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Novel progesterone receptor modulators with gene selective and context-dependent partial agonism

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ABSTRACT

Progesterone receptor (PR) modulators are used in contraception and post-menopausal hormone therapy, and are under clinical development for reproductive disorders such as uterine fibroids and endometriosis. Development of tissue selective PR modulators (SPRMs) with reduced side effects and improved pharmacology represents a large unmet medical need in the area of women's health. One approach to addressing this need is to focus on the two PR isoforms PR-A and PR-B. In vitro and in vivo studies have revealed both distinct as well as overlapping gene regulation and functional responses of the two PR isoforms that suggests that PR-A selective modulators may retain a desired biological profile. We have identified a chemical series of 4-(4-chlorophenyl)-substituted piperazine carbimidothioic acid esters (PCEs) that have partial PR agonist activity and selectively activate some PR-A isoform regulated genes in T47D cells. However, full microarray analysis in these cells does not predict a global isoform selective profile for these compounds, but rather a unique gene-selective profile is observed relative to steroidal progestins. Using multiplexed peptide interaction profiling and co-activator recruitment assays we find that the mechanism of partial agonism is only partly defined by the ability to recruit known co-activators or peptides but also depends on the cell and promoter context of the gene under investigation. The data demonstrate global consequences of mechanistic and functional differences that can lead to selective biological responses of novel steroid receptor modulators.

1. Introduction

The pharmaceutical uses of progestins are primarily oral contraception, post-menopausal hormone therapy, and treatment of reproductive disorders such as uterine fibroids and endometriosis [1]. Numerous steroidal progestins have been developed with varying degrees of potency, efficacy and steroid receptor selectivity [2]. For example, the potent

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Abbreviations: PR, progesterone receptor; PRE, progesterone response element; PCE, piperazine carbimidothioic acid ester; P4, progesterone; MPA, medroxyprogesterone acetate; TMG, trimegestone; NETA, norethindrone acetate; AR, androgen receptor; ER, estrogen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; SRC, steroid receptor coactivators; PPL, periplakin; Hig2, Hypoxia inducible gene 2; TF, tissue factor; NDRG1, n-myc downstream regulated gene 1.

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synthetic progestin medroxy progesterone acetate (MPA) exhibits weak androgen receptor (AR) agonism while other less potent progestins, such as drospirenone, exhibit some mineralocorticoid receptor (MR) and AR antagonism. On the other end of the functional spectrum are PR antagonists such as RU-486 (mifepristone) and ZK-98,299 (onapristone) that have utility in reproductive medicine [3]. These compounds are also proven tools for unraveling the molecular mechanisms of hormone action via modulation of the progesterone receptor (PR), a member of the nuclear hormone receptor superfamily of ligand activated transcription factors [4].

More recently, PR ligands such as asoprisnil have been described that result in mixed agonist/antagonist profiles depending on the cell, tissue or specific promoter context [5]. Similar to the case with selective estrogen receptor modulators (SERMs), the modulation of PR function by different ligands results in a spectrum of activities that is believed to result, at least in part, from discrete differences in ligand-induced conformations [6]. It is generally accepted that the unique structures of these novel ligand-receptor complexes are responsible for the partial agonism or antagonism that can vary not only with the end-point, but also with the cell-type, tissue or organism under investigation.

The PR is expressed as two isoforms, PR-A and PR-B, a result of alternative translational initiation sites. Multiple promoters in the PR gene give rise to a heterogeneous mixture of mRNA products containing initiation codons for either PR-A or the longer PR-B isoform [7]. Thus, the two isoforms are identical except for an additional 165 amino acids on the amino terminus of PR-B. In most progestin-responsive tissues both isoforms are co-expressed, although their relative expression levels may vary considerably with hormone cycles [8], under pathological conditions [9], or depending on the cell-type within a given tissue [10]. It is well known that the two isoforms possess distinct functional properties in cell-based systems, and several models describe these actions including differential cofactor recruitment and the presence of a third activation function (AF-3) within the PR-B unique sequence [11]. More recently, distinct gene activation profiles for the PR isoforms have been generated in several cell types expressing the PR isoforms independently [12,13]. However, the work of Conneely and colleagues using mouse genetic models have described unique in vivo roles for the PR isoforms in multiple tissues [14]. For instance, PR-A is necessary and sufficient for both ovulation and the anti-estrogenic function of progesterone in the uterus [15]. The sum of these findings suggest that selective pharmaceutical targeting of PR isoforms, particularly PR-A, may translate to unique tissue-selective functions and potentially greater therapeutic benefits [16].

In this study we describe the identification of a novel class of non-steroidal selective progesterone receptor modulators (SPRMs). We began by identifying non-steroidal compounds capable of PR-dependent transcriptional agonism. Then, using a panel of mRNA readouts previously identified as PR-isoform-selective, we quantified gene selectivity in terms of both transcriptional efficacy and potency. A series of compounds, piperazine carbimidothioic acid esters (PCE), were found that function as partial agonists. This partial agonism correlates with a reduced efficacy in co-activator recruitment. Peptide interaction profiling supports the SPRM classification demonstrating recruitment of both co-activator and co-repressor peptides. The gene selective properties of this series suggest a preference for activation of some PR-A selective genes and further suggest that a gene-based approach to drug discovery can serve as a starting point for identifying new molecules with potential for improved therapeutic profiles.

2. Materials and methods

2.1. Reagents and cells

All compounds used in this study were obtained from the Wyeth compound room. T47D cells (ATCC, Manassas, VA) were grown in DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 2% charcoal stripped fetal calf serum (Hyclone, Logan, UT). COS7 cells (ATCC) were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% charcoal stripped serum. Transfection assays were performed using the Fugene 6 transfection reagent (Roche, Indianapolis, IN) with DNA complexes formed in Opti-Mem I reduced serum media (Invitrogen, Carlsbad, California). Luciferase reporter assays were performed using the Luciferase Assay System (Promega, Madison, WI) along with the Cell Culture Lysis Reagent (Promega, Madison, WI) in Microlite 2+ white assay plates (Thermo Labsystems, Franklin, NJ).

