A Benzodiazepine Ligand with Improved GABA$_A$ Receptor \( \alpha \)-5-Subunit Selectivity Driven by Interactions with Loop C

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ABSTRACT

The family of GABA$_A$ receptors is an important drug target group in the treatment of sleep disorders, anxiety, epileptic seizures, and many others. The most frequent GABA$_A$ receptor subtype is composed of two \( \alpha \)-, two \( \beta \)-, and one \( \gamma \)-2-subunit, whereas the nature of the \( \alpha \)-subunit critically determines the properties of the benzodiazepine binding site of those receptors. Nearly all of the clinically relevant drugs target all GABA$_A$ receptor subtypes equally. In the past years, however, drug development research has focused on studying \( \alpha \)-5-containing GABA$_A$ receptors. Beyond the central nervous system, \( \alpha \)-5-containing GABA$_A$ receptors in airway smooth muscles are considered as an emerging target for bronchial asthma. Here, we investigated a novel compound derived from the previously described imidazobenzodiazepine SH-053-2'F-R-CH$_3$ (SH53d-ester). Although SH53d-ester is only moderately selective for \( \alpha \)-5-subunit-containing GABA$_A$ receptors, the derivative SH53d-acid shows superior (>40-fold) affinity selectivity and is a positive modulator. Using two-electrode voltage clamp electrophysiology in Xenopus laevis oocytes and radioligand displacement assays with human embryonic kidney 293 cells, we demonstrated that an acid group as substituent on the imidazobenzodiazepine scaffold leads to large improvements of functional and binding selectivity for \( \alpha \beta \gamma \)-2 over other \( \alpha \beta \gamma \)-2 GABA$_A$ receptors. Atom level structural studies provide hypotheses for the improved affinity to this receptor subtype. Mutational analysis confirmed the hypotheses, indicating that loop C of the GABA$_A$ receptor \( \alpha \)-subunit is the dominant molecular determinant of drug selectivity. Thus, we characterize a promising novel \( \alpha \)-5-subunit–selective drug candidate.

SIGNIFICANCE STATEMENT

In the current study we present the detailed pharmacological characterization of a novel compound derived from the previously described imidazobenzodiazepine SH-053-2'F-R-CH$_3$. We describe its superior (>40-fold) affinity selectivity for \( \alpha \)-5-containing GABA$_A$ receptors and show atom-level structural predictions to provide hypotheses for the improved affinity to this receptor subtype. Mutational analysis confirmed the hypotheses, indicating that loop C of the GABA$_A$ receptor \( \alpha \)-subunit is the dominant molecular determinant of drug selectivity.

Introduction

GABA$_A$ receptors are GABA-gated chloride channels that are expressed in neurons, glial cells, and several non-neuronal cell types (Gladkevich et al., 2006; Mizuta et al., 2008; Olsen and Sieghart, 2008; Barragan et al., 2015; Wan et al., 2015) wherein they influence a variety of cellular processes through ligand-gated chloride flux. These receptors are pentamers of subunits that are comprised of different subunit classes. The existence of six \( \alpha \)-, three \( \beta \)-, three \( \gamma \)-, one \( \delta \)-, one \( \epsilon \)-, one \( \theta \)-, one \( \pi \)-, and three \( \rho \)-subunits in mammalian systems gives rise to an enormous theoretical diversity of GABA$_A$ receptor subtypes with distinct subunit composition and unique pharmacological properties. Because of the presence of multiple GABA$_A$ receptor subunits in individual cells, however, an unequivocal identification of a receptor subtype is difficult (Olsen and Sieghart, 2008). So far, the native expression of only 11 subunit combinations has been conclusively demonstrated. In addition, there is strong evidence for the existence of several other subunit combinations, the number of which is slowly increasing (Olsen and Sieghart, 2008).

GABA$_A$ receptors in the central nervous system (CNS) are the site of action of a variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, and anesthetics, which allosterically modulate GABA-induced currents eliciting anticonvulsant, sedative-hypnotic, and anxiolytic effects (Brickley and Mody, 2012; Rudolph and Möhler, 2014; Sieghart, 2015). In addition to these synthetic drugs, a wide range of natural products (Khom et al., 2010; Lorenz et al., 2010; Hanrahan et al., 2011) as well as some endogenous agents, such as neuroactive steroids (Beelli and Lambert, 2005), the endocannabinoid 2-arachidonoyl glycerol (Sigel et al., 2011), histamine (Saras et al., 2008; Fleck et al.,...
2012), and dopamine (Hoerbelt et al., 2015), modulate GABA<sub>A</sub> receptors.

The benzodiazepine binding site is located at the extracellular α1+γ2 interface of the GABA<sub>A</sub> receptors (Sigel and Lüscher, 2011; Richter et al., 2012). Thus, the potency and efficacy of benzodiazepine site ligands are dependent on the subtype of both of these subunits. In the CNS, benzodiazepine ligands exert mostly anxiolytic, anticonvulsive, sedative hypnotic, and myo-relaxant properties (Sieghart, 2015; Sieghart and Savić, 2018). The individual effects of benzodiazepines seem to be predominantly mediated by distinct GABA<sub>A</sub> receptor subtypes, and drugs specifically interacting with these subtypes are thus expected to exhibit quite selective pharmacological and behavioral effects (Möhler, 2011).

One of the first relatively α5β3γ2-selective positive allosteric modulators reported was the imidazobenzodiazepine SH-053-2-F-R-CH3 (SH53d-ester), featuring a window of separation between the modulation of α5β3γ2 receptors and α1, α2, or α3β3γ2 receptors (Savić et al., 2010). In electrophysiological experiments, SH53d-ester is selective for α5 receptors at low nanomolar concentration. At 100 nM concentration, this compound markedly enhances GABA-elicited currents at α1β3γ2, α2β3γ2, α3β3γ2, and α5β3γ2 receptors (Fischer et al., 2010; Savić et al., 2010). Concomitantly, this compound has moderate affinity for the benzodiazepine binding site of α5β3γ2 receptors and low affinity for α1, α2, or α3β3γ2 receptors (Fischer et al., 2010).

