Allosteric Activation of the Follicle-stimulating Hormone (FSH) Receptor by Selective, Nonpeptide Agonists*

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The pituitary glycoprotein hormones, luteinizing hormone and follicle-stimulating hormone (FSH), act through their cognate receptors to initiate a series of coordinated physiological events that result in germ cell maturation. Given the importance of FSH in regulating folliculogenesis and fertility, the development of FSH mimetics has been sought to treat infertility. Currently, purified and recombinant human FSH are the only FSH receptor (FSH-R) agonists available for infertility treatment. By screening unbiased combinatorial chemistry libraries, using a cAMP-responsive luciferase reporter assay, we discovered thiazolidinone agonists (EC50's ≈ 20 μM) of the human FSH-R. Subsequent analog library screening and parallel synthesis optimization resulted in the identification of a potent agonist (EC50 = 2 nM) with full efficacy compared with FSH that was FSH-R-selective and -dependent. The compound mediated progesterone production in Y1 cells transfected with the human FSH-R (EC50 = 980 nM) and estradiol production from primary rat ovarian granulosa cells (EC50 = 10.5 nM). This and related compounds did not compete with FSH for binding to the FSH-R. Use of human FSH/thyroid-stimulating hormone (TSH) receptor chimeras suggested a novel mechanism for receptor activation through a binding site independent of the natural hormone binding site. This study is the first report of a high affinity small molecule agonist that activates a glycoprotein hormone receptor through an allosteric mechanism. The small molecule FSH receptor agonists described here could lead to an oral alternative to the current parenteral FSH treatments used clinically to induce ovulation for both in vivo and in vitro fertilization therapy.

Follicle-stimulating hormone (FSH)2 is a glycoprotein hormone produced by the anterior pituitary that plays a key role in stimulating ovulation and spermatogenesis. Like other members of the glycoprotein hormone family (luteinizing hormone, chorionic gonadotropin, and thyroid-stimulating hormone (TSH)), FSH is a heterodimeric protein of ~30,000 Da that consists of a common α-subunit joined noncovalently to a hormone-specific β-subunit. FSH activity is mediated through binding to the FSH receptor (FSH-R), which belongs to family 1 of the large 7-transmembrane-spanning, G protein-coupled receptor (GPCR) superfamily. The glycoprotein hormone receptors are unique among the members of the GPCR family, because they recognize protein hormones and are dominated by a large N-terminal extracellular region (366 amino acids in the case of the FSH-R) that is the predominant site of hormone binding (1, 2) and is required for signal transduction.

The binding of FSH to its receptor results in the activation of adenyl cyclase through heterotrimeric G proteins. Interaction of the activated FSH-R with G1, initiates the cAMP signaling cascade (3). The ability of the FSH-R to activate adenyl cyclase was exploited to generate a Chinese hamster ovary (CHO) FSH-R reporter cell line (4–6). Screening of a collection of diverse chemotypes led to the identification of compounds that activated this reporter cell line but were inactive on CHO cells lacking the human FSH-R. The activity of these compounds on receptor chimeras, which contained only a small portion (TM1 through TM3) of the FSH-R, suggests action through a site distinct from the large N-terminal extracellular domain, the region reported to be the predominant site of hormone binding (1, 2).

Reported effects of FSH and FSH-R defects provide evidence for the importance of this glycoprotein hormone and its receptor on female fertility. Mutations in FSH-R have been linked to clinical cases of infertility. Nonfunctional FSH-Rs have been found to cause resistant ovary syndrome, which results in infertility in women (7). The FSH-β gene knock-out female mouse is infertile because of inhibition of folliculogenesis prior to antral follicle formation (8). In addition, FSH-R gene knock-out female mice are also infertile because of arrested folliculogenesis (9). In males, FSH regulates the function of testicular Sertoli cells. However, the importance of FSH in male fecundity is less clear, as clinical reports of mutations to either FSH or its receptor describe reduced fertility but not complete sterilization.

