Pharmacological profile of MEN 11066, a novel potent and selective aromatase inhibitor

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Abstract

MEN 11066 is a new non-steroidal compound which potently inhibits human placenta (Kᵢ = 0.5 nM) and rat ovarian (Kᵢ = 0.2 nM) aromatase in vitro. In vivo, a single oral dose of 0.3 mg kg⁻¹ significantly decreased uterus weight in immature rats after stimulation of uterus growth by androstenedione. MEN 11066 reduced in a dose-dependent manner plasma estradiol levels in adult female rats treated with pregnant mare serum gonadotropin (PMSG). After 2 weeks of repeated daily treatment in adult rats, a significant decrease in uterine weight was observed together with a 65% decrease in plasma estradiol, whereas plasma levels of testosterone, progesterone, aldosterone, corticosterone, cholesterol, LH and FSH were not affected. The lack of any effect by MEN 11066 on adrenal steroids was confirmed by the unchanged plasma corticosterone and aldosterone levels in immature rats and also in adult rats when the repeated treatment with MEN 11066 (15 days) was followed by the administration of a synthetic ACTH analogue. No change in 11-hydroxylase or 21-hydroxylase activities was produced in vitro by the addition of 10⁻² M MEN 11066. Fifteen-day treatment with MEN 11066 did not produce changes in several rat hepatic enzymatic activities involved in the metabolism of xenobiotics. These results demonstrated that MEN 11066 is a potent inhibitor of aromatase which does not interfere with the cytochrome P450 involved in the synthesis of other steroids or in the metabolism of xenobiotics.

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I. Introduction

It has long been known that estrogens have a permissive role in a number of neoplastic diseases, especially breast and endometrial cancer in women. A variety of endocrine therapies, both surgical and pharmacological, aimed at preventing the estrogen action have been attempted in these diseases [1,2]. Among them, antagonism at the level of the estrogen receptors and inhibition of estrogen synthesis represent the most direct approaches currently adopted in post-menopausal women. After menopause, the main production of estrogens occurs in peripheral tissues, mainly in muscle and adipose tissue. This source of estrogens is not centrally regulated by the release of pituitary gonadotropins [3], unlike ovarian estrogen synthesis (predominantly in the fertile woman). Therefore, long-lasting blockade of estrogen receptors or of estrogen synthesis is not expected to produce any rebound increase in plasma estrogen synthesis in post-menopausal women. Tamoxifen, an estrogen receptor antagonist, is presently used as a standard first line adjuvant endocrine therapy to prevent recurrence and metastasis of breast cancer in post-menopausal women, especially those presenting estrogen receptor positive tumors. However, in time, a fairly high percentage of patients becomes resistant to the drug and the disease progresses [4]. Aromatase inhibitors have been consistently reported to be effective in ameliorating the conditions in about 30–40% of patients no longer responding to tamoxifen [5].

Cytochrome P450 aromatase (CYP19) is the enzyme mediating the conversion of the steroidal C-19 androgens to C-18 estrogens, i.e. the final and rate-limiting step in the biosynthetic cascade of estrogens [6]. Selective aromatase inhibitors were reported in the early 1970s [7] and reached the clinic 10 years later [8]. Aminoglutethimide, a first generation inhibitor, has been successfully used as a non-steroidal, orally active inhibitor of steroidogenesis. However, due to its limited potency and weak selectivity...
A novel clinical application of aromatase inhibitors has recently been suggested [10,11] for management of some cases of couples with infertility in which gonadotropin stimulation of the female partner is used to achieve multiple follicle development. Indeed, aromatase inhibition resulted in successfully inducing ovarian hyperstimulation, in inducing ovulation in anovulatory women with polycystic ovary syndrome and in ameliorating ovarian response in poor responders to gonadotropin treatment [10,11]. In addition, administration of letrozole in combination with FSH, was able to reduce the dose of the gonadotropin necessary for ovarian hyperstimulation [11]. Blockade of estrogen synthesis in the early part of the menstrual cycle would increase gonadotropin secretion by decreasing hypothalamus and pituitary estrogen levels and thus the estrogen negative feedback [10,11]. On the other hand, the failed conversion of androgens to estrogens and the consequent accumulation of intraovarian androgens may increase follicular sensitivity to FSH during the early stages of the follicular development [10,11], as already suggested [12–14].

