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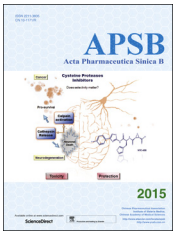


REVIEW

# Mechanism-based design of 2,3-benzodiazepine inhibitors for AMPA receptors


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**Abstract** 2,3-Benzodiazepine (2,3-BDZ) compounds represent a group of structurally diverse, small-molecule antagonists of (*R*, *S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) receptors. Antagonists of AMPA receptors are drug candidates for potential treatment of a number of neurological disorders such as epilepsy, stroke and amyotrophic lateral sclerosis (ALS). How to make better inhibitors, such as 2,3-BDZs, has been an enduring quest in drug discovery. Among a few available tools to address this specific question for making better 2,3-BDZs, perhaps the best one is to use mechanistic clues from studies of the existing antagonists to design and discover more selective and more potent antagonists. Here I review recent work in this area, and propose some ideas in the continuing effort of developing newer 2,3-BDZs for tighter control of AMPA receptor activities *in vivo*.



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## 1. Ionotropic glutamate receptors and AMPA receptor subtype

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). Glutamate acts through glutamate receptors, which are divided into ionotropic glutamate receptors (iGluRs)[1] and metabotropic glutamate receptors (mGluRs)[2]. iGluRs are ligand-gated ion channels, whereas mGluRs are G-protein-coupled receptors. iGluRs can be subdivided into three classes or subtypes after their prototypic exogenous ligands: *N*-methyl-D-aspartic acid (NMDA), (*R*, *S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) and kainic acid (KA), respectively[1]. iGluRs are indispensable for brain development and activities such as memory and learning. Dysregulation of these receptors by excessive receptor activities, known as excitotoxicity, and/or elevated expression has been implicated in a variety of neurological disorders and diseases, such as epilepsy, stroke, amyotrophic lateral sclerosis (ALS) and Alzheimer's disease[1]. Antagonists of iGluRs are therefore drug candidates for potential treatment of these neurological diseases. For example, using inhibitors to block excessive AMPA receptor activity has been shown to be neuroprotective in various disease settings[3], such as ischemia-induced cell death[4], cocaine withdrawal[5] and partial-onset seizures[6] (by perampanel, an FDA approved drug branded as "Fycompa").

Among the three subtypes of iGluRs, AMPA receptors are known to mediate fast excitatory neurotransmission in CNS. Each of the AMPA receptor subunits, GluA1–4 (or previously named as GluR1–4)[7], can form homomeric channels by itself or assemble into heteromeric channels with other subunits in a tetrameric assembly[8]. AMPA receptors are post-transcriptionally modified[9,10] by alternative splicing[11] and RNA editing[12]. RNA splicing and editing are developmentally regulated, and generate additional, functionally different receptors[13–15].

## 2. AMPA receptor antagonists

Over the past two decades, there has been a significant progress in developing small-molecule antagonists targeting AMPA receptors. NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione), a classic competitive antagonist[16], came out in late 1980s. Since then, additional competitive antagonists based on the quinoxaline template have been made to improve potency, selectivity and water solubility at the molecular level[17,18]. However, most, if not all, of these competitive antagonists show cross activity with kainate receptors (see reviews in Refs. 3 and 13). This may not be surprising given that competitive antagonists bind to the same site or "orthosteric" site to which endogenous ligand glutamate binds.

A group of antagonists that are more selective towards AMPA receptors are 2,3-benzodiazepine derivatives, also known as GYKI compounds[3,19]. The development of 2,3-BDZs has attracted more attention, although other groups or non-2,3-BDZ types of non-competitive antagonists were also synthesized, such as phthalazine derivatives[20]. Since the first 2,3-BDZ or GYKI 52466 appeared more than two decades ago[21], there have been hundreds of compounds publically reported. Mostly through radio–ligand binding experiments, these compounds are thought to be non-competitive inhibitors. Mechanistically, noncompetitive antagonists are considered better suited for a more selective blockade of AMPA receptors, because noncompetitive antagonists bind to a regulatory site(s) distinct to the agonist site and their actions should not depend on the concentration of an agonist. It should be pointed out that all of these small-molecule compounds are typically drug-like and amenable to chemical optimization for oral bioavailability and favorable pharmacokinetic properties.

