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Human Progesterone Receptor A and B Isoforms in CHO Cells. II. Comparison of Binding, Transactivation and ED_{50} Values of Several Synthetic (Anti)progestagens *In Vitro* in CHO and MCF-7 Cells and *In Vivo* in Rabbits and Rats

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The human progesterone receptor A and B isoforms (hPR-A and hPR-B) were stably transfected in Chinese Hamster Ovary (CHO) cells in the presence or absence of the mouse mamma tumor virus (MMTV) promoter and luciferase (LUC) reporter gene. In this way four stably transfected CHO cell lines, i.e. hPR-A, hPR-B, hPR-A-MMTV-LUC and hPR-B-MMTV-LUC cells, were prepared. hPR-A and -B isoforms were compared by binding and transactivation analysis for 14 progestagens and 7 antiprogestagens. Thereby Org 2058 was used as standard in both agonistic and binding assays and Org 31710 in antagonistic assays. The obtained data were compared with relative binding affinities (RBA) for both hPR-A and -B, which are present in human breast tumor MCF-7 cells, and with biopotency estimations with McPhail tests in rabbits and ovulation inhibition and pregnancy interruption tests in rats. The relative binding affinities of 14 progestagens and 7 antiprogestagens towards hPR-A, hPR-B or hPR-A/B of either CHO or MCF-7 cells were highly correlated with respect to ranking. This was also shown by the high correlation coefficients between the RBA's of hPR-B and hPR-A in CHO cells ($r = 0.983$) and between those of hPR-B of CHO and hPR A/B of MCF-7 cells ($r = 0.957$). The transactivation data of the 14 progestagens and 7 antiprogestagens for the hPR-B-MMTV-LUC cells were compared with those for hPR-A-MMTV-LUC cells and showed no differences between both cell lines with exception of the progestagens Org 32704 and 33766 and the antiprogestagen Org 33245. Therefore only the relative agonistic activities (RAA) and relative antagonistic activities (RANTA) of hPR-B-MMTV-LUC cells were compared with RBA values of hPR-B, showing a high similarity in ranking for the tested compounds, and high correlation coefficients of $r = 0.91$ and $r = 0.97$, respectively. Remarkably, RBA's were 1.6 fold higher than RAA's and RANTA's. These *in vitro* RBA, RAA and RANTA values for hPR-B were checked for their pharmacological relevance by *in vivo* biopotency measurements with the 14 progestagens and 7 antiprogestagens in rabbits and rats. The *in vitro* binding and transactivation potencies of progestagens appeared to be very predictive for *in vivo* analysis on endometrium proliferation in rabbits in the McPhail test with correlation coefficients of $r = 0.81$ and $r = 0.87$, while ovulation inhibition in rats correlated less well with $r = 0.516$ and $r = 0.65$. On the other hand, the antiprogestagenic potencies found with binding and transactivation assays had a good correlation with the potencies in the pregnancy interruption test in rats for all antiprogestagens tested, being $r = 0.849$ and $r = 0.744$, respectively. In conclusion, the binding and transactivation potencies for the tested compounds in hPR-A

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and -B containing cell lines showed in general a good resemblance. The transactivation studies with hPR-B-MMTV-LUC cells indicated that ranking of compounds was fairly identical to binding analysis and could be used for pre-screening of the (anti)-progestagenic bioactivity in the McPhail test in rabbits, the ovulation inhibition test and the pregnancy interruption test in rats. Therefore this transactivation assay can replace binding assays. Moreover, direct pre-screening of agonists, antagonists and partial antagonists is even possible in this *in vitro* bioassay, making transactivation assays for a particular class of chemical compounds to a valuable pre-screening tool for *in vivo* studies. **© 1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

The progesterone receptor (PR), belonging to the superfamily of nuclear receptors [1], is relatively unique among members of the steroid receptor family, since PR is expressed in two isoforms in chickens, humans and rats [2, 3]. Thus, also in human breast tumor and endometrium cell lines two PR isoforms, hPR-A and -B, have been identified [2, 4–6]. Human PR-A is a truncated form of hPR-B and lacks 164 N-terminal amino acids [7]. The isoforms originate from two in-frame ATG codons [8–10]. Both forms induce progestagen-mediated gene transcription. However, their relative efficacies vary according to the promoter context [8, 11]. Human PR-B appears to be more efficient in inducing mouse mammary tumor virus (MMTV) gene transcription, while so far hPR-A is only able to induce progestin transactivation of the ovalbumin gene [11]. Moreover, a change in hPR-A and -B mediated transcription can also be influenced by the cell context, suggesting that the ratio of hPR-A vs -B or the existence of additional transcription factors may modulate their transcription efficiencies [11–16]. Finally, also different synthetic antiprogestagens, like RU 38486 and ZK 98299, can induce in combination with cyclic AMP a difference in cellular hPR-A or -B mediated reactivity in T47D cells, leading for hPR-A to complete antagonism with both antagonists and for hPR-B to partial antagonism with RU 38486 and pure antagonism with ZK 98299 [17].

For screening purposes, we would like to know whether hPR-A or -B in combination with a specific reporter system in one cell context would lead to changes in reactivity between different progestagens and antiprogestagens. For this purpose the so-called co-transfection assay is a very useful relatively new technical development. With this technique reconstituted cell systems can be prepared, in which one receptor and/or receptor dependent reporter plasmid can be (co)transfected into a mammalian host cell. In order to study both hPR-A and -B isoforms independently with several synthetic progestagens and antiprogestagens, Chinese Hamster Ovary (CHO) cells were stably (co)-transfected with either hPR-A or -B in the presence and absence of the mouse mamma tumor virus (MMTV) promoter and the luciferase (LUC) reporter gene [18]. In this study several pregnane derived progestagens, 19-nortestosterone derived progestagens, new five membered spiromethylene 17-ring compounds as well as some antiprogestagens with 17α-prop(yn)yl and five membered spiro(methylene) 17-ring substituents were analyzed (see Figs 1 and 2). Both hPR-A and -B containing cell lines were characterized with these compounds with respect to binding characteristics, transactivation and inhibition of transactivation. The binding data for hPR-A and -B in CHO cells were compared with binding data of human breast tumor MCF-7 cells, which contain both hPR-A and -B isoforms in almost equal amounts. Furthermore, the meaning of the pharmacological relevance of the tested agonists and antagonists in the transactivation assays was studied by comparing these *in vitro* data with *in vivo* data in a McPhail test in rabbits and an ovulation inhibition or pregnancy interruption test in rats. In all these *in vivo* studies compounds were administered subcutaneously.

MATERIALS AND METHODS

Materials

All steroids were obtained from N.V. Organon (Oss) and the chemical structures are given in Figs 1 and 2. Trypsin was obtained from Flow Laboratories (Irvine), Dulbecco's Modified Eagles Medium/Nutrient Mixture F-12 (DMEM/HAM F12 medium in a ratio of 1:1) from Gibco (Paisley), characterized foetal calf serum and defined bovine calf serum supplemented from Hyclone (Utah), 96 well plates from Greiner (Nürtingen) and 96 well white view plates and Luclite from Packard (Meriden). Tritiated Org 2058 (s.a. 1.7 Tbq/mmol) was obtained from Amersham ('s-Hertogenbosch).

