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Review Article

Novel positive allosteric modulators of $GABA_A$ receptors

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CONTENTS

Introduction

$GABA_A$ receptors are the major inhibitory neuronal ion channels in the mammalian brain. They are members of the ligand-gated ion channel superfamily that includes nicotinic acetylcholine, glycine and serotonin type 3 receptors. The $GABA_A$ receptor is a pentameric membrane protein with an integral chloride ion selective ion channel. Biochemical purification (1) and subsequent cloning of the $GABA_A$ receptor revealed a total of 16 different mammalian subunits named $\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}3}$, δ, ε, π and θ (2-8). The major $GABA_A$ receptor isoform is likely to be composed of α_1, β_2 and γ_2 subunits (2, 3, 9, 10). Heterologous expression demonstrated that the combination of α and β subunits produces GABA-gated currents, but coexpression of a γ subunit is required for benzodiazepine sensitivity of the expressed receptors (11).

Increase in $GABA_A$ receptor function results in sedation/hypnosis, muscle relaxation, anxiolysis and prevention of convulsions, whereas a decrease in $GABA_A$ receptor function results in opposite effects. Activation of the receptor is achieved by interaction of GABA with its particular recognition site which induces opening of the channel. For this, site-specific antagonists are also known (Table I). The opening of the channel in the presence of GABA can be influenced by many compounds interacting at allosteric modulatory sites situated elsewhere on the receptor complex (12) (Table I). A number of such sites have been described, including that for benzodiazepines (13), barbiturates, picrotoxin and neurosteroids and very recently ROD compounds (14-20). Ligands of modulatory sites may be classified in three groups: positive and negative allosteric modulators and antagonists. Some of the modulators can also induce channel opening in the absence of GABA, *e.g.*, barbiturates.

In some cases, the action of a modulator is at least partially dependent on the presence of a certain subunit isoform. Thus, classical benzodiazepines only act at receptors containing α_1, α_2, α_3 or α_5, and not on those containing α_4 or α_6 (21). Only β_2 and β_3, but not β_1 containing receptors respond strongly to loreclezole, and a point mutation in β_2 to the amino acid residue present in the homologous position in β_1, β2N265S, makes the receptor much less responsive to this compound (22, 23). It is clear that drugs acting on a specific subtype pattern of $GABA_A$ receptors will have a unique action. This is supported by observations in transgenic mice expressing diazepam insensitive receptor subunit isoforms, that have been investigated for loss of diazepam effects (24-26). These latter experiments have indicated that α_1 containing receptors mainly mediate sedative effects and that the α_2 subunit mainly may be responsible for anxiolytic effects. The first subunit specific ligand of the benzodiazepine binding site to reach the market was zolpidem, followed by zaleplon. However, both of these compounds are mainly active at receptors containing α_1 (27-30), thus including the major isoform of the receptor. Many other subunit specific ligands of the benzodiazepine binding site have been described (*e.g.*, 31), but none of them to our knowledge has thus far reached the market.

ROD compounds

Searching for novel ligands of the GABA binding site of $GABA_A$ receptors, the competitive antagonist bicuculline was used as a structural template. This search failed. About 10 substances structurally similar to bicuculline were investigated for their activation of and competitive inhibition at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors expressed in *Xenopus* oocytes (32, 33). None of the compounds was an agonist or an antagonist at the GABA

Table I: Ligands of the $GABA_A$ receptor.

Agonist	Competitive antagonist	Noncompetitive antagonist	Modulators	Antagonist of modulator
GABA	Bicuculline	Picrotoxin	Benzodiazepines (+)	Ro 15-1788
Muscimol		TBPS	β-Carbolines (–)	Ro 15-1788
			Loreclezole (+)	
			Neurosteroids (+)	
			Pentobarbital (+)	
			α-EMTBL (+)	
			ROD compounds (+)	ROD178B
			EA compounds (+)	

Modulation depends in many cases on the subunit combination so that stimulation (+) and inhibition (–) are only general indications.

