Rescue of cell surface expression and signalling of mutant follicle-stimulating hormone receptors

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Disclosure Summary

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Abstract

Mutations in G protein-coupled receptors (GPCRs) underlie numerous diseases. Many cause receptor misfolding and failure to reach the cell surface. Pharmacological chaperones are cell-permeant small-molecules that engage nascent mutant GPCRs in the endoplasmic reticulum, stabilising folding and 'rescuing' cell surface expression. We previously demonstrated rescue of cell surface expression of luteinising hormone receptor mutants by an allosteric agonist. Here we demonstrate that a similar approach can be employed to rescue mutant follicle-stimulating hormone receptors (FSHRs) with poor cell surface expression using a small-molecule FSHR agonist, CAN1404. Seventeen FSHR mutations described in patients with reproductive dysfunction were expressed in HEK 293T cells and cell surface expression was determined by ELISA of epitope-tagged FSHRs before/after treatment with CAN1404. Cell surface expression was severely reduced to ≤18% of wild-type (WT) for eleven, modestly reduced to 66–84% of WT for four and was not reduced for two. Of the eleven with severely reduced cell surface expression, restoration to ≥57% of WT levels was achieved for six by treatment with 1 μM CAN1404 for 24 h and a corresponding increase in FSH-induced signalling was observed for four of these, indicating restored functionality. Therefore, CAN1404 acts as a pharmacological chaperone and can rescue cell surface expression and function of certain mutant FSHRs with severely reduced cell surface expression. These findings aid in advancing the understanding of the effects of genetic mutations on GPCR function and provide a proof of therapeutic principle for FSHR PCs.

Keywords: G protein-coupled receptor mutations, follicle-stimulating hormone receptors, reproductive dysfunction, pharmacological chaperones

1.0 Introduction

G protein-coupled receptors (GPCRs) mediate the majority of signal transduction by cell surface receptors (1), and dysfunction of GPCR signalling is consequently implicated in numerous physiological/pathophysiological processes (2-5). Dysfunction may be through changes in the biosynthesis and/or secretion of ligands and/or cognate GPCRs by alterations in cellular regulation or genetic mutations. Dysfunction through inactivating genetic mutations in GPCRs are classified into five categories: Class I (defective receptor biosynthesis), Class II (impaired trafficking to the cell surface), Class III (ligand binding-deficient), Class IV (defective receptor activation) and Class V (not know defects/no defects) (6). There is evidence that Class II mutations are the most common (7-9). These cause misfolding of the nascent receptor protein, with consequent intracellular retention and degradation, thus resulting in diminished cell surface expression.

Following mRNA translation by ribosomes on the rough endoplasmic reticulum (ER), nascent GPCR proteins are directed into the ER lumen, which houses folding chaperone proteins responsible for regulating protein folding and assembly (10, 11). Once correctly folded, GPCRs travel to the Golgi apparatus for further processing/post-translational modification and trafficking to the cell surface (12). A cellular quality control system (QCS) is responsible for regulating GPCR trafficking to the cell surface (13) by scrutiny of newly synthesised proteins for complete processing and folding. When a protein is not correctly folded, unfolded protein response (UPR) pathways are activated and the protein is retained in the ER while further folding is attempted. If this continues to be unsuccessful, the protein is targeted for degradation via ER assisted degradation (ERAD) pathways.

Importantly, intracellularly retained Class II mutant GPCRs, which are not degraded, generally maintain at least a degree of their intrinsic ligand binding and signal transduction functionality (14). This indicates that stabilisation of folding will allow misfolded GPCRs to bypass the QCS and reach the cell surface to facilitate ligand binding and receptor activation. The possibility of achieving this has been realised by the discovery of pharmacological chaperones, which are cell-permeant compounds that rescue the cell surface expression of intracellularly retained mutant proteins. These hydrophobic small-molecules are able to cross the cell membrane, bind the nascent mutant proteins in the ER, stabilise their correct folding, and thereby evade recognition by the QCS to avoid subsequent activation of UPR and ERAD processes (14, 15).

Although this is an embryonic field, pharmacological chaperones have already been described for several disease-causing GPCR mutants (reviewed in 15) and, in two instances (for vasopressin V_2 receptors and gonadotropin-releasing hormone receptors (GnRHRs)), these discoveries have been translated into *in vivo* demonstration of efficacy (16,17). Indeed, this approach to mutant protein "repair" appears to be more easily implemented than gene repair as demonstrated by success in treating cystic fibrosis (18).

Follicle-stimulating hormone receptors (FSHRs) play an important role in the hypothalamic-pituitary-gonadal (HPG) axis of the reproductive system and are expressed on granulosa cells in the ovaries of females (where their activation stimulates aromatase conversion of thecal cell-derived androgens to regulate maturation of ovarian follicles) and Sertoli cells in the testes of males (which support spermatogenesis). Consequently, inactivating FSHR mutations can result in reproductive pathologies including hypergonadotropic hypogonadism, primary/secondary amenorrhea and incomplete development of secondary sex characteristics (19, 20). In males, FSHR deficiency results in less severe phenotypes with reduced testicular size and sperm production, but the retention of fertility (19, 20).

We have previously identified a pharmacological chaperone, Org 42599 (renamed LHR-Chap), capable of rescuing cell surface expression of Class II mutants of the luteinising hormone receptor (LHR/LHCGR) (21, 22).The human FSHR is evolutionarily and structurally related to the human LHR (63% similarity and 48% identity for the entire sequence) (23), and an LHR small-molecule agonist closely related to LHR-Chap was capable of rescuing mutant FSHRs with poor cell surface expression (24). However, the effect was limited, and only one mutant (of several tested) displayed rescue. Furthermore, rescue of mutant FSHRs with an LHR agonist would be impractical therapeutically due to the concomitant non-specific activation, and possible densensitisation, of the LHR or premature stimulation of ovulation in *in vitro* fertilisation protocols.

We reasoned that we may be able to more effectively rescue cell surface expression of Class II mutant human FSHRs using FSHR-selective cell-permeant small-molecule agonists developed as orally-active allosteric alternatives to FSH for assisted reproductive technologies (25-28). Literature and patent databases have described a suite of small-molecule substituted dihydrobenzoindazoles, hexahydraquinolones, chromenopyrazloes, thiazolidinones, benzamides, hydroxyquinolines and aminoalkylamines, which act as agonists or antagonists at the human FSHR (25, 26, 28-31). The agonists had been developed for induction of folliculogenesis for *in vitro* fertilisation and antagonists as potential contraceptives. In the present study, we set out to explore the spectrum of described mutant FSHRs with poor cell surface expression that could be rescued by one of these novel FSHR small-molecules, CAN1404 (a dihydrobenzoindazole analogue originally described by Organon in the published patent WO2011/012674 (29)).

2.0 Materials and Methods

2.1 Materials

Follicle-stimulating hormone (FSH; GONAL-f) was produced by Merck Serono (Modugno, Italy). We initially tested a series of FSHR small-molecule analogues for their ability to rescue cell surface expression of selected mutant human FSHRs. A small-molecule FSHR agonist, CAN1404, (4-(cyclobutanecarbonyl)-1,4-diazepan-1-yl)(8-isopropoxy-7-methoxy-1-(thiophen-3-yl)-4,5-dihydro-1H-benzo[g]indazol-3-yl)methanone (Figure 1), was found to be efficacious. Of the panel of compounds tested, CAN1404 had the greatest effect on mutant FSHR cell surface expression, with respect to both number of mutants affected, and the degree of increase observed. CAN1404 is one of a family of dihydrobenzoindazole derivatives, originally described by Organon in the published patent WO2011/012674 and designated 2g in example 2 (29). It has high selectivity for the FSHR (confirmed using a homogeneous time-resolved fluorescence assay with Chinese hamster ovary cells stably expressing the human FSHR, LHR or thyroid stimulating hormone receptors (TSHR) [as described 31], in which it was found to stimulate cAMP production with a potency of 0.14, 58 and 2043 nM respectively). The compound was re-synthesised and the structure confirmed by mass spectrometry and nuclear magnetic resonance spectroscopy. The purity was >95%.

Primary mouse monoclonal anti-FLAG® M2 antibody (#F1804; RRID: AB_259529 1 mg/ml), was from Sigma-Aldrich (St Louis, MO, USA), goat anti-mouse IgG (H+L)-HRP Conjugate (#170-6516; RRID: AB_11125547) from Bio-Rad Laboratories (Hercules, CA, USA) and AlexaFluor® 488-conjugated goat anti-mouse secondary antibody (#ab150117, RRID:AB_2688012) from Abcam (Cambridge, UK). cDNA

encoding WT human FSHR was obtained from the Bloomsburg University cDNA Resource Centre (www.cdna.org, Accession number NM_000145.4). Expression vectors encoding CRE-luciferase (pCRE-luc), renilla-luciferase (pRL-TK; Renilla-luc) and empty vector (pcDNA3.1-) were from Clontech Laboratories (Mountain View, CA, USA), Promega (Madison, WI, USA) and Invitrogen (Carlsbad, CA, USA), respectively. A mammalian expression vector encoding $G\alpha_{16}$ was a kind gift from Dr Anna Aragay Combas of the Institut de Biologia Molecular de Barcelona.

2.2 Methods

2.2.1 Identification and characterisation of published human FSHR mutations

Seventeen naturally occurring inactivating mutations of the human FSHR were identified through literature review. Missense and in-frame deletion mutations in the mature receptor protein were selected as potential candidates for pharmacological chaperone rescue (Figure 2 and Table 1). Mutations M512I and R634H were identified in patients with spontaneous ovarian hyperstimulation syndrome (46, 53) suggesting that these are activating. However, following *in vitro* analysis, both mutant receptors had reduced cAMP generation compared to WT FSHR when exposed to high levels of FSH (46, 53), indicating that they are inactivating mutations and they were therefore included in this study.

