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# Comparative evaluation of androgen and progesterone receptor transcription selectivity indices of 19-nortestosterone-derived progestins

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**Abstract**

Synthetic 19-nortestosterone-derived progestins show affinity for the androgen receptor (AR) and retain varying degrees of androgenic activity. In this study, AR- and progesterone receptor (PR)-dependent transcriptional activation induced by norethisterone (NET), levonorgestrel (LNG) and gestodene (GSD), and their 5α-reduced derivatives, including limited trypsin digestion of AR in the presence of natural and synthetic progestins were investigated. The results confirmed the progestogenic activity of the three 19-nortestosterone derivatives, which decreases after reduction of the 4-ene-double bound. These compounds were able to activate AR-dependent reporter gene expression, LNG and GSD being the stronger activators. 5α-Reduction of LNG and GSD did not change their androgenic transcriptional activity; however, the activation of AR by 5α-NET was four-fold higher than NET. The highest selectivity transcriptional index, as a measure of progestogenicity versus androgenicity, was obtained for NET. The 5α-reduced derivatives had values significantly lower than those of their parent compounds. Non-reduced and 5α-reduced 19-nortestosterone progestins induced virtually identical proteolysis fragmentation patterns of the AR to those observed with DHT.


*Keywords:* Androgenic effects; Androgen receptor; Contraception; Gene expression; Synthetic progestins

## 1. Introduction

Oral contraceptives have been shown to induce metabolic changes, particularly in lipid and carbohydrate metabolism [1]. These metabolic disturbances are thought to be associated with the intrinsic androgenicity of progestins [2], which together with the androgenic cosmetic side-effects, represent the primary reasons for discontinuing their oral contraceptive use [3]. The androgenic potencies of synthetic progestins derived from 19-nortestosterone and their relative binding affinities for the AR have been previously reported [4–6]. In these studies, partial structural requirements for AR binding, such as the differential susceptibility of 19 nor-androgens/-progestins to 5α-reduction, have been reported; however, they are still far from being well defined. In as much as selectivity index studies for the major progestins used in oral contraception showed significantly higher indices for the relatively new progestins, receptor binding studies do not allow differentiation between agonist or antagonist effects, including hormonal metabolism, pharmacokinetics and dynamics; therefore, observations made from binding parameters can only be taken as a relative reference for biological potency. In this study, AR- and PR-dependent transcriptional activation by 19-nortestosterone-derived progestins currently used in oral contraceptive formulations (levonorgestrel, norethisterone and gestodene) was compared as an experimental strategy to re-evaluate their corresponding progestational and androgenic potencies. In addition, limited proteolysis of in vitro produced human AR was used to study ligand-induced changes in receptor conformation.

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## 2. Materials and methods

### 2.1. Reagents

Non-radioactive progesterone (P), 5α-dihydrotestosterone (DHT) and trypsin (type III) were purchased from Sigma (St. Louis, MO). $^{3}$[H]chloramphenicol (specific activity 38.9 Ci/mmol) was purchased from DuPont NEN Research products (Boston, MA). Unlabeled RU486 (RU 38486, mifepristone) was a gift from Roussell Uclaf (Romainville, France). Radioactivity was determined in a Beckman LS6500 scintillation system (Beckman Instruments, CA) using Biodegradable Counting Scintillant (Amersham, CA) as counting solution. Cell culture medium was purchased from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was from Hyclone Laboratories Inc. (Logan, UT). Authentic NET (17α-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) and GSD (13β-ethyl-17α-ethynil-17β-hydroxy-4,15-gonadien-3-one) by Schering AG (Berlin, Germany). Synthesis of the corresponding 5α-dihydro (5α-NET, 5α-LNG and 5α-GSD) metabolites, including the description of their physical and spectroscopic constants have been previously described [7–9]. All other solvents and reagents used were of analytical grade.

### 2.2. Plasmids

The pLEN-hPR$_B$ was generated by inserting the full-length human PR$_B$ cDNA into the *Bam*HI site of the pLEN vector as previously described [10]. The expression vector containing the coding sequence of the AR (pSVhAR.BHEXE) [11,12] was kindly provided by Dr. E.L. Young (National University of Singapore). The androgen and progesterone responsive reporter plasmid (PRE-E1b-CAT) contains an oligonucleotide containing a progesterone/androgen response element upstream of the adenovirus E1b TATA box fused to the chloramphenicol acetyltransferase (CAT) gene as previously described [10,13].

