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The Novel Progesterone Receptor Antagonists RTI 3021–012 and RTI 3021–022 Exhibit Complex Glucocorticoid Receptor Antagonist Activities: Implications for the Development of Dissociated Antiprogestins*

B. L. WAGNER†, G. POLLIO, P. GIANGRANDE‡, J. C. WEBSTER, M. BRESLIN, D. E. MAIS, C. E. COOK, W. V. VEDECKIS, J. A. CIDLOWSKI, AND D. P. MCDONNELL

Department of Pharmacology and Cancer Biology (B.L.W., G.P., P.G., D.P.M.), Duke University Medical Center, Durham, North Carolina 27710; Molecular Endocrinology Group (J.C.W., J.A.C.), NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709; Department of Biochemistry and Molecular Biology (M.B., W.V.V.), Louisiana State University Medical School, New Orleans, Louisiana 70112; Ligand Pharmaceuticals, Inc. (D.E.M.), San Diego, California 92121; Research Triangle Institute (C.E.C.), Chemistry and Life Sciences, Research Triangle Park, North Carolina 27709

ABSTRACT

We have identified two novel compounds (RTI 3021–012 and RTI 3021–022) that demonstrate similar affinities for human progesterone receptor (PR) and display equivalent antiprogestenic activity. As with most antiprogestins, such as RU486, RTI 3021–012, and RTI 3021–022 also bind to the glucocorticoid receptor (GR) with high affinity. Unexpectedly, when compared with RU486, the RTI antagonists manifest significantly less GR antagonist activity. This finding indicates that, with respect to antiglucocorticoid function, receptor binding affinity is not a good predictor of biological activity. We have determined that the lack of a clear correlation between the GR binding affinity of the RTI compounds and their antagonist activity reflects the unique manner in which they modulate GR signaling. Previously, we proposed a two step "active inhibition" model to explain steroid receptor antagonism: 1) competitive inhibition of agonist binding; and 2) competition of the antagonist bound receptor with that activated by agonists for DNA response elements within target gene promoters. Accordingly, we observed that RU486, RTI 3021–012, and RTI 3021–022, when assayed for PR antagonist activity, accomplished both of these steps. Thus, all three compounds are "active antagonists" of PR function. When assayed on GR, however, RU486 alone functioned as an active antagonist. RTI 3021–012 and RTI 3021–022, on the other hand, functioned solely as "competitive antagonists" since they were capable of high affinity GR binding, but the resulting ligand receptor complex was unable to bind DNA. These results have important pharmaceutical implications supporting the use of mechanism based approaches to identify nuclear receptor modulators. Of equal importance, RTI 3021–012 and RTI 3021–022 are two new antiprogestins that may have clinical utility and are likely to be useful as research reagents with which to separate the effects of antiprogestins and antiglucocorticoids in physiological systems. (*Endocrinology* **140:** 1449–1458, 1999)

THE STEROID HORMONE progesterone is a key regulator of the processes involved in the development and maintenance of reproductive function (1). However, the efficacy of antiprogestins as treatments for brain meningiomas, breast cancer, uterine fibroids, and endometriosis have implicated progesterone in the pathology of these diseases (2–9). Consequently, although a relatively new class of molecules, the antiprogestins are likely to have a wide range of clinical applications. The most widely used antiprogestin, RU486 (mifepristone), was originally developed as an antiglucocorticoid but was subsequently shown to be a potent and effective antiprogestin (10). As an antiprogestin, RU486 is used to induce medical abortions and as a missed menses inducer (11, 12). For these applications, the drug is given acutely and, consequently, the antiglucorticoid activity is unlikely to cause any lasting side effects. For chronic administration, however, such as would be required for most endocrinopathies, it is likely that the antiglucocorticoid activity of these compounds would not be desirable. Therefore, there has been a great deal of interest in developing compounds that will inhibit progesterone receptor (PR) transcriptional activity but do not interfere with the biological actions of glucocorticoids.

All of the currently available antiprogestins are steroidal in nature and are derived from a 19-nor testosterone backbone (10, 13, 14). It is likely that nonsteroidal antiprogestins with improved selectivity will be developed. In their absence, efforts to dissociate antiprogestational from antiglucocorticoid activity have been limited to modifications of existing steroidal antiprogestins. Unfortunately, a selective steroidal antiprogestin has not yet emerged. We believe that progress in this area has been limited by the approach that

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Address all correspondence and requests for reprints to: D. P. McDonnell, Department of Pharmacology and Cancer Biology, Duke University Medical School, Box 3813, Durham, North Carolina 27710. E-mail: McDon016@acpub.duke.edu.
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has been used in the past to screen for dissociated antiprogestins. Typically, *in vitro* receptor binding assays, assessing PR/GR selectivity, have been used to guide medicinal chemistry. This approach has not yet yielded a dissociated antiprogestin as it has been found that most compounds that display a reduced GR binding activity exhibit a commensurate decrease in affinity for PR (10). This observation suggested that a more predictive screen for novel antiprogestins was needed, one that did not discriminate based on receptor binding affinity, but rather on the ability of a compound to differentially affect PR or GR signaling.

Much of the justification for a mechanism-based approach to develop dissociated antiprogestins has come from our previous studies on the mechanism of action of PR agonists and antagonists (15–18). In these earlier studies, we identified two classes of antiprogestins that interact with similar, though distinct, regions within the PR ligand binding domain, resulting in unique alterations in PR structure (18). Subsequently, it was determined that members of one class of antiprogestins identified exhibited pure antiprogestenic activity in all contexts examined, whereas members of the second class functioned as antiprogestins in most contexts but had the ability to function as partial agonists in others (18). A potential molecular explanation for the differential activity of these two classes of antagonists was revealed when it was determined that the pure antiprogestins permitted the formation of high affinity interactions of PR with the nuclear receptor corepressors SMRT and NCoR, whereas the tissue selective antiprogestins (mixed agonists) formed weak associations with the same proteins. Importantly, overexpression of either corepressor had a pronounced effect on the activity of the PR mixed agonists where complete suppression of the partial agonist activity of these compounds was achieved. Cumulatively, these findings indicated that although the two classes of antiprogestins displayed similar PR binding affinities, they were mechanistically different. Based on this observation, which established a link between PR structure and biological activity, we considered that it may be possible to identify compounds that interact with both PR and GR but may not affect the transcriptional activity of these receptors in a similar manner. Therefore, in this study we used a series of mechanism based approaches to screen libraries of high affinity steroidal antiprogestins for compounds with reduced antiglucocorticoid activity.

