

**Identification of determinants required for agonistic and
inverse agonistic ligand properties at the ADP receptor P2Y₁₂**

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Abbreviations

ADP β S, adenosine 5'-O-2-(thio)diphosphate; AppNH₂, Adenosine-5'-(amido)diphosphate;
ATP γ S, adenosine 5'-O-(thiotriphosphate); GPCR, G protein-coupled receptor; IP, inositol
phosphate; LiAc, lithium acetate; LiCl, lithium chloride; MeS-ADP, 2-(methylthio)adenosine
5'-diphosphate; MeS-ATP, 2-(methylthio)adenosine 5'-triphosphate; PEP, phosphoenol
pyruvate; TNP-ADP, 2'-(OR-3')-O-(trinitrophenyl) adenosine 5'-diphosphate; TM,
transmembrane helix; WT, wild type; 2I-ATP γ S, 2-Iodo-adenosine-5'-(thio)-triphosphate

Abstract

The ADP receptor P2Y₁₂ belongs to the superfamily of G protein-coupled receptors (GPCRs) and its activation triggers platelet aggregation. Therefore, potent antagonists, e.g. clopidogrel, are of high clinical relevance in prophylaxis and treatment of thromboembolic events. P2Y₁₂ displays an elevated basal activity *in vitro* and as such, inverse agonists may be therapeutically beneficial compared to antagonists. Only a few inverse agonists of P2Y₁₂ have been described. To expand this limited chemical space and improve understanding of structural determinants of inverse agonist-receptor interaction, we screened a purine compound library for lead structures using wild type (WT) human P2Y₁₂ and 28 constitutively active mutants. We found that ATP and ATP derivatives are agonists at P2Y₁₂. The potency at P2Y₁₂ was 2-(methylthio)-ADP > 2-(methylthio)-ATP > ADP > ATP. Determinants required for agonistic ligand activity were identified. Molecular docking studies revealed a binding pocket for the ATP derivatives that is bordered by transmembrane helices 3, 5, 6 and 7 in the human P2Y₁₂, with Y¹⁰⁵, E¹⁸⁸, R²⁵⁶, Y²⁵⁹ and K²⁸⁰ playing a particularly important role in ligand interaction. *N*-Methyl-anthraniloyl modification at the 3'-OH of the 2'-deoxyribose leads to ligands (mant-dATP, mant-dADP) with inverse agonist activity. Inverse agonist activity of mant-dATP was found at the WT human P2Y₁₂ and half of the constitutive active P2Y₁₂ mutants. Our study showed that, in addition to ADP and ATP, other ATP derivatives are not only ligands of P2Y₁₂ but also agonists. Modification of the ribose within ATP can result in inverse activity of ATP-derived ligands.

Introduction

The ADP receptor P2Y₁₂ is a G_i protein-coupled receptor and a key player in platelet aggregation (Hollopeter et al., 2001). Inactivating mutations in P2Y₁₂ are responsible for bleeding disorders in humans and dogs (Boudreaux and Martin, 2011; Cattaneo, 2005; Cattaneo et al., 2003; Daly et al., 2009; Fontana et al., 2009; Hollopeter et al., 2001; Remijn et al., 2007; Shiraga et al., 2005). With significant relevance in pathophysiology, P2Y₁₂ is also the major target of the antithrombotic drugs ticlopidin and clopidogrel. The thienopyridine clopidogrel is a prodrug which requires the cytochrome P450 2C19 enzyme for its conversion to an active thiol metabolite. Several mechanisms of antagonistic action have been proposed for the active metabolite of clopidogrel, including interaction with extracellular cysteine residues of P2Y₁₂ (Ding et al., 2003) and receptor dimer disruption (Savi et al., 2006). Although very specific and effective, clopidogrel produces a variable platelet inhibition based on genetic polymorphisms and drug interactions (Munoz-Esparza et al., 2011; Nawarskas and Clark, 2011). This has triggered the search for alternative P2Y₁₂ blockers such as prasugrel, cangrelor and ticagrelor. The latter two compounds are ATP analogues and bind reversibly at P2Y₁₂ (Storey, 2011).

P2Y₁₂ displays a high constitutive activity when expressed *in vitro* (Chee et al., 2008; Schulz and Schoneberg, 2003). Therefore, inverse agonists may be therapeutically beneficial compared to antagonists. Because only a few inverse agonists of P2Y₁₂ have been described (Ding et al., 2006), we, therefore, screened for compounds that reduce the basal activity of constitutively active P2Y₁₂ mutants.

Functional characterization of P2Y receptors and their mutants in mammalian expression systems is problematic because of the abundance of endogenous nucleotide receptors, nucleosidases and nucleotide release. In previous experiments, we and others demonstrated that the human P2Y₁₂ is functionally expressed in the yeast system (Pausch et al., 2004; Schulz and Schoneberg, 2003) which lacks such problems. Numerous constitutively

activating mutations have been described for GPCRs in natural or recombinant systems, but only a few have been reported for P2Y receptors (Ding et al., 2006). From over 1000 single point mutations, we identified 28 constitutively active P2Y₁₂ mutants. Screening a purine compound library, we discovered several new agonists and inverse agonists for the WT P2Y₁₂ and constitutively active mutants, respectively.

Materials and Methods

Materials

If not stated otherwise, all standard substances were purchased from Sigma–Aldrich, Merck and Care Roth. Cell culture material was obtained from Sarstedt. MeS-ADP (2-(methylthio)adenosine-5'-diphosphate trisodium salt hydrate), salmon sperm DNA, lithium acetate (LiAc) dihydrate, lithium chloride (LiCl), PEG 3350 (cat. no. P-3640) and apyrase from potatoe (Grade III) were obtained from Sigma-Aldrich (Munich, Germany). MeS-ADP (P2Y₁₂ agonist) was dissolved in water and aliquots of stock solutions (10 mM) were stored at -20 °C. Yeast medium components were purchased from Sigma-Aldrich (Munich, Germany) and from BD Biosciences (Heidelberg, Germany). Restriction enzymes were purchased from New England Biolabs (Frankfurt a. M., Germany), primers were synthesized by Life Technologies (Darmstadt, Germany) and P2Y₁₂ mutant libraries were provided by Sloning BioTechnology (Puchheim, Germany). The adenine nucleotide library was from Jena Bioscience (Jena, Germany). For compound details see <http://www.jenabioscience.com/images/7c63e6fc71/LIB-101.pdf>. We additionally included GTP, adenine, adenosine, GDP, GTP, GTP γ S, IMP, and xanthine (all from Sigma) in our pharmacological screenings (further referred to as “purine compound library”).

Generation of P2Y₁₂ mutants

Mutants were generated by subcloning SlonoMax-SINGLE libraries (synthesized double-stranded DNA fragments containing individual mutants, fragment sizes 100 – 150 bp) via unique endogenous or silently introduced restriction sites. P2Y₁₂ mutants were introduced into the yeast expression plasmid p416GPD (provided by Dr. Mark Pausch, Wyeth Research, Princeton, NJ, U.S.A.) and transformed into *E. coli* DH5 α (Life Technologies, Darmstadt, Germany). Plasmids from individual clones were isolated (plasmid preparation kit; Promega,

Mannheim, Germany) and mutations were identified by DNA sequencing. Because full coverage was not achieved after sequencing 96 clones, missing mutants (4 mutants/position on average) were generated by PCR-based site-directed mutagenesis using mutant-specific mutagenesis primers.

Expression and functional testing of P2Y₁₂ mutants in yeast and mammalian cells

The *Saccharomyces cerevisiae* yeast strain MPY578t5 (provided by Dr. Mark Pausch) was used for yeast expression and functional testing of the P2Y₁₂ mutants. Cells were transformed with plasmid DNA using the LiAc/salmon sperm carrier DNA/PEG method. In brief, an overnight culture grown at 30 °C in YPAD (yeast extract, peptone, dextrose medium with adenine) was diluted to an optical density of 0.2 at 600 nm (OD_{600 nm}) in 50 ml YPAD. This culture was incubated at 30 °C until the OD_{600 nm} reached 0.7-0.9. Cells were then harvested (2,500 g for 5 min at room temperature) and washed once with 25 ml of water. The pellet was dissolved in 700 µl of LiAc (100 mM) and incubated for 10 min at 30 °C. A pellet of 50 µl from the yeast cell suspension was then mixed with 90 µl PEG 3350 (50 % w/v), 13.5 µl LiAc (1 M), 18.75 µl salmon sperm carrier DNA (2.0 mg/ml), 2.75 µl of sterile water and plasmid DNA (1 µg) before being incubated for 30 min at 30 °C, then for 30 min at 45 °C.

For selection of constitutively active clones, cells were plated on agar plates not containing uracil and histidine (U/H). After incubation at 30 °C for 4 days, clones were prepared for concentration-response curves. Cells transformed with P2Y₁₂ mutants were pre-cultured for 2 days at 30 °C in U/H with 10 µM MeS-ADP. To remove MeS-ADP, cells were washed twice with water and grown in U/H overnight without MeS-ADP. The yeast cell suspension was then diluted to an OD_{600 nm} of 0.1. From this cell suspension, 100 µl were pipetted into each well of a 96-well plate and to this, 100 µl of a 2x agonist solution or medium was added. Background growth was suppressed by the addition of 20 mM 3-aminotriazole. Mutants were

screened for growth and/or constitutive activity at 10 μ M MeS-ADP. All positive mutants were further evaluated through MeS-ADP concentration-response (growth) curves.

The purine compound library was screened for agonists and inverse agonists at the WT P2Y₁₂ and constitutively active mutants. 100 μ l of the respective yeast cell suspension (OD_{600 nm} = 0.1) was pipetted into each well of a 96-well plate and, to these samples, 100 μ l of a 2x ligand solution or medium was added. OD measurements were performed 24 and 48 hours later. Compounds identified to be agonists or inverse agonists were further characterized in concentration response setups. IC₅₀ and EC₅₀ values were calculated using GraphPadPrism 4 software (GraphPad).

