Inhibitory Effect on $^3$H-Diazepam Binding and Potentiating Action on GABA of Ethyl Loflazepate, a New Minor Tranquilizer

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Accepted January 7 1985

Abstract—A new benzodiazepine compound, ethyl loflazepate (ethyl-7-chloro-2,3-dihydro-5-(2-fluorophenyl)-2-oxo-1H,1,4-benzodiazepine-3-carboxylate; CM6912) was studied using in vitro experimental systems for its displacement activity on $^3$H-diazepam binding to the synaptosomal membrane fraction of rat cerebrum and potentiating action on GABA. CM6912 inhibited the specific binding of $^3$H-diazepam by 25%, 75% and 90% at concentrations of 0.01 μM, 0.1 μM and 1 μM, respectively, while its metabolites CM6913 and CM7116, at 0.1 μM, completely inhibited the binding. Concentrations for 50% inhibition (IC50) were 25 nM for CM6912, 3.2 nM for CM6913 and 1.4 nM for CM7116. These results suggest that the metabolite CM7116 is stronger than its parent compound in displacing the $^3$H-diazepam binding, and they also suggest that the long-lasting anti-anxiolic action of CM6912 might be due to the in vivo formation of CM7116. CM6912, CM7116 and diazepam potentiated the suppressive action of GABA on spontaneous spikes of Purkinje cells in guinea pig cerebellar slices in a dose-dependent manner. Concentrations for 50% suppression (IC50) were 96.0 μM for GABA alone, 75.0 μM for GABA plus diazepam (5 μM), 78.9 μM for GABA plus CM6912 (5 μM) and 60.8 μM for GABA plus CM7116 (5 μM). These findings suggest that CM6912 and CM7116 may potentiate the postsynaptic inhibitory action of GABA in a manner similar to and probably more strongly than diazepam.

Since the first introduction of chlordiazepoxide by the Roche Co. in 1957, many benzodiazepine derivatives such as diazepam, nitrazepam, etc. have been commercialized in many countries. Some of these benzodiazepines are clinically utilized as anti-anxiety drugs (minor tranquilizers), and others are used as sleep inducers. Ethyl loflazepate (CM6912, ethyl-7-chloro-2,3-dihydro-5-(2-fluorophenyl)-2-oxo-1H,1,4-benzodiazepine-3-carboxylate; Fig. 1) is one of the benzodiazepine derivatives synthesized first at the Clin-Midy Institute of the Sanofi Co. in France.

As regards to the pharmacological activities of this compound, some in vivo experiments with laboratory animals (1-3) and clinical studies (4, 5) including double blind tests have been carried out, and CM6912 is now established as an anti-

![Chemical structures of CM6912, CM6913 and CM7116 and in vivo transformations.](image-url)
For the purpose of further clarifying the pharmacological properties of CM6912, quantitative studies utilizing in vitro experimental systems were carried out in the present study. Particular interest was directed to the interaction with benzodiazepine receptors (6–11) and the potentiating actions on γ-aminobutyric acid (GABA) of not only CM6912 but also its metabolites CM6913 and CM7116 (Fig. 1).

Materials and Methods

1. Displacement of \(^3\)H-diazepam binding:
The preparation of the cerebral membrane fraction and the binding assay were carried out according to the method of Squires and Braestrup (6). Briefly, the rat cerebrum was homogenized with 15 volumes of 0.32 M sucrose and centrifuged at 2,000×g for 5 min; then the supernatant was centrifuged at 30,000×g for 10 min. The pellet was suspended in 50 volumes of 50 mM Tris-HCl buffer (pH 7.4) and stored at -20°C. For binding assays, 250 μl of the membrane suspension was incubated for 30 min at 4°C with 10 μl of \(^3\)H-diazepam solution (specific activity=40 Ci/mMole, New England Nuclear, the final concentration of diazepam=1.65 nM) and with 1.0 μl of a drug solution. Then the incubation was terminated by adding 5 ml of ice-cold 50 mM Tris-HCl buffer, and the mixture was immediately filtered under aspiration through a Whatman GF/C glass filter. The filter was washed with 5 ml of 50 mM Tris-HCl buffer (pH 7.4), dried and placed in a scintillation vial for radioactivity counting. Assays were carried out in duplicate.
The non-specific binding of \(^3\)H-diazepam was similarly measured but in the presence of 2 μM unlabeled diazepam. The value of the specific binding was calculated by subtracting the non-specific binding from the total binding. The non-specific binding was 5–10% of the specific binding.

Drugs tested were CM6912, CM7116 and diazepam. These drugs were first dissolved in ethanol and diluted with warmed Krebs-Ringer bicarbonate medium (37°C) to give a final concentration of 5 μM or 10 μM, the final ethanol concentration being 0.06% (13 mM). GABA was dissolved in Krebs-Ringer bicarbonate medium to give final concentrations of 0.05, 0.075, 0.1 and 0.125 mM.

