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# AROMATASE IN THE HUMAN CHORIOCARCINOMA JEG-3: INHIBITION BY R 76 713 IN CULTURED CELLS AND IN TUMORS GROWN IN NUDE MICE

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**Summary**—The aromatase enzyme and its inhibition by R 76 713 were characterized in the JEG-3 choriocarcinoma cell line in culture and in JEG-3 tumors grown in nude mice. Optimal cell culture parameters and enzyme reaction conditions for the determination of aromatase activity were established. Under these conditions, *in vitro* JEG-3 aromatase was inhibited by R 76 713 with $IC_{50}$-values of $7.6 \pm 0.5$ nM and $2.7 \pm 1.1$ nM using 500 nM of androstenedione and testosterone as substrate respectively. The $K_m$-value of the aromatase enzyme with androstenedione as substrate was $62 \pm 19$ nM; with testosterone as substrate, a value of $166 \pm 27$ nM was found. In the presence of increasing concentrations of R 76 713, the $K_m$-values increased while the $V_{max}$ remained unchanged. Using androstenedione and testosterone as substrate Lineweaver–Burk analysis of the data showed $K_i$-values for R 76 713 of $0.43 \pm 0.06$ nM and $0.47 \pm 0.39$ nM respectively. R 76 713 appeared to competitively inhibit the JEG-3 aromatase. Aromatase could easily be measured in homogenates of JEG-3 tumors grown in nude mice and showed $K_m$-values similar to those found for JEG-3 cells *in vitro*. $IC_{50}$-values for inhibition of tumor aromatase by R 76 713 were also similar to those found in cultured cells. Tumor aromatase measured *ex vivo*, 2 h after a single oral administration of R 76 713 was dose-dependently inhibited. An $ED_{50}$-value of 0.05 mg/kg was calculated. The JEG-3 choriocarcinoma proved to be a useful aromatase model enabling the comparative study of aromatase inhibition *in vitro* and *in vivo*.

## INTRODUCTION

Aromatase, a cytochrome *P*-450-dependent enzyme, is responsible for the formation of estrogens from their androgen precursors. In premenopausal women the enzyme is mainly found in the ovaries, whereas in postmenopausal women adipose tissue is the main site of aromatization [1]. Selective inhibition of the aromatase enzyme is one alternative for the treatment of estrogen-dependent diseases such as breast cancer, endometriosis and uterine fibroids with R 76 713 being a newly developed non-steroidal competitive aromatase inhibitor. This compound inhibits aromatase both *in vitro* and *in vivo* with very high potency and selectivity [2, 3] and induces near complete tumor regression in DMBA and NMU induced mammary carcinoma in rats [4, 5]. The first results obtained in male and in premenopausal female volunteers indicate that R 76 713 is capable of reducing the plasma estradiol levels after a single oral dose [6]. Research on aromatase inhibitors has led to a considerable interest in suitable *in vitro* and *in vivo* aromatase models. *In vitro*, placental or ovarian microsomes and granulosa cells have been used as a source of aromatase. However, problems of availability and reproducibility are not uncommon to these systems and the experiments are often very time-consuming. These problems could be solved by an aromatase containing continuous homogeneous (cancer) cell culture system. If appropriately chosen, these cells could have an additional advantage in being able to induce tumor growth in nude mice; thus enabling direct comparison of *in vitro* data with that generated *in vivo* or *ex vivo*. While there exists a limited

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*Abbreviations:* $IC_{50}$: drug concentration producing 50% inhibition *in vitro*; $V_{max}$: maximal velocity of an enzyme catalyzed reaction; $K_i$: dissociation constant of the enzyme–inhibitor complex; DMBA: 7,12-dimethylbenz-(*a*)anthracene; NMU: dimethylnitrosourea; DMSO: dimethylsulfoxide; PMSG: pregnant mare's serum gonadotrophin; SEM: standard error of the mean; 4OHA: 4-hydroxyandrostenedione; AG: aminoglutethimide.

number of reports on aromatase activity in MCF-7 cells and MDA-MB-231 breast cancer cells [7] as well as in some ovarian carcinoma cell lines [8], the amount of aromatase enzyme in these cells seems to be too low for routine determinations.

