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Report

Multiple actions of synthetic 'progestins' on the growth of ZR-75-1 human breast cancer cells: An *in vitro* model for the simultaneous assay of androgen, progestin, estrogen, and glucocorticoid agonistic and antagonistic activities of steroids

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Key words: progestins, steroid receptors, breast cancer, androgens, estrogens, glucocorticoids, ZR-75-1 cells

Abstract

This study was designed to assess the multiple steroid receptor mediated activities of a series of synthetic 'progestins' on breast cancer cell growth, using the human ZR-75-1 cell line which possesses functional estrogen (ER), androgen (AR), and glucocorticoid (GR) receptors as well as progesterone (PgR) receptors. Four 17-hydroxyprogesterone derivatives (chlormadinone acetate, CMA; cyproterone acetate, CPA; medroxyprogesterone acetate, MPA; and megestrol acetate, MGA) and two 19-nortestosterone derivatives (norethindrone, NRE, and norgestrel, NRG) were thus investigated.

Based on the requirement of estrogens for PgR-mediated antiproliferative effects and the reversal of PgR-mediated action by insulin, it was found that although all 'progestins' could inhibit ZR-75-1 cell growth through the PgR at low concentrations, the relative contribution of this receptor in cell growth control is highly variable between compounds. The quantitative importance of PgR-mediated inhibition of cell proliferation was inversely related to the amplitude of the androgenic effects induced by the compounds, the AR-mediated effects increasing in the order CPA < MGA < CMA < NRE < NRG < MPA. The specificity of these androgenic effects is further supported by their reversal upon addition of the antiandrogen hydroxyflutamide. In addition, the 17-hydroxyprogesterone derivatives, but not the 19-nortestosterone derivatives, had glucocorticoid activities at high (micromolar) concentrations, as shown by reversal of growth inhibition by the antagonist RU486 in the presence of saturating concentrations of 5α-dihydrotestosterone. All 'progestins' tested, except MPA and NRE, also had some antiglucocorticoid activity, NRG being the most potent in this respect. Finally, NRE and NRG exerted a marked mitogenic effect in estrogen-free medium which was clearly mediated through the ER as shown by the competitive reversal of their action by the steroidal antiestrogen EM-139.

The present results show that growth measurements of the human breast cancer cells ZR-75-1 permit, with the appropriate steroid additions, the assay of progestin, androgen, estrogen, and glucocorticoid agonistic as well as antagonistic activities of test compounds. The present study shows, somewhat surprisingly, that while the AR is almost completely responsible for the action of MPA at low concentrations, the majority of the action of NRE, NRG, and MGA is also exerted through AR, while the androgenic action of CPA plays a

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lower role in the growth inhibition induced by this compound. Such a model should be of great help in designing more specific steroid drugs and in better understanding the role of the different steroid classes which can be used to control the growth of hormone-sensitive cancer. The present data also indicate that 'progestin' is an inappropriate name for MPA, NRE, NRG, MGA, CMA, and CPA, which all possess other and sometimes more potent steroidal activities than those related to interaction with the progesterone receptor.

Abbreviations: CMA – chlormadinone acetate [17α-acetoxy-6-chloropregna-4, 6-dien-3, 20-dione], CPA – cyproterone acetate [17α-acetoxy-6-chloro-1α,2α-methylene-pregna-4, 6-dien-3, 20-dione], DEX – dexamethasone [9-fluoro-11β, 17, 21-trihydroxy-16α-methyl-pregna-1, 4-dien-3, 20-dione], DHT – 5α-dihydrotestosterone [17β-hydroxy-5α-androstan-3-one], E_2 – estradiol [estra-1, 3, 5 (10)-trien-3, 17β-diol], EM 139 – [*N*-n-butyl-*N*-methyl-11-(16α-chloro-3, 17β-dihydroxyestra-1, 3, 5 (10)-triene-7α-yl) undecanamide], MGA – megestrol acetate [17α-acetoxy-6-methylpregna-4, 6-dien-3, 20-dione], MPA – medroxyprogesterone acetate [17α-acetoxy-6-methylpregn-4-en-3, 20-dione], NRE – norethindrone [17β-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one], NRG – norgestrel [13β-ethyl-17β-hydroxy-18, 19-dinor-17α-pregn-4-en-20-yn-3-one], OHF – hydroxyflutamide (SCH 16423) [α, α, α-trifluoro-2-methyl-4'-nitro-m-lactotoluidide], R1881 – methyltrienolone [17β-hydroxy-17α-methyl estra-4, 9, 11-trien-3-one], R5020 – promegestone [17α, 21-dimethyl-19-norpregna-4, 9-dien-3, 20-dione], RU486 – [17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)-estra-4, 9-dien-3-one], triamcinolone acetonide – [9-fluoro-11β, 21-dihydroxy-16α, 17(1-methylethylidenebis < oxy >) pregna-1, 4-dien-3, 20-dione]

Introduction

Much attention has been given during the last decade to the use of synthetic 'progestins', especially megestrol acetate (MGA) and medroxyprogesterone acetate (MPA) (Fig. 1), for the treatment of advanced breast cancer. In fact, the 30% to 40% response rates obtained with the various 'progestins' [1] compare favorably with those reported for other endocrine therapies, especially tamoxifen, the most widely used agent in the management of breast cancer [2].

There is little information available, however, on the mechanism(s) of action of progestins in breast cancer tissue. *In vivo* studies with carcinogen-induced mammary tumors in the rat have shown a growth-promoting effect of low doses of progesterone [3–5], probably in synergism with estrogens [6]. On the other hand, conflicting results have been obtained on the role of progestins in human breast cancer models in culture, either mitogenic [7, 8] or antiproliferative effects [8–10] having been reported. In the ZR-75-1 human breast cancer cell line [11], the synthetic progestin R5020 exerts a progesterone receptor-specific inhibition of cell proliferation only in the presence of estrogens, a phenomenon which is reversed by the addition of physiological concentrations of insulin [12].