### 2.3. Transfections and reporter gene assays

HeLa cells were plated the day before transfections, at a density of $2.5 \times 10^5$ cells/well/six-well plate as previously described [10]. Transfections were performed in triplicate using PolyFect$^{TM}$ (QIAGEN Inc., Valencia, CA) according to the protocol provided by the manufacturer. Briefly: serum-free media (0.1 ml) was aliquoted and DNA added (1 μg of the reporter gene plasmid and 0.025–0.5 μg of the expression vector depending upon whether AR or PR assays were being performed), after vortexing, 10 μl of PolyFect$^{TM}$ reagent was added and vortexed for 10 s. Following incubation at room temperature for 5–10 min, 0.6 ml of supplemented DMEM-HG was added to each sample. The medium containing the transfection complexes was added to the cell monolayer, which had previously been rinsed with PBS. The plates were incubated for 3 h at 37 °C in 5% $CO_2$. After incubation, 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 medium containing the compounds of interest at various concentrations ($10^{-12}$ to $10^{-6}$ M). Dimethyl sulphoxide (DMSO) or ethanol (EtOH) were used as vehicle. CAT activity using 5 μg of protein, 10 μg of butyryl coenzyme-A (Sigma), $2 \times 10^5$ cpm of xylene-extracted [$^{3}$H]chloramphenicol in 0.25 M Tris–HCl, pH 8.0, was assayed as previously described [10,14,15]. Stimulatory concentration ($EC_{50}$) values were obtained by non-linear regression analysis using sigmoidal fitting with a sigmoidal dose–response curve with the aid of a scientific graphing software. Statistical significance was determined using two tailed *t*-test.

### 2.4. In vitro translation and partial protease digestion

The generation of the in vitro expression vector for the expression of AR (pcDNAHisARmcs) in rabbit reticulocyte lysates has been previously described [16]. The in vitro synthesis of recombinant AR using T7 RNA polymerase and the T7 TnT kit (Promega) has been previously described [16,17]. Briefly: in vitro translation reactions were carried out in rabbit reticulocyte lysates according to the manufacturers instructions (Promega) in the presence of L-[$^{35}$S] methionine (>1000 Ci/mmol, Amersham, Buckinghamshire, UK) using 1 μg of expression plasmid. In vitro translated receptor (4.5 μl) was incubated in the presence of $10^{-7}$ M of the corresponding steroid at room temperature for 10 min. Partial proteolytic digestion was performed by incubation with 0.5 μl trypsin (50 μg/ml) for 10 min at room temperature. One to two microliters of the various reactions were mixed with 5 μl SDS loading buffer and denatured at 100 °C for five min. The samples were analyzed by 10% SDS–PAGE [18].

## 3. Results

### 3.1. Transcriptional activation through PR and AR of 19-nortestosterone-derived progestins and their A-ring reduced derivatives

The progestogenic and androgenic activities of each of the synthetic progestins, including those of their 5α-reduced metabolites are shown in Fig. 1. PR- and AR-mediated agonistic activity upon transcriptional activation was studied by incubation in the presence of increasing concentrations ($10^{-12}$ to $10^{-6}$ M) of either NET, LNG or GSD and their corresponding A-ring reduced derivatives (5α-NET/-LNG/-GSD). Similar concentrations of natural hormones

