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Getting personal: Endogenous adenosine receptor signaling in lymphoblastoid cell lines

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ABSTRACT

Genetic differences between individuals that affect drug action form a challenge in drug therapy. Many drugs target G protein-coupled receptors (GPCRs), and a number of receptor variants have been noted to impact drug efficacy. This, however, has never been addressed in a systematic way, and, hence, we studied real-life genetic variation of receptor function in personalized cell lines. As a showcase we studied adenosine A_{2A} receptor (A_{2A}R) signaling in lymphoblastoid cell lines (LCLs) derived from a family of four from the Netherlands Twin Register (NTR), using a non-invasive label-free cellular assay. The potency of a partial agonist differed significantly for one individual. Genotype comparison revealed differences in two intron SNPs including rs2236624, which has been associated with caffeine-induced sleep disorders. While further validation is needed to confirm genotype-specific effects, this set-up clearly demonstrated that LCLs are a suitable model system to study genetic influences on A_{2A}R response in particular and GPCR responses in general.

1. Introduction

The majority of therapeutic drug targets to date are within the G protein-coupled receptor (GPCR) superfamily, a class of membrane-bound proteins [1,2]. As such, GPCRs have been widely and intensively studied for the development of new therapeutics. Among the most well-studied members of this group are the adenosine receptors, a family comprising of 4 different subtypes: A_1, A_{2A}, A_{2B} and A_3 [3]. The various subtypes have been implied in a broad range of diseases and (patho)-physiological conditions, such as a variety of respiratory and inflammatory conditions for the A_{2A} or cardiovascular disorders for the A_1 [4]. Likewise, a wide variety of compounds selectively activating, inhibiting or modulating these receptors are available to date [3,4]. Some of these have even been or are currently in clinical trials [3,4]. Adenosine itself has been long approved for treatment of supraventricular tachycardia [3] and one A_{2A}R antagonist, istradefylline, has made it to the market as adjuvant drug therapy for Parkinson's disease in Japan [5].

In the emerging era of personalized medicine, it is paramount for drug development to better understand the effects of a drug

Abbreviations: hA_1AR, human adenosine A_1 receptor; $hA_{2A}AR$, $A_{2A}R$, human adenosine A_{2A} receptor; $hA_{2B}AR$, human adenosine A_{2B} receptor; hA_3AR, human adenosine A_3 receptor; ADORA2A, adenosine A_{2A} receptor gene; AR, adenosine receptor; cAMP, cyclic adenosine 5′-monophosphate; CB2, cannabinoid receptor 2; CI, Cell Index; Δ CI, Δ cell index or delta cell index; DMSO, dimethylsulfoxide; FCS, fetal calf serum; EBV, Epstein–Barr Virus; EC_{50}, half maximal effective concentration; EC_{80}, 80% maximal effective concentration; GPCR, G protein-coupled receptor; IC_{50}, half maximal inhibitory concentration; K_i, equilibrium inhibition constant; LCL, lymphoblastoid cell line; NTR, Netherlands Twin Register; PBS, phosphate buffered saline; RTCA, real-time cell analyzer; SNP, single nucleotide polymorphism.

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not only in the overall population, but in the individual patient as well [6]. Genetic differences between individuals can affect drug action. Accordingly, several examples linking GPCR polymorphisms to diseases and drug response variation already exist [7–11], which include many commonly targeted GPCRs [11] such as purinergic [12,13], cannabinoid [9,10] and adenosine [14–16] receptors. Specifically for the adenosine A_{2A} receptor, Single Nucleotide Polymorphisms (SNPs) have been associated with for instance anxiety [17,18], caffeine intake [17], or vigilance and sleep [14]. Despite these examples of statistical association of genotype and condition, as well as extensive mutational characterization of the adenosine receptors, little is known about the direct functional effect of receptor polymorphisms or SNPs. Therefore, an ideal set-up would be to use patient-derived material as a model system to study the influence of polymorphisms on receptor response.

Lymphoblastoid cell lines (LCLs) are one of the most common choices for storing a person's genetic material [19,20] and can be used to study GPCR function as has been shown recently [21]. For example, Morag, Kirchheiner [22] studied the influence of a few GPCR antagonists on LCL growth. We recently published an even more direct way of measuring receptor function, including agonist and antagonist concentration-effect curves [21]. Using a newly developed, highly sensitive label-free cellular assay technology [21,23,24], we have shown that it is possible to measure an individual's GPCR response in LCLs using the cannabinoid receptor 2 as example [21]. In such label-free assays one can monitor drug effects on an intact cell in real-time, rather than being limited to a static, one-molecule-detection of ligand binding or second messenger accumulation, as is usually employed in GPCR and adenosine receptor research [3,23–25].

In the current study we have applied this label-free methodology to assess personal adenosine A_{2A} receptor function in LCLs. We characterized A_{2A}R signaling with various types of ligands including endogenous and synthetic agonists, partial agonist and antagonists, among which istradefylline. To allow conclusions about genotype in relation to receptor response, we compared responses between the individuals of a family of four from the Netherlands Twin Register [26]. This family consisted of two genetically unrelated individuals, the parents, as well as their children, which were monozygotic twins. Confirming the comparability of monozygotic twins' responses is one of the standard ways to control for genotype-unrelated effects, and thereby assess a system's suitability for genetic studies [26,27].

2. Material and methods

2.1. Chemicals and reagents

Fibronectin from bovine plasma, Roswell Park Memorial Institute (RPMI) 1640 cell culture medium (25 mM HEPES and $NaHCO_3$), NECA, adenosine and ATP were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). CGS21680, ZM241385 and CCPA were purchased from Abcam Biochemicals (Cambridge, United Kingdom), Cl-IB-MECA from Tocris Bioscience (Bristol, United Kingdom) and istradefylline from Axon Medchem (Groningen, The Netherlands). BAY60-6583 was synthesized in-house. LUF compounds were synthesized as described by van Tilburg, von Frijtag Drabbe Kunzel [28] for LUF5448 and LUF5631, van Tilburg, Gremmen [29] for LUF5549 and LUF5550 and Beukers, Chang [30] for LUF5834. All other chemicals and reagents were of analytical grade and obtained from commercial sources, unless stated otherwise.

2.2. Lymphoblastoid cell line generation

The lymphoblastoid cell lines (LCLs) were generated from participants of the Netherlands Twin Register (NTR, VU, Amsterdam, The Netherlands) [26]. The LCLs were generated by the Rutgers Institute (Department of Genetics, Piscataway, NJ, USA) using a standard transformation protocol [26], according to a previous publication [21]. Peripheral B-lymphocytes were transformed with Epstein–Barr Virus (EBV) by treatment with filtered medium from a Marmoset cell line in the presence of phytohemaglutinin (PHA) during the first week of culture [19,20,31]. Cultures were maintained for 8–12 weeks to expand the EBV transformed lymphocytes and subsequently cryopreserved.

