Thermal Unfolding of GPCRs

Application Note NT-PR-008

nanoDSF: Label-free Thermal Unfolding Assay of G Protein-Coupled Receptors for Compound Screening and Buffer Composition Optimization

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Abstract

A thermal unfolding based assay using low volume differential intrinsic tryptophan scanning fluorimetry (nanoDSF) was applied to study the stabilizing effects of ligands on G protein-coupled receptors (GPCRs). GPCRs are the fourth largest superfamily in the human genome and are the largest class of targets for drug discovery. The system has been validated using human adenosine A2A receptor (A2AR). A2AR binds natural (adenosine and caffeine) and synthetic ligands with different affinities to mediate a variety of physiological and pharmacological responses. Several well-characterized ligands were used for the unfolding experiments. The ΔT_m shift values obtained from nanoDSF analysis and traditional ligand binding studies correlate well with each other. We further characterized a second human GPCR target (test-GPCR) for which traditional cysteine-reactive DSF has been problematic. nanoDSF demonstrated that small molecule ligands can stabilize the detergent-solubilized receptor, thus showing the target GPCR is active in a selected detergent and lipid-free environment. In addition, we report a buffer composition screen to further stabilize the receptor in its detergent environment for biophysical assays.

Based on our results, we show that the nanoDSF technology will allow the development of an automated screening platform in a label-free environment to evaluate a large number of compounds for lead discovery and to improve receptor stability for biophysical assays by screening buffer conditions.

Introduction

G protein-coupled receptors (GPCRs) are implicated in numerous diseases and are the target of many drug treatments. Obtaining sufficient quantities of purified receptors for biophysical and structural characterization has been a challenge, and is still proving to be difficult. Until recently, of the almost thousand proteins in this family, only a few crystallographic structures have been solved (Figure 1) [1].

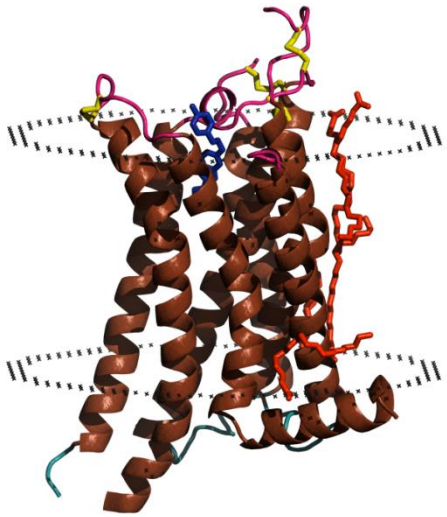

Figure 1: Structure of the human A2AR bound with the potential Parkinson's drug ZM241385 (blue). Several lipid molecules are seen in the X-ray structure (red). Four native disulfide bonds are also indicated (yellow). The seven transmembrane helices are colored in brown; the extracellular and intracellular loops are shown in pink and turquoise, respectively.

Biophysical results using samples of GPCRs, in general, are difficult to obtain because of the receptors' flexibility and their conformational heterogeneity. For biophysical and structural characterization studies, GPCRs need to be extracted from a lipid environment using surfactants and purified to homogeneity in the presence of stabilizing surfactants and a suitable buffer environment. Receptors can also be rapidly denatured upon concentration in absence of a high affinity ligand. Typically, receptor variants, surfactants, buffer compositions (pH, additives, lipids) and ligands are screened to optimize receptor stability for biophysical and structural analysis as well as lead discovery (Figure 2). Here we use the label-free differential scanning fluorimetry (nanoDSF) of the Prometheus NT.48 to determine the thermal stability of two GPCRs in the presence of a variety of different ligands and in a broad range of buffer systems.

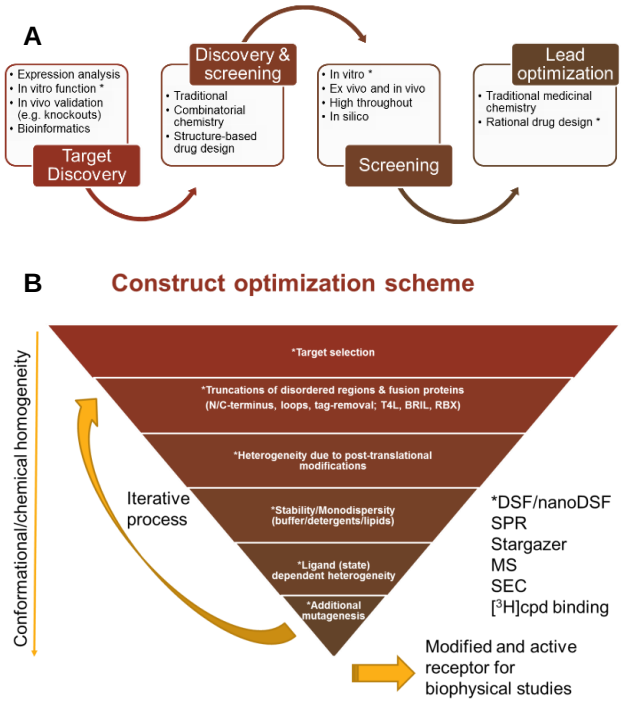

Figure 2: Use of nanoDSF in early lead discovery, screening assays and structural biology. A. Biophysical assays used in the early lead discovery process. **B.** Typical construct optimization cycle diagram of a GPCR target for structural biology. The nanoDSF assay can be used for various points in *in vitro* screening of compounds and construct selection and optimization for biochemical and biophysical assays such as X-ray crystallography (examples of assay possibilities are marked as *).

