MOLECULAR AND PHARMACOLOGICAL PROPERTIES OF A POTENT AND SELECTIVE NOVEL NONSTEROIDAL PROGESTERONE RECEPTOR AGONIST TANAPROGET


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Progesterone receptor (PR) agonists have several important applications in women’s health, such as in oral contraception and postmenopausal hormone therapy. Currently, all PR agonists used clinically are steroids. Due to their interactions with other steroid receptors, steroid metabolizing enzymes, or other steroid signaling pathways, these drugs can pose significant side effects in some women. Efforts to discover novel nonsteroidal PR agonists with improved biological properties led to the discovery of Tanaproget (TNPR). TNPR binds to the PR from various species with a higher relative affinity than reference steroidal progestins. In T47D cells, TNPR induces alkaline phosphatase activity with an EC₅₀ value of 0.1 nM, comparable to potent steroidal progestins such as medroxyprogesterone acetate (MPA) and trimegestone (TMG), albeit with a reduced efficacy (~60%). In a mammalian two-hybrid assay to measure PR agonist-induced interaction between steroid receptor co-activator-1 (SRC-1) and PR, TNPR shows similar potency (EC₅₀ value of 0.02 nM) and efficacy to MPA and TMG. Importantly, in key animal models such as the rat ovulation inhibition assay, TNPR demonstrates full efficacy and an enhanced progestational potency (30-fold) when compared to MPA and TMG. Furthermore, TNPR has relatively weak interactions with other steroid receptors and binding proteins and little effect on CYP-450 metabolic pathways. Finally, the three dimensional crystal structure of the PR ligand binding domain with TNPR has been delineated to demonstrate how this nonsteroidal ligand achieves its high binding affinity. Therefore, TNPR is a structurally novel and very selective PR agonist with an improved preclinical pharmacological profile.

Progesterone (P₄) plays a pivotal role in female reproduction. It is involved in the regulation of uterine development and differentiation, implantation, ovulation, and mammary gland development (1-3). Progesterone exerts its physiological roles primarily through the progesterone receptor (PR), a member of the nuclear receptor superfamilly of transcription factors (4, 5). The PR consists of two isoforms, PR-A and PR-B, which are derived from a single gene through alternative usage of promoters and translation initiation sites (6, 7). Divergent as well as overlapping functions of the two PR isoforms have been identified both in vitro and in vivo (2, 8-14).

The molecular mechanism of PR actions has been extensively studied in the last decade. It is now generally accepted that binding of P₄ causes the PR to undergo conformational changes, phosphorylation, dimerization, and interaction with its target genes (15-19). The conformational change induced by P₄ binding also facilitates the interactions between the PR, coregulators and basal transcriptional factors, eventually leading to altered gene expression (18,19). It is hypothesized that PR conformational changes conferred by different PR ligands will lead to various biological...
responses. In addition to its genomic action, PR has been shown to elicit biological effects through other signaling pathways. It has been demonstrated that PR can interact with the Src/Ras MAP kinase pathway to modulate cellular activities in a ligand dependent manner (21-23). Furthermore, ligand independent PR actions involving other signaling cascades have been shown in vitro and in vivo (24-28). Recently, G-protein coupled membrane progesterone receptors have been identified (29, 30). Furthermore, P₄ and/or steroidal progesterin metabolites have been shown to interact with a variety of non-PR pathways such as the GABAₐ receptor (31-33). Therefore, PR ligands have potential to elicit a wide variety of biological and pharmacological responses.

PR agonists, i.e. progestins, have many therapeutic uses in women’s health, such as in oral contraception, hormone therapies, and for the treatment of certain reproductive disorders. In the past few decades, many progestins have been developed. Most of them and all of those in clinical usage are steroidal compounds. Even though these compounds are effective in achieving their intended efficacy endpoints, such as inhibition of ovulation in contraception and suppression of estrogen induced uterine epithelial proliferation in hormone therapy, they also carry unwanted side effects, either due to interactions with other closely related steroid receptors, steroid metabolizing enzymes, or other signaling pathways. As a first step to circumvent some of these drawbacks, we set out to identify novel nonsteroidal structures of PR ligands that would maintain the required biological activity while reducing side effects often associated with steroidal progestins. In this report, we describe the biological and molecular characterization of a novel PR agonist tanaproget (TNPR, Fig. 1) that demonstrates improved pharmacological properties compared to traditional steroidal progestins. We also have delineated the first crystallographic structure of PR ligand binding domain with a nonsteroidal ligand.

