FOLLICLE-STIMULATING HORMONE GLYCOFORM INTERACTIONS WITH
THE FSH RECEPTOR

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FOLLICLE-STIMULATING HORMONE GLYCOFORM INTERACTIONS WITH THE FSH RECEPTOR

The following committee have examined the final copy of this Thesis for form and content and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with a major in Biological Sciences.

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DEDICATION

To my parents, my beautiful wife Maha, my beloved son Marwan, whom I love: with him I am well pleased, my sister, my brothers and to my Egyptian pharaohs ancestors who developed the first system of writing.
Life is journey . . . from birth to death. Countries change, people change, even we change and our thoughts change too, but life keeps going and you have to put up with it!
AKNOWLEDGEMENT

I would like to thank Dr. Bousfield for his endless support. To Dr. Bousfield, I want to sincerely thank you for hosting me at your laboratory and for all the effort you have exerted teaching me structural biology principles and techniques over the last year and half as a graduate student. Joining your laboratory was a beautiful experience. It was so much fun learning all of this techniques from you and from other laboratory individuals. Thank you so very much for everything.

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Finally, I would like to thank my wife and my son. Thank you for supporting me, motivating me and standing being away from you. To Maha, thank you for everything, thank you for being in my life and moreover, I loved you once, I love you still, I always have and always will.
ABSTRACT

Follicle-stimulating hormone (FSH) is a gonadotropic hormone produced by the anterior pituitary, which plays a major role in follicular development leading up to ovulation and spermatogenesis. FSH possesses two dissimilar subunits, α and β, like other members of the glycoprotein hormone family. Human FSH exists as a heterogeneous mixture of glycoforms, differing in the number and location of two β subunit N-glycans. FSH acts through its cognate receptor to initiate a series of physiological events required for granulosa cell proliferation and differentiation in the ovary, as well as Sertoli cell function in the testis. Preliminary studies have documented thiazolidinone derivatives activate FSH receptors expressed in Chinese hamster ovarian (CHO) cells and rat granulosa cells, however, the mechanism of action for this compound is not yet determined. The objective of our studies is to extend the previous investigations regarding one of these molecules, called compound 5, and study its ability to increase FSH/FSH receptor complex internalization rate. We believe that at least a portion of the increased FSH binding in the presence of Compound 5 results from increased internalization of the FSH-receptor complex.

We analyzed the kinetics of FSH/FSH receptor complex endocytosis using $^{125}$I-hFSH$^{21}$, $^{125}$I-hFSH$^{24}$ and $^{125}$I-eFSH glycoform tracers in the presence and absence of Compound 5 using recombinant hFSHR-expressing CHO cells as a model system. We also performed modeling studies of Compound 5 interactions with the FSH receptor, which suggested a potential binding site on the FSH receptor for this molecule, which was distinct from the large N-terminal extracellular domain. This study is the first report of increased internalization rate of FSH/FSH receptor complexes resulting from exposure to Compound 5.
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<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CG</td>
<td>Chorionic Gonadotropin</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts Per Minute</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxyl terminal end of protein</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovarian</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>eFSH</td>
<td>Equine Follicle-Stimulating Hormone</td>
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<tr>
<td>FSH</td>
<td>Follicle-Stimulating Hormone</td>
</tr>
<tr>
<td>FSHR</td>
<td>Follicle-Stimulating Hormone Receptor</td>
</tr>
<tr>
<td>FSHR&lt;sub&gt;ECD&lt;/sub&gt;</td>
<td>FSH receptor extracellular domain</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-Acetylglucosamine</td>
</tr>
<tr>
<td>GRKs</td>
<td>G protein receptor kinases</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G protein coupled receptors</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-Releasing Hormone</td>
</tr>
<tr>
<td>hFSH</td>
<td>Human Follicle-Stimulating Hormone</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
<tr>
<td>LRRs</td>
<td>Leucine-rich repeats</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>ml</td>
<td>Milliliter</td>
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<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>mm</td>
<td>Millimeter</td>
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<tr>
<td>MW</td>
<td>Molecular Weight</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<tr>
<td>nM</td>
<td>Nanomolar</td>
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<tr>
<td>N-terminal</td>
<td>Amino-terminal end of protein</td>
</tr>
<tr>
<td>N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>Pmol</td>
<td>Picomole</td>
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<tr>
<td>QSAR</td>
<td>Quantitative Structure-Activity Relationship</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SRP</td>
<td>Signal recognition particle</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane Domain</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid-Stimulating Hormone</td>
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<tr>
<td>uM</td>
<td>Micromole</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
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# LIST OF SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
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<tbody>
<tr>
<td>$\alpha$</td>
<td>Alpha</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Beta</td>
</tr>
<tr>
<td>$^\circ$</td>
<td>Degree</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Micro</td>
</tr>
<tr>
<td>$\AA$</td>
<td>Angstrom</td>
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Introduction

Gonadotropins comprise a family of glycoprotein hormones responsible for gonadal regulation and metabolism in all vertebrate species. The glycoprotein hormone family, in turn, belongs to the cystine knot growth factor superfamily, which is characterized by the presence of a folding motif, that consists of three disulfide bonds [1]. Two disulfide bonds form a ring, while the third bond passes through the ring. The resulting protein structure consists of two hairpin loops, designated L1 and L3 on one side of the ring and a single loop, L2, on the opposite side. The cystine knot is essential for proper 3D structure because these relatively small proteins have no hydrophobic core [2]. The glycoprotein hormone family includes follicle-stimulating hormone (FSH), luteinizing hormone (LH), chorionic gonadotropin (CG), and one other hormone, thyroid-stimulating hormone (TSH) [1, 3, 4]. The glycoprotein hormone receptors comprise a subgroup of the G protein-coupled receptor (GPCR) family A, which consists of TSH, FSH, and lutropin/choriogonadotropin (LH/CG) receptors [5]. Both FSH and its cognate receptor are N-glycosylated glycoproteins.

Glycosylation is divided into two types. The first type is called N-glycosylation because it involves linkage of an oligosaccharide to a nitrogen atom of an asparagine residue. The second type is called O-glycosylation because it involves linkage to an oxygen atom in threonine or serine residues [6]. In eukaryotes, N-glycan addition to the polypeptide chain occurs in the endoplasmic reticulum (ER), while O-glycan addition and most glycan modifications occur in the Golgi, before the protein is secreted. N-glycosylation usually occurs co-translationally in the ER at the consensus sequon Asn-X-Ser/Thr/Cys where X is any amino acid except proline [7]. In mammalian cells, proteins are typically translocated across the ER membrane in a co-translational mode by the ER protein translocon, comprised of the protein-conducting channel protein, Sec61, and additional complexes involved
in nascent chain processing and translocation [8-10]. As it is in intimate contact with the translocon, the oligosaccharyl-transferase complex catalyzes co-translational N-glycosylation as the protein nascent chain enters the ER lumen [8, 10]. N-glycosylation is often essential for the expression and folding of a glycoprotein [11]. The initial 14-residue glycan, transferred en bloc in the ER, is first degraded to a 10-residue intermediate that is transferred to the Golgi apparatus, where it is further degraded to a 6-residue core, which is then elaborated in the Golgi apparatus. Failure to degrade the high mannose precursor results in high mannose glycans. Initiation, but failure to complete the degradation process leads to hybrid type glycans, while elaboration of the glycan core leads to a large number of possible structures, designated complex glycans [11]. Microheterogeneity, results from populations of structurally heterogenous glycans attached at the same glycosylation site [12]. Glycosylation macroheterogeneity results from the absence of glycans at a specific glycosylation site or sites. FSH N-glycosylation exhibits microheterogeneity in both subunits and macro-heterogeneity in the β subunit (Figs. 1 and 2).

**Follicle-Stimulating Hormone**

FSH is necessary for female gamete production and successful reproduction in all mammals [13]. However, for males the necessity for FSH is species-dependent. In the mouse, FSH and FSH receptor knockouts resulted in reduced testis size, but fully fertile males [14, 15]. Naturally occurring mutations in the hormone or its receptor in human males are associated with sterility due to failed or impaired spermatogenesis [16].

FSH is a heterodimeric protein, consisting of a common α subunit non-covalently associated with a hormone-specific β subunit. Each glycoprotein hormone β subunit is expressed by a separate gene. The individual β subunit structures differ, which provides biological specificity to each hormone. However, sequence homology between β subunits is
observed, specifically at regions responsible for the cystine knot motifs and regions which interact with the common α subunit [17]. The β subunit possesses a fourth loop, which embraces the α subunit and stabilizes the heterodimer. As this loop is secured by a disulfide bond, the term seatbelt loop has been applied to it [18], although some authors use the term cystine noose [19].

Both FSH subunits have two potential N-glycosylation sites and all four are of the Asn-Xaa-Thr type, that should exhibit efficient carbohydrate attachment [7]. The α subunit is always decorated with N-glycans at both sites in FSH and all other glycoprotein hormones [20]. Surprisingly, FSHβ glycosylation exhibits partial glycosylation at both potential glycosylation sites, which can affect FSH biological activity because glycans at each glycosylation site have specific functions (Fig. 1).