On the other hand, it is now well established that in addition to binding to progesterone receptors (PgR), most synthetic progestational agents bind with significant affinity to androgen (AR) and glucocorticoid (GR) receptors, and induce biological actions specifically determined by these individual receptor systems [13–19]. A better understanding of the multiple endocrine activity of synthetic 'progestins' is required not only for their more rational use in the therapy of breast and endometrial cancers, but also to evaluate the consequences of the long-term use of oral contraceptives on the etiology and pathophysiology of gynecologic malignancies [20].

Precise analysis of the biological actions of synthetic 'progestins' having affinity for many steroid receptors would ideally require the selection of *in vitro* models possessing functional receptors for all major classes of steroids. In the present study, we have chosen the ZR-75-1 human breast cancer cell line, which possesses functional receptors for estrogens, androgens, progesterone, and glucocorti-

coids [11], in order to compare the relative contribution of the different steroid receptor systems in the control of cell proliferation by synthetic 'progestins'. While estrogens are strongly mitogenic in ZR-75-1 cells [21] and specifically regulate the expression and/or the secretion of several proteins [22], androgens [23], glucocorticoids [Hatton A-C, Labrie F, unpublished results; 24] as well as 'progestins' [25], inhibit their proliferation through specific interactions with their respective receptors.

On the basis of these characteristics, we have recently demonstrated that MPA strongly inhibits ZR-75-1 cell proliferation mainly through its interaction with the AR, the contribution of PgR-specific effects on cell growth being significant only at subnanomolar concentrations [17]. We now extend the investigation of this approach by comparing the effect of MPA on cell growth with three other 17α-hydroxyprogesterone (17-OHP) derivatives, namely megestrol acetate (MGA), chlormadinone acetate (CMA), and cyproterone acetate (CPA), and also two 19–nortestosterone (19-NT) derivatives used as oral contraceptives in women, namely norethindrone (NRE) and norgestrel (NRG) (Fig. 1). Furthermore, we have compared the potency and specificity of the ER-, PgR- and AR-mediated action of these compounds on ZR-75-1 cell proliferation with their ability to compete for the specific binding of the appropriate radioligands in intact cell monolayers. The aim of this study was to assess with precision the different steroid receptor-mediated activities of the above-mentioned synthetic 'progestins' using the regulation of ZR-75-1 human breast cancer cell growth as the specific parameter of response.

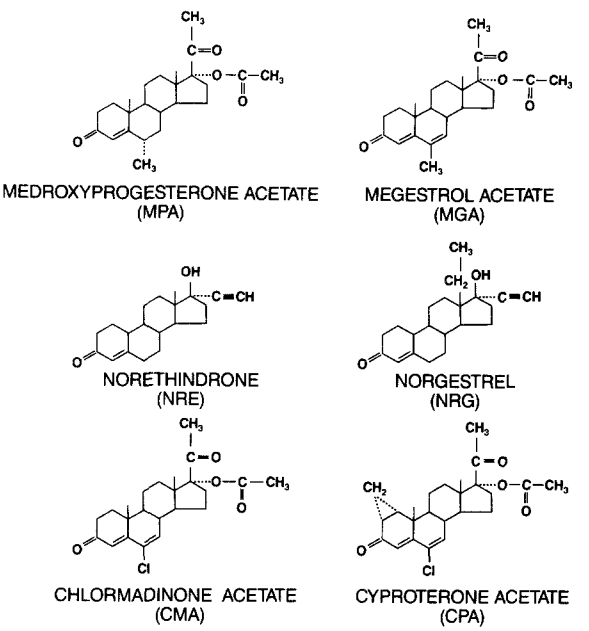

Fig. 1. Structure, trivial name, and abbreviations used for the six synthetic 'progestins' studied.

Materials and methods

Chemicals

Dexamethasone (DEX), triamcinolone acetonide, diethylstilbestrol, and the synthetic 'progestins' CMA, MGA, MPA, NRE, and NRG were purchased from Sigma, while CPA was a gift from Dr. F. Neumann (Schering AG, Berlin). Estradiol (E_2) and 5α-dihydrotestosterone (DHT) were obtained from Steraloids (Pawling, NY). The non-steroidal antiandrogen hydroxyflutamide (OHF) [26, 27] was generously provided by Drs. J. Nagabushin and R. Neri (Schering Corporation, Kenilworth, NJ). The antiprogestational, antiglucocorticoid, and antiandrogenic steroid RU486 [28, 29] was a gift from Roussel-UCLAF (Romainville, France), while the steroidal antiestrogen EM-139 was synthesized in our laboratory [30].

[2, 4, 6, 7-^{3}H]estradiol-17β ([^{3}H]E_2) (sp. act. 101 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). [17α-methyl-^{3}H]R1881 (sp. act. 87 Ci/mmol) and [17α-methyl-^{3}H] R5020 (sp. act. 86.5 Ci/mmol) as well as unlabeled R1881 and R5020 were obtained from New England Nuclear (Lachine, Québec, Canada).

Maintenance of stock cell cultures

All media and supplements for cell culture were from Sigma, except for fetal bovine serum (FBS) which was obtained either from HyClone (Logan, UT) or Flow Laboratories. ZR-75-1 human breast cancer cells were obtained from the American

Type Culture Collection (Rockville, MD) at their 83rd passage and routinely grown in phenol red-free [31] RPMI 1640 medium supplemented with 10 nM E_2, 2 mM L-glutamine, 1 mM sodium pyruvate, 15 mM Hepes, 100 IU penicillin per ml, 100 μg streptomycin sulfate per ml, and 10% (v/v) FBS as described [23]. Cell cultures used in the present study were between passages 88 and 100 and were subcultured weekly.

