Effect of Phenothiazines on Activated Macrophage-Induced Luminol-Dependent Chemiluminescence

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Abstract. The inhibitory effect of some phenothiazine neuroleptics (chlorpromazine, levomepromazine, thioridazine, promethazine and trifluoperazine) on the ability of rat peritoneal macrophages to produce O_2^- during phagocytosis was investigated The superoxide radical release was estimated by measuring the luminol-dependent chemiluminescence (CL) The effect of drugs was studied in the concentration range of 0 1–100 μmol/l

Additional experiments to determine the ability of the drugs to scavenge O_2^- were carried out They included measuring the effect of phenothiazines on the luminol-dependent CL in systems with enzymatically (xanthine-xanthine oxidase) and non-enzymatically (KO_2) generated O_2^- The ability of phenothiazines to scavenge O_2^- was additionally tested by a "non-luminescence" method in which the superoxide concentration was determined spectrophotometrically by the reduction of nitro blue tetrazolium to formazan

All drugs tested decreased significantly CL of stimulated macrophages at concentrations greater than 1 μmol/l The C_{50} values were between 0 45 and 1 74 μmol/l

Also phenothiazines were found to act as scavengers of O_2^- However, this effect occurred at significantly higher drug concentrations The C_{50} values for 50% scavenging of O_2^- in systems with different sources of O_2^- were in the concentration range of 5–160 μmol/l

These results suggested that phenothiazines predominantly affected the ability of macrophages to produce O_2^- during phagocytosis The findings may provide some insight into the untoward effects of the drugs tested

Key words: Chemiluminescence – Free radicals Macrophages – Phenothiazines – Superoxide

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Abbreviations: O_2^- superoxide radical, KO_2 potassium superoxide CL chemiluminescence C_{50} – drug concentration which causes 50% reduction of the parameter reflecting the concentration of O_2^- in the system PMNLs polymorphonuclear leukocytes, CPZ chlorpromazine, LVPZ – levomepromazine TRDZ thioridazine PMZ promethazine, TFPZ trifluoperazine, PBS phosphate buffer solution HBSS Hanks balanced salt solution, OZ opsonized zymosan CL-I chemiluminescence index CL SI chemiluminescence scavenging index SPh SI spectrophotometric scavenging index LP – lipid peroxidation

Introduction

It is well known (Johnston et al 1978) that macrophages activated by various stimuli release reactive oxygen species, such as superoxide radical hydrogen peroxide, etc which are thought to be major effector molecules mediating the killing of microorganisms (Nathan et al 1979) On the other hand in practical medicine treatment with different drugs could considerably affect the macrophages' ability to generate reactive oxygen species, and consequently their bactericide activity (Duncker and Ullman 1986 Itoh et al 1989) Phenothiazine derivatives are widely used in contemporary medicine (Baldessarini 1992) and have been extensively investigated There is limited evidence for their potential to modulate the generation of reactive oxygen species by phagocytes Taniguchi et al (1988) reported that trifluoperazine and chlorpromazine markedly inhibited superoxide production of rabbit PMNLs at concentrations higher than 10 μmol/l Also it has been demonstrated (Kelder et al 1991) that chlorpromazine inhibited the respiratory burst of human PMNLs as recorded by lucigenin dependent chemiluminescence

In all these investigations, however superoxide production was determined by measuring the superoxide concentration in the system This is correct for systems only in which superoxide, once released, does not interact with the drug tested

There was no direct evidence that phenothiazines are scavengers of O_2^- However, it has been shown that chlorpromazine inhibited lipid peroxidation in liposomes (Bindoli et al 1988), rat synaptosomes (Sram and Binkova 1992) and liver microsomes (Rider et al 1988) Since the initiation of lipid peroxidation needs an increased production of O_2^-, these results seem to imply that chlorpromazine (and possibly also other phenothiazines) is able to scavenge O_2

In the context of these data, the aim of the present work was to test the possibility of phenothiazines scavenging O_2^- generated in a system of activated macrophages This investigation enabled us to estimate the real modulation effect of phenothiazines on the ability of macrophages to produce O_2^-

Materials and Methods

Chemicals and preparations

The following phenothiazines were studied chlorpromazine, levomepromazine, thioridazine, promethazine and trifluoperazine Chlorpromazine, thioridazine promethazine hydrochlorides and trifluoperazine dihydrochloride were obtained from Sigma (St Louis, MO, USA) Levomepromazine (2-methoxy-10-[3-dimethylamino-2-methylpropyl]-phenothiazine) hydrochloride was kindly donated by Pharmachim Co (Sofia, Bulgaria) Zymosan was purchased from Serva Feinbiochemical (Heidelberg, Germany) Potassium superoxide (KO_2) and most of the other reagents were obtained from Fluka Chemie AG (Buchs, Switzerland), and were of finest grade

