Discovery of a Novel, Potent, and Orally Active Nonpeptide Antagonist of the Human Luteinizing Hormone-Releasing Hormone (LHRH) Receptor

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Luteinizing hormone-releasing hormone (LHRH; pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is secreted in pulses from the hypothalamus and stimulates the anterior pituitary gland to release the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Ultimately, both of these hormones elicit gonadal production of sex steroids and gametogenesis. Since its discovery in 1971,[1] LHRH (synonymous with gonadotropin-releasing hormone), which has a pivotal role in the modulation of reproductive functions, and its synthetic analogues have attracted considerable scientific interest because of their usefulness in the treatment of endocrine-based diseases such as prostate cancer, breast cancer, endometriosis, uterine leiomyoma, and precocious puberty.[2] Several LHRH agonists, represented by leuprorelin (pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt),[3] are currently used in the treatment of the above conditions.[4−6] They bind to the LHRH receptor in the pituitary gonadotrophs inducing the synthesis and release of gonadotropins. Chronic administration of potent LHRH agonists, which are referred to as 'superagonists', depletes gonadotropins and subsequently down-regulates the receptor in the pituitary gonadotrophs, resulting in suppression of steroidal hormones in mammals. Due to this peculiar mechanism of action, however, LHRH agonists suppress gonadotropins and sex steroids only following chronic administration (2−4 weeks) and occasionally evoke the release of the gonadal hormones[2] in the first days of administration.

On the other hand, LHRH antagonists are expected to suppress gonadotropins from the onset and be devoid of this initial hormone surge. Consequently, intense research over the past 20 years has focused on the development of potent and safe antagonists. The relatively low potency and the adverse effects, due to histamine release, have been the main obstacles to their acceptance and clinical use. Recently, several peptide antagonists with low histamine-release properties were reported,[7] but they are still in clinical studies. Moreover, these peptide antagonists must be administered by daily subcutaneous injection, intranasal spray, or a suitable sustained delivery system because of their insufficient oral bioavailability.

We sought, therefore, to discover and develop a potent and orally active nonpeptide LHRH antagonist with therapeutic efficacy in humans. In this paper, we describe the design, synthesis, and biological properties of isopropyl 3-(*N*-benzyl-*N*-methylaminomethyl)-7-(2,6-difluorobenzyl)-4,7-dihydro-2-(4-isobutyrylaminophenyl)-4-oxothieno[2,3-*b*]pyridine-5-carboxylate hydrochloride (**1**, T-98475), a highly potent and orally active nonpeptide antagonist of the human LHRH receptor (Chart 1).

In our search for a novel nonpeptide LHRH antagonist, we first focused on a type II β-turn[5,6] involving residues 5−8 (Tyr-Gly-Leu-Arg) of LHRH that was considered to be a dominant structure for its binding to the receptor. Introduction of crucial functional moieties for receptor binding into a bicyclic heterocycle "scaffold", which mimics the relatively fixed main chain of the β-turn, would provide a nonpeptide ligand of the LHRH receptor. In fact, a number of heterocyclic compounds bearing important functional groups for receptor binding have been disclosed as synthetic nonpeptide antagonists of G protein-coupled receptors (GPCRs), such as cholecystokinin,[8] angiotensin II,[9] substance P,[10] vasopressin,[11] and endothelin.[12] Our approach toward the discovery of a lead molecule included "directed screening"[13] of our chemical library. For this purpose, compounds selected for their similarity to antagonists of other GPCRs were screened for their inhibitory effects on the specific binding of [^{125}I]leuprorelin to the human LHRH receptor[14] expressed in Chinese hamster ovary (CHO) cells.[15] This screening led to the identification of a thieno[2,3-*b*]pyridin-4(7*H*)-one derivative (**2**) that demonstrated significant inhibition of [^{125}I]leuprorelin binding (67% inhibition at 20 μM).[16] Comparison of the structure of compound **2** with the β-turn portion of LHRH suggested that the *p*-methoxyphenyl group and the ethyl ester group of **2** would correspond to the side chains of the Tyr and Leu residues, respectively. Furthermore, by considering the fact that substituting hydrophobic D-amino acids for the Gly residue of LHRH increases the activity,[6] we hypothesized that the *o*-methoxybenzyl moiety of **2** may mimic the side chains of such hydrophobic D-amino acids. According to this hypothesis as illustrated in Chart 1, compound **2** is lacking a functional group corresponding to the Arg residue of LHRH, and we thus attempted to introduce an appropriate amino function into the lead compound (**2**). We found that introduction of a basic amino moiety into the 3-methyl group of **2** increased the activity, and further modification resulted in the discovery of T-98475 (**1**).