The present study was designed to characterize MEN 11066, letrozole (4,4′-oxybis-[1H-1,2,4-triazol-1-yl]-1-methylenem bist-benzonitrile), fadrozole (4-[5,6,7,8-tetrahydroimidazo(1,5a)pyridin-5-yl]-benzonitrile–HCl) and anastrozole (2,2′-5-(1H-1,2,4-triazol-1-yl-methyl)-1,3-phenylene)bis-(2-methylpropanonitrile)) were synthesized at Menarini Ricerche. Unlabeled synthetic androgen R1881, [3H]R1881 (specific activity 83.5 Ci mmol⁻¹) and [1β-3H]androstenedione (specific activity 24.7 Ci mmol⁻¹) were purchased from NEN Life Science Products, Boston, USA. Radioimmunoassay kits for 17β-estradiol, progesterone, testosterone, aldosterone and cholesterol were from Medical System, Genova, Italy; the kit for corticosterone was from ICN Biomedicals, Costa Mesa, CA, USA. Rat luteinizing hormone (rLH) and follicle stimulant hormone (rFSH) kits were from Amersham International. Tetracosactide acetate (ACTH1–24), Synacthen® was from Novartis, Basle, CH. Bicalutamide was a generous gift from Astrazeneca, London, UK. Testosterone (T), dihydrotestosterone (DHT), androstenedione, triamcinolone acetonide, PMSG and the other chemicals were purchased from Sigma.

2.2. In vitro/ex vivo assays

2.2.1. Enzyme preparations

Microsomes for the in vitro aromatase activity assay were prepared from human full-term placentas and from the ovaries of Wistar rats pre-treated with PMSG, 200 IU subcutaneously every other day for 9 days. The minced tissues were homogenized in 50 mM Tris–HCl buffer, pH 7.4, containing 10 μM phenylmethylsulfonylfluoride (PMSF), using a Polytron PT 3000 homogenizer (Kinematica, Switzerland); the homogenate was centrifuged for 35 min at 10,000 × g and the supernatant was recentrifuged for 60 min at 105,000 × g; the final pellet was resuspended in 0.5 volumes of the above buffer, aliquoted and stored at −80 °C.

Adrenal mitochondria and microsomes, for the in vitro assay of 11β-hydroxylase and 21-hydroxylase activities, respectively, were prepared from male adult bovine or male Wistar rat tissues. The adrenals were homogenized in five volumes of 50 mM Tris–HCl buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA, using a Polytron PT 3000 homogenizer and then centrifuged for 10 min at 900 × g. The supernatant was centrifuged again at 9500 × g for 10 min to obtain the mitochondrial fraction, that was washed by resuspension in the homogenization buffer and recentrifugation. The final mitochondrial pellet was suspended in the buffer (2–4 mg protein ml⁻¹). For the rat preparation, the 9500 × g supernatant was directly used as the source of microsomal activity. For the bovine tissues, the 9500 × g supernatant was centrifuged at 105,000 × g for 60 min to obtain a microsomal pellet, which was then resuspended in the buffer (4–6 mg protein ml⁻¹). Aliquots of mitochondrial and microsomal suspensions were stored at −80 °C until used (but, nonetheless, for less than 6 months).

Microsomes for the assay of cytochrome P450 and by content and of cytochrome P450-linked monooxygenase activities were prepared from the frozen livers of rats sacrificed at the end of the 15-day repeated treatment.

2. Materials and methods

2.1. Materials

MEN 11066, letrozole (4,4′-oxybis-[1H-1,2,4-triazol-1-yl]-1-methylenem bist-benzonitrile), fadrozole (4-[5,6,7,8-tetrahydroimidazo(1,5a)pyridin-5-yl]-benzonitrile–HCl) and anastrozole (2,2′-5-(1H-1,2,4-triazol-1-yl-methyl)-1,3-phenylene)bis-(2-methylpropanonitrile)) were synthesized at Menarini Ricerche. Unlabeled synthetic androgen R1881, [3H]R1881 (specific activity 83.5 Ci mmol⁻¹) and [1β-3H]androstenedione (specific activity 24.7 Ci mmol⁻¹) were purchased from NEN Life Science Products, Boston, USA. Radioimmunoassay kits for 17β-estradiol, progesterone, testosterone, aldosterone and cholesterol were from Medical System, Genova, Italy; the kit for corticosterone was from ICN Biomedicals, Costa Mesa, CA, USA. Rat luteinizing hormone (rLH) and follicle stimulant hormone (rFSH) kits were from Amersham International. Tetracosactide acetate (ACTH1–24), Synacthen® was from Novartis, Basle, CH. Bicalutamide was a generous gift from Astrazeneca, London, UK. Testosterone (T), dihydrotestosterone (DHT), androstenedione, triamcinolone acetonide, PMSG and the other chemicals were purchased from Sigma.
study (see below), according to the procedure described above for placental microsomes, but using a PMSF-free buffer.

