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Estrogenic and Progestagenic Activities Coexisting in Steroidal Drugs: Quantitative Evaluation by *In Vitro* Bioassays With Human Cells

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The progestin-specific stimulation of alkaline phosphatase (AP) activity in cells of the T47D human breast cancer line was applied to the development of a sensitive microtiter plate bioassay for the quantitative evaluation of progestagenic and antiprogestagenic potencies of natural and synthetic compounds. Some of the steroids tested (viz. progesterone, medroxyprogesterone acetate, norethynodrel) behaved as full-agonists, capable of inducing AP activities to the same maximal levels (equal efficacy), while others (norethindrone, gestrinone, R5020, norgestrel, Org OD 14 and its 4-ene metabolite) behaved as partial agonists, eliciting lower maximal effects. Efficacy, EC_{50} values (concentrations at which they induce one-half of the maximal response) and "slope factors" serve to characterize agonistic effects. Relative progestagenic potencies among the full-agonists were evaluated by comparing EC_{50} concentrations. Several 19-nor synthetic progestins (norethynodrel, norethindrone, Org OD 14 and its 4-ene isomer, *dl*-norgestrel, *levo*-norgestrel, RU2323), but none of the tested progestins with the pregnane structure, showed intrinsic estrogenic activity, as evaluated by using a similar *in vitro* bioassay based on a previously reported estrogen-specific induction of AP in human endometrial adenocarcinoma cells of the Ishikawa Var-1 line. Maximal estrogenic effects of all the tested progestins with dual activity were as high as those of estradiol. However, these compounds widely varied in their EC_{50} values for estrogenic activity. Consequently, the *in vitro* bioassays can reveal differences in the ratio of progestagenic and estrogenic activities intrinsic to these compounds. The reduced capability of the partial agonists to exert progestagenic or estrogenic effects on AP expression may reflect an impeded, receptor-mediated action, a mechanism that would also account for their inhibitory effects on the induction of AP activity by full agonists. Partial progestagenic agonists were able to reduce the efficacy of a full agonist to their own partial maximal activity.

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INTRODUCTION

On the basis of the progestin-specific enhancement of alkaline phosphatase (AP) activity in T47D human breast cancer cells, recently reported by Di Lorenzo *et al.* [1], we have developed an *in vitro* bioassay for the measurement of progestagenic and antiprogestagenic activities. This simple, specific and sensitive method, which allows the analysis of concentration–response relations characterizing the effects of progestagenic compounds, is similar to a previously described assay for the measurement of estrogenic activities based on the estrogen-specific induction of AP in cells of the human endometrial adenocarcinoma line Ishikawa Var-1 [2–4].

Comparison of progestagenic effects of several synthetic progestins on T47D cells with their estrogenic effects on Ishikawa Var-1 cells, offered the opportunity to evaluate and compare relative progestagenic/estrogenic potencies of compounds possessing both activities.

MATERIALS AND METHODS

The *in vitro* assays for progestagenic and antiprogestagenic activities are based on the progestin-

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specific stimulation of AP activity in T47D cells distributed in a multiwell culture plate and exposed to test compounds at various concentrations for 72 h, followed by colorimetric measurement of *p*-nitrophenol (*p*NP) formed by hydrolysis of added *p*NP-phosphate (*p*NPP). This method is similar to *in vitro* bioassays, previously described in detail, for estrogenic and antiestrogenic activities based on the stimulation of AP in Ishikawa cells [2, 3].