2.2.2 Generation of mammalian expression vectors encoding WT and mutant FSHRs

The FSHR coding sequence was subcloned into the mammalian expression vector pcDNA3.1- and was tagged with an N-terminal FLAG epitope tag (FLAG-hFSHR), immediately downstream of a signal peptide sequence. That the FLAG tag had no detrimental effect on FSHR function was confirmed by comparison of the signalling responses between the tagged and untagged receptor, in which FSH had the same potency at both, and the FLAG-tagged

receptor elicited 71% of the maximal response of the untagged receptor (data not shown). FLAG-hFSHR was used to monitor WT FSHR expression and activity and as a template for the production of plasmids encoding mutant FSHRs by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Agilent, Santa Clara, USA) (see Table 2 for mutagenesis primer sequences). Mutations were verified by Sanger sequencing.

2.2.3 Cell culture and transfection

Human Embryonic Kidney (HEK 293T) cells (Cat# CRL-3216, RRID:CVCL_0063) from ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% (v/v) foetal calf serum (FCS) at 37ºC, 5% CO_2 and 95% humidity. Prior to cell seeding, culture plates were pre-treated with a 1:30 dilution of Matrigel (Thermo Fisher Scientific, Waltham, MA, USA) to aid cell attachment. Exogenous WT and mutant FLAG-hFSHR plasmids were transfected into HEK 293T cells using XtremeGENE HP (XTG) DNA transfection reagent (Sigma-Aldrich, St Louis, MO, USA) at a 2:1 (XTG:DNA) ratio.

2.2.4 Measurement of FSHR signalling

2.2.4.1 CRE-luciferase reporter gene assay

CRE- luciferase reporter gene assays were conducted as previously described (22). In brief, HEK 293T cells were seeded at $1x10^5$ cells/well in 24-well tissue culture plates and were transiently transfected with WT or mutant FSHRs (0.23 µg/well) in combination with Renilla luciferase (0.04 µg/well) and CRE-luciferase (0.23 µg/well). After 24 h, the cells were washed with phosphate-buffered saline (PBS), supplemented with 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ (PBS+) and were incubated in

serum-free media (DMEM supplemented with 10 mM HEPES) at 37ºC for a further 24 h. Cells were stimulated with a range of concentrations of FSH or CAN1404 in serum-free media for 24 h at 37°C, then washed twice before lysis and measurement of luciferase activity using a Dual-luciferase reporter gene assay kit (Promega, Madison, WI, USA) and FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany).

CRE-luciferase activity was divided by Renilla luciferase activity in each well and basal signal (measured in the absence of stimulating ligand) was subtracted and data calculated as a percentage of the average maximal FSH-stimulated value attained across all experimental repeats. Data were analysed using GraphPad Prism (Version 8) software (GraphPad Inc, San Diego, CA, USA) and were fitted to sigmoidal dose-response curves with a Hill coefficient of unity, from which EC_{50} and maximal response to FSH and CAN1404 were determined. Comparisons were made by Student's t-test, with $p<0.05$ considered significant.

2.2.4.2 IP Accumulation assay

Functionality of the mutant FSHRs was determined by $G\alpha_{16}$-linked inositol phosphate (IP) accumulation assay as previously described (21). This assay has been previously demonstrated to result in good correlation between cell surface receptor expression (i.e. density of agonist-accessible receptors) and functional response for the closely related LHR (7). This is indicative of little/no "receptor reserve", a phenomena that can result in submaximal receptor occupancy eliciting maximal responses. In brief, cells were plated at $1.5x10^5$ cells/well in 24-well plates and were co-transfected with plasmids encoding WT/mutant FSHRs (0.25 µg/well) and the

promiscuous $G\alpha_{16}$ G protein (0.25 µg/well). Following a 24 h incubation, media was removed and cells were incubated for a further 24 h in Media 199 (Thermo Fisher Scientific) supplemented with 2% FCS containing [^{3}H]-myoinositol (0.5 µCi/well). The media was aspirated and cells were then incubated with buffer I supplemented with 10 mM LiCl for 30 minutes at 37ºC, before stimulation with FSH (a range of concentrations for dose-response analyses, or 1 nM [equivalent to approx. EC_{50} in this assay, Figure 3B] for single-concentration analyses), CAN1404 (a range of concentrations for dose-response analyses) or appropriate vehicle (ultra-pure water) for 1 h at 37ºC and measurement of IP accumulation.

Data were normalised by calculating values as a ratio of the sum of all data values obtained within each assay replicate. The % of the average FSH response measured in cells expressing WT FSHR was then determined after subtraction of the basal response in these cells. Data were analysed using GraphPad Prism (Version 8) software. For dose-response analyses, data were fitted to sigmoidal dose-response curves with a Hill coefficient of unity, from which EC_{50} and maximal response to FSH and CAN1404 were determined and comparisons made by Student's t-test. Comparison of mutant responses in single-concentration experiments were made by one-way ANOVA, followed by Dunnett's post-test for comparison with the WT receptor, with $p<0.05$ considered significant.

For measurement of functionality of the mutant FSHRs following rescue by CAN1404, cells were incubated for 24 h with 1 µM CAN1404, or appropriate vehicle control (0.1% v/v DMSO) during the Media 199/[^{3}H]-myoinositol incubation. Cells were then washed in buffer I (DMEM supplemented with 20 mM HEPES and 0.1% BSA) to ensure removal of CAN1404 (one wash, incubation for 1 h in buffer I and then a final wash) prior to stimulation with 10 nM FSH (a concentration sufficient to

induce a maximal response in this assay, Figure 3B). All samples, regardless of treatment, underwent the same washing procedure.

Data were normalised by calculating values as a ratio of the sum of all data values obtained within each assay replicate. The % of the average FSH response measured in cells expressing WT FSHR was then determined after subtraction of the average basal response in these cells. Data were analysed using GraphPad Prism (Version 8) software by one-way ANOVA, followed by Tukey's post-test for comparison of the different treatments at each receptor, with $p<0.05$ considered significant.

2.2.4.2 cAMP ELISA

To confirm the observed effects on FSH-induced signalling of the rescued receptors also corresponded to the canonical $G\alpha_s$/cAMP accumulation pathway, a cAMP accumulation assay was performed using the WT FSHR and mutants A419T and P587H. Cells were plated at $1x10^5$ cells/well in 24-well plates and were transfected with plasmids encoding WT or mutant FSHRs (0.5 µg/well) before incubation for 24 h with 100 nM CAN1404, or appropriate vehicle control (0.1% v/v DMSO). Cells were then washed in culture media to ensure removal of CAN1404 (one wash, incubation for 3 h in culture media, with media changes every 1 h, and then a final wash). Cells were then stimulated with 10 nM FSH or appropriate vehicle (ultra-pure water), prepared in serum free media supplemented with 1 mM 3-isobutyl-1-methylxanthine, for 1 h at 37ºC before lysis of the cells with 0.1 M HCl and measurement of cAMP accumulation using a Direct cAMP ELISA kit (Enzo Life Sciences Inc., Farmingdale, NY, USA; RRID: AB_2890930).

Data were normalised by calculating values as a ratio of the sum of all data values obtained within each assay replicate. The % of the average FSH response measured in cells expressing WT FSHR was then determined after subtraction of the average basal response in these cells. Data were analysed using GraphPad Prism (Version 8) software by one-way ANOVA, followed by Tukey's post-test for comparison of the different treatments at each receptor, with $p<0.05$ considered significant.

2.2.5 Quantification of FSHR expression by receptor enzyme-linked immunosorbent assay (ELISA)

HEK 293T cells were seeded at $8x10^4$ cells/well in 48-well tissue culture plates and were transiently transfected with WT, mutant FSHRs/LHRs or empty vector (0.3 µg/well). 24 h post-transfection, cells were incubated with CAN1404 (1 µM for single concentration experiments, or a range of concentrations for dose-response analyses), a range of concentrations of LHR-Chap (for examination of effects on mutant LHR cell surface expression) or vehicle for 24 h at 37^oC. Following incubation, cell surface and total cellular receptor expression were measured by ELISA (using antibodies targeting the N-terminal FLAG epitope tag), as described previously (7, 21) with intact cells or cells permeabilised by incubation for 10 mins with cold methanol. For measurement of total cellular receptor expression, the following modifications were made to reduce non-specific/background signal: cells were blocked with DMEM supplemented with 10% FCS, 5% bovine serum albumin (BSA) and 5% milk powder), cells were incubated overnight at 4^oC with primary antibody, and a reduced concentration of secondary antibody was used (1:5000).

Data were normalised by calculating values as a ratio of the sum of all data values obtained within each assay replicate. Data from cells transfected with empty vector, used to measure non-specific signal, (approx. 19% and 47% of the signal obtained in WT receptor-transfected cells for cell surface and total cellular measurement, respectively) were subtracted from all sample readings and the data were expressed as a percentage of the average values measured for WT FSHR transfected cells across all experimental repeats. Data were analysed using GraphPad Prism (Version 8) software by two-sided t-test for comparison of vehicle treatment and CAN1404 treatment, or by one-way ANOVA, followed by Dunnett's post-test for comparison of mutant and WT receptors, with $p<0.05$ considered significant. For dose-response analyses, data were fitted to sigmoidal dose-response curves with a Hill coefficient of unity, from which EC_{50} and maximal responses were determined.

