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RESEARCH PAPER

Negative and positive allosteric modulators of the $P2X_7$ receptor

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Background and purpose: Antagonist effects at the $P2X_7$ receptor are complex with many behaving in a non-competitive manner. In this study, the effects of *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide (compound-17) and N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride (GW791343) on $P2X_7$ receptors were examined and their mechanism of action explored.
Experimental approach: Antagonist effects were studied by measuring agonist-stimulated ethidium accumulation in cells expressing human or rat recombinant $P2X_7$ receptors and in radioligand binding studies.
Key results: Compound-17 and GW791343 were non-competitive inhibitors of human $P2X_7$ receptors. Receptor protection studies using decavanadate and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) showed that neither compound-17 nor GW791343 competitively interacted at the ATP binding site and so were probably negative allosteric modulators of the $P2X_7$ receptor. GW791343 prevented the slowly reversible blockade of the human $P2X_7$ receptor produced by compound-17 and inhibited [^{3}H]-compound-17 binding to the $P2X_7$ receptor suggesting they may bind to similar or interacting sites. At rat $P2X_7$ receptors, compound-17 was a negative allosteric modulator but the predominant effect of GW791343 was to increase agonist responses. Antagonist interaction and radioligand binding studies revealed that GW791343 did not interact at the ATP binding site but did interact with the compound-17 binding site suggesting that GW791343 is a positive allosteric modulator of the rat $P2X_7$ receptor.
Conclusions: Compound-17 was a negative allosteric modulator of human and rat $P2X_7$ receptors. GW791343 was a negative allosteric modulator of the human $P2X_7$ receptor but at the rat $P2X_7$ receptor its predominant effect was positive allosteric modulation. These compounds should provide valuable tools for mechanistic studies on $P2X_7$ receptors.
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Keywords: $P2X_7$ receptor; ATP; BzATP; allosteric modulator; non-competitive antagonist

Abbreviations: BzATP, 2′- & 3′-*O*-(4benzoylbenzoyl) ATP; compound-17, *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide; GW791343, N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride; KN62, 1-[*N*,*O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; oxATP, periodate oxidized ATP; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid

Introduction

The P2X receptors are a family of ligand-gated cation channels activated by extracellular ATP. To date seven members of the family have been identified and shown to function either as homomeric or heteromeric combinations (North and Surprenant, 2000; North, 2002). The $P2X_7$ receptor for extracellular ATP differs from other family members, as it exhibits a considerable degree of plasticity in function and affects a wide range of cellular functions (North, 2002). Like other members of the P2X receptor family, it functions as an ATP-activated ligand-gated cation channel permeable to monovalent and divalent cations following brief (ms to s) exposures to ATP (Surprenant *et al*., 1996). However, with prolonged activation (s to min), the channel properties change dramatically and the channel either dilates (Surprenant *et al*., 1996) or couples to pannexin hemi-channels (Pelegrin and Surprenant, 2006) to enable cellular entry of molecules with a MW of up to 800 Da, including the ethidium molecule used to measure receptor function in this study.

The $P2X_7$ receptor has attracted considerable interest as a therapeutic target due to its potential involvement in pain and inflammatory disorders (Dell'Antonio *et al*., 2002; Chessell *et al*., 2005). This has lead to the identification of several structurally different classes of $P2X_7$ receptor antagonist (Baraldi *et al*., 2004; Romagnoli *et al*., 2005;

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Donnelly-Roberts and Jarvis, 2007) to complement the earlier P2X$_7$ receptor antagonists such as oxidized ATP (oxATP), 1-[*N*, *O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN62) (Gargett and Wiley, 1997) and brilliant blue G (also known as coomassie brilliant blue) (Jiang *et al*., 2000).

Recent studies have started to describe the pharmacological properties of several of these novel antagonists such as AZ11645373 (Stokes *et al*., 2006) and A-740003 (Honore *et al*., 2006). However, it is not clear if these newly described compounds are competitive P2X$_7$ receptor antagonists. Certainly, AZ11645373 did not produce clearly competitive antagonist effects (Stokes *et al*., 2006) and the mechanism of action of A-740003 was not reported (Honore *et al*., 2006). This may be relevant, as studies using KN62 have shown that it behaves in a non-competitive allosteric manner to block human P2X$_7$ receptors (Michel *et al*., 2006, 2007), whereas a recently described P2X$_7$ receptor antagonist, *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo [3.3.1.1^{3,7}]dec-1-ylacetamide (compound-17), was found to label the human P2X$_7$ receptor but did not appear to bind to the ATP binding site, suggesting an allosteric mechanism of action (Michel *et al*., 2007).

In the present study, we have further examined compound-17 and a structurally different P2X$_7$ receptor antagonist, N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride (GW791343, Figure 1), as described by Furber *et al*., 2000, in functional studies in order to better understand their mechanism of interaction with the P2X$_7$ receptor.

To aid with these studies, we have performed receptor protection studies with decavanadate, as previously described with KN62 and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (Michel *et al*., 2006). In those studies, we found that co-incubation of the rapidly reversible P2X$_7$ receptor antagonist decavanadate with the slowly reversible or irreversible P2X$_7$ receptor antagonists PPADS or oxATP followed by extensive washout, resulted in a marked concentration-dependent competitive reduction in the apparent potency of PPADS and oxATP. The competitive nature of this effect of decavanadate suggested that decavanadate, oxATP and PPADS interacted at the same site on the P2X$_7$ receptor, which is presumably the ATP binding site. In contrast, decavanadate had little effect on the potency of KN62, suggesting that KN62 interacted at a site distinct from that recognized by decavanadate and so presumably distinct from the ATP binding site.

The present studies reveal that compound-17 and GW791343 are potent, but non-competitive, antagonists of human P2X$_7$ receptors and appear to be negative allosteric regulators of the human P2X$_7$ receptor. Furthermore, GW791343 appears to function as a positive allosteric regulator of the rat P2X$_7$ receptor.

GW791343

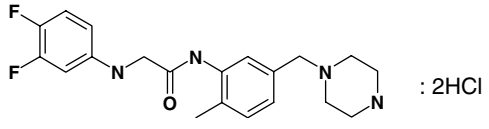

Figure 1 Structure of GW791343. GW791343, N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride.

Methods

Cellular ethidium accumulation measurements

Studies were performed as described previously (Michel *et al*., 2006). Briefly, HEK293 cells, expressing rat or human recombinant P2X$_7$ receptors, were grown (18–24 h) to form a completely confluent monolayer in poly-L-lysine pre-treated 96-well plates (Costar, High Wycombe, UK). Studies were performed using assay buffers comprising (in mM): HEPES 10, *N*-methyl-D-glucamine 5, KCl 5.6, D-glucose 10, $CaCl_2$ 0.5 (pH 7.4) and supplemented with either 280 mM sucrose (sucrose buffer) or 140 mM 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. All solutions were aspirated using 25-gauge bevelled syringe needles to provide complete solution removal. In all studies, the final assay volume was 100 μl. The majority of studies were performed at room temperature (19–21 °C). In one study, the effect of GW791343 at rat P2X$_7$ receptors was studied at 4 °C in sucrose buffer.

Evaluation of antagonist effects

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 μM final assay concentration). After agonist addition, incubations were continued until approximately 10–30% of maximal dye accumulation occurred. In sucrose buffer, the agonist incubation time was 1.5 and 2 min when studying the rat and human P2X$_7$ receptors, respectively. In NaCl buffer, agonist incubation times were 8 and 16 min for studies on the rat and human P2X$_7$ receptors, respectively. Reactions were rapidly terminated by addition of 25 μl of 1.3 M sucrose assay buffer containing 5 mM 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). In one study to examine the effect of GW791343 on the rat P2X$_7$ receptor at 4 °C in sucrose buffer, the effects of GW791343 were examined with either 0, 5 or 40 min pre-incubation period. For these studies, the subsequent agonist exposure period was 2 min.

In studies to measure antagonist dissociation from the human P2X$_7$ receptor, cells were incubated with antagonist at room temperature for 40 min and then the antagonist solution was aspirated and 350 μl of NaCl assay buffer was added. The wash step was repeated twice (total wash time of 8 min). Thereafter, cells were incubated for varying times before a final aspiration followed by addition of 100 μl of a mixture of ATP (1 mM) and ethidium. After 8 min agonist

exposure, cellular accumulation of ethidium was measured as described above. In some studies, 37 °C wash buffer was used and ATP-stimulated ethidium accumulation measured after a 4-min incubation period at 37 °C.

Protection of $P2X_7$ receptors from the slowly reversible blockade by $P2X_7$ receptor antagonists (receptor protection studies)
In these receptor protection studies to examine the ability of compounds to affect the slowly reversible blockade produced by antagonists such as PPADS or compound-17, cells were pre-incubated with NaCl assay buffer or the first, more rapidly reversible, antagonist (decavanadate or GW791343) for 10 min before addition of various concentrations of the second, more slowly reversible, antagonist in the absence or continued presence of the first antagonist. After a 30-min further co-incubation period, the wells were aspirated and 350 μl of NaCl assay buffer, at room temperature, was added. This wash step was repeated two more times (8 min wash period). The cells were incubated for various times as indicated in the Results section and the wells were aspirated and a mixture of 100 μM ethidium and ATP was added (0.5 or 1 mM for studies on rat or human $P2X_7$ receptors, respectively). After 4 min (rat) or 8 min (human) agonist exposure, reactions were terminated and cellular accumulation of ethidium was determined as described above.

