Evaluation of a Stable Gonadotropin-Releasing Hormone Analog in Mice for the Treatment of Endocrine Disorders and Prostate Cancer

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) receptor agonists have wide clinical applications including the treatment of prostate cancer and endocrine disorders. However, such agonists are characterized by poor pharmacokinetic properties, often requiring repeated administration or special formulations. Therefore, the development of novel peptide analogs with enhanced in vivo stability could potentially provide therapeutic alternatives. The pharmacological evaluation of a bioactive peptide [Des-Gly^{10},Tyr^5(OMe),D-Leu^6,Aze-NHEt^9]GnRH, analog 1, is presented herein and compared with leuprolide. Peptide stability was evaluated using mouse kidney membrane preparations, followed by a liquid chromatography-tandem mass spectrometry-based approach that afforded identification and quantification of its major metabolites. The analog was significantly more stable in vitro in comparison with leuprolide. In vitro and in vivo stability results correlated well, encouraging us to develop a clinically relevant pharmacokinetic mouse model, which facilitated efficacy measurements using testosterone as a biomarker. Analog 1, an agonist of the GnRH receptor with a binding affinity in the nanomolar range, caused testosterone release in mice that was acutely dose-dependent, an effect blocked by the GnRH receptor antagonist cetrorelix. Repeated dosing studies in mice demonstrated that analog 1 was well tolerated and had potency similar to that of leuprolide, based on plasma and testis testosterone reduction and histopathological findings. Analog 1 also shared with leuprolide similar significant antiproliferative activity on androgen-dependent prostate cancer (LNCaP) cells. On the basis of pharmacokinetic advantages, we expect that analog 1 or analogs based on this new design will be therapeutically advantageous for the treatment of cancer and endocrine disorders.

Introduction

Androgen deprivation therapy with gonadotropin-releasing hormone (GnRH) analogs is considered to be the first-line treatment for patients with prostate cancer. In early-stage prostate cancer, disease development and progression are mediated by androgens and growth and survival factors that promote cell proliferation and inhibit apoptosis. A significant proportion of patients develop locally advanced or metastatic disease not amenable to surgery or radiotherapy (Labrie et al., 2005). Recent advances suggest that GnRH analogs not only inhibit testicular androgen secretion via the pituitary-gonadal axis but are also capable of direct inhibition of tumor growth, exerting specific (receptor-mediated) antiproliferative, antimitogenic, and antimetastatic activities on cancer cells (Montagnani Marelli et al., 2006, 2007). Because several types of cancer cells overexpress the GnRH receptor, novel structures based on GnRH analogs that act as carriers to cancer cells, coupled with cytotoxic molecules (e.g., doxorubicin), have been prepared and evaluated in preclinical cancer models with promising results (Mezo and Manea, 2010).

Chronic administration of GnRH analogs results in the

ABBREVIATIONS: GnRH, gonadotropin-releasing hormone; LC, liquid chromatography; MS/MS, tandem mass spectrometry; LNCaP, lymph node carcinoma of the prostate; IS, internal standard; MS, mass spectrometry; HSA, human serum albumin; MRM, multiple reaction monitoring; EPI, enhanced product ion; QC, quality control; AUC, area under the curve.

desensitization of the GnRH receptor and, hence, medical castration (Maudsley et al., 2004; Pawson et al., 2008). Moreover, according to their "dual pharmacology," GnRH analogs are also characterized by direct anticancer potential via their interference with growth and survival factors, such as insulin-like growth factor-I, epidermal growth factor, and keratinocyte growth factor (Culig et al., 1994; Montagnani Marelli et al., 2007). GnRH receptor agonists currently in clinical use (e.g., leuprolide) are superior to the native hormone (GnRH) in terms of potency because of their high receptor affinity and improved proteolytic stability (Berger et al., 1991). However, GnRH analogs are still susceptible to the action of proteolytic enzymes (Haviv et al., 1992) and have limited absorption (Zheng et al., 1999) and low bioavailability (Adjei et al., 1993). As a consequence, these peptides are administered intramuscularly or subcutaneously and as depot formulations (Sennello et al., 1986; Perez-Marreno et al., 2002).

During the first days of their administration, GnRH receptor agonists are accompanied by the "flare phenomenon," in which the pituitary-gonadal axis is stimulated, thus resulting in the extensive release of gonadotrophs (10- and 5-fold increases in luteinizing hormone and follicle-stimulating hormone concentrations, respectively) and testosterone (Johns et al., 1990; Labrie et al., 2005). The flare phenomenon is an undesirable side effect that promotes metastasis. In the clinic, it can be managed by combined androgen blockade, which includes the coadministration of an antiandrogen (e.g., flutamide) and a GnRH receptor agonist (Wirth et al., 2007). As an alternative to GnRH receptor agonists, peptide antagonists have been synthesized and developed over the years. The administration of GnRH receptor antagonists does not cause the flare phenomenon, a considerable advantage in comparison with GnRH receptor agonists, but these agents have been associated with other side effects such as acute inflammation and require higher doses compared with agonists (Labrie et al., 2005; Kirby et al., 2009).

Considering the importance of hormonal therapy for the treatment of prostate cancer and the current needs for improved therapeutic approaches, we decided to focus on efforts to discover pharmacokinetically superior and possibly equipotent novel GnRH peptide analogs. Although superagonists have well established clinical benefits, their in vivo stability remains a limiting factor that most likely prevents them from exerting any direct effects on tumors (extrapituitary) or causing rapid desensitization of the GnRH receptor. After the development and establishment of in vitro and in vivo assays, coupled with liquid chromatography (LC)-tandem mass spectrometry (MS/MS) methodologies (Sofianos et al., 2008), analog 1, a potent stable peptide, was chosen from a screen among other GnRH receptor peptide agonists/antagonists. In this article, we report our findings regarding analog 1, [Des-Gly^{10},Tyr^5(OMe),D-Leu^6,Aze-NHEt^9]GnRH, a molecule with the conformational features required for agonistic activity (Matsoukas et al., 1997; Laimou et al., 2010) and enhanced in vitro and in vivo stability compared with leuprolide.

Although analog 1 has reduced binding affinity on the GnRH receptor compared with leuprolide, repeated dosing studies in mice demonstrated that analog 1 was well tolerated and had potency similar to that of leuprolide. Analog 1 was also endowed with significant antiproliferative activity on prostate cancer cells (LNCaP), similar to that exerted by

leuprolide. On the basis of its pharmacokinetic advantages, it is likely that the peptide analog in question or analogs based on this new design will be therapeutically advantageous for the treatment of endocrine disorders or prostate cancer. Such pharmacokinetic advantages can be particularly valuable with respect to local/direct extrapituitary effects that will be essential for more effective treatment of cancer.

Materials and Methods

Chemicals/Biological Reagents. [Des-Gly^{10},D-Leu^6,Pro-NHEt^9]-GnRH (leuprolide acetate salt), Ac-D-2-Nal-4-chloro-D-Phe-β-(3-pyridyl)-D-Ala-Ser-Tyr-D-Cit-Leu-Arg-Pro-D-Ala-NH$_2$ (cetrorelix acetate salt), and [Des-Gly^{10},D-Ala^6,Pro-NHEt^9]GnRH [internal standard (IS), acetate salt] were purchased from Bachem AG (Bubendorf, Switzerland). [Des-Gly^{10},Tyr^5(OMe),D-Leu^6,Aze-NHEt^9]GnRH (analog 1, acetate salt) was synthesized by the Department of Chemistry, University of Patras, Patras, Greece (Laimou et al., 2010). All solvents were LC-MS grade. Acetonitrile (Riedel-de Haën, Buchs SG, Switzerland), water (Sigma-Aldrich GmbH, Munich, Germany), ammonium acetate (Fluka, Buchs, Switzerland), formic acid (Fluka), human serum albumin (HSA) (Sigma-Aldrich GmbH), dexamethasone (Riedel-de Haën), testosterone (Riedel-de Haën), Trizma base (Sigma-Aldrich GmbH), sodium chloride (Fluka), sodium phosphate dibasic (Riedel-de Haën), and β-NaDPH were purchased from Sigma-Aldrich GmbH. Dithiothreitol was purchased from AppliChem GmbH (Darmstadt, Germany). Mouse plasma (CD-1) was purchased from Charles River Laboratories Italia s.r.l. (Calco, Italy). Tissues (kidneys and testes) were obtained from male C57BL/6N mice. Chinese hamster ovary-K1 cells were purchased from NEN (Waltham, MA). ^{125}I-(D-Trp^6)-GnRH was purchased from PerkinElmer Life and Analytical Sciences. The androgen-dependent LNCaP-FGC (lymph node carcinoma of the prostate-fast growing colony) cell line was obtained from American Type Culture Collection (Manassas, VA). RPMI 1640 medium was purchased from Seromed, Biochrom KG (Berlin, Germany), and fetal bovine serum was obtained from Invitrogen (Paisley, Scotland).

