

## Antioxidant Effect of Xanthinyl-7-Acetic Acid Derivative on SOD Activity under Condition of Nitrosative Stress in Vitro

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### Abstract

During recent years  $\text{NO}\cdot$  and  $\text{ONOO}^-$  are considered to be a key pathophysiological factor of some basic diseases of the central nervous system (ischemia, stroke, neurodegenerative diseases, etc.). Therefore search for antioxidants which would reduce  $\text{NO}\cdot$  and  $\text{ONOO}^-$  toxicity seems to be very topical nowadays. In this study, we provide experimental evidence of antioxidant activity of the hydrazide of 8-benzylaminotheophyllinyl-7-acetic acid (C-3 compound) (synthesized at Department of Biological Chemistry, Zaporozhye State Medical University, Zaporozhye, Ukraine).

The investigation was conducted in vitro in the test of  $\text{NO}\cdot$  formation. Authors suggest that inactivation of superoxide dismutase (SOD) under condition of nitrosative stress plays an important role. We studied effect of C-3 compound on

SOD activity under condition of excessive NO $\cdot$  and ONOO $^-$  production. NO $\cdot$  induction was performed under the action of light on sodium nitroprusside Na $_2$ [Fe(NO)(CN) $_5$ ] $\times$ 2H $_2$ O. The investigation of substances was carried out in supernatant obtained from the brain of white Wistar rats (male, 200-250 g). For nitrosive stress modeling dinitrosyl-Fe $^{2+}$  cystein complex was utilized.

Obtained results showed that compound C-3 had pronounced antioxidant properties in conditions of nitrosative stress modeling and caused protective effect on SOD activity. This is the experimental rationale of further investigation of C-3 compound properties in vivo as potential antioxidant.

**Keywords:** Nitric oxide, sodium nitroprusside, superoxide dismutase, theophyllinyl-7-acetic acid derivative

## Introduction

Nitric oxide (NO $\cdot$ ) is short-life reactive free radical which plays the role of a universal modulator of various physiological functions of animals including the central nervous system (CNS) under both normal and pathological conditions. NO participates in regulation of blood circulation, neurotransmission, memory formation, in modulation of endocrinal function and behavioral activity [1]. In CNS pathology NO $\cdot$  may manifest itself as a pathogenic factor in cerebral ischemia, stroke, neurodegenerative diseases, epilepsy, etc [2]. Participation of NO $\cdot$  in the ischemic disorder development is dissimilar. Apparently, in acute stroke under neuronal NO-synthase activation NO $\cdot$  plays neuroprotective role, but then, especially in reperfusion (recirculation) period NO $\cdot$  effect becomes neurotoxic (especially under inducible NO-synthase activation and L-arginine deficit [3, 4]. Just in that very moment in a cell a 'nitrosative stress' develops. 'Nitrosative stress' is a part of oxidative stress [5]. It results in metal and thiol group nitration in protein molecules and causes the dysfunction of them. It also results in nucleic acid fragmentation, reduction of NAD $^+$  and ATP level in a cell, in inhibition of mitochondrial enzyme function [6].

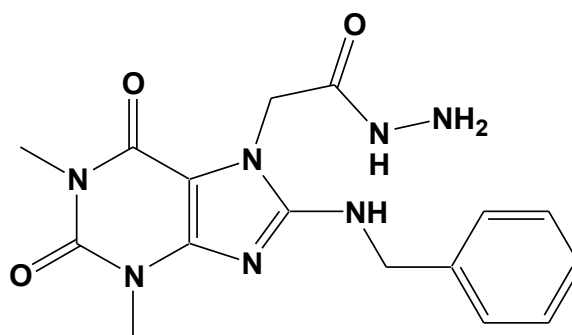
Main neurotoxic factor of nitrosive stress is a peroxynitrite (ONOO $^-$ ), which appears by NO $\cdot$  excess and superoxide radical interaction. A peroxynitrite molecule as a *cis*-isomer is relatively stable; it is able to diffuse at some cell body diameter

distance. As a strong oxidant ONOO $^-$  has a high cytotoxicity. It undergoes enzyme's sulfhydryl groups of protein fragments, receptors, ionic channels, DNA, and proteolipids to oxidative modification. Peroxynitrite reacts with metals of superoxide dismutase's active center and results in high toxic ion of nitrozonium (NO $_2^+$ ) formation which nitrosizes phenol groups of aminoacids (including tyrosine). The mechanism of impulse transmission in CNS thus breaks and the mechanism of apoptosis by means of peroxynitrite starts [6-8].