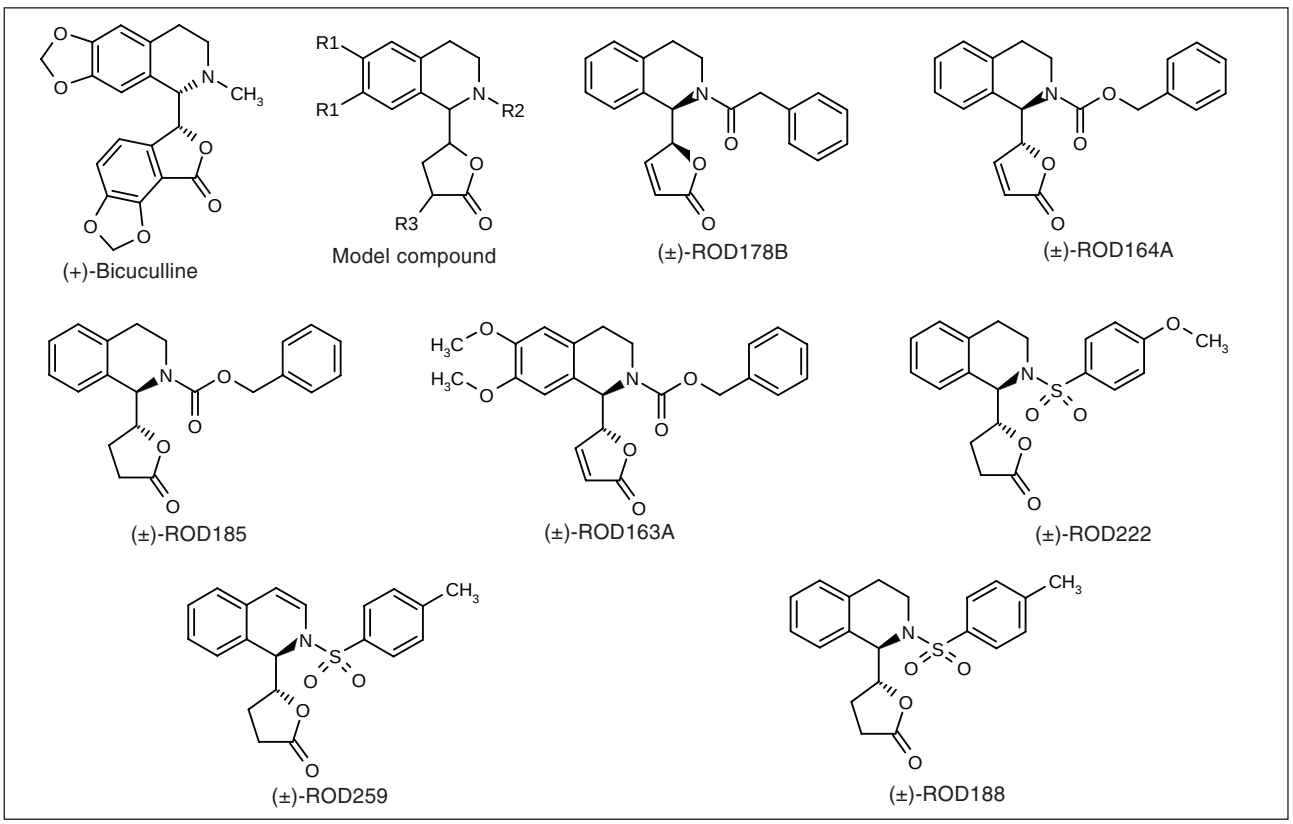


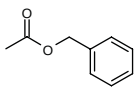
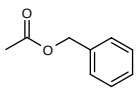
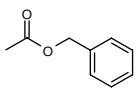
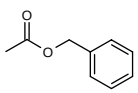
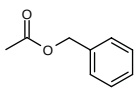
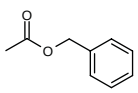
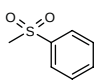
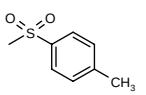
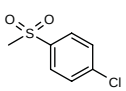
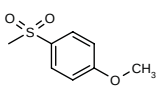
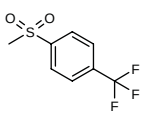
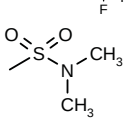
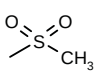
Fig. 1. Structures of ROD compounds.