The human trophoblast is a unique tissue which performs many varied endocrine functions to support fetal survival and development and exhibits aromatase activity. Cell lines derived from malignant trophoblasts which also demonstrate significant aromatase activity include the JAR [9] as well as the JEG-3 cell line. The JEG-3 is one of six clonally derived lines of human choriocarcinoma (malignant trophoblast) isolated and described by Kohler and associates [10]. Individual clonal lines have retained the capacity to synthesize human chorionic gonadotrophin (HCG), human chorionic somatomammotrophin (HCS) and progesterone as well as to aromatize androgens. In the present study, we describe the kinetics of the aromatase enzyme in the human choriocarcinoma cell line JEG-3 and characterize the inhibition of aromatase by R 76 713. We also evaluate the *ex vivo* effects of R 76 713 on aromatase in JEG-3 tumors, grown in nude mice.

## EXPERIMENTAL

### *Materials*

R 76 713 (6-[(4-chlorophenyl)(1*H*-1,2,4-triazol-1-yl)methyl]-1-methyl-1*H*-benzotriazole) was synthesized at the Department of Organic Synthesis of the Janssen Research Foundation (Beerse, Belgium). Aminoglutethimide was from Ciba-Geigy (Basel, Switzerland) and 4-hydroxyandrostenedione from Sigma (St Louis, U.S.A.). MEM REGA 3 medium, fetal calf serum, Hanks balanced salt solution, trypsin/EDTA, $NaHCO_3$ and L-glutamine were supplied by GIBCO (Ghent, Belgium). NADPH and Norit A were obtained from Serva (Heidelberg, Germany). Glucose-6-phosphate dehydrogenase and glucose-6-phosphate were supplied by Boehringer Pharma (Brussels, Belgium). [1$\beta$,2$\beta$-$^3$H]Androstenedione (41.8 Ci/mmol, 1546.6 GBq/mmol), [1$\beta$-$^3$H]androstenedione (25.4 Ci/mmol, 939.8 GBq/mmol) and [1$\beta$,2$\beta$-$^3$H]testosterone (53.5 Ci/mmol, 1979.5 GBq/mmol) were from New England Nuclear (Brussels, Belgium). Dextran T70 was purchased from Pharmacia (Brussels, Belgium). Female nude CD-1 mice were obtained from Charles River (Sulzfeld, Germany).

### *Methods*

#### *Cell culture*

Choriocarcinoma cells were obtained from the American Type Culture Collection (Rockville, U.S.A.) at passage number 126. For the experiments described in this study, cells from passage numbers 127–157 were used. Cells were cultured in MEM REGA-3, supplemented with 10% fetal calf serum, 2 mM glutamine and 2.8 g/l sodium bicarbonate at 37°C in a humidified 95% air, 5% $CO_2$ atmosphere in 175 $cm^2$ plastic cell culture flasks. Medium was changed twice weekly. Once a week, cells were subcultured at a split ratio of 1:30 using trypsin/EDTA solution.

#### *Determination of aromatase activity in cultured cells*

Aromatase activity was determined by measuring the amount of $^3H_2O$ formed during aromatization of [$^3$H]androstenedione or [$^3$H]-testosterone [11]. 800 $\mu$l aliquots of a cell suspension in medium without fetal calf serum were incubated at 37°C in the presence of the substrate and the inhibitor or its solvent. In the assay blanks, the cell suspension was replaced by an equal volume of the culture medium without serum. The total volume of the incubation mixture was always 1 ml. The substrate consisted of [1$\beta$,2$\beta$-$^3$H]androstenedione, [1$\beta$-$^3$H]androstenedione or [1$\beta$,2$\beta$-$^3$H]testosterone. In some experiments, a mixture of [1$\beta$,2$\beta$-$^3$H]androstenedione and unlabelled androstenedione or a mixture of [1$\beta$,2$\beta$-$^3$H]testosterone and unlabelled testosterone was used. For [1$\beta$-$^3$H]androstenedione the $^3$H-distribution (as determined by $^3$H-NMR) was 25.8% 1$\alpha$ and 74.2% 1$\beta$. For [1$\beta$,2$\beta$-$^3$H]testosterone the $^3$H-distribution was 14% 1$\alpha$, 43% 1$\beta$, 11% 2$\alpha$ and 32% 2$\beta$. R 76 713, aminoglutethimide and 4-hydroxyandrostenedione were dissolved in 100% DMSO, and further diluted in medium without serum. After addition to the incubation mixtures, final DMSO concentrations were always equal to or less than 0.1%. Following incubation, the incubation mixture was extracted with 8 ml of dichloromethane by brief agitation. The layers were separated by centrifugation for 10 min at 658 *g* (2000 rpm, Heraeus Minifuge T). A 500 $\mu$l aliquot of the aqueous layer was treated with 500 $\mu$l of a charcoal suspension (0.25 g dextran and 2.5 g Norit A in 100 ml of water) and centrifuged for 10 min at 1360 *g* (3300 rpm, Sorvall RC-3B). 700 $\mu$l of

