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# Mechanism and Site of Inhibition of AMPA Receptors: Pairing a Thiadiazole with a 2,3-Benzodiazepine Scaffold

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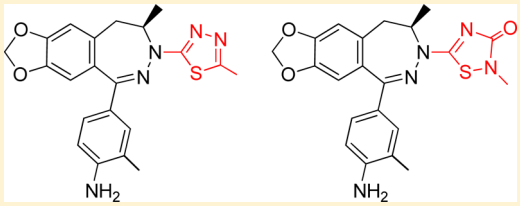
Supporting Information

**ABSTRACT:** 2,3-Benzodiazepine compounds are synthesized as drug candidates for treatment of various neurological disorders involving excessive activity of AMPA receptors. Here we report that pairing a thiadiazole moiety with a 2,3-benzodiazepine scaffold via the N-3 position yields an inhibitor type with >28-fold better potency and selectivity on AMPA receptors than the 2,3-benzodiazepine scaffold alone. Using whole-cell recording, we characterized two thiadiazolyl compounds, that is, one contains a 1,3,4-thiadiazole moiety and the other contains a 1,2,4-thiadiazole-3-one moiety. These compounds exhibit potent, equal inhibition of both the closed-channel and the open-channel conformations of all four homomeric AMPA receptor channels and two GluA2R-containing complex AMPA receptor channels. Furthermore, these compounds bind to the same receptor site as GYKI 52466 does, a site we previously termed as the "M" site. A thiadiazole moiety is thought to occupy more fully the side pocket of the receptor site or the "M" site, thereby generating a stronger, multivalent interaction between the inhibitor and the receptor binding site. We suggest that, as a heterocycle, a thiadiazole can be further modified chemically to produce a new class of even more potent, noncompetitive inhibitors of AMPA receptors.



**KEYWORDS:** *AMPA receptors, mechanism of inhibition, thiadiazolyl benzodiazepine compounds*

Pairing two dissimilar structural scaffolds is a valuable strategy of inhibitor and drug design. For example, if one of the scaffolds is an inhibitor and is known to bind to a site on its target, whereas the second scaffold is a synthetically extendable entity for further structural transformations, pairing them could generate a functionally diverse template for making a series of new inhibitors. Furthermore, if the second scaffold is properly chosen and linked, all the new compounds would retain the same mechanism of action and the same site of binding, but potentially with higher potency. A higher potency could result from a fuller occupation of the receptor binding site, which in return helps enumerate multivalent interaction between the inhibitor and the receptor within the binding site. This approach may be especially attractive for developing inhibitors for membrane proteins or receptors whose structures of inhibitor binding sites are not yet known. Here we describe the design and mechanistic characterization of a new structural template that combines a thiadiazole with a 2,3-benzodiazepine scaffold.

The first structural scaffold is the 2,3-benzodiazepine (2,3-BDZ), which defines a class of small molecule compounds synthesized to inhibit the $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (they are also known as GYKI compounds).[1−4] BDZ derivatives are more selective toward AMPA receptors, as compared with other classes of inhibitors such as quinoxalinedione compounds (e.g., NBQX and ZK200775)[5,6] or polyamine inhibitors derived from spider and wasp toxins.[7] BDZ compounds are effective as anticonvulsants in seizure models[8−11] and as neuroprotective agents in both focal[12] and global ischemia.[13]

AMPA receptors are a subtype of the glutamate ion channel receptor family, with the other two subtypes being the *N*-methyl-D-aspartate (NMDA) and kainate receptors.[14−17] AMPA receptors play essential roles in the function of the mammalian central nervous system (CNS), such as synaptic plasticity and neuronal development.[14−16] Elevated expression of AMPA receptors and/or excessive receptor activation at glutamatergic synapses have been implicated in both acute and chronic neurological disorders, such as cerebral ischemia, epilepsy, Parkinson's disease and amyotrophic lateral sclerosis.[14−17] Therefore, developing inhibitors of AMPA receptors to control excessive receptor activities has been a long pursued strategy for drug discovery for potential treatment of these neurological diseases.

The second scaffold is thiadiazole. The choice of thiadiazole as a heterocyclic scaffold is based on the following hypothesis. Using a detailed structure−activity relationship (SAR) examination and DIStance COmparison (DISCO) method, Rezessy and Sólyom[18] proposed two similar four-point pharmacophore models for 2,3-BDZ compounds. Both models contain two

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donor sites, a donor atom, and a hydrophobic center. The two models differ at one of the donor sites: in type 1 pharmacophore model, this site can be represented by, for example, the lone pair of electrons of the N-3 atom in GYKI 52466 (see its chemical structure in Figure 1); in type 2 model,

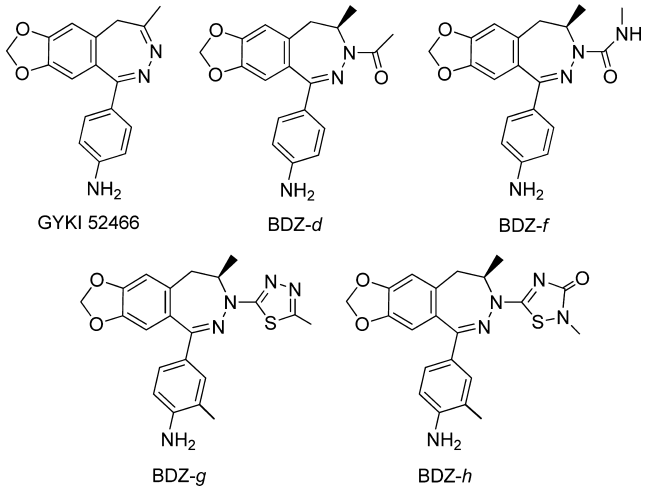


**Figure 1.** Chemical structures of the 2,3-benzodiazepine derivatives used in this study: GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine); BDZ-*d* (GYKI 53773, LY 300164, (*R*)-7-acetyl-5-(4-aminophenyl)-8-methyl-8,9-dihydro-7*H*-1,3-dioxolo[4,5-h][2,3]benzodiazepine); BDZ-*f* (GYKI 53784, LY 303070, (*R*)-5-(4-aminophenyl)-8-methyl-7-(*N*-methylcarbamoyl)-8,9-dihydro-7*H*-1,3-dioxolo[4,5-h][2,3]benzodiazepine; BDZ-*g*, GYKI 47409, (*R*)-5-(4-amino-3-methyl-phenyl)-8-methyl-7-(5-methyl-1,3,4-thiadiazol-2-yl)-8,9-dihydro-7*H*-1,3-dioxolo[4,5-h][2,3]-benzodiazepine; BDZ-*h*, GYKI 47654, (*R*)-5-(4-amino-3-methylphenyl)-8-methyl-7-(2-methyl-3-oxo-2,3-dihydro-1,2,4-thiadiazol-5-yl)-8,9-dihydro-7H-1,3-dioxolo[4,5-h][2,3]benzodiazepine.

this donor site can be defined by, for example, the lone pair of electrons from a heteroatom of heterocycles, such as GYKI 47261 [6-(4-aminophenyl)-8-chloro-2-methyl-11H-imidazo-[1,2c] [2,3]benzodiazepine][19] and some azolo-condensed 2,3-BDZs.[18] Based on favorable inhibitory properties of these earlier compounds, Rezessy and Sólyom[18] proposed that heterocycles at the N-3 position may generate a stronger interaction with their target sites, as predicted by the type 2 pharmacophore model.

