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JL 13, a Potential Successor to Clozapine, Is Less Sensitive to Oxidative Phenomena

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The oxidation behaviour of JL 13, a promising antipsychotic, was investigated in comparison with clozapine and loxapine, by measuring their direct "radical scavenging" abilities and their efficacies in inhibiting the lipid peroxidation. In the lipid peroxidation system, the reactivity of these compounds with free radicals produced by γ-irradiation of linoleic acid may be presented as follows: JL 13 = loxapine < clozapine. In two enzymatic systems (HRP/GSH and HRP/H_2O_2/GSH) which generate the thiyl free radicals, clozapine produces a strong enhancement of the thiyl-radical EPR signal intensity while JL 13 and loxapine exhibit no or minimal effect on this signal. The redox potential values for the three derivatives confirm the spectrophotometric and EPR results. Following this study, we show that JL 13, although presenting a preclinical clozapine-like profile, appears less sensitive to oxidation than clozapine.

During the last twenty years, the search for safer clozapine-like analogues has known a strong development and even constitutes the major effort in the modern psychopharmacology (1,2,3). In this context, we pursued for the last few years, a pharmacochemical study of diarylazepine analogues related to clozapine (4,5).

Currently, the advantages of clozapine as antipsychotic are well recognized in clinic (6). It is effective against negative schizophrenia and also in treatment-resistant patients (7). Nevertheless, the use of clozapine remains heavy and costly due to an important blood monitoring during the treatment. Indeed, clozapine induces severe haematological disorders (8).

Abbreviations: HRP, horseradish peroxidase; GSH, glutathione; DTPA, diethylpentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance.

Our searches have been focused on the discovery of an original drug possessing the clinical advantage of clozapine without its side effects mainly agranulocytosis. Now, we have selected a leader compound, JL 13, a pyridobenzoxazepine derivative, bioisostere of clozapine (Figure 1). JL 13 possesses a very interesting preclinical profile that allows to predict a clozapine-like profile with less side effects (5,9-12).

In 1991, Fischer and coworkers proposed a correlation between the oxidability of clozapine through the free radical formation and its agranulocytosis potential (13). Following this hypothesis, we tested various dibenzoazepine derivatives in a peroxidase-catalysed oxidation procedure (14). We demonstrated that diazepine or phenothiazine analogues were readily oxidized by peroxidase systems unlike oxazepine or thiazepine derivatives like loxapine or clothiapine respectively which were insensitive to this oxidasic system (14).

In this paper, we report some results concerning the oxidability of JL 13 in various procedures compared to clozapine and loxapine.

MATERIALS AND METHODS

Reagents. Horseradish peroxidase (HRP), glutathion (GSH) were obtained from Boehringer Mannheim, diethylpentaacetic acid (DTPA) was from Sigma and hydrogen peroxide (H_2O_2) was from UCB. Phosphate buffer (0.05M $NaH_2PO_4.2H_2O$ and Na_2HPO_4), 2-thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were obtained from Merck. Chelex 100 resin (200-400 mesh, sodium form) was from Biorad. 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was from Aldrich and purified with activated charcoal as previously described (15). Clozapine and loxapine (as succinate) were a generous gift from Sandoz Basel and Cyanamid Benelux Brussels respectively. JL 13 was synthesized in the laboratory of Medicinal Chemistry of the University of Liège. Drugs were dissolved in DMSO daily. All other chemical products were of analytical grade.

Spectrophotometric study. The stock solutions of JL 13, clozapine, and loxapine were prepared in DMSO and diluted at the final concentration of 0.1mM in phosphate buffer pH 7.4 (total volume 2 mL).

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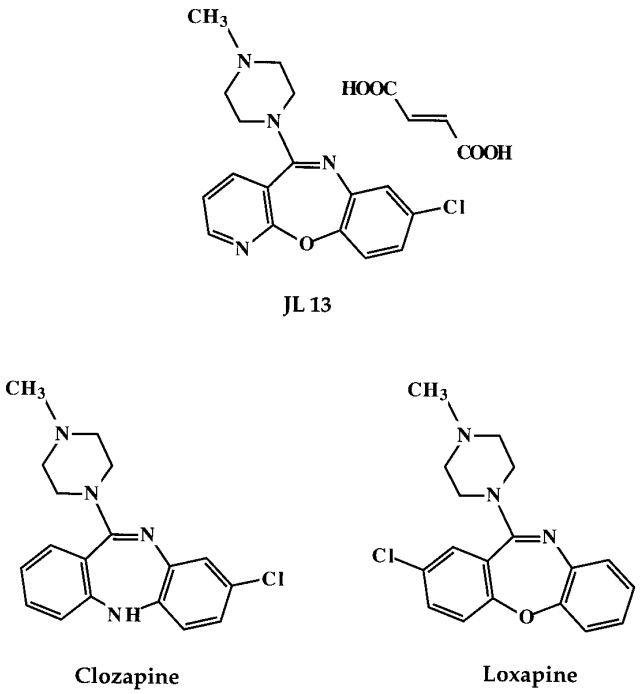

FIG. 1. Chemical structure of JL 13, clozapine, and loxapine.