the final supernatant were added to 10 ml of Optiphase "Hi Safe II" (Packard) and counted for radioactivity in a Packard Tri-carb-4530 liquid scintillation analyzer.

*Tumor model in nude mice*

Female athymic nude (Nu/Nu) CD-1 mice were housed in aseptic isolation box-cages on sterile bedding. The animals were provided with autoclaved chow and tapwater *ad libitum*. Mice were handled and injected using aseptic procedures in a laminar flow cabinet. JEG-3 cells were maintained in culture as described above. The cells were collected on ice using trypsin/EDTA solution. Nude mice (±25 g) were subcutaneously injected in the subinguinal region with $1.5 \times 10^6$ JEG-3 cells (in 0.2 ml medium without fetal calf serum). Mice were treated when tumors were well established. R 76 713 was dissolved in 20% PEG and administered orally by gavage, with control animals receiving 20% PEG. 2 h after drug administration, the mice were killed by decapitation, tumors were removed, weighed and snap frozen in liquid nitrogen.

*Aromatase measurement in JEG-3 tumors*

The JEG-3 tumors were defrosted on ice, homogenized in 0.1 M Tris–HCl pH 7.4 with an Ultra-Turrax T25 (dilution: 50 mg original wet wt of tissue/ml Tris-buffer) and the homogenate was centrifuged for 10 min at 2310 *g* (5000 rpm in a Sorval RC-5B Refrigerated Superspeed centrifuge). Determination of aromatase activity was based on the same principle as for measurement in cultured cells but the culture medium was replaced by 0.1 M Tris–HCl pH 7.4. 700 $\mu$l aliquots of the homogenate were incubated at 37°C with an energy-regenerating system (consisting of 0.2 mM NADPH, 1 U/ml glucose-6-phosphate dehydrogenase, 2.5 mM glucose-6-phosphate and 2 mM $MgCl_2$) in the presence of the substrate and the inhibitor or its solvent. Incubation, extraction, separation and counting were performed exactly as described above.

*Data analysis*

Lineweaver–Burk plots and slope re-plots were calculated by linear regression analysis. *Ex vivo* data were analysed using the Mann–Whitney U-test. Significance was defined at a level of $P < 0.05$.

## RESULTS

*Reaction conditions*

To determine the linearity of aromatase activity as a function of cell concentration, cell suspensions varying in concentration from 50,000 cells/ml to 900,000 cells/ml in medium without fetal calf serum were incubated for 1 h at 37°C. The substrate consisted of 10 nM [1$\beta$,2$\beta$-$^3$H]androstenedione or 10 nM [1$\beta$,2$\beta$-$^3$H]testosterone. The cell concentration curve was linear for all the cell concentrations tested (Fig. 1A and B). To determine the optimal incubation time a cell suspension of 200,000 cells/ml was incubated for 15–150 min at 37°C. The substrate consisted of 10 nM [1$\beta$,2$\beta$-$^3$H]-androstenedione or 10 nM [1$\beta$,2$\beta$-$^3$H]testosterone. The time curve with androstenedione as the substrate was linear up to 90 min (Fig. 1C) and with testosterone it was linear for all time periods tested (Fig. 1D). For determination of aromatase activity, a cell suspension of 200,000 cells/ml and an incubation time of 1 h were chosen. Under these conditions, conversion of substrate was always less than 10%.

