

Allosteric Regulation of the Follicle-Stimulating Hormone Receptor

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Follicle-stimulating hormone receptor (FSHR) belongs to the leucine-rich repeat family of the G protein-coupled receptor (GPCR), which includes the glycoprotein hormone receptors luteinizing hormone receptor, thyrotropin receptor, and other LGRs 4, 5, 6, and 7. FSH is the key regulator of folliculogenesis in females and spermatogenesis in males. FSH elicits its physiological response through its cognate receptor on the cell surface. Binding of the hormone FSH to its receptor FSHR brings about conformational changes in the receptor that are transduced through the transmembrane domain to the intracellular region, where the downstream effector interaction takes place, leading to activation of the downstream signaling cascade. Identification of small molecules that could activate or antagonize FSHR provided interesting tools to study the signal transduction mechanism of the receptor. However, because of the nature of the ligand-receptor interaction of FSH-FSHR, which contains multiple sites in the extracellular binding domain, most of the small-molecule modulators of FSHR are unable to bind to the orthosteric site of the receptors. Rather they modulate receptor activation through allosteric sites in the transmembrane region. This review will discuss allosteric modulation of FSHR primarily through the discovery of small-molecule modulators, focusing on current data on the status of development and the utility of these as tools to better understand signaling mechanisms. (*Endocrinology* 159: 2704–2716, 2018)

Follicle-stimulating hormone receptor (FSHR) is a leucine-rich repeat (LRR)-containing class A G protein-coupled receptor (GPCR) belonging to the subfamily of LRR-containing G protein-coupled receptors (1–3). FSHR contains a large extracellular domain (ECD) consisting of the LRR at the N-terminal end and a hinge region at the C-terminal end that connects to the transmembrane domain (TMD) (4, 5). The TMD contains seven α -helices that are connected through three extracellular loops and three intracellular loops (6). The TMD continues into a short cytoplasmic tail (1–3). Each region of the receptor plays distinct roles in structure activity and function of the receptor. It is well established that the orthosteric site for the hormone binding is within the ECD (7, 8). Fan and Hendrickson (9) in 2005 determined the crystal structure of the FSHR ECD complexed with FSH. Based on this structure, it was

suggested that FSH binds to FSHR like “hands clasped” on the concave surface of the FSHR (9). However, this model did not include the hinge region, which is considered to play critical roles in receptor activation based on various biochemical studies (10–12). More recent studies strongly suggest unliganded ECD acts as a tethered inverse agonist imposing an inhibitory effect through the hinge region, which is converted to a stimulatory response upon ligand binding (13). The crystal structure of FSH bound to the entire ECD as determined by Jiang *et al.* (14, 15) confirmed the importance of the hinge region for activation of the receptor. This work identified a function for sulfated Tyr-335 (sTyr) in the hinge region of FSHR to act as a second interaction site with FSH (15). Based on this model, it was suggested that binding of FSH to the concave surface of LRR exposes the sTyr-binding pocket in the FSH ligand. Following this

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Abbreviations: CGR, chorionic gonadotropin receptor; ECD, extracellular domain; FSH-CTP, FSH-c-terminal peptide; FSHR, follicle-stimulating hormone receptor; GPCR, G protein-coupled receptor; hFSHR, human follicle-stimulating hormone receptor; LGR, leucine-rich repeat-containing G protein-coupled receptor; LRR, leucine-rich repeat; NAM, negative allosteric modulator; PAM, positive allosteric modulator; sTyr, sulfated Tyr-335; TMD, transmembrane domain; TZD, thiazolidinone.

unmasking of the sTyr pocket, the receptor sTyr is lifted into the new ligand pocket, lifting the hinge region, thereby releasing the inhibitor effect and facilitating activation of the receptor (15, 16).

Studies by site-directed mutagenesis, chimeric receptors, and naturally occurring mutations have demonstrated the critical role of extracellular loops and intracellular loops in FSHR function (6, 12, 17, 18). They play key roles in ligand binding, cell surface receptor trafficking, signaling, and interaction with adaptor proteins (6). Similar to other GPCRs, the FSHR C-terminal tail plays a key role in trafficking of the receptor to cell surface through the highly conserved F(X)₆LL motif (19). The C-terminal tail contains sites for posttranslational modification, including palmitoylation, receptor phosphorylation, interaction with β -arrestin and hence, internalization (6).

It is widely accepted that upon FSH binding to its receptor, a number of intracellular signaling pathways are activated in a parallel or sequential manner in line with other GPCRs (20). In addition to the widely studied canonical cAMP/PKA pathway (21, 22), FSH-FSHR has been reported to trigger protein kinase B/Akt (23–25), serum and glucocorticoid-induced kinase (23), p38 MAPK (26), ERK1/2 (24, 27), EGFR (28, 29) activation, and Ca²⁺ (30, 31) and inositol triphosphate production (32). FSHR interacts with GPCR kinases and β -arrestins (33–37), in addition to other interacting proteins such as adaptor proteins containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif (38). Alternate glycosylation of FSH conveys distinct signals that are transduced by the receptor-transduction system as either stimulatory or inhibitory intracellular events via promiscuous G protein coupling (39). So, an integrative view of the signaling network induced by activation of FSHR, rather than the individual pathway, is critical to gain a better understanding on the physiological outcome from this interaction (40). To tackle these questions, a prerequisite is the availability of scientific tools like reliable and quantitative assays and agents to elicit distinct response upon stimulation of the receptor. In this respect, small-molecule allosteric modulators of FSHR will have a critical role in unraveling the FSHR signaling mechanism.