Cell growth experiments

ZR-75-1 cell cultures in the late exponential growth phase were harvested with 0.05% trypsin/0.02% EDTA (w/v) (Sigma) and resuspended in phenol red-free RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 15 mM Hepes, and antibiotics as described above, plus 5% (v/v) dextran-coated-charcoal-treated calf serum (SD medium) and, where indicated, 500 ng of bovine insulin per ml. Cells were then plated in Linbro 24-well plastic culture plates (2 cm^2/well) at a final density of 4×10^3 cells/cm^2 and allowed to adhere to substrate for 48 h. Steroids and steroid antagonists were then added from 1000 × to 10,000 × concentrated stock solutions in 99% redistilled ethanol to fresh SD medium, and cells were incubated for 12 or 15 days with the indicated additions, with medium changes every other day. At the end of the incubation period, cell cultures were trypsinized and cell number was determined with a Coulter counter (model ZM).

Competition studies of estrogen, androgen, and progestin specific uptake

The specific uptake of [^{3}H]E_2, [^{3}H]R1881, and [^{3}H]R5020 by intact ZR-75-1 cells in the presence of various competitors for determination of their relative binding affinity for estrogen, androgen, and progestin specific binding sites, respectively, was measured essentially as described [21, 23, 32]. Briefly, ZR-75-1 cells were grown to near confluence in 24-well culture plates in SD medium supplemented with 1 nM E_2 for the determination of progesterone specific binding sites, or otherwise without steroid addition. Cell monolayers were then incubated in triplicate for 60 min with 500 μl of phenol red-free RPMI 1640 supplemented as for SD medium, except that serum was replaced by 0.1% (w/v) fatty acid-free bovine serum albumin (Sigma), plus either [^{3}H]E_2 (3 nM), [^{3}H]R1881 (6 nM), or [^{3}H]R5020 (6 nM), and increasing concentrations (0.25 nM to 25 μM) of selected competitors. Triamcinolone acetonide (4.5 μM) and dexamethasone (500 nM) were included in the incubation medium used for the determination of [^{3}H]R1881 and [^{3}H]R5020 specific uptake, respectively, in order to block radioligand binding to PgR (R1881) and GR (R5020). The subsequent procedure for the extraction of specifically bound radioligand at the end of the incubation period was performed as described [32], except that the 30-min incubation step with sucrose buffer was omitted.

Calculation and statistical analysis

Apparent IC_{50} values were calculated using an iterative least-square regression [33], while apparent inhibition constants (Ki) were measured according to Cheng and Prusoff [34]. The apparent dissociation constant (Kd) of the different competitors for radioligand specific uptake in intact cells was calculated as recommended by Munson and Rodbard [35]. All data are presented as means (symbols) ± SEM (bars) of triplicate determinations from representative experiments. When SEM was smaller than the symbol used, only the symbol is shown.

Results

PgR activity

As mentioned above, the addition of insulin completely reverses the inhibition due to the interaction of R5020 with PgR in ZR-75-1 cells [12]. Moreover, the antiproliferative effect of R5020 is observed only under E_2-stimulated conditions [12]. These characteristics of ZR-75-1 cell growth allowed us to study the contribution of PgR in the

