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Nonsteroidal progesterone receptor ligands with unprecedented receptor selectivity

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Abstract

We have characterized a series of nonsteroidal progesterone receptor ligands, the tetrahydropyridazines. Compounds in this series, exemplified by RWJ 26819, demonstrate high affinity and unprecedented specificity for the progesterone receptor relative to other steroid hormone receptors. Like steroidal progestins, RWJ 26819 induces binding of the receptor to a progesterone response element in vitro, and stimulates gene expression in and proliferation of T47D human breast cancer cells. When administered to rabbits orally or subcutaneously, the compound induces histological changes in the uterine lining comparable to those induced by levonorgestrel. It also inhibits ovulation in monkeys. Though less potent in cells and in animal models than would be predicted from binding affinity alone, their enhanced selectivity suggests that they could be effectively used in a clinical setting. Most of the tetrahydropyridazines synthesized are progestin agonists or mixed agonists and antagonists in vitro; however, one compound with antagonist activity in the rabbit uterine transformation assay has been identified.

Keywords: Nonsteroidal progesterone receptor ligands; Tetrahydropyridazines; Receptor selectivity

1. Introduction

Steroidal progestins used in combination with estrogens in oral contraceptives represent one of the most widely used clinical therapies. The potential therapeutic value of progesterone receptor modulators for contraception and a wide variety of gynecological indications is well established [1]. Despite the safety profile of these compounds, there exist at least three areas for potential improvement. First, progestins used as oral contraceptives exhibit a range of androgenic effects that may modify the beneficial effects of estrogens on coronary heart disease [2]. Second, use of some oral contraceptive products can increase the incidence of breakthrough bleeding, which is a nuisance to women and leads to reduced compliance [3]. Although the cause of breakthrough bleeding is not well understood, it is possible that progesterone agonists with greater receptor and target organ selectivity would provide more acceptable contraceptive options. Third, steroidal compounds are not well tolerated by many patients, therefore, a nonsteroidal progesterone receptor modulator may be preferred in this patient population.

In addition to the potential for improved selectivity of progesterone agonists, there are several areas for potential improvement of progesterone receptor antagonists (PRMs, for progesterone receptor modulators). The contraceptive potential of PRMs has been demonstrated in monkeys by ovulation suppression and prevention of fertilization [4]. In addition, clinical studies

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have demonstrated the potential of PRMs for treatment of endometriosis and uterine leiomyoma [1,5,6]. However, several studies have established that steroidal PRMs possess antiglucocorticoid activity [7–9]. It is generally accepted that a significant improvement of PRMs would result if this activity were reduced. A PRM with reduced antiglucocorticoid activity would likely provide a promising clinical option for contraception and for treatment of uterine disease. Despite the tremendous potential of these compounds, little progress has been reported in the discovery of selective progesterone receptor modulators.

Nonsteroidal ligands for the estrogen and androgen receptors have been characterized previously [10]. Nonsteroidal compounds generally have improved receptor selectivity relative to their steroidal counterparts. For example, the steroid cyproterone acetate was the first androgen receptor antagonist developed, but it had significant activity on the progesterone receptor. However, Casodex (bicalutamide), a nonsteroidal androgen antagonist, has been shown to be selective for the androgen receptor and possesses neither agonistic or antagonistic activity on the progesterone, glucocorticoid, mineralocorticoid, or estrogen receptors [11]. However, nonsteroidal ligands for the progesterone receptor with the potential to modulate progestin action in women are rare. We have discovered a novel series of nonsteroidal progesterone receptor ligands, the tetrahydropyridazines [12,13] (Combs et al., manuscript in preparation), that demonstrate unprecedented progesterone receptor selectivity and potency in several in vitro and in vivo test models. Tetrahydropyridazines that act as progesterone receptor agonists or as progesterone receptor modulators will be described.

2. Materials and methods

2.1. Reagents

Tissue culture medium (RPMI 1640) for T47D cells (American Type Culture Collection, Manassas, VA; ATCC # 45528) and IM-9 cells (ATCC # CCL-159), fetal calf serum and Hepes-buffered sterile saline (HBSS) were purchased from Life Technologies (Rockville, MD). The radioligands [^{3}H]R5020 (progesterone receptor), [^{3}H]R1881 (androgen receptor), [^{3}H]dexamethasone (glucocorticoid receptor) and [^{3}H]diethylstilbestrol (estrogen receptor), as well as unlabeled R5020, were purchased from NEN Life Science Products (Boston, MA). [^{3}H]thymidine and cold thymidine were also obtained from NEN Life Science Products. Unlabelled steroids used to determine nonspecific binding and as reference compounds (progesterone, dihydrotestosterone, dexamethasone, and diethylstilbestrol), dimethyl sulfoxide (DMSO), polyethylene glycol, γ-globulin, formalin and trypsin were purchased from Sigma (St. Louis, MO) or ICN Biomedicals (Costa Mesa, CA). R5020 was obtained from NEN Life Science Products.