binding site, but surprisingly one of them was found to be a weak positive allosteric modulator of the $GABA_A$ receptor. In several rounds of chemical synthesis and functional analysis, a number of more active modulators, code named ROD compounds, were identified (14). The structure of some selected compounds and their effect on the current induced by GABA are shown in Figure 1 and Table II (17, 18). Four structural features seem important for stimulatory activity: 1) R_1 is preferably H, 2) the *threo* stereoisomers turn out to be more active than the bicuculline-like *erythro* isomers, 3) a saturated bond is preferred in the lactone ring, and 4) R_2 has to be either aryloxycarbonyl (ROD1 compounds) or *p*-substituted *N*-arylsulfonyl (ROD2 compounds). ROD1 and ROD2 compounds seem to use different sites (19) as evidenced in the following.

Evidence for two families of ROD compounds

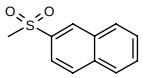
ROD164A, ROD185, ROD222, ROD259, (+)-ROD188, ROD163A and ROD178B are discussed here in more detail. All ROD compounds described here except ROD178B and ROD163A allosterically enhance currents activated by GABA in recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. Threshold of current stimulation was 0.1-1 μM for all stimulatory compounds studied.

Table II: Stimulation of GABA-elicited currents of recombinant α1β2γ2 $GABA_A$ receptors expressed in Xenopus *oocytes by some selected ROD compounds.*

Compound	R_1	R_2	R_3	Lactone ring	Stimulation[a]
(±)-ROD173A[d]	–OCH_2O–	CH_3	CH_3	Unsaturated	–3 ± 2%
(±)-ROD173B					–7 ± 1%
(±)-ROD70A	OCH_3	CH_3	CH_3	Unsaturated	–8 ± 1%
(±)-ROD70B					–10 ± 2%
(±)-ROD166A	–OCH_2O–		H	Unsaturated	215 ± 9%[b]
(±)-ROD166B					128 ± 21%[b]
(±)-ROD163A	OCH_3		H	Unsaturated	18 ± 3%
(±)-ROD163B					54 ± 6%
(±)-ROD169A	OCH_3		CH_3	Unsaturated	24 ± 5%
(±)-ROD169B					60 ± 26%
±)-ROD164A	H		H	Unsaturated	281 ± 20%
(±)-ROD164B					225 ± 39%
(±)-ROD213A	H		CH_3	Unsaturated	130 ± 4%
(±)-ROD213B					208 ± 4%
(±)-ROD185	H		H	Saturated	351 ± 10%
(±)-ROD212	H		H	Saturated	423 ± 32%
(±)-ROD188	H		H	Saturated	579 ± 61%
(±)-ROD211	H		H	Saturated	479 ± 12%
(±)-ROD222	H		H	Saturated	535 ± 7%
(±)-ROD220	H		H	Saturated	90 ± 8%
(±)-ROD261	H		H	Saturated	62 ± 7%
(±)-ROD242	H		H	Saturated	32 ± 4%

Continued

Table II: Stimulation of GABA-elicited currents of recombinant $\alpha1\beta2\gamma2$ $GABA_A$ receptors expressed in Xenopus oocytes *by some selected ROD compounds (Cont.).*

Compound	R_1	R_2	R_3	Lactone ring	Stimulation[a]
(±)-ROD263	H		H	Saturated	118 ± 24%[c]
(±)-ROD262	H		H	Saturated	353 ± 30% (48% at 10 μM)

[a]Current stimulation was determined electrophysiologically at a drug concentration of 100 μM in *Xenopus laevis* oocytes expressing $\alpha21\beta2\gamma2$ $GABA_A$ receptors as previously described (21, 32). Average of minimally 3 assays ± SEM; [b]Assayed at 50 μM; [c]Assayed at 10 μM; [d]"A" refers to molecules having the *threo* configuration; "B" refers to molecules having the *erythro* (*i.e.*, bicuculline-like) configuration. When unspecified, the molecule has the *threo* configuration.