2.2.6 Immunofluorescence to visualise FSHR expression/cellular localisation

HEK 293T cells were seeded on sterile glass coverslips, in 24-well tissue culture plates, at a density of $4x10^4$ cells/well and were transiently transfected with WT, mutant FSHRs/LHRs or empty vector (0.5 µg/well). 24 h post transfection, cells were incubated with CAN1404 (1 µM) or vehicle for 24 h at 37^{o}C. Following incubation, cells were fixed with 4% PFA for 15 min at room temperature, washed with PBS three times and permeabilised with 0.2% triton X-100 for 1 h at room temperature. The cells were washed three times with PBS and incubated for 60 minutes at room temperature in PBS supplemented with 2% BSA prior to incubation with mouse anti-FLAG primary antibody (1:300 dilution, in PBS supplemented with 2% BSA). The cells were then washed a further three times with PBS, incubated with AlexaFluor®

488 conjugated goat anti-mouse secondary antibody (1:200 dilution, in PBS supplemented with 2% BSA) for 1 h at room temperature in the dark before an additional three washes with PBS. The coverslips were then mounted onto glass slides using ProLong™ Diamond Antifade mountant containing DAPI (Life Technologies, Carlsbad, USA) before visualisation using an LSM 800 confocal microscope and Zen software (Zeiss, Oberkochen, Germany) at x64 magnification.

3.0 Results

3.1 WT FSHR stimulation by CAN1404 and FSH

Dose response analysis of CAN1404 was conducted to determine the EC_{50} at the human WT FSHR, to establish the dose required to employ it as a pharmacological chaperone. Cells transfected with WT FSHR were stimulated with a range of concentrations of FSH or CAN1404 and the response measured using a CRE-luciferase reporter gene assay (Figure 3A) or $G\alpha_{16}$-linked IP accumulation assay (Figure 3B). In the CRE-luciferase assay, CAN1404 elicited stimulation of the FSHR with an EC_{50} of 1.8 nM compared with 6.5 pM for FSH and the maximal responses elicited by both ligands were not different (p=0.14, Student's t-test). Interestingly, although CAN1404 exhibited a similar potency in the IP accumulation assay (5.8 nM), the maximal response elicited was much lower (16% of the FSH response). This observation could be reflective of 'biased agonism' or could be indicative of a high degree of 'receptor reserve' in the CRE-luciferase assay, which is supported by the observation that FSH has a much greater potency in the CRE-luciferase assay than the IP assay (6.5 pM and 0.6 nM, respectively), while the potency of CAN1404 was the same in both assays (p=0.13, Student's t-test).

As pharmacological chaperone activity usually requires a dose of at least 100-fold higher than the EC_{50} observed in signalling assays (54), treatment with 1 µM

CAN1404 was selected for screening of CAN1404 effects on mutant receptor cell surface expression.

3.2 Confirmation of loss-of function of FSHR mutants

Analysis of the FSH responsiveness of the mutant FSHRs revealed no differences in the basal activity of the mutant receptors compared to the WT FSHR (data not shown) and confirmed that the majority (15/17) resulted in loss-of-function as the level of response elicited by FSH was significantly reduced (Figure 4). For thirteen (A189V, N191I, V221G, D224V, P348R, D408Y, I418S, A419T, P519T, R573C, P587H, F591S and L601V) the response elicited was <20% of the WT response, while mutants I411N and A575V exhibited responses of 41-44% of WT. For mutants M512I and R634H, FSH induced responses were not significantly different to the WT FSHR (although not significant, the response elicited by R634H was lower than that observed at the WT receptor) (Figure 4).

3.3 Identification of FSHR mutants with reduced cell surface expression

Quantitative analysis of mutant FSHR expression at the cell surface by receptor ELISA assay showed that the majority (88%; 15/17) of mutant FSHRs (A189V, N191I, V221G, D224V, P348R, D408Y, I418S, A419T, M512I, P519T, A575V, P587H, F591S, L601V and R634H) had significantly reduced cell surface expression compared to the WT receptor (Figure 5A). However, there were varying degrees of reduced cell surface expression. Eleven mutants (A189V, N191I, D224V, P348R, D408Y, I418S, A419T, P519T, A575V, P587H and F591S) had very poor cell

surface expression levels (0-18% of WT; Figure 5A). Four mutants (V221G, M512I, L601V and R634H) had moderately reduced cell surface expression (66-84% of WT; Figure 5A), and just two (I411N and R573C) exhibited no reduction in cell surface expression (indeed, mutant I411N actually had higher cell surface expression than the WT receptor) (Figure 5A).

Total receptor expression levels, measured in permeabilised cells, indicated that most of the mutant FSHRs are expressed at similar levels to the WT FSHR (Figure 5B), supporting the notion that the mutations are largely affecting cell surface localisation rather than receptor expression and/or degradation. Seven mutants with poor cell surface expression did have statistically significantly decreases in total cellular receptor expression, with mutants A189V, N191I, D408Y, I418S, A419T and F591S having total expression 66-73% of WT and mutant P587H 50% of WT.

3.4 Effects of CAN1404 on FSHR mutants with poor cell surface expression

For the current studies, our attention is exclusively focused on the FSHR mutants that are poorly expressed on the cell surface and the ability of the small-molecule agonist CAN1404 to rescue their cell surface expression. When the eleven FSHR mutants with poor cell surface expression (≤18% of WT) were incubated with CAN1404, the cell surface expression of six (D408Y, A419T, P519T, A575V, P587H and F591S) was significantly increased to 57-89% of WT, with four of these (D408Y, A419T, A575V and F591S) being rescued to levels of 76-89 % of WT (Figure 6). Mutant N191I exhibited a slight increase in cell surface expression (from 0% - 12% of WT levels) following treatment with CAN1404, but this difference was not statistically significant ($p=0.067$; Figure 6). The remaining four mutants with poor cell

surface expression (A189V, D224V, P348R and I418S) failed to show any improvement of expression at the cell surface despite treatment with CAN1404 (Figure 6). Incubation with CAN1404 also had no effect on cell surface expression of the WT FSHR (Figure 6).

To confirm these results, the cellular expression/localisation of one of the mutant receptors, A419T, was examined by immunofluorescence in the presence/absence of CAN1404 treatment. Unlike the wild-type receptor which displayed good cell surface expression, in the absence of CAN1404, mutant A419T was located intracellularly and there was a marked redistribution to the cell surface following 1 µM CAN1404 treatment (Figure 7).

Dose-dependence of the CAN1404 rescue of cell-surface expression of mutant FSHRs was determined by incubation of cells expressing mutants D408Y and A419T with a range of concentrations of CAN1404 and measurement of receptor cell surface expression. Both mutant receptors exhibited a dose-dependent increase in cell surface expression (Figure 8). The potency of rescue by CAN1404 was not significantly different for the two mutant receptors (92 nM, D408Y and 309 nM, A419T; p=0.14, Student's t-test) and maximal rescue was observed upon treatment with approx. 30 µM of CAN1404. Although sub-maximal levels of rescue of cell surface expression were observed with the 1 µM CAN1404 utilised for screening of the mutants in Figure 6, this concentration was sufficient to elicit a robust degree of rescue for both mutants tested (73% and 84% of the maximal rescue for mutants A419T and D408Y, respectively).

3.5 FSH-induced signalling of CAN1404-rescued mutants

As rescue of cell surface expression of mutant receptors does not necessarily translate to rescue of function, FSH-signalling of the six mutants that demonstrated robust increases in cell surface expression following CAN1404 treatment was determined. Cells were pre-incubated in the presence or absence of CAN1404 and, following washing, were incubated in the presence or absence of FSH. As CAN1404 is a FSHR agonist, pre-incubation for 24 h to facilitate rescue of cell surface expression, would elicit a concurrent stimulation of cAMP accumulation that would hamper measurement of any further FSH-induced signalling of the rescued receptors by the CRE-luciferase assay. To overcome this, receptor activity was measured by $G\alpha_{16}$-linked inositol phosphate accumulation assay as, unlike FSH, CAN1404

elicits a poor response when measured via this pathway (Figure 3B). Thus, we were able to monitor rescue of receptor function with CAN1404 and monitor signalling response to the native ligand, FSH.

There was no difference in basal signalling for the WT or any mutant receptors, and CAN1404 pre-incubation had no effect on the basal signalling measured for the majority of the receptors, confirming that this compound could be successfully washed off following 'rescue' of the mutant receptors (Figure 9A). The exception being mutant A575V, which exhibited a small increase in signal (22% of the WT response to FSH) following pre-incubation with CAN1404 alone. In the absence of CAN1404 pre-incubation, FSH elicited a robust response in cells expressing the WT FSHR, while little or no FSH-induced response was observed in cells expressing the mutant receptors (Figure 9A). In the absence of CAN1404 pre-incubation, mutant A575V did respond to FSH stimulation, but this response was minimal (20% of the WT FSH response).

Neither the WT FSHR nor mutants D408Y and P587H exhibited any change in FSH-induced response following CAN1404 treatment. However, for mutants A419T, P519T and F591S no FSH-induced responses were measured in the absence of CAN1404 but following pre-incubation with this compound, responses of 69% (A419T), 36% (P519T) and 31% (F591S) of the WT response were measured (Figure 9A). The FSH-induced response in cells expressing mutant A575V was increased from 20% of WT to 51% of WT following CAN1404 pre-incubation.

To confirm that the effects observed using the $G\alpha_{16}$-linked inositol phosphate accumulation assay were representative of the effects seen via the canonical $G\alpha_s$/cAMP accumulation pathway, two of the mutants, A419T (which showed good 'rescue' of FSH-induced signalling following CAN1404 treatment) and P587H (which showed no increase in FSH-induced signalling following CAN1404 treatment), were selected and FSH-induced cAMP accumulation was examined in the presence/absence of CAN1404 treatment (Figure 9B). Although a lower concentration of CAN1404 and more extensive 'washing-out' was required in order to reduce the background cAMP response measured following CAN1404 incubation alone, the data obtained essentially mirror those observed using $G\alpha_{16}$-linked inositol phosphate accumulation as a read-out.

