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Effect of the aromatase inhibitor, MEN 11066, on growth of two different MCF-7 sublines

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Abstract

The racemate compound MEN 11066 (1-[(benzofuran-2-yl)(4′-cyanophenyl)methyl]-1*H*-1,2,4-triazole) and its enantiomers, (+)-MEN 11623 and (−)-MEN 11622, showed potent and selective aromatase activity on human placental microsomes. In addition, to better evaluate their potency as anticancer drugs, the compounds were assayed on testosterone-induced cell proliferation to measure their ability in inhibiting oestrogen-dependent tumour growth. Two different sublines originated from the human breast carcinoma MCF-7 were used. One, named MCF-7(tumour aromatase) (TA), that had maintained its intrinsic aromatase activity, was more sensitive to estradiol or testosterone-induced growth than the second subline named MCF-7(human placental aromatase) (hPA). The latter had been transfected with the human placental aromatase cDNA, after recognizing that the parental cells had aromatase activity reduced to undetectable levels. The MEN compounds completely reverted the testosterone-induced proliferation in both MCF-7(TA) and MCF-7(hPA) cells, while they did not affect the estradiol-triggered proliferation as a proof of their specificity for aromatase enzyme. Interestingly, MCF-7(TA) cells were more susceptible to the effects of aromatase inhibitors than the MCF-7(hPA) cell. These data suggest the efficacy of aromatase inhibitors in breast cancer when the growth dependency from oestrogen is high and a relatively low aromatase activity may be extremely important for tumour development. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Aromatase inhibitor; Breast carcinoma; Oestrogen; Androgen

1. Introduction

The presence of oestrogen in breast cancer cells is considered to be a critical factor for growth of this type of tumour (Henderson and Canellos, 1980). In premenopausal women, the main source of oestrogen synthesis is the ovary. After menopause, the principal sites of oestrogen synthesis are peripheral tissues, such as fat, skin and muscle (Miller et al., 1990). In these tissues, aromatase cytochrome P-450, a microsomal enzyme, is the rate-limiting step in the synthesis of oestrogen (Tan and Muto, 1986). It has been shown that, in postmenopausal women, the concentrations of circulating estrone and estradiol are insufficient to saturate the breast cancer oestrogen receptors and that intracellular concentrations of these hormones in breast cancer cells are > 10-fold higher than in plasma (Kitawaki et al., 1992). This suggests that oestrogen may be directly produced by cancer cells and that blockade of intratumoural aromatase may have drastic consequences on tumour growth.

Several groups have demonstrated that about 60–70% of breast carcinomas contain detectable quantities of aromatase (Miller, 1986; Silva et al., 1989, Lipton et al., 1987); clinical evidence indicates that differences in disease outcome are associated with the presence or absence of this enzyme activity (Miller and O'Neill, 1987). Therefore, a role of the intratumoural aromatase in the development of breast cancer has been postulated. For this reason, aromatase inhibitors have been widely used in the treatment of postmenopausal breast cancer patients and have been reported to be effective in ameliorating the conditions of 30–40% of such patients (Santen, 1991; Brodie, 1991). Discovering new aromatase inhibitors is, therefore, of great importance.

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In this study, we describe a new aromatase inhibitor, the racemate compound MEN 11066 (1-[(benzofuran-2-yl)(4′-cyanophenyl)methyl]-1*H*-1,2,4-triazole), and its enantiomers, (+)-MEN 11623 and (−)-MEN 11622, which show selective and specific ability in blocking the activity of human placental enzyme. In addition, the activity of these compounds, in comparison with anastrozole (2,2′-[5-(1*H*-1,2,4-triazol-1-ylmethyl)-1-3-phenylene]bis(2-methylpropiononitrile), a third-generation, triazole-based aromatase inhibitor (Buzdar et al., 1996; Harper-Wynne and Coombes, 1999) was assessed in the androgen-dependent tumour cell proliferation as a model to prove the physiological relevance of intratumoural aromatase and, therefore, to better evaluate the potential use of aromatase inhibitors as anticancer drugs.

The oestrogen-dependent human breast cancer cell line, MCF-7, was derived from a pleural effusion taken from a patient with metastatic breast cancer (Soule et al., 1973). Various MCF-7 sublines, with peculiar characteristics, have been originated from the primary culture and, in addition, transfected cell lines have been constructed. In this paper, two MCF-7 sublines — the first one, named MCF-7(tumour aromatase) (TA), has an intrinsic tumour aromatase activity and the second, named MCF-7(human placental aromatase) (hPA), obtained by transfection with a human placental aromatase cDNA of an MCF-7 subline with undetectable aromatase activity (Zhou et al., 1990) — have been characterized for their responsiveness to steroid hormones and used to evaluate the potency of the new aromatase inhibitors. The difference in the responses to oestrogen and androgens of these tumour cells may add new important information to the knowledge of breast cancer development and, in particular, about the role of the intrinsic intratumoural aromatase and the effects of its inhibition.

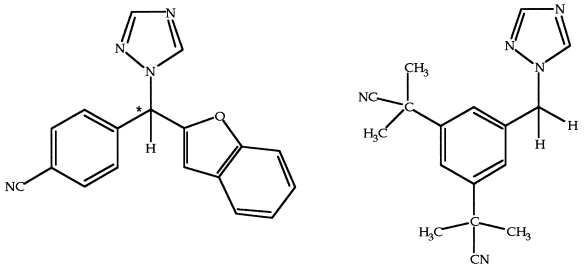

Fig. 1. Chemical structures of MEN 11066, (−)-MEN 11622, (+)-MEN 11623 and anastrozole.

