

RESEARCH PAPER

Mechanism of action of species-selective P2X₇ receptor antagonists

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Background and purpose: AZ11645373 and *N*-{2-methyl-5-[(1*R*, 5*S*)-9-oxa-3,7-diazabicyclo[3.3.1]non-3-ylcarbonyl]phenyl}-2-tricyclo[3.3.1.1.3,7]dec-1-ylacetamide hydrochloride (compound-22) are recently described P2X₇ receptor antagonists. In this study we have further characterized these compounds to determine their mechanism of action and interaction with other species orthologues.

Experimental approach: Antagonist effects at recombinant and chimeric P2X₇ receptors were assessed by ethidium accumulation and radioligand-binding studies.

Key results: AZ11645373 and compound-22 were confirmed as selective non-competitive antagonists of human or rat P2X₇ receptors respectively. Both compounds were weak antagonists of the mouse and guinea-pig P2X₇ receptors and, for each compound, their potency estimates at human and dog P2X₇ receptors were similar. The potency of compound-22 was moderately temperature-dependent while that of AZ11645373 was not. The antagonist effects of both compounds were slowly reversible and were not prevented by decavanadate, suggesting that they were allosteric antagonists. Indeed, the compounds competed for binding sites labelled by an allosteric radio-labelled P2X₇ receptor antagonist. The species selectivity of AZ11645373, but not compound-22, was influenced by the nature of the amino acid at position 95 of the P2X₇ receptor. *N*²-(3,4-difluorophenyl)-*N*¹-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride, a positive allosteric modulator of the rat receptor, reduced the potency of compound-22 at the rat receptor but had little effect on the actions of AZ11645373.

Conclusions: AZ11645373 and compound-22 are allosteric antagonists of human and rat P2X₇ receptors respectively. The differential interaction of the two compounds with the receptor suggests there may be more than one allosteric regulatory site on the P2X₇ receptor at which antagonists can bind and affect receptor function.

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Abbreviations: BzATP, 2'- & 3'-O-(4benzoylbenzoyl) ATP; compound-22, *N*-{2-methyl-5-[(1*R*, 5*S*)-9-oxa-3,7-diazabicyclo[3.3.1]non-3-ylcarbonyl]phenyl}-2-tricyclo[3.3.1.1.3,7]dec-1-ylacetamide hydrochloride; DR, dose-ratio; ECD, extracellular domain; GW791343, *N*²-(3,4-difluorophenyl)-*N*¹-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride; KN62, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-*L*-tyrosyl]-4-phenylpiperazine; PPADS, pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid

Introduction

The P2X₇ receptor is a ligand-gated cation channel gated by extracellular ATP (North, 2002). The receptor plays a key role in the release of the pro-inflammatory cytokine

interleukin-1 β from immune cells (Ferrari *et al.*, 2006) and has been implicated in inflammatory disease and pain disorders (Chessell *et al.*, 2005; Honore *et al.*, 2006; Donnelly-Roberts and Jarvis, 2007). This has generated considerable interest in developing antagonists of the P2X₇ receptor and recently the properties of several of these have been described. These include AZ11645373 (Stokes *et al.*, 2006), A740003 (Honore *et al.*, 2006), compound-17 (Michel *et al.*, 2008a), *N*-{2-methyl-5-[(1*R*, 5*S*)-9-oxa-3,7-diazabicyclo[3.3.1]non-3-ylcarbonyl]phenyl}-2-tricyclo[3.3.1.1.3,7]dec-1-ylacetamide hydrochloride (compound-22, Furber *et al.*, 2007), *N*²-(3,4-difluorophenyl)-*N*¹-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride (GW791343)

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(Michel *et al.*, 2008a) as well as numerous compounds described in patents (Baraldi *et al.*, 2004; Romagnoli *et al.*, 2005; Donnelly-Roberts and Jarvis, 2007).

A key requirement for the development of such antagonists as therapeutic agents is selectivity, which AZ11645373 and A-740003 exhibit to a high degree (Honore *et al.*, 2006; Stokes *et al.*, 2006). This is somewhat surprising as agents that block the effects of ATP might have been expected to suffer from limited selectivity or specificity. However, the mechanisms of action of AZ11645373 and compound-22 were not determined in the preliminary reports using these compounds and this may be important as a number of P2X₇ receptor antagonists have recently been shown to be allosteric antagonists that do not bind at the ATP-binding site. Thus, in receptor protection studies using the rapidly reversible competitive P2X₇ receptor antagonist, decavanadate, compounds, such as SB203580 (AD Michel, unpubl. obs.), 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62) (Michel *et al.*, 2006a) and compound-17 (Michel *et al.*, 2008a), produced a long-lasting blockade of the receptor that was minimally affected by pretreatment with decavanadate. In contrast, decavanadate competitively protected the P2X₇ receptor from the long-lasting blockade produced by agents that interact at the ATP-binding site such as pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) and periodate oxidized ATP (Michel *et al.*, 2006a).

Many antagonists are also selective for human, as compared with the rat, P2X₇ receptor (Humphreys *et al.*, 1998; Hibell *et al.*, 2001; Stokes *et al.*, 2006, Donnelly-Roberts and Jarvis, 2007) and we have identified the amino acid at position 95 of the receptor as a key determinant of the species selectivity of the allosteric antagonists GW791343, KN62 and SB203580 (Michel *et al.*, 2008b). This residue does not affect the species selectivity of PPADS, which is instead determined by the amino acid at position 126 and residues between amino acids 264 and 304 (Michel *et al.*, 2008b).

The initial reports describing AZ11645373 and compound-22 did not examine their mechanism of action in any detail but AZ11645373 did appear to function as a non-competitive inhibitor as it reduced maximal responses to ATP (Stokes *et al.*, 2006). As slowly reversible antagonists such as PPADS that act at the ATP-binding site (Michel *et al.*, 2006a), as well as negative allosteric antagonists (Michel *et al.*, 2008a), decrease maximal responses, it is not possible from the published data to determine if AZ11645373 is a slowly reversible competitive antagonist or a negative allosteric antagonist.

In this study we have carried out further studies using AZ11645373 and compound-22 both to determine their mechanism of action and to further elucidate the factors determining their species selectivity. We have found that both are negative allosteric antagonists of the P2X₇ receptor and that they appear to interact with the receptor in different ways.

Methods

Recombinant receptors

The nomenclature used for describing the P2X₇ receptor conforms to the Journal's guidelines (Alexander *et al.*, 2008). Studies on the human (Rassendren *et al.*, 1997), dog (Acces-

sion Number, EU334661.1; Roman *et al.*, 2007) and rat (Surprenant *et al.*, 1996) P2X₇ receptors were performed using HEK293 cells expressing the recombinant receptors (Fonfria *et al.*, 2008) whereas studies on guinea-pig (Fonfria *et al.*, 2008) and mouse (Young *et al.*, 2006) P2X₇ receptors were performed using the BacMam expression system previously described (Fonfria *et al.*, 2008).

The generation of HEK293 cells expressing chimeric rat-human P2X₇ and human-rat P2X₇ receptors has been described previously (Michel *et al.*, 2006b). Briefly, the human-rat P2X₇ receptor comprises amino acids 1–255 of the human P2X₇ receptor and amino acids 256–595 of the rat P2X₇ receptor while the rat-human P2X₇ receptor comprises amino acids 1–255 of the rat P2X₇ receptor and amino acids 256–595 of the human P2X₇ receptor.

The generation of the other chimeric P2X₇ receptors has been described previously (Michel *et al.*, 2008b). Briefly, the rat extracellular domain (ECD) P2X₇ receptor (rat ECD) is a human receptor in which the entire ECD between amino acids 48 and 332 has been replaced with the corresponding rat residues. We also examined chimeric P2X₇ receptors in which small sequences (25–42 amino acids) of the human P2X₇ receptor were replaced with the corresponding residues from the rat P2X₇ receptor. These were termed the domain 1 (rat residues 72–95), domain 2 (rat residues 108–136), domain 3 (rat residues 154–183), domain 4 (rat residues 212–244) and domain 5 (rat residues 264–304) P2X₇ receptors. Several single point mutant receptors were used in which a single residue in the human P2X₇ receptor was replaced with the corresponding residue from the rat P2X₇ receptor. These were the lysine 72 to threonine (K72T), aspartate 74 to asparagine (E74N), asparagine 78 to glycine (N78G), lysine 81 to threonine (K81T), serine 86 to glycine (S86G), phenylalanine 95 to leucine (F95L) and arginine 126 to glycine (R126G) P2X₇ receptors. The effects of compounds were also evaluated at a histidine 155 to tyrosine (H155Y) receptor but this was produced in a human P2X₇ receptor that also contained a histidine 270 to arginine mutation (H270R).

Cell culture and viral transductions

Studies on the human, rat, dog, human-rat, rat-human, rat L95F and domain 3 P2X₇ receptors were performed using HEK293 cells stably transfected with the recombinant receptor. The guinea-pig, mouse, rat ECD, other chimeric domain swaps and single point mutant P2X₇ receptors were transiently expressed in human osteosarcoma U-2 OS cells using the BacMam viral transduction method (Fonfria *et al.*, 2008). For the later studies wild-type human and rat P2X₇ receptors were also expressed in U-2 OS cells for comparative purposes. The BacMam viral transductions for ethidium accumulation studies were conducted as described previously (Fonfria *et al.*, 2008). Briefly, U-2 OS cells were maintained in adherent culture conditions in the presence of Dulbecco's modified Eagle's medium : nutrient mixture F-12 supplemented with Glutamax (DMEM: F12 + Glutamax, Invitrogen) and 10% foetal bovine serum (PAA laboratories GmbH, Austria) at 37°C, 5% CO₂. One day prior to assay, cells were harvested from the culture flasks using 0.05% trypsin/EDTA (Invitrogen, Paisley, UK) and resuspended at a concentration of ~ 750 × 10³

cells·mL⁻¹ in culture media in the presence of the various BacMam viruses (1–2 × 10⁸ plaque forming units·mL⁻¹ of the BacMam virus stock in culture media). Cells (70–80 000) were plated into individual wells of poly-L-lysine pretreated 96-well plates (Costar, High Wycombe, UK) and the plates were incubated at 37°C, 5% CO₂ overnight. HEK293 cells stably expressing the human, rat, dog, human-rat, rat-human or domain 3 recombinant P2X₇ receptors were prepared in a similar manner except that the BacMam virus was omitted.

