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ACCELERATED COMMUNICATION

β-Carboline γ-Aminobutyric Acid$_A$ Receptor Inverse Agonists Modulate γ-Aminobutyric Acid via the Loreclezole Binding Site as well as the Benzodiazepine Site

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SUMMARY

The benzodiazepine site on the γ-aminobutyric acid$_A$ (GABA$_A$) receptor is the principal site of action for a number of structurally diverse compounds, including the β-carbolines, many of which bind with high affinity. The apparent reversal of inhibition and potentiation by high concentrations of methyl-6,7-dimethoxy-4-ethyl-β-carboline (DMCM) and other β-carbolines has been reported by several groups and is insensitive to the benzodiazepine antagonist Ro 15–1788. By using α6-containing receptors, which have low affinity for benzodiazepines, we observed robust potentiation of GABA$_A$ responses by micromolar concentrations of DMCM and other β-carbolines that is dependent on the β subunit variant. The β subunit-dependent potentiation by the anticonvulsant loreclezole is dependent on a single amino acid in the putative transmembrane 2 region. By using single point mutations that discriminate the loreclezole site, we show that potentiation by DMCM is also dependent on the presence of the same amino acid, Asn^{290}, in β2 or β3 (serine in β1), providing evidence that the low affinity site for β-carboline potentiation is the loreclezole site. The potentiation is independent of the α subunit and is more pronounced on α6-containing receptors due to the lack of DMCM inhibition via the benzodiazepine site. In addition, the potentiation observed is competitive with that of loreclezole, and other β-carbolines, such as ethyl-β-carboline-3-carboxylate and propyl-β-carboline-3-carboxylate, act in a similar manner. The finding that β-carbolines can act via the loreclezole site as well as the benzodiazepine site suggests that a wider variety of compounds may act via this site and shows that compounds can interact with more than one modulatory site on the GABA$_A$ receptor.

GABA$_A$ receptors are known to be a heterogeneous population of several subtypes made up from different combinations of α1–6, β1–3, γ1–3, and δ subunits uniquely distributed throughout the brain (1, 2). Although similar in structure, each subtype displays different pharmacological properties dependent on its molecular structure (3). Benzodiazepines, which bind to the GABA$_A$ receptor, can modulate the channel by increasing or decreasing channel opening. Many β-carbolines bind to the benzodiazepine site on the GABA receptor with high affinity; one of the most commonly described is DMCM, which is an inverse agonist that inhibits GABA channel opening by reducing the frequency of channel opening (4). Several previous reports describing the DMCM modulation of GABA receptors have demonstrated a biphasic action, in which the GABA response is inhibited at low concentrations but potentiated at high concentrations (5–7). A recent report has shown this potentiation to be insensitive to the benzodiazepine antagonist Ro 15–1788 and to be present on receptors lacking a γ2 subunit, which is required for benzodiazepine modulation (7). In the present study, we show that the potentiation by DMCM is entirely dependent on the β subunit isoform, being present on β2- and β3- but not β1-containing receptors. Potentiation by the anticonvulsant loreclezole is similarly dependent on β2 and β3 and acts via a novel binding site (8) that is determined by a single aspartic acid residue located in the putative channel lining transmembrane 2 region (9). Single point mutants differing at this residue were used to study modulation by DMCM, and we show that DMCM potentiation is dependent on the same amino acid residue in the β subunit, indicating that the potentiation by β-carbolines observed at micromolar concentrations occurs via the same site as that by produced by loreclezole.

ABBREVIATIONS: GABA, γ-aminobutyric acid; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline; β-CCE, ethyl-β-carboline-3-carboxylate; β-CCP, propyl-β-carboline-3-carboxylate; HEPES, 4-(2-hydroxythyl)-1-piperazineethanesulfonic acid.