2.3. Cell culture

LCLs from a family of four individuals, two parents (genetically unrelated; called Parent 1 and Parent 2) and their monozygotic twin (genetically equal; called Twin 1 and Twin 2), were used for the experiments presented in this manuscript. According to culture conditions described in a previous publication [21], cryopreserved cells were thawed and resuscitated. LCLs were grown as suspension cells in RPMI 1640 (25 mM HEPES and $NaHCO_3$) supplemented with 15% FCS, 50 mg/ml streptomycin, 50 IU/ml penicillin, at 37 °C and 5% CO_2 and were subcultured twice a week at a ratio of 1:5 on 10 cm ø plates. LCLs were disposed of after maximally 120 days in culture.

2.4. qPCR

RNA from LCLs was isolated using RNeasy Mini kit (QIAGEN, Venlo, The Netherlands). The RNA was treated with optional on column DNase digestion using DNase I (QIAGEN) and converted to cDNA using Superscript III (Invitrogen, Bleiswijk, The Netherlands). cDNA was run on custom designed 384 well qPCR plates from Lonza (Copenhagen, DK), in accordance with a previous publication [32]. These plates contained primers for 379 GPCRs as well as 3 RAMPs, together with primers for Rn18s and genomic DNA (Primers are listed in Engelstoft et al. [32]). Genomic DNA sample was used as calibrator and the relative copy number was calculated as stipulated previously [32].

2.5. Label-free whole-cell analysis (xCELLigence RTCA system)

2.5.1. Instrumentation principle

Cellular assays were performed using the xCELLigence RTCA system [23] in accordance with previously published protocols [21,33]. Briefly, the real-time cell analyzer (RTCA) measures the whole-cell responses using a detection system based on electrical impedance. Impedance is generated through cell attachment to gold electrodes embedded on the bottom of the microelectronic E-plates, which changes the local ionic environment at the electrode-solution interface. Relative changes in impedance (Z) are recorded in real-time and summarized in the so-called Cell Index (CI), a dimensionless parameter. The CI at any given time point is defined as $(Z_i - Z_0)\ \Omega/15\ \Omega$, where Z_i is the impedance at each individual time point. Z_0 represents the baseline impedance in the absence of cells, which is measured prior to the start of the experiment and defined as 0. As cells adhere to the electrodes, impedance and the corresponding CI increase proportionally. Changes in cell number and degree of adhesion, as well as cellular viability and morphology are directly reflected in the impedance profile [23,24]. Such cellular parameters are also affected upon activation of GPCR signaling, thereby allowing real-time monitoring of cellular signaling events [23].

2.5.2. General protocol

xCELLigence assays on LCLs were performed in accordance with a previously published protocol [21] with minor modifications. Briefly, cells were seeded onto fibronectin-coated E-plates (10 μg/ml) at 80,000 cells/well. All cell counts were performed using Trypan blue staining and a BioRad TC10 automated cell counter. E-plates were placed into the recording station situated in a 37 °C and 5% CO_2 incubator and impedance was measured overnight. After 18 h, cells were stimulated by a GPCR ligand or vehicle control in 5 μl, unless specified otherwise. As compound solubility required addition of dimethylsulfoxide (DMSO), the final DMSO concentration upon ligand or vehicle addition was kept at 0.25% DMSO for all wells and assays.

For agonist screening purposes, cells were stimulated with agonist concentrations corresponding to $100 \times K_i$ value for their respective receptors [4]. For the partial agonist screen, all partial agonists as well as reference agonist CGS21680 were tested at a concentration of 1 μM.

Agonist concentration–response curves were generated by stimulating cells with increasing concentrations of the respective agonist. For antagonist assays, cells were pre-incubated for 30 min with 5 μl of vehicle control or the respective antagonist at increasing concentrations. Subsequently, cells were challenged with a submaximal agonist concentration of CGS21680 that was equal to the agonist's EC_{80} value (100 nM) or vehicle control. Generally, compound dilutions for concentration–response curves were generated using the digital TECAN dispenser (Tecan Group, Männedorf, Switzerland).

2.6. Data analysis

Data were analyzed as stipulated in the previous protocol [21]. Briefly, experimental data were obtained with RTCA Software 1.2 (Roche Applied Science). Ligand responses were normalized to Δ cell index (Δ CI) and exported to GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) for further analysis. Vehicle control was subtracted as baseline to correct for any agonist-independent effects. Peak responses were defined as highest Δ CI (Max ΔCI) observed within 60 min after compound addition. When stipulated, area under the curve (AUC ΔCI) within those 60 min was used as an additional parameter to analyze response height. Peak values and experimental Δ CI traces were used for construction of bar graphs or concentration–effect curves by nonlinear regression and calculation of IC_{50}, EC_{50} and EC_{80} values. K_i values for antagonists were calculated using the Cheng–Prusoff equation [34] using the concentration of the agonist (CGS21680, 100 nM) and EC_{50} value corresponding to each cell line.

All values obtained are means of at least three independent experiments performed in duplicate, unless stated otherwise. Statistical significance was determined by comparison of the means of multiple data sets by a one-way ANOVA, followed by Tukey's post hoc test for comparison of all columns or a Dunnett's post hoc test when comparing to control or reference compound.

2.7. Processing of SNPs and genetic data

SNP data for the four individuals were obtained from the Genomes of the Netherlands consortium (http://www.nlgenome.nl/) of which the Netherlands Twin Register is part of and analyzed in-house using PLINK, an open-source whole genome association analysis toolset [35,36].

3. Results

3.1. Label-free assays enable detection of adenosine A_{2A} receptor signaling in LCLs

The standard applications of label-free technologies such as the xCELLigence for GPCRs generally require adherent cell systems [23,24,33]. LCLs are suspension cells for which we have developed a protocol in which fibronectin coating of the plate wells allowed the LCLs to adhere [21]. With this approach we confirmed the presence or absence of adenosine receptor subtypes by testing selective agonists using LCLs of one individual as example (parent 2). These agonists included selective ligands such as CCPA for hA_1AR, CGS21680 for $hA_{2A}AR$, BAY60-6583 for $hA_{2B}AR$, Cl-IB-MECA for hA_3AR and the unselective agonist NECA. To ensure full receptor occupancy, we tested the compounds at concentrations corresponding to $100 \times K_i$ value for their respective receptor [4]. An example of resulting xCELLigence traces is provided in Fig. 1.

