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Effects of Two Classes of Progestagens, Pregnane and 19-Nortestosterone Derivatives, on Cell Growth of Human Breast Tumor Cells: I. MCF-7 Cell Lines

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The effects of two classes of progestagens, e.g. pregnane [Org 2058, medroxyprogesterone acetate (MPA), R5020, progesterone (PROG)] and 19-nortestosterone derived progestagens [3-ketodesogestrel (KDG), levonorgestrel (LNG), gestodene (GES), norethisterone (NE), Org 30659] on proliferation of three estradiol (E_2)-dependent human breast tumor MCF-7 cell lines of different origin [Van der Burg (B), Litton bionetics (L) and McGrath (M)] were studied. The pregnane derivatives hardly stimulated cell growth at 10^{-6} M in MCF-7 B and L cells except for Org 2058 in B cells, whereas in M cells a statistically significant growth induction was observed except for PROG. The 19-nortestosterone derivatives induced cell growth at doses at 10^{-7} M or higher in all three cell lines. NE, GES and Org 30659 were more potent stimulators than KDG and LNG at 10^{-7} M. E_2 already showed maximal stimulation at 10^{-10} M. For all three cell lines, the effects and ranking of the individual progestagens were similar. Antiprogestagens, like RU 38486 and Org 31710 could not block these stimulatory effects while antiestrogens like 4-hydroxytamoxifen and ICI 164,384 could. This suggests that cell growth by the above-mentioned progestagens occurs via an interaction with the estrogen receptor. Indeed, displacement studies with cytosol from MCF-7 M cells revealed that at very high concentrations NE, GES and Org 30659 were able to displace 50% of the radiolabelled E_2, while KDG and LNG could not. Relative binding affinities (RBAs) were 0.010, 0.025 and 0.015% for NE, GES and Org 30659, respectively. The effect of the two classes of progestagens on cell proliferation was also investigated at several dose levels in combination with E_2 (10^{-10} M) in the MCF-7 B cell line. This resulted in a statistically significant inhibition of cell growth with R5020, MPA and most of the 19-nortestosterone derivatives at concentrations of 10^{-8} M. Org 2058 and NE did not have any influence on E_2-induced growth. The inhibitory effects could not be blocked by antiprogestagens. In summary these studies with 3 subclones of MCF-7 cells show that the pregnane derived progestagens stimulate growth only in one subclone, whereas the 19-nortestosterone derived progestagens do so in all three subclones. The progestagens possess estrogenic activity only at high pharmacological doses, being 10,000 times weaker than estradiol. In combination with estrogens most progestagens gave a reduction of E_2-stimulated growth in the B subclone.

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INTRODUCTION

An association between breast tumor development and reproductive hormones has been suspected since in 1896 Beatson discovered the beneficial effect of ovariectomy in some patients with metastatic breast cancer [1]. Nowadays, it is well known that estrogens promote the growth of breast tumor cells [2]. On the other hand, the involvement of progestagens, alone or in combination with estrogens, on the development, growth and differentiation of breast tumor cells has only recently been described in rats pretreated with 7,12-dimethylbenzanthracene [3]. For humans, data from Anderson *et al.* [4] even implied that progestagens rather than estrogens induced cell proliferation of breast tumor cells. A recent epidemiological review [5], however, showed no consistent evidence for the association of progestagens and breast cancer.

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Whether compounds may have a stimulatory or inhibitory effect on breast cancer development or growth is often evaluated in studies with established breast cancer cell lines, like MCF-7, T47D and ZR-75-1. Under optimal culture conditions an overwhelming amount of evidence with these estrogen responsive cell lines is obtained, whereby estradiol (E_2) induces a dose-dependent increase in cell proliferation [6–15]. This enhancement of growth can be inhibited by antiestrogens, such as 4-hydroxytamoxifen and ICI 164,384 [15, 16]. Regulation of growth of human breast tumor cells by progestagens is still controversial. Both growth stimulation, inhibition and no effect at all were found in a variety of studies. These differences depend among others on the kind of cell line used, the chosen cell culture conditions, selection of specific cell clones, the choice of sera and sera conditions as well as on the presence or absence of phenol red in the culture medium. Some synthetic progestagens alone are able to stimulate cell proliferation between 10^{-9} and 10^{-6} M in MCF-7 [10, 17, 18], T47D [19–21] and ZR-75-1 cells [12], whereas in combination with E_2, progestagens are able to inhibit cell growth at 10^{-9} M in T47D [7–9, 14] and ZR-75-1 cells [12]. In addition to these findings, Coletta *et al.* [22, 23] demonstrated a specific GES binding protein in MCF-7 and T47D cells. This protein was suggested to be involved in the reduction of cell growth by the synthetic progestagen gestodene (GES), but not with others like 3-ketodesogestrel (KDG), R5020 and medroxyprogesterone acetate (MPA).

In the present study the above mentioned controversial data have been reanalyzed under standardized conditions in three MCF-7 cell lines of different origin, while more synthetic compounds were included to demonstrate whether the pregnane and 19-nortestosterone derivatives differed in activation and/or inhibition of MCF-7 cells. For the class of pregnanes Org 2058, progesterone (PROG), R5020 and MPA were used, while for the class of 19-nortestosterone derivatives norethisterone (NE), levonorgestrel (LNG), KDG, GES and Org 30659 were included. Three MCF-7 cell lines of different origin, i.e. Van der Burg *et al.* [6] (B; passage number 330), Litton Bionetics (L; passage number 138) and McGrath (M; passage number 326), were used and cultured under similar conditions in phenol-red free medium with dextran-coated charcoal treated serum in the presence or absence of E_2 and the above mentioned progestagens.

EXPERIMENTAL

Materials

The following steroids E_2, Org 2058, PROG, R5020, MPA, NE, LNG, KDG, GES, Org 30659 and antiprogestagens, RU 38486 (Mifepristone) and Org 31710 were obtained from N.V. Organon (Oss, The Netherlands). The antiestrogens, 4-hydroxytamoxifen and ICI 164,384 were kindly provided by Dr A. E. Wakeling, Zeneca Pharmaceuticals (Macclesfield Cheshire, U.K.). The chemical structures of the pregnane and 19-nortestosterone derivatives are given in Fig. 1. Dulbecco's Modified Eagles Medium/nutrient mixture F-12 (DMEM/HAM F12 medium in a ratio of 1:1) was obtained from Gibco (Paisley, U.K.), fetal bovine calf serum from Bocknec (Ontario, Canada), 24 well plates from Nunc (Roskilde, Denmark). [2,4,6,7,16,17-^{3}H]E_2 (sp.act. 5.81×10^3 GBq/mmol) and [6,7-^{3}H]Org 2058 (sp.act. 1.7×10^3 GBq/mmol) was obtained from Amersham (Amersham, U.K.) and [1,2,4,5,6,7-^{3}H]5α-dihydrotestosterone (5α-DHT) (sp.act. 4.07×10^3 GBq/mmol) (NEN, U.K.). All other chemicals were of analytical grade.

Cell culture

Three sub cell lines of MCF-7 cells were used in the experiments. The first MCF-7 (B) cell line was kindly provided by Dr B. Van der Burg (Hubrecht Lab., Utrecht, The Netherlands), who obtained it from Dr C. Quirin-Stricker (Institut de Chimie Biologique, Faculté de Médicine, Strasbourg, France). The second cell line (L) was obtained from Litton Bionetics (American Type Culture Collection, low passage of McGrath cell line) and the third cell line (M) from Dr M. McGrath (Michigan Cancer Foundation, U.S.A.). The passage numbers were respectively 330, 138 and 326. The cells were cultured under a humidified atmosphere of air/5% CO_2 (normoxia) at 37°C in 80 cm^3 sealed polystyrene flasks on phenol-red free DMEM/HAM F12 medium (PFM) supplemented with 5% fetal calf serum (FCS). The cells were passaged once a week with trypsin and EDTA. Cultures were free of mycoplasma contamination, as checked at regular intervals.

