A Low Molecular Weight Agonist Signals by Binding to the Transmembrane Domain of Thyroid-stimulating Hormone Receptor (TSHR) and Luteinizing Hormone/Chorionic Gonadotropin Receptor (LHCGR)\textsuperscript{a,b,c}

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Many cognate low molecular weight (LMW) agonists bind to seven transmembrane-spanning receptors within their transmembrane helices (TMHs). The thienopyrimidine org41841 was identified previously as an agonist for the luteinizing hormone/chorionic gonadotropin receptor (LHCGR) and suggested to bind within its TMHs because it did not compete for LH binding to the LHCGR ectodomain. Because of its high homology with LHCGR, we predicted that thyroid-stimulating hormone receptor (TSHR) might be activated by org41841 also. We show that org41841 is a partial agonist for TSHR but with lower potency than for LHCGR. Analysis of three-dimensional molecular models of TSHR and LHCGR predicted a binding pocket for org41841 in common clefts between TMHs 3, 4, 5, 6, and 7 and extracellular loop 2 in both receptors. Evidence for this binding pocket was obtained in signaling studies with chimeric receptors that exhibited improved responses to org41841. Furthermore, a key receptor-ligand interaction between the highly conserved negatively charged E3.37 and the amino group of org41841 predicted by docking of the ligand into the three-dimensional TSHR model was experimentally confirmed.

These findings provide the first evidence that, in contrast to the ectodomain, ligands were mapped to their extracellular amino termini (11, 12). Therefore, it cannot be assumed that LMW ligands bind to the transmembrane domain of 7TMRs.

In this study, computer-based docking using three-dimensional models of LHCGR and TSHR identified a putative binding pocket for org41841 in a cleft between the transmembrane helices (TMHs) close to ECL2. Validation of this proposed binding site provides the first evidence that a LMW agonist can activate GPHRs by interaction with the transmembrane core.

**MATERIALS AND METHODS**

Construction of Vectors and Site-directed Mutagenesis of TSHR—cDNA for human TSHR was amplified by PCR from hTSHR-pSVL (13) and inserted into the pcDNA3.1(−)hygromycin vector using restriction sites XhoI and BamHI. The analogous EcoRI/EcoRV fragment in wild type hLHR-pGS5 (14) was inserted into the pcDNA3.1(−)hygromycin vector using restriction sites BamHI and XhoI. Constructs were confirmed by sequencing (MWG Biotech). Mutations were introduced into hTSHR-pcDNA3.1 via the QuikChange site-directed mutagenesis kit (Strategene). PCR products containing mutations were digested with EcoRI and Eco91I (MBI Fermentas) and used to replace the analogous Eco81I/Eco91I fragment in wild type hTSHR-pcDNA3.1. All constructs were verified by sequencing (MWG Biotech).

Cell Culture and Transfection—HEK-EM 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 10 μg/ml streptomycin (Invitrogen) at 37 °C in a humidified 5% CO2 incubator. Cells were transiently transfected in 24-well plates (7.5 × 10⁶ cells per well) with 0.4 μg of DNA/well using FuGENETM 6 reagent (Roche Applied Science) according to the manufacturer’s protocol.

Determination of Cell Surface Expression—After transfection, cells were cultured for 48 h, harvested using 1 mL EDTA/1 mL EGTA in phosphate-buffered saline, and transferred to Falcon 2058 tubes. Cells were washed once with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% NaN3 (binding buffer), incubated for 1 h with a 1:200 dilution of mouse anti-human TSHR antibody (Serotec) in binding buffer, washed twice, and incubated for 1 h in the dark with a 1:200 dilution of an Alexa Fluor 488-labeled F(ab’), fragment of goat anti-mouse IgG (Molecular Probes) in binding buffer. Before FACS analysis (FACS Calibur, BD Biosciences), cells were washed twice and fixed with 1% paraformaldehyde. Receptor expression was estimated by fluorescence intensity and transfection efficiency was estimated from the percentage of fluorescent cells.

