Free Rad. Res., Vol. 29, pp. 151–157
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Antioxidant Properties of Clozapine and Related Neuroleptics

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Accepted by Prof. H. Sies

(Received 28 January 1998; In revised form 20 April 1998)

The antioxidant properties of clozapine and other related molecules were evaluated with the crocin bleaching test both in aqueous and non-aqueous environment. The tests of microsomal lipid peroxidation and carbonyl formation were also used. In aqueous solution, chlorpromazine and trifluoperazine appear particularly effective in the bleaching of crocin, while serotonin has an efficacy intermediate between those of phenothiazines and clozapine. The latter drug, on the other hand, in a non-aqueous medium shows an antioxidant power comparable to that of butylated hydroxytoluene, indicating that its antioxidant properties are better expressed in a hydrophobic environment of the type present in a biological membrane. In fact, in lipid peroxidation induced in microsomal membranes, clozapine, chlorpromazine, trifluoperazine and serotonin act as very good antioxidants; at low concentrations, clozapine appears to be the most efficient after butylated hydroxytoluene. Similarly, all these compounds markedly inhibit protein carbonyl formation, clozapine being one of the most efficient. Thus, under different *in vitro* experimental conditions, the neuroleptic drugs chlorpromazine and trifluoperazine and the antipsychotic substance clozapine act as very effective antioxidants; this property might, at least in part, be responsible for the physiological and clinical effects observed *in vivo*.

Keywords: Antioxidants, carbonyl groups, clozapine, crocin bleaching test, lipid peroxidation, neuroleptics

Abbreviations: BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; ABAP, 2,2′-azo-*bis*(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azo-*bis*(2,4-dimethylvaleronitrile); Trolox c, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; DMF, N,N-dimethylformamide; Clozapine, 8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo [b,e] [1,4] diazepine

INTRODUCTION

The central nervous system is particularly sensitive to oxidative stress since, compared to other tissues, its membrane lipids are rich in polyunsaturated fatty acids; in addition, the brain exhibits a low catalase activity and also superoxide dismutase and glutathione peroxidase are present in moderate amounts.[1] Furthermore, some areas such as *substantia nigra* and *globus pallidus* are rich in iron that appears to play an important role in brain metabolism.[1] After injury, part of the iron pool can be easily released and can

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interact with ascorbic acid present at high concentrations in the grey and white matter,[2] thereby promoting lipid peroxidation. There is growing evidence that free radicals might mediate neuronal death in diseases such as Parkinson's disease,[3–5] Alzheimer disease,[3] amyotrophic lateral sclerosis,[3] Hallervorden-Spatz disease,[6,7] and schizophrenia.[8] Iron has been implicated in the pathogenesis of Parkinson's disease since it enhances free radical formation that causes lipid peroxidation and cell death; some pigmented neurons are able to survive only in the presence of a low iron content.[4,5] There are also data indicating that oxidative stress acts as a mediator of excitotoxic cell death;[9] in addition, the oxidation products of catecholamines (aminochromes) might give rise, possibly through a redox cycling process, to the production of oxygen-derived free radicals.[10,11]

Antioxidants are able to protect against cell death[12,13] and, in general, the definition of the antioxidant characteristics of the molecules involved in brain metabolism might be important both for understanding pathophysiological mechanisms and for devising pharmacological strategies to slow down neuronal degeneration. In a previous paper[11] we have reported that neuroleptic drugs and serotonin are able to inhibit the oxidation of dopamine and lipid peroxidation; these properties might be related to their physiological and clinical effect on mental illness. In this paper the properties of serotonin and melatonin and of the well-known antioxidants trolox c and BHT were compared to the antioxidant properties of the traditional neuroleptic compounds chlorpromazine and trifluoperazine and of the antipsychotic drug clozapine. Nevertheless, the latter drug, despite its therapeutical effects, causes agranulocytosis in some users, possibly through a highly specific immune-mediated reaction.[14] According to Fischer *et al.*,[15] clozapine can be metabolized both by horseradish peroxidase and human myeloperoxidase to a free radical form giving rise to superoxide anion and adducts with glutathione and proteins potentially responsible for the occurrence of agranulocytosis. The antioxidant properties and the potential free radical damage of clozapine and other antioxidant molecules are discussed.

