



UNIVERSITÀ  
DEGLI STUDI  
FIRENZE

## FLORE

# Repository istituzionale dell'Università degli Studi di Firenze

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

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

*Original Citation:*

Pharmacological profile of MEN 11066, a novel potent and selective aromatase inhibitor / MURATORI M.; LIPPI A.; MANCINA R.; IAFRATE E.M.; CIRILLO R.; LOPEZ G.; BIGIONI M.; M. MAGGI; CRISCUOLI M.; MAGGI C.A.. - In: JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY. - ISSN 0960-0760. - STAMPA. - 84:(2003), pp. 503-512.

*Availability:*

The webpage <https://hdl.handle.net/2158/311594> of the repository was last updated on

*Terms of use:*

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

*Publisher copyright claim:*

La data sopra indicata si riferisce all'ultimo aggiornamento della scheda del Repository FloRe - The above-mentioned date refers to the last update of the record in the Institutional Repository FloRe

(Article begins on next page)



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

M. Muratori<sup>a</sup>, A. Lippi<sup>b</sup>, R. Mancina<sup>a</sup>, E.M. Iafrate<sup>c</sup>, R. Cirillo<sup>c</sup>, G. Lopez<sup>c</sup>, M. Bigioni<sup>c</sup>,  
M. Maggi<sup>a</sup>, M. Crisculi<sup>b,\*</sup>, C.A. Maggi<sup>b</sup>

<sup>a</sup> Department of Clinical Physiopathology, University of Florence, V. le Pieraccini 6, I-50139 Firenze, Italy

<sup>b</sup> Preclinical Development, Department of Pharmacology, Menarini Ricerche S.p.A., Via Sette Santi 3, I-50131 Firenze, Italy

<sup>c</sup> Department of Pharmacology, Menarini Ricerche S.p.A., Via Tito Speri 10, Pomezia, I-00040 Rome, Italy

Received 31 July 2002; accepted 20 January 2003

### Abstract

MEN 11066 is a new non-steroidal compound which potently inhibits human placenta ( $K_i = 0.5$  nM) and rat ovarian ( $K_i = 0.2$  nM) aromatase in vitro. In vivo, a single oral dose of  $0.3$  mg kg<sup>-1</sup> 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 $\beta$ -hydroxylase or 21-hydroxylase activities was produced in vitro by the addition of  $10$   $\mu$ 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.

© 2003 Elsevier Science Ltd. All rights reserved.

**Keywords:** Breast cancer; Inhibition; Aromatase; Steroid hormones; Cytochrome P450; Rat uterus

### 1. 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 pro-

duce 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

\* Corresponding author. Tel.: +39-055-5680691; fax: +39-055-5680510.  
E-mail address: mcrisculi@menarini-ricerche.it (M. Crisculi).

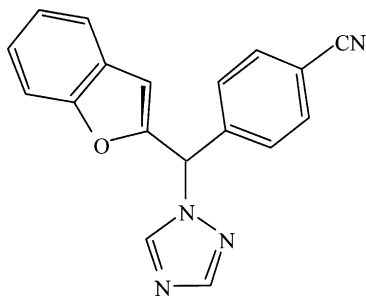


Fig. 1. Chemical structure of MEN 11066.

for CYP19, aminoglutethimide causes many adverse reactions and is often poorly tolerated. On the other hand, this compound resulted in quite effective tumor regression in patients with breast cancer. Hence, the search for more potent and selective aromatase inhibitors has received much attention in the last two decades [3,9].

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 (1-[(benzofuran-2-yl)(4'-cyanophenyl)-methyl]-1H-1,2,4-triazole; Fig. 1), a novel potent and selective aromatase inhibitor, in *in vitro* and *in vivo* tests, in comparison with some second and third generation non-steroidal inhibitors of CYP19, i.e. fadrozole, anastrozole and letrozole.

## 2. Materials and methods

### 2.1. Materials

MEN 11066, letrozole (4,4'-[1H-1,2,4-triazol-1-yl-methylene]-bis-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-methylpropionitrile]) were synthesized

at Menarini Ricerche. Unlabeled synthetic androgen R1881, [<sup>3</sup>H]R1881 (specific activity 83.5 Ci mmol<sup>-1</sup>) and [<sup>1</sup>β-<sup>3</sup>H]androstenedione (specific activity 24.7 Ci mmol<sup>-1</sup>) 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 (ACTH(1–24), 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<sup>-1</sup>). 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<sup>-1</sup>). 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 *b*<sub>5</sub> 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

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 [ $1\beta$ - $^3\text{H}$ ]androstenedione (9–300 nM), the cofactor NADPH (0.5 mM) and the inhibitors (1–10 nM) or their vehicle, in a total volume of 200 µl of 50 mM Tris–HCl buffer, was incubated at 37 °C. Ten or thirty minutes after the addition of placental or ovarian microsomes, respectively, the reaction was stopped by adding 200 µl of 1 mM HgCl<sub>2</sub>. Next, 400 µl of 1% charcoal suspension were added and the mixture was centrifuged at 2000 × *g* for 15 min. Then, 400 µl of supernatant were placed into scintillation vials containing 4 ml of Cytosint (ICN Biochemicals). Released tritiated water was measured by liquid scintillation (2200 CA β-counter, Packard).  $K_m$  and  $V_{max}$  values were calculated by fitting the curves of reaction rate versus substrate concentration by the non-linear regression program Ultrafit (Biosoft, UK).

