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Lack of Estrogenic Potential of Progesterone- or 19-Nor-progesterone-derived Progestins as Opposed to Testosterone or 19-Nor-testosterone Derivatives on Endometrial Ishikawa Cells*

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Estrogen receptors of human endometrial cancer Ishikawa cells were found to be present in moderate amounts (160–200 fmol/mg protein), and to specifically bind moxestrol (R2858) with a very high affinity characterized by a K_d around 60 pM, when measured under equilibrium conditions. The binding specificity respected a decreasing order as follows: estradiol (E_2: 100%) > 4-hydroxy-tamoxifen (4OHTAM: 52.7%) > estriol(E_3: 5.7%) > estrone(E_1: 2.1%) > TAM (0.2%). The induction of alkaline phosphatase activity (APase) used as an estrogen-specific response, confirmed the intrinsic estrogenicity of progestins derived from 19-nor-testosterone (19NT): norethindrone (NOR), norethynodrel and levonorgestrel, at concentrations ranging from 10^{-8} to 10^{-6} M. The effect of NOR was partially blocked by the antiestrogen 4OHTAM, which was also partially agonistic in this model, but neither by the antiprogestin mifepristone (RU486) nor by the aromatase inhibitor aminoglutethimide. A simulatory effect was also detected at 10^{-7} or 10^{-6} M with ethindrone, the testosterone- (T) derived progestin homologous to NOR, and with both androgenic parent-compounds, i.e. T and 19NT themselves. In contrast, progesterone (P) derivatives like medroxyprogesterone acetate (MPA) and chlormadinone acetate (CMA) remained totally inactive, as well as 19-nor-progesterone (19NP) itself or its progestagenic derivatives: ORG 2058 and nomegestrol acetate (NOM). Structure–activity relationships deduced from these studies suggest that it is not the absence of the 19-methyl group which can account for the estrogenic potential of the so-called "19-norprogestins", but rather their steroid structure derived from T in a broad sense (including the 19NT derivatives), as opposed to the non-estrogenic therapeutic progestins derived from P like MPA or CMA, or from 19NP like NOM.

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INTRODUCTION

The estrogenic potential of progestins derived from 19-nor-testosterone (19NT) has recently been emphasized following studies at the cellular level on human estrogen-dependent breast [1–4] or endometrial [5, 6] cancer cell lines tested in estrogen-free conditions. The weak estrogenic properties of this class of compounds had been recognized very early in the process of defining their endocrinological profile in animal models, as recently recalled and reviewed by Edgren [7]. However, *in vivo* animal data did not appear to bear the same relevance as direct *in vitro* evidence on human material to point to the risk associated with an intrinsic estrogenic potential.

The list of progestins tested positive *in vitro* in this regard includes only 19NT derivatives: norethynodrel (NEL), 7α-methyl-NEL (Org OD 14), norethindrone (NOR), 7α-methyl-NOR, levonorgestrel (LNG), gestrinone, gestodene, 3-ketodesogestrel and 7α-methyl-19NT [1–6]. Only one control progestin has

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reproducibly been tested negative in different studies: medroxy progesterone acetate (MPA), a progesterone (P) derivative [1–3, 6]. P itself was inactive on endometrial carcinoma Ishikawa cells [6], as was ORG 2058, a 19-nor-progesterone (19NP) derivative on MCF-7 breast cancer cells [3]. Ambiguous results were reported with another 19NP derivative, promegestone (R5020), because at the same concentration of 10^{-6} M, it was inactive on Ishikawa cells [6] and only slightly stimulatory on MCF-7 cell growth and on an associated estogen receptor (ER) sensitive reporter gene system [1].

The aim of the present study was to confirm and extend the detection of intrinsic estrogenic properties among different classes of progestins derived either from 19NT, P or 19NP, utilizing the induction of alkaline phosphatase (APase) activity of human endometrial adenocarcinoma Ishikawa cells, as originally described by Littlefield *et al.* [8].

MATERIALS AND METHODS

Materials

Promegestone (R5020; 17α,21-dimethyl-19-norpregn-4,9-dien-3,20-dione), moxestrol (R2858) and [^{3}H]R2858 (87 Ci/mmol), were obtained from NEN, Du Pont de Nemours (Paris, France). Mifepristone (RU486) was from Cold Spring Biochemicals Ltd (Wilmington, DE, U.S.A.). ORG 2058 (16α-ethyl-21-hydroxy-19-norpregna-4-en-3,20-dione) and [^{3}H]-thymidine (85 Ci/mmol) were purchased from Amersham (Les Ulis, France). 19NP was from Organon (Oss, The Netherlands), ethindrone from Roussel Uclaf (Romainville, France) and 4-hydroxytamoxifen (4OHTAM) from Chimifar (Monaco). Nomegestrol acetate (NOM: 17α-acetoxy-6-methyl-19-norpregn-4,6-dien-3,20-dione), 17αOH-NOM and 17αOH-19NP were synthesized by Théramex (Monaco).

