity of GTN (500 μM), PETN (100 μM) and RDX (300 μM) that caused 57–63% cell death. However, we failed to observe any protective effect. Thus, the cytotoxicity of the above studied nitroester and *N*-nitramine explosives is not caused by the oxidative stress. Next, we attempted to examine a possible link between the cytotoxicity of nitroesters and *N*-nitramines and their hydride-accepting potency, the heat of formation of the hydride adduct ($\Delta Hf(R\text{-}ON(OH)O^-)$ or $\Delta Hf(R_1, R_2{>}N\text{-}N(OH)O^-)$ (Table), which relatively well described their reactivity towards *Enterobacter cloacae* PB2 PETN reductase, a representative of a unique class of flavoenzymes which may perform the biodegradation of nitroesters [11]. However, we did not observe a correlation between ΔHf and log cL_{50} ($r^2 < 0.200$) in this case. The introduction of compound lipophilicity (log *P*) as a second variable also did not improve the correlation. Although it has been claimed that certain mammalian cytochromes P-450 may be partly responsible for the degradation of RDX [14], the inhibitors of cytochromes P-450 1A1 and 2E1, 5.0 μM α-naphthoflavone and 1.0 mM izoniazide also did not show the protective effects against the cytotoxicity induced by GTN, PETN, and RDX.

In contrast to the calculated ΔHf of hydride-adduct formation (Scheme, step *a*) which describes the energetics of reduction of nitroesters and *N*-nitramines *in vacuo*, i. e., it may mimick their reduction in the hydrophobic active center of PETNR and related flavoenzymes [11], the voltammetric reduction potential of nitroesters and *N*-nitramines at pH 7.0, $E_{p(7)}$ may reflect their reduction energetics in the aqueous media [15, 16]. Thus, this parameter also deserves

Table. **Concentrations of aliphatic nitroesters and *N*-nitramines causing 50% death of splenocytes (cL_{50}), their heats of formation of hydride adducts ($\Delta Hf(R\text{-}ON(OH)O^-)$ or $\Delta Hf(R_1,R_2{>}N\text{-}N(OH)O^-)$ [11], their voltammetric reduction potentials at pH 7.0 ($E_{p(7)}$, vs. Ag/AgCl), and their octanol / water partition coefficients (log *P*)**

No.	Compound	cL_{50}, μM	$\Delta Hf(R\text{-}ON(OH)O^-)$ or $\Delta Hf(R_1,R_2{>}N\text{-}N(OH)O^-)$, kJ mol^{-1}	$E_{p(7)}$, V	log *P*
1.	*N*-nitro-diethanolamine dinitrate	18 ± 2.0	–228.3[a], –203.3[b]	–0.835, –1.101	1.36
2.	1,6-Hexanediol dinitrate	28 ± 2.0	–198.3[a]	–1.076	3.50
3.	Pentaerythritol tetranitrate (PETN)	38 ± 4.0	–243.9[a]	–0.770	2.90
4.	1,4-Butanediol dinitrate	52 ± 5.0	–203.9[a]	–1.230	2.44
5.	Diglycerol tetranitrate	59 ± 4.0	–218.4[a], –252.7[c]	–0.565, –0.738	2.79
6.	Ethylene-1,2-dinitramine	63 ± 5.0	–166.4[b]	<–1.400	–0.60
7.	1,2-Butanediol dinitrate	85 ± 9.0	–199.8[a], –200.6[c]	–1.300	2.44
8.	Mannitol hexanitrate	109 ± 15	–239.2[a], –280.8[c]	–0.830	4.04
9.	Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)	140 ± 12	–255.2[a]	–0.976	–0.50
10.	Xylitol pentanitrate	148 ± 10	–250.1[a], –282.2[c]	–0.960	3.42
11.	1,2-Propylene glycol dinitrate	156 ± 15	–202.3[a], –206.7[c]	–0.768	1.84
12.	1,2-Pentanediol dinitrate	175 ± 20	–201.7[a], –203.4[c]	–0.863	2.99
13.	Erythritol tetranitrate	175 ± 25	–258.8[a], –284.7[c]	–0.847	2.81
14.	Glycerol trinitrate (GTN)	187 ± 20	–236.6[a], –249.4[c]	–1.007	2.19
15.	Inositol hexanitrate	200 ± 25	–288.9[a]	–0.780	3.87
16.	Nitroguanidine	250 ± 30	–225.9[b]	<–1.400	–1.19
17.	Diethyleneglycol dinitrate	≥250	–197.9[a]	–0.705	1.37
18.	1,2-Hexanediol dinitrate	>250	–200.5[a], 202.1[c]	–0.883	3.50
19.	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)	>250	–273.6[b]	–0.998	–0.73
20.	Ethylene glycol dinitrate	>250	–204.5[a]	–0.634	1.57

[a] Primary nitrate group.

