Published in final edited form as:

Neuropharmacology. 2013 January ; 64(1): 45–52. doi:10.1016/j.neuropharm.2012.06.008.

FUNCTIONAL ANALYSIS OF A NOVEL POSITIVE ALLOSTERIC MODULATOR OF AMPA RECEPTORS DERIVED FROM A STRUCTURE-BASED DRUG DESIGN STRATEGY

Jonathan E. Harms^a, Morris Benveniste^b, John K. F. Maclean^c, Kathryn M. Partin^a, and Craig Jamieson^d

Jonathan E. Harms: jonathan.harms@colostate.edu; Morris Benveniste: mbenvensiste@msm.edu; John K. F. Maclean: john.maclea@merck.com; Craig Jamieson: craig.jamieson@strath.ac.uk

^aDepartment of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523-1617

^bNeuroscience Institute, Morehouse School of Medicine, 720 Westview Drive SW, Atlanta, GA 30310-1495

^cMerck Research Laboratories, 33 Avenue Louis Pasteur, Boston, MA 02115

^dDepartment of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow, G1 1XL

Abstract

Positive allosteric modulators of α -amino-3-hydroxy-5-methyl-isoxazole-propionic acid (AMPA) receptors facilitate synaptic plasticity and can improve various forms of learning and memory. These modulators show promise as therapeutic agents for the treatment of neurological disorders such as schizophrenia, ADHD, and mental depression. Three classes of positive modulator, the benzamides, the thiadiazides, and the biarylsulfonamides differentially occupy a solvent accessible binding pocket at the interface between the two subunits that form the AMPA receptor ligand-binding pocket. Here, we describe the electrophysiological properties of a new chemotype derived from a structure-based drug design strategy (SBDD), which makes similar receptor interactions compared to previously reported classes of modulator. This pyrazole amide derivative, JAMI1001A, with a promising developability profile, efficaciously modulates AMPA receptor deactivation and desensitization of both flip and flop receptor isoforms.

1. Introduction

Glutamate is the primary excitatory neurotransmitter in the mammalian CNS. One of glutamate's primary target receptors, the AMPA family of ion channels, is responsible for mediating neural processes involved in learning and memory (reviewed in (Kessels and Malinow, 2009; Traynelis et al., 2010)). Furthermore, it has been shown that these processes, including synaptic plasticity and long-term potentiation (LTP), can be enhanced by modulatory drugs acting directly on AMPA receptors (Black, 2005; Ward and Harries, 2010). Because of such positive effects, AMPA receptors and their allosteric modulators

© 2012 Elsevier Ltd. All rights reserved.

Corresponding author: Kathryn M. Partin, Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523-1617; 970-491-1563; Kathy.partin@colostate.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

represent key targets for treatment of cognitive disorders, ranging from Alzheimer's disease and Parkinson's disease to schizophrenia and ADHD (Morrow et al., 2006).

AMPA receptors are hetero-tetrameric membrane proteins, composed of various combinations of GluA1, 2, 3 and 4 subunits. These subunits have a large extracellular domain, a transmembrane domain that gates mono- and divalent cations, and a cytoplasmic domain that encodes post-translational modification and scaffolding sites (Traynelis et al., 2010). The extracellular region of the channel contains an N-terminal domain and a ligand binding core (LBC) in each subunit (reviewed in Mayer, 2011). Both the N-terminal domain and LBC have intrasubunit associations such that the four subunits form a dimer of dimers in these regions (Sobolevsky et al., 2009). Both agonist and positive allosteric modulators bind to amino acids contained in this LBC (Armstrong and Gouaux, 2000; Sun et al., 2002). The LBC is formed from an upper lobe, domain 1 (D1), and a lower lobe, domain 2 (D2), which are brought together when agonist binds within the cleft formed between the domains (referred to as a "clamshell"). D1 and D2 are connected by "hinge" residues that are thought to impart stability upon the closed-cleft, agonist-bound conformation. Once agonist is bound, the channel will likely open and then may desensitize or close. Desensitization putatively results from a structural rearrangement of the interface between the paired LBCs of different subunits. Alternatively, upon brief exposure to glutamate, the channel may deactivate, such that the LBC cleft reopens and glutamate dissociates.

Positive modulators bind to the LBC, within a water-accessible cleft that lies at the interface between two protomers and is formed in part from the clamshell hinge residues that connect D1 and D2 (Ahmed et al., 2010; Jin et al., 2005; Sun et al., 2002; Timm et al., 2011). This allosteric modulatory site has been described as five overlapping subsites: the central subsite, A, lies across the axis of symmetry between the protomers and is formed by hinge residues (connecting D1 and D2 of one protomer), including Pro494, Ser497, and Ser729 (Ptak et al., 2009). Subsite B is an exposed, hydrophilic pocket formed by residues Tyr424, Phe495, Ser497, Lys763, and Ser729, whereas subsite C is a deep, hydrophobic pocket lined by residues Ile481, Lys493, and Leu751. Subsites B and C are self-contained within each protomer such that there are two B subsites and two C subsites per dimer, whereas there is only one, shared A subsite. It has been suggested that differential occupancy of these subsites by the various classes of positive modulator determines their different mechanisms of action. The classes of positive modulator compounds that have been characterized include: the benzothiadiazides (cyclothiazide, IDRA21, BPAM-97), the benzamides (aniracetam and CX614), the benzoxainones, and the biarylsulfonamides (LY404187, CMPDA, CMPDB; Fernandez et al., 2006; Jin et al., 2005; Krintel et al., 2012; Mueller et al., 2011; Sun et al., 2002; Timm et al., 2011). Although new and higher affinity compounds are being identified at a rapid pace, there appears to be a significant lag in the ability to translate novel compounds tested *in vitro* to viable targets for clinical trials (Lynch et al., 2011; Swanson, 2009; Ward et al., 2010) with only a few AMPA receptor positive modulators making it into clinical trials in the U.S. (CX516, Lynch et al., 1997; CX717, Wesensten et al., 2007; <http://clinicaltrials.gov/ct2/results?term=AMPAkine>).

