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[^{3}H]Org 43553, the First Low Molecular Weight Agonistic and Allosteric Radioligand for the Human Luteinizing Hormone Receptor.

Laura H. Heitman, Julia Oosterom, Kimberly M. Bonger, Cornelis M. Timmers, Peter H. G. Wiegerinck, Adriaan P. IJzerman

Division of Medicinal Chemistry (L.H.H., A.P.IJ), Leiden/Amsterdam Center for Drug Research and Department of bio-organic synthesis (K.M.B.), Leiden Institute of Chemistry, University of Leiden, P.O. Box 9502, 2300 RA Leiden, The Netherlands

Molecular Pharmacology (J.O.), Medicinal Chemistry (C.M.T.) and Process Chemistry (P.H.G.W.), Organon BioSciences, P.O. Box 20, 5340 BH Oss, The Netherlands

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[^{3}H]Org 43553, a New Allosteric Radioligand for the LH Receptor.

A.P. IJzerman

Division of Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research, University of Leiden, P.O. Box 9502, 2300 RA Leiden, The Netherlands

Phone: +31- (0)71 527 4651.

Fax: +31- (0)71 527 4565.

ijzerman@lacdr.leidenuniv.nl

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ABBREVIATIONS: 7-TM, seven-transmembrane; cAMP, cyclic adenosine-5'-monophosphate; CHO, Chinese hamster ovary; CHOhLHr_luc, human LH receptor and luciferase reporter gene transfected in CHO cells; CRE-luc, cAMP-response-element luciferase reporter gene; FSH, follicle-stimulating hormone; GPCR, G protein-coupled receptor; hCG, human chorionic gonadotropin; HMW, high molecular weight; LMW, low molecular weight; PBS, phosphate-buffered saline; recLH, recombinant luteinizing hormone; SAR, structure-activity relationship; TSH, thyroid-stimulating hormone.

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ABSTRACT

The luteinizing hormone (LH) receptor plays a pivotal role in reproduction. The high-molecular-weight (HMW) hCG and LH are the endogenous ligands of this receptor and bind to its large N-terminus. The present study characterizes the binding of a new low-molecular-weight (LMW) radioligand, [^{3}H]Org 43553, at the LH receptor. Equilibrium saturation and displacement assays were developed and optimized. Specific binding of [^{3}H]Org 43553 to CHO-K1 cell membranes expressing the human LH receptor and a CRE-luciferase reporter gene was saturable with a K_D value of 2.4 ± 0.4 nM and a B_{max} value of 1.6 ± 0.2 pmol/mg protein. Affinities of five LMW analogues of Org 43553 were determined. All displaced the radioligand competitively with K_i values ranging from 3.3 - 100 nM. Lastly, the potency of these compounds in a cAMP-induced luciferase assay was also determined. There was a high correlation between affinity and potency ($r = 0.99$; $P < 0.0001$) of these compounds. In the search for LMW ligands, which bind allosterically to the seven-transmembrane (7-TM) domain of the LH receptor, a HMW radioligand (e.g. [^{125}I]hCG) is not suitable as it is not displaced by a LMW compound. Therefore, [^{3}H]Org 43553, a new radioligand with good binding properties, allows screening for new LMW ligands that mimic the action of the endogenous hormone at the LH receptor.

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The luteinizing hormone (LH) receptor is a member of the glycoprotein hormone receptor family within class A of G protein-coupled receptors (Vassart et al., 2004). A unique feature of the LH receptor is that it recognizes two endogenous ligands with high molecular weight, namely human chorionic gonadotropin (hCG) and LH. Both hormones bind with high affinity and selectivity to the large N-terminus of the receptor (Smits et al., 2003). Together with other gonadotropins, LH and hCG play a pivotal role in reproduction in which LH is responsible for ovulation induction in women and control testosterone production in men, whereas hCG maintains the early stages of pregnancy (Ascoli et al., 2002). Gonadotropins are currently used clinically in infertility treatment. Here, either urinary or recombinant gonadotropins are used, which need to be administered by parenteral (subcutaneous or intramuscular) injection (Loumaye et al., 1996). The advantage of low molecular weight (LMW) agonists is that they have the potential to become orally available drugs (Van Straten et al., 2002). This will alleviate the necessity of parenteral administration, which may result in enhanced patient compliance and convenience in comparison to current methods.

In the past few years, medicinal chemists have therefore been challenged to find LMW ligands for receptors that have high molecular weight (HMW) endogenous ligands (e.g. polypeptides and protein hormones). Although LMW ligands have already been described for the gonadotropin hormone receptors (Van Straten et al., 2002; Van Straten et al., 2005), radioligands have not thus far. Small molecule radioligands have been reported for other receptors with HMW endogenous ligands, for example, an antagonist for the corticotropin-releasing factor$_1$ receptor (Zhang et al., 2003), an agonist for the

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insulin receptor (Zhang et al., 1999) and an agonist for the glucagon-like peptide$_1$ receptor (Knudsen et al., 2007).

This paper describes for the first time the pharmacological characterization of the human luteinizing hormone receptor transfected in Chinese hamster ovary (CHO) cells using a tritium-labeled form of a small molecule ligand, [^{3}H]Org 43553 (see Table 2 for its chemical structure). Org 43553 is one of a series of thieno[2,3-d]pyrimidine derivatives that showed agonistic LH activity in a functional assay (Hanssen and Timmers, 2003). Org 43553 was chosen to be labeled with tritium, because it was one of the more potent compounds from this screen. The kinetic and equilibrium binding characteristics of the new radioligand were determined, and compared to those of the radiolabeled natural hormone hCG. [^{3}H]Org 43553 was also used in a displacement assay with four other thienopyrimidines and one quinazoline derivative. Their affinities correspond well to their potency in generating a cAMP response. Thus, this LMW radioligand provides a useful tool to further understand the interactions of the LH receptor with small molecule ligands.

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MATERIALS AND METHODS

Materials. Org 41841, Org 41247, Org 42619, Org 43311, Org 43553, Org 43983, (compounds **1**-**6**, respectively) and recLH were provided by Organon BioSciences (Oss, The Netherlands), where the Org-compounds were synthesized as described previously (Gerritsma et al., 2000; Timmers and Karstens, 2002; Hanssen et al., 2003; Hanssen and Timmers, 2003). Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO, U.S.A.), whereas BCA protein assay reagent was from Pierce Chemical Company (Rockford, IL, U.S.A.). [^{125}I]hCG (5966 Ci/mmol) was purchased from Perkin Elmer Life Sciences Inc. (Boston, MA, U.S.A.). Chinese Hamster Ovary (CHO-K1) cells stably expressing the human luteinizing hormone (LH) receptor and cAMP-response-element luciferase reporter gene (CRE-luc) were kindly provided by Organon BioSciences (Oss, The Netherlands). All other chemicals and cell culture materials were obtained from standard commercial sources.

Cell Culture. CHO cells with stable expression of the human LH receptor and CRE-luc (CHOhLHr_luc) were grown in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium (1:1) supplemented with 5 % normal adult bovine serum, streptomycin (100 µg/ml), penicillin (100 IU/ ml) at 37 °C in 5 % CO2. The cells were subcultured twice weekly at a ratio of 1:15. For membrane preparation the cells were subcultured 1:10 and transferred to large 15-cm diameter plates.

Membrane Preparation. Cells were detached from the plates by scraping them into 5 ml phosphate-buffered saline (PBS), collected and centrifuged at 700 g (3000 rpm) for 5 min. Pellets derived from 30 plates were pooled and resuspended in 20 ml of ice-cold 50

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mM Tris-HCl buffer containing 2 mM $MgCl_2$, pH 7.4. An UltraThurrax was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100,000 g (31,000 rpm) in a Beckman Optima LE-80K ultracentrifuge at 4 °C for 20 min. The pellet was resuspended in 10 ml of the Tris buffer and the homogenization and centrifugation step was repeated. Tris buffer (12 ml) was used to resuspend the pellet and the membranes were stored in 500 µL aliquots at –80 °C. Membrane protein concentrations were measured using the BCA (bicinchoninic acid) method with BSA as a standard (Smith et al., 1985).