2. Materials and methods

2.1. Drugs and reagents

The aromatase inhibitors anastrozole (2,2′-[5-(1*H*-1,2,4-triazol-1-ylmethyl)-1-3-phenylene]bis(2-methylpropiononitrile), MEN 11066 (1-[(benzofuran-2-yl)(4′-cyanophenyl)methyl]-1*H*-1,2,4-triazole) (Fig. 1) were synthesized at the Chemistry Department of Menarini Ricerche, Pomezia, Italy. The synthesis of MEN 11066 yelded a 94% pure racemic mixture. The resolution of the single enantiomers was achieved by preparative chiral high-performance liquid chromatography (HPLC), with a Chiralcel OD stationary phase (Okamoto et al., 1988). The labor-intensive chromatographic resolution was chosen because of the lack in the molecule of strong basic or acidic groups that could make possible a fractional crystallization with a chiral reagent. To purify single enantiomers, a 20×250 mm Chiralcel OD (Daicel Chemical Ind.) semipreparative column was used with repetitive HPLC runs, loading 30 mg of racemic mixture in 1 ml eluent per run. The eluent was 70/30 hexane/isopropyl alcohol (Mallinckrodt Baker), pumped at a flow rate of 4 ml/min with a Delta Prep 2000 chromatograph (Waters, Milford, MA) equipped with a Knauer RI Detector at $16\times$. The fast eluting enantiomer ($k' = 2.27$ − (+)-MEN 11623) was obtained at 98.9% enantiomeric eccess; the slow eluting enantiomer ($k' = 2.63$ − (−)-MEN 11622) was obtained at 96.3% enantiomeric eccess. Then, the purified enantiomers were dried under vacuum.

All the inhibitors were dissolved at a concentration of 10^{-2} M in ethanol and stored at −20°C. These storage conditions prevent the racemization of the enantimoners for at least 6 months. Dilutions were then made in the appropriate experimental buffers.

Androstenedione, estradiol, estrone, testosterone and tamoxifen were purchased from Sigma (St. Louis, MO, USA).

1β-[^{3}H]androstenedione and 7β-[^{3}H]androstenedione were purchased from NEN Life Science Products (Boston, USA).

2.2. Enzyme preparations

Microsomes for the in vitro aromatase activity assay were prepared from human full-term placentas. The minced tissues were homogenized in 50 mM Tris–HCl buffer, pH 7.4, containing 10 μM phenylmethylsulfonylfluoride, using a Polytron PT 3000 homogenizer (Kinematica, Switzerland); the homogenate was centrifuged for 35 min at $10{,}000 \times g$ and the supernatant was recentrifuged for 60 min at $105{,}000 \times g$; the final pellet was resuspended in 0.5 volumes of the above buffer, aliquoted and stored at −80°C.

2.3. Aromatase activity on microsome preparation

The reaction mixture, containing the enzyme (2–4 μg of human placental microsomes), the substrate 1β-[^{3}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 minutes after the addition of placental microsomes, the reaction was stopped by adding 200 μl of 1 mM $HgCl_2$. Four hundred microlitres of 1% charcoal suspension were then added and the mixture was centrifuged at $2000 \times g$ for 15 min. Four hundred microlitres of supernatant were placed into scintillation vials containing 4 ml of Cytoscint (ICN Biochemicals). Released tritiated water was counted by liquid scintillation (2200 CA counter, Packard Instruments, USA). K_m and V_{max} values were calculated by fitting the curves of reaction rate vs. substrate concentration by the nonlinear regression program Ultrafit (Biosoft, UK).

K_i values were obtained according to the Cheng and Prusoff (1973) equation for competitive inhibition: $K_i = [i]/\{(K_p/K_m) - 1\}$, where: $[i]$ is the inhibitor concentration and K_p is the K_m value calculated in the presence of the inhibitor.

2.4. Culture of human breast carcinoma MCF-7(TA) and MCF-7(hPA) cells

MCF-7 cells were a kind gift from Dr. F. Zunino, Istituto Tumori, Milan, Italy. In our laboratory a subline able to proliferate in the presence of testosterone was selected and to indicate the presence of an intrinsic tumour aromatase activity the cells were named MCF-7(TA). These cells were routinely cultured in high glucose (4500 mg/l) Dulbecco's modified Eagle medium containing 10% heat-inactivated (65°C, 30 min) foetal bovine serum with 5 mM HEPES, 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in an atmosphere of 5% CO_2. The human breast carcinoma MCF-7 3(2) cells, transfected with human placental aromatase cDNA (Zhou et al., 1990) were a kind gift from Dr. Shiuan Chen, Beckman Research Institute of the City of Hope, Duarte, CA, USA. We renamed these cells as MCF-7(hPA) for better comprehension. The cells were routinely maintained in Eagle's minimal essential medium (MEM) containing 10% heat-inactivated foetal bovine serum with 5 mM HEPES, 2 mM L-glutamine, 600 μg/ml G418 at 37°C in an atmosphere of 5% CO_2. All reagents were purchased from Gibco Laboratories (Grand Island, NY).

2.5. Cell growth and DNA synthesis

To observe the effects of steroids on cell proliferation and DNA synthesis, step-down conditions were used, accomplished by growing cells for 7 days in phenol red-free MEM containing 10% dextran-coated charcoal stripped foetal calf serum (oestrogen-depleted medium) with 5 mM HEPES and 2 mM L-glutamine. After this 7-day growing in oestrogen-depleted medium, MCF-7(TA) and MCF-7(hPA) were plated on 24-well tissue culture plates (2.5 or 6×10^4 and 3 or 4×10^4 cells/well for MCF-7(TA) and MCF-7(hPA), respectively) in oestrogen-depleted medium and left to adhere for 24 h. In the experiments, to assay the steroid-dependent growth, the cells were incubated in oestrogen-depleted medium containing various concentrations (0.0001–100 nM) of estradiol, androstenendione, testosterone or estrone. The incubation medium was replaced twice a week. Proliferation and DNA synthesis were then measured at various time points as described below. In the experiments, to assay the potency of aromatase inhibitors, the cells were stimulated with testosterone or androstenedione 100 nM in the absence or the presence of various concentrations (0.001–100 nM) of anastrozole, MEN 11066, MEN 11623 or MEN 11622. The incubation medium was replaced twice a week. The cells were then counted after 12 or 13 days of stimulation for MCF-7(TA) and MCF-7(hPA), respectively.

