1   **Structural basis for subtype-specific inhibition of the P2X7 receptor**

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7   **Abstract**: The P2X7 receptor is a non-selective cation channel activated by extracellular

8   adenosine triphosphate (ATP). Chronic activation of P2X7 underlies many health problems such

9   as pathologic pain, yet we lack effective antagonists due to poorly understood mechanisms of

10 inhibition. Here we present crystal structures of a mammalian P2X7 receptor complexed with

11 five structurally-unrelated antagonists. Unexpectedly, these drugs all bind to an allosteric site

12 distinct from the ATP-binding pocket in a groove formed between two neighboring subunits. This

13 novel drug-binding pocket accommodates a diversity of small molecules mainly through

14 hydrophobic interactions. Functional assays propose that these compounds allosterically

15 prevent narrowing of the drug-binding pocket and the turret-like architecture during channel

16 opening, which is consistent with a site of action distal to the ATP-binding pocket. These novel

17 mechanistic insights will facilitate the development of P2X7-specific drugs for treating human

18 diseases.

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20 **Impact Statement:** Crystal structures of a mammalian P2X7 receptor and structure-based

21 functional studies reveal a novel drug-binding site and subtype-specific conformational

22 rearrangements required for channel opening.

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Introduction

Chronic pain is a major public health epidemic, debilitating more than 10% of adults globally with afflictions like persisting headaches, lower back pain, and rheumatoid arthritis (Gold and Gebhart, 2010; Goldberg and McGee, 2011). While some patients are responsive to commonly used analgesics, many are resistant to treatment (Dworkin et al., 2007; Finnerup et al., 2010). The P2X7 receptor, an extracellular ATP-gated ion channel predominantly expressed in immune cells of the blood and the brain (Surprenant et al., 1996; Habermacher et al., 2016), is an emerging target for treating refractory pain (Chessell et al., 2005; Gum et al., 2012; Burnstock, 2013; North and Jarvis, 2013; Sperlagh and Illes, 2014). Over the last decade, enormous effort has been made to develop a number of structurally distinct P2X7-specific antagonists, some of which have been demonstrated to alleviate chronic pain in animal models (Honore et al., 2006a; McGaraughty et al., 2007; Bartlett et al., 2014). However, mechanisms of action for these drugs remain poorly understood, hampering the development of effective therapeutic compounds for human patients (Keystone et al., 2012; Stock et al., 2012; Bhattacharya and Biber, 2016).

P2X receptors are trimeric ligand-gated ion channels that facilitate extracellular-ATP mediated signaling along with the G protein-coupled P2Y receptors (North, 2002; Burnstock, 2014). The P2X7 receptor belongs to the P2X receptor family, however, it was originally identified as a unique ATP-receptor named "the P2Z receptor", as it harbors many characteristics distinct from P2X and P2Y receptors (Di Virgilio, 1995). For instance, the P2X7 receptor requires an unusually high concentration of ATP (EC_{50} ≥1 mM under physiological ion concentrations) for initial activation (Yan et al., 2010), its channel activity is facilitated by prolonged or repeated ATP applications (Surprenant et al., 1996), and it opens a membrane pore large enough for molecules up to ~900 Da to permeate (Steinberg et al., 1987; Nuttle and Dubyak, 1994; Yan et al., 2008). While crystal structures of the P2X3 and P2X4 receptors have uncovered common mechanisms such as ATP-binding and gating for the P2X receptor family (Kawate et al., 2009; Hattori and Gouaux, 2012; Mansoor et al., 2016), many questions continue

51   to exist regarding subtype specific mechanisms, especially for the enigmatic P2X7 receptor

52   subtype. How do structurally-unrelated drugs antagonize only the P2X7 receptor but not the

53   other P2X subtypes? Do they target an activation mechanism unique to the P2X7 subtype?

54   Here we identified and mapped the binding site for the P2X7-specific inhibitors for the first time

55   using X-ray crystallography, and demonstrated by electrophysiological experiments that those

56   inhibitors allosterically abrogate conformational changes associated with P2X7 receptor

57   activation.

58

59   **Results**

60   ***Architecture of a mammalian P2X7 receptor***

61   To define the structural basis for drug binding, we first sought to determine the crystal structures

62   of a mammalian P2X7 receptor in the presence of five structurally distinct antagonists (A740003

63   (Honore et al., 2006a), A804598 (Donnelly-Roberts et al., 2009), AZ10606120 (Michel et al.,

64   2007), GW791343 (Michel et al., 2008a; Michel et al., 2008b), and JNJ47965567 (Bhattacharya

65   et al., 2013)). Using fluorescence detection size exclusion chromatography (FSEC) (Kawate and

66   Gouaux, 2006), we screened full-length and a series of C-terminally truncated versions of nine

67   human P2X7 (hP2X7) orthologues (72-89% identical), and found that an artificially truncated

68   version of the panda (*Ailuropoda melanoleuca*) P2X7 receptor (pdP2X7) not only expressed

69   better than other orthologues in insect cells but also remained trimeric and monodisperse in

70   detergents commonly used for crystallography (Figure 1A). Whole cell patch clamp recordings

71   confirmed that pdP2X7 presents comparable characteristics to hP2X7 (Figure 1B-G). The

72   truncated pdP2X7 was further optimized to obtain a construct termed pdP2X7$_{cryst}$ (Δ1-21/Δ360-

73   600/N241S/N284S/V35A/R125A/E174K) that we used to solve the crystal structures at ~3.5 Å

74   resolution. pdP2X7$_{cryst}$ exhibited slower deactivation and no obvious current facilitation (run-up)

75   after repeated ATP applications (Figure 1C and F).

76   Overall, a single subunit of the P2X7 receptor resembles the "dolphin-like" shape of

77   zebrafish P2X4 (zfP2X4; 45% identical to pdP2X7) (Kawate et al., 2009; Hattori and Gouaux,

78   2012) and human P2X3 (hP2X3; 38% identical to pdP2X7) (Mansoor et al., 2016), the other

79   P2X receptor subtypes with known architecture (Figure 2A and Figure 2-Figure Supplement 1A).

80   The P2X7 structure obtained in the absence of antagonists (apo-form) likely represents a closed

81   conformation, as the transmembrane helices constrict the channel gate at residues G338, S339,

82   and S342 (Figure 2B and Figure 2-Figure Supplement 1B). Structural resemblance of the

83   transmembrane helices between our current P2X7 and the zfP2X4 in the closed state (root-

84   mean-square displacement (RMSD) of the protomers is 2.8 Å) also supports that our apo-

85   structure represents a closed conformation (Figure 2-Figure Supplement 1C). Likewise,

86   antagonist-bound P2X7 structures represent the same closed conformation with a RMSD of less

87   than 0.5 Å to the apo structure, indicating that these drugs likely stabilize a resting closed state.

88

89   ***A novel drug binding pocket***

90   Surprisingly, all five structurally-unrelated compounds bind in the same pocket formed between

91   neighboring subunits, which is juxtaposed with the ATP-binding pocket (Figure 2C-D and Figure

92   2-Figure Supplement 2). This drug-binding pocket is surrounded by thirteen residues projecting

93   mainly from β-strands (β4, β13 and β14) in the upper body domains of the neighboring subunits

94   (Figure 3A). While the precise distances and angles between the side-chains and the drugs

95   cannot be determined at the current resolutions (~3.2-3.6 Å), electron density was clear enough

96   to localize and orient the side chains of the drug-coordinating residues (Figure 3-Figure

97   Supplement 1 and Video1). Drug binding seems to be mediated mainly by hydrophobic

98   interactions, especially at positions deep within the cavity, involving F95, F103, M105, F293,

99   and V312. Despite structural diversity, all five P2X7 antagonists fit within the drug-binding

100   pocket (Figure 3B), highlighting that the size and the shape of the drug-binding pocket play

101   major roles in determining the affinity and specificity of the drugs. Indeed, the equivalent pocket

102   in the P2X4 receptor is too narrow to accommodate the smallest P2X7 antagonist, A804598,

103   even though it is similarly hydrophobic to that of the P2X7 receptor (Figure 3-Figure Supplement

104   2 and 3). Thus, we suggest that the differences in the size of the inter-subunit hydrophobic

105   pocket is the major factor that confers P2X7-specific binding of the inhibitors.

106   We further validated the drug-receptor interactions by site-directed mutagenesis on the

107   drug-binding residues. To facilitate robust and systematic data collection, we monitored cellular

108   uptake of the fluorescent dye, YO-PRO-1, as a proxy for receptor activity (Surprenant et al.,

109   1996) (Figure 3-Figure Supplement 4A). Consistent with the crystal structures, the mutants,

110   F88A, F95A, F103A, M105A, F108A, and V312A, showed increased IC_{50} values (see Table 1

111   for IC_{50} values on the wildtype), supporting that these residues play important roles in drug

112   binding (Figures 3C and Figure 3-Figure Supplement 4B). In particular, interaction with F103 is

113   crucial for the inhibitory action of all five drugs.

114

Allosteric non-competitive inhibition

116   Binding of all five drugs to a site distinct from the ligand-binding pocket suggests that these

117   compounds act as non-competitive inhibitors. However, previous studies using cell-based Ca^{2+}

118   influx and IL-1β release assays proposed that three of those compounds, A740003, A804598,

119   and JNJ47965567, compete against ATP-binding to inhibit the P2X7 receptor (Honore et al.,

120   2006a; Donnelly-Roberts et al., 2009; Bhattacharya et al., 2013). To clarify the working

121   mechanism for each drug, we measured the dose responses of the P2X7-mediated YO-PRO-1

122   uptake in the presence of each drug at multiple concentrations. We used BzATP—an artificial

123   but potent agonist of P2X7 receptors—for these experiments to achieve saturating responses in

124   the presence of concentrated antagonist. For each drug, dose response curves fit well with a

125   non-competitive inhibition model (Kenakin, 2006), but poorly with a competitive model (Figure 3-

126   Figure Supplement 5). Furthermore, the Schild plots displayed non-linear relationships,

127 especially at higher concentrations, consistent with a non-competitive mechanism (Figure 3D)

128 (Schild, 1947).

129   To confirm the non-competitive mode of inhibition, we performed an ATP-binding assay

130 on purified P2X7 receptor pretreated with each drug. We exploited fluorescence anisotropy

131 (Figure 3-Figure Supplement 6A-C) using a fluorescently-labeled ATP analogue (Alexa-ATP),

132 which is as potent as ATP and is capable of triggering P2X7 channel opening at 10 μM in the

133 absence of divalent cations (Figure 3E). At this concentration, Alexa-ATP gave rise to

134 fluorescence anisotropy when incubated with P2X7 in a dose-dependent manner (Figure 3-

135 Figure Supplement 6D). Notably, drug-treated P2X7 receptor exhibited similar levels of

136 fluorescence anisotropy, which drastically decreased in the presence of unlabeled-ATP (Figure

137 3F and Figure 3-Figure Supplement 6E). Together, these experiments strongly support that all

138 five P2X7 antagonists are allosteric non-competitive inhibitors.