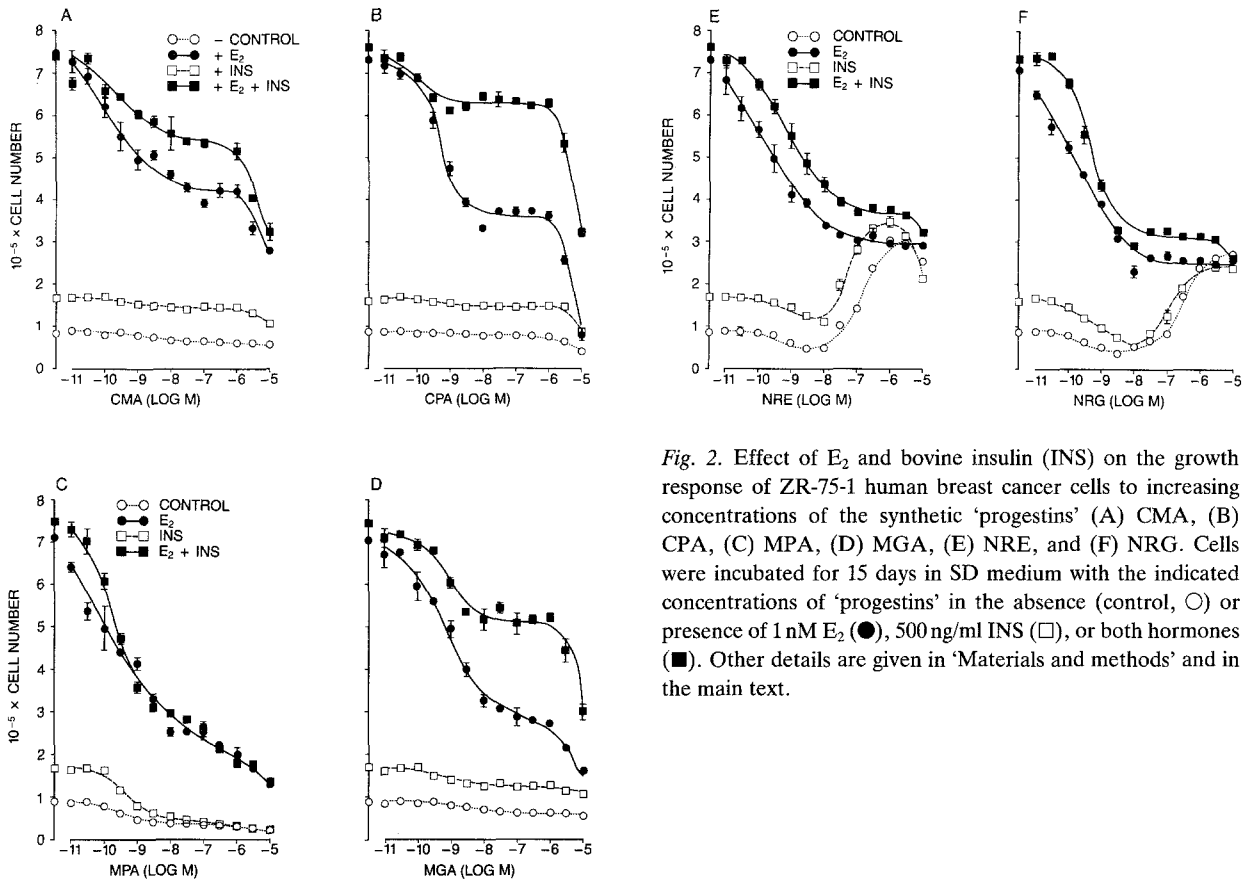

Fig. 2. Effect of E_2 and bovine insulin (INS) on the growth response of ZR-75-1 human breast cancer cells to increasing concentrations of the synthetic 'progestins' (A) CMA, (B) CPA, (C) MPA, (D) MGA, (E) NRE, and (F) NRG. Cells were incubated for 15 days in SD medium with the indicated concentrations of 'progestins' in the absence (control, ○) or presence of 1 nM E_2 (●), 500 ng/ml INS (□), or both hormones (■). Other details are given in 'Materials and methods' and in the main text.

effect of the six selected progestins by evaluating the effect of insulin and/or estrogen addition on the growth response measured at the end of a 15-day incubation of ZR-75-1 cells with the test compounds.

Table 1. Relative contribution of PgR-mediated growth inhibition in the action of synthetic 'progestins' on ZR-75-1 cell proliferation

Compound	Insulin-reversible effect (% of total growth inhibition)
CMA	36*
CPA	66
MPA	0
MGA	50
NRE	12
NRG	9

* Calculated as 100 × (1-[(Nci – Npi)/(Nc-Np)]), where Nc, Np, Nci, and Npi are cell number values determined in the presence of E_2 (1 nM) only, of E_2 + progestin (at 30 nM), E_2 + insulin (500 ng/ml), and E_2 + insulin + progestin, respectively. Data are those presented in Fig. 2.

As shown in Fig. 2 (*A* to *F*), the 6 'progestins' had very different patterns of effects on the proliferation of ZR-75-1 cells. In the absence of both E_2 and insulin, growth rate was very slow and only MPA (Fig. 2*C*) and the two 19-NT derivatives (NRE and NRG, Fig. 2*E, F*) decreased cell number at low concentrations ($p < 0.01$). NRE and NRG behaved very differently from MPA, their effect being growth-inhibitory up to about 3 to 10 nM while a stimulatory effect was observed at higher concentrations ranging between 10 and 1000 nM (Fig. 2*E, F*).

The addition of insulin to incubation media increased cell proliferation by about 2-fold, while not changing significantly the pattern of inhibition induced by the 'progestins' in the absence of E_2. Insulin, on the other hand, had little or no significant effect on maximal cell number values ob-

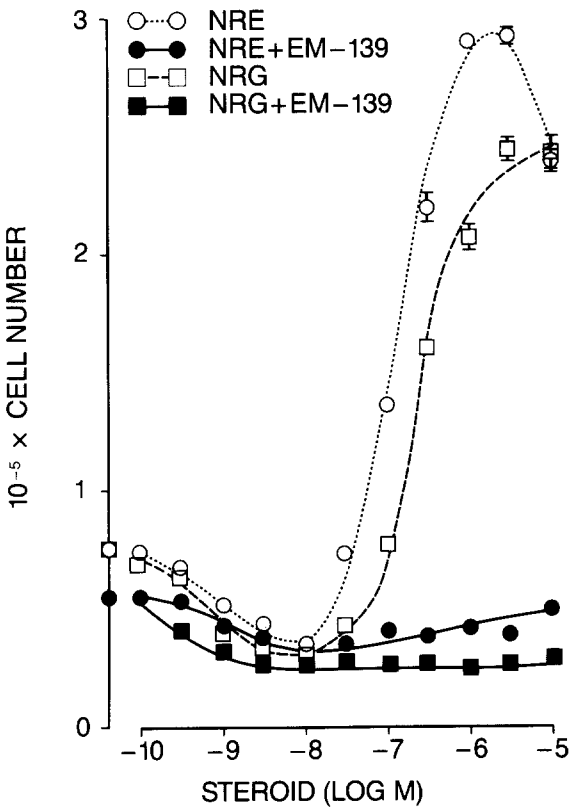

Fig. 3. Reversal of the mitogenic effect of NRE and NRG by the pure steroidal antiestrogen EM-139 in ZR-75-1 human breast cancer cells. Cells were incubated for 12 days in insulin- and estrogen-free SD medium with the indicated concentrations of NRE (○, ●) or NRG (□, ■) in the absence (*light* symbols) or in the presence (*dark* symbols) of EM-139 (300 nM).

tained in ZR-75-1 cells incubated with E_2 (1 nM), which increased cell growth by about 8.5- and 4.5-fold relative to insulin-free and insulin-induced cells, respectively. The effect of insulin on cell growth was most striking with the 6-chloro-17-OHP derivatives (CMA and CPA; Fig. 2*A, B*) and with MGA (Fig. 2*D*), thus indicating the value of their PgR-mediated action (Table 1, Fig. 2). In fact, the PgR-mediated inhibitory effect was calculated in the following order of potency: CPA > MGA > CMA > NRE > NRG > MPA. In the presence of E_2 alone, growth inhibition induced by the CPA, MGA, and CMA reached an intermediary plateau between 1–3 nM and 1 μM, while higher concentrations (> 1 μM) abruptly decreased cell number.

The important reversal by insulin or growth inhibition induced by CPA, MGA, and CMA clearly indicates that a substantial part of the antiproliferative action of these 'progestins' is mediated through interaction with the PgR. Nevertheless, the reversal by insulin was only partial and low concentrations of these steroids were still inducing significant growth inhibition in the presence of insulin, thus indicating participation of additional steroid receptor systems in their biological action. There was also a small but significant reversal by insulin of growth inhibition induced by all concentrations of the oral contraceptives NRE and NRG (Fig. 2*E*, F).

