



## Increased plasma membrane expression of human follicle-stimulating hormone receptor by a small molecule thienopyr(im)idine

Jo Ann Janovick<sup>a</sup>, Guadalupe Maya-Núñez<sup>b</sup>, Alfredo Ulloa-Aguirre<sup>a,b</sup>, Ilpo T. Huhtaniemi<sup>c</sup>, James A. Dias<sup>d</sup>, Pieter Verboost<sup>e</sup>, P. Michael Conn<sup>a,b,f,\*</sup>

<sup>a</sup> Oregon National Primate Research Center, Beaverton, OR, USA

<sup>b</sup> Research Unit in Reproductive Medicine, Hospital de Ginecología "Luis Castelazo Ayala", Instituto Mexicano del Seguro Social, Mexico, D.F., Mexico

<sup>c</sup> Department of Reproductive Biology, Imperial College London, Hammersmith Campus, Du Cane Road, London W12 NN, UK

<sup>d</sup> Wadsworth Center, New York State Department of Health, David Axelrod Institute, 120 New Scotland Avenue, Albany, NY 12208, USA

<sup>e</sup> Department of Pharmacology, Schering-Plough Corporation, P.O. Box 20, 5340 BH Oss, The Netherlands

<sup>f</sup> Departments of Physiology and Pharmacology, and Cell and Developmental Biology, Oregon Health Sciences University, Portland, OR, USA

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### ABSTRACT

A thienopyr(im)idine (Org41841) activates the luteinizing hormone (LH) receptor but does not compete with the natural ligand binding site and does not show agonistic action on the follicle-stimulating hormone receptor (hFSHR) at sub-millimolar concentrations. When this drug is preincubated at sub-micromolar concentrations with host cells expressing the hFSHR, and then washed out, binding analysis and assessment of receptor–effector coupling show that it increases plasma membrane expression of the hFSHR. Real-time PCR shows that this effect did not result from increased hFSHR mRNA accumulation. It is possible that Org41841 behaves as a pharmacoperone, a drug which increases the percentage of newly synthesized receptor routing to the membrane. Like pharmacoperones for other receptors, this drug was able to rescue a particular mutant hFSHR (A<sup>189</sup>V) associated with misrouting and endoplasmic reticulum retention, although other mutants could not be rescued. This is potentially the first member of the pharmacoperone drug class which binds at a site that is distinctive from the ligand binding site.

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### 1. Introduction

A pharmacoperone (from “pharmacological chaperone”) is a small molecule that enters cells and serves as a molecular scaffold to promote correct folding of otherwise-misfolded mutant proteins within the cell (Conn et al., 2002; Janovick et al., 2002). Misfolded proteins are frequently retained by the cellular quality control system (QCS) of the endoplasmic reticulum, and do not reach their normal site of function (Ulloa-Aguirre et al., 2004; Bernier et al., 2004) and may result in disease (Castro-Fernandez et al., 2005). For some plasma membrane receptors, a percentage of the nascent wild-type (WT) proteins also undergo misfolding and are retained; this event may serve a regulatory role (Conn et al., 2006a). Pharmacoperones may increase expression at the plasma membrane by increasing the percentage of protein that the QCS recognizes as “correct,” as shown for a number of G protein-

coupled receptors, including the gonadotropin releasing hormone receptor (GnRHR), the vasopressin type 2 receptor, and the  $\delta$  opioid receptor (Petäjä-Repo et al., 2000, 2002; Conn et al., 2007). Virtually by definition, pharmacoperones must physically interact with receptors and receptor mutants. For this reason, efforts to identify such molecules have often begun with peptidomimetic antagonists since these are sufficiently small and hydrophobic to enter cells. A small number of agonists have also been studied (Petäjä-Repo et al., 2002). The selection strategies for screening for either agonism or antagonism have been useful for identifying pharmacoperones but have led to the discovery of pharmacoperone molecules that also compete for the binding site of the natural ligand. Accordingly, this approach for identifying pharmacoperones will necessarily lead to drugs that will have to be removed from the receptor in order to allow endogenous regulatory control; this requirement is an undesirable characteristic for therapeutic use. We sought to consider whether pharmacoperones could be identified that would not compete with the natural ligand and would, therefore, lead to advantageous compounds for therapeutic development. Some human (h) follicle-stimulating hormone receptor (FSHR) mutations, in particular, seem to affect the intracellular traf-

\* Corresponding author at: ONPRC/OHSU, 505 NW 185th Avenue, Beaverton, OR 97006, USA. Tel.: +1 503 690 5297; fax: +1 503 690 5569.