MATERIALS AND METHODS

Reagents-Progesterone (P₄), dexamethasone (DEX), flutamide, 17α-ethinyl estradiol (EE), testosterone propionate (TP), medroxy progesterone acetate (MPA), norethindrone (NET), hrombin, glutathione, AEBSF, protease inhibitor cocktail, CHAPS, dithiothreitol, rifampicin, and ampicillin were purchased from Sigma Chemical Co. (St. Louis, MO). Mifepristone (RU486) was purchased from the Shanghai Organic Chemical Institute (Shanghai, China). Trimegestone (TMG) was obtained from Aventis Pharmaceuticals (Bridgewater, NJ). Tissue culture media DMEM/F12, MEM, and DMEM were obtained from GibcoBRL (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). ³H-R5020 was purchased from NEN Life Science Products, Inc. (Boston, MA). Tanaproget (TNPR; WAY-166989), and levonorgestrel (LNG) were prepared by the Chemical and Screening Sciences Department at Wyeth. 3-ketodesogestrel (3-KDG) was obtained from JanaPharma, Germany. IPTG (Isopropyl-beta-D-thiogalactopyranoside) was from Fisher Scientific (Pittsburgh, PA). GSTrap FF (glutathione-Sepharose) and HiTrap SP FF (sulfopropyl-Sepharose) were from Amersham Bioscience (Piscataway, NJ). NuPage 4-12% Bis-Tris gels were from Invitrogen (Carlsbad, CA). PEG 3350 (50% w/v) and propanediol (40% v/v) were from Hampton Research. BL21 Escherichia coli cells were from Stratagene (La Jolla, CA). All other chemicals were purchased from Sigma.

Cell Culture and In Vitro Assays-Human breast carcinoma cell lines T47D and MCF-7, human lung carcinoma cell line A549, mouse skin fibroblast cell line L929, and monkey kidney cell line COS7, were obtained from American Type Culture Collection (Rockville, MD). T47D and MCF-7 cells were maintained in DMEM/F12, A549 cells in DEF/NPM, L929 and COS7 cells in DMEM, all with 10% FBS. Cells were passed 2-3 times every week.

T47D alkaline phosphatase assay-The effect of TNPR and reference steroids on alkaline phosphatase activity in T47D cells was determined as described (34, 35). Briefly, cells were plated in 96-well plates at 20,000 cells/well in DMEM/F12 with 10% FBS. After overnight culture, the medium was changed to phenol red-free DMEM/F12 containing 2% charcoal-stripped FBS (experimental medium). The next day, cells were treated with test compound in the absence (agonist mode) or presence (antagonist mode) of 1 nM progestosterone in the experimental medium. Twenty-four hours after treatment, cellular alkaline phosphatase activity was measured using p-nitrophenyl phosphate as a substrate. Optical
density measurements were taken at 5-minute intervals for 30 min at a test wavelength of 405 nm.

**PR binding assay**—Uterine tissues from the rat, monkey, and rabbit were homogenized in 10 mM Tris buffer (pH 7.4) with 20 mM sodium molybdate and 1 mM dithiothreitol while T47D cells were homogenized in 20 mM HEPES buffer (pH 7.6) with protease inhibitors (aprotinin 0.5 µg/ml, leupeptin 0.5 µg/ml, pepstatin A 0.7 µg/ml, and PMSF 0.5 mM). After centrifugation at 100,000 g for 1 h (4°C), the supernatant containing PR was collected and measured for protein concentration. The PR competition-binding assay was done with 100 µg cytosolic protein, 3 nM [3H]-R5020 and increasing concentrations of test compounds. Following overnight incubation at 4°C, free and bound [3H]-R5020 were separated by using 1% charcoal/0.05% dextran 69K in Tris/EDTA buffer (pH 7.4). Total and bound [3H]-R5020 were counted in a Beckman LS6500 Scintillation Counter (Beckman Instruments, Inc., Fullerton, CA).

**Protease digestion assay**—The protease digestion analysis was performed essentially as described in (36, 37) with minor modifications. The plasmid pT7/BPRB, kindly provided by Dr. B. W. O’Malley (Baylor College of Medicine, Houston, TX), was used to generate [35S]-radiolabeled PR-B using a TNT T7 Quick Coupled Transcription/Translation System according to the manufacturer’s protocol (Promega, Madison, WI). After the translation reaction, an aliquot (30 µl) of the lysate was incubated for 10 min in the absence or presence of ligands at a final concentration of 100 nM. Aliquots (5 µl) of the ligand treated receptor mixture were then incubated with a trypsin solution (Worthington Biochemicals, Freehold, NJ), giving various final concentrations of the enzyme (0, 25, 50, 75 µg/ml). After incubation at room temperature for 10 min, the digestion reaction was terminated with the addition of 20 µl gel denaturing buffer and boiling for 5 min. The digestion products were separated on a 4-12% Bis-Tris NuPAGE gel (BioRad, Hercules, CA). After electrophoresis the gel was treated with a 50% (vol/vol) methanol-10% acetic acid (vol/vol) solution for 30 min and immersed in Amplify (Amersham) for 30 min. The gel was then dried under vacuum and the radiolabeled products were visualized by autoradiography.

**PR / SRC-1 Mammalian two hybrid assay-COS7**—Cells were transfected with PR LBD in the GAL4 DNA binding domain plasmid pM (Clontech, Palo Alto, CA), full-length SRC-1 or SRC-3 in the VP16 activation domain plasmid pVP16 (Clontech) and a GAL4 responsive luciferase reporter (5xGALuas) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were treated with test compounds for 24 h and luciferase activity was measured on a Victor2 luminometer (Perkin Elmer Life Sciences) using the Luciferase reporter assay kit (Promega).

**Adenovirus HRE-Luciferase Reporter Assays**—Luciferase reporter assays using cell lines expressing endogenous steroid receptors (MCF-7 for ER, A549 cells for GR, and L929 cells for AR) were used as described previously (34) to examine potential cross interactions between TNPR and these steroid receptors.

