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Are circulating gonadotropin isoforms naturally occurring biased agonists? Basic and therapeutic implications

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Abstract The gonadotropins, luteinizing hormone, human chorionic gonadotropin and follicle-stimulating hormone, are key regulators of reproduction. As a result of this function, they have been the focus of research for many years. Isolated or recombinant proteins have been successfully used therapeutically for the treatment of infertility; and, in the case of compounds that block gonadotropin activity, for their potential utility in contraception. Until recently, selective small molecules modulating gonadotropin receptor activity have proven difficult to identify. The gonadotropins are glycoproteins that are released into the plasma as differently glycosylated isoforms and bind to specific G protein-coupled receptors. The degree of glycosylation on the gonadotropins has been shown to be important for the biological activities of these hormones and is differentially regulated depending on the steroidal status. Recent data from the study of glycosylated variants of LH, hCG and FSH have revealed that these isoforms have distinct signaling properties that allow for gonadotropin pleiotropic signals to be transduced effectively at the level of the receptor. Thus, glycosylated variants of the gonadotropins behave as biased agonists. Recently, newly developed, small molecule, synthetic allosteric compounds have been identified that are capable of mimicking this biased signaling. This opens the door to development of orally available, drug-like therapies for reproductive disorders that offer similar pleiotropic richness as that offered by the complex, endogenous hormones.

Keywords GPCR · FSH · LH · Biased agonist · Infertility · Ovulation induction · Contraception

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1 Introduction

In all species the drive to survive and reproduce are prime stimuli for expression of defined behaviors. Thus, the search for food and mates represent major investments in time for those individuals of reproductive age. This has resulted, through evolution, in an intricately balanced internal system that strictly regulates the metabolism/production of energy as well as reproductive competencies. Indeed, these two necessary systems have been shown to be intimately associated. In order to tightly regulate these systems, nature has evolved a family of hormonal mediators that act to coordinate external (environmental) and internal (physiological) cues to provide homeostasis and a suitable physiological state for continuation of the species. Some of the key players in the physiological control of metabolism and reproduction are the glycoprotein hormones: luteinizing hormone (LH), human chorionic gonadotropin (hCG), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH) [1].

Three of these hormones (LH, FSH, TSH) are synthesized and released from the anterior pituitary. Human chorionic gonadotropin is synthesized in the placenta during pregnancy. Regardless of their source, the glycoprotein hormones are released as heterodimeric proteins that possess a common alpha subunit but have hormone-specific beta subunits. They are also post-translationally modified via glycosylation, and the level of glycosylation seems crucial to their physiological

actions [2, 3]. The gonadotropins (LH, hCG and FSH) have been shown to circulate as a series of glycosylated isoforms that vary in their complexity of glycosylation. Understanding the role of glycosylation in the physiological function of glycoprotein isohormones has been a focus of research for nearly four decades. However, the full appreciation of the potential of this post-translational modification has not been achieved until relatively recently. A full discussion of the role of glycosylation is out of scope of this review but it is important to describe, in general, the importance of post-translational processing on the function of these hormones; particularly since the glycosylation pattern has a profound effect on the biology of the gonadotropins. Tight regulation of glycosylation of gonadotropins suggests an important physiological role for the presence of glycosylated variants of LH, hCG and FSH [4–9]. However, the understanding how this modification conveys specific attributes to the function of the hormones is not without controversy. Conflicting data have existed in this field with several investigators reporting no effect of glycosylation on binding [10–12], but with others noting heightened receptor affinity by deglycosylated species [13, 14]. More recent work suggests that glycosylation plays an important role in determining the three-dimensional conformation of these ligands, and thus, potentially affects the interaction between hormone and receptor [15]. *In vivo*, glycosylation of the gonadotropins has been shown to be physiologically important to plasma half-life and immunoreactivity. Hyperglycosylated variants of hCG have been associated with trophoblast invasion and have been suggested as useful early markers for the occurrence of Down's Syndrome in fetuses [16].

The bioactivity of various glycosylated isoforms of the gonadotropins has been appreciated for some time. All three gonadotropins have displayed increased biological activity of more basic forms of the hormone (as determined by isoelectric focusing and apparent pI) compared to more acidic isoforms. This may be reflective of the higher affinity of the less glycosylated forms of LH, hCG and FSH for their receptors, but this has not been shown definitively. Interestingly though, glycosylation-dependent changes in the three dimensional conformation of the hormone support the view that glycosylation plays a crucial role in determining how the receptors interact with their gonadotropin ligands [13, 17].

Gonadotropin action is mediated at the target cell surface by gonadotropin-specific receptors that are members of the Class A, rhodopsin-like, G protein-coupled receptor (GPCR) family [18]. Gonadotropin receptors are unique among this family of GPCRs in that they possess long extracellular domains (>300 amino acids), which are required for binding of ligand; and relatively short, cytoplasmic carboxy-terminal tails [19, 20] involved in intracellular signaling. The extracellular domains of gonadotropin receptors are characterized by numerous leucine rich repeats that have been shown to be important for binding of the respective ligands to the receptors [20]. Along with the thyroid stimulating hormone receptor (TSHR), the relaxin receptors and three orphan receptors (LGR4, 5 and 6), the gonadotropin receptors comprise a sub-family of Class A GPCRs containing these leucine rich repeat structural motifs [21]. Similar to their hormone ligands, the luteinizing hormone / human chorionic gonadotropin receptor (LH/hCGR) and follicle-stimulating hormone receptor (FSHR) also contain sugar residues on their extracellular domains. Elegant studies, using mutation of the Asp residues on which glycosylation occurs, have determined that glycosylation is responsible for proper folding of the receptor during protein synthesis [22, 23]. This role is more apparent for the FSHR than the LH/hCGR [24]. Interestingly, and in contrast to the gonadotropins, the glycosylation states of the receptors do not seem to have an effect on ligand binding affinity or activation of signal transduction pathways [25].