In an effort to expedite the development of novel positive AMPA receptor modulators, a unique structure-based drug design (SBDD) approach in combination with a screening-led hit identification campaign has been used to discover compounds with positive modulatory effects and optimize them for use as potential therapeutics (Jamieson et al., 2010a; Jamieson et al., 2010b; Jamieson et al., 2011). The benefits of such a design strategy allow for the rapid identification of new chemotypes and their expedient optimization into relevant drug-like compounds. In this study, we confirm SBDD as an effective method for designing a targeted lead compound through the study of the pyrazole amide derivative JAMI1001A ((2-(2-(4-(hydroxymethyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl)acetamido)-4,5,6,7

tetrahydrobenzo[b]thiophene-3-carboxamide). Further, we reveal surprising biophysical properties of JAMI1001A that challenge the current dogma of the consequence on function of occupancy of specific subsites within the positive allosteric modulatory site.

2. Materials and Methods

2.1. Compound Synthesis

The pyrazole amide derivative JAMI1001A (**Compound 2** from Jamieson, et al., 2011) was synthesized using the synthetic approaches outlined in our earlier publication (Jamieson et al., 2010b). Briefly, (3-(trifluoromethyl)-1*H*-pyrazol-4-yl)methanol was treated with potassium *tert*-butoxide (1.2 eq) in DMF for 30 minutes before 2-(2-chloroacetamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxamide (1 eq) was added. The reaction mixture was heated to 60 °C for 4 hours before being concentrated to dryness and the residue purified by flash column chromatography, eluting with 40% ethyl acetate/hexane to furnish the target compound as a white solid.

2.2. X-ray crystallography

Crystals were grown as described by Armstrong et al. (1998) and subsequently by Jamieson et al. (2010a). Co-crystals were prepared by soaking JAMI1001A (100 mM) with seed crystals for 24h prior to isolation and data collection. Coordinates and structure factors have been deposited in the Protein Data Bank for the complex of JAMI1001A with the AMPA LBC (4FAT.pdb).

2.3 AMPA receptor recombinant cDNAs

The flip and flop isoforms of rat GluA2 cDNAs were expressed in pRK, a CMV expression vector, fused in-frame with yellow fluorescent protein. cDNAs were a gift of Dr. Peter Seeburg (University of Heidelberg, Germany). “WT” GluA2 cDNA was produced from the original plasmids, into which the pore mutation (R₆₀₇Q) was made, using site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit, Stratagene; La Jolla, CA). Mutant receptors conduct current robustly and have an inwardly rectifying current-voltage relationship, whereas receptors with arginine at position 607 express currents that are too small to measure with conventional patch-clamp electrophysiology (Hume et al., 1991; Verdoorn et al., 1991).

2.4. HEK293 cell culture and transient transfection

Passages 38–45 human embryonic kidney 293 (HEK293) cells (CRL 1573; American Type Culture Collection; Manassas, VA) were cultured in 35mm polystyrene dishes (Becton-Dickinson and Company; Lincoln Park, NJ). Cells were transiently transfected using PolyJet reagent (SignaGen Laboratories; Gaithersburg, MD) with yellow-fluorescent-protein (YFP) fused in-frame to the C-terminus of wild type GluA2 flip or wild type GluA2 flop AMPA receptor DNA (1 µg/dish). Media was changed 4–5 hours post transfection and NBQX was added to a final concentration of 10–20 µM to prevent toxicity.

2.5. Patch-clamp electrophysiology

Currents were recorded 1–2 days after transfection from cells expressing a moderate level of fluorescence from the YFP tag. Outside-out membrane patches were held under voltage-clamp at –60 mV using an Axopatch 200B amplifier (Molecular Devices; Union City, CA). Patchmaster software (version 2.43; HEKA Instruments Inc.; Bellmore, NY) controlled data acquisition and movement of a two barrel flowpipe perfusion system driven by a piezo-electric device (Burleigh Instruments; Fishers, NY). Micropipettes (TW150F; 2–5 MΩ; World Precision Instruments; Sarasota, FL) contained the following intracellular solution (in

mM): 135 CsCl, 10 CsF, 10 HEPES, 5 Cs₄BAPTA, 1 MgCl₂, and 0.5 CaCl₂, pH 7.2. Patches were perfused at 0.2 mL/min with solutions emitted from a two-barrel flow pipe made with theta tubing (BT150-10; Sutter Instruments; Novato, CA). One barrel contained control solution (in mM): 145 NaCl, 5.4 KCl, 5 HEPES, 1 MgCl₂, 1.8 CaCl₂, with 0.01 mg/mL phenol red, pH 7.3. The other barrel contained L-glutamate (10 mM) dissolved in the control solution. For drug studies, each barrel additionally contained either 100 μ M cyclothiazide (CTZ; Tocris Bioscience; Bristol, UK), 100 μ M CX614 (Cortex Pharmaceuticals, Inc; Irvine, CA), or 100 μ M JAMI1001A. CTZ and CX614 were dissolved in DMSO. JAMI1001A was dissolved in control solution. After attaining whole-cell voltage clamp, outside-out patches were pulled from cells, raised off the dish and positioned near the interface between the glutamate-free and glutamate-containing solutions of the flowpipe. Agonist applications were achieved by stepping into and out of the glutamate containing solution for either 1 or 500 ms to test for deactivation and desensitization, respectively. Solution exchange was measured for each patch by measurement of junction potentials after obliterating the patch at the end of the experiment. Solution exchange times were approximately 500 μ s. Responses were digitized at 20 kHz, and stored on an iMac computer (Apple, Inc.; Cupertino, CA) using an ITC-16 interface (HEKA Instruments Inc.; Bellmore, NY) connected through a USB-16 adapter (HEKA Instruments Inc.; Bellmore, NY).

