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A Time-Resolved FRET Cell-Based Binding Assay for the Apelin Receptor

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Analogues of apelin-13 carrying diverse spacers and an ad hoc DY647-derived fluorophore were designed and synthesized by chemoselective acylation of α-hydrazinopeptides. The resulting probes retain very high affinity and efficacy for both the wild-type and SNAP-tagged apelin receptor (ApelinR). They give a time-resolved FRET (TR-FRET) signal with rare-earth lanthanides used as donor fluorophores grafted onto the SNAP-tagged receptor. This specific signal allowed the validation of a binding assay with a high signal-to-noise ratio. In such an assay, the most potent sub-nanomolar fluorescent probe was found to be competitively displaced by the endogenous apelin peptides with binding constants similar to those obtained in a classical radioligand assay. We have thus validated the first TR-FRET cell-based binding assay for ApelinR with potential high-throughput screening applications.

Introduction

The APJ receptor, a class A G protein-coupled receptor (GPCR), has been paired with its cognate ligand apelin, a 36-residue peptide[1] derived from a single 77-residue precursor known as preproapelin.[2,3] Several apelin peptides of various lengths are produced, including apelin-36, apelin-17 (**1**), apelin-13, and its pyroglutamate analogue **2** (Table 1).[2,4,5] These peptides inhibit forskolin-induced cyclic AMP (cAMP) production in cells expressing either the human[2,6] or rat apelin receptor (ApelinR),[7,8] and are potent inducers of ApelinR internalization in a clathrin-dependent manner.[6,9]

Table 1. Amino acid sequences and binding properties of **1** and **2**.[8,16]

Peptide	Sequence	K_i [nM][a]
1	KFRRQRPRLSHKGPMPF-OH	0.04 ± 0.002
2	pERPRLSHKGPMPF-OH	0.37 ± 0.14

[a] Values are the mean ± SEM of three independent experiments performed in triplicate.

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Apelin and its receptor are widely distributed in the brain[3,7,10] where they co-localize with arginine vasopressin (AVP) in magnocellular neurons,[4,11] but they are also present in the cardiovascular system.[12] Thus, various studies have demonstrated the role of apelin in the maintenance of body fluid homeostasis[5,4,13] and cardiovascular functions.[9,14,15] The positive inotropic, vasodilator, and diuretic effects[4] of apelin suggest that the ApelinR could be a new therapeutic target for the treatment of heart failure and water retention. However, these studies are hampered by the lack of nonpeptidic ApelinR agonists.

To date, very few nonpeptidic ligands of the ApelinR have been described (Figure 1). In an attempt to discover the first nonpeptidic ApelinR agonist, we established an original Förster resonance energy transfer (FRET)-based binding assay that consists of screening libraries of fluorescent small organic compounds against enhanced green fluorescent protein (EGFP)-fused GPCRs.[16,17] The application of this strategy to ApelinR resulted in the discovery of E339-3D6 (**3**), which behaves as a partial agonist with regard to cAMP production and as a full agonist with regard to ApelinR internalization.[8,16] The structure–activity relationship (SAR) studies around **3** led to the identification of two ligands, LIT1349 (**4**) and LIT1109 (**5**), with different pharmacological profiles.[18] Compound **4** is a full agonist with regard to cAMP production and receptor internalization, whereas **5** behaves as a biased agonist toward cAMP production.[18] More recently, by using a β-arrestin-based functional assay, a high-throughput screen of the NIH small molecule collection (330 600 compounds) led to the identification of ML221, which was described as an ApelinR functional antagonist with regard to cAMP production and β-arrestin recruitment.[19] Small-molecule agonists were also reported, such as a pyrazole[20] and triazole[21] series with sub-micromolar efficacy

