Comparison of 7 α -methyl-19-nortestosterone effectiveness alone or combined with progestins on androgen receptor mediated-transactivation

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Abstract

7 α -methyl-19-nortestosterone (MENT) is an androgen with potent gonadotropin inhibitory activity and prostate-sparing effects. These attributes give MENT advantages over testosterone as a male contraceptive, but, as in the case of testosterone, a partial dose-dependent suppression of spermatogenesis has been observed. Combination of testosterone or MENT with synthetic progestins improves the rate of azoospermia; however, it is unknown whether these combinations affect hormone androgenicity or exert synergistic effects via gestational or androgenic interaction. Herein, using transactivation assays, we examined the ability of MENT alone or combined with several 19-nor-derived synthetic progestins to activate androgen receptor (AR)-dependent gene transcription. In addition, the capability of 7 α -methyl-estradiol (7 α -methyl-E₂), an aromatized metabolite of MENT, to transactivate gene transcription via estrogen receptor α (ER α ; ESR1) or ER β (ESR2) was also investigated. As expected, MENT induced gene transactivation through either the progesterone receptor (PGR) or the AR. MENT was as efficient as progesterone in activating PGR-mediated reporter gene expression, but it was ten times more potent than testosterone and dihydrotestosterone in activating of AR-driven gene expression. The addition of increasing concentrations of other 19-nortestosterone derivatives (norethisterone or levonorgestrel) did not affect, in a significant manner, the ability of MENT to activate AR-dependent reporter gene transcription. The same results were obtained with different cell lines. 7 α -Methyl-E₂ resulted in potent estrogen activity via both ER subtypes with efficiency similar to natural E₂. These results suggest that the addition of 19-nortestosterone-derived progestins, as a hormonal adjuvant in male fertility strategies for effective spermatogenic suppression, does not display any detrimental effect that would interfere with MENT androgenic transcriptional activity.

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Introduction

Androgen administration to men has two main purposes, as hormonal replacement therapy to support a wide spectrum of physiological needs and as a contraceptive agent for its ability to suppress both gonadotropins and spermatogenesis. Most of the currently available preparations include natural testosterone or its 17 β -hydroxyl ester derivatives, the effects of which on muscle, bone, prostate, sexual behavior and gonadotropin regulation, among others, reflect those of testosterone itself. In general, the biological impact of testosterone is derived from the sum of testosterone actions and its metabolites, mainly 5 α -dihydrotestosterone (DHT) and estradiol (E₂).

In contrast to E₂, whose actions have desirable effects in adult men (Zmuda *et al.* 1993, Bagatell *et al.* 1994), DHT has not yet been shown to display any known beneficial effect, but it is considered to be a permissive factor in the progression of benign hyperplasia of the prostate, balding, and acne (Mooradian *et al.* 1987). An ideal synthetic androgen for men should have the advantages of testosterone in terms of its anabolic effects but with some metabolic restrictions such as not undergoing 5 α -reduction to DHT, but otherwise aromatization to estrogens. In this regard, it is well known that the addition of a 7 α -methyl group to 19-nortestosterone increases its androgenic and anabolic actions (Kumar *et al.* 1999), but prevents

5 α -reduction (Agarwal & Monder 1988, Kumar *et al.* 1992). Therefore, the synthetic androgen 7 α -methyl-19-nortestosterone (MENT) seems to be the compound of choice, since it has the beneficial effects of testosterone without the local androgenic side effects of DHT (Sundaram *et al.* 1994). When administered through implants to healthy men, MENT showed a potent suppressive effect on testicular function and, as in the case of testosterone, its effects on pituitary gonadotropins and sperm counts were dose related (Suvisaari *et al.* 1997). A number of studies have shown that combination of androgens and androgenic progestins such as levonorgestrel (LNG), etonogestrel (ENG), and norethisterone (NET), which are all 19-nortestosterone derivatives, increases the rate of azoospermia. This combined method allows reduction of the high dose of testosterone needed to suppress gonadotropins and its secondary adverse effects, while maintaining the beneficial peripheral actions of testosterone and, therefore, its potential utility as a male contraceptive (Meriggiola & Bremner 1997, Anderson & Baird 2002, Meriggiola *et al.* 2005, 2006, Meriggiola & Pelusi 2006, Walton *et al.* 2007). Although it is well known that some progestins can effectively suppress gonadotropin secretion and have a direct inhibitory effect on Leydig cell steroidogenesis, it remains to be seen whether the hormonal combination has an additive agonist-antagonist androgenic effect. Accordingly, we have studied the functional abilities of MENT alone or in combination with several progestins to transactivate androgen receptor (AR)-dependent gene transcription.

