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Pharmacological Characterization and Modeling of the Binding Sites of Novel 1,3-bis(pyridinylethynyl)benzenes as Metabotropic Glutamate Receptor 5-selective Negative Allosteric Modulators

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Running title: Binding Site Characterization of Novel NAMs on mGluR5

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Nonstandard abbreviations: 7TMD, 7-transmembrane domain; BHK cells, Baby Hamster Kidney cells; BisPEB, 1,3-bis(pyridinylethynyl)benzene; GPCR, G protein-coupled receptor; mGluR, metabotropic glutamate receptor; MPEP, 2-methyl-6-(phenylethynyl)pyridine; NAM, negative allosteric modulator; OD, optical density; SEM, standard error of mean; TMH, transmembrane helix

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Abstract

Metabotropic glutamate receptor subtype 5 (mGluR5) is a potential drug target in neurological and psychiatric disorders, and subtype-selective allosteric modulators have attracted much attention as potential drug candidates. In this study the binding sites of three novel MPEP-derived negative allosteric modulators, 2-, 3- and 4-BisPEB, have been characterized. 2-, 3-, and 4-BisPEB are 1,3-bis(pyridinylethynyl)-benzenes and differ only by the position of the nitrogen atom in the pyridine rings. Despite their high structural similarity, 2-BisPEB (1,3-bis(pyridin-2-ylethynyl)-benzene, nitrogen atom in ortho position), with an IC_{50}-value in the nanomolar range, is significantly more potent than the 3- and 4-pyridyl analogues. Mutational analysis, directed by a previously published mGluR5 homology model, was used to determine key residues for the ligand-receptor interactions that may explain the potency differences of 2-, 3-, and 4-BisPEB. Residues I651, P655, Y659, N747, W785, F788, Y792, S809, and A810 were found to have critical roles for the activity of one or more of the three BisPEBs and the reference compound MPEP. The mutational data suggest that the higher potency of 2-BisPEB is due to hydrogen bonding to S809 as the S809A mutation made 2-BisPEB equipotent to 3- and 4-BisPEB (IC_{50}: 1–2.5 μM). The potency of MPEP was also greatly affected by S809A (52-fold) suggesting that a S809-mediated hydrogen bond is also a key interaction between MPEP and mGluR5. Potential binding modes of 2-, 3- and 4- BisPEB obtained by molecular docking to the mGluR5 homology model provides a structural context for the reported major mutational effects.

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Introduction

Metabotropic glutamate receptors (mGluRs) belong to Class C of the G protein-coupled receptor (GPCR) superfamily. They consist of eight subtypes divided into three groups based on sequence similarity, signal transduction pathway and pharmacology. They are organized in dimers and each protomer has a large amino-terminal Venus Flytrap domain, encompassing the orthosteric site, connected to the transmembrane domain through a cysteine-rich region (Bräuner-Osborne et al., 2007; Nakanishi and Masu, 1994; Niswender and Conn, 2010; Pin et al., 2003). No drugs directly targeting mGluRs have so far been marketed despite numerous studies supporting the role of mGluRs in several diseases. One example is mGluR subtype 5 (mGluR5), which has been proposed as a target for disorders like anxiety (Spooren and Gasparini, 2004; Swanson et al., 2005), depression (Palucha and Pilc, 2007), Parkinson's disease (Johnson et al., 2009), and schizophrenia (Conn et al., 2009). Due to a highly conserved orthosteric site (Wellendorph and Bräuner-Osborne, 2009) it has proven difficult to develop potent subtype-selective orthosteric ligands despite the fact that several crystal structures of the Venus Flytrap domain are available for several mGluR subtypes (mGluR1 (Kunishima et al., 2000; Tsuchiya et al., 2002), mGluR5 (PDB # 3LMK), mGluR3, and mGluR7 (Muto et al., 2007)). As a consequence, efforts have shifted towards the identification of allosteric modulators binding in the less conserved 7-transmembrane domain (7TMD). This has resulted in the identification of several subtype-selective and potent allosteric modulators (Galambos et al., 2010; Ritzen et al., 2009; Zhang et al., 2010).

In addition to being subtype-selective, which in itself is a desired property for new drugs, allosteric modulators act by fine-tuning the existing receptor activation as they in general do not have intrinsic agonist activity at the wild type receptor (Goudet et al., 2004; Noetzel et al., 2012). This means that allosteric receptor modulation is dependent on the presence of the endogenous ligand and therefore does not affect the spatio-temporal signal patterns, which is especially important when targeting the highly complex CNS neuronal network (Bridges and Lindsley, 2008; Christopoulos et al., 2004; Gregory et al., 2011).

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Several Class A GPCRs have been co-crystallized with diffusible ligands bound to the 7TMD. The first one was the β_2 adrenergic receptor published in 2007 (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007). Since then others like the β_1 adrenergic (Warne et al., 2008), adenosine A_{2A} (Jaakola et al., 2008), dopamine D_3 (Chien et al., 2010), chemokine CXCR4 (Wu et al., 2010), histamine H_1 (Shimamura et al., 2011), and muscarinic M_2 (Haga et al., 2012) receptor structures have been determined. There are, however, no crystal structures available of the 7TMD of mGluR5 or any other Class C GPCR. This limits the structural insights into allosteric binding pockets located within the 7TMD and thereby the possibilities for rational discovery and design of new drug candidates. In lack of target crystal structures, mutational studies combined with homology modeling and/or ligand analog series represent a powerful tool for obtaining important structural information regarding the identity and spatial arrangement of key residues essential for ligand-receptor interactions (Ballesteros and Palczewski, 2001; Gregory et al., 2011). This approach has previously been used for mGluR5 in delineation of the binding site of 2-methyl-6-(phenylethynyl)pyridine (MPEP), a selective negative allosteric modulator (NAM) (Malherbe et al., 2003; Pagano et al., 2000), as well as other allosteric modulators (Chen et al., 2008; Chen et al., 2007; Malherbe et al., 2006; Mühlemann et al., 2006).

In the present study mutational analysis, directed by a previously published mGluR5 homology model (Kaae et al., 2012), was used to determine key residues for the ligand-receptor interactions that may explain the potency differences of three novel MPEP-derived NAMs, 2-, 3- and (1,3-bis(pyridin-4-ylethynyl)-benzene (2-, 3- and 4-BisPEB) (Figure 1). The NAMs were first presented in a recent paper by Kaae et al. in which they were referred to as compound 19-21 (Kaae et al., 2012). These three NAMs only differ in the position of the nitrogen in the pyridine rings; however, this subtle variation in chemical structure leads to a more than 80-fold potency difference when tested on a stably expressing mGluR5 cell line (Kaae et al., 2012). Uncovering the structural determinants causing the large difference in potency is an important contribution towards understanding the allosteric binding pocket. MPEP was chosen as a reference for mutational studies as this is a well-characterized ligand and shares structural elements with the three BisPEBs. Several interacting residues were identified including I651, P655, Y659, N747, W785, F788, Y792, S809, and A810. Mutations of some residues

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affected all four NAMs, whereas others had differential effects revealing clear differences in NAM-receptor interactions. The mutational analysis was combined with molecular docking to give a structural explanation for the observed potency differences of the three BisPEBs. These results suggest that the potency difference between the three BisPEBs may be explained by a hydrogen bond between 2-BisPEB and S809. S809 is also suggested to form a strong hydrogen bond to MPEP, a new interaction not previously published as far as these authors are aware.