Methods

Cell culture. MCF-7 cells were obtained from Dr. McGrath (Michigan Cancer Foundation, U.S.A.). The selected CHO cells, derived from CHO K1 cells obtained from the American Type Culture Collection

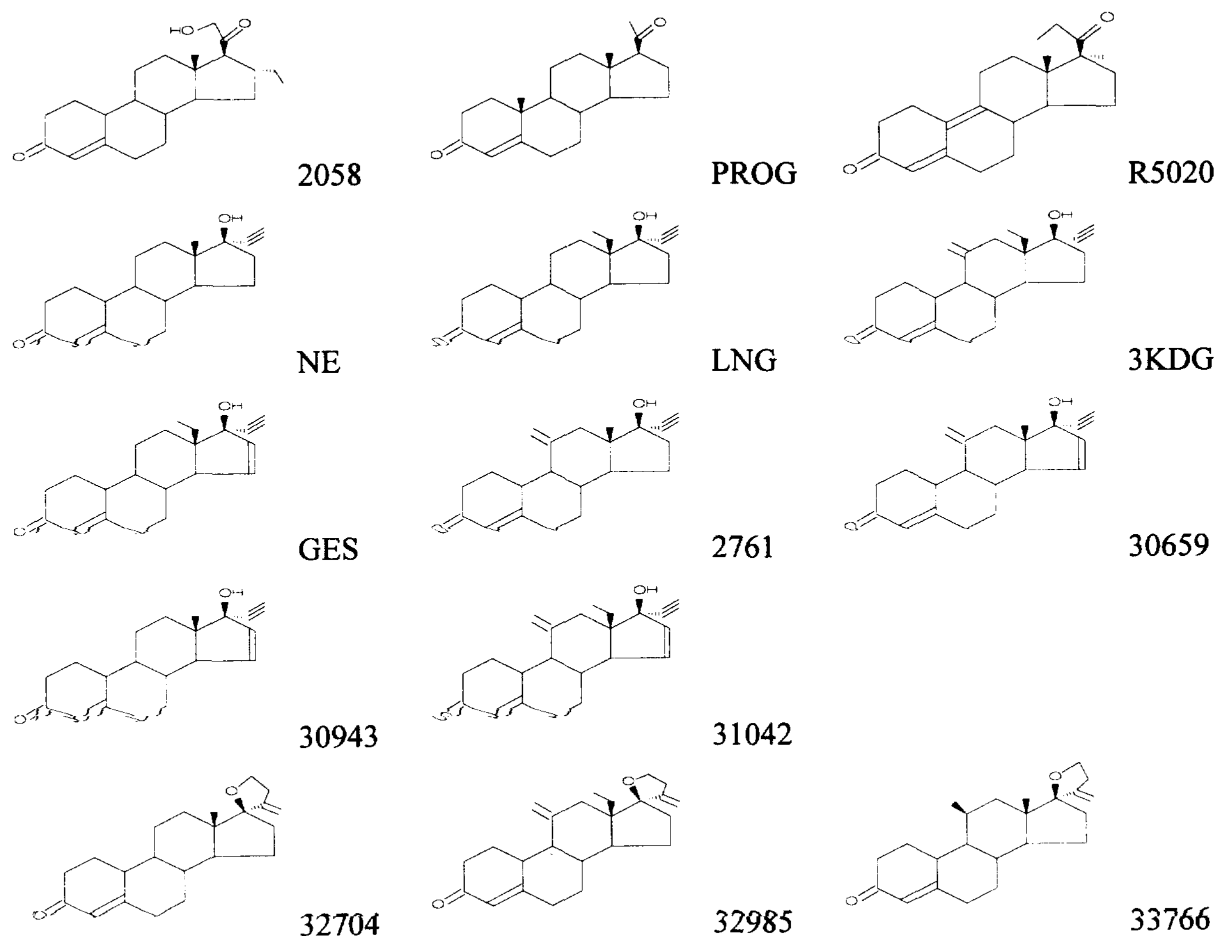


Fig. 1. Chemical structures of several progestagens.

(Rockville, MD, U.S.A.) and containing hPR-A (clone B6), hPR-B-Tag (clone 93), hPR-A-MMTV-LUC (clone A10-17) or hPR-B-MMTV-LUC (clone 1E2-A2), were cultured in medium with charcoal-treated supplemented defined bovine calf serum. The preparation and selection of these four cell lines is described by Dijkema *et al.* [18]. MCF-7 cells were cultured in medium with characterized foetal calf serum. All cell lines were cultured at 37°C in Roux flasks (175 cm^3) flushed with 5% CO_2 in air until pH 7.2–7.4 was reached. Complete medium was refreshed every two or three days. One day before harvesting MCF-7 cells, these cells were cultured on charcoal-treated characterized foetal calf serum.

Displacement studies. For displacement analysis CHO cells containing hPR-A or -B and MCF-7 cells containing hPR-A and -B were used. The cells were harvested by trypsinization and centrifugation at 2,000 N kg^{-1} for 10 min. The cells were washed once with culture medium and the resultant pellet was resuspended in medium or TEGM-buffer (10 nM Tris–HCl buffer pH 7.4 supplemented with 1 mM EDTA, 10 mM sodium molybdate and 250 mM sucrose) and frozen at −70°C before use as cytosolic preparations. The cell pellet was allowed to thaw after which the cells were homogenized using a Dounce all-glass homogenizer. The homogenate was ultra-centrifuged at 1,000,000 N kg^{-1} for 45 min at 4°C. The supernatant (cytosol) was collected in cooled glass tubes and used for the experiments. Prior to use the cytosol was diluted with buffer solution to a final receptor concentration of 1:25 for hPR-A CHO, 1:100 for hPR-B CHO cells and 1:25 for hPR A/B MCF-7 cells.

The assays were carried out in triplicate in 96 well microtiter plates and Org 2058 was used as radioligand. For displacement studies 30 μl of labelled radioligand (final concentration 1.9 nM) in TEGM-buffer were added to a well with 50 μl of cytosol. Non-specific binding and total binding was measured by the simultaneous addition of 20 μl TEGM-buffer with or without unlabelled ligand (1 μM final concentration), while displacement was measured with various concentrations (1:2:4 dilution) of the standard and compounds of interest. Thereby three on-following dilutions with a ratio of 1:2:4 for every standard and compound were used in the dilution range of 0.121, 0.242, 0.485, 0.97, 1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 nM. The assays were terminated by the addition of a dextran-coated char-

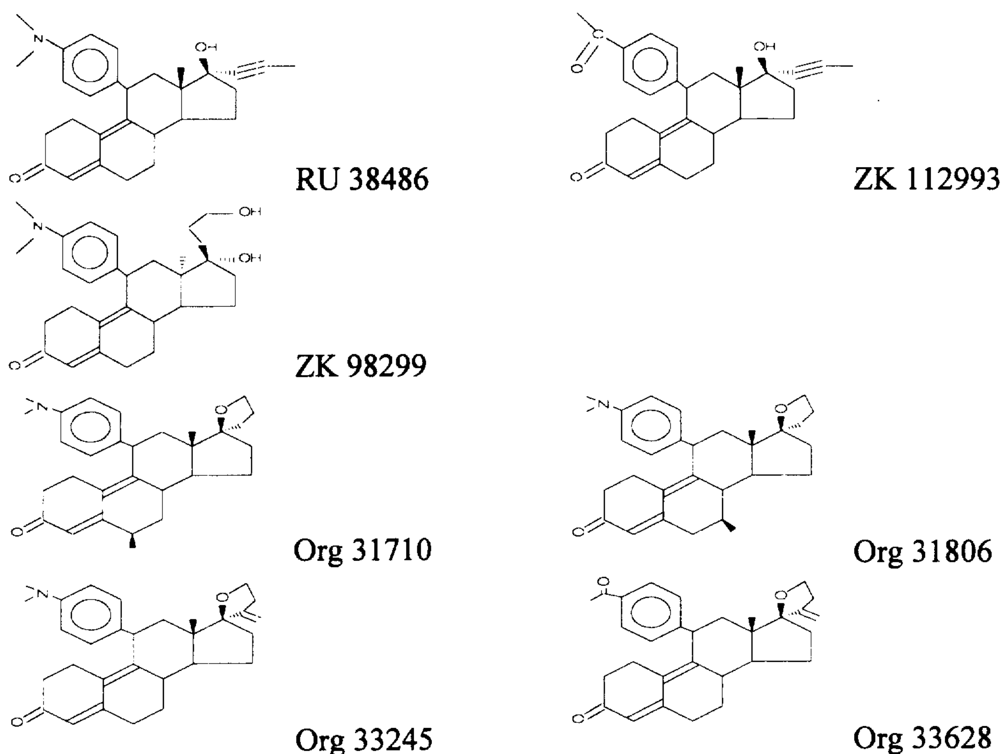


Fig. 2. Chemical structures of several antiprogestagens.

coal solution, which binds unbound ligand. The plates were shaken for exactly 5 min at 4°C and centrifuged at 8,000 N kg^{-1} for 5 min at 4°C. Finally 100 μl of each supernatant was counted in a Topcount microplate scintillation counter (Packard). Specific binding was determined afterwards by subtracting non-specific from total binding. The relative binding affinities of the compounds were obtained by analysis by a 3-point parallel line assay [19]. The mean RBA value with standard deviation (SD) of different independent tests was calculated.