*Effect of culture confluency on aromatase activity*

JEG-3 cells were subcultured in T175 tissue culture flasks and grown under standard conditions. At different moments during a 10-day period, cultures were trypsinized and the total amount of cells per flask was determined using a hemocytometer. Standard cell suspensions (200,000 cells/ml) were prepared and aromatase activity was measured. The substrate consisted of 470 nM unlabelled androstenedione and 30 nM [1$\beta$,2$\beta$-$^3$H]androstenedione. Non-specific release of $^3H_2O$ was measured in a parallel incubation using $10^{-6}$ M R 76 713 and was substracted from all measured values. Aromatase activity varied according to cell confluency. In low density cultures aromatase activity was almost undetectable. Aromatase activity was slightly higher during the log-phase of cell growth and increased dramatically at confluency (Fig. 2).

*Aromatase inhibition by R 76 713*

Under the reaction conditions described above JEG-3 cells were incubated with R 76 713 in a concentration range varying from $10^{-11}$ to $10^{-5}$ M. The substrate consisted of 470 nM unlabelled androstenedione and 30 nM [1$\beta$,2$\beta$-$^3$H]-androstenedione or 470 nM unlabelled testoster-

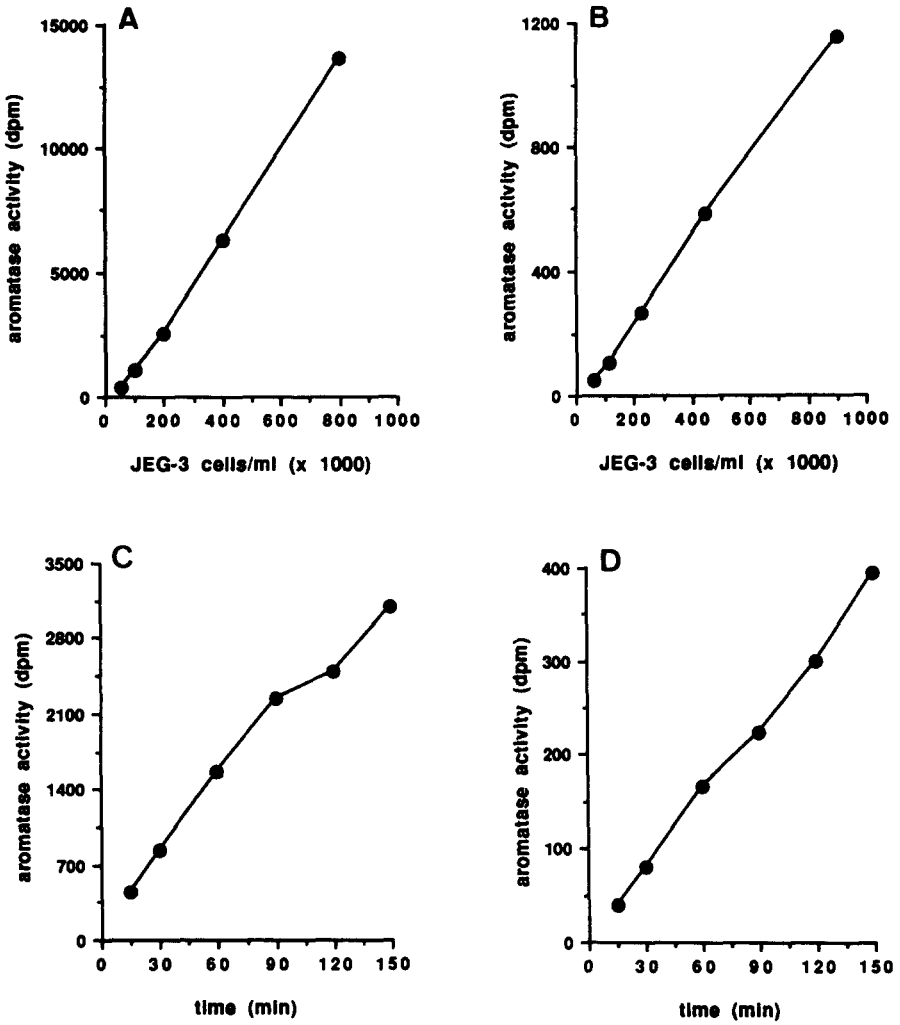


Fig. 1. Effect of cell concentration and incubation time on aromatase activity in JEG-3 choriocarcinoma. Upper panel: increasing cell concentrations were incubated for 1 h with 10 nM [1$\beta$,2$\beta$-$^3$H]androstenedione (A) or 10 nM [1$\beta$,2$\beta$-$^3$H]testosterone (B). Lower panel: a fixed cell concentration (200,000 cells/ml) was incubated for different periods of time with 10 nM [1$\beta$,2$\beta$-$^3$H]androstenedione (C) or 10 nM [1$\beta$,2$\beta$-$^3$H]testosterone (D). Results are expressed as means of duplicate determinations.