2.6. Data analysis

IGOR Pro 6.2 (Wavemetrics; Oswego, OR) software was used for data analysis. Traces were averaged (4–10 traces per sweep) and a single exponential was fit to the trace. I_{Peak} was calculated using an average of three points. $I_{\text{SS}}/I_{\text{Peak}}$ was calculated using an averaged steady state current (SS).

2.7. Kinetic simulations

Simulations of currents under voltage clamp to an AMPA receptor model were performed using code originally written by John Clements (Benveniste et al., 1990), revised and converted by M.B. to an Igor Pro XOP. Receptor state occupancies at each time point were determined numerically. The change in each state occupancy was calculated according to first order reaction rate kinetics for transitions into and out of each state, as detailed in Benveniste *et al.* (1990). This was done iteratively at least 20 times per time point. With the present model, flip and flop differences were largely replicated by simply changing the rate of onset of desensitization by 10-fold. Simulations, fitting and related analyses were run on a Macintosh MacBook Pro computer utilizing Igor Pro 6.2.

3. Results

3.1. Structure of JAMI1001A

JAMI1001A is a pyrazole amide system derived from lead “**Compound 1**” as described in (Jamieson et al., 2011). JAMI1001A is a U-shaped molecule of 402 Da molecular weight (Figure 1) that contains a hydrophobic trifluoromethyl group attached to the 3-position of a pyrazole ring (shown in green), and a hydrophobic tetrahydrobenzothiophene moiety (shown in blue). A hydrophilic carboxamide (red) extends from the 3-position of the tetrahydrobenzothiophene motif. The extended distal structures are connected by a linear acetamide chain that forms the bottom of the “U” shape. The structure of JAMI1001A differs from other positive allosteric modulators of AMPA receptors such as the benzamide or sulfonamide systems in its lack of central aromatic rings.

3.2. X-ray crystallography structure of JAMI1001A bound to the AMPA receptor GluA2 S1S2 domain

JAMI1001A lies at the dimer interface between two ligand-binding cores (Figure 2). The long axis of the molecule lies perpendicular to the axis of symmetry. The interactions between the compound and the GluA2 LBC are predominately mediated by hydrophobic contacts, with most of these contacts lying at the distal ends of the compound facilitated by the trifluoromethyl system on the pyrazole ring and the cycloalkane ring of the tetrahydrobenzothiophene, respectively (Figure 2A). Specific interactions between the LBC and the ligand which can be discerned are made by a hydrogen bond between the pendant hydroxyl at the 4-position of the pyrazole motif and the main chain of Ser729 and side chain of Ser754; or with the main chain of Pro494 (Figure 2B). An additional interaction exists between the carboxamide of the cycloalkane ring with the sidechain of Ser497.

A recent structural study suggests that relative occupancy of the modulator-binding pocket sub-domains influences the mechanisms of action of allosteric modulators (Ptak et al., 2009). For instance, the central subsite, A, lies across the axis of symmetry between the protomers and is occupied by the planar rings of CX614 (Figure 2D), and other biarylsulfonamide compounds (Timm et al., 2011). Hinge residues within subsite A (connecting domain 1 and domain 2 of one protomer), particularly Pro494, Ser497, and Ser729 form hydrogen bonds with these compounds and are believed to contribute to stabilization of closed clamshell conformations (Jin et al., 2005). Subsite B, or B' on the opposing protomer, is an exposed, hydrophilic pocket formed by residues Tyr424, Phe495, Ser497, Lys763, and Ser729, and interacts with the sulfonamide groups of CTZ (Figure 2C; Sun et al., 2002). The norbornenyl group of CTZ inserts into the hydrophobic pocket of subsite C, which is lined by residues Ile481, Lys493, and Leu751.

The prototypical benzamide, CX614, which occupies only subsite A, efficaciously modulates channel deactivation and desensitization (Figure 2D). Cyclothiazide, which occupies subsites B, B', C and C', with only partial occupancy of subsite A, has little effect on channel deactivation, with a robust effect on channel desensitization (of the flip isoform). Because structural studies have indicated that occupancy of subsites B and C may impart more efficacious allosteric modulation (Ptak et al., 2009; Timm et al., 2011), it is interesting to note that the central portion of JAMI1001A occupies subsite A, and the distal ends of the compound occupy subsites C and C'. Ser729, which demarcates subsites B and C, also makes direct contact with JAMI1001. Based on the structural model of the positive allosteric modulator binding site, one might predict that the critical interactions between the distal portions of JAMI1001A – the tetrahydrobenzothiophene domain that overlaps with the norbornenyl ring of cyclothiazide at subsite C, and the trifluoromethyl domain that overlaps with the sulfonamides at subsite C' - could potentially contribute to the strong modulation of both deactivation and desensitization.

The pendant hydroxyl of the distal pyrazole ring of JAMI1001A interacts with the sidechain hydroxyl of Ser754, the alternatively spliced residue underlying pharmacological selectivity of other positive allosteric modulators with splice isoform differences (Kessler et al., 2000; Ahmed et al., 2010). The flip isoform of the LBC was crystalized in this study; however, if the Ser754 residue is mutated *in silico*, the amide of Asn754 is also predicted to interact with the same hydroxyl of JAMI1001A. This would predict a lack of isoform selectivity of JAMI1001A.

