Nestorone®: a progestin with a unique pharmacological profile

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Abstract

Nestorone® (Nestorone 16-methylene-17α-acetoxy-19-norpregn-4-ene-3,20-dione), formerly referred to as ST 1435, is a potent progestin when given parenterally via sustained release formulations. The pharmacological profile of Nestorone was compared with that of levonorgestrel and 3-keto-desogestrel by steroid receptor binding studies and by in vivo bioassays in rats and rabbits. 3-Keto-desogestrel showed the highest binding affinity to progesterone receptors (PR) followed by Nestorone, levonorgestrel, and progesterone. The binding affinity of Nestorone to androgen receptors (AR) was 500- to 600-fold less than that of testosterone. However, both levonorgestrel and 3-keto-desogestrel showed significant binding (40 to 70% of testosterone) to AR. None of the progestins bound to estrogen receptors (ER).

The progestational activity of Nestorone, levonorgestrel, and progesterone was compared using McPhail index in immature rabbits and pregnancy maintenance and ovulation inhibition tests in rats after subcutaneous (s.c.) administration. In all three tests, Nestorone was the most potent progestin. The progestational activity of Nestorone was also compared after oral and s.c. administration in rabbits. The potency of Nestorone was over 100-fold higher upon s.c. administration than via the oral route. The androgenic activity of progestins, based on the stimulation of ventral prostate (androgenic target) and levator ani (anabolic target) growth in castrated immature rats, showed good correlation with their binding affinity to AR. Nestorone showed no androgenic or anabolic activity. Nestorone did not bind to sex hormone binding globulin (SHBG), whereas both levonorgestrel and 3-keto-desogestrel showed significant binding to SHBG. The estrogenic/antiestrogenic activity of Nestorone was investigated in immature ovariectomized rats. In contrast to estradiol and levonorgestrel, Nestorone showed no uterotrophic activity in ovariectomized rats. Despite significant binding to glucocorticoid receptors (GR), Nestorone showed no glucocorticoid activity in vivo. It is concluded that a strong progestational activity, combined with lack of androgenic, estrogenic, and glucocorticoid-like activities, confer special advantages to Nestorone for use in contraception and hormone replacement therapy.

Keywords: Progestin; Androgenic activity; Pharmacology

1. Introduction

The widespread use of progestins alone or in combination with estradiol for female contraception and hormone replacement therapy has generated renewed interest in the development of new progestins with high selectivity indices (ratio of progestational to androgenic activity) [1,2]. Nestorone® (16-methylene-17α-acetoxy-19-norpregn-4-ene-3,20-dione) Nestorone, a 19-norpregnasterone derivative, is a potent progestin when administered parenterally [3]. Upon oral administration, Nestorone undergoes rapid metabolism and inactivation, thereby making it suitable for use in nursing women during lactation [4]. Several clinical trials have been performed with Nestorone administered via subdermal implants [3,5,6]. Nestorone was found to be very effective in controlling fertility at low doses [6]. So far, no alterations in liver function and lipid and carbohydrate metabolism have been observed in women using Nestorone implants [7-9]. Many of the synthetic steroids, in addition to their primary hormonal effects, exhibit some actions typical of other steroids. Indeed, some of the side effects of a compound can be related to such ancillary activities. Thus, we compared the progestational, androgenic, and estrogenic activities of Nestorone with some 19-nortestosterone-derived progestins using receptor binding studies and in vivo bioassays. In addition, we investigated the glucocorticoid-
like activity of Nestorone and levonorgestrel in adrenalecto-
mized rats.

2. Materials and methods

2.1. Materials

Nestorone was custom synthesized by Gideon Richter, Budapest, Hungary. Levonorgestrel was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other steroids were obtained from Steraloids Inc., Wilton, NH, USA. 3-Keto-desogestrel (an active metabolite of desogestrel) was provided by Dr. A. Moo-Young, Population Council, New York. Chemicals and solvents were of reagent grade. Radiolabeled steroids were purchased from NEN Research Products, Boston, MA, USA. All chemicals used were of analytical reagent grade.

