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X-ray Structures of Progesterone Receptor Ligand Binding Domain in Its Agonist State Reveal Differing Mechanisms for Mixed Profiles of 11β-Substituted Steroids

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Scott J. Lusher[‡1], Hans C. A. Raaijmakers[‡], Diep Vu-Pham[§], Bert Kazemier[§], Rolien Bosch[‡], Ross McGuire[‡], Rita Azevedo[‡], Hans Hamersma[‡], Koen Dechering[§], Arthur Oubrie[‡], Marcel van Duin[‖], and Jacob de Vlieg[‡]**

*From the Departments of [‡]Molecular Design and Informatics, [§]Molecular Pharmacology and DMPK, and [‖]Women's Health, MSD, P. O. Box 20, 5340 BH, Oss, The Netherlands and the **Computational Drug Discovery Group, Radboud University, 6526 HP, Nijmegen, The Netherlands*

Background: Understanding the molecular basis for the mixed profiles of progesterone receptor (PR) ligands will benefit future drug design.
Results: Two differing mechanisms for the induction of mixed profiles by 11β-steroids are described.
Conclusion: Subtle electrostatic and steric factors explain the differing PR activities of 11β-steroids.
Significance: These observations will impact future drug-design strategies for PR and potentially other nuclear receptors.

We present here the x-ray structures of the progesterone receptor (PR) in complex with two mixed profile PR modulators whose functional activity results from two differing molecular mechanisms. The structure of Asoprisnil bound to the agonist state of PR demonstrates the contribution of the ligand to increasing stability of the agonist conformation of helix-12 via a specific hydrogen-bond network including Glu^{723}. This interaction is absent when the full antagonist, RU486, binds to PR. Combined with a previously reported structure of Asoprisnil bound to the antagonist state of the receptor, this structure extends our understanding of the complex molecular interactions underlying the mixed agonist/antagonist profile of the compound. In addition, we present the structure of PR in its agonist conformation bound to the mixed profile compound Org3H whose reduced antagonistic activity and increased agonistic activity compared with reference antagonists is due to an induced fit around Trp^{755}, resulting in a decreased steric clash with Met^{909} but inducing a new internal clash with Val^{912} in helix-12. This structure also explains the previously published observation that 16α attachments to RU486 analogs induce mixed profiles by altering the binding of 11β substituents. Together these structures further our understanding of the steric and electrostatic factors that contribute to the function of steroid receptor modulators, providing valuable insight for future compound design.

Modulation of the progesterone receptor (PR)[2] is the mechanism of action for an array of medications and continues to be a fertile area for research, with special focus on the development of mixed profile compounds (1–3). Mixed profile modulators of PR are characterized by decreased transcriptional activity compared with full agonists and increased transcriptional activity compared with full antagonists. Partial agonists, often referred to as selective progesterone receptor modulators (SPRMs), have the potential to treat a variety of women's health conditions (4–7) with improved safety and treatment profiles compared with full agonists or antagonists.

Asoprisnil (Fig. 1), demonstrating a mixed agonist/antagonist profile depending on tissue type, was the first SPRM to progress to late stage clinical development for the treatment of uterine fibroids and endometriosis (8–14). However, the mixed profiles of SPRMs are often poorly reflected in classical *in vitro* models (15). In our hands Asoprisnil is a full PR antagonist in cell-based transactivation assays (PR agonist $EC_{50} > 100$ nM, PR antagonist EC_{50} 0.14 nM with 96% efficacy compared with a standard reference (16)) but can be characterized as a SPRM by differences in *in vivo* models such as the McPhail test (measuring endometrial proliferation in immature rabbits) compared with the full antagonist RU486 (17). It has also been shown clinically that Asoprisnil mediates unique endometrial effects in healthy premenopausal women (17). Difficulties in characterizing SPRMs *in vitro* have historically made their identification and characterization problematic, but traditional methods of steroid receptor drug discovery, designed to identify agonists and antagonists, are now being supplemented by new design approaches and assay types, including peptide recruitment and gene expression, better suited to characterizing mixed profile compounds (15).

The shared domain structure of steroid receptors, such as PR, includes a variable N-terminal domain, a highly conserved DNA binding domain, and a moderately conserved ligand binding domain (LBD). The LBD combines a number of functions, including hormone binding, receptor dimerization, and binding to other co-modulating proteins that play a role in the control of transcription. Specifically, gene activation requires the

The atomic coordinates and structure factors (codes 4A2J and 4APU) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

[1] To whom correspondence should be addressed. E-mail: s.lusher@esciencecenter.nl.

[2] The abbreviations used are: PR, progesterone receptor; SPRM, selective progesterone receptor modulators; LBD, ligand binding domain; r.m.s.d., root mean square deviation; Org3H, 11-pyridinylphenyl steroids (17β-cyclopropylcarbonyl-16α-ethenyl-11β-[4-(3-pyridinyl)phenyl]-estra-4,9-dien-3-one).

recruitment of co-modulating proteins to a region of the surface of the LBD formed by helices 3, 5, and 12. The position of helix-12 can be influenced by the nature of the ligand bound to the receptor, allowing ligands to influence the binding of co-modulators and consequently gene activation. Our understanding of ligand binding to PR has been improved by a number of x-ray crystallography studies (18–23) that have also furthered our understanding of the molecular mechanisms underpinning antagonism and partial agonism (16, 17, 24–26). In particular, it is well accepted that clashes between some ligands and Met^{909} in helix-12 is a major contributing factor to reduced agonistic activity.

One important recent publication describes the x-ray structure of Asoprisnil bound to the antagonist conformation of PR in the presence of the co-repressors NCoR and SMRT (17). The authors report the PR LBD in a conformation divergent from the classical agonist state explaining the reduced agonistic activity of the compound compared with full agonists. However, the structure gives only limited explanation for the compound increased agonistic activity compared with other fully antagonistic 11β-steroids. The x-ray structure of Asoprisnil bound to the antagonistic conformation of PR shows the ligand polar oxime group to be in close contact to hydrophobic residues in the two described co-repressors (NCoR and SMRT) (17). RU486 has a less polar dimethyl amine group in the position equivalent to the Asoprisnil oxime, leading the authors to hypothesize that RU486 might make stronger hydrophobic contacts to co-repressors and, therefore, facilitate their recruitment, thus explaining the differing biological activity of the two compounds.

The same report demonstrates that Asoprisnil bound PR does recruit co-activators such as SRC1. The ability for the Asoprisnil-PR complex to recruit co-activators suggests the complex is able to adopt an agonistic conformation in addition to the antagonist conformation seen when in complex with co-repressors. This is in line with the equilibrium model for partial agonism (16, 27), which suggests that mixed profile compounds are able to partially stabilize their receptors in the agonist conformation compared with the complete stabilization elicited by full agonists. This results in altered patterns of co-modulator recruitment and modified biological outcomes. It also suggests that although the agonistic conformation of PR bound to Asoprisnil may not be the lowest energy, it remains biologically meaningful.

