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SHORT COMMUNICATION

Synthesis and pharmacological characterization of a europium-labelled single-chain antagonist for binding studies of the relaxin-3 receptor RXFP3

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Abstract Relaxin-3 and its endogenous receptor RXFP3 are involved in fundamental neurological signalling pathways, such as learning and memory, stress, feeding and addictive behaviour. Consequently, this signalling system has emerged as an attractive drug target. Development of leads targeting RXFP3 relies on assays for screening and ligand optimization. Here, we present the synthesis and in vitro characterization of a fluorescent europium-labelled antagonist of RXFP3. This ligand represents a cheap and safe but powerful tool for future mechanistic and cell-based receptor–ligand interaction studies of the RXFP3 receptor.

Keywords R3B1-22R · Europium · Relaxin family peptide receptor-3 (RXFP3) · Relaxin-3

Introduction

The relaxin family peptide receptor-3 (RXFP3) is a classic peptide ligand GPCRs and the cognate receptor for the neuropeptide relaxin-3 (Liu et al. 2003b). RXFP3 is highly expressed in the brain, particular in the olfactory bulb, paraventricular and supraoptic nuclei, hypothalamus, hippocampus, septum and amygdala (Liu et al. 2003b; Sutton et al. 2004). Functional studies in rodents suggest that the relaxin-3/RXFP3 system is involved in the control of several inter-related modalities, such as stress and arousal, feeding and metabolism, and learning and memory formation (Banerjee et al. 2010; Hida et al. 2006; Kuei et al. 2007; Ma et al. 2009; McGowan et al. 2005, 2006; Ryan et al. 2013a). Recently, it was shown that relaxin-3/RXFP3 also regulate alcohol intake and relapse-like behaviour in rats (Ryan et al. 2013b). Consequently, RXFP3 has emerged as a promising target for neurological modulators as treatment for addiction, depression and anxiety (Smith et al. 2014).

Relaxin-3 (R3), is a peptide hormone consisting of two peptide chains (B and A) that are held together by disulfide bonds in a compact fold (Rosengren et al. 2006). Relaxin-3 is poorly selective for RXFP3, also activating RXFP1 (Sudo et al. 2003) and RXFP4 with high affinity (Liu et al. 2003a; Sutton et al. 2004). The cross-reactivity and overlapping expression profiles of the receptors in the brain (Liu et al. 2003a, b; Ma et al. 2006) have driven the development of RXFP3-selective analogues to investigate physiological functions of RXFP3. A chimeric variant with a relaxin-3 B-chain and INSL5 A-chain (R3/I5) does not target RXFP1 (Liu et al. 2005) while a single-chain antagonist, R3B1-22R, which is a truncated variant of the relaxin-3 B-chain, is fully selective for RXFP3 (Haugaard-Kedstrom et al. 2011). R3B1-22R does not rely on complex chain combination and hence is a promising lead molecule, but significant further optimization is required to improve stability in vivo and ability to pass the blood brain barrier.

Traditionally, fluorophores or hazardous radioactive chemicals have been used to label ligands for receptor interaction studies. Lanthanides are promising alternatives compared to the use of organic fluorophores for screening

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and biodistribution analysis, when high autofluorescence is a problem. Lanthanides are naturally weak fluorophores. However, when the lanthanide ions form chelates with organic ligands, the complex exhibits unique fluorescent properties, such as a sharp emission band (typically 10–20 nm), large Stokes shift, long emission lifetimes compared to the nanosecond range for traditional organic reagents. The long-lived luminescence offers a signal-to-noise ratio advantage and improved signal sensitivity, which enables the use of time-resolved fluorescent (TRF) spectroscopy (Diamandis 1988; Handl and Gillies 2005; Josan et al. 2011). Taking advantage of the physical properties of lanthanide chelates, in combination with a dissociation-based enhancement assay called DELFIA (dissociation enhanced lanthanide fluoroimmunoassay), we have developed a robust binding assay for the detection of ligands binding to RXFP3, using time-resolved fluorescence measurement.

