Title: Biological Characterization of a Novel, Orally Active Small Molecule Gonadotropin

Releasing Hormone (GnRH) Antagonist Using Castrated and Intact Rats.

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Running Title: Biological Characterization of Nonpeptide GnRH Receptor Antagonists

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Abbreviations: GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; GnRH-A, [D-Ala6, des-Gly10]proethylamide9-GnRH.

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Abstract

GnRH receptor antagonists have potential in treating numerous hormone-dependent pathologies including cancers of the prostate, breast and ovary, endometriosis and fertility disorders. An unmet clinical need exists for an orally available GnRH receptor antagonist. Guided by structure activity relationships, ligand-based targeted library designs, and biomarker measurements our discovery efforts have yielded a novel, small molecule GnRH receptor antagonist 5-[(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthalenyl)methyl]-N-(2,4,6-trimethoxyphenyl)-2-furamide (CMPD1). CMPD1 bound with low nanomolar affinities to human, rat and mouse GnRH receptors (6.0, 3.8, 2.2 nM, respectively). CMPD1 was more than 100-fold selective for GnRH receptors versus various G-protein-coupled receptors and other enzymes and ion channels. In cells expressing recombinant rat GnRH receptors, CMPD1 was a competitive antagonist of GnRH-stimulated increases in extracellular acidification rates in Cytosensor® microphysiometer assays. In cells expressing recombinant human GnRH receptors, CMPD1 was a potent inhibitor of GnRH-stimulated total inositol phosphate accumulation. The effects of CMPD1 on circulating levels of LH and testosterone were studied in castrated and intact male rats, respectively. Intravenous and oral administration of CMPD1 dose-dependently suppressed GnRH-mediated elevations of LH in castrated male rats and testosterone in gonad-intact male rats. Moreover, CMPD1 when given at 20 mg/kg IV to intact male rats inhibited the elevations of LH and testosterone stimulated by superagonist of GnRH, [D-Ala^6, des-Gly^{10}]GnRH (GnRH-A). These data suggest that CMPD1 is a potent, selective, orally active GnRH receptor antagonist that may have potential application as a therapeutic agent for treating hormone-dependent cancers and diseases.

Gonadotropin-releasing hormone (GnRH) is a neuroendocrine decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$) synthesized in the neurovascular terminals of the hypothalamus and is secreted in a pulsatile manner directly into the hypophyseal portal blood supply. GnRH selectively binds specific receptors on the membranes of the anterior pituitary gonadotroph cells to stimulate synthesis and release of the gonadotropic hormones (luteinizing hormone, LH; and follicle-stimulating hormone, FSH). LH and FSH stimulate gonadal production of sex steroids and gametogenesis, respectively.

GnRH, as the primary regulator of LH, is consequently the primary regulator of the sex hormones testosterone and estrogen. GnRH and its analogs have stimulated much interest because of their potential therapeutic benefit in treating sex-hormone-dependent diseases such as prostate, ovarian and breast cancer as well as endometriosis, uterine fibroids, benign prostate hyperplasia, fertility disorders and precocious puberty (Huirne and Lambalk, 2001). In contrast to GnRH agonists, which are associated with an initial surge in LH and testosterone commonly referred to as "flare", GnRH antagonists competitively block the GnRH receptor without functionally inducing the release of gonadotropins. GnRH antagonists are more potent suppressors of bioactive LH and FSH than are agonists (Pavlou, 1991). Although antagonists do not require receptor down-regulation as GnRH analogues do, a marked down-regulation of pituitary GnRH receptors does occur with chronic administration of GnRH antagonists (Halmos, 1996).

The development of GnRH antagonists has trailed due to lack of potency, histamine-mediated side effects, poor aqueous solubility and low bioavailability. GnRH agonists and antagonists developed to date are injectable or depot formulations of GnRH peptide analogues (Bouchard, 1996). Although other laboratories have reported active nonpeptide GnRH antagonists (Walsh et al, 2000; Devita et al, 2001; Ashton et al, 2001a,b; Zhu et al, 2002a,b), few have shown oral activity in animal models (Cho et al, 1998, Besecke et al, 2001; Ashton et al, 2001c). An unmet clinical need still exists for an orally available GnRH receptor antagonist. Guided by structure activity relationships (Anderson et al., 2000, Luthin et al., 2002a,b) and biomarker

measurements our discovery efforts have yielded a novel, potent, orally active GnRH receptor antagonist CMPD1(Fig.1).

CMPD1 binds with low nanomolar affinity to human, rat and mouse GnRH receptors and suppresses: 1) GnRH-mediated elevations of LH in castrated male rats 2) the superagonist analog of GnRH, [D-Ala^6, des-Gly^{10}]GnRH (GnRH-A) stimulated elevations of LH and testosterone in gonad-intact male rats; and 3) circulating testosterone in gonad-intact male rats.

Methods

Chemicals and Reagents. Synthesis of CMPD1 was previously described in Anderson et al., 2000. pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2 (GnRH), Ac-D-2-Nal-p-chloro-D-Phe-β-(3-pyridyl)-D-Ala-Ser-Lys(nicotinoyl)-D-Lys(nicotinoyl)-Leu-Lys(isopropyl)-Pro-D-Ala-NH2 (antide) and the superagonist peptide, [D-Ala6, des-Gly10]proethylamide9-LHRH (GnRH-A) were purchased from Bachem (Torrance, CA). Cell Culture media was purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was from Omega Scientific, Inc. (Tarzana, CA). G418 and penicillin/streptomycin were from Gemini (Calabasas, CA).   Newborn calf serum was from Summit Biotech (Fort Collins, CO). DMSO and cremophor EL were from Sigma, (St. Louis, MO). Labrasol was from Gattefosse, (Westwood, NJ).

Cell Culture. The cDNA for mouse and human GnRH receptor was cloned into the plasmid expression vector, pcDNA 3.1 (In Vitrogen, San Diego, CA), and stably transfected into HEK 293 cells. Both the human and mouse receptors are modified versions of the native human and mouse receptors in that they are chimeric receptors expressing the cytoplasmic carboxyl terminal tail of a variant of the human GnRH receptor identified in the Expressed Sequence Tag (EST) database (Millar et al., 1999). The modifications help to increase receptor expression on the cell surface, without affecting binding affinity or functional activation of the receptors by GnRH (Flanagan et al., 1999 and personal communication from S. Sealfon). GH_3 cells, stably expressing recombinant rat GnRH receptors (GGH_3) were provided by Dr. William Chin (Harvard Medical School, Boston, MA). These cells also endogenously express TRH receptors coupled to inositol phosphate turnover and have been extensively characterized (Kaiser et al., 1997). HEK 293 cells stably transfected with mouse or human GnRH receptors, as described above, were grown in Dulbecco's high-glucose, modified Eagle's medium (DMEM) supplemented with 0.2% G418, 10% fetal bovine serum (FBS) and 100U/mL penicillin/streptomycin. GGH_3 cells were grown in low glucose DMEM containing: 100U/mL penicillin/streptomycin, 0.6% G418 and 10% heat-inactivated FBS.

Effect of CMPD1 on Cell Viability. The effect of CMPD1 on cell viability was examined. Cells (GGH$_3$ or 293 cells containing human GnRH receptors) were harvested using PBS/EDTA. Cells (10^7 cells/ml) were tested in the presence or absence of CMPD1 (10 μM) in 1% DMSO final concentration for 30 minutes at 37 °C prior to assessment of the ability of cells to exclude trypan blue.

Cell Membrane Preparation. HEK 293 cells containing mouse or human receptors, or rat pituitaries (Pel Freez Biologicals, Rogers, AR) were homogenized in buffer A containing: 50 mM Tris (pH 7.4), 0.32 M Sucrose, 2 mM EGTA, 1 mM PMSF, 5 μg/ml aprotinen, 5 μg/ml Pepstatin A, and 1 μg/ml leupeptin. Homogenized cells were centrifuged at 4°C at 20,000 x g for 25 min, re-suspended in buffer A and re-centrifuged at 4°C at 20,000 x g for an additional 25 min. Total membrane protein was determined with a BCA kit (Pierce, Rockford, IL). Membranes were stored at –70 °C at a final membrane protein concentration of approximately 5 mg/ml.

Radioligand Preparation. The radioiodinated agonist analog of GnRH, [des-Gly10,D-Ala6]GnRH ethylamide (^{125}I-GnRH-A) was used as the radioligand. Preparation of this radioligand has been previously described (Anderson et al., 2000).

