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ENDOCRINE AND ANTITUMORAL EFFECTS OF R 76 713 IN RATS

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Some effects of daily oral administration of a new non-steroidal aromatase inhibitor on the pituitary-gonadal and adrenal functions were investigated in female rats. At doses of 1 mg/kg twice daily or higher, R 76 713 lowered plasma estradiol levels to the range measured after ovariectomy. Plasma progesterone levels and uterine weights decreased whilst LH levels increased but to a lesser extent than after ovariectomy. The other hormonal data show that long-term administration of R 76 713 does not modify the gluco- and mineralocorticoid hormone levels even at the highest dose studied (20 mg/kg, 4 h after treatment). Furthermore, both ovariectomy and R 76 713 treatment (1 and 5 mg/kg twice a day) induced almost complete regression of 9,12-dimethyl-1,2-benzanthracene-induced mammary carcinoma in rats. The appearance of new tumors during the treatment period was completely inhibited by R 76 713 whilst multiplicity of the remaining tumors was dramatically reduced.

KEY WORDS: R 76 713, rat, endocrine and antitumoral effects.

INTRODUCTION

Estrogens have been implicated in several benign and malignant disorders. Aromatase inhibitors, which block the conversion of androgens into estrogens might be of value in the treatment of such diseases.[1,2] Indeed, aminogluthetimide, a piperidine derivative and 4-hydroxyandrostenedione, a steroid, have been found to be of therapeutic value in the treatment of metastatic breast cancer in postmenopausal women.[3–5] However, more potent and selective aromatase inhibitors, free of any androgenic and estrogenic activity should be useful therapeutic tools to treat estrogen-dependent disease. Recently, we described a new non-steroidal aromatase inhibitor, R 76 713. This triazole derivative is a very potent inhibitor of aromatase in rat ovarian homogenates,[6] rat and human ovarian granulosa cells,[7,8] human stromal cells from adipose tissue[8] and human placental microsomes.[9] In all these systems, 50% reduction of the aromatase activity was reached at a drug concentration of about 3 nmol/l. In rat ovarian homogenates, as well as in primary culture of human stromal cells and human ovarian granulosa cells, R 76 713 competitively inhibited the aromatization of androstenedione or testosterone with K_i values (dissociation constant of the enzyme-inhibitor complex estimated by Lineweaver-Burk analysis) varying between 0.1 and 1.6 nmol/l.[6,8]

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In pregnant mare serum gonadotropin-primed female rats, a single oral administration of 0.05 mg/kg of R 76 713 lowered plasma estradiol levels by more than 90%.[7] In cynomolgus monkeys, peripheral conversion of labeled androstenedione into estrone was decreased by 85%, 4 to 5 h after a single intravenous dose of 0.003 mg/kg of R 76 713.[10]

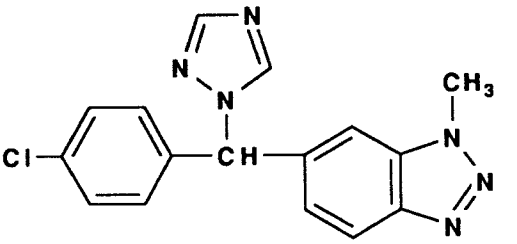
In vitro and/or *in vivo*, R 76 713, at doses at least 500 fold higher than that needed for aromatase inhibition, is also devoid of any effect on cholesterol, progestins, androgen, gluco- and mineralocorticoid biosynthesis.[7-10] It is also free of estrogenic and androgenic[7] as well as antifungal[9] activity.

The first results obtained in male and in premenopausal female volunteers indicated that R 76 713 lowered plasma estradiol levels by more than 50% for almost 24 h after single oral dosing of 5 and 20 mg, respectively.[10] In the present report, we describe some endocrine effects of long term oral administration of R 76 713 in female rats and report on the antitumoral effects of R 76 713 on 9,12-dimethyl-1,2-benzanthracene (DMBA)-induced tumors in rats.

MATERIALS AND METHODS

Test Compound

R 76 713 (Janssen Research Foundation; 6-[(4-chlorophenyl)(1*H*-1,2,4-triazol-1-yl)methyl]-1-methyl-1*H*-benzotriazole, was dissolved in 40% hydroxypropyl-β-cyclodextrine in water for the endocrinological studies and suspended in water for the DMBA-induced mammary carcinoma experiments.



