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Effects of Two Classes of Progestagens, Pregnane and 19-Nortestosterone Derivatives, on Cell Growth of Human Breast Tumor Cells: II. T47D Cell Lines

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Two classes of progestagens, e.g. pregnane [Org 2058, progesterone (PROG), R5020, medroxyprogesterone acetate (MPA)] and 19-nortestosterone derived progestagens [norethisterone (NE), levonorgestrel (LNG), 3-ketodesogestrel (KDG), gestodene (GES), Org 30659] were studied for their effect on cell growth of two human breast tumor T47D cell lines of different origin, i.e. from ATCC (A) and Sutherland (S) *et al.* [Sutherland *et al.*, *Cancer Res.* 48 (1988) 5084–5091]. The effect of estradiol (E_2) and progestagens alone as well as the combined effect of E_2 (10^{-10} M) and progestagens were investigated at several dose levels. Compared with E_2-induced growth at 10^{-10} M, pregnane and 19-nortestosterone derived progestagens at 10^{-6} M alone did enhance cell growth in T47D-A cells up to 25 and 100% respectively, whereas in T47D-S cells they did not influence growth. All these progestagens at 10^{-6} M did not affect E_2-induced growth in T47D-A cells, whereas in T47D-S cells they completely reduced cell proliferation at doses between 10^{-10} and 10^{-8} M. The involvement of progestagen (PR) and estrogen (ER) receptors with respect to growth stimulation was studied by using specific antihormones. In T47D-A cells, the antiprogestagens RU 38486 and Org 31710 could not block progestagen-induced growth. Antiestrogens, like 4-hydroxytamoxifen and ICI 164,384, inhibited the 19-nortestosterone derivative-induced cell growth by approx. 50%. Remarkably, both antiprogestagens alone could also inhibit E_2-induced growth in T47D-A cells by about 50%. In T47D-S cells, E_2-induced cell growth was completely blocked by both antiprogestagens and antiestrogens. Both antiprogestagens in T47D-S cells were equipotent to 4-hydroxytamoxifen and 10-fold more potent than ICI 164,384. In conclusion pregnane and 19-nortestosterone-derived progestagens stimulated cell growth in T47D-A cells at high unphysiological concentrations, whereas they did not affect cell growth in T47D-S cells. The 19-nortestosterone derivative induced growth in T47D-A cells could partially be inhibited by antiestrogens. In T47D-A cells, E_2-induced cell growth was not influenced by both classes of progestagens, whereas in T47D-S cells all tested progestagens, antiprogestagens, and antiestrogens inhibited E_2-induced cell growth completely. These results with T47D cells as well as those obtained previously with MCF-7 cells show that subclones of cell lines may respond differently to various types of progestagens in the presence and absence of estrogens.

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INTRODUCTION

For estrogens, it is well known that they promote the growth of breast tumor cells both *in vivo* and *in vitro* [1]. For progestagens, however, contradictive evidence exists for the role of progestagenic activity from *in vivo* [2–4] and *in vitro* [5–14] studies. The data show that progestagens can be stimulatory, inhibitory and without an effect. This inconsistency in effects may be due to differences in cell lines (MCF-7, T47D, ZR-75-1), cell origin, cell passage numbers, culture conditions, the usage of different sera batches and most importantly the choice of the progestagen.

In a previous study by Schoonen *et al.* [15], in which human breast tumor MCF-7 cells from three different origins were analysed, pregnane derived progestagens, i.e. Org 2058, progesterone (PROG), R5020 and medroxyprogesterone acetate (MPA), did not stimulate

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or hardly stimulated cell growth at 10^{-6} M, while 19-nortestosterone derived progestagens, like norethisterone (NE), levonorgestrel (LNG), 3-ketodesogestrel (KDG), gestodene (GES) and Org 30659 induced cellular proliferation at concentrations of 10^{-7} M and higher. KDG and LNG appeared to be less estrogenic than NE, GES and Org 30659. This stimulatory effect of the 19-nortestosterone derivatives could not be counteracted by antiprogestagens, but was inhibited by antiestrogens. It suggested that the proliferative effects were mediated by the estrogen receptor (ER). This study of Schoonen *et al.* [15] was in line with studies of others with MCF-7 [6, 8, 10] and T47D cells [13], but differed for T47D cells from those of Sutherland *et al.* [5], Clarke and Sutherland [14], Gill *et al.* [10] and Hissom *et al.* [11]. Sutherland *et al.* [5] and Clarke and Sutherland [14] described for a T47D subclone a growth stimulating effect of MPA and Org 2058 during a short incubation period of 12 h in the presence of both charcoal treated and untreated sera, whereas growth inhibiting effects with these progestagens were observed during a long incubation period of more than 48 h. Such growth inhibiting effects of progestins were also demonstrated with R5020 by Gill *et al.* [10] and Hissom *et al.* [11] on T47D cell lines, but the authors did not use charcoal treated sera.

