

## Different Benzodiazepines Bind with Distinct Binding Modes to GABA<sub>A</sub> Receptors

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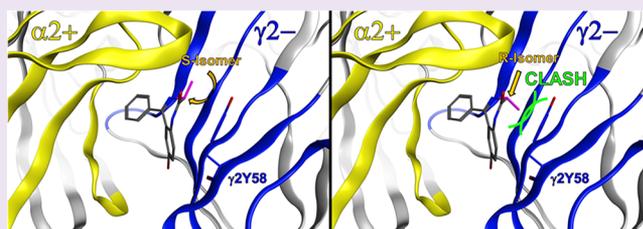
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### Supporting Information

**ABSTRACT:** Benzodiazepines are clinically relevant drugs that bind to GABA<sub>A</sub> neurotransmitter receptors at the  $\alpha$ +/ $\gamma$ 2- interfaces and thereby enhance GABA-induced chloride ion flux leading to neuronal hyperpolarization. However, the structural basis of benzodiazepine interactions with their high-affinity site at GABA<sub>A</sub> receptors is controversially debated in the literature, and *in silico* studies led to discrepant binding mode hypotheses. In this study, computational docking of diazepam into  $\alpha$ +/ $\gamma$ 2- homology models suggested that a chiral methyl group, which is known to promote preferred binding to  $\alpha$ 5-containing GABA<sub>A</sub> receptors (position 3 of the seven-membered diazepine ring), could possibly provide experimental evidence that supports or contradicts the proposed binding modes. Thus, we investigated three pairs of R and S isomers of structurally different chemotypes, namely, diazepam, imidazobenzodiazepine, and triazolam derivatives. We used radioligand displacement studies as well as two-electrode voltage clamp electrophysiology in  $\alpha$ 1 $\beta$ 3 $\gamma$ 2-,  $\alpha$ 2 $\beta$ 3 $\gamma$ 2-,  $\alpha$ 3 $\beta$ 3 $\gamma$ 2-, and  $\alpha$ 5 $\beta$ 3 $\gamma$ 2-containing GABA<sub>A</sub> receptors to determine the ligand binding and functional activity of the three chemotypes. Interestingly, both imidazobenzodiazepine isomers displayed comparable binding affinities, while for the other two chemotypes, a discrepancy in binding affinities of the different isomers was observed. Specifically, the R isomers displayed a loss of binding, whereas the S isomers remained active. These findings are in accordance with the results of our *in silico* studies suggesting the usage of a different binding mode of imidazobenzodiazepines compared to those of the other two tested chemotypes. Hence, we conclude that different chemically related benzodiazepine ligands interact via distinct binding modes rather than by using a common binding mode.



Since the discovery of the first benzodiazepine chlordiazepoxide in the late 1950s, this class of drugs has emerged rapidly. In 2008, >5% of the U.S. adult population used benzodiazepines<sup>1</sup> in the treatment of conditions such as insomnia, anxiety, and acute epileptic seizures or as presurgery medication. Benzodiazepines act by positive allosteric modulatory (PAM) enhancement of GABA-induced chloride ion flux through GABA<sub>A</sub> receptors.<sup>2</sup> GABA<sub>A</sub> receptors are pentameric ligand-gated ion channels, and a majority of these receptors are composed of two  $\alpha$  subunits, two  $\beta$  subunits, and one  $\gamma$  subunit,<sup>3</sup> typically  $\gamma$ 2, as approximately 75–80% of all GABA<sub>A</sub> receptors contain  $\gamma$ 2.<sup>4</sup> Nearly all of the clinically relevant benzodiazepine drugs target all GABA<sub>A</sub> receptor subtypes equally. However, in the past few years, research has focused on the role of  $\alpha$ 5-containing GABA<sub>A</sub> receptors. Although those receptors are rare (<5% of total) with the distribution restricted to hippocampus,<sup>5</sup> they are considered promising drug targets for both positive and