The discovery of association of glycoprotein hormone receptors with each other (oligomerization) [26–30] in the context of adapter and scaffolding proteins [31–34] and the understanding of biased signaling have put into better perspective earlier observations concerning promiscuous signaling of these receptors, and provide the basis for a physiological explanation of the observed experimental phenomena. It is now well documented that most GPCRs have the capability of signaling *via* multiple pathways in a given cell type. For many years, this hypothesis was poorly understood and was thought to be an artifact of recombinant cell systems despite the many receptors that demonstrated such behavior in primary cell systems as well [35]. With the recent development of allosteric agonists, antagonists and modulators to GPCRs, more light has been shed on this concept; e.g., direct targeting of specific signaling pathways has been demonstrated for various ligands [36–38]. This phenomenon has most recently been termed, biased signaling [Fig. 1; [38]]. Simply put, the concept of biased signaling describes the ability of ligands to direct specific and distinct biological responses *via* activation of select signaling pathways in a ligand-specific manner. There are many receptors that are known to associate with multiple naturally occurring ligands. Since varying glycosylation of gonadotropin isoforms is known to alter their physicochemical properties, one can consider gonadotropin isohormones as different ligands with potentially subtle, but unique association with their cognate receptors [36]. In addition, interaction of these diverse ligands with the receptor would result in multiple ligand-receptor conformations, which in turn lead to the observed activation of differing biological signaling pathways for LH, hCG and FSH [13, 27, 39]. Thus, it may be possible to envision gonadotropin isoforms as potential, naturally occurring

biased agonists for their receptors [36, 37]. Taken together, recent revelations concerning gonadotropin action add another level of complexity to earlier observations concerning signaling *via* these receptors. These data have provided new mechanistic insights into the basis for observed phenomena, highlighting the level of fine tuning possible with these complex signaling cascades and allow for a variety of mechanisms that may lead to pleiotropic signaling of the glycosylated isoforms of gonadotropins.

2 Gonadotropin receptors activate multiple signaling pathways

The concept that agonists and antagonists of a given receptor could selectively activate specific portions of a receptor's full repertoire of signaling arose in the 90's with the identification of promiscuous activation of signal transduction pathways by GPCRs [36, 38, 40–43]. These pioneering observations revealed new properties of G protein-coupled receptors reflecting additional pleiotropism to signaling and began a new era dedicated to further understanding the complexities of signal transduction for this family of receptors.

The molecular basis for biased agonism lies in the stabilization of conformation(s) of the receptor which increases the affinity of the biased agonist-receptor complex for a distinct and specific signaling pathway over another [Fig. 1; [44]]. Since GPCRs primarily utilize G proteins as signal transducers, biased agonism would imply ligand-dependent preference of the ligand-receptor complex for a specific G-protein over another. Since GPCR signaling is not exclusive *via* G-proteins, biased agonism is not restricted to G-protein signaling, and recent descriptions of biased ligand-mediated activation of non-G-protein-dependent signaling of GPCRs have appeared, such as is the case with β-arrestin signaling. Initially, ligand-dependent recruitment and association of ligand-receptor complexes with β-arrestin was thought to represent a feedback mechanism; whereby, by desensitizing the receptor-ligand complex response, the cell could protect itself from chronic activation of the GPCR. However, recent studies by a number of groups have demonstrated that desensitization only represents the tip of the iceberg in arrestin signaling, as β-arrestin is capable of activating a variety of signal transduction pathways. For example, β-arrestin signaling provides another mechanistic link between GPCRs and direct ERK activation [45–48]. GPCR/ERK cross-talk has been described for several different receptors when activated by synthetic allosteric agonists, or in interactions of the receptor with antagonists or inverse agonists. Natural ligands can also induce this type of cross-talk between GPCRs and ERK signaling cascades through different modes including G-proteins and trans-activation, in which activation of a GPCR stimulates

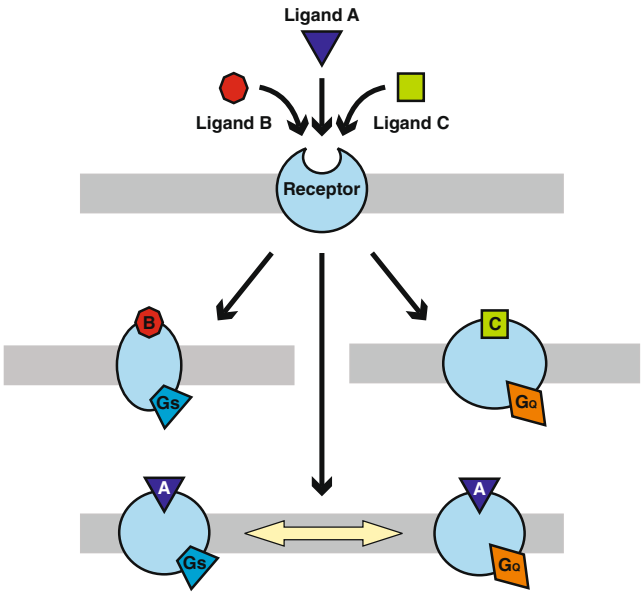

Fig. 1 A schematic diagram of biased signaling at a GPCR. Three ligands interact with the same receptor with similar or different affinities. Each ligand stabilizes the receptor in a different conformation that results in diverse affinities of the ligand-receptor complex for association with different G-protein transducers. Some ligands can activate limited signaling pathways (Ligands B and C), while others are capable of activating multiple pathways (Ligand A). Activation of ligand-specific signaling pathways leads to ligand-specific, distinct biological activities

signaling by tyrosine kinase receptors through phosphorylation [49, 50]. Indeed, both the CCK [51] and EP4 receptors [52] have been shown to signal preferentially *via* different signaling pathways following binding of different native ligands leading to multiple biological responses that are specific to the ligand. In the case of CCK, the prohormone, CCK-58, displayed differential signaling and functional activities in pancreatic acinar cells and in gall bladder from those observed with CCK-8. CCK-8 was thought to be the relevant biologically active form of CCK, since CCK-8 and CCK-33 show little difference in their biological properties. However, CCK-58 demonstrated additional biological activities *in vivo* than CCK-8 including differential binding characteristics at the CCK-A receptor depending on the cell type analyzed [53]. These data suggest a cell context dependent biased agonism of the CCK-A receptor to two endogenous ligands [51]. Similar observations have been made for the EP4 receptor, where different natural prostaglandin ligands for the EP4 receptor were shown to have preferential associations with different G protein α subunits using bioluminescence energy transfer (BRET) [54]. These ligands displayed full agonism for the various signaling pathways activated although PGD2 showed significantly less ability to activate β-arrestin recruitment.

