

Y-931, a Novel Atypical Antipsychotic Drug, Is Less Sensitive to Oxidative Phenomena

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The oxidation behavior of Y-931, a potent atypical antipsychotic drug, was compared with that of clozapine and olanzapine. In two enzymatic systems (horseradish peroxidase (HRP)/glutathione (GSH) and HRP/H₂O₂/GSH) which generate thyl radicals, clozapine markedly strengthened the electron paramagnetic resonance (EPR) signal for the radical. Olanzapine, Y-931 and the major metabolites (compounds 1—3) had no or minimal effect on the intensity of this signal. In addition, the redox potential values for the three derivatives were in accord with the EPR spin trapping results. In toxicological experiments in human leukocytes, a concentration-dependent toxicity was observed when neutrophils were incubated with clozapine (1—10 μmol/l) and H₂O₂ (1 mmol/l). However, Y-931 and olanzapine did not show remarkable toxicity under the conditions.

Key words Y-931; clozapine; olanzapine; agranulocytosis; EPR; redox potential

Clozapine (Fig. 1) is a dibenzodiazepine atypical antipsychotic drug whose main advantages include a lack of extrapyramidal side effects (EPS) such as tardive dyskinesia¹ and its effectiveness in otherwise “typical antipsychotic drugs treatment-resistant” patients.² Despite these advantages, the use of clozapine is restricted because of a relatively high incidence (0.8%) of lethal agranulocytosis.^{3,4} The exact mechanism of this adverse reaction is not known, yet clinical observations suggest an immune-mediated hypersensitivity reaction.⁵ In general, drug-induced hypersensitivity reactions appear to be linked to the metabolic activation of the drug by reactive metabolites. A good correlation exists between the incidence of agranulocytosis and drug activation by myeloperoxidase (MPO) or horseradish peroxidase, which is present in both polymorphonucleocytes and bone marrow cells.⁶ Olanzapine, which is a thienobenzodiazepine derivative structurally related to clozapine, is an effective antipsychotic agent.⁷ Interestingly, despite the structural similarity between clozapine and olanzapine, no cases of agranulocytosis have been reported with the clinical use of olanzapine.⁸

Recently, we reported that Y-931 (8-fluoro-12-(4-methylpiperazin-1-yl)-6*H*-[1]benzothieno[2,3-*b*][1,5]benzodiazepine),^{9–11} which was developed in our laboratory, showed a pattern of interaction with various neurotransmitter receptors similar to that of clozapine. In addition, Y-931 did not cause catalepsy (a correlate of EPS), despite its potent antagonistic activity of apomorphine-induced hyperlocomotion or conditioned avoidance response (a correlate of antipsychotic effects). Furthermore, Y-931 more potently prevented neurotoxicity (neuronal vacuolization) induced by MK-801, a non-competitive NMDA antagonist, in the rat retrosplenial cortex (a correlate of blockade ability for the NRH (NMDA receptor hypofunction)) than did clozapine or haloperidol. These findings suggest that Y-931 would be a novel antipsychotic drug not only with the potential to ameliorate NRH, but also with a low risk of EPS.

Recently, with investigations into the oxidability and the toxicity of antipsychotic agents, exciting progress has been

made in anticipating drug-induced agranulocytosis. In 1991, Fischer and coworkers proposed a correlation between the oxidability of clozapine through the formation of free radicals and its agranulocytosis potential.¹² Furthermore, the redox potential values for clozapine, JL13 and loxapine were in accord with spectrophotometric and electron paramagnetic resonance (EPR) results.¹³ In this paper, we report on the oxidability of Y-931, clozapine and olanzapine.

Experimental

Chemicals Horseradish peroxidase (HRP), glutathione (GSH) and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Diethylpentaacetic acid (DTPA) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan) and purified with activated charcoal as previously described.¹⁴ Phosphate buffer (0.05 M NaH₂PO₄·2H₂O and Na₂HPO₄), and H₂O₂ were obtained from Wako Pure Chemical Industries Co. (Osaka, Japan). Y-931, metabolites of Y-931 (compounds 1—3), clozapine and olanzapine were synthesized in our laboratory. All drugs and metabolites of Y-931 were dissolved in DMSO daily.

EPR Spin Trapping Experiments The experiments were performed in phosphate buffer (50 mmol/l, pH 7.4) containing DMPO (10 mmol/l) in a total volume of 1 ml. The reaction was started after the addition of drug (100 μmol/l, dissolved in DMSO, maximum final concentration; 1%) to a complete system containing: HRP (25 μg/ml), GSH (10 mmol/l), and DTPA (0.5 mmol/l). We also prepared another peroxidation system by adding, to the precedent system, H₂O₂ at a final concentration of 1 mmol/l. In each case, the reaction mixture was then immediately transferred into a quartz flat cell (JEOL Co., Tokyo, Japan, No. 4220-01040) in the EPR cavity. After 4.5 min, the EPR signal corresponding to the spin adduct DMPO/glutathionyl (GS·) free radical was recorded on a Varian E-line E-109E. EPR spectra were recorded at room temperature with the following settings: microwave power 20 mV, modulation frequency 100 kHz, modulation amplitude 1.000 G, center of field 3400 G, time constant 128 ms and scan rate 150 G.