Preparation of [^{3}H]Org 43553. The tritiation of Org 43553 was carried out by RC Tritec AG (Teufen, Switzerland). In short, 10 mg of 5-amino-2-methylsulfanyl-4-[3-(2-morpholin-4-yl-acetylamino)-phenyl]-thieno[2,3-d]pyrimidine-6-carboxylic acid tert-butylamide, Org 43553, was dissolved in 250 µl THF containing 0.1 % (v/v) water under nitrogen atmosphere. This solution was stirred for about 20 min, while cooling the flask to – 78 ºC. Then 120 µl of 1.3 M sec-butyllithium in hexane/cyclohexane (final concentration 10 eq.) was added drop wise. The color of the solution changed from yellow to dark red/brown, indicative for the molecule to be deprotonated. The solution was stirred for another 2 h at – 78 ºC. Next, the reaction was quenched with 20 Ci (i.e. an excess) of tritiated water at – 78 ºC. The color of the solution changed back to yellow, showing that the deprotonated molecule was tritiated. The reaction mixture was stirred for another 1.5 h at – 78 ºC after which the reaction mixture was allowed to warm up to room temperature in about 30 min. Next a large excess of diethyl ether (50 ml) was added. The organic layer was washed twice with water and once with brine, dried and concentrated under reduced pressure. The residue was dissolved in ethanol to give a

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solution of 250 mCi crude [^{3}H]Org 43553 in 25 ml ethanol. This solution was then purified by high-performance liquid chromatography on a Symmetry C_{18} column eluting with acetonitrile/water (40/60 (v/v) containing 0.1 % TFA) at Organon BioSciences (Oss, The Netherlands). After purification 140 mCi [^{3}H]Org 43553 with a radiochemical purity ≥ 95 % and a specific activity of 16.6 Ci/mmol was obtained.

Radioligand Displacement and Saturation Assays. *[^{3}H]Org 43553* Membrane aliquots containing 20 µg protein were incubated in a total volume of 100 µl assay buffer (25 mM Tris-HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.1 % BSA) at 30 °C for 1 h. For saturation experiments, total binding was determined at increasing concentrations (0.2-20 nM) of [^{3}H]Org 43553, whereas nonspecific binding was determined at three concentrations of radioligand in the presence of 10 µM Org 43553 and analyzed by linear regression. Displacement experiments were performed using ten concentrations of competing ligand in the presence of 20 nM [^{3}H]Org 43553. Non-specific binding was determined in the presence of 10 µM Org 43553 and represented approximately 50 % of the total binding. [^{3}H]Org 43553 did not bind specifically to membranes prepared from CHO cells lacking the LH receptor. Total binding was determined in the presence of buffer and was set at 100 % in all experiments, whereas non-specific binding was set at 0 %. Incubations were terminated by dilution with 1 ml ice-cold Tris-HCl buffer. Bound from free radioligand was immediately separated by rapid filtration through Whatman GF/B filters using a Millipore manifold. Filters were subsequently washed three times with ice-cold wash buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.05 % BSA). Filter-bound radioactivity was

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determined by scintillation spectrometry (Packard Tri-Carb 2900TR) after addition of 3.5 ml of Packard Emulsifier Safe.

[^{125}I]hCG Membrane aliquots containing 15 µg protein were incubated in a total volume of 100 µl assay buffer (25 mM Tris-HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.1 % BSA) at 30 °C for 2½ h. For [^{125}I]hCG, displacement experiments were performed using ten concentrations of recLH in the presence of 80,000 cpm (~0.1 nM) radioligand. Non-specific binding was determined in the presence of 50 U/ml (~70 nM) recLH and represented approximately 50 % of the total binding. [^{125}I]hCG did not bind specifically to membranes prepared from CHO cells lacking the LH receptor. Total binding was determined in the presence of buffer and was set at 100 % in all experiments, whereas non-specific binding was set at 0 %. Incubations were terminated by dilution with 1 ml ice-cold Tris-HCl buffer. Bound from free radioligand was immediately separated by rapid filtration through Whatman GF/C filters pre-soaked with 0.25 % poly(ethyleneimine) (PEI) for 1 h using a Millipore manifold. Filters were subsequently washed three times with ice-cold wash buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.05 % BSA). Filter-bound radioactivity was determined in a γ-counter (Wallac, Wizard 1470).

Radioligand Association and Dissociation Assays. Association experiments were performed by incubating membrane aliquots containing 20 µg protein in a total volume of 100 µL assay buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.1 % BSA) at 30 °C for 3 h with 80,000 cpm of [^{125}I]hCG or for 90 min with 20 nM [^{3}H]Org 43553. The amount of radioligand bound to the receptor was measured at various time intervals during incubation. Dissociation experiments were performed by

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preincubating membrane aliquots containing 20 µg protein in a total volume of 100 µl assay buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.1 % BSA) at 30 °C for 2½ h with 80,000 cpm [^{125}I]hCG or for 1 h with 20 nM [^{3}H]Org 43553, respectively. After preincubation, dissociation was initiated by addition of 50 U/ml recLH for [^{125}I]hCG. For [^{3}H]Org 43553 dissociation was initiated by addition of 10 µM Org 43553 (control), 10 ml assay buffer (100-fold dilution) or 50 U/ml recLH or combinations thereof, as explained in the Results section. The amount of radioligand still bound to the receptor was measured at various time intervals for a total of 240 min ([^{125}I]hCG) or 180 min ([^{3}H]Org 43553). Incubations were terminated and samples were obtained and analyzed as described above.

Luciferase Assays. CHOhLHr_luc cells were grown as described above. On the day of the assay, cells were washed with PBS and then harvested using trypsol (0.25 % (w/v) in PBS containing 4.4 mM EDTA). Cells were resuspended in assay medium consisting of DMEM and F12 (1:1) supplemented with 1 µg/ml insulin and 5 µg/ml apo-transferrin. Typically, a well contained 30 µL of test compound, 30 µL of assay medium and 30 µl cell suspension containing 7.5×10^5 cells/ml. Luciferase assays were performed using ten concentrations of test compound. Basal activity was determined in the presence of assay medium and represented approximately 10 % of the maximal activity. Maximal receptor activity was determined in the presence of 1 nM recLH and was set at 100 % in all experiments, whereas basal activity was set at 0 % in all experiments. After 4 h stimulation, 50 µl of luclite® (PerkinElmer, Groningen, The Netherlands) was added to each well for detection of luciferase protein and plates were left at room temperature for

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30 minutes in the dark. Finally, the luminescence signal was quantified on a Microbeta Trilux 1450 Luminescence Counter (PerkinElmer, Groningen, The Netherlands).

Data Analysis. All binding data were analyzed using the non-linear regression curve-fitting program GraphPad Prism v. 4.02 (GraphPad Software Inc, San Diego, CA, U.S.A.). EC_{50} values were directly obtained from the dose-response curves and inhibitory binding constants (K_i values) were derived from the IC_{50} values according to $K_i = IC_{50}/(1 + [C]/K_d)$ where [C] is the concentration of the radioligand and K_d its dissociation constant (Cheng and Prusoff, 1973). The K_d value of [^{3}H]Org 43553 at CHOhLHr_luc membranes was obtained by computer analysis of saturation curves. Dissociation constants, k_{off}, were obtained by computer analysis of the exponential decay of either [^{125}I]hCG or [^{3}H]Org 43553 bound to the receptor. Association rates were calculated according to the equation $k_{on} = (k_{obs} - k_{off})/[L]$, where k_{obs} was obtained by computer analysis of the exponential association of either [^{125}I]hCG or [^{3}H]Org 43553 bound to the receptor and [L] is the amount of radioligand used for the association experiments. All values obtained are means of at least three independent experiments performed in duplicate.

