PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 74 (2000) 213–222

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Hormonal properties of norethisterone, 7α-methyl-norethisterone and their derivatives

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Accepted 17 July 2000

Abstract

Norethisterone (NET) is a progestagenic compound with very weak androgenicity and estrogenicity. These low androgenic and estrogenic activities may be attributed to NET itself or induced by metabolites of NET. In order to improve the bioactivity of NET, the effects of a 7α-methyl substitution were studied. Thus this study has two objectives: first the comparison between biological activities of NET and 7α-methyl-NET (MeNET), and second the biological activity of tentative metabolites of NET and those of MeNET. The metabolites consist of a 3-keto-, 3α- or 3β-hydroxy-group located next to a carbon 4 to 5 double bond (Δ^4) or a 5α-hydrogen atom. The 7α-methyl substitution was of special interest as it prevents 5α-reduction. The biological activities of NET, MeNET and their potential metabolites were assessed by in vitro binding, transactivation and proliferation assays on progesterone (PR), androgen (AR), estrogen (ER) and glucocorticoid (GR) receptors and by in vivo progestagenic McPhail, androgenic Hershberger, estrogenic Allen–Doisy tests and combined estrogenic and progestagenic ovulation inhibition tests. NET is a compound with five- to eight-fold weaker PR binding and transactivation activities than the reference compound Org 2058 (100%) and two-fold stronger than progesterone. Binding and transactivation activities of NET for AR (DHT = 100%) are 3.2 and 1.1%, respectively, for ER none (E_2 = 100%) and for GR below 1% (DEX = 100%). MeNET is 1.5- to two-fold less progestagenic and ten- to 20-fold more androgenic than NET, while it does not show activity for ER and GR. The relative binding affinity of 5α-NET was seven-fold lower for PR and 1.5-fold higher for AR than for NET, while in transactivation assays 5α-NET was only active at levels below 1% for all tested receptors. 3β-Hydroxy-(5α-reduced)-metabolites showed clear ER binding and transactivation activities, while 3α-hydroxy-(5α-reduced)-metabolites did hardly possess these characteristics. These hydroxy metabolites did not bind or activate other receptors. Substitution of 7α-methyl to NET metabolites led to similar characteristics, but with higher activities for AR and ER and weaker activity for PR. The outcome of in vivo tests showed a remarkable effect for MeNET. Progestagenic activity in rabbits appeared for NET equipotent to or eight-fold higher than for MeNET, after subcutaneous or oral treatment, respectively. On the other hand, MeNET showed in rats a ten-fold higher androgenicity and eight-fold higher estrogenicity than NET. Ovulation inhibition was induced at very low oral or subcutaneous dose levels, being 120- or ten-fold lower than for NET, respectively. The estrogenicity can also be induced by 3α- or 3β-hydroxy metabolites of MeNET, which are 15 or even more than 40-fold stronger than those of NET, respectively. In conclusion, after the introduction of a 7α-methyl substituent to NET an increased estrogenicity and androgenicity and a reduced progestagenic activity was found. The in vivo estrogenicity is mainly due to 3β-hydroxy-MeNET and to a lesser extent to 3α-hydroxy-MeNET, while the androgenicity and progestagenicity are most likely caused by MeNET itself. Since the 7α-methyl substituent inhibits 5α-reductase, 5α-reduced MeNET metabolites can be excluded from biological activities. As MeNET is a very effective ovulation inhibitor, due to its mixed progestagenic and estrogenic profile, a further reduction of androgenicity of MeNET may yield new contraceptives with an attractive profile for contraception.

Keywords: Norethisterone; 7α-Methyl-norethisterone; Progesterone receptor; Androgen receptor; Estrogen receptor; Glucocorticoid receptor; Progestagenic activity; Androgenic activity; Estrogenic activity; Steroid conversion

1. Introduction

Norethisterone (NET) is a synthetic 19-nortestosterone derivative, that is widely used for birth control

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0960-0760/00/$ - see front matter
PII: S0960-0760(00)00125-4

and for climacterial complaints in several pharmaceutical formulations with or without estradiol or ethinylestradiol [1–4]. Further NET is well-known for its progestagenic effect on hypothalamus and/or pituitaries by the abolishment of the mid-cycle surge of gonadotropins, leading to an impairment of ovulation [5,6]. Moreover, endometrium proliferation is induced, which can prevent endometrial complaints [7,8]. Small substituents can have a major influence on biological activities. For instance, substitution of an 18-methyl group to NET results in the compound levonorgestrel, which has much higher progestagenic and androgenic properties [9–11]. Addition of a 7α-methyl group to 19-nortestosterone results in higher androgenic and anabolic activities [12,13]. In this study, we investigated the influence of such a 7α-methyl substituent, but now on NET, to establish the effects on the biological activity. The main aims were: (1) to compare the biological activities of NET with those of 7α-methyl-NET (MeNET); and (2) to compare the biological activities of the tentative metabolites of NET and MeNET.

