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# Molecular Pharmacology of Gonadotropins

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**Gonadotropins have been studied in biological systems for decades and many of their properties are well defined. These include pharmacological properties such as affinity, stability, and pharmacokinetics also used to characterize drugs. Technologies applied to research on gonadotropins have led to the creation of hormone analogs with alterations to one or more of these properties. Some of these analogs have potential therapeutic applications. A challenge to realizing this potential is the accurate prediction of how these compounds will perform in humans. This could be facilitated by advances in biological models and the understanding of specific effects of the hormones on their receptors.**

**Key Words:** Gonadotropin pharmacology; selectivity; affinity; pharmacokinetics; protein engineering.

## Introduction

It has been almost a century since the laboratories of Cushing and Aschner performed the first pituitary ablation studies, implicating this gland as a primary regulator of vertebrate reproduction *(1,2)*. A meticulous series of studies by P. E. Smith at Columbia further demonstrated the existence of anterior pituitary substances essential for the function of various endocrine organs *(3)*. Smith's work also demonstrated the ability of pituitary extracts to augment physiological functions such as growth and gamete production. The gonadotropic effects of pituitary extracts were soon associated with two distinct fractions *(4,5)*. These endocrine factors became designated as follicle-stimulating hormone (FSH, follitropin) and luteinizing hormone (LH, lutropin). In females, follitropin promotes proliferation and differentiation of ovarian granulosa cells and follicles. These effects include promoting the synthesis of estrogen from androgens *(6)*. Lutropin promotes the function of ovarian thecal cells, including the androgen production essential for estrogen synthesis. Lutropin also acts to trigger ovulation and the subsequent formation of the corpus luteum from the follicle. In males, FSH promotes the functions of Sertoli cells of the testis and has an essential role in the onset of spermatogenesis. Lutropin supports Leydig cell functions and stimulates the production of testosterone. A third gonadotropin hormone was discovered in the urine of pregnant women *(7)* and was later shown to be produced by cells of the human placenta *(8)*. This hormone became designated as chorionic gonadotropin (CG, choriogonadotropin). Most vertebrates have only FSH and LH, whereas primates and some perissodactyls (equines) also have a CG. The primate CG evolved by duplication and modification of the LH beta subunit gene *(9)* and apparent redeployment of subunit expression to the blastocyst and placenta. The primary function identified for CG in primates is to promote ovarian corpora lutea production of progesterone essential for maintenance of the uterine environment supporting pregnancy. The three gonadotropins—FSH, LH, and CG—are all glycoproteins *(10)* comprised of dissimilar subunits designated alpha and beta *(11, 12)*. The alpha subunit is the same across hormones within a species, while the beta subunits are unique to each hormone. Together with thyrotropin (TSH) these proteins comprise the family of vertebrate glycoprotein hormones *(13)*.

Following the discovery of these gonadotropic extracts, there were immediate efforts to adapt them for the treatment of infertility. A superb account of these research studies, technology developments, and associated practice decisions has recently been published by Lunenfeld *(14)*. In parallel to the development and refinement of gonadotropins as clinical medicines, there have been extensive advances in the understanding of molecular pharmacology, i.e., receptor binding, signal transduction, and pharmacokinetics. These and developments around protein engineering and high-throughput drug discovery have potentially set the stage for new treatment options. Engineering approaches introduced in studies of gonadotropins have included removal of glycosylation sites *(15)*, chimeric substitutions of amino acids *(16)*, fusions with the C-terminal peptide of hCG *(17)*, single-chain analogs in which alpha and beta subunits are fused into a single polypeptide sequence *(18)*, creation of intermolecular disulfides to cross-link the hormone subunits *(19)*, introduction of basic amino acid to alter binding potency *(20)*, and the fusion of dimerization domains to the hormone subunits to stabilize the complex *(21)*. The relationship of these approaches to the molecular pharmacology of the hormones, and challenges that face mobilization of this work for efficient drug discovery, are the subject of this review.

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The pharmacology of the gonadotropins can be subdivided into the same properties that describe the molecular pharmacology of drugs. These properties include affinity (binding to target receptors), intrinsic efficacy (modifying effects of hormone on the receptor), stability, pharmacokinetics, bioavailability, and toxicology *(22)*. Affinity and intrinsic efficacy are properties of the hormones themselves, while the other characteristics are influenced by biological and experimental contexts.