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RESULTS

Binding assay optimization. The assay conditions for [^{3}H]Org 43553 binding to CHOhLHr_luc membranes followed a general radioligand binding protocol in our laboratory (Heitman et al., 2006). We started our optimization efforts with [^{3}H]Org 43553 and 15 µg of protein in a simple buffer of low ionic strength (25 mM Tris HCl, pH 7.4) to which 2 mM $MgCl_2$ was added as is often done with agonist radioligands. Figure 1 summarizes the results of these experiments in which we studied further buffer components, filters and filter pretreatment, and membrane concentration. Firstly, a relatively high concentration (20 nM) of [^{3}H]Org 43553 was needed for an appreciable window of specific binding, which was greatly improved by the addition of BSA or CHAPS. It was decided to continue with 0.1 % BSA in the assay buffer. Secondly, the initial choice to use uncoated GF/B glass fiber filters to separate free from membrane-bound radioligand resulted in the highest specific binding; coating with PEI was not favorable. Next, the amount of protein used was increased to 20 µg, as this yielded a desired window of more than 1500 dpm. Initial kinetic association experiments taught us that the optimal incubation time was 60 min at 30 ºC.

Radioligand Saturation Experiments. Saturation binding assays were performed with [^{3}H]Org 43553. The results of a representative saturation experiment are shown in Figure 2. Binding of [^{3}H]Org 43553 to membranes of CHO cells expressing the human LH receptor was saturable and best described by a one-site model. The K_D value and B_{max} value obtained from the saturation experiments were 2.4 ± 0.4 nM and 1.6 ± 0.2 pmol/mg protein, respectively. The K_D value for [^{3}H]Org 43553 obtained with these experiments was used to derive K_i rather than IC_{50} values for analogues of Org43553 (see below). In

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the presence of 70 nM recLH, the K_D value was unaffected, while the B_{max} value was decreased by 24 ± 2 % (Figure 2), indicative for a non-competitive interaction.

Specific binding for [^{125}I]hCG was also demonstrated on these cell membranes. A consistent difference between total and non-specific binding was observed, but a plateau of specific binding was never reached at the radioligand concentrations used. The use of higher concentrations was considered prohibitively expensive, and spiking the samples with cold ligand yielded ambiguous results. Thus, it appeared impossible to determine K_D and B_{max} values for [^{125}I]hCG from these equilibrium studies. Therefore, the K_D value of [^{125}I]hCG was derived from its kinetic parameters as described in the next paragraph.

Kinetic Association and Dissociation Experiments. Subsequently, the kinetic behavior of [^{3}H]Org 43553 was studied and compared to that of [^{125}I]hCG. The kinetics of both radioligands were determined at 30 ºC on CHOhLHr_luc cell membranes. The binding of [^{3}H]Org 43553 and [^{125}I]hCG reached equilibrium after approximately 60 min and 150 min, respectively (Figures 3 and 4). Notably, equilibrium binding remained stable for both radioligands for at least 4 h (data not shown). [^{3}H]Org 43553 binding was reversible after the addition of 10 µM Org 43553 and complete dissociation was achieved after approximately 2 h as shown in Figure 3. The addition of an excess of recLH after association only resulted in a partial dissociation of [^{3}H]Org 43553. When recLH was already present during the preincubation, the B_{max} value of [^{3}H]Org 43553 was decreased by approximately 25 % (cf. Figure 2) and the dissociation rate was 1.7 ± 0.1-fold increased. The dissociation rate of the radioligand obtained by the 'infinite dilution' method (k_{off} = 0.020 ± 0.006 min^{-1}), however, was equal to the rate obtained in the control experiment in which dissociation was initiated by the addition of cold ligand only

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(Table 1). The further addition of 10 μM Org 43553 or 70 nM recLH in the infinite dilution experiment did not alter the dissociation rate of [^{3}H]Org 43553, as examined at 60 min of dissociation. For [^{125}I]hCG this was somewhat different: the addition of 70 nM recLH resulted in dissociation of this radioligand, however, after 4 h only 50 % was displaced (Figure 4). In Table 1, the association (k_{on}) and dissociation (k_{off}) constants are listed, as well as the apparent equilibrium dissociation constant (K_D), obtained from the control experiments represented in Figures 3 and 4. [^{125}I]hCG had a lower dissociation rate constant than [^{3}H]Org 43553, namely 0.0084 ± 0.001 min^{-1} compared to 0.020 ± 0.001 min^{-1}, respectively. The k_{obs} values obtained by analysis of the exponential association curves of both radioligands, together with the k_{off} values allowed to determine the kinetic association rate constants, k_{on} (Table 1). The dissociation binding constants (K_D) of the radioligands were derived from the dissociation and association rates. [^{125}I]hCG had a 60-fold higher affinity for the LH receptor than [^{3}H]Org 43553, 0.064 nM compared to 4.1 nM, respectively. The latter is in good agreement with the K_D value (2.4 nM) obtained in the saturation analysis.

Radioligand Displacement Assays. After this characterization of the radioligand, the affinities of non-labeled compounds **1**-**6** for the human luteinizing hormone receptor were determined (Table 2 and Figure 5). Displacement experiments were carried out with [^{3}H]Org 43553 on CHOhLHr_luc membranes, since the small molecule compounds were not able to displace [^{125}I]hCG from the human luteinizing hormone receptor (Table 2). Compound **1** (Org 41841) was potent, displacing [^{3}H]Org 43553 with a K_i value of 17 ± 5 nM. Replacement of the S-Me group of **1** with a phenyl group had a negative effect on receptor affinity (**2**, Org 41247). Enlargement of the meta-oriented substituent on the 4-

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phenylgroup had a positive impact, resulting in compounds **3-5** (Org 42619, 43311 and 43553, respectively) with a 4- to 10- fold higher affinity. Replacement of the thienyl moiety in the scaffold of **1** (Org 41841) by a phenyl ring (**6**, Org 43983) resulted in an approximately five-fold lower affinity. This value was comparable to the affinity of compound **2** (Org 41247). Org 43553 was chosen to be labeled with tritium, because it had a proven nanomolar potency in the functional assay and it was assumed to also exhibit a nanomolar binding affinity. In comparison, the endogenous ligand, recLH, was able to displace [^{125}I]hCG with a 3-fold higher affinity of 0.61 ± 0.1 nM, whereas it only modestly displaced [^{3}H]Org 43553 at a high concentration (Table 2). Labeling Org 43553 also had a practical reason, because the relatively acidic proton next to the morpholino-group was easily exchanged with tritium without a need for the synthesis of precursor molecules.

Agonistic Activity and Selectivity at the LH Receptor. In addition to radioligand displacement experiments, cAMP-induced luciferase assays were performed with compounds **1**-**6**. It follows from Figure 6 that all compounds were able to almost fully activate the receptor in comparison to recLH (87-95 %). Their potencies were determined from the relatively steep dose-response curves and ranged from 1.3 nM (**4**) to 119 ± 18 nM (**2**) (Table 2). In comparison, the endogenous ligand, recLH, had an EC_{50} value of 78 ± 2 pM in this cellular assay. There was a high correlation between data obtained from binding and functional assays ($r = 0.99$; $P < 0.0001$) (Figure 7). To obtain a full selectivity profile Org 43553 was examined on 59 different drug targets, of which 39 GPCRs. At the other glycoprotein hormone GPCRs, Org 43553 was at least ten-fold

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selective for the LH receptor (data not shown). LH receptor selectivity was more than 3000-fold for all other targets.

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DISCUSSION

The present study describes the first small molecule radioligand for the LH receptor. [^{3}H]Org 43553 has a thienopyrimidine core and literature survey reveals that this scaffold is a widely used pharmacophore, for example, as a serotonin receptor ligand (Modica et al., 2004), kinase inhibitor (Dai et al., 2005), and antimicrobial agent (Chambhare et al., 2003). The substitution pattern can be diverse, however, resulting in selectivity towards different targets. Org 43553 is selective for the LH receptor though, at least tenfold when compared with other glycoprotein hormone receptors, and more than 3000-fold selective for a whole panel of drug targets, including serotonin receptors.