Upon ligand binding, the gonadotropin/gonadotropin receptor complex interacts predominantly with the Gs

signaling pathway [5]. This leads to the subsequent activation of adenylate cyclase resulting in an increase in intracellular cAMP levels [55–57]. It is clear today that this view of signaling for gonadotropins is simplistic, and several investigators have observed that the gonadotropin receptors also transduce signaling *via* other pathways (Fig. 2a). The LH/hCG receptor is known to activate both adenylate cyclase activity and IP3 production *in vitro* [15, 58, 59]. Similarly, the FSHR has been shown to activate not only Gs, but also Gi [36] and the IP3 signaling cascade [60, 61]. Some have suggested previously that promiscuity of gonadotropin receptors represented an artifact of the *in vitro*

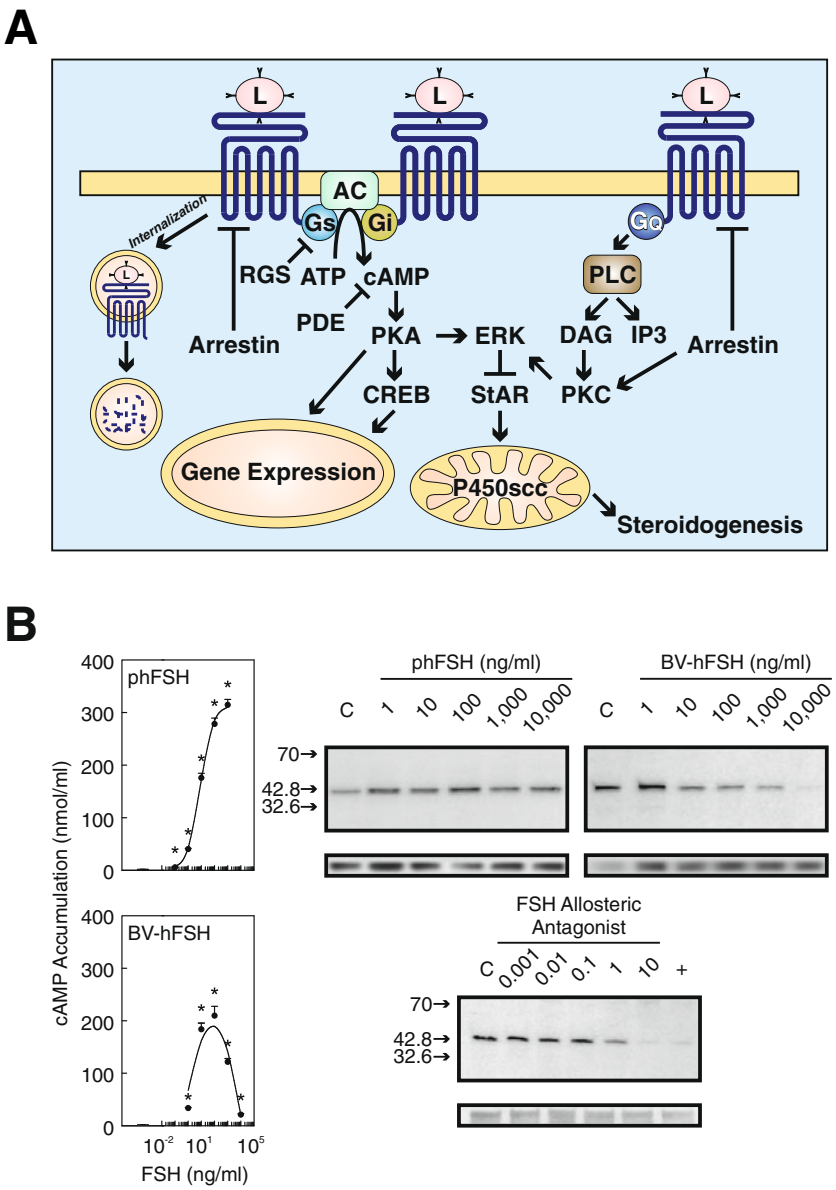

Fig. 2 Panel (**a**). Gonadotropin receptors activate multiple signaling pathways. Both FSH and LH have been shown to primarily activate adenylate cyclase (AC) *via* interactions with Gs. In addition, they also activate phospholipase C (PLC) through association with Gq. FSH has also been shown to signal through Gi depending on the glycosylated variant bound to the receptor, and the degree of glycosylation seems to be finely tuned by the hormonal status. Activation of these pathways leads ultimately to regulation of gene expression (e.g., aromatase) and steroidogenesis. The recruitment of β-arrestin to the ligand-bound receptor leads to receptor internalization, thus dampening the stimulus. Panel (**b**). Glycosylated variants of FSH have different biological activities. Highly glycosylated, sialylated purified pituitary human FSH (phFSH) increases cAMP production inducing a sigmoidal dose-response curve [36]. In contrast, the less complex glycosylated variant (BV-hFSH) increases cAMP levels inducing a bell-shaped dose-response relationship. The down-turn in the dose-response curve with BV-hFSH, is dependent on activation of Gi as measured by an ADP-ribosylation assay [37]. This property of BV-hFSH is recapitulated with an allosteric biased agonist (concentrations in μM) that appears as a functional antagonist in cell-based assays. Figures reprinted from [36, 37], with permission. Figure from (36) *Copyright 1997, The Endocrine Society*, Figure from (37) *Copyright 2008, Academic Press*

system used to uncover these activities [35]. However, activation of multiple signaling cascades occurs not only in engineered cells, but also in endogenous tissues. For instance, the FSHR activation of the Gi signaling pathway is observed not only in CHO cells [36], but also in primary granulosa cells [36] and primary osteoclasts [62]. Furthermore, whereas the ability of gonadotropin receptors to activate various signaling pathways differs, each receptor displays a preference for the Gs signaling pathway. However, in each case, the activation of secondary signaling mechanisms also occurs under physiological conditions and within the physiological range of plasma hormone concentrations. This is supported by recent reports of ligand-induced selectivity in β-arrestin signaling and ERK activation by the FSHR. These observations demonstrated quite clearly the ability for biased signaling of the gonadotropin receptors [63]. Overall, these observations suggest that gonadotropin receptors, like many other GPCRs [44], induce a complex pattern of cell activation upon binding to their respective ligand even within the physiological context.