**Figure 1. FSH glycosylation microheterogeneity and function.** The glycans localized at each N-glycosylation site by glycopeptide mass spectrometry are shown diagrammatically [21, 22]. The functions for each glycan are indicated. Drawing provided courtesy of Dr. Bousfield.
Asn\textsuperscript{52} oligosaccharides are essential for full activation of the LH and FSH receptors [23-25]. Asn\textsuperscript{78} glycans are essential for α subunit folding and stability, as they stabilize the hairpin turns of loops L1 and L3 [26], which are stabilized by a disulfide bond in the β subunit [19]. The FSHβ N-glycans both prolong survival in the circulation [27] and appear to attenuate receptor activation, possibly by limiting the number of FSH binding sites that can be occupied [28]. At the turn of the century, our laboratory discovered FSHβ variants resulting from loss of either one or both of the FSHβ N-glycans [20]. Eventually, four hFSH variants were identified by differences in mobility detected in FSHβ western blots (Fig. 2). FSH\textsuperscript{24} possessed all 4 potential N-glycans and was the most abundant glycoform in most hFSH preparations [29-31]. FSH\textsuperscript{21}, the first glycoform identified, lacked βAsn\textsuperscript{24} glycan, and was also observed in most hFSH preparations. FSH\textsuperscript{18} lacked βAsn\textsuperscript{7} glycan, but was only detected in FSH\textsuperscript{21}-enriched preparations. FSH\textsuperscript{15} lacked both FSHβ glycans and has only been detected by mass spectrometry.

**Figure 2. Human FSH glycoform models.** The FSHA (green) and FSHβ (yellow) subunits are shown as cartoons. The N-glycans are shown as red spheres and the same basic glycan is shown at each position. A. hFSH\textsuperscript{24}, which all 4 N-glycans are attached to the 4 potential glycosylation sites (each Asn residue
represented as blue spheres). B. hFSH\textsuperscript{21} lacking βAsn\textsuperscript{24} glycan. C. hFSH\textsuperscript{18} lacking βAsn\textsuperscript{7} glycan. D. hFSH\textsuperscript{15} lacking both βAsn\textsuperscript{7&24} glycans.

FSH isoforms were discovered by evaluating FSH size heterogeneity in rhesus monkey pituitary extracts via Sephadex G-100 gel filtration chromatography, which appeared to reflect changes in sialic acid [32, 33]. Subsequently, FSH isoforms were studied by charge separation techniques, such as zone electrophoresis, isoelectric focusing, and chromatofocusing, to exploit the differential charges provided by variation in sialic acid content [34]. Our laboratory tried to use chromatofocusing to separate biantennary FSH glycans from tri- and tetra-antennary glycans. Instead, FSHβ glycosylation variants were discovered [35]. Subsequently, when FSH glycoforms, purified by chromatofocusing, were evaluated by western blotting, most isoform fractions consisted of mixtures of hFSH\textsuperscript{21} and hFSH\textsuperscript{24} [35, 36]. Glycopeptide mass spectrometry showed the glycan populations at αAsn\textsuperscript{52} and βAsn\textsuperscript{24} were virtually identical in all isoform fractions, which contradicted the underlying assumption that FSH charge variants differ in the oligosaccharide populations attached to each glycosylation site [36]. It is quite difficult to show FSH glycosylation macroheterogeneity representing hFSH glycoforms combined with microheterogeneity resulting from the 30 over 100 glycans attached to the four Asn residues on both α and β subunits. One approach combined glycans detected by site-specific glycopeptide mass spectrometry with the 3D structure of hFSH (Fig. 1). However, these studies underestimated the number of glycans present and provided a potentially misleading impression that glycan populations differed at each site. A more recent mass spectrometric analysis of oligosaccharides released in a site-specific manner from both FSHα subunit glycosylation sites and from either Asn\textsuperscript{7} or both FSHβ glycosylation sites indicated glycan populations were largely identical on the same subunit but differed in that fucose was almost completely lacking in FSHα glycans, while fucose was found in most FSHβ glycans. FSH macro and microhet-
Heterogeneity are illustrated in Fig. 3, which combines elements of Figs. 1 and 2. The resulting figure illustrates only three glycoforms, FSH$^{24}$, FSH$^{21}$, and FSH$^{18}$, as these appear to be the physiologically relevant FSH variants [37]. The same glycan populations were found on both FSH$\alpha$ glycosylation sites. The situation was complicated for the FSH$\beta$ subunits, as the FSH$\beta^{24}$ glycans were removed from both Asn residues simultaneously. The argument that both populations were the same resulted from FSH$\beta^{21}$ possessing the same glycan population as FSH$\beta^{24}$ even though the former was derived from a single N-glycosylation site, while the latter were derived from two.

![Figure 3. Macroheterogeneity and microheterogeneity in FSH glycoforms.](image)

Recent studies showed that a hypo-glycosylated hFSH$^{21/18}$ preparation consisting of a mixture of both hypo-glycosylated glycoforms, FSH$^{21}$ and FSH$^{18}$, that was derived from hLH preparations, was more active than fully-glycosylated hFSH (hFSH$^{24}$) in vitro [38]. Specifically, this highly active hFSH$^{21/18}$ preparation exhibited 10-fold higher apparent af-
finity for FSHR binding than highly purified hFSH\textsuperscript{24}, associated more rapidly with FSH receptors [30] and occupied 2- to 3-fold more FSH binding sites than hFSH\textsuperscript{24} [28]. In the KGN human granulosa tumor cell line, a recombinant GH\textsubscript{3}-hFSH\textsuperscript{21/18} preparation was also significantly more active than hFSH\textsuperscript{24}, consistent with its greater FSHR binding activity [39].

Another naturally occurring FSH glycosylation variant, eFSH, is used in our laboratory as a surrogate for the most active hypo-glycosylated human FSH preparations, because it is even more active than hLH-derived hFSH\textsuperscript{21/18} [38]. All seven eFSH preparations purified in this laboratory consist of >90% hFSH\textsuperscript{18} and <10% hFSH\textsuperscript{24} (Dr. G.R. Bousfield, Wichita State University, personal communication).

\textit{Follicle-Stimulating Hormone Receptor (FSHR)}

The glycoprotein hormone receptors, form a subgroup of GPCR family A, which consists of TSH, FSH, and lutropin/choriogonadotropin (LH/CG) receptors [5]. The general structural topology of the glycoprotein hormone receptors is similar to that of other GPCRs. While the N-terminal extracellular region, or ectodomain is much larger than a typical family A GPCR, the intracellular C-terminal loop, with intervening transmembrane domain, consisting of 7 transmembrane (TM) helices connected by 3 intracellular loops and 3 extracellular loops, follow the highly conserved pattern found in other GPCRs [5]. The high affinity binding region for the hormones, TSH, LH, CG and FSH [40], was identified experimentally localized to the ectodomain leucine-rich repeats (LRRs) [41-43]. The primary structure of the human FSHR consists of 694 residues, including a 17-residue leader sequence [44]. In contrast, the human rhodopsin receptor consists of only 348 residues. All known glycoprotein hormone receptors possess a large N-terminal ectodomain, belonging to the leucine-rich repeat family [45]. In the human FSHR, this domain comprises the first 366 residues of the primary structure. The ectodomains can be divided into two subdo-
mains: a high affinity hormone binding subdomain primarily found in the N-terminal region, and a second subdomain located on the C-terminal region of the ectodomain, that possesses a sulfated tyrosine residue associated with receptor activation [45]. Both subdomains form an integral structure consisting of leucine-rich repeats [45]. This is followed by a partially characterized loop possessing the sulfated tyrosine residue that engages a gap between the FSH α and β subunits. The sTyr binding site appears as a consequence of engaging the high affinity binding site in the N-terminal subdomain. The ectodomain is an important drug target for the development of novel therapeutics for assisted reproduction [46]. The first crystal structure for the FSHR high affinity FSH binding site complexed with FSH (PDB code: 1XWD) was published in 2005 [19]. Subsequently, the structural features of the FSHR LRRs were confirmed by crystal structures of complexes between the TSHR LRRs (PDB code: 3G04) and activating [47] or inactivating [48] autoantibodies (PDB code: 2XWT), respectively.

The most recent crystal structure of FSH bound to the entire FSHR ectodomain provided new possibilities for understanding how FSH binds to and activates its cognate receptor [49]. Given the importance of FSH-FSHR activation in regulating fertility, much effort is expended investigating the physiological interaction between this ligand and receptor and how glycosylation of FSH affects or modulates its activity. Determination of how glycans affect FSH binding to its receptor and how different FSH glycoforms interact with their cognate receptor will give us better insight into ligand-receptor interaction, which is of pivotal importance for designing new ligands with therapeutic potential. In order to study these interactions, the three-dimensional structure of the target receptor can be most helpful. In this regard we will use the structure of the complex consisting of FSH bound to the entire ectodomain of the FSHR (PDB code: 4AY9). One of the FSH ligand models will
be used to build human FSH glycoform models. Each model will be docked to monomeric and trimeric forms of the FSHR\textsubscript{ECD}. The docking experiments will help us understand how each ligand is introduced to the receptor and how the glycans may affect their binding.