Determination of Intracellular Cyclic AMP Accumulation—Transfected cells were cultured for 48 h before incubation for 1 h in serum-free Dulbecco’s modified Eagle’s medium containing 1 mM 3-isobutyl-1-methylxanthine (Sigma) and bovine TSH (0–1.8 M) (Sigma) or human LH (0.1–100 ng) or org41841 (0–100 μM) in a humidified 5% CO₂ incubator. Highly purified human LH was purchased from Dr. A. Parlow and the NIDDK National Hormone and Pituitary Program. Following aspiration of the medium, cells were lysed using lysis buffer 1 of the cAMP Biotrak Enzymeimmunoassay System receptor; 7TMR, seven transmembrane-spanning receptor; LMW, low molecular weight; TMH, transmembrane helix; FACS, fluorescence-activated cell sorter; CCR5, CC chemokine receptor 5.
FIGURE 1. Differences in the proposed LMW ligand-binding site between TSHR and LHCGR provided experimental evidence that the thienopyrimidine org41841 signals by binding to the transmembrane domain. A, structure of org41841. B, alignment of selected amino acid residues of TSHR and LHCGR. Residues that contribute to the binding pocket are shown in bold and underlined if they differ between the two receptors. C, homology models for the transmembrane domain of TSHR and LHCGR. The transmembrane pocket is located within the extracellular half of the transmembrane helical bundle between TMH3 (cyan), TMH4 and TMH5 (green), and TMH6 and TMH7 (blue) close to ECL2 (green). Residues that differ between the two receptors and line the binding pocket are indicated for TSHR (L570 hidden, F5.42, Y6.54) and the corresponding positions for LHCGR (F515 hidden, T5.42, F6.54) in red. The cleft is covered by ECL2 and the three junctions of TMH4/ECL2, ECL2/TMH5, and TMH6/ECL3, which contain additional six interacting residues that differ between TSHR (I560, P5.34, A5.36, L5.37, A5.38, I6.59 in light magenta) and LHCGR (V505, T5.34, S5.36, Q5.37, V5.38, A6.59 in light magenta). The side chains of the differing residues, which line and cover the binding cleft, are generally less bulky in LHCGR than in TSHR. Molecular Connolly surfaces generated for the putative transmembrane-binding site and clipped at a common Z-clipping plane amid the binding clefts indicate that their shapes and volumes are different and larger at LHCGR (violet molecular surface) than at TSHR (yellow molecular surface) and, therefore, allow org41841 to have preferred binding to LHCGR. D, experimental evidence for the proposed binding pocket by TSHR/LHCGR chimera M9, comparison of activation of TSHR and M9 by TSH and org41841. Amino acid residues that were replaced in M9 are underlined in B. Intracellular cAMP accumulation in response to increasing concentrations of TSH or org41841 was determined. Data are expressed as percent of maximum response of TSHR to TSH (100 milliunits/ml). The data are presented as mean ± S.E. of three independent experiments, each performed in duplicate.
Activation of TSHR and Mutant Receptors by org41841

HEK-EM 293 cells were transiently transfected with TSHR or mutant TSHRs. Functional assays were carried out as described under "Material and Methods." Maximum cAMP production is given as mean ± S.E., expressed in % TSH stimulation of TSHR at 1.6 μM (100 milliunits/ml) TSH. EC₅₀ values and 95% confidence intervals were calculated from dose–response curves using the GraphPad Prism 4 software. Data are summarized from three independent experiments, each carried out in triplicate. The pcDNA3.1(+)hPgymycin vector was used as a control. M3 is L570F/F5.42T/Y6.54F. M9 is I560V (LHCGR: V505), L570F (LHCGR: F515), P5.34T, A5.36S, L5.37Q, A5.38V, F5.42T, Y6.54F, and I5.59A, ND.