All media, sera and antibiotics for cell culture were obtained from GIBCO-Life Technologies (Cergy-Pontoise, France). Other not listed compounds and reagents were from Sigma (St Quentin Fallavier, France).

The purity of progestins are certified as greater than 99% by thin layer chromatography (TLC) for chlormadinone acetate, MPA and NEL. The purity of NOM is routinely checked by UV spectrometry and always exceeds 99%. Promegestone is certified as 98% pure by TLC. NOR and LNG are respectively purer than 99.5% by TLC and pure at 99.6% by HPLC. The other progestins are not certified with a defined threshold, but appear as monospot on TLC.

Cell culture

Ishikawa cells were kindly provided by Dr E. Gurpide (Department of Obstetrics, Gynecology and Reproductive Science, Mount Sinaï School of Medicine, New York, NY, U.S.A.) Cells were routinely grown in Dulbecco's modified Eagle's medium containing L-glutamine (4 mM) and glucose (4.5 g/l), and supplemented with penicillin–streptomycin (5000 IU–5 mg/l), insulin (0.6 μg/ml), transferrin (5 μg/ml) and phenol red (15 mg/l) and maintained in a humidified atmosphere of 7% CO_2 and 93% air at 37°C. Medium also contained 10% of decomplemented foetal calf serum (dFCS, by heat inactivation for 1 h at 56°C). Dextran-coated charcoal (DCC) treatment was further applied to obtain a steroid-stripped decomplemented serum (DCC-dFCS) for use in estrogen-free cell growth experiments.

Stocks were passaged once weekly to maintain continuous exponential growth. The cells used in these studies were from passages 5–20 in our laboratory. Cells were plated in 175 cm^2 flasks for binding studies, in 24-well microplates for proliferation assays or in 96-well microplates for APase measurements.

When needed, steroids or test substances were dissolved in ethanol and added to the culture medium (0.1% v/v) to obtain the final concentrations described in the figure legends. Ethanol (0.1% v/v) was also added to control cells.

Binding assay on ER from Ishikawa cells

On the day of the binding assay, the culture was removed from the flasks and the cells were washed twice with ice-cold phosphate buffered saline (PBS), harvested by scraping in TED buffer (10 mM Tris, 10 mM sodium molybdate, 1.5 mM EDTA, 1 mM DTT, pH 7.4 with HCl) (2 ml/175 cm^2 flask) and collected as a suspension. The cell contents of four flasks were pooled and homogenized in an all glass Dounce apparatus. The homogenate was centrifuged at 105,000 *g* for 1 h at 4°C. The supernatant cytosol was diluted in TED buffer (1.5–1.7-fold) and was immediately used for the ER binding assay. The cytosolic protein concentrations were determined by the method of Bradford [9] using bovine serum albumine (BSA) as a standard; they routinely ranged from 0.5 to 1.2 mg/ml.

For saturation analysis, cytosol samples were incubated with increasing concentrations of [^{3}H]R2858 (moxestrol) as described in the legend of Fig. 1. A parallel set contained a 1000-fold molar excess of estradiol (E_2) for non specific binding (NSB) determination. After 4.5 h of incubation at 25°C, bound and free steroid fractions were separated by incubation with DCC in ice-cold TED-buffer (2.5% charcoal, 0.25% dextran) for 10 min at 4°C. Incubations with DCC were terminated by centrifugation at 4000 *g* for 10 min, and aliquots were submitted to radioactive counting of the receptor-bound fraction. Data were plotted according to the method of Scatchard [10] after NSB substraction.

For relative binding affinity (RBA) determination, cytosols were incubated at 25°C with 1 nM of

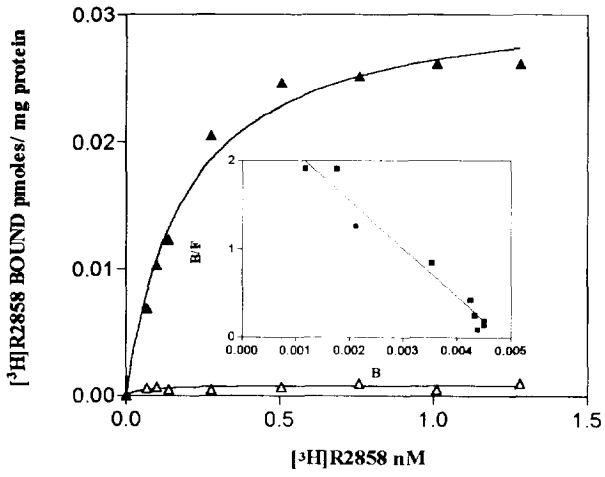

Fig. 1. Binding of [³H]R2858 to Ishikawa cell cytosol. Triplicate aliquots of cytosols (1.15 mg/ml protein) were incubated at 25°C for 4.5 h with 0.01–0.20 nM of [³H]R2858 for the determination of total binding. Incubations with [³H]R2858 in the presence of a 1000-fold molar excess of E_2 were made in parallel for the determination of non-specific binding (NSB, open symbols). The specifically bound radioactivity (closed symbols) was plotted after substraction of NSB and analysed according to Scatchard (inset). Each value is the mean of a triplicate determination for a representative experiment where K_d was 0.061 nM and B_{max} 0.161 pmol/mg protein.