## Affinity

Affinity describes the tendency of the hormone to remain bound to the receptor and is often expressed as the equilibrium dissociation constant, $K_{eq}$, a ratio of dissociation and association rates for the hormone–receptor complex *(22)*. The value of $K_{eq}$ corresponds to the concentration of hormone at which 50% of the receptors are occupied. Specific, saturable binding sites for gonadotropins are well established in the ovary and testis *(23–26)*. Two of the hormones, LH and CG, bind to the same receptor, while FSH has a separate receptor. Extensive studies of gonadotropins and receptor-bearing tissues from different species established that $K_{eq}$ values of the hormones for their primary receptors are typically in the low-nanomolar concentration range *(27)*.

Subsequent studies showed that the hormone receptors signaled through G proteins, adenyl cyclase, and cAMP (reviewed in ref. *13*). Cloning and sequencing of the receptors revealed them to be members of the G protein–coupled receptor family *(28–30)*. In addition to the canonical prediction of seven-transmembrane helices that define GPCRs, the gonadotropin receptors have large amino-terminal extracellular domains containing leucine-rich repeats. Both of the hormone subunits are required for receptor binding and appear to contact the receptor *(13)*. The major binding site in the receptor appears to be the extracellular domain, as this portion of the receptors has nearly the same affinity and selectivity for hormone binding as does the full-length receptor *(31)*. This study also showed that this region dictates the selectivity of hybrid FSH:LH receptors.

Association of macromolecules is strongly influenced by factors affecting diffusion processes, while dissociation is influenced by the nature of energetic interactions between the molecules. These interactions involve ionic, dipole, hydrogen bond, and van der Waals contributions *(22)*. Structural modifications to the hormone or receptor affecting the presence or orientation of such interacting groups would be expected to alter affinity, and this has been seen in a wide range of chemical modification and mutagenesis studies *(13, 32–34)*. Some of these studies have also used protection or epitope mapping to identify amino acid residues likely to be in or near the receptor contacts. As a result of these studies, multiple regions of the hormone have been implicated in receptor binding. The recent elucidation of the crystal structure of FSH bound to a non-signaling fragment of the FSH receptor is consistent with some, but not all, of these biochemical and molecular biology studies *(35,36)*. Future studies designed to disprove one or more of the existing models can hopefully provide a concrete solution to the complete hormone-receptor binding interaction.

Most modifications that alter hormone binding result in a loss of affinity. However, three types of modification have been shown to enhance binding, raising the prospect of hormone analogs with increased potency and/or recruitment of additional binding activities into the proteins. The first of these modifications to be recognized was that removal of negatively charged sialic acid groups from the hormone oligosaccharides could increase receptor binding affinity by almost 10-fold *(37)*. However, the utility of this effect is undermined by the increase in affinity of the modified hormones for asialogylcoprotein receptors of the liver, which rapidly clear desialylated glycoproteins from the circulation *(38)*.

More recently, it has been shown that substantial increases in binding activity can be achieved by alterations to the overall charge of the gonadotropins and TSH by mutagenesis to substitute basic amino acids into the alpha *(39)* or beta subunits *(40)*. These changes may stabilize the hormone–receptor complex, changing the off-rate and affinity. Because sialic acid content is not altered, the resulting analogs are not expected to be compromised in half-life.

A third method for enhancing binding affinity is the substitution of amino acids from one hormone sequence into another *(16)*. Certain mutations result in the recruitment of one *(41)* or more *(42)* additional binding activities into the resulting hybrid or chimeric hormones. These and related studies have generated a variety of combinations of FSH, LH, and TSH activities in hormone analogs. Another approach to combine hormone binding activities into a single protein has been the generation of triple domain hormone analogs that contain two different beta subunits plus an alpha subunit *(43)*.

## Intrinsic Efficacy

Along with affinity, intrinsic efficacy is a system-independent property useful to predict drug or hormone activity in new contexts based on prior observation. As such it can be a useful property for decision-making in drug discovery. Whereas affinity is fairly straightforward to assess, intrinsic efficacy can be a much harder property to define. Borrowing again from drug-receptor theory presented by Kenakin *(22)*, the intrinsic efficacy of a hormone or hormone analog can be defined as its tendency to modify the receptor. This would entail a change from one receptor isoform population (for example, an inactive form, R) to another isoform population (such as an active form, R*). This could be achieved by either "conformational selection," i.e., a ligand having affinity preference for a pre-existing R*, or by "conformational induction," i.e., a ligand promoting the conversion of R into R* *(22)*. In practice the measurement