Cell Cultures. Cells (passages 35–40) were routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine (1 mM), and antibiotics (100 IU of penicillin G sodium and 100 μg/ml streptomycin sulfate) in a humidified atmosphere of 5% CO$_2$-95% air. In these culture conditions, the duplication period of LNCaP cells was 48 h.

Animals. All procedures were approved by the bioethics committee of the institution and the local competent authority on the basis of the European Directive 86/609 on the protection of animals used for experimental and other scientific purposes. Male mice of the C57BL/6N inbred strain (Charles River Laboratories Italia s.r.l.) at the age of 12 weeks were randomly assigned to groups of five or six animals. Animal handling occurred before experiments, during which labeling and weighing took place. Mice were fasted overnight (12 h) before dosing for pharmacokinetic studies. Dosing solutions of leuprolide and analogs in saline were administered intraperitoneally. Blood sampling was performed by cardiac puncture under isoflurane anesthesia. Blood samples (500 μl) were collected into low retention (Axygen, Inc., Union City, CA) microtubes containing 10 μl of heparin (5000 IU/ml) and subsequently centrifuged (10 min at 3000 rpm) for plasma preparation. Plasma was stored in a −80°C freezer until sample extraction and analysis. Tissues (kidneys and testes) were removed after the animals were sacrificed using anesthesia by isoflurane and cervical dislocation and were washed with phosphate-buffered saline, weighed, and placed in a −80°C freezer until analysis.

In Vitro Stability: Mouse Kidney Membranes. Mouse kidney membrane preparations were obtained on the basis of the procedure described by Sofianos et al. (2008). Kidneys were washed with PBS, weighed, and homogenized (0.1 g wet weight/ml) in buffer A (100 mM

NaCl, 20 mM phosphate, pH 7.4, 0.1 mg HSA/ml, and 1 mM dithiothreitol) by a Teflon-glass homogenizer. After centrifugation at 1500g for 5 min, the supernatant was centrifuged for 60 min at 100,000g (Sorvall Discovery 100SE ultracentrifuge; Hitachi, Yokohama, Japan). The supernatant was discarded, and the pellet was washed by addition of buffer B (100 mM NaCl, 20 mM phosphate, pH 7.4, and 0.1 mg HSA/ml) followed by centrifugation at 100,000g for 60 min. After the supernatant was discarded, the pellet was washed by addition of buffer C (100 mM NaCl and 20 mM phosphate, pH 7.4) and the protein content was determined by the Bradford assay (Bradford, 1976). Incubation mixtures consisted of 500 ng/ml peptide (leuprolide and/ or analog 1) and 4 mg/ml protein in a final volume of 250 μl of mouse kidney homogenate. Additional control samples (no kidney membranes) were prepared for each of the time points tested. The reaction was stopped by acetonitrile addition, and samples were stored at -80°C.

Sample extraction (samples left at room temperature to thaw for 20 min) was performed by the addition of 250 μl of acetonitrile-water (50:50) with 0.1% formic acid, followed by brief vortexing and sonication. Proteins were precipitated by stepwise addition of 250 and 500 μl of ice-cold acetonitrile followed by brief vortexing. Samples were subsequently centrifuged (12,000g) for 10 min, and the supernatant was evaporated in a SpeedVac (SPD1010; Thermo Fisher Scientific, Waltham, MA) for 90 min at 50°C. An aliquot of 100 μl of mobile phase (see below) was added to each sample, and the sample was vortexed and transferred to a 96-well plate for analysis. An 1100 series system (Agilent Technologies, Waldbrown, Germany) equipped with a binary pump, autosampler, vacuum degasser, and temperature-controlled column compartment was used for analyte separation by high-performance liquid chromatography. The mobile phase consisted of solvents A (10% acetonitrile, 90% water, 2 mM ammonium acetate, and 0.1% formic acid) and B (90% acetonitrile, 10% water, 2 mM ammonium acetate, and 0.1% formic acid). An Atlantis dC18 μm, 2.1 $\times$ 50 mm column (Waters, Milford MA) was used at a flow rate of 0.3 ml/min. A linear gradient (run time of 15 min) from 95% A-5% B to 50% A-50% B over 5 min and 50% A-50% B up to 9 min was used for the chromatographic separation of peptides of interest. Mass spectrometry was performed on an API 4000 Q-TRAP LC-MS/MS system fitted with a TurboIonSpray source and a hybrid triple quadrupole hybrid linear ion trap mass spectrometer (Applied Biosystems, Concord, ON, Canada). The instrument was operated in positive ion mode under the following conditions: IonSpray voltage, 5500 V; source temperature, 550°C; curtain gas (nitrogen) at 20; collision gas (nitrogen) at 5; ion source gas 1 (air) at 40; and ion source gas 2 (air) at 45 (all arbitrary units). Analog 1 (mol. wt. 1209.4 g/mol) was monitored by using the multiple-reaction monitoring (MRM) transitions of m/z 605.7 $\rightarrow$ 249.2 and 605.7 $\rightarrow$ 285.4. The MRM transitions of m/z 605.5 $\rightarrow$ 249.1 and 605.7 $\rightarrow$ 221.1 were used for the detection of leuprolide (mol. wt. 1209.4 g/mol).

In vitro peptide stability was determined as a function of time; using the corresponding MRM transitions for each analyte, the peak areas for analog 1 and/or leuprolide (peptide concentration of 500 ng/ml) at $t = 0$ were set as 100%. Those peak areas were compared with the peak areas derived from samples at $t = 0.5$, 1, and 2 h. The mean intra-assay coefficient of variation based on control samples (500 ng/ml peptide) was less than 10%. The interassay coefficients of variation for the determination of leuprolide and analog 1 were 6.6 and 6.9%, respectively. Peptide monitoring by LC-MS/MS ensured specificity because both peptides had characteristic retention times (6.79 $\pm$ 0.01 min for leuprolide and 7.19 $\pm$ 0.01 min for analog 1) and corresponding MRM transitions. In addition, analyses of blank samples confirmed the absence of interference and established the selectivity of the assay.

Metabolism Studies: Mouse Kidney Membranes. After incubation of analog 1 with mouse kidney membrane preparations, metabolites were identified by a modification of the LC-MS/MS-based approach described above using the instrument's information-dependent acquisition feature and combining Q3 scanning followed by enhanced product ion (EPI). Information-dependent acquisition criteria were set so that a number of the most intense peaks (up to 8) would be selected for EPI analysis with a collision energy of 45 $\pm$ 5 V (leuprolide) and/or 45 $\pm$ 10 V (analog 1). The semiquantitative analysis for the major metabolites identified herein was achieved by using the MRM-positive mode, as described above. Two major metabolic products were identified: a tripeptide (M-III) and a pentapeptide (M-V). M-III (mol. wt. 452.4 g/mol) was monitored based on the MRM transitions of m/z 453.4 $\rightarrow$ 176.1 and 453.4 $\rightarrow$ 110.2. The MRM transitions of m/z 688.8 $\rightarrow$ 150.2 and 688.8 $\rightarrow$ 263.2 were used for the detection of M-V (mol. wt. 687.8 g/mol).