[³H]R2858 for 4.5 h alone or in the presence of unlabelled compounds: estradiol, estriol, estrone, tamoxifen (TAM) and 4OHTAM. NSB was measured in parallel in the presence of a 200-fold molar excess of

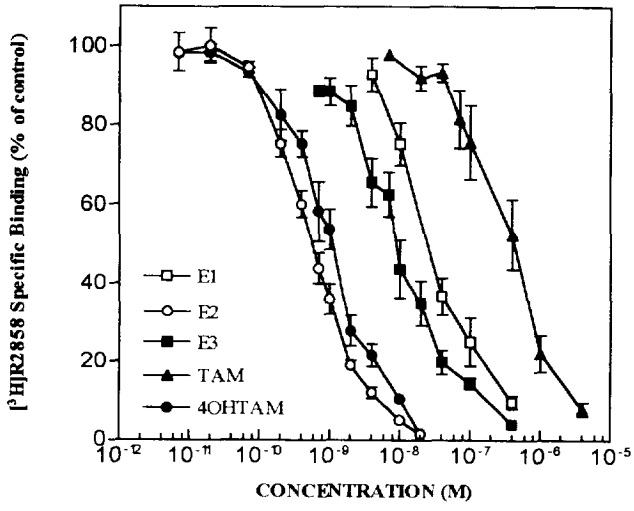

Fig. 2. Specificity of E_2 binding to Ishikawa cell ER. Triplicate aliquots of cytosols were incubated for 4.5 h at 25°C with 1 nM [³H]R2858 alone or together with 10^{-11}–10^{-5} M of unlabelled compounds. [³H]R2858 binding was determined by DCC assay as described in Materials and Methods. Non-specific binding was assessed by a 200-fold molar excess of E_2. Data points: means ± SEM of 4–6 separate experiments each run in triplicate.

Table 1. Relative binding affinities (RBA) for the ER of Ishikawa cells: competition against 1 nM of [³H]R2858 for 4.5 h at 25°C

	IC_{50} (nM)	RBA (%)
E_2	0.63 ± 0.06 (6)	100
4OHTAM	1.30 ± 0.20 (6)	52.7 ± 5.2**
E_1	10.8 ± 1.8 (4)	5.7 ± 0.5**
E_3	26.3 ± 3.3 (4)	2.1 ± 0.2**
TAM	420.1 ± 108.8 (5)	0.2 ± 0.05**

Means ± SEM for (n) determinations. IC_{50} is the approximate concentration of competitor causing 50% inhibition of [³H]R2858 binding; **$P < 0.01$ compared to E_2.

E_2. Samples were then submitted to DCC separation and radioactive counting.

Alkaline phosphatase assay

Ishikawa cells were plated at a density of 5×10^4 cells/cm² in 96-well microplates 48 h before studies. The next day, the medium was replaced by fresh medium containing 5% DCC-dFCS without phenol red. After an additional 24 h period, test substances were added. At the end of a 3 day treatment, the microplates were inverted to discard the growth medium and rinsed by immersion in PBS. The wash procedure was repeated once more. Afterwards, the inverted plates were gently blotted on a paper towel, covered and placed at −80°C for at least 15 min. Next, the cells were thawed at room temperature for

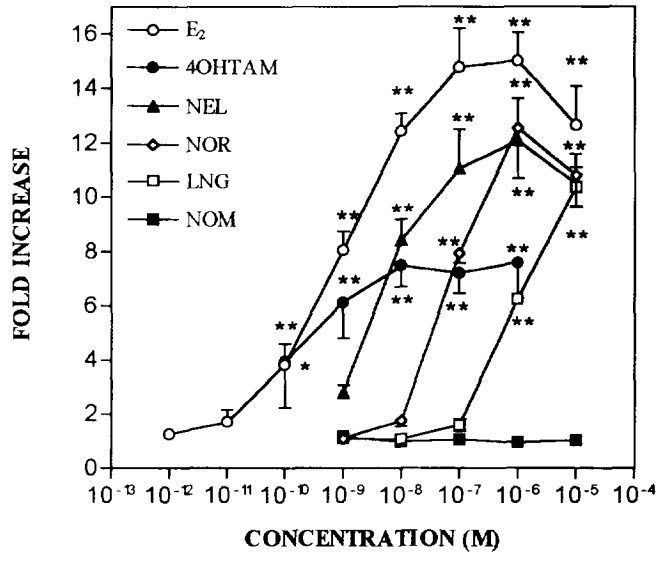

Fig. 3. Stimulation of the APase activity of Ishikawa cells by estradiol, 4OHTAM and 19NT-derived progestins: concentration–response relationships. Exponentially growing cells were plated in 96-well dishes as described in Materials and Methods. APase activity was measured after 3 days of incubation in the presence of the indicated concentrations of test compounds. Each point represents the mean ± SEM of 5 determinations each made in octuplicate wells in 5 different microplates. *$P < 0.05$ and **$P < 0.01$ as compared to control.