2.3. Enzyme assays

2.3.1. Aromatase activity

The reaction mixture, containing microsomal protein (2–4 µg from human placental or 20 µg from rat ovarian microsomes), the substrate [1β-3H]androstenedione (9–300 nM), the cofactor NADPH (0.5 mM) and the inhibitors (1–10 µM) or their vehicle, in a total volume of 200 µl of 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol and 10 mM sodium molybdate. Homogenization of the tissue was performed using an ultra-Turrax and a glass–Teflon personal computer. The mobile phase was composed of 0.1 M NaH2PO4, pH 2.5, acetonitrile and methanol in the ratio 60:40 (v/v). The dried extract was reconstituted in the assay buffer. Protein concentration was measured according to Lowry et al. [16].

2.3.2. 21-Hydroxylase activity

CYP21A1 activity was measured in the 9500 g supernatant (for rat adrenals) or in the microsomes (for bovine adrenals) as the rate of conversion of progesterone to 11-deoxycorticosterone. Supernatant (1 mg protein) or microsomes (0.2 mg protein) were pre-incubated for 5 min at 37°C with progesterone (150 nM) and inhibitor in 450 µl of 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 1.2 mM MgCl2, 0.6 mM KCl, 15 mM NaCl and 2.5 mM CaCl2 [16]. Fifty microliters of 0.25 mM NADPH were added to start the reaction, which was terminated 15 min later by adding 0.25 ml of 1N HCl and centrifuging to precipitate proteins. The supernatant (40 µl) was analyzed for the formation of corticosterone by HPLC. HPLC conditions were the same as above, except for the mobile phase, that was composed of 0.1 M NaH2PO4, pH 2.5, and acetonitrile in the ratio 60:40 (v/v).

2.3.3. 11β-Hydroxylase activity

CYP11B1 activity was measured in mitochondria from rat and bovine adrenals as the rate of conversion of 11-deoxycorticosterone to corticosterone. Mitochondria (0.2 mg protein) were pre-incubated for 5 min at 37°C with 11-deoxycorticosterone (150 nM) and inhibitor in 450 µl of the same buffer as used for 21-hydroxylase activity. Fifty microliters of 0.25 mM NADPH were added to start the reaction, which was terminated 15 min later by adding 0.25 ml of 1N HCl and centrifuging to precipitate proteins. The supernatant (40 µl) was analyzed for the formation of corticosterone by HPLC. HPLC conditions were the same as above, except for the mobile phase, that was composed of 0.1 M NaH2PO4, pH 2.5, and acetonitrile in the ratio 60:40 (v/v).

2.3.4. Hepatic cytochromes and microsomal monooxygenases

Rat liver cytochrome P450 and hsp content were measured by the spectrophotometric method of Omura and Sato [17]. Aminoxyprine and erythromycin demethylase activities were assayed by colorimetric quantitation of formaldehyde [18]. Assay for 7-ethoxycoumarin-O-deethylase activity was performed by fluorimetric determination of 7-hydroxycoumarin [19]. Ethoxysresorufin-O-deethylase and pentoxysresorufin-O-depentylase activities were measured by fluorimetric determination of resorufin [20]. Protein concentration was measured according to Lowry et al. [21].

2.3.5. Plasma hormones

Plasma levels of steroid (17β-estradiol, progesterone, testosterone, aldosterone, corticosterone and cholesterol) and peptide (LH and FSH) hormones were determined by commercially available radioimmunoassay kits, according to the manufacturers’ instructions. A purification and concentration step was needed to measure plasma testosterone in female rats: to the samples were added four volumes of diethyl ether, mixed by gentle inversion for 15 min and then centrifuged for 5 min at 2000 rpm. The aqueous phase was frozen in dry ice and the organic phase was recovered and evaporated to dryness under a nitrogen stream. The dried extract was reconstituted in the assay buffer.
2.4. In vivo studies

2.4.1. Effects on androstenedione-induced uterus development in immature female rats

Three groups of eight pre-puberal female rats (22 days old) were treated subcutaneously with androstenedione (30 mg kg\(^{-1}\)) for two consecutive days. Two of these groups were orally treated (three times: 24 h before and 1 h after the first dose of androstenedione and 1 h after the second one) with MEN 11066 (3 mg kg\(^{-1}\)) and letrozole (1 mg kg\(^{-1}\)). In each of them, 3 groups of 10 cycling female rats were daily treated with oral doses of either inhibitor or with the vehicle (see above) for 2 weeks. Twenty-four hours after the last treatment, the animals were killed by decapitation, trunk blood was collected and heparinized plasma was stored at \(-20^\circ\text{C}\) until analyzed.