## 3. Rapid kinetic characterization of mechanism of inhibition of AMPA receptors and their antagonists

To develop better regulatory molecules like antagonists, we have to do kinetic studies to gain a better understanding of the mechanism of inhibition of AMPA receptors. Measuring the kinetic rate constants of AMPA receptor channel opening has turned out to be quite challenging. First, a measurement must be compatible with a cellular system where the glutamate-induced current can be followed. This is because AMPA receptors are transmembrane proteins, and upon binding to glutamate, small cations like $Na^+$ and $K^+$ (sometime with $Ca^{2+}$ as well) permeate through the channel, thereby generating electrical current—this is the only direct, functional signal that can be followed. To date, an AMPA receptor channel can be conveniently expressed in a heterologous expression system, such as human embryonic kidney (HEK)-293 cells, to be studied with the use of an electrophysiological recording technique[22]. Second, the rising phase of the macroscopic current (from a single cell that expresses AMPA receptors) is fast in that an AMPA channel opens in the microsecond (μs) time region[23]; on the millisecond (ms) time scale, however, the channel becomes desensitized or transiently inactivated, while glutamate remains bound[24]. These intrinsic, fast channel gating properties of AMPA receptors have made it extremely difficult to apply solution exchange or single-channel recording technique to a study of the rate of channel opening.

To overcome relatively slow diffusion and mixing of glutamate with AMPA receptors on a cell surface, delivered by commonly used fast solution exchange techniques, we have developed a laser-pulse photolysis technique with the use of a photolabile, but biologically inert, glutamate precursor or caged glutamate (*γ-O*-(*α*-carboxy-2-nitrobenzyl glutamate)[25]. Upon photolysis, the caged glutamate liberates glutamate with a $t_{1/2}$ of $\sim 30\ \mu s$[26]. The ensemble channel-opening events from individual receptors on the cell surface can be synchronized, and the glutamate-induced current can be rapidly measured using whole-cell recording[22]. As a result, the channel-opening phase can be measured within the μs time region or before the channel-desensitization reaction that occurs in the ms time domain. Using this technique, we are able to characterize the effect of an inhibitor on the channel-opening ($k_{op}$) and channel-closing ($k_{cl}$) rate constants of an AMPA receptor for elucidation of the mechanism of inhibition[25]. For instance, an open-channel blocker will affect only $k_{cl}$ but not $k_{op}$, whereas a noncompetitive inhibitor will affect both $k_{op}$ and $k_{cl}$ [25,26]. The major difference between radio-ligand binding study of a mechanism and ours is that the former is an equilibrium study where a receptor is already desensitized if a radiolabeled agonist is used. If a radiolabeled antagonist [27] is used in the absence of agonist, one can only characterize the inhibitory property of an antagonist with the closed-channel state, but not the open state, and the mode of action of that antagonist must be *a priori*.

## 4. Mechanistic insights: general properties of noncompetitive sites on GluA2

Using rapid kinetic techniques, including the laser-pulse photolysis technique, to time the channel activation process, we have investigated a series of 2,3-BDZs[25,26]. Some important structure–

activity features have been revealed. (a) Our studies show that 2,3-BDZs are noncompetitive inhibitors with variable mechanistic features associated with their sites of interaction on AMPA receptors. By a double-inhibition experiment, we found that there are three noncompetitive sites on an AMPA receptor (Fig. 1). Compounds with a 4-methyl group on the 2,3-benzodiazepine ring bind to the "M" site (red color)[28]; those with 4-carbonyl group bind to the "O" site (blue color)[26,29], whereas those with the 7,8-ethylenedioxy ring (green color), instead of 7,8-methylenedioxy ring like "O" site compounds, bind to the "E" site[30]. There is no allosteric interaction among these sites. (b) Interestingly, the inhibitors that bind to the "M" or the "E" site prefer to inhibit the closed-channel state of a receptor, whereas those that bind to the "O" site prefer to inhibit the open-channel state. (c) These sites have different properties. For instance, acylation of compounds at the *N*-3 position of the 2,3-benzodiazepine ring for the "M" site increases potency, but decreases the potency for those that bind to the "O" site with the same acylating group[28,29]. (d) The most potent 2,3-BDZs in our series are in the "M" site group[31]. Some of these compounds have the highest potency in literature. Furthermore, the "M" is steroselective, which prefers compounds with the *R* configuration for the C-4 methyl group[25]. Chirality can introduce higher selectivity, and possible specificity as well for controlling the target function *in vivo* (the "M" site is the only site that is chiral).