Table 2. Lack of estrogenicity of pregnane derivatives as revealed by APase assays in Ishikawa cells

Progestin	Fold increase (control = 1)		
	10^{-7} M	10^{-6} M	10^{-5} M
Medroxyprogesterone acetate	1.07 ± 0.04 (6)^{NS}	1.05 ± 0.06 (5)^{NS}	0.94 ± 0.02 (6)^{NS}
Chlormadinone acetate	1.04 ± 0.05 (6)^{NS}	1.04 ± 0.02 (6)^{NS}	0.91 ± 0.04 (5)^{NS}
ORG 2058	0.97 ± 0.05 (6)^{NS}	1.03 ± 0.04 (6)^{NS}	1.07 ± 0.04 (6)^{NS}
Promegestone (R5020)	1.05 ± 0.04 (6)^{NS}	1.06 ± 0.07 (10)^{NS}	4.50 ± 0.14 (10)**

Means ± SEM (n). ^{NS}, $P > 0.05$ and **$P < 0.01$ as compared to control cells grown in absence of estrogens. Control stimulation by estradiol 10^{-8} M was 11.92 ± 0.75 (12)-fold over control ($P < 0.01$).

5–10 min. The microplates were then placed on ice, and 50 µl of an ice-cold solution containing 5 mM *p*-nitrophenyl phosphate, 0.24 mM $MgCl_2$, and 1 M diethanolamine (pH 9.8) was added in each well. The yellow colorimetric reaction was started by warming at room temperature and allowed to develop for 30 min. Individual well absorbance measurements were performed with a microplate reader set at 405 nm.

Statistical analysis

Data are reported as the mean ± SEM and analysed by one-way analysis of variance, multiple range and Student's *t*-test.

RESULTS

Ishikawa cells contain specific and high affinity ER

Concentration–response curves (Fig. 1) for [^{3}H]R2858 binding to cytosolic receptors showed that saturation occurs from 1 nM. When data of three different experiments were plotted according to the method of Scatchard [10], a single class of high-affinity binding sites was demonstrated, with a K_d of 59.5 ± 0.9 pM. The number of binding sites (B_{max}) ranged from 0.161 to 0.207 pmol/mg protein (0.184 ± 0.013), and the NSB was less than 2% of the total binding.

The specificity of these binding sites was assessed in competition studies by incubating cytosols with 1 nM [^{3}H]R2858 together with unlabelled compounds in concentrations covering a 6-log range (Fig. 2). The ability of some estrogens and antiestrogens to compete for binding was shown to follow a decreasing order: $E_2 > 4OHTAM > E_3 > E_1 > TAM$ (Table 1).

19NT-derived progestins stimulate APase activity, but not NOM, a 19NP-derived progestin

In a first set of experiments, maximal stimulation of APase activity was achieved after 4 days of exposure to E_2 at concentrations ranging from 10^{-7} to 10^{-6} M, reaching a 15-fold increase over basal levels displayed by cells grown in the absence of any estrogen (Fig. 3). The calculated concentration corresponding to half-maximal stimulation (EC_{50}) was equal to 5.3 10^{-10} M. The active metabolite of TAM *in vitro*, 4OHTAM, behaved as a partial agonist when tested alone: a plateau of maximal APase activity was observed for concentrations of 10^{-8} M and above at a level equivalent to 40% of the maximal effect of E_2 (Fig. 3).

The 19NT-derived progestins were nearly full agonists in this model with an EC_{50} equal to 3.6×10^{-9} M for NEL, 6.4×10^{-8} M for NOR and 6.2×10^{-7} M for LNG. By contrast, NOM did not exert any effect on APase activity, even at 10^{-5} M (Fig. 3).

Pregnane derivatives do not stimulate APase activity as compared to androstane derivatives

In a second set of experiments (Table 2), two other potent P-derived progestins: ORG2058 and R5020, as well as two potent 19NP-derived progestins: MPA and chlormadinone acetate (CMA), were shown to be totally inactive on APase activity, except for a slight increase with R5020 at the very high concentration of 10^{-5} M. The parent-compound of NOM, i.e. 19NP itself, and two chemical intermediates, 17αOH-19NP and 17α-OH-NOM, also remained without effect on this parameter (Table 3).

