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# Interactions between loreclezole, chlormethiazole and pentobarbitone at $GABA_A$ receptors: functional and binding studies

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**1** Interactions were investigated between loreclezole, chlormethiazole and pentobarbitone as potentiators of depolarization responses mediated by γ-aminobutyric $acid_A$ ($GABA_A$) receptors on afferent nerve terminals in the rat cuneate nucleus *in vitro*. These drugs were also compared as modulators of [$^3$H]-flunitrazepam (FNZ) binding to synaptic membranes prepared from rat whole brain homogenate.

**2** In rat cuneate nucleus slices, the drugs shifted muscimol log dose–response lines to the left in an approximately parallel fashion with the result that 200 μM chlormethiazole potentiated muscimol responses by $0.567 \pm 0.037$ log unit (mean ± s.e.mean, $n=4$) while loreclezole gave a maximal potentiation at 10 μM of only $0.121 \pm 0.037$ ($n=6$) log unit and $0.071 \pm 0.039$ ($n=22$) at 50 μM.

**3** While 50 μM chlormethiazole and 30 μM pentobarbitone showed no significant interactions between each other when potentiating muscimol responses in combination, 50 μM loreclezole in combination with either chlormethiazole or pentobarbitone attenuated their potentiating effects, possibly by inducing desensitization of $GABA_A$ receptors.

**4** In the [$^3$H]-FNZ binding studies on well-washed membranes, loreclezole enhanced binding to a maximum of $47.3 \pm 2.83$% of control (mean ± s.e.mean, $n=3$) at 300 μM. Scatchard analysis revealed no change in $B_{max}$ but a decrease in $K_D$ for [$^3$H]-FNZ from $3.9 \pm 0.29$ nM to $2.7 \pm 0.10$ nM (mean ± s.e.mean, $n=4$) in the presence of 100 μM loreclezole. In contrast, 100 μM chlormethiazole caused no potentiation. A small component of the enhancement by loreclezole could be blocked by 100 μM bicuculline and could also be blocked by 100 μM chlormethiazole. It seems likely that the effects on [$^3$H]-FNZ binding are due predominantly to direct actions of the drugs on the $GABA_A$ receptor and are separate from the GABA-potentiating effects.

**5** The results indicate distinctly different profiles of action for loreclezole, chlormethiazole and pentobarbitone on $GABA_A$ receptors.

**Keywords:** Loreclezole; chlormethiazole; pentobarbitone; $GABA_A$ receptors; [$^3$H]-flunitrazepam binding; cuneate nucleus

## Introduction

Much evidence suggests that γ-aminobutyric $acid_A$ ($GABA_A$) receptors are the targets of a variety of pharmacologically important drugs, such as benzodiazepines, barbiturates, steroids, general anaesthetics, convulsants acting at the picrotoxin site and chlormethiazole (for review, see Sieghart, 1995). These classes of drug each act on specific binding sites, which can allosterically interact with each other, to modulate the activity of the $GABA_A$ receptors. Only for the benzodiazepines (Wafford *et al*., 1994) and barbiturates (Thompson *et al*., 1996) amongst these drugs is there strong evidence for the modulatory action being dependent on the subunit composition of the receptors.

A more recent addition to the list of $GABA_A$ modulators is loreclezole ((Z)-1-(β,2,4-trichlorostyryl)-1*H*-1,2,4-triazole), a broad spectrum anticonvulsant. It inhibited [$^3$H]-phenyl-4-t-butyl-2,6,7-trioxabicyclo(2,2,2)octane (TBOB) binding to the picrotoxin site on $GABA_A$ receptors with an $IC_{50}$ of 3 μM (Van Rijn & Willems-van Bree, 1993), behaved more like a barbiturate than a benzodiazepine in two *in vivo* models (Ashton, *et al*., 1992) and was not sensitive to the benzodiazepine receptor antagonist, flumazenil (Dawson *et al*., 1994). As a GABA potentiator, loreclezole has been demonstrated to be highly selective for receptors containing the β2 or β3 subunit over those containing the β1 subunit (Wafford *et al*., 1994), and this unique subunit dependence was determined by a single amino acid, β2 Asn-289 (β3 Asn-290) (Wingrove *et al*., 1994).