one and 30 nM [1$\beta$,2$\beta$-$^3$H]testosterone. Figure 3 shows the obtained inhibition curves. $IC_{50}$-values of 7.6 ± 0.5 nM and 2.7 ± 1.1 nM were calculated with androstenedione and testosterone as the substrate (mean ± SD of three independent experiments performed in duplicate).

The calculated $IC_{50}$-values are shown in Table 1 together with the values obtained for aminoglutethimide (AG) and 4-hydroxyandrostenedione (4OHA).

*Lineweaver–Burk plots*

Non-inhibited aromatase activity was measured with either [1$\beta$-$^3$H]androstenedione or [1$\beta$,2$\beta$-$^3$H]testosterone as the substrate, varying in concentration from 12.5 to 300 nM. Inhibited aromatase activity was obtained in the presence of fixed concentrations of R 76 713. Figure 4 shows a typical Lineweaver–Burk plot with [1$\beta$-$^3$H]androstenedione as the substrate. From three independent experiments (performed in duplicate) a $K_m$-value of aromatase for androstenedione of 62 ± 19 nM (mean ± SD) was calculated. From a re-plot of the slopes of the Lineweaver–Burk plots obtained with different concentrations of R 76 713, a $K_i$-value for R 76 713 of 0.43 ± 0.06 nM (mean ± SD) was obtained. For [1$\beta$,2$\beta$-$^3$H]testosterone as the substrate, a representative Lineweaver–Burk plot is shown in Fig. 5. From three independent experiments performed in duplicate a $K_m$-value of aromatase for testosterone of 166 ± 27 nM was found. For R 76 713 a $K_i$-value of 0.47 ± 0.39 nM (mean ± SD) was calculated.

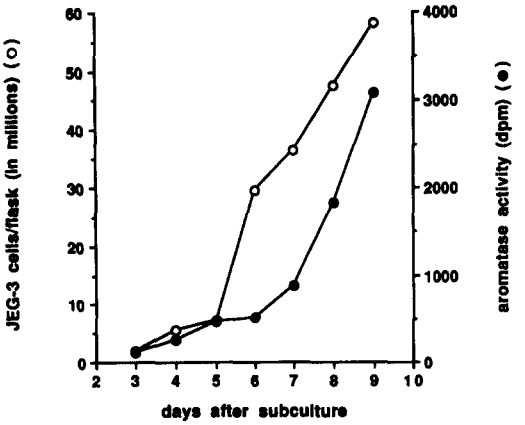


Fig. 2. Effect of culture confluency on aromatase activity in JEG-3 cells. On different days after subculturing cell density was determined and aromatase activity was measured in a cell suspension of 200,000 cells/ml using a mixture of 470 nM unlabelled androstenedione and 30 nM [1$\beta$,2$\beta$-$^3$H]-androstenedione. Results are expressed as means of duplicate determinations.

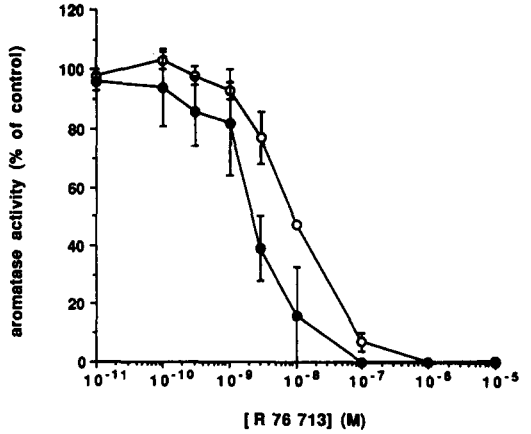


Fig. 3. Aromatase inhibition by R 76 713 in JEG-3 choriocarcinoma cells. Cell suspensions of 200,000 cells/ml were incubated for 1 h with either a mixture of 470 nM unlabelled androstenedione and 30 nM [1$\beta$,2$\beta$-$^3$H]androstenedione (○) or a mixture of 470 nM unlabelled testosterone and 30 nM [1$\beta$,2$\beta$-$^3$H]testosterone (●). Results are expressed as mean ± SD of three experiments performed in duplicate.