FSHR Allosteric Modulators

Infertility affects 15% of reproductive-age couples (41). FSH is the most important among the therapeutics in the clinic for treatment of infertility and is used primarily for women to induce controlled ovarian stimulation, while certain populations of infertile men also benefit from FSH treatment (42–44). The therapeutic protein FSH must be administered by injection, which is inconvenient

and stressful, resulting in low patient compliance. It has been realized, for the past decade, that receptors can be modulated at allosteric sites that are away from the orthosteric sites to bring about beneficial effect (45). This spurred interest in several pharmaceutical companies to develop small-molecule allosteric modulators of FSHR that could be orally administered, thereby improving the convenience of patients. The preference of patients to use orally administered drug has been successfully addressed by replacing injectable treatments in rheumatoid arthritis (anti-TNF agents vs JAK inhibitors) (46, 47) and multiple sclerosis (interferons vs teriflunomide) (48). In addition to the improved convenience of oral therapy and compliance of patients, creation of a drug product using a small molecule will not require extensive bioreactors, as are needed for protein production, nor will it require the cold supply chain needed to store and transport human FSH. Although FSHR antagonist development has lagged behind that of allosteric agonists, antagonists have the potential to offer a highly selective, nonsteroidal approach for contraception with fewer side effects than the currently available steroid-based contraceptives.

Allostery is a phenomenon describing the ability of interactions occurring at one site of a protein to modulate interactions at a spatially distinct site of the same molecule in a reciprocal manner (20). There is ample evidence over the past decade from independent groups that demonstrate synthetic small molecules are able to mimic the effects of protein hormones on their receptor by acting at allosteric sites (49–51). These modulators can either positively or negatively affect the receptor activation. Based on the current nomenclature, four types of allosteric ligands have been described: potentiators or positive allosteric modulators (PAMs), antagonists or negative allosteric modulators (NAMs), and allosteric agonist (alloagonist) and silent allosteric modulators (52). An allosteric ligand that potentiates an agonist-mediated response is referred to as a PAM, whereas one that inhibits activity is known as a NAM (53). Neutral allosteric ligands or silent allosteric modulators occupy allosteric sites yet elicit no functional response but can block the action of PAMs and NAMs (53). Development of small-molecule agonists or antagonists for receptors that are activated by large peptides or proteins has been challenging for various reasons that are elaborated below.

During the last two decades, a number of activating and inactivating mutations, single nucleotide polymorphisms, and spliced variants of the FSHR gene have been reported in selected infertile cases (54–56). Further, structural alterations provoked by mutation can lead to abnormal misfolding and may lead to retention/degradation of membrane proteins including GPCRs resulting in diseases (57). Several strategies including

pharmacological intervention through pharmacochaperones offer a promising method to correct the defective misdirected protein to the plasma membrane. This strategy has been successfully demonstrated in serotonin transporter (58), ABC transporter ABCB1 (59), vasopressin receptor 2 (60), GnRH receptor (61), DP1 receptor (62), and CFTR (63, 64). Mutant LH/chorionic gonadotropin receptor (CGR) A593P and S616Y cause Leydig cell hypoplasia in men due to retention of the receptor in endoplasmic reticulum (65). A small-molecule allosteric agonist Org42599 facilitated rescue of the mutant receptors to the cell surface and responsiveness to LH (66). In FSH receptor (FSHR), A189V mutation has been classified as loss of function due to the lack of expression of the receptor in the plasma membrane (67–70). Interestingly, LH receptor (LHR) agonist Org-41841, which had no agonistic effect on FSH receptor (FSHR), was able to increase the expression of the A189V mutant receptor to the surface as well as that of the wild-type FSHR (71). This is quite unexpected that an analog with no effect on signaling of the FSHR was able to rescue the mutant receptor to increase the expression of wild-type FSHR to the cell surface. This suggests that even though there is no signaling, LHR agonist can bind to the receptor at an allosteric site of FSHR and behave as pharmacochaperone. The pharmacological approach of using small molecules as pharmacochaperone to correct sorting of misfolded receptor to the plasma membrane could be a unique therapeutic opportunity for treatment of patients having these mutations.

Advances in high-throughput assays, cellular screening, and detection technologies have improved the tools for biologists to describe the effect of unique compounds. In parallel, the use of combinatorial chemistry has helped to diversify libraries to enrich for small-molecule modulators for FSHR. Stable cell lines such as Chinese hamster ovarian cells have been engineered to provide highly sensitive and reproducible screening assays (cAMP assays or luciferase-based luminescence assays) that are good tools to screen several thousands of molecules. Nevertheless, it has been previously reported that FSHR density can modulate the balance between distinct signal transduction pathways (72). Despite the value of engineered cell lines for high-throughput screening, it remains imperative to confirm that molecules obtained from overexpressed systems exert anticipated activity in physiologically relevant primary cellular assays such as estradiol production by granulosa cell culture in the case of FSHR (73). There are several excellent articles on development of small-molecule modulators of glycoprotein receptors (73–76). The objective of this work is to focus on the allosteric modulators of FSH receptor with recent advancement in using these as tools to understand FSHR biology.