**SHBG Competition-Binding Assay—SHBG**—Competition-binding assay was run based upon published methods (38, 39) with modifications. Briefly, human blood was collected in SST VACUTAINER tubes (Becton-Dickinson, Bedford, MA) and kept at room temperature for 30 min before centrifugation at 1000 g for 10 min (4°C). The serum was aliquoted and stored at -76°C. Human serum was diluted 20-60 times with TG buffer (10 mM Tris and 10% glycerol, pH 7.4, at room temperature) depending on the source of the serum, for a total [3H]-DHT binding of about 3,000 cpm. Seven hundred µl of dextran-coated charcoal (DCC, 1% charcoal, 0.05% dextran 69K in Tris/EDTA buffer, pH 7.4) was added to every 1000 µl of diluted serum and incubated on ice for 1 h, followed by centrifugation at 2000 g for 20 min. The SHBG competition-binding assay was performed with 100 µl of the supernatant, 8 nM [3H]-DHT and increasing concentrations of test compounds. Following overnight incubation at 4°C, free and bound [3H]-DHT were separated by using 0.5% DCC. Bound [3H]-DHT was counted in a Beckman LS6500 Scintillation Counter (Beckman Instruments, Inc., Fullerton, CA).

**Cytochrome P450 3A4 Induction Assay in DPX2 Cells**—The induction potential of CYP3A4 enzyme by TNPR was evaluated in a DPX2 cell line by Puracyp, Inc. (Carlsbad, CA). DPX2 cell line is a stably transformed tumor cell line. The enhancer of CYP3A4 (PXRE) and human PXR are stably integrated into the tumor cells. The incubations...
were performed in 96-well microtiter plates in a high-throughput manner. TNPR and 3-ketodesogestrel (3-KDG) were incubated at 1, 5, 10, and 25 µM concentrations in DPX2 cell line. Rifampicin (RIF), a known inducer of CYP3A4 was used as positive control. Induction of CYP3A4 was assessed by monitoring reporter gene activity and by comparing results to analogous cells treated with solvent. **Rat Ovulation Inhibition Model-**All animal studies were conducted under approved Wyeth Research Animal Care and Use Committee protocols. Animals were housed under a 12-hour light/dark cycle and fed a casein-based Purina Laboratory Rodent Diet 5K96 (Richmond, IN) and water *ad libitum* unless otherwise indicated.

Ovulation inhibition experiments were run as described (40). Briefly, random cycling mature female Sprague-Dawley rats (~200 g) were obtained from Charles River Laboratory (Boston, MA). Rats were synchronized for estrus with 2 µg of LHRH (in phosphate buffered saline containing 0.1% bovine serum albumin) administered subcutaneously (sc) per rat at 0900 h and again at 1600 h. Animals were allowed to rest for 8 days before the administration of test compounds. Animals were then grouped, with 7 to 9 rats per treatment group. The morning of the ninth day following LHRH treatment, the rats were treated with test compounds once daily, by gavage, for 4 consecutive days. The animals were euthanized the morning following the last treatment. Oviducts were removed, placed between 2 glass slides, and viewed through a dissecting microscope to count ova. The number of animals presenting ova in the oviduct from each treatment group and the number of ova in the oviduct of each animal were recorded.

**TNPR/PR-LBD Crystal Structure Analysis**

*Gene Construct for Expression of PR Ligand Binding Domain (PR-LBD)*-PR-LBD was expressed in *Escherichia coli* from a plasmid carrying the LBD coding sequence (residues 675-933, GS...KK) as a fusion protein with glutathione-S-transferase (GST). A thrombin cleavage site separates the GST and LBD regions. The construct is essentially the same as that described by Williams and Sigler (41).

**Cell Growth and Induction of Expression for PR-LBD**-Growth and expression were performed in a B. Braun Biotech Biostat C 10 liter fermenter. A 100 ml preculture was used to inoculate 10 liters of fermenter salts, glucose, ampicillin, trace metals and yeast extract media. Cells were grown overnight at 25°C. Fifteen minutes prior to induction, the vessel temperature was dropped to 15°C and 5 ml of 66 mM P₄ was added. Cells were induced with 2.4 g of IPTG (1.0 mM final concentration) at an A₆₀₀ value of 5.4. Five ml of 66 mM P₄ was added at induction and every fifteen minutes after induction throughout the entire expression (4 h total, 660 µM final concentration of P₄). After 4 h of induction, a final A₆₀₀ value of 8.0 had been obtained. The expression yielded approximately 160 g of wet cell weight and the protein of interest was approximated at 5-7% of total cell protein by SDS-PAGE.

**Purification of PR-LBD (Complexed with TNPR)**-Cell lysis and isolation of soluble protein: Twenty g of frozen cells were suspended in 300 ml of 50 mM HEPES (pH 7.3), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 5 mM dithiothreitol (DTT) with 0.33 mM aminoethyl-benzensulfonyl fluoride (a protease inhibitor), 0.3 ml of “protease inhibitor cocktail” (Sigma 8849) and 5 µM P₄. P₄ or TNPR were added to solutions from stocks of 50 mM in dimethyl sulfoxide. Cells were broken by passage through a microfluidizer (Midrofluidics, Newton, MA). Cell debris and aggregated GST/PR-LBD were removed by centrifugation for 2.5 h (SS34 rotor – Sorvall RC5B (or 5°C) – 18,500 rpm [approx. 40,000 RCF]) CHAPS was added to 1.5% and the solution was filtered (cellulose nitrate – 0.45 micron) and stored overnight.

