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POTENCY AND SELECTIVITY OF THE AROMATASE INHIBITOR R 76 713. A STUDY IN HUMAN OVARIAN, ADIPOSE STROMAL, TESTICULAR AND ADRENAL CELLS

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Summary—The effects of R 76 713 on steroidogenesis were studied in primary cultures of four different human cell types, i.e. ovarian granulosa cells, adipose stromal cells, testicular cells and adrenal cells.

In human granulosa cells aromatization of [1β,2β-^{3}H]androstenedione (as measured by the release of tritiated water) showed a K_m (Michaelis constant) of 78 nM. R 76 713 competitively inhibited aromatization with a K_i (dissociation constant of the enzyme-inhibitor complex) of 1.6 nM.

In human adipose stromal cells aromatization was measured by following the conversion of androstenedione to estrone and 17β-estradiol. In this system a K_m for aromatization of androstenedione of 10.8 nM was found. R 76 713 again showed competitive kinetics with a K_i-value of 0.14 nM.

In human testicular cells the synthesis of the androgens testosterone, androstenedione and dehydroepiandrosterone was only inhibited by drug concentrations exceeding 10^{-6} M. At 10^{-5} M of R 76 713, steroid concentrations were lowered to 56, 64 and 81% of the control for testosterone, androstenedione and dehydroepiandrosterone respectively. Concomitantly, a slight increase in the levels of pregnenolone (138% of the control) and progesterone (133% of the control) was seen.

In human adrenal cells the synthesis of cortisol and aldosterone was slightly affected by R 76 713 also at concentrations exceeding 10^{-6} M. At 10^{-5} M of R 76 713 the concentrations of cortisol and aldosterone were lowered to respectively 59 and 51% of the control. At the same drug concentration the precursors 11-deoxycortisol and 11-deoxycorticosterone rose to 189 and 147% of the control.

These results show that in primary cultures of human cells, R 76 713 is a very potent aromatase inhibitor with a selectivity of at least 1000-fold compared to other steps in steroidogenesis.

INTRODUCTION

Several benign and malignant diseases in women are estrogen-dependent. Amongst these are breast cancer, endometriosis and uterine fibroids [1, 2]. Reduction of circulating estrogen levels might be of value in the treatment of these diseases. In the case of benign disease, this kind of treatment would have the advantage of being completely reversible.

Estrogens are synthesized from their androgen precursors by aromatase, a microsomal cytochrome *P*450-regulated enzyme system [3]. In premenopausal women this enzyme is mainly found in the ovarian granulosa cells whereas in postmenopausal women the adipose tissue is the main site of aromatase activity [4].

Aminoglutethimide was the first aromatase inhibitor to be used in the treatment of postmenopausal breast cancer. The clinical results, obtained with this drug demonstrated that aromatase inhibition is a valid alternative to the use of

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Abbreviations: Steroid-6-CMO:BSA—steroid-6-carboxymethyloxime, coupled to bovine serum albumin; DMSO—dimethylsulfoxide; hMG—human menopausal gonadotrophin; IC_{50}—drug concentration, producing 50% inhibition; IU—international unit; K_i—dissociation constant of the enzyme-inhibitor complex; V_{max}—maximal velocity of enzyme-catalyzed reaction.

anti-estrogens [5, 6]. However, aminoglutethimide is a rather weak aromatase inhibitor and the compound is not selective since it inhibits several other enzymes involved in adrenal steroidogenesis. Moreover, it is not free of other significant side-effects at therapeutic doses [7]. As a result of this, several new aromatase inhibitors (both steroidal and non-steroidal) have been developed over the past few years and at least two of these (4-hydroxyandrostenedione and CGS 16949A) are being evaluated clinically [8, 9].

Recently, we described a new non-steroidal aromatase inhibitor, R 76 713. This compound is a very potent inhibitor of aromatase in rat ovarian homogenates [10] and in rat granulosa cells *in vitro* as well as in the rat *in vivo* [11]. This new aromatase inhibitor was also shown to be highly selective towards aromatase inhibition, both *in vitro* in primary cultures of rat cells and in the rat *in vivo* [11, 12]. The first results obtained in male and in premenopausal female volunteers indicate that R 76 713 is capable of reducing the levels of plasma estradiol after single oral dosing [12].

In the present report we describe the *in vitro* potency and selectivity of R 76 713 using primary cultures of four different human steroid-producing cell types, i.e. ovarian granulosa, adipose stromal, testicular and adrenal cells.

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 Chemical Research Centre of the Janssen Research Foundation. The compound was dissolved in DMSO and diluted in 1% DMSO. Final solvent concentrations were always equal to or less than 0.1%.