Chemistry. The synthetic route of compound **1** (T-98475) is shown in Scheme 1. Reaction of ethyl 2-amino-4-methyl-5-phenylthiophene-3-carboxylate (**3**)[17] with diethyl ethoxymethylenemalonate, followed by alkaline hydrolysis of the 3-ethyl ester moiety and subsequent ring closure, produced the thieno[2,3-*b*]pyridine **4** according to the procedure in the literature.[18] Benzylation of **4** occurred predominantly at the 7-position, and the

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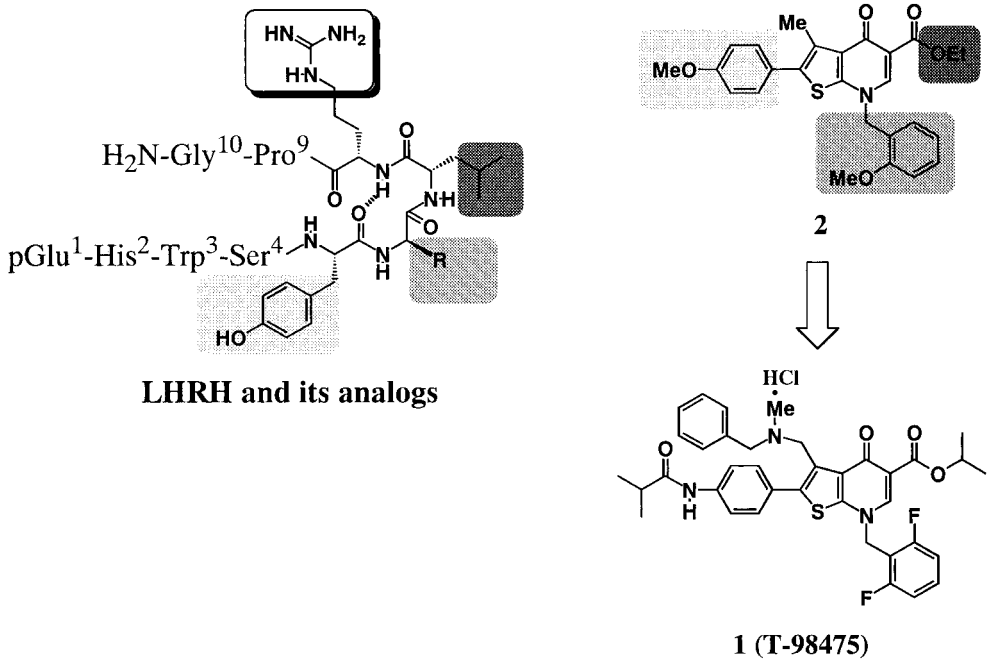
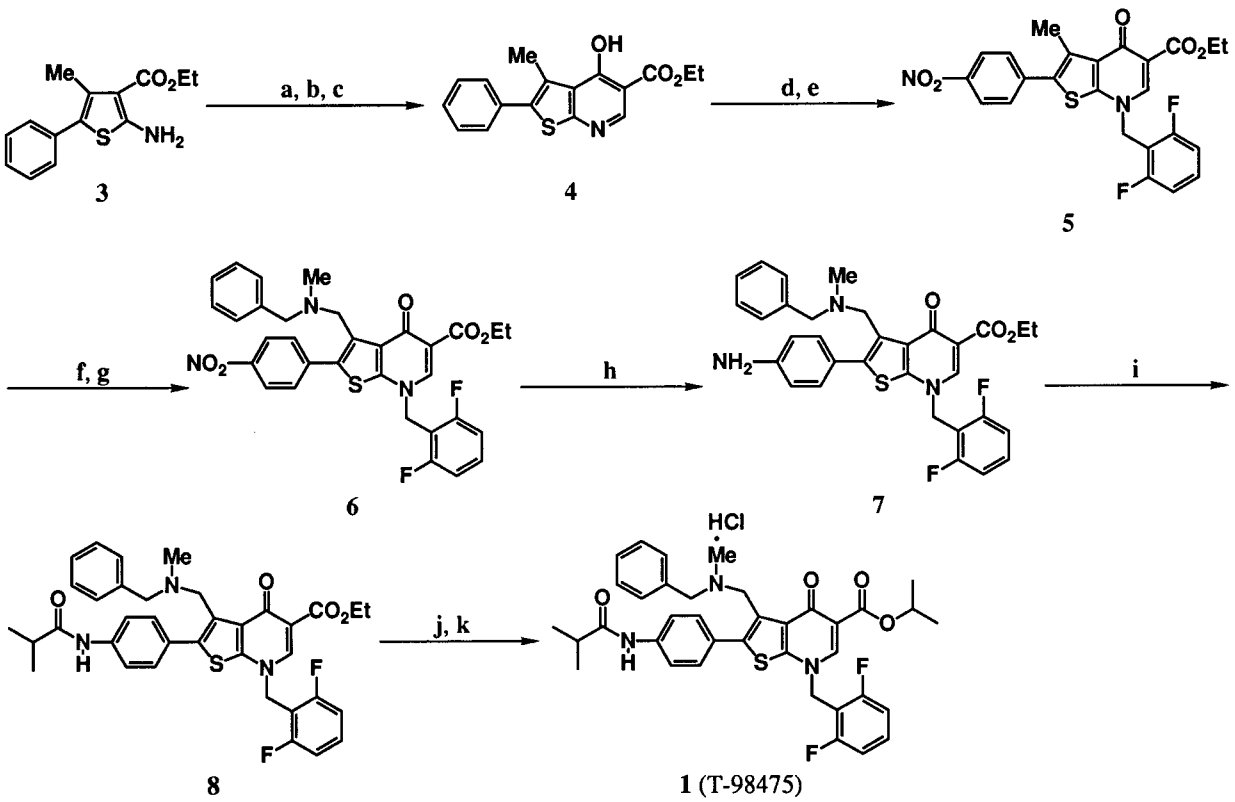
Chart 1