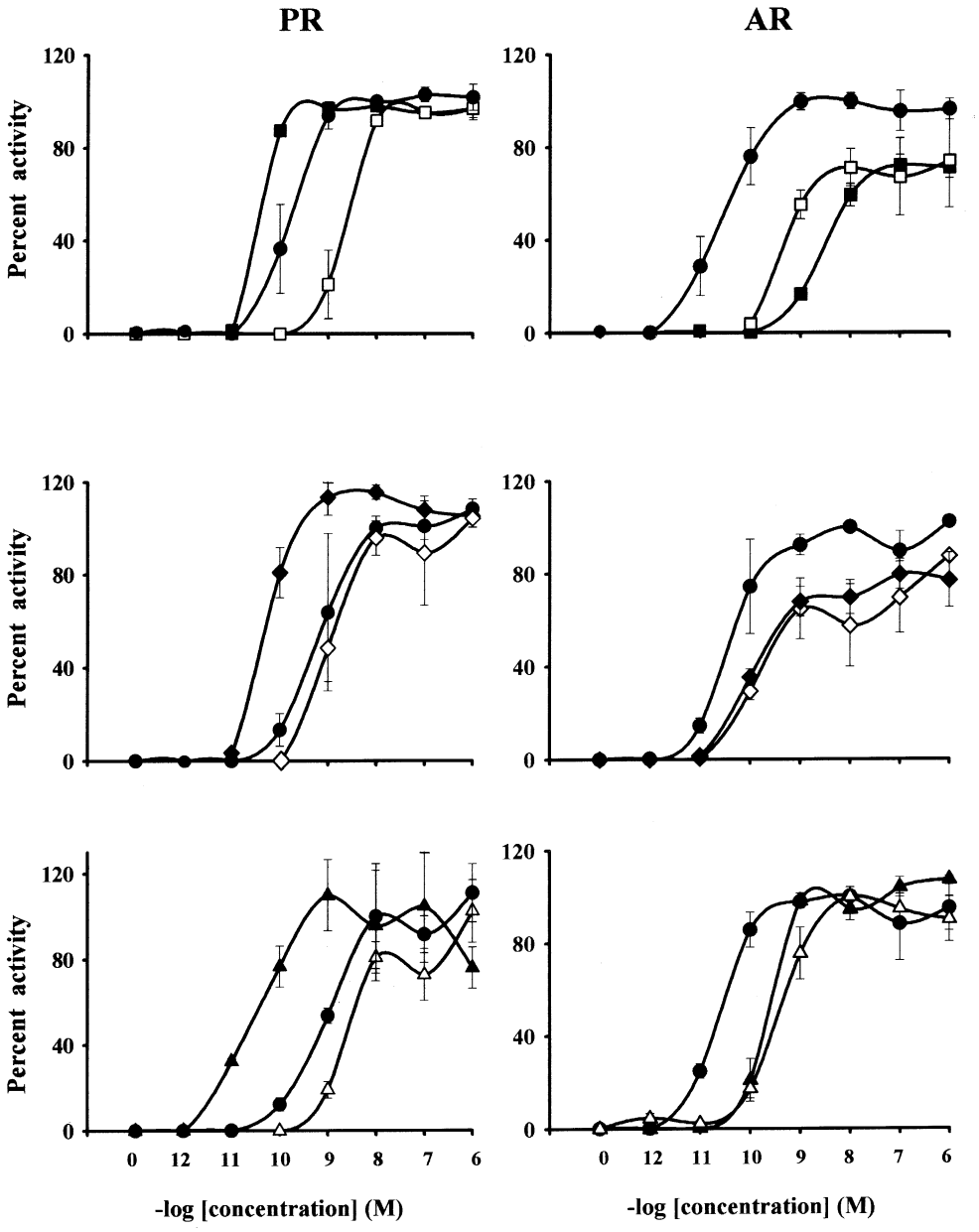


Fig. 1. Transcriptional activation via the progesterone receptor (PR) and androgen receptor (AR). HeLa cells were transiently transfected using the PRE-Elb-CAT reporter plasmid and the expression vectors for PR or AR, respectively. Cells were cultured in the absence or presence of increasing concentrations ($10^{-12}$ to $10^{-6}$ M) of NET (■), LNG (◆), GSD (▲), 5α-NET (□), 5α-LNG (◇), or 5α-GSD (△). CAT activity was determined in 5 μg of cellular protein. Natural P and DHT (●) were used as positive controls for the PR and AR, respectively. Each point represents the mean ± S.D. of three independent experiments and values are expressed as the percentage of the CAT activity induced by $10^{-8}$ M P and DHT (100%), respectively. Each experiment was run in triplicate.

progesterone (P) or dihydrotestosterone (DHT) were used as positive controls for PR- or AR-mediated reporter gene induction, respectively. As shown, all the non A-ring reduced progestins tested were more potent transcriptional activators than P, and reduction of the 4-ene-double bond was followed by a significant reduction of their receptor-mediated transcriptional activities. As judged by the $EC_{50}$ values (Table 1), obtained from data shown in Fig. 1, GSD was the most effective compound in activating transcription through the PR, with similar values for NET and LNG. The data also indicated that all the compounds behave as full PR agonists.