In an attempt to identify the basis for the ligands retained agonistic activity compared with full antagonists such as RU486 (Fig. 1), we have used a previously described soaking technique (16, 26) to determine the x-ray structure of the PR-Asoprisnil complex in its agonist state. This has allowed us to identify an interaction between the 11β-benzaldoxime of Asoprisnil and Glu^{723} that helps partially stabilize helix-12 in its agonist conformation.

To establish if this mechanism is universal for 11β-substituted steroids, we have also solved the x-ray structure of an in-house SPRM from a class of 11-pyridinylphenyl steroids (17β-cyclopropylcarbonyl-16α-ethenyl-11β-[4-(3-pyridinyl)-phenyl]-estra-4,9-dien-3-one) that we refer to as Org3H (Fig. 1), with a previously disclosed mixed PR profile (PR agonist EC_{50} 0.66 nM with 47% efficacy, PR antagonist EC_{50} 0.61 nM with 38% (28)).

Elucidation of Org3H in complex with PR revealed a second mechanism to explain its mixed profile compared with full antagonists, with Org3H making a reduced clash with Met^{909} due to an induced fit around Trp^{755}, which itself now clashes with Val^{912} in helix-12. As a further consequence, the flipping of Trp^{755} results in formation of an additional new subpocket. Together these structures improve our molecular understanding of the important steric and electrostatic factors contributing to the mixed profile seen for many PR modulators.

MATERIALS AND METHODS

Expression and Purification of PR-LBD—The PR LBD, comprising residues 678–933, was cloned in pET15b (Novagen). Expression was performed in *Escherichia coli* BL21(DE3) star (Invitrogen) in 2×YT medium by overnight induction at 20 °C in the presence of 10 μM OrgA (Fig. 1). OrgA is a member of a compound class described as glucocorticoid receptor antagonists (28) but is a relatively potent PR partial agonist whose activity is described in a recent article (26). Bacteria were lysed in buffer A (50 mM Tris, pH 7.8, 250 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol) with 0.4 mM Pefablock (Roche Applied Science) and 50 μM OrgA and purified on nickel nitrilotriacetic acid. Fractions were eluted with buffer A with 100 mM imidazole. Elution fractions were collected and treated with 2.5 wt/wt % thrombin (Kordia) overnight at 4 °C to cleave the N-terminal His tag. Thrombin was removed by adding benzamidine-Sepharose (GE Healthcare), centrifuging for 10 min at 5000 × *g*, and harvesting the supernatant. To make the final crystallization sample, the protein was dialyzed to buffer A to which 1 mM EDTA, 1 mM dithiothreitol, and 10 μM OrgA were added and subsequently concentrated in a stirring cell to about 4 mg/ml as measured by its absorption at 280 nm. The sample was stored at −70 °C in aliquots of 50 μl.

Crystallization—Crystals of the PR LBD in complex with OrgA were grown at room temperature from 3.5-μl drops hanging over a mother liquor of 20–30% polyethylene glycol 3350, 0.1 M Hepes, pH 6.5, 100 mM Mg_2SO_4, and 10% (v/v) glycerol. Drops consisted of 2 μl of protein sample and 1 μl of mother liquor and 0.5 μl of 40% 1,3-propanediol. Kite-shaped crystals usually appeared after about 3 days.

Ligand Replacement—Crystals of the PR LBD in complex with OrgA were collected and transferred to mother liquor to which 0.25 mM concentrations of either Asoprisnil or Org3H were added. 0.25 mM is a significant excess of either compound. The crystals were stored in a sitting drop at room temperature. The solutions surrounding the crystals were replaced by fresh solution 10 times over a period of 2 weeks. After this period, the crystals were frozen and transferred for data collection.

Data Collection—Diffraction data were collected using a Rigaku rotating-anode x-ray generator operating at 100 mA and 50 kV. The dataset for the PR-Asoprisnil complex was collected to 2.08 Å at 100 K and processed with mosflm/scala, and the data for the PR-Org3H complex were collected to 1.9 Å at 100 K and processed with d*TREK/scala (29). Structures were solved and refined using the CCP4i interface of the CCP4 software suite (30). Data are summarized in Table 1. Although of

TABLE 1
Final crystallographic data and refinement statistics
Data in parentheses indicate the last resolution shell.

Structure	PR-Asoprisnil	PR-Org3H
Space group	$P2_1$	$P2_1$
Unit cell (Å^3)	58.61 64.70 69.99	57.66 64.37 70.47
β-Angle (°)	95.7	96.3
Resolution (Å)	43.3-2.08 (2.19-2.08)	47.4-1.90 (2.00-1.90)
Completeness (%)	99.8 (100)	100 (99.9)
Rmerge	0.103 (0.496)	0.082 (0.729)
Mn^{2+} (I/σ)	6.3 (2.0)	8.7 (2.1)
Multiplicity	3.1 (3.0)	4.1 (4.0)
R factor/Rfreeb	20.4/25.1	20.8/24.5
Atoms	4217	4289
Protein atoms	3967	4047
Ligand atoms	66	64
Water molecules	78	173
Other molecules *e.g.* sulfate	5	5
r.m.s.d. bonds, Å	0.011	0.078
r.m.s.d. angles (°)	1.2	1.05
B-factors (average Å^2)		
Main chain	36.0	40.5
Side chain	38.2	42.0
Water molecules	22.5	30.8
Ligand A chain	30.9	50.1 (OrgA)
Ligand B chain	29.3	37.5 (Org3H)
Wilson B-factor (Å^2)	29.3	38.5
PDB identifier	4A2J	4APU

similar resolution, the Asoprisnil dataset is somewhat weaker than the Org3H dataset, and less water molecules were used describe the electron density.

Modeling and Visualization—All figures have been generated using PyMOL (The PyMOL molecular graphics system, Schrodinger, LLC). Ligand structures were optimized using the semi-empirical quantum mechanical MOPAC module in YASARA (31). YASARA was also used to calculate ligand volumes, with all figures representing the volume of the solvent accessible surface.

RESULTS AND DISCUSSION

Size of 11β Group Does Not Correlate to Antagonistic Activity—The generally accepted mechanism for introducing PR antagonism into steroidal and non-steroidal compounds is via the attachment of a bulky group at the steroids 11β position or an equivalent position in non-steroidal compounds. This group should clash with helix-12 and preclude it from adopting its agonistic conformation. Impeding helix-12 from adopting its agonistic position prevents the correct formation of the AF-2 surface and thus excludes the binding of co-activators. This mechanism is supported by the x-ray structures of PR bound to RU486 (16) and Asoprisnil (17) and structures of related receptors such as the AR and glucocorticoid receptor (32–34). However, it has become clear to us that not all bulky groups are equal in this respect and that more subtle factors must determine how successfully each substituent is able to preclude helix-12. For example, the side chain of the antagonist RU486 has a volume of 436 Å^3, which although larger than the side chain of the SPRM Asoprisnil at 385 Å^3, is somewhat smaller than the side chain of the second SPRM Org3H at 504 Å^3.

