Effect of Some Psychotropic Drugs on Luminol – Dependent Chemiluminescence Induced by O_2^-, $^\bullet OH$, HOCl

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We studied antioxidant activity of six neuroleptics (chlorpromazine, levomepromazine, promethazine, trifluoperazine and thioridazine) and two antidepressants (imipramine and amitriptyline) in the range of concentration of 10^{-7}–10^{-4} M. We applied luminol-dependent chemiluminescence to test the ability of these drugs to scavenge the biologically relevant oxygen-derived species: hydroxyl radical, superoxide radical, hypochlorous acid *in vitro*. We found that the phenothiazines were powerful scavengers of hydroxyl and superoxide radicals. Chlorprothixene, amitriptyline and imipramine had no scavenge activity to the superoxide radical. All drugs showed a moderate scavenger effect on hypochloric anion.

Introduction

Reactive oxygen radicals have been implicated in pathophysiology of many neurologic disorders and brain dysfunction (Kontos and George, 1985; Siesjo *et al.*, 1989; Chan, 1994). The evidence shows that reactive oxygen radicals are involved in brain injuries such as cerebral ischemia and reperfusion (Chan, 2001). It has been demonstrated that approximately 2% to 5% of the electron flow in isolated brain mitochondria produces superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2) (Boveris and Chance, 1973). These constantly produced reactive oxygen species (ROS) are reduced by enzyme and nonenzyme antioxidant protective systems of organism. Far more reactive hydroxyl radicals ($^\bullet OH$) are generated at disturbance of the balance upon increase of the concentration of O_2^- and H_2O_2. The invasion of phagocytes at the spot of the damaged or inflamed tissue results in local increase of ROS concentration. Together with the increased production of O_2^- and H_2O_2, HOCl is also produced. HOCl might inactivate α_1-antiproteinase at the spot of inflammation and it contributes to protiolitic damages though HOCl has a secondary role on bactericide effect of phagocytes.

The phenothiazines – their most widely known representative being chlorpromazine (CPZ) – and their metabolites are distributed in many body tissues reaching highest concentration in brain, lung, liver and spleen (AHFS Drug Information 89). The common therapeutic plasma concentration of these drugs is usually in the range of 0.05–5 μM in the brain the concentration is about two times bigger (Krushkov and Lambev 1993). The main therapeutic effect of CPZ is realized mostly at treatment of psychic disorders. It is established however that CPZ inhibits lipid peroxidation *in-vivo* (Roy *et al.*, 1984) and *in vitro* (Slater, 1968). CPZ and trifluoperazine (TFPZ) protect the myocardial phospholipids from LP connected with ischemic tissue damages (Janero and Burghardt, 1989). As far as initiation and development of LP is connected with availability of ROS, the manifestation of antioxidant properties could be ascribed to the ability of drugs to scavenge ROS.

Our aim was to establish the capability of a group widely used psychotropic drugs to interact with ROS *in vitro* by means of luminol-dependent chemiluminescence (CL). The present paper covers the investigations on the ability of neuroleptics: chlorpromazine (CPZ), promethazine (PMZ), levomepromazine (LVPZ), thioridazine (TRDZ), tri-

Abbreviations: O_2, superoxide radical; KO_2, potassium superoxide; $^\bullet OH$, hydroxyl radical; ROS, reactive oxygen species; NaOCl, sodium hypochlorite; CL, chemiluminescence; CPZ, chlorpromazine; LVPZ, levomepromazine; TRDZ, thioridazine; PMZ, promethazine; TFPZ, trifluoperazine; CPX, chlorprothixene; AMI, amitriptyline; IMI, imipramine; PBS, phosphate buffer solution; CL-SI, chemiluminescence scavenging index; LP, lipid peroxidation.

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fluoperazine (TFPZ), chlorprothixene (CPX) and antidepressants: imipramine (IMI), amitriptyline (AMI) to scavenge O_2^-, $^\bullet$OH and HOCl.

Materials and Methods

Chemicals and preparations

The following psychotropic drugs were studied: phenothiazines (chlorpromazine, levomepromazine, thioridazine, promethazine and trifluoperazine), three-cycled antidepressants-imipramine and amitriptyline, and thioxanthene neuroleptic-chlorprothixene. Chlorpromazine, thioridazine, promethazine hydrochlorides and trifluoperazine dihydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Levomepromazine (2-methoxy-10-[3-dimethylamino-2-methylpropyl]-phenothiazine) hydrochloride was kindly donated by Pharmachim Co. (Sofia, Bulgaria). Chlorprothixene, amitriptyline and imipramine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium hypochlorite and most of the other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and were of finest grade.

