ISSN 0012-5008, Doklady Chemistry, 2019, Vol. 486, Part 2, pp. 152–155. © Pleiades Publishing, Ltd., 2019.
Russian Text © The Author(s), 2019, published in Doklady Akademii Nauk, 2019, Vol. 486, No. 4.

CHEMISTRY

The Reactivity of 3-Hydroxy-6-methyl-2-ethylpyridine 2-Nitroxysuccinate and Reference Drugs in Model NO-Generating Systems

O. V. Pokidova[a,*], E. V. Batova[b], A. P. Sadkov[a], A. B. Eremeev[a], B. S. Fedorov[a†], and A. I. Kotelnikov[a,c]

Presented by Academician S.M. Aldoshin January 14, 2019

Received February 13, 2019

Abstract—The ability of 3-hydroxy-6-methyl-2-ethylpyridine 2-nitroxysuccinate (**I**) to generate nitrite ions (NO_2^-) and nitrogen monoxide (NO) in model systems with cysteine (Cys) and deoxyhemoglobin (Hb) has been studied. Compound **I** has been found to release NO_2^- and NO more efficiently than Nicorandil. The accumulation rate of NO_2^- in the system of Cys with **I** is by a factor of 1.5 higher than that with Nitroglycerin. It has been shown that, in contrast to Nitroglycerin, compound **I** is not reduced by Hb.

DOI: 10.1134/S0012500819060016

At present time, an important task of public health is to decrease cardiovascular morbidity and mortality caused mainly by ischemia. Pharmacotherapy of ischemia consists in the combined intake of pharmaceuticals that exhibit vasodilatory, diuretic, and attenuating action. Organic nitrates used in medicine practice, such as, for example, Nitroglycerin, Nicorandil, and Isosorbide dinitrate, are good vasodilating agents and, therefore, are widely used in ischemia therapy. It was found that the biological effect of organic nitrates is caused by the production of nitrogen monoxide (NO), and their reduction to NO involves a number of substrates, including thiols, ascorbate, aldehyde dehydrogenase, etc. [1, 2]. Resulting NO molecules activate guanylate cyclase, which finally, via a cascade of intracellular reactions, leads to the vasodilatory effect [3].

An original polyfunctional compound, 3-hydroxy-6-methyl-2-ethylpyridine 2-nitroxysuccinate (**I**) (Fig. 1) prepared at the Institute of Problems of Chemical Physics, RAS [4], combines the properties of organic nitrate with efficient antioxidant activity [5] and is a promising drug for the treatment of ischemia. This compound also behaves as a good inhibitor of phosphodiesterase [6], an enzyme, which, along with guanylate cyclase, is a key component to control the level of cyclic nucleotides. Thus, the concurrent effect of **I** on these two enzyme systems allows one to predict its high cardioprotective and anti-ischemic activity.

In this work, we assessed the activity of **I** as an NO donor. The reducing agent used was cysteine (Cys), whose reaction mechanism with organic nitrates has been studied [7]: thionitrates form at the first stage, which further transform into nitrite ions (NO_2^-) and NO. It has been reported [8] that the rate of NO_2^- formation from organic nitrates in the reaction with Cys correlates with their vasodilatory activity. Thus, the efficiency of vasodilatory drugs can be judged from the NO_2^- formation rate in organic nitrate–Cys system. The activity of compound **I** was compared with that of drugs used in clinical practice: Nitroglycerin and Nicorandil (Fig. 1).

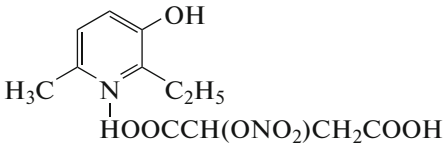
The most convenient and widely used method for the quantitative determination of NO_2^- is the Griess test [9]. The test is based on the determination of the optical density of aniline azo dye (proportional to NO_2^- concentration) resulting from the reaction of NO_2^- with sulfanylamide (SA) and *N*-(1-naphthyl)ethylenediamine (NEDA).