In order to classify the pharmacological mechanism of action of loreclezole, we compared its actions with those of chlormethiazole since there is some structural resemblance between loreclezole and chlormethiazole (Figure 1). We compared these compounds on (a) rat cuneate nucleus slices where chlormethiazole has been shown to potentiate responses to muscimol (Harrison & Simmonds, 1983) and (b) on the modulation of [$^3$H]-flunitrazepam binding where, unusually for a $GABA_A$ potentiator, chlormethiazole did not enhance the binding (Cross *et al*., 1989) but, at high concentrations, inhibited it (Moody & Skolnick, 1989). Some further comparisons were also made with pentobarbitone. A preliminary account of a part of this work has been presented (Zhong & Simmonds, 1996).

In parallel with this work, comparisons between loreclezole, chlormethiazole and pentobarbitone were made on a seizure model and additional ligand-binding sites on the $GABA_A$ receptors (Green *et al*., 1996).

## Methods

### *Functional study*

Experiments were performed on slices of medulla oblongata containing the dorsal funiculus and cuneate nucleus, prepared from male Wistar rats (100–150 g) as previously described (Simmonds, 1978; Harrison & Simmonds, 1983). Each slice was placed in a two-compartment bath so that the dorsal funiculus projected through a grease-filled gap in a barrier se-

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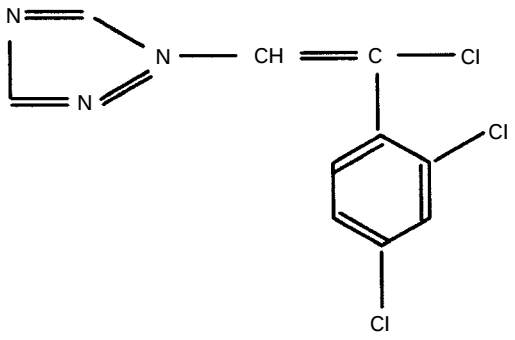
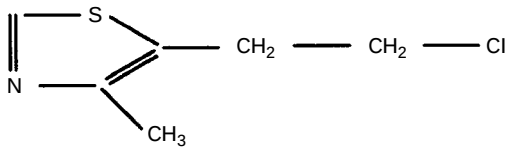



Loreclezole




Chlormethiazole

**Figure 1** Structures of loreclezole and chlormethiazole.

parating the two compartments. The slices were superfused with Krebs medium at room temperature and only the compartment containing the terminals of the dorsal funiculus within the cuneate nucleus was superfused with drugs incorporated into the Krebs medium. The potential difference between the two compartments was recorded continuously and the negativity induced in the drug-perfused compartment was interpreted as a depolarization of the terminals of dorsal funiculus fibres projecting through the barrier. Responses were measured at their peak amplitudes.

Muscimol was used routinely as the $GABA_A$ receptor agonist. To minimize the problem of desensitization, control responses in the lower part of the log dose-response curve were used and these were routinely obtained from 2 min superfusions of 1.25 and 2.5 $\mu$M muscimol. In each experiment, where two drugs were tested both singly and in combination, a muscimol control curve was first obtained, then one drug was superfused for 30 min before and during the redetermination of responses to muscimol. The same procedure was then carried out with a combination of the two drugs. The muscimol dose-response lines were always displaced in an approximately parallel fashion so each experiment gave a single value of the shift of the muscimol log dose-response line by the modulatory drug (see Harrison & Simmonds (1983) for a fuller description).