NET treatment at high dosages leads to weak additional androgenicity and estrogenicity, which can be attributed to NET itself or to one of its metabolites [14,15]. Indeed, several mono- and disulphated as well as mono- and diglucuronidated metabolites of NET have been detected in urine from NET treated women [16,17]. In unconjugated form these NET (or MeNET) metabolites are represented by 5α- and 5β-reduced NET (5α-NET or 5β-NET) and by 3α- and 3β-hydrogenated 5α-NET and 5β-NET, leading to 3α,5α-NET, 3β,5α-NET, 3α,5β-NET and 3β,5β-NET or their corresponding MeNET metabolites (Figs. 1 and 2). These steroid conversions of NET or MeNET may take place in the liver, but also in the pituitary, endometrium, prostate, vagina and breast. The enzymes involved in these metabolic processes are 5α- and 5β-reductase as well as 3α- and 3β-hydroxysteroid dehydrogenase (HSD). Earlier studies by Larrea et al. [14], Mendoza et al. [15] and Pasapera et al. [18] revealed that 5α-NET metabolites, but not 5β-NET metabolites, were the biological active compounds for uteroglobulin and progesterone receptor expression in rabbit uteri. Moreover, 3β-hydrogenation in combination with 5β-NET appears to be unlikely or very low [19]. In order to evaluate whether NET, MeNET or one of its tentative metabolic and biologically active 5α-reduced variants, i.e. 5α-NET, 3α,5α-NET, 3β,5α-NET or their MeNET analogues, induce these androgenic or estrogenic effects, these specific compounds were synthesized. Besides these compounds, also the influence of direct 3α- or 3β-hydrogenation of NET and MeNET was studied.

The 7α-methyl group was introduced, as a tool to prevent 5α-reduction [13,20]. Comparison of metabolism of tritiated 7α-methyl-19-nortestosterone with that of testosterone and 19-nortestosterone demonstrated that in liver, prostate and epididymis the enzyme 5α-reductase is inactive with respect to a 7α-methyl substituted compound. Moreover, the addition of a 7α-methyl group results in higher androgenic and anabolic activities [12,13], without adverse effects on the prostate and seminal vesicles. To obtain more insight in the role of NET, MeNET and their tentative metabolites, these compounds were studied in vitro on steroid receptor binding assays on progesterone (PR), androgen (AR), estrogen (ER), and glucocorticoid (GR) receptors. With the same receptors, their functional agonistic and antagonistic in vitro transactivation was assessed with state of the art bioassays in Chinese Hamster Ovary (CHO) cells, and more in particular the ER-mediated cell proliferation assay with human breast tumor cells. The in vivo properties of these compounds were established in vivo in progestagenic McPhail, androgenic Hershberger, estrogenic Allen–Doisy tests, and combined progestagenic and estrogenic ovulation inhibition tests after both oral and subcutaneous treatment.

2. Materials and methods

2.1. Materials

The following steroids norethisterone (NET), 3β-hydroxy-NET (3β-NET), 5α-hydrogen-NET (5α-NET),

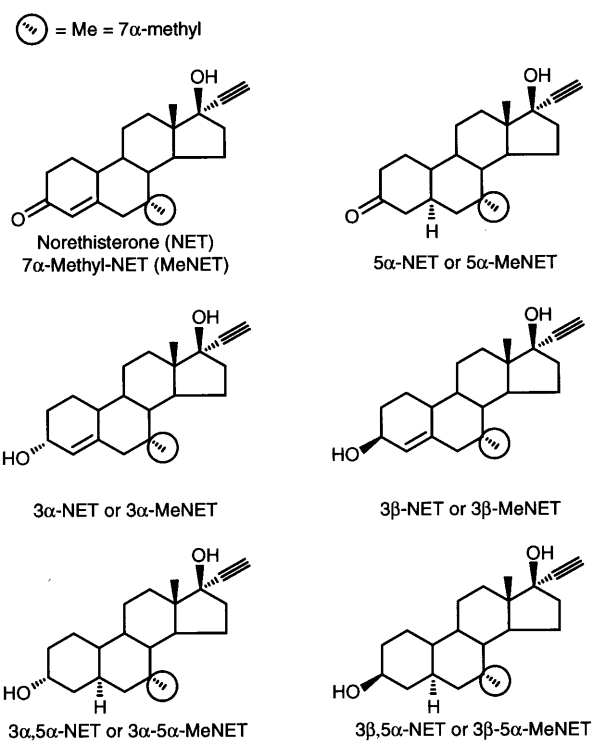

Fig. 1. The chemical structure of norethisterone (NET), 7α-methyl-NET (MeNET) and their metabolites. The 7α-methyl substituent is marked by a circle.

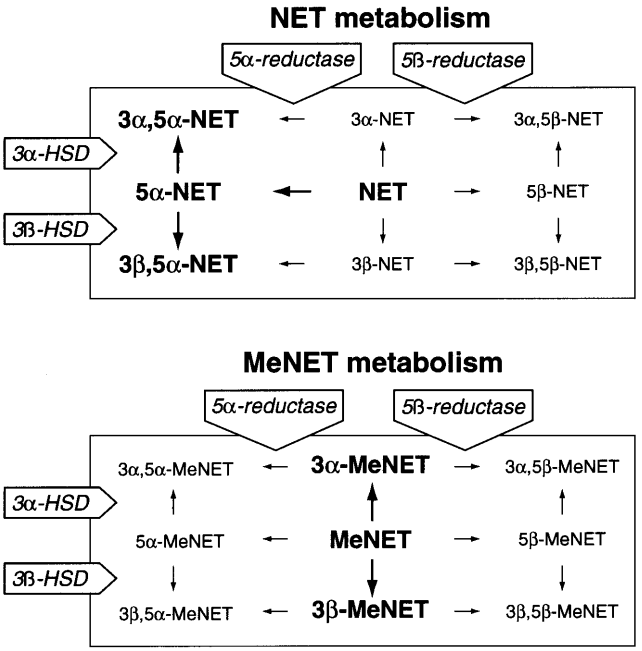

Fig. 2. Scheme of possible metabolic conversions of Norethisterone (NET) and of 7α-Methyl-NET (MeNET) by the enzymes 3α- and 3β-hydroxysteroid dehydrogenase (HSD) and 5α- and 5β-reductase with in bold the most plausible biological routes for NET (top) and MeNET (bottom).

