Two types of antiprogestins identified by their differential action in transcriptionally active extracts from T47D cells

Ludger Klein-Hitpass*, Andrew C.B.Cato[1], David Henderson[2] and Gerhart U.Ryffel
Institut für Zellbiologie, Tumorforschung, Universitätsklinikum Essen, Hufelandstrasse 55, D-4300 Essen 1, [1]Kernforschungszentrum Karlsruhe, Institut für Genetik und Toxikologie, Postfach 3640, D-7500 Karlsruhe and [2]Research Laboratories of Schering AG, Department of Experimental Andrology and Oncology, Müllerstrasse 170-178, D-1000 Berlin 65, FRG

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ABSTRACT

Transcriptionally active nuclear extracts from human breast carcinoma cells (T47D) were used to compare the action of progestins and several antiprogestins of the 11β-aryl substituted steroid series on the DNA-binding properties and the *trans*-activating potential of progesterone receptor (PR) *in vitro*. Using the gel-shift assay we identified a novel type of antiprogestin (ZK98299, type I), which in contrast to type II antiprogestins, including RU486, does not induce binding of PR to progesterone response elements (PREs). In competition experiments excess of type I antiprogestin inhibits induction of DNA binding of PR by progestins and type II antiprogestins suggesting that its binding to PR interferes with the formation of stable receptor dimers. Moreover, we demonstrate that the antagonistic action of ZK98299 can be fully mimicked *in vitro* by using cell-free nuclear extracts from T47D cells and a 'simple' test promoter. In contrast, type II antiprogestins known to induce certain promoters *in vivo* exert strong agonistic effects on *in vitro* transcription of the test template used.

INTRODUCTION

Progesterone receptor (PR) is a member of the family of ligand-inducible transcription factors which are characterized by a zinc-finger DNA-binding domain and a C-terminal hormone binding domain (reviewed in 1, 2, 3, 4). PR function is triggered by binding of the specific hormone and involves unmasking of the DNA-binding domain and receptor dimerization (5). Once activated through binding of progesterone, PR binds to specific progesterone response elements (PREs) located in the vicinity of the target genes. PREs have been identified in several progesterone-inducible genes (2) and are bound by PR with high affinity and specificity *in vitro* (5, 6, 7, 8, 9, 10). Binding of the receptor increases the rate of transcription initiation at the target promoter through interactions with components of the transcription initiation complex (11). Possibly, this process requires a specific cofactor, which might act as a mediator between steroid receptors and the transcriptional apparatus (12).

Direct transcriptional activation of genes by progestins can be specifically inhibited by antiprogestins. Such compounds counteracting steroid action are invaluable tools in the study of the mechanism of receptor function. Due to their antiproliferative effects (13, 14, 15, 16) antiprogestins have also found applications in the treatment of cancer (17). RU486, a potent progesterone antagonist (18), is currently used as a contraceptive and abortive drug, since it blocks progesterone dependent nidation and the maintenance of pregnancy (19). RU486 acts only in cells expressing PR and efficiently competes progesterone binding to the receptor (reviewed in 20). In accordance with *in vitro* DNA binding studies (6), transient transfection studies, however, indicated that RU486 promotes binding of PR to PREs *in vivo* analogous to the cognate hormone (21, 22, 23). It thus has been postulated that the antiprogestin-induced PR conformation, although able to bind to the regulatory sequences at target promoters, is not able to activate transcription of progesterone-inducible genes (21). However, the molecular mechanism underlying RU486 action is not understood. Antiprogestins that interfere at a different stage with receptor function have not been described yet.

Using nuclear extracts from a breast carcinoma cell line (T47D) expressing high levels of PR (13) we have, therefore, examined the action of a series of antiprogestins of the 11β-aryl substituted family. These RU486-related compounds, which include ZK98299, ZK98734 and ZK112993 (24) had originally been designed to find antiprogestins that exhibit lower antiglucocorticoid activity than RU486 (20). All these drugs exhibit affinities for PR comparable to RU486 and the agonist progesterone and efficiently compete progesterone binding to rabbit uterus PR *in vitro* (20), inhibit progestin induction of MMTV-CAT expression in T47D cells (25, 26; Henderson and Bengtson, unpublished) and are effective inhibitors of nidation in guinea pigs (20, 27). Our results show that one antiprogestin (ZK98299) with a change from trans- to cis-fusion between the C- and D-rings of the steroids (24) acts at a different stage than

* To whom correspondence should be addressed

RU486. Moreover, we demonstrate that the inhibitory action of antiprogestin ZK98299 can be mimicked in a progesterone-regulatable cell-free transcription system. In contrast to ZK98299, the three other antiprogestins investigated, including RU486, failed to antagonize progestin induced *in vitro* transcription but exhibited strong agonistic effects which correlated with their ability to induce PR binding to PREs *in vitro*.

MATERIALS AND METHODS

Steroids

Dexamethasone, 17β-estradiol (E2) and progesterone were obtained from Sigma, RU486 (=RU38486) from Roussel-Uclaf and R5020 from New England Nuclear. ZK98299 [11β-(4-dimethylaminophenyl)-17α-hydroxy-17β-(3-hydroxypropyl-13α-methyl-4,9-gonadiene-3-one], ZK112993 [11β-(4-acetyl-phenyl)-17β-hydroxy-17α-(1-propinyl)-4,9-estradiene-3-one] and ZK98734 [11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(3-hydroxy-1(z)-propenyl)-estra-4,9-diene-3-one] were synthesized at Schering AG (Berlin).

