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The Relationship Between Affinity of Progestins and Antiprogestins for the Progesterone Receptor in Breast Cancer Cells (ZR-PR-LT) and Ability to Down-regulate the Receptor: Evidence for Hetero-specific Receptor Modulation via the Glucocorticoid Receptor

H.W. van den Berg, M. Lynch and J.H.J. Martin

In a human breast cancer cell line (ZR-PR-LT) we have found a poor overall correlation between affinity of progestins and anti-progestins for the progesterone receptor (PGR), concentration required for receptor down-regulation and anti-proliferative potency. Medroxyprogesterone acetate (MPA) and the anti-progestin RU 38.486, which possess glucocorticoid and antiglucocorticoid activity, respectively, cause receptor down-regulation at lower concentrations than their Kd_i for [^{3}H] ORG 2058 binding sites. In addition dexamethasone markedly down-regulates PGR at concentrations which fail to interact with PGR suggesting that heterospecific modulation of PGR occurs via the glucocorticoid receptor. In contrast the progestin ORG2058 and the anti-progestin ZK 98.299 caused 50% PGR down-regulation at a concentration (EC_{50}) 50-fold higher than their Kd_i values. ZK 112.993 was 500-fold more potent at PGR down-regulation than ZK 98.299 but had only a 5-fold higher affinity for PGR. Anti-proliferative concentrations of progestins/anti-progestins showing were generally higher than either Kd_i values or EC_{50} values.
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INTRODUCTION

HORMONAL THERAPY of breast cancer may include the use of progestins, generally as a second line modality following failure of anti-oestrogen treatment. Both medroxyprogesterone acetate (MPA) and megestrol acetate induce remission rates (30–40%) comparable to other forms of endocrine therapy. The mechanism of action of progestins in the treatment of breast cancer is unclear, and hence there are no clearly defined criteria for identification of patients likely to respond. Progesterone receptor (PGR) status of tumour cells has been reported to be a poor indicator of response both *in vitro* [1–3] and *in vivo* [4]. MPA has both glucocorticoid and androgenic actions and *in vitro* studies have suggested that MPA may act primarily via receptors for these two classes of steroids [3].

Several reports [5–7] have indicated that a number of recently developed anti-progestins also show anti-proliferative activity towards murine breast cancer and these agents also have variable anti-glucocorticoid activities. RU 38.486 (mifepristone) has shown some activity against human breast cancer [8, 9] but side-effects consistent with its anti-glucocorticoid activity were noted. ZK 98.299 (Onapristone, Schering AG) and ZK 112.993 are of particular interest in that they have low anti-glucocorticoid activity and in murine tumour models appears to act via PGR inducing terminal differentiation and cell death [7].

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In this study we have investigated the ability of a number of progestins and anti-progestins to down-regulate PGR (an indicator of receptor activation) and correlated these findings with the agents' affinity for PGR, anti-proliferative action and known glucocorticoid/anti-glucocorticoid properties. As our experimental model we have used an oestrogen independent variant of the ZR-75-1 human breast cancer cell line designated ZR-PR-LT. This line is unusual in that it fails to express binding sites characteristic of Type 1 oestrogen receptor but synthesises high basal levels of PGR [10]. It therefore provides an excellent model for the study of progestin/anti-progestin action without the need for oestrogen induction of PGR. Our results indicate a poor overall correlation between affinity of progestins/anti-progestins for PGR and ability to down-regulate the receptor and indicate for the first time that PGR may be subject to down-regulation following ligand interaction with the glucocorticoid receptor. In addition, important differences were noted in the biological activities of ZK 98.299 and ZK 112.993.

MATERIALS AND METHODS

Cells

ZR-PR-LT cells arose as a result of long term culture of the well characterised oestrogen receptor (ER) and PGR positive human breast cancer line ZR-75-1 in the absence of all known oestrogenic activity [10]. ZR-PR-LT cells are routinely maintained in this selection medium which consists of phenol red-free RPMI 1640 medium supplemented with 5% heat treated and charcoal stripped serum. These cells fail to express binding sites characteristic of the type 1 oestrogen receptor, are not growth stimulated by oestradiol but express elevated levels

of PGR (approximately 1400 fmol/mg protein) which are not further induced by oestrogen treatment.

