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ORIGINAL PAPER

Allosteric modulators of glycoprotein hormone receptors: discovery and therapeutic potential

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Abstract The glycoprotein hormones, luteinizing hormone, follicle-stimulating hormone and thyroid stimulating hormone, are important regulators of reproductive and metabolic processes. However, because of the nature of their ligand-receptor interactions that contain multiple contact sites, classical small molecule drug discovery strategies have not been successful. However, recent advances in screening and combinatorial chemistry strategies have identified chemical series that act allosterically as positive, negative or mixed modulators of the glycoprotein hormone receptors. This review will discuss the discovery and highlight the currently known series of allosteric modulators to this therapeutically important family of G-protein coupled receptors. Lastly, we will present potential mechanisms whereby the different series could modulate receptor function in the context of currently held theory and known structure of G protein-coupled receptors.

Keywords Glycoprotein hormone receptors · Allosteric modulators · G protein-coupled receptors · LH · FSH · TSH · Partial agonist

Introduction

The glycoprotein hormones luteinizing hormone (LH)/human chorionic gonadotropin (hCG), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), are members of a small but crucial family of proteins that regulate reproduction and metabolism. These proteins have a long evolutionary history since similar proteins appear in far more primitive species such as anemones, worms, and fish [1]. This suggests that these proteins have been key to the evolutionary success of many species. The physiological significance of these hormones in humans is well documented in the clinical literature which has detailed the effects of naturally occurring mutations in these hormones or their receptors. These reports help us understand the physiological roles of these important ligand-receptor systems and also demonstrate the potential therapeutic utility of developing small molecule agonists and antagonists that interact with these systems.

The glycoprotein hormones are heterodimeric proteins that are composed of α and β subunits that are synthesized and secreted from specific (gonadotropes and thyrotropes) cells of the anterior pituitary in response to the releasing stimuli provided by hypothalamic hormones [2, 3]. The alpha subunit is common to all the glycoprotein hormones whereas receptor selectivity is determined by the hormone-specific beta subunits. Another common feature of this family of proteins is the presence of multiple glycosylation sites on both the alpha and beta subunits [2, 3]. Interestingly, the degree of glycosylation added to these hormones varies depending upon the physiological state and therefore, they are found in the plasma as a series of isoforms that vary in glycosylation complexity.

Historically, it has been accepted that glycosylation complexity has an impact on the overall acidity of each isoform with more complex variants (higher degree of terminal sialylation and sulfation) possessing more acidic isoelectric points (pI) [4, 5] with less terminally sialylated/sulfonated isoforms more basic in pI. Chromoatofocusing has been used as a way of purifying these differently

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glycosylated isoforms. Recently though, Bousfield and colleagues have generated data demonstrating that chromatofocusing does not separate isoforms on the basis of glycan structure suggesting that the isoelectric point is not determined by the glycosylation structure [6]. Nevertheless, the physiological significance of these glycosylated variants is suggested by data demonstrating that the degree of glycosylation that occurs within the anterior pituitary synthetic cells is regulated by exogenous factors including ovarian steroids [7, 8]. Tight regulation of this secondary protein processing of glycoprotein hormones suggests an important physiological role for the presence of glycosylated variants of TSH, LH, and FSH. Numerous reports have detailed the effects of partial or complete deglycosylation on the action of these hormones [3, 9, 10]. The data in this area are varying with some noting no effects on binding [3, 11–13], but others noting increased binding affinity [10, 14]. However, recent work in this area using more sophisticated separation techniques strongly suggest that hormone glycosylation does play a significant role in receptor binding potentially via alteration in the three-dimensional conformation of the protein [14]. There is a significant effect on signaling. Indeed, the use of a baculovirus expression system to create partially glycosylated isoforms of FSH has shown that glycosylation can change the pharmacological properties of the hormone via alterations in interaction of the FSH receptor with the G protein signaling machinery [2, 13]. In addition, numerous reports have found a distinct difference in the observed bioactivity of basic isoforms of glycoprotein hormones as compared to acidic isoforms [2, 3].