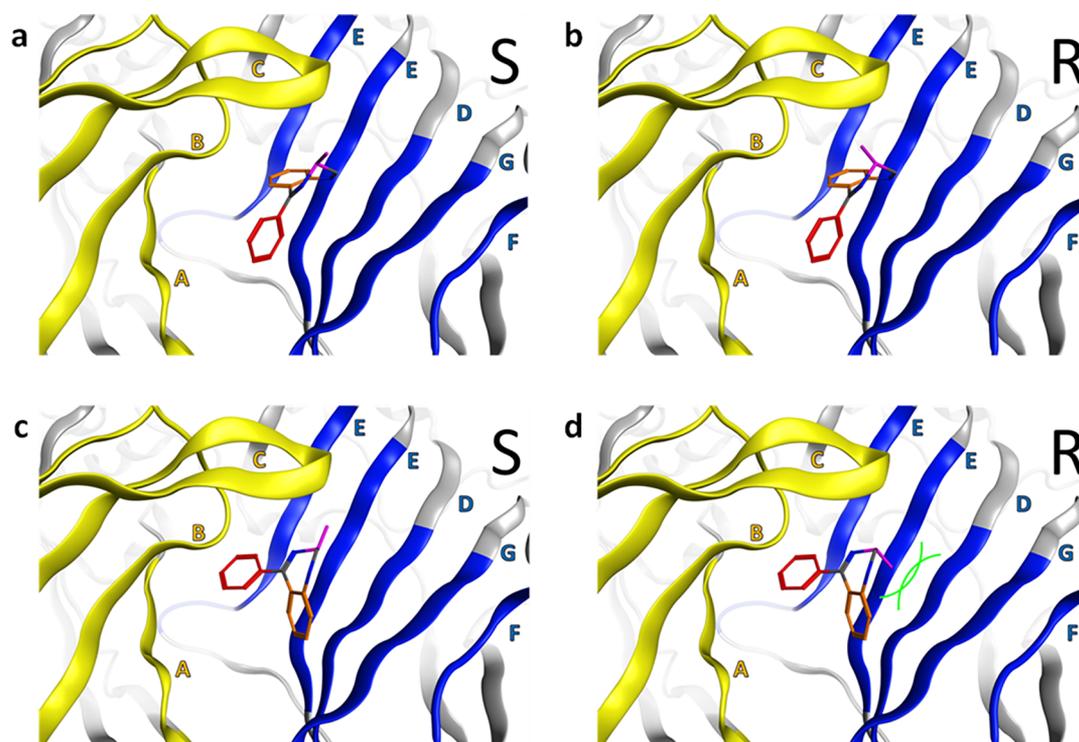
negative allosteric modulators<sup>6</sup> and could be useful for the treatment of cognitive and/or mood disorders.

For structure-guided development of subtype-selective ligands, knowledge of their binding modes in the pocket is required. Classical benzodiazepines, such as diazepam, midazolam, and similar compounds, bind at the  $\alpha$ +/ $\gamma$ - interface of  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3-, or  $\alpha$ 5- and  $\gamma$ 2-containing receptors. However, the exact molecular interactions of benzodiazepines at the GABA<sub>A</sub> receptor subunits have not been well characterized and remain under debate in the literature. It is generally assumed that all benzodiazepines interact with the receptor in a “common” binding mode. *In silico* studies, however, led to controversial hypotheses about the exact nature of this common binding mode.<sup>7–10</sup> Current studies suggest three possible common binding mode poses (CBM I,

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**Figure 1.** General BM I and BM II orientations of the benzodiazepine core scaffold at the extracellular  $\alpha+\gamma_2-$  site. Color codes for the protein ribbon: yellow for the  $\alpha$  subunit and blue for the  $\gamma_2$  subunit. Binding site forming segments (strands and loops A–F) are labeled with Arabic letters. (a and b) BM I orientation of the benzodiazepine core scaffold at the  $\alpha+\gamma_2-$  interface. The fused phenyl ring is colored orange, while the pendant phenyl ring is colored red. The crucial methyl group is colored purple, indicating sufficient space for the *R* isomer and the *S* isomer. (c and d) BM II orientation of the benzodiazepine core scaffold at the  $\alpha+\gamma_2-$  interface. The color coding of the ligand is as in panels a and b. The two phenyl rings changed positions if we compared the BM I and BM II orientations. The crucial methyl group is colored purple, indicating the *R* isomer has a steric clash with segment G of the  $\gamma_2$  subunit while the *S* isomer does not. Green curved lines indicate van der Waals radii, which illustrate the steric clash.

CBM II, and CBM III) for binding of diazepam to the pocket.<sup>11</sup> Richter et al. favor CBM I because it is supported by experimental evidence and could be used to identify further high-affinity ligands.<sup>11</sup> In contrast, Middendorp et al.<sup>12</sup> found evidence of CBM II and were also able to identify a number of active compounds by a virtual screening approach using this molecular orientation. This seemingly paradoxical situation can be reconciled by the pseudosymmetry that relates these two binding modes' pharmacophores for diazepam.<sup>13</sup>

In the study presented here, we aimed to find experimental evidence that favored one binding mode or the other. A closer examination of binding modes BM I and BM II found in our previous study<sup>11</sup> suggested that substitutions on the seven-membered diazepine ring could interfere with the BM II poses more severely than with the BM I poses. We therefore selected three scaffolds for docking and three corresponding pairs of substituted enantiomers for experimental testing. For the *in silico* docking, an improved homology model could be used, because the crystal structure of the human  $\beta_3$ -homopentameric GABA<sub>A</sub> receptor was resolved by Miller and Ariescu in 2014<sup>14</sup> and was selected as a template.