[b] N-nitramine group.

[c] Secondary nitrate group.

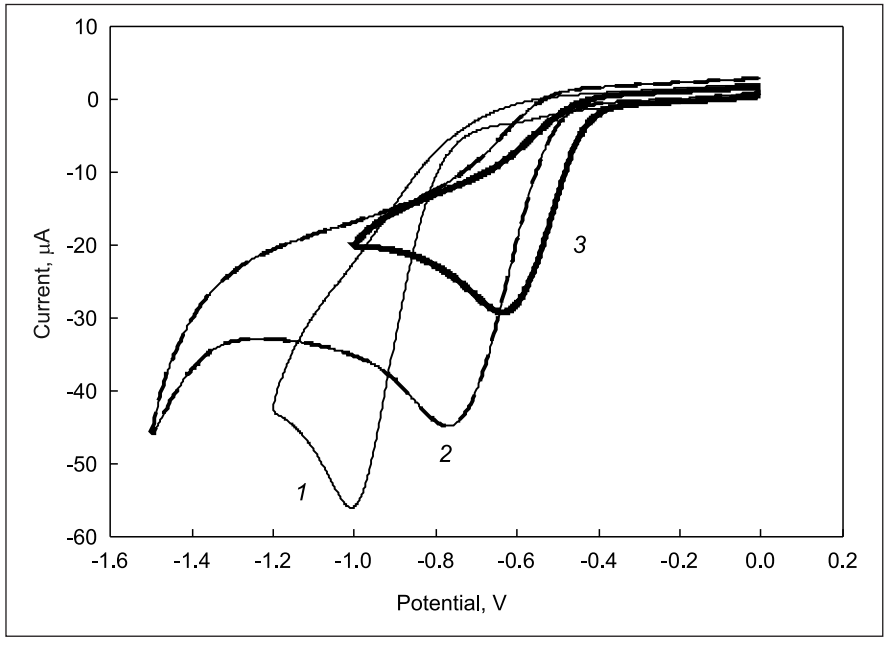


Fig. 2. Cyclic voltammograms ($v = 50$ mV/s) of electrochemical reduction of glycerol trinitrate (GTN) (*1*), 1,2-propylene glycol dinitrate (*2*), and ethylene glycol dinitrate (*3*) at 1.0 mM compound concentration and pH 7.0. The potentials are given vs. Ag/AgCl

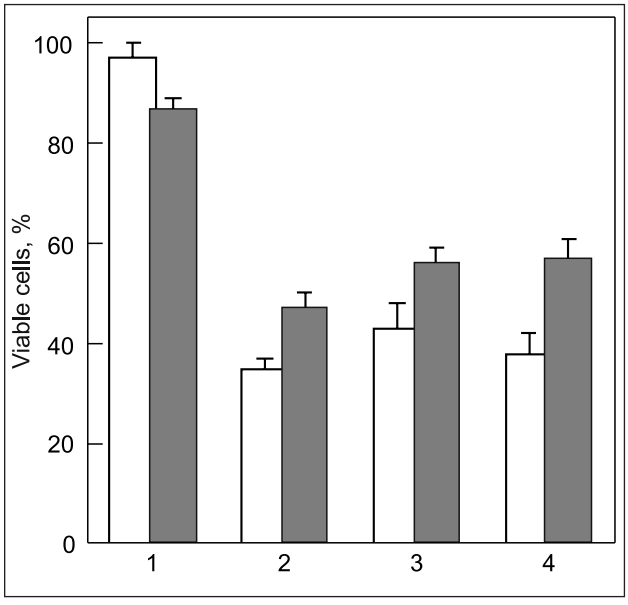


Fig. 3. Protective effects of tetraethylthiuram disulfide (1.0 μM) on the cytotoxicity of aliphatic nitroesters and *N*-nitramines to splenocytes. Conditions: control splenocytes (1), RDX (300 μM) (2), GTN (500 μM) (3), PETN (100 μM) (4). Open bars, no additions, filled bars – addition of tetraethylthiuram disulfide, $n = 3$, $p < 0.02$ for open vs. filled bars (2–4)