MATERIALS AND METHODS

Clozapine was a kind gift of Sandoz Pharma AG (Basel, Switzerland). Serotonin, chlorpromazine, trifluoperazine, melatonin and clozapine N-oxide were supplied from Sigma Chem. Co. (St. Louis, MO, USA). Crocin was isolated from commercial saffron according to Friend and Mayer[16] and its concentration was estimated using an $\varepsilon_M = 1.33 \times 10^5\,M^{-1}\,cm^{-1}$ at 440 nm.[17] The crocin bleaching test is based essentially on the procedure described by Bors *et al.*,[17] modified according to van Amsterdam *et al.*[18] and Tubaro *et al.*[19] The modification consists of the uses of diazocompounds (ABAP or AMVN) to produce peroxyl radicals and solvents of different polarity in order to perform the analysis of both hydrophilic and lipophilic compounds.[18,19] The test was carried out at 40°C in 5 mM phosphate buffer (pH 7.4) containing 5% ethanol when the peroxyl radical generating reaction was started with ABAP or dimethylformamide/toluene (4:1) when AMVN was used. Crocin was 12 μM and the various antioxidants were added at increasing concentrations starting from 6 μM. The azoderivatives were added from a fresh 0.5 M solution to obtain a final concentration of 5 mM. The rate of crocin bleaching was estimated spectrophotometrically by following the decrease of absorbance at 440 nm; the rate was linear after a lag time of approximately 2 min. Bleaching rates were plotted according to the equation: $v_0/v = 1 + k_a/k_c \times [A]/[C]$ where v_0 is the basal bleaching rate of crocin in the absence of antioxidant, v is the bleaching rate of crocin in the presence of antioxidants, [A] is the concentration of antioxidant and [C] is the concentration of crocin. k_a is the rate constant for the reaction of the

peroxyl radical with the antioxidant and k_c is the rate constant for the reaction of the peroxyl radical with crocin. The plot results in a straight line intersecting the ordinate at unit. The slope of this line is the ratio of the rate constants k_a/k_c and indicates the relative capacity of different molecules to interact with the hydroperoxyl radical.

Liver microsomes were prepared according to Ernster and Nordenbrand.[20] Microsomal lipid peroxidation was measured as malondialdehyde formation.[21] Carbonyl content was estimated according to Levine *et al.*[22] Proteins were measured by the biuret test.[23]

RESULTS AND DISCUSSION

The antioxidant capacity of the neuroleptic agents and drugs was measured with the crocin bleaching test based on the competition of crocin bleaching by the various substances in the presence of a free radical generating system.[17–19] In Figure 1(a) the relative antioxidant potency is reported and compared to that of trolox c, the hydrosoluble form of vitamin E, which is considered the most effective agent in reacting with crocin.[17] The phenothiazines, chlorpromazine and trifluoperazine appear to be particularly

