Chapter X

Pharmacoperones for misfolded gonadotropin receptors

Claire L. Newton[1 *] and Ross C. Anderson[1]

[1] Centre for Neuroendocrinology and Department of Immunology, Faculty of Health Sciences, University of Pretoria, PO Box 2034, Pretoria, 0001, South Africa

***** Corresponding author:
Dr Claire Newton
Centre for Neuroendocrinology and Department of Immunology, Faculty of Health Sciences, University of Pretoria, PO Box 2034, Pretoria, 0001, South Africa
Tel: +27 (0) 12 319 2501
Email: claire.newton@up.ac.za

CONTENTS

X.1 Abstract

The gonadotropin receptors (luteinizing hormone receptor; LHR and follicle – stimulating hormone receptor; FSHR) are G protein-coupled receptors (GPCRs) that play an important role in the endocrine control of reproduction. Thus genetic mutations that cause impaired function of these receptors have been implicated in a number of reproductive disorders. Disease-causing genetic mutations in GPCRs frequently result in intracellular retention and degradation of the nascent protein through misfolding and subsequent recognition by cellular quality control machinery. The discovery and development of novel compounds termed pharmacological chaperones (pharmacoperones) that can stabilise misfolded receptors, and restore trafficking and plasma membrane expression are therefore of great interest clinically and promising *in vitro* data describing the pharmacoperone rescue of a number of intracellularly retained mutant GPCRs has provided a platform for taking these compounds into *in vivo* trials. Thienopyrimidine small molecule allosteric gonadotropin receptor agonists (Org 42599 and Org 41841) have been demonstrated to have pharmacoperone activity. These compounds can rescue cell surface expression and in many cases, hormone responsiveness, of a range of retained mutant gonadotropin receptors. Should gonadotropin receptor-selectivity of these compounds be improved, they could offer therapeutic benefit to subsets of patients suffering from reproductive disorders attributed to defective gonadotropin receptor trafficking.

Key words: *gonadotropin receptors, pharmacoperones, thienopyrimidines, Org 42599, Org 41841, Org 43553, luteinizing hormone receptors, intracellular retention, follicle-stimulating hormone receptor*

Abbreviations*: 7-TM, seven transmembrane; BH4, tetrahydrobiopterin; BiP, binding immunoglobulin protein; CFTR, cystic fibrosis transmembrane conductance regulator; CHO, Chinese hamster ovary; ECD, ectodomain; ECL, extracellular loop; ER, endoplasmic reticulum; FSH, follicle-stimulating hormone; FSHR follicle-stimulating hormone receptor; GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; GPCR, G protein-coupled receptor; Grp94, glucose-regulated protein; H8, helix 8; hCG, human chorionic gonadotropin; HPG, hypothalamic-pituitary-gonadal; hMG, human menopausal gonadotropin; ICL, intracellular loop; LCH, leydig cell hypoplasia; LH, luteinising hormone; LHR/LHCGR, luteinising hormone receptor; OHSS, ovarian hyperstimulation syndrome; PAH, phenylalanine hydroxylase; PDI, protein disulphide isomerase; pharmacoperone, pharmacological chaperone; QCS, quality control system; TM, transmembrane; TSHR, thyroid-stimulating hormone receptor; UPR, unfolded protein response; V2R, vasopressin receptor 2.*

X.2 Introduction

The gonadotropin receptors, luteinising hormone/chorionic gonadotropin receptor (LHCGR/LHR) and follicle-stimulating hormone receptor (FSHR) play an important role in the hypothalamic-pituitary-gonadal (HPG) endocrine axis, which governs the control of reproduction. The FSHR and LHR are members of the G protein-coupled receptor (GPCR) superfamily of cell surface signalling proteins and, with the thyroid-stimulating hormone receptor (TSHR), they comprise the glycoprotein hormone receptor subfamily. The glycoprotein hormone receptors are unique in that, in addition to the seven transmembrane (7-TM) domain that is characteristic of all GPCRs, they have large (>300 amino acids) extracellular N-terminal ectodomains (ECDs), which consists of a series of leucine-rich repeats stabilised by disulphide bridges. The ECD and 7-TM domain regions are joined by a "hinge region", which is believed to confer an inhibitory effect on the 7-TM domain. Upon hormone binding to the ECD, the inhibitory constraint on the 7-TM domain is released, facilitating receptor activation. (Fan and Hendrickson, 2005; Jiang et al., 2012; Jiang et al., 2014).The members of the glycoprotein hormone receptor subfamily share a high degree of homology, particularly within their 7-TM domains where sequence identity is 68-72%, compared to their ECD regions where sequence identity is 39-46% (Vassart et al., 2004).

The glycoprotein hormone receptors interact with large heterodimeric glycoprotein hormone ligands, which comprise of a common α subunit in combination with a hormone-specific β subunit. The cognate hormone ligand for the FSHR is follicle-stimulating hormone (FSH), while the LHR interacts with both luteinising hormone (LH) and human chorionic gonadotropin (hCG). LH and FSH, are produced in gonadotrope cells of the anterior pituitary in response to hypothalamic release of gonadotropin-releasing hormone (GnRH), while hCG is secreted from the placenta of pregnant women. These gonadotropins interact with LHRs and FSHRs, predominantly expressed in gonadal tissues (although expression has been reported in extragonadal tissues), where they stimulate gonadal development, gametogenesis and production/secretion of sex hormones.

In females activation of LHRs expressed on ovarian thecal cells results in the production/secretion of androgens which pass to the neighbouring granulosa cells where activation of FSHRs stimulates their conversion to estrogen via synthesis of the enzyme aromatase. Activation of FSHRs expressed on ovarian granulosa cells also stimulates follicular growth and peptide hormone (inhibin) secretion. As mature follicles develop, LHRs become expressed and activation by an ovulatory 'LH surge' from the pituitary triggers egg release and the conversion of the residual follicle into the corpus luteum. Activation of LHRs in the corpus luteum by LH (or hCG should pregnancy occur) stimulates secretion of progesterone, which prepares the endometrium for implantation and placental development. In males, activation of FSHRs expressed on testicular Sertoli cells stimulates spermatogene-

sis (along with androgens) and peptide hormone (inhibin) secretion, while activation of LHRs on testicular Leydig cells results in the production/secretion of androgens. *In utero*, hCG stimulates a mid-gestational peak in androgen production by the testes is important for the development and maturation of the male genitalia. In both sexes, the HPG axis is briefly activated shortly after birth (mini-puberty) and this activation is associated with genital growth in males and follicle maturation in females. Following this period, the HPG axis remains quiescent until the onset of puberty. At puberty, gonadotropin levels increase in response to pulsatile GnRH secretion, and the resulting steroid hormone production is crucial for gonadal maturation and development of secondary sex characteristics.

Due to their vital role in the hormonal control of reproduction, genetic mutations that disrupt the function of the gonadotropin receptors or their hormone ligands result in perturbation of the HPG axis and a range of reproductive phenotypes. Interestingly mutations of these receptors illustrate the sexually dimorphic role of these hormones, with mutations of the LHR having a more profound effect in males, while mutations of the FSHR have a more profound effect in females. Activating mutations of the LHR result in a familial form of male-linked precocious puberty, while inactivating mutations result in impaired sexual differentiation (Leydig cell hyperplasia; LCH) in males and anovulation, amenorrhea and reduced fertility in females (Themmen APN, and Huhtaniemi, 2000; Huhtaniemi and Themmen, 2005; Desai et al., 2013). Mutations of the FSHR, and activating mutations in particular, appear to be less common that for the LHR, possibly due to the resultant phenotypes being less clear than for the LHR mutations. Activating mutations have been linked to spontaneous ovarian hyperstimulation syndrome (OHSS) in women and inactivating mutations result in follicular arrest or ovarian dysgenesis in women and impaired sperm quality in males (Themmen APN, and Huhtaniemi, 2000; Huhtaniemi and Themmen, 2005; Desai et al., 2013). For further details of LHR and FSHR inactivating mutations identified in human patients refer to Chapter XXX).

X.3 Thienopyrimidine gonadotropin analogues

Due to their crucial role in reproduction and fertility, gonadotropins and their analogues are administered exogenously to females undergoing assisted reproductive therapy, to enable controlled ovarian stimulation prior to *in vitro* fertilisation or intracytoplasmic sperm injection. The conventional technique involves an initial down-regulation of endogenous gonadotropin levels by prolonged administration of a GnRH agonist (or shorter administration of a GnRH antagonist), followed by a stimulatory phase where folliculogenesis is induced by daily administration of FSH. Upon follicle maturation, ovulation is stimulated by the administration of LH/hCG and oocytes are collected. Gonadotropins used for these therapies were traditionally extracted from the urine of pregnant women (hCG) or postmenopausal women (human menopausal gonadotropin; hMG, which con-

tains LH and FSH activity), but these preparations have now been largely replaced by recombinantly produced gonadotropins, allowing more controlled dosing and personalised treatment regimens. However, the pharmaceutically industry has made considerable efforts to produce orally active non-peptide gonadotropin analogues to improve convenience of treatment and to avoid deleterious side-effects such as OHSS.

The most advanced series of compounds to be developed in this regard are the thienopyrimidines. In 2002 a group from NV Organon (subsequently Schering-Plough Research Institute and currently Merck Research Laboratories) reported the first orally active non-peptidic low molecular weight agonist for the LH receptor (van Straten et al., 2002). A high-throughput screen using a Chinese hamster ovary (CHO) cell line stably expressing human LHR and a luciferase reporter gene containing a cAMP-response element promotor, identified a thieno[2,3-*d*]pyrimidine as having agonist activity at the LH receptor. Lead optimisation resulted in a thienopyrimidine, Org 41841 (Figure X.1A), which had an EC_{50} of 20 nM in the CHO-LHR assay and stimulated testosterone production in cultured mouse Leydig cells and ovulation in female mice (van Straten et al., 2002). Org 41841 has since been demonstrated to have partial agonist activity at the TSHR (with approximately 35-fold lower potency than at the LHR), and activity at the FSHR only at very high concentrations (Moore et al., 2006; Jaschke et al., 2006).

Further lead optimisation of Org 41841 lead to the development of another thienopyrimidine, Org 43553 (and its trifluoracetic acid salt form Org 42599) (Figure X.1B), with high potency ($EC_{50} =$ 5 nM) and good activity at the LHR (van Koppen et al., 2008). Although Org 43553 displays no activity at the TSHR, it is able to activate the FSHR (with approximately 32-fold lower potency than at the LHR) (van Koppen et al., 2008) and has good oral bioavailability (van de Lagemaat et al., 2009). In an *ex vivo* ovulation induction assay using cultured mouse follicles 1 μM Org 43553 was able to stimulate maximal levels of ovulation and progesterone production similar to that seen using hCG and was able to induce testosterone production in primary Leydig cell cultures with high potency (van de Lagemaat et al., 2009). The compound's *in vivo* activity and oral bioavailability has also been demonstrated using female rodent ovulation induction models and measurement of testosterone stimulation in male rats. Oral administration at concentrations $\geq$50 $mgkg^{-1}$ resulted in levels of testosterone comparable to those produced after treatment with 1000 IU hCG subcutaneously (van de Lagemaat et al., 2009). Human studies in healthy females have confirmed the safety and tolerability of Org 43553 and another related thienopyrimidine (Org 43902) and have successfully demonstrated their ability to induce ovulation in pituitary-suppressed women receiving recombinant FSH to induce follicular maturation (Gerrits et al., 2013).