In studies on the rat $P2X_7$ receptor to examine the interaction between the more slowly reversible antagonists (for example PPADS) and the more rapidly reversible antagonists (for example GW791343 or decavanadate), the antagonist pre-incubation and co-incubation steps were performed using sucrose buffer, as this facilitated the study due to the higher potency of PPADS in sucrose than in NaCl buffer. Thereafter, the antagonist washout and agonist exposure procedures were performed as described above.

Interaction studies with $P2X_7$ receptor antagonists
In studies to investigate the interaction between GW791343 and other $P2X_7$ receptor antagonists at the rat $P2X_7$ receptor, cells were pre-incubated with NaCl assay buffer or 10 μM GW791343 for 10 min before addition of various concentrations of the other antagonist in the absence or continued presence of 10 μM GW791343. After a further 40 min incubation period, the ATP and ethidium mixture was added. After 4 min ATP exposure, cellular ethidium accumulation was measured as described above.

Radioligand binding studies
The radioligand binding studies using [^{3}H]-compound-17 were performed as described previously (Michel *et al*., 2007). Briefly, studies were performed using membranes prepared from HEK293 cells expressing human or rat recombinant $P2X_7$ receptors. The radioligand, [^{3}H]-compound-17, was used at a concentration of 2–3 nM. Incubations were for 60 min at room temperature in a final assay volume of 200 μl of 50 mM Tris HCl buffer containing 0.01% bovine serum albumin (pH 7.4 at room temperature) and were terminated by vacuum filtration. Nonspecific binding was defined using 10 μM 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 EC_{50} or IC_{50} 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, the data from the Schild studies were also analysed to calculate antagonist pIC_{50} values at each agonist concentration, as this provided some quantitative estimate of antagonist potency. To graphically represent the effect of agonist concentration on antagonist pIC_{50}, the agonist concentration was expressed relative to its EC_{50} value at the various receptor orthologues (logarithm (agonist concentration/agonist EC_{50})). This enabled a simpler comparison of antagonist pIC_{50} values between the species orthologues and simplified the comparison between antagonist pIC_{50} values determined using ATP or BzATP as agonist.

In the receptor protection studies using decavanadate, the IC_{50} of the slowly reversible antagonists to block ATP responses after co-incubation with decavanadate and extensive washout was determined in control cells and in cells pretreated with various concentrations of decavanadate. For each concentration of decavanadate, a dose ratio for the slowly reversible antagonist was calculated as the ratio of the IC_{50} of the antagonist determined in the presence and absence of decavanadate. The log (dose ratio-1) estimates from these studies were plotted against the log of the decavanadate concentration in order to construct a form of Schild plot (Figure 3d) to represent the effects of decavanadate on the IC_{50} of the slowly reversible antagonist.

In all studies, the data are the mean ± s.e.mean 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, KN62, oxATP, PPADS, reactive black 5 and sodium orthovanadate were obtained from Sigma (Poole, UK). All culture media were obtained from Invitrogen (Paisley, Scotland), whereas other reagents were obtained from VWR (Loughborough, UK). [^{3}H]-Compound-17 was from Tritec, Switzerland (specific activity was 2.1 TBq mmol^{-1} and purity was >99% by HPLC). Decavanadate solutions were prepared as described previously (Michel *et al*., 2006). Compound-17 and GW791343 were synthesized in the Chemistry Department of GSK, Harlow. GW791343 is the dihydrochloride salt of example No. 1 of the patent WO 2000071529 A1 (Furber *et al*., 2000). KN62, compound-17 and GW791343 were dissolved in dimethyl sulphoxide as a 10 mM solution and were stored as frozen aliquots at −20 °C until required.

Results

Compound-17 is a non-competitive and slowly reversible antagonist of human and rat P2X$_7$ receptors

Compound-17 was a non-competitive antagonist of the human P2X$_7$ receptor decreasing responses to both ATP (Figure 2a) and BzATP (data not shown) when studied in NaCl buffer. The compound was also a non-competitive antagonist of the human P2X$_7$ receptor in sucrose buffer (data not shown). In NaCl buffer, compound-17 also acted as a non-competitive antagonist of responses to ATP (Figure 2b) and BzATP (data not shown) at the rat P2X$_7$ receptor. Although compound-17 produced a small increase in the ATP and BzATP EC_{50} at the rat P2X$_7$ receptor, the slope of the Schild plot was significantly less than unity in each case (0.50 ± 0.07 and 0.74 ± 0.06 for ATP and BzATP, respectively).

A comparison of antagonist pIC_{50} values at human and rat P2X$_7$ receptors indicated that compound-17 possessed higher potency as an antagonist of the human P2X$_7$ receptor, although the degree of selectivity depended upon the agonist concentration employed (Figure 2c).

The antagonist effects of compound-17 at the human P2X$_7$ receptor were slow in offset when studied at room temperature (Figure 2d), although the compound was not an irreversible antagonist as its effects did reverse when dissociation was examined at 37 °C (Figure 2d). PPADS exhibited a similar profile (Figure 2d).

Compound-17 is a negative allosteric regulator of the human and rat P2X$_7$ receptor

The slow offset of action of compound-17 precluded any interpretation of its mechanism of action from the studies in Figure 2 but did enable the compound to be studied in receptor protection studies with decavanadate as previously performed with KN62 and PPADS (Michel *et al*., 2006).

For the present receptor protection studies, a NaCl buffer was used and we first confirmed that decavanadate was able to produce a competitive protection of the P2X$_7$ receptor

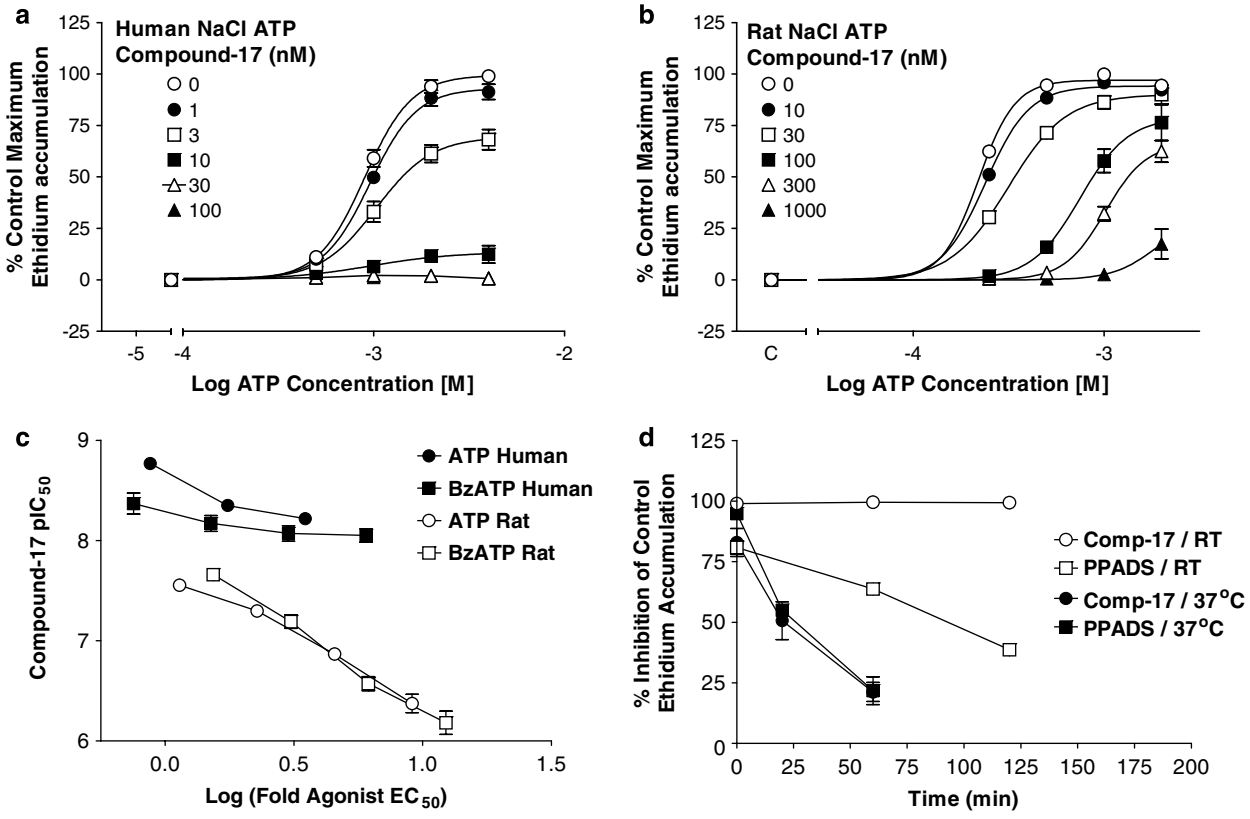

Figure 2 The effect of compound-17 on agonist-stimulated ethidium accumulation in HEK293 cells expressing rat and human recombinant P2X$_7$ receptors. (**a**) Concentration–effect curves to ATP in the presence of various concentrations of compound-17 measured in HEK293 cells expressing human recombinant P2X$_7$ receptors measured in a NaCl buffer. (**b**) Concentration–effect curves to ATP in the presence of various concentrations of compound-17 measured in HEK293 cells expressing rat recombinant P2X$_7$ receptors measured in a NaCl buffer. (**c**) pIC_{50} values for compound-17 measured in HEK293 cells expressing human or rat recombinant P2X$_7$ receptors. Studies were performed in NaCl buffer using BzATP or ATP as agonist. pIC_{50} values were calculated at each agonist concentration and are plotted as a function of the logarithm of the (agonist concentration/agonist EC_{50}) (Fold Agonist EC_{50}) for each condition. (**d**) Reversibility of compound-17 or PPADS effects at human P2X$_7$ receptors measured in NaCl buffer. Cells were equilibrated with antagonist for 40 min at room temperature or 37 °C and washed extensively. ATP-stimulated ethidium accumulation was subsequently measured after incubation at room temperature or 37 °C for the indicated times. The concentrations of compound-17 were 30 and 10 nM at room temperature and 37 °C, respectively, and for PPADS were 300 and 1000 nM at room temperature and 37 °C, respectively. These concentrations were not supra-maximal and were the lowest concentrations of antagonist that produced close to maximal inhibition of responses. The data are the mean ± s.e.mean of 3–4 experiments. BzATP, 2′- & 3′-*O*-(4benzoylbenzoyl) ATP; compound-17, *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}] dec-1-ylacetamide; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid.