Hormones and antagonists

The synthetic progestin ORG 2058 was obtained from Amersham International plc; Medroxyprogesterone acetate (MPA) and dexamethasone were obtained from the Sigma Chemical Co.; RU 38.486 (Mifapristone), ZK 98.299 (Onapristone) and ZK 112.993 were kindly supplied by Dr Horst Michna (Schering, Berlin).

Competitive binding analyses

The ability of the agents under study to displace [^{3}H] ORG 2058 (specific activity 1.5–2.2 TBq/mmol) from PGR was investigated using a whole cell binding assay as previously described [10]. Briefly, ZR-PR-LT cells (2×10^5) were plated into 24-well dishes and allowed to attach for 24 h. Medium was then removed and replaced with medium containing 1.4 nmol/l or 0.25 nmol/l [^{3}H] ORG 2058 in the presence or absence of competing ligand (10^{-12}–10^{-5} mol/l). Following an incubation period of 1 h at 37°C medium was removed, wells washed twice with ice-cold phosphate-buffered saline and radioactivity extracted in 1 ml ethanol. Radioactivity was assessed by liquid scintillation counting and data expressed as % [^{3}H] ORG 2058 specifically bound after allowing for non-specific binding, which was taken as radioactivity bound in the presence of 10^{-5} mol/l ligand. Sigmoid radioactive ligand displacement curves were linearised using a logit-log plot and concentration of unlabelled ligand resulting in 50% displacement of [^{3}H] ORG2058 (IC_{50}) determined from the intercept on the abscissa. Slope factors were also calculated to indicate whether more than one class of binding sites was involved. The Kd for the competing ligand (Kd_i) was determined from the relationship $Kd_i = IC_{50}/[(1 + (L)/K_d)]$ where (L) = concentration of [^{3}H] ORG 2058 and K_d = K_d of ORG 2058 for PGR, determined from saturation binding experiments [10].

Receptor down-regulation

ZR-PR-LT cells were plated in 24-well dishes as described above and exposed to hormone or antagonist (10^{-12}–10^{-6} mol/l) for 6 days. PGR expression was determined using a single point binding assay with 0.25 nmol/l [^{3}H] ORG 2058 as radioactive ligand. On several occasions experiments were repeated using full saturation binding analyses [10] to confirm that the degree of reduction in PGR expression determined by single point assay accurately reflected that obtained by saturation binding analysis.

Cell proliferation studies

The effects of hormones and antagonists on cell proliferation were determined as follows. Cells (5×10^4/well) were plated into 24 place multiwell dishes and allowed to attach for 24 h. Routine growth medium was then replaced with medium containing drug (10^{-8}–10^{-6} mol/l) and cells counted in triplicate at 0, 6, and 9 days during continuous treatment using a Coulter Counter Model D. For the sake of clarity, effects of treatment are presented as cell number as a % of control cell number at day 9.

RESULTS

Figure 1(a) shows displacement of [^{3}H] ORG 2058 (free concentration 1.4 nmol/l) from specific binding sites on ZR-PR-LT cells by the hormones/anti-hormones under investigation. Logit-log transformation of the data is shown in Fig. 1(b) and the slope factor exceeded unity for all drugs studied.