In proliferation studies, at the end of each experimental time, the medium was aspirated and the cells detached by incubation for 15 min at 37°C with 50 μl versene and 150 μl trypsin-EDTA per well. The cells were then diluted in the vital colorant trypan blue and counted in a haemocytometer counting chamber (Newbauer).

For DNA synthesis evaluation, at the end of each experimental time, the cells were pulsed for 1 h with 500 μl/well of [^{3}H]methyl-thymidine (specific activity, 82.5 Ci/mmol, NEN-Du Pont De Nemours Italiana, Cologno Monzese (MI), Italy) at a concentration of 1 μCi/ml. At the end of incubation, the cells were treated with 1 ml of cold 5% trichloroacetic acid for 10 min at 4°C. The acid supernatant was removed and the cells were washed with double-distilled H_2O (three times, 2 ml each time). The cells were removed from each well with 0.5 ml of 0.1 N NaOH and incubated for 15 min. Each sample was then neutralized with 0.1 N HCl in the scintillation vials. The radioactivity in the vials was quantified by a liquid scintillation counter (Packard, 2002).

2.6. Conversion of [^{3}H]androstenedione by human breast carcinoma MCF-7(TA) and MCF-7(hPA) cells: HPLC analysis

Cells (approximately 5×10^6), in the complete medium, were seeded in 250 ml cell culture flask. After 72 h of culture, cells were washed twice with phenol red-free MEM and then incubated with 3 ml of phenol red-free MEM containing 5 mM HEPES, 2 mM L-glutamine and 200 nM 7β-[^{3}H]androstenedione. After 7, 24 or 48 h of incubation at 37°C, incubation medium was transferred to plastic tubes and stored at −30°C until analysis. Cells were detached by incubation for 15 min at 37°C with versene and trypsin-EDTA and were then diluted in the vital colorant trypan blue and counted in a haemocytometer counting chamber (Newbauer).

Steroid extraction from the incubation medium (3 ml) was carried out with 5 ml of diethyl ether for three times; the pooled ether extracts were brought to dryness under N_2

prior to redissolution with 70% acqueous methanol and injection into the HPLC system.

The HPLC system consisted of a 126 model pump (Beckman Instruments, INC) connected to a Supersphere 100 RP-18 reverse phase column (E. Merk, Darmstadt) protected by its precolumn. UV absorbance at 214 nm was monitored with a model 486 detection (Waters Millipore, Milan, Italy). Radioactivity was analyzed by a Flo-one/Beta Series A-200 radioactivity detector (Radiomatic Instruments, USA), with 3:1 ratio of Ultima-Flo M cocktail (Packard) to mobile phase. Mobile phase was composed of aqueous 0.1% trifluoroacetic acid, and acetonitrile in the ratio 65/35 v/v. Flow rate was set at 0.8 ml/min.

2.7. Data analysis

Statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by Tukey test, if not differently stated.

Curves of aromatase inhibitor activity on testosterone-induced MCF-7(TA) or MCF-7(hPA) cell proliferation were fitted using the computer program ALLFIT (De Lean et al., 1978) that provides the IC_{50} values and related statistical analysis.

3. Results

3.1. Characterization of aromatase inhibitors on human placental microsomes

The kinetic parameters for the aromatization of 1β-[^{3}H]androstenedione by human placenta aromatase were: $K_m = 5.1 \pm 0.4$ nM and $V_{max} = 18 \pm 1$ pmol of released tritiated water per mg protein per min (mean of three values ± S.E.). When tested at increasing concentrations from 1 to 10 nM, all the aromatase inhibitors produced graded increases of K_m, but no significant variation of V_{max} values. This indicates a competitive inhibition and allowed calculation of the K_i values according to Cheng