The chemiluminescence reagent was prepared by dissolving luminol in a small amount of 0.01 M NaOH. Then the solution was diluted to 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.

Drugs were dissolved in PBS, pH 7.4. Their final concentrations in the samples investigated are shown in the Figure legends.

Methods

Luminol-dependent CL was used for registration of ROS (Allen, 1975; Allen and Loose, 1976; Itoh *et al.*, 1989). For this purpose an LKB 1251 luminometer (Bioorbit, Turku, Finland) set at 37 °C was used. It was connected with an AT-type computer via serial interface and MultiUse program ver. 1.08 (Bioorbit, Turku, Finland) was used for collecting of the data. Four types of CL assays were used.

Luminol-dependent CL in a system of xanthine-xanthine oxidase-generated superoxide (assay I)

One ml PBS, pH 7.4, containing 0.1 mM luminol, 1 mM xanthine and the drug at concentrations as indicated in the figure legends were used. In the control sample, drug was omitted. Each sample was incubated for 10 min. at 37 °C. Then, 20 μl of xanthine oxidase (100 IU/l in PBS) were added (one unit xanthine oxidase will convert 1.0 μmol of xanthine to uric acid per min at pH 7.5 at 25 °C). Subsequently, the CL signal was measured for 5 min. The ratio of CL in the presence and in the absence of the drug was termed CL scavenging index (CL-SI).

Luminol-dependent CL in a system of potassium superoxide (KO_2) – produced superoxide (assay II)

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 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 milliseconds. The ratio of CL in the presence and in the absence of the drug was termed CL scavenging index (CL-SI).

Luminol-dependent CL in a system of iron-dependent hydroxyl radical formation (assay III)

One ml samples of PBS, pH 7.4, containing: 0.1 mM luminol, 0.1 mM Fe^{3+}, 0.1 mM EDTA, 0.1 mM ascorbate, 1 mM H_2O_2 and either of 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. The ratio of CL in the presence and in the absence of the drug was termed CL scavenging index (CL-SI).

Luminol-dependent CL in a system of NaOCl-generated hypochlorite (assay IV)

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. The ratio of CL in the presence and in the absence of the drug was termed CL scavenging index (CL-SI).

Results and Discussion

The effects of drugs on the luminol-dependent CL in a system with enzymatically generated O_2^- (assay I) are shown in Fig. 1. We assume that the CL ratio in the presence and in the absence of the tested drugs should show the O_2^- scavenging properties of the drugs. Therefore, this ratio was termed CL-SI (chemiluminescence scavenging index).

At concentrations above 1 μM, phenothiazines decreased moderately CL-SI in a xanthine-xanthine oxidase system. The effect was most distinct for CPZ, while being vague for TFPZ. These results possibly indicate that phenothiazines were able to scavenge O_2^-.

Drug-induced decrease of the luminol-dependent CL in a system of xanthine-xanthine oxidase-generated superoxide radicals may be due to the well-known fact that phenothiazines are inhibitors of a number of enzymes (Lullmann-Rauch and Scheid, 1975; Abdalla and Bechara, 1994; Motohashi, 1995). There are no data showing that xanthine oxidase is among them. Nevertheless, the ability of drugs to scavenge O_2^- was tested also in a system with potassium superoxide – generated O_2^- (assay II). In this case, the release of O_2^- is an extremely fast process, and CL was registered by the "flash assay" option of the MultiUse program every 50 milliseconds. The results obtained are summarized in Fig. 2a. We did not find any significant changes of CL-SI below drug concentrations of 10 μM. At higher concentrations, CPZ, PMZ and TFPZ slightly reduced CL-SI. At the concentration of 0.1 mM, the phenothiazines decreased CL-SI 2.5 to 6.5-fold.

Figurs 1b. and 2b. show data concerning the effect of the CPX, IMI and AMI on the luminol-dependent CL in a xanthine-xanthine oxidase and KO_2 system of O_2^- generation. The results obtained demonstrate that the CPX, AMI and IMI in the systems do not affect substantially the CL-response. The results obtained show that at concentrations lower than 10 μM, the investigated drugs do not exhibit a detectable scavenging effect.

The formation of $^{\bullet}OH$ is achieved in most of the O_2^- generating systems, in particular by activated phagocytes. The main determining factor of the actual toxicity of O_2^- and H_2O_2 for the cells is connected with the availability of metal ions, catalysing the formation of $^{\bullet}OH$ radicals (Halliwell and Gutteridge, 1986).

