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Effects of genetic variation in the *P2RX7* gene on pharmacodynamics of a $P2X_7$ receptor antagonist: a prospective genotyping approach

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Analysis of allelic variation in large populations has identified numerous single nucleotide polymorphisms (SNPs) in the *P2RX7* gene. *In vitro* transfection has demonstrated SNP-dependent alterations (gain or loss) of $P2X_7$ receptor function, as measured by ATP-induced ethidium uptake and interleukin-1β production.

WHAT THIS STUDY ADDS

- We provide definitive evidence of SNP-dependent alteration in $P2X_7$ receptor pharmacodynamics to a specific antagonist (GSK1370319A) in a small sample of prospectively genotyped subjects. These effects on drug response underline the importance of genetic stratification in drug development of $P2X_7$ receptor antagonists.

AIMS

To investigate the effects of two single nucleotide polymorphisms (SNPs) in the human $P2X_7$ receptor gene (*P2RX7*) – 1068G>A (A348T) and 1513A>C (E496A) – on $P2X_7$ receptor function, using a specific receptor antagonist (GSK1370319A) and prospective genetic stratification.

METHODS

Lipopolysaccharide- and ATP-stimulated interleukin-1β production was determined in the presence or absence of GSK1370319A in blood culture from 32 prospectively genotyped subjects.

RESULTS

There was approximately 6.7-fold difference ($P < 0.0001$) in IC_{50} for inhibition of ATP-stimulated interleukin-1β release by GSK1370319A between individuals with the homozygous gain- (1068A) and loss-of-function (1513C) genotypes (expressing the 348T, 496E and 348A, 496A alleles, respectively).

CONCLUSIONS

Leukocyte $P2X_7$ receptors had significantly altered pharmacodynamic responses to a specific antagonist (GSK1370319A), directly related to SNP genotype.

Introduction

There is considerable interest in use of human bioresource databases, based on ethically approved collections of DNA, for prospective recruit-by-genotype approaches to experimental medicine and clinical trials designed to investigate the functional consequences of allelic variation in a gene of interest [1–4]. We have employed this approach to investigate the genetically based functional variation in the $P2X_7$ receptor.

The $P2X_7$ receptor is a purinergic nonselective cation channel receptor gated by extracellular ATP [5, 6]. The receptor-associated ion channel appears to change permeability to cations during prolonged agonist exposure either by channel dilatation or by coupling to the pannexin hemichannel [7]. $P2X_7$ receptor signalling is important in inflammasome formation, leading to cleavage-induced maturation of caspase-1 and subsequent release of interleukin-1β (IL-1β) and interleukin-18 following stimulation with endogenous receptor agonist, ATP [8, 9]. The $P2X_7$ receptor is expressed on many cell types, including monocytes/macrophages, erythrocytes, osteoblasts, lung mast cells, fibroblasts and certain neuronal tissues. Involvement of the $P2X_7$ receptor in IL-1β processing and release has led to considerable interest in development of selective $P2X_7$ receptor antagonists for the treatment of inflammatory disorders and pain [10] and has motivated therapeutic development of selective receptor antagonists, including GSK1370319A (*N*-[(2,4-dichlorophenyl)methyl]-1-methyl-5-oxo-L-prolinamide).

The *P2RX7* gene located on chromosome 12q24 [symbol approved by the HUGO Gene Nomenclature Committee (HGNC)] [5] encodes the P2X receptor subtype 7 ($P2X_7$) and is highly polymorphic, with 29 nonsynonymous amino-acid-altering single nucleotide polymorphisms (SNPs) reported [11]. Some *P2RX7* SNPs have been associated with mood disorders, susceptibility to infections and bone diseases in humans [12]. Previous studies have reported effects of multiple *P2RX7* SNPs on receptor expression and functional response to ATP [13], but SNP-dependent pharmacogenetic effects remain relatively poorly characterized.

