Biochemical Pharmacology 82 (2011) 1709–1719

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Discovery of a novel mechanism of steroid receptor antagonism: WAY-255348 modulates progesterone receptor cellular localization and promoter interactions

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ARTICLE INFO

Article history:
Received 16 May 2011
Accepted 4 August 2011
Available online 11 August 2011

Keywords:
Transcriptional regulation
Nuclear localization
Peptide profiling
Chromatin immunoprecipitation
Nonsteroidal selective progesterone receptor modulator (SPRM)

ABSTRACT

WAY-255348 is a potent nonsteroidal progesterone receptor (PR) antagonist previously characterized in rodents and nonhuman primates. This report describes the novel mechanism by which WAY-255348 inhibits the activity of progesterone. Most PR antagonists bind to and block PR action by inducing a unique "antagonist" conformation of the PR. However, WAY-255348 lacks the bulky side chains or chemical groups that have been associated with the conformation changes of helix 12 that lead to functional antagonism. We show that WAY-255348 achieves antagonist activity by binding to and subsequently preventing progesterone-induced nuclear accumulation, phosphorylation and promoter interactions of the PR. This effect was concentration dependent, as high concentrations of WAY-255348 alone are able to induce nuclear translocation, phosphorylation and subsequent promoter interactions resulting in partial agonist activity at these concentrations. However, at lower concentrations where nuclear accumulation and phosphorylation are prevented, the progesterone-induced DNA binding is blocked along with PR-dependent gene expression. Analysis of the PR conformation induced by WAY-255348 using a limited protease digestion assay, suggested that the WAY-255348 bound PR conformation was similar to that of a progesterone agonist-bound PR and distinct from steroidal antagonist-bound PR conformations. Furthermore, the recruitment and binding of peptides derived from nuclear receptor co-activators is consistent with WAY-255348 inducing an agonist-like conformation. Taken together, these data suggest that WAY-255348 inhibits PR action through a novel molecular mechanism that is distinct from previously studied PR modulators and may be a useful tool to further understanding of PR signaling pathways. Development of therapeutic molecules with this 'passive' antagonism mechanism may provide distinct advantages for patients with reproductive disorders or PR positive breast cancers.

1. Introduction

Progesterone (P4), synthesized by the ovary in response to hypothalamic and pituitary signals, is one of several essential hormones regulating the female reproductive cycle. Elevated P4 levels during the secretory phase lead to down-regulation of mitogenic estrogen action in the uterus and endometrial differentiation in preparation for oocyte implantation. During pregnancy, P4 levels rise even further to maintain pregnancy. Progesterone is also critical in mammary gland development, and in contrast to an anti-proliferative action in the reproductive tract conveys a proliferative paracrine signal in this tissue [1,2].

The progesterone receptor (PR) mediates the effects of the steroid hormone P4. It is a member of the nuclear hormone receptor superfamily of ligand activated transcription factors and is a common target of pharmaceutical intervention. The two predominant isoforms, PR-A and PR-B, formed from different protein translation start-sites, are co-expressed in most PR positive cell types. Although the two isoforms share overlapping functionality in vitro and in vivo, both quantitative and qualitative isoform selective activities have been well documented [3,4].

Therapeutic use of PR modulators is widespread and the development of PR ligands has been the subject of several recent reviews [5,6]. Steroidal PR agonists, derived from the natural hormone and other steroid scaffolds, are predominantly used in

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doi:10.1016/j.bcp.2011.08.006

oral contraceptives and postmenopausal hormone therapy. PR antagonists, such as RU-486 or mifepristone, and selective PR modulators (SPRMs) such as asoprisnil, have demonstrated clinical efficacy in reproductive medicine [7,8].

In many cell-based models of PR action, the non-liganded, native PR, like several other steroid receptors, is found both in the cytoplasm and nucleus and undergoes a rapid nuclear localization upon ligand binding. Interestingly, functionally distinct ligands – agonists, antagonists, and SPRMs – all cause nuclear localization of PR. The resulting biological effect depends upon the distinct conformation of the ligand-bound PR complex which necessitates subsequent interactions with gene promoter elements, transcription factors and co-activators [9,10]. A true molecular understanding is complicated by the existence of promoter-, cell-, and tissue-selective factors and elements each shown to contribute to overall functional responses.

WAY-255348 is a novel nonsteroidal progesterone receptor antagonist whose structure and pharmacological characterization were recently described [11]. However, during the course of characterization of this compound, peculiar 'biphasic' dose–response curves were observed where WAY-255348 behaved as an antagonist at low concentrations and a partial agonist at high concentrations. We hypothesized that the molecular mechanism of this compound actions were distinct from previously characterized PR modulators. Using a number of mechanistic tools to evaluate conformational changes and functional responses, we demonstrate the existence of a novel mechanism of action. Our data suggests that a 'conformation-centric' view of nuclear receptor action omits other important modes by which to achieve mechanistic and ultimately therapeutic differentiation.

2. Materials and methods

2.1. Reagents

Progesterone (P4) was purchased from Sigma Chemical Co. (St. Louis, MO). RU-486 was purchased from Shanghai Institute of Organic Chemistry. [^{3}H]-P4 was purchased from NEN Life Sciences (Boston, MA). Tissue culture media DMEM/F12, DMEM, glutamax and penn-strep were obtained from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). All other chemicals were purchased from Sigma (St. Louis, MO). Human breast carcinoma cell lines T47D were obtained from American Type Culture Collection (Rockville, MD). The isoform specific T47D cell lines, T47D-B6 (PR-B selective) and T47D-A14 (PR-A selective) were a gift from Dr. D. Edwards (Baylor College of Medicine).

2.2. Competition binding assays

The whole cell binding assay has been previously described in detail [11,12]. Briefly, T47D cells were treated with 1 nM [^{3}H]-P4 along with the indicated compounds or vehicle control for 3 h at 37 °C. Cells were washed 3 times with media, harvested with scintillation fluid and cpm measured on a Microbeta counter (PerkinElmer, Shelton, CT). The fluorescent polarization assay (green assay kit) was purchased from Invitrogen (Carlsbad, CA) and carried out according to their recommended protocol using a purified baculovirus expressed GST–PR–LBD fusion protein supplied. For each assay the IC_{50} values were calculated using a nonlinear logistic model from the SAS 8.2 statistics software (SAS Institute, Gary, NC) in combination with Microsoft Excel 2000.

2.3. Protease digestion assays

The protease digestion analysis was performed essentially as described with minor modifications [13]. The plasmid pT7BPRB, kindly provided by Dr. O'Malley (Baylor College of Medicine), was used to generate [^{35}S]-radiolabeled PR-B using the TNT T7 Quick Coupled Transcription/Translation System according to the manufacturer's protocol (Promega, Madison, WI). After the translation reaction, an aliquot (5 μl) of the lysate was incubated for 1 h at room temperature (RT) in the absence or presence of each ligand at the indicated concentrations. The receptor was then incubated with chymotrypsin (Sigma, St. Louis, MO), at a final concentration of 50 μg/ml. After incubation at RT for 20 min, the digestion reaction was terminated with the addition of gel denaturing buffer and boiling for 5 min. The digestion products were separated on a 4–12% Bis–Tris NuPAGE gel (Invitrogen). After the electrophoresis the gel was treated with a 50% (vol/vol) methanol–10% acetic acid (vol/vol) solution for 30 min and immersed in Amplify (GE Healthcare, Piscataway, NJ) for 30 min. The gel was then dried under vacuum and the radiolabeled products were visualized by autoradiography.

2.4. Nuclear-cytoplasmic fractionation

A Nuclear Extract Kit (cat#40140) from Active Motif (Carlsbad, CA) was used to separate nuclear and cytoplasmic fractions. Cells grown to 90% confluence on 100 mm dishes were treated for 1 h with ligands at 37 °C in phenol red free DMEM containing 5% serum. The cells were washed, scraped, and pelleted in 1× PBS containing phosphatase inhibitors. The cell pellet was resuspended in 1× Hypotonic Buffer and incubated on ice for 15 min. Detergent was added and cell suspension was vortexed on high for 10 s prior to centrifugation at 14,000 × *g* for 30 s at 4 °C. The supernatant was removed and stored at −80 °C as 'cytoplasmic' fraction. Complete lysis buffer containing 1 mM DTT and 1:100 dilution of Protease Inhibitor Cocktail (cat#P8340) from Sigma (St. Louis, MO) was added to nuclear pellet and vortexed on high for 10 s. The pellet suspension was then left at 4 °C to rock on a platform at 150 rpm for 30 min. Suspension was then vortexed again for 30 s on high before centrifugation at 14,000 × *g* for 10 min at 4 °C. Supernatant was saved at −80 °C as 'nuclear' fraction.