For many years, FSH has been used as a model to understand the role of glycosylation in determining glycoprotein hormone function. Several years ago, we noted that differently glycosylated variants of hFSH could induce activation of both the Gs and Gi signaling pathways [36, 37]. The phenomenon appeared as a bell-shaped concentration-response curve in *in vitro* assay systems for insect cell expressed hFSH (BV-hFSH) (see bottom-left panel in Fig. 2b). While initially puzzling, we devised a series of experiments to try to understand the mechanisms underpinning this bell-shaped concentration-response curve. Using pertussis toxin, we were able to block the down-turn in the dose-response relationship, indicating that the descending phase of the curve for the BV-hFSH was due to activation of Gi at higher concentrations of the hormone [36]. These pharmacological relationships had been described previously for other receptors such as the catacholamines and adenosine receptors [40–42], but the situation was unique for the gonadotropins, since the ligands were simply different in their glycosylation pattern. In the case of the insect cell expressed hFSH (BV-hFSH in Fig. 2b), glycosylation was terminated at short branched mannose residues, and the protein displayed a more basic migration pattern in chromatofocusing (Arey, unpublished data). This different pattern of glycosylation in BV-hFSH is due to the nature of the glycosylation machinery of the recombinant system used for protein expression [64]. Subsequent experiments using an ADP-ribosylation assay, along with immunoprecipitation and Western blotting of specific G-proteins, revealed that these pharmacological responses were definitively associated with activation of specific G-proteins [Fig. 2b, [37]]. Moreover, these responses are physiologically relevant as they exist in the animal. When administered to immature female rats, both purified pituitary hFSH (phFSH) and BV-hFSH produced similar pharmacological profiles as those observed *in vitro* (e.g., sigmoidal dose-dependent activation for phFSH, left panel of Fig. 3; and bell-shaped dose-response for the BV-hFSH middle panel of Fig. 3). When added together, however, BV-hFSH behaved as an antagonist of the native purified material as shown in the right panel of Fig. 3. These effects, when the hormones were administered alone or in combination, completely recapitulated the observed effects *in vitro*, both in the recombinant receptor overexpressing system and in primary cells [36]. It is important to emphasize that *in vivo*, we measure an integrated response such as the weight of two organs, the ovary and the uterus (see Fig. 3). These data demonstrate that the activities observed in signaling are directly translated into organ growth responses and illustrate the ability of the biased ligand (e.g., BV-hFSH) to elicit a different response pattern than that of the native ligand (phFSH). However, these differential responses were not evident for all glycosylated variants, as we had tested several variants that did not exhibit dose-response relationships similar to the BV-hFSH and deglycosylated hFSH (Arey, data not shown). These observations were subsequently confirmed by others [15, 63]. Interestingly, similar glycosylation-dependent signal biasing has been noted for other secreted glycoproteins [e.g. IL22 and BMP6, [65, 66]] including LH/hCG [11, 15]. Ligand glycosylation has also been suggested to be required for LH/hCG receptor dimerization [15, 28]. In the case of BMP6, detailed mutagenesis around key asparagine residues has revealed the importance of glycosylation in interactions with its receptor [66]. In the case of IL22, a single fucose residue on Asn54 was shown to be required for full efficacy of the cytokine at its receptor. It is worth mentioning that the binding kinetics of the receptor were altered by more complex glycosylation at this site [65]. Similar but more dramatic effects of glycosylation on binding kinetics have also been noted for erythropoeitin [67]. These examples lay the foundation for the concept that a variety of related natural ligands talk to the receptor by inducing specific receptor conformations and that glycosylation plays a role in aiding in this stabilization, thus transducing specific signals that are unique to a given physiological state. Similar activities were ultimately discovered for other GPCRs [44]. Therefore, from a mechanistic viewpoint, there is strong support for the notion that alteration of the glycosylation pattern on glycoprotein hormones leads to biased ligands that direct activation of one signaling pathway over another. The data support the notion that more basic isoforms of the gonadotropins bind with a higher affinity but are “less” bioactive. This may be due to different signaling than that induced by more sialylated acidic isoforms that may