Radioligand Binding Assays. Membranes were diluted to 0.01-0.5 mg/ml (depending upon the species of receptor) with assay buffer containing: 50 mM HEPES (pH 7.4), 1 mM EDTA, 2.5 mM MgCl$_2$, and 0.1% BSA. For saturation binding, membranes were incubated with approximately 0.0009-2.0 nM of ^{125}I-GnRH-A in a final volume of 200 μl in the presence or absence of GnRH (100 nM) for 1 hr at room temperature. For competition binding, membranes (diluted to utilize similar receptor numbers between assays) were incubated with approximately 0.04-0.06 nM ^{125}I-GnRH-A in the presence or absence of competing agents (0.1 – 10,000 nM) in a total volume of 200 μl in 96-well polypropylene plates for 1 hr at room temperature. Assays were stopped by rapid filtration onto 96-well GF/C filters soaked in 0.1% polyethylenimine (PEI) utilizing a Packard 96-well cell harvester. Filters were washed three times with ice-cold PBS (50 mM NaPO$_4$, 0.9% NaCl, 2 mM MgCl$_2$, and 0.02% NaN$_3$, pH 7.4). 35 μl of scintillation cocktail was

added to each filter well and filters were counted on a Packard Topcount. Control dose-response curves were generated to GnRH (0.1 nM-100 nM) in each competition binding experiment.

Binding Specificity Experiments. In order to assess for selectivity, CMPD1 was tested at 1 μM in duplicate in multiple assays including the Peripheral Side Effect Profile™ (SEP™) by Novascreen® (A Division of Oceanix Biosciences Corporation, Hanover, MD). Ki values were determined in assays in which CMPD1 inhibited $\geq$ 50% of specific binding when tested at 1 μM. Additional selectivity experiments were performed, as previously described (Luthin et al., 1999).

Microphysiometry. Use of the Cytosensor® Microphysiometer (Molecular Devices, Sunnyvale, CA) to assess GnRH-stimulated increases in extracellular acidification rates has been previously described (Anderson et al, 2000). GGH$_3$ cells were seeded in low-buffered minimal essential media (MEM, Sigma) containing 25 mM NaCl and 0.1% BSA at a density of 500,000 cells/capsule onto the polycarbonate membrane (3 μm porosity) of cell capsule cups (Molecular Devices, Sunnyvale, CA). Capsule cups were transferred to sensor chambers where cells were held in close apposition to a silicon sensor within a sensor chamber, which measures small changes in pH in the microvolume of the sensor chamber. Low-buffered medium was pumped continuously across the cells at a rate of approximately 100 μl/min from one of two fluid reservoirs. A selection valve determined which reservoir from which fluid was perifused onto the cells.

Cells were maintained at 37 °C on a two-minute flow cycle with cells being perfused with media for 80 seconds followed by 40 seconds in which the flow of media was stopped. During this 40-sec interval, acidification rates were measured for a 30-sec interval. In this fashion, a single acidification rate was calculated every two minutes. The Cytosensor® Microphysiometer unit contains eight such sensor units, allowing for eight simultaneous experiments to be performed. Each unit was individually programmed utilizing a computer linked to the system.

GGH$_3$ cells were initially equilibrated in the low-buffered MEM media for a period of 30-60 min in which basal acidification rates (measured as μV/sec), in the absence of any stimuli, were monitored. When the basal rate of acidification changed by less than ten percent over a period of

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twenty minutes, experiments were initiated. Time course experiments were performed to determine the optimal time for agonist exposure prior to acidification rate measurement and the duration of exposure needed to obtain peak acidification responses to various agonists. From these time course experiments, it was determined that cells should be exposed to GnRH peptide agonists at least one minute prior to collection of acidification rate data. Peak acidification rates usually occurred in the first two-minute exposure cycle.

To test the ability of CMPD1 to inhibit GnRH-stimulated increases in extracellular acidification, cells were pretreated for 20 min with cell media containing vehicle (1% DMSO) or CMPD1 (10, 30 or 100 nM final concentration in 1% DMSO) prior to stimulation with GnRH (1—1000 nM).

Total Inositol Phosphates Measurement. Approximately 200,000 cells/well of either GGH_3 or 293 cells containing human GnRH receptors were plated onto 24-well tissue culture plates (pretreated with poly-lysine) using DMEM media. The following day, cells were loaded with [3H]myoinositol (0.5 Ci/ml) for 16-18 hours in inositol-free medium. The medium was aspirated and the cells rinsed with serum-free DMEM. 293 cells were treated for 30 min at 37 °C with vehicle (1% DMSO in DMEM) or CMPD1 (0.1 – 1000 nM) prior to stimulation with GnRH (1 nM) for 45 minutes at 37 °C in a total volume of 1 mL containing 10 mM LiCl. To assess specificity of CMPD1, similar experiments were performed in GGH_3 cells that endogenously express TRH receptors. GGH_3 cells were pretreated with CMPD1 as described above and then stimulated with TRH (3 nM). As a control in each experiment, concentration response curves to GnRH (0.01 – 1000 nM; 293 or GGH_3 cells) or TRH (0.1 —1000 nM; GGH_3 cells) were generated. The media was replaced with 1 mL ice-cold 10 mM formic acid, which stopped the reaction and also served to extract cellular lipids. Inositol phosphates were separated by ion-exchange chromatography on Dowex columns, which were washed with 2.5 mL of 10 mM myoinositol and 10 mM formic acid. The columns were then washed with 5 mL of 60 mM sodium formate and 5 mM borax, and total inositol phosphates were eluted with 5 mL 1M ammonium formate, 0.1 M formic acid. The column eluates were added to liquid scintillation vials containing 15 ml of scintillation cocktail and were counted by liquid scintillation counting.

***In vitro* Data Analysis.** Cytosensor® Microphysiometer data were normalized utilizing Cytosoft® software (Molecular Devices, Sunnyvale, CA). EC_{50} values for agonists and IC_{50} values for CMPD1 were generated utilizing Prism™ (version 3.0, GraphPad Software, San Diego, CA), a computer graphics and statistics program. Binding inhibition constants (Ki) and antagonist equilibrium dissociation constants (KB) were calculated according to Cheng and Prussof (1973). pA2 values determined in Cytosensor® Microphysiometer experiments were calculated according to a method by Schild (1949). Values shown in tables are means ± SE of at least three replicate experiments. The computational modeling studies were all carried out in Silicon Graphics O2 Workstation.

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Plasma Protein Binding. The plasma protein binding of CMPD1 was measured by equilibrium dialysis against 100mM phosphate buffer pH7.4 at 10 μM concentration of the compound in a 100ul volume of rat plasma in triplicate. Following a 15hr dialysis compound was extracted from plasma and buffer with ACN and samples were analyzed by HPLC as described below. The unbound fraction of CMPD1 was estimated from buffer/plasma concentration ratio.

Animals. Adult male Sprague-Dawley (SD) rats were purchased from Harlan Sprague Dawley (San Diego). All animals were maintained in a temperature- controlled room (22 ± 2°C) with a photoperiod of 12 hr light/12 hr dark (lights on at 0600h). Rat chow (Teklad rat diet) and tap water were provided ad libitum. Protocols followed the "Principles of Laboratory Animal Care" (NIH publication of #85-23, revised 1985). Male SD (200-225g) rats were castrated via the scrotal approach under halothane anesthesia. All castrated animals were housed two per cage and allowed 14 days post-operative recovery prior to study. Intact male SD (300-325g, ~90 days old) animals for testosterone studies were single-housed to avoid the dominant male effect (Johnson and Everitt, 1988). Animals were handled on a daily basis and administered water by oral gavage to acclimate them to the experimental procedures. Three days prior to study, animals were anesthetized with halothane and instrumented with indwelling jugular vein microrenathane cannula. Details of the cannulation procedure have been described previously (Harms and Ojeda, 1974). On study day, animals were allowed to acclimate to the procedure room while residing in their home cage. An extension catheter was attached to the indwelling jugular vein cannula to facilitate remote sampling. Basal blood samples were withdrawn from all animals between 7:00-7:30am. Considering the circadian rhythm of testosterone (Mock, 1978), multiple samples were collected during the course of the experiment. Blood samples (400μL) were drawn into heparin containing tubes at multiple time points post treatment. Blood was centrifuged immediately, plasma collected and stored in -20° freezer until assayed.

Castrated Male Rat Model. Immediately following basal sampling, a single dose of vehicle or CMPD1 was administered intravenously (1.0, 5.0 or 10 mg/kg) or oral gavage (20 or 100 mg/kg).

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Intact Male Rat Model. For GnRH-A stimulation studies, vehicle or CMPD1 (20 mg/kg) was administered via intravenous infusion 5 minutes prior to GnRH-A (40 ng/kg;iv).

For testosterone efficacy studies, immediately following basal sampling, a single dose of vehicle or CMPD1 was administered intravenously (5.0, 10 or 20 mg/kg) or oral gavage (20, 50 or 100 mg/kg).

CMPD1 was formulated as a 10% DMSO, 10 % Cremophor EL and 80 % saline solution for intravenous administration. CMPD1 was prepared for oral administration as a 50 % Labrasol and 50% water solution.

Sample Analysis. LH and testosterone were measured using DSL-4600 ACTIVE LH coated-tube or DSL-4000 ACTIVE Testosterone coated-tube radioimmunoassay assay kits from Diagnostic Systems Laboratories, Inc. Webster, Texas.

Concentration of test compound in plasma was determined as follows. Plasma was immediately separated (100 µl) and compound was extracted with ethyl acetate (1.4 ml) containing internal standard. The supernatant was dried under nitrogen, the residue reconstituted in 80 µl of mobile phase and 50 µl was analyzed by HPLC.