Protein concentrations were determined using BCA Protein Assay Kit (cat#23225) from Pierce Biotechnology (Rockford, IL). BSA standards and a 1:5 dilution of sample were added to a 96 well plate in triplicate. Working reagent was added to all and plate was incubated at 37 °C for 30 min. Absorbance was read at 562 nm using a SpectraMax Plus spectrophotometer from Molecular Devices (Sunnyvale, CA).

2.5. Western blots

4× LDS sample buffer was added to samples and ran on NOVEX 4–12% Bis–Tris gels then transferred to 0.45 μm nitrocellulose membranes, all from Invitrogen (Carlsbad, CA). Membranes probed with primary Anti-Human PR Mouse mAb (Clone PgR1294) from DakoCytomation (Carpinteria, CA) or Anti-Human PR (Phospho-Ser190) (from Santa Cruz Biotechnology (Santa Cruz, CA) overnight at 4 °C in TBST with 5% dry milk, then incubated with secondary ECL Anti-Mouse IgG HRP-linked antibody from GE Healthcare (Piscataway, NJ) for 1 h at room temperature. Bands detected with SuperSignal West Pico Chemiluminescent Substrate from Pierce Biotechnology (Rockford, IL) using Biomax MR Film from Kodak (Rochester, NY). Image-J software (National Institutes of Health, http://rsb.info.nih.giv/ij/) was used for quantification of expression (Figs. 7 and 8). For quantification of data in Fig. 5, visualization was completed by chemiluminescence using the Dura Extended West Chemi-Reagents (Pierce, Rockford, IL). The FluorChem SP system was used to develop digital images of the luminescent protein bands along with the associated AlphaEase software to complete densitometry.

2.6. Fluorescence imaging

Cells were plated in 10 cm dishes at approximately 50% confluency and transfected with 0.2 μg of YFP–PR–B fusion protein expression plasmid (gift of Dr. D. Edwards) and incubated overnight in serum free media. Following a 1 h incubation with vehicle (0.1% DMSO), 10 nM RU-486 and either 100 nM or 3 μM of WAY-255348, whole-cell fluorescence was imaged using a Nikon TE-DH100W fluorescence microscope equipped with a digital camera (Nikon DXM1200). All images were taken at 20× magnification with identical light and camera settings. The experiment was repeated twice.

2.7. Molecular modeling

Docking calculations were performed using the QXP software package [31]. The PR/P4 X-ray structure was used for docking after initial refinement with constrained simulated annealing dynamics calculations. Once the binding site model was generated, docking was performed using the QXP Monte Carlo docking algorithm mcdock. In general, 1000 Monte Carlo steps were sufficient for the energy scores to converge. The top pose was selected for further analysis. Visualization of the docking results was done using the PYMOL program [32].

2.8. Multiplex assay

Unique fluorescently coded avidin-conjugated Luminex beads (25 μl of 1×10^7 beads/ml) were incubated with each of the biotinylated peptides (25 μl of 25 μM stock) overnight at 4 °C. The beads were then washed twice with multiplex buffer (50 mM KCl, 50 mM Tris pH 8.0) and incubated with D-biotin (100 μM) for 30 min at room temperature to block any remaining unbound avidin sites. Following two additional washes, the beads were pooled and resuspended in 6 ml of buffer to make a 2× concentrated stock. The assay was performed for 2 h at room temperature in 100 μl volume consisting of multiplex buffer, 10 nM GST–PR LBD protein, 0.1% BSA, 2 mM DTT, 0.8 μg/ml anti-GST phycoerythrin antibody and 1× peptide beads along with the test compounds. The plates were read on the Luminex instrument using standard settings with a minimum of 50 events quantified for each bead per well. Data were normalized by subtracting the background mean fluorescence intensity, determined with vehicle alone, from the mean fluorescence intensity obtained with each compound treatment.

2.9. Cell growth and treatment

T47D cells were exponentially grown in growth media (DMEM/F12, 10% FBS, 1% Glutamax, 1% Penn/Strep). Cells were transferred in 48 well dishes containing Plating media (DMEM/F12, 5% stripped FBS, 1% Glutamax and 1% Penn/Strep) and treated with compounds or vehicle. For PR antagonist experiments P4 was premixed with appropriate doses of WAY-255348 before being added to the cells. Cells were treated for 16 h. RNA was isolated by Trizol extraction.

2.10. PRE-luciferase assay

Ninety-six well plates of T47D cells were infected for 3 h at 37 °C with a 1:1000 dilution of the PRE-luciferase adenovirus [12]. After a 2 h recovery, the cells were treated with the indicated compounds for an additional 20 h. The cells were washed once in PBS, harvested in cell culture lysis reagent (cat#153A1, Promega, Madison, WI) and transferred to white 96 well plates. Luciferase activity was analyzed using the luciferase assay system (Promega, cat#E4550) on a Victor2 luminometer (PerkinElmer, Shelton, CT). The dose response curves were fit using a nonlinear logistic model from the SAS 8.2 statistics software (SAS Institute, Cary, NC) in combination with Microsoft Excel 2000.

2.11. RT-PCR analysis

A 2 μl aliquot of sample RNA was added to 350 μl RLT buffer and DNased on Column (Qiagen, Valencia, CA). The concentration of the DNased RNA was determined by OD_{260} on a NanoDrop Spectrophotometer, then diluted to 12–20 ng/μl. RT was performed for 2 h at 37 °C using a High-Capacity cDNA Archive Kit (ABI, Foster City, CA). cDNA samples were stored at −80 °C until QPCR was performed. For determination of gene expression, cDNA was diluted in DEPC–H_2O and 100 ng samples (in 50 μl of volume) were mixed with 50 μl of TaqMan PCR Master Mix (ABI, Foster City, CA). Reaction conditions were: 50 °C for 2 min, 94.5 °C for 10 min, and 40 cycles of: 97 °C (30 s)/59.7 °C (1 min). Primers and probes were ordered as assays on demand from ABI (Foster City, CA) and included *ADD3* (Hs00249895_m1), *ATP1A1* (Hs00167556_m1), *S100P* (Hs00195584_m1) and *SGK* (Hs00178612_m1).

For data analysis, all samples were normalized to four endogenous controls, *GAPDH* (Hs99999905_m1), *GUSB* (Hs99999908_m1), *B2M* (Hs99999907_m1) and *18S* (Hs99999901_s1). For each gene in each sample ΔCT values were calculated using average CT of test gene minus the average CT of the average of the four endogenous control genes. Expression values were multiplied by a constant value such that genes with CT values of background (CT > 35) had an expression value of ~1. This allowed for easy determination of signals above background. Log_2 fold changes were determined in Expressionist 4.0 (GeneData, Basel, Switzerland).

2.12. Chromatin immunoprecipitation

T47D cells were treated with vehicle, 10 nM P4, 1 nM P4 + 1 nM RU-486 or 10 nM RU-486 for 1 h. Cells were fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Chromatin was isolated by adding lysis buffer followed by disruption with a Dounce homogenizer. Lysates were sonicated and the DNA sheared to an average length of 300–500 bp. Genomic DNA (input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a Nanodrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantitation of the total chromatin yield.