139

140 ***P2X7 specific conformational change during channel activation***

141 We propose that the five studied drugs antagonize the P2X7 receptor through a common

142 mechanism and that the unique inter-subunit cavity may be a critical locus for functional

143 regulation. Interestingly, the inter-subunit cavity formed by β13 and β14 in the upper body

144 domain is much wider in P2X7 than in zfP2X4 or in hP2X3 (Figure 4A-C) (Mansoor et al., 2016).

145 Furthermore, this "turret-like" structure and the cleft corresponding to the P2X7 drug-binding

146 pocket remain relatively occluded in zfP2X4 and in hP2X3 after activation by ATP (Figure 4A-C

147 and Figure 4-Figure Supplement 1) (Mansoor et al., 2016). Do the drug-binding pocket and the

148 turret in P2X7 become narrower during channel activation? To explore the involvement of the

149 inter-subunit cavity in P2X7 receptor activation, we monitored movement of the cavity residues

150 with and without ATP. We first created a series of single cysteine mutants in the drug-binding

151 pocket (Figure 4D) and measured ATP-gated channel activity after applying a large cysteine-

152 reactive agent (MTS-TPAE; Mw: 447 Da). We reasoned that modification of a cysteine residue

153 with a bulky moiety should interfere with the conformational changes necessary for channel

154 opening, thereby resulting in diminished channel activity. When MTS-TPAE was applied in the

155 absence of ATP, four cysteine mutants (F103C, K110C, T308C, and I310C) showed irreversible

156 current reduction (Figure 4E and F), consistent with the idea that the covalently bound MTS-

157 TPAE at these positions hinders the conformational changes required for channel opening.

158 When MTS-TPAE was applied in the presence of ATP, on the other hand, none of the cysteine

159 mutants presented significant current reduction (Figure 4E and F). These results indicate that at

160 least four residues in the drug-binding pocket are more accessible to MTS-TPAE in the closed

161 state than in the open state. Our data therefore suggest that the drug-binding pocket narrows

162 upon ATP binding and that such a conformational rearrangement is crucial for P2X7 channel

163 opening.

164   To examine the movement of the turret during P2X7 activation, we took advantage of a

165 cysteine mutation at Y295, whose side chain faces the center of the turret (Figure 5A). Because

166 two of the three introduced cysteines may form a disulfide bond, we pretreated the cells with a

167 reducing agent prior to cysteine accessibility experiments. Under these conditions, MTS-TPAE

168 diminished the Y295C channel activity by ~80% in the absence of ATP but to a lesser extent

169 (~40%) in the presence of ATP (Figure 5B and C). These data support that the turret also

170 narrows during P2X7 activation. Widening of the turret upon ATP-binding is unlikely, as 1) MTS-

171 TPAE had no effect on the Y295C mutant prior to treatment with a reducing agent (Figure 5D),

172 indicating formation of a disulfide bond that would bring the two neighboring subunits closer

173 (Cβ-Cβ distance would change from ~14 Å to ~4 Å) and 2) Y295C mutant exhibited comparable

174 current density with the wildtype in the absence of a reducing agent (Figure 5-Figure

175 Supplement 1A). Notably, narrowing of the inter-subunit space seems unique to the P2X7

176 receptor, as suggested by the crystal structures of P2X3 and P2X4 solved in the presence of

177 ATP. Indeed, MTS-TPAE application either in the presence or absence of ATP did not exhibit

178 current reduction for cysteine mutants of the counterpart residues in the P2X4 receptor (Figure

179 5B and E, Figure 5-Figure Supplement 1B and C).

180   To confirm that allosterically-bound P2X7 inhibitors prevent the turret from narrowing

181 upon ATP-binding, we obtained the crystal structure of the P2X7 receptor in the presence of

182 both ATP and A804598 (Figure 6A-D and Figure 6-Figure Supplement 1). Consistent with the

183 activation mechanisms proposed for P2X3 and P2X4, ATP-binding brings the dorsal fin domain

184 towards the head domain and pushes the left flipper domain away from the ATP-binding pocket

185 (Figure 6C). These movements are coupled with widening of the lower body domain (Figure 6D),

186 though to an extent in which the transmembrane helices remain closed. In contrast, little

187 conformational change was observed for the upper body domain including the turret and the

188 drug-binding pockets (Figure 6A), supporting that the turret closure is essential for P2X7

189 channel opening. Altogether, our data suggest that P2X7 receptors undergo unique

190 conformational rearrangements where both the drug-binding pocket and the turret in the P2X7

191 receptor narrow upon ATP-binding. Because such conformational changes are required for

192 channel opening, binding of the P2X7 antagonists preclude these constrictions, thereby

193 efficiently blocking receptor activation (Figure 7 and Video 2).

194

195 **Discussion**

196 The presented crystal structures uncover the unique inter-subunit cavity in the upper body

197 domain of the P2X7 receptor. Our data suggests that this cavity shrinks during activation, which

198 allows the lower body domain to widen further. Indeed, the ATP-binding left flipper domain is

199 connected with the pore-lining transmembrane helix through the turret composed of two β-

200 strands (β13 and β14), which supports that movements of the upper body domain are tightly

201 coupled with the channel opening (Figure 2A). Although the lower body domain in our

202 ATP/A804598-bound structure does seem to widen to some extent upon ATP-binding, the turret

203   diameter remains locked due to A804598 binding, rendering the accompanying movement of

204   the pore-lining transmembrane helices insufficient for channel opening (Figure 6 and video 2).

205   Closure of the turret, therefore, seems to be a prerequisite for full-widening of the lower body

206   domain, which is necessary for channel opening. Interestingly, the turrets of both P2X3 and

207   P2X4 are narrower even in the closed state, which may facilitate subsequent conformational

208   changes in the lower body domain. Obviously, the crystal structure of an ATP-bound, open-state

209   P2X7 would strengthen our hypothesis. However, it is technically challenging to obtain such a

210   crystal structure, as ATP-bound P2X7 receptors are prone to aggregation in a detergent

211   containing solution. Future efforts to uncover molecular details about the open state of P2X7

212   may yield further insight into subtype-specific activation mechanism.

213   Our data demonstrate that all five tested drugs are allosteric and non-competitive

214   inhibitors, which is in contrast to the previous studies showing that A740003, A804598, and

215   JNJ47965567 are competitive inhibitors (Honore et al., 2006b; Donnelly-Roberts et al., 2009;

216   Bhattacharya et al., 2013). This discrepancy may be attributed to the high agonist

217   concentrations (i.e. up to 3 mM ATP or 1 mM BzATP) required for obtaining reliable EC_{50} values

218   in the presence of antagonists. With such a high concentration of agonists, we noticed that YO-

219   PRO-1 uptake activity starts to decline ~10 min after agonist application. To minimize potential

220   experimental artifacts, we took advantage of the initial rates instead of total counts at a certain

221   time point used by the previous studies. Nevertheless, the Schild plots of these three

222   compounds appear to be linear at lower concentrations (Figure 3D), making it difficult to rely

223   solely on the Schild regression analysis for determining the mode of drug action. We therefore

224   provided extra evidence of non-competitive allosteric drug action using 1) curve fitting with

225   known models of competitive and non-competitive inhibition, 2) ligand-binding assays in the

226   presence of antagonists, and 3) the crystal structure of P2X7 obtained in the presence of both

227   an agonist and an antagonist.

228   Combined with the crystal structures, information about the drug-receptor specific

229   interactions provide an excellent platform for rationally improving physicochemical properties of

230   currently available P2X7 antagonists. For example, replacing the phenyl group in JNJ47965567

231   with a slightly bulkier group may increase the binding affinity of this compound, as that would fill

232   the void around residue F95. Likewise, low potency of AZ10606120 and GW791343 (Table 1)

233   may be overcome by replacing chemical moieties with those fit better into the drug-binding

234   pocket. Our data also suggest that it may be possible to attach an extra chemical group to the

235   site away from the residue interacting with F103 without affecting binding of the P2X7

236   antagonists. This leads to an interesting possibility that these drugs could be conjugated with

237   cell penetrating peptides or specific ligands expressed on endothelial cells, which may improve

238   the delivery of drugs across the blood-brain barrier (Chen and Liu, 2012). In addition,

239   identification of an unexpected binding pocket within P2X7, that is absent in the closely related

240   P2X4, should allow for engineering of isoform-specific drugs. Development of a new generation

241   of P2X7 antagonists with increased clinical efficacy has the potential to have a significant impact

242   on the treatment of chronic pain and inflammation.

243

244 **Materials and Methods**

245 *Construct design*

246 Ten mammalian P2X7 genes (human: Q99572.4; giant panda: XP_002913164.1; horse:

247 XP_001495622.1; cattle: NP_001193445.1; dog: NP_001106927.1; rabbit: XP_002719791.1;

248 rat: Q64663.1; mouse: Q9Z1M0.2; opossum: XP_001373213.1; guinea pig: NP_001166578.1)

249 were synthesized based on the protein sequences (GenScript) and subcloned into a modified

250 pFastBac baculovirus expression vector (Invitrogen) that harbors a Strep-tag and EGFP at the

251 N-terminus (pNGFP-FB3). These P2X7 constructs were expressed in Sf9 insect cells and

252 screened using the FSEC strategy (Kawate and Gouaux, 2006). Briefly, Sf9 cells at a cell

253 density of 1.0×10^6 cells/mL in 6 well plates were infected with 20 μl/well of P1 virus (10^6-10^7

254 PFU/mL), harvested after 48 hours, and solubilized in 150 μl of S1 buffer (1% n-Dodecyl-β-D-

255 Maltopyranoside (DDM; Anatrace) and Halt protease inhibitor cocktail (ThermoFisher) in

256 phosphate-buffered saline; (pH 7.4)) for 30 min at 4°C. The soluble fractions were collected by

257 ultra-centrifugation at 87,000 x g for 30 min and analyzed by FSEC. Because the full-length

258 P2X7 receptors were poorly expressed in Sf9 cells and did not crystallize in our initial trials, C-

259 terminally truncated versions of the nine P2X7 receptor orthologues (~240 residues removed

260 from the C-terminus) were rescreened by FSEC. Based on the sharp and symmetrical peak

261 profile, the C-terminally truncated panda P2X7 (pdP2X7) was selected for crystallization trials. A

262 series of terminal deletions and glycosylation mutants of pdP2X7 were screened for improving

263 the expression level, stability, and monodispersity. The best behaved construct (Δ1-21/ Δ360-

264 600/N241S/N284S) formed crystals in the $P4_2$ space group that diffracted to ~7 Å, which was

265 sufficient for obtaining an initial electron density map by molecular replacement using the crystal

266 structure of the zebrafish P2X4. To improve crystal packing, we screened combinations of point

267 mutants at the predicted crystal contact sites for crystallization/diffraction behavior. Finally, we

268   obtained a construct termed pdP2X7$_{cryst}$ (Δ1-21/ Δ360-600/N241S/N284S/V35A/R125A/E174K),

269   which formed crystals in a different space group (I2$_1$3) that diffracted to ~3.5 Å.