E-mail address: [connm@ohsu.edu](mailto:connm@ohsu.edu) (P.M. Conn).

ficking of the receptor with consequent intracellular sequestration (Rannikko et al., 2002; Nechamen and Dias, 2003). A good example is the conserved Ala<sup>189</sup>Val mutation which inactivates the receptor through this mechanism. Our attention was directed to Org41841, since this molecule was reported to bind a conserved region of the luteinizing hormone (LH) receptor (LHR) without competing for the LH binding site, and potentially acts as an allosteric modulator of the binding site or the site of interaction with the effector (van Straten et al., 2002).

## 2. Experimental procedures

### 2.1. Materials

pcDNA3.1 (Invitrogen, San Diego, CA), human FSHR (UMR cDNA Resource Center, University of Missouri-Rolla, Rolla, MO; [www.cdna.org](http://www.cdna.org)), FSH (Serono, Inc., Rockland, MD), Org41841 (MW = 403; Organon, Oss, The Netherlands), DMEM, OPTI-MEM, Lipofectamine, phosphate-buffered saline, Trizol Reagent (Invitrogen), competent cells (Promega, Madison, WI), and Endofree maxi-prep kits (Qiagen, Valencia, CA) were obtained as indicated. Other reagents were obtained from commercial sources and were of the highest degree of purity available. The identity of all cDNA mutants and the correctness of all PCR-derived coding sequences were verified by ABI PRISM 3130 Genetic Analyzer, according to manufacturer's instructions (Applied Biosystems, Foster City, CA).

### 2.2. Receptor and mutant vectors

WT hFSHR in pcDNA3.1 was obtained as noted above (UMR); other human wild-type FSH receptors (i.e. in vectors other than pcDNA3.1) and mutant hFSHR cDNAs for transfection were obtained from the laboratories of the authors (Nechamen and Dias, 2003, 2000; Hong et al., 1998) or from the laboratories of Drs. Tae Ji (University of Kentucky) and Aaron Hsueh (Stanford University). The cAMP dose–response curves, binding and real-time PCR graphs all used WT hFSHR in pcDNA3.1.

### 2.3. Transient transfection

Cos-7 cells were cultured in growth medium (DMEM, 10% fetal calf serum, 20 µg/ml of gentamicin) at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere. For transfection of WT or mutant receptors into Cos-7 cells, 5 × 10<sup>4</sup> cells were plated in 0.25 ml of growth medium (Dulbecco's Modified Eagle Medium; DMEM) in 48-well Costar cell culture plates for cAMP assays. Twenty-four hours after plating, the cells were washed once with 0.5 ml of OPTI-MEM and then transfected with 5 ng/well of WT or mutant receptor with 95 ng of pcDNA3.1 (empty vector) to keep the total amount of DNA at 100 ng/well. Lipofectamine was used according to the manufacturer's instructions. Five hours after transfection, 0.125 ml of DMEM with 20% fetal calf serum and 20 µg/ml of gentamicin were added. Twenty-three hours after transfection the medium was replaced with 0.25 ml of fresh growth medium. Where indicated, a range of Org41841 (0–4 µg/ml, as indicated) in 1% dimethylsulfoxide (DMSO, “vehicle”) was added for 4 h in respective media to the cells and then removed 18 h before agonist treatment.

### 2.4. Binding assays

Cos-7 cells were cultured and plated in growth medium as described (Uribe et al., 2008) except that 10<sup>5</sup> cells in 0.5 ml of growth medium were added to 24-well Costar cell culture plates. Twenty-three hours after transfection the medium was removed and replaced with 0.5 ml of fresh growth medium with ORG 2 µg/ml or DMSO vehicle for 4 h at 37 °C. Cells were then washed twice with DMEM and maintained in culture for 18 h in DMEM–10% FCS. Thereafter, the medium was removed, replaced with fresh medium, and allowed to continue incubation at 37 °C for 1 h. After the pre-incubation period, the medium was removed and serum-free DMEM containing 20 ng/ml (about 2 nM) [<sup>125</sup>I]-human FSH (specific activity: 28–30 µCi/µg protein) or [<sup>125</sup>I]-FSH plus 1 µg/ml unlabeled human pituitary FSH (to assess for non-specific binding) was added to each well. Hormone was allowed to bind for 1 h at 37 °C before the cultures were placed on ice and washed twice with 0.5 ml cold PBS. Cell surface radiolabeled FSH was eluted by incubating for 20 min in ice-cold 50 mM glycine/100 mM NaCl pH 3.0. The radioactivity present in the acid wash was counted for 2 min in a γ-counter (Packard, Downers Grove, IL).