Endocrine Effects of R 76 713 in Rats

Ninety female Wistar rats weighing 250 g were kept under constant conditions and received a standard pelleted food and water *ad libitum*. A light-dark cycle of 12 h was employed. A first experiment including 50 rats was designed to evaluate the effects of long term administration of R 76 713 on the pituitary-gonadal axis. Groups of 10 animals were administered R 76 713 by gavage for 12 days at the following doses: 1 or 5 mg/kg once and twice daily or vehicle solution (once daily). After 12 days of treatment, the rats were sacrificed by decapitation in the morning (between 8 and 11 a.m.), i.e. 12 and 24 h after the last drug or vehicle administration.

To further evaluate the effects of R 76 713 on the gluco- and mineralocorticoid hormones 3 groups of 10 rats each received single daily oral administration of vehicle or 5 or 20 mg/kg of R 76 713. After 12 days, they were sacrificed 4 h after the last treatment, a time when the maximal hormonal effects of the drug were to be expected. In the same experiment, 10 rats were ovariectomized and were given daily admini-

stration of the vehicle. This latter group of animals was included in order to evaluate the effects of ovariectomy on the pituitary and ovarian functions.

In all rats, uterus-, ovarian-, adrenal- and body weight were recorded. Blood was collected on EDTA (0.03 mol/l, pH 7.4) in chilled tubes, immediately centrifuged at 4°C and plasma was stored at −20°C. Plasma LH concentrations were measured in all samples using the NIADDK rat LH kit (National Hormone and Pituitary Program, Baltimore, MD, USA) and were expressed as ng/ml in terms of NIADDK rat LH-RP2.

Plasma estradiol levels were measured using an antibody-coated [^{125}I]-estradiol radioimmunoassay kit (Baxter Travenol, Brussels, Belgium). Plasma progesterone was determined using direct radiomimmunoassay kits with iodinated tracer and coated antibodies (Coat-a-Count, Diagnostic Products Corporation, Medico-Service, Benelux, Dilbeek, Belgium). Furthermore, in the samples obtained 4 h after the last treatment, plasma renin activity (generated angiotensin I, nmol/l/h at 37°C), aldosterone, corticosterone and 11-deoxycorticosterone levels were measured as described previously.[11]

Antitumoral Effect of R 76 713 on DMBA-induced Mammary Carcinoma

Female Sprague-Dawley rats (IFFA CREDO, Lyon, France) were gavaged at 50 days of age with 200 mg/kg of DMBA (Sigma, St. Louis, MO, USA) dissolved in sesame oil. The animals had been deprived of food but not of water for 6 h prior to DMBA administration and received 0.9% saline containing 6% glucose to drink afterwards for 8 days.[12,13]

Six weeks after DMBA administration, the mammary glands were palpated at weekly intervals. After 64 days, animals with one or more tumors of 10 mm diameter or more were randomized to various treatment groups of 5 to 8 animals each. R 76 713 was given orally at a dose of 1 or 5 mg/kg body weight twice daily. Groups of control and ovariectomized rats received the vehicle only. Ovariectomy was performed concomitantly with the onset of treatment. The number and size of the tumors were recorded twice a week and the length and width of the biggest tumor was measured conventionally with calipers twice weekly. The tumor volume was calculated using the equation: Volume = Length × Width.[2]