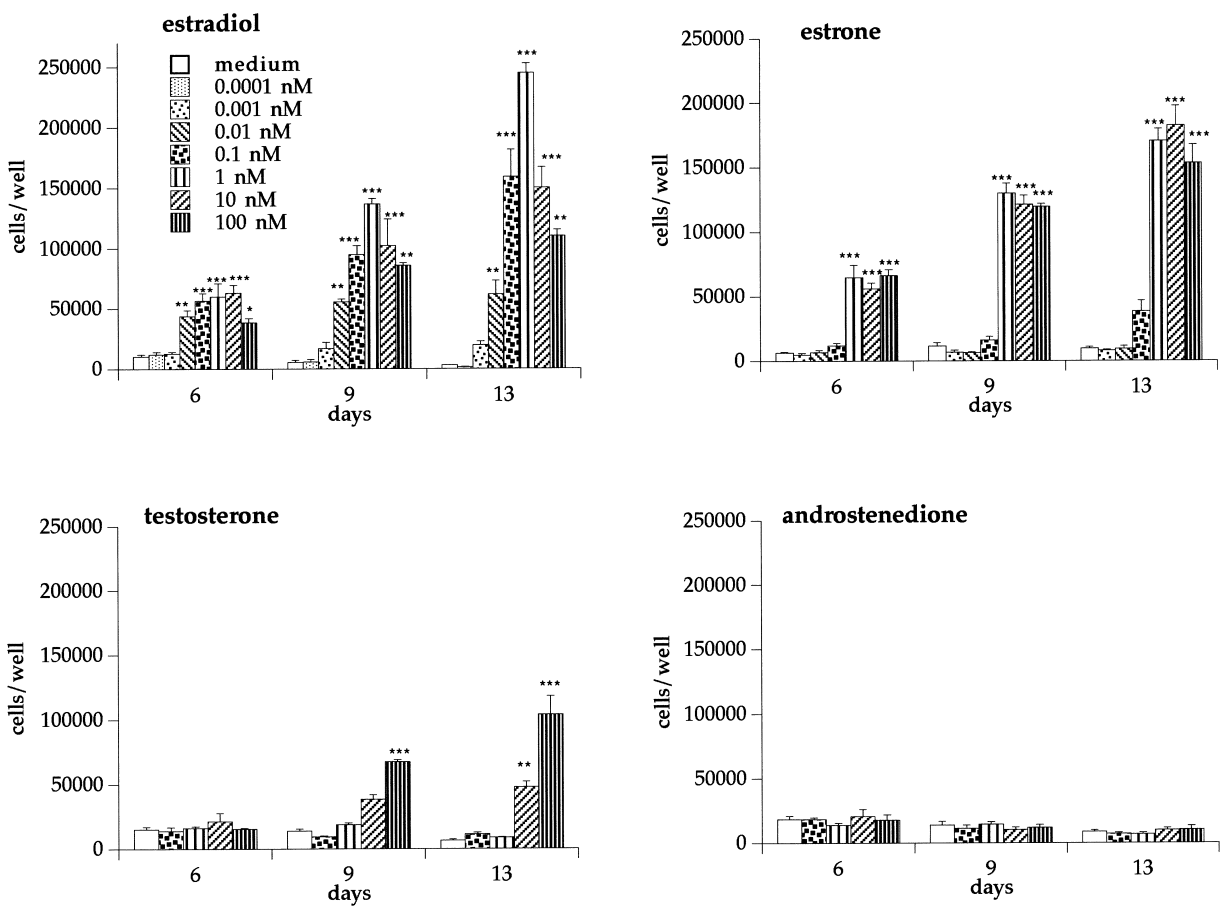

Fig. 2. Effects of steroid hormones on human breast carcinoma MCF-7(TA) cell proliferation. MCF-7(TA) cells were plated at 25,000 cells/well and stimulated in oestrogen depleted medium with various concentrations (0.0001–100 nM) of estradiol, estrone, testosterone and androstenedione. Incubation medium was replaced twice a week. Cells were counted in a haemocytometer counting chamber (Newbauer) after 6, 9 and 13 days. The data represent mean ± S.D. of triplicate determinations of one representative experiment out of two. $^{*}P < 0.05$ vs. medium alone; $^{**}P < 0.01$ vs. medium alone; $^{***}P < 0.001$ vs. medium alone.

and Prusoff (see Methods). MEN 11066 was a very potent inhibitor of human aromatase ($K_i = 0.098 \pm 0.01$ nM; $n = 3$) resulting at least 15-fold more active than the reference compound anastrozole ($K_i = 1.12 \pm 0.03$ nM; $n = 3$). MEN 11623 corresponding to the (+) enantiomer of MEN 11066 was at least 10-fold more active ($K_i = 0.079 \pm 0.01$ nM; $n = 3$) than the (−)-MEN 11622 ($K_i = 1.8 \pm 0.06$ nM; $n = 3$).

3.2. Effects of estradiol, estrone, androstenedione and testosterone on MCF-7(TA) and MCF-7(hPA) cell proliferation and DNA synthesis

The effects of steroid hormones on cell growth were assayed on two different sublines originated from the human breast carcinoma MCF-7: MCF-7(TA) and MCF-7(hPA).

In oestrogen-depleted medium, both cell lines were sensitive to estradiol, but their proliferative responses were different. MCF-7(TA) cells were extremely dependent on the presence of oestrogen in the medium: when subjected to further 6 to 13 days of oestrogen deprivation, MCF-7(TA) cells were unable to grow. In the presence of estradiol (0.01–100 nM), a significant cell number increase was observed at 6 and 13 days from the beginning of stimulation (Fig. 2). Estradiol response had the characteristics of a bell-shaped curve with a peak response at 1 nM, producing a 23-fold increase in the cell number over control.

On the other hand, MCF-7(hPA) cells cultured in oestrogen-depleted medium for 14 days, after the pretreatment period, did not show signs of suffering and even a little proliferation was observed in a few cases. When 0.01–100 nM estradiol was added, an increase in cell number was observed after 7-day stimulation only at a concentration of 10 nM; after 14 days, the increase in cell number was statistically significant at concentrations ranging from 0.1 to 100 nM (Fig. 3). The maximum increase in the MCF-7(hPA) cell number was only about threefold above control, indicating a lower sensitivity to estradiol-induced proliferation, as compared to MCF-7(TA) cells. In general, the proliferative response, in terms of magnitude