Transactivation studies. For transactivation studies hPR-A-MMTV-LUC and hPR-B-MMTV-LUC stably co-transfected CHO cells were used. Cells were seeded at 5×10^3 cells/well in a 96 well white view plate and incubated during 48 h in medium with charcoal-treated supplemented defined bovine calf serum at 37°C in 5% CO_2 in air in an incubator. Steroids for treatment were diluted in ethanol and before addition to the cells diluted to such a concentration that in the wells only 1% ethanol was present. This 1% ethanol concentration was not toxic to the CHO cells, as tested by luciferase activity measurements and trypane blue exclusion. After 16 h of preculturing of the cells, the steroid dilutions were added towards the cells and incubated overnight. Then part of the medium was removed and LucLite added for cell lysis and luciferase measurement. Luciferase activity was measured in a Topcount luminescence counter during 3 min per plate.

– Relative agonistic activity studies were carried out with various concentrations of the standard Org 2058 (1:2:4 dilutions, see above) and compounds of interest. The relative agonistic activities of the compounds were obtained by analysis by a 3-point parallel line assay [19]. The mean RAA value with SD of different independent tests was calculated.

– Relative antagonistic activity studies were carried out with various concentrations of the standard Org 31710 (1:2:4 dilutions, see above) and compounds of interest in combination with a fixed amount of agonist Org 2058 (10^{-9} M for hPR-A-MMTV-LUC and 10^{-10} M for hPR-B-MMTV-LUC). The relative antagonistic activities of the compounds were obtained by analysis by a 3-point parallel line assay [19]. The mean RANTA value with SD of different independent tests was calculated.

In vivo tests. With the McPhail test endometrium differentiation was scored in rabbits after subcutaneous administration of the compounds [20]. The ED_{50} dose at a score of 2 was determined and the potency against Org 2058 (100%) was recalculated.

With the ovulation inhibition test in rats, the number of animals per group of six rats were scored with complete, half or no ovulation inhibition after subcutaneous treatment of the compounds [20]. The ED_{50} dose giving 50% ovulation inhibition was determined and the potency against Org 2058 (100%) was recalculated.

With the pregnancy interruption test, the number of embryos and implantation sites were counted to

calculate the percentage of pregnancy interruption after subcutaneous treatment with the antiprogestagens [21]. The ED_{50} dose giving 50% pregnancy interruption was determined and the potency against Org 31710 (100%) was recalculated.

General statistics. The mean data were all compared with linear regression analysis for binding of hPR-A versus -B and hPR-B versus hPR A/B (MCF-7 cells) as well as for transactivation and inhibition of transactivation for hPR-A versus -B and for hPR-B versus *in vivo* data of McPhail, ovulation inhibition or pregnancy interruption tests. From each linear regression analysis, the correlation coefficient could be calculated.

RESULTS

Relative binding affinities of 14 progestagens and 7 antiprogestagens

The relative binding affinities (RBA's) of several characteristic (anti)progestagens were analyzed for hPR-A and hPR-B in cytosol of CHO cells and compared with the RBA values of those obtained for hPR A/B in cytosol of MCF-7 cells. The (anti)progestagens tested can be divided into the following 4 classes:

(1) pregnane derived progestagens, i.e. Org 2058, progesterone (PROG) and R5020 (promegestone);

(2) 19-nortestosterone-17α-ethinyl derived progestagens, i.e. norethisterone (NE), levonorgestrel (LNG), 3-ketodesogestrel (3-KDG), gestodene (GES), Org 2761, 30659, 30943 and 31042;

(3) new five membered spiro-(methylene) 17-ring structures, i.e. agonists Org 32704, 32985 and 33766 and antagonists like Org 31710, 31806, 33245 and 33628;

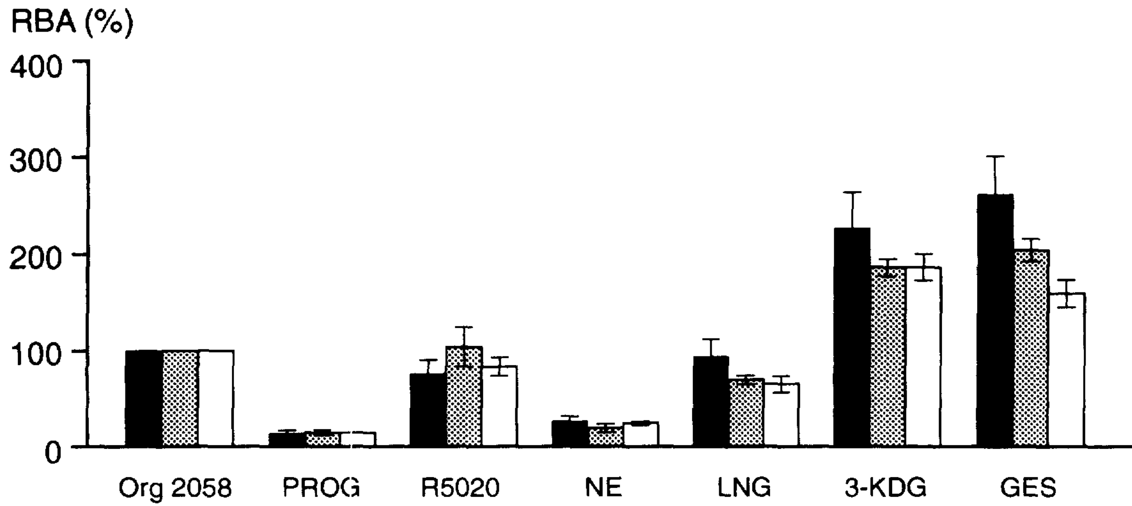
(4) several other well-known antiprogestagens, like RU 38486 (Mifepristone), ZK 98299 (Onapristone) and ZK 112993.

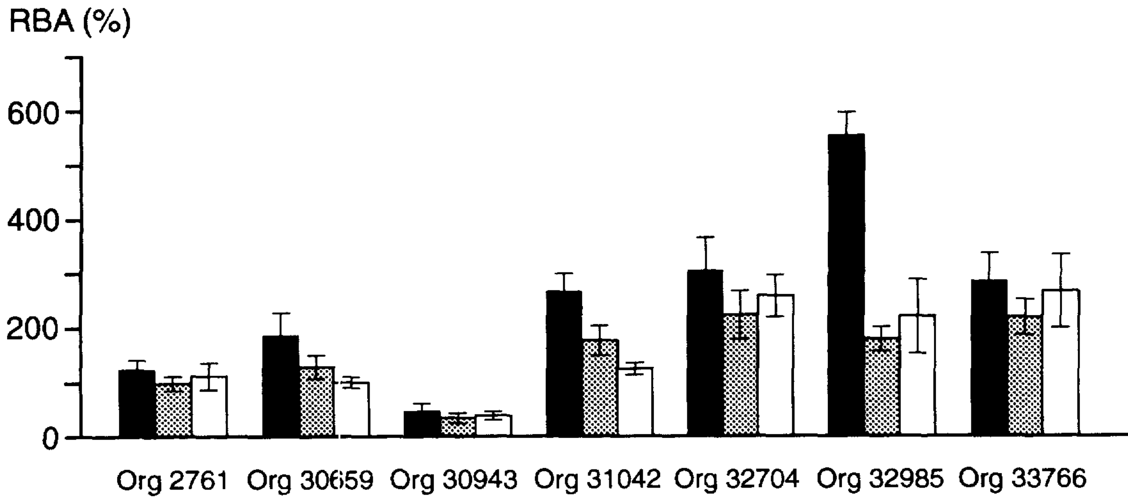
The obtained overall mean RBA values (Org 2058 = 100%) with hPR-A and hPR-B in CHO and hPR A/B in MCF-7 cells are shown in Table 1 and Fig. 3. There is in general a very good resemblance between the individual data of three to seven independent tests for each cell line, as shown by the standard deviations (SD). The RBA's of hPR-B in CHO cells of the progestagens and antiprogestagens tested are very comparable in magnitude and ranking with those of MCF-7 cells. Only GES and Org 31042 formed exceptions as these compounds were slightly more potent for hPR-B in CHO cells than for hPR A/B in MCF-7 cells. Comparison of the RBA's of hPR-B with that of hPR-A in CHO cells showed significantly higher RBA values with hPR-A for the progestagens LNG, 3-KDG, GES, Org 31042 and Org 32985 and for the antiprogestagens Org 31710 and 31806, while the other compounds had comparable RBA values. Although the antiprogestagens Org 31710 and 31806 demonstrated significantly higher RBA values with hPR-A than with hPR-B, this