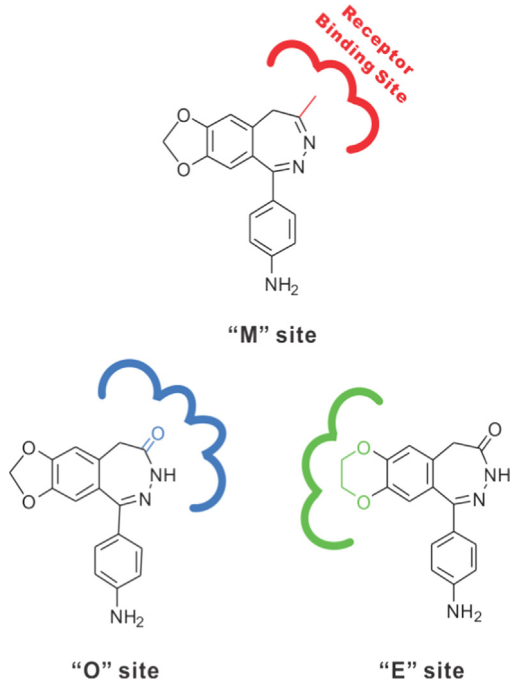


**Figure 1** A schematic representation of the three noncompetitive binding sites on the an AMPA receptor for 2,3-benzodiazepine compounds. Shown in color are three key chemical groups that interact with partially drawn receptors, which define their sites of binding on the receptor. The presence of both the C-4 methyl and the 7,8-methylenedioxy moiety in the 2,3-benzodiazepine structures defines the "M" site (red color). Shown is GYKI 52466 bound to the "M" site. The interaction between the receptor and an inhibitor at the "M" site is stereoselective in that the "M" site preferentially recognizes and accommodates those compounds with a C-4 methyl group in the *R* configuration. Replacing the C-4 methyl with a carbonyl group results in 2,3-benzodiazepin-4-ones that bind to the "O" site (blue color). Shown here is 1-(4-aminophenyl)-3,5-dihydro-7,8-methylenedioxy-4 *H*-2,3-benzodiazepin-4-one bound to the "O" site. Increase of the 7,8-methylenedioxy ring size into the 7,8-ethylenedioxy one renders the resulting compound binding to the "E" site (green color). Shown here is 1-(4-aminophenyl)-3,5-dihydro-7,8-ethylenedioxy-4 *H*-2,3-benzodiazepin-4-one bound to the "E" site.

## 5. Mechanistic insights: general properties of 2,3-BDZs

Those 2,3-BDZs that are inhibitors of AMPA receptors showed no appreciable cross activity on either NMDA or kainate receptors. However, there are only four compounds among those that we have investigated thus far that also show significant activity on GluA3 or GluA4[26,28–31]. However, there exists a trend that if a compound is inhibitory on AMPA receptors, the potency is generally in the rank order of GluA2 ~ GluA1 >> GluA3 ~ GluA4[25] (see the exception below with one special type of 2,3-BDZ). Furthermore, most of inhibitors have a slightly higher potency on GluA2 than on GluA1[32]. These results suggest that the noncompetitive sites for these compounds on GluA1 and GluA2 are similar but are different on either GluA3 or GluA4. This conclusion is based on the assumption that these 2,3-BDZs do not change their structures or conformations when binding from one AMPA receptor to the other. As such, a compound acts as a "structural probe" to measure the similarity or difference in the same type of the noncompetitive site, like the "M" site, among the four AMPA receptor subunits. It should be noted that our study did not reveal the locations of these sites. Furthermore, there is no structural data on noncompetitive sites on AMPA receptors in literature.

