Stobadine — a novel scavenger of free radicals

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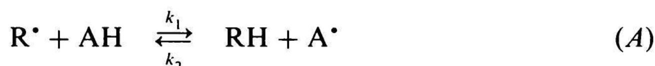
Dedicated to Professor Ľ. Treindl, DrSc., in honour of his 60th birthday

A new antiarrhythmic, antihypoxic drug — stobadine tested on its antioxidative activity is an effective inhibitor of lipid peroxidation and a potent scavenger of $^{\bullet}OH$ radicals. A stable radical derived from stobadine with a well resolved ESR spectrum was obtained indicating the conversion of its amino group to the corresponding nitroxyl radical. The effective antioxidant and scavenger activity of stobadine is attributed to its amino group in position 5.

Новое антиаритмическое и антигипоксическое лекарство — стобадин, исследуемое на антиокислительную активность, является эффективным ингибитором переокисления липидов и эффективной ловушкой радикалов $^{\bullet}OH$. Был получен из стобадина устойчивый радикал с хорошо разрешенным ЭПР спектром, что свидетельствует о превращении амино-группы стобадина в соответствующий нитроксильный радикал. Эффективная активность стобадина как антиоксиданта и ловушки радикалов приписывается наличию амино-группы в положении 5.

Free radicals generated in organisms as a consequence of ionizing radiation, metabolism of xenobiotics or postischemic reperfusion were found to induce damage of membrane lipids, proteins or nucleic acids, which may play a significant role in many diseases and aging [1]. Under pathological conditions the natural enzymic (*i.e.* redox metalloenzymes) and nonenzymic (*i.e.* ascorbic acid, tocopherols, glutathione) defense systems in the organism are overwhelmed. The need for drugs that suppress the free radical production is therefore raising [2].

Usually the scavengers react with free radicals at high rate, thus forming stable radicals, *i.e.* k_1 is high and k_2 is low in the following reaction



$$R^{\bullet} + AH \underset{k_2}{\overset{k_1}{\rightleftarrows}} RH + A^{\bullet} \qquad (A)$$

where $R^{\bullet}$ and RH represent radical and nonradical form of the substrate, respectively, and $A^{\bullet}$ and AH denote the molecule of scavenger in oxidized and

reduced form, respectively. Radical $A^{\bullet}$ or its products are frequently observable by ESR spectroscopy.

Stobadine, (−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido[4,3-*b*]indole (*I*), in the form of dichloride, a new drug with pyridoindole structure [3] (Scheme 1) was found to exhibit antiarrhythmic [4] and antihypoxic effects [5] in myocardium. In both effects free radicals might be involved, because it has been shown that there is a burst of free radicals during postischemic reperfusion [6, 7] and several antioxidants were found to suppress reperfusion arrhythmias [8]. Recently stobadine was found to decrease the extent of postischemic lipid peroxidation in rat brains [9], a process which is initiated and propagated by free radicals [10]. Moreover, the ability of stobadine to generate stable radicals was established in preliminary experiments [11, 12].

The aim of the present study was to provide a more detailed insight into the molecular basis of the free radical scavenging activity of the stobadine.

Experimental

Chemicals

Stobadine was provided by courtesy of Dr. *L. Beneš* and was prepared as described in [3]. The spin trap 5,5′-dimethyl-1-pyrroline *N*-oxide (DMPO) (Janssen, Chimica) was purified with charcoal and its concentration in stock solution was determined spectrophotometrically ($\varepsilon_{294\ \mathrm{nm}} = 770\ \mathrm{m^2\ mol^{-1}}$ in ethanol [13]). Egg yolk lecithin (phosphatidylcholine), isolated according to the method of *Singleton et al.* [14] was provided by courtesy of Dr. *P. Balgavý*. All other chemicals were of anal. grade obtained from commercial sources.

Preparation of liposomes

Lecithin solution (20 mg) in the mixture with $CHCl_3$—CH_3OH ($\varphi_r = 2:1$) was evaporated under a stream of nitrogen *in vacuo*. The dried samples were hydrated with 500 mm^3 of buffer: *c* ($mmol\ dm^{-3}$): 145 NaCl, 5 KCl, 1.4 $MgCl_2$, 1 $CaCl_2$, 5 Hepes; pH 7.4. Multilamellar liposomes were prepared by vortexing the suspension for 1—3 min. Stobadine was added and its final content was 1 mole % of lipids.