Protein–DNA complexes were immunoprecipitated with Anti-PR (SC-+7208, Santa Cruz Biotechnologies, Santa Cruz, CA). Genome-wide location analysis of PR DNA binding was performed using the Mouse Promoter 1.0R Array (Affymetrix, Santa Clara, CA) as a service provided by Genpathway (San Diego, CA). Selective PR-binding sites (PRBSs) were further validated using QPCR. Quantitative PCR (Q-PCR) reactions were carried out in triplicate using SYBR Green Supermix according to the manufacturer's recommended protocol (Bio-Rad, Hercules, CA). The resulting signals were normalized for primer efficiency by carrying out Q-PCR for each primer pair using input DNA. Experimental C_t values were converted to copy numbers detected by comparison with a DNA standard curve run on the same PCR plates. Copy number values were then normalized for primer efficiency by dividing by the values obtained using input DNA and the same primer pairs [34]. Error bars represent standard deviations calculated from the triplicate determinations. The PRBSs validated include sequences −2547 bp upstream of the transcription start site (TSS) of *ADD3*, −454 bp upstream of the TSS of *CLDN8*, and −3822 bp upstream of the TSS of *HDAC11*. The primer sequences for each location are as follows:

ADD3 −2547: forward primer, 5′-GCATGGAGAAAGAGGAAGAGA-3′; reverse primer, 5′-TCAAGGGACTTTACCCTAAACA-3′.

CLDN8 −454: forward primer, 5′-AGAATGTGGCTTCCCAGGTA-3′; reverse primer, 5′-GCTGTTGAAATGCCAAAGTG-3′.

HDAC11 −3822: forward primer, 5′-ACGAGGCTGGAGGAATCTC-3′; reverse primer, 5′-GTGGGAAACCCTGGTGAGT-3′.

Genome-wide RNA expression analysis was performed using the Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA). Treatments were identical to those used for the ChIP on Chip analysis except that the incubation time was extended to 6 h. Detailed analysis of the ChIP on chip and the expression analysis will be reported elsewhere (Jelinsky and Yudt).

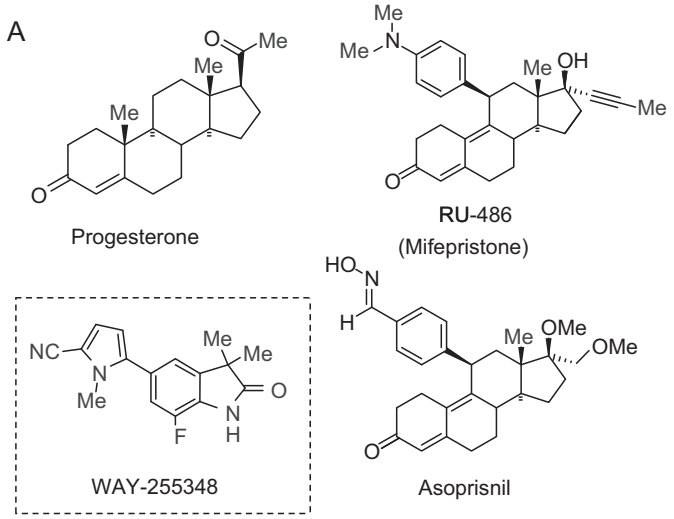
3. Results

3.1. WAY-255348 is a novel non-steroidal PR modulator

WAY-255348 is a novel non-steroidal PR modulator that can bind to PR and whose design, synthesis and structure–activity relationship have been previously published [11]. The chemical structure is shown in Fig. 1A along with the structures of the steroidal ligands used in these studies. Two parallel competitive binding assays confirmed specific binding activity. WAY-255348 competed [^{3}H]-P4 in a whole-cell binding (WCB) assay, and WAY-255348 competed P4 in a fluorescence polarization (FP) competition binding assay that utilized a purified PR ligand binding domain (Fig. 1B). The two binding assays gave similar qualitative results although the WCB assay was more sensitive in general, and gave significantly lower IC_{50} values particularly for RU-486 and asoprisnil.

WAY-255348 blocked PR mediated expression of a luciferase reporter assay under control of a progesterone responsive element (PRE) [12] in T47D cells. WAY-255348 was a potent antagonist, with an IC_{50} of 1.77 ± 0.38 nM against a concentration of 1 nM P4. The IC_{50}'s for RU-486 and asoprisnil were 0.040 ± 0.004 nM and 0.054 ± 0.009 nM respectively (Fig. 2A). However, at concentrations above 100 nM, WAY-255348 displayed partial agonist activity, achieving approximately 50% the maximum activity of P4 (Fig. 2A).

WAY-255348 has agonist activity and increased PR mediated activation of luciferase expression in the absence of exogenous P4 ligand. High concentration of WAY-255348 (>300 nM) induced luciferase expression, but lower concentrations did not (Fig. 2B). WAY-255348 mediated activation of luciferase was significantly less then P4 mediated activation but significantly higher than RU-486 or asoprisnil mediated activation. P4 induced luciferase expression 123.3-fold with an EC_{50} of 0.10 ± 0.01 nM while high concentrations of WAY-255348 induced expression 52.5-fold (43% of P4) with an estimated EC_{50} of 1.04 ± 0.04 μM. In contrast both RU-486 and asoprisnil only induced activity 3.2- and 11.7-fold respectively (2.6% and 9.4% of P4) which did not change as the concentration was increased. The agonist activity of both P4 and WAY-255348 are antagonized by RU-486 (Fig. 2B, inset).



B

Binding Assay (IC50's)		
Compound	WCB (nM)	FP (nM)
Progesterone	5.5 ± 0.3 (n=3)	20.0 ± 3.4 (n=5)
RU-486	0.8 ± 0.1 (n=13)	11.1 ± 1.7 (n=4)
Asoprisnil	1.0 ± 0.3 (n=2)	17.5 ± 4.9 (n=2)
WAY-255348	12.1 ± 3.4 (n=6)	29.1 ± 11.0 (n=3)

Fig. 1. Structure and binding analysis of WAY-255348. (A) The structure of WAY-255348 and the steroidal compounds used in these studies. (B) Competition binding analysis of the same 4 compounds. Both a whole cell binding assay (WCB) and commercial fluorescence polarization assay (FP) were carried out. The mean IC_{50}'s calculated (nM) are shown with SEM and number of replicates.

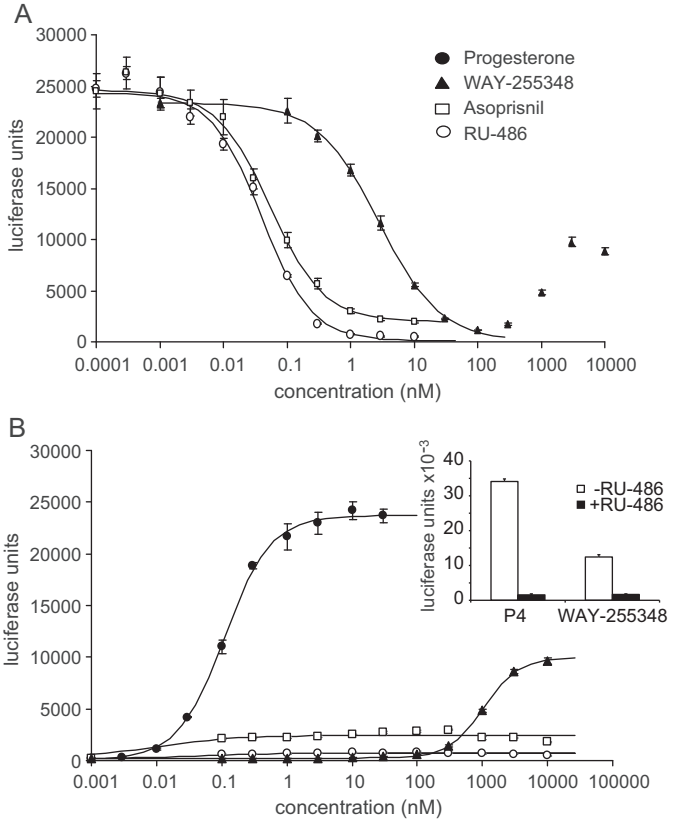


Fig. 2. Functional activity of WAY-255348 in the PRE-luciferase assay. (A) Antagonist mode. T47D cells were infected with a PRE-luc adenovirus and treated with RU-486, asoprisnil and WAY-255348 in semi-logarithmic serial dilutions for 16 h in the presence of 1 nM P4 to generate IC_{50}'s. (B) Agonist mode. PRE-luc infected T47D cells were treated with the same three compounds in A and are compared with progesterone. Data shown are representative of at least 3 independent experiments for each compound. The agonist activity of P4 and WAY-255348 are antagonized by RU-486 (B, inset). T47D cells were treated with P4 (10 nM) or WAY-255348 (10 μM) with (solid bar) or without (open bar) 100 nM RU-486 as above and analyzed for luciferase activity.

