Supplementary data
(For online publication)

Discovery of 2-aminoimidazole and 2-aminoimidazolyl-thiazoles as non-xanthine human adenosine A<sub>3</sub> receptor antagonists: Structure-Activity Relationships and Molecular Modeling studies

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Experimental section

Table-S1: Binding affinities of 2-aminoimidazole derivatives for human adenosine receptors

![Chemical Structure]

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>6a-6l Binding experiments</th>
<th>Adenylyl cyclase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>hA₁</td>
<td>hA₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hA₂</td>
<td>hA₃</td>
</tr>
<tr>
<td>6a</td>
<td>4-FC₆H₄</td>
<td>4-MeC₆H₄</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>6b</td>
<td>4-FC₆H₄</td>
<td>4-OMeC₆H₄</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>6c</td>
<td>4-FC₆H₄</td>
<td>4-ClC₆H₄</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>6d</td>
<td>4-ClC₆H₄</td>
<td>4-MeC₆H₄</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>6e</td>
<td>4-ClC₆H₄</td>
<td>4-C₅H₄N</td>
<td>&gt; 100</td>
<td>32.6 (25.8 – 41.1)</td>
</tr>
<tr>
<td>6f</td>
<td>C₆H₅</td>
<td>4-SO₂MeC₆H₄</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>6g</td>
<td>4-CF₃C₆H₄</td>
<td>4-ClC₆H₄</td>
<td>&gt; 100</td>
<td>39.0 (27.5 – 55.4)</td>
</tr>
<tr>
<td>6h</td>
<td>2-ClC₆H₄</td>
<td>4-MeC₆H₄</td>
<td>&gt; 100</td>
<td>15.2 (12.5 – 18.4)</td>
</tr>
<tr>
<td>6i</td>
<td>3-ClC₆H₄</td>
<td>4-MeC₆H₄</td>
<td>&gt; 100</td>
<td>22.7 (21.6– 23.7)</td>
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<tr>
<td>6j</td>
<td>3-Cl 4-FC₆H₄</td>
<td>4-ClC₆H₄</td>
<td>&gt; 100</td>
<td>34.8 (31.7- 38.1)</td>
</tr>
<tr>
<td>6k</td>
<td>4-FC₃C₆H₄</td>
<td>4-C₅H₄N</td>
<td>&gt; 100</td>
<td>27.8 (22.1 – 34.9)</td>
</tr>
<tr>
<td>6l</td>
<td>4-FC₆H₄</td>
<td>4-C₅H₄N</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

a Displacement of specific [³H]CCPA binding at human A₁ receptors expressed in CHO cells.

b Displacement of specific [³H]NECA binding at human A₂A receptors expressed in CHO cells.

c Displacement of specific [³H]HEMADO binding at human A₃ receptors expressed in CHO cells.

d Inhibition of NECA-stimulated adenylyl cyclase activity at human A₂B receptors expressed in CHO cells.

Data are expressed as geometric means, with 95% confidence limits in parentheses.

Biology
Receptor-radioligand binding studies were performed as previously described by Klotz et al. The membranes for radioligand binding were prepared from CHO cells stably transfected with human adenosine receptor subtypes in a two-step procedure. In a first low-speed step (1,000x g) cell fragments and nuclei were removed. The crude membrane fraction was sedimented from the supernatant at 100,000x g. The membrane pellet was re-suspended in the buffer used for the respective binding experiments, frozen in liquid nitrogen and stored at -80°C. For the measurement of adenylyl cyclase activity only one high-speed centrifugation of the homogenate was used. The resulting crude membrane pellet was re-suspended in 50mM Tris/HCl, pH 7.4 and immediately used for the cyclase assay.

For agonist radioligand binding at A<sub>1</sub> adenosine receptors 1 nM [³H]CCPA was used, whereas 10 nM [³H]NECA were used for A<sub>2A</sub> receptors. For A<sub>3</sub> adenosine receptors the newly developed high-affinity agonist [³H]HEMADO was used.