T47D cells, kindly supplied by Dr L. Y. Murphy (Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada) were maintained in Dulbecco's Modified Eagle's medium (DMEM No. D7777, Sigma Chemical Co., St Louis, MO) supplemented with penicillin 100 U/l, streptomycin 100 μg/l (GIBCO laboratories, Grand Island, NY), and 10% fetal bovine serum (Hyclone Laboratories, Logan, UT). Cells were plated at 1.5×10^6 cells/tissue culture dish (100 × 20 mm, 64 cm^2; Falcon, Becton Dickinson & Co., Lincoln Park, NY) and replated weekly. Twenty four hours before the start of an experiment, when cells were near confluency, the medium was replaced by DMEM supplemented with antibiotics and 5% calf bovine serum (Cellect, ICN Biomedicals Inc., Costa Mesa, CA) treated with dextran-coated charcoal (ct-CS) for 30 min at 55°C to remove endogenous steroids as described previously [5]. On the day of the experiment, cells were harvested by using 0.25% trypsin and plated in 96-well flat-bottom Microtest III tissue culture plates (Falcon) at a density of 50,000/well in 100 μl aliquots.

Test compounds, e.g. progesterone (P), estradiol (E_2), diethylstilbestrol (DES), medroxyprogesterone acetate (6α-methyl, 17-hydroxy-progesterone) (MPA) and *levo*-norgestrel (4-estren-17α-ethynyl-18-homo-17β-ol-3-one) (*levo*-NG) were purchased from Sigma Chem. Co (St Louis, MO); norethynodrel [5(10)-estren-17α-ethynyl-17β-ol-3-one](NE), its 4-ene isomer (norethindrone, NET) and *dl*-NG were obtained from Steraloids Inc (Wilton, NH); Org OD 14[(7α 17α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one] and its 4-ene isomer (4-ene Org OD-14) were gifts from Organon International b.v. (Oss, The Netherlands); gestrinone (13β-ethyl-17α-ethyl-17-hydroxy-gona-4,9,11-triene-3-one) (R2323), promegestone (17,21-dimethyl-19-nor-4,9 pregnandiene-3,20-dione) (R5020) and mifepristone [17β-hydroxy-11β-(4-dimethylaminophenyl-17α-(1-propyl)-estra-4,9-dien-3-one] (RU486) were gifts from Roussel-Uclaf (Romainville, France). In preparation for assays, these compounds were dissolved in ethanol and diluted in DMEM–5% ct-CS to appropriate concentrations (0.1% final ethanol concentration). Fifty microliter aliquots were delivered to each well to obtain a final volume of 150 μl/well. After addition of test compounds, the cells were incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air for 72 h. At the end of the incubation period, growth medium was removed and the plates were rinsed by gentle immersion and swirling in 2 l of phosphate-buffered saline (0.15 M NaCl, 10 mM sodium phosphate, pH 7.4). The plates were transferred to another tray containing 2 l of phosphate-buffered saline and rinsed again. The buffered saline was shaken out and the inverted plates were blotted on a paper towel. The covers were replaced and the plates were kept at −80°C for at least a 15 min period followed by thawing at room temperatute for 10 min. The plates were then placed on ice and 50 μl of an ice-cold solution consisting of 5 mM *p*NPP (Sigma), 0.24 mM $MgCl_2$ and 1 M diethanolamine (pH 9.8) was added to each well. Formation of *p*NP at room temperature was monitored periodically at 405 nm in a Titertek Multi scan Plus MKII plate reader at various times, from 30 to 100 min. Semilogarithmic plots of optical density readings vs test compound concentrations were obtained by using a KaleidaGraph software. The same program generated values for maximal effects elicited by each compound, for "slope factors" [6] and for concentrations at which one-half of the observed maximal increase in AP activity was obtained (EC_{50} values).