To determine the stability of ATP in the 24-h yeast assay we performed a phosphoenolpyruvate (PEP)/pyruvate kinase test. Thus, human WT P2Y₁₂ expressing yeast cells were grown identically to the previous assays. 100 μ l of the respective yeast cell suspension (OD_{600 nm} = 0.1) was pipetted into each well of a 96-well plate and 100 μ l of a 2x ATP solution or medium was added. Further, 2 mM PEP and 2 μ l of a pyruvate kinase solution (final 6 U/ml) were added every 5 h to the experimental solution (the total volume additional contained 100 mM imidazol, 5 mM MgCl₂, pH = 7.15 to assure proper pyruvate kinase function). In case ADP is formed due to degrading, PEP is utilized by the pyruvate kinase to produce ATP. Then, the concentration of PEP in the medium was monitored over 24 h using a coupled optical enzyme-test. Thus, 50 μ l yeast medium harvested after 0 h, 4 h and 24 h (or 1 mM phosphoenolpyruvate for control purposes) were incubated with 0.7 ml assay buffer (100 mM imidazol, 5 mM MgCl₂, pH = 7.15), 1.5 μ l lactate dehydrogenase (10 U/ml), 1 μ l pyruvate kinase (10 U/ml), 8 μ l of a 100 mM ADP solution and 8 μ l 2 mM NADH. NADH concentration was determined photometrically at 340 nm.

For expression in mammalian cells, Chinese hamster ovary (CHO)-K1 cells were grown in Dulbecco's modified Eagle's medium (DMEM supplemented with 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin) at 37 °C in a humidified 5 % CO₂

incubator. A CHO-K1 cell line stably expressing the chimeric G protein $G\alpha_{qi4}$ (Kostenis et al., 2005) was established. Transient transfection experiments of CHO-K1 cells with the respective P2Y₁₂ constructs and inositol phosphate (IP₁) accumulation assays were performed as described (Schulz and Schoneberg, 2003). In brief, $G\alpha_{qi4}$ -stable cells were seeded into twelve-well plates (about 0.15×10^6 cells/well), transiently transfected and about 48 h after this labeled with 2 μ Ci/ml of [*myo*-³H]inositol (Perkin Elmer). After a 16 h-labeling period, cells were washed once with serum-free DMEM containing 10 mM lithium chloride (LiCl) and then incubated for 60 min at 37 °C with serum-free DMEM containing 10 mM LiCl with or without a compound. After this time, the assay medium was removed, and the reaction was stopped by adding 0.3 ml of 0.1 N NaOH, followed by a 5 min-incubation at 37 °C. The alkaline solution was then neutralized by adding 0.1 ml of 0.2 M formic acid, and the IP₁ fraction was isolated by anion exchange chromatography as described (Berridge, 1983) and counted on a liquid scintillation counter.

For cAMP assays, transfected cells were labeled with [³H]adenine (2 μ Ci/ml; PerkinElmer) for 12 h and washed once in serum-free DMEM containing 1 mM 3-isobutyl-1-methylxanthine (Sigma–Aldrich), followed by incubation in the presence of the indicated compounds and forskolin (10 μ M) for 1 h at 37°C. Reactions were terminated by aspiration of the medium and addition of 1 ml of 5% (w/v) trichloroacetic acid. The cAMP content of cell extracts was determined by anion exchange chromatography as described (Salomon et al., 1974).

To measure label-free receptor activation, a dynamic mass redistribution (DMR) assay (Corning Epic® Biosensor Measurements) with stably transfected Human Embryonic Kidney (HEK) cells (HEK-FlpIn, P2Y₁₂ in pcDNA5/FRT) was performed as described previously (Ritscher et al., 2012; Schroder et al., 2010). Briefly, cells were seeded into fibronectin-coated Epic 384-well microplates (60,000 cells per well) and exposed to the various compounds. In DMR measurements, polarized light is passed through the bottom of the

biosensor microtiter plate, and a shift in wavelength of reflected light is indicative of intracellular mass redistribution triggered by receptor activation. DMR was recorded as a measure of cellular activity over 60 min. Agonist-induced DMR is concentration-dependent and concentration-effect curve were calculated from response peak maxima (approximately 6 min after adding the compound) of optical traces.

Generation of a P2Y₁₂ comparative model and ligand docking

A comparative model of P2Y₁₂ was constructed using the protein structure prediction software package, Rosetta version 3.2 (Leaver-Fay et al., 2011). The X-ray crystal structure of CXCR4 (Protein Data Bank ID: 3ODU) (Gupta et al., 2001) was chosen as a template based on its high similarity to P2Y₁₂ (e-value of $3e^{-15}$ with a sequence coverage of 90%) according to a search using NCBI BLASTP on sequences from the Protein Data Bank (PDB) (*Supplemental Figure S1*). CXCR4 and P2Y₁₂ also share a conserved disulfide bond between the N-terminal C17 and C270 in extracellular loop 3 (Deflorian and Jacobson, 2011). The backbone coordinates of CXCR4 were retained in the comparative model of P2Y₁₂, while the loop coordinates were built in Rosetta using Monte Carlo Metropolis (MCM) fragment replacement combined with cyclic coordinate descent loop closure. In brief, ϕ - ψ angles of backbone segments from homologous sequence fragments from the PDB are introduced into the loop regions. After the fragment substitution, small movements in the ϕ - ψ angles are performed to close breaks in the protein chain. The resulting full sequence models were subjected to eight iterative cycles of side chain repacking and gradient minimization of ϕ , ψ , and χ angles Rosetta Membrane (Yarov-Yarovoy et al., 2006).

Ligand docking into the comparative model of P2Y₁₂ with ADP, ATP, MeS-ADP, MeS-ATP, mant-ADP, mant-ATP, mant-dADP and mant-dATP was performed with Rosetta Ligand (Davis and Baker, 2009; Meiler and Baker, 2006). Each ligand was allowed to sample

docking poses in a 5 Å radius centered at the putative binding site for ADP, determined by averaging the coordinates of critical residues for ligand recognition: R²⁵⁶, Y²⁵⁹ and K²⁸⁰ (Hoffmann et al., 2008). Once a binding pose had been determined by the docking procedure, 100 conformations of the ligand created by MOE (Molecular Operating Environment, Chemical Computing Group, Ontario, Canada) were tested within the site. Side-chain rotamers around the ligand were optimized simultaneously in a MCM simulated annealing algorithm. The energy function used during the docking procedure contains terms for van der Waals attractive and repulsive forces, statistical energy derived from the probability of observing a side-chain conformation in the PDB, hydrogen bonding, electrostatic interactions between pairs of amino acids, and solvation assessing the effects of both side-chain/side-chain interactions and side-chain/ligand interactions. For each ligand, over 3,000 docked complexes were generated and clustered for structural similarity using bcl::Cluster (Alexander et al., 2011). The lowest energy binding pose from the five largest clusters for each ligand were used for further analysis. The change in free energy with and without ligands bound to P2Y₁₂ was calculated for each residue in the receptor. Residues with the greatest difference in predicted energy are suggested to be important for ligand interaction (*Supplemental Figure S2*).

Results

Expression of the human P2Y₁₂ in yeast and determination of agonist specificity

In previous experiments, we and others have already demonstrated that human P2Y₁₂ can be functionally expressed in the yeast system. In this system, P2Y₁₂-expressing yeast grows in 96-well cell plates and regular OD measurements are taken. OD values measure cell growth, which is used as a strong indicator for receptor activity.

The WT P2Y₁₂ was functionally tested with the compound library for agonists. P2Y₁₂ expressed in yeast showed a similar EC₅₀ value for MeS-ADP (EC₅₀ value: 6 nM, Figure 1) as when expressed in mammalian cells ranging from low nanomolar to 25-80 nM concentrations (Bodor et al., 2003; Ding et al., 2006; Hoffmann et al., 2008; Simon et al., 2002; Zhang et al., 2001; Zhong et al., 2004). ADP was about 500 fold less potent to MeS-ADP, which is consistent with previous findings showing an about 30 fold to 1000 fold lower potency in mammalian cells (Bodor et al., 2003; Simon et al., 2002; Zhang et al., 2001). In addition to the highly potent agonist MeS-ADP, we identified further P2Y₁₂ agonists: ATP and MeS-ATP. ATP was a partial agonist at the human P2Y₁₂ when expressed in yeast (Figure 1A). EC₅₀ values were ranked: MeS-ADP < MeS-ATP < ADP < ATP. We considered the possibility that the registered ATP activity might be due to the fraction of the nucleotides converted to ADP derivatives by nucleotidases or hydrolysis and quantified the possible decay of ATP during the 24-h assays. Thus, we indirectly quantified ATP degradation in the assay using the PEP/pyruvate kinase system. The pyruvate kinase catalyzes the transfer of a phosphate group from PEP to ADP, yielding one molecule of pyruvate and one molecule of ATP. PEP concentration in the medium is therefore a measure for degraded ATP (see Materials and Methods). We found that PEP concentration in the yeast medium remained almost unchanged during 24 h yeast growth ($\Delta E_{0\text{ h}} = 0.44$, $\Delta E_{4\text{ h}} = 0.44$, $\Delta E_{24\text{ h}} = 0.48$). Only 3.4 % of PEP (initial concentration 2 mM) was utilized by pyruvate kinase for ATP generation. This indicated high stability of ATP (96.6 %) in the assay over 24 h. The

functionality of the pyruvate kinase to convert ADP to ATP was verified in control experiments performed in parallel.

The agonistic properties of the adenine nucleotides were verified in the mammalian cell line COS-7 and CHO cells (data not shown) where the human P2Y₁₂ was co-expressed with the chimeric G α_{q14} protein, which redirects receptor activation to the phospholipase C/inositol phosphate (IP) pathway (Kostenis et al., 2005). Since ATP produces a cellular response via endogenous nucleotide receptors in most cell lines we performed additional measurements of P2Y₁₂ activation on stably transfected mammalian HEK with a dynamic mass redistribution assay (Corning Epic® Biosensor Measurements) (Schroder et al., 2010). Responses of endogenous nucleotide receptors were subtracted from the specific response of P2Y₁₂-transfected cells. As shown in Figure 1B, the concentration-response curves were similar to the data from the yeast expression system except we found that ATP was a full agonist in this mammalian expression system. We also performed Epic measurements in P2Y₁₂-stably transfected astrocytoma cells 1321N1, which should not express nucleotide receptors (Filtz et al., 1994). However, ATP-mediated responses in 1321N1 cells were less than in HEK cells having no advantage over transfected HEK cells. In sum, the yeast expression system is free of endogenous nucleotide receptors and, therefore, the most straight forward system to use in analyzing P2Y receptors. P2Y₁₂ expressed in yeast displays pharmacological properties very comparable to mammalian expression systems. Our screening revealed additional compounds with agonistic activity at P2Y₁₂: ADP β S, TNP-ADP, ATP γ S, 2I-ATP γ S, and AppNH₂ (Table 2). We did not follow the pharmacology of these ADP and ATP derivatives further, but they support the fact that derivatives of ATP, as well as of ADP, also have agonistic activities at the human P2Y₁₂.