3.6 FSHR-selectivity of CAN1404-rescue

As CAN1404 exhibits agonist activity at the LHR (albeit with >400-fold lower potency than at the FSHR), the effects of this compound on the cell surface expression of mutant LHRs was examined. A mutant LHR, T461I, that has poor cell surface expression but has been shown to be very responsive to the LHR pharmacological chaperone, LHR-Chap (21), was selected. Cells expressing this mutant LHR were treated with a range of concentrations of CAN1404 or

LHR-Chap and receptor cell surface expression was measured. While LHR-Chap was able to elicit a robust increase in cell surface expression of this mutant LHR, CAN1404 did not. Indeed, only a minor increase in cell surface expression was observed after treatment with very high concentrations (10 μM) of CAN1404 (Figure 10).

4.0 Discussion

4.1 The majority of human FSHR mutations result in decreased cell surface expression

Quantification of cell surface expression of the seventeen described human FSHRs with inactivating mutations revealed that the majority (15/17, 88%) gave rise to significantly decreased cell surface expression when compared to WT. Of these, eleven had very poor cell surface expression (≤18% of WT) and the remaining four had moderately impaired cell surface expression (66-84% of WT). This is remarkably similar to our findings examining twenty LHR mutants, in which 90% (18/20) of the mutants had reduced cell surface expression with thirteen of these having very poor cell surface expression (<10% of WT). The observation that most mutations result in impaired cell surface expression is not surprising since most constituent amino acids are likely to be involved in intramolecular interactions that configure the three-dimensional structure of the receptor, in contrast to the relatively few amino acids that are involved in ligand binding or interaction with intracellular signalling proteins.

The distribution of FSHR mutations which give rise to very poor cell surface expression (<18% of WT) was seven in the seven-transmembrane domain (7-TMD), one in the hinge region linking the extracellular leucine-rich repeat domain (LRRD) of the glycoprotein hormone receptors with the 7-TMD, and three in the LRRD. Again, this mirrors our findings examining LHR mutants, of which eight of the thirteen mutants with very poor cell surface expression were in the 7-TMD, one in the hinge region and four in the LRRD (7).

Unsurprisingly, those mutants with severely reduced cell surface expression also had a corresponding severely impaired response to FSH stimulation (although mutant A575V had a slightly higher response that would be expected based on the level of cell surface expression of this mutant receptor).

Total receptor content of the cell, although reduced for some of the poorly expressed mutants, was never less than 50% of WT levels and, in all but one case, was >65% of WT levels, indicating that the major contributor to poor cell surface expression was a failure to traffic to the cell surface. Confirmation of this suggestion will require comprehensive and complex interrogation of cellular events underlying the phenomenon as described by Hiramatsu and colleagues (55). Whatever the mechanisms, the development of pharmacological chaperones which stabilse mutant GPCRs and facilitate their trafficking to the cell surface has considerable therapeutic application for pathologies for which there is no other pharmacological treatment, such as for the FSHR and LHR mutations.

Mutants V221G, L601V and R573C which had unimpaired/only moderately impaired cell surface expression, exhibited very poor responses to FSH stimulation suggesting that the non-functionality of these mutants is likely to be primarily due to deficiencies in hormone binding and/or hormone-induced receptor signalling. Mutant I411N (which had unimpaired cell surface expression) only exhibited a modest signalling response suggestive of deficiencies in hormone binding and/or hormone-induced receptor signalling. Mutants M512I and R634H had moderately decreased cell surface expression but were able to elicit a robust signalling response. Both of these mutations were originally described in patients with spontaneous ovarian hyperstimulation syndrome and were thus thought to be activating mutations. Previous *in vitro* analysis revealed a reduced response to FSH stimulation (46, 53). However, the decreases observed were moderate (R634H) (53) or dependent on the signalling output measured (M512I) (46) and the present findings support the conclusion that these mutations are unlikely to be responsible for the observed phenotypes in these patients.

4.2 Rescue of cell surface expression and signalling of mutant FSHRs with CAN1404

Pilot screening studies on six FSHR-interactive small-molecules indicated that CAN1404, a dihydrobenzoindazole derivative with agonist activity at the FSHR (Figure 3), was efficacious in increasing cell surface expression of some FSHR mutants with poor cell surface expression. Of the panel of compounds tested in this preliminary screen, CAN1404 was able to increase cell surface expression of the most FSHR mutants tested, and to the greatest degree. This compound was therefore selected for further analysis.

Treatment with 1 µM CAN1404 for 24 hours was utilised for initial screening of CAN1404 effects on mutant receptor expression and in dose-response analyses, this concentration was shown to rescue cell surface expression of two of the mutant receptors to ≥73% of maximum. This duration of treatment and concentration of the pharmacological chaperone is the same as that found to be effective for rescue of mutant LHRs with the pharmacological chaperone LHR-Chap (21, 22). Subsequent dose-reponse analyses examining the effects of CAN1404 on cell surface expression of the mutant receptors confirmed that the potency of 'rescue' of cell surface expression by this compound was lower than that observed when measuring receptor activation and that incubation with 1 µM CAN1404 is sufficient to elicit >75% of maximal 'rescue'.

We elected to study the possible rescue of all eleven human FSHR mutants with poor cell surface expression (≤18% of WT) by incubation with CAN1404, but did not study the effects on the four loss-of-function FSHR mutants with moderately impaired expression. As these moderately expressed mutants retain reasonably

good cell surface expression (66-84% of WT), it is likely that they have impairments in binding and/or signalling that contribute to their loss-of–function. Cell surface expression of six of the eleven mutants was increased to 57-89% of WT, four of which achieved 76-89% of the cell surface expression of the WT receptor. The success rate is therefore in accordance with our findings in rescuing LHR mutants, in which five of the thirteen mutants with very poor cell surface expression (<10% of WT) exhibited a significant increase in cell surface expression upon treatment with LHR-Chap (21). There does not appear to be a correlation between the original level of cell surface expression of the mutants and the ability of CAN1404 to restore their cell surface expression. However, for both the LHR and FSHR, all of the well-rescued mutations were in the 7-TMD – six for the FSHR and five for the LHR. The LRRD and hinge region mutants could not be rescued for both receptors.

It is of note that, using receptor activity as a readout, a previous study observed a small increase in FSH-induced signalling of the WT FSHR and LRRD mutant A189V following treatment with a small-molecule LHR agonist (24). However, in the present study, similar increases for the WT/A189V FSHRs were not observed when receptor cell surface expression was measured directly.

Measurement of functionality of the ‘rescued’ mutant receptors indicated that, of the six mutants for which cell surface expression was increased to >57% of WT levels by CAN1404 treatment, four (A419T, P519T, A575V and F591S) also had a significant increase in FSH-induced signalling following CAN1404 incubation. The magnitude of the increase in FSH-induced signalling (to 31-69% of WT response) was less than that of the increase in cell surface expression (to 57-89% of WT levels) for these mutants. This is likely due to the mutations having effects on receptor functionality

(ligand interaction/signal transduction) in addition to effects on receptor conformation. Furthermore, CAN1404 interaction with these mutant receptors may be stabilising a conformation that is not fully receptive to activation by the native hormone. Nonetheless, these data support the fact that these mutant receptors are functional when rescued to the cell surface.

It is interesting to note that, unlike the WT FHSR and the other mutant receptors, mutant A575V exhibited increased signalling after pre-incubation with CAN1404. Indeed, a similar degree of signalling was measured when cells expressing this mutant receptor were stimulated with a high concentration of FSH. It is possible that this mutation results in increased binding affinity/activation potency so that any residual CAN1404 remaining following washing is sufficient to achieve some signalling response. Regardless of the reasons for this observation, a clear increase in FSH-induced signalling is still observed following CAN1404 treatment over and above that induced by CAN1404 or FSH alone.

Although mutants D408Y and P587H demonstrated a robust increase in cell surface expression in response to CAN1404 treatment, this was not translated to an increase in FSH responsiveness, suggesting that these mutations cause severe deficiencies in receptor functionality. Indeed, mutation D408Y affects the very highly conserved aspartate at position 2.50 (Ballesteros-Weinstein numbering system; 56) of TMH2, which is often involved in the allosteric binding of sodium ions and whose mutation has been shown to cause loss of agonist-dependent signalling in a number of GPCRs (57). Similarly, mutation P587H affects a very highly conserved residue (position 6.50) of TMH6. This proline is part of the conserved CWxP GPCR motif (CMAP in the FSHR) and confers a kink to TMH6 important for coupling ligand

binding to conformational changes within the helical bundle that result in subsequent receptor activation and signal transduction (58).

The mechanisms underlying the ability to rescue cell surface expression of mutant FSHRs, or not, is not immediately apparent. However, the six rescued mutants are all located in TMH2 or TMH6, except for P519T (ECL2). Four of them (A419T, P519H, P587H and F591S) are located close to a well-described allosteric small-molecule binding site of the glycoprotein hormone receptors (25, 59-61) within the 7-TMD region. Assuming that CAN1404, like other described small-molecules for the glycoprotein hormone receptors, interacts within this pocket, a plausible proposition is that the conformational disturbances induced in TMHs by mutations could feasibly be corrected by direct contacts with these helices by CAN1404. The conformational changes of the other two rescued mutations (D408Y and A575V), which are located in the more intracellular part of the 7-TMD, are possibly indirectly influenced by rigid body movements of their respective helices (TMH2 and TMH6) upon CAN1404 binding. Following this reasoning, it is also understandable that binding of CAN1404 is unable to overcome misfolding due to mutations in the hinge region (P348R) or the distant LRRD (A189V, N191I and D224V). There is no clear explanation why I418S cannot be rescued by CAN1404, as it is directly adjacent to A419T, which is rescued well. It is possible that the allosteric ligand binding site may be altered by this mutation in such a way that the biophysical properties necessary for the binding or action of CAN1404 are also disturbed. Future studies will focus on docking the available spectrum of small-molecule analogues to a molecular model of the FSHR, along with mutagenesis studies of both the postulated FSHR small-molecule binding sites and the interpreted conformational disturbances generated by various FSHR mutations to further explore these predictions. In parallel, determining the ability of

these compounds to rescue cell surface expression of the range of FSHR mutants, will provide a rich tapestry for revealing the mechanisms underlying pharmacological chaperone rescue of FSHR mutants. Information derived from these studies may provide insight for the design of pharmacological chaperones for FSHR and for GPCRs in general.