of intrinsic efficacy can be difficult. Most detectable effects of gonadotropins on their receptors depend on signal transduction mechanisms that convert these effects into detectable changes in one or more measurable properties of the system. Examples of these outputs include protein binding (e.g., arrestin association with receptor), enzyme activation (e.g., GTPase activity), second messenger elevation (e.g., cAMP, $Ca^{2+}$, or phosphatidylinositol levels), protein phosphorylation, or changes to mRNA or protein levels. Each of these downstream effects is dependent on multiple other features of the experimental system, in addition to the receptor itself. The ability of a system to translate or amplify intrinsic efficacy into a detectable response is termed stimulus–response coupling *(22)*. Effects proximal to the receptor, such as G protein activation or second messenger generation, tend to be closely tied to receptor occupancy, while effects resulting from signaling components more distant from the receptor reflect the process of signal amplification and are less closely tied to receptor occupancy. As a result, different readouts within a system (e.g., cAMP vs steroid production) can respond differently (showing full or partial agonism) to the same hormone preparations acting through the same receptor *(27,44)*. The earliest work on the biological effects of gonadotropins, already cited above, made use of physiological readouts many steps removed from receptor activation. Most studies employ readouts such as cAMP, cAMP-driven gene expression, or steroidogenesis, from which it can be difficult to assess intrinsic efficacy.

An extensive amount of knowledge has been generated about the effects of gonadotropins in various cell and animal systems and the reader is directed to recent reviews on this subject *(45,46)*. However, there remain challenges for understanding which specific effects of hormones on their receptors translate into their essential clinical uses and influence clinical responses.

Despite the difficulties in inferring intrinsic efficacy from the studies published to date, there is clear evidence that this property of the hormones can be altered. Removal of N-linked carbohydrate from gonadotropins by chemical treatment *(47,48)* or mutagenesis *(15)* converts the proteins into partial agonists. The observation that some of these preparations behaved as antagonists in certain assays prompted their investigation as possible clinical agents to regulate hormone activity. However, to date the residual partial agonist activity in these preparations has precluded their employment as effective antagonists in vivo *(49,50)*. Recently, the combination of deglycosylation and increased structural rigidity (through intermolecular disulfides) resulted in partial agonists with much lower efficacy *(51)*, approaching more closely what would be needed for effective antagonism in vivo.

## Stability

Gonadotropin heterodimers appear to be extremely stable having a very slow rate of dissociation *(52)*. Nonetheless, two technologies have emerged to potentially enhance stability, one of which has yielded molecules that are undergoing clinical testing.

A hormone design favoring association of subunits into heterodimers is the fusion of alpha and beta subunits into a single chain, first developed for FSH *(18)*. In these constructs the alpha and beta subunits are expressed as a single polypeptide, typically with an intervening linker. Some of these proteins have receptor binding and activation properties similar to the native hormones. In addition, the use of single chain designs eliminates free subunit populations that can complicate quantitation and other analyses in experiments using unpurified or partially purified recombinant protein preparations. As will be discussed below, hormone analogs incorporating the single-chain feature have been advanced into testing in humans.

Another strategy has been to directly stabilize the heterodimer quaternary structure through the introduction of disulfide bonds between alpha and beta subunits *(19)*. Some of these constructs exhibit binding and activity very similar to the wild-type hormones, while having superior resistance to conditions that dissociate the heterodimer, such as low pH. The design of cross-linked constructs can be aided by structures or high-quality homology models of the hormones. However, an additional strategy has recently been developed to introduce intermolecular disulfide cross-links without having to know the three-dimensional structure of the proteins to be linked *(53)*.

## Pharmacokinetics

Relatively few pharmacokinetic studies have been done on the hormones, and much of the literature is a mix of different preparations (differing purities of urinary extracts along with purified recombinant hormones), routes of administration (iv, im, sc), methods of measurement (immunoassay, bioassay), and parameters reported (e.g., distribution half-lives, elimination half-lives).

Studies in animals have described the half-life of plasma LH to be on the order of 20 min, while the half-life of FSH is several hours (reviewed in ref. *32*). The short half-life of LH is consistent with the presence of hourly LH pulses during parts of the reproductive cycle, while FSH does not exhibit this pattern. The difference in half-lives can be attributed, at least in part, to differences in the terminal groups on the N-linked oligosaccharides of FSH and LH. Whereas FSH has mainly sialic acid at the termini of its oligosaccharides, LH contains a substantial proportion of oligosaccharides in which the terminal groups are sulfate *(54)*. Sulfated hormones are ligands for clearance by a specific receptor found in the liver, resulting in their abbreviated half-life *(55)*.

A significant advance has been the determination of pharmacokinetic properties of the human hormones in humans in studies performed in support of product development. Human FSH administered iv has a distribution half-life of

about 2–3 h in humans, and a terminal (elimination) half-life of 15–17 h as measured by immunoassay *(56)*. A review of studies using different routes of administration (im or sc) showed a consistent terminal half-life of about 30–45 h across seven different papers using various urinary and recombinant human FSH preparations *(57)*. Fewer data are available for LH and CG. Comparison of results from studies using iv administration of the recombinant hormones in humans' shows that the terminal half life of CG *(58)* is about three times that of LH *(59)*, 30 h vs 10 h.

