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Effects of combined and sequential treatment with tamoxifen and the aromatase inhibitor vorozole on 7,12-dimethylbenz(a) anthracene-induced mammary carcinoma in the rat

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Abstract The aromatase inhibitor vorozole dose-dependently inhibited the growth of dimethylbenz(a)anthracene (DMBA)-induced mammary carcinoma in the rat. An oral dose of 5 mg/kg per day brought about growth inhibition and reduction of tumor multiplicity similar to that produced by ovariectomy. Tamoxifen (8 mg/kg per day) also reduced tumor growth, albeit to a lesser extent than did ovariectomy. Concomitant administration of varying doses of tamoxifen with the fully effective dose of vorozole (5 mg/kg per day) tended to be less effective than ovariectomy or vorozole alone. This is likely to be due to the estrogen-agonistic effects of tamoxifen. Combination of tamoxifen with the partially effective dose of vorozole (1 mg/kg per day) gave results comparable with those obtained for either of these compounds used in monotherapy. Combining tamoxifen with a marginally active low dose of vorozole (0.2 mg/kg per day) resulted in a minor additional growth inhibition as compared with that obtained with this dose of vorozole alone. Sequential treatment with tamoxifen (8 mg/kg per day) for 42 days and vorozole (5 mg/kg per day) for 42 days, and vice-versa, was performed with a drug-free interval of 14 days between treatments. Tumors regressing under vorozole therapy relapsed when subsequently treated with tamoxifen. In contrast, vorozole further reduced tumor volumes in rats previously treated with tamoxifen. Furthermore, monotherapy with tamoxifen as well as the two sequential tamoxifen-vorozole treatment schedules were in most cases less effective than vorozole monotherapy in inhibiting both tumor growth and tumor multiplicity. Although extrapolation of these findings in cycling animals to the clinical situation, involving postmenopausal women, is not straightforward, these results warrant further studies in preclinical models. Moreover, clinical trials comparing the most effective aromatase inhibitors with tamoxifen in previously untreated postmenopausal patients with breast cancer may also be warranted.

Key words Rat · Aromatase · Vorozole · Tamoxifen · Breast cancer

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Introduction

Approximately one-third of postmenopausal women with recurrent breast cancer respond to endocrine therapies. Both antiestrogens and aromatase inhibitors aim at reducing the estrogen supply to cancer cells, albeit through different mechanisms. Tamoxifen and its analogs compete for the estrogen receptor, whereas aromatase inhibitors block the conversion of androgens to estrogens [2, 15, 18]. The combination of such compounds might achieve a better suppression of the biological effects of estrogens. Tamoxifen also acts, however, as a partial estrogen agonist, particularly in the presence of low endogenous estradiol concentrations [11], and conflicting results have been reported on the antitumoral effects of the combination of tamoxifen with several aromatase inhibitors in carcinogen-induced mammary tumors in rats [3, 19, 21, 23] as well as in clinical trials [18].

On the other hand, 35–53% of postmenopausal patients with metastatic breast cancer responding to tamoxifen in first-line treatment show a second response to another endocrine agent, e.g. an aromatase inhibitor, and 5–25% of tamoxifen nonresponders benefit from second-line endocrine therapies [18].

The majority of preclinical and clinical studies with aromatase inhibitors have been performed with aminoglutethimide. This drug has several drawbacks, such as a lack of selectivity as well as a poor toxicity and tolerability profile [6, 18]. Newer aromatase

inhibitors with markedly improved activity and selectivity and favorable pharmacokinetic and tolerability profiles have been developed [2, 6].

The present paper describes the antitumoral and, in part, endocrine effects of concomitant and sequential treatment with tamoxifen and vorozole, a potent and selective nonsteroid aromatase inhibitor of the third generation [4, 7, 10, 16, 20, 22], on the growth of established dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rats.

Materials and methods

Test compounds

Vorozole (+)-(S) {6-[(4-chlorophenyl)(1*H*–1, 2, 4-triazol-1-yl)methyl]-1-methyl-1*H*-benzotriazole} and tamoxifen {trans-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-*N*,*N*-dimethyl-ethylamine} were dissolved in 20% polyethylene glycol (PEG 400) in water for oral dosing by gavage.

Tumors and animals

Female Sprague-Dawley rats (IFFA CREDO, Lyon, France) were orally gavaged at 50 days of age with 200 mg/kg 7,12-dimethylbenz(a)anthracene (DMBA, Sigma Chemical Co., St. Louis, Mo.) dissolved in sesame oil. The animals had been deprived of food but not water for 6 h prior to DMBA administration and received 0.9% saline containing 6% glucose to drink for 10 days afterward. Beginning at 6 weeks after DMBA administration, the mammary glands were palpated at weekly intervals for 4 weeks. Animals with one or more tumors (approximately 10 mm in diameter) were randomized to various treatment groups. Each treatment group consisted of 8–15 rats.