Plasmids

Construction of TATA (pLovTATA in ref. 11) and its derivative PRE_2TATA containing two PREs upstream of the TATA box has been previously described (11). TATA derivatives containing shorter G-free cassettes of about 200 or 300 bp in length were generated by Bal31 digestion from the Sma I site 3′ to the G-free cassette. After Bal31 digestion the plasmid was digested with Eco RI and the isolated promoter fragment religated into Eco RI/Sma I digested vector.

To generate β-globin, plasmid pG1B (39) was digested with Eco RI and Pvu II and the resulting 240 bp Eco RI-Pvu II fragment containing a SV40 72 bp repeat and rabbit β-globin sequences from −109 to −10 was isolated. This fragment was ligated into the vector fragment of the TATA derivative containing a G-free cassette of about 300 bp in length, which had been generated by Sac I digestion, T4-polymerase treatment and subsequent Eco RI digestion.

Cell culture and preparation of nuclear extracts

T47D cell stock cultures were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin/streptomycin (100 U/ml), 1 mM glutamine and bovine insulin (0.6 μg/ml). If not otherwise indicated, cells to be used for extract preparation were cultured for 4 to 5 days in medium which contained charcoal-stripped (40) fetal calf serum. Three and one day prior to harvesting the medium was changed.

Nuclear extracts were prepared according to a modification (41) of the method of Shapiro et al. (42). Additionally, all buffers used during extract preparation contained protease inhibitors: 0.1 mM phenylmethylsulfonyl flouride, 0.1 mM benzamidine, leupeptin (1 μg/ml), pepstatin A (1 μg/ml) and aprotinin (0.5 μg/ml). Extracts routinely contained 5 to 10 mg protein per ml.

Gel retardation

Typical binding reactions contained (10 μl volume) : 0.1 to 0.2 g ($1-2\times10^4$ cpm) PRE oligonucleotide labeled with [γ ^{32}P]-ATP and polynucleotide kinase, 0.5 μg pBR322 digested with Hinf I, 0.5 μl nuclear extract (2.5 to 5 μg protein), 25 mM HEPES-KOH (pH 7.6), 60 mM KCl, 10% (v/v) glycerol, 2.5% (w/v) Ficoll 400, 1 mM $MgCl_2$, 2 mM DTT, 0.15 mM EDTA and 0.05 mM EGTA. For preincubation with hormones or antihormones 4 μl aliquots of nuclear extract were diluted to 18 μl with dialysis buffer (42) containing 0.1 M KCl. Hormones were added in a volume of 2 μl to yield the indicated concentrations. After incubation on ice for one hour 2.5 μl aliquots were added to 7.5 μl of binding mixture to yield standard binding conditions. Binding reactions were carried out at room temperature for 20 min, separated in 4% polyacrylamide gels as described (43), fixed in 10% acetic acid and autoradiographed. The PRE oligonucleotide used as probe and competitor was obtained by annealing the two complementary strands 5′-AGC-TTAGAACACAGTGTTCTCTAGAG-3′ and 5′-GATCCTC-TAGAGAACACTGTGTTCTA-3′. The sequence of the ERE oligonucleotide used (EREwt 27mer) has been published previously (43).

In vitro transcription

Transcription reactions (20 μl volume) contained : 25 mM HEPES-KOH (pH 7.6), 10% (v/v) glycerol, 60 mM KCl, 6 mM $MgCl_2$, 2 mM DTT, 5 mM creatine phosphate, 0.6 mM ATP, 0.6 mM CTP, 5 μM UTP, 5 μCi of [α-^{32}P]-UTP, 0.1 mM 3′-O-methyl-GTP, 15 U RNase inhibitor, 15 U ribonuclease T1, 4 μl nuclear extract and transcription templates as indicated. Preincubation with hormones/antihormones was performed in 10 μl volume containing 4 μl nuclear extract, 5 μl nuclear dialysis buffer (42) and 1 μl of hormone/antihormone or, as control, 1 μl of carrier (10% ethanol). After incubation for 60 min on ice transcription was initiated by addition of DNA templates and nucleotides and the reaction continued for 45 min at 30°C. Transcripts were processed and analyzed as described (11).

RESULTS

Effects of progestins and antiprogestins on the formation of protein-PRE-complexes in gel-shift assays

The ability of PR present in T47D nuclear extracts to form stable high-affinity complexes with PREs in the gel-shift assay is greatly stimulated by pretreatment with progestins or the antiprogestin RU486 (6). This system that appears to reflect the *in vivo* mechanism was chosen to examine the effects of the novel antiprogestins on the binding of PR to PREs. Nuclear extract from T47D cells grown for 4 days in medium containing charcoal-treated serum was preincubated with various steroids for one hour on ice. Subsequently aliquots were incubated with labeled PRE oligonucleotides in the presence of nonspecific competitor DNA for 20 min at room temperature. These binding reactions were then subjected to nondenaturing polyacrylamide gelelectrophoresis to separate protein-PRE-complexes from free probe. As shown in Fig. 1, extract treated *in vitro* with progesterone (lane 3) or the synthetic agonist R5020 (lane 4) formed several protein-PRE-complexes of slightly different mobility, presumably PR-PRE-complexes (marked by triangles), which were not detectable with untreated extract (lanes 2 and 9). In accordance with results reported earlier (6), RU486 also induced formation of several protein-PRE-complexes (lane 5, marked with triangles) which, however, displayed slightly increased mobilities compared to progestin induced complexes. Pretreatment of the extracts with antiprogestin ZK98734 (lane 6) or ZK112993 (lane 7) induced protein-PRE-complexes that were undistinguishable from RU486-induced complexes. In contrast, preincubation with ZK98299 did not induce any protein-PRE-complexes, indicating that this antiprogestin might act in a manner distinct from RU486, ZK98734 and ZK112993. Neither the