We investigated the ability of the selected drugs to interact with $^{\bullet}OH$ in a system containing the Fe^{2+}-EDTA complex (Fig. 3). In this system $^{\bullet}OH$ are formed in the water phase in which the rest of the participants of the system also are – drugs and luminol. Strongest $^{\bullet}OH$ scavenging properties are

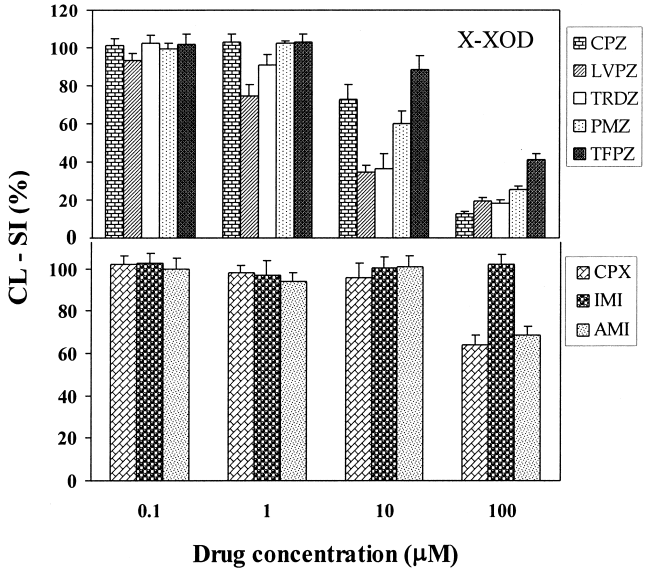

Fig. 1. Effect of psychotropic drugs on the luminol-dependent CL in a system of xanthine-xanthine oxidase- generated O_2^- (assay I). The reaction mixture contained 1 ml phosphate buffer solution (PBS), pH 7.4, 0.1 mM luminol, 1 mM xanthine and drug at concentrations as indicated. In the control sample drug was omitted. After addition of 20 μl xanthine oxidase (100 IU/l in PBS) the chemiluminescence was measured for 5 min (one unit xanthine oxidase will convert 1.0 μmol of xanthine to uric acid per min at pH 7.5 at 25 °C). The ratio of CL in the presence and in the absence of the drugs was termed CL-SI.

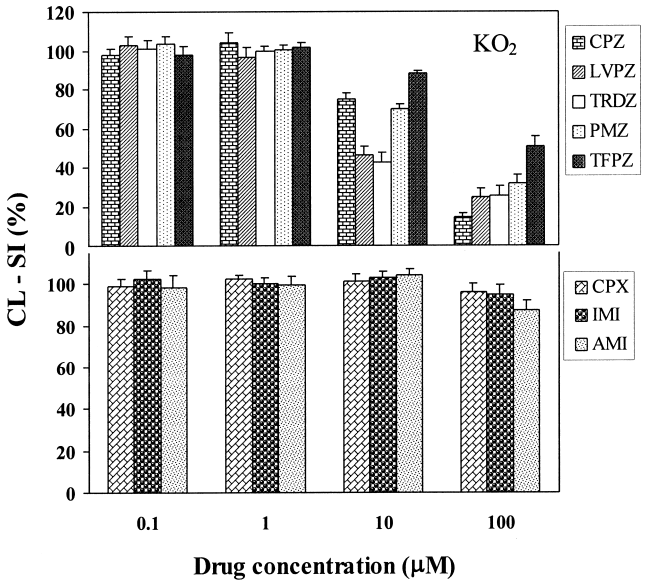

Fig. 2. Inhibition by psychotropic drugs of the luminol-dependent CL in a system with KO_2-generated superoxide radicals by phenothiazines (assay II).
The sample cuvette contained 1 ml phosphate buffer solution (pH 7.4), 0.1 mM luminol and drug. In control sample the drug was omitted. The chemiluminescence was registered after addition of 20 μl KO_2 (1 mM in dimethyl sulfoxide). For CL-SI see Fig. 1.