Compounds carrying an aryloxycarbonyl at R_2, such as ROD164A, ROD185 and ROD178B, were grouped in the ROD1 class of compounds. Compounds carrying a *p*-substituted *N*-arylsulfonyl at R_2, such as ROD222 and ROD259, were grouped in the ROD2 class of compounds. In contrast to ROD2 compounds, ROD1 compounds competitively displace [^{3}H]-flunitrazepam in radioactive ligand binding studies with low or moderate affinity (19). Enhancement of currents elicited by GABA by ROD1, but not ROD2 class compounds, was antagonized by the benzodiazepine antagonist Ro15-1788. The enhancement by ROD2, but not that by ROD1 compounds, was additive to that by the benzodiazepine diazepam. Thus, based on these observations, it was tempting to postulate that ROD1, but not ROD2 compounds, are novel ligands of the benzodiazepine binding site. Surprisingly, ROD1 as well as ROD2 compounds are active at recombinant $\alpha_1\beta_2$ receptors. This is in contrast to benzodiazepines (11, 33, 34). Results from an analysis of the inhibition of various concentrations of the ROD1 type compound ROD164A by the benzodiazepine antagonist Ro15-1788 were not compatible with a competitive interaction. Therefore, ROD1 type compounds not only act at the benzodiazepine binding site, but also at another site, which we call R_1. ROD178B inhibited current stimulation by both ROD1 and ROD2 compounds. Analysis of the inhibition by ROD178B of current enhancement by ROD164A and ROD259 argue for independent sites, R_1 and R_2, as inhibition at the R_1 site seemed to be of competitive nature. In contrast, inhibition at the R_2 site seemed to be due to a negative allosteric modulation (19). Only weak or no effects at all on radioactive ligand binding using [^{3}H]-muscimol or [^{35}S]-*tert*-butylbicyclophosphorothionate were seen for all compounds (19).

Specific properties of (+)-ROD188 and ROD163A

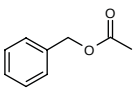
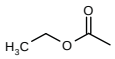
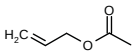
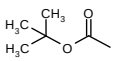
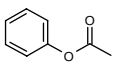
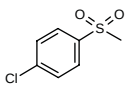
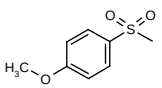
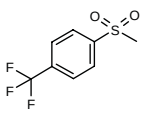
(+)-ROD188 was further investigated (16). (+)-ROD188 strongly stimulated currents elicited by either pentobarbital or 5α-pregnan-3α-ol-20-one (3α-OH-DHP), in line with a mode of action different from that of barbiturates or neurosteroids as channel agonists. Allosteric stimulation by (+)-ROD188 was similar in $\alpha_1\beta_2$N265S as in unmutated $\alpha_1\beta_2$, while that by loreclezole was strongly reduced. These observations further substantiate that ROD compounds act at novel sites. The subunit requirement for stimulation was also investigated. Stimulation by (+)-ROD188 was largest in $\alpha_6\beta_2\gamma_2$ ($\alpha_6\beta_2\gamma_2$ >> $\alpha_1\beta_2\gamma_2$ ≥ $\alpha_5\beta_2\gamma_2$ > $\alpha_2\beta_2\gamma_2$ ≥ $\alpha_3\beta_2\gamma_2$), indicating a unique subunit isoform specificity. Evidence was also provided that (+)-ROD188 acts not only on recombinant $GABA_A$ receptors, but in systems closer to the *in vivo* situation. Namely, miniature inhibitory postsynaptic currents (mIPSC) in cultured rat hippocampal neurons, caused by spontaneous release of GABA, showed a prolonged decay time in the presence of 30 μM (+)-ROD188, indicating an enhanced synaptic inhibitory transmission.