HPLC analysis. Samples were analyzed on a Betabasic C18 column, 3 µm, 4.6x50 mm (Western Analytical Products, Inc., Murrieta, CA) using a Hewlett-Packard HPLC system (model 1100, Palo Alto, CA). A linear gradient of 50 to 70 % ACN in 10 mM ammonium phosphate buffer pH 7 over 10 min was used with UV detection at 260 nm. The flow rate was 1 ml/min.

***In vivo* Data analysis.** LH pulse frequency increases after castration (Ellis and Desjardins, 1982) potentially contributing to the variability in plasma LH levels. LH data were expressed as percentage change from baseline, which was defined as the initial basal blood sample. Luteinizing hormone and testosterone levels were expressed as means $\pm$ SEM. Significant differences between groups of animals were determined by two-way analysis of variance with *post hoc* Tukey multiple comparisons at each particular time point (SPSS software, Chicago, IL). Differences were considered significant if $p < 0.05$.

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Results

Radioligand Binding Experiments. In saturation binding experiments, the radioligand, ^{125}I-GnRH-A, bound to one high affinity site on human, rat and mouse membranes with Kd values of 0.54 ± 0.07, 0.78 ± 0.23, and 0.86 ± 0.13 nM, respectively. Receptor expression levels (Bmax) for the recombinant human, rat pituitary or recombinant mouse receptors were 700 ± 140, 56 ± 11, and 19900 ± 780 fmol receptor/mg protein, respectively. Results from competition binding experiments for GnRH, GnRH-A, antide and CMPD1 are shown in Table 1.

Specificity of CMPD1. CMPD1 was examined in >40 receptor, channel and enzyme counterscreen assays performed at Novascreen® and Cerep. CMPD1 bound to human D2 dopamine, 5-HT$_{2a}$ serotonin serotonin and L-type Ca^{2+} channels with high nanomolar to low micromolar affinities. No detectable inhibition was noted at 1 μM in 43 other assays.

Cytosensor® Microphysiometer Assays for functional assessment of CMPD1.

In Cytosensor Microphysiometer assays, GnRH stimulated increases in extracellular acidification with an EC$_{50}$ value of 33 ± 6.4 nM and Hill slope of 0.93 ± 0.07 (N=4). CMPD1 produced no change in basal extracellular acidification rates (data not shown). In the presence of CMPD1 (Fig. 2), there was a parallel, rightward shift of the concentration-response curves to GnRH without a decrease in the maximum response to GnRH. In four experiments, the mean K$_B$ ± SE for CMPD1 was 9.1 ± 2.4 nM (Hill slope was 1.1 ± 0.09). The GnRH-stimulated increases in extracellular acidification in GGH$_3$ cells could be inhibited by the GnRH peptide antagonist, antide (data not shown).

Assessment of CMPD 1 in Inositol Phosphate Assays. GnRH stimulated a concentration-dependent increase in total [^{3}H]inositol phosphate accumulation in 293 cells expressing human GnRH receptors (Fig. 3). This response to GnRH had an EC50 of 0.6 ± 0.15 nM (N=10) with a Hill coefficient of 0.98 ± 0.05. CMPD1 produced no change in basal levels of inositol phosphates (data not shown). CMPD1 produced a concentration-dependent inhibition of the response to GnRH (Fig. 3). In 6 similar experiments, the mean KB ± SE was 25 ± 0.9 nM (Hill slope = 0.9 ±

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0.05) for CMPD1 at the human receptor. This response to GnRH was inhibited dose-dependently by antide (Luthin et al., 2002a). Inhibition by CMPD1 of TRH-stimulated increases in total inositol phosphates in GGH$_3$ cells was also investigated. TRH produced a concentration-dependent stimulation of total inositol phosphate accumulation in GGH$_3$ cells (EC$_{50}$ of 2.4 ± 0.16; N=4). At concentrations up to 10 uM of CMPD1, there was no appreciable inhibition of TRH-stimulated inositol phosphate accumulation (data not shown).

Cell Viability. The effect of CMPD1 on viability of GGH$_3$ and 293 cells containing human GnRH receptors was examined. Cell viability was assessed prior to and after treatment with 1% DMSO, CMPD1 in 1% DMSO, or media alone. There was no significant effect of vehicle (1% DMSO) or CMPD1 on cell viability (data not shown).

Plasma protein binding. The plasma protein binding of CMPD1 in rat was estimated to be > 99.9%.

Efficacy in castrated male rat model. Intravenous doses of 1, 5 and 10 mg/kg CMPD1 produced significant time and dose-dependent suppression of LH levels by 0.5 hr after injection in comparison to vehicle controls (Fig. 4). LH levels were undetectable with 10 mg/kg at 0.5 hr and returned to baseline 6 hr post dosing. Oral administration of CMPD1 produced sustained dose-dependent suppression of LH for up to 8 hours (Fig. 5). Compared to vehicle, doses of 20 and 100 mg/kg CMPD1 produced 60 and 100 % suppression of LH levels respectively. LH levels returned to pre-treatment levels 24 hours post dose. Plasma concentration of CMPD1 in castrated rats was not measured in this study however previously we have shown that the plasma concentration of CMPD1 is 3 times greater in castrated rats compared to intact rats (Iatsimirskaia et al., 2002).

GnRH-A stimulated LH and Testosterone. Pretreatment with 20 mg/kg CMPD1 inhibited GnRH-A stimulation of LH and produced an attenuation and rightward shift of testosterone (Fig. 6a & b). Lower doses of CMPD1 (5.0 and 10 mg/kg) inhibited LH at the time points measured but did not inhibit testosterone secretion at the time points measured (data not shown).

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Efficacy in intact male rat model. In the vehicle treated rats, a normal circadian rhythm of testosterone was observed. Intravenous doses of 5, 10 and 20 mg/kg CMPD1 produced significant time and dose-dependent suppression of testosterone levels within 0.5 hr after injection in comparison to vehicle controls (Fig. 7). Testosterone levels were below castrate levels (0.5 ng/ml) at 1 hr and 2 hr post treatment with 10 and 20 mg/kg, respectively (Fig. 7). Testosterone levels returned to normal within 6 hr post dosing. Oral administration of CMPD1 produced sustained dose-dependent suppression of testosterone for up to 24 hrs (Fig. 8). Compared to vehicle, doses of 20, 50 and 100 mg/kg CMPD1 produced castrate testosterone levels while only the highest dose maintained castrate levels. The suppression of testosterone was reversible within 24-30 hr post treatment (data not shown). The plasma concentration of CMPD1 required to suppress testosterone was 0.4 μM while > 2 μM was required to maintain castrate levels of testosterone in the intact rat (Fig. 9).

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Discussion

Development of GnRH receptor antagonists will represent a new class of drugs with the potential to be used for the same indications as GnRH agonists but with fewer untoward effects. GnRH agonists and antagonists developed to date are limited to injectable or depot formulations of GnRH peptide analogues. Development of non-peptide orally active GnRH receptor antagonists will provide an alternative route of administration and may offer advantages over peptide analogs. Many groups have reported discovery of nonpeptide GnRH receptor antagonists. However, only a few groups have reported oral efficacy of their compounds (Ashton et al, 2001c; Besecke et al, 2001; Cho et al, 1998). We reported limited oral bioavailability of a compound of a similar structural class (Luthin et al, 2002b). We have improved upon this series and increased oral bioavailability (Iatsimirskaia et al. 2002).

In saturation binding assays, ^{125}I-GnRH-A bound to a single class of high affinity receptors. In competition binding assays, GnRH-A bound with comparable affinity to human, rat and mouse receptors, whereas GnRH and antide exhibited marked species differences, but correspond well with historical values reported in the literature for these peptides at these receptors (Chi et al., 1993; Flanagan et al., 1994; Rivier et al., 1992; Perrin et al., 1993). In contrast, CMPD1 bound with similar low nanomolar affinities to recombinant human and mouse and rat pituitary GnRH receptors.

In functional assays with cells expressing either recombinant rat or human GnRH receptors, this CMPD1 was a reversible, competitive antagonist of GnRH-stimulated increases in extracellular acidification or inositol phosphate production, whereas it did not inhibit TRH-stimulated increases in inositol phosphates in GGH$_3$ cells that endogenously express TRH receptors. Since neither CMPD1, nor its vehicle (1% DMSO) produced an effect on cell viability, it is suggested that the antagonism of GnRH-mediated responses by CMPD1 in both GGH$_3$ and 293 cells was receptor-mediated and not due to a cytotoxic effect of CMPD1 on the

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cells. Antagonist dissociation constants (K_B) corresponded well with the binding affinity of CMPD1 to rat and human receptors.

In specificity assays, CMPD1 was greater than 100x selective for most proteins. CMPD1 had low micromolar or high nanomolar binding affinities at human 5-HT$_{2a}$ serotonin, D$_2$ dopamine, and L-type Ca^{2+} channels. Whether the relatively low binding affinity of CMPD1 to serotonin 5-HT$_{2a}$, D$_2$ dopamine receptors and L-type Ca^{2+} channels will have any relevance or impact on the therapeutic potential of this or similar compounds remains to be determined. A note of caution is that since many of the specificity assays utilized tissues containing potentially many mixed receptor populations from multiple species, the true affinity of CMPD1 may be different on recombinant human proteins. It is conceivable that the limited cross-reactivity with these receptors has more of an impact on rat as these were measured on the human variant. Further studies will be needed to address those issues.