2.2. Adenovirus and plasmid vectors

The progesterone response element (PRE) adenovirus construct contains two copies of the consensus PRE element in front of the thymidine kinase (TK) promoter driving luciferase production [17]. The cDNA sequences encoding the ligand-binding domains (LBD) of the PR (aa 632–933), ER (aa 303–595), MR (aa 669–984), GR (aa 485–777) and AR (aa 644–920) were each cloned into the mammalian two-hybrid GAL4 DNA-binding domain plasmid pM (Clontech, Mountain View, CA). The full-length open reading frames of the steroid receptor coactivators SRC-1 and SRC-3 or the C-terminal half of SRC-2 (aa 620–1121) were cloned into the mammalian two-hybrid VP16 activation domain vector pVP16 (Clontech, Mountain View, CA). The GAL_{UAS} luciferase reporter plasmid was created by transferring the five copies of the 17 base-pair GAL_{UAS} upstream of the adenovirus E1b minimal promoter from the pG5CAT plasmid (Clontech, Mountain View, CA) to the pGL3 basic reporter plasmid (Promega, Madison, WI).

2.3. Whole cell binding assay

T47D cells were treated with 1 nM [^{3}H]-progesterone (PerkinElmer, Shelton, CT) 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 measured on a Microbeta counter (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.

2.4. PRE-luciferase assay

96 well plates of T47D cells were infected for 3 h at 37 °C with a 1:1000 dilution of the PRE-luciferase adenovirus. 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, and transferred to white 96 well plates. Luciferase activity was analyzed using the Luciferase assay system (Promega, Madison, WI) 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.

2.5. Mammalian one and two-hybrid assays

In the MR, GR, and ER one-hybrid cross-reactivity assays, 96-well plates of COS-7 cells were co-transfected with 50 ng per well of the indicated steroid receptor LBD fused to the GAL4 DBD plasmid along with the GAL_{UAS} luciferase reporter plasmid. The PR and AR two-hybrid assays were performed similarly but with the addition of 50 ng per well coactivator VP16 plasmid. All transfections were performed in 96 well plates with 0.5 ul per well of Fugene6 for 20 h at 37 °C according to the manufacturer's protocol. Fresh media was added and the cells were treated with the compounds indicated for an additional 20 h at 37 °C. Antagonist mode assays for MR, GR, ER and AR were performed in the presence of 3 nM aldosterone, 5 nM dexamethasone, 5 nM 17β-estradiol (E_2) and 10 nM dihydrotestosterone (DHT), respectively. The cells were washed once in PBS, harvested in Cell Culture Lysis Reagent, and transferred to white 96 well plates. Luciferase activity was measured using the Luciferase Assay System 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.

2.6. Quantigene branched DNA assay

The Quantigene branched DNA assay (Panomics, Fremont, CA) was used to quantify the expression of PR isoform-selective genes. This alternative to quantitative RT-PCR (taqman) offers several advantages including greater throughput, better precision, no PCR amplification steps, and the ability to work straight from cell lysates. T47D cells were treated with the indicated compounds overnight at 37 °C. Cells were washed once with PBS, lysed using the supplied buffer and subjected to one freeze/thaw cycle at −80 °C. Upon thawing, probe sets were added to detect the following genes: tissue factor or TF (accession# NM_001993), periplakin or PPL (accession# NM_002705), N-myc downstream-regulated gene 1 or NDRG1 (accession# NM_006096), and hypoxia inducible protein 2 or HIG2 (accession# NM_013332). The alkaline phosphatase chemiluminescent readout was measured on the Victor2 luminometer (PerkinElmer, Shelton, CT) and the results were expressed as a percentage of the maximum gene expression level achieved with P4 treatment. 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.

2.7. Expression profiling

RNA was isolated with the addition of 600 μl of TRIzol, purified by chloroform extraction and further purified with an RNAeasy column (Qiagen, CA). For all samples, RNA quantity was determined by absorbance at 260 nm (NanoDrop, Wilmington, DE). Five micrograms of total RNA were used to generate biotin labeled cRNA using an oligo T7 primer in a reverse transcription reaction followed by *in vitro* transcription reaction with biotin labeled UTP and CTP. Ten micrograms of cRNA were fragmented and hybridized to HGU133 2.0 Plus arrays (Affymetrix, Santa Clara, CA) representing >55,000 transcripts. Hybridized arrays were stained according to the manufactures' protocols on a Fluidics Station 450 and scanned on an Affymetrix scanner 3000. All array images were visually inspected for defects and quality. Arrays with excessive background, low signal intensity, or major defects within the array were eliminated from further analysis. Signal values were determined using Gene Chip Operating System 1.0 (GCOS, Affymetrix). For each array, all probe sets were normalized to a mean signal intensity value of 100. The default GCOS statistical values were used for all analyses. Signal values and absolute detection calls were imported into Genesis 2.0 (GeneLogic, Gaithersburg, MD) or Decision Site 8.1 (TIBCO, Palo Alto, CA).

A qualifier was considered detectable if the mean expression was greater than 50 signal units and the percentage of samples with a Present (P) call as determined by GCOS default settings was greater than or equal to 67%. A qualifier was considered to be regulated if the difference between treated and vehicle met the following criteria; (1) the qualifier had to be detected in at least 67% of the samples in either the vehicle or treated samples, (2) the fold change was at least 1.7, and (3) the *p*-value based on an Welch test had to be ≤0.01. These conditions were met by 1320 qualifiers, which were used for further analysis. Genes that are regulated by at least two of the three steroidal progesterone receptor agonists, P4, norethindrone acetate (NETA) or trimegestone (TMG), defined a progestin-regulated gene set. A total of 813 qualifiers met these conditions.

2.8. Multiplex

The PR multiplex assay utilized 43 unique fluorescently coded low capacity avidin modified Luminex beads (RADIX Biosolutions, Georgetown, TX) along with 42 biotinylated nuclear receptor cofactor peptides or D-biotin as shown in Table S1 (Anaspec, San Jose, CA). 2500 per well of each bead were incubated with 12.5 μM biotinylated peptide or D-biotin overnight at 4 °C. The beads were then washed twice with multiplex buffer (50 mM TRIS pH 8.0, 50 mM KCl) and incubated with 100 μM D-biotin (Invitrogen, Carlsbad, CA) for 30 min at room temperature to block any remaining free avidin. Following two additional washes, the beads were pooled and resuspended in 50 μl per well of multiplex buffer. The assay was performed for 2 h at room temperature in 100 μl per well volume containing multiplex buffer, 50 ul peptide

beads, 10 nM GST PR LBD protein (Invitrogen, Carlsbad, CA), 80 ng anti-GST phycoerythrin antibody (Martek, Columbia, MD), 0.1% BSA, 2 mM DTT, and the test compounds. The plates were read on the Luminex 100 IS instrument (Luminex, Austin, TX) 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. Hierarchical clustering was performed in Spotfire DecisionSite 8.1.1 (TIBCO, Palo Alto, CA) using the UPGMA clustering method with Euclidian distance similarity measure and average value ordering.