270

271   ***Expression and purification***

272   pdP2X7$_{cryst}$ was expressed as an EGFP-fusion protein using a baculovirus-insect cell system.

273   Sf9 cells were infected at 3.5-4.0 x 10^6 cell/mL with 25-30 mL/L P2 virus (10^7-10^8 PFU/mL) for

274   one day at 27°C and for another two days at 18°C. Cells were harvested by centrifugation at

275   2,040 x g, washed with PBS, and resuspended with PBS containing leupeptin (0.5 μg/mL),

276   aprotinin (2 μg/mL), pepstatin A (0.5 μg/mL), and phenylmethylsulfonyl fluoride (0.5 mM). The

277   cells were broken by nitrogen cavitation at 600 psi using a 4635 cell disruption vessel (Parr

278   Instrument). After removing unbroken cells and debris by centrifugation at 12,000 x g for 10 min,

279   the membrane fraction was collected by centrifugation at 185,000 x g for one hour and

280   solubilized in S2 buffer (2% Triton X-100 (Anatrace) in PBS (pH 7.4)) for one hour. The soluble

281   fraction was collected after centrifugation at 185,000 x g for one hour and incubated with

282   StrepTactin Sepharose High Performance resin (GE Healthcare) for 30 min using a batch

283   procedure. The resin was transferred into a gravity column (Bio-rad) and washed with 10

284   column volumes of W buffer (100 mM Tris-HCl; pH 8.0, 150 mM NaCl, 1 mM EDTA, and 0.5

285   mM DDM), and pdP2X7$_{cryst}$-EGFP was eluted with E buffer (100 mM Tris-HCl; pH 8.0, 150 mM

286   NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, 15% glycerol, and 0.5 mM DDM). The N-terminal

287   EGFP and the strep tag was removed by incubating the P2X7$_{cryst}$-EGFP with human thrombin

288   (HTI; 1/30 w/w) overnight. P2X7$_{cryst}$ was isolated by size exclusion chromatography using

289   Superdex 200 (GE Healthcare) in C buffer (50 mM Tris-HCl; pH 7.4, 150 mM NaCl, 15%

290   Glycerol, and 0.5 mM DDM). The peak fractions were pooled, concentrated to 10 mg/mL, and

291   used for crystallization after ultracentrifugation at 265,000 x g for 20 min. All purification steps

292   were carried out at 4 ºC or on ice.

293

Crystallization

P2X7$_{cryst}$ was crystalized using the hanging drop vapor diffusion method by mixing 1:1 (v/v) ratio of protein and reservoir solutions at 4°C. The apo crystals appeared in two weeks and were fully grown in 3-4 months using a reservoir solution containing 100 mM HEPES (pH 7.0), 100 mM NaCl, 4% ethylene glycol, 15% glycerol, 29% PEG-400, 0.1 mg/mL lipid mixture (60% POPE, 20% POPG, and 20% cholesterol). The P2X7$_{cryst}$ crystals with antagonists reached their maximum sizes in about two months using reservoir solutions containing 100 mM HEPES or Tris (pH 6.0-7.5), 100 mM NaCl, 4% ethylene glycol, 15% glycerol, 27-32% PEG-400 or 31-36% PEG-300, 0.1 mg/mL lipid mixture (60% POPE, 20% POPG, and 20% cholesterol), and 1 mM P2X7 antagonists (AZ10606120, JNJ-47965567, A804598, GW791343 (Tocris Biosciences), and A740003 (Sigma Aldrich)). For heavy atom derivatization, A740003 bound P2X7$_{cryst}$ crystals were soaked into a reservoir solution containing 1 mM K$_2$IrCl$_6$ and 0.5 mM DDM overnight at 4°C. For determination of the ATP/A804598 bound P2X7 structure, crystals of P2X7$_{cryst}$ were grown in 33% PEG-400, 100 mM MES (pH 6.5), 100 mM NaCl, 5% Glycerol, and 1 mM A804598 for 1 month at 4°C. Cubic-shaped crystals were soaked with 1 mM ATP overnight. Crystals were flash-frozen in liquid nitrogen for data collection.

Structure determination

X-ray diffraction data were collected using synchrotron radiation at the Cornell High Energy Synchrotron Source (beamline F1) and Advanced Photon Source at Argonne National Laboratory (beamlines 24ID-C and E). The following X-ray wave length was used for each data set: Apo: 1.1051 Å (24ID-C); A740003: 0.9792 Å (24ID-E); A804598: 0.9774 Å (F1); AZ10606120: 0.9782 Å (F1); GW791343: 0.9782 Å (F1); JNJ47965567: 0.9774 Å (F1); A740003/K$_2$IrCl$_6$: 1.1051 Å (24ID-C); ATP/A804598: 0.9791 Å (24ID-C). Diffraction data were indexed, integrated, and scaled using XDS (Kabsch, 2010), and merged using AIMLESS (Evans

319   and Murshudov, 2013) in the CCP4 suite (Winn et al., 2011). Low resolution electron density

320   map (~7 Å) of the $P4_2$ crystal was obtained by molecular replacement (RFZ=7.9, TFZ=9.3, and

321   LLG=257) using the zebrafish P2X4 model (PDB code: 4DW0) with the program PHENIX

322   (Adams et al., 2010). Initial phase information of the A740003 bound pdP2X7$_{cryst}$ ($I2_13$ space

323   group) was obtained by MR-SAD with PHENIX using the datasets from Ir-derivatized crystals

324   and the zebrafish P2X4 structure (PDB code: 4DW0) as a searching model. Model building was

325   carried out manually using COOT (Emsley and Cowtan, 2004) and the structure was refined

326   using PHENIX. The final model was used as a searching template in molecular replacement for

327   solving the structures of the apo state as well as the antagonist bound forms of pdP2X7$_{cryst}$ with

328   AZ10606120, JNJ47965567, A804598, GW791343. For ATP/A804598bound form, A804598-

329   bound structure was used as a searching model. Model quality was assessed using PHENIX

330   where stereochemistry and R-values are satisfactory (Table 2). Molecular models presented in

331   the figures and videos are created using the PyMOL Molecular Graphics System, Version 1.8

332   Schrödinger, LLC (Acquas et al., 1998). The atomic coordinate files have been deposited in the

333   Protein Data Bank under the accession codes 5U1L (apo), 5U1U (A740003-bound), 5U1V

334   (A804598-bound), 5U1W (AZ10606120-bound), 5U1X (JNJ47965567-bound), 5U1Y

335   (GW791343-bound), and 5U2H (ATP/A804598-bound).

336

337   ***YO-PRO-1 uptake assay***

338   Human embryonic kidney (HEK293) cells were maintained in DMEM medium (Gibco)

339   supplemented with 10% FBS (Atlanta Biologicals), and 10 μg/mL gentamicin (Gibco). HEK293

340   cells were split into 6 well plates at a cell density of 2.0×10^6 cells/well and incubated overnight.

341   Three hours after transfection with 2 μg of the full length pdP2X7 in pIM2 vector (IRES-mCherry;

342   modified from pIE2 vector) using jetPRIME reagent (Polyplus), cells were trypsinized,

343   transferred into poly-D-Lysine coated black-walled 96 well plates (Corning) at 7.5×10^4 cells/well,

344 and incubated for 24 hours. Cells were washed with assay buffer (2 mM KCl, 0.1 mM CaCl$_2$, 13

345 mM Glucose, 147 mM NaCl, 10 mM HEPES (pH 7.3)) and were incubated with 5 µM YO-PRO-1

346 Iodide (Invitrogen), in the presence or absence of antagonists at multiple concentrations at 37°C

347 for 10 min. Upon application of 1 mM ATP (final), uptake of YO-PRO-1 was recorded with 1 min

348 intervals by following the fluorescence change using a Synergy HT multi-detection microplate

349 reader (Bio-Tek; Ex: 485 nm/20, Em:528nm/20, sensitivity 60). To obtain the dose dependent

350 uptake inhibition by antagonists, the initial rates of YO-PRO-1 uptake were plotted against

351 multiple concentrations of the P2X7 specific drugs. The inhibition curves from five independent

352 measurements were fitted to the Hill equation using Origin software (Originlab) to determine the

353 IC$_{50}$ values.

354

355 **Cell line generation**

356 HEK293 (CRL-1573) cell lines were purchased from the American Type Culture Collection

357 (ATCC, Manassas, VA), and therefore were not further authenticated. Mycoplasma

358 contamination test was confirmed to be negative at ATCC.

359

360 **Data analysis**

361 Dose response curves of the YO-PRO-1 uptake experiments were fitted with either competitive

362 or non-competitive inhibition models using Origin 6.0 software (OriginLab) as previously

363 descried (Kenakin, 2006). For the competitive antagonism model, we used the equation:

$$Response = \frac{[A]/KA}{[A]KA(1 + \tau) + [B]/KB + 1}$$

364 where [A], [B] are the concentrations of BzATP and antagonists, respectively; K$_A$ and K$_B$ are the

365 equilibrium dissociation-constant of BzATP and antagonists, respectively. Dose response

366 curves without antagonist were fitted with this equation, which gives the values K$_A$=28 µM, and τ

367 =0.031. K$_B$ was then determined using the dose response curves in the presence of

368   antagonists. The resulting K_B value for each antagonist was; JNJ: 1.7 nM; A80: 15 nM; A74: 24

369   nM; AZ10: 56 nM; GW: 3.0 μM. For the non-competitive inhibition model, we used the equation:

370

371    $$Response = ([A]^n \tau^n Emax)/([A]^n \tau^n + ([A](1 + \alpha[B]/K_B) + K_A[B]/K_B + K_A)^n$$

372

373   where n is the Hill coefficient, and Emax is the maximum initial rate. K_A, τ, n values were

374   determined using the data without antagonists (K_A=220 μM, τ=10, and n=2.6) and the rest of the

375   parameters were determined by curve fitting of data with different concentrations of antagonists

376   (α=1 and K_B value for each antagonist was: JNJ: 0.14 μM; A80: 0.57 μM; A74: 1.2 μM; AZ10:

377   3.4 μM; GW: 266 μM). Schild regression analysis was performed as described previously

378   (Wyllie and Chen, 2007). Briefly, concentrations of BzATP at half maximal initial rates of YO-

379   PRO-1 uptake in the presence (EC_{50}') or absence (EC_{50}) of antagonists were determined by

380   fitting the dose responses with the Hill equation. Dose ratio $r=EC_{50}'/EC_{50}$ was calculated for

381   various antagonist concentrations and *r-1* values were plotted against the antagonist

382   concentrations in log scale to obtain Schild plots.