Materials and methods

Peptide synthesis

R3B1-22R was assembled on PAL-PEG-PS resin by Fmoc-based solid phase peptide chemistry using a CS Bio CS336X synthesizer and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU)/*N*,*N*-diisopropylethylamine (DIPEA) protocol. 20 % piperidine in DMF (v/v) was used for deprotection. 3eq DTPA-tetra(tBu-ester) (Macrocyclics) was manually coupled to the peptide chain using 8eq HBTU and 8eq DIPEA. The peptide was cleaved off the resin using TFA/TIPS/H_2O (95/2.5/2.5 %) for 2 h at 22 °C. The cleavage mixture was evaporated and peptide precipitated in ice-cold diethyl ether. Crude peptide was purified by RP-HPLC on a C_{18} column using a 1 % min^{-1} gradient consisting of Buffer A (99.95 % H_2O and 0.05 % TFA) and Buffer B (90 % acetonitrile, 10 % H_2O and 0.045 % TFA). Analytical RP-HPLC and ES-MS were used to confirm the purity and the molecular weight of the peptide. 2eq Eu(III)chloride hexahydrate (Sigma-Aldrich) and DTPA-R3B1-22R were dissolved in H_2O and incubated for 15 min at 22 °C. Europium-loaded peptide (Eu-DTPA-R3B1-22R) was purified by RP-HPLC on either a C_{18} or C_8 column using a 1 % min^{-1} gradient of TEAA buffer (Buffer A: 50 mM triethylammonium acetate, pH 6.5, Buffer B: 10 % Buffer A and 90 % acetonitrile). Purity and molecular weight were confirmed by analytical RP-HPLC and MALDI-TOF MS. Direct detect (Merck), which is based on infrared spectroscopy, was used to determine the peptide content. Native R3 (acid form) was synthesized as previously described (Rosengren et al. 2006).

Binding assays

CHO cells stably expressing RXFP3 were plated onto precoated poly-Lysine 96-well viewplates for binding assays as previously described (Haugaard-Kedstrom et al. 2011). For saturation binding, media was aspirated off and cells were washed with PBS before incubation with increasing concentrations of Eu-DTPA-R3B1-22R (0.1–50 nM). Non-specific binding was determined in the presence of 1 μM unlabelled R3B1-22R. For competition binding, 5 nM Eu-DTPA-R3B1-22R was utilized in the presences of increasing concentrations of non-labelled R3B1-22R or R3 relaxin. Cells were washed with PBS followed by addition of europium enhancement solution DELFIA (PerkinElmer). The fluorescent measurements were recorded using 340 nm excitation and 614 nm emission. Each experiment was repeated at least three times and one data point represents the mean of triplicates.

Statistics

All statistical analysis was evaluated with the Prism 5 software. Binding data are presented as mean ± SEM. The saturation binding data were fitted to a one site-binding model in Prism 5. Competitive binding experiments were fitted to a one site-competitive model and pKi calculated using the K_d value of 28 nM determined from the saturation binding.

Results

Peptide synthesis and characterization

R3B1-22R was prepared by Fmoc-based solid phase peptide synthesis using standard methods and single couplings throughout, and purified using a single run of RP-HPLC, resulting in a high yield and high purity (>95 %) product. The coupling of the lanthanide chelate, DTPA, was successfully achieved on resin using HBTU as coupling reagent. After purification DTPA-R3B1-22R was incubated with an excess of $EuCl_3$ for 15 min, after which a complex between DTPA and Eu^{3+} was formed (Fig. 1a). The final peptide product was evaluated and characterized by MALDI-TOF–MS [observed (*m*/*z*) 3146.18, theoretical (*m*/*z*) 3146.39] and RP-HPLC (Fig. 1b), using a TEAA-based buffer, pH 6.5, to prevent the liberation of europium from the DTPA.