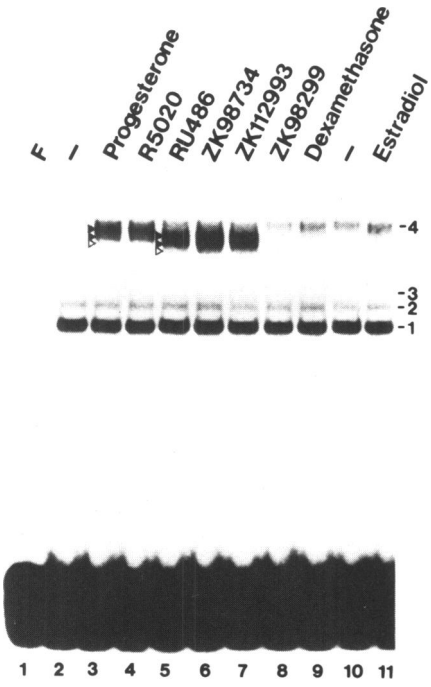

Fig. 1. Effects of hormones and antihormones on the formation of protein-PRE-complexes in T47D nuclear extract. Nuclear extract was incubated with hormone and antihormones and analyzed by gel retardation. Concentrations during preincubation were 10^{-5} M for Dex and 2×10^{-6} M for all other compounds. Lane 1 shows the PRE probe without protein, lanes 2 and 10 complexes formed in untreated extract. Protein-PRE-complexes induced by hormone or antihormone treatment are marked with filled and open triangles.

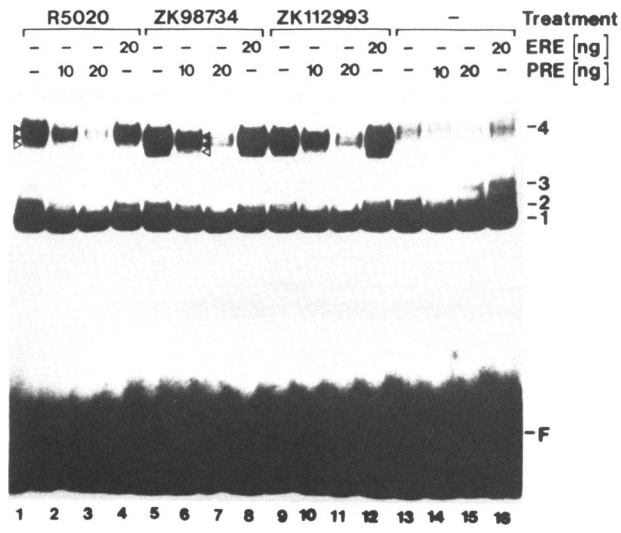

Fig. 2. Protein-PRE-complexes induced by R5020 and antiprogestins display sequence specificity. Nuclear extract from T47D cells was preincubated with 10^{-6} M R5020 (lanes 1–4), 10^{-6} M ZK98734 (lanes 5–8), 10^{-6} M ZK112993 (lanes 9–12) or without any hormone (lanes 13–16). Subsequently, aliquots were added to binding mixtures containing labeled PRE as a probe, nonspecific competitor DNA and specific competitor oligonucleotides as indicated. Binding reactions were analyzed by gel retardation as described in Materials and Methods. ERE, estrogen response element.

synthetic glucocorticoid dexamethasone (lane 9) nor estradiol (lane 11) had any effect on complex formation in T47D nuclear extract.

Protein-PRE-complexes induced by R5020 (Fig. 2, lanes 1 to 4), ZK98734 (lanes 5 to 8), ZK112993 (lanes 9–12) and RU486 (not shown) displayed sequence specificity since their formation could be inhibited by addition of excess unlabeled PRE oligonucleotides but not ERE oligonucleotides. In contrast to the progestin- or antiprogestin-induced complexes the formation of PRE-protein-complexes 1, 3 and 4, which were already present in untreated controls, could not be preferentially inhibited by PRE oligonucleotides (lanes 13–16) and were thus considered to be due to nonspecific interactions. One minor complex (complex 2) which was formed independently of hormone-treatment (compare lanes 1 and 13) exhibited sequence specificity. However, the protein(s) forming this complex was not bound by two different monoclonal antibodies directed against PR (see Fig. 3) and is thus most likely not formed by authentic PR.