The glycoprotein hormone receptors are G protein-coupled receptors (GPCR) that belong to the Class A, rhodopsin-like, family of GPCRs based on sequence homology [15]. These receptors are characterized by long amino-terminal extracellular domains (>300 aa) that are required for binding of ligand, seven lipophilic, membrane-spanning domains, and relatively short, cytoplasmic carboxy-terminal tails [16, 17]. The extracellular domains of the glycoprotein hormones are characterized by numerous leucine rich repeats (LRR) that have been shown to be important in the binding of the receptors with their respective ligands [16]. There are currently three subclasses of glycoprotein hormone-like receptors which are recognized as: Family A, the glycoprotein hormone receptors; Family B, orphan receptors; Family C, relaxin-binding receptors [18–20]. All of these receptors contain numerous LRRs, although the Family B receptors have a significantly greater number of these repeats within their structure. The Family C receptors also contain low-density lipoprotein (LDL) receptor motifs at the amino terminus of their extracellular domains that are bridged to the LRR region by a linker sequence [21]. The biological significance of these LDL receptor-like motifs is not currently understood. Similar to their hormone ligands, glycoprotein hormone receptors also contain sugar residues on their extracellular domains. Elegant studies of the contribution of this glycosylation via mutation of the Asp residues acting as glycosylation sites have found that the glycosylation is responsible for proper folding of the receptor during protein synthesis [22, 23]. This is more so for TSHR and FSHR than for LH/hCGR [24]. Overall, the glycosylation state of the receptor does not seem to have an effect on ligand-binding affinity or activation of signal transduction pathways [25].

On ligand binding, the glycoprotein hormones interact predominantly with the Gs signaling pathway [26]. This leads to the concomitant activation of adenylate cyclase and increase in cAMP [27–29]. However, we (and others) have also noted that the adenylate cyclase pathway is not the only signaling pathway that can be activated by glycoprotein hormones (Fig. 1). Our laboratory has shown that recombinant, partially glycosylated isoforms of human FSH can induce interaction of the FSHR with a pertussis toxin sensitive pathway depending upon the glycosylation state of the ligand bound. The pharmacological result of this promiscuity of signaling is that partially glycosylated variants of recombinant hFSH act as partial agonists, stimulating cAMP and estradiol production in cultured granulosa cells at low concentrations (<10 ng/ml) but at moderate- to- high concentrations (>10 ng/ml) these isoforms act to lower cAMP and estradiol production. Others have noted the activation of the Gi signaling pathway by the FSHR in osteoclasts [30]. The FSHR has also been shown to activate the IP3 signaling pathway [31, 32]. Similarly, the LH receptor has been reported to not only activate adenylate cyclase activity but also to stimulate IP3 production in vitro [33]. Lastly, the TSHR has also been reported to stimulate both cAMP and IP3 production through activation of Gs and Gq/11 [34]. These data suggest that glycoprotein hormones induce a complex pattern of cell activation on binding to their respective receptors.

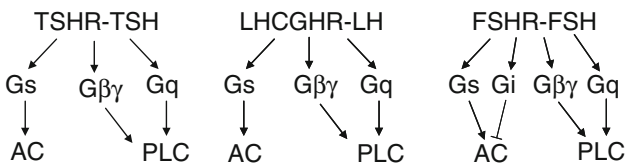

Fig. 1 Schematic diagram of signaling pathways activated by glycoprotein hormone receptors. The TSHR and LH/hCGR have been shown to activate adenylate cyclase (AC) and phospholipase C (PLC) via interaction with the G proteins Gαs (Gs) and Gαq (Gq). In addition, some data suggest that the G protein β/γ complex can also activate PLC. The FSHR has been shown to both activate and inhibit adenylate cyclase in an isoform-dependent manner following interaction with Gs and GαI (Gi). The FSHR has also been reported to activate PLC via Gq

This complexity raises the hurdle for the development of synthetic small molecules in terms of achieving agonist efficacy.

Mutations and polymorphisms of both the LH and FSH systems lead to reproductive abnormalities including sterilization or reduced fecundity and altered reproductive development [35]. Inactivating mutations in the LHR have been associated with a wide range of reproductive dysfunction and altered development including in its most severe form, Leydig cell hypoplasia resulting in pseudohermaphroditism [35, 36]. However, in less severe cases, inactivating mutations can lead to amenorrhea and interrupted breast development in females [37]. In contrast, activating mutations can lead to precocious puberty in males [38, 39] but are thought to have relatively little phenotypic impact on females [35, 40]. Activating mutations or polymorphisms of the FSHR that change binding properties have been shown to lead to ovarian hyperstimulation syndrome [41–44]. Inactivating mutations of the FSHR have been shown to lead to a retarded follicular development with associated amenorrhea and sterility [45, 46]. In men, these mutations lead to varying degrees of altered sperm production, although these mutations do not seem to affect pubertal development. Perhaps most importantly, these affected men are not azoospermic [35, 47]. Data suggest that FSH is required for spermatogenesis, and when the FSH activity is completely absent there is infertility [48].