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Materials and Methods

Materials – 2-, 3- and 4-BisPEB were synthesized as previously described (Kaae et al., 2012). L-glutamate and murine anti-FLAG antibody solution (M2) were purchased from Sigma (St. Louis, MO), HRP-conjugated secondary anti-mouse antibody was from Dako (Glostrup, Denmark), Fenobam was acquired from Ascent Scientific (Bristol, United Kingdom), MPEP and ^{3}H-MPEP were from Tocris (Bristol, United Kingdom) and American Radiolabeled Chemicals (St. Louis, MO), respectively. Fluo-4 NW Calcium Assay Kit was purchased from Molecular Probes (Eugene, OR), and bicinchoninic acid assay kits were acquired from Thermo Scientific (Waltham, MA).

Cell culturing and transfection – Baby hamster kidney (BHK) cells were cultured in DMEM with GlutaMAX-I supplemented with 10% diafiltered FBS, 100 units/ml penicillin and 100 µg/ml streptomycin and maintained at 37 °C and 5% CO_2. Cells were transiently transfected by the calcium phosphate method at 40-60% confluency using 20 µg, 50 µg, and 165 µg DNA per 100 mm, 150 mm, and 245 mm petri dish, respectively, and 5-6 hours incubation at 37 °C and 5% CO_2. One day post transfection cells were exposed to G418 either by seeding cells into multi-well plates (for Ca^{2+} mobilization assay and cell surface ELISA) or by changing growth media (for membrane preparation for binding assay) using regular growth media supplemented with 1 mg/ml G418. Ca^{2+}-mobilization assays, cell surface ELISA and membrane preparation were performed two days post transfection.

Plasmids and mutagenesis – The human mGluR5a was tagged with a FLAG epitope (DYKDDDDK) immediately after the signal peptide between amino acids 21 and 22 by overlap PCR to allow for measurement of cell surface expression. The FLAG-tagged mGluR5a was subcloned into pcDNA3.1(+) and used as template for the mutagenesis reactions. Point mutations were generated using 5'-end phosphorylated primers designed according to the principle of the Stratagene QuikChange Multi Site-Directed Mutagenesis Kit, or partially overlapping primer pairs designed according to the principle described by Zheng et al. (Zheng et al., 2004). The genotype of all mutants was confirmed by sequencing by Eurofins MWG Operon (Ebersberg, Germany).

Membrane preparation and ^{3}H-MPEP binding assay – Membranes were essentially prepared as described previously by Malherbe et al. (Malherbe et al., 2003), however, using HEPES-based instead

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of Tris-based buffers. Membranes were resuspended in binding assay buffer (15 mM Tris-HCl buffer containing 120 mM NaCl, 100 mM KCl, 25 mM $CaCl_2$, and 25 mM $MgCl_2$, pH 7.4, 2.5 µg/ml leupeptin, 10 µg/ml benzamidine) before freezing in aliquots at -80 °C and determining total protein concentration using a bicinchoninic acid assay kit. For binding studies, membranes were thawed and resuspended in binding assay buffer to a final concentration of 20 µg protein/well. ^{3}H-MPEP, binding assay buffer (total binding), 10 µM Fenobam (non-specific binding) and membranes were mixed for saturation binding. ^{3}H-MPEP, cold ligand (2-, 3- and 4-BisPEB) and membranes were mixed for competitive binding studies. Membranes were harvested onto GF/C filters after 1 hour incubation at room temperature as described by Malherbe et al. (Malherbe et al., 2003). 40 µl/well scintillation fluid was added, and the filters were counted on a Wallac MicroBeta Trilux (PerkinElmer, Waltham, MA) after a 2 hour delay minimum. Total radioactivity was measured for each ^{3}H-MPEP concentration on a Tri-Carb (PerkinElmer, Waltham, MA) for calculating the actual concentration as well as evaluating ligand depletion. Ligand depletion (>10%) was observed for some of the lower ^{3}H-MPEP concentrations. However, excluding these points from the data set had no significant effect on graph parameters such as K_d and B_{max}. It was therefore concluded that ligand depletion did not constitute a problem.

Ca^{2+} mobilization assay – The day before the assay transiently transfected cells were seeded in 96-well plates at 60,000 cells/well using regular growth media supplemented with 1 mg/ml G418. On the day of the assay cells were incubated at 37 °C and 5% CO_2 with HEPES-buffer (Hank's Balanced Salt Solution with calcium and magnesium containing 20 mM HEPES pH 7.4) supplemented with 1% BSA for minimum 4 hours prior to dye loading. After the 4 hours of incubation cells were washed once with HEPES-buffer after which 50 µl/well dye loading solution was added and cells were incubated for 1 hour at 37 °C and 5% CO_2. The Fluo-4 NW Calcium Assay Kit dye loading solution was prepared by dissolving the content of Component A according to the manufacturer's instructions using probenecid-buffer (HEPES-buffer supplemented with 2.5 mM probenecid). After dye loading cells were washed once with probenecid-buffer followed by addition of 100 µl/well probenecid-buffer and room temperature incubation in the dark for minimum 30 min. Glutamate-stimulated increases in intracellular Ca^{2+}-concentration were measured on a FDSS7000 imaging-based plate reader

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(Hamamatsu, Hamamatsu City, Japan) using excitation and emission filters of 480 nm and 540 nm, respectively.

Cell Surface ELISA – The day before the assay transiently transfected cells were seeded in poly-D-lysine coated 48-well plates at 180,000 cells/well using regular growth media supplemented with 1 mg/ml G418. On the day of the assay cells were washed twice with wash buffer (PBS supplemented with 1 mM $CaCl_2$) followed by a 30 min. incubation with blocking solution (50 mM Tris-buffer, pH 7.4, supplemented with 3% BSA and 1 mM $CaCl_2$). Blocking solution was replaced by 150 µl/well murine anti-FLAG antibody solution, M2, diluted 1:1000 in blocking solution, and cells were incubated for 2 hours. After incubation cells were washed 5 times with wash buffer and then incubated for 2 hours with 150 µl/well HRP-conjugated secondary anti-mouse antibody, diluted 1:1000 in blocking solution, after which they were washed 5 times with wash buffer. All the above-mentioned steps were carried out at 16–18 °C to avoid receptor internalization (von Zastrow and Kobilka, 1994). After the final wash cells for ELISA measurements were incubated with 230 µl/well 3,3',5'5-tetramethylenbenzidine for 3 min. at room temperature after which 230 µl/well 0.5 N H_2SO_4 was added to terminate the oxidation of 3,3',5'5-tetramethylenbenzidine. 200 µl/well was transferred to a clear flat-bottom 96-well plate and OD_{450} absorbance was read on a Multiskan Ascent (Labsystems, Vienna, VA). Experiments were conducted 3 times in duplicate. For every plate prepared for ELISA measurements an identical plate was prepared for protein measurements to evaluate whether mutant expression affected cell viability. These plates were handled the same way as the ELISA plates except that antibody incubations were replaced with blocking solution incubations. The cells were lysed with 50-100 mM NaOH and filtered after the final wash, and total protein concentration of the filtrate was measured using bicinchoninic acid assay kits. We found no significant difference between total protein concentration for cells expressing the wild type or mutant receptors ($p = 0.32$).