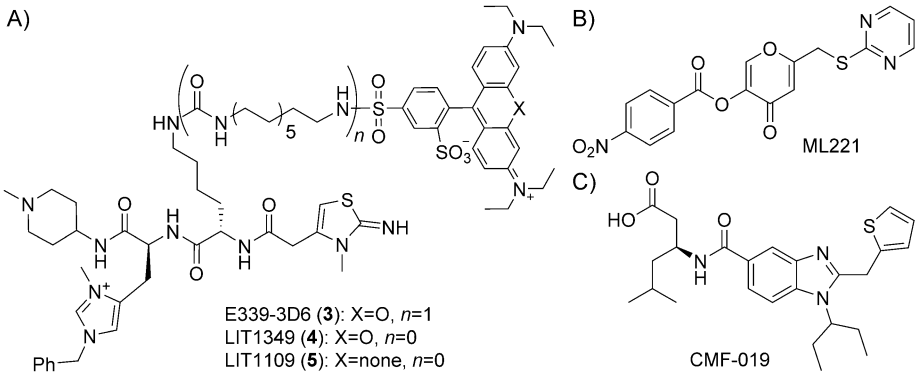


Figure 1. Nonpeptidic ligands described in the literature: A) Agonists (**3** and **4**) or biased agonist (**5**) with regard to inhibition of cAMP production.[16,18] B) Antagonist (ML221) with regard to inhibition of cAMP production and recruitment of β-arrestin.[19] C) Biased agonist toward G protein signaling over β-arrestin recruitment.[22]

and affinity for the ApelinR, and CMF-019, which was reported as the first biased small-molecule apelin agonist toward G protein signaling over β-arrestin recruitment.[22]

To accelerate the discovery of other potent small-molecule agonists, it is important to develop innovative assays that are readily amenable to high-throughput screening (HTS). Thus, the FRET-based assay that we developed does not require any washing steps and enables the convenient and rapid monitoring of ApelinR–ligand interactions. However, the significant intrinsic fluorescence of cells combined with the spectral overlap between the donor (EGFP) and acceptor (Lissamine rhodamine B) emission spectra largely impair the signal-to-noise (S/N) ratio and limits the applicability of this assay to HTS.

To overcome these limitations, we decided to develop a time-resolved FRET (TR-FRET)-based assay using rare-earth lanthanides with long emission half-lives as FRET donors. This assay is based on Tag-lite® technology, which combines homogeneous time-resolved fluorescence (HTRF) detection with a covalent labeling technology called SNAP-tag.[23,24] This approach provides significant benefits for HTS over classical radioligand binding assays.[25] It also opens the way to the study of GPCR oligomerization using cell lines[24,26] or native tissues.[27] However, the prerequisite to set up such an assay is the ad hoc design and synthesis of high-affinity fluorescent ligands for the receptor.

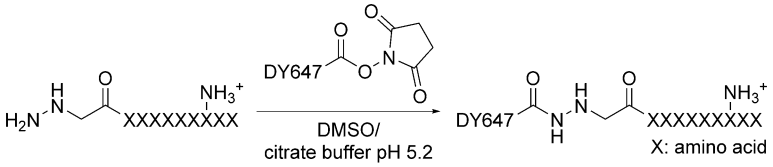
Relying on the recently described far-red fluorescent ligands with sub-nanomolar affinities for ApelinR,[28] herein we report the development of a TR-FRET-based binding assay for this receptor. Competition experiments were performed in the presence of **1** and **2** as competitors to fully validate the specificity of the assay. The latter represents the first TR-FRET-based binding assay for ApelinR described to date with potential HTS application.