4.3 Concluding remarks

The current study demonstrates that the majority of FSHR coding region mutations result in loss of cell surface expression of the receptor protein and that a small-molecule agonist, CAN1404, can act as an FSHR-selective pharmacological chaperone and rescue cell surface expression of 7-TMD-located mutations. Furthermore, many of these 'rescued' mutant receptors display increased FSH-signalling following treatment with the pharmacological chaperone. These findings therefore provide a proof of therapeutic principle for FSHR PCs. Unlike inactivating mutations of the neurokinin B and GnRH receptors, which can be overcome by treating patients with LH and FSH, there is no current pharmacological treatment for FSHR (or LHR) inactivating mutations. Further development of such compounds could have therapeutic potential in the treatment of infertile patients harbouring FSHR 7-TMD mutations.

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Data availability

Some or all data generated or analysed during this study are included in this article.

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Figure Legends

Figure 1: Structure of CAN1404

Chemical structure of CAN1404, a dihydrobenzoindazole derivative.

Figure 2: Snake plot representation of the human FSHR showing naturally occurring inactivating mutations reported to give rise to reproductive dysfunction in humans

The positions of the FSHR inactivating mutations identified in patients with reproductive dysfunction that were considered for this study are indicated in black and the residue numbers labelled. The positions of an additional six inactivating mutations reported after initiation of this study and one mutant for which mutagenesis was not successful are indicated in grey. Circular residues indicate those involved in helical structures, connecting lines indicate cysteine bridges and the dashed line indicates the predicted signal peptide cleavage site. ECL – extracellular loop; ICL – intracellular loop; TMH – transmembrane helix; H8 – helix 8, LRRD – leucine-rich repeat domain.

Figure 3: Activation of FSHR by FSH and CAN1404

*Wild-type FSHR-transfected HEK 293T cells were stimulated with a range of concentrations of FSH or CAN1404 and FSHR activation was measured using **A** a CRE-luciferase reporter gene assay or **B** a $G\alpha_{16}$-linked IP accumulation assay. Data are presented as a percentage of the average maximal FSH response (set at 100%) after subtraction of basal (B_0) response (signal measured in the absence of stimulating ligand) and are presented as mean ± SEM from **A** three or **B** five*

independent experiments, in which each data point was performed in duplicate. FSH concentrations have been calculated assuming an average mass of 35.5 kDa.

Figure 4: Response of mutant FSHRs to FSH stimulation

*Receptor signalling was measured using a $G\alpha_{16}$-linked IP accumulation assay HEK 293T cells transiently expressing FLAG-tagged wild-type (WT) or mutant FSHRs. Cells were incubated in the absence or presence of 1 nM FSH for 1 h and IP accumulation was measured. Data are presented as a percentage of the average FSH response at the WT FSHR (set at 100%) after subtraction of basal (B_0) response (signal measured in the absence of stimulating ligand) and are presented as mean ± SEM from three independent experiments, in which each data point was performed in triplicate. ***, p<0.001, for comparison with WT FSHR (one-way ANOVA followed by Dunnett's post-test).*

Figure 5: Cell surface and total cellular receptor expression of mutant FSHRs

*Receptor expression was measured using an ELISA assay targeting the N-terminal FLAG epitope tag in **A** intact (cell surface expression) or **B** permeabilised (total cellular expression) HEK 293T cells transiently expressing FLAG-tagged wild-type (WT) or mutant FSHRs. Mutants with no reduction in cell surface expression (those with expression not lower than the WT FSHR), mutants with moderately reduced cell surface expression (those with cell surface expression 66-84% of that of the WT FSHR) and mutants with severely reduced cell surface expression (those with cell surface expression ≤18% of that of the WT FSHR) are indicated. Data are presented as a percentage of WT FSHR expression (set to 100%) after subtraction of*

*nonspecific signal, measured in empty-vector transfected cells. Data are presented as mean ± SEM from three independent experiments, in which each data point was performed in triplicate. * p=0.01-0.05, ** p<0.01 and *** p<0.001, one-way ANOVA followed by Dunnett's post-hoc test for comparison of mutant receptors with WT FSHR.*

Figure 6: Cell surface expression of WT and eleven mutant FSHRs poorly expressed at the cell surface in the absence and presence of CAN1404

*Receptor expression was measured using an ELISA assay targeting the N-terminal FLAG epitope tag in intact HEK 293T cells transiently expressing FLAG-tagged wild-type (WT) or mutant FSHRs. Cells were treated in the absence (white bars) or presence (black bars) of CAN1404 (1 μM) for 24 hours prior to measurement of receptor expression. Data are presented as a percentage of WT FSHR expression (set to 100%) after subtraction of nonspecific signal, measured in empty-vector transfected cells. Data are presented as mean ± SEM from three independent experiments, in which each data point was performed in triplicate.** p<0.01 and ***, p<0.001, Student's t-test, for comparison of CAN1404 and vehicle treated cells.*

Figure 7: Localisation of WT and D408Y FSHRs in the presence and absence of CAN1404

Cells expressing wild-type (WT) or D408Y mutant FSHRs were incubated in the presence of vehicle (-) or 1 μM CAN1404 (+) for 24 h before fixation, permeabilisation, fluorescent labelling, and confocal imaging. FLAG-tagged FSHRs are labelled in green and cell nuclei (DAPI-stained) in blue.

Figure 8: Dose-dependent effects of CAN1404 on the cell surface expression of mutant FSHRs

Receptor expression was measured using an ELISA assay targeting the N-terminal FLAG epitope tag in intact HEK 293T cells transiently expressing FLAG-tagged wild-type (WT) or D408Y or A419T mutant FSHRs. Cells were treated in the absence or presence of a range of concentrations of CAN1404 for 24 hours prior to measurement of receptor cell surface expression. Data have been presented as a percentage of WT FSHR expression in the absence of CAN1404 (set to 100%) and are presented as mean ± SEM from three independent experiments, in which each data point was performed in triplicate.

Figure 9: FSH-stimulation of WT and 'rescued' mutant FSHRs in the absence and presence of CAN1404 pre-incubation

*Receptor signalling was measured using **A** a $G\alpha_{16}$-linked IP accumulation assay or **B** a cAMP ELISA in HEK 293T cells transiently expressing FLAG-tagged wild-type (WT) or mutant FSHRs. Cells were treated in the absence (white bars and black bars) or presence (grey bars and striped bars) of CAN1404 (**A** 1 μM or **B** 100 nM) for 24 hours prior to washing. Cells were then stimulated in the absence (white bars and grey bars) or presence (black bars and striped bars) of 10 nM FSH and **A** IP accumulation or **B** cAMP accumulation was measured. Data are presented as mean ± SEM from **A** four independent experiments, in which each data point was performed in triplicate or **B** three independent experiments, in which each data point was performed in duplicate. *, p=0.03 and ***, p<0.001, for comparison of*

vehicle/vehicle and vehicle/FSH treated cells; $^{\phi\phi}$, p<0.01 and $^{\phi\phi\phi}$, p<0.001, for comparison of vehicle/FSH and CAN1404/FSH treated cells; $^{\psi}$, p=0.02 , for comparison of vehicle/vehicle and CAN1404/vehicle treated cells (one-way ANOVA followed by Tukey's post-test).

Figure 10: Dose-dependent effects of CAN1404 on the cell surface expression of WT and T461I LHRs

Receptor expression was measured using an ELISA assay targeting the N-terminal FLAG epitope tag in intact HEK 293T cells transiently expressing FLAG-tagged wild-type (WT) or T461I mutant LHRs. Cells were incubated in the absence or presence of a range of concentrations of CAN1404 or LHR-Chap for 24 hours prior to measurement of receptor cell surface expression. Data have been presented as a percentage of WT LHR expression in the absence of compound (set to 100%) and are presented as mean ± SEM from three independent experiments, in which each data point was performed in triplicate.

Table 1: FSHR mutants described in reproductive dysfunction

Mutation	Location	Hom/ Het	Phenotype	Results from previous *in vitro* analyses			Ref.	Results from present study		
				FSH-induced Signalling	Cell surface expression	FSH binding		FSH-induced signalling	Cell surface expression	Effects of CAN1404
G15D	*SP*	*Cpd Het*[1]	*POI (ROS), hypergonadotropic secondary amenorrhea*	*Severely reduced*	*Severely reduced*	*ND*	*(32)*	*ND*	*ND*	*ND*
I61N	*LRRD*	*Cpd Het*[2]	*POI (ROS), hypergonadotropic primary/secondary amenorrhea*	*Severely reduced*	*Reduced*	*ND*	*(33)*	*ND*	*ND*	*ND*
I160T	*LRRD*	*Cpd Het*[3]	*Hypergonadotropic secondary amenorrhea*	Severely reduced	Severely reduced	Severely reduced	*(34)*	*ND*	*ND*	*ND*
A189V	LRRD	Hom/ Cpd Het[4]	Hypergonadotropic primary amenorrhea with ovarian dysgenesis/POI (XY: spermatogenic failure)	Severely reduced	ND	Severely reduced	(35, 36)	Severely reduced	Severely reduced	No rescue
N191I	LRRD	Het	Normal	Severely reduced	ND	ND	(37)	Severely reduced	Severely reduced	No rescue
V221G	LRRD	Het	Hypergonadotropic	Reduced	Similar to	Reduced	(38,	Severely	Reduced	ND

			primary amenorrhea		WT		39)	reduced		
D224V	LRRD	Cpd Het[5]	Hypergonadotropic primary amenorrhea	Severely reduced	Severely reduced	Severely reduced	(40)	Severely reduced	Severely reduced	No rescue
P348R	Hinge	Hom	Hypergonadotropic primary amenorrhea	Severely reduced	ND	Severely reduced	(41)	Severely reduced	Severely reduced	No rescue
D408Y	TMH2	Hom	Hypergonadotropic primary amenorrhea	Reduced	Reduced	ND	(42)	Severely reduced	Severely reduced	Rescue of cell surface expression but not signalling
I411N	TMH2	Het	PCOS	Similar to WT	ND	ND	(43)	Reduced	Similar to WT	ND
I418S	TMH2	Hom	POI, hypergonadotropic primary amenorrhea	ND	ND	ND	(44)	Severely reduced	Severely reduced	No rescue
A419T	TMH2	Cpd Het[4]	POI, hypergonadotropic primary amenorrhea	Severely reduced	ND	Similar to WT	(36)	Severely reduced	Severely reduced	Rescue of cell surface expression and signalling
A433D	*ECL1*	*Hom*	Hypergonadotropic primary amenorrhea (*XY: micropenis)*	*ND*	*ND*	*ND*	*(45)*	*ND*	*ND*	*ND*