The results of this paper show that [^{3}H]Org 43553 is a highly potent and selective agonistic radioligand that represents a novel tool for the screening of low molecular weight ligands for the LH receptor. The radioligand's receptor binding was saturable with a high affinity (K_D = 2.4 ± 0.4 nM). Kinetic experiments showed that both association and dissociation were much faster for [^{3}H]Org 43553 than for [^{125}I]hCG (Table 1). In addition, [^{125}I]hCG did not fully dissociate from the receptor. This has been described before for [^{125}I]hCG, where dissociation of specific binding was only 40 % after 8 h at 37 ºC (Henderson et al., 1984). This pseudo-irreversibility of dissociation most likely precluded the determination of the radioligand's K_D and B_{max} values from equilibrium saturation studies in the present experimental set-up. Others have reported a K_D value of 0.1 nM for [^{125}I]hCG, when binding to intact COS-7 cells transiently expressing the rat LH receptor was measured (Angelova et al., 2003; Bhowmick et al., 1996). This value is in good agreement with the K_D-value of 0.064 nM obtained from the kinetic experiments presented here. The dissociation of another glycoprotein hormone, thyroid-stimulating

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hormone (TSH), however, was shown to be complete and much faster, already at 22 ºC (Powell-Jones et al., 1981). In the kinetic comparison in the present study the binding of [^{3}H]Org 43553, unlike that of [^{125}I]hCG, was fully reversible. This feature rendered [^{3}H]Org 43553 a more suitable radioligand for further displacement studies.

To further explore the binding characteristics of this novel radioligand, assays were performed where the dissociation was initiated by the 'infinite dilution' method (Christopoulos et al., 1997). A 100-fold ('infinite') dilution gave a comparable dissociation rate as the rate obtained by the addition of excess cold Org 43553 (Figure 3), which suggests that maximal radioligand dissociation was achieved and that Org 43553 binds to a single non-interacting site. Infinite dilution in the presence of excess cold Org 43553 or recLH did not alter the dissociation rate of the radioligand, further proof of Org 43553's binding to a single, non-interacting site. To get more insight into the fact that recLH showed some displacement of Org 43553, the effect of recLH on the dissociation of [^{3}H]Org 43553 was monitored. As shown in Figure 3, recLH induces some dissociation of the radioligand, which correlates with the effect seen on the saturation (Figure 2) and displacement equilibrium binding of [^{3}H]Org 43553 (Table 2).

Characterization of the new radioligand was continued by radioligand displacement assays with non-labeled Org 43553 and a number of derivatives. Recently, a high throughput screen identified compounds with a thieno[2,3-*d*]pyrimidine core as potent and selective receptor agonists for the LH receptor (Van Straten et al., 2002). It was shown that the amide group at position 6 of the heterocyclic core was crucial for low nanomolar activity. From this series Org 41841 (**1**) was the most potent. In addition, a more bulky meta-substituent on the phenyl group at position 4 resulted in more potent

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compounds (Hanssen and Timmers, 2003; Hanssen et al., 2003). The brief structure-activity relationship (SAR) study presented here shows that the affinity of Org 41841 can be improved 5-fold through meta substitution, e.g. resulting in Org 43553 (Table 2). When compound **1** is compared to compound **3**, it becomes clear that the introduction of an additional H-bond donor by the amide-group in combination with steric bulk from the benzoyl group is favorable for receptor affinity. Introduction of the ethanolamine- (**4**) and morpholinogroup (**5**) was allowed, and helped to increase water solubility. The compounds were not able to displace [^{125}I]hCG in a binding assay (Table 2), which is in accordance with similar experiments on the FSH receptor (Van Straten et al., 2005). Recently, it was shown in docking and mutational studies that Org 41841 has its putative binding site in the seven-transmembrane (7-TM) part of the receptor (Jaschke et al., 2006; Moore et al., 2006), unlike the presumed binding of hCG and LH to the N-terminus of the receptor. This is in line with many other class A GPCRs, for example the adenosine A_{2A} (Kim et al., 1995), D_3 dopamine (Alberts et al., 1998) and the GnRH receptor (Soderhall et al., 2005), which bind their cognate ligands in the 7-TM domain as retinal in the rhodopsin receptor (Palczewski et al., 2000). Therefore, we assume that all small molecule ligands reported here also bind in this domain of the LH receptor, explaining why the endogenous ligand, [^{125}I]hCG, is not displaced. Conversely, recLH has some effect on [^{3}H]Org 43553 displacement. It is feasible that the recLH-occupied rather than 'empty' receptor is seen differently by LMW compounds such as Org43553.

In the cAMP-mediated luciferase assay, the compounds studied exhibited a rank order of potency similar to that observed in binding experiments (Figure 5). Although the endogenous ligand, recLH, was more potent, the small molecule ligands are potent in

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binding (K_i-values ranging from 3.3 – 100 nM) and in functional assays (EC_{50}-values ranging from 1.3 – 119 nM).

In conclusion, equilibrium saturation and displacement, and kinetic association and dissociation assays have been performed to elucidate the binding characteristics of the first small molecule radioligand, [^{3}H]Org 43553, at the human LH receptor. It was shown that a high correlation exists between affinity and activity of low molecular weight ligands. Moreover, the development of this binding assay will aid in the identification and elucidation of the SAR of newly synthesized small molecule LH receptor ligands.

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Legends for figures

Figure 1. Optimization of specific [^{3}H]Org 43553 binding to CHOhLHr_luc membranes. The effect of buffer composition, filter and filter treatment (with or without 0.25 % PEI), and protein amount on the amount of specific radioligand binding was surveyed. Bar graph presentation of the results from a single experiment performed in duplicate. These experiments were repeated one more time with similar outcome. White bars: 15 µg of protein, 20 nM [^{3}H]Org 43553, grey bars: 15 µg of protein, 20 nM [^{3}H]Org 43553 and 0.1% BSA, black bars: 20 nM [^{3}H]Org 43553, 0.1 % BSA, GF/B filters.

Figure 2. Saturation of [^{3}H]Org 43553 to luteinizing hormone receptors in the absence (control) or presence of 70 nM recLH. The control specific binding (■) was determined by subtracting the non-specific binding (▼) from the total binding (□) curve. The control K_D value was 2.4 ± 0.4 nM and the B_{max} value was 1.6 ± 0.2 pmol/mg protein (n = 5). A similar experiment was performed in the presence of 70 nM recLH, of which only the specific binding is shown (o). Representative graphs from one experiment performed in duplicate.

Figure 3. Association and dissociation kinetics of [^{3}H]Org 43553 binding to CHO-K1 membranes expressing the human luteinizing hormone receptor at 30ºC. Dissociation was either initialized by the addition of 10 µM Org 43553 (■; control) or 70 nM recLH (▼) or dilution in 100 volumes of assay buffer (□). Representative graphs from one experiment performed in duplicate (see Table 1 for kinetic parameters of control experiment).

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Figure 4. Association and dissociation kinetics of [^{125}I]hCG binding to CHO-K1 membranes expressing the human luteinizing hormone receptor at 30ºC. Representative graphs from one experiment performed in duplicate (see Table 1 for kinetic parameters).

Figure 5. Displacement of [^{3}H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes. Representative graphs from one experiment performed in duplicate (see Table 2 for affinity values).

Figure 6. Concentration-effect curves of recLH and low molecular weight ligands for cAMP-mediated luciferase production through human luteinizing hormone receptors. Representative graphs from one experiment performed in duplicate (see Table 2 for EC_{50} values).

Figure 7. Comparison between the logarithms of affinity values (M) of [^{3}H]Org 43553 binding and of EC_{50} values (M) obtained in luciferase assays for human luteinizing hormone receptors stably expressed on CHO-K1 cells ($r = 0.99$; $P < 0.0001$).

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Table 1

Association (k_{on}) rate, dissociation (k_{off}) rate constants and the apparent dissociation constant (K_D) of radiolabeled hCG and Org 43553.