Recent studies to unveil the mystery of the human FSHR structure and function suggest that reevaluation of the regulatory effects of FSH glycosylation on FSHR binding and signaling is a must. FSHR was first considered to exist as a monomer [50] with a mature receptor molecular weight of 74 kDa [51]. Fluorescence energy transfer and pull-down experiments have revealed it to possess at least a dimeric form [52-54]. However, there is biochemical evidence that hFSHRs exist as higher order combinations [53, 54]. Furthermore, the crystal structure of the complete FSHR extracellular domain revealed a trimeric structure [49]. Although the FSHR high affinity site dimeric model showed receptor dimerization via the extracellular domain in the crystal structure and provided ultracentrifugation evidence that such a dimers could exist in solution [19], this model was not supported by studies aimed at testing the dimerization mechanism [54]. The more recent complete FSHR\textsubscript{ECD}/FSH complex crystal structure consists of FSHR amino acid residues 17-291 and 331-357. In addition to the known hormone binding site, the FSHR\textsubscript{ECD} structure reveals an additional interaction with the hormone via a conserved sulfotyrosine (sTyr) residue at position 335 [49]. Tyrosine sulfation is already known as mandatory for receptor activation, and it had been reported that a sTyr motif was located downstream of the leucine-rich repeats (LRRs), close to the top of the first transmembrane helix [55]. This post-translational modification was shown to be important for high-affinity hormone binding and receptor activation [55]. Furthermore, it has been reported in the same study that when FSH first binds to the high affinity hormone-binding subdomain of FSHR it undergoes a conformational change forming a sTyr-binding pocket. Subsequent FSHR sTyr binding to the FSH pocket in the second step of a two-step mechanism eventually leads to receptor activation.
Jiang et al. [49] reported that the FSHR<sub>ECD</sub> may exist as a trimer, at least in the crystal structure. This trimeric model provides a means to rationalize the mechanism for αAsn<sup>52</sup> glycosylation effects on FSH binding to its receptor [46]. In the proposed trimeric FSHR model αAsn<sup>52</sup> potentially inhibited FSH binding because only one glycan could occupy the central pocket formed by the trimer at a time. They subsequently reported that FSHR bound three times more selectively deglycosylated αAsn<sup>52</sup>-mutant-FSH than fully-glycosylated recombinant FSH [46]. However, αAsn<sup>52</sup>-deglycosylated FSH is not a naturally occurring variation in hFSH glycosylation. FSHβ subunits lacking either one of the two potential N-glycans also exhibited 2- to 3-fold greater binding to rat, human, and bovine FSHRs [38] despite possessing αAsn<sup>52</sup> glycans that should prevent more than one FSH ligand from binding to each FSHR complex. For both LH and FSH receptors, αAsn<sup>52</sup> glycans are essential for maximal signal transduction and they may also play a role in heterodimer stability [23-25]. The latter has been suggested as the reason for partial activation of LHR by αAsn<sup>52</sup>-dg-hCG [46]. The fact that both α and β subunit N-glycans appear to reduce the number of FSH binding sites available gave us the idea to study the effect of glycosylation on FSH-FSHR binding using molecular modeling. Such an investigation could help explain the negative cooperativity observed for glycoprotein hormones binding to their receptor [52]. Negative cooperativity implies binding of FSH to a high-affinity site of an already occupied receptor, which requires that the receptor be at least in the dimeric state and the second FSH ligand binding would necessarily trigger a conformational change in the receptor that would result in the dissociation of bound FSH [52]. However, the mechanism of how FSH glycoforms interact with their cognate receptor is yet to be determined.


**Trafficking of the FSH/FSHR complex**

Although there are a number of studies characterizing the internalization and intracellular sorting of gonadotropins [56-64], available information on the fate of the FSH-FSHR complex is much more limited and it is not clear if internalization of FSHR occurs constitutively in the absence of ligand occupancy [63-66]. During the past few years, several studies have elucidated some features of the post-endocytotic trafficking of the porcine, rat, mouse and human gonadotropin receptors [57, 59, 61, 63, 64, 67, 68]. FSHR displays a polarized distribution in its target cells [69] and also can mediate the transcytotic transfer of their respective ligands from the basolateral to the apical surface [65, 70]. Furthermore, most of the internalized human FSH/FSHR complexes accumulate in endosomes and subsequently recycle back to the cell surface [63]. Generally, FSHR internalization is initiated by phosphorylation by G protein receptor kinases (GRK), recruitment of arrestin, accumulation in clathrin-coated pits, endocytosis, sorting, and recycling [64]. FSH binding to FSHR induces the exchange of GDP for GTP from the stimulatory subunit (Gαs) of the Gs protein heterotrimer [64]. Upon binding GTP, the G protein heterotrimer dissociates and Gαs activates adenylate cyclase, while the βγ dimer activates phospholipase Cβ and recruits GRKs. These are the canonical pathways for FSH activation [64]. The FSHR intracellular loops 1 and 3 are subject to phosphorylation by GRKs and subsequent arrestin binding [64]. Moreover, FSHR undergoes ubiquitination, enabling its sorting into recycling vesicles with bound FSH or fused with lysosomes where unliganded FSH is degraded [64]. In principle, the recycled unoccupied receptor can engage FSH for a second round of activation [64].

We studied FSH/FSHR complex internalization rates using a CHO-cell line expressing recombinant hFSHR as a model system [63, 71]. The initial goal was to compare an unamplified response to FSH binding in an effort to confirm faster receptor binding on
the part of hypo-glycosylated FSH was attended by rapid receptor activation. We also evaluated Compound 5, an allosteric modulator of FSHR activity for its effect on FSH/FSHR internalization [72].

**Compound 5 as an allosteric modulator of FSHR**

The pituitary glycoprotein hormones act through their cognate receptors to initiate a series of physiological events that result in germ cell maturation [72]. Regarding the importance of FSH in regulating folliculogenesis and fertility, the development of FSH mimetics has been sought to use in fertility treatments [72]. In 2006, Yanofsky et al.[72] first reported a high affinity small molecule agonist that activated glycoprotein hormone receptors through an allosteric mechanism [72]. In their study, they used thiazolidinone compounds numbered from 1 to 5 [72]. Two of these were reported to bind to the FSHR 7TM domain [72, 73] unlike FSH, which binds the FSHR ectodomain. It has been reported that only a small portion of the FSHR, spanning TM1 through TM3, is sufficient to elicit a response to the thiazolidinone compounds [73]. Recently, it has been reported that Compound 5 (Fig. 4) stimulated cAMP production in a dose-dependent manner, activated AKT and ERK signaling pathways, and induced estradiol production in cultured rat granulosa cells [74].

![Figure 4. Compound 5.](image-url)
Initial studies with Compound 5 in CHO-cells expressing recombinant human FSH, LH or TSH receptors documented selective activation of the FSH receptor, but neither LH nor TSH receptors [72]. In CHO cells that express recombinant FSHR, Compound 5 stimulated cAMP production, and recruited β-arrestin in a dose-dependent manner [74]. Receptor activation by Compound 5 was reportedly brought about by occupying an independent binding site on the FSHR, in a region spanning TM1 through TM3 [72]. This site was identified by creating chimeric receptors combining portions of non-responsive TSHR with complementary portions of Compound 5-responsive FSHR. A question remaining from these studies was whether the compound interacted with the transmembrane helices or with exoloop 1. When a different non-steroidal allosteric modulator was used to study changes in FSHR internalization rate, no changes in FSHR internalization rate were encountered when a largely FSH${^{24}}$ preparation was used as tracer [75]. However, in the presence of Compound 5, an increase in pH 3-resistant binding seemed to account for much of the increased binding associated with $^{125}$I-eFSH. Given this information, we decided to study the effect of Compound 5 on FSH glycoform-induced FSHR internalization using the classic approach with cultured CHO-cells expressing the hFSHR. We also employed molecular modeling and homology modeling to build a FSHR 7TM domain model and docked Compound 5 to identify a potential allosteric binding pocket. In this regard, we created, improved and refined homology model for the human FSHR based on the crystal structure of the β2 adrenergic receptor (β2AR) (PDB code: 3SN6) [76] as a template, as there is no human FSHR crystal structure available yet. β2AR was chosen as a template as it shares as many residues with glycoprotein hormone receptors 7TM domains as any other template structure according to the alignment [45], it’s the only G-protein coupled receptor with a known structure in an active state in complex with G-protein αβγ heterotrimer (PDB code:
Furthermore, β2AR binds external agonists and antagonists; therefore it is an appropriate model for drug discovery [45].

**Molecular Modeling: An Introduction**

Molecular modeling is one of the fastest growing fields in science [77]. It may vary from building and visualizing simple molecules in three dimensions (3D) to performing complex computer simulations on large proteins and nanostructures [77]. Molecular modeling is a collection of computer-based techniques for driving, representing and manipulating the structures and reactions of molecules, and those properties that are dependent on 3D structure [77]. Techniques in molecular modeling are applicable to computational chemistry, drug design, computational biology, nanostructures, and material science [77]. In scientific studies, models are built after simplification of the object or event they intend to represent. These should be verified and, if necessary, modified [78]. Protein modeling is the prediction of a three-dimensional structure for a protein for which only the amino acid sequence is known [78]. Either comparative modeling (homology modeling) is used, with the use of a similar protein structure [79], or predicting the three-dimensional structure of a protein directly from its amino acid sequence [80]. These two methods are widely applied in the field of molecular modeling. Moreover, molecular modeling is used to investigate the structure, dynamics, surface properties and thermodynamics of inorganic, biological and polymeric systems. Docking methods are essentially computer algorithms that give information on the possible binding site and binding mode of a ligand to a macromolecule or about macromolecule interactions with each other, such as protein-protein interactions [81]. Each docked compound is given a score, which is used to select the potentially most active compounds [82]. Virtual screening is the process of searching for potential ligands of a particular target between thousands or even millions of compounds that are synthesized in silico (in computer) [83]. QSAR (quantitative structure-activity relationship) is also used in
defining effective structural properties of potential drugs. This method finds a suitable equation that would summarize the physicochemical properties of a set of ligands. The resulting equation could then be used in order to predict the effectiveness in terms of activation or inhibition of novel compounds [84]. Each of these techniques is widely used and a combination of these methods is usually applied when using molecular modeling.

