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Influence of the substitution of 11-methylene, Δ^{15}, and/or 18-methyl groups in norethisterone on receptor binding, transactivation assays and biological activities in animals

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Abstract

The profile of norethisterone and newly developed derivatives thereof were assessed by in vitro binding and transactivation assays on progesterone (PR) as well as on androgen (AR) receptors and by subcutaneous treatment in in vivo models. The following in vivo models were performed: A McPhail test for progestational activity in immature rabbits, an ovulation inhibition test in cycling rats and a Hershberger test for androgenic activity in immature orchidectomised rats. The compounds tested were: norethisterone (NET), 11-methylene-NET (11-NET), Δ^{15}-NET (15-NET), 18-methyl-NET (18-NET, Levonorgestrel, LNG), 11-methylene-Δ^{15}-NET (11,15-NET), 11-methylene-18-methyl-NET (11,18-NET, 3-keto-desogestrel, Etonogestrel, ETG), (Δ^{15}-18-methyl-NET (15,18-NET, Gestodene, GSD) and 11-methylene-Δ^{15}-18-methyl-NET (11,15,18-NET). Compared to the non-substituted compound NET, the binding to and agonistic activity via PR was increased for all the three mono-substituted compounds, although the stimulatory effect of 15-NET was only twofold. Compounds with 18-methyl in combination with Δ^{15} (GSD), with 11-methylene (ETG) or with both combined showed clear synergistic effects, leading to equipotent compounds. If the 18-methyl group was lacking as in 11,15-NET, potency was lower than for ETG or GSD, but higher than for 18-NET (LNG). A correlation coefficient of 0.9 was found between binding affinity and agonistic potency. With respect to the AR binding and transactivation activities, the 18-methyl group potentiated androgenic in vitro activity (LNG). The 11-methylene group increased relative binding affinity in NET, but reduced androgenic activity clearly when also other substituents were present (11,15-NET, ETG and 11,15,18-NET). The Δ^{15} bond alone did not change the binding in NET, but decreased androgen binding, induced by the 18-methyl substituent, in GSD and 11,15,18-NET. Transactivation activity was also diminished in the compounds having a Δ^{15} bond. In the McPhail test mono-substitution of NET increased the progestagenic in vivo activity three to five times. Bi- and tri-substitution enhanced the activity further. With respect to ovulation inhibition mono-substitution of NET resulted in three to nine times more potent compounds, whereas bi- and tri-substitution increased potency further, except for 11,15-NET, which was as active as 11-NET. The relative progestagenic potencies in the McPhail and ovulation inhibition tests, correlated significantly with those of the relative binding affinity values (correlation coefficient of 0.91 and 0.93, respectively) and relative transactivation activity values (0.88 and 0.81) for the PR. In the Hershberger test, all the compounds increased androgenic activity with respect to growth of ventral prostate weight compared to NET, with the exception of 11,15-NET and 11,15,18-NET. The androgenic activity was negligible for these latter compounds. The androgenicity of both 18-NET (LNG) and 15,18-NET (GSD), on the other hand, was significantly higher than that of 11,18-NET (ETG). The results of this in vivo test are in line with the AR binding and transactivation activity values (correlation coefficients of 0.86 and 0.88). In addition, selectivity indices were calculated by dividing the progestational potencies by androgenic potencies for both in vitro and in vivo assays. ETG and GSD had clearly higher in vitro and in vivo indices than the other compounds with NET and LNG having the lowest indices. Because the androgenicity of 11,15-NET and 11,15,18-NET was very low, no exact selectivity ratios could be calculated for these compounds. From these experiments we may conclude that small structural modifications exert enhancement of progestational activity and a clear reduction in androgenicity leading to very selective progestagenic compounds. The influence of bi-substitution is additive over mono-substitution, whereas tri-substition is not additive. The three substituents (11-methylene, Δ^{15} bond and 18-methyl) increase the progestational activity over that of NET and combinations of these substituents result in an even further increase in activity

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The effect on androgenic activity of the three substituents is more complex: the 18-methyl group similar as in LNG increases the androgenic activity, whereas the 11-methylene group increases RBA in NET, but reduces androgen binding in combination with any other substituent (ETG, 11,15-NET and 11,15,18-NET). The Δ^{15} bond had no effect in NET, but decreased androgen binding, induced by the 18-methyl substituent, in GSD and 11,15,18-NET. The effects of these eight progestagens in in vitro binding and transactivation studies are very representative for in vivo endometrium proliferation in rabbits, ovulation inhibition in rats and growth of ventral prostate in orchidectomised rats.

Keywords: Steroid receptors; Competition studies; Progestational activity; Androgenic activity; McPhail test; Ovulation inhibition; 3-keto-desogestrel; Etonogestrel; Levonorgestrel; Desogestrel; Gestodene

1. Introduction

Norethisterone (NET) forms the basic structure of the newly developed 19-nortestosterone derivatives, which are widely used in oral contraceptives and hormone replacement therapies for climacteric complaints. The addition of a methyl group at carbon atom 18 of NET results in Levonorgestrel (LNG), which has been shown to be a much more potent progestagen [1,2] with increased androgenicity [2]. Since it was assumed in the past that oral contraceptives, containing progestagens with intrinsic androgenic properties, may cause atherosclerosis, hypertension and other arterial diseases [3,4], new entities of progestagens were developed.

