Mechanism of Action of Antipsychotics, Haloperidol and Olanzapine *in vitro.*

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ABSTRACT

Schizophrenia affects 1-1.5% of people in the United States alone. Haloperidol (HP), a butyrophenone and a typical antipsychotic, has been used as an antipsychotic drug in human. Unfortunately, the therapeutic effects of HP also come with severe extrapyramidal side effects, resulting in movement disorders in patients. Olanzapine, a new atypical neuroleptic, seems to have better efficacy, with less severe adverse effects. There has been increasing evidence of the role of reactive oxygen species (ROS) and oxidative stress in the pathogenesis of Schizophrenia. We therefore hypothesized that the differences between HP and Olz could be partly because of the differences in the oxidative stress they cause. We studied the pro-oxidant and antioxidant effects of these two drugs *in vitro* and examined the mechanism of their cytotoxicity in a neuronal cell model using PC-12 cells. HP was found to be ineffective as a superoxide radical scavenger but appeared to be a potent scavenger of hydroxyl radicals with a rate constant of ~6.78 X 10^9 $M^{-1}s^{-1}$. Olz on the other hand was found to scavenge hydroxyl radical at a rate of 34.1 X 10^9 $M^{-1}s^{-1}$. This was shown using the hydroxyl radical dependent deoxyribose degradation assay and EPR spin trapping methods. HP was also found to quench singlet oxygen in a dose-dependent manner. HP was found to enhance the microsomal lipid peroxidation in a dose-dependent manner and at 10 µM it augmented the lipid peroxide accumulation by 100% whereas Olz, at the same concentrations had trivial effects. Light microscopy and two cytometric apoptotic/viability probes (7-aminoactinomycin D and Annexin-V) were employed to evaluate mechanisms of drug-induced cell death in PC-12 pheochromocytoma cells exposed to HP or Olz. At low dose (50 µM), HP was more cytotoxic than Olz. At high concentrations (150 µM) each of these antipsychotic drugs caused a significant increase in cell death that was readily detectable by all the techniques. Light microscopy with trypan blue staining indicated that necrosis was the predominate form of cell death with both drugs. Apoptotic cells were rarely observed by microscopy in vehicle or drug-exposed cells. Further, no increase in early cellular apoptosis was observed using the Annexin-V probe. 7AAD and Annexin-V both showed drug-related increases in the late apoptotic/necrotic cell death window. These data, along with the cytologic evaluations suggest that cell death in PC-12 pheochromocytoma cells exposed to HP or Olz may primarily be necrotic in nature, rather than apoptotic. Because Olz at a low dose was less cytotoxic and was found to have lower pro-oxidant action than HP the secondary effects manifested in patients with chronic treatment with HP may, at least in part, be attributed to the pro-oxidant effects of the drug.

DEDICATION

I would like to dedicate this work to my family, for their support and encouragement throughout my program here. My parents, my brother and sisters, who were always there for moral support. To my husband, for his unconditional love and patience and his constant encouragement, when the going was tough.

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Thanks to the Janssen foundation for their generous gift of haloperidol and the analogs, and to Eli Lilly Corporation for kindly providing me with olanzapine.

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LIST OF ABBREVIATIONS

7AAD- 7-aminoactinomycin-D
ADP- Adenosine diphosphate
ANOVA-Analysis of Variance
ATCC- American Tissue and Cell Culture
BSA-Bovine Serum Albumin
CO_2- Carbon Dioxide
DMEM- Dulbecco's Minimum Essesntial Medium
DMPO- 5,5-Dimethyl-1-pyrroline-n-oxide
DNA- Deoxyribonucleic acid
DTPA- diethylenetriaminepentaacetic acid
EDTA- Ethylenediamminetetraacetic acid
EPR- Electron Paramagnetic Resonance
EPS- Extrapyramidal Symptoms
FACS- Flow activated cell sorter
FBS-Fetal Bovine Serum
FCS- Fetal Calf Serum
$FeCl_3$- Ferric Chloride
FITC- Fluorescein Isothiocyanate
H_2O_2- Hydrogen Peroxide
HCl- Hydrochloric acid
HP- Haloperidol
HS- Horse Serum
MDA- Malondialdehyde
MPTP-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NaN3-Sodium Azide
NOR- Nor compound of haloperidol
OLZ- Olanzapine

PBS- Phosphate Buffered Saline

PC-12- Rat pheochromocytoma (cell line)

PI-Propidium Iodide

RHP- Reduced Haloperidol

ROS- reactive Oxygen Species

SEM-Standard Error of Mean

TBA- Thiobarbituric Acid

TCA- Trichloroacetic Acid

TEMP- 2,2,6,6-teramethylpiperidine

TEMPO- 2,2,6,6-teramethylpiperidine oxide

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BACKGROUND AND LITERATURE REVIEW

1.1: Schizophrenia and antipsychotics:

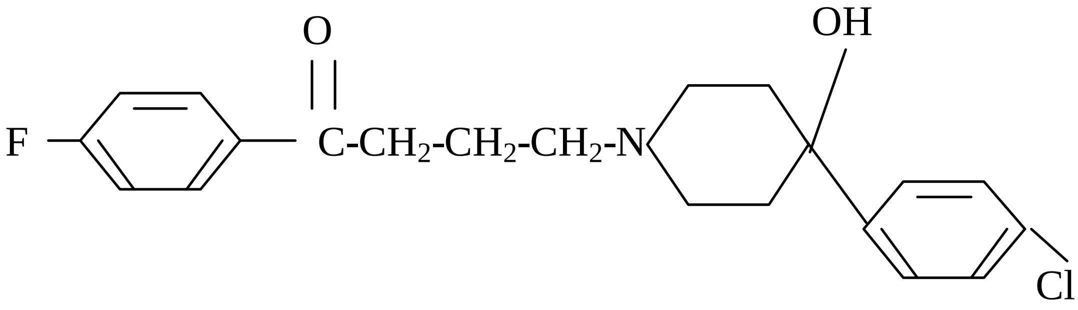
Schizophrenia is a debilitating disorder of the central nervous system. Its symptoms have been divided into two classes: positive symptoms, including hallucinations, delusions and conceptual disorganization; and negative symptoms, including social withdrawal, blunted affect, and poverty of speech (Donaldson *et al.*, 1983). This disorder reduces the ability of the individual to interact with the society. The typical neuroleptics used to treat schizophrenia are highly effective, but are associated with severe extrapyramidal side effects (EPS). The most predominant among these symptoms are dystonia, parkinsonian-like syndrome, and tardive dyskinesia. These extrapyramidal side effects have been and still are major concerns in the society, as the very drug that treats the patients for schizophrenia leaves them with life long disabilities.

Haloperidol, the most widely used typical antipsychotic is extremely efficient in treating the positive and negative symptoms of psychoses and schizophrenia. Long-term use of the drug, however, results in an irreversible motor disorder involving the orafacial muscles and the extremities, which has been a source of major concern, in the medical community (Andreasson, 1996). Recently, there has been development of the so-called atypical antipsychotic drugs. These atypical drugs seem to have similar clinical efficiency as the typical antipsychotics, but with minimal or no extrapyramidal symptoms (Borison, 1997). Clozapine was the first atypical antipsychotic drug introduced, but its use was restricted because of the fatal agranulocytosis associated with it. A few of the new ones to join the group are olanzapine, sertindole and quietapine which are equally potent as clozapine with out apparent agranulocytosis or any other major adverse effects (Borison, 1997). Because reactive oxygen species (ROS) are known to cause cellular injury that lead to various pathophysiological process, including neuropsychotic disorders, the current study was under taken to compare the pro-oxidant and the anti-oxidant profile of a typical antipsychotic haloperidol (HP), and an atypical antipsychotic olanzapine (OLZ).

1.2: Typical antipsychotic drugs:

The standard antipsychotic drugs like haloperidol, chlorpromazine, and perphenazine, have been used for a long time to treat psychotic diseases. Despite significant advantages provided by these drugs, responders to these drugs have to deal with the residual symptomatology that interferes significantly with their social and occupational functioning (Breier *et al.*, 1991). Some may develop disfiguring, disabling, and potentially life-threatening adverse effects, including parkinsonian symptoms, tardive dyskinesia, and neuroleptic malignant syndrome (Baldessarini, 1988; Levenson, 1985; Sovner et al., 1978), whereas others are resistant to the treatment totally.

Haloperidol: Haloperidol is a typical potent neuroleptic drug or a major tranquilizer. It was originally synthesized in 1956, clinically tested and in the market by 1960. Haloperidol has been used clinically in psychiatry, obstetrics, and anesthesiology, and its pharmacology has been extensively reported (Janssen, 1967; Kudo and Ishizaki, 1999; Ichikawa and Meltzer, 1999). Chemically, haloperidol belongs to the butyrophenone series of neuroleptic compounds, and the structure is as shown below.



Haloperidol, is a tertiary amine that tends to form interphase between water/air or water/lipid at very low concentrations of the order of 10^{-7} M. It has been shown that neuroleptic drugs tend to decrease the permeability of a variety of biological membranes for various inorganic and organic molecules, including water, and that they exert this effect in minute concentrations (Seeman & Bialy, 1963). That is to say that these drugs act like potent membrane permeability blockers. HP is a dopamine antagonist. Its major site of action is the dopamine D_2 receptors, which has high affinity for the drug. The average dose of HP is about 20 mg/day per person.

<u>Pharmacokinetics</u>: Mean elimination $t_{1/2}$ for HP is about 17.9 ± 6.4 hr. The mean distribution $t_{1/2}$ is anywhere between 0.19 ± 0.07 and 2 ± 1 hr after 0.125 mg/kg IV dosage. After 0.5 mg/kg oral dosage, mean absorption $t_{1/2}$ is about 0.37 ± 0.18 hr. Bioavailability of drug is about 0.65 ± 0.14 after oral doses (Holley et al. 1983).

<u>Metabolism</u>: Soujdin et al., 1967 (Soudijn *et al.*, 1967) showed that the major pathway of metabolism of HP is oxidative N-dealkylation yielding p-fluorobenzoyl-propionic acid. HP is metabolized mainly by the liver cytochrome P450 3A (CYP 3A) system (Igarashi *et al.*, 1995). Forsman and colleagues, reported the presence of reduced HP (RHP) as a major metabolite in the plasma of patients (Forsman & Larsson, 1978). The formation of these compounds is NADPH dependent. The pyridinium metabolite (HPP^+) was identified by Subramanyam and coworkers (Subramanyam *et al.*, 1991; Subramanyam *et al.*, 1990). Fang and Gorrod (1991) showed that the dehydrated product of HP, haloperidol tetrahydropyridine (HTP) serves as an intermediate in the metabolism of HP to HPP^+ (Fang & Gorrod, 1991). It is now known that the CYP 3A metabolizes HP into its pyridinium metabolite (HPP^+) via the specific isozymes CYP 450 3A4 (Fang *et al.*, 1997; Usuki *et al.*, 1996).

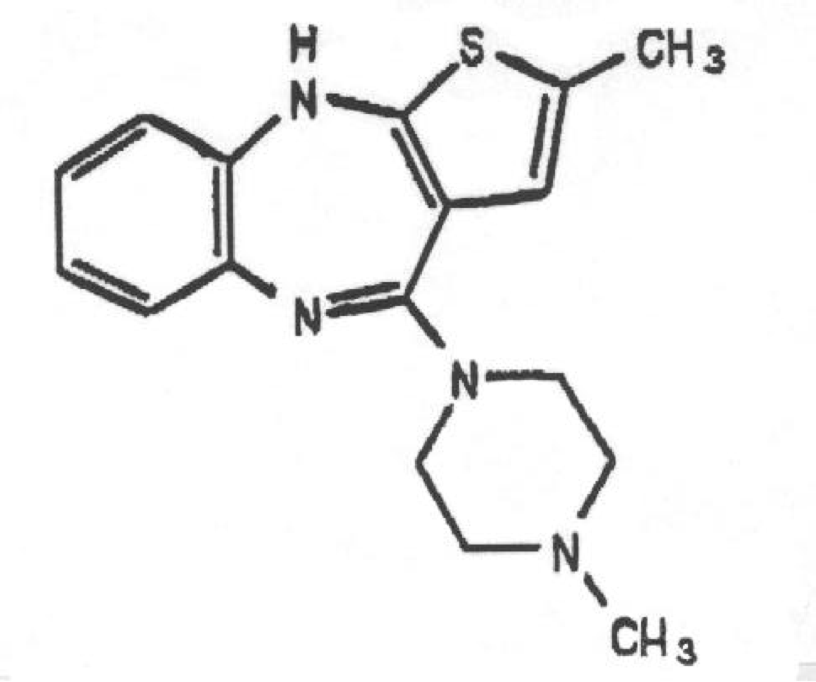
1.3: Atypical antipsychotic drugs

The term atypical antipsychotic refers to drugs that do not cause catalepsy in animals and extrapyramidal symptoms or tardive dyskinesia in humans. Unlike typical antipsychotics, which increase levels of prolactin (Petty, 1999; Gruen et al, 1978), the atypical antipsychotic drugs have a minor or no effect on plasma prolactin levels are effective in the treatment of negative symptoms, and may be effective in non-responders to classical neuroleptics (Nordstrom *et al.*, 1998).

In 1990, FDA approved clozapine as an antipsychotic medication for those with treatment resistant schizophrenia. Because of a lower propensity of causing extrapyramidal symptoms and raising serum prolactin levels, it was referred to as an atypical antipsychotic. In spite of being highly efficient, it is associated with a major adverse effect. Clozapine is associated with symptoms such as agranulocytosis, seizures, weight gain and sedation (Baldessarini & Frankenburg, 1991). Research is currently ongoing at the pharmaceutical industries at developing an agent that has superior efficacy on the patients, with favorable effects and acceptable adverse effect profile and cost effective.

A few of the drugs developed recently by various pharmaceutical companies, that meet the above needs are olanzapine, sertindole and quietapine. In the current study we focussed on olanzapine, which is marketed by Eli Lilly Corp. as Zyprexa^{TM}.

Olanzapine: The FDA approved Olanzapine, an antipsychotic drug manufactured by the Eli, Lily and company, in October 1996, for the treatment of psychotic disorders. It is a thieno-benzodiazepine analog with the chemical name of 2-methyl-4-(4-methyl-1-piperazinyl)-10 thieno [2,3-b][1,5] benzodiazepine. Olz is a yellow crystalline solid and practically insoluble in water. Its structure is as given below.



Behavioral pharmacological *in vivo* studies show that olanzapine is an antagonist of dopamine, serotonin, and acetylcholine (Moore *et al.*, 1993; Tye *et al.*, 1992). This receptor profile parallels that of Clozapine (Borison, 1995; Moore *et al.*, 1992).

Dose: The antipsychotic efficacy of olanzapine (Zyprexa) was demonstrated in the dose range of 5-20 mg/day.

Pharmacology: Olanzapine displays a very broad pharmacological profile, with potent activity at dopamine, serotonin, muscarinic, histamine and adrenergic receptors (Bakshi & Geyer, 1995; Bymaster *et al.*, 1996; Coyle, 1996; Fuller & Snoddy, 1992; Moore et al., 1992; Saller & Salama, 1993; Stockton & Rasmussen, 1996; White & Wang, 1983). Its antagonism to muscarinic receptors may explain its anticholinergic properties. Animal behavioral studies show that olanzapine has atypical antipsychotic characteristics, by virtue of its *in vitro* receptor profile (Bakshi & Geyer, 1995; Coyle, 1996; Fuller & Snoddy, 1992; Moore et al., 1992; Saller & Salama, 1993; Stockton & Rasmussen, 1996; White & Wang, 1983). The initial animal screening tests suggested that olanzapine possessed antipsychotic efficacy by virtue of its dopaminergic blocking properties. Furthermore, the animal tests suggest that the clinical efficiency with minimum EPS could be due to its specific action on the firing of the A10 region of the hippocampus the brain. The animal behavioral and electrophysiological studies show that at low doses, it might act as an atypical antipsychotic whereas at very high doses, it might resemble the typical antipsychotic.

Pharmacokinetics: There is complete absorption of olanzapine after oral administration. The maximum concentration (C_{max}) and the time required to reach (t_{max}) after single dose of 12 mg in six healthy male subjects were 11 ± 1 ng/mL and 4.9 ± 1.8 hrs, respectively (Obermeyer *et al.*, 1993).

Metabolism and excretion: It is metabolized extensively in humans via glucuronidation, allylic hydroxylation, N-oxidation, N-dealkylation and a combination thereof. This is the most important pathway both in terms of contribution to drug related circulating species and as an excretory product in the species (Kassahun *et al.*, 1997). The major metabolites found in humans are 10-N-glucuronide and 4-desmethylolanzapine (Kando *et al.*, 1997). *In vitro* evaluations of the human cytochrome P450 isoenzymes involved in the formation of the three major metabolites of olanzapine have found that CYP 1A2, CYP 2D6, and the flavin containing mono-oxygenase system are involved in the oxidation of olanzapine (Ring *et al.*, 1996).

The major route of elimination seems to be urine (first pass metabolism) in humans (Kassahun et al., 1997). It displays linear kinetics over the clinical dosing range. The systemic clearance of olanzapine takes about 26.1 ± 12.1 hrs The plasma elimination half-life ($t_{1/2b}$) is 33.1 ± 10.3 hrs (Obermeyer *et al.*, 1993). Compared with young men, young women demonstrated decreased clearance. Similarly, elderly subjects showed a decreased clearance compared to younger patients (Bergstrom *et al.*, 1995).

1.4: Oxidative stress and Schizophrenia :

There has been increasing evidence, implicating oxidative stress as a causative factor in neuropsychotic disorders including schizophrenia (Lohr, 1991; Mahadik & Scheffer, 1996; Ramchand *et al.*, 1996). Free radicals have been implicated in the pathogenesis and clinical course of neuropsychiatric disorders such as schizophrenia and in the development of tardive dyskinesia (Cadet, 1988). There have been reports of membrane pathologies and alterations in membrane phospholipids, essential fatty acids and signal transduction (Horrobin *et al.*, 1994; Van Kammen *et al.*, 1989), which are believed to be ROS mediated. Increased superoxide dismutase (SOD) activity has been reported in the red blood cells of schizophrenic patients by some groups (Abdalla *et al.*, 1986; Reddy *et al.*, 1993; Vaiva *et al.*, 1994; Wang, 1992). Abnormal activity of catalase (CAT) has also been reported (Abdalla et al., 1986; Reddy et al., 1993, 1991; Vaiva et al., 1994; Wang, 1992). Similar results have also been reported from other labs (Glazov & Mamtsev, 1976; Reddy et al., 1991). Sohal et al. (1992) have shown decreased CAT and increased SOD in schizophrenic patients (Sohal & Orr, 1992). Increased blood levels of malondialdehyde have been found in schizophrenic patients relative to normal controls (Guliaeva *et al.*, 1988; Reddy & Yao, 1996).