3α-hydroxy-5α-hydrogen-NET (3α,5α-NET), 3β-hydroxy-5α-hydrogen-NET (3β,5α-NET), 7α-Methyl-NET (MeNET), 3α-hydroxy-MeNET (3α-MeNET), 3β-hydroxy-MeNET (3β-MeNET), 5α-hydrogen-MeNET (5α-MeNET), 3α-hydroxy-5α-hydrogen-MeNET (3α,5α-MeNET), 3β-hydroxy-5α-hydrogen-MeNET (3β,5α-MeNET), as well as the standards estradiol-17β (E_2), 5α-dihydrotestosterone (DHT), dexamethasone (Dex), Org 2058 and the antihormones Org 31710 (antiprogestagen), Org 34116 (antiglucocorticoid), cyproterone acetate (antiandrogen) and ICI 164,384 (antiestrogen) were obtained from N.V. Organon (Oss, The Netherlands). The standards ethinyl estradiol, 17β-estradiol benzoate (E_2-B) and methyltestosterone (MT) were obtained from Diosynth (Oss, The Netherlands). The chemical structures of the 19-nortestosterone derivatives are given in Fig. 1.

Trypsin was obtained from Flow Laboratories (Irvine, Scotland), Dulbecco's Modified Eagles Medium/Nutrient Mixture F-12 (DMEM/HAM F12 medium in a ratio of 1:1) from Gibco (Paisley, UK), characterized foetal calf serum (FCS) and defined bovine calf serum supplemented (dBCS) from Hyclone (Utah, USA), 96 well plates from Greiner (Nürtingen, Germany) and 96 well white culture plates and LucLite from Packard (Meridan, USA). Tritiated Org 2058 (s.a. 1.7 Tbq/mmol), and Dex (s.a. 3.29 Tbq/mmol) were obtained from Amersham ('s-Hertogenbosch, The Netherlands) and E_2 (s.a. 4.66 Tbq/mmol) and DHT (s.a. 4.07 Tbq/mmol) from NEN (du Pont, 's-Hertogenbosch, The Netherlands). All other chemicals were of analytical grade.

2.2. Cell lines

MCF-7 cells for binding assays were obtained from Dr McGrath (Michigan Cancer Foundation, USA) and for proliferation from Dr B. van der Burg (Hubrecht Lab., Utrecht, The Netherlands). IM-9 cells were obtained from Dr M. Lesniak (National Institute of Health, USA). These cells were cultured in medium with FCS. The CHO cells, derived from CHO K1 cells obtained from the American Type Culture Collection (Rockville, MD, USA), contained hER-RO-LUC (clone 2B1-1E9), hAR-MMTV-LUC (clone 1G12-A5-CA), hPR-B-MMTV-LUC (clone 1E2-A2), or hGR-MMTV-LUC (clone B4-8), and these cells were cultured in medium with charcoal-treated dBCS. All cell lines were cultured at 37°C in Roux flasks (205 cm^3) flushed with 5% CO_2 in air until pH 7.2–7.4 was reached. Complete medium was refreshed every two or three days. One day before harvesting MCF-7 cells, these cells were cultured on charcoal-treated FCS.

2.3. Animals

The Harlan Sprague Dawley/Central Institute for the Breeding of Laboratory Animals of the Dutch Organisation for Applied Scientific Research (HSD-CPB), Zeist, The Netherlands supplied:

(1). SPF-bred immature male HSD/Cpb:ORGA rats;
(2). SPF-bred young female HSD/Cpb:ORGA rats;
(3). SPF-bred female HSD/Cpb: ORGA rats with known fertility;
(4). immature female HSD/Cpb:CH rabbits.

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). They were fed daily with 50 g pelleted food (LKK-20, Hope Farms) and had free access to tap water.

2.4. Pharmaceutical formulations

A suspension of the compounds or the reference compounds were given in an aqueous solution of gelatin (5 g/l) and mannitol (50 g/l) (gel.mann.) for oral treatment of rats. Rabbits were treated using a tablet

formulation of the following constituents: potato starch 10%, magnesium stearate 0.5% and tabletting powder with 2% amylopectin to 100%. For subcutaneous administration of rats and rabbits the compounds were dissolved in arachis oil (100 g/l) in the presence or absence of a small amount of benzyl alcohol.

3. In vitro studies

3.1. Displacement studies

For displacement analysis MCF-7 and IM-9 cells were used. The cells were cultured, harvested and cytosolic receptor preparations prepared as described by Schoonen et al. [21]. Prior to use the cytosol of 1 gram of cell material was diluted with buffer solution to a final receptor concentration of 1:10 for hER, 1:5 for hAR and 1:20 for hPR in MCF-7 cells and 1:20 for hGR in IM-9 cells. Samples were counted in a Topcount microplate scintillation counter (Packard). Specific binding was determined by subtracting non-specific from total binding. The relative binding affinities of the compounds were obtained by analysis by a three-point parallel line assay [22]. The mean RBA values with standard deviations (SD) of different independent tests were calculated.

