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NEW NON-STEROIDAL AROMATASE INHIBITORS: FOCUS ON R76713

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Summary—R76713 is a novel triazole derivative which selectively blocks the cytochrome *P*450-dependent aromatase. In human placental microsomes, in FSH-stimulated rat and human granulosa cells and in human adipose stromal cells, 50% inhibition of estradiol biosynthesis was obtained at drug concentrations of 2–10 nM. In PMSG-injected female rats, R76713 lowered plasma estradiol levels by 50 and 90% 2 h after single oral doses of 0.005 and 0.05 mg/kg respectively. After 1 mg/kg, estradiol levels were suppressed by 90% for 16 h. In male cynomolgus monkeys, R76713 dose-dependently (0.03–10 μg/kg) inhibited peripheral aromatization with an ED_{50} of 0.13 μg/kg without altering metabolic clearance rates and conversion ratios. *In vitro* R76713 had no effect on other *P*450-dependent steroidogenic enzymes up to 1000 nM at least. In rats, LHRH-, ACTH- and sodium-deprived diet stimulated plasma testosterone, corticosterone and aldosterone levels were not modified 2 h after single oral administrations of R76713 (up to 20 mg/kg). Furthermore, R76713 did not show any *in vitro* or *in vivo* estrogenic or antiestrogenic property. R76713 also induced regression of DMBA-induced mammary tumors after daily oral administration of 1 mg/kg b.i.d.

In male volunteers ($n = 4$), a single oral dose of 5 and 10 mg lowered median plasma estradiol levels from 70 pM to the detection limit of the assay (40 pM) 4, 8 and 24 h after intake whereas no changes were detected after placebo administration. In premenopausal women ($n = 15$), receiving a single oral dose of 20 mg, median plasma estradiol levels decreased from 389 pM (before) to 168, 133 and 147 pM, 4, 8 and 24 h after intake whereas they remained above 420 pM after placebo ($n = 7$).

INTRODUCTION

Reducing the supply of estrogens to cells is one of the major aims in the endocrine therapy of breast cancer. In premenopausal women, estrogens are mainly synthesized in the ovarian granulosa cells whereas, in postmenopausal women, the major pathway of estrogen production is the conversion of circulating androgens to estrogens mainly by peripheral adipose tissue [1]. The conversion of testosterone and androstenedione to estradiol and estrone, respectively, is mediated by aromatase, an enzyme complex which involves a NADPH-cytochrome *P*450-reductase and a cytochrome *P*450. Inhibition of aromatase can be achieved by steroidal compounds which block the active site of the enzyme without being converted into estrogens. One of the substrate analogues, 4-hydroxyandrostenedione, has been extensively studied and evaluated in clinical trials [2].

A second class of aromatase inhibitors consists of nitrogen heterocyclic derivatives which bind to the iron of the heme moiety and to the apoprotein of the enzyme. For example, aminoglutethimide, a piperidine derivative, blocks estrogen production *in vitro* [3] and inhibits the peripheral conversion of adrenal androgens to estrogens in postmenopausal women [4]. Clinical studies have confirmed that aminoglutethimide causes response rates similar to adrenalectomy in women with breast cancer [5]. Recently, several new non-steroidal aromatase inhibitors have been described and some of these are currently under pharmacological and/or clinical evaluation [6–14]. Careful characterization of their potency and specificity, particularly for adrenal steroid biosynthesis, and of their effects on steroid receptors are needed to provide a rationale for further development of these drugs.

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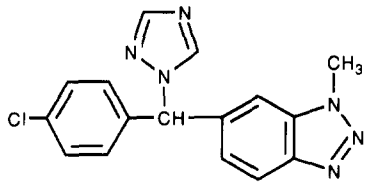
Abbreviations: LHRH: luteinizing hormone-releasing hormone; IC_{50}: drug concentration producing 50% inhibition *in vitro*; ED_{50}: drug concentration producing 50% reduction of plasma steroid levels.