Tamoxifen or vorozole treatment schedules

In the first group of experiments (experiments 1–3), combined treatment for 42 days with different doses of tamoxifen and vorozole was compared with monotherapy with either compound. Tamoxifen was given orally at a dose of 8 (experiment 1), 2 (experiment 2), and 0.5 mg/kg (experiment 3) once daily at 8 a.m. in combination with once-daily oral vorozole doses of 5, 1, and 0.2 mg/kg at 4 p.m. The monotherapy for tamoxifen and vorozole followed the same schedule: tamoxifen was given at 8 a.m. and the vehicle, at 4 p.m.; vorozole was given at 4 p.m. and the vehicle, at 8 a.m.

In the second group (experiment 4), the effects of sequential treatment with tamoxifen and vorozole were studied. Tamoxifen was gavaged at a dose of 8 mg/kg once daily for 42-days followed by 42 days of vorozole (5 mg/kg) treatment, and vice-versa. A 14-day washout period was inserted between the two 42-day treatment regimens. Monotherapy with either compound was performed for 98 days, with an interruption of 14 days occurring between days 42 and 56. Control and ovariectomized rats were included in all trials and received the vehicle by oral gavage twice daily (8 a.m. and 4 p.m.). No vehicle was given to the rats during the 14-day drug-free period. Ovariectomy was performed and treatment was started when tumors had reached a volume of approximately 1 cm^3 (day zero of treatment). The number and the size of the tumors were recorded once a week, and the length and the width of the tumors were measured conventionally with calipers. The volume of each individual tumor was calculated using the equation Volume = Length $\times$ Width^2. The total cumulative tumor volume was calculated for each animal.

At the end of the experiments, the animals were killed by decapitation at between 17 and 20 h after the last drug administration. Tumors were removed and weighed and the cumulative tumor weight was calculated for each rat. Serum obtained from the rats of experiment 1 (high dose of tamoxifen in combination with vorozole) was collected and stored at $-20\,^{\circ}C$ for hormone determinations.

Serum progesterone levels were measured by a direct radioimmunoassay kit using antibody-coated tubes and an iodinated tracer (Coat-a-Count; Diagnostic Products Corporation, Dilbeek, Belgium). Serum estradiol was assayed by a direct radioimmunoassay kit using a double-antibody procedure and [^{125}I]-estradiol (Estradiol-2, Clinical Assays, Sorin Biomedica, Saluggia, Italy). These assays were validated for rat serum. Experimental values equal to or below the detection limit were set equal to the detection limit (25 pmol/l for estradiol and 0.3 nmol/l for progesterone).

Plasma androstenedione was measured after extraction into pentane using [1, 2-^{3}H]-androstenedione (NEN, Dreieich, Germany; spec. act. 1.5 TBq/mmol) and an antibody raised in rabbits against androstenedione hemisuccinate coupled to bovine serum albumin (Laboratoire d'Hormonologie, Marloie, Belgium). The main cross-reacting steroids were androsterone (4.6%), dehydroepiandrosterone (1.4%), and testosterone (1.4%). Activated charcoal was used to separate bound and free steroids. Serum concentrations of luteinizing hormone (LH) were determined using the rat LH kit issued by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (National Hormone and Pituitary Program, Baltimore, Md.) and iodinated tracers obtained from Chemicon International Inc. (Temecuta, Calif.; spec. act., 120 Ci/μg). The results are expressed in nanograms per millimeter in terms of National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases rat LH-RP_2.

Statistical analysis

Data on tumor volumes measured during the experiment and on tumor numbers and tumor weights as well as hormone levels determined at the end of the experiments were analyzed for significance by the Mann-Whitney *U*-test (two-tailed) using the StatView II software package (Abacus Concepts, Inc.). All rats that showed evaluable tumors at the end of the study were included. Animals with ulcerated tumors, or rats that died during the experiment were not used for statistical analysis. The rate of "dropouts" was similar between vehicle-treated and experimental groups.

Data on cumulative tumor volume and growth curves were compared using the distribution-free test developed by Koziol et al. [12]. In short, this method treats growth curves as a whole and is based on multivariance rank statistics. All references to statistical significance apply to the $P \leq 0.05$ level.

Results

Concomitant tamoxifen and vorozole treatments

The results are compiled in Figs. 1–3 and Table 1. Ovariectomy inhibited median tumor growth by nearly 100% in all experiments (Figs. 1–3) and significantly reduced ($P \leq 0.05$) tumor multiplicity (Table 1). Vorozole at 5 mg/kg per day inhibited tumor growth