Figure 2 shows kinetic curves of NO_2^- accumulation for **I** and Nitroglycerin. The reaction began with

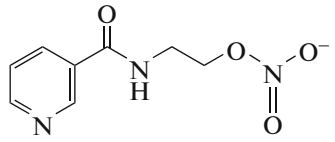
† Deceased.

[a] *Institute of Problems of Chemical Physics, Russian Academy of Sciences, Chernogolovka, Moscow oblast, 142432 Russia*
[b] *Ivanovo State University, Ivanovo, 153000 Russia*
[c] *Moscow State University, Moscow, 119991 Russia*
**e-mail: pov@icp.ac.ru; olesia16@mail.ru*

3-Hydroxy-6-methyl-2-ethylpyridine 2-nitroxysuccinate (I)



Nicorandil



Nitroglycerin

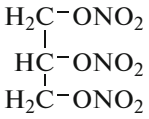


Fig. 1. Chemical structure of organic nitrates.

addition of organic nitrates to Cys and conducted at 23°C with stirring for 5 h under anaerobic conditions. The final Cys and organic nitrates concentration in the reaction mixture was 10^{-3} M and 9×10^{-4} M, respectively. After certain time intervals, 0.5-mL aliquots of the reaction mixture were sampled and placed in vials containing 1.5 mL of a 0.5% SA solution (Sigma) in 0.25 M HCl. The vials were incubated for 5 min at ambient temperature, and 1 mL of a 0.02% NEDA solution (MP Biomedicals) in 0.5 M HCl was added. Ten minutes later, the optical density was determined at 540 nm on a Specord M-40 spectrophotometer. The concentration of NO_2^- was calculated using a calibrating curve built toward sodium nitrite.

It was found that the reaction of I with Cys causes the efficient evolution of NO_2^-: 27.21 ± 4 μM NO_2^- formed over 5 h (Fig. 2).

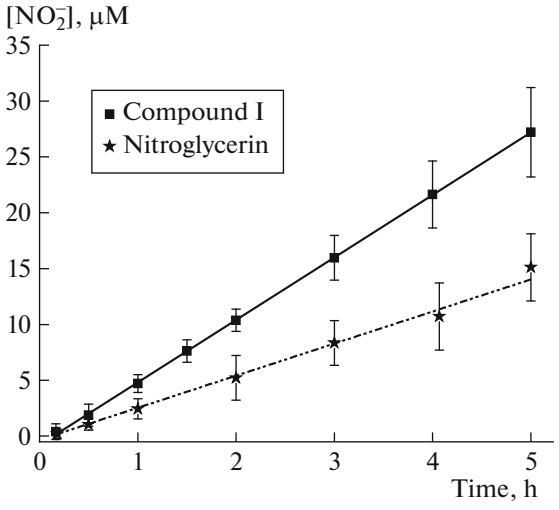


Fig. 2. Kinetics of NO_2^- accumulation in the system of 9×10^{-4} M **I** or Nitroglycerin with 10^{-3} M Cys. Dots show experimental data. The solvent is 0.05 M phosphate buffer, pH 7.0, temperature 23°C.

In experiments with Nitroglycerin, 15.12 ± 3 μM NO_2^- formed over 5 h (Fig. 2). Consequently, the activity of **I** is by a factor of 1.5 higher than that of Nitroglycerin.

In contrast to the Cys–**I** system, the amount of NO_2^- formed in the Cys–Nicorandil model system is much lower within the experiment error. After 19 h, the reaction produces 0.75 μM NO_2^- in this system. Thus, the activity of Nicorandil is much lower than that of Nitroglycerin and compound **I**.

Deoxyhemoglobin (Hb), which is one of the basic sources of NO_2^- reduction, plays an important role in the conversion of NO_2^- into NO [10]. It should be noted that Hb can undergo redox reactions with certain organic nitrates. It is known that Nitroglycerin reacts with Hb to form NO_2^- and methemoglobin [11]. Therefore, the intravenous injection of Nitroglycerin may result in methemoglobinemia.