Peptide agonists and antagonists

The data described above provide evidence toward the utility of identifying agonists and antagonists to the glycoprotein hormone receptors for potential therapeutic purposes. The first attempts at identifying these types of molecules naturally fell to producing small peptides based upon sequences within the ligand or receptor that could have biological activity. In terms of the gonadotropin receptors, Sluss, Krystek and colleagues published data suggesting that peptides as small as 4 amino acids in length could act as partial agonists or antagonists of the FSHR. Their work focused on a small domain of the FSH β subunit between amino acids 33 and 53, which was duplicated in the sequence of epidermal growth factor that had been shown to weakly inhibit FSH binding to the FSHR (potency $\approx$20,000 times less potent than hFSH) [49, 50]. Their data suggested that this region comprised two key interaction sites for FSH with its receptor such that synthesis of two tetrapeptide sequences (TRDL and KTCT) could both independently compete for binding of FSH to the FSHR. Creating a peptide spanning the region encompassing both of these sequences significantly improved potency, suggesting equivalent importance of these two regions on FSHR recognition of its hormone. When administered to cycling mice, a large dose (200 mg/kg, i.p.) of the TRDL peptide alone was shown to induce persistent estrus as determined by vaginal cytology and circulating levels of estradiol. These effects were not seen when peptides corresponding to other regions of the hormone were administered. These studies helped us to understand the critical regions associated with glycoprotein hormone recognition of receptor and this was a step forward in developing smaller ligands capable of blocking FSH action.

Perhaps one of the most interesting observations leading to our understanding of ligand-receptor interactions in the glycoprotein hormone family came from the observation that Graves' disease (hyperthyroid) patients possess circulating auto-antibodies to the TSHR that act as receptor agonists [51–53]. It was subsequently noted that certain hypothyroid states are also caused by circulating auto-antibodies to the TSHR [51, 53]. It was assumed that these antibodies acted as antagonists to the TSHR. These observations were subsequently confirmed by the creation of monoclonal antibodies to the TSHR that shared similar properties to the circulating auto-antibodies for the TSHR [54]. Given the discrete nature of antibody epitopes and the mutual competition between hormone and antibody for the receptor, it was hypothesized that small peptides interacting with glycoprotein hormone receptors could be designed as synthetic agonists and antagonists. Initial attempts to understand critical regions of interaction between glycoprotein hormones and their receptors involved mutation of the glycoprotein hormone dimer or synthesis of peptides from the α or β subunits of the hormones which were assessed for their ability to block binding of the native hormone to its receptor. However, unlike studies of the receptor for the decapeptide, gonadotropin-releasing hormone (GnRH), over the last 30 years only sparse reports have surfaced identifying peptide components of the naturally occurring ligands that could interact with the extracellular binding of the domains of the glycoprotein hormone receptors and thus block ligand binding or activate signaling. This may be due to a large contact surface of the glycoprotein hormone receptors with their ligands that requires interactions between the glycoprotein hormone receptor and residues on both the α and β subunits [55].

Some of the crucial steps forward in this regard were provided by Szkudlinski, Weintraub, and colleagues who showed through site-directed mutagenesis of the TSH α/β dimer that one could produce molecules that possessed dramatically increased potency and efficacy as compared to the naturally occurring hormone [56, 57]. The result of their work led to the creation of TSH analogs containing

several mutations in both the α and β subunits that had up to 1000-fold better receptor-binding affinity and 100-fold improved in vivo activity. Further analoging around these structures as well as information gleaned from naturally occurring mutations to the glycoprotein hormones themselves led researchers to identify analogs that could not only bind the glycoprotein hormone receptors but also to act as agonists or super-agonists of the receptors [58].

Despite the obvious increase in understanding of glycoprotein hormone action, efforts to develop peptide-based molecules have failed to lead to a viable therapeutic because of poor oral efficacy of these peptides. This was mainly due to the peptidic nature of these molecules which have poor oral bioavailability. Therefore, peptide approaches have been successful in developing both agonists and antagonists, but none of these lent themselves to the breakthrough profile of an orally active q.d. or b.i.d. therapeutic.