These clinical results were consistent with long-standing expectations about the relative pharmacokinetics of the three different hormones, i.e., that LH would have the shortest half-life, CG the longest, and FSH intermediate. The difference between CG and LH is largely attributable to the CG beta subunit having one more N-linked oligosaccharide than LH, as well as there being the 35-amino-acid C-terminal peptide extension in CG beta (along with four O-linked oligosaccharides not present in LH). Fusion of the C-terminal peptide from human CG onto the C-terminus of FSH results in a prolongation of circulating half-life and biological effect in rodents *(17)* and humans *(60)*. Administration of a single dose of FSH-CTP resulted in peak FSH levels 3–5 d postdosing and a transient stimulation of follicle growth that peaked about 7 d postdosing *(61)*. Similar effects on half-life in rodents (twofold increase) and accompanying increased biological activity have also been reported for single-chain FSH analogs containing N-linked glycosylation sites in the linker between the alpha and beta subunits *(62,63)*.

## Bioavailability

Gonadotropin drugs must be given by injection (im or sc) to reach their target tissues in sufficient concentration to have useful effects. It is not yet possible to administer protein drugs by other routes and retain high efficacy and safety. Good bioavailability with oral administration typically requires use of a non-peptide small-molecule drug.

Despite early reports of peptide fragments with potent hormone activity (for example, ref. *64*), no peptide analogs of the gonadotropins have been shown to have useful biological activity. Partial minimization of the hormones has been reported by Moyle, who created hybrid alpha–beta subunits by exchange of cys–knot loops *(21)*, but these constructs involved the fusion of additional cross-linking domains to compensate for loss of the seatbelt and also had greatly reduced activity compared to the native hormones.

More recently, there have been several reports of non-peptide small-molecule agonists *(65,66)* or antagonists *(67, 68)* with low micromolar or even submicromolar activity in cell-based assays. These molecular "hits" from screens of the FSH receptor provide encouragement that it will become possible to develop small molecule drugs to regulate gonadotropin receptors.

## Challenges

The earliest studies described at the start of this review were performed in animals. Much of the subsequent work has been done using reductionist approaches including isolated cell lines and, in some cases, isolated proteins. Most of the in vivo studies that have been done employed rodents and procedures initially developed to detect and qualify hormone preparations, rather than to mimic reproductive physiology of humans. These studies have yielded tremendous insight into the molecular properties of these components. But there is an opportunity to augment this work by reinforcing studies set in the appropriate biological context. Possible directions include (a) using complementary transgenic mouse strains in a manner akin to model organism work, as already described by Matzuk and colleagues *(69,70)*; (b) placing additional emphasis on other models, especially non-human primates, other animals with reproductive cycles more similar to human, and other monoovulatory models *(71,72)*. In addition to supporting the development of these models, it may be important to also support the generation of species-specific hormone reagents.

A second challenge is to get the complete structural picture of the hormone–receptor interaction. It has been 30 years since the invention of captopril, the first drug achieved using principles of chemical design *(73)*, and this ensuing time has shown that direct design of drugs is extremely difficult. Although the current hormone and hormone–receptor structures represent great advances, they are very remote from being suitable to support a directed design effort as they lack (a) the active part of the receptor, (b) a clear understanding of mechanism of activity, (c) a clear (or potential) binding site for small-molecule agents. To actually guide small molecule design, the ideal solution is to have one or more structures in which active compounds are bound, with which to facilitate inference of SAR and pharmacophore properties. The idea of using a single structure may be increasingly out of step with more modern models of how proteins work, such as the recent evidence that GPCRs are best modeled as ensembles of structures that may be associated with different aspects of efficacy and stimulus–response coupling *(74)*.

Finally, it may be important to shift the focus from the hormone and receptor to the biological phenomenon in which they participate. There are already examples of how this might lead to more tractable targets for therapeutic intervention such as control of hormone production *(75)* or control of target tissue responsiveness to hormone *(76)*. In his review *(14)* of the use of gonadotropins in clinical practice, Lunenfeld quotes from Hamblen *(77)* an early vision that "to permit effective therapy of hypo-functioning ovaries, a gonadotropin should evoke, in sequence, follicle stimulation, ovulation and corpus luteum development, and these phenomenon should be in physiologic order compatible with fertility and conception." While this vision has largely been

realized using human recombinant gonadotropins, it remains a useful guide for future research and drug discovery in this field.

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