This inconsistency suggests that steric contributions alone do not guarantee the generation of full antagonists. It also suggests that other factors must be involved in the inducement of mixed profiles and that understanding these factors may help in the future design of SPRMs. This promoted us to review the available x-ray studies and generate new x-ray structures to address this question.

Equilibrium Model for Steroid Receptor Function Suggests Both Agonistic and Antagonistic Conformations Are Relevant for Mixed Profile Compounds—It has been known since the early 1990s that agonists and antagonists induce different conformational changes in PR (35, 36) with the demonstration that the C-terminal is able to adopt two different positions corresponding to the biological activity of the ligand (35, 37). Both of these conformations are distinct from the conformation of the unliganded. It was later shown that a third conformation could be detected in the presence of 16α-substituted analogs of RU486 (38) known to be mixed-profile PR modulators. It has since been postulated that full agonists promote interaction with co-activators, and full antagonists change the conformation of the receptor to inhibit co-activator binding in favor of co-repressor binding (10). In relation to this, mixed-profile compounds induce an intermediate state of interaction between receptor and co-modulators (38–40). The equilibrium model for nuclear receptor agonism/antagonism suggests that mixed profile ligands partly stabilize the agonist conformation of their receptor (16, 27) and allow co-activators to bind but with less efficacy than full agonists. The ability for the Asoprisnil-PR complex to recruit co-activators (17) suggests the complex is able to adopt an agonistic conformation in addition to the antagonist conformation seen when in complex with co-repressors. It has also been shown for the steroid receptors that the presence or absence of co-modulating proteins can alter the specific conformation of structures resulting from x-ray crystallography even after co-expression studies. For example, the x-ray structure of genistein bound to the estrogen receptor-β has been reported in an agonist conformation in the presence of co-activator but also in a conformation similar to the classic antagonist conformation in the absence of co-activator (41, 42). The binding of co-modulating proteins is itself a driving force in the equilibrium and not just a consequence. The relative balance of co-activator and co-repressor expression within a given target cell determines the relative agonist *versus* antagonist activity of mixed-profile compounds (40). Although the previously reported structure of PR bound to Asoprisnil (from co-expression studies) shows the receptor in an antagonist conformation, it is in the presence of co-repressor. The ability of the PR-Asoprisnil complex to recruit co-activators indicates the agonist conformation of the receptor bound to Asoprisnil is both viable and biologically meaningful. This conclusion led us to utilize our previously described PR soaking method (16, 26) to study Asoprisnil bound to a fixed agonistic conformation of PR to determine whether this structure would give additional insight into the molecular basis for Asoprisnil retained agonism compared with RU486.

Asoprisnil Successfully Replaces OrgA during Soaking Experiments—OrgA is a nonsteroidal compound (Fig. 1) with a chemical structure distinct from 11β-steroids whose PR activity and binding is well described in an earlier publication to which we refer readers (26). PR forms homodimers endogenously and also after crystallization. Fig. 2 shows the electron density around Asoprisnil from the B monomer of a new x-ray structure of PR in its agonistic conformation. The clarity of the

electron density around the ligand allows us to conclude with a high degree of confidence that OrgA has been completely replaced by Asoprisnil during the soaking. The exchange is complete for both monomers with the ligand in an almost identical binding mode. To aid in comparison with the previously published RU486 structure in which the ligand soaks into only the B monomer, we will describe only the equivalent monomer from the new PR-Asoprisnil complex.

Helix-12 Is Well Ordered in Its Agonist Conformation—This PR Asoprisnil complex is quite different from the public PR-Asoprisnil structures (PDB access codes 2OVH and 2OVM) and from the PR-RU486 structure (PDB access code 2W8Y). The difference between the previously published PR-Asoprisnil structures and this new structure is mainly located between residues 881 and 924, as the classic antagonistic displacement of helix-12 does not occur in this new structure (Fig. 3). Excluding the C-terminal residues after helix-11, the PR-agonist and PR-antagonist structures bound to Asoprisnil show a root mean square deviation (r.m.s.d.), calculated for their Cα atoms, of 0.65 Å. Helix-12 is well ordered and in the agonist conformation compared with the poorly ordered helix-12 as observed when bound to RU486. This is consistent with Asoprisnil reduced antagonistic activity and its ability to recruit co-activators. There is, however, disorder in region 895–905, the connecting loop between helix-11 and helix-12 of both monomers in the crystal. The loops of both monomers touch through a symmetry contact and are also disordered in both PR-antagonist structures bound to Asoprisnil.

Soaking Asoprisnil into PR-Agonist Crystals Results in Same Ligand Binding Mode as Co-expression—Despite the new PR-Asoprisnil structure being in an agonistic conformation rather than the antagonistic conformation of the previously reported PR-Asoprisnil structures (17), the binding mode of the ligand is comparable. Asoprisnil orients itself almost identically within the agonist and antagonist conformations of PR, making the same contacts with the receptor. The 3-keto group makes the classic interactions to Gln^{725} and Arg^{766} typical for oxosteroids, with the 17α attachment occupying a hydrophobic pocket consisting of Leu^{715}, Leu^{718}, Phe^{794}, Leu^{797}, Met^{801}, and Tyr^{890} (Fig. 4). This pocket, referred to as the 17α pocket, has previously been described for a structure of PR bound to mometasone furoate (21) and appears to provide additional room for ligand expansion irrespective of agonism or antagonism.

No direct or indirect polar interaction exists between the ligand and Asn^{719}. The presence of a water-bridged interaction between steroidal ligands and Asn^{719} has been described in other PR-ligand complexes, including the binding of RU486 and norethindrone (26), but the 17β-methoxy group of Asoprisnil sterically precludes the presence of a water molecule at this position.

As can be seen in Fig. 5, the steroid scaffolds of Asoprisnil from both the PR-agonist and PR-antagonist complexes are relatively well overlaid with a small shift downward seen in Asoprisnil bound to the agonist conformation of PR. The 17α and 17β attachments of both compounds are also well overlaid. The most significant difference between Asoprisnil bound to the agonist conformation and the antagonist conformation of PR is a 0.9 Å adjustment in the position of the oxime group. In the agonistic complex the oxime and steroid core appear to sit lower to reduce the clash with Met^{909} in helix-12. Because of the displacement of helix-12 in the antagonistic complex, this clash is not possible.