3. Results

3.1. *Discovery of novel PR ligands – piperazine carbimidothioic acid esters*

During a high-throughput screening (HTS) campaign for new progesterone receptor agonist ligands, a series of 4-(4-chlorophenyl)-substituted-piperazine carbimidothioic acid esters (PCE) was identified (Fig. 1a). These non-steroidal compounds are structurally distinct from any other previously reported PR ligands [2,18]. The three compounds highlighted in this study are, respectively, the benzyl (PCE-1), 2-methylallyl (PCE-2), and methyl (PCE-3) esters of 4-(4-chlorophenyl) piperazine-1-carbimidothioic acid (See Fig. 1).

A whole cell competition-binding assay was used to demonstrate direct binding of the PCEs to the human PR. The PCEs have moderate relative binding affinity for PR, with IC_{50} values of 330 and 350 nM respectively for PCE1 and PCE2, compared with 0.5 nM for the potent antiprogestin RU-486 (Fig. 1b) and 3 nM for P4 (data not shown). The third molecule, PCE3, is approximately one order of magnitude weaker than the others (3.3 μM) and is likely due to the smaller size of the methyl substituent compared with the benzyl and isopropylene groups on PCE1 and PCE2 respectively (Fig. 1a). Most of the remaining studies will focus on PCE1 and PCE2. PCE3 is consistently one-half to one order of magnitude less potent than the other two among all the PR assays.

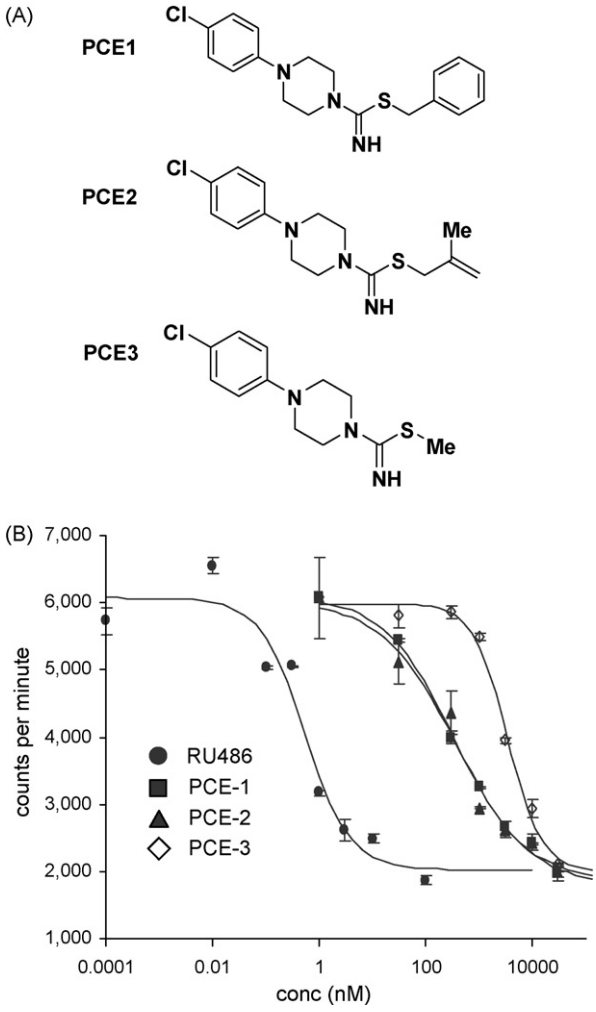


Fig. 1 – Piperazine carbimidothioic acid esters (PCEs) bind to the progesterone receptor.
(A) Structures of the PCEs used in this study.
(B) Whole cell competition-binding assay in T47D cells. Cells were incubated with the indicated compounds along with 1 nM [^{3}H]-P4 for 3 h at 37 °C. IC_{50} values were calculated using the statistical software SAS-Excel: RU486, 0.5 nM; PCE-1, 330 nM; PCE-2, 350 nM; PCE-3, 3.3 μM.

3.2. *Functional and mechanistic analysis of PCE compounds*

We next evaluated the functional activity of the PCEs in a progesterone response element luciferase (PRE-luc) reporter gene assay in T47D cells. The cells used in these studies expressed similar levels of PR-A and PR-B as assessed by western blot (data not shown). The data in Fig. 2 show that the PCEs are partial PR agonists with efficacy (y-axis) roughly 50% that of progesterone in this assay. Furthermore, the PCEs have reduced potency (x-axis) with EC_{50} values of 27 nM, 23 nM and 270 nM respectively for PCE1, PCE2 and PCE3 (Fig. 2a), consistent with the direct binding described in Fig. 1. RU-486, a well-characterized steroidal PR antagonist, can completely antagonize the PCE induced PRE-Luc activity providing evidence that PCE mediated induction of PRE-Luc activity is a PR-mediated mechanism (Fig. 2b).

The mechanism of action of the PCE compounds was evaluated in a mammalian two-hybrid assay that quantifies the interaction between the PR ligand binding domain (LBD) and the co-activator proteins SRC-1 and SRC-3. The PR has been shown to utilize both of these co-activators in several cell and animal models [19]. The PCE-bound PR can recruit the co-activators SRC-1 and SRC-3, although with reduced efficacy (y-axis) in comparison with P4 (Fig. 3). Interestingly, the reduced efficacy observed in this assay is consistent with the efficacy observed in the PRE reporter gene studies in Fig. 2, indicating that a weaker or partial recruitment of co-activators is a likely explanation for the reduced efficacy or partial agonism observed with the full-length PR in the PRE reporter assay. In assays performed without the coactivator plasmid, the PR LBD plasmid alone showed 150 fold less activity with P4, and no activity with the PCEs (data not shown).