Experimental protocol

MCF-7 cells were seeded in 24 well plates of polysterene (3×10^3 cells/well) and cultured under normoxia at 37°C over a period of 9 days on PFM supplemented with 10% dextran-coated charcoal treated fetal calf serum (CTS). After 2 days the media were changed with PFM supplemented with CTS in the presence or absence of the appropriate steroid concentrations. All steroid predilutions were made in ethanol. The final ethanol concentration in medium never exceeded 0.2%. After 5 and 7 days, PFM and CTS with or without steroids was again changed by media of the same constitution. After 9 days the media were removed and cells were stored at −70°C until DNA measurements were performed. The total DNA content was assessed by fluorescent staining with Hoechst 33342 in a Perkin-Elmer luminescence spectrometer model LS 50 [24, 25]. During these experiments both estrogen-treated and untreated cells showed continuous logarithmic cell growth during the

whole experiment for all three cell lines. The cells did not reach confluency in these experiments.

Relative binding analysis of progestagens to estrogen receptor (ER), androgen receptor (AR) and progestagen receptor (PR)

MCF-7 (M) cells were used for the estimation of the relative binding affinity (RBA) with the above mentioned steroids for ER, AR and PR. Hereto MCF-7 cells were cultured in PFM and 5% FCS, followed by culturing in cell factories for 8 days on PFM and 10% CTS (for ER and AR) or one day (for PR) before cells were harvested by trypsin treatment. After centrifugation at 8,000 N/kg and removal of the medium, 1 g of cells was resuspended in 5 ml of TEDMS buffer [Tris–HCl (10 mM); pH 7.4, supplemented with EDTA (1 mM), 1,4-dithioerythritol (1 mM), 10 mM sodium molybdate hydrate (10 mM) and sucrose (250 mM)] and stored at −70°C until further use. Cytosol was prepared by homogenization of the cell

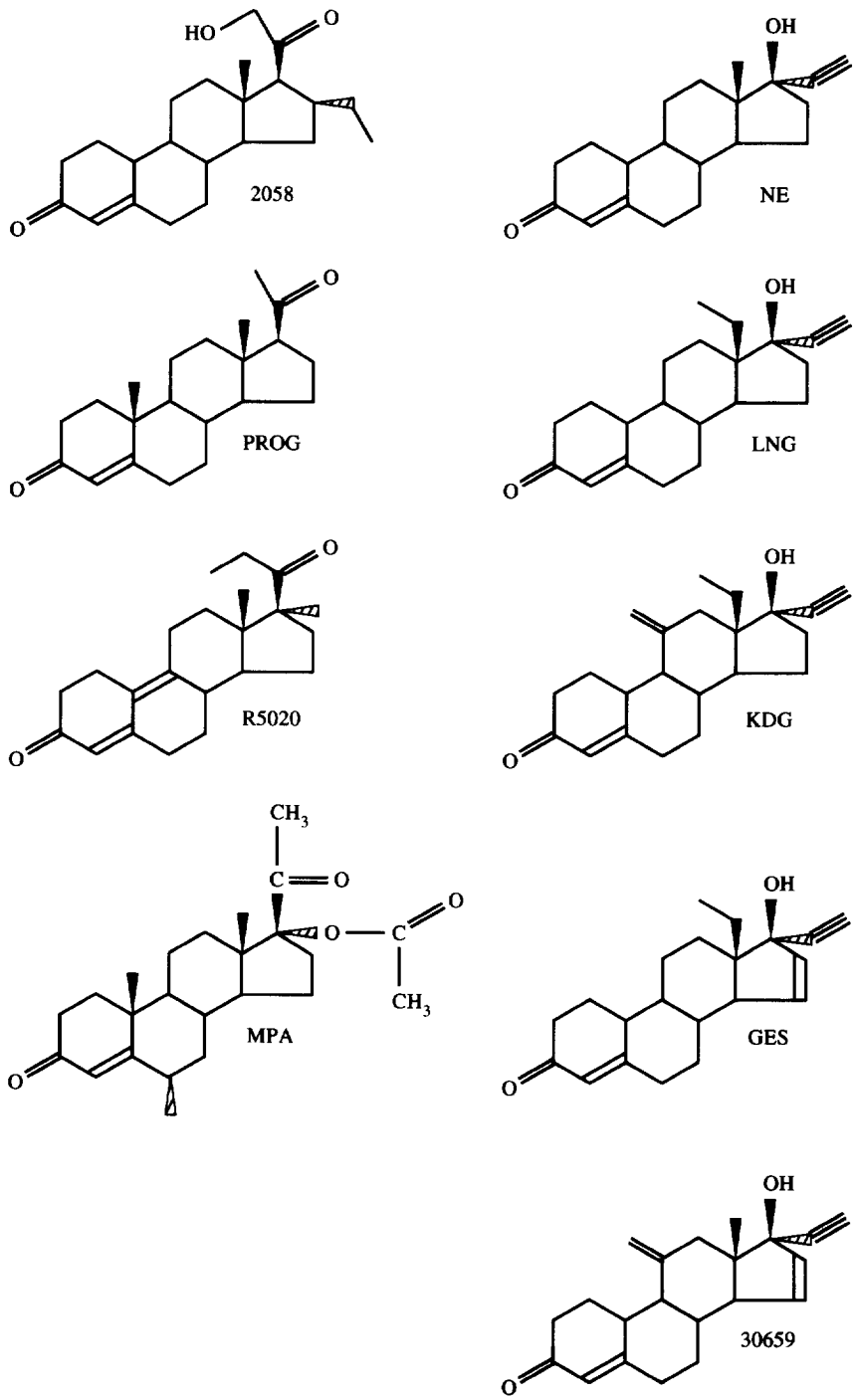

Fig. 1. Steroid structures of pregnanes (left), i.e. ORG 2058, PROG (progesterone), R5020 (promegestone) and MPA (medroxyprogesterone acetate); and 19-nortestosterone derived progestagens (right), i.e. NE (norethisterone), LNG (levonorgestrel), KDG (3-ketodesogestrel), GES (gestodene) and ORG 30659.

fraction in a Dounce all-glass homogenizer, followed by centrifugation at 1,000,000 N/kg for 45 min at 4°C in a Sorvall centrifuge, type OTD 50. The supernatant was decanted and diluted to a final w/v ratio of 1/10 for ER, 1/5 for AR and 1/25 for PR. This cytosol was used for overnight competition binding assays at 4°C with [^{3}H]E_2 (1.0 nM), [^{3}H]5α-DHT (1.9 nM) and [^{3}H]Org 2058 (1.9 nM) in the presence of test and reference compounds (10^{-10}–10^{-5} M). Assays were terminated by the addition of cold dextran-coated charcoal solution (0.25% Norit A and 0.025% dextran T-70) in TEDMS buffer for 10 min at 4°C, followed by centrifugation for 5 min at 8,000 N/kg in a Sorvall centrifuge, Type RC-3, to separate bound from free hormone. Finally, the supernatant was taken for quantification of the bound hormone in a Packard tricarb scintillation counter, model 2450. IC_{50} values were calculated after logit transformation.

Statistical analysis of the experiments with cell growth

The present studies were performed in two independent blocks. For each treatment and block the geometric mean of four measurements was calculated. Statistical analysis of these geometric means was carried out with an ANOVA for a randomized block design with the logarithmic transformed geometric mean per treatment and block as response variable. For each treatment with test compound, the ratio of the geometric mean of test compound over that of the control level or reference compound with 95% confidence intervals was calculated. Afterwards the ratio was multiplied by the geometric mean (μg DNA/ml) of the control level or reference compound. Test compounds for which the calculated interval does not include the geometric mean of the control level or reference compound are statistically different ($P < 0.05$) from the control level or reference compound. In the figures the geometric means (μg DNA/well) are given together with the recalculated confidence limits (μg DNA/well).

RESULTS

Statistical analysis

In Table 1 a representative example of the used calculation procedure is only given for E_2, Org 2058 and NE in two independent assays (blocks). As shown for E_2, Org 2058 and NE, the geometric mean at each concentration within one test has SD values ranging between 1 and 21%. Although the growth response rates between experiments I and II varied considerably, the SEM of the overall geometric mean was only 12.5%, as was calculated in the ANOVA for a randomized block design. This ANOVA was chosen as statistical analysis in order to correct for block effects in the responses of the two independent experiments, leading for both experiments to the calculation of an overall mean growth potency in comparison to the control and overall 95% confidence intervals. In the figures the mean geometric mean is presented together with the recalculated confidence intervals (μg DNA/well) as described in the statistical procedure. These results are also presented in the figures in which the experimental data are depicted. In Table 1 the growth ratios for several compound concentrations of the individual experiments are also presented. These values show that the relative responses in the two experiments are very similar. The mean of these values is mainly given by other investigators. When the potencies as calculated by ANOVA are compared with the mean growth ratio (see Table 1) the end result is very similar, although the variation as calculated in ANOVA is larger.