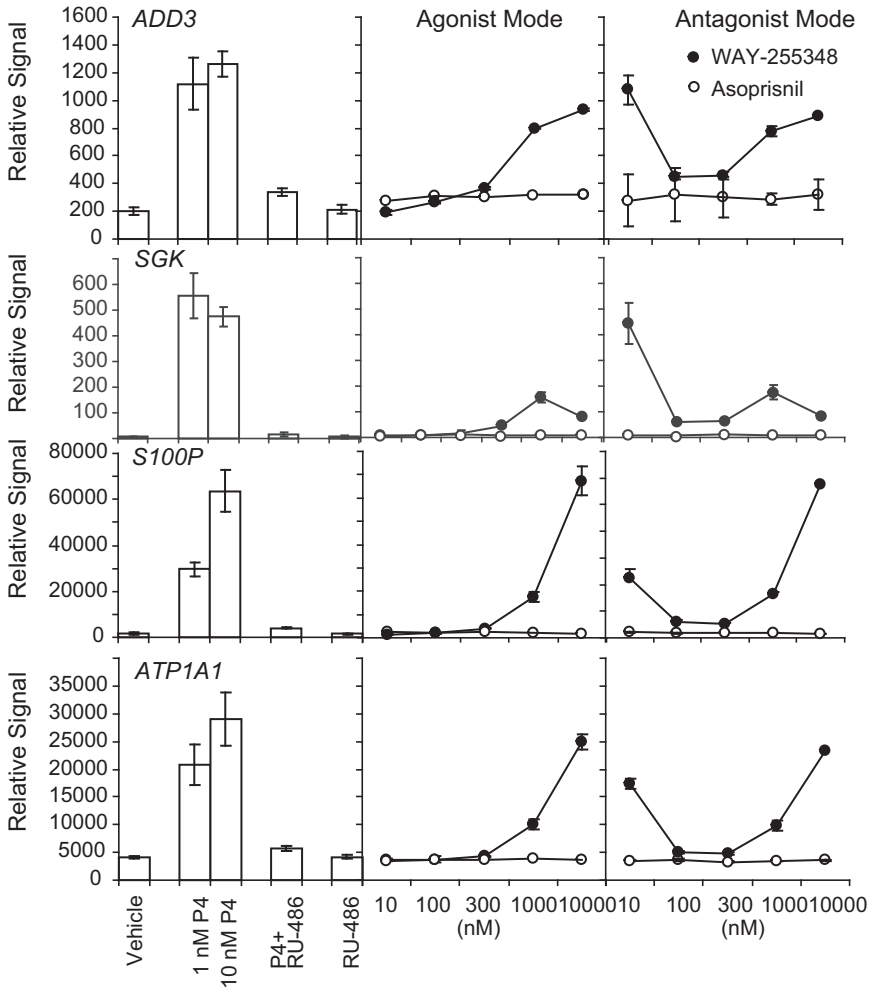


Fig. 3. Effect of WAY-255348 on endogenous gene expression in T47D cells. Messenger RNA expression levels for adducin 3 (*ADD3*), serum-glucocorticoid regulated kinase (*SGK*), S100 calcium binding protein P (*S100P*), and ATPase, Na+/K+ transporting, alpha 1 polypeptide (*ATP1A1*) were determined by qRT-PCR. Control treatments (vehicle, P4 and RU-486) are shown as bar graphs on the left side of each row of data. T47D cells were then treated with a serial dilution of WAY-255348 and asoprisnil in both agonist (compound alone) and antagonist (compound in the presence of 1 nM P4) modes. Values represent the mean relative signal ± SD, $N = 3$.

3.2. Gene expression analysis of WAY-255348 partial agonist activity

Progesterone (1 or 10 nM) treatment increased RNA expression of four well-established P4 regulated genes in T47D cells (Fig. 3). RU-486 effectively blocked P4 mediated activation of these genes. Consistent with the PRE-luciferase results, low concentrations of WAY-255348 (<300 nM) blocked P4 mediated induction (antagonistic activity) but high concentration of WAY-255348 (<300 nM) induced expression (agonist activity) of all four genes. The maximal amount of agonism achieved relative to P4 (percent efficacy) is gene-dependent (Fig. 3). Interestingly, unlike in the luciferase assay RU-486 or asoprisnil treatment did not induce expression of either of these genes, perhaps reflective of the reduced sensitivity (fold-change) of these endogenous marker gene responses to P4 relative to the luciferase reporter assay.

3.3. WAY-255348 binding to PR induces an 'agonist' conformation

Partial protease digestion, which gives a coarse measure of receptor conformation [13] and molecular modeling suggested WAY-255348 induced an agonist conformation. P4 specifically protected a 30 kDa fragment of PR from partial proteolysis, while both RU-486 and asoprisnil predominantly protected a 27 kDa fragment in addition to the 30 kDa fragment. At concentrations (100 nM) that completely antagonize P4 activity, WAY-255348 did not protect either fragment from proteolysis. At concentrations where WAY-255348 exhibits partial agonist activity, WAY-255348 only protected the 30 kDa fragment, an activity similar to P4 (Fig. 4A).

Based on the crystal structure of asoprisnil bound PR [14], the large substituent on C11 of the steroid backbone of asoprisnil bound to PR prevents helix 12 of PR to fold over the ligand pocket and PR is forced into an antagonist conformation. In contrast, molecular modeling of WAY-255348 suggested the molecule is able to fit in the P4 ligand pocket and keep PR in an agonist confirmation (Fig. 4C).

3.4. Effect of WAY-255348 binding on PR cellular localization and phosphorylation

WAY-255348 effected cellular localization and phosphorylation of PR in several cell lines including T47D wild type (wt) cells expressing both PR-B and PR-A isoforms (Fig. 5A) or T47D cells engineered to express the isoforms independently (Fig. 5B and C). Vehicle treated cells distributed PR in both the cytoplasm and nucleus. Treatment with 10 nM progesterone (P4) or with the steroidal PR antagonists RU-486 (10 nM) caused a nuclear accumulation of PR and decreased cytoplasmic concentration. Treatment with the steroidal PR modulator asoprisnil or with the

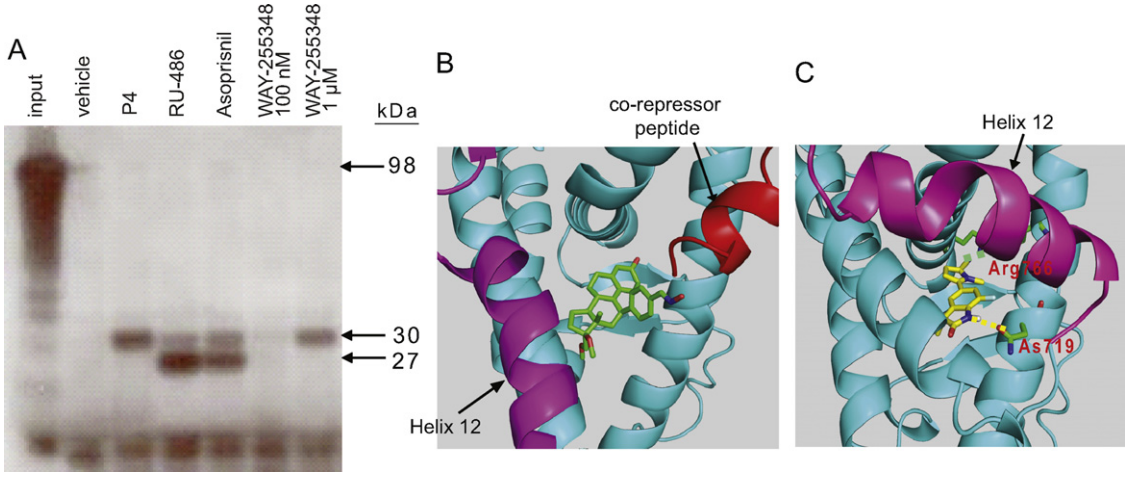


Fig. 4. Conformational analysis of WAY-255348-bound PR. (A) A protease digestion assay was used to evaluate ligand-induced conformations of PR on a macro scale visualized by autoradiography. The undigested, [^{35}S]-met-labeled PR is shown in the input lane. Chymotrypsin digests following vehicle, 100 nM progesterone (P4), 100 nM RU-486, 100 nM asoprisnil (Aso) and the indicated concentrations of WAY-255348 were separated by SDS-PAGE. The approximate MWs (kDa) of the full-length and two protected fragments are shown. The data shown is representative of 3 or more separate experiments for each compound. (B) X-ray structure of PR LBD bound to asoprisnil and the co-repressor SMRT peptide. Note helix 12 is shifted away from the ligand pocket in an antagonist-like conformation to accommodate the pendant 11-β benzaoldoxime substituent of asoprisnil. (C) docked orientation of WAY-255348 in the PR/Progesterone binding site. Helix 12 is in the agonist conformation and is able to partially cover the ligand binding pocket when agonists lacking bulky side chains are bound. Only key residues involved in ligand interaction are shown.