| Construct | Activation (% in TSH stimulation of TSHR) | EC₅₀ (95% confidence interval) |
|-----------|-----------------------------------------|-------------------------------|
| TSHR      | 23.1 ± 5.9                              | 7700 (5300–11,300)            |
| L570F     | 16.0 ± 2.5                              | 800 (210–2700)                |
| F5.42T    | 42%                                     | 13,400 (2–45,000)             |
| Y6.54F    | 66%                                     | 14,300 (2–45,000)             |
| L570F/F5.42T | 16.7 ± 3.5                      | 1000 (380–2700)               |
| L570F/Y6.54F | 25.5 ± 6.6                      | 1000 (400–4100)               |
| F5.42T/Y6.54F | 36%                                      | 15,600 (2–45,000)             |
| M3        | 35.5 ± 4.4                              | 6800 (2800–16,900)            |
| M9        | 99% ± 15.1                              | 2700 (1700–4500)              |
| E3.37A    | 3.3 ± 1.2                               | ND                            |

*Estimated maximum response at 100 μM org41841.

# Table 1

**ACCELERATED PUBLICATION:** Low Molecular Weight Agonist Binding at TSHR and LHCGR

(Amersham Biosciences). The cAMP content of the cell lysate was determined using the manufacturer’s protocol. Data were analyzed using GraphPad Prism 4 for Windows.

Synthesis of org41841 (N-tet-Butyl-5-amino-4-(3-methoxyphenyl)-2-(methylthio)thieno[2,3-d:pyridine-6-carboxamide)—The synthesis of org41841 was performed as published (5). Analysis by C8 reversed phase liquid chromatography-mass spectrometry using a linear gradient of H₂O with increasing amounts of CH₃CN (0–17 min, 30% → 70% CH₃CN at a flow rate of 1 ml/min: tᵣ 13.5 min) found greater than 95% purity by peak integration. ¹H NMR (CDCl₃): 8.14 (s, 9H), 2.65 (s, 3H), 3.86 (s, 3H), 5.99 (br s, 2H), 7.07–7.26 (m, 3H), 7.41–7.47 (m, 1H); mass spectrometry (time-of-flight); m/z = 403.1262 (M + H)⁺ (theoretical 403.1257).

Structural Bioinformatics and Molecular Modeling Studies—The initial three-dimensional structure of the serpentine domain of TSHR and LHCGR was established on the basis of the three-dimensional structure of bovine rhodopsin (15). (Protein Data Bank entry codes: 1F88, 1HZX, ILH9) (16). Several receptor-specific corrections were made based on sequence alignments using SeqLab (Wisconsin Package, Version 10.2, Accelrys Inc., San Diego, CA). The design of the amino acids in the transmembrane domain was based on the Ballesteros-Weinstein nomenclature (17). In rhodopsin, interactions of the side chains of two consecutive threonines with the helical backbone of the preceding residues caused a structural bulge in TMH2. In TSHR, interactions of the side chains of two consecutive threonines with the helical backbone of the preceding residues caused a structural bulge in TMH2. Therefore, a construct (M9) was engineered with six additional mutations replacing TSHR residues with the corresponding residues of LHCGR from the region that covers the binding cleft. Thus, M9 contained the model were validated by checking the geometry by PROCHECK (23) and during the Molecular Dynamics run (overall backbone root mean square deviation 1.8 Å). Molecular Connolly surfaces were generated for the interior transmembrane residues using the MOLCAD module of the TRIPOS package.