2.4.2. Effects on PMSG-stimulated estradiol synthesis in female adult rats

Four groups of 5-15 cycling female rats received five subcutaneous injections of PMSG (pregnant mare’s serum gonadotropin, 100 IU in 0.1 ml of sterile saline) on alternate days. Twenty-four hours after the last injection, three groups were treated orally with MEN 11066 at 0.1, 0.3 and 3 mg kg\(^{-1}\) doses, respectively. The fourth group received the vehicle (stimulated control, see above). A fifth group received subcutaneous physiological saline and oral vehicle (unstimulated control). Twenty-four hours after treatment, the animals were killed by decapitation and trunk blood was collected. Heparinized plasma was stored at \(-20^\circ\text{C}\) until analyzed.

2.4.3. Selective effects of repeated treatment in immature rats

Three groups of eight pre-puberal female rats (18 days old) were treated for 7 days with daily oral doses of MEN 11066 (3 mg kg\(^{-1}\)) or vehicle (see above). Twenty-four hours after the last dose, the animals were killed by decapitation and trunk blood was collected. Heparinized plasma was stored at \(-20^\circ\text{C}\) until analyzed.

2.4.4. Selectivity of the effects of repeated treatment on estradiol hormones in mature rats

Two similar studies were carried out in which MEN 11066 (3 mg kg\(^{-1}\)) was separately compared with anastrozole (3 mg kg\(^{-1}\)) and letrozole (1 mg kg\(^{-1}\)). In each of them, 3 groups of 10 cycling female rats were daily treated with oral doses of either inhibitor or with the vehicle (see above) for 2 weeks. Twenty-four hours after the last treatment, the animals were killed by decapitation, trunk blood was collected and heparinized plasma was stored at \(-20^\circ\text{C}\) until analyzed. ovaries, uteruses, adrenals, kidneys, liver and heart were removed and weighed. Livers were frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\). Hepatic microsomes were prepared as described above, within 3 weeks of freezing.

2.4.5. Effects of repeated treatment with MEN 11066 on plasma hormone levels after a challenge with Synacthen®

Two groups of 10 mature female rats were treated for 2 weeks with vehicle or oral MEN 11066 (3 mg kg\(^{-1}\)) as described above, but 24 h before sacrifice they were stimulated with the synthetic ACTH analogue Synacthen® (1 mg kg\(^{-1}\), subcutaneously). Post-mortem plasma hormone levels were assayed.

2.4.6. Statistics

Statistical significance was calculated by ANOVA test, followed by the Tukey test (Instat for Macintosh, GraphPad Software). Binding data were analyzed using the computerized program LIGAND [24].

3. Results

3.1. In vitro studies

3.1.1. Inhibition of aromatase activity

The kinetic parameters for the aromatization of 1\(\beta\)-\(\text{H}\)androstenedione by human placenta aromatase were: \(K_m = 31 \pm 6\) nM and \(V_{max} = 16 \pm 3\) pmol of released \(^2\text{H}2\text{O}\) per mg protein per min. The values calculated for rat ovarian aromatase were \(K_m = 16 \pm 7\) nM and \(V_{max} = 23 \pm 2\) pmol per mg protein per min (data are mean \pm S.E.M. of three independent determinations).

Fig. 2 reports the double reciprocal plots of aromatase activity in human placenta and rat ovarian microsomes, in the absence and in the presence of MEN 11066 and reference inhibitors (all being compared at the concentration of...
5 nM). When tested at increasing concentrations from 1 to 10 nM, all the inhibitors produced graded increases in $K_m$, but no significant variation of $V_{\text{max}}$ values. This evidence of a competitive inhibition allowed calculation of the $K_i$ values reported in Table 1. MEN 11066 is a very potent inhibitor both of human and rat aromatase, ranking close to letrozole.

### Table 1

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Human placenta</th>
<th>Rat ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEN 11066</td>
<td>$0.53 \pm 0.15$</td>
<td>$0.22 \pm 0.07$</td>
</tr>
<tr>
<td>Fadrozole</td>
<td>$2.4 \pm 0.6$</td>
<td>$0.25 \pm 0.06$</td>
</tr>
<tr>
<td>Letrozole</td>
<td>$0.51 \pm 0.13$</td>
<td>$0.15 \pm 0.03$</td>
</tr>
<tr>
<td>Anastrozole</td>
<td>$1.8 \pm 0.6$</td>
<td>$1.9 \pm 0.7$</td>
</tr>
</tbody>
</table>

$K_i$ values (mean±S.E.M., nM) of three to five determinations are reported.