In the case of AR-mediated transcriptional activation, all the tested compounds showed intrinsic androgenic agonistic activity. As shown in Fig. 1, LNG and GSD were stronger than NET but weaker than DHT in transactivation of gene expression. With the exception of NET, in which 5α-reduction increased its androgenic activity four-fold, A-ring reduc-

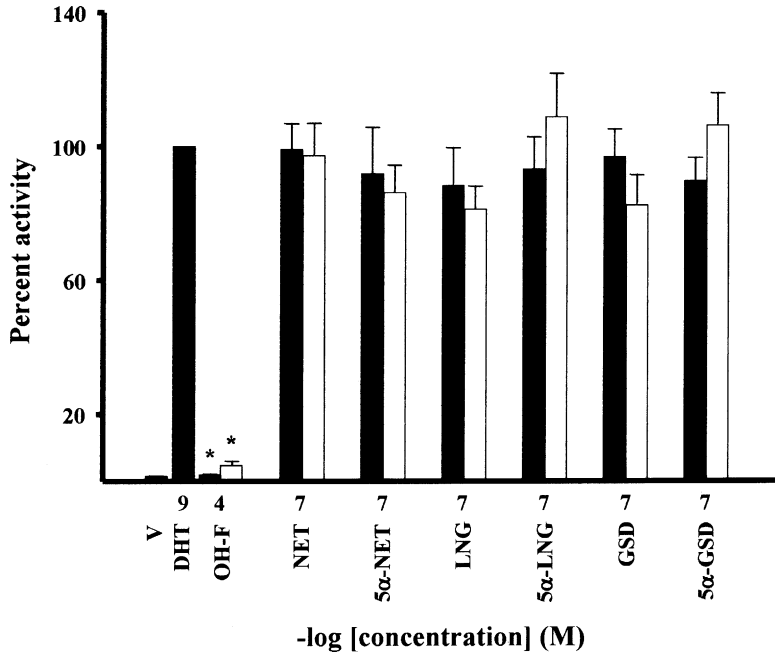


Fig. 2. Effects upon DHT-dependent transcriptional activation of non-reduced and 5α-reduced 19-nortestosterone progestins. HeLa cells, transiently co-transfected with pSVhAR.BHEXE and PRE-E1b-CAT as described in Section 2, were incubated with DHT ($10^{-9}$ M), NET, LNG, GSD or their corresponding 5α-reduced derivatives ($10^{-7}$ M), including the antiandrogen hydroxyflutamide (OH-F) ($10^{-4}$ M) (closed bars). Parallel incubations with DHT ($10^{-9}$ M) in the presence of $10^{-7}$ M of the indicated ligands or $10^{-4}$ M OH-F are represented by open bars. CAT activity was determined in 5 μg of cellular protein. Bars represent the mean ± S.D. of two experiments and values are expressed as the percentage of the CAT activity induced by 1nM DHT (100%). Each experiment was ran in triplicate. Responses below 5% of those induced with 1 nM DHT were obtained in incubations in the absence of ligand (V). $^{*}P < 0.001$ vs. DHT.

tion of these steroids did not significantly change their abilities to activate transcription (Table 1). According to the $EC_{50}$ values derived from Fig. 1, the following androgenicity ranking was obtained for the three A-ring non-reduced progestins: LNG > GSD > NET. No transcriptional activation, above that obtained with the vehicle, was observed with P and DHT through the AR and PR, respectively (data not shown).

As shown in Fig. 2, all compounds acted as agonists and none of them, especially the 5α-reduced derivatives altered or inhibited in a significant manner the DHT-mediated reporter gene expression. The antiandrogen hydroxyflutamide (OH-F) inhibited the effects of DHT, including those of NET, LNG and GSD.

Table 1
Transcriptional stimulatory concentrations ($EC_{50}$) of natural and 19-nortestosterone-derived ligands for the PR, AR and androgen-to-progestin (AR/PR) $EC_{50}$ ratios

| Ligand | PR (mol/L) | AR (mol/L) | AR/PR |
|---|---|---|---|
| $P_4$ | $8.76 \times 10^{-10}$ | – | – |
| DHT | – | $2.75 \times 10^{-11}$ | – |
| NET | $8.16 \times 10^{-11}$ | $2.07 \times 10^{-9}$ | 25.4 |
| GSD | $1.83 \times 10^{-11}$ | $2.37 \times 10^{-10}$ | 12.95 |
| LNG | $6.07 \times 10^{-11}$ | $1.05 \times 10^{-10}$ | 1.73 |
| 5α-GSD | $2.30 \times 10^{-9}$ | $3.61 \times 10^{-10}$ | 0.157 |
| 5α-LNG | $9.93 \times 10^{-10}$ | $1.48 \times 10^{-10}$ | 0.148 |
| 5α-NET | $1.80 \times 10^{-8}$ | $5.22 \times 10^{-10}$ | 0.029 |