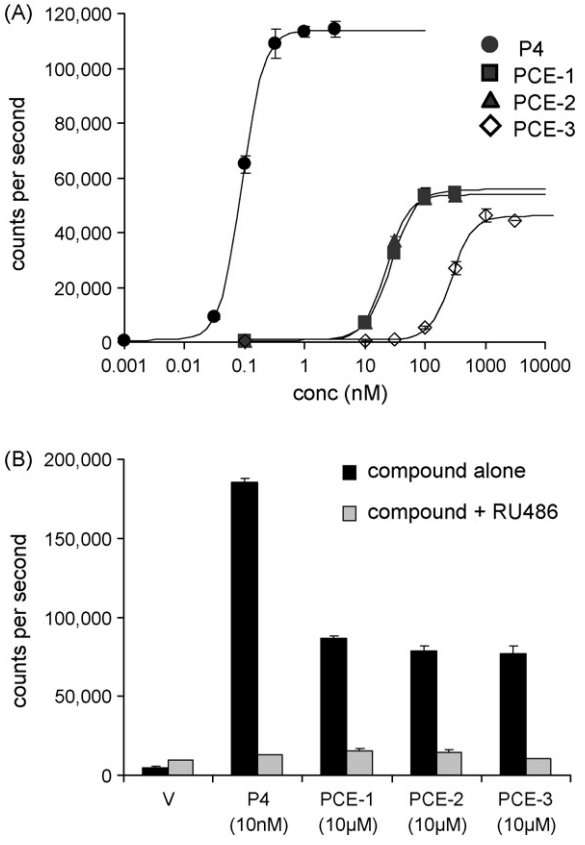


Fig. 2 – PCEs are partial agonists of the PR.
The PCEs exhibit approximately 50% agonist efficacy in the PRE reporter assay and are inhibited by the PR antagonist, RU486. (A) Dose response curves of P4 and the PCEs in the PRE luciferase assay. EC_{50} values were calculated using SAS-Excel: P4, 0.09 nM; PCE-1, 27 nM; PCE-2, 23 nM; PCE-3, 270 nM. A representative experiment is shown from three separate experiments. (B) To show that the PCE compounds, like P4, induce PR agonist activity, and this activity can be antagonized by RU486, T47D cells were infected with an adenovirus PRE-luciferase and treated with vehicle (V), 10 nM P4, or 10 μM of the indicated PCE compounds in the absence or presence of 300 nM RU486 for 16 h.

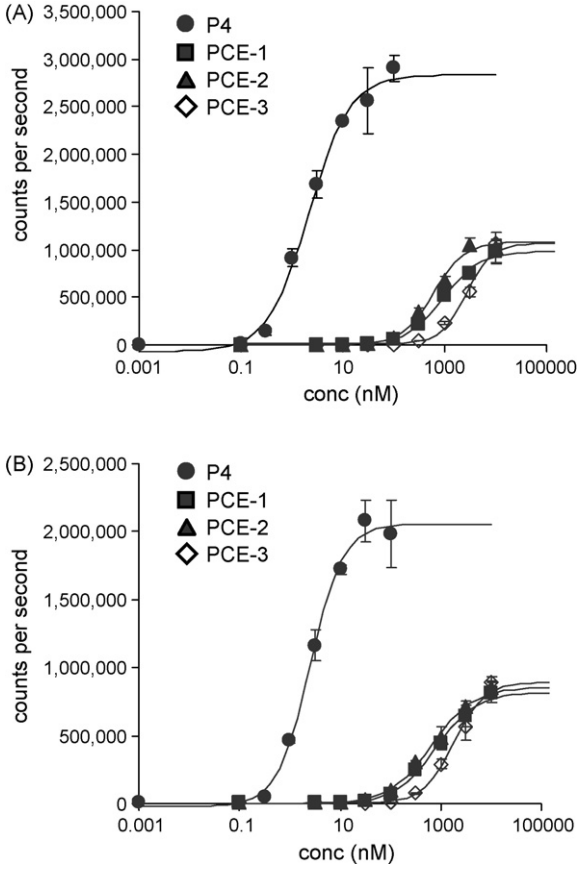


Fig. 3 – PR bound to PCEs recruits p160 coactivators.
PCE compounds are partial agonists in the PR/p160 mammalian two-hybrid assay. COS-7 cells were transfected with expression constructs for the GAL4-DBD fused to the PR-LBD, and the coactivator SRC-1 or SRC-3 fused to the VP16 activation domain, along with a $GAL4_{UAS}$-luciferase reporter construct. The cells were treated with serial dilutions of P4 or the PCE compounds. (A) Interaction of PR-LBD with steroid receptor coactivator 1 (SRC-1). EC_{50} values with 95% confidence intervals (CI) were calculated: P4, 2.1 nM (95% CI: 1.4–3.2 nM); PCE-1, 940 nM (95% CI: 730 nM–1.2 μM), 34% efficacy; PCE-2, 610 nM (95% CI: 420–890 nM), 35% efficacy; PCE-3, 2.7 μM (95% CI: 2.3–3.0 μM), 37% efficacy. (B) Interaction of PR-LBD with steroid receptor coactivator 3 (SRC-3). EC_{50} values were calculated: P4, 2.6 nM (95% CI: 1.8–3.6 nM); PCE-1, 790 nM (95% CI: 600 nM–1.0 μM), 41% efficacy; PCE-2, 650 nM (95% CI: 510–840 nM), 43% efficacy; PCE-3, 1.8 μM (95% CI: 1.5–2.2 μM), 45% efficacy.

3.3. *Steroid receptor selectivity of PCE compounds*

Cross-reactivity of steroid receptor modulators with other members of the receptor family is a common and major hurdle in the drug discovery process. To assess the steroid receptor functional selectivity of the PCE compounds, their ability to stimulate or attenuate the activity of the receptors for androgens (AR), estrogens (ER), glucocorticoids (GR) and mineralocorticoids (MR) was evaluated. The GAL4 mammalian hybrid system was used in one-hybrid (ER, GR, MR) or two-hybrid (AR, PR) format to measure transcriptional effects of PCEs in both agonist and antagonist modes. The AR and PR LBD constructs required a coactivator to produce a suitable window in these assays. The results are summarized in Table 1. No agonist activity and only partial antagonism is observed at the highest dose of 10 μM for AR, GR, MR, and ERalpha. The most significant antagonist activity was seen in the MR assay, with 74% and 70% inhibition at 10 μM PCE1 and PCE2, respectively. MR antagonism is also seen with many steroidal progestins [20].

3.4. *Selective activation of PR-A regulated genes by PCEs in T47D cells*

Having shown direct binding and selective activity of the PCE compounds for the PR, we next determined potential receptor

Table 1 – Receptor cross-reactivity. Mammalian one-hybrid assays were performed using the glucocorticoid (GR) mineralocorticoid (MR), and estrogen (ER) receptors. Two-hybrid assays were used for the progesterone (PR) and androgen (AR) receptors.