**Energy minimization**

The method, which minimizes the potential energy of a molecular model, is known as energy minimization. This technique is used to optimize a molecular structure to find the local minimum starting from an initial conformation. Energy minimization results in an optimized arrangement of electrostatic interactions, hydrogen bonding and van der Waals contacts (based on the initial structure). After the sequence structure alignment, the insertion of loop regions and addition of amino acid side-chains, the protein itself is complete in terms of all atoms being present, but there will often remain steric clashes and distorted bonds in the resulting models. Therefore, the goal of an energy minimization is to relax the worst conflicts in the resulting structure and find an energetically more favorable conformation of the system in order to be able to start simulation and docking studies [85].

**Docking Studies**

The ability of a protein to bind to another protein or to a different ligand in a highly specific manner is an important feature of many biological processes. The characterization of the structure and the energetics of molecular complexes is thus a key factor for understanding biological functions. Therefore, molecular docking is a key tool in structural molecular biology and computer-assisted drug design. This strategy used to predict the predominant binding mode, and affinity of a ligand with a protein of known 3D structure or a homology model of a related protein for which no 3D structure exists.
Hypotheses

Our studies had four specific aims, each testing a different hypothesis.

**Aim 1: Characterize the dissociation of FSHR dimer/oligomers into monomers.**

Our *working hypothesis* is that hFSH binding can change the conformation of FSHRs, thereby increasing the number of binding sites for FSH by dissociating oligomers into monomers.

**Aim 2: Model FSH glycoform interaction with the FSH receptor ectodomain.**

Our *working hypothesis* is that the glycosylation status of each FSH glycoform affects its binding to the FSH receptor. We examined how different FSH glycoform models interacted with the FSH receptor ectodomain, as a complete receptor model was not available.

**Aim 3: Study the effects of Compound 5 on the internalization rates for FSH glycoforms bound to the FSHR.** Our *working hypothesis* is that at least a portion of the increased FSH binding in the presence of compound 5 results from increased internalization of the FSH-receptor complex.

**Aim 4: Model Compound 5 interaction with the FSH receptor.** Our *working hypothesis* is that Compound 5, binds exclusively to the FSHR transmembrane helices, either TM2 or TM3. We used homology modeling and docking studies to propose a putative binding site of Compound 5 in the receptor TMD.
Materials and Methods

Materials

The hormone preparations, hFSH\textsuperscript{24}, hFSH\textsuperscript{21}, and eFSH were prepared in our laboratory [31, 86]. The small molecule FSHR modulator, compound 5, was obtained from EMD Serono, Billerica, MA [87].

SDS-PAGE and western blot analysis

Cells were solubilized with RIPA buffer (phosphate-buffered saline, 1% nonidet P-40, 0.5% sodium deoxycholate, 1% SDS). Membranes were isolated from CHO cells by centrifugation following homogenization in 0.25 M Sucrose, 20 mM Tris, pH 7 [88]. The membrane preparations from 5 million cells were resuspended in 500 µl RIPA buffer. The soluble supernatants were diluted with 5X SDS sample buffer. Samples were subjected to SDS-PAGE using 0.75 mm thick 7.5 % polyacrylamide slab gels prepared using the Bio-Rad (Hercules, CA) Mini-PROTEAN II gel casting system, under reducing conditions [89]. Each well was loaded with 10 µl, which represented 80,000 cells or with 10 µl Bio-Rad MW prestained protein standards (Hercules, CA). Once the gels were loaded, they were placed in a Promega mini gel apparatus, supplemented with cold running buffer and allowed to run for 45 minutes at 200 V. Following electrophoresis, the gel apparatus was disassembled and the gels were placed in a blotting sandwich consisting of a GE Healthcare (Piscataway, NJ) ImmobilonP polyvinylidene fluoride (PVDF) membrane sandwiched between two pieces of Whatman filter paper. Proteins were transferred from the polyacrylamide gel to PVDF membrane by electroblotting for 2 hours at 100 V at 4°C, with constant stirring [29]. Following electroblotting, the transfer apparatus was disassembled, and the PVDF membrane washed for 5 minutes each, with three changes of distilled water, and allowed to dry overnight.
Western blotting followed our published protocol [38]. Dried blots were probed with a solution of 5% dried non-fat milk dissolved in western blot buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 0.05% Tween 20) containing anti-hFSHR monoclonal antibody, 106.105 (diluted 1:2500), for one hour. Following incubation with the primary antibody, the membrane was rinsed with Millipore Elix water three times for five minutes each on a shaking table. Following washing, the bound primary antibody on the blot was probed for 30 minutes with goat anti-mouse IgG-HRP complex (Invitrogen, Grand Island, NY), diluted 1:2500, in 5% dried non-fat milk in western blot buffer. Following incubation with the secondary antibody, the blot was rinsed three times with water following the same protocol listed above. Following the rinse, each blot was treated with Amersham ECL Plus western blot detection reagents (GE Healthcare Life Sciences, Piscataway, NJ), as per the manufacturer’s protocol. The resulting chemiluminescence was measured using a Bio-Rad (Hercules, CA) VersaDoc 4000 imaging system. Immunoreactive band density was determined using the software package Quantity One (Bio-Rad, Hercules, CA) running on an Apple (Cupertino, CA) Macintosh G4 computer under the OS X operating system.

Blue Native-PAGE and western blot analysis

Cells were solubilized with RIPA buffer, as described above. The soluble supernatant was diluted 1:1 with Blue Native sample buffer. Samples were subjected to BN-PAGE using 10% polyacrylamide slab gels [90, 91], then transferred to a PVDF membrane and probed with anti-hFSHR monoclonal antibody, 106.105, following the same protocol as described in the previous section.

Comparing the internalization rate of hFSH with eFSH

Samples of FSH glycoforms were iodinated by Dr. Vladimir Butnev using the Chloramine T procedure [92] modified for FSH [93]. The specific activities of hFSH24, hFSH21, and eFSH tracers were 35.6, 40.5, and 45.3 µCi/µg, respectively.
Internalization was measured following a standard protocol using plated cells expressing FSHR [94, 95]. We used a CHO cell line that was transformed to express hFSHR attached to 12-well tissue culture plates. In each well, we cultured 5 million CHO cells and allowed them to plate down overnight in F12 cell culture medium, in a 37°C incubator (Thermo Scientific, Asheville, NC). The next day, we aspirated the culture medium, then added radioligand assay (RLA) buffer containing one of the 3 tracers alone, or combined with a FSHR allosteric modulator, Compound 5 or the DMSO carrier, for 3, 6, 9, 12 and 15 minutes at 37°C. The tracers used were $^{125}$I-hFSH$_{21}$, $^{125}$I-hFSH$_{24}$, or $^{125}$I-eFSH at a concentration of 25 ng/ml. Non-specific binding was determined in the presence of 1000-fold excess unlabeled FSH. Incubations were carried out in a tissue culture incubator (Thermo Scientific, Asheville, NC). The final concentration of Compound 5 was 1 µM. Following addition of the last tracer samples, unbound tracer was removed by aspiration and each well rinsed twice with 1 ml RLA buffer. Surface-bound hormone, was released by incubating the cell with 4°C isotonic, pH 3, buffer (50 mM glycine; 100 mM NaCl, pH 3.0) [64] for 2-4 minutes, then collecting the buffer in 12 x 75 mm tubes to count the released hormone. Cells were then washed with 1 ml RLA buffer before solubilization with 250 µl of 0.1 N NaOH [63]. The cell extracts were placed in 12 x 75 mm tubes to determine the amount of hormone that was internalized. Both sets of tubes were counted in a Perkin-Elmer (Waltham, MA) model 2040 Wizard$^2$ gamma counter, with a counting efficiency of 80%. Internalization was expressed as the ratio of internalized and surface specifically bound $^{125}$I-FSH at each time point. The rate of internalization (ke) was calculated from the slope of the line of a plot of the internalized radioactivity vs. the surface bound hormone [96]. The slopes of these lines were calculated by linear regression analysis using the software program Prism 5 for Macintosh (GraphPad, Inc., San Diego, CA). Data from three experiments were analyzed by two-way ANOVA using Prism 5.
Modeling studies on human FSH glycoform interaction with the human FSHR

- **Human FSH glycoform model building**

  Human FSH was extracted from PDB code 4AY9 [49], including 4 GlcNAc residues to serve as a carbohydrate fragment templates, using PyMol (Schroedinger, Inc., New York City, NY) [97]. The extracted, single-molecule FSH pdb file was uploaded to the GlyProt Web-based tool (http://www.glycosciences.de/modeling/glyprot_/php/main.php) [98]. This tool identified 4 potential N-glycosylation sites, then added 4 identical complex N-glycans (GlcNAc4Man3Gal2Neu5Ac2) to the protein model and the resulting glycoprotein model was subjected to adding hydrogen atoms and apply energy minimization. The resulting glycoprotein model was rendered with PyMol to illustrate a theoretical fully-glycosylated hFSH24 molecule. Four other glycoform models were prepared by omitting the addition of one or two N-glycans: hFSH21 (omitting βAsn24 glycan), hFSH18, (omitting βAsn7 glycan) hFSH15, (omitting βAsn7 and βAsn24 glycans), and N52dg-hFSH (omitting αAsn52 glycan).