Table 1. Mean of relative binding affinities with standard deviation for several progestagens and antiprogestagens to the human progesterone receptor A or B in CHO cells and hPR (A and B) in MCF-7 cells

Code	RBA's of hPR-A CHO cells (mean ± SD)	RBA's of hPR-B CHO cells (mean ± SD)	RBA's of hPR MCF-7 cells (mean ± SD)	Ratio (hPR-B/hPR-A)	Ratio (hPR-B/hPR)
			Progestagens		
Org 2058	100.00	100.00	100.00	1.00	1.00
PROG	14.0 ± 3.0	14.6 ± 2.7	14.6 ± 0.4	1.04	1.00
R5020	75.8 ± 14.9	104.0 ± 20.5	83.5 ± 9.5	1.37	1.25
NE	27.2 ± 4.6	19.5 ± 4.5	24.5 ± 1.8	0.71	0.79
LNG	94.0 ± 18.1	70.0 ± 4.4	65.5 ± 8.8	0.75	1.07
3-KDG	227.8 ± 36.8	186.8 ± 9.2	187.0 ± 13.7	0.82	1.00
GES	262.8 ± 39.8	204.8 ± 11.8	159.8 ± 14.5	0.78	1.28
Org 2761	125.2 ± 16.1	98.5 ± 12.7	111.5 ± 24.6	0.79	0.88
Org 30659	186.4 ± 41.3	128.8 ± 21.5	100.5 ± 10.0	0.69	1.28
Org 30943	48.0 ± 13.4	34.6 ± 9.7	38.8 ± 7.5	0.72	0.89
Org 31042	267.5 ± 33.4	176.4 ± 27.1	125.3 ± 11.1	0.66	1.41
Org 32704	306.0 ± 60.9	224.0 ± 44.3	259.2 ± 39.0	0.73	0.86
Org 32985	556.7 ± 41.9	179.3 ± 22.2	221.2 ± 68.8	0.32	0.81
Org 33766	286.7 ± 52.5	220.0 ± 32.7	268.1 ± 67.9	0.77	0.82
			Anti-progestagens		
RU 38486	17.4 ± 2.8	11.4 ± 3.3	7.7 ± 1.3	0.65	1.48
ZK 98299	1.3 ± 0.5	0.8 ± 0.1	0.5 ± 0.2	0.64	1.75
ZK 112993	43.5 ± 6.6	31.2 ± 13.3	30.0 ± 5.0	0.72	1.04
Org 31710	27.8 ± 7.3	13.1 ± 2.3	12.5 ± 0.6	0.47	1.05
Org 31806	26.8 ± 3.6	16.2 ± 4.2	11.9 ± 1.2	0.61	1.36
Org 33245	29.8 ± 4.6	36.5 ± 6.5	26.0 ± 7.0	1.22	1.41
Org 33628	105.4 ± 35.7	96.7 ± 29.3	72.2 ± 10.6	0.92	1.34

Also the ratio's between mean RBA in hPR-A vs hPR-B in CHO cells and hPR-A vs hPR (A + B) in MCF-7 cells are given.





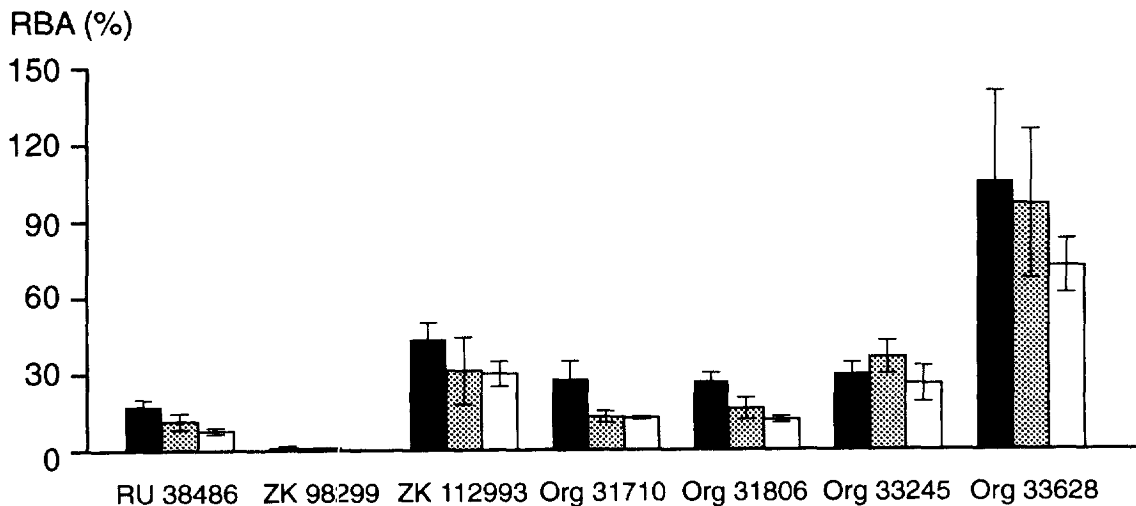


Fig. 3. Mean of relative binding affinities (RBA) with standard deviation for several progestagens and antiprogestagens to the human progesterone receptor A (black bars) or B (hatched bars) in CHO cells and hPR (A and B; open bars) in MCF-7 cells.

change did not influence the ranking of the seven antiprogestagens tested. Remarkably, all agonistic compounds with a 5 membered-spiromethylene 17-ring structure like Org 32704, 32985 and 33766 had RBA values that were above 220% with hPR-A and -B isoforms in CHO and hPR A/B in MCF-7 cells with only one exception for Org 32985 with hPR-B in CHO cells being 179.3%. On the other hand, the RBA value with hPR-A was even 556.7%. This may implicate that these new five membered spiromethylene 17-ring substituted progestagens generally bind with a higher affinity to the progesterone receptor than pregnane and 19-nortestosterone-17α-ethinyl derivatives.

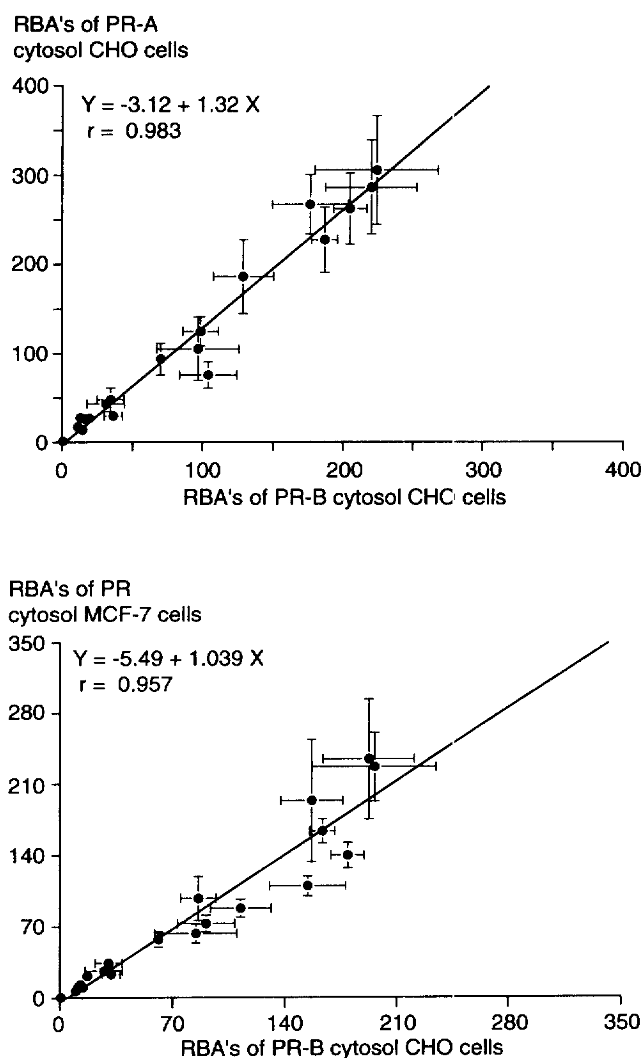


Fig. 4. Correlation between the relative binding affinities of several progestagens and antiprogestagens to hPR-A and hPR-B in cytosol from CHO cells (top) and to hPR-B in cytosol from CHO cells versus that of hPR-A and -B isoforms in MCF-7 cells (bottom) with the standard deviation included.