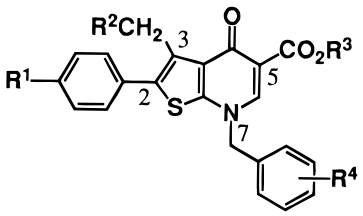
Scheme 1[a]



[a] Reagents: (a) diethyl ethoxymethylenemalonate, 120 °C (92%); (b) KOH−EtOH, dioxane, 70 °C (98%); (c) PPE, 120 °C (60%); (d) 2,6-difluorobenzyl chloride, K_2CO_3, KI, DMF, room temperature (90%); (e) $NaNO_3$, concd H_2SO_4, 0 °C (71%); (f) NBS, AIBN, CCl_4, reflux (79%); (g) *N*-benzylmethylamine, *N*,*N*-diisopropylethylamine, DMF, room temperature (91%); (h) iron powder, concd HCl, EtOH, 0 °C (96%); (i) isobutyric anhydride, triethylamine, CH_2Cl_2, room temperature (quant); (j) $Ti(O^iPr)_4$, ^{i}PrOH, room temperature (82%); (k) 10 M HCl−EtOH, 0 °C (quant).

resultant thieno[2,3-*b*]pyridin-4(7*H*)-one was selectively nitrated at the para position of the 2-phenyl ring to afford **5**. Radical bromination of the 3-methyl group of **5**, followed by installation of the *N*-benzylmethylamino moiety, furnished the nitro compound **6**. Reduction of **6** with iron powder-concentrated hydrochloric acid produced the amine **7**, which was converted to the amide **8** by acylation with isobutyric anhydride. The preparation of **1**[19] was accomplished by transesterification of **8** with titanium(IV) isopropoxide−2-propanol[20] and sub-

Table 1. Structures and Binding Affinities of Thieno[2,3-*b*]pyridin-4-ones



					IC_{50} (nM)[a]	
compd	R^1	R^2	R^3	R^4	human[b]	rat[c]
9	MeO	MeNH	Et	2-OMe	2000	43%[d]
10	MeO	$PhCH_2N(Me)$	Et	2-OMe	70	34%[d]
11	MeO	$PhCH_2N(Me)$	Et	2,6-F	6	800
7	NH_2	$PhCH_2N(Me)$	Et	2,6-F	4	500
1 (T-98475)	^{i}PrCONH	$PhCH_2N(Me)$	^{i}Pr	2,6-F	0.2	60

[a] All data are expressed as means of three or more determinations. [b] Binding to the cloned human receptor; see ref 20. [c] Binding to membrane fractions of rat pituitary; see ref 21. [d] Percent inhibition at 1 μM.

sequent treatment with 10 M hydrogen chloride−ethanol. In addition, compounds **2**, **9**, **10**, and **11** were prepared using a procedure analogous to that depicted in Scheme 1.