Results

Transactivation of gene transcription via progesterone receptor and AR by MENT and progestins

Because of known high affinity of MENT for both progesterone receptor (PGR) and AR, we were interested in investigating the effects of MENT on transactivation of transcription via these two receptors. The effects of MENT on receptor-mediated reporter gene transcription were compared with those obtained with progesterone (P₄), DHT, or testosterone respectively. PGR-mediated activity was studied in the presence of increasing concentrations (1×10^{-12} to 10^{-6} M) of P₄ or MENT as shown in Fig. 1A. In these experiments we also tested, as an additional positive control, another synthetic progestin, nestorone, a highly selective 19-norprogesterone derivative with no androgenic and estrogenic activities (Kumar *et al.* 2000). As shown, MENT exhibited 2.38 times more transcriptional activity than P₄, with effective doses (ED₅₀) of 7.46×10^{-10} and 1.78×10^{-9} M for MENT and P₄ respectively. As shown in Fig. 1A, nestorone was shown to be 100 times more potent than P₄ with an ED₅₀ of 6.25×10^{-12} M. Incubations in the presence of mifepristone (RU486)

Figure 1 (A) Transcriptional activation via the progesterone receptor (PGR). HeLa cells were transiently transfected using the PRE-Elb-CAT reporter plasmid and the expression vector for PGR. Cells were cultured in the presence of increasing concentrations (10^{-12} to 10^{-6} M) of P₄ (filled circle), MENT (filled triangle), and nestorone (filled diamond). CAT activity was determined in 5 μ g cellular protein. Natural P₄ and nestorone were used as natural and synthetic positive controls for the PGR. Each point represents the mean $\pm$ s.d. of three independent experiments and values are expressed as the percentage of the CAT activity induced by 10^{-9} M P₄ (100%). (B) Effects of mifepristone (RU486), a PGR antagonist on PGR-driven reporter gene transcription. Transfected HeLa cells were incubated in the presence of 1×10^{-9} M of P₄, nestorone, and MENT with or without RU486. No reporter gene transcription was observed in control incubations in the presence of E₂ or in the presence of P₄ in non-PGR transfected cells. * $P < 0.005$ vs vehicle (V); ** $P < 0.005$ vs control incubations without RU486. The value obtained with P₄ at the dose of 1×10^{-9} M was taken as 100% transcriptional activity. MENT, 7 α -methyl-19-nortestosterone.

resulted in the inhibition of MENT and nestorone effects upon PGR-driven transcriptional activation (Fig. 1B). No effects, different from control vehicle, were observed with mifepristone alone or with DHT, including with P₄ on cells not transfected with PGR.

A similar experiment but using cells transfected with the AR is shown in Fig. 2A. The androgenic activity of MENT was compared with those of DHT and testosterone. All three androgens significantly increased the reporter activity in a dose-dependent manner. In this assay, MENT was about 100 times more potent than testosterone as shown by the calculated ED₅₀ values

Figure 2 (A) Transcriptional activation via the androgen receptor (AR). HeLa cells were transiently transfected using the PRE-Elb-CAT reporter plasmid and the expression vector for AR. Cells were cultured in the presence of increasing concentrations of testosterone (filled circle), DHT (filled square), MENT (filled triangle) and nestorone (filled diamond). Natural testosterone and DHT were used as natural positive controls for the AR. Each point represents the mean $\pm$ the s.d. of three independent experiments and values are expressed as the percentage of CAT activity induced by 1×10^{-9} M DHT (100%). Nestorone, which does not bind to the AR, was used as a negative control. (B) Effects of flutamide (FLUT), an AR antagonist, on AR-driven reporter gene transcription. Transfected HeLa cells were incubated in the presence of 1×10^{-9} M of testosterone, DHT, and MENT, with or without FLUT. * $P < 0.005$ vs vehicle (V); ** $P < 0.005$ vs control incubations without FLUT. The value obtained with DHT at the dose of 1×10^{-9} M was taken as 100% activity. DHT, 5 α -dihydrotestosterone; MENT, 7 α -methyl-19-nortestosterone; CAT, chloramphenicol acetyltransferase.