Hanks' balanced salt solution without phenol red and bicarbonate (Sigma) was dissolved in redistilled water to 290 mOsm and the pH value was adjusted to 7 4 with disodium phosphate

The chemiluminescence reagent was prepared by dissolving luminol in a small amount of 0 01 mol/l NaOH Then the solution was diluted with 50 mmol/l PBS to give luminol concentration of 1 mmol/l, and the pH was adjusted to 7 4 with 0 01 mol/l HCl

Drugs were dissolved in PBS pH 7 4 Their final concentrations in the samples investigated are given in the figure legends

KO_2 was dissolved in anhydrous dimethyl sulfoxide (Aldrich-Chemie Steinheim, Germany) to concentration of 1 mmol/l The solution was stored under nitrogen and was used not later than 2 hours after the preparation

Opsonized zymosan was prepared according to Allen (1986), and suspended in HBSS to concentration of 20 mg/ml

Preparation of peritoneal macrophage suspension

Male albino Wistar rats (180 200 g) were used The rats were injected intraperitoneally with 20 ml HBSS After 1 h the remaining solution was sucked out from the peritoneal cavity The red blood cells were removed by a brief (20 seconds) osmotic shock The peritoneal exudate cells were washed 3 times in HBSS, tested for viability with trypan-solution (4 g/l) and then resuspended to yield of 5×10^6 cells/ml

Methods

O_2^- was registered by measuring the luminol-dependent CL (Allen and Loose 1976 Itoh et al 1989) For this purpose, LKB 1251 luminometer (Bioorbit Turku Finland) set at 37 °C was used It was connected to an AT-type computer via serial interface, and MultiUse program ver 1 08 (Bioorbit) was used to collect data

Three types of CL assays were used

Assay I (luminol dependent CL of OZ stimulated macrophages)

In this case, the sample cuvette contained 100 μl luminol solution, 600 μl HBSS 100 μl drug solution (100 μl HBSS for controls), and 100 μl cell suspension Each sample (pH 7 4) was incubated for 10 min Then, 100 μl of OZ were added using a programmed automatic dispenser and CL was registered for 40 min

Assay II (luminol dependent CL in a system of xanthine-xanthine oxidase generated superoxide)

One ml PBS pH 7 4 containing 0 1 mmol/l luminol, 1 mmol/l 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 Subsequently the CL signal was measured for 5 min

Assay III (luminol-dependent CL in a system of KO_2-produced superoxide)

The assay was carried out using 1 ml samples of PBS pH 7 4, containing 0 1 mmol/l luminol and the drug (in control sample drug was omitted) The CL was measured immediately after addition of 20 μl KO_2 solution The release of superoxide in this case is a fast process Therefore CL was measured using the 'flash assay" option of the MultiUse program, every 50 milliseconds To get some additional information, drugs were tested by the following non luminescence method

Assay IV (spectrophotometric registration of O_2^- generated by KO_2)

The inhibitory effect of the tested drugs on the reduction of NBT to formazan in a system of KO_2-generated O_2^- was studied For this purpose, we added 20 μl KO_2 solution to 1 ml of 50 mmol/l PBS, pH 7 4 containing 0 04 mmol/l NBT and the tested drugs The reaction mixture was vigorously mixed after the addition of KO_2 solution and the absorbance at 560 nm was measured using a Shimadzu UV 260 spectrophotometer Assays were repeated five times for each drug and control

Statistics

For multiple group comparisons, one way analysis of variance (ANOVA) was employed followed by Bonferroni's test for truly significant differences Statistical significance was defined at $P < 0\ 05$ The statistical procedures were performed with GraphPad InStat software, version 2 04, USA Data are expressed as mean ± S D

Results

In our initial experiments, we studied the effect of drugs on the CL in a system of zymosan activated peritoneal macrophages (assay I) The ratio (in percentage) between CL in the presence and in the absence of the tested drugs was termed CL-I (CL index) (Fig 1) As is well seen, at concentrations higher than 1 μmol/l all drugs markedly decrease the luminol-dependent CL in such a system At drug