Binding of Eu-DTPA-R3B1-22R to RXFP3 monitored by fluorescence measurements

The pharmacological profile of Eu-DTPA-R3B1-22R was characterized by both saturation binding and competitive

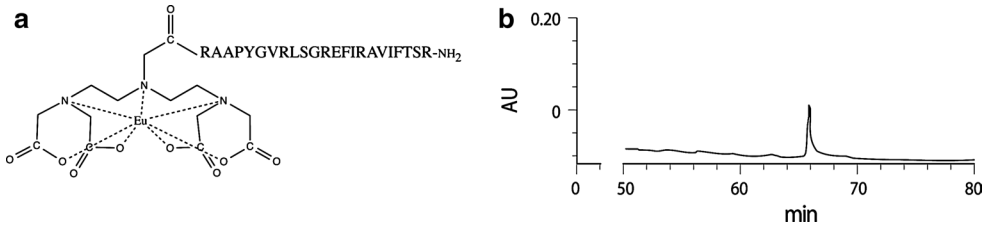

Fig. 1 **a** Primary structure of Eu-DTPA-R3B1-22R. Eu^{3+} and DTPA form a highly stable complex under basic conditions, as the multiple amine and carboxylate coordinating centre is wrapped around the metal ion. **b** RP-HPLC profile of purified Eu-DTPA-R3B1-22R. The analysis was run on a C_{18} column using a 1 % min^{-1} binary buffer system

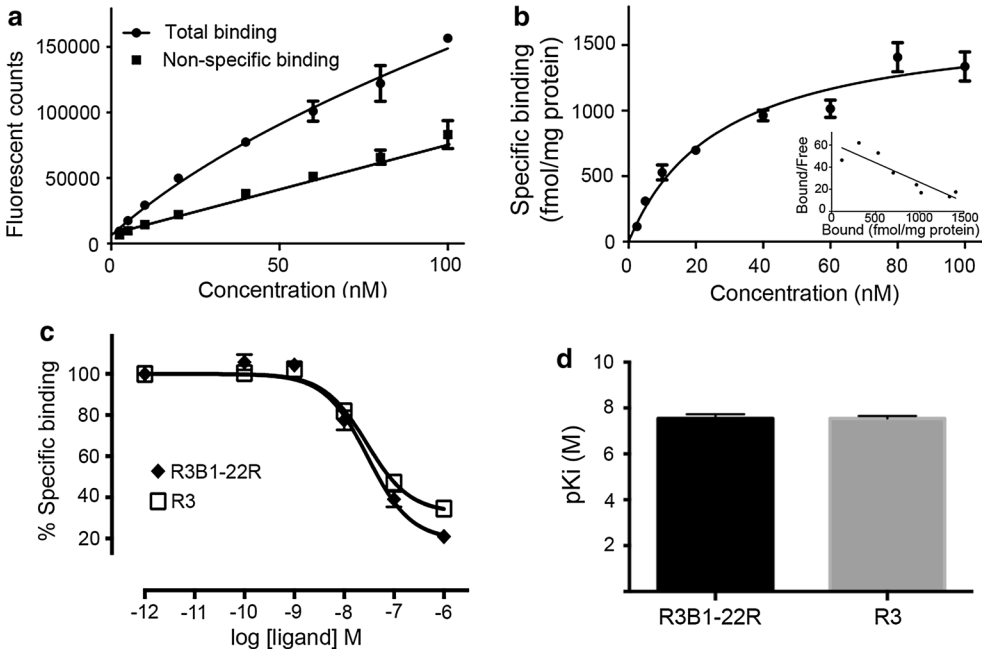

Fig. 2 Characterization of the binding of Eu-DTPA-R3B1-22R to RXFP3 monitored by fluorescence. **a** Saturation binding experiments were performed to determine the dissociation constant, K_d, between Eu-DTPA-R3B1-22R and RXFP3. K_d was determined by fitting a one-binding site model to increasing concentration of Eu-DTPA-R3B1-22R. Non-specific binding was determined in the presence of 1 μM unlabelled R3B1-22R. **b** Specific binding and corresponding receptor density. A representative Scatchard plot (*inset*) displays a linear correlation, confirming a one-site binding mode for Eu-R3B1-22R and RXFP3. **c** Comparison of agonist (R3) and antagonist (R3B1-22R) competition binding using Eu-R3B1-22R. **d** Pooled binding affinities, expressed as pKi values, for R3 and R3B1-22R which display no significant difference between agonist and antagonist binding. Data are presented as mean ± SEM of triplicates from at least three independent experiments