ER activity

The contribution of the other steroid receptor systems in the regulation of ZR-75-1 cell growth by the synthetic 'progestins' was next assessed. Since none of the compounds studied had previously been reported to have any significant affinity for the ER [14], we were intrigued by the observation of a marked mitogenic effect induced by the 19-NT derivatives in the absence of estrogens. In fact, only estrogens had previously been found to have trophic effects in ZR-75-1 cells under the conditions used in the present studies. As illustrated in Fig. 3, the growth stimulation observed with NRE and NRG in an estrogen- and insulin-free medium is likely due to an activation of the ER, since the addition of the antistrogen EM-139 completely reversed the stimulatory effect of both compounds.

AR and GR activity

In order to analyze the interactions of the synthetic 'progestins' with the AR and GR in their inhibitory action on cell growth, we took advantage of the additivity of the anti-proliferative effects of androgens and glucocorticoids in this cell line [23; Hatton AC, Labrie F, unpublished data). Thus, one can saturate AR with 5α-dihydrotestosterone (DHT) and then measure the effect on cell proliferation resulting from the addition of a putative glucocorticoid. On the other hand, the effect of a putative

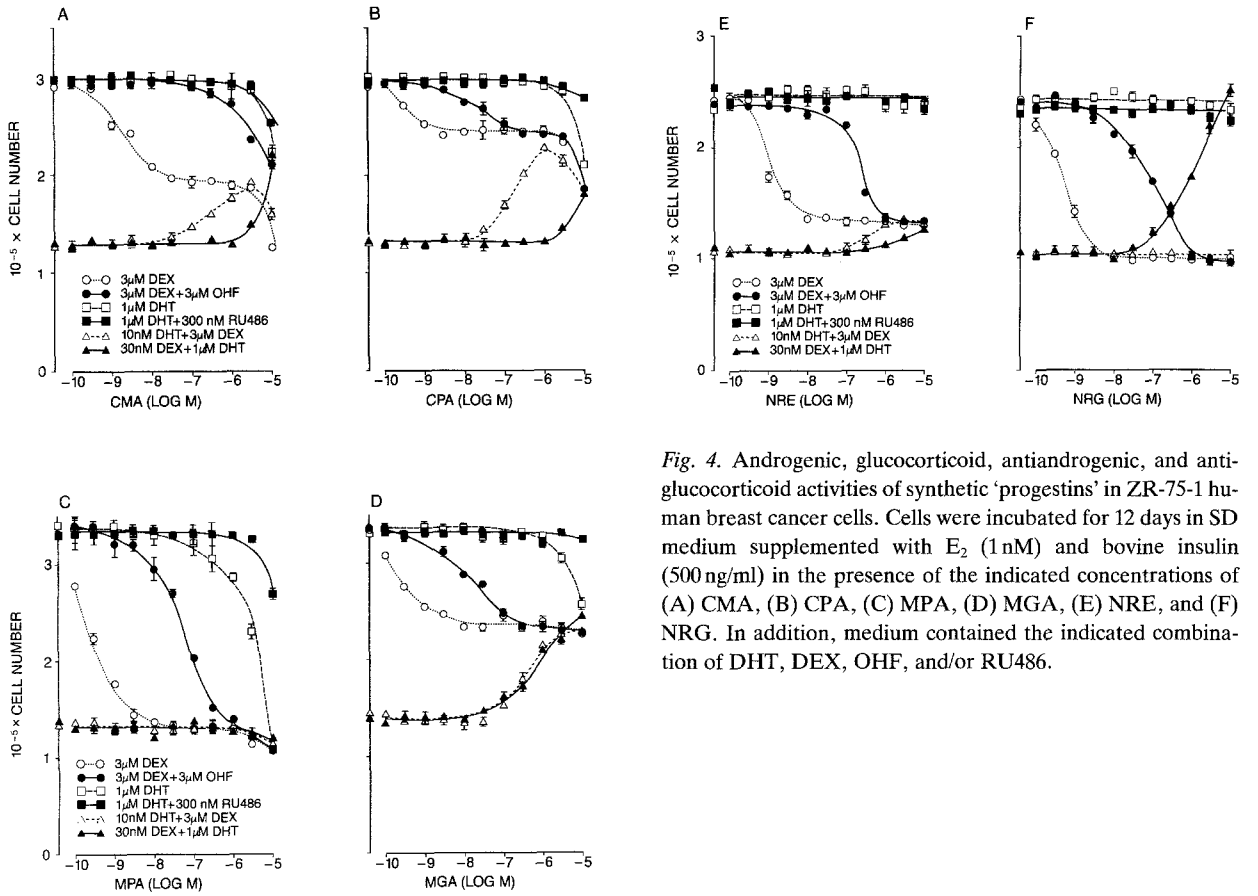

Fig. 4. Androgenic, glucocorticoid, antiandrogenic, and antiglucocorticoid activities of synthetic 'progestins' in ZR-75-1 human breast cancer cells. Cells were incubated for 12 days in SD medium supplemented with E_2 (1 nM) and bovine insulin (500 ng/ml) in the presence of the indicated concentrations of (A) CMA, (B) CPA, (C) MPA, (D) MGA, (E) NRE, and (F) NRG. In addition, medium contained the indicated combination of DHT, DEX, OHF, and/or RU486.

androgen can similarly be measured following saturation of GR by dexamethasone (DEX). The specificity of the growth-inhibitory activity thus observed with the test compound can also be further assessed by its reversibility using the appropriate antagonist (i.e. antiglucocorticoid or antiandrogen). The potent antiglucocorticoid RU486 could not reliably be used alone as a measure of GR-mediated effects, since it is also a potent antiprogestin and a moderately active antiandrogen [28, 29]. However, as shown previously [28], RU486 can nevertheless be used for measurement of antiglucocorticoid activity in the presence of the saturating concentration of the androgen used in this study.