Neither Org 43553 nor Org 41841 (or their salt variants) compete with hormone for binding to the LHR (Heitman et al., 2008; Newton et al., 2011), which

was the first indication that these compounds act in an allosteric manner at a site distinct from the natural hormone binding site. Using chimeric receptors in which the ECD and 7-TM domains of the TSHR and LHR had been interchanged, the allosteric site of interaction of Org 43553 with the LHR was investigated in more detail and was found to be located within the 7-TM region (van Koppen et al., 2008). Computational modelling of ligand-receptor docking has subsequently been used to further elucidate the allosteric site of action of these low molecular weight thienopyrimidines. Using these methods, a putative glycoprotein hormone receptor allosteric binding pocket for these compounds was identified with contacts in transmembrane domains (TMs) 3, 4, 5, 6, and 7 and extracellular loop (ECL) 2 (Moore et al., 2006; Jaschke et al., 2006; Neumann et al., 2009; Haas et al., 2011; Heitman et al., 2012; Hoyer et al., 2013).

X.4 Intracellular retention of mutant gonadotropin receptors

Inactivating mutations in GPCRs can be classified as: Class I (defective receptor biosynthesis, which includes mutations that truncate the receptor prematurely), Class II (defective trafficking to the cell surface), Class III (defective ligand binding), Class IV (defective receptor activation, which includes those unable to achieve an active conformation and those unable to couple to and/or activate G proteins) and Class V (mutants with no known defects) (Tao, 2006). Classically, GPCR mutations were thought to result in impaired ligand binding (Class III defects) or disruption of intracellular signalling (Class IV defects), but it is now apparent that many inactivating GPCR mutations result in a failure to reach the cell surface (Class II defects) due to misfolding of the nascent receptor protein., and subsequent recognition by cellular quality control systems resulting in intracellular retention and ultimately degradation.

GPCRs, such as the glycoprotein hormone receptors, are synthesized by ribosomes on rough endoplasmic reticulum (ER), where they are folded before entering the secretory pathway for further post-translational modification and trafficking to the cell surface. Misfolding can occur through deviation from “normal” physiological parameters or through genetic mutation that causes disruption of the proteins tertiary/quaternary structure. Indeed, protein misfolding has been implicated as the causative factor in a large number of diseases caused by genetic mutation and a study examining thousands of disease-causing missense mutations across a spectrum of pathophysiologies predicted that almost 30% could be attributed to protein instability/misfolding (Sahni et al., 2015).

Misfolded proteins can have altered or aberrant functions, can aggregate, or can simply be rendered non-functional, all of which can result in cell stress and/or disease. The cell has a quality control system (QCS) in place to attempt to stabilise/re-fold these misfolded proteins (termed the unfolded protein response; UPR).

The QCS recognises various indicators of misfolding, such as unpaired cysteines and exposed hydrophobic residues. Endogenous molecular chaperone proteins then attempt to refold the misfolded protein, but, should misfolding persist the proteins are targeted for degradation. In the case of GPCRs, such as the gonadotropin receptors, misfolding results in the receptors being retained in the ER and/or degraded, rather than being trafficked to the cell surface where they can interact with their hormone ligands (Morello et al., 2000; Mizrachi and Segaloff, 2004). (See Chapter XXX for more details regarding gonadotropin receptor synthesis and trafficking). Diseases caused by retention of GPCR mutants are numerous, and include retinitis pigmentosa, nephrogenic diabetes insipidus, reproductive dysfunction, and obesity (Beerepoot et al., 2017).

As mentioned above (Section X.2), inactivating mutations of gonadotropin receptors result in varying degrees of reproductive dysfunction. To date, approximately 34 naturally occurring inactivating genetic mutations of the LHR gene have been described in patients suffering from varying degrees of reproductive dysfunction (Huhtaniemi and Themmen, 2005; Kossack et al., 2013; Rivero-Muller et al., 2015; Newton et al., 2016) (also see Chapter XXX). A recent study examined 20 of these mutations (excluding those which cause reduced transcript levels, disruption of the signal peptide, frame shifts affecting large proportions of the receptor sequence, premature termination, or severe truncation of the receptor protein, which are clearly explainable) and demonstrated that impaired cell surface trafficking is the most common defect, with 13/20 (65%) of mutations resulting in severely reduced cell surface expression (<10% of wild-type receptor levels), and another resulting in cell surface expression of 23% of wild-type levels (**Thienopyrimidine allosteric agonists of the glycoprotein hormone receptors. A.** *N-tert-butyl-5-amino-4-(3-methoxyphenyl)-2-(methythio)thieno[2,3-d] pyrimidine-6-carboxamide (Org 41841) and* ***B.*** 5-amino-2-methylsulfanyl-4-[3-(2-morpholin-4-yl-acetylamino)-phenyl]-thieno[2,3-*d*]pyrimidine-6-carboxylic acid *tert*-butylamide (Org 43553/Org 42599).

Figure X.2) (Newton et al., 2016). Of these 14 mutations, six are located in the receptor ECD (I114^{LRR4}F, V144^{LRR5}F, F194^{LRR7}V, Del Exon 8$^{LRR8/9}$, Del Exon 10^{Hinge} and C343^{Hinge}S)[1] and eight in the 7-TM domain (T392^{ICL1}I, I374$^{1.47}$T + T392^{ICL1}I, T461$^{3.47}$I, L502$^{4.61}$P, C543$^{5.55}$R, A593$^{6.59}$P, Del L608$^{7.36}$-V609$^{7.37}$ and S616$^{7.46}$Y)[2] (Newton et al., 2016). A 7-TM domain missense mutation, I415$^{2.60}$T, and a frameshift, which results in the last 83 amino acids of the receptor being re-

[1] Superscripts indicate location of the mutation in the ectodomain structure. LLR, leucine rich-repeat; Hinge, hinge region

[2] Superscripts indicate location of the mutation in the 7-TM domain. Where mutated residues fall in transmembrane helices, residue numbering refers to the Ballesteros-Weinstein numbering system (Ballesteros and Weinstein, 1992). ECL, extracellular loop; ICL, intracellular loop; H8, helix 8.

placed with 21 different amino acids, have also been demonstrated to cause severe intracellular receptor retention (Kossack et al., 2013; Rivero-Muller et al., 2015).

Fewer (approximately 18) naturally-occurring inactivating mutations of the FSHR have been described (Kotlar et al., 1997; Huhtaniemi and Themmen, 2005; Desai et al., 2013; Uchida et al., 2013; Bramble et al., 2016; Hugon-Rodin et al., 2017) (also see Chapter XXX). However, similar to the LHR, of the 14 that have been functionally characterised with respect to their cell surface expression, the majority (9/14; 64%) have been found to cause severe intracellular receptor retention. These include four mutations located within the ECD ($I160^{LRR6}T$, $A189^{LRR7}V$, $N191^{LRR7}I$, and $D224^{LRR9}V$)[1] and five in the 7-TM domain ($D408^{2.50}Y$, $P519^{ECL2}T$, $A575^{6.38}V$, $F591^{6.54}S$ and $R634^{H8}H$,)[2] (Beau et al., 1998; Touraine et al., 1999; Rannikko et al., 2002; Gromoll et al., 2002a; Meduri et al., 2003; Desai et al., 2015; Bramble et al., 2016; Hugon-Rodin et al., 2017 and our own unpublished data).

As expected, the degree of retention of the mutant receptors correlates well with the severity the reproductive phenotype observed in patients. All of the intracellularly retained LHR and FSHR mutants have substitutions or deletions of residues or regions with a high degree of conservation between the different glycoprotein hormone receptors and across different species, indicative of their structural/functional importance, particularly within the ECD region which has a greater sequence divergence between the different receptors. Indeed many of the reported mutations in ECD residues would be predicted to disrupt the densely packed hydrophobic core and beta-sheet structure of the LLR regions (e.g mutations I114F of the LHR or I160T of the FSHR) or the cysteine bond network important for conferring stability to this region (e.g. mutation C343S of the LHR). Deletion of large portions would also be expected to compromise the structure of this domain (e.g. deletion of exons 8 and 10 of the LHR). In addition, three of the identified mutations (F194V of the LHR and A189V and N191I of the FSHR are located within a highly conserved 'AFNGT' motif of the glycoprotein hormone receptors which spans residues 193-197 and 189-193 of the LHR and FSH ECD regions, respectively, and which encompasses an N-linked glycosylation site (NGT). The integrity off this motif appears to be very important for correct receptor folding/trafficking, and the presence of glycosylation of the asparagine may also play a role in folding and maturation of the glycoprotein hormone receptors (Davis et al., 1995; Davis et al., 1997; Gromoll et al., 2002b).

Within the 7-TM domain many of the observed mutations would be predicted to disrupt TM helix conformation/structure for example through disruption of hydrophobic interactions (e.g mutations I415T of the LHR and F591S of the FSHR), introduction of charged residues (e.g. mutation C543R of the LHR), 'helix kinking' prolines (e.g. mutations A593P and L502P of the LHR) or residues with bulky sidechains (e.g mutations S616Y and T461I of the LHR and A575V of the FSHR), or through deletion of helix residues (e.g deletion of L608-V609 of the

LHR). Two of the identified mutations are located within the membrane proximal region of helix 8 of the intracellular C-terminal tail of the receptors (mutation R634H in the FSHR and a frameshift, which results in the last 83 amino acids of the receptor being replaced with 21 different amino acids of the LHR). These mutations cause loss or disruption of the reversed BBXXB motif (BXXBB) of the FSHR and conserved $F(X)_6LL$ motif of LHR, respectively. In the FSHR the BXXBB motif has been shown to be important for cell surface trafficking (Timossi et al., 2004) while the $F(X)_6LL$ motif is critical for trafficking of many GPCRs from the ER to the cell surface (Duvernay et al., 2004; Duvernay et al., 2009).

Mutations in gonadotropin receptor genes follow an autosomal recessive pattern of inheritance. Thus, in the majority of cases, the mutations in these receptors have been identified in homozygous or compound heterozygous individuals. However, in some cases (e.g. I114F of the LHR) mutation of only one allele was detected. This is suggestive of additional unreported mutation(s) (in other genes or unsequenced non-coding portions of the gonadotropin receptor genes) that contribute to the patient phenotype. Alternatively, these mutations may act in a dominant negative manner, resulting in retention of wild-type receptor by retained mutant receptors. Indeed, dominant negative effects of intracellularly retained mutants on wild-type FSHRs and LHRs have been reported (Zhang et al., 2009; Zarinan et al., 2010).

X.5 Pharmacological chaperones (pharmacoperones)

A number of approaches have been investigated to attempt to overcome protein misfolding including use of chemical chaperones, which act non-specifically to stabilise protein folding and/or prevent aggregation, and include osmolytes such as glycerol, trehalose, and hydrophobic compounds like 4-phenylbutyrate (Ringe and Petsko, 2009). However, a lack of specificity and/or limitations relating to the very high doses that are required have hindered the transition of many of these chemical chaperones into clinical trials, despite positive data from animal models of protein misfolding disease such as Huntington's disease, prion disease, and neurodegenerative disease (Cortez and Sim, 2014).