The castrated male rat is a sensitive and specific model for evaluating GnRH antagonists (Heber, 1982, Puente, 1986)). Removal of the testes produces a model with GnRH-mediated elevations of circulating LH. This mechanism of action of the hypothalamic-pituitary-gonadal axis is well defined (Ellis and Desjardins, 1984). Suppression of LH in this model following administration of a GnRH receptor antagonist reflects blockade of the GnRH receptor. A single IV dose of CMPD1 suppressed LH levels in a dose-dependent manner. LH suppression was transient and levels returned to pretreatment values within 6 hours. A single oral dose also suppressed LH levels in a dose-dependent manner. The highest dose completely suppressed LH for at least 8 hours. The effect was reversible as demonstrated by return to baseline LH levels 24 hours after treatment. The profound effects of CMPD1 on LH in the castrated rat model, in which LH depends on GnRH, provides evidence that the biological activity of CMPD1 is a consequence of GnRH receptor blockade.

GnRH-A stimulation was used to evaluate the effect of CMPD1 under pharmacological manipulation. LH and testosterone concentrations were elevated following GnRH-A stimulation. Pretreatment with 20 mg/kg CMPD1 inhibited GnRH-A stimulated LH. The testosterone

response was attenuated but not inhibited. There was a rightward shift observed in the testosterone time course response in the CMPD1 treated animals. The transient effect of CMPD1 administered by IV injection combined with the temporal nature of the hypothalamic-pituitary-gonadal (HPG) axis components likely contributed to the shift. It does seem clear that CMPD1 interrupted GnRH-A stimulated LH and testosterone through a GnRH receptor-mediated mechanism.

The activity of CMPD1 in the castrated rat model and the GnRH-A stimulated model provide strong confidence in the mechanism of action of our small molecule GnRH receptor antagonist. However, the intact male rat is a more physiological predictor of the pharmacodynamic properties of CMPD1. The difficulties associated with animal models and stress sensitive hormones are well known. It has been documented that some of the procedures commonly used in endocrine studies such as anesthesia, fasting, surgery may affect the hormone levels being studied (Howland, 1974). Luteinizing hormone and testosterone are sensitive to stressors**.** Numerous reports are conflicting about the effects of stressors on the HPG axis even when the same species and stressors are utilized (Howland, 1974, Kruhlich, 1974, Collu, 1984, Mann, 1990). It is, however, accepted that stressors do elicit changes in circulating LH and testosterone and the type of stress used, duration and severity cause different stress-induced changes. Considering the susceptibility of LH and testosterone to stress, the studies described in this report were conducted under conditions to minimize stress. Animals were handled daily and water was given by oral gavage to acclimate the animals to experimental procedures. Instrumented animals used for multiple sampling studies were allowed a minimum of 3 days postoperative recovery. Time courses were designed with consideration given to the daily rhythmicity of testosterone in rats. Animals were allowed to acclimate to the procedure room. The procedure room was kept quiet throughout the study. Sample volumes were kept at a minimum and an equal volume of saline was administered immediately following blood sampling. Our efforts to optimize experimental conditions to minimize stress were rewarded as shown by the normal testosterone levels and undisturbed diurnal rhythm in control animals (Mock, 1978). A

single IV dose of CMPD1 suppressed testosterone in a dose-dependent manner. Testosterone suppression was transient but castrate levels were achieved for 2 hours with 10 & 20 mg/kg. A single oral dose of CMPD1 completely suppressed testosterone levels to castrate and the highest dose maintained castrate levels below 0.5 ng/ml for at least 12 hours. The plasma concentration of CMPD1 required to suppress testosterone is estimated to be > 0.4 μM while the efficacious concentration required to maintain castrate levels of testosterone in the intact rat was determined to be > 2 μM. The apparent discrepancy between the in vitro potency and in vivo efficacious concentration is likely due to high plasma protein binding estimated to be $> 99.9\%$. Plasma concentration of CMPD1 in castrated rats was not measured in this study however previously we have shown that the plasma concentration of CMPD1 in intact rats was 3 times lower compared to castrated rats (Iatsimirskaia et al., 2002). Oral bioavailability was lower in intact male rats (8%) versus castrated rats (24%) (Iatsimirskaia et al., 2002). The pharmacokinetic profiles also differed. Details of this finding and a full discussion regarding the pharmacokinetics and metabolism of CMPD1 in intact and castrated rats are described (Iatsimirskaia et al., 2002).

We have shown that CMPD1 is a potent, orally active, non-peptide GnRH receptor antagonist with in vivo efficacy capable of suppressing castration-induced elevations of LH, GnRH-A stimulated LH and testosterone secretion and endogenous testosterone levels. CMPD1 may have potential application as a therapeutic agent for treating hormone-dependent cancers and other hormone-dependent maladies. The advantages GnRH receptor antagonists may provide over agonist therapy remain to be established. An orally available dosage form would be instrumental in establishing GnRH receptor antagonist therapy as a new "standard of care" for patients with hormone-dependent diseases.

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Footnotes

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Figure Legends

Figure 1. CMPD1 structure.

Figure 2. Effect of CMPD1 on GnRH-stimulated increases in extracellular acidification in GGH_3 cells. GGH_3 cells were plated onto Cytosensor® capsule cups and equilibrated as described in Methods. Cells were treated for 20 minutes with vehicle (1% DMSO) or CMPD1 dissolved in 1% DMSO at 10, 30 or 100 nM prior to 4-min stimulation with GnRH (concentrations shown). Concentration response curves to GnRH were plotted with Prism™ software. Inset: Schild Analysis of experiment depicted. The pA2 value for the experiment shown was 8.2 calculated from the formula: $\log (dr - 1) = \log [B] - \log KB$ in which dr is the dose-ratio of the response in the presence of antagonist divided by the response in the absence of antagonist; [B] is the concentration of the antagonist; and KB is the equilibrium dissociation constant for the receptor-antagonist complex. In four experiments, the mean $KB \pm SE$ was 9.1 ± 2.4 nM (Hill slope was 1.1 ± 0.09).

Figure 3. Inhibition by CMPD1 of GnRH-stimulated increases in total inositol phosphate production. 293 cells containing human GnRH receptors were plated onto 24-well plates as described in *Methods*. Cells were treated with CMPD1 at the concentrations shown prior to stimulation with GnRH (1 nM). A control concentration response curve to GnRH (0.01 nM – 100 nM) was generated in each experiment. Values shown are from one typical experiment performed in duplicate ($\pm$ range). In 6 similar experiments, the mean $\pm$ SE KB was 25 ± 0.9 nM (Hill slope = 0.9 ± 0.05) for CMPD1 at the human receptor.

Figure 4. LH time course in castrated male rat. A single intravenous dose of CMPD1 at 1.0, 5.0 or 10 mg/kg dose-dependently suppressed LH. * p <0.5 CMPD1 vs vehicle.

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Figure 5. LH time course in castrated male rat. CMPD1 administered by oral gavage 20 and 100

mg/kg dose-dependently suppressed LH. * p <0.5 CMPD1 vs vehicle.

Figure 6a. CMPD1 suppressed GnRH-A stimulated LH. CMPD1 (20 mg/kg) was administered

by intravenous infusion 5 minutes prior to GnRH-A (40 ng/kg; iv) stimulation.

Figure 6b. CMPD1 suppressed GnRH-A stimulated testosterone. CMPD1 (20 mg/kg) was

administered by intravenous infusion 5 minutes prior to GnRH-A (40 ng/kg; iv) stimulation.

Figure 7. Testosterone time course in intact male rat. A single intravenous dose of CMPD1 at

5, 10 and 20 mg/kg dose-dependently suppressed testosterone. * p <0.5 CMPD1 vs vehicle.

Figure 8. Testosterone time course in intact male rat. CMPD1 administered by oral gavage 20,

50 and 100 mg/kg dose-dependently suppressed testosterone.

Figure 9. Plasma concentration of CMPD1 in intact rats.

Table 1. Binding Constants (Ki) and Hill Coefficients of Various GnRH Peptide and

Nonpeptide Compounds at recombinant human, rat pituitary and recombinant mouse

receptors.

Compound	Human Ki (nM)	Human Hill Coefficient	Rat Ki (nM)	Rat Hill Coefficient	Mouse Ki (nM)	Mouse Hill Coefficient
GnRH	0.8 ± 0.14	0.83 ± 0.07	26 ± 3.6	0.77 ± 0.05	7.5 ± 1.5	0.82 ± 0.07
GnRH-A	0.2 ± 0.04	1.3 ± 0.16	0.51 ± 0.11	0.76 ± 0.04	0.52 ± 0.018	0.77 ± 0.17
Antide	0.25 ± 0.03	1.02 ± 0.04	0.06 ± 0.007	0.92 ± 0.13	0.025 ± 0.0025	1.06 ± 0.1
CMPD1	6.0 ± 0.8 (N=6)	0.9 ± 0.14	3.8 ± 0.8 (N=6)	0.9 ± 0.06	2.2 ± 0.4 (N=5)	0.9 ± 0.07

Values are means $\pm$ SE of at least three replicate experiments performed in duplicate. K_i
values were calculated according to Cheng and Prussof (1973).