	Control	PCE1	PCE2	PCE3
PR/SRC-1				
EC50 ± SE (nM)	2.1 ± 0.40 (P4)	940 ± 110	610 ± 110	2700 ± 170
Efficacy (%)	100	34	35	37
AR/SRC-2				
EC50 ± SE (nM)	20 ± 5.6 (DHT)	–	–	–
Efficacy (%)	100	<1	<1	<1
IC50 ± SE (nM)	25 ± 5.1 (OH-FLUT)	No fit	No fit	No fit
Inhibition (%)	92	39	36	35
GR				
EC50 ± SE (nM)	4.8 ± 0.62 (DEX)	–	–	–
Efficacy (%)	100	<1	<1	<1
IC50 ± SE (nM)	0.20 ± 0.046 (RU486)	No fit	No fit	–
Inhibition (%)	99	34	31	<1
MR				
EC50 ± SE (nM)	3.0 ± 0.14 (ALD)	–	–	–
Efficacy (%)	100	<1	<1	<1
IC50 ± SE (nM)	3.9 ± 0.70 (SPIR)	>3000	>3000	No fit
Inhibition (%)	99	74	70	37
ERα				
EC50 ± SE (nM)	1.3 ± 0.13 (E2)	–	–	–
Efficacy (%)	100	<1	<1	<1
IC50 ± SE (nM)	0.88 ± 0.06 (ICI)	–	–	–
Inhibition (%)	100	2	<1	<1

The EC50 is the concentration of ligand resulting in half-maximal response. The IC50 is the concentration of competing ligand inhibiting 50% of the control agonist response when run in the antagonist mode. The concentration of each control ligand used in the antagonist modes is listed in the Section 2.

isoform-selective activation. We monitored compound induced transcript levels for two putative PR-A selective genes (PPL and Hig2) and two putative PR-B selective genes (TF and NDRG1) in T47D cells expressing both PR isoforms [21–23]. Fig. 4 shows normalized relative expression of the four isoform-selective genes following treatment with P4, PCE1 and PCE2. While P4 activates expression of all four genes, PCE1 and PCE2 selectively activate PPL and HIG2. Note that for both PCE1 and PCE2, the level of activation of PPL and Hig2 is only about 50% of the activation by P4. However, TF is regulated less than 20% of P4, and NDRG1 basal levels decrease with PCE treatment, indicative of an inverse agonist effect of these compounds on this gene.

3.5. *Global gene expression profiling of PCE compounds in T47D cells*

To more fully characterize the properties of the PCEs, we used transcriptional profiling on compound treated wild type T47D cells expressing both PR-A and PR-B. This system appears to be more appropriate for this type of evaluation than one where the PR isoforms are expressed independently, since for the majority of cases in vivo, both isoforms are co-expressed.

T47D cells were treated with PCE1, PCE2, P4 and two additional steroidal progestins – trimegestone (TMG) and norithendrone acetate (NETA), and dexamethasone (DEX) as a control treatment. It has been previously shown that the steroidal progestins predominantly regulate the same set of genes [21]. We therefore identified a set of 'progestin signature genes' as those that were regulated by steroidal progestins in this study as described in the methods. The bar chart in Fig. 5 summarizes both the total number of genes regulated by each treatment as well as the number that are 'progestin signature genes' as defined above. The PCEs are less active in regulating transcription than P4 but are very specific for the regulation of progestin signature genes (Fig. 5b).

K-means clustering [24] grouped the data into sets containing genes with similar expression patterns. A number of interesting patterns emerge including a set of genes regulated only by P4, genes regulated equally by P4 and PCE, and genes regulated by only PCE. Based on this analysis, we decided to organize the regulated genes into four groups. Representative genes for each group are shown in Fig. 5C and the list of some of the top regulated genes are shown in Table 2.

3.6. *Multiplex peptide interaction analysis of PCE bound PR*

Previous work has shown that conformation-sensing peptides can be used in a multiplex assay to obtain an interaction "fingerprint" for a nuclear receptor bound to various ligands [25]. A panel of 42 unique peptides (25-mers) representing the binding motifs of various co-activators, co-repressors, transcription factors and other interacting proteins was used to assess the conformation of PCE-bound PR LBD. Progesterone-bound PR efficiently binds many co-activator peptides, and reproducibly dissociates basal interaction with a SMRT co-repressor peptide. In contrast, PR antagonist bound PR (such as

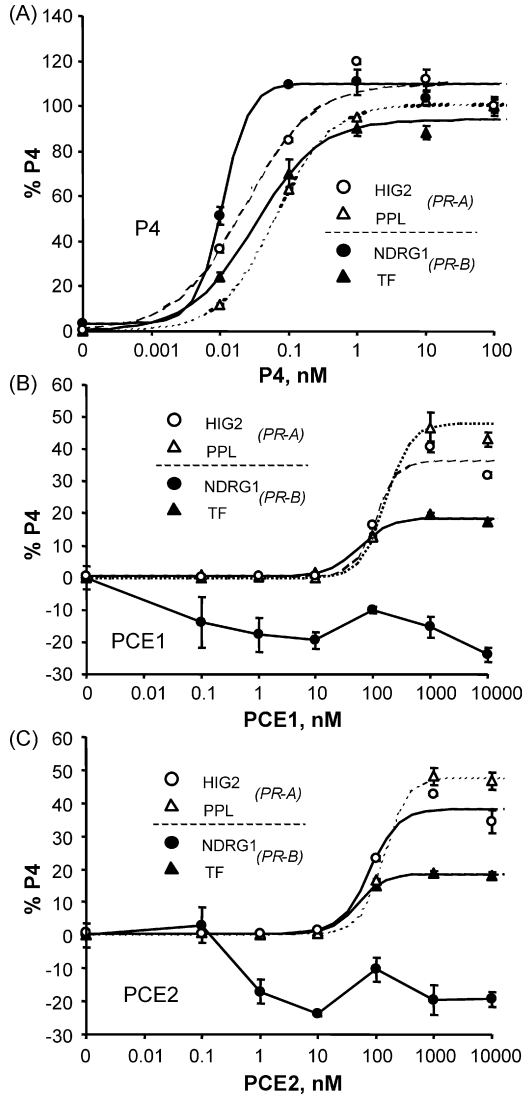


Fig. 4 – PCE compounds selectively induce PR-A regulated genes.

