DOI 10.1007/s11094-017-1517-3
Pharmaceutical Chemistry Journal, Vol. 50, No. 11, February, 2017 (Russian Original Vol. 50, No. 11, November, 2016)

CONJUGATION OF SUCCINATE TO CHITOSAN INCREASES THE COCHLEAR CYTOPROTECTIVE EFFECT

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Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 50, No. 11, pp. 9 – 12, November, 2016.

Original article submitted February 9, 2016.

N-Succinylchitosan (SC) with degree of substitution 0.86 was prepared by reacting chitosan with succinic anhydride. The otoprotective potential of a single i.v. injection of a solution (0.3%) of the sodium salt of SC in 60 rats (males, Wistar, 220 – 250 g) was studied. A solution of meglumine sodium succinate (MSS) at an equivalent concentration was used as a reference. The drugs were administered 2 h or 5 min prior to induction of acute injury of the auditory analyzer by acoustic stimulation (AS). The condition of hearing was estimated by studying optoacoustic emission (OAE) at the frequency of distortion product 1 h, 24 h, and 7 d after the AS. SC showed a more pronounced protective effect with early preventive administration ($p < 0.02$; ANOVA with Bonferroni correction). Conversely, MSS exhibited its protective properties only with immediate administration. A comparison of the two drug administration modes showed that the OAE amplitude was depressed more after MSS administration than after SC injection ($p < 0.05$; Tukey's test). It was concluded that conjugation to chitosan prolonged the elimination half-life of succinate and improved its access to cochlear back-barrier tissues.

Keywords: otoprotection, targeted drug delivery, elimination half-life increase, permeability of blood—cochlear barrier, *N*-succinylchitosan, meglumine sodium succinate.

Traditional drug-delivery methods for otoneurological pathologies struggle with the inability to cross the blood–cochlear barrier [1]. Similar complicated problems with drug distribution are studied with respect to targeted delivery [2]. Strategic development of enhanced permeability and control of pharmacokinetic parameters and pharmacodynamic activity result in the expected organ-oriented drug properties that are related to reaching the target [3]. This concept provides fundamentally new access pathways to target organs and has been used to produce positive ototropic properties *in vivo* in known metabolically active but cochlear-inactive drugs [4].

The biocompatible polymer chitosan is one of the most popular experimental materials for creating drug delivery systems [5, 6]. Attempts were made to employ it in drug constructs with the ability to access organs with tissues functioning as blood—tissue barriers, in particular, the blood—brain barrier [7, 8]. In turn, we intended to develop a drug carrier based on chitosan conjugates that would provide nonspecific (i.e., not through receptors) penetration of low-molecular-mass drugs to the spiral organ (SO). Herein, the ability of *N*-succinylchitosan in aqueous solution to act as a system for delivering succinate to intracochlear structures was studied.

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EXPERIMENTAL CHEMICAL PART

The conjugate of chitosan and succinate, i.e., the sodium salt of *N*-succinylchitosan (**I**), was selected for the studies. A sample was synthesized from crab chitosan (**II**) (ZAO Bioprogress, molecular mass 83,200, degree of deacetylation 0.95) by the literature method [9]. Thus, **II** (0.5 g, 2.8×10^{-3} mol N) was dissolved in HOAc (20 mL, 1%), treated with a solution of succinic anhydride (0.8 g, 8×10^{-3} mol) in Me_2CO (7 mL), and stirred at 20°C for 7 h. The product was precipitated, rinsed with Me_2CO, dissolved