Small-Molecule Modulators as Tools for Pharmacological Profiling of FSHR

The first published small-molecule FSHR agonists, thiazolidinones (TZDs), were identified through screening of a combinatorial library using an FSHR reporter assay (77). Subsequent parallel synthesis resulted in a potent agonist that was very specific for FSHR with no cross reactivity with LHR or TSHR (78). Compounds identified from this screen, as expected, did not compete with FSH binding to the receptor. By using FSHR/TSHR chimeric mutant receptors, the binding site for these compounds were identified to be allosteric based on the requirement of the FSHR transmembrane (TM) regions 1 to 3 for activity (78).

One of the many challenges facing development of small-molecule modulators of FSHR is the presence of a very narrow structure-activity relationship of these molecules to the activated receptor. Thus, subtle replacement of the acetylene bridge [shaded orange in Fig. 1(a)] with methoxy as in compound 2 generates an inverse agonist (79). On the other hand, replacing a dialkoxyphenyl side chain with an indole group and adding thiophene to compound 1 [gray areas in Fig. 1(a)] results in an antagonist, as for compound 3 (79). The authors demonstrated that the agonist stimulated FSHR through the G_s pathway, whereas compound 3 activated G_i -mediated signaling to inhibit cAMP (79). Further, compound 3 not only inhibited recombinant human FSH-induced estradiol secretion in rat granulosa cells [Fig. 1(b)]; it was also very potent in blocking estradiol produced by compound 1 and two other FSHR agonists with different chemical scaffolds developed in our group [Fig. 1(c)] (80, 81). This suggests that all three different chemical scaffolds bind to similar sites or regions within the TMD and the antagonist induces a distinct conformation in the receptor that inhibits the activity of the agonists. We had further demonstrated compound 1 was capable of activating alternate signaling pathways such as pAkt and pERK in addition to cAMP and stimulating *in vitro* cumulus expansion and *in vitro* follicle growth (24). Compound 1 was also very effective in recruiting β -arrestin to human FSHR (hFSHR) (14). The compound, was able to stimulate follicular development in rats when delivered through an Alzet pump. Despite mimicking FSH action *in vitro*, TZD was not suitable for development as an oral agent, as the compound was metabolized rapidly and was not orally bioavailable, demonstrating the steep hurdles required for drug development of FSHR agonists (24). However, it is quite fascinating that a small-molecule allosteric modulator can activate the breadth of action of the large protein FSH, leading to physiologically relevant

