Antioxidant Properties of Galantamine Hydrobromide

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The antioxidant properties of galantamine hydrobromide ((4α,6β)-4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6H-benzofuro[3a,3,2-ef][2]benzazepin-6-ol hydrobromide) were studied *in vitro*, using luminol-dependent chemiluminescence and spectrophotometry. It was found that this compound was a scavenger of reactive oxygen species (ROS). By comparing the antioxidant effects of galantamine ((4α,6β)-4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6*H*-benzofuro[3a,3,2-ef][2]benzazepin-6-ol), galantamine hydrobromide, narwedine (4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6*H*-benzofuro[3a,3,2-ef][2]benzazepin-6-one), and narwedine hydrobromide it was found that the antioxidant activity depended on the enolic OH group in the molecule. The presence of a quaternary nitrogen in the compound increased the strength of the scavenging effect. It is proposed that the antioxidant properties observed *in vitro* may contribute to the therapeutical effect of galantamine hydrobromide on patients with brain degeneration.

Key words: Galantamine Hydrobromide, Narwedine, Antioxidant Properties

Introduction

Galantamine hydrobromide is traditionally used in the stimulation of the synaptic acetylcholine – meditated neurotransmission (Santos *et al.*, 2002). During the past few years it has been tested as a drug against the most common cause of dementia – the Alzheimer's Disease (Ventura and Sternon, 2001; Schneider, 2002). As a result the quality of life of patients with mild to moderate AD symptoms remarkably improved (Rockwood *et al.*, 2001; Wilkinson and Murray, 2001). Therefore the opportunity to switch from treatment with other anti-AD drugs to galantamine is discussed (Getsios *et al.*, 2001; Gasser and Gasser, 2001).

Latest clinical trials (Pitchumoni and Doraiswamy, 1998; Tuppo and Forman, 2001) and *in vitro* research (Arlt *et al.*, 2001) supported an increasing evidence that free radical-induced oxidative damage play a role in the pathogenesis of AD (Pitchumoni and Doraiswamy, 1998; Babior, 2000; Raha *et al.*, 2000; Blass, 2001; Halliwell, 2001; Ozcankaya and Delibas, 2002; Xie Z. *et al.*, 2002). The brain is especially sensitive to oxidative damage because of its high content of easily oxidized fatty acids, high use of oxygen, and low levels of endogenous antioxidants (Rokyta, 1996; Tuppo and Forman, 2001; Behl and Moosmann, 2002). Any substance capable of penetrating the blood-brain barrier, should affect the brain degeneration by control on the oxidative stress. Therefore, the antioxidant properties of drugs used in the therapy of the

Abbreviations: Ach, acetylcholine; AchE, acetylcholinesterase; AD, Alzheimer's disease; ROS, reactive oxygen species; $^{\bullet}OH$, hydroxyl radical; $^{\bullet}O_2^-$, superoxide radical; HClO, hypochloric acid; Gal., galantamine (4α,6β)-4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6*H*-benzofuro[3a,3,2-ef][2]benzazepin-6-ol; Gal. HBr, galantamine hydrobromide (4α,6β)-4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6*H*-benzofuro[3a,3,2-ef][2]-benzazepin-6-ol hydrobromide; Narw., narwedine 4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6*H*-benzofuro[3a,3,2-ef][2]benzazepin-6-one; Narw. HBr, narwedine hydrobromide 4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6*H*-benzofuro[3a,3,2-ef][2]benzazepin-6-one hydrobromide; PBS, K, Na-phosphate buffer; KO_2, potassium superoxide; CL, chemiluminescence; CL-SI, chemiluminescence scavenging index; SPh-SI, spectrophotometric scavenging index.

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central nervous system (CNS) are of great importance.

The aim of the present investigation is to measure *in vitro* the antioxidant properties of galantamine hydrobromide (Gal. HBr), and to examine the influence of the molecular structure on the interactions with reactive oxygen species.

Materials and Methods

Materials

Galantamine, galantamine hydrobromide, narwedine and narwedine hydrobromide were synthesized and purified (Krikorian *et al.*, 2000). Analyses of the samples by infrared spectroscopy, nuclear magnetic resonance and mass spectroscopy, showed a 98–99% content of the desirable products and 1–2% water. The polarimetric analysis indicated 95% L-component in the racemic mixtures.