some interest with respect to the cytotoxicity of the compounds investigated (Table). It is suggested that the standard potential for a net two-electron reduction of GTN and 1,2-propylene glycol dinitrate (compound 11, Table) in the aqueous media at pH 7.0–12.0 may be high, 0.48–0.82 V vs. NHE, however, their electrochemical reduction proceeds irreversibly, with the overvoltage exceeding –1.0 V [15]. On the other hand, the voltammetric characteristics of other aliphatic nitroesters and *N*-nitramines are not well studied. Here we examined the cyclic voltammetry of compounds 1–20 (Fig. 2), finding that their reduction peak potentials ($E_{p(7)}$) vary from –0.565 V to –1.300 V (Table, Fig. 2). In certain cases, the cyclic voltammograms display several reduction peaks (Table). However, we also did not observe the correlation between $E_{p(7)}$ and log cL_{50} ($r^2 < 0.200$), and the introduction of compound log *P* as a second variable also did not improve the correlation.

Summing up, our data do not imply that the cytotoxicity of aliphatic nitroesters and *N*-nitramines in splenocytes is associated with the oxidative stress, or it is related to their hydrige-accepting properties, or to the ease of their electrochemical reduction. On the other hand, NADH-dependent aldehyde dehydrogenase (ADH, EC 1.2.1.10) was claimed to be at least partly responsible for the vasorelactant properties of GTN [17]. In this case, a net two-electron reduction of GTN (Scheme) accompanied by a release of nitrite is perfomed by a reduced catalytic disulfide moiety of this enzyme [18]. We found that the inhibitor of ADH, tetraethylthiuram disulfide (1-(diethylthiocarbamoyldisulfanyl)-*N*,*N*-diethylmethanethioamide), although being toxic to splenocytes (cL_{50} = 50 ± 10 μM), at low concentrations partly protected from the cytotoxicity of GTN, PETN, and RDX (Fig. 3). Analogous protecting effects of tetraethylthiuram disulfide were observed against the cytotoxicity of compounds (6) and (16) (Fig. 1) (data not shown). Thus, at present we may suggest that the cytotoxicity of aliphatic nitroesters and *N*-nitramines is partly attributed to their bioreductive activation by ADH, which initiates the intracellular formation of nitrite that is further converted into NO. However, the substrate specifity of nitrate reductase reactions of ADH, which is possibly responsible for the observed differences in the cytotoxicity of examined compounds, is not characterized so far. Other possibly important enzymatic mechanisms of their bioactivation also need to be elucidated in the future.

CONCLUSIONS

We found that the cytotoxicity of a large number of structurally diverse high energy aliphatic nitrate esters and *N*-nitramines in mouse splenocytes cells is not the oxidative stress dependent, and is not related to the calculated heats of their hydride adduct formation, or voltammetric reduction peak potentials, or lipophilicity. On the other hand, their cytotoxicity is partly decreased by an inhibitor of aldehyde dehydrogenase, tetraethylthiuram disulfide. It shows an involvement of this enzyme in bioreductive activation of nitrate esters and *N*-nitramines with the liberation of nitrite, which is further converted to NO.

