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Global gene expression profiling of progesterone receptor modulators in T47D cells provides a new classification system

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ABSTRACT

Progesterone receptor modulators (PRMs) play an important role in women's health. They are widely used in oral contraception or hormone therapy, and provide an attractive treatment approach for gynecological disorders such as uterine leiomyomas, endometriosis or breast cancer. Due to the broad range of activities, various studies were conducted to assess progesterone receptor antagonists (PAs) and selective progesterone receptor modulators (SPRMs) with respect to progesterone receptor (PR) agonistic and antagonistic activities in vivo. These properties are not always adequately reflected in classical in vitro models, especially differences in the agonistic potential of SPRMs, such as asoprisnil, J1042, and J912, and mixed antagonists, such as mifepristone, are not sufficiently substantiated. The effects of PRMs upon gene expression in progesterone target tissues such as breast epithelium and uterus are poorly understood. This study compares the properties of PR ligands using mammalian two-hybrid assays and gene expression profiling. The protein–protein interaction analyses in HeLa cells provide for specific ligand-induced PR conformations, whereas Affymetrix GeneChip HG-U133Plus2.0 analyses in T47D breast cancer cells indicate the transcriptional activity on the level of target genes. The analyses comprise the pure agonist R5020, the non-steroidal PR modulator PRA-910, SPRMs (J1042, asoprisnil, J912), the mixed antagonist mifepristone, classical antagonists (onapristone, ZK 137316) and the pure antagonist lonaprisan to consider all types of ligands described before. Marginal differences were identified in coactivator interaction profiles at all, but significant differences between SPRMs and PR antagonists (PAs) were observed in recruiting the LXXLL-motif containing peptide (LX-H10), very similar to in vivo activities in endometrial transformation in the rabbit (McPhail test). Global gene expression profiles demonstrated progesterone-independent effects for all PR modulators examined and emphasised similarities of asoprisnil and J1042 compared to J912 and all types of PR antagonists. In summary, the data support the popular concept of PR modulator classification in agonists, selective progesterone receptor modulators, mixed and pure antagonists. It further refines previous classification models and accentuates unique effects for each PR modulator.

1. Introduction

Progesterone is a key regulator of normal female reproductive functions, such as ovulation, uterine and mammary gland development, decidualisation, implantation and maintenance of pregnancy [1,2]. The diverse effects of progesterone on the female reproductive target tissues are mediated by the progesterone receptor (PR), a member of the nuclear receptor (NR) family of ligand-dependent transcription factors [3,4]. Human PR is expressed from a single gene as two proteins, PR-A and PR-B [5], with distinct functional activities in vitro and in vivo [6,7]. Both isoforms directly bind to DNA at progesterone response elements (PRE) and recruit coregulators which enhance or repress transcription via interaction with the general transcription apparatus [8–11]. A number of coactivators, including the p160 family, have been described to be important for PR activity. Many of these proteins contain a signature LXXLL motif (NR box), which is necessary and sufficient

Abbreviations: FBS, fetal bovine serum; FC, fold change; GO, gene ontology; HRT, hormone replacement therapy; NR, nuclear receptor; PA, progesterone receptor antagonist; PCA, principle component analysis; PR, progesterone receptor; PRM, progesterone receptor modulator; SPRM, selective progesterone receptor modulator.

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to permit PR interaction [12]. Hydrophobic binding pockets for cofactors are induced by ligands which create a conformational change in the PR [13], thus modulating transcriptional activity.

Clinically, the PR is an important therapeutic target. Many ligands have been synthesised ranging from pure agonists (e.g. the synthetic progesterone-analogue R5020), which mimic progesterone effects, to pure antagonists (e.g. lonaprisan (ZK 230211)), which completely reverse progesterone effects. Currently, all PR modulators (PRMs) used clinically are steroids, although novel non-steroidal compounds with distinct biological properties have been generated in recent years [14,15].

Synthetic PR ligands are widely used for oral contraception and menopausal hormone replacement therapy (HRT) [16–18]. In particular, PR antagonists and SPRMs have been demonstrated to be efficacious in the treatment of breast cancer [19,20] and gynecological disorders such as endometriosis [21,22] and uterine leiomyomas [23,24].

The term SPRM is designated to compounds with mixed agonistic/antagonistic properties depending on the specific tissue type. The molecular mechanism of SPRM-induced gene regulation is poorly characterised. The most conclusive evidence for a partial agonistic activity of SPRMs, such as J1042, asoprisnil or J912, comes from studies in the McPhail test. This test assesses the endometrial proliferation and transformation in immature rabbits. In contrast to PR antagonists such as mifepristone or onapristone, which behave as pure antagonists in this assay, SPRMs display partial agonistic properties [25,26]. Other in vivo models, such as compound treatment induced alterations of uterine and vaginal morphology in guinea pigs, show a mosaic of progesterone agonist and antagonist effects for SPRMs [26–28]. Paradoxically, the guinea pig as a very sensitive in vivo model for residual progestagenic activity shows partial agonism for the antagonist mifepristone, too [26]. Recent studies further reveal partial agonism of mifepristone in ovariectomised mice and lead to its designation as a mixed antagonist [29]. This heterogeneity with respect to the ratio of PR agonistic to PR antagonistic activity in vivo, which is observed for SPRMs, and partially also for mifepristone, is difficult to reconcile with in vitro data. However, it suggests a distinction between SPRMs, mixed antagonists such as mifepristone and pure antagonists such as lonaprisan. Several years ago [25,30], PR ligands were classified based on in vitro activities such as DNA binding, transactivating activity and behavior in the presence of protein kinase A activators (cAMP). A more profound and specific characterisation of each type of ligand is highly desirable.

In order to elucidate PR modulators mode of action on the one hand, and to refine previous classification systems on the other hand, firstly, a coactivator interaction study was performed to provide for specific ligand-induced PR conformations. Secondly, a global gene expression analysis using Affymetrix GeneChip HG-U133Plus2.0 arrays was conducted to compare individual ligand effects on trancription. The analyses include the pure PR agonist R5020, the non-steroidal PR modulator PRA-910, designated SPRMs (J1042, asoprisnil and J912), the mixed antagonist mifepristone, classical antagonists (onapristone, ZK 137316) and the pure antagonist lonaprisan (ZK 230211) in order to consider representative members of all ligand classes described before. We observed distinct interaction profiles of SPRMs and antagonists for the LXXLL-motif containing peptide (LX-H10) and fundamental differences in the gene expression profiles of T47D cells after treatment. Furthermore, candidate marker genes for SPRMs were identified and confirmed by quantitative real-time PCR. The data support and refine previous classifications and suggest it may be possible to distinguish PR ligands based on unique gene expression profiles.

2. Materials and methods

2.1. Cell culture

The human breast carcinoma cell line T47D was obtained from the American Type Culture Collection (ATCC; LGC Promochem, Wesel, Germany). The cells were maintained in phenol red-free RPMI 1640 (PAA Laboratories, Cölbe, Germany) supplemented with 10% FBS and 100 U/ml penicillin, 0.1 mg/ml streptomycin and 4 mmol/L L-glutamine (all from Invitrogen, Karlsruhe, Germany). The human steroid receptor-free neuroblastoma SK-NM-C cell lines stably transfected with the human PR-A or PR-B and the mammalian mammary tumor virus promoter linked to the LUC reporter gene, resulting in clone C23.43 and VIII-1.1, were generated in the lab of U. Fuhrmann (Bayer Schering Pharma AG, Berlin, Germany) [31,32]. Cells were maintained in minimum essential medium (MEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 4 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate (PAA Laboratories). The human cervix carcinoma cell line HeLa was obtained from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany). The cells were maintained in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Fetal bovine serum (FBS) was purchased from BioWhittaker, Inc. (Walkersville, MD, USA). All cell lines were cultured at 37 °C with 5% CO_2.

2.2. Treatments for gene expression analysis

T47D cells (1,000,000 per dish) were seeded onto 60 cm^2 dishes in 10% FBS containing phenol red-free RPMI 1640 and were allowed to attach for 24 h. Cells were then washed twice with PBS and medium was changed to phenol red-free RPMI 1640 containing 5% charcoal-stripped FBS. After hormonal starvation for 20 h, equipotent concentrations of PR modulator (10 nM, except PRA-910 in a concentration of 100 nM) or vehicle (0.1% DMSO) was added for 8 h. PR antagonists (lonaprisan (ZK 230211), ZK 137316, onapristone, mifepristone (RU486)) and SPRMs (J912, mesoprogestin (J1042), asoprisnil (J867)) as well as standard agonist promegestone (R5020) and non-steroidal PR modulator PRA-910 were synthesised by the Department of Medicinal Chemistry at Bayer Schering Pharma Research (Berlin, Germany).