R 76713

Fig. 1. Chemical structure of R76713 (6-[(4-chlorophenyl)(1H-1,2,4-triazol-1-yl)methyl]-1-methyl-1H-benzotriazole).

In this paper, we summarize the biochemical and preclinical data on a new potent and specific inhibitor of aromatase, R76713 (Fig. 1).

IN VITRO STUDIES

Inhibition of aromatase activity

The comparative effects of aminoglutethimide and R76713 on pFSH-stimulated estradiol and progesterone production were evaluated in primary cultures of rat granulosa cells [15]. Aminoglutethimide lowered estradiol production by 50% at a concentration of 3.9 ± 2.8 μmol/l (mean $\pm$ SD of 3 experiments in triplicate) and reduced progesterone production by 22% at 10 μmol/l. R76713 is about 1300-fold more potent in blocking estradiol production with an IC_{50} of 3.0 ± 0.2 nmol/l (mean $\pm$ SD of 4 experiments in triplicate) whilst progesterone biosynthesis was not affected at all up to a drug concentration of 10 μmol/l. Very similar IC_{50}-values were found in rat ovarian homogenates, cultured human ovarian granulosa and adipose stromal cells as well as in human placenta microsomes (Table 1). In rat ovarian homogenates and in primary culture of human granulosa and human adipose stromal cells, R76713 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 [16, 17]. Spectral studies using human placenta microsomes suggested that R76713 not only coordinates to the heme-iron of the cytochrome *P*450 but also binds to the apoprotein [18].

Specificity for the other cytochrome P450-dependent reactions of steroid biosynthesis

Measurements of progesterone production in the supernatant of cultured rat granulosa cells showed that medium concentrations of 10 μmol/l of R76713, in contrast to aminoglutethimide, do not alter the enzymes located prior to progesterone formation. Indeed, R76713 does not affect the 14α-demethylation of lanosterol, a key step in the formation of cholesterol in mammals, or the side-chain cleavage enzyme (Table 1). It also does not alter ergosterol biosynthesis in fungi [19].

Similarly, no effects on testicular 17-hydroxylase/17,20-lyase or on the adrenal 21- and 11-hydroxylase complexes were found up to a concentration of 1 μmol/l. Fifty percent inhibition of androgen, gluco- and mineralocorticoid biosynthesis was only reached at a concentration of about 10 μmol/l or higher (Table 1).

Table 1. Comparative effects of R76713 on the main cytochrome *P*450-dependent reactions of ovarian, testicular and adrenal steroid biosynthesis

	Tissue	Test system	IC_{50} (nmol/l)	Ref.
Aromatase	Human placenta microsomes	Conversion of [^{3}H]androstene-dione to [^{3}H]estrone	2.7	18, 19
	Cultured human ovarian granulosa cells	^3H_2O production	10.0	16
	Cultured human adipose stromal cells	Estrone and estradiol production	3.7	16
	Cultured rat granulosa cells	^3H_2O production	3.0	15
	Rat ovarian homogenates	^3H_2O production	5.1	17
Cholesterol biosynthesis (14-demethylase)	Rat liver subcellular fractions Cultured human lymphocytes	[^{14}C]Mevalonate or [^{3}H]acetate incorporation into cholesterol	> 10,000	18, 19
Cholesterol side-chain cleavage	Bovine adrenal cortex mitochondria Rat testis mitochondria	Formation of reduced *P*450–CO complexes	> 10,000	18, 19
17α-Hydroxylase-17,20-lyase	Rat testis microsomes	[^{14}C]Pregnenolone conversion to [^{14}C]androgens	8000	18, 19
	Cultured rat and human testicular cells	Production of androgens and progestins	> 10,000	15, 16
21-Hydroxylase	Cultured rat and human adrenal cells	11-Deoxycortisol and 11-deoxycorticosterone production	> 10,000	15, 16
11-Hydroxylase	Bovine and adrenal cortex mitochondria	Conversion of [^{3}H]11-deoxycortisol and [^{3}H]11-deoxycorticosterone to [^{3}H]cortisol and [^{3}H]corticosterone	> 10,000	18, 19
	Cultured rat adrenal cells	Corticosterone and 11-deoxycorticosterone production	$\geq$ 10,000	15
	Cultured human adrenal cells	Production of aldosterone, cortisol and their precursors	$\geq$ 10,000	16