Results and Discussion. Compounds **1**, **2**, **7**, **9**, **10**, and **11** were evaluated for inhibition of [^{125}I]leuprorelin binding to the cloned human LHRH receptor and membrane fractions of the rat anterior pituitary.[21] The results are summarized in Table 1 and are discussed based on the affinity to the human receptor unless otherwise mentioned. Introduction of a basic amino moiety, important for receptor binding, into the 3-position of **2** induced a significant increase in affinity. In fact, the methylaminomethyl derivative (**9**) had a higher affinity than the initial lead (**2**). Optimization of the amino moiety at the 3-position provided us with the first breakthrough and led to the *N*-benzylmethylaminomethyl derivative (**10**), which was ca. 30-fold more potent than **9**. The structure−activity study of the 7-subsitituent revealed that ortho-substituted benzyl moieties were favorable. Among them, the 2,6-difluorobenzyl derivative (**11**) exhibited excellent affinity with a half-maximal inhibition concentration (IC_{50}) of 6 nM. Transposition of the *p*-methoxy group on the 2-phenyl ring into the ortho or meta position was detrimental for the binding affinity. Other para substituents, e.g., amino (**7**), maintained affinity almost comparable to methoxy (**11**). Acylation of the *p*-amino group and transesterification, however, produced another boost in activity. Compound **1** had a 20-fold higher affinity compared to **7**, and its IC_{50} value was 0.2 nM. From these results, **1** (T-98475) was selected for further in vitro and in vivo evaluation.

As is shown in Figure 1, **1** (T-98475), leuprorelin, and LHRH produced a concentration-dependent displacement of the specific binding of [^{125}I]leuprorelin to the cloned human LHRH receptor. Compound **1** had a 50-fold higher affinity than LHRH for the human receptor. In addition, **1** was virtually equipotent to the superagonist leuprorelin in this assay (see Table 2). Compound **1** also inhibited LHRH-stimulated arachidonic acid release from CHO cells expressing the human LHRH receptor with a pA_2 value of 9.4 (data not shown). Furthermore, **1** had insignificant binding to other GPCRs of biologically active peptide ligands such as

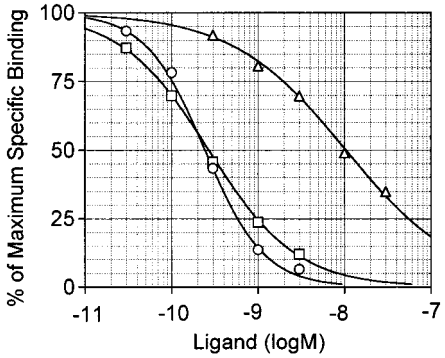


Figure 1. Inhibition of [^{125}I]leuprorelin binding to the human LHRH receptor by T-98475 (**1**) (○), leuprorelin (□), and LHRH (△).

Table 2. Species Specificity of T-98475 (**1**) Binding to the LHRH Receptors

	IC_{50} (nM)[a]		
compd	human[b]	monkey[c]	rat[c]
LHRH	10	6	7
leuprorelin	0.3	0.8	0.5
T-98475 (**1**)	0.2	4	60

[a] All data are expressed as means of three or more determinations. [b] Binding to the cloned human receptor; see ref 16. [c] Binding to membrane fractions of monkey and rat pituitaries; see ref 21.

angiotensin II, cholecystokinin, endothelin, neurotensin, substance P, thyrotropin-releasing hormone (TRH), and others. Thus, these results indicate that **1** (T-98475) is a potent and specific antagonist of the LHRH receptor in vitro.

To gain insight into the molecular basis for the interaction of **1** (T-98475) with LHRH receptors, a model for the human LHRH receptor was constructed (Figure 2, left).[22] Figure 2, right, shows the binding mode of **1** to the modeled receptor.[23] The bottom part of the ligand-binding pocket of the receptor is mainly hydrophobic and constitutes the binding site of the 2,6-difluorobenzyl and thieno[2,3-*b*]pyridin-4-one moieties of **1**. The structure−activity relationship of peptide LHRH analogues demonstrated the importance of the positively charged Arg residue of LHRH for high-affinity binding to the LHRH receptor.[6] Mutagenesis studies of the mouse LHRH receptor revealed a critical role for Glu301 in recognizing Arg of LHRH.[24] In the binding mode shown in Figure 2, right, the positively charged amino group of **1** is located in close proximity to Asp302 (equivalent to Glu301 of the mouse receptor) in the seventh transmembrane domain of the human receptor. Compound **1** might thus share one of the critical residues for receptor binding with LHRH.

All the compounds had higher affinities for the human receptor than for the rat receptor (Table 1). Therefore, species specificity of the binding of **1** (T-98475) to the LHRH receptors was investigated and compared to those of peptide ligands, LHRH and leuprorelin. The binding affinities of **1** to monkey and rat pituitary membranes[21] were 20- and 300-fold less potent than for the human receptor, whereas LHRH and leuprorelin had almost the same affinities in these species as in the human (Table 2). Accordingly, we chose a cynomolgus monkey and its tissues for evaluation of in vitro and in vivo functional activities of **1** (T-98475).