Radioligand	**k_{on} (nM^{-1} min^{-1})[a]**	**k_{off} (min^{-1})[a]**	**K_D (nM)[b]**
[^{125}I]hCG	0.13 ± 0.006	0.0084 ± 0.0006	0.064
[^{3}H]Org 43553	0.0051 ± 0.0007	0.021 ± 0.001	4.1

[a] The values of the kinetic association and dissociation rate constants were obtained by analysis of the exponential association and dissociation of either [^{125}I]hCG or [^{3}H]Org 43553 bound to human luteinizing hormone receptors.

[b] The dissociation constant was defined as the ratio of k_{off}- and k_{on}-values.

Values are means (± S.E.M.) of at least three separate assays performed in duplicate.

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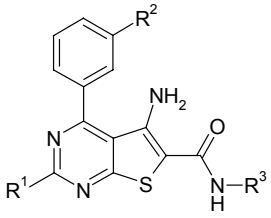
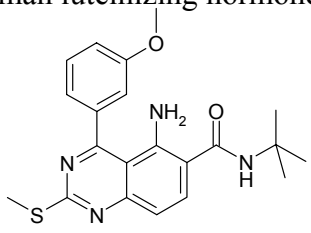
Table 2

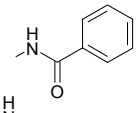
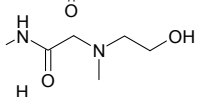
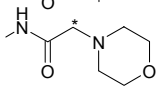
Receptor affinity assessed with either [^{3}H]Org 43553 or [^{125}I]hCG, and receptor activity of compounds **1**-**6** and recombinant LH at the human luteinizing hormone receptor.



1-5



6

Compound	**R^1**	**R^2**	**R^3**	**[^{3}H]Org 43553 binding[a]**	**[^{125}I]hCG binding[b]**	**EC$_{50}$ (nM)[c]**
1 (Org 41841)	SMe	OMe	*t*Bu	17 ± 5	1 (0-1)	13 ± 1
2 (Org 41247)	Ph	OMe	*i*Pr	100 ± 6	4 (0-8)	119 ± 18
3 (Org 42619)	SMe		*t*Bu	4.7 ± 0.7	0 (0-0)	3.0 ± 0.5
4 (Org 43311)	SMe		*t*Bu	4.1 ± 0.7	4 (0-7)	1.3 ± 0.4
5 (Org 43553)	SMe		*t*Bu	3.3 ± 0.3	7 (6-8)	1.7 ± 0.2
6 (Org 43983)	-	-	-	82 ± 17	0 (0-0)	111 ± 3
recLH	-	-	-	32 (31-33)[d]	0.61 ± 0.1[e]	0.078 ± 0.002

***** Position of the tritium substitution in Org 43553.

[a] Displacement of specific [^{3}H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes (K_i ± S.E.M. (nM), n ≥ 3, duplicate) or [d] % displacement of specific [^{3}H]Org 43553 binding at 70 nM recLH (n = 4, duplicate).

[b] % Displacement of specific [^{125}I]hCG binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes at 10 μM concentrations (n = 2, duplicate) or [e] displacement of specific [^{125}I]hCG binding (K_i ± S.E.M. (nM), n = 3, duplicate)

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[c] cAMP-mediated luciferase activity in CHO-K1 cells that stably express the human luteinizing hormone receptor and CRE-luciferase reporter gene (mean ± S.E.M., $n \geq 3$, duplicate).

Figure 1

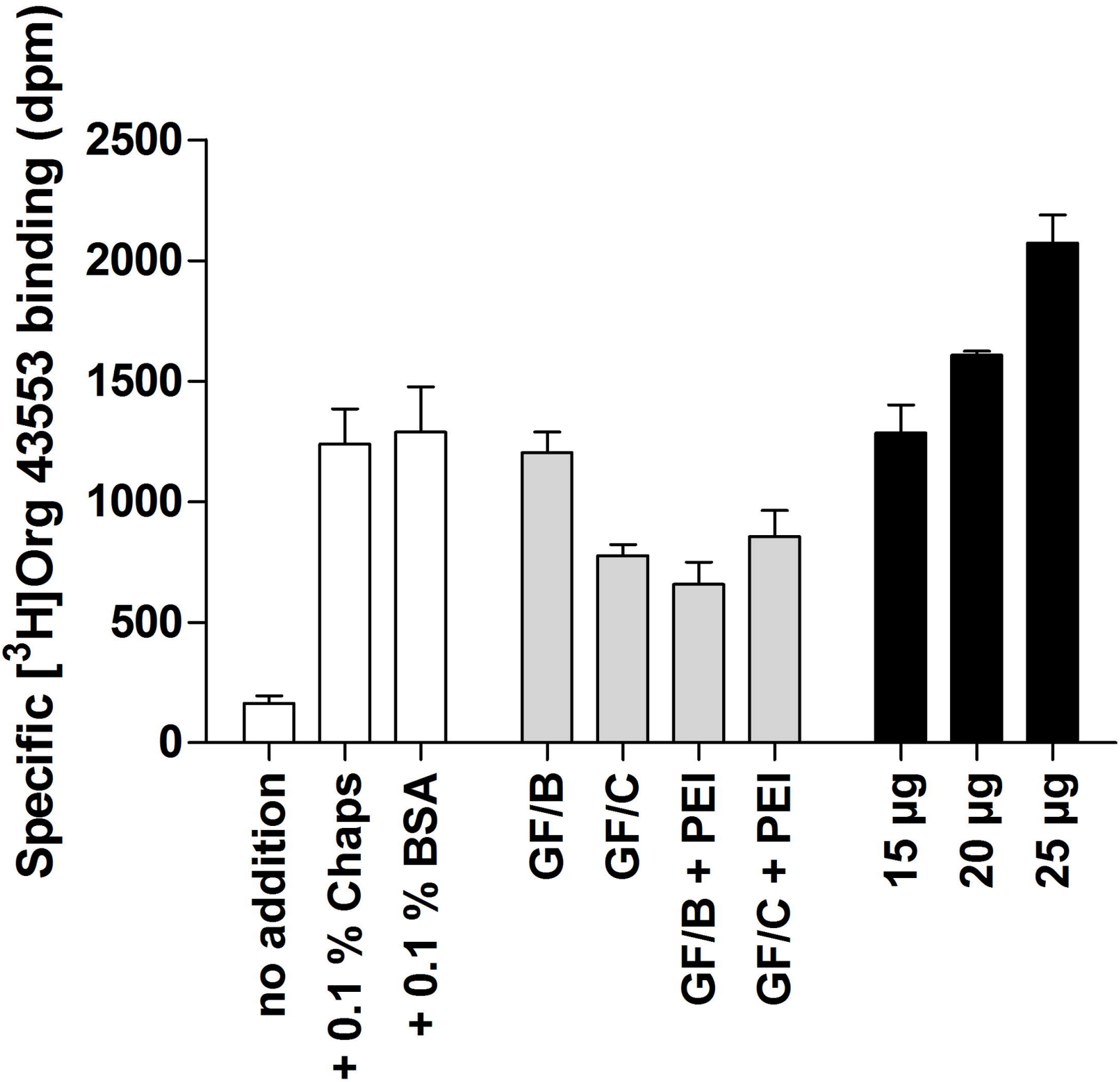

Specific [3H]Org 43553 binding (dpm)
2500
2000
1500
1000
500
0
no addition
+ 0.1 % Chaps
+ 0.1 % BSA
GF/B
GF/C
GF/B + PEI
GF/C + PEI
15 µg
20 µg
25 µg