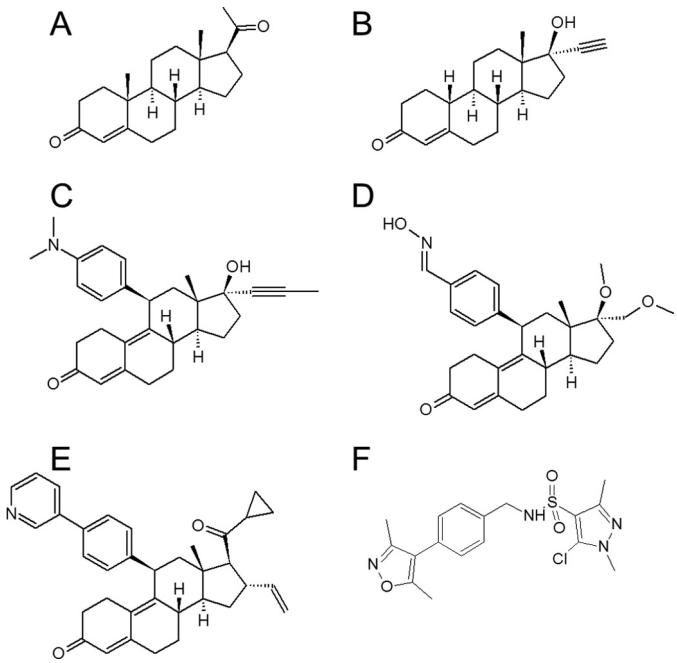

FIGURE 1. **The structures of progesterone (*A*), norethindrone (*B*), RU486 (*C*), Asoprisnil (*D*), Org3H (*E*), and OrgA (*F*) are shown.**

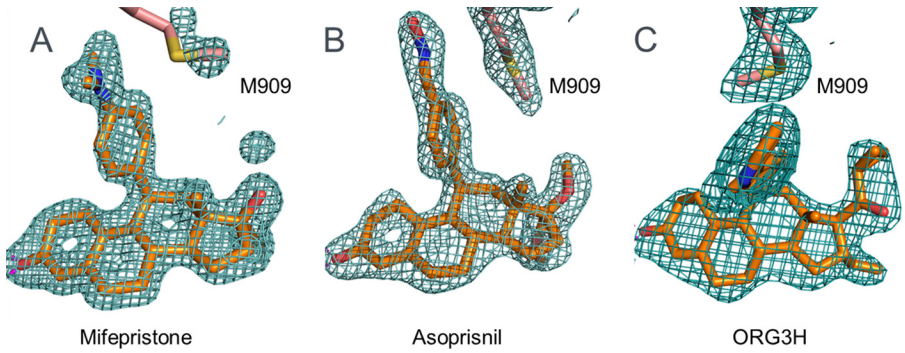

FIGURE 2. **$2F_o - DF_c$ OMIT electron density maps around the ligand and Met^{909} for RU486 (*A*), Asoprisnil (*B*), and Org3H (*C*) are shown at 1.0 δ.** Electron densities suggest a more stable conformation of Met^{909} in the Asoprisnil and Org3H structures compared with the RU486 complex.

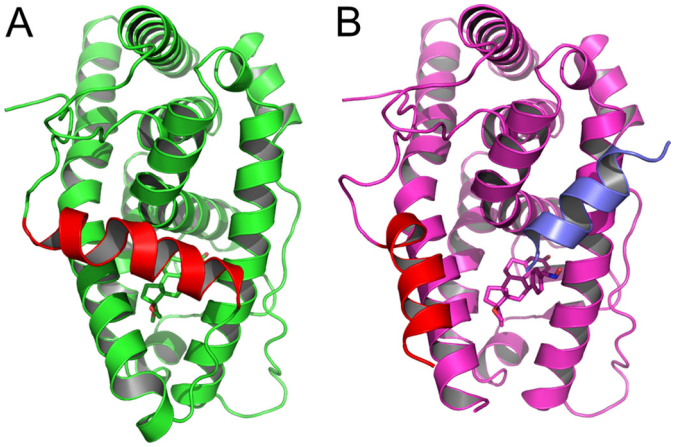

FIGURE 3. *Panel A* shows the secondary structure of the PR-Asoprisnil complex generated by a soaking experiment described in this article. Helix-12 is colored *red* and is oriented in the classic agonist conformation. *Panel B* shows the secondary structure of a previously described PR-Asoprisnil complex generated (PDB code 2OVH) by co-expression with the ligand. Helix-12 is colored *red* and is shifted from the agonist position to allow the binding of a co-repressor peptide, colored *blue*. Protein-ligand complexes were aligned using the Motif function in YASARA (31), and images were generated using PyMOL.

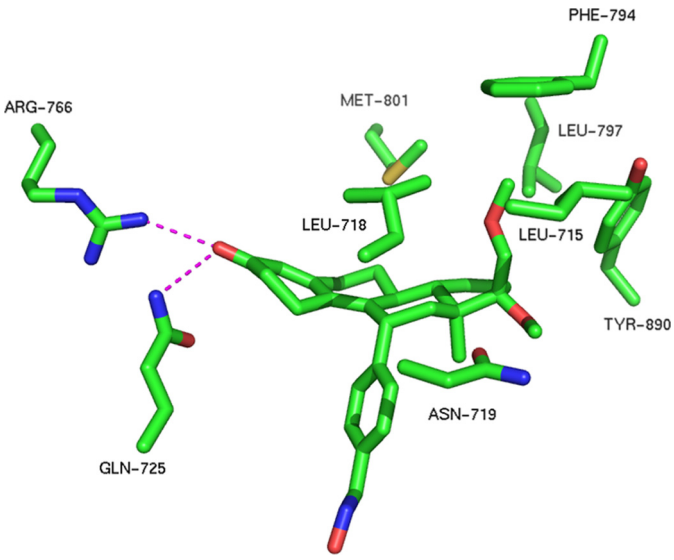

FIGURE 4. **Binding mode of Asoprisnil with hydrogen bonding from Gln^{725} and Arg^{766} to the ligands 3-keto group, highlighted in *magenta*.** Residues forming a hydrophobic pocket surrounding the 17α group are also shown.

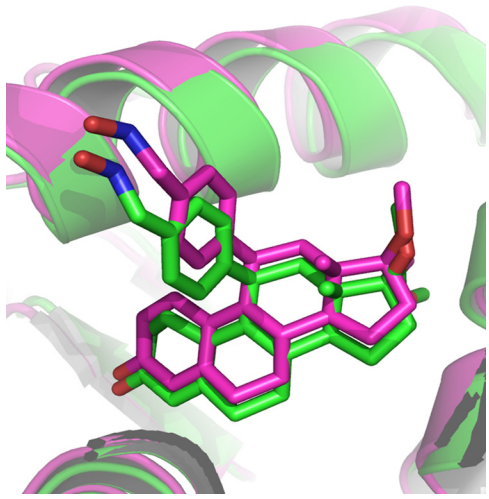

FIGURE 5. **Asoprisnil from the PR-agonist conformation is shown (carbons are colored *green*) and overlaid with Asoprisnil from the PR-antagonist conformation (carbons are colored *magenta*).** Asoprisnil from the PR-agonist conformation sits slightly lower in the pocket compared with Asoprisnil from the PR-antagonist conformation.