Results

1. Displacement of \(^3\)H-diazepam binding:
As shown in Fig. 2, the specific binding of \(^3\)H-diazepam was inhibited by 25%, 75% and almost completely by 0.01 μM, 0.1 μM and 1 μM of CM6912, respectively. The concentration of CM6912 to achieve 50% inhibition (IC50) was 25 nM. Both CM6913 and CM7116 at 0.01 μM also inhibited the \(^3\)H-diazepam binding by about 80%, but CM7116 appeared to be more potent than CM6916 at all concentrations tested. At
0.1 \mu M, both CM6913 and CM7116 exhibited nearly complete inhibition. The values of IC50 were 3.2 nM for CM6913 and 1.4 nM for CM7116.

Although not shown in Fig. 2, the IC50 values for the other benzodiazepines measured in a similar manner were 10 nM for diazepam, 3.4 nM for nitrazepam and 1.4 nM for lorazepam. Therefore, the potency of CM6912 to inhibit the \(^3\)H-diazepam binding was weaker than that of diazepam, while CM6913 was as potent as nitrazepam, and CM7116 was comparable with lorazepam.

2. Suppressive action of GABA on spontaneous spike discharges of cerebellar Purkinje cells and the potentiating effect thereon of CM6912, CM7116 and diazepam: Spontaneous spike discharges of Purkinje cells are known to be rapidly and reversibly suppressed by superfused GABA (12, 13). Such a suppression is usually followed by a partial recovery even in the presence of GABA (12, 13). The maximum inhibition of the spike discharge frequency was employed in this study.

Typical dose-response curves for the inhibitory action of GABA and the effect of diazepam are shown in Fig. 3. The control dose-response curve was clearly shifted to the left by the presence of 5 \mu M or 10 \mu M diazepam, indicating that the suppressive action of GABA was potentiated by this benzodiazepine.

As shown in Fig. 4, CM6912 or CM7116 also shifted the control curve to the left in a dose-dependent manner and in parallel. No antagonism was observed at all.

The values of percentage inhibition for GABA in the absence and presence of diazepam, CM6912 or CM7116 are shown in Table 1. Concentrations of GABA to show 50% inhibition (IC50) in the absence and presence of each benzodiazepine are listed in Table 2. The IC50 values were 96.0 \mu M for GABA alone, and 75.0 \mu M, 78.9 \mu M and 60.8 \mu M in the presence of diazepam.
CM6912 and CM7116 (all 5 ,WM), respectively, indicating the potentiation of the inhibitory action of GABA. The values of IC50 became smaller when the concentration of each benzodiazepine was raised to 10 i M. These results indicate that CM6912 is comparable with diazepam in potentiating the action of GABA, and they also indicate that CM7116 is slightly more effective than CM6912.

![Fig. 4](image)

**Fig. 4.** Log dose-response curves for the suppressive action of GABA on the frequency of spontaneous spikes of cerebellar Purkinje cells and the potentiating effects of CM6912 and CM7116 (5 ,M and 10 ,M). The values plotted are the means ±S.E.M. of 6–10 observed data for CM6912 and CM7116 (see Table 1). For further explanations, refer to Fig. 3 legend.

**CM6912 and CM7116 (all 5 ,M), respectively, indicating the potentiation of the inhibitory action of GABA. The values of IC50 became smaller when the concentration of each benzodiazepine was raised to 10 ,M. These results indicate that CM6912 is comparable with diazepam in potentiating the action of GABA, and they also indicate that CM7116 is slightly more effective than CM6912.**

**Discussion**

Behavioral pharmacological methods have been usually utilized for examining whether a compound has psychotropic activities or not, whenever it is expected to have anti-anxiety, neuroleptic or anti-depressant activity. However, individual variations in experimental animals and difficulties in setting consistent in vivo experimental conditions are unavoidable problems in this pharmacological approach.

On the other hand, in vitro experimental systems such as those used in the present study enable us not only to perform quantitative studies but also to set constant experimental conditions, resulting in high reproducibility. Taking these advantages of the in vitro system into consideration, the results of the present study are discussed below.

1. **Binding assays with benzodiazepine receptors:** The presence of specific receptors for benzodiazepines in the brain was first discovered by Braestrup et al. (6–11) in 1977. The binding abilities of various benzodiazepines to such receptors have been demonstrated to be closely correlated with their pharmacological potencies as minor tranquilizers. It is also known that the high-affinity specific binding of 3H-diazepam is displaced by various benzodiazepines not only in a stereospecific manner but also in parallel to their pharmacological potencies. Thus, it was thought that the measurement of the inhibitory ability of CM6912 to the 3H-diazepam binding may be an effective approach to estimate the psychotropic activity of this compound.