Addition of the compounds induced changes in cellular morphology that were recorded in real-time. Typically, agonist addition resulted in an immediate increase of impedance to a peak level which gradually decreased toward a plateau within 30 min. Responses were normalized to the subtype unselective agonist NECA for reference. Overall, $hA_{2A}AR$ selective agonist CGS21680 gave the highest response which was close to the response to NECA itself, as would be expected from the expression data which

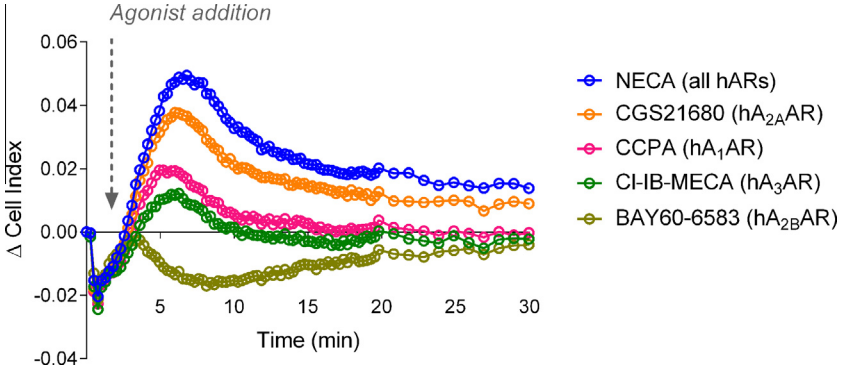

Fig. 1. Adenosine receptor agonist screen. Cells were seeded onto fibronectin-coated wells (10 μg/ml) at 80,000 cells/well. After 18 h of growth, cells were stimulated with AR ligands at concentrations corresponding to $100 \times K_i$ value for their respective receptor [4]. CCPA (83 nM) for hA_1AR at, CGS21680 (2.7 μM) for $hA_{2A}AR$, BAY60-6583 (36 μM) for $hA_{2B}AR$ and Cl-IB-MECA (140 nM) for hA_3AR were compared to the unselective hAR agonist NECA. Unselective NECA was tested a concentration of 14 μM which is at least $100 \times K_i$ or more for all ARs. Representative xCELLigence traces of a baseline-corrected ligand response are given of one individual (parent 2), where time point 0 represents the time of ligand addition. Data are from at least three separate experiments performed in duplicate. Statistical differences of compound responses to NECA were analyzed using a one-way ANOVA with Dunnett's post hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Response heights normalized to NECA (100 ± 1%) were for CCPA: 35 ± 5%***, CGS21680: 67 ± 11%, BAY60-6583: −40 ± 14%**** and Cl-IB-MECA: 39 ± 10%**.

showed that $hA_{2A}AR$ is the highest expressed in LCLs while the other three subtypes were expressed to a much lower extent (receptor expression family mean ± SEM was $hA_{2A}AR$ 21.87 ± 5.41, hA_1AR 1.35 ± 0.85, $hA_{2B}AR$ 0.88 ± 0.35 and hA_3AR 0.40 ± 0.37, calculated using a normalization factor derived from all genes expressed above genomic DNA levels, in accordance with a previous publication by Engelstoft et al. [32]). In fact, CGS21680 was the only compound whose response did not differ significantly from NECA. CCPA, the hA_1AR agonist, and hA_3AR agonist CL-IB-MECA gave small responses (Fig. 1), most likely caused by a modest activation of $A_{2A}R$ at the concentrations used. While all other agonists displayed a positive impedance response, BAY60-6583 gave a small positive peak followed by a decline to a negative impedance plateau. Responses to all agonists from LCLs of a second individual, parent 1, gave comparable results in terms of conclusion of receptor subtype presence (*data not shown*).

3.2. $A_{2A}R$ agonist and antagonist responses compare well between monozygotic twins and their parents

Subsequently, the label-free methodology was applied to compare adenosine A_{2A} receptor related responses between LCLs derived from the four different individuals. We characterized $A_{2A}R$ signaling with various types of ligands, including the endogenous agonist adenosine as well as the synthetic non-selective agonist NECA and $A_{2A}R$ selective agonist CGS21680. All three agonists displayed a similar shape of and height in response, both within each cell line and between individuals. An example of such a response is depicted in Fig. 2A. The corresponding concentration–response curves are shown in Fig. 2B-D. In a similar manner, concentration-inhibition curves for $A_{2A}R$ antagonists ZM241385 and istradefylline were obtained. An example trace of such an agonist/antagonist experiment is in Fig. 3A while the concentration-inhibition curves are represented in Fig. 3B and C. All pEC_{50} and pIC_{50} values for the LCLs of the four individuals are summarized in Table 1. From the pIC_{50} values we derived affinity (pK_i) values for both antagonists using the Cheng–Prusoff equation. For ZM241385 these values were 8.29 ± 0.11, 9.00 ± 0.09, 8.88 ± 0.05 and 9.08 ± 0.08 for parent 1, parent 2, twins 1 and 2. pK_i values for istradefylline were 6.84 ± 0.17, 7.67 ± 0.07, 7.47 ± 0.05 and 7.88 ± 0.07, respectively.

3.3. $A_{2A}R$ partial agonist responses are measurable in LCLs

Finally, we tested a number of partial agonists synthesized in house, all at a concentration of 1 μM. An example trace of partial agonist and CGS21680 responses for LCLs of one individual is in Fig. 4A. Some partial agonists (LUF5549 and LUF5631) displayed high efficacy in this cell system, as their maximum response almost equaled that of the full agonist CGS21680 with 112 ± 9% and 95 ± 11%, respectively. LUF5448 and LUF5550 however showed robust partial agonistic behavior of 64 ± 5% and 40 ± 5% of maximal efficacy (Fig. 4A). Partial agonist LUF5834 gave a different shape of response, which was marked by a negative peak followed by a negative impedance plateau, which differed significantly from any other partial agonist or reference full agonist CGS21680 (Fig. 4A). Its maximum response was therefore at −17 ± 8%.

3.4. A_{2A} partial agonist response differs between individuals

In order to further demonstrate the sensitivity of the label-free technology combined with LCLs, one partial agonist was chosen to obtain concentration–response curves. LUF5448 was chosen as a suitable candidate as it displayed robust partial agonistic behavior with a maximum effect of approx. 50% of the reference full agonist CGS21680. An example xCELLigence trace is provided in Fig. 4B while the corresponding concentration–response curves for the four individuals are summarized in Fig. 4C. Interestingly, while three of the individuals gave very comparable curves and pEC_{50} values, one of the parents differed significantly from all (Table 1), with an approx. tenfold higher potency (pEC_{50} value). LUF5448