The adenosine A2AR is a class A GPCR, and is important for neurotransmission, coronary blood flow and respiration. It is blocked by caffeine and is the subject of much research after epidemiological evidence suggested that coffee drinkers have a lower risk of Parkinson's disease [2]. Selective compounds are likely to be useful for the treatment of pain, cancer, Parkinson's disease and Huntington's disease [3].

We first validated the nanoDSF assay using a well-behaving adenosine A2AR variant as a model system. The wild-type amino acid sequence of A2AR contains long disordered regions and post-translational modifications. These are important for receptor functionality and signaling but detrimental for structural biology due to inherent conformational flexibility. There are various modified and stable A2AR constructs described in the literature. We used an engineered A2AR - apocytochrome b562RIL (BRIL) fusion variant rather than agonist/antagonist-trapped variants because of their preferential binding bias towards agonized or antagonized states of the receptor.

After assay validation we used another GPCR target as a test case (test-GPCR) and show that a number of parameters such as ligands, detergents and buffer additives can be assessed for their effectiveness on enhancing receptor thermostability.

Results

Assay development and validation with Adenosine A2A receptor

The thermal stability of A2A-BRIL protein in different detergents was measured using CPM-DSF according to the method described previously (Figure 3) [4]. From the inflection point in a sigmoidal curve, an apparent T_m can be calculated, signifying the temperature at which 50 % of the A2A-BRIL is unfolded. CPM dye specifically binds to the cysteine amino acid which often resides in the hydrophobic folds of the protein. Unbound CPM dye is non-fluorescent but becomes fluorescent upon binding with reactive cysteines.

To validate the nanoDSF technology, we compared binding of ZM241385, a high affinity A2AR subtype specific antagonist, to samples without ligand (apo). As expected, addition of ZM241385 increased the thermostability of A2A-BRIL by 8.3 °C when compared to the apo samples (Figure 4 and

Table 1). The stability effect is in very good agreement with the CPM-DSF assay and with published data under similar conditions. We further tested various commercially available additional ligands to A2AR, including antagonists and agonists (Figure 4 and Table 1). The assay clearly picks up the high affinity ligands and the main rank order between ΔT_m and literature values is maintained.

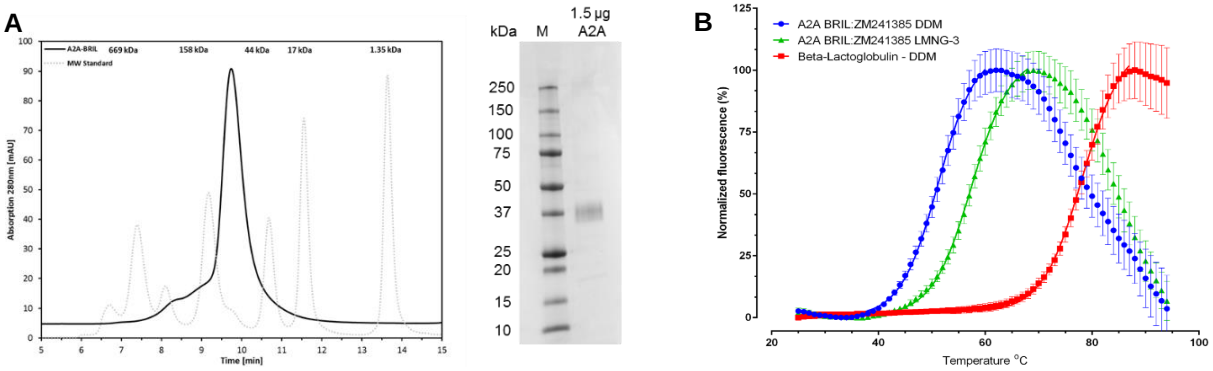

Figure 3. nanoDSF validation with A2AR. A. A2A-BRIL protein quality used in the assay development analyzed by SDS-PAGE and SEC. **B.** Reference CPM-DSF assay ΔT_m curves of A2A-BRIL in presence of ZM241385 using DDM (51 °C) and LMNG (58 °C) as detergents, beta-Lactoglobulin is shown as reference in DDM (78 °C).