Both forms of PR are present in PR-PRE-complexes induced by progestins and several antiprogestins

To confirm that the protein-PRE-complexes induced by the progestin R5020 and the antiprogestins RU486, ZK98734 and ZK112993 indeed contain PR, monoclonal antibody AB52 which specifically recognizes PR (28), was included in some of the binding reactions. Addition of AB52 to the reactions resulted in an upshift of all protein-PRE-complexes induced by R5020 (Fig. 3, lane 2), RU486 (lane 5), ZK98734 (lane 8) and ZK112993 (lane 11), whereas migration of complexes 1 to 3 was

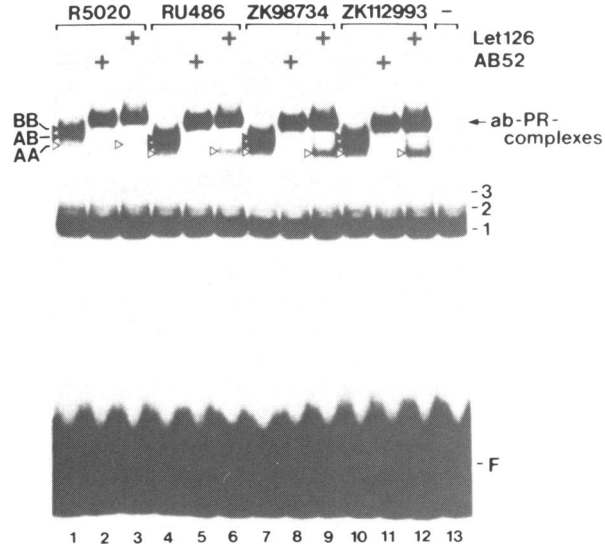

Fig. 3. Progestin and antiprogestin induced protein-PRE-complexes contain both forms of PR. Extracts pretreated with 10^{-6} M R5020 (lanes 1–3), RU486 (lanes 4–6), ZK98734 (lanes 7–9) or ZK112993 (lanes 10–12) were added to binding mixtures containing monoclonal antibodies AB52 or Let126 as indicated. After incubation for 20 min at room temperature samples were analyzed as in Fig. 1. Lane 13 shows protein-PRE-complexes formed in untreated extract. Filled triangles indicate PR form B containing complexes (AB, BB), whereas the open triangles indicates complexes containing form A receptor (AA) only (see Results).

not influenced. These results clearly show that the complexes induced by R5020 or the various antiprogestins were formed by PR.

T47D cells express two isoforms (A and B) of PR (28, 29). Form A represents an amino-terminally truncated version of form B, which originates from shorter mRNAs initiating at an

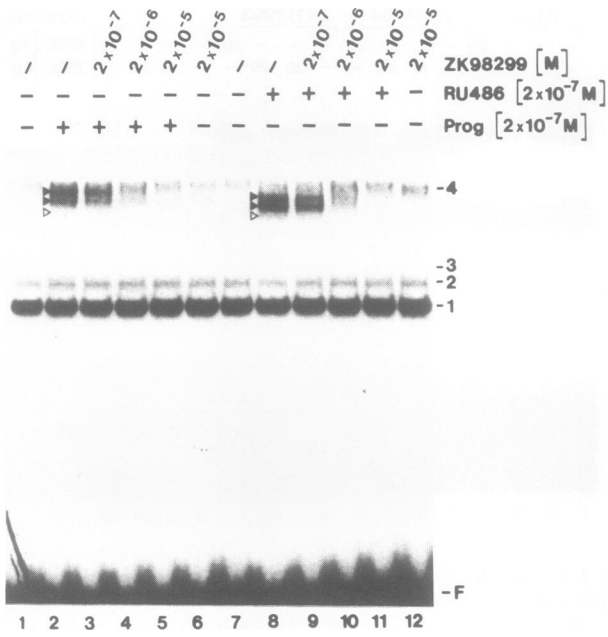

Fig. 4. Antiprogestin ZK98299 inhibits induction of PR-PRE-complexes by progesterone and RU486. Nuclear extract was preincubated with 2×10^{-7} M progesterone or RU486 and different concentrations of ZK98299 as indicated above each lane and assayed by gel retardation as in Fig. 1.

internal promoter present within the PR gene (30). To analyze whether both forms are present in progestin- and antiprogestin-induced complexes, monoclonal antibody Let126 (31) was added to binding reactions containing T47D extract pretreated with R5020 or antiprogestins. Since the epitope for Let126 is located within the aminoterminal part of form B receptor that is absent in form A, binding of this antibody should only decrease the mobility of form B containing complexes. Addition of saturating amounts of Let126 to R5020-induced complexes resulted in an upshift of the two major PR complex species (BB, AB), while the mobility of the third one (AA) was not changed (Fig. 3, lane 3). Since complex AA was upshifted by AB52 (Fig. 3, lane 2) which binds both forms of PR (28), this strongly suggests that complex AA does not contain form B receptor but rather represents form A homodimers. In accordance with the different molecular weights of form A and form B PR, we speculate that the most slowly migrating PR-PRE-complex (BB) is formed by form B homodimers, whereas the complex of intermediate mobility (AB) might represent form A/form B heterodimers. As shown in Fig. 3, complexes AA were induced by progestins as well as all three antiprogestins tested indicating that both PR forms bind these compounds. The ratio of AA complexes to form B containing complexes (BB, AB) in progestins and antiprogestin treated samples did not vary significantly, excluding the possibility that antiprogestins specifically induce only one form of PR.