Recently, a novel class of molecules termed pharmacological chaperones (pharmacoperones) have been described. These molecules are cell-permeant and can interact specifically with nascent misfolded target proteins, stabilising their folding, preventing degradation, and therefore can facilitate 'rescue' of function. While research surrounding development of these compounds as therapeutics is still in its infancy, recent discoveries imply that the mode of action of some existing therapeutics may be through chaperoning activities. For example, in phenylketonuria, a metabolic disorder arising from defects in the phenylalanine hydroxylase (PAH) enzyme, a subset of patients harbouring mutations in the PAH gene

have been found to respond to treatment with tetrahydrobiopterin (BH4). BH4 is a cofactor for PAH, and while the mechanism of action is probably multifactorial, there is evidence that BH4 may be stabilising misfolded mutant PAH, thus preventing its degradation (Pey et al., 2004).

A small number of clinical trials have also explored the therapeutic potential of novel pharmacoperones to treat other diseases resulting from protein misfolding. For example, in cystic fibrosis, a Del F508 mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) ion channel, which inhibits folding and cell membrane trafficking, is present in at least one allele in 90% of patients (Rowe and Verkman, 2013). Phase III clinical trials examining the efficacy of a combination drug (Orkambi) that contains Lumacaftor (a pharmacoperone that rescues Del F508 CFTR trafficking to the cell membrane) and Ivacaftor (a potentiator of CFTR) demonstrated a modest improvement in lung function and reduced rates of pulmonary exacerbations (Wainwright et al., 2015; Kuk and Taylor-Cousar, 2015).

GPCRs make excellent targets for development of novel pharmacoperone therapies because (i) they are responsible for the majority of signal transduction across mammalian cell membranes such that their dysfunction commonly results in disease and (ii) genetic mutations of GPCRs frequently cause misfolding (Oksche and Rosenthal, 1998; Newton et al., 2016). Indeed in 2000, Morello *et al.* described the first pharmacoperone targeting a GPCR; vasopressin receptor 2 (V2R). They demonstrated that a deletion mutation (Del L62-R64) identified as causative for nephrogenic diabetes insipidus (NDI) resulted in a loss of cell surface expression, and subsequently no hormone binding, or receptor signalling, but that upon treatment with a cell-permeant V2R antagonist (SR121463A), cell surface expression was restored, and importantly the rescued receptor responded to hormone following rescue (Morello et al., 2000). They then went on to demonstrate that the same compound could increase cell surface expression of seven additional mutants (Morello et al., 2000). Pharmacoperones have since been described for several disease-causing mutations of GPCRs including LHR and FSHR (Janovick et al., 2009; Newton et al., 2011) (see Section 0 for details), rhodopsin (Noorwez et al., 2003), melanocortin 4 receptor (Fan and Tao, 2009), gonadotropin-releasing hormone receptor (GnRHR) (Janovick et al., 2002; Janovick et al., 2013), calcium sensing receptor (Huang and Breitwieser, 2007), G protein-coupled glucagon receptor (Yu et al., 2012), α1, β1 and β2 adrenergic receptors (Canals et al., 2009; Kobayashi et al., 2009; Lan et al., 2012), bradykinin B1 receptor (Fortin et al., 2006), dopamine receptor 4 (Van Craenenbroeck et al., 2005), κ and δ opioid receptors (Petaja-Repo et al., 2000; Chen et al., 2006) and D-type prostanoid 1 receptor (Labrecque et al., 2013).

Studies examining GPCR pharmacoperone activity *in vivo* are limited. However, a recent study by Janovick *et al.* demonstrated that the hypogonadotropic hypogonadal phenotype of knock-in transgenic mice harbouring an intracellularly re-

tained E90K mutant of the GnRHR could be partially rescued by pulsatile treatment with a small-molecule cell-permeant GnRHR antagonist (IN3). Following a 30 day pulsatile treatment regime, increases in sperm concentration and improvements in sperm morphology were observed in males along with increased expression of steroidogenic enzymes (Janovick et al., 2013). Furthermore, in a human study using a small cohort of patients harbouring V2R mutations (R137H, W164S, and Del G185-W193) causative of NDI, an orally-active cell-permeant small molecule (Relcovaptan; SR49059), able to rescue cell surface expression and function of mutant V2Rs, was shown to have beneficial effects on urine volume and osmolality measured during a 48 hour study period (Bernier et al., 2006).

X.6 Thienopyrimidines as pharmacoperones for misfolded gonadotropin receptors

X.6.1 Pharmacoperones for luteinising hormone receptor mutants

The thienopyrimdine, Org 42599, (a trifluoracetic acid salt of Org 43553, an allosteric agonist of the LHR - see section X.4 for details) has been found to act as a pharmacoperone for mutant LHRs. Two inactivating mutant LHRs, A593P and S616Y were identified in patients suffering from impaired reproductive function. A593P, located in TM6 of the LHR, is associated with a very severe phenotype and was identified in two homozygous 46, XY siblings who had pseudohermaphroditism as a result of Leydig cell hypoplasia. They were phenotypically female, with female external genitalia but presented with primary amenorrhea and lack of breast development. It was found that the patients had short blind-ending vaginas, no uterus or fallopian tubes, high LH and low testosterone levels, and histological analysis of their gonads revealed testis with normal Sertoli cells but no mature Leydig cells (Kremer et al., 1995). Their 46, XX sibling, who suffered from amenorrhea but who had normally developed primary and secondary sex characteristics, was also found to have this mutation (Toledo et al., 1996). S616Y, located in TM7 of the LHR, is associated with a milder phenotype and has been identified in both homozygous and compound heterozygous 46, XY males who presented with a micropenis and primary hypogonadism (Latronico et al., 1996; Laue et al., 1996).

In vitro characterization of these mutant receptors revealed that they were able to stimulate little/no cAMP in response to hormone (Kremer et al., 1995; Toledo et al., 1996; Latronico et al., 1996; Laue et al., 1996; Newton et al., 2011; Newton et al., 2016) (**Figure X.3**). Radioligand binding assays also demonstrated substantially reduced maximal hormone binding to the mutant receptors in spite of hormone binding affinities comparable to wild-type receptor (Kremer et al., 1995; Laue et al., 1996; Latronico et al., 1996; Newton et al., 2011; Newton et al., 2016). Examination of their cellular localization in transfected cells using confocal microscopy revealed that, while the wild-type receptor is expressed at high levels and was predominantly located at the cell surface, both mutant receptors are expressed at much lower levels and are predominantly intracellularly located (Mizrachi and Segaloff, 2004; Newton et al., 2011) (

Figure X.4, left-hand panels) and co-localise with a fluorescently labelled ER marker (our own unpublished observations). Retention of the mutant receptors in the ER in an 'immature' form (with endoglycosidase H-sensitive high mannose-containing N-linked carbohydrates) has also been indicated by Western blotting analyses of cells transfected with wild-type and mutant receptors (Mizrachi and Segaloff, 2004).

Incubation of cells expressing A593P and S616Y mutant receptors with Org 42599 increases the amount of mutant receptor localized at the cell surface (

Figure X.**4**, right-hand panels) (Newton et al., 2011) and also the total level of mutant receptor expression and the proportion present in a 'mature' form (Newton et al., 2011). As has been demonstrated for the GnRH receptor (Janovick et al., 2007), pharmacoperone rescue of mutant LHRs can occur even when protein synthesis has been inhibited (our own unpublished observations). This finding suggests that Org 42599 facilitates the folding and transport of the pool of misfolded receptor retained within the ER thereby increasing the trafficking of receptor protein to the cell surface and reducing the amount of receptor targeted to degradation pathways.

The pharmacoperone 'rescue' of cell surface expression of the S616Y and A593P mutant receptors, as measured by an increase in number of hormone-binding sites on intact cells, is both concentration and time-dependent, with maximal increases seen after 24h with 1-10 μM Org 42599 (Newton et al., 2011) (**Figure X.5**). Interestingly, the 'rescue' of the mutant receptors by Org 42599 is transient and upon removal of the pharmacoperone, cell surface expression of the mutant receptors decreases back to pre-treatment levels (Newton et al., 2011). This phenomenon has not been examined in detail but could reflect turnover of the LHR protein at the cell surface.

Unlike the A593P mutant, for which no cell surface expression or mature receptor could be measured, the S616Y mutant LHR did display a small degree of cell surface expression and presence of a small amount of receptor in a 'mature' form in the absence of pharmacoperone treatment, indicating that these two mutant receptors are not retained to the same degree (Mizrachi and Segaloff, 2004; Newton et al., 2011; Newton et al., 2016). That these two mutants are handled differently by the cellular QCS is also indicated by the observation that they associ-

ate differentially with ER-resident molecular chaperone proteins (Mizrachi and Segaloff, 2004) suggesting that they are able to attain different stages of their folding/maturation pathway. Unlike the wild-type receptor, the S616Y and A593P mutant receptors interact with binding immunoglobulin protein (BiP), a stress protein that aids folding and translocation within the ER and in transporting misfolded proteins to the proteasome. In addition, the A593P mutant receptor interacts with another stress protein, 94 kDa glucose-regulated protein (Grp94), but, unlike the wild-type receptor and S616Y mutant, does not interact with protein disulphide isomerase (PDI), an enzyme involved in protein folding in the ER through catalysis of disulphide bonds (Mizrachi and Segaloff, 2004). Interestingly, the pharmacoperone effects of Org 42599 are most pronounced with cells expressing the less severely retained S616Y mutant (Newton et al., 2011). These observations are in agreement with previous studies examining intracellularly retained rat LHR mutants demonstrating that incubation of cells at reduced temperatures to facilitate protein folding increased their cell surface expression and that mutants with some degree of cell surface expression exhibited a greater ability to be rescued than those that were more profoundly retained (Jaquette and Segaloff, 1997), presumably due to more extensive misfolding.

Examination of the effects of Org 42599 incubation on a range of 20 naturally-occurring LHR mutants has revealed a spectrum of responses to pharmacoperone treatment from no response through to full restoration of wild-type receptor cell surface expression levels (our own unpublished observations). The variation in responses is likely due to (i) the location of the mutation within the receptor structure (as only mutations located in, or adjoining to, the areas of the 7-TM domain with which Org 42599 makes contact, and therefore can be stabilized through its interactions (TMs 3, 4, 5, 6, 7 and ECL2), are able to respond to pharmacoperone treatment) and (ii) the degree of misfolding elicited by the mutation. It should be noted that, although Org 42599 is able to stabilize mutations in the TM domains with which it makes contact, one caveat would relate to any mutations that cause direct disruption of its allosteric binding pocket. Mutations which disrupt important trafficking motifs would also be unlikely to be responsive to pharmacoperone treatment. For example, a frameshift mutation that results in the last 83 amino acids of the LHR being replaced with 21 different amino acids, and ablates the highly conserved F(X)$_6$LL motif within Helix 8 at the membrane-proximal end of the C-terminal tail, causes severe intracellular retention (due to loss of this trafficking motif important for mediating receptor transport from the ER to the cell surface) is not 'rescued' by treatment with Org 42599 (Rivero-Muller et al., 2015).

It is important to note that rescue of cell surface expression of mutant receptors does not predetermine rescue of their function as this would assume that the mutations do not impair binding/signalling of the receptor in addition to affecting their trafficking to the cell surface. However, rescued functionality of S616Y mutant receptors has been demonstrated by increased hormone response measured in cells expressing these receptors, after pre-incubation with Org 42599 at a concentra-

tion/time selected to minimize any direct effects of Org 42599 on cAMP generation (Newton et al., 2011) (Figure X.6). Examination of the functionality of a range of pharmacoperone-responsive LHR mutants has since revealed that this gain in functionality is not universal, and a subset of 'rescued' mutants remain non-functional even when their cell surface expression has been restored (our own unpublished observations), presumably due to perturbation of their signal transduction capabilities.