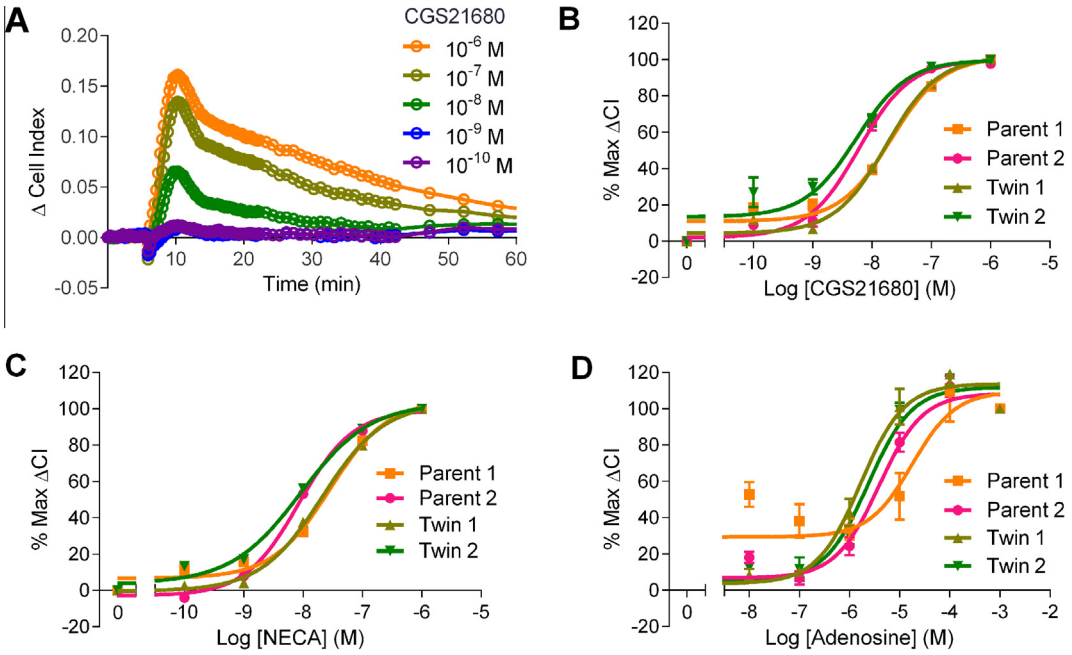

Fig. 2. Characterization of full agonist responses in LCLs from a family of four from the NTR. The family consists of two genetically unrelated individuals, parent 1 and 2, and their children which are a monozygotic twin (twin 1 and twin 2). Cell lines were stimulated with endogenous agonist adenosine [1 nM–100 μM], synthetic agonists NECA or CGS21680 [100 pM–1 μM] 18 h after seeding (80,000 cells/well). Representative example of a baseline-corrected concentration-dependent CGS21680 response (A). Concentration–response curves for CGS21680 (B), NECA (C) and adenosine (D) were derived from peak Δ cell index (Δ CI) within 60 min after agonist addition (see Methods). Data in B–D represent the means ± SEM of at least three separate experiments performed in duplicate.

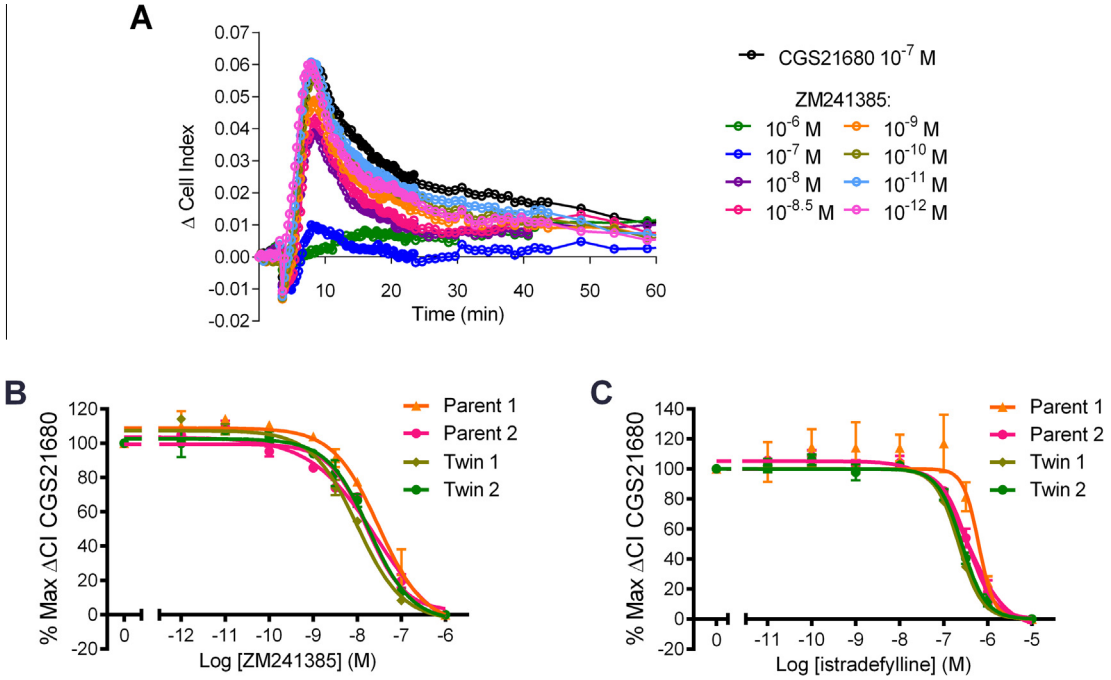

Fig. 3. Characterization of $A_{2A}R$ antagonist responses in LCLs from a family of four from the NTR. The family consists of two genetically unrelated individuals, parent 1 and 2, and their children which are a monozygotic twin (twin 1 and twin 2). For antagonist curves, cell lines were pre-incubated for 30 min with increasing concentrations of ZM241385 [10 pM–10 μM] before stimulation with CGS21680 [EC_{80}: 100 nM] 18 h after seeding (80,000 cells/well). Representative example of a baseline-corrected concentration-dependent response to ZM241385 (A). Concentration–response curves for ZM241385 (B) and istradefylline (C) were derived from peak Δ cell index (Δ CI) values within 60 min after agonist addition. Data in B and C represent the means ± SEM of at least three separate experiments performed in duplicate.

Table 1
Overview of the pEC_{50} and pIC_{50} values of Adenosine, NECA, CGS21680, ZM241385, istradefylline and LUF5448 for the tested individuals' LCLs. Data represent the means ± SEM of at least three separate experiments performed in duplicate. Statistical analysis was performed with a one-way ANOVA with Tukey's post hoc test. Asterisks highlight statistical differences to the other individuals (P1 = parent 1; P2 = parent 2; T1 = Twin 1; T2 = twin 2). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Ligand	pEC_{50}/pIC_{50} (M)				
	Literature	Parent 1	Parent 2	Twin 1	Twin 2
Adenosine (Endogenous agonist)	6.51 [44]	6.34 ± 0.32	5.59 ± 0.13	5.94 ± 0.12	5.82 ± 0.16
NECA (Full non-selective agonist)	8.60 ± 0.02 [33] 7.59 ± 0.33 [47]	7.54 ± 0.07 **T2; ***P2	8.06 ± 0.04 **T1; ***P1	7.68 ± 0.04 *T2; **P2	7.92 ± 0.07 *T1; **P1
CGS21680 (Full selective agonist)	8.42 ± 0.05 [33] 8.18 ± 0.36 [39]	7.61 ± 0.14	8.20 ± 0.09	7.76 ± 0.08	8.30 ± 0.42
ZM241385 (Antagonist/inverse agonist)	8.80[a] [4]	7.52 ± 0.15	7.55 ± 0.17	8.01 ± 0.07	7.73 ± 0.10
Istradefylline (Antagonist/inverse agonist)	7.92[a] [48]	6.21 ± 0.09 *P2; **T1; ***T2	6.45 ± 0.04 *P1	6.66 ± 0.02 **P1	6.59 ± 0.03 ***P1
LUF5448 (Partial agonist)	8.62 ± 0.19 [33]	8.69 ± 0.11 **all	7.60 ± 0.11 **P1	7.69 ± 0.08 **P1	7.76 ± 0.26 **P1

[a] K_i.

behaved as a typical partial agonist on all cell lines with a % Max ΔCI of CGS21680 of 66 ± 7% for parent 1, 70 ± 2% for parent 2 and 67 ± 2% and 54 ± 4% for twins 1 and 2, respectively.