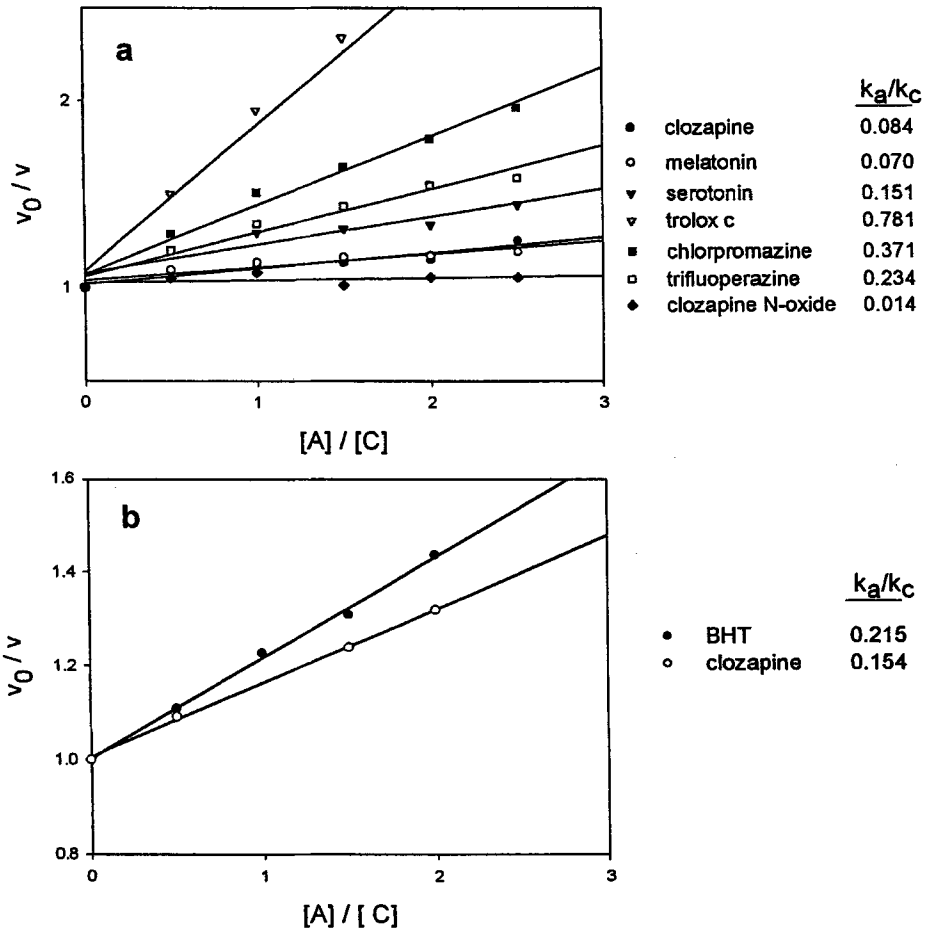

FIGURE 1 Competition plot of crocin and antioxidant substances for hydroperoxyl radicals derived from ABAP (a) or AMVN (b) subjected to thermal decomposition. Antioxidants were utilized at concentrations ranging from 6 to 30 μM (a) or from 10 to 50 μM (b). The slope of the straight line (k_a/k_c) indicates the relative capacity of the various substances to interact with the peroxyl radical (see also Materials and Methods). Data are the mean of 4 different experiments.

effective, while serotonin has an efficacy intermediate between that of phenothiazines and clozapine. The latter drug and melatonin show k_a/k_c values of 0.084 and 0.070, respectively. Clozapine N-oxide, a major catabolite of clozapine,[24] does not exhibit antioxidant capacity suggesting that even small alterations of the molecule can have an important role in restraining the antioxidant properties. Interestingly, the latter compound possesses practically no activity towards serotoninergic receptors.[24]

All the measurements were performed in an aqueous environment of phosphate buffer and the free radicals were generated from ABAP. Since clozapine is scarcely soluble in water, the crocin bleaching test was performed in DMF/Toluene and the free radical initiator was AMVN. Under these conditions the antioxidant power of clozapine is comparable to that of BHT (Figure 1(b)) since the k_a/k_c of BHT and clozapine are 0.215 and 0.154, respectively. These results indicate that the antioxidant properties of clozapine are more evident in a hydrophobic environment such as that present in a biological membrane.

The crocin test allows the quantitative measurement of the relative rate constants between hydroperoxyl forms and the compound under investigation, and also indicates a fundamental requirement for an antioxidant i.e. the generation of a relatively stable free radical species. To know how much an antioxidant molecule is active in an environment such as that of a biological membrane, the classical test of inhibition of the microsomal lipid peroxidation can be utilized since it allows a direct measurement of the antioxidant effect in a membrane. Lipid peroxidation was induced in liver microsomes by the system NADPH/Fe^{2+}/ADP. As shown in Figure 2 chlorpromazine, trifluoperazine, serotonin and clozapine are good antioxidants and, at low concentrations, clozapine appears to be the most efficient after BHT. The antioxidant and free radical scavenging properties of phenothiazines have been characterized both in model systems