Series of 3–5 experiments were carried out at appropriate concentrations (1–1000 pM for MPA and R5020; 1 or 5–5000 pM for NET, northynodrel, 4-ene Org OD 14, *levo*-NG and *dl*-NG; 10–10,000 pM for Org

Table 1. Progestagenic effects of steroidal progestins on AP activity in T47D cells

Compound	Relative maximal AP activity [mean ± SD (*n*) (conc.)]				EC_{50} (pM) [mean ± SD (*n*)]	"Slope factor". [mean]
MPA	100 ± 10	(18)	[1 nM]		150 ± 27 (7)	2.8 ± 1 0
Norethynodrel	102 ± 15	(18)	[5 nM]	(NS)	880 ± 150 (5)	3.0 ± 0.21
Progesterone	89 ± 20	(18)	[1 μM]	(NS)	25000 ± 11000 (3)	0.69 ± 0 061
Norethindrone	81 ± 11	(18)	[5 nM]	(*)	580 ± 86 (5)	2.8 ± 0.57
R5020	71 ± 5 3	(9)	[5 nM]	(*)	160 ± 37 (5)	3.0 ± 0.27
Org OD 14	70 ± 16	(18)	[10 nM]	(*)	1300 ± 280 (5)	2.5 ± 1 0
4-ene Org OD14	63 ± 14	(18)	[5 nM]	(*)	510 ± 50 (5)	2.1 ± 0 54
dl-Norgestrel	52 ± 11	(18)	[1 nM]	(*)	330 ± 19 (5)	3 1 ± 0.79
levo-Norgestrel	45 ± 11	(15)	[1 nM]	(*)	120 ± 18 (4)	2 5 ± 0.58
R2323	41 ± 15	(18)	[1 nM]	(*)	830 ± 300 (4)	1 6 ± 0.71

[*Test compound vs MPA $P < 0.001$, NS: not significant]

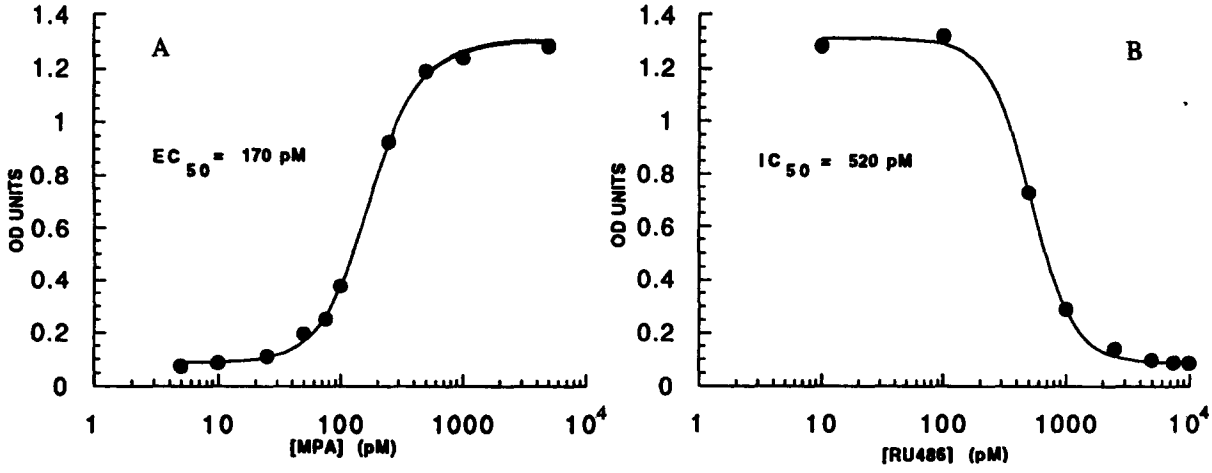

Fig. 1. (A) Concentration dependence of MPA effects on AP expression in T47D cells. (B) Concentration dependence of inhibitory effects of RU486 on AP induction by MPA (1 nM) in T47D cells.

OD 14 and R2323; 10 pM–5 μM for P), using 10 different dilutions for each assay. These experiments yielded values for maximal AP activities achievable with each compound (efficacy), EC_{50} values and "slope factors".