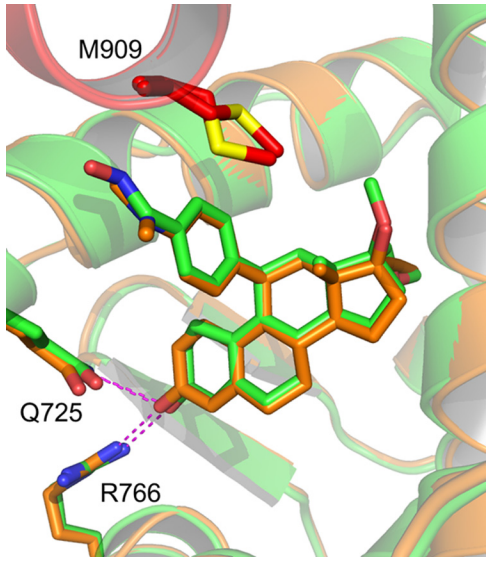

FIGURE 6. **Overlaid structures of PR-agonist structure bound to RU486 (carbons are colored *orange*) compared with PR-agonist structure bound to Asoprisnil (carbons are colored *green*).** Hydrogen bonds between the ligand keto groups to Gln^{725} and Arg^{766} are shown in *magenta*. Helix-12 is colored *red* with Met^{909} highlighted in both complexes (carbons are colored *red*). The ligands overlay closely and appear to sterically impact on Met^{909} to a similar degree.

Met^{909} Is Better Ordered in Asoprisnil Complex Compared with Equivalent RU486 Structure—In the past our group published an x-ray structure of RU486 bound to the agonist conformation of PR (16), which clearly demonstrated disorder of Met^{909} in helix-12 due to a clash with the ligand (Fig. 2*A*). This clash resulted in the destabilization of helix-12, measured by an increase in the b-factors of the helix compared with helix-12 bound to previously described full agonists. This provided a compelling model to explain the antagonistic activity of RU486.

Met^{909} in the PR-agonist complex bound to Asoprisnil does adopt a modified conformation compared with its position bound to other full agonists, but the new rotamer is well ordered, as can be seen when comparing the electron density of each complex shown in Fig. 2. The increased order of helix-12 and particularly Met^{909} in the Asoprisnil structure is consistent with the increased agonistic activity of the ligand compared with RU486.

As we investigated the molecular basis for this difference, we considered the possibility that the reduced volume of Asoprisnil oxime and its linear rather than branched nature, compared with the dimethylamine of RU486, may reduce its clash with Met^{909}. However, comparison of the overlaid ligands did not suggest a significant difference in their spatial arrangements around the position of Met^{909} (Fig. 6), and therefore, other factors must be responsible that will be discussed in the following section.

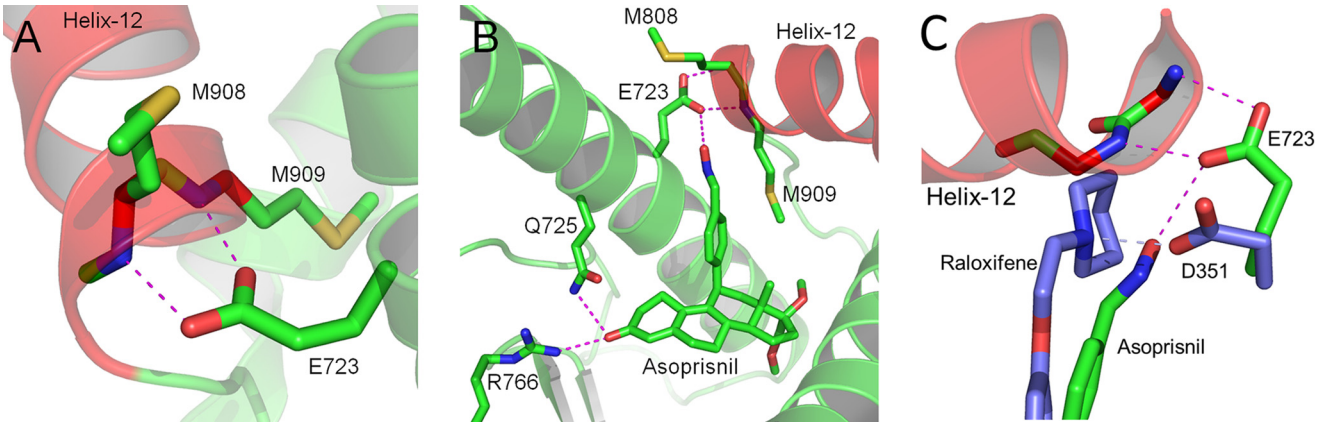

FIGURE 7. *Panel A* illustrates the stabilizing interaction between Glu^{723} and helix-12 (colored *red*) by hydrogen bonding to the backbone of Met^{908} and Met^{909}. *Panel B* shows the binding mode of Asoprisnil bound to the agonist conformation of PR with ligand to receptor hydrogen bonds shown in *magenta*. Interactions between Glu^{723} and helix-12 (shown in *red*) are also shown. *Panel C* shows a comparison between the effect of binding Asoprisnil (*green*) to PR and raloxifene (*blue*) to estrogen receptor α. Binding of Asoprisnil to PR contributes the stabilizing interactions between Glu^{723} and the backbone of helix-12. On the other hand, the binding of raloxifene to estrogen receptor α results in Asp^{351} losing its interactions with the backbone of helix-12 as it now prefers to re-orientate and make a salt bridge to the ligands basic amine.

Asoprisnil Oxime Group Contributes to Stabilization of Helix-12 in Its Agonistic Conformation—As previously described (16, 43), Glu^{723} in helix-3 plays an important role in stabilizing the agonist conformation of helix-12 by hydrogen bonding to main chain amines in both Met^{908} and Met^{909} (Fig. 7*A*). In addition, Glu^{723} is also able to make a hydrogen bond to a water molecule in the bulk solvent. In the antagonistic conformation of PR bound to Asoprisnil, Glu^{723} points away from the ligand to make polar contacts with the solvent and, due to the displacement of helix-12, no longer makes stabilizing interactions to Met^{908} and Met^{909}.