383

384   ***Ligand binding experiment***

385   GFP fused pdP2X7$_{cryst}$ (P2X7-GFP) was purified in a buffer containing 150 mM NaCl, 50 mM

386   Tris-HCl (pH 7.4), 15% glycerol, and 0.5 mM DDM as described in "Expression and purification."

387   GFP-tagged pdP2X7$_{cryst}$, which is substantially more stable than pdP2X7$_{cryst}$, was used in this

388   experiment as it does not interfere with the fluorescence properties of Alexa-ATP (Figure 3-

389   Figure Supplement 5B). P2X7-GFP (100 μM) was pre-incubated with each P2X7 specific

390   antagonist (100 μM) for 30 min at room temperature. P2X7-GFP was then incubated with 10 μM

391   ATP-Alexa 647 (Thermo Fisher Scientific) at 30°C for 10 min, which was required to obtain a

392   stable background prior to the fluorescence measurement. Fluorescence anisotropy was

393   measured at 30°C using FluoroMax 4 fluorimeter (Horiba) with excitation and emission

394   wavelengths of 590 nm and 670 nm, respectively. For binding competition experiments, various

395   concentrations of ATP ranging from 10 µM to 10 mM (pH was adjusted to 7.0 with NaOH) were

396   added from 100X solutions. Fluorescence anisotropy <r> was defined as:

$$<r>=\frac{IVV - G * IVH}{IVV + 2 * G * IVH}$$

397   where I_{VV} and I_{VH} are the fluorescence intensities with the excitation polarizer mounted vertically

398   and the emission polarizer mounted vertically or horizontally, respectively. *G* is defined as:

$$G = \frac{IHV}{IHH}$$

399   where I_{HV} and I_{HH} are the fluorescence intensities with the excitation polarizer mounted

400   horizontally and the emission polarizer mounted vertically or horizontally, respectively.

401

402   ***Electrophysiology***

403   HEK293 cells were split onto glass coverslips in 6 well plates at 1×10^5 cells/well and incubated

404   at 37°C overnight. Cells were transfected with 1 µg of the full length pdP2X7 (wildtype or

405   mutants) or the full length mP2X4 (wildtype or mutants) in pIE2 vector using FuGENE6

406   (Promega). Cells were used 18-32 hours after transfection for measuring the P2X receptor

407   activities using the whole cell patch clamp configuration. Membrane voltage was clamped at -60

408   mV with an Axopatch 200B amplifier (Axon Instruments), currents were filtered at 2 kHz (eight-

409   pole Bessel; model 900BT; Frequency Devices) and sampled at 10 kHz using a Digidata 1440A

410   and pCLAMP 10.5 software (Axon Instruments). The extracellular solution contained 147 mM

411   NaCl, 10 mM HEPES, 13 mM Glucose, 2 mM KCl, 0.1 mM $CaCl_2$, (pH 7.3). The pipette solution

412   contained 147 mM NaCl, 10 mM HEPES, 10 mM EGTA, which was adjusted to pH 7.0 using

413   NaOH. Whole cell configuration was made in an extracellular solution supplemented with 2 mM

414    $CaCl_2$ and 1 mM $MgCl_2$ and the extracellular solutions were rapidly exchanged to the solutions

415   containing desired concentrations of ATP using a computer-controlled perfusion system (RSC-

416   200; Bio-Logic). Because pdP2X7 substantially runs up (Figure 1B and E), we measured the

417   channel activity after treating the cells with 1 mM ATP for at least 20 s. For testing the effects of

418   P2X7 specific antagonists on pdP2X7, these drugs were incubated with ATP (1 mM) for 1 min.

419   Concentrations of the drugs were: A740003: 600 nM; A804598: 180 nM; AZ10606120: 2.3 μM;

420   GW791343: 50 μM; JNJ47965567: 136 nM. For the cysteine accessibility studies on pdP2X7,

421   0.1 mM MTS-TPAE (Tronto Chemical Reserach) was perfused for 10 s either in the absence or

422   presence of 1 mM ATP. For probing mP2X4 accessibility in the closed state, 0.1 mM MTS-

423   TPAE was applied for 10 s and application of 10 μM ATP for 1 s was used to measure channel

424   activity. For mP2X4 accessibility in the open state, 5 μM ATP was applied for 9 s and 0.1 mM

425   MTS-TPAE was applied for 3 s. For measuring cysteine accessibility of pdP2X7/Y295C or

426   mP2X4/F296C mutants, cells were treated with 10 mM dithiothreitol (DTT) for 5 min prior to

427   recording. To normalize the channel activities from multiple experiments, the ratio between

428   channel activity before and after MTS-TPAE application was calculated for each cell. Since

429   mP2X4 rapidly runs down, these ratios were further normalized to the control channel activities

430   measured on the same cell in the absence of MTS-TPAE. Stock solutions of 100 mM MTS-

431   TPAE were prepared freshly every 5 hours in water and stored on ice and were diluted to the

432   desired concentrations in the extracellular solution immediately before each recording. All

433   recordings were performed using more than five cells.

434

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615

Figure legend

617 **Figure 1. Characterization of pdP2X7.** (**A**) FSEC traces (Ex: 488 nm and Em: 509 nm) for the

618 full length (blue) and the truncated (green) pdP2X7. (**B**) and (**C**) Whole cell patch clamp

619 recordings from the wildtype pdP2X7 (B) and pdP2X7$_{cryst}$ (C) triggered by 1 mM ATP. (**D**) ATP

620 dose responses of pdP2X7 (square) and pdP2X7$_{cryst}$ (circle) determined by the whole cell patch

621 clamp experiments. The plots were made using the normalized means of five independent

622 experiments and the error bars represent SEM. Dose response curves were fit with the Hill

623 equation. EC$_{50}$ values of pdP2X7 and pdP2X7$_{cryst}$ were 122 μM and 40 μM, respectively. (**E**) and

624 (**F**) Whole cell patch clamp recordings from pdP2X7 (E) and pdP2X7$_{cryst}$ (F) expressed in

625 HEK293 cells. Each trace represents the pdP2X7 mediated current triggered by 1 s applications

626 of 100 μM ATP from the same patch. The numbers below the traces indicate the number of

627 repeated ATP applications. (**G**) Whole cell patch clamp recordings of pdP2X7 triggered by 1 mM

628 ATP in the presence of different P2X7 specific antagonists. Drugs were applied for 1 min in the

629 presence of ATP. Membrane was held at -60 mV. Concentrations of the drugs were; A740003:

630 600 nM; A804598: 180 nM; AZ10606120: 2.3 μM; GW791343: 50 μM; JNJ47965567: 136 nM.

631

632 **Figure 2. Drug-binding pocket of the P2X7 receptor.** (**A**) Cartoon representation of a

633 "dolphin-like" single subunit of the apo pdP2X7 structure. Fourteen beta strands are labeled as

634 β1-14. Each domain is colored consistent with the previous studies for better comparison

635 (Kawate et al., 2009; Hattori and Gouaux, 2012). (**B**) Cartoon representation of the trimeric

636 pdP2X7 structure viewed from the side. The black box indicates an approximate location of the

637 upper body domains shown in (C) and (D). (**C**) Side view of the upper body domains exhibiting

638 A804598 binding sites with respect to the ATP-binding pockets (orange dashed lines). A804598

639 is shown as CPK spheres. (**D**) Top view of the apo pdP2X7 structure with respect to the ATP-

640 binding pockets (orange dashed lines) and one of the drug-binding pockets (green dashed line).

641

Figure 2-Figure Supplement 1. Structural comparison between pdP2X7 and zfP2X4. (**A**)
Superposition of the apo closed pdP2X7 monomer (green) onto the apo closed zfP2X4 structure
(4DW0; gray). The two structures were superposed against each other at the body domain
using Lsqkab software (Kabsch, 1976). Notable differences between these two structures are
highlighted in orange. (**B**) Stick representations of the pdP2X7 gate residues. Three TM2
helices are shown as cartoon in green. (**C**) Superposition of the transmembrane helices of the
apo closed pdP2X7 monomer (green) onto the apo closed zfP2X4 structure (gray) Root-mean-
square displacement of the Cα positions between monomeric pdP2X7 and zfP2X4 is 2.8 Å.

650

Figure 2-Figure Supplement 2. Electron density around the P2X7 specific antagonists. (**A**)
2Fo-Fc map (contoured at σ=2.0) around the drug-binding pocket (orange) in the apo closed
pdP2X7. (**B**) Fo-Fc map (contoured at σ=3.0) around the P2X7 specific antagonists. Each drug
is depicted as a stick representation. (**C**) Chemical structure of each P2X7 antagonist is shown.

655

Figure 3. Coordination of the P2X7 specific antagonists. (**A**) Stick representations of the
indicated P2X7 specific antagonists (green) and the binding residues in the drug-binding pocket.
Structural frame of each subunit is depicted as a cartoon representation in a different color.
Oxygen atoms are shown in red and nitrogen atoms are shown in blue. (**B**) Surface
representation of the drug-binding pocket with CPK sphere representation of each drug (green).
Yellow and light blue surfaces represent two of the three subunits. The third subunit was omitted
for clear representation. (**C**) Fold changes in IC$_{50}$ values of the P2X7 specific inhibitors against
YO-PRO-1 uptake activity on alanine mutants of the drug coordinating residues. Increased IC$_{50}$
values in some of the alanine mutants indicate that these residues bind to the P2X7
antagonists. (**D**) Schild plots against drug concentrations over a range of three orders of

666   magnitude. Plots for all five of the different P2X7 specific antagonists are non-linear, supporting

667   that these drugs work non-competitively. (**E**) Whole cell patch clamp recordings of the wildtype

668   pdP2X7 triggered by 10 μM ATP (black) or 10 μM ATP-Alexa (red). The holding potential was -

669   60 mV. (**F**) Fluorescence anisotropy derived from 10 μM ATP-Alexa without protein (white), with

670   pdP2X7 (black), or with pdP2X7 in the presence of 1 mM ATP (grey). The concentration of

671   pdP2X7 was 100 μM. Concentrations of the drugs were: A740003: 600 nM; A804598: 180 nM;

672   AZ10606120: 2.3 μM; GW791343: 50 μM; JNJ47965567: 136 nM. The dots and the bars

673   represent the means of five independent experiments and the error bars represent SEM.