**Isolation of GST/PR-LBD**-The solution was passed over two 5 ml columns (in tandem) of GSTrap FF at about 1 ml/min. Resin was washed with 50 ml of 50 mM HEPES (pH 7.3), 150 mM NaCl, 5 mM EDTA, 10% glycerol, then with 50 ml of same solution containing 50 µM TNPR. GST/PR-LBD was eluted with 12 mM reduced glutathione in 50 mM HEPES (pH 7.3), 100 mM NaCl, 10% glycerol, 0.1% octyl-β-glucoside, 50 µM TNPR. Fractions of 5 ml were collected and pooled after location of the GST/PR-LBD by SDS-PAGE (generally a pool of 30-40 ml). Thrombin was added to 25,000 HNMIH units/ml; the solution was filtered (cellulose nitrate – 0.45 micron) and stored overnight.

**Isolation of PR-LBD**-The solution was diluted with 4 volumes of 10 mM HEPES (pH 7.3), 10% glycerol, 5 mM DTT, 0.1% octyl-β-glucoside, 50 µM TNPR. The solution was passed over 1 ml
column of SP FF at 1 ml/min. The column was washed with 5 ml of 10 mM HEPES (pH 7.3), 20 mM NaCl, 10% glycerol, 0.1% octyl-ß-glucoside, 1 μM TNPR. PR-LBD was eluted from the column with a 15-m1 gradient of sodium chloride, running from 20 to 220 mM (other components: directly above). Fractions of 1 ml were collected, PR-LBD was located by SDS-PAGE and those fractions containing PR/LBD at 1-2 mg/ml were used directly for crystallization.

**Crystallization:** Crystals were grown by hanging drop vapor diffusion at 18°C in drops containing 2.0 μl protein stock solution (5 mg/ml protein, 10 mM HEPES pH 7.3, 10% glycerol, 5 mM DTT, ~100 mM NaCl, 0.1% octyl-ß-glucoside, 1 μM TNPR) mixed with 1.0 μl well solution (8% PEG 3350, 300 mM MgSO4, 50 mM PIPES pH 6.5, 10% glycerol) and 0.5 μl 1,3-propanediol (40% v/v) and equilibrated against 1 ml well solution. Diamond shaped crystals grew in 2-6 weeks, measuring ~50 μm across.

**Data Collection and Processing:** Crystals belong to the space group P21 with unit cell parameters a = 57.52 Å, b = 64.50 Å, c = 70.41 Å, and β = 95.76°, and contain two molecules of PR LBD in the asymmetric unit, resulting in a solvent content of 44%. Crystals were drawn through a solution of 20% ethylene glycol and 80% well solution, and cooled rapidly in liquid nitrogen. Diffraction data were collected using a Rigaku rotating anode X-ray generator and recorded on a RaxisIV detector. Intensities were integrated and scaled using the programs Denzo and Scalepack (42).

**Phasing, Model building and Refinement:** The structure was determined by molecular replacement using the PR-LBD/P structure (41) as the search model. After several iterative cycles of refinement using CNX (43) and Refmac5 (44) and model improvement building using QUANTA (Molecular Simulations, Inc.) and Coot (45), TNPR was placed and refined. Final R_{work} and R_{free} values of 17.88% and 22.81% were obtained (Table 1). Figures were prepared in PyMol (Fig.8, 9) and Coot (Fig.10). (44, 45).

**Statistical Evaluation of Results:** For all in vitro studies, each experiment was run in triplicate. Experiments were repeated at least three times. Values are presented as means +/- SEM for different replicates. In T47D alkaline phosphatase assay, a dose response curve was generated for dose (X-axis) vs. the rate of enzyme reaction (slope) (Y-axis) for test compounds. Square root-transformed data were used for analysis of variance and nonlinear dose response curve fitting for PR and SHBG competition binding assays and T47D cell alkaline phosphatase assay, whereas log transformation was used for luciferase reporter assays. Huber weighting was used to down-weight the effects of outliers. EC_{50} or IC_{50} values were calculated from the re-transformed values. JMP software (SAS Institute, Inc.) was used for both one-way analysis of variance and non-linear dose response analysis.

**RESULTS**

**PR Activity of TNPR in T47D Cells:** The in vitro PR activity of TNPR was evaluated in the T47D alkaline phosphatase assay. The compound was tested along with reference steroidal progestins MPA and TMG. As shown in Fig. 2, TNPR showed potent PR agonist activity with an EC_{50} value of 0.15 +/- 0.01 nM (n=3), similar to MPA (EC_{50} =0.12 +/- 0.01 nM, n=3) and TMG (0.09 +/- 0.02 nM, n=3). TNPR had a reduced efficacy (~60%) as compared to the steroid compounds in this assay. However, as shown later, TNPR is fully efficacious in the rat ovulation inhibition model.

**TNPR Promotes PR and SRC-1 Interaction in Mammalian Cells:** In a mammalian two hybrid assay to determine the interaction between SRC-1 and PR induced by PR agonists, TNPR showed similar potency and efficacy to MPA, TMG, LNG and was approximately 50-fold more potent than P_{4} (Fig. 3). Similar results were obtained when SRC-3 was used in the two-hybrid assay (data not shown).