At the same time superoxide dismutase (SOD) is undergone to oxidative modifications (both reversible and irreversible) by peroxynitrite such as interaction with metalloproteinic center of enzymes and probable formation of nit-

rozole complex. SOD activity inhibition enforce neurotoxicity phenomenon [6, 9]. Therefore research of antioxidants which would reduce  $\text{NO}^\cdot$  and  $\text{ONOO}^-$  toxicity (especially towards SOD) seems to be very topical nowadays.

In the previous work we studied antioxidant activity of xanthinyl-7-acetic acid derivatives using in vitro methods of estimation [10]. During those experiment we found that the most pronounced activity had hydrazide of 8-bezylaminotheophyllinyl-7-acetic acid (compound C-3, figure 1), that had been synthesized by us earlier from theophylline [10]. The aim of this article is investigation of antioxidant activity of C-3 compound in vitro in the test of  $\text{NO}^\cdot$  formation. Also we studied its protective effect on SOD activity under conditions of excessive  $\text{NO}^\cdot$  and  $\text{ONOO}^-$  production.



**Figure 1.** Structure of 8-benzylaminotheophyllinyl-7-acetic acid (compound C-3)

## Materials and Methods

### Nitrosative stress modulation

$\text{NO}^\cdot$  induction was performed under the action of light on sodium nitroprusside sample (the light source 300W, wave  $> 425\text{nm}$ ). The light ray was focused on the sample with the help of lens. To remove thermal effect the ray was directed through the water filter. Water solution of sodium nitroprusside  $\text{Na}_2[\text{Fe}(\text{NO})(\text{CN})_5] \times 2\text{H}_2\text{O}$  (1,0 mM) irradiation (wave length = 1 mm) in quartz flask lasted for 30 min. Efficiency of  $\text{NO}^\cdot$  generation (control) and antioxidant activity of the investigated substances was measured by oxidation rate of ascorbic acid (40 mM, wave length = 265 nm). The substance was added to the samples before irradiation at a concentration of  $10^{-6}$  M. Optical density was measured before and after irradiation [11].

Investigation of the substance was carried out in supernatant obtained from the brain of white Wistar rats (male, 200-250 g). After decapitation the brain was removed and cooled in isotonic solution of sodium chloride, then homogenized in 5 volumes of 50 mM tris-HCl buffer (pH=7,4,  $t=5^\circ\text{C}$ ) containing 0,5 mM of EDTA and 1mM of dithiotreithole. Obtained homogenate was centrifuged in 11,000 g for 30 min,  $t=4^\circ\text{C}$ . For nitrosive stress modeling dinitrozolic complex of

$\text{Fe}^{2+}$  and cysteine was utilized. This one is a stable  $\text{NO}^\cdot$  complex which may be considered as a transport form of this radical with a longer  $\text{NO}^\cdot$  inhibition activity [6, 7].  $\text{NO}^\cdot$ -cysteine complex was added into supernatant (content of protein 5 mg/ml) at a concentration 100 mM and was incubated for 10 min at  $4^\circ\text{C}$ . The investigated substances were introduced before incubation at a concentration  $10^{-6}\text{M}$ . For nitrosive stress and the investigated substances estimation in vitro protein oxidative modification products were determined.

#### **Estimation of protein oxidative modification products**

Protein sedimentation with 20% solution of trichloroacetic acid was performed in supernatant aliquot. 1,0 ml of solution of 0,1 M 2,4-dinitrophenylhydrazine in 2 M HCl was added to denaturated protein and was incubated at  $37^\circ\text{C}$  for 60min. Then the samples were centrifuged at 3,000 g for 20 min. Sediment was washed with ethanol:ethylacetate mixture (1:1) 3 times. The obtained sediment was dried in thermostatic apparatus at  $40^\circ\text{C}$  and then it was dissolved in 3,0 ml of 8 M urea. For better solubility 0,01 ml of 2M HCl was added. Optic density of dinitrophenylhydrazones was registered at wave length of 274 nm (aldehydes) and 363 nm (carboxyles). Rate of protein oxidative modification was expressed in units of optical density per 1g of protein [12].