- **Model preparation**

  The hormone and receptor fragment models were prepared for the docking process. This involved deletion of the natural ligand and water, addition of missing hydrogen atoms, terminal residue treatment, and energy minimization of the hydrogen atoms, keeping heavy atoms fixed. Mostly, energy minimization ended up finding a local minimum structure. To avoid ending up in a local minimum, the system was heated in a subsequent step [99]. Monomeric and trimeric FSHR_ECD models were prepared in the same manner.

- **Identification of binding site**

  The hormone binding site, was determined using Tripos Sybyl-X v 2.1 (Certara USA, Inc., St Louis, MO) by docking the human FSH glycoforms to the receptor. Proto-
mols (opaque contour surfaces in electrostatic potential mode) are the binding site of the receptor and were generated by selecting the specific residues present within the ligand-binding pocket. Alternatively, the docking software algorithm checked the surface of the receptor for prospective pockets where ligands were likely to bind. The protomol generation was performed using the Surflexdoc module of Sybyl-X that employed a threshold setting of 0.5, Bloat setting of 2.0 and Radius of 3 Å [100].

- **FSH Docking**

  The FSH glycoform models were prepared, as above, using GlycoProt. To each model 2-3 complex N-glycans were attached these models and were docked to the FSHR_{ECD} monomeric or trimeric models using Sybyl-X. The selected poses represented a single FSH glycoform docked to either FSHR_{ECD} monomeric or trimeric models to study the effects of glycans on FSH binding to its cognate receptor. Docking was performed using the Surflexdoc module of Sybyl-X and allowing complex flexibility, while keeping heavy atoms fixed.

**Homology modeling and docking studies of Compound 5 interaction with the FSHR**

- **Target identification**

  The active, Gαs-bound model of the β2AR (PDB code: 3SN6) [76] was chosen as the template to build the FSHR 7TM domain model for the reasons mentioned in the introduction [45]. We also prepared a complete model of human FSHR, which was built using Sybyl-X to combine the FSHR ectodmain (PDB code: 4AY9) with the 7TM homology model. The two models were used to identify potential Compound 5 binding sites.

- **Sequence alignment and Homology modeling**

  The corresponding sequences of FSHR and β2AR were retrieved from the UniProtKB database ([http://www.uniprot.org/](http://www.uniprot.org/)) [101]. The sequences were aligned using the
align tool available at the UniprotKB website. The online protein model builder Expassy SWISS Model workspace (http://swissmodel.expasy.org/workspace/) [102, 103] automated homology model generation module was employed to build the FSHR-TMD model. An automated Blast search was performed for a suitable template for the sequences submitted for model generation. Model builder performed an energy minimization of the final model. The models were validated using QMEAN and all the amino acid residues had acceptable scores above zero.

- **Model validation**

  Although all available information had been integrated during generation of the homology models, errors inevitably occurred that reduced the applicability of the models. These included errors in the target template alignment, errors in loop regions due to lack of structural information and low stereochemical quality. The geometrical parameters of the created models were evaluated and compared with those obtained from the native structure of the templates using the PROCHECK program [104, 105].

- **Identification of a potential allosteric site in the FSHR model**

  The potential allosteric site was determined using Sybyl-X by docking Compound 5 onto each receptor model. Protomols (opaque contour surface in electrostatic potential mode) were generated by selecting specific residues present within the ligand-binding pocket of the created model. Alternatively, the docking software algorithm checked the surface of the receptor for any prospective pocket where ligand was likely to bind. The protomol generation was performed using the Surflexdoc module of Sybyl-X employing a threshold of 0.5, Bloat of 2.0 and Radius of 3 Å [100].
Docking

The Compound 5 model was prepared by Pymol and was docked against the FSHR transmembrane domain and FSHR_ECD monomer models using Sybyl-X. The selected poses each represented a single Compound 5 molecule docked to the model. Docking was performed using the Surflexdoc module of Sybyl-X allowing complex flexibility and keeping heavy atoms fixed.
Results

**Aim 1: Characterize the dissociation of FSHR dimer/oligomers into monomers.** We examined the electrophoretic patterns of FSHR dimers/oligomers using western blots probed with monoclonal antibody 106.105, which is an anti-hFSHR antibody that recognizes residues 300-315 of the hFSHR [106]. As hFSH21 exhibited greater binding to FSHR than FSH24 [30] and the trimeric model indicated only one FSH molecule could bind per FSHR trimer, we hypothesized that greater hFSH21 binding may result from dissociation of FSHR dimer/oligomers. First, we examined how much extract was necessary to detect the FSHR in western blotting experiments using samples derived from solubilizing 5 million CHO-hFSHR cells. We were able to detect FSHR in samples representing 0.4-2.4% of the extract, which was equivalent to 20,000-120,000 cells. In the course of this study, we also found that boiling the samples resulted in aggregation of the FSHR receptor (Fig. 5A).

In the next experiment, we compared several buffers to see which would be suitable to extract the FSHR (Fig. 5B). We followed the recommendation to heat the samples at 60°C for 5 minutes, rather than 100°C for 2 minutes, before electrophoresis [64]. All extracts treated at the lower temperature produced a major band of immunoreactivity that migrated between 75 and 82 kDa (see Fig. 5B, lanes 2, 4, and 6). Boiling resulted in a mixture of, high MW immunoactivity greater than 120 kDa along with an apparent receptor fragment band at 50 kDa (Fig. 5B, lanes 3 and 7). RIPA solubilizing buffer produced a single 77 kDa band for the presumed monomeric FSHR. The PBS and Igebal extracts both exhibited two bands, an apparently more abundant 78 kDa band along with a less abundant 73 kDa band (Fig. 5B lanes 4 and 6). The immunoreactive patterns resembled those previously reported for Igebal-extracted cells expressing hFSHR [53]. No immunoactivity was present in the boiled PBS extract sample lane,
while the sample denatured at 60°C possessed detectable FSHR. The reason for this discrepancy is unknown, as the experiment was not repeated.

Figure 5. Western blot analysis of denatured FSHR preparations. A. Human FSH receptor western blot of increasing amounts of RIPA buffer extracts of 5 million CHO cells expressing hFSH receptor (0.1 ml/million cells). Lane 1, BioRad 5 µl MW markers; lane 2, 2.5 µl CHO-hFSHR cell extract representing 20,000 cells; lane 3, 5 µl cell extract or 40,000 cells; lane 4, 10 µl cell extract or 80,000 cells; lane 5, 15 µl cell extract or 120,000 cells. B. Effects of temperature and solubilizing buffer on migration of the FSHR. Lane 2, RIPA buffer extract heated to 60°C; lane 3, RIPA buffer extract boiled; lane 4, PBS extract heated to 60°C; lane 5, PBS extract boiled; lane 6, Igebal-doc extract heated to 60°C; lane 7, Igebal-doc extract boiled. All lanes were loaded with the same concentration (13.75 µg/5 µl).

We compared SDS-PAGE with blue native gel electrophoresis (Fig. 6). Once again, SDS-PAGE of reduced samples showed only 77 kDa monomeric receptor and 50 kDa receptor fragment bands (Fig. 6A). There was some high molecular weight immunoreactivity, however, no discrete forms were obvious, making it impossible to quantify them. When blue native gel electrophoresis was employed, only a high molecular weight FSHR band was detected in each extract (Fig. 6B). Curiously, the intact cell band (lane 1) migrated more slowly than the membrane fraction band (lane 2). We were not able to detect the dissociation of FSHR dimer/oligomers to monomers as neither electrophoretic system could detect both forms of the receptor.
Figure 6. Comparison of hFSHR separated by SDS-PAGE and Blue Native PAGE. A Western blot of SDS-PAGE separated FSHR isolated from intact CHO-hFSHR cells or membrane fractions derived from these cells. Lane 1, MW markers, as indicated; lane 2, CHO-hFSHR cell extract (5 million cell extract); lanes 3 & 4, membrane fractions solubilized in RIPA buffer); lane 5, untransformed CHO cells; lane 6, MW markers. All lanes were loaded with the cell or membrane 10 µl extract equivalent to 500,000 cells. B. Western blot analysis of Blue Native electrophoretically separated hFSHR extracted from CHO-hFSHR cells or membrane fractions. Lane 1, CHO-hFSHR cell extract; lane 2, CHO-hFSHR membrane fraction; lane 3, wt CHO cell extract; lane 4, MW markers. All lanes were loaded with 10 µl extract equivalent to 500,000 cells.

Aim 2: Model FSH glycoform interaction with the FSH receptor ectodomain.

We now report the results of docking studies involving human FSH glycoform models interacting with a nearly complete extracellular domain model of their cognate receptor. First, we separated the 3 FSH ligands from the trimeric receptor complex shown in Fig. 7A. This step revealed the FSHR entire ectodomain trimeric structure, containing 3 FSH binding sites (one of these sites is highlighted as the protomol Fig. 7D). The C-terminal end of the FSHR_ECD that connects it to the TMD is highlighted. The FSH binding sites were identified in two studies [19, 49]. The model also includes the putative hinge region (arrow), which was reported as an essential region for signal transduction [107]. The hinge region did not form a separate region, as was anticipated, rather, most of it formed an integral part of the FSHR_ECD in the form of an extension of the LLR region [49]. This region also in-
cludes a large loop possessing the sulfotyrosine residue, which is required for activation of this receptor family [55].

**Figure 7. Receptor preparation and protomol generation.** A. The structures were extracted from the FSH-FSHR_{ECD} complex (PDB code 4AY9) as the search model. The receptor ECD trimer is shown as a green surface, the FSH peptide moiety as a red surface and FSH GlcNAc residues as blue surfaces. B. The FSHR_{ECD} trimer after removing its ligands. C. FSH bound to FSHR_{ECD} monomer extracted from 4AY9. D. FSHR_{ECD} with protomol shown as a magneta surface. The protomol was generated using the Surflexdoc module of Sybyl-X with the following settings: threshold-0.5, bloat-2.0, and radius-3 Å.