(MENT, 3.42×10^{-12} M; DHT, 7.0×10^{-11} M; and testosterone, 4.15×10^{-10} M). All transcriptional responses were significantly inhibited by the addition of flutamide (Fig. 2B). No effects, different from control vehicle, were observed with flutamide alone or P₄ or with DHT on cells not transfected with AR.

As shown in Fig. 3, both NET and LNG induced reporter gene transcription through the AR and PGR. Through both receptors, LNG induced significantly higher transcriptional responses than NET and they were significantly inhibited in the presence of flutamide or RU486 respectively.

Effects of addition of progestins on MENT-induced transcriptional activation

The potency of MENT on AR-driven reporter gene transcription was investigated in the presence of increasing concentrations of selected progestins. The potency of MENT was expressed as the ratio of androgen to P₄-driven transcriptional activation (ED₅₀ indexes) as a measure of androgenicity vs progestogenicity (selectivity index). The results were compared with the selectivity index of MENT obtained in the absence or presence of progestins. Figure 4 shows the induction of AR-mediated reporter transcription by increasing concentrations of MENT (1×10^{-11} to 1×10^{-8} M) in the presence or absence of NET and LNG at a concentration equal to their ED₅₀ to activate androgen-driven reporter expression (2.07×10^{-9} and 1.05×10^{-10} M for NET and LNG respectively). The activity of DHT at the concentration of 1×10^{-9} M was taken as 100% response. The addition of NET and LNG did not modify the ability of MENT to activate androgen-driven reporter gene transcription (ED₅₀ = 6.04×10^{-12} and 2.23×10^{-12} M for NET and LNG respectively). Incubations of MENT (1×10^{-11} to 1×10^{-8} M) in the presence of increasing concentrations of NET or LNG (1×10^{-10} to 1×10^{-8} M) did not result in a significant change in the MENT selectivity index compared with that obtained in the absence of progestins (Table 1). Incubations in the presence of increasing concentrations of nestorone, a synthetic progestin, which does not bind with the AR and used as a negative control, did not affect MENT-induced reporter transcriptional activity. Similar results on the effects of addition of progestins on MENT induced AR-mediated transactivation in HeLa cells were also observed in an AR transfected PC3 prostate cell line (data not shown) and in HEK-293 and T47D AR-positive cells (Fig. 5A and B respectively).

Transactivation of estrogen receptors by the aromatized MENT metabolite 7 α -methyl-E₂

In vitro, MENT undergoes aromatization to the active estrogen 7 α -methyl-E₂ (LaMorte *et al.* 1994); it was therefore thought important in this study to investigate

Table 1 Transcriptional effective stimulatory dose (ED₅₀) and the androgen-to-progestin (AR/PGR^a) indexes of 7 α -methyl-19-nortestosterone (MENT) alone or combined with 19-nor-derived progestins.

Ligand (M)	MENT-ED ₅₀ AR (M)	MENT-ED ₅₀ -index AR/PGR ^a
MENT	3.42×10^{-12}	5.13×10^{-3}
MENT + NET ⁻¹⁰	2.59×10^{-12}	3.47×10^{-3}
MENT + NET ⁻⁹	3.52×10^{-12}	4.72×10^{-3}
MENT + NET ⁻⁸	4.02×10^{-12}	5.39×10^{-3}
MENT + LNG ⁻¹⁰	2.77×10^{-12}	3.71×10^{-3}
MENT + LNG ⁻⁹	1.74×10^{-12}	2.33×10^{-3}
MENT + LNG ⁻⁸	1.63×10^{-12}	2.18×10^{-3}

^aMENT-PGR ED₅₀ = 7.46×10^{-10} M.