Dulbecco's Modified Eagle Medium/Ham's F12 1:1 (DMEM/F12), Eagles' Minimum Essential Medium (MEM REGA3), Medium 199, foetal calf serum, Hanks' Balanced Salt Solution (Ca- and Mg-free), bovine serum albumin (fraction V), sodiumbicarbonate, Hepes, glutamine, penicillin/streptomycin sulphate and Fungizone were supplied by GIBCO (Gibco Europe, Ghent, Belgium). Collagenase (Worthington, 127 IU/mg) was obtained from Cooper Biomedical (Brussels, Belgium). Ficoll-Paque, dextran T-70 and OptiPhase HiSafe II scintillator were from Pharmacia-LKB (Brussels, Belgium). Glucose was supplied by Janssen Chimica (Beerse, Belgium). Norit A was from Serva (Heidelberg, W. Germany). Ascorbic acid and calcium-chloride were obtained from Merck (Darmstadt, W. Germany). Androstenedione and dexamethasone were purchased from Sigma (St Louis, U.S.A.). $ACTH_{1-24}$ (Cortrosyn) was from Organon (Brussels, Belgium).

[1,2-^{3}H]aldosterone (50 Ci/mmol), [1β,2β-^{3}H]androstenedione (43 Ci/mmol), [1,2-^{3}H]dehydroepiandrosterone (51.5 Ci/mmol), [1,2-^{3}H]11-deoxycortisol (53.5 Ci/mmol), [6,7-^{3}H]17β-estradiol (51 Ci/mmol), [6,7-^{3}H]estrone (48.8 Ci/mmol), [1,2-^{3}H]17α-hydroxyprogesterone (42.3 Ci/mmol), [1,2-^{3}H]progesterone (55.2 Ci/mmol) and [1β,2β-^{3}H]testosterone (42.5 Ci/mmol) were obtained from NEN (Brussels, Belgium). [1,2-^{3}H]cortisol (50 Ci/mmol), [1α2α-^{3}H]11-deoxycorticosterone (36 Ci/mmol) and [7-^{3}H]pregnenolone (21 Ci/mmol) were from Amersham (Brussels, Belgium).

Aromatase activity in human granulosa cells

Human granulosa cells were obtained from women, undergoing an *in vitro* fertilization programme. Follicular development was induced by treatment with hMG and hCG. Follicular fluid was aspirated by ovarian follicle puncture using vaginal ultrasound under local anaesthesia. The number of follicles aspirated varied from 3 to 19 per patient. After screening for oocytes the fluid was immediately transported to the laboratory at room temperature.

Granulosa cells were obtained as previously described [13] with the following modifications. Upon arrival the follicular fluid was diluted 1:4 with culture medium (Dulbecco's Modified Eagle Medium/Ham's F12 1:1 supplemented with 1 g/l sodiumbicarbonate, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin sulphate and 2.5 μg/ml Fungizone) and centrifuged for 5 min at 200 ***g***. The cell pellet was resuspended in 20 ml of culture medium and treated with 0.1% collagenase for 30 min at 37°C. After this incubation, 6–7 ml of the suspension were layered on top of 3 ml of sterile Ficoll-Paque and centrifuged for 5 min at 600 ***g***. The granulosa cells, concentrated at the medium-Ficoll interface were collected and washed with culture medium by resuspension and centrifugation for 5 min at 200 ***g***. The cell pellet was resuspended in culture medium and the cells were counted in a hemocytometer. Cell viability as determined by the Trypan Blue exclusion method was usually between 70 and 80%.

500 μl aliquots of the cell suspension containing 40,000 viable cells were incubated for the indicated periods of time in 24-well plates at 37°C in a 5% CO_2-atmosphere with [1β,2β-^{3}H]androstenedione or a mixture of labeled and unlabeled androstenedione as aromatase substrate and different concentrations of R 76 713 or its solvent. At the end of incubation, the medium was transferred to glass test tubes and extracted with 8 ml of chloroform by brief agitation. The layers were separated by centrifugation for 10 min at 688 ***g***. A 300 μl aliquot of the aqueous layer was treated with 300 μl of a charcoal suspension (0.25 g dextran and 2.5 g norit in 100 ml water) and centrifuged for 10 min at 1360 ***g***. 500 μl of the final supernatant was counted for radioactivity with 8 ml of scintillator in a Packard Tri-Carb 1500 liquid scintillation analyzer. Assay blanks (in which the cells were omitted) were always run in parallel and their values were subtracted from the results to correct for

formation of ^3H_2O due to non-specific loss of tritium at the C2-position of [1β,2β-^{3}H]androstenedione.

Synthesis of estrone and 17β-estradiol in human adipose stromal cells

Subcutaneous abdominal adipose tissue was obtained from women undergoing laparatomy for diverse gynecological reasons. The tissue was immediately immersed in sterile normal saline and transported to the laboratory at room temperature.

