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Characterization of ligand binding to melanocortin 4 receptors using fluorescent peptides with improved kinetic properties

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ABSTRACT

Melanocortin 4 (MC_4) receptors are important drug targets as they regulate energy homeostasis, eating behaviour and sexual functions. The ligand binding process to these G protein-coupled receptors is subject to considerable complexity. Different steps in the complex dynamic regulation can be characterized by ligand binding kinetics. Optimization of these kinetic parameters in terms of on-rate and residence time can increase the rapid onset of drug action and reduce off-target effects. Fluorescence anisotropy (FA) is one of the homogeneous fluorescence-based assays that enable continuous online monitoring of ligand binding kinetics. FA has been implemented for the kinetic study of melanocortin MC_4 receptors expressed on budded baculoviruses. However, the slow dissociation of the fluorescently labelled peptide NDP-α-MSH does not enable reaching equilibrium nor enable more in-depth study of the binding mechanisms. To overcome this problem, two novel red-shifted fluorescent ligands were designed. These cyclized heptapeptide derivatives (UTBC101 and UTBC102) exhibited nanomolar affinity toward melanocortin MC_4 receptors but had relatively different kinetic properties. The dissociation half-lives of UTBC101 ($\tau_{1/2}$=160 min) and UTBC102 ($\tau_{1/2}$=7 min) were shorter compared to that what was previously reported for Cy3B-NDP-α-MSH ($\tau_{1/2}$=224 min). The significantly shorter dissociation half-life of UTBC102 enables equilibrium in screening assays, whereas the higher affinity of UTBC101 helps to resolve a wider range of competitor potencies. These two ligands are suitable for further kinetic screening of novel melanocortin MC_4 receptor specific ligands and could complement each other in these studies.

1. Introduction

Melanocortin receptors are G protein-coupled receptors (GPCRs) for melanocortins, which compose a key modulatory system for multiple physiological roles (Cone, 2000). Among five subtypes, melanocortin 4 (MC_4) receptors are known for their role in the regulation of body weight, sexual functions and neuroprotective actions (Giuliani et al., 2012; Wikberg and Mutulis, 2008).

The studies of these receptors on a molecular level require specific and high-affinity reporter ligands. Gaining insight into the optimal binding mechanisms helps to determine the best drug-like compounds for treatment (Fang, 2012; Swinney, 2011). In our previous work, we revealed that ligand binding to melanocortin MC_4 receptors is governed by a complex dynamic regulation (Kopanchuk et al., 2006, 2005). These findings are in agreement with the reports that binding events often consist of several steps of conformational adjustments (Meyer-Almes, 2016). For simplicity, such situations are often reduced to one of the two main mechanisms — induced-fit or conformational selection (Del Castillo and Katz, 1957; Leff, 1995). In addition, multimerization, allosteric binding sites and binding of other effector molecules have also been found to potentially influence the binding of melanocortin MC_4 receptors (Biebermann et al., 2003; Chapman and Findlay, 2013; Elsner et al., 2006; Kopanchuk et al., 2006, 2005; Nickolls and Maki, 2006). Studying ligand binding kinetics assists with developing knowledge about the mechanisms of binding processes. Further, optimization of drug candidates' kinetic parameters in terms of residence time and on-rates can increase efficacy as well as selectivity and reduce mechanism-based toxicity (Copeland et al., 2006; Hoffmann et al., 2015; Meyer-Almes, 2016; Swinney, 2006, 2004).

The radioligand binding assay has been an indispensable method for ligand binding studies for a long time (Paton and Rang, 1965). Although, this method is powerful, its usage in the studies of ligand binding kinetics is limited due to the need to separate free and bound ligands at every time point. The homogenous nature of fluorescence-based methods allows continuous online monitoring without the disturbing separation step (Stoddart et al., 2015). One of the promising

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fluorescence methods for kinetic high throughput screening is fluorescence anisotropy (FA) because it can provide automated, economical, rapid and convenient screenings (Jameson and Ross, 2010).

FA has been successfully applied in kinetic studies of ligand binding to several GPCRs (Huwiler et al., 2010; Junge et al., 2010; Kecskés et al., 2010; Tõntson et al., 2014; Veiksina et al., 2010). Among them, kinetic rate constants of ligand binding have been determined for melanocortin MC_4 receptors using the fluorescently labelled high-affinity melanocortin MC_4 receptor agonist NDP-α-MSH (Veiksina et al., 2014, 2010). However, it has already been established with radioligand binding assays that this ligand's slow dissociation imposes limitations for reaching equilibrium (Kopanchuk et al., 2006). Consequently, there is a need for fluorescently labelled ligands with faster binding kinetics for melanocortin MC_4 receptors. In this study, we identified two novel fluorescent ligands (UTBC101 and UTBC102) that exhibit nanomolar affinity binding to melanocortin MC_4 receptors and faster dissociation kinetics. These ligands could become valuable tools for kinetic screening of melanocortin MC_4 receptor specific ligands.

2. Materials and methods

2.1. Human MC_4 receptors and recombinant baculoviruses

The construction and generation of recombinant baculoviruses encoding human melanocortin MC_4 receptors was performed as previously described (Veiksina et al., 2010). Budded baculoviruses expressing human neuropeptide Y receptors Y1 (NPY1) were used as a control and the recombinant baculoviruses were generated using the same scheme (Veiksina et al., 2014). Briefly, *Spodoptera frugiperda* (Sf9) cells (Invitrogen Life Technologies, Paisley, UK) were used for homologous recombination of baculoviruses via transfection. The virus was amplified in a suspension culture at a multiplicity of infection (MOI) of 0.1 until the titre reached 10^8 infectious virus particles/ml. Virus titres were determined with a cell size-based assay (Janakiraman et al., 2006) that was slightly modified for routine use (Veiksina et al., 2015), where cell sizes are determined by a Cell and Particle Counter (Z2™ Series COULTER COUNTER®; Beckman Coulter). High-titre virus stocks were harvested at ~72 h post-infection by centrifugation at 1000g for 10 min. The supernatant containing baculoviruses were stored at 4 °C for short-term deposition and at –80 °C for long-term deposition.