RESULTS and DISCUSSION

Activation of TSHR and LHCGR signaling was determined by measuring accumulation of intracellular cAMP, org41841 (Fig. 1A) acts as a partial agonist of LHCGR with an EC₅₀ of 220 nM (95% confidence interval: 140–360 nM) and a maximum response 34 ± 2.1% of that stimulated by LH. Due to the high sequence homology between TSHR and LHCGR within the transmembrane core, we tested org41841 and found that it is also a partial agonist for TSHR with a maximum response 23% of TSH but with lower potency (EC₅₀ 7700 nM) than for LHCGR (Table 1). Amino acid alignments between TSHR and LHCGR (Fig. 1B) and computer-based docking using three-dimensional models identified a putative binding pocket for org41841 in a cleft between TMHs 3, 4, 5, 6, and 7 close to ECL2 in both receptors (Fig. 1C). The binding model is smaller for TSHR than for LHCGR and two transmembrane helical residues, F5.42 (LHCGR: T5.42) and Y6.54 (LHCGR: F6.54), and residue L570 in ECL2 (LHCGR: F515) differ in the central cores of the predicted binding pockets (Fig. 1C). Furthermore, the binding pocket of TSHR is characterized by strong hydrophobic and bulky residues at the junctions between TMH4/ECL2, ECL2/ TMH5, and TMH6/ECL3 that form an extracellular cover over the binding cleft. In contrast, generally less hydrophobic and/or bulky residues are involved at the junctions between TMH4/ECL2, ECL2/ TMH5, and TMH6/ECL3 that form an extracellular cover over the binding cleft. In TSHR, responses of the double mutants to TSH were similar to TSHR (supplemental Table 1). In contrast, responses of the double mutants to TSH were similar to TSHR (supplemental Table 1). However, F5.42T/Y6.54F exhibited improved efficacies relative to TSHR in response to org41841 (Table 1).

Expression levels for double mutants were 60% for L570F/F5.42T, 85% for L570F/Y6.54F, and 125% for F5.42T/Y6.54F relative to TSHR. Basal activities for these double mutants were elevated, but activation by TSH was similar to TSHR (supplemental Table 1). In contrast, responses of the double mutants to org41841 showed increases in potencies and/or efficacies. L570F/F5.42T and L570F/Y6.54F revealed an improved EC₅₀ of 1000 nM for org41841 (Table 1). F5.42T/Y6.54F exhibited an increase in efficacy for org41841 (Table 1). M3, in which all three residues within the TMHs and ECL2 were exchanged, exhibited cell surface expression of 98% and 4.2-fold elevated basal activity compared with TSHR and responded to TSH like the native receptor (supplemental Table 1). M3 responded to org41841 with similar potency but increased efficacy relative to TSHR (Table 1).

The putative binding pocket is covered by ECL2 and adjacent residues of the three junctions of TMH4/ECL2, TMH5/ECL2, and TMH6/ECL3, which contain six different residues between TSHR (I560 in ECL2, P5.34, A5.36, L5.37, A5.38, and I5.59) and LHCGR (V505 in ECL2, T5.34, S5.36, Q5.37, V5.38, and A6.39) (Fig. 1B). Therefore, a construct (M9) was engineered with six additional mutations replacing TSHR residues with the corresponding residues of LHCGR from the region that covers the binding cleft. Thus, M9 contained nine substitutions: I560V (LHCGR: V505), L570F (LHCGR: F515), P5.34T, A5.36S, L5.37Q, A5.38V, F5.42T, Y6.54F, and I5.59A. Mutant M9 was expressed on the cell surface at near 100% of TSHR. Activation of this construct by TSH revealed a small reduction in potency (EC₅₀ = 9.3 nM), but the maximal response to the native ligand was not affected (supplemental Table 1, Fig. 1D). Importantly, M9 responded to org41841 with an improved EC₅₀ of 2700 nM and a greatly improved efficacy for signaling to 99% of the maximal value observed for TSH stimulation of TSHR (Table 1, Fig. 1D). Thus, org41841, which is only a partial agonist with 22% of TSH activity at TSHR, acts as a full agonist for M9. This further supports the proposed binding pocket. In M9, all but one of the 6 residues that were changed in the region that covers the binding cleft were either less hydrophobic and/or less bulky than in TSHR and M9 lacks a proline (P5.34T) known for its impact on backbone conformation. Consequently, the three TMH/ECL junctions in M9 are differently packed than in TSHR and provide more space for the ligand and less hydrophobic properties at the extracellular cover of the small ligand-bind-
Intracellular cAMP accumulation was determined in response to increasing concentrations of TSH or org41841 and is expressed as percent of maximum response of TSHR to TSH (100 milliunits/ml). The data are presented as mean ± S.E. of three independent experiments, each performed in duplicate.