3.5. Genotype differences between the four individuals

SNP data for the four individuals were obtained from the Genomes of the Netherlands consortium and analyzed in-house using PLINK, an open-source whole genome association analysis toolset [35,36]. SNPs within the boundaries of the ADORA2A gene as defined by human genome overview GRCh37 were selected. Based on GRCh37 and dbSNP information (http://www.ncbi.nlm.nih.gov/SNP/), SNPs were further annotated according to position (e.g., intron, exon) and SNP type (e.g., missense, synonymous). The genotype differences of the individuals used in this study are summarized in Table 2.

4. Discussion

It is well established that label-free technologies can be applied to investigate GPCR signaling in heterologous as well primary adherent cell systems [23,24,33]. For instance, the xCELLigence system has successfully been applied to study ligand effects on the cannabinoid receptor 2 (CB2) and the metabotropic glutamate receptor 1 (mGluR1) using recombinant Chinese hamster ovary (CHO) cells [37]. Similarly, $A_{2A}R$ signaling has been studied in

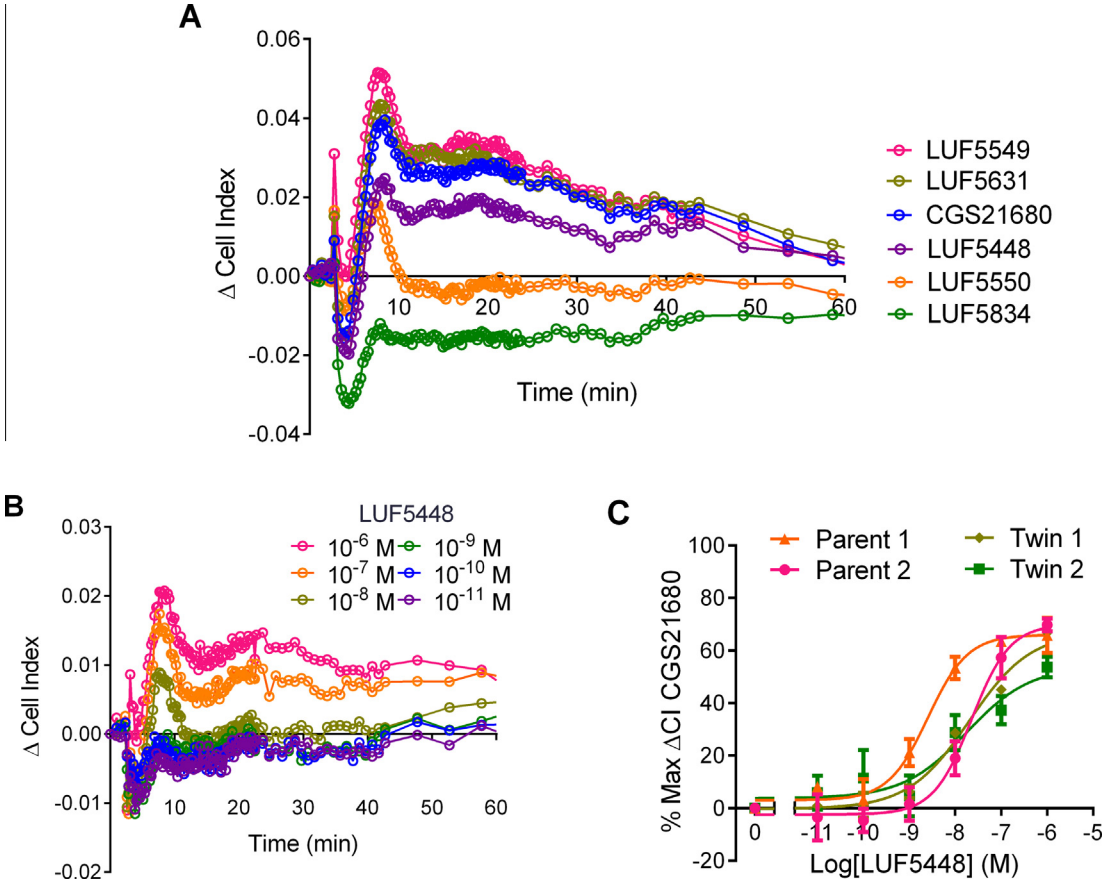

Fig. 4. $A_{2A}R$ partial agonist responses in LCLs. Cells were stimulated 18 h after seeding (80,000 cells/well) with $A_{2A}R$ partial agonists as well as full agonist CGS21680 [all at 1 μM] for reference. (A) Representative example of a baseline-corrected response is given from one individual (parent 2). Maximal responses of partial agonists compared to CGS21680 were 112 ± 9% for LUF5549, 95 ± 11% for LUF5631, 64 ± 5%* for LUF5448, 40 ± 5%*** for LUF5550 and −17 ± 8%**** for LUF5834. Statistical differences from CGS21680 were assessed with a one-way ANOVA with Dunnett's post hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (B) Representative example of a baseline-corrected response of $A_{2A}R$ partial agonist LUF5448 [10 pM–1 μM] for one individual (parent 2). (C) Concentration–response curves for all four individuals were derived from peak Δ cell index (Δ CI) within 60 min after agonist addition, normalized to CGS21680 as reference. Data are representative examples or means ± SEM of at least three separate experiments performed in duplicate.

Table 2
SNP genotype differences within the ADORA2A gene between the four individuals included in this study. The heterozygous differences of parent 1 to the other individuals are underlined. Data were obtained from the NTR and analyzed in-house.

SNP	Genotype		
	Parent 1	Parent 2	Twins
rs34999116	**T C**	C C	C C
rs5751869	A G	A G	G G
rs5760410	A G	A G	G G
rs5751870	T G	T G	G G
rs5751871	T G	T G	G G
rs9624470	A G	A G	G G
rs11704959	A C	C C	A C
rs2298383	T C	T C	C C
rs3761420	A G	A G	G G
rs3761422	C T	C T	T T
rs2267076	C T	C T	T T
rs11704811	T C	C C	T C
rs17650801	G G	A G	G G
rs4822489	G T	G T	T T
rs2236624	**C C**	T C	T C
rs5751876	C T	C T	T T

HEK293hA_{2A}AR cells using selective agonists as well as partial agonists [33]. While only such recombinant cell lines have been used to study $A_{2A}R$ signaling using label-free technology, $A_{2A}R$ function has been studied in some endogenous cell types using other, more traditional assays [38–40]. However, studying a person's $A_{2A}R$ response using a personal cell line such as the LCLs has not been possible up until now, and is therefore a translational step further toward precision medicine.