Figure 1. Structure of CMPD1.
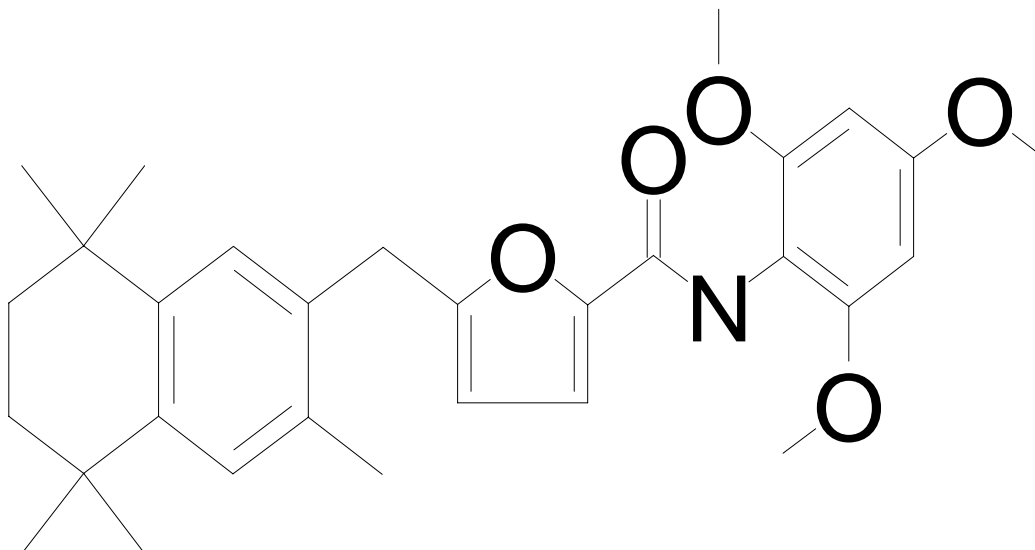
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Figure 2. Effect of CMPD1 on GnRH-stimulated increases in extracellular acidification in GGH3 cells.
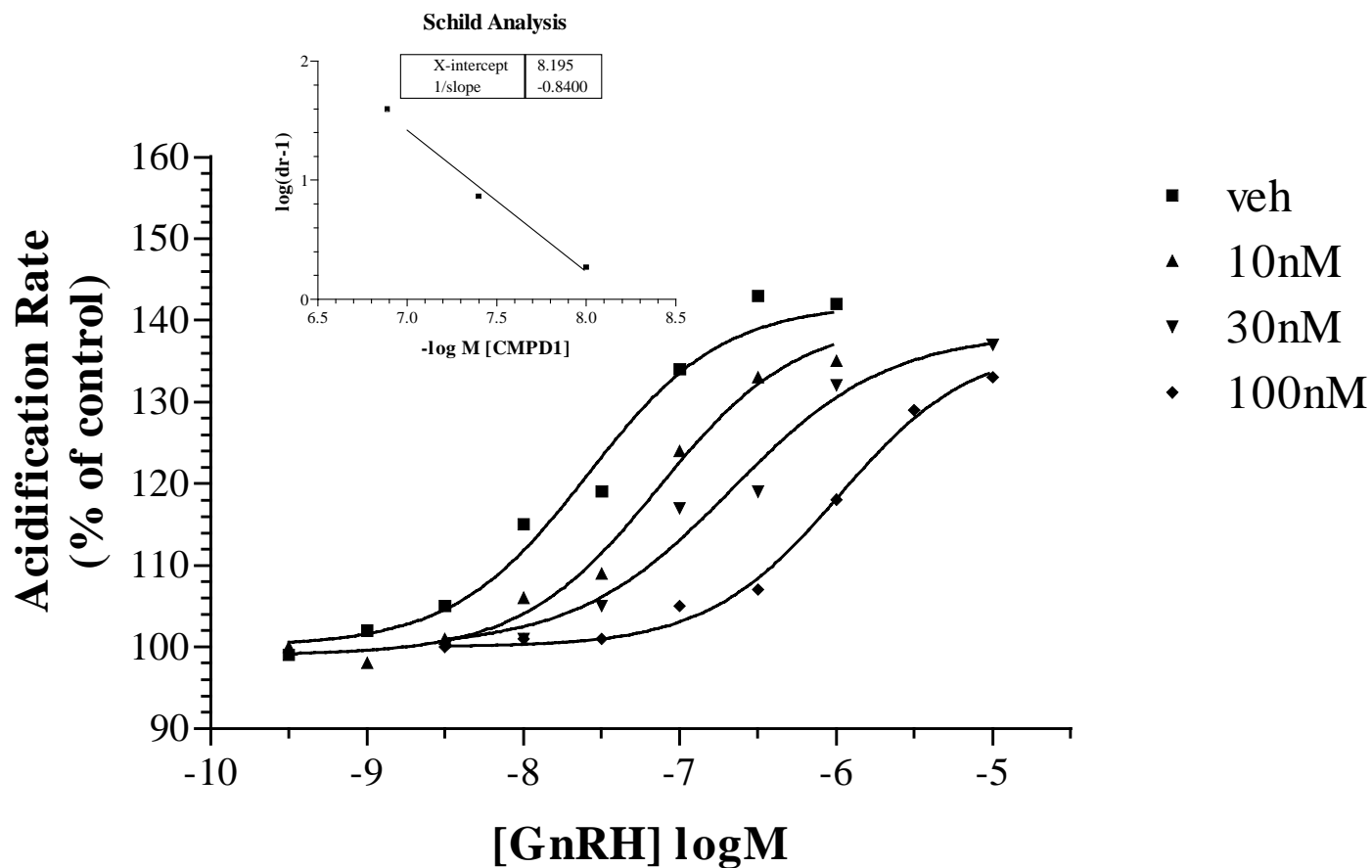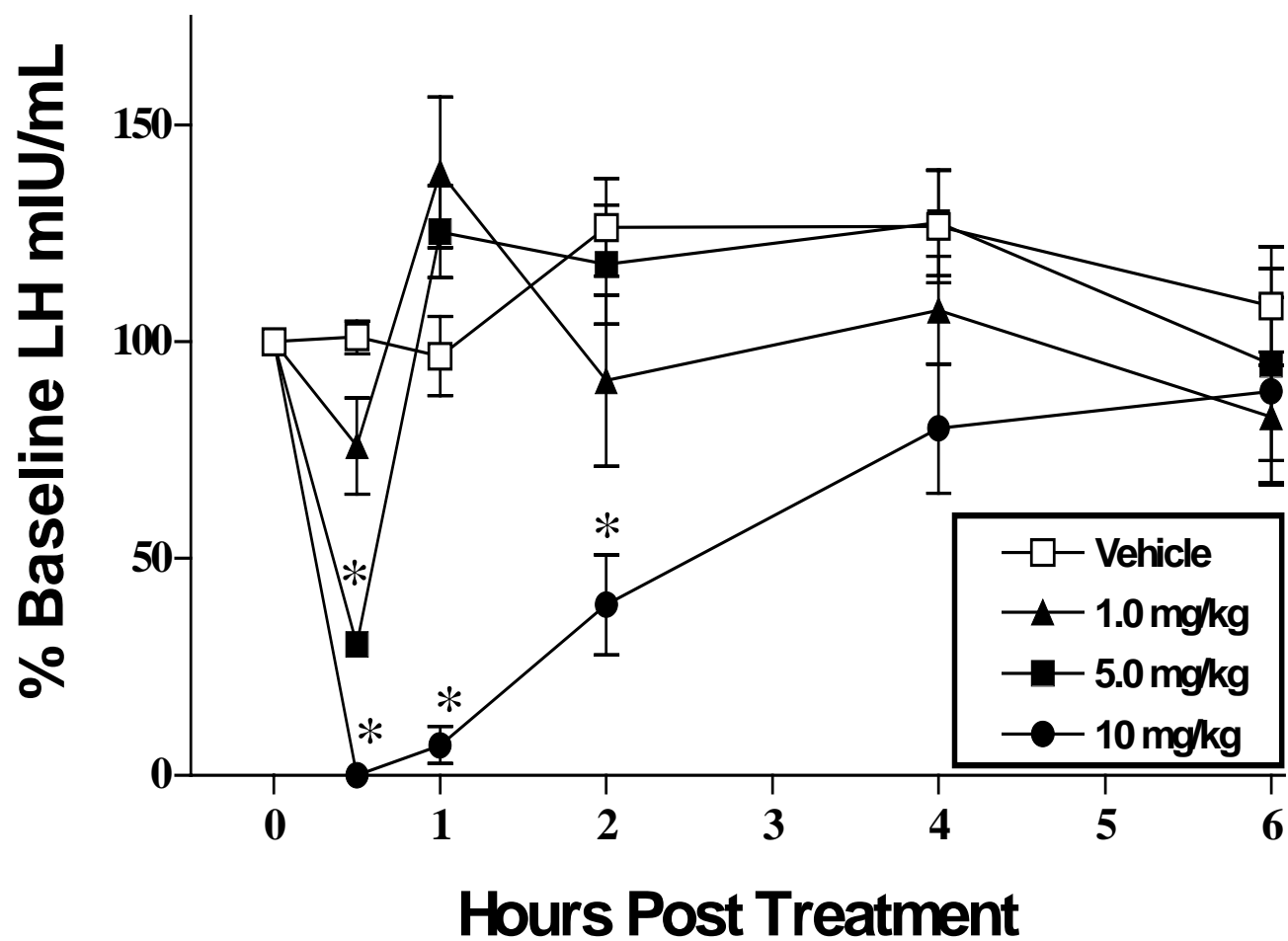
Anderes et al. JPET/2002/046656