1.5: Reactive oxygen species:

Free radicals are highly reactive entities with an unpaired electron in their outer orbital. Reactive oxygen species (ROS) consist of species like superoxide anion, hydroxyl radical, singlet oxygen species and hydrogen peroxide, hypochlorous acid, etc. ROS are produced ubiquitously during all aerobic metabolic processes. These reactive species can cause damage to proteins, lipids, membrane, and deoxyribonucleic acid (DNA). Free radicals accumulated in the tissues by various metabolic functions have a deteriorating effect on the central nervous system and contribute to aging (Sastre *et al.*, 2000). Free hemoglobin acts as a natural Fenton's reagent in the body, thus being a source of hydroxyl radicals, detrimental to the membranes and lipids (Sadrzadeh *et al.*, 1984). Oxyradicals are produced during regular metabolism (e.g. oxyradical production during metabolism of dopamine) and/or metabolism of xenobiotic substances (metabolism of various drugs and other toxicants). It is also possible during metabolism, that instead of generation of a reactive oxygen species, a xenobiotic substance might itself get transformed into a reactive species. The prime example of this is metabolism of carbon tetrachloride, CCl_4 (McCay *et al.*, 1984). It metabolizes to trichloromethyl radical species. These radical species are responsible for induction of lipid peroxidation in rat liver microsomes (McCay et al., 1984). Reactive oxygen species, such as superoxide, are also produced during the one electron transfer to oxygen in the mitochondrial electron transport chain (Liu, 1997; Turrens, 1997). On the other hand, inhibition of the respiratory chain, especially at complex-I also results in the production of ROS (Hodnick *et al.*, 1994; Hodnick *et al.*, 1986).

Clinical studies have shown that schizophrenic patients have lower metabolic rates in cortical and subcortical structures in the brain relative to controls (Wiesel, 1992). Other metabolic changes such as reduced levels of creatinine kinase in schizophrenic brains have also been reported, suggesting alterations in local concentrations of ATP (Klushnik *et al.*, 1991). Burkhardt et al. (Burkhardt *et al.*, 1993) found that neuroleptics like HP, chlorpromazine and other similar drugs inhibit NADH:ubiquinone oxidoreductase (complex -I) *in vitro*. They suggested that inhibition of complex-I could potentially be the underlying cause of the irreversible extrapyramidal disturbances observed with neuroleptic treated. Recently, Prince et al. (1997) confirmed the above findings and further showed that the atypical antipsychotic drug, clozapine did not inhibit complex-I. This may be a defining characteristic of atypicality**.**

Under physiological conditions there is a complex antioxidant defense system consisting of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, which provides protection from damaging effects of these ROS. Many small molecular weight antioxidants such as vitamin E, vitamin C, glutathione also protect the cell against oxidative injury. A critical balance between the ROS and the antioxidant defense is essential. Any tilt in this balance causes the organism to be under oxidative stress (Halliwell, 1993). Oxidative stress has been shown to cause both apoptotic as well as necrotic cell death (Mark *et al.*, 1995; O'Neill & Kaltschmidt, 1997; Richter *et al.*, 1995).

There are various ways by which oxidative stress manifests itself. Few of the most obvious manifestations are:

a) Induction of lipid peroxidation and upregulation of antioxidant enzymes -ROS can damage macromolecules such as proteins, lipids and nucleic acids The toxicity is manifested in form of lipid peroxidation (Darley-Ushmar & Halliwell, 1996; Halliwell,1993). Upregulation of antioxidant defense enzymes like superoxide dismutase (SOD), catalase (CAT) is observed immediately after oxidative insult (Lawler & Powers, 1998).

b) Nuclear transcription factor NF- kappaB: Induction of transcription factors like NFκB and other genes like Bcl-2 and c-jun occurs following oxidative stress**.** NFκB is a transcription factor (DNA-binding protein) that regulates the expression of multiple immune and inflammatory genes (Baeuerle & Henkel, 1994). NF-κB is normally held in the cytoplasm in an inactive form bound to an inhibitory protein, IκB, of which several types are recognized (Baeuerle & Henkel, 1994). Upon activation by a wide range of external stimuli IκB is phosphorylated by an as yet unknown protein kinase, then ubiquinated and degraded by the proteasome (Hellerbrand *et al.*, 1998). This allows NFκB to translocate into the nucleus where it binds to the κB consensus sequence the most common form of which is GGGACTTTCC (O'Neill & Kaltschmidt, 1997). The commonest complex that is activated in the mammalian system is the p50/RelA heterodimer. Many stimuli activate NFkB in cells, one of which is oxidative stress, mainly hydrogen peroxide (O'Neill & Kaltschmidt, 1997), as antioxidants such as pyrollidine dithiocarbamate (PDTC) and N-acetyl cysteine (Nac) can block the effect (Schreck *et al.*, 1992). The signals that lead to activation of the kinase are not fully understood. It is generally believed that reactive oxygen species such as H_2O_2 might be common messengers (Baeuerle & Henkel, 1994; Schoonbroodt *et al.*, 1997). Antioxidants are known to inhibit NFκB activation uniformly (Lahdenpohja *et al.*, 1998). Many target genes for NFκB have been identified, including genes for cytokines such as TNF, IL-2, IL-6 and enzymes such as inducible forms of nitric oxide synthase and the antioxidant enzyme Mn-SOD (Baeuerle & Henkel, 1994). Also recent studies have demonstrated that knocking out NFκB sensitizes cells to apoptosis induced by TNF (Abbadie *et al.*, 1993). NFκB has also been shown to be activated by triggers of necrotic cell death (Li *et al.*, 1997). At the molecular level, HP specifically induced the DNA binding activity and the transcriptional activity of the redox-sensitive transcription factor NFkB. This enhanced NFkB activity could be blocked by the neuroprotective antioxidants (Post et al., 1998)

c) <u>MAP kinase activation:</u> Mitogen activated protein kinases (MAPKs) were originally described as serine/threonine kinases that are activated commonly by various growth factors and tumor promoters in mammalian cultured cells. They are thought to be the key molecules in the signaling processes stimulated by the growth factors and differentiating factors (Nishida & Gotoh, 1993). The mammalian MAP kinases can be subdivided into the extracellular signal-regulated kinases (ERKs), the Jun N-terminal kinases (JNKs), and the p38 MAP kinases (Kummer *et al.*, 1997). MAPKs/p38 kinases are activated by various cellular stressors including oxidative stress (Clerk *et al.*, 1998; Guyton *et al.*, 1996; Kummer et al., 1997). p38 phosphorylates and activates the transcription factors ATF-2 and MEF2C, which implies that it plays a role in transcriptional regulation (Han *et al.*, 1997; Raingeaud *et al.*, 1996). It has been demonstrated that a direct link between the MAP kinase signal transduction pathway and ROS provides a unifying mechanism for activation of early and late response genes by inducers of oxidative stress such as H_2O_2 (Stevenson *et al.*, 1994). It has also been shown that oxidative stress is responsible for the activation of MAPK signal transduction pathway (Stevenson et al., 1994). There is also good experimental evidence for a connection between the NFkB and p38 pathways which may occur further downstream in the cell nucleus (Schulze-Ostoff *et al.*, 1997). Long lasting changes triggered by short-lived extracellular signals are known to be mediated by the induction of immediate early genes that code for transcription factors. Early gene induction is not restricted to the mitogenic response, and can be induced by depolarization and neurotransmitters (Herschman, 1991). Esteve et al. (1995) have shown increased mRNA expression of the proto-oncogenes c-fos and jun B in the striatum of rats treated with HP.

1.6: <u>Haloperidol and oxidative stress</u>:

Chronic treatment of HP is known to induce oxidative stress due to increased turnover of dopamine (Shivkumar and Ravindranath, 1993). Haloperidol is cytotoxic to primary hippocampal neurons, C6 glioma cells and NCB20 cells (Behl et al., 1995). It was demonstrated that it causes necrotic death rather than apoptotic. Vilner and Bowen (1993), reported the cytotoxic nature of HP but have not specified the type of cell death. Behl et al. (1996) in their investigations have demonstrated that amyloid beta resistant cells were resistant to HP toxicity, implying the role of free radicals in HP-induced cell death. Also, Bcl-2 prevents cell death caused by HP (Lezoualc'h *et al.*, 1996), which also implicates free radicals as a cause of the cell death. Typical neuroleptics such as HP and chlorpromazine are known to cause oxidative stress (Behl et al.,1996; Shivkumar and Ravindranath, 1993), which is thought to be responsible for its extrapyramidal side effects (Cadet *et al.*, 1986). It has also been shown that increasing doses of HP in rats (Shivkumar & Ravindranath, 1992) attenuates the extrapyramidal side effects caused by the same drug. As far as the atypical antipsychotics are concerned, there have been no reports so far on their cytotoxic/cyto-protective effects.

1.7: Apoptosis and Necrosis

Cell death can follow two pathways: apoptosis or necrosis. Apoptosis is derived from a Greek term meaning: the falling of leaves”. Kerr and associates (Kerr et al., 1972) first coined it as a term denoting cell death. It has unique biochemical, cytological and molecular features, by virtue of which this can be distinguished from necrosis. Apoptosis results in the condensation of the chromatin, and the fragmentation of DNA. Early apoptosis is marked by membrane changes such as the translocation of the phosphatidyl serine (PS, a phospholipid) from the inner membrane to the outer membrane. The cells also shrink in size during apoptosis. These are in contrast to the phenomenon of necrosis, which is a passive event, caused due to serious injury or trauma to cells. During necrosis, the cells often swell in size. Apoptosis is a normal physiologic process in living organisms. There is no inflammatory response triggered by cells dying via apoptosis, and there is minimal damage to the surrounding cells (Kerr et al., 1972). This is because apoptosis activates certain endonucleases that fragment the DNA and pack them into apoptotic bodies, which are eliminated by macrophages thus minimizing inflammation. In sharp contrast, a consequence of necrosis is inflammation of surrounding cells and tissues. Programmed cell death is sometimes used to describe apoptosis. This is because apoptosis triggers the activation of specific genes in a very ordered fashion.

Initial studies used DNA fragmentation as the hallmark to detect apoptosis (Kerr et al., 1972; Duke et al, 1983; Wyllie et al, 1980), which was visualized using gel electrophoresis. However, recently the reliability of that test has been challenged, and it has been shown that DNA laddering is not always indicative of apoptosis (Cohen et al. 1992). The use of various DNA binding dyes and fluorochrome labelled dyes in flow cytometry is becoming increasingly popular. Donner and associates (Donner et al., 1999) have compared the kinetics of various techniques used today to detect apoptosis. They reported that FITC-Annexin-V staining and 7-amino actinomycin D (7AAD) assays were comparable as markers of early apoptosis, and that cytology (using cytospins) could be used as supporting evidence for early apoptotic studies. The newly developed CASY-1 cell counter and analyzer system was also used in that study.

Haloperidol has been known to cause cell death in various cell lines and has been mostly been shown to be necrotic in nature (Vilner and Bowen, 1995; Behl et al.1996). The cytotoxic effects of olanzapine on cells, however are not known yet. Our study compares the effect of haloperidol and olanzapine on PC-12 cells and the type of cell death occurring. For our study we decided to use the FITC-Annexin V staining assay and the 7AAD assay using flow cytometry, cytology and the CASY-1 cell counter to study apoptosis. FITC-Annexin-V binding assay is based upon the translocation of the PS from the inner membrane to the outer membrane, whereas the 7AAD assay is based upon the binding of the dye at the nicked DNA.

1.8: Cell line used for research

Clonal cell line-PC-12, commonly known as pheochromocytomas, was used in the following experiments. The use of clonal cell lines in neurobiological research offers the advantage that its genetically determined and specific functions remain intact through numerous passages in culture (Brautigam et al., 1985). The advantage of using clonal cell lines over preparations of organ tissues is the fact that a genetically stable cell material is involved of which sufficient quantities for assays can be obtained in culture (Brautigam *et al.*, 1985). The clone PC-12 has been of special interest to the pharmacologists and toxicologists as it possesses the gene-dependent regulation for the synthesis of all enzymes that are characteristic for a dopaminergic or noradrenergic neuron (Brautigam et al., 1985). PC-12 cells, because of their resemblance to sympathetic neurons and their precursors, are a widely accepted neuronal model system (Greene & Tischler, 1976; Greene & Tischler, 1982; Saltiel and Decker, 1994; Tischler et al. 1990). The presence of a dopamine receptor of the D2 subtype has been described in PC-12 cells (Courtney *et al.*, 1991; Inoue *et al.*, 1992), which makes it an ideal cell line to study the effects of antipsychotic drugs.

Significance:

Schizophrenia affects 1-1.5% of people in the United States. Haloperidol (HP) was introduced in 1956, and since then has been the mainstay of treatment for schizophrenia and other psychotic diseases, in spite of its drastic adverse effects. HP specifically binds to the D_2 receptors, which is believed to be responsible for its therapeutic effects. It is also believed that oxidative stress may be responsible for the extrapyramidal side effects associated with its use. Olanzapine (Olz) is a relatively new drug in the market and its therapeutic and secondary effects are not fully characterized.

Olz has been found to be especially useful in treating refractory schizophrenic patients. Long-term use of Olz has been shown to reduce rate of relapse in the patients. Its (Olz) mode of action is attributed to its selective dopamine antagonism, specifically D_4 receptors, and serotonin antagonism (specifically $5HT_2$ receptors). Although, it has proved to be very efficient in treating schizophrenia, with no or minimal side effects, further research on OLZ is needed for the long term effects of this drug, and for the medical community to be more receptive to the newer treatments. This study would answer the question as to whether or not haloperidol and olanzapine are cytotoxic to neuronal cells and compare their magnitude of oxidative stress they may cause.

Hypothesis and Specific Aims:

The overall objectives of this study were to correlate the therapeutic and extrapyramidal side effects of the antipsychotic drugs, HP and Olz, to their antioxidant and pro-oxidant properties. It was hypothesized that both HP and Olz are effective antioxidants and are capable of protecting the neuronal cells against oxidative stress as seen in schizophrenic patients and HP has more pro-oxidant properties than Olz. This fact might attribute to the extrapyramidal side effects observed in patients treated with these drugs. Furthermore, the pro-oxidant effects of these drugs are related to their cytotoxicity in neuronal cells.

The specific aims of the study were:

1**. To study the the pro-/anti-oxidant properties of the typical neuroleptic haloperidol, and compare it with atypical neuroleptic olanzapine *in vitro*.** The antioxidant effects of these drugs were demonstrated by monitoring their scavenging ability on:

(a) superoxide anions,
(b) hydrogen peroxide,
(c) hydroxyl radicals, and
(d) singlet oxygen.

The pro-oxidant effects were monitored by examining their effects on microsomal membrane lipid peroxidation system.

2. **To investigate the mechanism of cyto-toxicity of HP and OLZ treatment on neuronal cells.**

The mechanism of cell death, apoptotic versus necrotic, were evaluated using rat pheochromocytoma, PC-12 cell line. Two commonly used probes for apoptotic detection were employed namely,

Annexin-V/Propidium Iodide staining and 7-Aminoactinomycin D staining, along with cytology, trypan blue exclusion test and the light scatter analysis by an electron cell counter.

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CHAPTER 1

Haloperidol: Scavenger of reactive oxygen species and enhancer of Microsomal Lipid Peroxidation

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ABSTRACT

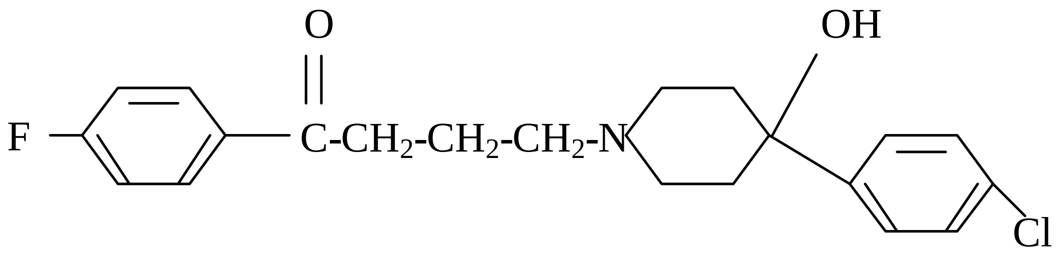
Haloperidol (HP), a butyrophenone with a potent sigma ligand, has been used as an antipsychotic drug in humans. Unfortunately, the therapeutic effects of HP also come with severe extrapyramidal side effects, resulting in movement disorders in patients. There has been increasing evidence of the role of reactive oxygen species (ROS) and oxidative stress in the pathogenesis of Schizophrenia. We investigated the pro-oxidant and antioxidant properties of HP *in vitro*. HP was found to be ineffective as a superoxide radical scavenger but appeared to be a potent scavenger of hydroxyl radicals with a rate constant of ~6.78 X 10^9 $M^{-1}s^{-1}$. Electron paramagnetic resonance (EPR) spectroscopy in combination with spin trapping techniques, using a Fenton type reaction and 5,5-Dimethyl-1-pyrroline-n-oxide (DMPO) as a spin trap, HP was found to cause a dose-dependent inhibition of DMPO-OH adduct formation. HP was also found to quench singlet oxygen in a dose-dependent manner. Singlet oxygen was generated using a photochemical reaction with rose bengal as photo-sensitizer and was trapped using 2,2,6,6-teramethylpiperidine (TEMP). The amount of HP required to inhibit 50% of singlet oxygen-dependent TEMPO production was 2.5 µM. Some of its analogs, such as reduced HP and nor compound were also effective in scavenging both the .OH and singlet oxygen. HP was found to enhance the microsomal lipid peroxidation in a dose-dependent manner and at 10 µM it augmented the lipid peroxide accumulation by 100%. We propose that HP exerts its beneficial effects, at least in part, through its ability to scavenge ROS and the side effects on the other hand may, in part, be due to the peroxidation of membrane lipids.