The ratio between the mean RBA values of hPR-B versus hPR-A in CHO and hPR-B versus hPR A/B in MCF-7 cells (Table 1) for all (anti)progestagen classes tested was always between 0.65 and 1.53 with exception of Org 32985 (0.32), ZK 98299 (0.64), Org 31710 (0.47) and Org 31806 (0.61) for the ratio of hPR-A vs hPR-B and ZK 98299 (1.75) for the ratio of hPR-B and hPR A/B. Although the mean RBA values of ZK 98299 differed between the cell lines, ZK 98299 gave always the lowest ranking and very low RBA values with all three cell lines. The difference found for Org 31710, 31806 and 32985 might be due to higher binding affinity for the hPR-A isoform than for the hPR-B isoforms as a result of the 5 membered spiro(methylene) 17-ring structure.

Linear regression analysis for the mean RBA values of hPR-B versus hPR-A of CHO and of hPR B of CHO cells versus hPR A/B of MCF-7 cells for 21 individual compounds gave the following line parameters $Y = -3.12 + 1.32X$ and $Y = -5.49 + 1.039X$ with correlation coefficients of $r = 0.983$ and $r = 0.957$ ($P \ll 0.001$) (Fig. 4). The regression coefficient indicates that RBA values for hPR-B of CHO cells and hPR A/B of MCF-7 cells are very comparable, while all RBA values of hPR-A of CHO cells are higher than those of hPR-B. However, in general with respect to ranking no relevant differences in binding potencies between the progestagens and antiprogestagens tested were observed.

Relative agonistic activities (RAA) of several progestagens and antiprogestagens

The progestagenic activity of progestagens and antiprogestagens was assessed in an *in vitro* bioassay with recombinant CHO cells stably co-transfected with hPR-A or -B isoforms in combination with the MMTV promoter and the fire fly luciferase reporter gene. The progestagenic transactivation activity of the enzyme luciferase by a test compound is mediated via hPR and compared with the standard Org 2058 (100%). As shown in Table 2 and Fig. 5 all progestagens tested caused transactivation of the enzyme luciferase. On the other hand, none of the antiprogestagens tested and neither 5α-dihydrotestosterone, dexamethasone, estradiol or aldosterone induced transactivation of the enzyme luciferase at concentrations below 10^{-8} mol/l (not shown). For the progestagens tested the following ranking in RAA values from low to high was observed for hPR-A and hPR-B containing cell lines:

hPR-A: PROG < NE < R5020 = Org 2761 = Org 30943 < LNG = Org 32985 = 3-KDG = Org 30659= Org 31042 < GES < Org 32704 < Org 33766.

hPR-B: PROG<NE< R5020=Org 2761=LNG= Org 30943 < Org 32985 ≤ 3-KDG = Org 30659= Org 31042 < Org 32704 = GES < Org 33766.

The five membered spiromethylene 17-ring substituents Org 32704 and 33766 were the most potent

Table 2. Mean of relative agonistic activities with standard deviation for several progestagens to hPR-A-MMTV-LUC or hPR-B-MMTV-LUC cells as well as the ED_{50} and relative potency values for endometrium proliferation in rabbits in the McPhail test and ovulation inhibition in rats

Code	RAA's of hPR-A CHO cells (mean ± SD)	RAA's of hPR-B CHO cells (mean ± SD)	McPhail		Ovulation	
			ED_{50} (μg/kg/day)	potency (%)	ED_{50} (μg/kg/day)	potency (%)
Org 2058	100	100	12	100.0	12	100.0
PROG	6.5 ± 0.7	5.5 ± 0.2	375	3.2	3000	0.4
R5020	31.5 ± 3.5	42.5 ± 14.2	16	75.0	N.D.	
NE	15.0 ± 1.4	12.0 ± 1.0	63	19.0	500	2.4
LNG	55.5 ± 10.6	36.0 ± 1.0	16	75.0	60	20.0
3-KDG	104.5 ± 7.8	86.3 ± 16.3	8	150.0	24	50.0
GES	136.5 ± 16.3	141.0 ± 14.1	8	150.0	24	50.0
Org 2761	28.0 ± 2.8	33.5 ± 3.5	12	100.0	42	28.6
Org 30659	108.0 ± 11.3	105.7 ± 9.2	6	200.0	42	28.6
Org 30943	22.5 ± 2.1	38.0 ± 2.9	24	50.0	128	9.4
Org 31042	94.5 ± 14.8	101.0 ± 9.3	6	200.0	12	100.0
Org 32704	230.0 ± 39.9	135.5 ± 4.5	8	150.0	36	33.3
Org 32985	101.6 ± 7.8	74.0 ± 13.5	12	100.0	144	8.3
Org 33766	640.0 ± 61.0	170.0 ± 30.0	4	300.0	16	75.0

ND = not determined.
With the relative potency estimations Org 2058 (100%) was used as standard.

transactivators with RAA's of 230 and 640% for hPR-A and of 135.5 and 170% for hPR-B. The 19-nortestosterone derived progestagens GES, 3-KDG, Org 31042, Org 30659, Org 30943, LNG, Org 2761 and NE had decreasing RAA values in the range from 136.5 to 15.0% for hPR-A and from 141 to 12% for hPR-B. PROG belonging to the pregnane derived progestagens had the weakest RAA of 6.5 for hPR-A and 5.5% for hPR-B, while R5020 had RAA values of 31.5 and 42.5%, respectively.

The RAA values of hPR-A and -B in CHO cells for the above mentioned agonists (except Org 32704 and 33766) were compared with each other by linear regression, revealing the following line parameter $Y = 3.92 + 0.90X$ with a correlation coefficient of $r = 0.956$ ($n = 12$). This indicates that there was a highly statistical significant correlation ($P < 0.001$) between both agonistic transactivation assays with hPR-A and hPR-B-MMTV-LUC cell lines. Furthermore, the regression coefficient shows that there was no difference in RAA values between both assays.

Therefore only RAA values obtained with hPR-B-MMTV-LUC cells were compared with the RBA values of hPR-B. Linear regression analysis for these two assays showed a line parameter of $Y = -4.8 + 0.627X$ and a correlation coefficient of $r = 0.91$ ($n = 14$). This implicates that RAA data are roughly 0.627 times lower than their RBA values. The potency ranking between both assays only differed for Org 2058, R5020 and Org 32985.

Relative antagonistic activities (RANTA) of several progestagens and antiprogestagens

The antiprogestagenic activity of progestagens and antiprogestagens was assessed in an *in vitro* bioassay with recombinant CHO cells stably co-transfected with hPR-A or -B isoforms in combination with the MMTV promoter and the fire fly luciferase reporter gene. The antiprogestagenic activity of a test compound for inhibition of transactivation of the enzyme luciferase is mediated via hPR and compared with the standard antiprogestagen Org 31710 (100%). All progestagens tested did not inhibit transactivation of luciferase activity induced by 10^{-10} (hPR-B) or 10^{-9} M (hPR-A) of Org 2058, neither did 5α-dihydrotestosterone, dexamethasone, estradiol or aldosterone. On the other hand, all strong and weak antiprogestagens tested could inhibit transactivation dependent on the dose levels used (Table 3 and Fig. 5). For the antagonists tested the ranking of RANTA values for both hPR-A-MMTV-LUC and hPR-B-MMTV-LUC cells was as follows: ZK 98299 < Org 31710 = RU 38486= Org 31806 < Org 33245 ≤ ZK 112993 < Org 33628. The ranking as well as the potencies did only differ for Org 33245 and ZK 112993. Org 33245 appeared to be 1.5-fold more active in hPR-A-MMTV-LUC than hPR-B-MMTV-LUC cells. The ranking of the tested compounds was also comparable to the ranking of RBA values. For the RBA's of hPR-B in CHO cells the ranking only differed for RU 38486 and Org 31710. However, these compounds are equipotent in both assays.