2.3. Transactivation assay

SK-NM-C VIII-1.1 cells (10,000 per well) were seeded onto 96-well dishes in MEM containing 5% charcoal-stripped FBS. After 48 h PR ligands were added and incubation was continued for 24 h. To determine agonistic activity, cells were cultured in the presence of increasing concentrations (10^{-11} to 10^{-6} M) of R5020, PRA-910, PR antagonists (lonaprisan, ZK 137316, onapristone, mifepristone) and SPRMs (J912, J1042, asoprisnil). As a negative control for reporter gene induction, cells were cultured in medium containing vehicle (0.1% DMSO). To determine antagonistic activity, cells were treated with increasing concentrations of PR modulator in combination with 100 pM R5020. Medium was removed and 160 μl of luminescence reporter gene assay system Steadylite HTS (Perkin Elmer, Inc., Waltham, MA, USA) was added to each well. Plates were incubated for 15 min at room temperature to ensure complete cell lysis and luciferase reaction, and were read in a TopCount NXT (Perkin Elmer, Inc.). LUC expression was given as a normalised response value relative to the maximal LUC expression produced by the reference agonist R5020. Data were analysed to obtain the maximum efficacy, EC_{50} and IC_{50} values using Sigma Plot 8.0 software.

2.4. Mammalian two-hybrid assay

HeLa cells (10,000 per well) were seeded onto 96-well dishes in phenol red-free DMEM containing 5% charcoal-stripped FBS. After 24 h cells were transfected with FuGENE 6 following the manufacturer's specifications (Roche, Mannheim, Germany). Transfection mix (10 μl) containing 4 ng pCMV-GAL4/cofactor, 2 ng pCMX-VP16/PR or pCMV-NFκB/PR and 75 ng pFR-luc expression plasmid (Stratagene; La Jolla, CA, USA) in threefold volume of FuGene 6 filled up with OptiMEM (Invitrogen, Karlsruhe, Germany) was added to the medium and cells were permitted to recover overnight. Medium was removed and cells were treated with R5020, PRA-910, PR antagonists (lonaprisan, ZK 137316, onapristone, mifepristone) and SPRMs (J912, J1042, asoprisnil) in increasing concentrations (10^{-12} to 10^{-7} M) for 24 h. Cells were then subjected to the transactivation assays procedure to obtain luciferase expression as described in Section 2.3.

2.5. Affymetrix GeneChip® expression profiling experiments

Total RNA of T47D cells was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. A DNase I (Qiagen) digestion step was included to eliminate genomic DNA. The quality of the total RNA was checked for integrity with RNA LabChips on the Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., USA) and for concentration on the Peqlab NanoDrop (Peqlab Biotechnology, Erlangen, Germany). Total RNA in an amount of 2 μg was used to prepare biotinylated and fragmented cRNA following the instruction of the Affymetrix One-Cycle Target Labeling protocol and individual samples were hybridised on the Affymetrix GeneChip HG-U133Plus2.0 arrays. Chips were scanned using a GeneChip Scanner 3000 7G, and scanned images were extracted using the Affymetrix GCOS Software. In total, $N = 55$ arrays were performed, as $n = 5$ biological replicates for all treatments and $n = 10$ biological replicates for DMSO controls were applied to Affymetrix GeneChip hybridisation and analyses. Expression analyses were performed using the Expressionist Pro 4.0 software (Genedata AG, Basel, CH). The quality of the data files (CEL format) containing probe level expression data were analysed with the Expressionist Refiner software. Subsequently, refined CEL files were condensed with MAS5.0 and LOWESS normalised using all experiments as reference.

2.5.1. Unsupervised analysis

Principle Component Analysis (PCA) showing the relationships between individual samples was performed using the Expressionist Analyst Pro 4.0 software.

2.5.2. Supervised analysis

The gene expression data were subjected to pairwise comparisons using the Expressionist Analyst Pro 4.0 software (Genedata AG, Basel, CH). Statistical analyses included pairwise comparisons between control samples treated with vehicle (0.1% DMSO) and compound treated samples. Probe sets were regarded to be regulated if they were outside of the triangular region in the Volcano plot (a plot of fold change (FC) versus *t*-test *p*-value) with the corner values of an fold change of five or higher and a *t*-test *p*-value analogous to ST *Q*-value <0.01.

2.6. TaqMan® quantitative RT-PCR assays

Total RNA from T47D cells was prepared using QIAshredder and RNeasy Mini Kit (Qiagen). Double-stranded cDNA was synthesised from 5 μg total RNA using SuperScript III First-Strand Synthesis System (Invitrogen). The expression levels of selected SPRM-regulated genes (FC > 2) were analysed using TaqMan® Gene Expression Assays from Applied Biosystems (#Hs00827141_g1 (AMIGO2, **NM_181847.3**), #Hs01651960_m1 (DEFB32, **NM_207469.1**), #Hs00173681_m1 (GPRC5A, **NM_003979.3**), #Hs00158421_m1 (KCNJ3, **NM_002239.2**), #Hs00174969_m1 (PTHLH, **NM_198964.1**), #Hs00232313_m1 (ZBTB16, **NM_001018011.1**); Foster City, CA, USA). Gene-specific primers and probes were used with Platinum qPCR SuperMix-UDG (Invitrogen) and were incubated at 50 °C for 2 min followed by 10 min at 95 °C, and then 40 cycles of PCR as follows: 95 °C for 15 s, then 60 °C for 1 min in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Data were analysed using the Sequence Detector Version 2.0 software (Applied Biosystems) and normalised to the cycle threshold (CT) value of the housekeeping gene Cyclophilin A (TaqMan® Gene Expression Assay from Applied Biosystems, #Hs99999904_m1 (PPIA, **NM_021130.3**)) using the ΔΔCT-method. Fold changes were determined by pairwise comparison of treatment versus vehicle.

3. Results

3.1. Antagonistic activity in a cellular transactivation assay

In general, the agonistic and antagonistic activity of synthetic PR modulators can be characterised by cellular transactivation assays. SK-NM-C cells stably expressing the full-length PR-isoform B and a luciferase reporter gene driven by the mammalian mammary tumor (MMTV) promoter were used for this analysis. The ligand-induced PR transactivation was tested for the selected groups of PR antagonists and modulators, including the non-steroidal ligand PRA-910 [33–35], the previous described selective progesterone receptor modulators (J1042, asoprisnil, J912), which exhibit tissue selective agonistic activity in vivo in some experimental settings [26], the mixed antagonist mifepristone, that exerts agonistic potential with respect to specific cellular cues [29], classical antagonists (ZK 137316, onapristone) and the pure antagonist lonaprisan. Cells were treated with increasing concentrations of the PR ligand alone for agonistic profiles or in the presence of 100 pM R5020 for antagonistic profiles. All activities were calculated relative to standard agonist R5020 or standard antagonist mifepristone, respectively. No differences in transactivating activity between pure antagonists, mixed antagonists and designated SPRMs were observed. Neither mifepristone, ZK 137316, onapristone, lonaprisan (Fig. 1A, C; Table 3), nor J1042, asoprisnil, and J912 (Fig. 1B, D; Table 3) exhibited any agonistic activity, but full antagonistic effects in this type of assay. The SPRMs displayed antagonistic potency comparable to mifepristone. However, PR antagonists differed in potency and lonaprisan was demonstrated to be the most potent antagonist. In contrast, PRA-910 displayed reduced antagonistic efficacy (62%) and partial agonistic effects (52% efficacy, Table 3). The potency of PRA-910 was markedly lower compared to the standards mifepristone or R5020, respectively (Fig. 1A, C).

3.2. Different interaction profiles with the LXXLL-motif of coactivators

Activation or inhibition of progesterone receptor action is a consequence of PR modulator-induced alterations in the receptor conformation. Conformational changes are responsible for the formation of a receptor surface with binding pockets and thus affect the ability of the PR to interact with the DNA, but also with coactivators and corepressors. Using a mammalian two-hybrid assay system, the interaction properties of full-length PR isoforms and an assortment of cofactors were analysed for selected PR modulators. In this system, plasmids expressing the full-length human PR fused to the strong transactivation domain of NFκB in pCMV-AD or the strong transactivation domain of VP16 in pCMX, respectively,