In the agonistic conformation of PR-bound Asoprisnil, Glu^{723}, as expected, stabilizes the agonist position of helix-12 but, additionally, makes a hydrogen bond to the ligand oxime group (Fig. 7*B*). In this new state Glu^{723} strengthens the hydrogen-bond network that stabilizes helix-12 in its agonistic position. This interaction is not duplicated on binding of RU486, which suggests the agonist conformation of PR bound to Asoprisnil is more stable than the agonist conformation of PR bound to RU486. This relative increased stability of the agonist conformation when bound to Asoprisnil explains the ability of this complex to recruit co-activators and suggests that the existence of the interaction with Glu^{723} pushes the receptor equilibrium toward agonism compared with RU486.

Glu^{723} Is Equivalent to Asp^{351}, Residue Crucial for Agonist/Antagonist Balance in Estrogen Receptor α—Glu^{723} in PR is equivalent, based on sequence and structure alignments, to Asp^{351} in the estrogen receptor α, which plays a similar role in stabilizing the agonist conformation of helix-12. The importance of this residue has been demonstrated by studying the naturally occurring D351Y mutation and other synthetic mutations that result in loss of receptor agonistic activity, consistent with the residues normal role in stabilizing the agonist conformation of the receptor. Selective modulators of estrogen receptor α, referred to as SERMs and including raloxifene and tamoxifen, contain an important basic amine function that is almost ubiquitous among this drug class. The role of this nitrogen is to form a salt bridge to Asp^{351}, requiring the amino acid to adopt a new conformation and prevent it from undertaking its usual function of stabilizing the agonistic conformation of helix-12 (27, 41, 44–51, 53).

SERMs are, therefore, well characterized examples of ligands whose mixed profiles can partly be attributed to disrupting the stabilizing interaction of the conserved acidic residue at this position. We believe that Asoprisnil is the first characterized example of a ligand whose mixed profiles can be attributed to strengthening this same stabilizing interaction.

Interaction with Glu^{723} Is Unlikely to Explain Partial Agonism of Org3H—The interaction between Asoprisnil and Glu^{723} is dependent on the presence of the 11β-benzaldooxime group and as such is unlikely to be a universal model for mixed profile compounds. For example, Org3H is a SPRM from a class of steroids incorporating 11β-pyridinylphenyl groups and, therefore, lacks the hydrogen-bond donating capacity of the Asoprisnil oxime substituent. In an attempt to explain its partial agonism, we selected Org3H as a candidate for characterization via x-ray crystallography following the same soaking procedure as we have described for Asoprisnil.

Org3H Is Bound to Monomer B but Not to Monomer A—As is the case for our PR-Asoprisnil structure, the asymmetric unit contains two copies of the PR-LBD. Visual inspection of the electron density within the ligand binding pocket of the structure clearly shows that monomer B contains Org3H but OrgA is still present in monomer A and has not been replaced during the soaking. A similar observation was made when RU486 soaked into the B monomer of PR-norethindrone crystals but failed to displace the original ligand from monomer A (16). As Asoprisnil displaced OrgA in both monomers, we can conclude that monomer A is accessible in the crystal but must have lower affinity for Org3H or a slower rate of ligand entry compared with monomer B. As hypothesized in the RU486 study, this difference is likely to arise from conformational differences in the two crystallographically independent PR LBDs in the crystals. These differences are most prominent around the loop 785–808, a region previously hypothesized to be a route of entry for the ligand during the soaking experiments (16).

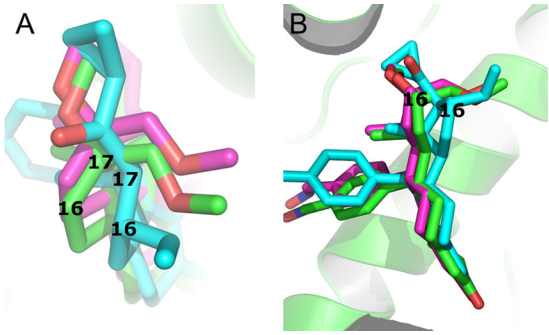

FIGURE 8. ***Panels A* and *B* show the overlaid structures of Asoprisnil (carbons are in *green*), RU486 (carbons are in *orange*), and Org3H (carbons are in *cyan*).** The secondary structure of the PR-Asoprisnil structure is shown as a schematic representation in *green*. The 17α side chains of Asoprisnil and RU486 overlay relatively closely with the 16α substituent of Org3H due to the displacement of this compounds D-ring. In *A*, positions 16 and 17 of Org3H and Asoprisnil are labeled. In *B*, position 16 of Org3H and Asoprisnil was labeled.

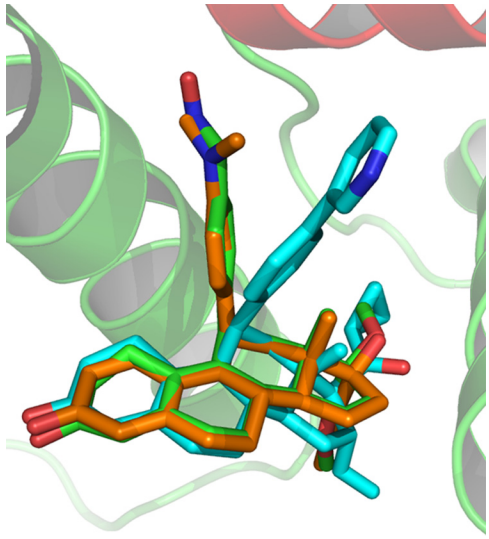

FIGURE 9. **Overlaid structure of Asoprisnil (carbons are in *green*), RU486 (carbons are in *orange*), and Org3H (carbons are in *cyan*).** The secondary structure of the PR-Asoprisnil structure is shown as a schematic representation in *green*. The 11β substituents of Asoprisnil and RU486 overlay closely, but the 11β substituent of Org3H is clearly divergent.

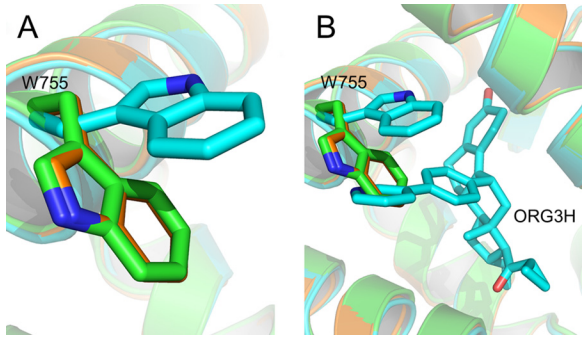

FIGURE 10. ***Panel A* shows Trp^{755} from the Asoprisnil-bound complex (carbons are in *green*) and from the RU486-bound complex (carbons are in *orange*) showing the normally observed rotamer for this residue.** In contrast, Trp^{755} from the Org3H bound complex is shown (carbons are in *cyan*), demonstrating its ligand induced movement. *Panel B* shows a similar scene to *panel A* but with Org3H superimposed to show the clash it would make to Trp^{755} if the amino acid was not shifted.