### 2.5. Radioreceptor assay

The binding affinity of WT hFSHR was determined in an equilibrium displacement binding assay (Uribe et al., 2008). Cultured Cos-7 cells were plated in 60 mm plates (Costar, Cambridge, MA) (750,000 cells per plate) and transfected with 0.75 µg/well of the hFSHR cDNA as described above. Twenty-four hours after the start of transfection, Org41841 (2 µg/ml) or vehicle (DMSO) was added for 4 h. Cells were then washed twice with DMEM and maintained in culture for 18 h in

DMEM–10% FCS. Thereafter, cells were detached with 2.5 mM EDTA, washed twice with PBS, resuspended in radioreceptor assay buffer (50 mM Tris pH 7.5, 25 mM MgCl<sub>2</sub>, and 0.3% BSA), and added to 12 mm × 75 mm culture tubes containing [<sup>125</sup>I]-FSH in a 0.3 ml reaction volume. Cell numbers were adjusted (150,000 cells/tube for Org41841-treated cells and 273,000 cells/tube for the control cells) in order to give a comparable cpm of [<sup>125</sup>I]iodo-FSH binding that allow to accurately compare K<sub>d</sub> values between DMSO and Org41841 stimulation. Increasing amounts (0–100 ng/tube) of unlabeled pituitary FSH competed with [<sup>125</sup>I]iodo-FSH for binding to the FSHR. Reactions were incubated 18 h at room temperature with continuous shaking. After the incubation period, 3 ml cold radioreceptor assay buffer was added to each tube and the cells were centrifugated at 1200 × g for 30 min at 4 °C. Radioactivity in the pellets was determined in a γ-counter. Quantitation of cAMP

Forty-eight hours after the start of transfection, the cells transfected with WT hFSHR or mutant receptor cDNAs were washed with DMEM containing 0.1% BSA (Irvine Scientific, Santa Ana, CA) and 20 µg/ml gentamicin (DMEM–BSA–gentamicin). The cells were then exposed for 2 h to medium or FSH (1–100 ng/ml) in DMEM–BSA–gentamicin containing 0.2 mM methyl isobutylxanthine (MIX) to prevent degradation of cAMP by inhibiting phosphodiesterases. After stimulation, the medium from each well was collected in tubes containing sufficient theophylline (also a phosphodiesterase inhibitor) for a final concentration of 1 mM. The samples were heated (95 °C) for 5 min to destroy phosphodiesterases. The RIA of cAMP was performed by a modification (Stanislaus et al., 1994) of the method of Steiner et al. (1972), with the addition of the acetylation step described by Harper and Brooker (1975). Cyclic AMP antiserum C-1B (prepared in our (PMC) laboratory; Stanislaus et al., 1994) was used at a titer of 1:5100. This antiserum showed less than 0.1% cross-reaction with cGMP, 2', 3'-cAMP, 5'-cAMP, 3'-cAMP, ADP, GDP, ATP, CTP, MIX, or theophylline.

### 2.7. RNA extraction for real-time PCR

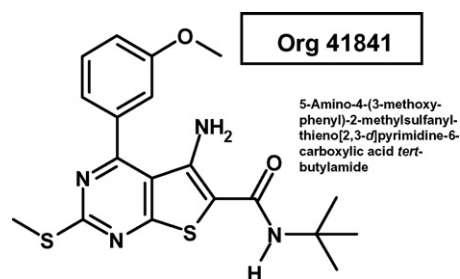
Cos-7 cells were plated in a 6-well culture plate at 2 × 10<sup>5</sup> cells per well. Cells were transfected as described above, with 20 ng WT hFSHR plus 380 ng empty vector per well. Twenty-three hours after transfection the medium was replaced with fresh growth medium. Where indicated, Org41841 (dose–response curves) in 1% DMSO (vehicle) was added for 4 h in growth media to the cells, then removed 18 h before RNA extraction. Cells were washed twice with ice-cold PBS on ice. Trizol Reagent was added to the cells for extracting total RNA and processed according to the manufacturer's instructions. Reverse transcription was performed using 1 µg total RNA and SuperScript III Reverse Transcriptase (Invitrogen) for single strand cDNA according to the supplier's protocol.