3.2. Transactivation studies

For transactivation studies the above described stably transfected CHO cells were used [11,23]. Steroids for treatment were diluted in ethanol and diluted with medium to such a concentration that in the wells of the 96-well white culture plate only 1% ethanol was present. 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. Then part of the medium was removed and LucLite added for cell lysis and luciferase measurement. Luciferase activity was measured in a Topcount luminescence counter. Relative agonistic activity (RAA) and relative antagonistic activity (RANTA) studies were carried out with various concentrations of the standards (1:2:4 dilutions) and compounds of interest. The relative (ant)agonistic activities of the compounds were obtained by analysis by a three-point parallel line assay [22]. The mean RAA and RANTA values with S.D. of different independent tests were calculated.

3.3. Estrogen induced cell proliferation

MCF-7 cells were seeded in 24 well plates, cultured and treated as described by Schoonen et al. [21]. A small modification in the total culture period was a change from 9 to 7 days.

4. In vivo studies

4.1. Progestagenic activity in immature rabbits

The progestational activity of the compounds was assessed as described by McPhail [24] and modified by de Visser et al. [6]. The minimal active dose (MAD) was considered to be the total dose at which the mean McPhail index attained a value of 2.0.

4.2. Androgenic-anabolic activity in orchidectomised rats

The test for androgenic activity was determined on the basis of the ventral prostate growth, with the Hershberger test [25], which is slightly modified by de Visser et al. [6]. The results are presented as MAD: the daily dose at which the ventral prostate growth in weight was 1.8 times higher than the placebo value.

4.3. Estrogenic activity in ovariectomised rats

The estrogenic activity of the compounds in rats was determined according to Allen and Doisy [26] and slightly modified by de Visser et al. [6]. The total dose at which 50% of the animals showed one or more positive smears is given as MAD.

4.4. Ovulation inhibition in rats

Ovulation inhibition in rats was determined according to de Visser et al. [6]. An ovulation inhibition of 60% was considered as the MAD.

5. Results

5.1. In vitro studies

In Table 1 relative binding affinity (RBA) values of NET, MeNET and their potential metabolites for PR, AR, ER and GR are given for cytosolic assays. In Table 2 the relative agonistic activity (RAA) values of NET, MeNET and their metabolites for PR, AR, ER and GR in combination with their respective promoter and luciferase reporter system are given.

5.1.1. Human progesterone receptors

MeNET showed RBA and RAA values of 10.4% and 8.0%, respectively, which was two- and 1.5-fold lower than that of NET. For 5α-NET and 5α-MeNET, RBA values were, respectively, seven- or 1.8-fold lower with respect to NET and MeNET, while RAA values were more than 100- and 12-fold lower. The 3β-hydroxy NET and 3α- and 3β-hydroxy MeNET metabolites had five- to ten-fold reduced RBA and RAA values with

respect to their parent compounds, while their respective 5α-reduced metabolites showed RBA and RAA values that were even more than 30- to 100-fold lower, respectively.

5.1.2. Human androgen receptors

The 7α-methyl substitution in MeNET led to RBA and RAA values of 29.1 and 20.3%, respectively, being ten- and 20-fold higher than that of NET. For the tentative 5α-reduced metabolites of NET and MeNET, RBA values were, respectively, 1.6-fold increased and equal to their parent compounds, while RAA values were equal and ten-fold lower. The 3β-hydroxy NET and 3α- and 3β-hydroxy MeNET metabolites had RBA and RAA values, that were five- to ten-fold reduced with respect to their parent compounds. Their respective 5α-reduced metabolites showed RBA and RAA values that were even more than 30- to 100-fold lower.

5.1.3. Human estrogen receptors

Neither NET nor MeNET did bind or transactivate via the estrogen receptor. Also the tentative 5α-reduced metabolites of NET and MeNET were very weak binders and activators with relative potencies of less than 0.7%. Especially the 3β-hydroxy NET and MeNET, but also 3α-hydroxy MeNET, showed clear binding and transactivation potencies. The MeNET metabolites were three to four times stronger than the NET metabolites and the 3β-hydroxy metabolites of MeNET were six-fold more potent than their 3α-hydroxy metabolite. Their respective 5α-reduced metabolites appeared to be 0.6- to three-fold weaker, while again the 3β-hydroxy metabolites appeared to be six- to 14-fold more potent than their 3α-hydroxy counterparts.

5.1.4. Human glucocorticoid receptors

NET and MeNET as well as their metabolites showed only a very weak binding of less than 0.8% or did not bind at all. None of these compounds showed transactivation activity.

5.1.5. Antagonistic properties of the steroid receptors

The relative antagonistic activity (RANTA) values of NET, MeNET and their metabolites were also assessed for the same set of steroid receptors in the same cell lines. PR was only weakly inhibited by 5α-NET, 3ß,5α-NET, 3β-MeNET, 3α,5α-MeNET, and 3β,5α-MeNET with relative potencies in the range from 0.2 to 0.7%. AR and GR were inhibited weakly by some of the compounds with relative potencies in the range from 1.5 to 6.3%. However, the observed inhibitions were only detected at very high concentrations exceeding levels of 10^{-6} and even 10^{-5} M, while for ER no inhibition was identified with any of these compounds.

5.1.6. Cellular proliferation of MCF-7 cells

The effects of NET, MeNET, 3ß-NET as well as of 3α- and 3β-MeNET were also studied on estradiol-induced cell growth with MCF-7 cells. After 2 days of steroid independent growth, 5 days of steroid treatment followed (Table 3). NET and MeNET were 20 000- and 4000-fold less potent than estradiol, respectively, while 3β-NET, 3α-MeNET and 3β-MeNET were only 30, 25 and four times less potent, respectively. The proliferating activities of these compounds correlate reasonably well with their respective RBA and RAA values (Table 3). The 5α-reduced metabolites were not studied in these assays.