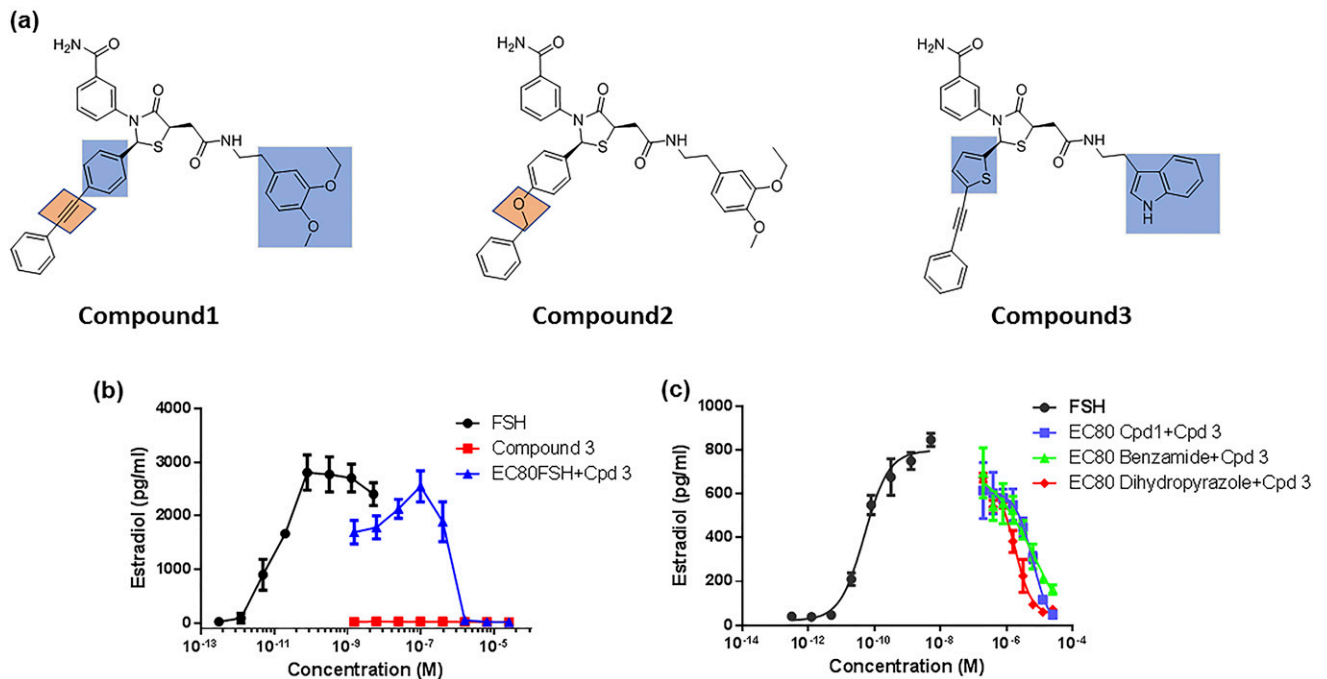


Figure 1. TZD series of FSHR agonist. (a) Chemical structure of TZD compounds that display distinct activity on FSHR. Orange shaded area is the difference between compound 1 and 2, whereas gray shaded areas represent the changes in compound 3 from compound 1. (b and c) Antagonist effect of compound 3 in rat granulosa cells. Granulosa cells isolated from diethylstilbestrol-primed immature rat were stimulated with FSH or test compounds for 24 hours, and estradiol in the supernatant was quantitated by ELISA as described earlier (24). (b) Estradiol in the culture supernatant following incubation of varying doses of FSH or compound 3 or compound 3 in the presence of 80% maximal effective concentration (EC80) of FSH. (c) Inhibition of estradiol secretion by compound 3 on EC80 of compound 1 or on other FSHR agonists from different chemical series, benzamide and dihydroprazole.

outcomes. Thus, these molecules, though not suitable for therapeutic development, serve as great tools for understanding the basic biology of FSHR structure-function relationships.

PAM With Oral *In Vivo* Activity

The major driver for FSHR small-molecule development is to improve the convenience of the available treatments for both female and male infertility. There have been several reports that describe the activity of small-molecule allosteric agonist activity *in vitro*; however, it has been quite a challenge to develop a small molecule with druglike properties and that can stimulate FSHR *in vivo* through a convenient route, preferably by oral administration. The first such compounds were hexahydroquinolines (82, 83). Org-214444 is a stereoselective, highly lipophilic compound stimulating cAMP with nanomolar potency and stimulating estradiol secretion in human granulosa cells like FSH (83). Most importantly, Org-214444 was very effective in stimulating follicular development at 1 mg/kg in rats, as demonstrated by the presence of ova in the oviduct following human chorionic gonadotropin administration (83). This is the first report of a small molecule stimulating

folliculogenesis through oral administration. Org-214444 behaved as an alloagonist by enhancing FSH response by 3.5-fold, through a mechanism that included increasing the number of available FSHR binding sites for FSH, as suggested by Jiang *et al.* (14, 16). This unmasking of FSHR will be discussed in more detail later in this review. However, further development of this molecule was not reported. Perhaps owing to high lipophilicity, this compound was not suitable to be developed as a drug.

Gerrits *et al.* (84) reported results from a first-in-human study testing of dihydropyrroloisoquinoline, MK-8389, defined in preclinical models as an oral FSHR agonist. This is the first report that evaluated a small-molecule oral FSHR agonist in women. The molecule was safe and well tolerated in women; however, it failed to demonstrate an increase in ovarian follicular development but caused an increase in thyroid hormone release (84). The clinical design involved pituitary suppression by oral contraceptive Marvelon during stimulation with the MK-8389. Marvelon is a combination contraceptive containing desogestrel, a synthetic progestin, and ethinyl estradiol. There are several studies from the early 1980s and 1990s demonstrating that high levels of progesterone can have direct negative impact on the ovary, reducing its

ability to respond to FSH (85–88). Thus Marvelon had the unintended consequence of diminishing the ovarian response to MK-8389. In fact, Goodman and Hodgen (89) observed antifolliculogenic action of exogenously administered progesterone despite hypersecretion of FSH in monkeys. In the MK-8389 clinical study, doses >20 mg caused moderate increase in inhibin levels, suggesting evidence of some early follicular development at higher doses (84). Unfortunately, due to the unexpected effect of MK-8389 on thyroid response, doses >40 mg were not tested in this study (84). Remarkably, when this same clinical design was used in the first-in-human studies with FSH-c-terminal peptide (CTP) (Elonva), similar diminished ovarian response was observed as compared with another Elonva study without oral contraceptive during FSH-CTP treatment (90, 91). At a much higher dose of FSH-CTP in the presence of oral contraceptive, the anticipated ovarian response was observed, so the development of this protein was continued (90, 91). Therefore, it is highly likely that MK-8389 could have stimulated an ovarian response in the absence of the contraceptive, at lower doses than the thyroid response. Although the MK-8389 study failed to demonstrate the agonistic activity on follicular development, the progression to first-in-human marks an encouraging development in small-molecule FSHR discovery, and this study paves the way for design of future clinical trial protocols to avoid unintended consequence of progesterone at the ovary.

NAMs

The selective expression of the FSHR in the ovary has been the motivation for developing an FSHR antagonist as a unique, effective contraceptive, in a unique regimen. Several commercial groups pursued development of small molecules to inhibit FSH-mediated effects. Today, the most widely used oral contraceptives are steroid based, which have substantial effects in multiple organ systems (92). Due to the highly restricted expression of FSHR in the granulosa cells of the ovary, an FSHR antagonist is anticipated to be devoid of effects on other tissues. In 1986, prior to our understanding of orthosteric or allosteric antagonists, a short peptide sequence (Thr-Arg-Asp-Leu) was shown to inhibit FSH binding and FSH-stimulated effect, albeit at very high concentrations (93). Arey *et al.* (94) provided the first report on identification of a selective nonpeptide FSHR antagonist. This compound, diazonaphthylsulfonic acid, associates with FSHR via the ECD of the receptor. This is the only nonpeptide small molecule so far reported to bind to the ECD of FSHR. The compound inhibited FSH-mediated cAMP and steroidogenesis *in vitro* and was capable of

blocking ovulation in mature rats at the highest dose (100 mg/kg IP) (94). Low efficacy of this compound did not permit further development for contraception. In a later study, van de Lagemaat *et al.* (95) demonstrated that administration of a short-acting low-molecular-weight FSHR agonist during the follicular phase of the estrous cycle inhibited ovulation by premature luteinization of unruptured follicles in rat and pig.