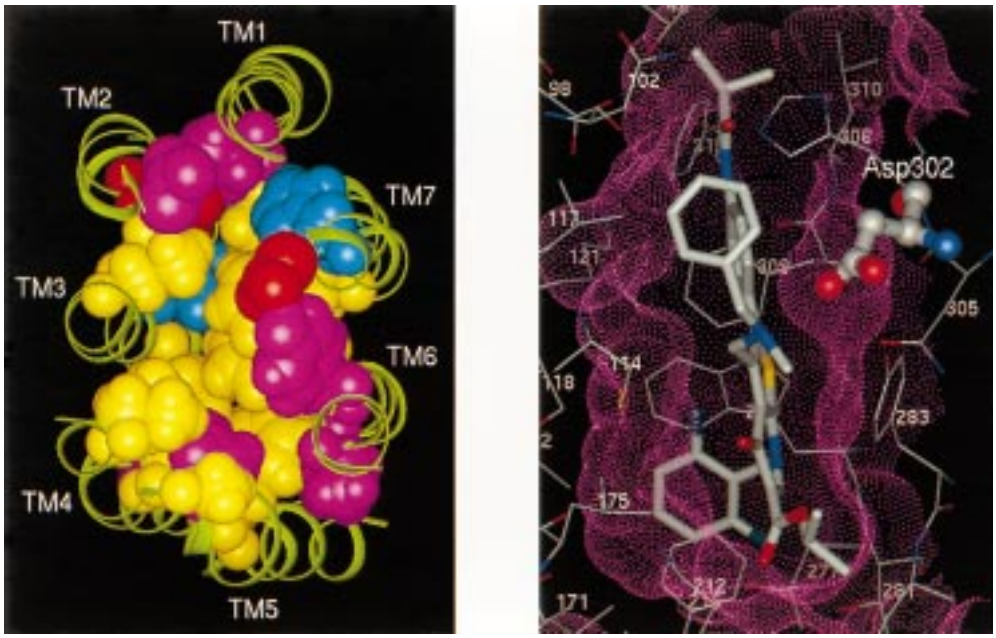


Figure 2. Left: Three-dimensional model of the human LHRH receptor viewed from the extracellular side. Right: Detailed view of the interaction between the human LHRH receptor (thin bonds) and T-98475 (**1**) (thick bonds).

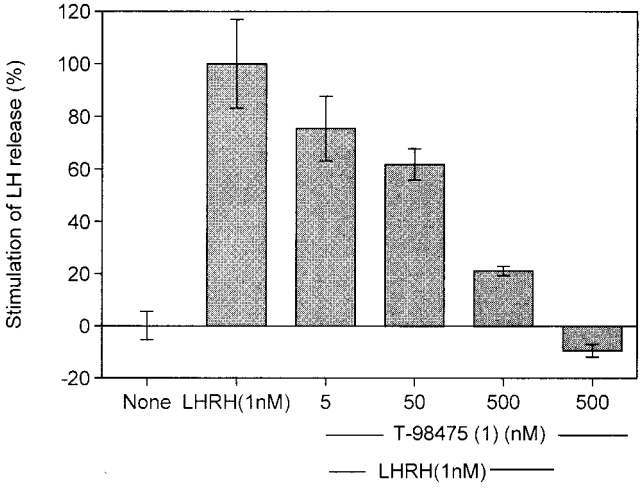


Figure 3. Inhibitory effects of T-98475 (**1**) on LHRH-stimulated LH release from monkey pituitary cells. Values shown are the mean ± SEM ($n = 4$).

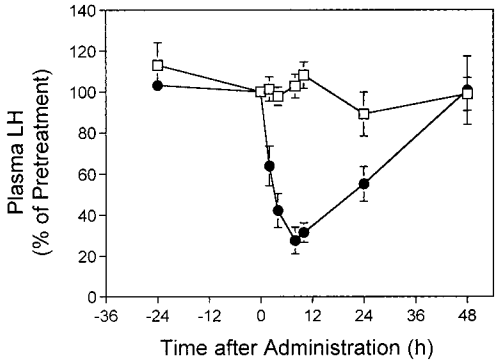


Figure 4. Suppression of plasma LH concentrations after oral administration of T-98475 (**1**) in castrated male cynomolgus monkeys: T-98475 (**1**) (60 mg/kg) suspended in 0.5% methylcellulose (●) ($n = 4$) and 0.5% methylcellulose alone (□) ($n = 5$). Values shown are the mean ± SEM.

First, the inhibitory effects of **1** on LHRH-stimulated LH release were examined using cultured cynomolgus monkey pituitary cells.[25] Compound **1** had no agonistic effect and markedly attenuated the stimulation of LH release induced by 1 nM LHRH in a concentration-dependent manner (Figure 3). The concentration of **1** that inhibited 50% of the stimulation of LH release (IC_{50}) was approximately 100 nM and 25-fold higher compared to that of receptor binding.