Table 1
Mean of relative binding affinity (RBA) values with standard deviation (S.D.) of norethisterone (NET), 7α-methyl-NET (MeNET) and their metabolites to the progesterone, androgen and estrogen receptor in cytosol of human breast MCF-7 cells and to the glucocorticoid receptor in that of leukemic IM-9 cells using Org 2058, 5α-dihydrotestosterone (DHT), estradiol (E_2) and dexamethasone (Dex) as standards[a]

Steroids	Progesterone receptor (Org 2058 = 100%)	Androgen receptor (DHT = 100%)	Estrogen receptor (E_2 = 100%)	Glucocorticoid receptor (Dex = 100%)
Norethisterone metabolites				
NET	21.5 ± 5.3 (31)	3.2 ± 1.4 (36)	N.B. (4)	0.8 ± 0.2 (2)
3β-NET	2.0 ± 1.0 (8)	0.3 ± 0.1 (4)	11.4 ± 2.0 (10)	N.B. (3)
5α-NET	3.0 ± 0.3 (2)	5.1 ± 1.7 (4)	0.2 ± 0.1 (2)	0.1 ± 0.1 (2)
3α,5α-NET	0.2 ± 0.1 (2)	0.1 ± 0.1 (2)	0.5 ± 0.1 (2)	N.B. (2)
3β,5α-NET	0.2 ± 0.1 (2)	N.B. (2)	7.8 ± 1.1 (2)	0.2 ± 0.1 (3)
7α-Methyl-NET metabolites				
MeNET	10.4 ± 2.2 (9)	29.1 ± 11.4 (21)	N.B. (4)	0.2 ± 0.1 (3)
3α-MeNET	1.8 ± 0.8 (4)	2.9 ± 0.4 (2)	5.5 ± 0.8 (6)	N.B. (3)
3β-MeNET	1.0 ± 0.3 (4)	2.1 ± 0.3 (2)	29.4 ± 7.5 (9)	N.B. (3)
5α-MeNET	6.0 ± 0.9 (2)	30.7 ± 11.9 (3)	0.7 ± 0.1 (2)	0.2 ± 0.1 (2)
3α,5α-MeNET	0.2 ± 0.1 (2)	0.8 ± 0.5 (3)	1.5 ± 0.2 (2)	N.B. (1)
3β,5α-MeNET	0.3 ± 0.1 (2)	0.3 ± 0.1 (3)	20.7 ± 2.4 (2)	N.B. (2)

[a] N.B. = No Binding at 10^{-6} M; between parenthesis = number of experiments.

Table 2
Mean of relative agonistic activity (RAA) values with standard deviations (S.D.) of norethisterone (NET), 7α-methyl-NET (MeNET) and their metabolites to the progesterone, androgen, estrogen and glucocorticoid receptor in transactivation assays with CHO cells using Org 2058, 5α-dihydro-testosterone (DHT), estradiol (E_2) and dexamethasone (Dex) as standards[a]

Steroids	Progesterone receptor (Org 2058 = 100%)	Androgen receptor (DHT = 100%)	Estrogen receptor (E_2 = 100%)	Glucocorticoid receptor (Dex = 100%)
Norethisterone metabolites				
NET	12.4 ± 3.30 (9)	1.1 ± 0.3 (32)	N.T. (2)	N.T. (2)
3β-NET	1.8 (1)	0.2 ± 0.1 (6)	4.6 ± 0.85 (20)	N.T. (2)
5α-NET	0.2 ± 0.01 (2)	0.8 ± 0.5 (3)	0.5 ± 0.05 (2)	N.T. (2)
3α,5α-NET	0.1 ± 0.02 (3)	0.3 ± 0.1 (3)	0.4 ± 0.07 (3)	N.T. (2)
3β,5α-NET	0.1 ± 0.02 (3)	0.1 ± 0.1 (3)	2.5 ± 1.15 (6)	N.T. (2)
7α-Methyl-NET metabolites				
MeNET	8.0 ± 1.70 (2)	20.3 ± 6.6 (30)	N.T. (3)	N.T. (2)
3α-MeNET	1.2 ± 0.90 (2)	2.8 ± 1.70 (2)	3.1 ± 0.7 (21)	N.T. (2)
3β-MeNET	1.6 ± 1.80 (2)	2.0 ± 0.50 (2)	19.0 ± 2.6 (16)	N.T. (2)
5α-MeNET	0.7 ± 0.03 (2)	2.5 ± 0.50 (2)	0.4 ± 0.09 (3)	N.T. (2)
3α,5α-MeNET	0.2 ± 0.06 (3)	0.5 ± 0.28 (3)	0.7 ± 0.05 (2)	N.T. (2)
3β,5α-MeNET	N.T. (2)	N.T. (3)	6.9 ± 0.54 (3)	N.T. (2)

[a] N.T. = no transactivation at 10^{-7} M; between parenthesis = number of experiments.

5.2. In vivo studies

In Table 4 the in vivo progestagenic Mc-Phail, androgenic Hershberger, estrogenic Allen–Doisy tests and combined progestagenic and estrogenic ovulation inhibition test for NET, MeNET and their metabolites are given in MAD after subcutaneous and oral administration. Dose response curves for NET and MeNET are given in Fig. 3.