2.2. Receptor binding assays

2.2.1. Progesterone receptor

T47D human breast cancer cells were cultured in RPMI 1640 medium containing 10% (v/v) charcoal-treated fetal bovine serum. The cells were recovered from flasks, washed with HBSS and centrifuged. The cells were resuspended in buffer containing 0.01 M Tris, pH 7.4, 1 mM sodium molybdate, 1.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol (TEDG) at a concentration of 25×10^6 cells/ml, and were disrupted with a Dounce/teflon homogenizer in TEDG. The homogenate was centrifuged at 34 000 × *g* for 1 h (Ti90, Beckman Instruments, Fullerton, CA) and the supernatant was diluted for assay to a concentration of 2.5×10^5 cell equivalents/ml to prepare the receptor preparation. Rabbit uterine cytosol was prepared as described for the estrogen receptor below. For assay, a dilution of receptor preparation that yielded 30–40% binding of the labeled ligand was used. A 0.05 ml aliquot of diluted receptor preparation was combined with 0.3 ml buffer, 0.05 ml [^{3}H]R5020 (final concentration 0.4 nM) and 0.05 ml test compound dissolved in DMSO (final concentration 5% (v/v)). Following a 4 h incubation at 4°C in siliconized deep well polypropylene minitube blocks (Beckman Instruments), 0.18 ml polyethylene glycol (40% (w/v) PEG in TEDG) and 0.05 ml human γ-globulin (10% (w/v) in TEDG) were added to the wells. The protein was precipitated onto double thick 'B' glass fiber filter mats (Wallac, Gaithersburg, MD) using a TomTec harvester (Wallac). After the filters were air dried overnight, they were placed in cassettes for counting on a BetaPlate scintillation counter (Wallac Inc.).

2.2.2. Glucocorticoid receptor

IM-9 human lymphoma cells were cultured in spinner flasks containing RPMI 1640 medium for 5 days. The cells were collected by centrifugation at 500 × *g*, resuspended in TEDG and processed as described above for the progesterone receptor. The diluted receptor preparation was brought to a total volume of 0.5 ml with 0.4 nM [^{3}H]dexamethasone, and either unlabeled dexamethasone to determine nonspecific binding, buffer to determine specific binding, or test compound. After an 18 h incubation at 4°C, a 0.5 ml aliquot of dextran-coated charcoal was added to separate bound from free, and the tubes were centrifuged at 1500 × *g* for 10 min. A 0.5 ml aliquot was removed from the tube and added to a scintillation vial containing 3.5 ml EcoLume (ICN Biomedicals) and the vials were counted in a

Packard scintillation counter (Packard Instrument, Downers Grove, IL).

2.2.3. Androgen receptor

To obtain androgen receptor from the ventral prostate for binding assays, mature male rats were surgically castrated one day prior to the removal of the gland. The prostates from 25 animals were pooled, a volume of TEDG equal to the weight of the prostates was added, and the prostates were minced at 4°C with a fine scissors. Subsequently, the minced prostates were diluted 2-fold with additional TEDG and homogenized on ice with a Polytron PT3000 homogenizer (Brinkmann Instruments, Westbury, NY) at a speed setting of three for ten equal bursts. The homogenate was centrifuged at 34 000 × *g* for 1 h at 4°C (Ti90) and the supernatant was saved for receptor binding assays. The androgen receptor binding assay was performed as described above for the glucocorticoid receptor binding assay.

2.2.4. Estrogen receptor

Uteri were obtained from immature New Zealand White rabbits and were minced in an equal volume of TEDG buffer. The minced tissue was subsequently diluted 2-fold with additional TEDG and homogenized with a Polytron homogenizer on ice at a speed setting of three for ten equal bursts. The homogenate was centrifuged at 34 000 × *g* for 1 h at 4°C (Ti90) and the supernatant was saved for receptor binding assays. The estrogen receptor binding assay was performed as described above for the glucocorticoid receptor binding assay.

2.3. Whole cell binding assay

T47D cells were incubated with test compounds or vehicle (1% (v/v) DMSO) and 0.4 nM [^{3}H]R5020 in medium containing serum for 24 h at 37°C in a 5% (v/v) CO_2 atmosphere. Following incubation, cells were washed in HBSS, then treated with trypsin. They were filtered onto Filtermat A glass fiber filters (Wallac) using a TomTec harvester. Filters were counted in a BetaPlate scintillation counter.

2.4. Electrophoretic mobility shift assay

Electrophoretic mobility shifts assays were performed as described previously [14]. Briefly, the B form of the human progesterone receptor was translated from the plasmid pT7βhPRB [15] using the TNT translation kit from Promega (Madison, WI). Reticulocyte lysate (4 μl) was incubated with test compound at room temperature for 10 min in a reaction containing 80 mM potassium chloride, 1 mM dithiothreitol and 10% (v/v) glycerol. Next, 200 pg (1×10^6 cpm) of ^{32}P-labeled progesterone response element oligonucleotide were added, followed by incubation for another 10 min. Samples were loaded on a 4% (w/v) polyacrylamide gel; after electrophoresis, the gel was dried and exposed to X-ray film.

