



## **A<sub>2A</sub> Adenosine Receptor NanoBRET® Competitive Binding Assay**

### **Introduction**

The A<sub>2A</sub> adenosine receptor (A<sub>2A</sub>AR) is one of the four adenosine receptor subtypes expressed in the human body (A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>). It participates in the downregulation of the immune system and has been identified as a promising target for treating several diseases, reaching phase II clinical trials for different cancers when used in combination with other immunocheckpoints inhibitors.<sup>1</sup> In their common quest to provide novel and robust screening methods as alternatives to radioligand binding, Celtarys Research and PROMEGA have joined their technologies to foster G protein-coupled receptor (GPCR) drug screening. A NanoBRET® competitive binding assay<sup>2</sup> was developed utilizing a new Celtarys fluorescent ligand in collaboration with Professor Kevin Pflieger's research group at the University of Western Australia, where the assay design and optimization was performed.

GPCRs are one of Celtarys' key targets. Using the previously developed fluorescent A<sub>2A</sub>AR probe CELT-300 as reference, Celtarys designed and optimized a new fluorescent ligand adapted to the NanoBRET® technology, employing NanoBRET® 590 Dyes commercialized by PROMEGA. This ligand was tested and validated by Prof. Pflieger's research group in NanoBRET® competitive binding assays.

### **Combining two technologies into one**

The NanoBRET® Target Engagement (TE) technology developed by Promega relies on bioluminescence resonance energy transfer (BRET) from a NanoLuc® luciferase genetically fused to a target and a fluorescent tracer that binds to the same target. This proximity-based approach utilizes fluorescent tracers in a competitive binding format to quantify interactions between compounds and their cognate targets in intact cells, in real time. In conjunction with Promega's NanoBRET® TE technology, Celtarys has applied its unique semi-combinatorial conjugation technology to develop optimal fluorescent ligands, keeping the activity of the labeled pharmacophore, while optimizing linker composition and conformation, eventually conjugating bright and photostable fluorophores, to obtain the highest detectable signal. Thanks to this technology, the time required for probe development is significantly reduced, leading to fluorescent



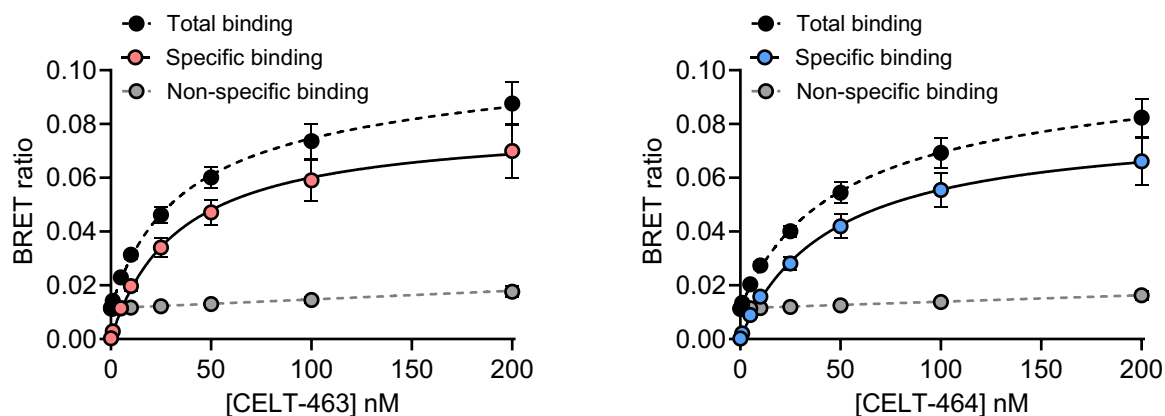
structures able to show the best performance in the desired assay. Here, Celtarys' unique conjugation technology was applied to generate NanoBRET® TE tracers with varying linker compositions and lengths to ensure optimal performance in NanoBRET® TE assays.

## Results

The combination of these two technologies, together with the extensive experience of Prof. Pflieger's research group, led to the development of two new tracers that can be used to verify **target engagement and calculate ligand affinity** for A<sub>2A</sub>AR in a NanoBRET®-based competitive binding assay. The two compounds identified are **CELT-463 and CELT-464**, both bearing the same pharmacophore and fluorophore (NanoBRET® 590 Dye) but different linkers.

### Saturation binding experiments on NanoLuc®-tagged A<sub>2A</sub> receptors.

To start the development of the assay and identify the best concentrations of CELT-463 and CELT-464 to be employed in competitive binding experiments, saturation binding assays were performed, showing consistent curves, high specific binding and optimal signal to noise ratio (Figure 1).