Ester-to-amide substitution in SH53d-ester led to MP-III-022 with improved selectivity, efficacy, and kinetic behavior as a positive modulator of GABA<sub>A</sub> receptors containing the α5 subunit (Stamenic et al., 2016). Accordingly, binding affinities of MP-III-022 (Ki) for the different receptor subtypes α1β3γ2, α2β3γ2, α3β3γ2, and α5β3γ2 expressed in human embryonic kidney (HEK) 293 cells were 850, 360, 660, and 55 nM, respectively. TEV electrophysiology in oocytes revealed allosteric modulation of MP-III-022 in α5β3γ2 GABA<sub>A</sub> receptors with efficiencies of 300% at 100 nM while being nonmodulatory (α1) or only weakly modulatory at α2- and α3-containing receptors.

Clinically applied drugs targeting GABA<sub>A</sub> receptors are mainly used for their effects on the human CNS (Sieghart, 2015). However, considerable interest in these receptors expressed in peripheral tissues as potential therapeutic targets has emerged (Gladkevich et al., 2006; Mizuta et al., 2008; Sengupta et al., 2014; Barragan et al., 2015; Gallos et al., 2015; Wan et al., 2015). Of specific interest are GABA<sub>A</sub> receptors expressed in airway smooth muscle (ASM) and their ability to induce relaxation of an established contraction, which could have enormous clinical implications in bronchoconstrictive diseases, such as asthma. It was shown that the α5-prefering chiral imidazobenzodiazepine SH53d-ester relaxes ASM and enhances chloride currents in cultured ASM cells (Gallos et al., 2015), thus suggesting that benzodiazepine-sensitive α5β2-containing receptors are present.

The derivative of SH53d-ester, which we characterize here (called SH53d-acid, formerly known as compound 2), has also been tested in this assay and shown to be effective (Forkuo et al., 2017), whereas a detailed presentation of the compound’s pharmacology was still missing. In the current study we now provide this detailed pharmacological characterization of SH53d-acid and describe its superior (>40-fold) affinity selectivity for α5-containing GABA<sub>A</sub> receptors. Computational docking provides a hypothesis for the improved affinity to this receptor subtype. Using mutational analysis, we were able to identify loop C of the GABA<sub>A</sub> receptor α-subunit as the dominant molecular determinant of drug selectivity.

### Materials and Methods

#### Compounds.

SH-053-2 F-R-CH<sub>3</sub> = SH53d-ester: (R)-8-ethyl-6-(2-fluorophenyl)-4-methyl-4H-benzo[f][imidazo[1,5-a][1,4]diazepine-3-carboxylic acid ethyl ester; SH53d-acid: (R)-8-ethyl-6-(2-fluorophenyl)-4-methyl-4H-benzo[f][imidazo[1,5-a][1,4]diazepine-3-carboxylic acid; Fig. 1. All compounds listed above were synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin–Milwaukee. For details of SH53d-acid synthesis, see “compound 2” in Forkuo et al. (2017).

[^3H]Flunitrazepam (specific activity 83 Ci/mmol) and [^3H]Ro 15-4513 (specific activity 49.5 Ci/mmol) were purchased from Perkin Elmer New England Nuclear (Waltham, MA). Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4, benzodiazepine-2-one) was from Nycomed (Opfikon, Switzerland). Standard chemicals came from Sigma-Aldrich (St. Louis, MO).

**GABA<sub>A</sub> Receptor Subunits and Mutated Subunits.** cDNAs of rat GABA<sub>A</sub> receptor subunits α1, α2, α3, α5, β3, and γ2 (GenBank accession numbers: NM_183326.2, NM_01135779.2, NM_017069.3, NM_017285.1, NM_017065.1, NM_183327.1) were cloned into pC11 expression vectors. The mutant subunits (for details on the nomenclature of the mutated amino acids see Supplemental Fig. 1 and Supplemental Table 2) were constructed using the QS Site-Directed Mutagenesis Kit (New England Biolabs) following manufacturer’s instruction using the primers designed with the NEBaseChanger online tool (https://nebasechanger.neb.com) and listed in Table 1. The mutated subunits were confirmed by sequencing (Table 1).

**Culturing of Human Embryonic Kidney 293 Cells.** HEK 293 cells (American Type Culture Collection ATCC CRL-1574) were maintained in Dulbecco’s modified Eagle medium (high glucose, GlutaMAX supplement, Gibco 61965-059; ThermoFisher, Waltham, MA) supplemented with 10% fetal calf serum (F7524; Sigma-Aldrich), 100 U/ml penicillin-streptomycin (Gibco 15140-122; ThermoFisher), and minimum Eagle’s medium (Nonessential Amino Acids Gibco 11410-035; Thermofisher) on 10-cm cell culture dishes (Cell*; Sarstedt, Nümbrecht, Germany) at 37°C and 5% CO<sub>2</sub>.

HEK 293 cells were transfected with cDNAs encoding rat GABA<sub>A</sub> receptor subunits subcloned into pC1 expression vectors. The ratio of plasmids used for transfection with the calcium phosphate precipitation method (Chen and Okayama, 1987) was 3 μg α (1, 2, 3, or 5); 3 μg β3: 15 μg γ2 per 10-cm dish. Medium was changed 4–6 hours after transfection. Cells were harvested 72 hours after transfection by scraping into phosphate-buffered saline. After centrifugation (10 minutes, 12,000g, 4°C) cells were resuspended in 50 mM Tris-citrate (pH 7.1), homogenized with an ULTRA-TURRAX (IKA, Staufen, Germany), and centrifuged (20 minutes, 50,000g). Membranes were washed three times in TC50 as described above and frozen at −20°C until use.