Human menopausal gonadotropin, a 1:1 mixture of FSH and luteinizing hormone, has been used successfully clinically to induce ovulation in both in vivo and in vitro fertilization therapy. Human menopausal gonadotropin is purified from the urine of postmenopausal women and must be administered by injection. Recombinant human FSH has recently become available for parenteral human therapy. Treatment typically involves multiple injections, which can have a negative impact on patient compliance and initiation of this course of treatment. Consequently, patients being treated for infertility may prefer an orally administered small molecule that mimics the activity of FSH. Therefore, we have set out to identify nonpeptide synthetic agonists to the hFSH-R for potential use in novel infertility treatments. A class of thiazolidinone agonists was identified through unbiased combinatorial chemistry library screening. Additional optimization through analog library screening and parallel synthesis led to the development of a potent FSH-R-selective and -dependent agonist with full efficacy to FSH. The thiazolidinone agonists appear to activate the FSH-R through

1 The abbreviations used are: FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone; hFSH-R, human FSH receptor.
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an allosteric mechanism, which was determined by the use of human FSH-R/TSH-R chimeras.

EXPERIMENTAL PROCEDURES

Cell Lines

Chinese hamster ovary (CHO) K-1 cells were used to engineer a clonal cell line that stably expresses the human FSH-R. cDNA for the human FSH-R was cloned into the p/H9251/H11001/12CA5-KH expression vector as described (10) and transfected into CHO-K1 cells by electroporation. After G418 selection, cells were stained with fluorescein isothiocyanate-labeled 12CA5 antibody, and those expressing the FSH-R were collected by fluorescence-activated cell sorting. Individual clones were examined for the ability to produce cAMP in response to purified human FSH (Cortex Biochem). Clone 3D2 was identified as a stable clone that responded to purified human FSH with an increase in cAMP production. In addition, the cloned human FSH-R gene was transfected along with a 6XCRE-luciferase plasmid (11) into CHO K-1 cells by electroporation. Following G418 selection, cells were stained with fluorescein isothiocyanate-labeled 12CA5 antibody, and those expressing the FSH-R were collected by fluorescence-activated cell sorting. Clone 1D7 was identified as a stable clone giving a robust luciferase reporter gene response to human FSH. Reporter cell lines expressing each of the chimeric receptors described below were generated in the same manner as for the CHO FSH-R 6XCRE-luciferase cell line. A mouse adrenocortical tumor cell line (Y1 cells) purchased from American Type Culture Collection (Manassas, VA) was engineered to stably express the full-length human FSHR cDNA (provided by Dr. Kerry Koller) as described previously (12).

Generation of FSH-R/TSH-R Chimeric Constructs

The FSH-R α+T8-12CA5-KH plasmid construct was available from previous work (10). TSH-R was amplified by PCR from cDNA made from a human thyroid gland poly(A)+ RNA library (Clontech) using oligonucleotide primers generated from the GenBank™ sequence M32215, and cloned into the α+T8-12CA5-KH plasmid at the Xhol and NotI restriction sites. The correct sequence was verified by automated DNA sequencing. Chimeric constructs were made combining the FSH-R and TSH-R. Oligomers were designed to engineer restriction sites into the most highly conserved regions of the first and second transmembrane regions of each receptor. No new amino acid codons were added when the restriction sites were inserted.

TSH-R/FSH-R Chimera —The construct contained the TSH-R from amino acids 1–407 and the FSH-R from amino acids 360–678 of the respective mature proteins. NheI sites were added at amino acids 405–407 for the TSH-R(LLA)/FSH-R amino acids 357–359(ILA).

FSH-R/TSH-R Chimera —The construct contained the FSH-R from amino acids 1–356 and the TSH-R from amino acids 405–743 of the respective mature proteins. NheI sites were added at amino acids 435–437 for the FSH-R(LLA)/TSH-R amino acids 405–407(ASE).

F/T II Chimera —The construct contained the FSH-R from amino acids 1–437 and the TSH-R from amino acids 486–743 of the respective mature proteins. NheI sites were added at amino acids 435–437 for the FSH-R(ASE)/TSH-R amino acids 482–485(ASE).

F/T III Chimera —The construct contained the TSH-R from amino acids 1–407, the FSH-R from amino acids 435–442, and then the TSH-R from amino acids 490–743. The same NheI site as in FSH-R/TSH-R was used, and HpaI sites were added at amino acids 442–444 of the FSH-R(TLT)/TSH-R amino acids 490–492(TLT).