After receptor ECD preparation and protomol generation, we used the human FSH glycoform models, described above, to perform the docking studies. In these experiments we studied the effects of removing three different N-glycans, one at a time, on the binding of FSH glycoforms. We also evaluated whether or not the glycans were in close proximity to each other by visual inspection and the structural differences based on the glycans attached to each FSH glycoform complex and how this affected their binding to either the FSHR trimeric or monomeric models. The βAsn^7 glycan was close to the βAsn^24 glycan, but not to the αAsn^52 glycan.
**Figure 8. Docking of hFSH glycoforms to the FSHR\textsubscript{ECD} monomer.** The receptor fragments are shown as green surfaces, FSH amino acids as red surfaces and carbohydrates as blue surfaces. All FSH glycoforms were generated as described in the legend to Fig. 7. 

A. Molecular model of fully-glycosylated FSH\textsuperscript{24} docked to the FSHR\textsubscript{ECD} monomer, viewed from the side. 

B. Molecular model of N\textsuperscript{52}dg-FSH glycoform docked to a FSHR\textsubscript{ECD} monomer. 

C. Molecular model of the glycoform, hFSH\textsuperscript{21}, docked to a FSHR\textsubscript{ECD} monomer. 

D. Molecular model of the glycoform, hFSH\textsuperscript{18}, docked to a FSHR\textsubscript{ECD} monomer. 

E. Molecular model of the glycoform, hFSH\textsuperscript{15}, docked to a FSHR\textsubscript{ECD} monomer.

FSH\textsuperscript{24} docked to the monomeric FSHR\textsubscript{ECD}, as shown in Fig. 8A, illustrates the largely protein-protein interaction between the glycoprotein pair. The red FSH\textsuperscript{24} protein moiety almost completely obscured the green colored FSHR extracellular domain. All four blue glycans were visible and partially obscured the FSH peptide moiety. As FSH was interposed between the receptor and the glycans, there was little obvious oppor-
tunity for these glycans to inhibit binding. Docking FSH to its receptor appeared to involve the glycan-deficient, top portion of the molecule, as seen in Fig. 9A, to slip under the “thumb” loop located in the non-LLR hinge region. Although there is no crystal structure for the unoccupied FSHR\text{ECD}, it is likely the visible part of the loop is as flexible as the missing portion, particularly since the sulfo-Tyr binding site in FSH does not appear until after high affinity binding to the LLR [49]. In any case, the FSH peptide moiety was interposed between αAsn\textsuperscript{78} glycan and the thumb. When αAsn\textsuperscript{52} glycan was eliminated (Fig. 8B) little additional FSHR\text{ECD} could be seen, as this glycan was off to the side of the FSH model in this orientation. The same was true for elimination of each of the other glycans (Figs. 8C-8E).

![Figure 9. Effect of FSH glycosylation on FSH binding to trimeric receptor. A. Molecular model of a single fully-glycosylated FSH\textsuperscript{24} molecule binding to an FSHR\text{ECD} trimer, viewed from the top. The receptor trimer is shown as a green surface, FSH amino acids are shown as a red surface and FSH carbohydrates as blue surfaces. All FSH glycoforms used were generated by the GlyProt tool available online at the Glycosciences.De web site. B. A single FSH glycoform model, with the αAsn\textsuperscript{52} glycan omitted, docked to a FSHR\text{ECD} trimer. C. Molecular model of the glycoform, hFSH\textsuperscript{21}, docked to a FSHR\text{ECD} trimer. D. Molecular model of the glycoform, hFSH\textsuperscript{18}, docked to a FSHR\text{ECD} trimer. E. Molecular model of the glycoform, hFSH\textsuperscript{15}, docked to a FSHR\text{ECD} trimer.](image)

When FSH glycoforms were docked with the trimeric FSHR\text{ECD} model (Fig. 9), there was greater opportunity for steric hindrance because the glycan-rich back side of FSH engaged to the concave, high affinity binding site of one receptor now had to avoid the
convex side of an adjacent FSHR\textsubscript{ECD}. Docked to the trimeric model, the glycans appeared to fill most of the free space between two adjacent FSHR\textsubscript{ECDs} (Fig. 9A). As suggested by Jiang and colleagues [108, 109], the αAsn\textsuperscript{52} glycan partially filled the center of the receptor trimer. Presumably, this would prevent docking a second FSH glycoform model. Elimination of αAsn\textsuperscript{52} glycan removed all carbohydrate from the trimer center (Fig. 9B). Elimination of αAsn\textsuperscript{24} glycan potentially reduced steric clash with the FSHR\textsubscript{ECD} not involved in high affinity binding (Fig. 9C). Elimination of αAsn\textsuperscript{7} glycan appeared to limit steric clash to αAsn\textsuperscript{52} glycan alone (Fig. 9D), similar to the situation with docked FSH\textsuperscript{15} (Fig. 9E). In conclusion, the fewer glycans attached to Asn\textsuperscript{24} or Asn\textsuperscript{7}, the less potential for steric hindrance impeding receptor binding, which is exhibits higher receptor binding by the glycoform [38]. This is also consistent with faster binding to the receptor. If this is attended by immediate receptor activation it should lead to the higher reported levels of biological activity [38, 110]. To study an unamplified response to FSH binding, we characterized the internalization rates for FSH glycoforms.

**Aim 3: Study the effects of Compound 5 on the internalization rate for FSH glycoforms bound to the FSHR.** The first experiment was performed in order to determine if FSHβ glycosylation affected the internalization rate of the FSH-FSHR complex. The second experiment was designed to determine the effect of Compound 5, an allosteric modulator of the FSHR, on the internalization rate of the same three FSH-FSHR complexes.

We measured the surface bound radioactivity and internalized radioactivity for each tracer. Representative results are shown in Fig. 10. There was not much difference between the internalized and surface bound FSH\textsuperscript{21} and FSH\textsuperscript{24} preparations in the absence of Compound 5 (Figs. 10A&B). This was consistent with the relatively low bio-
logical activity of the only hFSH$^{21}$ preparation available for this study. The results for a highly active hypo-glycosylated FSH preparation, eFSH, revealed significantly higher receptor binding activity. Both surface bound and internalized eFSH tracer exceeded the surface binding for both FSH$^{24}$ and FSH$^{21}$ (Fig. 10C).

Figure 10. Surface binding and internalization of FSH glycoform preparations. Surface bound, pH 3-sensitive, $^{125}$Iodine cpm indicated by the solid lines and internalized, pH 3-resistant, cpm indicated by the dashed lines. A. $^{125}$I-hFSH$^{24}$ tracer. B. $^{125}$I-hFSH$^{21}$ tracer. C. $^{125}$I-eFSH tracer.

The ratio of internalized and surface, specifically bound $^{125}$I-FSH at each time point was calculated and plotted against time (Fig. 11). The rate of internalization ($k_e$) was calculated from the slope of the resulting line [58], which was calculated by linear regression analysis. There was no significant difference between the internalization rates for any of the glycoform preparations. When we employed Compound 5 in our experiments, all the internalization rates were doubled (Table 1) and now the eFSH internalization rate significantly exceeded those of hFSH$^{24}$ and hFSH$^{21}$. 
Figure 11. Binding and internalization of FSH glycoforms to FSHR. Effects of Compound 5 on the endocytic rate (Ke) of hFSH$^{21}$, hFSH$^{24}$ and eFSH during endocytosis.

Table 1. Endocytic rates for FSH-FSHR complexes in the presence and absence of the allosteric modulator Compound 5. Average endocytic rates for $^{125}$I-hFSH$^{21}$, $^{125}$I-hFSH$^{24}$ and $^{125}$I-eFSH. Mean ± St. Dev. for triplicate determinations using 25 ng/ml tracer. Two way analysis of variance was performed to test data significance. The superscript $^a$ means no significant difference without Compound 5, $^b$ means significance difference with Compound 5, and $^c$ means eFSH internalization rate is higher than other glycoforms used.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Ke (min$^{-1}$) without Compound 5</th>
<th>Ke (min$^{-1}$) with Compound 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-hFSH$^{24}$</td>
<td>$0.016 \pm 0.003^a$</td>
<td>$0.033 \pm 0.007^b$</td>
</tr>
<tr>
<td>$^{125}$I-hFSH$^{21}$</td>
<td>$0.019 \pm 0.003^a$</td>
<td>$0.038 \pm 0.007^b$</td>
</tr>
<tr>
<td>$^{125}$I-eFSH</td>
<td>$0.026 \pm 0.003^a$</td>
<td>$0.044 \pm 0.009^b,c$</td>
</tr>
</tbody>
</table>
Aim 4: Model Compound 5 interaction with the FSH receptor. To evaluate Compound 5 interaction with FSHR, we created an improved and refined homology model for the human FSHR transmembrane domain based on the crystal structure of the β2-adrenergic receptor (β2AR, PDB code: 3SN6, [76]) as a template. The β2AR structure was chosen as a template because it shares as many residues with glycoprotein hormone receptor 7TM domains as any other template structure according to a sequence alignment performed by others [45]. In Fig. 12, we show the aligned sequences of FSHR residues 359-695 with the β2AR sequence. In addition, the β2AR is the only G-protein coupled receptor structure showing the active state conformation in complex with a G-protein αsβγ heterotrimer [76]. Furthermore, the β2AR binds external agonists and antagonists; therefore it is an appropriate model for drug discovery [45].