In the present study, the effects of pregnane and 19-nortestosterone derivatives alone as well as in combination with E_2 on cell proliferation were investigated for the human breast tumor T47D cell line. Two subclones of this T47D cell line were used, i.e. a clone from ATCC (A) and one clone from Sutherland (S) *et al.* [5]. The same culture conditions were used as previously with MCF-7 cells [15], i.e. phenol-red free medium with dextran-coated charcoal-treated sera in the presence or absence of E_2 and several steroids from the two described classes of 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 chemical structures of the progestagens were presented before [15]. The antiestrogens, 4-hydroxytamoxifen and ICI 164,384 were kindly provided by Dr. A. E. Wakeling, Zeneca Pharmaceuticals (Macclesfield, Cheshire, U.K.). 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) and 24 well plates from Nunc (Roskilde, Denmark). All other chemicals were of analytical grade.

Cell culture

Two sub cell lines of T47D, i.e. one from the American Type Culture Collection (Rockville, MD, U.S.A.) and another kindly provided by Dr Rob Sutherland (Cancer Biology Division, Garvan Institute of Medical Research, St Vincents Hospital, Darlinghurst, Australia), were used in these experiments. The cells were cultured and maintained as described previously [15].

Experimental protocol

T47D cells were seeded in 24 well plates of polysterene (3×10^3 cells/well) and cultured as described before by Schoonen *et al.* [15].

Statistical analysis of cell growth experiments

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. The statistical evaluation of the data is described in more detail by Schoonen *et al.* [15].

RESULTS

Effect of progestagens alone on cell growth

As shown in Figs 1 and 2 for T47D-A and S cells, E_2-stimulated already growth at 10^{-11} or 10^{-10} M after 7 days of treatment with a maximal effect at 10^{-9} M. In T47D-A cells (Fig. 1), 19-nortestosterone derivatives stimulated cellular proliferation at 10^{-8} M significantly and reached at 10^{-6} M a level comparable to that of E_2-induced growth at 10^{-10} M. So, the growth inducing potency of the 19-nortestosterone derivatives in comparison with that of E_2 is at least 10,000 times lower in this ATCC cell line. The pregnanes, Org 2058, PROG and R5020 at 10^{-6} M, had a slight but significant stimulatory effect of 15–20% of E_2-induced growth. The effect of MPA appeared to be somewhat stronger. In the T47D-S cells (Fig. 2), neither the pregnanes nor the 19-nortestosterone derivatives induced cell growth at the concentrations tested.

In order to study the involvement of PR and/or ER on these progestagenic cell growth effects in T47D-A

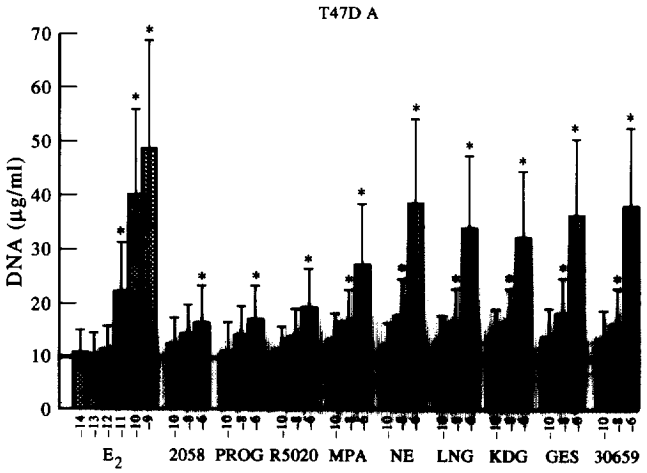

Fig. 1. Dose-dependent effects of estradiol (E_2) (10^{-14}–10^{-9} M) and several progestagens (10^{-10}, 10^{-8} and 10^{-6} M) on the cell growth of T47D-A cells. For E_2, Org 2058 (2058), progesterone (PROG), promegestone (R5020), medroxyprogesterone acetate (MPA), norethisterone (NE), levonorgestrel (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 control level is not within the confidence intervals.