Data analyses – Prism 5.0a (GraphPad Software) was used for data fitting and analysis.

Total binding data from saturation binding studies were corrected for non-specific binding and fitted to the equation: $Y=(B_{max}\times[^3\text{H-MPEP}])/(K_d+[^3\text{H-MPEP}])$ for estimation of K_d, and $Y=\text{Bottom}+(\text{Top-Bottom})/(1+10^{((\text{Log(EC50)-log([3H-MPEP]))}\times\text{Hill slope})})$ for estimation of the Hill slope. Data from competitive

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binding studies were fitted to the equation: $Y=Bottom+(Top-Bottom)/(1+10^{((\log(IC50)-\log([NAM]))\times Hill\ slope)})$. K_i-values for the individual NAMs were calculated from the fitted IC_{50}-value and the K_d-value of ^{3}H-MPEP, estimated from the saturation binding studies, using the Cheng-Prusoff equation: $K_i=IC_{50}/(1+[^3H\text{-}MPEP]/K_d)$. Experiments were conducted 2-3 times in duplicate.

Data from glutamate concentration-response experiments were normalized and fitted to: $Y=Bottom+(Top-Bottom)/(1+10^{((\log(EC50)-[Glutamate])\times Hill\ Slope)})$. Data from NAM concentration-response experiments were normalized and fitted to: $Y=Bottom+(Top-Bottom)/(1+10^{((\log(IC50)-\log([NAM]))\times Hill\ Slope)})$. Experiments were conducted 2-16 times in duplicate. Mutants were compared to wild type using one-sample t-test (for mutants with NAM $IC_{50} \geq 45$ µM, the highest possible concentration due to solubility issues) or one-way ANOVA followed by Dunnett's multiple comparison test.

Molecular docking – 2-, 3-, 4-BisPEB and MPEP were built in Maestro (version 9.2, Schrödinger, LLC, New York, NY, 2011) and minimized in MacroModel (version 9.9, Schrödinger, LLC, New York, NY, 2011) with default settings. The backbone-dependent rotamer library included in PyMOL (The PyMOL Molecular Graphics System, version 1.5, Schrödinger, LLC, New York, NY, 2011) was applied to N747 and S809 in our mGluR5 homology model (Kaae et al., 2012) to identify the most likely side chain conformation for each residue with the hydrogen bonding donor groups pointing into the putative binding site. The receptor grids for docking in Glide (version 5.8, Schrödinger, LLC, New York, NY, 2011) were prepared as previously described (Kaae et al., 2012). 2-, 3-, 4-BisPEB and MPEP were docked into the mGluR5 homology model using the XP scoring function, a scaling factor of 0.7 for all ligand atoms and asking for maximum 10 output poses. We selected the highest-ranking output poses that rationalize as much as possible of our and referenced mutational results as the most plausible binding mode.

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Results

2-, 3-, 4-BisPEB displace ^{3}H-MPEP. MPEP binds to an allosteric site in the 7TMD of mGluR5 (Malherbe et al., 2003; Pagano et al., 2000), and competitive binding studies showed that all three BisPEBs can displace ^{3}H-MPEP (Figure 2) with relative affinities similar to the order of potencies found in the functional Ca^{2+} mobilization assay (Table 1). This is consistent with the three BisPEBs binding in the 7TMD like MPEP, which is further supported by the mutational data.

Mutational studies – To elucidate the binding modes of the four NAMs 14 residues were selected for mutation based on the previously published docking of 2-BisPEB into our mGluR5 homology model (Kaae et al., 2012) and on previously published mutational data for MPEP (Malherbe et al., 2003; Pagano et al., 2000). The selected residues were either modeled by us to interact directly with 2-BisPEB, located in close proximity to the suggested interaction partners, or found to have pronounced effects (≥ 10-fold) on the affinity and/or potency of MPEP or Methoxy-MPEP in previous studies (Malherbe et al., 2003; Pagano et al., 2000). Complementing mutational strategies were designed to remove or introduce side-chains with favorable and unfavorable interactions.

Mutational effects on cell surface expression, glutamate potency and efficacy – The mutants were characterized by transiently transfecting BHK cells with wild type and mutant mGluR5 and measuring increases in intracellular Ca^{2+}-concentration upon glutamate stimulation. The majority of the mutants responded to glutamate with efficacies and potencies not statistically significant different from wild type (E_{max} and EC_{50} values are given in Table 2). The mutants, for which glutamate potency was significantly affected, were T632P, R648E, W785A, and A810V (lower glutamate potency) and Y792A and A813F (higher glutamate potency). The differences were, however, less than approximately 3-fold numerically. Cell surface ELISA was performed to investigate whether lower efficacy or lack of glutamate-stimulated response of some mutants were due to lower cell surface expression or non-/dysfunctional receptors. Optical density (OD) values are given in Table 2 as a measure for cell surface expression. The two non-responding mutants (G628F and F788D) gave OD responses comparable to mock transfected cells (0.072 ± 0.001 (Mean ± SEM), n = 7) indicating that the mutants were not expressed at cell surface level in a sufficient amount to mediate a cellular response. It was found that the glutamate-stimulated response levels (relative E_{max}) in general

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correlated with cell surface expression of the mutants giving a linear-regression R^2-value = 0.81 (Supplemental Figure 1).

Mutational effects common for all four NAMs – To measure antagonistic activity, NAMs were automatically added to the cells expressing wild type or mutant mGluR5 prior to stimulation with 10 µM L-glutamate (upper dynamic range of the concentration-response curves). When tested on the wild type receptor, 2-BisPEB was > 25 times as potent as the two other BisPEBs, although still approximately 10 times less potent than MPEP.

