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Structure–activity relationship of etomidate derivatives at the $GABA_A$ receptor: Comparison with binding to 11β-hydroxylase

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ABSTRACT

At the $GABA_A$ receptor, low concentrations of etomidate potentiate the inhibitory effect of GABA on specific binding of the *closed channel ligand* [^{3}H]ethynylpropylbicycloorthobenzoate ([^{3}H]EBOB). Here, we present SARs for etomidate and structurally related compounds inducing this effect. In the absence of GABA, similar SARs, but 14–20 times weaker potencies were observed. We discuss these SARs in comparison to the much higher potencies of these compounds as inhibitors of 11β-hydroxylase.

The anaesthetic effect of (*R*)-1-(1-phenylethyl)-1*H*-imidazole-5-carboxylic acid ethyl ester [etomidate, (*R*)-**2**] is mediated by a still poorly understood allosteric regulatory site at the $GABA_A$ receptor,[1,2] the most abundant receptor for the main inhibitory neurotransmitter γ-aminobutyric acid (GABA). In addition, (*R*)-**2** and structurally related compounds influence several other biological targets that have in common their relatedness to steroid substrates or cofactors.[3] For example, prolonged sedation with etomidate results in a pronounced inhibition of adrenal steroidogenesis,[4] due to the specific interaction with the enzyme 11β-hydroxylase (CYP11B1)[5] via the binding site for its steroid substrate deoxycorticosterone. Likewise, structurally related azoles act as fungicides by targeting the biosynthesis of ergosterol, a major component of fungal membranes.[6] Whether the action of etomidate at the $GABA_A$ receptor is also mediated by one of the steroid binding sites of this receptor[7] currently is not known.

The methyl ester metomidate (*R*)-**1** and the ethyl ester (*R*)-**2**, radiolabelled with ^{11}C, bind with nanomolar affinities to 11β-hydroxylase and have been introduced as radiotracers for PET imaging of the adrenal cortex and its tumours.[8] Recently, we have characterized specific binding of [^{131}I]4-I-(*R*)-**1** [i.e., (*R*)-**7** labelled with ^{131}I] to 11β-hydroxylase on rat adrenal membranes and described SARs of several analogues of (*R*)-**1** and (*R*)-**2**.[9] To identify possible similarities and differences in the SARs, and thus, in the structure of the respective binding pockets, here we investigate the potencies of the same compounds as allosteric ligands of $GABA_A$ receptors, using specific binding of the radioligand [^{3}H]EBOB[10,11] to rat cortical membranes. This radioligand binds specifically and with high affinity to the $GABA_A$ receptor complex in its inactive state (chloride channel closed) and can be allosterically displaced from its binding site by activating the receptor by GABA, GABA-mimetic compounds, or low concentrations of GABA combined with compounds enhancing the potency of GABA, as for example benzodiazepines, neuroactive steroids, or etomidate. It is believed that the modulation of the binding of *closed channel ligands* such as [^{3}H]EBOB or the more commonly used [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) reflects the functional interaction of GABA or GABAergic drugs with the $GABA_A$ receptor complex.[12]

Neuronal membranes were prepared from the frontoparietal cortices of male, adult Sprague-Dawley rats, using EDTA to aid removal of endogenous GABA,[13] and stored as aliquots at −80 °C until use. For binding assays, an aliquot was thawed, diluted with assay buffer (50 mM Tris·HCl, 150 mM NaCl, pH 7.4) and centrifuged. Resuspended membranes were incubated in glass vials (triplicates) with 2 nM [^{3}H]EBOB (30 Ci/mM; PerkinElmer, Boston MA, USA)[14] for 2 h in a 25 °C water bath, in the absence or presence of GABA (1 μM)[15] and various concentrations of the test compounds; non-specific binding (NB) was obtained in the presence of 50 μM picrotoxin. Membranes with bound radioligand were collected with a Brandel harvester (48 places) on glass fibre filter presoaked in buffer. The filter was washed three times with 5 ml cold buffer, transferred into scintillation vials and, after addition of liquid scintillation cocktail (PPO & POPOP in toluene, Rotiszint 11, Carl Roth, Karlsruhe, Germany), shaken vigorously and subse-