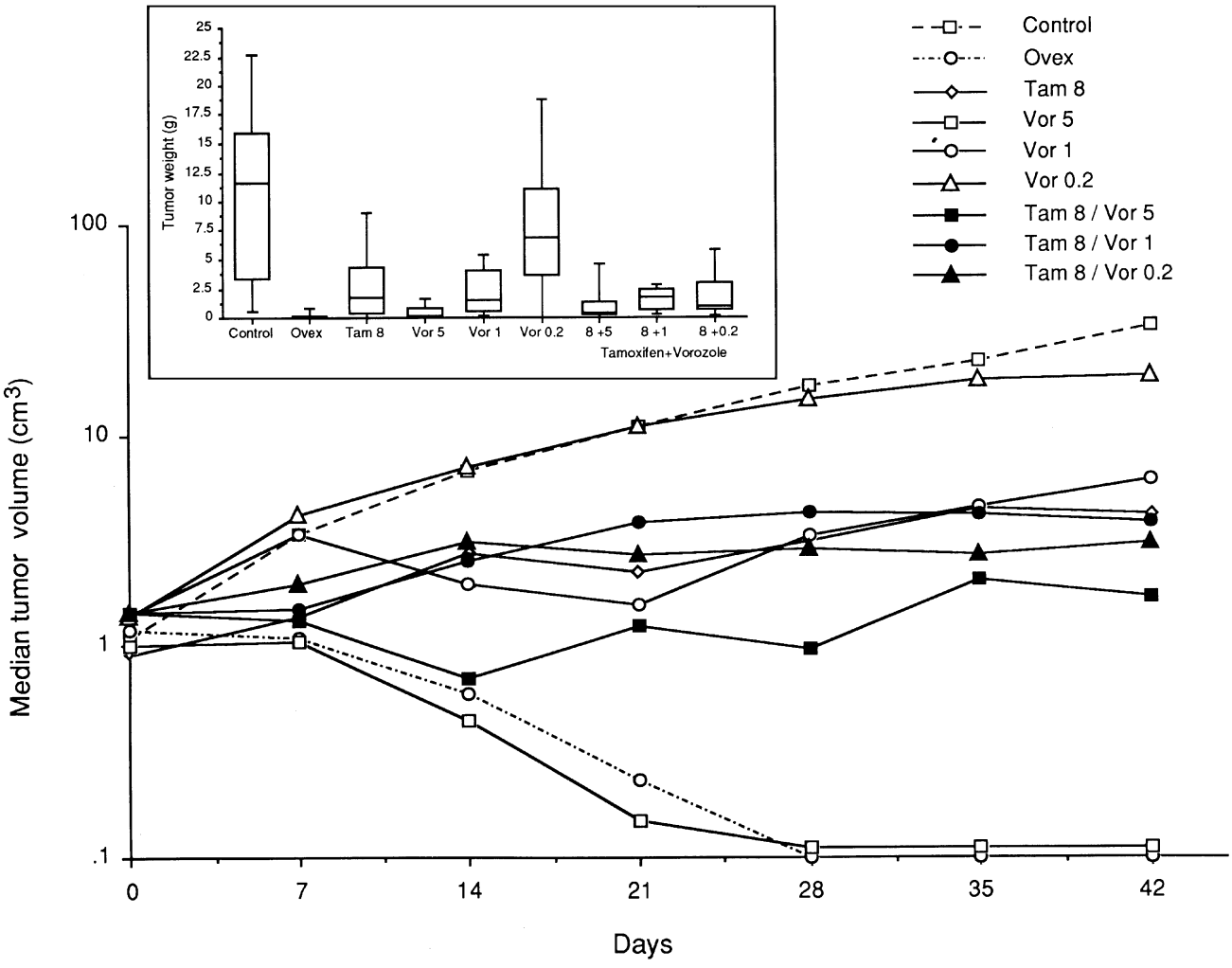

Fig. 1 Effect of ovariectomy (*Ovex*), treatment with 8 mg/kg tamoxifen (*Tam 8*), 5 mg/kg vorozole (*Vor 5*), 1 mg/kg vorozole (*Vor 1*), 0.2 mg/kg vorozole (*Vor 0.2*), and concomitant treatment on the growth of DMBA-induced rat mammary carcinoma. Ovariectomy was performed or treatment was initiated as soon as tumors had reached a volume of approximately 1 cm^3. The rats were treated daily by oral gavage for 42 days. Median cumulative tumor volumes are shown. Median, 25th to 75th, and 10th to 90th percentiles of cumulative tumor weights at day 42 are shown in the *insert*

and reduced tumor multiplicity to the same extent as did ovariectomy ($P \leq 0.01$).

At 1 mg/kg per day, vorozole significantly inhibited tumor growth and multiplicity (reduction of 74–95% in median tumor weight), whereas the limited decrease in tumor growth observed after administration of 0.2 mg/kg vorozole (41–68% of median tumor weight) did not reach the level of statistical significance. The three doses of tamoxifen (0.5, 2, 8 mg per kg) dose-dependently reduced tumor growth and multiplicity, albeit to a lesser extent than did ovariectomy.