3.1.2 Inhibition of 21-hydroxylase and 11β-hydroxylase activities

As reported in Table 2, none of the inhibitors, tested at 100 μM, affected the 21-hydroxylation of progesterone by either the bovine or the rat enzyme.

Fadrozole at 10 μM consistently inhibited the conversion of desoxycorticosterone to corticosterone—a step mediated by 11β-hydroxylase—and completely blocked the reaction at 100 μM. MEN 11066 displayed an inhibitory effect (about 40% inhibition) only at 100 μM, while anastrozole and letrozole were ineffective up to 100 μM.
Table 2
In vitro selectivity of aromatase inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (μM)</th>
<th>CYP21A1 (adrenal microsomes)</th>
<th>CYP11B1 (adrenal mitochondria)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bovine</td>
<td>Rat</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>MEN 11066</td>
<td>100</td>
<td>104</td>
<td>95</td>
</tr>
<tr>
<td>Fadrozole</td>
<td>100</td>
<td>150</td>
<td>135</td>
</tr>
<tr>
<td>Anastrozole</td>
<td>100</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>Letrozole</td>
<td>100</td>
<td>100</td>
<td>111</td>
</tr>
</tbody>
</table>

Effects of MEN 11066 and reference aromatase inhibitors on 21-hydroxylase (CYP21A1) and 11β-hydroxylase (CYP11B1) activities. Percent variation of control activities, as the mean of two determinations, is reported.

3.2 In vivo studies

3.2.1 Effects on androstenedione-induced uterus development in immature rats

The estrogens formed by the action of peripheral aromatase on exogenous androstenedione produced a 133% increase in the uterus weight above baseline (189 ± 11 mg versus 81 ± 4 mg), in pre-puberal rats. MEN 11066 dose-dependently antagonized this effect (Fig. 3). To exclude that this effect of MEN 11066 was due to an antagonistic action at the level of the androgen receptors (AR), we performed binding assay with the radiolabeled AR ligand [3H]R1881, in cytosolic preparations of human prostate. Competition experiments were carried out using [3H]R1881 in the presence or in the absence of increasing concentrations of MEN 11066 and several AR ligands: cold R1881, DHT, T and bicalutamide. Fig. 4 shows displacement curves obtained by LIGAND analysis [24]. Results from two separate experiments indicates that R1881 (Kd = 0.4 ± 0.18 nM), DHT (Kd = 0.14 ± 0.06 nM), T (Kd = 3.6 ± 1.4 nM), and the AR antagonist bicalutamide (Kd = 424 ± 169 nM), completely displaced [3H]R1881 binding. Conversely, MEN 11066 did not compete for [3H]R1881 binding at any concentration tested (up to 100 μM) indicating the inability of MEN 11066 to bind to the androgen receptor.

3.2.2 Effects on PMSG-stimulated 17β-estradiol synthesis in female adult rats

The repeated treatment of rats with horse gonadotropin dramatically increased the ovarian synthesis of 17β-estradiol. Estradiol plasma concentration attained values about 30 times higher than baseline (1009 ± 122 pM versus 36 ± 12 pM). A single oral treatment with MEN 11066 dose-dependently (0.1–3 mg kg−1) reduced this effect, 24 h after dosing (Fig. 5). Blockade of aromatase was virtually complete at a dose of 3 mg kg−1 of MEN 11066.

3.2.3 Selective effects of repeated treatment in immature rats

A 7-day treatment with MEN 11066 at 3 mg kg−1, a dose fully active in inhibiting peripheral aromatase-dependent androstenedione conversion to estrogen (see above), did not affect the biosynthesis of adrenal steroids in pre-puberal rats (Fig. 6).

3.2.4 Selectivity of the effects of repeated treatment on steroid hormones in mature rats

MEN 11066 (3 mg kg−1) was compared with anastrozole (3 mg kg−1) and letrozole (1 mg kg−1) in two separate studies. In both cases, MEN 11066 significantly reduced the uterus relative weight by approximately 20% (Table 3). This effect of MEN 11066 was similar to that produced by the same dose of anastrozole. Letrozole, despite being administered at a three times lower dose level, produced a stronger (69% inhibition) reduction in this parameter. The decrease in uterus weight appeared related to the reduction of plasma 17β-estradiol concentrations, that amounted to roughly 65% for both MEN 11066 and anastrozole, but was virtually complete for letrozole (hormone levels were below detection limit, 5 pM) (Table 4). The 2-week treatment with 3 mg kg−1 per day of MEN 11066 did not produce any significant effect on the weight of the other organs, except for the slight decrease (10%) of liver relative weight. Letrozole significantly increased the relative weight of ovaries (by 11%) and decreased that of adrenals (by 24%). MEN 11066 did not significantly affect plasma concentration...
Fig. 4. Effect of increasing concentrations of MEN 11066 and several known AR ligands on [3H]R1881 (1 nM) binding to human prostate homogenates. Cytosol preparations of prostate were incubated in the presence of [3H]R1881 and increasing concentrations of the corresponding unlabelled ligand (β/β17033), DHT (β/β17039), T (β/β17004), bicalutamide (β/β17009) and MEN 11066 (β/β17010). B/T: bound to total ratio for [3H]R1881.