Non-specific binding of [³H]CCPA was determined in the presence of 1 mM theophylline, in the case of [³H]NECA and [³H]HEMADO 100 µM R-PIA was used. K<sub>i</sub>-values from competition experiments were calculated with the program SCTFIT. ³

Radioligand binding at A<sub>2B</sub> adenosine receptors is problematic as no high-affinity ligand is available for this subtype. Therefore, inhibition of NECA-stimulated adenylyl cyclase activity was determined as a measurement of affinity of compounds. No detectable interaction of the compounds under investigation with the A<sub>2B</sub> AR was observed.

References:


Molecular modeling studies
**Software overview**

General molecular modeling operations, such as drawing of ligand structures, partial charges computation, energetic analysis and visual inspection of docking poses, were performed using the MOE suite (Molecular Operating Environment, version 2015.1001) [1]. Shape similarity studies were conducted with the vROCS application of the OpenEye Scientific Software [7]. GOLD (Genetic Optimization for Ligand Docking, version 5.2) suite [2] was used for docking simulations. Calculation of ligand partial charges was performed with the software MOPAC [3] as implemented in the MOE suite. CHIMERA [4] was used for image production and GNUPlot 4.6 [5] to construct plots. Video assembly was carried out using MEncoder [6]. Molecular modeling studies have been performed on a 8 CPU (Intel® Xeon® CPU E5-1620 3.70 GHz) linux workstation.

**Three-dimensional structures of Adenosine Receptors**

A shape similarity comparison was conducted among compound 12b, chosen as representative of the series under examination, and all the ligands retrieved from A_{2A} AR PDB structures available to date. Consequently, 3UZC [8] crystallographic structure was chosen because its co-crystallized ligand, 4-(3-amino-5-phenyl-1,2,4-triazin-6-yl)-2-chlorophenol, showed highest ROCS Tanimoto Combo value for compound 12b. Homology models of A_{3} and A_{1} ARs, constructed using 3UZC structure as a template, were retrieved from the Adenosiland web-platform [9,10]. The protein residues have been named according the generic Ballesteros Weinstein numbering system [11] throughout this work.

**Molecular docking**

MOE-Builder tool was employed to model three-dimensional structures of ligands and the MOE-Protonate-3D [12] tool was used to predict ionization states of ligands and proteins. Newly added protein hydrogens were minimized using Amber12EHT force field, while ligand structures were subjected to MMFF94x energy minimization, fixing the root mean square (rms) gradient cutoff at 0.1 kcal mol\(^{-1}\) \(\text{A}^\text{2}\). Taking advantage of a docking benchmark previously performed in our laboratory [13,14], GOLD docking tool [2] was selected as conformational search program and GoldScore as scoring function. For each compound, 20 docking runs were performed on each
receptor subtype, searching in a sphere of 20 Å radius centered on Asn6.55 residue. After computing Amber12EHT partial charges for receptor atoms and PM3/ESP atomic partial charges for ligands poses, electrostatic and van der Waals interactions were calculated with MOE.

**Interaction Energy Fingerprints (IEFs)**

Protein residues within 4.5 Å of the selected pose of compound 12b were identified as defining the binding pocket of the receptor. Per residue electrostatic and hydrophobic interactions (named IEele and IEhyd, respectively) were calculated between each binding site residue and the ligand, using MOE [13,15]. In particular, IEele, measured in kcal/mol, were computed with Amber12EHT force field. Instead, IEhyd, expressed by an adimensional score (the higher the better), were computed as contact hydrophobic surfaces. The data were plotted on a heat map, called Interaction Energy Fingerprints (IEFs), in which residues are reported on the x-axis and the energetic values are rendered by a colorimetric scale. As regards IEele, colors from blue to red depict energy values ranging from negative to positive ones; for IEhyd, colors from white to dark green represent scores going from 0 to positive values.

**MMsDocking video maker**

A in-house tool, named MMsDocking video maker, was used to produce videos with the aim to facilitate the visualization and analysis of data obtained from the docking simulations. The videos show for each ligand the selected docking pose, scoring values, experimental binding data and per residue IEhyd and IEele of most relevant residues. IEhyd and IEele were computed as previously described and plotted with GNUPLOT [5], molecular representation of docked compounds were obtained using CHIMERA [4] software, 2D depictions of the compounds were drawn with RDKit toolkit [16]. Images were assembled into videos using MEncoder[6].