Thus, in the presence of excess androgen (1 μM DHT) in the presence of E_2 and insulin, glucocorticoid effects can be assessed with precision and with no interference by the other receptors. The same applies to study of the role of AR when the cells are incubated in the presence of excess glucocorticoid (3 μM DEX), in the presence of E_2 and insulin. As demonstrated by detailed kinetic studies, 1 μM DHT and 3 μM DEX exert maximal inhibitory effects on the AR and GR, respectively.

In addition, the possible antagonistic activities of 'progestins' mediated through the AR and GR were determined by saturating both receptor systems with DHT and DEX with one ligand being in far greater excess than the other, in order to allow reversal through a single chosen receptor at a time. The results obtained using this approach are summarized in Fig. 4 (A–F) and Table 2. All experiments were performed with ZR-75-1 cells grown in E_2-supplemented media containing insulin in order to prevent the PgR-mediated effect of 'progestins' on cell growth.

Androgenic activity. In the presence of a maximal concentration of DEX (3 μM), increasing concen-

trations of all 'progestins' tested further decreased cell number to variable degrees (Fig. 4), an effect which was competitively reversed by addition of the pure non-steroidal antiandrogen hydroxyflutamide (OHF) [26, 27], thus indicating mediation by the AR. As shown in Table 2, the amplitude of the androgenic inhibitory activity relative to that of a maximally effective concentration of DHT (10 nM), indicates that MPA and NRG behave as complete and near-complete full androgen agonists, respectively, while the other steroids have submaximal agonistic activities in this system with the following decreasing order of potency: NRE > CMA ~ MGA > CPA. However, all 'progestins' exerted half-maximal androgenic effects at similar IC_{50} values (0.2–3 nM).

Glucocorticoid activity. The glucocorticoid inhibitory activity of the 'progestins' studied was assessed by measuring the effect of each compound in the presence of a concentration of DHT (1 μM) which, by itself, maximally inhibited cell growth through the AR while preventing any demonstrable antiandrogenic action in the range of progestin concentrations used. Under these conditions, MPA, MGA, CPA, and CMA significantly inhibited cell proliferation at high concentrations ($\geq$ 100 nM) in decreasing order of potency. Only MPA was able to inhibit cell proliferation to the same extent as 30 nM DEX in the range of concentrations used. The addition of RU486 (300 nM), while having by itself no effect on growth inhibition induced by DHT, potently reversed the antiproliferative effect of MPA and of the other less potent 'progestins'. At the concentration used, NRE and NRG had no demonstrable glucocorticoid effect. Thus, in addition to their AR-mediated effects on cell growth, MPA, and to a much lesser extent high concentrations of MGA, CMA, and CPA, behave as glucocorticoid agonists in the regulation of ZR-75-1 cell growth.

Antiandrogen activity. In the presence of high DEX (3 μM) and low DHT (10 nM) levels, increasing concentrations of CPA, CMA, MGA, and NRE stimulated cell growth significantly in order of increasing potency. MPA and NRG, on the other hand, had no significant effect on cell number up to about 3 μM under the same conditions. Interestingly, the cell number values obtained with increasing concentrations of CPA, CMA, MGA, and NRE were almost superimposable on those obtained by the androgenic action of the same steroids (in the absence of DHT and presence of 3 μM DEX). Thus, the concentration-dependent increase in cell number induced by CPA, CMA, MGA, and NRE in ZR-75-1 cells incubated with DEX and DHT in a 300:1 ratio is most likely due to a partial antiandrogenic activity of these compounds, while MPA and NRG show no antiandrogenic action.

Antiglucorticoid activity. On the other hand, when the concentrations of DHT and DEX were increased and decreased, respectively, by a factor of 100 in order to detect a possible antiglucocorticoid

Table 2. IC_{50} and apparent inhibition constant (Ki) values of the AR-mediated growth inhibition by synthetic 'progestins' and their relative agonistic/antagonistic activities in ZR-75-1 human breast cancer cells

Compound	AR-mediated activity			
	IC_{50} (nM)	Maximal agonistic* (% of maximal DHT effect)	Ki (nM)	Antagonistic** (% of maximal DHT effect)
CMA	3	65	3	35
CPA	~0.3	27	1.5	59
MPA	0.3	100	–	0
MGA	0.2	58	0.9	47
NRE	0.8	79	1.4	21
NRG	0.5	100	–	0

* The relative agonistic AR-mediated activity of a progestin was calculated according to: $100 \times [(n_c - n_p)/(n_c - n_{dht})]$ where n_c, n_p, and n_{dht} are the mean cell number values measured in ZR-75-1 cells incubated with DEX (3 μM) only, DEX + 10 nM progestin, and DEX + 10 nM DHT, respectively, as determined in Fig. 4.

** The relative antagonistic AR-mediated activity of a progestin was calculated as follows: $100 \times (n_{dht+p} - n_{dht})/(n_c - N_{dht})]$ where n_{dht+p} is the maximal mean cell number value obtained when increasing concentrations of 'progestins' were added to ZR-75-1 cells incubated with DEX (3 μM) + DHT (10 nM) (see preceding note for the definitions of N_{dht} and n_c).

effect, MGA, CMA, CPA, and NRE had partial effects while NRE completely reversed the effect of 30 nM DEX at high concentrations. A strong indication is thus provided by the present data that MPA and NRG behave as pure glucocorticoid agonist and antagonist, respectively, while CMA, CPA, and especially MGA have mixed glucocorticoid-antiglucocorticoid activities.