In Vivo Stability and Pharmacokinetic Studies. Analog 1 was administered to male C57BL/6N mice at 1 mg/kg i.p., and blood samples were obtained at $t = 1$, 2, 4, and 6 h postdose ($n = 5$). In a parallel test group ($n = 5$), leuprolide was administered at 1 mg/kg. For the quantification of peptides in plasma, stock solutions of leuprolide or analog 1 were prepared in acetonitrile-water (50:50) with 0.1% formic acid in the range of 0.5 to 5000 ng/ml. A linear peptide analog of both peptides was used as the IS at the concentration of 600 ng/ml. A set of solutions at two different concentrations (50 and 500 ng/ml) was prepared, serving as the quality control (QC) samples. Calibration curves (0.5–500 ng/ml for leuprolide and 5–2500 ng/ml for analog 1) and QC samples (50 and 500 ng/ml) were prepared in plasma by addition of 20 μl of leuprolide or analog 1 solutions and 25 μl of the IS solution to 50 μl of mouse plasma. Samples were prepared for LC-MS/MS analysis by protein precipitation as described above. Peptide quantification was performed in the MRM-positive mode. For leuprolide and IS detection, mass spectrometry-based quantification methods were developed and validated previously (Sofianos et al., 2008). Analog 1 was monitored in mouse plasma using the MRM transitions of m/z 605.7 $\rightarrow$ 249.2 and 605.7 $\rightarrow$ 221.4. A nine-point calibration curve was constructed using a $1/x$ weighted linear regression model ($r = 0.999$). On the basis of concentrations at 50 and 500 ng/ml, the intra-assay coefficient of variation for the assay was less than 6% for leuprolide detection and less than 5% for the analysis of analog 1. The interassay coefficients of variation for analog 1 and leuprolide were 6 and 7%, respectively.

Assay specificity and selectivity was ensured by using an LC-MS/MS methodology (retention time and MRM transitions characteristic of the analyte) and analyzing blank samples to test for interferences. Based on QC samples at 50 and 500 ng/ml, the accuracy of the assays for the determination of analog 1 and leuprolide in mouse plasma was 100 and 97%, respectively. The lower limit of quantification for both peptides in mouse plasma was 0.5 ng/ml, defined as the concentration that yielded a peak with a signal/noise ratio of 10 and at least 3 times the response compared with signal from blank extracts (samples not spiked with peptide). The accuracy and precision of the measurement at the concentration of 0.5 ng/ml were within $\pm$20%.

Binding to the Human GnRH Receptor. A screening protocol was followed for the ranking of GnRH analogs (including analog 1) in terms of their binding affinities to the human GnRH receptor. In brief, competition binding studies were performed according to the method of PerkinElmer Life and Analytical Sciences. Frozen membrane homogenates from Chinese hamster ovary-K1 cells stably expressing the human GnRH receptor, purchased from PerkinElmer Life and Analytical Sciences, were thawed and diluted in assay buffer (25 mM Hepes, pH 7.4, 10 mM MgCl$_2$, 1 mM CaCl$_2$, and 0.5% bovine serum albumin) at a protein concentration of 0.07 mg/ml. Aliquots of diluted membrane suspension (150 μl) were incubated with increasing concentrations of GnRH analogs in the presence of 0.2 nM ^{125}I-(D-Trp^6)-GnRH (PerkinElmer Life and Analytical Sciences) in a final volume of 0.2 ml. The mixtures were incubated at 27°C for 1 h and then filtered using a Brandel cell harvester through Whatman GF/C glass fiber filters, presoaked for 1 h in 0.5% polyethylenimine at 4°C. The filters were washed nine times with 0.5 ml of ice-cold 50 mM Tris-HCl, pH 7.4, and the filters were assessed for radioactivity in a gamma counter (LKB Wallac 1275 minigamma,

80% efficiency). Specific ^{125}I-(D-Trp^6)-GnRH binding was defined as total binding less nonspecific binding in the presence of 1000 nM (D-Trp^6)-GnRH. Data for competition binding were analyzed by nonlinear regression analysis, using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA), and log IC$_{50}$ values were obtained by fitting the data from competition studies to a one-site competition model. The K_i values were determined from competition data and the equation, $K_i = IC_{50}/(1 + L/K_D)$ (Cheng and Prusoff, 1973), where L is the concentration of radioligand and K_D is the affinity of radioligand binding, determined from saturation studies performed by Perkin-Elmer Life and Analytical Sciences and under the same experimental conditions used in the present study.

To further confirm the binding to the GnRH receptor, analog 1 was evaluated in a radioligand binding assay by Ricerca Biosciences, LLC (Concord, OH) as described in the literature (Halmos et al., 1996), using human recombinant Chem-1 cells, 0.05 nM ^{125}I-(D-Trp^6)-GnRH (ligand), and 1 μM (D-Trp^6)-GnRH (nonspecific ligand). Reference standards were run as an integral part of each assay to ensure the validity of the results obtained. IC$_{50}$ values were determined by a nonlinear, least-squares regression analysis using MathIQ (ID Business Solutions Ltd., Guildford, Surrey, UK). The K_i values were calculated (Cheng and Prusoff, 1973) using the observed IC$_{50}$ of the tested compound, the concentration of radioligand used in the assay, and the historical values for the K_D of the ligand (obtained experimentally at Ricerca Biosciences, LLC).

Efficacy Studies (Testosterone Quantification in Mouse Plasma). The ability of analog 1 to elicit testosterone release (GnRH receptor agonism) in mice was investigated and compared with that of leuprolide. Analog 1 was administered to male C57BL/6N mice at 1 mg/kg, and blood sampling was conducted by cardiac puncture under isoflurane anesthesia at $t = 1$, 2, 4, and 6 h postdose ($n = 5$). The same approach was followed in the case of vehicle (saline)- and leuprolide (1 mg/kg)-treated animals ($n = 5$). Peptides and testosterone were measured in mouse plasma by an LC-MS/MS-based assay. Stock solutions of testosterone (0.125–250 ng/ml) and dexamethasone (served as the IS for testosterone quantification) were prepared in acetonitrile-water (50:50) with 0.1% formic acid. Calibration curves (0.2–100 ng/ml) and QC samples (1, 5, 10, and 40 ng/ml) were prepared by adding 20 μl of testosterone solutions and 25 μl of dexamethasone solution (200 ng/ml) to 50 μl of female CD-1 mouse plasma. Female CD-1 plasma was devoid of testosterone and, hence, was used for the construction of the standard curves and QC samples. A similar extraction protocol (protein precipitation) and chromatographic conditions as described above were used to achieve the separation of testosterone, dexamethasone, and peptides. Quantification was performed in the MRM-positive mode. For the detection of testosterone (mol. wt. 288.4 g/mol), the MRM transitions of m/z 289.4 → 109.1 and 289.4 → 97.2 were optimized, and dexamethasone (mol. wt. 392.5 g/mol) was monitored using the MRM transition of m/z 393.5 → 237.2. A nine-point calibration curve was constructed using a $1/x$ weighted linear regression model ($r = 0.993$).

On the basis of QC samples at 1 and 10 ng/ml, the intra- and interassay coefficients of variation were less than 10 and equal to 7%, respectively. The accuracy of the testosterone assay was 98%. The retention times (expressed in minutes) obtained were characteristic of the analytes: 8.92 ± 0.02 (testosterone), 7.92 ± 0.01 (dexamethasone), 6.69 ± 0.01 (analog 1), 6.22 ± 0.02 (leuprolide), and 6.67 ± 0.01 (IS). The absence of interference was further confirmed by analyzing blank samples (female CD-1 mouse plasma). The lower limit of quantification for testosterone in mouse plasma was 0.05 ng/ml, defined as the concentration that yielded a peak with a signal/noise ratio of 10 and at least 3 times the response compared with signal from blank extracts (samples not spiked with testosterone). The accuracy and precision of the measurement at the concentration of 0.05 ng/ml were within ±20%. The LC-MS/MS method that was developed and applied to our study ensured the simultaneous quantification of testosterone and peptide agonists in both plasma and testis from the same limited sample.

Dose-Response Study. To investigate and establish a dose-response relationship on the administration of analog 1 in mice, analog 1 was dosed intraperitoneally to male C57BL/6N mice. A dose-response relationship was also investigated and determined for leuprolide. The peptides were dosed at 0.1, 1, 10, and 1000 μg/kg, and blood samples were obtained by cardiac puncture under isoflurane anesthesia at $t = 1$ h postdose ($n = 5$). The same approach was followed in the case of vehicle (saline)-treated animals ($n = 5$).

