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# *In vivo* evidence against the existence of antiprogestins disrupting receptor binding to DNA

(progesterone receptor/agonists–antagonists)

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**ABSTRACT** **The binding of a steroid hormone to its receptor elicits a sequence of events: activation of the receptor (probably through dissociation from a complex of heat shock proteins), dimerization, binding to hormone responsive elements, and finally modulation of gene transcription. RU 486, the first antiprogestin studied, has been shown to act at the last step of this sequence: provoking an inefficient binding of the receptor to hormone responsive elements. Recently, based on *in vitro* studies, it has been proposed that ZK 98299 was the prototype of a second class of antiprogestins that were supposed to act through disruption of the binding to DNA. We have devised methods allowing us to study the various steps of agonist or antagonist action *in vivo*. We show here that RU 486 and ZK 98299 have the same effects on receptor activation, dimerization, and binding to hormone responsive elements; differences in their action are explained by the 10-fold difference in their affinity for the receptor (ZK 98299 having the lower affinity).**

Recently, there has been a great interest in the study of antiprogestins, for two main reasons. (*i*) They have been shown to have a great therapeutic potential: interruption of early pregnancy (1, 2), postcoital contraception (3), triggering of labor (4), and treatment of hormone-dependent tumors (5–7). (*ii*) Antiprogestins have also been used as potent tools to decipher the molecular mechanisms of action of the hormone (8, 9).

Initially, it was shown (10, 11) that RU 486 provokes the binding of the receptor to hormone responsive elements (HREs). But the conformation of these complexes probably differs from that of agonist–receptor complexes since they do not trigger activation of transcription (10, 11). More recently, *in vitro*, studies of ZK 98299–receptor complexes have shown the absence of binding to HREs (12). This has led to the suggestion that ZK 98299 was an example of a second class of antiprogestins that acted by preventing the formation of DNA–receptor complexes (12–14).

The binding of the receptor to HREs implicates several steps. The first step, called activation, is probably due to the dissociation of heat shock proteins and leads to the unmasking of the DNA binding domain (15, 16). It is followed by receptor dimerization that involves mainly the ligand binding domain (17–19). The third step is the binding of the dimers to the HREs (10, 11). We have devised methods allowing us to examine in intact cells the occurrence of all these steps (10, 17). Using such an analysis, we have observed that RU 486 and ZK 98299 differ in their affinity toward the receptor but not in their mechanism of action.

## MATERIALS AND METHODS

**Chemicals.** RU 486 {(11$\beta$,17$\beta$)-11-[4-(dimethylamino)phenyl]-17-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one} and R 5020 [(17$\beta$)-17-methyl-17-(1-oxopropyl)estra-4,9-dien-3-one] were gifts from D. Philibert (Roussel-Uclaf). ZK 98299 [11$\beta$-(4-dimethylaminophenyl)-17$\alpha$-hydroxy-17$\beta$-(3-hydroxypropyl)-13$\alpha$-methyl-4,9-gonadien-3-one] was a gift from H. Michna (Schering).

[$^3$H]R 5020 (86.3 Ci/mmol; 1 Ci = 37 GBq) was purchased from DuPont/NEN.

**Plasmids.** Plasmid nomenclature is as follows. Derivatives denoted with a $\Delta$ lack the receptor segment delineated by the numbered amino acids. When the epitope recognized by the antibody Let126 is deleted (aa 25–103), the mutant is called "Let−." Plasmids containing the intact rabbit progesterone receptor (PR) cDNA (pKSV-rPR), wild-type receptor Let−, and mutants PR$\Delta$593–640, PR$\Delta$373–546, PR$\Delta$638–642, and PR$\Delta$663–930 have been described (10, 17).

Mutant PR$\Delta$593–640,Let− was constructed by cleavage of the mutant PR$\Delta$593–640 at the proper restriction sites, purification of the restriction fragment encompassing the deletion, cleavage of the wild-type Let− receptor at the same restriction sites, and ligation of the purified restriction fragment with the wild-type Let− receptor.