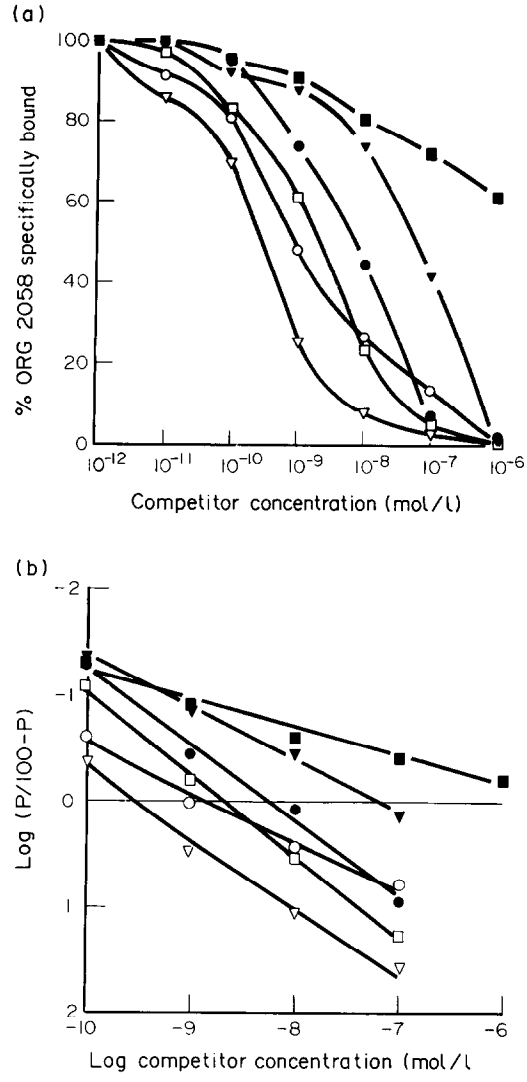

Fig. 1. (a)Displacement of [^{3}H] ORG 2058 (free concentration 1.4 nmol/l) from specific binding sites in ZR-PR-LT cells. ○–○ ORG 2058; ▽–▽ MPA; □–□ RU 38.486; ●–● ZK 112.993; ▼–▼ ZK 98.299; ■–■ dexamethasone. (b)Logit-log plot of data shown in Fig. 1(a). P = per cent competition of specific binding in the presence of a given concentration of inhibitor.

Since the most common cause of logit-log plots with slopes greater than one is interaction of the tracer with more than one class of binding site, we surmised that [^{3}H] ORG 2058 was also interacting with the glucocorticoid receptor and we repeated this experiment using a lower free concentration of [^{3}H] ORG 2058 (0.25 nmol/l). The results are shown in Fig. 2(a) and logit-log plots (Fig. 2b) had slopes close to unity. At this lower concentration of [^{3}H] ORG2058 dexamethasone only displaced a significant proportion of labelled progestin at a concentration of 10^{-7} mol/l and a logit-log plot for this ligand could not be constructed. A comparison of Figs 1 and 2 reveals that at the higher free radioactive ligand concentration MPA had the highest affinity for ORG 2058 binding sites followed by RU 38.486 > ZK 112.993 > ZK 98.299 > dexamethasone. This rank order was maintained when the [^{3}H] ORG 2058 concentration was reduced to 0.25 nmol/l with the exception that RU 38.486 had a higher affinity for [^{3}H] ORG 2058 binding sites than MPA.

Figure 3 demonstrates the ability of the compounds investi-

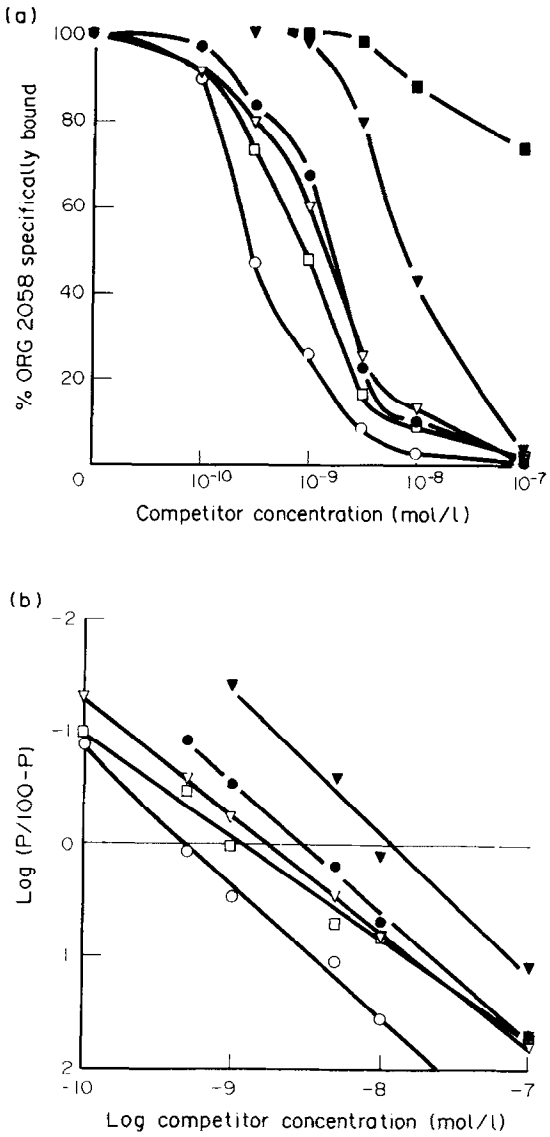

Fig. 2. (a)Displacement of [^{3}H] ORG 2058 (free concentration 0.25 nmol/l) from specific binding sites in ZR-PR-LT cells. ○–○ ORG 2058; ▽–▽ MPA; □–□ RU 38.486; ●–● ZK 112.988; ▼–▼ ZK 98.299; ■–■ dexamethasone. (b)Logit-log plot of data shown in Fig. 1(a). P = per cent competition of specific binding in the presence of a given concentration of inhibitor.