INTRODUCTION

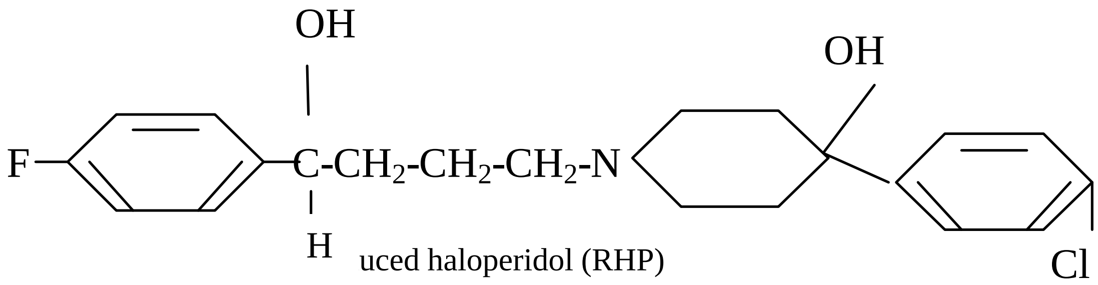
Haloperidol (HP), a member of the butyrophenone class of drugs, is commonly used in humans for the treatment of various psychotic disorders such as mania, psychoses, Gilles de la Tourettes' syndrome and Schizophrenia (1). Since the introduction of the drug in early 1960s in the United States, it has gained popularity and has been increasingly used against both acute and chronic Schizophrenic patients (2). HP has also been shown to be very effective in the treatment of acute and chronic brain syndrome, delirium tremens, Huntington's chorea, and manic depressive psychosis, and in controlling hallucinations and paranoid symptomatology (2,3). The clinical potency of HP is thought to be closely related to its ability to bind to D_2 dopaminergic receptor (4). The sigma ligand of the drug appears to be essential for the neuroprotective property (5). HP was shown to bind the D_2 receptors in the hippocampus, and thereby reducing the excitability of cells in CA1 (one of the longitudinal lobes of the hippocampus) and as a result altering hippocampal modulation of brainstem areas via descending projection (6). HP appears to inhibit ATP gated ionic current thereby leading to attenuation of intracellular Ca^{2+} ($[Ca]_i$) rise in PC12 cells, that is independent of dopamine receptor-mediated mechanisms (7). The competence of HP as a maintenance neuroleptic drug in preventing psychotic relapse in chronic Schizophrenia patients has been well-established (8).

Apart from being an antipsychotic drug, HP appears to have other beneficial effects. It has been reported to be a good analgesic, sometimes obviating the need for narcotics, during surgery (2). Certain studies have shown that HP attenuates the Ehrlich carcinoma (9,10) in mice in a dose-dependent manner. It was also found to be very effective in lowering the intraoccular pressure in normal and glaucomatous eyes, thus has greater premise for the treatment of glaucoma (11).

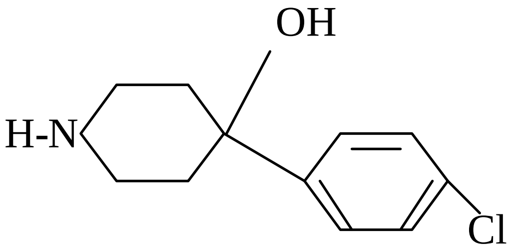
Oxidative stress has been implicated in the etiology of psychosis in certain individuals. The antioxidant defense appears to be compromised at the onset of psychosis and oxidative injury has been implicated as a contributing factor in the pathogenic cascade of schizophrenia (12, 13). The use of neuroleptics, especially butyrophenones, is limited by their tendency to produce a range of extrapyramidal movement disorders such as Parkinsonism, akathisia, dystonia, and finally chronic tardive dyskinesia (14). It was also suggested that the depletion of essential fatty acids from erythrocyte membrane is possibly the result of the schizophrenic process rather than antipsychotic drug treatment (13). Moreover, lipid peroxidation has been implicated as a causative factor in the development of tardive dyskinesia and other motor disorder movements (15). Because production of excess oxyradicals can overwhelm the body's antioxidant defenses resulting in cellular damage including peroxidation of lipids, oxidation of proteins and damage to DNA (12, 16), we developed the hypothesis that HP might exert its beneficial effects, in part, by scavenging free radicals. In this study, we present evidence that HP and some of its analogs, such as reduced HP (RHP) and nor compound (see structural formula below), are potent inhibitors of hydroxyl radicals and singlet oxygen, and are capable of augmenting membrane lipid peroxidation.



Haloperidol (HP)



uced haloperidol (RHP)



Nor compound of haloperidol (NOR)

Scheme 1: Structural formula of HP, RHP and NOR

MATERIALS AND METHODS

HP was obtained from RBI, Natick, MA. Its analogs were generous gifts from Janssen Pharmaceuticals, Beerse, Belgium. 5,5-Dimethyl-1-pyrroline N-oxide (DMPO), bovine superoxide dismutase, cytochrome c (Type III), trichloroacetic acid, thiobarbituric acid (TBA), tartaric acid (solvent for the drugs), adenosine diphosphate (ADP), ferric chloride, and deoxyribose were obtained from Sigma (St. Louis, MO). 2,2,6,6-teramethylpiperidine (TEMP) was obtained from Aldrich (Milwaukee, WI). All other materials were purchased at the highest available purity.

Preparation of liver microsomes: The microsomes were prepared as described by Das and Misra (17). Briefly, bovine liver was collected on ice after sacrifice, and was minced with scissors. The minced liver was homogenized for 3 minutes in a blender with 5 volumes of ice-cold 0.15M potassium phosphate buffer, pH 7.6, and filtered through a triple-layered cheese cloth. The filtrate was centrifuged at 24,000 X g for 10 minutes to remove mitochondria, nuclei, and cell debris. The supernatant was then centrifuged at 100,000 X g for 90 minutes, and the microsomal pellet was collected. The microsomes were washed by resuspending in 0.15 M phosphate buffer, pH 7.6 to the original volume and by sedimenting for 90 minutes at 100,000 X g. The washed microsomal pellet was then resuspended in Tris-HCl buffer, pH 7.6, to yield a final concentration of 10 mg microsomal protein/ml. The washing procedure removes most contaminants of hemoglobin, superoxide dismutase, and catalase from microsomal protein. Protein concentration was determined by Bradford protein assay using bovine serum albumin as the standard (18).

Assay of Lipid Peroxidation: Lipid peroxidation was determined as thiobarbituric acid (TBA)-reacting products as described (19). The experimental conditions were similar to Castilho et al. (20) with slight modifications. Briefly, the reaction mixture, containing 3 mg of microsomal protein in 0.05 M Tris-HCl buffer, pH 7.6, 1 mM ADP, 50 µM ferric chloride, 100 µM ascorbate was incubated at 37^{o} C for 15 minutes. Lipid peroxidation was initiated by the addition of 50 mM H_2O_2 and terminated by addition of 2 ml of 0.5% TBA (w/v) and 2% trichloroacetic acid (TCA). This mixture was heated at 95^{o}C for 10 minutes. Three ml of n-butanol was added after cooling and the mixture was vortexed for 30 s. Samples were centrifuged, and the supernatants, containing MDA, were read at 535 nm against a blank, which contained all reagents except H_2O_2**,** and were quantitated by using an extinction coefficient of 1.56 X10^{5} M^{-1}cm^{-1} (19).

EPR studies: Hydroxyl radicals were generated in a Fenton type reaction. The hydroxyl radicals generated were detected as DMPO-OH spin adducts exhibiting a well-characterized 1:2:2:1 signal pattern with $A_N = A_{Hb}$ =14.92 G (17). Effects of HP and reduced HP were studied in reaction mixtures that contained the following reagents at the final concentration: 32.2 µM H_2O_2, 32 µM $FeSO_4$, 3.22 mM purified DMPO in 0.2M boric acid-borax buffer, pH 7.8. The reaction was initiated by addition of ferrous sulfate. Various levels of HP and reduced HP were used in the above system. The generation of hydroxyl radicals was observed as DMPO-OH adduct on a Bruker D-200 X-Band EPR spectrometer. Scan conditions, unless otherwise indicated were as follows: microwave frequency, 100 kHz; modulation amplitude, 2.0 G; modulation frequency, 100 kHz; time constant, 0.64 s, scan time, 200s; receiver gain, 1X10^{6}; center field setting, 3483 G. The signal height of the second peak was used to calculate the percent inhibition.

Determination of Rate Constant: The degradation of deoxyribose by hydroxyl radicals and the production of a pink chromogen monitored spectrophotometrically has been used to determine the rate constants of various compounds and are in agreement with rate constants determined by the pulse radiolysis method (18, 21, 22). Hence in this study we have used the deoxyribose method (21) to determine the rate constant for reaction of $^{\cdot}$OH with HP and its analogs (reduced HP, and nor compound). Reaction mixtures contained, in a final volume of 1.0 ml, the following reagents at the final concentrations stated: deoxyribose (2.8 mM), potassium phosphate buffer, pH 7.4 (20 mM), EDTA (30µM), H_2O_2 (1 mM), and ascorbate (100 µM) and $FeCl_3$ (50µM). Solutions of $FeCl_3$ and ascorbate were prepared immediately before use in de-aerated water. Reaction mixtures were incubated at 37^{o}C for one hour, and one ml of 0.5 % TBA (w/v) and 1 ml of 2% TCA (w/v) were added, and mixtures were heated at 80^{o} C for 30 minutes. The rate of deoxyribose degradation was found to be constant over the 1 hr incubation period.

Photolysis EPR studies: The generation of singlet oxygen by photochemical reactions of rose bengal was studied by EPR spectroscopy using 2,2,6,6-tetramethylpiperidine (TEMP) as a singlet oxygen trap (23). The characteristic EPR spectral pattern of three lines of equal intensity of TEMPO nitroxide radical was observed (A_N = 17.2 G and g = 2.0056) when air-saturated aqueous solution of rose bengal (40 µM) was irradiated with TEMP (65 mM) in 0.05 M potassium phosphate buffer, pH 7.8, with 10^{-4} M EDTA. Photolysis studies were performed at room temperature in quartz capillary tubes irradiated for various time periods at a distance of 30 cm from the lens of a Viewlex VR-25 remote-controlled slide projector.

X-ray studies: Because iron was present in the above procedures, to exclude the possibility of the metal effects we have examined the oxidation of deoxyribose in an assay performed in a metal free environment. The phosphate buffer (100 ml) was passed twice through Chelex-100 column (1cm X 25 cm) to eliminate any trace of iron. All containers including reaction vessels were polyethylene material because there is possibility that glass may leach some metals. The hydroxyl radicals were generated by X-irradiation of water. Hydroxyl radicals were produced radiolytic cleavage of water, using a Minishot cabinet X-ray machine, model M-110-NH, TFI Corp., West haven, CT. Inhibition of the hydroxyl radical dependent deoxyribose degradation by the drugs was studied by the TBA method. Thus, 2.8mM deoxyribose and 0.05M phosphate buffer, pH 7.8 were X-irradiated at a dose of 5 Gy/min for various time periods. The reaction was stopped by the addition of 2% TCA and 0.5 % (w/v) TBA. The reaction mixture was then heated to 90^{o}C for 30 minutes and the TBA-reactive products generated during degradation of deoxyribose were monitored spectrophotometrically at 535 nm. The accumulation of TBA reactive products was found to be linear over a range of 0-40 minutes (data not shown). Based on that we X-irradiated the samples for 30 minutes for all subsequent experiments.

Iron chelation studies: Direct iron chelation assays were performed using calcein, a fluorescence dye, and diethylenetriaminepentaacetic acid (DTPA), an iron chelator, as described (24). Calcein was used as a probe, whose florescence is rapidly and stoichiometrically quenched by divalent metals such as Fe(II). Addition of DTPA, a known iron chelator, removes iron from the solution and augments the fluorescence. The florescence of calcein was monitored at excitation wavelength of 487 nm and emission wavelength of 517 nm. The reaction mixture contained 50µM calcein in 0.25M Tris-HCl buffer, pH 7.6. Iron was added (2µM) in the form of ferrous ammonium sulfate or 2µM ferric chloride. Florescence was recorded for 3 minutes and then either DTPA (250 µM) or drugs (1mM) was added. Florescent reading was recorded every minute for an additional 7 minutes.

Statistical analysis:
All experiments were performed at least three times and in triplicates. One way ANOVA was used to perform the statistical analysis.

RESULTS

Effect of HP on microsomal lipid peroxidation

Lipid peroxidation has been implicated as a causative factor in the development of tardive dyskinesia and other motor disorder movements (15). We have investigated the role of HP on microsomal lipid peroxidation. The accumulation of lipid peroxides was monitored as TBA-reactive products. As shown in Fig.1, HP was found to enhance microsomal lipid peroxidation in a dose-dependent manner and as little as 10 µM HP caused 100% enhancement of lipid peroxide formation. It is possible that the augmentation of membrane lipid peroxidation by HP may, at least in part, be responsible for the extrapyramidal side effects observed in patients treated with this drug. Unfortunately the RHP and NOR could not be tested in this system because of only a limited amounts of the compounds available to us.

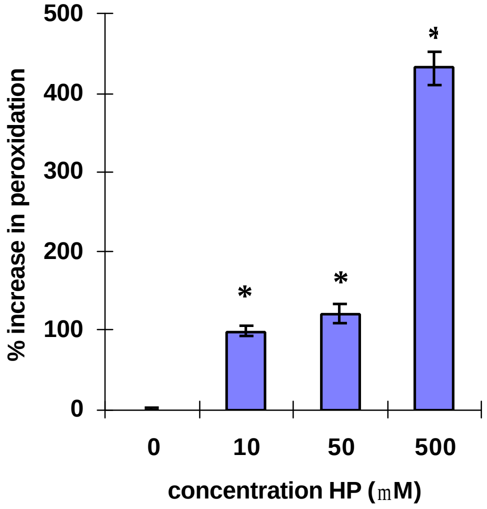


Figure 1: *Effect of HP on lipid peroxidation*: The experimental conditions are as described under “Materials and Methods”. The reaction mixture (total 1 ml) containing 3 mg of microsomal protein in 0.05 M Tris-HCl buffer, pH 7.6, 1 mM ADP, 50 µM ferric chloride, 100 µM ascorbate was incubated at 37^{o} C for 15 minutes. Lipid peroxidation was initiated by the addition of H_2O_2 and was terminated by the addition of 2 ml of 0.5% TBA, and 2% trichloroacetic acid. *A p value ≤ 0.01 was considered significant.

Effect of HP on reactive oxygen species (ROS) - Because ROS are known to be involved in lipid peroxidation processes and oxidative injury is thought to contribute to the pathogenic cascade of schizophrenia (12), we investigated the role of HP and its analogs in various ROS generating systems.

Superoxide anions are known to be produced when xanthine oxidase acts on xanthine in the presence of molecular oxygen. The superoxide anion so generated has the ability to reduce ferricytochrome *c*, and this has been taken advantage of to measure superoxide dismutase activity (25). We tested the effects of HP, RHP and the NOR in this system**.** At 0.1-2 mM concentrations these agents were found to be neither effective in scavenging superoxide anions nor did they augment the generation of superoxide radicals. These compounds do not alter xanthine oxidase activity as monitored by the accumulation of uric acid in the absence of cytochrome *c* (data not shown). We further tested the superoxide scavenging activities of HP in an epinephrine autoxidation assay. Thus, when 2.8 mM epinephrine was added to a 0.05 M bicarbonate buffer at pH 10.2 and rate of oxidation of epinephrine to form adrenochrome was monitored at 480 nm (25), a linear rate of accumulation of adrenochrome, after a short lag, was observed. HP was found to have little effect on the rate of adrenochrome formation at 0.1 and 2 mM. These results indicate that HP is neither an effective superoxide scavenger nor a generator of superoxide radicals.

Hydroxyl radicals, produced in a Fenton type reaction ($Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+}$), yield spin adducts with DMPO (26). Thus, as presented in figure 2 inset**,** a well characterized

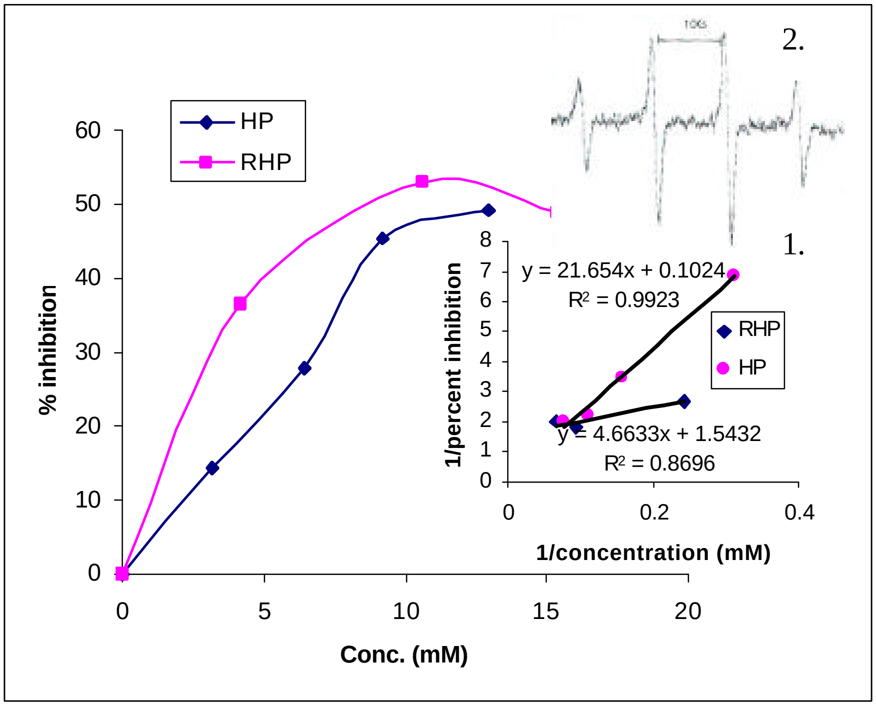


Figure 2: *Effect of HP(haloperidol) and RHP(reduced haloperidol) on the formation of DMPO-OH adducts.* The experimental conditions are as described under "Material and Methods." The DMPO-OH adduct was recorded immediately. Receiver gain was 1X 10^6, and the scan rate was 200s. Other EPR parameters were as described under "Materials and Methods." *Inset* 1 shows the double reciprocal plot of the above data. *Inset* 2 shows the characteristic DMPO-OH signals.