**Cell Culture.** Simian COS-7 cells (20) were grown under 5% $CO_2$/95% air at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum (GIBCO), 2 mM glutamine, and 1 mM pyruvate. Confluent cells were harvested by treatment with 0.25% trypsin/5 mM EDTA.

**Transfection.** Cells were seeded 1 day before transfection in Dulbecco's modified Eagle's medium supplemented with 10% charcoal-treated fetal calf serum. Transfections were performed by calcium phosphate coprecipitation (21) with various amounts of DNA in different studies. The precipitate was removed after 20 h and the cells were incubated for 24 h with fresh medium containing hormone when indicated.

**Chloramphenicol Acetyltransferase (CAT) Assays.** To test antihormone effects on the biological activity of the wild-type receptor, $1.5 \times 10^6$ cells were transfected with expression vector encoding for PR (pKSV-rPR) (10 $\mu$g/ml), the reporter plasmid MMTV–CAT (10 $\mu$g/ml; MMTV is mouse mammary tumor virus), and pCH 110 plasmid (Pharmacia; 5 $\mu$g/ml), which encodes *Escherichia coli* $\beta$-galactosidase, as described (10). Cells were incubated 24 h prior to harvesting with 1 nM R 5020 and various concentrations of the antagonist (0.1 nM–1 $\mu$M).

To study the inhibition of activity of the constitutive receptor by wild-type receptor complexed to antihormone, expression vector (1 $\mu$g/ml) encoding the constitutive mutant receptor (PR$\Delta$663–930), pKSV-rPR (1 $\mu$g/ml), salmon sperm DNA (3 $\mu$g/ml), MMTV–CAT (10 $\mu$g/ml), and pCH110 (5 $\mu$g/ml) were used as described (10). Cells were incubated

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Abbreviations: PR, progesterone receptor; HRE, hormone responsive element; CAT, chloramphenicol acetyltransferase; MMTV, mouse mammary tumor virus.

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with various concentrations of antagonist, as indicated, 24 h prior to harvesting.

Cellular extracts were prepared as described (22). Proteins were assayed by the micro BCA technique (Pierce). CAT assays were incubated for 60 min using 300 μg of protein as described (22). β-Galactosidase assays were performed as described to monitor transfection efficiency (23). The percentage of chloramphenicol converted per unit of β-galactosidase activity was calculated. Each point is the mean of three experiments.

**Hormone Binding Studies.** Approximately $1.5 \times 10^6$ cells were transfected with pKSV-rPR (10 μg/ml) and salmon sperm DNA (10 μg/ml). After 40 h, the cells were collected using a rubber policeman, pooled in warm (37°C) phosphate-buffered saline, and centrifuged. After two washes in warm phosphate-buffered saline, the cell pellets were resuspended in warm Dulbecco's modified Eagle's medium devoid of serum, including 1 nM [$^3$H]R 5020 and various concentrations of antagonists as indicated. The cells were incubated at 37°C under 5% $CO_2$/95% air for 2 h. All the following procedures were carried out at 4°C. The cells were collected by centrifugation and washed in ice-cold phosphate-buffered saline. The cell pellets were washed in ice-cold 1.5 mM EDTA/10 mM Tris·HCl, pH 7.4/30% (vol/vol) glycerol. Hormone–receptor complexes were extracted from the pellet by a treatment with 1.5 mM EDTA/1 M NaCl/10 mM Tris·HCl, pH 7.4/30% glycerol. For each fraction, total radioactivity was measured in aliquots mixed with a scintillation solution (Ultima Gold, Packard). Unbound hormone was adsorbed with dextran-coated charcoal as described (24) and radioactivity in aliquots of the supernatants was measured to determine the concentration of hormone–receptor complexes.

**Immunofluorescence.** Approximately $5 \times 10^5$ cells were plated on fibronectin-precoated chamber slides (Nunc) as described (17). For receptor activation studies, cells were transfected with PRΔ638–642 mutant (60 μg/ml) and salmon sperm DNA (30 μg/ml).