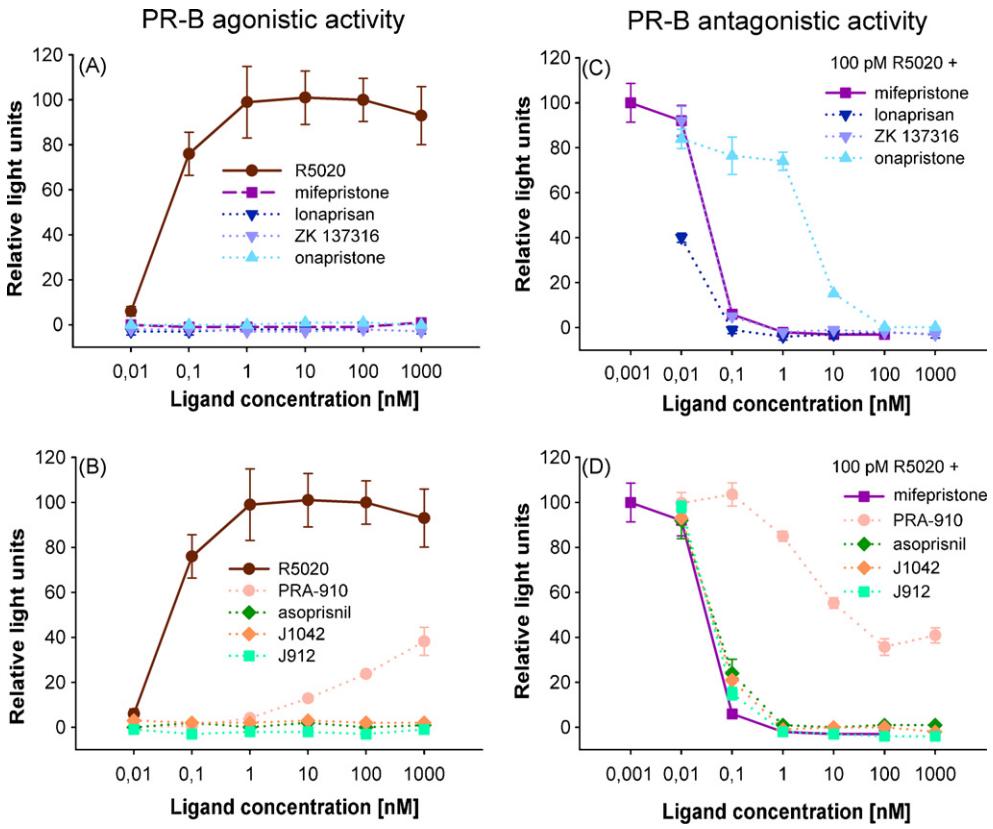

Fig. 1. Similar transcriptional activity of PR antagonists and SPRMs in transactivation assays in SK-NM-C VIII-1.1 cells. (A and B) Agonistic activity of ligand-bound PR-B. Cells were treated for 24 h with vehicle or increasing concentrations (10^{-12} to 10^{-7} M) of PR modulator. (C and D) Antagonistic activity of ligand-bound PR-B. Cells were treated for 24 h with 100 pM R5020 in combination with increasing concentrations (10^{-12} to 10^{-7} M) of PR modulator. Results are triplicates of three independent experiments.

were used in combination with plasmids expressing the respective cofactor fused to the GAL4-DNA binding domain in pCMV-BD. The ability of the PR/NFκB or PR/VP16 fusion to activate transcription from a GAL4-responsive reporter plasmid (pFR-Luc) was utilised as a readout for the interaction between PR and the cofactor. Analysis of human cell lines with low or no endogenous expression of progesterone receptor isoforms A and B (HeLa, SK-NM-C, PC-3, MCF-7, HEK-293 cells) identified HeLa cells as the cell line with the lowest expression level of selected coactivators and corepressors on both mRNA and protein level. Furthermore, HeLa cells were identified to have low basal GAL4-mediated transcriptional activity (data not shown).

Tested cofactors included members of the p160 family of steroid receptor coactivators (SRC-1, SRC-2 and SRC-3) [36–38] in full-length and truncated forms (ligand binding domain). In a phage peptide library of $(X)_7LxxLL(X)_7$ peptides differing in sequences flanking the LxxLL core motif of coactivators which is critical for nuclear receptor interaction, the LX-H10 peptide was identified to be useful in determining receptor selectivity [9,39]. Therefore, PR interaction with the LX-H10 peptide was analysed to investigate PR isoform-specific recruitments. The corepressors NCoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) were used in truncated form (interaction domains) [40].

For all types of ligands interactions of PR isoforms A and B with coactivators and corepressors were observed (data not shown). These interactions were not significantly different for designated SPRMs (J1042, asoprisnil, J912) and all types of antagonists (mifepristone, lonaprisan, ZK 137316, onapristone), except for LX-H10 peptide interaction. Antagonist-bound PR-B did not exhibit any recruitment of LX-H10 peptide (Fig. 2A; Table 3), in contrast, SPRM-bound PR did (Fig. 2B; Table 3). The maximum efficacy of LX-H10 peptide interaction observed for asoprisnil- as well as J1042-bound PR-B was about 100% and similar to agonist R5020, whereas J912-induced recruitment of LX-H10 was significantly lower (14%). Thus, in the mammalian two-hybrid in vitro assay, asoprisnil and J1042 differed from PR antagonists and J912. Secondary, the potency of recruiting activity was stronger for R5020- than for SPRM-bound PR. An isoform-specific recruitment of LX-H10 as reported by Giangrande et al. [9] was not observed.

3.3. Gene expression profiles

In order to assess whether the reported differences in in vivo activities and the divergent effects on PR-LX-H10 peptide interaction directly reflect on the gene expression profiles of PR modulators, RNA isolated from T47D cells treated with the different PR ligands was hybridised onto Affymetrix GeneChip HG-U133Plus2.0 arrays. The study was performed in the progesterone-responsive human mammary T47D cell line. T47D cells express high levels of functional PR-B and -A under basal conditions which allows analysis of progestin action in the absence of estradiol. Furthermore, reference studies of other global PR ligand expression profiling studies in T47D cells have been published [41–44] and offered a thorough basis for the experimental design. Thereby, the selected time point of 8 h of treatment was determined from previous experiments to provide for consistently high gene expression of known PR target genes. Cells were treated with concentrations of PR ligand leading to full efficacy in transactivation assays (10 nM, except for the non-steroidal PR modulator PRA-910 (100 nM); see Fig. 1). The initial simultaneous assessment of the gene expression values in treated cells (see Section 2.5) revealed uniform overall expression of transcripts, indicating expression profiles which were consistent with established standards for gene expression analyses.

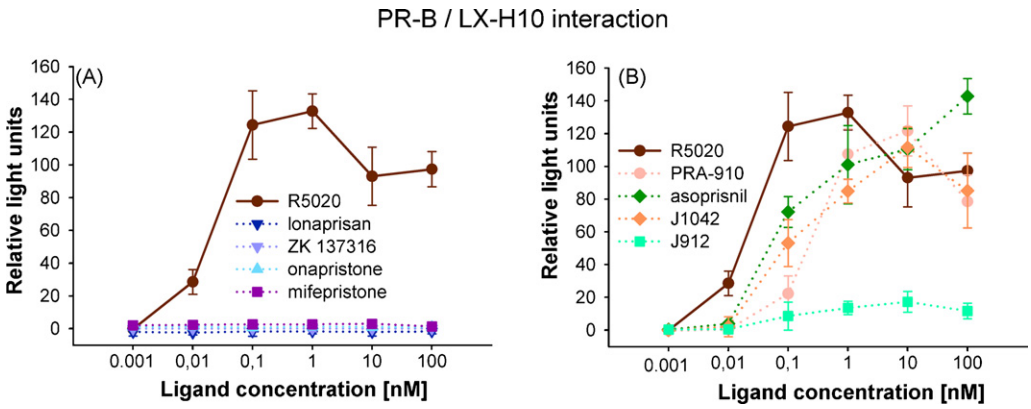

Fig. 2. Dissociated effects of SPRMs and PR antagonists for the induced PR-B interaction with the LXXLL-motif bearing LX-H10 peptide in a mammalian two-hybrid assay system in HeLa cells. Transiently transfected cells were treated for 24 h with vehicle or increasing concentrations (10^{-12} to 10^{-7} M) of PR antagonist (A) or SPRM (B). Results are triplicates of three independent experiments.

3.4. Supervised and unsupervised analyses

The principle component analysis (PCA) revealed that samples clustered according to treatment and that the different treatment groups were separated from each other (Fig. 3A, B). PCA depicts the variance in gene expression profiles between samples. On the three-dimensional graphic, the distance between two plotted spheres is inversely proportional to the degree of similarity between the gene expression profiles of these two groups, using all probe sets on the Affymetrix GeneChip HG-U133Plus2.0 array. Firstly, all vehicle controls ($n = 10$) and all treatment samples (each $n = 5$) formed distinct cluster. The largest variance between samples from different treatments was observed for R5020 and vehicle control, the smallest variance for antagonists and vehicle control (Fig. 3A). PR modulator PRA-910 samples were most similar to R5020, but had a lower variance to vehicle control samples. Samples from J1042 and asoprisnil treatment were observed to cluster collectively in a fourth group, slightly apart from all types of PR antagonists. Interestingly, J912 plotted much closer to antagonists than to J1042 and asoprisnil samples. In another angular field, a separation with low, but significant variance was also observed between J912, mixed, classical and pure antagonist samples (Fig. 3B).

Hierarchical clustering analysis was conducted using gene expression data of replicate treatment samples based on pairwise comparisons (treatment versus vehicle) which resulted in a combined list of 199 probe sets, which are significantly regulated by all types of antagonists, J912, asoprisnil and J1042 (fold change >2 and *t*-test *p*-value analogous to ST *Q*-value <0.01). In cluster areas III, IV and VI, R5020 and PRA-910 samples segregate from antagonists and SPRMs according to trend and intensity of gene regulation (Fig. 4). Partially, J1042 and asoprisnil treated samples cluster with R5020 and PRA-910 samples as indicated by areas II and V, confirming our previously reported observation of separation from antagonists in PCA (Fig. 3A). J912 treated samples were observed to cluster with antagonists predominantly and therefore, segregate from J1042 and asoprisnil. Nearly similar effects for all types of ligands were found in cluster areas I, VII and VIII, except onapristone acting as a very particular PR ligand.