Measurement of Redox Potential Voltammetric measurements were made using a BAS100B/W and CV-50W system. The experiments were performed with three working electrodes, and one reference electrode (Ag/AgCl in 3 mol/l KCl). The working electrodes were made of glassy carbon (GCE-BAS, diameter 3 mm). Before each recording, the surface of the electrode was made smooth by rubbing with soft paper. Voltammograms were recorded in phosphate buffer (0.1 mol/l, pH 7.4) at a scan rate of 25 mV/s and room temperature. Similar measurements were made acetate

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buffer (0.25 mol/l, pH 4.7). The oxidability of each compound was compared on the basis of its peak potential (Ep). The drugs were first dissolved in methanol: the solutions were made up of the drug (0.1 mmol/l) in a buffer containing 2% methanol. GSH (10 mmol/l solution) was dissolved in the acetate buffer (pH 4.7) or the phosphate buffer (pH 7.4) in the absence of methanol.

Isolation of Human Leukocytes Neutrophils were isolated from venous blood of healthy volunteers ($n=4$) by centrifugation as described in detail previously.¹⁵ Peripheral blood mononuclear cells (2×10^6 cells/ml) were re-suspended with HBSS containing 10% heat-inactivated fetal bovine serum, 4 mmol/l glutamine, 60 $\mu\text{g/ml}$ penicillin, and 100 $\mu\text{g/ml}$ streptomycin and then distributed onto 12-well plates. The plates were incubated for 2 h at 37 °C in an incubator (5%, CO₂).

Toxicity of the Reactive Intermediates of Y-931, Olanzapine and Clozapine in Human Neutrophils Each drug was dissolved in PBS (100 μl , pH 7.4). H₂O₂ (1 mmol/l, dissolved in PBS, pH 6.0, 100 μl) was added. Immediately, neutrophils (4×10^6 in 1 ml of HBSS) were added to the reactive metabolite solution. After incubating at 37 °C for 2 h, cells were stained with 0.1% (w/v) trypan blue and counted using a hemocytometer.

Results

To study the reactions of clozapine, olanzapine, Y-931 and the major metabolites (compounds 1–3, Fig. 1) with the thiyl free radicals, we used two enzymatic systems; HRP/GSH and HRP/H₂O₂/GSH, in the presence of DTPA and DMPO (Table 1). In both systems, a typical spectrum characterized by a four-line EPR signal corresponding to the DMPO/GSH spin adduct of the thiyl radical was obtained (Fig. 2). The adduct resulted in a distinctive EPR spectrum (a^{N} (coupling constant of nitrogen)=15.4 G and $a^{\text{H}}\beta$ (cou-

pling constant of hydrogen)=16.2 G) with hyperfine splitting similar to that reported by Harman *et al.*¹⁶ In the HRP/GSH system, the addition of clozapine at 0.1 mmol/l led to a 2-fold increase in signal intensity for the thiyl radical. On the other hand, olanzapine, Y-931 and the metabolites of Y-931 at the same concentration were insensitive to this oxidation system. Similar results were obtained in the HRP/H₂O₂/GSH system but a strong increase in amplitude for the DMPO-thiyl spin adduct was observed after these drugs were added due to the presence of H₂O₂. Notably, the addition of clozapine led to an enhancement of signal intensity of 30-fold. However, the other drugs had only a weak effect on the generation of thiyl radicals. Thus, in both systems, Y-931 and its metabolites were more stable than clozapine.

Values of oxidation potential obtained with the glassy carbon electrode are listed in Table 2. A pH of 7.4 was chosen for physiological reasons, while pH 4.7 was selected for its buffering capacity, for its weak interference with the electrochemical reaction, and also because it permits detection of a cation radical which is highly unstable under neutral or basic conditions. In terms of oxidation potential at pH 4.7, the compounds ranked as follows (from 374 to 538 mV): olanzapine < Y-931 < clozapine. At pH 7.4, the values were in the range of 202 to 409 mV. The ranking for oxidation potential at pH 7.4 was quite similar to that at pH 4.7 as reported in Table 2. Thus, olanzapine was more rapidly oxidized than clozapine and Y-931. The oxidation potential of GSH at pH