Different ROD compounds seem to have different subunit specificities. While ROD163A is almost inactive at $\alpha_1\beta_2\gamma_2$, it acts in a stimulatory manner on other subunit combinations ($\alpha_5\beta_2\gamma_2$ > $\alpha_3\beta_2\gamma_2$ > $\alpha_6\beta_2\gamma_2$ ≥ $\alpha_2\beta_2\gamma_2$ >> $\alpha_1\beta_2\gamma_2$) (14, unpublished observations). As mentioned before, classic benzodiazepines do not modulate α_6 containing receptors. An additional ROD compound not discussed here was almost inactive at $\alpha_1\beta_2\gamma_2$ and behaved as a negative allosteric modulator at $\alpha_5\beta_2\gamma_2$ (E.S., unpublished observations).

EA compounds

3,3-Dialkylbutyrolactones, thiobutyrolactones and related compounds potentiate currents mediated by $GABA_A$ receptors and are anticonvulsive. These compounds, best represented by α-EMTBL, were largely developed by Covey and Ferrendelli during the past 20 years (35-43). We decided to further investigate substituted butyrolactones. These compounds differ structurally from α-EMTBL by the presence of 1) an oxygen instead of a sulfur atom in the ring, 2) two *n*-propyl groups instead of a methyl and ethyl group at the α (*i.e.*, C-3) position, and 3) a substituted amine function at the β (*i.e.*, C-4) position. In several rounds of chemical synthesis and

Table III: Effect of EA compounds on GABA-elicited currents in Xenopus *oocytes expressing α1β2γ2 $GABA_A$ receptors.*

Compound	R_1	R_2	R_3	Stimulation[a] (μM)
(±)-EA2		H	H	0 ± 1% (200)
(±)-EA16C		allyl	H	+73 ± 18% (200)
(±)-EA16		H	allyl	–1 ± 3% (200)
(±)-EA17		allyl	allyl	+115 ± 17% (200)
(±)-EA18 (*R*)-EA18 (*S*)-EA18		propyl	propyl	+140 ± 29% (50) +279 ± 47% (100) +104 ± 4% (100)
(±)-EA36		propyl	propyl	+426 ± 8% (200)
(±)-EA35		propyl	propyl	+765 ± 61% (200)
(±)-EA44		propyl	propyl	+46 ± 5% (200)
(±)-EA59		propyl	propyl	+330 ± 35% (200)
(±)-EA34		propyl	propyl	+420 ± 33% (200)
(±)-EA31		propyl	propyl	+23 ± 8% (5)
(±)-EA33		propyl	propyl	–22 ± 10% (200)
(±)-EA32		propyl	propyl	–44 ± 1% (200)

[a]Determined electrophysiologically in *Xenopus laevis* oocytes expressing α1β2γ2 $GABA_A$ receptors as previously described (31, 32). Average of three assays ± SEM.

functional analysis at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors expressed in *Xenopus* oocytes, a number of active modulators, code named EA compounds, were identified. The structure of some selected compounds and the current stimulation caused by them are summarized in Figure 2 and Table III (15, 20). Threshold of current stimulation was about 2 μM for (±)-EA18, (±)-EA34 and (±)-EA35. Comparing current stimulation at the same concentration, (±)-EA34 and (±)-EA35 were clearly more potent than α-EMTBL. Three structural features seemed important for activity: 1) addition of alkyl or dialkyl substituents at C-3, 2) addition of an *N*-carboxamide or carbamate (but not *N*-sulfonyl) group at C-4, 3) at least in (±)-EA18 the (*R*)-enantiomer was approximately 2.5 times more active than the (*S*)-enantiomer (20). Only weak or no effects at all on radioactive ligand binding using [^{3}H]-muscimol, [^{3}H]-flunitrazepam or [^{35}S]-*tert*-butylbicyclophosphorothionate were seen (20).