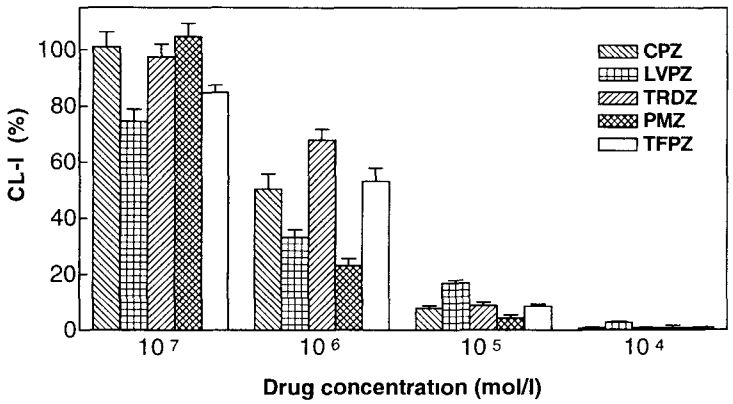

Figure 1. Decrease of the luminol-dependent CL of peritoneal macrophages in the presence of phenothiazines (assay I) The sample cuvette contained 100 μl luminol solution (1 mmol/l) 600 μl HBSS (290 mOsm pH 7 1) 100 μl drug solution or 100 μl HBSS in control and 100 μl cell suspension (5×10^6 cells/ml) After addition of 100 μl OZ (20 mg/ml) the chemiluminescence was recorded for 10 min The ratio (in percentages) of CL in the presence and in the absence of the tested drugs was termed CL I

concentration of 0 1 mmol/l the CL-I was in the range of 0 7 3%

It is well known (Allen and Loose 1976) that luminol-dependent chemiluminescence reflects the concentration of superoxide radicals in the reaction mixture Therefore it may be suggested that phenothiazines somehow decrease the O_2^- content in the system of activated macrophages At least two different mechanisms may be involved drug induced changes of the ability of macrophages to produce O_2^- during phagocytosis (real modulation effect of the drugs on the bactericidal activity of the macrophages), and/or drug induced scavenge of O_2^- generated Initially, we studied the O_2^--scavenging effect of phenothiazines The effects of drugs on the luminol-dependent CL in a system with enzymatically generated O_2^- (assay II) are shown in Fig 2 We believed that the CL ratio in the presence and in the absence of the tested drugs in this system would reflect the O_2^--scavenging properties of the drugs Therefore, this ratio was termed CL-SI (chemiluminescence scavenging index)

It was established that, at concentrations above 1 μmol/l, all drugs decreased moderately CL-SI in a xanthine-xanthine oxidase system The effect was most distinct for chlorpromazine while being vague for trifluoperazine These results possibly mean 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 (Lullmann-Rauch and Scheid 1975, Abdalla and Bechara 1994, Motohashi 1995)

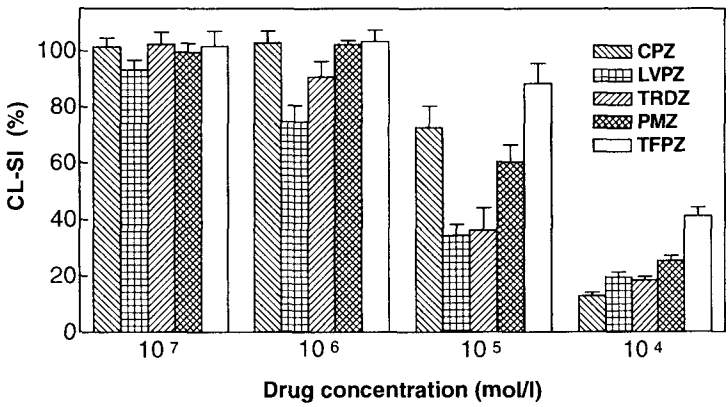

Figure 2. Effect of phenothiazines on the luminol-dependent CL in a system of xanthine-xanthine oxidase- generated O_2^- (assay II) The reaction mixture contained 1 ml PBS pH 7 4 0 1 mmol/l luminol, 1 mmol/l xanthine and drug at concentrations as indicated In the control sample drug was omitted After addition of 20 μl xanthine oxidase (100 IU/l in PBS) the chemiluminescence was measured for 5 min The ratio of CL in the presence and in the absence of the drugs was termed CL SI

that phenothiazines are inhibitors of a number of enzymes There are no data showing that xanthine oxidase is among them Nevertheless, the ability of phenothiazines to scavenge O_2^- was tested also in a system with KO_2 generated O_2^- (assay III) 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 Typical time courses of the luminol-dependent CL in the system of KO_2-generated O_2^- are shown in Fig 3, and the results obtained are summarized in Fig 4 We did not find any significant changes of CL-SI below drug concentrations of 10 μmol/l At higher concentrations, chlorpromazine, promethazine and trifluoperazine slightly reduced CL-SI At the concentration of 0 1 mmol/l, the tested drugs decreased CL-SI 2 5 to 6 5-fold

