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Development of Selective LH Receptor Agonists by Heterodimerization with a FSH Receptor Antagonist

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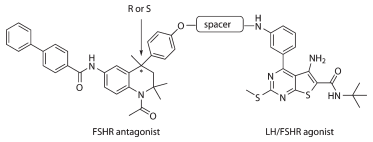
ABSTRACT The structural resemblance of the luteinizing hormone receptor (LHR) and follicle-stimulating hormone receptor (FSHR) impedes selective agonistic targeting of one of those by low molecular weight (LMW) ligands. In the present study, we describe a series of dimeric ligands consisting of a LMW agonist with dual activity on the FSHR and the LHR linked to a selective FSHR antagonist. Biological evaluation shows these compounds to be potent and selective LHR agonists, since no agonistic activity on the FSHR was observed. Equimolar mixing of the monomeric counterparts did not yield the pharmacological profile observed for the heterodimeric ligands, and FSHR agonism of the monomeric LHR agonist was still observed. The here-described results show that ligands with unique pharmacological profiles can be obtained by dimerizing monomeric molecules with distinct apposite properties.

KEYWORDS Luteinizing hormone receptor, follicle-stimulating hormone receptor, G-protein-coupled receptor, dimeric ligands, bivalency



The luteinizing hormone receptor (LHR)[1] and follicle-stimulating hormone receptor (FSHR)[2] are glycoprotein hormone receptors[3,4] that belong to the large family of G-protein-coupled receptors (GPCRs) and are important mediators in human reproduction. Upon activation by luteinizing hormone (LH), the LHR initiates a signal cascade resulting, among others, in androgen production. In females, FSHR activation by FSH induces aromatase release in the ovaries, upon which androgens are converted to estrogens. Both LH and FSH are members of the so-called glycoprotein hormone family and are heterodimers composed of an identical α-subunit and a hormone-specific β-subunit. The LHR can be agonized independently by yet another glycoprotein hormone, namely, the human chorionic gonadotropin (hCG), and for this reason, the LHR is also referred to as the hCG receptor or CGR.[1] The hCG glycoprotein combines the LHR/FSHR α-subunit with yet another β-subunit. During reproductive cycles, both LHR and FSHR are often activated at the same time. In exerting control over human fertility, selective access to either the LHR- or the FSHR-mediated signaling pathway is desirable. The unique β-subunits of the glycoprotein hormones provide high selectivity in their binding to the extracellular N terminus of their respective receptors, and thus, selective therapeutic control in either LHR- or FSHR-mediated signaling can be attained by making use of recombinant LH/hCG or FSH, respectively.

A major research objective in pharmaceutical research on human fertility is to identify low molecular weight (LMW) agonists and antagonists that are selective for either LHR[5−7] or FSHR.[8−12] Relevant recent discoveries stemming from such research and at the basis of the here-presented studies are the LMW glycoprotein hormone receptor ligands, Org 41841 (**1**)[5] and (*R*)-tetrahydroquinoline (**2**).[8] Compound **1** is a potent LHR agonist that appears to bind to LHR at a site distinct from that employed by the natural glycoprotein hormone LH, namely, within the trans-membrane region of the receptor. Although at much higher concentrations, Org 41841 (**1**) also agonizes FSHR, and thus, the compound does not emulate the glycoprotein hormone receptor selectivity featured by LH itself. Interestingly, selective FSHR antagonists have been reported.[8,12] For instance, tetrahydroquinoline derivative (*R*)-**2** is a potent FSHR antagonist for which no activity on other glycoprotein hormone receptors has been reported yet. Compound (*R*)-**2** is a negative allosteric modulator and antagonizes the FSHR by binding to the trans-membrane region and is thus noncompetitive with respect to the glycoprotein ligand FSH.