$K_i$  values were obtained according to the Cheng and Prusoff [15] equation for competitive inhibition:  $K_i = [i] / \{(K_p / K_m) - 1\}$ , where  $[i]$  is the inhibitor concentration (1, 2.5, 5 and 10 nM) and  $K_p$  is the  $K_m$  value calculated in the presence of the inhibitor.

#### 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 nmol) and inhibitor in 450 µl of 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 1.2 mM MgCl<sub>2</sub>, 0.6 mM KCl, 15 mM NaCl and 2.5 mM CaCl<sub>2</sub> [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 11-deoxycorticosterone by HPLC. The HPLC system consisted of a model 465 autosampler (Kontron Instruments, Milan, Italy) and a model LC-9A pump (Shimadzu, Kyoto, Japan) connected to a Superspher 100 RP18 reversed-phase column (250 mm × 4.6 mm, particle size 4 µm, Merck, Darmstadt, Germany) protected by its pre-column. Peak detection was through a UV spectrophotometer (SPD-10, Shimadzu, Tokyo, Japan) set at 240 nm. Signals from the detector were analyzed by the Class LC-10 software (Shimadzu) on a model ProLinea 3/25s Compaq personal computer. The mobile phase was composed of 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 2.5, acetonitrile and methanol in the ratio 50:40:10 (v/v). The flow rate was set at 0.8 ml/min.

#### 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 nmol) 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 NaH<sub>2</sub>PO<sub>4</sub>, 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  $b_5$  content were measured by the spectrophotometric method of Omura and Sato [17]. Aminopyrine and erythromycin demethylase activities were assayed by colorimetric quantitation of formed formaldehyde [18]. Assay for 7-ethoxycoumarin-*O*-deethylase activity was performed by fluorimetric determination of 7-hydroxycoumarin [19]. Ethoxyresorufin-*O*-deethylase and pentoxyresorufin-*O*-deethylase 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 (rLH and rFSH) 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.

#### 2.3.6. Binding assay

Binding assay on prostate tissue was performed as previously reported [22]. Human prostate specimens were collected and frozen in liquid nitrogen. Frozen tissues were allowed to thaw on ice prior to homogenization. All subsequent procedures were carried out at 0–4 °C. Charred material was removed and the tissues were, finally, minced with scissors, suspended in cold TEDGMO (10 mM Tris–HCl, pH 7.4, containing 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol and 10 mM sodium molybdate). Homogenization was performed using an ultra-Turrax and a glass–Teflon

homogenizer. Homogenates were centrifuged at  $600 \times g$  for 10 min and the supernatant further centrifuged at  $100,000 \times g$  for 60 min. The resulting cytosol was fractionated into portions and stored at  $-80^\circ\text{C}$ . Protein content was determined by the Bradford method [23] using BSA as standard. The cytosolic fractions, appropriately diluted to 100  $\mu\text{l}$  containing 0.4 mg of proteins, were incubated overnight at  $4^\circ\text{C}$  in a final volume of 250  $\mu\text{l}$  in TEDGMO with [ $^3\text{H}$ ]R1881 ( $10^{-9}\text{M}$ ) in the absence or presence of increasing concentrations of cold ligands. Homologous and heterologous competition curves were obtained using R1881 ( $10^{-11}$  to  $10^{-6}\text{M}$ ), DHT ( $10^{-11}$  to  $10^{-6}\text{M}$ ), T ( $10^{-10}$  to  $10^{-6}\text{M}$ ), bicalutamide ( $10^{-10}$  to  $10^{-4}\text{M}$ ), and MEN 11066 ( $10^{-10}$  to  $10^{-4}\text{M}$ ). To prevent R1881 binding to progesterone receptor,  $10^{-6}\text{M}$  triamcinolone acetonide was added to each tube. Separation of bound and unbound ligand was achieved by a 15 min treatment with a 500  $\mu\text{l}$  suspension of dextran (0.05%) coated charcoal (0.5%) in 10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 1 mM dithiothreitol at  $4^\circ\text{C}$ . The charcoal was pelleted by centrifugation for 10 min at  $1500 \times g$  and 600  $\mu\text{l}$  were counted in Instagel plus (Packard, Meriden, CT, USA) using a  $\beta$ -counter.