Cultures of adipose stromal cells were established as previously described [14] with several modifications. The tissue was weighed and minced with scissors. It was then suspended (1 g of tissue per 20 ml of solution) in Hanks' balanced salt solution containing 40 mg/ml bovine serum albumin, 2 mg/ml glucose and 1 mg/ml collagenase. The suspension was gently shaken for 60 min at 37°C. The digested tissue was then filtered through 200 μm nylon gauze. The filtrate was centrifuged at 400 *g* for 5 min. The floating layer of fat was removed and the pellet kept aside. The remaining fluid was again centrifuged. The second pellet was combined with the first and washed twice by resuspension in culture medium and recentrifugation. The final cell pellet was suspended in culture medium consisting of Eagles' minimum essential medium supplemented with 2.2 g/l sodiumbicarbonate, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin sulphate, 2.5 μg/ml Fungizone and 10% foetal calf serum. Cells were grown to confluency in 75 cm^2 plastic tissue culture flasks in a humidified 5% CO_2-atmosphere with a medium change every two days. At confluency, cells were trypsinized and subcultured at a split ratio of 1:5. Only the first five passages of each cell line were used for the experiments.

Confluent monolayers were trypsinized and the cells were plated in 6 well tissue culture plates at a density of 30,000 cells/well. The cells were allowed to reach confluency. Then dexamethasone was added (250 nM final concentration) to stimulate the aromatase activity [15] and the cells were further incubated for 24 h. At the end of this incubation the medium was changed to serum-free medium containing 250 nM dexamethasone, the indicated concentration of androstenedione as the substrate and different concentrations of R 76 713 or its solvent. The cells were then incubated for the indicated periods of time, after which the culture supernatants were assayed for estrone and 17β-estradiol.

Estrone and 17β-estradiol were determined by direct radiommunoassay using [^{3}H]steroids and dextran-coated charcoal separation of bound and free radioligand. The antisera used were raised in male New Zealand white rabbits against steroid-6-CMO:BSA conjugates. The estrone antibody has a cross-reactivity of 1% with 17β-estradiol and less than 0.01% with estriol, androstenedione, testosterone, progesterone and dexamethasone. The cross-reactivity of the 17β-estradiol antibody is 2% with estrone, 0.41% with estriol and less than 0.01% with androstenedione, testosterone, progesterone and dexamethasone. The sensitivity of the standard curve was 10 pmol/l and 20 pmol/l for estrone and 17β-estradiol respectively. Interassay coefficients of variation were 7.8% for estrone and 8.2% for 17β-estradiol. Intra-assay coefficients of variation were 5.5% and 5.9% for estrone and 17β-estradiol respectively.

Steroid synthesis in human testicular cells

Human testis tissue was obtained from patients undergoing orchiectomy as primary therapy for metastatic prostate carcinoma. The patients were anaesthetized with pentothal. None of them had been taking any specific medication.

The tissue was immediately immersed in culture medium (Medium 199 containing 2 mg/ml bovine serum albumin, 0.6 g sodiumbicarbonate, 100 IU/ml penicillin and 100 μg/ml streptomycin sulphate) and transported to the laboratory at room temperature. Testicular cell suspensions were obtained and incubated as previously described [16]. Testosterone, androstenedione, progesterone, 17α-hydroxyprogesterone, pregnenolone and dehydroepiandrosterone were determined directly on the culture supernatants by radioimmunoassay using [^{3}H]steroids and dextran-coated charcoal separation of bound and free radioligand. Characterization and specificity of the antisera have been published elsewhere: testosterone [17], androstenedione [18], progesterone [19], 17α-hydroxyprogesterone [20]. Pregnenolone and dehydroepiandrosterone were measured with commercially available antisera (Radioassay Systems Laboratories, Carson, U.S.A.) as previously described [16]. The cross-reactivity of the pregnenolone antiserum is 50% with pregnenolone-sulphate, 2% with 17α-hydroxypregnenolone, 1.5% with progesterone and less than 0.1% with all other testicular steroids. The dehydroepiandrosterone antiserum has a cross-reactivity of 1.18% with dehydroepiandrosterone-sulphate, 0.32% with androstenedione and less than 0.1% with the other steroids.

Steroid synthesis in human adrenal cells

Human adrenal tissue was obtained during organ-prelevation, immediately immersed in sterile normal saline and transported to the laboratory at room temperature. After removal of the capsule, the cortex was separated from the medulla, minced with scissors and gently shaken for 30 min at 37°C in culture medium (Eagles' minimum esssential medium supplemented with 20 mM Hepes, 2 mM glutamine, 5 mg/ml bovine serum albumin, 8 mM calcium-chloride and 2 mM ascorbic acid) containing 200 IU/ml of collagenase. The digested tissue was filtered through 200 μm nylon gauze; the filtrate was kept apart and the digestion procedure was repeated with the remaining tissue. After the second digestion, the combined filtrates were centrifuged for 10 min at

100 *g*. The sedimented cells were washed twice by resuspension in culture medium and centrifugation. The final cell pellet was resuspended in culture medium and the cells were counted in a hemocytometer. Cell viability as determined by the Trypan Blue exclusion method was usually around 70%

500 μl aliquots of the cell suspension containing 75,000 viable cells were incubated for 15 h in 24-well plates at 37°C in a 5% CO_2-atmosphere with 100 ng/ml $ACTH_{1\text{-}24}$ and different concentrations of R 76 713 or its solvent. At the end of incubation, the tissue culture plates were centrifuged for 10 min at 1610 *g*, the supernatants were removed and stored at −20°C.