2.2. Budded baculovirus preparation

The production of budded baculoviruses that display melanocortin MC_4 receptors on their membrane envelope was carried out by infection of 500 ml of Sf9 cell suspension at a density of 2×10^6 cells/ml with a high-titre recombinant baculovirus at MOI=6. The suspension was incubated in an Erlenmeyer flask (BD Falcon™ Erlenmeyer Flasks, Fisher Scientific) for ~72 h with an agitation of 115 rpm at 27 °C in a non-humidified environment. The supernatant containing budded baculoviruses was collected by centrifugation of the cell suspension at 1000*g* for 10 min. The collected budded baculoviruses were then centrifuged at 48,000*g* for 40 min at 4 °C. The final pellet containing budded baculoviruses was washed with 1 ml Dulbecco's phosphate-buffered saline (DPBS) and suspended in modified Krebs-Ringer buffer (135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mg/ml BSA, 11 mM Na-Hepes, 0.1% Pluronic F-127, and Complete EDTA-Free Protease Inhibitor Cocktail according to the manufacturer's description [Roche Applied Science], pH 7.4) in a ratio of 1:30 as the initial cell suspension volume. This suspension was denoted as “the budded baculovirus preparation.” Aliquots were stored at –80 °C until further use.

2.3. Homogeneous budded baculovirus/FA-based assay system

2.3.1. Determination of fluorescence anisotropy

Novel cyclic Cy3B-labelled fluorescent ligands UTBC101 (Cy3B-Nle-c[Asp-His-DNal(2′)-Arg-Trp-Lys]-NH_2) and UTBC102 (Ac-Lys(Cy3B)-c[Asp-His-DNal(2′)-Arg-Trp-Lys]-NH_2) were custom synthesized by CEPEP EESTI OÜ (Estonia). The novel ligands were stored in dimethyl sulfoxide (DMSO) at –20 °C. The concentration of the fluorescent ligands was confirmed by the absorbance of Cy3B (ε_{558}=130,000 M^{-1} cm^{-1}). The general framework for the characterization of ligand-receptor interactions with the FA method with the implementation of the homogenous budded baculovirus technology has been previously described, based on the example of melanocortin MC_4 receptors (Veiksina et al., 2014). In short, the FA measurements with fluorescent ligands and budded baculoviruses were performed on a PHERAstar (BMG Labtech, Germany) or Sunergy™ NEO (BioTek, USA) microplate reader. A black 96-well half area, black flat bottom polystyrene NBS microplate (Corning, Product No. 3993) was found to give optimal results (Veiksina et al., 2010). An optical module with excitation and emission filters of 540 nm (slit 20 nm) and 590 nm (slit 20 nm) was used, respectively. The sample was excited with parallel polarized light and emitted light was simultaneously measured in the parallel ($I_{||}$) and the perpendicular ($I_{\perp}$) plane, relative to the excitation source. Erythrosine B was used as a standard for the correction of sensitivities of different channels (G factor) (Thompson et al., 2002). Any background fluorescence (originating from budded baculoviruses, unlabelled ligands, buffer components or light scattering) measured in the absence of a fluorescent ligand was subtracted separately from all channels (Owicki, 2000). The function of parallel and perpendicular intensities ($I_{||}$ and $I_{\perp}$) of emitted polarized light, expressed as fluorescence anisotropy r (Eq. (1)), was used as a measure for the determination of ligand binding to the receptor (Jablonski, 1960):

$$r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}}, \quad (1)$$

where the denominator $I_{||}+2I_{\perp}$ stands for the total intensity of the emission excited by parallel polarized light. The value of FA is increased as the mobility of the fluorescent ligand decreases upon binding to a larger moiety; i.e., the receptor. All experiments were carried out in the kinetic mode in a total volume of 100 µl using Krebs-Ringer buffer as an assay buffer at 27 °C, which is physiologically relevant temperature for Sf9 cells. Fluorescence intensities were measured at appropriate time points after the addition of the budded baculovirus preparation to the reaction medium, which contained the fluorescent ligand (UTBC101 or UTBC102) with or without a competing ligand.

2.3.2. Saturation and kinetic assays

In all measurements, a parallel set of conditions was included to distinguish binding of the labelled ligand to nonspecific or specific sites. The total binding of the fluorescent ligand was measured in the absence and nonspecific binding in the presence of a fixed high concentration of unlabelled NDP-α-MSH (3 µM, Tocris Bioscience, UK) or SHU9119 (3 µM, BachemAG, Switzerland). The specific binding was defined as the difference between these values. Signals were background-corrected (see paragraph 2.3.1).

The binding saturation of the fluorescent ligand to MC_4 receptors was measured by varying the concentration of ligand specific MC_4 receptor binding sites (0–5.6 nM for UTBC101 and 0–8.1 nM for UTBC102) at fixed tracer concentrations (0.2–1.7 nM range of UTBC101 and 0.4–5.8 nM range of UTBC102), which covers area close to their binding affinities. The reaction was initiated by the addition of the budded baculovirus preparation. The fluorescent ligand binding was determined after 3 h (UTBC101) or 0.5 h (UTBC102) of incubation of the budded baculovirus preparation with the fluorescent ligand. In preliminary experiments different incubation periods were