It is generally accepted that a valuable correlation exists between relative binding affinity (RBA) values of steroid hormones in vitro and their biological activity in vivo. However, if a compound binds to the receptor, it remains to be assessed whether it acts as an agonist or an antagonist. The transactivation assay is in this respect a very useful test in which both agonistic and/or antagonistic activity can be established [5,6]. The introduction of structural modifications into the steroid skeleton of NET at carbons 11, 15 or 18 increased the RBA to the progesterone receptor [7–9]. One compound out of this series is 3-keto-desogestrel (Etonogestrel, ETG), which possesses an extra 11-methylene group in addition to the 18-methyl group as in LNG. ETG and/or its precursor molecule Desogestrel (DSG) showed a higher in vitro and in vivo progestational activity in various bioassays than LNG and NET [1,2,7,8,10]. To obtain biological activation of DSG, this compound must first be converted into its biologically active metabolite ETG. ETG, in turn, can bind to the progesterone receptor [10]. Another compound in this series is Gestodene (GSD), which is LNG modified with a double bond between carbon atoms 15 and 16. GSD acts far stronger at the pituitary level than LNG, but is equipotent to ETG [11].

Some 19-norsteroids, and especially the 18-methyl substituted compounds, have a very low bioavailability in rats after oral application. As far as known all eight progestagens, derived from the three substituents on NET and the combinations thereof, have never been studied in extensive comparative studies for the estimation of progestational and androgenic activities in vivo. Such studies have only been completed with respect to their binding affinity values to the progestagen and androgen receptor [12]. Hoppen and Hammann [8] presented the progesterone and androgen RBA's of eight progestagens structurally derived from NET, including NET, LNG, ETG and GSD, while Schoonen et al. [6] published the progestagenic in vitro and in vivo values for a selection of these progestagens. The aim of the present study was to assess the influence of substitution of 11-methylene (11-NET), a double bond at carbon 15–16 (Δ^{15}, 15-NET) and 18-methyl (18-NET) and all possible combinations of these substitutions in NET on binding with human progesterone (PR) and androgen (AR) receptors from breast tumor MCF-7 cells. As a second approach the transactivation activity of these compounds was assessed with Chinese hamster ovarian (CHO) cells transfected with the human progesterone and androgen receptors. Moreover, to eliminate the effect of metabolism, the potencies of their in vivo progestational uterotrophic, ovulation inhibiting and androgenic activity were assessed after subcutaneous (sc) administration.

2. Materials and methods

2.1. Animals

SPF bred HSD/Cpb:ORGA rats and Chinchilla (HSD/Cpb:CH) rabbits were obtained from Harlan Sprague Dawley/Central Institute for the Breeding of Laboratory Animals of the Netherlands Organisation for Applied Scientific Research (HSD-CPB), Zeist, The Netherlands. The rats were housed in light and temperature controlled rooms (14 h light–10 h dark; 21–23°C). Tap water and pelleted food (RMH-B, Hope Farms, Linschoten, The Netherlands) were given ad libitum. The rabbits were housed in light and temperature controlled rooms (14 h light–10 h dark; 19–21°C), fed daily with 50 g pelleted food (LKK-20, Hope Farms) and had free access to tap water.

2.2. Cell lines

Human breast tumor MCF-7 cells were provided by Dr. C.M. McGrath (Michigan Cancer Foundation,

USA). The CHO cells, derived from CHO K1 cells obtained from the American Type Culture Collection (Rockville, MD, USA) contained hPRB-MMTV-LUC (clone 1E2-A2) or hAR-MMTV-LUC (clone 1G12-A5-CA). These cells were cultured in medium with charcoal-treated defined bovine calf serum (dBCS, Hyclone). All cell lines were cultured at 37°C in Roux flasks with 5% CO_2 in air until pH 7.2–7.4 was reached. Complete medium was refreshed every 2 or 3 days. One day before harvesting, MCF-7 cells were cultured on charcoal-treated fetal calf serum.

2.3. Ligands and compounds

[^{3}H]Org 2058 (450 Gbq/mmol) and [^{3}H]5α-dihydrotestosterone (DHT; 5,3 TBq/mmol) were purchased from Amersham Int., UK and Du Pont de Nemours, Boston, USA, respectively. The 19-nortestosterone derivatives (see Fig. 1 for the structures) were synthesized by the Organic Chemistry Department, N.V. Organon, Oss, The Netherlands. For the in vitro experiments the compounds were dissolved in ethanol and for the in vivo experiments the compounds were dissolved in arachis oil and administered sc. The dosages used in the experiments are indicated in the figures.