To a mixture containing: 200 mg of CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate), 0.2 mL of linoleic acid and 100 mL of chelexed phosphate buffer was added 20 μL (0.1 mM) of the drug. The mixture reaction was γ-irradiated (*from Cesium 137 source at the rate of 10000 rads*). Then, 0.5 mL of the irradiated solution was added to 0.5 mL TCA and 2 mL of TBA (26 mM) in 50 mM of Tris-HCl pH 7. After extraction with 2 mL of n-butanol, the absorbance of each sample was measured by monitoring changes by using the thiobarbituric method (16).

The absorbance was measured at 540 nm with n-butanol as internal reference on Perkin-Elmer Lambda 15 UV:VIS spectrophotometer.

EPR spin trapping experiments. The experiments were performed in 50 mM phosphate buffer (pH 7.5) containing 100 mM DMPO in a total volume of 1 mL. Reaction was started after addition of drug to the complete system containing: HRP (25 mg/mL), GSH (10 mM), and DTPA (0.5 mM). We also used another peroxidation system by adding, in the precedent system, hydrogen peroxide (H_2O_2) at final concentration of 1 mM. In each case, the reaction mixture was then immediately transferred into quartz flat cell in the EPR cavity. All measurements were recorded on the Bruker spectrometer ESP 300 E (Bruker Karlshure, Germany). EPR spectra were monitored at room temperature with following instrumental settings: microwave power 20 mW, modulation frequency 100 KHz, modulation amplitude 1.012 Gauss, center of field 3480 Gauss and sweep width 100 Gauss. Resulting scan time for 5 scans was 7 min. The other parameters are reported in the legends of figures.

Redox potential measurements. Voltammetric measurements have been realized using a CV 27 voltammograph (BAS West Lafayette) connected to a Hewlett-Packard 7090A x-y recorder. The experiments were performed in a three electrode cell containing the working electrode, a platinum auxiliary electrode, and a Ag/AgCl in 3 M KCl reference electrode. The working electrode was made of glassy carbon (GCE-BAS diameter 3 mm). Before each voltammogram, the electrode surface was smoothed on a soft polishing cloth in the presence of alumina suspension. Voltammograms were recorded in phosphate buffer 0.1 M, pH 7.4 at a scan rate of 25 mV/s and at room temperature. Similar measurements were made at pH 4.7 in 0.25 M acetate buffer. The oxidability of the compound was compared on the basis of their half peak potential (Ep 1/2).

RESULTS

Spectrophotometric Procedure

The results obtained from the oxidation of linoleic acid emulsion indicated that clozapine inhibits this peroxidation by reacting with free radicals produced in the mixture reaction by γ-irradiation (Figure 2). Thus, we observed a strong decrease (31.02%) of the absorbance value in the presence of 0.1 mM clozapine. The addition of 0.1 mM JL 13 or loxapine to the complete system induced less inhibitory effect on the absorbance than that seen with clozapine (72.18% and 74.83% for JL 13 and loxapine respectively) (Figure 2). The effect of clozapine is very significatively different to that of JL 13 ($p<0.0001$) and of loxapine ($p=0.0004$). Between JL 13 and loxapine, minimal difference is observed ($p<0.06$).

Peroxidase-Catalysed Oxidation

To study the reactions of clozapine, JL 13, and loxapine with the thiyl free radicals, we used two enzymatic systems; i)HRP/GSH and ii)HRP/H_2O_2/GSH, in the presence of DTPA and spin trap DMPO. In both systems, the typical spectrum characterized by a four-line EPR signal correrresponding to the DMPO/GSH spin adduct of thiyl radical was observed (Fig-

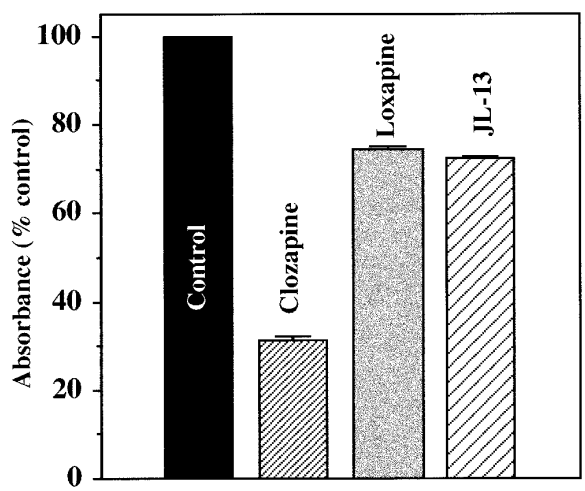

FIG. 2. The influence of JL 13, clozapine and loxapine on the linoleic acid oxidation induced by γ-irradiation. This reaction was monitored by following the absorbance values versus the linoleic acid oxidation which was taken as control value (100%) at the visible wavelength of 540 nm (mean values ± SD, n=3). Statistical data (from Student t test) are mentioned in the text.