Antiprogestin ZK98299 competitively inhibits induction of DNA-binding by progesterone or RU486

In contrast to RU486, ZK98734 and ZK112993, the antiprogestin ZK98299, which antagonizes progestin induction of MMTV promoter driven CAT expression in T47D cells (Henderson and Bengtson, unpublished), did not induce stable binding of PR to the PRE (Fig. 1). This strongly suggests that ZK98299 might act by preventing the induction of the DNA-binding activity of

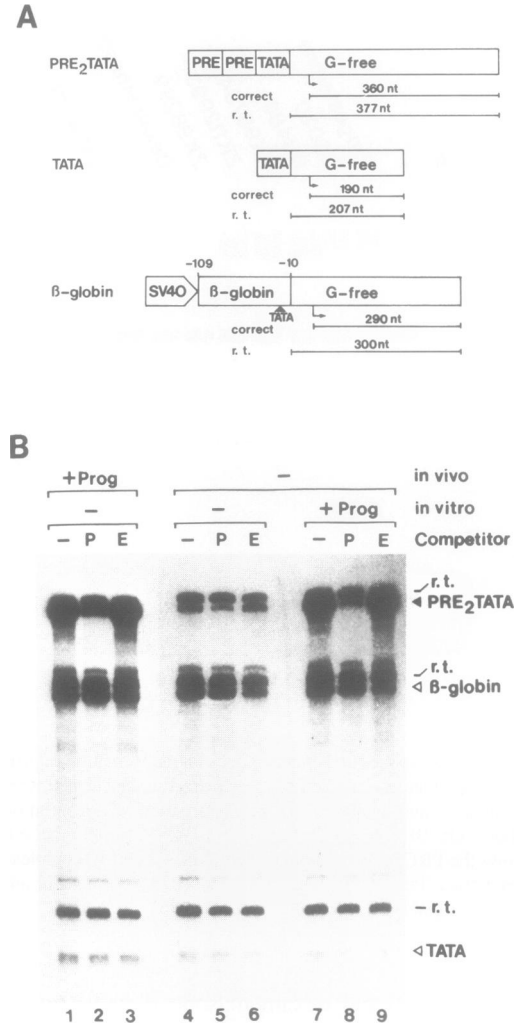

Fig. 5. Effect of progesterone treatment *in vivo* and *in vitro* on cell-free transcription in T47D nuclear extract. A) The structure of the test genes containing G-free cassettes of different lengths is schematically represented. The expected sizes of transcripts correctly initiated about 30 bp downstream of the TATA boxes are indicated. Ribonuclease T1 digestion of read through transcripts initiated upstream of the G-free cassette gives rise to G-free transcripts corresponding to the length of the cassettes (r. t.). PRE, progesterone response element; TATA, TATA-box element. B) Extracts were prepared from cells treated for 2 hours with 10^{-6} M progesterone (lanes 1–3) and from cells grown in medium containing charcoal-treated serum (lanes 4–9). *In vitro* transcription was performed as described in Materials and Methods. Reactions contained 160 ng each of PRE_2TATA and TATA as well as 80 ng of β-globin. The extract used in lanes 7–9 was preincubated with 10^{-6} M progesterone *in vitro* (+ Prog) for one hour on ice. One hundred ng of PRE (P) or ERE (E) competitor oligonucleotides corresponding to a 60-fold molar ratio of oligonucleotide over PREs in the PRE_2TATA template were added prior to the addition of hormone. The positions of correctly initiated transcripts and read through (r. t.) signals are indicated. The autoradiograph was overexposed to reveal the correctly initiated transcripts of the TATA template.

PR. To show this directly extracts were incubated with progesterone (Fig. 4, lanes 2 to 5) or the antiprogestin RU486 (lanes 8 to 11) and increasing amounts of ZK98299 simultaneously. In both cases addition of ZK98299 inhibited the formation of PR-PRE-complexes in a concentration dependent manner. Complete inhibition of progesterone- and RU486-induced PR binding occurred at 100-fold molar excess of ZK98299 (lanes 5 and 11, respectively). These data confirm that ZK98299 binds to PR with an affinity comparable to progestin and RU486 and provide evidence that ZK98299 acts

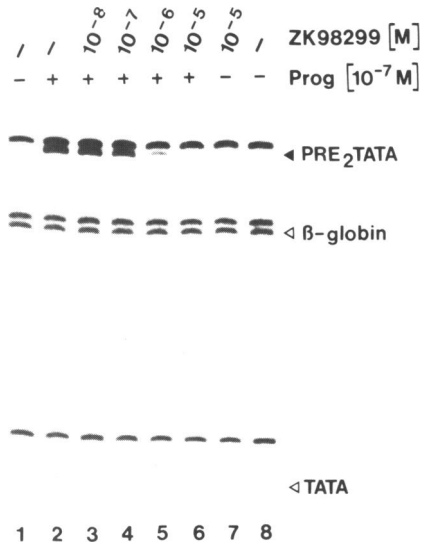

Fig. 6. Antiprogestin ZK98299 inhibits progesterone induction of PRE_2TATA transcription *in vitro*. Nuclear extract from cells grown in medium supplemented with charcoal-treated serum was preincubated with 10^{-7} M progesterone (lane 2) or mixtures of 10^{-7} M progesterone and increasing concentrations of ZK98299 (lanes 3–6) for one hour on ice. Control samples received 10^{-5} M ZK98299 only (lane 7) or the corresponding amount of solvent (lanes 1 and 8). *In vitro* transcription was performed as in Fig. 5. The position of correctly initiated transcripts of each test template is indicated. Longer exposures of this autoradiograph showed equal levels of correctly initiated TATA transcripts.