2.5. T47D cell proliferation, CAT and alkaline phosphatase assays

T47D cell proliferation assays were performed as described previously [14]. Briefly, cells in microtiter plate wells (5000 cells per well) were incubated in the presence or absence of 150 pM R5020 and test compound for 16 h. Cells were then incubated with [^{3}H]thymidine for 4 h. Cold thymidine was added and the cells were washed in HBSS, then treated with trypsin. They were filtered onto Filtermat A filters using a TomTec harvester, then counted in a BetaPlate scintillation counter.

Chloramphenicol acetyl transferase (CAT) assays were performed using T47D cells stably transfected with an MMTV-CAT reporter [14]. Cells were incubated in the presence or absence of 1 nM R5020 and test compound for 24 h prior to CAT assay using an enzyme-linked immunosorbence (ELISA) kit from Roche Molecular Biochemicals (Indianapolis, IN). CAT activity in cell extracts was normalized to protein concentration, which was determined using BCA protein assay reagent (Pierce Chemical, Rockford, IL). For both the ELISA and the protein assay, absorbance determinations were made using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA).

T47D cell alkaline phosphatase assays were performed as follows. T47D cells were plated at 25 000 cells per well of a white microtiter plate. After 48 h cells were washed twice in HBSS and treated for 30 min with room temperature 5% (v/v) formalin. After another wash, an alkaline phosphatase assay was performed on the fixed cells using the Great EscAPe SEAP kit from Clontech (Palo Alto, CA). The assay was incubated for 1 h prior to reading on an MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA).

2.6. Rabbit uterine transformation assay

Endometrial stimulation in rabbits was measured as previously described [16,17]. Immature rabbits were treated subcutaneously with estrogen for 6 days, followed by 5 days treatment with a progestin and/or test compound. At the end of the study, uteri were removed and prepared for histological examination. Endometrial thickness and luminal folding were graded according to McPhail [16] by computer-assisted morphometric analysis using visual scoring or a Presage CV-6 image processing system.

2.7. Ovulation inhibition in monkeys

The effects of test compounds on ovulation were determined in cynomolgus monkeys as described previously [18]. Adult primates were treated from days 2–22 of the menstrual cycle with daily intramuscular injections of test compound. Daily femoral blood samples were collected and analyzed for estradiol and progesterone. The occurrence of ovulation was inferred from the patterns of estradiol and progesterone concentrations.

3. Results

We evaluated over 600 related compounds in the tetrahydropyridazine chemical series for inhibition of [^{3}H]R5020 binding to the progesterone receptor. Initial synthesis of this series of compounds and preliminary structure–activity relationships for binding to the progesterone receptor have been discussed previously [12]. The structure–activity relationships that contributed to high affinity binding have recently been summarized (Combs et al., manuscript in preparation). The lead compound in this series, RWJ 26819 (Fig. 1), was further profiled for in vitro and in vivo agonist activity and for receptor selectivity.

Radioiodinated RWJ 26819 was prepared by incorporation of the radioisotope during compound synthesis. The radiolabeled compound was used to compare its receptor binding parameters with standard steroidal progestins. Unlike steroids, RWJ 26819 does not adsorb well to charcoal, and displays high nonspecific binding to polystyrene. Therefore, separation of bound [^{125}I]RWJ 26819 from free was accomplished by chromatography over LH-20 polypropylene minicolumns. [^{125}I]RWJ 26819 bound to progesterone receptors with an affinity ($K_d = 0.12$ nM) that is comparable to the binding affinity of R5020 (a synthetic progesterone

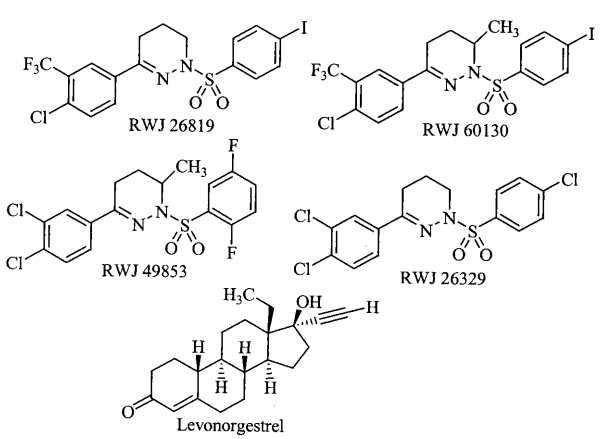

Fig. 1. Structures of the tetrahydropyridazines used in this study. The structure of levonorgestrel is shown for comparison.

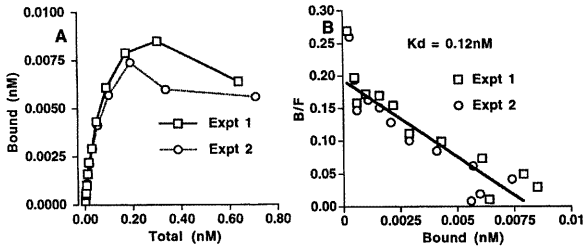

Fig. 2. Saturation (A) and Scatchard (B) analysis of [^{125}I]RWJ 26819 binding to progestin receptors from rabbit uterine cytosol. Increasing concentrations of iodinated RWJ 26819 were incubated with uterine cytosol and a binding assay performed in the presence or absence of excess unlabeled RWJ 26819. Specific binding counts were converted to concentration of bound ligand and are plotted in (A). Scatchard transformation of the data is shown in (B). Two separate experiments were performed.

agonist; $K_d = 0.05$ nM) (Fig. 2). Scatchard analysis revealed that [^{125}I]RWJ 26819 bound to a single site, and that its binding was saturable.