1:2:2:1 pattern of DMPO-OH was obtained when 32.2 µM H_2O_2, 32 µM $FeSO_4$, 3.22 mM purified DMPO, 0.01 mM tartaric acid (solvent for the drugs) in 0.2M boric acid-borax buffer, pH 7.8. The EPR signal of DMPO-OH was stable for several minutes. Addition of 1 mM thiourea inhibited the signal almost completely (data not shown). The effect of HP and reduced-HP were tested in this system. As shown in the Fig. 2, HP and RHP inhibited the DMPO-OH adduct formation in a dose-dependent manner.

The percent inhibition was calculated from signal heights of second peak and presented as percent of control. The molar concentration of HP and reduced HP required to cause 50% inhibition of the rate of DMPO-OH adduct formation was found to be 12.9 and 10.58 mM, respectively. When these data were presented on reciprocal coordinates (Fig. 2, *inset*) inhibition in all cases appeared to be kinetically linear.

If the inhibition as shown in Fig.2 is truly a reflection of interaction of these agents with $^{\cdot}OH$, then identical results should be obtained with a different assay. That this was the case is illustrated in Fig. 3 where a different assay, a deoxyribose colorimetric assay (21), was adopted. In this assay a mixture of $FeCl_3$-EDTA, H_2O_2, and ascorbic acid at pH 7.4, generates $^{\cdot}OH$ radicals that can be detected by their ability to degrade the sugar deoxyribose into fragments generating a pink chromogen upon heating with TBA at low pH.

$$Fe^{3+}\text{-EDTA} + \text{Ascorbate} \rightarrow Fe^{2+}\text{-EDTA} + \text{oxidized ascorbate} \qquad \text{(Rxn. 1)}$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + {}^{\cdot}OH + OH^- \qquad \text{(Rxn. 2)}$$

$${}^{\cdot}OH + \text{deoxyribose} \rightarrow \text{degraded sugar} \xrightarrow{\text{TBA}} \text{color product} \qquad \text{(Rxn. 3)}$$

The $^{\cdot}OH$ so generated (reaction 2) is equally accessible to deoxyribose (the detector molecule) and to any other scavenger of $^{\cdot}OH$ added. Thus, the ability of a substance to inhibit competitively with deoxyribose under these conditions is a measure of its ability to scavenge $^{\cdot}OH$ and can be used to calculate the rate constant for reaction of $^{\cdot}OH$ (21). HP, RHP, and the NOR, were able to compete with deoxyribose effectively in preventing the TBA-reactive color product formation in a dose-dependent manner (Fig. 3A). The second order rate constants for the reaction for HP, NOR and RHP with $^{\cdot}OH$ were calculated (21) and were found to be 6.78 X 10^9, 4.57 X 10^9, and 6.51 X 10^9 $M^{-1}s^{-1}$, respectively (Fig. 3B). Control experiments showed that none of the drugs interfered with the assay system. Thus, when 1 mM each of the drug was added to the reaction

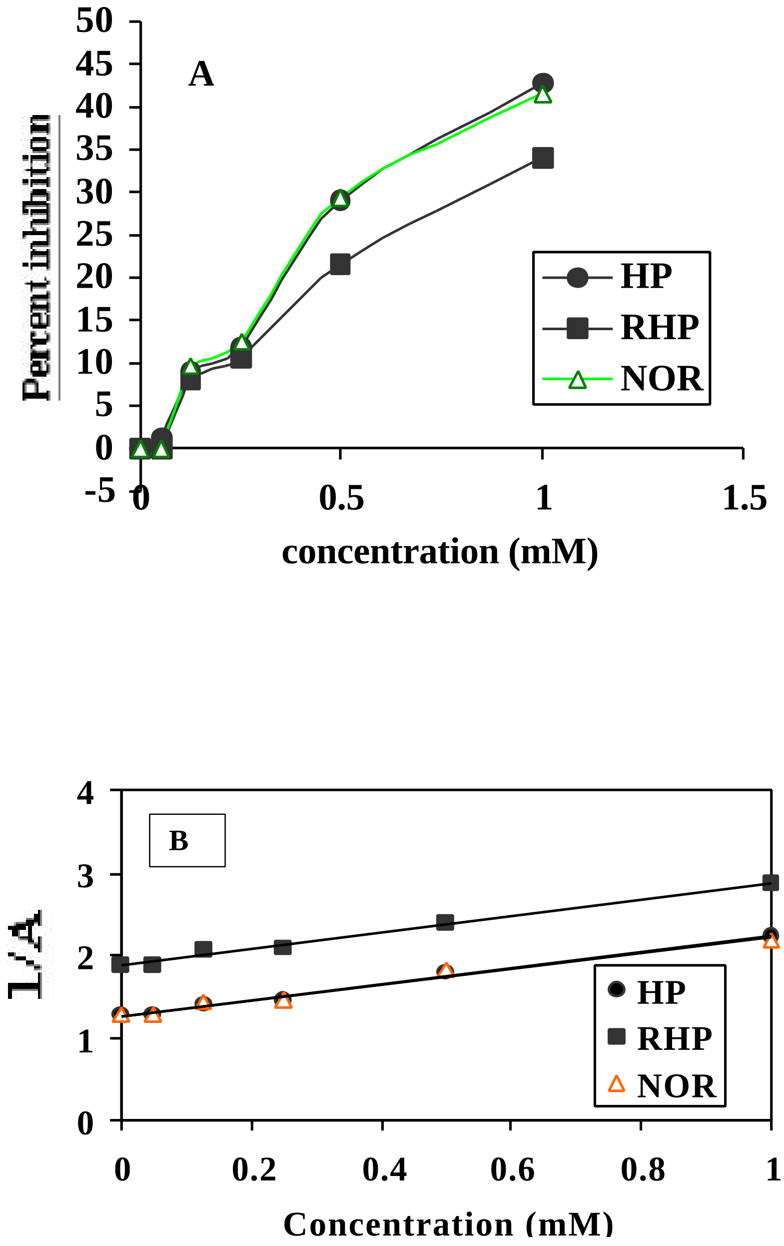


Figure 3: *Hydroxyl radical scavenging by HP and its analogs: determination of rate constants*. Deoxyribose degradation in the presence of various concentrations of HP and its analogs was followed as described under "Materials and Methods" using a final deoxyribose concentration of 2.8 mM in the reaction mixture. A: Inhibition of deoxyribose degradation by HP, reduced HP, and nor compound; B: Determination of rate constant. The rate constant was determined from the slope of the line (k = slope X $k_{[DR]}$ X [DR] XA) as described in the text yielding the value of HP, 6.78 X 10^9; NOR, 4.57 X 10^9; RHP, 6.51 X $10^9 M^{-1}s^{-1}$.

Further, when these drugs were allowed to react with the $^{\bullet}$OH generating system in the absence of deoxyribose, no TBA-reactive products were observed at 535 nm. In this system, HP, NOR and RHP were found to scavenge hydroxyl radicals at rate constants of 6.78 X 10^9, 4.57 X 10^9, and 6.51 X 10^9 $M^{-1}s^{-1}$, respectively. This implies that HP and its analogs are highly efficient hydroxyl radical scavengers.

Because singlet oxygen is thought to be involved in the lipid peroxidation process, we investigated the role of HP in a known singlet oxygen generating system. The effect of HP on singlet oxygen was monitored by EPR spectroscopic technique using TEMP as singlet trap (23). The formation of TEMPO, as a nitroxyl radical, by the attack of singlet oxygen on TEMP resulted in a characteristic EPR spectral pattern of three equal intensity lines with a splitting constant of A_N = 17.2 G and g value of 2.0056, respectively (Fig 4 *inset*). The effect of HP on

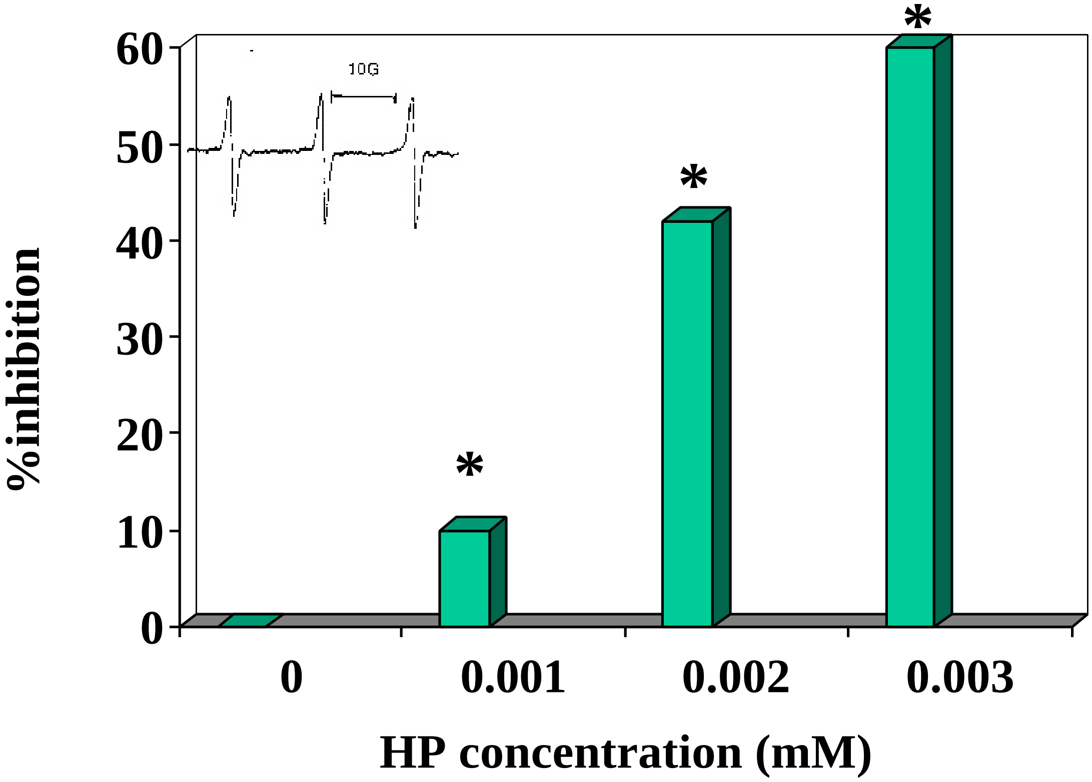


Figure 4: *Effect of HP on the formation of TEMPO adducts.* The experimental conditions were as described under "Material and Methods." *Significant at $p \leq 0.001$. *Inset:* Representative EPR signals of TEMPO.

singlet oxygen was studied in this system. The percent inhibition was calculated from the signal intensity of first peak of the EPR signal as compared with the control. As shown in Fig. 4, HP was found to be a potent scavenger of the singlet oxygen species. At a concentration of 3 µM HP inhibited over 60% of TEMPO signal. Because of limited availability of RHP and NOR, these compounds were not studied in this system.

Although HP and its analogs were found to scavenge ${}^{\cdot}OH$ in both the EPR and the deoxyribose assay, both these assays used H_2O_2 and Fe^{2+} in a Fenton type system to generate the radical. Therefore, there is a reason to believe that these drugs could directly interact with H_2O_2 and/or Fe^{2+} and could lower their concentration in the system, thus reduce the rate of generation of ${}^{\cdot}OH$ and appear to inhibit the ${}^{\cdot}OH$ dependent reaction. In order to lessen the likelihood of this subtle artifact, we have investigated the effects of HP and its analogs in a iron-free system as well as

their direct effects on both H_2O_2 and Fe^{2+}. Thus, ${}^{\cdot}OH$ were generated by X-irradiation of water (passed through Chelex-100 column) and allowed to react with deoxyribose before reacting with TBA.

$$H_2O \xrightarrow{h\nu} {}^{\cdot}OH + e^-_{aq} + H^+$$

The accumulation of TBA reactive products was found to be linear over a range of 0-40 minutes (data not shown). Based on that we X-irradiated the samples for 30 minutes for all subsequent experiments. A dose response curve (X-ray dose vs. TBA reacting product) that was linear upto 200Gy X-ray was obtained (data not shown). In this system, at 150 Gy, the malondialdehyde (MDA) (TBA reactive products) in the absence of any drugs was found to be 15.2 nmole/Gy. Addition of 1mM of HP, RHP and NOR was found to inhibit the formation of TBA-reactive products at 33.0, 33.0 and 30.0 %, respectively (Table 1). These data indicate that the scavenging of hydroxyl radicals in the deoxyribose assay was not because of the drugs chelating iron or decomposing H_2O_2 and

Table 1: Percent Inhibition of deoxyribose degradation as determined by X-ray studies. The experimental conditions were as described under "Materials & Methods."

Drug (1mM)	MDA nmole/Gy	% Inhibition
Control	15.2×10^6	0
HP	10.2×10^6	32.7
RHP	10.2×10^6	32.7
NOR	10.7×10^6	29.2

more likely due to a direct effect of the drugs on hydroxyl radicals. However, it does not rule out completely the interaction of H_2O_2 in this system, because ${}^{\cdot}OH$ can dismute to form H_2O_2 and both the e^-_{aq} and $H^{\cdot}$ can react with molecular O_2 to generate O_2^- and eventually produce H_2O_2 as follows:

$$
\begin{gathered}
{}^{\cdot}OH + {}^{\cdot}OH \rightarrow H_2O_2 \\
O_2 + e^-_{aq} \rightarrow O_2^- \\
O_2 + H^{\cdot} \rightarrow H^+ + O_2^- \\
O_2^- + O_2^- \rightarrow H_2O_2
\end{gathered}
$$

Therefore the following studies were conducted to rule out this possibility. On the basis of the absorption curves of peroxide solution (27), the activity of catalase can be determined by direct measurements of the decrease in absorbance at 240nm caused by the decomposition of H_2O_2 by the enzyme. By replacing catalase with HP, RHP or NOR, no such change in decomposition of H_2O_2 was observed when 2 mM H_2O_2 was allowed to react on 0.5 or 1 mM of these compounds in 0.01 M phosphate buffer, pH 7.0 for 10 minutes at 25^{o}C (data not shown).

To further confirm and rule out the possibility of HP chelating iron, direct iron chelation studies were performed using calcein as a fluorescence probe as described (24). The effects of HP and its analogs were studied in this assay. As shown in Fig 5, addition of Fe^{2+} decreased the calcein florescence with time up to 3 minutes and then slowly came to a plateau (line 1). In a similar experiment, addition of DTPA, an iron chelator, at the end of this reaction negated the effects of iron and caused an increase in florescence as shown in line 3. If HP reacts with Fe^{2+}, in a manner which leads to the formation of Fe^{2+} -HP complex, then adding of HP to calcein + Fe^{2+} reaction mixture should result in an enhancement of florescence, similar to DTPA (line 3). The results shown in Fig. 5 demonstrate that this was not the case. Thus as shown in line 2, up to 1mM HP and its analogs (data not shown) did not enhance the florescence. Further, when 2 µM ferric

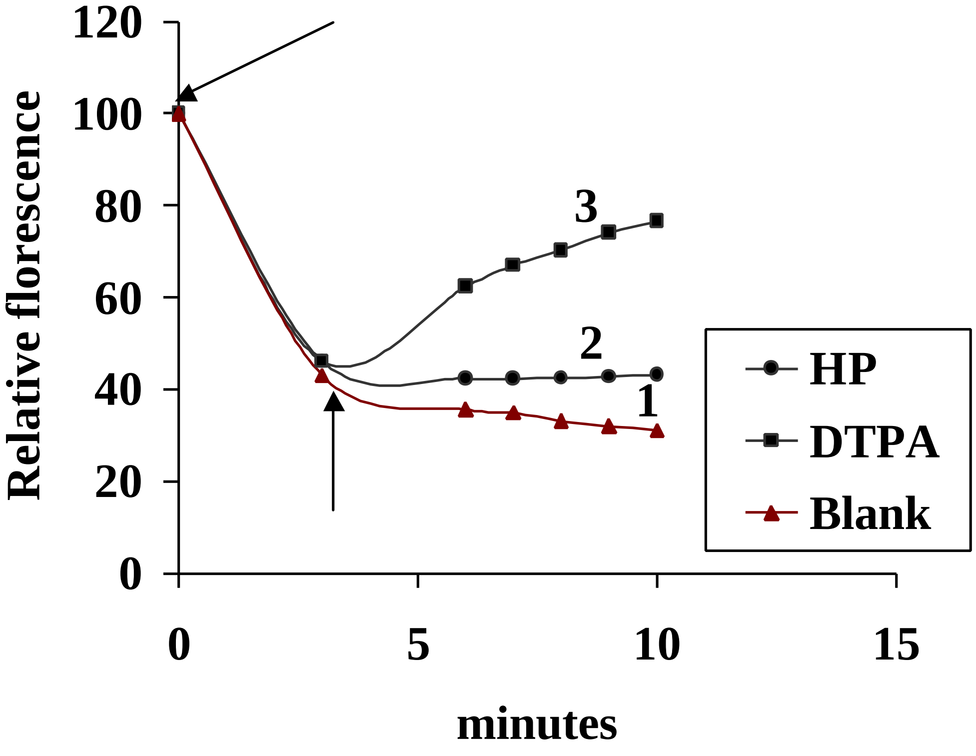
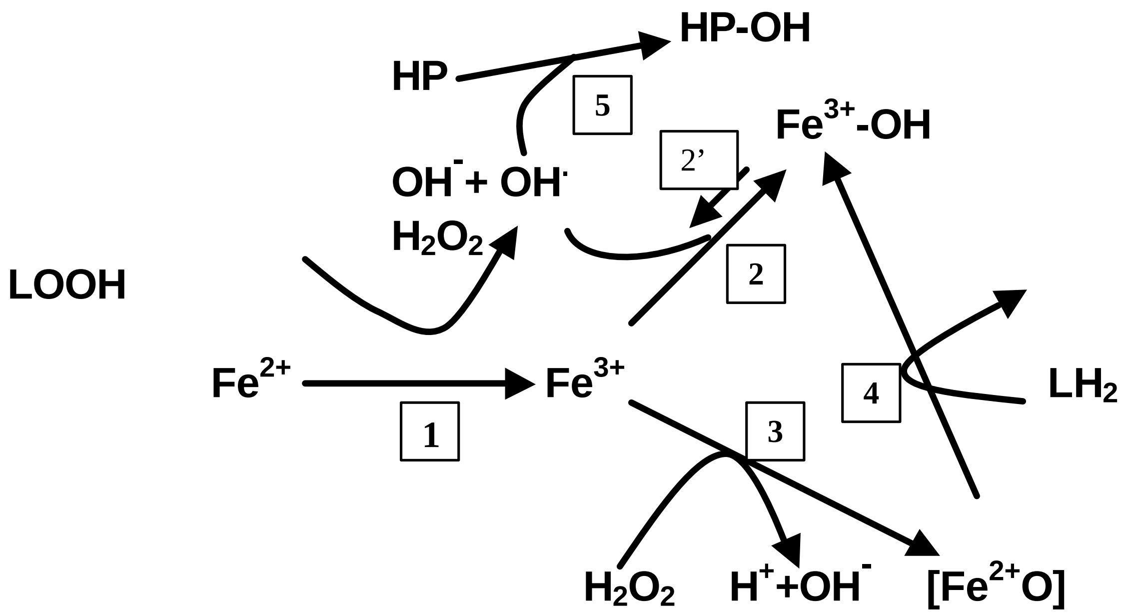


Figure 5: *Effects of HP and its analogs on calcein florescence:* The reaction mixture contained: 50µM calcein, 2mM ferrous ammonium sulfate in 0.25M tris-HCl, pH 7.6. At zero time (1st arrow), Fe^{2+} was added and at 3 minutes (2nd arrow) either DTPA or the drugs were added.

chloride was added in place of ferrous ammonium sulfate a 50% slower rate of fluorescence decay was observed and 1 mM HP and its analogs had no detectable effect (data not shown). These data indicate that HP and its analogs do not interact with Fe^{3+} or Fe^{2+} under these experimental conditions.