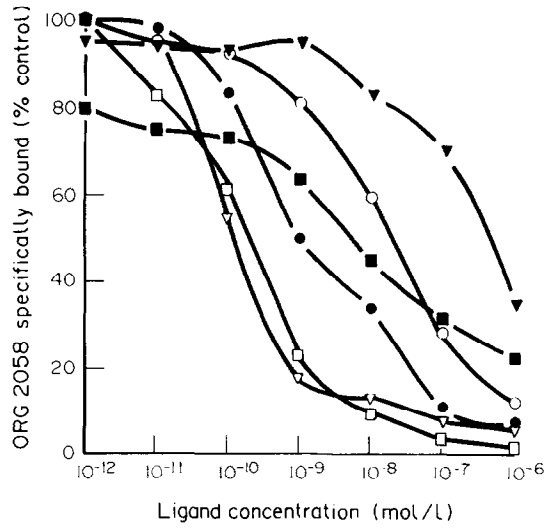

Fig. 3. Reduction in PGR expression by increasing concentrations of progestins/anti-progestins and dexamethasone. PGR expression was determined by single-point assay following a 6 day continual exposure as described in the text. For definition of symbols, see Fig. 2.

gated to down-regulate PGR in ZR-PR-LT cells over a 6 day treatment period. MPA and RU 38.486 were equipotent and the rank order of potency (MPA = RU 38.486 > ZK 112.993 > dexamethasone > ORG 2058 > ZK 98.299) differed from the rank order of affinities for [^{3}H] ORG 2058 binding sites (Fig. 2a and b).

Table 1 compares the concentrations of ligands required to down-regulate PGR expression by 50% (EC_{50}) with the affinity of the ligands for [^{3}H] ORG 2058 binding sites (Kd_i) calculated for both concentrations of free [^{3}H] ORG 2058. Apparent Kd_is of RU 38.486, ZK 98.299 and ZK 112.993 were similar for both concentrations of free [^{3}H] ORG 2058. However, the apparent affinity of MPA for [^{3}H] ORG 2058 binding sites was 30-fold higher when the free tracer concentration was 1.4 nmol/l compared to the value obtained with a free concentration of 0.25 nmol/l. There were marked discrepancies between the affinity of certain ligands for [^{3}H] ORG 2058 binding sites and potency in down-regulating PGR. Thus, MPA and RU 38.486 caused a 50% reduction in PGR expression at a concentration 10-fold and 4–5-fold less, respectively, than their Kd_is calculated using the lower [^{3}H] ORG 2058 free concentration and dexamethasone down-regulated PGR at concentrations which failed to displace [^{3}H] ORG 2058 from binding sites. Conversely, compared to its Kd_i for [^{3}H] ORG 2058 binding sites, a 50-fold higher concentration of ZK 98.299 was required to down-regulate PGR by 50%. ORG 2058 itself also required a 50-fold increase in Kd value to down-regulate PGR by 50%. In contrast there was reasonable agreement between EC_{50} and Kd_i for ZK 112.993.

Figure 4 shows the anti-proliferative effects of ligands towards ZR-PR-LT cells. MPA was the most effective agent, with the three anti-progestins RU 38.486, ZK 98.299 and ZK 112.993 being approximately equipotent. However, none of the agents

Table 1. Affinity of ligands for PGR and efficacy in down-regulating receptor

Drug	EC_{50}	Kd_i (1.4 nmol/l)	Kd_i (0.25 nmol/l)
		Kd of ORG 2058 for	
ORG 2058	15	PGR = 0.3 nmol/l	
MPA	0.12	0.04	1.2
RU 38.486	0.12	0.2	0.55
ZK 98.299	500	8.8	9.2
ZK 112.993	1.1	1.1	1.8
Dexamethasone	5.0	630	—

All values expressed as nmol/l concentrations. Apparent Kd_i values are given for free [^{3}H] ORG 2058 concentrations of 1.4 and 0.25 nmol/l.
EC_{50} = Concentration required to down-regulate PGR expression by 50% over 6 days. Kd_i = Kd for competitor calculated from the relationship $Kd_i = IC_{50}/1 + [(L)/Kd]$ where (L) = concentration of [^{3}H] ORG 2058 and IC_{50} = concentration required to compete for 50% of [^{3}H] ORG 2058 binding.