Concomitant administration of tamoxifen with the fully effective dose of vorozole (5 mg/kg) tended to be less effective than ovariectomy or vorozole alone. The growth curves were not statistically significantly different from each other, with the exception of inhibition of tumor growth with vorozole alone as compared with vorozole plus tamoxifen at 0.5 mg/kg ($P = 0.047$). Significantly lower tumor weights and multiplicity were recorded for vorozole 5 mg/kg alone as compared with vorozole plus tamoxifen (0.5 and 2 mg/kg). The combination of tamoxifen with vorozole (1 mg/kg) gave results similar to those found for each compound used as monotherapy. Combining tamoxifen at various doses with a marginally active, low dose of vorozole (0.2 mg/kg) resulted in a minor additional growth inhibition in terms of tumor weight as compared with vorozole alone ($P \leq 0.04$).

Sequential tamoxifen and vorozole treatment

Both monotherapies as well as sequential therapy regimens significantly reduced tumor growth (Fig. 4; $P \leq 0.007$) and multiplicity (Table 1). No statistically

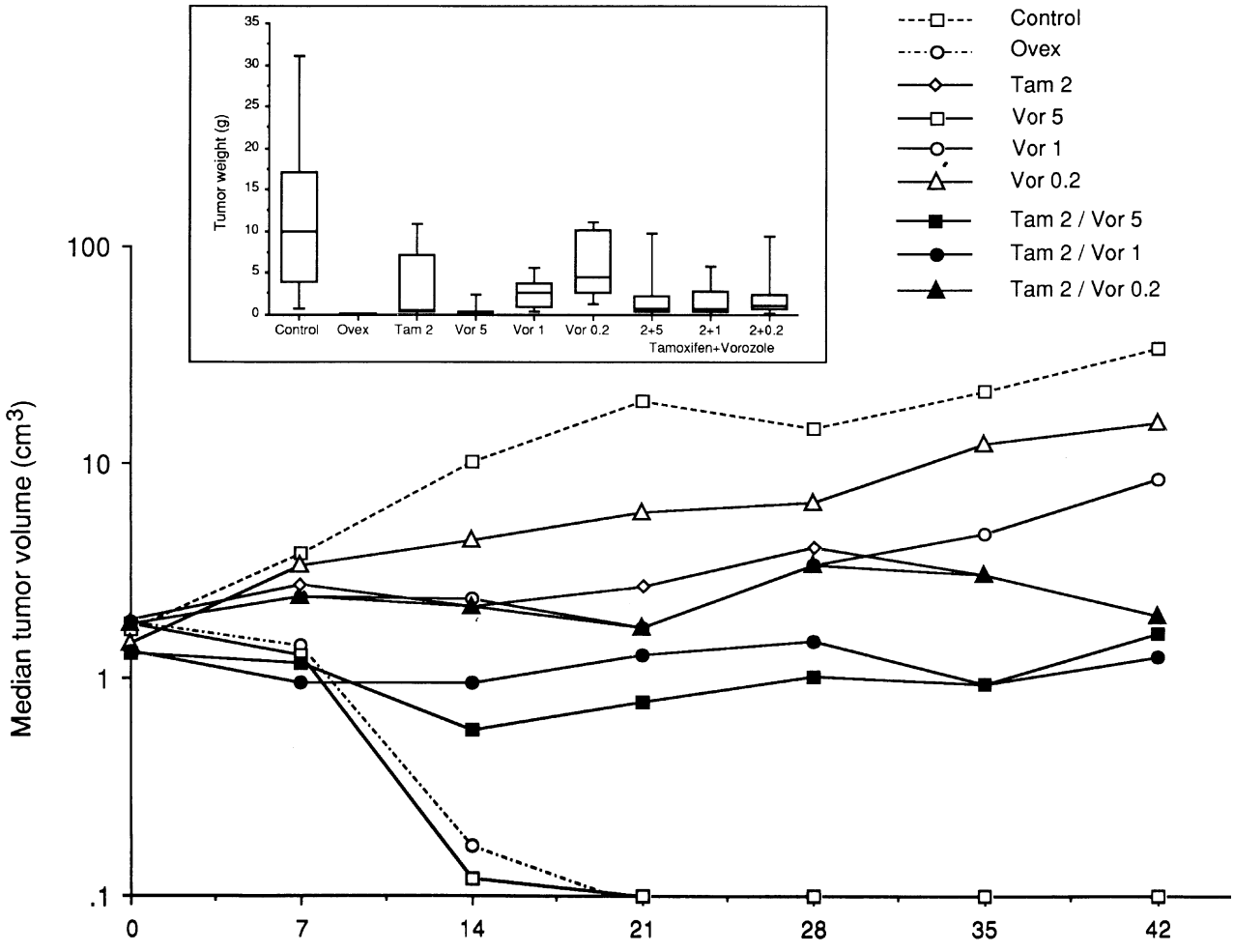

Fig. 2 Effect of ovariectomy (*Ovex*), treatment with 2 mg/kg tamoxifen (*Tam 2*), 5 mg/kg vorozole (*Vor 5*), 1 mg/kg vorozole (*Vor 1*), 0.2 mg/kg vorozole (*Vor 0.2*) and concomitant treatment on the growth of DMBA-induced rat mammary carcinoma. Ovariectomy was performed or treatment was initiated as soon as tumors had reached a volume of approximately 1 cm^3. The rats were treated daily by oral gavage for 42 days. Median cumulative tumor volumes are shown. Median, 25th to 75th, and 10th to 90th percentiles of cumulative tumor weights at day 42 are shown in the *insert*