The Krebs medium contained (mM): NaCl 118, KCl 2.1, $KH_2PO_4$ 1.2, $CaCl_2$ 2.5, $MgSO_4$ 2.2, $NaHCO_3$ 25 and glucose 11 and was continuously gassed with 95% $O_2$: 5% $CO_2$ to give pH 7.4. Muscimol was prepared as a 10 mM solution in distilled water and diluted into the Krebs medium just before use. Chlormethiazole and pentobarbitone were dissolved directly in the Krebs medium. Loreclezole was first dissolved in a little acetone and then diluted into the Krebs medium. The final concentration of acetone never exceeded 0.1% which had no effect on muscimol responses in this preparation.

### *Binding study*

*Membrane preparation* Crude rat brain synaptic membranes were prepared as described previously (Prince & Simmonds, 1992). Briefly, male Wistar rats (150–200 g) were killed by decapitation and their brains homogenized in 20 volumes of ice-cold wash buffer (5 mM Tris-HCl, 1 mM EDTA, pH 7.4 at 4°C) for 1 min, with an Ultra Turrax homogenizer. The homogenate was centrifuged for 10 min at 1000 x*g* at 4°C (Beckman J2–21 M/E centrifuge). The supernatant was collected and centrifuged for 20 min at 48,000 x*g*. The resultant pellet was resuspended in wash buffer and centrifuged for 20 min at 48,000 x*g* a further three times. The final pellet was resuspended in wash buffer at a concentration of 2–3 mg protein $ml^{-1}$ and then frozen at −20°C until required (14 days maximum). The protein yield was about 20 mg protein $g^{-1}$ brain.

On the day of the assay, aliquots of 8 ml of stored tissue were thawed and washed twice with 20 ml distilled water and twice with the assay buffer (50 mM Tris, 150 mM NaCl, pH 7.4 at 4°C) by centrifugation for 20 min at 48,000 x*g*. The final pellet was resuspended in assay buffer to give a protein concentration of approximately 2 mg $ml^{-1}$, as determined by the Biorad assay (Biorad Laboratories Ltd).

### *[$^3$H]-flunitrazapam (FNZ) binding*

*Concentration-effect relationship* One hundred microlitre aliquots of membranes (0.4 mg protein $ml^{-1}$) were pre-incubated with various concentrations of drugs for 10 min at 37°C (Prince & Simmonds, 1992). Then 50 $\mu$l of [$^3$H]-FNZ was added to each tube to give a final concentration of 1 nM and a total volume of 500 $\mu$l assay buffer. The samples were incubated for 60 min at 4°C. The binding reaction was terminated by addition of 2 ml ice-cold wash buffer, followed by rapid filtration through Whatman GF-C filters by use of a Brandel Cell Harvester. The filters were washed 4 times with 2 ml ice-cold wash buffer and the bound radioactivity quantified by conventional liquid scintillation techniques. Non-specific binding, which was less than 5% of the total binding, was determined by addition of 10 $\mu$M unlabelled flunitrazepam.

*Saturation curve* One hundred microlitre aliquots of membranes (0.4 mg protein $ml^{-1}$) were incubated with 0.1–20 nM [$^3$H]-FNZ for 60 min at 4°C in the presence or absence of loreclezole (100 $\mu$M) in a total sample volume of 500 $\mu$l. The binding reaction was terminated by the addition of ice-cold wash buffer, followed by rapid filtration and washing, as described above. Non-specific binding, which was less than 5% of the total binding, was determined for each concentration of [$^3$H]-FNZ by addition of 10 $\mu$M unlabelled FNZ.

Loreclezole was initially dissolved in acetone which was present in all samples at a final concentration of 1%. Chlormethiazole and bicuculline ((−)-bicuculline methochloride) were dissolved directly in assay buffer. Unlabelled FNZ was initially dissolved in DMSO (dimethyl sulphoxide) or acetone then diluted in assay buffer.

### *Chemicals*

[$^3$H]-FNZ (82.0 Ci $mmol^{-1}$) was obtained from Du Pont, U.K. Chlormethiazole and loreclezole were gifts from Astra and Janssen, respectively. Pentobarbitone, muscimol, (−)-bicuculline methochloride, unlabelled FNZ and other chemicals were obtained from Sigma.