The expression of putative PR isoform-regulated genes in wild type T47D cells was examined using the QuantiGene branched DNA assay. The four genes used are periplakin (PPL) and hypoxia-inducible gene 2 (HIG2), two PR-A regulated genes; and tissue factor (TF) and N-myc downstream-regulated gene 1 (NDRG1), two PR-B regulated genes. For comparisons among the genes, data for each gene is expressed as a percentage of the expression obtained by treatment with 100 nM P4. (A) P4 treatment with the PPL (EC_{50} = 0.06 nM), HIG2 (EC_{50} = 0.02 nM), TF (EC_{50} = 0.03 nM) and NDRG1 (EC_{50} = 0.01 nM). (B) PCE-1 treatment measuring PPL (EC_{50} = 170 nM, efficacy = 47%), HIG2 (EC_{50} = 110 nM, efficacy = 47%), TF (EC_{50} = 60 nM, efficacy = 20%), and NDRG1 (no agonist activity). (C) PCE-2 treatment with PPL (EC_{50} = 130 nM, efficacy = 48%), HIG2 (EC_{50} = 77 nM, efficacy = 43%), TF (EC_{50} = 49 nM, efficacy = 19%), and NDRG1 (no agonist activity). Data shown are averages of duplicate wells and plots shown are representative of four independent experiments. One-way ANOVA analyses were performed on the data from the 1 and 10 μM treatments of PCE compounds, normalized to the P4 response. For the 10 μM treatments, all comparisons among the four genes were statistically significant ($p < 0.05$). For the 1 μM treatments, all comparisons were statistically significant with the exception of PPL to HIG2, the two PR-A regulated genes ($p > 0.2$).

RU-486) does not bind to the co-activator peptides but does show increased interaction with the SMRT peptide. The PCE compounds bind to both co-activator and co-repressor peptides (Fig. 6) to a greater extent than the SPRM asoprisnil with which it clusters.

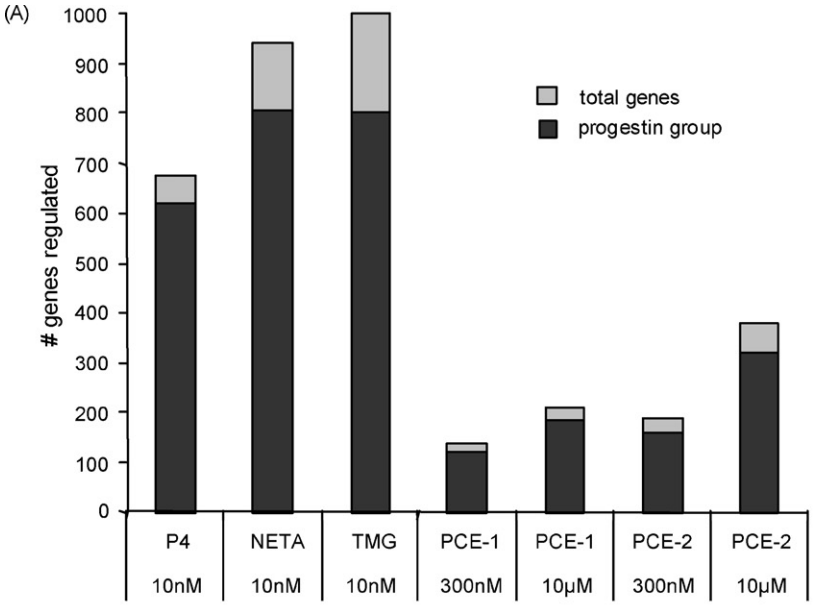
4. Discussion

In this report we describe a series of PCEs as novel PR modulators. The compounds bind to the PR with weaker affinity than steroidal progestins, and are partial agonists on the classical PRE-luciferase transcription assay in T47D cells that can be fully antagonized with the steroidal antagonist RU-486. Their partial agonist activity directly correlates with a partial efficacy in the recruitment of p160 coactivators. The PCEs are selective for PR, demonstrating no activity on ER and GR and weak antagonism of MR at very high concentration (10 μM). Using a panel of putative PR-A- and PR-B-selective genes, we show a potential isoform selective profile. A global transcriptional profiling analysis further supports a unique gene-selective transcriptional fingerprint for these molecules that is further corroborated in a peptide interaction profile demonstrating the ability of PCE-bound PR to recruit both co-activator and co-repressor peptides.

In our search for novel selective PR modulators (SPRMs), we limited our interest to non-steroidal molecules on the assumption that steroidal compounds are less likely to exhibit gene selective profiles and more likely to cross-react with other steroid receptors. That non-steroidal compounds might provide a greater likelihood for finding selective compounds comes from published data indicating highly overlapping gene sets for common steroidal progestins [21] as well as our own data with steroidal progestins in the Quantigene branched DNA assay (Berrodin and Yudt, unpublished data). Compounds were determined to be selective if they had 40% or greater efficacy relative to P_4 on at least one gene and at least a 2:1 ratio in maximal efficacy between the two genes. Using these criteria, of the initial 122 hits from our non-selective progestin screen, less than 5% were selective when tested across the four genes used in Fig. 5.

4.1. *Molecular mechanisms of partial agonism and gene selectivity*

The partial efficacy and potency observed for the PCEs in the PRE-luc reporter gene assay is also observed in the mammalian two-hybrid assay using the PR-LBD and co-activators SRC-1 and SRC-3. It appears that the reduced transcriptional efficacy in the reporter gene assay can be correlated to a reduced binding efficacy (B_{max}) with co-activators. This is further demonstrated in the multiplex peptide profiling



(B)

	Dose	Total genes regulated	Progestin genes regulated	% Progestin genes
P4	10 nM	675	619	92
NETA	10 nM	942	808	86
TMG	10 nM	999	801	80
PCE-1	300nM	138	121	88
PCE-1	10μM	211	183	87
PCE-2	300nM	187	160	86
PCE-2	10μM	381	317	83