3.3 JAMI1001A is an efficacious modulator of GluA2 deactivation and desensitization

Although previous studies had characterized some of the chemical and biological properties of the new chemotype (Jamieson et al., 2010a; Jamieson et al., 2010b), insight into its

molecular mechanism of action requires higher resolution electrophysiological recordings of channel activity. Specifically, recording the response to a brief (1 ms) pulse of glutamate is thought to be a proxy for channel “deactivation”, a process that entails channel closing, the opening of the cleft between the upper and lower domains of the ion channel that forms the agonist-binding “clamshell”, and dissociation of glutamate from its binding site. This process of terminating a response to glutamate is predicated on a synaptic response in which glutamate is only briefly presented to postsynaptic AMPA receptors. Desensitization is the process by which the two ligand binding cores forming a dimer dramatically rearrange their interactions, putatively disrupting non-bonded interactions to relieve strain induced by channel opening, thereby uncoupling the occupied agonist binding site from the channel pore in the lipid bilayer (Sun et al., 2002). This process is recapitulated by applying a prolonged (500 ms) pulse of glutamate to induce receptor desensitization. The effects of JAMI1001A on channel deactivation and desensitization were assessed by measuring current reduction following 1 or 500 ms pulses of glutamate, in the absence and presence of modulator, measured on outside-out membrane patches from HEK293 cells transiently expressing the flip and flop isoforms of GluA2.

As previously published, CTZ has little effect on channel deactivation of either the flip or flop isoforms of AMPA receptors (Arai et al., 1996; Mitchell and Fleck, 2007; Partin et al., 1996), whereas CX614 slows deactivation significantly of the flop but not the GluA2 flip isoform (Jin et al., 2005). JAMI1001A slows channel deactivation of both GluA2 isoforms by approximately 1.5-fold (Figures 3A and 4A). This deactivation modulation profile for JAMI1001A clearly differs from other known channel modulators in its lack of isoform selectivity. Because different populations of neurons express specific repertoires of subunits and splice isoforms (Geiger et al., 1995), resulting in neuron-specific efficacy of modulation (Xia and Arai, 2005), the physiological consequence of the lack of isoform specificity of the JAMI1001A compound may be quite important.

The lack of isoform selectivity of JAMI1001A was also apparent when measuring channel desensitization (Figure 3B). The ratio of the steady-state current to the initial peak current is a measure of the efficacy of modulation, such that a ss/pk ratio of one would indicate a complete inability of the channel to desensitize during a prolonged pulse of glutamate. JAMI1001A blocked desensitization to virtually the same extent in both the flip and flop isoforms (Figures 3B and 4B). This is consistent with structural data, discussed in Section 3.2, indicating that direct interactions exist between Ser754 and may exist between Asn754 and JAMI1001A.

3.4 Mechanism of action of JAMI1001A

In JAMI1001A, the absence of aromatic rings that occupy subsite A may have mechanistic implications. It has been hypothesized that the aromatic rings of aniracetam, CX614 and “CMPDA” or “CMPDB” (Jin et al., 2005; Timm et al., 2011) allow hydrophobic interactions that stabilize the hinges of the clamshell formed by the upper and lower lobes of the ligand binding core. In the present study, JAMI1001A has an acetamide linker which could potentially interact with the channel in the same way and, therefore, may modulate channel deactivation. Macroscopic rates of channel deactivation (the decay of the current after a brief pulse of agonist) can be influenced by allosteric modulation of different conformational transitions. For example, the decay is slowed by agonists that have a higher affinity (Kessler and Arai, 2006), thereby slowing agonist dissociation (K_{off}). Alternatively, the rate of decay can be slowed by a slowing of the channel closing (α). Finally, the rate of decay can be changed by slowing the entry into the desensitized state (Mitchell and Fleck, 2007; Partin et al., 1996), although the impact on deactivation by this mechanism is very modest (Timm et al., 2011).

Computational modeling of conformational transitions is useful in assessing potential mechanisms of action of allosteric modulators. Recent studies on two novel allosteric modulators of channel deactivation and desensitization, CMPDA and CMPDB, led to the conclusion that the slowing of channel deactivation arose from an independent mechanism rather than as a consequence of their actions on channel desensitization (Timm et al., 2011). Figure 5A and Table 1 show an analogous, albeit simplified, gating scheme that has been used in a computational simulation of electrophysiological currents (Figure 5B). Because of the simplified scheme, this model may not recapitulate all kinetic data acquired for GluA2. However, this model is the simplest scheme that can reproduce the given experimental data, where both modulator bound and modulator unbound states need to be considered. Although several different solutions for parameter values could be possible, our parameter space has been limited by approximating peak and steady state dose response curves for GluA2 in the absence of modulator, by approximating the macroscopic deactivation time constant in the absence and presence of modulator, and by approximating the kinetics of onset and recovery of desensitization in the absence and presence of modulator. We have also used more complicated models for other modulators and come to a similar conclusion, namely: more than one mechanism is being invoked for the dual effects on deactivation and desensitization (Timm et al, 2011).

In this model, the decays of deactivation and desensitization in the absence of modulator are 0.9 ms and 1.4 ms, respectively. Slowing $k'_{glu\ off}$ by two-fold to simulate the effects of 10 μ M JAMI1001A slows the rate of channel deactivation to 1.0 ms, less than JAMI1001A does experimentally, and has little effect on channel desensitization. Similarly, slowing the onset of channel desensitization by 500-fold results in “blocking” desensitization, but also slows channel deactivation only to 1.1 ms. Thus, adjusting either channel rate independently does not simulate the results shown in Figure 3. However, adjusting both rates together, accurately replicates the physiological data (τ_{Deact} =1.6 ms; τ_{Des} =211 ms; I_{SS}/I_{Peak} =0.6). These simulations indicate that two independent processes of modulation are required to replicate JAMI1001A data (Figure 5B). Our data suggest that the hydrophobic contacts made by JAMI1001A on modulator pocket subsite A has an independent and efficacious impact on channel deactivation. One possibility is that the unique occupancy of subsite A *together* with subsite C, as well as its direct interaction with the main chain of Ser729 (which lies at the border between subsites B and C), accounts for the efficacious modulation of deactivation. This would have important consequences to future SBDD efforts in terms of designing new classes of modulator.