Having demonstrated that RWJ 26819 bound to the progesterone receptor, we next evaluated its species and receptor selectivity. The IC50s for displacement of [^{3}H]R5020 binding to the progesterone receptor from various mammalian species are shown in Table 1. RWJ 26819 demonstrated selectivity for progesterone receptors from rabbit, monkey and human tissues (IC50s $<$ 20 nM), compared to progesterone receptors prepared from rat (IC50 $>$ 100 nM), mouse, guinea pig and hamster tissues (IC50s $\gg$ 100 nM). In addition to species selectivity, RWJ 26819 demonstrated remarkable selectivity for binding to the progesterone receptor over other steroid hormone receptors (Table 2). RWJ 26819 demonstrated 10 000-fold greater selectivity for binding to the rabbit progesterone receptor over the androgen receptor; this compares to a 15-fold greater selectivity of progesterone and 2-fold greater selectivity of levonorgestrel for binding to their receptor over the androgen receptor. RWJ 26819 also demonstrated 1800-fold and 200-fold greater selectivity for binding to the progesterone receptor over the estrogen receptor (rabbit) and glucocorticoid receptor (human), respectively. By comparison, the selectivity of progesterone for its receptor relative to the estrogen receptor (rabbit, 100-fold) and glucocorticoid receptor (human, 27-fold) is much less. Collectively, these data demonstrate the species and receptor selectivity of RWJ 26819 relative to steroidal agonists such as progesterone and levonorgestrel. This level of selectivity was a feature of all of the tetrahydropyridazines.

Progesterone agonists have been shown previously to enhance binding of the occupied receptor to a progesterone response element (PRE) encoded upstream of target genes. The ability of R5020 and RWJ 26819 to induce binding of the progesterone receptor to a PRE-containing oligonucleotide was examined by elec-

trophoretic mobility shift analysis (Fig. 3). R5020 stimulated a concentration-dependent increase in the amount of receptor binding to the PRE-containing oligonucleotide that was easily detectable at a concentration of 10 nM. The concentration of RWJ 26819 required to induce a shift in the PRE-containing oligonucleotide comparable to 10 nM R5020 was at least 100-fold greater. These data indicate that although RWJ 26819 has impressive receptor binding affinity, the concentration required to induce a functional receptor-DNA complex is significantly higher.

Progesterone agonists have previously been shown to stimulate proliferation of T47D human breast cancer cells, as indicated by [^{3}H]thymidine incorporation [19,20]. To determine the potency of RWJ 26819 and related compounds relative to R5020 in cell-based assays, stimulation of T47D breast cancer cell proliferation was examined. During short term exposure to a progestin (16–18 h), the incorporation of [^{3}H]thymidine into T47D cells was increased by R5020, levonorgestrel, and progesterone in a concentration-dependent manner (Fig. 4). The potency with which these steroidal ligands induced [^{3}H]thymidine incorporation was proportional to their known in vitro and in vivo activities. A similar stimulation of [^{3}H]thymidine incorporation was induced by RWJ 26819, although the concentration of RWJ 26819 (74 nM) required to achieve two-fold stimulation was 200- to 1000-fold greater than the concentration required of the steroidal ligands.

Stimulation of [^{3}H]thymidine incorporation involves multiple genetic changes in the cell. To assess the effects of our compounds on a single progesterone-responsive gene, stimulation of the endogenous alkaline phosphatase enzyme was next compared. R5020 at a concentration of 5 nM stimulated a 20-fold increase in alkaline phosphatase activity from T47D cells. A concentration of 0.04 nM stimulated a 2-fold increase in alkaline phosphatase activity (Fig. 5). By comparison, 500 nM RWJ 26819 stimulated a 14-fold increase in alkaline phosphatase activity from the cells, and required a 300-fold greater concentration (13 nM) to stimulate a 2-fold increase in enzyme activity.

A common predictor of progestin activity is the evaluation of ligand-dependent stimulation of the exogenous reporter gene, chloramphenicol acetyl transferase (CAT) driven by the mouse mammary tumor virus promoter (MMTV) in T47D cells [21]. In this system, R5020 stimulated a maximum seven-fold increase in CAT production from T47D cells containing a stably integrated MMTV-CAT construct, and a concentration of 0.025 nM R5020 stimulated a two-fold increase in CAT production (Fig. 6). In contrast to R5020, the tetrahydropyridazines were weak activators of MMTV-CAT transcription. The concentration of RWJ 26819 that stimulated CAT production 2-fold was 1000 nM, approximately 4000-fold greater than the concentration of R5020 required. Two other tetrahydropyridazines structurally related to RWJ 26819 were also tested for their ability to stimulate transcription of MMTV-CAT. RWJ 60130 and RWJ 49853 (Fig. 1) were also weak stimulators of MMTV-CAT transcription, with RWJ 60130 demonstrating the greatest activity of the three compounds tested. The results shown in Figs. 3–6 show that the potency of tetrahydropyridazines in general is 100- to 1000-fold less than the potency of a steroidal progestin like R5020 in functional in vitro assays.