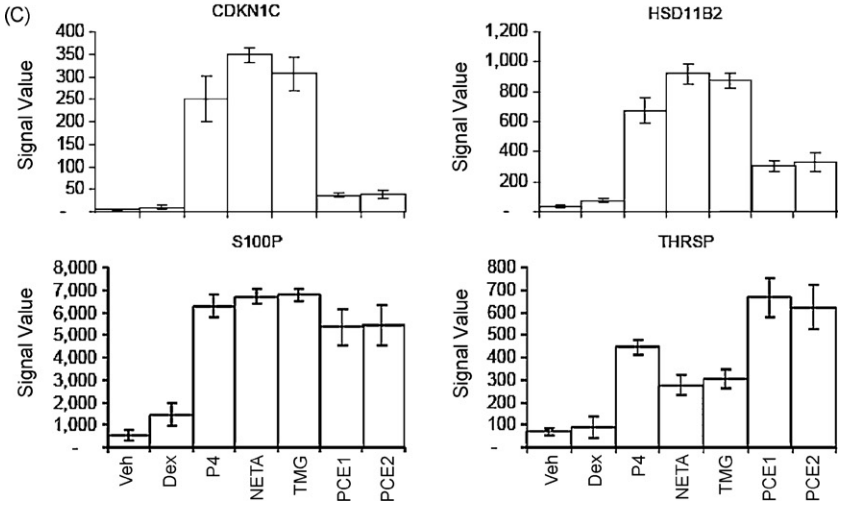


Fig. 5 – PCE compounds regulate fewer genes, but are very specific for progestin activity.
Expression profiling was used to determine the number of genes regulated by various progestins and the PCE compounds. Three replicate analyses were performed for each treatment. A gene was considered regulated if treatment resulted in a 1.7 fold or greater change in expression ($P \leq 0.01$). A progestin 'signature-gene' is defined as regulated by at least two of the three steroidal progestins used: progesterone (P4), norethindrone acetate (NETA) and trimegestone (TMG). (B) Numbers of genes in bar chart in A. indicating % P genes. (C) Top regulated gene from each of the four clusters described in Table 2. CDKN1C is cyclin dependent kinase inhibitor 1C (p57, Kip2), HSD11B2 is hydroxysteroid (11-beta) dehydrogenase 2; S100P is S100 calcium binding protein P; and THRSP is thrombospondin. Signal values were determined by microarray analysis using the default settings in GCOS1.0 (Affymetrix, Santa Clara, CA).

Table 2 – Comparison of genes regulated by P4 and PCE1, PCE2. Clustering of gene sets from the transcriptional profiling study in T47D cells with steroidal progestins and PCE1 and PCE2. The top regulated genes by P4 are shown and compared to PCE effect (% P4).

Gene name	Unigene accession #	%P4	P4 fold	PCE1 (% P4)	PCE2 (% P4)
P4 activity with little or no PCE activity					
CDKN1C	Hs.106070	100	48.4	13	13
DSIPI	Hs.522074	100	20.9	7	8
FKBP1B	Hs.306834	100	8.8	26	23
IL20RA	Hs.445868	100	6.7	2	3
SLPI	Hs.517070	100	5.5	21	14
PDK4	Hs.8364	100	5.2	24	25
DNAJB4	Hs.380282	100	5.1	22	32
SOD2	Hs.487046	100	4.8	20	21
SRD5A1	Hs.552	100	4.4	21	19
CBR1	Hs. 88778	100	3.6	16	14
SGEF	Hs.240845	100	2.9	5	11
EGF	Hs.419815	100	2.6	9	19
P4 activity > PCE (33–70%)					
HSD11B2	Hs.1376	100	20.1	47	43
GPR153	Hs.531581	100	15.9	42	44
MAFB	Hs.169487	100	11.8	42	35
PPL	Hs.192233	100	9.2	70	61
CEBPD	Hs.440829	100	7.7	45	47
TM4SF11	Hs.200821	100	7.3	42	49
FKBP5	Hs.407190	100	7.2	54	55
DUSP1	Hs.171695	100	6.6	33	29
ATP1A1	Hs.371889	100	6.1	44	43
MPHOSPH10	Hs.201676	100	6.0	42	46
CLDN8	Hs.162209	100	5.8	33	38
NET1	Hs.25155	100	5.8	44	46
P4HA2	Hs.519568	100	5.6	62	64
MT1X	Hs.374950	100	5.5	57	66
MT1X	Hs.374950	100	3.9	65	101
ST3GAL4	Hs.504251	100	5.0	55	60
ADD3	Hs.501012	100	4.3	49	52
ANKRD15	Hs.493272	100	4.0	47	51
CITED1	Hs.40403	100	3.9	44	45
ATP1B1	Hs.291196	100	3.9	62	71
CD9	Hs.114286	100	3.7	56	56
P4 activity equivalent to PCE activity					
S100P	Hs.2962	100	12.5	85	84
ABLIM3	Hs.49688	100	7.9	117	126
PACSIN1	Hs.520087	100	5.7	81	84
ACSL1	Hs.406678	100	5.6	86	96
CITED4	Hs.355820	100	5.1	112	105
SCUBE2	Hs.523468	100	4.6	89	86
CRAT	Hs.12068	100	3.8	82	77
KIBRA	Hs.484047	100	3.7	98	79
PCE activity greater than P4 activity					
THRSP	Hs.546310	100	6.4	147	159
ARF4L	Hs.183153	100	3.0	161	152
BLNK	Hs.444049	100	2.2	159	145
EDG8	Hs.501561	100	1.9	269	327

analysis where many co-activator-derived peptides have a similar reduced interaction potential (efficacy) with PCE bound to PR. However, it is clear from the transcriptional profiling analysis that this reduced interaction efficacy on one or more co-activators is insufficient to predict functional response on all genes. For example, the data in Table 2 show many genes are fully activated by PCE compounds and others are not regulated at all. Furthermore, the PCE-induced PR-peptide interaction profile includes significant recruitment of a co-repressor peptide and divergence from asoprisnil and progesterone in relation to NRIP1 and other transcription factor-derived peptide interactions indicating a unique PCE-PR conformation. Future studies analyzing a diverse group of full-length interaction partners will likely further elucidate and ultimately aid in understanding and predicting the biological importance and implications of these interactions.

The expression profiling study demonstrates how gene regulation by steroid hormone receptors is a very context-