Several steroids were determined directly on the culture supernatants by radioimmunoassay using [^{3}H]steroids and dextran-coated charcoal separation of bound and free radioligand. Androstenedione, dehydroepiandrosterone, progesterone and 17α-hydroxyprogesterone were measured as described above. Characterization and specificity of the antisera to 11-deoxycortisol and 11-deoxycorticosterone have been published elsewhere [21]. Cortisol and aldosterone were measured with commercially available antisera (Radioassay Systems Laboratories, Carson, U.S.A.) as previously described [22]. The main cross-reactivities of the cortisol antiserum are 11.4% with 21-deoxycorticosterone, 8.9% with 11-deoxycortisol, 1.6% with corticosterone, 0.1% with 11-deoxycorticosterone and less than 0.1% with all other adrenal steroids. The aldosterone antiserum has a cross-reactivity of less than 0.1% with all adrenal steroids.

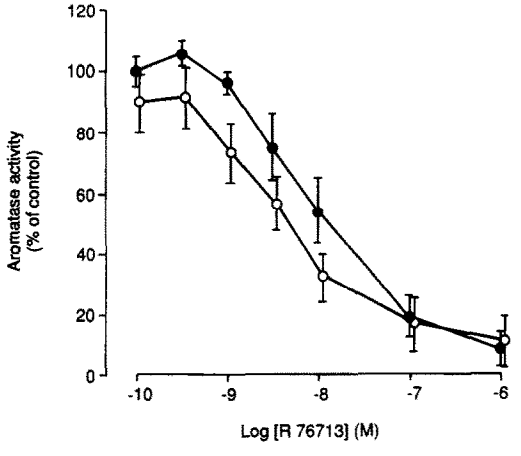

Fig. 1. Inhibition by R 76 713 of aromatization of adrostenedione in human granulosa cells (●) and human adipose stromal cells (○). In the granulosa cells aromatase activity was measured as the amount of ^3H_2O released from [1β,2β-^{3}H]androstenedione. In the adipose stromal cells aromatase activity was calculated as the sum of estrone and estradiol synthesized from androstenedione. Results are expressed as the mean ± SD of 4 (granulosa) or 5 (stromal cells) independent experiments, each performed in triplicate. Mean control values were 35 pmol/10^5 cells/24 h for granulosa cells and 70 pmol/mg protein/24 h for adipose stromal cells.

Protein determination

Protein was measured using the Bio-Rad protein assay with bovine serum albumin as the standard.

Data calculation and analysis

Lineweaver–Burk plots and slope replots were calculated by linear regression analysis. Where indicated, data were statistically analyzed using the Student's *t*-test. Significance was defined at the level of $P < 0.05$.

RESULTS

The potency of R 76 713 as an aromatase inhibitor in human granulosa cells and human adipose stromal cells is shown in Fig. 1. In the human granulosa cells aromatization was followed by measuring the formation of tritiated water during a 24 h incubation period in the presence of increasing concentrations of R 76 713. The substrate consisted of 30 nM [1β,2β-^{3}H]androstenedione plus 470 nM unlabeled androstenedione. An IC_{50}-value of 10.0 ± 5.8 nM (mean ± SD, 4 independent experiments each performed in triplicate) was calculated. In the human adipose stromal cells, aromatase activity was calculated as the sum of estrone and 17β-estradiol (as measured by radioimmunoassay) formed from androstenedione (250 nM final concentration) during a 24 h incubation period. Basal aromatization of androstenedione by the adipose stromal cells was low (< 5 pmol estrogen/mg protein/24 h). After stimulation with dexamethasone the synthesis of estrone and 17β-estradiol increased reaching values for total estrogen production of up to 70 pmol/mg protein/24 h. The stimulation was maximal in the early passages of the cells and decreased in later passages. In the presence of increasing concentrations of R 76 713, a concentration-dependent inhibition curve was obtained. From this curve an IC_{50}-value of 3.7 ± 1.2 nM was calculated (mean ± SD, 5 independent experiments each performed in triplicate).

To further characterize the inhibition by R 76 713, both cellular systems were analyzed kinetically. In the human granulosa cells, aromatization, using 50 nM [1β,2β-^{3}H]androstenedione as the substrate, was linear for more than 3 h (using 40,000 cells per well) and for cell densities of more than 100,000 cells per well (using a 2 h incubation time) (results not shown). Therefore, incubation of 40,000 cells per well for 2 h was adopted as the optimal reaction condition, resulting in less than 10% conversion of substrate to product. Using these conditions, the human granulosa cells were incubated with [1β,2β-^{3}H] androstenedione varying in concentration from 50 to 400 nM in the absence or presence of 1, 4 and 8 nM of R 76 713. Figure 2 shows the Lineweaver–Burk analysis of one representative experiment, with replot of the slopes. From three independent experiments (each performed in triplicate) a K_m for aromatization