This *in silico* docking confirmed the crucial position at the methyl group of the seven-membered diazepine ring. This methyl group, when oriented in the *R* configuration, is known to promote  $\alpha_5$  subtype selectivity of imidazobenzodiazepines such as SH-053-2'-F-R-CH<sub>3</sub>.<sup>15</sup> Here, we predict that the very same methyl group in this position could additionally be used as a chemical reporter to provide insights into the molecular interaction profile of benzodiazepine ligands and provide a

means to rank candidate binding modes for given specific ligands and chemotypes.

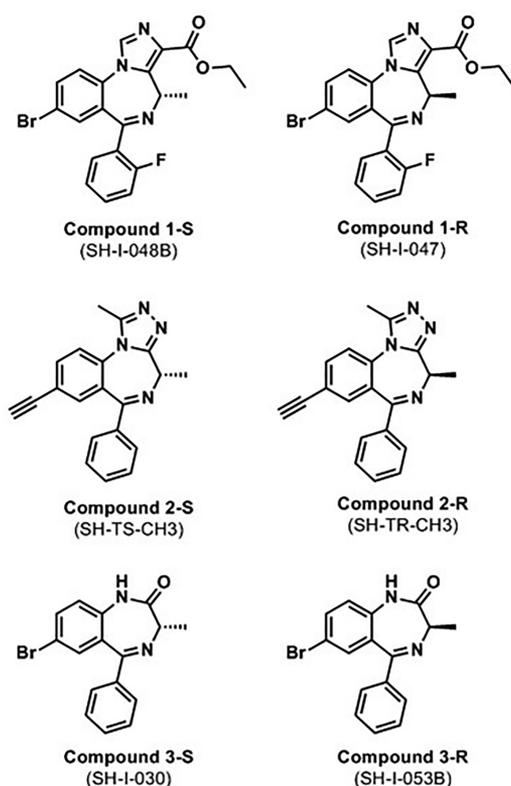
We investigated a set of three stereoisomeric pairs in radioligand displacement assays and with the two-electrode voltage clamp method. Interestingly, our data gave diverging results for the different chemical scaffolds, suggesting that different benzodiazepine ligands use distinct binding modes rather than a common binding mode. In the accompanying manuscript,<sup>13</sup> we also present evidence of different binding mode usage based on an orthogonal mutational approach.

## RESULTS AND DISCUSSION

### Bound State Models Reveal a Crucial Methyl Group.

A closer inspection of our previous examination of docking of diazepam to the  $\alpha+\gamma-$  interface of GABA<sub>A</sub> receptors<sup>11</sup> indicated that substitutions on the seven-membered diazepine ring of the benzodiazepine scaffold could more severely interfere with the published benzodiazepine binding mode BM II binding pose than with the BM I binding pose (see Figure 1). We reasoned that this fact could possibly be used to find experimental evidence supporting one binding mode or the other.

In this study, we therefore selected three pairs of stereoisomers for our binding and electrophysiological studies, each with a chiral methyl group at position 3 of the seven-membered diazepine ring in the *R* or *S* configuration. Our test compounds belong to three different structural classes (see Figure 2): a pair of imidazobenzodiazepines, SH-I-048B and SH-I-047 (termed 1-S and 1-R, respectively), a pair of



**Figure 2.** Chemical structures of the three stereoisomer pairs. The *S* isomers are depicted in the left column, and the corresponding *R* isomers in the right column. IUPAC nomenclature as well as the numbering of the C atoms in the structures is shown in Figure S1.

triazolam derivatives, SH-TS-CH3 and SH-TR-CH3<sup>16</sup> (termed 2-*S* and 2-*R*, respectively), and a pair of diazepam derivatives, SH-I-030 and SH-I-053B (termed 3-*S* and 3-*R*, respectively).

To correlate the computational results with the binding studies, we updated and revisited the *in silico* approach for the prediction and validation of binding modes. Because there is no crystal structure of hetero-oligomeric GABA<sub>A</sub> receptors, we have to rely on homology modeling based on the published  $\beta 3$  homo-oligomer.<sup>14</sup> We chose for the computational study to investigate the  $\alpha 2+/\gamma 2-$  interface as being representative for the “diazepam-sensitive” class of benzodiazepine binding sites due to the rising interest in  $\alpha 2$ -selective PAM ligands, in the treatment of not only anxiety disorders but also schizophrenia, depression, and pain disorders.<sup>5,17</sup>