## Results

### *Functional study on the cuneate nucleus*

*Effects of chlormethiazole, pentobarbitone and loreclezole individually* The time-courses of the responses to muscimol and the overall appearance of the responses were very similar to those previously described for this preparation (e.g. Simmonds & Turner, 1985). In agreement with earlier results (Harrison & Simmonds, 1983; Simmonds & Turner, 1987), chlormethiazole dose-dependently potentiated responses to muscimol on the cuneate nucleus (Figure 2, $P<0.0001$, one-way ANOVA) and a submaximal concentration of pentobarbitone, 30 $\mu$M gave a similar potentiation to 50 $\mu$M chlormethiazole.

However, loreclezole showed a rather different profile (Figure 3). At 5, 10 and 20 $\mu$M, loreclezole gave quite small leftward shifts. At 10 and 20 $\mu$M, the shifts were significant ($P<0.05$, $P<0.01$, respectively, Student's $t$ test). At 50 $\mu$M, the responses were very variable, ranging from a clear potentiating effect to an antagonizing effect and the mean shift was not significant.

*Effect of chlormethiazole, pentobarbitone and loreclezole applied in pairs* When pentobarbitone 30 $\mu$M was co-applied with chlormethiazole 50 $\mu$M, the leftward shifts induced by these two drugs were very similar to the sum of their effects when applied separately (Figure 4). However, when loreclezole 20 $\mu$M and 50 $\mu$M were co-applied with pentobarbitone 30 $\mu$M, the total shifts were less than the sums of their individual effects. As the concentration of loreclezole increased, the total shift decreased and, with loreclezole 50 $\mu$M, was significantly different from the predicted sum of the individual effects ($P<0.05$, Student's $t$ test). Similarly, when loreclezole 10 $\mu$M and 50 $\mu$M were co-applied with chlormethiazole 50 $\mu$M, the total shifts were less than the sums of their individual effects and, with loreclezole 50 $\mu$M, this difference was significant ($P<0.05$, Student's t test).

These results suggest that chlormethiazole and pentobarbitone have no interaction between them when potentiating responses to muscimol. However, loreclezole attenuated the effects of chlormethiazole and pentobarbitone.

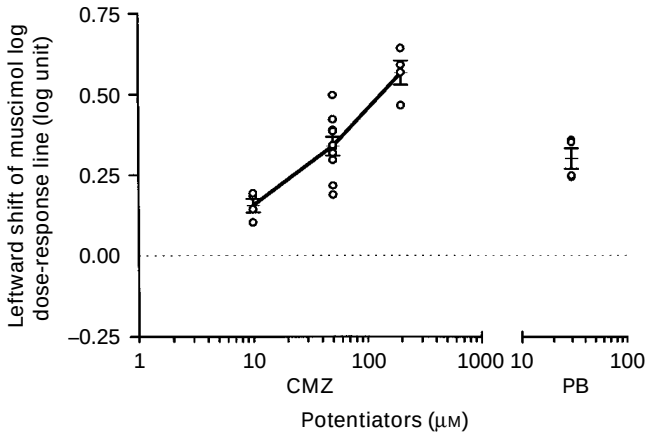



**Figure 2** Potentiation by chlormethiazole (CMZ) and pentobarbitone (PB) of responses to muscimol, expressed as the leftward shift (log unit) of the muscimol log dose-response line. The results of individual experiments as well as means ± s.e.means (vertical lines) of 4 to 10 experiments are shown.