It should also be noted that a number of nucleotides and nucleosides (e.g. AMP, GTP, cAMP, adenosine) which do not activate P2Y₁₂ in mammalian expression systems (Zhang et al., 2001) did not activate P2Y₁₂ expressed in yeast.

The comprehensive compound library allowed identification of all determinants necessary for agonist function at P2Y₁₂ (*Supplemental Table S1*). Compounds showing significant agonistic activity are given in Table 2. Substitutions which are not compatible with agonistic activity at the human P2Y₁₂ (at least in two tested compounds) are listed in Table 3. The results for agonistic activity can be roughly summarized for the three major nucleotide components (base, ribose, phosphate group):

1. The purine ring is absolutely required. Some modifications (methylthio, iodo) at the 2-position of adenine are tolerated, but guanine- and inosine-based nucleotides are not agonistic.
2. Deoxidation of the ribose is not tolerated. The trinitrophenyl modification (TNP-ADP) is tolerated.
3. Adenine nucleotides with two or three phosphate residues are agonistic, while < 2 phosphate residues or cyclic phosphates are insufficient for agonistic activity. Some substitutions of phosphate moieties as in ADPβS, ATPγS, and AppNH₂ are tolerated.
4. Adenine nucleotide multimers (P1-(5'-Adenosyl) P3-(5'-adenosyl) triphosphate, P1-(5'-Adenosyl) P4-(5'-adenosyl) tetraphosphate, P1-(5'-Adenosyl) P5-(5'-adenosyl) pentaphosphate, P1-(5'-Adenosyl) P6-(5'-adenosyl) hexaphosphate) displayed no agonistic activity.

Structural model of agonist binding

To estimate whether the different agonists may have similar binding properties, we simulated binding by docking the agonists into the comparative model of P2Y₁₂ (Figure 2 and Figure 6). The model suggested that ADP, ATP, MeS-ADP, MeS-ATP, mant-ADP, mant-ATP, mant-dADP and mant-dATP bind in the site bordered by transmembrane helices (TM) 3, 5, 6 and 7. Ligands were oriented such that the phosphate groups generally pointed towards TM 3 and 7, forming hydrogen bonds with Y¹⁰⁵ and K²⁸⁰. Adenosine rings frequently interacted with

the hydrophobic residues on TM 5, namely L¹⁸⁴, V¹⁸⁵ and F¹⁷⁷ in the second extracellular loop. In agreement with previous docking studies, R²⁵⁶ and K²⁸⁰ were found to be critical residues in the ADP binding pocket (Deflorian and Jacobson, 2011; Ignatovica et al., 2011). R²⁵⁶ frequently interacts with the hydroxyl groups and the oxygen from the furanose. K²⁸⁰ is demonstrated to interact with the negatively charged phosphate groups of the ligands. In addition to the R²⁵⁶ and K²⁸⁰, Y¹⁰⁵, E¹⁸⁸ and Y²⁵⁹ are consistently found to interact with the ligand. Y¹⁰⁵ and E¹⁸⁸ form hydrogen bonds with the phosphate tail, while Y²⁵⁹ seems to stabilize the adenine.

Identification of constitutively active mutants

It is still impossible to predict mutations leading to constitutive activity of a given GPCR. Furthermore, at positions where some mutations activate the GPCR, not all mutations will result in constitutive receptor activation (Bakker et al., 2008; Lalueza-Fox et al., 2007). Therefore, screening of mutant libraries is required. Mutations induced via error-prone derived mutant libraries cannot provide mutational saturation of every codon, and instead, most alleles will contain more than one mutated codon (Li et al., 2007; Thor et al., 2008). Recent advances in gene synthesis technology (see Methods) have made it possible to generate comprehensive mutant libraries.

Here, we mutated every single position to all possible amino acids in a receptor region known from other GPCRs to be sensitive for mutational induced constitutive activity. In sum, 1,254 P2Y₁₂ mutants were generated covering 66 positions (amino acid position 236 – 301) of the receptor and yielding 28 constitutive active mutants at 10 positions (positions are given in Figure 3). Most mutations were found at positions that faced the lipid, while three positions faced the receptor pore (F²⁴⁶, F²⁴⁹ and N²⁹⁰) and three were near the C-terminal receptor tail (F²⁹⁹, F³⁰⁰ and L³⁰¹). All data are available and organized in a P2Y₁₂ mutant database (<http://www.ssfa-7tmr.de/p2y12>).

Identification of mant-dATP as inverse agonist at constitutively active P2Y₁₂ mutants

Constitutively active mutants were expressed and the purine compound library was tested for inverse agonists. *N*-Methyl-anthraniloyl- (mant-) dATP reduced basal activity of many constitutively active P2Y₁₂ mutants (Table 1). For several mutants, mant-*N*⁶-Methyl-ATP was also an inverse agonist (see Table 1). There is no obvious structural overlap or difference between the mutants at which the different inverse agonists act or do not act.

Inverse agonist activity was studied at F²⁵⁴L in more detail. As shown in Figure 4A, mant-dATP suppressed basal activity in a concentration-dependent manner with an IC₅₀ value in a μM range. Interestingly, the potency of mant-dADP was lower compared to mant-dATP (see Figure 4A). Both the deoxy- and the mant- modifications are required, since mant-ATP and dATP had no effect on basal activity of P2Y₁₂ mutants. It should be noted that the basal activities of several mutants (V²⁴⁴E, F²⁴⁶C, F²⁴⁶G, F²⁴⁶P, F²⁴⁶S, F²⁴⁶T, I²⁴⁷F, F²⁴⁹Y, N²⁹⁰W, N²⁹⁰Y, F²⁹⁶I, F²⁹⁶L, F²⁹⁶V, F²⁹⁹I, F²⁹⁹V, L³⁰¹C, L³⁰¹G, L³⁰¹T) were not reduced by mant-dATP or any other compound tested. It is known already that WT P2Y₁₂ displays increased basal activity when compared to non-transfected mammalian cells (Schulz and Schoneberg, 2003).

To verify that mant-dATP mediates its inverse agonistic activity at the constitutive activity of the WT P2Y₁₂ expressed in mammalian cells as well, CHO-K1 cells were co-transfected with chimeric Gα_{qi4} and IP accumulation assays were performed. As shown in Figure 4B, the WT P2Y₁₂ displayed a high basal activity and MeS-ADP increased IP levels only 2-fold. Mant-dATP almost completely blocked basal IP formation at the WT P2Y₁₂ and N³⁰⁰F (Figure 4B). Also in cAMP inhibition assays at CHO-K1 cells, mant-dATP displayed strong inverse agonistic activity on the inhibition of basal cAMP formation at the WT P2Y₁₂ (Figure 4C).

It has been shown that some cell lines release receptor-function relevant amounts of nucleotides into the cell culture medium (Lazarowski et al., 1997; Parr et al., 1994). This may account for high basal activity of P2Y₁₂ heterologously expressed in mammalian cell lines.

Therefore, we performed similar control experiments with CHO-K1 cells stably transfected with $G\alpha_{q4}$. As shown in the *Supplemental Figure S3*, $G\alpha_{q4}$ -CHO-K1 cells transiently transfected with P2Y₁₂ presented an increased basal IP₁ level compared to cells transfected with the control plasmid (GFP). Incubation with apyrase did not reduce this elevated IP₁ level. This clearly indicates that P2Y₁₂ does induce signal transduction by intrinsic active receptor conformation and not by nucleotides released from the cells into the medium. Proper apyrase function was demonstrated by loss of ADP action on P2Y₁₂.

Mant-dATP is most likely an orthosteric ligand at P2Y₁₂

To evaluate whether mant-dATP mediates its inverse agonistic action through an orthosteric or an allosteric binding site, the ADP-concentration-response curves at F²⁵⁴L were determined in the presence of different concentrations of mant-dATP. As shown in Figure 5A, increasing concentrations of mant-dATP shifted the concentration-response curves to higher ADP concentrations. Similar results were obtained for mant-dADP, but with lower potency (Figure 5B). This competition is indicative of an orthosteric binding modus for the inverse agonists. Although, functional and docking data (see below) support orthosteric binding we cannot rule out the possibility of an allosteric binding of the inverse agonists given the limited concentration range of mant-dATP investigated herein.

Structural model of inverse agonist binding

Using our P2Y₁₂ model we investigated whether mant-dATP can dock into the agonist binding pocket of P2Y₁₂ and if specific interactions may explain inverse agonistic activity (Figure 6). As with the other ATP derivatives, mant-dATP sits between TMH 3, 5, 6 and 7 with Y¹⁰⁵ and K²⁸⁰ forming hydrogen bonds with the phosphate tail and R²⁵⁶ stabilizing the oxygen connecting the furanose to the mant group. Unlike ATP, the extra bulk of the mant group is further stabilized by interactions with I²⁵⁷, H²⁵³ and Q²⁶³. However, similar

interactions are seen with mant-ATP, which does not exhibit inverse agonism. We conclude that the inverse agonistic activity is likely not the result of a different binding pose. It is possibly caused by smaller scale modulations in the strengths of specific interactions between ligand and protein. Pin-pointing these changes to reveal the mechanism behind the inverse agonistic activity are beyond the accuracy of the present comparative model but will be the focus of future mutational studies.

Discussion

We used a genetically modified yeast strain (Pausch et al., 2004) to heterologously express and functionally test the human ADP receptor P2Y₁₂. This expression system offers some advantages over mammalian cells lines, specifically in characterizing nucleotide receptors, because it lacks endogenous nucleotide receptors. ADP and MeS-ADP are full agonists in this expression system with EC₅₀ values of 2.8 μM and 6 nM, respectively (Figure 1A). Screening a purine compound library we identified ATP and some derivatives as partial agonists at P2Y₁₂ in addition to ADP and its derivatives (*Supplemental Table S1*). The agonistic activity of ATP was not only found in the heterologous yeast expression system but also in different mammalian cell lines and signaling assays.