In terms of expected model accuracy, the  $\alpha 1+$ ,  $\alpha 2+$ , and  $\alpha 3+$  interfaces are more closely related to the experimentally determined  $\beta 3+/\beta 3-$  interface than the  $\alpha 5+/\gamma 2-$  interface, and reliabilities of all three would be expected to be comparable. For the docking procedure, we employed the three achiral scaffolds from the six molecules, which we studied experimentally, and added the respective (*R*)- and (*S*)-methyl groups to the candidate binding modes. This procedure avoids excessive false positive *in silico* docking solutions for the experimentally tested compounds. In addition, docking the unsubstituted parent compounds of the chiral ligands facilitates direct comparison with the previous docking studies in which mainly achiral compounds have been studied. Subsequent *in silico* derivatization of the docked ligands into the six test compounds led to predictions that were tested in the experimental part of this study.

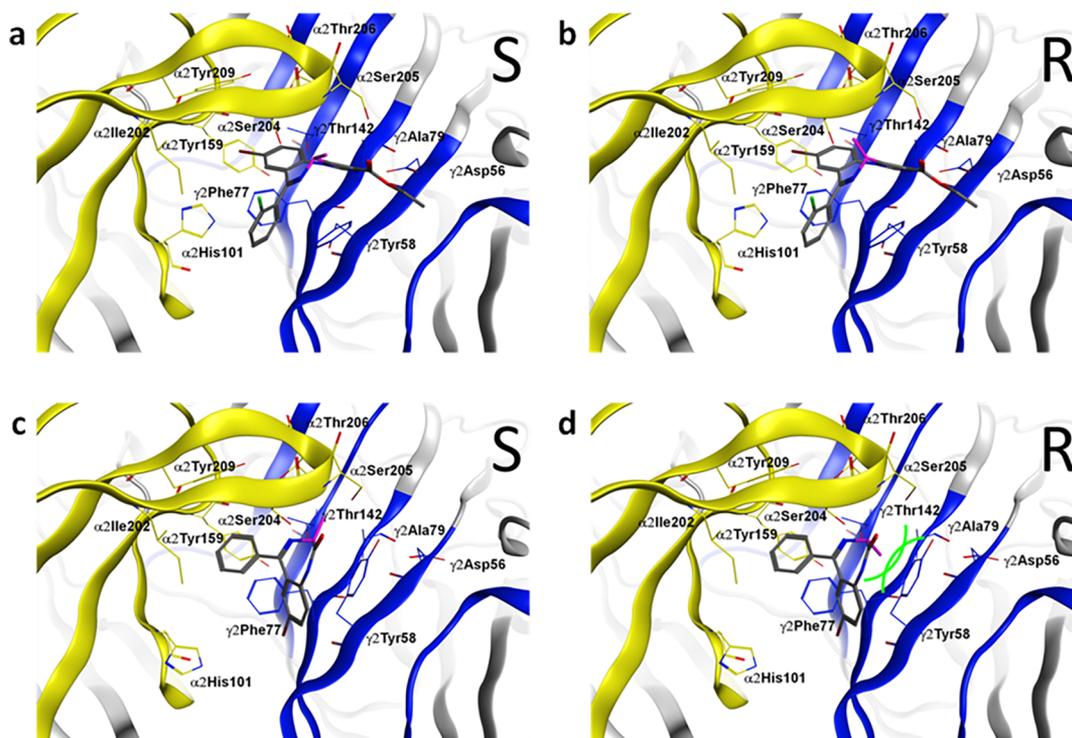
Computational docking of the three achiral parent ligands into our  $\alpha 2+/\gamma 2-$  homology model using GOLD<sup>18</sup> resulted in the two previously reported binding mode orientations, BM I and BM II,<sup>11</sup> for all ligands tested. Careful analysis of the two binding modes supported our initial hypothesis that the introduction of a methyl group into the seven-membered diazepine ring could serve as tool for examining binding mode orientations of different benzodiazepine ligands. In detail, the methyl group at the diazepine ring leads to two distinct isomers, namely, an *R* isomer and an *S* isomer. According to our model, the methyl groups of both isomers are pointing in the direction of the more flexible loop C if the ligands bind in a BM I orientation, and thus, both isomers should be active (see panels a and b of Figure 3). On the contrary, if the ligands use a BM II orientation, methyl groups of the two isomers are pointing in substantially different directions. While for the *S* isomer the methyl group is also directed toward the flexible loop C (see Figure 3c), the methyl group of the *R* isomer is directed toward the more rigid segment G of the  $\gamma 2$  subunit, which leads to a steric clash with a bulky tyrosine residue ( $\gamma 2$ Tyr58) (see Figure 3d). Hence, *in silico* docking leads to the prediction that for ligands using BM I, *R* and *S* isomer analogues should both be binding with high or at least intermediate affinity and differ only mildly in affinity, while for BM II binding scaffolds, the *R* isomer should be inactive or display a marked drop in affinity compared to that of the *S* isomer.

