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COMPARATIVE EFFECTS OF THE AROMATASE INHIBITOR R76713 AND OF ITS ENANTIOMERS R83839 AND R83842 ON STEROID BIOSYNTHESIS *IN VITRO* AND *IN VIVO*

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Summary—R76713 (6-[(4-chlorophenyl)(1H-1,2,4-triazol-1-yl)methyl]-1-methyl-1H-benzotriazole) is a selective, non-steroidal aromatase inhibitor containing an asymmetric carbon atom. In this paper, we compare the effects of R76713 (racemate) with its enantiomers R83839 (the levo-isomer) and R83842 (the dextro-isomer) on steroid biosynthesis in rat cells *in vitro* and in the rat *in vivo*.

In rat granulosa cells, aromatase activity was inhibited by 50% at concentrations of 0.93 nM of R76713, 240 nM of R83839 and 0.44 nM of R83842, revealing a 545-fold difference in activity between both enantiomers.

Up to 1 μM, none of the compounds had any effect on steroid production in primary cultures of rat testicular cells. Above this concentration all three compounds showed a similar slight inhibition of androgen synthesis with a concomitant increase in the precursor progestins, indicative for some effect on the 17-hydroxylase/17,20-lyase enzyme. In rat adrenal cells none of the compounds showed any effect on corticosterone synthesis. At concentrations above 1 μM there was an increase in the levels of 11-deoxycorticosterone pointing towards an inhibition of the 11-hydroxylase enzyme. This increase was more pronounced for R83839 than for R76713 and R83842.

In vivo, in PMSG-primed rats, R83842 reduced plasma estradiol by 50%, 2 h after oral administration of 0.0034 mg/kg, whereas 0.011 mg/kg of R76713 and 0.25 mg/kg of R83839 were needed to obtain the same result.

Oral administration of up to 20 mg/kg of the compounds did not significantly affect plasma levels of adrenal steroids in LHRH/ACTH-injected rats. Plasma testosterone was lowered at 10 and 20 mg/kg of R83842 and at the highest dose (20 mg/kg) of R76713 and R83839.

In conclusion, the present study shows that the aromatase inhibitory activity of R76713 resides almost exclusively in its dextro-isomer R83842. R83842 exhibits a specificity for aromatase as compared to other enzymes involved in steroid biosynthesis of at least a 1000-fold *in vitro* as well as *in vivo*. This confirms the extreme selectivity previously found for the racemate.

INTRODUCTION

Several benign and malignant diseases in man and woman are estrogen-dependent. The obvious therapy for these diseases is to take away the hormonal driving force. This can pharmacologically be achieved by either inhibiting the hormonal synthesis or by blocking the hormone receptors [1, 2].

Estrogens are synthesized from their androgen precursors by the cytochrome *P*450-dependent aromatase enzyme complex [3]. Recently we reported on R76713, a new highly active, non-steroidal aromatase inhibitor. This compound is a very potent inhibitor of aromatase in rat ovarian homogenates, in human placental microsomes, in rat and human granulosa cells and in human adipose stromal cells *in vitro* [4–7] as well as in the rat and the monkey *in vivo* [6, 8]. This new aromatase inhibitor is also highly selective towards aromatase inhibition

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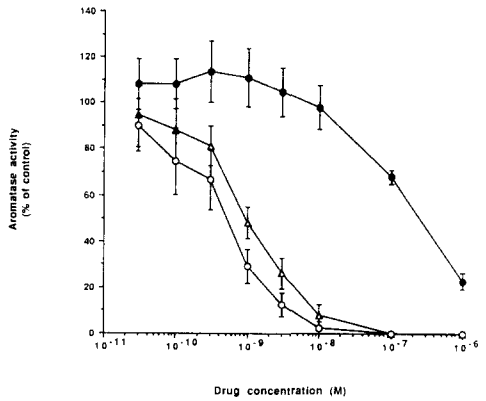

Fig. 1. Inhibition by R76713 (△), R83839 (●) and R83842 (○) of aromatase activity in granulosa cells, isolated from PMSG-primed rats. Each point represents the mean ± SD of five independent experiments, each performed in triplicate. Control aromatase activity was 22.7 ± 16.7 pmol/10^6 cells/24 h.

both *in vitro* in primary cultures of rat and human cells and in the rat *in vivo* [6, 7, 9]. R76713 shows antitumoral activity in DMBA- and NMU-induced mammary tumors in the rat and induces regression of the volume of endometrial autotransplants in a rat experimental endometriosis model [8, 10].