674   Asterisks indicate significant differences from wildtype or the no protein control ($P < 0.01$)

675   determined by one way ANOVA followed by Dunnett's test.

676

677   **Figure 3-Figure Supplement 1. Side-chain electron density near the drug-binding pocket.**

678   (**A-C**) 2Fo-Fc electron density map of the JNJ47965567-bound P2X7 structure contoured at

679   σ=1.0. Electron density of β13 (A) and β14 (B) in the upper body domain, and the α-helix near

680   the drug-binding pocket (C) are shown in red. The amino acid residues are depicted as stick

681   representations. Red represents oxygen and blue represents nitrogen.

682

683   **Figure 3-Figure Supplement 2. Equivalent position of the drug-binding pocket in the P2X4**

684   **receptor is too narrow for P2X7 antagonist to fit in.** (**A**) Surface representation of the apo

685   pdP2X7 receptor viewed from the side. Light blue, yellow, and light pink represent each subunit.

686   Green residues are the A804598 binding residues (F88, F95, F103, M105, F108 and Y295).

687   Light pink subunit is presented as semi-transparent surface for visualizing the yellow and blue

688   subunits. (**B**) Zoomed in drug-binding pocket of the pdP2X7 shown in (A). (**C**) CPK sphere

689   representation of A804598 at the same magnification and orientation as the drug binding site

690   shown in (B). (**D**) Equivalent pocket in apo zfP2X4. The green residues represent counterparts

691   of the A804598 binding residues (W87, I94, F103, L105, M108, and F289).

692

693   **Figure 3-Figure Supplement 3. Sequence alignment of P2X7 and P2X4 receptors.** Amino

694   acid sequences of the panda P2X7 (pdP2X7: XP_002913164.1), the human P2X7 (hP2X7:

695   Q99572.4), the mouse P2X4 (mP2X4: NM_011026.3), and the zebrafish P2X4 (zfP2X4:

696   AF317643.1). Starting and ending points of the pdP2X7$_{cryst}$ are indicated by arrows. The drug

697   surrounding residues are highlighted in green. Mutations at N-linked glycosylation sites (N241S

698   and N284S) and at crystal contact sites in the P4$_2$ crystal form (V35A, R125A, and E174K) are

699   indicated. The C-terminal sequences beyond 359 AA are omitted for simplicity.

700

701   **Figure 3-Figure Supplement 4. YO-PRO-1 uptake assays on the alanine mutants of the**

702   **drug coordinating residues.** (**A**) pdP2X7-mediated YO-PRO-1 uptake triggered by 1 mM ATP

703   (blue). Vector control (red) shows little accumulation of YO-PRO-1. (**B**) Dose responses of the

704   wildtype and each alanine mutant for the five P2X7 specific antagonists are shown. YO-PRO-1

705   uptake was initiated with 1 mM ATP application. IC$_{50}$ values are obtained by fitting the dose

706   responses with the Hill equation. The circles represent the mean values of five independent

707   experiments and the error bars represent SEM.

708

709   **Figure 3-Figure Supplement 5. The P2X7 specific antagonists are allosteric non-**

710   **competitive inhibitors.** (**A**) and (**B**) BzATP dose response of the wildtype pdP2X7 in the

711   presence of different concentrations of antagonists. Normalized initial rates of YO-PRO-1

712   uptake are plotted and the dose response curves are fitted with a non-competitive (A) or

713   competitive (B) inhibition model. The circles represent the means of five independent

714   experiments and the error bars represent SEM. (**C**) Fitting statistics for the non-competitive and

715   the competitive inhibition models. SS indicates the sum of squared difference. Both R-squared

716   values and the p-values from the F-test support that the drugs used in this study are allosteric

717   non-competitive inhibitors.

718

719   **Figure 3-Figure Supplement 6. Fluorescent anisotropy experiments.** (**A**) Schematic

720   representation of the fluorescence anisotropy experiment. (**B**) Fluorescence emission spectra

721   for 10 μM ATP-Alexa (red) and 100 μM GFP-tagged pdP2X7$_{cryst}$ (green). Excitation wavelength

722   was 590 nm. (**C**) A SDS-PAGE gel demonstrating that the GFP-tagged pdP2X7$_{cryst}$ is pure. (**D**)

723   Fluorescence anisotropy plotted against concentrations of the pdP2X7. Polarized emission from

724   10 μM ATP-Alexa in response to binding to P2X7 was recorded and fitted with the Hill equation

725   with the slope (n) of 0.90. (**E**) Fluorescent anisotropy decreases as ATP concentration

726   increases, indicating that ATP and ATP-Alexa competes for binding to pdP2X7. Competitive

727   binding of ATP supports that ATP-Alexa binds specifically at the ATP-binding pocket. The

728   circles represent the mean values of five independent experiments and the error bars represent

729   SEM.

730

731   **Figure 4. Drug-binding pocket narrows during P2X7 activation. (A)** Cartoon representation

732   of the turret formed by β13 and β14. P2X7 (apo), zfP2X4 (apo), and ATP-bound zfP2X4 are

733   shown as the top (left) and the side (right) views. (**B**) Dot representations of the internal-space

734   along the molecular threefold axis running through the center of the apo closed pdP2X7 (top),

735   the apo closed zfP2X4 (4DW0; middle), and the ATP-bound zfP2X4 (4DW1; bottom). The dot

736   plots are made using the HOLE program (Smart et al., 1996). Purple: >2.3 Å; green: 1.15-2.3 Å;

737   red: <1.15 Å. (**C**) Central pore radii of the three P2X crystal structures shown in (B). (**D**) Surface

738   representation of the residues in the drug-binding pocket tested for accessibility. (**E**) Whole cell

739   patch clamp recordings of the wildtype pdP2X7 and the I310C mutant triggered by 1 mM ATP.

740   MTS-TPAE (1 mM) was applied in the absence (left) or presence (right) of ATP for 10 s to probe

741 the accessibility in the closed or open state, respectively. The membrane was held at -60 mV.

742 (**F**) Normalized channel activities after MTS-TPAE treatment during the closed (grey) or open

743 (black) states. The bars represent the means of five or more independent experiments

744 (numbers above the bars indicate the n value) and the error bars represent SEM. Asterisks

745 indicate significant differences from the widltype control (P < 0.01) determined by one way

746 ANOVA followed by Dunnett's test.

747

748 **Figure 4-Figure Supplement 1. Volume of the P2X7 drug-binding pocket is larger than the**

749 **corresponding clefts in P2X3 or P2X4. (A-C)** Volumes of the drug-binding pocket in P2X7 (A),

750 and equivalent regions in P2X4 (B) and P2X3 (C) are depicted as mesh representation based

751 on the filled water molecules using HOLLOW (Ho and Gruswitz, 2008). Green mesh represents

752 the volume in apo, closed state and the red mesh represents the volume in ATP-bound, open

753 state. Side view (upper panel) and the top view (lower panel) include cartoon representation of

754 the corresponding P2X structures to locate the cleft.

755

756 **Figure 5. The turret closes during P2X7 activation.** (**A**) Surface representation of the P2X7

757 receptor viewed from the side highlighting the location of Y295 (red) facing the center of the

758 turret (blue). Only two of the three subunits are shown for clarity. (**B**) Summary of MTS-TPAE

759 accessibility. Normalized channel activities after MTS-TPAE treatment indicate that Y295C in

760 P2X7 is more accessible in the closed state than in the open state. The bars represent the

761 means of more than five independent experiments (numbers above the bars indicate the n

762 value) and the error bars represent SEM. Asterisk indicates significant difference in normalized

763 channel activity between the cells treated with MTS-TPAE in the closed and in the open states

764 (P < 0.01) determined by Dunnett's test. N.S. indicates not significant. (**C**) and (**D**) Whole cell

765 patch clamp recordings of the P2X7/Y295C mutant triggered by 1 mM ATP. MTS-TPAE (100

766 µM) was applied in the absence (left) or presence (right) of ATP for probing the accessibility in

767   the closed or open state, respectively. Cells were used before (D) or after treating with 10 mM

768   DTT for 5 min (C). Membrane was held at -60 mV. (**E**) Whole cell patch clamp recordings of the

769   P2X4/F296C mutant triggered by 10 μM ATP. Cells were treated with 10 mM DTT for 5 min

770   before recordings.

771

772   **Figure 5-Figure Supplement 1. Equivalent position of the drug-binding pocket in the P2X4**

773   **receptor is not accessible.** (**A**) Current densities of the pdP2X7 wildtype (left) and the Y295C

774   mutant (right) in the absence (gray) or presence (black) of 10 mM DTT. Currents were obtained

775   by whole cell patch clamp recordings triggered by 1 mM ATP. Membrane potential was held at -

776   60 mV. The bars represent the means (numbers above the bars indicate the n value) and the

777   error bars represent SEM. N.S. indicates there is no significant difference between the wildtype

778   and Y295C (P=0.216 without DTT; P=0.389 with DTT) by the t-test. (**B**) Whole cell patch clamp

779   recordings of the wildtype mouse P2X4 (mP2X4) and the T312C mutant triggered by 10 μM

780   ATP (closed state) or 5 μM ATP (open state). MTS-TPAE (1 mM) was applied in the absence

781   (left) or presence (right) of ATP to probe the accessibility in the closed or open state. The

782   membrane was held at -60 mV. (**C**) Normalized channel activities after MTS-TPAE treatment in

783   the closed (grey) or open (black) states. Residues in parenthesis indicate the counterpart

784   residues in pdP2X7, whose cysteine mutants exhibit current reduction by MTS-TPAE

785   application in the closed state. The bars represent the means of five or more independent

786   experiments (numbers above the bars indicate the n value) and the error bars represent SEM.

787   There was no statistically significant differences from the widltype control (P > 0.05) judged by

788   one way ANOVA followed by Dunnett's test.

789

790   **Figure 6. A804598 prevents conformational changes in the upper body domain triggered**

791   **by ATP-binding.** (**A**) Cartoon representations indicate little conformational change upon ATP-

792   binding in the upper body domain. Each domain of the ATP/A804598-bound P2X7 is colored

793   according to Figure 2 and the overall structure is shown in (B). Apo P2X7 structure is presented

794   in gray for comparison (A, C and D). (**C**) and (**D**) ATP-binding evokes conformational

795   rearrangement in the ATP-binding pocket (C) and in the lower body domain (D). The arrows

796   highlight the direction of the domain movement upon ATP binding.