significant difference was observed between the growth curves generated for the various treatment groups except for a slight improvement in growth inhibition that was observed after vorozole monotherapy as compared with sequential vorozole plus tamoxifen treatment ($p = 0.025$). Vorozole given after tamoxifen also tended further to decrease tumor volumes. In contrast, tamoxifen was not capable of decreasing the median tumor volume following tumor relapse during the 14-day drug-free interval after the cessation of vorozole (Fig. 4). Furthermore, monotherapy with tamoxifen as well as the two sequential treatment regimens were somewhat less effective in reducing the numbers and weights of tumors than was either vorozole alone or ovariectomy (Fig. 4; Table 1).

Endocrine effects of combined tamoxifen vorozole treatment

As expected, vorozole at 5 mg/kg reduced serum estradiol levels to the detection limit of the assay. It also markedly decreased progesterone concentrations and increased serum LH and androstenedione levels (Table 2). Vorozole at 1 and 0.2 mg/kg did not markedly alter plasma estradiol levels at 17–20 h after the final vorozole dose. Since the duration of maximal aromatase inhibition does not exceed 12 h at these doses, this finding was not unexpected. Tamoxifen mainly reduced LH and progesterone levels. Combination of the two compounds resulted in androstenedione and LH levels similar to those observed after vehicle treatment. The serum estradiol and progesterone levels achieved with vorozole plus tamoxifen were similar to those measured after treatment with vorozole alone.