Materials and Methods

Alkaline phosphatase assay

T47D cells were seeded into 96-well plates at a density of 10,000 cells/well in RPMI media supplemented with 10% FCS. Following a 24-h incubation, the cells were washed and fresh medium containing 2% FCS and ligand (10^{-6}–10^{-9} M) was added. The treated cells were incubated with ligand for 48 h, washed, and fixed with 5% formalin at room temperature for 30 min. Cells were subsequently washed and assayed for alkaline phosphatase activity as described previously (18, 19).

Mammalian transfections and luciferase assays

HeLa and T47D cells were maintained in MEM and RPMI supplemented with 10% FCS, respectively. Cells were plated in 24-well plates, 24–48 h before transfection. HeLa cells were transiently transfected for 3 h with a total of 3 μg of DNA per triplicate using Lipofectin. T47D cells were similarly transfected with Lipofectin for 2 h. After transfection, the cells were immediately washed and incubated with the designated ligands for 24 or 48 h. The cells were then lysed and analyzed for luciferase and β-galactosidase activity as previously reported (20).

Cell viability

CEM-C7 cells were maintained in RPMI 1640 media containing 10% dialyzed, heat-inactivated FBS. Cells were seeded at 1×10^5 to 3×10^5 cells per ml in 6-well plates and incubated with the designated ligands for 72 h. Following the incubation, 500 μl of cells were removed and the number of viable cells was assayed using trypan blue exclusion.

Relative binding affinities

All procedures were performed using a Biomek 1000 automated workstation (Beckman Coulter Instruments, Inc., Fullerton, CA). Tenfold serial dilutions (10^{-6}–10^{-10}) of the compound to be tested were prepared in a 10 mM Tris (pH 7.6) 0.3 MKCl, 5 mM DTT solution. A 100 μl aliquot of each dilution was transferred to a polystyrene tube containing 5 nM [^{3}H] progesterone or [^{3}H] dexamethasone (Amersham, Arlington Heights, IL). To each tube either PR containing extracts from baculovirus (20 μg total protein) or GR containing extracts from MDA-231 cells (250 μg total protein) were added and incubated overnight at 4 C. Hydroxylapatite slurry (100 μl) in 10 mM Tris (pH 7.6) and 2 mM DTT were added and the tubes were incubated for an additional 30 min at 4 C, after which they were centrifuged to recover the pellets. Hydroxylapatite pellets were washed four times with 1% Triton X-100, 10 mM Tris (pH 7.6), 5 mM DTT after which they were resuspended in 800 μl Ecoscint A scintillation fluid (National Diagnostic, Manville, NJ), and the activity was measured on a LS60001C scintillation counter (Beckman Coulter Instruments, Inc., Fullerton, CA).

Immunohistochemistry

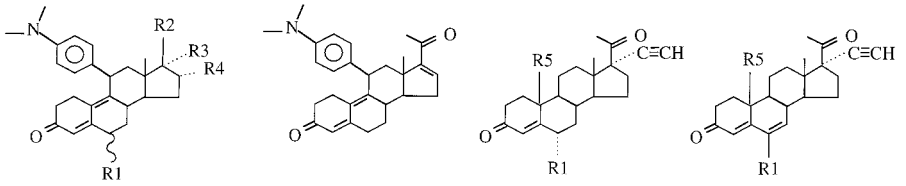
The subcellular distribution of human GR transiently transfected into COS-1 cells has been previously described (21). Briefly, COS-1 cells (African Green Monkey Kidney, ATCC) were grown in DMEM (Gibco BRL, Gaithersburg, MD) containing 9 mg/ml glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin, and supplemented with 2 mM glutamine and 10% of a 1:1 mixture of FCS/calf serum (FCS:CS) (Irvine Scientific, Santa Ana, CA). Cultures were maintained at 37 C in a humidified atmosphere of 5% CO_2. The cells were passed every 3–4 days and were maintained in culture for no longer than 15 passages. Cells were transfected by the commercial agent DMRIE C (Gibco BRL) as per manufacturer's instructions. Cells were incubated with the appropriate DNA/DMRIE C mixture for 4 h and placed in DMEM supplemented with steroid-stripped FCS:CS and further incubated at 37 C for 24 h. Transfected cells were then placed in two-chamber glass slides and incubated for an additional 24 h and then treated with 100 nM hormone or vehicle for 1 h. Cells were fixed and processed for immunohistochemical staining as previously described (21).

Results

PR ligands can be classified into either of three mechanistically distinct groups

As an initial step in this study, we screened a series of steroidal PR ligands to identify compounds that displayed agonist, antagonist, or mixed agonist activity on PR. It was anticipated that this would allow the identification of mechanistically unique PR antagonists that could function as dissociated antiprogestins or which could serve as leads for additional synthetic chemistry. The structures of the compounds evaluated in this study are shown in Table 1. Previous studies with these compounds indicated that they could be separated into one of three groups based on how they interacted with PR (18). In this study, we evaluated whether the biological activity of these compounds reflected these mechanistic classifications. This was accomplished by evaluating each compound for agonist and antagonist ac-

TABLE 1. Structures of PR agonists and antagonists



RU486, 1-3,5-7 **4** **8-9** **10**

Compound	R1	R2	R3	R4	R5
RU486	-H	-OH	$-C\equiv C-CH_3$	-H	-
1. 3021-002	-H	-OH	$-CH_2N_3$	-H	-
2. 3021-003	-H	-OH	$-CH_2OCH_3$	-H	-
3. 3021-012	-H	$-COCH_3$	-OAc	-H	-
4. 3021-023	-	-	-	-	-
5. 3021-020	$-\alpha CH_3$	$-COCH_3$	-H	$-C_2H_5$	-
6. 3021-021	$-\beta CH_3$	$-COCH_3$	-H	$-C_2H_5$	-
7. 3021-022	-H	$-COCH_3$	-H	$-C_2H_5$	-
8. 2207-222	-H	-	-	-	-H
9. 2207-225	$-CH_3$	-	-	-	$-CH_3$
10. 2207-226	$-CH_3$	-	-	-	$-CH_3$