Recently, nanobodies have been used to study GPCR structure function (96, 97). FSHR nanobodies were identified by whole cell panning of a synthetic library (98). These nanobodies did not compete with FSH for binding the FSHR, but inhibited FSH-mediated cAMP through its interaction at the allosteric site (98). FSHR nanobody antagonists could be an interesting development for unique contraceptive agents. Nanobodies are a rather radically different way to inhibit FSHR function through allosteric interactions; however, to date, nanobodies have not been successfully formulated for oral administration. Dias *et al.* (99, 100) characterized a series of FSHR NAMs developed at Addex. These NAMs inhibited FSH-induced cAMP production in Chinese hamster ovarian—human FSHR cells and rat granulosa cells (99, 100). Interestingly some of the NAMs displayed biased antagonistic activity on steroid induced by FSH (99, 100). Based on these studies, the authors demonstrated that a NAM capable of contraception needed to inhibit both progesterone and estradiol production. These properties are discussed in more details in the next section of this review.

Biased Signaling of FSHR Allosteric Modulators

It is widely accepted that following FSHR activation, multiple signaling pathways are stimulated in parallel or in a sequential manner. The interaction of activated and phosphorylated FSHR with several receptor-associated proteins, including β -arrestins (101–104) and adaptor proteins containing PH domain, PTB domain, and leucine zipper motif (38, 105–107), is crucial for regulation of their signaling and functional outcomes. These associated proteins regulate several signaling pathways (38, 108), including Erk, inositol triphosphate, and Ca^{2+} . It is now generally known that GPCRs adopt multiple inactive and active conformations that engage distinct signal transduction mechanisms (20). Although these signaling pathways can be activated or inhibited by balanced ligands, a biased ligand has the capacity to distinguish among the partners that associate with the receptors to trigger a subset of biased responses (108, 109). This feature of GPCR signaling may allow development of ligands that specifically modulate effectors

supporting a desired action. Over the past few years, a range of structural, biochemical, and cellular studies have revealed unique insights into FSHR signaling. Some of these findings (discussed below) have come as a surprise and therefore have the potential to significantly refine the conceptual framework of the FSHR system. Availability of small-molecule allosteric modulators of glycoprotein hormone receptor as a tool have enhanced our knowledge in the multiple dimensions of the signaling pathways of the receptors.

As discussed earlier, most of the small-molecule modulators of FSHR are allosteric in nature, and they bind to the TMD of the receptor as demonstrated for TZD (78). By binding to the allosteric site, these small-molecule ligands induce different conformations of FSHR, thereby leading to biased signaling. Thus, by making subtle modification to the TZD core, a wide range of biased ligands could be synthesized, from an agonist stimulating cAMP to an inverse agonist to an antagonist activating the G_i pathway (79). These data suggest it is possible to obtain small molecules in a single chemical core with a wide range of activities. Similar bias in FSHR signaling has been demonstrated using FSH glycoforms (39, 110).

In an effort to develop an FSHR antagonist, Dias *et al.* (99, 100) examined a series of NAMs with very interesting properties. In the first study, ADX61623 was able to inhibit FSH-induced cAMP and progesterone in granulosa cells, but the compound was not effective in blocking FSH-induced estradiol secretion, whereas another NAM, ADX49626, was a pure antagonist in blocking both steroids (99). This suggests that ADX61623 stabilized a conformer of the receptor that inhibits the G_s pathway and progesterone production but had no effect on estradiol, unlike ADX49626, which was a pure antagonist. This was the first report of biased signaling downstream to a second messenger. Interestingly, both these NAMs increased ^{125}I -FSH binding to HEK-hFSHR cells without affecting internalization of the receptor due to increased receptor capacity. Based on the crystal structure of complete ECD with FSH, Jiang *et al.* (14, 16) proposed that the increased binding of FSH caused by these NAMs is due to opening up of the trimer to allow additional binding of FSH. In a second study, Dias *et al.* (100) reported on the antagonistic effect of ADX68692 on cAMP and progesterone and estradiol secretion in granulosa cells. Another FSHR NAM, ADX68693, exhibited an antagonistic effect on cAMP and progesterone but synergized with FSH to increase estradiol secretion from granulosa cells (100). Based on these data, it can be assumed that a range of FSHR conformers can be established by the small-molecule FSHR NAMs to achieve alternative associations of