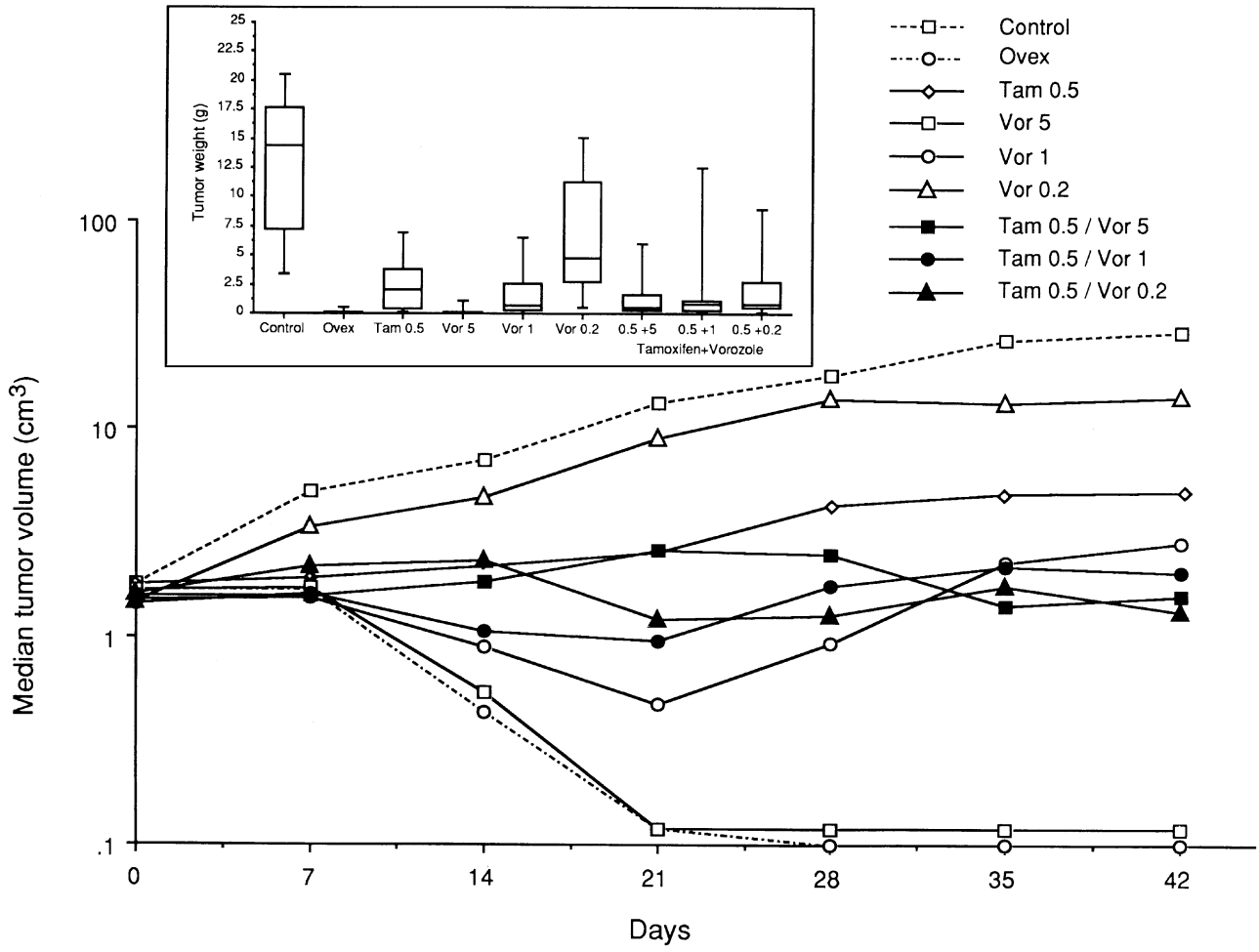

Fig. 3 Effect of ovariectomy (*Ovex*), treatment with 0.5 mg/kg tamoxifen (*Tam 0.5*), 5 mg/kg vorozole (*Vor 5*), 1 mg/kg vorozole (*Vor1*), 0.2 mg/kg vorozole (*Vor 0.2*), and concomitant treatment on the growth of DMBA-induced rat mammary carcinoma. Ovariectomy was performed or treatment was initiated as soon as tumors had reached a volume of approximately 1 cm^3. The rats were treated daily by oral gavage for 42 days. Median cumulative tumor volumes are shown. Median, 25th to 75th, and 10th to 90 percentiles of cumulative tumor weights at day 42 are shown in the *insert*

Discussion

The antitumoral and hormonal effects of combined and sequential vorozole and tamoxifen treatment regimens were investigated in cycling female rats bearing DMBA-induced mammary carcinoma. Concomitant administration of tamoxifen with vorozole slightly impaired the antitumoral effects of the fully effective vorozole dose (5 mg/kg) but tended to improve the effects of the marginally effective dose of vorozole (0.2 mg/kg). A possible explanation for this observation might be the partial estrogen-agonistic effect of tamoxifen in the absence of circulating estradiol (caused by the 5 mg/kg dose of vorozole) and/or the slight increase in prolactin levels induced by tamoxifen [19, 21]. The additive effects of tamoxifen with the low dose of vorozole might be explained by the partial inhibition of estrogen production by vorozole, enabling tamoxifen to act as an antiestrogen.

Similarly, the combination of tamoxifen with a steroidal aromatase inhibitor, 4-acetoxy-4-androstene-3,17-dione, in the same DMBA model was less effective than the aromatase inhibitor given as a single agent [3]. In a similar model using nitrosomethylurea as the carcinogen, the incidence of tumor regression following combined treatment with tamoxifen and formestane (4-hydroxyandrostenedione) was lower than that achieved with formestane alone [21]. In contrast, tamoxifen combined with exemestane, another steroid aromatase inhibitor [23], or with fadrozole, a non-steroid aromatase inhibitor [19], was more effective than either drug alone. The latter studies were performed with a marginally active dose of the aromatase

Table 1 Effects of combined and sequential tamoxifen/vorozole treatments on the multiplicity of DMBA-induced mammary carcinomas in female rats (*Exp.* Experiment, *Tam* tamoxifen)

	Number of tumors/rat (median and range)				
	Exp. 1 Tam 8 mg/kg Day 42	Exp. 2 Tam 2 mg/kg Day 42	Exp. 3 Tam 0.5 mg/kg Day 42	Exp. 4 Day 42	Exp. 4 Day 98
Control ($n = 9$–14)	5 (2–10)	6 (1–8)	5.5 (1–15)	4 (2–9)	5 (3–10)
Ovariectomy ($n = 8$–14)	0 (0–1)*^{2}	0 (0–0)*^{2}	0 (0–2)*^{2}	0 (0–1)*^{2}	0 (0–3)*^{2}
Tamoxifen ($n = 8$–12)	2.5 (0–11)$^{++}$	1 (0–5)*^{1,*4}	2 (0–5)*^{1,*4}	1 (0–4)*^{2,*3}	1 (0–6)*^{2,*3}
Vorozole 5 mg/kg per day ($n = 11$)	0 (0–7)*^{2}	0 (0–1)*^{2}	0 (0–2)*^{2}	0 (0–2)*^{2}	1 (0–5)*^{2}
Vorozole 1 mg/kg per day ($n = 14$)	2.5 (0–7)*^{1}	2.5 (1–4)*^{1}	2 (0–3)*^{2,*3}	–	–
Vorozole 0.2 mg/kg per day ($n = 14$)	4.5 (1–15)	4 (1–8)	4 (0–12)*^{4}	–	–
Tamoxifen + vorozole 5 mg/kg per day ($n = 9$–12)	2 (0–3)*^{2} ($n = 12$)	1 (1–3)*^{1,*5} ($n = 9$)	1.5 (0–5)*^{1,*5}(n =11)	–	–
Tamoxifen + vorozole 1 mg/kg per day ($n = 9$–11)	2 (0–4)*^{2} ($n = 11$)	2.5 (0–8) ($n = 9$)	1.5 (1–7)*^{1} ($n = 11$)	–	–
Tamoxifen + vorozole 0.2 mg/kg per day ($n = 9$–13)	2 (0–6)*^{2} ($n = 13$)	2.5 (0–5)*^{1} ($n = 9$)	1 (0–4)*^{2,*6}($n = 11$)	–	–
Tamoxifen followed by vorozole ($n = 15$)	–	–	–	1 (0–6)**$^{,++,\circ}$	1 (0–4)**
Vorozole followed by tamoxifen ($n = 15$)	–	–	–	1 (0–2)**	2 (0–4)**$^{,+}$