from the slowly reversible antagonist effects of PPADS in this buffer. As can be seen (Figure 3a), when cells expressing human P2X$_7$ receptors were incubated with decavanadate and PPADS, extensively washed and then tested 15 min after the start of antagonist washout, the presence of decavanadate during the pre- and co-incubation period resulted in a concentration-dependent reduction in the subsequently measured pIC$_{50}$ for PPADS. A Schild plot of the data (Figure 3d) was linear (Slope $= 0.98 \pm 0.03$) and produced a pA$_2$ for decavanadate of 5.73 ± 0.08, similar to its pA$_2$ of 6.2 as an antagonist of BzATP-induced responses in NaCl buffer (Michel *et al.*, 2006).

In contrast, using the same procedure, decavanadate only partially protected the human P2X$_7$ receptor from the slowly reversible blockade of responses produced by compound-17 (Figures 3b and d). A maximal shift in the compound-17 inhibition curve was produced by 30 μM decavanadate and increasing the decavanadate concentration 10-fold to 300 μM did not produce any further shift in the inhibition curve, and this resulted in a curvi-linear Schild plot (Figure 3d), which illustrates the ceiling of effect for decavanadate against the inhibitory effects of compound-17. This was similar to results previously obtained with KN62 in sucrose buffer (Michel *et al.*, 2006) and which were also observed in NaCl buffer in this study (Figures 3c and d). Taken together, these data suggest that compound-17 is not a competitive P2X$_7$ receptor antagonist but is a negative allosteric modulator of the human P2X$_7$ receptor in functional studies.

In receptor protection studies on the rat P2X$_7$ receptor, decavanadate produced a competitive blockade of the slowly reversible antagonist effect of PPADS similar to that observed at the human P2X$_7$ receptor, but had no effect on the slowly reversible antagonist effect of compound-17 (data not shown).

GW791343 is a non-competitive antagonist of the human P2X$_7$ receptor

In cells expressing human P2X$_7$ receptors, GW791343 inhibited agonist-stimulated ethidium accumulation in both sucrose and NaCl buffer, although the effects differed slightly between the buffers (Figure 4). In NaCl buffer, GW791343 reduced the maximal response to both ATP and BzATP, but there was little effect on agonist potency except for a

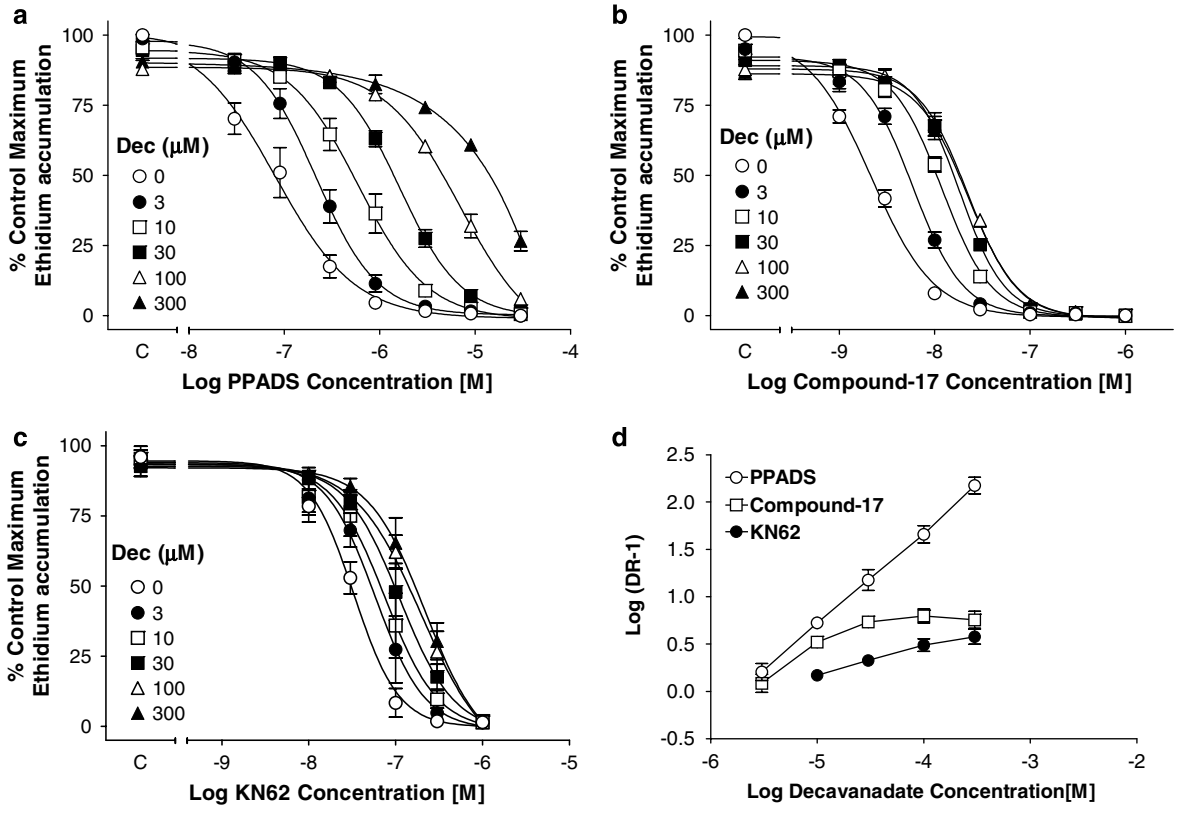

Figure 3 Receptor protection studies to investigate the interaction of decavanadate with PPADS, compound-17 or KN62 in HEK293 in cells expressing human P2X$_7$ receptors. Studies were performed in NaCl buffer. Cells were incubated with decavanadate for 10 min prior to addition of PPADS, compound-17 or KN62. After a further 30 min co-incubation, cells were washed extensively and 1 mM ATP-stimulated ethidium accumulation was measured 15 min after the start of antagonist washout. (**a**) Inhibition curves for PPADS in cells pre-incubated with the indicated concentrations of decavanadate. (**b**) Inhibition curves for compound-17 in cells pre-incubated with the indicated concentrations of decavanadate. (**c**) Inhibition curves for KN62 in cells pre-incubated with the indicated concentrations of decavanadate. (**d**) IC$_{50}$ values for each antagonist were measured in the absence and presence of the indicated concentrations of decavanadate and are presented in the form of a Schild Plot where dose ratio = IC$_{50}$ of compound in the presence of decavanadate/IC$_{50}$ of compound in the absence of decavanadate. The data are the mean ± s.e.mean of 3–4 experiments. Compound-17, *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide; KN62, 1-[*N*,*O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid.