Similarity to the action of loreclezole

(*R*)-EA18 was also tested at $\alpha_1\beta_1$ and $\alpha_1\beta_2$ recombinant receptors. The compound was 3- to 4-fold more active at $\alpha_1\beta_2$ receptors than at $\alpha_1\beta_1$ receptors. Interestingly, this profile resembles that of the anticonvulsant loreclezole (22). In order to test this hypothesis, the effect of (*R*)-EA18 on GABA currents in the mutated $\alpha_1\beta_2$N265S receptor, which shows a diminished response to loreclezole (22, 23), was studied. Current stimulation

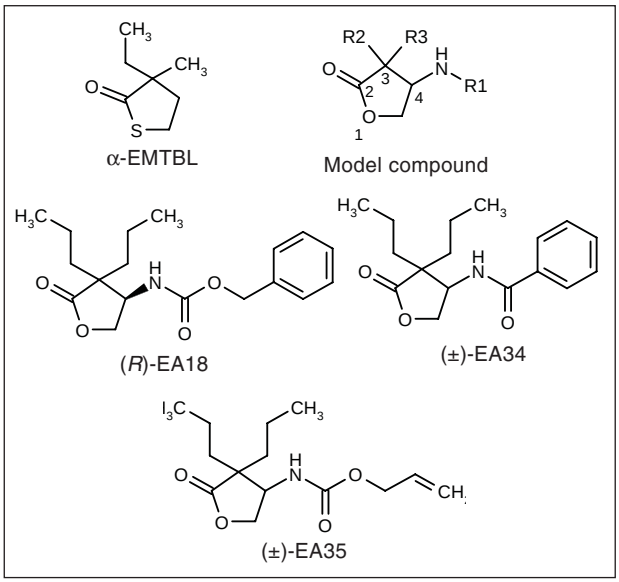


Fig. 2. Structures of EA compounds.

by (*R*)-EA18 was also greatly decreased in this case, again suggesting similarity in action with loreclezole. (±)-EA35 showed similar behavior. Interestingly, stimulation by (±)-EA34 was not significantly affected by the same mutation. Thus, at least some ((*R*)-EA18, (±)-EA35) but not all ((±)-EA34) of the butyrolactones analyzed may share similarity in their action to the anticonvulsant loreclezole (20).

Preliminary experiments indicate that at least (±)-EA35 also acts at recombinant $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_5\beta_2\gamma_2$ and $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptors, in addition to $\alpha_1\beta_2\gamma_2$ (E.S., unpublished observations).

Predictive power of radioactive ligand binding studies

For both groups of compounds, ROD and EA, no correlation of modulatory activity with radioactive ligand binding could be observed using either [^{3}H]-muscimol, [^{3}H]-flunitrazepam or [^{35}S]-*tert*-butylbicyclophosphorothionate as ligand. In the past, radioactive ligand binding has often been used to predict functional effects on the $GABA_A$ receptor, especially for ligands of the benzodiazepine binding site. Screening of the new compounds using only radioactive ligand binding assays would have missed their modulatory power altogether. At least in the present case, only the much more time-consuming electrophysiological measurements were able to identify active compounds.

Conclusions

In summary, we discuss here evidence for the presence of additional allosteric sites on the $GABA_A$ receptor. It remains to be shown where the novel sites reside. It also remains to be established whether the described compounds have an action at targets other than $GABA_A$ receptors. The $GABA_A$ receptor subunit specificity, as far as has been investigated, seems unique. This feature provides hope that the three new families of modulators of the $GABA_A$ receptor will find clinical applications. The positive allosteric modulators have a wide potential as centrally active drugs, especially as sedatives, anxiolytics, muscle relaxants and antiepileptics, and the negative allosteric modulators as cognitive enhancers.

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