R76713 is a triazole derivative, containing an asymmetric carbon atom. In the present report, we compare the effects of R76713 (the racemate) with those of its enantiomers R83839 (the levo-isomer) and R83842 (the dextroisomer) on steroid biosynthesis in rat cells *in vitro* and in the rat *in vivo*.

EXPERIMENTAL

Test compounds

R76713 (racemate), R83839 (levo-isomer) and R83842 (dextro-isomer) were synthesized in the Department of Organic Synthesis of the Janssen Research Foundation. For *in vitro* tests the compounds were dissolved in dimethylsulfoxide (DMSO) at an initial concentration of 10^{-2} M and further dilutions were made in 1% (v/v) DMSO in water. Final solvent concentrations during incubation were equal to or less than 0.1% (v/v). For *in vivo* experiments, the drugs were dissolved in 100% PEG 200.

In vitro experiments

Rat granulosa cells were isolated and cultured and aromatase activity was measured as described before [11] but the extraction was performed with dichloromethane instead of chloroform. The effects of the test compounds in primary cultures of rat testicular and rat adrenal cells were evaluated as described in [6].

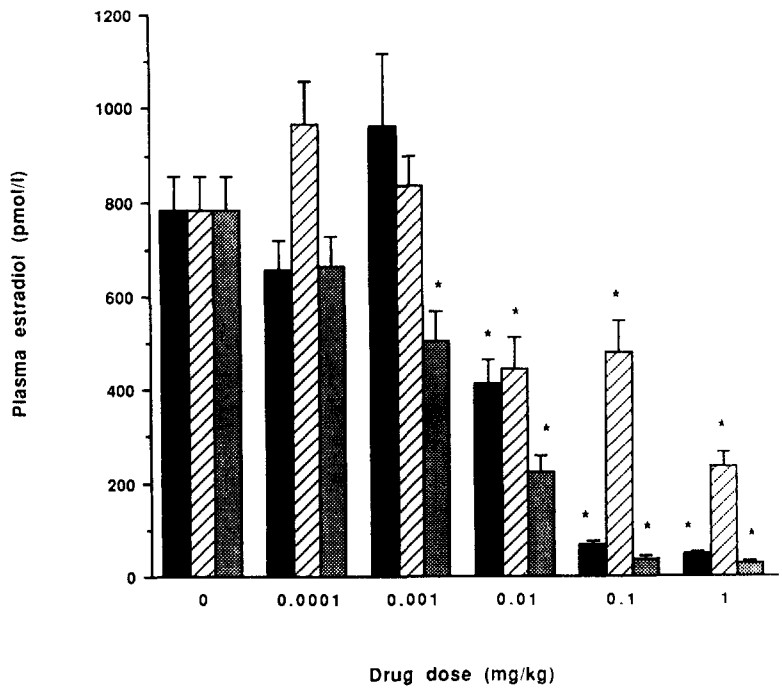

Fig. 2. Effects of R76713 (filled bars), R83839 (hatched bars) and R83842 (speckled bars) on plasma estradiol levels in PMSG-injected rats, 2 h after a single oral drug administration. Results are represented as mean ± SEM (n = 12 in each group). *Statistically different from the control ($P < 0.05$).

In vivo experiments

The effects on estradiol synthesis in PMSG-injected rats were assessed as described in [6]. Plasma estradiol was measured using a direct fluorescence immunoassay (DELFIA, LKB-Pharmacia, Brussels, Belgium).

The effects on testicular and adrenal steroid synthesis were studied as described in [6]. Plasma aldosterone was measured using a direct radioimmunoassay with antibody-coated tubes and ^{125}I-labelled tracer (ALDOCTK-2, Sorin Feuter, Brussels, Belgium).

Statistical analysis

In vitro data were analyzed using Student's t-test. *In vivo* results were analyzed using the Mann–Whitney U-test. Significance was defined at the level of $P < 0.05$.