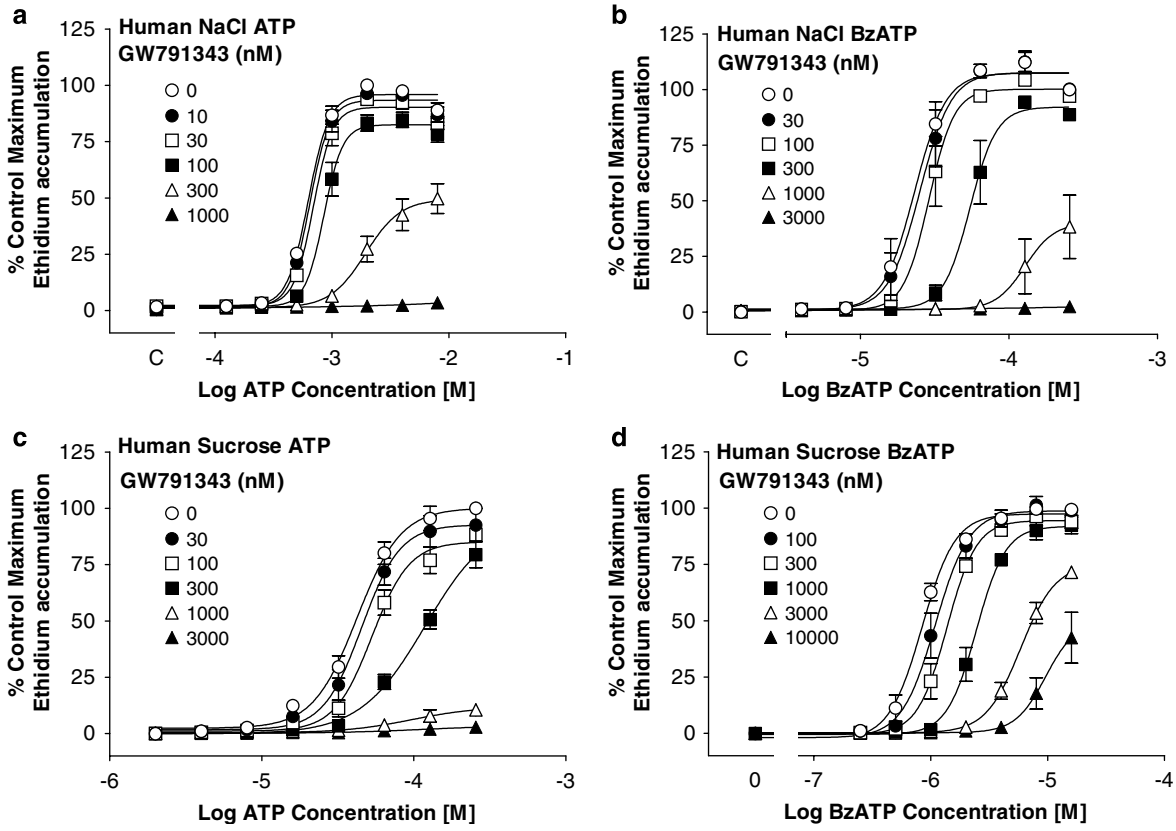

Figure 4 The effect of GW791343 on agonist-stimulated ethidium accumulation in HEK293 cells expressing human recombinant P2X$_7$ receptors. (**a**) Concentration–effect curves to ATP in the presence of various concentrations of GW791343 in HEK293 cells expressing human recombinant P2X$_7$ receptors measured in a NaCl buffer. (**b**) Concentration–effect curves to BzATP in the presence of various concentrations of GW791343 in HEK293 cells expressing human recombinant P2X$_7$ receptors measured in a NaCl buffer. (**c**) Concentration–effect curves to ATP in the presence of various concentrations of GW791343 in HEK293 cells expressing human recombinant P2X$_7$ receptors measured in a sucrose buffer. (**d**) Concentration–effect curves to BzATP in the presence of various concentrations of GW791343 measured in HEK293 cells expressing human recombinant P2X$_7$ receptors measured in a sucrose buffer. The data are the mean ± s.e.mean of 3–4 experiments. BzATP, 2′- & 3′-*O*-(4benzoylbenzoyl) ATP; GW791343, N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride.

decrease in the presence of 300–1000 nM GW791343 (Figures 4a and b). GW791343 also reduced maximal responses to ATP and BzATP in sucrose buffer, although this effect was more marked when using ATP as agonist (Figures 4c and d). In sucrose buffer, GW791343 produced a slight decrease in ATP potency at 300 nM (Figure 4c) and decreased BzATP potency at concentrations of 300 nM to 10 μM (Figure 4d).

For GW791343, there was an approximately linear relationship between pIC_{50} values and logarithm (agonist concentration/agonist EC_{50}) at the human P2X$_7$ receptor, with pIC_{50} values decreasing as agonist concentration was increased (Figure 5a). At low agonist concentrations, pIC_{50} values were similar in both buffers and with both agonists (pIC_{50} approximately 6.9–7.2). However, at higher agonist concentrations, the pIC_{50} values deviated depending on agonist and assay buffer. In general, pIC_{50} values were higher when using ATP as agonist than when using BzATP and were higher in NaCl than in sucrose buffer.

The data in Figure 4d were also analysed using a Schild plot, as GW791343 produced a quantifiable rightward shift in the BzATP concentration–effect curve. When BzATP EC_{50} values were calculated without constraining the parameters of the curve fits, the Schild plot possessed a slope of 0.82 ± 0.04, which was significantly less than unity (Figure 5b). When the BzATP EC_{50} values were calculated by constraining the maximal responses for each BzATP concentration–effect curve to 100%, then the slope of the Schild plot was 0.96 ± 0.04 and was not significantly different to unity (Figure 5b). When the studies shown in Figure 4d were repeated using a wider range of BzATP and GW791343 concentrations, it could be more clearly seen that GW791343 reduced the maximal response to BzATP and also that 10 and 30 μM GW791343 produced similar shifts in the BzATP concentration–effect curve (Figure 5c). Taken together, these data suggest that GW791343 is a non-competitive antagonist of the human P2X$_7$ receptor.

GW791343 is a negative allosteric modulator of the human P2X$_7$ receptor

The reversal of GW791343 effects at the human P2X$_7$ receptor was relatively slow with appreciable inhibition of responses after a 15 min washout (data not shown). This enabled receptor protection studies to be performed using decavanadate as for compound-17 and PPADS. However, when cells were pre-incubated with decavanadate and then

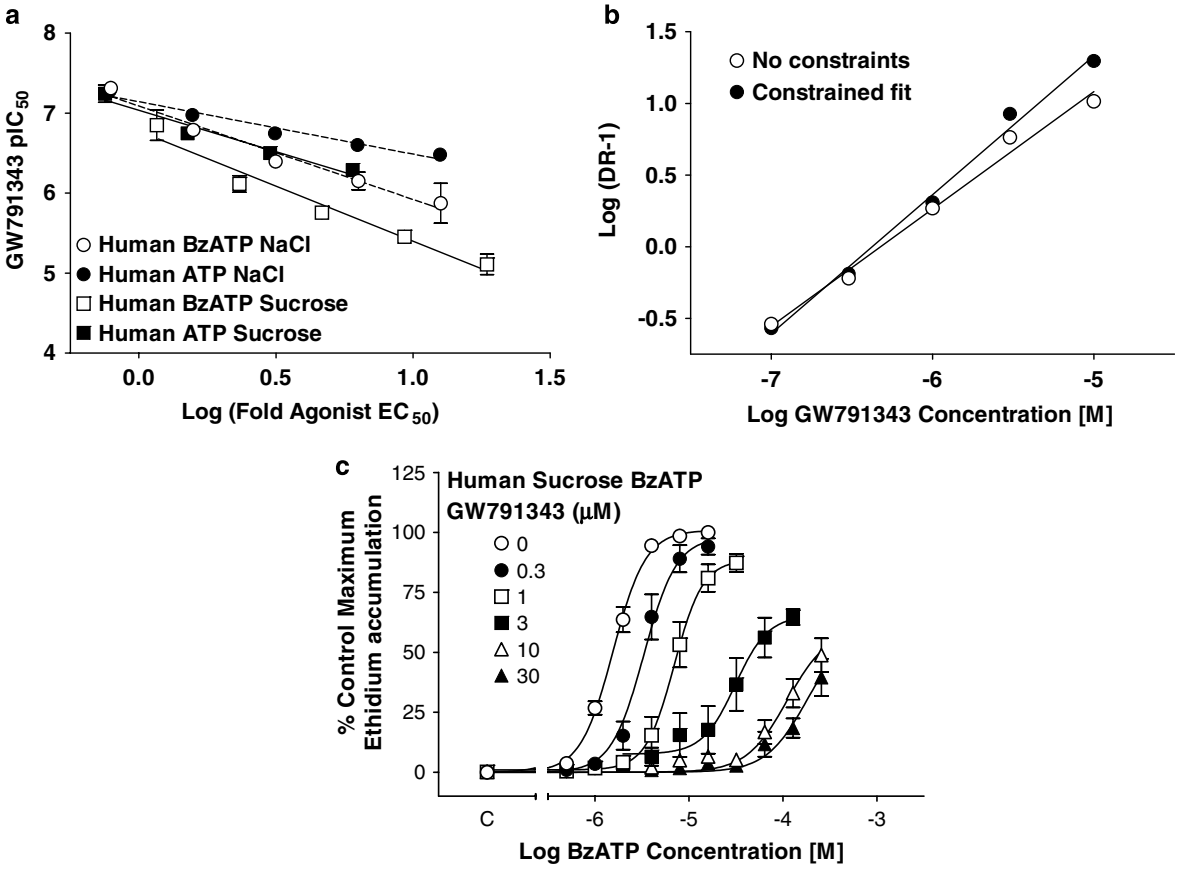

Figure 5 The effect of GW791343 on agonist-stimulated ethidium accumulation in HEK293 cells expressing human recombinant P2X$_7$ receptors. (**a**) pIC$_{50}$ values for GW791343 measured in HEK293 cells expressing human recombinant P2X$_7$ receptors. The pIC$_{50}$ values from the data in Figure 4 were calculated at the various agonist concentrations and are plotted as a function of the logarithm of the (agonist concentration/agonist EC$_{50}$) (Fold Agonist EC$_{50}$) for each condition. (**b**) Schild plot of the data from Figure 4d. The concentration–effect curves to BzATP were analysed with either no constraints or were constrained, so that the maximal response in all cases was 100%. (**c**) The effect of GW791343 on concentration–effect curves to an extended range of BzATP concentrations in HEK293 cells expressing human recombinant P2X$_7$ receptors. The study was performed in sucrose buffer. The data are the mean ± s.e.mean of 3–5 experiments. BzATP, 2′- & 3′-*O*-(4benzoylbenzoyl) ATP; GW791343, N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride.

co-incubated with both GW791343 and decavanadate, there was no effect of decavanadate on the persistent inhibitory effects of GW791343 measured 15 min after antagonist washout (Figure 6a).