*$^{1}P \leq 0.05$; *$^{2}P \leq 0.005$ versus vehicle; *$^{3}P \leq 0.05$; *$^{4}P \leq 0.005$ versus ovariectomy; *$^{5}P \leq 0.05$ versus vorozole 5 mg/kg per day; *$^{6}P \leq 0.05$ versus vorozole 0.2 mg/kg per day

inhibitors, which is in agreement with our data on low-dose vorozole (0.2 mg/kg).

Whether these findings can be extrapolated to the clinical situation remains to be elucidated. Indeed, the DMBA model may be considered a "premenopausal" model, and the metabolism of tamoxifen is not identical between species, which can lead to differences in the agonist/antagonist balance of tamoxifen and its metabolites. Several randomized trials in postmenopausal breast-cancer patients have compared tamoxifen with tamoxifen plus aminoglutethimide, a first-generation aromatase inhibitor [1, 8, 13, 17]. A higher response rate was observed with the combination in one trial, but no survival benefit was observed. Other trials were inconclusive with regard to the response rate, and toxicity was greater in patients receiving the combination. Aminoglutethimide is known to be a nonselective aromatase inhibitor belonging to the first-generation agents whose side effects are well known, whereas the clinical profile of the combination of tamoxifen with a new, potent, selective, and well-tolerated aromatase inhibitor such as vorozole might be different. The issue of tamoxifen's estrogenic properties when the drug is used in the context of estrogen deprivation remains to be clarified.

In our DMBA-induced mammary carcinoma model, the results of sequential treatment with vorozole and tamoxifen suggest that vorozole might further reduce the tumor volume in animals previously treated with tamoxifen. Tamoxifen, however, is incapable of decreasing volumes of tumors relapsing after cessation of vorozole administration in animals.

These results are in agreement with the clinical situation, whereby about 50% of tamoxifen responders experience a secondary response to aminoglutethimide, whereas only 25% of patients receiving aminoglutethimide as first-line treatment benefit from second-line tamoxifen therapy [14, 18]. The response to vorozole given as second-line treatment after tamoxifen may also result from a "withdrawal response" related to the emergence of tamoxifen-dependent tumors induced by tamoxifen treatment [5, 9]. The finding that monotherapy with vorozole proved to be slightly more effective in reducing DMBA-induced tumors than did sequential tamoxifen/vorozole treatment raises again the issue of the slightly estrogenic properties of tamoxifen and warrants further clinical studies comparing the most effective aromatase inhibitors with tamoxifen in other preclinical models. Moreover, since vorozole is an effective and well-tolerated second-line therapy in postmenopausal patients with breast cancer [7, 10, 16], clinical studies comparing the most effective aromatase inhibitors with tamoxifen in previously untreated postmenopausal patients with breast cancer may also be warranted.