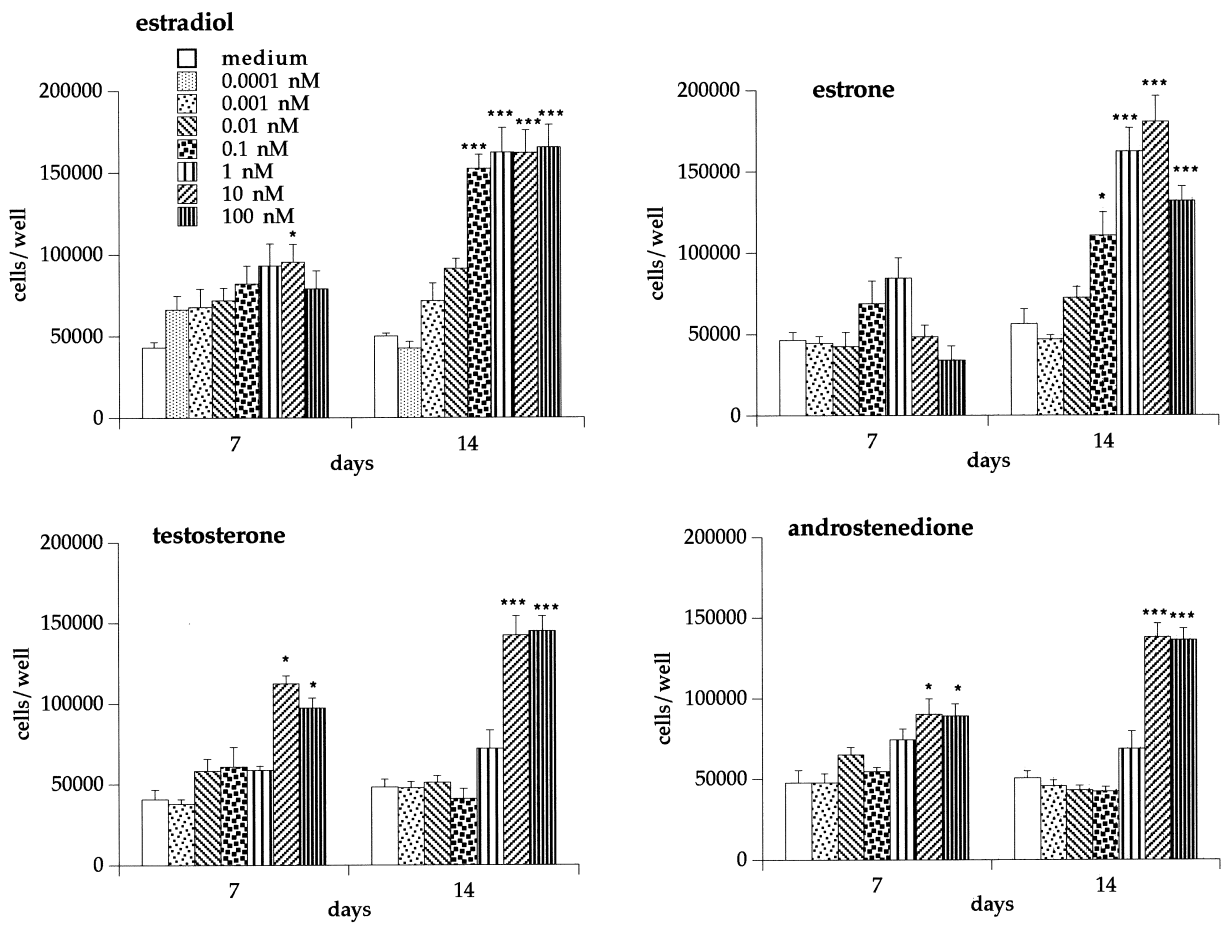

Fig. 3. Effects of steroid hormones on human breast carcinoma MCF-7(hPA) cell proliferation. MCF-7(hPA) cells were plated at 30,000 cells/well and stimulated in oestrogen-depleted medium with various concentrations (0.0001–100 nM) of estradiol, estrone, testosterone and androstenedione. Incubation medium was replaced twice a week. Cells were counted in a haemocytometer counting chamber (Newbauer) after 7 and 14 days. The data represent mean ± S.D. of triplicate determinations of one representative experiment out of two. $^{*}P < 0.05$ vs. medium alone; $^{**}P < 0.01$ vs. medium alone; $^{***}P < 0.001$ vs. medium alone.

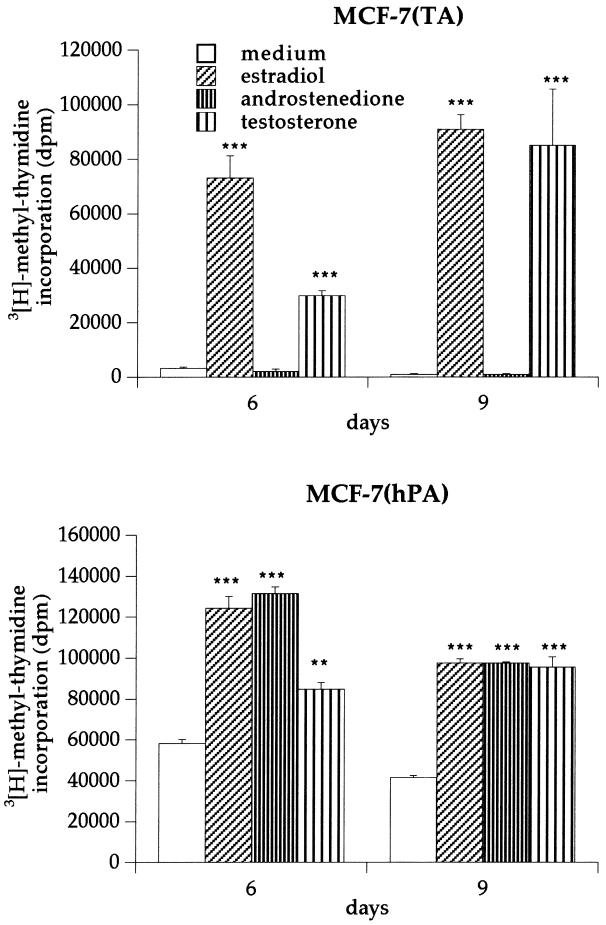

Fig. 4. Effects of steroid hormones on DNA synthesis of human breast carcinoma MCF-7(TA) and MCF-7(hPA) cells. MCF-7(TA) and MCF-7(hPA) cells were plated at 25000 and 30000 cells/well, respectively, and stimulated in oestrogen-depleted medium with estradiol (10 nM), androstenedione (100 nM) and testosterone (100 nM). Incubation medium was replaced twice a week. At the indicated time points, cells were pulsed with [^{3}H]methyl-thymidine for 1 h and the incorporation in DNA was then measured. The data represent mean ± S.D. of triplicate determinations. $^{**}P < 0.01$ vs. medium alone; $^{***}P < 0.001$ vs. medium alone.

of effect, triggered by steroids in MCF-7(hPA) was less that that observed in MCF-7(TA), suggesting that MCF-7(hPA) cells represent a phenotype of a more aggressive steroid-independent breast tumour.

