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RESEARCH PAPER

Identification of novel positive allosteric modulators and null modulators at the $GABA_A$ receptor α+β– interface

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BACKGROUND AND PURPOSE

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the mammalian brain and the target of many clinically important drugs interacting with different binding sites. Recently, we demonstrated that CGS 9895 (2-(4-methoxyphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one) acts as a null modulator (antagonist) at the high affinity benzodiazepine binding site, but in addition elicits a strong enhancement of GABA-induced currents via a novel drug binding site at the extracellular α+β– interface. Here, we investigated 32 structural analogues of CGS 9895 for their ability to mediate their effects via the α1+β3– interface of $GABA_A$ receptors.

EXPERIMENTAL APPROACH

$GABA_A$ receptors were expressed in *Xenopus laevis* oocytes and investigated by the two-electrode voltage clamp method.

KEY RESULTS

We not only identified compounds with higher efficacy/potency than CGS 9895 for stimulating GABA-induced currents via the α1+β3-binding site, but also discovered compounds acting as null modulators at this site. Most of the compounds also acted as null modulators via the benzodiazepine binding site of $GABA_A$ receptors. But some of the positive allosteric modulators or null modulators exclusively exerted their action via the α+β– binding site.

CONCLUSION AND IMPLICATIONS

Pyrazoloquinolinones and pyrazolopyridinones represent the first prototype of drug candidates mediating benzodiazepine like modulatory effects via the α+β-interface of $GABA_A$ receptors. The discovery of null modulators acting as inhibitors of the plus modulators provides a highly useful tool for the discovery of additional classes of compounds that can modulate $GABA_A$ receptors via this site, which may lead to novel therapeutic principles.

LINKED ARTICLE

This article is accompanied by Varagic *et al.*, pp. 384–399 of this issue. To view this article visit http://dx.doi.org/10.1111/bph.12153

Abbreviations

CI, confidence interval; $GABA_A$ receptors, GABA type A receptors; MTSEA-biotin (MB), N-Biotinylaminoethyl methanethiosulfonate; PLS, partial least squares; QSAR, quantitative structure activity relation; TEV, Two electrode voltage clamp

Introduction

GABA type A receptors ($GABA_A$ receptors) are the major inhibitory transmitter receptors in the brain. They are chloride channels that can be opened by GABA and are composed of five subunits that can belong to different subunit classes. A total of 19 subunits have been identified in the mammalian brain giving rise to an enormous diversity of $GABA_A$ receptor subtypes with different pharmacological properties (Olsen and Sieghart, 2008; Sieghart, 1995). The majority of $GABA_A$ receptors, however, is composed of two α, two β and one γ subunit. $GABA_A$ receptors are the site of action of a variety of pharmacologically and clinically important drugs such as benzodiazepines, barbiturates, neuroactive steroids, anaesthetics and convulsants that allosterically modulate GABA-induced currents (Sieghart, 1995).

So far, binding sites for only a few of these drugs have been unequivocally identified on these receptors (Olsen and Sieghart, 2008). However, a modelling study (Ernst *et al.*, 2005) indicated the presence of multiple solvent accessible pockets within the $GABA_A$ receptor that could function as possible drug binding sites. Simultaneous drug interaction with several of these binding sites can explain the extremely complex pharmacology of these receptors. Benzodiazepines are the best characterized drugs that interact with $GABA_A$ receptors. They act by modulating ongoing GABAergic activity via the allosteric high affinity benzodiazepine binding site at $GABA_A$ receptors (Richter *et al.*, 2012), in contrast to other drugs that can also directly open the $GABA_A$ receptor-associated chloride channel at higher concentrations (Sieghart, 1995). The high affinity benzodiazepine binding site is located in the extracellular domain of $GABA_A$ receptors, at the α+γ– interface (Sigel, 2002; Ernst *et al.*, 2003), whereas the two GABA binding sites of these receptors are located at the two β+α– interfaces (Smith and Olsen, 1995) (Figure 1).

Very recently, it was demonstrated that the high affinity benzodiazepine binding site ligand CGS 9895 behaves as a null modulator via this site, and in addition, exerts a low potency positive modulatory action at $GABA_A$ receptors via a newly discovered drug binding site at the extracellular α+β– interface (Ramerstorfer *et al.*, 2011). A low affinity flurazepam binding site has been identified previously at this interface (Baur *et al.*, 2008). The CGS 9895 binding site at the α+β– interface is homologous to the benzodiazepine binding site at the α+γ– interface and is thus strongly influenced by the type of α subunit present in the receptor. Drugs interacting with the α+β– interface should be able to modulate αβ, αβγ, αβδ, αβε, αβπ and αβθ receptors and should thus exhibit a much broader action than benzodiazepines. Nevertheless, such drugs might also be able to distinguish between different receptor subtypes depending on the exact α and β subunit type forming their binding site (Sieghart *et al.*, 2012).

Here, we investigated 32 structural analogues of CGS 9895 for their ability to mediate their effects via the α+β– interface (Ramerstorfer *et al.*, 2011). We identified compounds displaying not only higher efficacy and apparent potency than CGS 9895, but also for the first time discovered compounds that act as null modulators at the α+β– binding site. In the accompanying paper (Varagic *et al.*, 2013), we describe the effects of 16 of these compounds at various αxβ3 or αxβ3γ2 $GABA_A$ receptor subtypes.

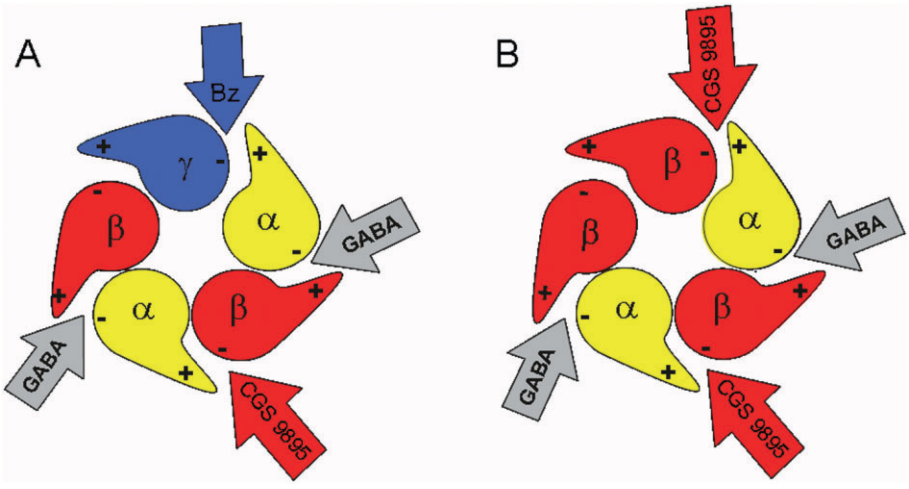


Figure 1

Top view onto the extracellular domain of $GABA_A$ receptors. Each subunit features a plus (+) and a minus (–) side. Binding sites for GABA are located at the interfaces formed by the '+' side of the β and the '–' side of the α subunits. (A) α1β3γ2 receptors composed of one γ2, two α1 and two β3 subunits. The binding site for benzodiazepine ligands (Bz) is located at the interface formed by the '+' side of the α and the '–' side of the γ subunit. The CGS 9895 (compound 3) binding site is located at the interfaces formed by the '+' side of the α and the '–' side of the β subunit. (B) α1β3 receptors composed of two α1 and three β3 subunits. To avoid interaction with the benzodiazepine binding site, we used $GABA_A$ receptors composed of α1 and β3 subunits only. Such receptors are assumed to be composed of three β and two α subunits (Tretter *et al.*, 1997; Farrar *et al.*, 1999; Baumann *et al.*, 2001), and should, thus, have two β3+/α1– interfaces (GABA binding sites), two α1+/β3– interfaces and one β3+/β3– interface, but no benzodiazepine binding site.