### 2.8. Real-time PCR

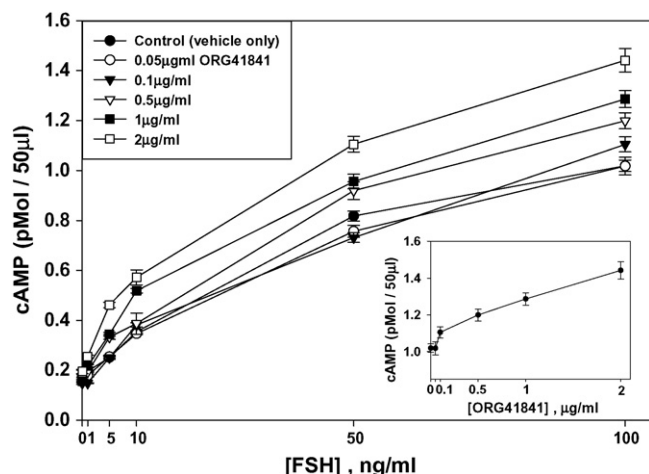
The sequence of the human FSHR (accession # BC118548) was used to design primers and TaqMan probe. The forward primer sequence was (5'-CTATCACCTTGGAAAGATGGCATA-3'), the reverse primer was (5'-AATCCAGCCATCACCATGA-3') (Invitrogen, Carlsbad, CA), the TaqMan MGB probe was labeled 5'-6FAM-CGCATGCCATGCAGCTGGAGTGG-MGBNFQ-3' (Applied Biosystems, Foster City, CA). The Applied Biosystems 7900HT Fast Real-time PCR System and reagents kit were used for generating the real-time PCR data. Primers and TaqMan probes for Monkey β-Actin was used as the experimental internal control for normalizing the data. The target gene (hFSHR) primers and control gene primers were used at 300 and 80 nM final concentration, respectively. Probes were used at 250 nM final concentration. Quantitative standard curves were generated by serial dilutions of 1:10 of the RT product ranging from 1 to 10 ng and were used for determining the Ct values in nanograms using the straightline extrapolation. Samples were run in duplicate in at least four separate experiments.

### 2.9. Statistics

Data were analyzed with one-way analysis of variance and then Student–Newman–Keuls test (SigmaStat 3.1; Jandel Scientific Software); *p* < 0.05



**Fig. 1.** Chemical structure and name of Org41841, the thienopyr(im)idine used in these studies.

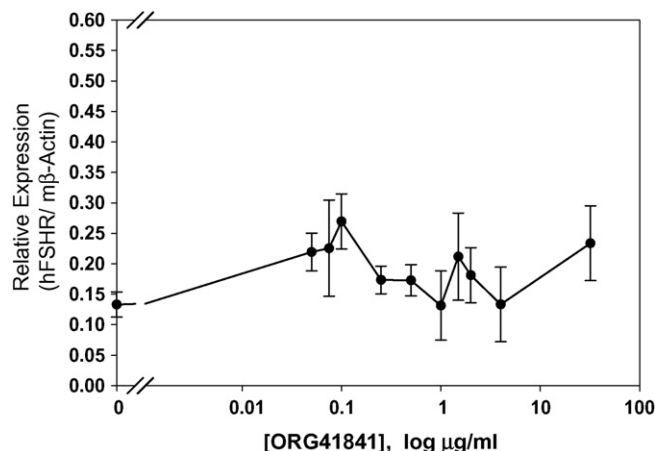


**Fig. 2.** The effect of pre-incubation of cells with various concentrations of Org41841 on production of cyclic AMP in cells expressing WT hFSHR. The drug was removed 18 h prior to addition of FSH. Cyclic AMP was determined by RIA as described in Section 2. The inset shows the dose–response curve of Org41841 when the amount of FSH in the challenge is 100 ng/ml.

was considered significant. Dissociation constant ( $K_d$ ) and binding sites were calculated from the dose–response displacement curves using the software GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA).