Lipid peroxidation assay

Liposomes with or without stobadine were incubated in 50 °C water bath under air atmosphere. At various time aliquots of 2 mg of lipids were taken, dissolved in 3 cm^3 of

ethanol and UV spectra at $\lambda = 215$ nm and 233 nm were measured using Specord M 40 (Zeiss, Jena) UV VIS spectrometer. The relative extent of lipid peroxidation was calculated according to [15] as the ratio of absorbances $A_{233\,\text{nm}}/A_{215\,\text{nm}}$ which reflects conjugated dienes formation in the fatty acyl chains of lipids. The interference of stobadine with the conjugated dienes at $\lambda = 233$ nm in the concentration used did not influence the results significantly.

Spin trapping

$^{\bullet}$OH radicals were generated by a Fenton-type reaction

$$Fe^{2+}\text{—ADP} + H_2O_2 \rightarrow Fe^{3+}\text{—ADP} + OH^- + {}^{\bullet}OH \qquad (B)$$

A sample preparation was carried out as described in [16]. Following aqueous solutions, prepared immediately before application were used: $c/(\text{mmol dm}^{-3})$: 20 ADP, 1 $FeSO_4$, 85 DMPO, 90 H_2O_2. The solutions were added as follows: 10 mm^3 ADP, 10 mm^3 $FeSO_4$, 30 mm^3 buffer 0.1 M-NaCl, 0.025 M-$NaHCO_3$; then HCl until pH = 7.4 ± 0.3 was reached or stobadine in buffer; then 10 mm^3 DMPO, vortex 10 s, 10 mm^3 H_2O_2, vortex 10 s. The final concentration of DMPO in the samples was 12.1 mmol dm^{-3}. Sample tubes were kept at 37 °C and 20 s elapsed between the addition of each component. After the addition of the last component and 30 s of incubation at 37 °C samples were rapidly frozen in liquid nitrogen. The ESR spectra in X band were recorded on Bruker ESR spectrometer 200D in the glass capillaries at 25 °C 1 min after the melting of the sample; modulation amplitude 0.063 mT, sweep width 6 mT, time constant 0.1 s, sweep time 100 s, microwave power 9.9 mW.

Generation of radical from stobadine

Stobadine, when oxidized with *e.g.* *p*-NO_2-C_6H_4COOOH or *tert*-BuOOH, forms free radicals observable by ESR spectroscopy. Following procedure gave the best resolved ESR spectrum depicted in Fig. 4. Stobadine dichloride was suspended in the $NaHCO_3$—water—benzene mixture. The benzene layer was dried over Na_2SO_4. To approximately 0.5 cm^3 of 10^{-3} M stobadine solution in benzene 50 mg of PbO_2 and 30 mm^3 *tert*-BuOOH were added. The ESR spectra were measured at room temperature on a Bruker spectrometer equipped with Aspect 2000 computer.

Results and discussion

The lipid peroxidation of lecithin liposomes evaluated as the absorbance change at $\lambda = 233$ nm ($A_{233\,\text{nm}}/A_{215\,\text{nm}}$) caused by the formation of conjugated dienes is shown in Fig. 1. In the control samples, *i.e.* in the absence of stobadine a continuous progress of lipid peroxidation was found, whereas in the presence of stobadine no peroxidation changes were observed. Action of stobadine as an

inhibitor of lipid peroxidation is consistent with the decreased lipid peroxidation in rat brains after postischemic reoxygenation reported by *Štolc* and *Horáková* [9]. While in the experiments on animal tissues a variety of different mechanisms (*i.e.* stimulation or inhibition of some enzymes responsible for the redox state of organism) could lead to the inhibition effect, our results confirm that stobadine itself inhibits lipid peroxidation in a nonenzymic way.