5.2.1. Progestagenic activity in immature rabbits (McPhail test)

5.2.1.1. Subcutaneously. NET and MeNET showed similar progestagenic efficacy when given at 63 μg/kg. The potency of 3α-MeNET was eight times less than that of MeNET, whereas none of the other tested NET or MeNET metabolites showed progestagenic activity at a dose of 1 mg/kg or higher.

5.2.1.2. Oral. NET attained a MAD at 250 μg/kg. Substitution of a 7α-methyl group reduced this progestational activity by eight-fold. At 2 mg/kg a score of 2 was obtained with MeNET, but at higher doses the effect diminished (Fig. 3). All the other 5α-reduced metabolites with or without 3α- or 3β-hydroxy groups were less active than the respective parent compound.

5.2.2. Androgenic activity in orchidectomised rats (Hershberger test)

5.2.2.1. Subcutaneously. Introduction of the 7α-methyl group into NET increased the androgenic activity, determined on the ventral prostate, by eight-fold. On the other hand, 3β-NET was two-fold more potent than NET. Comparison of the results of the other 5α-reduced metabolites of NET and MeNET with or without 3α- or 3β-hydroxy groups showed that these compounds were less active than the respective parent compound.

5.2.2.2. Oral. MeNET had about eight times the activity of NET on the ventral prostate. With exception of 3α-MeNET, which was equipotent or two-fold more potent than MeNET, all other metabolites showed equal or reduced androgenic potencies with respect to the parent compounds.

5.2.3. Estrogenic activity in ovariectomised rats (Allen–Doisy test)

5.2.3.1. Subcutaneously. In order to obtain an estrogenic response in 50% of the rats a dose of 4 and 0.25 mg/kg was needed for NET and MeNET, respectively. 3β-Hydrogenation increased the estrogenic potency by 16- to 8-fold, respectively. 5α-Reduction increased the activity of NET about four times, whereas such a change in MeNET showed only a two-fold increase. However, if also 3α- or 3β-hydrogenation was introduced this potency increase disappeared again.

5.2.3.2. Oral. MeNET was about 50 times more estrogenic than NET. In contrast to subcutaneous treatment 3β-NET showed now only a two-fold potency improvement. The other metabolites were almost as active as the parent compounds, whereas in case of 5α-MeNET a ten-fold reduction in activity was observed.

5.2.4. Ovulation inhibition in rats

Both progestagens and estrogens can contribute to a blockade in ovulation. This means that this assay is not specific and compounds with low progestagenic and high estrogenic potencies, like some NET derivatives, will behave as estrogenic compounds with respect to ovulation inhibition (Fig. 3).

5.2.4.1. Subcutaneously. MeNET and 3β,5α-MeNET were ten times more potent than NET. 3β-MeNET was the strongest compound with respect to ovulation inhibition, being even four-fold more potent than MeNET. All the other metabolites were less active than the parent compounds.

5.2.4.2. Oral. Introduction of a 7α-methyl group in NET resulted in a 120 times higher ovulation inhibition. 3β-NET was about eight times more effective than NET, while the other NET metabolites were approximately equipotent to NET. The MADs of MeNET and its metabolites were almost equal, except for 5α-MeNET, which was four times less active.

6. Discussion

NET is described as a compound with progestagenic activity [2–4]. Braselton et al. [27], Orme et al. [1], and Sahlberg et al. [16] have shown with in vivo metabolism studies that 3α- or 3β-hydroxy and/or 5α- and 5β-reduced metabolites of NET are formed, since they identified their sulphated and/or glucuronidated products in blood and urine samples. Others, like Larrea et al. [14] and Mendoza et al. [15], have demonstrated that especially the 5α-reduced NET metabolites, but not the 5β-reduced NET metabolites, are the biologically active compounds. Therefore in the present study these 5α-reduced NET metabolites were investigated. In order to establish the influence of the 7α-methyl substitution on in vivo metabolism both NET and MeNET as well as their potential metabolites were studied for their respective progestagenic, androgenic, estrogenic or combined estrogenic and progestagenic activities in in vivo studies by McPhail, Hershberger, Allen–Doisy and ovulation inhibition tests, respectively. The direct effects of all these tested compounds on PR, AR, ER and GR were demonstrated by binding analysis, by in vitro bioassays with luciferase measurement in CHO based transactivation assays and by in vitro proliferation bioassays with MCF-7 cells. With the in vitro bioassays both agonistic and antagonistic effects can be visualised.

In the tests for progestagenic activity the introduction of a 7α-methyl group to NET led to lower RBA and RAA values for PR. 3α- or 3β-Hydrogenation and 5α-reduction of both compounds showed lower in vitro activity compared to the respective parent compounds. This lower in vitro activity of MeNET did not result in lower in vivo activity after subcutaneous treatment. Both NET and MeNET showed even similar subcutaneous progestagenic activities. However, after oral treatment NET was eight times more potent than MeNET. Moreover, the progestagenic effect of the latter compound diminishes at higher dosages, probably due to the relatively strong estrogenic activity of a metabolite of MeNET (see later in the Allen–Doisy tests), which antagonizes this progestagenic effect. Also at lower dose levels during oral treatment, this estrogenicity of MeNET metabolites is reflected in diminished regression of the dose response of MeNET compared with NET. This difference, however, disappears after subcutaneous treatment, suggesting that metabolites of MeNET are synthesized at much lower amounts, making inhibition of the progestagenic response impossible. These data together confirm that both NET and MeNET are true progestagenic compounds. With respect to the progestagenic activity 7α-