Applicability of this label-free technology to LCLs is, however, not entirely straightforward due to their suspension cell nature. Nonetheless, adherence levels after coating of the wells with fibronectin were sufficient to allow monitoring of receptor responses, as was demonstrated by testing adenosine receptor ligands (Fig. 1). Activation of $A_{2A}R$ receptors led to a typical increase in impedance often seen for GPCR ligands in LCLs. For instance, P2Y receptors (Ensembl family: ENSFM00760001715026) are abundantly present on many cell types, including LCLs [41,42], which has made ATP a reference agonist for testing of functional LCL responses [21]. Interestingly, both adenosine receptor agonists and ATP display the same shape of response, which was also comparable to the response to cannabinoid receptor 2 (CB2) agonists as seen in an earlier publication [21]. Herein we showed that LCL densities of 50,000 cells/well were sufficient for detection of a robust CB2 as well as P2Y receptor response [21]. In the present study seeding densities were increased to 80,000 cells/well to obtain a window sufficient for $A_{2A}R$ partial agonist characterization.

It is well known that $A_{2A}R$ are expressed in immune cells, including lymphocytes and LCLs [38,43], which was confirmed in

this study by both receptor expression levels in the qPCR experiments and the responses to selective adenosine receptor agonists in the label-free assay (Fig. 1). The results from these tests indicated that $A_{2A}R$ are the only adenosine receptors highly expressed in LCLs. This was further confirmed by the comparability of the responses of all three full agonists tested in this paper. The endogenous ligand adenosine as well as subtype unselective NECA and $A_{2A}R$ selective CGS21680 had comparable responses (Fig. 2) suggesting these were all mediated through the $A_{2A}R$. Similarly, antagonist responses were also measurable for all four different individuals (Fig. 3), strengthening the conclusion that responses are mediated through $A_{2A}R$ only.

While it is straightforward to confirm that an impedance response is a specific receptor-mediated effect with recombinant cell lines, namely by simply using the untransfected parental cell line as negative control [33,37], this is not possible in cell lines with endogenous receptor expression. Therefore, for LCLs the most reliable way is to confirm overall receptor pharmacology with receptor subtype-selective agonists and antagonists. By showing that the $A_{2A}R$ selective antagonists ZM241385 and istradefylline competed with and blocked the signal of the $A_{2A}R$ selective agonist CGS21680 (Fig. 3), we confirmed that the impedance effects indeed originate from an $A_{2A}R$ response.

Overall, agonist pEC_{50} values for agonists were within a log unit from previously reported literature values obtained with standard functional assays on heterologous cell lines (Table 1). For instance, adenosine itself is within that range as it has been reported with an EC_{50} value of 310 nM in a cAMP assay on $hA_{2A}AR$ [44]. For the antagonists, the calculated pK_i values of ZM241385 and istradefylline were also within the range of previously published values. This calculation corrects for the fact that the same concentration of agonist was used during the assay, corresponding to the EC_{80} of CGS21680, while the efficacy of this agonist differed slightly between cell lines.

Following this characterization of full agonists and antagonists to verify the presence and functional relevance of $A_{2A}R$, a number of partial agonists were tested to demonstrate the sensitivity of the system. The set-up was well able to measure partial agonist effects on LCLs, quite comparable to our previous study on HEK293hA_{2A}AR cells (20). Interestingly, while most agonists induced an increase in impedance with a single peak in LCLs, there were two agonists which gave rise to a different shape of response. Both BAY60-6583 and the partial agonist LUF5834 responses were marked by a small peak followed by a negative impedance plateau, rather than one positive peak (Figs. 1 and 4). Interestingly, both BAY60-6583 and LUF5834 belong to a structurally distinct class of non-ribose agonists, as opposed to all other agonists tested in this paper. Hence, it seems that non-ribose agonists, while equally able to activate the $hA_{2A}AR$, give rise to a different cellular response than the more common ribose-containing agonists. This was not observed in the heterologous HEK293hA_{2A}AR cell line where partial agonist LUF5834 had been tested previously [33], which highlights the differences of using an unmodified human cell line when characterizing compound effects. In fact, efficacies and signaling of ligands can differ under artificial or heterologous conditions due to a number of factors [23,45]. Receptor overexpression, differences in intracellular metabolic conditions as well as products from other genes could modify cellular responses. Unfortunately, most studies of receptor function involve artificially expressed receptors in heterologous cell systems, such as CHO or HEK cells [3,33]. While useful for high-throughput screening and fundamental research, such systems are far from the real-life situation in an individual. To move further toward the physiological situation, it is essential to study receptor function in a more endogenous setting such as LCLs. This is especially true when attempting to understand how polymorphisms may functionally affect the receptor and therefore the drug response of an individual.

Employing the LCLs, we investigated genotype effects on receptor response by comparing the effects of various types of $A_{2A}R$ ligands between the individuals of a family of four from the Netherlands Twin Register, which consisted of two genetically unrelated individuals, the parents, and their children, which were monozygotic twins. Overall, the results were comparable between all individuals. Analyzing and confirming the comparability of results obtained in monozygotic twins is one of the standard ways in genetic studies to control for genotype-unrelated effects, and assess a system's suitability for genetic studies [26,27]. As expected, the twins did not differ significantly from each other, with exception of their pEC_{50} values for NECA ($p < 0.05$; Table 1). Interestingly, NECA was also the only ligand for which all individuals differed significantly in their pEC_{50} values. As monozygotic twins are genetically identical, these differences could not be related to genetic effects and therefore precluded any further conclusion about differences between the parents. However, parent 1 showed significant differences on two occasions, when all other three individuals, including the monozygotic twins, were comparable. This was the case with istradefylline as well as with the partial agonist LUF5448. While with istradefylline the difference was rather marginal within half a log unit, the potency shift (approx. tenfold higher) for LUF5448 was much more pronounced for parent 1. Partial agonists are deemed more sensitive to system-related differences in receptor function, for instance in receptor expression or downstream coupling, than full agonists or antagonists [29]. Therefore, the difference in potency possibly reflects subtle changes introduced by the genetic differences between individuals. While none of the four individuals had non-synonymous SNPs in the ADORA2A gene (Table 2), there were some heterozygous differences present in non-coding SNPs. Two SNP differences were in line with the pEC_{50} and pIC_{50} changes, namely in which only parent 1 differed while parent 2 and the twins showed the same genotype and response. These were rs34999116 where parent 1 is heterozygote for the minor allele and rs2236624 where parent 1 is homozygote for the minor allele. Interestingly, the C-allele of rs2236624, which is located in intron 4 of the ADORA2A gene, has been associated with vigilance and sleep, while the CC genotype has been associated with anxiety in autism patients [15,16,14]. The TT genotype has been associated with pharmacotherapy-related toxicities in acute lymphoblastic leukemia [46]. Several studies have proposed a subtle effect on receptor expression as possible mechanism, as this intron SNP has intermediate regulatory potential [16,46]. As we did not observe significant differences in receptor mRNA levels in our qPCR experiments, this regulation may affect the subsequent translation. Changes in receptor expression may affect G protein coupling efficiency, for which a partial agonist is more sensitive than a full agonist.