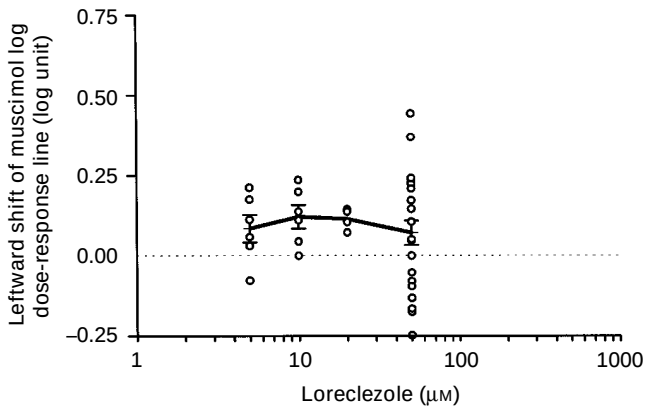



**Figure 3** Effects of loreclezole on responses to muscimol, expressed as the leftward/rightward shift (log unit) of the muscimol log dose-response line. The results of individual experiments as well as means ± s.e.means (vertical lines) of 4 to 22 experiments are shown.

## *Binding studies*

*Enhancement by loreclezole of [$^3$H]-FNZ binding* Loreclezole considerably enhanced [$^3$H]-FNZ binding (Figure 5). The concentration-effect relationship had a threshold at 3 $\mu$M and a maximal 47.3 ± 2.83% increase in binding at 300 $\mu$M (mean ± s.e.mean, $n=3$).

In saturation curve analysis, loreclezole 100 $\mu$M significantly decreased the $K_D$ ($P<0.01$, Student's $t$ test) but not $B_{max}$ of [$^3$H]-FNZ binding (Figure 6). Therefore, the enhancement by loreclezole of [$^3$H]-FNZ binding was due to an increase in binding affinity with no change in the numbers of binding sites.

*Effect of chlormethiazole on the enhancement by loreclezole of [$^3$H]-FNZ binding* At 100 $\mu$M, chlormethiazole did not significantly change [$^3$H]-FNZ binding (1.5 ± 2.35% change from control, mean ± s.e.mean, $n=8$) (Figure 5). However, in the

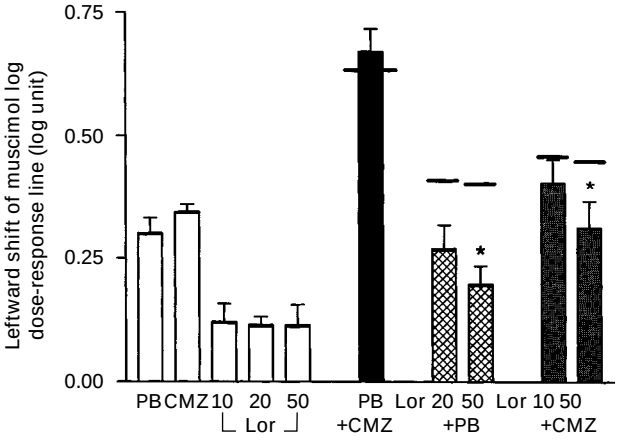



**Figure 4** Effects of combinations of drugs on responses to muscimol, expressed as the leftward shift (log unit) of the muscimol log dose-response line. The open bars show the effects of (from left to right) pentobarbitone 30 $\mu$M (PB), chlormethiazole 50 $\mu$M (CMZ), loreclezole (Lor) 10, 20 and 50 $\mu$M. The shaded columns show the effects of combinations of drugs (from left to right): pentobarbitone 30 $\mu$M + chlormethiazole 50 $\mu$M (PB + CMZ), pentobarbitone 30 $\mu$M + loreclezole 20 $\mu$M (Lor 20 + PB), pentobarbitone 30 $\mu$M + loreclezole 50 $\mu$M (Lor 50 + PB), chlormethiazole 50 $\mu$M + loreclezole 10 $\mu$M (Lor 10 + CMZ), chlormethiazole 50 $\mu$M + loreclezole 50 $\mu$M (Lor 50 + CMZ). For the combinations of drugs, the predicted dose-ratios assuming simple additive effects with no interactions, i.e. additions of the individual log shifts, are shown as short horizontal lines above the actual shifts obtained. Comparisons of the predicted and actual shifts were made by Student's $t$ test (*$P<0.05$). The results are the means ± s.e.means (vertical lines) of 4 to 10 experiments.