On the other hand, the effect of phenothiazines on the luminol-dependent CL (assays II and III) may be due, at least in part to interaction of drugs with excited luminol molecules and/or with the light emitted If this were the case, the result would be a drop in CL in the presence of the drugs, even if they were not O_2^- scavengers at all Therefore, a "non-luminescent" study (assay IV) was carried out In this case, the concentration of O_2^- in the system was measured by a NBT-test The ratio (in percentage) of the increase of absorbance at 560 nm in the presence and in the absence of the drug was termed SPh-SI (spectrophotometric scavenging index), (Fig 5) The data obtained were similar to those obtained from the "luminescent" experiments (assays II and III) The drugs had no effect below the concentration of 1 μmol/l Trifluoperazine did not cause any changes of the SPh-SI

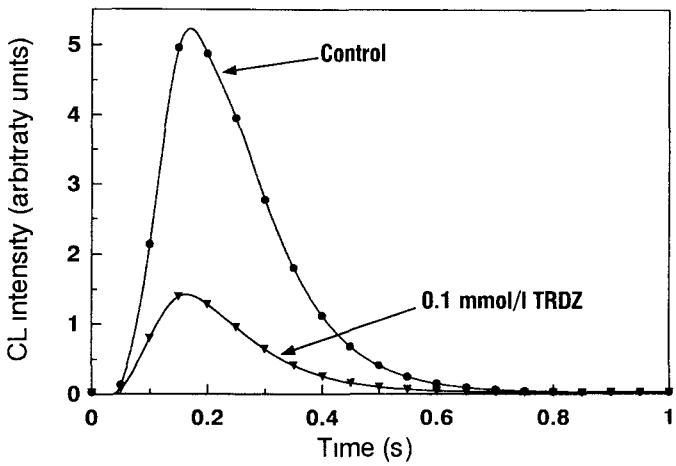

Figure 3. Typical luminol-dependent CL time courses in a system with KO_2-generated O_2^- (assay III) The sample cuvette contained 1 ml PBS (pH 7 4), 0 1 mmol/l luminol and drug In control sample the drug was omitted The chemiluminescence was registered after addition of 20 μl KO_2 (1 mmol/l in dimethyl sulfoxide)

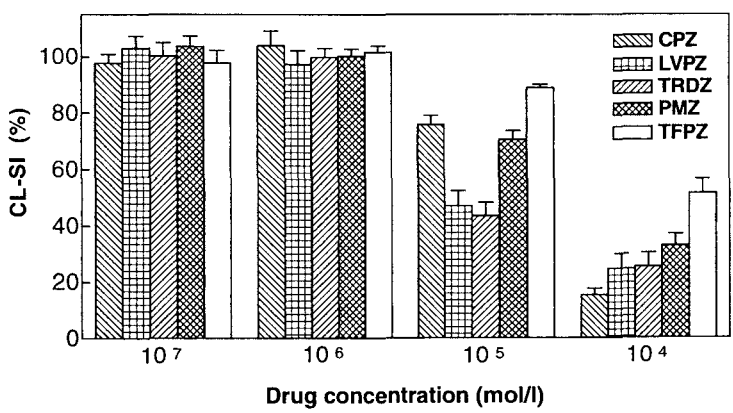

Figure 4. Inhibition by phenothiazines of the luminol-dependent CL in a system with KO_2-generated superoxide radicals by phenothiazines (assay III) The sample cuvette contained 1 ml PBS (pH 7 4), 0 1 mmol/l luminol and drug In control sample the drug was omitted The chemiluminescence was registered after addition of 20 μl KO_2 (1 mmol/l in dimethyl sulfoxide) The ratio of CL in the presence and in the absence of the drugs was termed CL-SI

even at 10 μmol/l At such concentration, the other caused insignificant decrease in SPh-SI Further increase of the drugs concentration led to well expressed reduction