To test the potential of the thiadiazolyl benzodiazepine template for developing better AMPA receptor inhibitors, here we investigate the mechanism and the site of inhibition of AMPA receptors by two thiadiazolyl benzodiazepines.[20,21] For simplicity, we name these two compounds as BDZ-*g* with a 1,3,4-thiadiazole moiety and BDZ-*h* with a 1,2,4-thiadiazole-3-one moiety (see their structures and chemical names in Figure 1 and its legend; see also the Supporting Information). We predict that both BDZ-*g* and BDZ-*h* bind to the same noncompetitive site, which we have previously termed as the "M" site on the AMPA receptor.[22] This prediction is based on the fact that the two compounds contain both 7,8-methylenedioxy moiety and a C-4 methyl group on the 2,3-diazepine ring, the key features for BDZ compounds that bind to the "M" site.[22] Since a thiadiazole is covalently coupled at the N-3 position of the 2,3-benzodiazpine ring (Figure 1), we further predict that the two thiadiazolyl benzodiazepine compounds have higher potency than GYKI 52466, the prototypic compound without any N-3 derivatization. This prediction is based on our finding that for those 2,3-BDZs that bind to the "M" site, addition of functional groups at the N-3 position yields compounds with higher potency.[22,23]

For hypothesis testing, we include GYKI 52466 as our control, along with two other compounds, all of which share both 7,8-methylenedioxy moiety and a C-4 methyl group on the 2,3-diazepine ring (Figure 1). Because GYKI 52466 is also a representative compound for type 1 pharmacophore, whereas the two thiadiazolyl benzodiazepine compounds are designed based on the type 2 pharmacophore model, our results provide a comparison between the two pharmacophore models.[18] We further characterized the inhibitory potency of the two thiadiazolyl benzodiazepine compounds with AMPA receptors in both homomeric and heteromeric forms. Thus, the selectivity profiles for the two thiadiazolyl benzodiazepine compounds are also established. The implication of the similarity and difference in the receptor binding sites that accommodate a thiadiazole scaffold among all AMPA receptor subunits is further suggested.

## RESULTS AND DISCUSSION

**Experimental Design.** In this study, we assessed the pairing of two different thiadiazole derivatives with the 2,3-BDZ scaffold that contains C-4 methyl group, that is, BDZ-*g* and BDZ-*h*, and characterized their mechanism of inhibition and the site of binding on AMPA receptors. BDZ-*h* has a different 5-membered, thiadiazole structure, as compared with BDZ-*g* (Figure 1). Therefore, our results would permit us to assess the potential difference in inhibitory properties from varying a thiadiazole scaffold. To achieve this goal, we included the following receptors and compounds in our experiments.

(a) To determine the effect of pairing a thiadiazole moiety with the 2,3-benzodiazepine ring, we included three more 2,3-BDZ compounds for comparison: GYKI 52466, BDZ-*d* (also known as talampanel), and BDZ-*f* (Figure 1). GYKI 52466 is routinely used as the standard for evaluating new 2,3-benzodiazepine derivatives.[2] It should be also noted that the C-4 methyl group on the diazepine ring of all the compounds used in our study (Figure 1), except GYKI 52466, was in the *R* configuration. This is because the "M" is stereoselective to the C-4 methyl group of a 2,3-benzodiazepine compound with an endismic ratio of >10-fold.[22] The inclusion of these compounds was to test if the thiadiazole was better than traditional N-3 derivatives, which are acyl groups.

(b) We tested BDZ-*g* and BDZ-*h*, together with all the compounds (Figure 1), with each of the GluA1, GluA2Q, GluA3, and GluA4 homomeric AMPA receptor channels. GluA1−4 are the four subunits of the AMPA receptor subtype.[15] The GluA2 AMPA receptor subunit is exclusively subject to RNA editing at the glutamine/arginine (Q/R) site.[24] Like GluA1, GluA3, and GluA4, the unedited Q isoform of GluA2 can form functional, calcium-permeable channels, but the edited or the R isoform of GluA2 cannot (see detail below). Thus, the experiments with GluA1, GluA2Q, GluA3, and GluA4 would allow us to determine whether BDZ-*g* and BDZ-*h* were inhibitors of AMPA receptors, and if so, whether they were better than the other, structurally similar 2,3-BDZ compounds (Figure 1). Experimentally, we used the whole-cell recording technique and measured the inhibitory potency all the compounds with the AMPA receptors mentioned above. Each of the receptors was transiently expressed in human embryonic kidney (HEK) 293 cells for the measurement. The relative potency of these compounds would allow us to


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determine their selectivity to each of the AMPA receptor subunits.

(c) To objectively compare the selectivity of each of the compounds, we chose the "flip", an alternatively spliced isoform[25] of every AMPA receptor channels for our assay. We note, however, that all the BDZ inhibitors that we studied before show no preference in inhibiting the "flip" over the "flop" isoform of GluA2 receptor channels.[22,23,26−30]

(d) We further assayed all the compounds with both kainate receptors (i.e., GluK1 and GluK2 homomeric channels) and NMDA receptors (i.e., GluN1a/GluN2A and GluN1a/GluN2B heteromeric channels) to find out whether the two compounds had any cross activity. GluN1a/GluN2A and GluN1a/GluN2B are two dominant NMDA receptor complexes in vivo.[15] None of the subunits, however, can form functional channels by itself.[31]

(e) Our previous studies show that the 2,3-benzodiazepine compounds that bind to the "M" site prefer to inhibit the closed-channel conformation of GluA2.[22,23,26,29] Therefore, we further tested BDZ-*g* and BDZ-*h* with both the open-channel and the closed-channel conformations of AMPA receptors. To do this, we varied glutamate concentration to "titrate" the receptor conformation. For example, the use of a saturating glutamate concentration correlates to the opening of ∼96% of the GluA2 channel population; or ∼96% of the GluA2 will be in the open conformation, given that the channel-opening probability of GluA2Q$_{flip}$ is ∼0.96.[32] On the other hand, a 100 $\mu$M glutamate correlates to the induction of ∼4% of the channels into the open form[32] or the majority of the channel population is still in the closed-channel conformation. This can be readily viewed in a dose−response relationship.[32] Thus, using 96% and 4% of the fraction of the open-channel conformation as the way to set up glutamate concentrations for the assay allowed us to not only measure the potency of an inhibitor with the two conformations of a specific AMPA receptor channel but also compare quantitatively the inhibitory potency among all AMPA receptor channels.

**BDZ-*g* and BDZ-*h* are Potent, Selective AMPA Receptor Antagonists.** Using whole-cell recording, we found that BDZ-*g* and BDZ-*h* inhibited each of the GluA1$_{flip}$, GluA2Q$_{flip}$, GluA3$_{flip}$, and GluA4$_{flip}$ AMPA receptor homomeric channels expressed in HEK-293 cells (Figure 2). An example is shown in Figure 2a where the glutamate-induced whole-cell current amplitude was reduced in the presence of BDZ-*g*. Based on the percentage of the amplitude in the presence ($A_I$) and absence ($A$) of an inhibitor, we compared the relative potency of all of the compounds we tested. Specifically, as the N-3 position of GYKI 52466 was derivatized and the chain length of the N-3 derivation grew longer, that is, from BDZ-*d* to BDZ-*f*, the potency of the resulting compounds became stronger (Figure 2b). However, GYKI 52466, BDZ-*d*, and BDZ-*f* largely limited their ability of inhibition to GluA1 and GluA2 in that none inhibited either GluA3 or GluA4 as strongly as they did with respect to either GluA1 or GluA2 (Figure 2b). In contrast, both BDZ-*g* and BDZ-*h* inhibited each of the four AMPA receptor homomeric channels equally strongly (Figure 2b). Furthermore, BDZ-*g* and BDZ-*h* inhibited both the open-channel (lower panel, Figure 2b) and the closed-channel (upper panel, Figure 2b) conformations of AMPA receptors almost equally strongly. These results show that BDZ-*g* and BDZ-*h* were the most potent inhibitors among this group, and that the two inhibitors exhibited roughly the same rank order of potency on each of the AMPA receptors (Figure 2b).