**Radioligand Binding Assay.** Frozen HEK 293 membranes were thawed, resuspended in TC50, and incubated for 90 minutes at 4°C in a total of 500 μl of a solution containing 50 mM Tris-citrate buffer, pH = 7.1; 150 mM NaCl; and 2 nM [^3H]Flunitrazepam or 5 nM [^3H]Ro 15-4513 in the absence or presence of 5 μM diazepam or 50 μM Ro 15-1788 (to determine nonspecific binding; final DMSO concentration 0.5%). Membranes were filtered through Whatman GF/B filters (GE Healthcare, distributed by VWR Austria) and rinsed twice with 4 ml of ice-cold 50 mM Tris-citrate buffer. Filters were transferred to scintillation vials and subjected to scintillation counting after the addition of 3 ml Rotiszint Eco plus liquid scintillation cocktail (Lacant, Graz, Austria). Nonspecific binding determined in the presence of 5 μM diazepam or 50 μM Ro 15-1788 was subtracted from total binding to determine specific binding.
To determine the equilibrium binding constant $K_0$ for the various receptor subtypes, membranes were incubated with various concentrations of the radioligand in the absence or presence of an inhibitor. Saturation binding experiments were analyzed using the equation $Y = B_{\text{max}} \times X/(K_0 + X)$. Nonlinear regression analysis of the displacement curves used the equation: $\log(\text{inhibitor})$ vs. response — variable slope $Y = 100/(1 + 10^{(\log IC_{50}\times X)} \times \text{Hill slope})$) with Top = 100%; Bottom = 0%; and Hill slope = −1. Both analyses were performed using GraphPad Prism version 8.3.0 for Mac OS X (GraphPad Software, La Jolla, CA; www.graphpad.com). Drug concentrations resulting in half-maximal inhibition of specific $[3H]$-ligand binding (IC$_{50}$) were converted to $K_i$ values by using the Cheng-Prusoff relationship (Cheng and Prusoff, 1973), $K_i = IC_{50}/(1 + (S/KD))$, with $S$ being the concentration range of the compound. pH was measured in the buffer alone, in the same buffer containing GABA and/or drugs. Drugs were diluted from DMSO solutions, resulting in a final concentration of 0.1% DMSO. The GABA concentration eliciting 5-50% of the maximal GABA response (EC$_{50}$) was determined at the beginning of each experiment. Maximum currents measured in mRNA-injected oocytes were in the microampere range for all receptor subtypes. Compounds were coapplied with GABA until a peak response was observed. Enhancement of the chloride current was defined as $(I_{\text{GABA+Comp}}/I_{\text{GABA}}) - 1$, wherein $I_{\text{GABA+Comp}}$ is the current response in the presence of a given compound, and $I_{\text{GABA}}$ is the control GABA current. Between two applications, oocytes were washed in ND96 for up to 15 minutes to ensure full recovery from desensitization. SH53d-acid did not affect the pH of the measuring buffer (7.51−7.52) over the whole concentration range of the compound. pH was measured in the buffer alone, in GABA EC$_{50}$, and in GABA EC$_{50}$ containing SH53d-acid at 1 nm to 10 μM. Since the measurement buffer keeps the pH constant, we can be sure that over the dose-response curve, the protonation state of the compound did not change. Beyond this observation, we cannot draw any conclusions about the influence of the protonation state on the activity. It seems reasonable to assume that this compound was, as organic acid, deprotonated at physiologic pH. As has been discussed in Forkuo et al., (2018), this is beneficial for topical application. All recordings were performed at room temperature at a holding potential of −60 mV using a Dagan CA-1B Oocyte Clamp or a Dagan TEV-200 A TEV amplifier (Dagan Corporation, Mineapolis, MN). Data were digitized using a Digidata 1322 A or 1550 data acquisition system (Axon Instruments, Union City, CA), recorded using Clampex 10.5 software (Molecular Devices, Sunnyvale, CA), and analyzed using Clampfit 10.5. Data were analyzed using GraphPad Prism v.6 and plotted as concentration-response curves. These curves were normalized and fitted by nonlinear regression analysis to the equation $Y = \text{bottom} + \text{top-bottom}/(1 + 10^{(\log EC_{50}\times X)} \times \text{Hill})$, wherein $EC_{50}$ 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 ± S.D. from at least three oocytes of two or more oocyte batches. Statistical significance was calculated using an extra sum of squares $F$ test. $P$ values of $<0.05$ were accepted as statistically significant.

### Computational Docking

The experimental structures 6HUO, 6D6T, and 6A96 containing the α1β2 interface were employed for this.

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**Table 1**

| Cloning primers |  |
|-----------------|--|
| 5'-a3-T215P-FW  | CTATGCCTATG-CCAAGGCTGGAAG |
| 5'-a3-T215P-RV  | CTGGCCAACTTCAGTGGGAC |
| 5'-a3-S257T-FW  | GATATCAGGAGCTATGCAGG |
| 5'-a3-S257T-RV  | TCTGTCCAAATGACATGAC |
| 5'-a5-197T-FW   | TTTAGCTACAATTCCAGGAAG |
| 5'-a5-197T-RV   | TCTGGCAATTTCAGGGGAGG |
| 5'-a5-238S-FW   | AACATCAGGACAGACAGG |
| 5'-a5-238S-RV   | CTCAGTGTCCTACTGTCG |

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**Fig. 1.** Chemical structures of the two compounds.
study. The 6A96 structure does not contain a benzodiazepine site. The a5 subunit’s extracellular domain of 6A96 overlaps well with the α1 subunits in 6HUO, 6D6T, and 6D6U. Thus, since benzodiazepine-bound states are of interest, we used 6HUO and 6D6T to generate homology models of the extracellular α5+γ2- interface on the basis of the alprazolam- and flumazenil-bound experimental structures using Modeler (Sali and Blundell, 1993) for the subsequent docking. Molecular docking was performed with the program GOLD (Jones et al., 1997). The numbering of the amino acids in the figures corresponds to mature human protein UniProtKB-accession numbers P14867 for α1 and P31644 for a5 (for comparison between rat and human amino acid numbering, see Supplemental Table 2). The putative binding site was set around the β carbon atom of the α5Ser209 that lies on the loop C with a cutoff distance of 12 Å. Five amino acids of the γ2-subunit and five of the α5-subunit were selected to have flexible side chains: γ2Asp56, γ2Tyr58, γ2Phe77, γ2Thr142, and γ2Arg144 and α5Tyr163, α5Thr208, α5Ser209, α5Thr210, and α5Tyr213, respectively. Soft potentials were chosen for α5Thr208, α5Ser209, α5Thr210, and α5Gly211 to simulate backbone flexibility of loop C. The ligand was docked with the protonated carboxylic acids group fully flexible and the seven-ring restrained to what is seen in the experimental structures (a control run with a flexible seven-ring gave very similar results). The poses were scored with GoldScore and rescoring with ChemScore, as implemented in GOLD. For each ligand and each protein, the top-100 (GoldScore) poses were generated with default settings.