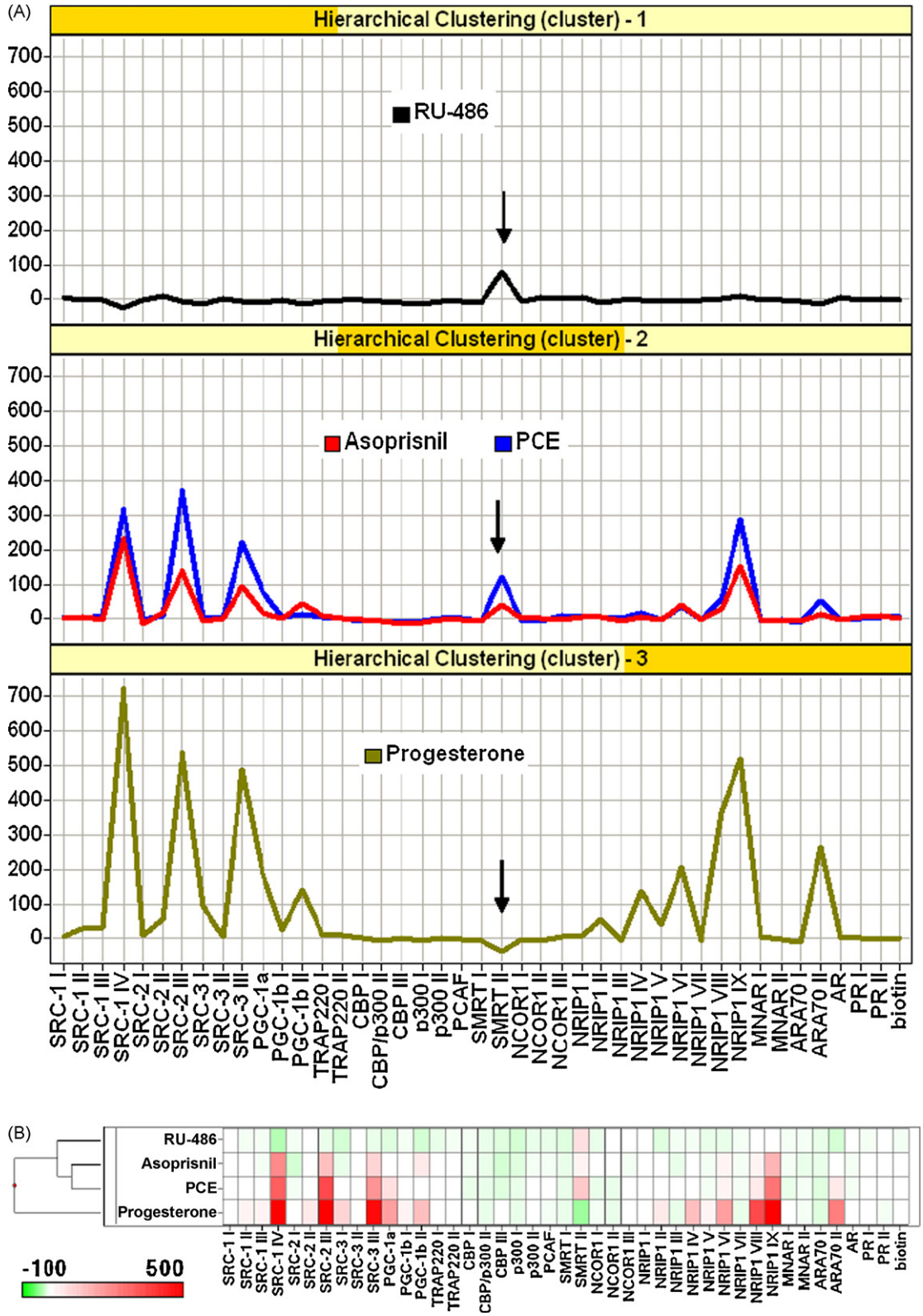


Fig. 6 – Peptide interaction profile for PR.

The multiplex PR cofactor assay was performed using the 43 Luminex beads coupled to 42 peptides or D-biotin as described in Table S1. 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. Red indicates an increase and green a decrease from basal peptide interaction.

dependent process. Similar studies have been done with SERMs [26] but have yet to sufficiently predict biological outcomes. At present, a large amount of data obtained from global expression profiling cannot be adequately interpreted because of the difficulty in translating to relevant biology.

The dynamics of any particular gene regulation as a result of different ligand-PR complexes cannot be captured in the single time point of our expression studies here. It is also possible that some differences may be the result of altered expression of secondary genes by modulation of primary gene levels. For example, several early factors involved in cell cycle such as CDKN1, EGF are more strongly regulated by P4, and may have greater effects on secondary genes than the PCEs. Perhaps some of the 'tissue-selective' actions of nuclear receptor modulators in general can be traced to differential effects on a subset of directly regulated 'early' genes.

Our initial aim was to use a gene-selective screening approach to quantify isoform selectivity by measuring potency and efficacy of putative isoform-selective gene endpoints in the same cell. We quickly realized several complications in this approach that may inadvertently bias results. First, we chose genes that were initially described as isoform-selective in altered cell lines [23] that were also strongly regulated in our T47D cells expressing both receptors. We made the assumption that the isoform selectivity is present in the parental wild type T47D cell line expressing both PR isoforms. Of the four genes used in Fig. 4, only tissue factor (TF) has any known physiological significance in hormone action, i.e. regulation of hemostasis in the endometrium [27], so no in vivo data are available to support the importance of these endpoints in progestin biology. We also know that selectivity is certainly not equivalent to specificity and that a gene may be 'selectively' regulated to a greater extent by one isoform, but both isoforms can modulate its expression to some significance. Although the PCEs regulate a set of four genes consistent with reported isoform selectivity [22,23], the results from our full array do not support a completely PR-A-selective profile. For example ACSL1 (also known as FACL1) is reported to be a PR-B-selective gene by the same criteria we chose the others [22], yet is regulated by the PCEs with almost 100% the efficacy of P4 – certainly not what one would expect with a PR-A-selective modulator. One may therefore choose to call them 'context-selective modulators' (COSMOs). Future studies, particularly in vivo experiments, will discern the nature of these compounds, whether they are a truly novel class of selective modulators or simply weaker or partial agonists that manifest in gene selective properties. Either way, the development of PR modulators with decreased side-effect profiles, regardless of the molecular mechanism, remains a clear unmet medical need and an important women's health issue.

The sum of nuclear receptor research over the past decade or so has led to the appreciation of the so-called ''4 C's'' – compound, conformation, co-regulators and context [28]. Essentially, the nature of the compound binding to the receptor determines the tertiary structure, or conformation, of the liganded complex. This complex then serves as a platform for interactions with co-regulators (the quaternary structure), which in the dynamic context of particular gene promoter elements, drives the transcriptional regulation of the target gene.

In this manuscript we describe the identification of a new compound class that induces a unique PR conformation and co-regulator peptide interaction profile. We demonstrate in T47D cells how these features have broad functional effects on gene regulation that appears to depend on the context of the specific promoter in question. The results provide mechanistic insights to the modulation of PR action by novel ligands that may ultimately be reflected in expanded therapeutic options and a better understanding of the limitations of drugs targeting this receptor. The challenge remains in predicting a desired biology based on a broad array of both gene regulation and protein/peptide interactions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.10.016.

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