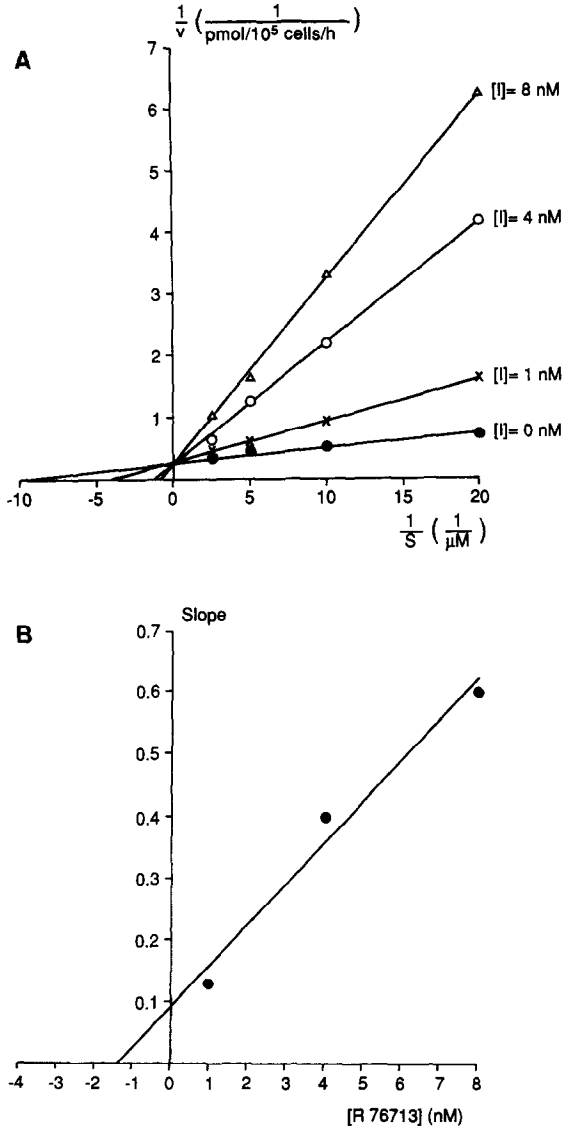

Fig. 2. Lineweaver–Burk analysis (A) with replot of the slopes (B) of aromatase inhibition by R 76 713 in human granulosa cells. Aromatase activity was measured as the amount of ^3H_2O formed during incubation of the cells with increasing concentrations of [1β,2β-^{3}H]androstenedione in the absence or presence of 1, 4 and 8 nM of R 76 713. The data are taken from one representative experiment, each point being the mean of triplicate determinations.

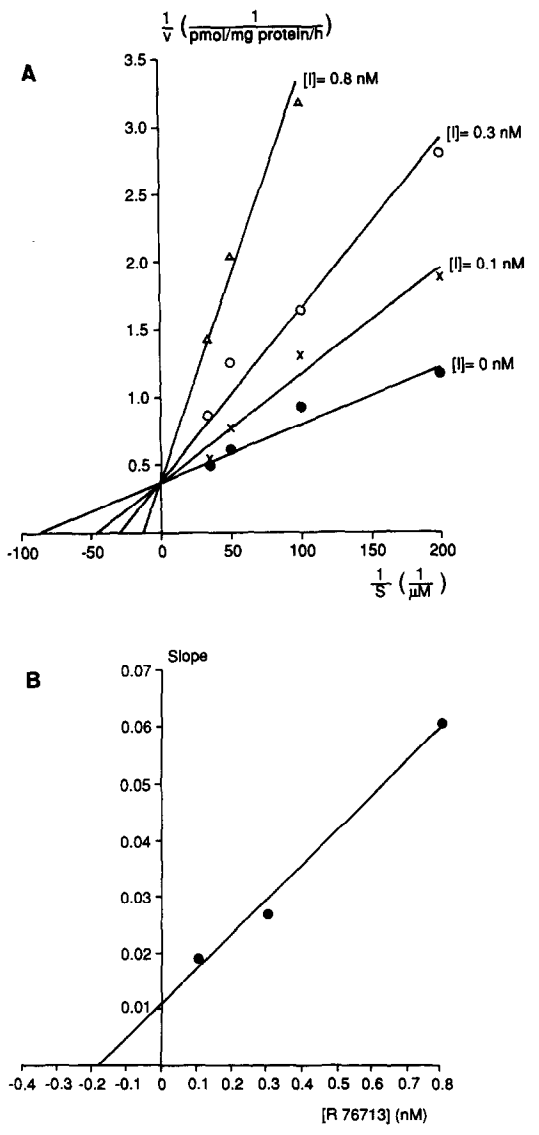

Fig. 3. Lineweaver–Burk analysis (A) with replot of the slopes (B) of aromatase inhibition by R 76 713 in human adipose stromal cells. Aromatase activity was measured as the sum of estrone and 17β-estradiol synthesized from increasing concentrations of androstenedione in the absence or presence of 0.1, 0.3 and 0.8 nM of R 76 713. The data are taken from one representative experiment, each point being the mean of triplicate determinations.

of androstenedione of 78 ± 24 nM (mean $\pm$ SD) was calculated. Using the known ^{3}H-distribution of [1β,2β-^{3}H]androstenedione (48% in 1β as determined by ^{3}H-NMR analysis) a V_{max} of 4.7 ± 4.2 pmol/100,000 cells/h (mean $\pm$ SD) could be derived. In the presence of increasing concentrations of R 76 713 the K_m increased while the V_{max} remained unchanged. From the replot of the slopes a K_i of 1.6 ± 0.6 nM (mean $\pm$ SD) was determined.