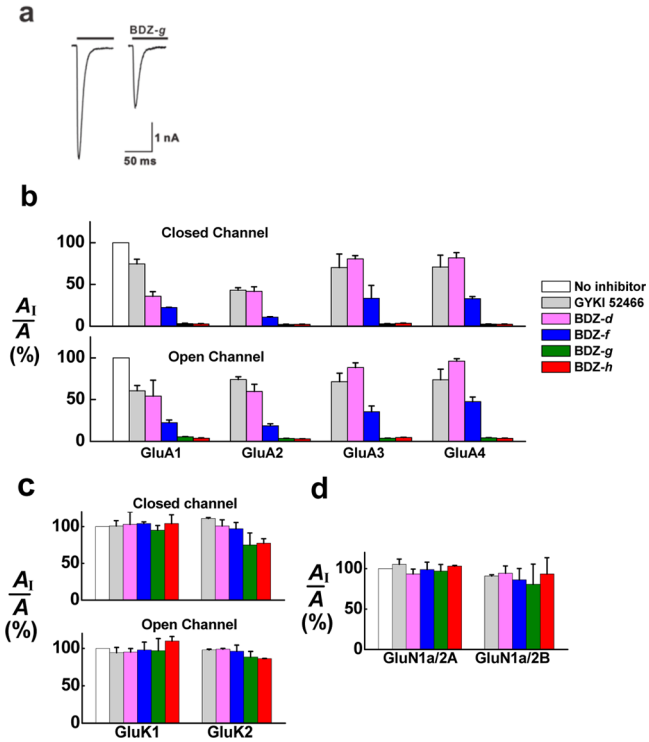


**Figure 2.** (a) A pair of representative whole-cell current response of GluA2Q$_{flip}$ receptors, expressed in an HEK-293 cell, to 3 mM glutamate in the absence (left) and presence (right) of 0.5 $\mu$M BDZ-*g*. The whole-cell recording was at −60 mV, pH 7.4, and 22 °C. The inhibitory effects of the 2,3-BDZs are shown on the AMPA (b), kainate (c), and NMDA receptors (d). An inhibitory effect of a compound on any of these receptors is shown as the percentage of the current response in the presence and absence of that compound ($A_I/A$). Each point is an average of at least three measurements from three cells. Specially, 50 $\mu$M glutamate was used for assaying with the closed-channel state of GluA1$_{flip}$ and kainate receptors, and 100 $\mu$M for the others; 2 mM glutamate was used for assaying with the open-channel state of GluA1$_{flip}$ and the kainate receptors, and 3 mM glutamate for all others receptors. The NMDA receptors were tested only with 50 $\mu$M glutamate and 100 $\mu$M glycine (as in panel d). All the compounds were tested at 20 $\mu$M except BDZ-*g* and BDZ-*h* on AMPA receptors. Because of their strong potency, we used 1 $\mu$M for both compounds with all four AMPA receptors. However, we calibrated the $A_I/A$ to a 20 $\mu$M percentage for comparison with all other compounds in the same plot.

To determine whether BDZ-*g* and BDZ-*h* were AMPA receptor specific, we further tested BDZ-*g* and BDZ-*h*, along with all other compounds, with homomeric GluK1 and GluK2 kainate receptor channels (Figure 2c), and GluN1a/2A and GluN1a/2B NMDA receptor channels (Figure 2d). As seen, no significant inhibition of these channels by any of the compounds was observed.

**Characterization of the Overall Inhibition Constants of BDZ-*g* and BDZ-*h* on AMPA Receptors.** To quantitatively characterize the potency, we determined the inhibition constants for BDZ-*g* and BDZ-*h* with both the open-channel and the closed-channel conformations for all four AMPA receptor homomeric channels. Specifically, we measured the ratio of the current amplitude in the absence and presence of an inhibitor ($A/A_I$) as a function of the inhibitor concentration (an example of the data analysis is shown in Figure 3). For BDZ-*g*, we estimated, using eq 1 (see Methods), from the $A/A_I$ plot, an inhibition constant of 0.5 ± 0.1 $\mu$M for


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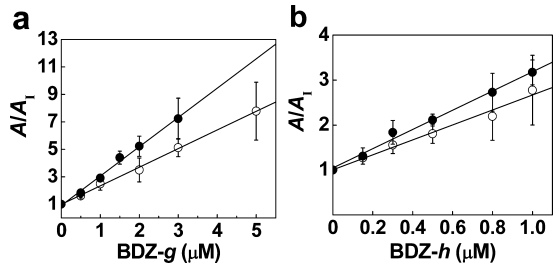


**Figure 3.** (a) Effect of BDZ-*g* on the whole-cell current amplitude of GluA2Q$_{flip}$ receptors obtained from the solution flow technique. A $K_I$ of 0.5 ± 0.1 μM was determined for the closed-channel state (100 μM glutamate, ●), whereas a $\overline{K_I}$ of 0.7 ± 0.1 μM was obtained for the open-channel state (3 mM glutamate, ○). (b) Effect of BDZ-*h* on the whole-cell current amplitude of GluA2Q$_{flip}$ receptors obtained from the same technique. A $K_I$ of 0.5 ± 0.1 μM was determined for the closed-channel state (100 μM glutamate, ●), whereas a $\overline{K_I}$ of 0.6 ± 0.1 μM was obtained for the open-channel state (3 mM glutamate, ○). All the inhibition constants were determined using eq 1. In both (a) and (b), each data point is the average of at least three separate measurements from different cells.

the closed-channel ($K_I$) and 0.7 ± 0.1 μM for the open-channel state ($\overline{K_I}$) of GluA2Q$_{flip}$ receptors (Figure 3a). For BDZ-*h*, the $K_I$ was found to be 0.5 ± 0.1 μM for the closed-channel state, whereas a $\overline{K_I}$ of 0.6 ± 0.1 μM was estimated for the open-channel state likewise (Figure 3b). These inhibition constants are summarized in Table 1, together with their respective inhibition constants for the rest of the homomeric AMPA receptor channels (the specific $A/A_I$ plots are not shown).