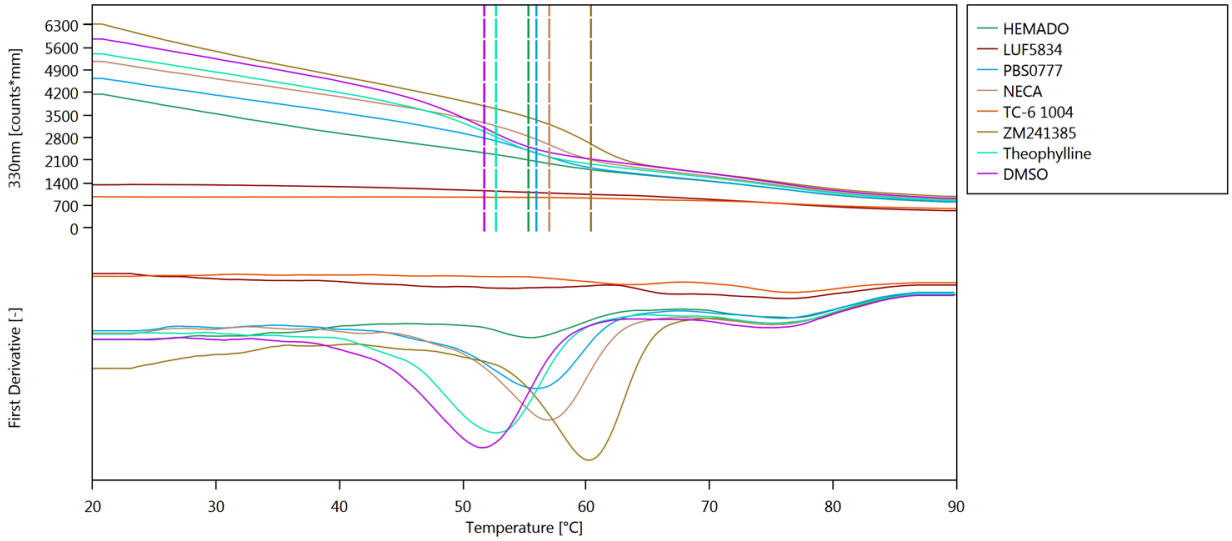

Figure 4: Typical nanoDSF unfolding curves using A2A-BRIL as a reference for class A GPCRs. Recordings of tryptophan fluorescence at 330 nm are shown in the top half of the graph and the corresponding first derivative is plotted in the bottom half. Inflection points (equivalent to the T_m) are shown as vertical lines.

Test Case: screening compound and buffer conditions for stability with a class A GPCR (test-GPCR)

We used a second human class A GPCR BRIL fusion protein as a test case to further verify the application of nanoDSF in compound and buffer stability screening. Previously, we were not able to obtain reliable melting curves for human test-GPCR constructs using a conventional CPM-DSF assay. The CPM-DSF assay worked reasonably well with the rat isoform of test-GPCR using a sample quality similar to the human receptor. We speculated that the lack of cysteine residues in the transmembrane network of the human isoform which are required for CPM fluorescence probe reactivity could be one reason for the lack of melting curves in the CPM-DSF assay. We used a native-PAGE unfolding assay as a reference point for the thermal stability of human test-GPCR. In this assay, protein oligomerization and aggregation is checked after incubation of the sample at different temperatures in native-PAGE. After 30 min incubation, human test-GPCR demonstrated half aggregation at approximately 40 °C (apo) and 60 °C (#22 tool compound for human) (Figure 5), respectively.

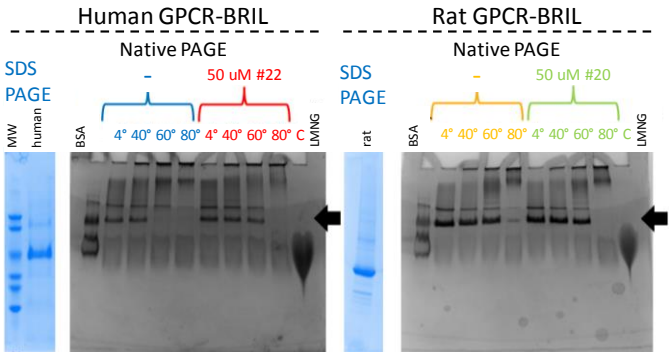

Figure 5: Comparison of human and rat test-GPCR. Upper panel: Complementary native PAGE stability assay for ligand screening using human and rat test-GPCR; CPM-DSF is not working for the human isoform. **Lower panel:** Analysis of monomeric band thermal aggregation in native PAGE. Apoprotein band insensitivity (marked as "🡐") at 4 °C was used as a reference (100 %). Samples were incubated 30 min on ice, at 40 °C, 60 °C or 80 °C with and without high affinity ligand prior to analysis.