**TNPR Binds to PR with High Affinity:** In the competition-binding assay, TNPR displaced R5020 (a synthetic steroidal progestin) binding to the human PR with high relative affinity. The IC_{50} values (50% inhibition of 3 nM [3H]-R5020 binding to the human PR) for TNPR were 1.7 compared to 11.2 and 7.8 nM for MPA and TMG, respectively (Fig. 4). TNPR also showed a 630 fold higher affinity to the monkey, rat, and rabbit PR than MPA and TMG (Table 2).

**Other Steroid Receptor Activity of TNPR in vitro:** TNPR was tested for potential effects via the estrogen receptor (ER), androgen receptor (AR), and glucocorticoid receptor (GR) using hormone response element reporter assays in MCF-7, L929, and A549 cells, respectively (34). TNPR did not exhibit any agonist activity for these receptors.
when tested up to 10 \( \mu \text{M} \) and showed relatively weak GR antagonist activity with an \( IC_{50} \) value of 40 nM. The reference GR antagonist RU-486 had an \( IC_{50} \) value of about 1 nM in this assay. TNPR also showed modest ER antagonist activity with an \( IC_{50} \) value of greater than 1000 nM. Thus the in vitro selectivity of TNPR was more than 250 fold for the PR over the other steroid receptors tested. Furthermore, TNPR did not show any GR antagonist activity at 10 mg/kg in the rat. (data not shown).

**TNPR Does Not Compete for SHBG Binding**-

SHBG competition-binding assay was used to determine if TNPR competes with \(^3\text{H}-\text{DHT} \) for SHBG binding. While unlabeled DHT, LNG, and NET competitively inhibited \(^3\text{H}-\text{DHT} \) binding with \( IC_{50} \) values of 1.3, 15.2, and 72.0 nM respectively, TNPR did not significantly compete for the binding at a concentration up to 10,000 nM (Fig. 5).

**TNPR Does Not Induce Cytochrome P450 3A4 in DPX2 Cells**-

In the DPX2 cells TNPR did not increase luciferase activity at concentrations up to 25 \( \mu \text{M} \), while the synthetic progesterin 3-ketodesogestrel (3-KDG) showed a dose response induction on CYP3A4 (Fig. 6). The fold induction over the control by 3-KDG was 1.7, 3.7, 8.3, and 21.3 at 1, 5, 10, and 25 \( \mu \text{M} \), respectively. Under the same conditions, the positive control RIF showed 10.0, 21.6, 29.5, and 25.0-fold CYP3A4 induction at 1, 5, 10, and 25 \( \mu \text{M} \), respectively. These data indicate that 3-KDG induces CYP3A4, while TNPR does not. Therefore, it is less likely that TNPR will interact with drugs that are metabolized by CYP3A4.

**Partial Proteolytic Analysis of TNPR Bound PR**-

Partial protease digestion as described by Allan et al. (36) was carried out to determine the gross conformational changes of PR upon TNPR binding. As shown in Fig. 7, TNPR bound PR provided a trypsin digestion pattern that was similar to PR bound with \( P_4 \) or TMG but distinct from that of RU-486 bound PR. Partial digestion with chymotrypsin also demonstrated similar peptide patterns among TNPR-, \( P_4 \)- and TMG-bound PR that were different from that of RU-486-bound PR (data not shown).

**TNPR/PR LBD Crystal Structure Analysis**-

In order to better understand the molecular interaction of TNPR with the PR receptor, we determined the crystal structure of TNPR bound ligand-binding domain of human progesterone receptor. The structure was solved by the Molecular Replacement method, using \( P_4/\text{PR} \) complex structure (41) as a search model. TNPR binds within the same steroidal binding pocket defined by the \( P_4/\text{PR} \). The crystallographic asymmetric unit of TNPR/PR contains two ligand bound monomers that form a dimer. This dimer interface is composed of residues from a-helices 11 and 12 and is similar to the dimer reported for \( P_4/\text{PR} \) (Fig. 8, 41). A superposition of the \( P_4/\text{PR} \) dimer with TNPR/PR gave an average root mean square deviation (RMSD) of 0.25 \( \AA \) for all equivalent C alpha atoms. The two monomers, A and B, which form the dimer in the asymmetric unit of TNPR/PR have an average RMSD of 0.87 \( \AA \) for all C alpha atoms. These differences, also seen in the \( P_4/\text{PR} \) structure, are located at a crystal packing interface on the loop between a-helix 6 and a-helix 7.

Thus, TNPR binding does not perturb the tertiary structure of PR relative to \( P_4 \) as overall differences between monomers in the TNPR/PR structure are statistically more significant than differences between the \( P_4/\text{PR} \) and TNPR/PR complexes.

This is consistent with the limited protease digestion analysis indicating that TNPR triggered similar PR conformational changes to those of \( P_4 \) and TMG bound PR. Ligand induced adjustment in the PR binding pocket has also been observed in the recently solved x-ray structures of norethindrone and mometasone furoate (47) where local shifts in both protein main chain and side chain conformations allow these ligands to be accommodated.