### *Aromatase in JEG-3 tumors*

JEG-3 tumors developed readily in the nude mice. In about 70% of the injected animals, the tumors were well established 15–30 days after inoculation.

Two large tumors were used to characterize aromatase activity. Lineweaver–Burk plots were obtained as described above with [1$\beta$-$^3$H]-androstenedione as the substrate (Fig. 6). The tumors showed $K_m$-values of aromatase for

Table 1. $IC_{50}$-values for aromatase inhibition by R 76 713 in JEG-3 choriocarcinoma cells and comparison to the values obtained for aminoglutethimide (AG) and 4-hydroxy-androstenedione (4OHA). The substrate consisted of a mixture of 470 nM unlabelled androstenedione and 30 nM [1$\beta$,2$\beta$-$^3$H]androstenedione or a mixture of 470 nM unlabelled testosterone and 30 nM [1$\beta$,2$\beta$-$^3$H]testosterone. Results are expressed as mean ± SD of three experiments performed in duplicate

| | $IC_{50}$-value (nM) | |
|---|---|---|
| | Androstenedione | Testosterone |
| R 76 713 | 7.6 ± 0.5 | 2.7 ± 1.1 |
| AG | 34,000 ± 24,000 | 30,000 ± 500 |
| 4OHA | 83 ± 15 | 130 ± 60 |

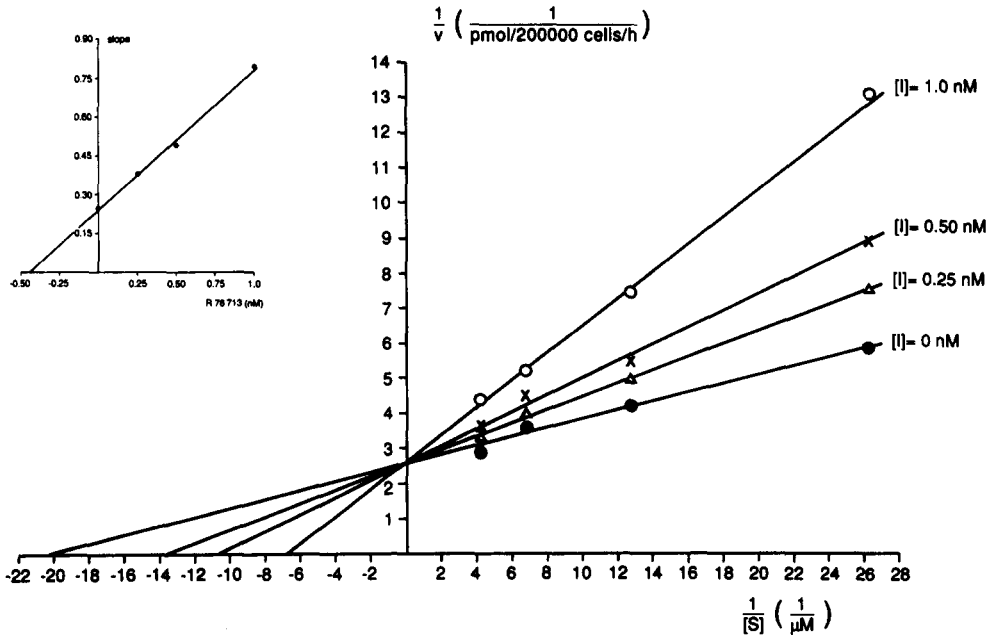


Fig. 4. Kinetic analysis of aromatase inhibition in JEG-3 choriocarcinoma cells. Lineweaver–Burk plot using [1$\beta$-$^3$H]androstenedione in the absence and presence of increasing concentrations of R 76 713. Insert: re-plot of the slopes ($r = 0.995$).