Results and Discussion

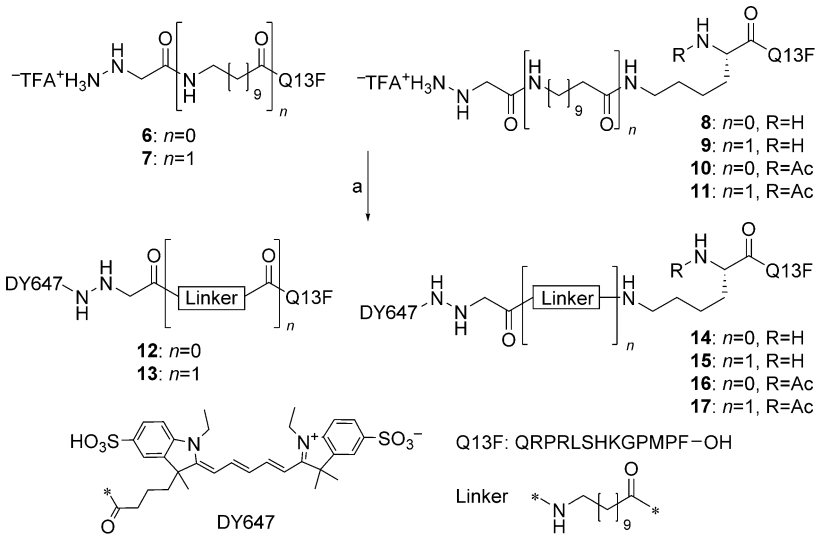
Design and synthesis

As previously reported,[6] the pyroglutamylated or free N-terminal amino forms of apelin-13 both display the same binding affinity for ApelinR and the same ability to inhibit forskolin-induced cAMP production, suggesting that the α-amino group of apelin does not interact with ApelinR. Therefore, we decided to incorporate the dye either at the N-terminal part of apelin-13 or linked to the Nε atom of an extra N-terminal lysine. The free amino or acetylated forms of this latter were envisaged to investigate the influence of a positive charge on the binding affinity. In addition, to evaluate the potential steric clash between the dye and the binding pocket of the receptor, the dye was introduced either directly on the peptide or through an aminoundecanoic acyl linker. The DY647 cyanine dye (Scheme 2 below) was selected as fluorophore owing to its fluorescence properties (λ_{abs} = 648 nm and λ_{em} = 664 nm in HEPES buffer pH 7.4) compatible with HTRF assay. The solid-phase strategy usually requires a large excess of reagents to drive the reaction to completion. Thus, due to the cost of the dye, its incorporation on the peptide was envisaged in solution by acylation of fully deprotected and purified hydrazino-peptides with the commercially available *N*-hydroxysuccinimidyl ester of DY647 (Scheme 1).[29,30]

Thus, six hydrazinopeptides **6**–**11** were first obtained by following a solid-phase approach as previously described[28] (see



Scheme 1. General strategy for the synthesis of fluorescent conjugates by chemoselective acylation of hydrazinopeptides.



Scheme 2. Synthesis of fluorescent conjugates **12**–**17** by chemoselective acylation of hydrazinopeptides **6**–**11**. *Reagents and conditions*: a) DY647-NHS, DMSO/citrate-phosphate buffer 25 mM, pH 5.2.[28]

Supporting Information), and involved in chemoselective acylation with the *N*-hydroxysuccinimide ester of DY647 dye in citrate-phosphate buffer (pH 5.2) for 3 h (Scheme 2). These experimental conditions enabled the protection of the ε-amino group of Lys6 ($pK_a \approx 10$) through protonation, and the discrimination between the ε-amino and α-hydrazino ($pK_a \approx 6$) groups. In addition, the very mild acylation conditions were found to be fully compatible with the chemical stability of both the dye and the hydazino-peptides. The six fluorescent conjugates **12**–**17** were thus obtained in 37–55% isolated yields and were evaluated for their binding affinities to the ApelinR. The influence of the location of the dye (either at the N terminus of peptides or linked to the Nε amino group of an extra N-terminal lysine) and the presence of a linker on the affinity of the resulting conjugates were carefully investigated.

Table 2. Spectral and binding properties of fluorescent peptides **12**–**17**.