That MeS-ATP and ATP bind to the human P2Y₁₂ has been shown (Savi et al., 2001) but the ligand properties of ATP at P2Y₁₂ are controversially discussed, ranging from antagonism (Bodor et al., 2004; Springthorpe et al., 2007) to agonism (Barnard and Simon, 2001; Simon et al., 2002). These contrary results are likely due to differences in mammalian expression systems and functional assays used. Introduction of a 2'-methylthio group increased ligand potency at P2Y₁₂ and made ATP a highly potent full agonist (Figure 1A) consistent with previous findings (Simon et al., 2002; Zhang et al., 2001). Through ligand docking into a structural comparative model of P2Y₁₂, ATP derivatives are found to bind in a similar binding site. While our structural P2Y₁₂ model is not at the resolution to reveal what fine-structural requirements are essential to turn a nucleotide into an agonist at P2Y₁₂, specific

residues critical to ligand interaction can be predicted from the model. Notably, we find that for six of the seven residues indicated to be significant for ligand interaction that are also in the mutant database (H²⁵³, I²⁵⁷, Y²⁵⁹, T²⁶⁰, Q²⁶³, T²⁶⁴ and K²⁸⁰), mutation of the residues to any other amino acid results in a loss of WT function (see our P2Y₁₂ mutant database: <http://www.ssfa-7tmr.de/p2y12>). Therefore, there is agreement between the residues predicted to be critical for agonist function through docking studies and experimental results. Our model and the docking studies are consistent with the fact that ATP fits into the same binding pocket as well-characterized agonists.

These findings raise a relevant question whether ATP can serve as P2Y₁₂ agonist also *in vivo*. The ATP-to-ADP ratio in human platelet dense granules is approximately two (Cattaneo et al., 2000; Weiss et al., 1979). If one assumes that ATP and ADP secretion from dense granule undergo with the same kinetics, previous data suggest that the surface concentration of ADP following thrombin stimulation will transiently reach 7–10 μM (Beigi et al., 1999). This is sufficient for activation of the platelet P2Y₁₂ by ADP but also by ATP and consistent with feed-forward autocrine/paracrine activation of platelet responses.

Many WT GPCR, such as histamine receptors, thyrotropin receptor and melanocortin receptors present high basal activity (Seifert and Wenzel-Seifert, 2002). In contrast to antagonists inverse agonists suppress both agonist-dependent and -independent activity and are therefore developed in priority. For example, many β-blockers and atropine are inverse agonists at β₁-adrenoceptors and muscarinic acetylcholine receptors, respectively (Baker et al., 2011; Thor et al., 2009). Therapeutically used P2Y₁₂ ligands are high affinity antagonists but inverse activity was described only for the experimental P2Y₁₂ blocker AR-C78511 (Ding et al., 2006; Vasiljev et al., 2003). AR-C78511 is a 2-alkylthio-substituted ATP analog but, in contrast to mant-dATP, has no modification at the 2' or 3' OH residues of the ribose. Mant-dATP binds most likely at the orthosteric ligand-binding site and inverse agonistic activity mutually depends on the desoxyribose, since mant-ATP lacks inverse agonistic activity. At

present we cannot explain or predict inverse activity, even with a receptor model in hand, since the pharmacological properties of a ligand are the result of a tight interplay of the ligand and the receptor molecule. It is however of interest that, as for AR-C78511 (Springthorpe et al., 2007), modification of an ATP backbone resulted again in an inverse agonist (mant-dATP). This also supports our findings that P2Y₁₂ naturally recognizes not only ADP but also ATP and binding of ATP and other ATP derivatives induce conformational changes within P2Y₁₂.

In sum, we clearly show that, in addition to ADP and ATP, some ATP derivatives are not only ligands of P2Y₁₂ but also agonists. Keeping with an ATP/ADP ratio >1 *in vivo* and the small differences in concentration response curves (see Figure 1B), P2Y₁₂ should rather be referred to as adenine nucleotide receptor without suggesting ADP specificity. Modification of the ribose within ATP can result in inverse activity of ATP-derived ligands.

Authorship Contributions

Participated in research design: P. Schmidt, E. N. Dong, J. Meiler, T. Schöneberg

Conducted experiments: P. Schmidt, L. Ritscher, T. Hermsdorf

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Performed data analysis: P. Schmidt, E. N. Dong, L. Ritscher, T. Hermsdorf, T. Schöneberg

Wrote or contributed to the writing of the manuscript: P. Schmidt, E. N. Dong, J. Meiler, T. Schöneberg

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Footnotes

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Conflict of interest

The authors have declared that no conflict of interest exists.

Figure Legends

Figure 1. Nucleotide agonists at the human P2Y₁₂.

A) The human P2Y₁₂ was transformed into yeast cells and incubated with different concentrations of P2Y₁₂ agonists. Receptor activation-dependent growth was measured as OD_{600 nm} after 24 h. Data are given as mean \pm SD of three independent experiments all performed in triplicate. **B)** For label-free measurements of receptor activation, a dynamic mass redistribution assay (Corning Epic® Biosensor Measurements) with stably transfected HEK cells was performed essentially as described previously (Schroder et al., 2010). The response is shown 6 min after compound application. The response of each compound at non-transfected HEK cells was subtracted from the respective response at P2Y₁₂ transfected HEK cells. Data are presented as mean \pm S.E.M. of two independent experiments, each carried out in triplicate.

Figure 2. P2Y₁₂ docked in complex with agonists ADP, ATP, MeS-ADP and MeS-ATP.

The docked binding poses in the comparative model of P2Y₁₂ for agonists **A)** ADP, **B)** ATP, **C)** MeS-ADP and **D)** MeS-ATP in relation to residues Y¹⁰⁵, E¹⁸⁸, R²⁵⁶, Y²⁵⁹ and K²⁸⁰. All side chains within the binding site important for ligand interaction according to calculations of free energy change with and without ligands bound to P2Y₁₂ are highlighted in red in the model and also shown in relation to the 2-D ligand depiction.

Figure 3. Position and basal activity of constitutively active P2Y₁₂ mutants.

A) The position of constitutively active mutations in TM6 and TM7 are depicted. Basal activities of the individual mutants expressed in yeast are given in the table. Data are presented as mean \pm SD of three independent experiments, each carried out in triplicate. The

basal activity of the WT P2Y₁₂ was OD_{600 nm}: 0.074 ± 0.016. Complete functional data are available and organized in a P2Y₁₂ mutant database (<http://www.ssfa-7tmr.de/p2y12>).

B) The comparative model of P2Y₁₂ based on the CXCR4 template is depicted. Residues producing constitutively active mutants on TM6 and TM7 are highlighted in red. Residue side chains facing the pore of the receptor (F²⁴⁶, F²⁴⁹ and N²⁹⁰) are shown in sticks.

Figure 4. *Mant-dADP and mant-dATP are inverse agonists at constitutively active P2Y₁₂ F²⁵⁴L.*

A) Yeast cells expressing F²⁵⁴L were incubated with increasing concentrations of the indicated compounds and yeast growth was measured after 24 h incubation. The ligand-induced decrease of basal activity of F²⁵⁴L is shown relative to the basal activity of the WT P2Y₁₂ (OD_{600 nm} = 0.074; set to 0%) and the basal activity of F²⁵⁴L (OD_{600 nm} = 0.165; set to 100%). Data are given as mean ± SD of three independent experiments all performed in triplicate. **B)** To evaluate inverse agonist specificity, CHO-K1 cells, stably expressing the chimeric G-protein G_{α_{qi4}}, were transfected with plasmids encoding GFP (control) or the human ADP receptor. IP formation under basal conditions (*white*), in the presence of 10 μM MeS-ADP (*black bars*) and in presence of 100 μM mant-dATP (*light grey bars*). The basal IP for GFP was 321 cpm/well and set 0%, the basal IP for WT P2Y₁₂ was 970 cpm/well and set 100%. Data are presented as mean ± SD (cpm/well) of three independent experiments, each carried out in duplicate. **C)** Forskolin-induced cAMP levels in CHO-K1 cells stably expressing human ADP receptor were determined under basal conditions (*white bars*), in the presence of 10 μM MeS-ADP (*black bars*) and in presence of 100 μM mant-dATP (*light grey bars*). The decrease of basal activity of WT P2Y₁₂ is shown relative to GFP basal activity (7486 cpm/well; set 0%) and basal activity of WT P2Y₁₂ (4533 cpm/well; set 100%). Data are given as mean ± SD of three independent experiments all performed in triplicates.

Figure 5. Mant-dADP and mant-dATP are most likely orthosteric ligands at P2Y₁₂ F²⁵⁴L.

To evaluate the modus of inverse agonist binding, ADP-concentration-response curves at F²⁵⁴L-transformed yeast cells were determined in the presence of 0, 10 and 100 μ M mant-dATP (**A**) and mant-dADP (**B**). Data are given as mean \pm SD of three independent experiments all performed in duplicate.

Figure 6. P2Y₁₂ docked in complex with agonists mant-ADP and mant-ATP and inverse agonists, mant-dADP and mant-dATP.

The docked binding poses in the comparative model of P2Y₁₂ for agonists **A**) mant-ADP and **B**) mant-ATP and the inverse agonists **C**) mant-dADP and **D**) mant-dATP in relation to residues Y¹⁰⁵, E¹⁸⁸, R²⁵⁶, Y²⁵⁹ and K²⁸⁰. All side chains within the binding site important for ligand interaction according to calculations of change in free energy with and without ligands bound to P2Y₁₂ are highlighted in red and shown in relation to the 2-D ligand depiction.

Tables

Table 1. Mant-dATP is an inverse agonist at different constitutively active mutants.