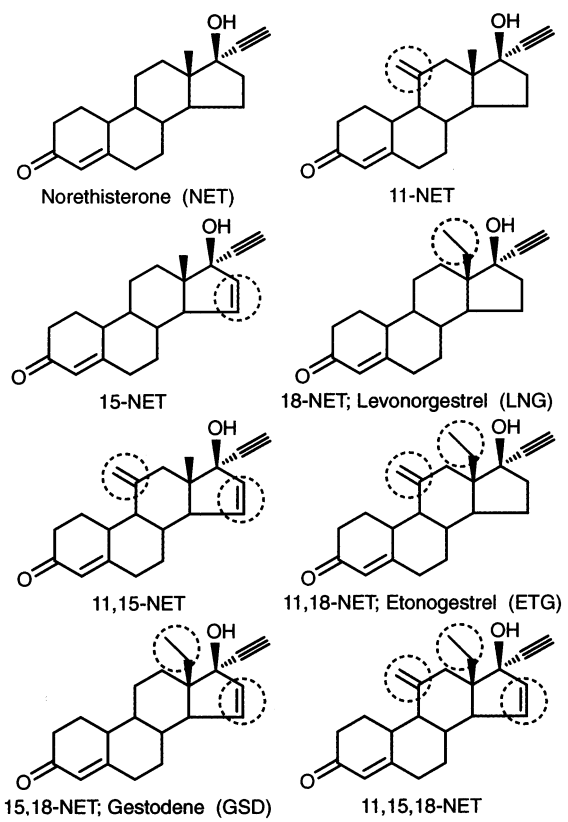

Fig. 1. Structures and chemical names of the progestational compounds used in this study.

2.4. Receptor binding

Receptor binding was carried out with MCF-7 cells as described by Schoonen et al. [9]. Prior to use 1 g of cells was homogenized with 5 ml of buffer solution, and thereafter diluted to a final receptor concentration of 1:20 for hPR and undiluted for hAR. Samples were counted in a Topcount microplate scintillation counter (Packard). Specific binding was determined by subtracting non-specific from total binding.

2.5. Transactivation studies

For the relative agonistic activity the above described stably transfected CHO cells were used [5,6]. Steroids for treatment were first diluted in ethanol and finally with medium to such a concentration that in wells of 96 well white culture plate only 1% ethanol was present during cell incubation. Thereafter cells were seeded at 5×10^4 cells/well and incubated during 16 h in medium with charcoal-treated dBCS at 37°C in 5% CO_2 in air in an incubator. Subsequently, part of the medium was removed and LucLite added for cell lysis and luciferase measurement in a Topcount luminescence counter. RAA activity studies were carried out with various concentrations of the standards (1:2:4 dilutions) and compounds of interest.

2.6. McPhail test for progestational activity in immature rabbits

The test was carried out as described by Overbeek and de Visser [13]. In short, immature female rabbits weighing 800–1300 g were primed sc with estradiol benzoate (2 μg/day/rabbit) in arachis oil for 8 days. Subsequently the progestational compounds were administered twice a day for 5 days. Animals were killed by pentobarbitone (60 mg/rabbit/intravenously). The uterus was dissected and two different parts of each horn were stained with haematoxylin-eosin. The endometrium differentiation was scored using the McPhail index (0–4). The McPhail test was performed using 3 blocks and 2 rabbits/block/dose.

2.7. Ovulation inhibition in rats

The experiment was done as described by Van der Vies and de Visser [1]. Mature female rats with a regular 4 days estrous cycle and weighing 270–425 g were used. Twice daily treatment was started at estrous and lasted 5 days. In the morning of day 6 the animals were killed with CO_2 gas and the oviducts were dissected and microscopically examined for the presence of ova (with or without granulosa cells). The test was performed in 2 blocks and 3 rats/block/dose.

Table 1
Relative binding affinities (RBA) and relative agonistic activities (RAA) for eight progestational compounds for the human progesterone and androgen receptors in MCF-7 and CHO cells. Org 2058 and DHT were used as reference compounds for the progesterone and androgen receptor, respectively[c]

Compound	Progesterone receptor		Androgen receptor	
	RBA MCF-7 cells	RAA CHO cells	RBA MCF-7 cells	RAA CHO cells
NET	22 ± 1.0^{b} (31)	12 ± 1.3^{b} (9)	3.2 ± 0.2^{b} (34)	1.1 ± 0.1^{b} (32)
11-NET	106 ± 7.1^{b} (24)	39 ± 4.0^{b} (4)	5.0 ± 0.9^{b} (9)	1.3 ± 0.1^{b} (10)
15-NET	44 ± 3.4^{b} (15)	34 ± 3.9^{b} (4)	2.5 ± 0.2^{b} (6)	[a]
18-NET (LNG)	80 ± 2.9^{b} (27)	42 ± 4.9^{b} (4)	10.3 ± 1.4^{b} (22)	5.1 ± 0.4^{b} (30)
11,15-NET	139 ± 5.6^{b} (22)	122 ± 14 (4)	2.0 ± 0.3^{b} (7)	0.4^{b} (1)
11,18-NET (ETG)	192 ± 5.4 (93)	112 ± 12 (8)	6.2 ± 0.8 (28)	2.6 ± 0.1 (21)
15,18-NET (GSD)	188 ± 8.8 (37)	151 ± 6.3^{b} (8)	5.2 ± 0.5 (12)	6.1 ± 1.6^{b} (2)
11,15,18-NET	209 ± 17 (14)	112 ± 12 (6)	2.2 ± 0.1^{b} (6)	[a]

[a] = No competition at 10^{-7} M.
[b] = Statistically significantly different from ETG ($P < 0.05$).
[c] Data are given as means ± SEM and the number of experiments is given between brackets.