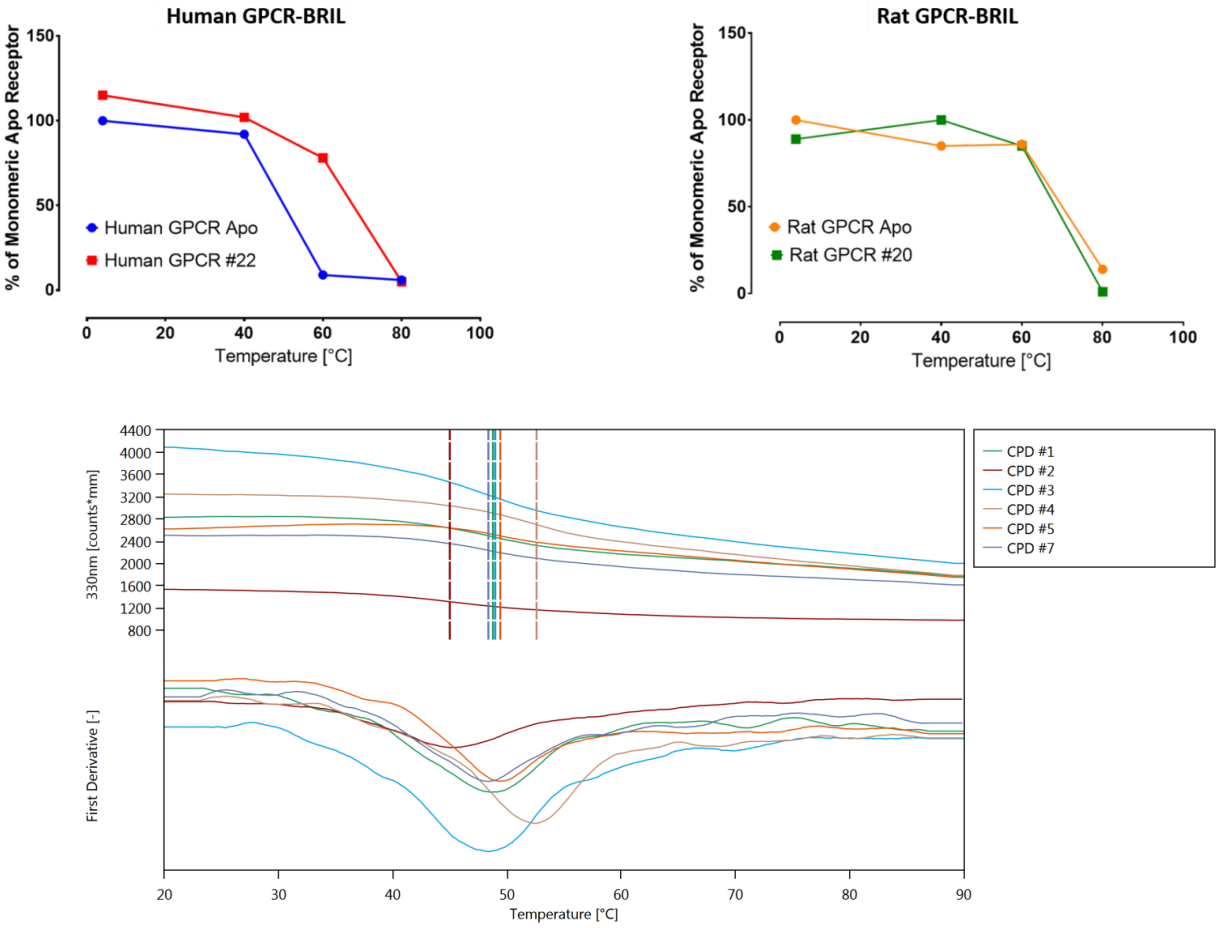

Figure 6: Typical nanoDSF unfolding curves using test-GPCR. Recordings of tryptophan fluorescence at 330 nm are shown in the top half of the graph and the corresponding first derivative is plotted in the bottom half. Inflection points (equivalent to the T_m) are shown as vertical lines.

We were able to obtain melting curves for the test-GPCR in nanoDSF experiments (Figure 6) and used the Prometheus NT.48 system (NanoTemper Technologies) to further characterize the thermal stability of the test-GPCR in presence of 22 selected compounds (Table 2) and different buffer systems (Table 3).

The selected compounds have µM to nM affinity towards human test-GPCR in the functional Ca^{2+}-FLIPR assays. IC50 values obtained from Ca^{2+}-FLIPR assays are reported in Table 2. The rank order of compounds with sub-µM affinity in the calcium reporter assay and strong ΔT_m shift in nanoDSF assay are in good correlation (Table 2). As expected, high µM affinity compounds do not have a pronounced stability effect on the purified receptors (Figure 7 and Table 2), whereas three out of five high affinity compounds show a pronounced increase in thermostability of the receptor (compound #17, #19 and #22). Unexpectedly, compounds #10 and #16 did not show a strong effect on the thermostability of the test-GPCR despite their high affinity in the Ca^{2+}-FLIPR assay.