Talampanel is a good example to illustrate that our results are useful. Talampanel is perhaps the best known 2,3-BDZ[33,34]. Recently, a much anticipated phase II clinical trial of this compound for ALS (sponsored by Teva Pharmaceutical Industries) ended without statistically significant efficacy. At the molecular level, very few mechanistic details for talampanel were previously known[3,33]. We found that talampanel inhibits GluA1 and GluA2, albeit only weakly, but virtually does not inhibit either GluA3 or GluA4[25]. Yet, spinal motor neurons that undergo neurodegeneration, the pathogenic hallmark of ALS, actually express GluA3 and GluA4, in addition to GluA1 and GluA2[35]. Thus, if the molecular properties of both the AMPA receptors and talampanel are used for selecting a single inhibitor as a drug candidate for an ALS clinical testing, talampanel is not ideal.

We have recently reported 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 either 2,3-benzodiazepine scaffold alone[31] or talampanel[25]. This result suggests that the "side pocket" surrounding the "M" site on the receptor is able to accommodate a larger size of the *N*-3 derivative such as a thiadiazole. One such compound we characterized[31] is shown to inhibit GluA1, 2Q, 3 and 4 AMPA receptors with roughly a $K_I$ value of about 0.5 μmol/L. It also inhibits equally strongly both the closed-channel and the open-channel forms of these AMPA receptors.

## 6. Mechanism-based design of potent 2,3-BDZs

The mechanistic clues we have learned so far can be used to design more potent, site-specific 2,3-BDZs. Some of the superior mechanistic features from one site may be also used to design 2,3-BDZs that bind to other sites. Here are some predictions and ideas.

First, our studies thus far have shown that the majority of the potent 2,3-BDZs are in the "M" site group and thiadiazolyl-2,3-BDZs are the most potent of all[31]. We therefore hypothesize that a thiadiazole moiety occupies more fully the side pocket of the "M" site, thereby generating a stronger, multivalent interaction with the receptor site. Thiadiazole is a heterocyclic ring system, and chemical modifications are expected to yield a large group of thiadiazolyl derivatives from which more potent inhibitors should be found.

Second, the same thiadiazole scaffold can be also explored for the synthesis of "E" site compounds. In addition, the type 2 pharmacophore model[36] should be refined along with three distinct noncompetitive sites on AMPA receptors (note that the "O" site and "E" site compounds differ by a single atom enlargement of the 7,8-methylenedioxy ring). The impact of the increase in ring size from a dioxole to a dioxane moiety on the binding site for the resulting compounds might reflect the intimate molecular contact between the 7,8-position and the receptor site. Therefore, a modulation of the dioxole ring, *i.e.*, the methylene group of the methylenedioxy function, may be used to explore undiscovered, noncompetitive binding sites as well. For example, the 7,8-methylenedioxy moiety can be replaced with 7,8-ethylidenedioxy or 7,8-isopropylidenedioxy moieties. Additionally, this ring enlargement should be explored with the "M" site compounds.

Third, kinetic evidence on both the loosely bound receptor–inhibitor intermediate and the structure–activity relationship of the "M" and "E" site compounds suggests that the closed-channel state is more flexible and more accommodating to 2,3-BDZs. Targeting the closed-channel state by designing compounds that bind to the "M" site and the "E" site is therefore a way to develop more potent compounds. In this context, thiadiazolyl-2,3-BDZs may be the best antagonists in that their ability of blocking AMPA receptors is independent of agonist concentrations[31].

## 7. Developing selective antagonists of AMPA receptors

Improving selectivity (especially towards AMPA receptor subunits) may be the most significant challenge of all. This challenge can be illustrated in the following way.

Absence of high selectivity towards a disease-linked receptor or site requires the use of a higher dosage of the drug to achieve a sufficient local concentration for efficacy. Yet higher dosing promotes the occurrence of nonspecific toxicity and other adverse side effects, causing collateral toxicity to healthy cells/tissues[37]. Toxicity and a lack of efficacy are the two most common factors that drug candidates fail[38]. This problem becomes especially appreciable when competitive antagonists are used as drugs. Competitive antagonists must have a sufficiently high affinity for the target to displace an endogenous ligand from the same binding site, and must also have a sufficiently high concentration near the vicinity of the target to be effective, especially when the concentration of ligand or agonist is high. This means a higher dose must be administered and maintained. In addition, if the target is a member of isoform family and the ligand activates all isoforms, the use of a competitive antagonist generally does not lead to selective control of the target.