Compd	λ_{max} [nm][a] Abs	Em	Linker (*n*)	R	K_i [nM][b]
12	651	665	0	–	1.70 ± 0.20
13	651	665	1	–	1.51 ± 0.08
14	651	665	0	H	1.15 ± 0.24
15	651	665	1	H	0.44 ± 0.09
16	651	665	0	Ac	4.77 ± 0.70
17	649	665	1	Ac	2.17 ± 0.17

[a] The absorption and emission parameters were measured in HEPES/0.1% BSA, pH 7.4; the excitation wavelength was set at 620 nm. [b] The inhibition constants of fluorescent compounds were determined with membranes from CHO cells stably expressing the human ApelinR using radioactive [^{125}I]**2**.

SAR studies

The affinities of **12**–**17** for the ApelinR were first assessed by competition binding experiments on Chinese hamster ovary (CHO) cell membranes stably expressing the human ApelinR, using [^{125}I][pyroGlu1]-apelin-13 ([^{125}I]**2**), iodinated by the Bolton–Hunter method on lysine 8, as radioligand (Table 2). The SAR study performed on the six ligands demonstrates that, as expected, the N-terminal part of apelin-13 is the position of choice to incorporate the dye, resulting on nanomolar conjugates (K_i range: 0.44–4.77 nM). On the other hand, the presence of an aminoundecanoic acyl linker between the peptide and the dye has no significant impact on affinity (**12** and **13**, K_i: 1.70 and 1.51 nM, respectively). Finally, the presence of a free α-amino group at the N-terminal part of the labeled peptides **14** and **15** results in a significant increase in affinity of the fluorescent conjugate (K_i: 1.15 vs. 4.77 nM for **14** and **16**, and K_i: 0.44 vs. 2.17 nM for **15** and **17**, respectively). This result could be ascribed to the contribution of the α-amino group of the lysine residue to an additional interaction with the ApelinR that is not present with peptide **2**. Altogether, this SAR study enabled the discovery of **15**, which is the first subnanomolar ($K_i = 0.44 \pm 0.09$ nM) far-red fluorescent ligand for the ApelinR.

Effect of probe 15 on IP production

The functional activity of probe **15** was evaluated by the measurement of inositol phosphate (IP) accumulation following cell incubation with increasing concentrations of **15**, and was compared with that obtained with peptide **2** (Figure 2). To this end, wild-type (WT) ApelinR was transfected in HEK293 cells with a chimeric G protein, Gqi9,[31] which allows the receptor to activate phospholipase C upon agonist stimulation, thereby triggering the IP cascade. As shown in Figure 2, stimulation of WT ApelinR with probe **15** induced IP accumulation (Figure 2A) as efficiently as native peptide **2** (Figure 2B), with EC_{50} values of 1.20 ± 0.01 vs. 1.13 ± 0.18 nM, respectively. Thus, probe **15** behaves as full agonist with regard to IP production, in the same way as endogenous peptide **2**, demonstrating that

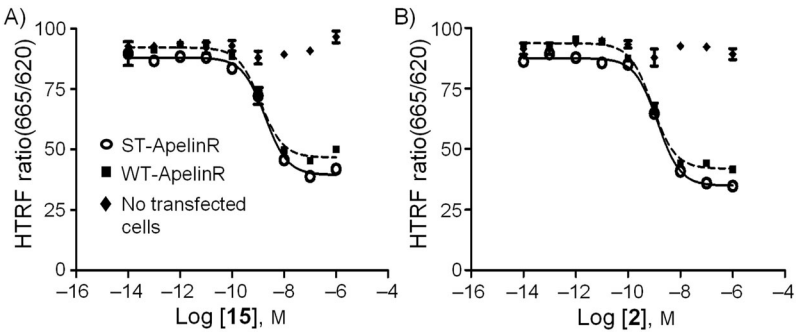


Figure 2. Effects of A) **15** and B) **2** on IP accumulation in HEK293 cells transfected with a chimeric G protein Gqi9 and ST or WT ApelinR. IP was quantified using the HTRF IP-One Gq assay kit (see Experimental Section). Results are expressed as the mean ± SEM of three independent experiments performed in triplicate.

the incorporation of DY647 on apelin-13 has no impact on either the affinity or the functional activity of the resulting fluorescent probe.