of hormones other than 17β-estradiol, whereas anastrozole decreased progesterone levels (by 40%) and letrozole markedly diminished aldosterone (by 80%) and corticosterone (by 56%) levels. No effect on the release of the pituitary gonadotropins FSH and LH was observed with any inhibitor.

3.2.5. Effects of repeated treatment with MEN 11066 on plasma hormone levels in rats challenged with Synacthen®

The repeated treatment (15 days) with MEN 11066 did not affect the plasma aldosterone response elicited by Synacthen® in vehicle-treated rats (1481 ± 86 pg ml⁻¹ versus 1575 ± 93 pg ml⁻¹, n = 10, P > 0.05), and even increased corticosterone release by 50% (645 ± 66 ng ml⁻¹ versus 429 ± 32 ng ml⁻¹, P < 0.05). As expected, plasma 17β-estradiol concentrations were markedly reduced (11 ± 4 pM versus 44 ± 11 pM, P < 0.05) and cholesterol levels were unchanged (54 ± 3 mg dl⁻¹ versus 63 ± 8 mg dl⁻¹, P > 0.05).

3.2.6. Effects on hepatic drug metabolizing enzymes

MEN 11066, after a 2-week treatment with a daily oral dose of 3 mg kg⁻¹, did not produce any effect on rat liver cytochrome P450 and b5 content and on P450-dependent rat liver monoxygenases, as assessed in two independent studies (Table 5). On the other hand, anastrozole, at the same dose regimen, significantly increased the activity of aminopyrine demethylase.

![Fig. 5. Effect of a single oral treatment with MEN 11066 on plasma estradiol in PMSG-treated rats. Results are mean ± S.E.M. (n = 5, for basal and PMSG/vehicle; n = 5, for PMSG/0.1 mg kg⁻¹ MEN; n = 7, for PMSG/0.3 mg kg⁻¹ MEN). Significantly different from basal value in non-PMSG-stimulated rats (tP < 0.01). Significantly different from control, PMSG-stimulated rats (tP < 0.01).](image-url)

![Fig. 6. Effect of a 7-day oral treatment with 3 mg kg⁻¹ MEN 11066 on plasma adrenal steroids in pre-puberal rats. Results are mean ± S.E.M. (n = 8).](image-url)
Table 3
Effects of a 2-week oral treatment of aromatase inhibitors on body weight and on relative weight of selected organs in mature female rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>Uterus (mg/100g)</th>
<th>Ovaries (mg/100g)</th>
<th>Adrenals (mg/100g)</th>
<th>Kidneys (g/100g)</th>
<th>Liver (g/100g)</th>
<th>Heart (g/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>262 ± 4</td>
<td>197 ± 14</td>
<td>44 ± 2</td>
<td>42 ± 2</td>
<td>0.60 ± 0.01</td>
<td>3.4 ± 0.1</td>
<td>0.30 ± 0.00</td>
</tr>
<tr>
<td>MEN 1066</td>
<td>271 ± 3</td>
<td>161 ± 8*</td>
<td>48 ± 3</td>
<td>38 ± 1</td>
<td>0.59 ± 0.01</td>
<td>3.1 ± 0.0</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>Letrozole</td>
<td>292 ± 4*</td>
<td>64 ± 2</td>
<td>49 ± 3*</td>
<td>32 ± 1*</td>
<td>0.55 ± 0.01</td>
<td>3.2 ± 0.1</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>Study 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>221 ± 4</td>
<td>246 ± 22</td>
<td>66 ± 5</td>
<td>33 ± 3</td>
<td>0.8 ± 0.0</td>
<td>3.0 ± 0.0</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>MEN 1066</td>
<td>234 ± 3*</td>
<td>188 ± 15*</td>
<td>71 ± 6</td>
<td>30 ± 2</td>
<td>0.7 ± 0.0</td>
<td>2.7 ± 0.1*</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>Anastrozole</td>
<td>238 ± 4*</td>
<td>184 ± 12*</td>
<td>76 ± 5</td>
<td>31 ± 2</td>
<td>0.7 ± 0.0</td>
<td>2.9 ± 0.1</td>
<td>0.34 ± 0.01</td>
</tr>
</tbody>
</table>

MEN 1066 (3 mg kg\(^{-1}\) per day) was compared with letrozole (1 mg kg\(^{-1}\) per day) in study 1 and with anastrozole (3 mg kg\(^{-1}\) per day) in study 2. Data are reported as mean ± S.E.M. (n = 10).