potent non-steroidal agonist tanaproget [15] resulted in similar translocation (data not shown). However, treatment with 100 nM WAY-255348 (348L), a dose in which WAY-255348 behaves as an antagonist in these cells (Fig. 3), failed to significantly alter the cellular distribution of PR in wt and PR-A expressing cells and only slightly changed PR distribution in the PR-B cells (Fig. 5A–C). In contrast, high concentration of WAY-255348 (3000 nM), caused nuclear localization of PR similar to the activity seen with P4 and RU-486. In addition, treatment of HEK293 or COS-7 cells transiently expressing PR-B, with antagonist concentration (<300 nM) of WAY-255348 caused little change in cellular localization, but treatment of these cells with agonist concentration of WAY-25534 (>1000 nM) caused nuclear localization of PR (data not shown). Incubation times from 1 h up to 12 h showed similar results under all of the conditions examined.

Cellular localization of PR was confirmed by fluorescence microscopy using transfected PR-B-GFP fusion protein in COS-7 cells. In vehicle treated cells, the fluorescently tagged receptor is

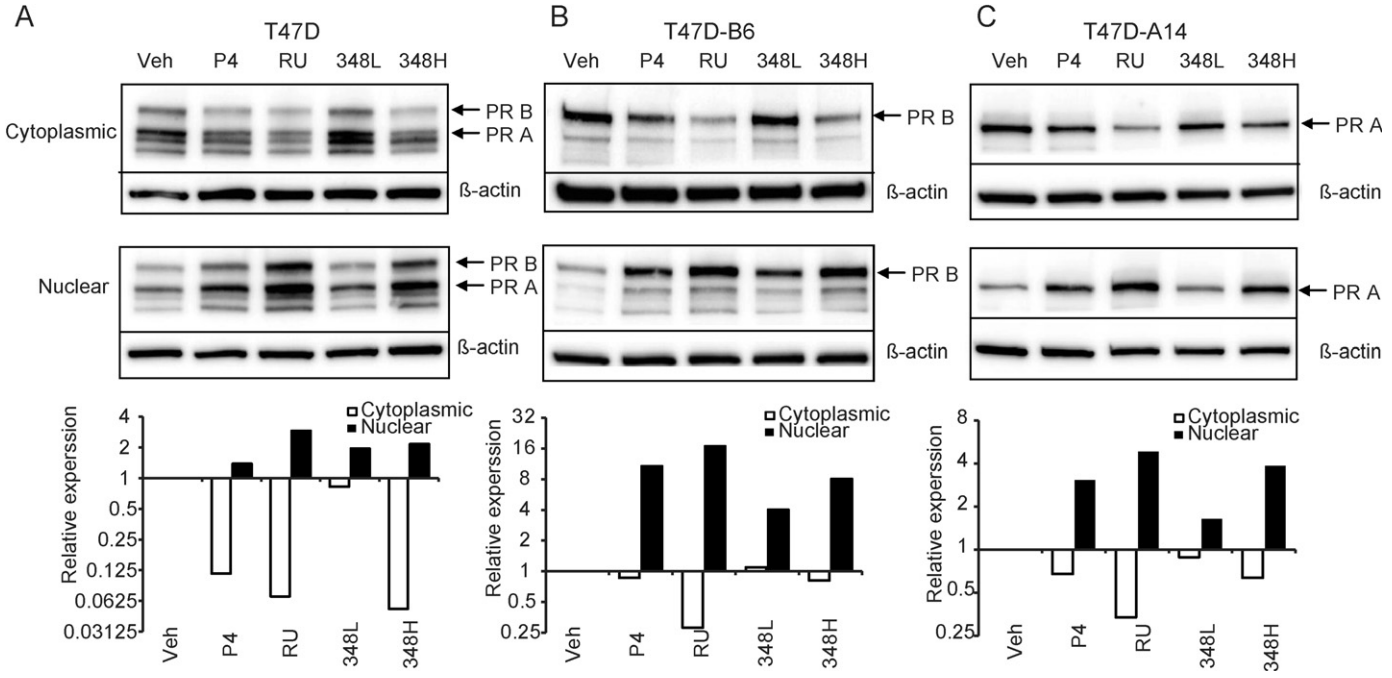


Fig. 5. Effect of WAY-255348 on localization and phosphorylation of PR. Ligand induced changes in PR nuclear/cytoplasmic distribution were measured by western blot analysis of the fractioned cell compartments. (A) WT T47D cells expressing both PR-A and PR-B were treated for 1 h with the vehicle (0.1% DMSO), 10 nM progesterone (P4), 10 nM RU-486 (RU) and 100 nM (348L) or 3000 nM (348H) of WAY-255348. Following fractionation of cytoplasmic and nuclear extracts using commercial reagents and protocol, 15 μg of protein was loaded in each lane. The PR antibody recognizes both isoforms. β-Actin was used as loading control. The relative expression of PR was quantified below the blots using AlphaEase software and presented relative to vehicle on a $\log_2$ scale such that a increase or decrease in receptor localization is indicated by changes above or below the reference value of 1 for vehicle, respectively. (B) Same conditions as (A) but using the T47D-B6 cells expressing only PR-B. (C) Same as (A) and (B), but using the T47D-A14 cells expressing only PR-A.