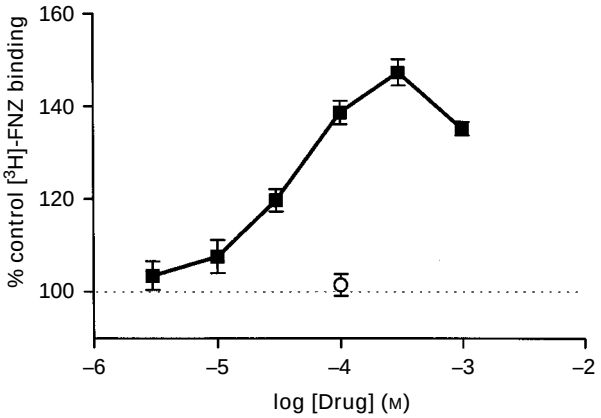



**Figure 5** Concentration-effect relationship for the enhancement by loreclezole of [$^3$H]-flunitrazepam ([$^3$H]-FNZ) binding (■). The effect of 100 $\mu$M chlormethiazole is also shown (○). Data points are the means ± s.e.means of 3 experiments.

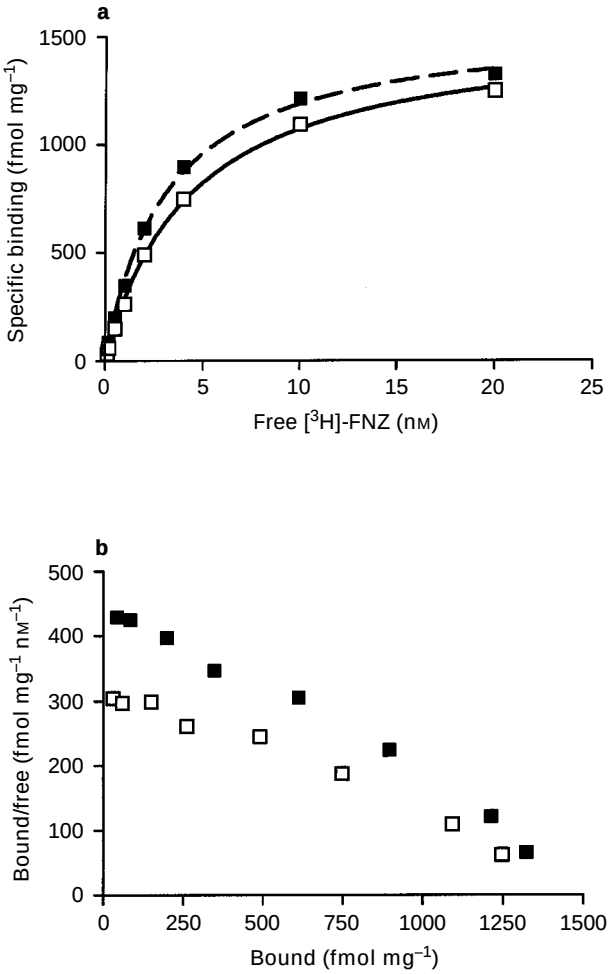



**Figure 6** Saturation curve (a) and Scatchard plot (b) for [$^3$H]-flunitrazepam ([$^3$H]-FNZ) binding in the presence (■) and absence (□) of loreclezole (100 $\mu$M) from a typical experiment. The results from 4 experiments (means ± s.e.means) showed that, in the absence of loreclezole, [$^3$H]-FNZ binding had a $K_D$ of 3.9 ± 0.29 nM and a $B_{max}$ of 1149 ± 30 fmol mg$^{-1}$; in the presence of loreclezole (100 $\mu$M), [$^3$H]-FNZ binding yielded a $K_D$ of 2.7 ± 0.10 nM*, and a $B_{max}$ of 1204 ± 15 fmol mg$^{-1}$ (*significantly different from control value, $P<0.05$).

presence of chlormethiazole 100 $\mu$M, loreclezole gave significantly smaller enhancements (Figure 7), the decreases being significant ($P<0.05$, Student's $t$ test) with loreclezole 100 $\mu$M, 300 $\mu$M and 1 mM. These results suggest that chlormethiazole can partially block the potentiating effect of loreclezole on [$^3$H]-FNZ binding.