### 3. Results

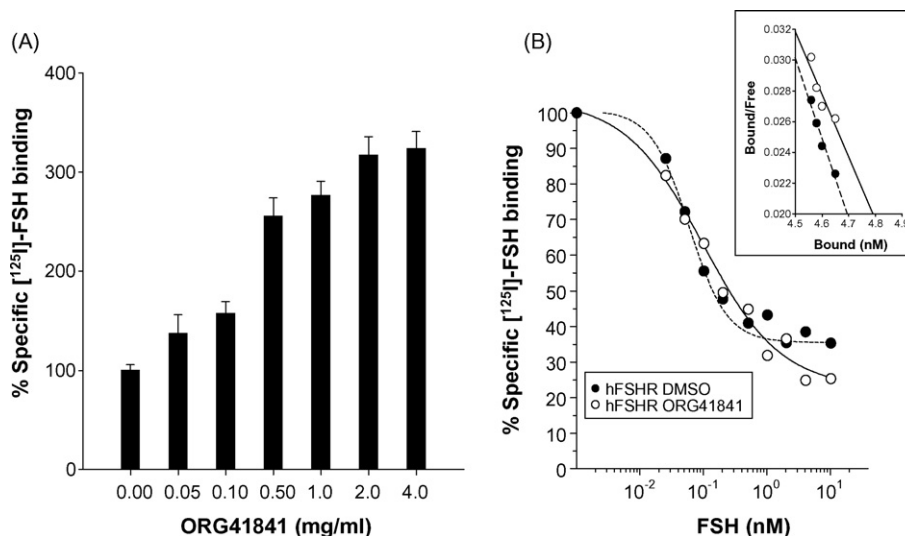
Fig. 1 shows the chemical structure of Org41841, a thienopyr(im)idine that activates the LH receptor (van Straten et al., 2002). This molecule was selected from a high-throughput screening approach and was interesting to us since the procedure identified only those molecules that were selective hLHR agonists with no comparable activity on hFSHR, human corticotropin-releasing factor receptor 1, or human thyrotropin (TSH) receptor (van Straten et al., 2002). This molecule then became the first example of a low molecular weight agonist for a gonadotropin receptor with *in vivo* efficacy after oral administration. Of special interest to us, this molecule did not displace  $^{125}$ I-LH binding



**Fig. 4.** Lack of a consistent effect of Org41841 on accumulation of WT hFSHR mRNA. The data suggest that pre-incubation with this drug does not alter mRNA accumulation of this receptor. Real-time PCR was conducted as described in Section 2.

to the LH receptor, suggesting allosteric activation and that the binding of this structure might be in the evolutionarily conserved monoamine-related binding pocket of the seven transmembrane-segment (TMS) domains rather than the large extracellular domain where the natural ligand binds. Indeed, a subsequent study relying on chimeras and modeling techniques (Jäschke et al., 2006) revealed a binding pocket in common clefts between TMS 3, 4, 5, 6, and 7 and the extracellular loop (ECL) 2 in both the hLH and hTSH receptors (in the TSH receptor, the compounds bind to the same clefts with a 35-fold lower affinity to the hTSHR than to the hLHR). We considered the possibility that Org41841 might be an example of a chemical structure that could bind to this receptor (in the TMS region) – although with low specificity – in a region that was distinct from the endogenous ligand binding site at the N-terminus.

Fig. 2 shows the effect of pre-incubation of cells with various concentrations of Org41841 on cyclic AMP production in cells expressing the WT hFSHR. The drug was removed 18 h prior to addition of FSH. The data indicate that pre-incubation with the drug



**Fig. 3.** (A) Representative experiment showing the relative binding of  $^{125}$ I-FSH to the WT hFSHR in Cos7 cells pre-treated with either DMSO (zero concentration) or 0.05–4.0 mg/ml Org41841. (B) Representative radioligand binding assay. Cells were assayed for  $^{125}$ I-FSH binding in the presence of increasing concentrations of nonradioactive FSH. The results were analyzed by Scatchard plot (inset) to determine the  $K_d$ . Similar results were found in two additional experiments. The results shown in (B) are not corrected for cell number (see Section 2).

increases the amount of cyclic AMP produced in response to a subsequent challenge with hFSH. The inset shows the dose–response curve of Org41841 when the amount of FSH in the challenge is fixed at 100 ng/ml.