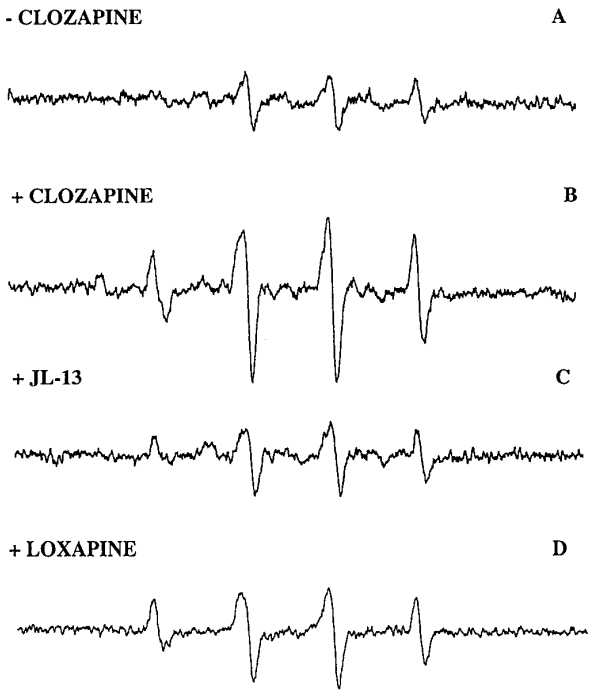

FIG. 3. Spin adduct formation with thiyl radicals generated by HRP/GSH system. The influence of 0.1 mM clozapine (B), 0.1 mM JL 13 (C), and 0.1 mM loxapine (D) on the DMPO-thiyl spin adduct intensity. HRP and GSH served as source of thiyl radicals. The spectrum in the absence of drugs is shown in (A). Hyperfine splitting parameters of the EPR spectrum were as follows: a^N = 15.4 G; $a^{H\beta}$ = 16.2 G (as previously reported (17)). Receiver gain: 2.10^4; time conversion: 81.92 ms; time constant: 164 ms; and number of scans was 5.

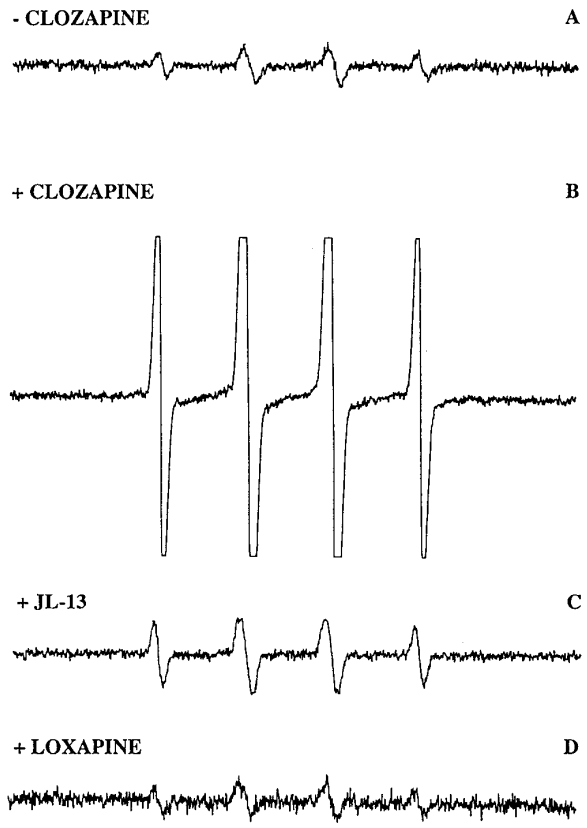

FIG. 4. The effect of similar concentrations (0.1 mM) of clozapine (B), JL 13 (C), and loxapine (D) on the DMPO-thiyl spin adduct generated by enzymatic system containing HRP/H_2O_2/GSH (A). The hyperfine splitting parameters were the same as in Figure 3. Receiver gain: 2.10^4; time conversion: 40.96 ms; time constant: 164 ms; and number of scans was 3.

ure 3A and Figure 4A). The adduct resulted in a distinctive EPR spectrum (a^N (coupling constant of nitrogen) = 15.4 G and $a^H\beta$ (coupling constant of hydrogen) = 16.2 G) with hyperfine splitting similar to those previously reported (17).