IN VIVO STUDIES

Effects of R76713 on pituitary–gonadal axis

In pregnant mare's serum gonadotropin-primed female rats, a single oral administration of 0.005 mg/kg of R76713 lowered plasma estradiol levels by 50% [15]. At a dose of 1 mg/kg, plasma estradiol levels were lowered by more than 90% for at least 16 h [15]. During chronic treatment, administration of 1 mg/kg of R76713 twice daily reduced plasma estradiol levels to the same extent as did ovariectomy [20, 21]. Plasma progesterone levels also decreased but to a lesser extent than after ovariectomy (Fig. 2). In response to the inhibition of estrogen production, LH slightly increased, but again, less than after ovariectomy. This might be related to the higher plasma levels of progesterone determined in the animals receiving R76713 as compared to those measured in ovariectomized animals.

Inhibition of peripheral aromatization

Since most of the circulating estrogens in postmenopausal women and in men are of peripheral origin, the effects of R76713 on peripheral aromatization were evaluated in male cynomolgus monkeys using a double label isotope-primed-constant infusion technique [22]. Various dosages of the drug (0.03–10 μg/kg dissolved in 10% w/v hydroxypropyl-β-cyclodextrine) or vehicle were injected intravenously 90 min before the start of the infusion of radiolabeled steroids. Blood was drawn from the saphenous vein 4–5 h after drug administration, and plasma steroids were isolated and purified by high pressure liquid chromatography [21, 22]. Fifty percent inhibition of peripheral aromatization was obtained at an intravenous dose of 130 ng/kg of R76713 (Fig. 3). There was no specific effect of R76713 either on the metabolic clearance rates of androstenedione and estrone or on the conversion ratios between the androgens and between the estrogens [21].

Specificity for the cytochrome P450-dependent reactions of steroid biosynthesis in vivo

The *in vivo* selectivity of R76713 was tested by measuring plasma steroid levels in LHRH/ACTH-injected rats 2 h after single oral administration of drug doses of up to 20 mg/kg [15]. Effects of R76713 on mineralocorticoid activity were studied in rats fed a sodium deprived diet for 3 weeks preceding treatment with up to 20 mg/kg of R76713 [26]. In both studies, no changes in testosterone, corticosterone or aldosterone were detected (Fig. 4). Furthermore, neither the precursors of the androgens (progesterone) and of corticosterone (11-deoxycorticosterone) nor the plasma renin activity levels were altered by R76713 treatment [15, 26]. Similar results were obtained after 12 days daily oral treatment (up to 20 mg/kg) in female rats, sacrificed 4 h after the last treatment, a