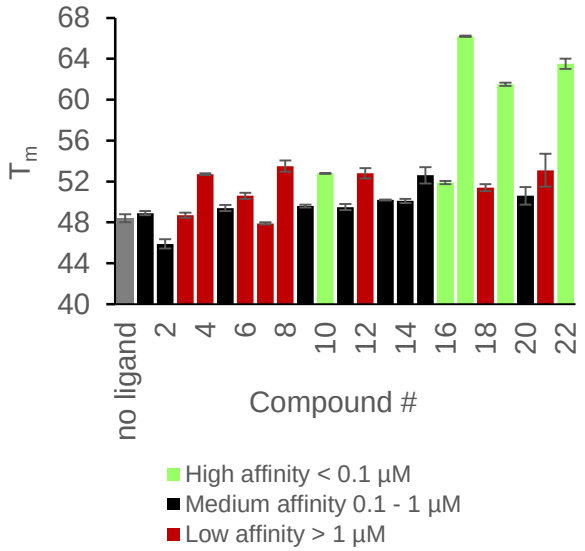

Figure 7: Summary of thermal stabilization of test-GPCR by selected compounds (see also Table 2).

We wanted to further optimize the buffer conditions in the presence of compound #17 which showed the highest ΔT_m in our nanoDSF assay. We constructed a limited matrix screen with different pH values and sodium chloride concentrations (Figure 8 and Table 3).

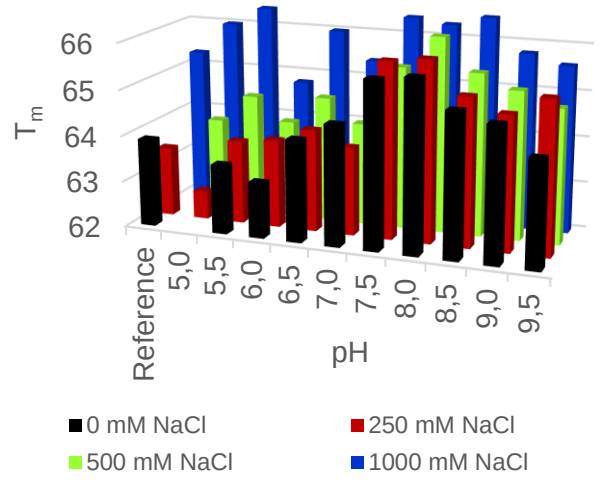

Figure 8: Buffer composition and osmolality screen for human test-GPCR. Receptor was purified in presence of 10 µM compound #17. Best buffer condition ΔT_m 2.6 °C. Receptor-detergent micelles apparently prefer a high osmotic buffer and slightly alkaline buffer.

The receptor:#17:LMNG complex preferred a neutral and slightly alkaline pH buffer (ΔT_m + 1.7 °C). The transition was lost in low pH buffer < 5.5. Notably, the receptor complex was most stable in the presence of high sodium chloride concentration (ΔT_m + 2.6 °C).

Conclusion

In this case study, we demonstrate the usage of the NanoTemper Technologies instrument Prometheus NT.48 in defining thermal unfolding properties of two class A GPCRs for further ligand screening, biophysical characterization and structural biology.

The system was validated with human adenosine A2AR-BRIL fusion protein by detecting changes in intrinsic tryptophan fluorescence at two wavelengths. T_m values of the receptor: ligand complexes could be determined for the majority of the selected ligands. A small portion of the selected ligands showed strong autofluorescence or fluorescence quenching and was not suitable for nanoDSF. Overall, the determined T_m values show a good agreement with published and in-house data with commonly used CPM-DSF assays. Sample consumption and assay time is comparable to standard CPM-DSF assays. A major advantage of the nanoDSF technology compared to CPM-DSF assay is the label-free assay format and straightforward assay development.

We successfully applied nanoDSF for a test case where the standard CPM-DSF assay did not yield reliable and accurate determinations of T_m values. Using nanoDSF, we were able to confirm a rank order of ligands verified with in-house radioligand competition and Ca^{2+}-FLIPR functional assays. We could further improve the receptor stability by including a matrix buffer screen in our assay development for the class A test-GPCR.

Material and Methods

Protein preparation

A2A-BRIL

A2A-BRIL was prepared as previously described [5]. In brief, Sf9 cells in SF-4 Baculo Express ICM (BioConcept) at a density of 2.0x10^6/mL were infected with high-titer recombinant Baculovirus (FlashBac system) at MOI (multiplicity of infection) of 10. Cells were harvested by centrifugation 72 h post infection and stored at -80 °C until use.

Cell membranes were prepared by thawing the pellet in hypotonic buffer containing 10 mM HEPES pH 7.5, 10 mM $MgCl_2$, 20 mM KCl and EDTA-free complete protease inhibitor cocktail (Roche). Cells were broken with a Polytron PT1300D (Kinematica)

and centrifuged at 45.000 RPM in a Ti45 rotor (Beckman Coulter) for 45 min. Membranes were washed extensively by repeating this step 2-3 times with hypotonic buffer, followed by 2-3 washing steps using a high osmotic buffer containing 10 mM HEPES pH 7.5, 1000 mM NaCl, 10 mM $MgCl_2$, 20 mM KCl and EDTA-free complete protease inhibitor cocktail (Roche). Purified membranes were resuspended in buffer containing 10 mM HEPES pH 7.5, 10 mM $MgCl_2$, 20 mM KCl and 40 % Glycerol (w/v) and stored at -80 °C until further use.