Figure 3 Transcriptional activation via the androgen receptor (AR) and progesterone receptor (PGR) by NET and LNG in HeLa cells respectively. Incubations were done in the presence of 1×10^{-9} M of agonist with or without 1×10^{-7} and 1×10^{-4} M of RU486 and FLUT respectively. * $P < 0.05$ vs LNG; ** $P < 0.05$ vs incubations in the absence of antagonists. The value obtained with DHT and P_4 at the dose of 1×10^{-9} M was taken as 100% transcriptional activity. No effects upon transcription were observed with antagonists only. NET, norethisterone; LNG, levonorgestrel; FLUT, flutamide; DHT, 5 α -dihydrotestosterone.

the ability of this estrogen to activate transcription via both estrogen receptor (ER) subtypes. Figs 6 and 7 show the estrogen agonist activity of both E_2 and 7 α -methyl- E_2 in terms of transactivation via the ESR1 or ESR2 respectively. As can be seen, the aromatized metabolite of MENT activated reporter gene transcription through both receptors with similar ED_{50} values (8.04×10^{-12} and 1.27×10^{-10} M for the ESR1 and ESR2 respectively), to those observed with E_2 (7.69×10^{-12} and 1.84×10^{-10} M for the ESR1 and ESR2 respectively). Incubations in the presence of ICI, an ER antagonist, resulted in a significant inhibition of 7 α -methyl- E_2 effects upon transcription via the ESR1 (Fig. 6B) and ESR2 (Fig. 7B). No effects, different from the control vehicle, were obtained with ICI alone, nestorone or with E_2 on cells not transfected with ER subtypes.

Discussion

Addition of a 7 α -methyl group to 19-nortestosterone significantly enhances its androgenic potency and ability to bind to the AR; however, this 7 α -methyl-substituted steroid is not reduced by prostatic 5 α -reductase (Liao *et al.* 1973). Indeed, in contrast to testosterone, MENT is less potent on male accessory sex glands compared with its ability to suppress gonadotropins; MENT otherwise can be aromatized to 7 α -methyl- E_2 (LaMorte *et al.* 1994). These observations indicate that MENT, as a component for androgen replacement therapy or male contraceptive, has health-promoting effects, particularly by avoiding hyper-stimulative effects on the prostate, including prostate-specific antigen (Kumar *et al.* 1992, Morali *et al.* 1993, Cummings *et al.* 1998, Anderson *et al.* 2003, von

Eckardstein *et al.* 2003, Walton *et al.* 2007), and those derived from MENT aromatization (LaMorte *et al.* 1994, Anderson *et al.* 1999).

Suppression of gonadotropin secretion with exogenously administered testosterone and therefore testicular activity is important for hormone-based male contraception approach. In this regard, MENT when administered to healthy men leads to gonadotropin suppression and inhibition of spermatogenesis making it potentially useful as a male contraceptive (von Eckardstein *et al.* 2003). In the case of testosterone, combination with gonadotropin-inhibiting agents such as progestins results in a more effective formulation for spermatogenic suppression than testosterone alone (Bebb *et al.* 1996). Recently, Walton *et al.* (2007) showed that combination of MENT with a progestin resulted in rapid inhibition of spermatogenesis similar to the combined testosterone formulation and pointed out the importance of the

Figure 4 Dose-response curves for MENT (filled triangle), DHT (filled square), and MENT plus NET (filled circle) or LNG (filled diamond) on AR-dependent gene reporter activation. HeLa cells cotransfected with PRE-Elb-CAT reporter plasmid and the expression vector for AR were incubated in the presence of increasing concentrations of MENT alone or in combination with a single dose of 2.07×10^{-9} M NET (A) or 1.05×10^{-10} M LNG (B). These doses corresponded to their respective ED_{50} value of NET and LNG to activate AR-dependent gene transcription respectively. Each point represents the mean $\pm$ s.d. of three independent experiments and values are expressed as the percentage of the CAT activity induced by 1×10^{-9} M DHT (100%). DHT, 5 α -dihydrotestosterone; MENT, 7 α -methyl-19-nortestosterone; CAT, chloramphenicol acetyltransferase.

synthetic progestin dose over the androgen dose in improving spermatogenesis suppression. However, subjects treated with four MENT implants exhibited higher and longer spermatogenesis suppression (von Eckardstein *et al.* 2003) than subjects treated with two MENT implants plus two ENG implants (Walton *et al.* 2007). Four MENT implants induced azoospermia or severe oligozoospermia (sperm count $<1 \times 10^6/\text{ml}$) in 82% and 100% of subjects during the first and second 6-month periods of treatment respectively. Thus, during the last 6 months of treatment, MENT alone was as effective as testosterone undecanoate plus NET enanthate administered at 8-week intervals (Meriggiola *et al.* 2005).