Fig. 3A shows a representative  $^{125}\text{I}$ -FSH radioligand binding assay which indicates increased specific binding to its cognate receptor after pre-incubation of cells with increasing amounts of Org41841. As shown in Fig. 3B, pre-treatment with Org41841 did not alter the affinity of the hFSHR to bind labeled agonist ( $K_d$  for the hFSHR from Org41841-treated cells,  $11.4 \pm 6.5$  nM; vehicle,  $8.8 \pm 5.1$  nM,  $p > 0.05$ ); nevertheless, the number of binding sites

after correction for cell number significantly ( $p < 0.05$ ) increased almost by two-fold (Org41841-treated cells,  $4.7 \pm 0.68$  nM/150,000 cells; vehicle,  $2.6 \pm 0.35$  nM/150,000 cells).

Fig. 4 shows the lack of a consistent effect of Org41841 on accumulation of WT hFSHR mRNA. The data suggest that pre-incubation with this drug does not alter mRNA accumulation of this receptor. Accordingly, the increased hFSHR at the plasma membrane appears to indicate that Org41841 either increases stabilization of the receptor molecule at the plasma membrane or promotes routing to the plasma membrane.

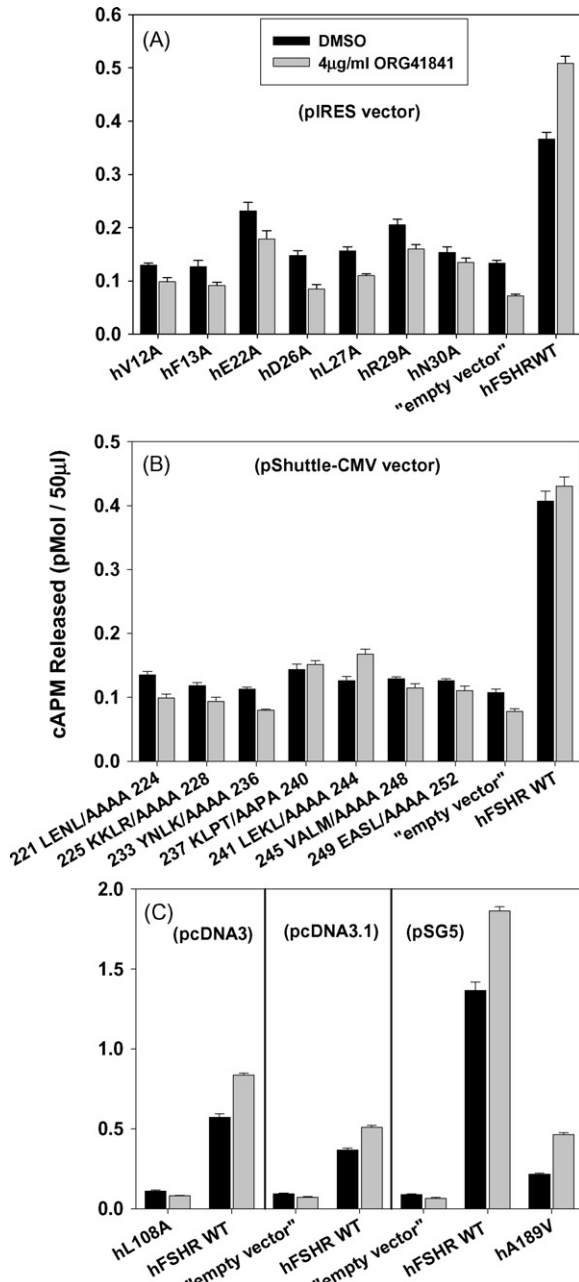
Fig. 5 shows efforts to use Org41841 to rescue a range of mutants at diverse sites in the molecule, some of which are known to be recognized as misrouted and retained in the endoplasmic reticulum. After transfection and incubation in Org41841, cellular responses were assessed in the presence of 100 ng FSH. Of the mutants, only Ala<sup>189</sup>Val, which appears to affect the intracellular trafficking of the receptor with consequent intracellular sequestration (Rannikko et al., 2002; Nechamen and Dias, 2003), could be rescued by Org41841.