For oligomerization studies, wild-type Let− receptor (50 μg/ml), cytoplasmic receptor form (PRΔ593–640) (10 μg/ml), and salmon sperm DNA (30 μg/ml) were used. Immunofluorescence studies were performed as described (17), using monoclonal antibody Let126 (25) at 18 μg/ml. The same results were obtained using a different nuclear monomer (PRΔ373–546) deleted of the Mi60 epitope and a different cytoplasmic receptor form (PRΔ593–640,Let−). In this case, the cytoplasmic monomer was visualized using the monoclonal antibody Mi60 (26). A fluorescein-conjugated rabbit anti-mouse antibody (Dakopatts, Glostrup, Denmark) was added at a 1:40 dilution. Photographs were taken on a Leitz microscope, with Fujichrome 1600 ASA film. In each case, three transfections were performed. The subcellular localization of the mutant PR was observed in 100 cells. Staining was considered nuclear (or cytoplasmic) when it was exclusively nuclear (or cytoplasmic) or stronger in the nucleus (or cytoplasm) than in the cytoplasm (or nucleus).

## RESULTS

**Comparison of the Inhibitory Activity of RU 486 and ZK 98299.** This activity was studied by measuring the inhibition of the biological effect of an agonist and by analyzing the inhibition of the binding to the receptor of this agonist.

COS-7 cells were transfected with an expression vector encoding the PR and with a reporter gene (MMTV–CAT construct). The CAT activity was measured in the presence of a fixed concentration of the agonist R 5020 and various concentrations of the antagonists RU 486 and ZK 98299. As shown in Fig. 1*A*, there was a 10-fold difference in the inhibitory activity of both compounds (RU 486 acting at the lower concentration).

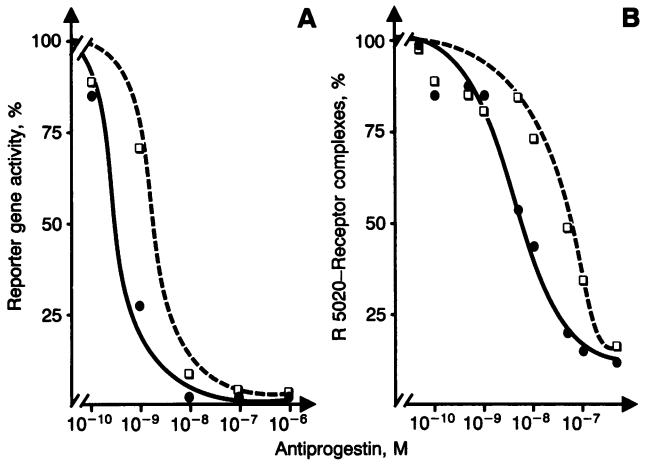


FIG. 1. Comparison of the inhibitory activity of RU 486 (●) and ZK 98299 (□). (*A*) COS-7 cells were cotransfected with an expression vector encoding the PR (pKSV-rPR) and a reporter gene (MMTV–CAT). Cells were incubated 24 h prior to harvesting with 1 nM R 5020 and various concentrations (0.1 nM to 1 μM) of RU 486 and ZK 98299. The relative CAT activity was determined; 100% CAT activity is the percent of acetylation obtained in the absence of antagonist. (*B*) COS-7 cells were transfected with pKSV-rPR. After 40 h, the cells were harvested and incubated with 1 nM [$^3$H]R 5020 and various concentrations (0.1 nM to 1 μM) of RU 486 and ZK 98299. Hormone–receptor complexes were measured. The mean of three experiments was calculated; 100% is the concentration of [$^3$H]R 5020–receptor complexes in the absence of antagonist.

We then used the same system to measure receptor binding inhibition. The cells were incubated with a constant concentration of [$^3$H]R 5020 and increasing concentrations of both antagonists (Fig. 1*B*). Again we observed that RU 486 was 10-fold more efficient than ZK 98299 in displacing the radioactive agonist.