Figure 2

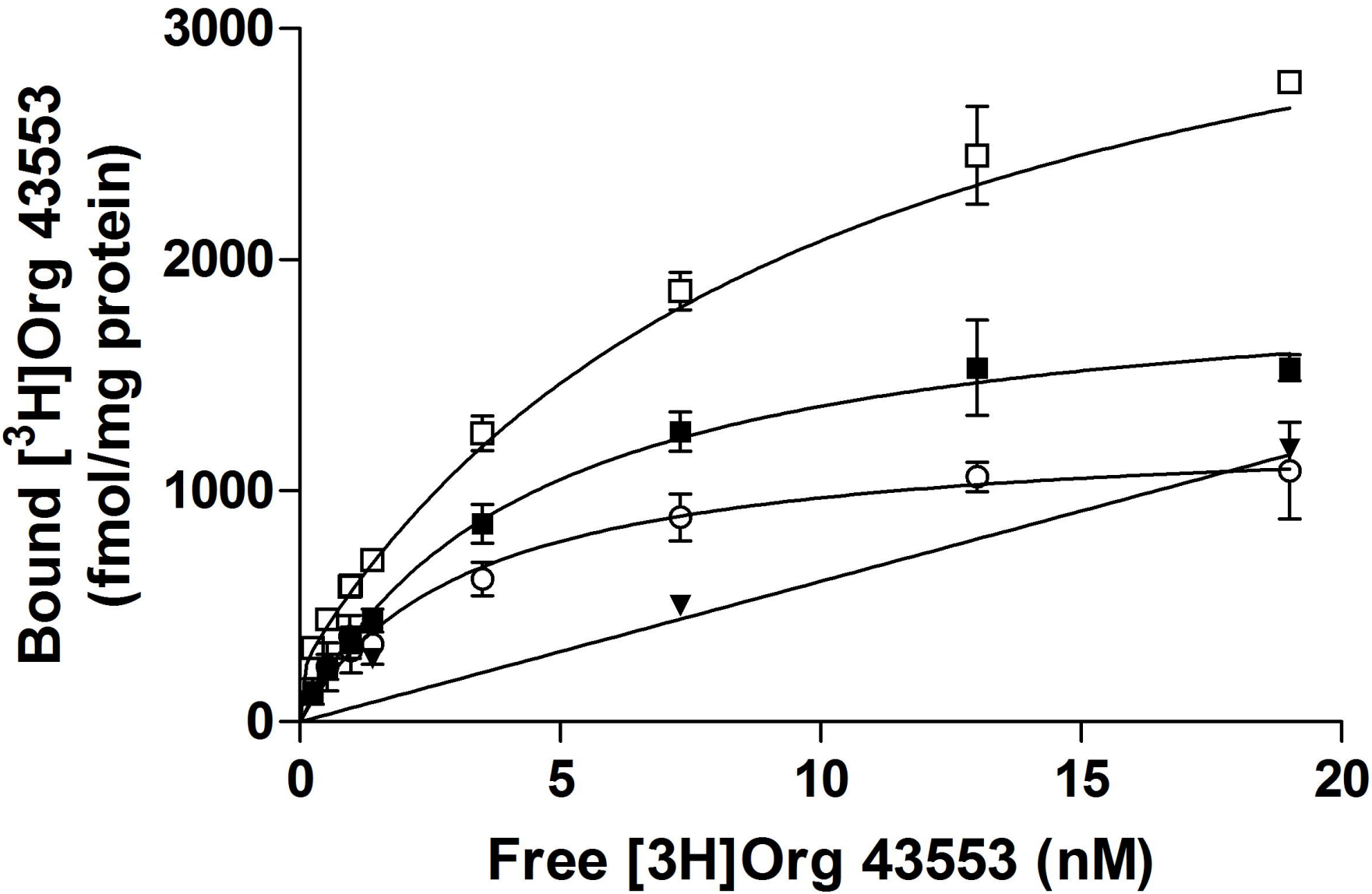

Bound [3H]Org 43553 (fmol/mg protein)
3000
2000
1000
0
0
5
10
15
20
Free [3H]Org 43553 (nM)

Figure 3

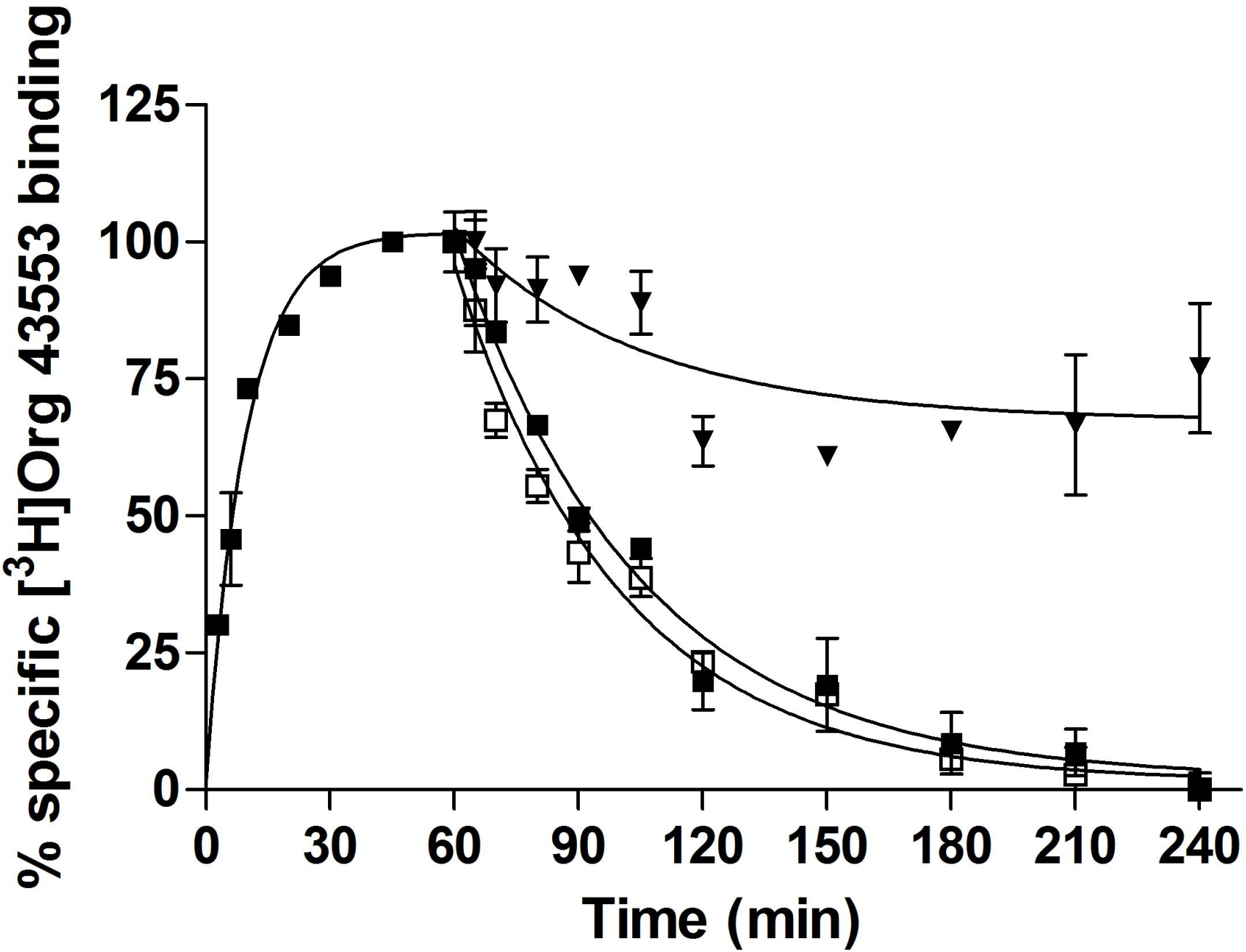

% specific [3H]Org 43553 binding
125
100
75
50
25
0
0
30
60
90
120
150
180
210
240
Time (min)