DISCUSSION

In the present study, we have demonstrated that HP enhances microsomal lipid peroxidation (Fig. 1) and scavenges some of the ROS such as hydroxyl radical and singlet oxygen (Figs. 2-4). The hydroxyl radical scavenging ability of HP was found to be similar to some of its analogs such as RHP and NOR. The hydroxyl radical scavenging activities of these compounds were demonstrated both in the EPR spectroscopy in combination with spin trapping techniques as well as in the deoxyribose degradation assays (Figs 2-4). Thus when the ·OH radicals were generated in a Fenton-type reaction and detected as DMPO-OH adduct by EPR spectroscopic techniques, HP and RHP inhibited DMPO-OH signal in a dose-dependent manner. The hydroxyl radical scavenging effects of these compounds were also shown by their ability to protect. OH-dependent deoxyribose degradation (Figs 3). HP was also shown to scavenge singlet oxygen as evident by its ability to inhibit the TEMPO adduct signal in the EPR studies (Fig 4). These data indicate that HP is both a pro-oxidant augmenting membrane lipid peroxidation and an effective antioxidant that scavenges both hydroxyl radicals and singlet oxygen. Thus, the beneficial effects of HP in patients, at least in part, are possibly due to the ROS scavenging properties, and the secondary effects could be due to its pro-oxidant properties. These observations can be discussed and explained most conveniently by proposing a sequence of reactions. As shown in Scheme 2, lipid peroxidation is associated with Fenton system in the consequence of the interaction of membrane lipids with ferryl-ion [FeO^{2+}], which is secondary to the formation of ·OH. The step 1 presented in the scheme is similar to Fenton's reagent (2). The existence of such a mechanism would not be unlikely because an aqueous solution of Fe^{2+} and O_2 is capable of promoting a variety of other oxidative reactions such as hydroxylation of aromatic compounds (29-31), modification of proteins (32), and generation of bio- and chemiluminescence (33, 34). [FeO^{2+}] was also seen to be capable of hydrogen abstraction from many organic compounds (35, 36). The augmentation of lipid peroxide by HP is explained in Scheme 2.



Scheme 2: proposed mechanism for Lipid Peroxidation

As shown in the reaction scheme (step 1), hydroxyl radicals would be a product of this reaction without interacting directly with lipids (LH_2) to form lipid hydroperoxide (LOOH). The ferric iron could react with H_2O_2 to form ferryl-ion $[FeO^{2+}]$, as shown in step 3, which could form LOOH upon reacting with LH_2 (step 4). In the presence of HP, the Fe^{3+}-OH formed in reaction 4 would be used up, as in steps 2’ and 5 to form HP-OH, thus would accelerate the production of LOOH in step 4. In the absence of HP, the step 2 will predominate generating Fe^{3+}-OH thus, would slow down the step 4 to generate LOOH. The augmentation of lipid peroxide by HP, is therefore the expected result.

Of course, it is possible that one or more of the intermediates formed during hydroxylation of HP could be highly reactive that could augment lipid peroxidation. If these intermediates could directly interact with lipids to cause lipid peroxidation, the $^{\cdot}OH$ scavenging action of HP would influence the rate of oxidation of lipids. It does however establish that HP could be a potent antioxidant, but could cause membrane lipid peroxidation via its reactive intermediate or via allowing to form iron-oxygen complex, such as ferryl-ion (37). The ferryl-ion has also received considerable attention as a reactive species capable of replacing $^{\cdot}OH$ in oxidative damage (38). The results of Jeding et al. (39) could also be explained in this mechanism where HP was shown to accelerate the arachidonic acid peroxidation by heme proteins. Evidence in support of this hypothesis could also include elevated levels of lipid peroxidation on HP treated rats (40) and in psychotic patients (41, 42).

Although numerous investigations have shown that Fenton reagents hydroxylate aromatic substrates (43, 44), the interpretations have assumed free $^{\cdot}OH$ to be the reactive intermediate. Hage et al. (43) has shown that several iron complexes in combination with hydrogen peroxide catalytically hydroxylate aromatic substrates. The base-induced nucleophilic addition of H_2O_2 to the electrophilic iron center yields the reactive intermediate of Fenton reagents (Fe-OOH complex) that reacts with aromatic rings to give their hydroxylated derivatives. When this is coupled with the recognition that the hydroxylation of aromatic molecules is a fundamental process in biology (e.g. phenylalanine hydroxylase, tyrosine hydroxylase and type I drug metabolism process by cytochrome P450 system), there is a clear need for a better understanding of Fe^{2+}/H_2O_2 chemistry in hydroxylation of HP. Because both the RHP, a metabolite produced *in vivo* (45) and the NOR were almost equally effective in scavenging $^{\cdot}OH$, we propose that hydroxylation of the chlorophenyl ring of HP may be associated with its antioxidant properties.

Several theories have been presented for the variety of side effects including reversible Parkinsonism and TD in some individuals following long term treatment of some of the patients with HP. Although dopamine receptor super-sensitivity has been noted for the side effects of the drug (46), the TD appears to persist in some patients following termination of drug treatment (47). It has been shown that the biotransformation of HP to HPP^+, to a phenolic pyridinium type species, occurs in humans treated with HP (47, 48). The HPP^+ so generated appears to display neurotoxic properties (49, 50) resembling those of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-derived pyridinium metabolite MPP^+. We have shown earlier (51, 52) that conversion of MPTP to MPP+ by monoamine oxidase-B (MAO-B) generates ROS**.** Boismenu, et al. (53) have demonstrated the formation of hydroxyl radical adducts of the neurotoxin MPP+ using striatal microdialysis techniques. Therefore the extrapyramidal side effects observed in some patients following long term treatment with HP may, at least in part, be associated with excessive generation of ROS, which in the presence of iron or iron-containing proteins (39) would lead to cell dysfunction and even cell death. There is evidence that HP causes oxidative toxicity in clonal hippocampal cells via the induction of intracellular peroxide accumulation followed by depletion of intracellular glutathione. The lipophilic antioxidant vitamin E and other antioxidants blocked the immediate rise in peroxides and prevented HP-induced cell death (54, 55). Hemoglobin and myoglobin can accelerate free radical damage in the presence of H_2O_2, and this could occur in vivo after brain hemorrhage (56, 57).

With respect to the involved mechanism, we propose that HP may have a dual mode of action. (1) It could block the highly reactive $^{\cdot}OH$ -dependent neurodegeneration, and (2) under the conditions of high intracellular peroxide levels, such as when accumulates in certain parts of brain (54) after neuroleptic treatment, would react with free or bound forms of iron to cause peroxidation of membranes. The later mechanism has been causally related to neuroleptic-induced increase in free radical production resulting in degeneration of susceptible neurons (58, 59) leading to secondary complications, such as TD, of neuroleptic treatment. ROS-mediated lipid peroxidation has been implicated in altered synaptic transmission, with decreased transport of dopamine and γ-aminobutyric acid (GABA) in synaptosomes (60) and decreased GABA receptor-gated chloride flux in synaptic vesicles (61) in rat brain. Increased levels of TBA-reactive products have been found in the cerebrospinal fluid of neuroleptic treated patients (41, 62) and also in plasma of schizophrenic patients with (15) or without (63) TD. Support for lipid peroxidative damage in patients with movement disorders comes also from reports of amelioration of

TD after treatment with vitamin E, a lipid soluble antioxidant (15, 59, 64). It has been suggested that adjunctive use of vitamin C in schizophrenia works synergistically to reduce some psychiatric symptom (65).

Regarding the possible clinical implications of the present findings, it is of note that therapeutic plasma concentration of HP is ~0.05 µM (66, 67), which is orders of magnitude lower than the concentration used in this study. However, plasma concentrations do not necessarily reflect local cerebral concentrations. Thus high levels of HP, a lipophilic drug, at specific target sites are possible. Moreover, our certain experimental conditions where HP is supposed to compete with high concentrations of deoxyribose or with spin traps that interact with $^{\cdot}OH$ at almost diffusion-controlled rate needed high levels of HP to show any effect. Taken together, our findings suggest that high levels of HP could potentiate oxidative stress in neuronal cells resulting in irreversible membrane damage, which among other clinical symptoms may lead to tardive dyskinesia in certain vulnerable patients. The cytoprotective effects of the drug in treating schizophrenia may be attributed to its antioxidant properties.

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CHAPTER-2

***<u>TITLE</u>*: CYTOTOXIC EFFECTS OF HALOPERIDOL AND OLANZAPINE IN PC-12 PHEOCHROMOCYTOMA CELLS: DRUG-INDUCED APOPTOSIS OR NECROSIS?**

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ABBREVIATED TITLE: CYTOTOXICITY OF HALOPERIDOL AND OLANZAPINE

KEY WORDS: CYTOTOXICITY; PC-12 PHEOCHROMOCYTOMA;
HALOPERIDOL; OLANAPINE; ANNEXIN-V
Manuscript to be submitted to Cytometry is in preparation

ABSTRACT

Haloperidol (HP) and Olanzapine (Olz) are neuroleptic drugs used widely in humans for the treatment of schizophrenia and other psychotropic disorders. Some of these drugs are known to cause cell dysfunction in neuronal cells. We employed light microscopy , two flow cytometric apoptotic/viability probes (7-aminoactinomycin D (7AAD) and Annexin-V), along with light scatter analysis techniques to evaluate the mechanisms of drug-induced cell death in cultured PC-12 pheochromocytoma cells exposed to haloperidol (HP) or olanzapine (OLZ). Each of these antipsychotic drugs caused a significant increase in cell death that was readily detected by all three techniques. Further, increase in early cellular apoptosis was observed using the Annexin-V probe as well as 7AAD. The results of 7AAD assay were comparable to trypan blue viability results. 7AAD and Annexin-V also detected different levels of early apoptosis in PC-12 cells exposed to the cytotoxic agents, H_2O_2, which was used as a positive control. 7AAD and Annexin-V both showed HP and Olz-related increases in the late apoptotic/necrotic cell death window. These data suggest that cell death in PC-12 pheochromocytoma cells exposed to HP or Olz occurred both via apoptotic as well as necrotic pathways.

INTRODUCTION

Schizophrenia is a debilitating disorder of the central nervous system. Its symptoms have been divided into two classes: positive symptoms, including hallucinations, delusions and conceptual disorganization; and negative symptoms, including social withdrawal, blunted affect, and poverty of speech (Donaldson et al. 1983). As a result, this disorder reduces the ability of the schizophrenic individual to interact with society. The typical neuroleptic drugs, used to treat schizophrenia are highly effective, but are also associated with potentially severe extrapyramidal side effects (EPS). The most predominant among these symptoms are dystonia, Parkinsonian-like syndrome, and tardive dyskinesia.

HP, the most widely used typical antipsychotic drug, is effective in treating both the positive and negative symptoms of psychoses, Tourrette's syndrome and schizophrenia. Further, it is commonly used as a tranquilizer following major surgeries to treat post-operative delerium. HP have been used clinically in psychiatry, obstretics, and anesthesiology (Janssen, 1967; Kudo and Ishizaki, 1999; Ichikawa and Meltzer, 1999). Long-term use of this drug, however, can result in an irreversible motor disorder involving the orafacial muscles and the extremities, which have been a source of major concern (Andreasson, 1996). Recently, there has been development of the so called "atypical antipsychotic drugs". These drugs appear to have similar clinical efficacy as the typical antipsychotics, but with minimal or no extrapyramidal symptoms (Borison et al. 1995). Clozapine was the first such drug introduced, but its use has been restricted because of the potential for development of fatal agranulocytosis sometimes associated with it. The atypical antipsychotic drugs olanzapine (Olz), sertindole and quietapine are equally potent as clozapine but do not produce agranulocytosis (Borison et al. 1997).

Extrapyramidal side effects of HP may result from direct neurotoxic effects of this agent, resulting in cytotoxity. For instance, HP has been found to be cytotoxic to C6 glioma *in vitro* at very low concentrations (100μM; Vilner et al. 1993,1995). However limited information is available, regarding the mechanisms of haloperidol cytotoxicity and the potential cytotoxicity of atypical antipsychotic drugs. Therefore investigating the potential cytotoxic effects of HP and Olz leading to cell death in a neuronal cell line rat pheochromocytoma cell line, PC-12, could provide some useful information concerning cell death issues with these two drugs. The PC-12 cell line was chosen for these studies because these cells resemble sympathetic neurons and their precursors, possess a dopamine receptor of the D2 subtype (Greene & Tischler, 1976, 1982; Courtney et al. 1991), and are accepted as a neuronal model system to study neurotoxic effects of antipsychotic drugs.

Cell death follows two different pathways, necrosis and apoptosis. These two types of cell death differ from each other by unique biochemical, cytological and molecular events. Currently many sensitive detection techniques are employed to differentiate apoptosis from necrosis. These are based upon well-defined cellular events that occur during cell death. In the present study, four different techniques were employed to examine cytotoxicity in PC-12 cells exposed to a typical (HP) and an atypical (olanzapine;Olz) antipsychotic drug. These techniques included: 1) light-scatter analysis to detect changes in cell size associated with apoptosis or necrosis; 2) light microscopy and trypan blue staining which permitted visual quantification of viable cells and dead cells; 3) use of Annexin-V and propidium iodide to flow cyometrically quantify viable cells and to detect translocation of phospatidyl serine (PS) from the inner membrane to the outer membrane (the latter being an early marker of apoptosis (Vermes et al. 1995); and 4) 7-aminoactinomycin D (7AAD) via flow cytometry to detect end-nicks in fragmented DNA.

The focus of the study was to evaluate the potential cytotoxic effects of HP and Olz on cultured neuronal cells. Further, another object was to assess the potential application of two commonly employed apoptotic probes to the cultured PC-12 cell line. Each of these antipsychotic drugs caused a significant increase in cell death that was readily detectable by all three assays. At lower concentration (50 μM), Olz is less cytotoxic to the PC-12 cells but at high concentrations Olz is equally toxic to the cells.

MATERIALS AND METHODS

Cell Culture: PC-12 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Rockville, MD) 5% Horse Serum (Intergen, NY) and 10% Fetal Cafl Serum (Intergen, NY) in a humidified 37^oC incubator and 5% CO_2. Cell viability was assessed using the standard trypan blue exclusion assay. PC-12 cells were obtained from the ATCC, Rockville, MD. HP was purchased from Research Biochemicals Inc., Natick, MA and Olz was received as a generous gift from Eli Lilly and Co., Indianapolis, IN.

Determination of optimal PC-12 cell culture conditions: Cell proliferation assay was evaluated in the PC-12 cells using the Alamar Blue assay to determine optimal densities for cell growth (Ahmed et al. 1994). Briefly, the dye is added in an oxidize form (blue in color) and is reduced (red color) as the cells proliferate. Differences in the specific absorbance of the oxidized form (600 nm)/or specific fluorescence (530 nm and emission wavelength of 590 nm) reflect the level of proliferation. Cells were plated in a round bottom 96 well plate (Corning, NY) at varying densities. Alamar Blue dye (20 µl; Accumed International Inc., Westlake, OH) was added to plate wells and the plates were placed in the incubator at 37^{o}C and 5% CO_2. Forty-eight hours after the dye was added, the plates were removed from the incubator and evaluated as previously described (Ahmed et al. 1994; Gogal et al.1999). Briefly, The plate was read in a Cytofluor (Perspective Biosystems, Framingham, MA) with an excitation wavelength of 530 nm and emission wavelength of 590 nm.

Annexin-V cell death probe: The Annexin-V Fluos staining kit (Roche Bioscience, Palo Alto, CA) was purchased and used in accordance with the manufacturer's recommended procedure. The cells were cultured with the indicated drugs at 5 x 10^5 cells/well in a 96 well plate (Corning) for 23 hours in a 37^{o}C incubator with 5% CO_2. Following the incubation, cells were rinsed once with PBS and evaluated as per Annexin-V kit instructions. Briefly, the cells were resuspended in 100 µl kit binding buffer. To each tube, 50 µl of the Annexin V-FITC- binding buffer (0.5 µg/ml) and 50 µl of kit propidium iodide (0.125 µg/ml) were added. Each tube was gently mixed and incubated at 23^{o}C for 15 min in the dark. Cells were analyzed for uptake of fluorescent probe using a Coulter EPICS XL flow cytometer (Hileah, FL). The excitation wavelength was 488 nm with an emission at 625 nm. Three staining-intensity regions were identified. A large population of dull staining cells was evident, representing viable cells. In addition, a small population of intermediate and large population of intense stained cells were present, representing early apoptotic and late apoptotic/dead/necrotic cells, respectively. The values were reported as percentages of total cells counted (i.e., 5000 events).