In order to reduce variability and obtain accurate data on relative efficacies, several drugs were tested in a single 96-well plate (a column of 8 wells for each compound and an appropriate blank). In order to assure a temporal linearity, the color development period during the hydrolysis of *p*NPP was limited to the time when the optical density corresponding to the wells with the highest concentration for MPA reached 1.2–1.4 OD units [2]. These single plate experiments were replicated 18 times in order to allow for statistical analyses by paired Student's *t*-test, performed with the True Epistat computer program (Epistat Services, Richardson, TX).

A similar test was performed with the same compounds and Ishikawa Var-1 cells, in order to determine their relative maximal estrogenic effect on AP activity. Methodology concerning the use of Ishikawa Var-1 cells for the measurement of estrogenic effects has been described in a recent publication [3], which also presented EC_{50} values corresponding to the estrogenicity of several progestins but did not include data on maximal effects.

RESULTS

Progestagenic activities

Table 1 shows relative values for maximal progestagenic effects of various progestins on AP activity in T47D cells, as determined in multiple experiments performed in parallel. The concentrations used to measure maximal effects for each drug were chosen on the basis of data from concentration dependence curves generated during the determination of EC_{50} values, as illustrated for MPA in Fig. 1(A). Statistical analysis by paired Student's *t*-test indicates that MPA, norethynodrel and P are full agonists in this system. In contrast, the maximal effects induced by NET and the 4-ene metabolite of Org OD 14, which is more progestagenic than Org OD 14, are significantly lower than the MPA effect (Table 2).

Table 1 also includes EC_{50} concentrations and "slope functions" determined from measurements of responses elicited by the same compounds tested at various concentrations. As evident from these data, the synthetic progestins tested have EC_{50} values which are much lower that the EC_{50} of progesterone.

Estrogenic activity of progestins

Table 3 presents data, obtained by measuring AP activity in Ishikawa Var-1 cells, on maximal estrogenic

Table 2. Statistical significance (paired Student's t-test) on maximal AP activies in T47D cells

	MPA	P	NE	NET	OD14	R5020	4-ene OD14	*dl*-NG	*l*-NG	R2323
MPA	•	NS	NS	xxx	xxx	xxx	xxx	xxx	xxx	xxx
P		•	NS	NS	xxx	NS	xxx	xxx	xxx	xxx
NE			•	xxx	xxx	NS	xxx	xxx		xxx
NET				•	xxx	NS	xxx	xxx		xxx
Org OD-14					•	NS	xxx	xxx	xxx	xxx
R5020						•	NS	xxx		xxx
4-ene Org OD-14							•	x		xxx
dl-NG								•		x
levo-NG									•	NS

***$P < 0.001$, *$P < 0.01$

Table 3. Estrogenic effects of some steroidal progestins on AP activity in Ishikawa Var-1 cells

Compound	Relative maximal AP activity [(mean ± SD)(*n*) (conc.)]		EC_{50} [mean ± SD]	"Slope factor" [mean ± SD]
Estradiol	100 ± 3.0 (8)	[1 nM]	8.7 ± 4.2 pM	0 86 ± 0.18
Org OD-14	105 ± 14 (8)	[100 nM]	7 0 ± 3 5 nM	1.2 ± 0 19
Norethynodrel	102 ± 9.5 (8)	[1 μM]	14 ± 3 2 nM	1.4 ± 0.17
4-ene Org OD-14	98 ± 13 (8)	[1 μM]	59 ± 9.8 nM	1.3 ± 0 32
R2323	98 ± 13 (8)	[1 μM]	140 ± 54 nM	1.7 ± 0.27
Norethindrone	96 ± 13 (8)	[1 μM]	200 ± 44 nM	1.5 ± 0 23
levo-Norgestrel	102 ± 6.4 (4)	[100 nM]	0 84 ± 0.19 μM	1.4 ± 0 26
dl-Norgestrel	89.3 ± 3.1 (4)	[7 5 μM]	2.9 + 0.7μM	1 2 + 0 26

effects relative to E_2, EC_{50} values and "slope factors" of various progestins.