Based on the magnitude of inhibition constants (Table 1), the following conclusions were drawn. (i) That BDZ-*g* and BDZ-*h* each inhibited both the open-channel and the closed-channel states of AMPA receptors was consistent with the notion that both compounds are noncompetitive inhibitors.[22,23,26,29,30] On the other hand, an uncompetitive or an open-channel blocker would only inhibit the open-channel, but not the closed-channel, state. In contrast, a competitive inhibitor would only inhibit the closed-channel, but not the open-channel state.[22,23,26,29,30] (ii) The fact that $\overline{K_I} \approx K_I$ for either BDZ-*g* or BDZ-*h* suggested that each of the two compounds inhibited the closed-channel and the open-channel states of an AMPA receptor equally strongly (Table 1). (iii) Because BDZ-*g* and BDZ-*h* were found to inhibit all four AMPA receptor channels noncompetitively, they were supposed to bind to the "M" site; in other words, the "M" site exists in all four AMPA receptor homomeric channels. (iv) Acylation of the 2,3-benzodiazepine ring at the N-3 position has been a standard of functionalizing a 2,3-benzodiazepine ring.[2,33] Our results, however, showed that coupling a thiadiazole heterocycle to the N-3 position generated inhibitors, that is, BDZ-*g* and BDZ-*h*, which were more potent than simply coupling acyl groups to the same position, that is, BDZ-*d* and BDZ-*f*. This outcome was predicted by the type 2 pharmacophore model.[18]

It should be noted that acylation of the same group (i.e., an N-methyl carbamoyl group) at the N-3 position increases potency for compounds that bind to the "M" site,[23] but decreases potency for compounds that bind to the "O" site.[29] Compounds that bind to the "M" site share a C-4 methyl (i.e., an azomethine feature on the diazepine ring)[23] whereas those that bind to the "O" site have a C-4 carbonyl group (i.e., a ε-lactam feature).[26] Yet both types of compounds contain a methylenedioxy group on the benzene ring of the 2,3-diazepine skeleton (Figure 1). As a comparison, the compounds that bind to the "E" site have also a C-4 carbonyl group but with 7,8-ethylenedioxy ring, rather than 7,8-methylenedioxy ring structure.[22]

**BDZ-*g* and BDZ-*h* Bind to the Same Site or the "M" Site on GluA2Q$_{flip}$.** BDZ-*g* and BDZ-*h* were predicted to bind to the "M" site, because both compounds contain a C-4 methyl and a methylenedioxy groups on the diazepine ring.[22,23,26] To test this prediction, we performed a double-inhibitor experiment (see Methods). In this experiment, two inhibitors were applied onto a receptor together. The concentration of one inhibitor was kept constant while the concentration of the other was varied. An apparent inhibition constant obtained from the two-inhibitor experiment or from the slope of the $A/A_{I,P}$ plot (upper solid line in any one of Figure 4a–c) was compared to that obtained from the one-inhibitor experiment or from the slope of the $A/A_I$ plot (lower solid line in Figure 4a–c) (see Methods for eqs 1 and 2 for calculation of inhibition constants, where I and P represent two different inhibitors). The comparison of an inhibition constant obtained from one-inhibitor experiment with that from two-inhibitor experiment would allow us to determine whether the two inhibitors bound to the same or two different sites. Using this method, we previously reported that there are three noncompetitive sites for 2,3-benzodiazepine compounds on GluA2Q$_{flip}$, one of which is for GYKI 52466 and any compound that shares both a C-4 methyl group and a 7,8-methylenedioxy ring on the 2,3-diazepine scaffold.[22,23]

Using the same method, we tested BDZ-*g* with GYKI 52466 on the closed-channel state of GluA2Q$_{flip}$ receptors (Figure 4a). As seen, the double-inhibition constant, $K_I'$, was determined to be 0.5 μM (upper solid line in Figure 4a), which was identical to $K_I$ of 0.5 μM for BDZ-*g* alone (lower solid line in Figure 4a). That the $K_I$ value or the slope of the plot did not change was consistent with the prediction that BDZ-*g* and GYKI 52466 competed for binding to the same site on GluA2Q$_{flip}$. If they bound to two different sites, the apparent inhibition would be

**Table 1. Inhibition Constants of BDZ-*g* and BDZ-*h* on Homomeric AMPA Receptors**

| | inhibition constants (μM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | GluA1$_{flip}$[a] | | GluA2Q$_{flip}$[b] | | GluA3$_{flip}$ | | GluA4$_{flip}$ | |
| inhibitor | $K_I$ | $\overline{K_I}$ | $K_I$ | $\overline{K_I}$ | $K_I$ | $\overline{K_I}$ | $K_I$ | $\overline{K_I}$ |
| BDZ-*g* | 0.7 ± 0.1 | 1.2 ± 0.1 | 0.5 ± 0.1 | 0.7 ± 0.1 | 0.6 ± 0.1 | 0.8 ± 0.1 | 0.6 ± 0.1 | 0.9 ± 0.1 |
| BDZ-*h* | 0.6 ± 0.1 | 0.8 ± 0.1 | 0.5 ± 0.1 | 0.6 ± 0.1 | 0.7 ± 0.1 | 1.0 ± 0.1 | 0.5 ± 0.1 | 0.8 ± 0.1 |

[a]50 μM and 2 mM glutamate were chosen for measuring the inhibition constants of both compounds with the closed-channel and the open-channel states of GluA1$_{flip}$, respectively. [b]100 μM and 3 mM glutamate were chosen for measuring the inhibition constants of both compounds with the closed-channel and the open-channel states of GluA2Q$_{flip}$, GluA3$_{flip}$ and GluA4$_{flip}$, respectively.


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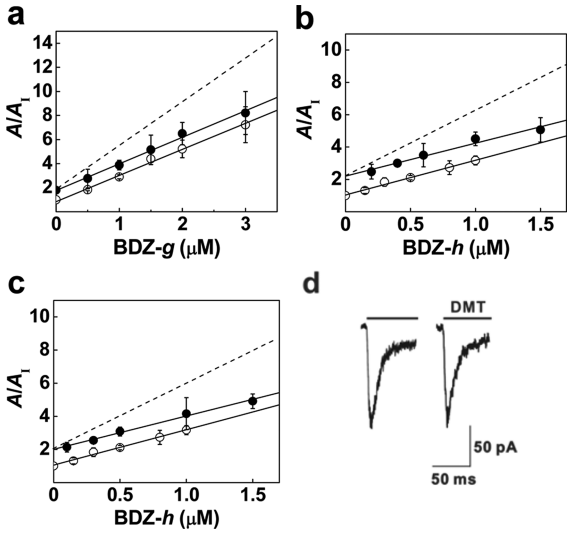