Although this genetic variation does not provide causal evidence that response differences as observed in the LCLs from these individuals are directly related to these SNPs, the experimental results show that the chosen methodology and set-up are capable of picking up individual differences in receptor signaling for the $A_{2A}R$. Although $A_{2A}R$ function has been studied in endogenous cell types [38–40], we made a further step toward both physiologically relevant conditions and personalized medicine by enabling the study of a person's $A_{2A}R$ response using a combination of LCLs from a family of four from the NTR and a non-invasive label-free cellular assay.

It is increasingly recognized that genetic differences between individuals form a large challenge in drug therapy indeed. In our study of real-life genetic variation of $A_{2A}R$ signaling, we found that partial agonist potency differed significantly for one individual with genotype differences in two intron SNPs, one of which has

previously been associated with caffeine-induced sleep disorders. While further validation is needed to confirm genotype-specific effects, this set-up clearly demonstrated that LCLs are a suitable model system to study genetic influences on $A_{2A}R$ and GPCR responses in general. LCLs express a wide range of other 'drugable' GPCRs, besides the $A_{2A}R$, CB2 and P2Y receptors investigated in this and earlier studies [21,43]. Therefore, screening receptor responses in LCLs may help to provide the mechanistic link between polymorphisms of various GPCRs and the individual variation in drug response.

Data access

The LCLs used in this study were kindly provided within the framework of this collaboration [26] and are part of the Netherlands Twin Register (NTR; http://www.tweelingenregister.org/en/), and part of the Center for Collaborative Genomic Studies on Mental Disorders (NIMH U24 MH068457-06). Data and biomaterials (such as cell lines) are available to qualified investigators, and may be accessed by following a set of instructions stipulated on the National Institute of Mental Health (NIMH) website (https://www.nimhgenetics.org/access_data_biomaterial.php).

Disclosure declaration

The authors declare that no competing interests exist.

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References

[1] J.P. Overington, B. Al-Lazikani, A.L. Hopkins, How many drug targets are there? Nat. Rev. Drug Discov. 5 (2006) 993–996.
[2] A.P. Russ, S. Lampel, The druggable genome: an update, Drug Discov. Today 10 (2005) 1607–1610.
[3] B.B. Fredholm, A.P. IJzerman, K.A. Jacobson, J. Linden, C.E. Muller, International union of basic and clinical pharmacology. LXXXI. Nomenclature and classification of adenosine receptors – an update, Pharmacol. Rev. 63 (2011) 1–34.
[4] K.A. Jacobson, Z.G. Gao, Adenosine receptors as therapeutic targets, Nat. Rev. Drug Discov. 5 (2006) 247–264.
[5] T. Muller, The safety of istradefylline for the treatment of Parkinson's disease, Expert Opin. Drug Saf. 1–7 (2015).
[6] A.J. Venkatakrishnan, X. Deupi, G. Lebon, C.G. Tate, G.F. Schertler, M.M. Babu, Molecular signatures of G-protein-coupled receptors, Nature 494 (2013) 185–194.
[7] M.A. Davies, V. Setola, R.T. Strachan, D.J. Sheffler, E. Salay, S.J. Hufeisen, et al., Pharmacologic analysis of non-synonymous coding h5-HT2A SNPs reveals alterations in atypical antipsychotic and agonist efficacies, Pharmacogenomics J. 6 (2006) 42–51.
[8] S.J. Docherty, O.S. Davis, C.M. Haworth, R. Plomin, U. D'Souza, J. Mill, A genetic association study of DNA methylation levels in the DRD4 gene region finds associations with nearby SNPs, Behav. Brain Funct. 8 (2012) 31–44.
[9] H. Ishiguro, Y. Horiuchi, M. Ishikawa, M. Koga, K. Imai, Y. Suzuki, et al., Brain cannabinoid CB2 receptor in schizophrenia, Biol. Psychiatry 67 (2010) 974–982.
[10] D. Tong, S. He, L. Wang, L. Jin, P. Si, X. Cheng, Association of single-nucleotide polymorphisms in the cannabinoid receptor 2 gene with schizophrenia in the Han Chinese population, J. Mol. Neurosci. 51 (2013) 454–460.
[11] W. Sadee, E. Hoeg, J. Lucas, D. Wang, Genetic variations in human G protein-coupled receptors: implications for drug therapy, AAPS PharmSci. 3 (2001) 54–80.
[12] R. Buscher, A. Hoerning, H.H. Patel, S. Zhang, D.B. Arthur, H. Grasemann, et al., P2Y2 receptor polymorphisms and haplotypes in cystic fibrosis and their impact on Ca2+ influx, Pharmacogenet. Genomics 16 (2006) 199–205.
[13] A. Wesselius, M.J. Bours, Z. Henriksen, S. Syberg, S. Petersen, P. Schwarz, et al., Association of P2Y(2) receptor SNPs with bone mineral density and osteoporosis risk in a cohort of Dutch fracture patients, Purinergic Signal. 9 (2013) 41–49.
[14] S. Bodenmann, C. Hohoff, C. Freitag, J. Deckert, J.V. Retey, V. Bachmann, et al., Polymorphisms of ADORA2A modulate psychomotor vigilance and the effects of caffeine on neurobehavioural performance and sleep EEG after sleep deprivation, Br. J. Pharmacol. 165 (2012) 1904–1913.
[15] E. Childs, C. Hohoff, J. Deckert, K. Xu, J. Badner, H. de Wit, Association between ADORA2A and DRD2 polymorphisms and caffeine-induced anxiety, Neuropsychopharmacology 33 (2008) 2791–2800.
[16] C.M. Freitag, K. Agelopoulos, E. Huy, M. Rothermundt, P. Krakowitzky, J. Meyer, et al., Adenosine A(2A) receptor gene (ADORA2A) variants may increase autistic symptoms and anxiety in autism spectrum disorder, Eur. Child Adolesc. Psychiatry 19 (2010) 67–74.
[17] P.J. Rogers, C. Hohoff, S.V. Heatherley, E.L. Mullings, P.J. Maxfield, R.P. Evershed, et al., Association of the anxiogenic and alerting effects of caffeine with ADORA2A and ADORA1 polymorphisms and habitual level of caffeine consumption, Neuropsychopharmacology 35 (2010) 1973–1983.
[18] C. Hohoff, E.L. Mullings, S.V. Heatherley, C.M. Freitag, L.C. Neumann, K. Domschke, et al., Adenosine A(2A) receptor gene: evidence for association of risk variants with panic disorder and anxious personality, J. Psychiatr. Res. 44 (2010) 930–937.
[19] L. Sie, S. Loong, E.K. Tan, Utility of lymphoblastoid cell lines, J. Neurosci. Res. 87 (2009) 1953–1959.
[20] M. Sugimoto, H. Tahara, T. Ide, Y. Furuichi, Steps involved in immortalization and tumorigenesis in human B-lymphoblastoid cell lines transformed by Epstein–Barr virus, Cancer Res. 64 (2004) 3361–3364.
[21] J.M. Hillger, J. Schoop, D.I. Boomsma, P. Eline Slagboom, A.P. IJzerman, L.H. Heitman, Whole-cell biosensor for label-free detection of GPCR-mediated drug responses in personal cell lines, Biosens. Bioelectron. 74 (2015) 233–242.
[22] A. Morag, J. Kirchheiner, M. Rehavi, D. Gurwitz, Human lymphoblastoid cell line panels: novel tools for assessing shared drug pathways, Pharmacogenomics 11 (2010) 327–340.
[23] N. Yu, J.M. Atienza, J. Bernard, S. Blanc, J. Zhu, X. Wang, et al., Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors, Anal. Chem. 78 (2006) 35–43.
[24] Fang Y. Label-Free, Label-free receptor assays, Drug Discov. Today Technol. 7 (2011) e5–e11.
[25] M. Rocheville, J. Martin, J. Jerman, E. Kostenis, Mining the potential of label-free biosensors for seven-transmembrane receptor drug discovery, Prog. Mol. Biol. Transl. Sci. 115 (2013) 123–142.
[26] G. Willemsen, E.J. de Geus, M. Bartels, C.E. van Beijsterveldt, A.I. Brooks, G.F. Estourgie-van Burk, et al., The Netherlands Twin Register biobank: a resource for genetic epidemiological studies, Twin Res. Hum. Genet. 13 (2010) 231–245.
[27] K. Silventoinen, A. Jelenkovic, R. Sund, C. Honda, S. Aaltonen, Y. Yokoyama, et al., The CODA twins project: the cohort description of collaborative project of development of anthropometrical measures in twins to study macro-environmental variation in genetic and environmental effects on anthropometric traits, Twin Res. Hum. Genet. 18 (2015) 348–360.
[28] E.W. van Tilburg, J. von Frijtag Drabbe Kunzel, M. de Groote, A.P. IJzerman, 2,5′-Disubstituted adenosine derivatives: evaluation of selectivity and efficacy for the adenosine A(1), A(2A), and A(3) receptor, J. Med. Chem. 45 (2002) 420–429.
[29] E.W. van Tilburg, M. Gremmen, J. von Frijtag Drabbe Kunzel, M. de Groote, A.P. IJzerman, 2,8-Disubstituted adenosine derivatives as partial agonists for the adenosine A2A receptor, Bioorg. Med. Chem. 11 (2003) 2183–2192.
[30] M.W. Beukers, L.C. Chang, J.K. von Frijtag Drabbe Kunzel, T. Mulder-Krieger, R. F. Spanjersberg, J. Brussee, et al., New, non-adenosine, high-potency agonists for the human adenosine A2B receptor with an improved selectivity profile compared to the reference agonist N-ethylcarboxamidoadenosine, J. Med. Chem. 47 (2004) 3707–3709.
[31] G. Miller, M. Lipman, Release of infectious Epstein–Barr virus by transformed marmoset leukocytes, Proc. Natl. Acad. Sci. U.S.A. 70 (1973) 190–194.
[32] M.S. Engelstoft, W.M. Park, I. Sakata, L.V. Kristensen, A.S. Husted, S. Osborne-Lawrence, et al., Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells, Mol. Metab. 2 (2013) 376–392.
[33] D. Guo, T. Mulder-Krieger, A.P. Ijzerman, L.H. Heitman, Functional efficacy of adenosine A(2)A receptor agonists is positively correlated to their receptor residence time, Br. J. Pharmacol. 166 (2012) 1846–1859.
[34] Y. Cheng, W.H. Prusoff, Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction, Biochem. Pharmacol. 22 (1973) 3099–3108.
[35] S. Purcell, B. Neale, K. Todd-Brown, L. Thomas, M.A. Ferreira, D. Bender, et al., PLINK: a tool set for whole-genome association and population-based linkage analyses, Am. J. Hum. Genet. 81 (2007) 559–575.
[36] S. Purcell, PLINK v1.07, <http://pngu.mgh.harvard.edu/purcell/plink/>.
[37] P. Scandroglio, R. Brusa, G. Lozza, I. Mancini, R. Petro, A. Reggiani, et al., Evaluation of cannabinoid receptor 2 and metabotropic glutamate receptor 1 functional responses using a cell impedance-based technology, J. Biomol. Screen. 15 (2010) 1238–1247.

