Use of chimeras, point mutants and molecular modelling to map the antagonist binding site of 4,4',4'',4'''-(carbonylbis(imino-5,1,3-benzenetriylbis-(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid (NF449) at P2X1 receptors for ATP.

Louise K. Farmer¹, Ralf Schmid² and Richard J. Evans¹*

¹Department of Cell Physiology & Pharmacology, ²Department of Biochemistry, University of Leicester, Leicester, U.K.

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*author for correspondence. Tel.:44-116-227-7057; Fax:44-116-252-5045; E-mail:rje6@le.ac.uk.

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The abbreviations used are: NF449 (4,4',4'',4'''-(carbonylbis(imino-5,1,3-benzenetriylbis-(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid), PPADS (pyridoxal-phosphate-6-azophenyl-2,4-Disulfonate).

Background: NF449 is a selective P2X1 receptor antagonist.

Results: Chimeric/mutant P2X1/4 receptors and molecular docking provided models of NF449 binding.

Conclusion: NF449 is co-ordinated by the core ATP binding site, the base of the cysteine rich head and dorsal fin region.

Significance: The study provides an insight into selective antagonism of P2X receptors that may aid rational drug design.

P2X receptor subtype selective antagonists are promising candidates for treatment of a range of pathophysiological conditions. However in contrast to high resolution structural understanding of agonist action at the receptors comparatively little is known about the molecular basis of antagonist binding. We have generated chimeras and point mutations in the extracellular ligand binding loop of the human P2X1 receptor, that is inhibited by NF449, suramin and PPADS, with residues from the rat P2X4 receptor that is insensitive to these antagonists. There was little or no effect on the sensitivity to suramin and PPADS at chimeric P2X1/4 receptors indicating that a significant number of residues required for binding of these antagonists are present at the P2X4 receptor. Sensitivity to the selective P2X1 receptor antagonist NF449 was reduced ~60 and ~135 fold in chimeras replacing the cysteine rich head and the dorsal fin region below it in the adjacent subunit. Point mutants identified the importance of four positively charged residues at the base of the cysteine rich head and two variant residues in the dorsal fin to high affinity NF449 binding. These six residues were used as the starting area for molecular docking. The four best potential NF449 binding poses were then discriminated by correspondence with the mutagenesis data and an additional mutant to validate the binding of one lobe of NF449 within the core conserved ATP binding pocket and the other lobes co-ordinated by positive charge on the cysteine rich head region and residues in the adjacent dorsal fin.

P2X receptors comprise a family of ATP-gated ion channels made from the homo- and hetero-trimeric assembly of seven receptor subunits (P2X1-7) with intracellular amino and carboxyl termini, two transmembrane spanning segments and a large extracellular ligand binding loop (1). In the 20 years since the receptors were first cloned (2,3) the roles of defined P2X receptor subtypes in a range of physiological processes and pathophysiological conditions have been determined (4,5). This has highlighted the therapeutic potential of P2X receptor subtype selective antagonists. One target is the P2X1 receptor that is expressed on platelets; receptor knockout mice had normal bleeding responses but were protected from thrombosis (6), and
overexpression of the receptor in platelets was pro-thrombotic (7). The selective P2X1 receptor antagonist NF449 \((4,4',4'',4''\text{-}(\text{carbonylbis}(\text{imino}-5,1,3\text{-benzenetriylbis-}(	ext{carbonylimino}))\text{tetrakis-benzene-1,3-disulfonic acid}))\) had no effect on normal bleeding in mice, but reduced platelet aggregation in a model of thromboembolism and reduced thrombus size following laser-induced injury of mesenteric arterioles (8). Thus P2X1 receptor selective antagonists would protect from thrombosis, heart attack and stroke without affecting normal haemostasis.

The crystalization of the zebrafish P2X4 receptor in an ATP bound conformation provided structural insight into the agonist binding site at P2X receptors (9) and is supported by a range of mutagenesis studies (10-13). The P2X receptor subunit structure resembles a dolphin with a cysteine rich head, body, flippers, dorsal fin and transmembrane fluke (14). ATP binds at the inter-subunit interfaces in pockets formed by the head, upper body and left flipper of one subunit with the lower body and dorsal fin of the adjacent one (9). However there is currently little or no structural information on the site(s) of antagonist binding at P2X receptors. Previous studies highlighted roles of residues around the agonist binding pocket involved in the action of the relatively non-selective P2X receptor antagonists suramin and PPADS (pyridoxal-phosphate-6-azophenyl-2,4-disulphonate)(15-17). For the P2X1 receptor selective antagonist NF449, mutagenesis based approaches identified positive charges at the base of the cysteine rich head region, as contributing to high affinity binding (18,19). However the full extent of the binding site remains to be determined. In this study we have used chimeras to assess the contribution of variations in the extracellular loop to antagonist action. To optimise the approach we have replaced sections of extracellular loop of the human P2X1 (hP2X1) receptor with the corresponding parts of the rat P2X4 (rP2X4) receptor that is essentially insensitive to the antagonists NF449, suramin and PPADS (15,20-22). Site directed mutagenesis was then used to identify individual residues that contribute to NF449 action that were the starting point for \textit{in silico} antagonist docking and validation.