In studies to measure compound effects in radioligand-binding studies, U-2 OS cells were grown to confluence in T175 cm² flasks and the media replaced with fresh growth media containing BacMam virus (1–2 × 10⁸ plaque forming units·mL⁻¹). The cells were incubated overnight, harvested using Versene (Invitrogen, Paisley, UK) and membranes prepared as described previously (Michel *et al.*, 2007). Similar studies to determine the effects of agonists in radioligand-binding studies were performed using membranes prepared from HEK293 cells stably expressing the human, rat or domain 3 chimeric recombinant P2X₇ receptors, using the same methods as described above for the U-2 OS cells.

Cellular ethidium accumulation measurements

Studies were performed as described previously (Michel *et al.*, 2006a; Fonfria *et al.*, 2008) using assay buffers comprising (in mmol·L⁻¹): HEPES 10, N-methyl-D-glucamine 5, KCl 5.6, D-glucose 10, CaCl₂ 0.5 (pH 7.4) and supplemented with either 280 mmol·L⁻¹ sucrose (sucrose buffer) or 140 mmol·L⁻¹ NaCl (NaCl buffer). Before use, growth media was completely removed from the cells and they were rinsed with 350 µL of the appropriate assay buffer which was also removed before performing assay additions. In all studies the final assay volume was 100 µL and studies were performed at room temperature of 19–21°C.

Cells were incubated with antagonist for 40 min before addition of a mixture containing the agonists, ATP or 2'- & 3'-O-(4benzoylbenzoyl) ATP (BzATP), and ethidium bromide (100 µmol·L⁻¹ final assay concentration). After agonist addition, incubations were continued until approximately 10–30% of maximal agonist-stimulated dye accumulation occurred. Reactions were rapidly terminated by addition of 25 µL of 1.3 mol·L⁻¹ sucrose assay buffer containing 5 mmol·L⁻¹ reactive black 5 and cellular accumulation of ethidium was determined by immediately measuring fluorescence (excitation wavelength of 530 nm and emission wavelength of 620 nm) from below the plate with a 96 well plate fluorescence reader (FlexStation, Molecular Devices, Wokingham, UK).

Protection of P2X₇ receptors from the slowly reversible blockade by P2X₇ receptor antagonists (receptor protection studies)

These studies were performed exactly as described previously (Michel *et al.*, 2008b). Briefly, cells expressing rat or human P2X₇ receptors were incubated with decavanadate for 10 min prior to addition of AZ11645373, compound-22 or PPADS. After a 40 min co-incubation, the antagonists were extensively washed over a 15 min period before measuring ATP-stimulated ethidium accumulation as described above. The

studies were performed using NaCl buffer and for studies on the rat and human P2X₇ receptors, the ATP concentrations were 0.5 mmol·L⁻¹ and 2 mmol·L⁻¹ respectively.

Interaction studies with GW791343

These studies were performed exactly as described previously (Michel *et al.*, 2008b). Briefly, cells expressing rat P2X₇ receptors were incubated with 30 µmol·L⁻¹ GW791343 for 10 min prior to addition of AZ11645373 or compound-22. After a 40 min co-incubation period, ATP-stimulated ethidium accumulation was measured as described above.

Radioligand-binding studies

The radioligand-binding studies using [³H]-compound-17 were performed as described previously (Michel *et al.*, 2007). Briefly, membranes prepared from U-2 OS cells transduced with human, rat or chimeric recombinant P2X₇ receptors were incubated with the compounds and the radioligand, [³H]-compound-17 (2–3 nmol·L⁻¹) for 60 min at room temperature in a final assay volume of 200 µL of 50 mmol·L⁻¹ Tris HCl buffer containing 0.01% bovine serum albumen (pH 7.4 at RT). Reactions were terminated by vacuum filtration. Non-specific binding was defined using 10 µmol·L⁻¹ compound-17.

Data analysis

Individual concentration-effect or inhibition curves from each experiment were fitted to a four-parameter logistic function to determine the maximum and minimum responses and to calculate the EC₅₀ or IC₅₀ values and the Hill slope. For graphical purposes, most concentration-effect and inhibition curves are presented as a percentage of the maximal response obtained in the control group.

As the compounds produced non-competitive antagonist effects in the Schild studies (see Figure 1A,D), the data from the Schild studies were also analysed to calculate antagonist pIC₅₀ values at each agonist concentration as this provided some quantitative estimate of antagonist potency. To graphically represent the effect of agonist concentration on antagonist pIC₅₀, the agonist concentration was expressed relative to its EC₅₀ at the various receptors [logarithm (agonist concentration/agonist EC₅₀)]. This enabled a simpler comparison of antagonist pIC₅₀ values between the species orthologues and chimeric receptors (see Figure 1F). In addition, the relationship between pIC₅₀ and [logarithm (agonist concentration/agonist EC₅₀)] was linear (Figures 1F and 2F) and so, in order to provide a statistical comparison of antagonist potency between the various receptors, the data for each experiment were analysed by linear regression and the antagonist pIC₅₀ at the agonist EC₅₀ was calculated from the fit. In graphical terms, this corresponded to the pIC₅₀ at 0 on the X-axis (Figure 1F) and this value is referred to as the normalized pIC₅₀.

The data for the decavanadate protection experiments were analysed as described previously (Michel *et al.*, 2006a). Briefly, the IC₅₀ of the antagonists to block ATP responses was determined in control cells and in cells pretreated with the various concentrations of decavanadate. For each concentration of

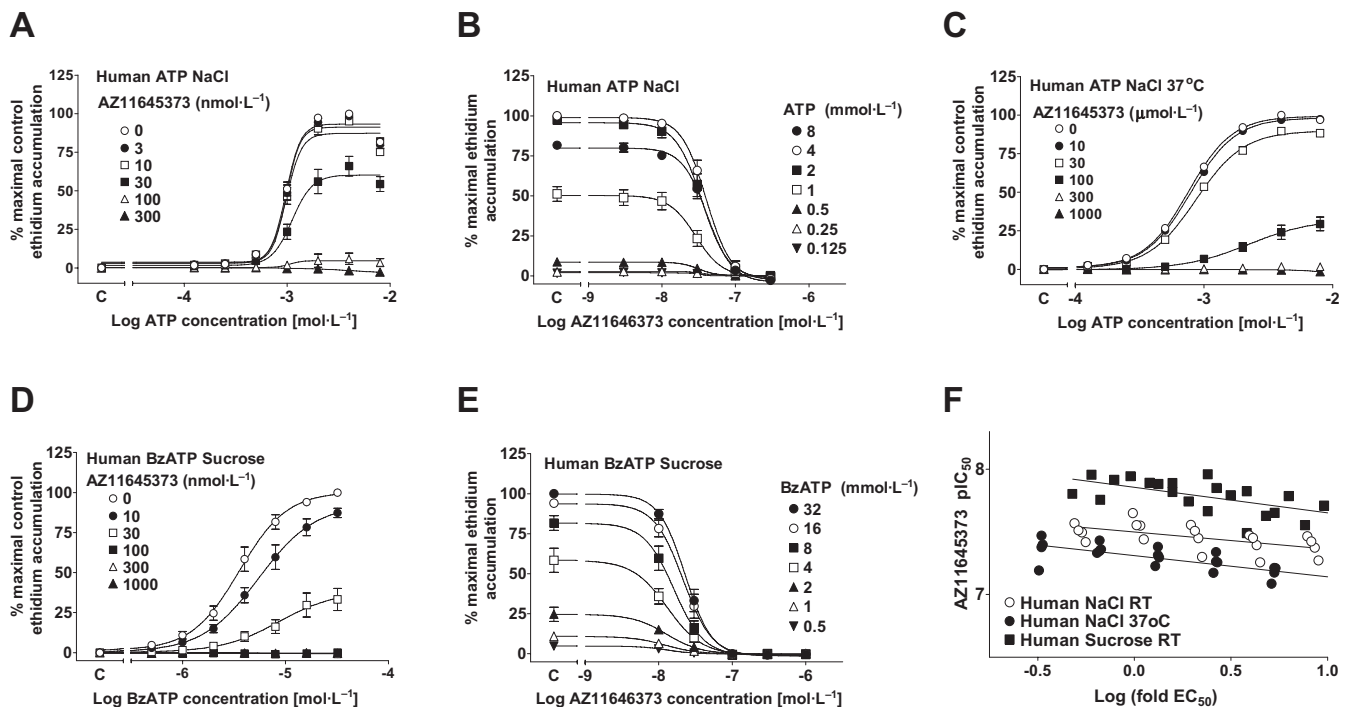


Figure 1 The effect of AZ11645373 at the human P2X₇ receptor in ethidium accumulation studies. HEK293 cells expressing the human recombinant P2X₇ receptor were pre-incubated for 40 min with the indicated concentrations of AZ11645373 before measuring agonist-stimulated ethidium accumulation. (A) The effect of AZ11645373 on ATP responses in NaCl buffer at room temperature. (B) Transposition of the data from panel (A) to illustrate the effect of AZ11645373 on responses to ATP. (C) The effect of AZ11645373 on responses in NaCl buffer at 37°C. (D) The effect of AZ11645373 on BzATP responses in sucrose buffer at room temperature. (E) Transposition of the data from panel (D) to illustrate the effect of AZ11645373 on responses to BzATP. (F) The data from panels A, C and D were analysed to calculate the pIC₅₀ of AZ11645373 at each concentration of agonist (RT, room temperature). Agonist concentration is expressed relative to agonist EC₅₀ at each receptor such that Log (fold EC₅₀) represents logarithm (agonist concentration/agonist EC₅₀). Basal ethidium accumulation in the absence or presence of AZ11645373 is indicated on the X-ordinate as C in (A,C,D). In (B,E) the response to agonist in the absence of AZ11645373 is indicated on the X-ordinate as C. The data are the mean ± SEM of three to four separate experiments. BzATP, 2'- & 3'-O-(4benzoylbenzoyl) ATP.

decavanadate, a dose-ratio (DR) was calculated as the ratio of the IC₅₀ of the compound determined in presence of decavanadate and in its absence. The log (DR-1) estimates from these studies were plotted against the log of the decavanadate concentration in order to construct a form of Schild plot to represent the effects of decavanadate on the antagonist IC₅₀.

Statistical comparisons were made using Student's *t*-test or one-way ANOVA followed by Tukey's *post-hoc* test. Differences were assessed as significant when $P < 0.05$. In these studies the data are the mean ± SEM of three to five experiments. All curve fitting and statistical analysis was performed using GraphPad Prism 3 (San Diego, CA, USA).