Four mutations (I651F, Y659A, F788W, and A810V) affected the potency of all four NAMs. The effect of I651F was strong for all four (>20-fold). The effect of Y659A was also strong, although the range was wider (14-fold for 2-BisPEB to 120-fold for MPEP). The effects of F788W and A810V were statistically significant for all NAMs, but of varying magnitude. The effect of F788W ranged from >20-fold for 3- and 4-BisPEB to a modest 3.3-fold for 2-BisPEB, similarly the effect of A810V ranged from 63-fold for MPEP to 3.6-fold for 4-BisPEB. Five mutations (T632A, Y659F, G748L, Y792F, and A813F) displayed general minor effects and were therefore not considered of direct relevance for the interaction between any of the NAMs and mGluR5.

Mutations with differential effects on the four NAMs – The largest effect for 2-BisPEB was seen when mutating S809. S809F and S809A resulted in a 63-fold and 19-fold decrease in potency, respectively. W785A and N747A both led to an 11-fold decrease in potency for 2-BisPEB, whereas N747S led only to a 5.4-fold decrease in potency. W785A decreased potency of 2-BisPEB 11-fold. T632P and R648E had a potentiating effect of minor magnitude (IC_{50} = 0.16 and 0.15 relative to wild type, respectively).

Substantial effects for the potency of 3-BisPEB were seen for P655S (>20-fold), N747A (>20-fold), N747S (11-fold), and Y792A (>20-fold). The effect of R648E was statistically significant, but the shift in potency was only ~3-fold, and R648 is therefore not considered to be directly involved in the interactions between 3-BisPEB and mGluR5.

The only statistically significant mutation affecting 4-BisPEB potency without affecting the three other NAMs was F788A (~21-fold). The effects of W785A and Y792A were also statistically

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significant, but the shifts in potency were ~3-fold. W785A and Y792A are therefore not considered to be directly involved in the interactions between 3-BisPEB and mGluR5.

Of the mutations without a common effect on all tested NAMs, only one (N747A) was without statistical significant effect on the potency of MPEP. For some of the other mutations, however, the effect on potency was of limited magnitude (7.4-fold for P655S, and 4.2-fold N747S, and 8.1-fold for Y792A). Like for 2-BisPEB, the two mutations T632P and R648E had a minor potentiating effect on MPEP-mediated inhibition of mGluR5 activity (IC_{50} = 0.26 and 0.12 relative to wild type, respectively). For other mutations, however, a relatively large decrease in potency was observed. These were W785A (280-fold), F788A (27-fold), S809F (490-fold), and S809A (52-fold).

Modeled binding modes of 2, 3-, 4-BisPEB and MPEP. To put our experimental results into a structural context 2-, 3-, 4-BisPEB, and MPEP were docked into the mGluR5 homology model to generate plausible binding modes that rationalize the mutational results. Though asking for 10 output conformations, the extra-precision (XP) docking protocol used presented only four or less possible binding modes for each NAM. For 2-BisPEB we observed four different output poses and since only the second ranked pose was able to rationalize the most pronounced mutational effect it was selected as the most likely binding conformation (Figure 3a-c). For 3- and 4-BisPEB we observed very limited variation between the output poses and the highest ranked poses were selected as potential binding modes (Figure 3c). Docking of MPEP resulted in two completely different output poses; the highest-ranking pose (pose 1) was located between TMHs 3, 5 and 6 and the other pose (pose 2) in the area between TMHs 2, 3, 6 and 7 (Figure 3d). Both MPEP poses are within interaction distance of about half of the residues showing effect on MPEP inhibition and therefore have similar plausibility. While the docking results for MPEP identified two sub-sites in the cavity, the three other NAMs, due to their larger size and bent shape, bridge both sub-sites occupying a larger area between TMHs 2, 3, 5, 6 and 7. Thus, consistent with the results from the ^{3}H-MPEP binding assay the potential binding conformations of all four compounds overlap (Figure 3c-d).

The model with the docked compounds can be acquired by contacting the corresponding author.

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Discussion

We set out to explain the large potency differences between three novel MPEP-derived mGluR5 NAMs with high structural similarity. We characterized the binding sites by combining extensive mutational studies with ligand docking into a homology model. Competitive binding studies showed that 2-, 3-, and 4-BisPEB can displace ^{3}H-MPEP consistent with binding pockets located in the 7TMD. This is further supported by mutations in the 7TMD affecting the potency of MPEP, 2-, 3- and/or 4-BisPEB (Table 1), and the obtained docking poses (Figure 3). Even though 2-, 3- and 4-BisPEB are derived from MPEP (Kaae et al., 2012), their sizes and shapes result in binding modes distinct from that of MPEP. The mutational effects and structural interpretation of the BisPEBs and MPEP are therefore discussed separately.

Two mutations, I651F and Y659A, had pronounced effects on the potency of the three BisPEBs suggesting that these residues are involved in shaping the binding sites of all three NAMs. The mGluR5 homology model places I651 in the central part of the binding pockets (Figure 3a-c), and introducing a phenylalanine likely occludes the site hindering ligand binding. Y659 is placed in the bottom of the binding pocket between TMHs 3, 5, and 6 and our suggested binding modes indicate ligand-receptor π-π interactions with this residue. This is consistent with the fact that Y659F did not affect IC_{50} of the BisPEBs while Y659A increased IC_{50} $\geq$14-fold.

Despite high structural similarity, 2-BisPEB was found to be >25 times as potent as 3- and 4-BisPEB. Given that the only structural difference is the nitrogen atom position in the pyridine rings (Figure 1), the explanation for the varying potencies is likely to be found among hydrogen bonding partners. Residues T632, R648, N747, Y792, and S809 were identified as potential candidates based on our previously published docking of 2-BisPEB (Kaae et al., 2012). The T632A, T632P, R648E, and Y792F mutations gave no indications of these residues being hydrogen bonding partners of any of the three BisPEBs (Table 1). However, mutation of two hydrophilic residues, N747 and S809, resulted in differential effects on the three BisPEBs (Table 1): The two N747 mutants primarily affected 3-BisPEB and to a minor degree 2-BisPEB while the S809 mutations affected only 2-BisPEB. According to our docking studies these effects can be explained by different hydrogen bonding patterns of the three BisPEBs based on the position of the pyridine nitrogens (Figure 3c): Only 2-

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BisPEB can form a direct hydrogen bond to S809 and the vicinity to N747 indicates a possible water-mediated hydrogen bond; 3-BisPEB can interact with N747 directly; and 4-BisPEB displays no hydrogen bonds. The mutational data as well as the docking studies suggest that the different interaction patterns to S809 are responsible for the higher potency of 2-BisPEB and results in a shifted binding mode for 2-BisPEB compared to 3-, and 4-BisPEB (Figure 3c). Thus, the S809A mutation is expected to result in a binding mode for 2-BisPEB similar to that of the two other NAMs consistent with the similar IC_{50} values for 2-BisPEB on the S809A mutant (1.5 μM) and 3-, and 4-BisPEB on the wild-type receptor (2.2 and 2.1 μM, respectively). Such a shift in binding mode would also allow introduction of bulk at residue 809 without steric clash explaining the relatively subtle effect of the S809F vs. S809A mutation on 2-BisPEB (~3-fold) comparable with the effects of S809F vs. wild type on 3-, and 4-BisPEB (Table 1).