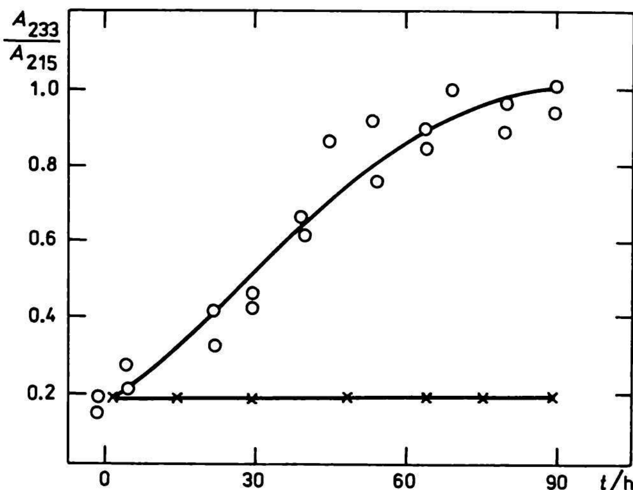


Fig. 1. Dependence of the relative lipid peroxidation (A_{233}/A_{215}) on the incubation time at 50 °C. Sample without (○) and with (×) added stobadine.

Further we tested the ability of stobadine to scavenge hydroxyl radicals which are thought to be responsible for the initiation of lipid peroxidation [17]. A system known as a powerful source of ˙OH radicals — a Fenton reaction — was chosen. Because ˙OH radicals are extremely reactive, their lifetime is very short (7×10^{-10} s in the system of 1 M linoleate [18]), and so they cannot be detected by the ESR directly. Therefore a spin trap DMPO was used. The spectrum of the DMPO—OH adduct is shown in Fig. 2*a*. The measured spectra are typical of the DMPO—OH adducts with the hyperfine splitting constants $a_N = a_H = 1.49$ mT [19]. The concentration of the trapped ˙OH in the samples without stobadine was in the range 1—10 μmol dm^{-3} as calibrated with the stable radical TEMPO (2,2,6,6-tetramethylpiperidine *N*-oxyl). The signal intensity of the DMPO—OH adduct reflects the relative ˙OH radical concentration in the system. In the presence of 0.0121 M stobadine solution the height of the DMPO—OH signal was markedly decreased (Fig. 2*b*). The change of the relative DMPO—OH signal intensity with the stobadine concentration is shown

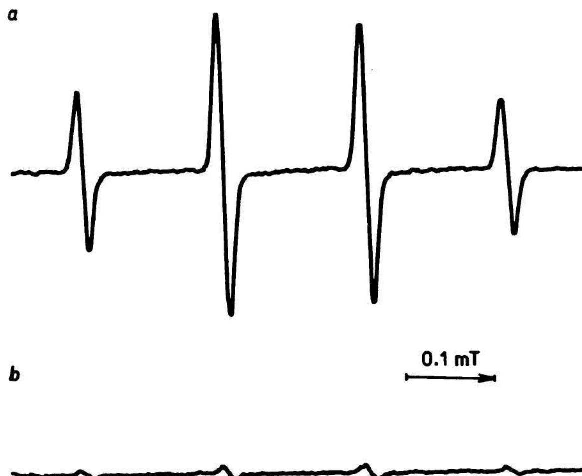


Fig. 2. ESR spectra of the DMPO—OH adduct obtained by a Fenton reaction without (*a*) or with (*b*) added stobadine at equimolar ratio with DMPO and concentrations of 12.1 mmol dm^{-3}.

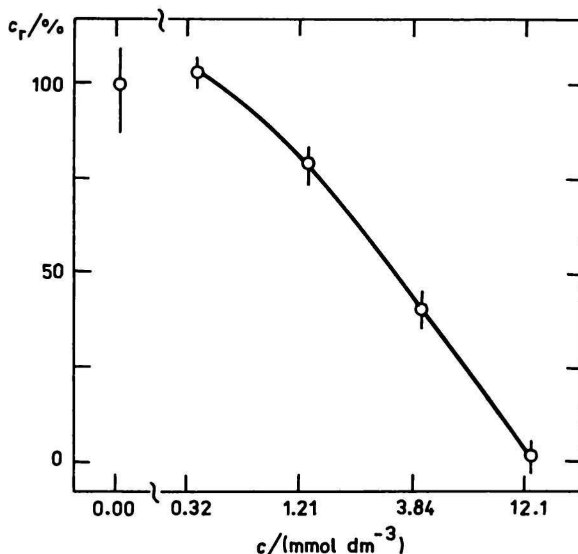


Fig. 3. The change of the relative DMPO—OH signal intensity c_r with the concentration of stobadine (c_r is the ratio of the peak heights of ESR spectra of the DMPO—OH spin-adduct with and without stobadine).

in Fig. 3. Most probably the decreased amount of trapped $^{\bullet}OH$ radicals is a consequence of the competition for $^{\bullet}OH$ radicals between stobadine and DMPO, the reaction of the former with $^{\bullet}OH$ being more favourable

$$\text{DMPO} + {}^{\bullet}\text{OH} \xrightarrow{k_1} \text{DMPO—OH}^{\bullet} \qquad (C)$$

$$\text{STO—H} + {}^{\bullet}\text{OH} \xrightarrow{k_2} \text{STO}^{\bullet} + \text{H}_2\text{O} \qquad (D)$$

where STO—H and $STO^{\bullet}$ represent stobadine and stobadine radical, respectively.