*Effect of bicuculline on the enhancement by loreclezole of [$^3$H]-FNZ binding* On well washed membranes, there was very little endogenous GABA left, as evidenced by the fact that 100 $\mu$M bicuculline failed to reduce [$^3$H]-FNZ binding significantly (−3.9 ± 1.96% from control, mean ± s.e.mean, $n=3$). Nevertheless, it was possible that part of the potentiating action of loreclezole on [$^3$H]-FNZ binding was due to potentiation of a subthreshold GABA tone. Therefore, in this series of experiments, 100 $\mu$M bicuculline was routinely used.

The influence of 100 $\mu$M bicuculline on the concentration–effect relationship for 60 min exposure to loreclezole is shown in Figure 7. In the presence of bicuculline 100 $\mu$M loreclezole gave a smaller enhancement of [$^3$H]-FNZ binding than in the absence of bicuculline, the decreases with 30 $\mu$M, 300 $\mu$M and 1 mM loreclezole being significant ($P<0.05$, Student's $t$ test). These results suggest that there are bicuculline-sensitive and bicucullin-insensitive components in the enhancement by loreclezole of [$^3$H]-FNZ binding.

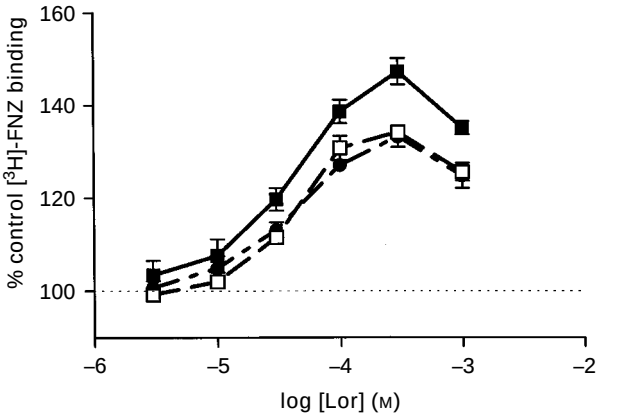



**Figure 7** Concentration-effect relationships for the enhancement of [$^3$H]-flunitrazepam ([$^3$H]-FNZ) binding by loreclezole (Lor) alone (■), and in the presence of bicuculline 100 $\mu$M (□) or chlormethiazole 100 $\mu$M (●). Data points are the means ± s.e.means (vertical lines) of 3 to 4 experiments.

*Effect of bicuculline and chlormethiazole on the enhancement by loreclezole of [$^3$H]-FNZ binding* Since the concentration–effect relationships of loreclezole in the presence of chlormethiazole or bicuculline were superimposed on each other, we were interested to find out if the two components identified by chlormethiazole and bicuculline were the same. A combination of 100 $\mu$M chlormethiazole and 100 $\mu$M bicuculline did not depress the enhancement by loreclezole of [$^3$H]-FNZ binding any further than either chlormethiazole or bicuculline on its own (data not shown).

*Enhancement by pentobarbitone of [$^3$H]-FNZ binding* Pentobarbitone did not enhance [$^3$H]-FNZ binding until fairly high concentrations (> 300 $\mu$M) were reached. Neither chlormethiazole nor bicuculline had any significant influence on the concentration–effect relationship of pentobarbitone (data not shown).