Results

SH53d-Acid has Superb Affinity, Efficacy, and Selectivity. Since the ester-to-amide substitution in SH53d-ester led to MP-III-022 with improved binding and efficacy selectivity, more substritutes were explored. Here, we present an acid-substituted compound with superior affinity selectivity. To directly compare the properties of this novel compound SH53d-acid with its parent compound SH53d-ester, both were measured at identical conditions side-by-side in radioligand binding assays as well as in two-electrode voltage clamp experiments. Although SH53d-acid still displays low affinity for α1, α2, or α3β3γ2 receptors (in the micromolar range) in radioligand binding assays, the affinity toward α5β3γ2 is approximately 10-fold higher than the parent compound, now being in the low nM range (see Fig. 2B; Table 2). SH53d-acid has superior (>40-fold) affinity selectivity for α5-over α1-containing GABA_A receptors, which is much more selective than MP-III-022 (15-fold) and SH53d-ester (9-fold). There is also a clear window of separation between α5- and α3-containing GABA_A receptors with an affinity selectivity of 16-fold in SH53d-acid compared with 12-fold in MP-III-022, whereas it is only 7-fold in SH53d-ester. Compared with α3-containing GABA_A receptors, the differences in affinities are 13-fold (SH53d-acid), being nearly equal to the parent compound SH53d-ester (15-fold), whereas MP-III-022 has the smallest difference (6-fold). In two-electrode voltage clamp experiments, we not only observed a similar affinity shift but also greatly enhanced efficacy in modulating GABA-induced currents in α5-containing receptors compared with the other receptors analyzed (Fig. 2D; Table 3). The SH53d-acid has a wider window of separation, with nearly no modulatory activity in the non-α5 subtypes up to 30 nM (see Table 3). As is also the case for diazepam, the efficacy is higher in α3-containing receptors compared with α2-containing receptors, but unlike diazepam, it is highest in α5-containing receptors (Savić et al., 2010) (Fig. 2; Tables 2 and 3).
sought to narrow down the influence of amino acids that are different among α isoforms but do not contribute to the α5 unique properties. In segment C, the α5- and α1-subunits differ in two pocket-forming amino acids (see Fig. 3), whereas the α5- and α3-subunits differ only in one of these. We therefore chose to compare the two subunits that share more amino acids in the pocket and proceeded to the mutational analysis with a side-by-side comparison of the α5- and α3-subunits as described in the Materials and Methods.

**Choice of Radioligand: 3H-Flunitrazepam Proves to Be Unsuitable.** Based on our computational analysis we decided to generate mutated subunits: In the B-loop amino acids, proline and threonine were exchanged between α3 and α5, generating an α3T215P- and an α5P197T-subunit. Likewise, in the C-loop, amino acids serine and threonine were switched, generating α3S257T and α5T239S. Those mutated α-subunits were cotransfected with β3γ2 in HEK 293 cells, and the receptors formed were analyzed via radioligand binding experiments.

Equilibrium binding assays showed that receptors composed of α5P197Tβ3γ2 bind [3H]flunitrazepam with similar high affinity as α5(wt)β3γ2 (K_D: 1.9 ± 0.2 and 2.2 ± 0.3 nM, respectively). In contrast, α5(T239S)β3γ2 receptors lost this high-affinity binding and showed a 10-fold shift in K_D to 23.4 ± 6.1 nM (see Fig. 5).

Radioligand displacement studies cannot be performed using a low-affinity ligand. Therefore, α5T239Sβ3γ2 receptors could not easily have been analyzed using [3H]flunitrazepam, and we needed to search for another radioligand: Casula et al. (2001) described similar mutants in their publication and showed that the benzodiazepine Ro 15-4513 had a much higher affinity to the mutated receptors compared with flunitrazepam. We therefore decided to test [3H]Ro 15-4513 as possible radioligand, and equilibrium binding assays were performed on transfected HEK 293 cells. As shown in Table 4, [3H]Ro 15-4513 exhibited high-affinity binding for all constructs/receptors tested.

Conversion Mutations Confirm Loop B Is Not the Determinant of the Selectivity. We transfected HEK 293 cells with various GABA_A receptor subunit combinations and performed radioligand displacement assays with 5 nM [3H]Ro 15-4513 and increasing concentrations of SH53d-acid. The concentrations resulting in half-maximal inhibition of radioligand binding were converted into K_i values by using the Cheng-Prusoff relationship, and the respective K_D values were given in Table 4.

Figure 6B shows the comparison of the K_i values obtained from the B-loop mutant α5P197T and α3T215P compared with wild-type receptors (Panel A). As can be seen, the compound SH53d-acid still binds to α5-mutant receptors at very high (nanomolar) concentrations and to α3-mutant receptors at micromolar concentrations. It seems that proline 197 in the B loop of the benzodiazepine binding pocket does not contribute to the α5-selective binding properties of SH53d-acid.