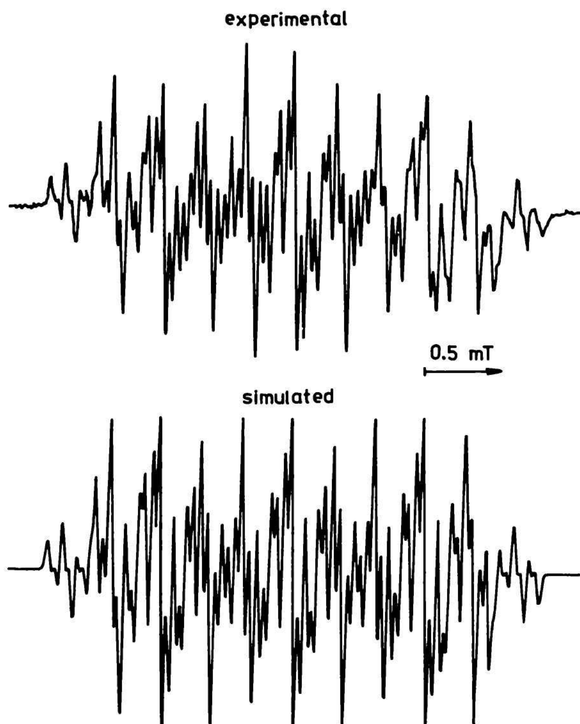
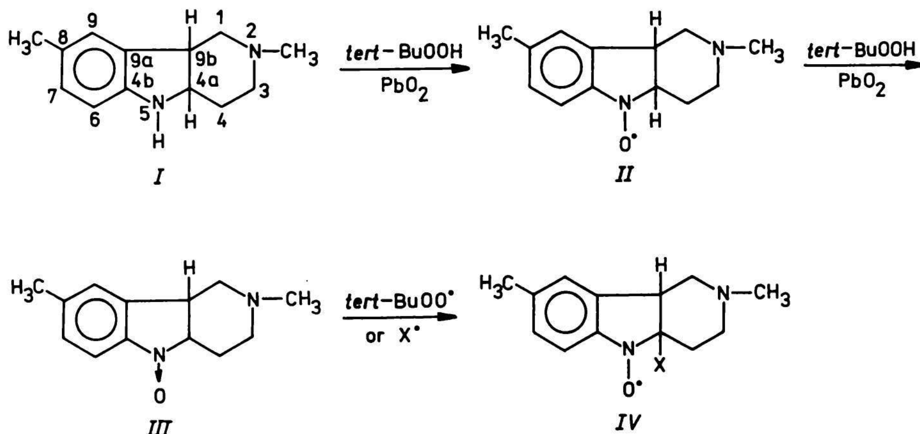


Fig. 4. Experimental and simulated ESR spectrum of radical observed in the oxidation of stobadine with PbO_2—*tert*-BuOOH mixture in benzene.

The experimental ESR spectrum observed in oxidation of stobadine, along with its simulation, is given in Fig. 4. It was simulated by means of the following splitting constants a_{H}/mT: 0.04, 0.093, 0.107, 0.30, 0.337, 0.337, 0.337, and $a_{\text{N}} = 0.925$ mT. Their assignment is based on the assumption that the amino group of stobadine *I* in position 5 (Scheme 1) is in the first step oxidized to nitroxyl radical of *II*. This corresponds to a phenylnitroxyl type of radicals, substituted in *ortho* position with an alkyl chain. Consequently, in agreement with the common data in the literature [20], $a_{\text{N}} = 0.925$ mT was assigned to

$a_{N\text{-}5}(NO) = 0.925$ mT. The next highest splitting constant of three equivalent protons belongs to the methyl protons in *para* position, $a_{H\text{-}8} = 0.337$ mT, and the next highest one to the proton in *ortho* position, $a_{H\text{-}6} = 0.30$ mT. Two splitting constants with around 0.1 mT are assigned to *metha* positions: $a_{H\text{-}7} = 0.1$, $a_{H\text{-}9} = 0.093$. The remaining splitting constant $a_H = 0.04$ mT can only be assigned to the protons in 4a or 9b position. Generally, in similar nitroxyl radical structures the protons in β position to nitroxyl group have much higher splitting constants than 0.04 mT and are in the range from 0.15 mT up to 2 mT.