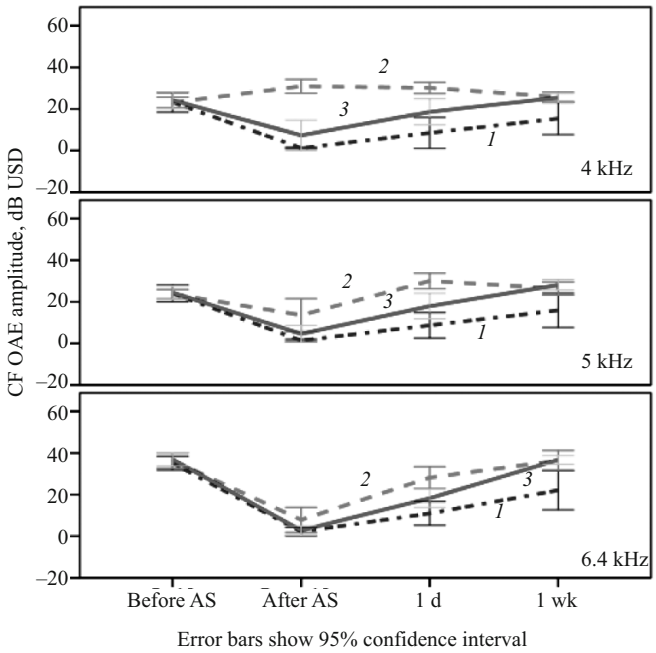

Fig. 1. Dynamics of CF OAE change at studied frequencies after injection of compounds 5 min before acoustic stimulation. Control (*1*), **III** (*2*), and **I** (*3*).

in $NaHCO_3$ solution (50 mL, 3%), dialyzed against distilled H_2O for 3 d, and lyophilized in a CoolSafe 110-4 freeze dryer (LaboGene ApS, Denmark).

Elemental analyses were performed on a Vario Micro Cube CHNS analyzer (Elementar, Germany). The degree of substitution was calculated using:

$$C3 = \frac{1}{n}\left[\left(\frac{\omega_C}{\omega_N}\right)_{CX} - \left(\frac{\omega_C}{\omega_N}\right)_{\text{chitosan}}\right]\frac{M_N}{M_C} =$$

$$= \frac{1}{4}\left[\frac{39.13}{4.78} - \frac{40.97}{7.80}\right]\frac{14.01}{12.01} = 0.86,$$

where ω is the mass fraction; *M*, molar mass of the corresponding element; *n*, number of C atoms in the substituent (for **I**, *n* = 4). The succinic-acid content in the sample was 2.93 mmol/g according to the elemental analyses. The reference drug was meglumine sodium succinate (MSS, **III**), the source of which was a pharmacopoeial preparation based on it, Reamberin (NTFF Polisan, Russia).

Compound **I** was injected as a solution (0.3%) in normal saline (0.9% NaCl solution). Preliminary experiments on the acute toxicity of **I** showed that this was the maximum possible concentration for parenteral administration. The official Reamberin preparation (1.5% solution of **III**) was diluted five times with normal saline in order to prepare a solution of **III** (44.7 mM) with the equivalent number of succinate groups. The solutions were sterilized by UV light for 1 h.

EXPERIMENTAL BIOLOGICAL PART

The work used 60 male Wistar rats (220 – 250 g, Rappolovo nursery, Russia) that were somatically healthy with normal otoscopic patterns and tympanometric parameters.

Exposure to acoustic stimulation (AS) by a 5-kHz tonal signal of intensity 110 – 112 dB with continuous action for 2 h was used to model acute injury to the auditory analyzer [10]. Sound testing was carried out in a free sound field in a locally constructed acoustic chamber (0.75 m^3).

Six animal groups (10 rats in each) were randomly selected. Groups 1 and 2 (controls) received normal saline + AS; groups 3 and 4, a solution (0.3%) of **III** (12 – 14 mg/kg, 9 μmol of succinate) + AS; groups 5 and 6, a solution (0.3%) of **I** (12 – 14 mg/kg, 9 μmol of succinate) + AS.

The ototropic effect of the tested compounds was studied using two versions of preventive administration relative to the created AS, i.e., early and immediate (2 h and 5 min, respectively, before the AS). The solutions (1 mL) were infused slowly into a tail vein.

The functional condition of the auditory analyzer was assessed from the optoacoustic emission (OAE) amplitude on the carrier frequency (CF) at three frequencies in the range 4 – 6.4 kHz. A Neuro-Audio instrument with Neuro-Audio. NET software (Neurosoft Inc., Russia) was used. OAE was recorded for 24 h before the experiment and 1h, 24 h, and 7 d after AS.

All manipulations of animals (OAE study, i.v. injections) were performed under general anesthesia with i.p. injection of Zoletil 100 preparation (6 mg/kg).