2.2. Determination of binding affinities to steroid hormone receptors

2.2.1. Progesterone receptors (PR)

Female Sprague–Dawley rats (150 g, b.wt.) were ovariec-
tomized and 1 week later, injected daily for 3 days (i.p.) with 1 μg 17β-estradiol (E2) to induce synthesis of PR. The animals were killed on day 4, and uteri were collected over dry ice. Uteri were minced into small pieces and homogenized in 10 mM Tris-HCl buffer, pH 7.4, containing 1.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 10 mM sodium molybdate, 10% glycerol (v/v), and 1 mM phenylmethylsulfonyl fluoride (PMSF), using a Polytron PT-10 homogenizer. The homogenate was centrifuged for 20 min at 1000 × g in a refrigerated centrifuge. The supernatant was centrifuged for 90 min at 10 000 × g in a refrigerated Beckman ultracentrifuge. The cytosol was removed and stored at −70°C until used. Aliquot (100 μl) of cytosol were incubated overnight at 4°C in glass tubes with [3H]promegestone, with or without cold steroids, at different concentrations. The bound and free radioactive activity was separated by the dextran-charcoal method [10]. The supernatant was de-
canted into scintillation vials and counted for radioactivity with 5 ml of Beckman Ready Safe® scintillation fluid in a Packard TriCarb Liquid Scintillation Counter. The percentage of radioligand bound in the presence of competitor, compared to that bound in its absence, was plotted against the concentration of unlabeled steroid. The molar concentrations of steroid competitor that reduced radioligand binding by 50% (ED50) were determined graphically. The ratio of these concentrations, multiplied by 100, was termed the relative binding affinity (RBA).

2.2.2. Androgen receptors (AR)

Male Sprague–Dawley rats were castrated through a scrotal incision and were killed the next day. The ventral prostate was collected over ice, minced into small pieces, homogenized and the cytosol fraction was prepared. Aliquots of cytosol were incubated at 4°C for 18 h with [3H]dimethyl-19-nortestosterone (Mibolerone), in the presence or absence of cold steroids. The bound and free radioactivity was separated by the hydroxylapatite method [11].

2.2.3. Estrogen receptors (ER)

Immature female rats (18–21 days old) were killed, and the uteri were removed and homogenized in buffer. The cytosol was prepared as described above. Aliquots of cytosol were incubated with [3H]estradiol, with or without cold steroids. The bound and free fractions were separated by the dextran-charcoal method.

2.2.4. Glucocorticoid receptors (GR)

Calf thymus cytosol was used as the source of GR. Aliquots of cytosol were incubated with 6,7-[3H]triamcinolone acetonide with or without cold dexamethasone or progestins. The percentage bound radiolabeled triamcinolone was determined by separating the bound and free fractions by the dextran-charcoal method.

2.3. Determination of binding to sex hormone binding globulin (SHBG)

Sex steroids, especially estrogens and androgens, are bound to SHBG in circulation. Several synthetic progestins also show binding to SHBG. Thus, the binding of Nestorone and other progestins to SHBG was studied by methods described earlier [12]. Third trimester pregnancy serum was used as the source of SHBG and [3H]testosterone was used as the radioactive ligand. Dextran-charcoal was used to separate bound and free radioactivity.

3. Evaluation of biologic activity: progestational activity

3.1. Endometrial transformation test

Immature female rabbits (700–800 g) were primed with estradiol (5 μg/day s.c. for 6 days) and assigned to treatment groups. They were injected s.c. with progestins for five consecutive days. In a separate experiment, the progestational activity of Nestorone administered via the oral or s.c. route was compared. One day after the last progestin ad-
ministration, the rabbits were killed by asphyxiation with CO2. The uteri were excised and weighed. Part of each uterus was fixed in Bouin’s solution and processed for microscopic examination. The uterine sections were graded for progestagenic activity according to McPhail Index [13].

3.2. Maintenance of pregnancy in ovariectomized rats

Adult female rats were placed in suspended cages with proven fertile males. The next morning, animals were ex-
Two days later, the rats were killed, the oviducts were excised and sandwiched between two glass slides, and the ectomized on day 8. On the day of surgery, and daily through day 21 of pregnancy, 1 μg of estrone together with varying doses of progestins was injected (s.c.). A negative control group received estrone only. On day 22, all animals were killed by asphyxiation with CO₂, the uteri were exposed, and the number of live fetuses in each uterine horn was recorded.

3.3. Ovulation inhibition in rats

Adult female rats (200–250 g) showing three consecutive 4-day cycles were used for this assay. On the day of diestrus (at 1:00 p.m.), they were injected s.c. with steroids in 0.2 ml of vehicle (cottonseed oil containing 5% ethanol). Two days later, the rats were killed, the oviducts were excised and sandwiched between two glass slides, and the number of ova counted under the microscope.