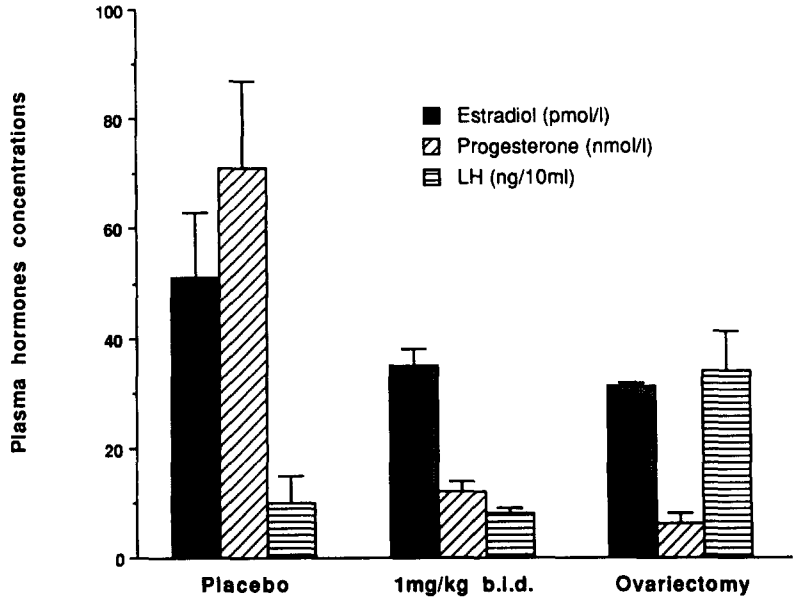


Fig. 2. Effects of oral administration of R76713 (1 mg/kg twice daily) or vehicle for 12 days on plasma estradiol, progesterone, LH and FSH levels in female rats. Each column represents the mean ± SEM of 10 animals, sacrified 4 h after the last drug administration.

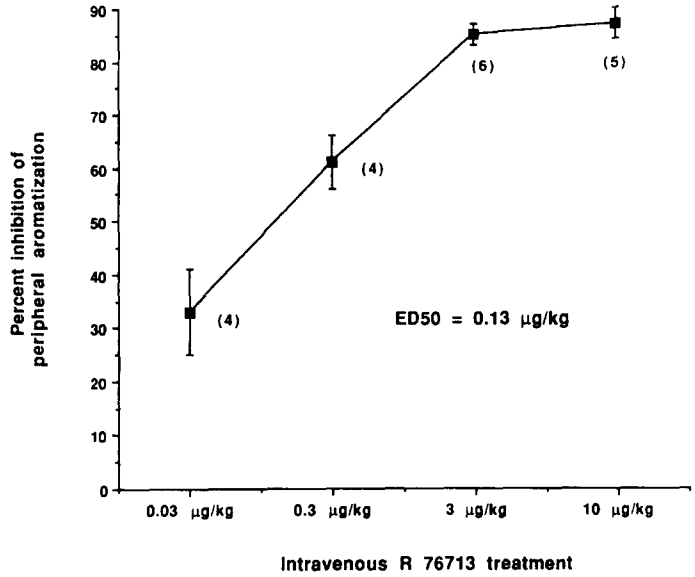


Fig. 3. Inhibition of peripheral aromatization in male cynomolgus monkeys (*Macaca fascicularis*). R76713 or vehicle was intravenously administered 90 min before infusion of radiolabeled steroids. Conversion of androstenedione to estrone was measured 4–5 h after dosing. The numbers in parentheses indicate the number of monkeys used. In vehicle-treated monkeys, $1.35 \pm 0.11\%$ of androstenedione was converted to estrone ($n = 7$).

time when the maximal hormonal effects of the drug were to be expected [20].

EFFECTS OF R76713 ON THE STEROID RECEPTORS

In vitro, R76713 (up to 10 μmol/l) did not alter the specific binding of estrogens, progestins, glucocorticoids and androgens to their respective receptors [15]. This was confirmed *in vivo* for the estrogen receptor by measuring the ability of R76713 to induce rat uterine ornithine decarboxylase activity (ODC) and uterine growth in immature rats. At doses up to 1 mg/rat s.c. (33 mg/kg), R76713 neither induced ODC activity nor increased uterine weight (Table 2).