TR-FRET-based binding assay

Taking advantage of the spectroscopic properties and the potency of **15**, the first TR-FRET-based binding assay for ApelinR was developed. Tag-lite® technology requires the use of a SNAP-tag enzyme fused to the N-terminal part of a GPCR to enable its subsequent labeling with Lumi4-Tb-donor-derivatized benzylguanine substrate (SNAP-Lumi4-Tb).[32] Although previous reports have suggested that the N-terminal modification of the GPCR does not alter its functional response,[33,34] it was important to verify that the functional properties of the newly expressed SNAP-tagged (ST) ApelinR were similar to those determined for the WT ApelinR. Therefore, the ability of ST ApelinR to induce IP accumulation upon stimulation by endogenous peptide **2** was measured and compared with WT ApelinR. As shown in Figure 2B, peptide **2** induced similar IP accumulation in HEK293 cells expressing either WT or ST ApelinR, with EC_{50} values of 0.75 ± 0.12 vs. 1.13 ± 0.18 nM, respectively. These results show that the ST fusion does not affect the functional response of ApelinR. Similarly to peptide **2**, increased concentrations of **15** induced IP accumulation in HEK293 cells expressing either WT or ST ApelinR with EC_{50} values of 1.20 ± 0.01 and 1.65 ± 0.15 nM, respectively (Figure 2A).

Next, to validate the binding properties of ST ApelinR, the dissociation constant (K_d) for probe **15** was determined by saturation experiments in plotting specific TR-FRET signal as a function of compound **15** concentration (Figure 3A). The resulting K_d value for **15** (0.08 ± 0.01 nM) is in the same subnanomolar range as its K_i value determined by classical radioli-