Yeast cells expressing different basal active mutants were incubated with a 10 μ M purine compound library to identify inverse agonists. In a screen of over 80 adenine nucleotides and their derivatives mant-dATP and mant-*N*⁶-Methyl-ATP showed inverse activity on several constitutively active mutants. All the mutants listed showed activation (> 2-fold above increased basal activity) upon stimulation with ADP, MeS-ADP, ATP and mant-ADP. dATP and mant-ATP had no significant effects on the mutants.

position	mutation	inverse activating substances (fold over basal ≤ 0.8)
F ²⁴⁶	V	mant-dATP
F ²⁵⁴	I	mant-dATP, mant- <i>N</i> ⁶ -Methyl-ATP
	L	mant-dATP, mant- <i>N</i> ⁶ -Methyl-ATP
	V	mant-dATP
F ²⁹⁶	A	mant-dATP
	C	mant-dATP, mant- <i>N</i> ⁶ -Methyl-ATP
	M	mant-dATP
F ³⁰⁰	N	mant-dATP
L ³⁰¹	N	mant-dATP
	M	mant-dATP

Table 2. Structure of compounds with agonistic properties at the WT human P2Y₁₂

TNP: 2'-(OR-3')-O-(trinitrophenyl)

backbone	substances	side chain				
		R1	R2	R3	R4	R5
	ADP	H	OH	OH	O	OH
	ADPβS	H	OH	OH	S	OH
	TNP-ADP	H	C ₆ N ₃ O ₈ H ₄		O	OH
	AppNH ₂	H	OH	OH	O	NH ₂
	ATP	H	OH	OH	O	PO ₄ H ₂
	ATPγS	H	OH	OH	O	PSO ₃ H ₂
	2I-ATPγS	I	OH	OH	O	PSO ₃ H ₂

Table 3. ADP modifications not compatible with agonistic activity at the WT human P2Y₁₂

The table summarizes the modifications at the ADP backbone which are not compatible with agonistic activity at the WT human P2Y₁₂ at in least two different adenine nucleotides of the compound library (see *Materials and Methods*).

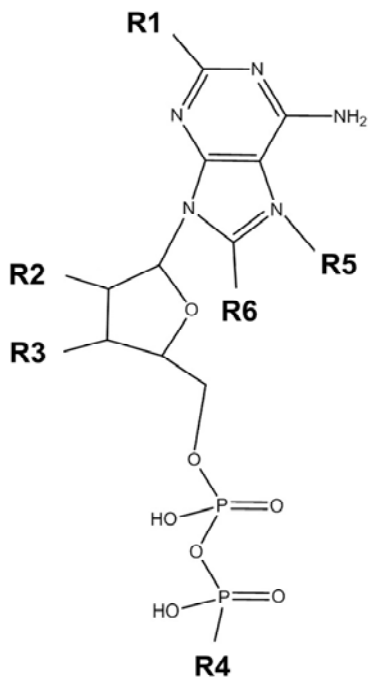
backbone	position	side chain
	R1	OH
	R2	H ₂
	R3	H ₂
		CH ₃
		I
	R4	NH ₂
		CH ₃
R6	Br	

Figure 1

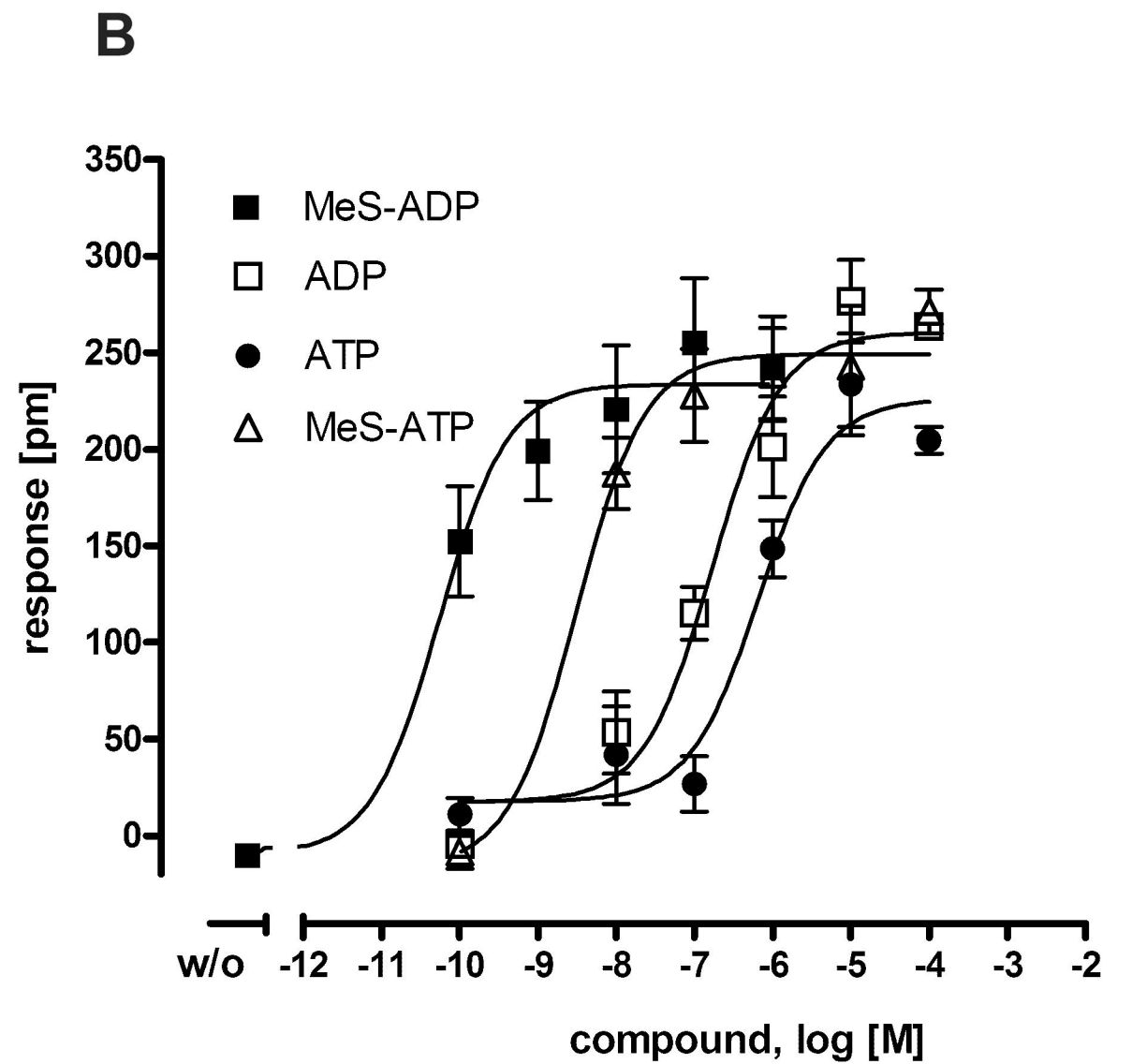
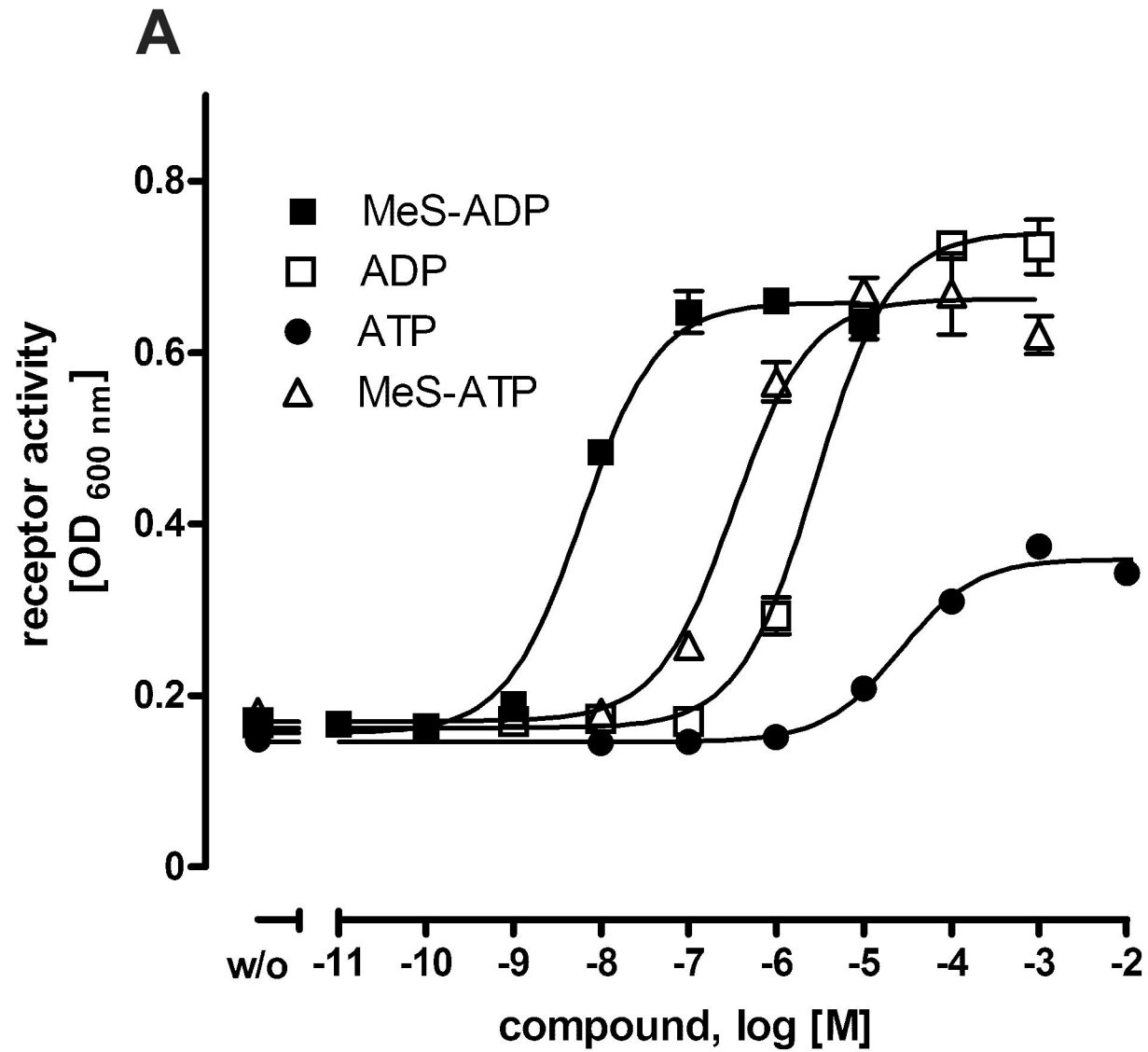
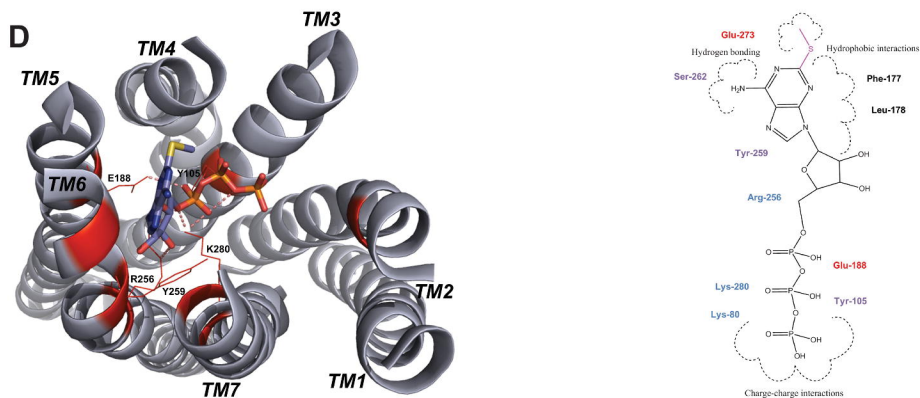
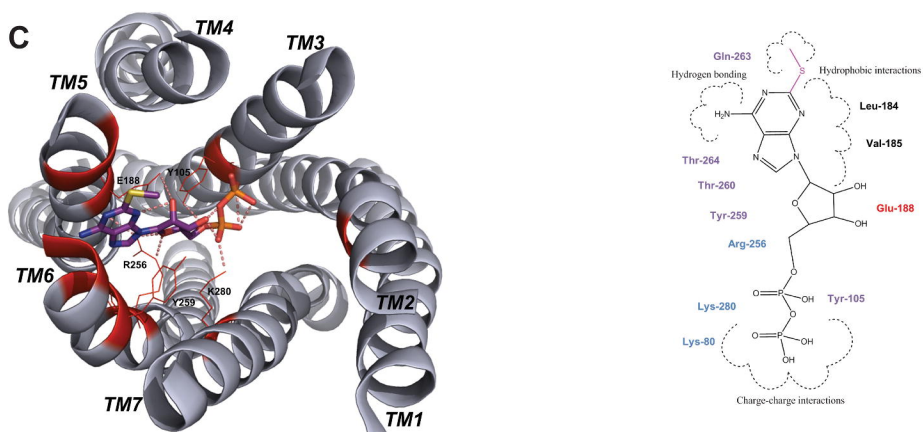
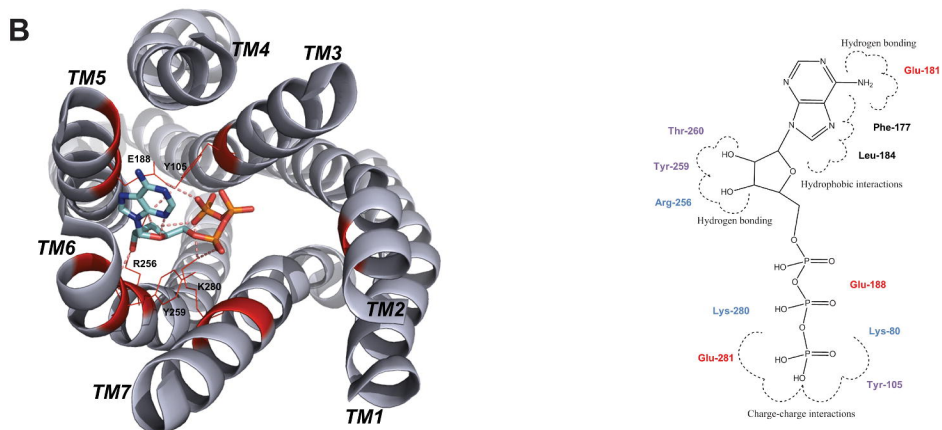
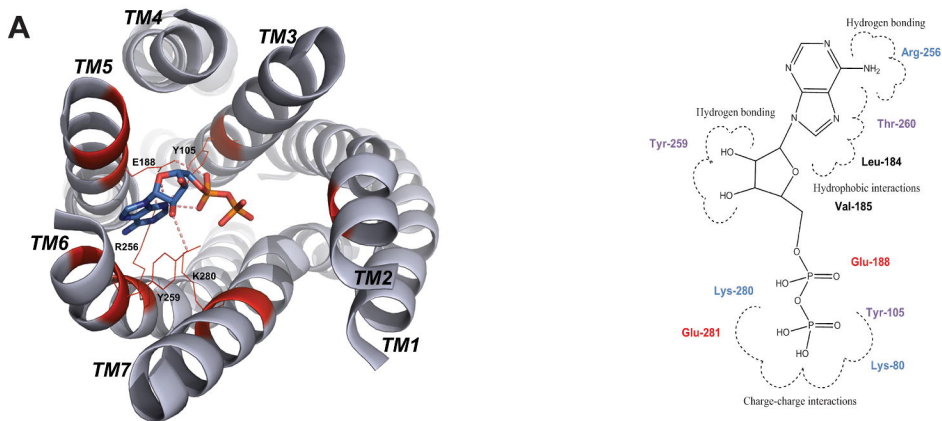