RESULTS AND DISCUSSION

In vitro

The aromatase inhibitory activities of R76713 and of its enantiomers were evaluated in granulosa cells isolated from PMSG-injected

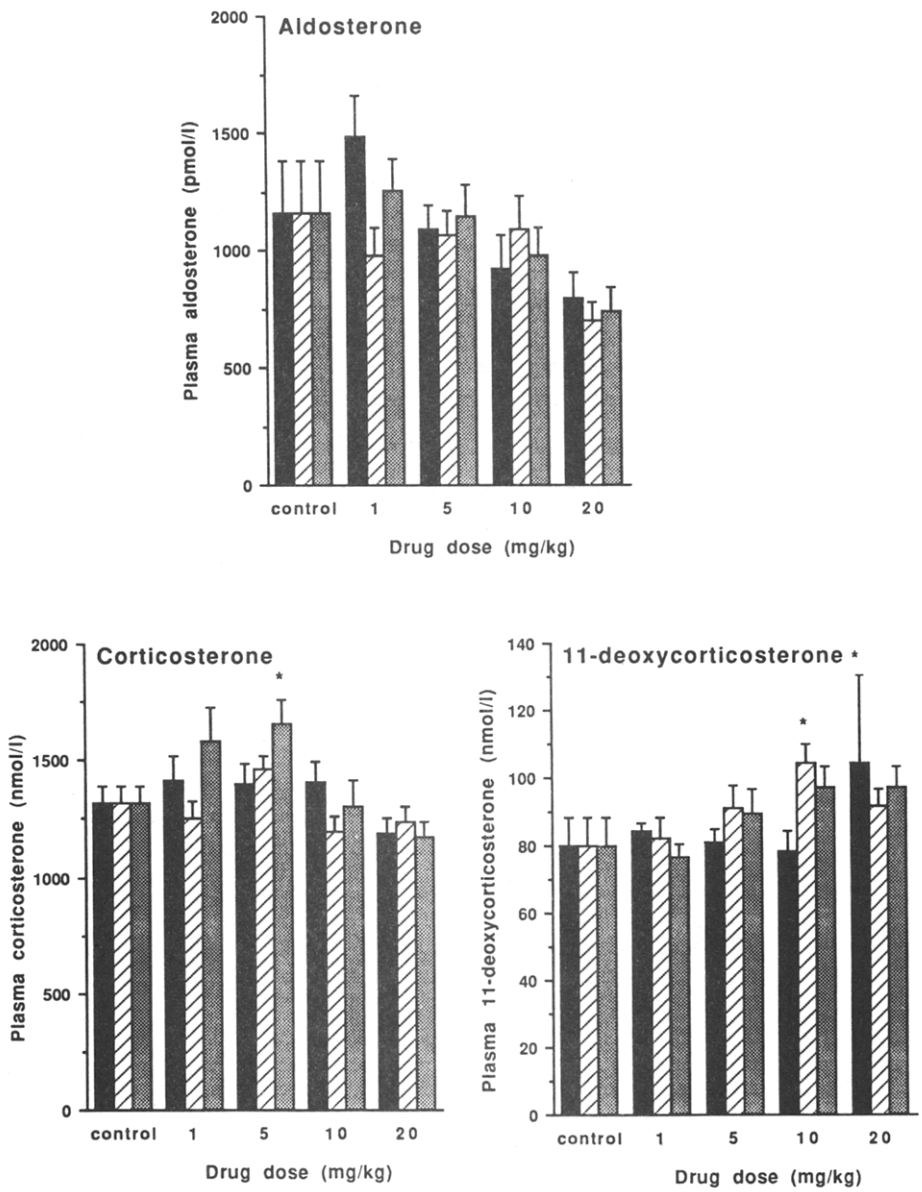

Fig. 3. Effects of R76713 (filled bars), R83839 (hatched bars) and R83842 (speckled bars) on plasma levels of testicular steroids in LHRH/ACTH-injected rats, 2 h after a single oral drug administration. Results are represented as mean ± SEM (n = 12–18 in each group). *Statistically different from the control ($P < 0.05$).

rats. From the inhibition curves (Fig. 1) the following IC_{50}-values (drug concentrations producing 50% inhibition) were calculated: 0.93 ± 0.26 nM for R76713, 240 ± 31 nM for R83839 and 0.44 ± 0.21 nM for R83842 (mean $\pm$ SD; five independent experiments performed in triplicate). This revealed a 545-fold difference in potency between both enantiomers.

The *in vitro* selectivity of R76713 and of its enantiomers was compared by measuring steroid production in rat testicular and adrenal cells after incubation in the presence of increasing concentrations of the drugs. The results of these experiments are summarized in Table 1.