The different binding mode of the three BisPEBs also results in one of the pyridine rings of 2-BisPEB being closer to W785 and moves the central aromatic ring further away from F788 (Figure 3c). Thus, the selective effect of W785A on 2-BisPEB may be explained by more optimal π-π interactions between W785 and 2-BisPEB compared to 3-, and 4-BisPEB. The opposite may be true for mutations of F788, which affect only 3-, and 4-BisPEB, and not 2-BisPEB.

The P655S, Y792A and A810V mutations, which also had differential effects on the BisPEBs, are more difficult to explain from our docking studies. P655 is located in the vicinity of N747 (Figure 3) and as P655S affects the IC_{50} of only 3-BisPEB it could indicate that the introduced serine interacts with N747 and prevents the direct hydrogen bond to 3-BisPEB discussed above. Y792 is predicted to interact with the central aromatic ring of the BisPEBs and since the binding modes of 3-, and 4-BisPEB overlap perfectly in this area (Figure 3c) the model does not offer an explanation for the selective effect by Y792A on 3-BisPEB. Furthermore, none of the predicted binding modes (also applies to MPEP) display interactions to A810 (Figure 3) and the occlusion of the binding site indicated by A810V remains unexplained by our model. This indicates that TMH7 in mGluR5 may be slightly rotated compared to the Class A GPCRs used as templates for our homology model. Such a rotation would, in addition to S809, also position A810 in the vicinity of the suggested binding poses enabling the model to explain the mutational effects of the A810V mutation.

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MPEP was included as a reference compound as the three BisPEBs were designed as MPEP analogs. Our molecular docking only returned two different potential MPEP binding poses, however, neither of the poses alone can rationalize the major mutational effects on MPEP potency: Pose 1 is within favorable van der Waals contact distance of P655, Y659, W785 and Y792 while pose 2 is in contact with I651, F788, Y792 and displays a hydrogen bond to S809 (Figure 3d). Thus, each of the possible MPEP binding poses offers an explanation for the mutational effects of four residue positions making them equally plausible. Detailed examination of the mGluR5 homology model shows that W785 divides the putative binding cavity into the two sub-sites occupied by the MPEP docking poses (Figure 3d). This tryptophan is conserved in 73% and 69% of Class C (including all mGluRs) and Class A GPCRs, respectively. It is located in the middle of TMH6 and shapes the lower part of the 7TM binding cavity. Class A GPCRs have been explored by solving a number of crystal structures and so far the tryptophane has been shown to display only two different conformations (Cherezov et al., 2007; Chien et al., 2010; Haga et al., 2012; Jaakola et al., 2008; Rasmussen et al., 2007; Rosenbaum et al., 2007; Shimamura et al., 2011; Warne et al., 2008; Wu et al., 2010). Both the W785 conformation incorporated in our mGluR5 model and the alternative conformation observed in one Class A GPCR crystal structure (Haga et al., 2012) separates Y659 and S809 into different parts of the binding cavity. Both of these residues are suggested as important interaction partners for MPEP, as they were for 2-BisPEB, with Y659 as a potential π-π interaction partner and S809 as a potential hydrogen bonding partner. However, W785 makes it impossible to obtain a binding mode of MPEP that interacts directly with both residues.

The binding site of MPEP and a close structural analog, Methoxy-MPEP, has been explored in two previous studies by similar approaches and with similar qualitative effects for identical or equivalent mutations (Malherbe et al., 2003; Pagano et al., 2000). Both studies, however, report a single binding mode of MPEP based on their homology models that can rationalize all of their major mutational effects. Our homology model differs from those of Pagano and Malherbe by use of a sequence alignment based on all Class A, B and C GPCRs (Kaae et al., 2012), we use different templates, and retain side chain conformations of conserved residues. Furthermore, as we make use of a validated docking program, as opposed to manual docking, it is not surprising that we obtain different binding

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modes for MPEP. The MPEP binding mode reported by Malherbe et al. is located just below P655 and W785 (P654 and W784 in the Malherbe rat mGluR5 model) and is in contact with residues from both TMHs 5 and 7. However, in our model this area does not present a cavity that can accommodate MPEP. Pagano et al. reports a MPEP binding mode that interacts with both Y659 and S809 without the obstruction by W785. Such a binding mode is possible in their model because Y659 is located in the second helical turn of TMH3, compared to the fifth helical turn in our model, positioning Y659 and S809 much closer to each other without W785 in between.

Our mGluR5 homology model and predicted BisPEB binding modes show remarkably good consistency with our experimental data suggesting that it may be used to rationalize structure-activity-relations data for NAMs at mGluR5. However, the model and MPEP binding modes differ significantly from those previously reported and two different MPEP binding modes are necessary to rationalize all the mutations with significant effect on MPEP IC_{50}. A detailed explanation of the mode of action for MPEP inhibition is beyond the scope of this work, but our results could indicate that two different binding sites may exist. Another possible explanation could be that some of the residues showing effect on MPEP inhibition, e.g. S809 and A810, do not affect binding, but regulate access to the binding site as previously suggested (de Graaf and Rognan, 2009). That some residues are involved in early events in the ligand binding pathway, but not in the final ligand binding, has also been shown by molecular dynamics simulation studies (Dror et al., 2011).

In conclusion, we have identified nine residues, I651, P655, Y659, N747, W785, F788, Y792, S809 and A810, as important molecular determinants for the activity of the studied NAMs. Three of these residues, I651, N747 and S809, have not previously been implicated in negative allosteric modulation of mGluR5, and especially S809 is highlighted as a potential important interaction partner. Our results show that the several fold higher potency of 2-BisPEB compared to 3- and 4-BisPEB might be explained by a direct hydrogen bond between S809 and 2-BisPEB. The homology model used in the present study differs from other mGluR5 models previously published, but it can rationalize the structure-activity-relations observed for the three BisPEB NAMs. Our model can also serve as starting point for studies of other NAMs, e.g. fenobam.

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Authorship Contributions

Participated in research design: Mølck, Harpsøe, Pedersen, Nielsen, Mathiesen, and Bräuner-Osborne.

Conducted experiments: Mølck, Harpsøe, Gloriam, and Jimenez.

Contributed new reagents or analytic tools: Clausen, and Madsen.

Performed data analysis: Mølck, Harpsøe, Gloriam, and Pedersen.

Wrote or contributed to the writing of the manuscript: Mølck, Harpsøe, Gloriam, Clausen, Madsen, Nielsen, Mathiesen, and Bräuner-Osborne.

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Footnotes

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Figure Legends

Figure 1. Chemical structures of the studied compounds: 2-BisPEB (1,3-bis(pyridin-2-ylethynyl)benzene), 3-BisPEB (1,3-bis(pyridin-3-ylethynyl)benzene), 4-BisPEB (1,3-bis(pyridin-4-ylethynyl)benzene), and MPEP (2-methyl-6-(phenylethynyl)pyridine).