Materials

ATP, BzATP, ethidium bromide, PPADS, sodium orthovanadate and reactive black 5 were obtained from Sigma (Poole, UK). AZ11645373, compound-22, GW791343 and SB203580 were synthesized in the Chemistry Department of GSK, Harlow, UK. All culture media were obtained from Invitrogen (Paisley, Scotland) while other reagents were obtained from VWR (Loughborough, UK). [³H]-Compound-17 was from Tritec, Switzerland (specific activity was 2.1TBq·mmol⁻¹ and purity was >99% by HPLC). Decavanadate solutions were prepared as described previously (Michel *et al.*, 2006a).

Results

AZ11645373 is a selective, non-competitive, antagonist of human and dog P2X₇ receptors

Antagonist effects were studied in both sucrose and NaCl buffer as some receptors could only be studied in sucrose buffer and some antagonists have different potency in these two buffers (Hibell *et al.*, 2001). AZ11645373 was a potent antagonist of the human P2X₇ receptor whether studied in sucrose or NaCl buffer (Figure 1). In both buffers, AZ11645373 was a non-competitive antagonist reducing the maximal response to ATP and BzATP. The pIC₅₀ varied slightly with ATP or BzATP concentration, significantly ($P < 0.05$, *F*-test) decreasing as agonist concentration increased (Figure 1F), and there was an approximately linear relationship between pIC₅₀ and the logarithm of the agonist concentration. In order to provide a statistical comparison of potency between the various studies, we analysed the data in Figure 1F using linear regression and calculated the antagonist pIC₅₀ at the agonist EC₅₀. In graphical terms, this corresponded to the pIC₅₀ at 0 on the X-axis and is termed the normalized pIC₅₀. The potency of AZ11645373 at the human P2X₇ receptor in NaCl buffer when using ATP as agonist (normalized pIC₅₀ = 7.46 ± 0.04) was significantly lower ($P < 0.05$, one-way ANOVA followed by Tukey's *post-hoc* test) than in sucrose buffer using

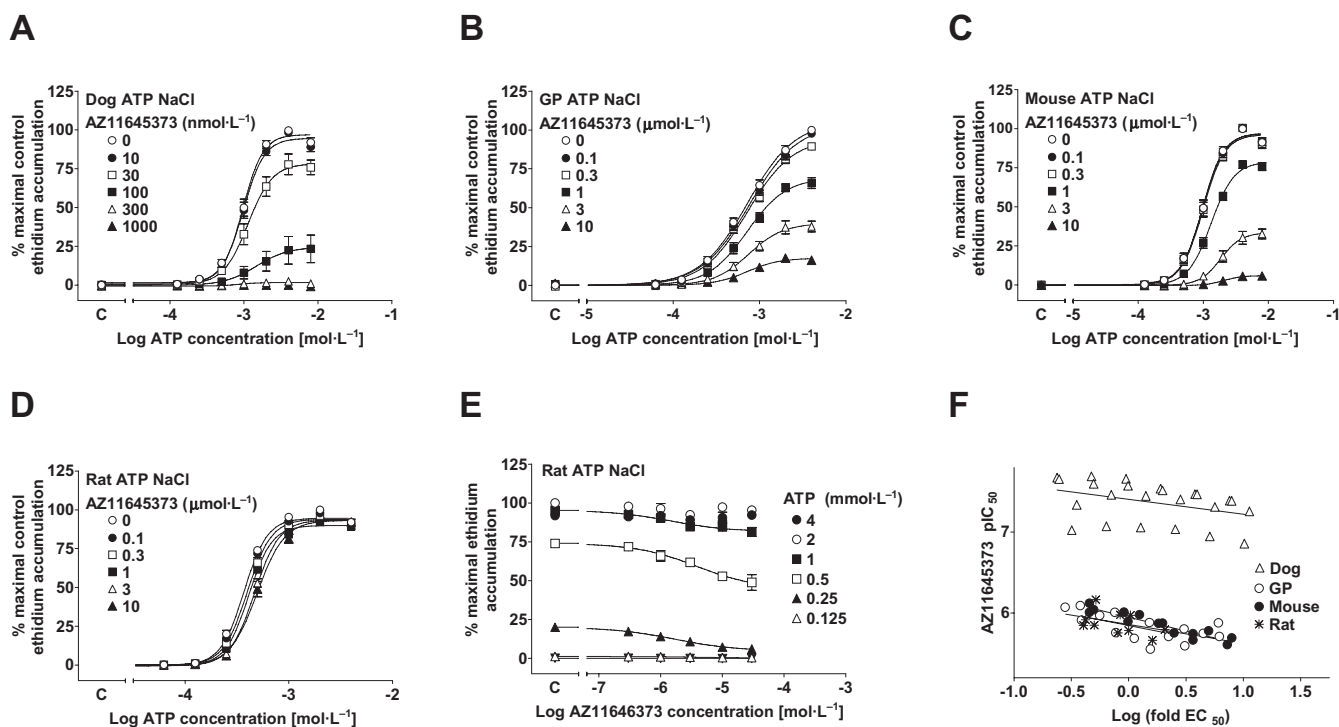


Figure 2 The effect of AZ11645373 at (A) dog, (B) guinea-pig (GP), (C) mouse and (D,E) rat P2X₇ receptors in ethidium accumulation studies. HEK293 cells expressing the dog (A) or rat (D) recombinant receptors or U-2 OS cells transduced with guinea-pig (B) or mouse (C) P2X₇ receptors were pre-incubated for 40 min with the indicated concentrations of AZ11645373 before measuring ATP-induced ethidium accumulation. Studies were performed in NaCl buffer at RT. (E) Transposition of the data from panel (D) to illustrate effect of AZ11645373 on responses to ATP at the rat receptor. (F) The data from (A–D) were analysed to calculate the pIC₅₀ of AZ11645373 at each concentration of agonist. Agonist concentration is expressed relative to agonist EC₅₀ at each receptor such that Log (fold EC₅₀) represents logarithm (agonist concentration/agonist EC₅₀). Basal ethidium accumulation in the absence or presence of AZ11645373 is indicated on the X-ordinate as C in (A–D). In (E) the response to agonist in the absence of AZ11645373 is indicated on the X-ordinate as C. The data are the mean ± SEM of three to four separate experiments.

BzATP as agonist (normalized pIC₅₀ = 7.87 ± 0.02) (Figure 1F). In NaCl buffer using ATP as agonist, there was no significant difference ($P < 0.05$, one-way ANOVA followed by Tukey's *post-hoc* test) in the potency of AZ11645373 at room temperature (normalized pIC₅₀ = 7.46 ± 0.04) and at 37°C (normalized pIC₅₀ = 7.31 ± 0.04) (Figure 1).

AZ11645373 was also a potent antagonist at the dog receptor (Figure 2A) where its normalized pIC₅₀ of 7.40 ± 0.13 (Figure 2F) was similar to that at the human receptor (7.46 ± 0.04). AZ11645373 was also an antagonist of guinea-pig and mouse receptors producing almost complete inhibition of responses at 10 μmol·L⁻¹ (Figure 2B,C). However, it was less potent than at the human or dog receptors and the normalized pIC₅₀ values at mouse and guinea-pig receptors were 5.81 ± 0.13 and 5.94 ± 0.06 respectively (Figure 2F).

AZ11645373 was a low potency antagonist at the rat P2X₇ receptor producing very little shift in the ATP (Figure 2D) or BzATP (data not shown but see Figure 3C) concentration-effect curves in NaCl buffer or that of BzATP in sucrose buffer (data not shown but see Figure 3D). AZ11645373 only appeared to inhibit responses at intermediate agonist concentrations in both NaCl and sucrose buffer (Figure 3) and at these intermediate agonist concentrations the inhibition of responses appeared to be incomplete with saturation of effect at the higher concentrations of AZ11645373 although we only examined the compound at concentrations up to

30 μmol·L⁻¹. The inhibition of agonist effects produced by AZ11645373 was modest but reproducible in two separate studies (Figure 3A,B) although the normalized pIC₅₀ determined using ATP as agonist in NaCl buffer varied between the studies (5.28 ± 0.05 and 5.90 ± 0.05) probably reflecting the difficulty in calculating pIC₅₀ values with modest and incomplete inhibition of responses.

AZ11645373 does not interact at the ATP-binding site

AZ11645373 produced a long-lasting inhibition of responses, with the inhibition of responses at 15 min after washout being the same as without washout (data not shown). This enabled AZ11645373 to be used in receptor protection experiments to determine if the rapidly reversible competitive antagonist decavanadate could affect the persistent antagonist effects of AZ11645373. Decavanadate had very little effect on the long-lasting inhibitory effects of AZ11645373 although it did produce a significant decrease in the pIC₅₀ of AZ11645373 at concentrations of 30, 100 and 300 μmol·L⁻¹ ($P < 0.05$, one-way ANOVA followed by Tukey's *post-hoc* test) although this was no more than twofold and the effects at these three doses were identical ($P > 0.05$, one-way ANOVA followed by Tukey's *post-hoc* test) (Figure 4A,C). These effects contrasted markedly with those observed with PPADS where decavanadate produced a more competitive shift in the