Inspection of the TNPR ligand binding pocket does reveal differences between the steroidal and the non-steroidal ligand binding pockets. Superposition of the \( P_4 \) structure shows that the 1-methyl-1H-pyrrole-2-carbonitrile ring of TNPR lies roughly between the A and B rings of \( P_4 \), and the 1,4-dihydro-3,1-benzoxazine-2-thione moiety (which is referred to as “benzoxazine”) lies just above the C and D rings of \( P_4 \) in the direction of \( P_4 \)’s protruding methyl groups (Fig. 9). TNPR presents its nitrile group as a hydrogen-bond acceptor to the amido group of Gln\textsuperscript{725} (3.0 \( \AA \) distance) and Arg\textsuperscript{766} (2.8 \( \AA \) distance) thereby preserving hydrogen bonding network previously seen between the 3-keto group of the steroids and these residues. The benzoxazine moiety occupies approximately the same space as rings C and D of \( P_4 \) with similar hydrophobic interactions. Small
adjustments in the position of the side chains of several residues lining the pocket, primarily Met\textsuperscript{756} (helix 5), Met\textsuperscript{759} (helix 5), and Met\textsuperscript{799} (helix 12) accommodate the difference in shape between P\textsubscript{4} and TNPR. The presence of a hydrogen bond donor at the distal end of the ligand allows the benzoxazine NH to form a hydrogen-bonds with the side chain oxygen of Asn\textsuperscript{719} (2.8 Å distance). The orientation of Asn\textsuperscript{719} side chain is fixed through hydrogen-bonding with the main chain oxygen atom of Glu\textsuperscript{904}, which contributes to securing the loop between helices 11 and 12 to the rest of the protein. Although previously a water mediated interaction has been reported between the C17 hydroxyl group of norethindrone and Asn\textsuperscript{719} residue (47), this novel direct interaction between the ligand and the receptor clearly offers additional affinity of TNPR to the PR.

TNPR presents its nitrile group and the benzoxazine NH for hydrogen-bonding with the protein and the scaffold is thus optimally positioned for hydrophobic contacts with the pocket. The hydrogen-bonding network and hydrophobic interactions are similar to the interaction of P\textsubscript{4} with the ligand binding pocket, but notably achieved with a completely independent small molecule scaffold (Fig. 9 and 10).

TNPR is accommodated by the PR ligand binding pocket better than the ligand binding pockets of the other closely related receptors such as GR and AR (48-50). In the PR, residues Phe\textsuperscript{794} (helix 7) is substituted by methionines in the GR and AR while Leu\textsuperscript{797} (helix 7) takes the place of the polar Gln of the GR and AR. Together these two residues contribute to the creation of a hydrophobic environment that can better accommodate the two methyl substituents of the benzoxazine ring. Both the GR and AR have several other conservative differences with respect to the PR among the hydrophobic residues which line the ligand binding pocket, possibly contributing to the selectivity of TNPR for PR.

**TNPR Is a Potent Ovulation Inhibitor in the Rat**—In the rat, TNPR inhibited ovulation in 100\% of animals at 0.03 mg/kg (ED\textsubscript{100}) when given orally. To achieve the same effect, the dose for MPA and TMG was 1 mg/kg (Fig. 11). Thus TNPR is about 30 fold more potent than these steroid progestins in a key efficacy endpoint for contraception.

**DISCUSSION**

While several nonsteroidal tissue selective estrogen receptor (ER) modulators with an improved pharmacological profile and enhanced clinical utility have been developed over the past few decades, to our knowledge, no nonsteroidal PR ligands have been in clinical development so far. In this report, we describe the identification and molecular and pharmacological characterization of a novel, nonsteroidal PR agonist TNPR. TNPR was initially identified as a PR agonist through a medicinal chemistry effort based upon its in vitro activity in T47D cells. It showed similar potency to potent steroid compounds such as TMG and MPA in the alkaline phosphatase assay in T47D cells. However, TNPR had reduced efficacy in this in vitro model (Fig.2 and 34). The biological relevance of this reduced efficacy in the breast cancer line is unclear and is currently under investigation using in vivo models of mammary gland development. In an artificial system using a mammalian two-hybrid system to evaluate the interaction between PR and coactivators such as SRC-1 and SRC-3, TNPR showed similar potency and efficacy as the two steroidal progestins but was much more potent than P\textsubscript{4} or NET (Fig.3 and data not shown).

TNPR showed higher relative binding affinity as compared to the tested reference steroid progestins in various species. It was about 4-6 fold more potent than TMG and MPA on human PR and up to 100 fold more potent than MPA on rat PR in the competition-binding assays. The increased binding affinity may be explained by the additional hydrogen bond between TNPR and PR as observed in the crystal structure discussed below. Importantly, TNPR demonstrated potent in vivo progestational activity. In the ovulation inhibition model in the rat, it was fully efficacious at 0.03 mg/kg, p.o., approximately 30-fold more potent that MPA and TMG (40). This enhanced progestational activity has been shown in other animal models (Z. Zhang et al., manuscript in preparation).

TNPR demonstrated over 250-fold selectivity for the PR versus other closely related steroid receptors. It only weakly interacted with GR in vitro and this activity was not detected in vivo at 10 mg/kg, a dose that is 300-fold higher than the efficacious dose (0.03 mg/kg in the rat ovulation inhibition model, Fig. 11 and data not shown). Some of the side effects (e.g. metabolic
changes) of the clinically used steroid progestins may be attributed to their interactions with other steroid receptors. For example, progestins with androgenic activity, such as LNG and MPA, have been shown to alter plasma lipid profile (51, 52). The improved selectivity profile of TNPR will likely reduce these side effects.