Both MCF-7(TA) and MCF-7(hPA) cells responded to estrone too. As shown in Figs. 2 and 3, MCF-7(hPA) cells were more sensitive to estrone than MCF-7(TA) cells; in fact, a significant proliferation of the former was observed with 0.1 nM estrone, whereas a 10-fold greater concentration of estrone was required to produce the same effect in the latter.

Both testosterone and androstenedione-induced cell proliferation in MCF-7(hPA) (Fig. 3). A significant stimulation was observed at high concentrations (10–100 nM) for both male sex hormones with a maximal response similar to that observed with estradiol. The growth of the MCF-7(TA) cell line was stimulated by testosterone, but not by androstenedione (Fig. 2). The stimulatory effect measured at 100 nM testosterone, although consistently smaller than that measured with the optimal estradiol concentration, induced, however, a 10-fold increase in cell growth over control.

In the presence of the estrogen-receptor competitor tamoxifen (0.1 nM), the proliferative effects induced by 100 nM testosterone in both cell types were completely inhibited (data not shown). This indicated that cell proliferation induced by male sex hormones is mediated via oestrogen receptor.

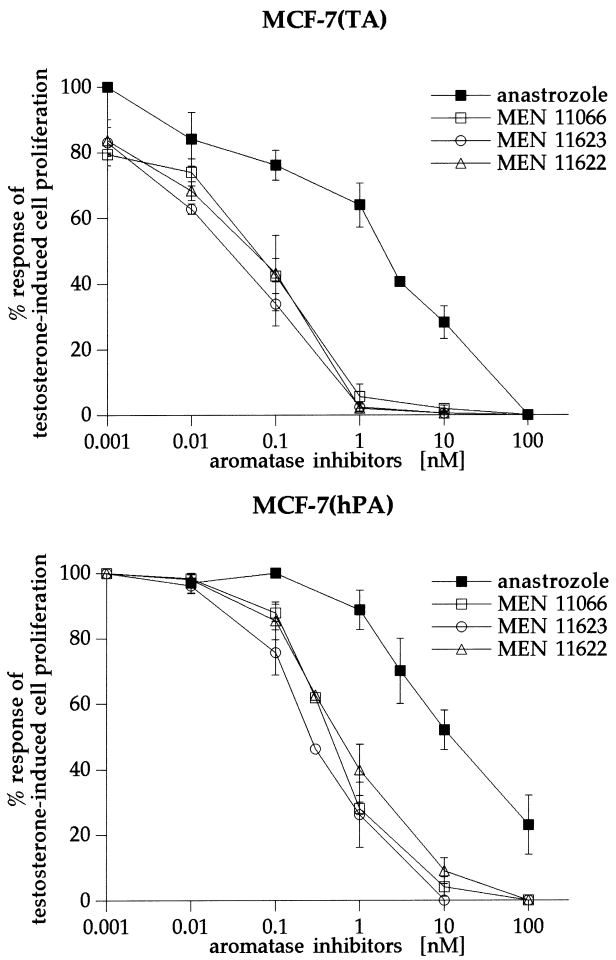

Fig. 5. Effects of aromatase inhibitors on testosterone-induced proliferation in human breast carcinoma MCF-7(TA) and MCF-7(hPA) cells. MCF-7(TA) and MCF-7(hPA) cells were plated at 60,000 and 40,000 cells/well, respectively, and stimulated in oestrogen-depleted medium with testosterone (100 nM). Variable concentrations of the aromatase inhibitors (0.001–100 nM) were coadministered with testosterone. Medium was replaced twice a week. Cells were counted in a haemocytometer counting chamber (Newbauer) after 12 days for MCF-7(TA) and 13 days for MCF-7(hPA). Data are presented as % of response obtained in cells stimulated with testosterone in the absence of aromatase inhibitors (MCF-7(TA) cells/well: 39,472 ± 2311 and 408,083 ± 42,754 for unstimulated and testosterone-stimulated cells, respectively ($n = 12$); MCF-7(hPA) cells/well: 82,694 ± 7531 and 236,305 ± 20,658 for unstimulated and testosterone-stimulated cells, respectively ($n = 12$)). The data represent mean ± S.D. of three independent experiments for each aromatase inhibitor assayed.

Table 1
Effects of aromatase inhibitors on testosterone-induced proliferation in human breast carcinoma MCF-7(TA) and MCF-7(hPA) cells

Aromatase inhibitors	Cell lines [IC_{50} (nM)]	
	MCF-7(TA)	MCF-7(hPA)
MEN 11066	0.05 ± 0.01	0.45 ± 0.06
MEN 11622	0.05 ± 0.02	0.57 ± 0.15
MEN 11623	0.03 ± 0.01	0.34 ± 0.09
Anastrozole	1.2 ± 0.5	7 ± 4

MCF-7(TA) and MCF-7(hPA) cells were stimulated in oestrogen depleted medium with testosterone (100 nM). Variable concentrations of aromatase inhibitors (0.001–100 nM) were coadministered with testosterone. Medium was replaced twice a week. Cells were counted in a haemocytometer counting chamber (Newbauer) after 12 days for MCF-7(TA) and 13 days for MCF-7(hPA).
The results are expressed as IC_{50} values calculated with the computer program ALLFIT by simultaneous analysis of curves from three independent experiments, each performed in triplicate.