Membranes were thawed in the presence of 4 mM Theophylline and 2.0 mg/mL iodoacetamide. After 30 min incubation on ice, membranes were solubilized in 50 mM HEPES pH 7.5, 800 mM NaCl, 10 % Glycerol (w/v), 1 % (w/v) Lauryl Maltose Neopentyl Glycol-3 (LMNG-3) / 0.2 % (w/v) cholesteryl hemisuccinate (CHS) and EDTA-free complete protease inhibitor cocktail (Roche) by stirring for 2 h at 4 °C. Unsolubilized material was removed by centrifugation at 150.000 xg for 45 min at 4 °C. Imidazole was added to the supernatant to a final concentration of 20 mM and incubated with TALON IMAC resin (Clontech) over night. The resin was washed with 10 column volumes (CV) of 50 mM HEPES pH 7.5, 800 mM NaCl, 10 % Glycerol (w/v), 20 mM Imidazole, 0.01 % LMNG-3/0.002 % CHS (w/v) and 2 mM Theophylline, 5 CV of 50 mM HEPES pH 7.5, 800 mM NaCl, 10 % Glycerol (w/v), 25 mM Imidazole, 0.01 % LMNG-3/0.002 % CHS (w/v) and 2 mM Theophylline, and 2 CV of 50 mM HEPES pH 7.5, 800 mM NaCl, 10 % Glycerol, 50 mM Imidazole, 0.01 % LMNG-3/0.002 % CHS (w/v) and 2 mM Theophylline. Bound receptor was eluted with 50 mM HEPES pH 7.5, 800 mM NaCl, 10 % Glycerol (w/v), 300 mM Imidazole, 0.01 % LMNG-3/0.002 % (w/v) CHS and 2 mM Theophylline in a minimal volume.

Purified receptor was buffer exchanged on a PD10 column (GE Healthcare) into 50 mM MES pH 7.5, 800 mM NaCl, 10 % Glycerol (w/v), 0.01 % LMNG-3/0.002 % CHS (w/v), 2 mM Theophylline and concentrated with 100 kDa MWCO Vivaspin concentrators (Sartorius) to 3 mg/mL.

Test-GPCR

Test-GPCR was prepared similar to A2A-BRIL. In brief, Sf9 cells in SF-4 Baculo Express ICM (BioConcept) at a density of $2.0x10^6$/mL were infected with high-titer recombinant Baculovirus (FlashBac system) at MOI (multiplicity of infection) of 10. Cells were harvested by centrifugation 72 h post infection and stored at -80 °C until use.

Cell membranes were prepared by thawing the pellet of 1 L expression culture in 40 mL hypotonic buffer containing 10 mM MES pH 6.0, 10 mM $MgCl_2$, 20 mM KCl and EDTA-free complete protease inhibitor cocktail (Roche). Cells were broken with a Polytron PT1300D (Kinematica), filled up to 300 mL with high salt buffer containing 50 mM MES pH 6.0, 1000 mM NaCl and centrifuged at 45.000 RPM in a Ti45 rotor (Beckman Coulter) for 45 min. Purified membranes were resuspended in buffer containing 10 mM MES pH 6.0, 10 mM $MgCl_2$, 20 mM KCl and 40 % Glycerol (w/v) and stored at -80 °C until further use.

Membranes were thawed on ice and solubilized in 50 mM MES pH 6.0, 800 mM NaCl, 10 % Glycerol (w/v), 1 % (w/v) Lauryl Maltose Neopentyl Glycol-3 (LMNG-3) shaking smoothly 3 h at 4 °C. Unsolubilized material was removed by centrifugation at 150.000xg for 45 min at 4 °C. The supernatant was incubated with TALON IMAC resin (Clontech) over night. The resin was washed with 20 CV of 50 mM MES pH 6.0, 800 mM NaCl, 10 % Glycerol, 50 mM Imidazole, 0.02 % LMNG. Bound receptor was eluted with 50 mM MES pH 6.0, 800 mM NaCl, 10 % Glycerol (w/v), 300 mM Imidazole, 0.02 % LMNG-3 in a minimal volume.

Purified receptor was buffer exchanged on a PD10 column (GE Healthcare) into 50 mM MES pH 6.0, 800 mM NaCl, 10 % Glycerol (w/v), 0.02 % LMNG-3, and concentrated with 100 kDa MWCO Vivaspin concentrators (Sartorius) to 1.3 mg/mL.