* P < 0.05 (significantly different from the vehicle-treated group).

Table 4
Effects of a 2-week oral treatment of aromatase inhibitors on plasma concentration of steroid and protein hormones in mature female rats

| Treatment | 17β-Estradiol (pmol l\(^{-1}\)) | Testosterone (nM) | Progesterone (nM) | Aldosterone (pg ml\(^{-1}\)) | Cortisol (ng ml\(^{-1}\)) | Cholesterol (mg ml\(^{-1}\)) | LH (ng ml\(^{-1}\)) | FSH (ng ml\(^{-1}\)) |
|-----------|-------------------------------|------------------|------------------|----------------------------|--------------------------|----------------------------|----------------|----------------|----------------|
| Study 1   |                               |                  |                  |                            |                          |                            |                 |                 |                 |
| Vehicle   | 110 ± 31                      | NA               | NA               | 289 ± 42                   | 381 ± 78                 | 50 ± 3                     | 4.2 ± 1         | 8.7 ± 0.5      |
| MEN 1066  | 32 ± 10*                      | NA               | NA               | 343 ± 41                   | 256 ± 28                 | 45 ± 3                     | 3.1 ± 0.2       | 10.6 ± 1.2     |
| Letrozole | NQ\(^{2}\)                    | NA               | NA               | 71 ± 20*                   | 166 ± 41*                | 52 ± 3                     | 5.0 ± 0.7       | 10.7 ± 0.7     |
| Study 2   |                               |                  |                  |                            |                          |                            |                 |                 |                 |
| Vehicle   | 120 ± 24                      | 4.3 ± 2.1        | 22.9 ± 3.8       | 246 ± 31                   | 337 ± 60                 | NA                         | 1.2 ± 0.1       | NA             |
| MEN 1066  | 43 ± 9*                       | 2.5 ± 0.4        | 18.7 ± 3.6       | 191 ± 18                   | 183 ± 39                 | NA                         | 1.5 ± 0.1       | NA             |
| Anastrozole| 40 ± 15*                     | 2.7 ± 1.1        | 13.6 ± 1.7\(^{2}\)| 210 ± 65                   | 256 ± 61                 | NA                         | 1.3 ± 0.1       | NA             |

MEN 1066 (3 mg kg\(^{-1}\) per day) was compared with letrozole (1 mg kg\(^{-1}\) per day) in study 1 and with anastrozole (3 mg kg\(^{-1}\) per day) in study 2. Data are reported as mean ± S.E.M. (n = 10). NQ, not assayed.

* P < 0.05 (significantly different from the vehicle-treated group).

Table 5
Effects of a 2-week oral treatment of aromatase inhibitors on liver cytochrome content and P450-dependent monooxygenases activities in mature female rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytochrome P450 (nmol mg(^{-1}))</th>
<th>Cytochrome b(_{5}) (nmol mg(^{-1}))</th>
<th>Aminopyrine demethylease (nmol mg(^{-1}) min(^{-1}))</th>
<th>Ethoxyresorufin deethylase (nmol mg(^{-1}) min(^{-1}))</th>
<th>Penityresorufin depentilase (nmol mg(^{-1}) min(^{-1}))</th>
<th>Ethoxyresorufin deethylase (nmol mg(^{-1}) min(^{-1}))</th>
<th>Ethoxyresorufin deethylase (nmol mg(^{-1}) min(^{-1}))</th>
<th>Erythromycin 1 min (nmol mg(^{-1}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.47 ± 0.08</td>
<td>0.44 ± 0.03</td>
<td>2.27 ± 0.24</td>
<td>0.59 ± 0.12</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>MEN 1066</td>
<td>0.46 ± 0.09</td>
<td>0.37 ± 0.04</td>
<td>1.82 ± 0.02</td>
<td>0.77 ± 0.11</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Letrozole</td>
<td>0.52 ± 0.07</td>
<td>0.40 ± 0.02</td>
<td>3.87 ± 0.39(^{2})</td>
<td>0.81 ± 0.42</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Study 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.51 ± 0.05</td>
<td>0.67 ± 0.09</td>
<td>3.30 ± 0.70</td>
<td>0.29 ± 0.09</td>
<td>1.49 ± 0.29</td>
<td>50.7 ± 14.9</td>
<td>0.36 ± 0.07</td>
<td>NA</td>
</tr>
<tr>
<td>MEN 1066</td>
<td>0.47 ± 0.05</td>
<td>0.69 ± 0.08</td>
<td>3.53 ± 1.06</td>
<td>0.34 ± 0.06</td>
<td>1.96 ± 0.38</td>
<td>54.9 ± 10.6</td>
<td>0.40 ± 0.10</td>
<td>NA</td>
</tr>
<tr>
<td>Anastrozole</td>
<td>0.53 ± 0.05</td>
<td>0.76 ± 0.06</td>
<td>5.30 ± 1.06(^{2})</td>
<td>0.52 ± 0.07(^{2})</td>
<td>15.2 ± 3.9</td>
<td>81.3 ± 22.7</td>
<td>0.43 ± 0.22</td>
<td>NA</td>
</tr>
</tbody>
</table>