**EXPERIMENTAL PROCEDURES**

P2X receptor constructs and the generation of chimeras and point mutants. The hP2X1 receptor cDNA was originally cloned from the bladder (23) and the rP2X4 receptor DNA was a gift of Dr. Francois Rassendren, (CNRS, Montpellier, France). A mutation (Y378A) was introduced to the rP2X4 receptor template to give more stable and reproducible currents (24). In order to design chimeras, regions of conservation of \(\geq\) three residues between the sequences of the hP2X1 and rP2X4 receptors were identified using a sequence line up that was generated using the protein BLAST program (NCBI) and manually corrected. Five of these conserved regions were chosen as borders to four initial sections (A-D) that were swapped between the receptors. Four of these were 100% conserved and easy to choose as borders to regions A-D using the line-up. There was no obvious island of conservation in a suitable location to form the border between section C and D, so a section of six amino acids with > 65% conservation located around the conserved cysteine residue 261 was chosen. The initial four regions swapped consisted of residues 56-132 in region A, 133-184 in B, 185-261 in C and 262-330 in D. Chimeras were generated using domain swapping PCR as described previously (25). Point mutations were made using the QuikChange mutagenesis kit (Stratagene). Mutations were verified by DNA sequencing by the Protein and Nucleic Acid Chemistry Laboratory services, University of Leicester.

The original chimera replacing region A (residues 56-132) was non-functional due to a lack of expression at the cell surface. This was then re-designed to exclude the residues closest to TM1 (56-62) from the swapped region. These residues were not located at the surface of the receptor and were therefore unlikely to be involved in NF449 binding. The region A chimera (X1-AX4) referred to in this paper consists of the hP2X1 receptor with residues 63-133 of the rP2X4 receptor. The mean peak current evoked to a maximal concentration of ATP was the same for the WT and all mutant receptors, with the exception of the X1-AX4 chimera which had an ~5-fold reduction in peak current compared to the hP2X1 receptor (p<0.0001).
Expression in Xenopus laevis Oocytes. cRNA was synthesised using the T7 mMessage mMACHINE kit (Ambion) and injected into stage V X. laevis oocytes as described previously (23). Oocytes expressing P2X receptors were stored at 16 °C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM sodium pyruvate, 5 mM HEPES, pH 7.6) with 50 µg.ml$^{-1}$ gentamycin for 3–7 days. For electrophysiological recordings gentamycin was not present and the 1.8 mM CaCl$_2$ was replaced with 1.8 mM BaCl$_2$.

Electrophysiological Recordings. Two-electrode voltage clamp recordings were carried out using a Geneclamp 500B amplifier with a Digidata 1322A analogue-to-digital converter and pClamp 8.2 acquisition software (Molecular Devices, Menlo Park, CA, USA) at a holding potential of -60 mV. ATP (Mg$^{2+}$ salt, Sigma) was applied via a U-tube perfusion system for 3 s at 5-10 min intervals (dependent on the P2X receptor) to allow for reproducible responses to be recorded. Antagonists were bath perfused in ND96 solution for 5 min before they were co-applied with an EC$_{50}$ concentration of ATP through the U-tube. To generate inhibition curves antagonists were co-applied with an EC$_{50}$ concentration of ATP to standardise any shift in ATP potency. Antagonists fully equilibrated with the receptor during the first application period as the level of inhibition was maintained on a second test application. The inhibitory effects of the antagonists were reversed in the washout period between agonist applications. Antagonists, at maximum concentrations used, were applied to all WT and mutant receptors in the absence of ATP and were seen to have no effect on the holding current. Suramin was from Sigma, PPADS from Tocris and NF449 from Abcam.

Data Analysis. Individual normalized concentration response curves were fitted with the Hill equation (variable slope) with GraphPad Prism 6. For agonists pEC$_{50}$ is the $-\log_{10}$ of the concentration giving 50% maximal response (EC$_{50}$ value), and IC$_{50}$ is the concentration of antagonist inhibiting the EC$_{50}$ concentration of ATP by 50%. pIC$_{50}$ is the $-\log_{10}$ of the IC$_{50}$ value. For the calculation of EC$_{50}$/IC$_{50}$ values and Hill slopes, individual concentration response curves were generated for each experiment and statistical analysis carried out on the data generated. In the figures, inhibition curves are fitted to the mean normalized data. Any significant differences between the WTs/chimeras/mutants (e.g. current amplitude, rise and desensitization/current remaining at the end of the ATP application, Hill slope, pEC$_{50}$, and pIC$_{50}$) were calculated by one-way ANOVA followed by Dunnett’s test. The software used was GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA), n $\geq$ 3 for all data points.