4. Discussion

Here, we describe the biophysical activity of a novel chemotype (pyrazole amide derivative) of AMPA receptor positive modulator. The compound was derived from a high throughput screen, followed by structure-based design using the available biostructural data (Jamieson et al., 2010a; Jamieson et al., 2010b; Jamieson et al., 2011). Optimization was driven by *in vitro* characterization of potency, solubility, selectivity and microsomal stability, allied with knowledge of how the ligands interacted with the receptor. *In vitro* studies were complemented with *in vivo* assays such as oral bioavailability and stability as measured by pharmacokinetics. These studies led to *in vivo* electrophysiological assessment of efficacy, measured primarily by looking at evoked single unit recordings in the hippocampus of rats, which had received an intravenous dose of a test compound. Although the optimization strategy led to the development of a novel chemotype, little information on the precise biophysical mechanism was known. Accordingly, our studies reinforce the value of the overall approach in finding new drugs to treat dysfunction of AMPA receptors which may contribute to mental illness and cognitive decline (reviewed in Carlson, 2012; Hashimoto, 2011; Lopez-Munoz and Alamo, 2011).

The most significant finding of the present study is that JAMI1001A somewhat unexpectedly modulates both channel deactivation and desensitization (Figures 3 and 4). Based on the overlap of the tetrahydrobenzothiophene domain of JAMI1001A with the norbornenyl group of cyclothiazide in the subsite C domain, and the absence of large hydrophobic substituents such as aromatic rings in subsite A of the allosteric binding pocket, it may have been predicted that JAMI1001A would impact desensitization with less effect on channel deactivation. Based on the structure, one might predict that JAMI1001A would prevent the rearrangement of the dimer interface necessary for the onset of channel desensitization but not affect deactivation, similar to cyclothiazide (Sun et al., 2002). However, this is not the case. Rather, through hydrogen bonds with residues in subsite A, and with only minimal hydrophobic interactions in subsite C to “stabilize” the closed cleft conformation, JAMI1001A is as effective as previously described modulators such as CX614 in blocking desensitization and slowing deactivation. Importantly, however, JAMI1001A lacks the isoform selectivity of CX614 (Figures 3 and 4). Recognizing that different brain regions express unique populations of AMPA receptors and their splice isoforms with differing efficacy of modulation (Geiger et al., 1995; Xia and Arai, 2005), the ability to synthesize drugs with predictable isoform efficacies may be a fundamentally important drug design goal for targetting modulators to neurological disease that globally affect the brain or preferentially affect specific brain regions.

Significant advances have been made in our understanding of the molecular basis through which positive allosteric modulators exert their biological effect on the AMPA receptor. These studies are complemented by an SBDD approach that provides a more extensive understanding of biophysical properties for novel compounds showing promising *in vivo* and *in vitro* properties, thereby possibly reducing the time from discovery to delivery as a therapeutic.

In conclusion, the synergistic combination of structural biology, receptor biophysics and modeling have led to a significant advance in our understanding of the specific mechanism of action of this novel class of AMPA receptor modulator. Detailed knowledge of how JAMI1001A and related compounds facilitate modulation of the receptor will undoubtedly have a significant impact on the design of additional new molecular entities derived through further evolution of the existing compounds.

Acknowledgments

NIHR01 MH064700 (KMP); University of Strathclyde John Anderson Research Leadership scheme and Tenovus Scotland (CJ); NINDS S11NS055883, (MB). The authors thank John Gieser for his contributions to the artwork. We thank Cortex Pharmaceuticals, Inc. for providing CX614.

Abbreviations

AMPA	alpha-amino-3-hydroxy-5-methyl-isoxazole-propionic acid
CTZ	cyclothiazide;2,4-benzothiadiazine-3-bicyclo[2.2.1]hept-5-en-2-yl-6-chloro-4-dihydro-2h-1
CX614	2H,3H,6aH-pyrrolidino(2,1-3',2')1,3-oxazino(6',5'-5,4)benzo(e)1,4-dioxan-10-one
JAMI1001A	2-(2-(4-(hydroxymethyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl)acetamido)-4,5,6,7 tetrahydrobenzo[b]thiophene-3-carboxamide)