Figure 4

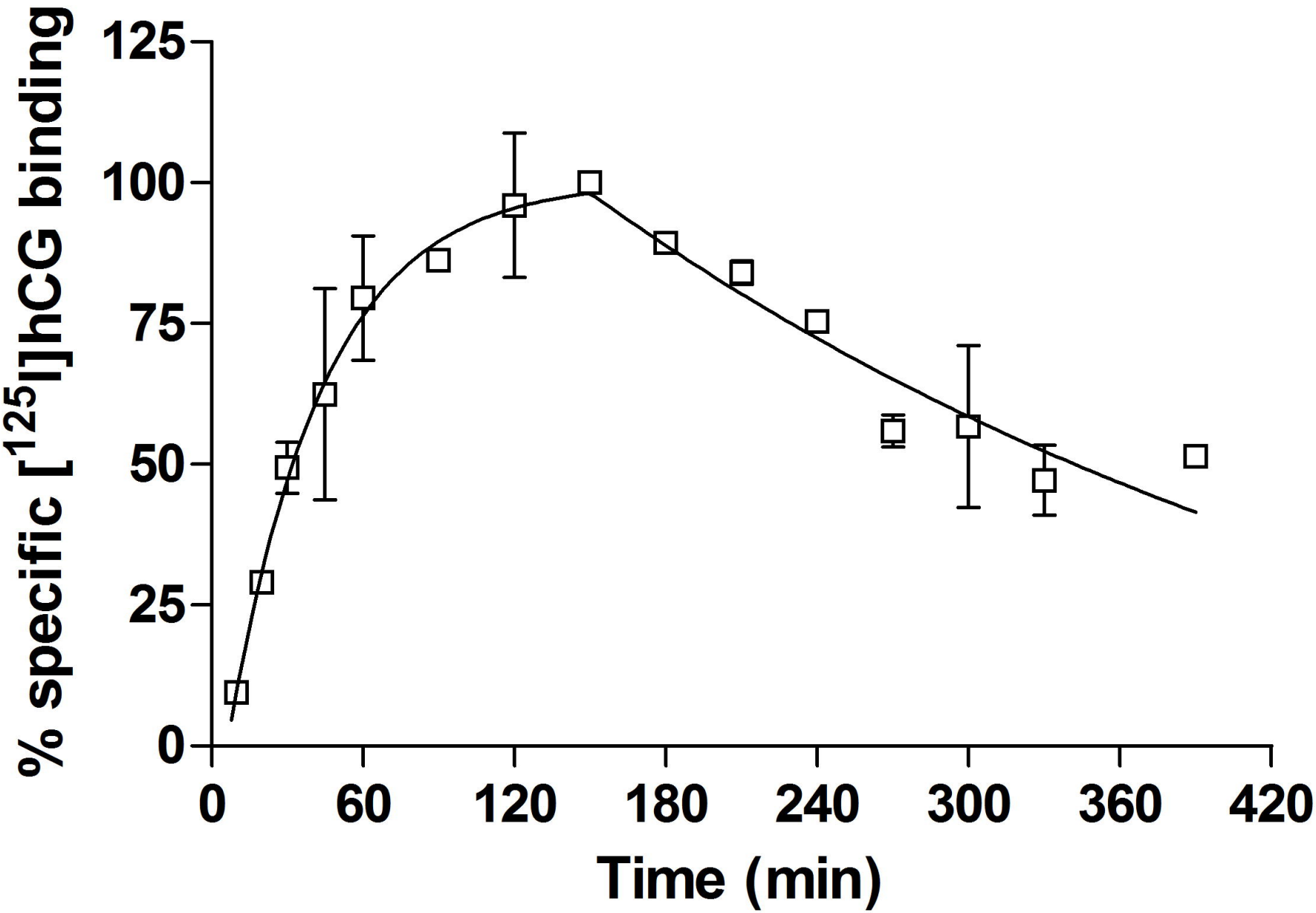

% specific [125I]hCG binding
125
100
75
50
25
0
0
60
120
180
240
300
360
420
Time (min)

Figure 5

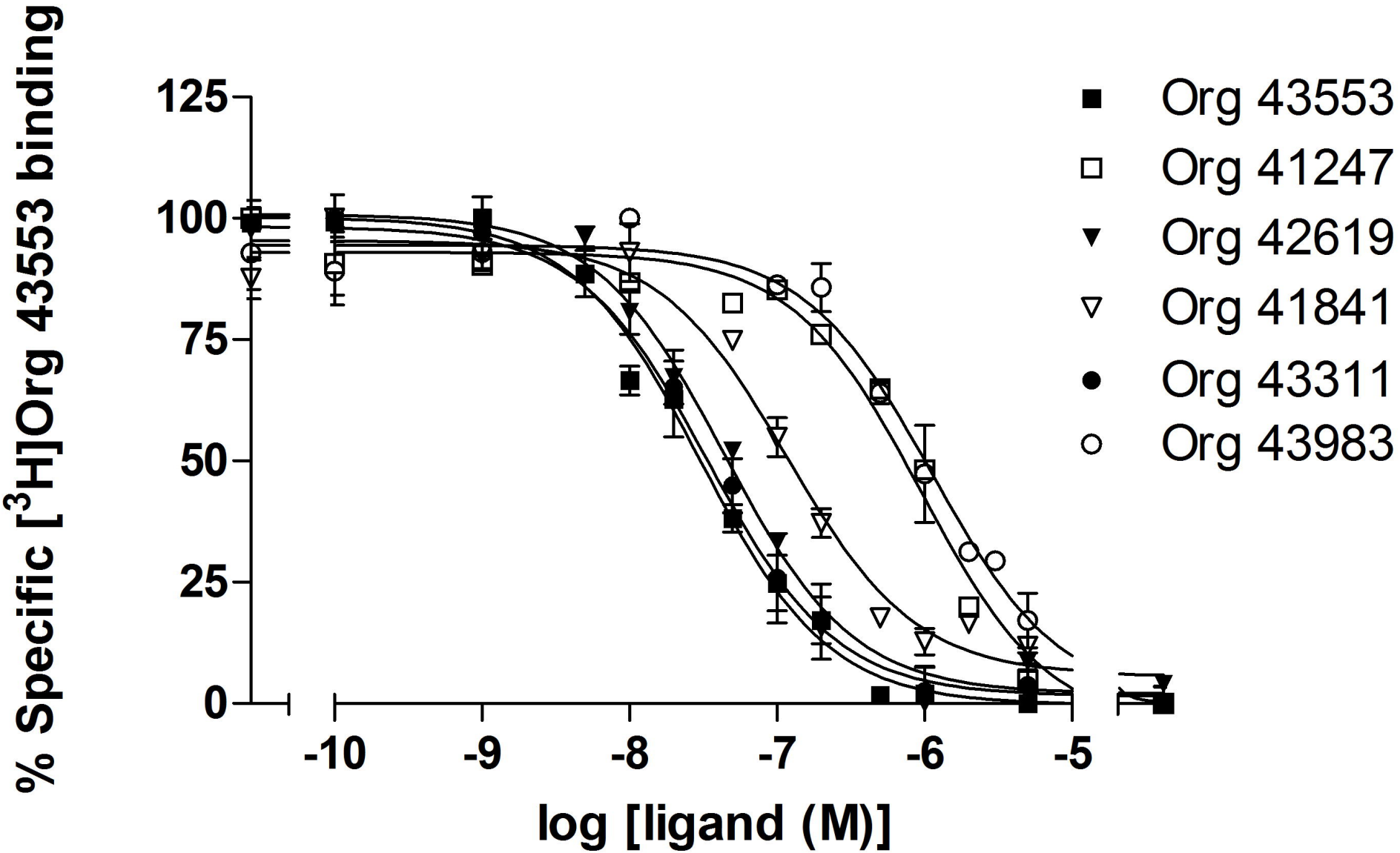

% Specific [3H]Org 43553 binding
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
log [ligand (M)]
Org 43553
Org 41247
Org 42619
Org 41841
Org 43311
Org 43983