Effect of progestagens alone on cell growth

As shown in Figs 2–4 almost similar results were obtained for all three MCF-7 (B,L,M) sublines used. E_2 enhanced growth after 7 days of steroid treatment at approximately 10^{-12} M with a maximal effect at 10^{-10} M in all three cell lines. In all three cell lines, the 19-nortestosterone derivatives NE, GES and Org 30659 appeared to be more potent in growth stimulation than KDG and LNG at a concentration of 10^{-7} M, while all compounds show growth stimulating effects at 10^{-6} M. In both B and L cells, none of the pregnanes used induced cell proliferation at concentrations of 10^{-6} M with exception of Org 2058 in B cells. In M cells, Org 2058, R5020 and MPA showed a modest, but statistically significant, activation at 10^{-6} M. The estrogenic activity in this respect of the 19-nortestosterone and some pregnane derivatives is at least 10,000 times weaker than that of E_2.

In order to study whether these progestagens mediated their effects on cell growth via PR and/or ER, specific antiprogestagens, i.e. Org 31710 with low antiglucocorticoid activity and RU 38486 with high antiglucocorticoid activity, as well as specific antiestrogens, i.e. 4-hydroxytamoxifen and ICI 164,384, were tested. These experiments were only performed with MCF-7 (B) cells. The antiprogestagens Org 31710 and RU 38486 hardly influenced the effect on growth of any progestagen (Fig. 5). RU 38486 and Org 31710 alone showed a tendency to increase cell growth, but these increases were not statistically significant. Both antiestrogens at a concentration of 10^{-8} M could only partially inactivate the growth induced by KDG and LNG (10^{-6} M), while even at these levels no reduction in cell proliferation was found with GES, Org 30659, NE (10^{-6} M) and E_2 (10^{-10} M) (Fig. 6). Both antiestrogens, at a concentration of 10^{-7} M, could completely inhibit the growth stimulatory effects of E_2 (10^{-10} M) and the 19-nortestosterone derivatives (10^{-6} M) (Fig. 7).

Effects of progestagens on cell growth in combination with E_2

The effects of progestagens on E_2-induced cell growth at 10^{-10} M were examined, since combined

Table 1. Dose-dependent induction of growth proliferation of MCF-7 cells by E_2, Org 2058 and NE is given as the mean amounts of DNA ± SD (also in %) (μg DNA/well) (n = 4), growth ratio values (%) for two independent experiments as well as their mean growth ratio (%). From the mean of the log transformed data of both experiments the geometric mean ± SEM (also in %) is recalculated and compared with the geometric mean of the control values without steroid treatments, leading to potency values with 95% confidence intervals and recalculated "95% confidence intervals" in μg DNA/well

	MCF-7 (B) cells					ANOVA results		
	Experiment I		Experiment II					
	Geometric mean DNA ± SD (n = 4) (μg DNA/well)	Growth ratio (%)	Geometric mean DNA ± SD (n = 4) (μg DNA/well)	Growth ratio (%)	Mean growth ratio ± SD (n = 2) (%)	Geometric mean DNA ± SEM (n = 2) (μg DNA/well)	Potency (%) with 95% confidence intervals	Geometric mean with 95% confidence intervals (μg DNA/well)
Control	3.23 ± 0.02 (1%)		10.16 ± 0.27 (3%)			5.73 ± 0.72 (12.5%)		
Estradiol								
10^{-14} M	2.50 ± 0.10 (4%)	77	9.40 ± 0.45 (5%)	93	85 ± 11.3	4.85 ± 0.61 (12.5%)	85 (59–120)	4.85 (3.38–6.88)
10^{-13} M	2.95 ± 0.21 (7%)	91	11.05 ± 0.57 (5%)	109	100 ± 12.7	5.71 ± 0.72 (12.5%)	100 (70–142)	5.71 (4.01–8.14)
10^{-12} M	5.05 ± 0.74 (15%)	156	18.38 ± 1.28 (8%)	180	168 ± 17.0	9.62 ± 1.21 (12.5%)	170 (120–240)	9.62 (6.88–13.75)
10^{-11} M	13.68 ± 2.04 (15%)	423	37.96 ± 3.30 (11%)	372	398 ± 36.0	22.74 ± 2.86 (12.5%)	400 (280–570)	22.74 (16.04–32.66)
10^{-10} M	13.94 ± 2.28 (16%)	431	44.61 ± 5.02 (14%)	436	434 ± 3.5	24.85 ± 3.12 (12.5%)	430 (300–620)	24.85 (17.19–35.52)
10^{-9} M	12.23 ± 1.08 (9%)	378	36.12 ± 4.52 (14%)	355	367 ± 16.3	21.01 ± 2.64 (12.5%)	370 (260–520)	21.01 (14.90–29.80)
Org 2058								
10^{-8} M	4.34 ± 0.31 (7%)	134	10.55 ± 0.31 (3%)	104	119 ± 21.2	6.77 ± 0.85 (12.5%)	118 (83–168)	6.77 (4.76–9.63)
10^{-7} M	4.25 ± 0.21 (5%)	131	11.10 ± 0.26 (2%)	109	120 ± 15.6	6.87 ± 3.49 (12.5%)	120 (84–171)	6.87 (4.81–9.80)
10^{-6} M	6.49 ± 1.30 (20%)	201	21.36 ± 1.43 (8%)	210	206 ± 6.4	11.76 ± 1.48 (12.5%)	210 (140–290)	11.76 (8.02–16.62)
NE								
10^{-8} M	3.28 ± 0.28 (8%)	101	12.51 ± 0.83 (7%)	123	112 ± 15.6	6.41 ± 0.81 (12.5%)	112 (78–159)	6.41 (4.47–9.11)
10^{-7} M	9.95 ± 0.79 (8%)	308	24.62 ± 1.68 (7%)	243	278 ± 46.0	15.65 ± 1.96 (12.5%)	270 (190–390)	15.65 (10.89–22.35)
10^{-6} M	11.84 ± 2.53 (21%)	366	32.15 ± 4.25 (13%)	316	341 ± 35.4	19.51 ± 2.45 (12.5%)	340 (240–480)	19.51 (13.75–27.50)

SEMs and confidence intervals are based on the ANOVA estimated coefficient of variation = 17.8% (degrees of freedom = 60).
The results are also given in Fig. 2.