**Distinct Binding Activity of *R* Stereoisomers.** To test our predictions, we performed radioligand displacement studies and compared the potencies of our six chiral benzodiazepine ligands for the inhibition of binding of [<sup>3</sup>H]flunitrazepam to  $\alpha 2\beta 3\gamma 2$  recombinant rat GABA<sub>A</sub> receptor subtypes (see Figure 4).

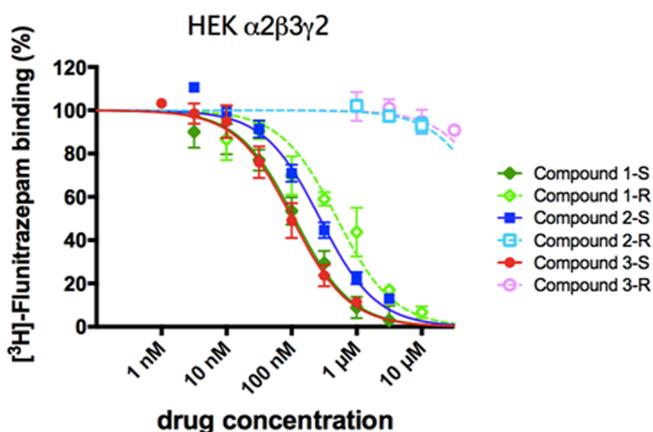
While for the *S* isomers (compounds 1-*S*, 2-*S*, and 3-*S*) intermediate to high binding affinities for the recombinant receptors were observed, which is in accordance with the results of our docking studies, the *R* isomers showed diverse binding behavior. Interestingly, only imidazobenzodiazepine compound 1-*R* retained its potential to displace the radioligand [<sup>3</sup>H]flunitrazepam, whereas compounds 2-*R* and 3-*R* were inactive, suggesting nonbinding. Generally, the *S* isomers display affinities higher than those of their *R* analogues.

To exclude a binding behavior unique to  $\alpha 2$ -containing receptors, we also performed similar radioligand displacement studies on other receptor combinations such as recombinantly expressed rat GABA<sub>A</sub> receptor subtypes  $\alpha 1\beta 3\gamma 2$ ,  $\alpha 3\beta 3\gamma 2$ , and  $\alpha 5\beta 3\gamma 2$  (see Figure S2). The obtained *K<sub>i</sub>* values are listed in Table 1.

Compounds 1-*S*, 2-*S*, 3-*S*, and 1-*R* potentially replaced [<sup>3</sup>H]flunitrazepam in all receptor subtypes, while compounds 2-*R* and 3-*R* failed to displace the radioligand in all receptors tested (see Table 1). However, we did observe differences in the potency rank order. Although compound 1-*S* showed a significant preference for  $\alpha 5$ -containing receptors (>4-fold), it also exhibited substantial affinity at the other receptor subtypes that decreased in the following order:  $\alpha 5 > \alpha 2 > \alpha 3 = \alpha 1$ . Its *R* isomeric analogue compound 1-*R* possessed slightly higher  $\alpha 5$  selectivity (~5-fold); however, it displayed slightly lower affinities in the tested receptor subtypes. Triazolam-like compound 2-*S* showed significant higher binding affinities in  $\alpha 2$  and  $\alpha 5$  GABA<sub>A</sub>R subtypes than in  $\alpha 1$  and  $\alpha 3$  subtypes. Diazepam-like compound 3-*S* showed low subtype selectivity. Compounds 2-*R* and 3-*R* failed to displace [<sup>3</sup>H]flunitrazepam



**Figure 3.** Distinct binding modes (BM I or BM II) of tested compounds 1-S, 1-R, 3-S, and 3-R at the  $\alpha 2/\gamma 2$ - interface. (a and b) Compounds 1-S and 1-R are both compatible with BM I in the binding pocket ( $\alpha 2$  subunit, yellow;  $\gamma 2$  subunit, blue; amino acids that were set flexible in the docking are rendered as sticks and labeled). The orientation of the methyl is colored purple. (c) For compound 3-S in BM II, the methyl group (purple) is sterically compatible ( $\alpha 2$  subunit, yellow;  $\gamma 2$  subunit, blue). (d) For compound 3-R in BM II, the methyl group (purple) is not tolerated and clashes with the  $\gamma 2$ Tyr58 on segment G in the binding pocket ( $\alpha 2$  subunit, yellow;  $\gamma 2$  subunit, blue); thus, this ligand is predicted to show reduced binding affinity. Green curved lines indicate van der Waals radii, which illustrate the steric clash.