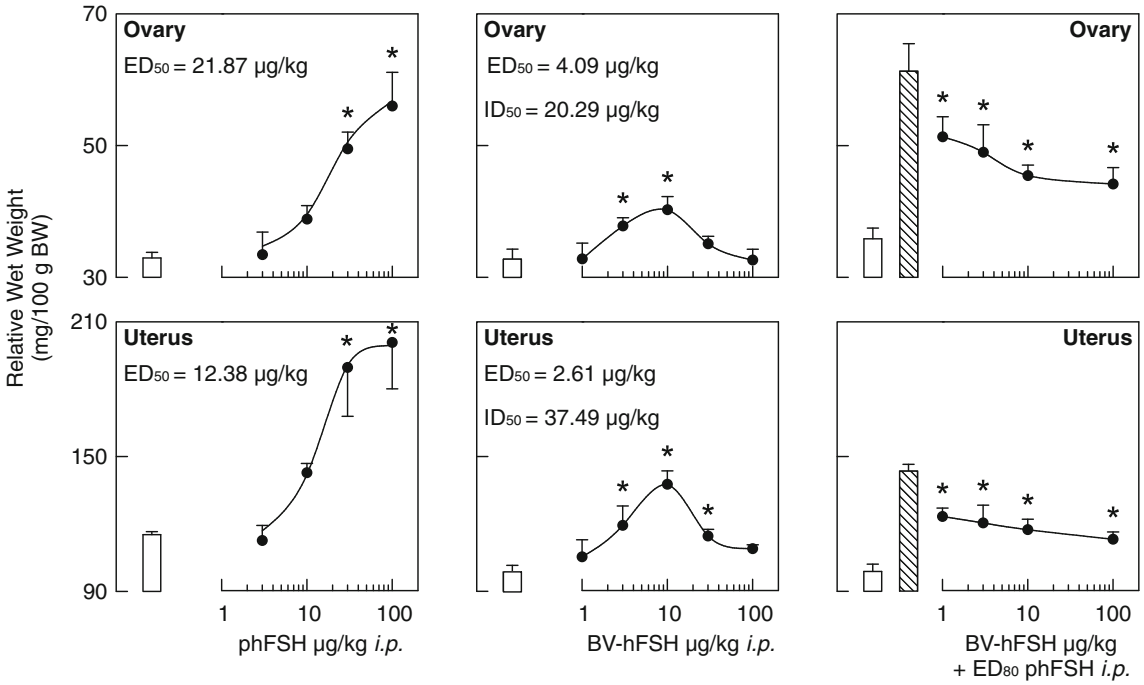

Fig. 3 Differently glycosylated variants of FSH produce markedly diverse activity on ovarian and uterine tissue weights *in vivo*. Immature female rats were treated *i.p.* with increasing doses of highly glycosylated purified pituitary hFSH (phFSH) or less glycosylated recombinant hFSH (BV-hFSH). The two preparations induce *in vivo* dose-response relationships similar to their *in vitro* bioactivities. Partial agonist activity of BV-hFSH is apparent in the center-top and -bottom panels. Treatment of immature rats with the ED_{80} of phFSH+ increasing doses of BV-hFSH reveals the inhibitory nature of BV-hFSH under high receptor activation conditions (right top and bottom panels, hatched bars are response to ED80 of phFSH alone), recapitulating *in vitro* bioactivity observed when tested under a similar pharmacological paradigm [36]

produce perceived differences in bioactivity. Put in terms of our model of biased signaling, different glycosylated variants interact with the receptor in subtle, but unique ways to result in different signaling and biological responses as noted in Fig. 4. There are data supporting the notion that the degree of glycosylation of gonadotropins is regulated by gonadal steroids [4, 60, 68, 69]. Furthermore, in menopausal women a higher predominance of more acidic FSH isoforms (more bioactive) has been demonstrated as compared with women of reproductive age [70, 71]. A similar phenomenon has been noted for hCG, where the presence of differently glycosylated isoforms of this gonadotropin changes with the stage of pregnancy [72]. Overall, the sum of the data indicate that receptor signaling has evolved to convey complex regulatory signals in response to varying ligands which are dynamically adjusted to accommodate to external/internal influences and ultimately maintain homeostasis.

3 Synthetic small molecule FSH agonists mimic biased signaling of FSH isoforms

One of the obvious implications of the observations described earlier is the impact they may have on the development of selective biased agonists/antagonists of the gonadotropin receptors for therapeutic uses. With the onset of improved screening technology, the application of new synthetic mechanisms and access to larger, more diverse chemical libraries, the last decade has seen numerous reports describing the identification of novel chemical series of synthetic small molecules modulating activity of gonadotropin receptors. Interestingly, these small molecule synthetic ligands have been found to possess a range of pharmacological activities; however, they are devoid of competitive binding with the gonadotropins themselves [73]. Therefore, these agents do not appear to alter the orthosteric site, eliciting their modulatory activities *via* allosteric sites present in the receptor.

The top row of molecules in Fig. 5 illustrates the chemical structures of some of the most notable synthetic agonists recently described for the gonadotropin receptors. These representative structures have been optimized, in most cases, from lower potency compounds that were identified in screening of large chemical libraries. The exception to this was the identification of the thiazolidinone class of FSHR agonists, which were first identified using a combinatorial chemistry approach [74]. In all cases, these compounds were optimized leading to improved potency analogs that approached the single digit nanomolar (nM)

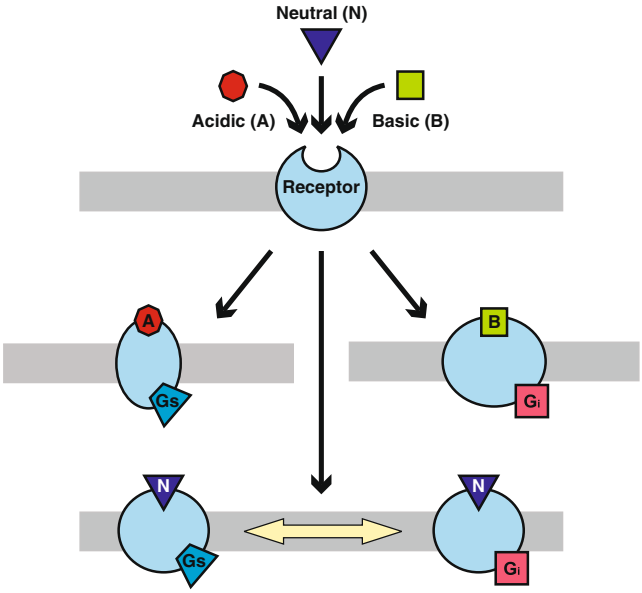

Fig. 4 A schematic diagram of potential biased signaling induced by different glycosylated isoforms of gonadotropins. More acidic (sialylated) isoforms direct signaling *via* the primary second messenger system driven by Gs association. However, neutral and more basic isoforms interact with the same receptor differentially. Each ligand stabilizes the receptor into a different conformation that results in diverse affinities of the ligand-receptor complex for association with different G-protein transducers. Activation of ligand-specific signaling pathways leads to distinct biological activities

range. The thiazolidinone compounds were further optimized for selectivity *versus* the other glycoprotein hormone receptors, whereas the pyrazole and thienopyridine/-pyrimidine chemotypes achieved less selectivity. The thienopyridine/-pyrimidine class of LH/TSH agonists were reported to have moderate potency *in vitro* with the majority of analogs possessing EC_{50}s in the range of 100–500 nM [75]. However, Org-41841 demonstrated significantly improved potency to other members of this class with an EC_{50} of 20 nM [75]. Furthermore, Org-41841 was also found to induce ovulation in 40% of animals when administered at 50 mg/kg. Recently, Jorand-Lebrun *et al.* [76] have reported a series of pyrazole compounds that act as LHR and LHR/FSHR partial agonists. The pyrazole LHR/FSHR agonist showed an approximate 7-fold selectivity for the LHR over the FSHR [77]. Interestingly, the efficacy reported for the LH and FSH related activities of this compound were slightly greater (73%) for the FSHR as compared to the efficacy for this compound at the LH receptor (53%), despite preferential LHR potency *in vitro*. This compound also demonstrated *in vivo* efficacy in a model of LH-induced testosterone production [76]. When administered intraperitoneally, the pyrazole compound induced an approximate 5-fold increase in serum testosterone levels. More recent efforts around the Org-41841 led to the identification of an LH selective agent (Org-43453), which has been used for induction of ovulation in humans ([78]; see a more detailed description below).