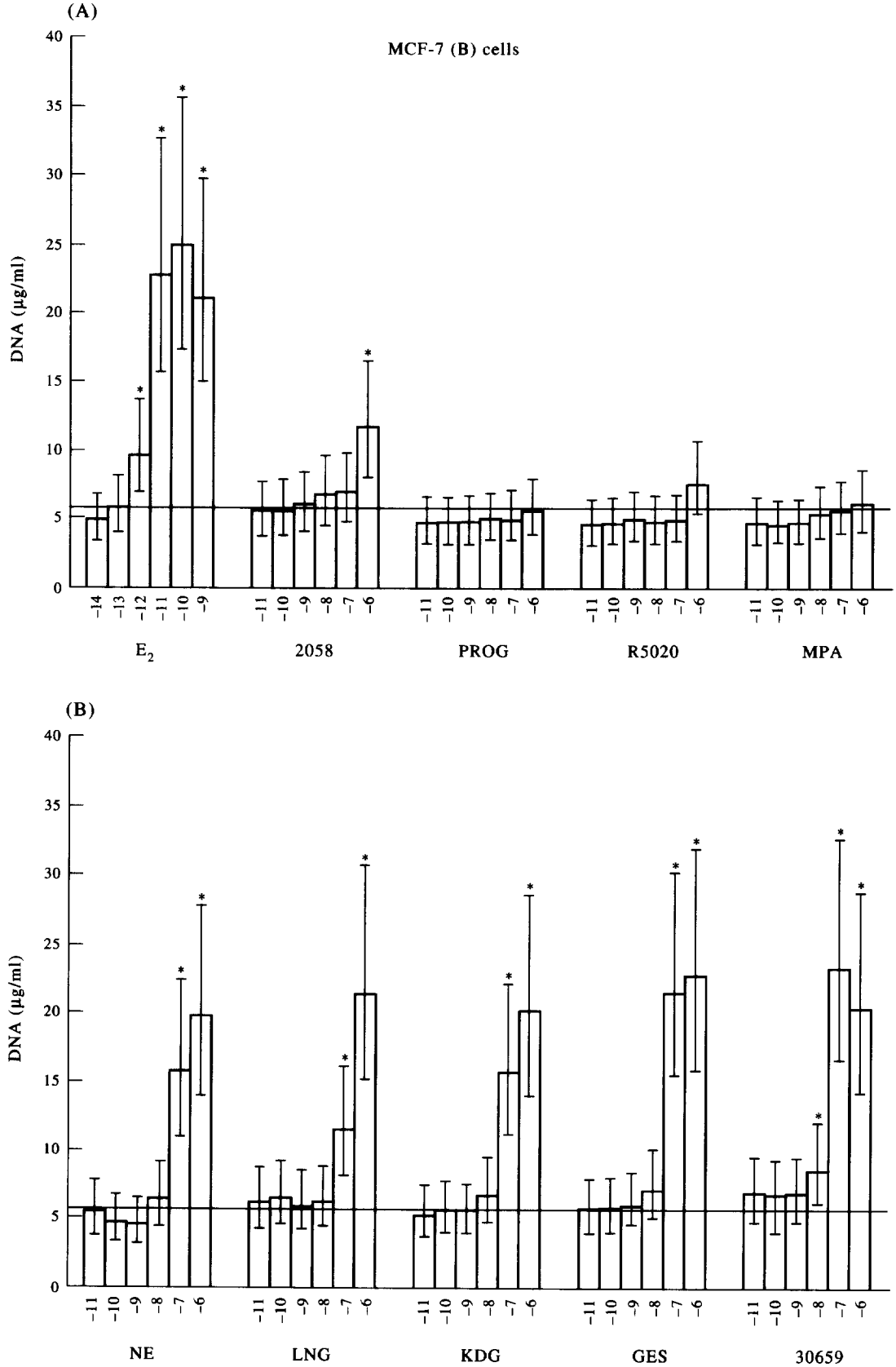

Fig. 2. The effect of E_2 and several progestagens on cell growth of MCF-7 (B) cells. For E_2, Org 2058 (2058), progesterone (PROG), promegestone (R5020), medroxyprogesterone acetate (MPA), norethisterone (NE), levonorgestrel (LNG), 3-ketodesogestrel (KDG), gestodene (GES), and Org 30659 (30659). Each bar represents the ratio of the geometric mean of test compound over that of the control level (black line) with the overall 95% confidence intervals, which are multiplied by the geometric mean (µg DNA/ml) of the control level. If a statistical significant difference of $P < 0.05$ (*) is found the confidence intervals do not cross the control level (black line).

effects of E_2 and PROG on breast tumor cell growth have also to be considered. In these studies PROG inhibited cell growth significantly to half-maximal levels at 10^{-6} M, R5020 at 10^{-7} M, and MPA, LNG, KDG, GES and Org 30659 at 10^{-9} M in MCF-7 (B) cells (Fig. 8). The most potent compound in this

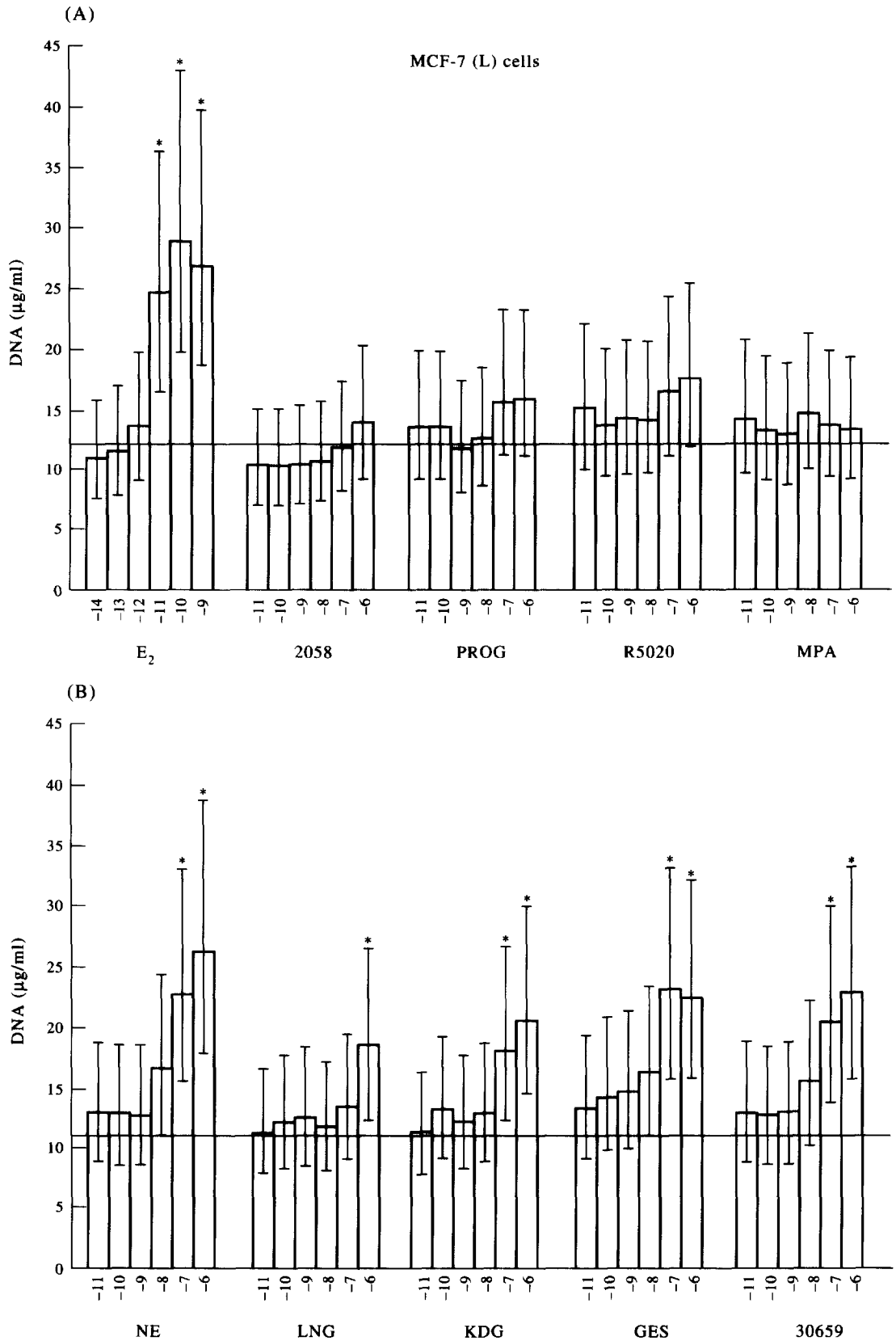

Fig. 3. The effect of E_2 and several progestagens on the cell growth of MCF-7 (L) cells. For an explanation of the symbols and graphics, see Fig. 2.

respect was MPA, which showed almost complete reduction of cellular growth at 10^{-6} M, whereas Org 2058 and NE hardly inhibited cell growth at 10^{-6} M. With MCF-7 (L) cells no inhibition with progestagens was observed, not even at 10^{-6} M (not shown).