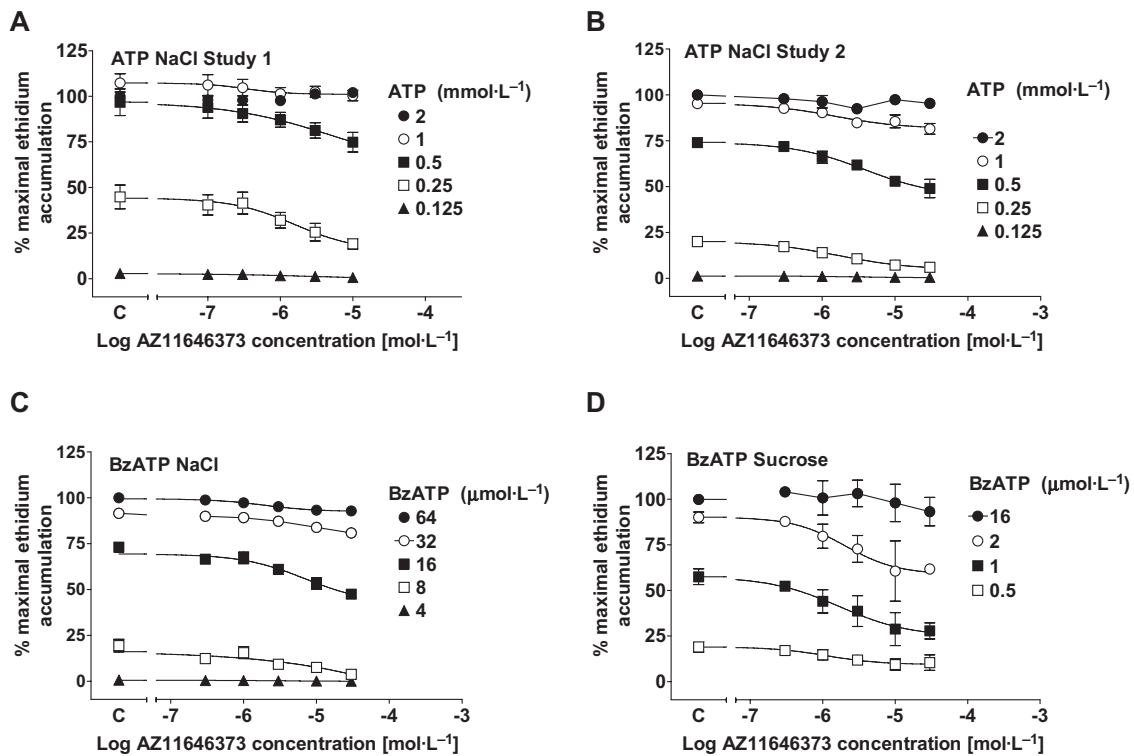


Figure 3 The effect of AZ11645373 at the rat P2X₇ receptor in ethidium accumulation studies. HEK293 cells expressing the rat recombinant receptor were pre-incubated for 40 min with AZ11645373 before measuring agonist stimulated ethidium accumulation. (A) The effect of AZ11645373 on responses to ATP in NaCl buffer in study 1. (B) The effect of AZ11645373 on responses to ATP in NaCl buffer in study 2. (C) The effect of AZ11645373 on responses to BzATP in NaCl buffer. (D) The effect of AZ11645373 on responses to BzATP in sucrose buffer. The response to agonist in the absence of AZ11645373 is indicated on the X-ordinate as C. The data are the mean \pm SEM of three to four separate experiments. BzATP, 2'- & 3'-O-(4benzoylbenzoyl) ATP.

PPADS inhibition curve (Figure 4B) and the resultant Schild plot of the data exhibited a slope of unity (Figure 4C, slope = 1.03 ± 0.03).

GW791343 is a positive allosteric modulator of the rat P2X₇ receptor that increases the potency and effect of ATP (Michel *et al.*, 2008a). In the presence of GW791343, the potency of the negative allosteric P2X₇ receptor modulator, compound-17, was reduced but the potency of compounds that act at the ATP-binding site, such as PPADS and decavanadate, was not affected (Michel *et al.*, 2008a). The ability of AZ11645373 to inhibit responses to equi-effective concentrations of ATP at the rat P2X₇ receptor was not detectably affected by $30 \mu\text{mol}\cdot\text{L}^{-1}$ GW791343 (Figure 4D,E) although the small magnitude of the AZ11645373 effect clearly complicated these studies.

Effects of AZ11645373 at human-rat chimeric receptors

Previous studies have identified amino acid 95 as a key determinant in the species selectivity of several P2X₇ receptor antagonists (see Introduction). To determine if this was also the case with AZ11645373, we examined its effects at chimeric human-rat receptors.

At the rat ECD receptor, as at the wild-type rat receptor, AZ11645373 had very little effect on responses to ATP (data not shown) suggesting its species selectivity was determined

through an interaction with the ECD of the P2X₇ receptor. The compound was a relatively potent antagonist of the human-rat receptor although these studies were complicated by the biphasic nature of the ATP concentration effect curve at this receptor (Figure 5A). To allow for this, the normalized pIC_{50} was calculated at ATP concentrations below $0.125 \text{ mmol}\cdot\text{L}^{-1}$, corresponding to the high affinity component of the ATP concentration-effect curve, and at concentrations of $0.25 \text{ mmol}\cdot\text{L}^{-1}$ and above, corresponding to the low affinity component of the ATP concentration-effect curve. However, the normalized pIC_{50} values were similar over each concentration range of ATP and were intermediate between the potency at the rat and the human receptors (Figure 5F). AZ11645373 was a very weak antagonist of BzATP effects at rat-human receptors (Figure 5B) where its normalized pIC_{50} value was less than at the rat receptor (Figure 5F). Taken together, this suggested that residues in the N-terminal 255 amino acids of the human receptor were more responsible for the species selectivity of AZ11645373.

The normalized pIC_{50} of AZ11645373 at the domain 1 receptor (6.44 ± 0.03) was significantly less than the value of 7.87 ± 0.02 at the human receptor (Figure 5C,F; $P < 0.05$, one-way ANOVA followed by Tukey's *post-hoc* test). However, the normalized pIC_{50} values at domain 2 (7.84 ± 0.05), domain 4 (7.57 ± 0.02) and domain 5 (7.59 ± 0.15) mutant receptors were not significantly different from the value of

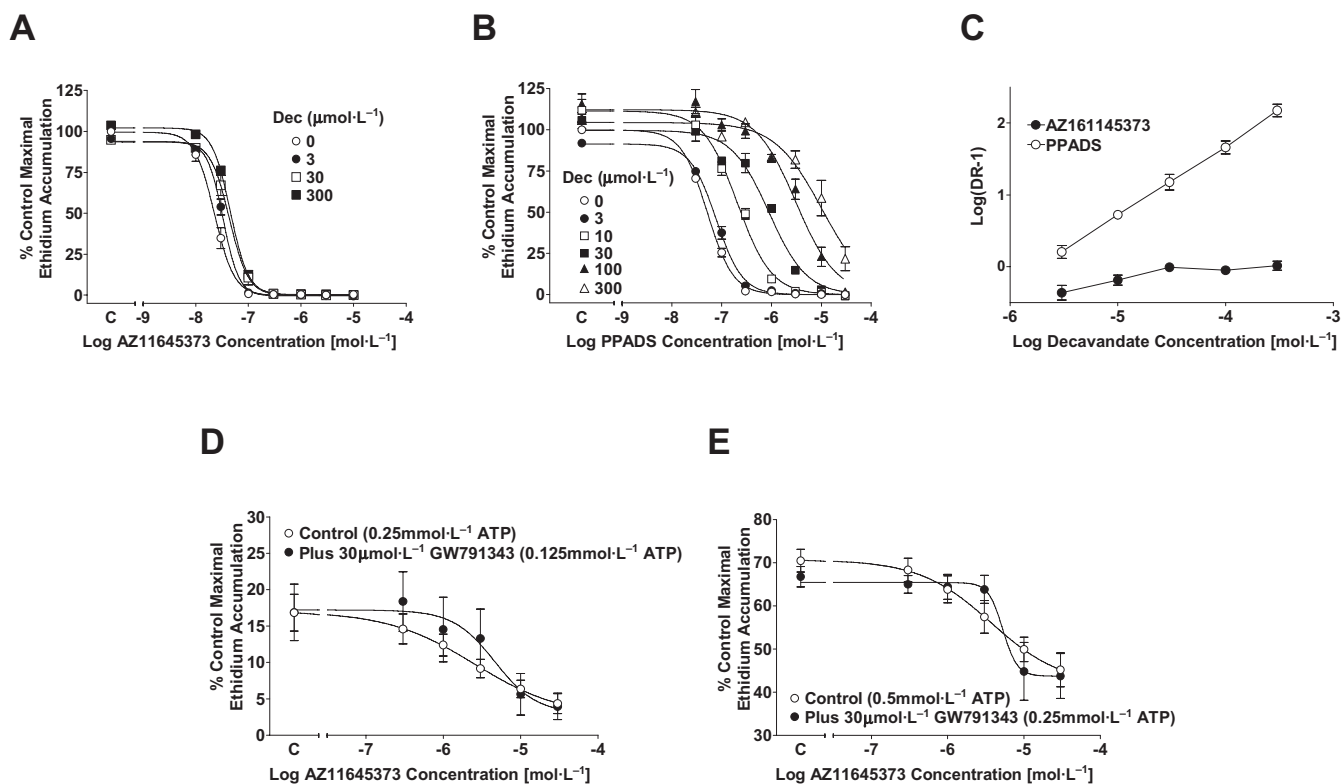


Figure 4 The interaction of AZ11645373 or PPADS with decavanadate or GW791343 in ethidium accumulation studies. (A–C) HEK293 cells expressing the human recombinant P2X₇ receptor were pre-incubated with the indicated concentrations of decavanadate (Dec) for 10 min prior to addition of AZ11645373 or PPADS. Following a further 40 min co-incubation the cells were washed before measuring 2 mmol·L⁻¹ ATP stimulated ethidium accumulation. (C) Schild plot for the interaction between decavanadate and AZ11645373 or PPADS. (D,E) HEK293 cells expressing the rat recombinant P2X₇ receptor were pre-incubated with 30 μmol·L⁻¹ GW791343 for 10 min and then co-incubated with GW791343 and AZ11645373 for a further 40 min before measuring ATP responses. The effect of 30 μmol·L⁻¹ GW791343 on AZ11645373 inhibition of responses to (D) a threshold or (E) a near maximal concentration of ATP is shown. The response to agonist in the absence of AZ11645373 is indicated on the X-ordinate as C in panels A, B, D and E. The data are the mean ± SEM of three to four separate experiments. GW791343, N²-(3,4-difluorophenyl)-N¹-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride; PPADS, pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid.

7.87 ± 0.02 at the human receptor (Figure 5D–F, $P > 0.05$, one-way ANOVA followed by Tukey's *post-hoc* test).

When AZ11645373 was evaluated at P2X₇ receptors with single point mutations within domain 1 (Figure 6), its normalized pIC₅₀ was only significantly reduced at the F95L mutant compared with the human receptor, although the normalized pIC₅₀ of 6.91 ± 0.06 was significantly higher than the value of 6.44 ± 0.03 at the domain 1 mutant ($P < 0.05$, one-way ANOVA followed by Tukey's *post-hoc* test).

Effect of AZ11645373 in radioligand-binding studies

AZ11645373 was a potent inhibitor of compound-17 binding to the human P2X₇ receptor but had no detectable inhibitory effect at the rat P2X₇ receptor and even slightly, but significantly ($P < 0.05$, one-way ANOVA followed by Tukey's *post-hoc* test), increased binding at 1 and 3 μmol·L⁻¹ (Figure 7). At the rat ECD chimeric receptor, the effects of AZ11645373 were similar to those at the rat receptor but the effects of AZ11645373 were reduced considerably at the domain 1 chimeric receptor and similarly reduced at the F95L mutant (Figure 7A–C). The effects of AZ11645373 at

the domain 2 and 4 receptors were not significantly different from those at the human P2X₇ receptor (Figure 7D,F) although at the domain 4 receptor the inhibition of binding produced at 10 μmol·L⁻¹ was less than at 1 and 3 μmol·L⁻¹ (Figure 7F). At the domain 3 receptor (Figure 7E) and domain 5 receptor (data not shown), AZ11645373 potency was slightly, but significantly, reduced when compared with the potency at the human receptor ($P < 0.05$, one-way ANOVA followed by Tukey's *post-hoc* test).