### 3.2. Effects of 19-nortestosterone-derived progestins on conformation of the AR

To study the ligand-induced transformation of the AR partial proteolytic analysis was used as an indirect assay [16]. To this end, in vitro produced AR was first incubated in the presence of each one of the three 19-nortestosterone progestins, including their A-ring reduced dihydro- and tetrahydro-derivatives followed by limited trypsinization. Protease resistant fragments were analyzed by SDS–PAGE and compared with those obtained with DHT. As shown in Fig. 3A, in the absence of ligand the receptor was totally degraded (lane 3). In the presence of DHT two protease resistant fragments (35 and 30 kDa) were observed (lane 2), similar results were obtained when AR was incubated in the presence of NET, LNG and GSD and their corresponding 5α-dihydro-derivatives (lanes 4–9). With increasing concentrations ($10^{-8}$ to $10^{-6}$ M) of NET, LNG or GSD a shift in the relative abundance of the 30 kDa proteolytic resistant fragment of the AR was observed (Fig. 3B) with no apparent changes in the 35 kDa fragment. The relative abundance of 30 kDa species was established by densitometric analysis normalizing the 30 kDa band intensity generated with

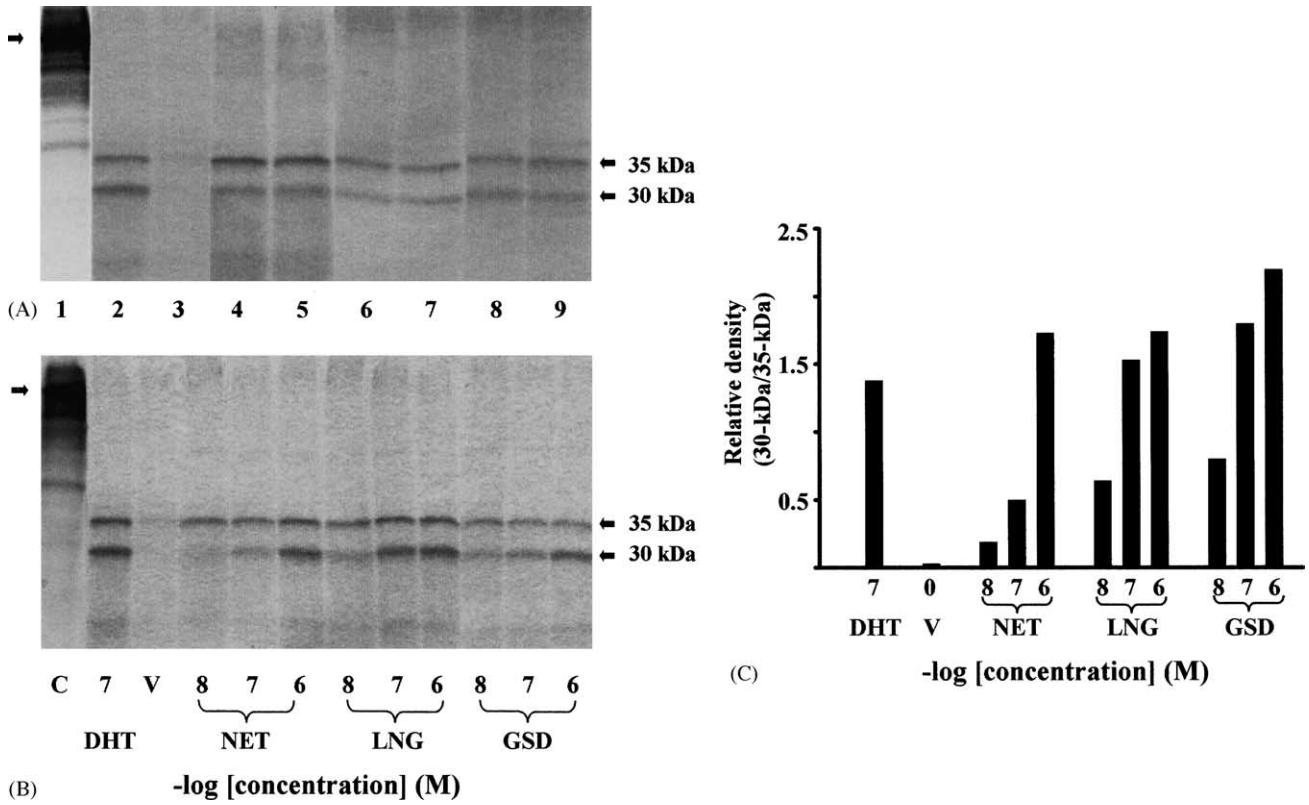


Fig. 3. SDS–PAGE of limited trypsinization products of the AR. (A) In vitro translated [$^{35}$S] methionine-labeled AR was incubated in the presence of DHT (lane 2), NET (lane 4), 5α-NET (lane 5), LNG (lane 6), 5α-LNG (lane 7), GSD (lane 8), or 5α-GSD (lane 9) before limited trypsin digestion. Control incubations were performed in the absence of trypsin (lane 1) or ligand (lane 3). All steroids were incubated at the same concentration of $10^{-7}$ M. Undigested AR and protease resistant fragments are indicated by the arrows. (B) In vitro translated [$^{35}$S] methionine-labeled AR was incubated in the presence of ligands at the indicated concentrations before trypsinization. Control incubations were also performed in the absence of trypsin (C) and ligand (V). Undigested AR and protease resistant fragments are indicated by the arrows. The relative abundance of the 30 kDa fragment normalized against the 35 kDa band under different concentrations of compounds is shown in (C).

diferent concentrations of NET, LNG or GSD with that obtained with their respective 35 kDa fragment (Fig. 3C). This shift indicated that the 30 kDa proteolytic resistant fragment was the one interacting with the ligand at the level of the ligand binding domain in the AR. Similar results were obtained with 5α-dihydro derivatives of NET, LNG and GSD, respectively (data not shown).