Methods

Two electrode voltage clamp (TEV)

In vitro transcription of mRNA was based on the cDNA expression vectors encoding for $GABA_A$ receptor subunits α1, β3 and γ2 (all from rat) (Ramerstorfer *et al.*, 2010). After linearizing the cDNA vectors with appropriate restriction endonucleases, capped transcripts were produced using the *mMESSAGE mMACHINE®* T7 transcription kit (Ambion, TX, USA). The capped transcripts were polyadenylated using yeast poly (A) polymerase (USB Corp., Cleveland, OH, USA) and were diluted and stored in diethylpyrocarbonate-treated water at –70°C.

The methods for isolating, culturing, injecting, and defolliculating of oocytes were identical with those described by E. Sigel (Sigel *et al.*, 1990). Mature female *Xenopus laevis* (Nasco, Fort Atkinson, WI, USA) were anaesthetized in a bath of ice-cold 0.17% Tricain (Ethyl-m-aminobenzoate, Sigma-Aldrich, St. Louis, MO, USA) before decapitation and removal of the frog's ovary. Stage 5 to 6 oocytes with the follicle cell layer around them were singled out of the ovary using a platinum wire loop. Oocytes were stored and incubated at 18°C in modified Barths' Medium [88 mM NaCl, 10 mM HEPES-NaOH (pH 7.4), 2.4 mM $NaHCO_3$, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.34 mM $Ca(NO_3)_2$] that was supplemented with 100 $U{\cdot}mL^{-1}$ penicillin and 100 $\mu g{\cdot}mL^{-1}$ streptomycin. Oocytes with follicle cell layer still around them were injected with an aqueous solution of mRNA. A total of 2.5 ng of mRNA per oocyte was injected. Subunit ratio was 1:1:5 for α1β3γ2 receptors and 1:1 for α1β3 receptors consisting of wild-type or mutated α1 subunit together with wild-type or mutated β3 subunit. After injection of mRNA, oocytes were incubated for at least 24 h for α1β3 receptors and for at least 36 h for α1β3γ2 receptors before the enveloping follicle cell layers were removed. Collagenase treatment (type IA; Sigma-Aldrich) and mechanical defolliculation of the oocytes was performed as described previously (Li *et al.*, 2003).

For electrophysiological recordings, oocytes were placed on a nylon-grid in a bath of Xenopus Ringer solution [XR, containing 90 mM NaCl, 5 mM HEPES-NaOH (pH 7.4), 1 mM $MgCl_2$, 1 mM KCl and 1 mM $CaCl_2$]. For current measurements the oocytes were impaled with two microelectrodes (2–3 MΩ) which were filled with 2 M KCl. The oocytes were constantly washed by a flow of 6 $mL{\cdot}min^{-1}$ XR that could be switched to XR containing GABA and/or drugs. Drugs were diluted into XR from DMSO-solutions resulting in a final concentration of 0.1% DMSO perfusing the oocytes. Drugs were pre-applied for 30 s before the addition of GABA, which was then co-applied with the drugs until a peak response was observed. Between two applications, oocytes were washed in XR for up to 15 min to ensure full recovery from desensitization. Maximum currents measured in mRNA injected oocytes were in the microampere range for all subtypes of $GABA_A$ receptors. To test for modulation of GABA induced currents by compounds, a GABA concentration that was titrated to trigger 3% of the respective maximum GABA-elicited current of the individual oocyte (EC3) was applied to the cell together with various concentrations of tested compounds. All recordings were performed at room temperature at a holding potential of –60 mV using a Warner OC-725C TEV (Warner Instrument, Hamden, CT, USA) or a Dagan CA-1B Oocyte Clamp or a Dagan TEV-200A TEV (Dagan Corporation, Mineapolis, MN, USA). Data were digitized, recorded and measured using a Digidata 1322A data acquisition system (Axon Instruments, Union City, CA, USA). Data were analysed using GraphPad Prism (La Jolla, CA, USA). Data for GABA dependent dose-response curves were fitted to the equation $Y = Bottom + (Top-Bottom)/1 + 10^{(LogEC50-X)*nH}$, where EC_{50} is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50% and *n*H is the Hill coefficient. Data are given as mean ± SEM from at least three oocytes of two or more oocyte batches. Statistical significance was determined by paired or unpaired Student's *t*-test and one-sample *t*-test, comparing the means with a hypothetical value of 100 (100% of GABA EC3 – control current) and a CI of $P < 0.05$.

N-Biotinylaminoethyl methanethiosulfonate (MTSEA) biotin – steric hindrance

A 2 mM MTSEA-biotin solution was freshly made in XR buffer containing the respective GABA-EC_3 concentration. Defolliculated oocytes were immediately immersed in the MTSEA-biotin solution for 3 min and washed with XR for 5 min. After the washing step, cells were used the same day for the electrophysiological recordings described above.

Molecular modelling and quantitative structure activity relation (QSAR)

Molecules were built using the builder module in MOE 2011.10 (Molecular Operating Environment; Chemical Computing Group, Montreal, Canada) and energy minimized using standard conditions (MMFF94x force field, adjust H and LP, gradient = 0.01, calculate force field partial charges). A database was built and a set of physicochemical parameters was calculated. These comprise a set of global descriptors for the compounds, such as log *P* (log *P*(o/w), (partition coefficient between octanol and water, a measure of lipophilicity), topological polar surface area, molar refractivity (a measure of the total polarizability), the water accessible surface area, the total hydrophobic surface area, the total polar surface area, as well as the hydrophobic surface area and the total polar van der Waal surface area. As descriptors for the substituents, Pi (contribution of a substituent to the lipophilicity of a compound) and Sigma-Hammett values (electron withdrawing strength relative to H) of substituents have been taken from (Hansch *et al.*, 1991).

QSAR analyses (partial least squares linear fit of pEC_{50} values as function of these descriptors) were performed as implemented in MOE 2011.10 using the above listed physicochemical descriptors. The quality of the models for predicting EC_{50} values was assessed and confirmed by standard statistical parameters (r2, RMSE) as well as by leave one out cross validation.

In an attempt to create classification models for separating compounds into positive modulator and null modulators, binary QSAR and a decision tree were performed. However, none of the classification models obtained showed satisfactory performance (data not shown).

Materials

GABA$_A$ receptor subunits and point mutations

cDNAs of rat GABA$_A$ receptor subunits α1, β3 and γ2S were cloned as described (Ebert *et al.*, 1996). The mutated construct α1V211C was a gift from E Sigel (Institute of Biochemistry and Molecular Medicine, Bern, Switzerland). For the generation of mutated β3 and γ2 subunits, these subunits were subcloned into pCDM8 expression vectors (Invitrogen, San Diego, CA, USA) as described previously (Tretter *et al.*, 1997). Mutated subunits were constructed by PCR amplification using the wild-type subunit as a template. For this, PCR primers were used to construct point mutations within the subunits by the 'gene splicing by overlap extension' technique (Horton *et al.*, 1993). The PCR primers for β3Q64C contained *XmaI* and *XhoI* restriction sites, which were used to clone the β3 fragments into pCI vector (Promega, Madison, WI, USA). The mutated subunits were confirmed by sequencing.

Compound synthesis

Twenty-three of the 33 compounds used in this study have been synthesized and published previously. Synthesis of the 10 investigated *LAU* compounds was conducted in analogy to previously outlined synthetic routes (Hoerlein *et al.*, 1979; Fryer *et al.*, 1993). In the case of the thiophene analog LAU168, a slightly modified protocol was followed (Elliott *et al.*, 1987) (see Supporting Information).