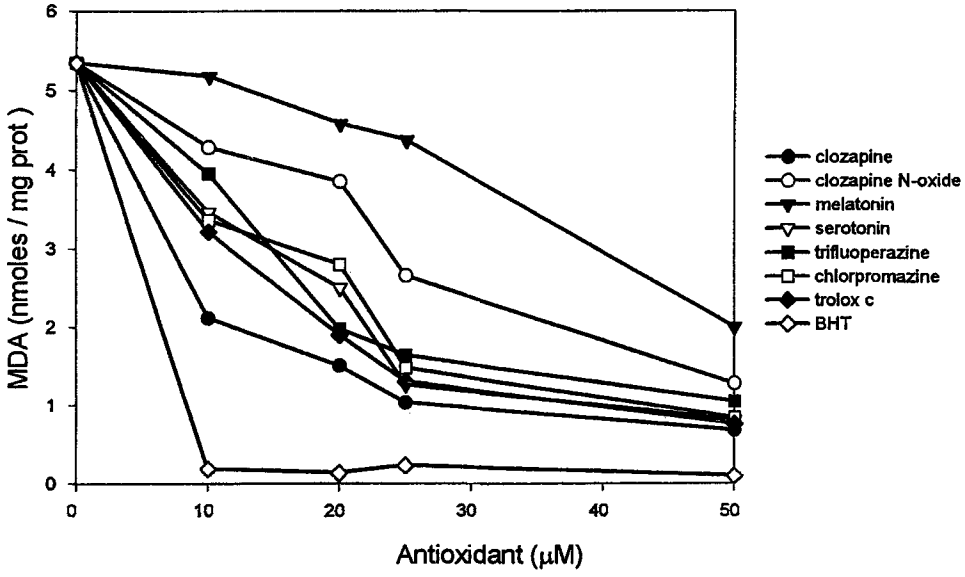

FIGURE 2 Inhibition of lipid peroxidation by increasing concentrations of clozapine and other antioxidants. Liver microsomes (0.5 mg/ml) were incubated at 30°C in 0.125 M KCl, 20 mM Hepes/Tris (pH 7.4). Malondialdehyde formation was initiated by the addition of 0.5 mM NADPH, 20 μM $FeCl_2$ and 0.2 mM ADP and determined as described under Materials and Methods. Data are the mean of 4 different experiments.

and *in vivo*.[1,25–28] In part, their antioxidant features were also dependent on the formation of hydroxylated derivatives generated during their metabolism by NADPH-dependent microsomal oxygenase.[29] We did not find evidence of hydroxylated products of phenothiazines in the samples treated with ABAP. However, hydroxylation might occur in the microsomal system during lipid peroxidation induced by NADPH/Fe^{2+}/ADP, thus reinforcing their antioxidant properties. Clozapine N-oxide and melatonin appear less efficient in inhibiting lipid peroxidation in agreement with the results obtained with the crocin bleaching test and with other investigations.[30]

Free radicals, in addition to polyunsaturated fatty acids, can also alter proteins, through the formation of carbonyl group derivatives via a variety of mechanisms. Moreover, the levels of carbonyl groups increase with age and in some pathological conditions[31] and hence the direct action on carbonyl group formation exerted by the various potential antioxidant drugs was examined. As shown in Table I, all the compounds tested, at 50 μM concentration, markedly inhibit carbonyl formation induced by NADPH/Fe^{2+}/ADP in rat liver microsomes. Trolox c and BHT completely inhibit the formation of these compounds while clozapine has an inhibitory effect of about 80%.

TABLE I Protein carbonyl group formation in rat liver microsomes treated with different antioxidants

	Carbonyl groups (nmol/mg protein)	% Inhibition
None	12.21 ± 0.66	
Chlorpromazine	5.99 ± 0.54	51
Clozapine	2.31 ± 0.88	81
Serotonin	2.84 ± 1.13	77
Trifluoperazine	2.33 ± 0.81	81
Melatonin	2.91 ± 1.20	76
Trolox c	1.39 ± 0.98	89
Butylated hydroxytoluene	0.40 ± 0.28	97

Rat liver microsomes (1 mg/ml) were incubated for 15 min at 25°C in 125 mM KCl, 20 mM Hepes/Tris (pH 7.4). Carbonyl group formation was stimulated by the addition of 0.5 mM NADPH, 0.2 mM ADP and 20 μM $FeSO_4$; antioxidants were 50 μM. Data are expressed as mean ± SE ($n = 4$).

From the reported data it appears that, in different *in vitro* experimental conditions, the neuroleptic drugs chlorpromazine and trifluoperazine and the antipsychotic substance clozapine act as efficient antioxidants; this property might, at least in part, be responsible for the physiological and clinical effects observed *in vivo*. Interestingly, clozapine shows an effectiveness close to that of antioxidant vitamins and free radical scavengers on interdose choreic dyskinesias induced by L-dopa and on dystonic form of tardive dyskinesia.[32–34] The present data suggest that the antioxidant properties of this atypical neuroleptic drug might be associated with its therapeutical and pharmacological efficacy within a wide range of human pathologies. Nevertheless, as reported in the Introduction, clozapine causes agranulocytosis[14] and can be metabolized to a free radical by both horseradish peroxidase and human myeloperoxidase.[15] The formation of free radicals might be the molecular basis responsible of agranulocytosis occurrence. Similarly, chlorpromazine[35] and the well-known antioxidants BHT and 2-tert-butyl-4-methoxyphenol (BHA)[36] that act as strong inhibitors of lipid peroxidation give rise, in the presence of horseradish peroxidase and hydrogen peroxide, to potentially toxic free radical forms. The latter are unable to further stimulate lipid peroxidation, but can form adducts with proteins or deplete glutathione. In conclusion, the characterization of these radical species and in particular the identification of their fate in a biological environment can give information about the mechanisms of toxicity that operate independently of the antiperoxidative properties.

Acknowledgments

This work was supported by grants from National Research Council of Italy (CNR). The authors wish to thank Professor Lauro Galzigna for the critical reading of the manuscript and Dr. E. Brunati, Sandoz, Milano, for the kind gift of clozapine.

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