Receptor affinities

In order to better define the various receptor-mediated activities of synthetic 'progestins' on ZR-75-1 cell proliferation, we determined the apparent affinities of the compounds studied for estrogen, androgen, and progesterone specific binding sites using specific radioligand uptake in intact cell monolayers [21, 32]. Attempts to determine the glucocorticoid specific binding sites present in the ZR-75-1 cells [11] using the same technique yielded inconsistent results which are probably due, at least in part, to a high level of non-specific binding of ^{3}H-labelled DEX. The apparent dissociation constants of the ligands studied towards ER, AR, and PgR present in intact ZR-75-1 cells, as well as the relative agonistic and antagonistic activities of the synthetic 'progestins' on a highly specific parameter of glucocorticoid action, namely the inhibition of CRF-induced ACTH secretion in rat pituitary corticotrophs, are shown in Table 3.

Only MGA and NRG, at high concentrations, were able to significantly compete for the specific uptake of [^{3}H]E_2, with relative binding affinities about 0.01 to 0.02% that of E_2. On the other hand, all synthetic 'progestins' displayed a high affinity for androgen and progesterone specific binding sites, although CPA was less potent. Except for CPA, there was reasonably good agreement between the observed IC_{50} values of 'progestin'-induced growth inhibition through interaction with the AR and the potency to compete for the specific uptake of [^{3}H]R1881. However, it should be mentioned that the effect on proliferation was measured after 12-day incubation period, while steroid uptake studies were performed after only 12 h in order to minimize steroid-induced changes in receptor levels. It is likely that some metabolites

Table 3. Apparent dissociation constants (Kd) of selected synthetic 'progestins' for the ER, AR, and PgR present in intact ZR-75-1 cells, as well as IC_{50} and apparent inhibition constant (Ki) values of their action on CRF-induced ACTH secretion in rat pituitary corticotrophs in culture

Steroid	ZR-75-1 cells			Rat pituitary (ACTH secretion) [b]	
	ER	AR	PgR	IC_{50} (agonistic action) (nM)	Ki (antagonistic action) (nM)
	Kd[a] (nM)	Kd[a] (nM)	Kd[a] (nM)		
CMA	150,000	2.8	3.1	> 1,000	200
CPA	150,000	13	14	100,000	300
MPA	> 150,000	2.2	2.9	100	> 300
MGA	5,300	8.1	3.0	900	400
NRE	> 150,000	3.2	3.2	100,000	0
NRG	3,660	1.7	3.6	0	300
E_2	0.60[c]	–	–	–	–
R1881	–	0.7[d]	–	–	–
R5020	–	–	3.3[e]	–	–

[a] Calculated from the IC_{50} value observed for the displacement of [^{3}H]E_2, [^{3}H]R1881, and [^{3}H]R5020 by the indicated competitors from estrogen (ER), androgen (AR), and progesterone (PgR) specific binding sites, according to Munson PJ and Rodbard D [35].

[b] From Raynaud J-P *et al.* [14]

[c] From Poulin R and Labrie F [21].

[d] From Poulin R *et al.* [23].

[e] From Poulin R and Labrie F [12].

formed during the 12-day incubation could be responsible for discrepancies between uptake and cell proliferation studies. It should be mentioned that the above-mentioned potency of the 'progestins' to inhibit cell proliferation at high concentrations through an apparently GR-mediated mechanism was parallel to the inhibitory action of the same compounds on CRF-induced ACTH secretion in rat pituitary corticotrophs. MPA was the most potent in this respect, while CPA and the two 19-NT derivatives were essentially inactive. Likewise, all 'progestins' studied, with the exception of NRE and MPA, displayed quite potent antiglucocorticoid activity on ACTH secretion, in keeping with our observations on the reversal of DEX-induced inhibition of growth in ZR-75-1 cells (Fig. 4).

Discussion

The present data show that while all the synthetic 'progestins' studied possess variable degrees of growth-inhibitory activity in breast cancer cells, their overall effect on cell proliferation results from interactions with up to four steroid receptor systems. Moreover, the present data demonstrate that for some compounds, the main inhibitory action is exerted through the androgen and not through the progesterone receptor.

Since no monospecific progesterone antagonist is available, we used an indirect but precise parameter, namely the reversal by insulin of PgR-dependent growth inhibition, to assess the contribution of PgR to the effect of synthetic 'progestins' on cell proliferation. Comparison of the relative importance of PgR- (Table 1) and AR- (Table 2) mediated action on ZR-75-1 cell growth for the series of compounds under study suggests an inverse relationship between the two activities ($r = -0.9644$ at a concentration of 30 nM for both androgens and 'progestins'). This raises the interesting possibility that androgens might inhibit PgR-mediated action through a specific interaction with the AR. Since androgens are also known to specifically suppress the expression of both ER [36] and PgR [37] in breast tumor cells, it would be interesting to study the possible AR-mediated down-regulation of these steroid receptors by androgenic 'progestins', as a potential mechanism for the apparent domination of AR- over PgR-dependent regulation of ZR-75-1 breast cancer cell growth. In fact, although MPA clearly possesses high affinity for the PgR, it is clear from Fig. 2C and 4C that at low and intermediate concentrations (1–100 nM), the inhibitory effect of the 'progestin' is mediated almost exclusively through the AR.

A somewhat unexpected finding from the present study is the significant estrogenic activity of the two 19-NT derivatives, namely NRE and NRG, on ZR-75-1 cell proliferation. The most likely explanation for this discrepancy is provided by the recently reported estrogenic activity of the 5α-reduced metabolites of NRE in ovariectomized female rats [38, 39]. These authors have found that 17α-ethinyl-5α-estran-3β, 17β-diol was more potent than its 3α-epimer, and that both metabolites could be recovered following incubation of estrogen target tissues with radiolabelled NRE [38]. In fact, both ring A-reduced metabolites of NRE have been found in the plasma [40, 41] and milk [42] of women following the administration of NRE. These observations coupled with the finding of 5α-reductase as well as 3α- and 3β-hydroxy-5α-steroid dehydrogenase activities in ZR-75-1 cells [43; Thériault C, Poulin R, Labrie F, unpublished observations], strongly suggest that NRE is likely to act as an estrogen in ZR-75-1 cells following its metabolism into 3(α,β), 5α-reduced derivatives. Based on the affinity for ER reported for the most potent NRE metabolite (i.e. its 3β,5α-reduced form) in rat uterine cytosol ($Kd = 46$ nM; [39]), it would appear that NRE is very extensively metabolized in ZR-75-1 cells, since the EC_{50} value of its mitogenic effect is observed at about 125 nM (Fig. 2). The same explanation may also hold for NRG [44], although no direct information is available on the bioactivity of its ring A-reduced metabolites. To our knowledge, this is the first report of the estrogenic activity of NRE and NRG in a human target tissue.