#### **Estimation of SOD activity**

Adrenaline's autooxidation in basic medium leading to superoxide radical formation lies in the base of this method. At SOD presence this reaction slows down [13]. 4,4 ml of 0,5 mM carbonate buffer (pH=10,2) is put into spectrophotometer flask with optical path of 1 cm. 0,1 ml of supernatant and 0,5 ml of adrenaline solution in citric acid (100 ml of bidistilate contains 192 mg of citric acid and 333 mg of adrenaline) is added to buffer. Kinetics of reaction is registered at 480 nm. SOD activity is expressed in units per mg of protein per min.

#### **Statistical analysis**

Results of the experiment were processed using STATISTICA<sup>®</sup> 6.0 licensed software for Windows (Stat Soft Inc NAXXR712D833214FANS). Results were represented as sample mean  $\pm$  standard error of the mean. Significant differences between trial groups were evaluated by means of Student t-test and Mann-Whitney U-test. For all types of analysis, the differences were deemed to be statistically significant at  $p \leq 0.05$

#### **Results and Their Discussion**

The modeled nitrosative stress in supernatant of rats' brain characterized by inhibition of antioxidant enzyme – superoxide dismutase activity, by increase in formation of protein oxidative modification products reacting with 2,4-dinitrophenylhydrazine. These reaction products showed the maximum of absorbtion at 270 and 363 nm. Thus SOD activity in the control test was decreased

on 53,89% in comparison with intact. Protein oxidative modification products found at 270 nm increased to 87,14%, and increased to 64,57% at 363 nm (table 1). The obtained data do not contradict the data obtained by other investigators which showed that the nitration of thiol groups of protein molecules (receptors, ionic channels), of low-molecular antioxidants (glutathione, methionine, etc) and of phenols occurred in nitrosative stress [4, 5, 14]. Antioxidant enzymes (their activity is inhibited by formation of nitrocomplexes with metal ions of enzyme's active center) were another object of nitrosative stress destructive influence.

Reduction of antioxidant enzyme activity and thiol antioxidant level,  $\text{NO}_2^+$  and

$\text{OH}^-$  production at  $\text{ONOO}^-$  decay result in significant increase of free radical oxidation [1, 4, 5, 15]. By many authors' data in nitrosive stress unfavorable conditions arise in a cell, tyrosine kinase activity is inhibited, protein phosphorylation breaks, enzymes of oxidation are inhibited (succinate dehydrogenase, malate dehydrogenase), proapoptotic factors of transcription are activated [2, 3, 6, 14, 16].

Addition of test compound to the incubation mixture before modeling of nitrosative stress at a concentration of  $10^{-6}$  M, led to the limiting of damaging effect of aggressive forms of nitrogen monoxide in relation to the SOD (table 1). It increased SOD activity of 83.94%, that on 60.97% higher than N-acetylcysteine effect, Compound C-3 also decreased the level of markers of oxidative protein modification – 2,4-dinitrophenylhydrazones on 34.35 % and 31.60 % respectively. In the same time addition of selective peroxynitrite scavenger – N-acetylcysteine to the incubation mixture decreased these markers on 13,74 % and 17,71 %.

Obtained results showed that compound C-3 had pronounced antioxidant properties in conditions of nitrosative stress modeling and caused protective effect on SOD activity. This is the experimental rationale of further investigation of C-3 compound properties in vivo as potential antioxidant.

## References

- [1] I. Fridovich, Fundamental aspects of reactive oxygen species, or what's the matter with oxygen, *Annals of the New York Academy of Sciences*, **893** (1999), 13-18. <http://dx.doi.org/10.1111/j.1749-6632.1999.tb07814.x>
- [2] C. Szobo, Physiological and pathophysiological role of nitric oxide in the central nervous system, *Brain Res. Bull.*, **41** (1996), 131-141. [http://dx.doi.org/10.1016/0361-9230\(96\)00159-1](http://dx.doi.org/10.1016/0361-9230(96)00159-1)
- [3] C.S. Parkins, A.L. Halder, M.F. Dennis et al., Involvement of oxygen free radicals in ischemia-reperfusion injury to Murine Turnours: Role of nitric oxide, *Free Radical. Res.*, **28** (1998), 271-281. <http://dx.doi.org/10.3109/10715769809069279>