Reporter Assay Using CHO 6XCRE-luciferase Cell Lines That Stably Produce Recombinant Human FSH-R or FSH-R/TSH-R Chimeras

Cells were placed at 100,000/well into a 96-well black-bottom microtiter plate (Packard) in DMEM/F12 (Invitrogen) without phenol red. Human FSH, human TSH (Cortex Biochem), or thiazolidinone compounds were added in DMEM/F12 without phenol red, and the plate was incubated at 37 °C in the presence of 5% CO2. After 4 h, an equal volume of LucLite reagent (Packard) was added, and the plate was counted in a TopCount scintillation counter (Packard).

cAMP Assay

CHO cells transfected with the human FSH receptor were placed at 100,000/well into a 96-well plate and incubated overnight at 37 °C in the presence of 5% CO2. After washing with DMEM/F12, cells were challenged with compounds for 30 min at room temperature in 100 µl DMEM/F12 containing 0.1% bovine serum albumin. At the end of the challenge period, the assay was terminated by the addition of 100 µl of 0.2 N HCl. The media were removed and subsequently assayed for cAMP by radioimmunoassay (Amersham Biosciences, RPA542).
Solid-phase Synthesis of Thiazolidin-4-ones

Compounds were prepared using either Fmoc-aminoethyl-pho-
tolinker NovaSyn® TG resin (Novabiochem) or ArgoGel®-Rink-Fmoc
resin (Argonaut Technologies) following standard protocols (13). Prod-
ucts were cleaved from resin by photolysis (λ = 365 nm) or by treatment
with TFA/CH₂Cl₂, as described previously and depicted in synthetic
Scheme 1, and purified by reversed-phase HPLC.

Rat Aromatase Bioassay and Y1/5E5/s3 Steroidogenesis Assay

The production of estradiol by primary rat granulosa cells and pro-
gestosterone by Y1/5E5/s3 cells after treatment with purified human FSH
or test compounds was determined as described previously (12).

RESULTS

Screening Combinatorial Small Molecule Libraries for FSH-R Ago-
nists Using a Reporter Gene Assay—Extensive screening of combinator-
ial small molecule libraries for compounds that would displace the
binding of [125I]FSH to the human FSH-R was unsuccessful. To screen
directly for small molecule activators of the FSH receptor, we employed
a reporter gene assay (4–6) in which the firefly luciferase gene was
placed downstream of a series of cyclic-AMP response elements (CRE)
to screen for compounds that activate adenylyl cyclase (3). As shown in
Fig. 1A, the CHO FSH-R 6XCRE-luciferase cell line, 1D7, responded in
a dose-dependent manner to challenge with human FSH. Maximal effi-
cacy for human FSH in this assay was ~10-fold greater than control
cells. The EC₅₀ for human FSH in this bioassay was ~110 pm (Fig. 1A).

We utilized this FSH-R luciferase reporter assay to screen a collection
of small molecule combinatorial libraries representing more than 20
core scaffolds created using solid-phase chemical synthesis methodologies (14, 15). Libraries were typically prepared by split synthesis on
TentaGel® resin equipped with a photocleavable linker (16), and com-
 pounds were screened in pools containing 10–36 compounds at an
estimated concentration of 1–5 μM/compound. More than 120,000
compounds were initially tested for agonist activity using the CHO

FIGURE 1. Identification of compounds with FSH-like activity by screening against CHO FSH-R 6XCRE luciferase cell line. A, response of CHO FSH-R 6XCRE-luciferase clone to human FSH. Cells were incubated in 96-well plates for 4 h at 37 °C, and the assay was developed using LucLite (Packard) as described under “Experimental Procedures.” B, structure of compounds in combinatorial library ACL1547. C, activity of compound 1 (open squares) and compound 2 (open diamonds) in reporter assay using the CHO FSH-R 6XCRE-luciferase 1D7 cell line. D, structures of thiazolidinone compounds 1 and 2.
FSH-R 6XCRE-luciferase reporter cell line. Activity was detected upon screening of ACL1547, a 960-member thiazolidin-4-one library (Fig. 1B). Individual compounds from active pools were re synthesized, purified, and tested in the CHO FSH-R reporter assay. Two compounds, 1 and 2 (Fig. 1D), were found to activate the luciferase reporter gene but exhibited low potency (EC_{50} \sim 20 \mu M) (Fig. 1C).