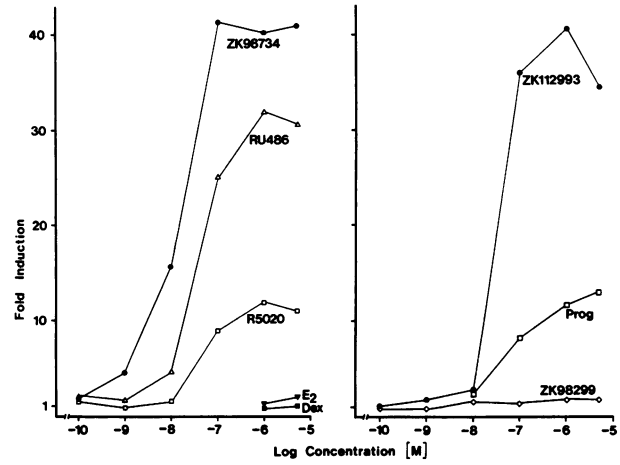

Fig. 7. Type II antiprogestins are strong agonists of PRE_2TATA transcription *in vitro*. Nuclear extract from T47D cells was preincubated with the indicated concentration of hormones and antihormones and *in vitro* transcription performed as described in Fig. 5. Appropiate autoradiographs were scanned with a laser densitometer. The signals representing correctly initiated PRE_2TATA transcripts were normalized by using the TATA signal as an internal control and plotted against steroid concentration. One-fold induction denotes the averaged signal of three control reaction lacking hormone or antihormone. E2, estradiol; Dex, dexamethasone; Prog, progesterone.

through antagonizing the progestin-induced conversion of PR to the DNA binding state. Our experiments thus identified ZK98299 as an antiprogestin with a yet undescribed mode of action. To distinguish the antiprogestins with different modes of action we defined two classes: type I, represented by ZK98299, which does not induce binding of PR to PREs, and type II, including RU486, ZK98734 and ZK112993, which induce binding of PR to PREs.

Progesterone treatment *in vivo* and *in vitro* stimulates the *trans*-activating potential of PR in a T47D cell derived *in vitro* transcription system

To further characterize the mode of action of the various antiprogestins we examined their effects on PR-activity in a hormonally regulated cell-free transcription system which uses T47D nuclear extracts as a source for general transcription factors and PR (7). The structure of the three different test genes which were transcribed simultaneously in all reactions is schematically given in Fig. 5 A. The first, PRE_2TATA (11), consists of two palindromic binding sites for PR (PREs) linked to a TATA-box element and a G-free cassette (32) of 377 bp in length. As demonstrated in a previous report (11), the activity of this promoter can be stimulated through binding of active PR. Correct initiation of transcription directed by the TATA-box occurs within the G-free cassette and results in a 360 nt transcript. A transcript of 377 nt indicates read through transcripts starting upstream of the G-free cassette trimmed by the G-specific ribonuclease T1 which was added to the reactions to reduce the background (32). The second test gene, TATA, contains the same TATA-box as PRE_2TATA linked to a shorter G-free cassette of about 207 bp. Due to the lack of PREs the activity of this test gene in T47D extracts was expected not to be influenced by PR. *In vitro* transcription of the TATA construct results in a correctly initiated G-free transcript of about 190 nt in length and a 17 nt longer transcript generated by ribonuclease T1 digestion of read through transcripts. Another nonresponsive internal control (β-globin) containing a SV40 72 bp repeat 5′ to the rabbit β-globin promoter from −109 to −10 directing transcription of a 300 bp G-free cassette generates a correctly initiated transcript of 290 nt and a read through transcript of 300 nt.

In extracts obtained from cells treated for 2 hours with 10^{-6} M progesterone in culture correctly initiated transcripts from all three test genes could be detected (Fig. 5B, lane 1). The level of PRE_2TATA transcripts was about 20-fold higher than the level of TATA transcripts indicating that the PREs in PRE_2TATA confer higher activity to the TATA box promoter. Moreover, addition of 100 ng of an oligonucleotide containing a binding site for PR (PRE) resulted in about 90 per cent inhibition of $PREE_2TATA$ activity (lane 2) but did not alter transcription of the TATA construct and the β-globin promoter. In contrast, ERE oligonucleotides known not to be bound by PR (6) had no effect on PRE_2TATA transcription (lane 3). Thus, we conclude that most of the high activity of the PRE_2TATA promoter can be attributed to transcriptional enhancement by a factor with the binding specificity known for PR.

For the analysis of antihormone action, however, an extract containing PR in a latent, but hormone inducible form would be more suitable. We, therefore, prepared nuclear extracts from T47D cells which were cultured for 4 days in medium containing serum that had been treated with charcoal to remove progesterone. In such extracts, the level of PRE_2TATA transcripts relative to TATA and β-globin transcripts was low compared to the activity in extracts from progesterone treated cells (Fig. 5B, compare lanes 1 and 4). Pretreatment of this extract with 10^{-6} M progesterone *in vitro* for one hour on ice exclusively stimulated PRE_2TATA transcription about 10-fold (compare lane 4 and 7). Stimulation of PRE_2TATA transcription by hormone could specifically be prevented by addition of PRE but not by ERE oligonucleotides (lanes 8 and 9). Addition of PRE oligonucleotides to untreated extracts inhibited PRE_2TATA transcription at most two-fold (lane 5).