2.8. Hershberger test for androgenic activity in immature orchidectomised rats

The test was performed according to the procedure described by Van der Vies and de Visser [1]. In brief, immature male rats weighing 50–80 g were orchidectomised under ether anesthesia. The progestational compounds were administered once daily for 7 days. The day after last treatment the animals were killed with CO_2 gas and the seminal vesicles and ventral prostate were weighed. The progestagens were suspended in an aqueous solution of gelatin (5 mg/ml) and mannitol (50 mg/ml). For this test four blocks or five blocks and two rats/block/dose were used in experiment 1 and 3 or in experiment 2, respectively.

2.9. Calculations and statistical analyses

2.9.1. In vitro assays

The relative binding affinity (RBA) and relative agonistic activity (RAA) values were calculated using the parallel line assay by plotting the ln B/B_t-B versus the logarithm of the dose competitor [14]. For the calculation of the mean values and the standard error of the means (SEM's) the cumulative results of the number of experiments (see Table 1) were combined. For the calculation of the in vitro selectivity indices the mean values of the RBA's and RAA's for the progesterone and androgen receptors were taken. The difference between compounds is regarded to be significant when a Student's t-test gives a $P < 0.05$.

2.9.2. In vivo assays

The mean score per dose was calculated in the McPhail test, transformed into logaritmic values and the log–response curves were made for the compounds. For every compound the effective dose at a score of 2 (minimum positive effect) was assessed (ED_{50}) by intrapolation with the best-fitted line in the linear part of the dose-response curve by using the method of least squares.

The results of the ovulation inhibition test were calculated by giving each ovulating rat a score of 0, postponed ovulation a score of 1 and absence of ovulation a score of 3 [1]. The score per group was expressed as percentage of the possible maximum. Log-dose responses were made and the dose giving a response of 50% was estimated (ED_{50}) by intrapolation as for the McPhail test.

Ventral prostate weights, being the most relevant parameter for androgenic activity in rats in the Hershberger test, were replaced by their logarithms and the log-dose vs $100 \times \log$ response curves were constructed. The best-fitted line of the linear part of the dose–response curves was calculated using the method of least squares and the dose giving an increase in percentage log-weight of 180 were calculated (ED_{180}).

The ratio of the doses giving a percentage increase of the ventral prostate of 180 and the effective dose in the McPhail and ovulation inhibition tests is used as in vivo selectivity index. In addition the calculated selectivity indices were compared to that of ETG (potency 1.0).

3. Results

3.1. Receptor studies

3.1.1. Progesterone receptor

The RBA values of the compounds for the PR in MCF-7 cells and the RAA values obtained in the CHO transactivation assays are presented in Table 1 and Fig. 2. NET, the basic structure of the 8 compounds, has

both the lowest affinity to the progesterone receptor (22% of Org 2058) and the lowest transactivation activity (12% of Org 2058). The introduction of an 11-methylene group (11-NET), a Δ^{15} bond (15-NET) or an 18-methyl group (LNG) gave a five-, two- and four-fold higher affinity to the progesterone receptor, respectively. The in vitro agonistic activity for these three derivatives increased approximately threefold. Addition of a Δ^{15} bond towards 11-NET (11,15-NET) results in a 6.5 times higher binding affinity and a 10 times higher transactivation activity compared with NET. Substitution of 11-methylene or a Δ^{15} bond or both substitutions in LNG (ETG, GSD and 11,15,18-NET) increased the RBA about 9 times to that of NET. The agonistic activity is about 9 times higher for ETG and 11,15,18-NET and 12 times for GSD.

3.1.2. Androgen receptor

The RBA and RAA values of NET for the AR were relatively low: 3.2 and 1.1% of DHT, respectively (Table 1, Fig. 2). Introduction of a Δ^{15} bond, with or without 11-methylene as well as 11-methylene and 18-methyl or an 11-methylene alone had no effect on RBA or RAA values. Substitution of a methyl group at C-18 (LNG) alone enhances the RBA values with a factor 3. The 11-methylene or the Δ^{15} bond (ETG and GSD), when introduced in LNG, reduced the RBA values by about two-fold to LNG. The in vitro agonistic activity of LNG and GSD, however, was comparable and 5–6 times higher than that of NET, whereas ETG was only 2.4 times more active than NET. Introduction of a Δ^{15} bond diminished transactivation activity.