Investigated compounds

The following compounds were used: **1.** (*CGS 8216*): 2-phenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **2.** (*CGS 9896*): 2-(4-chlorophenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **3.** (*CGS 9895*): 2-(4-methoxyphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **4.** (*XHe-III-063*): 2-(4-ethynylphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **5.** (*PWZ-009A1*): 7-methoxy-2-phenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **6.** (*PZ-II-029*): 7-methoxy-2-(4-methoxyphenyl)-2*H*-pyrazolo [4,3-*c*]quinolin-3(5*H*)-one; **7.** (*XHe-III-006c*): 7-bromo-2-(4-bromophenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **8.** (*XHe-II-087c*): 2-(4-bromophenyl)-6-(tert-butyl)-2H-pyrazolo [4,3-c]quinolin-3(5H)-one; **9.** (LAU 163): 8-chloro-2-phenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **10.** (LAU 156): 8-chloro-2-(4-methylphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **11.** (*PZ-II-028*): 8-chloro-2-(4-methoxyphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **12.** (LAU 161): 4-(8-chloro-3-oxo-3,5-dihydro-2H-pyrazolo[4,3-c]quinolin-2-yl) benzonitrile; **13.** (LAU 206): 8-chloro-2-(4-aminophenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **14.** (LAU 162): ethyl 4-(8-chloro-3,5-dihydro-3-oxo-2*H*-pyrazolo[4,3-*c*]quinolin-2-yl) benzoate; **15.** (LAU 157): 8-chloro-2-(4-nitrophenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **16.** (LAU 159): 8-chloro-2-(3-methoxyphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **17.** (*PWZ-007A*): 8-methoxy-2-phenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **18.** (LAU 176): 8-methoxy-2-(4-methoxyphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **19.** (LAU 177): 4-(8-methoxy-3-oxo-3,5-dihydro-2H-pyrazolo [4,3-c]quinolin-2-yl)benzonitrile; **20.** (*XHe-III-24*): 8-*tert*-butyl-2-(4-fluorophenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **21**. (*XHe-II-006*): 8-*tert*-butyl-2-(4-bromophenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **22.** (*XHe-II-17*): 8-*tert*-butyl-2-(4-ethynylphenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **23.** (*XHe-II-094*): 8-(tert-butyl)-2-(4-((trimethylsilyl) ethinyl)phenyl)-2H-pyrazolo[4,3-c]quinolin-3(5H)-one; **24.** *(XHe/ON-I)*: 8-(tert-butyl)-2-(4-(3-(trimethylsilyl)prop-1-yn-1-yl)phenyl)-2H-pyrazolo[4,3-c]quinolin-3(5H)-one; **25.** (*XHe-II-019*): 8-*tert*-butyl-2-(4-(5,5-dimethylhexa-1,3-diynyl) phenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **26.** (PB XHe): 2-(4-bromophenyl)-7,8-dimethyl-2H-pyrazolo[4,3-c] quinolin-3(5H)-one; **27.** (*XHe-II-098b*): 2-(4-bromophenyl)-7,7,10,10-tetramethyl-7,8,9,10-tetrahydro-2*H*-benzo[*g*] pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **28.** (*XHe-II-098a*): 2-phenyl-7,7,10,10-tetramethyl-7,8,9,10-tetrahydro-2*H*-benzo [*g*]pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **29.** (*XHe-II-098c*): 2-(4-chlorophenyl)-7,7,10,10-tetramethyl-7,8,9,10-tetrahydro-2*H*-benzo[*g*]pyrazolo[4,3-*c*]quinolin-3(5*H*)-one; **30.** (*CGS 20625*): 5,6,7,8,9,10-hexahydro-2-(4-methoxyphenyl)cyclohepta[*b*] pyrazolo[3,4-*d*]pyridin-3(2*H*)-one; **31.** (LAU 168): 2-(4-methoxyphenyl)-2H-pyrazolo[3,4-d]thieno[3,2-b]pyridin-3 (5H)-one; **32.** (*XHe-III-67*): 2-(4-((trimethylsilyl)ethynyl) phenyl)-2H-pyrazolo[4,3-c]pyridin-3(5H)-one; and **33.** (*XHe-III-56*): 2-(4-bromophenyl)-2*H*-pyrazolo[4,3-*c*]pyridin-3(5*H*)-one; Compounds 1, 2, 3, 30 were gifts from Ciba Geigy (Novartis, Basle, Switzerland). Compounds 4–8, 11, 17, 20–29, 32, 33 were synthesized and provided by the laboratory of Prof. James Cook. The LAU-compounds were synthesized for this study in the laboratory of Prof. Marko D. Mihovilovic; ROD 188: (5R)-5-{(1R)-2-[(4-methylphenyl) sulfonyl]-1,2,3,4-tetrahydroisoquinolin-1-yl}dihydrofuran-2 (3H)-one (gift from Prof. Robert Dodd); Diazepam, Ro15-1788 (Sigma-Aldrich).

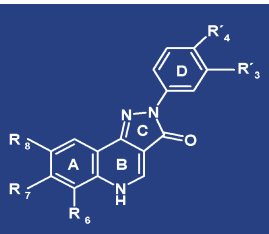
Results

Many structural analogues of CGS 9895 interact with the α+β– interface of GABA$_A$ receptors

CGS 9895 has been demonstrated to bind at two extracellular binding sites of GABA$_A$ receptors. At the α1+β2/3– interface (Figure 1), it acts as a positive allosteric modulator. At the high affinity benzodiazepine binding site – the α1+γ2– interface – it is a high affinity null modulator (Ramerstorfer *et al.*, 2011). To identify further compounds possibly mediating their effects via the α1+β3– interface and to exclude effects mediated via the high affinity benzodiazepine binding site at the α+γ– interface, we investigated 32 additional pyrazoloquinolinones/pyrazolopyridinones that are structurally analogous to CGS 9895 (compound 3) (Table 1), at receptors composed of α1 and β3 subunits (Figure 1B). Three of these compounds with the prefix CGS (compounds 1, 2 and 30) have been published as high affinity benzodiazepine site ligands (Brown *et al.*, 1984; Loo *et al.*, 1987; Williams *et al.*, 1989; Smith *et al.*, 2001; Ogris *et al.*, 2004). Nineteen other compounds have previously been generated as a series of symmetrically substituted pyrazoloquinolinones/pyrazolopyridinones, to probe the benzodiazepine binding site of different GABA$_A$ receptor subtypes (He *et al.*, 1999; Yu *et al.*, 1999; He, 2000). Nine representatives of this second group of compounds (compounds 4, 5, 6, 7, 11, 17, 20, 21

Table 1

Efficacy and potency of substituted pyrazoloquinolinones/ pyrazolopyridinones at α1β3 $GABA_A$ receptors, as well as their affinity for the benzodiazepine binding site of α1β3γ2 $GABA_A$ receptors.
Top: Pyrazolo[4, 3-c]quinolin-3-one nucleus; *Left:* Different substituents and whole chemical structure of pyrazoloquinolinones/ pyrazolopyridinones investigated; *Right, α1β3 receptor data:* Efficacy (10 μM) and potency (EC_{50} for positive modulators and IC_{50} for null modulators) of pyrazoloquinolinones/pyrazolopyridinones in recombinant rat α1β3 receptors expressed in *Xenopus laevis* oocytes, obtained by TEV electrophysiology. EC_{50}/IC_{50} values were computed by *GraphPad Prism*. Data represent means ± SEM (n = 4–12); *α1β3γ2 receptor data:* previously published affinities (K_i/IC_{50}) of the compounds for the benzodiazepine binding site of $GABA_A$ receptors (for references and methods used, see table footnote)



