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Equilibrium and kinetic selectivity profiling on the human adenosine receptors

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ABSTRACT

Classical evaluation of target selectivity is usually undertaken by measuring the binding affinity of lead compounds against a number of potential targets under equilibrium conditions, without considering the kinetics of the ligand–receptor interaction. In the present study we propose a combined strategy including both equilibrium- and kinetics-based selectivity profiling. The adenosine receptor (AR) was chosen as a prototypical drug target. Six in-house AR antagonists were evaluated in a radioligand displacement assay for their affinity and in a competition association assay for their binding kinetics on three AR subtypes. One of the compounds with a promising kinetic selectivity profile was also examined in a [^{35}S]-GTPγS binding assay for functional activity. We found that XAC and LUF5964 were kinetically more selective for the A_1R and A_3R, respectively, although they are non-selective in terms of their affinity. In comparison, LUF5967 displayed a strong equilibrium-based selectivity for the A_1R over the $A_{2A}R$, yet its kinetic selectivity thereon was less pronounced. In a GTPγS assay, LUF5964 exhibited insurmountable antagonism on the A_3R while having a surmountable effect on the A_1R, consistent with its kinetic selectivity profile. This study provides evidence that equilibrium and kinetic selectivity profiling can both be important in the early phases of the drug discovery process. Our proposed combinational strategy could be considered for future medicinal chemistry efforts and aid the design and discovery of different or even better leads for clinical applications.

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1. Introduction

Adenosine receptors (ARs) belong to the superfamily of G protein-coupled receptors (GPCRs), which represent the largest family of drug targets [1]. Four AR subtypes have been identified, namely A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R, according to their physiological effects in responding to adenosine, the endogenous ligand [2,3]. The A_1R and A_3R couple to a G_i protein and inhibit the enzyme adenylate cyclase, whereas the $A_{2A}R$ and $A_{2B}R$ stimulate this enzyme via a G_s protein [4]. ARs are distributed throughout the body and involved in a wide range of (patho-)physiological responses, and may be promising drug targets [5]. However, the ubiquitous distribution of ARs challenges the discovery of new ligands. Over the years, many efforts have been undertaken to yield selective agonists and antagonists for the each AR subtype [6]. This selectivity is generally evaluated based on dose-dependent assessments of activities (i.e., EC_{50} or K_i values) performed under equilibrium conditions. However, such equilibrium *in vitro* is rarely met in the body and the binding selectivity evolves over the course of treatment as a function of the temporal binding between the drug and the main and secondary targets [7]. Thus, the binding kinetics of the drug–target interaction, in particular, residence time (RT = $1 \cdot k_{off}^{-1}$), is gaining awareness, since it can provide detailed information under non-equilibrium situations [7]. Furthermore, accumulating evidence suggests that compounds with desired

Abbreviations: ARs, adenosine receptors; CGS15943, 9-chloro-2-(2-furanyl)-[1,2,4]triazolo [1,5-*c*]quinazolin-5-amine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CPA, N^6-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; IB-MECA, N^6-(3-Iodobenzyl)adenosine-5′-*N*-methyluronamide; GPCR, G protein-coupled receptor; k_1, the association rate constant of the radioligand; k_2, the dissociation rate constant of the radioligand; k_3, the association rate constant of the unlabeled ligand; k_4, the dissociation rate constant of the unlabeled ligand; NECA, 5′-*N*-ethylcarboxamidoadenosine; LUF5963, 2,6,8-triphenyl-9*H*-purine; LUF5964, 2,6-diphenyl-8-propyl-9*H*-purine; LUF5967, 6-phenyl-2-(*p*-tolyl)-9*H*-purine; PSB-11, (8*R*)-8-ethyl-1,4,7,8-tetrahydro-4-5*H*-imidazole[2,1-*i*]purin-5-one; RT, residence time; Theophylline, 1,3-dimethyl-7*H*-purine-2,6-dione; ZM241385, 4-(2-(7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-5-ylamino)ethyl)phenol; XAC, xanthine amine congener.

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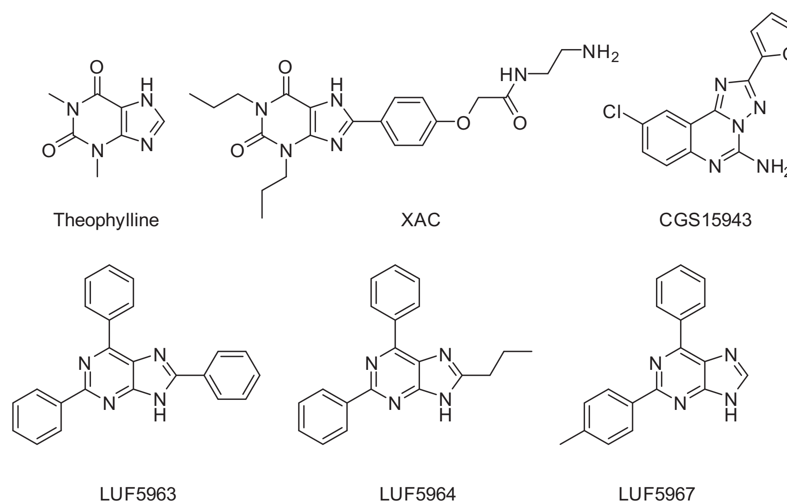

Fig. 1. Chemical structures of compounds used in the present study. Theophylline and XAC are xanthine-like adenosine receptor antagonists. CGS15943, LUF5963, LUF5964 and LUF5967 are non-xanthine adenosine receptor antagonists.

kinetic profiles can provide additional advantages. Compounds with long-lasting target occupancy may offer improved clinical efficacy, whereas compounds with fast dissociation kinetics from unwanted targets might show less side effects [8,9]. Given this, designing drugs with desirable selectivity profiles should therefore not only require an appropriate tuning of binding selectivity but also the modulation of kinetic selectivity [10]. However, such notion has been rarely taken into account and only few studies have touched upon the concept of kinetic selectivity profiling before.