Compound-22 is a selective non-competitive antagonist of the rat P2X₇ receptor

In contrast to AZ11645373, compound-22 was a more potent antagonist of rat than of human receptors (Figure 8A,B,F). The compound was a weak antagonist of the dog receptor and even weaker antagonist of the mouse and guinea-pig receptors (Figure 8C–E). The effects of compound-22 at human and dog receptors were clearly non-competitive as the maximal response was reduced (Figure 8A,C). At the rat receptor, compound-22 appeared to act in a more competitive manner (Figure 8B) as it reduced the ATP EC₅₀ and a pA₂ of 8.12 ± 0.20

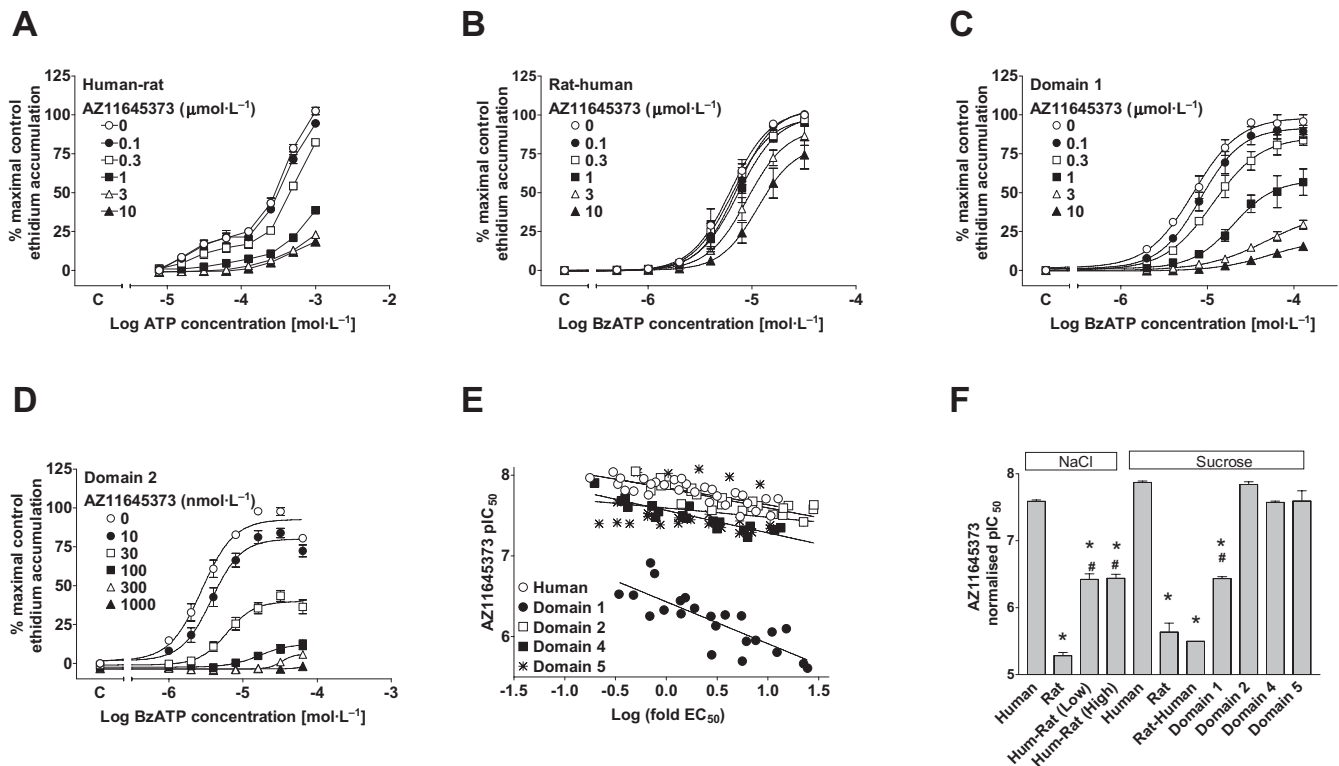


Figure 5 The effect of AZ11645373 on human rat chimeric receptors in ethidium accumulation studies. (A,B) HEK293 cells expressing chimeric human rat receptors or (C–E) U-2 OS cells transduced with chimeric human rat receptors were pre-incubated for 40 min with AZ11645373 before measuring agonist-induced ethidium accumulation. Studies were performed in (A,C,D,E) NaCl buffer using ATP as agonist or (B) sucrose buffer using BzATP as agonist. (E) The dependence of AZ11645373 pIC₅₀ on agonist concentration. The data from (A–D), and other studies, were analysed to calculate the pIC₅₀ of AZ11645373 at each concentration of agonist for each experiment. Agonist concentration is expressed relative to agonist EC₅₀ at each receptor such that Log (fold EC₅₀) represents logarithm (agonist concentration/agonist EC₅₀). (F) AZ11645373 potency at chimeric and wild-type P2X₇ receptors. The normalized pIC₅₀ for AZ11645373, which corresponds to the extrapolated pIC₅₀ for AZ11645373 at the agonist EC₅₀, was calculated from (E), and other studies, as described in the methods. *Significantly different to normalized pIC₅₀ determined at the human receptor using the same assay buffer, $P < 0.05$, one-way ANOVA followed by Tukey's *post hoc* test. #Significantly different to normalized pIC₅₀ determined at the rat receptor using the same assay buffer, $P < 0.05$, one-way ANOVA followed by Tukey's *post hoc* test. Basal ethidium accumulation in the absence or presence of AZ11645373 is indicated on the X-ordinate as C in (A–D). The data are the mean \pm SEM of three to four separate experiments. BzATP, 2'- & 3'-O-(4benzoylbenzoyl) ATP.

could be calculated. However, compound-22 reduced the maximal response to ATP and the slope of the Schild plot was significantly less than unity (0.36 ± 0.06).

Antagonist interaction studies with compound-22

Compound-22 produced a long-lasting inhibition of responses at the rat receptor with the inhibition of responses 15 min after washout being the same as without washout (data not shown). This enabled compound-22 to be used in receptor protection experiments with decavanadate as performed with AZ11645373. Decavanadate had no significant effect on the long-lasting inhibitory effects of compound-22 (Figure 9A, $P > 0.05$, one-way ANOVA followed by Tukey's *post-hoc* test). These effects contrast markedly with those observed with PPADS (Figure 2B,C).

In contrast to the data obtained with AZ11645373, the potency of compound-22 was reduced in the presence of GW791343 (Figure 9B,C,E). Thus, the normalized pIC₅₀ values in the absence or presence of $10 \mu\text{mol}\cdot\text{L}^{-1}$ GW791343 were 8.19 ± 0.02 and 7.22 ± 0.08 respectively.

Temperature and buffer sensitivity of effects of compound-22 at rat receptors

The antagonist potency of compound-22 at rat receptors against responses to ATP in NaCl buffer was moderately temperature sensitive with normalized pIC₅₀ values at 37°C and room temperature being 7.41 ± 0.02 and 8.19 ± 0.02 respectively (Figure 9B,D,E).

The potency and effect of compound-22 at rat receptors were modestly affected by assay conditions as the normalized pIC₅₀ against BzATP in sucrose buffer in U-2 OS cells (7.45 ± 0.10 see Figure 10) was lower than against ATP in NaCl buffer in HEK cells (8.19 ± 0.02 and 8.06 ± 0.05 in two separate studies, see Figures 8F and 9F).

Furthermore, compound-22 did not reduce responses to BzATP at rat P2X₇ receptors in sucrose buffer (Figure 10B) and even appeared to act in a more competitive manner as the slope of the Schild plot of the data (0.87 ± 0.14) was not significantly different to unity ($P > 0.05$, One sample *t*-test). Additional more direct comparisons would be required to determine if the compounds potency and effect was affected specifically by ionic conditions, agonist used or varied