3.5. Individual expression profiles

To specify the progesterone-independent effects of PR modulators as seen in the PCA, pairwise comparisons (vehicle versus treatment) were conducted. A total of 1981 significantly regulated genes was identified for R5020. In Table 1, the PR modulator-induced expression profiles were organised by similarity to R5020, based on the variance in the PCA (Fig. 3A). The entire number of significantly regulated genes in absence of progesterone is presented in the diagonal. Genes identified in two corresponding sets (column/row) were shown in the upper right. For example, eleven mifepristone-regulated genes were also regulated in J912-treated cells. Genes exclusively identified in one of the two sets (column/row) are shown in the lower left. The highest similarity was identified for genes regulated by J1042 and asoprisnil (54% overlap) and for genes regulated by the pure antagonist lonaprisan and the classical antagonist ZK 137316 (77% overlap). In general, all SPRMs

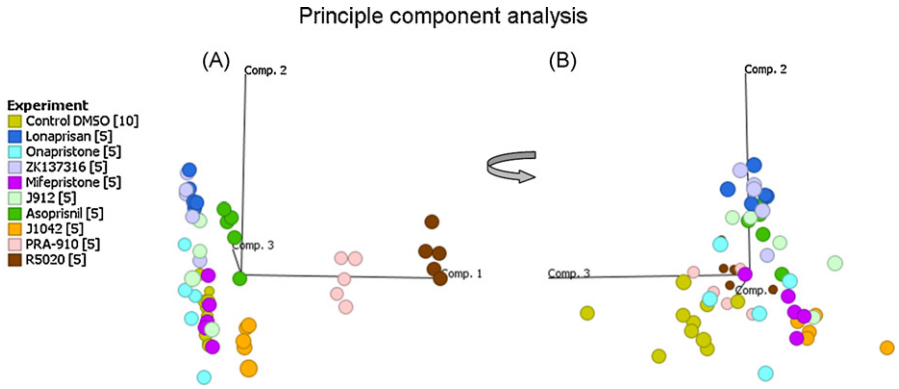

Fig. 3. Principle Component Analysis (PCA) of T47D cells after PR ligand treatment uncovering relationships between individual samples. Samples are coloured according to PR ligand treatment and the number of biological replicates is given in brackets. Each plotted sphere represents the expression profile of an individual sample based on the projection of the data on the first three principal components, accounting for most of the variability in the data (labeled axes). The PCA is shown for two different angular fields (A, B; 90°-rotation on component 2).

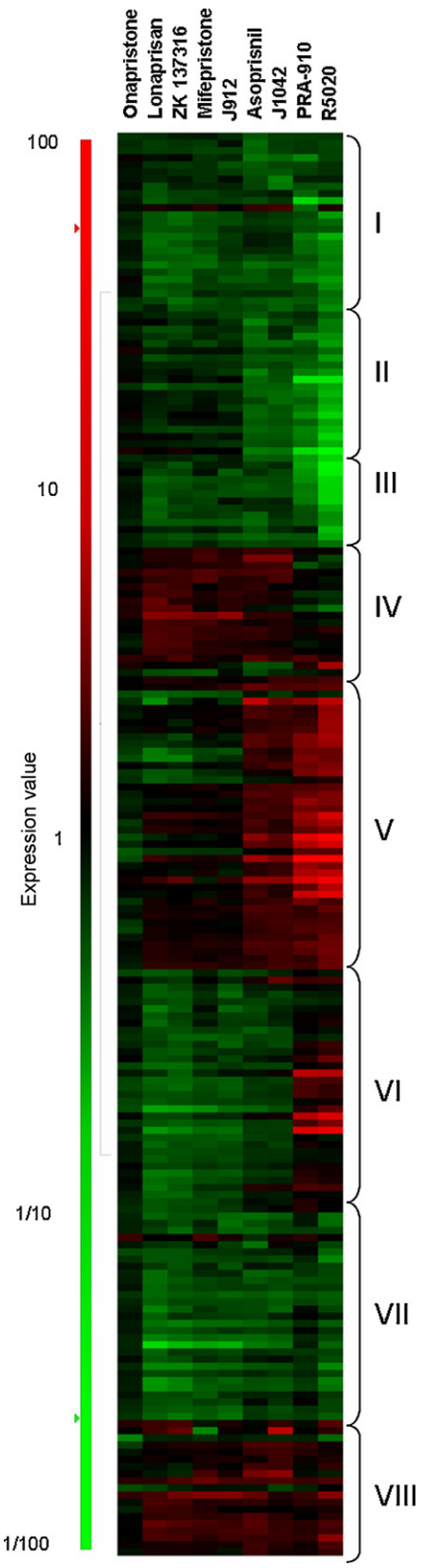

Fig. 4. Hierarchical clustering analysis of probe sets significantly regulated by PR antagonists and SPRMs. Fold change >2 and *t*-test *p*-value analogous to ST *Q*-value <0.01. Red: up-regulated versus vehicle control; green: down-regulated versus vehicle control.

and all antagonists displayed an overlap in regulated genes except for onapristone. The transcriptional effect of onapristone was minimal whereas other PR antagonists, J1042, asoprisnil and J912 clearly exhibited progesterone-independent effects on gene expression in T47D cells (Table 1).

The PR antagonists lonaprisan and ZK 137316 as well as J912 predominantly down-regulated gene expression (>80% of regulated genes). Mifepristone and asoprisnil tended to result in a down-regulation of genes (~60% of regulated genes). In contrast, more than 58% of regulated genes were up-regulated after treatment with J1042, PRA-910 and R5020.

3.6. SPRM regulated gene transcripts

J1042 and asoprisnil showed very similar profiles in previous analyses. In addition, the Venn intersection analysis identified 29 unique probe sets significantly changed in expression by asoprisnil and J1042, but not by J912, mixed antagonist mifepristone, classical antagonists ZK 137316 and onapristone or the pure antagonist lonaprisan (Table 2). Gene ontology (GO) assessment and sorting of differentially expressed genes into similar functional categories indicated that the products of these genes are mainly involved in signal transduction and membrane effects, transcriptional and nucleic acid processing activities, but also hormone activities, cell cycle control, metabolic processes and defense response. For one of the genes, C8orf13, the role in biological processes is still unknown.

3.7. Real-time PCR validation of asoprisnil- and J1042-regulated gene transcripts

Four up-regulated and two down-regulated genes were selected for confirmation of differential expression by using quantitative real-time PCR as an independent method. The genes chosen for validation were member 3 of subfamily J of potassium inwardly-rectifying channels (KCNJ3), member A of group 5 of family C of G protein-coupled receptors (GPRC5A), the adhesion molecule with immunoglobulin (Ig)-like domain 2 (AMIGO2), the parathyroid hormone-like hormone (PTHLH), the zinc finger and BTB domain containing protein 16 (ZBTB16) and defensin beta 32 (DEFB32). Therefore, expressed genes represent factors in different biological processes such as ion flux, membrane-bound receptor signaling, cell adhesion, hormone activity and transcription. Induction of KCNJ3- and GPRC5A-transcripts as well as repression of AMIGO2-transcripts were specific for J1042 and asoprisnil, as well as J912 (Fig. 5A–C); the latter was not apparent from global gene expression profiling (Table 2). Neither mixed nor pure antagonists affected transcription of these genes with statistical significance as expected from microarray analysis. In particular, the increase in KCNJ3 mRNA expression was exclusive for the SPRMs J1042, asoprisnil and J912. Besides the regulation of GPRC5A- and AMIGO2-transcript levels, R5020 and PRA-910 influenced the transcription of PTHLH, ZBTB16 and DEFB32 (Fig. 5D–F). Additionally, gene expression of AMIGO2 and ZBTB16 was shown to be regulated by R5020 and PRA-910 even stronger than by SPRMs. The effects of J912 on ZBTB16-, and also DEFB32- and PTHLH-transcript levels were minimal or absent. Therefore, J912 corresponded to PR antagonists with regard to the regulation of PTHLH, DEFB32 and ZBTB16, but to asoprisnil and J1042 with regard to the regulation of KCNJ3, GPRC5A and AMIGO2.

In addition, the antagonistic effects of asoprisnil, J1042, the mixed antagonist mifepristone and the pure antagonist lonaprisan were analysed. In combined treatments, R5020 was used in a concentration which was shown to evoke half maximal transactivation efficacy (EC_{50} = 100 pM) to enable sustained counter-regulation by the respective PR modulator. The SPRMs generally dominated the effects of agonists when applicated concomitantly. Similar effects

Table 1
PR modulator-regulated probe sets assessed by Affymetrix GeneChip analysis in T47D cells.