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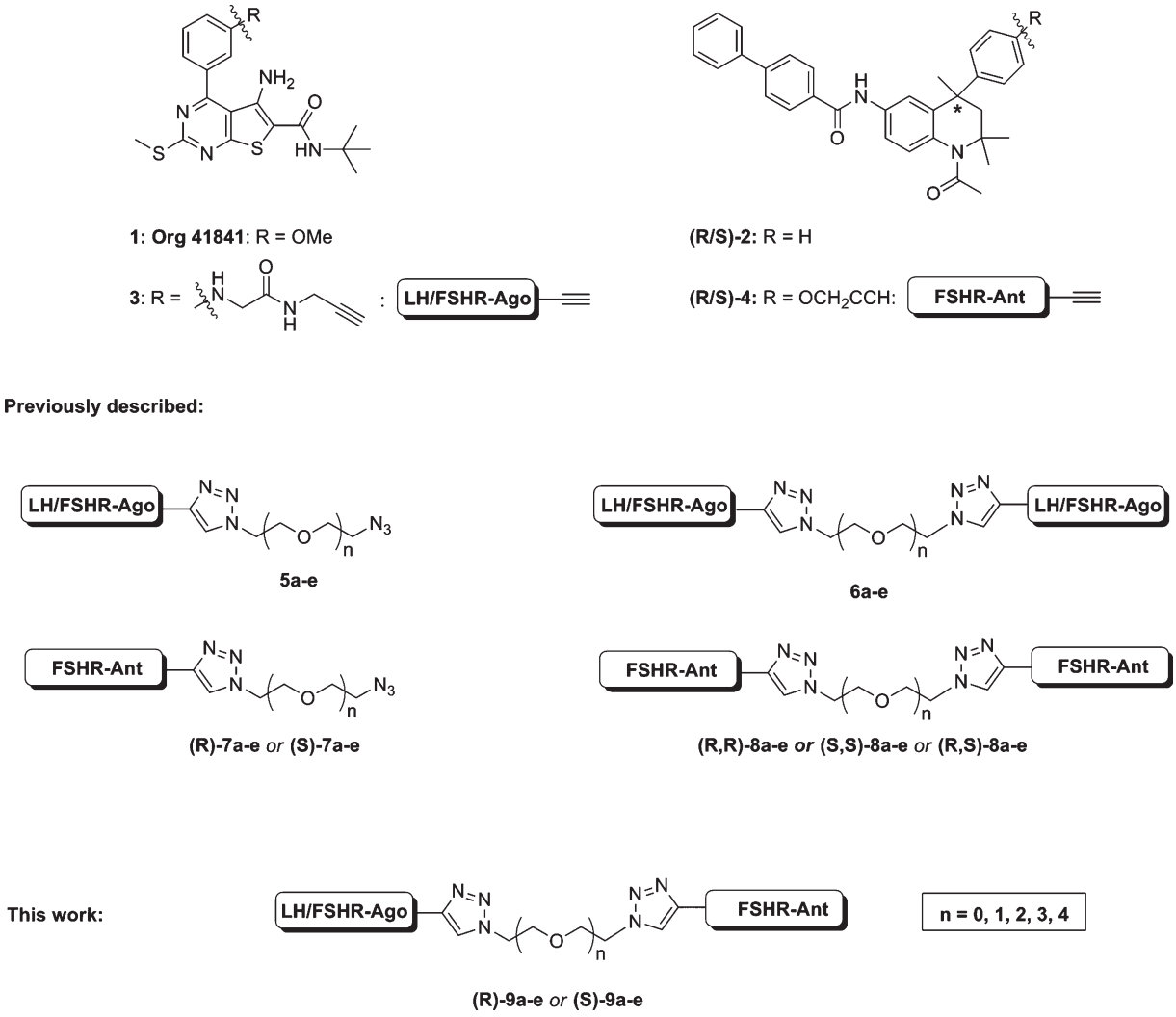

Figure 1. Monomeric and homo- and heterodimeric LHR and FSHR ligands subject to the here-described studies.

Ligand dimerization has recently emerged as an attractive strategy in the search for receptor ligands with optimal pharmaceutical properties. This holds true especially for GPCR-based research, since several GPCRs, including LHR and FSHR, were found to function at least in part as oligomeric assemblies.[13−18] We investigated the merits of the dimerization approach in both LHR agonistic[19] and FSHR antagonistic[20] settings, using compounds **1** and **2**, respectively, as starting points (Figure 1). With respect to the first, we previously established that acetylene derivative **3** agonizes the LHR and FSHR with potencies comparable to that of the parent compound, Org 41841 (**1**).[19] We then prepared homodimers **6** starting from acetylene derivative **3** and employing a double copper-catalyzed Huisgen [3 + 2] "click" cycloaddition with the appropriate ethylene glycol-derived bis-azides. We found that dimers **6** were less potent LHR agonists than compounds **1** and **3** but that they outperformed their monomeric counterparts **5**, composed of a single copy of **3** linked to ethylene glycol bis-azide by means of a single click event. In this study, we did not succeed in separating LHR agonism from FSHR agonism, since dimeric compounds **6** all possessed agonistic activity on the latter receptor albeit that the maximal agonistic effect of **6** was considerably lower for the FSHR. In a related study, we investigated the panel of homodimeric tetrahydroquinoline structures **8** on their FSHR antagonistic properties.[20] Compounds **8** were prepared from acetylene (*R*)-**4** [itself an FSHR antagonist with potency comparable to that of the parent compound (*R*)-**2**] using the aforementioned "click" strategy and employing the same set of ethylene glycol bis-azides. Although much less potent than compounds (*R*)-**2** and (*R*)-**4**, dimers **8** were capable of antagonizing the FSHR in the micromolar range. From the combined experiments and from the finding that the dimers were noncompetitive with the respective glycoprotein hormones, we concluded that dimeric structures **6** and **8**, although considerably more bulky as compared to their monomeric counterparts, still find access to the LHR/FSHR trans-membrane region. In all, the results invite the design of a set of heterodimeric structures that encompass both a LHR agonist entity and a