multiple downstream effectors. Interestingly, only ADX68692 exhibited *in vivo* antagonistic activity; however, ADX68693 was unable to block FSH-induced follicular development *in vivo* despite its improved oral bioavailability (100). This data led the authors to conclude that for development of an effective contraceptive, both arms of FSH-induced steroid production should be inhibited. The pharmacological profiles of these two NAMs (ADX68692 and ADX68693) were recently evaluated in LH/CGR with regards to G_s , β -arrestin, and steroid production (111). The molecules showed biased antagonism of steroid production that was distinct from FSHR depending on the cell type (111). Inhibition of testosterone production by these two NAMs was reported in both primary Leydig cells and a tumor Leydig cell line, whereas ADX68693 inhibited progesterone production only in primary Leydig cells but not in the cell line, and ADX68692 either had no effect on progesterone in a Leydig cell line or promoted progesterone production in primary Leydig cells (111). Taken together, these data provide evidence for the complexity of gonadotropin receptor signaling. De Pascali *et al.* (112) recently reported biased agonism with several different FSHR small-molecule agonists with respect to G_s and β -arrestin recruitment to the receptor, opening up an exciting prospect to dissect the complexity of FSHR signaling. The mechanism of biased response in GPCR can be due to the functional presence of specific signaling complexes formed by the ligand, the receptor, and directly interacting signaling partners. These partners provide a unique conformational stabilization that determines the signaling profile for each ligand-receptor pair (53, 113, 114). Despite significant advancement of GPCR biology, translating bias information into unique mechanistic insight for development of better therapeutic ligands is limited by time- and cell-dependent factors, and they herald unprecedented complexity in receptor-ligand signaling. Nevertheless, it is still important to quantify the signaling bias through mathematical modeling (108, 115, 116). Quantification of signaling bias helps to ensure comparison among ligands with similar potency on one endpoint and can be used to guide structure-activity studies and the selection of drug candidates.

Mechanism of Allosteric Regulation of FSHR

To date, results strongly suggest that the allosteric site for the interaction of low-molecular-weight FSHR modulators is in the TMD of the receptor. Direct evidence obtained using an FSHR-TSHR chimera approach indicated the importance of transmembrane 1, 2, and 3 for the binding of TZD (78). The general structure of GPCRs

are assumed to be similar to rhodopsin, which is often used as a template to map the three-dimensional structure of most GPCRs. In the glycoprotein hormone receptor, in general, two pockets, P1 and P2, are formed by positioning of the TMD helices (74). Heitman *et al.* (117) confirmed two binding sites in the TMD of LHR for small-molecule modulators. By *in silico* docking studies, putative binding sites for two LHR agonists were suggested to occupy distinct regions in the allosteric binding pocket (117). An antagonist was likely to interact with additional residues in TM2 and TM7, perhaps stabilizing

the receptor conformation in a restricted inactive state. This second allosteric site in LHR closely resembles the orthosteric site of A3R, whereas experimental evidence shows the LHR antagonist was able to bind to A3R (117). Interestingly, an FSHR small-molecule agonist developed at TocopheRx, in a selectivity screen of membrane proteins, specifically displaced binding of radiolabeled A3 agonist to A3R at very high concentration (118). This suggests a similarity between glycoprotein receptor and A3R in the TMD region for the allosteric binding pocket.