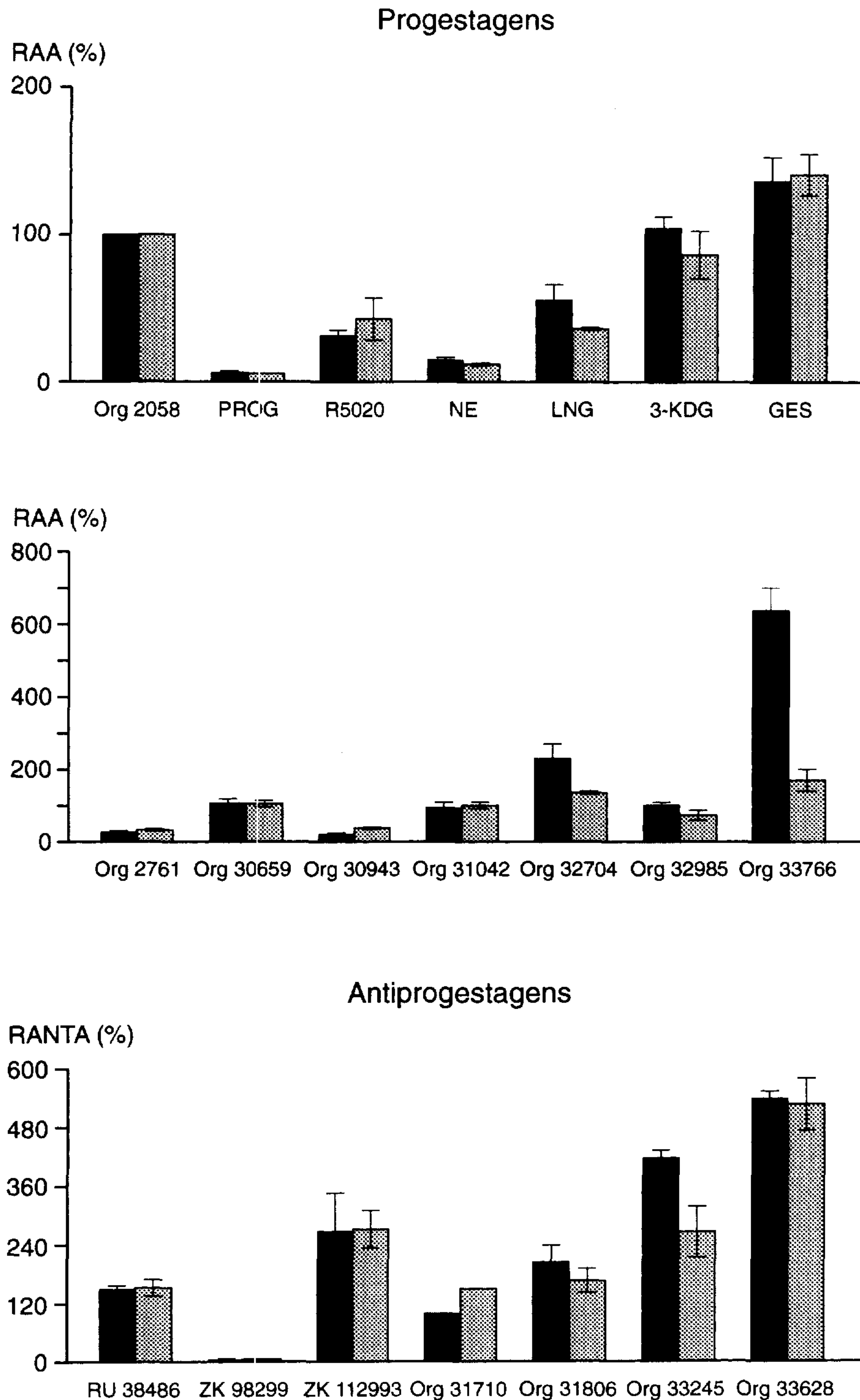


Fig. 5. Mean of relative agonistic activities (RAA) with standard deviation for several progestagens and mean of relative antagonistic activities (RANTA) for several antiprogestagens to the human progesterone receptor A (black bars) or B (hatched bars) in CHO cells in combination with the MMTV-LUC promoter.

The obtained RANTA values for hPR-A-MMTV-LUC and hPR-B-MMTV-LUC cells were compared with each other by linear regression. This lead to the following line parameter $Y = 4.02 + 1.07X$ and a correlation coefficient of $r = 0.942$, which is highly significant.

The obtained RANTA values were also compared by linear regression with RBA values of these antipro-

Table 3. Mean of relative antagonistic activities with standard deviation for several antiprogestagens to hPR-A-MMTV-LUC or hPR-B-MMTV-LUC cells as well as the ED_{50} and relative potency values in the pregnancy interruption test (PIT) in rats

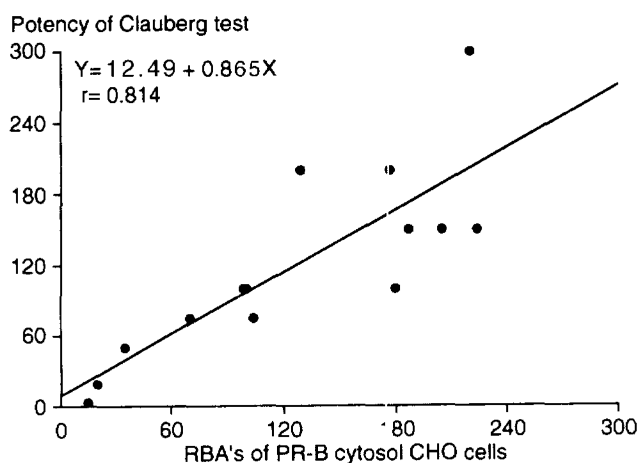
Code	RANTA's of hPR-A CHO cells (mean ± SD)	RANTA's of hPR-B CHO cells (mean ± SD)	PIT	
			ED_{50} (μg/kg/day)	potency (%)
RU 38486	151 ± 6.6	153 ± 17.1	750	267
ZK 98299	5 ± 2.1	5 ± 1.7	3000	67
ZK 112993	268 ± 78.0	272 ± 38.3	≤500	400
Org 31710	100	150	2000	100
Org 31806	205 ± 33.8	167 ± 24.6	1000	200
Org 33245	418 ± 15.0	266 ± 52.0	360	555
Org 33628	540 ± 14.1	528 ± 53.4	64	3125

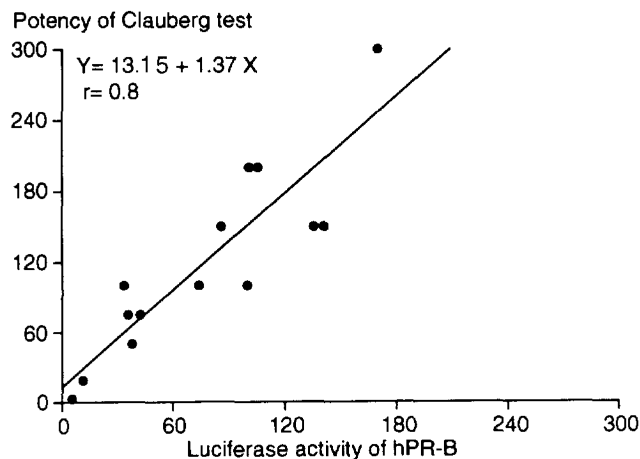
With the relative potency estimations Org 31710 (100%) was used as standard.