The mitogenic effect of steroids in MCF-7(TA) and MCF-7(hPA) cells was also confirmed by assessing the stimulation of new DNA synthesis, measured as incorporation [^{3}H]methyl-thymidine (Fig. 4). In addition, it is worth noting that the MCF-7(hPA) cells, at variance with MCF-7(TA) cells, retain the ability to synthesize new DNA even in the absence of steroids.

3.3. Effects of aromatase inhibitors on testosterone-induced MCF-7(TA) and MCF-7(hPA) cell proliferation

The aromatase inhibitors anastrozole, MEN 11066, MEN 11623 and MEN 11622 (0.001–100 nM for each drugs), were assayed on cell proliferation induced by 100 nM testosterone. As shown in Fig. 5, all aromatase inhibitors were able to completely revert the testosterone-induced cell proliferation both in MCF-7(TA) and MCF-7(hPA) cells. On the contrary, none of them, even at 100 nM, did affect the growth of estradiol-stimulated cells (data not shown), indicating that the effect was not due to aspecific toxicity but to a blockade of estrone/estradiol formation.

In both cell lines, anastrozole was less potent (1 to 2 orders of magnitude) than MEN 11066 and its enantiomers that showed comparable activity. IC_{50} values indicated that MCF-7(TA) cells are about 10-fold more sensitive to MEN compounds than MCF-7(hPA) cells (Table 1). When MCF-7(hPA) cell line was used, all the aromatase inhibitors assayed were also able to inhibit cell proliferation induced by 100 nM androstenedione with comparable or slightly greater potency (IC_{50} values expressed in nM were 0.15 ± 0.008, 0.17 ± 0.02, 0.09 ± 0.02 and 9 ± 3 for MEN 11066, MEN 11622, MEN 11623 and anastrozole, respectively, $n = 3$) than that measured with testosterone-triggered response.

3.4. Conversion of [^{3}H]androstenedione in MCF-7(TA) and MCF-7(hPA) cells.

Aromatase activity was measured after 7, 24 or 48 h of incubation of 200 nM [^{3}H]androstenedione with both cell lines. Based on relative retention times of authentic standards processed in parallel, the described HPLC analysis method allowed the determination of estrone, estradiol, testosterone and 5α-androstanedione, besides androstenedione, in the incubation medium. The proportions of these metabolites are reported in Table 2. The metabolism of androstenedione was maximal after 24-h incubation, and the most abundant metabolite detected with both cell lines, was 5α-androstanedione, presumably formed because of a high 5α-reductase activity (Macaulay et al., 1994). The quantitative distribution of metabolites was different with the two cell sublines used. With MCF-7(TA) cells, relatively low amounts of estradiol flanked estrone formation (in an approximate 1:1 ratio) and no testosterone could be detected. On the other hand, with MCF-7(hPA) cells, estradiol formed at a higher rate, being significantly more abundant (about sevenfold at all considered times, $P <$

Table 2
Time-dependent changes in the concentration of androstenedione and metabolites after incubation of [^{3}H]androstenedione with human breast carcinoma MCF-7(TA) and MCF-7(hPA) cells

Cell subline	Incubation time (h)	*N*	Analyte (% relative amount)				
			Androstenedione	Estradiol	Estrone	Testosterone	5α-Androstane-3,17-dione
MCF-7(TA)	7	3	46.9 ± 3.2	2.5 ± 0.2	0.3 ± 0.2	undetectable	46.2 ± 0.1
	24	3	18.1 ± 1.9	3.6 ± 0.2	2.5 ± 0.7	undetectable	63.1 ± 1.1
	48	2	15.7 ± 8.2	3.0 ± 1.3	3.0 ± 0.6	undetectable	51.9 ± 1.1
MCF-7(hPA)	7	3	41.2 ± 1.8	6.1 ± 2.3	undetectable	undetectable	50.3 ± 0.4
	24	4	9.0 ± 1.3	6.7 ± 1.8	0.9 ± 0.3	1.5 ± 0.3	68.2 ± 2.5
	48	3	14.4 ± 2.1	6.8 ± 1.7	1.0 ± 0.9	3.5 ± 0.7	54.4 ± 1.2

Cells were incubated in the culture medium with 200 nM 7β-[^{3}H]androstenedione at 37°C. At the indicated times, incubation medium was extracted thrice with diethyl ether. Extracted steroids were analysed by radiodetector-coupled HPLC. The relative amount of each metabolite is calculated as the percent ratio of the individual peak area to the total area of the five identified radioactive peaks. Data are expressed as mean values ± S.D.
In a control experiment, tritiated androstenedione was incubated in the culture medium without cells for 7, 24 and 48 h. In these conditions, no metabolites of androstenedione were detected.

0.01, Student *t*-test) than estrone, that accumulated significantly ($P < 0.01$) less than with MCF-7(TA) cells). Also testosterone formation was measurable with these cells. Overall, these data suggest a higher 17β-hydroxysteroid dehydrogenase activity in MCF-7(hPA) cells, as compared to MCF-7(TA) cells.

4. Discussion

The racemate compound MEN 11066 and its enantiomers, (+)-MEN 11623 and (−)-MEN 11622, are new potent aromatase inhibitors as demonstrated in the human placental microsome assay. In addition, these compounds were able to inhibit the androgen-dependent human breast carcinoma cell proliferation confirming that blocking conversion of androgens into oestrogen represents a suitable therapeutic strategy in the treatment of oestrogen-dependent tumours (Brodie, 1991; Santen, 1991). In view of the fact that cell populations of human tumours are heterogeneous with respect to hormonal response, growth rate, drug sensitivity and other biological characteristics according to the evolutionary stage of the tumour (Sutherland, 1988), these differences should be taken into account when studying the effects of anticancer drugs. For this reason, we have characterized two sublines of the human breast carcinoma MCF-7 as different models of oestrogen-dependent tumour growth in which to evaluate the potency of aromatase inhibitors.