binding in CHO cells stably expressing RXFP3. Eu-DTPA-R3B1-22R bound to RXFP3 expressing CHO cells in a specific and saturable manner consistent with a single binding site with a dissociation constant (K_d) of 27.9 ± 9.4 nM and B_{max} of 1597 ± 294 fmol/mg protein (Fig. 2a, b). Competition binding studies using non-labelled R3 B1-22R in the same cells utilizing various concentrations of Eu-DTPA-R3B1-22R demonstrated specific competition best fitted by a single binding site model with a K_i value of 28.4 nM ($n = 4$) (Fig. 2c, d). To rule out any significant difference in agonist and antagonist binding mode, competitive binding experiment were also performed using increasing concentration of relaxin-3 (agonist). Relaxin-3 demonstrate a K_i of 28.6 nM (pK_i 7.54 ± 0.12, $n = 3$), which is not significant different from R3B1-22R ($p > 0.05$ using one-way ANOVA). The parallel one-site competition binding curves suggest that the binding site for R3 B1-22R is at least in part overlapping with the binding site for R3.

Discussion

Relaxin-3 is a neuropeptide that together with its endogenous receptor, RXFP3, regulates neuroendocrine signalling

and has therapeutic relevance for several disease states (Smith et al. 2014). However, the development of RXFP3-selective ligands requires a robust and reliable receptor binding screening method. Previously, radioactive ^{125}I-relaxin-3 or ^{125}I-R3/I5 has been used. Although high-affinity binders (K_d 0.31 and 0.41 nM, respectively), these probes are associated with limited half-life, very high cost due to complex synthesis, and safety issues (Kuei et al. 2007; Liu et al. 2003b). Eu-labelled variants of the chimeric R3/I5 have recently been described, both synthetic and recombinantly produced variants. However, again use is limited by the requirement of either complex synthesis of two chains and directed disulphide bond formation (Shabanpoor et al. 2011) or several steps of enzymatic processing and purification steps prior to labelling (Zhang et al. 2013).

Using europium-labelled peptides for high-throughput screening has previously been highly successful for ligands targeting disease relevant targets, such as the vasopressin receptors, oxytocin receptor and protease activated receptor-2 (PAR_2) (Albizu et al. 2007; Hoffman et al. 2012). Here, we present a site-directed synthesis strategy where the chelating Eu-DTPA is conjugated to the N-terminus of the single-chain R3B1-22R, which results in high yield and purity.

The pharmacological profile of Eu-DTPA-R3B1-22R was characterized by both saturation binding and competitive binding and displays K_d and K_i values of 27.9 and 28.4 nM, respectively. Importantly, this K_i matches well with that obtained from competition binding studies with Eu-H3/I5 (Haugaard-Kedstrom et al. 2011) indicating that the DTPA-cage does not interfere with the ligand binding to RXFP3. Importantly, several other relaxin ligands have been fluorescent labelled using the Eu-DTPA strategy without altered receptor selectivity profile (Belgi et al. 2011; Shabanpoor et al. 2008, 2012). Competition binding experiments using cold R3B1-22R and R3 ligands demonstrate that this peptide is an excellent probe for screening of both antagonist and agonist compounds. Although its binding affinity is slightly less than the currently available two chain tracer variants, this should be put in the perspective of the simple synthesis and cheap cost of Eu-DTPA-R3B1-22R, which makes it ideal tool for characterizing RXFP3 ligands.

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Conflict of interest The authors declare that they have no conflict of interests.

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