Next, the in vivo pharmacological activity of **1** was investigated in the suppression of plasma LH concentrations in castrated male cynomolgus monkeys.[26] Castration elicited an elevation of the circulating LH levels in cynomolgus monkeys. After oral administration of **1** (60 mg/kg), suppression of plasma LH levels occurred in a time-dependent manner. Compound **1** exhibited more than 70% inhibition of plasma LH levels 8 h after administration, and this inhibitory effect lasted for more than 10 h at this dosage (Figure 4). Taking into account the large difference in the affinities of **1** between human and monkey receptors, a far smaller amount of **1** should be enough to suppress plasma LH levels in humans.

Conclusion. The results of in vitro and in vivo studies clearly demonstrate that **1** (T-98475) is the first potent and orally effective nonpeptide antagonist of the human LHRH receptor. It is expected that **1** (T-98475) will overcome the shortcomings of the current peptide antagonists that require daily injection and will provide a new class of useful therapeutic agents for the treatment of sex hormone-dependent pathologies.

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(15) The human LHRH receptor cDNA was cloned from a pituitary cDNA library using a rat LHRH receptor cDNA fragment as a probe. Briefly, a 658-bp rat LHRH receptor cDNA fragment was cloned from rat testis poly(A)$^+$RNA with reverse transcription and polymerase chain reaction methods with oligonucleotide primers (5′-TGAAGCCTGTCCTTGGAGAAATATGGC-3′ and 5′-AAAGTTGTAGAAGGCCTGATGCCACCA-3′) synthesized based on the sequence of the mouse LHRH receptor; see: Tsutsumi, M.; Zhou, W.; Millar, R. P.; Mellon, P. L.; Roberts, J. L.; Flanagan, C. A.; Dong, K.; Gillo, B.; Sealfon, S. C. Cloning and Functional Expression of a Mouse Gonadotropin-Releasing Hormone Receptor. *Mol. Endocrinol.* **1992**, *6*, 1163−1169. Then, a human pituitary λgt 11 cDNA library (Clontech, Palo Alto, CA) was screened with the [α-^{32}P]dCTP-labeled rat LHRH receptor cDNA fragment used as a probe. The hybridization of filters was performed at 55 °C in hybridization buffer (5×SSC, 10×Denhardt's solution, 0.1% SDS, 150 μg/mL heat-denatured salmon sperm DNA). The filters were washed in 0.2×SSC, 0.1% SDS at 50 °C, dried, and autoradiographed. A 1.1-kb *Eco*RI fragment was selected and subcloned into a pUC118 plasmid. The nucleotide sequence of the cloned human LHRH receptor cDNA was identical to that reported in the literature.[14] The human LHRH receptor cDNA was inserted into the pAKKO-111 expression vector, which had an SR α-promoter and a dihydrofolate reductase gene (*dhfr*) as a selection marker. The vector was introduced into (*dhfr*$^-$)CHO cells using calcium phosphate-mediated transfection. The transfected CHO cells were cultured in a selection medium, and a single colony expressing high levels of the receptor was isolated.

(16) The binding data in this text for all the compounds was obtained using the cloned human receptor expressed in CHO cells[15] for initial screening. The CHO cells expressing the human LHRH receptor (1×10^9 cells) were suspended in 5 mM EDTA-PBS and centrifuged. The pellet suspended in 10 mL of homogenate buffer (10 mM $NaHCO_3$, 5 mM EDTA, pH 7.5) was homogenized using a Polytron homogenizer. After the resulting homogenate was centrifuged for 15 min at 400*g*, the supernatant was centrifuged for 1 h at 100000*g*. The pellet, resuspended in 10 mL of assay buffer A [25 mM Tris, 1 mM EDTA, 0.1% bovine serum albumin (BSA), 0.03% NaN_3, 0.25 mM phenylmethanesulfonyl fluoride, 1 μg/mL pepstatin A, 20 μg/mL leupeptin, and 100 μg/mL phosphoramidon, pH 7.5], was centrifuged for 1 h at 100000*g*. The membrane fraction obtained as a pellet was suspended in 20 mL of assay buffer A and was stored at −80 °C. The protocol of the binding experiments was as follows: Labeled leuprorelin (0.15 nM) and the membrane fractions (0.2 mg/mL) of CHO cells expressing the human LHRH receptor were incubated at 25 °C for 60 min in 0.2 mL of assay buffer A in the presence of various concentrations of test compounds. The reaction was terminated by the addition of 2 mL of ice-cold assay buffer A, and bound and free ligands were separated by filtration through a poly(ethylenimine)-coated glass microfiber filter (Whatman, GF/F). The filter was washed twice with 2 mL of assay buffer A, and radioactivity was measured using a γ-ray counter. Specific binding was determined by subtraction of the nonspecific binding, which was measured in the presence of 1 μM unlabeled leuprorelin. The concentration of a test compound causing 50% inhibition of the specific binding (IC_{50} value) was derived by fitting the data into a pseudo-Hill equation: $\log[\%SPB/(100 - \%SPB)] = n[\log(C) - \log(IC_{50})]$, where %SPB is specific binding as a percentage of maximum specific binding, n is a pseudo-Hill constant, and C is the concentration of a test compound.