Maximal estrogenic activities were determined in parallel in multiple separate experiments at the concentrations listed in Table 3, found in preliminary studies to be sufficiently high for maximal stimulation. The maximal effect obtained with E_2 was not significantly different from that obtained with the progestins tested.

In contrast, the estrogenic potencies of the tested progestins varied considerably. For instance, *levo*-NG was considered to be 2- to 3-fold as potent as *dl*-NG as an estrogen since these 2 compounds have approximately equal efficacies and the EC_{50} of *levo*-NG was only one-third to one-half the EC_{50} for *dl*-NG in the *in vitro* bioassay.

Concurrent estrogenic-progestagenic activities in synthetic steroids

On the basis of the data shown in Tables 1 and 3, the estrogenic and progestagenic activities of these compounds can be compared to those of E_2 and MPA, respectively, considering both the maximal effects on AP activity they can elicit (efficacy) and their EC_{50} values. In fact, the similarity in the estrogenic efficacy of the 19-nor type of synthetic progestins tested, shown in Fig. 2, allows the estimation of their relative estrogenic potencies, based on the ratio of their EC_{50} values. Table 4 shows potencies relative to E_2 of the tested compounds. These data reveals a wide range of potential estrogenicity of synthetic progestins.

Figure 2 also shows that, in contrast with their full estrogenic efficacy, many of the synthetic progestins tested behave as partial progestagenic agonists. Interestingly, none of the C_{21} steroids with the pregnane structure characteristic of progesterone tested in these studies failed to show any estrogenic activity.