## 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 ( $30\text{ mg kg}^{-1}$ ) 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 at 0.03 and  $0.3\text{ mg kg}^{-1}$ , respectively. The inhibitor was first dissolved in ethanol ( $20\text{ mg ml}^{-1}$ ) and then diluted in sesame oil to the desired concentration to be dosed at  $2\text{ ml kg}^{-1}$ . The third group received the vehicle (stimulated control). A fourth group received subcutaneous physiological saline and oral vehicle (unstimulated control). Twenty-four hours after the last treatment, the animals were killed by decapitation and their uteri removed and weighed.

### 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  $3\text{ mg kg}^{-1}$  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 ( $3\text{ mg kg}^{-1}$ ) 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 steroid hormones in mature rats

Two similar studies were carried out in which MEN 11066 ( $3\text{ mg kg}^{-1}$ ) was separately compared with anastrozole ( $3\text{ mg kg}^{-1}$ ) and letrozole ( $1\text{ mg kg}^{-1}$ ). 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, uterus, 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<sup>®</sup>

Two groups of 10 mature female rats were treated for 2 weeks with vehicle or oral MEN 11066 ( $3\text{ mg kg}^{-1}$ ) as described above, but 24 h before sacrifice they were stimulated with the synthetic ACTH analogue Synacthen<sup>®</sup> ( $1\text{ mg kg}^{-1}$ , 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 [ $^3\text{H}$ ]androstenedione by human placenta aromatase were:  $K_m = 31 \pm 6\text{ nM}$  and  $V_{max} = 16 \pm 3\text{ pmol}$  of released  $^3\text{H}_2\text{O}$  per mg protein per min. The values calculated for rat ovarian aromatase were  $K_m = 16 \pm 7\text{ nM}$  and  $V_{max} = 23 \pm 2\text{ fmol}$  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

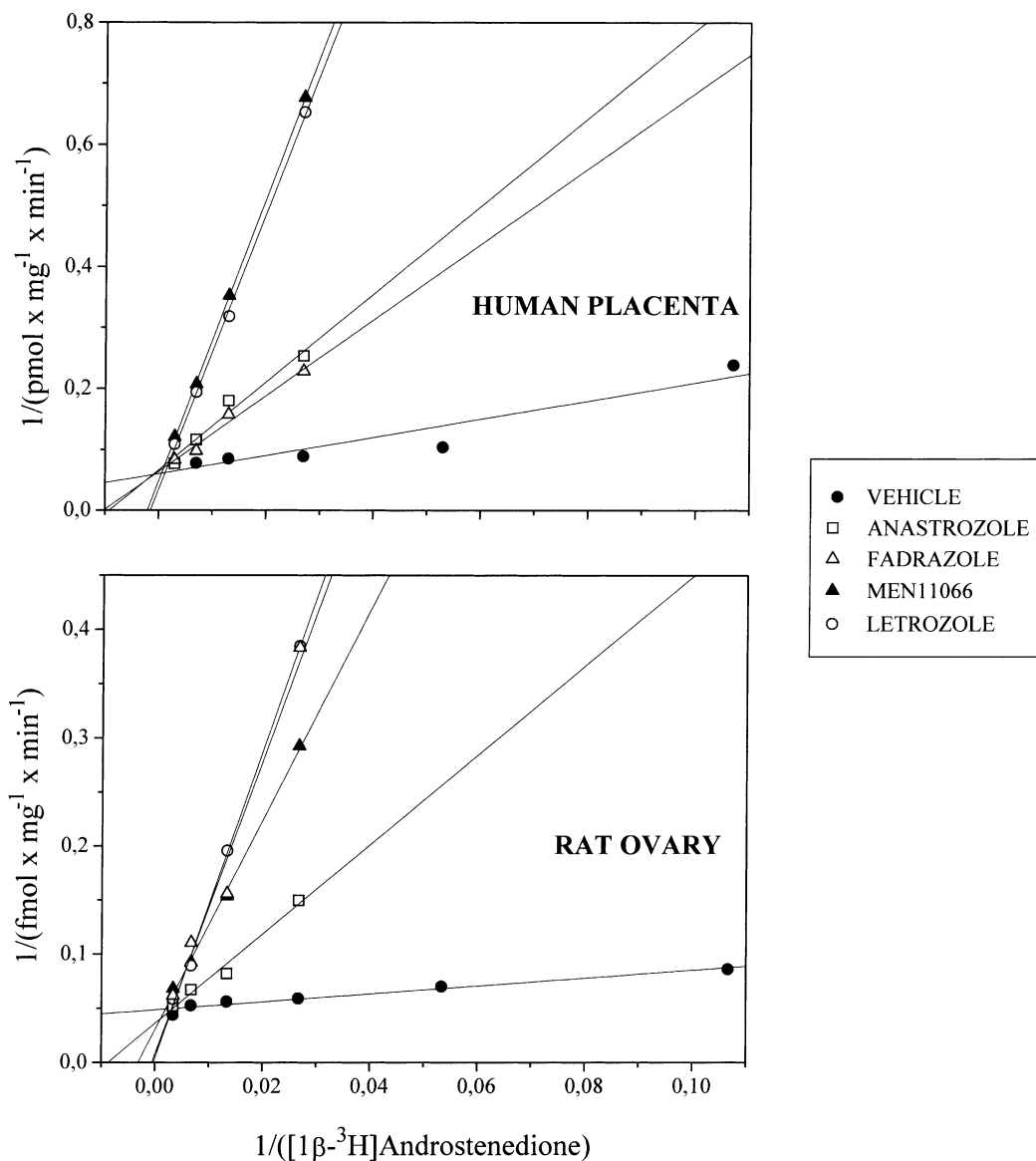


Fig. 2. Double reciprocal plots of aromatase activity in human placenta and rat ovarian microsomes in the absence and presence of MEN 11066 and reference inhibitors, all compared at a concentrations of 5 nM.