It is unclear why A<sup>189</sup>V should be the only mutant that was rescued by Org41841. There is, however, a highly conserved stretch of five amino acids (Ala-Phe-Asn-Gly-Thr) in the region of this mutation in all glycoprotein hormone receptors (Conn et al., 2007). The same (Ala to Val) transition in the human LHR exhibited similar functional alterations, i.e., intracellular sequestration and attenuated signal transduction as in the mutated FSHR (Rannikko et al., 2002). This mutant could be a glycosylation/trafficking defect mutant at Asn<sup>191</sup>. Ala<sup>189</sup> is juxtaposed to Asn<sup>191</sup>, and this change in the side chain may cause steric alterations impacting on the accessibility of Asn<sup>191</sup> to glycosyl transferases for the addition of dolichol-linked precursors. In principle Org41841 may minimize the effect of this event and promote trafficking to the plasma membrane, as it does for wild-type hFSHR.

#### 4. Discussion

A number of GPCRs are only fractionally expressed at their normal site of action compared with the total amount of protein that is translated. This is the case for several GPCRs and appears to be due to misfolding and retention in the endoplasmic reticulum (Wuller et al., 2004; Lu et al., 2004, 2003; Ulloa-Aguirre et al., 2004). In the case of the hGnRHR, control of routing is affected by either the formation of the Cys<sup>14</sup>-Cys<sup>200</sup> bridge (resulting in a GnRHR that is routed to the plasma membrane) or its failure to form (which results in ER retention) (Janovick et al., 2006). This process appears to have evolved under convergent evolutionary pressure even though it is wasteful of the protein produced (Ulloa-Aguirre et al., 2006). Moreover the delicate balance between the PM and ER results in high susceptibility to mutational disease (Conn et al., 2006a,b). The misfolded fraction can be rescued, as can many naturally occurring mutants of the hGnRHR and other GPCRs by specific pharmacoperone drugs that increase plasma membrane routing of the WT hGnRHR above that normally seen (Conn et al., 2006a).

Among the glycoprotein receptors, Pietilä et al. (2005) reported the ER-accumulation of immature underglycosylated WT rLHR and others (Fishburn et al., 1995) showed the impact of glycosylation on the dopamine D2 receptor on intracellular trafficking. In addition, some mutants of the hFSHR do not appear to traffic correctly to the plasma membrane (Nechamen and Dias, 2000, 2003; Rannikko et al., 2002), particularly when the affected residue compromises or involves endoplasmic reticulum export motifs (Timossi et al., 2004; Conn et al., 2007). Cohen et al., 2003 demonstrated that the hFSHR interacts with ubiquitin and that cell surface expression of the receptor can be increased by 30% by inhibition of the proteasome.



**Fig. 5.** Org41841 rescues WT hFSHR and mutant A<sup>189</sup>V ( $p = 0.002$ ), but is unable to rescue other mutants with mutation at diverse sites in the molecule (SEMs shown). After transfection and incubation in Org41841, cellular response was assessed by a challenge with 100 ng hFSH as described in Section 2. As mutants were obtained from different laboratories and were in different expression vectors, controls are shown with the corresponding WT hFSHR and the empty vector in each case.



In the present study, we found that treatment with the allosteric LHR agonist Org41841, increased cell surface membrane expression of the WT hFSHR. We selected Cos cells for these experiments because, although primate-derived (Gluzman, 1981), they are kidney cells and lack the constellation of endocrine receptors that characterize reproductive tissue and are extremely efficient at protein synthesis. While this choice minimized the chances that the drug under study interacted with a molecule other than the FSHR, which in turn caused up-regulation of the FSHR, we cannot eliminate this possibility altogether. In addition, activation of the FSHR requires millimolar concentrations of Org41841 (Organon, data not shown).

It is interesting that the interaction with Org41841 enhances the expression of WT hFSH at the plasma membrane, and could rescue mutant Ala<sup>189</sup>Val which appears to decrease plasma membrane expression by inhibition of intracellular trafficking of the receptor with consequent intracellular sequestration (Rannikko et al., 2002; Nechamen and Dias, 2003). In the case of the hGnRHR and the V2 receptor, pharmacoperone drugs are also able to increase both WT expression and expression of many of the corresponding mutants. We believe that this is the first report of a structure that appears to interact with a receptor at a site that is distinct from the naturally occurring ligand binding site and which results in increased WT receptor expression at the plasma membrane.

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