GnRH Receptor Agonism. To further confirm that testosterone release results from the binding of analog 1 to the GnRH receptor (agonism), analog 1 was dosed intraperitoneally to male C57BL/6N mice in the presence and absence of a known GnRH receptor antagonist, cetrorelix. Cetrorelix was dosed (intraperitoneally) at 1 mg/kg and after 15 min, analog 1 was dosed either at 1 or 10 μg/kg. In a different group, animals were treated with vehicle (saline) and after 15 min, analog 1 was administered either at 1 or 10 μg/kg. Likewise, GnRH agonism was investigated upon leuprolide dosing (at 1 μg/kg). Blood sampling was conducted by cardiac puncture under isoflurane anesthesia at $t = 1$ h postdose ($n = 5$). Quantification for peptide and testosterone was performed as described above. Cetrorelix (mol. wt. 1431.1 g/mol) was monitored by LC-MS/MS using the MRM transitions of m/z 716.2 → 154.3 and 716.2 → 569.4.

Cell Proliferation Assays. LNCaP cells were plated (100,000 cells/dish) in 10-cm dishes in standard culture medium. Cells were allowed to attach and start growing for 3 days; the seeding media were then changed to experimental media. Cells were treated, daily, for 7 days, with either leuprolide or with analog 1 (10^{-6}, 10^{-8}, and 10^{-10} M); the medium was changed every 2 days. At the end of the treatment, cells were harvested and counted by hemocytometer. The doses of the GnRH analogs have been chosen on the basis of previous articles from Dr. Limonta's laboratory (Limonta et al., 1992, 1999).

Efficacy Based on GnRH Desensitization and Testosterone Depletion. To investigate and determine the ability of analog 1 to cause testosterone ablation upon repeated administration, a long-term repeated dosing study was conducted. Analog 1 was dosed once a day intraperitoneally to male C57BL/6N mice at 500 μg/kg for 20 days ($n = 6$). Vehicle (saline)-treated animals served as a control group ($n = 6$). The pharmacological effect of analog 1 was compared with that of leuprolide ($n = 6$). Blood samples were obtained by cardiac puncture under isoflurane anesthesia 1) at day 0, $t = 2$ h postdose, 2) at day 9, $t = 2$ h postdose, and 3) at day 20. To further confirm testosterone depletion, blood samples were obtained at day 20, $t = 2$ h postdose. Testes were removed at days 0, 9, and 20 and weighed and subsequently histopathologically examined; testes were fixed in 10% formalin overnight at 4°C, followed by embedding, sectioning, and staining with hematoxylin and eosin and finally were examined under a light microscope. Analog 1, leuprolide, and testosterone concentrations were determined in both mouse plasma and testis by LC-MS/MS with minor modifications; the standard curve for the analysis of testosterone in testis was constructed using male mouse testis homogenate that was treated with activated charcoal for steroid depletion as described by Green and Leak (1987).

Statistical Analysis. The results presented herein are expressed as means ± S.D. Statistical analyses were performed by the Stat-Graphics Centurion 15.206S program. Statistical significance was determined by using Student's t test. Data from proliferation assays were analyzed by a Bonferroni test after one-way analysis of variance.

Results

Binding Data. The IC$_{50}$ and K_i for analog 1 were 16 and 14 nM, respectively. (D-Trp^6)-GnRH was used as a control for the study, giving IC$_{50}$ and K_i values of approximately 0.2 nM (the IC$_{50}$ for leuprolide is approximately 0.3 nM on the basis
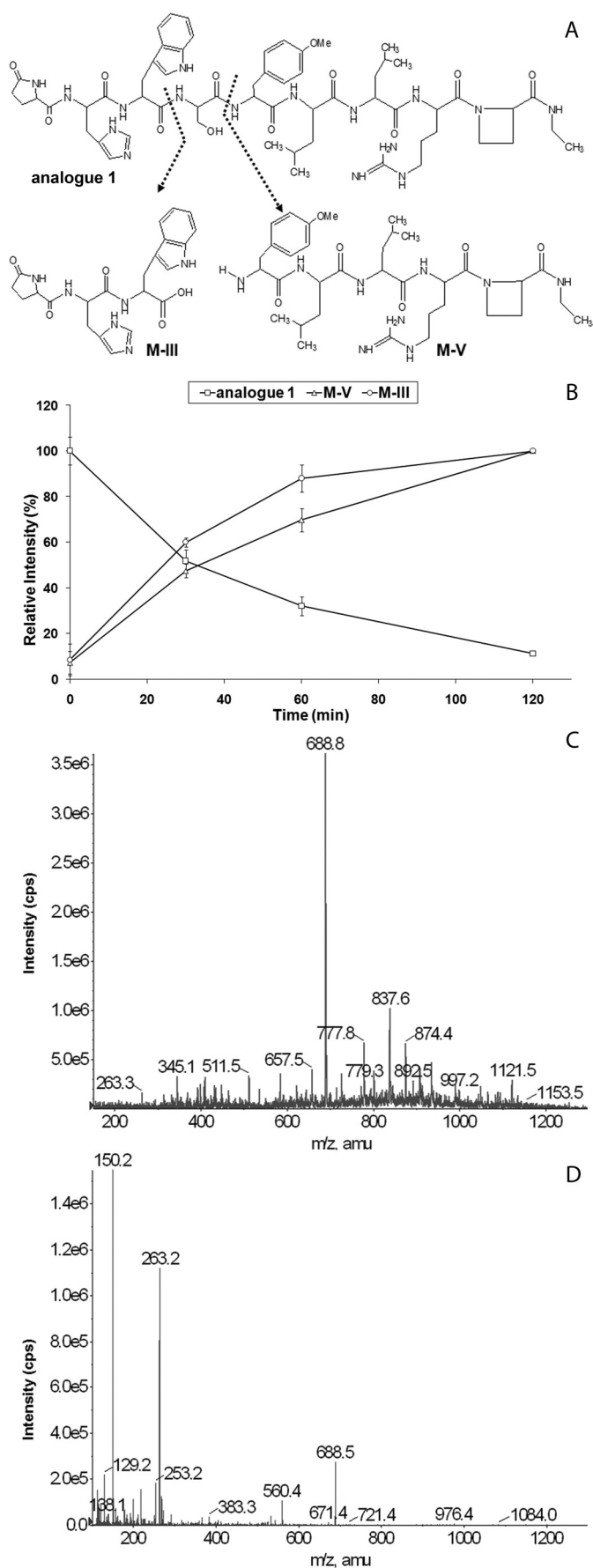
of historic data provided by Ricerca Biosciences, LLC). The results suggest that the biological activity of analog 1 is mediated through potent binding on the GnRH receptor.

In Vitro Stability and Metabolism Studies. The incubation of analog 1 with mouse kidney membrane preparations resulted in two major metabolic products: a tripeptide (M-III: pGlu[1]-His[2]-Trp[3]-OH) and a pentapeptide [M-V: Tyr-(OMe)[5]-D-Leu[6]-Leu[7]-Arg[8]-Aze[9]-NHEt]. The proposed structures of metabolites of analog 1 are shown in Fig. 1A. Our data support the susceptibility of the Trp[3]-Ser[4] and Ser[4]-Tyr[5] peptide bonds, a consistent finding based on studies that focus on GnRH and other analogs (Berger et al., 1991; Brudel et al., 1994). The approach described allowed the semiquantification of analog 1 (degradation of peptide) and M-III and M-V (formation of metabolites) as a function of time, depicted in Fig. 1B. Metabolite assignments were based on the MS and MS/MS spectra of analog 1 and its corresponding metabolites. The MS and MS/MS spectra for metabolite M-V (m/z at 688.8) are represented in Fig. 1, C and D. The major product ions at m/z 150.2 and 263.2, shown in Fig. 1D, correspond to the immonium ion of the methylated Tyr[5] and the a2 fragment ion of M-V. Likewise, the major product ions for M-III at m/z 110.2 and 221.1 correspond to the immonium ion of His[2] and the a2 fragment ion of M-III (data not shown). A leuprolide metabolism/in vitro stability study was also performed, in which two major metabolites were also detected: the tripeptide described above (M-III) and a pentapeptide (M-I: Tyr[5]-D-Leu[6]-Leu[7]-Arg[8]-Pro[9]-NHEt), being consistent with those metabolites reported in the literature (Ueno and Matsuo, 1991). Analog 1 and leuprolide share a rather common peptide sequence, with differences at position 5 (analog 1 is methylated at tyrosine) and position 9 (analog 1 has an azetidine instead of proline). On the basis of the structural similarities of analog 1 and leuprolide, as was expected, M-III was a common metabolic product for the two peptides.