Steroidal Core of Org3H Binds Differently Than Other Steroids—The binding of OrgA in monomer A is practically identical to the previously described PR structures containing OrgA and requires no further description (26). Org3H shares the classical interaction with Gln^{725} and Arg^{766} via its 3-keto group, with the A-ring in general binding in the conventional manner described for RU486 and Asoprisnil. In the second monomer Org3H shares the same scaffold as RU486 and Asoprisnil, but does not overlay as closely as these two ligands after binding to the receptor (Fig. 8). Although the conformation of the ligand is unchanged, the Org3H binding mode shows distinct unexpected differences, mostly around the D-ring, compared with the other steroids as it is shifted away from its typical position toward the 17α pocket. Org3H does not have a 17α attachment, as seen for RU486 and Asoprisnil, but the repositioning of the D-ring is significant enough that the vinyl group on position 16α of Org3H is oriented in a similar position to the 17α groups of RU486 and Asoprisnil, occupying a comparable space within the receptor as shown in Fig. 8, *A* and *B*. This overlay would not be possible if the steroid scaffold bound in the classic position.

Induced Fit around Trp^{755} Relieves Clash with Met^{909}—The scaffold carbon atoms at C11 in RU486, Asoprisnil and Org3H are all located at approximately the same position in their various co-crystals. The pendant attachments of RU486 and Asoprisnil, attached to C11, are also well overlaid and conserved. The phenyl group attached at C11 of Org3H is, however, significantly divergent from the other reference compounds (Fig. 9). The pyridinylphenyl fills a pocket resulting from a rearrangement of Trp^{755}.

Upon binding of Org3H, Trp^{755} rotates 120° around the bond between the residues Cα and Cβ bond and 100° around the bond between Cβ and Cγ (shown in Fig. 10, *A* and *B*). The new orientation of Trp^{755} now packs against Leu^{726} in helix-3, providing space to accommodate the side chain of Org3H (shown in Fig. 11, *A* and *B*). The capacity for Trp^{755} to adopt novel positions has been described when the receptor binds to non-steroidal compounds (24–26). In addition to these examples, the same plasticity has been noted for the equivalent residue, Trp^{741}, in AR (34, 54–59).

The pyridinylphenyl side chain is able to occupy a channel pointing toward His^{888} to which it makes a water-bridged H-bond from the ligand pyridine nitrogen shown in Fig. 12. In addition to His^{888} and the ligand, the conserved water is also able to hydrogen-bond to the backbones of Tyr^{753} and Met^{756}.

It has previously been observed that the Trp^{741} flip in AR (equivalent to Trp^{755} in PR) makes available a channel and potential interaction with His^{874} (equivalent to His^{888} in PR), including the presence of the conserved water molecule. Comparison of all publicly available PR structures shows that this channel has not previously been described for this receptor (Fig 13). It appears that the unusual orientation of Org3H steroidal core is required to allow the 11β side chain to adopt this position.

Effect of 16α Attachment Explains Structure Activity Relationship, Suggesting That This Substituent Induces Partial Agonism—McDonnell and co-workers (38) have previously reported the observation that 16α attachments on RU486 analogs result in partial agonist activity rather than the full antag-

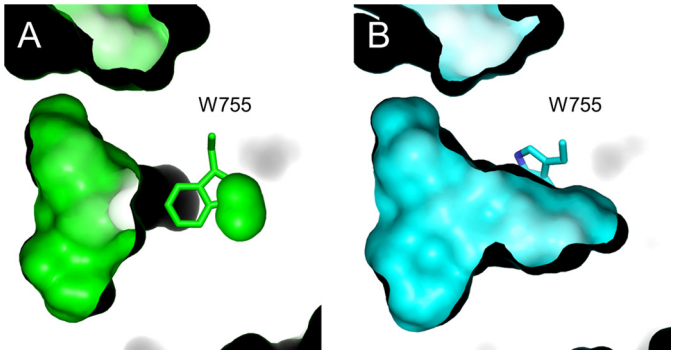

FIGURE 11. *Panel A* shows a stick representation of Trp^{755} and a surface representation of the binding pocket of the PR-agonist structure bound to Asoprisnil (ligand hidden for clarity). *Panel B* shows a stick representation of Trp^{755} and a surface representation of the binding pocket of the PR-agonist structure bound to Org3H (the ligand is hidden for clarity). The flipping of Trp^{755} in the Org3H-bound complex expands the overall size of the pocket and provides the opportunity for novel structure-based drug design.

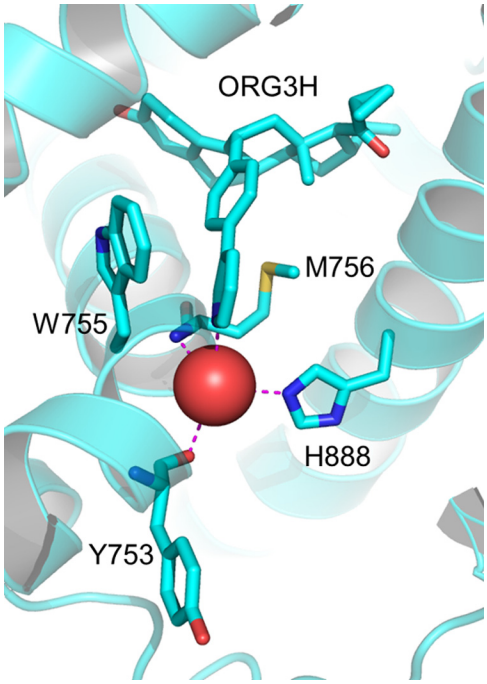

FIGURE 12. **Binding mode of Org3H with the water (oxygen is shown as a *red sphere*)-mediated hydrogen-bond to His^{888} is highlighted.** Additional interactions between the water molecule and Met^{756} are also shown.

onism that would otherwise be expected. They also suggest that the basis for this effect is by altering the manner in which the crucial 11β group of RU486 and its analogs interact with the receptor but that crystallographic data would be needed to confirm this. Our crystallography studies show that due to the 16α attachment on Org3H, its steroidal scaffold binds in an atypical fashion that consequently leads to the ligands 11β having a reduced clash with Met^{909}. Our PR-Org3H x-ray structure, the first of PR bound to a 16α-substituted PR modulator, supports McDonnell's hypothesis and suggests that the changing interaction between the receptor and the 11β groups they observed is due to the utilization of the newly formed pocket behind Trp^{755}.