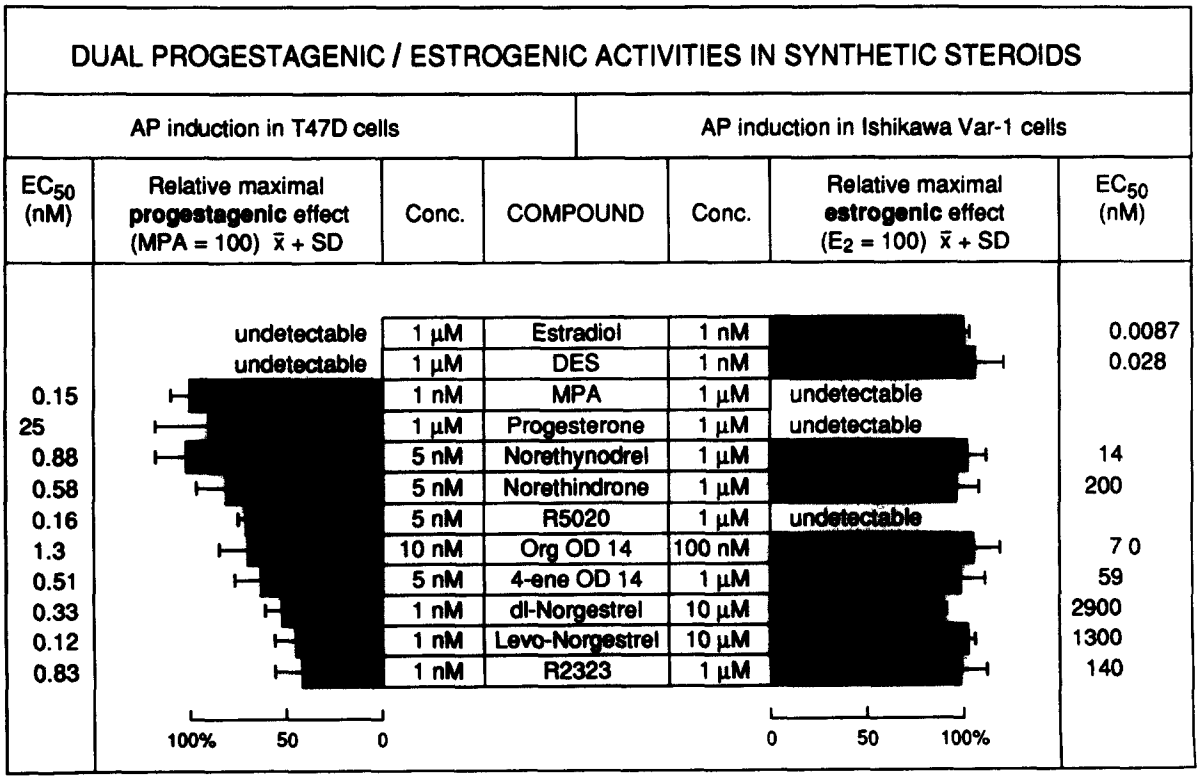

Fig. 2. Progestagenic and estrogenic effects of various progestins

Table 4. Estrogenic potencies of synthetic progestins relative to E_2

Compound	Estrogenic potency relative to E_2 (%)
Org OD 14	0.12
4-ene isomer of Org OD 14	0 015
Norethynodrel	0.062
Norethindrone	0.0043
R2323	0.0062
levo-Norgestrel	0.00103
dl-Norgestrel	0.00030

In vitro testing for antiprogestagenic activity

Figure 1(B) shows inhibition by RU486, an antiprogestin devoid of agonist activity, of the effects of MPA (1 nM) on AP activity in T47D cells. One half of the activity was inhibited at a 520 pM concentration of RU486 (IC_{50}) and complete inhibition could be achieved by using sufficiently high levels of the inhibitor.

Since the stimulation of AP activity by progestins in T47D cells is considered to be P receptor-mediated, the maximal effect obtainable with a mixture of a full agonist with a partial agonist at a concentration sufficiently large to prevent the binding of the full agonist to the receptor, would be expected to equal the maximal effect of the partial agonist. Such possibility is supported by the results shown in Fig. 3, which illustrates the effect on AP activity of MPA (1 nM), alone or in the presence of 100-fold molar excess of a partial agonist (Org OD 14, its 4-ene-metabolite, R2323, or *dl*-NG).

DISCUSSION

As evident from the data shown in Tables 1 and 3, graphically summarized in Fig. 2, the synthetic 19-nor progestins tested demonstrated to possess intrinsic estrogenic and progestagenic capabilities. Since these actions are receptor mediated, as evident from the inhibitory effects of antiestrogens and antiprogestins, these compounds can simultaneously act as progestins and estrogens activating both the estrogen receptor (ER) and the progesterone receptor (PR). Consequently, the overall effect of these drugs in systems responsive to both activities may reflect both estrogenic and progestagenic effects, which may be either antagonistic or synergistic. For instance, the overall effect that a drug possessing both potential activities may have on prostaglandin production by human endometrial cells, known to be enhanced by estrogens and diminished by progestins [7, 8], might be determined by the balance of its relative estrogenic and progestagenic potencies. Furthermore, its effects on receptor levels may be complex, as progestins reduce ER levels while estrogens increase PR concentrations.

Whether the overall effect of a drug possessing both activities is estrogenic or progestagenic may be predictable from the relative EC_{50} values corresponding to each of these activities. Thus, the EC_{50} for the progestagenic activity of the tested 19-nor progestins is much smaller than the EC_{50} for their estrogenic activity. Similarly, ethynylestradiol, an obvious estrogen, has a EC_{50} value for its estrogenic activity in the induction of AP activity in Ishikawa cells that is much lower than the EC_{50} for its progestagenic activity in the T47D system (data not shown). Further characterization of the agonistic actions is provided by "slope factors" [6]