In the human adipose stromal cells aromatization of androstenedione (5 nM final concentration) was linear for more than 4 h using initial culture densities of up to 30,000 cells/well (results not shown). An incubation period of 4 h was adopted as the standard procedure. Under these conditions less than 10% of the substrate was converted to product. Using this procedure, the adipose stromal cells were incubated with androstenedione varying in concentration from 5 to 30 nM in the absence or presence of 0.1, 0.3 or 0.8 nM of R 76 713. Figure 3 shows the Lineweaver–Burk analysis of one representative experiment, with replot of the slopes. From 3 independent experiments (each performed in triplicate) a K_m-value for aromatization of androstenedione of 10.8 ± 4.1 nM was calculated; the V_{max} was 4.2 ± 1.8 pmol/mg protein/h (mean $\pm$ SD). In the

presence of increasing concentrations of R 76 713 an increase in the K_m was seen with the V_{max} remaining constant. A replot of the slopes of the reciprocal plots revealed a K_i for R 76 713 of 0.14 ± 0.03 nM (mean $\pm$ SD).

In order to assess the specificity of R 76 713 as an aromatase inhibitor, the effects of the compound on steroidogenesis in human testicular and adrenal cells were studied. The results obtained by incubating human testicular cells with increasing concentrations of R 76 713 are shown in Fig. 4. The synthesis of the Δ^4 and Δ^5 androgens testosterone, androstenedione and dehydroepiandrosterone was unaffected by drug concentrations up to 10^{-6} M. Above this drug concentration the androgen concentrations were significantly lower than in the control cultures. At 10^{-5} M of R 76 713 an inhibition of 44, 36 and 19% was seen for testosterone, androstenedione and dehydroepiandrosterone respectively. There were further

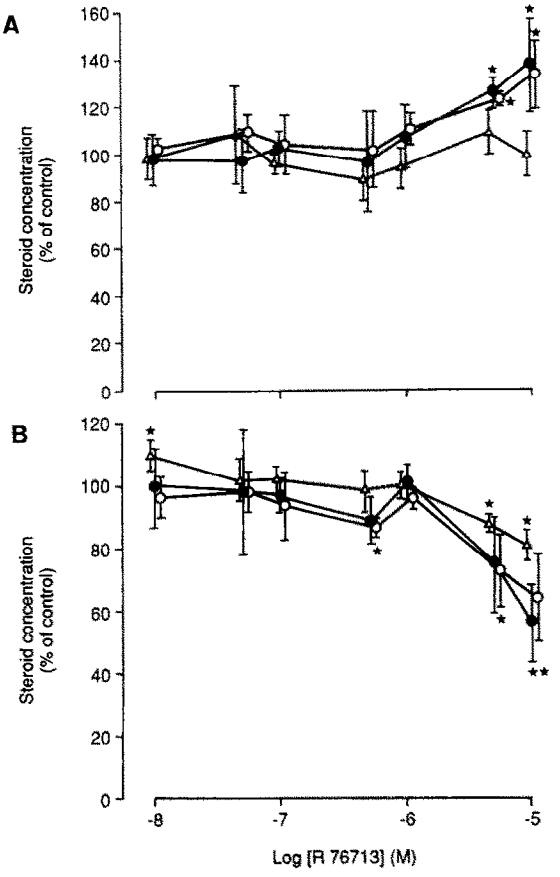

Fig. 4. Effects of R 76 713 on steroid biosynthesis in human testicular cells. Panel A: (●) pregnenolone, (○) progesterone, (△) 17α-hydroxyprogesterone. Panel B: (●) testosterone, (○) androstenedione, (△) dehydroepiandrosterone. Results are represented as the mean ± SD of three independent experiments, each performed in duplicate. *Significantly different from the control at $P < 0.05$. Control values (in pmol/10^6 cells/15 h) varied among the different experiments as follows: pregnenolone: 34–78; progesterone: 5–13; 17α-hydroxyprogesterone: 4–24; testosterone: 70–82; androstenedione: 6–16; dehydroepiandrosterone: 16–96.

significant changes in the concentration of androstenedione at 5×10^{-7} M of R 76 713 and in the concentration of dehydroepiandrosterone at 10^{-8} M of R 76 713 but no biological significance could be attributed to these changes. Together with the inhibition of androgen biosynthesis an increase in the levels of the precursors was seen. At 10^{-5} M of R 76 713 this increase reached values of 138 and 133% of the control for pregnenolone and progesterone respectively. The synthesis of 17α-hydroxyprogesterone was not affected by any of the drug concentrations tested.