To obtain a complete profile of ligand–receptor selectivity, we examined a series of AR antagonists (Fig. 1) and extensively studied both their affinity and kinetics on different AR subtypes. The association rate (k_{on}) and dissociation rate (k_{off}) constants of these antagonists were determined using competition association assays at the A_1R, $A_{2A}R$ and A_3R. Furthermore, one compound with promising kinetic selectivity was further tested in a [^{35}S]-GTPγS assay for functional evaluation.

2. Materials and methods

2.1. Materials

[^{3}H]-1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]-DPCPX, specific activity 120 Ci·mmol^{-1}) and [^{3}H]-4-(2-(7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-5-ylamino)ethyl)phenol ([^{3}H]-ZM241385, specific activity 50 Ci·mmol^{-1}) were purchased from ARC, Inc. (St. Louis, MO, USA). [^{3}H]-(8*R*)-8-ethyl-1,4,7,8-tetrahydro-4-5*H*-imidazole[2,1-*i*]purin-5-one ([^{3}H]-PSB-11, specific activity 56 Ci·mmol^{-1}) was a gift from Prof. C.E. Müller (University of Bonn, Germany). Unlabeled PSB-11 and *N*^{6}-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide (IB-MECA) were purchased from Tocris (Abingdon, UK). Unlabeled DPCPX and 5′-*N*-ethylcarboxamidoadenosine (NECA) was purchased from Sigma–Aldrich (Steinheim, Germany). *N*^{6}-Cyclopentyladenosine (CPA) and ZM241385 were from Abcam (Cambridge, UK). LUF5963, LU5964 and LUF5967 were synthesized in our laboratory [11]. Adenosine deaminase (ADA) was purchased from Boehringer Mannheim (Mannheim, Germany). Chinese hamster ovary cells stably expressing the human adenosine A_1 receptor (CHOhA_1R) were kindly provided by Prof. Steve Hill (University of Nottingham, UK). Human Embryonic Kidney 293 cells stably expressing the human adenosine A_{2A} receptor (HEK293hA_{2A}R) were a gift from Dr. J. Wang (Biogen, USA). Chinese hamster ovary cells stably expressing the human adenosine A_3 receptor (CHOhA_3R) were a gift from Dr. K-N Klotz (University of Würzburg, Germany). All other chemicals were of analytical grade and obtained from standard commercial sources.

2.2. Cell culture and membrane preparation

Cell culture and membrane preparation were performed as reported previously [12–14].

2.3. Radioligand binding experiments

All concentrations mentioned are final concentrations.

2.3.1. Radioligand displacement assays

Membrane aliquots containing the A_1R, $A_{2A}R$ or A_3R in a total volume of 100 μL of assay buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, supplemented with 0.1% CHAPS for the A_1R and $A_{2A}R$ or with 0.01% CHAPS and 1 mM EDTA for the A_3R, pH = 7.4) with 2 nM [^{3}H]-DPCPX, 3 nM [^{3}H]-ZM241385 or 9.5 nM [^{3}H]-PSB-11 in the presence of a competing ligand were incubated at 10 °C for 2 h or 3 h to ensure equilibrium was reached. Nonspecific binding was determined in the presence of 100 μM NECA and represented less than 10% of the total radioligand binding. Incubations were terminated by rapid vacuum filtration to separate the bound and free radioligand through Whatman GF/B filters (Whatman International, Maidstone, UK) with a Brandel harvester or through 96-well GF/B filter plates using a PerkinElmer Filtermate harvester (Perkin Elmer, Groningen, the Netherlands). Filters were washed three times with ice-cold wash buffer (50 mM Tris–HCl, pH 7.4, supplemented with 5 mM $MgCl_2$ for the A_1R and $A_{2A}R$ or 50 mM Tris–HCl, pH 7.4, supplemented with 5 mM $MgCl_2$ and 1 mM EDTA for the A_3R). The filter-bound radioactivity was determined by scintillation spectrometry using a PE 1450 Microbeta Wallac Trilux scintillation counter (PerkinElmer) for 96-well GF/B filter plates or by a liquid scintillation counter (Tri-Carb 2900 TR, PerkinElmer) for Whatman GF/B filters.

2.3.2. Kinetic radioligand binding experiments

2.3.2.1. A_1R. Association experiments were performed by incubating CHOhA_1R membranes in a total volume of 100 μL of assay buffer with 2 nM [^{3}H]-DPCPX at 10 °C. The amount of radioligand bound to the receptor was measured at different time intervals during incubation for 2 h at 10 °C. Dissociation experiments were

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