797

798   **Figure 6-Figure Supplement 1. P2X7 structure in the ATP/A804598 bound state.** (**A**) and

799   (**B**) Cartoon representations of the two protomers in the asymmetric unit. Clear ATP density was

800   observed in chain B (B) but not in chain A (A). (**C**) Fo-Fc map (contoured at $\sigma=3.0$) around the

801   ATP molecule in chain B.

802

803   **Figure 7. Mechanisms of P2X7 activation and inhibition.** Schematic representations of the

804   P2X7 receptor viewed from the top. Each color represents a different subunit. The drug-binding

805   pocket and the turret narrow during channel activation. P2X7 specific antagonists stabilize its

806   closed conformation by preventing the movement of these inter-subunit cavities.

807

808   **Video 1. Electron density of the drug-coordinating residues**. 2Fo-Fc electron density map of

809   the JNJ47965567-bound P2X7 structure contoured at $\sigma=1.0$. Electron density is shown as mesh

810   in firebrick red. JNJ47965567 (green) and amino acid residues (white) are depicted as stick

811   representations. Red represents oxygen and blue represents nitrogen.

812

813   **Video 2. Conformational differences between the apo and ATP/A804598-bound P2X7**

814   **structures.** Each domain of the ATP/A804598-bound P2X7 is colored according to Figure 2.

815   ATP and A804598 molecules are presented as CPK spheres.

816
817 **Table 1. IC_{50} value of each P2X7 inhibitor from the YO-PRO-1 uptake assay**

Inhibitor	A740003	A804598	AZ10606120	GW791343	JNJ47965567
IC_{50}	69.3 nM	21.7 nM	231 nM	8.9 μM	11.9 nM

818
819
820

821 **Table 2. Data collection and refinement statistics**

	pdP2X7$_{cryst}$	pdP2X7$_{cryst}$ - JNJ47965567	pdP2X7$_{cryst}$ -A740003	pdP2X7$_{cryst}$ - A804598	pdP2X7$_{cryst}$ AZ10606120	pdP2X7$_{cryst}$ - GW791343	pdP2X7$_{cryst}$- ATP/A804598
Data collection							
Space group	$I2_13$	$I2_13$	$I2_13$	$I2_13$	$I2_13$	$I2_13$	$P2_13$
Cell dimensions							
a, b, c (Å)	169.1, 169.1, 169.1	169.3, 169.3, 169.3	169.6, 169.6, 169.6	170.4, 170.4, 170.4	170.7, 170.7, 170.7	169.7, 169.7, 169.7	167.6, 167.6, 167.6
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	45.2 - 3.40 (3.52 - 3.40)*	45.3 - 3.20 (3.31 - 3.20)*	42.4 - 3.61 (3.73 - 3.61)*	42.6 - 3.40 (3.52 - 3.40)*	42.7 - 3.50 (3.63 - 3.50)*	45.4 - 3.30 (3.42 - 3.30)*	46.5 - 3.90 (4.04 - 3.90)*
R_{merge}	0.099 (1.80)	0.12 (0.95)	0.23 (1.71)	0.15 (1.49)	0.17 (2.21)	0.12 (1.72)	0.12 (2.17)
$I/\sigma I$	17.9 (1.30)	13.4 (2.90)	13.2 (1.65)	11.0 (1.71)	11.01 (1.16)	12.55 (1.34)	15.7 (1.25)
Completeness (%)	99.8 (98.8)	100 (100)	100 (100)	100 (100)	100 (100)	99.8 (99.4)	99.8 (99.2)
Redundancy	9.8 (9.9)	10.1 (10.4)	11.0 (11.0)	10.1 (10.0)	10.1 (9.9)	10.1 (10.2)	10.0 (10.3)
Refinement							
Resolution (Å)	45.2 - 3.40	45.3 - 3.20	42.4 - 3.61	42.6 - 3.40	42.7 - 3.50	45.4 - 3.30	46.5 - 3.90
No. reflections	11207	13487	9515	11478	10607	12383	14510
R_{work}/ R_{free} (%)	24.2/ 26.3	22.2/26.4	23.3/26.1	24.8/27.1	24.5/27.0	24.0/27.1	33.6/38.1
No. atoms	2304	2473	2384	2439	2454	2383	4118
Protein	2276	2382	2307	2359	2367	2313	3969
Ligand/ion	28	91	77	80	87	69	149
B-factors	124.4	103.6	97.8	96.5	114.9	119.0	140.0
Protein	123.8	102.9	97.1	95.9	114.5	118.5	139.8
Ligand/ion	171.1	124.1	120.6	116.9	128.0	137.4	145.2
R.m.s deviations							
Bond lengths (Å)	0.002	0.004	0.003	0.004	0.004	0.004	0.001
Bond angles (°)	0.49	0.73	0.68	0.76	0.68	068	0.51

822 *Highest resolution shell is shown in parenthesis.

A pdP2X7-ΔN/ΔC, pdP2X7

B pdP2X7_wt, 1 mM ATP, 1 nA, 5s

C pdP2X7$_{cryst}$, 1 mM ATP, 100 pA, 5s

D Normalized response vs ATP (μM), pdP2X7$_{cryst}$, pdP2X7

E ATP, 1 3 5 7 9 11 13 15 17 19 21 23 25 27, 1 nA, 10 s

F ATP, 1 8 9 10 11 12, 200 pA, 10 s

G A740003 / A74, A804598 / A80, AZ10606120 / AZ10, GW791343 / GW, JNJ47965567 / JNJ, ATP, 1 nA, 500 pA, 20 s

A
Upper body
β3
β2 β13
Right flipper
β4
β14
β5 β6
β7
Head
β10
β8 β1
β11
β12
Left flipper
Dorsal
fin
β9
Lower body
TM helices
(Fluke)
N   C

B
Out
In

C
β4 β14 β13
A804598
ATP binding
pocket

D
90°
Drug binding
pocket
ATP binding
pocket
A804598

A

A740003 — F108, Y295, V312, M105, F88, F103

A804598 — F108, Y295, M105, F88, F103

AZ10606120 — F108, Y295, V312, F293, F88, F103

GW791343 — F108, Y295, V312, F293, F88, F95, F103, W167

JNJ47965567 — F108, Y295, V312, M105, F88, F293, F95, W167

B

A740003   A804598   AZ10606120   GW791343   JNJ47965567

C

A740003 — Fold change in IC50 vs WT, F88A, F95A, F103A, M105A, F108A, K110A, E305A, I310A, V312A

A804598

AZ10606120

GW791343

JNJ47965567

D

R-1 vs Antagonist (nM); Slope=1; JNJ, A80, A74, AZ10, GW

E

10 μM ATP or 10 μM ATP-Alexa; 30 pA; 5s

F

Anisotropy vs ND, A74, A80, AZ, GW, JNJ; No protein, +P2X7, +P2X7+1 mM ATP

A

P2X7 (A804598)

P2X4 (apo)

P2X4 (ATP)

ATP

B

C

D

Drug binding pocket

MTS

T308
I310
V312
F103
F95
K110
F88
M105

E

WT (closed)

ATP   MTS-TPAE   ATP

1 nA
5 s

WT (open)

ATP

MTS-TPAE

1 nA
10 s

I310C (closed)

ATP   MTS-TPAE   ATP

1 nA
5 s

I310C (open)

ATP

MTS-TPAE

1 nA
10 s

F

Cys modification by MTS-TPAE

Closed
Open

A

Turret   MTS

Y295

Out

In

B

Cys modification by MTS-TPAE

Normalized activity

N.S.   *   N.S.   N.S.

☐ Closed
■ Open

6 5   6   6   5 5   5 5

WT   Y295C   WT   F296C
—— P2X7 ——   —— P2X4 ——

C P2X7/Y295C-closed (+DTT)   P2X7/Y295C-open (+DTT)

ATP   MTS-TPAE   ATP   MTS-TPAE
                                    ATP

1 nA   1 nA
5 s   10 s
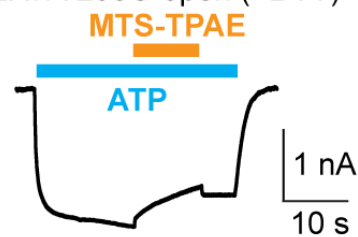
D P2X7/Y295C-closed (no DTT)   P2X7Y295C-open (no DTT)

ATP   MTS-TPAE   ATP   MTS-TPAE
                                    ATP

1 nA   500 pA
5s   10s
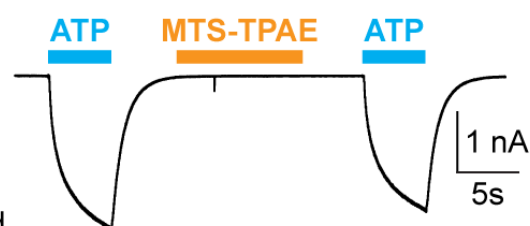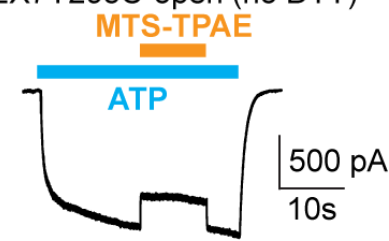
E P2X4/F296C-closed (+DTT)   P2X4/F296C-open (+DTT)

ATP   MTS-TPAE   ATP   MTS-TPAE
                                    ATP

0.5 nA   0.5 nA
5 s   3 s

A Upper body

90°

B

C ATP binding pocket

Head

Dorsal fin

Left flipper

D Lower body

Antagonist

Turret

ATP

Closed
(antagonist bound)

Closed
(apo)

Open

A

Head

Upper body

Dorsal fin

Left flipper

Right flipper

TM helices
(Fluke)

P2X7

P2X4

N   C

B

G338

S339   S342

C

TM2

TM1

C

90°

N   C

A Drug binding pocket in the apo structure

B A740003   A804598   AZ10606120   GW791343   JNJ47965567

C

A   β13

E301

K300

Y299

Y298

K297

A296

Y295

R294

F293

B   β14

N303

N302

V304

E305

K306

R307

T308

L309

I310

K311

C   α-helix near
drug-binding pocket

D89

D92

A91

T90

Y93

T94

F95

A

B pdP2X7 (Apo)

40°

C

A804598

~14 Å

~9 Å

D zfP2X4 (Apo)

Start of pdP2X7_cryst

22

35
A

TM1

```
pdP2X7  (1)   ---MPACCSWK--DVFQYETNKVLRIQSTNYGTIKWIFHVLVFSYIS-FALISDKRYQKKEPLISSVHTKVKGIAEVKAE
hP2X7   (1)   ---MPACCSCS--DVFQYETNKVTRIQSMNYGTIKWFFHVIIFSYVC-FALVSDKLYQRKEPVISSVHTKVKGIAEVKEE
mP2X4   (1)   ---MAGCCSVLGSFLFEYDTPRIVLIRSRKVGLMNRVVQLLILAYVIGWVFVWEKGYQETDSVVSSVTTKAKGVAVTNTS
zfP2X4  (1)   MSESVGCCDSVSQCFFDYYTSKILIIRSKKVGTLNRFTQALVIAYVIGYVCVYNKGYQDTDTVLSSVTTKVKGIALTNTS
```