Although the offset of the antagonist effect of GW791343 at the human P2X$_7$ receptor was slow, its dissociation rate was more rapid than either PPADS or compound-17, enabling the compound to be evaluated in the same manner as decavanadate for its ability to affect the persistent inhibitory effects of PPADS and compound-17. For these studies, cells were pre-incubated with buffer or GW791343 and then co-incubated with compound-17 or PPADS. The antagonists were removed by washing and ATP responses measured 45 min after beginning antagonist washout. The inhibitory effects of PPADS and compound-17 persisted for at least 45 min after washout (Figures 6b and c). The inhibitory effects of GW791343 were only slowly reversible (Figure 6c) but after 45 min had reversed sufficiently to determine if the presence of GW791343 during the pre-incubation period had affected the ability of compound-17 or PPADS to block responses. GW791343 produced appreciable blockade of the inhibitory effects of compound 17 (Figures 6c and d) but did not affect the inhibitory effects of PPADS (Figure 6b). Overall, these data suggest that GW791343 does not interact at the same site as PPADS or decavanadate, which is presumed to be the ATP binding site (Michel *et al.*, 2006), and so GW791343 appears to be a negative allosteric modulator of the human P2X$_7$ receptor.

GW791343 is a positive allosteric modulator of the rat P2X$_7$ receptor

In NaCl buffer, low concentrations (100–1000 nM) of GW791343 slightly reduced responses to some of the intermediate concentrations of BzATP (Figures 7a and b). However, this was not a very marked effect and there was some variability in the data. There was no clear inhibitory affect of GW791343 when using ATP as agonist (Figure 8a).

The predominant effect of GW791343 in NaCl was to enhance BzATP-induced ethidium accumulation at concentrations of 10 and 30 μM (Figures 7c and d). A more marked increase in agonist effect was observed when using ATP as agonist in NaCl buffer with GW791343 increasing the pEC$_{50}$ and maximal response to ATP at concentrations of 10 and 30 μM (Figure 8a). In sucrose buffer, GW791343 also increased responses when using ATP as agonist (Figure 8b),

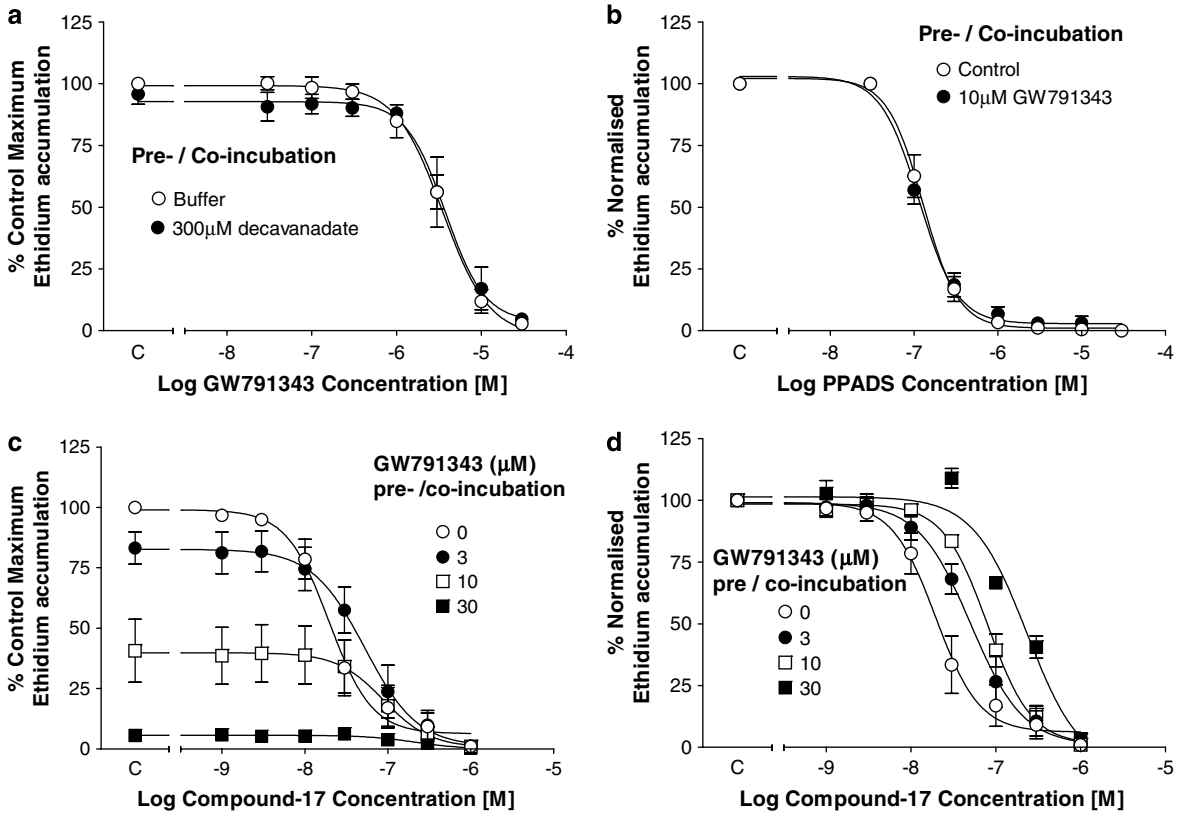

Figure 6 Receptor protection studies to investigate the interaction between antagonists in HEK293 cells expressing human recombinant P2X$_7$ receptors. Cells were pre-incubated with the indicated antagonist for 10 min prior to and then during a 30-min incubation with the antagonist indicated on the ordinate and then washed extensively before measuring 0.5 mM ATP-stimulated ethidium accumulation. (**a**) Inhibition curves for GW791343 in cells pre- and co-incubated with 300 μM decavanadate. Responses were measured 15 min after initiating the washout of antagonists. (**b**) Normalized inhibition curves for PPADS in cells pre- and co-incubated with 10 μM GW791343. Responses were measured 45 min after initiating the washout of antagonists. Note that the presence of GW791343 in the assay reduced the maximal response to ATP by approximately 60% as shown in (**c**) and so the data for each concentration of GW791343 were normalized to the response obtained to ATP in the absence of PPADS. (**c**) Inhibition curves for compound-17 in cells pre- and co-incubated with the indicated concentrations of GW791343. Responses were measured 45 min after initiating the washout of antagonists. (**d**) Normalized graph of the data from (**c**) to illustrate the effect of GW791343 on inhibition curves to compound-17. In this figure, the data for each concentration of GW791343 were normalized to the response obtained to ATP in the absence of compound-17. The data are the mean ± s.e.mean of 3–4 experiments. Compound-17, *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide; GW791343, N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid.

but variably affected responses to BzATP with effects observed in some but not all studies (data not shown). P2X$_7$ receptor-mediated ethidium accumulation is much more readily demonstrated in sucrose than in NaCl buffer, as agonist potency and rates of ethidium accumulation are much higher in sucrose than NaCl buffer. To investigate if the limited effects of GW791343 observed in sucrose buffer reflected the use of a system in which agonist effects are already maximal, we examined the effects of GW791343 in sucrose buffer at 4 °C, as agonist potency and rates of ethidium accumulation are much lower than at room temperature. When studied at 4 °C, GW791343 increased responses to both ATP (Figure 8c) and BzATP (data not shown). Furthermore, GW791343 increased agonist responses with just a 5 min antagonist pre-exposure (Figure 8d). Even with no pre-incubation, GW791343 increased responses to BzATP and ATP (data not shown).

In contrast to the persistent inhibitory effects of GW791343 at the human receptor, both the minor inhibitory effect and the potentiation of agonist effects by GW791343 at the rat P2X$_7$ receptor observed in studies in NaCl at room temperature were reversible when cells were washed extensively and tested 15 min after washout (data not shown). This enabled receptor protection studies to be performed to determine if GW791343 was interacting with the PPADS or compound-17 binding sites as both of these compounds produced a relatively persistent inhibition of responses at the rat P2X$_7$ receptor. The antagonist exposure in this study was performed in sucrose buffer, as the potency of PPADS is much higher in this buffer and so facilitated the studies. GW791343 had no inhibitory effect on the potency of PPADS (Figure 9a) but produced a decrease in the potency of compound-17 (Figure 9b).