Contrary to other studies demonstrating pharmacoperone-induced increases in cell surface expression of wild-type receptors, such as the human GnRH receptor (Janovick et al., 2003b; Finch et al., 2008), no increase in cell surface localization or number of hormone binding sites is observed following Org 42599 incubation of cells expressing the wild-type LHR (Newton et al., 2011). However, while cell surface expression of a number of wild-type GPCRs, including the human GnRH receptor, are only fractionally expressed at the cell surface and have a high degree of intracellular retention (in the case of the GnRH receptor, this is largely due to the absence of the long cytoplasmic C-terminal tail typical of most GPCRs and presence of a basic K^{191} residue in ECL2 (Janovick et al., 2003a; Finch et al., 2008)) the wild-type human LHR is expressed predominantly at the cell surface in a mature form in endogenous and exogenous stable expression systems (Tao et al., 2004; Lin et al., 2008), thus there is a limited pool of retained WT LHRs available for 'rescue'.

X.6.2 Pharmacoperones for follicle-stimulating hormone receptor mutants

Org 42599 (a trifluroacetic acid salt of the thienopyrimidine Org 43553) is an allosteric agonist of the LHR (see section X.3 for details) and acts as a pharmacoperone to 'rescue' cell surface expression of mutant LHRs (see section X.6.1 for more details). Although developed as an agonist for the LHR, this compound also has activity at the FSHR, albeit at 32-fold lower potency. Examination of the ability of Org 42599 to 'rescue' cell surface expression a range of intracellularly retained naturally-occurring and laboratory-generated FSHR mutations indicated that this compound is also able to act as a pharmacoperone at the FSHR, with the potency of rescue being proportional to the potency of activation of each of the two gonadotropin receptors (our own unpublished observations). Again, mutants receptive to 'rescue' are located in TM domains implicated in the glycoprotein hormone receptor allosteric binding site of the thienopyrimidines and, like the LHR, no effect on wild-type receptor cell surface expression was noted. However, contrary to these observations, Org 41841 (another thienopyrimidine LHR allosteric agonist closely related to Org 42599 (see section X.3 for details) that is only able to activate the FSHR at very high concentrations) has been shown to increase the number of FSH binding sites present on cells expressing wild-type FSHR 1.8-

fold, with no effect on hormone affinity (Janovick et al., 2009). In the same study, an Org 41841-induced increase in hormone response in cells expressing the intracellularly retained A189V mutant FSHR was also observed, although no effect was seen for a number of other FSHR mutants with mutations at diverse sites in the receptor (Janovick et al., 2009). Not discounting technical factors such as differences in cell lines or transfection methodologies utilised in these studies, these observations are surprising because (i) the A189V mutation is located in LLR7 of the FSHR ECD distant from the glycoprotein-hormone allosteric binding site and (ii) even at the wild-type FSHR, Org 41841 is only able to induce receptor activation at very high (millimolar) concentrations but increases in wild-type and A198V mutant receptor binding sites were observed at lower (micromolar) concentrations.

X.7 Conclusions and future perspectives

As discussed herein, the gonadotropin receptors (LHR and FSHR) play an important role in the endocrine control of reproduction and as such, inactivating mutations in these receptors has been implicated in a range of reproductive disorders. It has been traditionally assumed that inactivating mutations of GPCRs would disrupt hormone-binding or signal transduction capabilities of the receptors, but is now becoming clear that many inactivating mutations result in loss of expression of receptors at the cell surface – their functional site – likely due to misfolding of the nascent receptor protein and detection and intracellular retention/degradation by cellular quality control processes. This certainly appears to be the case for mutants of the gonadotropin receptors. Pharmacoperones able to restore cell surface expression of retained mutant receptors have enormous potential as novel therapeutics for treating patients suffering from disorders linked to 'misfolded' GPCR mutants and several examples of such ligands have been described and, in the case of pharmacoperones for mutant V2 vasopressin and GnRH receptors, their *in vivo* activities have been demonstrated.

The pharmacoperone 'rescue' of cell surface expression of disease-causing mutant LHRs and FSHRs has been demonstrated *in vitro* using low molecular weight, cell permeant allosteric thienopyrimidine LHR agonists (Org 42599 and Org 41841). These pharmacoperones have several advantages over many other pharmacoperones as: (i) they interact with the receptors at an allosteric biding site within the 7-TM domain and therefore do not compete with hormone binding at the ECD and (ii) they are agonists and therefore can function to rescue cell surface expression, activate the mutant receptors and allow increased activation of the receptors with endogenous ligands. They thus have the capacity to facilitate responses to both pharmacoperone and endogenous hormone *in vivo*. However, there are also a number of limitations. Firstly, only mutations located in regions of

the receptor that can be stabilised directly or indirectly by pharmacoperone binding will be responsive. In the case of the gonadotropin receptors which have two separate and distinct domains, it is unlikely that interactions with these pharmacoperones in the allosteric binding site in the 7-TM domain will be able to induce significant, if any, stabilisation of disruptions in the ECD domain. Secondly, rescue of cell surface expression does not predetermine rescue of functionality as this would assume that the mutations do not result in concurrent impairment of ligand binding or signal transduction in addition to structural destabilisation. Indeed, in the case of the LHR, increased functionality is not observed for all pharmacoperone-responsive mutants.

In addition to these general considerations, another limitation of pharmacoperones targeting the gonadotropin receptors are related to disease severity. As the gonadotropins play an important role in foetal gonadal development and sex steroid hormone production, mutations that are particularly disruptive and result in extreme phenotypes (such as complete pseudohermaphroditism in males due to severely inactivating LHR mutations) will inhibit this early development and pharmacoperone treatment. However effective pharmacoperone treatment is at restoring mutant receptor cell surface expression/functionality, it will likely be ineffective at restoring reproductive competence in these individuals. That being said, these compounds still have potential for the therapeutic treatment of patients suffering from milder reproductive dysfunction caused by 'responsive' mutations of the glycoprotein hormone receptors.

The studies described herein have demonstrated that thienopyrimidine gonadotropin analogues are able to act as pharmacoperones for mutant gonadotropin receptors. Relatively high concentrations of Org 42599 are required to observe pharmacoperone effects on LHR mutants and at these concentrations, this compound would also have significant FSHR activity. Similarly, at concentrations of Org 41841 required to see pharmacoperone activity at the FSHR, a high degree of LHR activation will be elicited. Although there may be some situations where activity at both gonadotropin receptors may be desirable, modifications to impair improvements in receptor selectivity would be required to enhance their therapeutic potential. It has also been demonstrated that heterodimeric molecules comprising of a derivative of Org 41841 with dual LHR/FSHR agonist activity linked to a selective FSHR antagonist retained LHR activity (albeit with reduced potency) but had no FSHR activity (Bonger et al., 2010). It is therefore feasible that similar dimeric molecules could be utilized to develop receptor-selective pharmacoperone therapeutics.

It is likely that any small-molecule, cell permeant, ligand that can interact with a GPCR will be able to stabilize its conformation to some extent and therefore have the potential to act as a pharmacoperone for destabilising mutations of that receptor. In addition to the thienopyrimidine class of gonadotropin analogues, several other small-molecule modulators of the glycoprotein hormone receptors have

been described (Nataraja et al., 2015). Therefore, there is potential for the re-purposing of these as pharmacoperones. Indeed, while pharmacoperones can be identified using high-throughput *in vitro* cell-based assays to screen compound libraries, re-purposing of existing small molecule agonists and antagonists to GPCRs is advantageous, as these compounds often bind with nanomolar affinities, and in the case of compounds identified for therapeutic application, such as the thienopyrimidines, they have frequently navigated Phase I toxicology and safety studies, theoretically expediting their clinical application as pharmacoperones.

X.8 References

Ballesteros JA, Weinstein H (1992) Analysis and refinement of criteria for predicting the structure and relative orientations of transmembranal helical domains. Biophys J 62:107-109.

Beau I, Touraine P, Meduri G, Gougeon A, Desroches A, Matuchansky C, Milgrom E, Kuttenn F, Misrahi M (1998) A novel phenotype related to partial loss of function mutations of the follicle stimulating hormone receptor. J Clin Invest 102:1352-1359.

Beerepoot P, Nazari R, Salahpour A (2017) Pharmacological chaperone approaches for rescuing GPCR mutants: Current state, challenges, and screening strategies. Pharmacol Res 117:242-251.

Bernier V, Morello JP, Zarruk A, Debrand N, Salahpour A, Lonergan M, Arthus MF, Laperriere A, Brouard R, Bouvier M, Bichet DG (2006) Pharmacologic chaperones as a potential treatment for X-linked nephrogenic diabetes insipidus. J Am Soc Nephrol 17:232-243.

Bonger KM, Hoogendoorn S, van Koppen CJ, Timmers CM, van der Marel GA, Overkleeft HS (2010) Development of Selective LH Receptor Agonists by Heterodimerization with a FSH Receptor Antagonist. ACS Med Chem Lett 2:85-89.

Bramble MS, Goldstein EH, Lipson A, Ngun T, Eskin A, Gosschalk JE, Roach L, Vashist N, Barseghyan H, Lee E, Arboleda VA, Vaiman D, Yuksel Z, Fellous M, Vilain E (2016) A novel follicle-stimulating hormone receptor mutation causing primary ovarian failure: a fertility application of whole exome sequencing. Hum Reprod 31:905-914.

Canals M, Lopez-gimenez JF, Milligan G (2009) Cell surface delivery and structural reorganization by pharmacological chaperones of an oligomerization-defective alpha(1b)-adrenoceptor mutant demonstrates membrane targeting of GPCR oligomers. Biochem J 417:161-172.

Chen Y, Chen C, Wang Y, Liu-Chen LY (2006) Ligands regulate cell surface level of the human kappa opioid receptor by activation-induced down-regulation and pharmacological chaperone-mediated enhancement: differential effects of nonpeptide and peptide agonists. J Pharmacol Exp Ther 319:765-775.

Cortez L, Sim V (2014) The therapeutic potential of chemical chaperones in protein folding diseases. Prion 8:28938. Epub 2014 May 12.

Davis D, Liu X, Segaloff DL (1995) Identification of the sites of N-linked glycosylation on the follicle-stimulating hormone (FSH) receptor and assessment of their role in FSH receptor function. Mol Endocrinol 9:159-170.

Davis DP, Rozell TG, Liu X, Segaloff DL (1997) The six N-linked carbohydrates of the lutropin/choriogonadotropin receptor are not absolutely required for correct folding, cell surface expression, hormone binding, or signal transduction. Mol Endocrinol 11:550-562.

Desai SS, Achrekar SK, Sahasrabuddhe KA, Meharji PK, Desai SK, Mangoli VS, Mahale SD (2015) Functional characterization of two naturally occurring mutations (Val514Ala and Ala575Val) in follicle-stimulating hormone receptor. J Clin Endocrinol Metab 100:E638-45.

Desai SS, Roy BS, Mahale SD (2013) Mutations and polymorphisms in FSH receptor: functional implications in human reproduction. Reproduction 146:R235-48.

Duvernay MT, Dong C, Zhang X, Zhou F, Nichols CD, Wu G (2009) Anterograde trafficking of G protein-coupled receptors: function of the C-terminal F(X)6LL motif in export from the endoplasmic reticulum. Mol Pharmacol 75:751-761.

Duvernay MT, Zhou F, Wu G (2004) A conserved motif for the transport of G protein-coupled receptors from the endoplasmic reticulum to the cell surface. J Biol Chem 279:30741-30750.