P2X1 receptor modelling and NF449 ligand docking. Homology models for the hP2X1 receptor were built based on the X-ray structure (4DW0) of the zfP2X4 receptor apo form as described previously (26). These models were used as receptors for ligand docking in GOLD (27). The antagonist NF449 was prepared for docking and energy minimized in Hypercube v8.0 (Hypercube Inc., Gainesville, FL, USA). The docking site in the hP2X1 receptor models was set to sample the regions defined by point mutations in the cysteine rich region and dorsal fin (K136, K138, R139, K140, T216, Q231). The receptor set-up treated lysine and arginine side-chains within the docking site as flexible by using rotamer ensembles. Docking poses were generated using the genetic algorithm implemented in GOLD. The dimensionless Astex Statistical Potential (ASP score) was used to rank the resulting NF449 docking poses. Poses with ASP scores $>35$ were visualized in pymol and further analysed for their interactions with the six residues mentioned above. Four docking poses (in the following referred to as poses A-D) showed interactions with most of the residues above and were used for further analysis.

Molecular Dynamics Simulations. The extracellular domains of the P2X1 models with NF449 in poses A-D were used as starting structures for molecular dynamics simulations. The molecular dynamics simulations were performed in amber 12 (28) with the ff99SB and GAFF force fields using AM1/BCC partial charges for NF449. Protein and antagonist were solvated in a cubic TIP3P water box and neutralised by adding counter ions. Before the simulations the systems were energy-
minimised in three steps (constraints on non-hydrogen atoms, constraints on non-solvent atoms, no constraints). The energy-minimised complexes were then equilibrated by 50 ps heating from 0 K to 300 K with weak restraints for all protein residues (2.0 kcal/mol Å²) followed by 100 ps density equilibration. Production runs were run for 10 ns with 2 fs time steps. For all simulations the SHAKE algorithm was used to constrain bonds between hydrogens and heavy atoms, and Langevin dynamics were used for temperature control. Trajectories were analysed with the amber ptraj module.

RESULTS

Characterization of ATP action at P2X1/4 receptor chimeras. Four chimeras replacing sections of the extracellular loop of the hP2X1 receptor with the corresponding part from the rP2X4 receptor were designed with the aid of sequence line-ups and homology models. Regions A-D each consisted of > 50 residues; C was the largest (76 aa), and B the smallest (51 aa)(the crossover points between regions were chosen to contain 3-6 conserved residues). The residues contained within each section were 63-132 in A, 133-184 in B, 185-261 in C and 262-330 in D (hP2X1 receptor numbering). The nomenclature for the chimeras indicates the part replaced e.g. X1-AX4 indicates the replacement of the A region of hP2X1 receptor with the corresponding region from the rP2X4 receptor. Of the residues in sections A, B and D, ~50% were conserved. Region C had more variance with only 35% conservation. Each of the chimeras contained residues in or around the ATP binding pocket. Regions A and B contained residues mainly located at the top of the receptor, above the ATP binding pocket (Figure 1). Region C was adjacent to and immediately below the ATP binding pocket and region D was mainly below the binding site, with some residues located at the apex of the receptor.

ATP evoked concentration dependent currents at hP2X1 and rP2X4 receptors (EC₅₀ ~ 1 and 10 µM respectively) that showed contrasting levels of desensitization to continued agonist application (19.7 ± 4.3 and 71.4 ± 4.5 % respectively remaining at the end of a 3s application of a maximal concentration of ATP) consistent with previous reports (15,20,21,23). The hP2X1 receptor based chimeras all gave robust reproducible responses to ATP (Figure 1, Table 1). For the X1-CX4 chimera ATP potency (EC₅₀ ~ 1 µM) and desensitization (25.3 ± 2.9 % current remaining at the end of a 3s application of maximal ATP) was indistinguishable from the hP2X1 WT receptor. The X1-DX4 chimera showed rP2X4 receptor agonist sensitivity (EC₅₀ ~ 10 µM) with desensitization that was intermediate between hP2X1 and rP2X4 receptors (40.1 ± 2.1 % peak current remaining at end of application, p<0.01). At chimeras X1-AX4 and X1-BX4 ATP was ~ 10 fold less potent than at the rP2X4 receptor (pEC₅₀ 4.43 ± 0.08, 4.28 ± 0.04, respectively, p<0.0001 compared to rP2X4 receptor pEC₅₀ 5.39 ± 0.10). Interestingly although these chimeras showed a similar large decrease in ATP potency compared to the WT hP2X1 receptor the time-course of X1-AX4 receptors (29.2 ± 11.4 % current remaining) was the same as for WT hP2X1 receptors whilst that of X1-BX4 was equivalent to that of the rP2X4 receptor (59.5 ± 2.9 and 71.4 ± 4.5 % current remaining at end of agonist application for X1-BX4 and rP2X4 respectively). These studies demonstrate a complex role of residues in the extracellular loop in determining responses to ATP, and that agonist potency and time-course can be regulated independently.