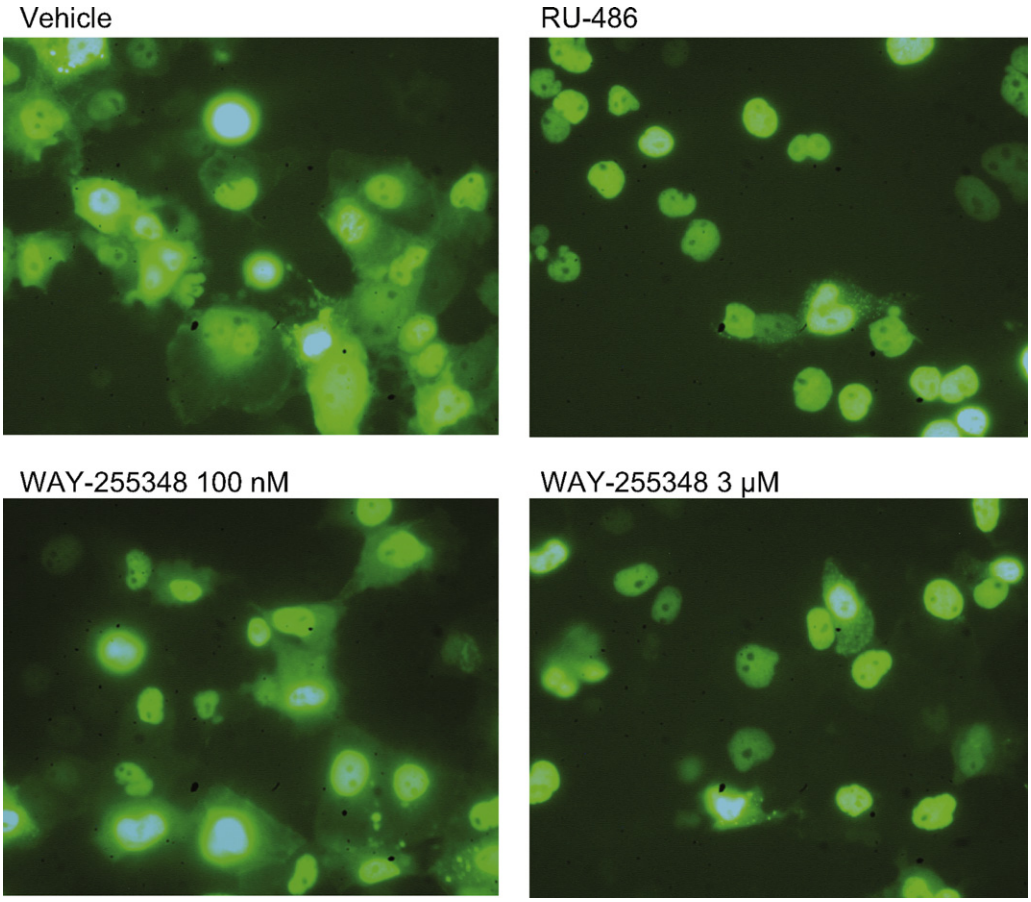


Fig. 6. Flourescence microscopy imaging of PR-B-GFP in transfected COS-7 cells. COS-7 cells were transiently transfected with an YFP-PR-B fusion protein expression plasmid and incubated overnight in serum free media. Following a 1 h incubation with vehicle (0.1% DMSO), 10 nM RU-486 and either 100 nM or 3 μM of WAY-255348, fluorescence was imaged using a Nikon TE-DH100W fluorescence microscope equipped with a digital camera. Cytoplsmic expression is indicated by diffuse patterns extending beyond the circular nuclear foci.

distributed throughout the cell and treatment with RU-486 (10 nM) rapidly (within 1 h) caused nuclear accumulation (Fig. 6, top panels). Treatment 100 nM WAY-255348 (antagonist efficacy) had little effect on nuclear localization while treatment with 3 μM (agonist efficacy) caused complete nuclear accumulation similar to RU-486 (Fig. 6, bottom panels).

P4 and RU-486 increased phosphorylation of nuclear PR (Fig. 7) while phosphorylated cytoplasmic PR was not detected (data not shown). Low concentration of WAY-255348 (100 nM) slightly increased phosphorylated receptor while high concentration (3000 nM) of WAY-255348 significantly increased nuclear phosphorylation of PR.

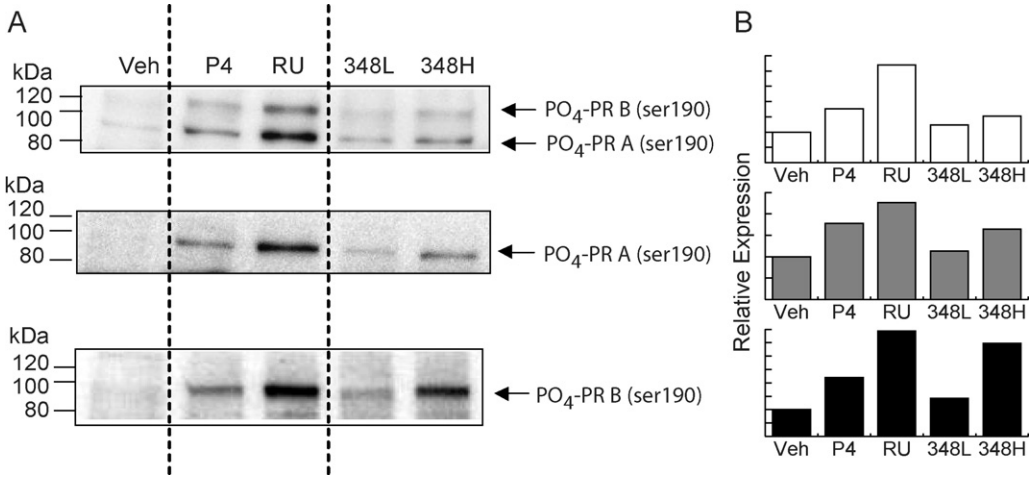


Fig. 7. Western blot analysis of phosphorylated PR in WT, B6 and A14 cells and competition of WAY-255348 with P4. (A) Western blot showing relative amounts of pPR-A and/or pPR-B (Serine 190) in the nuclear fraction of T47D-WT cells (top panel), T47D-A14 cells (middle panel), and T47D-B6 cells (lower panel) after treatment with PR ligands. Veh = vehicle, P4 = 10 nM progesterone, RU = 10 nM RU-486, 348L = 100 nM WAY-255348, 348H = 3 μM WAY-255348. B. The individual blots were quantified by densitometry (Image-J software) for each of the corresponding panels.

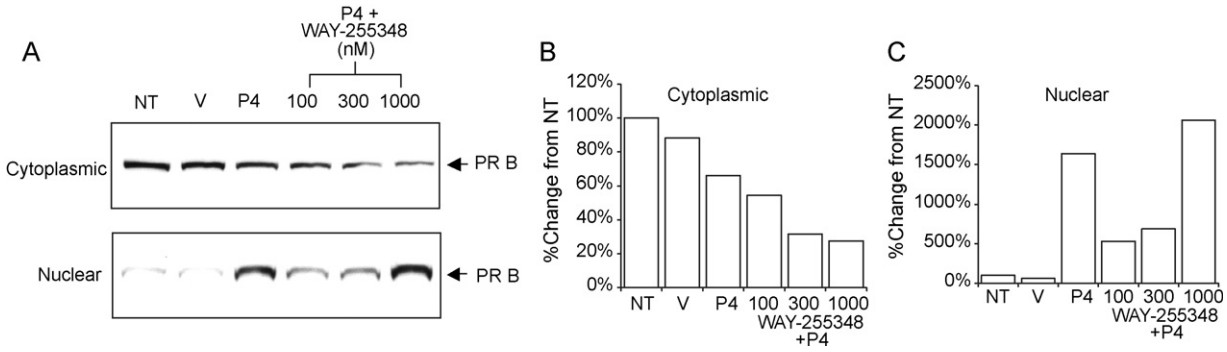


Fig. 8. WAY-255348 antagonizes P4 induced accumulation of nuclear PR. HEK293 cells were transfected with PR-B and treated with vehicle or the indicated ligands for 1 h prior to harvesting and separation of cytoplasmic and nuclear fractions. (A) Western blots for PR localization in untreated cells (NT), vehicle (V), 1 nM P4 alone and 1 nM P4 plus the indicated concentrations of WAY-255348. The individual blots were quantified by densitometry (Image-J software) for the (B) cytoplasmic and (C) nuclear and are representative of 3 or more independent experiments.

Finally, WAY-255348 blocked P4-indcued PR nuclear translocation. Using PR-B transfected HEK293 cells, nuclear accumulation of 1 nM P4 was effectively antagonized at low concentrations (100 nM and 300 nM) of WAY-255348. In contrast, high concentrations (1000 nM) of WAY-255348 did not prevent and may have enhanced nuclear localization of PR in the presence of 1 nM P4 (Fig. 8). This is consistent with the activity in cell-based functional assays (see Figs. 2 and 3).

3.5. WAY-255348 bound PR recruits co-activator peptides similar to a partial agonist

Peptides derived from nuclear receptor co-activators, co-repressors or other interacting proteins differentially interacted with PR bound with different ligands. P4 bound PR bound peptides derived from many nuclear receptor co-activators including SRC1, SRC2, SCR3, and NRIP1 and decreased binding to a peptide derived from the co-repressor SMRT (Fig. 9). In contrast, PR bound to RU-486 failed to recruit any co-activator peptide examined but did interact with the SMRT II co-repressor peptide (Fig. 9). Asoprisnil-bound PR bound both co-activator and co-repressor derived peptides, confirming its molecular definition as a SPRM [16]. WAY-255348-bound PR had similar peptide binding as P4-bound PR. Nine of the eleven peptides that interacted by P4-bound PR also interacted with WAY-255348-bound PR although with a weaker efficacy. Importantly, WAY-255348-bound PR reproducible decreased interaction with the SMRT II peptide similar to the decreased interaction observed with P4-bound PR. This decrease is a clear distinction from both RU-486 and asoprisnil which both recruited this co-repressor-derived peptide (Fig. 9A). Hierarchical clustering of the data further highlights the similarities and differences between WAY-255348 and the steroidal comparators (Fig. 9B).

