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Effect of venlafaxine on scavenging free radicals *in vitro*

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Abstract

OBJECTIVE: Venlafaxine (VLF) was examined as a potential donor of H atom(s) to scavenge hydroxyl and peroxy-type radicals generated under aerobic conditions by catalytic oxidation of ascorbate with Cu^{2+} ions. Kinetics of the electron-donor property of VLF was investigated by standard ABTS and DPPH assays. Electron paramagnetic resonance measurements were applied to prove/disprove the VLF ability to scavenge superoxide anion radical.

RESULTS: Results indicated that the drug venlafaxine was slightly capable of donating $^{\bullet}H$, this way VLF scavenged the in situ generated hydroxyl radicals. Under the experimental conditions VLF was not able to inhibit/retard the propagation of the peroxy-type radicals. Regarding to the drug electron donating property, VLF did not show any $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ radical quenching property. Venlafaxine was not effective in scavenging $O_2^{\bullet-}$.

CONCLUSION: Results of ABTS and DPPH assay showed a negligible redox activity of venlafaxine to both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. Venlafaxine was not capable of scavenging the superoxide anion radical generated in KO_2/DMSO system, which indicates that VLF is not an efficient electron/proton donor molecule.

INTRODUCTION

Venlafaxine (VLF; the drug base $C_{17}H_{27}NO_2$; Figure 1), proposed for the treatment of depression in 1993, is a serotonin-norepinephrine reuptake inhibitor (Zhang *et al.* 2014; Veľtishchev 2015). It is a relatively safe drug with minimal undesirable effects in adults and it is also used in the treatment of maternal depression during pregnancy and lactation (Dubovicky *et al.* 2012; Csaszar *et al.* 2014). According to Durand *et al.* (2012), VLF, which blocks sodium channels resulting in some neuroprotective effect, modulates also the oxidative stress in the nervous system. As stated, medical treatment with oral VLF can be beneficial to depression due to reducing free radical production in the brain and medulla. This way the treatment of depression by VLF may also play a role in pre-

ORIGINAL ARTICLE

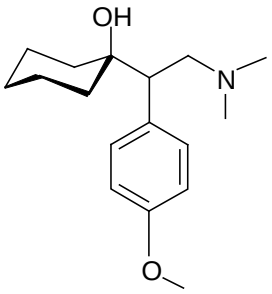

Fig. 1. Chemical structure of venlafaxine.

venting oxidative stress in the nervous system (Eren *et al.* 2007).

The imbalance between antioxidant- and oxidant-generating systems results in oxidative stress, which may contribute to pathological changes associated with neurodegeneration (Gasparova *et al.* 2014; Ujhazy *et al.* 2013). Although most of the oxygen used in brain tissue is converted to CO_2 and water, small amounts of oxygen are converted by mitochondria to reactive oxygen species (ROS). The easy oxidability of polyunsaturated fatty acids (PUFAs), the targets of ROS, make thus the brain cells sensitive to oxidative damage (Eren *et al.* 2007). However, as proved by studies in mice, VLF protected against stress-induced oxidative lipid and/or DNA damages in hippocampus, which also resulted in the decrease in serum 8-OHdG – 8-hydroxy-2'-deoxyguanosine. Hence, these results indicate that, in addition to its antidepressant properties, VLF has antioxidant and protective effects not only against the lipid peroxidation but also stress induced oxidative DNA-damage (Abdel-Wahab *et al.* 2011).

The oxidative damage in the tissues of the living organisms occurs permanently to lipids (e.g. in cellular membranes), proteins, DNA, and polysaccharides (e.g. in extracellular matrices). Nuclear and mitochondrial DNA oxidation results in production of 8-OHdG and/or 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). Reactive oxygen species are the compounds responsible for aforementioned uncontrolled damage reactions. Of those ROS, the primary reactant can be superoxide anion radical ($O_2^{\bullet -}$), which is classified as a prominent oxidant. The scheme presented below shows the primary and several other chemical reactions, which yield ROS:

$$\text{Molecular oxygen} \xrightarrow{\text{one electron reduction}} \text{superoxide anion radical } (O_2^{\bullet -})$$

$$O_2^{\bullet -} + O_2^{\bullet -} + 4H^+ \xrightarrow{\text{dismutation reaction}} 2\,H_2O_2$$

$$H_2O_2 + O_2^{\bullet -} \xrightarrow{\text{Haber-Weiss reaction}} HO^- + HO^{\bullet} + O_2$$