To determine the in vitro stability of analog 1 (and leuprolide) as a function of time, the compounds were incubated with mouse kidney membrane preparations as described. After extraction of the analytes and detection by the LC-MS/MS methodology, the peak areas for both peptides were set as 100% at $t = 0$. Those peak areas were subsequently compared with the peak areas derived from samples at $t = 0.5$ h, $t = 1$ h, and $t = 2$ h. Analog 1 was found to be significantly more stable in comparison with leuprolide; at $t = 2$ h, leuprolide was extensively metabolized by mouse kidney membrane preparations, whereas 11% of analog 1 remained intact (Fig. 2A). This in vitro system also allowed compound ranking of selected GnRH analogs. The absence of metabolism of the described peptides (leuprolide, analog 1) in mouse liver microsomes (data not shown) further supports the fact that the kidney is the major site for the metabolism of leuprolide and the analog described (Carone and Peterson, 1980).

Fig. 1. A, the proposed metabolic products after the incubation of analog 1 with mouse kidney membranes. B, peptide degradation and metabolite (M-III and M-V) formation were monitored as a function of time. C, MS spectrum characteristic of metabolite M-V. D, EPI spectrum of M-V of ion m/z 689. The major ions shown (m/z 150.2 and 263.2) correspond to the immonium ion of methylated Tyr[5] and the a2 fragment of M-V. amu, atomic mass units.
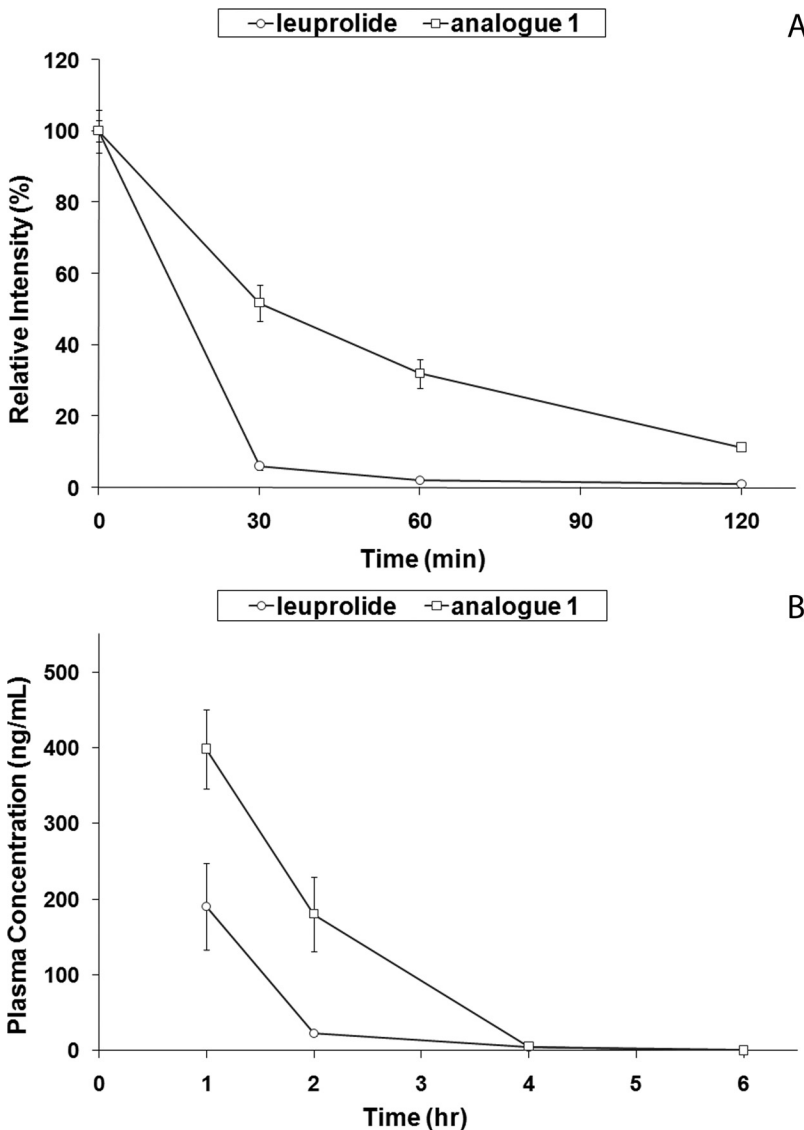
Fig. 2. A, the in vitro stability of analog 1 and leuprolide after peptide incubation with mouse kidney membrane preparations. B, the pharmacokinetic profiles (in vivo stability) of analog 1 and leuprolide after peptide administration in mice ($n = 5$) at 1 mg/kg i.p.

In Vivo Stability and Pharmacokinetic Studies. A pharmacokinetic study was conducted in mice for the evaluation of the in vivo stability of analog 1. In parallel, the pharmacokinetic profile of leuprolide was also evaluated. A graphical representation of peptide concentrations for both analog 1 and leuprolide in mouse plasma at $t = 1$ h, $t = 2$ h, $t = 4$ h, and $t = 6$ h postdose is depicted in Fig. 2B. Analog 1 exhibited a profound pharmacokinetic advantage compared with leuprolide on the basis of absolute plasma concentrations and areas under the curve (AUCs). Analog 1 dosing resulted in an $AUC_{1-6\,h} = 479.5$ ng · h/ml, whereas the AUC value for leuprolide dosing was $AUC_{1-6\,h} = 136.8$ ng · h/ml. Plasma peptide concentrations determined at all time points for both peptides tested showed relatively low intersubject variation. In this setting, the described in vivo pharmacokinetic protocol was also used as a comparative platform between leuprolide and other peptide analogs.

Functional Agonism of the GnRH Receptor-Testosterone Quantification in Mouse Plasma after Dosing in Mice. An efficacy study was conducted in which testosterone release was monitored upon the administration of analog 1 in mice (agonism of the GnRH receptor). Likewise, testosterone

concentrations were determined in the plasma of leuprolide-treated mice. Testosterone plasma concentrations as a function of time ($t = 1$–6 h postdose) are shown in Fig. 3A. Testosterone measurements in vehicle-treated mice were low as expected and in agreement with our previous studies (Sofianos et al., 2008). In comparison, testosterone concentrations in leuprolide-treated mice were significantly higher at $t = 1$ to 6 h postdose (***, $p < 0.001$ versus vehicle-treated mice). Testosterone release due to analog 1 dosing was suggestive of agonism (***, $p < 0.001$ at $t = 1$ h, $t = 2$ h, and $t = 4$ h versus vehicle-treated mice), showing a pattern similar to that with leuprolide treatment. In addition, Testosterone $AUC_{1-6\,h}$ values were equal to 244.1 and 154.9 ng · h/ml for leuprolide and analog 1 dosing, respectively.