Differences were found by comparing averages of test groups with the control and their starting values. The significance was evaluated using analysis of variance (ANOVA) and *a posteriori* pair comparisons using the Bonferroni correction and Tukey's criterion. Results were processed statistically using the SAS 9.3 software. Differences were considered statistically significant for $p < 0.05$.

RESULTS AND DISCUSSION

Lethal outcomes resulting from the acute toxicity of the tested preparations at the used doses were not observed in the test groups. AS with the selected parameters suppressed the OAE amplitude at all studied frequencies (4.0, 5.0, and 6.4 kHz) ($p < 0.05$ compared with the starting values). Its return independently to the starting values by the seventh day of the test indicated that this acoustic load depressed reversibly the auditory receptor functioning. However, this confirmation is correct only for that part of the cochlea that corresponds tonotopically to the reception zone of the studied range. Thus, the protective potential of the succinate compounds was assessed 1 h and 24 h after the AS.

Immediate injection of **III** (after 5 min) in studies 1 h after AS preserved the auditory receptor only in tonotopic zone

4 kHz ($p < 0.001$ compared with the control). The functional activity of the SO was restored at the two other frequencies ($p < 0.005$ compared with the control) after 24 h. The OAE amplitude after 24 h already agreed with the starting values at 4 and 5 kHz (Fig. 1).

Conversely, injection of **I** during the study after 1 h did not produce a protective effect at any of the studied frequencies. Protective action was observed at 4 and 5 kHz after 24 h ($p = 0.013$ and 0.027, respectively, compared with the control). The OAE parameter at 4 kHz was equal to the starting value and did not reach it at 5 and 6.4 kHz ($p = 0.024$ and 0.002).

Earlier (2 h before) injection of **III** provided a protective effect by 1 h after AS only at 4 kHz ($p = 0.012$ compared with the control). The OAE amplitude did not differ from the starting value. A protective effect was not observed at 5 and 6.4 kHz. The level of OAE amplitude depression at these frequencies corresponded to the control. The OAE amplitude after 24 h at all frequencies was the same as the control values and reflected natural restoration processes. The parameters at 4 and 5 kHz corresponded to the starting values whereas the otodepressive effect of the AS persisted at 6.4 kHz ($p < 0.05$) (Fig. 2).

Studies after 1 h demonstrated a protective effect at 4 and 5 kHz ($p \leq 0.001$ compared with the control) for the early injection regime of **I**. Injection of **I** after 24 h restored the OAE amplitude to the starting values at all frequencies. Differences from the parameters of the AS control group ($p_{4;5;6.4} < 0.001$) were observed.

The results from the two versions of injecting **I** and **III** were evaluated by comparing pairs. Compound **I** demonstrated a significantly more pronounced protective effect with early injection ($p < 0.002$, Bonferroni correction). Conversely, **III** exhibited a protective effect only with immediate injection ($p < 0.004$).

The degree of cochlear access of the tested compounds could be compared because they were injected at doses with equivalent amounts of succinate. For this, the injection version that demonstrated a greater number of frequencies with retained OAE amplitude (for **III**, with immediate injection; for **I**, with early injection after AS) was selected for each compound. It was found that the OAE amplitude at 5 and 6.4 kHz was retained more with injection of **I** than with that of **III** ($p < 0.05$; Tukey's criterion).

The cytoprotective antihypoxic effect of the pharmacological succinate preparations was explained by its physiological potential as a Krebs cycle substrate for energy metabolism [11]. This is significant for the cochlea because the molecular physiology of acoustic reception is regulated by controlled cellular oxidation, among other processes [12]. These become a source of cellular oxidative stress with acoustic loading of non-physiological intensity [13]. This molecular mechanism operates in the early pathogenesis stages of damage to excited tissue structures of the peripheral analyzer sections [14].