It should be pointed, however, that the above reactions do not occur without enzyme and/or transitional metal ion catalysis (e.g. $Fe^{2+/3+}$; $Cu^{1+/2+}$). Contrary to above listed (one-step) reaction, a lipid peroxidation is classifiable as a free-radical reaction consisting of 4 elementary reaction steps, namely: initiation, propagation, transfer, and termination.

Experimentally, *in situ* generation of $O_2^{\bullet -}$ has been simply accomplished by decomposition of KO_2 – potassium superoxide. As a well characterized generator of hydroxyl radicals, the Cu^{2+} catalyzed ascorbate autoxidation recently gains popularity (Weissberger *et al.* 1943; Harris *et al.* 1972; Biaglow *et al.* 1997; Hrabárová *et al.* 2009, 2012; Valachová *et al.* 2015), while for the free-radical reaction – leading during the propagation step to peroxy-type radicals – the oxidative degradation of the polysaccharide [high-molar-mass hyaluronan (HA)] has been proved as a proper choice (Hrabárová *et al.* 2009, 2012; Valachová *et al.* 2015).

The aim of this study was to assess the ability of venlafaxine to directly scavenge various ROS, namely superoxide anion radical, and $^{\bullet}OH$ or peroxy-type free radicals, along with proving/disproving the VLF drug electron/proton donating property.

MATERIALS AND METHODS

Chemicals

A hyaluronan (HA) sample (sodium salt) of molar mass 709.3 kDa (Lifecore Biomedical, Chaska, MN, USA), analytical purity grade NaCl and $CuCl_2{\cdot}2H_2O$ (Slavus, Bratislava, Slovakia), ascorbic acid and potassium persulfate ($K_2S_2O_8$ p.a. purity, max. 0.001% nitrogen, Merck, Darmstadt, Germany), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) (ABTS; purum, >99%) (Fluka, Seelze, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH; 95%, Aldrich, Germany), monohydrochloride salt of venlafaxine (Chemoz, Prag, Czech Republic, purity, 98.5%) were purchased. 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO; distilled prior to the application and stored at –18 °C) was the product of Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Dimethylsulfoxide (DMSO; SeccoSolv®, max. 0.025 % H_2O, 150 mL septum bottle, Merck) and potassium superoxide (KO_2; without further purification; Sigma-Aldrich) were purchased. Redistilled deionized high quality grade water, with conductivity of $<0.055\ \mu S{\cdot}cm^{-1}$, was produced using the TKA water purification system from Water Purification Systems, Niederelbert, Germany.

Venlafaxine action to superoxide anion radical generation

For the experiments with electron paramagnetic resonance (EPR) spectroscopy, the DMPO spin trapping agent was applied. The DMPO stock solution was prepared by dissolving 25 mL of DMPO in 1 mL of DMSO. Superoxide anion radical in DMSO was prepared by dissolving KO_2 (0.7 mg) in anhydrous DMSO (5 mL) by sonication for 10 min.

The monitoring of superoxide anion radicals was examined by spin trapping technique in an EPR X-band EMX spectrometer (Bruker, Rheinstetten, Germany)

at room temperature. A representative EPR spin trapping experiment with VLF sample was chosen using reaction conditions as follows: 200 mL of KO_2-DMSO solution was firstly rigorously mixed with 100 mL of 1.25 mM VLF in DMSO. Both solutions were rigorously purged with argon before and during the mixing. After that, 100 mL of DMPO-DMSO stock solution was added and the final solution was transferred under argon into a thin flat EPR quartz cell and EPR spectra were measured. The same experiment was performed with the reference sample, where only DMSO was used. The operational parameters of the EPR equipment were adjusted as follows: central field 3353 G, sweep width 80 G, time constant 10.24 ms, conversion time 10.24 ms, receiver gain 5.10^5, microwave power 10 mW, and modulation amplitude 2 G, number of scans 20.