tivity in PR-containing T47D cells on the endogenous progesterone-responsive alkaline phosphatase gene (19). Although the alkaline phosphatase gene is regulated by PR, it is not clear if this activity occurs in a direct or an indirect manner. As observed in Fig. 1A, progesterone administration induced significant alkaline phosphatase activity in this cell system. Compounds that, based on their effect on PR structure, were predicted to function as antagonists [RTI 3021–002 (RTI-002), RTI 3021–003 (RTI-003), and RTI 3021–012 (RTI-012)], exhibited no measurable agonist activity. Conversely, compounds that interacted with PR in a manner similar to progesterone [RTI 2207–222 (RTI-222), RTI 2207–225 (RTI-225), and RTI 2207–226 (RTI-226)] functioned as agonists. The PR ligands, RTI 3021–020 (RTI-020), RTI 3021–021 (RTI-021), and RTI 3021–022 (RTI-022), which induce unique structural alterations within the receptor, exhibited partial agonist activity in this assay, a result that distinguished them from agonists and antagonists. The classification of these compounds as partial agonists, as distinct from weak agonists, was confirmed by examining their ability to inhibit progesterone induced expression of alkaline phosphatase activity. As shown in Fig. 1B, the pure antagonists all functioned as potent PR antagonists and quantitative inhibition was achieved at concentrations as low as 100 nM. The partial agonist activity of RTI-020, -021, and -022 was confirmed by demonstrating that they inhibit progesterone activated PR transcriptional activity to a level equivalent to their maximal agonist activity. Although the direct measurement of alkaline phosphatase activity indicated that like progesterone, RTI-222, -225, and -226 function as PR agonists they may not function in an identical manner to progesterone in this assay. Specifically, it is noted that the maximal efficacy of the RTI agonists is significantly less than progesterone (Fig. 1A). Paradoxically, these compounds do not inhibit progesterone agonist activity when tested in the antagonist mode. As yet, we have been unable to explain this result. As shown below, however, additional experiments indicate that this particular activity of the RTI agonists may be unique to the alkaline phosphatase promoter.

It has previously been determined that the activity of the ER-mixed agonist tamoxifen is influenced by cell and promoter context (22, 23). In light of this, we decided to examine whether or not the partial agonist activity of RTI-020, -021, and -022 was likewise affected by the context in which it was assayed. To address this issue we evaluated the pharmacology of the PR-mixed agonists on a transfected MMTV promoter in PR-containing T47D cells and compared it to that of the pure agonists and antagonists. As observed in Fig. 2A, the pure antagonists and agonists functioned predictably. However, in this environment RTI-020, -021, and -022 do not exhibit measurable agonist activity (Fig. 2A) and at 100 nM all members of this class functioned as efficient antagonists of progesterone agonist activity (> 95% efficacy; Fig. 2B). Similar results were obtained in transfected CV-1 cells using the same experimental paradigm (data not shown). Taken together, these results confirmed that PR ligands could be separated into at least three functionally distinct classes. We proceeded, therefore, to assess the antiglucocorticoid activity of these newly identified PR antagonists and partial antagonists.

The compounds RTI-022 and RTI-012 differ in their ability to modulate PR and GR transcriptional activity

In the past, it was generally held that the ability of a nuclear receptor antagonist to inhibit transcriptional activity was determined solely by its affinity for its cognate receptor (1). If this were true, then binding selectivity would be the only way of generating pure antiprogestins that were GR sparing. It is becoming more apparent, however, that the effect of the

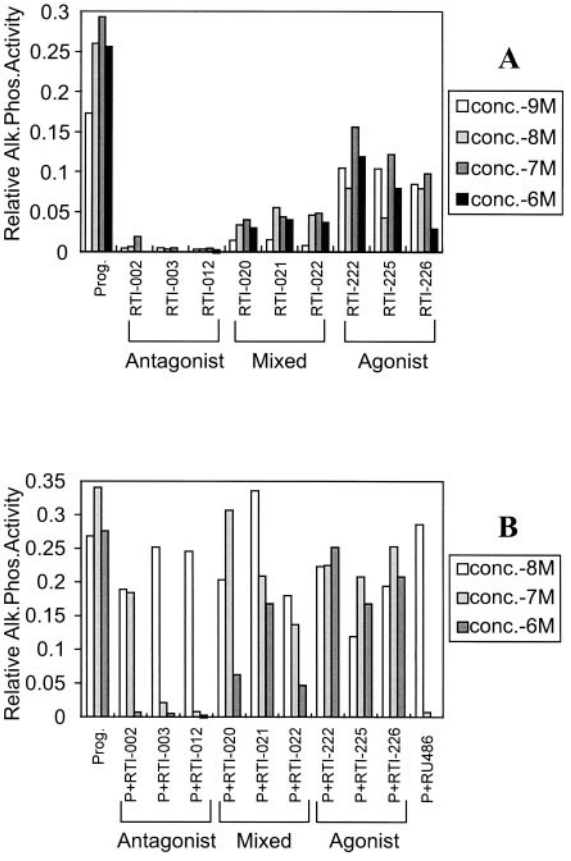


FIG. 1. Progesterone receptor ligands can be divided into three classes: agonists, mixed agonists and antagonists. The agonist and antagonist activities of the RTI series of PR ligands (the structures shown in Table 2) were assessed on the progesterone responsive alkaline phosphatase gene in T47D cells. T47D cells were incubated with the indicated ligands (A) alone to assay for agonist activity (10^{-6}–10^{-9} M) or (B) together with progesterone (10^{-7} M) to assay for antagonist activity (10^{-6}–10^{-8} M). After 48 h incubation with ligand, the cells were fixed and assayed for alkaline phosphatase activity. Each data point represents the average of triplicate determinations.

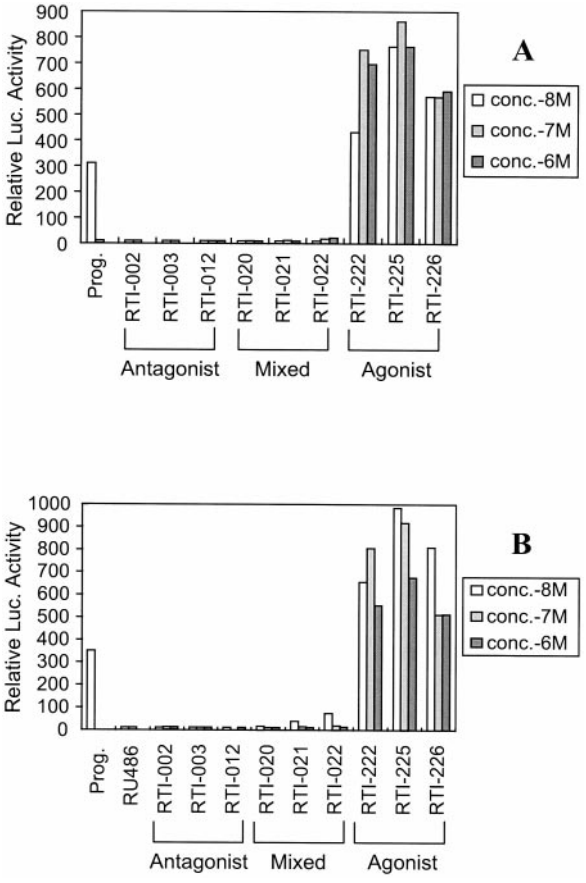


FIG. 2. PR mixed agonist activity is promoter dependent. The agonist and antagonist activity of a series of PR ligands was analyzed in PR-containing T47D human breast cancer cells that were transiently transfected with an MMTV-Luciferase reporter plasmid and a CMV-β-galactosidase expression plasmid for normalization. To assay agonist activity, transfected cells were incubated with (A) either 10^{-8} M progesterone or increasing concentrations of the indicated ligands (10^{-6}–10^{-8} M). Antagonist activity (B) was assessed by incubating cells with either 10^{-8} M progesterone alone or together with increasing concentrations of competing ligands as indicated (10^{-6}–10^{-8} M). Forty-eight hours post transfection, the cells were lysed and assayed for luciferase and β-galactosidase activities. The data points are averages of triplicate determinations.