Several synthetic progestins behave as weak androgens, particularly medroxyprogesterone acetate, LNG, and NET (Perez-Palacios *et al.* 1981, Bergink *et al.* 1983, Kloosterboer *et al.* 1988, Garcia-Becerra *et al.* 2004). Indeed, in a previous publication from this laboratory these compounds were able to activate AR-dependent reporter gene expression, with LNG acting as the strongest activator (Garcia-Becerra *et al.* 2004). In males, administration of P_4 or synthetic progestins, in addition to their inhibitory effects on Leydig cell steroidogenesis (El-Hefnawy & Huhtaniemi 1998) and 5α -reductase activity (Mauvais-Jarvis *et al.* 1974), inhibit gonadotropin secretion and suppresses spermatogenesis (Anderson & Baird 2002). Although the addition of some other antigonadotropic compound ensures effective suppression of gonadotropin secretion, it is still unknown whether the hormonal combination may or may not affect, in an agonist-antagonist fashion, the androgenic activity of MENT at the level of AR. This observation is of particular importance since androgens are required to maintain a number of physiological parameters in men, including, among others, muscle metabolism, erythropoiesis, sexual activity, and function of accessory sex organs. Therefore, we thought it was of interest to evaluate the ability of MENT alone or combined with other synthetic progestins to activate gene transcription via the AR.

In this study, we confirmed the androgenic and progestational activities of MENT. Both MENT and synthetic progestins activated AR- and PGR-dependent reporter gene transcription, with MENT being the strongest AR activator. We used the ratio of androgen to P_4 transcriptional activation potencies (ED_{50} indexes) of MENT, alone or in the presence of various progestins (selectivity index), as a measure of androgen vs progestogenic activity. Results were normalized against MENT's ED_{50} for PGR in the absence of progestins. The smaller the ratio, the greater the difference between the androgen-to- P_4 transcriptional responses. Changes in MENT selectivity index values were taken as indicating a decrease or increase in androgenic transcriptional activity. It was interesting to see that neither P_4 nor DHT activated transcription through the AR and PGR respectively.

In this study, none of the progestins with (LNG and NET) or without (nestorone) affinity for the AR was able to significantly modify the MENT selectivity index. These results indicated that synthetic progestins, at the doses tested, did not act as agonists or antagonists of MENT upon the AR-dependent transcriptional assay, suggesting that addition of progestins to actual or future MENT formulations will not interfere with their expected androgenic biological responses. It is also important to mention that although MENT does not undergo further metabolism to 5α -reduced metabolites, reduction of the 4-ene-double bond of LNG and NET does not change

Figure 5 Transcriptional activation via the androgen receptor (AR) by MENT in the presence or absence of NET and LNG in HEK-293 (A) and T47D cells (B). Incubations were done in the presence of 1×10^{-9} M of MENT in the absence or presence of NET (2.07×10^{-9} M) or LNG (1.05×10^{-10} M). Control incubations were carried out in the presence of 1×10^{-4} M flutamide (FLUT). * $P < 0.001$ vs vehicle (V); ** $P < 0.05$ vs NET; *** $P < 0.001$ vs incubations in the absence of antagonists. The value obtained with DHT at the dose of 1×10^{-9} M was taken as 100% transcriptional activity. No effects upon transcription were observed with the antagonist only. Each bar represents the mean $\pm$ s.d. of three independent experiments. DHT, 5α -dihydrotestosterone; MENT, 7α -methyl-19-nortestosterone; CAT, chloramphenicol acetyltransferase; NET, norethisterone; LNG, levonorgestrel; FLUT, flutamide.