P504S	*TMH4*	*Hom*	*POI (ROS), hypergonadotropic primary amenorrhea*	Severely reduced	*ND*	*ND*	*(32)*	*ND*	*ND*	*ND*
M512I	ECL2	Het	(sOHSS)	Reduced/ similar to WT (pathway dependent)	Similar to WT	ND	(46)	Similar to WT	Reduced	ND
P519T	ECL2	Hom	POI, hypergonadotropic primary amenorrhea	Severely reduced	Severely reduced	Severely reduced	(47)	Severely reduced	Severely reduced	Rescue of cell surface expression and signalling
R573C	TMH6	Cpd Het[3]	Hypergonadotropic secondary amenorrhea	Reduced	Similar to WT	Similar to WT	(34)	Severely reduced	Similar to WT	ND
A575V	TMH6	Hom	Hypergonadotropic primary amenorrhea	Severely reduced	Reduced	ND	(48, 49)	Reduced	Severely reduced	Rescue of cell surface expression and signalling
P587H	TMH6	Cpd Het[6]	Hypergonadotropic primary amenorrhea	Severely reduced	ND	ND	(50)	Severely reduced	Severely reduced	Rescue of cell surface expression but not signalling
F591S	TMH6	Het	Ovarian sex cord	Severely	ND	Reduced	(51)	Severely	Severely	Rescue of

			tumours	reduced				reduced	reduced	cell surface expression and signalling
L597I	*TMH6*	*Het*	*POI, hypergonadotropic primary amenorrhea*	*Reduced*	*Reduced*	*ND*	*(52)*	*ND*	*ND*	*ND*
L601V	ECL3	Cpd Het[5]	Hypergonadotropic primary amenorrhea	Severely reduced	Similar to WT	Similar to WT	(40)	Severely reduced	Reduced	ND
R634H	C-tail (Helix 8)	Het	sOHSS	Reduced	Reduced	ND	(53)	Similar to WT	Reduced	ND
P688T	*C-tail*	*Cpd Het[2]*	*POI (ROS), hypergonadotropic primary/secondary amenorrhea*	*Reduced*	*Similar to WT*	*ND*	*(33)*	*ND*	*ND*	*ND*

Abbreviations: Cpd Het – compound heterozygous; C-tail – cytoplasmic tail; ECL – extracellular loop; FSH – follicle-stimulating hormone; Het – heterozygous; Hom – homozygous; ICL – intracellular loop; LRRD – leucine-rich repeat domain; ND – not determined; POI – premature ovarian insufficiency; Ref – reference; ROS – resistant ovary syndrome; sOHSS – spontaneous ovarian hyperstimulation syndrome; SP – signal peptide; TMH – transmembrane helix; [2] Mutation G15D was identified in a compound heterozygous patient with a deletion of exons 1 and 2. [2] Mutations I61N and P688T were identified in the same compound heterozygous patient. [3] Mutations I160T and R573C were identified in the same compound heterozygous patient. [4] Mutations A419T and L189V were identified in the same compound heterozygous patient. [5] Mutations D224V and L601V were identified in the same compound heterozygous patient. [6] Mutation P587H was identified in a compound heterozygous patient in which the second allele was deleted. Italic script denotes mutations not included in the present study as they were identified after initiation of the present study (G15D, I61N, A433D, P504S, L597I and P688T), or for which construction of the expression vector encoding the mutant receptor was not successful (I160T).

Table 2: Mutagenesis primers

Mutation	Orientation	Oligonucleotide sequence
A189V	s	5' - catctagttgggttccattgaatacacagttgtgtatttcttgaatc - 3'
	as	5' - gattcaagaaatacacaactgtgtattcaatggaacccaactagatg - 3'
N191I	s	5' - ttcaagaaatacacaactgtgcattcattggaacccaactag - 3'
	as	5' - ctagttgggttccaatgaatgcacagttgtgtatttcttgaa - 3'
V221G	s	5’ - cttgaaatatctagaatgcctggtccagaggctccg - 3’
	as	5’ - cggagcctctggaccaggcattctagatatttaag - 3’
D224V	s	5' - ggatccttgttcttgaaataactagaatgactggtccagag - 3'
	as	5' - ctctggaccagtcattctagttatttcaagaacaaggatcc - 3'
P348R	s	5' - atgcatctggcttacgggagcaggtcacg - 3'
	as	5' – cgtgacctgctcccgtaagccagatgcat - 3'
D408Y	s	5’ - gattccaatgcagagataagcaaaggccaggttgc - 3’
	as	5’ - gcaacctggcctttgcttatctctgcattggaatc - 3’
I411N	s	5’ - gcaggtagattccattgcagagatcagcaaaggc - 3’
	as	5’ - gcctttgctgatctctgcaatggaatctaccgtc - 3’
I418S	s	5’ - tcaactgatgcactgagcagcaggtagattccaatg - 3’
	as	5’ - cattggaatctacctgctgctcagtgcatcagttga - 3’
A419T	s	5' - gcattggaatctacctgctgctcattacgtcagttgatatcca - 3'
	as	5' - tggatatcaactgacgtaatgagcagcaggtagattccaatgc - 3'
M512I	s	5’ - gatgctcacctttatgtagctgctgatgccaaaga - 3’
	as	5’ - tctttggcatcagcagctacataaaggtgagcatc - 3’
P519T	s	5’ - ctgtcaatatccatggtcaggcagatgctcacc - 3’
	as	5’ - ggtgagcatctgcctgaccatggatattgacag - 3’
R573C	s	5' - gagcatggccatgcacttggcgatcctgg - 3'
	as	5' - ccaggatcgccaagtgcatggccatgctc - 3'
A575V	s	5' - gtgaagatgagcatgaccatgcgcttggcga - 3'
	as	5' - tcgccaagcgcatggtcatgctcatcttcac - 3'
P587H	s	5’ - tggcaaagaaagaaatgtgtgccatgcagaggaag - 3’
	as	5’ - cttcctctgcatggcacacatttctttctttgcca - 3’
F591S	s	5' - gggaggcagaaatggcactgaaagaaatgggtgccatgc - 3'
	as	5' - gcatggcacccatttctttcagtgccatttctgcctccc - 3'

L601V	s	5' - ggacacagtgatgacgggcaccttgaggg - 3'
	as	5' - ccctcaaggtgcccgtcatcactgtgtcc - 3'
R634H	s	5’ - cagcagaatgaagaaatctctgtgaaagtttttggtaaagatggc - 3’
	as	5’ - gccatctttaccaaaaactttcacagagatttcttcattctgctg - 3’