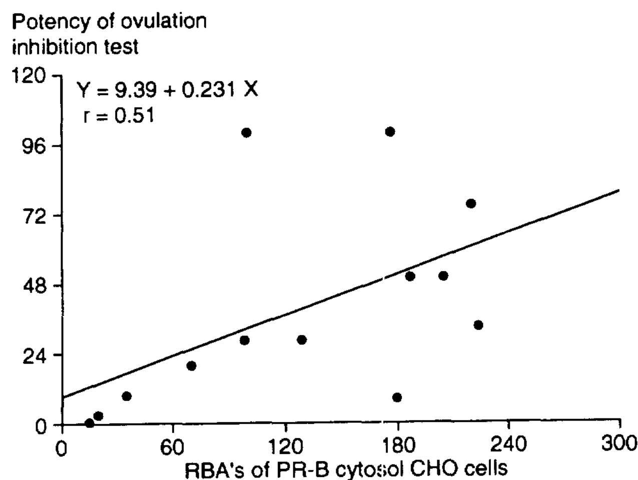
gestagens for hPR-B in CHO cells. In this comparison RBA binding data of hPR-B were normalized with Org 31710 as standard (100%). Linear regression analysis revealed a line parameter of $Y = 75.2 + 0.647X$ with a correlation coefficient of $r = 0.97$ ($n = 7$), respectively. This indicates that there

Relative binding affinities

Relative agonistic affinities







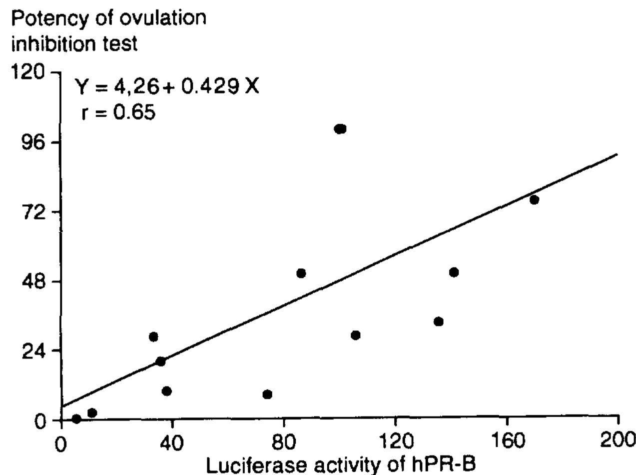


Fig. 6. Correlation between the relative binding affinities with hPR-B in CHO cells and ED_{50} dose levels for endometrium proliferation in rabbits according the McPhail test (upper left) and ovulation inhibition in rats (lower left) as well as between the relative agonistic activities with hPR-B-MMTV-LUC in CHO cells and ED_{50} dose levels for endometrium proliferation in rabbits (upper right) and ovulation inhibition in rats (lower right).

was a highly statistical significant correlation ($P < 0.001$) between both binding and antagonistic transactivation assays, in which binding values were approximately 1.6 fold higher than the transactivation values.

Correlation of RBA, RAA and RANTA values with ED_{50} *in vivo potencies in rabbits and rats*

To get an impression about the pharmacological relevance of the binding and transactivation assays, the (anti)progestagenic potencies of the compounds tested were compared with *in vivo* progestagenic potencies of these compounds after subcutaneous administration in rabbits and rats and with *in vivo* antiprogestagenic potencies after oral treatment in rats.

In rabbits, the McPhail test indicative for endometrium differentiation of progestagenic agonists was performed, leading to the following ranking in potency from low to high: PROG < NE < Org 30943 < LNG = R5020 < Org 2761 = Org 2058 = Org 32985 < 3-KDG = GES = Org 32704 < Org 30659 = Org 31042 < Org 33766. For PROG and NE the ED_{50} dose level was very high, being 375 and 63 μg/kg/day, respectively, while other compounds were active between 24 and 4 μg/kg/day. Org 30659 and 31042 were even active at 6 and Org 33766 at 4 μg/kg/day.

Although the ranking was not identical to that of the RBA and RAA values the correlation between RBA and biopotency values as well as between RAA and biopotency values was relatively high with correlation coefficients of $r = 0.81$ and $r = 0.87$ and line parameters of $Y = 12.49 + 0.865X$ and $Y = 13.15 + 1.37X$, respectively (Fig. 6). This indicates that there is a relative good agreement between RBA and RAA values versus data of the McPhail test.

In rats, ovulation inhibition was tested with progestagenic agonists, leading to the following ranking in potency values from low to high: PROG < NE < Org 32985 = Org 30943 < LNG < Org 2761 = Org 30659 = Org 32704 < 3-KDG = GES < Org 33766 < Org 2058 = Org 31042. For PROG and NE again very high ED_{50} dose levels of 1500 and 250 μg/kg/day were obtained, while also relative high ED_{50} dose levels were found now for LNG, Org 30943 and Org 32985, being 30, 64 and 72 μg/kg/day. Org 2058, 31042 and 33766 were the most potent ovulation inhibitors at 6, 6, and 8 μg/kg/day, respectively. Linear regression analysis was also carried out for RBA and biopotency values as well as for RAA and biopotency values. The obtained line parameters were $Y = 9.39 + 0.231X$ and $Y = 4.26 + 0.429X$ with correlation coefficients of $r = 0.51$ and $r = 0.65$, respectively (Fig. 6). This implicates that the correlation between the ovulation inhibition test versus the RBA and RAA values was far lower than for those with the McPhail test.

In rats, the antiprogestagens were tested with the pregnancy interruption test showing the following ranking in potency: ZK 98299 < RU 38486 = Org 31710 = Org 31806 = ZK 112993 < Org 33245 < Org 33628. This ranking was similar to that of the RBA and RAA values for hPR-B in CHO cells with exception of ZK 112993 which was less potent *in vivo* than *in vitro*. Still, linear regression analysis for RBA and biopotency values and RAA and biopotency values demonstrated a very good correlation between both data sets with correlation coefficients of $r = 0.849$ and $r = 0.744$ and line parameters of $Y = 1.00 + 0.71X$ and $Y = 1.16 + 0.621X$.

DISCUSSION

Progesterone receptor cDNA of the hPR-A or -B isoform was stably expressed in CHO cells as demonstrated with Western Blotting with monoclonal antibodies against hPR A/B and molecular weight determinations by Dijkema *et al.* [18]. Moreover, the hPR-A and -B isoforms of CHO cells were identical to the hPR A/B isoforms present in MCF-7 cells with respect to their molecular and biochemical resemblance as indicated by comparable dissociation constants [18].

MCF-7 cells were routinely used for (anti)progestagenic binding affinity estimations of new synthetic (anti)progestagens during many years within our lab. Replacement with CHO cells containing hPR-A or -B was only allowed if the obtained RBA values for (anti)progestagens would be comparable with those obtained with MCF-7 cells. Therefore RBA's of hPR-B in CHO cells were compared with those of hPR A/B in MCF-7 cells and hPR-A in CHO cells. The tested classes were:

(1) the natural and synthetic pregnane derived progestagens, including PROG, R5020 and Org 2058;

(2) the synthetic 19-nortestosterone-17α-ethinyl derived progestagens like NE, LNG, 3-KDG, GES, Org 2761, Org 30659, Org 30943 and Org 31042;

(3) the newly synthesized five membered spiro(-methylene) 17-ring derived progestagens like Org 32704, Org 32985 and Org 33766 and the antiprogestagens Org 31710, Org 31806, Org 33245 and Org 33628; and

(4) finally several other antiprogestagens, such as RU 38486, ZK 98299 and ZK 112993.