**Table 2**

| Compound       | α1     | α2    | α3     | α5     | α1/α5 |
|----------------|--------|-------|--------|--------|-------|
| SH53d-ester    | 1.9 ± 0.5 | 3.3 ± 0.6 | 1.6 ± 0.4 | 0.22 ± 0.03 | 9     |
| SH53d-acid     | 1.6 ± 0.44 | 0.53 ± 0.14 | 0.65 ± 0.22 | 0.039 ± 0.003 | 41    |
Affinity Change Induced by Conversion Mutations Confirm Loop C as Dominant Molecular Determinant of the Selectivity. Figure 6C shows the Ki values obtained from the C-loop mutants α3S257T and α5T239S. Exchanging the amino acid sequence from a threonine to a serine at position α5 239/α3 257 in the C loop of the subunits changed the binding properties of SH53d-acid in gaining nanomolar binding to the α3 mutant while shifting α5-mutant binding into the micromolar range. It can therefore be concluded that threonine 239 in the C loop of the benzodiazepine binding pocket contributes heavily to the α5-selective binding properties of SH53d-acid (for detailed statistical analysis see Supplemental Fig. 2). For the parent compound SH53d-ester, the influence of threonine 239 in the C loop is much less pronounced (see Supplemental Fig. 2).

In line with the Ki values, we also observe a right-shifted dose response in the modulation of α5T239S (EC50 of 555 vs. 60 nM in the wild type) and no change in EC50 for the B-loop mutant (see Fig. 6). Both mutants do not strongly impact efficacy (Fig. 6).

Discussion

Subtype-selective ligands of the benzodiazepine binding site of GABA\_A receptors are highly desirable both as research tools to study the role of individual receptor subtypes in neuronal circuits and CNS function (Drexler et al., 2013; Sieghart, 2015) and to selectively modulate these receptors in experiments investigating animal models of diseases as well as GABA effects in non-neuronal cells that express GABA\_A receptors. Because of the high homology of the six α subunits and particularly of the four diazepam-sensitive α1-, α2-, α3-, and α5-subunits, compounds with pronounced selectivity are still very rare (Rudolph and Möhler, 2014). One of the first α5β3γ2-selective compounds reported was L-655,708 (Quirk et al., 1996), a benzodiazepine negative allosteric modulator, which exerts its subtype selectivity via high-affinity binding while showing no change in efficacy (Casula et al., 2001). The imidazobenzodiazepine SH-053-2F-R-CH3 (SH53d-ester) proved to be the first α5β3γ2-selective positive allosteric modulator (Savić et al., 2010). Here, we present the pharmacology of a derivative of the α5-prefering compound SH53d-ester with improved GABA\_A \_α5-subunit–selective properties. This novel compound SH53d-acid displays a 40-fold–higher affinity toward α5-containing receptors. In addition, it shows pronounced efficacy selectivity: The maximal modulation of GABA EC3-5 achieved at high concentrations is 3-fold higher in α5β3γ2 compared with α1β3γ2. This combination of affinity and efficacy selectivity enables a specific concentration range (up to ~30 nM) in which other α-containing receptors are not yet modulated, whereas GABA currents in α5 receptors are markedly enhanced.

SH53d-acid has already been shown to be effective as an airway smooth muscle relaxant (Forkuo et al., 2017). Airway

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**TABLE 3**

Efficacy at α3β3γ2 receptor expressed in X. laevis oocytes given as % of control current at 30 nM concentration of the compound

| Compound     | α1     | α2     | α3     | α5     | α1/α5 |
|--------------|--------|--------|--------|--------|-------|
| SH53d-ester  | 105 (5.0 μM) | 108 (4.4 μM) | 112 (1.6 μM) | 138 (0.7 μM) | 1.3   |
| SH53d-acid   | 112 (1.3 μM) | 127 (0.4 μM) | 120 (2.4 μM) | 380 (0.09 μM) | 3.3   |

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**Fig. 3.** The benzodiazepine pocket of the cryo-EM structure (6D6T) and the human α5 subunit in 6A96. Color coding: Amino acids differing between α5 and α1 are marked in cyan. γ2-Subunit is light gray. α5- and α1-subunits are in pale yellow. Consecutive numbers are used in the images and the partial alignment to identify the depicted side-chains. (A) The benzodiazepine pocket of the α1 (pale yellow)/γ2 (gray) GABA\_A receptor with the pocket-forming amino acids in stick rendering. (B) The principal subunit of the benzodiazepine pocket of the α5-subunit. (C) Partial alignment of the α1- and α5-subunits, with the mutated amino acids marked by cyan boxes, pocket-forming amino acids from the γ2-subunit. For comparison with the rat amino acid numbering, see Supplemental Table 2.
smooth muscle cells contain several GABA\(\alpha\) receptor subunits, in which, among the \(\alpha\) subunits, only \(\alpha_4\) and \(\alpha_5\) are expressed (Mizuta et al., 2008; Gallos et al., 2015; Yocum et al., 2016). It has been demonstrated previously that targeting GABA\(\alpha\) receptors containing either one of these subunits leads to relaxation of precontracted ex vivo ASM from guinea pig, mouse, and human (Gallos et al., 2015; Yocum et al., 2016) and, indeed, so does SH53d-acid (Forkuo et al., 2017).

A combination of computational modeling, mutagenesis, and radioligand binding assays has been used to determine the amino acids responsible for the exceptional \(\alpha_5\)-subtype selectivity of SH53d-acid. The benzodiazepine ligand binding site is situated at the extracellular interface between adjacent \(\alpha\)- and \(\gamma\)-subunits and is being lined by amino acids located in seven so-called “loops.” Loops A, B, and C are on the principal (+) side of the \(\alpha\)-subunit, whereas loops D, E, F, and G are on the complementary (−) side of the \(\gamma\)-2-subunit (Richter et al., 2012; Middendorp et al., 2014). The amino acids of loop B (GSYAYT in the subunits \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\); GSYAYP in \(\alpha_4\), \(\alpha_5\), and \(\alpha_6\)) have been studied previously to understand how they are involved in ligand binding. Especially \(\alpha_1\)-alanine 160 seems to contribute to the benzodiazepine binding site, whereas \(\alpha_1\)-threonine 162 seems to be of less importance (Morlock and Czajkowski, 2011). In our experiments, this region in loop B did not contribute to the \(\alpha_5\)-selective binding properties of SH53d-acid. Our findings are in line with Moody and Jenkins (2018), who describe that mutations in loop B made no significant shifts in affinity and only modest changes in maximum whole-cell current modulation to benzodiazepine (such as midazolam) binding.