DMSO vs.	R5020	PRA-910	Asoprisnil	J1042	J912	Mifepristone	Onapristone	ZK 137316	Lonaprisan
R5020	1981	593	62	53	20	12	1	34	44
PRA-910	1388/52	645	37	37	7	4	1	18	27
Asoprisnil	1919/18	608/43	80	38	14	9	0	17	21
J1042	1928/17	608/33	42/32	70	6	8	0	7	11
J912	1961/10	638/23	66/16	64/24	30	11	0	23	23
Mifepristone	1969/13	641/21	71/16	62/17	19/14	25	0	16	15
Onapristone	1979/2	644/2	80/3	70/3	30/3	25/3	3	0	0
ZK 137316	1947/28	627/44	63/45	63/55	7/39	9/46	3/62	62	48
Lonaprisan	1937/45	618/62	59/68	59/78	7/66	10/71	3/89	14/41	89

Cells were treated with vehicle or 10 nM of PR ligand, except PRA-910 in a concentration of 100 nM, for 8 h. Entire number of significant regulated genes was shown in the diagonal (e.g. 30 genes regulated by J912). Genes found in both corresponding sets of column and row were shown in the upper right (e.g. 11 genes of J912-regulated genes were also observed in mifepristone-treated cells). Genes exclusively found in one of the two sets (column/row) were shown in the lower left (e.g. 19 genes of J912-regulated genes were not identified in mifepristone-treated cells, 14 genes of mifepristone-regulated genes were not observed in J912-treated cells). Cells were analysed in quintuplicate (for vehicle control 10-fold), time-separated experiments. Statistical significance was assessed by pairwise comparisons of treatment versus vehicle (FC > 2, Volcano: FC > 5 and p-value analogous to ST $Q < 0.01$ from t-test).

were obtained for PR antagonists. However, counter-regulation of R5020 effects was stronger for PR antagonists than for SPRMs.

4. Discussion

In this study, a representative selection of PR modulators was analysed for specific in vitro characteristics to provide a more reliable classification system. Synthetic PR ligands are clinically important and commonly used for the treatment of several gynecological diseases as well as in contraceptives and hormone replacement therapy. They elicit very specific effects, which could be evoked by different functional interactions and transcriptional activities. PR ligands exhibit a broad spectrum of activity ranging from pure antagonists, such as lonaprisan, via mixed agonist/antagonists, currently known as selective progesterone modulators (SPRMs), to pure agonists, such as R5020. In recent years, a classification in type I–III PR antagonists has been carried out [25,30] to differentiate between classical, mixed and pure antagonists (Table 3). Type I antagonists (e.g. onapristone) have been described to prevent PR binding to DNA. Type II antagonists (e.g. mifepristone) promote DNA binding and act as PR antagonists in transactivation assays under most circumstances. In the presence of protein kinase A activators (cAMP), they behave like PR agonists. Type III antagonists (e.g. lonaprisan) also promote a strong binding to DNA, but do not display any PR agonistic activity in the presence of cAMP. SPRMs (asoprisnil, J1042, J912) are all assigned as type II ligands in this model. Thereby, they are not differentiated from the mixed antagonist mifepristone, although they vary in some in vivo activities [26]. This demonstrates the prevalent problem for a reliable classification of PR ligands, especially in vitro with respect to their in vivo activities. Recent crystallographic observations for the SPRM asoprisnil have demonstrated that the functional changes which are associated with compound binding and which are matched by structural changes in the PR ligand binding domain, are antagonist-like [45,46]. Finally, classical in vitro assays including transactivation assays using the MMTV promoter (Fig. 1) fail to explain diverse in vivo characteristics and a distinction between PR antagonists such as mifepristone and SPRMs is not possible, yet.

This study identified interaction profiles with the LX-H10 peptide, which contains the LXXLL-motif of coactivators [9,39], as indicative for the agonist-like properties of SPRMs. Mammalian two-hybrid assays in HeLa cells demonstrated that J1042 and asoprisnil induce PR interactions with the LX-H10 peptide similar to R5020, whereas mixed as well as pure antagonists do not induce recruitment (Fig. 2). The potency and efficacy profiles for asoprisnil, J1042 and J912 correspond to the partial agonistic activity of the respective PR ligand in endometrial transformation in the rabbit (McPhail test) [27], which serves as a reference assay for PR modulator classification in vivo (Table 3). Thus, the LX-H10 peptide interaction profile correlates with specific in vivo properties, although the in vitro assay is rather artificial. The separation between SPRMs and PR antagonists might indicate different receptor conformations, which could lead to different hydrophobic binding pockets for cofactors and subsequently, to different in vivo activities by differential gene expression [47,48].

The effects of different cofactor recruitment on the gene expression profile elicited by nuclear receptors have extensively been described before [49]. This global gene expression profiling study further confirms previous results and demonstrates a ligand-dependent transcriptional regulation of PR target genes. Gene expression profiles of J1042 and asoprisnil in T47D cells are significantly different from R5020 and PRA-910, as expected. They are also different from mixed as well as pure antagonists, and interestingly, J912. In the principal component analysis (PCA), vehicle control, PR antagonists (onapristone, mifepristone, ZK 137316, lonaprisan), and J912 samples exert the strongest differential effects compared to R5020 samples. J1042 and asoprisnil expression profiles form a different cluster (Fig. 3) which is more similar to R5020 than antagonists are. J1042-regulated genes are most similar to asoprisnil-regulated genes. This is consistent with the special role of J1042 and asoprisnil in vivo, in particular considering their agonistic properties in the guinea pig and in endometrial transformation in the rabbit (McPhail test) [25,26]. The results from unsupervised hierarchical clustering as well as analyses for significant regulated genes confirm these findings. Notably, J912-modulated genes are most similar to lonaprisan and ZK 137316 target genes (Fig. 4; Table 1). Therefore, the highly antagonistic characteristics of J912 in T47D cell global gene expression profiles should be emphasised and its previous classification as 'weak' SPRM [50] needs to be relativised. Its weak 'SPRM'-like effects in the regulation of gene transcripts such as KCNJ3 and GPRC5A (Figs. 4 and 5A–C) and the antagonist-like effects in the regulation of gene transcripts such as PTHLH and DEFB32 demonstrate the difficulties to strictly classify PR modulators such as J912. In vivo J912 displays various agonistic properties in the guinea pig model, very similar to asoprisnil and J1042. However, the antagonistic potentials in endometrial transformation in the rabbit are stronger for J912 than for asoprisnil or J1042 [25,26]. Thus, in vivo and in vitro, the classification of J912 depends on the species-specific activity surveyed (Table 3). In addition to the induced LX-H10 peptide interaction which indicates different in vitro activities of SPRMs and PR antagonists (Fig. 2A, B), the induction of KCNJ3-transcription might be used to identify SPRM-like activity (Fig. 5A; Table 3). The inwardly rectifying potassium channel (KCNJ3, GIRK1) is activated via stimulation of G protein-coupled receptors and selective potassium influx could

Table 2
Categorical list of genes significantly regulated by J867 and J1042, but not by J912 and antagonists.

Identifier	Gene name	Gene symbol	Gene ID	Fold change								
				R5020	PRA-910	J867	J1042	J912	Mifepristone	Onapristone	ZK 137316	Lonaprisan
Hormone activity												
230746_s_at	Stanniocalcin 1	STC1	6781	−3.4	n.c.	−3.4	−2.4	n.c.	n.c.	n.c.	n.c.	n.c.
211756_at	Parathyroid hormone-like hormone	**PTHLH**	5744	n.c.	−4.4	−3.2	−2.8	n.c.	n.c.	n.c.	n.c.	n.c.
Membrane effects												
Cell adhesion/cytoskeletal interactions												
208353_x_at	Ankyrin 1	ANK1	286	n.c.	n.c.	2.2	2.1	n.c.	n.c.	n.c.	n.c.	n.c.
222108_at	Adhesion molecule with Ig-like domain 2	**AMIGO2**	347902	−31.8	−9.9	−2.9	−2.6	n.c.	n.c.	n.c.	n.c.	n.c.
Protein transporter												
213413_at	Stoned B-like factor	STON1	11037	−8.7	−3.0	−2.1	−2.1	n.c.	n.c.	n.c.	n.c.	n.c.
Ion channels												
233059_at	Potassium inwardly-rectifying channel, subfamily J, member 3	**KCNJ3**	3760	n.d.	n.d.	4.7	5.1	n.d.	n.d.	n.d.	n.d.	n.d.
Membrane organisation												
214255_at	ATPase, Class V, type 10A	ATP10A	57194	11.5	6.0	2.6	2.5	n.c.	n.c.	n.c.	n.c.	n.c.
227834_at	Taxilin beta	TXLNB	167838	−4.5	n.c.	−2.5	−2.3	n.c.	n.c.	n.c.	n.c.	n.c.
Signal transduction from membrane												
221245_s_at	Frizzled homolog 5 (Drosophila)	FZD5	7855	3.3	2.9	2.3	2.3	n.c.	n.c.	n.c.	n.c.	n.c.
214724_at	DIX domain containing 1	DIXDC1	85458	2.7	n.c.	2.3	2.2	n.c.	n.c.	−1.2	n.c.	n.c.
203108_at	G protein-coupled receptor, family C, group 5, member A	**GPRC5A**	9052	n.c.	n.c.	2.2	2.3	n.c.	n.c.	−1.5	n.c.	n.c.
215306_at	Luteinizing hormone/choriogonadotropin receptor	LHCGR	3973	−7.4	−4.5	−2.2	−2.7	n.c.	n.c.	n.c.	n.c.	n.c.
231120_x_at	Protein kinase (cAMP-dependent, catalytic) inhibitor beta	PKIB	5570	−5.7	−3.0	−2.4	−2.2	n.c.	n.c.	n.c.	n.c.	n.c.
223843_at	Scavenger receptor class A, member 3	SCARA3	51435	−5.1	−2.1	−2.1	−2.2	n.c.	n.c.	n.c.	n.c.	n.c.
Ca^{2+} binding proteins												
1553392_at	EF-hand calcium binding domain 3	EFCAB3	146779	4.7	3.9	2.5	2.1	n.c.	n.c.	−1.5	n.c.	n.c.
Nucleic acid and protein processing												
DNA replication/transcription/translation												
206045_s_at	Nucleolar protein 4	NOL4	8715	−10.3	−6.4	−2.2	−2.6	n.c.	n.c.	n.c.	n.c.	n.c.
Chaperones/protein folding												
203810_at	DnaJ (Hsp40) homolog, subfamily B, member 4	DNAJB4	11080	4.4	1.7	2.4	2.1	n.c.	n.c.	n.c.	n.c.	n.c.
Transcription factors												
228854_at	Zinc finger and BTB domain containing 16	**ZBTB16**	7704	119.9	108.8	7.1	5.9	n.d.	n.d.	n.d.	n.d.	n.d.
213293_s_at	Tripartite motif-containing 22	TRIM22	10346	62.9	21.5	4.5	3.4	n.d.	n.d.	n.d.	n.d.	n.d.
209211_at	Kruppel-like factor 5 (intestinal)	KLF5	688	2.7	2.3	2.7	2.4	n.c.	n.c.	n.c.	n.c.	n.c.
229228_at	cAMP responsive element binding protein 5	CREB5	9586	−4.4	−2.8	−2.8	−2.0	n.c.	n.c.	n.c.	n.c.	n.c.
Cell cycle												
41644_at	SAM and SH3 domain containing 1	SASH1	23328	3.7	4.5	2.0	2.3	n.c.	n.c.	n.c.	n.c.	n.c.
Metabolism												
227361_at	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	HS3ST3B1	9953	5.7	4.3	2.6	2.2	n.c.	n.c.	n.c.	n.c.	n.c.
Defense response												
243311_at	Defensin, beta 32	**DEFB32**	400830	6.9	4.1	6.6	3.6	n.d.	n.d.	n.d.	n.d.	n.d.
Unknown function												
226614_s_at	Chromosome 8 open reading Frame 13	C8orf13	83648	n.c.	−2.0	2.1	2.0	n.c.	n.c.	n.c.	n.c.	n.c.