Preliminary tests with the tetrahydropyridazines in vivo determined that these compounds had better activity in a rabbit model of endometrial transformation than would have been predicted based on their in vitro functional activity. RWJ 26819, progesterone, and levonorgestrel were compared for their ability to induce progesterone-dependent transformation of the rabbit uterus (Fig. 7). Five days subcutaneous administration of progesterone (0.4 mg/kg) or RWJ 26819 (1.0 mg/kg)

Table 1
Species selectivity of RWJ 26819 for binding to the progesterone receptor[a]

Receptor source	Progesterone IC50 (nM)	RWJ 26819 IC50 (nM)	Relative binding affinity of RWJ 26819 (progesterone = 1.0)
Rabbit uterus	4.0	1.0	3.0
Monkey uterus	6.0	2.0	3.0
Human breast (T47D cell line)	3.0	8.0	0.4
Human endometrium	3.0	16	0.2
Rat uterus	2.0	120	0.02
Guinea pig uterus	2.0	4100	0.0004
Hamster uterus	9.0	>10 000	NA[b]
Mouse uterus	9.0	>10 000	NA[b]

[a] Binding of progesterone or RWJ 26819 to the indicated receptors was measured as described in Section 2. IC50, concentration of RWJ 26819 that inhibited 50% of [^{3}H]R5020 binding. Relative binding affinity is the ratio of IC50's for progesterone and RWJ 26819.

[b] Not applicable because IC50 for RWJ 26819 >10 000 nM.

Table 2
Steroid receptor selectivity of RWJ 26819[a]

Receptor	IC50 (nM) concentrations					
Type	PR	PR	AR	AR	ER	GR
Species	Rabbit	Human	Rat	Rabbit	Rabbit	Human
Source	Uterus	T47D	Prostate	Prostate	Uterus	IM9
RWJ 26819	1.0	8.0	>10 000	>10 000	1800	>10 000
Progesterone	2.0	3.0	190	30	200	80
Levonorgestrel	1.0	NT	3.0	2.0	960	NT
RU486	3.0	1.0	10	NT	950	2.0

[a] Binding of the indicated compounds to various receptors was measured as described in Section 2. PR, progesterone receptor; AR, androgen receptor; ER, estrogen receptor; GR, glucocorticoid receptor; NT, not tested.

stimulated transformation of the rabbit endometrium as assessed by histological evaluation of the McPhail index [16]. The relative potency of RWJ 26819 compared to progesterone in this model was 0.25. Next, the potencies of levonorgestrel, an orally active progestin, and RWJ 26819 were compared following oral administration for five days. Levonorgestrel (0.05 mg/kg) or RWJ 26819 (2 mg/kg) induced similar half-maximal changes in endometrial transformation. The relative potency of RWJ 26819 was 0.026, or approximately 40-fold less potent than levonorgestrel.

Finally, the contraceptive potential of RWJ 26819 was evaluated in cynomolgus monkeys by monitoring the percentage of monkeys that failed to ovulate following 21 days of treatment (Fig. 8). RWJ 26819 and levonorgestrel both demonstrated an ability to block ovulation, although the dose of RWJ 26819 required for complete inhibition of ovulation was 1000-fold greater than the dose of levonorgestrel required.

Based on our ability to identify compounds from the tetrahydropyridazine series with agonist activity in vivo, experiments were conducted to determine if there were compounds that exhibited progesterone antagonist (PRM) activity. Preliminary results for tetrahydropyridazine antagonists (Table 3) demonstrated that several compounds had mixed agonist/antagonist activity in vitro in the T47D cell proliferation assay (Combs et al., manuscript in preparation), and a subset of these compounds were subsequently evaluated for antagonist activity in vivo. Progesterone antagonists such as mifepristone have been shown to reverse progesterone-induced transformation of the estrogen-primed rabbit uterus. All but one of the tetrahydropyridazines with progesterone antagonist activity in vitro failed to inhibit progesterone-stimulated transformation of the rabbit uterus (Table 3). RWJ 26329 (Fig. 1), a tetrahydropyridazine with poor efficacy as a progesterone antagonist in vitro, did inhibit progesterone-stimulated uterine transformation. Significantly, this was the only compound that lacked any agonist activity in the cellular assay.

Based on our results with RWJ 26819 and related tetrahydropyridazines, it appeared that the compounds demonstrated very good binding activity to the receptor in cell extracts, but when tested in cellular assays or in animals they lost potency. To determine if diffusion of the nonsteroidal compounds into a cell might serve to explain the drop in potency, we evaluated binding affinities of selected compounds in cytosolic and whole cell binding assays. Competition assays were performed in the presence of 5% (v/v) DMSO and 1% (v/v) DMSO with cytosolic preparations from T47D cells, and in the presence of 1% (v/v) DMSO with live T47D cells. The results are presented in Table 4. In contrast to the steroidal compounds, IC50s for the tetrahydropyridazines were markedly reduced in the whole cell binding assays.