References

- Ahmed AH, Ptak CP, Oswald RE. Molecular mechanism of flop selectivity and subsite recognition for an AMPA receptor allosteric modulator: structures of GluA2 and GluA3 in complexes with PEPA. *Biochemistry*. 2010; 49(13):2843–2850. [PubMed: 20199107]
- Arai A, Kessler M, Ambros-Ingerson J, Quan A, Yigeter E, Rogers G, Lynch G. Effects of a centrally active benzoylpyrrolidine drug on AMPA receptor kinetics. *Neuroscience*. 1996; 75:573–585. [PubMed: 8931020]
- Armstrong N, Gouaux E. Mechanisms for activation and antagonism of an AMPA-sensitive glutamate receptor: crystal structures of the GluR2 ligand binding core. *Neuron*. 2000; 28(1):165–181. [PubMed: 11086992]
- Armstrong N, Sun Y, Chen GQ, Gouaux E. Structure of a glutamate-receptor ligand-binding core in complex with kainate. *Nature*. 1998; 395:913–917. [PubMed: 9804426]
- Benveniste M, Clements J, Vyklicky L, Mayer ML. A kinetic analysis of the modulation of N-methyl-D-aspartic acid receptors by glycine in mouse cultured hippocampal neurones. *Journal of Physiology (London)*. 1990; 428:333–357. [PubMed: 2146385]
- Black MD. Therapeutic potential of positive AMPA modulators and their relationship to AMPA receptor subunits. A review of preclinical data. *Psychopharmacology (Berl)*. 2005; 179(1):154–163. [PubMed: 15672275]
- Carlson GC. Glutamate receptor dysfunction and drug targets across models of autism spectrum disorders. *Pharmacol Biochem Behav*. 2012; 100(4):850–854. [PubMed: 21315104]
- Fernandez MC, Castano A, Dominguez E, Escribano A, Jiang D, Jimenez A, Hong E, Hornback WJ, Nisenbaum ES, Rankl N, Tromiczak E, Vaught G, Zarrinmayeh H, Zimmerman DM. A novel class of AMPA receptor allosteric modulators. Part 1: design, synthesis, and SAR of 3-aryl-4-cyano-5-substituted-heteroaryl-2-carboxylic acid derivatives. *Bioorg Med Chem Lett*. 2006; 16(19):5057–5061. [PubMed: 16879964]
- Geiger JRP, Melcher T, Koh DS, Sakmann B, Seeburg PH, Jonas P, Monyer H. Relative abundance of subunit mRNAs determines gating and Ca^{2+} permeability of AMPA receptors in principle neurons and interneurons in rat CNS. *Neuron*. 1995; 15:193–204. [PubMed: 7619522]
- Hald H, Ahring PK, Timmermann DB, Liljefors T, Gajhede M, Kastrup JS. Distinct structural features of cyclothiazide are responsible for effects on peak current amplitude and desensitization kinetics at iGluR2. *J Mol Biol*. 2009; 391(5):906–917. [PubMed: 19591837]
- Hashimoto K. The role of glutamate on the action of antidepressants. *Prog Neuropsychopharmacol Biol Psychiatry*. 2011; 35(7):1558–1568. [PubMed: 20600468]
- Hume RI, Dingledine R, Heinemann SF. Identification of a site in glutamate receptor subunits that controls calcium permeability. *Science*. 1991; 253:1028–1031. [PubMed: 1653450]
- Jamieson C, Basten S, Campbell RA, Cumming IA, Gillen KJ, Gillespie J, Kazemier B, Kiczun M, Lamont Y, Lyons AJ, Maclean JK, Moir EM, Morrow JA, Papakosta M, Rankovic Z, Smith L. A novel series of positive modulators of the AMPA receptor: discovery and structure based hit-to-lead studies. *Bioorg Med Chem Lett*. 2010a; 20(19):5753–5756. [PubMed: 20805031]
- Jamieson C, Campbell RA, Cumming IA, Gillen KJ, Gillespie J, Kazemier B, Kiczun M, Lamont Y, Lyons AJ, Maclean JK, Martin F, Moir EM, Morrow JA, Pantling J, Rankovic Z, Smith L. A novel series of positive modulators of the AMPA receptor: structure-based lead optimization. *Bioorg Med Chem Lett*. 2010b; 20(20):6072–6075. [PubMed: 20817521]
- Jamieson C, Maclean JK, Brown CI, Campbell RA, Gillen KJ, Gillespie J, Kazemier B, Kiczun M, Lamont Y, Lyons AJ, Moir EM, Morrow JA, Pantling J, Rankovic Z, Smith L. Structure based evolution of a novel series of positive modulators of the AMPA receptor. *Bioorg Med Chem Lett*. 2011
- Jin R, Clark S, Weeks AM, Judman JT, Gouaux E, Partin KM. Mechanism of positive allosteric modulators acting on AMPA receptors. *J Neurosci*. 2005; 25(39):9027–9036. [PubMed: 16192394]
- Kessels HW, Malinow R. Synaptic AMPA receptor plasticity and behavior. *Neuron*. 2009; 61(3):340–350. [PubMed: 19217372]

- Kessler M, Arai AC. Use of [3H]fluorowillardiine to study properties of AMPA receptor allosteric modulators. *Brain Res.* 2006; 1076(1):25–41. [PubMed: 16256076]
- Kessler M, Rogers G, Arai A. The norbornenyl moiety of cyclothiazide determines the preference for flip-flop variants of AMPA receptor subunits. *Neuroscience Letters.* 2000; 287:161–165. [PubMed: 10854736]
- Krintel C, Frydenvang K, Olsen L, Kristensen MT, de Barrios O, Naur P, Francotte P, Pirotte B, Gajhede M, Kastrup JS. Thermodynamics and structural analysis of positive allosteric modulation of the ionotropic glutamate receptor GluA2. *Biochem J.* 2012; 441(1):173–178. [PubMed: 21895609]
- Lopez-Munoz F, Alamo C. Neurobiological background for the development of new drugs in schizophrenia. *Clin Neuropharmacol.* 2011; 34(3):111–126. [PubMed: 21586917]
- Lynch G, Granger R, Ambros-Ingerson J, Davis MC, Kessler M, Schehr R. Evidence that a positive modulator of AMPA-type glutamate receptors improves delayed recall in aged humans. *Experimental Neurology.* 1997; 145:89–92. [PubMed: 9184112]
- Lynch G, Palmer LC, Gall CM. The likelihood of cognitive enhancement. *Pharmacol Biochem Behav.* 2011; 99(2):116–129. [PubMed: 21215768]
- Mayer ML. Structure and mechanism of glutamate receptor ion channel assembly, activation and modulation. *Curr Opin Neurobiol.* 2011; 21(2):283–290. [PubMed: 21349697]
- Mitchell NA, Fleck MW. Targeting AMPA receptor gating processes with allosteric modulators and mutations. *Biophys J.* 2007; 92(7):2392–2402. [PubMed: 17208968]
- Morrow JA, Maclean JK, Jamieson C. Recent advances in positive allosteric modulators of the AMPA receptor. *Curr Opin Drug Discov Devel.* 2006; 9(5):571–579.
- Mueller R, Rachwal S, Lee S, Zhong S, Li YX, Haroldsen P, Herbst T, Tanimura S, Varney M, Johnson S, Rogers G, Street LJ. Benzotriazinone and benzopyrimidinone derivatives as potent positive allosteric AMPA receptor modulators. *Bioorg Med Chem Lett.* 2011; 21(20):6170–6175. [PubMed: 21889339]
- Partin KM, Fleck MF, Mayer ML. AMPA receptor flip/flop mutants affecting deactivation, desensitization and modulation by cyclothiazide, aniracetam and thiocyanate. *Journal of Neuroscience.* 1996; 16:6634–6647. [PubMed: 8824304]
- Ptak CP, Ahmed AH, Oswald RE. Probing the allosteric modulator binding site of GluR2 with thiazide derivatives. *Biochemistry.* 2009; 48(36):8594–8602. [PubMed: 19673491]
- Sobolevsky AI, Rosconi MP, Gouaux E. X-ray structure, symmetry and mechanism of an AMPA-subtype glutamate receptor. *Nature.* 2009; 462(7274):745–756. [PubMed: 19946266]
- Sun Y, Olson R, Horning M, Armstrong N, Mayer ML, Gouaux E. Mechanism of glutamate receptor desensitization. *Nature.* 2002; 417:245–253. [PubMed: 12015593]
- Swanson GT. Targeting AMPA and kainate receptors in neurological disease: therapies on the horizon? *Neuropsychopharmacology.* 2009; 34(1):249–250. [PubMed: 19079074]
- Timm DE, Benveniste M, Weeks AM, Nisenbaum ES, Partin KM. Structural and functional analysis of two new positive allosteric modulators of GluA2 desensitization and deactivation. *Mol Pharmacol.* 2011; 80(2):267–280. [PubMed: 21543522]
- Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R. Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev.* 2010; 62(3):405–496. [PubMed: 20716669]
- Verdoorn TA, Burnashev N, Monyer H, Seeburg PH, Sakmann B. Structural determinants of ion flow through recombinant glutamate receptor channels. *Science.* 1991; 252:1715–1718. [PubMed: 1710829]
- Ward SE, Bax BD, Harries M. Challenges for and current status of research into positive modulators of AMPA receptors. *Br J Pharmacol.* 2010; 160(2):181–190. [PubMed: 20423333]
- Ward SE, Harries M. Recent advances in the discovery of selective AMPA receptor positive allosteric modulators. *Curr Med Chem.* 2010; 17(30):3503–3513. [PubMed: 20738249]
- Wesensten NJ, Reichardt RM, Balkin TJ. Ampakine (CX717) effects on performance and alertness during simulated night shift work. *Aviat Space Environ Med.* 2007; 78(10):937–943. [PubMed: 17955941]