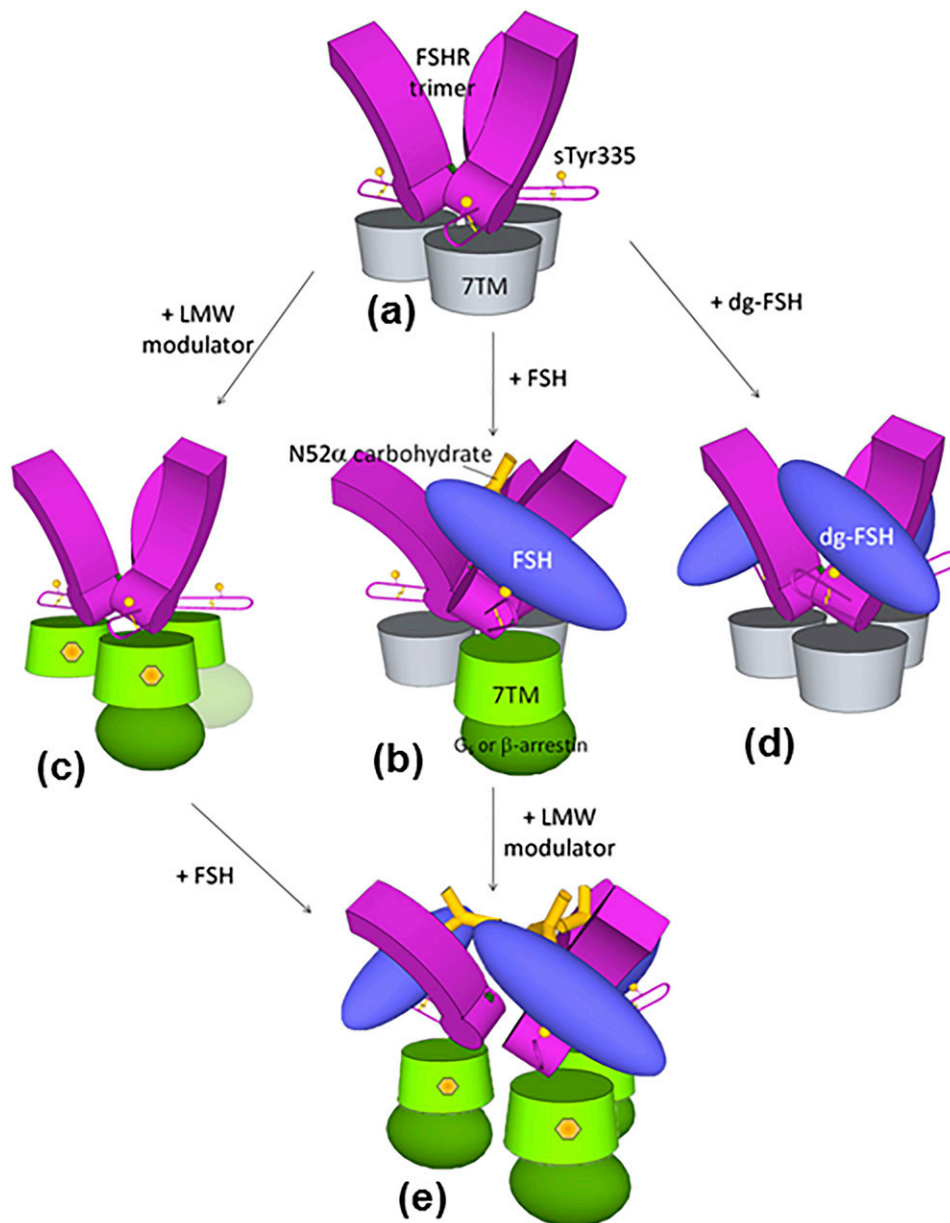


Figure 2. FSHR structure as determined by complex of FSH with complete ECD of FSHR. (a) FSHR exists as trimer in basal state. (b) Fully glycosylated FSH binds to one of the monomers and activates the receptor due to the presence of N52 α glycosylation. (c) Small-molecule FSHR agonists (yellow hexagons) bind to the allosteric site in 7TM and activate the receptor. (d) dg-FSH can bind to all three trimers but constrains the receptor in trimeric state, keeping it inactive. (e) In the presence of small-molecule FSHR agonist, the trimeric receptor opens up, allowing three FSH molecules to bind to each of the protomers and activating the receptor. Reproduced under Creative Commons CC BY 3.0 license from Jiang *et al.* (14). 7TM, 7-TMD; dg-FSH, deglycosylated FSH; LMW, low-molecular-weight.

Oligomerization of glycoprotein receptors is supported by several biochemical, pharmacological, and biophysical studies (119–124). As with many other GPCRs, glycoprotein hormone receptors form dimers or higher-order oligomers and even heterodimers between FSHR and LHR, as these two receptors are expressed in the same cell type (granulosa cells) depending on the differentiation status (late follicular phase and luteinization) of the cells (120, 123, 125, 126). The most recent crystal structure of ligand-bound ECD of FSHR provided evidence for the presence of trimeric receptor in the basal state [Fig. 2(a)] (14). The model predicts that the receptor is held in trimeric state, through interaction of ECD and TMD between the protomers. This model further predicts that only one FSH can bind to the trimeric receptor due to the bulky glycosylation of FSH. As a result, one unit of the trimer, the FSH-bound protomer, is activated [Fig. 2(b)]. In contrast, three molecules of deglycosylated FSH, due to the absence of bulky glycosylation, are capable of binding to all of the cognate receptor trimer unit, so the complex is maintained in trimeric state, keeping it inactive [Fig. 2(d)]. Interestingly, this model predicts that small-molecule agonist binding to the TMD can

dissociate the receptor in the TMD while activating the second messengers [Fig. 2(c)]. At the same time, this allows loosening of the interaction of the ECD in the trimeric receptor, permitting three molecules of FSH to bind to the receptor [Fig. 2(e)]. Additional experimental evidence confirms that small-molecule modulators were able to increase binding of FSH to FSHR (71, 83, 99). Similarly, coincubation of TZD with FSH caused a threefold increase in recruitment of β -arrestin to FSHR over FSH alone (14, 16). It is interesting that both agonist and antagonist can increase FSH binding to its receptor, while clearly the conformation stabilized by each ligand is unique to either promote or inhibit the signaling event. Thus, unraveling the molecular signaling mechanism of agonist vs antagonist would help us to develop unique therapeutics with specific properties.

Modeling of the TMD of FSHR, developed from the family A GPCR model, provides prediction of the allosteric binding pocket size and hydrophobicity (Fig. 3). In FSHR, there is a change in conformation of the TMD from basal (Fig. 3a) to the active state (Fig. 3b). The FSHR 7 TMD contains a large allosteric pocket on top of the TMD close to helices projecting from ECL1 and