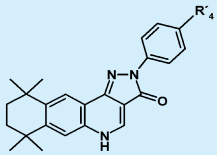
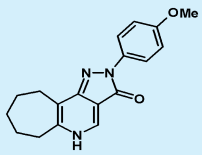
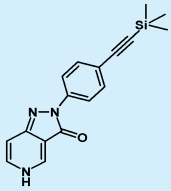
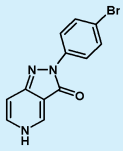
							α1β3	α1β3γ2
	Compounds	**R_8**	**R_7**	**R_6**	**R_4'**	**R_3'**	**10 μM**	**EC_{50} / IC_{50}* (μM)**	**K_i / IC_{50} (nM)**
1	CGS 8216	H	H	H	H	H	156 ± 18	*22 ± 10.2*	0.17 ± 0.01[a]
2	CGS 9896	H	H	H	Cl	H	245 ± 5	*11 ± 6.8*	0.5 ± 0.1[b]
3	*CGS 9895*	H	H	H	OMe	H	413 ± 25	9.5 ± 3.8	0.32 ± 0.5[a]
4	XHe-III-063	H	H	H	C ≡ CH	H	474 ± 29	4.3 ± 1.2	0.073[c]
5	PWZ-009A1	H	OMe	H	H	H	154 ± 3	*15 ± 4.6*	1.3[c]
6	PZ-II-029	H	OMe	H	OMe	H	213 ± 3	*26 ± 10.7*	0.3[c]
7	XHe-III-006c	H	Br	H	Br	H	110 ± 1	>10	34[c]
8	XHe-II-087c	H	H	*t*Bu	Br	H	196 ± 14	6.1 ± 1.5	7000[c]
9	LAU 163	Cl	H	H	H	H	151 ± 2	1.2 ± 0.2	N/D
10	LAU 156	Cl	H	H	CH_3	H	372 ± 40	2.3 ± 1.0	N/D
11	PZ-II-028	Cl	H	H	OMe	H	1079 ± 81	1.6 ± 0.4	0.2[c]
12	LAU 161	Cl	H	H	CN	H	300 ± 23	0.4 ± 0.1	N/D
13	LAU 206	Cl	H	H	NH_2	H	360 ± 36	0.6 ± 0.4	N/D
14	LAU 162	Cl	H	H	COOEt	H	140 ± 7	0.8 ± 0.4	N/D
15	LAU 157	Cl	H	H	NO_2	H	*n.s.*	7.5 ± 1.1*	N/D
16	LAU 159	Cl	H	H	H	OMe	117 ± 10	2.2 ± 1.9	N/D
17	PWZ-007A	OMe	H	H	H	H	244 ± 6	4.5 ± 0.3	0.1[c]
18	LAU 176	OMe	H	H	OMe	H	1058 ± 61	3.8 ± 0.2	0.14 ± 0.09[d]
19	LAU 177	OMe	H	H	CN	H	1063 ± 128	1 ± 0.1	0.75 ± 0.81[d]
20	XHe-III-24	*t*Bu	H	H	F	H	266 ± 28	*11 ± 1.7*	0.25[c]
21	XHe-II-006	*t*Bu	H	H	Br	H	216 ± 20	4.6 ± 1.9	4.7[c]
22	XHe-II-17	*t*Bu	H	H	C≡CH	H	180 ± 12	3.8 ± 1.2	3.3[c]
23	XHe-II-094	*t*Bu	H	H	C≡C$SiMe_3$	H	*n.s.*	33 ± 7.5*	329[c]
24	XHe/ON-I	*t*Bu	H	H	C≡CCH_2SiMe_3	H	112 ± 2	>10	N/D
25	XHe-II-019	*t*Bu	H	H	C≡C–C≡C*t*Bu	H	*n.s.*	25 ± 6.9*	273[c]
26	PB-XHe	CH_3	CH_3	H	Br	H	112 ± 1	>10	108[c]
27	XHe-II-098b				Br	H	*n.s.*	38 ± 7.9*	7000[c]
28	XHe-II-098a				H	H		*non-binder*	4000[c]
29	XHe-II-098c				Cl	H		*non-binder*	6000[c]
30	CGS 20625						391 ± 19	8.5 ± 2.1	0.5 ± 0.1[b]

Table 1

Continued

	Compounds	R_8	R_7	R_6	R_4'	R_3'	10 μM	α1β3 EC_{50} / IC_{50}* (μM)	α1β3γ2 K_i / IC_{50} (nM)
31	LAU 168							non-binder	N/D
32	XHe-III-67						484 ± 50	2.7 ± 0.6	>3000[c]
33	XHe-III-56						*n.s.*	35 ± 12.9*	1010[c]

*IC_{50} values obtained by the inhibition of [300 nM] compound 11 effect on GABA EC3 current; n.s., not significant; N/D, not determined.
[a]Ki: Displacement of [^{3}H]Ro15-1788 binding to human α1β3γ2 receptors (mean ± SEM, n = 3–6) (Smith *et al.*, 2001).
[b]Ki: Displacement of [^{3}H]flunitrazepam binding to mouse brain membranes without cerebellum (mean ± SD, *n* = 6–9) (Ogris *et al.*, 2004).
[c]Ki: Displacement of [^{3}H]Ro15-1788 binding to Ltk-cells expressing human α1β3γ2 receptors. Data are the means of two determinations, which differed by less than 10%. (He *et al.*, 1999; Yu *et al.*, 1999; He, 2000).
[d]IC_{50}: Displacement of [^{3}H]Ro15-1788 binding in a mouse cerebellar membrane (three times in triplicates; means ± SEM).
Italic EC_{50} values represent estimated values due to unsaturated compound dose response curves.

and 22) exhibited an affinity between 0.1 and 34 nM for the benzodiazepine binding site of α1β3γ2 $GABA_A$ receptors, whereas compounds 23, 25 and 26 exhibited an affinity between 100 and 300 nM (Table 1). Compounds 8, 27, 28, 29, 32 and 33 exhibited a drastically reduced affinity (1–7 μM) for this binding site (He *et al.*, 1999; Yu *et al.*, 1999; He, 2000; Smith *et al.*, 2001). No data on the affinity for the benzodiazepine binding site are available for compound 24, and the LAU compounds. The remaining 10 'LAU' compounds (9, 10, 12, 13, 14, 15, 16, 18, 19 and 31) were specifically synthesized in the context of the present study.

Neither compound 3, nor the other 32 compounds investigated were able to directly activate recombinant α1β3 receptors expressed in *X. laevis* oocytes. However, 24 of the 32 newly investigated compounds were able to robustly enhance GABA-induced currents at these receptors at 10 μM concentrations (Table 1). Whereas compounds 11, 18 and 19 enhanced GABA EC3 (GABA concentration eliciting 3% of the maximal GABA current) more than 10-fold, the effects of most of the other compounds were weaker (between 1.5- and fivefold stimulation of GABA EC3; Table 1).

In contrast to these positive modulators, compounds 15, 23, 25, 27, 28, 29, 31 or 33, did not significantly stimulate GABA EC3 at α1β3 receptors (Table 1).

To investigate whether these pyrazoloquinolinones/pyrazolopyridinones, similar to CGS 9895, mediate their effects via the extracellular α1+β3– interface, the effects of compound 11 were studied in more detail. This compound was selected due to its high efficacy for stimulation of GABA EC3 (to >1000%). In analogy to the previous study, we employed the substituted cysteine accessibility method to introduce a steric hindrance into the α1+β3– interface of α1β3 receptors (Ramerstorfer *et al.*, 2011). The point mutations α1V211C (loop C of the α1+side) and β3Q64C (loop D of the β3– side) have been shown previously to not significantly change the potency or efficacy of GABA for enhancing GABA-induced currents (Ramerstorfer *et al.*, 2011) at α1β3 or α1β3γ2 receptors. These mutations, however, partially reduced the effects of compound 11 in the absence of MTSEA-biotin. After incubation with MTSEA-biotin the effect of compound 11 was strongly further reduced ($P < 0.001$; Figure 2A, B). In contrast, no change in GABA EC3 current or in the