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  $V_{max}$  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.

### 3.1.2. Inhibition of 21-hydroxylase and 11 $\beta$ -hydroxylase activities

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

Fadrozole at 10  $\mu$ M consistently inhibited the conversion of desoxycorticosterone to corticosterone—a step mediated

Table 1  
In vitro inhibition of aromatase activity

Inhibitors	Human placenta	Rat ovary
MEN 11066	0.53 $\pm$ 0.15	0.22 $\pm$ 0.07
Fadrozole	2.4 $\pm$ 0.6	0.25 $\pm$ 0.04
Letrozole	0.51 $\pm$ 0.13	0.15 $\pm$ 0.03
Anastrozole	1.8 $\pm$ 0.6	1.9 $\pm$ 0.7

$K_i$  values (mean  $\pm$  S.E.M.; nM) of three to five determinations are reported.

by 11 $\beta$ -hydroxylase—and completely blocked the reaction at 100  $\mu$ M. MEN 11066 displayed an inhibitory effect (about 40% inhibition) only at 100  $\mu$ M, while anastrozole and letrozole were ineffective up to 100  $\mu$ M.

Table 2  
In vitro selectivity of aromatase inhibitors

Inhibitor	Concentration (μM)	CYP21A1 (adrenal microsomes)		CYP11B1 (adrenal mitochondria)	
		Bovine	Rat	Bovine	Rat
Control		100	100	100	100
MEN 11066	100	85	148	55	61
	10			104	
Fadrozole	100	135	150	9	7
	10			59	40
Anastrozole	100	108	104	99	100
	10			101	
Letrozole	100	91	138	111	97
	10			107	

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 ( $189 \pm 11$  mg versus  $81 \pm 4$  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

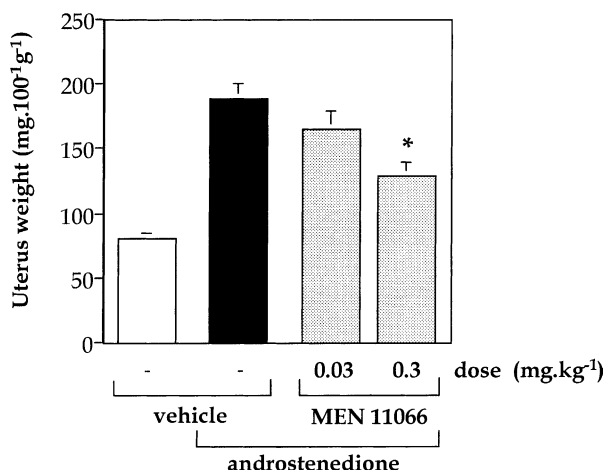


Fig. 3. Effect of oral treatment with MEN 11066 (see Section 2) on uterus weight in pre-puberal rats stimulated with  $30 \text{ mg kg}^{-1}$  subcutaneous androstenedione for two consecutive days. Results are mean  $\pm$  S.E.M. ( $n = 8$ ).

AR ligand [<sup>3</sup>H]R1881, in cytosolic preparations of human prostate. Competition experiments were carried out using [<sup>3</sup>H]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 ( $K_d = 0.4 \pm 0.18 \text{ nM}$ ), DHT ( $K_d = 0.14 \pm 0.06 \text{ nM}$ ), T ( $K_d = 3.6 \pm 1.4 \text{ nM}$ ), and the AR antagonist bicalutamide ( $K_d = 424 \pm 169 \text{ nM}$ ), completely displaced [<sup>3</sup>H]R1881 binding. Conversely, MEN 11066 did not compete for [<sup>3</sup>H]R1881 binding at any concentration tested (up to  $100 \mu\text{M}$ ) 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 ( $1009 \pm 122 \text{ pM}$  versus  $36 \pm 12 \text{ pM}$ ). A single oral treatment with MEN 11066 dose-dependently ( $0.1\text{--}3 \text{ mg kg}^{-1}$ ) reduced this effect, 24 h after dosing (Fig. 5). Blockade of aromatase was virtually complete at a dose of  $3 \text{ mg kg}^{-1}$  of MEN 11066.