Figure 2. 2- ( ), 3- (■), and 4-BisPEB (×) displace ^{3}H-MPEP. This was shown by competitive binding studies on membrane prepared from BHK cells transiently transfected with wild type mGluR5. The individual points represent mean ± SEM of normalized data pooled from 2-3 independent experiments (duplicate measurements) using 1-2 membrane batches. K_i was calculated using the Cheng-Prusoff equation and represent the mean from 2-3 independent experiments. K_D for ^{3}H-MPEP was found to be 2.9 nM (n = 3) by saturation binding studies.

Figure 3. Possible binding poses of the studied ligands obtained by molecular docking to the mGluR5 homology model selected by docking score and the number of mutations they can rationalize. a and b: Suggested binding mode of 2-BisPEB (cyan carbons) in the 7TMD as seen from the side (membrane view) and from the top (extracellular view), respectively. c: Comparison of the suggested binding modes of 2-, 3- (magenta carbons) and 4-BisPEB (green carbons). d: Two possible binding poses of MPEP with the best scoring pose (pose 1, yellow carbons) between TMHs 3, 5 and 6 and the second ranked pose (pose 2, purple carbons) between TMHs 2, 3, 6 and 7. TMHs 1, 5, 6, and 7 are represented as white cartoon with the mutated residues discussed in the text shown as sticks. TMHs 2, 3 and 4 are represented as thin, transparent and brown cartoon with residues discussed in the text shown as thin sticks. Dashed red lines indicate direct hydrogen bonds.

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Table 1. Mutational effect on the potencies of 2-, 3-, 4-BisPEB, and MPEP. BHK cells were transiently transfected with wild type and mutated mGluR5 using the calcium phosphate method. Intracellular Ca^{2+}-response was measured on an imaging-based plate reader two days post transfection. The potency is presented as IC_{50} and pIC_{50} and represents data from n independent transfections and experiments (duplicate measurements).

Statistics: Mutants were compared to wild type mGluR5 (pIC_{50}) by one-way ANOVA combined with Dunnett's multiple comparison test or by one-sample t-test for mutants with IC_{50} values ≥ 45 µM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

	2-BisPEB				**3-BisPEB**			
	IC_{50}, nM	IC_{50}/IC_{50}(wt)	pIC_{50}, M Mean ± SEM	n	IC_{50}, nM	IC_{50}/IC_{50}(wt)	pIC_{50}, M Mean ± SEM	n
wt	79	-	7.10 ± 0.07	19	2200	-	5.66 ± 0.07	19
T632A	83	1.1	7.08 ± 0.08	3	1800	0.90	5.75 ± 0.03	3
T632P	13	0.16	7.9 ± 0.3***	3	790	0.35	6.1 ± 0.1	3
R648E	12	0.15	7.92 ± 0.03***	3	590	0.27	6.23 ± 0.08**	5
I651F	3200	41	5.5 ± 0.1***	3	> 45000	>20	- ***	2
P655S	160	2.0	6.8 ± 0.1	3	> 45000	>20	- ***	2
Y659A	1100	14	5.97 ± 0.03***	3	> 45000	>20	- ***	2
Y659F	38	0.48	7.42 ± 0.01	3	1200	0.54	5.93 ± 0.09	3
N747A	830	11	6.08 ± 0.07***	3	> 45000	>20	- ***	2
N747S	430	5.4	6.37 ± 0.01***	3	25000	11	4.6 ± 0.2***	3
G748L	79	1.0	7.1 ± 0.2	3	4000	1.8	5.4 ± 0.3	3
W785A	890	11	6.05 ± 0.09***	3	4300	2.0	5.37 ± 0.09 ^	3
F788A	98	1.2	7.01 ± 0.02	3	1900	0.86	5.72 ± 0.06	6
F788W	260	3.3	6.59 ± 0.09*	3	> 45000	>20	- ***	2
Y792A	160	2.0	6.79 ± 0.03	3	> 45000	>20	- ***	3
Y792F	110	1.4	6.97 ± 0.04	3	2500	1.1	5.6 ± 0.2	3
S809F	5000	63	5.3 ± 0.2***	3	6300	2.9	5.2 ± 0.2	3
S809A	1500	19	5.81 ± 0.03***	6	2500	1.1	5.6 ± 0.2	3
A810V	790	10	6.1 ± 0.1***	3	> 45000	>20	- ***	3
A813F	120	1.5	6.91 ± 0.08	3	3400	1.5	5.47 ± 0.07	3

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Table 1 continued

	4-BisPEB				**MPEP**			
	IC_{50}, nM	IC_{50}/IC_{50}(wt)	pIC_{50}, M Mean ± SEM	n	IC_{50}, nM	IC_{50}/IC_{50}(wt)	pIC_{50}, M Mean ± SEM	n
wt	2100	-	5.67 ± 0.06	14	8.1	-	8.09 ± 0.05	19
T632A	3500	1.7	5.45 ± 0.05	3	4.5	0.56	8.35 ± 0.02	3
T632P	2500	1.2	5.61 ± 0.08	3	2.0	0.25	8.7 ± 0.1***	3
R648E	2000	1.0	5.7 ± 0.2	4	0.91	0.11	9.04 ± 0.06***	3
I651F	> 45000	>21	- ***	2	500	62	6.3 ± 0.2***	3
P655S	3200	1.5	5.5 ± 0.1	6	60	7.4	7.22 ± 0.04***	3
Y659A	> 45000	>21	- ***	2	950	120	6.02 ± 0.03***	3
Y659F	630	0.30	6.2 ± 0.1*	3	5.5	0.68	8.26 ± 0.09	3
N747A	2800	1.3	5.55 ± 0.08	6	13	1.6	7.88 ± 0.06	3
N747S	5000	2.4	5.3 ± 0.1	3	34	4.2	7.47 ± 0.06***	3
G748L	4000	1.9	5.4 ± 0.1	3	40	4.9	7.4 ± 0.2***	3
W785A	6000	2.9	5.22 ± 0.09*	3	2300	280	5.64 ± 0.08*** ^	3
F788A	~ 45000	~ 21	- ***	2	220	27	6.66 ± 0.06***	3
F788W	>45000	>21	- ***	2	79	9.8	7.1 ± 0.2***	3
Y792A	6300	3.0	5.20 ± 0.04*	3	66	8.1	7.18 ± 0.04***	3
Y792F	1600	0.76	5.8 ± 0.1	3	13	1.6	7.90 ± 0.04	3
S809F	5000	2.4	5.3 ± 0.1	3	4000	490	5.4 ± 0.1***	3
S809A	1000	0.48	6.0 ± 0.2	3	420	52	6.38 ± 0.09***	5
A810V	7600	3.6	5.12 ± 0.08**	3	510	63	6.29 ± 0.04***	3
A813F	3500	1.7	5.46 ± 0.03	3	10	1.2	7.99 ± 0.06	3

^Only partial inhibition of the glutamate response was observed

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Table 2. Mutational effect on glutamate-induced Ca^{2+}-response and cell surface expression of mGluR5. BHK cells were transiently transfected with wild type and mutated mGluR5 using the calcium phosphate method. Intracellular Ca^{2+}-response was measured on an imaging-based plate reader. Cell surface expression of wild type and mutated receptors was determined by cell surface ELISA. Results are presented as mean ± SEM and represent data from minimum two independent transfections. n state the number of experiments (duplicate measurements).