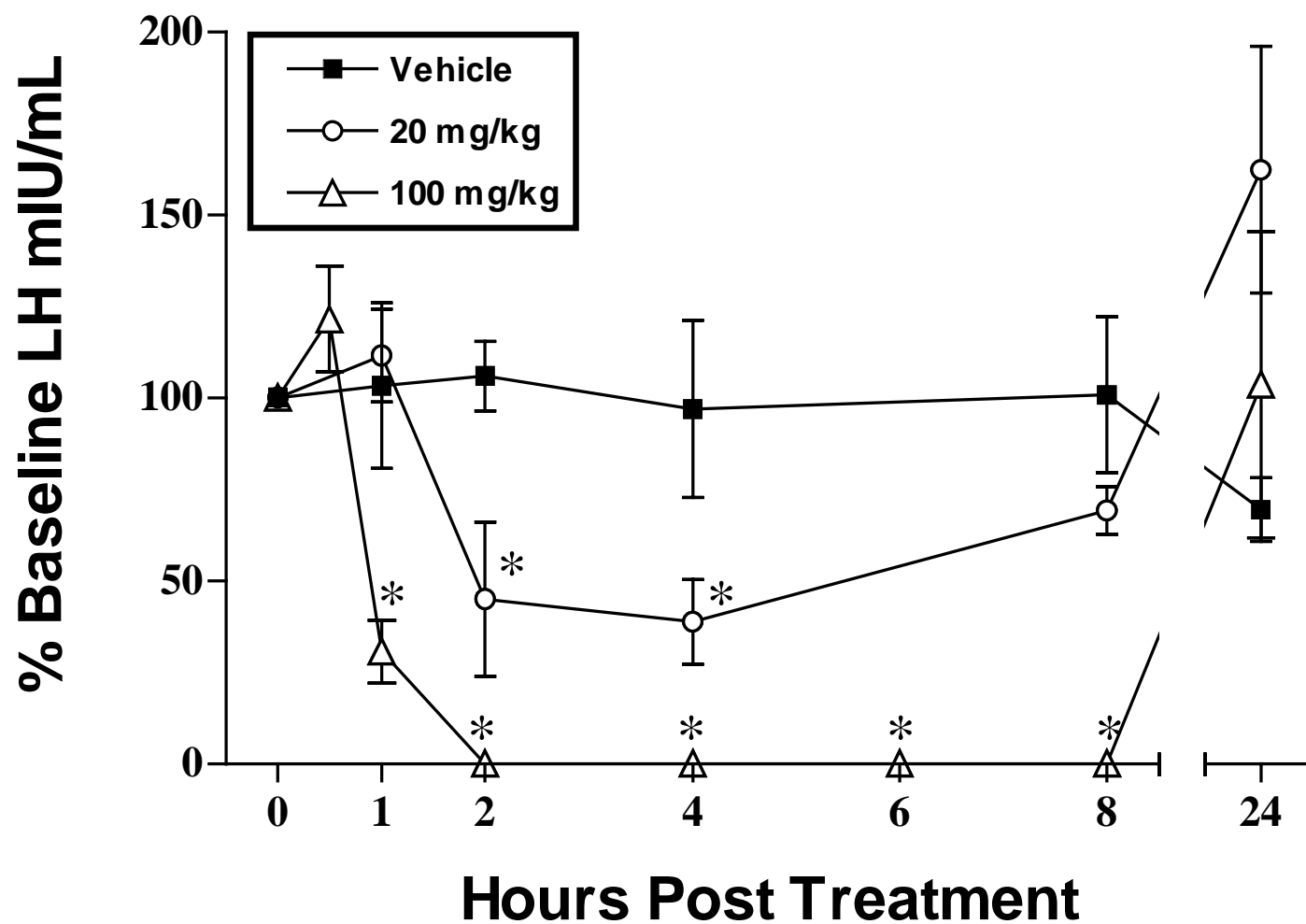Figure 3. Inhibition by CMPD1 of GnRH-stimulated increases in total inositol phosphate production.
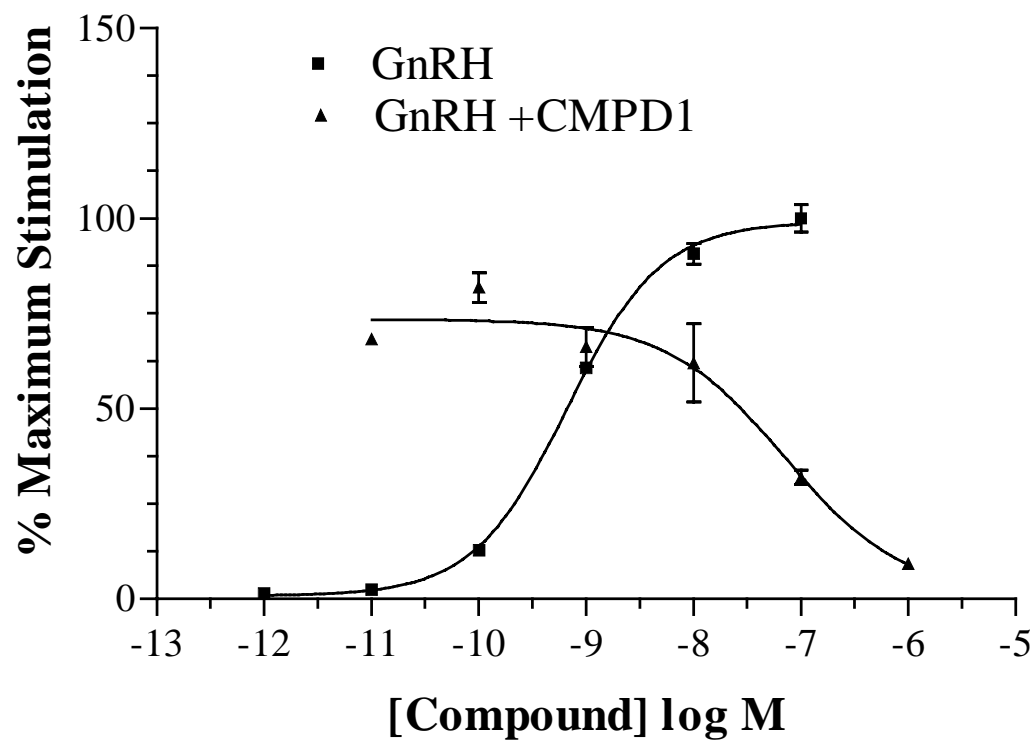
Anderes et al. JPET/2002/046656