3.2. McPhail test for progestational activity in immature rabbits

The results of all eight progestagens are given in Fig. 3. In Table 2 the effective dose values are presented. NET attained a mean effective score of 2.0 at a total sc dose of 64 μg/kg. A dose of 96 μg/kg resulted in a score of 2.7, whereas at a dose of 125 μg/kg the effect decreased. Mono-substitution with 11-methylene or 18-methyl (LNG) enhanced the potency about 5 times, while a Δ^{15} bond resulted in a 2.5-fold increase. A combination of two or three substituents doubled activity further. ETG, GSD, 11,15-NET and 11,15,18-NET were 12.5-fold more potent than NET.

3.3. Ovulation inhibition test

In Fig. 4 the results of all eight progestagens are given. The mono-substituted compounds were significantly more potent in the ovulation inhibition test than NET, which at a twice daily sc dose of 186 μg/kg inhibited ovulation by 50%. Substitution with an 11-methylene, Δ^{15} bond or 18-methyl increased the activity 9, 3 and 6 times. 11,15-NET was 9 times more potent

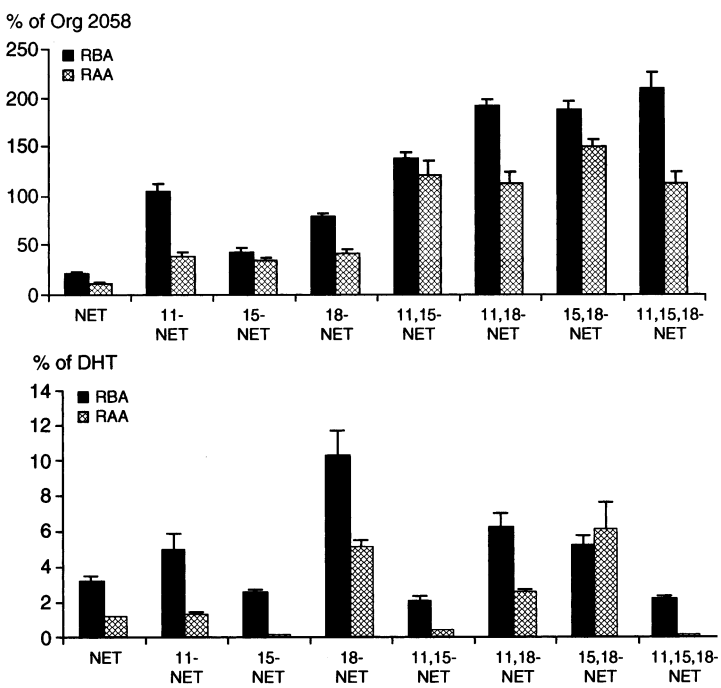

Fig. 2. Relative binding affinities (RBA) and relative agonistic activities (RAA) for 8 progestational compounds for the human progesterone (top) and androgen receptors (bottom) in MCF-7 (black bars) and CHO cells (hatched bars). Org 2058 and DHT were used as reference compounds for the progesterone and androgen receptor, respectively. Data are given as means ± SEM.

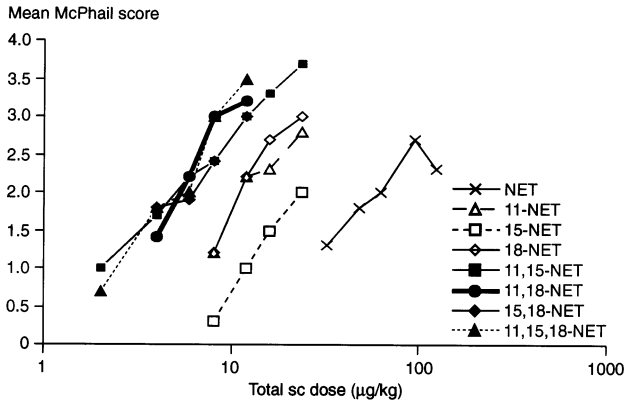

Fig. 3. The effective dose (μg/kg) for eight progestational compounds after subcutaneous administration in the McPhail test in immature rabbits.

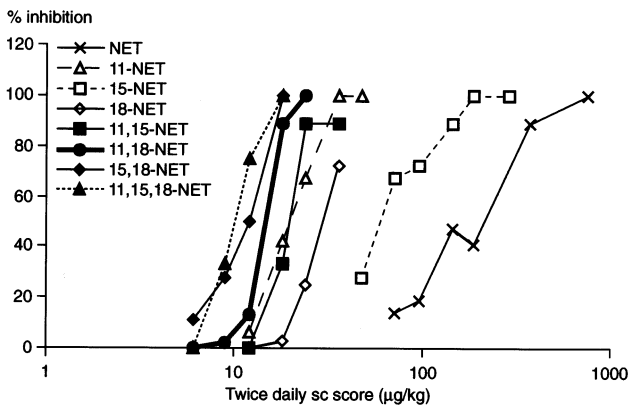

Fig. 4. The effective dose (μg/kg) for eight progestational compounds after subcutaneous administration in the ovulation inhibition test in normal cycling rats.

than NET, but significantly less active than ETG. GSD and 11,15,18-NET were the most potent compounds, possessing an activity significantly higher than that of ETG. Mono-substituted compounds were significantly less active than bi-substituted compounds. After statistical calculation it appeared that the potency of 11,15-NET was significantly lower than that of ETG, but GSD and 11,15,18-NET were significantly more potent than ETG.