Thermal stability screen

A2A-BRIL ligand stability screen

Samples were prepared in 96-well PCR plates on ice. The assay was carried out at a protein concentration of 0.20 mg/mL. Therefore, 2.5 µL of purified A2A-BRIL at 3.0 mg/mL were diluted with buffer containing 50 mM HEPES pH 7.5, 800 mM NaCl, 10 % Glycerol (w/v), 0.01 %LMNG-3/0.002 % CHS into a final volume of 33.75 µL. 3.75 µL of ligand stock solution (1 mM in DMSO) was added and samples were mixed thoroughly. Samples were manually loaded into nanoDSF Grade Standard Capillaries (NanoTemper Technologies) in triplicates and transferred to a Prometheus NT.48 nanoDSF device (NanoTemper Technologies). Thermal unfolding was detected during heating in a

linear thermal ramp (2.5 °C/min; 20 °C to 90 °C) with an excitation power of 80 %. Unfolding transition points were determined from changes in the emission wavelengths of tryptophan fluorescence at 330 nm, 350 nm and their ratios. Data was analyzed with the Prometheus PR. Control software (NanoTemper Technologies).

Test-GPCR buffer stability screen

Samples were prepared in 96-well PCR plates on ice. The assay was carried out at a protein concentration of 0.26 mg/mL. Therefore, 6.0 µL of purified test-GPCR at 1.5 mg/mL were diluted with buffer containing 5 mM MES pH 6.0, 800 mM NaCl, 10 % Glycerol, 0.02 %LMNG-3 into a final volume of 26.25 µL. 8.75 µL of the Solubility & Stability Screen 2 (Hampton Research) was added and samples were mixed thoroughly. Only wells A1-A12, F1-F12, G1-G12 and H1-H12 of the Solubility & Stability Screen 2 were used. Samples were manually loaded into nanoDSF Grade Standard Capillaries (NanoTemper Technologies) in triplicates and transferred to a Prometheus NT.48 nanoDSF device (NanoTemper Technologies). Thermal unfolding was detected during heating in a linear thermal ramp (2.5 °C/min; 20 °C to 90 °C) with an excitation power of 75 %. Unfolding transition points were determined from changes in the emission wavelengths of tryptophan fluorescence at 330 nm, 350 nm and their ratios. Data was analyzed with the Prometheus PR. Control software (NanoTemper Technologies).

Acknowledgments

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Tables

Table 1. Reference and commercial compounds screened using A2A-BRIL.
[1] Strongly autofluorescent compound
[2] Reference without compound

	Drug	Average T_m		ΔT_m	Rank	Lit. Ki	Rank
		°C	±SD	°C	ROC	nM	ROC
	No drug (DMSO)[2]	52.2	0.9				
1	HEMADO	55.0	0.5	2.8	6	1230	14
2	CGS21680 [1]	-				27	8
3	LUF5834	61.7	0.3	9.5	2	12	6
4	PBS0777	56.5	0.6	4.3	5	44	9
5	NECA	57.1	0.3	4.9	4	20	7
6	TC-6 1004	63.9	0.1	11.7	1	0.44	2
7	SCH58261 [1]	-				1.3	4
8	SCH442461 [1]	-				0.048	1
9	ZM241384	60.5	0.3	8.3	3	1	3
10	Theophylline	53.2	0.8	1.0	8	1700	
11	PBS1115	54.3	1.3	2.1	7	60	11
12	Caffeine	54.3	0.1	2.0	7	10200	15
13	Guanfacine	51.8	1.0	-0.4	9		
14	SRF36466	52.0	0.6	-0.2	9		
15	Adenosine	55.7	0.4	3.5	6	360	13
16	W7811 [1]	-					
17	MRS1334	50.4	1.3	-1.8	10	100	12
18	ZK756326	51.8	0.2	-0.4	9		
19	ANR94	54.6	1.0	2.4	7	46	10
20	Istradefyll	55.7	0.3	3.5	6	2.2	5

Table 2. Blinded compound screen for test-GPCR.
[1] Reference without compound
[2] Classification: 1: IC50 less than 100 nM. 2: IC50 between 101 nM and 1000 nM. 3: IC50 over 1 µM

Cpd	Average T_m		ΔT_m	Rank	IC50	Rank	Class[2]
	°C	±SD	°C	ROC	nM	ROC	
Apo[1]	48.4	0.8					
1	48.9	0.4	0.5	8	2066	17	3
2	45.9	0.9	-2.5	11	1427.5	15	3
3	48.7	0.5	0.3	9	4767.5	19	3
4	52.7	0.2	4.0	5	333.5	8	2
5	49.4	0.6	0.9	8	15918	20	3
6	50.6	0.6	2.2	7	118	6	2
7	47.9	0.2	-0.5	10	530	9	2
8	53.5	1.1	5.1	4	178	7	2
9	49.6	0.3	1.2	8	1169.5	14	3
10	52.8	1.6	4.4	5	41	3	1
11	49.5	0.6	1.1	8	21884	21	3
12	52.8	0.1	4.4	5	824.5	12	2
13	50.2	0.1	1.8	7	1625	16	3
14	50.1	0.4	1.7	7	2456	18	3
15	52.6	1.6	4.2	5	30000	22	3
16	51.9	0.3	3.5	5	32	2	1
17	66.2	0.1	17.8	1	28	1	1
18	51.4	0.7	3.0	6	789	11	2
19	61.5	0.3	13.1	3	43.5	4	1
20	50.6	1.7	2.2	7	1005	13	3
21	53.1	3.2	4.7	4	579	10	2
22	63.5	1.0	15.1	2	54	5	1

Table 3. Buffer composition and osmolality screen for human test-GPCR. Receptor was purified in presence of 10 µM compound #17.