7AAD cell death probe: We used 7AAD flow cytometric method to discriminate between live cells, early apoptotic and late apoptotic/dead/necrotic cells. This procedure made use of the fluorescent DNA binding agent 7AAD (Molecular Probes, Eugene, OR). We followed previously published methods (Donner et al. 1999; Gogal et al. 1999) as follows. Briefly, cells were treated with the indicated drugs at 5 x 10^5 cells/well in a 96 well plate for 23 hours in a 37°C incubator with 5% CO_2. Following the incubation, cells were rinsed once with PBS and 100 µl of 10.0 µg/ml 7-AAD in a cell stabilizing buffer (0.1% BSA, 0.1% NaN_3, 1.0% FBS in PBS) were added to wells containing 5 x 10^5 cells/well. The culture plates were incubated on ice for 20 min and the cells then evaluated as above for Annexin-V on a Coulter Epics XL flow cytometer. Apoptotic cells were identified and quantified based on the method of Schmid et al., (1994a,b). In brief, cells were gated based on size (forward scatter analysis) and granularity (side scatter analysis). The values were reported as percentages of total cells counted (i.e., 5000 events).

Trypan Blue cell viability: Trypan blue (Sigma, St. Louis, MO) exclusion was used to visually quantify numbers of dead and live cells under the microscope. This dye does not differentiate between apoptotic and necrotic cells, but permits gross determination of the number of dead cells and live cells. Early apoptotic cells are recognized as live cells (i.e., these cells do not uptake the viability dye).

Cell size evaluation: The CASY-1 cell counter (Scharfe System GmbH, Reutlingen, Germany) uses light scatter properties of cells to categorize cells based upon relative diameter and volume. Following drug exposures, cells were rinsed with PBS and then 20µl of the cells (control or treated) were added to 10 ml of sterile PBS (pH 7.2) and evaluated for changes in cell size that might be reflective of necrosis (cell swelling) or apoptosis (cell pyknosis).

Statistics: All assays were performed using triplicate well samples, and each experiment was repeated at least three times. Data were expressed as arithmetic mean ± SEM. Analysis of variance (ANOVA) was used with Dunnett's's t-test for comparison of groups, using StatView^{TM} (Berkeley,CA) to establish significant differences among groups. Results described as different in this paper indicate significantly different at $p \leq 0.05$. To be noted here is that in some cases, the standard error was extremely low, disabling the software to express in the bar graphs.

RESULTS

PC-12 cells: Optimization of proliferation with Alamar Blue: As shown in Fig.1, 125,000 cells showed increasing fluorescence for almost 50 hours. As the cell numbers increased, a dramatic lowering in fluorescence was observed within as little as 10 hours. This could possibly be due to clustering of cells in a single well. Hence in order to perform toxicity studies using prolonged incubation periods, 125,000 cells per well were used in all subsequent studies.

Viability test using Trypan Blue: Fig. 2 shows the viability of cells using the Trypan Blue exclusion test after 24 hrs. in culture. As shown in Fig.2 H_2O_2 (1mM) used as a positive control, killed almost all the cells in the system. Although the test enabled a gross determination of the percentage of live and dead cells, the percentage of apoptotic cells cannot be determined by this test. As shown in Fig.2, HP at both low (50 μM) and high dose (150 μM) significantly ($p=0.05$) reduced viabilities.

Measuring cell death with Annexin-V/PI double staining: Using the Annexin-V/PI double staining, viable, early and late apoptotic/dead cells were visualized on the flow. As shown in Fig. 3A, we observed that except the high concentration of HP (150 μM), all other groups showed a significant ($p\leq 0.05$) decrease in viability. Fig. 3B which shows the distribution of early apoptotic cells after a day in culture with the two antipsychotic drugs, there was seen to be an increase in the apoptotic cells with 50 μM HP, whereas there was a decrease in early apoptotic cells in the 150 μM HP treated group and a dramatically lower percentage of early apoptotic cells in the hydrogen peroxide treated group. In Fig. 3C, the distribution of late apoptotic or dead cells is shown. Only 150 μM HP shows a barely significant ($p\leq 0.05$) increase in the necrotic cells as compared to the control.

Measuring cell death with 7AAD: Using the 7AAD DNA binding dye, the viable, early apoptotic, and late apoptotic/necrotic cells were visualized on the flow cytometer. As can be seen in Fig. 4A, both low and high dose of HP, and hydrogen peroxide under the given experimental conditions, decreased the viability of PC-12 cells. The percent apoptotic cells increased significantly in the 50 μM HP treated group. No other treatment group showed any increase in apoptotic cell percentage (Fig. 4B). Distribution of late apoptotic or necrotic cells is shown in Fig. 4C. All treatment groups, except, 50 μM Olz showed significant increases in percentages.

Light scatter analysis of cultured PC-12 cells: Histograms from the electronic cell counter are shown in Fig. 5A-D. As is evident, the PC-12 cells showed a wide range of cell sizes (Fig. 5A), and in culture with either HP, OLZ or H_2O_2, a shift in peak towards smaller size is seen (Figs, 5B-D).

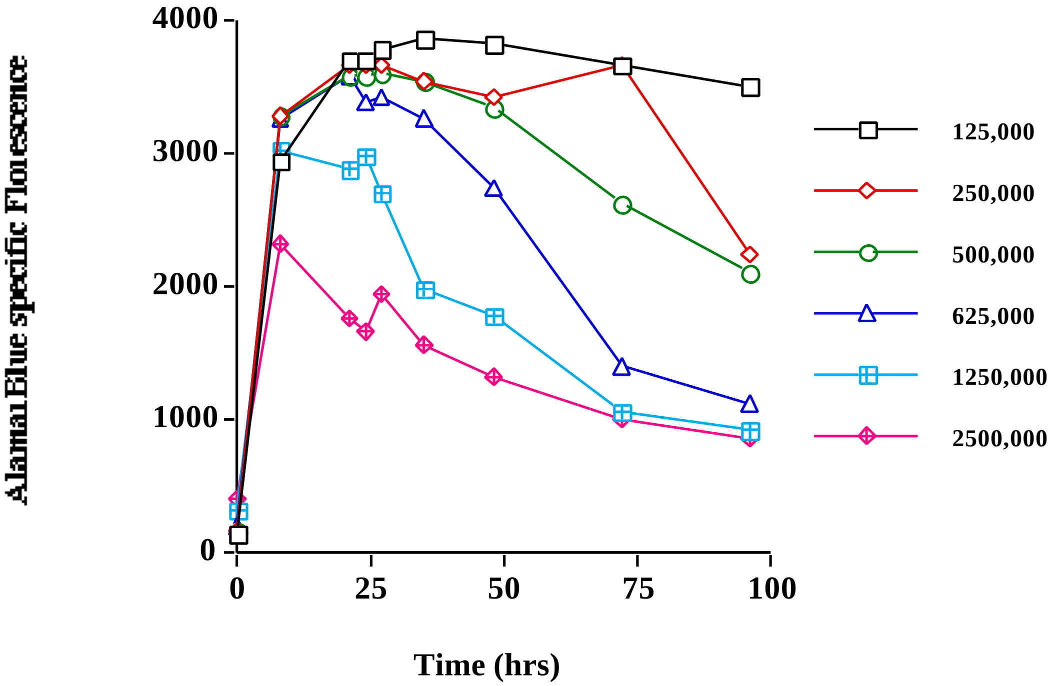

Figure1. *Determining optimal cell concentrations for studies, using Alamar Blue metabolic dye*. PC-12 cells were plated in 96 well plates at varying cell densities, with 10% alamar blue and thereafter incubated at 37^{o}C and 5% CO_2, 48 hours later, the plates were removed and monitored in the Cytofluor with a λex =530nm and λem = 590 nm.

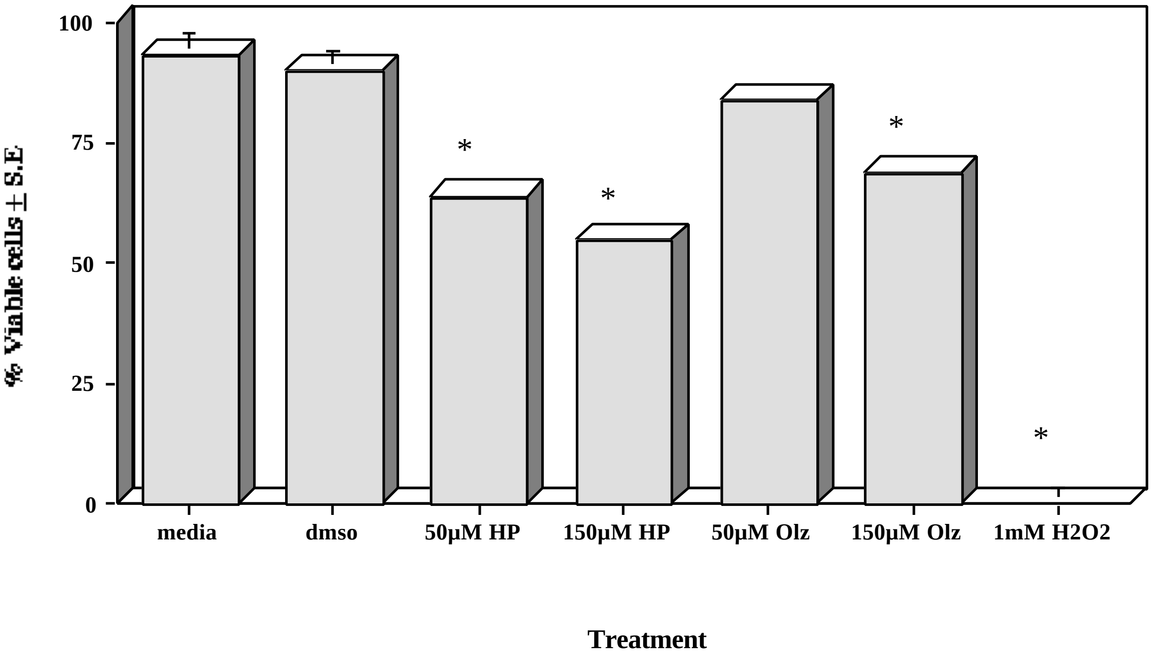

Figure 2. *Viability of PC-12 cells was determined by using trypan blue exclusion test & light microscopy as described under "Materials and Methods" (n=9, p*≤0.05).

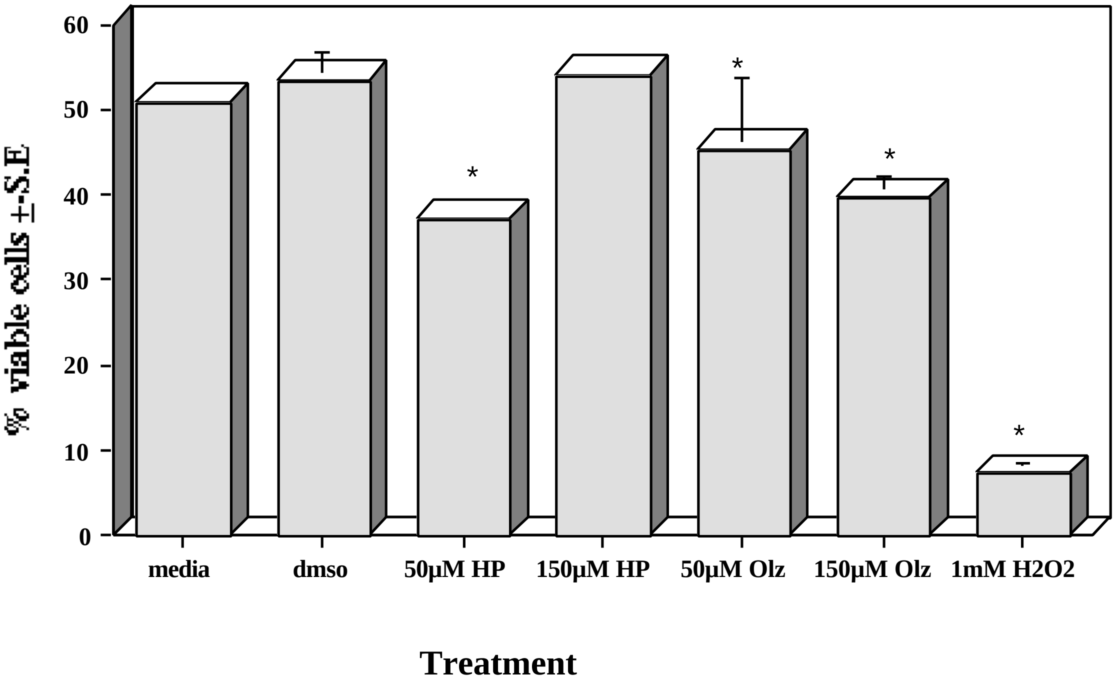

Figure 3A. *This figure represents the percentage of viable cells after the specified treatment.* PC-12 cells were plated on 96-well plates with medium and dmso/HP/Olz at concentrations indicated, and thereafter incubated at 37°C. 24 hrs later, the cells were centrifuged at 50 X g for 5 min. and rinsed with PBS, and 100µl of the Annexin/PI dye were added. The plate was incubated in dark for 30 min, and was analyzed flow cytometrically (n=9).

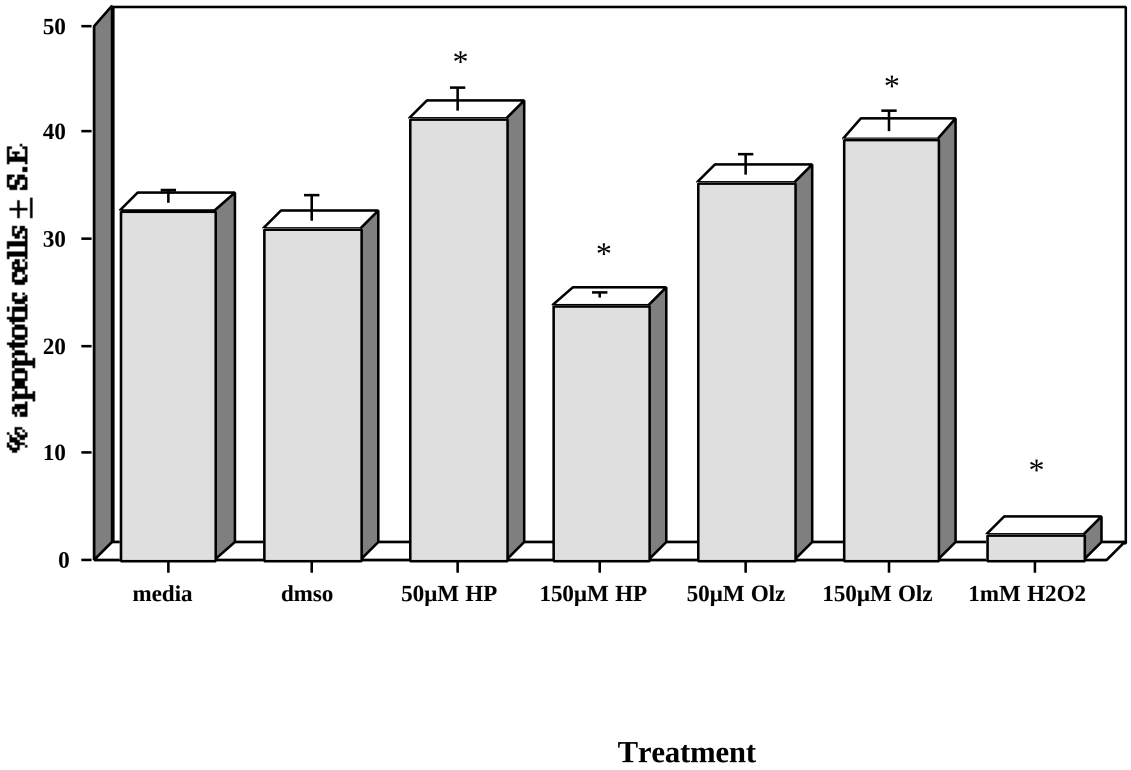

Figure 3B: *Shown above is the percentage of early apoptotic cells using Annexin-V/PI staining*. Experimental method is the same as described in Fig. 3A (n=9).

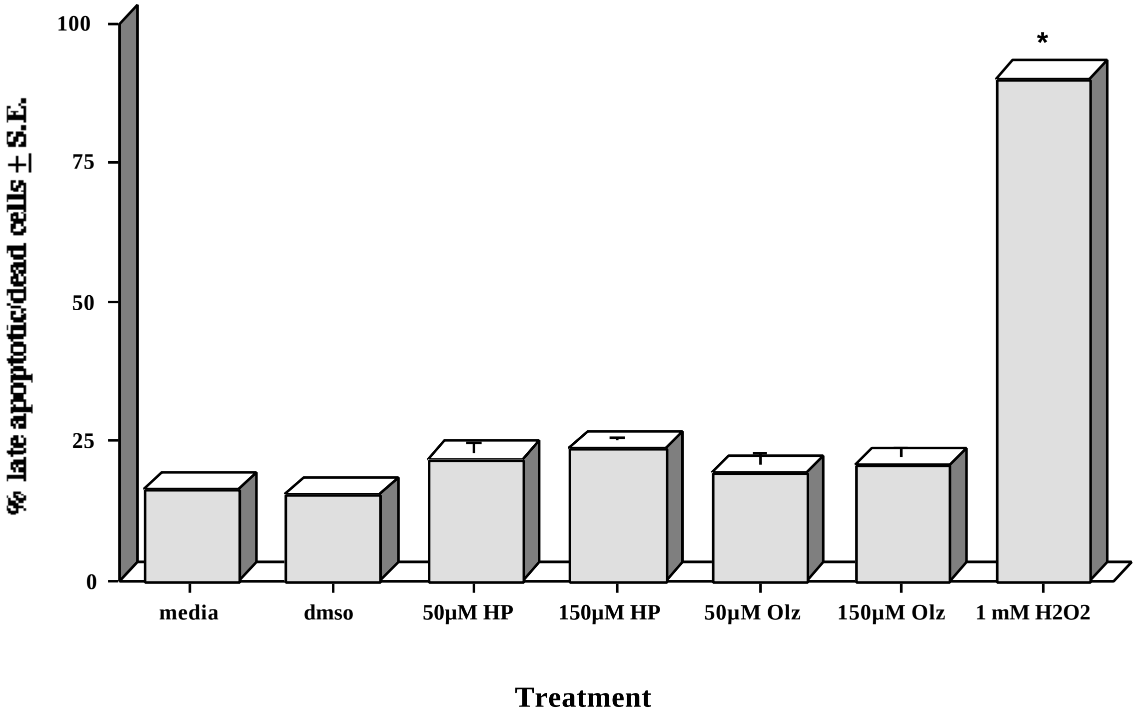

Figure 3C. *Represents the percentage of late apoptotic/dead cells using Annexin-V/PI staining*. Experimental method is the same as described in Fig. 3A (n=9).