MEN 1066 (3 mg kg\(^{-1}\) per day) was compared with letrozole (1 mg kg\(^{-1}\) per day) in study 1 and with anastrozole (3 mg kg\(^{-1}\) per day) in study 2. Data are reported as mean ± S.E.M. (n = 8). NQ, not assayed.

* P < 0.05 (significantly different from the vehicle-treated group).

(1.8-fold), ethoxyresorufin deethylase (1.8-fold), pentityresorufin depentilase (10-fold) and ethoxyresorufin deethylase (1.6-fold). Letrozole (1 mg kg\(^{-1}\) per day) produced a 1.7-fold increase in aminopyrine demethylase activity.

4. Discussion
Aromatase inhibitors are emerging as efficacious compounds for the treatment of estrogen-dependent breast cancer. Tamoxifen is presently used as first line endocrine
therapy based on its ability to block estrogen receptor action. However, tamoxifen exerts both estrogen antagonistic and agonistic effects depending on the tissue examined [25]. More specifically, endometrial cells are a target for its agonist actions and, in principle, are a potential risk for promoting uterine cancer. Moreover, in breast cancer cells, antagonistic properties can shift to agonistic ones [26] probably due to adaptive cellular processes [25]. The latter finding can partially explain the development of drug resistance observed in a number of patients as well as the lack of cross-resistance between tamoxifen and aromatase inhibitors in 25% of the patients initially responding to the estrogen antagonist and then relapsing [9]. On the contrary, non-steroidal aromatase inhibitors would not be expected either to exert antagonistic actions, or to act as growth factors either for uterine cells or for a relapsing breast cancer.

The goal in seeking compounds which inhibit aromatase activity was to obtain potent and selective molecules, without interference with the other cytochrome P450 enzymes involved in the steroidogenic process.

A novel clinical application for aromatase inhibitors is emerging [10,11]. Indeed, the successful use of letrozole in treatment to induce or increase ovulation, respectively, in anovulatory women and ovulatory women undergoing assisted reproduction techniques has been reported [10,11]. In such cases, the expected lack of anti and/or estrogenic properties of non-steroidal aromatase inhibitors represents an advantage in comparison to the traditional treatment with an anti-estrogen, commonly clomiphene citrate (CC). Indeed, the discrepancy between the ovulation induced by CC and conception rates as well as the higher incidence of miscarriage after treatment with CC, has been attributed to the negative anti-estrogenic actions of CC on endometrial function [27] and development [28].

The non-steroidal aromatase inhibitor MEN 11066 described in this study, demonstrated an in vitro potency as high as the third generation drug letrozole towards both human placental and rat ovarian enzyme. High inhibitory activity was confirmed also in vivo tests showing a strong direct inhibition of the cell growth of two sublines from human breast carcinoma showed that MEN 11066 is a potent inhibitor of aromatase that does not interfere with the P450 involved in the synthesis of other steroids. Furthermore, the in situ production of estrogens by tumor tissues in the treatment of estrogen-dependent diseases, such as breast cancer.

A recent study on cell sublines from human breast carcinoma showed that MEN 11066 exerts a strong direct inhibition of the cell growth induced by aromatizable androgen, an indication of the activity on tumor cell aromatase [30]. In conclusion, the results described herein demonstrate that MEN 11066 is a potent inhibitor of aromatase that does not interfere with the P450 involved in the synthesis of other steroids or in the metabolism of xenobiotics. Hence, this compound could be a suitable candidate for clinical studies in the treatment of estrogen-dependent diseases, such as breast cancer.

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References


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