Since ERE oligonucleotides had no effect at all on this activity we believe that it results from PR molecules activated by residual hormone in the medium. Taken together, these results prove that PR can be regulated by progesterone addition *in vitro* in this type of extract. This system, therefore, is suitable to investigate how antihormones interfere with the hormonal induction of the transcriptional activity of PR.

The antagonistic action of ZK98299 on PR-mediated transcription can be mimicked *in vitro*

To examine whether compounds with known antiprogestin activity are able to prevent progesterone action in the *in vitro* transcription system, nuclear extract was preincubated with 10^{-7} M progesterone or mixtures of 10^{-7} M progesterone and various concentrations of antihormone. Increasing concentrations of type I antiprogestin ZK98299 shown to interfere with the induction of DNA binding activity of PR (Fig. 4) inhibited the induction of PRE_2TATA transcription by progesterone in a dose dependent manner, whereas the internal controls were not changed (Fig. 6). At equimolar concentration of ZK98299 about 30% inhibition was observed (lane 4) and at 100-fold molar excess of ZK98299 over progesterone the level of PRE_2TATA transcription was reduced to basal activity (lane 6). When added alone (lane 7), the highest concentration of ZK98299 used in the competition experiment (10^{-5} M) had no effect on PRE_2TATA transcription. Thus, type I antiprogestin ZK98299 displays pure antiprogestin activity in the T47D cell derived cell-free transcription system.

All type II antiprogestins, including RU486, strongly induce PRE_2TATA transcription *in vitro*

Unexpectedly, the type II antiprogestins RU486, ZK98734 and ZK112993 failed to antagonize transcriptional stimulation of PRE_2TATA by progesterone. Addition of increasing concentrations of this group of antiprogestins to 10^{-7} M progesterone rather led to a further 2- to 3-fold increase of PRE_2TATA transcription (data not shown). As shown in Figure 8, antiprogestins RU486 (lane 5), ZK98734 (lane 3) and ZK112993 (lane 7) are able to stimulate PRE_2TATA transcription by themselves very strongly. The strong agonist activity exhibited by these compounds, quite obviously, explains their failure to antagonize the action of progesterone *in vitro*. Quantitation of dose-response experiments by densitometry revealed that RU486 induces PRE_2TATA about 30-fold, ZK98734 and ZK112993 about 40-fold at saturation (Fig. 7), whereas the progestins R5020 and progesterone induce about 12-fold. None of the type II antiprogestins tested influenced the activity of β-globin and TATA (Fig. 8). Together with the finding that the type II antiprogestins induce binding of PR to PREs, this suggests that the agonistic action of all compounds is a direct transcriptional effect mediated through binding of antiprogestin-PR-complexes to PREs. Estradiol and dexamethasone could not induce PRE_2TATA (Fig. 7) indicating a specific action of the various steroids in inducing transcription in the *in vitro* system.

Type I antiprogestin ZK98299 prevents agonistic effects of type II antiprogestins on PRE_2TATA transcription *in vitro*

As type I antiprogestin ZK98299 is able to competitively inhibit induction of PR binding to PREs by type II antiprogestin RU486 (Fig. 4), one has to expect that excess ZK98299 is able to prevent induction of PRE_2TATA transcription by RU486 *in vitro*. Fig. 8 illustrates that induction of PRE_2TATA transcription by RU486 as well as the other type II antiprogestins, ZK98734 and ZK112993, could efficiently be inhibited by a 100-fold molar excess of ZK98299. Thus, ZK98299 is not only able to prevent progestin-induced PRE_2TATA transcription *in vitro* (Fig. 6) but also capable to antagonize the agonistic effects of type II antiprogestins in the T47D derived cell-free transcription system.

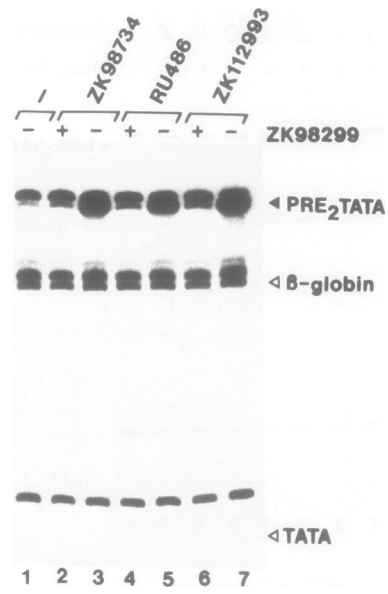

Fig. 8. Type I antiprogestin ZK98299 inhibits induction of *in vitro* transcription by type II antiprogestins. T47D nuclear extract was preincubated with (+) or without (−) 10^{-5} M ZK98299 and 10^{-7} M ZK98734 (lanes 2 and 3), 10^{-7} M RU486 (lanes 4 and 5) or 10^{-7} M ZK112993 (lanes 6 and 7). *In vitro* transcription was performed as in Fig. 5. Control activity is shown in lane 1.

