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Pharmacological profiling of sigma 1 receptor ligands by novel receptor homomer assays

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ABSTRACT

The sigma 1 receptor (σ_1R) is a structurally unique transmembrane protein that functions as a molecular chaperone in the endoplasmic reticulum (ER), and has been implicated in cancer, neuropathic pain, and psychostimulant abuse. Despite physiological and pharmacological significance, mechanistic underpinnings of structure-function relationships of σ_1R are poorly understood, and molecular interactions of selective ligands with σ_1R have not been elucidated. The recent crystallographic determination of σ_1R as a homo-trimer provides the foundation for mechanistic elucidation at the molecular level. Here we report novel bioluminescence resonance energy transfer (BRET) assays that enable analyses of ligand-induced multimerization of σ_1R and its interaction with BiP. Haloperidol, PD144418, and 4-PPBP enhanced σ_1R homomer BRET signals in a dose dependent manner, suggesting their significant effects in stabilizing σ_1R multimerization, whereas (+)-pentazocine and several other ligands do not. In non-denaturing gels, (+)-pentazocine significantly decreased whereas haloperidol increased the fraction of σ_1R multimers, consistent with the results from the homomer BRET assay. Further, BRET assays examining heteromeric σ_1R-BiP interaction revealed that (+)-pentazocine and haloperidol induced opposite trends of signals. From molecular modeling and simulations of σ_1R in complex with the tested ligands, we identified initial clues that may lead to the differed responses of σ_1R upon binding of structurally diverse ligands. By combining multiple *in vitro* pharmacological and *in silico* molecular biophysical methods, we propose a novel integrative approach to analyze σ_1R-ligand binding and its impact on interaction of σ_1R with client proteins.

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1. Introduction

The sigma 1 receptor (σ1R) is an intriguing transmembrane protein that does not share sequence homology to any known eukaryotic protein family, except for a fungal sterol isomerase (Hanner et al., 1996). It has been characterized as a molecular chaperone in the endoplasmic reticulum (ER) (Hayashi and Su, 2007), and may be a promising therapeutic target for several neuropsychiatric disorders (Kourrich et al., 2012; Maurice and Su, 2009). In addition, σ_1R has been shown to be involved in pain (Romero et al., 2016; Sanchez-Fernandez et al., 2017), psychostimulant abuse (Katz et al., 2017; Sabino et al., 2017), and neurodegenerative diseases (Maurice and Goguadze, 2017; Nguyen et al., 2017) among others (Albayrak and Hashimoto, 2017; Soriani and Rapetti-Mauss, 2017; Wang et al., 2017). Depending on the physiological readout, σ_1R ligands have been described as “agonists” or “antagonists”, as for G-protein coupled receptors (GPCRs). For instance, σ_1R “antagonists” demonstrate efficacy in counteracting neuropathic pain (Romero et al., 2016)

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and drug seeking behavior in stimulant abuse (Katz et al., 2017), whereas σ_1R "agonists" display favorable effects in depression (Fishback et al., 2010). However, it is worth noting that the efficacy distinction between "agonist" and "antagonist" has not reached consensus in a therapeutic context. For instance, in the development of antipsychotics both an "antagonist" (Ferris et al., 1986; Gilmore et al., 2004) and an "agonist" (Albayrak and Hashimoto, 2017) have been found to be beneficial. Furthermore, the underlying molecular mechanistic differences between agonists and antagonists have not been well characterized across different pathological contexts (Katz et al., 2016; Merlos et al., 2017), and it must be noted that some of the observations and interpretations are not monolithic (Katz et al., 2017). For example, the σ_1R ligand BMY 14802 was characterized as both an antagonist and an agonist (Schoenwald et al., 1995; Taylor et al., 1993). This discrepancy can be attributed to readouts and interpretations at different downstream effector levels. Indeed only a few studies have evaluated the efficacy of σ_1R ligands specifically at the level of σ_1R-σ_1R interaction (Gomez-Soler et al., 2014; Gromek et al., 2014; Mishra et al., 2015).

σ_1R has a multitude of client proteins and the interactions with them may influence the nature of ligand action and signaling outcome (Su et al., 2016). Thus, client protein coupling and subsequent effector activation or inactivation have been reported as the major biological function of σ_1R (Su et al., 2016). Among the different client proteins, in particular, binding immunoglobulin protein (BiP), also known as heat shock 70 kDa protein 5, has been well-characterized (Ha et al., 2014; Miki et al., 2015; Ono et al., 2013; Penas et al., 2011) and shown to regulate ER-originated events such as calcium release and receptor trafficking (Hayashi and Su, 2007).

To understand the molecular mechanism of ligands on σ_1R, simplified signaling-independent methods are expected to better categorize the ligands, which may require going beyond the canonical agonist/antagonist definitions. In light of recent studies on σ_1R homomerization in response to a variety of ligands (Gromek et al., 2014; Mishra et al., 2015), ligand-induced changes on receptor multimerization state may provide at least qualitative criterion for functional categorization. Recent crystal structures of ligand-bound σ_1R, which are solved in homo-trimers, have revealed its transmembrane topology as well as the ligand binding site (Schmidt et al., 2016). Even though discrepancies with previous reports with regard to the structural topology (Ortega-Roldan et al., 2015; Ossa et al., 2017) remain, the high-resolution structural information revealed by the crystal structures sets the path to fundamental understanding of biophysical and pharmacological properties of σ_1R at the molecular level. Specifically, the identification of the homomerization interface provides a framework to design the constructs that are feasible to study the ligand-induced changes of multimerization state with pharmacological assays.