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quently agitated for 20 min. Radioactivity was quantified in a liquid scintillation counter (Tri-Carb 2100 TR). IC_{50} values were obtained by fitting the results to the inhibition function $B(x) = B_o \times IC_{50}^{n_H}/(IC_{50}^{n_H} + x^{n_H}) + NB$, where x is the inhibitor concentration, B_o specific binding in absence of inhibitor, and n_H the Hill coefficient. IC_{50} values were obtained from at least three independent experiments.

Table 1 presents IC_{50} values of several etomidate derivatives and, for comparison, of the prototypic neurosteroids allopregnanolone and alfaxalone. The potency to increase the effect of a weakly acting low GABA concentration (1 μM)[15] grew with the size of the alcoholic part of the ester; the propyl ester (*R*)-**4** was equipotent to alfaxalone. At the phenyl ring, Br was best tolerated as a substituent [(*R*)-**9**]; larger substituents such as I [(*R*)-**6**, (*R*)-**7**, (*R*)-**8**] or hydroxymethyl [(*R*)-**11**] resulted in a reduction of potency. The methyl substituent at the chiral centre was essential; neither its omission (**12**), nor a change in configuration from *R* to *S* as in (*S*)-**1** was tolerated. Its elongation to ethyl [(*R*)-**13**] was without influence, but integration into an indane bicyclus [(*R*)-**15**] resulted in loss of affinity. Interestingly, this indane derivative has been described as 'essentially devoid of hypnotic activity'.[16]

Omission of GABA substantially reduced the potency of etomidate analogues to inhibit [^{3}H]EBOB binding. Only for (*R*)-**1**, (*R*)-**2**, (*R*)-**3**, (*R*)-**4**, (*R*)-**5**, (*R*)-**9**, and (*R*)-**13**, that is, the **7** most potent etomidate analogues, IC_{50} values <300 μM could be obtained. These IC_{50}s were highly correlated with the IC_{50}s of the same **7** compounds in the presence of GABA (Fig. 1), resulting in similar reduction factors (all ratios between 14 and 20).[17] This agrees with data demonstrating direct opening of the $GABA_A$ receptor-associated Cl^- channel only by rather high concentrations of etomidate. The limited SARs observed with these 7 compounds do not suggest structural leads to the differentiation of GABA-dependent and GABA-independent effects of etomidate-like drugs. An even stronger dependence on GABA was observed for the neurosteroids allopregnanolone (factor 50) and alfaxalone (factor 102; Table 1).

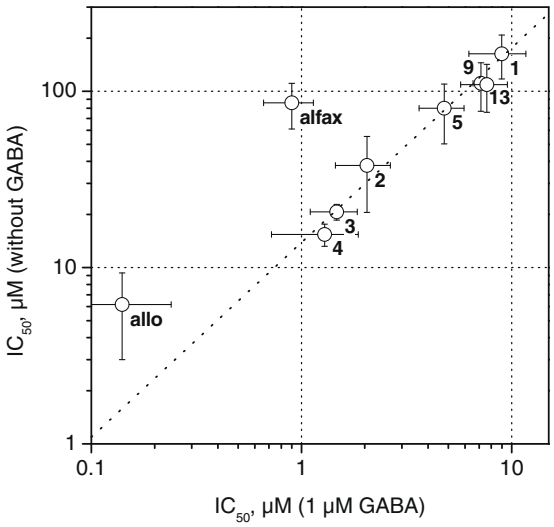
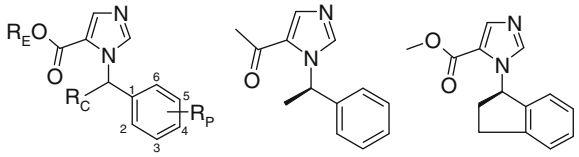