DISCUSSION

A novel type of antiprogestin interferes with the hormonal induction of DNA-binding of PR

In the present study we have compared the mechanism of action of progestins and several antiprogestins on the binding properties and the *trans*-activating potential of PR in cell-free extracts from T47D breast carcinoma cells. Based on the results obtained in the *in vitro* DNA-binding assay we identified two types of antiprogestins, (i) a novel type (type I), represented by ZK98299, that does not induce specific binding of PR to PREs but competitively inhibits induction of DNA binding by progestins as well as type II antiprogestins and (ii) those, including RU486, ZK98734 and ZK112993, that induce stable, high-affinity binding of PR to PREs (type II antiprogestins) analogous to progestins. Studies with antiestrogens have also identified compounds that induce binding activity of hER and one compound, ICI164,384, that at least in certain systems prevents binding of ER to DNA in a manner comparable to the action of ZK98299 (33, 34). Our results suggest that binding of ZK98299 to PR fails to elicit the structural alteration(s) of the steroid receptor necessary for the formation of stable receptor dimers. However, further characterization of the structural status of ZK98299-PR-complexes is required to rule out the possibility that these complexes form dimers unable to interact with DNA in a stable and sequence-specific manner. It will be of particular importance to determine whether the binding of ZK98299 to PR affects its interactions with heat shock proteins (hsp) or other components of the aporeceptor complex (19). Hsp90 has recently been shown to be important for glucocorticoid receptor activity (35).

Interestingly, our classification of antiprogestins according to their properties in the *in vitro* DNA-binding assay correlates with a major structural difference of these two types of compounds: In type II antiprogestins the natural trans-configuration of the C- and D-ring of steroids is maintained, whereas in ZK98299 these rings are fused in the cis-configuration (24). Whether this structural feature is responsible for the particular mode of action of ZK98299 can now be tested by studying more compounds with related structure.

PR-PRE-complexes induced by progestins and type II antiprogestins display different electrophoretic mobilities

In contrast to type I antiprogestin ZK98299, type II compounds induce stable and sequence-specific binding of PR to PREs *in vitro*. As indicated by work of the group of Milgrom (21, 22) and very recently proven by Meyer et al. (36), type II antiprogestin RU486 also induces binding of PR to PREs *in vivo*. Thus our *in vitro* DNA-binding assay perfectly reflects this step of the *in vivo* mechanism. In addition the mobility shift assay reveals that PR-PRE-complexes induced by type II antiprogestins can be distinguished from progestin-induced complexes, in that they display different electrophoretic mobilities. Among the various type II antiprogestins no differences in mobility and PR form A/form B ratios are detectable (Fig. 3). Distinct electrophoretic mobilities have also been observed for estrogen- and antiestrogen-induced ER-ERE-complexes and interpreted to reflect an altered structure of the antihormone-receptor complex (37).

Type I and type II antiprogestins have differential effects on *in vitro* transcription of PRE_2TATA

Using a novel cell-free transcription assay, in which faithful and efficient progestin stimulation of PR-driven transcription initiation can be detected, to investigate the molecular mechanism of antiprogestin action we obtained the predicted result for type I antiprogestin ZK98299. Since this compound competes with progestins for binding to the receptor but does not induce stable interactions of PR with the PREs in the template, which is absolutely required for trans-activation, ZK98299 competitively inhibits progestin induction of the regulated test gene (PRE_2TATA) when applied in excess (Fig. 6). In contrast, all type II antiprogestins were unable to antagonize progestin induction of PRE_2TATA *in vitro* transcription but displayed strong agonist activity (Fig. 7). This finding was rather surprising to us since no agonistic effect of RU486 had been found in a cell-free transcription system reconstituted by addition of purified rabbit uterus PR to HeLa cell nuclear extract (38). In addition our own studies have shown that all type II antiprogestins RU486 are unable to induce CAT mRNA synthesis in a stably transfected T47D cell clone carrying five copies of MMTV-CAT (Klein-Hitpass and Cato, unpublished). However, after completion of this work Meyer et al. (36) reported that RU486 can induce transcription of a PRE-tk-CAT gene construct but not MMTV-CAT in HeLa cells transiently expressing PR form B. Interestingly, PRE-tk-CAT and our antiprogestin-inducible *in vitro* transcription template, PRE_2TATA, share perfectly palindromic PREs as a common feature. Direct evidence for promoter context-dependent agonistic effects *in vitro*, however, is not possible yet since the MMTV-CAT construct is barely regulated in our T47D nuclear extracts. In any case, the T47D cell derived *in vitro* transcription system, which is able to fully mimick the antagonistic action of type I and agonistic effects of type II antiprogestins represents a useful complementary approach to differentiate the molecular mechanism of hormone and antihormone action.

Implications for the mechanism of action of type I antiprogestins

The identification of two distinct levels at which antiprogestins interfere with PR function will allow a further dissection of the steps involved in the mechanism of progesterone induced gene transcription. Based on our results we expect that type I antiprogestins have the potential to completely antagonize transcriptional enhancement by PR in humans, as they block the transition of PR to the state that can stably interact with PREs. Transcriptional repression of genes by agonist-PR-complexes (23), however, should be completely relieved in the presence of excess of ZK98299. This special mode of action of type I antiprogestin ZK98299 which might be responsible for the slightly different biological activities of RU486 and ZK98299 (16) together with its lower antiglucocorticoid activity (20) makes ZK98299 a compound with an interesting therapeutic potential.

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