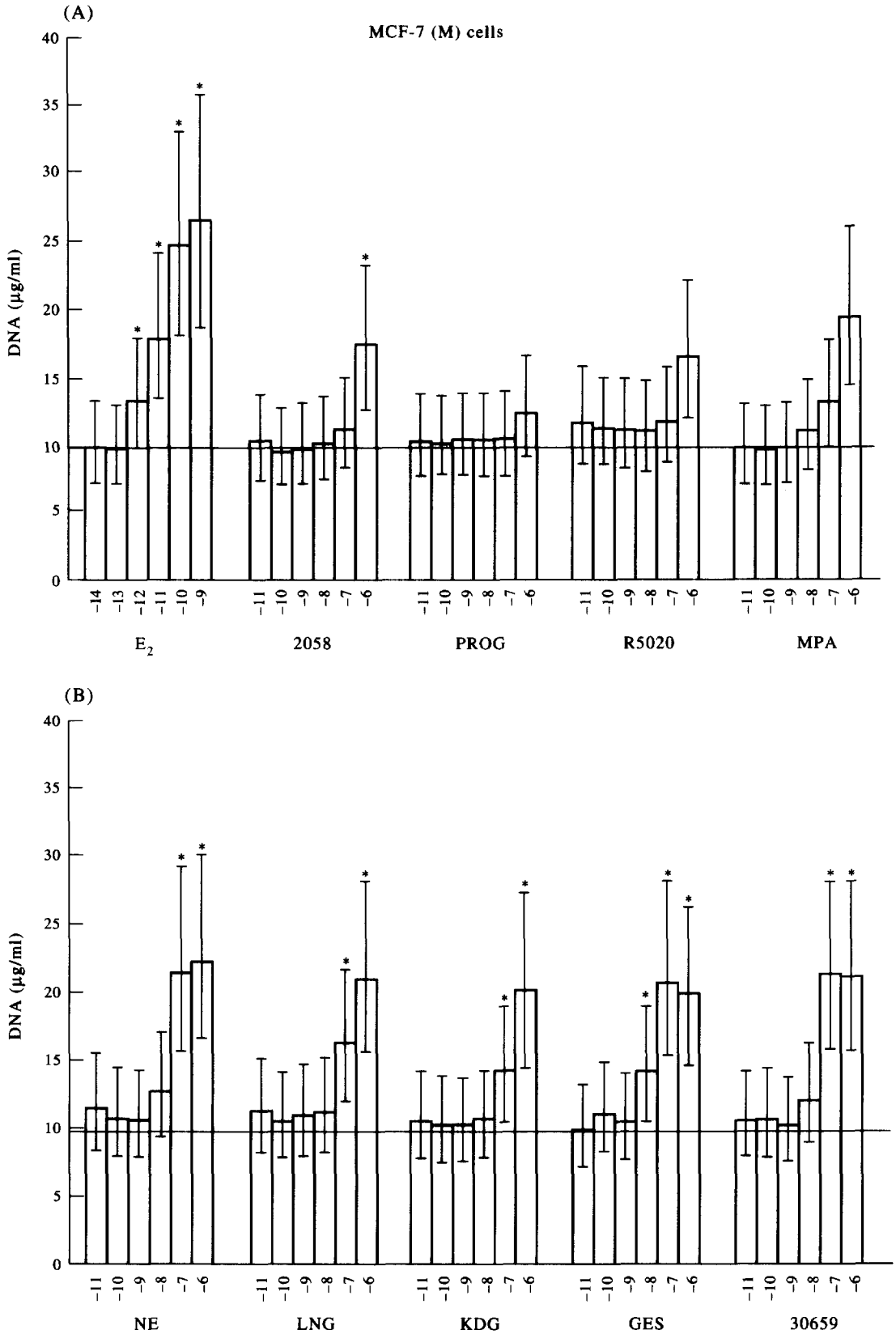

Fig. 4. The effect of E_2 and several progestagens on the cell growth of MCF-7 (M) cells. For an explanation of the symbols and graphics, see Fig. 2.

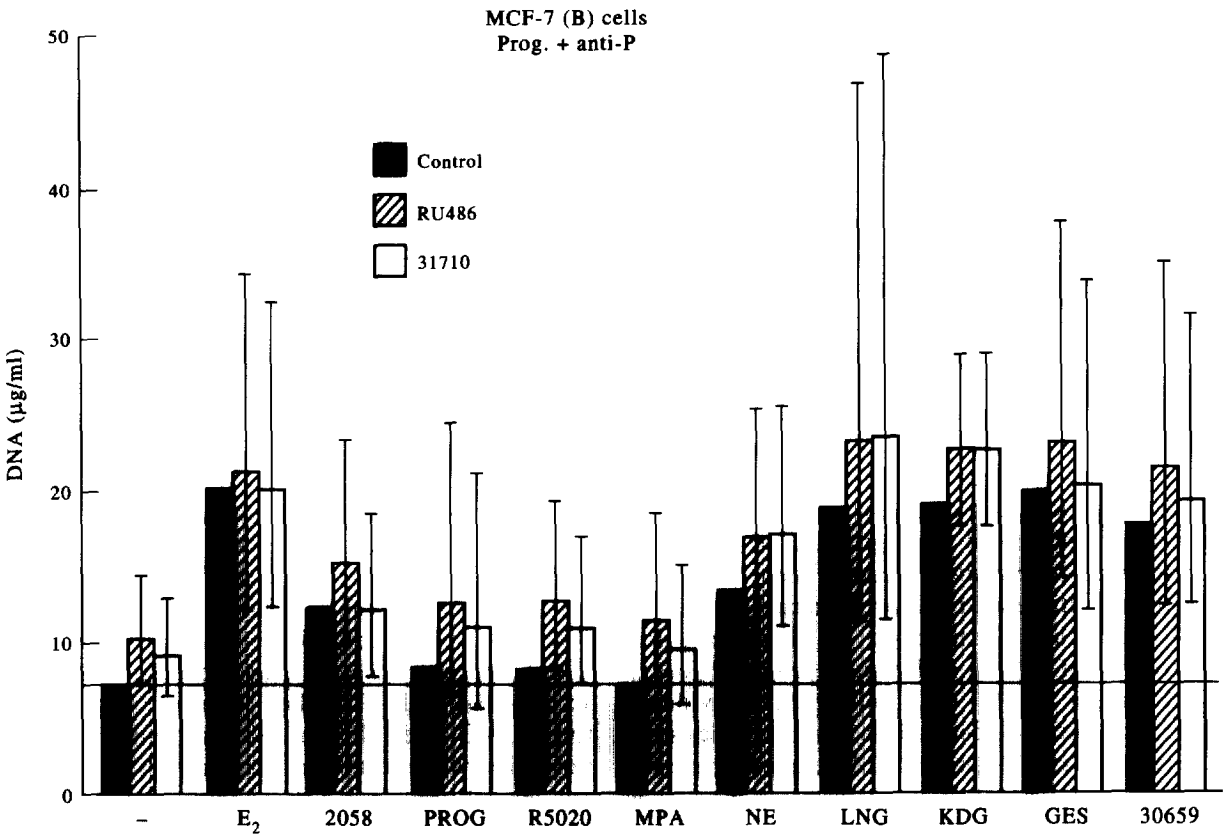

Fig. 5. The effect of antiprogestagens on the E_2 (10^{-10} M) and progestagen (10^{-6} M) induced cell growth of MCF-7 (B) cells. Black, hatched and open bars represent control levels without antiprogestagen, and treatments with RU 38486 and Org 31710 at 10^{-6} M, respectively. The hatched and open bars represent the ratio of the geometric mean of test compound over that of the reference compound (black bars) with the overall 95% confidence intervals, which are multiplied by the geometric mean (µg DNA/ml) of the reference compound. If a statistical significant difference of $P < 0.05$ (*) is found the confidence intervals do not cross the upper-side of the reference compound. For an explanation of the symbols, see Fig. 2.

The specificity of progestagenic inhibition of E_2-induced growth (10^{-10} M) mediated via PR or ER was tested with antiprogestagens or antiestrogens in combination with progestagens (10^{-8} M). The progestagen mediated growth inhibition of E_2-induced proliferation could be prevented by both antiprogestagens RU 38486 and Org 31710 at 10^{-6} M in the MCF-7 (B) cells (Fig. 9). As expected all E_2-induced growth was immediately inhibited by the antiestrogens 4-hydroxytamoxifen and ICI 164,384 at 10^{-7} M (results not shown).

RBA values of progestagens for ER, AR and PR

In Table 2 the RBA values of the used progestagens for ER, AR and PR are given. Since antiestrogens were able to inhibit the cellular proliferation of MCF-7 cells induced by the 19-nortestosterone derivatives, this effect could be due to binding of the progestagens to ER. The RBA values of these progestagens as well as of the pregnanes were therefore estimated for the cytosolic estrogen receptor. Table 2 shows that NE, Org 30659, GES and R5020 showed low binding to ER. All other progestagens did not show any cross-reactivity with ER. The following ranking in potency was obtained: GES > Org 30659 > NE > R5020, followed by the weak or non-competitive binders KDG, LNG, MPA, PROG and Org 2058.

The RBA values of AR for Org 2058, PROG and Org 30659 were all below 2.8% (Table 2), while those of NE, KDG, LNG, and GES were between 4.5 and 8.2%. MPA, on the other hand, had a relatively high binding value of 30%. The RBA values of LNG, Org 2058, MPA, Org 30659, R5020, GES and KDG for PR were between 81 and 192%, while NE and PROG show far lower RBA values being 21 and 11%, respectively.