[38] M.V. Sitkovsky, D. Lukashev, S. Apasov, H. Kojima, M. Koshiba, C. Caldwell, et al., Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors, Annu. Rev. Immunol. 22 (2004) 657–682.
[39] A. Desai, C. Victor-Vega, S. Gadangi, M.C. Montesinos, C.C. Chu, B.N. Cronstein, Adenosine A2A receptor stimulation increases angiogenesis by down-regulating production of the antiangiogenic matrix protein thrombospondin 1, Mol. Pharmacol. 67 (2005) 1406–1413.
[40] C. Escudero, P. Bertoglia, M. Hernadez, C. Celis, M. Gonzalez, C. Aguayo, et al., Impaired A2A adenosine receptor/nitric oxide/VEGF signaling pathway in fetal endothelium during late- and early-onset preeclampsia, Purinergic Signal. 9 (2013) 215–226.
[41] F. Jacob, C. Perez Novo, C. Bachert, K. Van Crombruggen, Purinergic signaling in inflammatory cells: P2 receptor expression, functional effects, and modulation of inflammatory responses, Purinergic Signal. 9 (2013) 285–306.
[42] D.H. Lee, K.S. Park, I.D. Kong, J.W. Kim, B.G. Han, Expression of P2 receptors in human B cells and Epstein–Barr virus-transformed lymphoblastoid cell lines, BMC Immunol. 7 (2006) 22–33.
[43] M. Vincent, K. Oved, A. Morag, M. Pasmanik-Chor, V. Oron-Karni, N. Shomron, et al., Genome-wide transcriptomic variations of human lymphoblastoid cell lines: insights from pairwise gene-expression correlations, Pharmacogenomics 13 (2012) 1893–1904.
[44] L. Yan, J.C. Burbiel, A. Maass, C.E. Muller, Adenosine receptor agonists: from basic medicinal chemistry to clinical development, Expert Opin. Emerg. Drugs 8 (2003) 537–576.
[45] R. Eglen, T. Reisine, Primary cells and stem cells in drug discovery: emerging tools for high-throughput screening, Assay Drug Dev. Technol. 9 (2011) 108–124.
[46] R. Franca, P. Rebora, N. Bertorello, F. Fagioli, V. Conter, A. Biondi, et al., Pharmacogenetics and induction/consolidation therapy toxicities in acute lymphoblastic leukemia patients treated with AIEOP-BFM ALL 2000 protocol, Pharmacogenomics J. (2015).
[47] G. Schulte, B.B. Fredholm, Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2, Mol. Pharmacol. 58 (2000) 477–482.
[48] H. Kase, Industry forum: progress in pursuit of therapeutic A2A antagonists: the adenosine A2A receptor selective antagonist KW6002: research and development toward a novel nondopaminergic therapy for Parkinson's disease, Neurology 61 (2003) S97–S100.