Materials and Methods

Oocyte expression. *Xenopus* oocytes were removed from anesthetized frogs and manually defolliculated with fine forceps. After mild collagenase treatment to remove follicle cells (type IA, 0.5 mg/ml for 8 min), the oocyte nuclei were directly injected with 10–20 nl of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, pH 7.0 [nitrocellulose filtered]) containing different combinations of human $GABA_A$ subunit cDNAs (20 ng/μl) engineered into the expression vector pCDM8 or pcDNAAmp. The single point mutants used were previously constructed for a study of loreclezole by Wingrove *et al.* (for mutagenesis methods, see Ref. 9). After incubation for 24 hr, oocytes were placed in a 50-μl bath and perfused with modified Barth's medium consisting of 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, and 2.4 mM $NaHCO_3$, pH 7.5. Cells were impaled with two 1–3 MΩ electrodes containing 2 M KCl and voltage-clamped between −40 and −70 mV. The cell was continuously perfused with saline at a rate of 4–6 ml/min, and drugs were applied in the perfusate. GABA modulators were preapplied for 30 sec before the addition of GABA. GABA was applied until the peak of the response was observed, usually within 30 sec. To prevent desensitization, at least 3 min was allowed between each GABA application for washing. Concentration-response curves were calculated using a nonlinear, least-squares fitting program to this equation: $f(x) = B_{max}/[1 + (EC_{50}/x)^{nH}]$, where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response, and n_H is the Hill coefficient.

Loreclezole was a gift from Janssen, and DMCM, β-CCE, and β-CCP were obtained from Research Biochemicals. All other compounds were obtained from Sigma Chemical Co.

Results and Discussion

Receptors containing α1, α2, α3, or α5 combined with a β and γ2 subunit possess a high affinity binding site for benzodiazepines. Those containing an α6, while retaining a high affinity for the benzodiazepine Ro 15–4513, exhibit dramatically reduced affinity for most benzodiazepine compounds (10, 11). β-Carbolines have long been known to interact with high affinity at the benzodiazepine binding site on $GABA_A$ receptors. A range of different efficacies are apparent, including agonists (abecarnil) and inverse agonists (DMCM, β-CCM). Several compounds have shown anomolous behavior at high concentrations; for example, DMCM and β-CCE appear to inhibit GABA-induced responses in frog isolated sensory neurons at concentrations of <3 μM but to enhance the current at higher concentrations (6).

When examining the β-carboline inverse agonist DMCM on the subunit combination α1β2γ2, we observed an inhibitory component with a high affinity (EC_{50} = 3.7 ± 0.92 nM; maximum inhibition, −40.9 ± 1.7%) correlating with the effects of DMCM binding at the benzodiazepine site (12). At concentrations of >1 μM, the concentration-response curve reversed and DMCM potentiated the GABA EC_{20}. With α6β2γ2S, however, no inhibition occurred, but at concentrations of ≥1 μM, DMCM potentiated the GABA EC_{20} (Fig. 1) with an EC_{50} of 1.7 μM. With [^{3}H]Ro 15–4513, the reported affinity for DMCM at the α6β3γ2 receptor combination is 0.18 μM, 10-fold higher than the apparent functional EC_{50}.[1] As the α6 subunit confers low affinity for benzodiazepines,

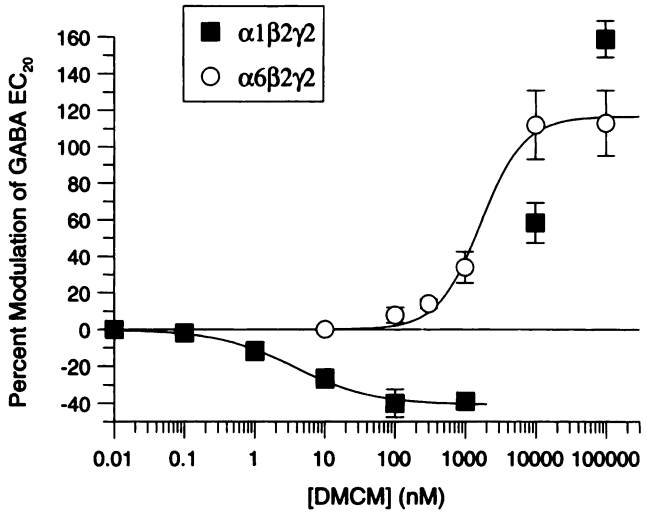

Fig. 1. Concentration-response curves for DMCM on the human $GABA_A$ receptor combinations α1β2γ2s (■) and α6β2γ2s (○) expressed in *Xenopus* oocytes. DMCM was coapplied with GABA EC_{20} values determined for each oocyte. Values are the mean ± standard error of at least four oocytes. Curves were fitted as described.