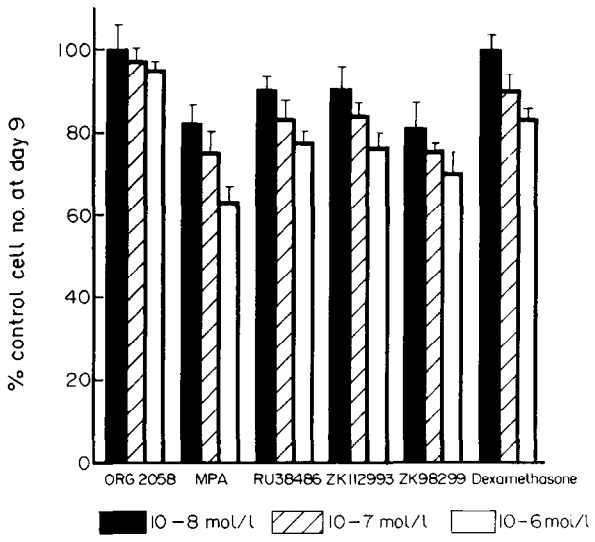

Fig. 4. Inhibition of ZR-PR-LT cell proliferation by progestins/antiprogestins and dexamethasone.

showed marked anti-proliferative activity within the concentration range studied, the maximum effect being a 40% reduction in cell number at day 9 following treatment with 10^{-6} mol/l MPA.

DISCUSSION

ZR-PR-LT cells, an oestrogen independent variant of the ZR-75-1 human breast cancer cell line, do not possess binding sites characteristic of type 1 ER but constitutively express PGR at a level similar to that achieved in the parent line following oestrogen priming [10]. In this respect this cell line resembles a variant of the T47D human breast cancer cell line [11] which has also been used to investigate PGR down-regulation without the need for prior oestrogen induction of receptor synthesis [12, 13]. To our knowledge, the data presented here are the first to correlate ligand affinity for PGR with potency in down-regulating receptor and inhibiting cell proliferation. The results obtained in this study for relative apparent affinities of ligands for PGR emphasise the need to use concentrations of radioactive ligand which will interact with one class of binding site only. At a free concentration of [^{3}H] ORG 2058 of 1.4 nmol/l, logit-log plots with slopes exceeding unity (Fig. 1b) indicated that competing ligands were displacing labelled radioactive ligand from more than one class of binding site. The ability of dexamethasone at concentrations of $< 10^{-8}$ mol/l to displace [^{3}H] ORG 2058 suggest that at this high free concentration [^{3} H] ORG 2058 was also interacting with the glucocorticoid receptor. This conclusion is supported by the observation that the apparent Kd_i for MPA, which has marked glucocorticoid activity, was 30-fold lower than that observed using 0.25 nmol/l [^{3}H] ORG 2058 (Table 1). RU 38.486, which has anti-glucocorticoid activity, also displayed a 2-fold higher apparent Kd_i at the higher free [^{3}H] ORG 2058 concentration.

Previous studies have shown that exposure of breast cancer cells in culture to the synthetic progestin R5020 results in rapid down-regulation of PGR [12, 13]. Maximum effects were observed within 12–30 h of exposure. In a series of time-course studies (data not shown) we have confirmed that PGR down-regulation in ZR-PR-LT cells is also rapid, with all ligands studied showing maximum effects within 4 h. This depression of PGR expression remained constant during 6 days continual exposure to ligand.