By comparison, the parent compound of NOR, i.e.

Table 3. Lack of estrogenicity of 19NP and of 19NP derivatives related to nomegestrol acetate on the APase activity of Ishikawa cells

Steroid	Concentration (M)	Fold increase (n)
Estradiol	10^{-8}	4.46 ± 0.30 (6)**
19-nor-progesterone	10^{-7}	1.00 ± 0.04 (6)^{NS}
19-nor-progesterone	10^{-6}	1.52 ± 0.10 (6)^{NS}
17αOH-19-nor-progesterone	10^{-7}	1.00 ± 0.03 (6)^{NS}
17αOH-19-nor-progesterone	10^{-6}	1.02 ± 0.04 (6)^{NS}
Nomegestrol (17α-OH)	10^{-7}	0.99 ± 0.02 (5)^{NS}
Nomegestrol (17α-OH)	10^{-6}	0.99 ± 0.02 (5)^{NS}

**$P < 0.01$. $^{NS}P > 0.05$ as compared to control cells grown in absence of estrogens.

19NT itself, the 17β-acetate esterified form of NOR (NOR-Ac) and two full C19 structural counterparts, namely ethindrone and testosterone (T), all exerted some degree of stimulation of APase activity at 10^{-7} or 10^{-6} M (Fig. 4). The following sequence of potency may be drawn from this study: NOR > NOR-Ac $\geq$ 19NT > ethindrone $\geq$ T.

In all these experiments, the stimulation induced by 10^{-8} M E_2 was taken as a positive control.

The stimulation of APase by NOR is a specific estrogenic response

The stimulation induced by 10^{-6} M NOR was partially antagonized by 10^{-7} and 10^{-6} M of 4OHTAM, down to a level not different from that obtained with 4OHTAM alone; the same concentrations of RU486 were without effect on NOR-induced APase activity (Fig. 5). Alone, RU486 remained inactive from 10^{-9} to 10^{-6} M (not shown).

Aromatase inhibition does not prevent NOR from stimulating APase activity

The aromatase inhibitor aminoglutethimide (AMG) was tested either alone or in combination with NOR on Ishikawa cells. AMG was totally devoid of any effect, neither on basal APase activity, nor on the concentration-related stimulation induced by NOR (Fig. 6).

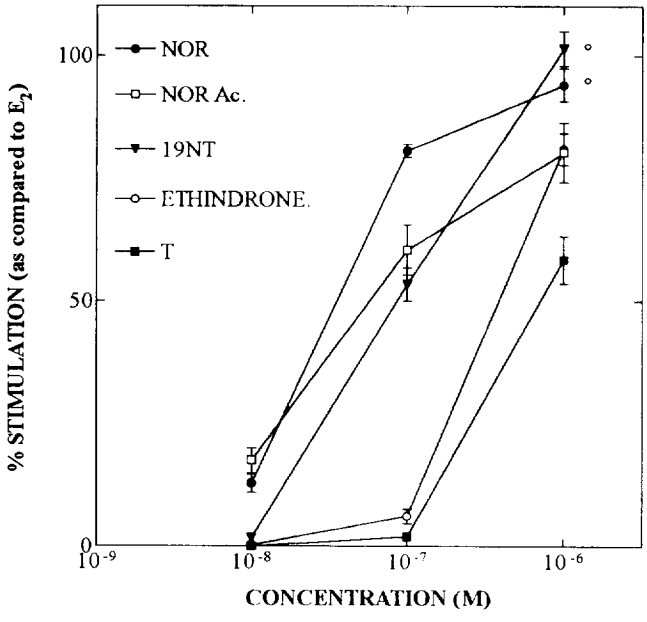

Fig. 4. Effect of androstane derivatives on the APase activity of Ishikawa cells. Exponentially growing cells were plated in 96-well dishes as described in Materials and Methods. APase activity was measured after 3 days of incubation in the presence of the indicated concentrations of test compounds. Each point represents the mean ± SEM of 5 determinations each made in octuplicate wells in 5 different microplates. Data are pooled from 3 different sets of 5 experiments each, in which the positive control stimulation induced by 10^{-8} M E_2 was 7.05 ± 0.61 when tested with T, 11.1 ± 0.80 with NOR-Ac and 12.6 ± 0.39 with NOR, ethindrone and 19NT. (○) NS ($P > 0.05$) as compared to stimulation by 10^{-8} M E_2 taken to be equal to 100%.