Antagonist action at hP2X1 receptor chimeras. A standardised EC₉₀ concentration of ATP at each receptor was used to characterize antagonist sensitivity. NF449 inhibited ATP responses with an IC₅₀ of ~ 1nM at the hP2X1 receptor and even at 1 µM had no effect at the rP2X4 receptor (as reported previously, (15,20,21,29)). The chimeras showed decreased NF449 sensitivity compared to the hP2X1 receptor (Figure 2). This was <5 fold for X1-AX4 and X1-DX4, ~ 60 fold for X1-BX4 and ~ 135 fold for X1-CX4 (Figure 2, Table 1).

The antagonists suramin and PPADS inhibited the hP2X1 receptor (IC₅₀ of ~ 1 µM) but were ineffective (100 µM) at the rP2X4 receptor (as reported previously, (15,20,21,29)). At chimera X1-CX4 sensitivity to suramin or PPADS was the same as at the hP2X1 receptor (Figure 3, Table 1). Paradoxically sensitivity to both suramin and PPADS was actually increased ~ 10 fold compared to the hP2X1 receptor for the X1-
DX4 chimera (p<0.0001, Figure 3, Table 1). For the remaining chimeras they showed either an ~ 4 fold increase for suramin (p<0.001) with no effect on PPADS (X1-AX4), or an ~ 3.5-fold decrease in PPADS inhibition (p<0.05) with no effect on suramin sensitivity (X1-BX4)(Figure 3, Table 1). The predominant finding of a lack of decrease in suramin or PPADS action at the chimeras contrasts with the decrease in NF449 sensitivity. This suggests that antagonist insensitivity at the rP2X4 receptor has multiple underlying molecular mechanisms dependent on the antagonist. The large decreases in NF449 sensitivity for chimeras X1-BX4 and X1-CX4 suggests that variant residues in these regions are likely to make an important contribution to NF449 action and were the focus of further studies.

**Decreased NF449 sensitivity in X1-BX4 chimera is due to removal of positive charge at the base of the cysteine rich head region.** The ~ 60 fold decrease in NF449 sensitivity at the X1-BX4 chimera was consistent with our previous study showing that mutating four positive charges at the base of the cysteine rich head region reduced sensitivity to the antagonist (19). However, as well as the four positive charges the region contained an additional 20 residues that were variant. To identify if it was solely the replacement of the positive charges that was responsible for the decrease in NF449 sensitivity of X1-BX4, or if other residues within region B were involved, the positive charges were mutated back into the X1-BX4 chimera (Figure 4). The mutant chimera incorporating the mutations to reintroduce the charges (S136K, D138K, T139R and H140K named X1-BX4(4+)) reduced ATP potency further (~85 fold from hP2X1 receptors and ~10 fold from X1-BX4; pEC\textsubscript{50} 3.6 ± 0.1, p < 0.0001 and 0.05 to P2X1 and X1-BX4 respectively) but interestingly returned the level of desensitization (15.7 ± 1.7 % current remaining at end of application) to hP2X1 receptor levels. These results show that the positively charged residues present in the cysteine rich head region of the WT hP2X1 receptor contribute to its characteristic fast time course and this is independent of effects on ATP sensitivity. The NF449 sensitivity of the X1-BX4(4+) mutant was returned to hP2X1 receptor levels (pIC\textsubscript{50} 9.28 ± 0.05 and 8.94 ± 0.04 respectively). This demonstrates the importance of the four positive charges at the base of the cysteine rich head region in antagonist action. In addition it suggests that the other 20 variant residues between hP2X1 and rP2X4 receptors in the B region are unlikely to account for the insensitivity to NF449 at the rP2X4 receptor.

As the presence of four positive charges reintroduced nanomolar NF449 sensitivity to the X1-BX4 chimera, it was hypothesised that their introduction to the rP2X4 receptor could make the receptor NF449 sensitive. The positive charges were therefore introduced at the equivalent positions of the rP2X4 receptor (S136K, D138K, T139R and H140K) to make the P2X4(4+) mutant. When 1 µM NF449 was co-applied with EC\textsubscript{90} ATP to the P2X4(4+) receptor no inhibition of the peak current was seen (103.5 ± 5.3 % of control). This shows that presence of the four charges alone is not enough to cause the rP2X4 receptor to be inhibited by NF449. This is consistent with previous studies on the hP2X2 receptor where introduction of the four positive charges had no effect on NF449 sensitivity (19) and indicates that a combination of residues are required for high affinity NF449 binding.