**Figure 4.** (a) Double-inhibitor experiment for GYKI 52466 and BDZ-*g* with the GluA2Q$_{flip}$ receptor (note that 100 $\mu$M glutamate was used for the assay, which reflected the closed-channel state of GluA2Q$_{flip}$). The concentration of GYKI 52466 was kept at 20 $\mu$M, while that of BDZ-*g* varied from 0.5 to 3 $\mu$M. The apparent double-inhibition constant, $K_I'$, was determined to be 0.5 ± 0.1 $\mu$M (filled circles, ●), as compared with $K_I$ of 0.5 ± 0.1 $\mu$M for BDZ-*g* alone (open circles, ○). The dashed line is the simulation of the $A/A_I$ plot on the assumption that the two inhibitors bound to two different sites with a double-inhibition constant of ∼0.3 $\mu$M (when GYKI 52466 was kept at 20 $\mu$M). The dashed line was generated using eq 3. (b) Double-inhibitor experiment for GYKI 52466 and BDZ-*h* with the GluA2Q$_{flip}$ receptor. The concentration of GYKI 52466 was kept at 20 $\mu$M, while that of BDZ-*h* varied from 0.2 to 1.5 $\mu$M. The glutamate concentration was $\mu$M 100, which corresponded to the closed-channel conformation. The apparent double-inhibition constant, $K_I'$, was determined to be 0.5 ± 0.1 $\mu$M (filled circles, ●), as compared with $K_I$ of 0.5 ± 0.1 $\mu$M for BDZ-*h* alone (open circles, ○). By the use of eq 3, the dashed line is generated that simulates the $A/A_I$ plot based on the assumption that the two inhibitors bound to two different sites with a double-inhibition constant of ∼0.3 $\mu$M (when GYKI 52466 was kept at 20 $\mu$M). (c) Double-inhibitor experiment for BDZ-*g* and BDZ-*h* with GluA2Q$_{flip}$. The concentration of BDZ-*g* was fixed at 0.5 $\mu$M, while that of BDZ-*h* varied from 0.1 to 1.5 $\mu$M. The apparent double-inhibition constant, $K_I'$, was determined to be 0.5 ± 0.1 $\mu$M (filled circles, ●), as compared with $K_I$ of 0.5 ± 0.1 $\mu$M for BDZ-*h* alone (open circles, ○). As in (b), the glutamate concentration was at 100 $\mu$M. The dashed line is the simulation of the $A/A_I$ plot based on the assumption that the two inhibitors bound to two different sites with a double-inhibition constant of ∼0.3 $\mu$M (when BDZ-*g* was kept at 0.5 $\mu$M). (d) A pair of representative whole-cell current response of the GluA2Q$_{flip}$ receptors to 100 $\mu$M glutamate in the absence (left) and presence (right) of 20 $\mu$M 2,5-dimethyl-1,3,4-thiadiazole.

"additive", generating a steeper slope (i.e., the dashed-line in Figure 4a and see Methods as well) or a stronger, apparent inhibition constant when both inhibitors were present. Similarly, the double-inhibition experiment showed that BDZ-*h* and GYKI 52466 also bound to the same site (Figure 4b). To confirm that BDZ-*g* and BDZ-*h* indeed bound to the same site, we further tested the inhibition of the whole-cell current response of GluA2Q$_{flip}$ in the presence of both BDZ-*g* and BDZ-*h*, as compared with BDZ-*h* alone (Figure 4c). As expected, this pair of compounds competed for binding to the same site on the GluA2Q$_{flip}$ receptor. This site, as we previously termed, was the "M" site.

Among all of the compounds we tested (Figure 1), the potency of either BDZ-*g* or BDZ-*h* is higher than either BDZ-*d* or BDZ-*f*, all of which are higher than GYKI 52466 (Table 1). All of these compounds have been shown to bind to the same site.[22,23,26,29,30] Given that GYKI 52466 lacks any N-3 acylating group (Figure 1), we hypothesize that a fuller occupancy of the side pocket or the cavity that accommodates an N-3 derivative at the "M" site gives rise to a higher potency. In either BDZ-*g* or BDZ-*h*, the N-3 derivative is a thiadiazole moiety, which is bulkier than an acetyl group as in BDZ-*d* or a longer, N-methyl-carbamoyl group as in BDZ-*f*. In fact, BDZ-*f* has a stronger potency than BDZ-*d*.[22,23]

It is not known whether a thiadiazole alone has any inhibitory activity on AMPA receptors or whether occupying only the side pocket alone is able to exert any inhibitory effect. To address this question, we tested whether 2,5-dimethyl-1,3,4-thiadiazole was sufficient to generate any inhibition on GluA2Q$_{flip}$. 2,5-Dimethyl-1,3,4-thiadiazole has the closest structural similarity with the thiadiazole moiety in BDZ-*g*. As shown (Figure 4d), the application of this thiadiazole analogue did not yield any inhibition on either the open-channel or the closed-channel conformation of GluA2Q$_{flip}$. Based on the assumption that 2,5-dimethyl-1,3,4-thiadiazole indeed bound to the same site or the same side pocket as the 1,3,4-thiadiazole group of BDZ-*g* bound, we conclude that binding of a thiadiazole scaffold alone to an AMPA receptor or occupying only the N-3 side pocket of the "M" site alone is ineffective to produce inhibition. Consequently, a higher potency from the coupled structural scaffolds, as compared with either GYKI 52466 or BDZ-*d* or BDZ-*f* alone, is most likely a result of a fuller occupancy of the N-3 side pocket by a thiadiazole structure. A fuller occupancy of the N-3 side pocket of the "M" site by a thiadiazole moiety might have generated additional multivalent interaction between a thiadiazole and the surrounding amino-acid residues of the receptor binding site. This conclusion is also consistent with the prediction by the type 2 pharmacophore model.[18]

We note, however, whether the 1,3,4-thiadiazole and the 1,2,4-thiadiazole-3-one moiety represent the most optimal interaction with or fit to the "M" site is not known. An answer to this question should await a detailed structural description of the interaction between BDZ-*g* or BDZ-*h* and the "M" site on an AMPA receptor. Currently, there is no structural information about a noncompetitive inhibitor bound to an AMPA receptor, nor the exact location of any of these noncompetitive sites, including the "M" site. Alternatively, more compounds in varying a thiadiazole structure can be prepared and characterized similarly to probe the side pocket of the "M" site.

**BDZ-*g* and BDZ-*h* Inhibit GluA2R-Containing AMPA Receptors.** We next investigated if BDZ-*g* and BDZ-*h* also inhibited GluA1$_{flip}$/GluA2R$_{flip}$ and GluA2Q$_{flip}$/GluA2R$_{flip}$ receptors, the two representative, GluA2R-containing AMPA receptor channels. The GluA2R subunit cannot form functional channels by itself but can assemble into functional channels with an AMPA receptor subunit in the Q isoform,[34−37] such as GluA1 and GluA2Q. The GluA2 subunit is present virtually only in the edited or R isoform in healthy adult tissue.[38,39] As such, GluA1/GluA2R is one of the main complex AMPA receptor forms found in vivo, such as in hippocampus.[40,41] Yet under certain disease conditions, such as amyotrophic lateral sclerosis (ALS),[42] the GluA2 Q/R editing is defective, thereby generating the unedited isoform or GluA2Q. Consequently,

GluA2Q/GluA2R is a possible configuration of AMPA receptors. Therefore, it would be useful to investigate whether these GluA2R-containing AMPA receptor complexes could be inhibited by compounds such as BDZ-*g* and BDZ-*h*.