88   95   103   108   125
                                 105   110   A

```
pdP2X7  (75)  ILENGMKKMVSGVFDTADYTFPLQ-GNSFFVMTNFIKTEGQQQGLCPDFPTRRTICSSDRGCKKGRMDPQSKGIQTGRCV
hP2X7   (75)  IVENGVKKLVHSVFDTADYTFPLQ-GNSFFVMTNFLKTEGQEQRLCPEYPTRRTLCSSDRGCKKGWMDPQSKGIQTGRCV
mP2X4   (78)  QLG-------FRIWDVADYVVPAQEENSLFIMTNMIVTVNQTQGTCPEIPDKTSICDSDANCTLGSSDTHSSGIGTGRCV
zfP2X4  (81)  ELG-------ERIWDVADYIIPPQEDGSFFVLTNMIITTNQTQSKCAENPTPASTCTSHRDCKRGFNDARGDGVRTGRCV
```

167   174
             K

```
pdP2X7  (154) VYKERLKTCEVSAWCPIEEVEDAPRPALLNSAENFTVLIKNNIDFPGHNYTTRNILPGVN----ITCTFHKTQNPQCPIF
hP2X7   (154) VYEGNQKTCEVSAWCPIEAVEEAPRPALLNSAENFTVLIKNNIDFPGHNYTTRNILPGLN----ITCTFHKTQNPQCPIF
mP2X4   (151) PFNASVKTCEVAAWCPVENDAGVPTPAFLKAAENFTLLVKNNIWYPKFNFSKRNILPNITTSYLKSCIYNARTDPFCPIF
zfP2X4  (154) SYSASVKTCEVLSWCPLEKIVDPPNPPLLADAENFTVLIKNNIRYPKFNFNKRNILPNINSSYLTHCVFSRKTDPDCPIF
```

241   284   295
  S   S   293   298   305 308

```
pdP2X7  (230) RLGDIFQETGDNFSDVAIQGGIMGIEIYWDCNLDGWFHHCRPKYSFRRLDDKTTNESLYPGYNFRYAKYYKENN-VEKRT
hP2X7   (230) RLGDIFRETGDNFSDVAIQGGIMGIEIYWDCNLDRWFHHCRPKYSFRRLDDKTTNVSLYPGYNFRYAKYYKENN-VEKRT
mP2X4   (231) RLGQIVADAGHSFQEMAVEGGIMGIQIKWDCNLDRAASHCLPRYSFRRLDTRDLEHNVSPGYNFRFAKYYRDLAGNEQRT
zfP2X4  (234) RLGDIVGEAEEDFQIMAVRGGVMGVQIRWDCDLDMPQSWCVPRYTFRRLDNKDPDNNVAPGYNFRFAKYYKNSDGTETRT
```

End of pdP2X7_cryst

310   359
  312   **TM2**

```
pdP2X7  (309) LIKVFGIRFDILVFGTGGKFNVIQLAVYIGSVISYFGLATVFIDILINTYSS-
hP2X7   (309) LIKVFGIRFDILVFGTGGKFDIIQLVVYIGSTLSYFGLAAVFIDFLIDTYSS-
mP2X4   (311) LTKAYGIRFDIIVFGKAGKFDIIPTMINVGSGLALLGVATVLCDVIVLYCMK-
zfP2X4  (314) LIKGYGIRFDVMVFGQAGKFNIIPTLLNIGAGLALLGLVNVICDWIVLTFMK-
```
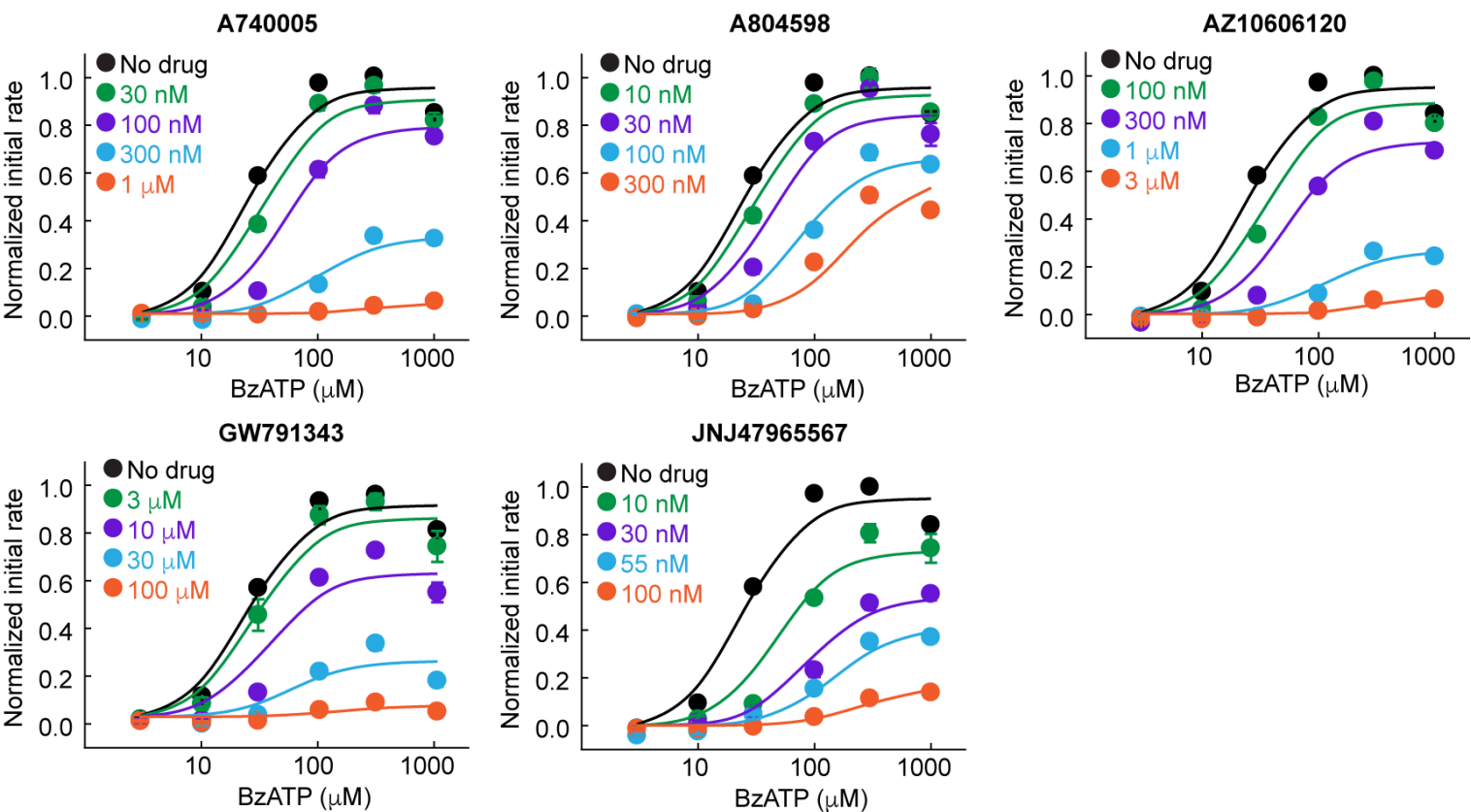
C-terminal domain