**Determination of role of variant regions in the X1-CX4 chimera to NF449 action.** The ~ 135-fold decrease in NF449 sensitivity at chimera X1-CX4 indicates that the variant residues in the region 185-261 contribute to antagonist action. The region swapped in chimera X1-CX4 is located below the cysteine rich head region. Given the importance of positive charge in the cysteine rich head region to NF449 sensitivity we reasoned that residues in the X1-CX4 chimera also important to NF449 sensitivity would be nearby. To test this we split the C region into two larger sub-chimeras X1-C\textalpha{X}4 (184-209) and X1-C\textbeta{X}4 (232-261) that were most distant from the head region and 4 smaller chimeras in closer proximity (X1-C\textgamma{X}4 (210-215), X1-C\textdelta{X}4 (216-220), X1-C\textepsilon{X}4 (221-226) and X1-C\textzeta{X}4 (227-231)(Figure 5). Chimers X1-C\textgamma{X}4 and X1-C\textdelta{X}4 had hP2X1 receptor ATP sensitivity, in contrast it was significantly reduced (p<0.0001) for the remaining chimeras, and in the case of X1-C\textdelta{X}4 and X1-C\textepsilon{X}4 to rP2X4 receptor levels (pEC\textsubscript{50} 5.21 ± 0.04 and 5.54 ± 0.06 respectively)(Table 2). For all the C
sub-chimeras the desensitization was equivalent to hP2X1 receptor levels (10-35% of peak current remaining at 3 s).

NF449 sensitivity was equivalent to hP2X1 receptor levels at X1-CαX4, X1-CβX4, X1-CγX4 and X1-CεX4 chimeras and reduced ~20 fold for X1-CδX4 and X1-ζX4 (p < 0.0001) (Figure 5, Table 2). No one chimera replicated the decrease in sensitivity to NF449 of the X1-CX4 chimera. This suggests, that it is a combination of residues within sub-chimeras of region C, closest to the four positively charged residues in the cysteine rich head region, that are contributing to NF449 sensitivity.

**Importance of individual variant residues in C chimera region to NF449 action.** For the sub-chimeras with a decrease in NF449 sensitivity (X1-CδX4 and X1-ζX4) individual point mutations of rP2X4 receptor variant residues within these regions were introduced to the hP2X1 receptor. The mutations made were T216S, L218I, F219Y, H220N, V229I and Q231R (Figure 6). All of the mutants were functional and showed hP2X1 receptor ATP sensitivity and levels of desensitization with the exception of H220N that had an ~ 8 fold decrease in ATP sensitivity (p<0.001) and reduced desensitization (current remaining at end of agonist application, 54.2 ± 5.1 % compared to 19.7 ± 4.3 % for hP2X1 receptors). There was no change in inhibition by NF449 compared to the hP2X1 receptor for the L218I, F219Y, H220N, V229I and Q231R mutants (Figure 6). The mutation at position 216, which mutated a threonine residue to a serine decreased NF449 sensitivity ~7 fold (p<0.05, Figure 6). The Q231R mutation, which introduced a positively charged residue beneath the four charges in the cyanine rich head region also showed a decrease in NF449 sensitivity of ~ 7-fold (p<0.01, Figure 6). These results highlight individual residues below the cyanine rich head region that contribute to NF449 sensitivity.

**Molecular modelling of NF449 binding and validation by site directed mutagenesis.** The six residues (K136, K138, R139, K140, T216 and Q231) that have been identified in the first round of site-directed mutagenesis as contributing to the inhibition of the hP2X1 receptor by NF449 were used to define the overall docking site on the hP2X1 apo form homology models, but no specific constraints were enforced. Ligand docking provides a series of alternative poses ranked by docking scores. For the interpretation of models, considering agreement with experimental data in addition to docking scores is paramount. All NF449 docking poses with ASP-scores >35 were visualised and filtered manually for their interactions with the six residues contributing to NF449 antagonism. The four poses matching both criteria have some key interactions with NF449 in common (see table 3). A characteristic feature of NF449 could be described as its four phenyl ‘arms’, each with two sulfonic acid groups in ortho and para position (ortho and para defined in relation to the amide linker). These negatively charged ‘arms’ would be expected to form salt bridges or hydrogen bonds with the receptor. All four docking poses show one ‘arm’ forming salt bridges with at least two of the positively charged residues of the ATP binding site (K68, K70, R292, K309). This suggests that NF449 partially occupies the ATP binding pocket accounting for its competitive antagonist characteristics. The majority of NF449 interactions with P2X1 however take place outside of the ATP binding pocket (as predicted due to the larger size of NF449). In all four poses a second ‘arm’ is involved in salt bridges with the block of positively charged residues at the base of the cysteine rich head region (K136, K138, R139, K140), though the specifics of these interactions vary between the four poses (see Figure 7). All four poses occupy the cavity under the cysteine rich head region close to site of ATP binding which could prevent the movement of the cysteine rich head region upon ATP binding (9). In poses A, C and D (and to a lesser extent in B) a third ‘arm’ of NF449 interacts with the side chains of T216 and Q231. It is also noteworthy that the conserved residue K215 contributes to salt bridges in all four poses.