Computational methods employing the 3D structures of GPCRs have become important tools for investigating these receptors and for drug discovery [111-113]. In order to build the FSHR TMD model, both the β2-adrenergic receptor and FSHR sequences were aligned in order to identify the amino acid residues that likely formed the seven transmembrane α-helices in the FSHR (Fig. 12). The homology model was obtained for the FSHR transmembrane domain based on the published X-ray structure for the β2AR (PDB code: 3SN6, [76]) using the procedure described under Methods (Fig. 13A). The homology model predicted transmembrane helical sequences exhibited 26% identity (value obtained after alignment of sequences using the Clustal W tool available at in the DNAStar Megalign software package).
**Figure 12.** Prediction of FSHR TM domains I-VII. Alignment of the FSHR C-terminal amino acid sequence, residues 359 to 695, with the human β2 adrenergic receptor primary structure, residues 33 to 413. Residues known to be included in the transmembrane helices of the active, G protein-bound β2AR receptor are colored yellow. Predicted FSHR transmembrane helix sequences are colored red. Sequence gaps are represented by dashes. The identity of each transmembrane helix is marked as “TM” followed by the helix number.

![Predicted FSHR Transmembrane Domains](image)

**Figure 13.** Homology model of the FSHR 7TM domain. A. Molecular model showing the transmembrane domains. TM1 is shown as a green cartoon, TM2 as a yellow cartoon, TM3 as a magenta cartoon, TM4 as a cyan cartoon, TM5 as a salmon cartoon, TM6 as an orange cartoon and TM7 as a blue cartoon.
Molecular model showing the FSHR 7TM domain model as a red cartoon, the previously identified allosteric binding region is shown as yellow cartoon, and the residues with which Compound 5 has hydrophobic interactions are shown in green spheres. The remaining TM helices are colored red.

Formal validation of the FSHR TMD model was evaluated by means of Ramachandran plots generated using PROCHECK [104, 105]. As shown in Figure 14, all helical amino acid residues were located in the region favoring right-handed α-helices. None of the residues were located in sterically disallowed regions. The crystal structure of the β2AR itself, displayed no residues in disallowed regions. Residues in most favored regions represented 90.1% of all modeled residues and 100% were within the fully allowed, additionally allowed, and/or generously allowed regions, thereby validating the model. A good quality model is expected to have over 90% of its residues in the most favored regions [104, 105].

Figure 14. Ramachandran plot of the FSHR 7TM domain model. The most favored regions are colored red, additional allowed, generously allowed and disallowed regions are indicated as yellow, light yellow and white fields, respectively. Residues marked with red squares have a bad conformation, which usually disappears during minimization and/or dynamics simulation.
The potential allosteric binding pocket for Compound 5 in the FSHR 7TM domain was determined as described before. Docking studies of Compound 5 predicted an allosteric site involving interactions in TM1 and TM2, but no interaction with exoloop 1, TM3, which addresses a question raised in the study the identified the C-terminal portion of TM1, all of TM2, exoloop 1, and the N-terminal portion of TM3 as the location of Compound 5 binding [72].

**Figure 15. Potential allosteric binding pocket in the FSHR 7TM domain with Compound 5 docked to it.** The FSHR 7TM domain model is shown as a red cartoon, the potential allosteric binding site shown as yellow cartoon, the residues that Compound 5 has hydrophobic interaction with are shown in green color and Compound 5 as a magenta stick.

The docking study identified three hydrophobic amino acid residues, Leu387, Leu404 and Ala407, as playing an important role in Compound 5 binding (Fig. 15).
Discussion

For technical reasons, the hypothesis that FSH glycoform binding caused FSHR dimer/oligomer dissociation was not tested because neither electrophoretic system revealed the presence of both monomeric and dimeric/oligomeric forms under the conditions explored in this study. The hypothesis that FSH glycans affected binding to its receptor was tested by docking FSH glycoform models to the FSHR extracellular domain model. The docking studies led us to conclude that glycans affect binding of FSH glycoforms to an oligomerized form of the FSH receptor, not the monomeric form. The hypothesis that increased binding to the FSHR by highly active FSH glycoforms in the presence of Compound 5 was partly due to increased internalization was supported by the doubling of the internalization rates for eFSH, however, doubling was noted for all glycoforms tested. The hypothesis that Compound 5 also increased the number of available binding sites was not tested directly, also for technical reasons. Compound 5 was docked to a model of the FSHR and the results identified a more restricted binding region in TM1-2.

When we tested three FSHR extraction conditions, SDS-PAGE under reducing conditions revealed two bands in the monomeric FSHR region of the gel after PBS and Igepal extraction. The latter outcome was consistent with published data for recombinant hFSHR [53]. In contrast, RIPA buffer extraction resulted in only a single band with intermediate mobility [114]. Substituting Coomassie Blue dye for SDS in blue native gel electrophoresis resulted in only FSHR oligomers with no detectable monomeric form. The fairly tight bands near the top of the blot did not indicate a range of oligomerization, as has been reported for the LHR [115]. Immunoreactivity was observed in the high molecular weight regions of SDS-PAGE western blots, consistent with the existence of multiple oligomeric forms of the receptor. However, no distinct bands were detected, possibly because dimer abundance may only be 15% and trimers-octamers less than 5% each, as compared
with ~60% monomeric receptor. In these circumstances, FSH binding would only provide a qualitative indication of FSHR conformational change, shifting from dimer/oligomerized receptor to monomeric receptor. As neither electrophoretic system revealed both monomeric and oligomeric FSHR, we were not able to test the hypothesis that FSH binding dissociated FSHR oligomers.

As with other GPCRs, the FSHR has been found to exist in a variety of oligomeric states [53, 54]. While cloning the FSHR appeared to establish the monomeric nature of the receptor [116, 117] shortly thereafter, a study indicated the molecular weight of the FSH/FSHR complex greatly exceeded the predicted mass for monomeric receptor [118]. Recent studies suggest FSHR is a functional dimer or trimer [46, 49, 53]. The first crystal structure for the FSHR high affinity FSH binding site complexed with FSH suggested the receptor may form dimers via interactions between the convex sides of their extracellular domains [19]. Subsequently, the structural features of the FSHR LRRs were confirmed by crystal structures of complexes between the TSHR LRRs (PDB code: 3G04) and activating [47] or inactivating [48] autoantibodies (PDB code: 2XWT), respectively. Crystallization of the entire extracellular domain complexed with FSH revealed a trimeric structure, which has potential to rationalize the effects of FSH glycosylation on receptor binding [46]. Significantly, the structure determination was followed up with biochemical studies that supported the trimeric model [46]. However, FSH glycoforms possess αAsn<sub>52</sub> glycans that are too large to permit simultaneous binding to such trimeric receptors. A high resolution fluorescent study of the LHR described monomeric receptors as well as oligomers ranging from 2 to more than 9 receptors clustered together which accounted for about 40% of the receptors observed [115]. Model building based on locations of the fluorescent tags showed receptors associated by their transmembrane domains. Receptor association via both extracel-
lular and transmembrane domains has been determined experimentally using ultracentrifugation and western blot analysis of the resulting fractions [54]. The fact that the FSHR may exist in oligomeric states may provide a mechanism for rationalizing the impact of N-glycosylation on FSH binding, provided accurate models of the monomeric and oligomeric forms can be obtained. The existence of a mixed population of monomeric and oligomeric FSHRs is supported by the observation of negative cooperativity during dissociation of FSH from its receptor [52]. In the absence of unlabeled FSH, $^{125}$I-FSH can remain bound to its receptor for at least 24 hr [119]. In the presence of at least 1000-fold excess unlabeled FSH, partial dissociation of previously bound $^{125}$I-hFSH takes place. However, 40-85% of the tracer remains bound in the presence of competitor. Since negative cooperativity implies the existence of an unoccupied ligand binding site for the cold FSH to bind, the resistance of some pre-bound $^{125}$I-FSH to dissociation suggests the existence of monomeric FSHRs. The latter have no additional FSH binding sites for the cold ligand to bind and induce a change in receptor conformation that dislodges previously bound FSH. These data imply that binding of FSH to the high-affinity site would necessarily trigger a conformational change in the receptor. This does not appear to occur in the extracellular domain, suggesting the connection between it and the TMD or between adjacent TMDs may be affected. Moreover, due to negative cooperativity of FSH binding to its receptor and based on the fact that $\alpha$Asn$^{52}$ glycans are too large to enable binding to FSHR trimers, we proposed a mechanism whereby, upon binding of FSH to its receptor, dissociation of the receptor complex occurs and monomeric receptors become available to bind more FSH. This mechanism could be supported by the fact that hypo-glycosylated FSH$^{21/18}$ preparations are known to bind FSHR faster than fully-glycosylated FSH$^{24}$ [38]. FSH$^{21/18}$ binds immediately upon exposure to FSHR and dissociates FSHR oligomers into monomers, thereby provid-
ing more FSH binding sites to free FSH\textsuperscript{21/18}. We sought to test this hypothesis by biochemical means, using both Western blots and gel filtration of solubilized receptors. Unfortunately, neither SDS-PAGE nor Blue Native electrophoresis could reveal both monomeric and oligomeric FSHR variants. It is possible that FSHR oligomers were so low in abundance that they were undetectable. In future experiments more receptor could be applied to the gel, as saturation binding studies were typically performed with 250,000 cells/tube, while Western blot samples were only 80,000 cells/well. Results from gel filtration experiments were even worse, as most of the receptor-bound FSH tracer was dissociated either during solubilization or chromatography.