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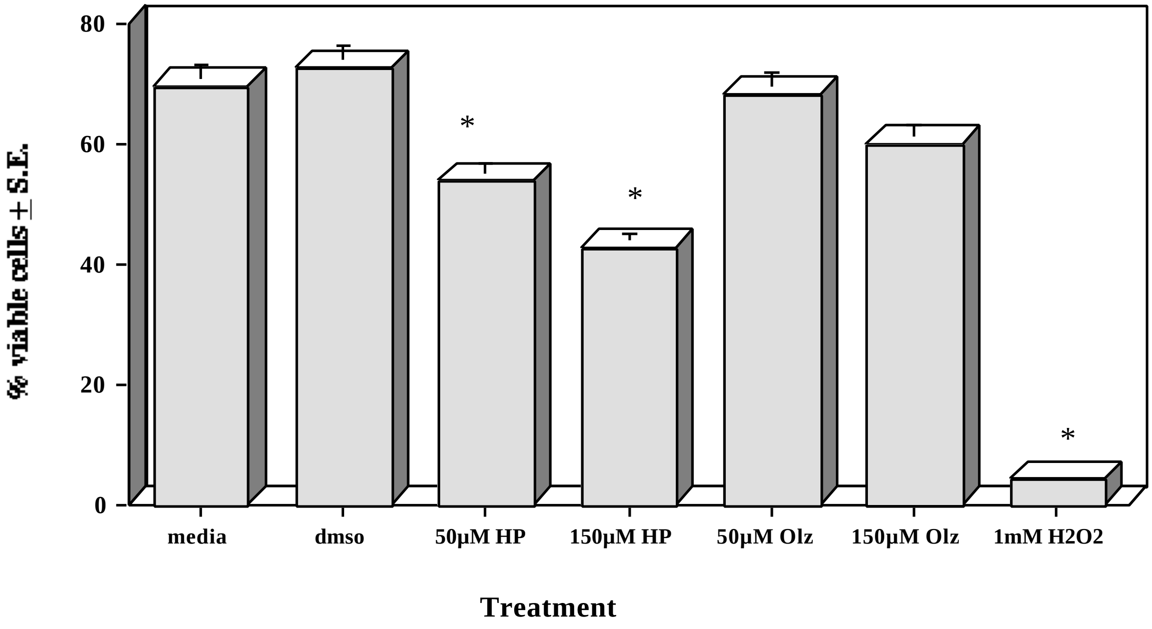


Figure 4A: *This figure represents the percentage of viable cells after the mentioned treatment*. PC-12 cells were plated on 96-well plates with medium and dmso/HP/Olz at concentrations indicated, and thereafter incubated at 37°C. 24 hrs later, the cells were centrifuged at 50 X g for 5 min. and rinsed with PBS, and 100µl of the 7AAD dye (0.5µg/well) was added. The plate was incubated in dark for 30 min, and was analyzed flow cytometrically. Data are represented as the means ± S.E (n=9).

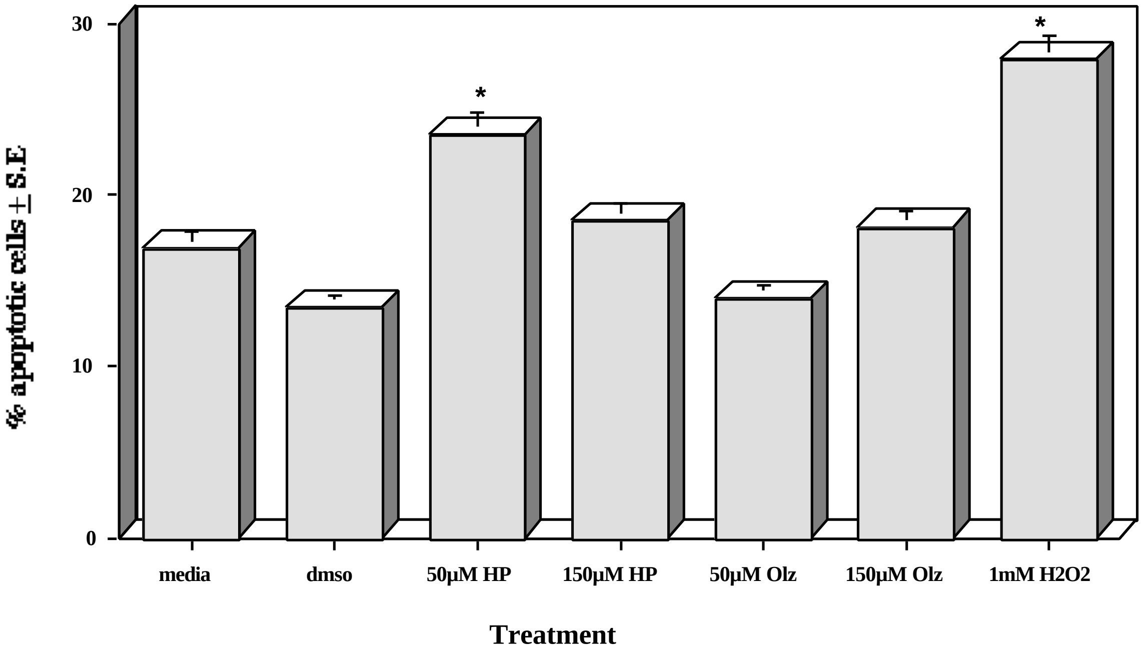


Figure 4B. *Represents the early apoptotic cells using 7AAD assay.* The experimental method is as described in Fig 4A. Data are represented as the means ± S.E (n=9).

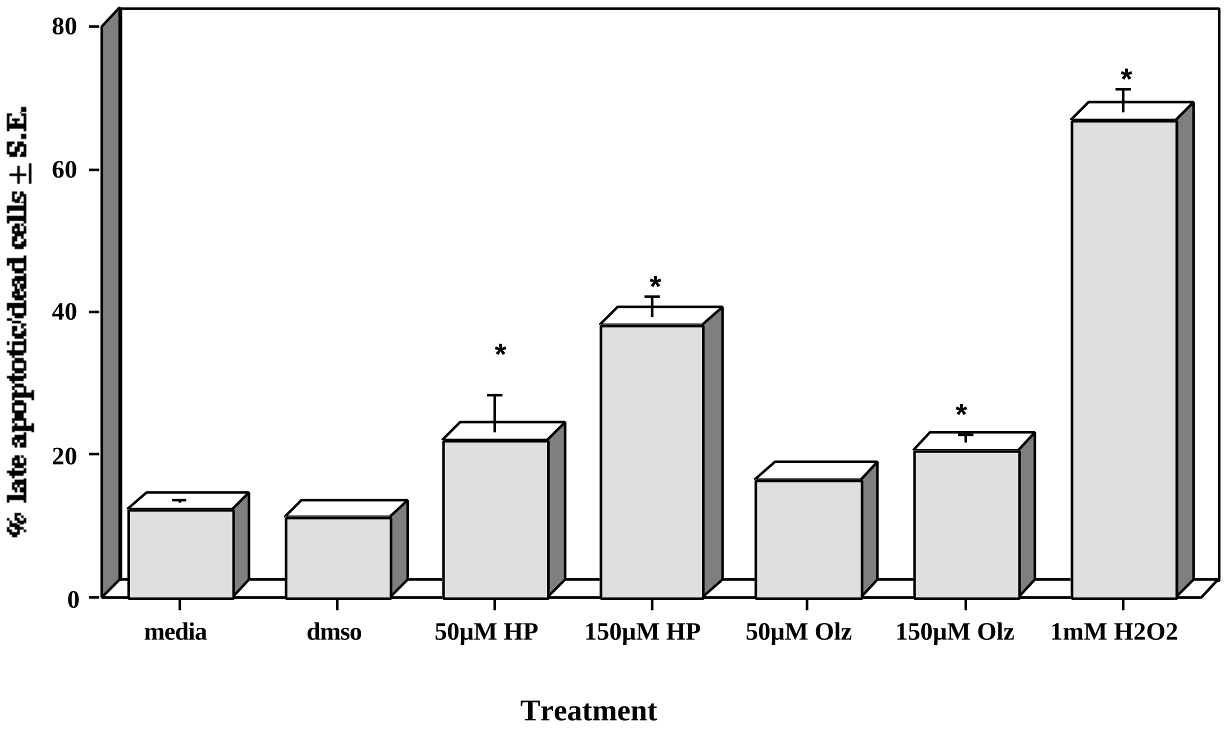
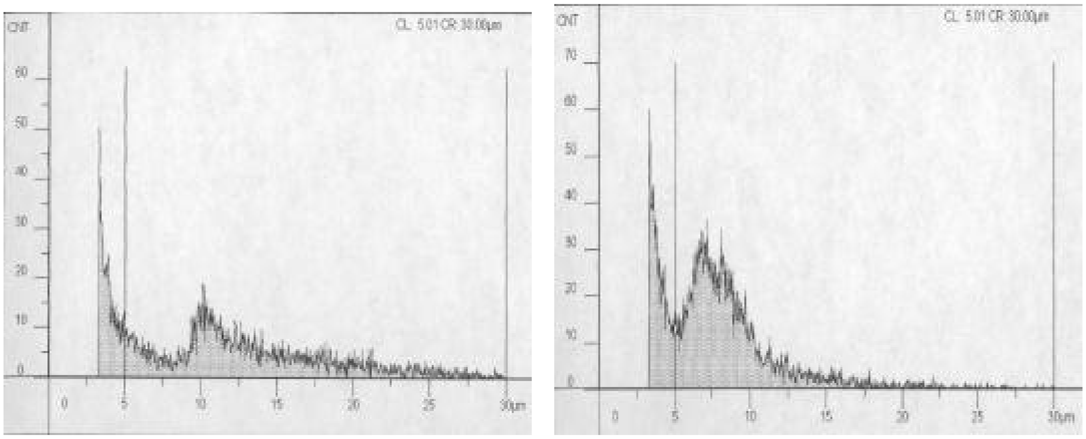


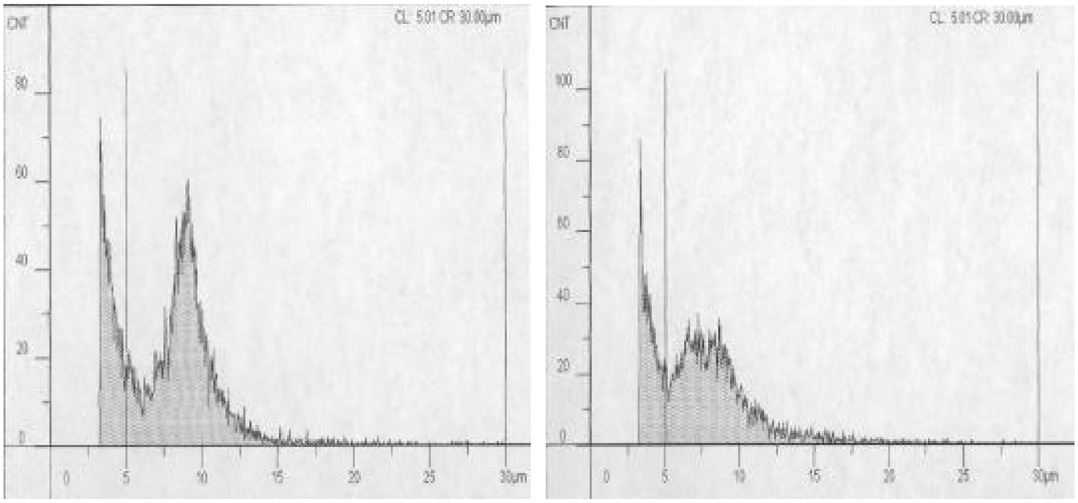
Figure. 4C. *Percentage of late apoptotic/dead cells*. The experimental method is as described in Fig 4A. Data are represented as the means ± S.E (n=9).

Figure 5. *CASY-1 analysis: Representative histograms from the CASY counter are presented*. Experimental method is as described under “Materials and Methods” section. An increase in larger cells are apparent, when the PC-12 cells are treated with H_2O_2 or high concentration of HP (150 µM), indicating that the cells may be undergoing necrosis
.



A:*Cell count with media only*

B: *Cell count with 150 mM HP*



C: *cell counts with 1 mM H_2O_2*

D: *Cell count with 50 mM HP*

DISCUSSION

In this study, we conducted a comparison of the two probes and evaluated the effect of HP and Olz in cultured PC-12 cells. 7AAD assay indicated about 30% early apoptosis with 1 mM H_2O_2 while Annexin-V assay showed < 3% (10x difference). Extensive vacuolization of the cells was observed in the low dose HP (50 μM) treatment groups. Both probes identified numerous cells that were either apoptotic, late apoptotic or necrotic. Also, apoptotic cells appear smaller while necrotic cells are initially swollen. Light scatter analysis from the CASY cell counter showed a shift towards the left which could be explained in two possible ways. Either the cell shrinkage during an apoptotic event or the cell debris, a consequence of necrosis shifted the peak towards the left. 7AAD was able to accurately quantify the viable cell populations, as compared to trypan blue. Both 7AAD and Annexin-V/PI stains detected notable increase in apoptotic cells in the HP and Olz treated groups. Unfortuantely due to technical difficulties in fixing the cells during cytospins, these results could not be co-related with light microscopic results (App.3).

Collectively, our results suggest that HP injures cells by apoptosis at lower doses and at higher doses, cell death is occuring via both necrosis and apoptosis. The necrosis observed with HP appears to support the previous reports (Behl et al. 1995, 1996). Olz at 50 µM produced less cytotoxicity to PC-12 cells than did the same concentration of HP. Both drugs at 150 µM concentrations, showed cytotoxicity in PC-12 cells. This is the first time, apoptosis has been shown to be involved in HP-related cell death.

Cytotoxicity has been observed in neuronal cells following in vivo and in vitro administration of HP in rodents and humans (Petzer et al. 2000; Eyles et al. 1996; Usuki et al. 1996; Castagnoli et al. 1999; Subramanyam et al. 1991; Van der Schyf et al. 1994; Van der Schyf et al. 1996; Avent et al. 1997; Lockhart et al. 1995; DeCoster et al. 1995). Also, Vilner et al (1995) have shown the cytotoxic effect of HP in cultured C6 glioma cells. It caused loss of processes, assumption of spherical shape, and cessation of cell division. Lezoualc'h et al. (1996) and Sagara (1998) have further shown the involvement of reactive oxygen species in the toxicity of HP. In *in vivo* studies, investigators were unsuccessful in finding MPTP (1-methyl-4-phenyl 1,2,3,4 tetrahydropyridine) like lesions in baboons treated with HP. Instead, there is evidence of lesions in the nucleus basalis of Mynert (Castagnoli et al.1999). Increased incidence of excitotoxic lesions related to HP (one year treatment) have also been reported. Specific mechanisms leading to HP-induced cell death, however, have remained poorly defined. An important first step in understanding such mechanisms is the determination of the type of cell death caused-necrotic or apoptotic or both.

The detection and quantitation of apoptotic cells is becoming increasingly important in the investigation of the role of apoptosis in cellular proliferation and differentiation and various disease processes. 7AAD and Annexin-V have been used to detect apoptosis in lymphocytes and numerous tumor cell lines (Philpott et al, 1996; Donner et al. 1999; Toba et al. 1996; Gogal et al. 2000; King et al. 2000).

Annexin-V/PI staining procedure uses double staining with Annexin-V and propidium iodide to differentiate between apoptotic and necrotic cells. Annexin-V is a Ca^{2+} dependent phospholipid binding protein with high affinity for phospholipid. Annexin-V can therefore be used as a sensitive probe to detect phospholipid exposure on the outer membrane of the cells, as it occurs early on in apoptosis. Recently, there has been a report that PS exposure may not always be an indicator of early apoptosis, as phosphatidyl serine translocation can either precede or follow DNA cleavage (King et al. 2000). The phospholipid exposure upon the outer leaflet of the cell membrane can also occur during necrosis. It is therefore important to differentiate between apoptotic and necrotic events. In this assay, simultaneous double staining with a vital dye propidium iodide enables to differentiate between the necrotic and the apoptotic cells positively.

7AAD is a fluorescent dye and binds to nicked DNA. In a flow cytometer, the apoptotic cells are detected using fluorescence and the forward light scatter. This is a quick and simple method and is inexpensive. Annexin-V FITC tagged kits utilizes the phenomenon of the translocation of phosphatidyl serine during early apoptosis. In lymphoid cells, both probes have been shown to be equally effective and compare well (Donner et al. 1999; Gogal et al. 2000).

In summary, both HP and Olz, when cultured with PC-12 cells resulted in increased cell death. Both the drugs showed a dose dependent toxicity. At lower concentrations, Olz was less cytotoxic as compared to HP, but at higher concentrations, both the drugs were cytotoxic at varying degrees. Employing the apoptotic probes 7AAD and Annexin-V and light microscopy, the cell death appeared to be both apoptotic as well as necrotic. Furthermore, the results also indicate that a battery of techniques be used simultaneously to get an accurate evaluation of the type of cell death mechanisms.