- [4] S. Pou, W. Pou, Generation of superoxide by brain nitric oxide synthase, *J. Biol. Chem.*, **267** (1992), 24173-24176.
- [5] A.N. Hausladen, NO and metalloproteins, *Eur. Cell. Biol.: Suppl.*, 48/75 (1998), 32-38.
- [6] A.A. Boldyrev, Role of reactive oxygen species in Functional activity of neuronal cells, *Success of Physiological Science*, **34** (2005), no. 3, 21-34.
- [7] I.F. Belenichev, Yu.I. Gubsky, E.L. Levitsky, S.I. Kovalenko, A.N. Marchenko, Antioxidant system of protection organism, *Modern problems of Toxicology*, **3** (2002), 24-31.
- [8] I.V. Victorov, Role of Nitric oxide and other free radicals in brain ischemic lesions, *Herald of Rus. Academy of Med. Sci.*, **6** (2000), 5-9.
- [9] I.F. Belenichev, E.L. Levitsky, S.I. Kovalenko, Yu.I. Gubsky, A.N. Marchenko, Products of free radical peroxides oxidation and method of their identification, *Modern Problems of Toxicology*, **4** (2002), 9-14.
- [10] K.V. Aleksandrova, I.F. Belenichev, A.S. Shkoda, S.V. Levich, D.N. Yurchenko, N.V. Buchtiyarova, Research of antioxidant properties of theophyllinyl-7-acetic acid derivatives, *Oxid Antioxid Med Sci.*, **3** (2014), no. 3, 187-194. <http://dx.doi.org/10.5455/oams.191214.or.078>
- [11] Yu.I. Gubsky, V.V. Dunaev, I.F. Belenichev, E.L. Levitsky, N.V. Litvinova, S.I. Kovalenko, N.V. Buhtiyarova, O.V. Afanasenko, Methods of estimation of physiologically active substances antioxidant properties under free radical processes initiation in vitro, *Kyiv: Avicenna*, **26** (2002).
- [12] I. Fridovich, *Handbook of Methods for Oxygen Radical Research*, Boca Paton, Florida, 1987, 256.
- [13] H.P. Misra, I. Fridovich, The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase, *J. Biol. Chem.*, **247** (1972), 3170-3175.
- [14] C. Edschiuch, D. Gilbert, C. Colton, *The Neurobiology of NO and OH*, Acad. Sci. New York, 1994, 140.
- [15] O. Seggent, B. Griffon, I. Morel et al., Effect of nitric oxide on iron-mediated oxidative stress in primary rat hepatocyte culture, *Hepatology*, **25** (1997), 122-127. <http://dx.doi.org/10.1002/hep.510250123>

[16] H. Nakazawa, Ch. Genka, M. Fujishima, Pathological aspects of active oxygens/free radicals, *Jap. J. Physiology*, **46** (1996), 15-32.  
<http://dx.doi.org/10.2170/jjphysiol.46.15>

Table 1. Influence of C-3 compound on the activity of SOD and content of products of oxidative modification of proteins in the brain supernatant of rats brain in conditions of nitrosative stress modeling in vitro ( $M \pm m$ )

Samples	SOD, units /mg of protein /min	Products of oxidative modification of proteins, u/mg of protein	
		Aldehydes, 270 nm	Carboxyles, 363 nm
Intact (n = 10)	260,7 ± 7,6	14,0 ± 0,11	22,3 ± 0,17
Control, DNIC, 100 $\mu$ M (n = 10)	120,2 ± 5,0 <sup>Δ</sup>	26,2 ± 0,21 <sup>Δ</sup>	36,7 ± 0,10 <sup>Δ</sup>
Test, DNIC + C-3, 10 <sup>-6</sup> M (n = 10)	221,1 ± 11,0 <sup>*#</sup>	17,2 ± 0,21 <sup>*#</sup>	25,1 ± 0,07 <sup>*#</sup>
Reference, DNIC + N- acetylcysteine, 10 <sup>-6</sup> M (n = 10)	147,8 ± 3,7 <sup>*</sup>	22,6 ± 0,12 <sup>*</sup>	30,2 ± 0,12 <sup>*</sup>

Remark:

1. <sup>Δ</sup> – p<0,05 in comparison with intact;
2. <sup>\*</sup> – p<0,05 in comparison with control;
3. <sup>#</sup> – p<0,05 in comparison with N-acetylcysteine.

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