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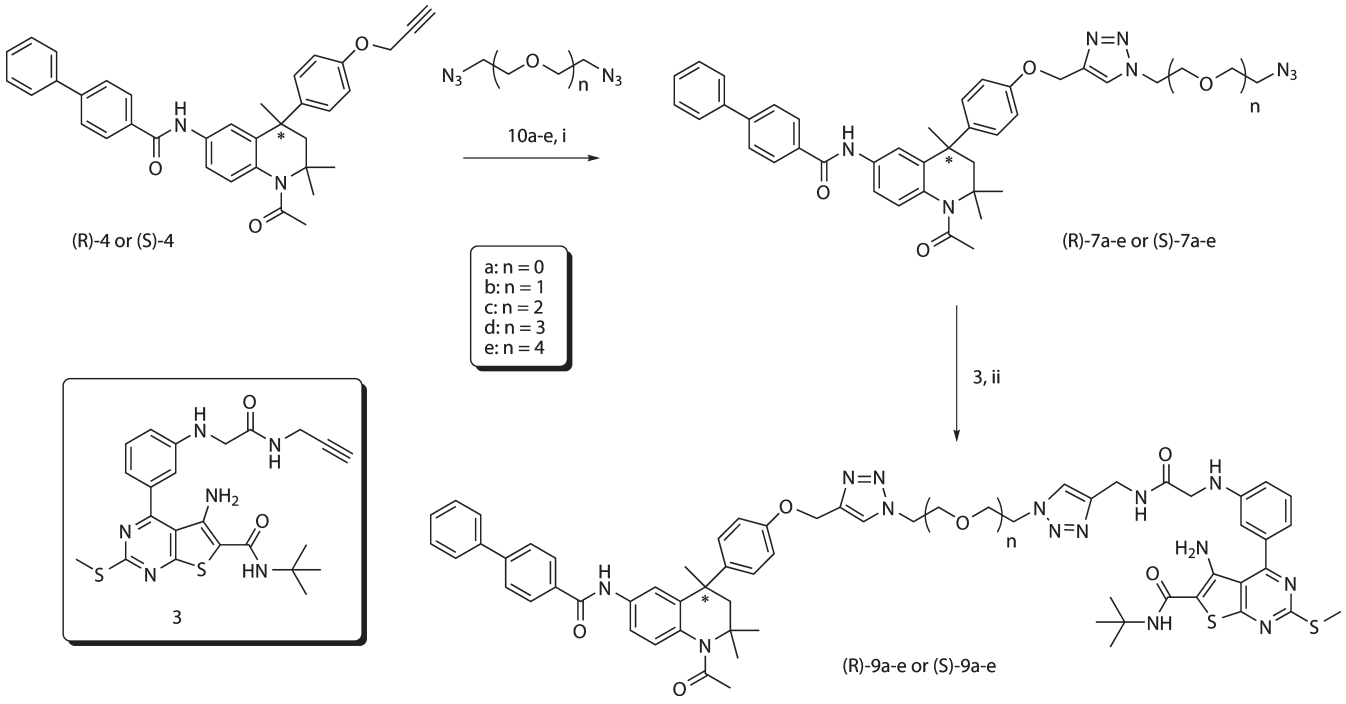

Figure 2. Synthesis of compounds **9a**−**e**. Conditions: (a) Bis-azide **10a**−**e** (5 equiv), $CuSO_4$ (0.2 equiv), sodium ascorbate (1 equiv), CH_2Cl_2/H_2O (33−56%). (b) Acetylene **3**, $CuSO_4$ (0.2 equiv), sodium ascorbate (1 equiv), CH_2Cl_2/H_2O (75−97%).