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CONCLUSION

We investigated the pro-oxidant and antioxidant properties of HP *in vitro*. HP was found to enhance membrane lipid peroxidation and scavenges the reactive oxygen species. Olanzapine also scavenges the hydroxyl radicals but does not significantly enhance membrane lipid peroxidation. HP was found to be ineffective as a superoxide radical scavenger but appeared to be a potent scavenger of hydroxyl radicals with a rate constant of ~6.78 X 10^9 $M^{-1}s^{-1}$. The rate constant of the reaction of Olz with hydroxyl radical was found to be much higher (31.4 X 10^9) than HP. Electron paramagnetic resonance (EPR) spectroscopy in combination with spin trapping techniques, using a Fenton type reaction and 5,5-Dimethyl-1-pyrroline-n-oxide (DMPO) as a spin trap, HP was found to cause a dose-dependent inhibition of DMPO-OH adduct formation. HP was found to quench singlet oxygen in a dose-dependent manner. Singlet oxygen was generated using photochemical reaction with rose bengal as photo-sensitizer and 2,2,6,6-teramethylpiperidine (TEMP) as trapping agent. The amount of HP required to inhibit 50% of singlet oxygen-dependent TEMPO production was found to be 2.5 µM. HP was found to enhance the microsomal lipid peroxidation in a dose-dependent manner. At 10 µM concentration, HP was found to augment the lipid peroxide accumulation by 100%. On the other hand Olz, upto 500 µM concentrations had trivial effects. Light microscopy and two cytometric apoptotic/viability probes (7-aminoactinomycin D and Annexin-V) were employed to evaluate mechanisms of drug-induced cell death in PC-12 pheochromocytoma cells exposed to HP or Olz. Each of these antipsychotic drugs caused a significant increase in cell death that was readily detectable by all three assays. At lower concentration (50 µM), Olz is less cytotoxic to the PC-12 cells but at high concentrations Olz is equally toxic to the cells.

Light microscopy with trypan blue indicated numerous dead cells with both the drugs, but could not discriminate between apoptotic versus necrotic cells. Viability with 7AAD was comparable to that of trypan blue exclusion studies. Both, 7AAD and Annexin-V both showed drug-related increases in the apoptotic as well as necrotic cell death window. Increase in early apoptotic cells was observed using the Annexin-V probe as well as 7AAD. These data suggest that cell death in PC-12 pheochromocytoma cells exposed to HP or OLZ may be both apoptotic as well as necrotic in nature.

Thus, in this study we found that

1. HP is both an antioxidant and a pro-oxidant
2. Olz is a better antioxidant and appears to have little pro-oxidant effects
3. The cytotoxicity sudies indicate that at lower dose (50 μM), HP appears to be more cytotoxic than Olz, whereas at high doses (150 μM), both HP and Olz seem to be equally toxic. The differences could be supported by the observation that at low concentrations, Olz did not significantly enhance membrane lipid peroxidation as compared to HP, and also that the rate constant of reaction with hydroxyl radical observed for Olz was higher than HP .
4. 7AAD was more accurate in quantifying the viability as well as apoptotic results, in agreement with the light microscopic studies. Both Annexin-V/PI and 7AAD indicated a combination of apoptotic as well as necrotic process in the dying PC-12 cells. The electronic cell counter CASY-1 was in agreement with the flow cytometric evaluations as it showed a shift towards the left in cells cultured with drugs or H_2O_2. Taken together, our findings suggest the high levels of HP could potentiate oxidative stress in neuronal cells which could lead to secondary effects (such as tardive dyskinesia) observed as a consequence of drug treatment. The therapeutic effects of these drugs in treating schizophrenia may be attributed to its antioxidant properties Furthermore, the atypical drug Olz is better than HP because it seems to have little pro-oxidant properties. The secondary effects manifested in patients with HP use may be minimized with the use of Olz.

FUTURE DIRECTION

1. Cytoprotective effects of the drugs will be investigated, in particular

a) protection against hydrogen peroxide mediated necrosis and

b) protection against DEX induced apoptosis.

2. It is possible that haloperidol and olanzapine might differ in their signal transduction pathway, and that could be the one of the reasons behind the differences in their observed adverse effects. For instance, haloperidol has been shown to activate the NF kappa B system. Evaluation of the effect of Olz on the NF kappa B system would throw some light on the mechanism. Also, actiavtion of MAP kinases would be studied with both these agents.

3. Acid of p-florophenyl compound and the nor-compound are the result of the the breakdown of haloperidol. We have already shown that the nor compound is a good inhibitor of the hydroxyl radical dependent deoxyribose degradation. We are yet to find out if the acid of the florophenyl compound does the same. It would give us an indication as to which end of the haloperidol is essential for its therapeutic action.

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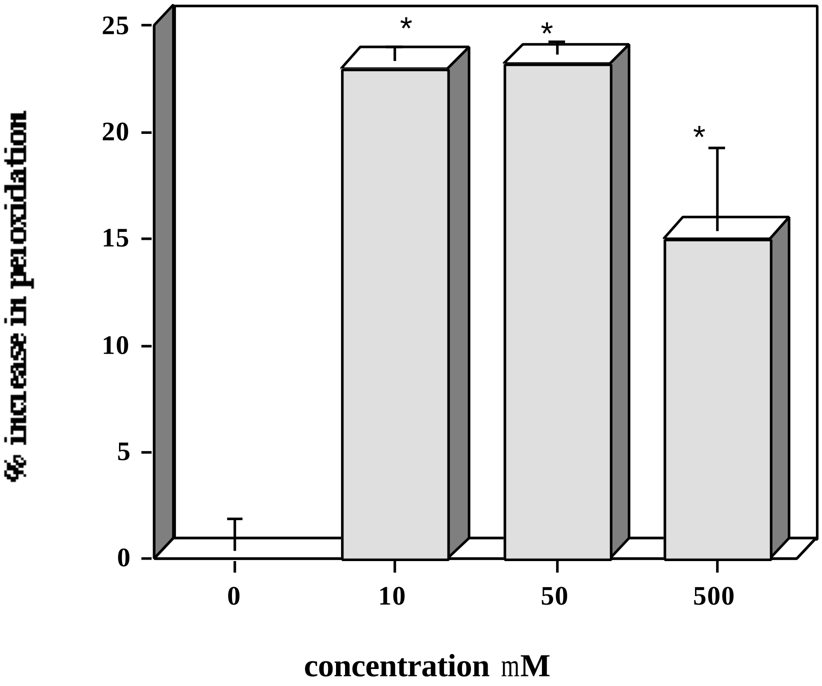
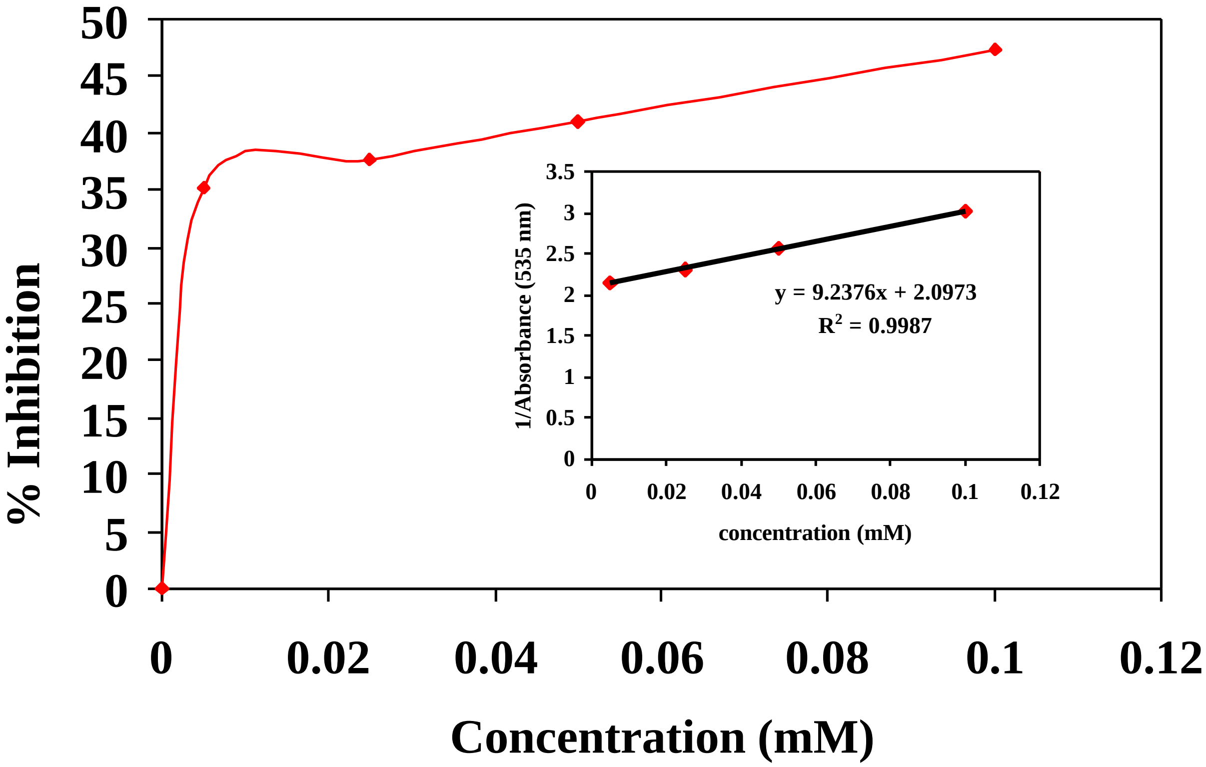
Appendix 1

Membrane lipid peroxidation studies with Olanzapine:
Effect of Olz on lipid peroxidation: The experimental conditions are as described under "Materials and Methods" in chapter 1. The reaction mixture (total 1 ml) containing 3 mg of microsomal protein in 0.05 M Tris-HCl buffer, pH 7.6, 1 mM ADP, 50 µM ferric chloride, 100 µM ascorbate was incubated at 37^{o} C for 15 minutes. Lipid peroxidation was initiated by the addition of H_2O_2 and was terminated by the addition of 2 ml of 0.5% TBA, and 2% trichloroacetic acid. *A p value ≤ 0.01 was considered significant.



Appendix 2

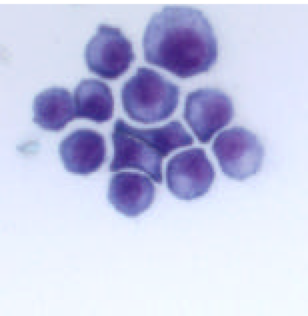
Hydroxyl radical scavenging by Olz: determination of rate constant. Deoxyribose degradation in the presence of various concentrations of Olz was followed as described under "Materials and Methods", Chapter 1, using a final deoxyribose concentration of 2.8 mM in the reaction mixture. A: Inhibition of deoxyribose degradation by Olz and determination of rate constant (inset). The rate constant was determined from the slope of the line (k = slope X $k_{[DR]}$ X [DR] XA) as described in the text (chapter 1) yielding the value of 31.4 X 10^9 $M^{-1}s^{-1}$



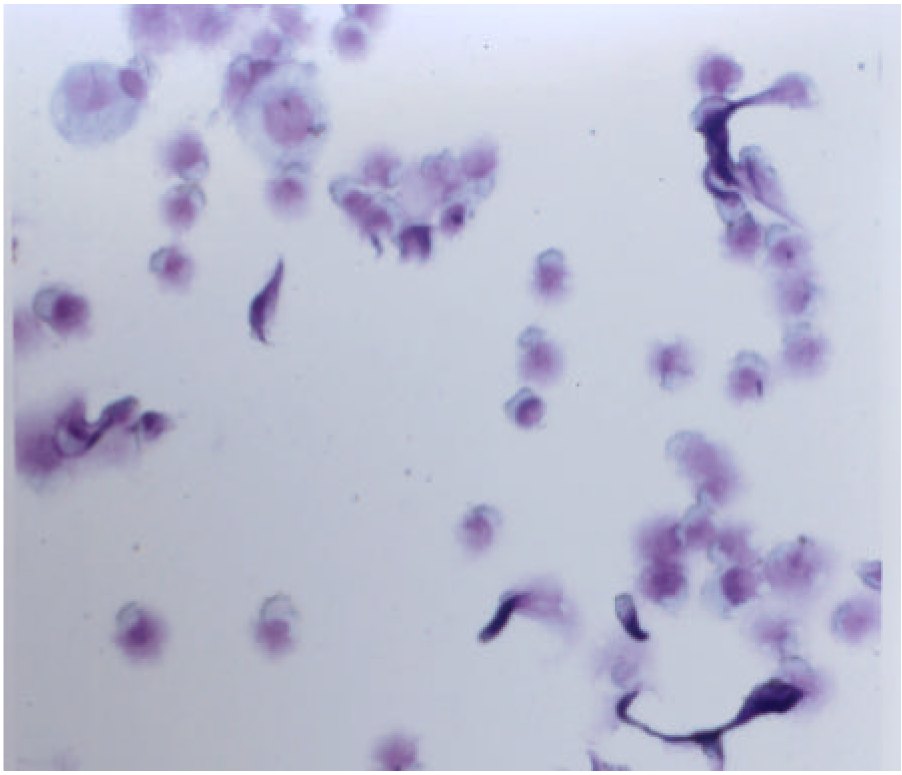
Appendix 3

Cytology: Cells treated with HP, Olz, hydrogen peroxide or vehicle were rinsed with PBS and examined following cytocentrifugation. Briefly, A 200 µl aliquot of 1.0 x 10^6 cells was collected and placed into a cyto-centrifugation chamber (Cyto-TEK, Sakura, Japan) containing 250 µl phosphate buffered saline (pH 7.2) and 50 µl of 15% Bovine Serum Albumin (BSA, Sigma) in saline. The cells were centrifuged at 50 X g, 23^{o}C for 7 min. The slides were fixed with 95% methanol and stained with Modified Wright stain (Sigma). Slides were coverslipped and sealed with Permount (Fisher Scientific). Cells were examined under light microscope for the presence of apoptotic bodies under oil immersion at 100x

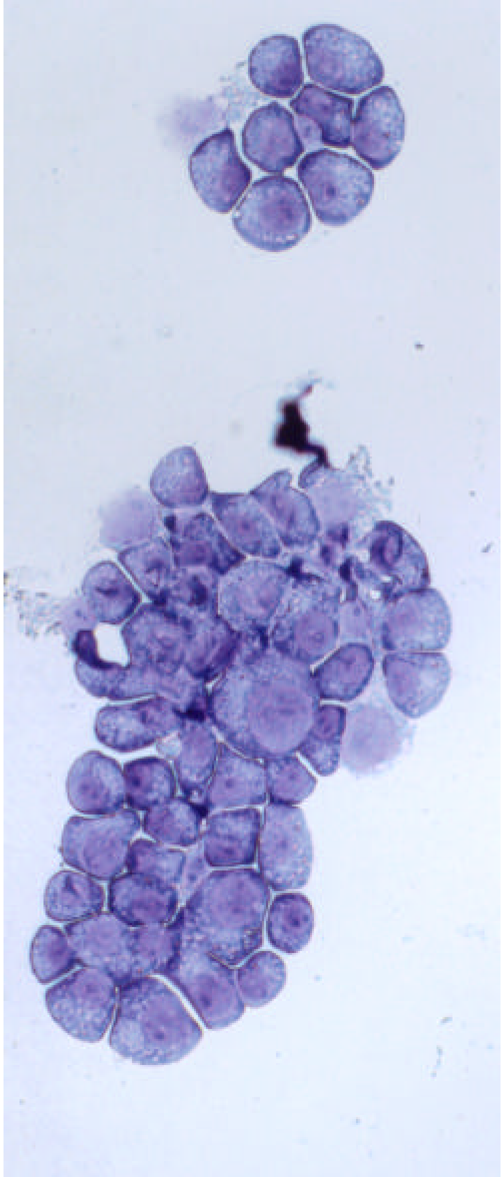
Fig 3: *Representative pictures from the cytologic and light microscopic evaluation of the HP and Olz treated PC-12 cells* (original magnification 100X):



A:*Healthy PC-12 cells*



B: *Necrotic PC-12 cells (as indicated by arrows) when cultured with high drug concentration of HP/Ol z or H2O2.*



C: *Highly granular cells after treatment with HP. Increased vacuolization is observed. Mostly all the cells show this phenomena.*

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Vita

Vijaylaxmi Mahapatra was born on February6, 1973 in Bareilly, India. In 1993, she recived her B.S from Osmania University, Hyderabad, India majoring in Mathematics, Physics, and Chemistry. In 1995, she received her M.S. in Organic Chemistry, specializing in Medicinal Chemistry from the same university. In 1995, she joined the graduate program at the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Polytechnic Institute and State University. She is married to Chinmaya , and is living in San Francisco, CA.

Honors/Awards:
Gold Medal winner in MS program from Osmania University, India.
Ranked second in BS Program from Osmania University, India.
Awarded teaching and research assistantship from Fall 1995 –Fall 1999.
Awarded the Raulet Scholarship in May 1996.
Awarded the Raulet Scholarship in Dec 1996.
Awarded the Raulet Scholarship in Dec 1997.
Awarded the Virginia Tech Graduate Student Association's travel award Spring 1999.
Ranked 2nd in the students presentations category at the Virginia Maryland Regional College of Veterinary Medicine (VMRCVM), Tenth Annual Research Symposium June 1998.

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Abstracts:
Haloperidol-induced Tardive Dyskinesia: Are reactive oxygen species involved? V.Mahapatra, Van Der Schyf, and H.P. Misra. Presented at the Society of Toxicology, annual meeting at Seattle Washington (March 1998).

The role of Haloperidol and its metabolites in scavenging hydroxyl radicals. V. Mahapatra and H.P Misra. Presented at The American Society Of Biochemistry and Molecular Biology (ASBMB) annual meeting at Washington, D.C. (May 1998)

The role of Haloperidol as a reactive oxygen species scavenger. V. Mahapatra and H.P. Misra presented at VMRCVM Tenth Annual Research Symposium, (Jun 1998).

A comparative study of a typical antipsychotic drug haloperidol and an atypical antipsychotic olanzapine in ameliorating oxidative stress. ? V. Mahapatra and H.P. Misra Presented at the Society of Toxicology, annual meeting at New Orleans, LO (March 1999)

Publications:

1. Haloperidol and Olanzapine induced cell toxicity on PC-12 rat pheochromocytoma cells. A comparison of 7AAD and Annexin V in the early detection of apoptosis in the

PC-12 cell line. Mahapatra V, Gogal R. and Misra, H.P. 2000 (Manuscript in preparation.)

2. Haloperidol: Scavenger of reactive oxygen species and enhancer of Microsomal Lipid Peroxidation. Mahapatra V. and Misra, H.P. 2000 (Submitted to J. Biol. Chem.)