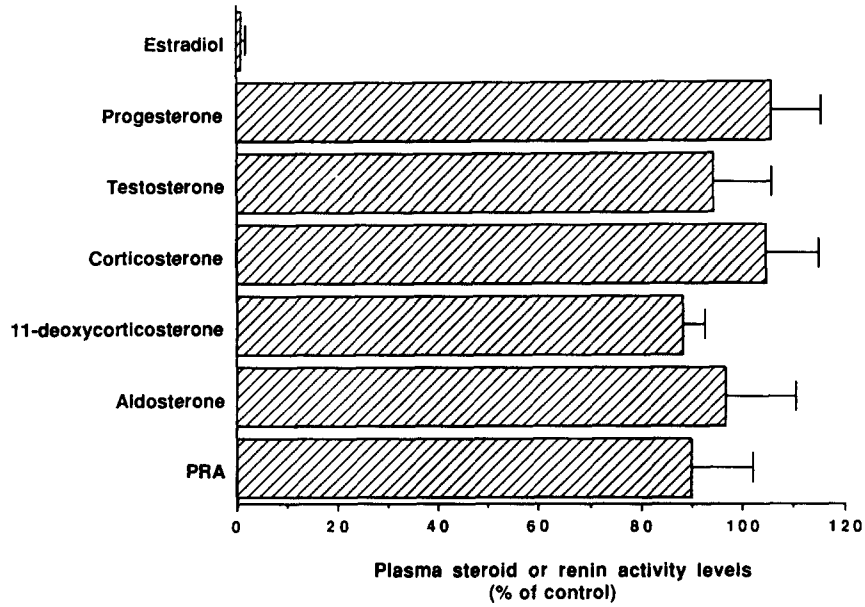


Fig. 4. Effects of R76713 (20 mg/kg) on plasma estradiol, testosterone, progesterone, corticosterone, 11-deoxycorticosterone, aldosterone and plasma renin activity levels in rats injected with pregnant mare's serum gonadotropin, LHRH or ACTH, or fed a sodium-deprived diet respectively. The animals ($n \geq 10$/group) were sacrified 2–4 h after drug administration. The results (mean $\pm$ SEM) are expressed as % of the control values measured in animals which had been treated with vehicle.

Table 2. Effects of R76713 and estradiol on uterine ornithine decarboxylase (ODC) activity and uterine wet wt in immature rats at 6 h post-dosing

Treatment	Dose (μg/rat)	Uterine ODC activity (pmol $^{14}CO_2$/mg protein/h)	Uterine wet wt (mg/10 g body wt)
Vehicle		22 ± 4	5.42 ± 0.19
R76713	10	19 ± 2	5.10 ± 0.19
R76713	100	14 ± 3	5.21 ± 0.20
R76713	1000	17 ± 3	5.98 ± 0.23
Vehicle		55 ± 7	5.48 ± 0.16
Estradiol	0.010	219 ± 54	5.55 ± 0.17
Estradiol	0.025	796 ± 71	7.08 ± 0.18
Estradiol	0.100	1177 ± 117	7.31 ± 0.38
Estradiol	10.000	1380 ± 119	7.74 ± 0.35

EFFECTS OF R76713 ON THE GROWTH OF AN ENDOMETRIAL AUTOTRANSPLANT MODEL OF EXPERIMENTAL ENDOMETRIOSIS

The activity of R76713 on experimental endometriosis was evaluated using a rat endometrial autotransplant model [23]. Both ovariectomy and three weeks oral treatment with R76713 (0.3 mg/kg and upwards) induced regression of endometrial volume (Fig. 5).

ANTITUMORAL ACTIVITY IN THE 7,12-DIMETHYLBENZ(A)ANTHRACENE (DMBA)- AND DIMETHYLNITROSOUREA (NMU)-INDUCED MAMMARY CARCINOMA MODELS

Antitumoral activity of R76713 was evaluated in female Sprague–Dawley rats treated with DMBA [24] and in Wistar rats treated with NMU [25]. In DMBA-treated rats, both ovariectomy and R76713 caused almost complete regression of tumors (Fig. 6). In most of the animals, tumors became undetectable after ovariectomy or daily oral administration of 2 × 5 and 2 × 1 mg/kg of R76713 [20]. The incidence of the tumors was also reduced by ovariectomy and both doses of R76713. In NMU-treated rats, dietary admixture of 40 and 160 mg R76713/100 g food, reduced tumor growth at least to the same extent as ovariectomy (Fig. 6).