3.4. Hershberger test for androgenic activity in immature orchidectomised rats

In Fig. 5 the percentage increase in ventral prostate weight was compared to the placebo-treated groups, using various doses of the progestational compounds. In addition the calculated dose is given at which a 180% (effective dose) increase is attained (Table 2). NET possessed low intrinsic androgenic activity: an effective dose of 2.4 mg/kg was found. Introducing a Δ^{15} bond into NET (15-NET) did hardly influence the androgenic effect, whereas 11-NET increased androgenicity by 1.8. The 18-methyl group in NET (LNG) resulted in the highest androgenicity: its effective dose was 4.6 times lower than that of NET. Combining 11-methylene with 18-methyl (ETG) diminished androgenicity 1.6 times, which activity was significantly lower than that of LNG. 18-Methyl with a Δ^{15} bond (GSD) was as active as LNG. After statistical calculation it appeared that the androgenicity of both compounds was significantly higher compared to that of ETG. 11,15-NET with or without 18-methyl showed hardly any activity in the Hershberger test and therefore no effective dose could be calculated.

3.5. Selectivity indices

The effective doses for progestagenic and androgenic potencies as found in the different assays are given in

Table 2
The effective dose (μg/kg) for eight progestational compounds after subcutaneous administration in the McPhail, ovulation inhibition and Hershberger test

Compound	McPhail test [a]	Ovulation inhibition test [b]	Hershberger test[c]
NET	64.0[g]	186[g]	2402[g]
11-NET	12.7[g]	19.9[g]	1302
15-NET	23.0[d]	65.0[g]	2100[g]
18-NET (LNG)	11.8[g]	28.7[g]	519[g]
11,15-NET	5.1	20.2[g]	[f]
11,18-NET (ETG)	5.4	13.5	812
15,18-NET (GSD)	5.5	10.7[g]	668[g]
11,15,18-NET	4.8	10.2[g]	[f]

[a] The effective dose was calculated as the dose at which: A McPhail score of 2 was attained; the dose is given at total dose administered over 5 days.
[b] An inhibition of 50% was attained; the dose given was administered twice daily.
[c] An increase of 180% in ventral prostate weight was attained; the dose was administered daily.
[d] No significance determined (score ≤ 2).
[f] Not detectable because of non-significant regression of test compound.
[g] Statistically significantly different from ETG ($P < 0.05$).

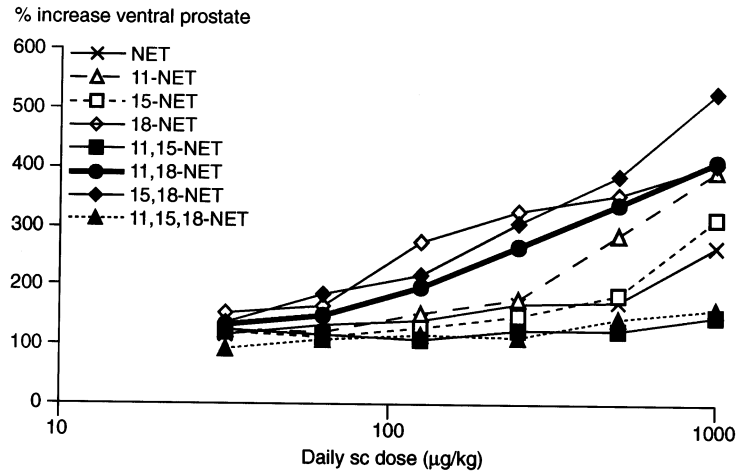

Fig. 5. The effective dose (µg/kg) for eight progestational compounds after subcutaneous administration in the Hershberger test in immature orchidectomised rats.

Table 2. In Table 3 the Progestagenic/Androgenic selectivity indices derived from receptor binding studies, transactivation assays and in vivo bioassays are presented. A good correlation between the results in the McPhail test and the ovulation inhibition test is found ($r = 0.99$). Therefore the indices obtained in two species are calculated: (1) results found in the rabbit in the McPhail test versus results in rat in the Hershberger test and; (2) results found in rat in the ovulation inhibition test versus results in rat in the Hershberger test.

NET showed in the in vitro bioassays for RBA and RAA selectivity indices of 6.7 and 11, respectively. Introduction of 11-methylene increased both indices 3 times, whereas 18-methyl had hardly any effect. The Δ^{15} bond had a weak enhancing effect on selectivity found for RBA, but in the transactivation test the ratio was 31 times higher than that of NET as a result of the low androgenic activity. ETG and GSD showed more favourable indices than LNG (3–4 times higher). Extremely high indices are found for 11,15-NET and 11,15,18-NET both due to their high progesterone receptor binding and very low androgen receptor binding.