To explore potential dosing regimens for efficacy studies, a dose-response relationship was established for both analog 1 and leuprolide treatment by dosing experiments in mice at 0.1, 1, 10, and 1000 μg/kg. Peptide plasma concentrations (1 h postdose) as a function of dose are depicted in Fig. 3B. Testosterone concentrations seemed to reach a plateau at doses higher than 10 μg/kg (data not shown). To demonstrate the specificity of the effects of testosterone release (i.e., bind-
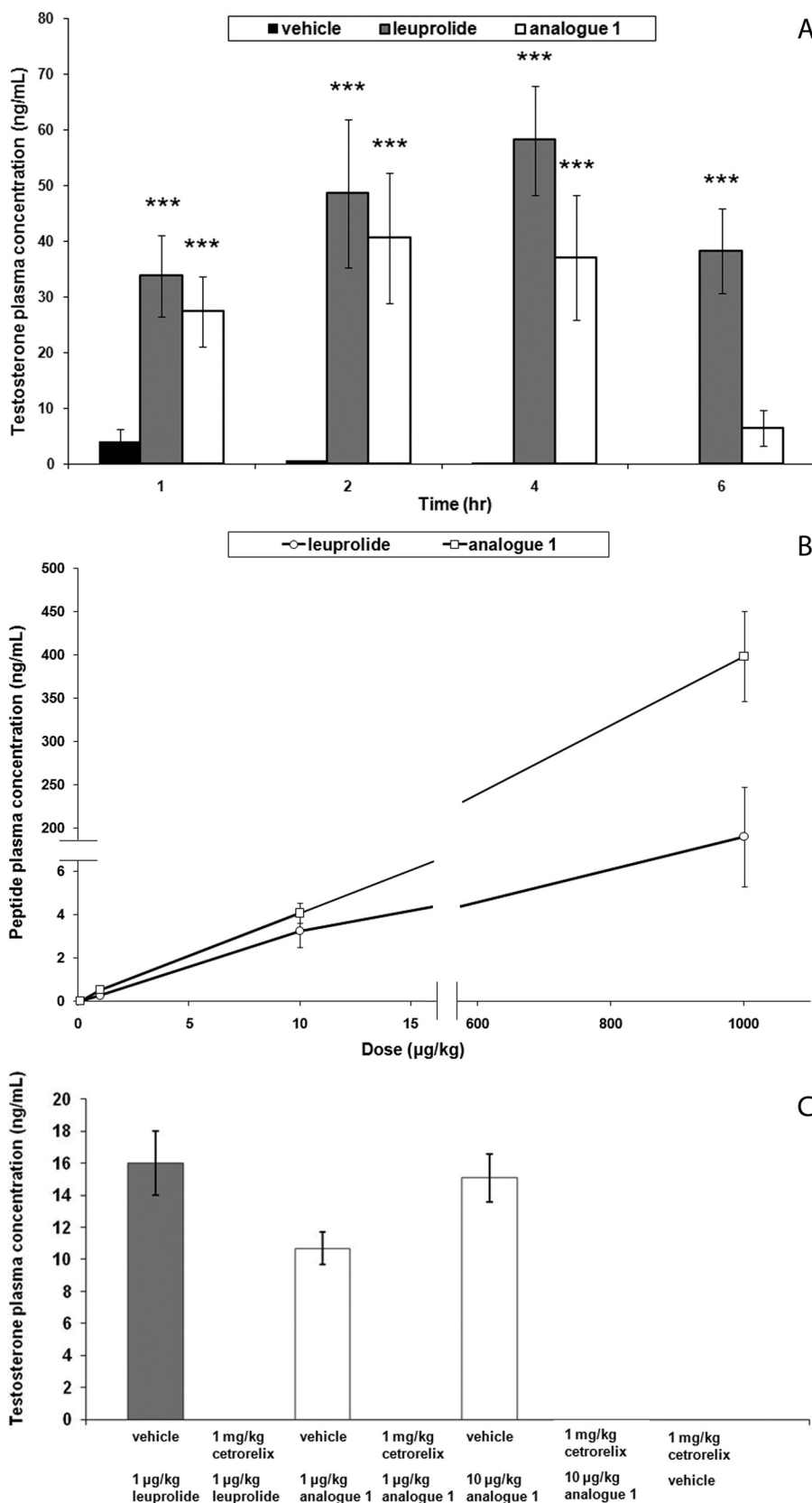
Fig. 3. A, testosterone release (functional agonism) upon analog 1 and leuprolide intraperitoneal dosing in mice ($n = 5$). The plasma testosterone concentration in vehicle-treated mice was low as expected and in agreement with previous studies. The testosterone concentration in leuprolide-treated mice was significantly higher at each time point tested (∗∗∗, $p < 0.001$ versus vehicle-treated mice). Testosterone release due to analog 1 dosing was suggestive of agonism (∗∗∗, $p < 0.001$ versus vehicle-treated mice at $t = 1$ h, $t = 2$ h, and $t = 4$ h), showing a pattern similar to that with leuprolide treatment. B, peptide plasma concentrations (1 h postdose) as a function of dose. In searching for appropriate doses for efficacy studies, a dose-response relationship was established for both analog 1 and leuprolide by dosing intraperitoneal experiments in mice at 0.1, 1, 10, and 1000 μg/kg ($n = 5$). Testosterone concentrations seemed to reach a plateau at doses higher than 10 μg/kg (data not shown). C, plasma testosterone concentration upon analog 1 and leuprolide dosing in mice ($n = 5$) in the presence and absence of cetrorelix to demonstrate the specificity of the effects of testosterone release. No response (no testosterone release) was observed in the presence of cetrorelix. Testosterone release is observed by the administration of peptides (either leuprolide or analog 1) in the absence of cetrorelix. Values are presented as the mean ± S.D.

ing to the GnRH receptor and agonism by analog 1), the dosing of analog 1 was evaluated in the presence and absence of cetrorelix (GnRH receptor antagonist). The administration of analog 1 in the absence of cetrorelix resulted in a robust plasma testosterone release. In contrast, no testosterone was released when analog 1 was administered after cetrorelix administration. A similar result was obtained upon leuprolide dosing in the presence and absence of cetrorelix (Fig. 3C).
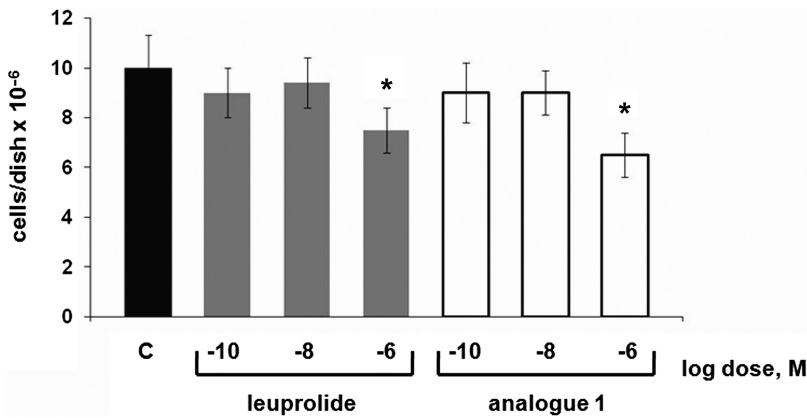
Fig. 4. Antiproliferative effect of analog 1 on LNCaP cells (hormone-dependent prostate cancer cell line). LNCaP cells were treated daily for 7 days with either leuprolide (positive control) or with analog 1 (10^{-10}–10^{-6} M). C, untreated controls. Results are expressed as the number of cells per dish and represent the results from three separate experiments. Values are presented as the mean ± S.D. ∗, $p <$ 0.05 versus C.

Antiproliferative Effect of Analog 1 on LNCaP Cells. The effect of analog 1 (10^{-10}–10^{-6} M) on the proliferation of the androgen-dependent LNCaP cells was investigated. Cells treated with the GnRH receptor agonist leuprolide served as the positive control. Analog 1 significantly inhibited the proliferation of LNCaP cells at 1 μM (∗, $p <$ 0.05 versus controls), showing a pharmacological activity similar to that of leuprolide (Fig. 4).