FSHR antagonist moiety interconnected through ethylene glycol spacers. With the aim to establish whether such structures would be able to agonize the LHR without agonizing the FSHR, we set about to the synthesis of heterodimeric structures **9** (Figure 2).

Treatment of acetylene functionalized pharmacophore **4** with 5 equiv of 1,2-diazidoethane **10a**, copper sulfate (0.2 equiv), and sodium carbonate (0.1 equiv) in a 1:1 mixture of methylene chloride and water afforded monomeric compound **7a** in 33% yield. The main side product in this reaction is homodimer **8a**, a side reaction that we could not suppress further, but **8a** and **9a** are readily separable by silica gel chromatography. In the next step, azide **7a** and acetylene derivative **3** are subjected to the same click conditions to yield heterodimer **9a** in 75%. Following the same two-step procedure and starting from the appropriate ethylene glycol bis-azides (**10b**−**e**), heterodimeric ligands **9b**−**e** were prepared in comparable overall yields.

We next set out to measure the LHR agonistic properties, the FSHR agonistic properties, and the FSHR antagonistic properties of the panel of heterodimeric compounds (*R*)-**9a**−**e**. As control compounds, we included the monomeric acetylene-modified compounds **3** and **4** in our assays.[19,20] As shown in entries 3−7 in Table 1, all heterodimers are potent, LHR agonists. Although not as potent as the parent compound **3**, all agonize the target receptor with an EC_{50} value in the nanomolar range. Importantly, whereas monomer **3** agonizes the FSHR (EC_{50} = 126 nM), no agonistic effect of any of the compounds (*R*)-**9a**−**e** on the FSHR at concentrations up to 10 μM were seen. In an antagonistic setup, the compounds composed of the shorter spacers [(*R*)-**9a** and (*R*)-**9b**, with n = 0 or 1] appear moderate FSHR antagonists, whereas the compounds in which the two pharmacophores are connected by longer spacers [n = 3−5, compounds (*R*)-**9c**−**e**] are low micromolar (IC_{50} values) FSHR antagonists. It should be noted, however, that these compounds did not antagonize FSHR-mediated signaling by 100% (I_{max}). A tentative conclusion at this stage appears that the intrinsic agonistic activity of the LHR selective pharmacophore based on the Org 41841 structure (**1**) toward the FSHR is counterbalanced by the presence of a dedicated FSHR antagonist within the dimeric structure. The affinity of the latter for the FSHR is such that the apparent binding affinity of the Org 41841 pharmacophore to this receptor is reduced.

To exclude that steric bulk, rather than FSHR antagonistic properties, is at the basis for exclusive LHR recognition, the agonistic activity of compounds **5c** and (*S*)-**9a**−**e** on both receptors was measured (Figure 3, entries 10−14 in Table 1). Compound **5c** stems from our previous research on homodimeric LHR agonists, and as we demonstrated before, this compound has significant FSHR agonistic properties.[19] Compounds (*S*)-**9a**−**e** encompass the distomer of the FSHR antagonist **2** [the stereoisomer at the marked (*) chiral carbon atom] and are otherwise the same as (*R*)-**9a**−**e**. Compounds (*S*)-**9a**−**e** are in fact LHR agonists with potencies comparable to that of (*R*)-**9a**−**e** but appear to exhibit a weak but significant FSHR agonistic property, much more so than observed for compounds (*R*)-**9a**−**e**. We previously[20] demonstrated that the (*S*)-enantiomer of parent compound **2** and its derivatives have no activity on the FSHR, and this result thus underscores our hypothesis that the lack of FSHR agonism in compounds (*R*)-**9a**−**e** is caused by the presence of the FSHR-specific (*R*)-tetrahydroquinoline antagonist in these structures.

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Table 1. Mean Agonistic Potency (EC_{50}) on the FSHR/LHR and Mean Antagonistic Potency (IC_{50}) and Maximal Inhibition (I_{max}) on the FSHR of Heterodimeric Compounds (*R*)-**9a**−**e**[a]

	compound		LHR agonistic assay EC_{50} (nM)[b,c]	FSHR agonistic assay EC_{50} (nM)[b] (E_{max})[d]	FSHR antagonistic assay IC_{50} (nM)[b] (I_{max})[e]
1	**3**		1	126 (78%)	NA
2	**4**		NA	NA	39
3	(*R*)-**9a**	$n = 0$	10	NA	65% at 10 μM
4	(*R*)-**9b**	$n = 1$	27	NA	75% at 10 μM
5	(*R*)-**9c**	$n = 2$	43	NA	920 (73%)
6	(*R*)-**9d**	$n = 3$	61	NA	1662 (65%)
7	(*R*)-**9e**	$n = 4$	55	NA	1023 (72%)
8	**7c**	$n = 2$	NA	NA	324
9	**5c**	$n = 2$	5	397 (81%)	NA
10	(*S*)-**9a**	$n = 0$	24	43% at 10 μM	NA
11	(*S*)-**9b**	$n = 1$	80	44% at 10 μM	NA
12	(*S*)-**9c**	$n = 2$	75	18% at 10 μM	NA
13	(*S*)-**9d**	$n = 3$	57	31% at 10 μM	NA
14	(*S*)-**9e**	$n = 4$	39	39% at 10 μM	NA