Xia YF, Arai AC. AMPA receptor modulators have different impact on hippocampal pyramidal cells and interneurons. *Neuroscience*. 2005; 135(2):555–567. [PubMed: 16125852]

Highlights

- JAMI1001A binds in the solvent accessible allosteric pocket that lies at the interface between AMPA receptor ligand-binding core dimers.
- The compound partially overlaps with the benzamide and biarylsulfonamides binding pocket (subsite A), but does so with an extended alkyl chain rather than planar rings. Distal moieties of JAMI1001A extend into subsite C, making similar interactions as benzothiadiazides such as cyclothiazide.
- JAMI1001A efficaciously blocks channel desensitization and slows deactivation of the flip and flop isoforms of GluA2.
- A simple kinetic model suggests that JAMI1001A independently modulates both the protein rearrangements necessary for channel desensitization and its recovery from the desensitized state, and the protein rearrangements necessary for channel deactivation (closed-cleft stability or channel gating).
- The structure-based drug design strategy successfully yielded an important, clinically relevant new class of positive allosteric modulator of AMPA receptors.

CTZ **CX614** **JAMI1001A**

Figure 1. Chemical structures of three positive allosteric modulators of AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid) receptors

CTZ, cyclothiazide (2,4-benzothiadiazine-3-bicyclo[2.2.1]hept-5-en-2-yl-6-chloro-4-dihydro-2h-1); CX614 (2H,3H,6aH-pyrrolidino(2,1-3',2')1,3-oxazino(6',5'-5,4)benzo(e)1,4-dioxan-10-one), a proto-typical benzamide compound; and JAMI1001A (2-(2-(4-(hydroxymethyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl)acetamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxamide), a new chemotype tested in this paper. Moieties discussed in the Results include: a trifluoromethyl group attached to a pyrazole ring (shown in green), a tetrahydrobenzothiophene moiety (shown in blue), and a carboxamide (red) which extends from the tetrahydrobenzothiophene. The extended distal structures are connected by a linear acetamide chain.

Figure 2. Structural comparison between JAMI1001A and CTZ or CX614

A, A single molecule of JAMI1001A lies at the interface between two AMPA receptor protomers, straddling the axis of symmetry. **B**, Contact map of JAMI1001A showing polar interactions with GluA2 (dotted black lines). The pendant hydroxyl of the pyrazole crystallizes in two orientations: one (shown as stick) interacts with Ser729 and the isoform-specific Ser754, the other (shown as line) with the mainchain of Pro494. An additional interaction is found between the sidechain hydroxyl of Ser497 with the carboxamide of JAMI1001A. **C**, Two molecules of CTZ lie at the dimer interface, either side of the axis of symmetry (left panel). There is significant overlap between moieties at the distal ends of JAMI1001A and CTZ, particularly in subsite C (green circles, right panels). **D**, A single molecule of CX614 lies at the interface, straddling the axis of symmetry (left panel). There is significant overlap between the planar rings of CX614 and the central, alkyl moiety of JAMI1001A, which occupies subsite A (blue ellipses; right panels). This figure was generated with pdb files from the RCSB Protein Data Bank using the The PyMol Molecular

Graphics System (version 1.3, Schrödinger, LLC). The following are shown in cartoon representation, aligned manually: Chains A and C of the 3H6T.pdb file for cyclothiazide (Hald et al., 2009), Chains D and F of the 2AL4.pdb file for CX614 (Jin et al., 2005), and Chain A of JAMI1001A (4FAT.pdb). A mask was applied to the CX614 subunit of 2AL4 so only one conformation of CX614 would be displayed. Modulators shown in complex are against Chains D and F of the 2AL4.pdb file at 60% transparency.