The effects of increasing concentrations of R 76 713 on steroid biosynthesis in human adrenal cells are shown in Fig. 5. The synthesis of androstenedione tended to decrease in the presence of R 76 713 at concentrations of 10^{-6} M and higher. At 10^{-5} M of R 76 713 an inhibition of 33% was seen. The synthesis of the other adrenal androgen, dehydroepiandrosterone remained unaltered at all drug concentrations tested. There were no significant changes in the levels of the precursors progesterone and 17α-hydroxyprogesterone. The levels of cortisol and aldosterone also decreased in the presence of R 76 713 at concentrations of 10^{-6} M and higher. At 10^{-5} M of R 76 713 the synthesis of cortisol and aldosterone was inhibited by 41 and 49% respectively. Concomitantly there was an increase in the levels of the precursors 11-deoxycortisol and 11-deoxycorticosterone reaching 189 and 147% of the control respectively.

DISCUSSION

The aim of the present report was to describe the potency and specificity of the new non-steroidal aromatase inhibitor R 76 713 using primary cultures of four different human cell types.

The potency of R 76 713 was assessed in two estrogen producing cell types. Human granulosa cells were chosen because they reflect the highly active ovarian estrogen synthesis in premenopausal women whereas human adipose stromal cells are indicative for postmenopausal peripheral aromatization (which is mainly located in the adipose tissue). R 76 713 very potently inhibited aromatization of androstenedione in both celluar systems with IC_{50}-values of 10 nM and 3.5 nM for the granulosa cells and the adipose stromal cells respectively. Previously, aromatase inhibition by R 76 713 has been characterized by IC_{50}-values of 3 and 5 nM in FSH-stimulated rat granulosa cells [11] and rat ovarian homogenates [10] respectively.

Drug inhibition curves at one fixed substrate concentration however give only a limited picture of enzyme inhibition. In order to better characterize aromatase inhibition by R 76 713 a kinetic analysis in both human cellular systems was performed.

Only very few data are available in the literature about the kinetics of aromatization in human granulosa cells. In our human granulosa cell system, aromatization of androstenedione was characterized by