Venlafaxine action to generated hydroxyl and peroxy-type radicals

Preparation of stock and working solutions

The HA sample (24.0 mg) was dissolved in aqueous NaCl for 24 h in the dark in two steps: first, 4.0 mL and after 6 h, 3.90 mL or 3.85 mL of 0.15 M NaCl solution was added when working in the absence or presence of venlafaxine, respectively. Solutions of ascorbate (16 mM) and cupric chloride (320 μM) were prepared also in 0.15 M aqueous NaCl.

Uninhibited/inhibited hyaluronan degradation

First, HA degradation was induced by the oxidative system comprising $CuCl_2$ (2.0 μM) and ascorbic acid (100 μM). The procedure was as follows: a volume of 50 μL of 320 μM $CuCl_2$ solution was added to the HA solution (7.90 mL), and the mixture was left to stand for 7 min 30 s at room temperature after stirring for 30 s. Then, 50 μL of ascorbic acid solution (16 mM) was added to the HA solution, stirred for 30 s and followed by an immediate addition into the viscometer Teflon® cup reservoir.

The procedures to investigate the H atom donating effectiveness of VLF were as follows:

i. A volume of 50 μL of 320 μM $CuCl_2$ solution was added to the HA solution (7.85, 7.8 or 7.7 mL), and the mixture, after a 30 s stirring, was left to stand for 7 min 30 s at room temperature. Then, 50, 100 or 200 μL of venlafaxine (16 mM) was added to the HA solution, followed by stirring again for 30 s. Finally, 50 μL of ascorbic acid solution (16 mM) was added to the solution, stirred for 30 s and followed by an immediate addition into the viscometer Teflon® cup reservoir.
ii. In the second experimental setting, a procedure similar to that described in i) was applied, however, after standing of the HA solution for 7 min 30 s at room temperature, 50 μL of ascorbic acid solution (16 mM) was added to the mixture and a 30 s stirring followed. After 1 h, finally 50, 100 or 200 μL of venlafaxine (16 mM) was added to the solution, followed by 30 s stirring. The solution mixture was then immediately transferred into the viscometer Teflon® cup reservoir.

Rotational viscometry

Dynamic viscosity of the reaction mixture (8 mL) containing HA (24.0 mg), ascorbate (100 μM) along with Cu^{2+} ions (2 μM) in the absence and presence of VLF (100, 200 and 400 μM) was monitored by a Brookfield LVDV-II+PRO digital rotational viscometer (Brookfield Engineering Labs., Middleboro, MA, USA) at 25.0±0.1 °C and at a shear rate of 237.6 s^{-1} for 5 h in the Teflon® cup reservoir (Valachová *et al.* 2014; Topolska *et al.* 2014).

Venlafaxine electron-donating action

ABTS assay

Radical-scavenging activity of VLF by donating electron(s) was measured according to a modified ABTS method (Re *et al.* 1999). To prepare $ABTS^{\bullet+}$ cation radical, an aqueous solution of $K_2S_2O_8$ (3.3 mg in 5 mL of H_2O) was added to ABTS (17.2 mg), and the resulting solution was freeze-stored for 14 h in the dark. Finally, the dark-green $ABTS^{\bullet+}$ radical cation solution (1 mL) was diluted with H_2O (60 mL) and used in the ABTS assays. The investigated sample composed of 2 mL of the diluted $ABTS^{\bullet+}$ solution with addition of 50 μL of aqueous venlafaxine solution (200, 100, 50 mM). UV/VIS spectra were recorded in the 2nd, 5th and 10th min using a UV-VIS S2000 spectrophotometer (Sentronic, Dresden, Germany) (Rapta *et al.* 2009; Hrabárová *et al.* 2010). Kinetics of scavenging $ABTS^{\bullet+}$ was performed in triplicate at the wavelength 730 nm.