High potency and high selectivity are two very desirable properties of an inhibitor. Between the two, however, a higher selectivity of an antagonist is perhaps more important than potency in the context of controlling the target activity. This is especially true for noncompetitive antagonists. For example, an apparent potency can be augmented by the use of multiple but selective drug molecules, provided they bind to different sites. In this scenario, an additive potency will be observed. If there is a positive allosterism between or among sites, the synergistic antagonism would be even stronger. In fact, the combinatorial use of different, but multiple drug molecules that bind to different noncompetitive sites of the same target, may actually provide an additional mechanism to control a target more selectively.

On the other hand, the reward of developing highly selective antagonists is also significant. In this context, finding subunit-selective antagonists for AMPA receptors should be particularly ideal. If there are multiple antagonists with varying degrees of subunit selectivity, a higher selectivity to target complex AMPA receptors in varying compositions and differential expression patterns *in vivo* will be possible. If multiple, separate subunits (or genes) are linked to a disease, a unique combination of single-subunit inhibitors can be used. These subunit-selective inhibitors provide a means for us to mix and match for a more quantitative and tighter control of a target function *in vivo*.

The use of a single, subunit-selective antagonist of AMPA receptors as a drug candidate could be also therapeutically beneficial, because there is evidence of unique AMPA receptor subunit involvement in various neurological conditions. For example, global ischemia significantly downregulates GluA2, but not other subunits, and such a downregulation is specific in vulnerable CA1 pyramidal neurons, which are specifically subject to ischemia-induced neurodegeneration[39]. Seizure downregulates GluA2 in CA1 and CA3 pyramidal neurons before the onset of neuronal death[40,41]. Significant RNA editing defect in GluA2 has been found in spinal motor neurons of ALS patients; in turn, $Ca^{2+}$-permeable GluA2Q isoform is generated[42]. In contrast, the editing efficiency in normal human control is near 100%[42]. A study of post-mortem samples from multiple sclerosis patients shows that GluA3 and GluA4 are expressed in astrocytes and MS active plaques and GluA1 is upregulated[43]. All these examples illustrate that one or some, but not all, of the AMPA receptor subunits in specific tissue regions are often involved in a disease. Therefore, blockade of excessive AMPA receptor activity and/or abnormal expression would be better achieved by selectively inhibiting those subunits or channels formed by these subunits. A generalized, promiscuous blockade of AMPA receptors would conceivably interfere with the normal function of AMPA receptors and cause side effects.

To date, none of the 2,3-BDZs are subunit-selective. However, the fact that the *N*-3 acylated "M" site compounds (or non-thiadiazole derivatives) prefer GluA1 and GluA2 subunits suggests that developing subunit-preferred 2,3-BDZs should be possible. In fact, a GluA1/2-preferred inhibitor can be useful in targeting GluA1/2 complex channels, given that the GluA1/2 AMPA receptor is a major receptor population found in mature hippocampus[44]. Furthermore, GluA1-containing AMPA receptors are driven into synapses by long-term potentiation (LTP) or calcium/calmodulin-dependent protein kinase II (CaMKII) activity[45]. In pursuing subunit-selective antagonists, we have explored the use of systematic evolution of ligands by exponential enrichment (SELEX) to "breed" RNA inhibitors or aptamers for AMPA receptors from a RNA library (*i.e.*, $\sim 10^{15}$ sequences)[46]. We have indeed isolated a GluA2-selective antagonist or an RNA aptamer[46]. This result demonstrated the potential of using SELEX to generate a novel class of RNA-based, subunit-selective AMPA receptor antagonists, alternative to small-molecule inhibitors. It should be also noted that RNA molecules are water soluble, and are supposedly less diffusible (chemically modified RNAs) after they are delivered locally. However, unlike small-molecule compounds such as 2,3-BDZs,

RNA aptamers cannot penetrate the blood–brain barrier, and are thus required to be delivered to CNS using other means, such as intrathecal injection.

Additional challenges and opportunities lie ahead. Solving structures of each of the AMPA receptor subunits to offer noncompetitive site information will help design better inhibitors. There are other types of AMPA receptor antagonists that are structurally different from 2,3-BDZs. One such compound is perampanel; it is a drug recently approved by FDA for treatment of partial-onset seizures[6]. Finding additional noncompetitive sites on AMPA receptors potentially offers additional opportunities for design of highly selective, potent inhibitors as efficacious drug candidates with little or no side effects.

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