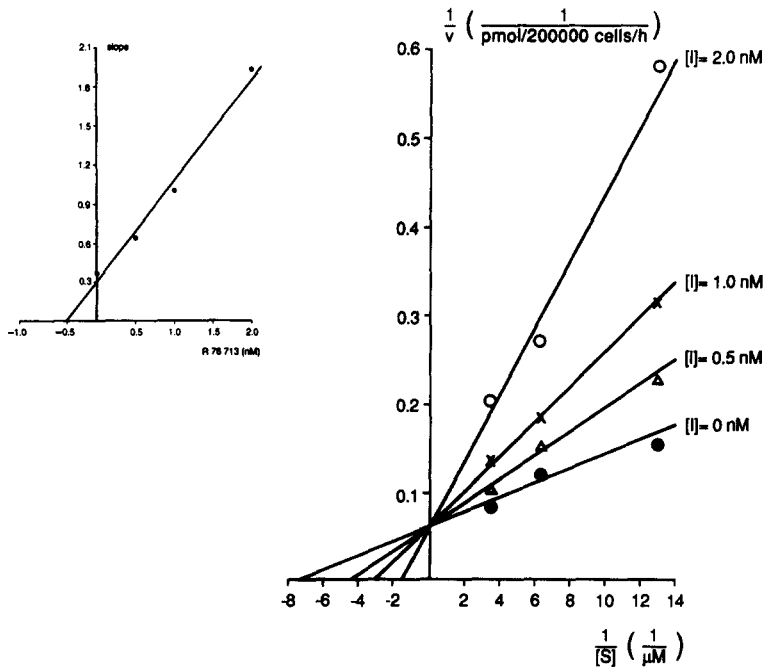


Fig. 5. Kinetic analysis of aromatase inhibition in JEG-3 choriocarcinoma cells. Lineweaver–Burk plot using [1$\beta$,2$\beta$-$^3$H]testosterone in the absence and presence of increasing concentrations of R 76 713. Insert: re-plot of the slopes ($r = 0.988$).

androstenedione of 54 nM and 63 nM and $IC_{50}$-values of 4.5 nM and 2.8 nM for R 76 713. The *ex-vivo* effects of R 76 713 on tumor aromatase were examined 2 h after a single oral dose. The results are shown in Fig. 7. Aromatase was significantly inhibited by 94 and 72.4% at drug doses of 5 and 0.25 mg/kg. At the lowest drug dose (0.005 mg/kg) aromatase activity was inhibited by 18.6% but this inhibition was not statistically significant compared to the control activity.

## DISCUSSION

JEG-3 cells are able to aromatize androgens into estrogens at such a rate that it is readily detectable using the tritiated water method. The amount of aromatase activity was not constant but increased with increasing culture confluency. This difference in aromatase activity was not just due to recovery after subculturing of the cells. Indeed, when subcultured cells were plated at different cell densities and aromatase activity

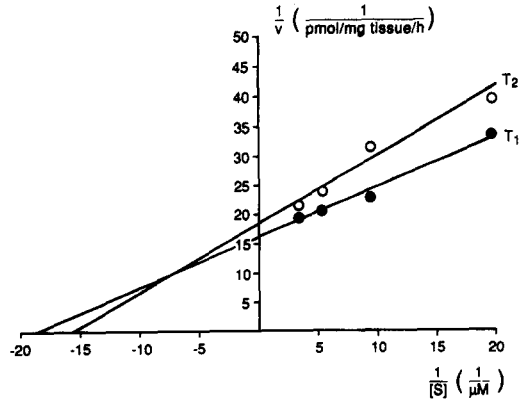


Fig. 6. Kinetic analysis of aromatase inhibition in two JEG-3 tumors (T1 and T2) grown in nude mice. Lineweaver–Burk plot using [1$\beta$-$^3$H]androstenedione.