3.6. WAY-255348 in the chromatin immunoprecipitation assay

Treatment with RU-486 and WAY-255348 had differential effects of DNA binding and RNA transcription of several progesterone-induced genes with identified promoter binding sites in T47D cells. P4 induced expression of the three genes tested (Adducin, *ADD3*; histone deacetylase 11 *HDAC11*; and claudin8, *CLDN8*). RU-486 blocked P4 induced mRNA expression while WAY-255348 partially blocked the P4 induced mRNA expression. We have previously identified PR binding sites upstream of the transcription start site of these genes (data not shown). Treatment with P4 increased PR binding upstream of these genes as measured by ChIP–PCR. RU-486 treatment did not block the P4 induced PR–DNA binding and even enhanced binding at some sites while treatment with WAY-255348 blocked P4 induced PR–DNA binding (Fig. 10).

4. Discussion

The data presented in this manuscript suggests that the mechanism by which WAY-255348 antagonizes the action of progesterone (P4) through the progesterone receptor (PR) is different from previously described compounds. Many extensively characterized PR modulators, from full antagonists like RU-486 to partial agonists like asoprisnil, produce a distinct PR protein conformation that is transcriptionally inert [7]. However, WAY-255348 induces a conformation distinct from these other antagonists, and is, in fact similar to that of P4 itself. Multiple lines of evidence including the protease digestion assay and peptide interaction profiles along with molecular modeling support this conclusion. The antagonism of progesterone action by WAY-255348 appears to be a result of inhibited nuclear localization, phosphorylation and subsequent DNA promoter interactions. This represents a novel mechanism to modulate PR activity. Previously studied PR ligands, including both functional agonists and antagonists, induce the nuclear accumulation and phosphorylation of the PR, and actively promote or inhibit transcriptional responses via favorable or unfavorable protein interactions while still interacting with promoter elements [18–20,30]. In contrast, our results suggest that WAY-255348 is acting as a "passive" antagonist, competitively preventing nuclear accumulation, phosphorylation and ultimately DNA binding and transcription.

Nevertheless, the precise mechanism for the concentration-dependent "functional switch" from antagonism at lower concentrations of WAY-255348 (<300 nM) to partial agonism at higher concentrations (>300 nM) remains to be further elucidated. Transcriptional activation, nuclear accumulation and receptor phosphorylation all track together as the compounds' functional profile changes at these high concentrations. There are several possible explanations for these data which are the subject of ongoing research.

One possible explanation for concentration-dependent functional switch is the existence of a second PR binding site for WAY-255348, i.e. a lower affinity agonist binding site distinct from the higher affinity antagonist site. However, this would not explain the unique effects on PR since the high affinity (antagonist) site competes with progesterone for binding at low nM concentrations (Fig. 1) and prevents nuclear translocation and phosphorylation. Thus the high affinity site competes for the LBD pocket but does not induce antagonist conformation changes. Furthermore, the high dose agonist activity of WAY-255348 can be blocked by RU-486

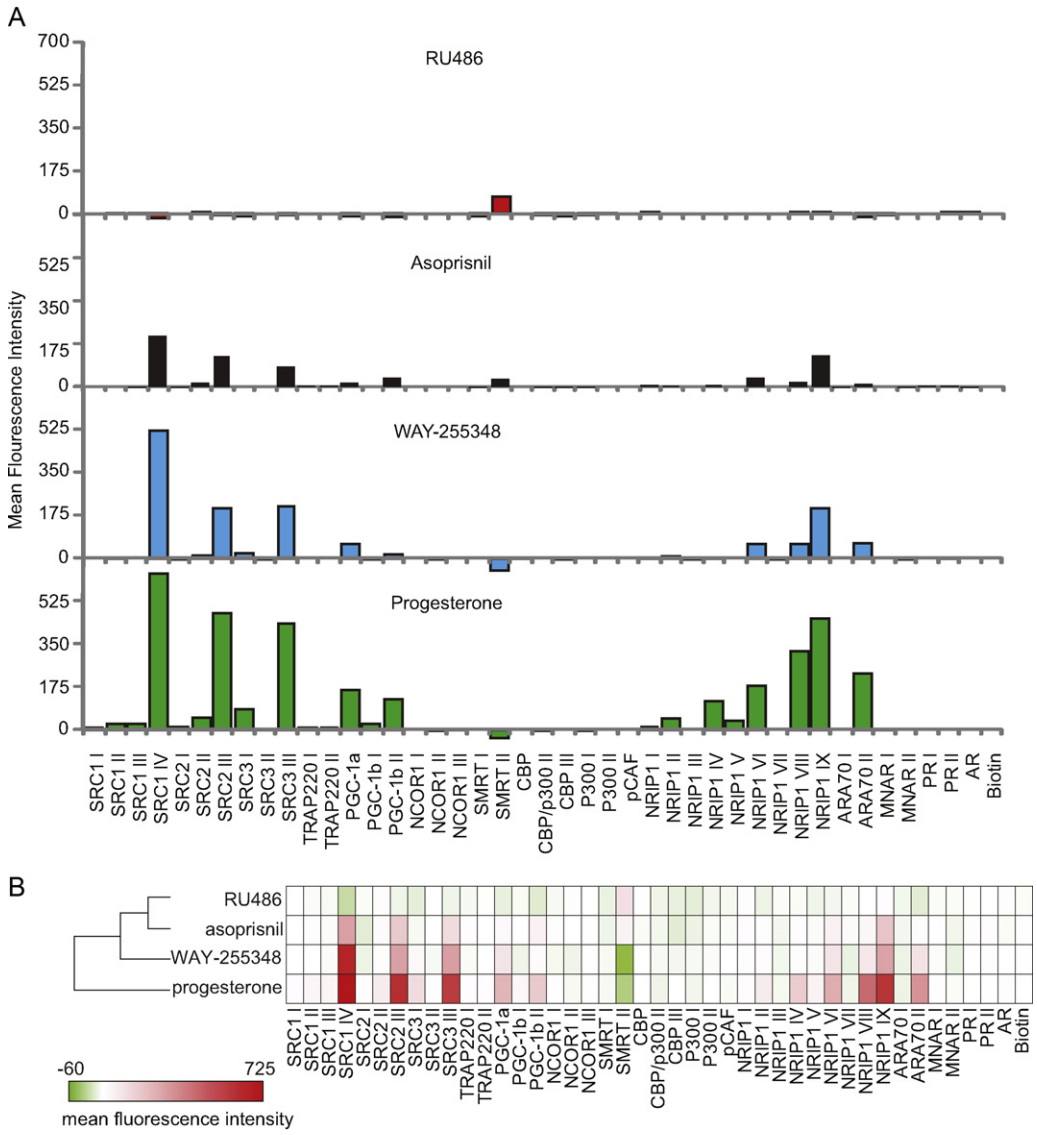


Fig. 9. Peptide interaction profile for PR. The multiplex PR cofactor assay was performed using the 43 Luminex beads coupled to 42 peptides or D-biotin as previously described [12]. Saturating concentrations of each compound were used (100 nM for P4, RU-486 and asoprisnil and 10 μM for WAY-255348). Data presented is mean fluorescence intensity, subtracting the background signal obtained with vehicle alone (1:1 DMSO:ethanol). (A) Hierarchical clustering using the UPGMA method was performed and the three major clusters are plotted. (B) Heat map illustrating the results of the hierarchical clustering. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.) Red indicates an increase and green a decrease from basal peptide interaction.

competition (suggesting common binding modes or dominance of the RU-486 site) and the agonist activity of WAY-255348 is not additive with P4 or other agonists, but rather converges to the activity of the bound ligand – further suggesting a common binding site. It should be noted that a similar 'functional switch' is observed with a mammalian two-hybrid assay using only the PR LBD and interactions with SRC1 co-activator (data not shown). In summary, these binding and functional studies suggest a competitive binding mode at one site.

A second explanation for the 'functional switch' could be a direct effect of WAY-255348 on the nuclear import/export machinery. However, this seems implausible given that this potential effect is overcome at high concentrations. To be sure, we found that WAY-255348 had no effect on TNF (1 ug/ml) stimulated translocation of p65 or on dexamethasone (10 nM) stimulated GR translocation (data not shown) in the same cell line. This argues that the effect, like the compound, is specific for PR. This is consistent with the lack of potent antagonist activity on other steroid receptors previously published [11].