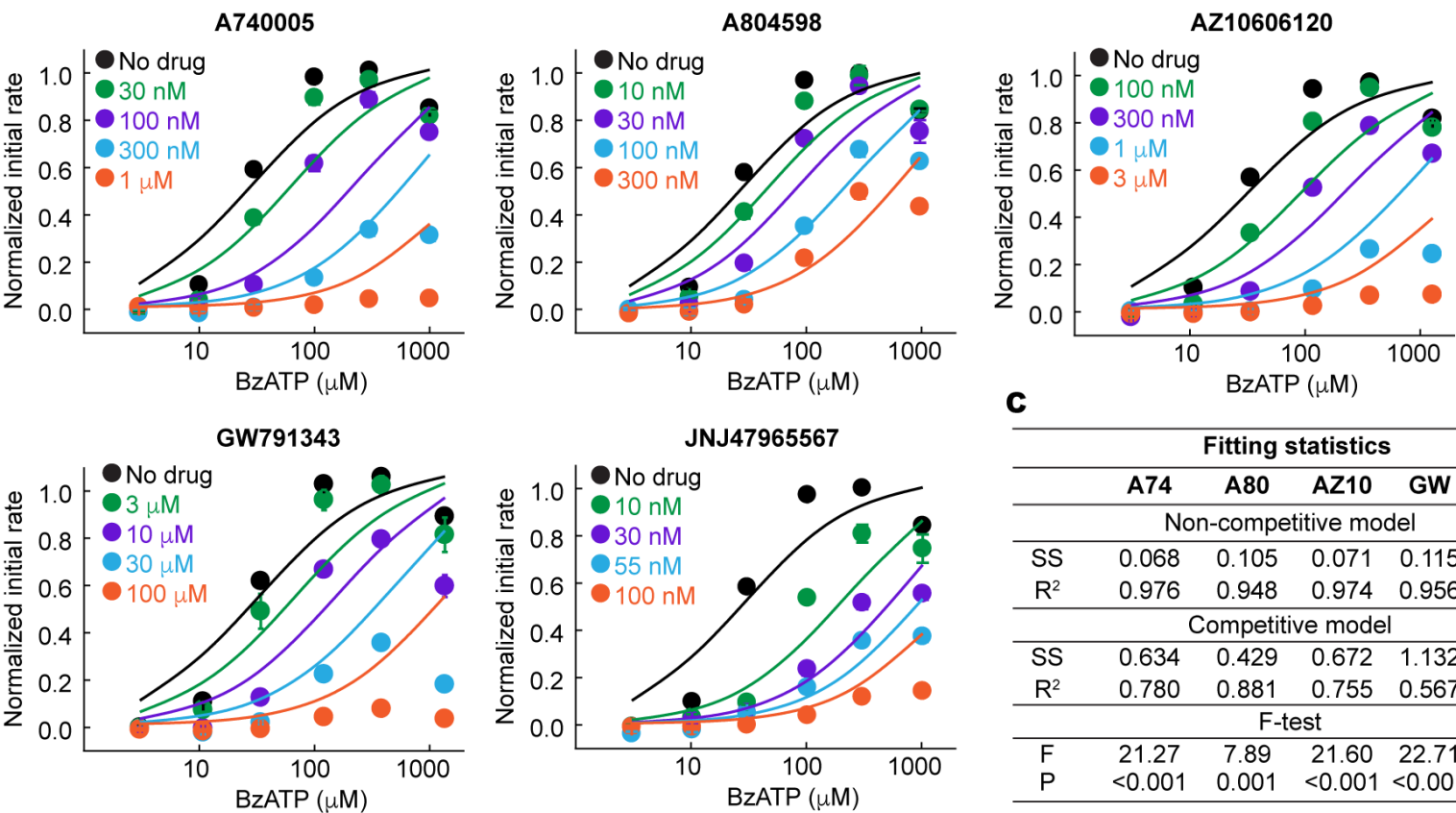**A** YO-PRO-1 uptake

B WT, F88A, F95A, F103A, M105A, F108A, K110A, E305A, E310A, V312A

Legend: JNJ (black), A80 (red), A74 (green), AZ10 (blue), GW (purple)
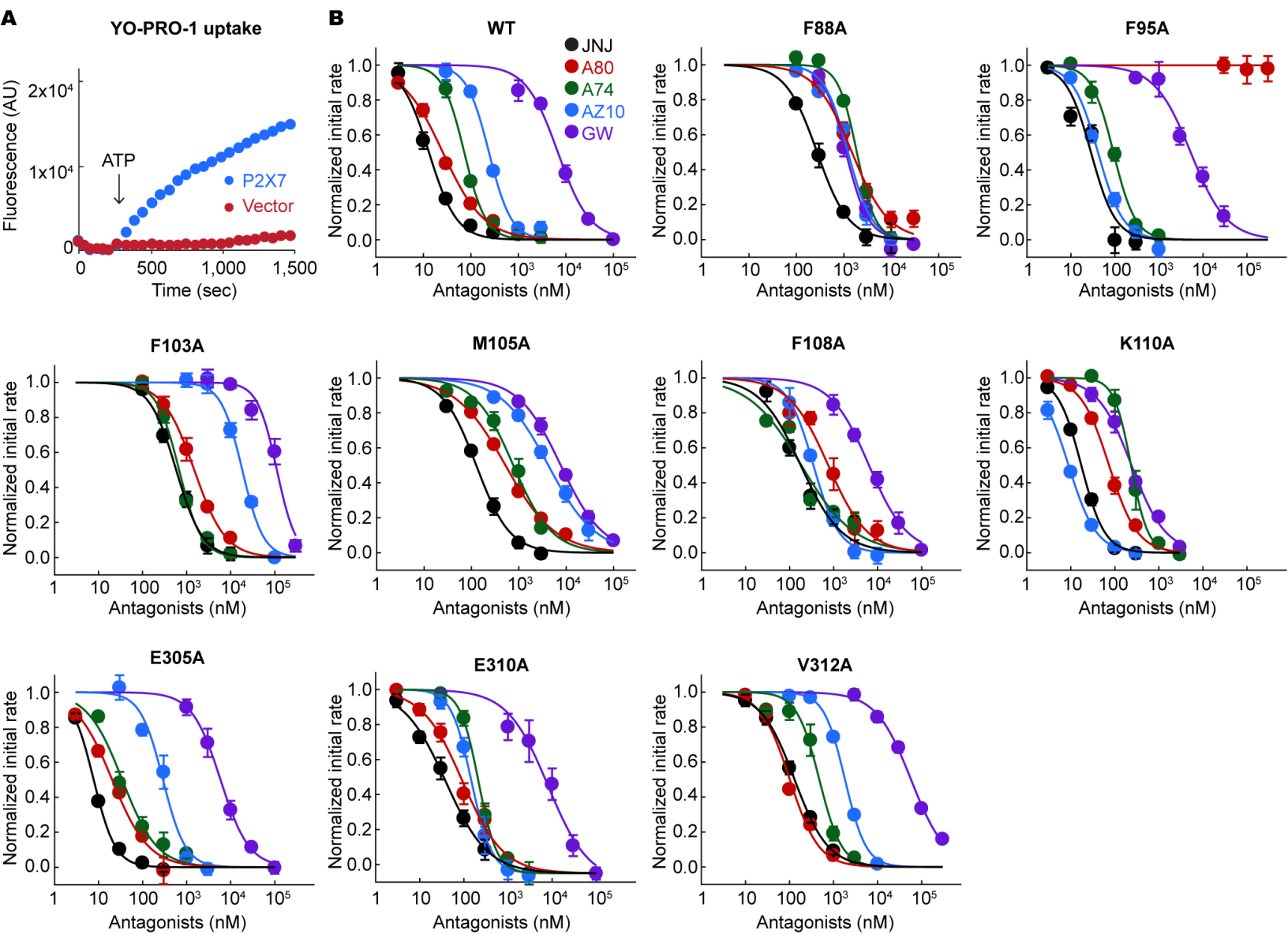
A Fitting with a non-competitive inhibition model
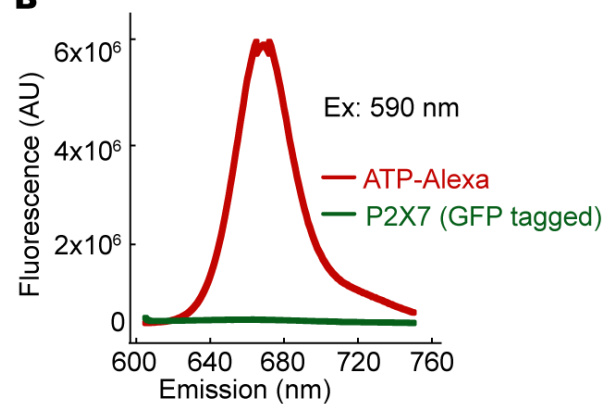
A740005

A804598

AZ10606120

GW791343

JNJ47965567

B Fitting with a competitive inhibition model

A740005

A804598

AZ10606120

GW791343

JNJ47965567

C

		Fitting statistics			
	A74	**A80**	**AZ10**	**GW**	**JNJ**
	Non-competitive model				
SS	0.068	0.105	0.071	0.115	0.061
R^2	0.976	0.948	0.974	0.956	0.972
	Competitive model				
SS	0.634	0.429	0.672	1.132	0.333
R^2	0.780	0.881	0.755	0.567	0.849
	F-test				
F	21.27	7.89	21.60	22.71	11.32
P	<0.001	0.001	<0.001	<0.001	<0.001

A Excitation polarizer / Antagonists / Emission polarizer

Excitation at 590 nm → V ↕ H → P2X7 (ATP-Alexa) → V ↕ H → Fluorescence anisotropy measurement at 670 nm

B

Fluorescence (AU)

Ex: 590 nm
— ATP-Alexa
— P2X7 (GFP tagged)

Emission (nm)

C

kDa
250
150
100
75
50
37
25

← P2X7 (GFP-tagged)

D

Anisotropy

n=0.90
R=0.999

P2X7 (μM)

E

Anisotropy

- no drug
- JNJ
- A80
- A74
- AZ10
- GW

ATP (mM)
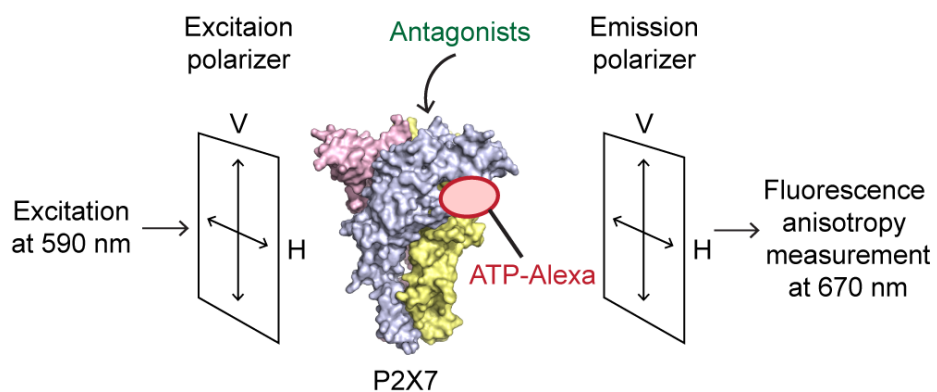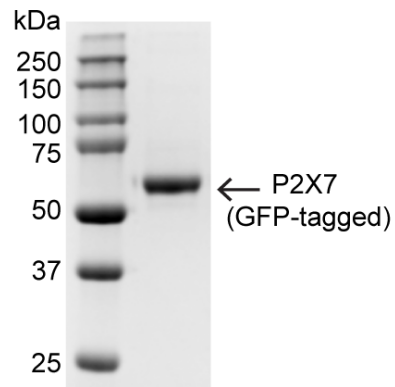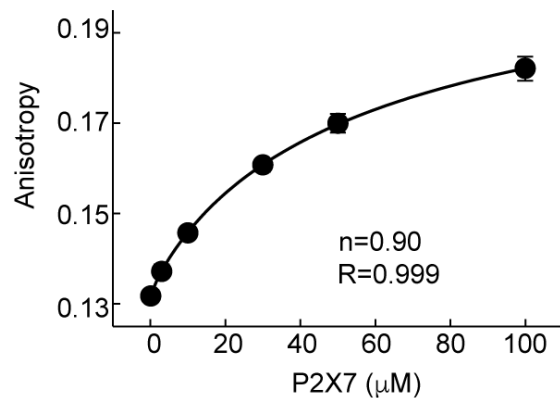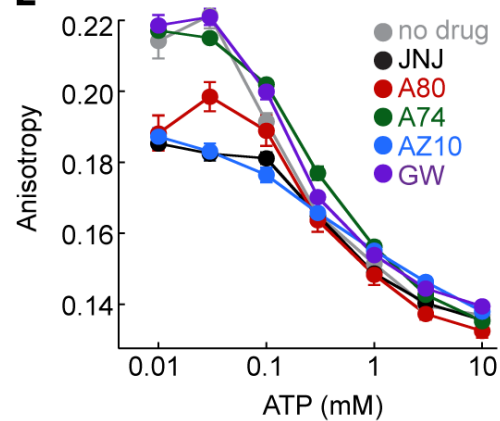
A P2X7

Apo-closed

90°

B P2X4

Apo-closed   ATP-open

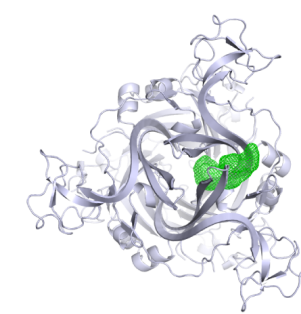90°   90°

C P2X3

Apo-closed   ATP-open

90°   90°

A

B

WT (closed)   WT (open)

T312C (closed)   T312C (open)

C

Cys modification by MTS-TPAE

A Chain A (unclear ATP density)

Upper body

A804598

Right flipper

Head

Dorsal fin

Left flipper

Lower body

TM helices (Fluke)

N   C

B Chain B (clear ATP density)

A804598

ATP

C