Development of small molecule modulators

In addition to the pursuit of peptide approaches to the development of therapeutics to the glycoprotein hormones, there has been a consistent effort to discover and develop small molecule synthetic compounds capable of affecting glycoprotein hormone receptors. However, until recently this approach has proven difficult. This is perhaps due to the nature of the receptor–ligand interactions that require multiple contact sites of the α/β dimer of the hormone ligands with the extracellular domain of their receptors. But with the onset of improved screening methodologies, the application of combinatorial chemistry, and access to more diverse chemical libraries, the last decade has seen numerous reports of the identification of novel chemical series of synthetic molecules affecting glycoprotein hormone receptors. These small molecule synthetic ligands have been found to possess a range of pharmacological activities.

Figure 2 illustrates the chemical structures of some of the most notable synthetic agonists recently described for the various glycoprotein hormone receptors. These representative structures in most cases have been optimized following initial identification of much lower potency leads through screening of large chemical libraries. The exception to this was the identification of the thiazolidinone class of FSHR agonists which were first identified through screening of combinatorial chemical scaffolds [59]. In all the cases, these compounds were optimized to improve potency into the double-digit or even single digit nanomolar (nM) range. The thiazolidinone compounds were further optimized for selectivity versus the other glycoprotein hormone receptors, whereas both the pyrazole and

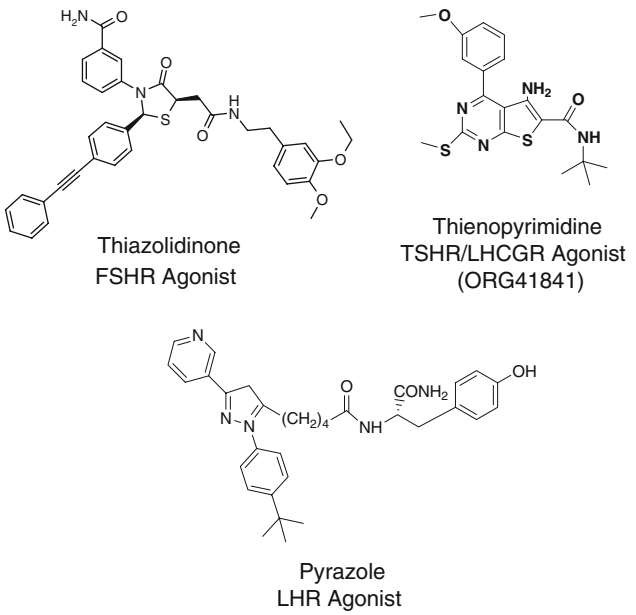

Fig. 2 Structures of published glycoprotein hormone receptor agonists. These compounds are representative of the chemotypes of synthetic small molecule glycoprotein hormone agonists reported for the FSHR, TSHR, and LHR

thienopyridine/-pyrimidine chemotypes achieved less selectivity. The thienopyridine/-pyrimidine class of TSH/LH 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 [60]. However, ORG41841 demonstrated significantly improved potency over other members of this class with an $EC_{50} = 20$ nM [60]. This compound was further tested for effects on ovulation. ORG-41841 was found to induce ovulation in 40% of animals when administered 50 mg/kg. Recently, Jorand-Lebrun et al. have reported a series of pyrazole compounds that act as LHR and LHR/FSHR partial agonists [61]. The pyrazole LHR/FSHR compound showed an approximate seven-fold selectivity for the LHR over the FSHR [62]. Interestingly, the efficacy reported for the LH- and FSH- related activities of this compound were slightly greater (73%) for the FSHR as compared with the efficacy for this compound at the LH receptor (53%) despite the preferential LHR potency. This compound also demonstrated in vivo efficacy in a model of LH-induced testosterone production [61]. When administered intraperitoneally, the pyrazole compound shown above induced an approximate five-fold increase in serum testosterone.

Perhaps the most potent synthetic agonists of glycoprotein hormone receptors are those identified to the FSHR. These molecules have been optimized to low nM and in some cases to high picomolar potency for induction of FSHR signaling not only in CHO cells expressing the hFSHR but also in primary cultures of rat granulosa cells

[63, 64]. The optimization of these chemical series to high potency and efficacy represents a significant achievement when one considers the size of these molecules in comparison to the size of the native ligands of these receptors. In the case of the FSH agonists, these compounds are more than 100-times smaller in terms of mass than the naturally occurring ligand. Thus, there is proof of principle that one can design small molecules capable of activating full efficacy in terms of primary signaling of a receptor with a large protein ligand. The question arises: How can this be accomplished? Studies on synthetic peptides, epitope mapping, mutations, and modeling all point to numerous contact sites for glycoprotein hormones with their respective receptors. These would argue against the development of small molecules being able to provide the structural changes required within the extracellular domain of the receptors in order to achieve full efficacy and low potency.