Sodium hypochlorite and most of the other reagents used were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and were of finest grade.

KO_2 was dissolved in anhydrous dimethylsulfoxide (Aldrich-Chemie, Steinheim, Germany) in a concentration of 1 mM. The solution was stored in nitrogen and was used not later than 2 h after preparation.

The chemiluminescence reagent was prepared by dissolving luminol in a small amount of 0.01 M NaOH. Then the solution was diluted to a luminol concentration of 1 mM with a 50 mM phosphate buffer solution (PBS), and the pH was adjusted to 7.4 with 0.01 M HCl.

The compounds tested were dissolved in PBS, pH 7.4 to concentrations of 1, 10 and 100 μM and their effect on the luminol-dependent chemiluminescence (CL) and on the reduction of Nitroblue Tetrazolium to formazan (NBT test) was examined.

The reagent for NBT test was prepared by dissolving Nitroblue Tetrazolium (0.04 mM), EDTA (0.005 wt%) and Na_2CO_3 (0.008 wt%) in PBS (pH 7.4).

Methods

The luminol-dependent chemiluminescence (CL) was used for registration of ROS (Allen, 1975; Allen and Loose, 1976), with an LKB 1251 luminometer (Bioorbit, Turku, Finland) set at 310 K, and connected with an AT-type computer. Data collection was performed by MultiUse program version 1.08 (Bioorbit, Turku, Finland). Three types of CL assays were used. The ratio of CL in the presence and absence of the compound investigated was named CL scavenging index (CL-SI) and presented in percent. To compare the results for different compounds and different assays the concentrations of the compounds causing 50% (C_{50}) decrease of the parameters (CL-SI) were calculated.

By measuring the reduction of Nitroblue Tetrazolium to formazan any possible interactions of the molecules with the luminol and/or the light emitted were monitored. The intensity of the absorbance of a sample divided by the intensity of the absorbance of the control sample, is presented in percent; this is the spectrophotometrical scavenging index (SPh-SI).

Assay I: Luminol-dependent CL in the system of potassium superoxide (KO_2)-produced superoxide

The assay was carried out using 1 ml samples of PBS, pH 7.4, containing 0.1 mM luminol and the drug (in control sample, drug was omitted). The CL was measured immediately after the addition of 20 μl KO_2 solution. In this case the release of the superoxide is a fast process. Therefore CL was measured using the "flash assay" option of the MultiUse program every 50 msec.

Assay II: Luminol-dependent CL in the system of iron-dependent hydroxyl radical formation

One ml samples of PBS, pH 7.4, containing: 0.1 mM luminol, 0.1 mM Fe^{3+} ($FeCl_3$), 0.1 mM EDTA, 0.1 mM ascorbate, 1 mM H_2O_2 and any of the tested drug at concentrations between 1 and 100 μM, or a buffer for the controls. The CL was measured using the "flash assay" option of the MultiUse program, every 50 milliseconds.

Assay III: Luminol-dependent CL in the system of NaOCl-generated HClO

The sample contained the following substances in 1 ml PBS: 0.1 mM luminol 0.06 mM NaOCl and the tested drug at concentrations between 1 and 100 μM, or a buffer for the controls. The chemiluminescence was registered after addition of

NaOCl using the "flash assay" option of the MultiUse program, every 50 milliseconds.

Assay IV: Reduction of NBT to formazan in the presence of K_2O-generated $^{\bullet}O_2^-$ radicals

The inhibitory effect of each synthesized compound on the reduction of NBT to formazan in presence of $^{\bullet}O_2^-$ was tested using Perkin-Elmer 552 UV-VIS spectrophotometer, which was connected to a PC. For this purpose, in a 1-ml sample vessel we introduced 20 μl 1 mM solution of KO_2 in dimethylsulfoxide and 980 μl of 50 mM PBS, pH 7.4, containing 0.04 mM NBT and the compound to be tested. The reaction mixture was vigorously mixed after the addition of the KO_2 solution and the absorbance at 560 nm measured. Five measurements of the compounds at each concentration were performed to calculate the experimental errors. The experiments were performed as it is described in Traykov *et al.* (1997).