Genes were organised by primary function according to Gene Ontology (GO). Statistical significance was assessed by pairwise comparisons of treatment versus vehicle control (FC > 2, Volcano: FC > 5 and *p*-value analogous to ST Q < 0.01 from *t*-test). Bold genes were validated independently by TaqMan® quantitative real-time PCR. n.c. = no change (FC = 1.0, ±1.1), n.d. = not detected.

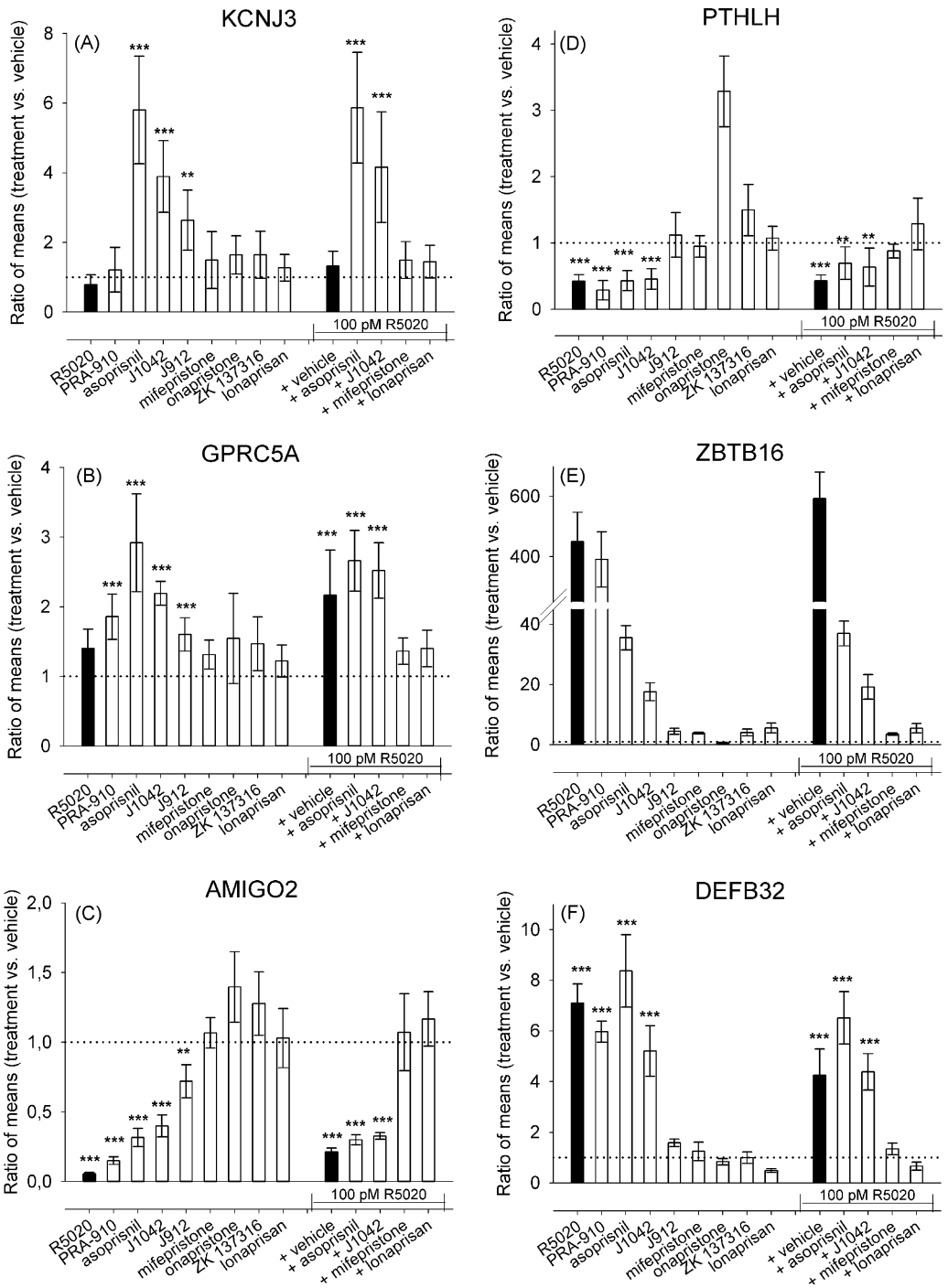

Fig. 5. Stratification marker of SPRMs validated via quantitative real-time PCR. Left part of diagram: Agonistic profile. T47D cells were treated with vehicle or 10 nM of PR ligand, except PRA-910 in a concentration of 100 nM, for 8 h. Right part of diagram: Antagonistic profile. T47D cells were treated with 100 pM R5020 plus vehicle or 10 nM of PR modulator for 8 h. RNA expression level of KCNJ3 (A), GPRC5A (B), AMIGO2 (C), PTHLH (D), ZBTB16 (E), and DEFB32 (F) was analysed via TaqMan® gene expression assay and normalised to the cycle threshold (CT) value of the housekeeping gene cyclophilin A using the ΔΔCT-method. Vehicle control was set to an arbitrary value of one. Results were represented as ratio of means (±C.I., p-value <0.05) of treatment versus vehicle from duplicates of three independent, time-separated experiments. Statistical significance was assessed by Student's t-test (**p-value <0.005; ***p-value <0.001).

represent a signalling pathway activated by PR ligands with special properties as demonstrated by SPRMs. Further investigation of the underlying mechanism of KCNJ3-induction and its relevance for the SPRMs function might be of interest.

The results confirm and extend previous gene expression studies on PR target genes, including previously described progesterone target genes such as periplakin (PPL) [41], hydroxysteroid (11-beta) dehydrogenase 2 (HSD11B2) [44], transforming growth factor beta-stimulated protein TSC-22 (TSC22D1), HSP90-binding immunophilin (FKBP51) and Na^+/K^+-ATPase alpha 1 subunit (ATP1A1) [43]. The comparison of steroidal and non-steroidal PR ligands supports the unique gene regulation profile of the non-steroidal PR modulator PRA-910 which has been described to be different from agonists and antagonists [42]. Its predominantly agonistic properties in gene expression and cofactor interaction analyses are consistent with previous reports on agonistic activity

Table 3
In vitro and in vivo activities for classification of PR modulators.