N°.	Buffer	NaCl	T_m (n=3)	±SD	ΔT_m
		mM	°C	°C	°C
1	Sample in purification buffer (reference)	0	63.9	0.3	
2	50 mM Sodium acetate trihydrate pH 4.5	0			
3	50 mM Sodium citrate tribasic dihydrate pH 5.0	0			
4	50 mM Succinic acid pH 5.5	0	63.5	0.2	-0.4
5	50 mM MES monohydrate pH 6.0	0	63.2	1.0	-0.7
6	50 mM BIS-TRIS pH 6.5	0	64.2	0.1	0.3
7	50 mM Imidazole pH 7.0	0	64.6	0.5	0.7
8	50 mM HEPES pH 7.5	0	65.6	0.9	1.7
9	50 mM Tris pH 8.0	0	65.7	0.4	1.7
10	50 mM BIS-TRIS Propane pH 8.5	0	65.1	1.0	1.2
11	50 mM AMPD pH 9.0	0	64.9	0.1	0.9
12	50 mM Glycine pH 9.5	0	64.3	0.9	0.3
13	Sample in purification buffer	250	63.5	0.4	-0.4
14	50 mM Sodium acetate trihydrate pH 4.5	250			
15	50 mMSodium citrate tribasic dihydrate pH 5.0	250	62.6	1.0	-1.4
16	50 mM Succinic acid pH 5.5	250	63.8	0.3	-0.1
17	50 mM MES monohydrate pH 6.0	250	63.9	0.3	0.0
18	50 mM BIS-TRIS pH 6.5	250	64.2	0.4	0.2
19	50 mM Imidazole pH 7.0	250	63.9	0.2	0.0
20	50 mM HEPES pH 7.5	250	65.8	1.4	1.8
21	50 mM Tris pH 8.0	250	65.9	0.8	2.0
22	50 mM BIS-TRIS Propane pH 8.5	250	65.2	0.6	1.3
23	50 mM AMPD pH 9.0	250	64.9	0.5	1.0
24	50 mM Glycine pH 9.5	250	65.3	0.1	1.4
25	Sample in purification buffer	250	63.7	0.3	-0.2
26	50 mM Sodium acetate trihydrate pH 4.5				
27	50 mM Sodium citrate tribasic dihydrate pH 5.0	500	64.0	0.3	0.0
28	50 mM Succinic acid pH 5.5	500	64.6	0.2	0.7
29	50 mM MES monohydrate pH 6.0	500	64.1	0.4	0.2
30	50 mM BIS-TRIS pH 6.5	500	64.7	0.3	0.7
31	50 mM Imidazole pH 7.0	500	64.2	0.2	0.3
32	50 mM HEPES pH 7.5	500	65.5	0.3	1.6
33	50 mM Tris pH 8.0	500	66.2	0.8	2.2
34	50 mM BIS-TRIS Propane pH 8.5	500	65.5	0.3	1.6
35	50 mM AMPD pH 9.0	500	65.2	0.4	1.3
36	50 mM Glycine pH 9.5	500	64.9	0.6	0.9
37	Sample in purification buffer	1000	65.3	0.3	1.3
38	50 mM Sodium acetate trihydrate pH 4.5	1000			
39	50 mM Sodium citrate tribasic dihydrate pH 5.0	1000	66.0	0.6	2.0
40	50 mM Succinic acid pH 5.5	1000	66.4	0.4	2.5
41	50 mM MES monohydrate pH 6.0	1000	64.8	0.9	0.9
42	50 mM BIS-TRIS pH 6.5	1000	66.0	0.1	2.0
43	50 mM Imidazole pH 7.0	1000	65.4	0.2	1.4
44	50 mM HEPES pH 7.5	1000	66.4	0.4	2.5
45	50 mM Tris pH 8.0	1000	66.3	0.2	2.4
46	50 mM BIS-TRIS Propane pH 8.5	1000	66.5	0.3	2.6
47	50 mM AMPD pH 9.0	1000	65.8	0.6	1.8
48	50 mM Glycine pH 9.5	1000	65.6	0.5	1.7

Best buffer condition ΔT_m + 2.6 °C. Receptor-detergent micelles apparently prefer a high osmotic buffer and slightly alkaline buffer.

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