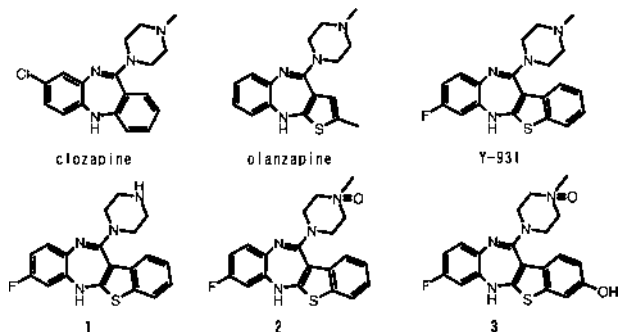


Fig. 1. Chemical Structure of Antipsychotic Drugs and Metabolites of Y-931 (1–3)

Table 1. Experimental Results Obtained from EPR

Drug	HRP/GSH (Intensity of signal)	HRP/H ₂ O ₂ /GSH (Intensity of signal)
Vehicle	73 ^{a)}	10.5 ^{a)}
Clozapine	159	295
Olanzapine	0	32
Y-931	28	93
Compound 1	28	74
2	35	83
3	0	90

^{a)} Value was obtained from a sample containing the complete enzymatic system (HRP/GSH or HRP/H₂O₂/GSH) without drug.

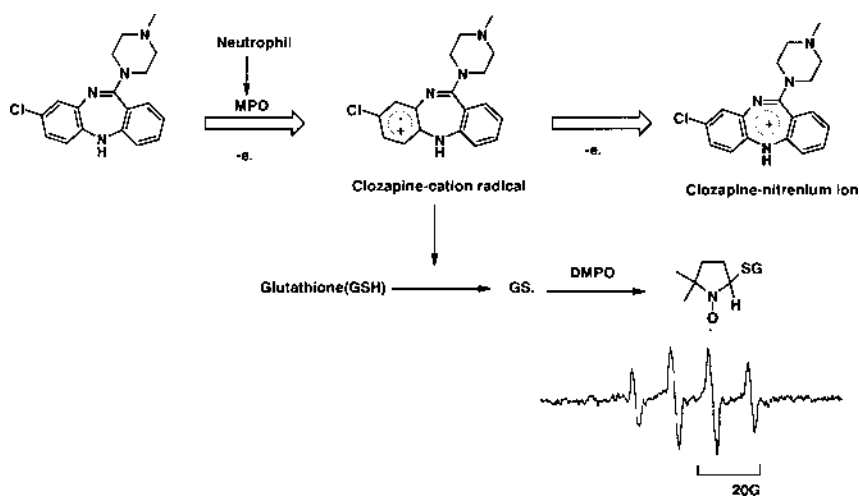


Fig. 2. Proposed Pathway for the Oxidation of Clozapine to Reactive Ion and Its Reaction with GSH

4.7 and pH 7.4 was 215 mV and 114 mV, respectively.

In a previous study,¹⁷⁾ although no toxicity was observed when NaOCl (20 $\mu\text{mol/l}$) or exogenous MPO (1 unit) was used as an activating reagent, significant toxicity was observed at a concentration of clozapine of 1 $\mu\text{mol/l}$ when H_2O_2 (1 mmol/l) was included in the incubations. Therefore, we used H_2O_2 as an activating reagent for these drugs. The addition of olanzapine or Y-931 to the reaction mixtures in the presence of H_2O_2 (1 mmol/l) had little effect on the toxicity, however, the addition of clozapine reduced the number of viable neutrophils (Fig. 3, left panel). No toxicity was observed with any drug alone at concentrations below 10 $\mu\text{mol/l}$ (Fig. 3, right panel).

Discussion

It is possible that drug-induced agranulocytosis is due to reactive metabolites generated by activated neutrophils or other cells that contain myeloperoxidase. Some indirect evidence supports a possible immune mechanism for clozapine-associated agranulocytosis but a direct toxicity of clozapine metabolites, or some combination of toxic and immune mechanisms, cannot be excluded.¹⁸⁾ Thus, the mechanism of clozapine-induced agranulocytosis and the best method of direct examination are not clear. Therefore, we examined the oxidability of these drugs using various procedures.

EPR-spectrometry with a spin trapping agent is a specific and sensible tool for the measurement of free radicals. In the presence of HRP with DMPO as the spin trapping agent, the oxidability of clozapine, olanzapine, Y-931 and metabolites