Figure 2

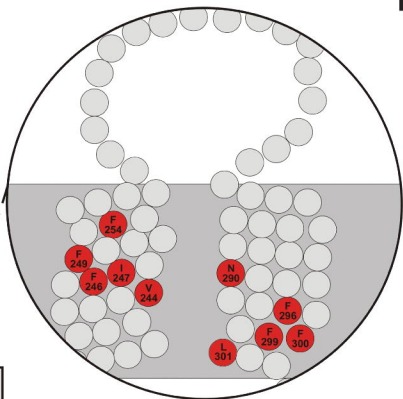
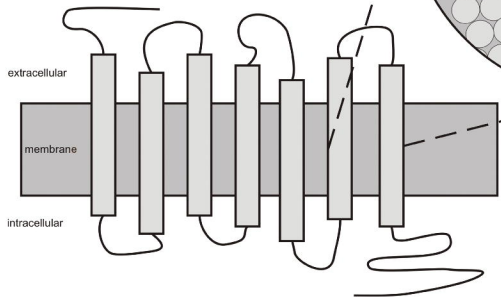


RESIDUE KEY
Non-polar, Hydrophobic
Polar, Negative Charge
Polar, Uncharged
Polar, Positive Charge

Figure 3

A

mutant	basal activity (% of wild type)
F ²⁵⁴ L	223.1±5.5
F ³⁰⁰ N	324.4±13.4



B

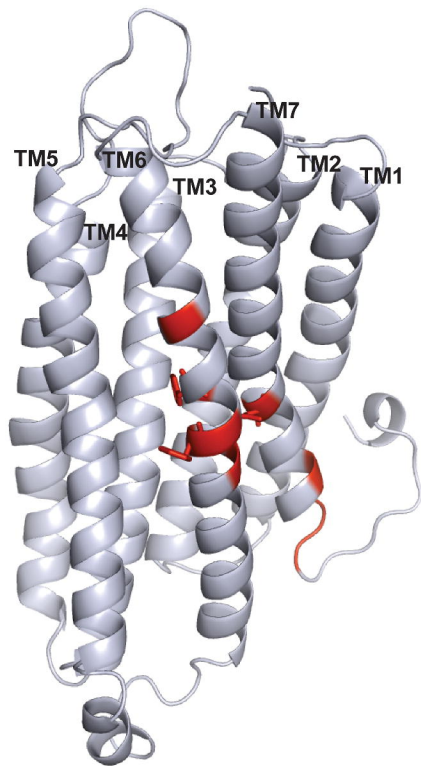


Figure 4

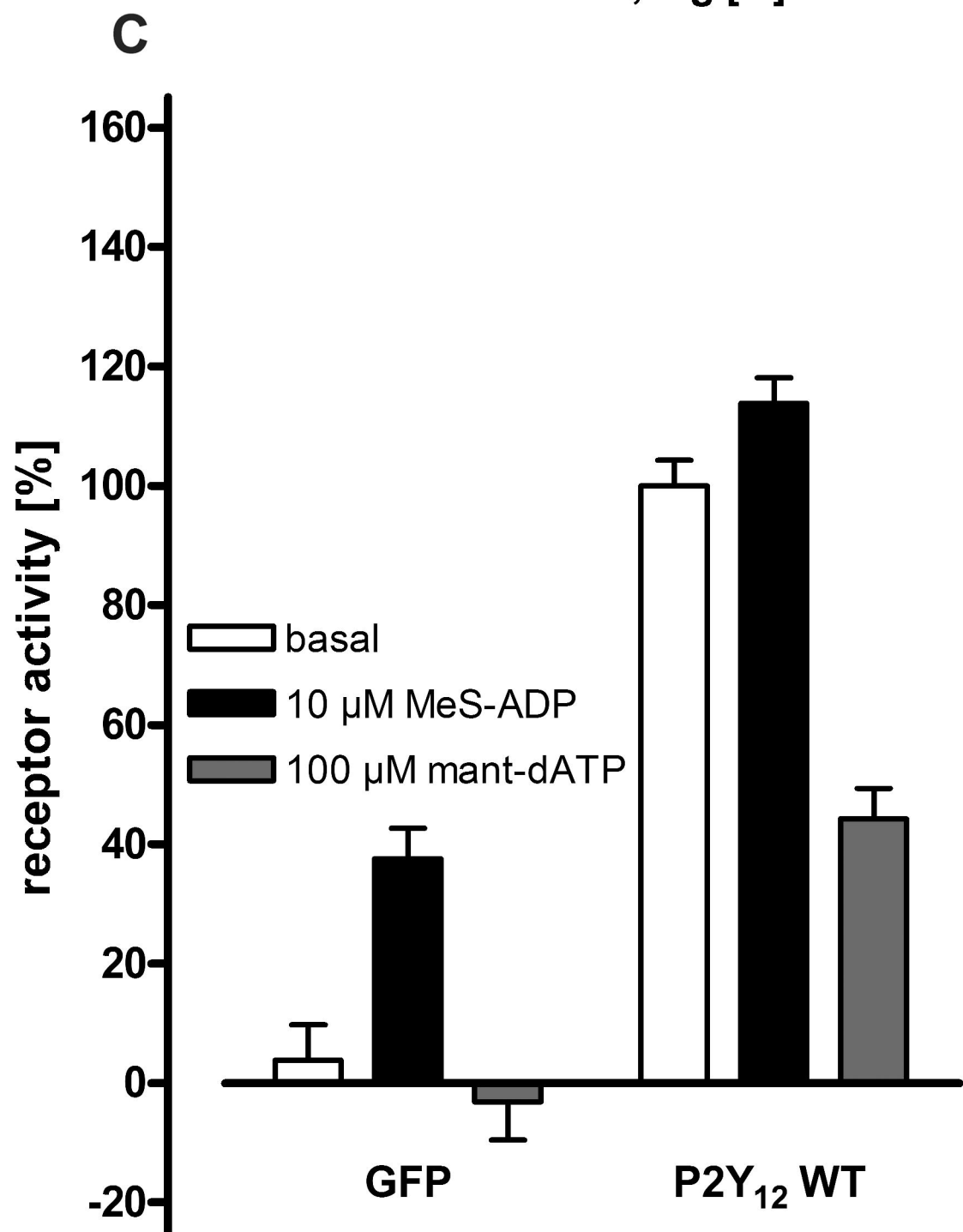
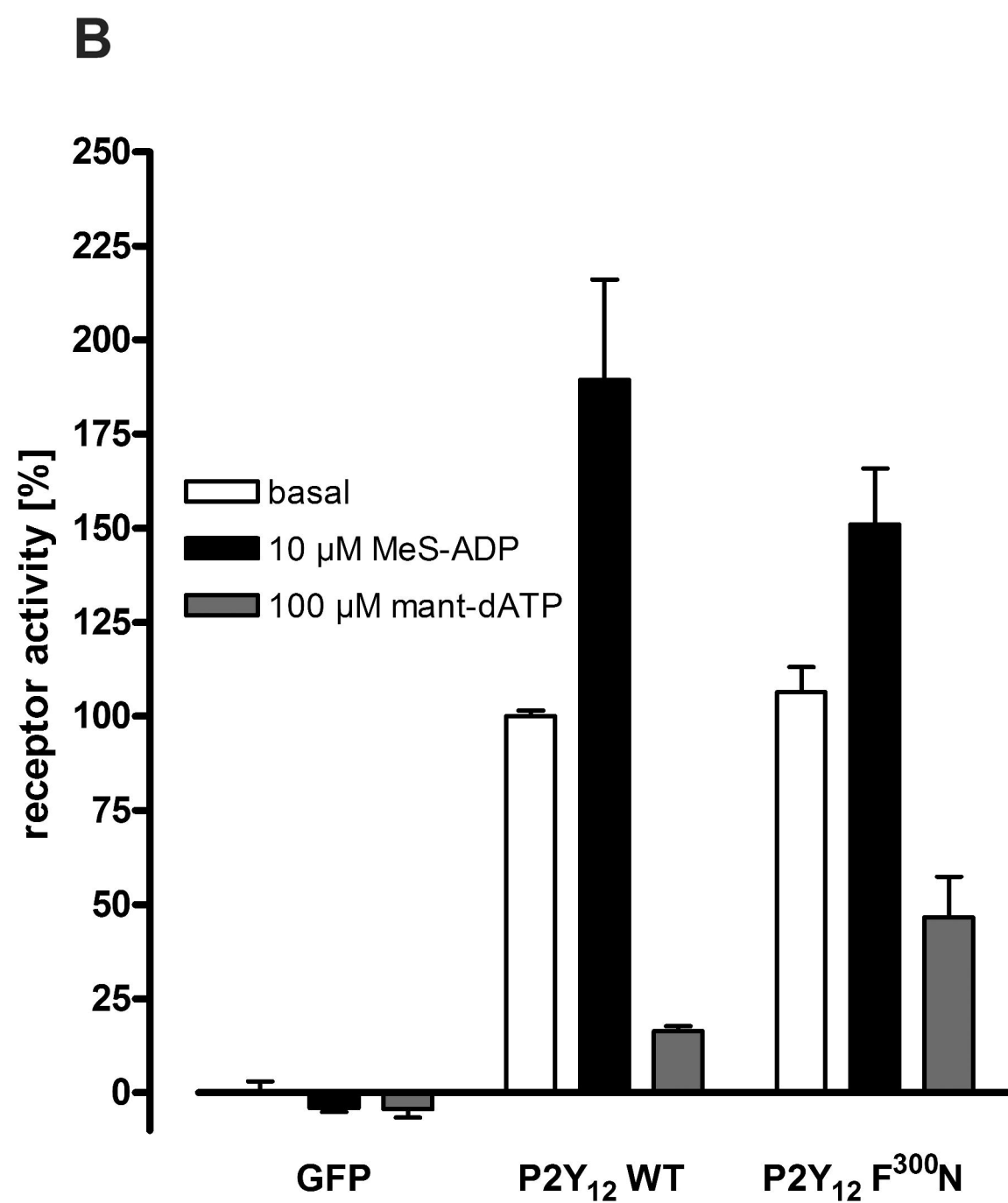
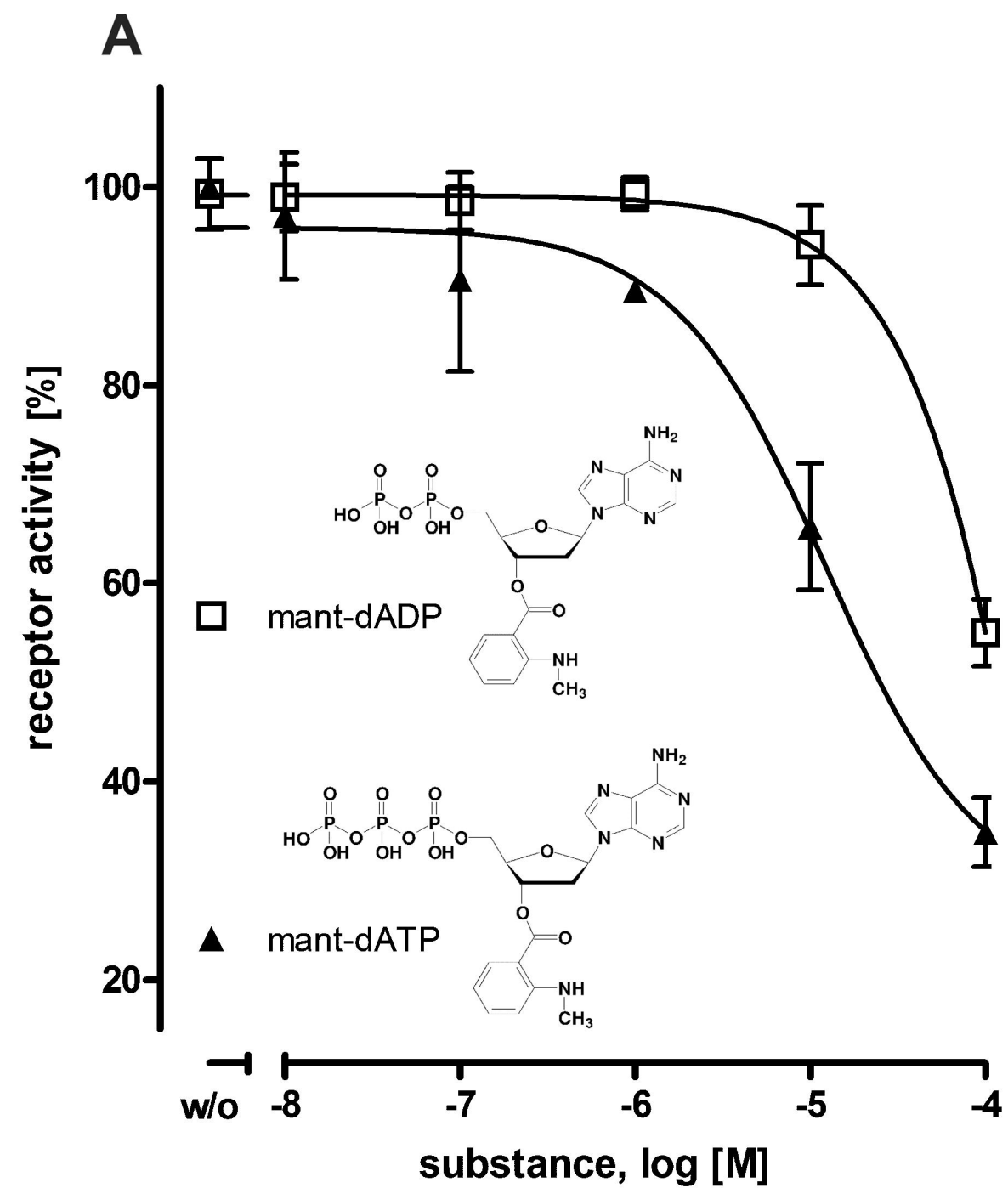