New Position of Trp^{755} Clashes with Helix-12—The divergent position of the Org3H side chain results in a reduced clash with

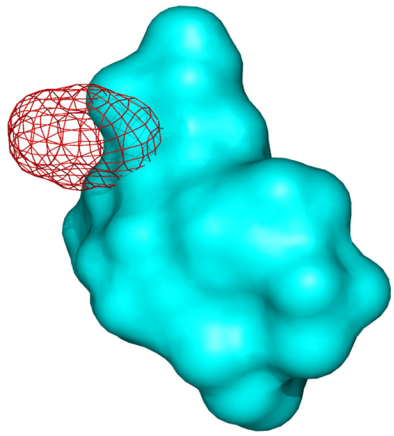

FIGURE 13. **The available PR LBD structures in the PDB were aligned using the MOTIF function in YASARA and the superimposed ligands extracted.** A molecular surface encompassing the sum of the ligands was calculated by PyMOL and shown is *cyan*. Org3H was added to the ligand alignment, and its molecular surface was calculated and displayed as a *red mesh*. The portion of the red mesh visible represents the additional space required for the tolerance of this ligand that has not previously been described for this receptor.

Met^{909} compared with RU486, which is in agreement with the increased agonistic activity of Org3H compared with RU486. Met^{909} adopts a different conformation compared with the rotamer reported when binding to the full agonist norethindrone (16, 21) but appears to be well tolerated (Fig. 2), even making favorable hydrophobic contacts to the phenyl in the ligands 11β attachment. This prompted us to hypothesize that the remaining clash with Met^{909} may not be significant enough on its own to explain the reduced agonistic activity Org3H compared with full agonists. We, therefore, continued examining the structure to identify if any other factors were at play.

In its new position the Trp^{755} side chain is directed toward Val^{912} in helix-12 and would clash with the position of this residue in either the RU486 or Asoprisnil (ago conformation) structures, as they would be within 2 Å. To relieve this potential clash, Val^{912} is pushed away (~1.5 Å average r.m.s.d. across all non-hydrogen atoms but as much as ~2.5 Å for some of the side-chain atoms), and we observed a shift of helix-12 not present in agonist structures or either the PR-agonist-Asoprisnil or PR-agonist-RU486 structures (Fig 14). We previously described a PR structure bound to a full antagonist, OrgB, that also induces a flipping of Trp^{755} (26) but does not result in the clash with Val^{912} or the displacement of helix-12. So far, the indirect destabilization of helix-12 independent of Met^{909} appears to be unique to this series of compounds and may explain why these compounds are clearly mixed profile as measured in cell-based *in vitro* assays, which typically characterize SPRMs as full antagonists.

Stabilized Receptor Conformations Provide Valuable Approach for Studying Mixed Profile Modulators—Understanding the molecular basis for mixed profile compounds is hampered by the difficulty in determining relevant co-crystal structures. Full agonists stabilize the receptor and, specifically, helix-12 in a conformation suited to binding co-activating proteins, and full antagonists stabilize the receptor in a conformation suited to binding co-repressing proteins. The apparent reason for the difficulty in co-crystallizing mixed profile compounds is that they do not fully stabilize the receptor in either conformation,

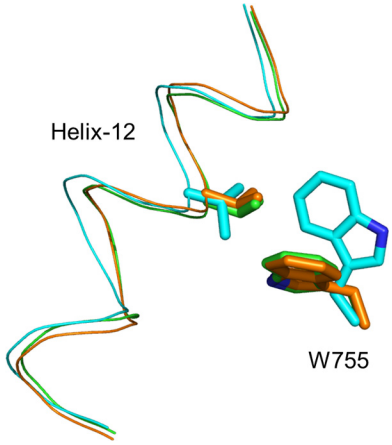

FIGURE 14. **Trp^{755} and helix-12 from Asoprisnil-bound complex (*green*) with Trp^{755} and helix-12 from RU486-bound complex (*orange*) compared with Trp^{755} and helix-12 from the Org3H-bound complex (*cyan*).** The alternate position of Trp^{755} from the Org3H complex would clash with Val^{912} from the Asoprisnil and RU486 complexes. This clash is relieved by a rotation of Val^{912} and a pushing away of the backbone of helix-12 in this region, suggesting a destabilization of the agonist position of helix-12.

adopting some degree of equilibrium between the two. This equilibrium allows mixed profile compounds to bind unique patterns of co-modulators compared with full agonists and antagonists, resulting in their potentially interesting biological effects. Unfortunately, as a result it also renders them poorly suited to co-crystallization studies (60). Recently we have seen the first publications describing methods to circumvent this problem either by introducing stabilizing mutations into the receptor (52, 56, 60) or by generating stable crystals of the receptor using a receptor stabilizing ligand and then exchanging this compound with other compounds of interest via soaking (16). Both approaches have the potential to dramatically increase our understanding of the biological mechanisms underpinning partial agonism and provide novel insight for drug optimization.

Implications of New PR Structures for Drug Design—All ligands elicit their behaviors by a combination of their steric and electrostatic character. The binding of PR agonists is the result of a well described combination of steric complementarity and specific electrostatic interactions, typically to Asn^{719}, Gln^{725}, and Arg^{766}. Our understanding of the additional interactions that differentiate full antagonists and SPRMs from full agonists have until now been limited to the steric properties of bulky 11β substituents. The binding mode of Asoprisnil to the agonist state of PR is the first description of how additional electrostatic factors, specifically the interaction with Glu^{723}, can alter biological properties of SPRMs. Altering the nature of that interaction by compound optimization has the potential to fine-tune the characteristics of SPRMs with the aim to improve their therapeutic response.

In addition to this new insight into the potential value of modifying SPRMs via an electrostatic approach, the PR-Org3H co-crystal also provides valuable new information regarding the steric influences that will also benefit future drug design. In particular we show how modifications away from the 11β group, such as the presence of 16α groups, can influence the effect of the classic bulky substituent. This complex is also the first indication that SPRMs can illicit their responses without directly impeding helix-12 or clashing with Met^{909}. A combination of the ligand-induced clash between Trp^{755} and Val^{912} in helix-12 and the limited clash with Met^{909} suggest a unique mechanism for the Org3H mixed profile. Overall we suggest specific new directions for the design of SPRMs including the exploration of the pocket behind Trp^{755}, modification of interactions with Glu^{723}, and the generation of indirect clashes with helix-12.

Conclusion—We present here two new PR structures bound to partial agonists. The structures demonstrate that two distinct mechanisms explain the mixed profiles of the two ligands, indicating that both steric and electrostatic factors can contribute to this mixed PR activity. The additional information that has been learned from the binding of Asoprisnil bound to the agonist conformation of PR illustrates the value of studying mixed profile compounds bound to both the agonist and antagonist conformations of their receptors as a useful tool for drug design. This also supports the growing use of stabilized receptor systems either by mutation or following soaking strategies as shown here to study otherwise inaccessible receptor-ligand complexes.

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