**SUPPLEMENTARY MATERIAL**

Videos SM1-SM2-SM3: Videos reporting the selected docking poses on A3, A2A and A1 adenosine receptors binding sites, respectively. Ligands are rendered by light-blue sticks, and protein residues by tan sticks. Protein residues mainly involved in binding are shown. For A2A and A1 receptors, in addition to highly interacting residues, residues at equivalent positions to those selected for A3 receptor are reported, to enable inter-receptors comparison. The heat maps depicted in the
background report the electrostatic and hydrophobic contributions of the selected residues (“ele” and “hyd” labels identify the major contribution type of the residue) by a colorimetric scale going from blue to green for negative to positive values. Hydrogen bonds are represented by yellow lines.

REFERENCES
1. Chemical Computing Group Inc. Molecular Operating Environment (MOE) [Internet]. Available from: http://www.chemcomp.com
2. Cambridge Crystallographic Data Centre: 12 Union Road, Cambridge CB2 1EZ, UK. GOLD suite, version 5.2 [Internet]; Available from: http://www.ccdc.cam.ac.uk
5. Gnuplot [Internet]. Available from: http://www.gnuplot.info/index.html
6. MEncoder [Internet]. Available from: http://www.mplayerhq.hu/design7/projects.html


Spectral data (LC-MS (M+1) +1H NMR and 13C NMR) of compounds (6a-6l) and (12a-12l)
Exact Mass: 295.11
Exact Mass: 311.11
Exact Mass: 311.08
Exact Mass: 298.06
Exact Mass: 341.08
Exact Mass: 365.05
Exact Mass: 311.08
Exact Mass: 311.08
Exact Mass: 349.02
+Q1 mass spectrum of Sample 1 (AP) of 170309.wiff (Ion Spray), subtracted (0.150 to 0.351 min)

**Exact Mass: 332.09**

(+Q1 mass spectrum of Sample 1 (AP) of 170309.wiff (Ion Spray), subtracted (0.150 to 0.351 min)

**Exact Mass: 282.09**

Method Name: N/A

Sample Number: N/A

Project: Example

Electronic Signature: no
+Q1: 0.485 to 0.703 min from Sample 13 (IT 1) of 110909.wiff (Ion Spray), subtracted (0.201 to 0.368 min)

Max: 2.8e6 cps

OCH₃
Exact Mass: 455.12
13C-NMR/ TH-3/ DMSO-d6
30.11.09

H2N

Ph

N

NH

F

N

INSTRUM:

F2 - Acquisition Parameters

SO2: 12288

SWM: 17985.61 Hz

FIDRES: 0.274339 Hz

A1: 1.8219598 sec

AM: 32768

BW: 27800 usec

TC: 0.00 usec

TH: 300 K

DE1: 0.02880000000 sec

DE2: 2.0000000000 sec

DELETA: 1.89999995 sec

TD: 1

--- CHANNEL I ---

NUC1: 13C

P1: 9.00 usec

P1: 1.00 (B)

SF01: 75.4752953 MHz

--- CHANNEL II ---

CPU/PW12

NUC2: 13C

PC: 80.00 usec

PL: 0.00 dB

PL: 16.00 dB

SF: 300.1312005 MHz

F2 - Processing parameters

SF: 300.1312005 MHz

WOW: EM

FW: 17.13

LB: 0.00 Hz

GC: 1.80

--- QC: 0.625 to 1.006 min from Sample 15 (IT 1) of 150000 wlf (Ion Spray), subtracted (0.217 to 0.586 min) ---

Max. 8.865 ppm.
+Q1: 0.318 to 0.620 min from Sample 2 (T1-11) of 281209.wiff (Ion Spray), subtracted (1.642 to 1.976 min)

Max. 3.4e6 cps

m/z, amu: 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800

Intensity, cps: 2.0e5, 4.0e5, 6.0e5, 8.0e5, 1.0e6, 1.2e6, 1.4e6, 1.6e6, 2.0e6, 3.0e6, 3.2e6, 3.4e6

Chemical structure: [Diagram of the molecule]