The first subline, named MCF-7(TA), was selected for its ability to proliferate in the presence of testosterone; the second one, named MCF-7(hPA), had been transfected with the human placenta aromatase cDNA (Zhou et al., 1990), after recognizing that the parental cells had aromatase activity reduced to undetectable levels. Both cell lines, MCF-7(TA) and MCF-7(hPA), increased their proliferative rate in the presence of estradiol and estrone. However, MCF-7(TA) cells were more sensitive than MCF-7(hPA) cells to estradiol-induced growth in terms of effective concentration threshold, rapidity of response and magnitude of effects. Responsiveness to testosterone was also observed in both cell lines and, again, MCF-7(TA) subline was more sensitive in terms of magnitude of response. The proliferation induced by testosterone was blocked by aromatase inhibitors demonstrating the existence of an intrinsic aromatase activity in MCF-7(TA) cells. Also, the proliferative effects of androstenedione, measurable only with MCF-7(hPA), can be attributed to its conversion to oestrogen as demonstrated by the block produced by aromatase inhibitors. These data, in accordance with the blocking effect of tamoxifen, confirmed that the cellular growth triggered by male sex hormones was mediated by their conversion into oestrogen.

The measurement of the metabolites, formed after incubation with [^{3}H]androstenedione, confirmed that aromatase activity was present in both the cell lines, but the different distribution of these metabolites helped in clarifying some apparently conflicting data. In fact, both cell lines were able to convert androstenedione into estrone; however, MCF-7(hPA) cells were able to convert estrone into estradiol and androstenedione into testosterone, while in MCF-7(TA), cells, no production of testosterone was detected and a consistent amount of estrone was not converted into estradiol. These data indicate a higher activity of the 17β-hydroxysteroid dehydrogenase enzyme in the MCF-7(hPA) cells, which accounts for the comparable mitogenic potency exerted by estrone and estradiol and also for their responsiveness to both male sex hormones, androstenedione and testosterone. Instead, MCF-7(TA) cells, although able to correctly aromatize androstenedione into estrone, were unable to consistently convert estrone and androstenedione into estradiol and testosterone, respectively. This was probably due to an impairment of the 17β-hydroxysteroid dehydrogenase enzyme. As a consequence of that, MCF-7(TA) cells were more responsive to estradiol than estrone, and between the male sex hormones, only testosterone acted as a growth factor. Although estrone can directly interact with estrogen receptor, similar to estradiol, higher concentrations of estrone were required to have a measurable proliferative effect. In MCF-7(TA) cells, aromatase probably produces an amount of estrone (from androstenedione) that is insufficient to reach the concentration threshold for a proliferative effect, while the amount of estradiol formed by testosterone aromatization is efficacious in view of the lower concentration required. Therefore, the MCF-7(TA) unresponsiveness to androstenedione is not related to a misfunction of the tumour aromatase enzyme: this conclusion is in keeping with the previous observation of no sequence difference of aromatase gene expressed in human tumour cells in comparison with that of the enzyme expressed in placenta (Zhou et al., 1993).

Thus, the MCF-7(TA) subline used in this study is a model of breast tumour extremely dependent from oestrogen for its growth and, therefore, the presence of an intrinsic intratumoral aromatase activity represents an advantage for its sustenance. On the contrary, the scarce oestrogen dependency shown by MCF-7(hPA) cells and the fact that they derived from cells that had lost intrinsic aromatase activity (Zhou et al., 1990) suggest that MCF-7(hPA) cells represent a more aggressive oestrogen-independent breast tumour phenotype with no need for aromatase activity. The peculiar characteristics of these cell lines have given us the opportunity to evaluate the role and efficacy of aromatase inhibitors in oestrogen-dependent proliferation of two different tumour models.

The MEN aromatase inhibitors were able to completely inhibit in a concentration-dependent manner, testosterone-triggered proliferation both with MCF-7(TA) and MCF-7(hPA) cells, while they did not affect the estradiol-induced cell growth, as a proof of their specificity for the aromatase enzyme. These data, while showing evidence of

the biological relevance of aromatase in breast cancer proliferation, highlight also the ability of aromatase inhibitors to effectively contrast the functionality of intratumoural aromatase, either intrinsic or of placental origin.

MEN 11066 and its enantiomers showed comparable activity in both systems, MEN 11623 being only slightly more potent. However, it was at least ten times more potent than MEN 11622 in inhibiting purified human placenta aromatase. The different experimental conditions, including the long-lasting exposure of these compounds to biological media during proliferation assays, may be the cause of these differences in potencies. In fact, racemization of the enantiomers could occur in the medium where the cells are grown and, as a consequence, differences in activity between them may be lowered. If racemization occurs not only in this in vitro cell culture condition but also in vivo, the separation of enantiomers may not be mandatory for developing these drugs. In any case, the MEN aromatase inhibitors were found to be markedly more potent than anastrozole, a drug currently marketed (Buzdar et al., 1996; Harper-Wynne and Coombes, 1999).

Interestingly, the values of IC_{50} calculated for all the aromatase inhibitors assayed, indicated that MCF-7(TA) are more sensitive than MCF-7(hPA) to these compounds. The greater sensitivity of MCF-7(TA) cells may be related to their greater sensitivity to oestrogen-dependent growth, although, we cannot rule out the presence of a lower amount of aromatase enzyme per cell. When using MCF-7(hPA) cells, in which aromatase has been artificially overexpressed and the cells are transforming into a more aggressive oestrogen-independent tumour phenotype, the potency of aromatase inhibitors might be underestimated.

The data suggest that the anticancer treatment with aromatase inhibitors may be extremely efficient in tumours highly responsive to oestrogen, as occurs in the early phase of breast cancer, supporting the possibility to use aromatase inhibitors in both neo-adjuvant and chemopreventive setting (Kelloff et al., 1998; Lubet et al., 1998).

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