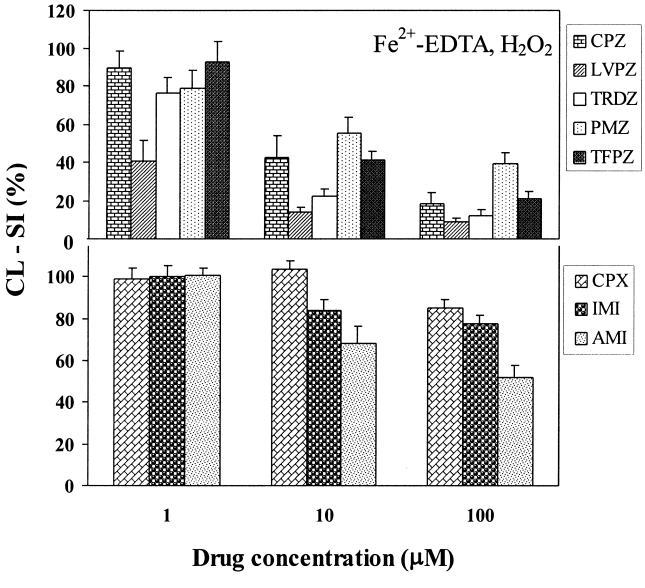

Fig. 3. Effect of drugs investigated on the luminol-dipendent CL in a system of iron ion-dependent hydroxyl radical formation (assay III).
One ml samples of phosphate buffer solution, pH 7.4, containing: 0.1 mM luminol, 0.1 mM Fe^{3+}, 0.1 mM EDTA, 0.1 mM ascorbate, 1 mM H_2O_2 and either of tested drug at concentrations between 1 and 100 μM, or a buffer for the controls. The CL signal was measured for 1 min. For CL-SI see Fig. 1.

shown by TRDZ and LVPZ at concentration 0.1 mM decreasing CL-SI about 12 and 8 times, respectively. PMZ reveals the least scavenger effect from the rest of the investigated phenothiazines. PMZ and AMI decreased the amount of $^{\bullet}OH$ in the system about 2 times. CPX and IMI at concentration of 0.1 mM show CL-SI 85% and 77%, respectively. This suggests a slight ability for interaction with $^{\bullet}OH$. Upon decrease of substance concentration, CL-SI decreased and at 1 μM for all substances it is in the interval 76–92%. Only LVPZ is an exemption – for it CL-SI hardly

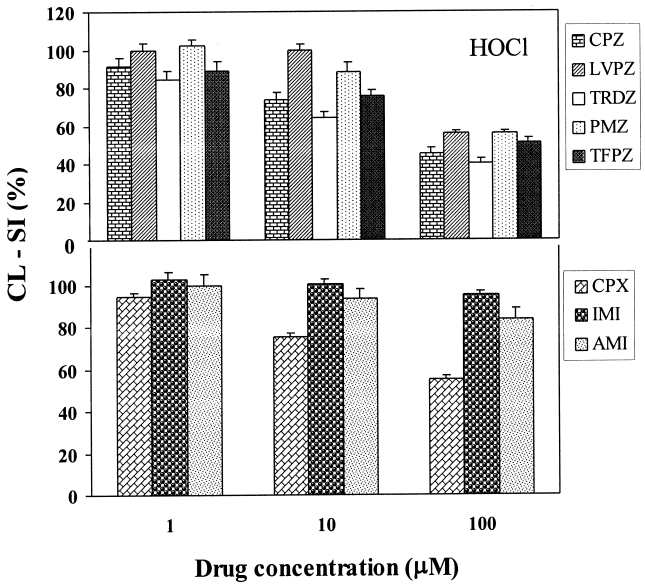

Fig. 4. Effect of drugs investigated on the luminol-dipendent CL in a system of NaOCl-generated hypochlorite (assay IV).
The sample contained the following substances in 1 ml phosphate buffer solution: 0.1 mM luminol 0.06 mM NaOCl and the tested drug at concentrations between 1 and 100 μM, or a buffer for the controls. The chemiluminescence was registered after addition of NaOCl using the "flash assay" option of the MultiUse program, every 50 milliseconds. For CL-SI see Fig. 1.

reached 41%. The results obtained indicate probably that all investigated drugs scavenge $^{\bullet}OH$ to a higher extent than O_2^-. CPZ (Bindoli *et al.*, 1988; Breugnot *et al.*, 1990), PMZ (Poli *et al.*, 1989) and TFPZ (Janero and Burghardt, 1989) are pointed out as good antioxidants. A possible explanation of these properties is the ability of the drugs to scavenge O_2^- and $^{\bullet}OH$ radicals.

It is known that CPZ protects α_1-antiproteinase from HOCl attack, which may occur upon phagocyte activation (Jeding *et al.*, 1995). The interaction of the drugs with hypochlorite ions (OCl^-) measured by luminol-dependent CL is presented on Fig. 4. The amount of OCl^- added to the model system is considered with the amount eliminated by phagocytes during phagocytosis – 60 μM (Gresham *et al.*, 1990; Suzuki *et al.*, 1991; 1992). At concentration 0.1 mM all neuroleptics investigated by us decrease CL-SI index by 2 times approximately. Concerning the antidepressants investigated, we did not find a significant interaction with OCl^- (Fig. 4b). There is a little evidence available for the capability for a direct interaction with hypochlorite of the drugs that we have investigated.

We emphasize that our results are obtained *in vitro* only. The value of these tests is that they enable one to investigate the possibility of direct antioxidant effects of compounds *in vivo*. The fact that such effects could be possible (as shown by *in vitro* testing) does not mean that they actually occur *in vivo*. Future studies that are underway will examine these questions.

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