Fan QR, Hendrickson WA (2005) Structure of human follicle-stimulating hormone in complex with its receptor. Nature 433:269-277.

Fan ZC, Tao YX (2009) Functional characterization and pharmacological rescue of melanocortin-4 receptor mutations identified from obese patients. J Cell Mol Med 13:3268-3282.

Finch AR, Sedgley KR, Caunt CJ, McArdle CA (2008) Plasma membrane expression of GnRH receptors: regulation by antagonists in breast, prostate, and gonadotrope cell lines. J Endocrinol 196:353-367.

Fortin JP, Dziadulewicz EK, Gera L, Marceau F (2006) A nonpeptide antagonist reveals a highly glycosylated state of the rabbit kinin B1 receptor. Mol Pharmacol 69:1146-1157.

Gerrits M, Mannaerts B, Kramer H, Addo S, Hanssen R (2013) First evidence of ovulation induced by oral LH agonists in healthy female volunteers of reproductive age. J Clin Endocrinol Metab 98:1558-1566.

Gromoll J, Schulz A, Borta H, Gudermann T, Teerds KJ, Greschniok A, Nieschlag E, Seif FJ (2002a) Homozygous mutation within the conserved Ala-Phe-Asn-Glu-Thr motif of exon 7 of the LH receptor causes male pseudohermaphroditism. Eur J Endocrinol 147:597-608.

Gromoll J, Schulz A, Borta H, Gudermann T, Teerds KJ, Greschniok A, Nieschlag E, Seif FJ (2002b) Homozygous mutation within the conserved Ala-Phe-Asn-Glu-Thr motif of exon 7 of the LH receptor causes male pseudohermaphroditism. Eur J Endocrinol 147:597-608.

Haas AK, Kleinau G, Hoyer I, Neumann S, Furkert J, Rutz C, Schulein R, Gershengorn MC, Krause G (2011) Mutations that silence constitutive signaling activity in the allosteric ligand-binding site of the thyrotropin receptor. Cell Mol Life Sci 68:159-167.

Heitman LH, Kleinau G, Brussee J, Krause G, Ijzerman AP (2012) Determination of different putative allosteric binding pockets at the lutropin receptor by using diverse drug-like low molecular weight ligands. Mol Cell Endocrinol 351:326-336.

Heitman LH, Oosterom J, Bonger KM, Timmers CM, Wiegerinck PH, Ijzerman AP (2008) [3H]Org 43553, the first low-molecular-weight agonistic and allosteric radioligand for the human luteinizing hormone receptor. Mol Pharmacol 73:518-524.

Hoyer I, Haas AK, Kreuchwig A, Schulein R, Krause G (2013) Molecular sampling of the allosteric binding pocket of the TSH receptor provides discriminative pharmacophores for antagonist and agonists. Biochem Soc Trans 41:213-217.

Huang Y, Breitwieser GE (2007) Rescue of calcium-sensing receptor mutants by allosteric modulators reveals a conformational checkpoint in receptor biogenesis. J Biol Chem 282:9517-9525.

Hugon-Rodin J, Sonigo C, Gompel A, Dode C, Grynberg M, Binart N, Beau I (2017) First mutation in the FSHR cytoplasmic tail identified in a non-pregnant woman with spontaneous ovarian hyperstimulation syndrome. BMC Med Genet 18:44-017-0407-6.

Huhtaniemi IT, Themmen AP (2005) Mutations in human gonadotropin and gonadotropin-receptor genes. Endocrine 26:207-217.

Janovick JA, Brothers SP, Cornea A, Bush E, Goulet MT, Ashton WT, Sauer DR, Haviv F, Greer J, Conn PM (2007) Refolding of misfolded mutant GPCR: post-translational pharmacoperone action in vitro. Mol Cell Endocrinol 272:77-85.

Janovick JA, Goulet M, Bush E, Greer J, Wettlaufer DG, Conn PM (2003a) Structure-activity relations of successful pharmacologic chaperones for rescue of naturally occurring and manufactured mutants of the gonadotropin-releasing hormone receptor. J Pharmacol Exp Ther 305:608-614.

Janovick JA, Maya-Nunez G, Ulloa-Aguirre A, Huhtaniemi IT, Dias JA, Verbost P, Conn PM (2009) Increased plasma membrane expression of human follicle-stimulating hormone receptor by a small molecule thienopyr(im)idine. Mol Cell Endocrinol 298:84-88.

Janovick JA, Stewart MD, Jacob D, Martin LD, Deng JM, Stewart CA, Wang Y, Cornea A, Chavali L, Lopez S, Mitalipov S, Kang E, Lee HS, Manna PR, Stocco DM, Behringer RR, Conn PM (2013) Restoration of testis function in hypogonadotropic hypogonadal mice harboring a misfolded GnRHR mutant by pharmacoperone drug therapy. Proc Natl Acad Sci U S A 110:21030-21035.

Janovick JA, Ulloa-Aguirre A, Conn PM (2003b) Evolved regulation of gonadotropin-releasing hormone receptor cell surface expression. Endocrine 22:317-327.

Janovick JA, Maya-Nunez G, Conn PM (2002) Rescue of Hypogonadotropic Hypogonadism-Causing and Manufactured GnRH Receptor Mutants by a Specific Protein-Folding Template: Misrouted Proteins as a Novel Disease Etiology and Therapeutic Target. J Clin Endocrinol Metab 87:3255-3262.

Jaquette J, Segaloff DL (1997) Temperature sensitivity of some mutants of the lutropin/choriogonadotropin receptor. Endocrinology 138:85-91.

Jaschke H, Neumann S, Moore S, Thomas CJ, Colson AO, Costanzi S, Kleinau G, Jiang JK, Paschke R, Raaka BM, Krause G, Gershengorn MC (2006) A low molecular weight agonist signals by binding to the transmembrane domain of thyroid-stimulating hormone receptor (TSHR) and luteinizing hormone/chorionic gonadotropin receptor (LHCGR). J Biol Chem 281:9841-9844.

Jiang X, Dias JA, He X (2014) Structural biology of glycoprotein hormones and their receptors: insights to signaling. Mol Cell Endocrinol 382:424-451.

Jiang X, Liu H, Chen X, Chen PH, Fischer D, Sriraman V, Yu HN, Arkinstall S, He X (2012) Structure of follicle-stimulating hormone in complex with the entire ectodomain of its receptor. Proc Natl Acad Sci U S A 109:12491-12496.

Kobayashi H, Ogawa K, Yao R, Lichtarge O, Bouvier M (2009) Functional rescue of beta-adrenoceptor dimerization and trafficking by pharmacological chaperones. Traffic 10:1019-1033.

Kossack N, Troppmann B, Richter-Unruh A, Kleinau G, Gromoll J (2013) Aberrant transcription of the LHCGR gene caused by a mutation in exon 6A leads to Leydig cell hypoplasia type II. Mol Cell Endocrinol 366:59-67.

Kotlar TJ, Young RH, Albanese C, Crowley WF,Jr, Scully RE, Jameson JL (1997) A mutation in the follicle-stimulating hormone receptor occurs frequently in human ovarian sex cord tumors. J Clin Endocrinol Metab 82:1020-1026.

Kremer H, Kraaij R, Toledo SP, Post M, Fridman JB, Hayashida CY, van Reen M, Milgrom E, Ropers HH, Mariman E (1995) Male pseudohermaphroditism due to a homozygous missense mutation of the luteinizing hormone receptor gene. Nat Genet 9:160-164.

Kuk K, Taylor-Cousar JL (2015) Lumacaftor and ivacaftor in the management of patients with cystic fibrosis: current evidence and future prospects. Ther Adv Respir Dis 9:313-326.

Labrecque P, Roy SJ, Frechette L, Iorio-Morin C, Gallant MA, Parent JL (2013) Inverse agonist and pharmacochaperone properties of MK-0524 on the prostanoid DP1 receptor. PLoS One 8:e65767.

Lan TH, Liu Q, Li C, Wu G, Lambert NA (2012) Sensitive and high resolution localization and tracking of membrane proteins in live cells with BRET. Traffic 13:1450-1456.

Latronico AC, Anasti J, Arnhold IJ, Rapaport R, Mendonca BB, Bloise W, Castro M, Tsigos C, Chrousos GP (1996) Brief report: testicular and ovarian resistance to luteinizing hormone caused by inactivating mutations of the luteinizing hormone-receptor gene. N Engl J Med 334:507-512.

Laue LL, Wu SM, Kudo M, Bourdony CJ, Cutler GB,Jr., Hsueh AJ, Chan WY (1996) Compound heterozygous mutations of the luteinizing hormone receptor gene in Leydig cell hypoplasia. Mol Endocrinol 10:987-997.

Lin CC, Clouser C, Peegel H, Menon B, Menon KM (2008) The extracellular domain of luteinizing hormone receptor dictates its efficiency of maturation. Biochem Biophys Res Commun 377:307-311.

Meduri G, Touraine P, Beau I, Lahuna O, Desroches A, Vacher-Lavenu MC, Kuttenn F, Misrahi M (2003) Delayed puberty and primary amenorrhea associated with a novel mutation of the human follicle-stimulating hormone receptor: clinical, histological, and molecular studies. J Clin Endocrinol Metab 88:3491-3498.

Mizrachi D, Segaloff DL (2004) Intracellularly located misfolded glycoprotein hormone receptors associate with different chaperone proteins than their cognate wild-type receptors. Mol Endocrinol 18:1768-1777.

Moore S, Jaeschke H, Kleinau G, Neumann S, Costanzi S, Jiang JK, Childress J, Raaka BM, Colson A, Paschke R, Krause G, Thomas CJ, Gershengorn MC (2006) Evaluation of small-molecule modulators of the luteinizing hormone/choriogonadotropin and thyroid stimulating hormone receptors: structure-activity relationships and selective binding patterns. J Med Chem 49:3888-3896.

Morello JP, Salahpour A, Laperriere A, Bernier V, Arthus MF, Lonergan M, Petaja-Repo U, Angers S, Morin D, Bichet DG, Bouvier M (2000) Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. J Clin Invest 105:887-895.

Nataraja SG, Yu HN, Palmer SS (2015) Discovery and Development of Small Molecule Allosteric Modulators of Glycoprotein Hormone Receptors. Front Endocrinol (Lausanne) 6:142.

Neumann S, Raaka BM, Gershengorn MC (2009) Human TSH receptor ligands as pharmacological probes with potential clinical application. Expert Rev Endocrinol Metab 4:669.

Newton CL, Anderson RC, Katz AA, Millar RP (2016) Loss of function mutations in the human luteinizing hormone receptor predominantly cause intracellular retention. Endocrinology :en20161104.

Newton CL, Whay AM, McArdle CA, Zhang M, van Koppen CJ, van de Lagemaat R, Segaloff DL, Millar RP (2011) Rescue of expression and signaling of human luteinizing hormone G protein-coupled receptor mutants with an allosterically binding small-molecule agonist. Proc Natl Acad Sci USA 108:7172-7176.

Noorwez SM, Kuksa V, Imanishi Y, Zhu L, Filipek S, Palczewski K, Kaushal S (2003) Pharmacological chaperone-mediated in vivo folding and stabilization of the P23H-opsin mutant associated with autosomal dominant retinitis pigmentosa. J Biol Chem 278:14442-14450.

Oksche A, Rosenthal W (1998) The molecular basis of nephrogenic diabetes insipidus. J Mol Med (Berl) 76:326-337.