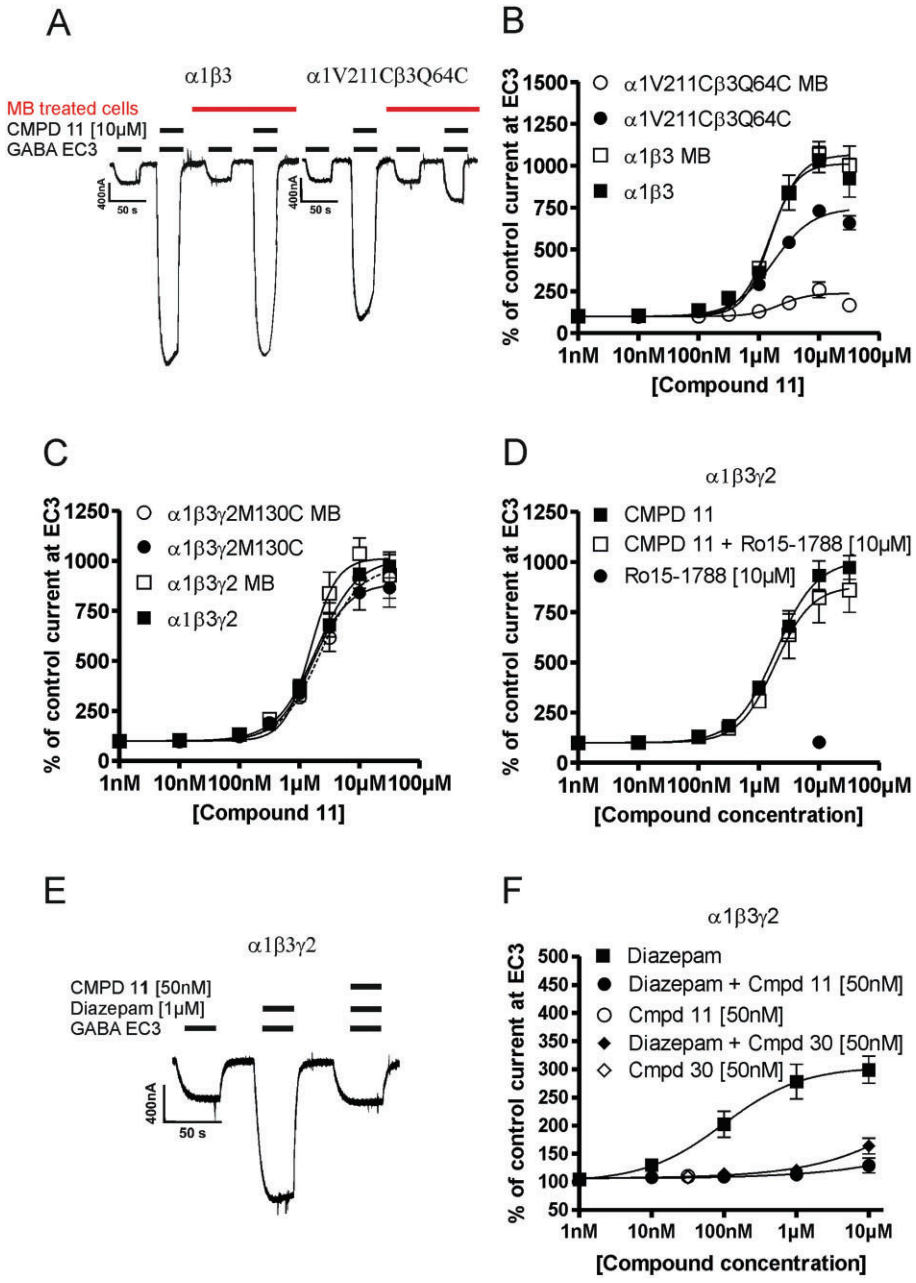


Figure 2

Effects of compound 11 on GABA EC3 at wild-type and mutated α1β3 and at α1β3γ2 receptors. (A) Left trace: Individual current traces of GABA EC3 in the absence or presence of 10 μM compound 11 in α1β3 wild-type receptors and in α1β3 wild-type receptors pre-incubated with MTSEA-biotin (MB); Right trace: Individual current traces at GABA EC3 in the absence or presence of 10 μM compound 11 in α1V211Cβ3Q64C receptors and in α1V211Cβ3Q64C receptors treated (pre-incubated) with MTSEA-biotin (MB). (B) Concentration-response curves of compound 11 in wild-type α1β3 receptors (■), α1β3 receptors pre-incubated with MTSEA-biotin (MB) (□), α1V211Cβ3Q64C receptors (●), and α1V211Cβ3Q64C receptors labelled by MTSEA-biotin (MB) (○).There was a slight reduction of compound 11 effect in mutated receptors (α1V211Cβ3Q64C) as compared with the wild-type α1β3 receptors. This effect was further significantly diminished after labelling of α1V211Cβ3Q64C receptors by MTSEA-biotin (100 nM – 30 μM; $P < 0.001$). (C) Concentration-dependent modulation of GABA EC3 control current by compound 11 in α1β3γ2 (■), wild-type α1β3γ2 receptors pre-incubated with MTSEA-biotin (MB) (□), α1β3γ2M130C (●) and α1β3γ2M130C receptors pre-incubated with MTSEA-biotin (○). Data represent means ± SEM ($n = 4–6$) (D) Concentration-dependent modulation of α1β3γ2 receptors by compound 11 in the absence (■) and the presence of 10 μM Ro15-1788 (□); the effect of Ro15-1788 on GABA EC3 current alone (●). The effect of compound 11 remained unchanged by the presence of Ro15-1788. Data represent means ± SEM ($n = 4–5$); (E) Individual current traces at GABA EC3 in the absence or presence of 1 μM diazepam, or 1 μM diazepam together with 50 nM compound 11. (F) Concentration-response curves for diazepam (■), or for diazepam applied together with 50 nM compound 11 (●) or compound 30 (◆) and the effect of 50 nM compound 11 (□) or compound 30 (◇) on the GABA evoked EC3 current alone, all measured in the same cell expressing α1β3γ2 receptors. Compound 11 or compound 30 at 50 nM concentrations drastically inhibited the positive modulatory effect of diazepam ($P < 0.001$). All experiments were performed four to eight times in oocytes of different batches. Data represent means ± SEM.

potency and efficacy of compound 11 was observed when wild-type α1β3 receptors were incubated with MTSEA-biotin (Figure 2A, B). These data indicated that compound 11 indeed exerts its action via the extracellular part of the α1+β3– interface. Using the same steric hindrance approach, we confirmed that other positive modulators from Table 1, such as compounds 32 and 8 (see below), and compounds 2, 10, 17, 21, 30, also act via the α1+β3– binding site (experiments not shown).

In other experiments, it was investigated whether compound 11, that additionally was able to inhibit [^{3}H]Ro15-1788 binding to *Ltk*-cells expressing human α1β3γ2 receptors with a Ki of 0.2 nM (He *et al.*, 1999), is mediating additional effects via the BZ binding site. Compound 11 dose dependently stimulated GABA EC3 in *X. laevis* oocytes expressing α1β3γ2 receptors (Figure 2C, D) and the efficacy of the compound as well as the shape of the dose-response curve was similar at α1β3 and α1β3γ2 receptors (see Figure 2B, C). The effects of compound 11 at α1β3γ2 receptors could not be significantly inhibited by 10 μM of the BZ-site antagonist Ro15-1788 (Figure 2D), nor by a steric hindrance approach targeting the BZ binding site of $GABA_A$ receptors via the γ2M130C mutation (Figure 2C) that previously has been demonstrated to inhibit diazepam effects at these receptors (Ramerstorfer *et al.*, 2011). These data strongly support the conclusion that compound 11 indeed exert its modulatory action solely via the extracellular part of the α1+β3– interface.

To investigate whether compound 11 similar to compound 3 (CGS 9895) is an allosteric null modulator at the BZ site (Ramerstorfer *et al.*, 2011), we first tested the effect of diazepam on the GABA EC3 current at α1β3γ2 receptors and after washing, repeated the diazepam dose-response curve in the presence of a low (50 nM) concentration of compound 11. This low concentration of compound 11 displayed a weak modulatory effect (to ~110% of 100% control current) on the GABA EC3 current. The potentiating effect of diazepam on the GABA EC3 current, however, was abolished by 50 nM of compound 11 down to the effects of this compound in the absence of diazepam (~110% of GABA EC3 control current, Figure 2E, F). Similar experiment was performed with 50 nM compound 30 that also almost completely abolished the diazepam effect (Figure 2F). These data indicated that compound 11 and 30, similar to compound 3 (Ramerstorfer *et al.*, 2011), act as a high affinity null modulators or extremely weak positive modulators at the benzodiazepine binding site, whereas at higher concentrations they stimulate $GABA_A$ receptors strongly via their second binding site at the α1+β3– interface.

Some compounds are null modulators at the α1+β3– binding site

As shown in Table 1, several compounds exhibited no modulation of GABA EC3 current at α1β3 receptors at 10 μM concentration. To explore whether they bind to the α1+β3– binding site as null modulators, we tested them by co-application with positive modulators of the α+/β– site. As indicated in Figure 3A, five compounds at 60 μM were able to more or less completely inhibit the effect of 300 nM of compound 11 at α1β3 receptors (compounds 15, 23, 25, 27, 33). To show that this is a specific effect, we also co-applied these compounds with modulatory ligands that do not elicit modulation via the α+/β– site (Ramerstorfer *et al.*, 2011). As expected, the GABA potentiating effects of ROD 188 (Thomet *et al.*, 2000; Figure 3B) or of valerenic acid amide (Khom *et al.*, 2010; data not shown) remained unaltered in the presence of compounds 23, 25, 27 or 33. These data indicated that these compounds were able to specifically block the effects of compound 11 mediated via the α1+β3– binding site without interfering with the action of compounds binding to an unrelated binding site of α1β3 receptors. These five compounds thus act as null modulators at the α1+β3– binding site. In contrast, compounds 28, 29 or 31, did neither inhibit the effects of 300 nM compound 11 at α1β3 receptors, nor those of 10 μM diazepam at α1β3γ2 receptors, and thus, are apparent non binders at these binding sites under these conditions.