**Potency Optimization through Analog Libraries**—One of the advantages of obtaining leads through a combinatorial library approach is that focused analog libraries can be rapidly generated in a search for compounds with improved properties. To rapidly identify more potent compounds, an encoded analog library (ACL1876) was generated using the same thiazolidinone scaffold employed in ACL1547, with 35 amino acids at the R1 position, 35 aldehydes at the R2 position, and 35 amines at the R3 position (17). The R1 and R2 positions were fully encoded using secondary amine tags (18), and the R3 pools were kept separate for screening purposes. The compounds were synthesized on 130-mm TentaGel resin bearing a photocleavable linker. This library of 42,875 compounds was screened as 35 pools containing 1,225 compounds each, at 30 beads/well (an estimated concentration of 800 nM/compound). Most of the pools lacked activity; however, one pool containing 3,4-dimethoxyphenethylamine at the amine position showed significant levels of activity. Single beads from this pool were distributed into separate wells, and the compounds were released by photolysis and tested for activity using the CHO FSH-R reporter assay. Beads from active wells were collected, and their tags were decoded to determine the structure of the active compounds. Two of these (compounds 3 and 4) were synthesized as pure compounds for further investigation (Table 1). Thiazolidinones 3 and 4 were found to act as full agonists in the reporter assay with potencies significantly greater than the initial lead compounds (EC_{50} values of 300 and 30 nM, respectively) but possessing an efficacy equivalent to that of hFSH (Fig. 2). Finally, parallel synthesis was used to generate additional analogs, resulting in identification of compounds 5 (EC_{50} = 2 nM), 6 (EC_{50} = 6 nM), and 7 (EC_{50} = 1 nM) as highly potent agonists in the reporter assay (Fig. 2).

**Activity of Thiazolidinone Compounds against Recombinant and Native Cell Lines**—Thiazolidinone compounds 5, 6, and 7 were also examined for their ability to stimulate cAMP production in a CHO cell line transfected with the human FSH-R sequence. Compound 5 elevated cAMP levels with an EC_{50} of 2 nM (data not shown). Both compounds 4 and 5 (at up to 100 \mu M concentration) failed to elevate cAMP levels or stimulate luciferase production in a number of control cell lines, including untransfected CHO cells and CRE-based reporter CHO cells expressing the calcitonin receptor, glucagon receptor, or the homologous human TSH receptor (data not shown).

To ascertain whether thiazolidinone 5 was an agonist of the rat FSH-R, compound 5 was tested for its ability to activate the native FSH-R found in primary cultures of rat ovarian granulosa cells (19, 20). These cells produce an FSH-dependent increase in estradiol production. Compound 5 was a full agonist, with an EC_{50} of 10.5 nM in this in vitro assay (Fig. 3A). Compound 5 also produced FSH-like responses in a mouse adrenal Y1 cell line transfected with the human FSH-R. This cell line produced a dose-dependent increase in progesterone upon stimulation with FSH resulting in an EC_{50} of 992 pM (Fig. 3B). Compound 5 was less potent (EC_{50} = 998 nM) but had an efficacy comparable to purified human FSH in stimulating progesterone production via the recombinant human FSH-R. Neither purified human FSH nor compound 5 stimulated progesterone production on parental Y1 cells (data not shown).

**Stereoselectivity of FSH Mimetic Compounds**—Racemic mercaptosuccinic acid was used in the solid-phase synthesis of thiazolidin-4-ones in the initial library and in subsequent focused chemistry that resulted in formation of four isomers of each compound. The cis and trans isomers could be separated by reversed-phased HPLC. Compounds 3, 4, and 5 are each a racemic pair of cis enantiomers. The corresponding racemic trans isomers of these three compounds were tested in the reporter assay and found to be at least 100-fold less active. Results from subsequent studies in which the pair of cis enantiomers was resolved by chiral HPLC or where the individual cis enantiomers were synthesized using optically active mercaptosuccinic acid indicated that the isomer having the 25,5R configuration was exclusively responsible for the FSH-mimetic activity. Thus, interaction of this compound class with the FSH-R appears to exhibit exquisite stereoselectivity.