	Previous classification		DNA binding	In vitro transactivation (MMTV promoter)		Mammalian two-hybrid (LX-H10 interaction)	Gene expression in T47D cells (8 h of treatment)				Endometrial transformation in the rabbit (McPhail test)
	Character	Type		Standard	+ cAMP		KCNJ3	AMIGO2	PTHLH	Global (PCA cluster)	
R5020	Agonist	n.d.	+	+++	+++	+++	↔	↓↓↓	↓↓	A	+++
PRA-910	Partial agonist	n.d.	+	+	n.e.	+++	↔	↓↓↓	↓↓	B	n.e.
Asoprisnil	SPRM	II	+	–	+	+++	↑↑↑	↓↓	↓↓	C	+++
J1042	SPRM	II	+	–	+	+++	↑↑	↓↓	↓↓	C	+++
J912	SPRM	II	+	–	+	+	↑	→	↔	D	+
Mifepristone	Antagonist	II	+	–	+	–	↔	↔	↔	D	–
ZK 137316	Antagonist	II	+	–	n.e.	–	↔	↔	↔	E	–
Lonaprisan	Antagonist	III	+++	–	–	–	↔	↔	↔	E	–
Onapristone	Antagonist	I	–	–	–	–	↔	↔	↔	D	–

+++ = full agonistic effect, + = effect, – = no effect, ↑ = up-regulated, ↓ = down-regulated, ↔ = no change, n.e. = not evidenced, n.d. = not determined.

in vitro [34]. However, these properties are contrary to its almost antagonistic activity in vivo [35,51,52].

In addition, the concept of steroidal PR antagonists subclassification [25] was confirmed by significant differences in the expression profiles of lonaprisan, ZK 137316, mifepristone and onapristone (Fig. 3B). The minimal regulation of transcription by onapristone is consistent with the previous model of type I antagonists preventing PR binding to DNA [30]. Mifepristone represents a type of antagonist with small agonistic potential (Fig. 4), similar to its species- and tissue-specific partial PR agonistic activities in vivo which have been described previously [29,53–56]. Although mifepristone is a well-characterised antagonist, binding to PR and actively impairing its gene-regulatory activity [57], it separates from pure antagonists such as lonaprisan, and also from ZK 137316 and onapristone. Lonaprisan-treated samples cluster apart, together with ZK 137316-treated samples (Fig. 3B). In contrast to onapristone, lonaprisan promotes PR binding to DNA, because it significantly affected transcription (Table 1). The predominant down-regulation of genes (>80%) is consistent with previous reports on lonaprisan to be a very strong antagonist [32] (Table 3).

In summary, the presented data support the concept of classifying PR modulators into pure agonists (R5020), selective progesterone receptor modulators (J1042 and asoprisnil), mixed antagonists (mifepristone), and pure antagonists (lonaprisan). However, they also point to transition states between SPRMs and antagonists for J912, and between mixed and pure antagonists for ZK 137316 (Table 3). The PR modulators seem to be capable to induce a continuum of differential PR signaling effects [26]. All tested compounds display unique activities under standardised conditions, which likely correlate with different in vivo properties. Moreover, some of the in vitro activities, like the induction of LX-H10 interaction and a gene expression fingerprint, might be useful to predict 'SPRM'-like activities in vivo.

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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.jsbmb.2008.11.015.

References

[1] J.D. Graham, C.L. Clarke, Physiological action of progesterone in target tissues, Endocr. Rev. 18 (4) (1997) 502–519.
[2] J.P. Lydon, F.J. DeMayo, C.R. Funk, S.K. Mani, A.R. Hughes, C.A. Montgomery Jr., G. Shyamala, O.M. Conneely, B.W. O'Malley, Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities, Genes Dev. 9 (18) (1995) 2266–2278.
[3] B.A. Lessey, P.S. Alexander, K.B. Horwitz, The subunit structure of human breast cancer progesterone receptors: characterization by chromatography and photoaffinity labeling, Endocrinology 112 (4) (1983) 1267–1274.
[4] D.J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, R.M. Evans, The nuclear receptor superfamily: the second decade, Cell 83 (6) (1995) 835–839.
[5] P. Kastner, M.T. Bocquel, B. Turcotte, J.M. Garnier, K.B. Horwitz, P. Chambon, H. Gronemeyer, Transient expression of human and chicken progesterone receptors does not support alternative translational initiation from a single mRNA as the mechanism generating two receptor isoforms, J. Biol. Chem. 265 (21) (1990) 12163–12167.
[6] O.M. Conneely, B. Mulac-Jericevic, J.P. Lydon, F.J. De Mayo, Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice, Mol. Cell Endocrinol. 179 (1–2) (2001) 97–103.
[7] B. Mulac-Jericevic, R.A. Mullinax, F.J. DeMayo, J.P. Lydon, O.M. Conneely, Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform, Science 289 (5485) (2000) 1751–1754.
[8] M. Beato, Gene regulation by steroid hormones, Cell 56 (3) (1989) 335–344.