Petaja-Repo UE, Hogue M, Laperriere A, Walker P, Bouvier M (2000) Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. J Biol Chem 275:13727-13736.

Pey AL, Perez B, Desviat LR, Martinez MA, Aguado C, Erlandsen H, Gamez A, Stevens RC, Thorolfsson M, Ugarte M, Martinez A (2004) Mechanisms underlying responsiveness to tetrahydrobiopterin in mild phenylketonuria mutations. Hum Mutat 24:388-399.

Rannikko A, Pakarinen P, Manna PR, Beau I, Misrahi M, Aittomaki K, Huhtaniemi I (2002) Functional characterization of the human FSH receptor with an inactivating Ala189Val mutation. Mol Hum Reprod 8:311-317.

Ringe D, Petsko GA (2009) What are pharmacological chaperones and why are they interesting?. J Biol 8:80.

Rivero-Muller A, Potorac I, Pintiaux A, Daly AF, Thiry A, Rydlewski C, Nisolle M, Parent AS, Huhtaniemi I, Beckers A (2015) A novel inactivating mutation of the LH/chorionic gonadotrophin receptor with impaired membrane trafficking leading to Leydig cell hypoplasia type 1. Eur J Endocrinol 172:K27-36.

Rowe SM, Verkman AS (2013) Cystic fibrosis transmembrane regulator correctors and potentiators. Cold Spring Harb Perspect Med 3:10.1101/cshperspect.a009761.

Sahni N, Yi S, Taipale M, Fuxman Bass JI, Coulombe-Huntington J, Yang F, Peng J, Weile J, Karras GI, Wang Y, Kovacs IA, Kamburov A, Krykbaeva I, Lam MH, Tucker G, Khurana V, Sharma A, Liu YY, Yachie N, Zhong Q, Shen Y, Palagi A, San-Miguel A, Fan C, Balcha D, Dricot A, Jordan DM, Walsh JM, Shah AA, Yang X, Stoyanova AK, Leighton A, Calderwood MA, Jacob Y, Cusick ME, Salehi-Ashtiani K, Whitesell LJ, Sunyaev S, Berger B, Barabasi AL, Charloteaux B, Hill DE, Hao T, Roth FP, Xia Y, Walhout AJ, Lindquist S, Vidal M (2015) Widespread macromolecular interaction perturbations in human genetic disorders. Cell 161:647-660.

Tao YX (2006) Inactivating mutations of G protein-coupled receptors and diseases: structure-function insights and therapeutic implications. Pharmacol Ther 111:949-973.

Tao YX, Johnson NB, Segaloff DL (2004) Constitutive and agonist-dependent self-association of the cell surface human lutropin receptor. J Biol Chem 279:5904-5914.

Themmen APN, Huhtaniemi IT (2000) Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. Endocr Rev 21:551-583.

Timossi C, Ortiz-Elizondo C, Pineda DB, Dias JA, Conn PM, Ulloa-Aguirre A (2004) Functional significance of the BBXXB motif reversed present in the cytoplasmic domains of the human follicle-stimulating hormone receptor. Mol Cell Endocrinol 223:17-26.

Toledo SP, Brunner HG, Kraaij R, Post M, Dahia PL, Hayashida CY, Kremer HT (1996) An inactivating mutation of the luteinizing hormone receptor causes amenorrhea in a 46,XX female. J Clin Endocrinol Metab 81:3850-3854.

Touraine P, Beau I, Gougeon A, Meduri G, Desroches A, Pichard C, Detoeuf M, Paniel B, Prieur M, Zorn JR, Milgrom E, Kuttenn F, Misrahi M (1999) New natural inactivating mutations of the follicle-stimulating hormone receptor: correlations between receptor function and phenotype. Mol Endocrinol 13:1844-1854.

Uchida S, Uchida H, Maruyama T, Kajitani T, Oda H, Miyazaki K, Kagami M, Yoshimura Y (2013) Molecular analysis of a mutated FSH receptor detected in a patient with spontaneous ovarian hyperstimulation syndrome. PLoS One 8:e75478.

Van Craenenbroeck K, Clark SD, Cox MJ, Oak JN, Liu F, Van Tol HH (2005) Folding efficiency is rate-limiting in dopamine D4 receptor biogenesis. J Biol Chem 280:19350-19357.

van de Lagemaat R, Timmers CM, Kelder J, van Koppen C, Mosselman S, Hanssen RG (2009) Induction of ovulation by a potent, orally active, low molecular weight agonist (Org 43553) of the luteinizing hormone receptor. Hum Reprod 24:640-648.

van Koppen CJ, Zaman GJ, Timmers CM, Kelder J, Mosselman S, van de Lagemaat R, Smit MJ, Hanssen RG (2008) A signaling-selective, nanomolar potent allosteric low molecular weight agonist for the human luteinizing hormone receptor. Naunyn Schmiedebergs Arch Pharmacol 378:503-514.

van Straten NC, Schoonus-Gerritsma GG, van Someren RG, Draaijer J, Adang AE, Timmers CM, Hanssen RG, van Boeckel CA (2002) The first orally active low molecular weight agonists for the LH receptor: thienopyr(im)idines with therapeutic potential for ovulation induction. Chembiochem 3:1023-1026.

Vassart G, Pardo L, Costagliola S (2004) A molecular dissection of the glycoprotein hormone receptors. Trends Biochem Sci 29:119-126.

Wainwright CE, Elborn JS, Ramsey BW (2015) Lumacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. N Engl J Med 373:1783-1784.

Yu R, Chen CR, Liu X, Kodra JT (2012) Rescue of a pathogenic mutant human glucagon receptor by pharmacological chaperones. J Mol Endocrinol 49:69-78.

Zarinan T, Perez-Solis MA, Maya-Nunez G, Casas-Gonzalez P, Conn PM, Dias JA, Ulloa-Aguirre A (2010) Dominant negative effects of human follicle-stimulating hormone receptor expression-deficient mutants on wild-type receptor cell surface expression. Rescue of oligomerization-dependent defective receptor expression by using cognate decoys. Mol Cell Endocrinol 321:112-122.

Zhang M, Feng X, Guan R, Hebert TE, Segaloff DL (2009) A cell surface inactive mutant of the human lutropin receptor (hLHR) attenuates signaling of wild-type or constitutively active receptors via heterodimerization. Cell Signal 21:1663-1671.

Figure Legends

Figure X.1 Thienopyrimidine allosteric agonists of the glycoprotein hormone receptors. A. *N-tert-butyl-5-amino-4-(3-methoxyphenyl)-2-(methythio)thieno[2,3-d] pyrimidine-6-carboxamide (Org 41841) and* ***B.*** 5-amino-2-methylsulfanyl-4-[3-(2-morpholin-4-yl-acetylamino)-phenyl]-thieno[2,3-*d*]pyrimidine-6-carboxylic acid *tert*-butylamide (Org 43553/Org 42599).

Figure X.2 The majority of mutant LHRs are intracellularly retained. Cell surface receptor expression was measured in intact cells expressing wild-type (filled bars), and **A.** retained mutant LHRs or **B.** partially/non-retained mutant LHRs (open bars), using an ELISA assay targeting N-terminal (extracellular) FLAG epitope tags of the receptors. Data are presented as percentage of the maximal expression measured for the WT receptor (set at 100%) after subtraction of nonspecific signal (measured in the presence of cells transfected with empty vector) and are mean ± SEM from at least three independent experiments. ***, $P < .001$; **, $P < .01$; *, $P < .05$, by one-way ANOVA followed by Dunnett's multiple comparison test, for comparison with WT.

Figure X.3 LH elicits little or no activation of cells expressing A593P or S616Y mutant LHRs. Measurement of cAMP accumulation by cAMP ELISA after 1 h stimulation in cells expressing WT (●), A593P mutant (■), or S616Y (▲) mutant LHRs over a range of concentrations of LH. Data were fitted by sigmoidal dose–response curves with Hill coefficients of unity. Data are presented as fold versus basal values for each receptor.

Figure X.4 Cellular localizations of mutant LHRs are altered after incubation with Org 42599. Cells expressing WT, A593P mutant, or S616Y mutant LHRs were incubated in the presence of vehicle (left panels) or 1 μM Org 42599 (right panels) for 24 h before fixation, immunocytofluorescent labelling, and confocal imaging. LH receptors are labelled in green and cell nuclei marker (DAPI) in blue. (Scale bar: 10 μm).

Figure X.5 Binding of ^{125}I-hLH to cell surface mutant LHRs is increased in a time- and concentration-dependent manner after incubation with Org 42599. Binding of ^{125}I-hLH to cells expressing WT, A593P mutant, or S616Y mutant LHRs was measured after incubation with Org 42599 (1 μM) for a range of incubation times **(A)** or for 24 h with a range of concentrations of Org 42599 **(B)**. After incubation with Org 42599, cells were washed once before incubation with radioligand. Data are presented as fold over binding in the absence of Org 42599

treatment and are mean ± SEM from at least three independent experiments. *P < 0.05 and **P < 0.01 (one-sample t test) for comparison with vehicle control (1.0-fold change, dotted line).

Figure X.6 LH stimulation of cells expressing S616Y mutant LHRs is increased by preincubation with Org 42599. cAMP accumulation was measured by cAMP ELISA after stimulation of cells expressing S616Y mutant LHRs with LH (3 nM) for 1 h at 37 °C after preincubation in the presence or absence of Org 42599 (0.1 μM) for 2 h and washing once for 1 h. Data are mean ± SEM from three independent experiments and are presented as percentage of the maximum LH response obtained in the absence of Org 42599 incubation.

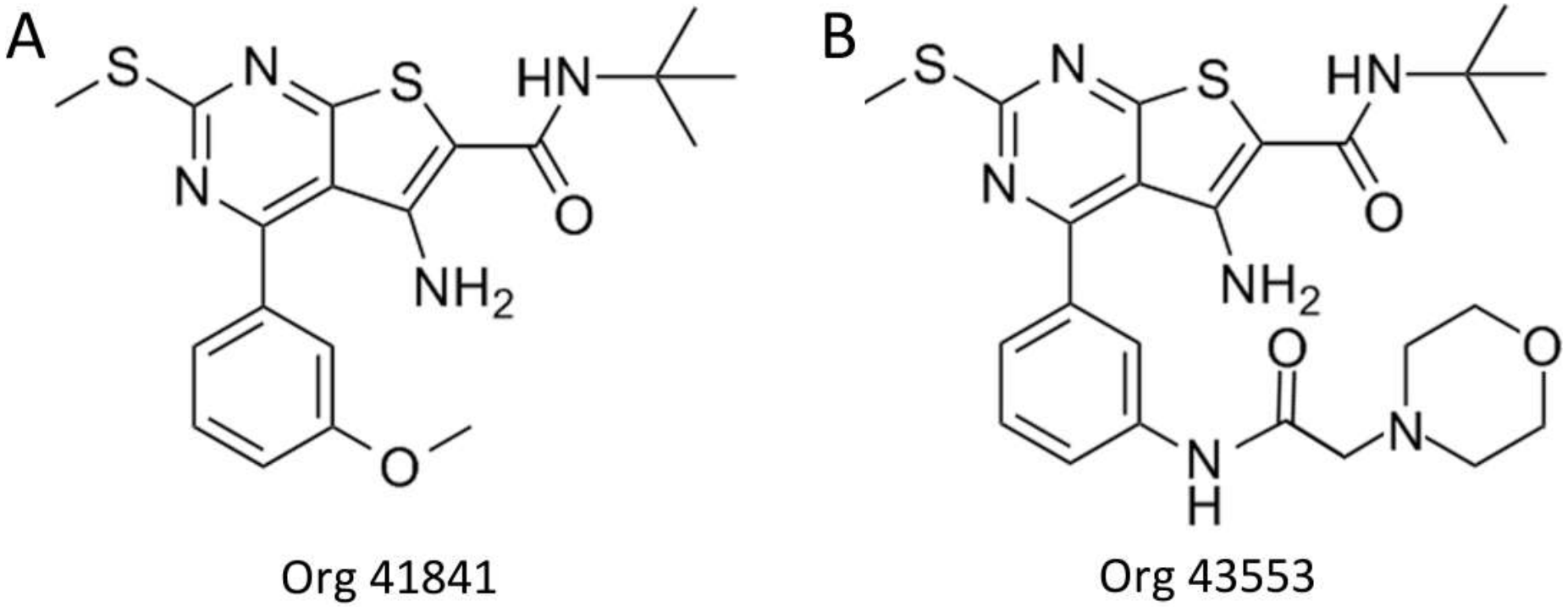

Figure X.1 Thienopyrimidine allosteric agonists of the glycoprotein hormone receptors. A. *N-tert-butyl-5-amino-4-(3-methoxyphenyl)-2-(methythio)thieno[2,3-d] pyrimidine-6-carboxamide (Org 41841) and* ***B.*** 5-amino-2-methylsulfanyl-4-[3-(2-morpholin-4-yl-acetylamino)-phenyl]-thieno[2,3-*d*]pyrimidine-6-carboxylic acid *tert*-butylamide (Org 43553/Org 42599).