## Discussion

Compared to chlormethiazole and pentobarbitone, loreclezole generated much smaller potentiations of muscimol responses in the cuneate nucleus. At high concentrations (50 $\mu$M), loreclezole induced very variable responses, ranging from a clear potentiating effect to an antagonizing effect. When 50 $\mu$M loreclezole was co-applied with pentobarbitone or chlormethiazole, it attenuated the effects of chlormethiazole and pentobarbitone. One explanation could be that loreclezole acted on the same site as chlormethiazole and pentobarbitone and acted as a partial agonist to antagonize their potentiating effects. However, the fact that chlormethiazole and pentobarbitone had no interaction between themselves when potentiating responses to GABA suggests that chlormethiazole and pentobarbitone do not share common binding sites. The most plausible explanation of the attenuations by loreclezole of the actions of chlormethiazole and pentobarbitone, and the variable responses induced by loreclezole on its own, is that loreclezole not only potentiates the muscimol responses but also induces desensitization of $GABA_A$ receptors. Direct evidence in support of this explanation has come from a recent study (Donnelly & Macdonald, 1996), in which it was clearly shown that loreclezole increases desensitization of $GABA_A$ receptor-mediated responses to GABA recorded intracellularly. In the present experiments, therefore, it seems likely that the muscimol-potentiating effect of loreclezole was largely obscured by a concomitant exacerbation of desensitization.

Loreclezole considerably enhanced [$^3$H]-FNZ binding to the crude synaptic membranes prepared from rat whole brain homogenate and this enhancement was due to an increase in

binding affinity rather than a change in the number of binding sites. Both chlormethiazole and bicuculline could block the enhancement by loreclezole of [$^3$H]-FNZ binding to a small extent. The combination of them failed to depress the enhancement by loreclezole any further than either chlormethiazole or bicuculline on its own. This suggests that the component that is sensitive to chlormethiazole is also sensitive to bicuculline and therefore GABA-dependent, and the remaining component is a direct effect of loreclezole which is neither dependent on GABA nor influenced by chlormethiazole. An analogous, bicuculline-resistant enhancement by loreclezole of the binding of the picrotoxin site ligand [$^{35}$S]-TBPS has been found (Sanna *et al*., 1996) and attributed to a direct effect of loreclezole on the $GABA_A$ receptor. The higher concentrations of loreclezole required for the enhancement of [$^3$H]-FNZ binding, compared with those that affected the potentiation of muscimol in the cuneate nucleus, also support the suggestion of a direct effect of loreclezole on [$^3$H]-FNZ binding which is distinct from the effects of loreclezole on responses to muscimol.

In contrast to loreclezole, chlormethiazole dose-dependently potentiated GABA responses in the cuneate nucleus but failed to enhance [$^3$H]-FNZ binding. Pentobarbitone, also a good potentiator in the functional study, did not enhance [$^3$H]-FNZ binding until concentrations in excess of 300 $\mu$M were reached. Chlormethiazole failed to influence this effect of pentobarbitone, a result in agreement with the findings of Moody & Skolnick (1989), but not with those of Cross *et al*. (1989). Bicuculline similarly did not reduce this effect of pentobarbitone. These observations further support the contention that, under the conditions of our [$^3$H]-FNZ binding experiments on well-washed whole brain membranes, with only low levels of endogenous GABA likely to have been present, it was predominantly the direct actions of drugs on the $GABA_A$ receptor that were seen.

In this study, the data on potentiation of muscimol responses were obtained on tissue from a lower level of the neuraxis than the tissue on which [$^3$H]-FNZ binding was studied. It is conceivable, therefore, that the spectrum of subunit compositions of the $GABA_A$ receptors differed between these two preparations. We do not know to what extent, if at all, this may have contributed to the different patterns of modulation of muscimol responses and [$^3$H]-FNZ binding. A further complication is the suggestion that loreclezole has two distinct sites of action on the $GABA_A$ receptor; a $\beta 2/3$ subunit-specific site for potentiation of GABA and a site for desensitization that does not depend on the type of $\beta$-subunit present (Donnelly & Macdonald, 1996). In view of the dominant influence of desensitization in our cuneate nucleus experiments, it is difficult to draw a firm conclusion on the question of a common site for the GABA-potentiating actions of loreclezole and chlormethiazole.

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