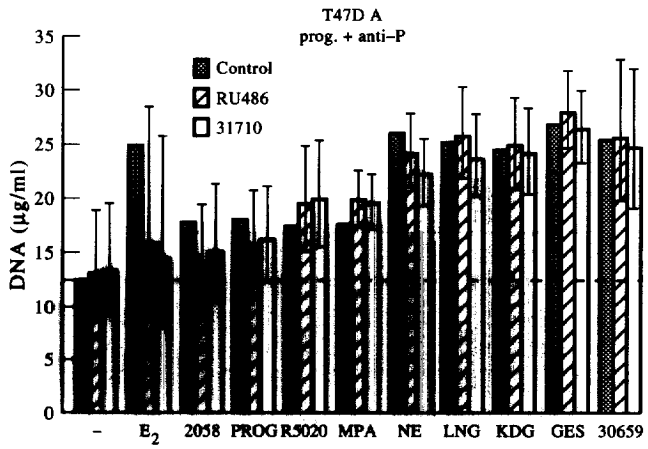

Fig. 3. Effect of antiprogestagens on the E_2 (10^{-10} M) and progestagen (10^{-6} M) induced cell growth of T47D-A cells. Condensed dotted, hatched and open bars represent control levels without antiprogestagens, and treatments with RU 38486 and Org 31710 at 10^{-6} M, respectively. The hatched and open bars represent the ratio of the geometric mean of test compound over that of the reference compound (condensed dotted 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 upper-side of the bar of the reference compound is not within the confidence intervals. For explanation of the symbols, see Fig. 1.

cells antiprogestagens, like Org 31710 with low antiglucocorticoid activity and RU 38486 with high antiglucocorticoid activity (Fig. 3), as well as antiestrogens, i.e. a partial antagonist 4-hydroxytamoxifen and a pure antagonist ICI 164,384, were tested (Fig. 4). Both antiprogestagens did not influence the effect on growth of any progestagen, but surprisingly both antiprogestagens alone at 10^{-6} M showed a decrease in E_2-induced cell growth. The antiestrogens at 10^{-7} M (Fig. 4) could inhibit the growth stimulatory effects of E_2 (10^{-10} M) and Org 2058 completely, while the growth induced by 19-nortestosterone derivatives (10^{-6} M) was only reduced by 50%. On the other hand, the growth induced with PROG and R5020 could only slightly, but not significantly be inhibited by antiestrogens, while MPA induced growth could not at all be inhibited by antiestrogens.

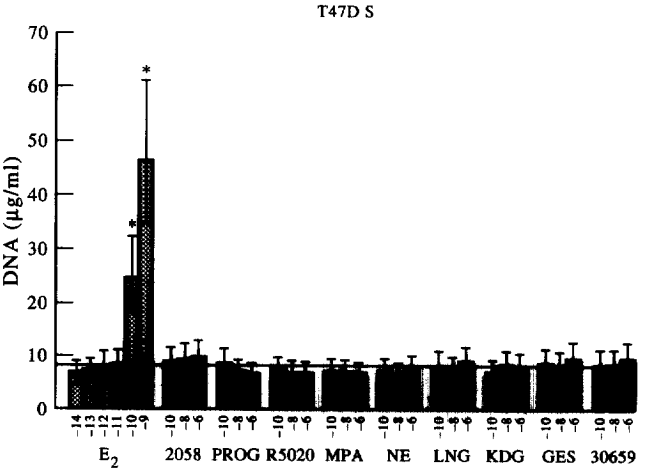

Fig. 2. Dose-dependent effects of estradiol (10^{-14}–10^{-9} M) and several progestagens (10^{-10}, 10^{-8} and 10^{-6} M) on cell growth of T47D-S cells. For explanation of the symbols, see Fig. 1.

Effects of progestagens on cell growth in combination with E_2

The effects of progestagens on E_2 (10^{-10} M) induced cell growth were also examined. With T47D-A cells neither pregnane nor 19-nortestosterone derived progestagens induced inhibition of E_2-stimulated