The answer becomes apparent when one studies the pharmacological properties of these compounds in more detail. One key feature of these small molecule agonists is that, regardless of the chemical series or receptor they interact with, none of them is capable of displacing native ligand binding despite the requirement of the expression of their respective target receptors in order to induce a signaling response. These data suggest that these synthetic agonists to glycoprotein hormone receptors must utilize, so-called "allosteric" sites, in order to activate the receptor-signaling mechanism. The idea of allosteric modulators to GPCRs has been well documented for other receptors, including serotonergic, dopaminergic and metabotropic glutamate, and calcium-sensing (CaSR) receptors as well as others [65]. The phenylalkylamine calcium-sensing receptor agonists and antagonists are some of the best characterized allosteric modulators to the long N-terminus GPCRs [66]. Both the glycoprotein hormone receptors and the CaSR are the members of this subclass of GPCRs. Similar to the glycoprotein hormone receptors, the CaSR also utilizes an extracellular binding domain within the N-terminus.

In addition to in vitro activity, some of the glycoprotein hormone receptor agonists also have displayed significant in vivo activity. The thienopyrimidine series of TSHR/LHR partial agonists displayed in vivo activity in terms of ovulation induction in female rats [60]. Similarly, the pyrazole series of LHR agonists also demonstrated significant in vivo activity [61]. The thiazolidinone class of FSHR agonists has been reported not to have in vivo activity despite single digit nM potency in vitro.

Figure 3 illustrates the chemical structures of representative antagonists to glycoprotein hormones reported to date. Interestingly, synthetic, small molecule antagonists have not been reported for the LHR but have been reported for both the FSHR and the TSHR. In terms of the FSHR, a number of different chemotypes have been reported including the naphthalene, sulfonic acid chemotype and the stilbene (bis)sulfonic acid chemotype [67, 68]. None of these chemotypes was found to be highly potent (IC_{50}s of approximately 1 μM in cAMP and aromatase assays). Although, they do demonstrate noncompetitive receptor-binding characteristics in competition binding experiments with the FSHR using ^{125}I-hFSH. The naphthalene, sulfonic acid compounds, demonstrated fairly good selectivity for the FSHR with very little competition for binding at either the LHR or TSHR up to 100 μM [67]. The stilbene (bis) sulfonic acids series were less selective demonstrating only approximately 30- to 40-fold selectivity for the FSHR over

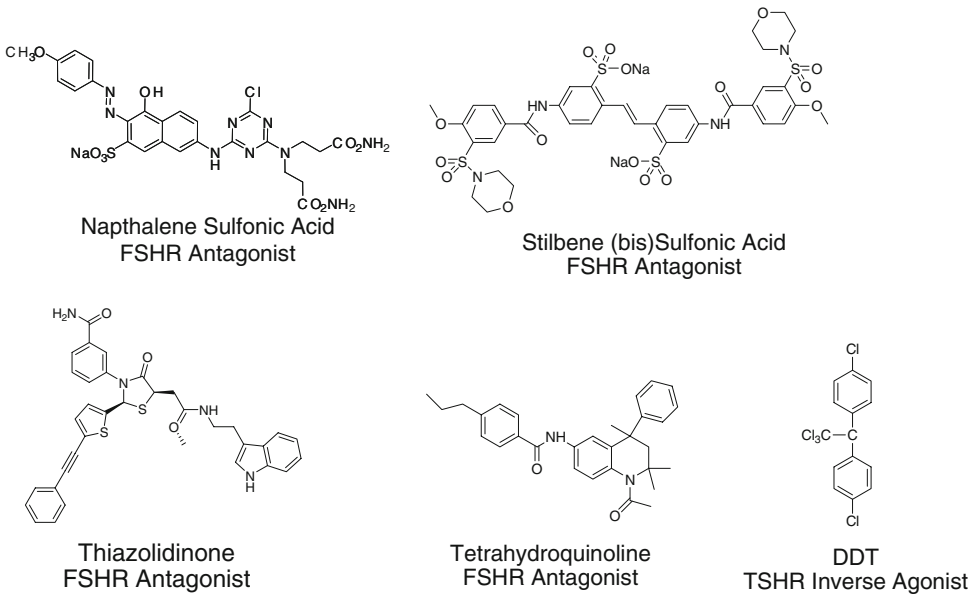

Fig. 3 Structures of published glycoprotein hormone receptor antagonists. The first reported small molecule synthetic antagonists to the glycoprotein hormones are represented by the naphthalene sulfonic acid and stilbene sulfonic acid chemotypes. The compounds displayed the ability to block FSH binding to its receptor at the extracellular domain. The thiazolidinone, tetrahydroquinoline, and TSHR inverse agonist do not compete for ligand binding to their receptor and are thought to interact with allosteric sites on their respective receptors. This has been demonstrated clearly only for the thiazolidinone chemotype thus far