The effect of BDZ-*g* was measured on the whole-cell current amplitude of $GluA2Q_{flip}/2R_{flip}$ receptors expressed in HEK-293 cells. A $K_I$ of 0.5 ± 0.1 μM was determined from the plot of the ratio of the current amplitude in the absence and presence of BDZ-*g* for the closed-channel conformation (Figure 5a) (note

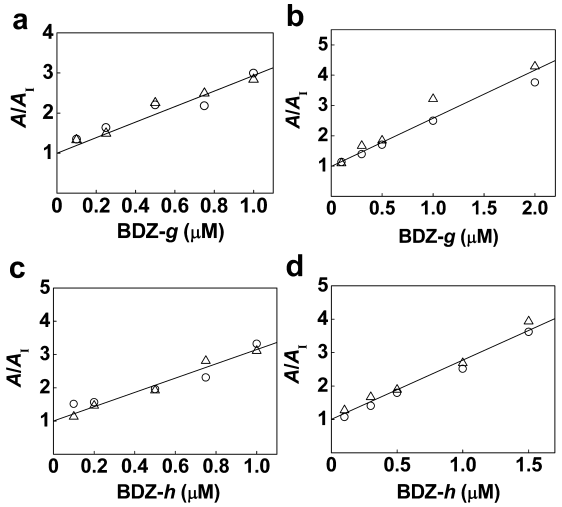


**Figure 5.** (a) Effect of BDZ-*g* on the whole-cell current amplitude of $GluA2Q_{flip}/2R_{flip}$ receptors as a function of BDZ-*g* concentration. For the assay with the closed-channel (○) and the open-channel (△) conformations, 75 μM and 3 mM glutamate concentrations were used, respectively. With the combined data, an inhibition constant of 0.5 ± 0.1 μM was determined using eq 1. (b) Effect of BDZ-*g* on the whole-cell current amplitude of $GluA1_{flip}/2R_{flip}$ receptors as a function of BDZ-*g* concentration. Here, 50 μM and 3 mM glutamate concentrations were used for the assay with the closed-channel (○) and the open-channel (△) conformations, respectively. From the combined data, a $K_I$ of 0.6 ± 0.1 μM was determined. Similarly, the effect of BDZ-*h* on the whole-cell current amplitude of both the $GluA2Q_{flip}/2R_{flip}$ (c) and the $GluA1_{flip}/2R_{flip}$ receptors (d) was determined. In (c), a $K_I$ of 0.5 ± 0.1 μM was determined from the data combining the points from the closed-channel state (75 μM glutamate, △) and the open-channel state (3 mM glutamate, ○). Likewise, a $K_I$ of 0.6 ± 0.1 μM was determined from the data in (d) combining the points of the closed-channel state (50 μM glutamate, △) and the open-channel state (3 mM glutamate, ○).

that 75 μM glutamate was used for this experiment, which corresponded ∼4% of the fraction of the open-channel population; the $EC_{50}$ value used for estimating the glutamate concentration was from unpublished data). Furthermore, a $\overline{K_I}$ of 0.5 ± 0.1 μM was estimated for the open-channel conformation (i.e., 3 mM glutamate was chosen for this experiment for the same reason as described above). Because the $K_I$ values for both the closed-channel and the open-channel conformations were identical, a $K_I$ of 0.5 ± 0.1 μM was estimated by using the combined data (Figure 5a). Similarly, the effect of BDZ-*g* on the whole-cell current amplitude of $GluA1_{flip}/2R_{flip}$ receptors was measured. From the combined data, a $K_I$ of 0.6 ± 0.1 μM was determined (Figure 5b). When the $A/A_I$ value for the closed-channel conformation (i.e., 50 μM glutamate) and the open-channel conformation (i.e., 3 mM glutamate) was individually analyzed, a $K_I$ of 0.6 ± 0.1 μM and 0.7 ± 0.1 μM would be obtained, respectively. These inhibition constants are also summarized in Table 2. Likewise, the

**Table 2. Inhibition Constants of BDZ-*g* and BDZ-*h* on GluA2R-Containing AMPA Receptors**

| | inhibition constants (μM) | |
|---|---|---|
| | $GluA2Q_{flip}/2R_{flip}$ [a] | $GluA1_{flip}/2R_{flip}$ [b] |
| inhibitor | $K_I$ | $K_I$ |
| BDZ-*g* | 0.5 ± 0.1 | 0.6 ± 0.1 |
| BDZ-*h* | 0.5 ± 0.1 | 0.6 ± 0.1 |

[a]75 μM and 3 mM glutamate were chosen for measuring the inhibition constants of both compounds with the closed-channel and the open-channel states of $GluA2Q_{flip}/2R_{flip}$, respectively. [b]50 μM and 3 mM glutamate were chosen for measuring the inhibition constants of both compounds with the closed-channel and the open-channel states of $GluA1_{flip}/2R_{flip}$, respectively.

inhibition constants for BDZ-*h* with $GluA2Q_{flip}/2R_{flip}$ receptors (Figure 5c) and the GluA1/GluA2R receptors (Figure 5d) were determined, and are also summarized in Table 2.

From these results, we conclude that (i) BDZ-*g* and BDZ-*h* inhibited both GluA1/GluA2R and GluA2Q/GluA2R, and did so with a similar potency as compared with GluA1 and GluA2Q, respectively (Tables 1 and 2); (ii) neither BDZ-*g* nor BDZ-*h* showed any preference in inhibiting the closed-channel over the open-channel state. This trend (Table 2) is identical to how BDZ-*g* and BDZ-*h* inhibit GluA1 and GluA2Q homomeric AMPA receptor channels (Table 1). However, our data do not allow us to conclude whether a BDZ compound binds to a Q isoform (i.e., GluA1 or GluA2Q) or the GluA2R isoform or both in order to exert inhibition of the GluA2R-containing channels. Different experiments and/or different techniques will be needed to address this question.

**Comparison of the Potency of BDZ-*g* and BDZ-*h* with Other BDZs.** The type 2 pharmacophore model predicts that a heteroatom in a heterocycle, near the C-4 and the N-3 region, is a key structural determinant in improving the inhibitory property of a 2,3-benzodiazepine compound.[18] A heteroatom in this region is predicted to generate a stronger interaction between the compound and the receptor binding site, such as through hydrogen bonding, than just a C-4 alkyl group alone.[18] The model was established based on earlier finding that some 4-oxo-2,3-BDZs and several heteroring-condensed 2,3-BDZs show higher activity than GYKI 52466.[18] The results from this study are consistent with the prediction in that coupling a 5-membered, thiadiazole scaffold through the N-3 position produces potent AMPA receptor inhibitors. In fact, BDZ-*g* and BDZ-*h* represent the most potent noncompetitive inhibitors of AMPA receptors ever reported.[2,3,22,23,26,29,30] It should be noted that a series of 5- and 6-membered heterocycles were previously synthesized, and the inhibitors with the highest activity were the 5-membered thiadiazolyl BDZs.[20,21] Furthermore, because both BDZ-*g*, which contains 1,3,4-thiadiazole moiety, and BDZ-*h*, which contains a 1,2,4-thiadiazole-3-one moiety (Figure 1) work equally well, we hypothesize that additional chemical modifications on a thiadiazole scaffold can be explored for generating even better thiadiazolyl benzodiazepine inhibitors.

As a 5-membered heterocyclic ring structure, a thiadiazole has indeed a high potential for chemical modifications. For instance, thiadiazoles are considered bioisosteres of oxadiazole, oxazole, pyrimidine, and so forth.[43] Substitution of a thiadiazole


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with its bioisostere can lead to different compounds with a prospect of improving biological activities, such as better liposolubility.[44] 1,3,4-Thiadiazoles are also mesoionic with dense, highly polarizable character that promotes stronger interaction with the amino-acid residues forming the receptor binding site. In practice, thiadiazoles have attracted a great deal of interest in synthesis of novel chemical compounds and drug candidates.[43] Compounds bearing thiadiazole rings are known to exhibit anticancer, anti-inflammatory, antibiotic, antiviral, and anticonvulsant activities.[43,45] In fact, there are a number of thiadiazole-containing drugs currently in clinical use, such as timolol,[46] a nonselective $\beta$-adrenergic antagonist and a drug widely used for glaucoma treatment.[47] Therefore, combining a thiadiazole ring structure with a 2,3-BDZ scaffold opens a new area of design and development of more potent, noncompetitive AMPA receptor inhibitors.