[9] P.H. Giangrande, E.A. Kimbrel, D.P. Edwards, D.P. McDonnell, The opposing transcriptional activities of the two isoforms of the human progesterone receptor are due to differential cofactor binding, Mol. Cell Biol. 20 (9) (2000) 3102–3115.
[10] L. Klein-Hitpass, S.Y. Tsai, N.L. Weigel, G.F. Allan, D. Riley, R. Rodriguez, W.T. Schrader, M.J. Tsai, B.W. O'Malley, The progesterone receptor stimulates cell-free transcription by enhancing the formation of a stable preinitiation complex, Cell 60 (2) (1990) 247–257.
[11] C.L. Smith, B.W. O'Malley, Coregulator function: a key to understanding tissue specificity of selective receptor modulators, Endocr. Rev. 25 (1) (2004) 45–71.
[12] D.M. Heery, E. Kalkhoven, S. Hoare, M.G. Parker, A signature motif in transcriptional co-activators mediates binding to nuclear receptors, Nature 387 (6634) (1997) 733–736.
[13] D.M. Tanenbaum, Y. Wang, S.P. Williams, P.B. Sigler, Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains, Proc. Natl. Acad. Sci. U.S.A. 95 (11) (1998) 5998–6003.
[14] Y. Dong, J.Y. Roberge, Z. Wang, X. Wang, J. Tamasi, V. Dell, R. Golla, J.R. Corte, Y. Liu, T. Fang, M.N. Anthony, D.M. Schnur, M.L. Agler, J.K. Dickson Jr., R.M. Lawrence, M.M. Prack, R. Seethala, J.H. Feyen, Characterization of a new class of selective nonsteroidal progesterone receptor agonists, Steroids 69 (3) (2004) 201–217.
[15] R.C. Winneker, A. Fensome, J.E. Wrobel, Z. Zhang, P. Zhang, Nonsteroidal progesterone receptor modulators: structure activity relationships, Semin. Reprod. Med. 23 (1) (2005) 46–57.
[16] H.B. Croxatto, A.M. Salvatierra, H.D. Croxatto, B. Fuentealba, Effects of continuous treatment with low dose mifepristone throughout one menstrual cycle, Hum. Reprod. 8 (2) (1993) 201–207.
[17] T. Luukkainen, O. Heikinheimo, M. Haukkamaa, P. Lahteenmaki, Inhibition of folliculogenesis and ovulation by the antiprogesterone RU 486, Fertil. Steril. 49 (6) (1988) 961–963.
[18] P.C. Ishwad, R.R. Katkam, I.N. Hinduja, K. Chwalisz, W. Elger, C.P. Puri, Treatment with a progesterone antagonist ZK 98.299 delays endometrial development without blocking ovulation in bonnet monkeys, Contraception 48 (1) (1993) 57–70.
[19] H. Michna, Y. Nishino, G. Neef, W.L. McGuire, M.R. Schneider, Progesterone antagonists: tumor-inhibiting potential and mechanism of action, J. Steroid Biochem. Mol. Biol. 41 (3–8) (1992) 339–348.
[20] G. Romieu, T. Maudelonde, A. Ulmann, H. Pujol, J. Grenier, G. Cavalie, S. Khalaf, H. Rochefort, The antiprogestin RU486 in advanced breast cancer: preliminary clinical trial, Bull. Cancer 74 (4) (1987) 455–461.
[21] L.M. Kettel, A.A. Murphy, A.J. Morales, A. Ulmann, E.E. Baulieu, S.S. Yen, Treatment of endometriosis with the antiprogesterone mifepristone (RU486), Fertil. Steril. 65 (1) (1996) 23–28.
[22] K. Chwalisz, R. Garg, R.M. Brenner, G. Schubert, W. Elger, Selective progesterone receptor modulators (SPRMs): a novel therapeutic concept in endometriosis, Ann. N. Y. Acad. Sci. 955 (2002) 373–388 (discussion 389–393, 396–406).
[23] K. Chwalisz, L. Larsen, C. Mattia-Goldberg, A. Edmonds, W. Elger, C.A. Winkel, A randomized, controlled trial of asoprisnil, a novel selective progesterone receptor modulator, in women with uterine leiomyomata, Fertil. Steril. 87 (6) (2007) 1399–1412.
[24] A.A. Murphy, L.M. Kettel, A.J. Morales, V.J. Roberts, S.S. Yen, Regression of uterine leiomyomata in response to the antiprogesterone RU 486, J. Clin. Endocrinol. Metab. 76 (2) (1993) 513–517.
[25] K. Chwalisz, R.M. Brenner, U.U. Fuhrmann, H. Hess-Stumpp, W. Elger, Antiproliferative effects of progesterone antagonists and progesterone receptor modulators on the endometrium, Steroids 65 (10–11) (2000) 741–751.
[26] W. Elger, J. Bartley, B. Schneider, G. Kaufmann, G. Schubert, K. Chwalisz, Endocrine pharmacological characterization of progesterone antagonists and progesterone receptor modulators with respect to PR-agonistic and antagonistic activity, Steroids 65 (10–11) (2000) 713–723.
[27] D. DeManno, W. Elger, R. Garg, R. Lee, B. Schneider, H. Hess-Stumpp, G. Schubert, K. Chwalisz, Asoprisnil (J867): a selective progesterone receptor modulator for gynecological therapy, Steroids 68 (10–13) (2003) 1019–1032.
[28] G. Schubert, W. Elger, G. Kaufmann, B. Schneider, G. Reddersen, K. Chwalisz, Discovery, chemistry, and reproductive pharmacology of asoprisnil and related 11beta-benzaldoxime substituted selective progesterone receptor modulators (SPRMs), Semin. Reprod. Med. 23 (1) (2005) 58–73.
[29] S.J. Han, S.Y. Tsai, M.J. Tsai, B.W. O'Malley, Distinct temporal and spatial activities of RU486 on progesterone receptor function in reproductive organs of ovariectomized mice, Endocrinology 148 (5) (2007) 2471–2486.
[30] L. Klein-Hitpass, A.C. Cato, D. Henderson, G.U. Ryffel, Two types of antiprogestins identified by their differential action in transcriptionally active extracts from T47D cells, Nucleic Acids Res. 19 (6) (1991) 1227–1234.
[31] U. Fuhrmann, C. Bengtson, G. Repenthin, E. Schillinger, Stable transfection of androgen receptor and MMTV-CAT into mammalian cells: inhibition of cat expression by anti-androgens, J. Steroid Biochem. Mol. Biol. 42 (8) (1992) 787–793.
[32] U. Fuhrmann, H. Hess-Stumpp, A. Cleve, G. Neef, W. Schwede, J. Hoffmann, K.H. Fritzemeier, K. Chwalisz, Synthesis and biological activity of a novel, highly potent progesterone receptor antagonist, J. Med. Chem. 43 (26) (2000) 5010–5016.
[33] S.G. Lundeen, Z. Zhang, Y. Zhu, J.M. Carver, R.C. Winneker, Rat uterine complement C3 expression as a model for progesterone receptor modulators: characterization of the new progestin trimegestone, J. Steroid Biochem. Mol. Biol. 78 (2) (2001) 137–143.
[34] Z. Zhang, S.G. Lundeen, Y. Zhu, J.M. Carver, R.C. Winneker, In vitro characterization of trimegestone: a new potent and selective progestin, Steroids 65 (10–11) (2000) 637–643.
[35] Z. Zhang, A.M. Olland, Y. Zhu, J. Cohen, T. Berrodin, S. Chippari, C. Appavu, S. Li, J. Wilhem, R. Chopra, A. Fensome, P. Zhang, J. Wrobel, R.J. Unwalla, C.R. Lyttle, R.C. Winneker, Molecular and pharmacological properties of a potent and selective novel nonsteroidal progesterone receptor agonist tanaproget, J. Biol. Chem. 280 (31) (2005) 28468–28475.
[36] Y. Kamei, L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S.C. Lin, R.A. Heyman, D.W. Rose, C.K. Glass, M.G. Rosenfeld, A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors, Cell 85 (3) (1996) 403–414.
[37] S.A. Onate, S.Y. Tsai, M.J. Tsai, B.W. O'Malley, Sequence and characterization of a coactivator for the steroid hormone receptor superfamily, Science 270 (5240) (1995) 1354–1357.
[38] A. Takeshita, P.M. Yen, S. Misiti, G.R. Cardona, Y. Liu, W.W. Chin, Molecular cloning and properties of a full-length putative thyroid hormone receptor coactivator, Endocrinology 137 (8) (1996) 3594–3597.
[39] C. Chang, J.D. Norris, H. Gron, L.A. Paige, P.T. Hamilton, D.J. Kenan, D. Fowlkes, D.P. McDonnell, Dissection of the LXXLL nuclear receptor–coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta, Mol. Cell Biol. 19 (12) (1999) 8226–8239.
[40] R.N. Cohen, S. Brzostek, B. Kim, M. Chorev, F.E. Wondisford, A.N. Hollenberg, The specificity of interactions between nuclear hormone receptors and corepressors is mediated by distinct amino acid sequences within the interacting domains, Mol. Endocrinol. 15 (7) (2001) 1049–1061.
[41] J.D. Bray, S. Jelinsky, R. Ghatge, J.A. Bray, C. Tunkey, K. Saraf, B.M. Jacobsen, J.K. Richer, E.L. Brown, R.C. Winneker, K.B. Horwitz, C.R. Lyttle, Quantitative analysis of gene regulation by seven clinically relevant progestins suggests a highly similar mechanism of action through progesterone receptors in T47D breast cancer cells, J. Steroid Biochem. Mol. Biol. 97 (4) (2005) 328–341.
[42] J.D. Bray, Z. Zhang, R.C. Winneker, C.R. Lyttle, Regulation of gene expression by PRA-910, a novel progesterone receptor modulator, in T47D cells, Steroids 68 (10–13) (2003) 995–1003.
[43] H.A. Kester, B.M. van der Leede, P.T. van der Saag, B. van der Burg, Novel progesterone target genes identified by an improved differential display technique suggest that progestin-induced growth inhibition of breast cancer cells coincides with enhancement of differentiation, J. Biol. Chem. 272 (26) (1997) 16637–16643.
[44] J.K. Richer, B.M. Jacobsen, N.G. Manning, M.G. Abel, D.M. Wolf, K.B. Horwitz, Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells, J. Biol. Chem. 277 (7) (2002) 5209–5218.
[45] K.P. Madauss, E.T. Grygielko, S.J. Deng, A.C. Sulpizio, T.B. Stanley, C. Wu, S.A. Short, S.K. Thompson, E.L. Stewart, N.J. Laping, S.P. Williams, J.D. Bray, A structural and in vitro characterization of asoprisnil: a selective progesterone receptor modulator, Mol. Endocrinol. 21 (5) (2007) 1066–1081.
[46] M.A. Pullen, N. Laping, R. Edwards, J. Bray, Determination of conformational changes in the progesterone receptor using ELISA-like assays, Steroids 71 (9) (2006) 792–798.
[47] G.F. Allan, X. Leng, S.Y. Tsai, N.L. Weigel, D.P. Edwards, M.J. Tsai, B.W. O'Malley, Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation, J. Biol. Chem. 267 (27) (1992) 19513–19520.
[48] J.A. Katzenellenbogen, B.S. Katzenellenbogen, Nuclear hormone receptors: ligand-activated regulators of transcription and diverse cell responses, Chem. Biol. 3 (7) (1996) 529–536.
[49] N.J. McKenna, B.W. O'Malley, Combinatorial control of gene expression by nuclear receptors and coregulators, Cell 108 (4) (2002) 465–474.
[50] K. Chwalisz, M.C. Perez, D. Demanno, C. Winkel, G. Schubert, W. Elger, Selective progesterone receptor modulator development and use in the treatment of leiomyomata and endometriosis, Endocr. Rev. 26 (3) (2005) 423–438.
[51] R.C. Winneker, D. Bitran, Z. Zhang, The preclinical biology of a new potent and selective progestin: trimegestone, Steroids 68 (10–13) (2003) 915–920.
[52] P. Zhang, E.A. Terefenko, A. Fensome, J. Wrobel, R. Winneker, S. Lundeen, K.B. Marschke, Z. Zhang, 6-Aryl-1,4-dihydro-benzo[d][1,3]oxazin-2-ones: a novel class of potent, selective, and orally active nonsteroidal progesterone receptor antagonists, J. Med. Chem. 45 (20) (2002) 4379–4382.
[53] Z. Liu, D. Auboeuf, J. Wong, J.D. Chen, S.Y. Tsai, M.J. Tsai, B.W. O'Malley, Coactivator/corepressor ratios modulate PR-mediated transcription by the selective receptor modulator RU486, Proc. Natl. Acad. Sci. U.S.A. 99 (12) (2002) 7940–7944.
[54] M.E. Meyer, A. Pornon, J.W. Ji, M.T. Bocquel, P. Chambon, H. Gronemeyer, Agonistic and antagonistic activities of RU486 on the functions of the human progesterone receptor, EMBO J. 9 (12) (1990) 3923–3932.
[55] C.A. Sartorius, L. Tung, G.S. Takimoto, K.B. Horwitz, Antagonist-occupied human progesterone receptors bound to DNA are functionally switched to transcriptional agonists by cAMP, J. Biol. Chem. 268 (13) (1993) 9262–9266.
[56] C.A. Beck, N.L. Weigel, M.L. Moyer, S.K. Nordeen, D.P. Edwards, The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signaling pathways, Proc. Natl. Acad. Sci. U.S.A. 90 (10) (1993) 4441–4445.
[57] E.E. Baulieu, On the mechanism of action of RU486, Ann. N. Y. Acad. Sci. 626 (1991) 545–560.