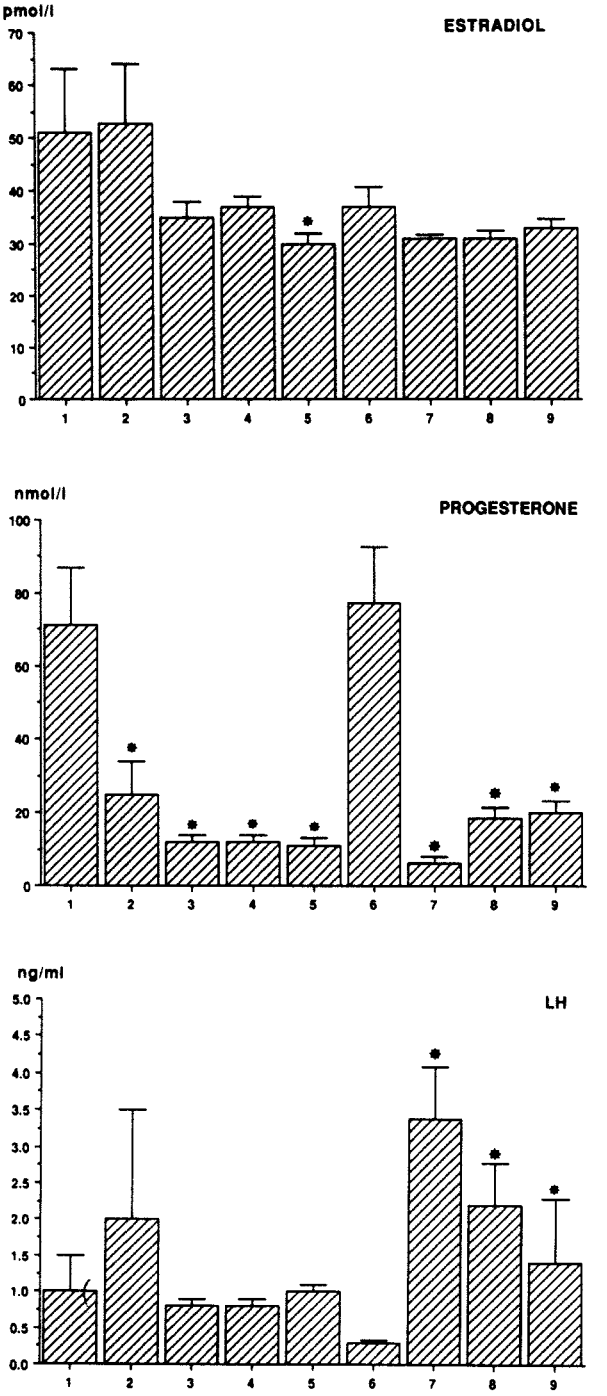
Statistical Analysis

Data from organ weights and plasma hormone concentrations were analyzed for significance by the Mann-Whitney U-test (two-tailed) with all references to statistical significance being at the ≤ 0.05 level.

RESULTS

Endocrine Effect of R 76 713 in female rats

The effects of ovariectomy and daily oral administration of R 76 713 or vehicle on plasma levels of estradiol, progesterone and LH are represented in Figure 1. Twenty four hours after the last single oral administration of 1 mg/kg, no change in estradiol was observed compared to control animals. At higher doses, plasma estradiol concentrations were close to the detection limit of the assay (30 pmol/l) and almost similar



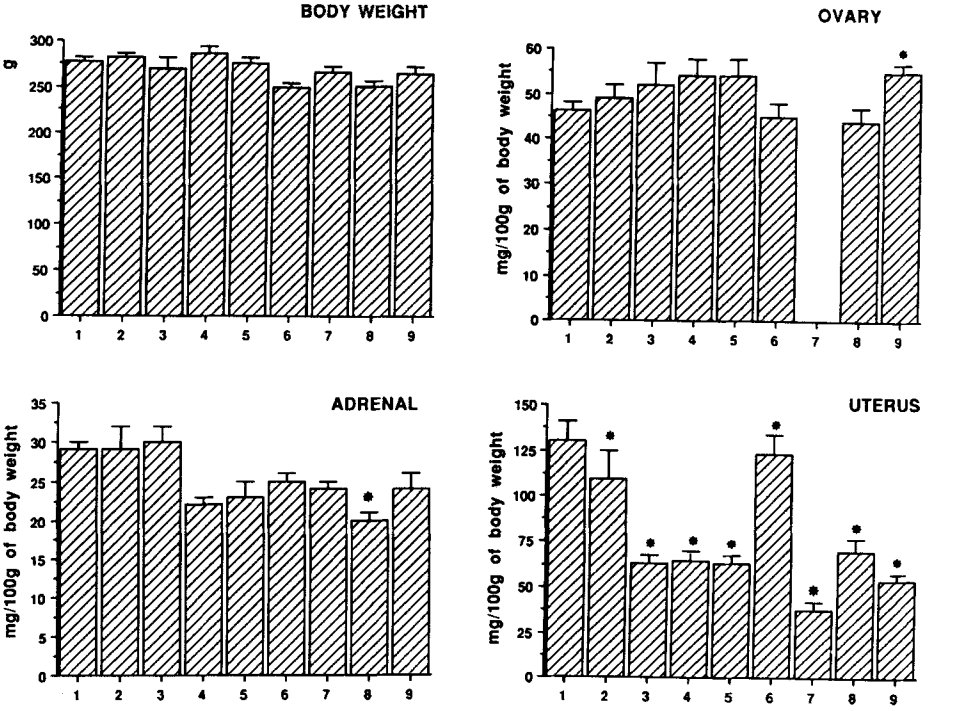


FIGURE 2 Effects of daily oral administration of R 76 713 or vehicle for 12 days on plasma renin activity (PRA), aldosterone, corticosterone and 11-deoxycorticosterone concentrations in female rats. Each column represents the mean ± SEM of 10 animals. $*p \leq 0.05$ versus the control group.