3.4. Androgenic activity

Immature male Sprague-Dawley rats (27–28 days old) were castrated under methoxyflurane anesthesia. They were then treated (s.c.) with testosterone (control) or progestins daily for 10 days. One day after the last injection, they were killed, and the ventral prostate and levator ani were removed and weighed. Serum LH levels were measured by homologous radioimmunoassay (RIA) using reagents supplied by the National Hormone and Pituitary Program, NICHD, Baltimore, MD, USA.

3.5. Estrogenic/antiestrogenic activity

Immature female rats were ovariectomized under methoxyflurane anesthesia, randomly distributed into treatment groups (n = 6 per group), and used 5 to 7 days later. Levonorgestrel or Nestorone (1 or 5 mg) was injected s.c. daily for 5 days. Vaginal lavages were examined microscopically to detect vaginal cornification. The animals were killed on day 6, and the uteri were removed, cleaned, blotted dry, and weighed. The antiestrogenic effects of levonorgestrel and Nestorone were determined in rats receiving 1.0 μg E₂. A positive control group received E₂ alone. Negative controls received the vehicle.

3.6. Glucocorticoid activity

Female Sprague-Dawley rats (175–200 g) were bilaterally adrenalectomized and supplied with 1% NaCl in drinking water. Four days after surgery, the animals were injected s.c. with dexamethasone or progestins for 3 days. They were killed 4 h after the last injection, and the livers were removed. Glycogen content and tyrosine transaminase (TAT) activity were determined by previously described methods [14,15].

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Relative binding affinitya</th>
<th>Selectivity index PR/AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>20 (50.3)</td>
<td>NB</td>
</tr>
<tr>
<td>Testosterone</td>
<td>NB</td>
<td>100 (7.4)</td>
</tr>
<tr>
<td>Levonorgestrel</td>
<td>100 (11.0)</td>
<td>70 (10.1)</td>
</tr>
<tr>
<td>3-Keto-desogestrel</td>
<td>220 (5.1)</td>
<td>40 (19.0)</td>
</tr>
<tr>
<td>Nestorone®</td>
<td>110 (10.3)</td>
<td>0.2 (6279)</td>
</tr>
</tbody>
</table>

*The nanomolar concentration of steroid competitors that reduced binding by 50% were determined. ED₅₀ values are shown in parentheses. The ratio of the reference compound to the test compound was expressed as the relative binding affinity. Levonorgestrel is used as reference compound for progesterone receptors (PR), and testosterone is used as reference compound for androgen receptors (AR). NB, no binding.

4. Results

4.1. Binding affinity to steroid receptors

Rat uterine cytosol preparation was used as the source of PR to compare the binding affinities. The relative binding affinity of 3-keto-desogestrel (220) was highest followed by Nestorone (110), levonorgestrel (100), and progesterone (20) (Table 1). A comparison of the binding to AR (rat ventral prostate) showed that all progestins studied had lower binding affinity than did testosterone (Fig. 1). The decreasing order of binding to AR was: testosterone, levonorgestrel, 3-keto-desogestrel, progesterone, and Nestorone. Compared to testosterone, the RBA of levonorgestrel to AR was 70%, whereas that of 3-keto-desogestrel was 40%. Nestorone did not show significant binding to AR (Table 1). None of the progestins showed significant binding to ER (data not shown). Nestorone showed significant binding (ED₅₀ = 56 nM) compared to the binding of dexamethasone (ED₅₀ = 21 nM) to calf thymus GR. Levonorg-
N. Kumar et al. / Steroids 65 (2000) 629–636

Fig. 2. Competitive inhibition of [3H]testosterone binding to sex hormone binding globulin (human trimester pregnancy serum) by testosterone and progestins. Each point represents the mean of three experiments.

Estrogens and 3-keto-desogestrel bound to GR with low affinity (data not shown).

The binding affinity of levonorgestrel (2.3 nM) and 3-keto-desogestrel (9 nM) to SHBG was significant but lower than that of testosterone (1 nM) (Fig. 2). Progesterone and Nestorone showed no binding to SHBG. Thus, the binding pattern of progestins to SHBG was similar to their binding to AR.