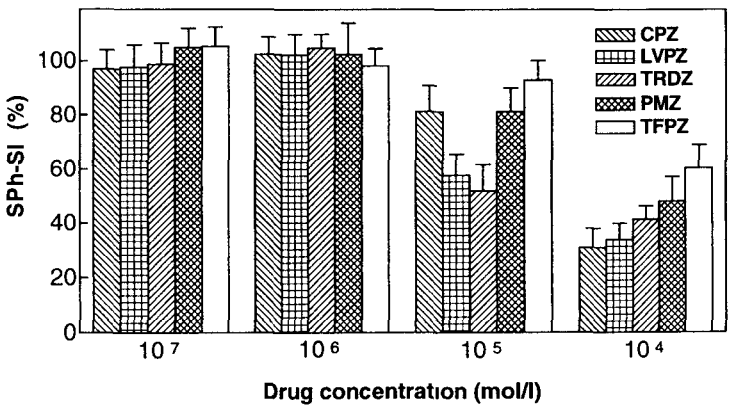

Figure 5. Scavenging of superoxide radicals by phenothiazines, determined by the 'non luminescent" NBT test (assay IV) The sample cuvette contained 1 ml of 50 mmol/l PBS (pH 7 4) 0 04 mmol/l NBT and drug After addition of 20 μl KO_2 (1 mmol/l in dimethyl sulfoxide) the change of absorbance at 560 nm was measured The ratio (in percentage) of the change of absorbance at 560 nm in the presence and in the absence of the drugs was termed SPh-SI

of this index Thus, 0 1 mmol/l chlorpromazine or levomepromazine decreased its value approximately 3 fold whereas there was a less than twofold decrease after trifluoperazine

Discussion

The dose-effect relations in Figures 1, 2 4 and 5 are sigmoid for all tested drugs To compare the results obtained from the assays, we therefore calculated the values of C_{50}, i e drug concentrations causing a 50% decrease of the parameters (CL-I for assay I, CL-SI for assays II and III, and SPh-SI for assay IV) reflecting the concentration of O_2^- in the respective reaction mixture (Table 1) The calculations used fitting of the data to the "sigmoid" model

$$CL - I(CL - SI \text{ or } SPh - SI) = 100/\left[1 + 10^{B(\lg C - \lg C_{50})}\right]$$

where B is the coefficient (hill slope), and C is the drug concentration

The results obtained in assays II, III and IV (columns 3, 4 and 5, respectively) clearly show that all the tested phenothiazines are able to scavenge O_2^- However, we did not find significant differences between the results obtained Nevertheless, it seems that C_{50} (assay II) $< C_{50}$ (assay III) $< C_{50}$ (assay IV) These differences and tendencies might be due to the different rates of O_2^- generation by the used O_2^- sources For the xanthine – xanthine oxidase system, the results might also be influenced by the drug-induced decrease of the xanthine oxidase activity Furthermore,

Table 1. Concentrations (C_{50}) of phenothiazines leading to 50% decrease of the parameter reflecting the O_2^- content in the reaction mixtures of assays I–IV The calculations were carried out by a non-linear regression analysis The correlation coefficient values R^2 were > 0 995 which indicates a high goodness of fit and non-significant deviation from the used model

Drugs	C_{50}, μmol/l			
	CL-I (assay I)	CL-SI (assay II)	CL-SI (assay III)	SPh-SI (assay IV)
Chlorpromazine	1 05 ± 0 12	21 70 ± 0 20	25 30 ± 3 60	44 90 ± 6 00
Levomepromazine	0 45 ± 0 01	4 96 ± 1 20	13 30 ± 4 80	27 20 ± 10 80
Thioridazine	1 74 ± 0 18	7 06 ± 2 18	12 20 ± 5 10	32 40 ± 18 60
Promethazine	0 85 ± 0 11	21 70 ± 6 20	37 20 ± 8 10	86 10 ± 21 50
Trifluoperazine	1 02 ± 0 15	71 50 ± 8 10	107 00 ± 12 00	157 00 ± 33 00

phenothiazines seem to be able to react with the excited luminol molecules and/or with the emitted light All these suggestions however, need further experimental verifications

As is well seen from column 2 in Table 1 (assay I), phenothiazines cause a well expressed decrease of the luminol-dependent CL in a system of activated macrophages In such a case, the C_{50} values reside within the range of 0 45 1 74 μmol/l This effect seems to be due at least to two types of reactions of the drugs The first one involves interactions with macrophages and as a result, the cell ability to produce O_2^- during phagocytosis is reduced This is an actual modulation effect of phenothiazines on the bactericidal activity of the macrophages The second interaction involves scavenging of O_2^- after its generation by the activated macrophages