Table 3
Mean of relative estrogenic growth potency with 95% confidence intervals of Norethisterone (NET), 7α-Methyl-NET (MeNET) and some of the potential metabolites for estrogen-induced cellular proliferation in human breast tumor MCF-7 cells using estradiol as standard (100%) in three to six independent assays

Steroids	Potency	95% Confidence intervals		RBA[a]	RAA[a]
		Lower limit	Upper limit		
Norethisterone metabolites					
NET	0.0055 (6)	0.0014	0.0096	N.B.	N.T.
3β-NET	3.24 (4)	0.86	5.61	11.4	4.6
7α-Methyl-NET metabolites					
MeNET	0.028 (4)	0.011	0.045	N.B.	N.T.
3α-MeNET	4.06 (3)	0.07	7.91	5.5	3.1
3β-MeNET	23.3 (3)	13.6	33.0	29.4	19.0

[a] Relative binding affinity (RBA) and relative agonistic activity (RAA) values are also indicated for these compounds. Between parenthesis = number of experiments.

Table 4
Mean active dose (MAD) in mg/kg body weight of norethisterone (NET), 7α-methyl-NET (MeNET) and their metabolites in progestagenic (McPhail), androgenic (Hershberger), estrogenic (Allen–Doisy) tests, and in a progestagenic and estrogenic (ovulation inhibition test) using Org 2058, progesterone, 20α-methyl-testosterone, and estradiol (E_2) as standards both after subcutaneous and oral administration.

Steroids	McPhail test		Hershberger test		Allen–Doisy test		Ovulation inhibition test	
	SC	Oral	SC	Oral	SC	Oral	SC	Oral
Norethisterone metabolites								
NET	0.063	0.25	2.50	20	4	8	0.235	12
3β-NET	>2	1	1.25	>20	0.250	4		1
5α-NET	>8	>8	5	20	1	>2	0.750	>6
3α,5α-NET	>4	>8	>5	10			<0.200	>6
3β,5α-NET	>8	>8	>5	20	1		1	6
7α-Methyl-NET metabolites								
MeNET	0.063	2	0.32	<2.50	0.250	0.160	0.025	0.100
3α-MeNET	0.500	4		1.25	0.250	0.500	≤0.050	0.100
3β-MeNET	>1	>4	1.25	2.50	0.032	0.250	<0.006	0.100
5α-MeNET	>4	>4	1.25	≤5	0.128	2	≤0.400	0.400
3α,5α-MeNET	>4	>4	<5	>5	0.200	0.500	0.200	>0.375
3β,5α-MeNET	>4	>4	>1.25	>5	0.250	0.125	0.024	0.200

methyl substitution of NET reduces the in vitro activity slightly, whereas it does not modify its subcutaneous treatment. The metabolites of MeNET with estrogenic potency appear to influence oral progestagenic activity. In agreement with the in vitro results 3α- and 3β-hydrogenation and/or 5α-reduction decreased the in vivo progestagenic activity both after subcutaneous and oral treatment.

In the tests for androgenicity the 7α-methyl substitution resulted in a compound which had 10- and 20-fold higher RBA and RAA values, respectively. In vivo an eight times increased activity was found irrespective of the way of administration, i.e. subcutaneous or oral. The 3α- and 3β-hydrogenated and/or 5α-reduced metabolites of NET and MeNET showed very low binding, transactivation or in vivo activity. However, 5α-NET and 5α-MeNET demonstrated similar binding activities as their respective parent compounds, whereas the transactivation activity of 5α-MeNET was eight-fold lower than that of MeNET. The MAD's of 5α-NET and NET were equal after both subcutaneous and oral treatment, while those of 5α-MeNET were equally active after oral, but four-fold less effective after subcutaneous administration compared with MeNET. Finally, a biological contribution of 5α-MeNET in vivo is very doubtfull due to the lack of 5α-reduction in vivo. The low transactivation and subcutaneous activity of 5α-MeNET as well as the prevention of metabolism during oral treatment may indicate, that androgenicity is an intrinsic feature of MeNET.

In the tests for estrogenicity NET and MeNET did not bind to or transactivate via ER. On the other hand, 3β-MeNET showed strong binding, transactivation, proliferation and in vivo subcutaneous and oral activity. 3α-MeNET showed far less activity in this respect. From these results it appears that addition of 7α-methyl to NET results in a compound with strong estrogenic properties and that metabolism into a 3β-hydroxy compound is more responsible for the estrogenic properties in contrast to its 3α-hydroxy counterpart. Hereto also the differences in MADs between oral and subcutaneous treatment of NET (8 vs. 4 mg/kg), 3β-NET (4 vs. 0.25 mg/kg), MeNET (160 vs. 250 μg/kg), 3α-MeNET (500 vs. 250 μg/kg) and 3β-MeNET (250 vs. 32 μg/kg) should be taken into consideration. The contrasting effects of NET and MeNET are apparently caused by fast liver metabolism of MeNET into 3β-MeNET and/or fast clearance from the blood of NET into sulphated and glucuronidated compounds, instead of into 3β-NET or 3β,5α-NET. This difference in metabolism is apparently due to the 7α-methyl substitution in MeNET, since this is the only position in which a chemical modification was introduced between NET and MeNET. The fact that 5α-reductase in the liver is prevented by a 7α-methyl substituent [20,17], likely improves the bioavailability of the 3α- and 3β-hydroxy metabolites of MeNET and causes the estrogenic activity. This outcome further implicates, that 3β-MeNET is responsible for the estrogenic properties in vivo. Such a profile is consistent with the data from binding, transactivation and proliferation assays and from Allen–Doisy tests.