to those measured in ovariectomized rats. In contrast, plasma progesterone levels were significantly lowered by all doses of R 76 713 used, even if they remained about 3-fold higher than that measured in ovariectomized rats (Figure 1). Plasma LH levels showed no significant variation in the groups of rats sacrificed 12 or 24 h after treatment but significantly increased 4 h after administration of 5 and 20 mg/kg of R 76 713. This is due to the low values observed in the control animals included in this group and might be related to the large individual fluctuations observed in non-synchronized rats or to the time of sacrifice (12 a.m. or 8 a.m.). Moreover, this LH increase was less pronounced than that observed after ovariectomy (Figure 1).

Plasma levels of the main gluco- and mineralocorticoids, as well as the amount of angiotensin I generated per ml plasma per hour at 37°C, were also measured 4 h after

FIGURE 1 Effects of daily oral administration of R 76 713 for 12 days on plasma estradiol, progesterone, and LH levels in female rats. The animals were treated between 8 and 9 a.m. (single daily dose) or around 8 a.m. and 8 p.m. (twice daily dose). Each column represents the mean ± SEM of 10 animals. $*p \leq 0.05$ versus respective control groups. 1: control rats, sacrificed 24 h after the last vehicle administration; 2: rats sacrificed 24 h after the last administration of 1 mg/kg, o.d.; 3: rats sacrificed 12 h afer the last administration of 1 mg/kg, b.i.d.; 4: rats sacrificed 24 h after the last administration of 5 mg/kg, o.d.; 5: rats sacrificed 12 h after the last administration of 5 mg/kg, b.i.d.; 6: control rats sacrificed 4 h after the last morning vehicle administration; 7: ovariectomized rats sacrificed 4 h after the last morning vehicle administration; 8: rats sacrificed 4 h after the last morning administration of 5 mg/kg, o.d.; 9: rats sacrificed 4 h after the last morning administration of 20 mg/kg, o.d.