**Figure 4.** Inhibition of binding of [ $^3$ H]flunitrazepam to recombinant  $\alpha 2\beta 3\gamma 2$  GABA<sub>A</sub> receptor subtypes. Membranes from HEK-293 cells transfected with the GABA<sub>A</sub> receptor subunit combination  $\alpha 2\beta 3\gamma 2$  were incubated with 2 nM [ $^3$ H]flunitrazepam in the presence of various concentrations of the six BZ site ligands. Data shown are the means  $\pm$  the standard error of the mean of three independent experiments each performed in duplicate.

binding at all GABA<sub>A</sub> receptor subtypes investigated even at high micromolar concentrations.

**Modulatory Effects Support Heterogeneous Activity Profiles of R Isomers.** Radioligand displacement assays provide information about the binding of a compound to only a specific binding site. To exclude the possibility that compounds 2-R and 3-R bind at a site different from the [ $^3$ H]flunitrazepam binding site (and are therefore unable to

compete with the radioligand), we decided to determine the efficacies of all our test compounds using two-electrode voltage clamp recordings on  $\alpha 2\beta 3\gamma 2$ -mRNA-treated *Xenopus laevis* oocytes (see Figure 5).

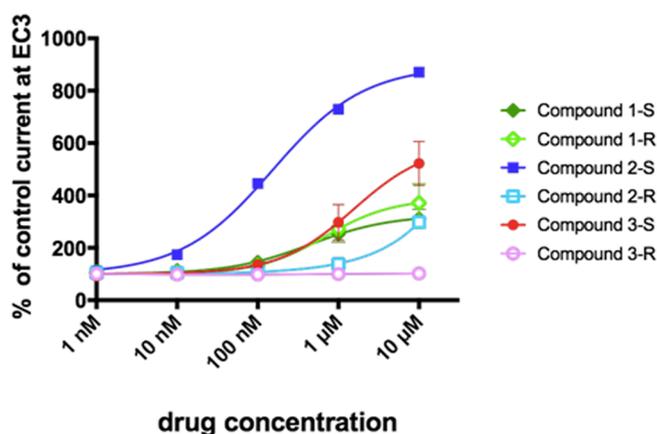
Electrophysiological experiments supported our radioligand binding assay findings. Both imidazobenzodiazepines 1-S and 1-R as well as compounds 2-S and 3-S interacted with GABA receptors. Two-voltage clamp recordings provide us with the additional knowledge that those drugs behaved as positive allosteric modulators as they potentiated the GABA-induced chloride ion flux. On the other hand, compounds 2-R and 3-R displayed complete loss of activity in the oocyte assay at drug concentrations of  $\leq 1 \mu\text{M}$ , indicating that they indeed do not interact with GABA receptors. Nevertheless, compound 2-R showed a slight positive modulation of the GABA-induced currents at concentrations of  $10 \mu\text{M}$ . Because at those concentrations the drug does not yet displace [ $^3$ H]-flunitrazepam (see Figure 4 and Figure S2), this modulatory interaction is most likely mediated through a binding site different from the classical benzodiazepine binding site at the  $\alpha +/\gamma -$  interface. One putative binding site could be the  $\alpha +/\beta -$  interface, as described by Ramerstorfer et al., because compounds that interact with both sites, the  $\alpha +/\beta -$  site and the BZ site, are known (e.g., pyrazoloquinolinones).<sup>21</sup> This hypothesis is supported by the fact that the previously published compound SH-I-048A,<sup>22</sup> which is structurally very similar to compound 3-S, displayed a biphasic concentration-response curve in two-voltage clamp recordings, also indicating an additional interaction site for benzodiazepine ligands.

In addition to the analysis of  $\alpha 2\beta 3\gamma 2$ -containing GABA<sub>A</sub> receptors, we also performed similar electrophysiological

Table 1. Affinities of Benzodiazepine Binding Site Ligands for Recombinant GABA<sub>A</sub> Receptors<sup>a</sup>

compound	K <sub>i</sub>			
	α1β3γ2	α2β3γ2	α3β3γ2	α5β3γ2
1-S (SH-I-048B)	190 ± 55 nM	67 ± 9 nM	136 ± 24 nM	17 ± 5 nM <sup>b</sup>
1-R (SH-I-047)	273 ± 41 nM	253 ± 31 nM	501 ± 79 nM	56 ± 8 nM <sup>c</sup>
2-S (SH-TS-CH3)	663 ± 21 nM	164 ± 15 nM	656 ± 110 nM	80 ± 4 nM <sup>d</sup>
2-R (SH-TR-CH3)	>10 μM	>10 μM	>10 μM	>10 μM
3-S (SH-I-030)	64 ± 2 nM	61 ± 10 nM	102 ± 7 nM	31 ± 5 nM
3-R (SH-I-053B)	>10 μM	>10 μM	>10 μM	>10 μM

