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

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Abstract

7α-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 progestational 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 7α-methyl-estradiol (7α-methyl-E2), an aromatized metabolite of MENT, to transactivate gene transcription via estrogen receptor α (ERα; ESR1) or ERβ (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 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. 7α-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.


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 17β-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 5α-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 5α-reduction to DHT, but otherwise aromatization to estrogens. In this regard, it is well known that the addition of a 7α-methyl group to 19-nortestosterone increases its androgenic and anabolic actions (Kumar et al. 1999), but prevents...
5α-reduction (Agarwal & Monder 1988, Kumar et al. 1992). Therefore, the synthetic androgen 7α-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-norprogestogen 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) 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.
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 nesterone, 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 transcription 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 7α-methyl-E2

In vitro, MENT undergoes aromatization to the active estrogen 7α-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) indexes of 7α-methyl-19-nortestosterone (MENT) alone or combined with 19-nor-derived progestins.

<table>
<thead>
<tr>
<th>Ligand (M)</th>
<th>MENT-ED50 AR (M)</th>
<th>MENT-ED50-index AR/PGRa</th>
</tr>
</thead>
<tbody>
<tr>
<td>MENT</td>
<td>3.42×10^{-12}</td>
<td>5.13×10^{-3}</td>
</tr>
<tr>
<td>MENT+NET^{-10}</td>
<td>2.59×10^{-12}</td>
<td>3.47×10^{-3}</td>
</tr>
<tr>
<td>MENT+NET^{-9}</td>
<td>3.52×10^{-12}</td>
<td>4.72×10^{-3}</td>
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<td>MENT+NET^{-8}</td>
<td>4.02×10^{-12}</td>
<td>5.39×10^{-3}</td>
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<td>MENT+LNG^{-10}</td>
<td>2.77×10^{-12}</td>
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<td>MENT+LNG^{-9}</td>
<td>1.74×10^{-12}</td>
<td>2.33×10^{-3}</td>
</tr>
<tr>
<td>MENT+LNG^{-8}</td>
<td>1.63×10^{-12}</td>
<td>2.18×10^{-3}</td>
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aMENT-PGR ED50 = 7.46×10^{-10} M.
The value obtained with DHT and P4 at the dose of $1 \times 10^{-9}$ M of agonist with or without $1 \times 10^{-7}$ and $1 \times 10^{-8}$ 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 P4 at the dose of $1 \times 10^{-9}$ M was taken as 100% transcriptional activity. No effects upon transcription were observed with antagonists only. NET, norethisterone; LNG, levonorgestrel; FLUT, flutamide; DHT, 5α-dihydrotestosterone.

Discussion

Addition of a 7α-methyl group to 19-nortestosterone significantly enhances its androgenic potency and ability to bind to the AR; however, this 7α-methyl-substituted steroid is not reduced by prostatic 5α-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 7α-methyl-E2 (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 hyperstimulative 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
syntentic 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 <1×10⁹/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 P₄ 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 progessional 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 P₄ transcriptional activation potencies (ED₅₀ 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 ED₅₀ for PGR in the presence of progestins. The smaller the ratio, the greater the difference between the androgen-to-P₄ 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 P₄ 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](image)

**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 (2.07×10⁻⁶ M) or LNG (1.05×10⁻¹⁰ M). Control incubations were carried out in the presence of 1×10⁻⁶ 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±s.d. of three independent experiments. DHT, 5α-dihydrotestosterone; MENT, 7α-methyl-19-nortestosterone; CAT, chloramphenicol acetyltransferase; NET, norethisterone; LNG, levonorgestrel; FLUT, flutamide.
significantly their androgenic transcriptional activities (Sundaram et al. 1995, Garcia-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.

In this study, we also tested the ability of 7α-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 7α-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...
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 7α-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 5α-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 182 780 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 7α-methyl-E2 and nestorone from Instituto Chileno de Medicina Reproductiva, Santiago, Chile. Authentic NET (17z-ethynyl-17β-hydroxy-4-gonen-3-one) and LNG (13β-ethyl-17α-ethynyl-17β-hydroxy-4-gonen-3-one) were kindly provided by Schering Mexicana S.A. (Mexico City, Mexico).

Plasmids

The expression vectors for hPGRβ (pCR3.1-hPGRβ), 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-A Gustafsson, Karolinska Institute (Huddinge, Sweden) respectively. The pLEN-hPGRβ was generated by inserting the full-length human PGRβ 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 3.0×10⁶ 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 µg/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 µg protein, 25 µg butyryl coenzyme-A (Sigma-Aldrich), 2×10⁵ 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 (Kd=1.8×10⁻⁹ 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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