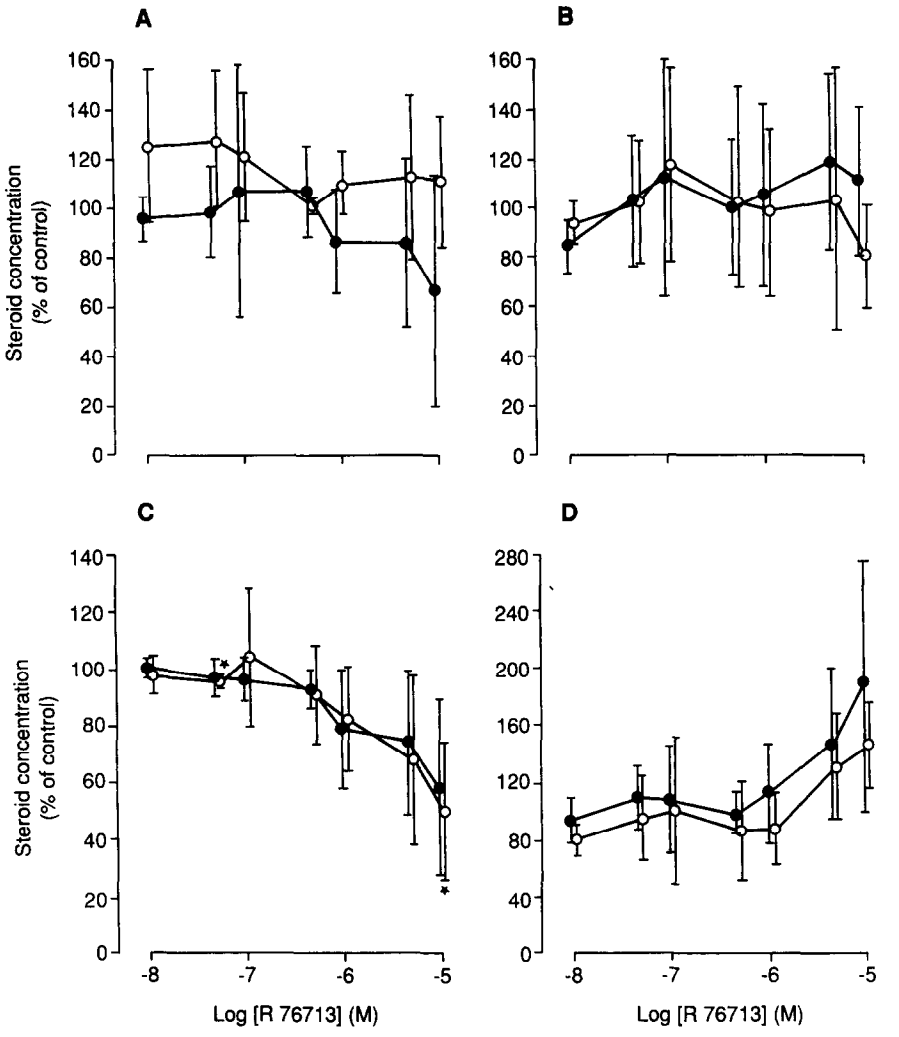

Fig. 5. Effects of R 76 713 on steroid biosynthesis in human adrenal cells. Panel A: (●) androstenedione, (○) dehydroepiandrosterone. Panel B: (●) progesterone, (○) 17α-hydroxyprogesterone. Panel C: (●) cortisol, (○) aldosterone. Panel D: (●) 11-deoxycortisol, (○) 11-deoxycorticosterone. Results are represented as the mean ± SD of three independent experiments, each performed in duplicate. *Significantly different from the control at $P < 0.05$. Control values (in pmol/10^6 cells/15 h) varied among the different experiments as follows: androstenedione: 84–126; dehydroepiandrosterone: 5–9; progesterone: 206–264; 17α-hydroxyprogesterone: 72–102; cortisol: 1760–3320; aldosterone: 88–96; 11-deoxycortisol: 1000–1080; 11-deoxycorticosterone: 826–1150.

a K_m-value of 78 nM. This is consistent with a value of approximately 50 nM which can be estimated from a study by Hillier *et al.*[23]. The K_m-value is slightly higher than the 25 nM, previously found for rat granulosa cell aromatase [24]. For aromatase inhibition by R 76 713, a K_i-value of 1.6 nM was found. This is in the same order of magnitude though slightly higher than the K_i-value of 0.7 nM, found in rat ovarian homogenates [10].

For aromatization of androstenedione in human adipose stromal cells a K_m-value of 10.8 nM was found. This value agrees very well with those reported by Ackerman *et al.* for human subcutaneous adipose stromal cells [14] but it is clearly lower than the value, found for the human granulosa cells. Aromatase inhibition by R 76 713 in the adipose stromal cells was characterized by a K_i of 0.14 nM. Again this value is lower than the K_i for R 76 713, found in the granulosa cells. Therefore, there seems to be a difference in kinetic parameters of aromatase in human granulosa cells as compared to human adipose stromal cells. While the exact reason for this is not clear, we have to keep in mind that neither of the cellular systems are used under basal conditions since the stromal cells are stimulated with dexamethasone *in vitro* and granulosa cells are stimulated *in vivo* during pretreatment for *in vitro* fertilization.

Aromatase inhibition by R 76 713 in both human granulosa and human adipose stromal cells showed competitive kinetics, confirming data previously found in rat ovarian homogenates. This type of kinetics, the lower K_i-value found in the adipose stromal cells and the fact that there is much less aromatase substrate available after menopause might

indicate that much lower doses of R 76 713 will be needed for treatment of postmenopausal women as compared to premenopausal women.

Competitive inhibition by non-steroidal aromatase inhibitors such as R 76 713 usually involves coordination of a nitrogen free electron pair with the haem-iron of the cytochrome *P*450, thereby effectively blocking the enzyme catalytic site [25, 26]. Since aromatase is only one of a series of cytochrome *P*450-regulated enzymatic reactions in steroid biosynthesis, the specificity of a new inhibitor towards this one enzyme is of the utmost importance.

The inhibition by R 76 713 of other steps in steroidogenesis was examined in cultures of human testicular and human adrenal cells. In the testicular cells the compound showed no effects up to concentrations of 10^{-6} M. Above this drug concentration, decreases in the androgen levels were noticed but even at 10^{-5} M of R 76 713, 50% inhibition was not reached. Concomitantly there was a slight increase in the levels of the progestin precursors. This indicates an effect of high concentrations of R 76 713 on the 17-hydroxylase/17,20-lyase enzyme complex, responsible for androgen synthesis. In the human adrenal cells a similar inhibition of androstenedione synthesis was seen while synthesis of dehydroepiandrosterone was unaffected even by the highest drug concentrations. In these cells there was also no significant rise of the progestin precursors. The synthesis of the corticoids cortisol and aldosterone was slightly lowered by high drug concentration. Again, 50% inhibition was not reached at 10^{-5} M of R 76 713. There was a concomitant rise in the levels of the precursors 11-deoxycortisol and 11-deoxycorticosterone. This suggests an inhibition by high concentrations of R 76 713 of the 11-hydroxylase enzyme.

Overall there was a difference of at least a 1000-fold between the concentration of R 76 713 needed for aromatase inhibition and the concentration inhibiting any other step in steroidogenesis. This confirms the specificity data previously obtained in rat cellular systems and in the rat *in vivo* [11, 12].

In conclusion, the present study shows that R 76 713 is a very potent inhibitor of both human ovarian and adipose tissue aromatase. Moreover, R 76 713 has an endocrinological profile which is highly selective towards aromatase inhibition. The compound may therefore prove to be of clinical importance for the treatment of estrogen-dependent diseases both in pre- and in postmenopausal women.

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