Efficacy Based on GnRH Desensitization and Testosterone Depletion. The effect of repeated dosing of analog 1 (500 μg/kg per day) was investigated in mice for 20 days and compared with effects caused by leuprolide. Testosterone concentrations in mouse plasma are summarized in Table 1. At day 0, peptide dosing resulted in significant testosterone release in mouse plasma compared with that in vehicle-treated mice (∗∗∗, $p <$ 0.001 for both analog 1- and leuprolide-treated mice). At day 9, plasma testosterone concentrations of peptide-treated mice were decreased compared with those of their counterparts at day 0 but were higher than those of vehicle-treated mice, suggesting that receptor desensitization had not been accomplished. At day 20, peptide dosing resulted in a decrease by almost 50% in plasma testosterone concentration (not statistically significant), compared with that in vehicle-treated mice. With respect to testis testosterone measurements, at day 0, peptide dosing resulted in significant testosterone release and high testis testosterone concentrations compared with those of vehicle-treated mice (∗∗∗, $p <$ 0.001 for both analog 1- and leuprolide-treated mice). After a 20-day repeated dosing regimen for both analog 1 and leuprolide, testis testosterone was significantly decreased (∗, $p <$ 0.05) in comparison with that in vehicle-treated mice (Fig. 5A). The testis weight of mice treated with 500 μg/kg analog 1 was decreased by 51% (∗∗∗, $p <$ 0.001 versus vehicle-treated mice), whereas no body weight losses were observed. Testis atrophy was also present in leuprolide-treated mice (Fig. 5B). Histopathological examination fur-

ther confirmed that analog 1 and leuprolide treatment resulted in remarkable atrophy of testis in mice. As shown in Fig. 6, the seminiferous epithelium became thinner and the number of the spermatogenic cells was decreased compared with that of the vehicle-treated mice.

Discussion

Based on the highly influential findings of Huggins (1963) and Schally et al. (1984), hormonal or chemical castration via the GnRH receptor became a well established strategy for the treatment of hormone-dependent cancers. Hormone-dependent cancers are not limited to the prostate and include those in other reproductive tissues such as the breast, ovaries, and endometrium. Numerous GnRH analogs have been synthesized in the context of the hormonal therapy for prostate cancer with the aim of achieving medical castration. Overall, systematic work has resulted in the synthesis of GnRH receptor agonists and antagonists, either peptides, linear or cyclic (Keramida et al., 2006), or small organic molecules (Anderes et al., 2003). More recently, degarelix, a novel GnRH receptor antagonist, was discovered. Degarelix, shown to be as effective as leuprolide in reducing testosterone in clinical trials without any evidence of systemic allergic reactions (Persson et al., 2009), was approved by the U.S. Food and Drug Administration and the European Medicines Agency for the treatment of advanced hormone-dependent prostate cancer (2009 and 2010, respectively). In addition, in the past few years diverse cytotoxic analogs targeting the GnRH receptor (cytotoxic radicals coupled to GnRH analogs that function as carriers) have been synthesized and evaluated with the intention of targeted chemotherapy (Mezo and Manea, 2010).

Although extensive research has been performed in the field of hormonal therapy, poor pharmacokinetic properties still characterize GnRH peptide analogs. Poor stability of GnRH analogs necessitates their subcutaneous administration (depot formulations) and leads to several side effects such as leukocytoblastic vasculitis (Turk et al., 2007) and injection site granulomas (Shiota et al., 2007). Most likely, GnRH analogs in clinical use achieve the desired pharmacological effects by action primarily on the pituitary and to a much lesser extent by direct antiproliferative effects on tumor cells. We hypothesize that stable analogs of such superagonists would be advantageous, either by allowing a reduction in dosing frequency or by allowing the use of analogs

TABLE 1

Testosterone concentrations in plasma after a 20-day repeated dosing of analog 1 or leuprolide

Data shown represent mean values ± S.D.

Days	Plasma Testosterone		
	Vehicle-Treated Mice	Leuprolide-Treated Mice	Analog 1-Treated Mice
	ng/ml		
0	0.16 ± 0.03	25.3 ± 4	20.9 ± 7
20	0.46 ± 0.37	0.20 ± 0.08	0.20 ± 0.17

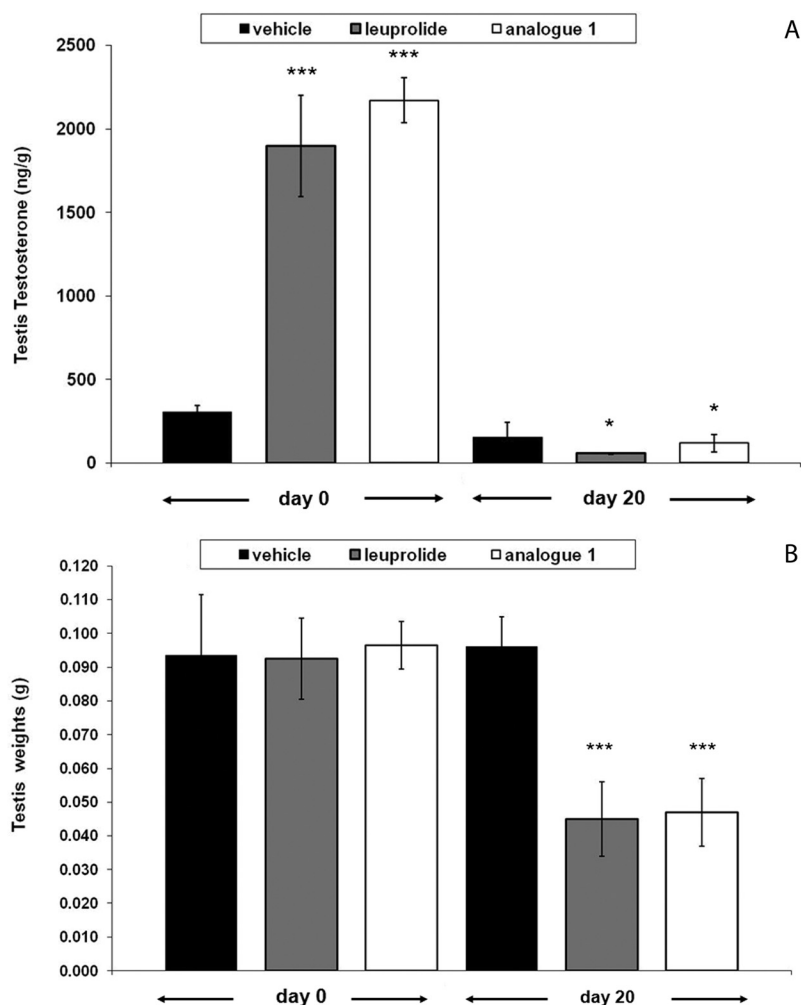**Fig. 5.** A, testis testosterone concentrations after analog 1 or leuprolide (500 μg/kg) dosing in mice for 20 days ($n = 6$). At day 0, high testis testosterone concentrations are obtained compared with those in vehicle-treated mice (***, $p < 0.001$ for both analog 1- and leuprolide-treated mice). After a 20-day repeated dosing regimen for both analog 1 and leuprolide, testis testosterone was significantly decreased below the basal testosterone concentrations (*, $p < 0.05$) as defined by testosterone measurements in vehicle-treated mice. B, testis atrophy as a result of peptide repeated dosing. The testis weight of mice treated with 500 μg/kg analog 1 was decreased by 51% (***, $p < 0.001$ versus vehicle-treated mice), whereas no body weight losses were observed. Testis atrophy was also present in leuprolide-treated mice. Values are presented as the mean ± S.D.

that act directly on the tumor, with possible additive effects and subsequent enhancements in efficacy.

Because prostate cancer is our research area of interest, a novel GnRH receptor peptide agonist, analog 1 was synthesized with the aim of enhancing in vivo stability based on the design of leuprolide, a potent drug for the treatment of prostate cancer and other endocrine disorders (e.g., precocious puberty). In this article, we have described experimental findings regarding evaluation of analog 1, using leuprolide as a positive control. The novel peptide analog exhibited significant in vitro and in vivo metabolic stability, a critical result suggesting that modifications at positions 5 (tyrosine methylation) and 9 (azetidine instead of proline) improve metabolic stability. In comparison with analog 1, leuprolide was metabolized at a faster rate by our in vitro system and after dosing in mice it was cleared rapidly from the plasma.