Results and Discussion

The measurements of the luminol-dependent chemiluminescence in presence of $^{\bullet}O_2^-$, $^{\bullet}OH$ and HOCl showed that galantamine hydrobromide is an antioxidant with strong influence of the concentration on the CL-SI values (Fig. 1). The spectrophotometric NBT- test proved that the effect of Gal. HBr on CL was not related to an interaction with exited luminol and/or with the light emitted. The calculated C_{50} values were 15 ± 2 μM, 83 ± 12 μM and 25 ± 4 μM for $^{\bullet}O_2^-$, $^{\bullet}OH$, and HOCl, respectively. Apparently galantamine hydrobromide is a scavenger of $^{\bullet}O_2^-$, $^{\bullet}OH$ and HOCl. The values of C_{50} suggested that the strength of the radical-scavenging effect decreased in the order $^{\bullet}O_2^- > HOCl > {}^{\bullet}OH$.

Antioxidant activities of four compounds: galantamine, narwedine, galantamine hydrobromide and narwedine hydrobromide were assayed. Galantamine (Fig. 2, structure **1**) and narwedine (structure **2**) were identical except for the enol group in galantamine transformed into a carbonyl group in narwedine. The corresponding hydrobromides (Fig. 2, structures **3** and **4**) showed the same difference. The difference between molecules of the galantamine (structure **1**) and galantamine hydrobromide (structure **3**) is in the partial charge, coordination symmetry and bonding of the nitrogen. The same difference shows up between narwedine (structure **2**) and its hydrobromide (structure **4**). The radical-scavenging properties were compared by measuring the luminol-dependent chemiluminescence in a system containing Fe^{3+}-EDTA, H_2O_2-generated $^{\bullet}OH$ radicals with 1, 10 and 100 μM PBS solutions of these compounds (Fig. 3). The antioxidant activity of the molecule disappeared after transformation of the enol group (galantamine) into a carbonyl group (nar-

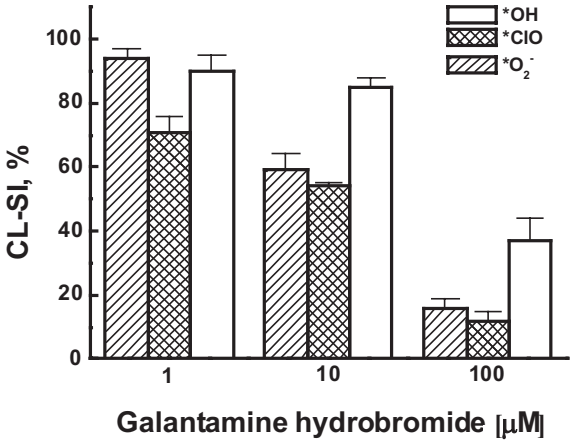

Fig. 1. Effect of the galantamine hydrobromide on the luminol-dependent chemiluminescence scavenging index (CL-SI) in presence of K_2O-generated $^{\bullet}O_2^-$ radicals, Fe^{3+}-EDTA-H_2O_2-generated $^{\bullet}OH$ radicals and NaOCl-generated HOCl.

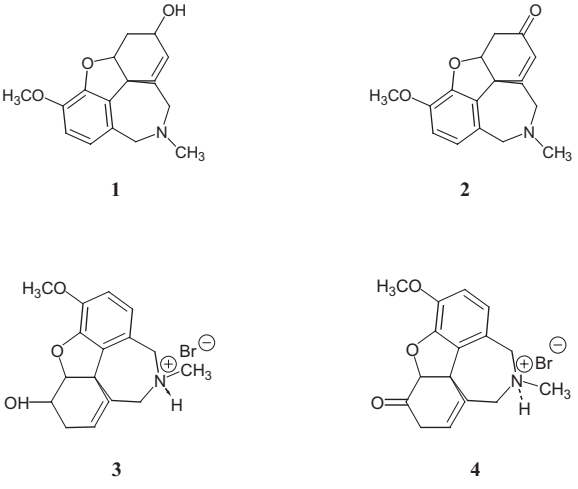

Fig. 2. Structural formulae of galantamine (**1**), narwedine (**2**), galantamine hydrobromide (**3**), and narwedine hydrobromide (**4**).