At the next stage of the work, we studied the NO-producing activity of organic nitrates in the Cys–organic nitrate–Hb model system. This system is convenient to use for the spectrophotometric registration of NO accumulation kinetics by the rate of formation of nitrosylated hemoglobin (HbNO) because Hb is a trap for NO: the rate of NO binding to Hb hemes is close to the diffusion rate, the binding constant is 3×10^{10} M^{-1} [12]. Moreover, HbNO shows a characteristic absorption spectrum: the Hb absorption maximum (556 nm) upon the formation of HbNO will split into two peaks (545 and 575 nm), which is convenient for the determination of conversion.

A homogeneous hemoglobin solution was isolated from fresh blood of experimental animals by the known procedure [13]. The obtained preparation was a solution of oxyhemoglobin in water with the concentration 4.2×10^{-5} M. To convert it into Hb, a solution of the preparation was purged in 10-mL vessels with an argon flow for 10 min.

All further experiments with organic nitrates, Cys, and Hb were conducted in an argon atmosphere at

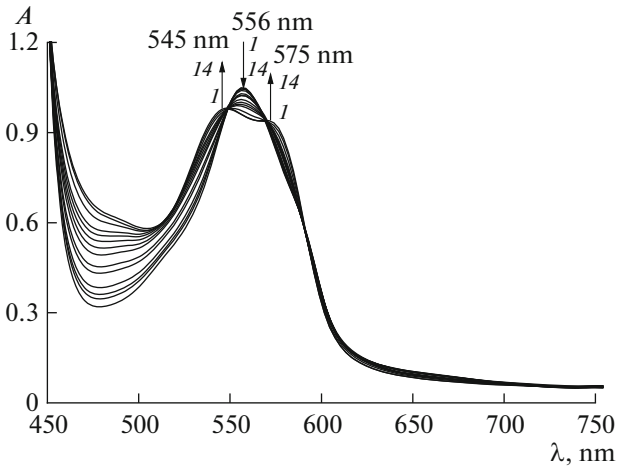


Fig. 3. Time variation of absorption spectra for the reaction of 2.5×10^{-4} M of **I** with 7×10^{-3} M Cys in the presence of 2.1×10^{-5} M Hb. The spectra were recorded for 24 h. The solvent is 0.05 M phosphate buffer, pH 7.0, temperature 23°C.

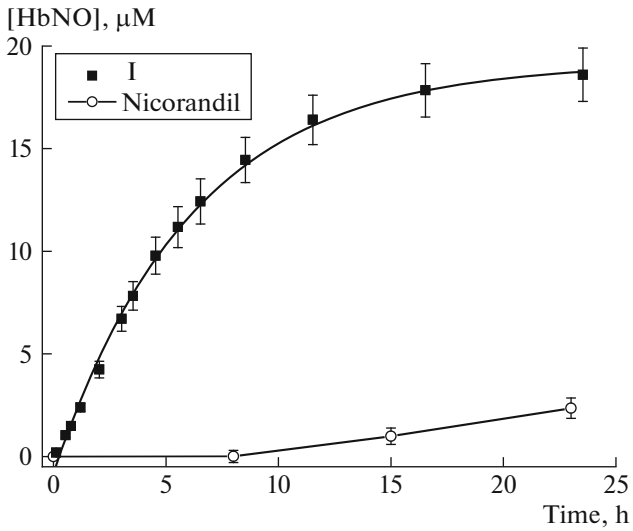


Fig. 4. Kinetic curves for the accumulation of HbNO in the Hb–organic nitrate–Cys system. Conditions are similar to those in the caption to Fig. 3.

23°C. It was established first of all that Hb in the absence of Cys does not reduce **I** and Nicorandil: the spectrum of Hb in the Hb–organic nitrate reaction system did not vary in time. Next, an experiment was conducted in the presence of Cys. For this purpose, an aliquot of an anaerobic Cys solution was added into a quartz cuvette containing 1.47 mL of an Hb solution. The reaction was initiated by the addition of organic nitrates in the cuvette, absorption spectra were recorded at certain time intervals for 24 h. The final concentrations were 2.5×10^{-4} M for organic nitrates, 7×10^{-3} M for Cys, and 2.1×10^{-5} M for Hb. A comparison cuvette contained 3 mL of the phosphate buffer pH 7.0. The decomposition of absorption spectra of the reaction system into components (Hb and HbNO) for the determination of component concentration was performed using the MathCAD.