Perhaps the most potent synthetic agonists of these receptors are those identified for the FSHR. These molecules have been optimized to low nM and, in some cases, high pM potency for activation of the FSHR not only in CHO cells expressing the hFSHR, but also in primary cultures of rat granulosa cells [37, 74]. The optimization of these chemical series to high potency and efficacy represents a significant achievement if one considers the large discrepancy in molecular weights between the native

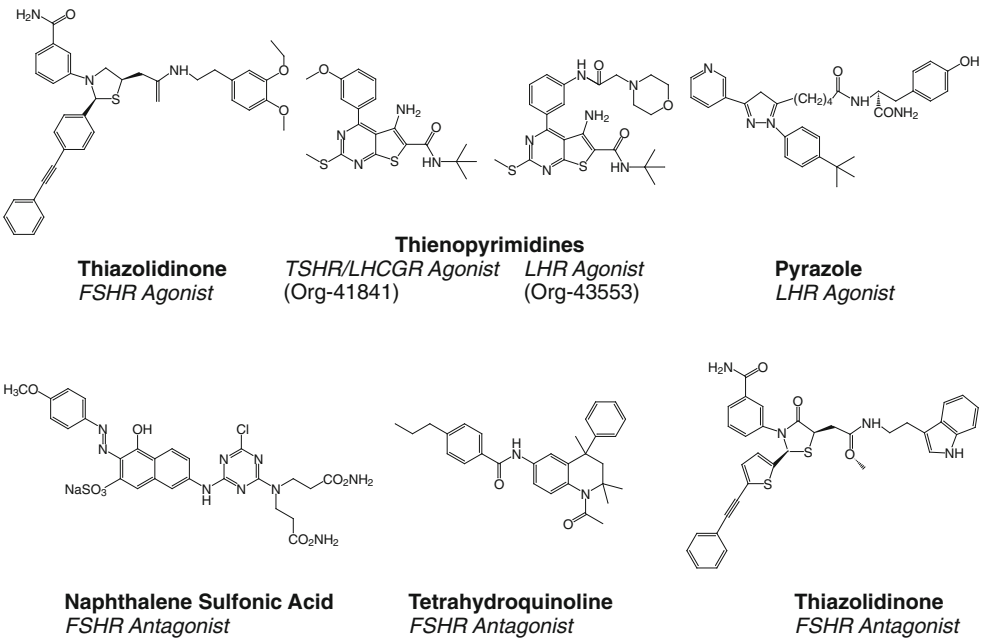

Fig. 5 Representative allosteric agonists (top) and antagonists (bottom) of the gonadotropin receptors derived from several different core structures. These agents show different degrees of selectivity among the related glycoprotein hormone receptors as identified in the figure and all show the appropriate activity when tested *in vitro*

ligand (MW of FSH~30kD) and these new chemical entities (in the 500 Da range). In addition to exhibiting high potency and behaving as full agonists when compared with the native hormone, some of these compounds have been found to activate specific signaling pathways, demonstrating biased signaling [37]. Therefore, these data demonstrate that one can design small molecules capable of activating full efficacy in terms of primary signaling of a receptor with a large protein ligand. More importantly, these observations indicate that the pleiotropism conveyed by the complex native ligand can also be recapitulated and modulated at will with these small molecules.

Representative antagonists to gonadotropins that have been reported to date are shown at the bottom of Fig. 5. Synthetic, small molecule antagonists of gonadotropin receptors have been only reported for the FSHR. A number of different chemotypes have been identified including the naphthalene, sulfonic acid chemotype and the stilbene (bis) sulfonic acid chemotype [79, 80]. Neither one of these chemotypes were found to be highly potent (IC_{50}s of approximately 1 μM in cAMP and aromatase assays), although they do demonstrate non-competitive receptor binding characteristics in experiments with iodinated phFSH [79]. The naphthalene, sulfonic acid compounds demonstrated fairly good selectivity for the FSHR with very little competition for binding at either the LHR or TSHR when studied up to 100 μM [79]. The stilbene (bis) sulfonic acids series were less selective demonstrating approximately 30- to 40-fold selectivity for the FSHR over the TSHR in a cAMP bioassay [80]. Interestingly, binding studies using an immobilized FSHR extracellular domain revealed that both of these chemotypes utilized a binding site within the extracellular domain that is unique from that present in the FSHR [79].

When tested in animals studies, both chemotypes were found to possess *in vivo* activity. The naphthalene sulfonic acid series dose-dependently inhibited ovulation in female rats treated for 4 days, achieving 100% contraception at 100 mg/kg. Similar efficacy has been observed with the stilbene (bis) sulfonic acids as well [80]. These compounds provided the first proof-of-concept for the development of non-peptide, small molecule antagonists to the FSHR for use as contraceptive agents. More recently, van Straten *et al.* [81] have described the synthesis of a new class of more lipophilic, tetrahydroquinoline FSHR antagonists. These compounds possess better potency as compared to the naphthalene sulfonic acid and stilbene (bis) sulfonic acid classes of antagonists with IC_{50}s ca. 10 nM in cAMP assays. Using a similar approach as Yanofsky *et al.* [74], van Koppen *et al.* [82] demonstrated that the thienopyrimidine class of LH/hCGR allosteric modulators interact in a similar region as the thiazolidinone FSH allosteric modulators and the pyridine/pyrimidine class of calcium-sensing receptor antagonists (the P2 pocket [73]) on their respective receptors. This confirms that the P2 pocket of GPCRs is an important site for directing therapeutically relevant functional agonists and antagonists [73, 83, 84]. These data suggest that these compounds are negative allosteric modulators/allosteric antagonists of the FSHR. We have also recently described additional allosteric antagonists that are analogs of the thiazolidinone class of FSHR agonists [37]. These agents are completely devoid of agonistic activity, but dose-dependently inhibit FSH-induced cAMP production and steroidogenesis *in vitro* with sub-micromolar potencies and 100% efficacy. Mechanistic studies revealed these compounds selectively activate the Gi-coupled pathways with a concentration—response relationship that parallels their effects on cAMP production in cells expressing the hFSHR (Fig. 2b; [37]), while demonstrating no ability to activate adenylate cyclase activity. Therefore, they behave as allosteric biased agonists that are perceived as functional antagonists, since they counteract the primary signaling mechanism of the receptor induced by the endogenous ligand. Since other analogs demonstrated strongly increased cAMP production in both whole cells and cell membranes, we studied whether the agents inhibited the labeling of Gi in CHO cells expressing the hFSHR. Similar to insect cell expressed hFSH, we found that certain thiazolidinone analogs were capable of activation of Gi (see bottom gel in panel B of Fig. 2; [37]). The ability of thiazolidinone analogs to block PTX-dependent ribosylation of Gi was correlated with their respective IC_{50}s in reducing cAMP accumulation. Thus, it has been proposed that these thiazolidinone analogs behave as allosteric antagonists of FSH *via* activation of a biased signal that directly competes (*i.e.* through Gi activation) with the production of cAMP induced by the cognate ligand [37]. Therefore, these agents are better defined as functional antagonists of the FSHR and can be seen as being pharmacologically independent from negative allosteric modulators, which act to reduce the affinity of the natural ligand for its receptor.