FIRST STUDIES IN VOLUNTEERS

In 4 groups of 4 healthy male volunteers each, single oral doses of 5 or 10 mg of R76713 lowered plasma estradiol levels from 70 pmol/l to the detection limit of the assay (30 pmol/l), 4 and 8 h after drug intake, whereas no changes were detected after placebo [26]. Peak plasma concentrations of R76713 were attained within 1 h after dosing. Peak plasma levels were 80 ± 16 and 156 ± 22 ng/ml (mean ± SD) for the 5- and 10-mg dose, respectively. Dose-normalized values of maximal concentrations and areas under the curve from 0 to 8 h were not significantly different, which is indicative of a linear dose-proportional increase in these parameters. After the peak of the 10-mg dose, plasma concentrations of R76713 decayed biphasically with mean sequential half-lives of 2.3 and 7.0 h.

In premenopausal women ($n = 15$) receiving a single oral dose of 20 mg, median plasma estradiol levels decreased from 389 pmol/l (before) to

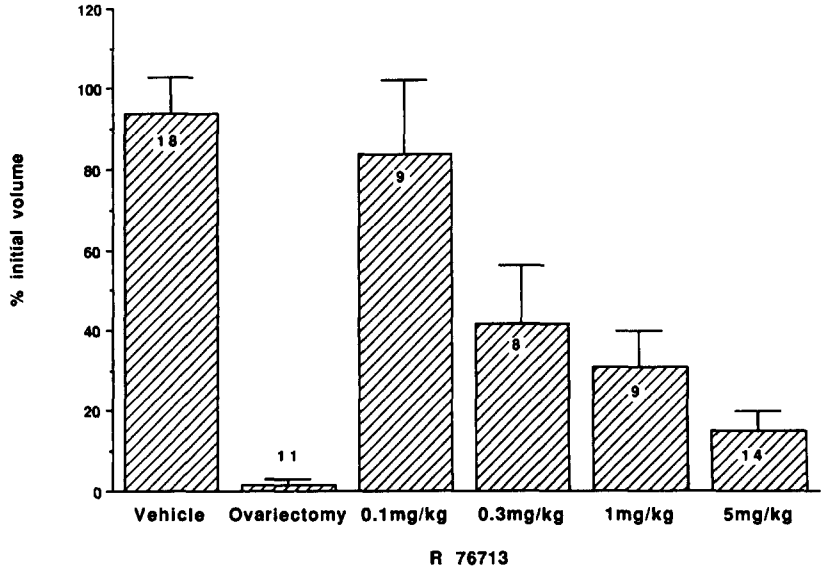


Fig. 5. Effects of R76713 in an endometrial autotransplant model of experimental endometriosis. The results (mean ± SEM) are expressed as percentage of the autotransplant volume measured on the day prior to beginning treatment. The numbers in the columns indicate the number of rats used.