Figure 1. Inhibition of [^{3}H]EBOB binding (mean IC_{50} ± SD) by etomidate dervatives (**numbers**; stereodescriptor *R* omitted for clarity), and by allopregnanolone (**allo**) and alfaxalone (**alfax**). For the etomidate derivatives, IC_{50}s without GABA (ordinate) are correlated, on logarithmic scales, with IC_{50}s in the presence of GABA (abscissa) (R = 0.996), while **allo** and **alfax** do not follow this trend.

Many of these etomidate analogues are approximately 3 orders of magnitude more potent as inhibitors of [^{131}I]4-I-(*R*)-**1** binding to 11β-hydroxylase on rat adrenal membranes.[9] A comparison reveals similarities and differences (Fig. 2). Some of the similarities may be the consequence of a parallel evolution of different proteins for

Table 1
Inhibition of [^{3}H]EBOB binding to rat cortical membranes, by structural analogues of etomidate; R_E indicates the alcoholic part of the ester, and R_C and R_P the substituents at the chiral centre and at the phenyl ring, respectively; IC_{50} values (μM) are means ± SD (number of independent experiments in parentheses)



1-13 **14** **15**

	R_E	R_C	R_P	IC_{50}		IC_{50} ratio (without/with GABA)
				1 μM GABA	without GABA	
(*R*)-**1**	CH_3–	CH_3–	—	8.97 ± 2.71 (5)	163 ± 46 (5)	19.6 ± 9.5 (5)
Etomidate, (*R*)-**2**	CH_3CH_2–	CH_3–	—	2.05 ± 0.60 (7)	38 ± 17 (6)	19.3 ± 6.8 (6)
(*R*)-**3**	$(CH_3)_2CH$–	CH_3–	—	1.47 ± 0.37 (3)	20.7 ± 2.1 (3)	14.6 ± 3.5 (3)
(*R*)-**4**	$CH_3CH_2CH_2$–	CH_3–	—	1.29 ± 0.57 (3)	15.4 ± 2.2 (3)	14.1 ± 7.6 (3)
(*R*)-**5**	FCH_2CH_2–	CH_3–	—	4.78 ± 1.15 (4)	80 ± 30 (4)	16.6 ± 3.2 (4)
(*R*)-**6**	CH_3–	CH_3–	3-I–	53 ± 13 (3)	>300	
(*R*)-**7**	CH_3–	CH_3–	4-I	37 ± 13 (3)	>300	
(*R*)-**8**	CH_3CH_2–	CH_3–	4-I	33 ± 5 (3)	>300	
(*R*)-**9**	CH_3–	CH_3–	4-Br–	7.62 ± 1.91 (3)	109 ± 33 (3)	15.6 ± 8.4 (3)
(*R*)-**10**	CH_3–	CH_3–	4-CH_3–	15.5 ± 5.5 (3)	>300	
(*R*)-**11**	CH_3–	CH_3–	4-$HOCH_2$–	169 ± 22 (3)	>300	
12	CH_3–	H–	—	157 ± 17 (3)	>300	
(*S*)-**1**	CH_3–	CH_3–	—	202 ± 46 (3)	>300	
(*R*)-**13**	CH_3–	CH_3CH_2–	—	7.15 ± 0.60 (3)	111 ± 34 (3)	15.8 ± 5.9 (3)
(*R*)-**14**	(see structure)	CH_3–	—	155 ± 36 (3)	>300	
(*R*)-**15**	CH_3–	(see structure)		122 ± 59 (3)	>300	
Allopregnanolone				0.14 ± 0.10 (5)	6.2 ± 3.2 (5)	50 ± 18 (5)
Alfaxalone				0.90 ± 0.24 (4)	86 ± 25 (4)	102 ± 41 (4)

In addition, the following compounds had no significant influence at 100 μM concentrations: (*R*)-(+)-1-(1-phenylethyl)-1*H*-imidazole-5-carboxylate [(*R*)-**16**, the free acid of (*R*)-**1**]; the methylamide of (*R*)-**16**; the 3-pyridyl derivative of (*R*)-**1**; and the 11β-hydroxylase inhibitors metyrapone and metyrapol.