the TSHR in a cAMP bioassay [68]. 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. However, these compounds did not demonstrate oral bioavailability but did provide the first proof of concept for the development of nonpeptide, small molecule antagonists to the FSHR for use as contraceptive agents. In addition to synthetic antagonists, the chemical DDT has been found to act as an inverse agonist for the TSHR [69, 70]. No other small molecules have been reported to have inverse agonist activity versus their respective receptors. In the case of the TSHR, this effect may be due to constitutive blockade of basal receptor signaling provided by the non-liganded N-terminal region of the receptor [71] that can somehow be relieved allosterically by small molecules such as DDT or Aroclor 1254 (not shown [69]).

More recently, van Straten et al. have described the synthesis of a new class of more lipophilic tetrahydroquinoline FSHR antagonists [72]. These compounds possess improved potency as compared with the naphthalene sulfonic acid and stilbene (bis) sulfonic acid classes of antagonists with IC_{50}s ca. 10 nM in cAMP assays. There was no report of in vivo activity for these compounds despite the improved potency. In addition, like the reported synthetic agonists, the tetrahydroquinoline compounds were not able to compete for binding with FSH to the FSHR. These data suggest that these compounds are negative allosteric modulators of the FSHR. We have also described recently negative allosteric modulators that are analogs of the thiazolidinone class of FSHR agonists [64]. These compounds are completely devoid of agonistic activity but are capable of dose-dependently inhibiting FSH-induced cAMP and steroidogensis in vitro. These compounds have achieved submicromolar potency and 100% efficacy. Since these compounds do not compete for FSH binding, we have studied how these compounds could potentially be blocking FSH action. We have shown that in the presence of these compounds the FSHR selectively activates the Gi signaling pathway with a dose–response relationship that parallels effects on cAMP production [13, 64].

In addition to these negative allosteric modulators, we have also identified thiazolidinone compounds that demonstrate partial agonist activity [64]. These compounds possess a pharmacological profile that is both agonistic and antagonistic, which gives rise to a bell-shaped dose–response curve in both signaling and steroidogenesis assays. Although the maximal efficacy of these compounds is somewhat lower than full agonists of this chemotype (50–60%), the agonist potencies of these compounds are similar. We have also demonstrated that the mechanism of this observed partial agonism is due, at least in part, to 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 based upon cAMP production and steroidogenesis. 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 [13, 64]. It is not known whether these molecules are capable of activating the IP3 signaling pathway. These data suggest that one can design allosteric modulators to GPCRs that can selectively target signaling pathways associated with a given receptor [64].

Functional mechanics of allosterism

As we have described, several different chemotypes have been reported to act as allosteric modulators of glycoprotein hormones. Some of these reports have described either positive allosteric modulators or negative modulators. Some chemotypes have also been shown to contain compounds that share a common core structure which can be modified to produce a wide range of pharmacological activities including partial agonism. Study of the crystal structure of the glycoprotein hormone receptors has demonstrated that the LRR of the extracellular domains of the glycoprotein hormone receptors bind to the α and β subunits of the hormones in a "hand-clasp" fashion [55]. In this interaction, physical association of key residues within the LRR domain of the receptor wrap around the hinged region of the hormone dimer. This interaction induces structural changes to both the receptor and the ligand. The predominant effect on the ligand is to stiffen the hormone dimer; this is achieved by movement of the FSHα C-terminus almost 180° to allow it to bury within the FSHR extracellular domain pocket [55]. Interestingly, this ordered movement of the FSHα domain involves numerous amino acid residues that are conserved amongst the glycoprotein hormones, suggesting that hormones of this family share a common binding mechanism with their respective receptors [55, 73, 74].