In rat testicular cells, the three compounds had no effect on hCG-stimulated testosterone production up to drug concentrations of 1 μM. Incubation with 10 μM resulted in a slight but not statistically significant inhibition of testosterone production (−30%). A similar profile was obtained for androstenedione. Levels of progesterone started to rise at drug concentrations above 1 μM, reaching a maximum of 392% for R76713, 358% for R83839 and 384% for R83842 at 10 μM. The levels of 17α-hydroxyprogesterone started to rise from 1 μM drug concentrations and reached maximum values of 473% for R76713, 395% for R83839 and 447% for R83842 at 10 μM. All this is indicative for a slight inhibition of the 17-hydroxylase/17,20-lyase at high drug concentrations with no clear difference in potency between R76713 and its enantiomers.

In dispersed rat adrenal cells, synthesis of corticosterone was not affected by any of the drugs up to concentrations of 10 μM. Levels of 11-deoxycorticosterone started to increase at 1 μM of R83839, reaching a maximum of 733% of the control at 10 μM of this compound. For R76713 and R83842 this increase was less pronounced and resulted in maximum values of 259 and 226%, respectively at 10 μM. Progesterone concentrations increased only very slightly. These results are indicative for a slight inhibition of the 11-hydroxylase enzyme which was more pronounced for R83839 than for R76713 and R83842.

In vivo

Ovarian estradiol synthesis in PMSG-injected rats. Two hours after a single oral administration of R76713 or one of its enantiomers plasma estradiol levels in PMSG-injected rats were dose-dependently lowered (Fig. 2). The effect of the racemate (R76713) started at a dose of 0.01 mg/kg whereas a dose of 0.001 mg/kg of R83842 already significantly reduced plasma estradiol levels. Both R76713 and R83842 reduced estradiol levels by more than 90% at 0.1 mg/kg. R83839 on the other hand was clearly less active; a dose of 1 mg/kg gave only 70% reduction. From the dose–response curves, the following ED_{50}-values (drug doses, needed to reduce plasma estradiol to 50% of the control) were calculated: 0.011 mg/kg for R76713, 0.25 mg/kg for R83839 and 0.0034 mg/kg for R83842. This means a 73-fold difference in potency between both enantiomers.

Steroid synthesis in LHRH/ACTH-injected rats. Figure 3 shows the effects on plasma testicular hormone levels in LHRH/ACTH-injected rats, measured 2 h after single oral dosing. Testosterone was significantly lowered by 10 and 20 mg/kg of R83842 and by 20 mg/kg of R76713 or R83839. This was accompanied by an increase in the levels of 17α-hydroxyprogesterone. The level of progesterone was

Table 1. Effects of two fixed doses of R76713, R83839 and R83842 on steroid biosynthesis in primary cultures of rat testicular and rat adrenal cells

	Steroid concentration (% of control)					
	10^{-6} M Drug concentration			10^{-5} M Drug concentration		
	R76713	R83839	R83842	R76713	R83839	R83842
Rat testicular cells						
Testosterone	90 ± 9	85 ± 11	100 ± 13	70 ± 10	70 ± 7	70 ± 11
Androstenedione	93 ± 17	81 ± 11	75 ± 9[a]	61 ± 9[a]	67 ± 12[a]	53 ± 6[a]
Progesterone	141 ± 26	153 ± 68	133 ± 36	392 ± 147[a]	358 ± 191[a]	384 ± 236[a]
17α-Hydroxyprogesterone	175 ± 17[a]	150 ± 32[a]	173 ± 13[a]	473 ± 112[a]	395 ± 92[a]	447 ± 109[a]
Rat adrenal cells						
Corticosterone	103 ± 13	105 ± 14	96 ± 3	88 ± 15	91 ± 17	96 ± 8
11-Deoxycorticosterone	119 ± 15	156 ± 41[a]	140 ± 5	259 ± 116[a]	733 ± 242[a]	226 ± 50[a]
Progesterone	105 ± 15	111 ± 4	95 ± 10	122 ± 22	131 ± 17[a]	116 ± 13

[a]Statistically different from the control ($P < 0.05$).

Results are expressed as mean ± SD of 5 independent experiments, each performed in duplicate.

Steroid synthesis levels in the controls (in pmol/10^6 cells/h): testosterone, 168 ± 84; androstenedione, 62 ± 8; progesterone (testicular), 8.7 ± 3.6; 17α-hydroxyprogesterone, 16 ± 4.2; corticosterone, 16,325 ± 7100; 11-deoxycorticosterone, 1600 ± 900; progesterone (adrenal), 1275 ± 375.