To determine the potency of the null modulators at the α1+β3– binding site, the GABA-stimulatory action of 300 nM of compound 11 was inhibited by increasing concentrations of each of the null modulators. IC_{50} values for these five null modulators were in the μM range and they varied from 7.5 to 38 μM (Figure 3C, Table 1). The most potent null modulator was compound 15. This compound reduced the effect of compound 11 already at 1 μM, and nearly completely inhibited this effect at a concentration of 60 μM (Figure 3C). In other experiments, it was demonstrated that these null modulators were also able to inhibit the action of several other α+/β– positive modulators (for 8 and 32, see below, for 3, 10, 12, 30 experiments not shown) at α1β3 receptors as well. These results support the steric hindrance data, and strongly suggest that all investigated compounds interact with the same binding site at the α+/β– interface.

The null modulators we identified exhibited not only low potency (IC_{50}) for inhibiting the effects mediated via the α+/β– binding site, but their affinity for the benzodiazepine binding site was also significantly lower (K_i between 300 and 7000 nM for the displacement of specific [^{3}H]Ro15-1788 binding to α1β2γ2 receptors) compared to that of most of the α+β– positive modulators (K_i below 1 nM; He *et al.*, 1999; Yu *et al.*, 1999; Smith *et al.*, 2001; Ogris *et al.*, 2004). Around 60 μM of these null modulators, however, displayed diverse effects. Compounds 23 and 25 with a higher affinity (about 300 nM) for the α1+/γ2– binding site, at 60 μM completely abolished diazepam potentiation of GABA-induced currents. In contrast, compound 33 (affinity of about 1000 nM) only partially reduced diazepam potentiation, whereas compound 27 (affinity of about 7000 nM) did not influence the diazepam effect at all (Figure 3D). These results agree with the relative affinity of these compounds for the BZ binding site and indicate that compound 27 is the first null modulator selective for the α+/β– binding site.

Some positive allosteric modulators at the α1+β3– site do not interact with the α1+γ2– site

In addition to the abovementioned null modulators 27 and 33, two other compounds (32 and 8) also have low affinity (K_i of >3 and 7 μM, respectively) for the benzodiazepine binding site (He *et al.*, 1999) and additionally mediate robust positive stimulation of the GABA EC3 current at α1β3 receptors. Compound 32 already modulates GABA EC3 current at α1β3 receptor at 100 nM (to 113 ± 2%), and at 10 μM it potentiates GABA EC3 current to 466 ± 40% (Table 1).

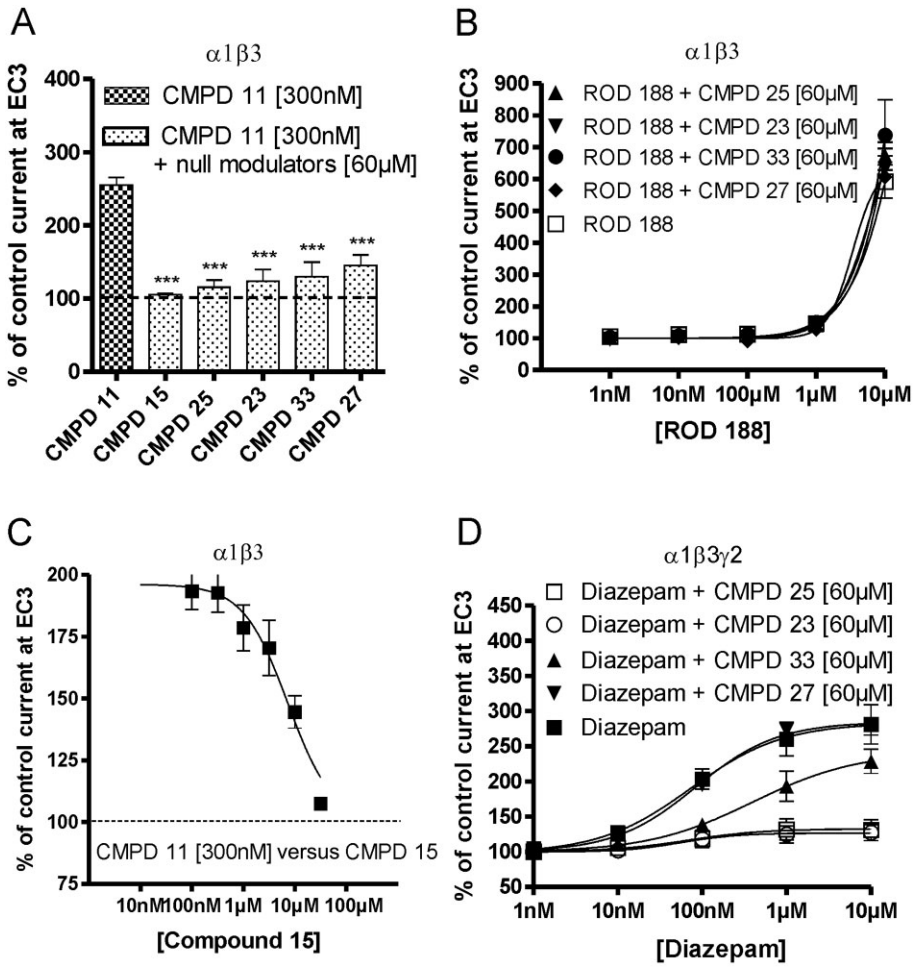


Figure 3

Several pyrazoloquinolinones/ pyrazolopyridinones are null modulators at the α1+β3– binding site. (A) The positive modulatory effect of 300 nM compound 11 (243 ± 11%) on GABA EC3 current (100%) in α1β3 receptors is significantly inhibited (***$P < 0.001$) by the co-application of 60 μM compound 15, compound 25, compound 23, compound 33, or compound 27. Data are expressed as means ± SEM ($n = 4–7$). (B) Concentration–response curves for ROD188 at α1β3 receptors (□) and ROD188 with co-applied 60 μM compound 25 (▲), 60 μM compound 23 (▼), 60 μM compound 33 (●) and 60 μM compound 27 (◆), indicating that the α1+β3– null modulators had no influence on the GABA enhancing effect of ROD188 in α1β3 receptors. (C) Compound 15 dose dependently inhibits the effects of 300 nM compound 11 on GABA EC3 in α1β3 receptors; IC_{50} values for compound 15 and those of the other four null modulators (compounds 25, 23, 33, 27), are mean values obtained from similar inhibition studies (curves not shown, $n = 4–6$), and are displayed in the Table 1. IC_{50} values were calculated using *GraphPad Prism* and a non-linear regression – one site competition curve. (D) Concentration–response curves for diazepam (■), and diazepam together with 60 μM compound 25 (□), compound 23 (○), compound 33 (▲) and compound 27 (▼) at α1β3γ2 receptors. The positive modulatory effect of diazepam on the GABA EC3 current was completely abolished by compound 25 and compound 23 ($P < 0.01$ at 100 μM-10 μM). Compound 33 decreased only partially the positive modulation of diazepam, whereas this effect remained almost unchanged in the presence of 60 μM compound 27. All experiments were performed four to seven times in oocytes of different batches. Data represent means ± SEM.

To confirm that the positive modulatory effects of compound 32 and compound 8 are mediated via the α+β– binding site we again used two approaches: the steric hindrance approach and the co-application with null modulators at the α+β– site. Results indicated that the positive modulatory effects of these compounds can be blocked by both the introduction of a steric hindrance at the α+β– binding pocket (Figure 4A, B) or by the α+β– binding site null modulator 33 (Figure 4C, D). These findings indicate that not only null modulators (compound 27) but also positive allosteric modulators (compounds 32 and 8) can be synthesized for the α+β– site that do not interact with the high affinity benzodiazepine binding site.