[a] None of the compounds showed antagonistic activity on the LHR. For comparison, the potencies of the previously prepared compounds **3**, **4**, **7c**, and **5c** and control compounds (*S*)-**9a**−**e** are listed. [b] EC_{50}/IC_{50} values are determined from two independent experiments performed in duplicate. SD is generally lower than 0.3. [c] All active compounds are full agonists for the LHR. [d] Maximal effect of the compounds on the FSHR. [e] I_{max} is the maximal percentage of inhibition of FSH-mediated signaling observed for the compound at 10 μM. NA, not active.

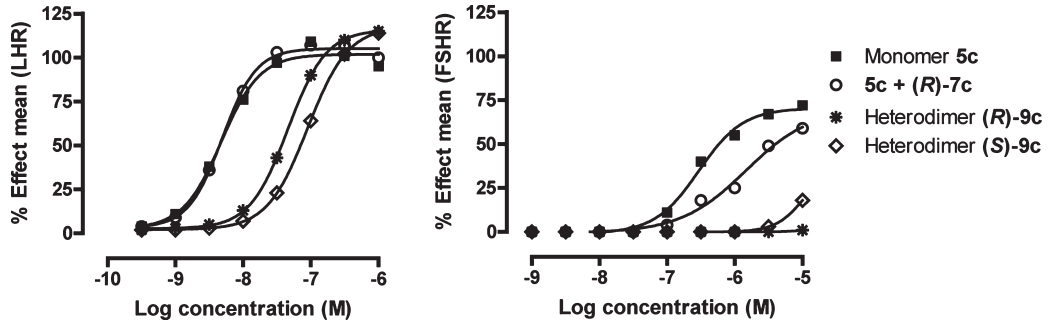

Figure 3. Concentration−effect curves (agonistic setup) of selected compounds on CHO cells expressing either the LHR (left) or the FSHR (right). Selected compounds are monomeric LH/FSHR agonist **5c** and heterodimeric FSHR antagonist-LH/FSHR agonist (*R*)-**9c** and (*S*)-**9c**. The mixture of FSHR antagonist **7c** and LH/FSHR agonist **5c** is also depicted. Concentration−effect curves were determined from two or three independent experiments performed in duplicate.

In a next experiment, we set out to establish whether LHR signaling, with concomitant FSHR silencing, could also be attained by mixing the two individual components instead of physically linking these. To this end, we mixed LH/FSHR agonist **5c** with monomeric FSHR antagonist **7c** (Figures 2 and 3). While the potency of this mixture on the LHR remains in the same order of magnitude as in the absence of (*R*)-antagonist, the potency on the FSHR is reduced significantly ($P < 0.05$). This is in accordance with inhibition of FSHR binding of the agonist **5c** by the antagonist **7c**. Thus, when the LH/FSHR agonist is interconnected to the active (*R*)-FSHR antagonist, agonistic activity on the FSH receptor is completely abolished up to 10 μM, whereas mixing the individual pharmacophores still results in significant FSHR agonistic activity. This demonstrates the additional effect of the recognition units in the dimeric ligand (*R*)-**9c** to interact with the FSHR.

In conclusion, we have demonstrated that covalently linking an agonist with dual activity on the LHR and the FSHR with a FSHR antagonist may provide compounds that are agonistic on the LHR and not on the FSHR. Although LHR agonistic potency is somewhat compromised when compared to the parent compounds, the gain is selective LHR activation with no observed FSHR activation. We here reveal that unique receptor selectivity profiles may be obtained by linking two pharmacophores with two distinct activities but undesired cross-reactivity. Such a receptor selectivity profile may otherwise be difficult to achieve with monomeric LMW compounds.

SUPPORTING INFORMATION AVAILABLE

Experimental procedures on the synthesis, analysis (NMR, HPLC, and MS), and biological evaluation of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions: ‖ These authors contributed equally to this work.

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