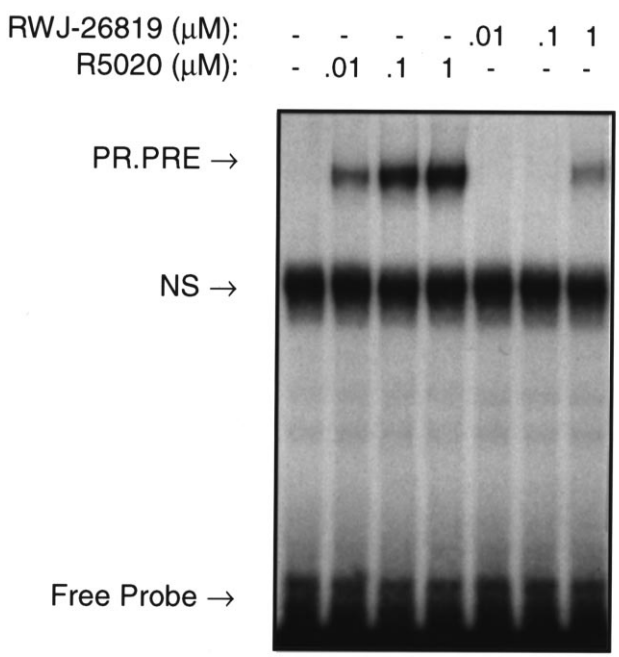

Fig. 3. RWJ 26819 induces DNA binding of the progesterone receptor. The in vitro translated B form of the progesterone receptor was incubated with labeled PRE oligonucleotide (Free Probe) and the indicated compounds. PR.PRE, progesterone receptor-DNA complex, NS, nonreceptor binding complex.

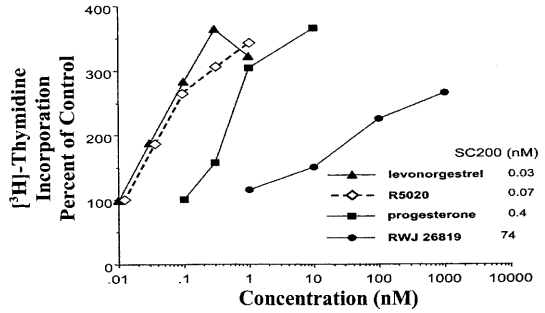

Fig. 4. RWJ 26819 stimulates proliferation of human T47D breast cancer cells. Cells were incubated with test compound for 16 h. Proliferation was monitored during a subsequent 4 h incubation with tritiated thymidine. Cells were filtered onto mats and radioactivity was counted. SC200 = concentration of compound required to stimulate cell proliferation 2-fold above control.

4. Discussion

In these experiments, we have demonstrated that RWJ 26819 and structurally related nonsteroidal progesterone agonists and progesterone receptor modulators display unusual selectivity for the progesterone receptor. In addition, we have shown that activation of the progesterone receptor in vitro by tetrahydropyridazines is markedly reduced compared to steroidal ligands, despite the similar receptor binding affinities. Third, we have shown that the potency of tetrahydropyridazines in vivo compared to steroidal compounds was greater than predicted from the results of in vitro evaluation.

Compounds from the tetrahydropyridazine series bind to the progesterone receptor with high affinity compared to androgen, estrogen and glucocorticoid receptors (Table 2, Fig. 2). Several steroidal progestins have previously been profiled for their affinity for the progesterone, androgen and glucocorticoid receptors. A ratio of androgen receptor binding affinity to progesterone receptor binding affinity of 50 or more may be considered an improvement over currently marketed

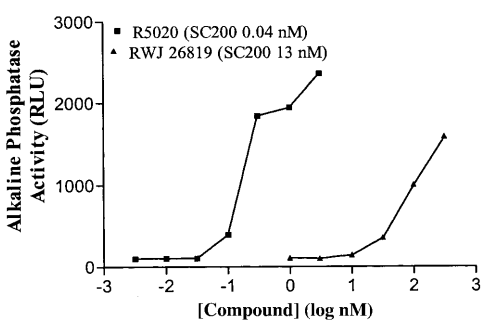

Fig. 5. Stimulation of T47D cell alkaline phosphatase activity by RWJ 26819. Cells were incubated with test compound for 48 h. Cells were fixed and permeabilized, then endogenous alkaline phosphatase activity was detected in situ using a chemiluminescent substrate. RLU, relative light units.