It was found in the present study that the specific binding of 3H-diazepam was inhibited 25% by CM6912 at 0.01 ,M and nearly completely inhibited at 1 ,M. It was also observed that CM7116 and CM6913, metabolites of CM6912, inhibited the 3H-diazepam binding by 80% at a low concentration of 0.01 ,M. These results, therefore, suggest that CM6912 has pharmacological properties common to benzodiazepine families. In our separate experiments, the IC50 value for diazepam has been found to be 10 nM, while those for CM6912, CM7116 and CM6913 were 25 nM, 1.4 nM and 3.2 nM, respectively (Fig. 2). As mentioned earlier and shown in Fig. 1, CM6912 is known to be rapidly metabolized after oral administration (14). Thus, it may be mostly in the form of CM6913 and CM7116 in the blood. Therefore, it may be expected that the in vitro pharmacological activity of CM6912 might be intensified by the in vivo formation of these metabolites and probably becomes stronger than that of diazepam (1). Since the IC50 values for nitrazepam and lorazepam are 3.4 nM and 1.4 nM, respectively (Y. Sakai and N. Namima, unpublished observation), both CM6913 and CM7116 may be thought to exhibit similar clinical potencies as either...
Table 1. Percentage inhibition of spike discharge frequency by GABA in the presence and absence of diazepam, CM6912 and CM7116

| GABA (mM) | +Nil (Control) | +Diazepam | +CM6912 | +CM7116 |
|-----------|----------------|-----------|---------|---------|
|           | 5 μM  | 10 μM    | 5 μM    | 10 μM   | 5 μM    | 10 μM   |
| 0.05      | 14.3±2.2 (24) | 24.6±9.8 (9) | 30.1±7.4 (9) | 22.2±3.2 (6) | 30.8±5.6 (7) | 37.4±6.2 (9) | 51.3±8.9 (10) |
| 0.075     | 32.0±3.4 (24) | 47.9±10.2 (9) | 54.0±9.5 (9) | 49.4±7.3 (7) | 54.0±6.6 (7) | 62.3±8.1 (9) | 72.2±10.5 (10) |
| 0.100     | 52.6±5.4 (24) | 79.7±9.9 (8) | 79.7±6.9 (9) | 70.0±9.8 (7) | 79.7±7.0 (7) | 74.2±6.7 (9) | 83.2±7.5 (10) |
| 0.125     | 78.1±5.8 (24) | 93.5±6.5 (8) | 96.4±2.5 (9) | 83.8±9.5 (7) | 93.4±3.5 (7) | 83.2±8.7 (9) | 88.5±6.7 (10) |

Significant differences from the control: *P<0.001, **P<0.005, ***P<0.010, #P<0.025, $P<0.050 and †P<0.100. The number of observations are in parentheses.

Table 2. IC50 values for the inhibitory action of GABA on spike discharge frequency in the presence and absence of diazepam, CM6912 and CM7116

| GABA alone (Control) | IC50 (μM) |
|----------------------|-----------|
|                      | 96.0      |
| GABA+Diazepam        |           |
| 5 μM                 | 75.0      |
| 10 μM                | 71.4      |
| GABA+CM6912          |           |
| 5 μM                 | 78.9      |
| 10 μM                | 70.7      |
| GABA+CM7116          |           |
| 5 μM                 | 60.8      |
| 10 μM                | 48.3      |
nitrazepam or lorazepam.

2. Potentiation of the action of GABA: It is known that benzodiazepines potentiate the action of GABA by acting either postsynaptically or presynaptically in GABAergic neurons (15–17), although detailed mechanisms are not clarified yet. On the other hand, it has also been demonstrated that benzodiazepines may displace the binding of glycine or strychnine to central glycine receptors, and such displacing activities of various benzodiazepines are correlated with their pharmacological potencies, although these findings do not necessarily mean that benzodiazepines exhibit their pharmacological actions by acting directly on glycineric neurons (18–20).

As regards to the potentiation by benzodiazepines of the action of GABA (17, 21), the following three mechanisms may be considered: 1) the increase of the synaptic release of GABA, 2) the blockade of the reuptake of GABA, and 3) the sensitization of postsynaptic receptors for GABA (22).

Previously we have reported (12) that chlordiazepoxide (10 μM) potentiates the inhibitory actions of externally applied GABA, β-alanine and taurine, but not of glycine, on the frequency of spontaneous spike discharges of Purkinje cells in cerebellar slices of guinea pigs. Based on this previous finding and also on the absence of the potentiating action of a GABA uptake inhibitor on 3H-GABA release as reported by Mitchell and Martin (23), it may be said that benzodiazepines augment the inhibitory action of GABA at the postsynaptic receptor site.

As shown in Fig. 4 and Tables 1 and 2, not only CM6912 but also its metabolite CM7116 potentiated the inhibitory action of GABA in a similar manner as diazepam (Fig. 3), indicating that both CM6912 and CM7116 have similar pharmacological properties as diazepam. According to the values of IC50, the potentiating potency of CM6912 was comparable with that of diazepam, while the metabolite CM7116 was more potent than diazepam. Thus, there is a close correlation between these electropharmacological findings and the results obtained by the binding assay.

Acknowledgement: We are grateful to Meiji Seika Co., Ltd., for supplying CM6912 and its metabolites for this work.

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