Received 11 January 2012
Accepted 20 March 2012

References

1. J. Abrams, *Arch. Intern. Med.*, **155**, 357 (1995).
2. Y. Meah, G. J. Brown, S. Chakraborthy, V. Massey, *Proc. Natl. Acad. Sci. USA*, **98**, 8560 (2001).
3. P. S.-Y. Wong, J. M. Fukuto, *Drug Metab. Dispos.*, **27**, 502 (1999).
4. F. Monteil-Rivera, A. Halasz, C. Groom, J.-S. Zhao, S. Thiboutot, G. Ampleman, J. Hawari, in: G. I. Sunahara, G. Lotufo, R. G. Kuperman, J. Hawari (eds.), *Ecotoxicology of Explosives,* CRC Press, Boca Raton, FL, USA, 5 (2009).
5. R. Podlipna, Z. Fialova, T. Vanek, *Plant Cell Tiss. Org.*, **94,** 305 (2008).
6. M. Nipper, R. S. Carr, G. R. Lotufo, in: G. I. Sunahara, G. Lotufo, R. G. Kuperman, J. Hawari (eds.), *Ecotoxicology of Explosives*, CRC Press, Boca Raton, FL, USA, 77 (2009).
7. M. S. Johnson, C. A. McFarland, M. A. Bazar, M. J. Quinn, E. M. LaFiandra, L. G. Talent, *Arch. Environ. Contam. Toxicol.*, **58**, 836 (2010).
8. N. Garcia-Reyero, T. Habib, M. Pirooznia, K. A. Gust, P. Gong, C. Warner, M. Wilbanks, E. Perkins, *Ecotoxicology*, **20**, 580 (2011).

9. N. Čėnas, A. Nemeikaitė-Čėnienė, J. Šarlauskas, Ž. Anusevičius, H. Nivinskas, L. Misevičienė, A. Marozienė, in: G. I. Sunahara, G. Lotufo, R. G. Kuperman, J. Hawari (eds.), *Ecotoxicology of Explosives,* CRC Press, Boca Raton, FL, USA, 211 (2009).
10. V. Miliukienė, N. Čėnas, *Z. Naturforsch*., **63c**, 519 (2008).
11. H. Nivinskas, J. Šarlauskas, Ž. Anusevičius, H. S. Toogood, N. S. Scrutton, N. Čėnas, *FEBS J.*, **275**, 6192 (2008).
12. H. Li, X. Liu, H. Cui, Y.-R. Chen, A. J. Cardounel, J. L. Zweier, *J. Biol. Chem*., **281**, 12546 (2006).
13. H. Li, H. Cui, X. Liu, J. L. Zweier, *J. Biol. Chem.*, **280**, 16594 (2005).
14. B. Bhushan, S. Trott, J. C. Spain, A. Halasz, L. Paquet, M. Hawari, *Appl. Environ. Microbiol.*, **69**, 1347 (2003).
15. M. H. Miles, D. A. Fine, *J. Electroanal. Chem*., **127**, 143 (1981).
16. N. P. Saravanan, S. Venugopalan, N. Senthilkumar, P. Santosh, B. Kavita, H. G. Prabu, *Talanta*, **69**, 656 (2006).
17. Z. Chen, J. Zhang, J. S. Stamler, *Proc. Natl. Acad. Sci. USA*, **99**, 8306 (2002).
18. D. Chen, Q. C. Cui, H. Yang, Q. P. Dou, *Cancer Res*., **66**, 10425 (2006).

Valė Miliukienė, Jonas Šarlauskas, Kastis Krikštopaitis, Algirdas Šaikūnas, Narimantas Čėnas

GALIMI AUKŠTOS ENERGIJOS ALIFATINIŲ NITROESTERIŲ IR *N*-NITRAMINŲ CITOTOKSIŠKUMO KELIAI PELIŲ SPLENOCITUOSE

S a n t r a u k a

Darbe pirmą kartą pateikti duomenys apie daugelio (n = 20) skirtingos struktūros aukštos energijos alifatinių nitroesterių ir *N*-nitraminų citotoksiškumą pelių splenocitų ląstelėms. Jų koncentracijos, sukeliančios 50 % ląstelių žūtį (cL_{50}), kito nuo 18 μM (*N*-nitro-dietanolamino dinitratas) iki >250 μM (oktahidro-1,3,5,7-tetranitro-1,3,5,7-tetrazocinas, etilenglikolio dinitratas). Junginių log cL_{50} nepriklausė nuo jų apskaičiuotųjų hidrido aduktų susidarymo šilumų, jų elektrocheminės redukcijos potencialų maksimumų ciklinėje voltamperometrijoje ir nuo jų lipofiliškumo. Taip pat jų citotoksiškumas nebuvo susijęs su oksidaciniu stresu. Antra vertus, junginių citotoksiškumą tam tikru laipsniu mažino aldehiddehidrogenazės (FK 1.2.1.10) inhibitorius tetraetiltiuramo disulfidas. Tai rodo, kad minėtas fermentas dalyvauja šių junginių bioredukcinėje aktyvacijoje susidarant nitritui, kuris toliau yra redukuojamas į NO.