ligand on overall receptor structure is an equally important determinant of biological activity. This has led to the concept that antagonists are "actively" involved in inhibiting receptor action (15, 18, 24, 25). If this model is correct, then binding affinity and antagonistic activity are not necessarily equivalent. The availability of a repertoire of novel, mechanistically different antiprogestins provided us with the reagents to test this model. For these specific studies, the pure antagonist RTI-012 and the mixed agonist RTI-022 were chosen for an analysis of their ability to inhibit GR transcriptional activity. These specific ligands were selected because they exhibit similar relative binding affinities (RBA) for both PR and GR, allowing a direct analysis of the role of "mechanism" in determining the relative GR/PR cross-reactivity of a PR ligand (Table 2). When compared with dexamethasone, it was observed that RTI-022, RTI-012, and RU486 (the standard used in our assays) had similar GR binding affinities (RBAs 5.7, 5.2, and 13.9, respectively) to the pure agonist dexamethasone. A similar analysis comparing the affinities of these compounds for PR indicated that, compared with progesterone, the RBAs for RTI-012, RTI-022, and RU486 were 12.7, 11.9, and 6.8, respectively. Thus, if ligand binding is the primary determinant of antagonist efficacy, then these compounds should display equivalent antiprogestenic and antiglucocorticoid activities. To test this hypothesis, we compared the ability of RU486, RTI-012, and RTI-022 to inhibit PR and GR transcriptional activity in transfected cells.

To assess the antagonist activity of RTI-012, RTI-022, and RU486, we transfected the PR/GR responsive reporter gene MMTV-LUC into T47D cells and assayed the ability of these compounds to inhibit the agonist activity of the synthetic progestin R5020. The results of this analysis, shown in Fig. 3, demonstrate that all three compounds are effective PR antagonists. In accord with the observed affinity differences, we noticed that the antagonist potency of RU486 was slightly greater than either of the two RTI compounds, which themselves behaved quite similarly in this assay. Thus, in this cell and promoter context, the *in vitro* PR binding affinity of these compounds and their PR antagonist efficacy match closely.

TABLE 2. Receptor binding characteristics

Analog	PR-A		GR		PR-RBA/GR-RBA[a]
	K_d (nM)	RBA	K_d (nM)	RBA	
Dexamethasone			9.5 ± 1.5	1	1
Progesterone	3.95 ± 0.25	1			1
RU486	0.58 ± 0.03	6.8	0.68 ± 0.06	13.9	0.48
RTI-012	0.31 ± 0.04	12.7	1.68 ± 0.18	5.7	2.3
RTI-022	0.33 ± 0.03	11.9	1.83 ± 0.16	5.2	2.2

Data shown as mean ± SEM (N = 2). [^{3}H] Progesterone was used as the ligand for PR and [^{3}H] dexamethasone for GR. Prep was Baculovirus extracts of PR-A and cytosolic extract from MDA-231 cells for GR. Total protein per tube was 20 μg and 250 μg for PR and GR, respectively. Incubation overnight at 4 C.

RBA, Relative binding affinity, progesterone and dexamethasone = 1.

[a] Ratio of RBA at progesterone receptor to RBA at glucocorticoid receptor. Values >1 favor affinity for progesterone receptor over glucocorticoid receptor.

Similar results were observed in HeLa cells in which PR and a PR-responsive promoter were cotransfected (data not shown).

We next performed a comparison of the ability of the selected compounds to inhibit GR transcriptional activity. This was accomplished by cotransfecting GR and the GR/PR responsive MMTV-LUC reporter gene into HeLa cells and assessing the ability of these compounds to inhibit dexamethasone-stimulated GR transcriptional activity. The results of this analysis are shown in Fig. 3B. As expected from its GR-binding affinity, RU486 functioned as an effective GR antagonist. Quite surprisingly, however, RTI-012 and RTI-022, whose affinities for GR were similar to each other and to that displayed by RU486, did not function as potent GR antagonists. Specifically, under the conditions of this assay the antagonist potencies of RU486 and RTI-012 differed by over 100-fold, whereas a greater than 1000-fold difference in potency was observed between RU486 and RTI-022. The differences between RTI-012 and RTI-022 may relate to subtle mechanistic differences between these compounds. Alternatively, it is possible that RTI-012 is converted to its 17α-OH metabolite, a transformation that would not express itself in the *in vitro* binding assays and may enhance its receptor binding affinity. These informative results indicated that, with respect to GR antagonism, there was a large discrepancy between GR antagonist efficacy and binding affinity. It must also be mentioned that neither RTI-012, nor RTI-022 exhibited any GR agonist activity when assayed on a GR-responsive promoter in transfected mammalian cells (data not shown).

RTI-012 and RTI-022 efficiently promote the interaction of PR, but not GR, with target gene promoters in vivo

We considered that one reason for the difference in GR antagonist efficacy manifest by RU486, RTI-022, and RTI-012 was that they were not equally effective at delivering GR to DNA. This possibility was tested by assessing the ability of these compounds to activate transcription of a GR-VP16 fusion plasmid. In this assay, GR/ligand complexes that bind DNA permit the activation of transcription by the VP16 activation domain contained within the chimeric GR. This approach was chosen as we and others have shown that the VP16 activation function, when used in the context of a receptor chimera, permits both agonists and antagonists to activate transcription upon DNA binding (22, 26). Thus, antagonists will function as agonists if they can deliver the chimera receptor to DNA. For this analysis, HeLa cells were transiently transfected with an expression vector encoding the GR-VP16 chimera together with one of two different GRE containing luciferase reporter vectors, MMTV-LUC or PRE-TK-LUC. As shown in Fig. 4A, both dexamethasone and RU486 efficiently delivered GR-VP16 to DNA. Interestingly, this is not the case when the assay is performed in the presence of either RTI-012 or RTI-022. Under the conditions of this assay, using saturating concentrations of test compounds, we observed that the GR/DNA binding activity of RTI-012 and RTI-022 was only 35% and 6%, respectively, of that observed in the presence of RU486 when assayed on the MMTV-LUC promoter. A similar result was observed when the assay was performed on the PRE-TK-LUC promoter. For comparative purposes, we performed the same type of assay using PR-VP16. The results of this analysis shown in Fig. 4 indicate that both RTI-022 and RTI-012 are capable of inducing high affinity PR-DNA interactions in a manner that is indistinguishable from RU486. Thus, we conclude from these results that the inability of RTI-012 and RTI-022 to efficiently deliver GR to DNA may explain their relatively weak GR-antagonist activity.