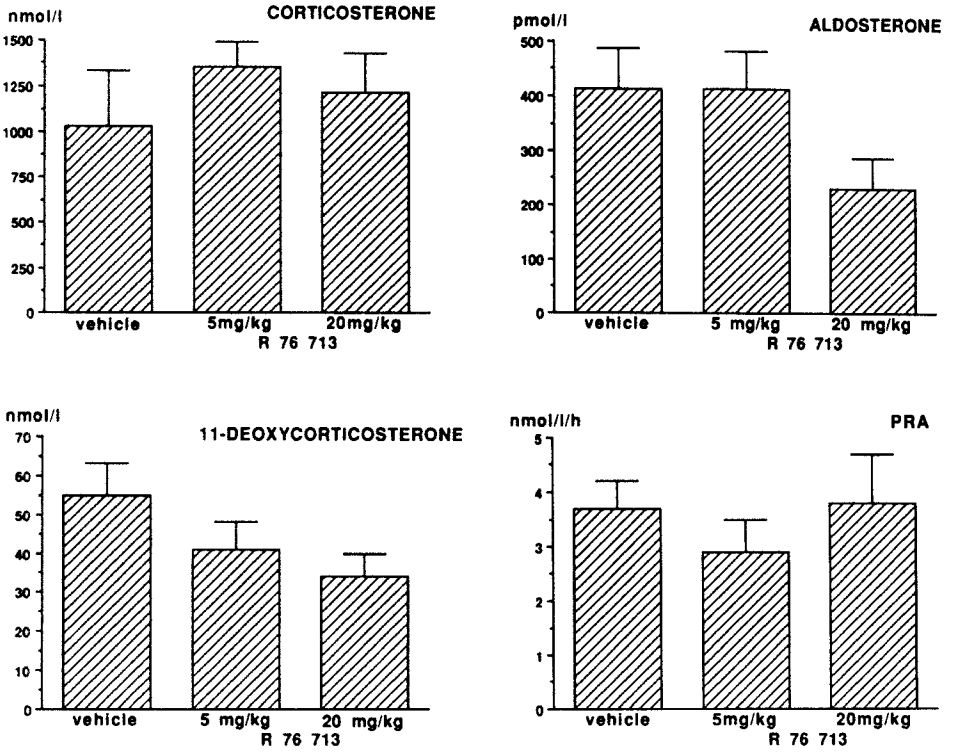


FIGURE 3 Effects of daily oral administration of R 76 713 for 12 days on body and organ weights in female rats. Each column represents the mean ± SEM of 10 animals. $*p \leq 0.05$ versus respective control groups. 1: control rats, sacrificed 24 h after the last vehicle administration; 2: rats sacrificed 24 h after the last administration of 1 mg/kg, o.d.; 3: rats sacrificed 12 h after the last administration of 1 mg/kg, b.i.d.; 4: rats sacrificed 24 h after the last administration of 5 mg/kg, o.d.; 5: rats sacrificed 12 h afer the last administration of 5 mg/kg, b.i.d.; 6: control rats sacrificed 4 h after the last morning vehicle administration; 7: ovariectomized rats sacrificed 4 h after the last morning vehicle administration; 8: rats sacrificed 4 h afer the last morning administration of 5 mg/kg, o.d.; 9: rats sacrificed 4 h after the last morning administration of 20 mg/kg, o.d.

single daily oral administration of 5 and 20 mg/kg of R 76 713 (Figure 2). These values were compared to those obtained in intact animals. At the highest dose used (20 mg/kg), plasma aldosterone and 11-deoxycorticosterone levels slightly decreased but the level of significance was not reached. No alteration was detected for the other parameters measured. Figure 3 represents the mean body weights and the relative uterine, ovarian and adrenal weights measured in all rats. A slight but not significant increase in body weight was observed after ovariectomy (+6.4%) and R 76 713 treatment (20 mg/kg, + 6.0%). No marked effects on adrenal and ovarian weight were detected with exception of a significant increase in ovarian weight at 20 mk/kg. In contrast, all doses of R 76 713 significantly reduced the relative uterus weight. The mean maximal decrease (− 58%) was observed after 20 mg/kg of R 76 713 but it did not reach the mean reduction level obtained in ovariectomized animals (− 69%). Furthermore, a single daily administration of 1 mg/kg of R 76 713 reduced the mean relative uterus weight by 16% only (Figure 3).

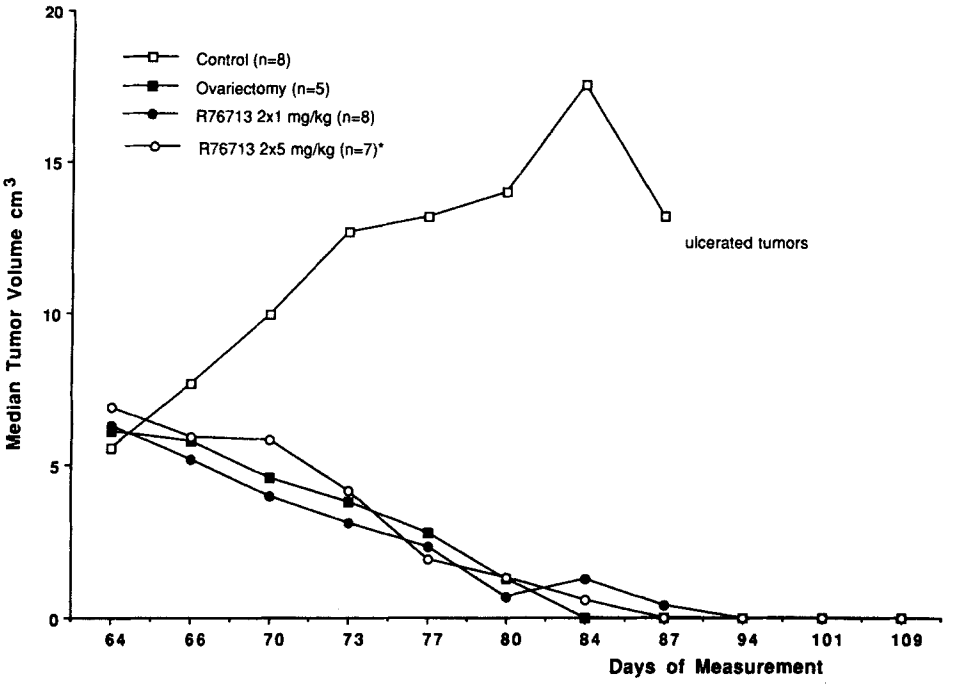


FIGURE 4 Effect of ovariectomy and treatment with R 76 713 on the growth of DMBA-induced rat mammary carcinoma. Ovariectomy was performed at day 64 after DMBA administration. Treatment with a dose of 1 and 5 mg/kg once or twice a day for the following 6 weeks was installed at the same day. Due to ulceration of the control tumor, measurements in that group were stopped at day 87. The medians of each group are connected. *: one rat accidently died on day 85.