4.2. Progestational activity

Three different bioassays were used to compare the progestational activity of Nestorone, levonorgestrel, and progesterone. 3-Keto-desogestrel was not available in sufficient amounts for the bioassays. In the endometrial transformation test, dose-dependent increases in both uterine weight as well as McPhail index was observed. Nestorone was the most potent followed by levonorgestrel and progesterone upon s.c. administration (Fig. 3). Doses that elicited approximately half-maximal response (ED50) of the McPhail index were 1 µg, 10 µg, and 100 µg for Nestorone, levonorgestrel, and progesterone, respectively. These estimates are approximate since the dose-response curves did not have the same slope. Thus, Nestorone was about 10× more potent than levonorgestrel and 100 times more potent than progesterone in this assay. The progestational activity of Nestorone was also compared in rabbits by oral and s.c. administration. Based on uterine weight increase and McPhail index, Nestorone was over 100-fold more potent when administered s.c. than by the oral route (Table 2).

In the pregnancy maintenance assay, Nestorone maintained pregnancy in 40 to 50% of rats at a dose of 0.03 to 0.1 mg/day. The minimal effective dose of Nestorone for maintaining pregnancy was 0.3 mg/rat/day. The minimal effective doses were 0.3 mg for levonorgestrel and 5 mg for progesterone (Table 3). The ovulation inhibition assay in cycling rats showed inhibition of spontaneous ovulation in a dose-dependent manner by the progestins (Fig. 4). Ovulation was completely inhibited by Nestorone, levonorgestrel, and progesterone at doses of 10, 20, and 900 µg/rat/day,

Table 2
Comparison of progestational activity of NES administered via oral or sc route in immature rabbits

<table>
<thead>
<tr>
<th>Treatment (n = 4)</th>
<th>Uterine weight (g) mean ± SE</th>
<th>Progestational activity Average McPhail index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.6 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Nestorone® 0.5 µg, sc</td>
<td>3.6 ± 0.4</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Nestorone® 5.0 µg, sc</td>
<td>6.5 ± 0.4</td>
<td>4.0 ± 0.0</td>
</tr>
<tr>
<td>Nestorone® 5.0 µg, oral</td>
<td>1.9 ± 0.6</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Nestorone® 50.0 µg, oral</td>
<td>3.1 ± 0.2</td>
<td>1.8 ± 0.3</td>
</tr>
</tbody>
</table>

Immature rabbits were primed with estradiol for 6 days and treated with Nestorone® for 5 days. On day 6, rabbits were killed and their uteri removed, weighed, and processed for histology. Endometrial transformation scores (McPhail index) were determined based on uterine changes.

Immature female rabbits were primed with estradiol for 6 days and treated with Nestorone® for 5 days. On day 6, rabbits were killed and their uteri removed, weighed, and processed for histology. Endometrial transformation scores (McPhail index) were determined based on uterine changes.

Table 3
Maintenance of pregnancy by progestins in ovariectomized pregnant rats treated with estrone

<table>
<thead>
<tr>
<th>Compounds administered</th>
<th>Dose (mg/day)</th>
<th>No. of rats</th>
<th>Rats pregnant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.3</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>Nestorone®</td>
<td>0.01</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Levonorgestrel</td>
<td>0.3</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>6</td>
<td>100</td>
</tr>
</tbody>
</table>

* Rats were ovariectomized on day 8 of pregnancy. Progestins and estrone (1.0 µg/day) were injected sc from day 8 to day 21. Rats were killed on day 22.
respectively. In cases where ovulation was not blocked, there were no significant differences in the number of ova shed. Thus, the inhibitory effect of progestins on ovulation appears to be an all-or-none phenomenon.

4.3. Androgenic/antiandrogenic activity

The androgenic activity of progestins was compared with that of testosterone. Both levonorgestrel and 3-keto-desogestrel increased the weights of ventral prostate and levator ani in immature castrated rats in a dose-dependent manner (Fig. 5). Levonorgestrel was more potent than 3-keto-desogestrel with testosterone being 2- to 3-fold more potent than the progestins. Relatively high doses of Nestorone (1 and 4 mg/day) had no effect on the androgen target tissues (Fig. 6). Also, Nestorone showed no antiandrogenic activity (Fig. 6). Both testosterone and levonorgestrel were able to suppress serum LH in a dose-dependent manner in castrated rats; whereas Nestorone had no effect (Fig. 6). Thus, Nestorone showed no androgenic or antiandrogenic activity at a dose (20 mg/kg/day), which is almost 10 000× the effective human dose for contraception (2 μg/kg/day).