Figure 5

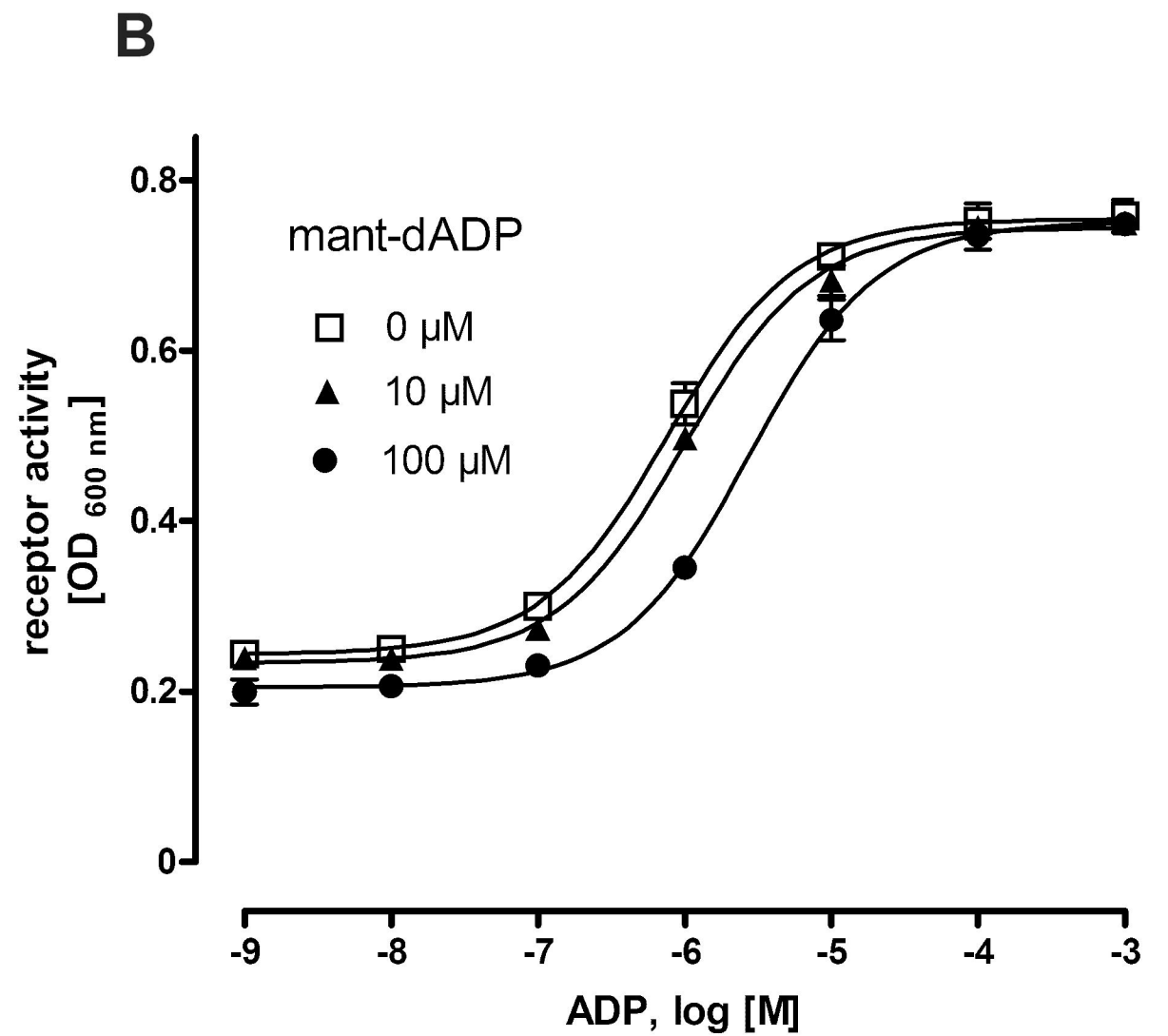
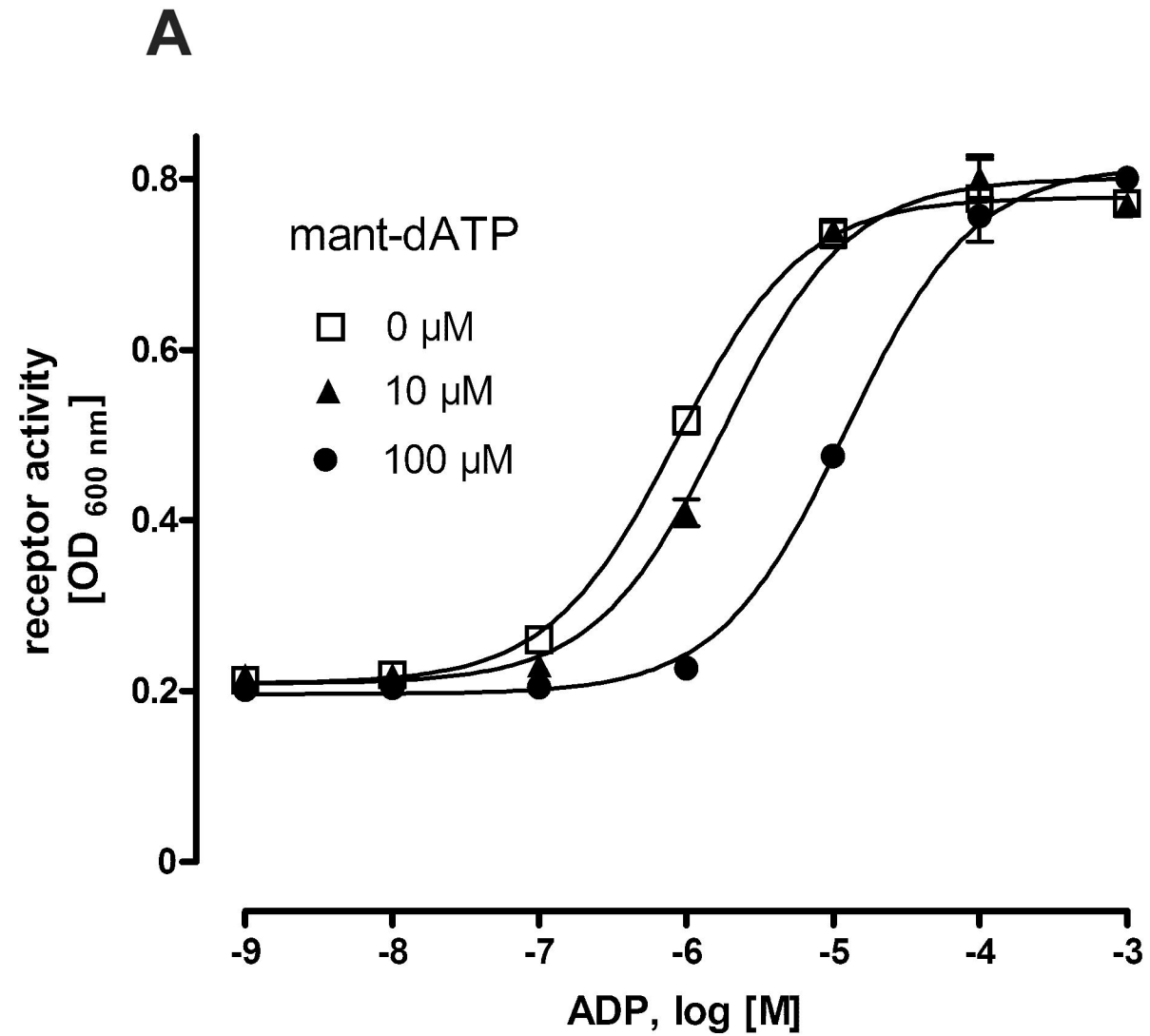
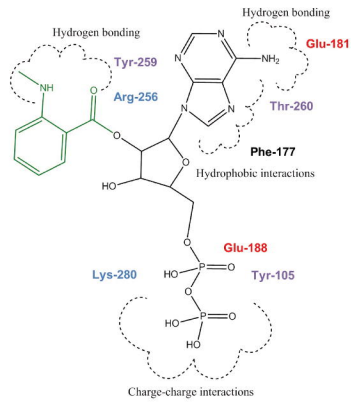
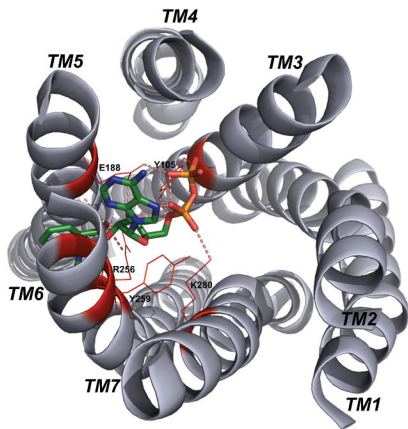
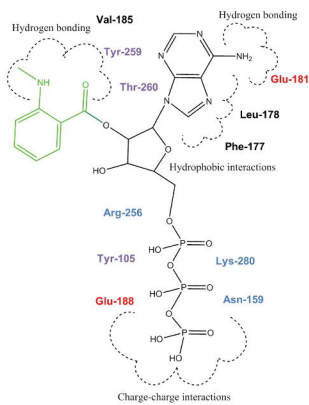
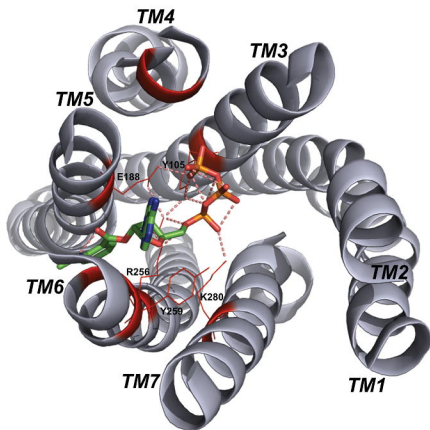


Figure 6

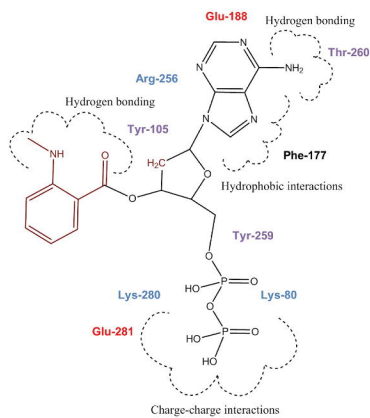
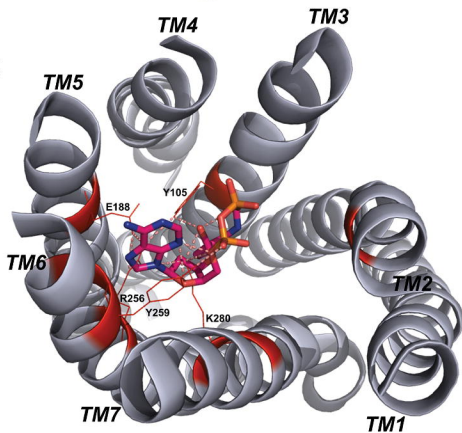
A



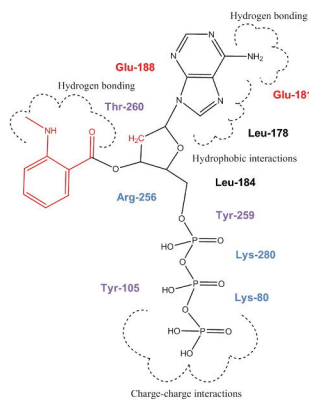
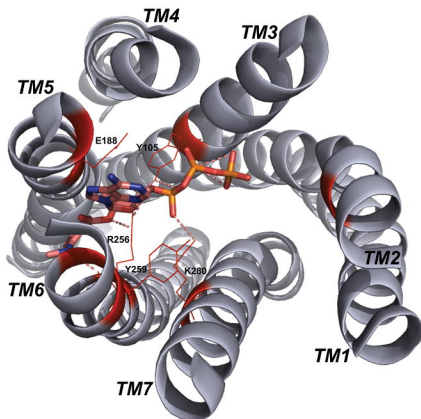
B



C



D



RESIDUE KEY	
Non-polar, Hydrophobic	Grey
Polar, Negative Charge	Blue
Polar, Uncharged	Grey
Polar, Positive Charge	Red