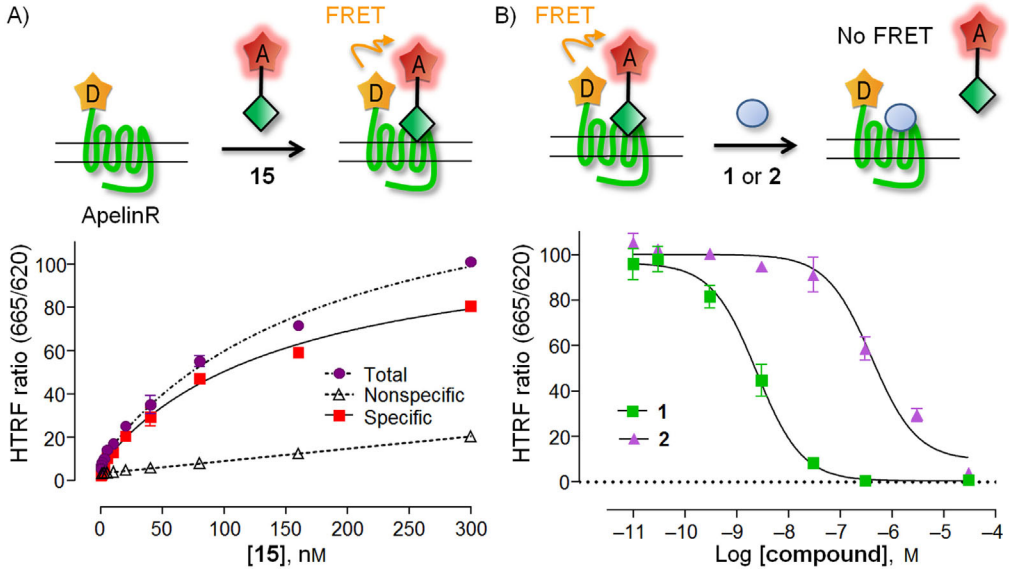


Figure 3. Pharmacological validation of probe **15**: A) Time-resolved FRET-based saturation experiments with cells expressing ApelinR fused to SNAP-tag incubated in the presence of SNAP-Lumi4-Tb to label the receptor; cells were then incubated in the presence of increasing concentrations of fluorescent ligand **15**, and TR-FRET signal (665/620 ratio) was plotted as a function of ligand concentration. B) TR-FRET competition binding assays were performed on the same cells as described in panel A. Cells were then incubated with a constant concentration of fluorescent ligand **15** as tracer, and increasing concentrations of **1** and **2** as competitors. TR-FRET signal intensity (665/620 ratio) was plotted as a function of competitor concentration. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate.

gand binding assay (0.44 ± 0.09 nM). Finally, competition experiments with **15** as tracer, and **1** and **2** as competitors were performed (Figure 3B). K_i values determined for **1** and **2** (0.026 ± 0.004 and 2.93 ± 0.20 nM, respectively) were close to those determined by radioligand binding assay (Table 1).[16] In addition, calculation of the statistical parameter Z' (0.7) confirmed the robustness of the assay. Altogether, these results validate the newly developed TR-FRET binding assay for ApelinR as a powerful and robust alternative to radioligand binding assays.[34]

Conclusions

The ApelinR controls a number of important physiological functions that remain to be further explored at the cellular level as well as in whole organisms. To this end, there is still a need for the rapid discovery of bioavailable ligands that can serve as useful pharmacological probes and precursors of drug candidates. One major bottleneck toward this goal is the availability of an efficient, robust, and safe binding assay amenable to HTS. We were able to design, synthesize, and characterize potent and specific fluorescent probes derived from apelin-13 compatible with the TR-FRET assay. Notably, the DY647 dye was advantageously introduced in solution by a chemoselective acylation of fully deprotected α-hydrazinopeptides derived from apelin-13. With the most potent fluorescent probe exhibiting sub-nanomolar affinity for the ApelinR, we developed the first TR-FRET binding assay to investigate ligand–ApelinR interactions at the cell surface with potential HTS applications.

Experimental Section

General methods. Reagents were obtained from commercial sources and were used without any further purification. Hydrazinopeptides **6**–**11** were synthesized as previously described (see Supporting Information).[28] SNAP-Lumi4-Tb (Ref: SSNPTBX) and IP-One Gq assay kit (Ref: 62IPAPEC) were purchased from Cisbio Bioassays (Codolet, France), and DY647-NHS was from Dyomics GmbH (Jena, Germany). $(Boc)_2NN(Boc)CH_2CO_2H$ was synthesized as previously described.[29] Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates. Flash chromatography was performed on silica gel pre-packed columns (30 μm, Interchim) or on RP18 (30 μm, Interchim) on a SPOT II ultima instrument from Armen. ^{1}H NMR spectra were recorded at 400 MHz on a Bruker Advance spectrometer. Chemical shifts are reported in parts per million (ppm), and coupling constants (*J*) are reported in hertz (Hz). Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) separations were performed on a C_{18} Ascentis Express column (2.7 μm, 4.6 mm × 75 mm) using a linear gradient (5–95% solvent B in solvent A over 7 min, flow rate: 1.6 mL min^{-1}, detection at 220 nm; solvent A: water/0.1% TFA, solvent B: acetonitrile/0.1% TFA). Semipreparative RP-HPLC separations were performed on a Waters Symmetry Shield RP-C_{18} column (7 μm, 19 mm × 300 mm) using a linear gradient (solvent B in solvent A (both as described above); flow rate: 20 mL min^{-1}; detection at 220 nm). Purified final compounds eluted as single and symmetrical peaks (thereby confirming a purity of ≥95%) at retention times (t_R) given below. Their identity was determined by high-resolution mass spectrometry (HRMS) performed on a Bruker MicroTof mass spectrometer, using electrospray ionization (ESI) and a time-of-flight (TOF) analyzer.