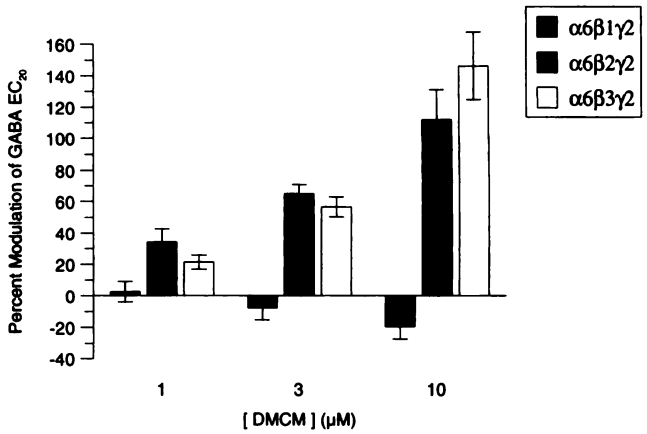

Fig. 2. Effects of increasing concentrations of DMCM on human $GABA_A$ receptor subtypes α6β1γ2s, α6β2γ2s, and α6β3γ2s expressed in *Xenopus* oocytes. DMCM was coapplied with GABA EC_{20} values determined for each oocyte. Values are the mean ± standard error of at least four oocytes.

α6-containing receptors were used to study the potentiating effects of DMCM in the absence of the inhibitory component.

The effects of DMCM at 1, 3, and 10 μM on the GABA EC_{20} were studied on receptors containing α6β1γ2S, α6β2γ2S, and α6β3γ2S (Fig. 2). As observed for β2-containing receptors, α6β3γ2 GABA responses were potentiated by micromolar concentrations of DMCM, with 10 μM eliciting 146 ± 21% potentiation on α6β3γ2 and 112 ± 19% on α6β2γ2. With α6β1γ2, however, no effect was observed at 1 μM, with slight inhibition at 3 and 10 μM (−19 ± 7.9%). We previously reported that the β subunit isoform does not influence benzodiazepine binding affinities or efficacies on cloned human $GABA_A$ receptors and that 300 nM DMCM produced equivalent inhibition on α1β1γ2, α1β2γ2, and α1β3γ2 receptors expressed in *Xenopus* oocytes (12). As can be seen in Fig. 2, however, at concentrations of >300 nM with the α6-containing receptor, DMCM had little effect on α6β1γ2 but produced large potentiation on α6β2γ2 and α6β3γ2, suggesting that

[1] K. L. Hadingham, K. A. Wafford, C. J. Bain, E. M. Garrett, R. P. Heavens, D. J. S. Sirinathsinghji, and P. J. Whiting. Cloning of cDNA encoding the human γ-aminobutyric acid type A receptor α6 subunit and characterization of the pharmacology of α6 containing receptors. Submitted for publication.

this potentiation does not correlate with activity through the benzodiazepine site. Previous experiments on cloned rat $GABA_A$ receptors have shown a reversal phenomena for DMCM at concentrations of >1 μM on α1β2γ2, α5β2γ2, and β2γ2, suggesting this to be independent of an α subunit (5). Recent experiments have also demonstrated that the β-carbolines DMCM, β-CCM, and β-CCP potentiate GABA responses in human embryonic kidney cells expressing rat α1β2γ2, α6β2γ2, and α1β2 at concentrations of >1 μM. This potentiation was not inhibited by the benzodiazepine antagonist Ro 15–1788 and was independent of the barbiturate and steroid site (7).