Ligand features driving potency and efficacy at the α1+β3-site

In this study, we report EC_{50} values for positive modulation ranging from 400 nM to ~30 μM, and efficacies ranging from 100% GABA EC3 (null modulators) to >1000% of GABA EC3 currents. In addition, we report IC_{50} values for the null modulators ranging from 7 to ~40 μM.

To investigate some basic structure-potency relationships, linear QSAR analysis was performed, relating log EC_{50} values to a set of global physicochemical parameters (see Methods) of all positive allosteric modulators for which EC_{50} values could be measured (22 in total). The best partial least squares (PLS) model retrieved showed a q2 value of 0.47, which is

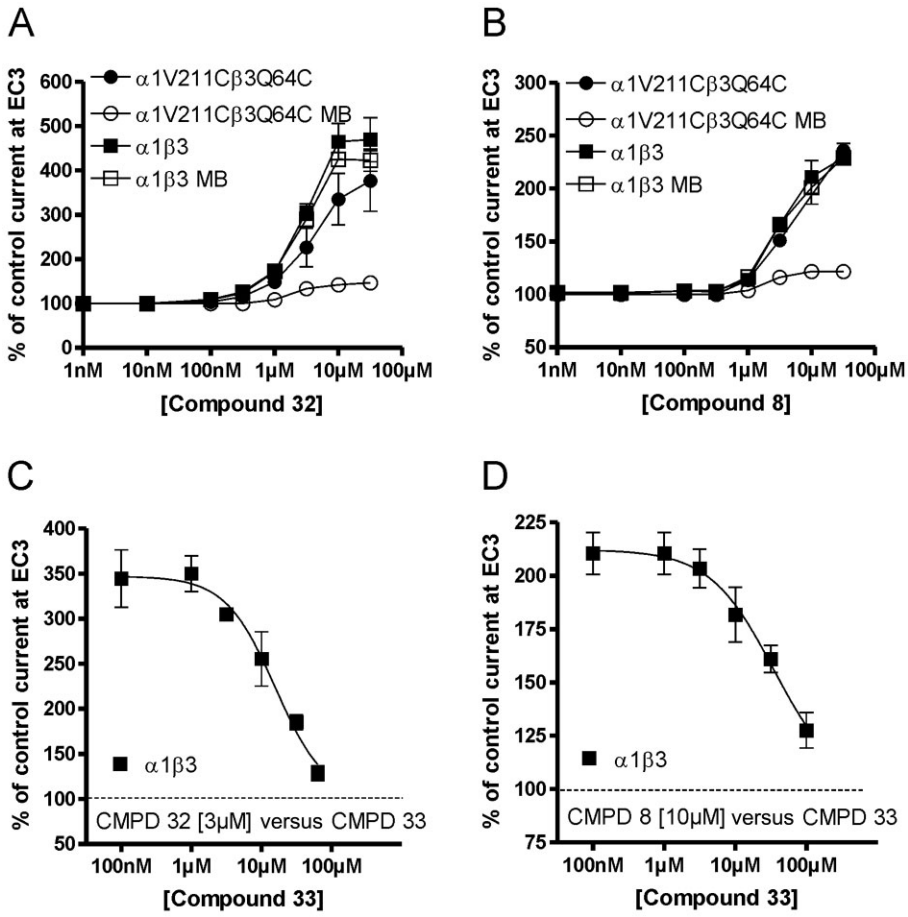


Figure 4

Pyrazoloquinolinones/ pyrazolopyridinones exhibiting either weak or no affinity for the benzodiazepine binding site exert their action via the α1+β3– binding site (A) Effects of compound 32, or (B) compound 8, on α1β3 (■), or on α1β3 pre-incubated with MTSEA-biotin(□) and, on α1V211Cβ3Q64C (●) receptors or α1V211Cβ3Q64C (MB), receptors labelled by MTSEA-biotin (○). A significant reduction of the effects of compound 32 or compound 8 was only observed in mutated receptors labelled with MTSEA-biotin (α1V211Cβ3Q64C MB) (at 1 μM $P < 0.05$; at 3, 10 and 30 μM $P < 0.001$ for both compounds). Data represent means ± SEM (n = 5–6). (C) The positive modulatory effects of 3 μM compound 32 (345 ± 32), or (D) of 10 μM compound 8 (211 ± 10) on GABA EC3 could be concentration-dependently inhibited by compound 33, an α+/β– binding site null modulator. Data represent means ± SEM (n = 4–5).

not considered as being significant. Global physicochemical parameters of the individual compounds thus cannot sufficiently explain the observed variance in the EC_{50} values. This indicates that the substituents introduced at the two aromatic rings influence the biological activity mostly via their local contribution rather than via modifying global physicochemical parameters of the compounds. In the next step, a subset of 20 compounds with the same basic scaffold was used for the calculations (excluding compounds 30 and 32 that show different basic scaffolds), whereby the pi, sigma-Hammett and MR substituent constants (defining lipophilicity, electron donating or withdrawing ability and molar refractivity, respectively) of the individual substituents R'_3, R'_4, R_6, R_7, and R_8 at the pyrazoloquinolinone scaffold, were used as descriptors. As the sigma-Hammett constant is encoding the electronic contribution of a given substituent to an aromatic system (electron donating and electron withdrawing), its value is dependent on the position of the substituent at the aromatic ring (ortho/ para vs. meta). While in ring D these positions are unambiguous (R'_4 is para, R'_3 is meta), in ring A, there are two possibilities. In the data matrix described above, we considered the position para to the quinoline nitrogen atom as para-position (R_8). Subsequently, R_7 is meta and R_6 is considered as ortho. In principle also R_7 could be assigned as para-position, which renders R_8 and R_6 to meta. Using the respective sigma-Hammett values, a significant model with q2 = 0.73 (r2 = 0.85) was obtained (see Supporting Information Diagram S1).

$$pEC_{50} = 0.50\ \text{sigma-}R_D - 0.34\ \text{pi-}R_D + 1.73\ \text{sigma-}R_A + 0.16\ \text{pi-}R_A + 5.16 \quad (1)$$

Legend: A and D stand for ring A and D. Sigma is a measure for electron withdrawing or donating property and pi a measure for lipophilicity (see Supporting Information Table S1).

The fact that both coefficients for sigma are positive indicates that on both aromatic rings electron withdrawing substituents are beneficial for high potency. Since the

lipophilicity coefficients (pi) are negative for substituents at ring D and positive for those at ring A, this descriptor apparently acts as space directed property. This indicates that on binding of the pyrazoloquinolinones into the α+β– pocket, presumably substituent R'_4 is in a more hydrophilic environment, while R_8 reaches into a hydrophobic pocket.

Finally, using only compounds showing a chloro substituent at ring A, the same trend (at ring D, electron withdrawing substituents with low lipophilicity are beneficial for activity) was observed (pIC_{50} = 0.39 sigma-RD – 0.39 pi-RD + 5.91; n = 7, r^2 = 0.71). Interestingly, in this case the m-OCH_3 derivative 16 might be considered as outlier, and using only the p-substituted derivatives 9–15 gave an almost perfect fit (r^2 = 0.91). However, as the biological activity range in this small subset is less than one order of magnitude, the QSAR models should only be taken as indicative for trends. For definite results, more detailed modelling studies with more compounds, covering a broader EC50 range, will be required in follow up studies. No model could be derived for a structure-efficacy relationship of the compounds.

Discussion

Here we investigated whether 32 structural analogues of compound 3 (CGS 9895) were also able to interact with the extracellular domain of the α1+β3– interface of $GABA_A$ receptors (Ramerstorfer *et al.*, 2011). Results indicated that similar to compound 3, none of these compounds was able to directly activate $GABA_A$ receptors in the absence of GABA, but 24 of the investigated compounds were able to significantly enhance GABA-induced currents at α1β3 receptors at 10 μM concentrations. The potency of these compounds for modulation of GABA-induced currents at α1β3 receptors varied between 0.4 and 26 μM. Similarly, the extent of modulation was significantly different for different compounds. Compounds 11, 18 and 19, dramatically stimulated GABA EC3 to >1000% of control current. Other compounds exhibited intermediate or weak efficacy at 10 μM concentrations.