It is clear from the evidence herein presented that all synthetic 'progestins' studied exhibit androgenic activity on ZR-75-1 breast cancer growth. Similar conclusions had previously been obtained

for some of these compounds using the rat ventral prostate weight [13, 15, 16], ornithine decarboxylase induction [16], prostatic binding protein mRNA levels [19], mouse Shionogi tumor growth [18], or mouse kidney β-glucuronidase [45] as endpoints of androgen action. However, the significant partial antiandrogenic activity of 'progestins' such as CMA and MGA has not always been observed [15, 16], possibly as a result of tissue or endpoint differences in androgen sensitivity. Other explanations for the divergent findings on the antiandrogenic effect of MGA, CMA, and MGA might pertain to *in vivo* metabolic conversion of these compounds [46], while 6-substituted, 17-OHP-derived synthetic 'progestins' appear to be metabolically stable in breast cancer cells in culture [47].

The AR-mediated activities demonstrated for a series of synthetic 'progestins' on ZR-75-1 cell proliferation illustrate in its simplest form the concept of 'impeded' agonists. Thus, weak androgens such as CPA, CMA, and MGA likely exert their antiandrogenic action in virtue of their incomplete activation of the AR, as shown by the convergence of their action on cell growth when measured in the absence (e.g. agonistic activity) and in the presence (eg. antagonistic activity) of DHT. In keeping with such an explanation, 'progestins' such as MPA and NRG, which induced as potent AR-mediated growth inhibition as DHT, did not exhibit any antiandrogenic activity in the presence of the latter steroid. It seems logical to suggest that maximal inhibitory effects on breast cancer cell growth will be obtained with pure agonistic compounds.

The fact that GR-mediated growth inhibition by synthetic 'progestins' was demonstrable only at concentrations exceeding 100 nM prevented the precise characterization of their relative glucocorticoid-antiglucocorticoid behavior. This finding is in keeping with the high dissociation rates of synthetic 'progestins' from the GR, these compounds showing a decreasing affinity for GR in a time-dependent manner [14, 48]. While the glucocorticoid activity of MPA has been well documented in a number of target tissues [13, 14, 16], including man [49], information on the agonistic and/or antagonistic effect of the other 'progestins' is more scanty. There is, however, a good parallelism between the relative glucocorticoid/antiglucocorticoid characteristics of these compounds measured in rat pituitary cells [14] and in ZR-75-1 cells.

The present results indicate that, in addition to indirect systemic effects, especially inhibition of gonadotropin secretion, the growth-inhibitory action of synthetic 'progestins' at the breast cancer cell level can be mediated directly through the AR and GR, as well as the PgR. The relative contribution of each receptor system in the *in vivo* antitumor action of these agents is difficult to predict. However, the assumption that the therapeutic efficacy of 'progestins' at the breast tumor cell level is correlated with their progesterone-like activity [1, 3, 49] should be revised to include the androgenic and potentially the glucocorticoid actions of these steroids [17, 23]. Interestingly, while most clinical trials have failed to find a significant correlation between objective response to progestin therapy and PgR status and/or content [1, 50–52], others have reported that only AR and ER contents were significantly associated with response rates and duration of remission [53]. In fact, as mentioned earlier, the present data indicate that the AR-mediated action (especially for compounds like MPA) is largely predominant over PgR-mediated effects [17].

That the information presented above about interaction of synthetic 'progestins' with AR applies to the current therapy of breast cancer is well supported by the plasma concentration of 'progestins' measured in patients treated with these compounds. Thus, serum levels of MPA measured 4–6 h after a morning dose of 500 mg were at 142 ng/ml (370 nM) (54). In patients who received megestrol acetate (160 mg), the serum levels were on average 355 ng/ml (795 nM). Since, as shown in Fig. 2C and 2D and Table 2, the half-maximal inhibitory effect of MPA and megestrol acetate mediated by AR is exerted at approximately 0.3 nM, the high doses used of the two steroids exert maximal androgenic effects. Moreover, the serum levels of MPA and MGA in women treated with these compounds are within the range of the GR-mediated inhibitory effects on cell growth (Fig. 4C and 4D) as well as ACTH secretion (Table 3).

The interaction of such doses of MPA with GR is also well supported by the usually complete inhibition of serum cortisol in patients receiving daily oral doses of 1500 mg MPA (55).

Our finding that NRG and NRE can exert local estrogenic stimulation of breast cancer cell growth at moderately high concentrations (30–1000 nM), an effect which is likely exerted following extensive metabolism to ring A-reduced metabolites, deserves careful consideration in view of the use of high doses (up to 80 mg/day) of NRE (in its acetylated form) in the progestin therapy of breast cancer [50, 56].

In conclusion, the present data show that synthetic 'progestins' derived from either 17-OHP or 19-NT can inhibit breast cancer cell growth through a combination of AR-, PgR-, and GR-mediated activities, according to the compound used. In addition, many of these compounds behave as pure or partial androgen and/or glucocorticoid antagonists, a behavior reflected by different maximal amplitudes of growth inhibition. Moreover, this study clearly illustrates that the ZR-75-1 cell line is a very useful model for assessing the complex interactions of steroids with ER, AR, GR, and PgR, and should help further understanding of the function of these receptors in the regulation of breast cancer.

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