In the HRP/GSH system, the addition of 0.1 mM clozapine led to an enhancement of thiyl radical EPR signal intensity of 2 times (Figure 3B). Otherwise, JL 13 and loxapine at the same concentration were insensitive to these oxidation systems (Figure 3C and 3D). Qualitatively, similar results were obtained in the second enzymatic system (HRP/H_2O_2/GSH) but a strong increase of DMPO-thiyl spin adduct amplitude (around 3 times) after clozapine addition was observed (Figure 4B) due to the presence of hydrogen peroxide, a powerful oxidant. In this second model, JL 13 and loxapine inducede only a weak effect on the thiyl radical generation (Figure 4C and 4D). The reactivity of the three derivatives on both enzymatic systems could be reported as follows: JL 13 = loxapine < clozapine.

Redox Potential

In both media, JL 13 presented a higher peak potential than clozapine although it presented a lipophilicity slightly superior to clozapine (Table 1). Lipophilicity is not the criterion for explaining this behaviour since chlorpromazine was also easily oxidized (14) while presenting a higher lipophilicity (data not shown). Thus, clozapine is more rapidly oxidized than JL 13. Loxapine presented an oxidation potential comparable to that of JL 13.

TABLE 1

Electrochemical Behaviour of JL 13, Clozapine, and Loxapine

	Acetate buffer 0.25M pH 4.7 Ep $\frac{1}{2}$ (mV vs Ag/AgCl)	Phosphate buffer 0.1M pH 7.4 Ep $\frac{1}{2}$ (mV vs Ag/AgCl)	log k′ (*)
JL 13	1040	860	0.919
Clozapine	505	395	0.795
Loxapine	1000	840	1.143

(*) determined by HPLC method using a Lichrospher C18 with a mixture of 30% phosphate buffer, pH 7.4 and 70% methanol as mobile phase.

DISCUSSION AND CONCLUSION

EPR-spectrometry in combination with spin trapping is a specific and sensible tool for the measurement of free radicals formation. In the presence of horseradish peroxidase with DMPO as spin trapping agent, JL 13, clozapine and loxapine differentialy reacted with thiyl radicals. Our data clearly demonstrated that clozapine was easily oxidized by increasing the thiyl radicals production. Such a result was in accordance with the work of Fischer (13) and our own experience (14). This effect was amplified in the presence of hydrogen peroxide which reinforced the oxidant power of the system strongly reacting with the drug. Otherwise, we observed that the addition of JL13 or loxapine in both systems had no or minimal effect on the EPR signal intensity.

Moreover, their effect on the oxidation of the linoleic acid emulsion showed an inhibitory effect for clozapine (31.02%) which was higher than that of loxapine (74.83%) and JL 13 (72.18%). This behavior can be explained by the fact that clozapine was able to react with free radicals and to form *in vivo* a free radical species (clozapine cation radical) as reported by Fischer's team (13). Thus, it clearly appears that JL 13 has no effect on the lipid oxidation and no radical scavenging activity is found in our experiments. From the electrochemical point of view, oxidation of clozapine was effective at much lower potential than JL 13 and loxapine. Actually, oxidation of clozapine occured at the tricyclic ring while the other two compounds were oxidized on the piperazine group (18). This should be attributed to a much higher availability of the lone pair of electron on the nitrogen and to the possible semi quinone or dimer formation during clozapine oxidation. The semi quinone derivatives was described for amodiaquine as a source of toxic intermediates (19,20).

Although the problem of clozapine-induced agranulocytosis is well documented, many hypotheses have been proposed to explain the appearance of such phenomenon (20,21). Oxidation of clozapine by activated human neutrophils provides reactive nitrenium ions that could be also responsible for clozapine-induced agranulocytosis (22). In such nitrenium ion, the positive charge is highly delocalized and can react with GSH to give GSH conjugates. In the case of JL 13, the formation of this ion is not possible as the delocalization found in clozapine (13,22) cannot exist in the JL 13 nucleus. This is confirmed in part by the works of Kauffmann and coworkers showing the differential electrochemical behaviour of these tricyclics (18).

Presently, there is no real predictive model for haematological side effects of neuroleptic drugs. Indeed, recently, the case of remoxipride has confirmed that some side effects are not easily detectable during clinical phases of development. Remoxipride was rapidly withdrawn because of aplastic anaemia (20).

In conclusion, although we do not possess any warranty concerning the future clinical development, we think that JL 13 could offer a promising alternative to clozapine.

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