If down-regulation were mediated solely via interaction with PGR than a close correlation might be expected between affinity of ligand (Kd_i) for PGR and the concentration required to down-regulate receptor expression by 50% (EC_{50}). Table 1 shows that this correlation held true only for the anti-progestin ZK 112.993. EC_{50} values for both MPA and RU 38.486 were lower than their affinity for PGR and both compounds also possess glucocorticoid and anti-glucocorticoid activities, respectively. A most unexpected finding was the effectiveness of dexamethasone in down-regulating PGR at concentrations which fail to significantly interact with that receptor (Fig. 2a and Table 1). The high potency of dexamethasone, MPA and RU 38.486 in down-regulating PGR suggest that a degree of heterospecific down-regulation is occurring via ligand interaction with the glucocorticoid receptor, although we cannot exclude the possibility that PGR down-regulation by MPA and RU 38.486 may occur as a result of very low ligand occupation of PGR. It is also of interest that the 'pure' progestin (ORG 2058) and ZK 98.299, which has a 10-fold lower anti-glucocorticoid activity than RU 38.486 [14], both have EC_{50} values for receptor down-regulation some 50-fold higher than their respective Kd_is for PGR. It is possible that complete occupation of available PGR by 'pure' progestins/anti-progestins is necessary for receptor down-regulation, or that very low levels of interaction with the glucocorticoid receptor activates heterospecific down-regulation. These considerations do not, however, explain the discrepancies between relative Kd_is/EC_{50}s for ZK 98.299 and ZK 112.993. The latter compound also has low anti-glucocorticoid activity and exhibits similar anti-tumour activity to ZK 98.299 in murine models [7]. Our findings with these new anti-progestins are of interest in light of a recent report which showed that ZK 98.299 complexed to PGR failed to induce specific binding of PGR to progesterone response elements (PRE) located adjacent to target genes, in contrast to RU 38.486 and ZK 112.993 [15]. ZK 98.299 differs structurally from the latter agents in that the C and D steroid rings are fused in the *cis* rather than the *trans* configuration. It is possible that the low potency of ZK 98.299 in PGR down-regulation may be in part explained by receptor down-regulation being dependent on ligand-receptor binding to PRE. Differences in the ability of agonist or antagonist-complexed oestrogen receptor to bind to or activate oestrogen response elements have also been described [16].

The importance of ligand interaction with PGR as a mediator of the anti-proliferative effects of progestins and anti-progestins remains a matter of some controversy. MPA is the most commonly used agent clinically and *in vitro* studies have suggested that anti-proliferative effects are mediated primarily via the androgen or glucocorticoid receptor [1, 3]. Clinically, presence of PGR in the primary tumour has been reported to be a poor indicator of response to the synthetic progestin megestrol acetate [4]. Whilst MPA displayed the most potent anti-proliferative effect towards ZR-PR-LT cells (Fig. 3) only a 20% reduction in proliferative rate was observed at 10^{-8} mol/l MPA, a 10-fold higher concentration than the Kd_i for PGR, and 100-fold higher than the concentration required to down-regulate PGR by 50%. PGR activation, as reflected by receptor down-regulation, thus appears to be a poor indicator of the anti-proliferative effects of MPA. Similar arguments apply to the anti-progestin RU 38.486 (Fig. 3 and Table 1) although a number of studies have indicated that RU 38.486 does exert its antiproliferative and anti-tumour activity via PGR [5–7, 17]. However, it is noteworthy that in the

latter *in vitro* study [17], the breast cancer cell lines used were extremely sensitive to RU 38.486 with marked anti-proliferative effects being observed with concentrations as low as 10^{-10} mol/l. As we show in this and an earlier study [10], elevated PGR expression by ZR-PR-LT cells does not confer a marked increase in sensitivity to progestins/anti-progestins compared to the parent line.

The newer anti-progestins, ZK 98.299 and ZK 112.993 have similar anti-proliferative effects towards ZR-PR-LT cells as RU 38.486 despite their reduced anti-glucocorticoid activity. ZK 98.299 and ZK 112.993 also show a 5-fold difference in Kd_i for PGR but a 500-fold difference in EC_{50} values (Table 1). These very different biological properties are not reflected in differences in anti-proliferative activity in this *in vitro* system. In summary, ZR-PR-LT cells are a useful model for studying the correlation between ligand affinity for PGR and receptor activation as measured by receptor down-regulation. We show for the first time that PGR may be heterospecifically modulated via ligand interaction with the glucocorticoid receptor. In future studies with novel progestins/anti-progestins it should be borne in mind that potent PGR down-regulation may indicate interaction with the glucocorticoid receptor. Our model may also be useful in distinguishing between the different classes of anti-progestins proposed by Klein-Hitpass *et al.* [15]. Our data would be consistent with the proposal [3] that MPA does not exert its anti-proliferative effects primarily via PGR. However, our results also suggest that *in vitro* systems may not consistently reflect the anti-tumour activity *in vivo* of the newer anti-progestins [5–7].