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(19) The physicochemical data of T-98475 (**1**) was as follows: ^{1}H NMR (free base) (300 MHz, $CDCl_3$) δ 1.28 (6H, d, J = 6.8 Hz), 1.36 (6H, d, J = 6.3 Hz), 2.10 (3H, s), 2.53−2.61 (1H, m), 3.65 (2H, s), 4.16 (2H, s), 5.19−5.27 (1H, m), 5.23 (2H, s), 7.00 (2H, t, J = 8.1 Hz), 7.10−7.26 (5H, m), 7.34−7.42 (1H, m), 7.63 (2H, d, J = 8.3 Hz), 7.78 (2H, d, J = 8.6 Hz), 8.29 (1H, s). The hydrochloride salt was obtained by treatment of the free base with 10 M HCl−EtOH to afford colorless needles (from EtOH−ether): mp 168−170 °C; IR (KBr) 3400, 2976, 1690, 1603, 1504, 1473 cm^{-1}; FAB-MS *m/e* 658 (MH^+). Anal. ($C_{37}H_{37}N_3O_4SF_2 \cdot HCl \cdot 0.5H_2O$) C, H, N.

(20) Imwinkelried, R.; Schiess, M.; Seebach, D. Diisopropyl (2S,3S)-2,3-*O*-isopropylidenetartrate (1,3-Dioxolane-4,5-dicarboxylic acid, 2,2-dimethyl-, bis(1-methylethyl)ester, (4R-trans)-). *Org. Synth.* **1987**, *65*, 230−235.

(21) The binding experiments of test compounds to the LHRH receptors of other species were performed using membranes prepared from the anterior pituitaries of male Wistar rats and cynomolgus monkeys. The protocol of the binding experiments was essentially the same as that using the cloned human receptor.[16] The membrane fractions of rat and monkey pituitaries were diluted with assay buffer A to 0.2 and 0.5 mg/mL, respectively. In addition, incubation with these membrane fractions was carried out at 4 °C for 90 min. For example, the preparation procedure of rat pituitary membranes was as follows: The anterior pituitaries obtained from 40 Wistar rats (male, 8 weeks old) were suspended in homogenate buffer (25 mM Tris, 0.3 M sucrose, 1 mM EGTA, 0.03% NaN_3, 0.25 mM phenylmethanesulfonyl fluoride, 10 U/mL aprotinin, 1 μg/mL pepstatin A, 20 μg/mL leupeptin, and 100 μg/mL phosphoramidon, pH 7.5) and homogenized using a Polytron homogenizer. After the resulting homogenate was centrifuged for 15 min at

700*g*, the supernatant was centrifuged for 1 h at 100000*g*. The pellet resuspended in 10 mL of assay buffer A was centrifuged for 1 h at 100000*g*. The membrane fraction obtained as a pellet was suspended in 10 mL of assay buffer A and was stored at −80 °C. The preparation of monkey pituitary membranes was performed in a similar manner.

(22) The LHRH receptor belongs to the G protein-coupled receptor family and consists of seven transmembrane segments, presumably adopting an α-helical conformation. Modeling procedures for the human LHRH receptor were similar to those already described; see: Yamamoto, Y.; Kamiya, K.; Terao, S. Modeling of Human Thromboxane A_2 Receptor and Analysis of the Receptor−Ligand Interaction. *J. Med. Chem.* **1993**, *36*, 820−825. First, a model for the human β_2-adrenergic receptor was constructed using its amino acid sequence and the known helix arrangement of bacteriorhodopsin; see: Henderson, R.; Baldwin, J. M.; Ceska, T. A.; Zemlin, F.; Beckmann, E.; Downing, K. H. Model for the Structure of Bacteriorhodopsin Based on High-Resolution Electron Cryo-Microscopy. *J. Mol. Biol.* **1990**, *213*, 899−929. Then, a model of the human LHRH receptor was built upon the scaffold of the β_2-adrenergic receptor. Figure 2, left, shows a three-dimensional model of the human LHRH receptor viewed from the extracellular side. Transmembrane helices are depicted as green ribbons, and residues constituting the putative ligand-binding pocket are shown with CPK models. Colors used for CPK models are red for acidic residues, cyan for basic residues, magenta for other hydrophilic residues, and yellow for hydrophobic residues.