**Mapping Site of Action Using FSH-R/TSH-R Chimeras**—The generation and characterization of chimeric receptors is a convenient way to identify the functional activity of specific domains. Glycoprotein hormone receptor chimeras have been used to map the regions responsible for ligand binding and signaling (21–23). To map the site of action of the thiazolidinone compounds on the human FSH-R, we constructed chimeric receptors using the human TSH-R. This glycoprotein hormone receptor possesses 39% homology to the human FSH-R in the N-terminal extracellular domain and 68% homology over the 7-transmembrane-spanning portion (24). The N-terminal extracellular domain of the human FSH-R was fused to the remainder of the human TSH-R to generate a FSH-R/TSH-R chimera (Fig. 4, upper left), and the N-terminal domain of the TSH-R was swapped onto the human FSH-R to generate a TSH-R/FSH-R chimera (Fig. 4, upper right). CHO 6XCRE-luciferase reporter cell lines were generated expressing each of these chimeric receptors. The parent cell line, CHO FSH-R 6XCRE-luciferase clone 1C2, responded to FSH with an EC_{50} of 110 pM but did not respond at all to TSH at concentrations ranging from 1 nM up to 10 \mu M. In the presence of compound 6, this cell line gave a maximal response equivalent to that produced by FSH, with an EC_{50} of 2.7 nM (Table 2). The CHO FSH-R/TSH-R 6XCRE-luciferase clone 2B6 responded to FSH with an EC_{50} of 200 pM (Table 2). Thus, the high affinity response of the FSH-R to FSH appears to require only the N-terminal ligand binding domain, as reported by others (30, 35, 38, 39). The responsiveness of this cell line to human TSH was studied. In these experiments, CHO FSH-R/TSH-R 6XCRE-luciferase clone 2B6 did respond to TSH with an EC_{50} of 310 nM; however, this cell line did not respond at all to compound 6 at concentrations up to 10 \mu M (Table 2).

CHO TSH-R/FSH-R 6XCRE-luciferase clone 3E7 responded to TSH with an EC_{50} of 24 nM (Table 2). These cells responded to thiazolidinone 6 with an EC_{50} of 200 nM (Table 2) and to thiazolidinone 7 with an EC_{50} of 76 nM. Thus, this cell line, which completely lacks the N-terminal FSH-binding domain, is able to respond to thiazolidinones 6 and 7.

To further define the site of action of compound 6, the chimeric receptor F/T II was generated. This construct contains FSH-R sequences through the first extracellular loop (N-terminal domain, first two transmembranes (TMs), first intracellular loop, and first extracellular loop), with the remainder of the receptor being TSH-R (Fig. 4, lower left). CHO F/T II clone 2G7 responded to FSH with an EC_{50} of 50 pM and responded to TSH only at concentrations above 200 nM, while responding to compound 6 with an EC_{50} of 87 nM (Table 2). Thus, it appears that the FSH sequences contained in the F/T II chimera are sufficient to confer high affinity binding of FSH and the thiazolidinone compound tested.

Finally, the receptor chimera F/T III was constructed, in which the N-terminal FSH-R domain from F/T II was replaced with the N-terminal domain of the human TSH-R (Fig. 4, lower right). F/T III contains only FSH-R sequences corresponding to TM1, the first intracellular
### Table 1

Potent thiazolidin-4-one FSH agonists identified from combinatorial analog libraries based on compounds 1 and 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
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<td>Compound 3</td>
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<td>Compound 7</td>
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compound 3, compound 4, compound 5.

FIGURE 2. Response of CHO FSH-R 6XCRE-luciferase clone 1D7 to human FSH and small molecule agonists. Cell-based reporter assays were performed as described under “Experimental Procedures.” ●, human FSH; X, compound 1; △, compound 4; ○, compound 3; ■, compound 5.

FIGURE 3. Steroid production from rat granulosa cells or Y1 cells transfected with the human FSH receptor that has been stimulated by purified human FSH or the thiazolidinone agonist 5. Estradiol production induced by purified human FSH (filled circles) or thiazolidinone agonist 5 (open circles) from primary cultures of rat granulosa cells. Granulosa cells were harvested from immature rats treated with 100 μg/kg diethylstilbestrol. Cells were challenged with increasing concentration of either purified human FSH or compound 1 for 18 h. The response of cells incubated in the absence of agonist is represented by the white bar. Estradiol was measured in the media by radioimmunoassay. *, p < 0.05 versus control by analysis-of-variance followed by Dunnett’s test. B, progesterone production induced by purified human FSH (filled circles) or the thiazolidinone agonist 5 (open circles) from Y1 cells transfected with the human FSH receptor. Cells were challenged with various concentrations of either purified human FSH or compound 5 for 18 h. The response of cells incubated in the absence of agonist (Control) is represented by the white bar. Progesterone was measured in the medium by radioimmunoassay. *, p < 0.05 versus control by analysis-of-variance followed by least significant difference test.