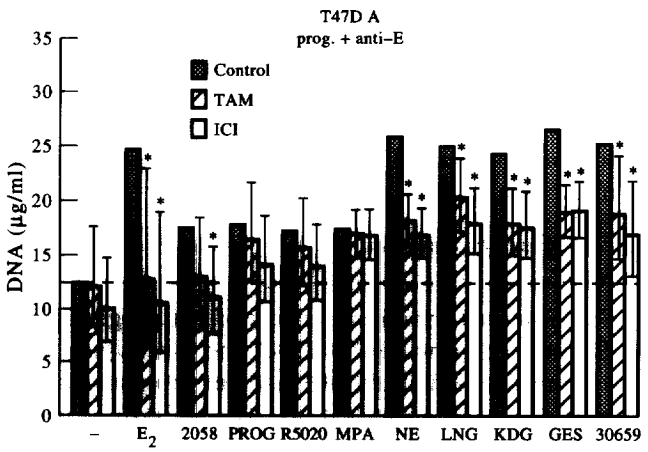

Fig. 4. Effect of antiestrogens on the E_2 (10^{-10} M) and progestagen (10^{-6} M) induced cell growth of T47D-A cells. Condensed dotted, hatched and open bars represent control levels without antiestrogens, and treatments with 4-hydroxytamoxifen and ICI 164,384 at 10^{-7} M, respectively. For explanation of the symbols, see Figs 1 and 3, respectively.

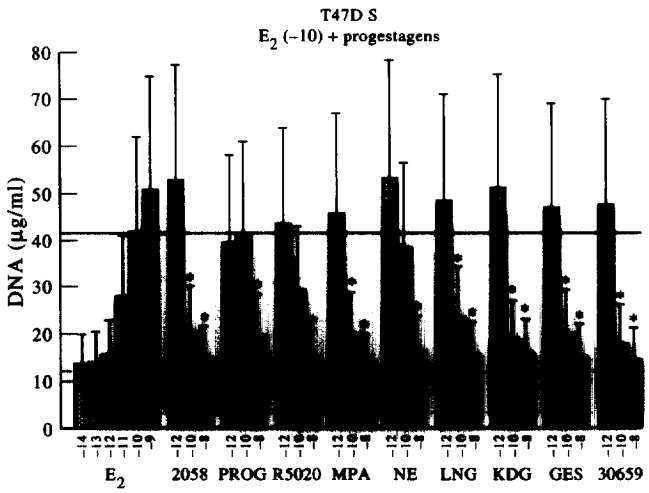

Fig. 5. Dose-dependent effects of several progestagens (10^{-12}, 10^{-10} and 10^{-8} M) in combination with E_2 (10^{-10} M) on cell growth of T47D-S cells. Each bar represents the ratio of the geometric mean of test compound over that of the control level (black line = E_2 at 10^{-10} M) with the 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 control level is not within the confidence intervals.

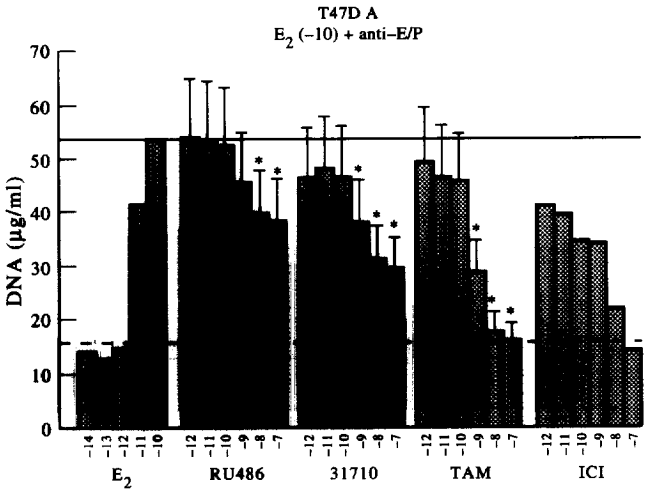

Fig. 6. Dose-dependent effects of 10^{-12}–10^{-7} M of antiprogestagens, i.e. RU 38486 and Org 31710, and of antiestrogens, i.e. 4-hydroxytamoxifen and ICI 164,384, in combination with E_2 (10^{-10} M) on cell growth of T47D-A cells. For explanation of the symbols, see Fig. 5.

growth (results not shown). With T47D-S cells, pregnane derived progestagens, like MPA and Org 2058, as well as 19-nortestosterone derivatives at 10^{-10} M, like LNG, KDG, GES, Org 30659, inhibited cell growth significantly (Fig. 5), while NE, PROG and R5020 at 10^{-10} M did not reduce growth significantly. However, all pregnane and 19-nortestosterone derivatives at 10^{-8} M inhibited this E_2-induced cell growth completely in T47D-S cells.