To further evaluate the interaction of GW791343 with other P2X$_7$ receptor antagonists at the rat P2X$_7$ receptor, the effect of several antagonists were tested in the absence and presence of a fixed concentration of 10 μM GW791343. For these studies, the concentration–effect curve to ATP was determined in the presence of various concentrations of decavanadate, PPADS or compound-17 in cells pretreated

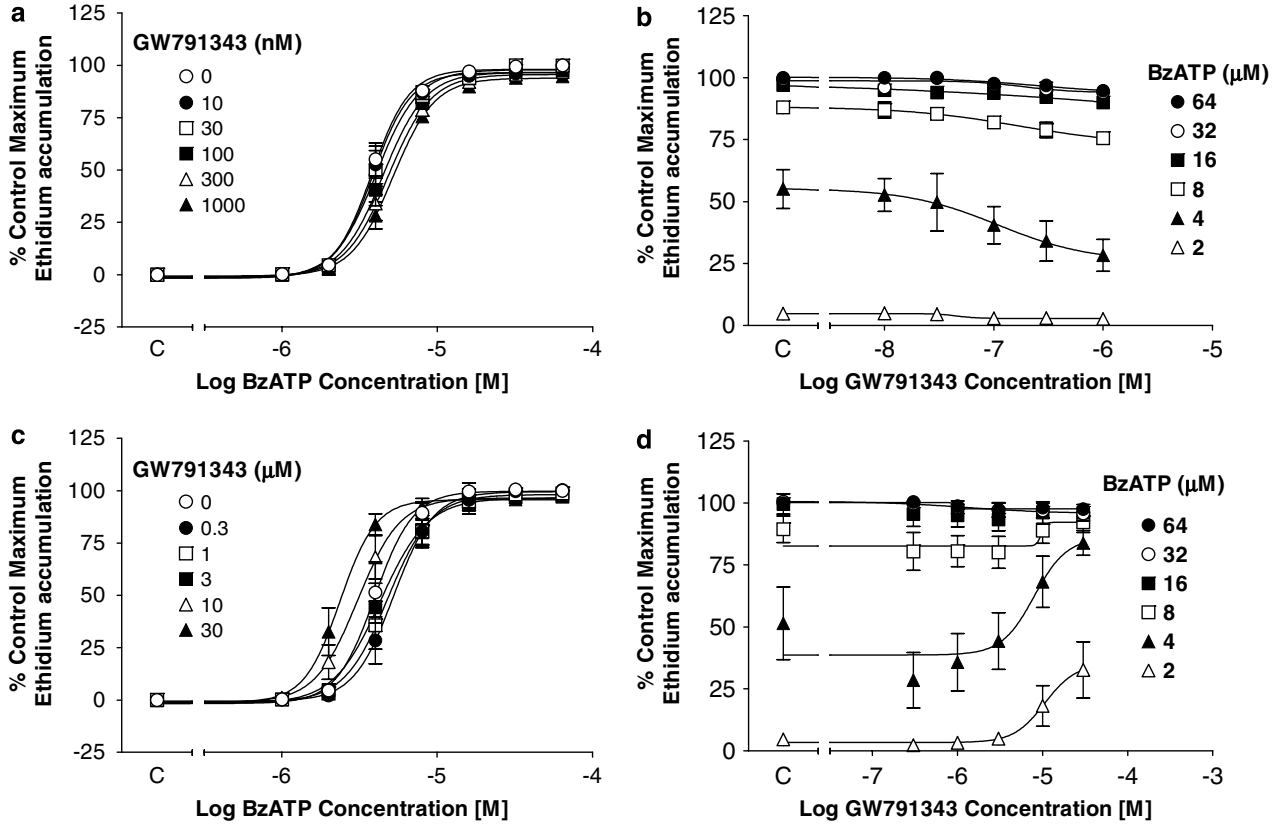

Figure 7 The effect of GW791343 on BzATP-stimulated ethidium accumulation in HEK293 cells expressing rat recombinant P2X$_7$ receptors. (**a**) Concentration–effect curves to BzATP in the presence of low concentrations of GW791343 in HEK293 cells expressing rat recombinant P2X$_7$ receptors measured in a NaCl buffer. (**b**) Transposition of the data in (**a**) to illustrate the effect of GW791343 on responses to BzATP. (**c**) Concentration–effect curves to BzATP in the presence of higher concentrations of GW791343 in HEK293 cells expressing rat recombinant P2X$_7$ receptors measured in a NaCl buffer. (**d**) Transposition of the data in (**c**) to illustrate the effect of GW791343 on responses to BzATP. The data are the mean ± s.e.mean of 3–4 experiments. BzATP, 2′- & 3′-*O*-(4benzoylbenzoyl) ATP; GW791343, N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride.

with buffer or 10 μM GW791343 for 10 min prior to addition of the other antagonists. The presence of 10 μM GW791343 had no effect on the pIC$_{50}$ values for decavanadate (Figure 10a) and did not affect the Schild plot of the data (Figure 10b). Similarly, the presence of 10 μM GW791343 did not affect the pIC$_{50}$ values for PPADS (Figure 10c) but reduced the pIC$_{50}$ values for compound-17 (Figure 10d).

Effect of GW791343 in radioligand binding studies

The studies above suggested that GW791343, similar to compound-17, may bind at a site on the P2X$_7$ receptor that is distinct from the ATP binding site. Consistent with this, GW791343 inhibited compound-17 binding to the human P2X$_7$ receptor with a pIC$_{50}$ of 6.14 ± 0.09 and appeared to be a competitive inhibitor as the Hill slope was close to unity (0.89 ± 0.06) and it inhibited binding to the same extent as compound-17 (Figure 11). This was in marked contrast to the effects of antagonists, such as oxATP, decavanadate or PPADS, which have little or no effect on binding (Michel *et al*., 2007). There appeared to be a slight potentiation of binding at 10 and 30 nM GW791343, although this was not significant (Figure 11). A potentiation of binding by low concentrations of compound-17 has been observed previously and may reflect positive cooperative interactions between subunits of the P2X$_7$ receptor complex (Michel *et al*., 2007).

Finally, GW791343 inhibited compound-17 binding to the rat P2X$_7$ receptor with a pIC$_{50}$ of 6.04 ± 0.10, which was similar to its potency at the human receptor. As in studies on the human receptor, the compound appeared to be a competitive inhibitor as it possessed a Hill slope of 0.99 ± 0.3, although it did not inhibit binding to the same extent as compound-17 over the concentration range examined. As with studies on the human P2X$_7$ receptor, low concentrations of GW791343 (10–30 nM) appeared to slightly potentiate radioligand binding but this was not significant (Figure 11).

The binding of [^{3}H]-compound-17 to human and rat P2X7 receptors is complex (Michel *et al*., 2007) and so the pIC$_{50}$ values were not converted to pK_i values. However, for reference, K_D values for [^{3}H]-compound-17 at the human and rat P2X$_7$ receptors were 1.4 and 19 nM, respectively. Comparison of the binding and functional data was also complex but the pIC$_{50}$ obtained in binding studies on the human receptor was almost 1 log unit lower than the pIC$_{50}$ of approximately 6.9–7.2 obtained using the lowest agonist concentrations in the functional studies.