Finally, a third model to describe the concentration-dependent functional switch involves the kinetics of ligand binding and conformational changes that precede phosphorylation and nuclear translocation. If WAY-255348 exhibits a higher off-rate than the conformational change rate, then at low concentrations, the compound may compete with progesterone or other ligands (with similar on-rates) but become unliganded by nature of a high off-rate before a stable induced conformation permits the subsequent steps leading to receptor activation (i.e. phosphorylation and translocation). This model could also explain the observations at high concentrations where the fast off-rate would be overcome by the presence of saturating compound levels and thus allow the receptor to adopt a stable conformational state necessary for nuclear translocation, phosphorylation and ultimately, transcriptional activation. Analysis of PR binding kinetics using a tritiated or otherwise labeled version of WAY-255348 is currently unavailable. However, this model is circumstantially supported by observations of previously studied non-steroidal PR modulators. The potent (subnanomolar) PR agonist tanaproget is changed to a weaker

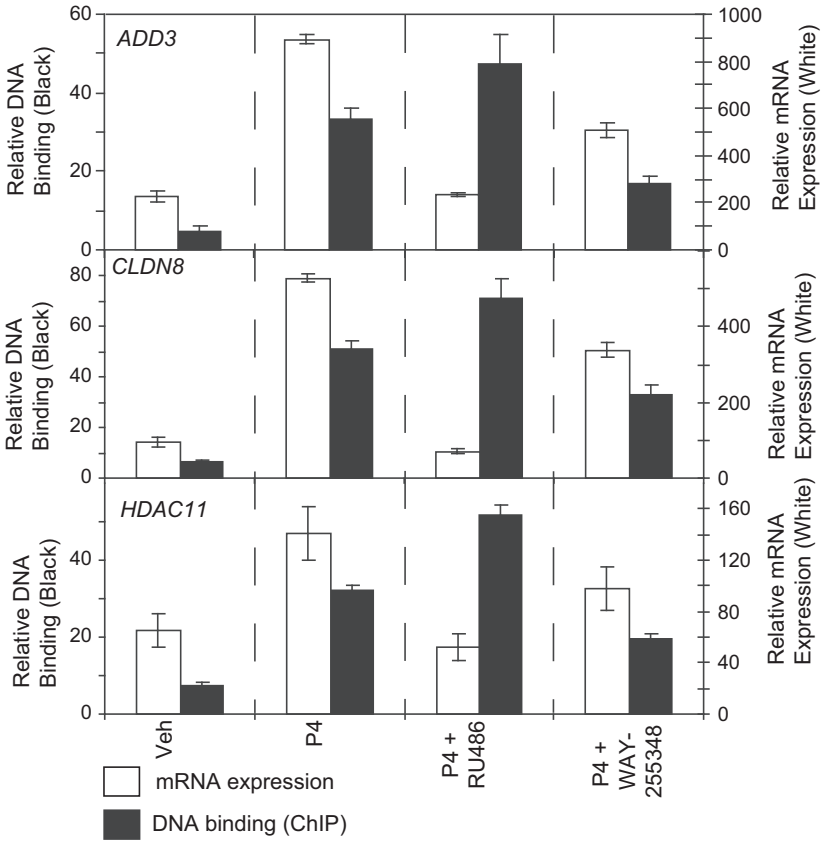


Fig. 10. Effect of WAY-255348 on DNA binding in P4 regulated gene promoters. ChIP–qPCR of progesterone receptor binding sites was determined in T47D treated cells. DNA binding to upstream sequences of three genes, adducin3 (*ADD3*), claudin8 (*CLDN8*), and histone deacetylase 11 (*HDAC11*), were determined by conventional PCR following ChIP (DNA binding, black bars) and are compared with microarray derived relative mRNA expression (white bars). Progesterone (P4) (1 nM) is compared to vehicle (0.1% DMSO) and 1 nM P4 plus either 10 nM of RU-486 or 300 nM of WAY-255348. Statistical significance relative to P4 treated samples is indicated ($^{\#}p < 0.05$; $^{*}p < 0.001$).

affinity (>10 fold) ligand upon very small changes in chemical structure [17]. Under the proposed kinetic model, this change most likely results in a weaker binding affinity by increasing the off-rate (or increasing the on-rate). However, in the proposed model, if the off-rate of the new compound is now greater than the rate of conformational changes, the profile is switched to an antagonist because of the inability or "instability" of the PR to translocate to the nucleus, become phosphorylated or interact with DNA promoter elements.

4.1. Comparison with other novel antagonist mechanisms

The mechanism we are describing for WAY-255348 is different from that of other PR antagonists. There are a number of published reports describing the difference in mechanism of action between RU-486 and the structurally similar steroidal compound ZK-98299 [22]. These two molecules were originally classified as type I (ZK, lack of DNA binding) and type II (RU, stable DNA binding) PR antagonists [23]. However, it was later found that the DNA binding off-rate for ZK is faster than for RU-486 and that the PR–ZK complex can induce DNA binding under some conditions [21,24]. The reasons for the different DNA binding kinetics are presumed to arise from conformational differences, although in the protease digestion assay, ZK is similar to RU and is able to protect the shorter 27 kDa "antagonist" band. ZK may even be more of a complete PR antagonist than RU, based on a greater ability to recruit co-repressors in a two-hybrid assay [25]. Our molecular modeling study demonstrates the physical ability of WAY-255348 to bind in agonist conformation positioning H12 to prevent co-repressor peptide recruitment, precisely as supported experimentally by our peptide affinity profiling. Thus, RU-486 and ZK-98299 are much different than WAY-255348 regarding mechanism of action.

The mechanism of action for WAY-255348 on PR is novel even in comparison with other steroid receptor antagonism models. The antiestrogen ICI 182780 was reported to disrupt ER nucleocytoplasmic shuttling [26]. However, this may be more of a consequence of the conformation, which is presumed to disrupt dimerization and destabilize the ER. Unlike WAY-255348 effects on PR, ICI induces a clear antagonist conformation of the ER, as shown by crystallography [27] and protease digestion [9] and does not induce agonist activity at higher concentrations. Similar discrimination and quantification of functional Vitamin D Receptor (VDR) conformations using limited protease digestion has also been successful for functionally distinguishing VDR modulators [33]. We also found that antagonist concentrations of WAY-255348 did not induce receptor turnover (data not shown) which is supported by the fluorescence microscopy studies shown with GFP–PR–B.

Another mechanism for steroid receptor antagonism was reported by Sasson and Notides [28] for the estrogen receptor. In their molecular and kinetic analysis of the mixed ER agonist/antagonist estriol (E3), the authors observed a biphasic dose response – similar to that reported here for WAY-255348 and the PR. The authors show that titration of a weak or partial agonist (estriol) results in dose-dependent antagonism of the full agonist estradiol (E2). The authors postulate a complex equilibrium involving monomeric vs. dimeric states, DNA-bound states, and ligand-bound states of the ER which ultimately results in a narrow concentration range of E3 that antagonizes the functional response of E2. Although this mechanism is similar to the current situation

with PR and WAY-255348 there are a couple of important distinctions. First, there is no reported effect of E3 on receptor localization, and second, the high dose agonist activity of WAY-255348 in cell-based assays is 1–2 orders of magnitude from the competition binding values. In the case of E3, the agonist activity of the compound alone is more closely coupled with its apparent binding affinity for the ER.

A recent publication regarding a second-generation androgen receptor antagonist implicates effects on cellular localization as either contributing factors or consequences of a potential novel mechanism of antagonism [29]. Similar to our results with WAY-255348 on PR, the ratio of nuclear vs. cytoplasmic AR was significantly lower in cells treated with these novel ligands when compared to known steroidal AR modulators. Furthermore, a distinct AR conformation as well as the prevention of a DNA-bound complex was observed with these ligands. Together, these results suggest there are novel approaches and mechanisms for steroid receptor antagonism that go beyond receptor binding and conformation changes.

In summary, the novel mechanism of antagonism presented here provides new avenues for nuclear receptor drug discovery. These data also suggest that a "conformation-centric" view of ligand–receptor interactions may not provide an appropriate or complete picture of the potential functional responses that can be elicited by the PR when complexed with a structurally unique ligand.

Acknowledgements

The authors would like to thank Dr. Dean Edwards for the T47D cell lines expressing either PR-A (A14) or PR-B (B6) independently and the YFP-hPR-B expression vector.

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