Loop C, on the other hand, has long been known to be important for ligand binding since it is more variable than the other loops (Michałowski et al., 2017). \(\alpha_1\)-Serine 206 (neighboring the serine mutated in our study) seems to interact

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**Fig. 4.** Comparison of alprazolam and flumazenil binding modes with representative results from the top-20 SH53d-acid poses. (A) 6HUO with alprazolam; (B) representative alprazolam-like (BMII) pose; (C) 6D6T with flumazenil; (D) representative flumazenil-like (BMII) pose. Color coding: pale yellow ribbons: \(\alpha_1/\alpha_5\)-subunits; gray ribbons: \(\gamma_2\)-subunit; stick rendering: O is red, N is blue. The Ser/Thr position in which \(\alpha_5\) sequence is uniquely featuring Thr is also shown in stick representation.

**Fig. 5.** \([3H]\)flunitrazepam equilibrium binding assays. Membranes from transfected HEK 293 cells were incubated with 1–20 nM (A and B) and 5–150 nM (C) \([3H]\)flunitrazepam in absence or presence of 5 \(\mu\)M diazepam (to determine nonspecific binding). Radioactivity bound to the membranes was determined after rapid filtration. Inserts show the Scatchard transformation of the results. Data represent a single experiment performed in duplicates each. Experiments were repeated three to four times with similar results.
physically with diazepam (Lüscher et al., 2012). Both α1-serine 206 as well as α1-tyrosine 209 are important in determining the binding affinities for ligands of the benzodiazepine binding (Buhr et al., 1997), and S206 has been shown to influence the efficacy of midazolam to modulate GABA-azepine binding (Buhr et al., 1997), and S206 has been shown determining the binding affinities for ligands of the benzodiazepine affinity while leaving binding unaffected (Morlock and Czajkowski, 2011).

In our study mutating the conserved serine SxTGEY in the subunits α1, α2, α3, α4, and α6 versus TSTGEY in α5 had a huge influence in shifting binding affinities, proving to be the dominant molecular determinant of drug selectivity. Although the S > T mutation is conserved, the threonine side chain has an additional methyl group and thus a larger volume and hydrophobic surface. It could be speculated that ligand burial is improved, leading to a more efficient change in conformation and higher affinity of binding.

Two recently released cryo-EM structures feature binding modes of flumazenil and alprazolam that are otherwise quite different but share the positioning of the imidazole ring, which bears the acid group. Computational docking of the SH53d-acid results in both predictions (i.e., the compound can assume a flumazenil-like or an alprazolam-like position in the pocket). In both cases, the acid group is in the vicinity of loop C. Thus, our docking results correlate well with experimental evidence but do not differentiate between these two known binding modes. The cryo-EM structures were obtained with human subunits, whereas our experimental data were obtained with rat subunits. The α isoforms are highly conserved across mammalian species (%ID between rat and human subunits is 98% for α1 and α3 and 96% for α5, respectively; the binding sites are 100% conserved), and both rodent and human subunits are broadly used to characterize benzodiazepines. Thus, the use of the human structural data is valid for the interpretation of our experimental data.

In the current study we provide the detailed pharmacological characterization of SH53d-acid and describe its superior (>40-fold) affinity selectivity for α5-containing GABA_A receptors. This makes SH53d-acid a potentially very useful research tool for applications, such as slice electrophysiology, because of its much wider window of selectivity. In addition, in the current study we were able to identify the molecular basis for drug selectivity: Computational docking combined with mutational analysis identified loop C of the GABA_A receptor

| Subunit combination | $K_D \pm S.D. (nM)$ | $a_3(wt)/b_3y_2$ | $a_3(T215P)/b_3y_2$ | $a_3(S257T)/b_3y_2$ |
|---------------------|------------------|-----------------|-----------------|-----------------|
| $K_D \pm S.D. (nM)$ | $10.45 \pm 4.5$  | $2.85 \pm 1.12$ | $13.57 \pm 0.73$ |
| $a_3(wt)/b_3y_2$   | $0.16 \pm 0.09$  | $0.23 \pm 0.04$ | $0.47 \pm 0.13$ |

Fig. 6. Inhibition constants (Ki) of SH53d-acid competition for [3H]Ro 15-4513 binding and TEV functional data in α5T239S-b3y2- and α5P197T-b3y2-injected oocytes. (A–C) Membranes from transfected HEK 293 cells were incubated with 5 nM [3H]Ro 15-4513 in the presence of various concentrations of SH53d-acid. The concentrations resulting in half-maximal inhibition of radioligand binding were converted into Ki values by using the Cheng-Prusoff relationship, and the respective Kp values were given in Table 4. For detailed statistical analysis, see Supplemental Fig. 3. (D and E) Concentration-response curves and respective fitting parameters of SH53d-acid in mutated αβy2 GABA_A receptors expressed in X. laevis oocytes. Values are given as mean ± S.D., n = 4 to 5 for at least two batches of oocytes.
α-subunit as the dominant molecular determinant of drug selectivity. Thus, we characterize a promising novel α5-subunit–selective drug candidate and suggest that this atom-level structural hypothesis can now be used toward a structure-guided design of further novel compounds with similarly pronounced selectivity and otherwise improved properties. As suggested based on in vivo data from murine asthma models (Forkuo et al., 2017), this compound might be similarly pronounced selectivity and otherwise improved a structure-guided design of further novel compounds with.

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Authorship Contributions

Participated in research design: Ernst, Scholze.
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Performed data analysis: Simeone, Koniuszewski, Steudle: Ernst, Scholze.
Wrote or contributed to the writing of the manuscript: Simeone, Koniuszewski, Ernst, Scholze.