Figure 3. JAMI1001A modulates AMPA receptor deactivation and desensitization

A, Modulation of a 1 ms pulse of 10 mM glutamate (deactivation, left) and of a 500 ms pulse of glutamate (desensitization, right) of the flip isoform of GluA2 by 100 μ M CTZ, 100 μ M CX614, and 100 μ M JAMI1001A in outside-out patches of transiently transfected HEK293 cells. Control (No Drug) responses are shown in light gray; modulated responses in black. **B**, Identical parameters measured for the flop isoform of GluA2.

Figure 4. JAMI1001A significantly modulates AMPA receptor deactivation

A, Mean data for a 1 ms pulse of glutamate demonstrates that JAMI1001A significantly modulates the kinetics of channel deactivation for both splice isoforms of GluA2. **B**, Mean data for 500 ms pulses of glutamate demonstrate that JAMI1001A significantly modulates the equilibrium desensitization for both splice isoforms of GluA2. n values are indicated in parentheses. A two-tailed student's t-test demonstrates significance, such that *, $p < .05$, **, $p < .01$, ***, $p < .001$.

Figure 5. Kinetic model of mechanism of action of JAMI1001A

A, A simplified gating scheme was used to test likely mechanism(s) of action of JAMI1001A. The model shows gating conformations in the absence (red) and presence (green) of an allosteric modulator. R, receptor; R_{des} , desensitized receptor; Glu and Glu₂, one and two molecules of bound glutamate, respectively; M, modulator; *, open channel. Transitions are indicated by number and rates are given in Table 1. **B**, Simulated responses to 1 ms or 500 ms of 10 mM glutamate, with no drug (gray) or in the presence of 10 μ M JAMI1001A (black), demonstrate that electrophysiological data can be well-recapitulated by slowing the rate of agonist dissociation ($k'_{glu\ off}$), and either slowing, the rate of entry into the desensitized state (δ) or increasing the rate of return from the desensitized state (γ). The

data in Figure 3 could not be simulated by changing a single rate constant. Thus, JAMI1001A appears to modulate both deactivation and desensitization.

Table 1

Rate constants used for kinetic simulations shown in Figure 5.

Alternative Nomenclature				
Transition Number	Transition		Forward Rate	Reverse Rate
	Forward	Ligand Reverse		
1	$2k_{glu\ on}$	Glu $k_{glu\ off}$	$15\ \mu\text{M}^{-1}\text{s}^{-1}$	$1,000\ \text{s}^{-1}$
2	$k_{glu\ on}$	Glu $2k_{glu\ off}$	$7.5\ \mu\text{M}^{-1}\text{s}^{-1}$	$2,000\ \text{s}^{-1}$
3	β	- α	$4,000\ \text{s}^{-1}$	$10,000\ \text{s}^{-1}$
4	δ	- γ	$500\ \text{s}^{-1}$	$20\ \text{s}^{-1}$
5	2δ	- γ	$1,000\ \text{s}^{-1}$	$20\ \text{s}^{-1}$
6	$k_{glu\ on}$	Glu $k_{glu\ off}$	$7.5\ \mu\text{M}^{-1}\text{s}^{-1}$	$1,000\ \text{s}^{-1}$
7	$k_{jami\ on}$	JAMI $k_{jami\ off}$	$0.1\ \mu\text{M}^{-1}\text{s}^{-1}$	$0.1\ \text{s}^{-1}$
8	$2k'_{glu\ on}$	Glu $k'_{glu\ off}$	$15\ \mu\text{M}^{-1}\text{s}^{-1}$	$1,000\ \text{s}^{-1}$
9	$k'_{glu\ on}$	Glu $2k'_{glu\ off}$	$7.5\ \mu\text{M}^{-1}\text{s}^{-1}$	$2,000\ \text{s}^{-1}$
10	β	- α	$4,000\ \text{s}^{-1}$	$10,000\ \text{s}^{-1}$
11	δ	- γ	$500\ \text{s}^{-1}$	$20\ \text{s}^{-1}$
12	2δ	- γ	$1,000\ \text{s}^{-1}$	$20\ \text{s}^{-1}$
13	$k'_{glu\ on}$	Glu $2k'_{glu\ off}$	$7.5\ \mu\text{M}^{-1}\text{s}^{-1}$	$1,000\ \text{s}^{-1}$
14	$k_{jami\ on}$	JAMI $k_{jami\ off}$	$0.1\ \mu\text{M}^{-1}\text{s}^{-1}$	$0.1\ \text{s}^{-1}$
15	$k_{jami\ on}$	JAMI $k_{jami\ off}$	$0.1\ \mu\text{M}^{-1}\text{s}^{-1}$	$0.1\ \text{s}^{-1}$
16	$k_{jami\ on}$	JAMI $k_{jami\ off}$	$0.1\ \mu\text{M}^{-1}\text{s}^{-1}$	$0.1\ \text{s}^{-1}$
17	$k_{jami\ on}$	JAMI $k_{jami\ off}$	$0.1\ \mu\text{M}^{-1}\text{s}^{-1}$	$0.1\ \text{s}^{-1}$

GLU, 10 mM glutamate; JAMI, 10 μM JAMI1001A, β , $k_{glu\ on}$ and $k_{glu\ off}$, respective forward and reverse microscopic rate constants for glutamate; $k'_{glu\ on}$ and $k'_{glu\ off}$, respective forward and reverse rate constants for glutamate in the presence of JAMI1001A; β , $k_{jami\ on}$ and $k_{jami\ off}$, respective forward and reverse microscopic rate constants for JAMI1001A binding; α , channel closing rate; β , channel opening rate; δ , rate of onset of receptor desensitization; γ , rate of recovery from channel desensitization.