Scheme 1

Therefore, we assume that the intermediately formed radical *II* is converted by its oxidation to nitrone (*III*). After trapping a radical (probably *tert*-BuOO$^•$, BuO$^•$ or an unknown radical X$^•$) it finally gives adduct *IV*. A similar nitroxyl radical→nitrone→nitroxyl radical conversion was found in [21] according to Scheme 1. Therefore we assigned the splitting constant of 0.04 mT to the proton 9b in structure *IV* ($a_{H\text{-}9b} = 0.04$ mT), as similar values were also found [20] for β protons in *ortho*-substituted phenylnitroxyl radicals.

References

1. *Free Radicals in Molecular Biology Aging and Disease.* (Armstrong, D., Sohal, R. S., Cutler, R. G., and Slater, F., Editors.) Raven Press, New York, 1984.
2. Rőth, E., Tőrők, B., Zsoldos, T., and Matkovics, B., *Basic Res. Cardiol. 80,* 530 (1985).
3. Štolc, S., Bauer, V., Beneš, L., and Tichý, M., *Czechoslov.* 229 06b; *Belg.* 894 1048; *Swiss* 651 754; *Span.* 553 017, and other Patents pending (1983).

4. Babuľová, A., Buran, Ľ., and Beneš, L., *Farmakol. Obzor 54*, 15 (1985).
5. Styk, J., Gabauer, I., Okoličány, J., Holec, V., Bauer, V., and Beneš, L., *Bratislavské Lekárske Listy 85*, 274 (1986).
6. Rao, P. S., Cohen, M. V., and Mueller, H. S., *J. Mol. Cell. Cardiol. 15*, 713 (1983).
7. Blasig, I. E., Ebert, B., and Löve, H., *Studia Biophys. 116*, 35 (1986).
8. Kato, K., Terao, S., Shimamoto, N., and Hirata, M., *J. Med. Chem. 31*, 793 (1988).
9. Štolc, S. and Horáková, Ľ., *Proc. 5th Eur. Workshop Clin. Neuropharmacol.*, p. 59. Bratislava, July 6—8, 1987.
10. Buege, J. A. and Aust, S. D., in *Methods in Enzymology*. (Fleisher, S. and Packer, L., Editors.) Vol. *52*, p. 17. Academic Press, New York, 1978.
11. Szőcsová, H., Beneš, L., Hloušková, Z., and Szőcs, F., *Cesk. Fysiol. 35*, 364 (1986).
12. Szőcsová, H. and Beneš, L., *Cesk. Fysiol. 37*, 274 (1988).
13. Green, M. J. and Hill, H. A. O., in *Methods in Enzymology*. (Packer, L. *et al.*, Editors.) Vol. *105*, p. 3. Academic Press, New York, 1984.
14. Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. J., *J. Am. Oil Chem. Soc. 42*, 53 (1965).
15. Klein, R. A., *Biochim. Biophys. Acta 210*, 486 (1970).
16. Floyd, R. A. and Lewis, C. A., *Biochemistry 22*, 2645 (1983).
17. Pryor, W. A. and Tang, R. H., *Biochim. Biophys. Res. Commun. 81*, 498 (1978).
18. Pryor, W. A., in *Free Radicals in Molecular Biology Aging and Disease*. (Armstrong, D., Sohal, R. S., Cutler, R. G., and Slater, F., Editors.) P. 13. Raven Press, New York, 1984.
19. Buettner, G. R., *Free Radicals Biol. Med. 3*, 259 (1987).
20. Landolt—Börnstein, *New Series*, Vol. *9*. *Magnetic Properties of Free Radicals*. Part c1. Springer-Verlag, New York, 1979.
21. Cholvad, V., Staško, A., Bučačenko, A. L., and Malík, Ľ., *Collect. Czechoslov. Chem. Commun. 46*, 823 (1981).

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