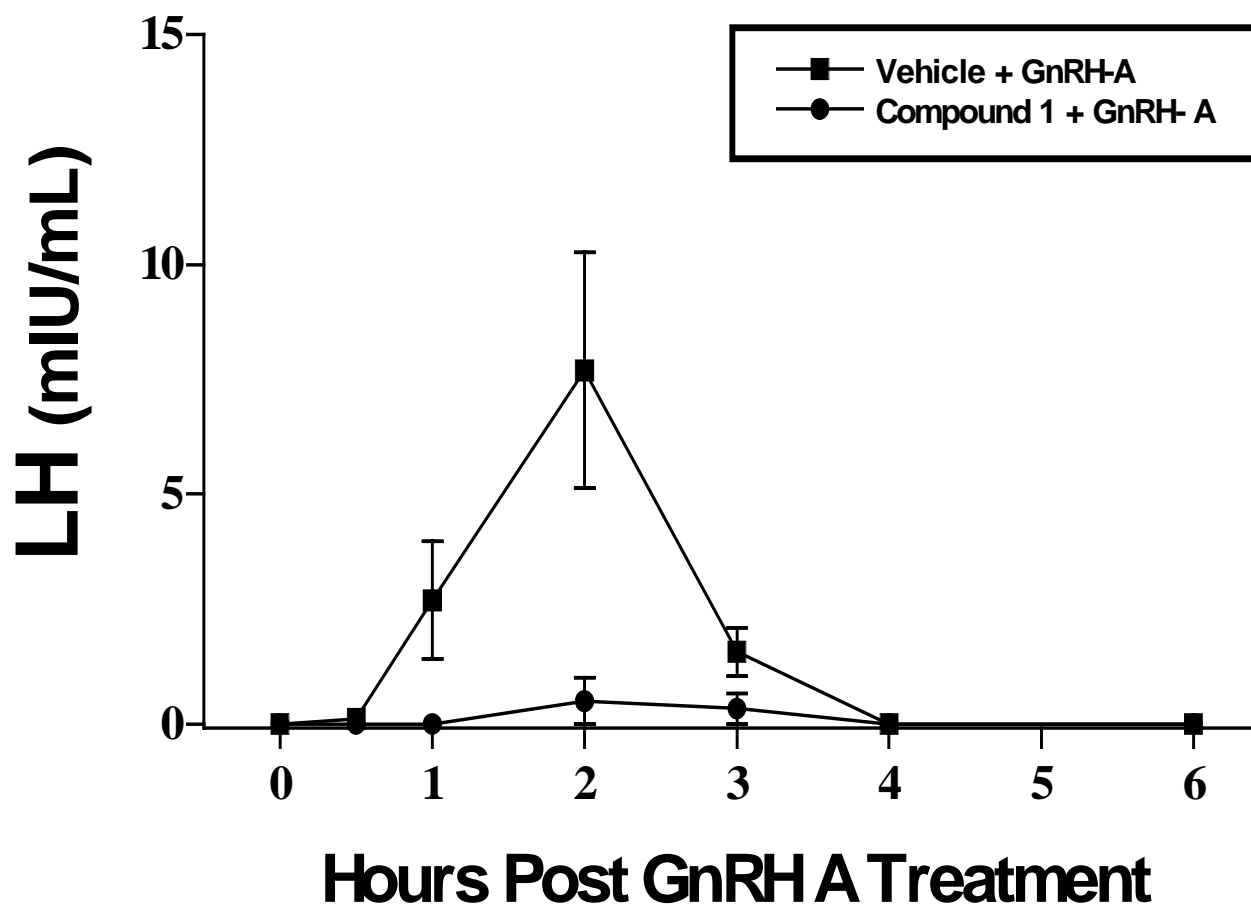Figure 4. LH in castrated male rat.

Anderes et al. JPET/2002/046656
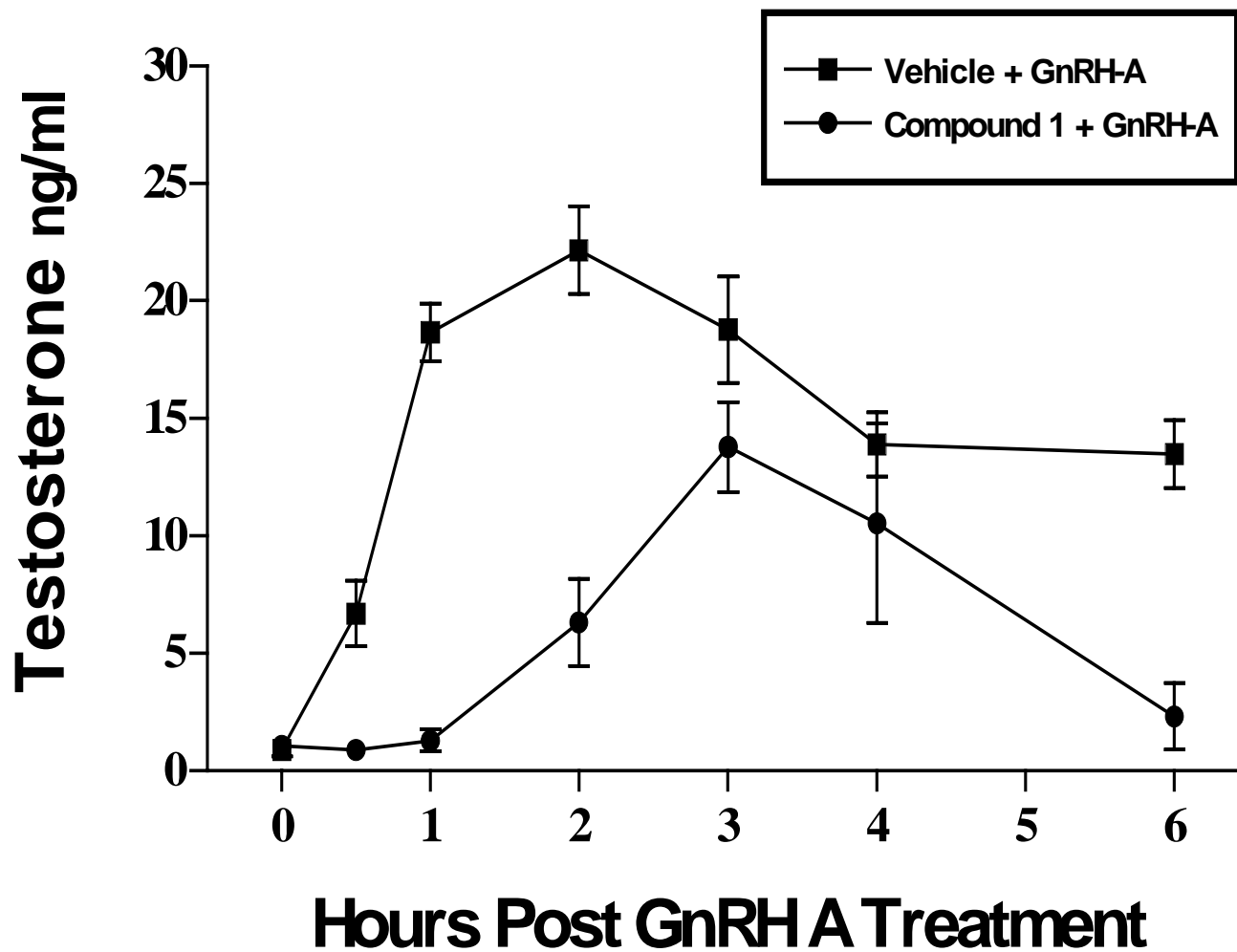
Figure 5. LH in castrated male rat.

Anderes et al. JPET/2002/046656

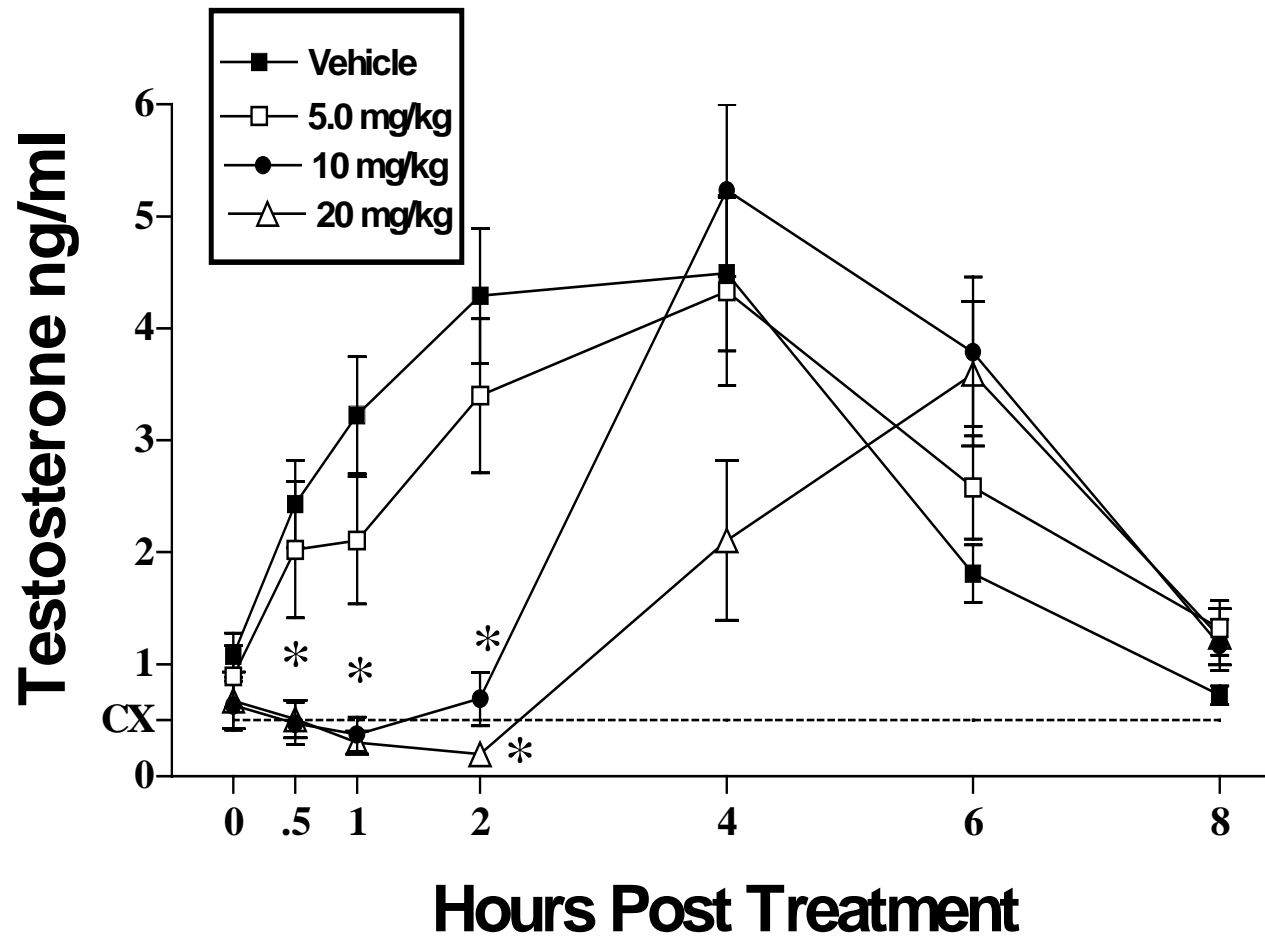Figure 6a. LH in intact male rat administered 20 mg/kg IV CMPD1 and GnRH A (40 ng/kg).