General procedure for the chemoselective acylation of compounds 12–17. Hydrazinopeptides **6**–**11** were dissolved in a citrate-phosphate buffer (25 mM, pH 5.2, 3 times the volume of the solution of DY647-NHS in DMSO), and the mixture was re-adjusted to pH 5.2 with Na_2HPO_4 (0.2 M) if needed. A solution of DY647-NHS in DMSO was added, and the mixture was stirred at room temperature under argon atmosphere. Completion of the reaction was monitored by analytical RP-HPLC (usually 1 h). The expected labeled compounds were isolated by semipreparative RP-HPLC on a SunFire RP-C_{18} column using a linear gradient of solvent B in solvent A. Fractions containing the products of interest were lyophilized and further checked by analytical RP-HPLC. For **12**: blue solid (554 nmol, 55%); HRMS (ESI) calcd for $C_{103}H_{154}N_{27}O_{24}S_3$ ($[M+3H]^{3+}$) 749.69407, found 749.69157. For **13**: blue solid (633 nmol, 65%); HRMS (ESI) calcd for $C_{114}H_{175}N_{28}O_{25}S_3$ ($[M+3H]^{3+}$) 810.74817, found 810.74752. For **14**: blue solid (339 nmol, 37%); HRMS (ESI) calcd for $C_{109}H_{167}N_{29}O_{25}S_3$ ($[M+4H]^{4+}$) 594.54625, found 594.54204. For **15**: blue solid (319 nmol, 46%); HRMS (ESI) calcd for $C_{120}H_{188}N_{30}O_{26}S_3$ ($[M+4H]^{4+}$) 640.33683, found 640.33617. For **16**: blue solid (473 nmol, 49%), HRMS (ESI) calcd for $C_{111}H_{169}N_{29}O_{26}S_3$ ($[M+4H]^{4+}$) 605.04894, found 605.04546. For **17**: blue solid (580 nmol, 58%); HRMS (ESI) calcd for $C_{122}H_{190}N_{30}O_{27}S_3$ ($[M+4H]^{4+}$) 650.83947, found 650.83750.

Radioligand binding assay. Membrane preparations from CHO cells stably expressing the human ApelinR were purchased from PerkinElmer (PerkinElmer, Wellesley, MA, USA). Membrane preparations (0.5 μg total mass of membranes per assay) were incubated for 1 h at 20 °C with 0.2 nM [^{125}I]**2** (PerkinElmer) in binding buffer (50 mM HEPES, 5 mM $MgCl_2$ pH 7.5, BSA 1%) alone or in the presence of the different compounds to be tested at various concentrations. The reaction was stopped by adding 4 mL of cold binding buffer and filtered on Whatman GF/C filters. After washing, radioactivity was counted with a Wizard 1470 Wallac γ-counter (PerkinElmer, Turku, Finland).

TR-FRET binding assay. This assay was performed as previously described.[22] Briefly, ready-to-use Tag-lite® ApelinR labeled cells (Cellcust, lot FRUSAPELI01, Cisbio Bioassays) from frozen aliquots were thawed, washed once in the Tag-lite® labeling medium (Ref: LABMED, Cisbio Bioassays), and distributed in white 384-well small-volume plates (5000 cells per well). For saturation assays, increasing concentrations (0–300 nM) of fluorescent ligand **15** diluted in the Tag-lite® labeling medium supplemented with 1% DMSO were added to the cells and incubated at room temperature for 3 h. For each concentration, nonspecific binding was determined by adding excess unlabeled apelin-13 diluted in the same buffer. For competition assays, increasing concentrations of competitor ligands **1** or **2** (from 0.01 nM to 30 μM) diluted in the Tag-lite® labeling medium supplemented with 1% DMSO were first added to the cells, then a fixed concentration of **15** (10 nM) was added, and the plate was incubated at room temperature for 3 h. In both assays, plates were read in an HTRF-compatible multi-well plate reader (Envision, PerkinElmer) with a classic HTRF protocol (excitation at 337 nM, donor emission measured at 620 nm and acceptor emission at 665 nm, 50 μs delay, 400 μs integration). The TR-FRET signal was collected both at 665 and 620 nm, and HTRF ratios were obtained by dividing the acceptor signal (665 nm) by the donor signal (620 nm) and multiplying this value by 10 000.