In the test for ovulation inhibition, both estrogens and progestagens can contribute to a blockade in ovulation. The low dose of MeNET needed for ovulation inhibition with respect to NET implies that the combined lower intrinsic progestagenic activity of MeNET together with its estrogenic activity, in which 3β-MeNET plays a pivotal role, leads to synergism and high oral afficacy of MeNET. The results of NET and

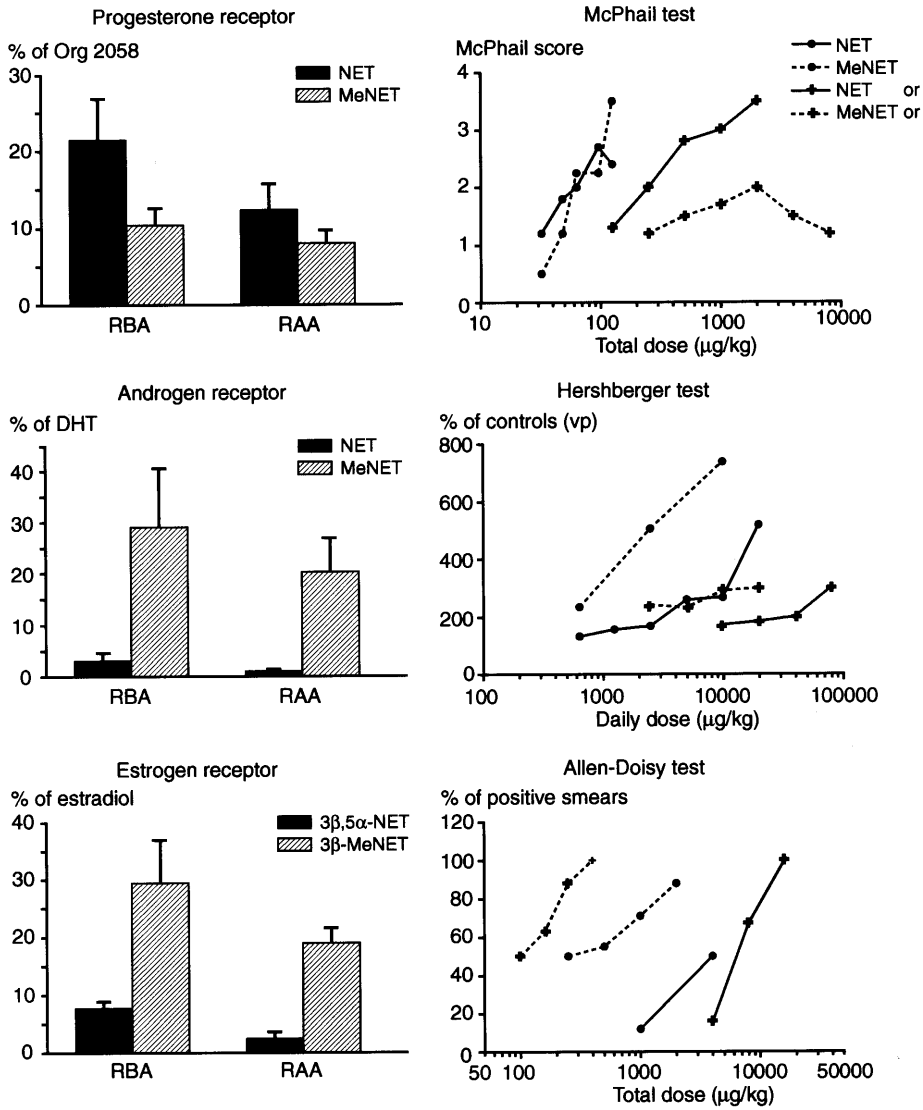

Fig. 3. The relative binding affinity (RBA) and relative agonistic activity (RAA) of norethisterone (NET) and 7α-methyl-NET (MeNET) for the progesterone and androgen receptor and the estrogenic metabolites 3β,5α-NET and 3β-MeNET for the estrogen receptor as well as the bioactivity of NET and MeNET in progestagenic, androgenic, estrogenic and ovulation inhibiting activity, respectively, in McPhail tests in rabbits, and Hershberger, Allen–Doisy and ovulation inhibition tests in rats after subcutaneous and oral administration.

MeNET and its metabolites, seen in the ovulation inhibition test, are in line with the progestagenic activity in the McPhail test and with the estrogenic activity in the Allen–Doisy test. If both the progestagenic and estrogenic in vitro effects of, respectively, MeNET and 3β-MeNET in binding, transactivation and proliferation assays are taken into account the in vivo data in this ovulation inhibition test may explain the strong ovulation inhibitory efficacy of MeNET.

In conclusion, after the introduction of a 7α-methyl substituent to NET an increased estrogenicity and androgenicity and a reduced progestagenic activity was found. The in vivo estrogenicity is mainly due to 3β-hydroxy-MeNET and to a lesser extent to 3α-hydroxy-MeNET, while the androgenicity and progestagenicity are most likely caused by MeNET itself. Since the 7α-methyl substituent inhibits 5α-reductase, 5α-reduced MeNET metabolites can be excluded from biological activities. As MeNET is a very effective ovulation inhibitor, due to its mixed progestagenic and estrogenic profile, a further reduction of androgenicity of MeNET may yield new contraceptives with an attractive profile for contraception.

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