While the four poses have key features in common, there are also differences. For example poses B, C and D predict an interaction of one NF449 ‘arm’ with H224, an interaction not present in pose A. Interestingly, H224 is part of chimera X1-CεX4 with a 4 fold decrease in NF449 sensitivity. To test whether this predicted
interaction can be confirmed, we generated the H224D mutant (to the rP2X4 receptor residue). The H224D mutation (no effect ATP sensitivity or time-course), which introduced a negatively charged residue beneath the four positively charged residues in the cysteine head region, produced an ~6-fold decrease (p < 0.05) in NF449 sensitivity compared to the hP2X1 receptor (Figure 6). This supports the idea that this residue is indeed involved in NF449 binding. Taken together with the data above, specifically the lack of favourable interactions between pose B and residues T216 and Q231, poses C and D are in better agreement with the experimental data than poses A and B.

**Analysis of NF449 binding with molecular dynamics simulations.** Molecular dynamics simulations are an efficient way of refining structural models, testing their stability and improving conformational sampling. To further analyse and refine the four predicted poses from ligand docking in an unbiased way, four 10 ns molecular dynamics simulations were run. The molecular dynamics trajectories of the extracellular domains of the P2X1 models with NF449 in poses A-D were analysed to test whether the interactions found by ligand docking are stable over the time course of the simulations.

All four poses maintained favourable interactions between NF449 and positively charged residues of the cysteine rich region (K85, K87, R88, K89), and NF449 and positively charged residues within the ATP binding site (K68, K70, R292, K309), but differences were found for interactions with T216, H224 and Q231. For pose A H224-N\(\delta\) and H224-N\(\epsilon\) are found within distances of 8-12 Å from the closest NF449-sulfonate group over the full time course of the simulation, too far for a strong charge interaction. The trajectory of pose B shows a stable interaction between a NF449-sulfonate group and H224 over the entire simulation, but favourable interactions of NF449 and residues T216 and Q231 are only present in less than 10% of the trajectory time points. Docking poses C and D occupy roughly the same space between the cysteine rich head and the ATP binding site. Over the time course of the simulations pose C maintained the interactions between NF449 sulfonate groups and T216 and H224, and to an extent to Q231 (Figure 7e). This was not the case for pose D, here none of these interactions were maintained. Hence, structural models derived from the trajectory of pose C are in best agreement with the experimental data.

**DISCUSSION**

P2X receptors share a conserved core ATP binding site that forms at the interface between adjacent subunits (9). Variations in amino acid sequence in the rest of the receptor give rise to a range of subtype dependent properties. The chimeras between hP2X1 and rP2X4 receptors in this study have been useful to identify whether a region contributes to differences in properties e.g. ATP sensitivity and desensitization. Replacing residues 185-261 (chimera X1-C4) of the hP2X1 receptor with those from the rP2X4 receptor had no effect on ATP-potency or desensitization demonstrating that variations in this region (below the ATP binding pocket) do not contribute to the differences in these properties. In contrast ATP sensitivity and desensitization was decreased (compared to hP2X1 receptors) at chimeras X1-BX4 and X1-DX4. It seems unlikely that these changes result from gross conformational effects on the receptor as chimera X1-BX4, that showed the largest decrease in ATP potency (~100 fold less than hP2X1 and ~10 fold less than rP2X4 receptors), had no effect on inhibition by suramin. These results suggest that variations in regions B and D (133-184 and 262-330) contribute to the differences in ATP responses. They also raise the possibility that regulation of agonist potency and time-course are inter-dependent. However this is not the case as chimera X1-AX4 had an ~100 fold decrease in ATP potency with no effect on desensitization. This supports studies that agonist sensitivity and time-course can be regulated independently (25,30). Our findings complement work showing the importance of residues throughout the intracellular, transmembrane and extracellular regions in the regulation of ATP responsiveness (4) and highlight that modifications throughout the whole receptor can regulate agonist binding and channel gating.

The >1,000 fold difference in inhibition to NF449, suramin and PPADS between hP2X1 (sensitive) and rP2X4 (insensitive) receptors was the starting point
for this study. The ≥ 60 fold decrease in NF449 antagonism at both X1-BX4 and X1-CX4 mutants showed that a chimeric approach could identify variant regions contributing to antagonist action. It was therefore surprising that none of the chimera showed a reduction in suramin sensitivity, and the affinity was actually increased ~ 10 fold for the X1-AX4 and X1-DX4 chimeras. A similar result was seen for the actions of PPADS with only a modest 3 fold decrease in sensitivity for X1-BX4, no change for X1-AX4 and X1-CX4, and an ~10 fold increase in affinity for X1-DX4. These results show that sections of the suramin and PPADS insensitive rP2X4 receptor can substitute for those of the hP2X1 receptor and a significant number of residues required for suramin and PPADS action are already present in the rP2X4 receptor. This is consistent with studies showing point mutations, or changing small sections, increased suramin and PPADS sensitivity at P2X4 receptors (15,16,31). For example PPADS sensitivity can be increased at the rat P2X4 receptor with the mutation of the negatively charged aspartate residue at position 249 to the positive lysine residue found at the equivalent position at the PPADS sensitive P2X1 and P2X2 receptors (15). The ~ 60 fold decrease in NF449 antagonism at the X1-BX4 chimera could be attributed to the replacement of four positive charges at the base of the cysteine rich loop as sensitivity was completely rescued in the X1-BX4(4+) chimera mutant. This is consistent with previous studies showing the importance of positive charge at this position in NF449 action (18,19). In addition the rescue of NF449 sensitivity at the X1-BX4(4+) chimera indicates that the other 20 variant residues in the BX4 segment are unlikely to contribute to sensitivity of the antagonist (consistent with ligand docking studies).