#### 3.2.3. Selective effects of repeated treatment in immature rats

A 7-day treatment with MEN 11066 at  $3 \text{ mg kg}^{-1}$ , 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 ( $3 \text{ mg kg}^{-1}$ ) was compared with anastrozole ( $3 \text{ mg kg}^{-1}$ ) and letrozole ( $1 \text{ mg kg}^{-1}$ ) 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,  $5 \text{ pM}$ ) (Table 4). The 2-week treatment with  $3 \text{ mg kg}^{-1}$  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

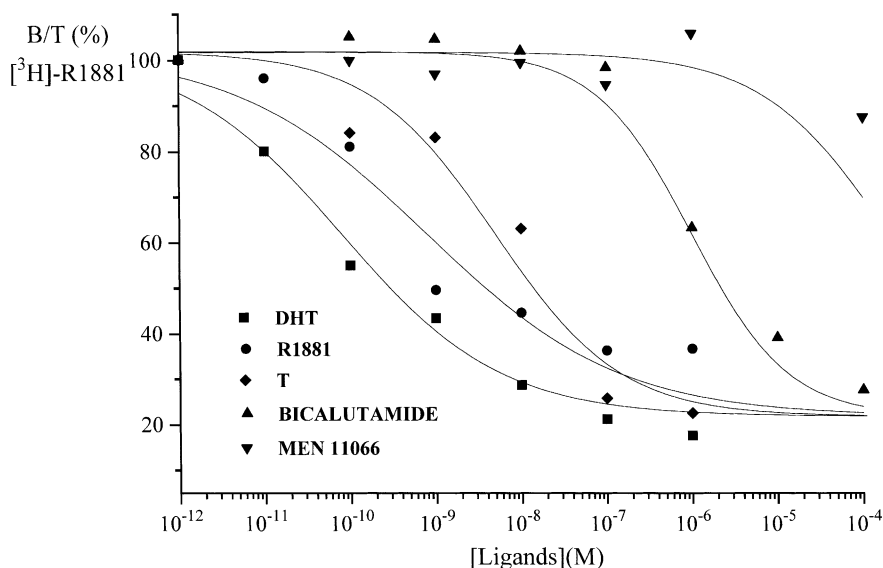


Fig. 4. Effect of increasing concentrations of MEN 11066 and several known AR ligands on [<sup>3</sup>H]R1881 (1 nM) binding to human prostate homogenates. Cytosol preparations of prostate were incubated in the presence of [<sup>3</sup>H]R1881 and increasing concentrations of the corresponding unlabelled ligand (●), DHT (■), T (◆), bicalutamide (▲) and MEN 11066 (▼). B/T: bound to total ratio for [<sup>3</sup>H]R1881.

of hormones other than 17 $\beta$ -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<sup>®</sup>

The repeated treatment (15 days) with MEN 11066 did not affect the plasma aldosterone response elicited

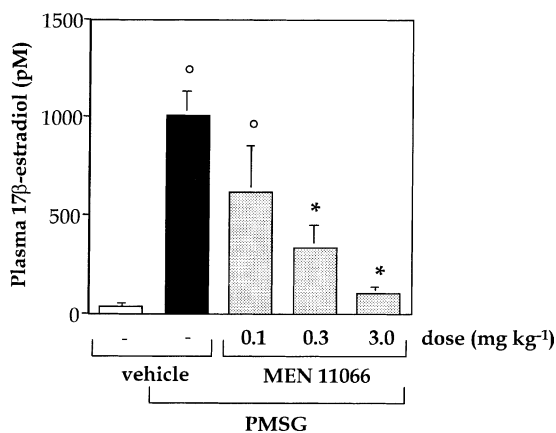


Fig. 5. Effect of a single oral treatment with MEN 11066 on plasma estradiol in PMSG-treated rats. Results are mean  $\pm$  S.E.M. ( $n = 15$ , for basal and PMSG/vehicle;  $n = 5$ , for PMSG/0.1 mg kg<sup>-1</sup> MEN;  $n = 7$ , for PMSG/0.3 mg kg<sup>-1</sup> MEN;  $n = 8$ , for PMSG/3.0 mg kg<sup>-1</sup> MEN). Significantly different from basal value in non-PMSG-stimulated rats ( $^{\circ}P < 0.01$ ). Significantly different from control, PMSG-stimulated rats ( $*P < 0.01$ ).

by Synacthen<sup>®</sup> in vehicle-treated rats ( $1481 \pm 86$  pg ml<sup>-1</sup> versus  $1575 \pm 93$  pg ml<sup>-1</sup>,  $n = 10$ ,  $P > 0.05$ ), and even increased corticosterone release by 50% ( $645 \pm 66$  ng ml<sup>-1</sup> versus  $429 \pm 32$  ng ml<sup>-1</sup>,  $P < 0.05$ ). As expected, plasma 17 $\beta$ -estradiol concentrations were markedly reduced ( $11 \pm 4$  pM versus  $44 \pm 11$  pM,  $P < 0.05$ ) and cholesterol levels were unchanged ( $54 \pm 3$  mg dl<sup>-1</sup> versus  $63 \pm 8$  mg dl<sup>-1</sup>,  $P > 0.05$ ).