DISCUSSION

Several progestagens were studied for their capacity to stimulate and/or inhibit the growth of MCF-7 cells of different origin. The data obtained showed that 19-nortestosterone derivatives alone, such as NE, LNG, KDG, GES and Org 30659, could stimulate cellular proliferation in all three lines of MCF-7 cells at high pharmacological doses of 10^{-7} and 10^{-6} M, but not at physiological concentrations. With respect to this growth stimulation NE, GES and Org 30659 demonstrated a higher growth stimulatory potency than KDG and LNG at 10^{-7} M. The pregnanes alone,

i.e. Org 2058, PROG, R5020 and MPA, did not induce cell growth in MCF-7 B and L cells with the exception of Org 2058 in B cells. However, all pregnanes, with the exception of PROG, induced a modest, but statistically significant, cell growth at 10^{-6} M in MCF-7 M cells. The different effects of progestagens in these cell lines may be due to the different origin of the cell lines, rather than by a diversity of cell culture conditions. As expected, the physiological substrate E_2 already induced MCF-7 cell growth at a level of 10^{-12} M with its maximal stimulation reached at 10^{-10} M.

The growth effects as observed for both classes of progestagens in the present study are completely in line with earlier studies with MCF-7 cells [10, 17, 18]. These investigations also demonstrated that pregnanes alone hardly influenced cell growth, whereas 19-nortestosterone derivatives alone, also including norethynodrel, stimulated cell proliferation of MCF-7 cells of the B and L subclone at pharmacological concentrations of 10^{-7} M and higher. Jeng and Jordan [10] and Jeng *et al.* [18] even deliver direct evidence that 19-nortestosterone derivatives have intrinsic estrogenic properties at 10^{-7} and 10^{-6} M, suggesting growth stimulating activities in MCF-7 breast tumor cells via ER since ER negative MDA-MB231, BT-20 and T47DC4 cells were non-responsive to E_2 or 19-nortestosterone derivatives. The real physiological importance of the growth stimulating effects of these progestagens at these non-physiological and pharmacological dosages of 10^{-7} and 10^{-6} M remains doubtful as serum levels of progestagens do not exceed 10^{-8} M [26].

The combined influence of E_2 and progestagens on growth of the three MCF-7 B and L cell lines was also investigated. E_2-induced growth was clearly reduced by approx. 60% in MCF-7 (B) cells by the synthetic progestagens KDG, LNG, GES and Org 30659. Besides those 19-nortestosterone derivatives, the pregnanes PROG, R5020 and MPA also inhibited cell growth. MPA even showed an almost complete reduction in cellular proliferation. Remarkably, a structurally related 19-nortestosterone derivative as NE and the pregnane derivative Org 2058 did not inhibit cell growth in these experiments.

An inhibition of cellular E_2-induced proliferation was previously demonstrated in T-47-D cells [7, 14] by R5020 as well as in ZR-75-1 cells with MPA, NE, LNG, cyproterone acetate and megestrol acetate [12]. In ZR-75-1 cells [12], the 19-nortestosterone derivatives LNG and NE had similar growth inhibiting potencies, while in the present study NE did not show any growth inhibition. On the other hand, MPA was the most potent compound for growth inhibition in ZR-75-1 cells [12] as it was in MCF-7 cells of the present study.

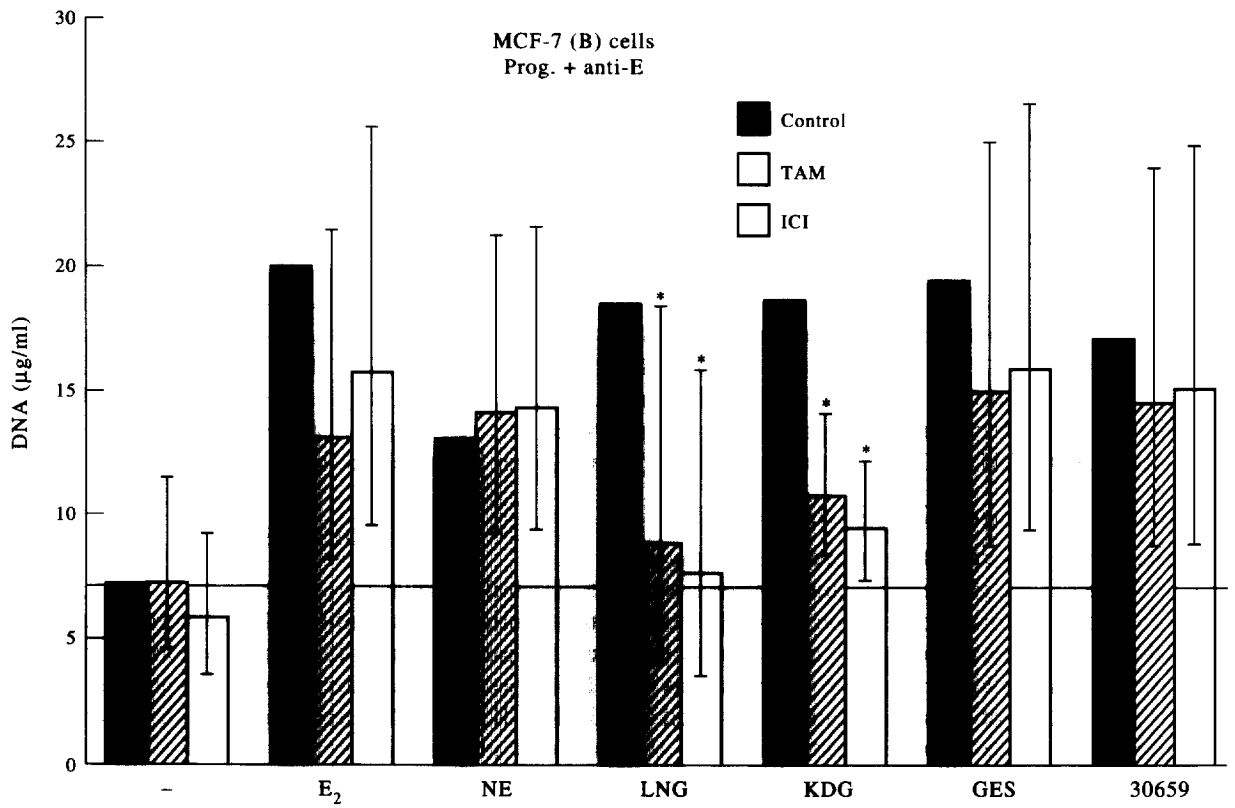

Fig. 6. The effect of antiestrogens on the E_2 (10^{-10} M) and 19-nortestosterone derived progestagen (10^{-6} M) induced cell growth of MCF-7 (B) cells. Black, hatched and open bars represent control levels without antiestrogen, and treatments with 4-hydroxy-tamoxifen and ICI 164.384 at 10^{-8} M, respectively. For an explanation of the symbols and graphics, see Figs 2 and 5, respectively.