Statistics: Mutants were compared to wild type mGluR5 (E_{max}, pEC_{50}, and OD) by one-way ANOVA combined with Dunnett's multiple comparison test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

	Mutational location	**Relative E_{max}**		**Glutamate potency**			**OD (cell surface ELISA)**	
		Mean ± SEM	n	EC_{50}, µM	pEC_{50}, M Mean ± SEM	n	Mean ± SEM	n
wt	-	100%		1.0	6.00 ± 0.02	26	0.90 ± 0.04	6
G628F	TMH2	N/A		N/A	-		0.094 ± 0.004 ***	3
T632A	TMH2	58 ± 8% **	3	1.8	5.75 ± 0.07	3	0.57 ± 0.03 ***	3
T632P	TMH2	67 ± 11% **	4	1.8	5.74 ± 0.09 *	4	0.72 ± 0.06 *	3
R648E	TMH3	23 ± 4% ***	6	3.2	5.50 ± 0.08 ***	6	0.16 ± 0.01 ***	3
I651F	TMH3	105 ± 9%	6	1.4	5.85 ± 0.05	6	0.93 ± 0.08	3
P655S	TMH3	77 ± 10% *	6	1.4	5.85 ±0.04	6	0.72 ± 0.02 *	3
Y659A	TMH3	81 ± 9%	6	1.0	6.02 ± 0.06	6	0.99 ± 0.05	3
Y659F	TMH3	91 ± 13%	4	1.1	5.96 ± 0.05	4	0.78 ± 0.04	3
N747A	TMH5	93 ± 10%	6	1.0	6.01 ± 0.04	6	0.84 ± 0.02	3
N747S	TMH5	108 ± 5%	5	0.9	6.04 ± 0.05	5	0.76 ± 0.08	3
G748L	TMH5	100 ± 5%	4	0.9	6.05 ± 0.07	4	0.94 ± 0.03	3
W785A	TMH6	59 ± 4% ***	6	2.5	5.60 ± 0.03 ***	6	0.54 ± 0.02 **	3
F788A	TMH6	102 ± 13%	5	1.4	5.86 ± 0.03	5	0.93 ± 0.03	3
F788D	TMH6	N/A		N/A	-		0.10 ± 0.01 ***	3
F788W	TMH6	84 ± 8%	6	0.9	6.05 ± 0.05	6	0.74 ± 0.04	3
Y792A	TMH6	58 ± 2% ***	6	0.6	6.24 ± 0.06 ***	6	0.60 ± 0.05 **	3
Y792F	TMH6	109 ± 3%	5	1.0	6.00 ± 0.04	5	0.6 ± 0.1 *	3
S809F	TMH7	37 ± 6% ***	5	1.3	5.89 ± 0.05	5	0.47 ± 0.06 **	3

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Table 2 continued

S809A	TMH7	85 ± 9%	6	1.0	6.02 ± 0.03	6	0.6 ± 0.2	3
A810V	TMH7	92 ± 7%	6	1.5	5.82 ± 0.04 *	6	0.8 ± 0.1	3
A813F	TMH7	69 ± 6% *	3	0.5	6.3 ± 0.2 ***	3	0.6 ± 0.1 **	3

N/A: These mutants did not respond to glutamate (based on observations from minimum two independent transfections and experiments).

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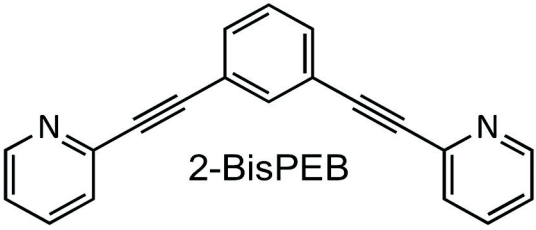
Figure 1



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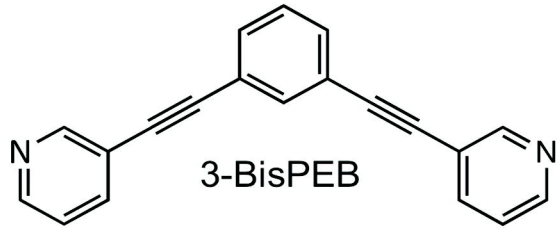
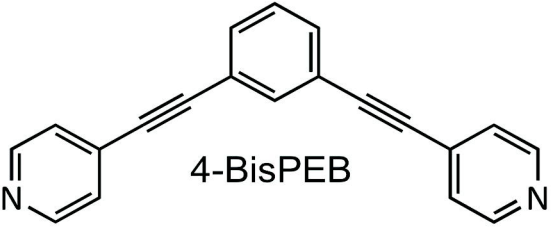
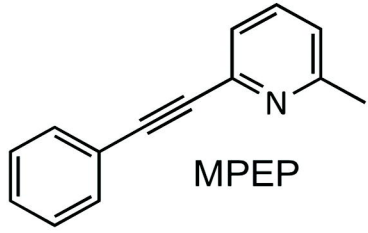