Antitumoral Effects of R 76 713 in DMBA-induced Mammary Carcinoma

Both ovariectomy and R 76 713 (at 1 and 5 mg/kg twice daily) caused complete regression of the tumors (Figure 4). Median tumor volume became undetectable after 84, 87 and 94 days of treatment after ovariectomy, 2 × 5 and 2 × 1 mg/kg of R 76 713, respectively. No significant differences in tumor growth were detected between ovariectomy and both R 76 713 treatment regimens. The multiplicity of the tumors was also significantly reduced by ovariectomy and both doses of R 76 713. At

TABLE 1
Effects of ovariectomy or R 76 713 treatment on the multiplicity of DMBA-induced mammary carcinoma. Ovariectomy was performed and treatment was started on day 64. °: one rat accidently died on day 85

	Number of tumors/rat (median and range)		
	Day 64	Day 92	Day 106
Control rats (n = 8)	2.5 (1–4)	5 (1–6)	5 (3–7)
Ovariectomized rats (n = 5)	2.5 (1–4)	0 (0–1)	0 (0–1)
R 76 713, 2 × 1 mg/kg/day (n = 8)	2.5 (2–5)	2 (0–4)	2 (0–1)
R 76 713, 2 × 5 mg/kg/day (n = 7)	2.5 (1–5)	0.5 (0–2)	0 (0–1)

the highest dosage used (2 × 5 mg/kg), the number of tumors was not significantly different from that observed after ovariectomy (Table 1). The appearance of new tumors was completely inhibited by all treatment regimens.

DISCUSSION

The data presented in this paper confirm the potency and specificity of R 76 713 as an aromatase inhibitor. A single oral administration of 1 mg/kg of this new triazole lowered plasma estradiol by more than 90% for almost 24 h in PMSG-primed rats.[7] After chronic administration of the same dose of R 76 713 in normal rats, plasma estradiol levels were lowered by 16% only, 24 h after the last treatment. This is likely to be related to a compensatory rise of gonadotropins. Yet, higher doses of R 76 713 lowered plasma estradiol levels close to the detection limit of the assay and to the values observed after ovariectomy. In contrast, progesterone levels were similarly lowered by all the doses of R 76 713 used, suggesting some interference with the luteal function. Since R 76 713 did not affect the biosynthesis of progesterone in cultured ovarian granulosa cells,[7,8] a direct effect on steroid production is unlikely. The gonadotropin increase, however, was less marked after R 76 713 treatment than after ovariectomy. This might be related to some hypothalamic or pituitary effect of R 76 713 or to an increased production of ovarian androgens, resulting from an inhibition of aromatase together with high gonadotropin levels. More detailed studies on the effects of R 76 713 on the pituitary-gonadal axis are currently running to clarify these points, particularly in synchronized cycling rats, in order to lower the large individual hormonal variations observed in our animals.

The other hormonal data presented confirm that R 76 713 does not modify the gluco- and mineralocorticoid hormone levels even at the highest dose studied (20 mg/kg, 4 h after treatment). These results are in complete agreement with previous *in vitro*[7-9] and *in vivo*[7] data.

The DMBA-induced tumor model in rats is commonly accepted as one of the most suitable animal models for estrogen-dependent mammary carincoma. The data obtained in the present study clearly demonstrate the potency of R 76 713 to induce remission of existing mammary carcinoma to almost complete regression. The appearance of new tumors during the treatment period was completely inhibited in both R 76 713-treated groups, whilst multiplicity of the remaining tumors was dramatically reduced.

No difference could be detected between the effects of ovariectomy and the highest dose of R 76 713 used (2 × 5 mg/kg/day) in any of the parameters recorded. These results are in good agreement with the endocrine data showing that daily oral administrations of 2 × 1 mg/kg/day or more reduce plasma estradiol levels for 24 h. Although more studies are needed to further characterize the antitumoral properties of R 76 713, the data obtained so far are promising for possible clinical application of this triazole.

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