The action of ATP at the X1-CX4 chimera was identical to the hP2X1 receptor however the sensitivity to NF449 was reduced ~ 135 fold. Sub-division of the chimera identified the “dorsal fin” region (14) below the positive charges of the cysteine rich head as contributing to the decrease in NF449 action. Within this region the point mutations (hP2X1-rP2X4) T216S and Q231R reduced NF449 sensitivity ~ 7 fold indicating that these variations contribute to the reduction in antagonist affinity. Interestingly at all other mammalian P2X receptor subunits (except P2X5) the residue corresponding to Q231 is a positive charge suggesting that this variation contributes in part to the selectivity of NF449 to P2X1 receptors. In addition there is some modest difference in NF449 sensitivity between species orthologues of the P2X1 receptor (18,32) that may result in part from variation in the residue at position 231 (human - Q, rat - N and mouse - S) and/or 216 (human – T, rat and mouse – K). Molecular modelling suggests a mechanistic explanation for the effect of T216S and Q231R mutations. In docking poses B-D one of the benzene disulfonic acid arms of NF449 is anchored between the side chains of T216 and Q231 so that T216 forms an H-bond with the sulfonic acid group in ortho position while Q231 interacts with the sulfonic acid group in para position (Figure 7D). Such an arrangement may be sterically less favourable for the T216S and Q231R mutants. Interestingly the NF449 analogs NF110 (sulfonic acid only in para position) and MK3 (sulfonic acid only in ortho position) show significantly lower Ki than NF449 (33). The bi-dental arrangement of T216 and Q231 seems ideal for binding NF449, but less so for its analogs with only one substituent. This may provide a rational for the observation that the Ki of NF449/P2X1 is ~800 fold lower than for NF110/P2X1 (250 fold lower for MK3/P2X1), in contrast to rP2X2 and rP2X3 where the Ki of NF449 is actually higher compared to its NF110 and MK3 analogs (33).

The identification of positive charges at the base of the cysteine rich head region and T216 and Q231 as being important in NF49 action was the starting point for molecular docking studies. These identified four clusters of solutions around the cysteine rich head region with part of the NF449 molecule being co-ordinated by residues within the core ATP binding pocket conserved between P2X receptor subunits. A common feature of the
models was the binding of part of NF449 within the agonist pocket. This is consistent with the antagonist action of NF449 at non-P2X1 receptor subunits, albeit with >1,000 fold reduced affinity at P2X2 (19,22), P2X3 (34), P2X4 (22) and P2X7 receptors (32). Direct overlap of part of the NF449 binding site would also account for the competitive nature of the antagonism (32).

Our work is a major step forward in understanding the specificity of NF449 antagonist action at the hP2X1 receptor. The combination of chimera, point mutants and molecular modelling suggests that the interplay of four key regions of interactions are of importance for NF449 binding to P2X1 receptors (Figure 7). The interaction of NF449 with positively charged residues of the ATP binding site cannot explain NF449 specificity, as these residues are essential for ATP binding and conserved across other P2X receptors. However, the positively charged residues K136, K138, R139 and K140 within the cysteine rich head region are exclusively found in P2X1 receptors. Similarly, T216 and Q231 residues are not conserved across the P2X receptor family and H224 corresponds to an aspartic acid in P2X2, P3X3 and P2X4. We have shown that these sites affect NF449 potency. As they are particular to hP2X1 receptors and not present in other P2X receptors we conclude that their interactions with NF449 are likely to be key to NF449 specificity and that they are of significant interest for the development of P2X1 receptor specific drugs.

Acknowledgements: We thank Manijeh Maleki-Dizaji for technical support.

References


Figures legends.

Figure 1. Characterisation of ATP at chimeric P2X Receptors. (a) Location of regions A-D on an ATP bound homology model of the P2X1 receptor. Regions A-D are shown in colour. Transmembrane domains are shown in dark grey. (b) Representative traces for WT and chimeric receptors at maximal ATP concentrations. The bar represents a 3s ATP application. Traces have been normalised to peak currents to allow for comparison. (c) Concentration response curves for ATP. Black stars indicate significant shifts in EC50 concentration from the WT P2X1 receptor, red stars from the P2X4 receptor and blue stars from both receptors. **** p<0.0001.