In the structure of 2,3-benzodiazepine scaffold for both BDZ-*g* and BDZ-*h*, the aminophenyl ring contains a 3-methyl group, whereas the rest of the compounds we used, such as GYKI 52466, do not (Figure 1). Earlier studies suggest that the main benefit of substituting a 3-methyl group on the 4-aminophenyl ring is that 2,3-benzodiazepine compounds with this extra 3-methyl group show slower metabolic rate in vivo.[3,48] As we have shown here, the presence of this methyl group on the aminophenyl ring apparently does not affect the site of binding for both BDZ-*g* and BDZ-*h*.

**Comparison of the Selectivity Profile of BDZ-*g* and BDZ-*h* with Other BDZ Compounds.** The comparison of the selectivity profile of BDZ-*g* and BDZ-*h* with other known inhibitors, such as BDZ-*d* and BDZ-*f*, on all AMPA receptor homomeric channels shows that BDZ-*g* and BDZ-*h* inhibit all four AMPA receptor subunits almost equally potently whereas BDZ-*d* and BDZ-*f* prefer to inhibit only GluA1 and GluA2. The difference in selectivity between BDZ-*g*/BDZ-*h* and BDZ-*d*/BDZ-*f* may be contributed by how fully the N-3 side pocket of the "M" site is occupied. Occupying this side pocket and interacting with the surrounding amino-acid residues on the receptor site is perhaps more important in producing effective inhibition on both GluA3 and GluA4 AMPA receptor channels. Therefore, the results from this study suggest that coupling a thiadiazole with a 2,3-benzodiazepine scaffold can be particularly useful in developing potent noncompetitive inhibitors targeting both GluA3 and GluA4 AMPA receptor subunits.

A fuller occupancy of the N-3 side pocket of the "M" site may be also the reason by which both BDZ-*g* and BDZ-*h* do not recognize the difference in receptor conformation or they inhibit both the closed-channel and the open-channel conformations indiscriminately. In contrast, other acyl group-containing 2,3-BDZs, such as BDZ-*d* and BDZ-*f*, prefer to inhibit the closed-channel over the open-channel conformation of the AMPA receptors.[22,23,26] These results may suggest that a fuller occupancy of the N-3 side pocket of the "M" site is more important in generating inhibition of the open-channel than the closed-channel conformation. An inhibitor that shows no conformational preference is capable of inhibiting an AMPA receptor with an apparent potency independent of glutamate concentration (note that this inhibitory property is consistent with the conclusion that BDZ-*g* and BDZ-*h* are noncompetitive inhibitors).

## CONCLUSION

As we have shown, pairing a thiadiazole with the 2,3-benzodiazepine scaffold that contains a C-4 methyl group can produce new AMPA receptor inhibitors that are far more potent than the original 2,3-benzodiazepine scaffold alone (i.e., GYKI 52466) without any appreciable activity on either kainate or NMDA receptors. If the inhibition constant is used as a measure of potency, BDZ-*g* is >28-fold more potent than GYKI 52466 on the same AMPA receptor (i.e., the $K_I$ value for BDZ-*g* and GYKI 52466 with the closed-channel conformation of GluA2Q$_{flip}$ is 0.5 $\mu$M and 14 $\mu$M, respectively) (if the $\overline{K_I}$ value for the open-channel conformation of GluA2Q$_{flip}$ is used for comparison, BDZ-*g* will be >40-fold better: $\overline{K_I}$ for BDZ-*g* and GYKI 52466 is 0.7 and 30 $\mu$M, respectively).[26] In fact, the two thiadiazolyl benzodiazepine compounds are the most potent, noncompetitive AMPA receptor inhibitors ever reported. The superior inhibitory properties of the two thiadiazolyl benzodiazepine compounds show the utility of the type 2 pharmacophore model. By this model, an N-3 linked thiadiazole ring as a potent donor moiety interacts with the "M" site. That the addition of a 5-membered heterocycle to a 2,3-BDZ results in a much more potent inhibitor than an acylating group may also suggest that a larger ring structure enables a much stronger, multivalent interaction of the inhibitor with the N-3 side pocket of the "M" site. The interaction at this donor site may be particularly important in both the GluA3 and GluA4 AMPA receptor subunits. Our results from this and earlier studies[22,23,26] support that the size of the donor moiety near the C-4 methyl and the N-3 position of the 2,3-benzodiazepine template, as predicted by the type 2 pharmacophore, also affects the potency of the resulting 2,3-BDZs. Because a thiadiazole moiety is a synthetically extendable scaffold, further structural transformations can readily lead to the generation of a series of new, potentially even better 2,3-BDZ inhibitors that bind to the "M" site on AMPA receptors.

## METHODS

**Chemicals and Inhibitors.** The synthesis of BDZ-*g* (GYKI 47409) was previously described,[20,21] and BDZ-*h* (GYKI 47654) was synthesized analogously (see the Supporting Information). GYKI 52466, BDZ-*d* (also known as talampanel), and BDZ-*f* used in this study (their chemical names are listed in the legend of Figure 1) were previously reported.[1,2,22,23,26] 2,5-Dimethyl-1,3,4-thiadiazole was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used in making buffers were from commercial sources.

**Cell Culture and Receptor Expression.** All receptors were transiently expressed in HEK-293S as described.[22,23] Briefly, HEK-293S cells were maintained in the Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Invitrogen), 100 units of penicillin/mL, and 0.1 mg streptomycin/mL (Sigma-Aldrich, St. Louis, MO). The humidified incubator where the cells grew was set at 37 °C, 5% $CO_2$. It should be noted that our study involved testing all of the compounds with AMPA, kainate, and NMDA receptors. In the transfection for homomeric AMPA receptors and kainate receptors, 2−30 $\mu$g of a desired cDNA plasmid was used, together with the plasmid that encoded green fluorescent protein (GFP) and another plasmid that encoded simian virus large T-antigen (TAg), at a ratio of 10:1:1. For the transfection of NMDA receptors (i.e., GluN1a/GluN2A and GluN1a/GluN2B heteromeric channels), a 1:1 ratio of NMDA receptor plasmids was used. The media in which NMDA receptors were maintained were supplemented with 1 mM ketamine.

We also tested our compounds with two representative GluA2R-containing AMPA receptors, that is, GluA1/GluA2R and GluA2Q/

GluA2R. In the transfection, we used the GluA2R plasmid in 4-fold excess, as compared to the plasmid amount of a Q isoform, to ensure the formation of GluA2R-containing AMPA receptor channels.[49] During recording, we further tested each of the cells for the current−voltage ($I-V$) relationship. The GluA2R-containing channels show a linear $I-V$ curve, whereas the homomeric GluA1, GluA2Q, GluA3, and GluA4 homomeric channels, and complex AMPA receptors lacking the GluA2R subunit all exhibit inwardly rectifying $I-V$ profiles.[49−51] Only those cells that showed a linear $I-V$ curve were used for recording.