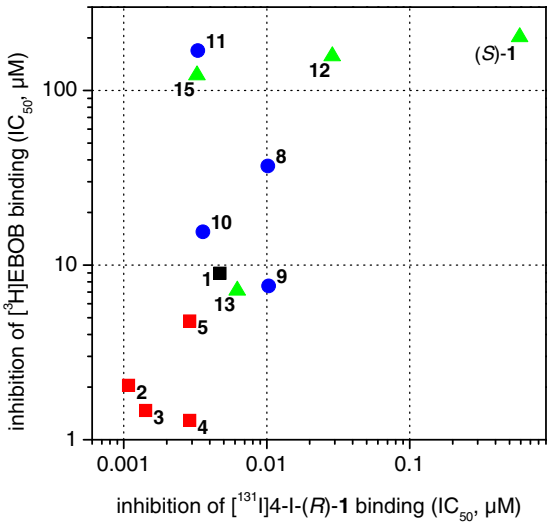


Figure 2. Comparison of IC_{50} values at the $GABA_A$ receptor (ordinate) and at 11β-hydroxylase[9] (abscissa) for derivatives of metomidate (**1**, black square) with increasing size of the alcoholic part of the ester (red squares), various substituents at the phenyl ring (blue circles), and modifications at the chiral centre (green triangles).

best recognition of a steroid as physiological ligand.[2,3] In both binding assays, increasing the length of the alcoholic part of the ester from methyl [(*R*)-**1**] to ethyl [(*R*)-**2**] increases potency, with no significant increase on further elongation (Fig. 2, red squares). However, at the phenyl group, substituents above a critical size corrupt the action on $GABA_A$ receptors, whereas inhibition of 11β-hydroxylase is only slightly influenced (Fig. 2, blue circles). Most strikingly, the hydroxymethyl derivative (*R*)-**11** was 20 times weaker than (*R*)-**1** at the $GABA_A$ receptor complex, but was an excellent inhibitor of [^{131}I]4-I-(*R*)-**1** binding.

Increasing GABA-induced Cl^- flux and binding to 11β-hydroxylase both prefer etomidate analogues with *R* over *S* configuration. However, rigidization by bridge formation between the chiral centre and the phenyl ring as in (*R*)-**15** is tolerated for 11β-hydroxylase inhibition (and for antifungal activity[16]), but not for potentiating the GABA effect on [^{3}H]EBOB binding (Fig. 2, green triangles). Samples of those two compounds with the most discordant results, of the hydroxymethyl derivative (*R*)-**11** and of the indanyl derivative (*R*)-**15**, were re-analysed by NMR, confirming their structures. Also these re-analysed samples exhibited low potencies as inhibitors of [^{3}H]EBOB binding in presence of 1 μM GABA. Given their weak potency at the $GABA_A$ receptor, they represent leads to 11β-hydroxylase inhibitors devoid of narcotic side effects.