DPPH assay

The DPPH• radical was prepared as follows: 2,2-diphenyl-1-picrylhydrazyl (1.1 mg) was dissolved in 50 mL of distilled methanol. The investigated sample composed of 2 mL of the diluted DPPH• solution with addition of 50 μL of venlafaxine methanolic solution (200, 100, 50 mM). UV/VIS spectra were reported in the 2nd, 5th and 10th min (Rapta *et al.* 2009, Hrabárová *et al.* 2010). Kinetics of scavenging DPPH• was performed in triplicate at the wavelength 517 nm.

RESULTS AND DISCUSSION

EPR monitoring of venlafaxine action to generated superoxide anion radicals

The solutions of KO_2 in aprotic solvents represent a well-defined source of superoxide anion radical (Poupko *et al.* 1973), as we also confirmed in KO_2/DMSO solutions *via* spin trapping experiments using DMPO (Figure 2). The EPR spectra obtained in (freshly prepared) deaerated KO_2/DMSO solutions in the presence of DMPO are fully compatible with the presence of the $\bullet DMPO\text{-}O_2^-$ spin adduct, which is characterized with a_N=1.289 mT, a_H=1.038 mT, a_H=0.130 mT; g=2.0059 (Li *et al.* 1988).

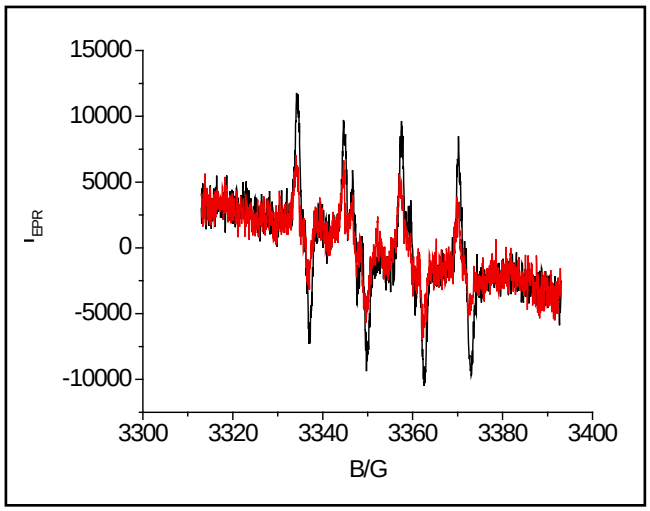

Fig. 2. EPR spectra of •DMPO-O_2^- spin adducts obtained at room temperature in freshly prepared KO_2/DMSO solutions under argon in the presence of DMPO for the reference (red) and for the sample containing VLF (black).

In the presence of VLF no decrease but a slight increase in •DMPO-O_2^- spin adducts was observed compared to the reference sample (DMSO) indicating that under the experimental conditions no scavenging of superoxide anion radicals by VLF has been evidenced.

Venlafaxine action to generated hydroxyl and peroxy-type radicals

One of the most harmful free-radical oxidants, present in organism, is the hydroxyl radical •OH. According to its electronegativity value, equaling 2.31 V, the •OH radical has an extremely high affinity to the atom (radical) of hydrogen •H. It means that, when investigating venlafaxine as a potential donor of •H, hydrogen atom(s) can be abstracted from the VLF molecule leading to a "venlafaxine radical".

The base of the drug, $C_{17}H_{27}NO_2$ can theoretically donate up to 27 atoms of •H, however the firmness of binding the hydrogen atom to the carbon/nitrogen/oxygen atoms is high. However, since the electronegativity of •OH is very high, the following reaction can occur in the aqueous solution:

$$\bullet OH + \bullet H_{(in\ VLF)} \rightarrow H_2O$$

It can be expected that the higher the concentration of the drug is, the higher antioxidative activity or more precisely the higher •H atom donating capacity of venlafaxine is.

Figure 3, left panel displays curve (black), which shows predominantly the efficacy of •OH on a "probe-indicator" – the macromolecule of hyaluronan (HA). It is known (Valachová *et al.* 2010, 2011a, b), that •OH initiates radical degradation of HA macromolecules, thereby the initial value of the dynamic viscosity of the HA solution gradually decreases from 10.38 mPa·s in time 0 min to 6.63 mPa·s in time 300 min. In case of a sufficient •H atom(s) donor capacity of VLF, the resulting value of the dynamic viscosity in time 300 min should not be 6.63 mPa·s but 10.38 mPa·s. Thus, in such a situation, one may state that VLF prevented degradation of the high-molar-mass HA. However, VLF protective action can be observed, as evidenced in Figure 3, left panel, green and blue curves, when the drug concentration was ≥200 mmol/L.