The aim of this study was to investigate the effects on $P2X_7$ receptor function of two SNPs in the human $P2X_7$ receptor gene (*P2RX7*); 1068G>A (A348T) and 1513A>C (E496A). These two SNPs have been described as having the greatest opposite functional effects in terms of induced IL-1β production [14, 15] and downstream inflammatory responses. The study was conducted using a specific receptor antagonist (GSK1370319A) and prospective genetic stratification from a human bioresource.

Methods

To investigate the effects of genetic variation in the *P2RX7* gene on functional response to GSK1370319A, we focused on two SNPs resulting in the amino-acid changes Glu496Ala (E496A; 1513A>C, rs3751143) and Ala348Thr (A348T; 1068G>A, rs1718119). The SNP 1513A>C is believed to confer loss of function and 1068G>A gain of function of the $P2X_7$ receptor, and they were selected for study on this basis. For 1513A>C, a full three-way cross was performed, comparing the homozygous loss of function (CC) with both heterozygous (AC) and dominant homozygous wild-type (AA), against a constant background of the homozygous wild-type (GG) of the 1068 variant. For 1068G>A, a two-way cross was performed, comparing homozygous gain of function (AA) with homozygous wild-type (GG), against a constant background of the homozygous (AA) 1513 variant (Table 1). Four other SNPs with putative functional effects at the $P2X_7$ receptor (rs1653624, rs17525809, rs2230911 and rs28360447) were all wild-type matched.

Four groups of eight genetically stratified, age- and sex-matched healthy European Caucasian volunteers were recruited from a bioresource maintained at GSK's Clinical Unit in Cambridge, UK. The bioresource has DNA extracted from peripheral venous blood samples from volunteers who had given consent to be genotyped at any gene of interest, and recontacted if their genetic status makes them potentially eligible to participate in further studies. Four hundred and seventy-nine volunteers were genotyped for the listed SNPs of interest in the *P2RX7* gene by a competitive allele specific PCR SNP genotyping system using Fluorescence Resonance Energy Transfer quencher cassette oligonucleotides (KBioscience, Hoddesdon, Hertfordshire, UK).

Ethical approval (LREC ref: 08/H0302/100, UK) and written informed consent were obtained prior to conducting this study. The $P2X_7$ receptor function was assessed in the presence or absence of GSK1370319A in a lipopolysaccharide (LPS)–ATP stimulation, IL-1β release whole-blood assay. Blood was collected from $P2X_7$-genotyped volunteers into citrate buffer (15% v/v) and incubated for 80 min with or without 1 μg ml^{-1} LPS (*Escherichia coli* serotype 7136; Sigma, St Louis, MO, USA) at 37°C. Thereafter, 50 μl aliquots of blood were added to 96-well plates together with 30 μl PBS or antagonist and the plates incubated for 40 min at 37°C, before adding 20 μl of ATP solution (final concentration 0.125–8.0 mM). The plates were mixed by shaking and incubated at 37°C for 30 min. Reactions were terminated by the addition of ice-cold HEPES-buffered RPMI-1640 (Invitrogen, Life Technologies Ltd., Paisley, UK). The 96-well plates were centrifuged at 300*g* for 10 min at 4°C, and the resulting supernatants were harvested, diluted appropriately, and their IL-1β content was determined using a human IL-1β ELISA (DLB50; R&D systems, Europe Ltd., Abingdon, UK) according to the manufacturer's instructions. In titration experiments, 4.0 mM ATP was found to provide optimal stimulation and was used in the reported experiments.