Data analysis and statistics. K_d values of the fluorescent and radioactive ligands were determined from saturation curves of the spe-

cific binding using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). K_i values of the compounds were determined from binding competition experiments according to the Cheng–Prusoff equation.[35] S/N calculations were performed by dividing the mean of the maximum value (λ_{max}) by that of the minimum value (λ_{min}) obtained from the sigmoid fits. Comparisons between values are reported as means ± SEM.

IP accumulation assay. The inositol phosphate (IP) accumulation assay was carried out 24 h after transitory transfection on adherent HEK293 cells in a black 96-well plate of a chimeric G protein Gqi9 and WT ApelinR (Cisbio Bioassays) or SNAP-ApelinR (Ref: PSNAPAGTRL1, Cisbio Bioassays). IP accumulation corresponding to the basal activity of the non-transfected cells was used as a control. IP1 accumulation was measured using an IP-One Gq assay kit (Ref: 62IPAPEC, Cisbio Bioassays). Briefly, cells were stimulated for 45 min at 37 °C with the ligand to be tested in 70 μL stimulation buffer. An anti-IP1 antibody labeled with cryptate (15 μL) and an IP1-d2 derivative (15 μL) were added to the cells and incubated for 1 h at room temperature. TR-FRET signals at 665 and 620 nm were detected using a PHERAstar (BMG Labtech) fluorescence reader with the same settings as described in the TR-FRET binding assay section above, and HTRF ratios were calculated using the same method.

Tag-lite membrane expression assay. HEK293 cells were transitory transfected with SNAP-ApelinR (Ref: PSNAPAGTRL1, Cisbio Bioassays) or pCDNA3.1 empty vector as control, in black 96-well plates, and then incubated for 24 h at 37 °C under 5% CO_2. The medium was removed from the 96-well plates, and 200 nM SNAP-Lumi4-Tb (Ref: SSNPTBX, Cisbio Bioassays) diluted in the Tag-lite® labeling medium (Ref: LABMED, Cisbio Bioassays) was added (50 μL per well). The labeled plates were incubated for 1 h at 37 °C under 5% CO_2. Excess SNAP-Lumi4-Tb was then removed by washing each well with Tag-lite® labeling medium (4×100 μL). TR-FRET signal was read with an HTRF-compatible reader in Tag-lite® labeling medium (100 μL per well) using the following time-resolved settings: delay 50 μs, integration time 400 μs, excitation at 337 nm, and signal collection at 620 nm.

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Conflict of interest

C.D., T.R., E.T., and E.D. are employees of Cisbio Bioassays (Codolet, France).

Keywords: apelin · chemical ligation · fluorescent probes · G protein-coupled receptors · TR-FRET assays

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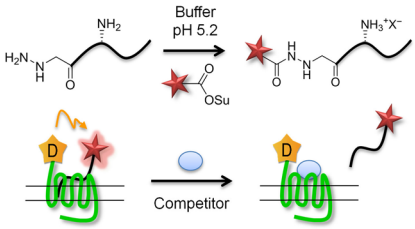
A Time-Resolved FRET Cell-Based Binding Assay for the Apelin Receptor



The search is on! There is still a need for the rapid discovery of apelin receptor ligands for use as pharmacological probes and precursors of drug candidates. As an alternative to radioligand binding assays, we report herein an unprecedented, efficient, robust, and safe time-resolved binding assay to investigate ligand–apelin receptor interactions at the cell surface with potential application for high-throughput screening.