**Receptor Activation by RU 486 and ZK 98299.** The PR is directed into the nucleus by a complex nuclear localization signal containing both hormone-dependent and constitutive elements (17). Deletion of 5 aa (aa 638–642) in the latter results in a PR mutant (PRΔ638–642) that is dependent on the hormone-provoked activation to be shifted from the cytoplasm to the nucleus (17). This is due to the fact that the remaining part of the nuclear localization signal lies in the second zinc-finger region (17). In the PRΔ638–642, unmasking of the DNA binding region ("activation") thus also conditions nuclear transfer. This unmasking may be enticed either by administration of hormone or by deletion of the entire ligand binding domain (see mutant PRΔ638–642, 663–930 in ref. 17).

We thus incubated PRΔ638–642-transfected COS-7 cells with increasing concentrations of both antagonists and counted the proportion of the cells in which the receptor had shifted into the nucleus. We observed that both antagonists determined receptor activation: 0.2 nM RU 486 provoked nuclear staining in 50% of the cells and 2 nM ZK 98299 produced the same effect (Fig. 2).

**Receptor Dimerization Under the Effect of RU 486 and ZK 98299.** We have shown (17) that receptor dimerization can be observed *in vivo* using the following experimental design. Cells are cotransfected with expression vectors encoding a cytoplasmic monomer (deleted of both constitutive and hormone-dependent nuclear localization signals) and a wild-type nuclear monomer. The latter is only deleted of the epitope recognized by monoclonal antibody Let126. Thus when using this antibody only the cytoplasmic monomer will be detected whereas the nuclear monomer will be invisible. In the absence of ligand, both monomers behaved independently, but if hormone is administered, oligomerization occurs and the

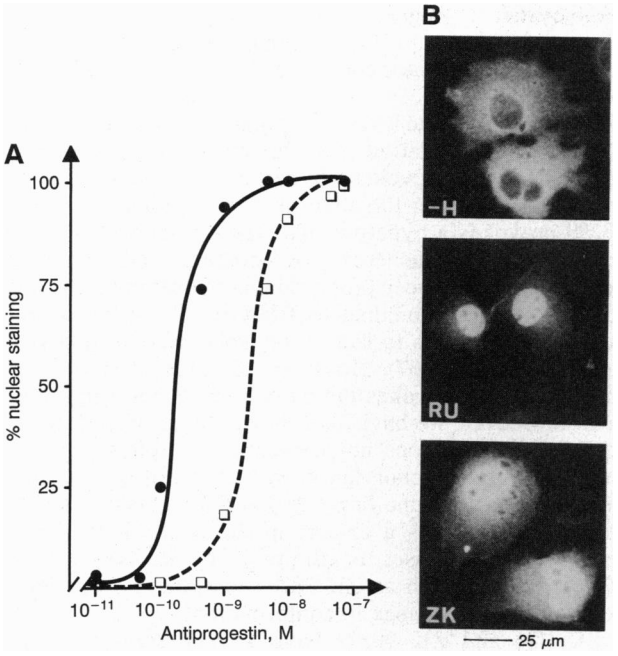


FIG. 2. Receptor activation by RU 486 (●) and ZK 98299 (□). (*A*) COS-7 cells were transfected with a plasmid encoding the mutant PRΔ638–642. Cells were treated 24 h prior to fixation with various concentrations of antiprogestins. The percentage indicates the number of cells with nuclear staining compared to total stained cells. (*B*) Immunofluorescence micrographs showing the effects of RU 486 and ZK 98299 on the subcellular localization of PRΔ638–642. Cells were transfected with the receptor encoding PRΔ638–642 and treated 24 h prior to fixation with no hormone (−H), RU 486 at 0.1 μM (RU), or ZK 98299 at 0.1 μM (ZK).