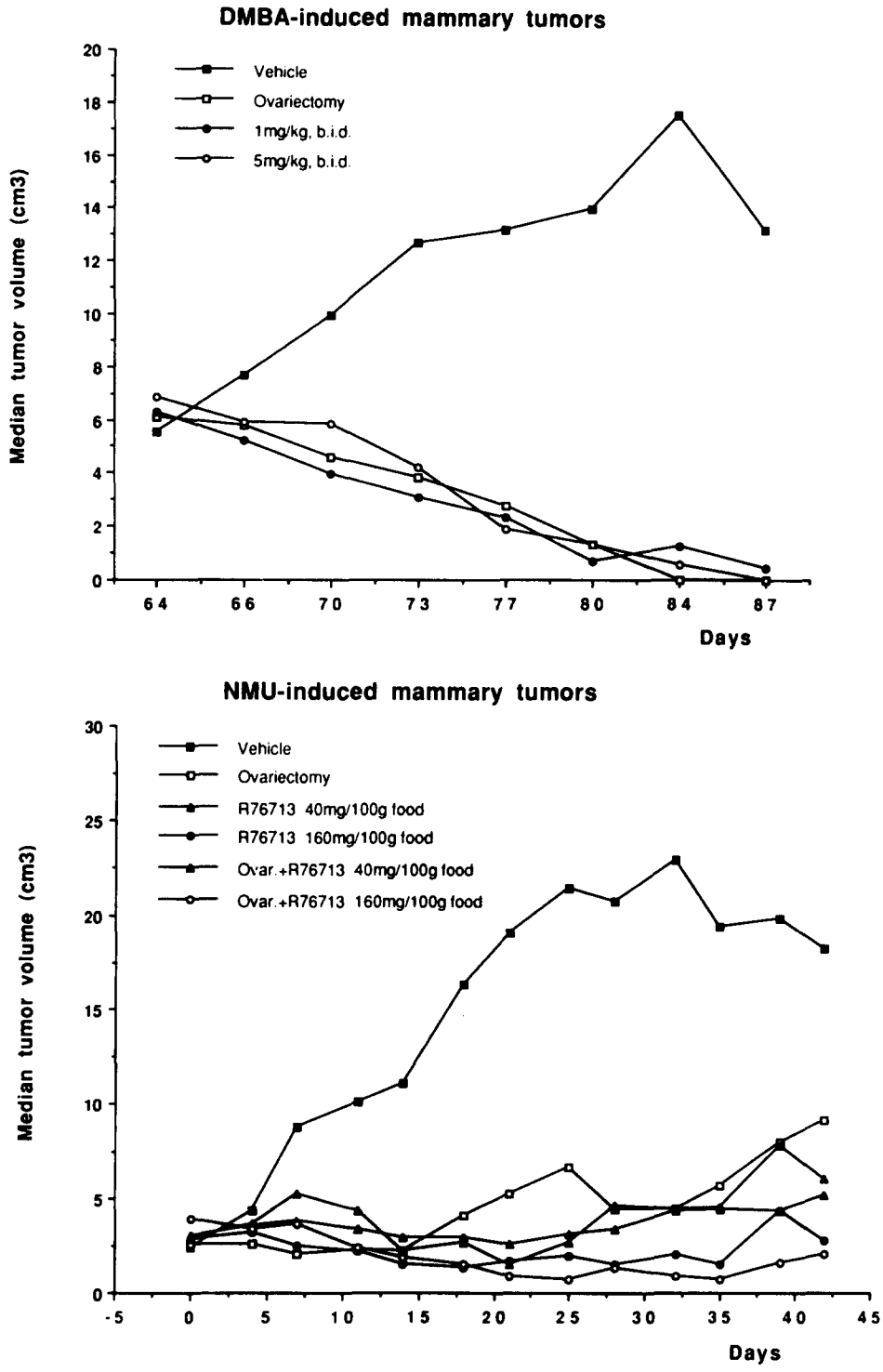


Fig. 6. Effects of ovariectomy and R76713 treatment on the growth of DMBA- and NMU-induced mammary carcinoma in Sprague–Dawley and Wister rats, respectively. DMBA: ovariectomy was performed at day 64 after DMBA administration. Treatment with a dose of 1 and 5 mg/kg twice a day for the following 6 weeks was begun on the same day. The medians of each group are connected ($n \geq 8$). NMU: R76713 was administered for a period of 6 weeks as a dietary admixture of 40 and 160 mg R76713/100 g food. Ovariectomy was performed concomitantly with the onset of treatment. The medians of each group ($n \geq 10$) are connected.

168, 133 and 147 pmol/l respectively at 4, 8 and 24 h after intake whereas they remained above 420 pmol/l after placebo ($n = 7$) [26].

In all volunteers studied, drug tolerance was perfect and there were no significant changes in hematological and biochemical safety

parameters in the treated groups as compared to the placebo group.

CONCLUSION

The results of biochemical and preclinical studies reported in this review show that R76713 is a very potent and specific inhibitor of ovarian and peripheral aromatase. Studies in animal mammary carcinoma and in experimental endometriosis models as well as the results of preliminary volunteer studies strongly warrant further clinical evaluation of this compound for the treatment of estrogen-related diseases in both pre- and postmenopausal women.

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