<sup>a</sup>The concentrations of drugs resulting in half-maximal inhibition of specific [<sup>3</sup>H]flunitrazepam binding (IC<sub>50</sub>) shown in Figure S2 were converted to K<sub>i</sub> values by using the Cheng–Prusoff relationship<sup>19</sup> and the respective published K<sub>D</sub> values for [<sup>3</sup>H]flunitrazepam binding (nanomolar).<sup>20</sup> Data presented are means ± the standard error of the mean of three independent experiments each performed in duplicate. Statistical evaluation was performed with one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple-comparison test. <sup>b</sup>*p* < 0.01 compared to α1. <sup>c</sup>*p* < 0.05 compared to α1. <sup>d</sup>*p* < 0.001 compared to α1.



**Figure 5.** Concentration–response curves of drug-induced enhancement of GABA currents. *X. laevis* oocytes were treated with mRNA for α2β3γ2-GABA<sub>A</sub> receptors and studied using two-electrode voltage clamp recordings. GABA-induced currents (3% of maximum) were modulated by addition of drug. Data are means ± the standard error of the mean of three independent experiments.

studies of other receptor combinations such as α1β3γ2, α3β3γ2, and α5β3γ2 (see Figure S3). In accordance with the previous data, compounds 1-S, 2-S, 3-S, and 1-R displayed a positive modulation in all receptor subtypes, while compounds 2-R and 3-R remained mainly modulatory silent.

In this study, we used a combined approach comprising computational ligand docking, radioligand binding assays, and functional two-electrode voltage clamp assays to revisit the common binding mode hypothesis for diazepam-derived ligands versus imidazobenzodiazepines with different substituents on the imidazole ring.

Overall, we found that our chiral benzodiazepine ligand 1-R, representing an ester-substituted imidazobenzodiazepine, binds to the GABA<sub>A</sub> receptor benzodiazepine binding site. This suggests a preference for the CBM I binding mode as described by Richter et al.<sup>11</sup> In contrast, the two R isomer compounds derived from diazepam or from triazolam are no longer able to bind to the benzodiazepine binding site. For these compounds, the CBM II binding mode seems to apply, as suggested by Middendorp et al.<sup>12</sup> In the accompanying manuscript,<sup>13</sup> an orthogonal approach to the one used here was employed, where an artificial benzodiazepine binding site was engineered into binary α1β3 receptors. In this study, imidazobenzodiazepines interacted with the artificial site in a manner completely different from that of diazepam, which was also investigated

with computational models and in turn interpreted as a sign of different binding site usage.

Our results are in line with previous experiments, in which it was demonstrated that the binding affinity, efficacy, and *in vivo* activity of a series of ligands with a chiral S-CH3 group at C-4 are different from those of the R-CH3 group.<sup>23,24</sup> For instance, the R isomer of SH-I-048A<sup>22</sup> [which is structurally very similar to the compound 3-S/3-R pair (SH-I-030/SH-I-053B) tested in this study] was almost completely inactive in oocyte assays.<sup>22</sup> On the other hand, the previously published compounds SH-053-2'F-S-CH3 and SH-053-2'F-R-CH3<sup>25</sup> (of which compounds 1-S and 1-R, respectively, are derivatives) both bind to GABA<sub>A</sub> receptors with high affinity, but the R isomer SH-053-2'F-R-CH3 displays 3–5-fold lower affinity in α1-, α3-, and α5-containing receptors and even 30-fold lower affinity in α2-containing receptors compared to those of the S isomer.<sup>23</sup>

The compounds tested have a rather low affinity for the benzodiazepine binding site of GABA<sub>A</sub> receptors. In addition, if we compare the substitutions on the basic benzodiazepine core (scaffold), the pairs of compounds differ at several positions. In Figure 2, the top row (scaffold 1) and the middle row (scaffold 2) differ in three substituents and are thus not a systematic variation on the scaffold (see also Figure S4). The middle row (scaffold 2) differs from the bottom row (scaffold 3) in two positions, which again is not a systematic variation. However, the Br versus ethinyl has little impact on affinity;<sup>7</sup> it can therefore be assumed that the presence or absence of the five-membered ring on the “north” end of the molecule is the dominant difference. It still would not be appropriate to generalize to other similar compounds and look for structure–activity relationships (SARs) based on these compounds; our results do indicate in fact that chemically similar compounds can display strikingly dissimilar binding properties and ligand similarity-based SAR extrapolations might fail.