To explore whether the inhibition of E_2-induced cell growth with these progestagens in T47D-S cells was mediated via PR or ER the effects of both antiprogestagens and antiestrogens were studied separately at dose levels of 10^{-6} and 10^{-7} M, respectively. As expected antiestrogens inhibited E_2-stimulated growth (results not shown), but surprisingly the antiprogestagens (10^{-6} M) could not counteract the cellular growth inhibiting effects of the progestagens (10^{-8} M) during 7 days of treatment. This aspect was therefore studied in more detail.

Effects of antiprogestagens on cell growth in combination with E_2

The observed inhibitory effects of antiprogestagens on E_2-induced growth in T47D-A and S cells gave rise to a more detailed study of these antiprogestagens in comparison with antiestrogens. In T47D-A cells, a growth reduction of approx. 50% was observed with antiprogestagens at 10^{-7} M and of 100% with antiestrogens at the same concentration (Fig. 6). In T47D-S cells, both antiprogestagens as well as the antiestrogen 4-hydroxytamoxifen already inhibited E_2-induced cell growth almost completely at 10^{-9} M, while the antiestrogen ICI 164,384 only became as active at 10^{-8} M (Fig. 7). This indicates that the antiprogestagens are also able to prevent cell growth just like antiestrogens, but surprisingly in this test model they show even 10 times higher potencies than the antiestrogen ICI 164,384.

DISCUSSION

The pregnane and 19-nortestosterone derived progestagens alone stimulated cell growth in T47D-A cells at unphysiological high concentrations of 10^{-6} M up to 25 and 100%, respectively, when compared with E_2-induced growth (100%) at a 10,000-fold lower concentration. These data are in line with those of other reports studying MCF-7 [6–8, 15] and ZR-75-1 cells

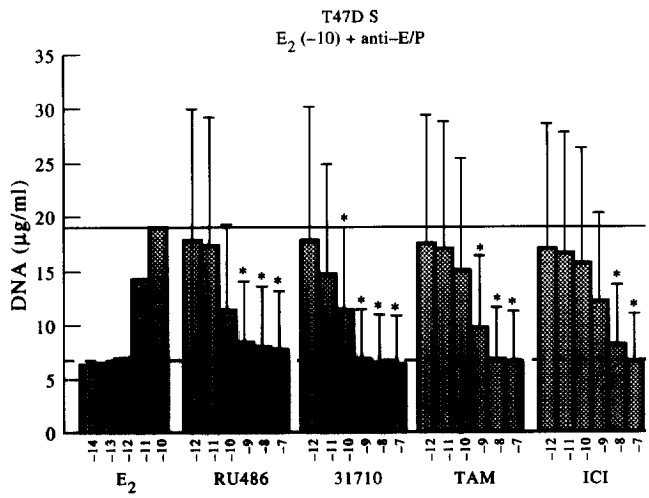

Fig. 7. Dose-dependent effects of 10^{-12}–10^{-7} M of antiprogestagens, i.e. RU 38486 and Org 31710, and of antiestrogens, i.e. 4-hydroxy-tamoxifen and ICI 164,384, in combination with E_2(10^{-10} M) on cell growth of T47D-S cells. For explanation of the symbols, see Fig. 5.

[16], in which pregnanes alone also hardly influenced cell growth and in which 19-nortestosterone derived progestagens alone stimulated cell growth at pharmacological concentrations of 10^{-8} M and higher. On the other hand, in T47D-S cells both classes of progestagens alone were unable to stimulate cellular proliferation at all concentrations tested, whereas they completely reduced E_2-induced cell growth at 10^{-8} M in this cell subline. However, E_2-induced cell growth in T47D-A cells was not changed by both classes of progestagens not even at concentrations of 10^{-6} M. Only a few other investigations confirm that progestagens can inhibit E_2-induced cell growth. This was demonstrated for R5020 with T47D cells [9, 12]; MPA, NE, LNG, cyproterone acetate and megestrol acetate with ZR-75-1 cells [16]; and LNG, KDG, GES, Org 30659, MPA and R5020 with a MCF-7 subline [15]. In these MCF-7 as well as in ZR-75-1 cells, MPA was a more potent inhibitor of cell proliferation than LNG and NE [15, 16]. That these effects in T47D-S cells are mediated via the PR can be illustrated by the dose-dependent inhibition of the progestagens used. The weakest inhibitors progesterone and NE have the lowest relative binding affinity (RBA) values for hPR with Org 2058 as radioligand, i.e. 11 and 21%, respectively [15]. The other progestagens have far higher RBA values of above 81 and up to 192% [15] being indeed more potent with respect to growth inhibition. So, in certain human breast tumor cell lines progestagens may act as inhibitors of E_2-induced cell growth by down-regulation of the ER levels or as activators by initiation of differentiation [14]. Whether the progestagens used are converted into estrogenic compounds is very unlikely, although it is known that 19-nortestosterone is converted *in vivo* into E_2. However, the absence of conversion of radiolabelled progestagens, like KDG, LNG and GES into aromatic compounds in MCF-7 cells makes aromatization less likely (unpublished results). In contradiction with the data from the present study with T47D-A and S cells and those of others [6, 10, 11, 15, 16], Coletta *et al.* [17, 18] described that GES alone could reduce cell growth in T47D and MCF-7 cells, whereas KDG, R5020 and MPA did not have any effect on growth. Although two different T47D-A and S sublines were used all progestagens alone could not inhibit cell growth. Therefore Coletta most likely used a very selective T47D subclone as a result of the chosen culture conditions. Finally, the observed difference in progestagenic sensitivity of cell growth between T47D-A and S cells in both the presence and absence of E_2 may be due to the use of different cell sublines, rather than to different culture conditions. The diversity between the cell sublines might be caused by different PR and ER ratios or combined PR and ER dependent transactivation mechanisms of growth inducing genes.