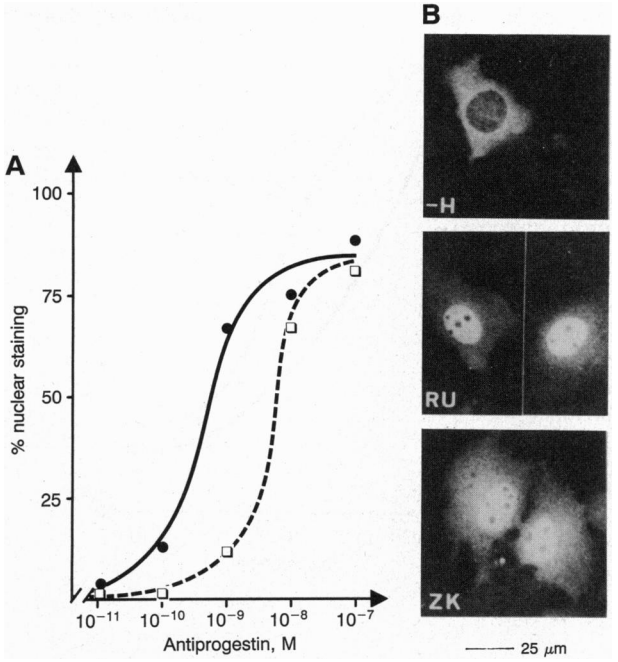


FIG. 3. Receptor dimerization under the effect of RU 486 (●) and ZK 98299 (□). (*A*) COS-7 cells were cotransfected with a plasmid encoding a cytoplasmic monomer (PRΔ593–640) and a wild-type monomer deleted of the epitope recognized by the monoclonal antibody Let126 (wild-type Let−). The cells were incubated with various concentrations of antiprogestins 24 h prior to fixation. The percentage indicates the number of cells with nuclear staining compared to total stained cells. Let126 antibody was used for the immunofluorescence study. (*B*) Immunofluorescence micrographs showing the effects of RU 486 and ZK 98299 on the oligomerization of PR. Cells were cotransfected with vectors encoding wild-type Let− (nuclear) and mutant PRΔ593–640 (cytoplasmic) monomers and treated 24 h prior to fixation with no hormone (−H), RU 486 at 0.1 μM (RU), or ZK 98299 at 0.1 μM (ZK). Let126 antibody was used as in *A*.

cytoplasmic monomer is carried "piggy back" into the nucleus (17). We thus administered increasing concentrations of both antagonists and observed the nuclear location of PRΔ593–640. As shown in Fig. 3, both antagonists provoked receptor dimerization; 0.6 nM RU 486 and 6 nM ZK-98299 were necessary to obtain half-maximal effects.

**Binding to HREs of PRs Complexed to RU 486 or ZK 98299.** A receptor deleted of the entire ligand binding region (PRΔ663–930) becomes constitutively active, at ≈60% of the maximal activity of wild-type receptor (10). It is of course totally unaffected by the presence of antiprogestins (ref. 10 and Fig. 4). However, when an expression vector encoding wild-type receptor is cotransfected and the cells are treated by RU 486, an inhibition of the activity of the constitutive receptor is observed (10). This is due to a competition by RU 486–wild-type receptor complexes at the level of the HREs. [It is not due to a trapping of the constitutive mutant by the RU 486–PR complexes because the constitutive mutant, deleted of the ligand binding domain, is unable to form a heterooligomer (17).] We thus transfected COS-7 cells with expression vectors encoding wild-type PR, PRΔ663–930, and the expression vector MMTV–CAT. Increasing concentrations of the antiprogestins were added and CAT activity was measured. As shown in Fig. 4, RU 486 at 0.01 nM inhibited 50% of the activity of the constitutive receptor, whereas 0.1 nM ZK 98299 was necessary to observe the same effect. Thus both antagonists, complexed to the PR, competed with the constitutive mutant, at the level of the target gene.