Figure 6 (A) ESR1-mediated gene reporter activation by 7 α -methyl-E₂. HeLa cells cotransfected with the estrogen-responsive reporter plasmid (ERE-E1b-CAT) and the expression vector for ESR1 were incubated in the presence of increasing concentrations of 7 α -methyl-E₂ (filled square) or E₂ (filled circle). (B) Additional control incubations were done in the presence of 7 α -methyl-E₂ or E₂ at the dose of 1×10^{-9} M with or without ICI, an ER antagonist. No reporter gene transcription was observed in incubations in the presence of nesterone or E₂ in non-ESR1 transfected cells. Each point represents the mean $\pm$ S.D. of three independent experiments and values are expressed as the percentage of the CAT activity induced by 1×10^{-9} M E₂ (100%). * $P < 0.005$ vs vehicle (V); ** $P < 0.005$ vs incubations in the absence of ICI. ESR1, estrogen receptor 1; CAT, chloramphenicol acetyltransferase.

significantly their androgenic transcriptional activities (Sundaram *et al.* 1995, García-Becerra *et al.* 2004) so as to have unwanted effects upon the prostate. Our results may also support promising approaches for male fertility regulation in terms of raising the dose of progestins to improve sperm suppression and minimize MENT non-reproductive androgenic effects (Anderson & Baird 2002, von Eckardstein *et al.* 2003, Walton *et al.* 2007).

Although the affinity of MENT and progestins for the AR was not estimated, particularly due to the lack of radiolabeled ligands, special attention should be paid to recent observations of Simons (2008) showing that changes in receptor sequence may alter transcriptional activities without affecting receptor binding affinities. This observation, suggesting that a single ligand concentration can differentially affect gene transactivation, should be taken into consideration when data obtained *in vitro*, as in this study, are thought to be

applied to *in vivo* conditions. In addition, receptor mutations may also increase affinity for coactivators or corepressors, as well as show differential activity depending on the use of promoters. In this study, instead of using different reporter gene constructs, we used different cell lines with or without endogenous AR expression. In these experiments similar if not identical results were obtained among all cell lines studied. However, additional experiments should be performed to investigate receptor structural conformations and the binding of selective co-regulatory proteins in the presence of different ligands, which will importantly contribute to a better understanding of MENT selective actions on the AR.

In this study, we also tested the ability of 7 α -methyl-E₂, the aromatized metabolite of MENT, to transactivate reporter gene expression via both ESR1 and ESR2. Our results confirmed and established the fully estrogenic activity of 7 α -methyl-E₂ throughout both ER subtypes. Although at present it is not known to what extent MENT is metabolized to an estrogenic compound *in vivo*, it is well known that metabolism of testosterone, particularly

Figure 7 (A) ESR2-mediated gene reporter activation by 7 α -methyl-E₂. HeLa cells were cotransfected and incubated in the presence of increasing concentrations of 7 α -methyl-E₂ (filled square) or E₂ (filled circle) as indicated in Fig. 6. (B) Control incubations were performed as indicated in Fig. 6. Each point represents the mean $\pm$ S.D. of three independent experiments and values are expressed as the percentage of the CAT activity induced by 1×10^{-9} M E₂ (100%). * $P < 0.005$ vs vehicle (V); ** $P < 0.005$ vs incubations in the absence of ICI. ESR1, estrogen receptor 1; CAT, chloramphenicol acetyltransferase.

to aromatized derivatives, is important in mediating its effects on several physiological processes, including control of gonadotropin secretion, maintenance of bone mass, and sexual behavior (Morali *et al.* 1977, Finkelstein *et al.* 1991, Vanderschueren *et al.* 2004). The fact that 7 α -methyl-E₂ resulted in a potent estrogenic activity via both ER subtypes suggests that some anabolic actions of MENT may be mediated by this metabolite. However, whether MENT biotransformation to an active estrogen is responsible for some androgen-dependent outcomes observed in animal and human studies is still uncertain.

In conclusion, this study demonstrated that combination of MENT and synthetic progestins with affinity for AR results in activation of gene transcription in a similar manner to that obtained with MENT alone. These results indicated that addition of progestins to MENT formulations for effective spermatogenic suppression, as a promising approach for male fertility regulation, may not interfere with the beneficial androgenic effects of MENT. Furthermore, the fact that MENT does not undergo 5 α -reduction, in addition to its ability to be aromatized to an estrogen metabolite with activity via both ER subtypes, makes MENT an ideal synthetic androgen for men.