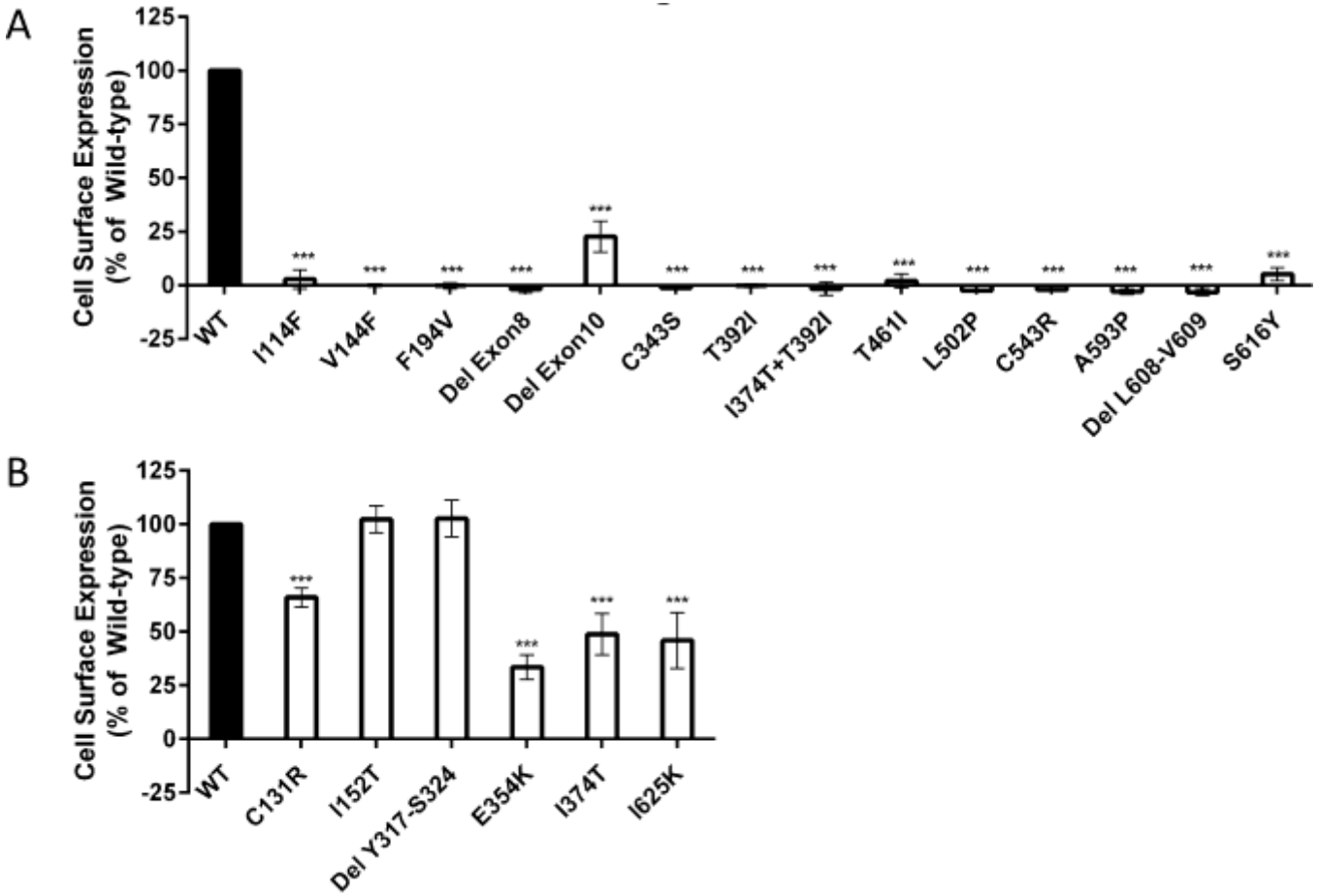

Figure X.2 The majority of mutant LHRs are intracellularly retained. Cell surface receptor expression was measured in intact cells expressing wild-type (filled bars), and **A.** retained mutant LHRs or **B.** partially/non-retained mutant LHRs (open bars), using an ELISA assay targeting N-terminal (extracellular) FLAG epitope tags of the receptors. Data are presented as percentage of the maximal expression measured for the WT receptor (set at 100%) after subtraction of nonspecific signal (measured in the presence of cells transfected with empty vector) and are mean ± SEM from at least three independent experiments. ***, P <.001; **, P<.01; *, P<.05, by one-way ANOVA followed by Dunnett's multiple comparison test, for comparison with WT.

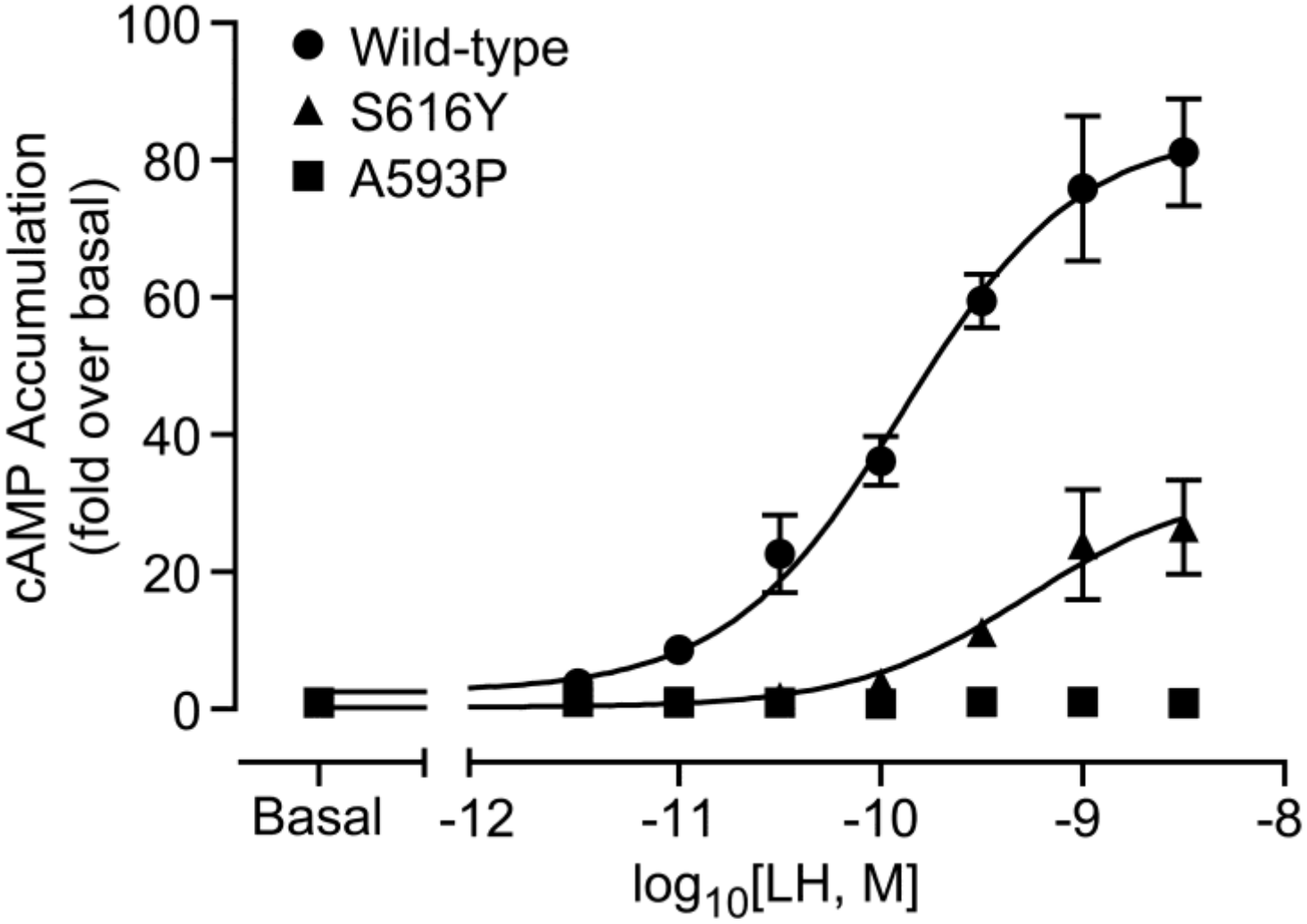

Figure X.3 LH elicits little or no activation of cells expressing A593P or S616Y mutant LHRs. Measurement of cAMP accumulation by cAMP ELISA after 1 h stimulation in cells expressing WT (●), A593P mutant (■), or S616Y (▲) mutant LHRs over a range of concentrations of LH. Data were fitted by sigmoidal dose–response curves with Hill coefficients of unity. Data are presented as fold versus basal values for each receptor.