In the study of compound **I** in the triple system Cys–organic nitrate–Hb, it was established that the efficient NO evolution occurs with the subsequent formation of HbNO. Figure 3 displays absorption spectra of this system. It is seen that a gradual decrease in the optical density is observed in the region of the Hb absorption maximum (556 nm), while a growth of the optical density is detected at 545 and 575 nm, which indicates the HbNO formation.

The process for Nicorandil takes place much slower: no two expressed peaks of HbNO form within a day.

Figure 4 displays kinetic curves of HbNO accumulation for the experiments described above. The incubation of the system with compound **I** for one day resulted in 18.6 μM HbNO; i.e., 88.6% of Hb converted into HbNO. Under similar conditions, Nicorandil showed a more prolonged action: only 2.36 μM HbNO formed over this time.

Thus, the data obtained in this work allow us to draw a conclusion that compound **I** in the reduction of Cys is by a factor of 1.5 more efficient than Nitroglycerin. In NO_2^-- and NO-producing systems, compound **I** is also a more efficient agent than Nicorandil. Compound **I**, in contrast to Nitroglycerin, is not involved in the redox reaction with Hb and consequently is not able to lead to methemoglobinemia.

Thus, our findings enable us to recommend compound **I** for further study as a promising drug for ischemia treatment.

FUNDING

This work was supported by State Contract no. 14.N08.12.0185 of November 2017 "Preclinical study of innovative drugs" under the Federal Research Program "Development of Pharmaceutical and Medical Industry of the Russian Federation until 2020 and for further period" and by State Contract no. 0089–2019–0014.

REFERENCES

1. Wang, P.G., Cai, T.B., and Taniguchi, N., *Nitric Oxide Donors: For Pharmaceutical and Biological Applications*, Weinheim: Wiley-VCH, 2005.
2. Page, N.A. and Fung, H.L., *J. Pharm. Sci.*, 2013, vol. 102, no. 9, pp. 3070–3081.
3. Ignarro, L.J., *Nitric Oxide Biology and Pathobiology*, San Diego: Academic, 2000.
4. RF Patent 2250210, 2010.
5. Neganova, M.E., Klochkova, S.G., Shevtsova, E.F., Bogatyrenko, T.N., Mishchenko, D.V., *Byul. Eksp. Biol. Med.*, 2018, vol. 166, no. 7, pp. 53–57.

6. Tat'yanenko, L.V., Dobrokhotova, O.V., Varfolomeev, V.N., Fadeev, M.A., Fedorov, B.S., Shtol'ko, V.N., and Mishchenko, D.V., *Khim.-Farm. Zh.*, 2014, vol. 48, no. 7, pp. 3–6.
7. Feelisch, M., *J. Cardiovasc. Pharmacol.*, 1991, vol. 17, no. Suppl. 3, pp. S25–S33.
8. Yeates, R.A., Laufen, H., and Leitold, M., *Mol. Pharmacol.*, 1985, vol. 28, pp. 555–559.
9. Fox, J.B., *Anal. Chem.*, 1979, vol. 51, no. 9, pp. 1493–1502.
10. Huang, Z., Shiva, S., Kim-Shapiro, D.B., and Patel, R.P., *J. Clin. Invest.*, 2005, vol. 115, no. 8, pp. 2099–2107.
11. Bennett, B.M., Nakatsu, K., Brein, J.F., and Marks, G.S., *Can. J. Physiol. Pharmacol.*, 1984, vol. 62, no. 6, pp. 704–706.
12. Antonini, E. and Brunori, M., Hemoglobin and myoglobin in the reactions with ligands, in: *North-Holland Research Monographs. Frontiers of Biology*, Amsterdam: North-Holland, 1971, vol. 21.
13. Geraci, G., Parkhurst, L.J., and Gibson, Q.H., *J. Biol. Chem.*, 1969, vol. 244, no. 17, pp. 4664–4667.

Translated by I. Kudryavtsev