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In conclusion, a bioinformatic comparison of the related receptors for TSH and LHCGR delineated the conformational space and orientations of the ligand in the pocket. To find optimal ligand-receptor interaction, a further restraint was used with respect to the experimentally determined contact point E3.37 (see below) and the potential complementary NH₂ group of the ligand. This docking of org41841 followed by molecular dynamics simulations allowed two preferred binding orientations within the binding cleft (supplemental Fig. S1). Common to both is the pivotal and experimentally supported role of the anchor-point E3.37 for hydrogen-bonding interaction with the ligand. In docking version A, the t-butyl group is oriented toward the transmembrane core between residues M6.48 and Y7.42 and the aryl-meta-methoxy group points toward F5.42. In docking version B, the t-butyl and aryl-meta-methoxy groups are oriented in opposite directions from their positions in A.

The predicted hydrogen bonding interaction between the amino group of the ligand and residue E3.37 in the receptor was tested. A mutant receptor with an alanine substitution at the highly conserved position E3.37 exhibited cell surface expression (93%) and response to TSH (Fig. 2, left) similar to TSHR. However, mutant E3.37A was unable to form the predicted hydrogen bond with and was not activated by org41841 (Fig. 2, right). The finding that loss of the hydrogen bond acceptor in mutant E3.37A disrupts receptor activation strongly supports the critical role of the predicted hydrogen bond between org41841 and E3.37 and thereby adds critical validation of the model.

In conclusion, a bioinformatic comparison of the related receptors for TSH and CG/LH allowed the successful prediction that a LMW ligand for one receptor would also activate the other. Utilizing the sequence differences and the fact that org41841 was less effective at TSHR than at LHCGR, this comparison provided experimental evidence for the proposed binding pockets within the TMHs, since changing TSHR residues to the corresponding residues of LHCGR in the proposed pocket consistently improved the response to org41841. Our results provide the first report of a LMW ligand for TSHR and direct evidence that this ligand binds within the transmembrane core to activate GPHRs.

Indeed, org41841 activates the TSHR with too low a potency to be clinically useful. Further experiment-based refinements of both the binding pocket and the orientation of the ligand within the pocket may guide the synthesis of new compounds by structure-based rational design and might lead to identification of highly potent LMW ligands for glycoprotein hormone receptors. LMW ligands of LHCGR and FSHR have the potential to become therapeutics for infertility treatment or oral contraception. It is noteworthy that in vivo efficacy of org41841 for LHCGR was demonstrated in an ovulation induction model (5) supporting the potential pharmacological utility of such synthetic ligands. Similarly, LMW antagonists of TSHR may have therapeutic potential for TSHR-mediated hyperthyroidism (24, 25), while agonists might replace injected recombinant TSH in diagnostic screening for thyroid cancer (26).