### 3.3. Transcription selectivity indices

Selectivity indices were obtained from the $EC_{50}$ values for each progestin reflective of their ability to activate transcription through the AR and PR, respectively. We took the androgen-to-progestin $EC_{50}$ transcription activation ratio (AR/PR) as a measure of progestogenicity versus androgenicity; thus, the higher the ratio the greater the difference between these two activities. As shown in Table 1, the highest selectivity index was found for NET followed by GSD and LNG. As expected, the lowest $EC_{50}$ AR/PR ratios were found for the 5α-reduced derivatives, particularly for 5α-NET in which the ratio was significantly lower than that obtained for the other A-ring reduced compounds.

## 4. Discussion

In the present study we investigated the ability of three synthetic progestins and their corresponding A-ring reduced metabolites to transactivate gene transcription through the AR and PR. Receptor binding affinities for these compounds have been previously reported for both receptors [4–6,8,19–21]; however, varying estimates may occur since the binding data usually comes from separate studies, as well as from different experimental conditions. Of particular importance is the observation that steroid receptors are mainly found associated with the nuclear fraction and in many of the studies the cytosolic fractions were used as the source of the receptors. The aim of the present study was to re-evaluate the androgenicity of three 19-nortestosterone-derived progestins widely used in hormonal contraception by means of a hormone-dependent transcriptional activation assays. This assay, in addition to estimating the hormone-dependent receptor activity upon transcription, allowed us to determine both agonistic and antagonistic properties of these compounds, which are not considered in receptor binding studies.

In this study we confirmed the potent progestin activity of 19-nortestosterone derivatives. As judged by the $EC_{50}$ of each compound, in terms of their ability to activate transcription through the PR, GSD was three to five times more potent than LNG and NET, which agreed with previous PR binding studies [5]. Reduction of the 4-ene-double bound in GSD, LNG and NET into their corresponding 5α-dihydro derivatives significantly decreased the ability of these compounds to activate PR-dependent gene transcription, indicating that A-ring reduction at the C-5 position results in a steric molecule, which either does not appropriately fit the ligand binding pocket, as has been previously suggested by receptor displacement studies [8,19], and/or interferes with receptor-dependent gene transcription, as reported previously [10,22]. These data agree with and may also offer a plausible explanation for the antagonist effects of 5α-NET on the previously reported progesterone-induced uteroglobin gene transcription and PR down-regulation in rabbit endometrium [23].