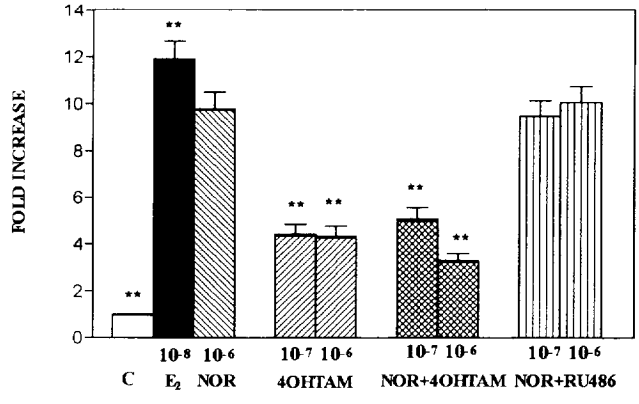

Fig. 5. Influence of 4OHTAM and of RU486 on the APase activity of Ishikawa cells stimulated by NOR. C, control cells grown alone without estrogen. E_2, cells grown with 10^{-8} M estradiol alone. NOR, norethindrone alone; NOR + 4OHTAM, cells grown in the presence of the indicated concentration of 4OHTAM plus 10^{-6} M NOR; NOR + RU486, cells grown in the presence of the indicated concentrations of RU486 plus 10^{-6} M NOR. Means ± SEM for 5 determinations each made in octuplicate wells in 5 different microplates. $^{**}P < 0.01$ as compared to NOR.

DISCUSSION

On the basis of APase induction in endometrial Ishikawa cells, the present study has clearly delineated two classes of compounds among progestins in terms of potential estrogenicity. But first of all, the specificity of

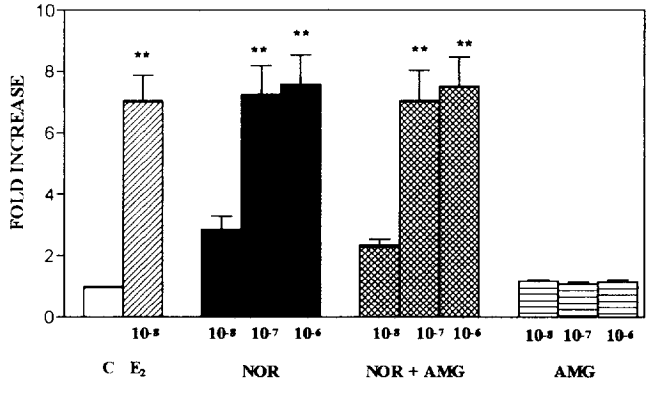

Fig. 6. Influence of aminoglutethimide (AMG) on the APase activity of Ishikawa cells stimulated by NOR. C, control cells grown alone without estrogens; E_2, cells grown with 10^{-8} M estradiol alone; NOR, cells grown with the indicated concentration (M) of norethindrone alone; AMG cells grown with the indicated concentrations (M) of aminoglutethimide alone; NOR + AMG, cells grown in the presence of the indicated concentration of NOR + 10^{-6} M AMG. Means ± SEM for 5 determinations each made in octuplicate wells in 5 different microplates. $^{**}P < 0.01$ as compared to control.

this model as an estrogenic yardstick had to be checked by binding, functional and inhibition studies.

The results reported here support the existence of a pool of high affinity estrogen binding sites in our strain of Ishikawa cells, with a K_d of 0.06 nM for the [^{3}H] synthetic estrogen moxestrol ([^{3}H]R2858) under equilibrium conditions [11]. The NSB was extremely low and the number of binding sites around 200 fmol/mg protein. In that way, our cells are different from the IK-90 subline of Ishikawa cells which did not display saturable binding sites for [^{3}H]E_2, and relate more closely to the estrogen receptor (ER) status of the original Ishikawa cell line, although the affinity and the number of binding sites for [^{3}H]E_2 were relatively different (K_d, 2.5 nM; B_{max}, 21.1 fmol/mg protein) [12]. The lower K_d presently observed fit better the value obtained with [^{3}H]E_2 in the human MCF-7 breast cancer cells [13]. Displacement of this high affinity ^{3}H-derivative of estradiol revealed the usual ranking of decreasing RBAs among natural hormones (E_2, E_3 and E_1), and a low IC_{50} for E_2 (0.6 nM). Then, the RBAs of TAM and 4OHTAM confirm the classical specificity of this Ishikawa cell ER since, firstly, TAM displayed a very small affinity [14] and, secondly, because its 4-hydroxylated metabolite was more than half as potent as E_2 in competing for ER binding [15].

Besides affinity properties, the APase activity of Ishikawa cells provided a sensitive and specific biological response to estrogenic stimulations, as originally described by Gurpide, Kuramoto, Markiewicz and coworkers [5, 6, 8, 16]. By comparison with their previous reports, the EC_{50} for E_2 was much higher in our study (0.5 nM) than the 0.006 nM reported in references [5, 6, 8]. However, the EC_{50} was very close to the IC_{50} displayed by E_2 (0.63 nM) against 1 nM [^{3}H]R2858 in our set of data. On the other hand, the maximal level of APase stimulation achieved with E_2 was similar, although some unexplained sporadic variability was encountered. The same positive control E_2 concentration of 10^{-8} M was used in our sets of experiments as in the first study by Holinka *et al.* [16]: they observed increases ranging from 3.4–20-fold with an overall mean stimulation factor of 9.4 [16], whereas our values ranged from 4.5–12.6-fold and gave a mean factor of 10.2. Later results from this group were expressed as relative values to the maximal estrogenic stimulation within each experiment, without mention of its absolute value [5, 6, 8], but representative experiments shown in figures usually displayed a 10–15-fold increase in APase activity [5, 8], which was also the most frequent order of magnitude measured in the present study.