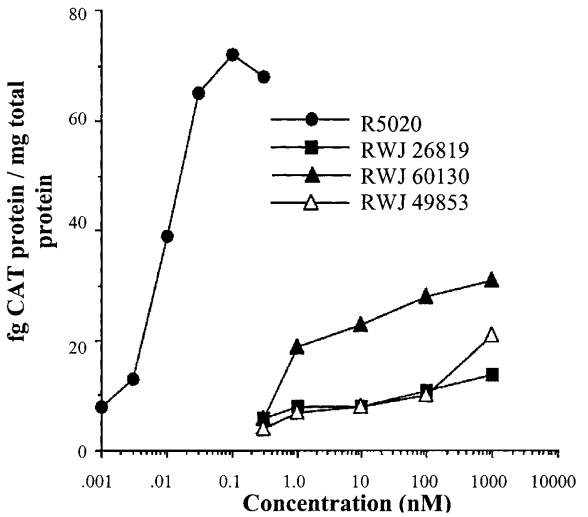

Fig. 6. Stimulation of MMTV-CAT activity in stably transfected T47D cells. Cells were incubated with test compound for 24 h. Cells were lysed and CAT activity was detected by ELISA.

steroids. For example, for norgestimate, this ratio is 25 [22], and this progestin is one of the more selective progestins available clinically today. The tetrahydropyridazine series in general demonstrated a ratio greater than 2500, indicating that a 100-fold higher dose of RWJ 26819 relative to levonorgestrel would be acceptable. In addition to potential androgenic activity of new progestins, selectivity for the glucocorticoid receptor is critical. For levonorgestrel, the ratio of glucocorticoid receptor binding affinity to progesterone receptor binding affinity was 27; but the ratio for RWJ 26819 was >1000. This improved activity ratio suggests that, on the basis of glucocorticoid activity, approximately 50-fold higher concentrations of RWJ 26819 would be acceptable.

Compounds from this series display strong species selectivity (Table 1). RWJ 26819 and its analogs bind preferentially to rabbit, monkey and human progesterone receptors, while the compounds demonstrate weak affinity for rodent progesterone receptors. This property of nonsteroidal ligands has not been reported previously. For example, compounds developed by

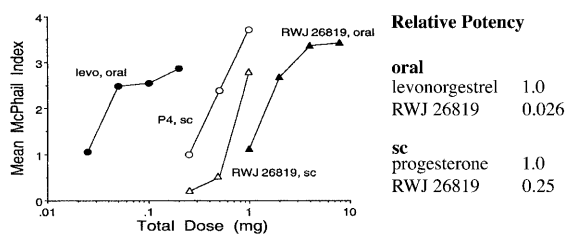

Fig. 7. RWJ 26819 stimulates the endometrium in immature estrogen-primed rabbits. Rabbits were treated with estrogen for 6 days, followed by test compound for 5 days. Uteri were removed and endometrial changes were scored histologically using the McPhail method. sc, Subcutaneous.

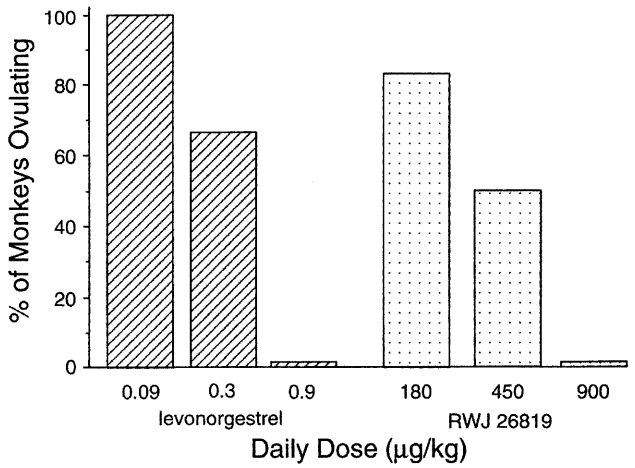

Fig. 8. RWJ 26819 inhibits ovulation in cynomolgus monkeys. Female monkeys were treated from days 2–22 of the menstrual cycle with intramuscular injections of test compound. Daily blood samples were taken for measurement of estrogen and progesterone levels, which were used to monitor ovulation.

Jones et al. [23] demonstrated similar binding affinities for progesterone receptors from rat and human sources. Therefore, it appears that the ligand binding pockets of the rabbit and primate receptors differ significantly enough from the receptor of rodents to affect tetrahydropyridazine binding. The crystal structure of the human receptor has recently been determined [24], but the nature of the interaction of the tetrahydropyridazines with the human and other species of receptor is unknown.

Despite the highly favorable binding affinity and selectivity of the tetrahydropyridazines, the in vitro activity of the compounds is less than would be predicted. This diminished activity was evident in cell-free DNA binding assays and in T47D cell-based assays. The tetrahydropyridazines stimulated proliferation of these cells, as well as endogenous alkaline phosphatase and exogenous CAT activities, in a concentration-de-

Table 4
Binding affinities of tetrahydropyridazines in cell-free and cell-intact assays[a]

RWJ	IC50 (nM)		
	5% (v/v) DMSO	1% (v/v) DMSO	1% (v/v) DMSO
	T47D cytosol	T47D cytosol	Whole T47D cells
26496	1.2	15	870
26497	27	60	470
26819	27	36	1100
27729	2.0	10	200
27886	5.0	13	2200
47126	15	41	430
47370	91	110	1600
47750	4.0	11	73
48085	29	36	130
60425	2.0	8.0	1100
61215	16	33	260
61239	52	450	280
61341	38	57	480
61680	2.0	6.0	200
62159	3.0	14	80
RU486	1.0	2.0	6.0

[a] Binding of the indicated compounds to the progesterone receptor was measured in T47D cell extracts and in whole cells. IC50s were determined in the presence of 5% (v/v) DMSO, which is the concentration of solvent used throughout this paper; and in the presence of 1% (v/v) DMSO, which was not toxic to the T47D cells.