### 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<sup>-1</sup>, did not produce any effect on rat liver cytochrome P450 and  $b_5$  content and on P450-dependent rat liver monooxygenases, 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

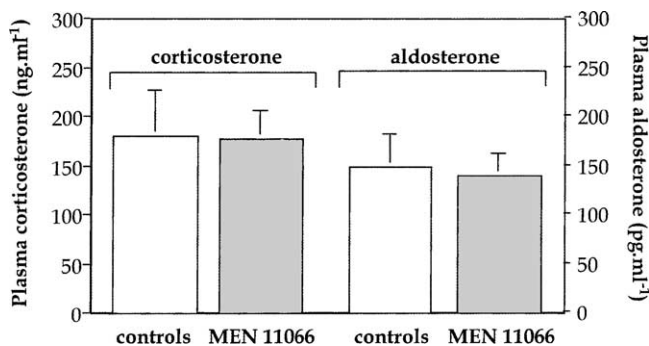


Fig. 6. Effect of a 7-day oral treatment with 3 mg kg<sup>-1</sup> MEN 11066 on plasma adrenal steroids in pre-pubertal rats. Results are mean  $\pm$  S.E.M. ( $n = 8$ ).

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

Treatment	Body weight (g)	Uterus (mg/100 g)	Ovaries (mg/100 g)	Adrenals (mg/100 g)	Kidneys (g/100 g)	Liver (g/100 g)	Heart (g/100 g)
Study 1							
Vehicle	262 ± 4	197 ± 14	44 ± 2	42 ± 2	0.60 ± 0.01	3.4 ± 0.1	0.30 ± 0.00
MEN 11066	271 ± 3	161 ± 8*	48 ± 3	38 ± 1	0.59 ± 0.01	3.1 ± 0.0	0.31 ± 0.01
Letrozole	292 ± 4*	61 ± 2*	49 ± 3*	32 ± 1*	0.55 ± 0.01	3.2 ± 0.1	0.30 ± 0.01
Study 2							
Vehicle	221 ± 4	246 ± 22	66 ± 5	33 ± 3	0.8 ± 0.0	3.0 ± 0.0	0.37 ± 0.01
MEN 11066	234 ± 3*	188 ± 15*	71 ± 6	30 ± 2	0.7 ± 0.0	2.7 ± 0.1*	0.34 ± 0.01
Anastrozole	238 ± 4*	184 ± 12*	76 ± 5	31 ± 2	0.7 ± 0.0	2.9 ± 0.1	0.34 ± 0.01

MEN 11066 (3 mg kg<sup>-1</sup> per day) was compared with letrozole (1 mg kg<sup>-1</sup> per day) in study 1 and with anastrozole (3 mg kg<sup>-1</sup> 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 (pM)	Testosterone (nM)	Progesterone (nM)	Aldosterone (pg ml <sup>-1</sup> )	Corticosterone (ng ml <sup>-1</sup> )	Cholesterol (mg ml <sup>-1</sup> )	LH (ng ml <sup>-1</sup> )	FSH (ng ml <sup>-1</sup> )
Study 1								
Vehicle	110 ± 31	NA	NA	289 ± 42	381 ± 78	50 ± 3	4.2 ± 1.4	8.7 ± 0.5
MEN 11066	32 ± 10*	NA	NA	343 ± 41	256 ± 28	45 ± 3	3.1 ± 0.2	10.6 ± 1.2
Letrozole	NQ*	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 11066	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*	210 ± 65	250 ± 61	NA	1.3 ± 0.1	NA

MEN 11066 (3 mg kg<sup>-1</sup> per day) was compared with letrozole (1 mg kg<sup>-1</sup> per day) in study 1 and with anastrozole (3 mg kg<sup>-1</sup> per day) in study 2. Data are reported as mean ± S.E.M. (*n* = 10). NA, not assayed. NQ, not quantifiable, below the limit of quantification.

\* *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

Treatment	Cytochrome P450 (nmol mg <sup>-1</sup> )	Cytochrome <i>b</i> <sub>5</sub> (nmol mg <sup>-1</sup> )	Aminopyrine demethylase (nmol mg <sup>-1</sup> min <sup>-1</sup> )	Ethoxycoumarin deethylase (nmol mg <sup>-1</sup> min <sup>-1</sup> )	Pentoxoresorufin deethylase (pmol mg <sup>-1</sup> min <sup>-1</sup> )	Ethoxoresorufin deethylase (pmol mg <sup>-1</sup> min <sup>-1</sup> )	Erythromycin demethylase (nmol mg <sup>-1</sup> min <sup>-1</sup> )
Study 1							
Vehicle	0.47 ± 0.08	0.44 ± 0.03	2.27 ± 0.24	0.59 ± 0.12	NA	NA	NA
MEN 11066	0.46 ± 0.09	0.37 ± 0.04	1.82 ± 0.02	0.77 ± 0.11	NA	NA	NA
Letrozole	0.52 ± 0.07	0.49 ± 0.02	3.87 ± 0.39*	0.81 ± 0.42	NA	NA	NA
Study 2							
Vehicle	0.51 ± 0.05	0.67 ± 0.09	3.30 ± 0.70	0.29 ± 0.09	1.49 ± 0.29	50.7 ± 14.9	0.36 ± 0.07
MEN 11066	0.47 ± 0.05	0.69 ± 0.08	3.53 ± 1.06	0.34 ± 0.06	1.96 ± 0.38	54.9 ± 10.6	0.40 ± 0.10
Anastrozole	0.53 ± 0.05	0.76 ± 0.06	5.50 ± 1.06*	0.52 ± 0.07*	15.2 ± 3.9*	81.3 ± 22.5*	0.43 ± 0.22