SHBG is a serum sex steroid binding protein produced by the liver that regulates the availability of androgens and estrogens to target tissues. It is well known that some synthetic steroids bind to and/or regulate the production of SHBG, thus affecting endogenous sex steroid bioavailability (53-55). For example, LNG competes for SHBG binding with an IC \(_{50}\) value of 16 nM (Fig. 5). It also dramatically reduces serum SHBG levels when given to primates (data not shown). Unlike some steroidal progestins, TNPR did not compete for SHBG binding at concentrations up to 10,000 nM and at a dose that is 100-fold its efficacious dose did not change serum SHBG levels in primates (Z. Zhang et al. manuscript in preparation). Therefore, TNPR offers advantages over steroidal progestins in that it will not affect blood SHBG levels and sex hormone availability to the target tissues.

Failures of contraceptives containing some steroidal progestins, although small, are known to result from interactions with other drugs. Likewise, some oral contraceptives/progestins are also known to affect the efficacy of other drugs (56-60). These drug-drug interactions are caused by modulation of drug metabolizing enzymes (61, 62). Steroidal progestins such as desogestrel are metabolized primarily by CYP3A4. CYP3A4 is known to be induced by several drugs that are sometimes co-administered with oral contraceptives. Elevated CYP3A4 concentrations can lead to lower efficacy of contraceptives. Results from recent metabolism studies clearly demonstrate that TNPR is superior to currently marketed steroidal progestins. For example, glucuronidation is the major metabolic pathway for TNPR and TNPR is not metabolized by CYP3A4 (data not shown). TNPR does not have the potential to induce CYP3A4, while 3-ketodesogestrel, a metabolite of desogestrel has the capacity to induce this isozyme. TNPR does not inhibit any of the major P450 enzymes (data not shown), while steroidal progestins have the potential to inhibit CYP 2C19, an isozyme responsible for the metabolism of proton pump inhibitors such as omeprazole (63). These characteristics of TNPR clearly demonstrate that unlike other steroidal progestins the efficacy of TNPR will likely not be affected by any concomitant drugs and TNPR should not interfere with the efficacy of other drugs.

The crystal structure of the PR/TNPR complex is the first reported PR LBD structure with a non-steroidal ligand. This structure shows that TNPR retains many of the key interactions of \( \text{P}_4 \) with the protein albeit from an unrelated chemical scaffold. As it is a fundamentally different compound, the common features of ligand binding between TNPR and \( \text{P}_4 \) delineate general features required for a potent PR agonist. Furthermore, TNPR interacts with the protein in novel ways. TNPR takes advantage of the ligand binding pocket even more effectively than \( \text{P}_4 \), with a novel hydrogen-bond as the basis for its greater affinity for PR (41). Comparisons with the GR and AR help understand the subtle differences that make the PR ligand binding pocket unique from those of other closely related steroid receptors and that TNPR takes advantage of to achieve greater than 250-fold selectivity for the PR (48-50).

In summary, TNPR is a structurally novel nonsteroidal PR agonist. It demonstrated potent PR agonist activity in vitro and in vivo. TNPR has an excellent receptor selectivity profile and has improved pharmacological properties. Due to its novel chemical structure, TNPR may offer other unique biological properties. In fact recent evidence shows that TNPR possesses some tissue selective properties (Z. Zhang et al. manuscript in preparation). The PR isoform selective effect of TNPR is currently under investigation. Finally, the crystal structure of PR LBD-TNPR has been resolved which support the potent activity of this nonsteroidal compound.

REFERENCES

**FOOTNOTE**

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The abbreviations used are: P₄, progesterone; PR, progesterone receptor; PR-LBD, PR ligand binding domain; TNPR, tanaproget; MPA, medroxyprogesterone acetate; TMG, trimegestone; 3-KDG, 3-ketodesogestrel; LNG, levonorgestrel; DEX, dexamethasone; TP, testosterone propionate; ER, estrogen receptor; AR, androgen receptor; GR, glucocorticoid receptor; HRE, hormone response element; SRC-1, steroid receptor coactivator-1; SHBG, sex hormone binding globulin; RIF, rifampicin; DMSO, dimethyl sulfoxide.
FIGURE LEGENDS

Fig. 1. Chemical structure of tanaproget (TNPR)

Fig. 2. Representative dose response curves in T47D alkaline phosphatase assay. T47 cells were plated at 20,000 cells/well in 96 well plates in DMEM/F12 with 10% fetal bovine serum (FBS). The medium was changed to DMEM/F12 (phenol red free) with 2% charcoal stripped FBS the next day and cells were treated with compounds over night. Cellular alkaline phosphatase activity was measured as described in Materials and Methods.

Fig. 3. Effects of TNPR, MPA, TMG, and P4 on PR-LBD/SRC-1 interaction in a mammalian two-hybrid assay. COS7 cells were transfected with PR LBD in the GAL4 DNA binding domain plasmid pM, full-length SRC-1 in the VP16 activation domain plasmid pVP16 and a GAL4 responsive luciferase reporter (5xGALuas) using Lipofectamine 2000. Cells were treated with test compounds for 24 hours and luciferase activity was measured as described in Materials and Methods.