We recently described a novel binding site on the $GABA_A$ receptor that is sensitive to the anticonvulsant loreclezole (8). This site is independent to that of the benzodiazepine, barbiturate, and steroid sites, being solely selective for receptors containing the β2 or β3 subunit over those containing the β1 subunit. This unique site is determined by a single asparagine residue in the proposed channel-lining transmembrane 2 region of the β2/β3, the corresponding amino acid in β1 being a serine (9). To test the hypothesis that DMCM acts via the same site as the anticonvulsant loreclezole, we used single amino acid mutants that have been shown to discriminate the potentiating effect of loreclezole (9). The mutant $\beta3Asn^{290}Ser$, which has been shown to reduce sensitivity to loreclezole, and the opposite mutation with the equivalent substitution in β1, $\beta1Ser^{290}Asn$, were expressed with α6 and γ2S and compared with wild-type α6β1γ2 and α6β3γ2 with respect to increasing concentrations of DMCM (Fig. 3a). Large potentiation occurred on α6β3γ2 and the $\alpha6\beta1Ser^{290}Asn\gamma2$ receptors; however, very little effect was seen on α6β1γ2 or $\alpha6\beta3Asn^{290}Ser\gamma2$ receptors. To show that this effect was not unique to α6 and a dependence on the β subunit existed in the presence of other α subunits, similar experiments were performed on α1β1γ2, $\alpha1\beta1Ser^{290}Asn\gamma2$, $\alpha1\beta3Asn^{290}Ser\gamma2$, and α1β3γ2 (Fig. 3b). At 1 and 3 μM DMCM, the inhibitory component due to binding at the benzodiazepine site predominated. At 10 μM, inhibition was still observed with receptors containing the $\beta1Asn^{290}Ser$ or $\beta3Asn^{290}Ser$; however, on α1β3γ2 and $\alpha1\beta1Ser^{290}Asn\gamma2$, ~30% potentiation of the GABA EC_{20} was seen, suggesting that similar to loreclezole, the equivalent β subunit dependence is observed independent of other α subunits.

To determine whether the potentiation by DMCM was additive with that of loreclezole, we applied a saturating concentration of loreclezole (10 μM) and observed potentiation of a submaximal GABA response (205 ± 19%, 10 experiments). After this response occurred, 100 μM DMCM was coapplied with 10 μM loreclezole to determine whether there was additional potentiation by DMCM. No additional potentiation was observed (196 ± 33%, three experiments) (Fig. 4), providing further evidence that DMCM was acting at the same site as loreclezole.

Other high affinity benzodiazepine site β-carbolines were also examined at equivalent concentrations on α6β3γ2 $GABA_A$ receptors (Fig. 5); both β-CCE and β-CCP potentiated GABA responses in a similar manner to DMCM. Potentiation by β-CCP was equal to that of DMCM at all concentrations tested, whereas β-CCE was slightly less effective, eliciting only 52 ± 6.2% potentiation at 10 μM compared with 146 ± 21% and 128 ± 32 with DMCM and β-CCP, respectively.

The recently cloned *Rdl* gene from *Drosophila* has been shown to encode a $GABA_A$ receptor subunit that forms homomeric receptors (13). The sequence shows many similarities to the mammalian β subunit; however, the homologous position for asparagine in β2 and β3, which confers loreclezole sensitivity, is not conserved but rather is taken up by a methionine residue. We have found that if the Asn^{290} of β3 is mutated to a methionine and expressed with α1 and γ2S, the resulting receptor loses all sensitivity to loreclezole up to 100 μM, being less sensitive than β1, which contains a serine in the equivalent position (Fig. 6a). The $\alpha1\beta3Asn^{290}Met\gamma2S$ receptor also was not potentiated by DMCM but gave equivalent inhibition to α1β1γ2s (Fig. 6b), providing more evidence that DMCM potentiation occurs via the loreclezole site.

Although the concentrations of loreclezole that potentiate GABA match effective doses *in vivo* (5–10 mg/kg), producing a plasma concentration of 8 μM (14), the behavioral effects of β-carbolines are primarily determined via their high affinity actions at the benzodiazepine site, where they are usually up