RTI-022 and RTI-012 differ from RU486 in their ability to efficiently induce nuclear translocation of GR

The results outlined above (Fig. 4) demonstrated that there were differences in the ability of antagonists to promote GR target promoter associations (RU486[tmt]RTI-012 > RTI-022). One explanation for this activity is that there were differences in the ability of these compounds to promote nuclear translocation. GR is unique among the nuclear receptors in that it resides in the cytoplasm of target cells in the absence of ligand (21). Upon binding an agonist such as dexamethasone, the receptor translocates to the nucleus where it exerts its regulatory activities (21). To test the effect of the RTI compounds on GR nuclear translocation we transfected COS-1 cells with an expression vector for GR and examined the cellular localization of the recombinant receptor using immunohistochemical techniques following treatment of the cells with selected agonists and antagonists. The results of this analysis are shown in Fig. 5. Both dexamethasone and RU486 promoted an efficient translocation of GR to the nuclear compartment of these cells. However, under the conditions of this assay both RTI-012 and RTI-022 were only partially active in this regard. We therefore concluded that RTI-012 or RTI-022 function predominantly as compet-

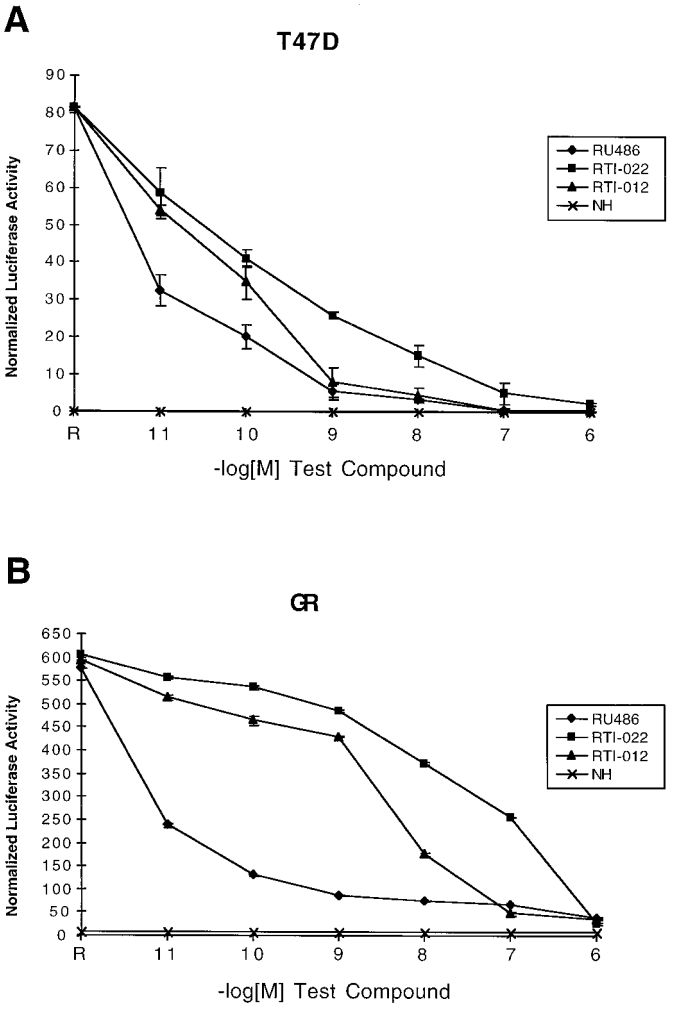


FIG. 3. RTI-012 and RTI-022 are potent antiprogestins that demonstrate weak antiglucocorticoid activity. A, The relative PR antagonist activity of RU486, RTI-012, and RTI-022 were compared in PR-containing T47D human breast cancer cells that were transiently transfected with an MMTV-Luciferase reporter plasmid and a CMV-β-galactosidase expression plasmid for normalization. To assay agonist activity, transfected cells were incubated with 10^{-8} M R5020 and increasing concentrations of the indicated antagonists (10^{-6}–10^{-11} M). Forty-eight hours post transfection, the cells were lysed and assayed for luciferase and β-galactosidase activities. The data points are averages of triplicate determinations. B, Antiglucocorticoid activity was analyzed in HeLa cells transiently transfected with a GR expression plasmid, the MMTV-Luciferase reporter plasmid, and a CMV-β-galactosidase plasmid for normalization. After transfection cells were treated with 10^{-9} M Dexamethasone (Dex) alone or in the presence of competing ligand as indicated (10^{-11}–10^{-6} M) for 48 h. Cells were lysed and assayed for luciferase and β-galactosidase activities. Each data point presented is the average of triplicate determinations.

itive antagonists on GR because the resulting receptor-ligand complexes cannot translocate efficiently to the nucleus and compete for DNA binding with agonist activated receptor.