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A benzodiazepine ligand with improved GABA<sub>A</sub> receptor α5-subunit-selectivity driven by interactions with loop C

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Supplemental Figure 1: Nomenclature of mutated subunits and sequence alignment

Supplemental Figure 1 shows the sequences of rat α3 and α5 subunits, as published by UniProtKB (accession IDs P20236 and P19969). The point mutated amino acids are highlighted in yellow. In the structural work, amino acids of the mature protein are counted without the signal peptide, while in the mutational analysis, amino acids are counted including the signal peptide. In addition a full alignment of human α1, α3 and α5 subunits is shown to support Figure 3 in the manuscript.

**GBRA3_RAT (P20236) signal peptide (SP): 28 amino acids**

10 20 30 40 50
MITTMWHPY VTRVGLLLLI SILPGTTGQQ ESRRQEPGDF VKQDIGGLSP
60 70 80 90 100
KHPDIPDDDS TDNITIFTRI LDRLLGDYDN RLRPLGDAV TEVKTIDYVT
110 120 130 140 150
SGPVSDTDM EYTIDVFRQ THWDERLKFDD GPMKILPLNN LLASKIWTPD
160 170 180 190 200
TPhNGKKS VAHNMTPNKL LRLVDNGTYY YMRRLTHAE CPMHLEDPFM
210 220 230 240 250
DDeviceKLFG SYATKEAEV YSWTLMKKE SVEAQDGSRL NQYDLLGHHV
260 270 280 290 300
GTEEEIRSTG EVYVMTHFH LKRKIGVFVI QTULPCITV MSLQVSFWLN
310 320 330 340 350
RESVPARTVF GVTTLTM TSLISARNSSL KAYATAMDW FMAVCYAFVF
360 370 380 390 400
SALIFPATVN YPTKRSAWE GKKVPAEM KKKTPAAPT KTSTTPNIVG
410 420 430 440 450
TTYPINLALD TEFSTISKA AAPSASSTPT VIASPKTTYV QDSPAETKTY
460 470 480 490
NSVSKVDKS RIIFVPLFAI FNLVYVATV NYRESAIKMI RKQ

**GBRA5_RAT (P19969) Signal peptide: 25 amino acids**

10 20 30 40 50
MDNGMLSRFI MTKTLVFC1 SMTLSSHFQP SQMPTSSVQD ETNDNITIPT
60 70 80 90 100
RILDQLDGY DNLRLPQLGE RITQVRDTLV VTSQFPVSTD EMEYTDVF
110 120 130 140 150
RQSWKDELRL RQGMQRPLG NLNALSIFRT PDTPFHNGKX SIAHNMTTPN
160 170 180 190 200
KLRLEDGQT LLYTMRLRTS AECPPQLEDF PMDAHACPLK FGSYAYENV
210 220 230 240 250
VYVTNGST KSVVVAEDG SRLMQHLMQ TVGTVENI S TGYITMTAH
260 270 280 290 300
PHLKRIGVYF VIQTLPCIM TVILQSFWF RNLRSVPART VFGVTTLTM
310 320 330 340 350
TTLISARNS LPKVAYATAM DWFIAVYAF VFSALIFMAT VNYPTKRGWA
360 370 380 390 400
WGGKALEAA KIKKKERLLI LNKSTNAPFT GKLTHPPNIP KEQLPDDGN
410 420 430 440 450
AVGTASIRAS EKTESESQKT YNSISKIDKM SRIVFPILFG TFNLVYVATY
460
LNREPVIKGA TSPK
Full alignment of human α1, α3 and α5 subunits
Supplemental Tables 1 and 2: Comparison of rat and human subunits

Throughout our manuscript, amino acids are counted with the signal peptide included and based on the rat sequences. In some published studies, human sequences are used and/or the signal peptide is not counted, and the first amino acid of the mature peptide numbered as “1”. In Table 1 the different length of the signaling peptides is shown, in Table 2 the amino acids analyzed in this study are compared between rat and human sequence.

Table 1: Length of signaling peptide
Length of the signaling peptide as published by UniProtKB (accession IDs P62813, P20236, P19969, P14867, P34903, and P31644)

|     | rat | human |
|-----|-----|-------|
| α1  | 27  | 27    |
| α3  | 28  | 28    |
| α5  | 25  | 31    |

Table 2: Summary of the homologous amino acids in Loops B and C

|     | rat with signal peptide | rat without signal peptide | human with signal peptide | human without signal peptide |
|-----|-------------------------|-----------------------------|---------------------------|-----------------------------|
| α1 B-loop | T 189                   | T 162                       | T 190                     | T 163                       |
| α3 B-loop | T 215                   | T 187                       | T 215                     | T 187                       |
| α5 B-loop | P 197                   | P 172                       | P 197                     | P 166                       |
| α1 C-loop | S 232                   | S 205                       | S 232                     | S 205                       |
| α3 C-loop | S 257                   | S 229                       | S 257                     | S 229                       |
| α5 C-loop | T 239                   | T 214                       | T 239                     | T 208                       |