REFERENCES
1. Ascoli, M., Fanelli, F., and Segaloff, D. L. (2002) Endocrin. Rev. 23, 141–174
2. Fan, Q. R., and Hendrickson, W. A. (2005) Nature 433, 269–277
3. Szudlinksi, M. W., Fremont, V., Ronin, C., and Weinstraub, B. D. (2002) Physiol. Rev. 82, 473–505
4. Vassart, G., Pardo, L., and Costagliola, S. (2004) Trends Biochem. Sci. 29, 119–126
5. van Straten, N. C., Schoonruis-Gerritsma, G. G., van Someren, R. G., Draaijjer, J., Adang, A. E., Timmers, C. M., Hansen, R. G., and van Boeckel, C. A. (2002) Chemico-biochem. 3, 1023–1026
6. van Straten, N. C., van Berkel, T. H., Demont, D. R., Karstens, W. J., Merkx, R., Oosterom, J., Schulz, J., van Someren, R. G., Timmers, C. M., and van Zandvoort, P. M. (2005) J. Med. Chem. 48, 1697–1700
7. Gershengorn, M. C., and Osman, R. (2001) Endocrinology 142, 2–10
8. Stenkamp, R. E., Teller, D. C., and Palczewski, K. (2005) Arch. Pharm. (Weinheim) 338, 209–216
9. Strader, C. D., Candelier, M. R., Hill, W. S., Sigal, I. S., and Dixon, R. A. (1989) J. Biol. Chem. 264, 13572–13578
10. Tunaru, S., Lattig, J., Kero, J., Krause, G., and Offermanns, S. (2005) Mol. Pharmacol. 68, 1217–1280
11. Malitschek, B., Schweizer, C., Keir, M., Heid, J., Froestl, W., Mosbacher, J., Kuhn, R., Henley, J., Joly, C., Fin, P. J., Kaupmann, K., and Bettler, B. (1999) Mol. Pharmacol. 56, 448–454
12. Tobudizu, E. C., Chen, C., and Beinborn, M. (2001) J. Biol. Chem. 276, 37787–37793
13. Libert, F., Lefort, A., Gérard, C., Parmentier, M., Perret, J., Ludgeat, M., Dumont, J. E., and Vassart, G. (1998) Biochem. Biophys. Res. Commun. 165, 1250–1255
14. Schulz, A., Schöneberg, T., Paschke, R., Schulte, G., and Gudermann, T. (1999) Mol. Endocrinol. 13, 181–190
15. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motifohs, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
16. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) Nucleic Acids Res. 28, 235–242
17. Ballesteros, J. A., and Weinstein, H. (1995) Methods Enzymol. 253, 366–425
18. Azaros, F. I., Reddows, S., Willingham, T., Wu, L., and Koup, R. A. (2001) Virology 287, 382–390
19. Dragic, T., Trzola, A., Lin, S. W., Nagashima, K. A., Kaijmo, F., Zhao, L., Olson, W. C., Wu, L., Mackay, C. R., Allaway, G. P., Sakmar, T. P., Moore, J. P., and Maddon, P. J. (1998) J. Virol. 72, 279–285
20. Lee, B., Sharron, M., Blainpant, C., Dornaz, B. J., Vakili, J., Setoh, P., Berg, E., Liu, G., Guy, H. R., Durell, S. R., Parmentier, M., Chang, C. N., Price, K., Tsang, M., and Doms, R. W. (1999) J. Biol. Chem. 274, 9617–9626
21. ter Laak, A. M., and Kuhnze, R. (1999) Receptors Channels 6, 295–308
22. Case, D. A. (2002) Acc. Chem. Res. 35, 325–331
23. Laskowski, R. A., Moss, D. S., and Thornton, J. M. (1993) J. Mol. Biol. 231, 1049–1067
24. McLachlan, S. M., Nagayama, Y., and Rapoport, B. (2005) Endocur. Rev. 26, 800–832
25. Paschke, R., and Ludgeat, M. (1997) N. Engl. J. Med. 337, 1675–1681
26. Woodmansee, W. W., and Haugen, R. R. (2004) Clin. Endocrinol. (Oxf) 61, 163–173

FIGURE 2. Evidence for a hydrogen bond between org41841 and TSHR residue E3.37. Comparison of activation of TSHR and mutant E3.37A by TSH and org41841. E3.37A confirmed at this position is required for org41841 activation of the TSHR. Intracellular cAMP accumulation was determined in response to increasing concentrations of TSH or org41841 and is expressed as percent of maximum response of TSHR to TSH (100 milliunits/ml). The data are presented as mean ± S.E. of three independent experiments, each performed in duplicate.