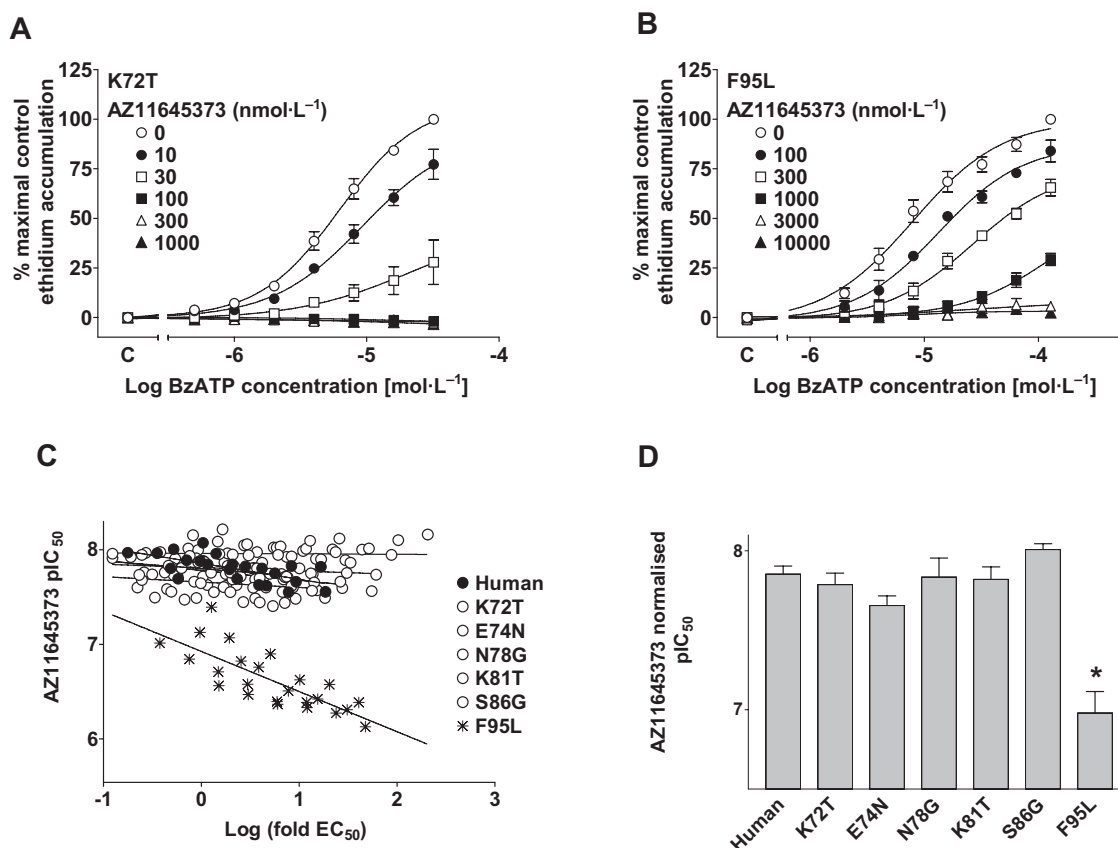


Figure 6 The effect of AZ11645373 on single point mutant receptors from within domain 1 of the P2X₇ receptor measured in ethidium accumulation studies. U-2 OS cells transduced with mutant receptors were pre-incubated for 40 min with AZ11645373 before measuring BzATP-induced ethidium accumulation. Studies were performed in sucrose buffer. The effect of AZ11645373 on BzATP responses in cells expressing (A) the K72T or (B) the F95L P2X₇ receptor. (C) The dependence of AZ11645373 pIC₅₀ on agonist concentration. The data from (A,B) and other studies were analysed to calculate the pIC₅₀ of AZ11645373 at each concentration of agonist. Agonist concentration is expressed relative to agonist EC₅₀ at each receptor such that Log (fold EC₅₀) represents logarithm (agonist concentration/agonist EC₅₀). (D) AZ11645373 potency at chimeric and wild-type P2X₇ receptors. The normalized pIC₅₀ for AZ11645373, which corresponds to the extrapolated pIC₅₀ for AZ11645373 at the agonist EC₅₀, was calculated from the studies in (C). *Significantly different to normalized pIC₅₀ determined at the human receptor, $P < 0.05$, one-way ANOVA followed by Tukey's *post hoc* test. Basal ethidium accumulation in the absence or presence of AZ11645373 is indicated on the X-ordinate as C in (A,B). The data are the mean \pm SEM of three to four separate experiments. BzATP, 2'- & 3'-O-(4benzoylbenzoyl) ATP.

depending on expression system. Nevertheless, it is clear that compound-22 did not act as a simple competitive antagonist.

Effect of compound-22 at chimeric receptors

Compound-22 was an antagonist of the rat-human P2X₇ receptor but was much less potent than at the rat, and even the human receptor, as it only inhibited responses at 3 and 10 $\mu\text{mol}\cdot\text{L}^{-1}$ (data not shown). At the human-rat P2X₇ receptor, compound-22 was an even weaker antagonist and only blocked responses at 10 $\mu\text{mol}\cdot\text{L}^{-1}$ (data not shown).

Compound-22 potency at the domain 1, domain 2, domain 4, F95L and R126G chimeric P2X₇ receptors was similar to that at the human P2X₇ receptor while its potency at the domain 5 P2X₇ receptor was significantly less than at the human receptor (Figure 10). The effects at the domain 3 P2X₇ receptor (normalized pIC₅₀ of 6.62 ± 0.05) were slightly more pronounced than at the human, wild-type, P2X₇ receptor examined in HEK cells (normalized pIC₅₀ of 6.36 ± 0.01). We

only examined one mutant within domain 3. This was the His155Tyr P2X₇ receptor where the normalized pIC₅₀ of 6.16 ± 0.02 was similar to the value of 6.20 ± 0.02 at the wild-type human P2X₇ receptor expressed in U-2 OS cells. This mutant also contains an Arg270His mutation but the normalized pIC₅₀ values of 6.17 ± 0.01 and 6.20 ± 0.02 at the Arg270 and His270 P2X₇ receptors, respectively, were not significantly different.

The effect of compound-22 was examined in radioligand-binding studies. These studies confirmed the rat P2X₇ receptor selectivity of compound-22 (Figure 11). The potency of compound-22 was identical at the rat ECD and rat receptor suggesting residues in the ECD were responsible for its species difference in potency. Compared with its effect at the human wild-type receptor, the effects of compound-22 were significantly increased at the domain 1, domain 2 and F95L mutant P2X₇ receptor (Figure 11B–D) and reduced at the domain 5 mutant receptor (Figure 11F). However, in each case the IC₅₀ values were only 1.5- to twofold different to those at the wild-type human P2X₇ receptor. There were no significant

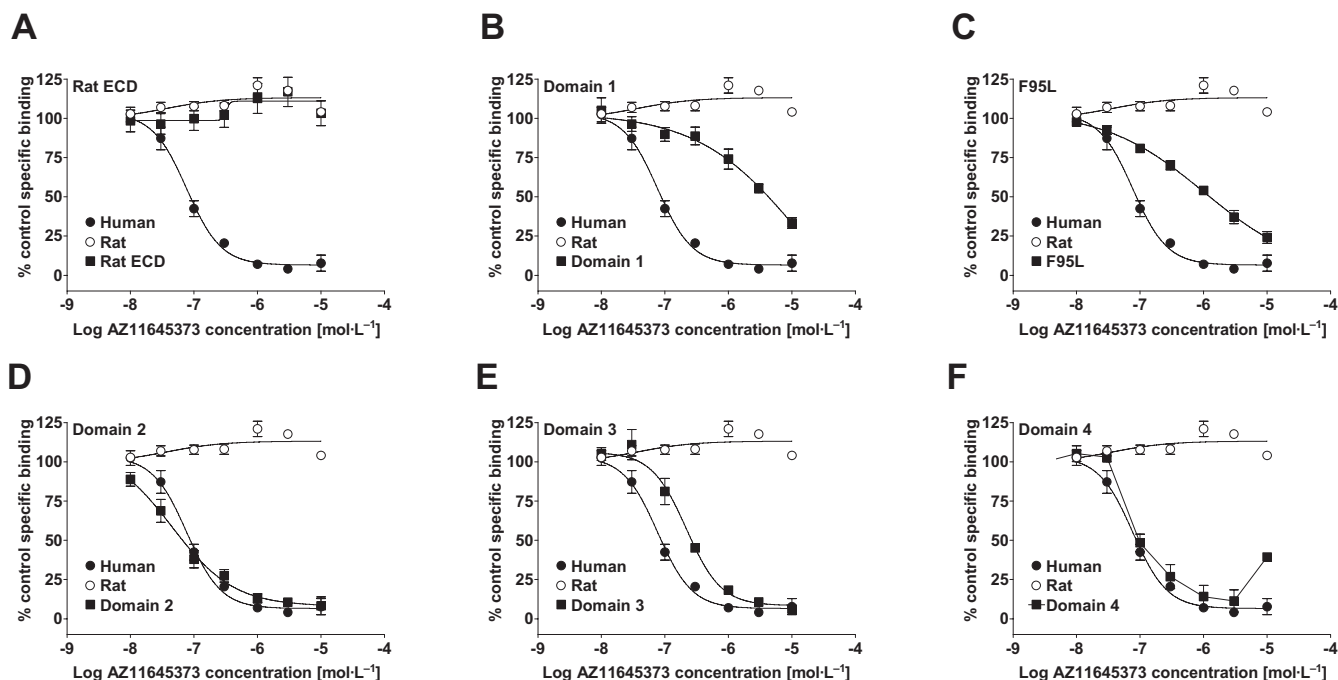


Figure 7 The ability of AZ11645373 to inhibit [³H]-compound-17 binding to membranes prepared from cells expressing human-rat recombinant chimeric P2X₇ receptors. Membranes were prepared from U-2 OS cells transiently transfected with recombinant P2X₇ receptors using BacMam virus. The radioligand concentration was 2 nmol·L⁻¹ and specific binding was defined with 10 μmol·L⁻¹ compound-17. The effect of AZ11645373 at wild-type rat and human receptors is compared with effects at (A) rat-ECD, (B) domain 1, (C) F95L, (D) domain 2, (E) domain 3 or (F) domain 4 P2X₇ receptors. The data are the mean ± SEM of three separate experiments. ECD, extracellular domain.

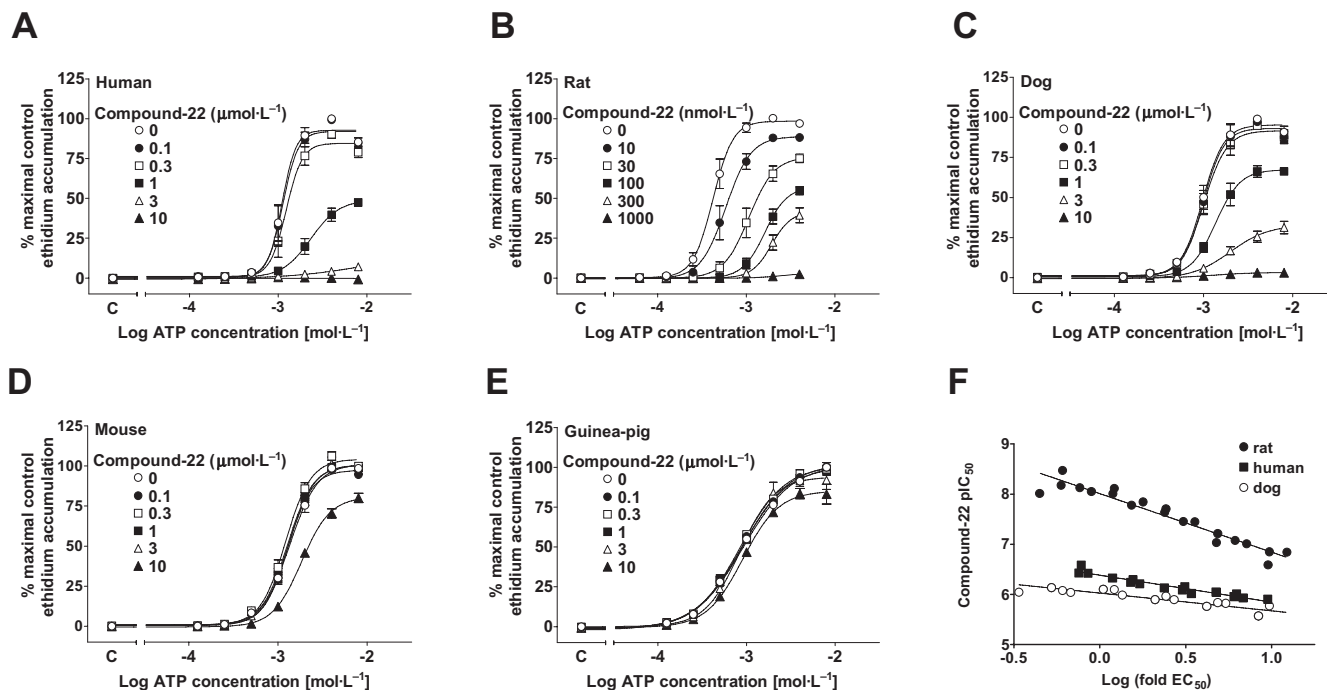


Figure 8 The effect of compound-22 at P2X₇ receptor species orthologues in ethidium accumulation studies. Studies were performed using HEK293 cells (A–C) or in U-2 OS cells transfected with P2X₇ receptors (D, E). Cells expressing (A) human, (B) rat, (C) dog, (D) mouse or (E) guinea-pig P2X₇ receptors were pre-incubated for 40 min with compound-22 in NaCl buffer before measuring ATP-induced ethidium accumulation in NaCl buffer at room temperature. (F) The data from (A–C) were analysed to calculate the pIC₅₀ of compound-22 at each concentration of agonist. Agonist concentration is expressed relative to agonist EC₅₀ at each receptor such that Log (fold EC₅₀) represents logarithm (agonist concentration/agonist EC₅₀). Basal ethidium accumulation in the absence or presence of compound-22 is indicated on the X-ordinate as C in (A–E). The data are the mean ± SEM of three to four separate experiments. Compound-22, *N*-[2-methyl-5-[(1*R*,5*S*)-9-oxa-3,7-diazabicyclo[3.3.1]non-3-ylcarbonyl]phenyl]-2-tricyclo[3.3.1.1.3,7]dec-1-ylacetamide hydrochloride.