Supplemental Figure 2: Detailed statistical analyses of SH53d-acid and SH53d-ester binding to mutated receptors
Membranes from transfected HEK 293 cells were incubated with 5 nM [³H]Ro 15-4513 in the presence of various concentrations of SH53d-acid or SH53d-ester. The concentrations resulting in half maximal inhibition of radioligand binding were converted into Ki values by using the Cheng-Prusoff relationship and the respective KD values given in Table 3 of the main manuscript. One-way ANOVA followed by Tukey’s multiple comparisons test was performed using GraphPad Prism version 8.3.0 for Mac OS X, GraphPad Software, La Jolla, California, USA, www.graphpad.com.
| Tukey's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value |
|----------------------------------|------------|--------------------|--------------|---------|-----------------|
| α3β3γ2 vs. α3β3γ2               | 1.197e+006 | 5.916e+006 to 1.602e+006 | Yes          | ****    | <0.0001         |
| α3β3γ2 vs. α5P197Tβ3γ2           | 1.191e+006 | 5.861e+007 to 1.796e+007 | Yes          | ****    | <0.0001         |
| α3P197Tγ2 vs. α5T239Sβ3γ2        | -3.163e-007| -9.215e-007 to 2.888e-007| No           | ns      | 0.5720          |
| α3β3γ2 vs. α5T215Pβ3γ2          | 4.684e-007 | -6.312e-008 to 9.999e-007| No           | ns      | 0.1030          |
| α3β3γ2 vs. α35257Tβ3γ2          | 1.006e-006 | 4.849e-007 to 1.518e-006| Yes          | ****    | <0.0001         |
| α3P197Tγ2 vs. α5P197Tβ3γ2       | -5.005e-009| -6.524e-007 to 6.414e-007| No           | ns      | >0.9999         |
| α5P197Tγ2 vs. α5T239Sβ3γ2       | 1.014e-006 | -2.180e-006 to -8.682e-007| Yes          | ****    | <0.0001         |
| α5P197Tγ2 vs. α35257Tβ3γ2       | -7.784e-007| -1.307e-006 to -1.498e-007| Yes          | **      | 0.0092          |
| α3P197Tγ2 vs. α5T215Pβ3γ2       | -1.905e-007| -7.507e-007 to 3.686e-007| No           | ns      | 0.8826          |
| α5P197Tγ2 vs. α3T215Pβ3γ2       | -1.508e-006| -2.155e-006 to -1.807e-007| Yes          | ****    | <0.0001         |
| α5P197Tγ2 vs. α3T215Pβ3γ2       | -7.725e-007| -1.302e-006 to -1.443e-007| Yes          | **      | 0.0098          |
| α3P197Tγ2 vs. α35257Tβ3γ2       | -1.890e-007| -7.452e-007 to -3.753e-007| No           | ns      | 0.8947          |
| α5T239Sγ2 vs. α3T215Pβ3γ2       | 7.847e-007 | 2.061e-007 to 1.363e-006| Yes          | **      | 0.0048          |
| α5T239Sγ2 vs. α35257Tβ3γ2       | 1.323e-006 | 7.524e-007 to 1.883e-006| Yes          | ****    | <0.0001         |
| α3T215Pβ3γ2 vs. α35257Tβ3γ2     | 5.379e-007 | 5.815e-006 to 1.018e-005| Yes          | *       | 0.0230          |
| Tukey's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary |
|----------------------------------|------------|--------------------|--------------|---------|
| $\alpha_{3}\beta_{3}\gamma_{2}$ vs. $\alpha_{5}\beta_{3}\gamma_{2}$ | $3.417e-06$ | $2.064e-06$ to $4.771e-06$ | Yes | **** |
| $\alpha_{3}\beta_{3}\gamma_{2}$ vs. $\alpha_{5}\beta_{197T}\beta_{3}\gamma_{2}$ | $2.909e-06$ | $1.555e-06$ to $4.263e-06$ | Yes | **** |
| $\alpha_{3}\beta_{3}\gamma_{2}$ vs. $\alpha_{5}T239S\beta_{3}\gamma_{2}$ | $2.473e-06$ | $1.119e-06$ to $3.827e-06$ | Yes | *** |
| $\alpha_{3}\beta_{3}\gamma_{2}$ vs. $\alpha_{3}T215P\beta_{3}\gamma_{2}$ | $1.074e-06$ | $-3.878e-07$ to $2.536e-06$ | No | ns |
| $\alpha_{3}\beta_{3}\gamma_{2}$ vs. $\alpha_{3}S257T\beta_{3}\gamma_{2}$ | $1.058e-06$ | $-2.960e-07$ to $2.411e-06$ | No | ns |
| $\alpha_{5}\beta_{3}\gamma_{2}$ vs. $\alpha_{5}\beta_{197T}\beta_{3}\gamma_{2}$ | $-5.080e-07$ | $-1.862e-06$ to $8.455e-07$ | No | ns |
| $\alpha_{5}\beta_{3}\gamma_{2}$ vs. $\alpha_{5}T239S\beta_{3}\gamma_{2}$ | $-9.440e-07$ | $-2.298e-06$ to $4.095e-07$ | No | ns |
| $\alpha_{5}\beta_{3}\gamma_{2}$ vs. $\alpha_{3}T215P\beta_{3}\gamma_{2}$ | $-2.343e-06$ | $-3.805e-06$ to $-8.809e-07$ | Yes | ** |
| $\alpha_{5}\beta_{3}\gamma_{2}$ vs. $\alpha_{3}S257T\beta_{3}\gamma_{2}$ | $-2.360e-06$ | $-3.713e-06$ to $-1.006e-06$ | Yes | *** |
| $\alpha_{5}P197T\beta_{3}\gamma_{2}$ vs. $\alpha_{5}T239S\beta_{3}\gamma_{2}$ | $-4.360e-07$ | $-1.790e-06$ to $9.175e-07$ | No | ns |
| $\alpha_{5}P197T\beta_{3}\gamma_{2}$ vs. $\alpha_{3}T215P\beta_{3}\gamma_{2}$ | $-1.835e-06$ | $-3.297e-06$ to $-3.729e-07$ | Yes | ** |
| $\alpha_{3}P197T\beta_{3}\gamma_{2}$ vs. $\alpha_{3}S257T\beta_{3}\gamma_{2}$ | $-1.852e-06$ | $-3.205e-06$ to $-4.980e-07$ | Yes | ** |
| $\alpha_{5}T239S\beta_{3}\gamma_{2}$ vs. $\alpha_{3}T215P\beta_{3}\gamma_{2}$ | $-1.399e-06$ | $-2.861e-06$ to $6.312e-08$ | No | ns |
| $\alpha_{5}T239S\beta_{3}\gamma_{2}$ vs. $\alpha_{3}S257T\beta_{3}\gamma_{2}$ | $-1.416e-06$ | $-2.769e-06$ to $6.199e-08$ | Yes | * |
| $\alpha_{3}T215P\beta_{3}\gamma_{2}$ vs. $\alpha_{3}S257T\beta_{3}\gamma_{2}$ | $-1.667e-08$ | $-1.479e-06$ to $1.445e-06$ | No | ns |