(23) The binding mode of T-98475 (**1**) to the modeled receptor was investigated with the automated docking program DOCK 3.5; see: (a) Shoichet, B. K.; Bodian, D. L.; Kuntz, I. D. Molecular Docking Using Shape Descriptors. *J. Comput. Chem.* **1992**, *13*, 380−397. (b) Meng, E. C.; Shoichet, B. K.; Kuntz, I. D. Automated Docking with Grid-Based Energy Evaluation. *J. Comput. Chem.* **1992**, *13*, 505−524. (c) Meng, E. C.; Gschwend, D. A.; Blaney, J. M.; Kuntz, I. D. Orientational Sampling and Rigid-Body Minimization in Molecular Docking. *Proteins* **1993**, *17*, 266−278. Flexibility of the compound was taken into account by searching the conformational database prepared for T-98475 (**1**). Figure 2, right (the highest scoring mode), shows a detailed view of the interaction between the human LHRH receptor (thin bonds) and T-98475 (**1**) (thick bonds). T-98475 (**1**) is drawn with atoms in light green (carbon), cyan (nitrogen), red (oxygen), sulfur (yellow), and dark green (fluorine). A solvent-accessible surface of the receptor molecule is shown as purple dots. Asp302 in the seventh transmembrane domain is drawn as a ball-and-stick model.

(24) Flanagan, C. A.; Becker, I. I.; Davidson, J. S.; Wakefield, I. K.; Zhou, W.; Sealfon, S. C.; Millar, R. P. Glutamate 301 of the Mouse Gonadotropin-Releasing Hormone Receptor Confers Specificity for Arginine 8 of Mammalian Gonadotropin-Releasing Hormone. *J. Biol. Chem.* **1994**, *269*, 22636−22641.

(25) The anterior pituitaries obtained from three male cynomolgus monkeys were diced and incubated at 37 °C for 1 h in a buffer (0.7 mM Na_2HPO_4, 137 mM NaCl, 5 mM KCl, 25 mM HEPES, and 50 μg/mL gentamycin sulfate) containing 0.4% collagenase (Boehringer Mannheim, Mannheim, Germany) and 10 μg/mL deoxyribonuclease (Sigma, St. Louis, MO); then they were treated with 0.25% pancreatin (Sigma) at 37 °C for 8 min. The pituitary cells were washed twice with Dulbecco's modified Eagle's medium containing 20 mM HEPES (DMEM-H), then seeded in plates (24 wells, 2×10^5/well), and cultured for 3 days in DMEM-H supplemented with 10% FCS. To evaluate T-98475 (**1**), the cells were pretreated with DMEM-H containing 0.2% BSA at 37 °C for 1 h. After the medium was removed, fresh DMEM-H containing 0.2% BSA and various concentrations of T-98475 (**1**) was added and the mixture incubated for 1 h. Then, LHRH (1 nM) was added and the mixture incubated for 3 more hours. The medium was harvested, and LH concentration was measured with bioassays using mouse testicular cells. The bioassay to determine LH concentration was performed as follows: Testicular cells were obtained from male BALB/c mice (8−9 weeks old). An assay buffer, DMEM-H containing 0.2% BSA, was used in all the steps in this experiment. The cells were washed, incubated at 37 °C for 1 h, and passed through a nylon mesh (70 μm). The cells (8×10^5 cells) were washed twice and then incubated in 0.4 mL of the assay buffer with samples (conditioned medium of monkey pituitary cells or monkey plasma) to be tested at 37 °C for 2 h. Equine LH (Sigma) was used as a standard. Then, the medium was harvested, and testosterone concentration was measured with radioimmunoassay (CIS Diagnostics, Ltd., Sakura, Chiba, Japan).

(26) Cynomolgus monkeys (male, 4−8 years old) were castrated more than 11 weeks prior to the examination. The monkeys were trained to sit in a primate-restraining chair for administration of the compounds. T-98475 (**1**) (60 mg/kg) suspended in 0.5% methylcellulose or 0.5% methylcellulose alone was orally administered to the monkeys (volume of the administration: 3 mL/kg). Blood samples (heparin-plasma) were collected from a femoral vein at 24 h before administration and at 0, 2, 4, 8, 10, 24, and 48 h after administration. LH concentrations in the plasma were measured with bioassays using mouse testicular cells.[25]

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