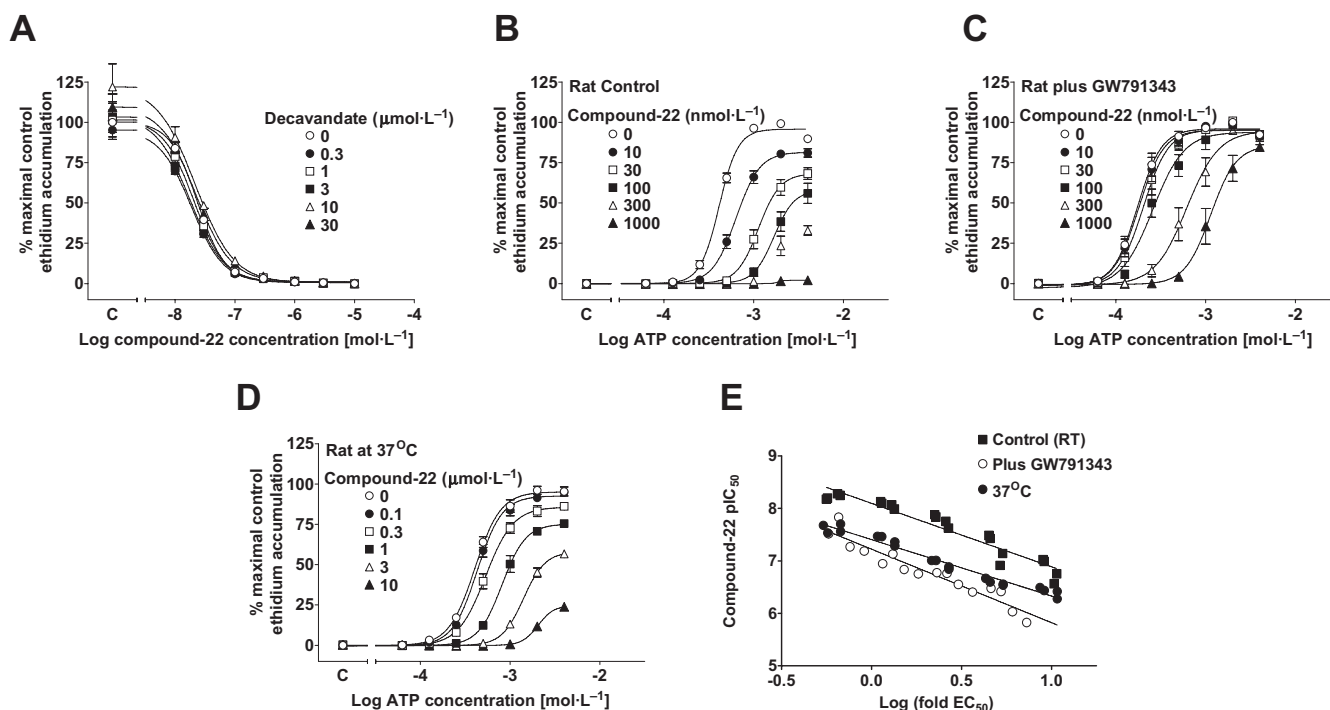


Figure 9 The effect of decavanadate, GW791343 or temperature on the antagonist effect of compound-22 at the rat P2X₇ receptor measured in ethidium accumulation studies. Studies were performed using HEK293 cells expressing the rat recombinant P2X₇ receptor. (A) Cells were pre-incubated with decavanadate for 10 min prior to addition of compound-22. After a 40 min co-incubation the cells were washed over a 15 min period before measuring the response to 0.5 mmol·L⁻¹ ATP. (B,C) Cells were incubated with compound-22 for 40 min before measuring ATP responses. Studies were performed at room temperature (B) without or (C) following pre-incubation with 30 μmol·L⁻¹ GW791343 for 10 min prior to addition of compound-22. (D) Studies were performed at 37°C. (E) The effect of temperature and GW791343 on the potency of compound-22. The data from (B–D) were analysed to calculate the pIC₅₀ of compound-22 at each concentration of agonist. Agonist concentration is expressed relative to agonist EC₅₀ under each condition such that Log (fold EC₅₀) represents logarithm (agonist concentration/agonist EC₅₀). The response to agonist in the absence of decavanadate (A) or compound-22 (B–D) is indicated on the X-ordinate as C. The data are the mean ± SEM of three to four separate experiments. Compound-22, *N*-[2-methyl-5-[(1*R*, 5*S*)-9-oxa-3,7-diazabicyclo[3.3.1]non-3-ylcarbonyl]phenyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide hydrochloride.

changes in pIC₅₀ at the domain 3 (Figure 11E) or the domain 4 (data not shown) P2X₇ receptors.

Discussion

In this study we have presented evidence that two previously described P2X₇ antagonists are negative allosteric modulators of the P2X₇ receptors and confirmed their species selectivity. We have also extended the information on their orthologue selectivity and shown that these two molecules may interact with different sites on the P2X₇ receptor.

AZ11645373 was recently described as a selective antagonist of the human P2X₇ receptor and its effects were examined in detail in electrophysiology, ion influx and cytokine release assays (Stokes *et al.*, 2006). However, it was a non-competitive antagonist and its mechanism of action was not identified (see Introduction). In this study, we have studied the effects of this compound in ethidium accumulation and radioligand-binding studies in order to better understand its mechanism of action. Our studies confirm the non-competitive mechanism of action at the human receptor and also demonstrate that its effects are only slightly affected by assay buffer composition or assay temperature. The latter point may be important as several P2X₇ antagonists (Fonfria *et al.*, 2005; A.D.

Michel, unpubl. obs.), as well as compound-22 (see below), display between three- and 10-fold lower affinities at the more physiologically relevant temperature of 37°C than at room temperature where most *in vitro* studies are performed.

AZ11645373 was confirmed as a highly selective antagonist of human, as opposed to rat, P2X₇ receptors in both ethidium accumulation and radioligand-binding studies. The compound only partially blocked responses at the rat receptor but produced a much more pronounced blockade of the guinea-pig and mouse receptors although the pIC₅₀ values were not very different between the three rodent orthologues. At the rat receptor, AZ11645373 produced partial and incomplete inhibition of responses which may suggest that it is acting in an allosteric manner (see below) and that the main difference between its effects at the rodent orthologues is in the magnitude of its efficacy to inhibit responses to ATP.

AZ11645373, like several other P2X₇ receptor antagonists (Chessell *et al.*, 1998; Michel *et al.*, 2008a), appeared to have a very slow offset of action when studied using the ethidium accumulation technique, with effects persisting unchanged for at least 15 min after washout. The compound appeared to have faster offset kinetics in electrophysiology studies where its offset was described as slow but with responses returning to 10% of control within 15–25 min (Stokes *et al.*, 2006). We do not know the reason for this difference in kinetics between