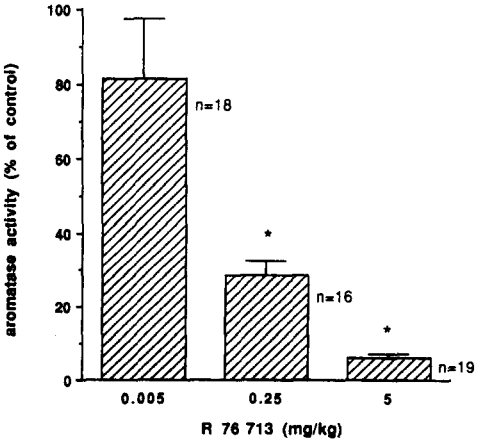


Fig. 7. Aromatase inhibition by R 76 713 measured *ex vivo* in JEG-3 tumors 2 h after a single oral drug administration. Results are expressed as mean SEM. Control aromatase activity was 77 ± 14 fmol/mg tissue/h (mean ± SD; $n = 15$). *: significantly different from the control value ($P < 0.05$).

was determined on the following day, the same cell density-dependent difference in aromatase activity was obtained (results not shown). Thus, as the rate of cell replication decreases, aromatase activity increases. This phenomenon, which was also reported for HCG production in JEG-3 cells [10], may reflect altered energy consumption with all energy needed for cell replication at non-confluency and redirected to more differentiated functions such as aromatase activity at confluency.

Aromatase inhibition curves obtained with increasing concentrations of R 76 713 in the presence of a fixed substrate concentration showed $IC_{50}$-values of 7.6 and 2.1 nM with androstenedione and testosterone respectively as the substrate. Under the same experimental conditions 4-hydroxyandrostenedione and aminoglutethimide showed values which were about 20 and 6000 times higher. The values found for R 76 713 correlate very well with the $IC_{50}$-values of 5.1 nM and 4.4 nM found in rat ovarian homogenates with androstenedione and testosterone respectively as the substrate [12] and with those previously found for rat and human granulosa cells as well as human adipose tissue stromal cells [2, 3, 13]. Aromatase activity was completely inhibited by $10^{-6}$ M R 76 713 and this inhibition by R 76 713 was completely reversible since JEG-3 cells incubated with R 76 713 for 1 h and subsequently thoroughly washed showed the same aromatase activity as cells treated with solvent (results not shown).

Aromatization of androstenedione or testosterone followed simple Michaelis–Menten kinetics. Lineweaver–Burk analysis of the data yielded linear plots and revealed $K_m$-values of 62 and 166 nM for androstenedione and testosterone respectively [12]. In the presence of increasing concentrations of R 76 713, the $K_m$-value ($K_{mapp}$) increased while the $V_{max}$ remained unchanged, indicating true competitive inhibition. This true competitive inhibition was confirmed by reduction of the aromatase activity to background level at the highest concentration of R 76 713 in the inhibition experiments. From the Lineweaver–Burk analysis $K_i$-values for R 76 713 of 0.43 and 0.47 nM with androstenedione and testosterone respectively as substrate were calculated. Again, these values correlate very well with the values found in rat ovarian homogenates (0.7 and 1.6 nM for androstenedione and testosterone) [12].

JEG-3 cells can be reproducibly grown as tumors in nude mice with about 70% of the inoculated mice developing tumors within 2–4 weeks. These tumors retain aromatase enzyme activity with kinetic properties, and sensitivity to R 76 713 similar to those found for the JEG-3 cells *in vitro*.

A single oral treatment with R 76 713, dose-dependently inhibited tumor aromatase activity measured *ex vivo*, 2 h after drug administration. A dose of 5 mg/kg of R 76 713 gave an almost complete inhibition while after a dose of 0.005 mg/kg aromatase activity was similar to that in control groups. An $ED_{50}$-value of 0.05 mg/kg was calculated for aromatase inhibition. This value is one order of magnitude higher than that previously found for lowering serum levels of oestradiol in PMSG-injected rats [2]. This difference may be due either to species differences in the pharmacokinetic properties of R 76 713 or to a different distribution of R 76 713 between different tissues (tumor vs ovary). Differences in substrate concentration (500 nM androstenedione in the *ex vivo* system versus endogenous androstenedione levels in PMSG-injected rats) may also explain this higher $ED_{50}$-value.

While peripheral aromatization in the adipose tissue is the main source of estrogen production in postmenopausal women, aromatase activity in tumors themselves could also play an important role in the maintenance and growth of tumor tissue but this issue is still in question [14]. The JEG-3 tumor grown in nude mice not only exhibits aromatase activity equivalent to that measured *in vitro* but tumor aromatase can be potently inhibited by R 76 713. The JEG-3 choriocarcinoma proved to be a good model to study inhibition of aromatase both *in vitro* and *ex vivo*.

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