## DISCUSSION

The hypothesis that ZK 98299 belonged to a distinct class of antiprogestins came mainly from gel-shift experiments in which no binding of receptor to HREs was observed (12). It is possible that the 10-fold lower affinity for the receptor of ZK 98299 when compared to RU 486 determines a faster dissociation of receptor–ZK 98299 complexes. This may lead to a dissociation of the receptor from the DNA during the electrophoresis, the latter being performed in nonequilibrium conditions.

Surprisingly, to our knowledge, there has been no report comparing the affinities of the PR for RU 486 and ZK 98299 in the same cells and under the same conditions. Most of the comparisons have been based on *in vitro* studies performed in different species (27–30), at different temperatures, and in different buffers (28, 31). For instance the simple addition of glycerol has been shown to markedly modify antagonist binding to receptor (31). Dramatic differences between *in vitro* and *in vivo* have also been reported: *in vitro*, the affinity of RU 486 for the rabbit PR is higher than that of R 5020; *in vivo*, the opposite is observed (27, 32). The composition of the buffer, the temperature, the membrane permeability of the cells, and the intracellular metabolism of the antagonist may explain these differences between *in vitro* and *in vivo* situations.

In the experiments described above we have always observed an ≈10-fold difference between the concentrations of RU 486 and ZK 98299 necessary to obtain the half-maximal effect on various receptor functions. However, for receptor activation and dimerization, these concentrations were 0.6 nM for RU 486 and 6 nM for ZK 98299, whereas for the competition for HREs with the constitutive receptor they were of 0.01 nM for RU 486 and 0.1 nM for ZK 98299. We have noticed (10) in previous experiments that this type of