Fig. 4. Representative PR competition binding curves for TNPR, MPA, and TMG using PR isolated from human T47D cells. The PR competition-binding assay was done with 100 µg T47D cell cytosolic protein, 3 nM 3H-R5020 and increasing concentrations of test compounds. Following overnight incubation at 4°C, free and bound 3H-R5020 were separated and measured as described in Materials and Methods.

Fig. 5. Representative SHBG competition binding curves for TNPR, LNG, and NET. The SHBG competition-binding assay was performed with 100 µl of diluted human serum, 8 nM 3H-DHT and increasing concentrations of test compounds. Following overnight incubation at 4°C, free and bound 3H-DHT were separated by using 0.5% DCC. Bound 3H-DHT was counted as described in Materials and Methods.

Fig. 6. TNPR does not induce cytochrome P450 3A4 in DPX2 cells. DPX2 cells stably transformed with the enhancer of CYP3A4 (PXRE) and human PXR were treated with TNPR and 3-ketodesogestrel (3-KDG) at 1, 5, 10, and 25 µM. Rifampicin (RIF), a known inducer of CYP3A4 was used as positive control. Induction of CYP3A4 was assessed by monitoring reporter gene activity and by comparing results to analogous cells treated with vehicle control.

Fig. 7. Protease digestion patterns of TNPR, P4, TMG and RU486 bound PR. In vitro translated and 35S-radiolabeled PR-B was incubated for 10 min in the absence or presence of ligands at a final concentration of 100 nM. Aliquots of the ligand treated receptor mixture were then incubated with a trypsin solution (0, 25, 50, 75 µg/ml). After incubation at room temperature for 10 min, the digestion products were separated on a 4-12% Bis-Tris NuPAGE gel. The gel was then tried under vacuum and the radiolabeled products were visualized by autoradiography.

Fig. 8. The dimer of the PR LBD/TNPR that is present in the crystallographic asymmetric unit. Single molecules of the PR LBD are shown in spectral colors with the N-terminus in blue and the C-terminus in red.

Fig. 9. Superposition of TNPR and progesterone-bound PR LBD structures based on all Cα backbone positions. The PR LBD/TNPR structure is shown in gold, and the PR LBD/P structure in blue. Side chains of residues participating in hydrogen-bonding with TNPR are shown.

Fig. 10. Electron density for TNPR and the surrounding residues in a 2Fo-1Fc map contoured at 1.3σ.
Fig. 11. Effects of TNPR, MPA, and TMG on ovulation in the rat. Estrous synchronized rats (n=8 or 9 animals/group) were treated with test compounds for 4 days. Ova in the oviduct were counted the morning after the last treatment. Data were presented as percentage of animals that ovulated in each dosage group. For each compound, experiments were repeated at least in two separate occasions with the same ED_{100} values.

**Table 1.** Statistics of X-Ray Diffraction Data Collection

<table>
<thead>
<tr>
<th>Data Collection</th>
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<tr>
<td>Crystal System</td>
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<td>R_{merge} a</td>
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<tr>
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</tr>
<tr>
<td>I/σ(I)</td>
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</tr>
</tbody>
</table>

**Phasing and Refinement**

| Model for molecular refinement | 1a28.pdb (PR-LBD/progesterone) |
| Construct (aa)                 | PR-LBD (675-933)               |
| Compound (ligand)              | TNPR                         |
| PR-LBD molecules per asymmetric unit | 2                              |
| Resolution range of refinement | 70.0 – 2.0 Å                  |
| R_{work} b                     | 17.88 %                       |
| R_{free} c                     | 22.81 %                       |
| Number of non-hydrogen protein atoms | 4072                        |
| Number of water molecules      | 179                           |
| RMS deviations from ideal bond lengths | 0.018 Å                   |
| RMS deviations from ideal bond angles | 1.583°                     |

* R_{merge} = |I_{h} - <I_{h}>| / I_{h}, where <I_{h}> is the average intensity over symmetry equivalents.
  Numbers in parentheses reflect statistics for the last shell.

b R_{work} = |F_{obs}|-|F_{calc}| / |F_{obs}|

c R_{free} is equivalent to R_{work}, but calculated for a randomly chosen 5% of reflections omitted from the refinement process.

**Table 2.** IC_{50} values (nM) of TNPR, MPA, and TMG in competition binding assays using a PR source from different species. Values represent the mean of at least 3 determinations.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source of progesterone receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>MPA</td>
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<tr>
<td>TMG</td>
<td>7.6</td>
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<tr>
<td>TNPR</td>
<td>1.7</td>
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</table>
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 8
Fig. 9
Fig. 11
Molecular and pharmacological properties of a potent and selective novel nonsteroidal progesterone receptor agonist tanaproget
Zhiming Zhang, Andrea M. Olland, Yuan Zhu, Jeff Cohen, Tom Berrodin, Susan Chippari, Chandrasekaran Appavu, Shen Li, James Wilhem, Raj Chopra, Andrew Fensome, Puwen Zhang, Jay Wrobel, Rayomond J. Unwalla, C. Richard Lyttle and Richard C. Winneker

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