Materials and Methods

Reagents

Non-radioactive P₄, DHT, E₂, testosterone, and trypsin (type III) were purchased from Sigma-Aldrich. [³H]Chloramphenicol (specific activity, 38.9 Ci/mmol) was purchased from DuPont NEN Research Products (Boston, MA, USA). Unlabeled RU-486 (RU 38486, mifepristone) was a gift from Roussel Uclaf (Romainville, France) and the antiestrogen ICI 162 780 was purchased from AstraZeneca. Radioactivity was measured in a Beckman LS 6500 scintillation system (Beckman Coulter, Brea, CA, USA) using Biodegradable Counting Scintillant (Amersham) as the counting solution. Cell culture medium was purchased from Life Technologies Corporation. Fetal bovine serum (FBS) was obtained from HyClone Laboratories, Inc. (Logan, UT, USA). MENT was kindly donated by the Population Council's Center for Biomedical Research (New York, NY, USA) and 7 α -methyl-E₂ and nestorone from Instituto Chinelo de Medicina Reproductiva, Santiago, Chile. Authentic NET (17 α -ethynyl-17 β -hydroxy-4-gonen-3-one) and LNG (13 β -ethyl-17 α -ethynyl-17 β -hydroxy-4-gonen-3-one) were kindly provided by Schering Mexicana S.A. (Mexico City, Mexico).

Plasmids

The expression vectors for hPGR_B (pCR3.1-hPGR_B), ESR1 (pCR3.1-hESR1), ESR2 (pCR3.1-hESR2), and AR (pCDNAHisARmcs) for *in vitro* transcription and translation have been described previously (Allan *et al.* 1992, Kraichely *et al.* 2000). In the case of transactivation assays, the expression vectors for human ESR1 and ESR2 (pCMV₅-hESR1 and pCMV₅-hESR2)

containing the coding sequence of the ESR1 and ESR2 were kindly provided by Drs B S Katzenellenbogen, University of Illinois (Urbana, IL, USA) and J-Å Gustafsson, Karolinska Institute (Huddinge, Sweden) respectively. The pLEN-hPGR_B was generated by inserting the full-length human PGR_B cDNA into the BamHI site of the pLEN vector. The pSVhAR.BHEXE vector was kindly provided by Dr E L Young (National University of Singapore). The estrogen-responsive reporter plasmid (ERE-E1b-CAT) contains a fragment of the vitellogenin A2 gene promoter (positions -331 to -87) upstream of the adenovirus E1b TATA box fused to the chloramphenicol acetyltransferase (CAT) gene (Allgood *et al.* 1993, Larrea *et al.* 2001). The androgen and P₄ responsive reporter plasmid (PRE-E1b-CAT) contains an oligonucleotide containing a P₄/androgen response element upstream of the adenovirus E1b TATA box fused to the CAT gene as described previously.

Transient transfections

HeLa, PC-3, HEK-293, and T47D human cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were plated the day before transfections, at a density of 3.0×10^5 cells/well per 6-well plate, in DMEM without phenol red (DMEM-HG), which was supplemented with 5% stripped FBS and 100 U/ml of penicillin and 100 μ g/ml streptomycin and incubated in 5% CO₂ at 37 °C. Transfections were performed as described previously (Larrea *et al.* 2001). Briefly, the medium containing the transfection complexes was added to the cell monolayer and after incubation at 37 °C for 3 h, the plates containing the transfection complexes were rinsed with PBS and 3 ml of supplemented DMEM-HG was added to each well. Twenty-four hours later, the medium was replaced with the same medium containing the compounds of interest at various concentrations (10^{-12} to 10^{-6} M). DMSO or ethanol was used as vehicle. CAT activity using 5 μ g protein, 25 μ g butyryl coenzyme-A (Sigma-Aldrich), 2×10^5 c.p.m. of xylene-extracted [³H]chloramphenicol in 0.25 M Tris-HCl, pH 8.0, was assayed as described previously (Larrea *et al.* 2001). Statistical significance was established by two-tailed *t*-test. Receptor binding assays on HeLa cells transfected with the PGR or AR demonstrated affinity constants ($K_d = 1.8 \times 10^{-9}$ and 0.32×10^{-9} M respectively), within values previously reported for these receptors under native conditions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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