Given this information, we decided to use molecular modeling studies in order to try to understand how glycosylation affects binding of different FSH glycoforms to the FSHR monomeric and trimeric models. In this regard, we used the structure of the complex consisting of FSH bound to the entire ectodomain of the FSHR (PDB code: 4AY9). This was derived from a recent X-ray crystallographic study of the FSHR\textsubscript{ECD}, which revealed a trimeric organization of extracellular domains, each occupied by a deglycosylated FSH molecule [46]. However, replacing the single $\alpha$\textsubscript{Asn}\textsuperscript{52} GlcNAc residue with a biantennary, sialylated glycan suggested that only one FSH molecule could occupy the trimer at a time [46]. This suggestion was supported by saturation binding data showing a 3-fold increase in binding by a recombinant FSH mutant lacking the $\alpha$\textsubscript{Asn}\textsuperscript{52} glycosylation site as compared with wild-type recombinant FSH [46]. However, studies from our laboratory indicate the naturally occurring loss of FSH$\beta$ N-glycans can also increase the FSHR occupancy 2- to 3-fold [38, 110]. Docking studies described above indicated that hypo-glycosylated FSH glycoforms could bind FSHR\textsubscript{ECD} trimers and place the $\alpha$\textsubscript{Asn}\textsuperscript{52} glycan in the middle of the trimeric cluster. One rationale for this was that hypo-glycosylated hFSH $\alpha$\textsubscript{Asn}\textsuperscript{52} glycans were
small enough to permit multiple FSH ligands to occupy trimeric FSHRs, based on more rapid migration of FSH$^{21/18}$ α-subunit bands during SDS-PAGE [38]. However, mass spectrometry studies revealed that none of the hypo-glycosylated FSH αAsn$^{52}$ glycans were small enough to permit multiple FSH binding to FSHR complexes [120].

FSH docked to the monomeric FSH$\text{ECD}_{\text{ECD}}$ illustrates the paradox that has bedeviled glycoprotein hormone studies for over 36 years [121]. The docked FSH$^{24}$ model obscured most of the FSH$\text{ECD}_{\text{ECD}}$ model, consistent with high affinity binding restricted to protein-protein interactions between FSH and its receptor [122, 123]. All 4 N-glycans associated with the fully-glycosylated FSH$^{24}$ glycoform were located on the back side of the receptor-bound FSH protein model where they should have minimal impact on FSHR binding. The “thumb” loop motif at the C-terminal end of the FSH$\text{ECD}_{\text{ECD}}$ provides an opportunity for glycans to hinder binding, however, this is located at the end of the FSH molecule where only one N-glycan, that at αAsn$^{78}$, is located. Moreover, loss of αAsn$^{78}$ glycan has no reported effects on FSH binding to its receptor [24, 25].

When a single FSH glycoform was docked to a FSHR trimer, the αAsn$^{52}$ glycan protruded into the center pocket formed by the trimeric ECD complex and was too large to enable more than one ligand bind to the occupied FSHR trimer as the presence of a second ligand would result in steric clash from overlapping of αAsn$^{52}$ glycans. The three glycans that appear to inhibit FSHR binding, αAsn$^{52}$, βAsn$^{24}$, and βAsn$^{7}$, are in a close proximity to each other. While none of these appeared capable of interfering with FSH binding monomeric FSH$\text{ECD}_{\text{ECD}}$, in the trimeric form of the receptor, each contributed to steric hindrance on the part of a neighboring extracellular domain not involved in high affinity FSH binding. It was obvious when docking the model with no αAsn$^{52}$ glycans that no overlapping glycans would be present in the center of the FSH$\text{ECD}_{\text{ECD}}$ trimer complex, which would allow it
to bind more ligands. From a physiological point of view, this observation is somewhat irrelevant, as this FSH glycoform does not exist in nature. The FSHβ glycan-deficient glycoforms possessed αAsn52 glycans that would occupy the center of a trimeric FSHRECD. Nevertheless, as hypo-glycosylated FSH preparations also occupied 2- to 3-fold more FSH binding sites in several different FSHR preparations, a different mechanism must permit these FSH variants to occupy more FSH binding sites.

We tested the effect of Compound 5, an allosteric modulator of the FSHR, on FSH binding and internalization in CHO-hFSHR expressing cell lines in short term experiments, using attached cells for up to 15 minutes in the presence and absence of Compound 5. No significant difference in internalization rate was measured when we compared two human pituitary FSH glycoform preparations, fully-glycosylated FSH24 and hypo-glycosylated FSH21/18. On the other hand, when we used eFSH, a surrogate for the most active hypo-glycosylated FSH21/18 preparations [30], we measured both increased FSH surface binding as well as an increased internalization of tracer. However, because the rate calculation involved plotting the ratio of internalized to surface-bound cpm, there was no significant difference for the eFSH internalization rate from those of the other human FSH glycoform preparation employed in this study. Surprisingly, the presence of compound 5 doubled internalization rates of all glycoforms tested. Although small molecules are known to increase binding of hFSH24 but not recombinant GH3-hFSH21 to the FSH receptor, we did not find a significant difference between the internalization rates for both of them. Compound 5 allosterically modulated FSHR by either dimer/oligomer dissociation or causing a conformational change to the FSHR. A conformational change can mean dissociation of a non-covalently organized complex or an actual change in shape. Both mechanisms can increase FSH access to the receptor.
Compound 5 has been reported to be an in vitro FSH receptor agonist that stimulates similar intracellular responses as FSH [74]. Initial studies of Compound 5 with recombinant CHO-cell lines expressing human FSHR, LHR, and TSHR revealed the selective activation of FSHR, but not LHR or TSHR [72]. Furthermore, Compound 5 stimulated cAMP production and recruit β-arrestin in a dose-dependent manner [72]. It has been reported that receptor activation by this allosteric modulator is brought out by occupying a binding site independent of the FSH binding site located in the transmembrane region, spanning TM1 through TM3 [72]. Given this information, first we tried to build a complete FSHR model using Sybyl X to combine the complete extracellular domain model with the transmembrane domain homology model and then dock Compound 5 to it, but with no success. We tried to dock Compound 5 to the FSHR_{ECD} model to see if it docked there instead of to the TMD homology model, but also with no success. We used the TMD homology model to predict a potential allosteric binding pocket for Compound 5.

Our molecular modeling results suggested that Compound 5 can bind a hydrophobic-rich region, spanning TM1 through TM2, while excluding exoloop 1. Compound 5 docked to the potential allosteric pocket and binds Leu387, Leu404 and Ala407. These data are consistent with previously published data regarding the binding site of Compound 5 [72] and as Compound 5 binds to an allosteric binding pocket of the FSHR 7TM domain, it may cause a conformational change in the FSHR that enables the binding of more FSH ligands. Compound 5 can cause conformational changes to FSHR either by dissociation of the oligomerized receptor complex providing more monomeric binding sites for FSH or by changing the conformation of FSHR oligomers to make it more accessible for more FSH ligands to bind. Such a mechanism is yet to be determined due to the absence of a biochemical technique that can reveal the transition of FSHR oligomers to monomers, if such a transition occurs. This is the first report that Compound 5 can increase the internalization
rate of FSH/FSHR complexes. Other laboratory have reported that similar compounds increase FSH tracer binding, but do not affect internalization rate [75].

Several studies from our laboratory [1, 4, 20, 38, 124-126] support the existence of least four human FSH glycoforms resulting from glycosylation macroheterogeneity [20]. While fully-glycosylated hFSH$^{24}$ can be obtained in the purified state, most hypo-glycosylated hFSH preparations consist of both hFSH$^{21}$ and hFSH$^{18}$. Hypo-glycosylated human FSH$^{21/18}$ preparations can be much more active than the fully-glycosylated human FSH$^{24}$ in vitro [38]. Loss of FSHβ glycans results in more active glycoforms that engage the FSHR more rapidly, exhibit a higher apparent affinity, and occupy more FSH binding sites [38]. The potential physiological significance of hypo-glycosylated FSH is underlined by the observation FSH$^{21}$ exhibits an age-related decrease in relative abundance about same time when fertility decreases in women. Loss of FSH αAsn$^{52}$ glycans also allows more partially deglycosylated FSH molecules to bind to FSH receptor complexes [46]. However, loss of this glycan is also attended by significantly reduced biological response to FSHR binding [24, 25].

A model for glycoprotein hormone activation has been proposed in which ligands bind with high affinity to the N-terminal extracellular domain and convert it from an inverse agonist into full agonist, activating the transmembrane-spanning portion of the receptor [127]. Substantial support for this model was provided by the crystal structure of the FSHR extracellular domain, as sTyr binding to FSH may relieve ECD-induced inhibition by lifting a helical segment the loop sTry resides in via disulfide bond connection [49]. This alteration in the extracellular domain conformation permits the TMD to adopt the active conformation leading to receptor activation. A weakness in this mechanism is that FSH glycans are unnecessary, as essentially deglycosylated FSH binds with high affinity to the receptor and undergoes a conformational change that creates the essential sTry binding
pocket. When sTyr binds FSH, ligand-mediated FSHR activation follows. However, FSH glycans, especially those attached to αAsn$_{52}$ are necessary for full receptor activation [24, 25]. Compound 5 probably acted directly on the FSH receptor TMD to stabilize the active receptor conformation. Whether Compound 5 also dissociated the receptor into monomers so the FSH ligands remains unknown.
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