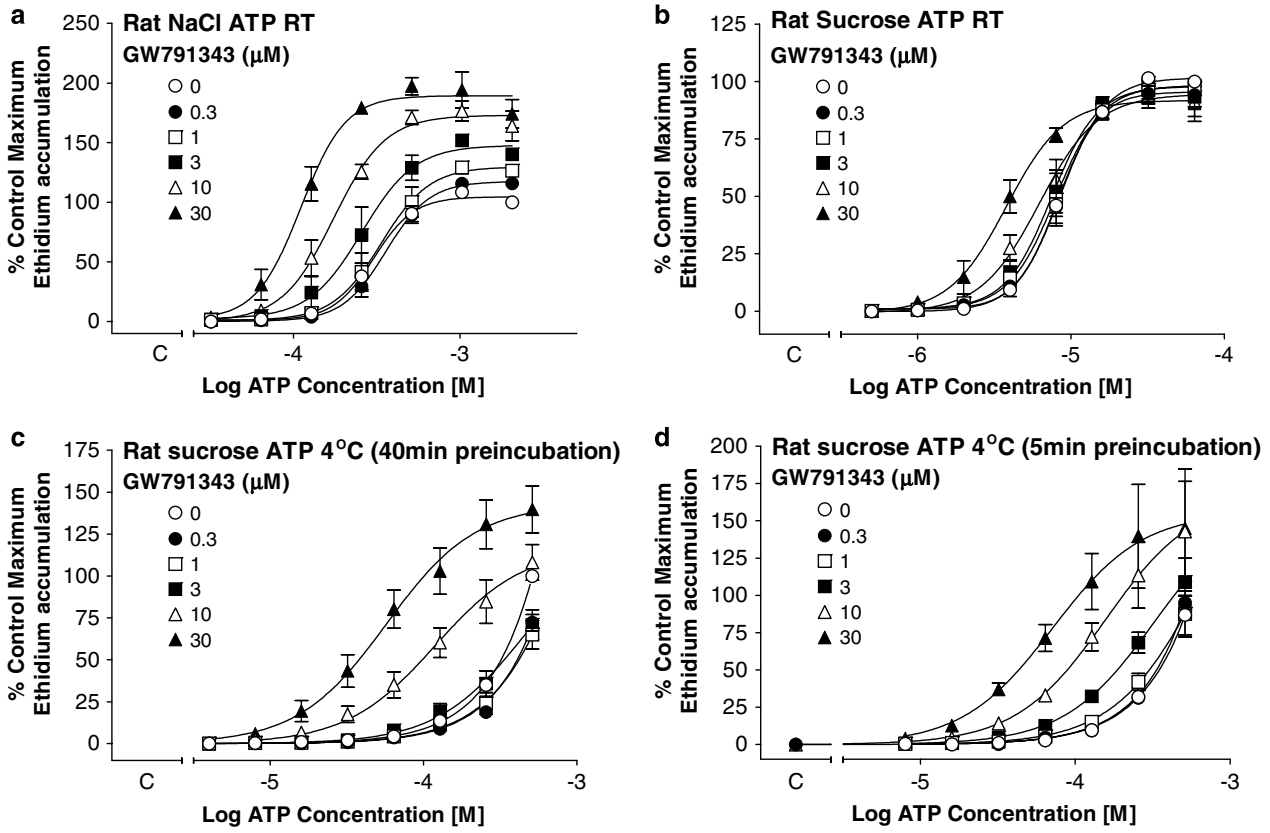

Figure 8 The effect of GW791343 on agonist-stimulated ethidium accumulation in HEK293 cells expressing rat recombinant P2X$_7$ receptors. (**a**) Concentration–effect curves to ATP in the presence of various concentrations of GW791343 in HEK293 cells expressing rat recombinant P2X$_7$ receptors measured in a NaCl buffer at room temperature. (**b**) Concentration–effect curves to ATP in the presence of various concentrations of GW791343 in HEK293 cells expressing rat recombinant P2X$_7$ receptors measured in a sucrose buffer at room temperature. (**c**) Concentration–effect curves to ATP in the presence of various concentrations of GW791343 in HEK293 cells expressing rat recombinant P2X$_7$ receptors measured in a sucrose buffer at 4 °C. (**d**) Concentration–effect curves to ATP in the presence of various concentrations of GW791343 in HEK293 cells expressing rat recombinant P2X$_7$ receptors measured in a sucrose buffer at 4 °C. The antagonist pre-incubation period was 5 min in (**d**) but 40 min in (**a**, **b**, **c**). The data are the mean ± s.e.mean of 3-4 experiments. GW791343, N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride.

Discussion

The main findings of this study were that two recently described P2X$_7$ receptor antagonists were negative allosteric modulators of the human P2X$_7$ receptor in functional studies and that GW791343 also produced a positive allosteric modulation of the rat P2X$_7$ receptor.

Previous studies have highlighted that the interaction of antagonists with the P2X$_7$ receptor is complex with considerable species differences in antagonist potency and many antagonists producing non-competitive or insurmountable effects (Hibell *et al*., 2001). Indeed, the original reports on KN62 demonstrated a complex antagonist effect that was inconsistent with it being a simple competitive antagonist (Humphreys *et al*., 1998; Wiley *et al*., 1998).

We have previously presented evidence from radioligand binding studies that compound-17 is an allosteric regulator of the human P2X$_7$ receptor (Michel *et al*., 2007). In this study, we found that compound-17 was a non-competitive antagonist of both human and rat P2X$_7$ receptors in functional studies as it reduced ATP and BzATP maximal effects with little effect on agonist potency. Although this action would be expected of a negative allosteric modulator, the very slow offset of action seen with this compound made interpretation of these data difficult, as a slowly reversible antagonist can also cause a suppression of maximal agonist responses. However, we were able to utilize the slow reversal of compound-17 to study its interaction with other P2X receptor antagonists to obtain supportive data for an allosteric mechanism of action. We have previously shown in functional studies that decavanadate produces a competitive antagonism of the slowly reversible effects of oxATP and PPADS in receptor protection studies and suggested that these three agents all bound at the ATP binding site of the P2X$_7$ receptor (Michel *et al*., 2006). We used a similar approach to evaluate the effects of compound-17 at the human P2X$_7$ receptor and found that decavanadate produced only a slight and saturating decrease in the potency of compound-17 in receptor protection studies. The partial interaction between compound-17 and decavanadate was of interest as there was evidence of a slight interaction between decavanadate and compound-17 in radioligand binding studies. This contrasted with the failure of PPADS or oxATP to affect compound-17 binding and may reflect limited cross talk between the decavanadate and compound-17 binding sites and suggest that decavanadate interacts with the P2X$_7$

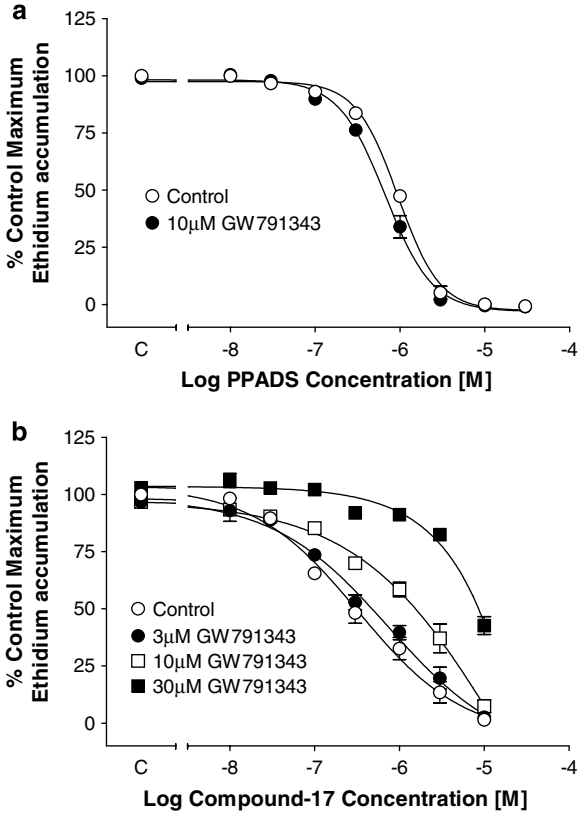

Figure 9 Receptor protection studies to investigate the interaction between antagonists in HEK293 cells expressing rat recombinant P2X$_7$ receptors. Cells were pre-incubated with GW791343 for 10 min prior to and during a 30-min incubation with the antagonist indicated on the ordinate. Cells were washed extensively before measuring 0.25 mM ATP-stimulated ethidium accumulation 15 min after initiating washout of the antagonists. (**a**) Inhibition curves for PPADS in cells pre- and co-incubated with buffer or 10 μM GW791343. (**b**) Inhibition curves for compound-17 in cells pre-incubated with buffer or the indicated concentrations of GW791343. The data are the mean ± s.e.mean of 3–4 experiments. Compound-17, *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide; GW791343, N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid.

receptor in a slightly different manner to either PPADS or oxATP. Compound-17 was also a non-competitive inhibitor of the rat P2X$_7$ receptor and receptor protection studies suggested that it was a negative allosteric modulator, as decavanadate had no effect on the slowly reversible antagonist effects of compound-17.

GW791343 was also a negative allosteric modulator of the human P2X$_7$ receptor. As with studies using compound-17, GW791343 predominantly produced a non-competitive antagonist effect at the human P2X$_7$ receptor. However, in sucrose buffer, GW791343 reduced BzATP potency and the effect could be construed as being competitive depending on the analysis method applied and the range of agonist concentrations employed. Thus, when a limited BzATP and GW791343 concentration range was utilized, and agonist concentration–effect curves were treated assuming no reduction in maximal effect, the Schild slope for the GW791343 antagonist effect was close to unity. However, such analysis was clearly flawed as further studies, using higher concentrations of BzATP, revealed that GW791343 produced a clear reduction in maximal effect and also revealed the evidence of a saturating effect of GW791343 on the concentration–effect curve to BzATP. These studies illustrate that caution must be exercised when studying the effects of P2X$_7$ receptor antagonists to identify their mechanism of action. Interestingly, compound 8h, an analogue of AZ11645373, was claimed to act as a competitive antagonist in ethidium accumulation studies (Alcaraz *et al.*, 2003). However, it was only examined over a limited concentration range, reduced maximal responses to BzATP and Schild analysis was not performed. Furthermore, AZ11645373 itself is a non-competitive antagonist (Stokes *et al.*, 2006).

Receptor protection studies with GW791343 at the human P2X$_7$ receptor provided further evidence that the compound was a negative allosteric modulator. Thus, decavanadate had little effect on the slowly reversible receptor blockade produced by GW791343. In addition, there was no effect of GW791343 on the persistent blockade of responses produced by PPADS but GW791343 could substantially reduce the potency of compound-17. Although these studies were hampered by the slow reversal kinetics of GW791343, additional binding experiments confirmed that GW791343 could directly interact with the compound-17 binding site on the human P2X$_7$ receptor and as this site is distinct from that recognized by ATP, it seems likely that GW791343 is a negative allosteric modulator of the human P2X$_7$ receptor (Michel *et al.*, 2007).

The effects of GW791343 at the rat P2X$_7$ receptor revealed a further level of complexity in antagonist effects at the P2X$_7$ receptor. Low concentrations of antagonist (10–100 nM) appeared to have little effect on agonist responses, although a detailed analysis revealed a modest inhibition of responses to intermediate concentrations of ATP or BzATP. The reason for this partial blockade is not known, although the pIC$_{50}$ value for blocking responses was close to that determined at the human P2X$_7$ receptor. Higher concentrations of GW791343 (3–30 μM) produced an increase in agonist potency or effect. This action could be due to some additional nonspecific effect of GW791343 or could reflect a positive allosteric enhancement of receptor function.