It should be noted that the enhanced metabolic stability observed with analog 1 compared with that with leuprolide is primarily due to methylation on the tyrosine residue, presumably due to steric hindrance at the site of cleavage (5–6 position) by endopeptidases. However, data from studies in our laboratory (not shown) suggest that the replacement of proline with azetidine at position 9 has an additive effect when it comes to the metabolic stability of analog 1. Regarding the reduced binding affinity of analog 1 compared with that with leuprolide, we believe that it is consistent with

analysis of ligand interactions with the GnRH receptor (Sealfon et al., 1997) according to which methylation of tyrosine on GnRH eliminates one of the two postulated hydrogen bonds with Arg[8], thus leading to reduced binding affinity (Laimou et al., 2010). Although no single residue is crucial for GnRH activity, the change of proline to azetidine in position 9 is not expected to significantly affect binding affinity. We established early on in our screening efforts an excellent correlation between in vitro data (incubation of peptide with mouse kidney membranes) and in vivo stability (administration of peptide in mice and plasma monitoring as a function of time). Because it has been shown that tissue-specific metabolic degradation of the native hormone (GnRH) by the metalloendopeptidase EP24.15 generates biologically active peptides (Cleverly and Wu, 2010), we consider understanding of the metabolism of new analogs an essential aspect of future compound optimization. It should be noted that although we have a good understanding of the metabolism of analog 1, the pharmacological significance of the metabolites presented in this article (M-III and M-V) has not been evaluated. However, based on the literature (M-III is also a metabolite of leuprolide), we hypothesize that those metabolites are not active.

A facile preclinical mouse model was developed in the context of our objectives. Intraperitoneal administration was selected, because oral administration was not practical due to the low
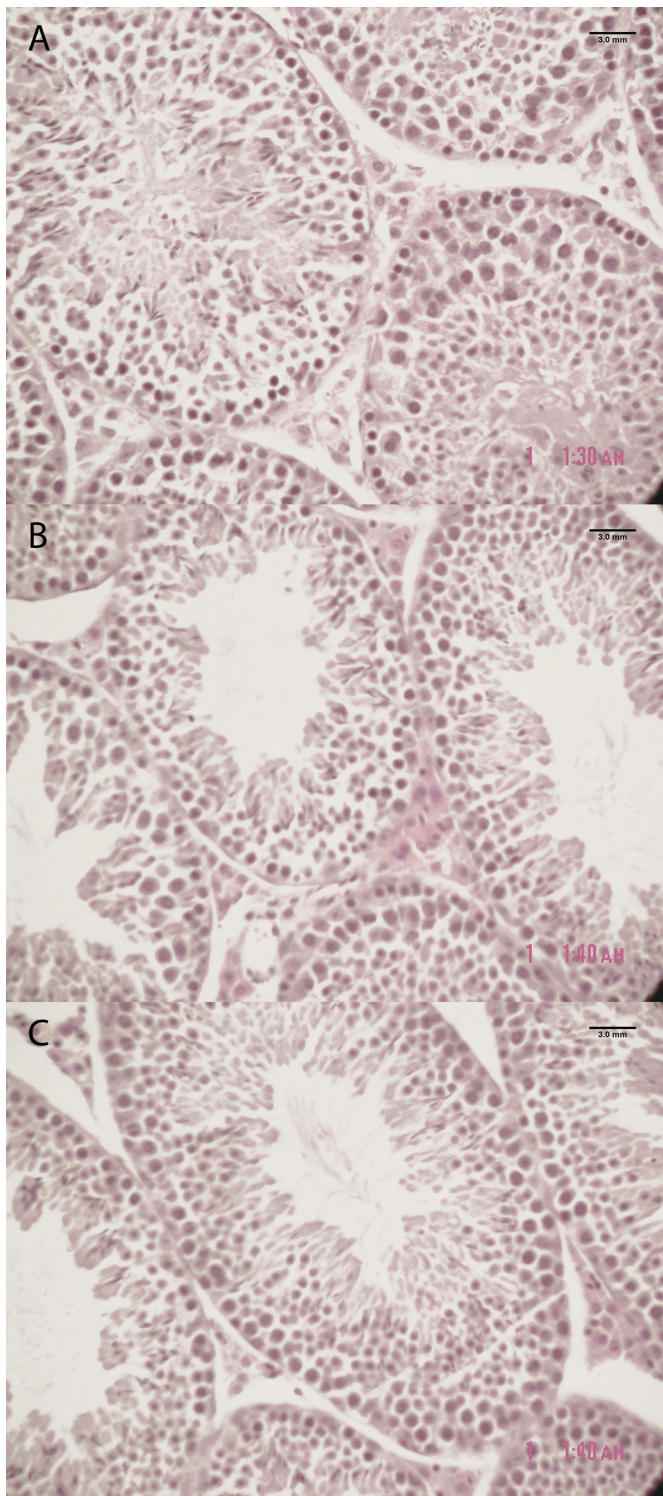
Fig. 6. A, testis from vehicle-treated mice demonstrating normal seminiferous epithelium (hematoxylin and eosin; original magnification, 400×). B, testis from mice treated with 500 μg/kg leuprolide. C, testis from mice treated with 500 μg/kg analog 1, demonstrating a significant decrease in spermatogenic cells (hematoxylin and eosin; original magnification, 400×).

bioavailability of the peptides that were tested. The LC-MS/MS-based quantification of the selected bioactive peptides and their corresponding metabolites as well as the selective monitoring of biomarkers (e.g., testosterone) in response to drug dose, in plasma and testes, combined with the appropriate preclinical

mouse model, represents a distinctive approach. The mouse model described in this article is particularly valuable, because 1) the human GnRH receptor is homologous to the mouse receptor (Millar et al., 2004), 2) information on in vitro and in vivo stability can be obtained with a relatively small amount of peptide (1–2 mg), 3) information on the GnRH receptor agonism (receptor specific in vivo modulation) can be obtained by using testosterone as a marker, 4) it allows the determination of the dosing regimen required for efficacy based on action on the pituitary, and 5) it can become the basis of follow-up experiments on genetically modified mouse animal models or other tumor xenografted mouse models (Sharpless and Depinho, 2006; Morgan et al., 2008).

The robust sensitive methodology that we developed for the quantification of testosterone in mouse plasma (0.05–100 ng/ml) or determination of testosterone in testis (2–2000 ng/g) gives us an excellent understanding of compound efficacy assessment. Because endogenous testosterone plasma concentrations of control animals are low (∼0.2 ng/ml), by using our methodology it is difficult to establish "castration" plasma values for treated animals, an anticipated effect after dosing. However, the high concentrations of testosterone in testis (average control values of 305 ng/g) allowed us to easily distinguish between control and treated values. After the 20-day treatment, average testis concentrations in leuprolide- or analog 1-treated animals were 58 and 118 ng/g, respectively. Moreover, the testis weights of the treated animals were significantly lower in comparison with those of the control group (atrophy induced by dosing), thus making the differences in testosterone testis concentration between control and peptide-treated animals even more pronounced. Although the binding affinity of analog 1 on the GnRH receptor was not as high as the binding affinity of leuprolide (∼15 versus <1 nM), the in vivo efficacy between the two analogs was similar (at the tested dose), suggesting that the enhanced stability or bioavailability of analog 1 compensates for binding affinity differences. Proliferation studies based on a hormone-dependent prostate cancer cell line (LNCaP cells) indicated that analog 1 was as potent as the superagonist leuprolide in terms of inhibiting cell proliferation. It is possible that analog 1 can play a significant role for the treatment of hormone-dependent cancers, by acting not only at the pituitary level (thus suppressing the pituitary-testicular axis) but also by exerting antitumor activity directly on cancer cells, as has been previously shown for other GnRH agonists (Maudsley et al., 2004; Montagnani Marelli et al., 2006). On the basis of the binding affinities of both analogs (leuprolide or analog 1), perhaps antiproliferative effects should have been expected at nanomolar concentrations. However, our studies with the LNCaP cell line showed that significant effects were only observed at the 10^{-6} M concentration. Although recent studies that used the superagonist D-Trp[6]-GnRH-I as a positive control show that with certain cell lines (e.g., transfected HEK293 cells), growth inhibition can be achieved at 5 nM agonist concentrations (Morgan et al., 2008); the same studies demonstrate elegantly that cell receptor levels and cell context are critical for cell responses. Thus, the potential of analog 1 for the treatment of prostate cancer will have to be further investigated by taking into consideration GnRH receptor levels and cell context and testing in animal models that address antiproliferative effects.

In conclusion, our belief is that the discovery of GnRH

analogs, such as analog 1 or analogs based on this new design with features that lead to enhanced in vivo stability and in vivo potency similar to that of leuprolide could result in valuable alternatives for the treatment of prostate cancer.

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