Abbreviations: s – sense; as – antisense

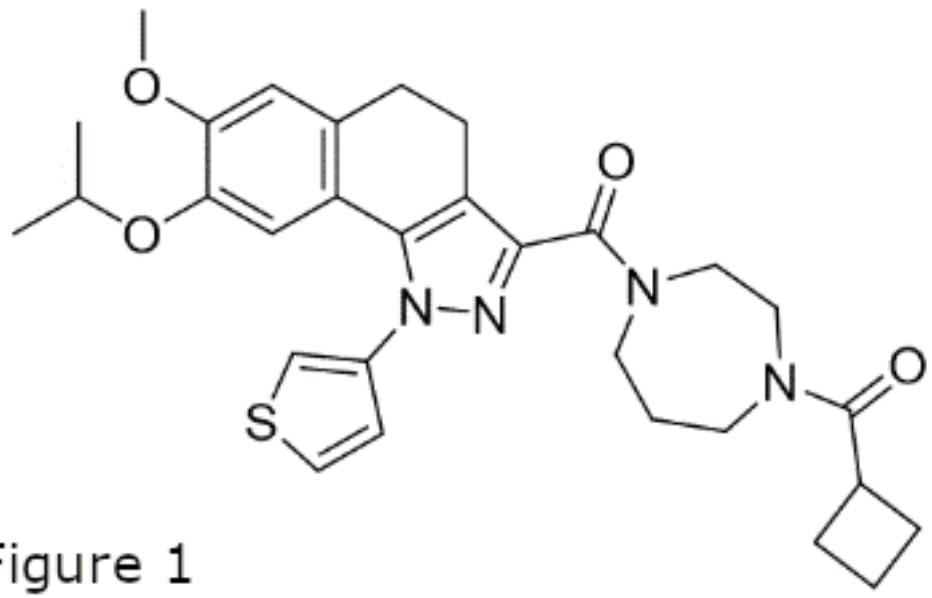
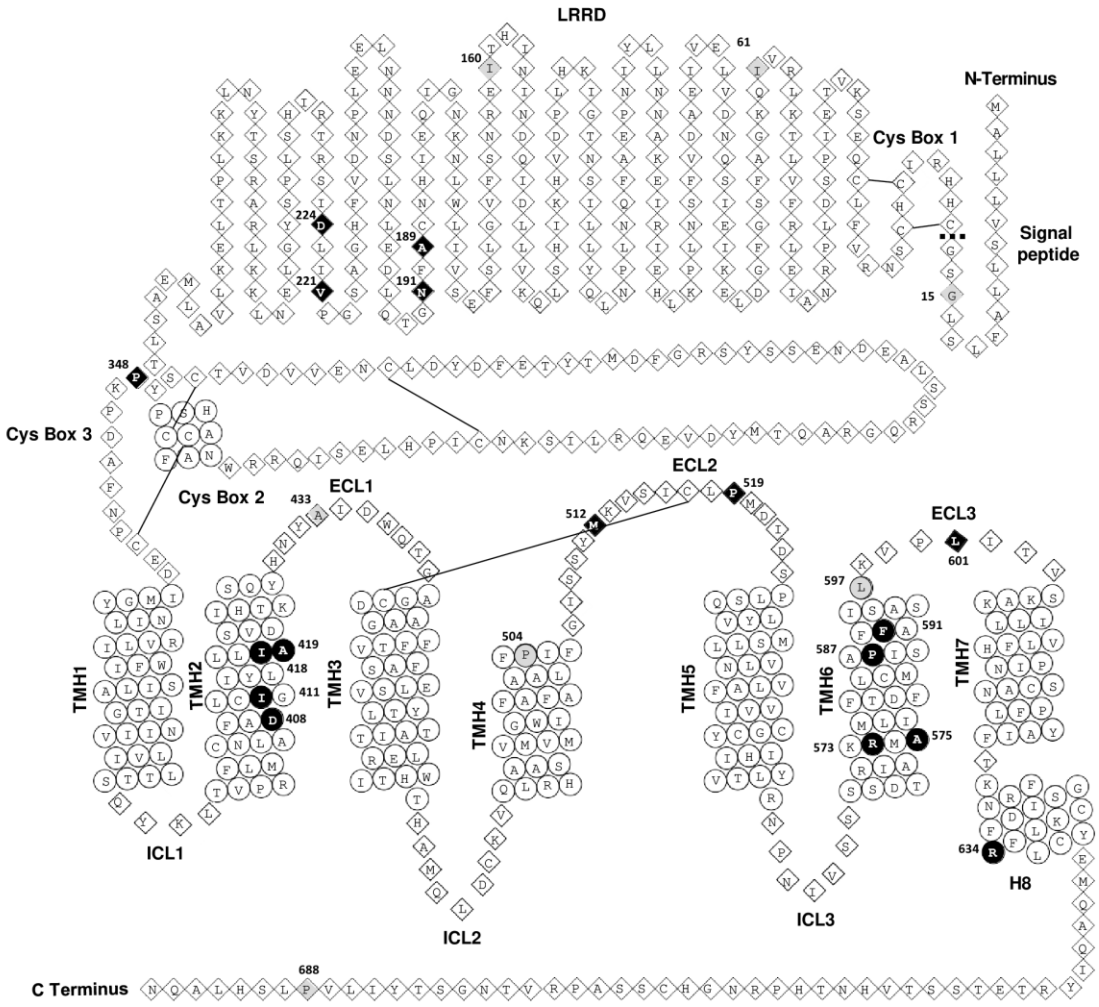


Figure 1

Figure 2


LRRD
N-Terminus
Cys Box 1
Signal peptide
Cys Box 3
Cys Box 2
ECL1
ECL2
ECL3
TMH1
TMH2
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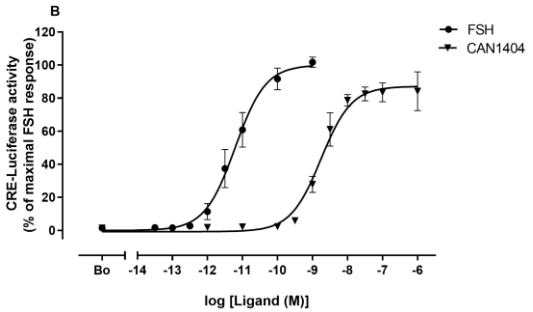
TMH4
TMH5
TMH6
TMH7
ICL1
ICL2
ICL3
H8
C Terminus
160
61
224
189
221
191
15
348
433
512
519
504
597
601
587
591
573
575
419
418
411
408
634
688

Figure 3


B
CRE-Luciferase activity
(% of maximal FSH response)
120
100
80
60
40
20
0
FSH
CAN1404
Bo
-14
-13
-12
-11
-10
-9
-8
-7
-6
log [Ligand (M)]

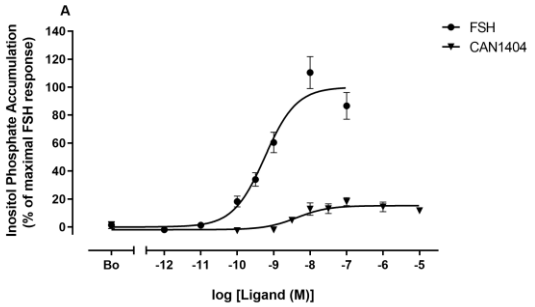

A
Inositol Phosphate Accumulation
(% of maximal FSH response)
140
120
100
80
60
40
20
0
FSH
CAN1404
Bo
-12
-11
-10
-9
-8
-7
-6
-5
log [Ligand (M)]

Figure 4

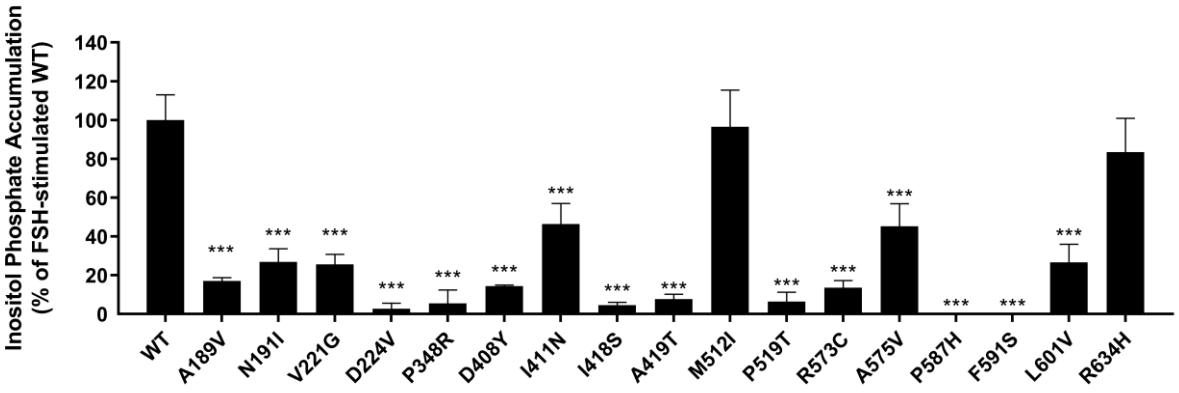

Inositol Phosphate Accumulation
(% of FSH-stimulated WT)
140
120
100
80
60
40
20
0
***
WT
A189V
N191I
V221G
D224V
P348R
D408Y
I411N
I418S
A419T
M512I
P519T
R573C
A575V
P587H
F591S
L601V
R634H

Figure 5

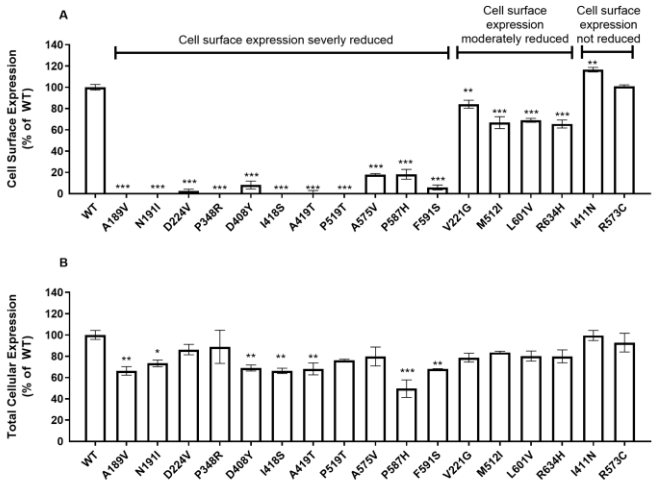

A
Cell surface expression severly reduced
Cell surface expression moderately reduced
Cell surface expression not reduced
Cell Surface Expression (% of WT)
140
120
100
80
60
40
20
0
WT
A189V
N191I
D224V
P346R
D408Y
I418S
A419T
P519T
A575V
P587H
F591S
V221G
M512I
L601V
R634H
I411N
R573C
B
Total Cellular Expression (% of WT)
140
120
100
80
60
40
20
0
WT
A189V
N191I
D224V
P346R
D408Y
I418S
A419T
P519T
A575V
P587H
F591S
V221G
M512I
L601V
R634H
I411N
R573C

Figure 6

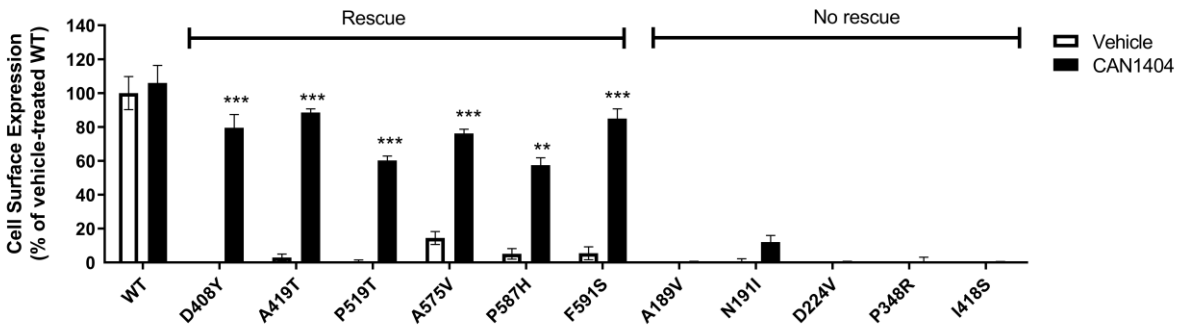

Rescue
No rescue
Vehicle
CAN1404
Cell Surface Expression
(% of vehicle-treated WT)
140
120
100
80
60
40
20
0
***
***
***
***
**
***
WT
D408Y
A419T
P519T
A575V
P587H
F591S
A189V
N191I
D224V
P348R
I418S