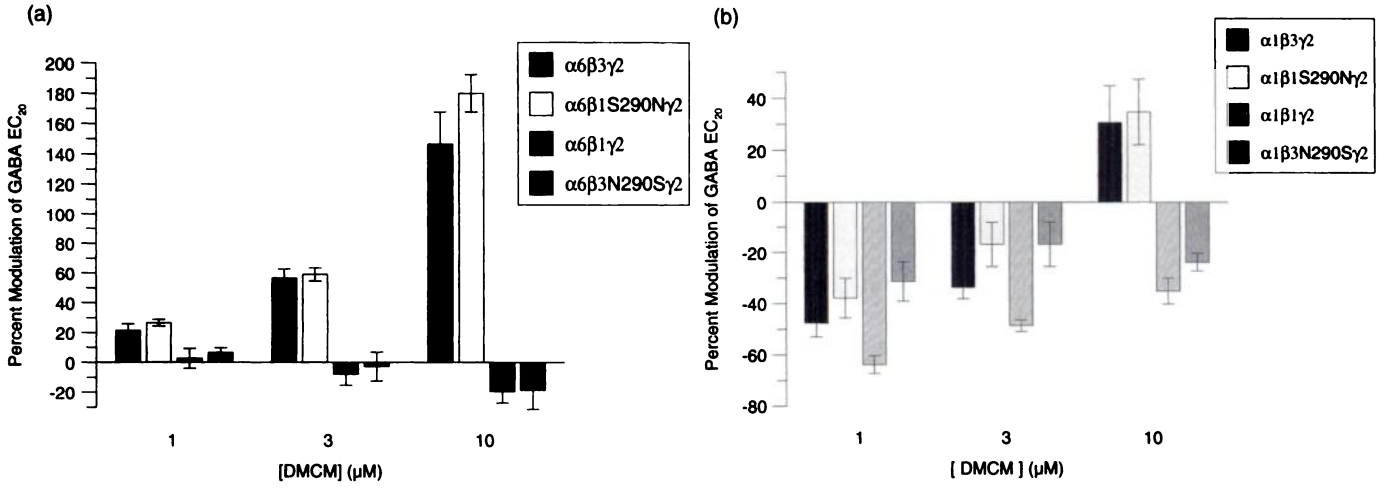

Fig. 3. Effects of increasing concentrations of DMCM on human $GABA_A$ receptors containing either (a) α6 or (b) α1 with β3, β1, and the single point mutant β subunits β1S290N and β3N290S which determine loreclezole sensitivity. DMCM was coapplied with GABA EC_{20} values determined for each oocyte. Values are the mean ± standard error of at least four oocytes.

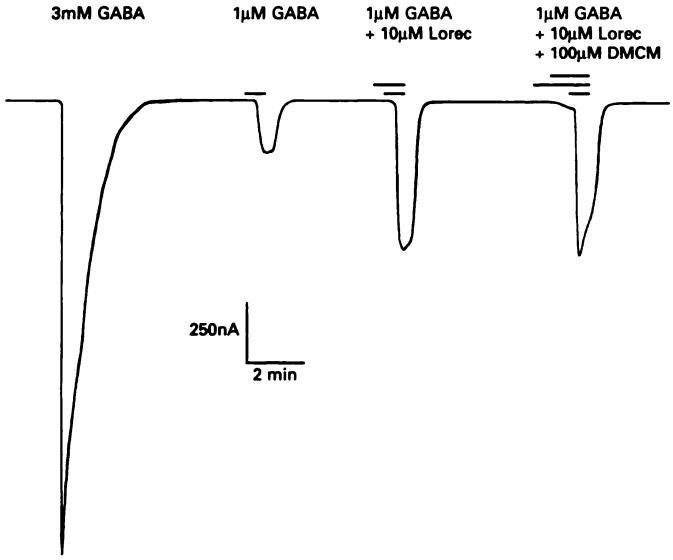

Fig. 4. Current responses from an oocyte expressing $\alpha1\beta2\gamma2$, demonstrating the nonadditive effects of DMCM and loreclezole (*Lorec*). A maximum concentration of GABA is followed by a concentration of 1 μM (~15% of maximum). This response is potentiated by 10 μM loreclezole; however, when 100 μM DMCM is coapplied with 10 μM loreclezole, no further potentiation is observed. *Bars above tracings*, application of compounds.