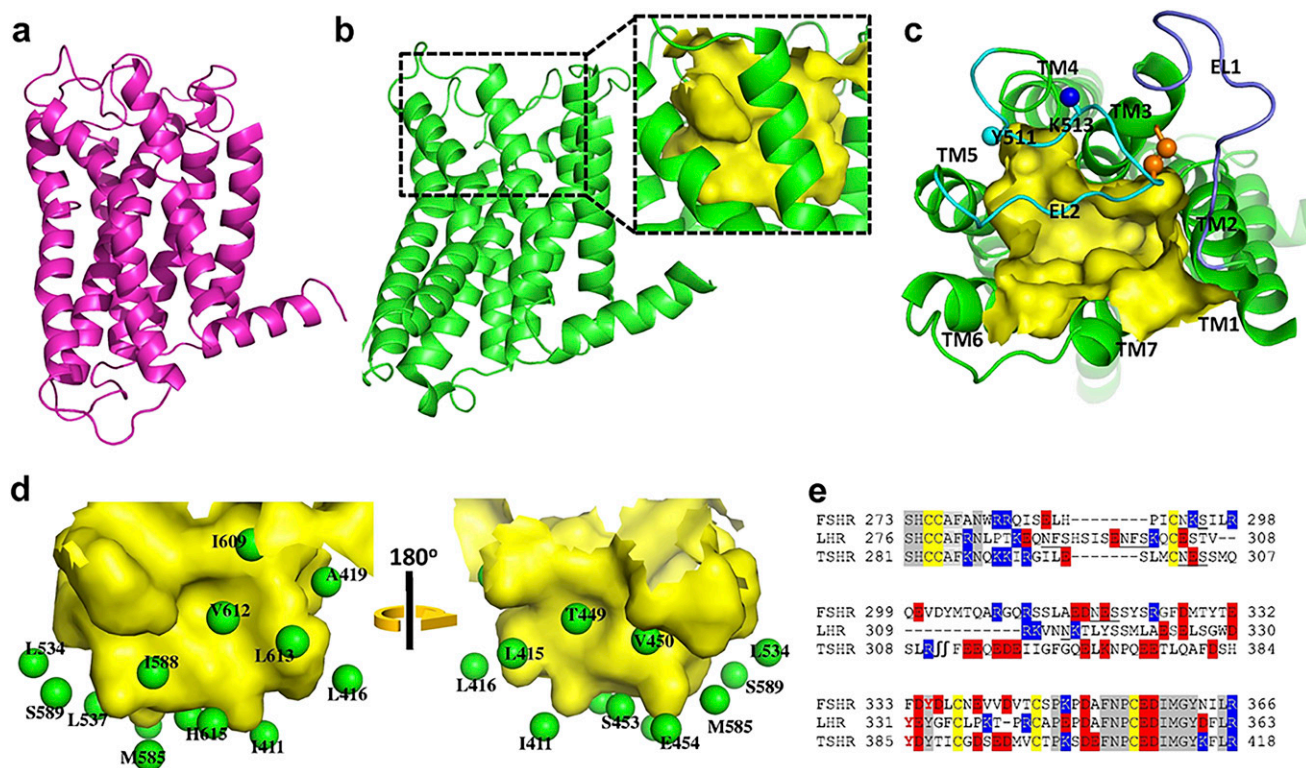


Figure 3. Molecular modeling of potential allosteric binding site of small-molecule ligand binding to FSHR TMD. (a) TMD in the inactive state of FSHR. (b) TMD during the activation state of FSHR. (c) Zoomed region is the potential pocket of allosteric binding site. (d) Front and back view of the binding pocket with the surrounding residues. (e) Alignment of charged residues in the hairpin loop in the signal specificity subdomain among the glycoprotein hormone receptors. Positive charges are in blue, negative are in red, cysteines are in yellow, and other conserved residues are shaded in gray. Gaps are represented by dash symbols. Reproduced under Creative Commons CC BY 3.0 license from Jiang *et al.* (16). EL, extracellular loop.

ECL2 (Fig. 3c). Figure 3d provides a closer view of the pocket surrounded by many residues including T449. Molecular modeling of the small-molecule LH/chorionic gonadotropin ligand allosteric binding pocket was proposed to be close to the upper portion of the TMD of LH/CGR, similar to the model of the FSHR allosteric site (117). Site-directed mutagenesis of FSHR with T449V showed loss of cAMP response by Org-214444, but FSH-mediated signaling was intact, indicating the importance of T449 for allosteric binding of small-molecule ligands (83). The hairpin loop of the hinge region contains highly charged residues within the glycoprotein hormone receptors (Fig. 3e), and they may interact with the extracellular loops. Identification of potential allosteric sites on FSHR reveal new opportunities to develop unique molecules to achieve selected drug action for this important class of target.

Summary and Future Directions

Application of allosteric modulators of FSHR for therapeutics as well as pharmacological tools to understand the basic signaling mechanism of the receptor is rather exciting. Discovery of biased agonism or antagonism has provided a paradigm shift, moving our view of receptor modulation from purely a single response to a more quantitatively diverse event culminating in the final physiological response. Signaling bias theoretically allows one pharmacological pathway that is beneficial, while avoiding the pathways associated with undesirable activity. Thus, developing a pharmacological ligand that can specifically activate the beneficial signaling event can lead to better therapeutics. Women undergoing infertility treatment often face the challenge of ovarian hyperstimulation syndrome, which can be, at times, life threatening. If we can design an allosteric modulator that does not have this undesirable effect, it would be a great boon to women going through infertility treatment, in addition to providing a more convenient route of administration. However, we still need to have a better understanding of the basic biology of the receptor. It is well established that FSHR signals through multiple signaling pathways; however, it is still not known which are critical for folliculogenesis vs ovarian hyperstimulation syndrome. With the availability of several new families of small-molecule allosteric modulators, we can address these challenging questions to help us better understand the basic biology of FSHR. Further, availability of several new reliable and quantitative assays to measure different coupling mechanisms including oligomerization of the receptor, internalization, and adaptor protein modulation will prove to be valuable tools in this endeavor. Understanding the multiple dimensions of

FSHR is in the horizon, and it is an exciting time to further explore this promising avenue for drug discovery and FSHR signaling.

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