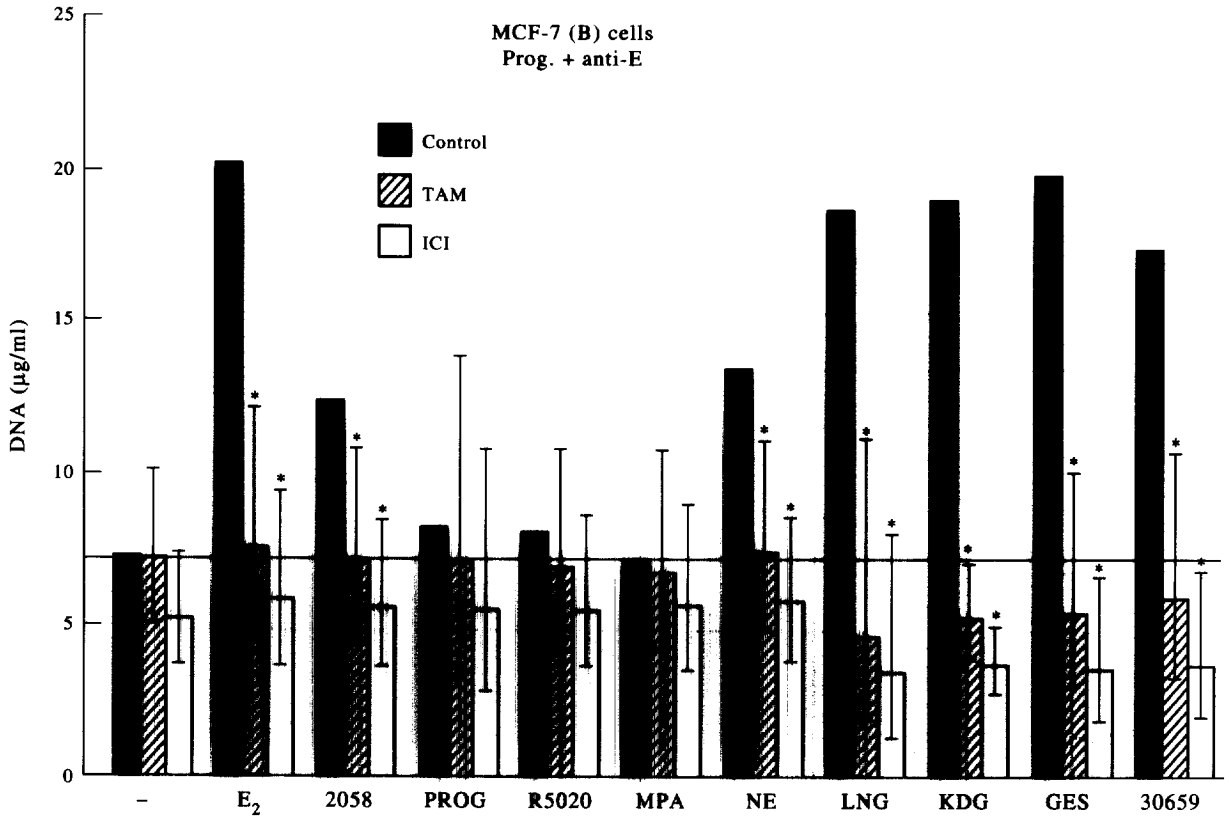

Fig. 7. The effect of antiestrogens on the E_2 (10^{-10} M) and progestagen (10^{-6} M) induced cell growth of MCF-7 (B) cells. Black, hatched and open bars represent control levels without antiestrogen, and treatments with 4-hydroxy-tamoxifen and ICI 164,384 at 10^{-7} M, respectively. For an explanation of the symbols and graphics, see Figs 2 and 5, respectively.

In contradiction to the present study and that of other [7, 17, 18], Coletta *et al.* [23] found growth inhibition with GES alone, but neither a stimulatory nor an inhibitory effect with KDG, R5020 and MPA in MCF-7 cells. However, the observed growth stimulatory effect of E_2 by Coletta after 8 days was only 1.2-fold, while general cellular proliferation under strictly controlled growth conditions is increased at least 2–5 fold in MCF-7 cells by 10^{-8} M E_2 [6, 9, 11, 17, 18]; this suggests that the culture conditions chosen by Coletta may have had a particular influence on the response of the subclone to estrogens. The authors claimed that this special growth inhibitory effect was due to a specific GES binding protein in MCF-7 and T-47-D cells, which was responsible for the regulation of TGF-β production, but the presence of a specific binding protein could not be confirmed with radiolabelled GES for both cell lines with three different methods of separation by Newton and Dickens [27]. The absence of such a GES binding site as well as the comparable actions of 19-nortestosterone derivatives as shown in the present study and those of others [7, 12, 17, 18] suggest that the cell line of Coletta was different [22, 23].

The growth stimulatory effects of estrogens and 19-nortestosterone derivatives demonstrated in the present study could not be inhibited by the antiprogestagen Org 31710 with a low antiglucocorticoid activity, or by the antiprogestagen RU 38486 with a high antiglucocorticoid activity. The involvement of progestagen and glucocorticoid receptors in this process of growth stimulation seems therefore very unlikely. RU 38486 and Org 31710 alone did not enhance cell growth significantly (Fig. 5) and the growth stimulating effects of RU 38486 mediated through the estrogen receptor as described by Jeng *et al.* [28] could not be confirmed here. On the other hand, the progestagen induced growth inhibition of E_2-induced proliferation could be prevented by both antiprogestagens RU 38486 and Org 31710 at 10^{-6} M in the MCF-7 (B) cells, indicating that in this process progesterone receptors are involved. So, in the presence of E_2, progestagens induce their activity via PR, whereas in the absence of E_2, 19-nortestosterone derivatives mediate their effects via ER. This contradiction is hard to explain, but may be caused by a better up-regulation of PR by E_2 than by 19-nortestosterone derivatives or may be due to higher competition of ER and PR for transactivation factors, a process also known as squelching.

The specific antiestrogens 4-hydroxytamoxifen and ICI 164,384 at 10^{-8} M, could not inhibit the maximal E_2-induced and/or "progestagenic"-induced cell growth by GES, Org 30659 and NE, whereas that of KDG and LNG could be inhibited partially. However,

at 10^{-7} M the antiestrogens completely inhibited these growth stimulatory effects. Other investigators also demonstrated that, all 19-nortestosterone derivative induced growth effects in MCF-7 [7, 17, 18] and ZR-75-1 [12] cells could be blocked by antiestrogens via the ER at concentrations of 10^{-7} M. Circumstantial

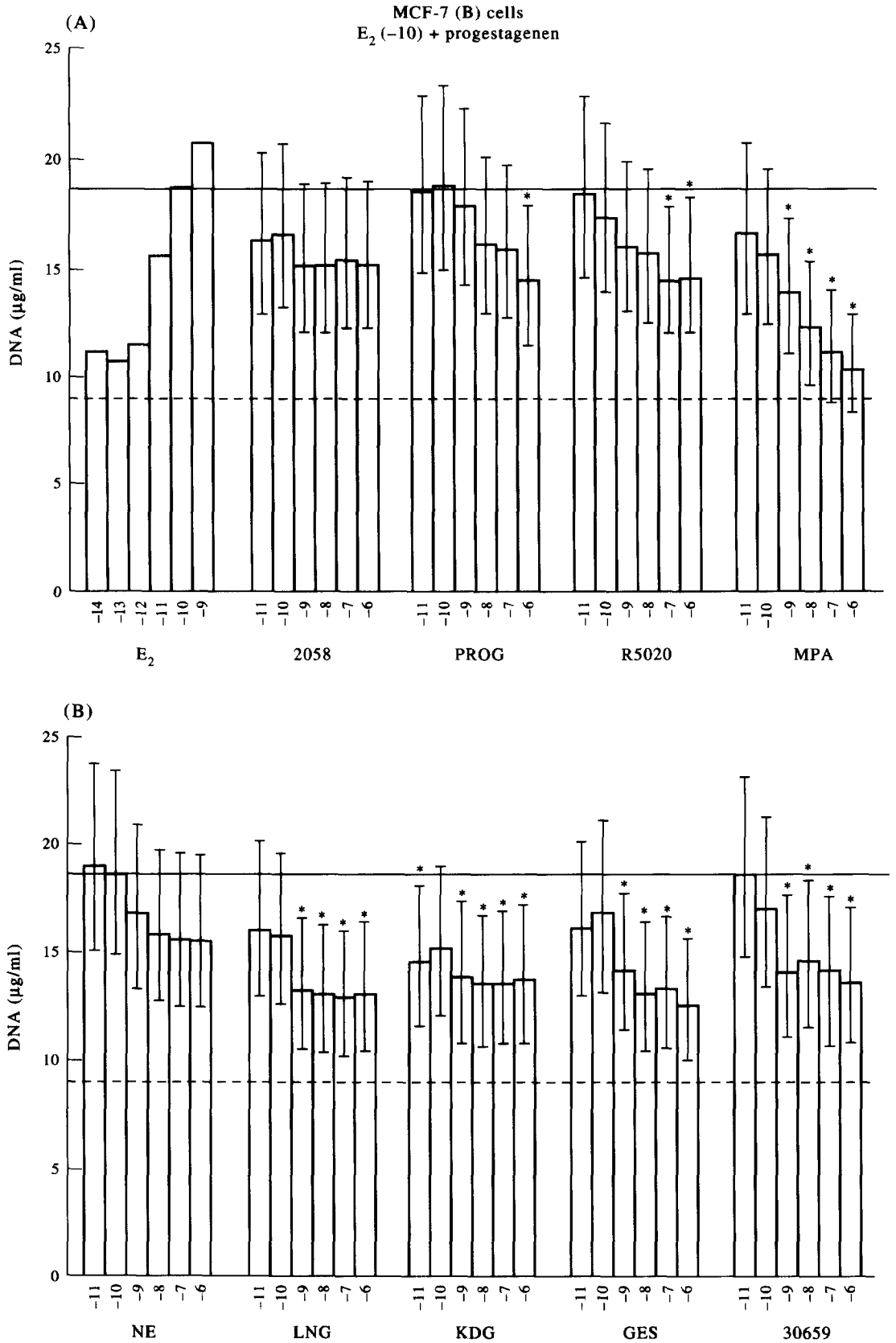

Fig. 8. The effects of several progestagens in combination with E_2 (10^{-10} M) on cell growth of MCF-7 (B) cells. For an explanation of the symbols and graphics, see Fig. 2.