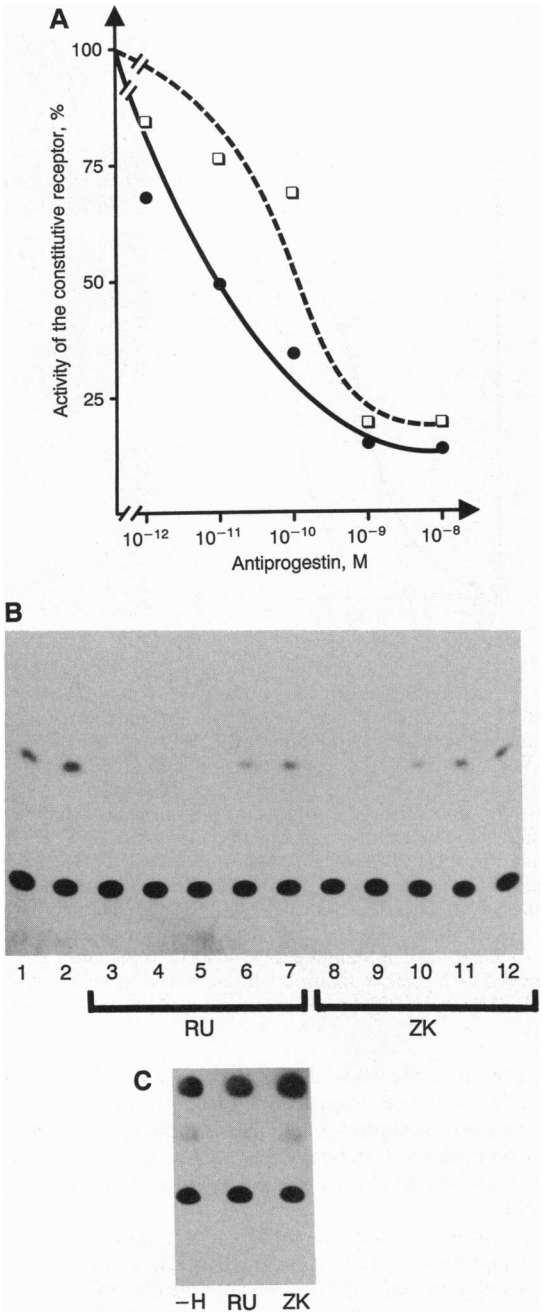


FIG. 4. Binding to HREs of PR complexed to RU 486 (●) and ZK 98299 (□). (*A*) Cells were cotransfected with a vector expressing the constitutive mutant (PRΔ663–930), the vector expressing wild-type receptor (pKSV-rPR), and the reporter plasmid MMTV–CAT. The cells were treated 24 h prior to harvesting with various concentrations of RU 486 and ZK 98299 (1 pM to 10 nM). CAT activity was measured; 100% CAT activity is the activity in the absence of antagonist. (*B*) Autoradiograph of a typical experiment (see *A*) of three experiments. Lanes: 1 and 2, control experiment, no antagonist was added; 3–7, cells were incubated with decreasing concentrations of RU 486 (10 nM, 1 nM, 0.1 nM, 10 pM, and 1 pM, respectively); 8–12, cells were incubated with decreasing concentrations of ZK 98299 (10 nM, 1 nM, 0.1 nM, 10 pM, and 1 pM, respectively). RU, RU 486; ZK, ZK 98299. (*C*) Absence of a direct effect of the antiprogestins on the constitutive PR. The cells were cotransfected with the vector expressing the constitutive mutant (PRΔ663–930) and the reporter plasmid MMTV–CAT. The cells were treated 24 h prior to harvesting with no hormone (−H), 1 μM RU 486 (RU), or 1 μM ZK 98299 (ZK). CAT activity was measured.

competition was observed for low occupation values of the receptor by the antagonist. This finding is probably related to the fact that the regulatory region of MMTV binds several dimers of the receptor (33) and that even partial occupation by antagonist–receptor complexes leads to total inhibition of activity (10).

The PR has a basal level of phosphorylation and undergoes a hyperphosphorylation after having bound the hormone (34–36). RU 486 provokes a hyperphosphorylation similar to that observed under the effect of the hormone whereas ZK 98299 provokes a hyperphosphorylation decreased ≈2-fold (ref. 37 and A. Chauchereau, H. Loosfelt, and E.M., unpublished data). It has been proposed that this difference was due to the absence of binding to DNA of ZK 98299–receptor complexes and thus to lack of phosphorylation by a DNA-dependent kinase (37). However, the present study shows that ZK 98299 provokes the binding of receptor to DNA *in vivo*. Moreover, we have also shown that *in vivo* disruption of DNA binding does not decrease hyperphosphorylation [normal hyperphosphorylation was observed with a mutant devoid of the first zinc-finger region (38)]. It is possible that the ligand provokes a change in the conformation of the receptor that increases its affinity for the kinase(s). Lower-affinity ligands such as ZK 98299 may be less efficient in determining this change in conformation.

RU 486 and ZK 98299 have a very similar chemical structure (39, 40); it was thus surprising that they might have completely different molecular mechanisms of action. These compounds differ only by the presence in ZK 98299 of a 13α-methyl group. This change provokes a modification of the relative orientation of the C and D cycles and a modification of the orientation of the substitutions at position 17. However, it induces only a minor modification in the position of the 4-(dimethylamino)phenyl-11β side chain (40). It has been shown that the latter is the main feature related to the antagonistic effect (41). Moreover, Gly-722 in human PR is of major importance for the binding of the antagonists to PR (8). This amino acid is specific of human and rabbit receptors and its replacement with a cysteine in the chicken PR explains the absence of binding to the receptor and the lack of activity of RU 486 in this species (8). It has been reported that ZK 98299 also binds to human (12, 37) and rabbit (30, 42) PRs but does not bind to the chicken PR (43), suggesting that it could contact the same amino acid(s) as RU 486 in the hormone binding domain. Although RU 486 and ZK 98299 have similar binding characteristics and seem to act by triggering the formation of inefficient receptor–HRE complexes, it remains possible that other molecules could be devised that could inhibit receptor action at the level of activation, dimerization, or binding to regulatory DNA regions.

The experiments described in this study also show a method of more general interest that allows one to analyze the effects of hormone antagonists *in vivo* in intact cells.

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