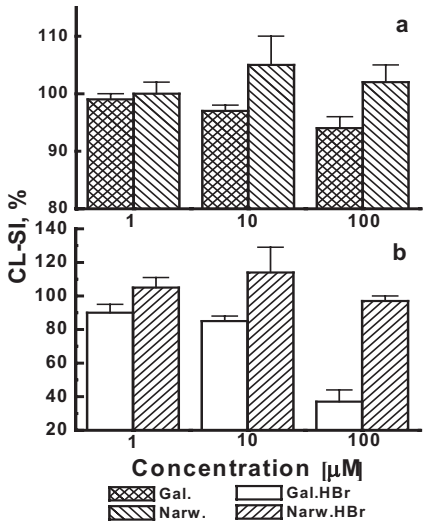

Fig. 3. Effect of the concentration of galantamine (a), narwedine (a), galantamine hydrobromide (b) and narwedine hydrobromide (b) on the luminol-dependent chemiluminescence in a system of Fe^{3+}-EDTA, H_2O_2-generated $^{\bullet}OH$ radicals.

wedine). The same effect was observed with galantamine hydrobromide and narwedine hydrobromide (Fig. 3b). The effect of the concentration of the quaternary ammonium salt on the CL was stronger when comparing galantamine (Fig. 3a) and galantamine hydrobromide (Figure 3b). Narwedine (Fig. 3a) and its corresponding hydrobromide (Fig. 3b) did not exhibit radical scavenger activity within the experimental error. The capability of galantamine and galantamine hydrobromide to scavenge ROS was related to the enol groups. Transformations of galantamine (Fig. 2, structure **1**) and narwedine (structure **2**) to the corresponding quaternary ammonium salts (structures **3** and **4**) resulted in transformation of the coordination symmetry, bonding and charge of the nitrogen. The transformation of the galantamine (structure **1**) to galantamine hydrobromide (structure **3**) was accompanied with a significant increase of the radical-scavenging effect (Fig. 3a, 3b). In the case of narwedine (Fig. 2, structure **2**) and its corresponding hydrobromide (structure **4**) no radical-scavenging effect was detected (Fig. 3a, 3b). Evidently, the quaternary coordinated positively charged nitrogen is not involved in the radical-scavenging activity, but is responsible for its increase.

The radical-scavenging property of galantamine hydrobromide was observed *in vitro*. The value of this result is that it enables one to investigate the possibility of direct antioxidant effect of the compound *in vivo*. The fact that such effect could be feasible (as shown in the *in vitro* testing) does not mean that it actually occur *in vivo*, and further studies are underway to examine this question.

Another important question is whether the concentrations at which the galantamine hydrobromide exerts antioxidant effects are relevant to the concentrations present *in vivo*. Taking into account the pharmacokinetics of galantamine hydrobromide (Michailova *et al.*, 1989) and its optimum therapeutical dose (between 16 and 24 mg) for AD patients (Wilkinson and Murray, 2001; Tariot, 2001; Lilienfeld, 2002), concentrations of about 10–15 μM of the drug should be expected in the blood plasma of the average adult person (Diem *et al.*, 1973; Lambev *et al.*, 2002). In addition, the ability of lipophilic drugs to concentrate within hydrophobic regions, such as the interior of membranes, must not be ignored too. It may be expected that the concentration range of galantamine hydrobromide used at the present *in vitro* investigation (1–100 μM) is probably close to the range of the possible therapeutical concentrations achieved in the clinically treated AD patients.

As the antioxidant activity was related to the enol group of the galantamine and galantamine hydrobromide, any chemical transformation of the OH group should affect the ability of the resulting compound to scavenge the ROS. If a substantial antioxidant effect of galantamine hydrobromide is proved *in vivo*, the enol group should be kept intact during the synthesis of new drugs based on galantamine.

Finally, if an *in vivo* effect is proved, other possible aspects of the antioxidant activity of the galantamine hydrobromide (such as inactivation of the enzyme systems implicated in the production or elimination of the free-radicals, control on phagocytosis, etc) should be investigated too.

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