A model has been proposed, based on the crystal structure of a bacterial enzyme, for interaction of (*R*)-**2** and its structural analogue fadrazole with 11β-hydroxylase.[18] In this model, amino acid residue F130 has been proposed to undergo π-π stacking interaction with the phenyl ring, and T318 to form a hydrogen bond with the ester carbonyl group of (*R*)-**2** (position numbers according to the human variant hCYP11B1). For activation of the $GABA_A$ receptor complex by etomidate, such detailed information on the location of a conceivable etomidate binding site is still missing. Up to now, no crystals of $GABA_A$ receptors or parts of it have been obtained to enable X-ray crystallographic analysis. In addition, each $GABA_A$ receptor is formed by five subunits, and pharmacologically relevant binding sites are often formed by subunit interfaces.[12] Finally, the GABA-potentiating and the GABA-independent direct effects of etomidate might involve separate binding sites. Site-directed mutagenesis studies[1] point to an important role of N265 at the extracellular end of the 2nd transmembrane domain (TM) of subunit β2 or β3,[19] especially for the direct effects of etomidate.[20] On the other hand, the photoreactive etomidate analogue [^{3}H]azietomidate labels two methionine residues,[21] one in the 1st TM of the α1 (M236 in the bovine, M235 in the murine α1 subunit), the other in the 3rd TM of the β3 subunit (M286). Unfortunately, it was not possible to investigate if such labelling occurred also in the 2nd TM of β2/β3, for methodological reasons.

The neurosteroid tetrahydrodeoxycorticosterone (THDOC) also stimulates chloride conductance at the $GABA_A$ receptor either directly (at high concentrations) or indirectly by potentiating the action of GABA (at low concentrations). Each of these effects seems to be mediated by a different set of amino acid residues. Interestingly, site-directed mutagenesis studies localize the direct, GABA-independent effect of THDOC at the α/β interface (T236 in α-, Y284 in β-subunits),[2,7] close to residues α1M235 and βM286 photo-labelled by [^{3}H]azietomidate.[21] Nevertheless, neurosteroids do not reduce this photolabelling, but rather enhance it,[22] probably due to the second, GABA-potentiating effect of neurosteroids mediated by other sites.[2,7] Our present data neither support nor discredit the possibility that etomidate binds to one of the steroid binding sites of $GABA_A$ receptors.

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13. Frontoparietal cortices (ca. 300 mg per rat) were dissected and stored at −80 °C. After thawing, tissue was homogenized in 100 parts of ice–cold 50 mM Tris acetate buffer (pH 7.0) containing 3 mM EDTA with a glass/teflon Potter-type homogenizer. After centrifugation (10 min 17.000 rpm), the pellet was resuspended in buffer without EDTA, centrifuged a 2nd time, resuspended, and centrifuged a 3rd time. The next suspension was warmed to 37 °C for 10 min in a water bath, cooled, and centrifuged a 4th time. The final suspension was divided into aliquots and stored at −80 °C. For the binding assay, each glass vial contained a membrane equivalent of ca. 1 mg original tissue.
14. [^{3}H]EBOB was delivered in absolute ethanol. Immediately before use, the amount needed for one assay was evaporated to dryness and taken up in assay buffer. The radioligand was stable in absolute ethanol, but unstable in aqueous solution (hydrolysis rate ca. 50% in 48 h).
15. In the presence of 1 μM GABA, the channel is in a slightly activated state, resulting in a moderate reduction of specific [^{3}H]EBOB binding by 20%. Neuroactive steroids and etomidate act as GABA potentiators and lower [^{3}H]EBOB binding further by increasing GABA affinity. Only at higher concentrations, they are able to open the channel by themselves, in the absence of GABA.
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17. ANOVA for IC_{50} ratios of all 7 etomidate analogues with detectable IC_{50}s in presence and absence of GABA resulted in $F_{6,20} = 0.40$ (n.s.). Adding allopregnanolone to the calculation raised this value to $F_{7,24} = 7.05$ ($p < 0.001$); *post hoc* Newman–Keuls test demonstrated that the ratio for

allopregnanolone was significantly higher than that for all etomidate analogues. If alfaxalone was included as well, we obtained $F_{8,27} = 12.6$ ($p < 0.001$); here, Newman–Keuls test indicated that the ratio for alfaxalone was significantly higher than that for allopregnanolone.
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19. For this and for all subsequent positions, numbering starts with the N-terminal amino acid of the mature protein, that no longer contains the signal sequence.
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