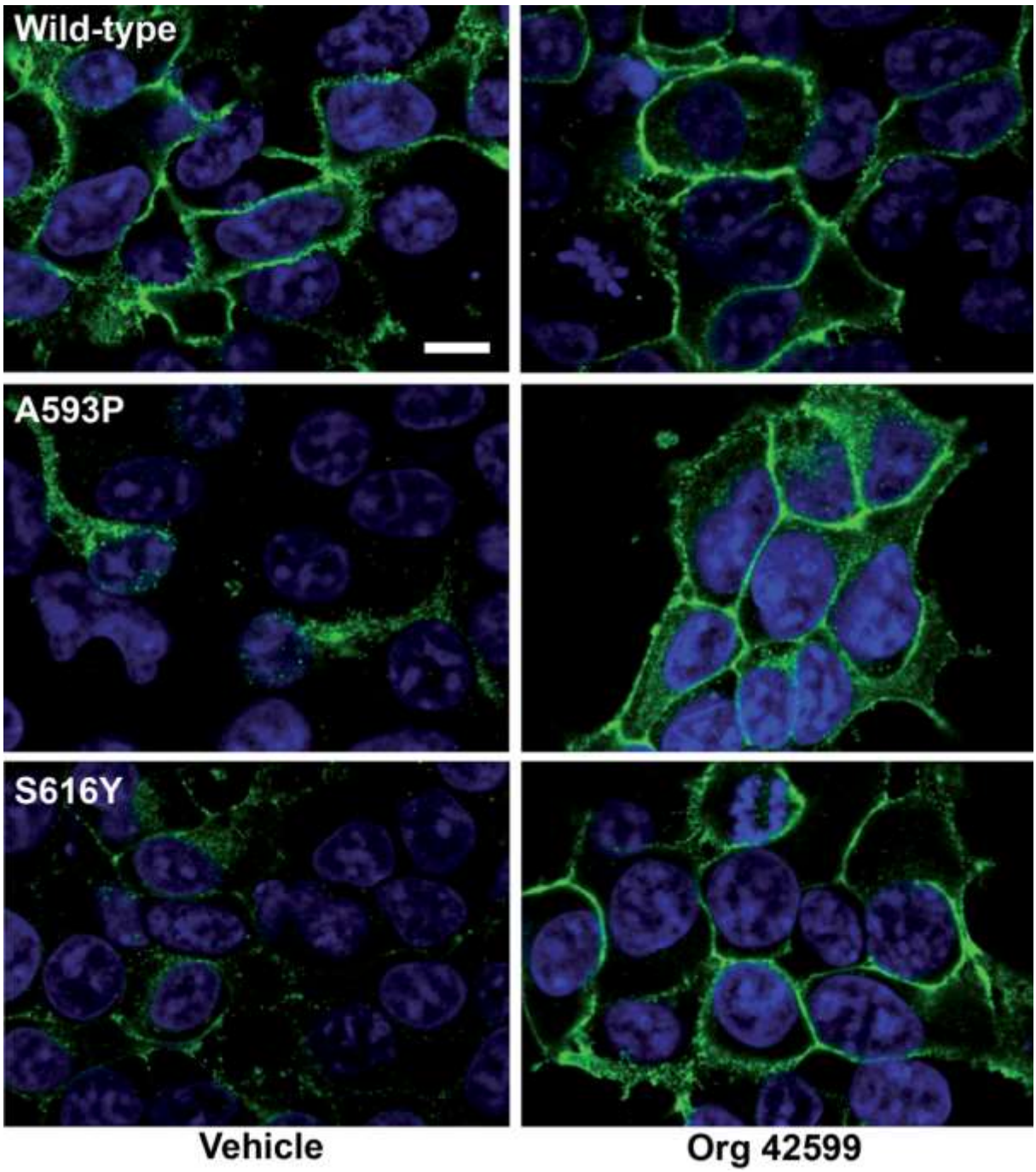

Figure X.4 Cellular localizations of mutant LHRs are altered after incubation with Org 42599. Cells expressing WT, A593P mutant, or S616Y mutant LHRs were incubated in the presence of vehicle (left panels) or 1 μM Org 42599 (right panels) for 24 h before fixation, immunocytofluorescent labelling, and confocal imaging. LH receptors are labelled in green and cell nuclei marker (DAPI) in blue. (Scale bar: 10 μm).

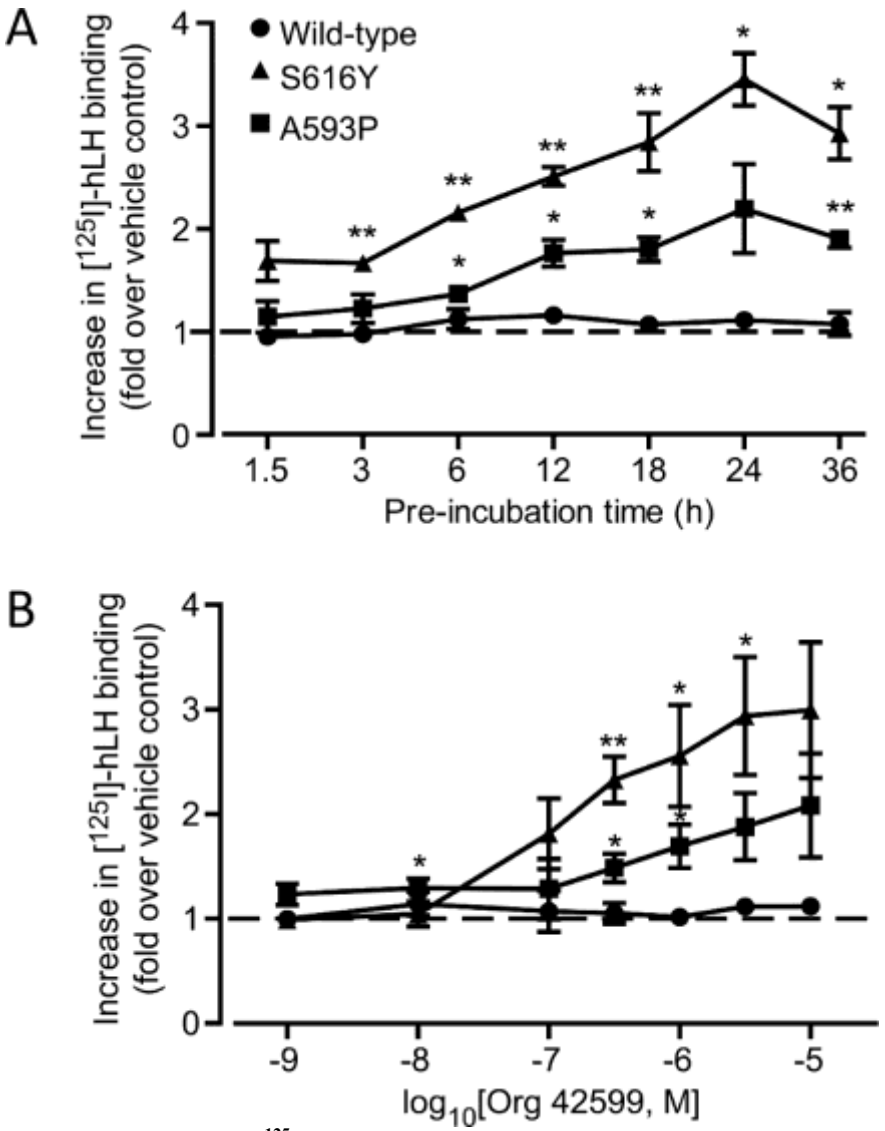

Figure X.5 Binding of ^{125}I-hLH to cell surface mutant LHRs is increased in a time- and concentration-dependent manner after incubation with Org 42599. Binding of ^{125}I-hLH to cells expressing WT, A593P mutant, or S616Y mutant LHRs was measured after incubation with Org 42599 (1 μM) for a range of incubation times **(A)** or for 24 h with a range of concentrations of Org 42599 **(B)**. After incubation with Org 42599, cells were washed once before incubation with radioligand. Data are presented as fold over binding in the absence of Org 42599 treatment and are mean ± SEM from at least three independent experiments. *P < 0.05 and **P < 0.01 (one-sample t test) for comparison with vehicle control (1.0-fold change, dotted line).

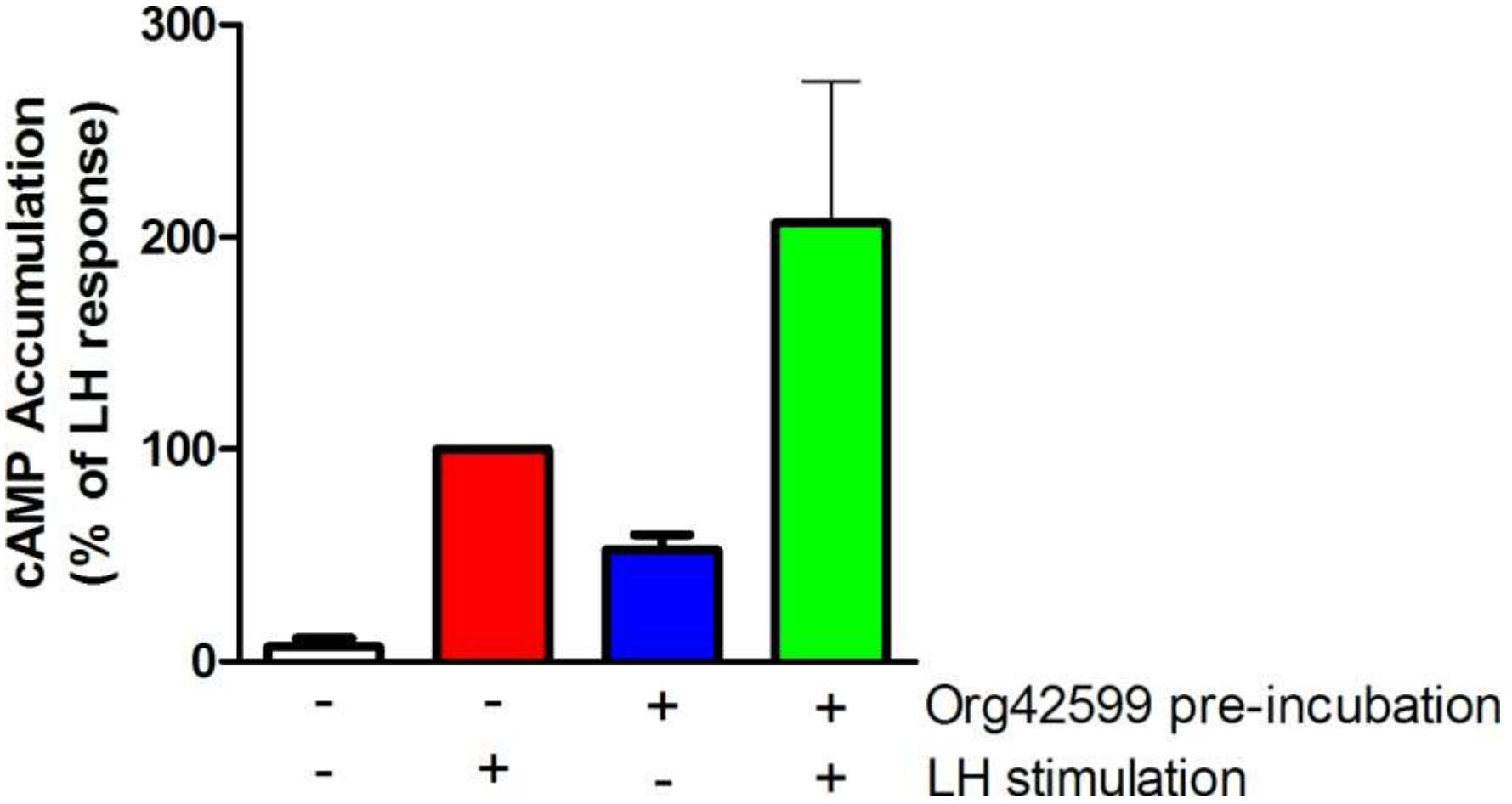

Figure X.6 LH stimulation of cells expressing S616Y mutant LHRs is increased by preincubation with Org 42599. cAMP accumulation was measured by cAMP ELISA after stimulation of cells expressing S616Y mutant LHRs with LH (3 nM) for 1 h at 37 °C after preincubation in the presence or absence of Org 42599 (0.1 μM) for 2 h and washing once for 1 h. Data are mean ± SEM from three independent experiments and are presented as percentage of the maximum LH response obtained in the absence of Org 42599 incubation.