The contribution of the latter interaction seems negligible Thus, any of the drugs have not shown O_2^- – scavenging properties at concentrations (0 45–1 74) μmol/l (Figs 2, 4 and 5) In addition, as is seen from columns 3, 4 and 5 of Table 1, the C_{50} values for 50% scavenging of O_2^- generated from different sources range within 5–157 μmol/l Therefore, the C_{50} values for the system of activated macrophages seems to be 10–100 times smaller than the C_{50} values for systems with xanthine-xanthine oxidase or KO_2 sources of O_2^-

The conclusion that phenothiazines at micromolar concentration may inhibit the ability of macrophage to produce O_2^- during phagocytosis is in good agreement with the data obtained by other investigators Taniguchi et al (1988) found that trifluoperazine and chlorpromazine, at concentrations higher than 10 μmol/l, markedly inhibit superoxide production of rabbit PMNLs Although their object (rabbit PMNLs), stimulus (chemotactic peptide *n*-formyl-methionyl-leucyl-

phenylalanine) and method for determining superoxide generation (ferricytochrome c reduction) were different from ours, their results are similar At concentration of 0 1 mmol/l, they found zero superoxide production for both drugs Similar to ours are also the results of Kelder et al (1991) They found that chlorpromazine inhibits the respiratory burst of human PMNLs measured with lucigenin-dependent CL They speculated that the inhibition was most probably due to some interference with processes in the cell, leading to a respiratory burst rather than to scavenging of the oxygen radicals generated that provoke luminescence Our results show that the scavenging properties of phenothiazines are not negligible and must be considered when doing investigations with superoxide sources in the presence of these drugs There has been a number of such studies published recently (Dijkstra et al 1985, Wolff 1986, Taniguchi et al 1988, Kelder et al 1991) in which, however, possible scavenging and quenching properties of the drugs tested were not taken into account

So far, we have not heard of investigations of modulation capabilities of levomepromazine, promethazine and thioridazine on the respiratory burst It could be suggested that they have similar properties as chlorpromazine and trifluoperazine but this would not be quite accurate Thus it was shown (Kelder et al 1991) that phenothiazines with a similar structure (chlorpromazine and chlorpromazine sulfoxide) have completely different modulation capabilities CPZ strongly inhibited the respiratory burst whereas chlorpromazine sulfoxide did not affect it at all Therefore results for levomepromazine, promethazine and thioridazine obtained in our experiments are almost as much novel as those established by other authors (Taniguchi et al 1988, Kelder et al 1991) for chlorpromazine and trifluoperazine

We could not identify papers which would report on the ability of phenothiazines to scavenge superoxide radical Chlorpromazine has been shown to inhibit moderately Fe^{2+}/ascorbate catalyzed LP in liposomes (Bindoli et al 1988) and rat synaptosomes (Sram and Binkova 1992) for concentrations greater than 10 μmol/l Since initiation and development of LP involve reactive oxygen species these results would suggest that chlorpromazine is able to scavenge them However, another explanation is also possible It is well known (Slater et al 1985, Halliwell 1990) that phenothiazines are able to bind iron ions Obviously, such a reaction could also lead to a drop in LP, even when the drug is not a scavenger Our results confirmed that phenothiazines have scavenging capabilities

The effect reported herein was obtained for an *in vitro* system So far, we can not say if it takes place *in vivo* If it does, these results could be important for practical medicine For a number of phenothiazines we found a sigmoid dose-dependent influence on the CL of stimulated macrophages with C_{50} within the range 0 47–1 74 μmol/l On the other hand, the therapeutic plasma concentrations of these drugs are 0 05–5 μmol/l (Dahl and Strandjord 1977, Benet and Williams 1992, Krushkov and Lambev 1993) Therefore, the results may give hints for untoward

effects of phenothiazine neuroleptics (Baldessarini 1992 Hollister 1992 Krushkov and Lambev 1993) which may be important in the therapy of immunocompromized patients Another important question is the antioxidant action of these drugs *in vivo* The scavenging effect established herein is not strong enough for therapeutic plasma concentrations, but the ability of lipophilic drugs to concentrate within hydrophobic regions (such as membrane interior) must not be ignored

The mechanism by which phenothiazines modulate the ability of macrophages to produce O_2^- during phagocytosis is still not clear It may involve drug-induced inhibition of calmodulin functions (Curnutte et al 1984, Morimoto et al 1986) Future studies that are underway will examine these possibilities

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