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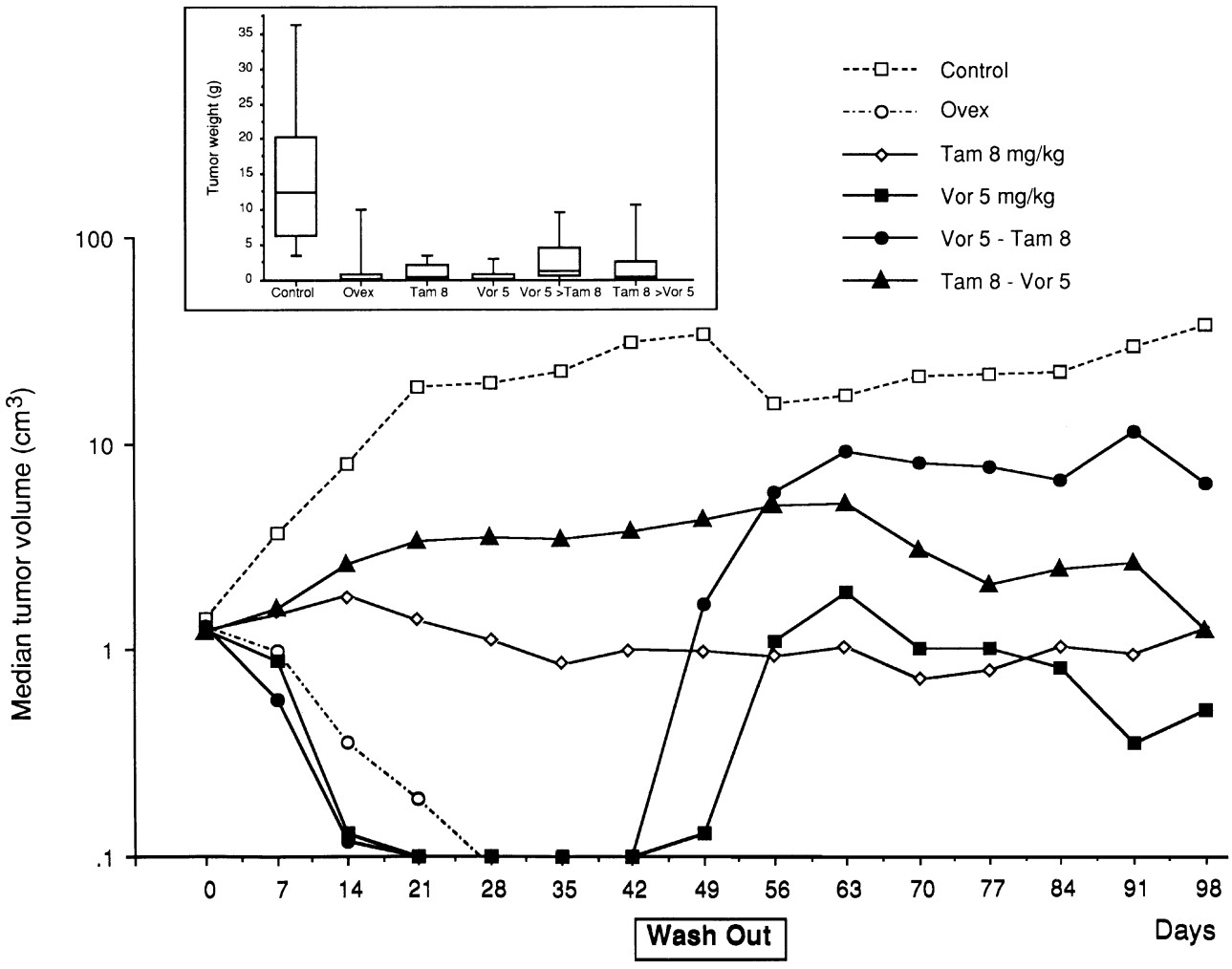

Fig. 4 Effect of ovariectomy (*Ovex*) and monotherapy with 8 mg/kg tamoxifen (*Tam 8*) or 5 mg/kg vorozole (*Vor 5*) for 42 days. A 14-day drug-free period was inserted, followed by vorozole or tamoxifen treatment for another 42 days. Sequential treatment was initiated for 42 days with tamoxifen (8 mg/kg) or vorozole (5 mg/kg). After the 14-day washout period (*Wash Out*), tamoxifen was substituted for vorozole, and vice-versa, and treatment was continued for 42 days. Ovariectomy was performed or daily oral treatment was initiated as soon as tumors had reached a volume of approximately 1 cm^3. Median cumulative tumor volumes are shown. Median, 25th to 75th, and 10th to 90th percentiles of cumulative tumor weights at day 96 are shown in the *insert*

Table 2 Endocrine effects of combined tamoxifen vorozole treatment in cycling female Sprague-Dawley rats bearing DMBA-induced mammary carcinoma; results are expressed as mean values ± SEM

	Estradiol (pmol/l)	Progesterone (nmol/l)	Androstenedione (nmol/l)	LH (ng/ml)
Control ($n = 13$)	46 ± 10	23 ± 6.2	1.7 ± 0.45	0.66 ± 0.11
Ovariectomy ($n = 13$)	≤ 25[a]	2.2 ± 0.35**	1.0 ± 0.15	8.1 ± 0.47**
Tamoxifen 8 mg/kg per day ($n = 12$)	34 ± 2.5	5.1 ± 1.5**	1.4 ± 0.22	≤ 0.49[a]*
Vorozole 5 mg/kg per day ($n = 11$)	≤ 25[a]	3.1 ± 0.53**	19 ± 5.8**	1.8 ± 0.24**
1 mg/kg per day ($n = 14$)	52 ± 17	6.9 ± 1.3*	10 ± 2.9**	1.2 ± 0.18**
0.2 mg/kg per day ($n = 14$)	58 ± 11	11 ± 2.9	4.0 ± 0.58**	0.82 ± 0.11
Tamoxifen + vorozole 5 mg/kg per day ($n = 12$)	≤ 25[a]	6.6 ± 2.7**	5.1 ± 2.7	0.78 ± 0.11
Tamoxifen + vorozole 1 mg/kg per day ($n = 11$)	31 ± 2.6	4.1 ± 0.56**	2.0 ± 0.52	0.70 ± 0.06
Tamoxifen + vorozole 0.1 mg/kg per day ($n = 13$)	31 ± 2.4	4.4 ± 0.56**	1.5 ± 0.31	0.66 ± 0.09

*$P \leq 0.05$; **$P \leq 0.005$ versus vehicle
[a]Detection limit of the assay

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