Figure 6

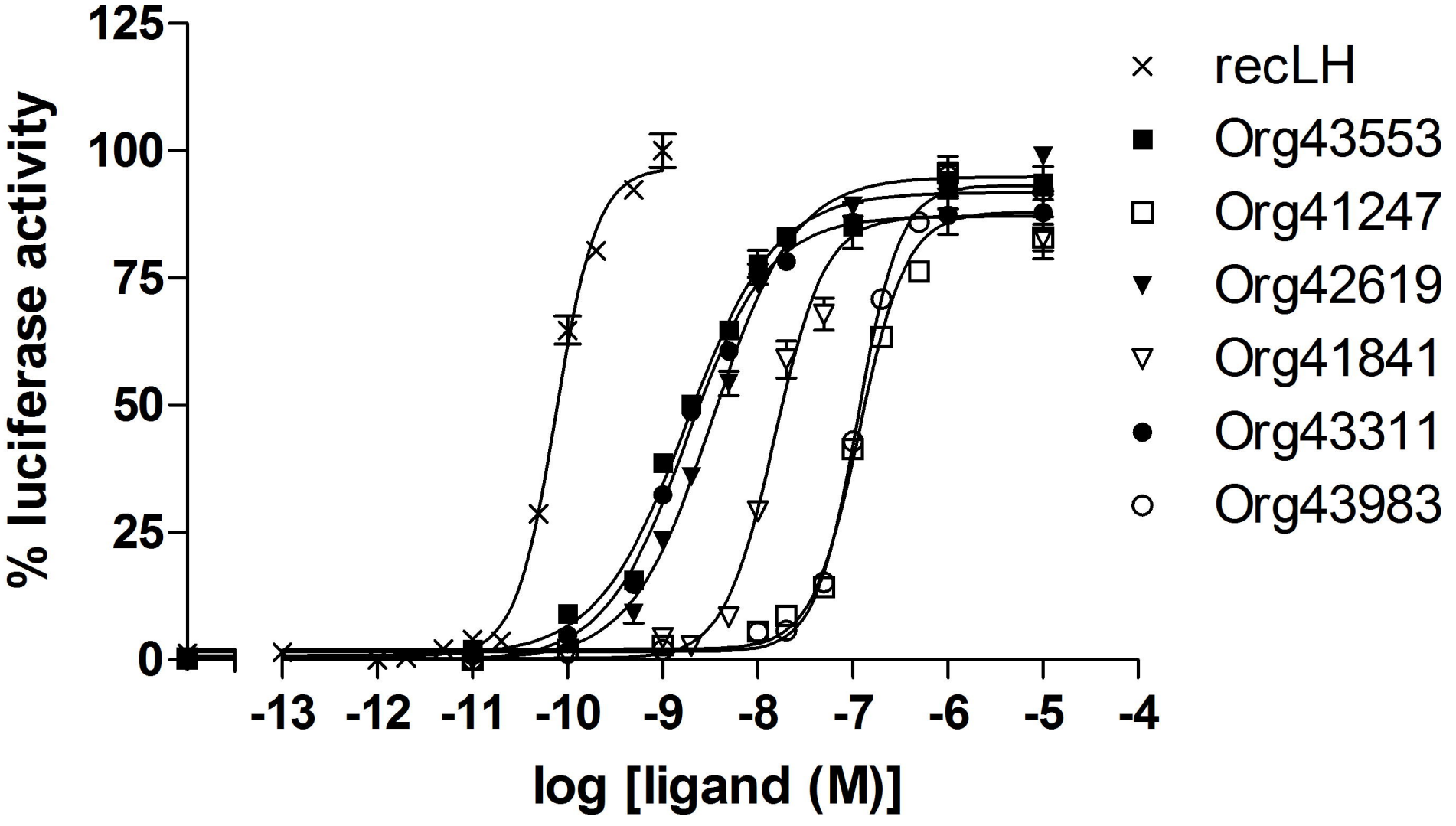
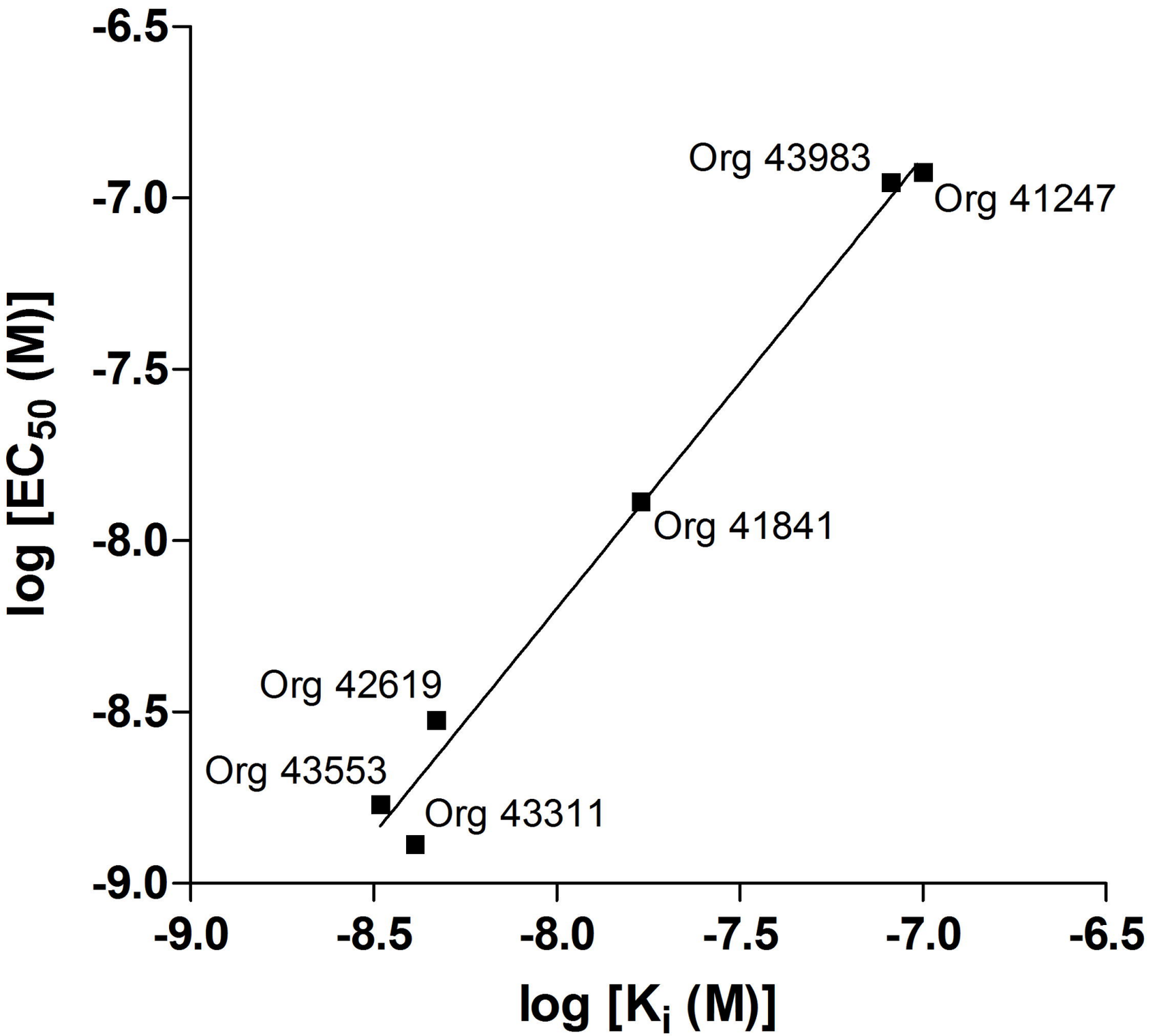

125
100
75
50
25
0
% luciferase activity
-13
-12
-11
-10
-9
-8
-7
-6
-5
-4
log [ligand (M)]
recLH
Org43553
Org41247
Org42619
Org41841
Org43311
Org43983

Figure 7


-6.5
-7.0
-7.5
-8.0
-8.5
-9.0
log [EC50 (M)]
Org 43983
Org 41247
Org 41841
Org 42619
Org 43553
Org 43311
-9.0
-8.5
-8.0
-7.5
-7.0
-6.5
log [Ki (M)]