RTI-022 exhibits weak antiglucocorticoid activity in GR-mediated apoptosis

The ability to develop compounds that effectively inhibit PR transcriptional activity but which do not inhibit GR actions is likely to facilitate the use of antiprogestins for the

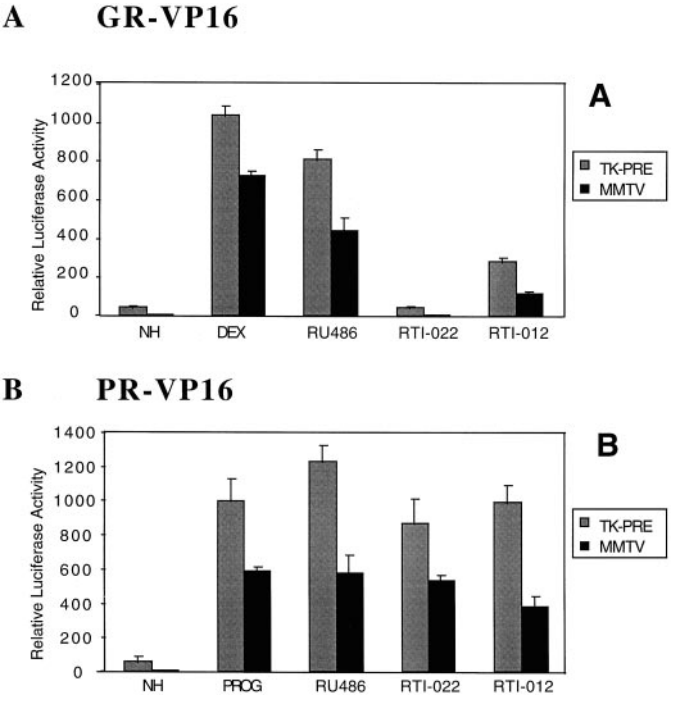


FIG. 4. RU486, RTI-022, and RTI-012 differ in their ability to facilitate interactions of PR and GR with their cognate target gene promoters. DNA binding ability was assayed by measuring the transcriptional activity of GR or PR fused to the VP16 activation domain on the PRE-containing luciferase reporter vectors MMTV-Luc or PRE-TK-Luc as indicated. For this analysis HeLa cells were transiently transfected with an expression vector for either (A) GR-VP16 or (B) PR-VP16, in combination with either a PRE-TK-Luc, or MMTV-Luc and a CMV-β-galactosidase normalization plasmid. Cells were incubated with dexamethasone (Dex), R5020, RU486, RTI-022, or RTI-012 (10^{-7} M) for 48 h followed by lysis and analysis for luciferase and β-galactosidase activities. Each data point represents the average of triplicate determinations. *Error* is represented as ($\pm$SEM).

treatment of several chronic diseases where inhibition of PR action is implicated. The molecular data presented thus far suggest that the RTI-012 and RTI-022 compounds may, if their pharmaceutical properties permit, be clinically useful compounds. To develop this hypothesis further we extended our studies to cell based models that may be more reflective of *in vivo* biological responses. We chose to use RTI-022 for these studies as it gave the largest separation between PR and GR antagonist activities and consequently would likely be the compound of choice for clinical development. Glucocorticoid agonists are effective in causing apoptosis in T-lymphoblasts, such as the human T-lymphoblastic cell line, CEM-C7, an event that is blocked by the antagonist RU486 (27). While GR transrepression of AP-1 activity has been implicated in Jurkat cells (28), GR-mediated up-regulation of GR and c-*jun* appears to regulate apoptosis in CEM-C7 cells (29, 30). Furthermore, suppression of GR-agonist induced up-regulation of c-*jun* gene expression using an antisense c-*jun* expression vector blocks the apoptotic response (30). We were interested, therefore, in assaying the ability of RTI-022 to prevent dexamethasone (Dex) induced apoptosis in this cell line. For this assay, CEM-C7 cells were grown in the presence of either vehicle, or dexamethasone alone, or together with increasing concentrations of the designated li-

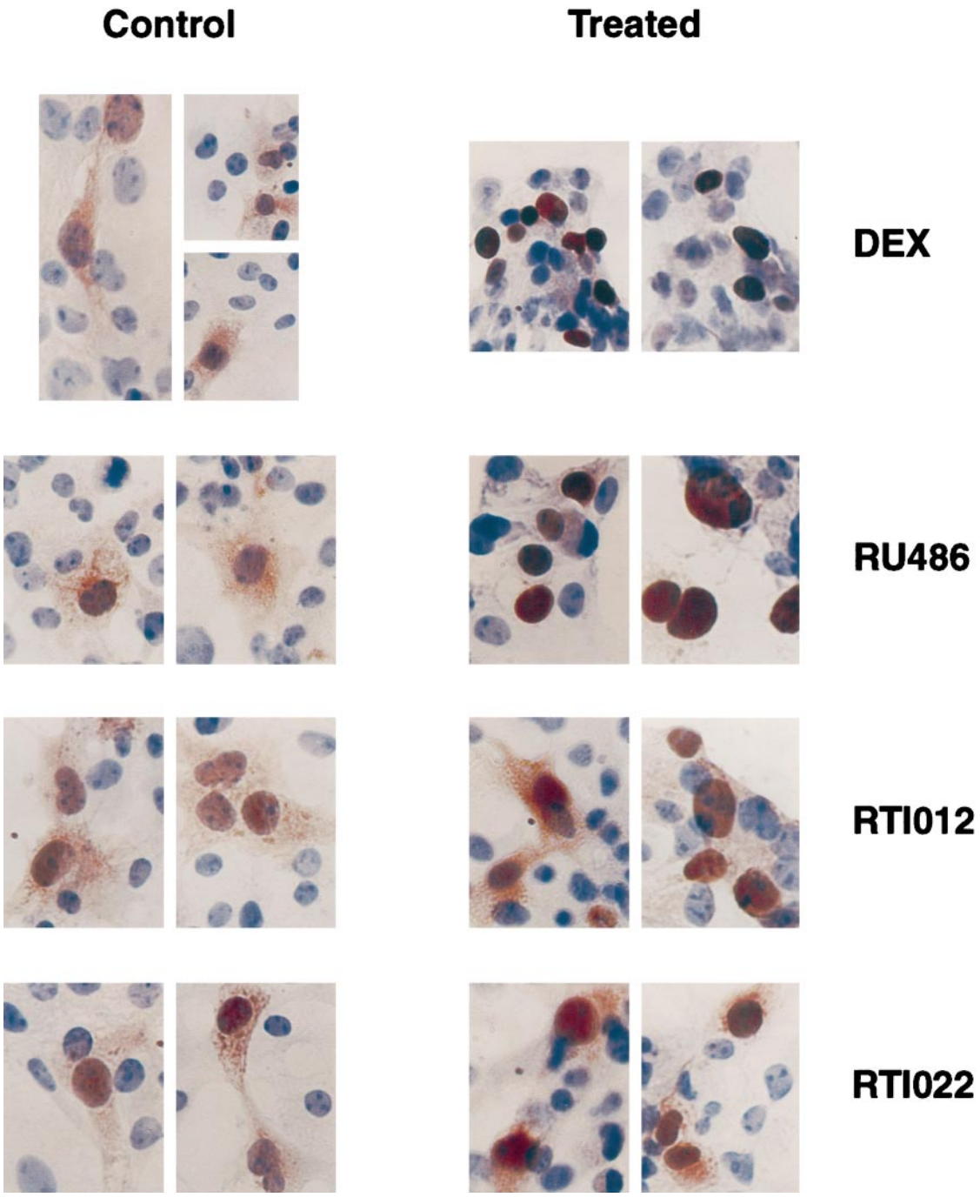


FIG. 5. Nuclear translocation of glucocorticoid receptor in the presence of RU486, RTI-022, or RTI-012. Wild-type human glucocorticoid receptor (hGR) complementary DNA was transiently expressed in COS-1 cells and treated with 100 nM hormone (Treated) or not (Control) for 2 h. Cells were fixed and subsequently incubated with an epitope purified GR specific antibody. Immunoreactivity was visualized using an avidin-biotin peroxidase stain. Photomicrographs were taken and then evaluated in a blind manner at 600× magnification using Kodak Royal Gold ASA-200.