As the desired biological effect of progestins in fertility regulation is related to their progestin selectivity, estimations of undesired hormonal activities are of importance in predicting the clinical effects and acceptability of these compounds. Regarding this, 19-nortestosterone-derived progestins are known to exhibit androgenicity, which is caused by their direct binding to receptors and also by their effects on SHBG [24], including the degree to which they bind to it [4]. Although these studies indicate a putative intrinsic androgenic property of these compounds, the translation of receptor binding data into biological activity is generally missing. In this study, an androgen-driven transcription activation assay was used and revealed that the three progestins studied had a significant intrinsic androgenic activity. Both LNG and GSD were active at a potency of about one third to one tenth of that of DHT and had comparable abilities to stimulate reporter gene transcription; however, they showed an $EC_{50}$ approximately 15-fold lower in comparison to that of NET. This observation agrees with a number of studies indicating that addition of the 18-ethyl group, as in the case of LNG, increases the binding affinity for the AR and the presence of an extra double bound at the C-15 position, as in GSD, has little if any further effect [5,6,25].

In tissues like prostate and seminal vesicles, testosterone is enzymatically biotransformed to DHT by the action of the enzyme 5α-reductase. The binding affinity of DHT for AR and its bioactivity is three to five times greater than of testosterone [26]. Since 19-nortestosterone-derived progestins undergo extensive in vitro and in vivo enzyme-mediated 5α-reduction [8,27,28], it was of interest to investigate the androgenic potencies of the 5α-dihydro metabolites upon androgen-mediated reporter gene activation and compare them with those of their parent compounds. The results demonstrated that 5α-reduction resulted in a slight but not significant increase in the $EC_{50}$ for LNG and GSD to activate transcription; however, in the case of 5α-NET there was a significant decrease in the $EC_{50}$ of approximately four-fold when compared to that observed with NET, but it was three-fold higher than that of 5α-LNG and 5α-GSD, and nearly 20-fold with respect to that of DHT. These results indicate that A-ring reduction may not be as critical, as in the case of testosterone, in potentiating the androgenic properties of 19-nortestosterone-derived progestins. Our results agree with others demonstrating that A-ring reduction of 19-nortestosterone derivatives did not increase their biological androgenic activities. Indeed, in this study, with the exception of 5α-NET, a decrease of 1.5-fold in androgenic potencies was observed. In addition, these observations are consistent with the postulate that the androgenic versus anabolic actions of 19-nortestosterone derivatives are dependent upon the relative biotransformation to their corresponding 5α-dihydro metabolites in target tissues [19,29].

In this study, we took the androgen-to-progestin-dependent transcriptional activation potency ($EC_{50}$) ratio as a measure of progestogenicity versus androgenicity for each of the synthetic steroids being investigated. The higher the ratio, the greater the difference between the progestational activity and the androgenic responses. In this study, the highest ratio was obtained with NET (25.4) and the lowest with LNG (1.73). In addition, all 5α-reduced metabolites of NET, LNG and GSD had values significantly lower than those of their parent compounds, which agree with this study and other previous communications [8,19] that 5α-reduction decreases the affinity for the PR. These observations, from the profile of androgen and progesterone transcriptional activation of each compound, suggest that NET possesses a better transcription-level selectivity index followed by GSD and LNG, even though GSD is a more potent progestogen than NET and LNG. However, in vivo metabolic biotransformation should be taken into consideration, particularly with NET where reduction of the 4-ene-double bond significantly decreases its progesterone-dependent transcriptional activity and increases its androgen receptor dependent transcriptional activity.

In this study, all 19-nortestosterone progestins, including their 5α-reduced derivatives, induced a conformational change in the AR, observed after partial proteolytic digestion of the in vitro translated protein. This change, which resulted in a dose-dependent specific 30 kDa protease-resistant fragment, was virtually identical to that observed with naturally occurring androgens, thus indicating the intrinsic androgenic agonist nature of these compounds. These results agree with those, also reported herein, at the transcriptional level, since ligand-induced conformational change in the ligand binding domain of the receptor precedes and is required for transcriptional activation [30].

Although pharmacokinetics and dynamics are not considered in this study, the results may offer a better estimation of the intrinsic hormonal properties of these progestins than those derived from receptor binding studies alone, and may also contribute to the understanding of the undesired metabolic side-effects of these compounds in humans.

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