loop, TM2, and the first extracellular loop. CHO FTIII 6XCRE-luciferase clone 1C9 cells did not respond to FSH at concentrations tested up to 100 nM. However, these cells responded to TSH with an EC₅₀ of 280 nM, and they responded to compound 5 with an EC₅₀ of 610 nM and to compound 7 with an EC₅₀ of 62 nM (Table 2). These thiazolidinone agonists appeared to activate FSH-R by interacting in an allosteric region (TM1 through the first extracellular loop) separate from the N-terminal domain.

DISCUSSION

The GPCR family of cell surface receptors is estimated to contain up to 1000 members. A large percentage of the current pharmacopoeia is comprised of compounds that interact with specific GPCRs. Thus, it is widely believed that existing and newly discovered members of this family represent attractive targets for future drugs. Historically, competitive radioligand binding assays have been utilized for high throughput screening for antagonists and agonists of GPCRs. Our search for a small, orally available mimic of FSH action began by screening for compounds that would competitively block the binding of radiolabeled FSH to CHO cells expressing the FSH receptor. Using membranes prepared from CHO FSH-R cells and a scintillation proximity assay format, we screened combinatorial small molecule libraries representing a total of 130,000 compounds at an estimated concentration of 1–5 μM/compound. We were unable to find any compounds that showed reproducible inhibition of FSH binding.

Clearly, the identification of a small molecule that can block the interaction between a large protein and its cell surface receptor presents a challenge. Much effort has been expended within the pharmaceutical industry in the search for small molecule antagonists of various growth factor receptors with little success, although a small molecule FSH-R antagonist was discovered using a radioligand binding assay (12). However, by screening directly for compounds with agonist activity, small molecule activators have been identified for the insulin receptor (25) and the granulocyte-colony-stimulating factor (G-CSF) receptor (26). Using a cell-based method similar to our approach, small molecule
**Allosteric Activation of FSH Receptor**

<table>
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<th>Table 2</th>
<th>Activity of FSH, TSH, and thiazolidinone compounds on CHO cell lines expressing chimeric receptors</th>
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<td>Receptor</td>
<td>Ligand (EC&lt;sub&gt;50&lt;/sub&gt;)</td>
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<tr>
<td>FSH-R</td>
<td>FSH</td>
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<tr>
<td>FSH-R/TSH-R</td>
<td>140 pM</td>
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<tr>
<td>TSH-R/FSH-R</td>
<td>200 pM</td>
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<tr>
<td>F/T II</td>
<td>ND</td>
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<tr>
<td>F/T III</td>
<td>50 pM</td>
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FSH-R agonists have been identified (27, 28). These compounds activate a CHO FSH-R reporter cell line and induce cAMP activity; however, their site of action remains unknown.

We constructed a reporter cell line expressing the FSH-R and the luciferase gene under a cAMP-responsive 6XCRE-based control element and established a high throughput reporter assay for compounds that would act as FSH agonists. Using this assay, we screened over 78,000 molecules in pools of 10–36/well from our collection of combinatorial small molecule libraries.

The lead thiazolidinone compounds, 1 and 2, showed activity in the 20–50 μM range using the FSH-R reporter assay. With the use of combinatorial analoguing efforts we rapidly identified potent analogs with activity in the low nanomolar range. To address the mechanism of action of these novel FSH agonists, we tested for their ability to compete with the binding of radiolabeled FSH to both cells expressing the FSH-R and to membranes prepared from such cells; the compounds were unable to block the binding of 125I-labeled FSH.