Figure 7

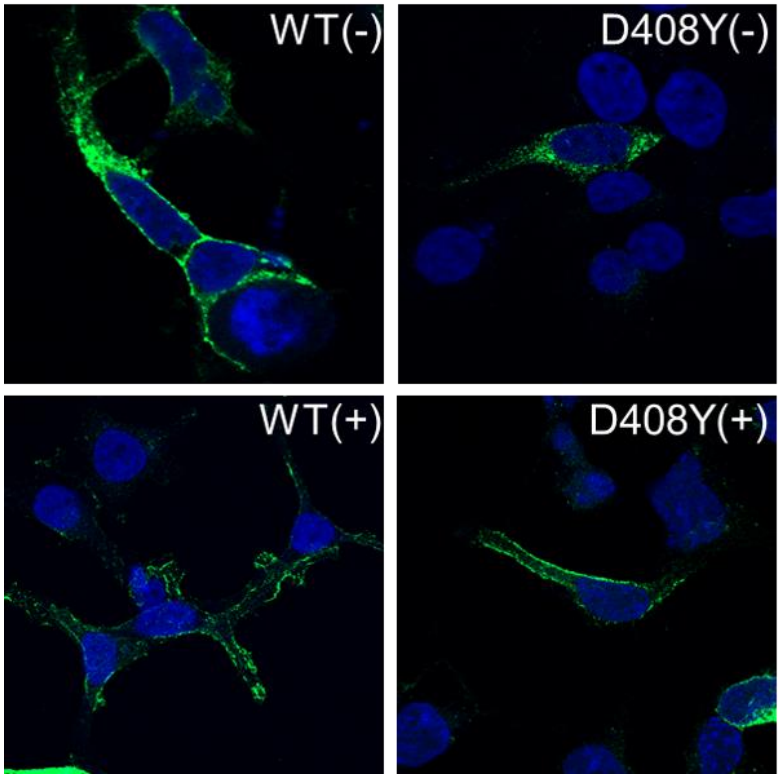


Figure 8

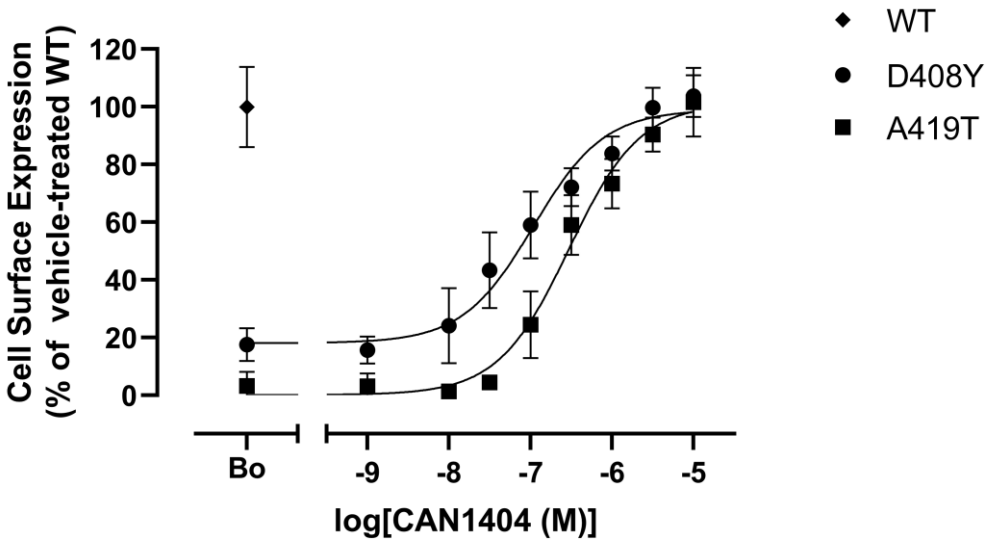
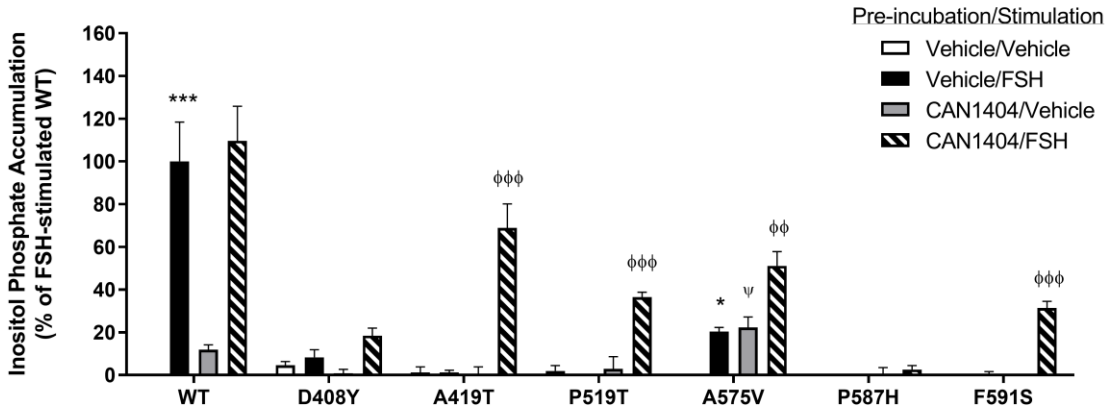

Cell Surface Expression
(% of vehicle-treated WT)
120
100
80
60
40
20
0
Bo
-9
-8
-7
-6
-5
log[CAN1404 (M)]
WT
D408Y
A419T

Figure 9

A


Inositol Phosphate Accumulation
(% of FSH-stimulated WT)
160
140
120
100
80
60
40
20
0
***
ϕϕϕ
ϕϕϕ
*
ψ
ϕϕ
ϕϕϕ
WT
D408Y
A419T
P519T
A575V
P587H
F591S
Pre-incubation/Stimulation
Vehicle/Vehicle
Vehicle/FSH
CAN1404/Vehicle
CAN1404/FSH

B

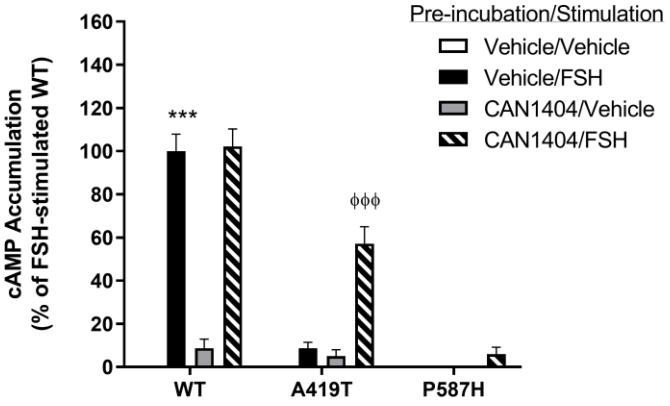
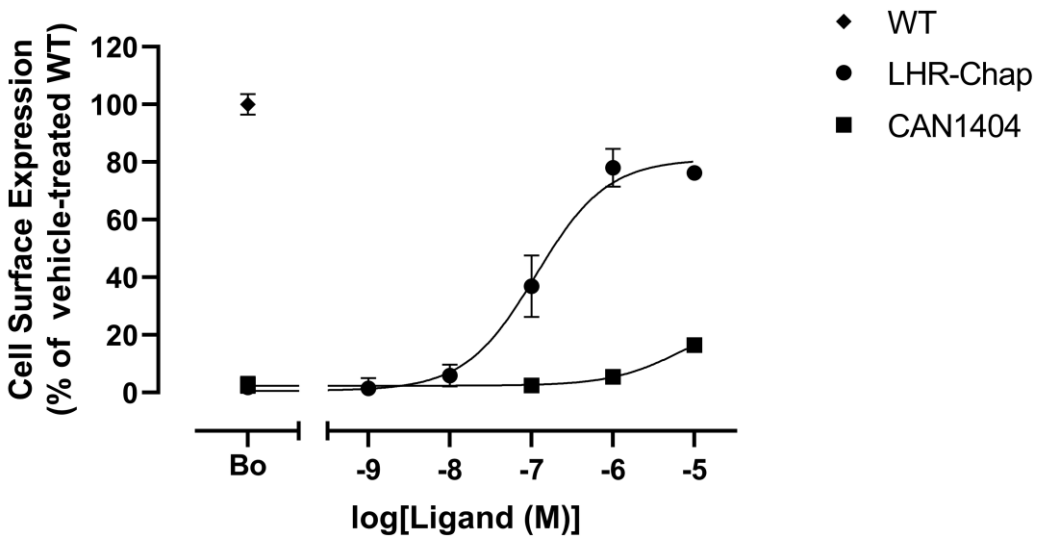

cAMP Accumulation
(% of FSH-stimulated WT)
160
140
120
100
80
60
40
20
0
***
ϕϕϕ
WT
A419T
P587H
Pre-incubation/Stimulation
Vehicle/Vehicle
Vehicle/FSH
CAN1404/Vehicle
CAN1404/FSH

Figure 10


Cell Surface Expression
(% of vehicle-treated WT)
120
100
80
60
40
20
0
Bo
-9
-8
-7
-6
-5
log[Ligand (M)]
WT
LHR-Chap
CAN1404