In addition to allosteric agonists and antagonists, thiazolidinone compounds that demonstrate partial agonist activity [37] have also been described. These partial agonists display a pharmacological profile that is a mix of both agonistic and antagonistic properties, resulting in bell-shaped dose response curves when evaluated in assays measuring signaling (cAMP accumulation) and steroidogenesis (estradiol accumulation). Although the maximal efficacy of these compounds is somewhat lower than full agonists of the thiazolidinone chemotype (50-60%), the agonist potencies for these compounds are in the same range as those for the full agonists. The mechanism of this observed partial agonism involves, at least in part, activation of multiple G-protein signaling pathways. That

is, at lower concentrations these compounds preferentially activate the Gs signaling pathway, whereas at concentrations above 100 nM these compounds increasingly activate the Gi signaling pathway. The result is a dose-response curve that reflects both of these activities in terms of downstream effectors (e.g. cAMP) and is similar to the activity of differently glycosylated isoforms of FSH [36, 37]. Whether these molecules are capable of activating the IP3 signaling pathway or other cascades associated with FSH signal transduction is not currently known. Overall, these observations demonstrate that one can design allosteric modulators of the same core structure that can selectively target signaling pathways associated with a given receptor producing biased signaling [37, 73]. Since these compounds activate the receptor in different ways, we refer to them pharmacologically as allosteric biased agonists, but they can be perceived as functional agonists and antagonists in order to reflect the overall effects they have on the biology of the target cell. It is not hard to imagine that such compounds could provide an improved selectivity and control in affecting the pharmacology needed in a therapeutic setting and usher in a new era in terms of improved drug efficacy and safety profiles.

4 Therapeutic uses for modulators of gonadotropin action

The concept of the pituitary as a master regulator of sexual function and, the demonstration of two pituitary gonadotropic entities, one enhancing follicular development and the other responsible for follicular rupture and the formation of the corpus luteum is now over 80 years old [85–88]. These classical studies established the basis for today's therapeutic application of gonadotropins, i.e., the induction of ovulation. In addition, they laid the groundwork for novel contraceptive therapies targeting the gonadotropin receptors.

Today gonadotropin treatment for induction of ovulation is indicated in anovulatory infertile women with hypogonadotropic amenorrhea and those with polycystic ovary syndrome that have failed to respond to less elaborate treatments for induction; the goal of the treatment being the development of a single mature follicle [89]. In these women, optimal clinical outcomes are obtained by concomitant administration of FSH and LH [89, 90], administration of hMG [91] or a combination of FSH and either recombinant LH [92, 93] or a low dose of hCG [94]. Once optimal follicular development has been achieved, induction of ovulation can be elicited by a variety of methods including administration of hCG either recombinant in origin (250 μg SC) or derived from urine (5,000–10,000 IU IM or SC), recombinant LH or even GnRH administration [89]. Further support of corpus luteum function is often needed, and this is accomplished by either administering progesterone or with a low dose of hCG (1,500–2,500 IU every 3–4 days). Potential complications of gonadotropin treatment are related to administration of higher doses than those required for the particular ovarian sensitivity, which may lead to superovulation, multiple pregnancy and the ovarian hyperstimulation syndrome. The latter consists of rapid ovarian enlargement and intraperitoneal effusion. In serious cases, hyperstimulation syndrome can result in renal failure, thromboembolic phenomena, adult respiratory distress syndrome and occasionally death [95]. hCG has a long half-life and this long duration of action has been associated with a higher risk for ovarian hyperstimulation syndrome [96]. In this respect, ovarian hyperstimulation syndrome has not been reported in patients treated with doses of 30,000 IU (this dose is equivalent to the 5,000 IU hCG used routinely) of recombinant LH [97]. Allosteric agonists and partial agonists could provide potential improvements over existing therapy through their ability to activate biased signals and potentially reduce the incidence of ovarian hyperstimulation syndrome. Furthermore, since different synthetic molecules naturally possess unique pharmacokinetic profiles, these molecules could ideally be designed with pharmacokinetics that optimize efficacy, while broadening the therapeutic index.

Another important consideration in ovulation induction paradigms is the need for parenteral, daily administration of the various gonadotropin preparations due to their lack of oral bioavailability. In itself, the need for daily injections comes associated with important compliance issues, and therefore, patients under ovulation induction regimes may favor oral therapies that mimic gonadotropin activity. Org-43553 (Figs. 5 and 6), a thienopyrimidine, is a low molecular weight LH receptor selective agonist (activity at the FSH and TSH receptors is detected with EC50s of 110 and 3000 nM, respectively [98]) with a Ki for the LH receptor of 3.3 nM [99]. In an engineered system *in vitro*, Org-43553 enhances cAMP production with an EC_{50} of 1.7 nM, while recombinant LH EC_{50} is 0.078 nM [99]. Binding of the compound to the LH receptor appears to be non-competitive, as addition of the compound in the presence of 70 nM of recombinant LH results in no change of the K_D, but a reduction of 24% in the B_{MAX} [99]. These observations suggest that the activity of the compound does not occur at the orthosteric site. In humans, a single oral dose of 300 mg Org-43553 induces ovulation [78]. This compound, therefore, would offer an oral alternative to current ovulation induction paradigms. In addition, because of its shorter half-life [ca. 3.4 hours after i.v. injection [98]] than hCG, which is used to elicit follicular rupture and ovulation, administration of Org-43553 may result in a reduced incidence of ovarian hyperstimulation syndrome [98].