It is not clear what subsequently occurs following ligand binding to generate the signaling event. Current theory holds that the binding event causes shifting within the molecular structure of the receptor resulting in a transmission of the ligand–receptor binding event to other parts of the receptor that favors interaction and activation of the G-protein signaling machinery. There have been numerous reviews on this topic over the years, which capture in detail

the mechanisms of changes in GPCRs during activation [75]. A full discussion of this topic is out of scope of this review. However, we will attempt to discuss in general terms potential mechanisms involved in the activation of glycoprotein hormone receptors in the presence of allosteric modulators. The first step in this process is to understand the general topology of GPCRs that may lend itself to accommodating a small molecule ligand. Generally speaking, this refers to crevices or pockets that can capture and stabilize interactions between the compound and its target. In this case, we are interested in identifying those pockets that might be formed in the three dimensional structure of glycoprotein hormone receptors that are not involved in hormone binding. For many years, the general structure of GPCRs has been assumed to be similar to that of rhodopsin, and researchers have used this model as a template to map the three-dimensional structure of the most known GPCRs. Therefore, using this approach we can identify two pockets within transmembrane regions of the glycoprotein hormone receptors that may be sites of binding of allosteric modulators. Figure 4 schematically illustrates the putative positioning of these pockets with reference to the transmembrane α-helices of the glycoprotein hormone receptors based on currently accepted models of GPCRs [76]. It should be noted that in the top-down diagram the extracellular loops have been removed for clarity. The presence of these loops would certainly provide additional residues for interaction with small molecule ligands. The two pockets formed by the positioning of the helices are termed P1 and P2. The P1 pocket is formed by the relative positioning of transmembrane helices III, IV, V, and VI, and to some extent helix VII. Due to the skewed vertical position of these helices (tilted away from each at the extracellular surface but toward each other on the cytoplasmic surface), a pocket that is formed can be accessed by small molecule ligands. Indeed, the P1 pocket formed by these helices is a common binding site for native small molecule ligands of GPCRs (e.g., β-adrenergic receptor) [77]. However, recently it has become apparent that in addition to the P1 pocket, a smaller pocket also exists within GPCRs, which is formed by the positioning of transmembrane helices I, II, III, and VII. This pocket, the P2 pocket, is predicted to be significantly smaller than the P1 pocket and to be much shallower. However, because of its composition it also is predicted to involve a more significant contribution from helix VII. This is important because it is thought that disruption of a stabilized complex between helices III, VI and, VII allows the association of the receptor with the G-protein signaling machinery. Helix three contains the "DRY" motif consisting of the sequence aspartic acid–arginine–tyrosine that is highly conserved within the GPCR superfamily. The aspartic acid residue within this sequence is hypothesized to associate with key cysteine residues in transmembrane helices VI and VII to form a coordinated stabilization between the three helices. Some have hypothesized that disruption of this stabilized complex allows movement of these helices in juxtaposition to one another to allow signaling to occur [76].

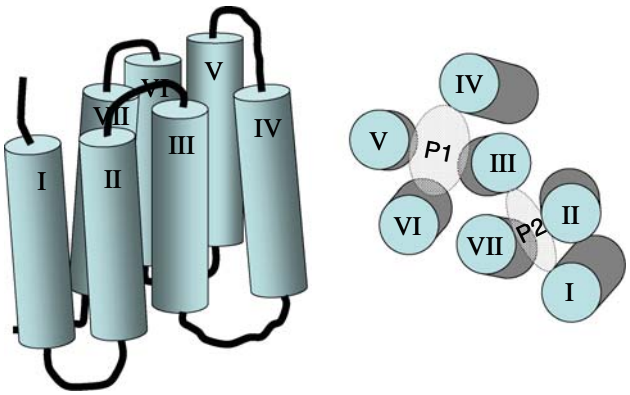

Fig. 4 Schematic diagram of the structure of the transmembrane domains of glycoprotein hormone receptors. The extracellular domain has been removed so as to provide a clear picture of the general positioning of the transmembrane domains of these receptors based on models for rhodopsin and the β-adrenergic receptor [77]. The top down view reveals two putative binding pockets for potential glycoprotein hormone receptor allosteric modulators, P1 and P2. P1 has been suggested to be the site of interaction of pyrazole, pyrimidine, and tetrahydroquinoline chemotypes but the thiazolidinone series has been shown to interact with the region of the P2 pocket