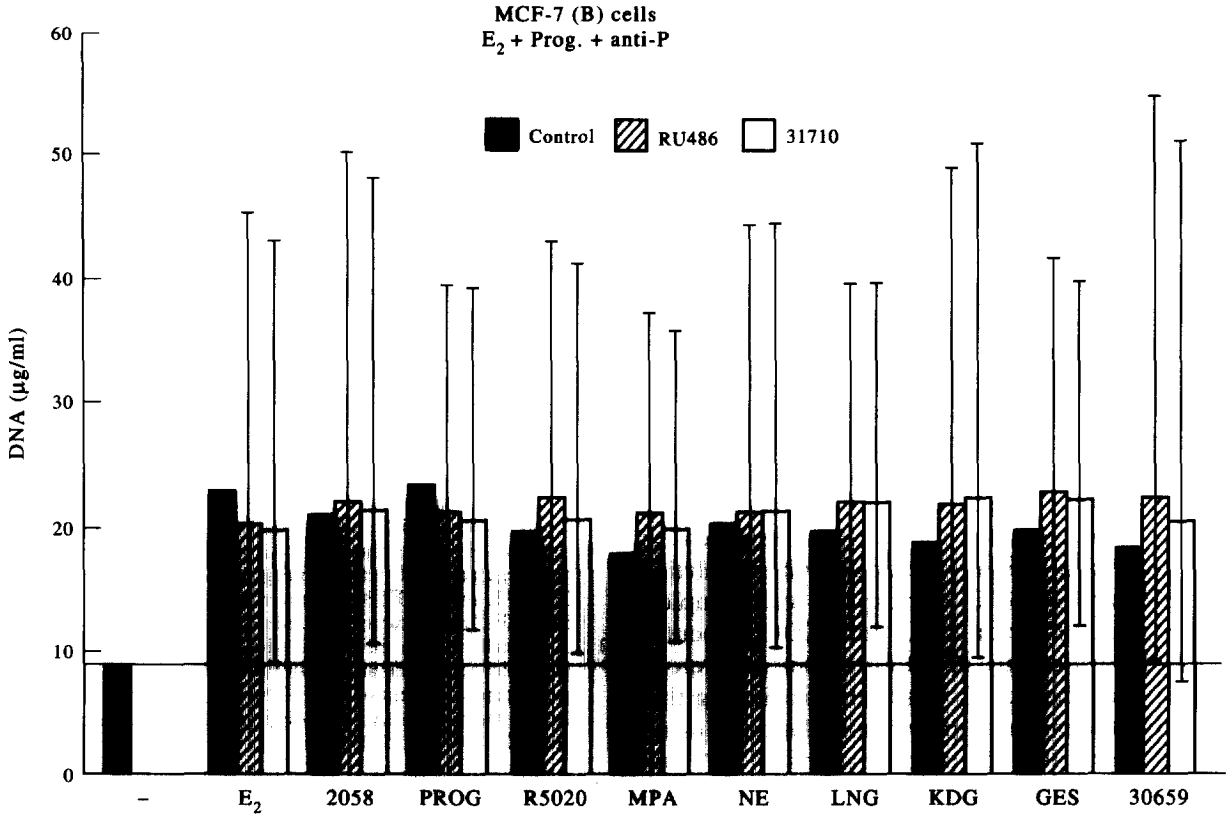

Fig. 9. The effects of antiprogestagens in combination with several progestagens (10^{-8} M) and E_2 (10^{-10} M) on cell growth of MCF-7 (B) cells. Black, hatched and open bars represent control levels without antiprogestagens, and treatments with RU 38486 and Org 31710 at 10^{-6} M, respectively. For an explanation of the symbols and graphics, see Figs 2 and 5, respectively.

evidence of ER involvement came from a study of Jeng *et al.* [18] and Kalkhoven *et al.* [29], showing that MCF-7 cells transiently transfected with a vitERE-tk-CAT construct were able to produce the enzyme CAT 24 h after treatment with E_2 (10^{-9} M), norethynodrel, GES (10^{-6} M) or KDG (10^{-6} M). This transactivation could be blocked by the antiestrogen 4-hydroxytamoxifen or ICI 164,384, indicating that these progestagens can really act as weak estrogens. These findings show that ER is clearly involved. The involvement of the AR is unlikely because the RBA values for AR of MPA, NE, LNG, KDG, GES and Org 30659 in MCF-7 cells do not correlate with the results obtained. MPA should have been much more potent than NE, LNG, KDG, GES and Org 30659. Such an effect was only observed with MPA at high concentrations of 10^{-7} or 10^{-6} M.

Table 2. Relative binding affinities of pregnanes and 19-nortestosterone derivatives to the estrogen, progesterone and androgen receptor in cytosol of human breast tumor cells (MCF-7 M) with respectively E_2, 5α-DHT or Org 2058 as standard (100%) and radioligand

Compounds		Estrogen receptor (E_2 = 100%)	Androgen receptor (5α-DHT = 100%)	Progesterone receptor (Org 2058 = 100%)
Pregnanes				
Org 2058		N.C.	0.14	100
Progesterone	(PROG)	N.C.	1.50	11
R5020		0.005	N.D.	153
MPA		N.C.	30.0	102
19-Nortestosterone derivatives				
Norethisterone	(NE)	0.010	6.0	21
Levonorgestrel	(LNG)	N.C.	8.2	81
3-ketodesogestrel	(KDG)	N.C.	4.5	192
Gestodene	(GES)	0.025	6.1	180
Org 30659		0.015	2.8	141

N.C., non-competitive; N.D., not determined.

On the other hand, DHT at 10^{-10} and 10^{-9} M inhibited growth of ZR-75-1 cells and T47D cells [12, 30], while it stimulated growth of MCF-7 cells [30], indicating that in our MCF-7 cells AR is not involved. Such an explanation can also be given for PR since the progestagens used even had RBA values in the range of 11–192%, implicating again effects at concentrations of 10^{-10} and 10^{-9} M.

The effects of 19-nortestosterone derivatives on cell growth alone as well as in combination with antiestrogens are compared with the RBA values for these compounds in displacement studies. The displacement experiments (see Table 1) showed that GES, Org 30659 and NE have higher binding affinities for ER than KDG and LNG, which is in line with the higher *in vitro* cell growth biopotencies of NE, GES and 30659 as well as with the observation that antiestrogens at 10^{-8} M could only partially block growth stimulation of NE, GES and 30659 in comparison with complete inhibition observed with LNG and KDG. About a 10,000-fold lower affinity to ER for 19-nortestosterone derivatives than that for E_2 was found, indicating that in relation to the maximal growth stimulation of estradiol at 10^{-11} and 10^{-10} M concentrations of 10^{-7} and 10^{-6} M of progestagens are needed to play a role under therapeutical conditions. Since such steroid levels will not be reached at tissue level, these effects are not thought to be of any clinical significance. Moreover since both binding data and growth stimulation show a similar potency ratio of 10,000-fold and conversion of radioactive 19-nortestosterone derivatives such as of LNG, KDG and GES at 10^{-9} and 10^{-6} M into estrogenic compounds was not observed in MCF-7 cells (unpublished results), it is unlikely that metabolites of 19-nortestosterone derivatives are responsible for the effects found on growth, which are mediated via the estrogen receptor.

In conclusion, the 19-nortestosterone derived progestagens demonstrate a very weak estrogenic activity in all three MCF-7 sublines, while pregnane derived progestagens possess this activity only in MCF-7 M cells. The estrogenic activity of 19-nortestosterone derived progestagens is only present at high pharmacological doses and is 10,000 times weaker than that of E_2. In combination with estrogens all 19-nortestosterone derived progestagens and most pregnane derived progestagens gave a statistically significant reduction of E_2-stimulated growth.

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