3-BisPEB

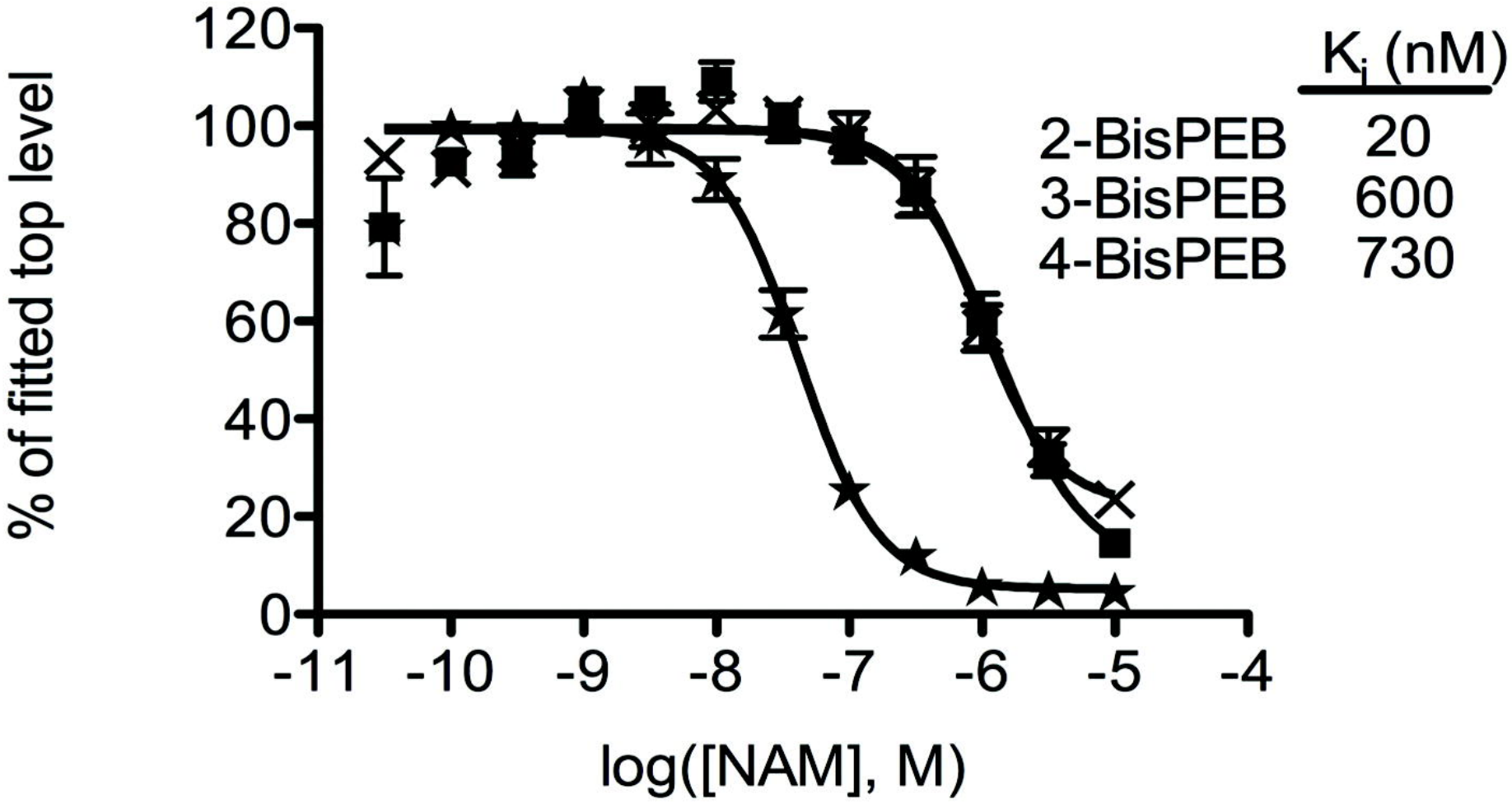


4-BisPEB



MPEP

Figure 2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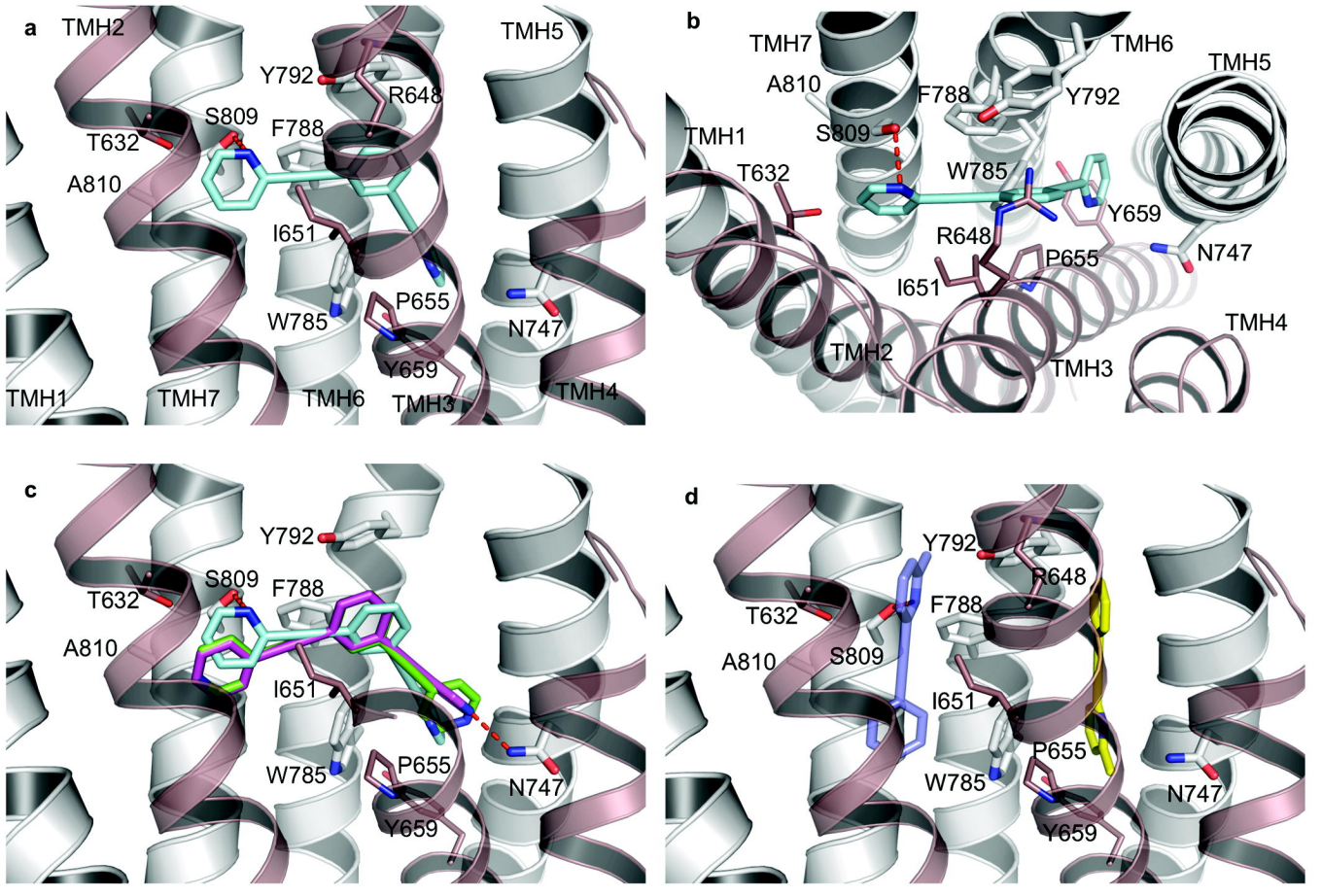
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log([NAM], M)
Ki (nM)
2-BisPEB 20
3-BisPEB 600
4-BisPEB 730

Figure 3 a-d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S809
F788
T632
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TMH1
TMH7
TMH6
TMH3
TMH4
b
TMH7
TMH6
TMH5
A810
F788
Y792
TMH1
S809
T632
W785
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P655
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c
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d
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