Apart from the EC_{50} estimates, the only other discrepancy arose from the small effect found with 4OHTAM as opposed to its inactivity reported so far [8, 16]. Nevertheless, our results are consistent with the findings of Jamil *et al.* [17] who tested the two antiestrogens, 4OHTAM and ICI 164,384, on Ishikawa cell growth and progesterone receptor (PgR) concentration, another estrogen-specific parameter [17]. They observed an increase in both cell numbers and PgR levels following incubation with 4OHTAM, but not with ICI 164,384. Furthermore, the response to 4OHTAM could be totally blocked by ICI 164,384, therefore demonstrating the partial agonistic estrogenic activity of the former compound as opposed to the purely antiestrogenic nature of the latter [17]. Those hints of residual estrogenic effects on endometrial cells are consistent with the risk of endometrial cancer attributed to long term treatments of breast disease by tamoxifen [18].

The results on progestins were in a remarkably good agreement both qualitatively and quantitatively with previous reports by Markiewicz *et al.* [5, 6] in the ranking of the three 19NT-derived progestins: NEL > NOR > LNG, and in the absence of activity observed at 10^{-6} M with R5020, a 19NP derivative, and with MPA, a P derivative. Our results extend the list of potentially estrogenic progestins to NOR-Ac, which was only slightly less active than NOR, and more interestingly to ethindrone, the structural equivalent of NOR with the undeleted C19-methyl group, i.e. a T derivative. Conversely, the list of progestins devoid of any effect on Ishikawa cell APase activity grew by one more P derivative: chlormadinone acetate, and by two 19NP-derived compounds: NOM and ORG 2058.

One may argue that the estrogenic potential of a given progestin might be masked by its antiestrogenic potency. However, it has been clearly demonstrated that APase activity is not sensitive to the usual antiestrogenic effects of P and of its synthetic analogs: the stimulation by E_2, or by an estrogenic 19NT-derived progestin such as gestrinone, was not altered by P, present even in 100,000-fold molar excess [5]. Furthermore, MPA, which is devoid of any estrogenic activity in this model and generally considered as an effective antiestrogenic agent [7], did not modify the enzymatic response to 10^{-8} M E_2 when added at 10^{-6} M in the incubation medium [16]. Thus, it was concluded that any increase in APase activity of Ishikawa cells results from a truly intrinsic feature present for some progestins and absent for others, and not from a balance between agonism and antagonism of an estrogenic mechanism.

As far as the parent-compounds themselves are concerned, 19NT and even T were effective in stimulating APase activity, whereas 19NP was not, a finding which raised the issue of an androgen-mediated mechanism. To our knowledge, the androgen receptor status of Ishikawa cells has not been reported so far, but a few functional studies are available. Littlefield *et al.* [8] did not report any effect with T because they tested it only up to 10^{-7} M [8]. At this concentration, T was also inactive in our study, but at 10^{-6} M the increase in APase activity induced by T just exceeded 50% of the effect of E_2 10^{-8} M. Holinka *et al.* [16] tested both T

and dihydrotestosterone (DHT) at 10^{-6} M: DHT was more effective than T, with respectively 65 and 10% of the stimulation obtained with E_2 10^{-8} M [16]. Paradoxically, the DHT-induced increase in APase activity was totally abolished by 10^{-6} M of 4OHTAM but only partially inhibited by a 100 times higher concentration of the pure antiandrogen, hydroxyflutamide [16], suggesting an estrogenic rather than an androgenic pathway for the action of DHT. In addition, the full C19 androgen of adrenal origin: 3β,17β-dihydroxy-androst-5-ene (Δ^5-androstenediol) was shown to be the only androgen able to stimulate the APase activity at 10^{-7} M [8], although it is a much weaker androgenic agent that DHT. Furthermore, the effect of Δ^5-androstanediol was effectively blocked by both antiestrogens, 4OHTAM and LY 156758, at an equimolar concentration, once more indicating the involvement of an estrogenic mediation [8].

Consequently, the induction of Ishikawa cells APase activity is recognized as a good predictor of an intrinsic estrogenic potential not only for progestins in particular, but also for other steroids in general.

In this respect, Δ^5-androstenediol is a good example of a T derivative which does not belong to the 19-nor family, which is not even a progestin, and which nevertheless does exert a full estrogenic effect with an EC_{50} in the same concentration range as NOR, the typical 19NT-derived progestin.