Interestingly, modeling and mutagenesis studies of the interactions between allosteric modulators of the glycoprotein hormone receptors suggest that the majority of these small molecules interact in the P1 pocket of these receptors. However, in the case of the FSHR, chimeric receptor studies suggest that the thiazolidinone positive and negative allosteric modulators involve association with the P2 pocket [63]. This is similar to what we have recently proposed for the tri-substituted pyridine/pyrimidine class of CaSR negative allosteric modulators [78, 79]. In these studies, we found that the region bracketed by transmembrane helices I, II, and III were critical for signaling by these ligands. The extracellular domain and the other transmembrane domains did not appreciably contribute to the signaling of the chimeric receptor in the presence of these ligands. Recently, Schwartz has hypothesized that one can achieve different pharmacological profiles from affecting this P2 pocket. He suggests that the depth that the ligand protrudes into this pocket has a significant impact on the biological activity of the compound: with negative modulators binding deeper in the pocket than positive modulators [76]. If this is the case, the thiazolidinone series of compounds represents a prime opportunity to study the merits of this hypothesis due to the similarity in chemical

structures in the face of drastically different pharmacological activities [64].

Therapeutic potential of synthetic modulators of glycoprotein hormone receptors

The glycoprotein hormones are important modulators of reproductive and metabolic functions in humans. As a result, the receptors for these proteins are an attractive target for the development of novel therapeutic intervention for a host of clinical situations. Unfortunately, until recently there has been little success in identifying synthetic, small molecules that could activate or block receptor function. Development of allosteric modulators of glycoprotein hormones opens a new avenue for the identification of novel therapeutics for these receptors.

In terms of the gonadotropin receptors, numerous examples of naturally occurring mutations to both the ligands and receptors support a clear opportunity to develop novel contraceptive therapies that could have potentially lessened the side-effects than the currently used standard, steroid-based contraceptive agents. This would be especially true in the case of FSHR negative allosteric modulators because of the relatively limited expression of the FSHR. One key advantage of this approach would be a theoretical improvement of the side-effect profile when using FSHR-targeted therapies in terms of improved cardiovascular risk because steroid-based contraceptive therapies have a well-documented effect in increasing the risk of venous thromboembolism. Naturally occurring mutations to the FSHR have been demonstrated to be involved in male and female infertility. These data provide a natural proof-of-concept for the use of negative allosteric modulators as contraceptive agents in both sexes. In addition, FSHR antagonists/negative modulators have also been proposed as potential treatments for ovarian epithelial cancers [80] because of their ability to inhibit folliculogenesis and estradiol production. Indeed, there are reports of heightened expression of FSHR in malignant ovarian epithelial tissue [81, 82].

Another use of FSHR allosteric modulators would be for infertility treatments, which could represent a significant step forward through an improved route of administration (orally vs. subcutaneous). Current infertility regimens require daily parenteral injection of recombinant or purified gonadotropin [83]. In this case, FSHR and LHR positive allosteric modulators could be used in IVT (in vitro fertilization) regimens. From the data discussed above, the thiazolidinone partial agonist modulators could prove to be an important new therapeutic given their pharmacological profile that would lend itself to better control and prevention of ovarian hyperstimulation during IVF treatment.

In addition to its role in regulating reproductive processes, the FSHR negative modulators could also prove to be useful for potential osteoporosis treatments. The role of FSH in regulating bone osteocytes is just emerging with a single report suggesting a role for an FSHR negative allosteric modulator in regulating osteoclastic bone resorption [30].

Graves' Disease is an important metabolic disease occurring with a frequency of 1–2% in Asian and Caucasian populations. The cause of Graves' disease is the presence of autoantibodies to the TSHR which overlap with the TSH binding site thus activating TSHR signaling. This leads to fluctuations in the disease in which there appear alternating periods of hyperthyroidism and hypothyroidism. Common therapeutic approaches for the treatment of this disease involve destruction of the thyroid by use of radioiodine or surgery. Although in patients under the age of 50, use of anti-thyroid drugs is often prescribed. These small molecules act to inhibit thyroid hormone synthesis or conversion of T_4 to T_3. However, these treatments have only modest success rates. Therefore, development of negative allosteric modulators to the TSHR may be helpful in the treatment of Graves' Disease and goiter associated with hyperthyroidism by offering a more selective therapeutic approach.

Summary

With the onset of new synthetic methods and a better understanding of GPCR function, new small molecule allosteric modulators to glycoprotein hormone receptors have emerged. Work in this area has demonstrated that positive and negative modulators can be generated that mimic the naturally occurring biological properties of native glycoprotein hormones. This represents a giant stride taken toward the development of orally active therapeutic agents that could affect such diverse disease states as infertility, hyperthyroidism, and cancer. However, the chemotypes thus far identified suffer from poor pharmacokinetic properties, and thus there remains work to be done in this area in order to deliver on the promise of selective, small molecule agonists and antagonists to the glycoprotein hormone receptors.

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