MEN 11066 (3 mg kg<sup>-1</sup> per day) was compared with letrozole (1 mg kg<sup>-1</sup> per day) in study 1 and with anastrozole (3 mg kg<sup>-1</sup> per day) in study 2. Data are reported as mean ± S.E.M. (*n* = 8). NA, not assayed.

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

(1.8-fold), ethoxycoumarin deethylase (1.8-fold), pentoxoresorufin deethylase (10-fold) and ethoxoresorufin deethylase (1.6-fold). Letrozole (1 mg kg<sup>-1</sup> 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 by *in vivo* tests showing a potency similar to anastrozole, albeit lower than letrozole. Indeed, MEN 11066 was able to decrease both androstenedione-induced uterine hypertrophy in immature rats and plasma level of estradiol after PMSG stimulation of mature female rats (a model reflecting ovarian aromatase activity).

Along with its potency, MEN 11066 exhibited high selectivity both in the *in vivo* and the *in vitro* tests. The fully active dose of this compound, even after repeated administration, did not affect the other cytochrome P450 enzymes involved in steroidogenesis, as demonstrated by the lack of effect on the corticosterone and aldosterone plasma levels in immature rats. Moreover, in a similar experimental model, in which adrenal steroidogenesis was challenged with Synacthen<sup>®</sup>, the corticosterone plasma level was not affected in mature female rats and even increased after MEN 11066 in ACTH challenged animals. The latter effect of MEN 11066 could

be due to a possible upstream accumulation of adrenal substrates as a consequence of the failed conversion of androstenedione to estrone. Thus, the increased corticosterone level is consistent with the ability of MEN 11066 to inhibit aromatase without interfering with other steroidogenic pathways.

It is important to note that, while MEN 11066 did not affect the weight of adrenals, letrozole reduced their weight. In addition, letrozole significantly decreased corticosterone and aldosterone plasma levels after 2 weeks of treatment, whereas MEN 11066 had no effect on the aforementioned steroids. Finally, the *ex vivo* tests reported that anastrozole and, to a lesser extent, letrozole changed the content of some hepatic drug metabolizing enzymes, diversely to MEN 11066.

Recently, Yue et al. [29], demonstrated the biological relevance of the *in situ* production of estrogens by tumor aromatase. They also reported that a local breast cancer estradiol production is a greater source of estrogens than uptake from plasma. Hence, another desirable feature for effective aromatase inhibitor is the ability to block a possible intra-tumoral production of estrogens. 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.

## Acknowledgements

The authors would like to thank Dr. Elisabeth Guerin for English editing of the manuscript.

## References

- [1] J.G.M. Klijn, B. Setyono-Han, M.M. Bontenbal, C. Seynaeve, J. Foekens, Novel endocrine therapies in breast cancer, *Acta Oncol.* 35 (Suppl. 5) (1997) 30–37.
- [2] J.N. Ingle, J.F. Krook, S.J. Green, T.P. Kubista, L.K. Everson, D.L. Ahmann, M.N. Chang, Randomized trial of bilateral oophorectomy versus tamoxifen in premenopausal women with metastatic breast cancer, *J. Clin. Oncol.* 4 (1986) 178–185.
- [3] A.M. Brodie, Aromatase, its inhibitors and their use in breast cancer treatment, *Pharmacol. Ther.* 60 (1993) 501–515.
- [4] A.M. Brodie, V. Njar, Aromatase inhibitors in advanced breast cancer: mechanism of action and clinical implications, *J. Steroid Biochem. Mol. Biol.* 66 (1998) 1–10.
- [5] W. Yue, R.J. Santen, Aromatase inhibitors: rationale for use following anti-estrogen therapy, *Semin. Oncol.* 23 (Suppl. 9) (1996) 21–27.
- [6] E.R. Simpson, M.S. Mahendroo, G.D. Means, M.W. Kilgore, M.M. Hinshelwood, S. Graham-Lorence, B. Amareh, Y. Ito, C.R. Fisher,