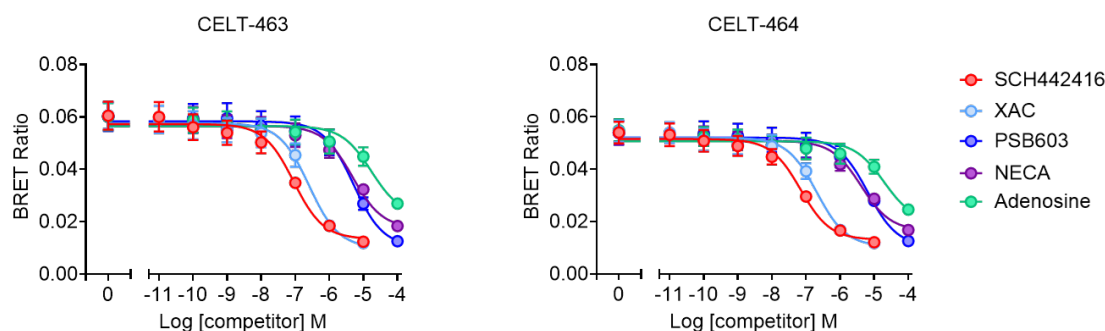


**FIGURE 1. SATURATION BINDING EXPERIMENT FOR CELT-463 ( $K_D = 33 \pm 4$  nM) AND CELT-464 ( $K_D = 44 \pm 5$  nM) USING HEK293FT CELLS TRANSIENTLY TRANSFECTED WITH SIGNAL PEPTIDE-NANOLUC®-A<sub>2A</sub>AR EXPRESSION VECTOR.** TRANSFECTED CELLS WERE TREATED WITH INCREASING CONCENTRATIONS OF CELT-463 OR CELT-464 IN THE PRESENCE (NON-SPECIFIC BINDING) OR ABSENCE (TOTAL BINDING) OF SCH 442416. SPECIFIC BINDING WAS CALCULATED BY SUBTRACTING NON-SPECIFIC BINDING FROM TOTAL BINDING (MEAN  $\pm$  SEM, N=6).

### NanoBRET® competitive binding assays on A<sub>2A</sub>AR with CELT-463 and CELT-464

These data were crucial to set the experimental conditions for competitive binding assays, in which we used 50 nM of the tracer, with increasing concentrations of various competitor compounds. A tracer concentration of

50 nM was selected as it produced a sufficiently large signal window to perform the competition experiment. As reported in Figure 2, the affinity of a heterogeneous set of reference compounds was measured employing CELT-463 or CELT-464 in NanoBRET®-based experiments to validate the assay performance.



**FIGURE 2. MEASUREMENT OF COMPETITIVE LIGAND BINDING TO A<sub>2A</sub>R USING TRACERS CELT-463 AND CELT-464 IN NANO BRET® ASSAYS FOR A SET OF REFERENCE COMPOUNDS.** CELLS EXPRESSING SIGNAL PEPTIDE-NANOLUC®-A<sub>2A</sub>AR WERE TREATED WITH 50 nM CELT-463 OR CELT-464 IN THE PRESENCE OF INCREASING CONCENTRATIONS OF VARIOUS COMPETITOR COMPOUNDS (MEAN ± SEM, N=6).

Both agonists and antagonists, selective or promiscuous for the 4 adenosine receptor subtypes, were included in this set. The data obtained were compared with those reported in literature previously employing radioligand binding assays (Table 1).

Functional activity	Compound	Selectivity	pIC <sub>50</sub> in NanoBRET		pKi in NanoBRET		pKi reported	Ref
			CELT-463	CELT-464	CELT-463	CELT-464		
Antagonist	SCH 442416	Selective against A <sub>2A</sub> AR	6.94 ± 0.14	7.04 ± 0.16	7.3	7.4	8.4 – 10.3	3
	XAC	Promiscuous for the 4 ARs	6.62 ± 0.03	6.70 ± 0.03	7.0	7.1	7.74	4
	PSB603	Selective against A <sub>2B</sub> AR	5.30 ± 0.07	5.16 ± 0.10	5.7	5.6	<5.0	5
Agonist	NECA	Promiscuous for the 4 ARs	5.28 ± 0.07	5.34 ± 0.07	5.7	5.7	6.9 – 8.7	6
	Adenosine	Promiscuous for the 4 ARs	ND	ND	ND	ND	6.5	7

**TABLE 1. SET OF REFERENCE COMPOUNDS TESTED FOR ASSAY VALIDATION, TOGETHER WITH THE REPORTED AND EXPERIMENTAL BINDING DATA. pKi VALUES WERE DERIVED FROM pIC<sub>50</sub> VALUES USING THE CHENG-PRUSOFF EQUATION<sup>8</sup>.**

As observed, the pKi values display a similar rank order of affinity compared to reported values, confirming that the NanoBRET® competitive binding assay on A<sub>2A</sub>AR with CELT-463 and CELT-464 is a reliable and valuable alternative to conventional methodologies.