Results

Dose–response relationships were constructed for ATP-induced IL-1β release (absolute amount in picograms per

Table 1

Significance of functional single nucleotide polymorphisms (SNPs) in the *P2RX7* gene on $P2X_7$ receptor sensitivity to agonist (ATP) and to antagonism by GSK1370319A

Group	E_{max} [pg ml^{-1} (95% confidence interval)]	g (slope) (95% confidence interval)	EC_{50} [mM (95%CI)]	*P* value (EC_{50})	Mean age (years ± SD; *n* = 8, 5 male, 3 female)	*P2RX7* Genotype ($P2X_7R$ amino acids)	Population frequency (%; *n* = 479)
			Response to ATP				
1	180 (140, 230)	2.0 (1.3, 2.8)	3.8 (2.2, 6.5)		45.5 ± 15.0	1513CC–1068GG (496AA–348AA)	2.7
2	150 (100, 190)	2.5 (1.7, 3.3)	1.1 (0.6, 1.9)	$P < 0.05$	46.3 ± 13.6	1513CA–1068GG (496EA–348AA)	30.5
3	130 (90, 180)	2.9 (2.1, 3.7)	0.9 (0.5, 1.5)	NS	48.3 ± 12.8	1513AA–1068GG (496EE–348AA)	66.8
4	160 (120, 200)	2.9 (2.1, 3.7)	0.5 (0.3, 0.9)		43.1 ± 10.9	1513AA–1068AA (496EE–348TT)	13.8

Group	E_{max} [pg ml^{-1} (95% confidence interval)]	g (slope) (95% confidence interval)	IC_{50} [mM (95% confidence interval)]	IC_{50} statistical analysis, *P* value (fold difference)
			Response to GSK1370319A	
1 (496AA) (LoF)	90 (150, 130)	1.2 (0.4, 2.0)	0.27 (0.18, 0.42)	
2 (496EA) (LoF_h)	130 (90, 170)	2.0 (1.2, 2.8)	0.66 (0.44, 1.00)	$P = 0.0023$ (2.44)
3 (496EE) (WT)	110 (70, 150)	3.2 (2.4, 4.0)	0.67 (0.46, 0.99)	NS
4 (348TT) (GoF)	170 (130, 120)	2.4 (1.6, 3.2)	1.82 (1.21, 2.72)	$P = 0.0033$ (2.71)
				$P < 0.0001$ (6.74)

Estimated EC_{50} values for ATP and IC_{50} values for GSK1370319A (with 95% confidence intervals) for each of the genetically defined dose–response curves. Model parameters were estimated using a three-parameter logistic function. ANOVA with pairwise contrasts was used to calculate the *t* statistic (28 degrees of freedom) from which probability (*P*) was determined. Statistical significance was shown between loss- and gain-of-function homozygotes (Lof and GoF), LoF heterozygotes (LoF_h) and wild-type (WT).

millilitre) and percentage inhibition of IL-1β release as a function of the concentration of GSK1370319A for each of the genetically stratified groups (Figure 1), and the drug concentration causing 50% inhibition of IL-1β production at 4 mM ATP was estimated (IC_{50}; Table 1). The maximal levels of IL-1β produced did not vary significantly between the groups, but the ATP concentration required was significantly higher for the loss-of-function homozygotes (1513CC and 1068GG). Conversely, for ATP, the amount of IL-1β produced was significantly higher for the gain-of-function homozygotes (1513AA and 1068AA). Loss-of-function genotype was associated with a leftward shift of the dose–response curve, whereas gain-of-function was associated with a rightward shift.

Discussion

The $P2X_7$ receptor is implicated in a number of disorders (reviewed in [16]), including inflammatory components of atherosclerosis, temporal lobe epilepsy, Parkinson's disease and Alzheimer's disease, diabetes and diabetic retinopathy, polycystic kidney disease, neurodegeneration and immunomodulation, and in neuropathic pain (where disruption of signalling via $P2X_7$ abolishes pain). Formation of the innate inflammasome via $P2X_7$ receptor engagement is a central process in initiating an inflammatory cascade, resulting in caspase-1-dependent release of IL-1β and interleukin-18 [9]. This is not only important in antibacterial immune function, but it also provides the basis of a physiologically and mechanistically relevant pharmacodynamic assay. We have demonstrated that the extent to which activation of such an immune response occurs is highly dependent on certain SNPs within the *P2RX7* gene. Leukocyte $P2X_7$ receptors had significantly altered functional capacity directly related to genotype. Thus, individuals homozygous for 1513C (expressing Ala496) had loss of function in terms of IL-1β production (i.e. increased sensitivity to GSK1370319A), whilst individuals homozygous for 1068A (expressing Thr348) showed gain of function, when tested against a controlled genetic background. In an HEK-293 transfection model, gain of function has been demonstrated using Thr348 [13], but to a lesser degree than we observe in *ex vivo* whole-blood assays.