The RBA's of hPR-B in CHO and hPR A/B in MCF-7 cells were comparable for all 21 compounds tested as demonstrated by linear regression. An small exception formed GES and Org 31042, that had slightly higher RBA values for hPR-B in CHO cells. The RBA's of hPR-B and hPR-A in CHO cells differed for much more compounds, being higher with hPR-A for LNG, 3-KDG, GES, Org 31042, Org 32985, Org 31710 and Org 31806 and leading to a linear regression slope of 1.32. The compounds

LNG, 3-KDG, GES, Org 31042 and Org 32985 all contained a 18-methyl substitution, while Org 31710 and Org 31806 contained 6β and 7β-methyl additions. Whether these methyl groups were due to the observed changes in binding values remains open for further research. The change in the RBA value of Org 32985 from 179.3% with hPR-B towards 556.7% with hPR-A was rather remarkable. This effect is most likely due to the five membered spiromethylene 17-ring substitution in combination with the 18-methyl and 11-methylene substitutions. Especially the five membered spiromethylene 17-ring substitution, which is also present in the progestagen agonists Org 32704 and Org 33766 enhanced binding in such a way that these latter compounds were among the most active binders with hPR-A, hPR-B and hPR A/B. On the other hand, the effect of the five membered spiromethylene 17-ring substitution with the antiprogestagens Org 33245 and Org 33628 was less obvious. Org 33628 was the most potent antiprogestagen, but this was inevitably caused by the 11β-acetophenon substitution. Although these CHO cells contain only one type of steroid receptor (hPR-A or -B) instead of hPR-A and -B isoforms in combination with estrogen and androgen receptor as in MCF-7 cells, a clear significant difference in RBA values for the 21 tested (anti)progestagens was not found. So, the presence of hPR-A or other receptors in MCF-7 cells did not influence RBA's for the compounds tested in MCF-7 cells as compared with those of hPR-B in CHO cells. This confirms that hPR-B of CHO cells can be used instead of hPR A/B of MCF-7 cells for progestagenic potency estimations.

In this study also the agonistic and antagonistic activities of several (anti)-progestagens were assessed with stably co-transfected CHO cell lines, containing the hPR-A or -B isoform together with the MMTV promoter and the fire fly luciferase gene. These CHO cell lines showed clear transactivation and transcription activities for the luciferase enzyme with progestagens only. Estradiol, 5α-dihydrotestosterone, dexamethasone, aldosterone and antiprogestagens like RU 38486, ZK 98299, ZK 112993, Org 31710, Org 31806, Org 33245 and Org 33628 did not induce the production of luciferase. On the other hand, the progestagen induced transactivation could be inhibited by all the antiprogestagens tested. Comparison of these data for hPR-A and -B cells showed hardly any difference in agonistic or antagonistic potency levels. This is very indicative for the idea that there is no difference in transactivation potency or inhibitory transactivation potency between hPR-A and -B with the MMTV promoter and luciferase reporter gene in CHO cells. However, 10-fold higher levels of Org 2058 were needed for maximal transactivation of hPR-A-MMTV-LUC cells than for hPR-B-MMTV-LUC cells. This is in agreement with earlier data on transactivation of the MMTV promoter with hPR-A and -B [11]. On the other hand, this effect may also have been due to lower receptor levels in hPR-A-MMTV-LUC than in hPR-B-MMTV-LUC cells. However, saturation analysis of these cell lines with Org 2058 demonstrated that this was not the case, since hPR-B-MMTV-LUC cells only contained twice as much receptor as hPR-A-MMTV-LUC cells [18].

An agonistic effect of RU 38486 with hPR-B-MMTV-LUC cells was not found, implying that these CHO cells responded differently than T47D cells from Horwitz group [22, 23]. With these T47D cells a weak agonistic activity was observed with RU 38486 in cells containing the hPR-B isoform in combination with the thymidine kinase promoter and chloramphenicol transferase (CAT) reporter gene. Such an agonistic effect of RU 38486 was lacking in T47D cells containing only hPR-A [22, 23]. Treatment of hPR-B containing T47D cells with both bromo cyclic AMP and RU 38486 showed a clear synergistic effect on the production of CAT expression. In hPR-B-MMTV-LUC CHO cells, simultaneous treatment with bromo cyclic AMP and RU 38486 also lead to a 10% enhancement of luciferase production (unpublished results).

Since there was no clear difference observed between the RAA and RANTA values of hPR-B-MMTV-LUC and hPR-A-MMTV-LUC cells, only the hPR-B-MMTV-LUC CHO cell line was used for comparison with the RBA values of hPR-B in CHO cells. The obtained data show that there is a rather good correlation in ranking of the RBA values of the tested (anti)progestagens with respect to the RAA and RANTA values in these CHO cells. The absolute potency value for the agonistic and antagonistic compounds is always 0.6 to 0.65 times less than the corresponding binding values with the same standard. This suggests that with transactivation other mechanistic processes such as receptor dimerization, receptor phosphorylation, interaction with the DNA and transcription factors play a role in the final potency of a compound. However, in binding studies only the interaction with the hormone binding site is important. As the ranking of compounds was not really changed, transactivation assays can replace binding assays. The advantage of these transactivation assays is that with these assays pure agonists and antagonists as well as partial antagonists can immediately be identified. Moreover, in the transactivation assays much less cell material is needed for a test if compared with binding analysis. For binding analysis at least 2×10^8 CHO cells are needed per test, whereas for both agonistic and antagonistic transactivation assays only 3×10^5 cells are used per assay.

Then only one essential point remains, whether the outcome of the *in vitro* transactivation assays are predictive for the *in vivo* situation. To evaluate this critical issue, the 14 progestagens were analyzed in an

endometrium differentiation test in the McPhail test in rabbits and an ovulation inhibition test in rats, while the antiprogestagens were tested for pregnancy interruption in rats. The ED_{50} potencies in the McPhail test for these progestagens were compared with the RBA and RAA values for hPR-B, showing a very good correlation between both comparisons with correlation coefficients of 0.81 and 0.87 ($n = 13$), respectively. This is indicative for a very high correlation between *in vitro* binding and transactivation assays versus *in vivo* endometrium proliferation in rabbits. The ED_{50} potencies in the ovulation inhibition test for these progestagens were compared with the RBA and RAA values for hPR-B, showing a much lower correlation between both comparisons with correlation coefficients of 0.516 and 0.65 ($n = 13$), respectively. So, there is correlation between *in vitro* binding and transactivation assays versus *in vivo* ovulation inhibition in rats, but it is less clear than in rabbits. Especially, compounds like Org 32704 and Org 32985 had a low *in vivo* activity in rats with respect to the transactivation and binding data of CHO cells. Org 33766 a structure related compound to Org 32704 and 32985 was much more potent *in vivo* in both rabbits and rats. This suggests that although the five membered spiromethylene 17-ring enhances binding and transactivation of all three compounds, it is not enough for *in vivo* stabilization. Therefore the addition of an 11β-methyl addition appears beneficial. The conclusion from this data set is that the effects of progestagens in *in vitro* transactivation and binding studies with hPR-B in CHO cells are very representative for *in vivo* endometrium proliferation in rabbits, whereas they are in a lesser way predictive for *in vivo* ovulation inhibition in rats. This discrepancy may be due to a difference in liver metabolism between rabbits and rats or to the progesterone receptor hormone binding site specificity among species. In rats both PR-A and B isoforms have been described [2, 3], whereas in rabbits so far only the PR-B isoform has been recognized in endometrium [24–26]. The antiprogestagenic activity in the pregnancy interruption test lead to ED_{50} potencies that were in line with the RBA and RANTA values. ZK 98299 had the lowest *in vivo* and *in vitro* potencies, followed by the equipotent compounds RU 38486, Org 31710 and Org 31806. ZK 112993 was *in vivo* as active as the latter three, but *in vitro* 1.7-fold more potent. The five membered spiromethylene 17-ring substituents were the most potent antiprogestagens in the pregnancy interruption test as well as in RBA and RANTA studies. So, also for the well-known antiprogestagens the inhibition of transactivation with CHO cells forms a predictive test for *in vivo* biopotency.

In conclusion, the binding and transactivation potencies for the tested compounds in hPR-A and -B containing cell lines showed in general a good resemblance. The transactivation studies with hPR-B-MMTV-LUC cells indicated that ranking of compounds was fairly identical to binding analysis and could be used for pre-screening of the (anti)-progestagenic bioactivity in the McPhail test in rabbits, the ovulation inhibition test and the pregnancy interruption test in rats. Therefore this transactivation assay can replace binding assays. Moreover, direct pre-screening of agonists, antagonists and partial antagonists is even possible in this *in vitro* bioassay, making transactivation assays for a particular class of chemical compounds to a valuable substitute for *in vivo* studies.

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