1. Braunsberg H, Coldham NG, Leake RE, Cowan SK, Wong W. Actions of a progestogen on human breast cancer cells: mechanisms of growth stimulation and inhibition. *Eur J Cancer Clin Oncol* 1987, **23**, 563–571.
2. Sutherland RL, Hall RE, Pang GYN, Musgrove EA, Clarke CL. Effect of medroxyprogesterone acetate on proliferation and cell cycle kinetics of human mammary carcinoma cells. *Cancer Res* 1988, **48**, 5084–5091.
3. Poulin R, Baker D, Labrie F. Androgen and glucocorticoid receptor mediated inhibition of cell proliferation by medroxyprogesterone acetate in ZR-75-1 human breast cancer cells. *Breast Cancer Res Treat* 1989, **13**, 161–172.
4. Robertson JFR, Williams MR, Todd J, Nicholson RI, Morgan DAL, Blamey RW. Factors predicting the response of patients with advanced breast cancer to endocrine (Megace) therapy. *Eur J Cancer Clin Oncol* 1989, **25**, 469–475.
5. Schneider MR, Michna H, Nishino Y, El Etraby MF. Antitumor activity of the progesterone antagonists ZK 98.299 and RU 38.486 in the hormone dependent MXT mammary tumor model of the mouse and the DMBA- and the MNU- induced mammary tumor models of the rat. *Eur J Cancer Clin Oncol* 1989, **25**, 691–701.
6. Michna H, Nishino Y, Neef G, McGuire WL, Schneider MR. Progesterone antagonists — tumor-inhibiting potential and mechanism of action. *J Steroid Biochem Mol Biol* 1992, **41**, 339–348.
7. Michna H, Schneider MR, Nishino Y, El Etreby MF. The antitumor mechanism of progesterone antagonists is a receptor mediated antiproliferative effect by induction of terminal cell death. *J Steroid Biochem* 1989, **34**, 447–453.
8. Romieu G, Maudelonde T, Ulmann A, *et al.* The antiprogestin RU 486 in advanced breast cancer: preliminary clinical trial. *Bull Cancer* 1987, **74**, 455–461.
9. Klijn JGM, De Jong FH, Bakker GR, Lamberts SWJ, Rodenburg CJ, Alexieva-Figusch J. Antiprogestins, a new form of endocrine therapy for human breast cancer. *Cancer Res* 1989, **49**, 2851–2856.
10. Vandenberg HW, Martin J, Lynch M. High progesterone receptor concentration in a variant of the ZR-75-1 human breast cancer cell line adapted to growth in oestrogen free conditions. *Br J Cancer* 1990, **61**, 504–507.
11. Horwitz KB, Mockus MB, Lessey BA. Variant T47D human breast cancer cells with high progesterone receptor levels despite estrogen and antiestrogen resistance. *Cell* 1982, **28**, 633–642.
12. Nardulli AM, Katzenellenbogen BS. Progesterone receptor regulation in T47D human breast cancer cells: analysis by density labeling of progesterone receptor synthesis and degradation and their modulation by progestin. *Endocrinology* 1988, **122**, 1532–1540.
13. Read LD, Snider CE, Miller JS, Greene GL, Katzenellenbogen BS. Ligand-modulated regulation of progesterone receptor messenger ribonucleic acid and protein in human breast cancer cell lines. *Mol Endocrinol* 1988, **2**, 263–271.
14. Neef G, Beier S, Elger W, Henderson D, Weichert R. New steroids with anti-progestational and antiglucocorticoid activities. *Steroids* 1984, **44**, 349–372.
15. Klein-Hitpass L, Cato ACB, Henderson D, Ryffel GU. Two types of antiprogestins identified by their differential action in transcriptionally active extracts from T47D cells. *Nucleic Acids Research* 1991, **19**, 1227–1234.
16. Sabbah M, Gouilleux F, Sola B, Redeuilh G, Baulieu E-E. Structural differences between the hormone and antihormone estrogen receptor complexes bound to the hormone response element. *Proc Natl Acad Sci USA* 1991, **88**, 390–394.
17. Bardon S, Vignon F, Chalbos D, Rochefort H. RU486, a progestin and glucocorticoid antagonist inhibits the growth of breast cancer cells via the progesterone receptor. *J Clin Endocrinol Metab* 1985, **50**, 692–697.

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