- M.D. Michael, Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis, *Endocr. Rev.* 15 (1994) 342–355.
- [7] W.C. Schwarzel, W.G. Kruggel, H.J. Brodie, Studies on the mechanism of estrogen biosynthesis. 8. The development of inhibitors of the enzyme system in human placenta, *Endocrinology* 92 (1973) 866–880.
- [8] P.E. Goss, T.J. Powles, M. Dowsett, G. Hutchison, A.M. Brodie, J.C. Gazet, R.C. Coombes, Treatment of advanced post-menopausal breast cancer with an aromatase inhibitor, 4-hydroxyandrostenedione: phase II report, *Cancer Res.* 46 (1986) 4823–4826.
- [9] R.J. Santen, H.A. Harvey, Use of aromatase inhibitors in breast carcinoma, *Endocr. Relat. Cancer* 6 (1999) 75–92.
- [10] M.F. Mitwally, R.F. Casper, Use of an aromatase inhibitor for induction of ovulation in patients with an inadequate response to clomiphene citrate, *Fertil. Steril.* 75 (2001) 305–309.
- [11] M.F. Mitwally, R.F. Casper, Aromatase inhibition improves ovarian response to follicle-stimulating hormone in poor responders, *Fertil. Steril.* 77 (2002) 776–780.
- [12] S.J. Weil, K. Vendola, J. Zhou, O.O. Adesanya, J. Wang, J. Okafor, C.A. Bondy, Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations, *J. Clin. Endocrinol. Metab.* 83 (1998) 2479–2485.
- [13] K.A. Vendola, J. Zhou, O.O. Adesanya, S.J. Weil, C.A. Bondy, Androgens stimulate early stages of follicular growth in the primate ovary, *J. Clin. Invest.* 101 (1998) 2622–2629.
- [14] S.J. Weil, K. Vendola, J. Zhou, C.A. Bondy, Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development, *J. Clin. Endocrinol. Metab.* 84 (1999) 2951–2956.
- [15] Y. Cheng, W.H. Prusoff, Relationship between the inhibition constant ( $K_i$ ) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction, *Biochem. Pharmacol.* 22 (1973) 3099–3108.
- [16] R.E. Kramer, S. Gallant, A.C. Brownie, The role of cytochrome P450 in the action of sodium depletion on aldosterone biosynthesis in rats, *J. Biol. Chem.* 254 (1979) 3953–3958.
- [17] T. Omura, R. Sato, The carbon monoxide binding pigment of liver microsomes, *J. Biol. Chem.* 239 (1964) 2370–2378.
- [18] Y.Y. Tu, C.S. Yang, High affinity nitrosamine dealkylase system in rat liver microsomes and its induction by fasting, *Cancer Res.* 43 (1983) 623–629.
- [19] A. Aitio, A single and sensitive assay of ethoxycoumarin deethylation, *Anal. Biochem.* 85 (1978) 488–491.
- [20] R.A. Lubet, R.T. Mayer, J.W. Cameron, R.W. Nims, M.D. Burke, T. Wolff, F.P. Guengerich, Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P450 by phenobarbital and other xenobiotics in the rat, *Arch. Biochem. Biophys.* 238 (1985) 43–48.
- [21] O.H. Lowry, N.J. Rosebrough, A.L. Farr, et al., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- [22] S. Granchi, G.B. Vannelli, L. Vignozzi, C. Crescioli, P. Ferruzzi, R. Mancina, M.C. Vinci, G. Forti, S. Filippi, M. Luconi, F. Ledda, M. Maggi, Expression and regulation of endothelin-1 and its receptors in human penile smooth muscle cells, *Mol. Hum. Reprod.* 8 (2002) 1053–1064.
- [23] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [24] P.J. Munson, D. Rodbard, LIGAND: a versatile computerized approach for characterization of ligand-binding systems, *Anal. Biochem.* 107 (1980) 220–239.
- [25] R.J. Santen, Long term tamoxifen therapy: can an antagonist become an agonist? *J. Clin. Endocrinol. Metab.* 81 (1997) 2027–2029.
- [26] M.M. Gottardis, V.C. Jordan, Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term anti-estrogen administration, *Cancer Res.* 48 (1988) 5183–5187.
- [27] J.M. Randall, A. Templeton, Cervical mucus score and in vitro sperm mucus interaction in spontaneous and clomiphene citrate cycles, *Fertil. Steril.* 56 (1991) 465–468.
- [28] Y. Gonen, R.F. Casper, Sonographic determination of a possible adverse effect of clomiphene citrate on endometrial growth, *Hum. Reprod.* 5 (1990) 670–674.
- [29] W. Yue, J.P. Wang, C.J. Hamilton, L.M. Demers, R.J. Santen, In situ aromatization enhances breast tumor estradiol levels and cellular proliferation, *Cancer Res.* 58 (1998) 927–932.
- [30] C. Palma, M. Criscuoli, A. Lippi, M. Muratori, S. Mauro, C.A. Maggi, Effect of the aromatase inhibitor, MEN 11066, on growth of two different MCF-7 sublines, *Eur. J. Pharmacol.* 409 (2000) 93–101.