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Figure 6b. Testosterone in intact male rat administered 20 mg/kg IV CMPD1 and GnRH A (40 ng/kg).
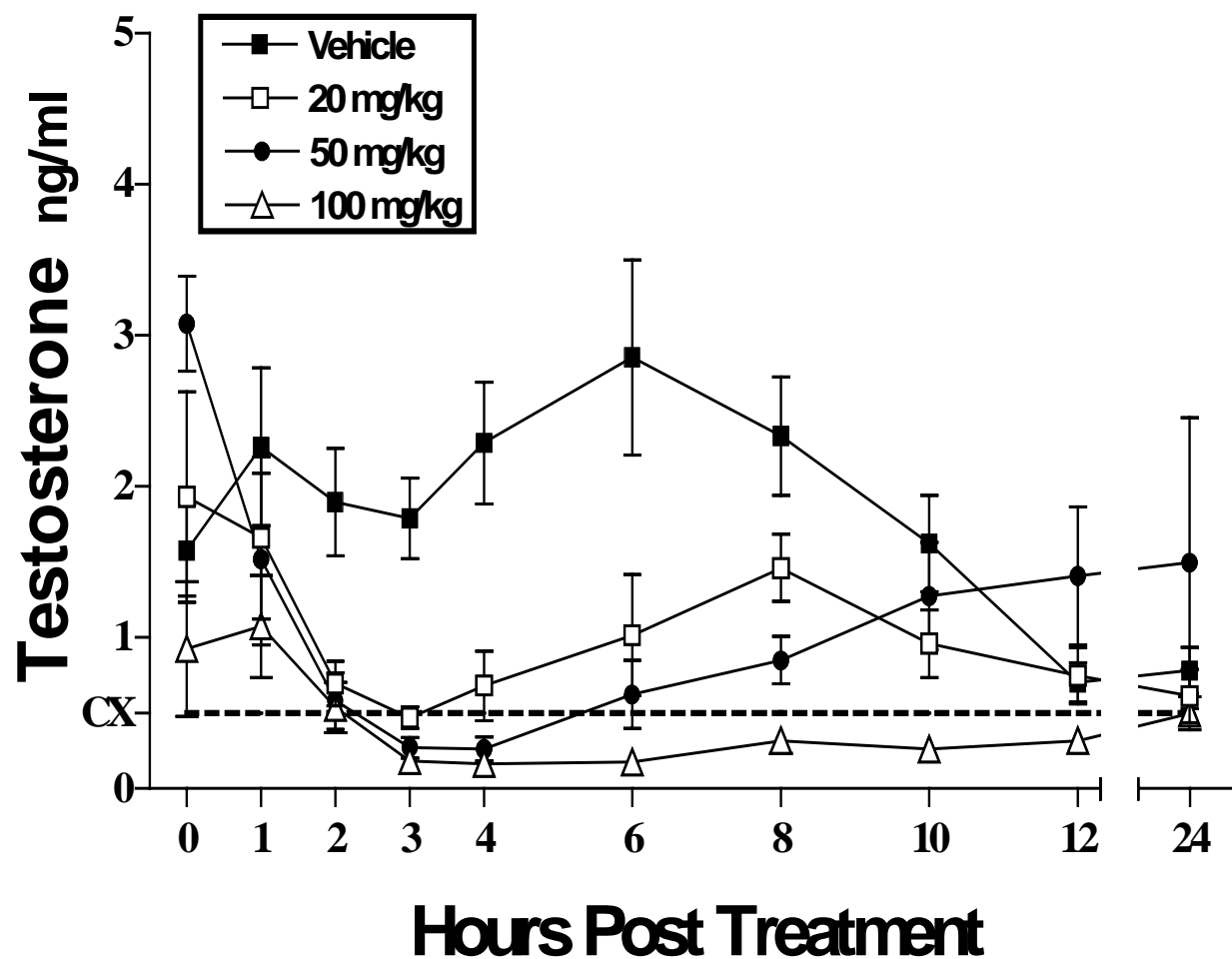
Anderes et al. JPET/2002/046656

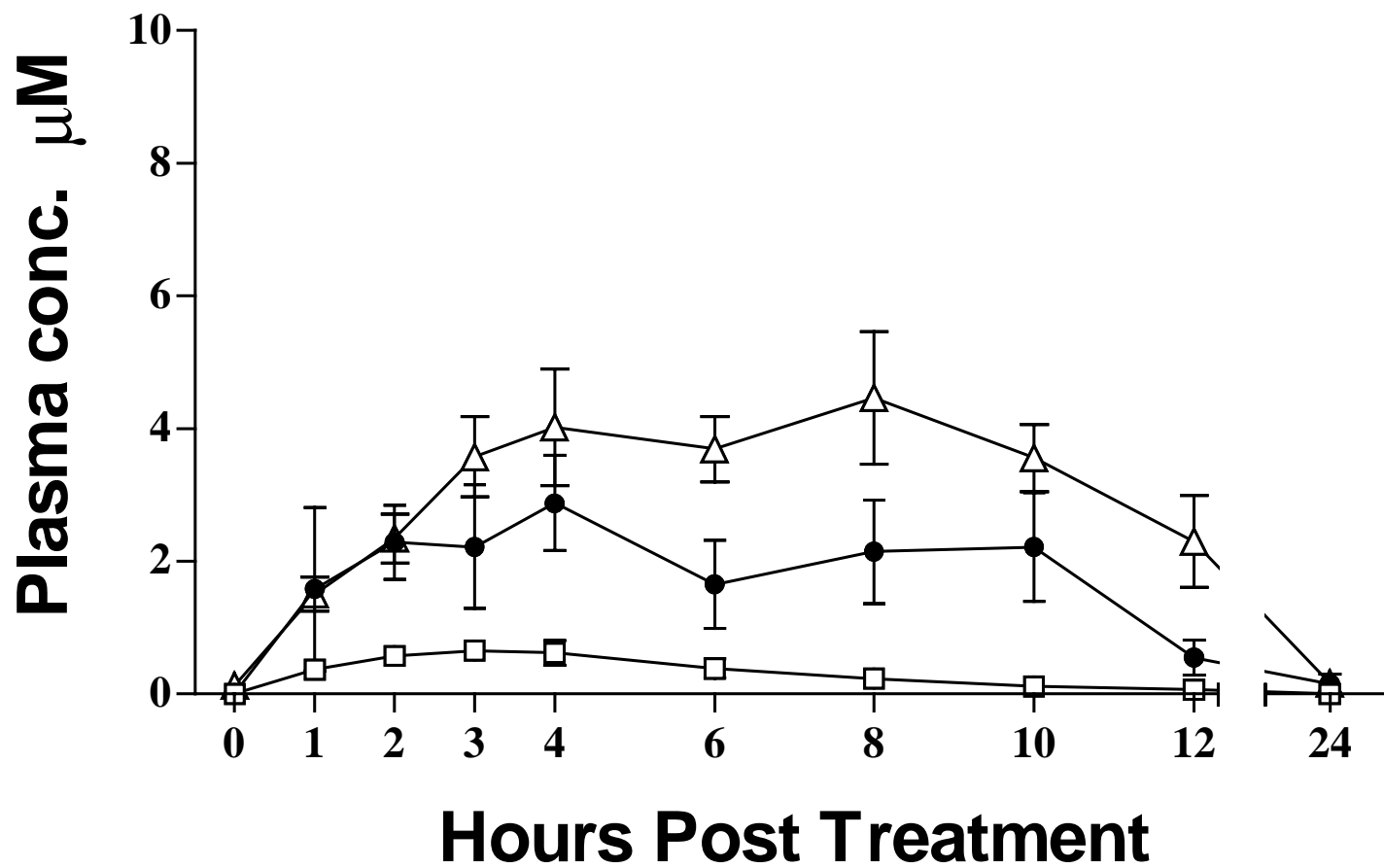Figure 7. Testosterone time course in intact male rat.

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Figure 8. Testosterone time course in intact male rat.

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Figure 9. Plasma concentration CMPD1 in intact male rat.

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