With respect to the in vivo Progestagenic/Androgenic selectivity indices, obtained in the McPhail test, ETG and GSD had clearly higher indices (150 and 121) than the other compounds of which NET and LNG had the lowest ratios (37 and 44). Using the progestational activity obtained in the ovulation inhibition test, these values were 60 and 62 for ETG and GSD and 13 and 18 for NET and LNG, respectively. The selectivity indices of 11,15-NET and 11,15,18-NET are also large, but could not be calculated accurately because of non-parallelism in the Hershberger test.

Comparison of in vitro and in vivo Progestagenic/Androgenic selectivity ratios using ETG as a reference leads to very similar values in all models (Fig. 6). The selectivity of NET, 15-NET and LNG was lower than that of ETG and that of GSD was comparable. The in vitro and in vivo selectivity of 11-NET was lower, but in the rat it was equal to ETG. Those of 11,15-NET and 11,15,18-NET were in vitro much higher, but because of lack of androgenicity their high in vivo selectivity could not be calculated precisely.

4. Discussion

Norethisterone (NET) forms the basic structure of newly developed 19-nortestosterone derivatives, which are widely used in oral contraceptives and for hormonal replacement therapies for climacteric complaints. Some of these newly developed progestagens with an 18-methyl (LNG) or 11-methylene-18-methyl (ETG) and Δ^{15}-18-methyl (GSD) are already used in OC's. The potential properties of these progestagens can be tested by in vitro binding and transactivation studies, but the potencies estimated in various in vivo bioassays are more relevant, because metabolism and pharmacokinetics of a drug may strongly influence their activities. However, although receptor binding and clinical data on these drugs have been published, data on the properties in animal studies are lacking [1,2,7,8].

The most important progestagens used from the 19-nortestosterone series are NET, LNG, ETG and GSD. These compounds differ only very slightly in structure (Fig. 1), but are quite different with respect to their progestational and androgenic properties. In the present study we investigated the influence of molecular modifications with 11-methylene, Δ^{15} and 18-methyl substituents in the NET structure on progestational and androgenic potencies in vitro (receptor and transactivation studies) and in vivo (bioassays) systematically. Part of the receptor binding data as presented here have

Table 3
Selectivity indices (progestagenic over androgenic activity) for eight progestational compounds found in in vitro and in vivo bioassays

Compound	In vitro		In vivo	
	Binding MCF-7 cells PR/AR	Transactivation CHO cells PR/AR	McPhail/Hershb.	Ovul.inh./Hershb.
NET	6.7	11	37	12.9
11-NET	21	30	102	65.4
15-NET	18	340	91	32.3
18-NET (LNG)	7.8	8	44	18.1
11,15-NET	69	305	[a]	[a]
11,18-NET (ETG)	31	43	150	60.1
15,18-NET GSD)	36	25	121	62.4
11,15,18-NET	95	1120	[a]	[a]

[a] Not determined due to the absence of ED 50 value for the androgenic activity in the Hershberger test.

been published before, using various different cell lines [6,8,12]. However, incomplete in vivo data have been provided in these studies. Other investigators used different routes of administration in the same study [15] or other species [16]. Very often a limited number of the presently used compounds are tested in one particular study [2,10,11,15,17–21]. We present here a complete overview of both the in vitro and in vivo data of individual 11-methylene, Δ^{15} bond and 18-methyl additions and combinations thereof, comprising ETG and GES, drug substances used in marketed products.

The progesterone receptor binding and agonistic activity increased for all three mono-substituted compounds, although the effect of Δ^{15} bond was far less potent than with the other two. Introduction of 11-methylene (ETG) or Δ^{15} (GSD) or both towards LNG did clearly show synergistic activity and equipotency for these three compounds. If the 18-methyl was lacking, as in 11,15-NET, the RBA value was lower than for ETG or GSD, but higher than for LNG. The results obtained in the transactivation tests correlated very well with those obtained in the binding assays (correlation coefficient 0.90). These results indicate that small modifications such as 11-methylene or 18-methyl have a large effect, whereas a Δ^{15} has a smaller influence on the binding affinity. Mono- and bi-substituents have additive effects in contrast to the tri-substitutions. The results in the transactivation assay were in line with those of the binding assay, although GSD was more active in the transactivation test than ETG, despite equipotent activities in binding studies.

The RBA values of LNG, ETG and GSD for the progesterone receptor are in agreement with those of Phillips et al. [15] using rabbit uterine cytosol preparations, whereas the results of Pollow et al. [18] differ from ours in that LNG and ETG exhibit equal affinity for the human cytosol receptor, which was higher than that of GSD. The ranking of the RBA's of the compounds for the progesterone receptor found by Hoppen and Hammann [8] in human premenopausal endometrium and our results obtained in MCF-7 cells are identical.