The genetically determined molecular basis of the altered function of the $P2X_7$ receptor is currently not well understood, but recent structure–function studies suggest that alterations in ATP and/or antagonist binding sites and

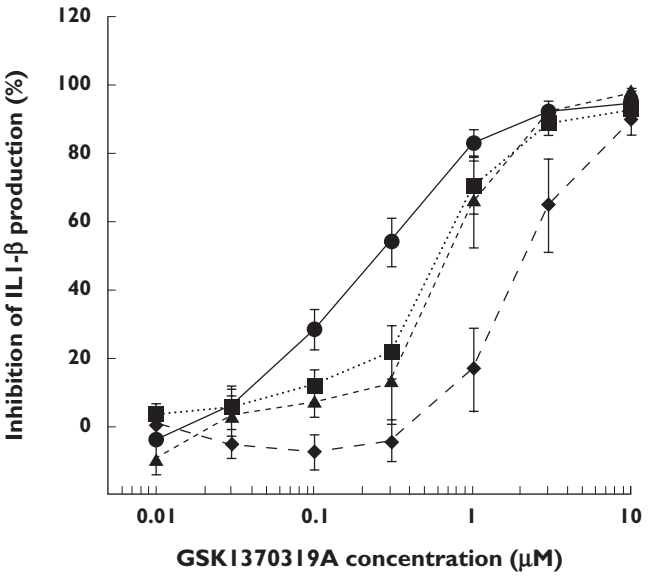

Figure 1

Influence of polymorphisms on pharmacodynamic effects of an antagonist at the $P2X_7$ receptor. Dose–response curves for interleukin-1β (IL-1β) production following lipopolysaccharide/ATP stimulation of whole blood, drawn from four genetically stratified groups of healthy volunteers ($n = 8$ per group), incubated in the presence of variable concentrations of the $P2X_7$ antagonist, GSK1370319A. Each data point represents the mean ± SEM of percentage IL-1β inhibition (derived from biological assay triplicates). 496AA-348AA (Loss of function hom) (–●–); 496EA-348AA (Loss of function het) (··■··); 496EE-348AA (Wild type) (- ▲-); 496EE-348TT (Gain of function hom) (–◆–)

allosteric modulation of ATP binding are all important determinants of receptor function [16]. Our results suggest that the SNP in *P2RX7* resulting in E496A substitution caused significant alteration in sensitivity to ATP, whereas both E496A and A348T together resulted in an almost sevenfold difference in response to the specific antagonist GSK1370319A between the extremes of gain or loss of receptor function.

$P2X_7$ receptor antagonists have been used in a number of clinical settings [16], and understanding their relative potency and pharmacogenetics is critical in establishing safe and effective criteria for dose setting. We provide evidence that the problem of lack of statistical power in addressing equivocal interpretation of genotype–phenotype association due to insufficiently stringent criteria [17], particularly in *ex vivo* and clinical pharmacogenetic studies, can be addressed by prospective genotyping. Use of DNA biobanks is increasing, e.g. http://www.cambridgebioresource.org.uk, and more widespread adoption of such platforms in clinical development may lead to greater personalization of medicines or genetic stratification of therapeutics in the longer term. As this study also demonstrates, if the drug target is accessible from a peripheral blood sample, then prospective stratification supports estimation of human pharmacogenetic effects on dose–response parameters of new compounds, before administration to man.

Competing Interests

S.M.McH., S.R., B.D., A.K., A.M.P., J.C.R., S.R.M., S.W., C.J.C. and E.T.B. are, or have been, employees of GSK and may hold shares in the company. The other authors have no competing interests to declare.

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