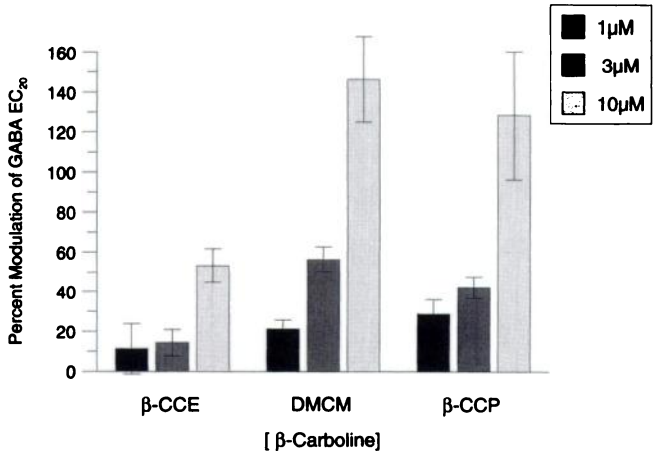

Fig. 5. Increasing concentrations of the different β-carbolines β-CCE, DMCM, and β-CCP all potentiate the human $GABA_A$ receptor $\alpha6\beta3\beta2s$ subtype in a dose-dependent manner at 1, 3, and 10 μM. β-Carbolines were coapplied with GABA EC_{20} values determined for each oocyte. Values are the mean ± standard error of at least four oocytes.

to 1000-fold more potent. As DMCM is proconvulsant, the administration of high doses is extremely anxiogenic and elicits seizures. As a consequence, studies using these high concentrations of β-carbolines have not been performed. Therefore, it is unlikely that the *in vivo* effects of DMCM can be accounted for by its binding at the loreclezole site. It may be that other β-carbolines, which lack efficacy at the benzodiazepine site, exert an anticonvulsant action via this site. It is currently unknown whether there is an endogenous ligand for the site; the only modulatory site on the receptor for which endogenous ligands have been demonstrated is the steroid binding site.

Several previous reports have demonstrated that DMCM potentiates receptors containing the γ1 subunit (15, 16). Potentiation on γ1-containing receptors is, however, unlikely to

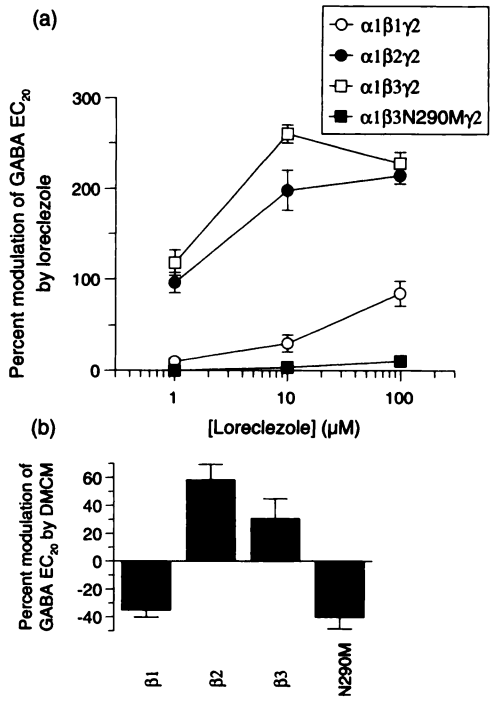

Fig. 6. Mutation of the Asn^{290} residue to a methionine produces a β3 subunit completely insensitive to loreclezole, as well as the potentiation by DMCM. a, Effects of 1, 10, and 100 μM loreclezole on oocytes expressing human $\alpha1\beta1\gamma2s$, $\alpha1\beta2\gamma2s$, $\alpha1\beta3\gamma2s$, and $\alpha1\beta3N290M\gamma2s$. b, Effects of 10 μM DMCM on receptors containing human $\alpha1\beta1\gamma2s$, $\alpha1\beta2\gamma2s$, $\alpha1\beta3\gamma2s$, and $\alpha1\beta3N290M\gamma2s$. DMCM and loreclezole were applied with GABA EC_{20} values determined for each oocyte. Values are the mean ± standard error of at least four oocytes.

be via the loreclezole site as these experiments were performed using the β1 subunit.

Several other β-carbolines exhibit a similar potentiation of $\alpha6\beta2\gamma2$ receptors expressed in *Xenopus* oocytes (Fig. 5), suggesting some kind of structure activity for compounds that act via the loreclezole site. Loreclezole has several structural features in common with the β-carbolines: the two hydrogen bond acceptors in the triazole group may mimic the ester group of the β-carbolines, and the lipophilic dichlorophenyl ring could mimic the benzofused ring of the β-carbolines. However, more structure activity experiments are necessary to determine the exact requirements for binding at this site. The discovery that β-carbolines can act via the loreclezole site provides a number of clues to the structural requirements for activity at this site. Further studies examining the structure activity of compounds active via the loreclezole site may provide a useful path to the discovery of future anticonvulsant or anxiolytic agents.

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