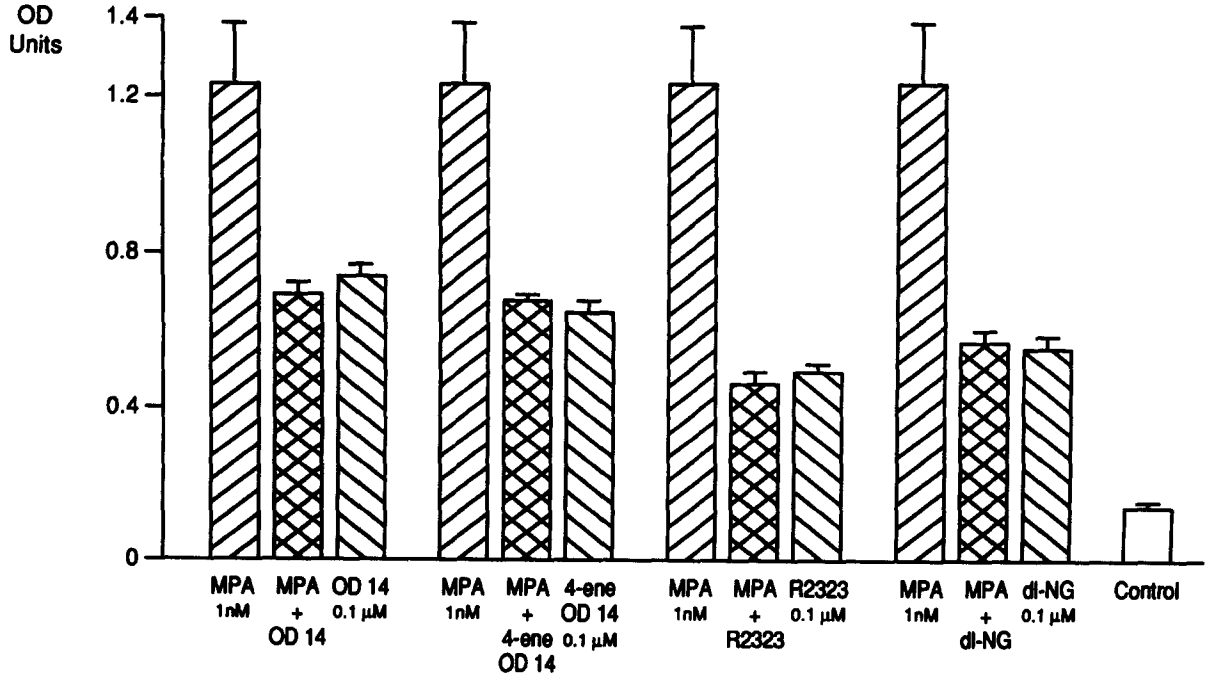

Fig. 3. Antagonistic effects of various progestagenic partial agonists on the effects of a full agonist (MPA 1 nM).

generated by the analysis of concentration–response curves and are also included in Tables 1 and 3.

Possible effects of the estrogenic components of progestins used for contraception and hormonal replacement therapy during menopause are of obvious importance. Jeng *et al.* [9] have reported that norethynodrel, NET and *levo*-NG stimulate proliferation of ER^+ cells but not of ER^- cells and showed that this effect can be blocked by antiestrogens but not by the antiprogestin RU486. On the basis of these results they suggested that the estrogenic activity detectable in 19-nortestosterone derivatives, but not in MPA or R5020 could account for the conflicting evidence linking breast cancer and the use of oral contraceptives.

Various *in vitro* approaches to the evaluation of estrogenic and progestagenic activities have been investigated in many laboratories as they offer the possibility of avoiding the use of animals and their relative simplicity and accuracy. One example of such approaches was provided by Shapiro *et al.* [10] who used fragments of human endometrium to compare potencies of synthetic progestins by measuring their effects on glycogen production and to evaluate relative affinities of the drugs for the PR. These authors also emphasized the advantage of using human tissue to obtain information of plausible relevance to clinical implications.

A feature of the *in vitro* bioassays that we are now reporting is that metabolic modifications of the tested compounds by enzymatic systems present in the T47D and the Ishikawa cells may mimick to some extent metabolic processes affecting steroidal drug effects in the human target tissues (breast and endometrium) from which those cells were originally derived by neoplastic transformation.

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