To understand the aforementioned statement, it is necessary to take into account that the used degradative system Cu^{2+} ions (2 μM) with ascorbate (100 μM) can theoretically generate maximally 100 μmol/L •OH, which could be inhibited by 100 or much less micromo-

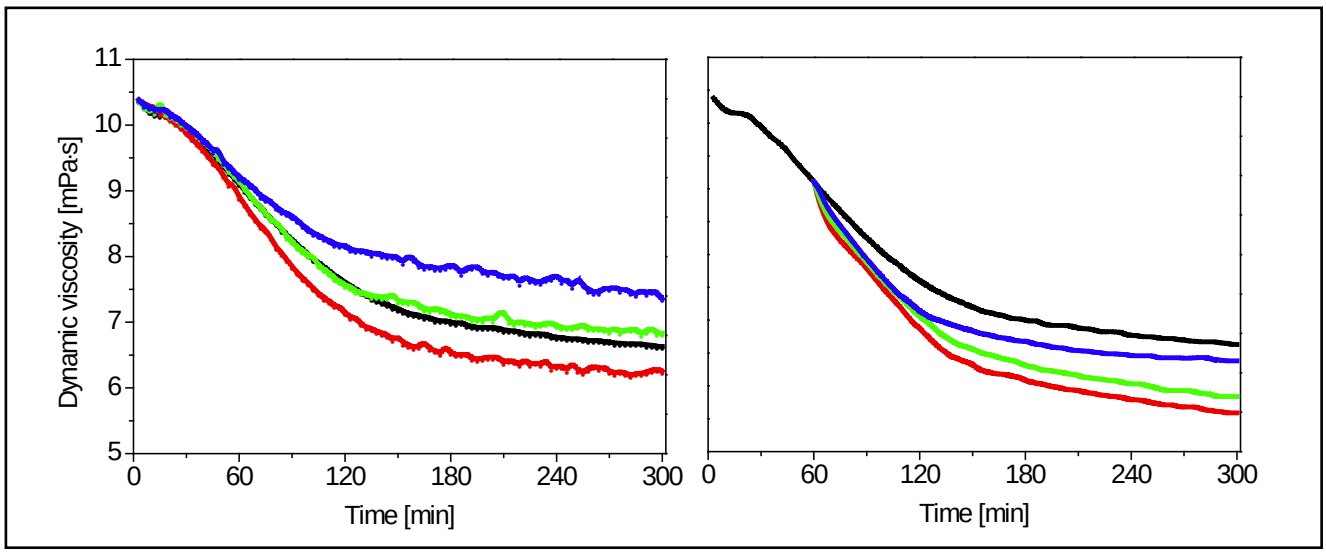

Fig. 3. Time-dependent decrease in dynamic viscosity of the HA solutions exposed to oxidative degradation by Cu(II) ions (2 μM) and ascorbate (100 μM) (black). The addition of venlafaxine before (left panel) and 1 h after initiating HA degradation (right panel). Concentrations of VLF were: 100 (red), 200 (green) and 400 mM (blue).