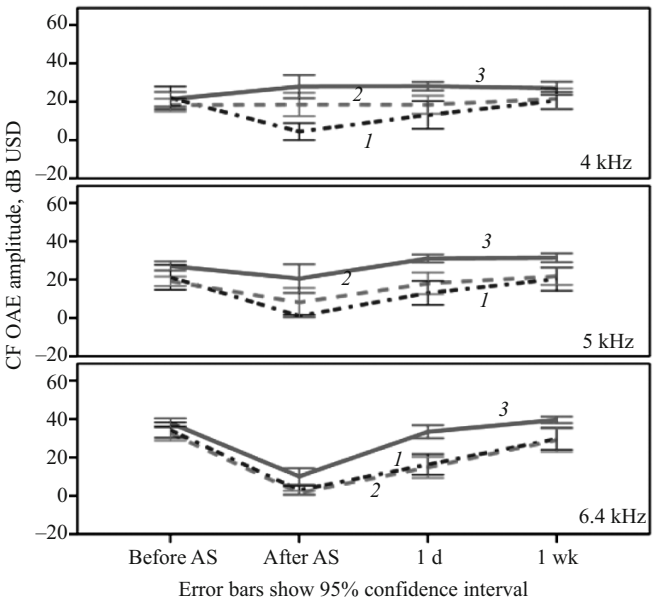

Fig. 2. Dynamics of CF OAE change at studied frequencies after injection of compounds 2 h before acoustic stimulation. Control (*1*), **III** (*2*), and **I** (*3*)

Thus, the expected antihypoxic effect of **III** was observed in our experiment. Its activity was evaluated indirectly from its protective effect in an acute injury model. The succinate compound, consistent with its short elimination half-life [15, 16], demonstrated protective potential only with injection immediately before AS, i.e., as close as possible to the traumatizing SO exposure. This feature of the effects of **III** confirmed indirectly that the molecular factors of the developed oxidative stress depressed the motility of hair cells and acted only at the same time as the AS itself and lost its pathogenic value after it [17].

Injection of **I** was associated with a positive ototropic effect in the same direction as **III**. In addition, injection of succinate as a conjugate with a high-molecular-mass compound had its own pharmacokinetic features. Thus, **I** with early injection showed a more pronounced protective effect in studies 1 and 24 h after injury. Immediate injection of **I** before AS had no protective effect. This indicated indirectly that succinate conjugated to a polymer resided significantly longer in the circulation than **III** and evidently had a longer elimination half-life. It was assumed that this also reduced its rate of intracochlear distribution with increasing oxidative stress when the antihypoxic potential of exogenous succinate could manifest a greater cytoprotective effect.

However, our observation of total protection of the SO with early preventive injection of **I** indicated that the two-hour period of systemic circulation of **I** from the time of injection to the creation of AS occurred without changes in the therapeutic succinate concentration. Recall that **III** injected under these conditions did not demonstrate its own cytoprotective effect.

Therefore, the narrow therapeutic window of low-molecular-mass succinate compounds was expanded for the conjugate of succinate with a polymer [15, 18]. Apparently, this was explained by the circulation of **I** during a certain (currently unknown) time interval in an inactive form and in turn hindered the manifestation by **I** of an instantaneous substrate—substituent antihypoxic (of metabolic character) effect.

Indications that conjugation with chitosan affected the degree of intracochlear access of succinate were observed in addition to the data confirming that the elimination half-life of succinate was increased in **I** [19]. Here, the nonspecific nature of this effect is noteworthy because drugs conjugated to chitosan are known to have increased penetration through another blood—tissue barrier, i.e., the blood—brain barrier [7]. The observed phenomenon surely deserves further research.

Thus, the model of acute acoustic injury to the auditory analyzer showed a positive ototropic effect for **I**. The observed restorative effect of **I** was similar to that of **III**, a compound that is the active pharmaceutical ingredient of antihypoxic succinate-containing preparations. Moreover, conjugation to a polymer enhanced new pharmacokinetic properties of succinate. Its elimination half-life increased. It penetrated more into tissues functioning as tissue barriers. We demonstrated this for the blood—cochlear barrier using a model of acute acoustic injury of the auditory analyzer during studies of the SO functional activity.

ACKNOWLEDGMENTS

The research was supported financially by the Russian Scientific Foundation (Project No. 14-15-00473, D. L. Sonin, A. A. Panevin, S. G. Zhuravskii) and the Russian Foundation for Basic Research (Project No. 15-04-0664, A. A. Golyshev, Yu. A. Skorik).

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