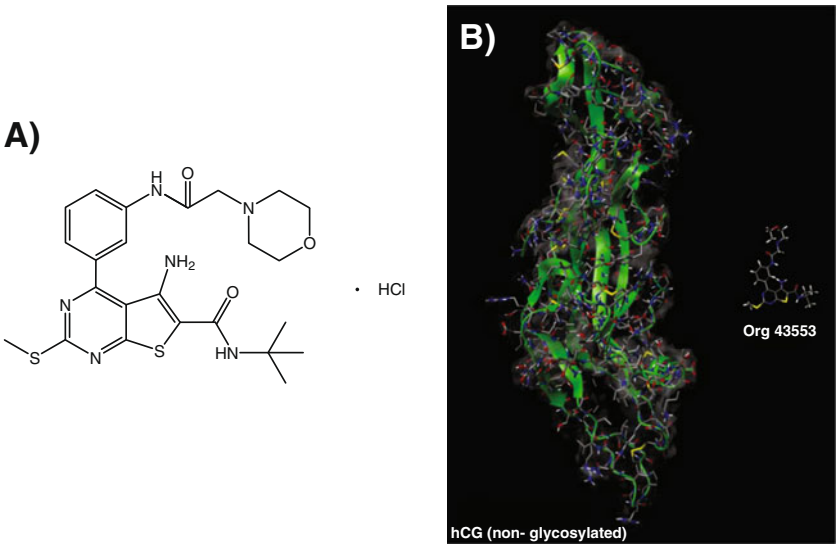

Fig. 6 Structure of Org-43553 (**a**), a small molecule (MW 515) LH/hCG receptor agonist that is effective in inducing ovulation. (**b**) Crystal structure of hCG and structural model for Org-43553. Notice the difference in size. Reprinted from [98], with permission

Org-43553 provides an alternative to recombinant LH or hCG in ovulation induction paradigms; however, FSH is also required to induce follicular maturation and development. In this respect, we have also described small molecules that selectively activate the FSH receptor (see Fig. 4, left structure in the upper row). This thiazolidinone compound induces cAMP production in a FSH receptor-expressing cell line with an EC_{50} of 2 nM and it is also active in primary cultures of rat ovarian granulosa cells by increasing estradiol secretion with an EC_{50} of 10.5 nM [74]. The small molecule FSH receptor agonists do not interact with the orthosteric site of the FSH receptor. Although, these compounds were not optimized for oral bioavailability, they provide proof-of-concept for the development of small molecule biased agonists to the FSHR. Similar to biased agonists of the LH/hCGR, FSHR biased agonists would potentially provide an opportunity for greater control over responses during assisted reproduction protocols by activating only specific portions of the full receptor signaling repertoire. Allosteric partial agonists that activate multiple conflicting pathways such as the thiazolidinone chemotypes directed to the FSHR could provide a means of protecting against hyperstimulation of receptor function through activation of inhibitory signaling pathways at higher concentrations.

Orally available biased agonists of gonadotropins would also be useful in the treatment of male hypogonadism resulting in infertility. While the use of LH agonists for male hypogonadism would not probably replace testosterone administration, the use of FSH agonists for the treatment of infertility (e.g., enhancement of spermatogenesis) would probably be more reasonable from a physiological point of view [100–102]. However, treatment effectiveness remains controversial (see [103] for a review). In contrast, the use of allosteric biased agonists of gonadotropin receptors for treating individuals with genetic mutations leading to aberrant receptor activity can also potentially be envisioned. In these cases, patients presenting with constitutively activating mutations of gonadotropin receptors may be aided by the administration of a biased allosteric functional antagonist or inverse agonist. Such would be the case in boys diagnosed with gonadotropin independent precocious puberty.

Whereas activation of LH and FSH receptors is useful in reproductive disorders, their blockade could potentially be used as novel approaches to contraception. With respect to blocking FSH action as a contraceptive strategy, the concept is borne out in the literature through reports of naturally occurring mutations in either the hormone or its receptor [104]. Similarly, knockout models have confirmed the importance of FSH in fertility. Inactivating mutations in either the ligand or the receptor leads to decreased fertility or infertility in females [105–108]. The effect of these mutations to render women completely infertile is well documented. However, studies of men with such mutations have led to conflicting reports. It is clear that disruption of the FSH system biological action will at the very least lead to reduced fertility in such subjects, but in some cases only reduced spermatogenesis or partial effects on sperm quality were found [107, 109, 110]. In contrast to these observations, data obtained in knockout mouse models and research in primates in some cases have led to drastic effects on male fertility [111–115]. Independently of their effectiveness as male contraceptives, small molecule FSH receptor blockers will provide a means to further research the role of FSH in spermatogenesis.

In the case of the female, administration of a naphthalene sulfonic acid compound completely inhibited ovulation at the highest dose tested in rats [79]. The inhibition of ovulation does not appear to be related to alterations in the number of or microscopic appearance of ovarian follicles or

corpora lutea. Because histological evidence is lacking on the effect of this agent on folliculogenesis, effects not directly related to the FSH receptor (such as injection-related periovarian inflammation or decreased growth rate) may have indirectly contributed to the ovulation inhibition. However, studies of the *in vitro* efficacy of this naphthalene sulfonic acid compound, taken together with the *in vivo* data, point to the ability of this compound to inhibit ovulation through the FSHR [79].

Overall the availability of small molecule agonists of both the LH and FSH receptor would open new avenues for more effective and simpler ovulation induction protocols. The fact that these agents can be given orally, provides a means to better compliance and ease of administration as well as potentially reduced cost as compared with the preparation of recombinant hormones. Moreover, the discovery of antagonists, particularly against the FSH receptor would allow a better understanding of FSH action on spermatogenesis and folliculogenesis opening the doors to their potential as a novel approach to contraception. This would be of particular novelty in its possible application to male contraception. Lastly, the ability to selectively target one or more signaling pathway(s) with an allosteric biased agonist should provide better control for the physician and an improved safety profile for those patients in need of reproductive therapies.

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