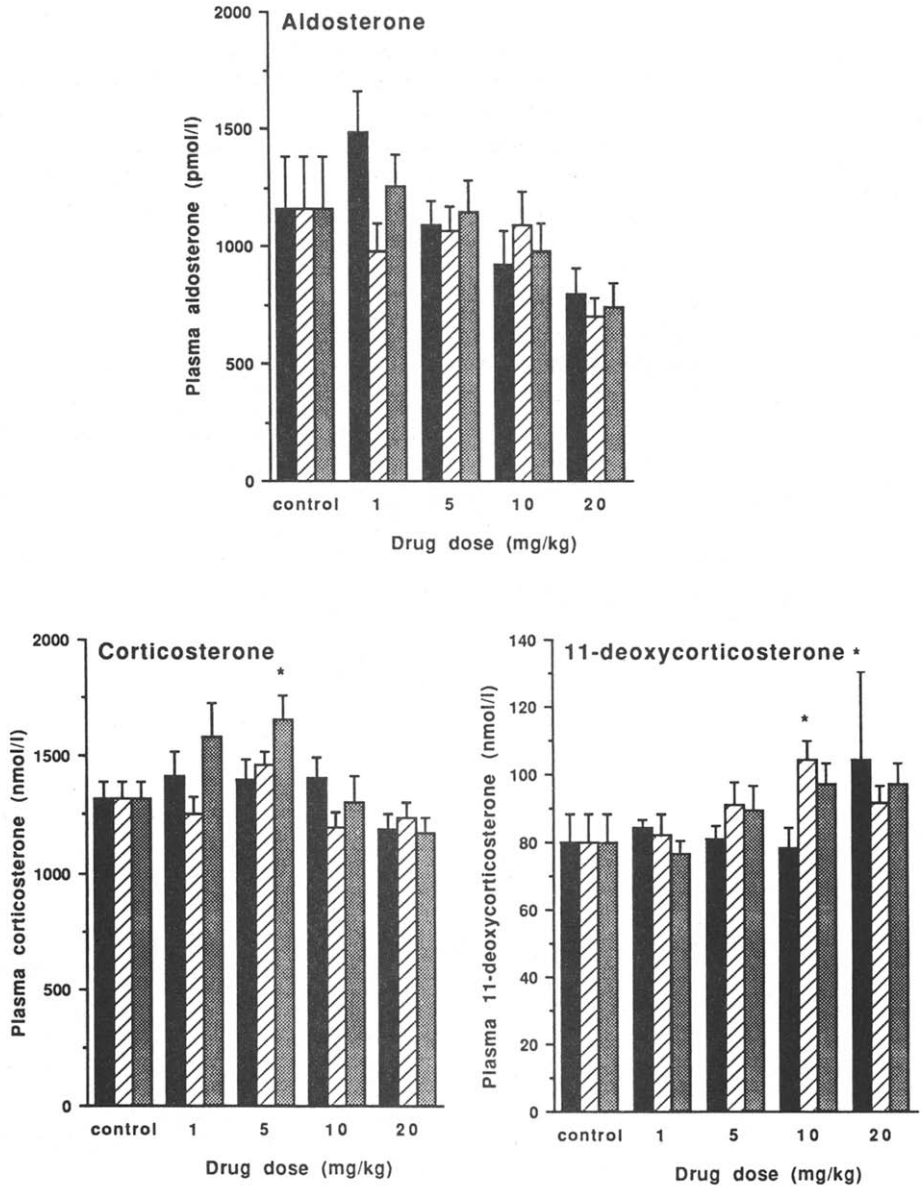

Fig. 4. Effects of R76713 (filled bars), R83839 (hatched bars) and R83842 (speckled bars) on plasma levels of adrenal steroids in LHRH/ACTH-injected rats, 2 h after a single oral drug administration. Results are represented as mean ± SEM (n = 12–18 in each group). *Statistically different from the control ($P < 0.05$).

significantly higher than the control value at 20 mg/kg of R83842 only.

The effects of R76713 and its enantiomers on plasma adrenal hormone levels in LHRH/ACTH-injected rats, measured 2 h after single oral dosing, are shown in Fig. 4. Corticosterone was not lowered by any of the drug doses tested. The levels of 11-deoxycorticosterone were significantly higher than the control values at 10 mg/kg of R83839 and at 20 mg/kg of R76713. Aldosterone levels tended to decrease at the highest drug doses but these changes were statistically not significant.

In conclusion, it was found that the enantiomers of R76713 have very different aromatase inhibitory activities. The dextro-isomer (R83842) is the more potent compound both *in vitro* and *in vivo*.

R83842 has almost no effect on testicular and adrenal steroid biosynthesis *in vitro* or *in vivo*. This confirms the extreme selectivity, previously found for the racemate.

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