## Conclusions

This fruitful three-way collaboration between PROMEGA, Celtarys Research, and The University of Western Australia led to the identification of two fluorescent ligands, CELT-463 and CELT-464, which were specifically designed for the development of a NanoBRET®-based methodology to screen the affinity of novel candidates targeting the A<sub>2A</sub> adenosine receptor. Both ligands worked well as NanoBRET® TE tracers in the NanoBRET® competitive binding assay, providing comparable assay window and cellular affinities for the reference compounds.

This proof-of-concept study demonstrates the applicability of Celtarys' chemistry in generating fluorescent ligands compatible with the NanoBRET® TE GPCR assay<sup>2</sup>. This facilitates the development of novel TE assays, which can be readily scaled to 384-well plates for screening of GPCR ligands, and positions Celtarys' chemistry as a valuable tool for advancing GPCR probe development and drug discovery.

## Methods:

### Cell culture and transfection:

1. HEK293FT cells were seeded in 10cm dishes at a density of approximately  $4.2 \times 10^6$  cells/well and maintained at 37 °C, 5% CO<sub>2</sub> in Complete Media (DMEM containing 0.3 mg/ml glutamine) supplemented with 10% fetal calf serum (FCS; Sigma Aldrich).
2. Transient transfection with Signal Peptide-NanoLuc®-A<sub>2A</sub>AR in pcDNA3 expression vector was carried out 24 h after seeding using FuGENE® 6 (Promega) according to manufacturer instructions.
3. 24 h post-transfection, cells were washed with PBS, detached using 0.05% trypsin/0.53 mM EDTA, resuspended in phenol red-free Complete Media containing 5% FCS and added to a poly-L-lysine-coated white 96-well microplate (Greiner Bio-One) at a density of approximately  $5 \times 10^4$  cells/well and maintained at 37°C, 5% CO<sub>2</sub>.

### Saturation binding assay:

1. Fluorescent ligands were serially diluted in 100% DMSO before being further diluted 5-fold using tracer dilution buffer (Promega), which itself was pre-diluted 3-fold in the vehicle (phenol red-free DMEM).
2. 24 h after seeding, media in 96-well microplates was replaced with the vehicle or the competitor ligand SCH442416 (10µM final concentration) followed by the fluorescent ligand (CELT-463 or CELT-464) and incubated at 37°C for 1 hour.

3. NanoBRET® Nano-Glo® Substrate (1:500 dilution, Promega) was added and mixed by orbital shaking prior to BRET measurement using the CLARIOstar microplate reader (BMG Labtech).
4. Filtered light emissions were sequentially measured for 1 s in the 'donor wavelength window' (410-490 nm) and 'acceptor wavelength window' (610 nm long pass) of each well.
5. The BRET ratio was calculated by taking the ratio of the acceptor emission over the donor emission.
6. Specific Binding was calculated by subtracting the Non Specific Binding (BRET ratio for a cell sample treated with labelled ligand and competitor ligand) from the Total Binding (BRET ratio for a second aliquot of the same cell sample treated with labelled ligand and vehicle).

#### Competitive binding assay

1. Fluorescent ligands (50 nM final concentration) were diluted in 100% DMSO before being further diluted 5-fold using tracer dilution buffer (Promega), which itself was pre-diluted 3-fold in the vehicle (phenol red-free DMEM).
2. 24 h after seeding, media in 96-well microplates was replaced with serially-diluted competitor ligands (SCH442416, XAC, Adenosine, NECA, PSB603) made in the vehicle.
3. Following addition of competitor ligands, the fluorescent ligand (CELT 463 or CELT 464) was added and incubated at 37°C for 1 hour.
4. NanoBRET® Nano-Glo® Substrate (1:500 dilution) was added and mixed by orbital shaking prior to BRET measurement using the CLARIOstar microplate reader.
5. Filtered light emissions were sequentially measured for 1 s in the 'donor wavelength window' (410-490 nm) and 'acceptor wavelength window' (610 nm long pass) of each well.
6. The BRET ratio was calculated by taking the ratio of the acceptor emission over the donor emission.

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