4.4. Estrogenic/antiestrogenic activity

 Estradiol, at a dose of 0.1 μg/day induced a significant increase in uterine weight (Fig. 7). Levonorgestrel (1 and 5 mg/day) also caused significant increase in uterine weight. In contrast, Nestorone showed no uterotrophic activity at similar doses. Combined administration of estradiol and levonorgestrel led to a synergistic increase in uterine weight; whereas Nestorone did not affect the uterotrophic activity of estradiol (Fig. 7). However, both levonorgestrel and Nestorone blocked E2-induced vaginal cornification, indicating antiestrogenic action.

4.5. Glucocorticoid activity

Nestorone showed significant binding affinity to calf thymus GR compared to dexamethasone. The thymolytic effect of Nestorone and levonorgestrel was evaluated in orchidectomized male and female rats in conjunction with the bioassays for androgenic and estrogenic activity. Only high doses of Nestorone (16 to 20 mg/kg b.wt.) caused significant thymic involution (data not shown). To further evaluate this activity, glucocorticoid-specific responses in the liver of adrenalectomized rats were studied. Dexamethasone at a dose of 40 μg caused a 2- to 3-fold increase in glycogen content and TAT activity in the liver; whereas both Nestorone and levonorgestrel at doses as high as 4 mg/kg had no effect on these parameters (Fig. 8). The results of the combined treatment with dexamethasone and Nestorone showed that Nestorone had no antiglucocorticoid activity. Thus, the lack of a glucocorticoid action of Nestor-
5. Discussion

Synthetic progestins, alone or in combination with estrogens, have been used as female contraceptives for a long time. Their use encompasses oral contraceptives, intrauterine devices, sustained-release injectables, and Silastic implants. Newer formulations of progestins in the form of transdermal gels or skin patches and vaginal rings are under development [16,17]. At present, the progestins most commonly used are norethindrone, levonorgestrel, 3-keto-desogestrel, and gestodene, which are derivatives of 19-nortestosterone. These progestins, with demonstrable androgenic activity, may be responsible for changes in the lipid profile, acne, and weight gain [18–20]. Thus, new progestins with less or no androgenic activity are being investigated [9,21].

At the Population Council, we are evaluating Nestorone (a norprogesterone derivative) alone for female contraception administered via Silastic® implants or vaginal rings and in combination with estradiol for hormone replacement therapy (HRT). Clinical testing of new contraceptive steroids requires the determination of their pharmacological effects to assess their effectiveness and side effects [22]. The pharmacological profile of Nestorone presented here has identified this progestin to be highly selective based on its binding to sex hormone receptors and its in vivo bioactivity.

An index based on the ratio between the desired effect (progestational activity) and the undesired effect (androgenic activity) of synthetic progestins has been used as a measure of selectivity. One measure of selectivity index is the ratio of progesterone to androgen receptor binding affinity [1]. The most commonly observed adverse effects of some progestins are attributed to their androgenic effect on lipid metabolism [23–25]. The binding of Nestorone to AR was negligible. It was 300- to 400-fold less than that of 3-keto-desogestrel and levonorgestrel. 3-Keto-desogestrel exhibited the highest binding affinity to PR whereas Nestorone and levonorgestrel exhibited similar binding affinity. The selectivity index based on the binding affinity to PR and AR was highest for Nestorone followed by 3-keto-desogestrel and levonorgestrel (Table 1). The binding affinities of some synthetic progestins to steroid receptors have been reported by others [26,27]. The results presented here are in agreement with the earlier reports. Based on the binding affinities, the selectivity profiles of some progestins have also been reported [1,28]. Of the 19-nortestosterone-derived progestins, 3-keto-desogestrel was considered very selective compared to levonorgestrel and norethindrone [1,25]. Even though 3-keto-desogestrel exhibited significant binding affinity to AR, its enhanced progestational activity conferred a high selectivity index on it [1]. Norgestimate, on the other hand, while showing no androgenic activity, had relatively
In summary, this report identifies Nestorone as a highly selective progestin for use in female contraception. In view of its high progestational potency and lack of androgenic and estrogenic activities, Nestorone could be safely used for long periods of time. The pharmacological profile of Nestorone conforms well to the safety and effectiveness of this progestin in the clinical trials reported so far.
Acknowledgments

We wish to acknowledge the help of Dr Richard P. Elye, Contraceptive and Reproductive Health Branch, NIH in arranging to analyze the uterine slides for McPhail index. We also wish to thank W. DeJesus and A. Marshall for technical assistance and M. Small for reviewing the manuscript.

References