A nonspecific or non-P2X$_7$ receptor-mediated effect seems unlikely for several reasons. First, the effect of GW791343 was reversible within 15 min and so does not appear to be a persistent nonspecific change in agonist effect. Second, the ability of GW791343 to increase agonist responses was also observed at 4 °C and after short exposures, suggesting that nonspecific metabolic effects are unlikely to underlie this action. Third, concentrations of GW791343 that enhanced receptor function (3–30 μM) were able to protect against the slowly reversible antagonist effects of compound-17 but had no effect on the blockade produced by PPADS. Similarly, when a high concentration of GW791343 (10 μM) that increased agonist responses was co-incubated with other antagonists, it had no effect on the potency of decavanadate or PPADS but decreased the potency of compound-17. Such differential effects seem unlikely to arise from a nonspecific

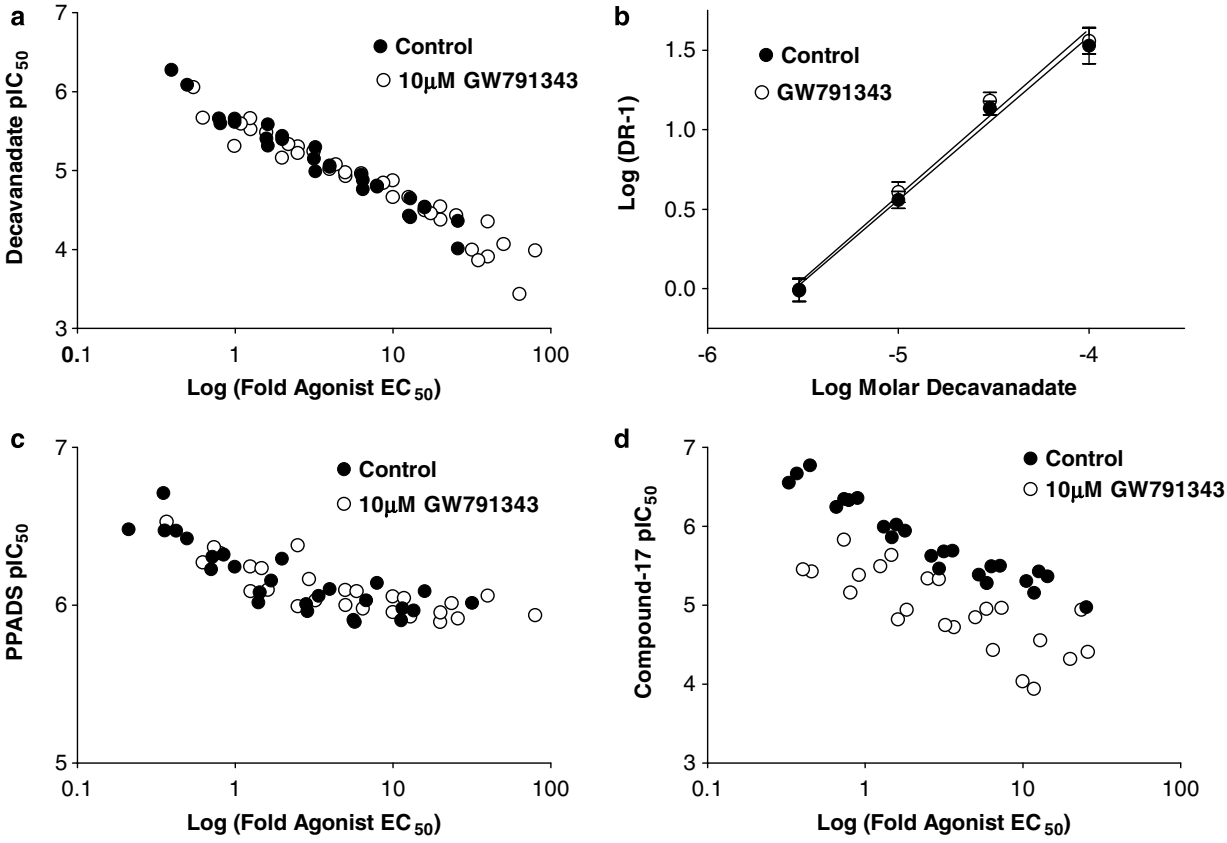

Figure 10 The effect of GW791343 on antagonist potency for blocking ATP-stimulated ethidium accumulation in HEK293 cells expressing rat recombinant P2X$_7$ receptors. The cells were first exposed to buffer or 10 μM GW791343 for 10 min before addition of the second antagonist. After a 40-min incubation period, an ATP concentration–effect curve was constructed and the antagonist pIC$_{50}$ was calculated at each concentration of ATP as illustrated in Figure 7. (**a**, **c**, **d**) The pIC$_{50}$ values are plotted as a function of the logarithm of the (agonist concentration/agonist EC$_{50}$) (Fold Agonist EC$_{50}$) for data obtained in the absence of GW791343 (filled symbols) or in the presence of 10 μM GW791343 (open symbols). The data are the individual data points from 3 to 4 separate experiments. (**b**) The Schild plot of the data for decavanadate. The data are the mean ± s.e.mean of three experiments. GW791343, N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]glycinamide dihydrochloride.

effect of GW791343. Finally, recent site-directed mutagenesis studies have revealed that the potentiation of agonist effect observed at the rat P2X$_7$ receptor can be transferred between rat and human receptors by exchange of a single amino-acid residue in the extracellular domain of the receptor (AD Michel, unpublished data). Overall, these various observations suggest that GW791343 enhanced receptor function through a positive allosteric interaction with the receptor.

The mechanism by which GW791343 enhances P2X$_7$ receptor function is not known, although it seems plausible that it increases receptor function by increasing agonist efficacy or increasing the rate of formation or opening of the large pore through which ethidium enters cells. Certainly, the extent of potentiation of responses by GW791343 was agonist dependent and was more pronounced against the partial agonist ATP than against the full agonist BzATP. Furthermore, the potentiation of responses produced by GW791343 was affected by assay buffer and temperature, and was much greater when receptor function was constrained either by ionic conditions (NaCl) or by temperature. These various observations suggest that binding of GW791343 facilitates channel opening following agonist activation rather than affecting agonist binding.

The finding of a compound that potentiates P2X$_7$ receptor-mediated responses is not that novel, as previous studies have also identified compounds that potentiate responses at P2X$_7$ receptors. These include tenidap at the mouse P2X$_7$ receptor (Sanz *et al*., 1998), bromoenol lactone at the rat P2X$_7$ receptor (Chaib *et al*., 2000) and hexamethylene amiloride under certain conditions at the mouse P2X$_7$ receptor (Hibell *et al*., 2001). Furthermore, polymyxin B (Ferrari *et al*., 2004, 2007), diverse lipids (Michel and Fonfria, 2007) and arachidonic acid (Alloisio *et al*., 2006) also increase P2X$_7$ responses, as do agents that affect tyrosine phosphorylation (Adinolfi *et al*., 2003). However, the mechanism of action for most of these compounds has not been examined in detail, although the effects of lipids do not show any clear structure–activity relationship and exhibit no species selectivity (Michel and Fonfria, 2007), suggesting that lipid effects are unlikely to arise through binding to a specific site on the P2X$_7$ receptor.

Overall, these data provide an increasingly complex view of antagonist effects at the P2X$_7$ receptor. It seems likely that

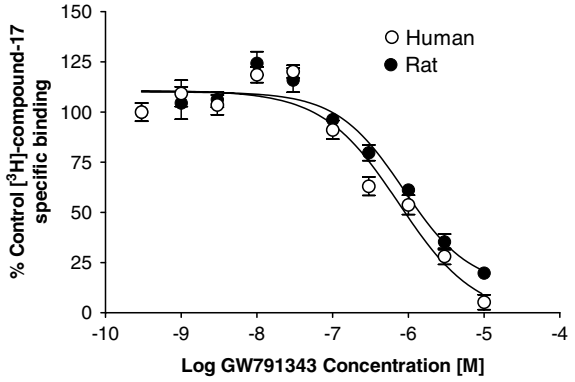

Figure 11 The effect of GW791343 on binding of [^{3}H]-compound-17 to human or rat P2X$_7$ receptors. Studies were performed using membranes prepared from HEK293 cells stably expressing the human or rat P2X$_7$ receptor. The radioligand concentration was 2 nM and specific binding was defined with 10 μM compound-17. The data are the mean ± s.e.mean of three experiments. Total binding and nonspecific binding values were 1589 ± 91 and 488 ± 54 d.p.m., respectively, in studies on the human receptor. Total binding and nonspecific binding values were 1832 ± 290 and 402 ± 63 d.p.m., respectively, in studies on the rat receptor. Compound-17, *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide; GW791343, N^2-(3,4-difluorophenyl)-N^1-[2-methyl-5-(1-piperazinylmethyl)phenyl]-glycinamide dihydrochloride.

both compound-17 and GW791343 bind to the P2X$_7$ receptor at a site that is distinct from the ATP binding site. Although it seems plausible that both compounds interact at the same site, further studies would be required to confirm this. More importantly, these data provide some insight into how some of the novel P2X$_7$ antagonists may affect receptor function. The limited, or lack of, interaction with the ATP binding site suggests that neither compound-17 nor GW791343 affected ATP binding directly, and it is more likely that these compounds affected the ability of ATP binding to produce a functional effect. In the case of low concentrations of GW791343 at the human and rat P2X$_7$ receptors, this resulted in an inhibition of receptor function, whereas higher concentrations of GW791343 enhanced the ability of agonists to activate the rat P2X$_7$ receptor.

An increasing number of P2X$_7$ receptor antagonists are being described in the literature (Donnelly-Roberts and Jarvis, 2007) and many of these are highly selective for human over rat P2X$_7$ receptors. It remains to be seen whether these compounds are competitive antagonists or allosteric regulators of the P2X$_7$ receptor and whether their species selectivity is a reflection of differences in binding affinity or differences in their efficacy to affect channel function.

Conflict of interest

The authors are employed by GlaxoSmithKline.

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