of Y-931 were investigated. Our data clearly demonstrated that clozapine was easily oxidized in the presence of HRP and GSH. This effect was amplified in the presence of hydrogen peroxide which reinforced the oxidant power of the system strongly reacting with the drug. As a result, we observed that the addition of olanzapine and Y-931 in both systems had no or minimal effect on the EPR signal intensity. In a previous paper, fluperlapine, well known to induce agranulocytosis, was indifferent to the enzymatic oxidation.¹⁹⁾ It therefore seems that metabolites of fluperlapine induce the side effect.^{20,21)} However, the metabolites of Y-931 had no or little effect on the oxidation in the HRP/GSH or HRP/ H_2O_2 /GSH system. In the electrochemical experiment, the order of the agents in terms of oxidability at pH 4.7 and pH 7.4 was clozapine < Y-931 < olanzapine. On the other hand, GSH had a lower oxidation potential (215 mV (pH 4.7), 114 mV (pH 7.4)) than any of these three drugs. Hence, in reactivity against GSH, the oxidized drugs (drug cation radical) ranked olanzapine < Y-931 < clozapine. Thus, the redox potential values for the three derivatives were in accord with the results of EPR. In the toxicological experiment in human leukocytes, when neutrophils were incubated with clozapine and hydrogen peroxide, significant toxicity toward them was observed. However, no toxicity was observed in the absence of H_2O_2 . The most likely explanation of this finding is that the hydrogen peroxide enters the cell and is used as a cofactor by intracellular enzymes (presumably MPO) to generate the cytotoxic clozapine-reactive metabolite. Furthermore, no toxicity was observed with Y-931 and olanzapine in the presence or absence of H_2O_2 . Therefore, it seems that Y-931 has little propensity to induce agranulocytosis, similar to olanzapine, in the clinic.

Recently, Liégeois *et al.* reported that the reduced toxicity with olanzapine compared to clozapine could result from a difference in dosage.²²⁾ Indeed, clozapine is generally administered in the range of 200–900 mg/d, while for olanzapine the usual dose is 10–20 mg/d. Consequently, the low therapeutic plasma concentrations of olanzapine (0.03–0.1 $\mu\text{mol/l}$)^{23,24)} compared to those of clozapine (0.2–1.6 $\mu\text{mol/l}$)²⁵⁾ might explain why olanzapine is not frequently associated with agranulocytosis in humans.

Table 2. Electrochemical Behavior of Clozapine, Olanzapine, Y-931, and GSH

	Acetate buffer 0.25 M pH 4.7 Ep (mV vs. Ag/AgCl)	Phosphate buffer 0.1 M pH 7.4 Ep (mV vs. Ag/AgCl)
Clozapine	538.8 (450) ^{a)}	409.9 (375) ^{a)}
Y-931	456.3	294.5
Olanzapine	374.0 (285) ^{a)}	202.8 (150) ^{a)}
Glutathione ^{b)}	215	114

a) Reference values (22). b) 10 mmol/l solution.

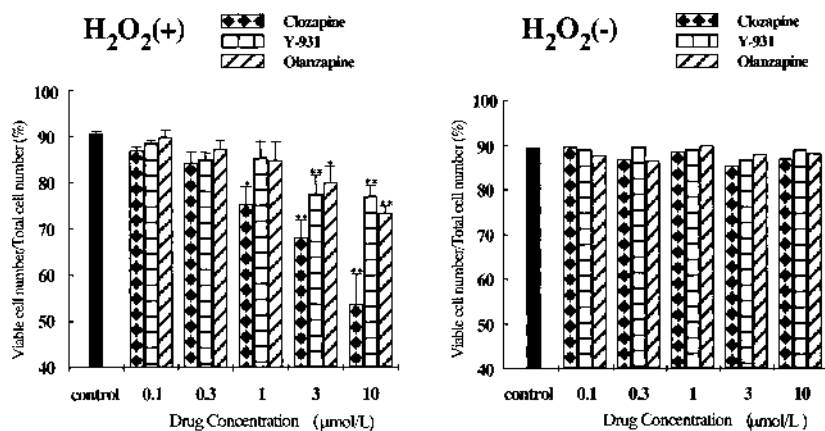


Fig. 3. Toxicity of Clozapine, Y-931 or Olanzapine Reactive Intermediates in Human Neutrophils

Drug concentrations varied between 0.1 and 10 $\mu\text{mol/l}$ as indicated. Hydrogen peroxide was used to generate the reactive intermediates: (left panel) reactive metabolites produced with hydrogen peroxide (1 mmol/l); (right panel) hydrogen peroxide was absent. Cells were incubated at 37 °C for 2 h. Results are expressed as a percentage of the number of viable cells present in untreated samples incubated at 37 °C for 2 h. Each bar represents the mean \pm S.E.M. of data from four human. * Significantly different from vehicle control ($p < 0.05$), Dunnet method. ** Significantly different from vehicle control ($p < 0.01$), Dunnet method.

There is no real predictive model for hematological side effects of antipsychotic drugs. Indeed, the recent case of remoxipride has confirmed that some side effects are not easily detectable during clinical phases of development. Remoxipride was withdrawn because it caused aplastic anaemia.^{26,27)}

In conclusion, Y-931 was less sensitive to oxidation than clozapine similar to olanzapine. Therefore, Y-931 might be an efficient antipsychotic drug with little propensity to induce agranulocytosis.

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