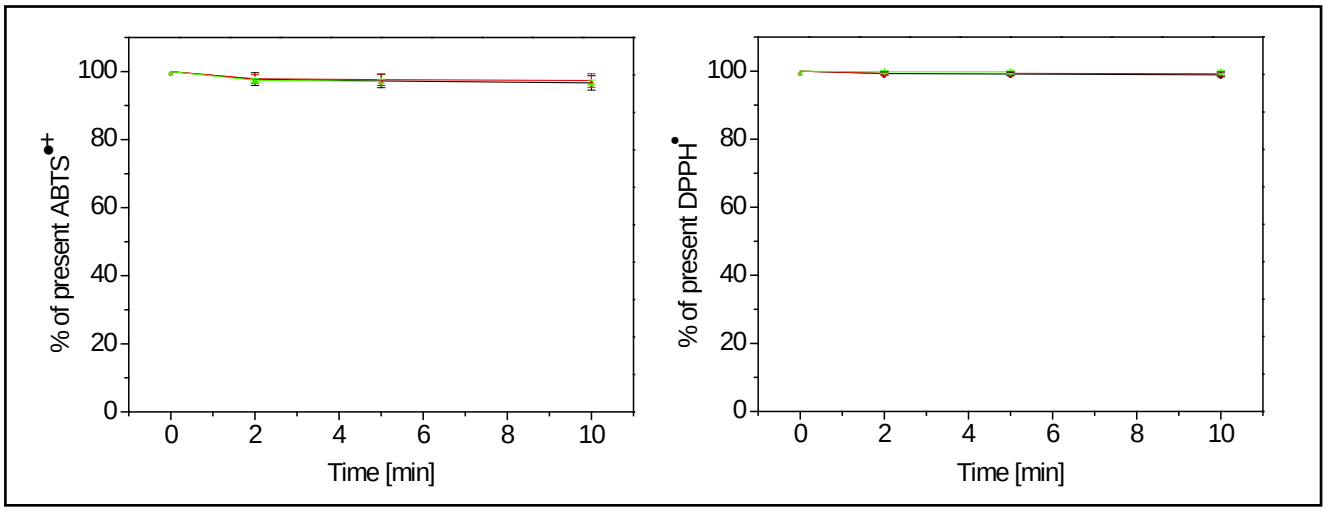

Fig. 4. Percentage of present ABTS$^{\bullet+}$ (left panel) and DPPH$^{\bullet}$ (right panel) after addition of venlafaxine at mM concentrations: 200 (black), 100 (red), 50 (green).

lar concentration of VLF. The fact of necessity of a valuable excess of VLF (i.e. ≥200 mmol/L) for inhibition of the degradation of HA macromolecules, it can be concluded that the drug venlafaxine is only a slight donor of •H. The greater inhibitory effect can be reached but a higher addition of VLF should be applied (Figure 3, left panel green and blue curves).

The results in Figure 3, right panel are similar to those in Figure 3, left panel however the drug was added not in time 0 min, i.e. when radical chain reaction begins but in time 60 min, i.e. when no ascorbate is present and the chain reaction of HA radicals continued due to the presence of peroxy- (and alkoxy-) type radicals (Valachová *et al.* 2010, 2011 a, b). The experimental results, represented in Figure 3, right panel, are indicative to the statement that VLF has (if any) practically no scavenging properties against peroxy- or alkoxy- type radicals.

Venlafaxine electron-donating property

Spectrophotometric ABTS and DPPH methods are routinely used for demonstration of electron donor properties of an agent, in our case venlafaxine, whereby its kinetics was monitored. If a certain amount of an electron donor type-antioxidant is added to a "probe-indicator" ABTS$^{\bullet+}$ or DPPH$^{\bullet}$, a series of reactions can be performed:

$$ABTS^{\bullet+} + \text{electron} \rightarrow ABTS$$
resp.
$$DPPH^{\bullet} + \text{electron} \rightarrow DPPH^{-}$$

The fact that the studied substance is an efficient electron donor, the aforementioned reactions can occur and the "probe-indicators" ABTS$^{\bullet+}$ or DPPH$^{\bullet}$ change color. However, Figure 4 displays that venlafaxine did not change the color of both ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ solutions.

CONCLUSION

The results of the two most common assays for determination of the electron/proton donating activity of samples based on scavenging DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals showed a negligible redox activity of venlafaxine to both DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ Additionally, no activity of venlafaxine to scavenge the superoxide anion radical $O_2^{\bullet-}$ was observed in KO_2/DMSO system again indicating that venlafaxine is not the efficient electron/proton donor molecule. A slight increase in •DMPO-O_2^- spin-trap adducts observed in the KO_2/DMSO/DMPO system in the presence of venlafaxine needs further investigation. However, we have shown in our dynamic viscosity tests using hyaluronan degradation induced by the oxidative system comprising $CuCl_2$ and ascorbic acid that venlafaxine at high concentrations manifested a limited capability to donor H$^{\bullet}$ atoms.

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