gands, after which cell viability was measured using trypan blue exclusion. The negative control, progesterone, which exhibits a much lower affinity for GR (30 nM) (Cook, C. E., data not shown) than the compounds we are investigating, did not prevent dexamethasone from inducing apoptosis (Fig. 6). As previously reported, RU486 completely prevented dexamethasone-induced apoptosis when these compounds were added in equimolar concentrations and gave 50% protection when added at a concentration 1/10th that of dexamethasone. This is the expected result given that RU486 has nearly a 13-fold higher affinity for GR than does dexamethasone. Interestingly, when assayed under the same conditions RTI-022, whose affinity for GR is only 2.5-fold less than RU486, required 50–100 times more compound to evoke the same response. Cumulatively, therefore, our data, emanating from both cotransfection and cell based assays, indi-

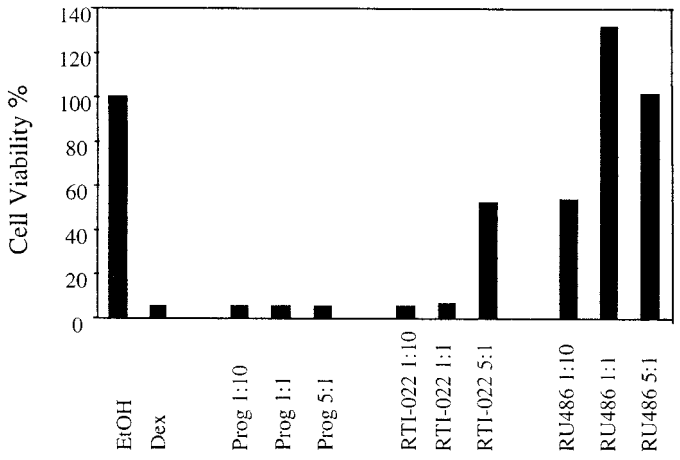


FIG. 6. RTI-022 is not a potent inhibitor of dexamethasone induced apoptosis. CEM-C7 cells were seeded at 300,000 cells/ml and grown in the presence of vehicle (EtOH), 1 μM dexamethasone, or 1 μM dexamethasone (Dex) with increasing concentrations (0.1, 1, or 5 μM) of RU486, progesterone (Prog) or RTI-022 (1:10, 1:1 and 5:1) molar ratios compared with dexamethasone (Dex). Following a 72-h incubation, cell viability was measured using trypan blue exclusion. The data are presented as % of viable cells remaining following ligand treatment compared with vehicle alone.

cate that PR antagonists can be developed that do not significantly impact GR signaling.

Discussion

Classical receptor theory predicts that the biological activity of an agonist, or an antagonist, is a reflection of its affinity for its target receptor (1). However, it is clear that ligand binding affinity is only one of many factors that influence the pharmacology of steroid receptor ligands (22, 31, 32). For instance, the high affinity ER-ligand tamoxifen can function as an ER-antagonist, partial agonist or a full agonist, depending on the cell context in which it is analyzed (23, 33). These data suggest that ER is not functioning in an identical manner in all cells. This concept appears not to be restricted to ER because we have recently determined that PR ligands can be classified into three distinct groups, pure agonists, mixed agonists or pure antagonists, and that the relative agonist/antagonist activities of the mixed agonists is determined, to a large extent, by the cell and promoter context in which transcriptional activity is assessed (18). Cumulatively, these studies on the molecular pharmacology of ER and PR suggest to us that it may be possible to use mechanism based approaches to discover novel steroid receptor ligands that display improved selectivity over existing compounds.

In this study, we undertook a molecular approach to understand the mechanism by which antiprogestins manifest antagonist activity on PR and GR. The currently available antiprogestins also function as effective antiglucocorticoids (13, 14). Thus, for applications that require chronic administration there is a medical need to develop dissociated antiprogestins; compounds that display no or reduced antiglucocorticoid activity (9). However, there has been little success in identifying antiprogestins that do not function as antiglucocorticoids (10, 13, 14). This may relate to the fact that the currently available antiprogestins are steroidal, derived from the same chemical backbone, and so may function by very similar mechanisms (13). The recent identification of a new class of PR mixed agonists, which interact with the PR hormone binding domain in a distinct manner, prompted us to reexamine the issue of GR cross-reactivity of PR antagonists (18). In this study, we profiled this new series of PR ligands and determined that the compounds RTI-022 and RTI-012 that functioned as potent PR antagonists *in vitro* exhibited significantly less GR antagonist activity than their receptor binding affinities would predict. To understand the discrepancy between binding affinity and biological potency, we compared the ability of RU486, RTI-022, and RTI-012 to facilitate the interaction of GR with target gene promoters. These studies revealed that neither RTI-012 or RTI-022 were as effective as RU486 at inducing nuclear translocation of GR. In contrast, however, RTI-012, RTI-022 and RU486 efficiently facilitated PR/DNA interactions and demonstrated comparable progesterone antagonist activities. Thus, although we previously had shown that RTI-012 and RTI-022 interact with different regions of the PR-ligand binding domain and do not inhibit PR-transcriptional activity in the same manner, they both efficiently delivered PR to DNA indicating that it was post DNA binding events that distinguished these compounds. When assayed on GR, we were surprised to find that RTI-012 and RTI-022, that displayed high affinity GR binding, were not potent antagonists. This was in great distinction to RU486, an affinity matched ligand, which functioned as a potent PR and GR antagonist. Thus, although we can classify compounds as PR agonists, antagonists or partial agonists based on how they interact with PR, these classifications do not predict the likely GR cross-reactivity of specific compounds. Thus, at this point, we believe that the unique chemical structures of RTI-012 and RTI-022 have some effect on GR that distinguishes them from RU486. This interesting possibility will be followed up in subsequent studies. Regardless, these data strongly support our hypothesis that binding affinity alone is not sufficient to predict the biological activity of a receptor antagonist.