Those various lines of evidence suggest that the abbreviated generic label of "potentially estrogenic 19-norprogestins" is a misnomer without the mention of the testosterone origin of the compounds, which in itself appears to be a sufficient structural feature to raise the suspicion of residual estrogenic properties. In addition, the assertion that the loss of the C19-methyl group does enhance the intrinsic estrogenic properties of steroids should be restricted to T derivatives, as indicated by the 10-fold increase in potency from T to 19NT and from ethindrone to NOR, and not applied to the P family of progestins.

How do T-derived steroids express their estrogenic potential? They may directly bind the ER with a small but biologically relevant affinity, which seems to be the case of Δ^5-androstenediol [19, 20], and perhaps also of NEL, 7α-methyl-NEL (Org OD 14) or 7α-methyl-NOR (Org OD 14 4-ene isomer) [21–28]. However, NOR, LNG, and most other 19NT-derived progestins are not considered to significantly bind the ER [21, 22], and therefore, are supposed to need to be converted into active metabolites in order to exert estrogenic effects. Aromatization is the first metabolic process to come to mind, since for example it would transform NOR into ethinyl-E_2, an estrogen reported to be 4.8 times more active than E_2 in the same Ishikawa cell APase assay [8]. Aromatization of NOR remains a controversial issue, despite several *in vitro* reports recently reviewed by Fotherby [23] and one *in vivo* estimate of 2% of conversion into ethinyl-E_2 in two perimenopausal women [24]. But it is not a mechanism involved in the estrogenic activity of NOR on Ishikawa cells because aromatase inhibition by aminoglutethimide did not modify their concentration-related response to this 19NT-progestin.

Thus, some other type of estrogenic metabolites must account for the stimulation of APase activity; they are to be found among the A-ring reduced NOR derivatives, namely the 5β- or 5α-dihydrosteroids or the four possible tetrahydrosteroids (3α,5α; 3β,5α; 3α,5β; 3β,5β), which altogether represent the bulk of its terminal degradation by-products, like for all other 19NT-progestins [23, 25]. The 5α-reduced form of NOR has been reported to have enhanced androgenic properties but no estrogenic activity, whereas the 3β-hydroxylated and 5α-reduced metabolite was clearly a weak estrogen [26, 27]. The same observation has been made for T itself, with 3β,17β-dihydroxy-5α-androstane being able to directly bind with moderate affinity the ER from calf and rat uterus; in the same set of data, the 3α-hydroxylated isomer (3α,17β-dihydroxy-5α-androstane) was a much weaker competitor [19]. The reduction of a ketone in position 3 of 7α-methyl-NEL (Org OD 14) into a 3-hydroxy group, independently of its α or β configuration, was also associated with an increase in estrogenic activity *in vitro* on normal human endometrium in culture [21]. Furthermore, the enzyme 3β-hydroxysteroid dehydrogenase/isomerase responsible for both the reversible oxido-reduction of the 3β-hydroxy group and the isomerization between Δ^4 and Δ^5 double bonds has been shown to modulate the estrogenic activity of Org OD 14 [28]. Together with the data on Δ^5-androstenediol [8, 19, 20], these sparse structure–activity relationships suggest the involvement of a free 3-hydroxy group (preferably in β-configuration) in the mediation of the estrogenic potential of T-derived compounds after metabolic reductions.

The free 17β-hydroxy group must be an even more important structural feature, because it is present prior to any metabolic transformation in all T and 19NT steroids shown *in vitro* to be estrogenic on Ishikawa cells. In the case of NOR-Ac, the 17β-acetate group is most probably hydrolyzed by an esterase like the one that cleaves free E_2 from E_2-17β-valerate or 17β-stearate [8]. It should also be recalled that when the 17β-OH of E_2 is switched to the 17α position, there is a 200-fold decrease in potency on the same Ishikawa cell APase model [8]. This 17β-OH requirement would nicely explain the lack of intrinsic estrogenic properties of P and 19NP steroids, because they all carry a 17β side-chain of 2 carbon atoms (3 for R5020).

In conclusion, as could have been predicted from initial *in vivo* animal studies in which no P-derived progestin has ever been found to display the slightest estrogenic effect [7], and from the similar pharmacological profile of at least one 19NP-derived progestin (NOM) [29, 30], no estrogenic potential was detected

in vitro on human endometrial Ishikawa cells among the representatives of the pregnane family. Results on promegestone (R5020) remain equivocal due to the very high concentration (10^{-5} M) needed to record a small response. By contrast, all androstane steroids derived from T were able to elicit to some extent an increase in APase activity, an estrogen-specific response of Ishikawa cells. The most active compounds were the 19NT-derived progestins, to which an intrinsic estrogenic potential definitely needs to be attributed and, moreover, restricted by comparison with the non-estrogenic P and 19NP progestagenic derivatives.

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