The involvement of progestagen receptors in growth regulation of T47D-A cells appeared to be unlikely, since both antiprogestagens Org 31710 and RU 38486 did not inhibit cell proliferation induced by 19-nortestosterone derived progestagens. Remarkably, both antiprogestagens could inhibit E_2-induced cell growth in this T47D-A cell line partially and in T47D-S cells completely. On the other hand, antiprogestagens did not influence E_2-induced cell growth in MCF-7 cells [6, 15]. This shows that at least for T47D-S cells and to a lesser extent for T47D-A cells PR is involved in growth regulation. Besides PR ER is also a growth mediator as was demonstrated by the inhibition of E_2-stimulated cell proliferation by the specific antiestrogens 4-hydroxytamoxifen and ICI 164,384 in both T47D-A and S cells. Moreover these antiestrogens were also able to reduce cell growth induced by 19-nortestosterone derived progestagens in T47D-A cells for 50% as in other studies [6–8, 15, 16], although in those studies a complete inhibition was found. The partial growth reduction in T47D-A cells of 50% could be explained partly by binding of these progestagens to estrogen receptors, as described previously by Schoonen *et al.* [15] and also suggested by others [6–8, 15]. This points to a more general mechanism of action of 19-nortestosterone derived progestagens via ER in MCF-7, ZR-75-1 and T47D-A human breast tumor cell lines, but not in T47D-S cells. A second explanation for the reduction of growth in the T47D sublines is probably caused by the dual interaction on the same promoter with ER and PR as transcription factors, as described by Sutherland *et al.* [5], Clarke and Sutherland [14] and Musgrove *et al.* [19]. These authors suggested that antiprogestagens might mediate their action via PR and its hormone responsive elements in a promotor of growth regulating genes that also contains estrogen responsive elements [14]. A third explanation for the different action of progestagens in T47D-S cells might be the biphasic progestagenic activity on growth. A short 12 h treatment with progestagens in this cell line induces growth stimulation, whereas a long 48 h treatment reduces or prevents cell proliferation [19]. As a consequence antiprogestagens are already active inhibitors of the growth stimulating effects of progestagens in the short 12 h treatment period and therefore also during the 48–96 h treatment. So besides antiestrogens, progestagens and antiprogestagens may also probably be used in certain breast tumors to prevent cell growth. Finally Jeng *et al.* [8], pointed at the fourth possibility, i.e. metabolism of antiprogestagens into 3β-hydroxylated and/or aromatized compounds leading to the production of (anti)estrogens. Whether this occurs needs further investigation.

In conclusion pregnane and 19-nortestosterone derivatives stimulated cell growth in T47D-A cells at high unphysiological concentrations, whereas they did not affect cell growth in T47D-S cells. The growth induced by 19-nortestosterone derivatives in T47D-A cells could partially be inhibited by both antiestrogens.

In T47D-A cells, E_2-induced cell growth was not influenced by both classes of progestagens, but was inhibited by antiprogestagens and antiestrogens, whereas in T47D-S cells all tested progestagens, antiprogestagens and antiestrogens inhibited this E_2-induced cell growth completely. The latter effect on T47D-S cells might explain why (anti)progestagens can reduce growth in certain breast tumors. These results with T47D cells as well as those obtained previously with MCF-7 cells show that subclones of cell lines may respond differently to various types of progestagens, antiprogestagens and progestagen/estrogen combinations.

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