Table 3
RWJ nonsteroidal progesterone receptor modulators[a]

RWJ	EC50 (nM)	IC50 (nM)	Maximum inhibition (%)	Rabbit uterine transformation	
				sc	Oral
26329	10 000	1.0	17	A at 8 mg/kg	A at 5 mg/kg
26407	370	0.5	72	NA at 8 mg/kg	NA at 8 mg/kg
26416	52	4.0	9.0	NA at 1 mg/kg	NA at 4 mg/kg
26441	30	3.0	83	NA at 8 mg/kg	NA at 8 mg/kg
26442	44	3.0	100	NA at 1 mg/kg	NA at 1 mg/kg
26497	650	2.0	59	NA at 1 mg/kg	NA at 8 mg/kg
26499	79	3.3	77	NA at 1 mg/kg	NA at 4 mg/kg
26821	410	0.9	72	NA at 1 mg/kg	NA at 8 mg/kg
26824	570	3.0	59	NA at 1 mg/kg	NA at 8 mg/kg
26825	63	4.8	84	NA at 1 mg/kg	NA at 4 mg/kg
27441	120	2.4	100	NA at 1 mg/kg	NA at 4 mg/kg
47126	36	6.3	90	NA at 4 mg/kg	NA at 8 mg/kg
47238	2.0	3.8	120	NA at 8 mg/kg	NA at 8 mg/kg

[a] IC50s and EC50s were determined in the T47D cell proliferation assay, in the presence and absence of progestin agonist, respectively. Compounds were judged to be active (A) or not active (NA) in the rabbit uterine transformation (anti-Clauberg) assay based on McPhail indices of the degree of endometrial thickness and luminal folding observed in the presence of compound and levonorgestrel (0.05 mg/kg). Compounds were administered subcutaneously (sc) or orally, daily for 5 days.

pendent manner, but in each case the response curve was shifted to the right relative to steroidal controls (Figs. 4–6). This is at least partially due to a reduced ability of the nonsteroidal ligands to enter the cell, as judged by side-by-side comparisons of binding potencies in whole cells versus in cell extracts (Table 4). However, the poor potency of RWJ 26819 (Fig. 3) and other tetrahydropyridazines (data not shown) in inducing DNA binding suggests that the compounds may not induce the optimal conformation of the progesterone receptor for dimerization. Dimerization is a prerequisite for efficient DNA binding by all steroid receptors, and correct formation of the dimerization interface requires the correct ligand-induced conformational changes in the ligand binding pocket [24,25]. Thus, it is possible that the modest in vitro functional activity of the tetrahydropyridazine series is due to a combination of poor cellular uptake and the formation of a suboptimal receptor conformation.

Despite this, the series continued to have potential because of its remarkable selectivity. When tested in vivo, RWJ 26819 was as potent as progesterone at stimulating transformation of the rabbit uterus, known to be a sensitive assay for progesterone (Fig. 7). In addition, RWJ 26819 was orally active at 2 mg/kg, whereas progesterone has little activity when administered orally. The oral activities of RWJ 26819 and levonorgestrel, a steroidal progestin that is a component of oral contraceptives, were comparable in this assay, though the nonsteroidal compound was 40 times less potent.

The favorable comparison of RWJ 26819 with levonorgestrel was less pronounced in the primate ovulation inhibition model. Here, RWJ 26819 was 1000-fold less potent than levonorgestrel. In primates, progesterone has been shown to act at the hypothalamic and pituitary level to inhibit the preovulatory surge of gonadotropins that induce ovulation. In addition, progesterone is likely to have local ovarian effects that may influence ovulation. In cell extracts, the binding affinity of RWJ 26819 for the primate progesterone receptor was similar to its affinity for the human and rabbit receptors. However, in vivo, the pharmacokinetics, pharmacodynamics and/or metabolism of RWJ 26819 must differ substantially between the monkey and rabbit models. There are no other reports comparing the effects of nonsteroidal progesterone receptor ligands in lower animals and in primates. To our knowledge, other nonsteroidal progesterone receptor ligands, such as cyclocymopol and quinololone derivatives [23,26], have not been tested in primates at all.

In summary, we have characterized the activity of a novel series of progesterone receptor ligands. The compounds demonstrate high affinity for the progesterone receptor and remarkable selectivity compared to related steroid hormone receptors. They are active in all progesterone receptor-dependent functional assays tested, though with reduced potency. Moreover, they are capable of transforming the uteri of rabbits and of inhibiting ovulation in monkeys. They were active whether given orally, subcutaneously or intramuscularly. Their reduced potency in the latter studies relative to steroids may be compensated for by their greatly enhanced selectivity for the progesterone receptor. Our work and that of others [23,26] shows that nonsteroidal progestins have significant advantages relative to steroids; nevertheless, more work is needed to improve their in vivo potency. This might now be done by exploiting the recently released crystal structure of the receptor ligand binding domain [24], which should help to identify structural features of the compounds that are required to contact critical activating regions of the receptor.

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