The studies presented here, and those of others, are compatible with the existence of two distinct types of antagonists, competitive and active antagonists (Fig. 7). Using GR antagonism as an example, we propose that the RTI compounds function only as competitive antagonists; a one-step process in which agonists and antagonists only compete for receptor binding. Possibly because of a specific conformational change, the resultant GR ligand complex does not enter the nucleus and therefore does not directly oppose the actions of residual agonist activated receptor. Because competitive inhibitors do not prevent agonist occupied receptors from binding DNA and activating transcription, their antagonist activity is governed mainly by affinity. In contrast to RTI-022 and RTI-012, RU486 functions as an active antagonist of GR transcriptional activity. Thus, RU486 not only competitively inhibits agonist binding to GR but permits the formation of a ligand-GR complex that can participate actively in the inhibition process. Specifically, these complexes can bind with high affinity to target gene promoters and block agonist activated receptor from interacting with its DNA-target site. In some contexts, members of this class of active antagonists can function as partial agonists; an event that can only occur

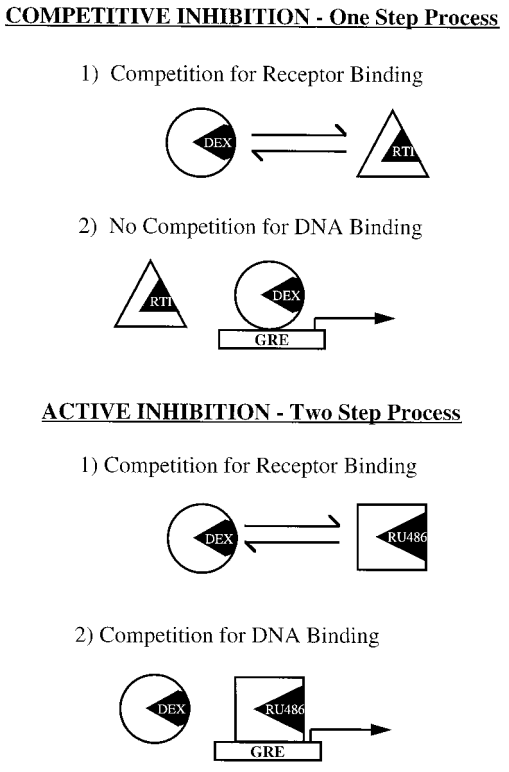


FIG. 7. Competitive *vs.* active inhibition of GR transcriptional activity. Competitive inhibition is a one-step process in which the antagonist competes with the agonist for receptor binding. Competitive inhibitors induce a conformational change in the receptor that is incompatible with DNA binding, preventing the antagonist occupied receptors from competing at the level of DNA binding. The two RTI compounds examined in this study thus function as competitive inhibitors of GR. Active inhibition is a two-step process in which 1) the antagonist competes with the agonist for receptor binding and 2) antagonist occupied receptors compete with agonist occupied receptors for binding to glucocorticoid responsive elements. The ability of PR and GR to recruit the transcriptional corepressors N-CoR and SMRT when occupied by active antagonists is likely to be important also. These proteins are part of a large complex that can de-acetylate histones H3 and H4 resulting in nucleosome condensation and transcriptional silencing.

when the receptor binds DNA. Indeed, Nordeen *et al.* (34) have demonstrated that RU486 can in fact manifest partial GR agonist activity in some contexts. Although the mechanism of this partial agonism remains to be determined, it is likely that differences in the expression of specific receptor associated proteins contribute significantly to the degree of agonist activity manifest by the RU486/GR complex. Therefore, in those contexts in which RU486 exhibits partial agonist activity, competitive inhibitors, at saturating doses, are more likely to function as pure antagonists. As a final note on this topic, we believe that because RU486, RTI-012, and RTI-022 efficiently deliver PR to DNA, they are functioning as active antagonists of PR transcriptional activity. This highlights the need to qualify the classification of a given compound with respect to a specific receptor.

One of the major findings of this paper is that, with respect to RTI-012 and RTI-022, there is a large discrepancy between the *in vitro* GR-binding affinity and antagonist potency. In the past, discrepancies of this nature were usually explained by differences in metabolism and/or pharmacokinetics; factors that are unlikely to be important in this case. However, we and others have defined a molecular mechanism that adequately explains active antagonism. Specifically, it has been determined that active antagonists like RU486 facilitate the interaction of PR and GR with the nuclear corepressors SMRT and N-CoR. The nuclear corepressors were originally identified as proteins that could bind to unoccupied TR and RAR located on target gene promoters and permit these receptors to function as transcriptional repressors (35, 36). Although the mechanism by which the corepressors exhibit their inhibitory activity remains under investigation, it appears that they are part of a multiprotein complex that is responsible for deacetylating histones H3 and H4 and facilitating a local condensation of chromatin (37–39). Recently we, and other groups, have been able to show that the influence of the corepressors is not restricted to the Class II nuclear receptors but that they are also an important part of PR, GR, and ER pharmacology (17, 40, 41). Specifically, it was shown that in the presence of pure antagonists, PR was capable of high affinity interactions with either N-CoR or SMRT (17, 41). Agonist binding abolished these interactions and partial agonists demonstrated an intermediate activity as expected (17). Thus, the model for active inhibition must be expanded to incorporate this new information. Specifically, an active antagonist such as RU486 can competitively bind to its target receptor, induce high affinity DNA binding and subsequently recruit an inhibitory complex that is capable of enzymatically altering chromatin structure. In support of this model, we have been able to show that the corepressor SMRT can interact with both PR and GR when activated by RU486 (17). Thus, it is likely that the reason why RTI-022 and RTI-012 function only as competitive antagonists of GR activity is that they are unable to translocate GR to the nucleus. Thus, the association of the receptor with the corepressor is prevented.

In summary, this work has led to the identification of RTI-022 and RTI-012, compounds that function as competitive antagonists of GR function and active antagonists of PR transcriptional activity. These mechanistic differences manifest themselves as a 1- to 400-fold discrepancy between binding and antagonist efficacy with respect to GR activity and comparable binding and antagonist potency on PR. Thus, a separation between GR and PR antagonism is afforded by virtue of differences in the mechanism of action of these compounds on the two different receptors. This result validates using a mechanism-based approach to develop dissociated antiprogestins that, when used in combination with traditional direct binding approaches, is likely to be a powerful combination in the discovery of dissociated antiprogestins. In addition to providing useful insights into the pharmacology of PR and GR, we believe that RTI-012 and RTI-022 will find use *in vivo*, both as research tools and hopefully as drugs where it is important to separate antiprogestenic from antiglucocticoid activities.

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