As far as the binding to the androgen receptor is concerned, it was shown that especially the 18-methyl group potentiated binding affinity (LNG). The 11-methylene group increased RBA in NET, but reduced androgen binding when any other substituent was present (11,15-NET, 11,18-NET and 11,15,18-NET). The Δ_{15} had no effect in NET, but decreased androgen binding, induced by the 18-methyl substituent, in GSD and 11,15,18-NET. Using the transactivation assay, LNG and GSD showed the highest androgenic agonistic activity and again the 11-methylene substituent diminished the effect in ETG, 11,15-NET and 11,15,18-NET. A Δ^{15} induced a lower agonistic in vitro activity, which resulted in a relatively low correlation coefficient of 0.77 to RBA values.

Of the NET-substituted marketed progestagens LNG, ETG and GSD, ETG has the lowest RBA for the androgen receptor in human mammary carcinoma cells [[8]; this study], which is in agreement with the results of Pollow et al. [18] and Phillips et al. [15] using rat prostatic cytosol assays, whereas Spona [16] reported that the RBA values of ETG for the mouse kidney cytosol androgen receptor was significantly higher than that of LNG. The data of the remaining compounds correspond very well with those of Hoppen and Hammann [8].

NET showed in the McPhail test a biphasic effect, due to its weak estrogenic activity, which causes an antiprogestagenic effect in this test [22,23]. In the McPhail test mono-substitution of NET increased the progestagenic in vivo activity 3–5 times (Table 2). Bi- and tri-substitution enhanced the activity further. With respect to ovulation inhibition mono-substition of NET resulted in 3–9 times more potent compounds, whereas bi- and tri-substitution increased potency further, except for 11,15-NET, which was as active as 11-NET.

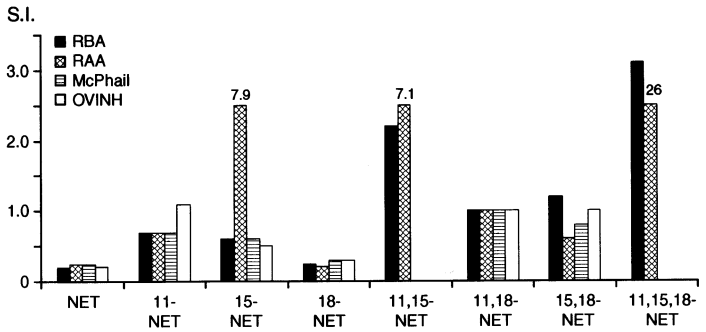

Fig. 6. Selectivity indices (progestagenic activity over androgenic activity) of eight progestagens determined in vitro and in vivo bioassays. 11,18-NET (ETG) is used as reference compound (selectivity is defined as 1.0).

This means that the lower in vivo activity found in the ovulation inhibition test corresponds better with the RBA than with the high activity in the McPhail test. The relative potencies in the McPhail and the ovulation inhibition tests determining progestational activity correlated very well with that of the RBA for the progesterone receptor (correlation coefficients of 0.91 and 0.93, respectively) and the in vitro agonistic activity (0.88 and 0.81). The conclusion from these data is that the effects of progestagens in in vitro binding and transactivation studies are very representative for in vivo endometrium proliferation in rabbits and ovulation inhibition in rats.

All compounds showed in the Hershberger test a higher androgenic activity with respect to growth of ventral prostate weight than the parent compound NET with the exception of 11,15-NET and 11,15,18-NET, for which androgenic activity became negligible at dose levels of 10 mg/kg. The androgenicity of LNG and GSD was significantly higher than that of ETG (Table 2). The results for this in vivo test are in line with the RBA and RAA values found for the androgen receptor (correlation coefficients of 0.86 and 0.88, respectively). Since the absolute figures for the androgenic activities can be misleading we calculated the selectivity ratios by dividing the progestational activity by the androgenic activity for both in vitro and in vivo potencies. Because the androgenic activity of 11,15-NET and 11,15,18-NET was very low or even absent, the selectivity of these compounds was therefore not presented as a ratio. The ranking of in vitro selectivity indices as found in this study did not deviate from those found by Hoppen and Hammann [8].

From these experiments we may conclude that small structural modifications exert enhancement of progestational activity and a clear reduction in androgenicity leading to very selective progestagenic compounds. The influence of bi-substitution is additive over mono-substitution, whereas tri-substition is not additive. The three substituents (11-methylene, Δ^{15} bond and 18-methyl) increase the progestational activity over that of NET and combinations of these substituents result in an even further increase in activity. The effect on androgenic activity of the three substituents is more complex: the 18-methyl group as present in LNG increases the androgenic activity, whereas the 11-methylene group increases RBA in NET, but reduces androgen binding in combination with any other substituent (ETG, 11,15-NET and 11,15,18-NET). The Δ^{15} bond had no effect in NET, but decreased androgen binding, induced by the 18-methyl substituent, in GSD and 11,15,18-NET. The effects of these eight progestagens in in vitro binding and transactivation studies are very representative for in vivo endometrium proliferation in rabbits, ovulation inhibition in rats and growth of ventral prostate in orchidectomised rats.

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