Using a steric hindrance approach (Ramerstorfer *et al.*, 2011), we demonstrated that similar to compound 3, the effect of compound 11 and that of compounds 2, 8, 10, 17, 21, 30, 32, could be blocked by MTSEA-biotin interacting with cysteines at the α1+ (α1V211C) and β3– (β3Q64C) interface, suggesting that all these compounds mediate their modulatory effects via the α1+β3– interface. Most of these compounds have been demonstrated previously to exhibit a nM affinity for the benzodiazepine binding site located at the α+γ2– interface of α1β3γ2 $GABA_A$ receptors. The observation that compound 11 exhibited a comparable action at α1β3 and α1β3γ2 receptors indicates that the interaction with the benzodiazepine binding site, at least for this compound, seems not to contribute to the overall modulation of α1β3γ2 receptors. This conclusion was tested by experiments indicating that the effects of compound 11 at α1β3γ2 receptors could not be inhibited by Ro15-1788, a high affinity null modulator at the benzodiazepine binding site, and could also not be altered by a steric hindrance approach targeting the benzodiazepine binding site (Figure 2C, D). The additional observation that compound 11, as well as compounds 30, or 3, were able to inhibit the effects of diazepam at α1β3γ2 receptors (Figure 2F, Ramerstorfer *et al.*, 2011) indicate that these compounds act as potent null modulators via the BZ site of α1β3γ2 $GABA_A$ receptors. Furthermore, these observations together imply that binding of null modulators at the high affinity benzodiazepine site is not allosterically affecting the receptors response to plus modulators acting via the α1+β3– site.

Whereas α1β3γ2 receptors contain one α1+β3– and one α1+γ2– interface, α1β3 receptors contain two α1+β3– interfaces (Figure 1). As discussed above, interaction of compound 11 with the α1+γ2– interface does not contribute to its effects at α1β3γ2 receptors, because the effects of this compound could not be blocked by 10 μM of the benzodiazepine site null modulator Ro15-1788 and could not be inhibited by steric hindrance experiments via the benzodiazepine binding site. Since the dose-response curves for compound 11 and compound 3 (Ramerstorfer *et al.*, 2011) as well as their efficacy is identical at receptors with one (α1β3γ2) or two α1+β3– interfaces (α1β3), it seems likely that already the first binding event triggers a conformational change that leads to the observed dynamic effect.

In this study, we also identified five compounds (compounds 15, 23, 25, 27, 33) that did not modulate GABA-induced currents at α1β3 receptors but were able to inhibit the action of positive allosteric modulators acting via the α1+β3– interface. These compounds thus represent the first five null modulators at the α+β– interface. Whereas compounds 23, 25 and 33 are null modulators at the α+β– interface as well as at the benzodiazepine binding site, compound 27 does not bind to the benzodiazepine binding site and thus represents the first null modulator with α+/β– binding site selectivity.

Similarly, compounds 32 and 8 exhibit a low affinity for the benzodiazepine binding site (>3 μM) but are positive allosteric modulators at α1β3γ2 receptors. These compounds thus, represent the first positive allosteric modulators that seem to exclusively act via the α+β– interface.

Almost all compounds investigated, with the exception of compound 30, 31 32 and 33 contained the compound 1 core structure, a pyrazoloquinolinone nucleus without substituents (Table 1), that itself only weakly modulated GABA EC3 currents at α1β3 receptors (to 156%). Different substituents at the A and D rings (Table 1) induced profound alterations in the efficacy of compounds at α1β3 receptors. Although deriving a quantitative structure-efficacy model was not possible, a comparison of the efficacy data reported in Table 1 between pairs and triplets of compounds differing from compound 1 only in positions R'_4 and/or R_8 reveals some interesting trends on how compound structure influences efficacy.

If H at position R'_4 of compound 1 is replaced by methoxy (compound 3) or ethinyl (compound 4), efficacy increases strongly. In contrast, if R'_4 is Cl (compound 2), efficacy stays low. If H at position R_8 of compound 1 is replaced by Cl (compound 9) efficacy also does not change. But, interestingly, Cl in R_8 dramatically enhances effects of further substitutions at position R'_4, as exemplified by methoxy (compound 11), resulting in one of the most active compounds at the α+β– binding site. Similarly, if H at position R_8 of compound 1 is replaced by methoxy as single substitution (compound 17) no change in efficacy is observed, but this substituent again dramatically influences effects of further

substitutions at position R'_4 by methoxy (compound 18) or CN (compound 19). Thus, obviously substituents in R_8 and R'_4 must act on biological activity in a complex concerted fashion. This is also exemplified by the finding that R_8 Cl and R_8 methoxy seem to be equivalent for efficacy as long as there is a methoxy at R'_4 (compounds 11 vs. 18). In case of a CN at R'_4, however, R_8 Cl and R_8 methoxy are not equivalent for efficacy (compounds 12 vs. 19). Finally, removing or replacing the A ring by different other moieties also results in changes of modulatory properties. Interestingly, as demonstrated by compounds 30 and 32, both removal of A and replacement of A by larger, non-planar moieties can yield ligands that modulate via the α+β– binding site.

Different trends also determine the potencies of the compounds as measured by their EC_{50} values. Here, a QSAR model of the contributions of functional groups in R_8 and R'_4 to EC50 could be established, indicating that electron withdrawing substituents on aromatic rings A and D, as well as lipophilic R_8 and hydrophilic R'_4 substituents are beneficial for high potency. The path forward will thus combine efficacy and potency promoting substituents in positions R'_4 and R_8 with α+β– selectivity promoting features such as the tBu group in R_6 from compound 8.

Most of the data presented here were generated using α1β3 receptors. These receptors not only served as a model system for investigating the interaction of pyrazoloquinolinones/pyrazolopyridinones with the α1+β3– interface but also have been demonstrated to actually exist in the brain (Mortensen and Smart, 2006). Drugs non-selectively interacting with the α+β– interface should be able to modulate αβ, αβγ, αβδ, αβε, αβπ and αβθ receptors and should thus exhibit a much broader action than benzodiazepine site ligands, which only interact with αβγ receptors. The broader action of such drugs might be especially beneficial for the treatment of epilepsy. Since drugs mediating their effects via the α+β– site act by enhancing ongoing GABAergic transmission only, they should exhibit low toxicity and be specifically active in brain areas with exaggerated excitatory and thus also GABAergic activity (Sieghart *et al.*, 2012).

Although the potency of the novel compounds interacting with the α+β– interface is still too low for a clinical development, the present study indicates that in addition to compound 3, other pyrazoloquinolinones/pyrazolopyridinones are also exerting their effects via the α+β– interface. Further modification of the pyrazoloquinolinones/pyrazolopyridinones structure might thus generate compounds with higher potency and a potential clinical application. In addition, the novel α+β– selective antagonist compound 27 can now be used for the identification of drugs from different structural classes mediating their positive or negative allosteric effects via this binding site.

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 **A.** Pyrazoloquinolinones scaffold; A ring: R_8 (para (*p*)-position), R_7 (meta (*m*)-position), R_6 (orto (*o*)- position); D ring: R'_4 (para-position), R'_3 (meta-position); **B.** QSAR analysis (structure-potency relationships): EC_{50} (potency – 50% of maximal effective concentration), pEC_{50} ($logEC_{50}$), sigma (Sigma-Hammet: the electronic contribution of a given substituent to an aromatic system – electron donating and electron withdrawing), pi (lipophilicity), MR (molar refractivity); pEC_{50} (predicted) – ($pEC_{50} = 0.50$ sigma-R_D – 0.34 pi-R_D + 1.73 sigma-R_A +0.16 pi-R_A + 5.16).

Diagram S1 pEC_{50} (predicted value) versus pEC_{50} (lab value): Performance of the QSAR model visualized on the linear plot: The small deviations of predicted EC_{50} versus lab values demonstrate the overall validity of the QSAR model. A and D stand for ring A and D. Sigma is a measure for electron withdrawing or donating property, and pi a measure for lipophilicity.