The existence of a “common” binding mode for all benzodiazepine ligands was until now well accepted in spite of the fact that the structure of those ligands can vary dramatically. The flunitrazepam-like compounds possess a 1,4-benzodiazepine nucleus with a 5-phenyl substituent, the midazolam-like compounds an imidazo ring with a 5-phenyl substituent, and the flumazenil-like compounds such as Ro15-4513 and Ro15-1788 an imidazo ring without a 5-phenyl substituent. It is therefore not surprising that some experimental evidence points to the fact that some drugs interact with GABA<sub>A</sub> receptors differently than others. In particular, the family of imidazobenzodiazepines seems to

possess structural binding requirements (such as the interaction with  $\gamma 2\text{Ala}79$ ) distinct from those of the classical benzodiazepines,<sup>26,27</sup> which is supported by our experiments.

In summary, we can conclude that our data suggest that there is not one single “common” binding mode for benzodiazepines in general; there are at least two different ones.

## METHODS

**Competition Binding Assays and Electrophysiological Experiments.** Radioligand displacement assays were performed in HEK-293 cells transfected with recombinant rat  $\alpha x\beta 3\gamma 2$  receptors ( $x = 1, 2, 3, \text{ or } 5$ ) using 2 nM [<sup>3</sup>H]flunitrazepam and 1 nM to 10  $\mu\text{M}$  investigated compound as described previously.<sup>20</sup> Two-electrode voltage clamp recordings were performed on *X. laevis* oocytes treated with mRNA for the desired subunit combination at a GABA concentration eliciting 3% of the maximum current.<sup>21</sup>

**Molecular Docking.** The homology model of the extracellular  $\alpha 2+\gamma 2-$  interface (BZ site) was based on 4COF<sup>14</sup> using Modeller<sup>28</sup> as described previously.<sup>29</sup> Molecular docking of the three ligands without their chiral methyl groups at position 3 (compounds 1–3 as depicted in Figure S1) was performed using GOLD.<sup>18</sup> The putative binding site was defined by a cutoff distance of 11.5 Å around residue  $\alpha 2\text{Ser}204$  of the C-loop of the  $\alpha 2$  subunit. In addition, we selected 10 amino acids with flexible side chains ( $\gamma 2\text{Tyr}58$ ,  $\gamma 2\text{Phe}77$ ,  $\gamma 2\text{Thr}142$ ,  $\alpha 2\text{His}101$ ,  $\alpha 2\text{Tyr}159$ ,  $\alpha 2\text{Ile}202$ ,  $\alpha 2\text{Ser}204$ ,  $\alpha 2\text{Ser}205$ ,  $\alpha 2\text{Thr}206$ , and  $\alpha 2\text{Tyr}209$ ) and set a soft potential to increase the backbone flexibility of the C-loop ( $\alpha 2\text{Ser}204$ ,  $\alpha 2\text{Ser}205$ ,  $\alpha 2\text{Thr}206$ , and  $\alpha 2\text{Gly}207$ ). To ensure convergence of the sampling, 100 genetic algorithm runs were performed. The ligands were built in MOE using the M conformation of the seven-membered ring that is supported by experimental studies. Before the docking run, the ligand was energetically minimized using the MMFF94 force field. The docking poses were rescored with the ChemScore fitness function implemented in GOLD. MOE Builder was used to derivatize the docked parent compounds with an (S)- or (R)-methyl at position 3.

**Investigated Compounds.** The synthesis of compounds 1-S (SH-I-048B), 1-R (SH-I-047), 2-S (SH-TS-CH3), 2-R (SH-TR-CHR), 3-S (SH-I-030), and 3-R (SH-I-053B) can be found in ref 16 and the Ph.D. Thesis of S. Huang.<sup>30</sup>

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b00144.

IUPAC nomenclature of the structures used in this study (Figure S1), [<sup>3</sup>H]flunitrazepam displacement assays with recombinant  $\alpha 1\beta 3\gamma 2$ ,  $\alpha 2\beta 3\gamma 2$ ,  $\alpha 3\beta 3\gamma 2$ , and  $\alpha 5\beta 3\gamma 2$  GABA<sub>A</sub> receptors (Figure S2), two-electrode voltage clamp recordings on oocytes expressing different GABA<sub>A</sub> receptors ( $\alpha 1\beta 3\gamma 2$ ,  $\alpha 2\beta 3\gamma 2$ ,  $\alpha 3\beta 3\gamma 2$ , and  $\alpha 5\beta 3\gamma 2$ ) (Figure S3), and comparison of the substitutions on the basic benzodiazepine core scaffold of the compound pairs investigated in this study (Figure S4) (PDF)

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## Notes

The authors declare no competing financial interest.

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