An obvious question that we posed is: What is the mechanism of activation of the FSH-R by the thiazolidinone compounds? The response is specific and stereoselective, requires the presence of the FSH-R, and occurs in the absence of FSH. However, a number of mechanisms can be postulated. FSH is known to bind with high affinity to the large extracellular domain on the FSH-R (1, 2). However, high-affinity binding and signaling appear to involve separate domains on the FSH-R (29, 30) and other glycoprotein hormone receptors (31). Allosteric modulators have also been reported for a number of 7-transmembrane GPCRs (32–34) and have been proposed as possible for glycoprotein hormone receptors on the basis of a reported separation of sites necessary for ligand binding and signal transduction. Recently, the crystal structure of human FSH bound to the extracellular portion of the FSH-R has been reported, along with data indicating that the active receptor complex exists as a dimer (35).

One possible mechanism for FSH stimulation of its receptor invokes high affinity binding to this N-terminal domain followed by receptor activation via interaction of the tethered FSH with the extracellular face of the 7-transmembrane core of the receptor similar to that proposed for proteinase-activated receptor-2 (PAR2) (36). A model for glycoprotein hormone activation has been proposed in which ligands bind with high affinity to the N-terminal extracellular domain and convert the extracellular domain from an inverse agonist into a full agonist, activating the transmembrane-spanning portion of the receptor (37). The thiazolidinone compounds could interact with the same site on the core of the receptor to promote such a conformational change in the receptor. If this proposed mechanism is accurate, it suggests the possibility that small molecule analogs could be generated that bind to the same site but lack the ability to activate the receptor, leading to effective antagonism. Such small molecule FSH antagonists could be effective oral contraceptives and useful for other therapies requiring hormonal modulation.

Alternatively, the thiazolidinone compounds could bind to an unrelated site on the FSH-R to cause an allosteric change in receptor conformation or the direct recruitment of G protein. If any of these mechanisms of drug action can be applied generally to other members of the GPCR family, it suggests that the screening of diverse collections of small molecules using cellular functional assays may be a very productive means of identifying new drug candidates.

FSH-R/TSH-R chimeras were generated to identify the regions responsible for FSH agonist receptor activation and signaling described herein. The results with the FSH-R/TSH-R and TSH-R/FSH-R chimera-expressing cell lines clearly show that whereas response to the hormones maps with the N-terminal extracellular domain, response to the thiazolidinone compounds maps with the transmembrane-spanning portion of the receptor. Activity on the F/T II chimera-expressing cell line shows responsiveness to FSH (as it contains the FSH-R N-terminal domain) and response to thiazolidinone compounds. By then swapping the N-terminal domains in the generation of F/T III, we were able to show that only a small portion of the FSH-R is required for thiazolidinone action. This region, spanning TM1 through TM3, is sufficient to elicit a response to the thiazolidinone compounds. Further definition of the site of action of these compounds would be helpful to determine whether the compounds are acting on the first extracellular loop or with one of the transmembrane-spanning regions.

This study represents the first reported small molecule high affinity agonist of the important class of 7-TM glycoprotein hormone receptors that appears to function through a novel allosteric mechanism. The small molecule FSH receptor agonists described here are a significant achievement toward the development of an oral alternative to the current injectable FSH treatments for clinical use to induce ovarian stimulation for both in vivo and in vitro fertilization therapy. In addition, the allosteric mechanism by which these agonists act may remedy the infertility that is attributable to FSH-R mutations in the N-terminal extracellular ligand binding domain.

**REFERENCES**

col. 125, 717–726
12. Arey, B. J., Deecher, D. C., Shen, E. S., Stevis, P. E., Meade, E. H., Jr., Wrobel, J., Frail,
Chem. 60, 7328–7333
Chem. 37, 1385–1401
196–206
209–277
22. Nagayama, Y., Wadsworth, H. L., Chazenbalk, G. D., Russo, D., Seto, P., and Rapo-
Metab. 2, 151–156
M. T., Pelaez, F., Ruby, C., Kendall, R. L., Mao, X., Griffin, P., Calaycay, J., Zierath, J. R.,
281, 257–259
27. Guo, T., Adang, A. E., Dolle, R. E., Dong, G., Fitzpatrick, D., Geng, P., Ho, K. K.,
Kulgen, S. G., Liu, R., McDonald, E., McGuinness, B. F., Saiouz, K. W., Valenzano,
1717–1720
50165–50175
33. Arey, B. J., Seethala, R., Ma, Z., Fura, A., Morin, J., Swartz, J., Vyas, V., Yang, W.,
13943–13947
(Oxf) Suppl. 3, 18–35 and 47–51

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