**Whole-Cell Current Recording.** The use of whole-cell current recording to characterize AMPA receptor inhibitors was previously described.[23,26,29] In brief, the glutamate induced whole-cell current was recorded on an Axopatch 200B at a cutoff frequency of 2−20 kHz by a built-in, 4-pole low-pass Bessel filter; the whole-cell current traces were digitized at a 5−50 kHz sampling frequency using a Digidata 1322A (Molecular Devices, Sunnyvale, CA). All recordings were with transfected HEK-293S cells that were voltage-clamped at −60 mV and 22 °C. pClamp 8 (Molecular Devices) was used for data acquisition. The electrode had a resistance of ∼3 MΩ and was filled with the following electrode solution: 110 mM CsF, 30 mM CsCl, 4 mM NaCl, 0.5 mM $CaCl_2$, 5 mM EGTA, and 10 mM HEPES (pH 7.4 adjusted by CsOH). The extracellular bath buffer contained 150 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4 adjusted by NaOH). In the experiments for NMDA receptors, the electrode solution contained 140 mM CsCl, 1 mM $MgCl_2$, 0.1 mM EGTA, and 10 mM HEPES (pH 7.2 adjusted by CsOH). The extracellular buffer contained 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 10 mM glucose, and 5 mM HEPES (pH 7.2 adjusted by NaOH).[52] As a NMDA receptor coagonist, 100 $\mu$M glycine was added to all the extracellular buffer and inhibitor solutions.

A solution flow device was used to deliver a free glutamate solution with or without an inhibitor as previously described.[23,26,29] To achieve full inhibition, we preincubated an HEK-293 cell with an inhibitor solution for at least 6 s.[23,26,29] For data analysis, the amplitude of the whole-cell current measured using the flow device was corrected for receptor desensitization.[53,54]

**Mechanistic Models, Equations, and Data Analysis.** A general, minimal mechanism of AMPA receptor channel opening was used for data analysis.[23,26,29]

$$A + L \overset{K_1}{\rightleftharpoons} AL_n \overset{\Phi}{\rightleftharpoons} \underset{\text{(open)}}{\overline{AL_n}}$$

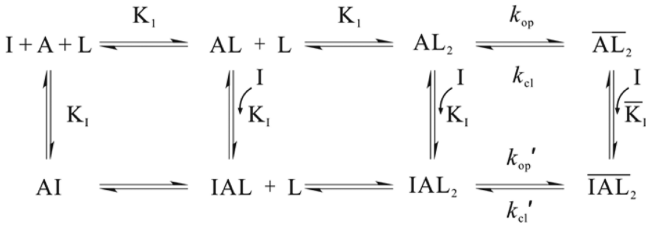
In the above scheme, $A$ represents the unliganded form of the receptor, $AL_n$ are the closed-channel forms of the receptor, and ($\overline{AL_n}$) represents the open-channel form. $K_1$ is the intrinsic dissociation constant of glutamate; $\Phi$, the channel-opening equilibrium constant; $I_A$, the current amplitude corresponding to a certain concentration of ligand; $I_m$, the current per mole of receptor sites; and $R_m$, the moles of receptor sites.

$$I_A = I_M R_M \frac{L^n}{L^n + \Phi(L + K_1)^n} = I_M R_M (\overline{AL}_n)_0 \quad \text{(a)}$$

In eq a, the number of glutamate molecules to bind to the receptor and to open its channel, $n$, can be from 1 to 4, assuming that a receptor is a tetrameric complex and each subunit has one glutamate binding site. Our earlier studies of the AMPA receptor channel opening, however, support the assumption that the binding of a minimum of two glutamate molecules per receptor complex is sufficient to open an AMPA receptor channel.[32,55−58] For simplicity and without contrary evidence, we further assume that glutamate binds with equal affinity or $K_1$ at all steps.[32,55−58] Using $n$ = 2, we define that $(\overline{AL_n})_0$ in eq a or $(\overline{AL_2})_0$ is expressed as a function of the fraction of all receptor forms.

$$(\overline{AL_2})_0 = \frac{\overline{AL_2}}{A + AL + AL_2 + AL_2} = \frac{L^2}{L^2(1 + \Phi) + 2K_1 L\Phi + K_1^2\Phi} \quad \text{(b)}$$

The effect of an inhibitor on the whole-cell current amplitude was used to determine an inhibition constant.[23,26,29] This was based on a minimal mechanism of inhibition of AMPA receptors by, for example, a noncompetitive model, where inhibitor, $I$, can bind to and inhibit various receptor forms, shown below. Thus, $K_I$ and $\overline{K_I}$ represent the inhibition constant for the closed- and the open-channel forms.




From the mechanism of inhibition, eq 1 was derived and was used to determine an inhibition constant from the effect of BDZ-*g* or BDZ-*h* on the amplitude of whole-cell current:

$$\frac{A}{A_I} = 1 + I\frac{(\overline{AL_2})_0}{K_I} \quad (1)$$

$A$ and $A_I$ are the whole-cell current amplitude in the absence and presence of an inhibitor. $(\overline{AL_2})_0$ was defined in eq *b*. It should be pointed out that eq 1 is also applicable for assaying competitive and uncompetitive inhibitors.[23,26,29]

A double-inhibitor experiment was designed to determine whether the two inhibitors (e.g., BDZ-*g* or BDZ-*h*) competed to binding to the same site or two different sites on the same receptor. In this experiment, two inhibitors were used simultaneously to inhibit a receptor. The effect of the two inhibitors, $I$ and $P$ in molar concentration, on the current amplitude was plotted as $A/A_{I,P}$ vs one inhibitor concentration. If one inhibitor is bound to a receptor and the binding of that inhibitor excludes the binding of the other (i.e., $A{\cdot}I$ and $A{\cdot}P$ are allowed but not $A{\cdot}I{\cdot}P$), the ratio of the current amplitude was given in eq 2 for the one-site model below.

$$\frac{A}{A_{I,P}} = \left(1 + \frac{P}{K_P}\right) + \frac{I}{K_I} \quad (2)$$

On the other hand, for a two-site model in which there are two sites, one for $I$ and the other for $P$ separately (i.e., $A{\cdot}I$, $A{\cdot}P$, and $A{\cdot}I{\cdot}P$ are all allowed), the ratio of the current amplitude is given in eq 1 for this two-site model.

$$\frac{A}{A_{I,P}} = \left(1 + \frac{P}{K_P}\right) + \left(1 + \frac{P}{K_P}\right)\frac{I}{K_I} \quad (3)$$

Experimentally, the concentration of one inhibitor was kept constant, while the concentration of the other was varied. The effect of two inhibitors on the whole-cell current amplitude was measured (see the data in Figure 4).

Origin 7 (Origin Lab, Northampton, MA) was used for data analysis and plotting. Unless otherwise noted, every data point used for data analysis and plotting was an average of at least three measurements collected from three different cells. The error reported refers to the standard error of the fits.

## ■ ASSOCIATED CONTENT

### Supporting Information

Additional information showing the synthetic scheme of BDZ-*h*, physical constants for both BDZ-*g* and BDZ-*h*, including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UV−vis and CD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

S.S. synthesized and characterized the compounds (when he worked at the IVAX Drug Research Institute in Budapest, Hungary). C.W., Y.H., and A.W. did all other experiments as described. C.W. did the majority of the experiments and data analysis. All authors contributed to the paper writing. L.N. directed the project and wrote the paper based on the draft written by C.W.


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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; 2,3-BDZ, 2,3-benzodiazepine; NMDA, *N*-methyl-D-aspartate; CNS, central nervous system; SAR, structure−activity relationship; DISCO, DIStance COmparison method; HEK-293S cells, human embryonic kidney 293S cells

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