Figure 2. NF449 Action at Chimeric P2X Receptors. (a) Representative traces showing the effect of NF449 (10 nM) on currents evoked by an EC90 concentration of ATP at Xenopus oocytes expressing WT and chimeric receptors. The bar indicates a 3s agonist/antagonist application. (b) NF449 inhibition curves at an EC 90 concentration of ATP. Stars indicate a significant shift from the hP2X1 receptor; * p<0.05, ** p<0.01, **** p<0.0001.

Figure 3. Suramin and PPADS antagonism at chimeric P2X receptors. (a) Comparison of suramin action at WT receptors and chimeras. (b) PPADS inhibition curves for chimeric and WT receptors. In all cases antagonist action was determined against an EC90 concentration of ATP. Stars indicate a significant shift from the WT P2X1 receptor. ** p<0.01, *** p<0.001.

Figure 4. Introduction of four charges re-introduced NF449 sensitivity to chimera X1-BX4. (a) Location of residues K136, K138, R139 and K140 on a P2X1 receptor homology model, docked ATP is shown in yellow. (b) NF449 inhibition curves showing the effect of reintroducing the positive charges to chimera X1-BX4, and NF449 action at the P2X4(4+) receptor. Stars indicate a significant difference in IC50 compared to the P2X1 receptor. **** p < 0.0001.

Figure 5. Effects of NF449 at C-region sub-chimeras. (a) Location of residues swapped to generate chimeras shown on a homology model of the P2X1 receptor; docked ATP is shown in yellow, transmembrane domains are in dark grey. (b) NF449 inhibition curves for sub-chimeras. Curves were generated at an EC 90 concentration of ATP. Stars represent a significant difference in IC50 compared to the P2X1 receptor. ** p < 0.01 **** p < 0.0001.

Figure 6. Effects of NF449 at point mutants. (a) Location of point mutated residues on a P2X1 receptor homology model, docked ATP is shown in yellow. Residues were mutated to the equivalent residue of the P2X4 receptor. (b) Histogram to show the fold change in NF449 inhibition compared to the WT P2X1 receptor at an EC90 concentration of ATP. Stars represent a significant difference * p<0.05 ** p < 0.01.

Figure 7. Docking poses for the NF449 P2X1 complex. (a) and (b) Overlay of the four docking poses A-D on the P2X1 receptor shown from different angles. The P2X1 receptor is shown in surface
representation highlighting the positively charged residues of the ATP binding site (K68, K70, R292, K309) in light blue, positively charged residues of the cysteine rich head region (K136, K138, R139, K140) in dark blue, T216 and Q231 in green, region X1-CγX4 (210-215, see text) in pale cyan and H224 in purple. NF449 poses A-D (A, red; B, orange; C pink; D, yellow) are shown as a mix of sphere representation (for the core of the poses) and stick representation (for the ‘arms’ of the poses). All four poses bind to the cleft between the cysteine rich head region, ATP binding site and dorsal fin. 

\(c\) Molecular structure of the NF449 sodium salt. \(d\) Snapshot of NF449 from the pose C trajectory. NF449 and K68, K70, K136, K138, R139, K140, T216, H224, Q231, R292 and K309 in stick representation. 

\(e\) Distances between NF449 sulfonate sulphur atoms and H220-Nε (black), T216-Oγ (green) and the amide nitrogen of Q231 monitored over the 10 ns molecular dynamics simulation of pose C indicating that NF449/H220 and NF449/T216 interactions are present in most of the frames, NF449/Q231 interactions are less stable in this simulation and only present between 1.5 and 4.5 ns. \(f\) Section of NF449 pose C interacting with the ATP binding site, potential salt bridges are indicated by black dotted lines. \(g\) Section of NF449 pose A and K136, K138 and R139 of cysteine rich head region. \(h\) Arm of NF449 pose D forming H-bonds with T216 and Q231 side chains.
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<tr>
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<th>Peak Current (nA)</th>
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<th>% ATP Current Remaining at 3s</th>
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<th>NF449 pIC&lt;sub&gt;50&lt;/sub&gt;</th>
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**Table 1 Properties of Chimeras A-D to ATP and Antagonists.** Antagonists properties were determined at an EC<sub>90</sub> concentration of ATP. Stars represent a significant difference from the hP2X1 receptor. ND not determined, ns not significant. * p<0.05, ** p<0.01, *** p < 0.001, **** p<0.0001.
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<th></th>
<th>Peak Current (nA)</th>
<th>ATP pEC\textsubscript{50}</th>
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<td>P2X1</td>
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Table 2 Properties of region C sub-chimeras to ATP and NF449. NF449 properties were determined at an EC\textsubscript{90} concentration of ATP. Stars represent a significant difference from the hP2X1 receptor. ND not determined, ns not significant. **** p<0.0001.
Table 3. Salt bridge and hydrogen bond interaction of the four docking poses A-D with key residues of the P2X1 receptor model. ‘o’ and ‘p’ refer to sulfonate groups in ortho and para position relative to the amide linker.

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Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
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