The bioluminescence resonance energy transfer (BRET) assay is a reliable protein-protein proximity assay. The methodology is capable of uncovering both distance changes between two proteins (Pfleger et al., 2006), and large conformational rearrangements within a protein such as ligand-induced conformational changes of GPCRs (Lohse et al., 2012). Importantly, BRET is suited for real-time kinetic tracking of the movements of labeled proteins. Here we develop novel BRET assays to characterize the ligand-induced changes in the homomerization of σ_1R and its interaction with BiP. The findings in σ_1R homomer BRET assay were validated by a biochemical assay, and were further shown to correlate with the results from computational modeling. Herein, we report the pharmacological characterizations of 8 known σ_1R ligands in a signaling-independent manner.

2. Materials and methods

2.1. σ_1R radioligand binding in guinea pig cortex

Male Hartley guinea pig cortices were dissected from freshly harvested brains (shipped cold in phosphate-buffered saline (PBS) buffer from BioReclamation IVT) and frozen at −80 °C for future use. On test day, thawed guinea pig cortices were suspended and homogenized in 20 vol (w/v) (10 mM Tris.HCl, 0.32 M Sucrose, pH 7.4 at 25 °C) with a glass-teflon apparatus and centrifuged (~1200 rpm) for 10 min at 4 °C. The supernatant was collected in a clean tube and the pellet re-suspended in 10 ml of cold buffer and centrifuged again (~1200 rpm) for 10 min at 4 °C. The supernatants were pooled together and centrifuged (20,000 rpm) for 15 min at 4 °C. The final pellet was suspended in ice-cold binding buffer at 50 mg/ml concentration (original wet weight). A Bradford protein assay (Bio-Rad, Hercules, CA) was used to determine the protein concentration present in the tissue preparation (1.25 mg/ml). All test compounds were freshly dissolved in 30% DMSO and 70% H_2O to a stock concentration of 1 mM or 100 μM. To assist the solubilization of free-base compounds, 10 μl of glacial acetic acid was added along with the DMSO (in place of 10 μl final H_2O volume). Each test compound was then diluted into 10 half-log serial dilutions using 30% DMSO as the vehicle. Radioligand competition experiments were conducted in 96-well plates containing 300 μl fresh binding buffer, 50 μl of diluted test compound, 100 μl of tissue preparation (125 μg/well total protein amount), and 50 μl of radioligand diluted in binding buffer ([^{3}H]-(+)-pentazocine: 3 nM final concentration, ARC, Saint Louis, MO). Nonspecific binding was determined using 10 μM PRE-084 and total binding was determined with 30% DMSO vehicle (3% DMSO final concentration). All compound dilutions were tested in triplicate and the competition reactions started with the addition of the tissue preparation and incubated for 120 min at room temperature. The reaction was terminated by filtration through Perkin Elmer Uni-Filter-96 GF/B, presoaked for 120 min in 0.05% polyethylenimine, using a Brandel 96-Well Plates Harvester Manifold (Brandel Instruments, Gaithersburg, MD). The filters were washed 3 times with 3 ml (3 × 1 ml/well) of ice cold binding buffer. 65 μL Perkin Elmer MicroScint 20 Scintillation Cocktail was added to each well and filters were counted using a Perkin Elmer MicroBeta Microplate Counter (calculated efficiency: 31%). IC50 values for each compound were determined from inhibition curves and K_i values were calculated using the Cheng-Prusoff equation; K_d values for [^{3}H]-(+)-pentazocine (σ_1R: 5.18 nM) and B_{max} (1091 fmol/mg) were determined via separate homologous competitive binding experiments. K_i values were determined from at least 3 independent experiments and are reported as mean ± SEM.

2.2. σ_1R radioligand binding in HEK293 cell membranes

HEK293T cells were grown as described below in section 2.4. Upon reaching 80–90% confluence, non-transfected HEK293T cells were harvested using pre-mixed Earle's Balanced Salt Solution (EBSS) with 5 mM EDTA (Life Technologies) and centrifuged at 3000 rpm for 10 min at 21 °C. The supernatant was removed and the pellet was resuspended in 10 ml hypotonic lysis buffer (5 mM $MgCl_2$, 5 mM Tris, pH 7.4 at 4 °C) and centrifuged at 20,000 rpm for 30 min at 4 °C. The pellet was then resuspended in fresh EBSS binding buffer made from 8.7 g/l Earle's Balanced Salts without phenol red (US Biological, Salem, MA) and 2.2 g/L sodium bicarbonate, pH to 7.4. A Bradford protein assay (Bio-Rad, Hercules, CA) was used to determine the protein concentration. On test day, the experiments were conducted in 96-well plates containing 300 μl fresh binding buffer, 50 μL of diluted test compound, 100 μL of