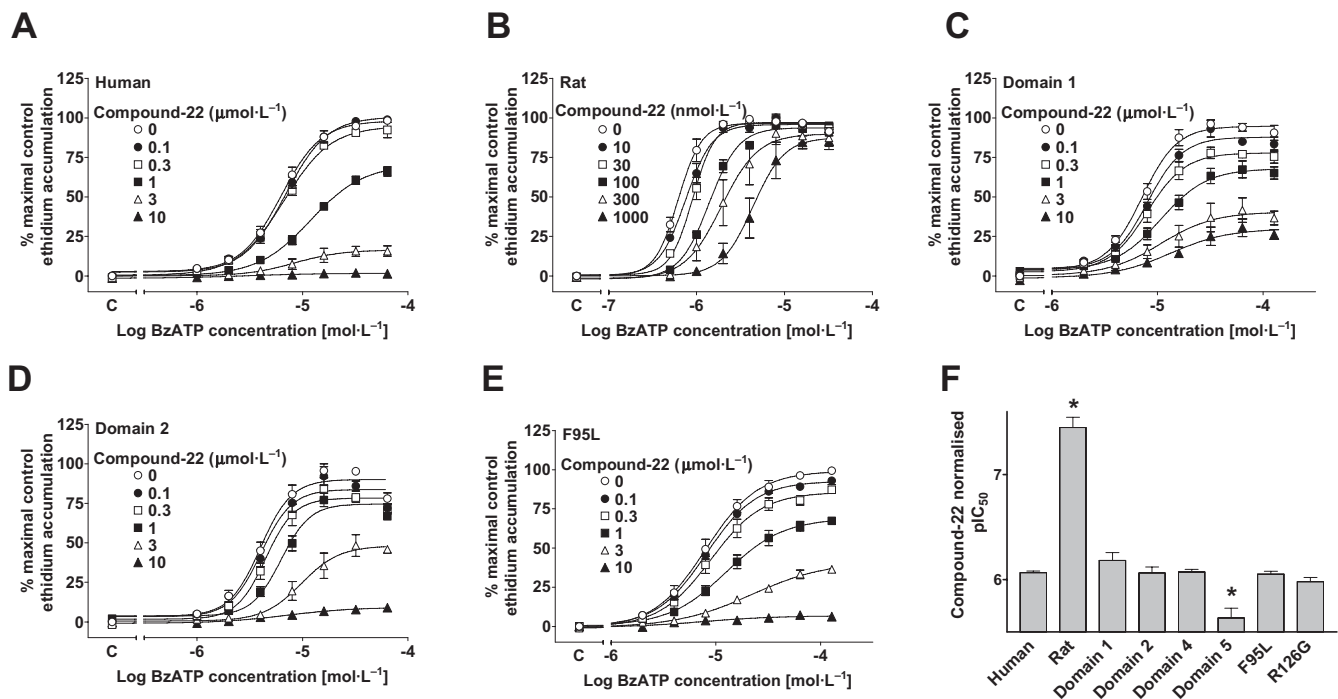


Figure 10 The effect of compound-22 on human rat chimeric P2X₇ receptors measured in ethidium accumulation studies. U-2 OS cells transfected with chimeric receptors were pre-incubated for 40 min with the indicated concentrations of compound-22 before measuring BzATP responses. Studies were performed in sucrose buffer. The effect of compound-22 is shown in cells expressing (A) human, (B) rat, (C) domain 1, (D) domain 2 or (E) F95L P2X₇ receptors. (F) The potency of compound-22 at chimeric and wild-type receptors. The normalized pIC₅₀ for compound-22, which corresponds to the extrapolated pIC₅₀ at the agonist EC₅₀, was calculated as described in the methods. *Significantly different to the normalized pIC₅₀ at the human P2X₇ receptor, $P < 0.05$, one-way ANOVA followed by Tukey's *post hoc* test. Basal ethidium accumulation in the absence or presence of compound-22 is indicated on the X-ordinate as C in (A–E). The data are the mean \pm SEM of three to four separate experiments. Compound-22, *N*-[2-methyl-5-[(1*R*, 5*S*)-9-oxa-3,7-diazabicyclo[3.3.1]non-3-ylcarbonyl]phenyl]-2-tricyclo[3.3.1.1.3,7]dec-1-ylacetamide hydrochloride.

studies but the slow offset of action did allow the compound to be used in receptor protection studies to explore its mechanism of action. In those studies, decavanadate had very little effect on the long-lasting inhibition produced by AZ11645373 and even the minimal effect it had saturated at concentrations of 30–300 $\mu\text{mol}\cdot\text{L}^{-1}$. This contrasted markedly with the pronounced and apparently competitive protection of the receptor by decavanadate from the persistent blockade produced by PPADS and was similar to the very limited effects of decavanadate on the persistent blockade of the receptor produced by the allosteric antagonist compound-17 in studies on the human receptor (Michel *et al.*, 2008a). This suggested that, like compound-17, AZ11645373 is an allosteric antagonist of the receptor. In this respect, site-directed mutagenesis studies also provided indirect evidence that AZ11645373 was acting as an allosteric antagonist as the potency of AZ11645373 was affected by the same residues that affected the positive allosteric modulator, GW791343, and the negative allosteric modulators SB203580 and KN62 (Michel *et al.*, 2008b). Thus, amino acid 95 was found to be a key residue in determining the allosteric effects of these allosteric agents and we found that this residue also contributed to the human/rat species selectivity of AZ11645373. Interestingly, although compound-17 and AZ11645373 both appear to be allosteric antagonists of the human P2X₇ receptor, they may interact with the receptor in a different manner, as GW791343 was

able to reduce the potency of compound-17 as an antagonist at the rat receptor (Michel *et al.*, 2008a) but had little effect on the actions of AZ11645373.

The pharmacological properties of compound-22 have not previously been presented in any detail but it represented a unique compound in being selective for rat over human P2X₇ receptors (Furber *et al.*, 2007). We were able to confirm this selectivity and, additionally, found that compound-22 had little or no detectable affinity for mouse or guinea-pig P2X₇ receptors and so also discriminated between rodent receptors. KN62 also discriminates between rat and mouse P2X₇ receptors (Humphreys *et al.*, 1998) and rat and guinea-pig P2X₇ receptors (Fonfria *et al.*, 2008) but compound-22 appears to be the most selective tool to date in this regard. Compound-22 also possessed low affinity for the dog P2X₇ receptor where its potency was similar to that at the human receptor. Indeed, the effects of both AZ11645373 and compound-22 at human and dog P2X₇ receptors were similar and contrasted with the marked species differences observed with the compounds between human and rodent P2X₇ receptors. We have recently characterized the dog recombinant P2X₇ receptor (Roman *et al.*, 2007) and found that its antagonist sensitivity was similar to the human P2X₇ receptor and so the present observations strengthen this correlation.

Compound-22, like AZ11645373, was a non-competitive P2X₇ receptor antagonist and also appeared to be acting in an

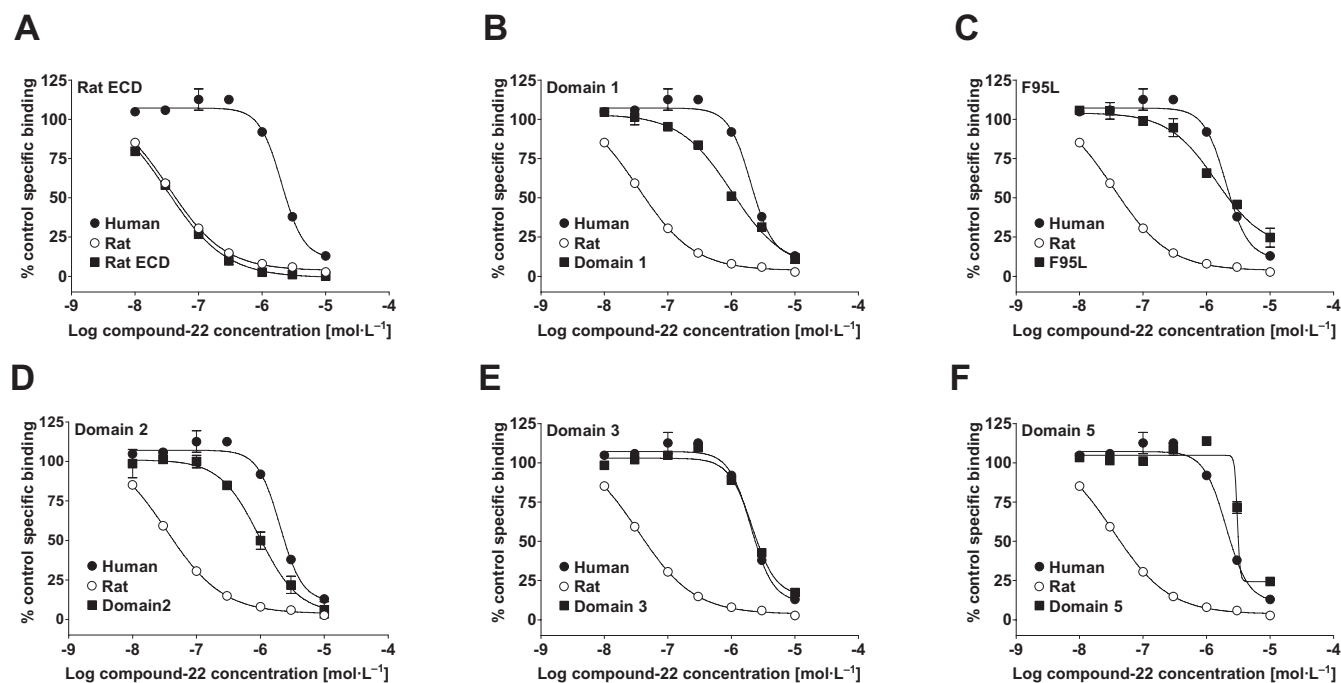


Figure 11 The ability of compound-22 to inhibit [³H]-compound-17 binding to membranes prepared from cells expressing human-rat recombinant chimeric P2X₇ receptors. Membranes were prepared from U-2 OS cells transiently transfected with recombinant P2X₇ receptors using BacMam virus. The radioligand concentration was 2 nmol·L⁻¹ and specific binding was defined with 10 μmol·L⁻¹ compound-17. The effect of compound-22 at wild-type rat and human receptors is compared with effects at (A) rat-ECD, (B) domain 1, (C) F95L, (D) domain 2, (E) domain 3 or (F) domain 5 P2X₇ receptors. The data are the mean ± SEM of three separate experiments. Compound-22, *N*-[2-methyl-5-[(1*R*,5*S*)-9-oxa-3,7-diazabicyclo[3.3.1]non-3-ylcarbonyl]phenyl]-2-tricyclo[3.3.1.1.3,7]dec-1-ylacetamide hydrochloride; ECD, extracellular domain.

allosteric manner. The allosteric nature of its effect was evident in receptor protection experiments where there was no detectable interaction between compound-22 and decavanadate which appears to bind at, or close to, the ATP-binding site (Michel *et al.*, 2006a). Furthermore, in radioligand-binding studies, compound-22 interacted with the sites labelled by the allosteric antagonist compound-17. In contrast, competitive P2X₇ receptor antagonists such as PPADS and decavanadate have little effect on the binding of compound-17 (Michel *et al.*, 2007). Finally, like compound-17 (Michel *et al.*, 2008a), the potency of compound-22 at rat P2X₇ receptors was reduced by GW791343.

Although AZ11645373 and compound-22 were both allosteric P2X₇ receptor antagonists, they may bind to the receptor in different ways as the compounds differed in several respects. First, compound-22 potency was temperature-dependent but this was not observed with AZ11645373. Second, in compound interaction studies, GW791343 had little detectable effect on the potency of AZ11645373 but reduced the potency of compound-22 10-fold. Third, the potency of AZ11645373 was affected in the domain 1 chimera and the human F95L single point mutant receptors. In contrast, the potency of compound-22 was not affected in functional studies on the human F95L receptor and only slightly affected in the binding studies (twofold change in potency). Interestingly, compound-22 had little effect in rat-human or human-rat receptors suggesting that multiple sites may be required for its binding to the P2X₇ receptor and that loss of either site results in a substantial loss of affinity for the receptor. This was not observed with AZ11645373. Taken together,

it seems likely that compound-22 and AZ11645373 are both allosteric inhibitors of the P2X₇ receptor but that each compound has a different mode of binding.

In conclusion, we have confirmed the species selectivity of the P2X₇ receptor antagonists, AZ11645373 and compound-22, and characterized their interaction with three other species orthologues. The latter studies provided further evidence for pharmacological similarities in the human and dog P2X₇ receptors and also identified quite marked species differences among rodent receptors in terms of their antagonist sensitivity. Furthermore, we have demonstrated that both AZ11645373 and compound-22 are allosteric antagonists, although they appear to bind at separate sites or induce their allosteric effects in different ways. It remains to be seen if the different mechanism of action will affect their potential therapeutic effects.

Conflict of interest

The authors are employed by GlaxoSmithKline.

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