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Tumor Growth-stimulating and Inhibiting Effects of Antiestrogens on the DMBA-induced Mammary Carcinoma of the Ovariectomized, Diethylstilbestrol-treated SD Rat. A Study on the Mechanism of Action of Antiestrogens*

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Abstract—*After ovariectomy DMBA tumor-bearing SD rats were treated with the estrogen DES, a combination of DES plus mammary tumor-inhibiting antiestrogen (tetramethylHES, 3,3'HES and tamoxifen respectively) or with tetramethylHES alone. Small doses of DES led to a stimulation of tumor growth showing a maximum effect at* 1 μg/kg/day, *whereas higher doses inhibited tumor growth. In the combination experiment only tetramethylHES reduced, i.e. antagonized, the DES effect. Tamoxifen and 3,3'HES, however, led to an increase of the tumor growth-stimulating and inhibiting effects of DES. Only the dose of* 10 mg/kg/day *of tetramethylHES led to a slight stimulation of the tumor growth in ovariectomized DMBA tumor-bearing rats. These results indicate that 3,3'HES and tamoxifen possibly unfold their mammary tumor-inhibiting activity by means of their estrogenic potency, whereas tetramethylHES might act as an antiestrogen, though the latter compound does not lack estrogenic activity completely.*

INTRODUCTION

RECENTLY we reported on the syntheses of two new mammary tumor-inhibiting antiestrogens, 1,1,2,2-tetramethyl-1,2-bis(4'-hydroxyphenyl) ethane (tetramethylHES) [1] and meso-3,4-bis(3'-hydroxyphenyl)hexane (3,3'HES) [2, 3]. They are derived from the synthetic estrogen hexestrol (Fig. 1).

The *in vitro* affinity of these compounds for the estrogen receptor is only slightly decreased compared to that of hexestrol [1–3]. *In vivo*, these two compounds showed a strong antiestrogenic effect, inhibiting estrone-stimulated mouse uterine growth [1–3]. On the liver of male chickens tetramethylHES strongly inhibited the estrogen-induced synthesis of the egg-yolk protein vitellogenin [4, 5]. *In vitro* 3,3'HES exhibited a dose-dependent growth inhibition on the MCF-7 human breast tumor cell line [2, 3, 6, 7]. *In vivo* tetramethylHES and 3,3'HES showed a strong inhibition of the tumor growth on the 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced hormone-dependent mammary adenocarcinoma of the Sprague–Dawley (SD) rat [1–3]. 3,3'HES produced a strong inhibition of the postmenopausal estrogen and progesterone receptor-positive human mammary carcinoma KA implanted in nude mice [6], and showed a strong decrease of tumor weight and a high percentage of cures on the MXT hormone-dependent transplantable mouse mammary adenocarcinoma [6].

As with all other hitherto known antiestrogens, these two compounds also exhibited estrogenic properties. High doses led to a stimulation of the uterine growth in the immature mouse. Seventy percent of the maximum estrone effect was reached with 3,3'HES [2, 3], whereas tetramethylHES led to only a 12% increase [1]. In the liver of male chickens, however, the latter

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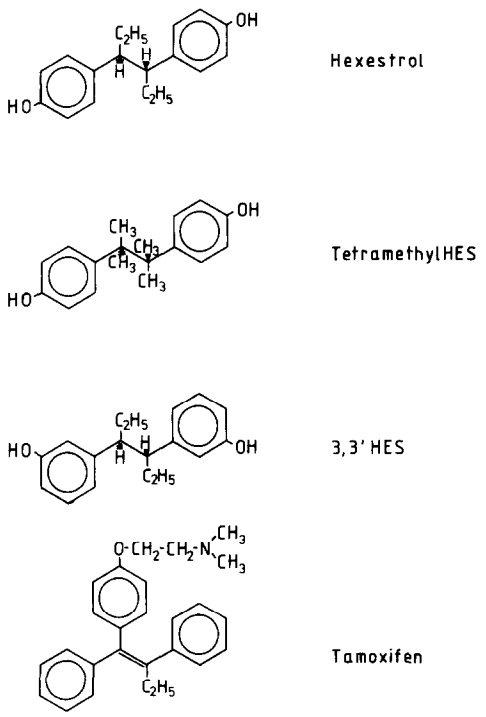

Fig. 1. Structural formulae of hexestrol and antiestrogens used in this study.

compound did not stimulate the synthesis of vitellogenin [4, 5].

As it is generally known that estrogens also inhibit the growth of hormone-dependent human and experimental mammary tumors, it cannot be excluded that 3,3'HES and tetramethylHES as well as all other antiestrogens are acting as estrogens inhibiting tumor growth.

The aim of the present investigation is to elucidate whether the antitumor activity of the antiestrogens tetramethylHES, 3,3'HES and tamoxifen is due to their estrogenic or their antiestrogenic activity.

MATERIALS AND METHODS

Chemicals

3,3'HES and tetramethylHES were synthesized in the author's laboratory [1, 2]. Tamoxifen was a gift of Dr. Ziegler, ICI, Heidelberg, F.R.G. Diethylstilbestrol (DES) was purchased from Merck, Darmstadt, F.R.G. and 9,10-dimethyl-1,2-benzanthracene (DMBA) was purchased from Sigma Chemie GmbH, München, F.R.G.

Induction of tumors

Virgin female Sprague–Dawley (SD) rats weighing 110–130 g were purchased from the Zentralinstitut für Versuchstierzucht, Hannover, F.R.G. The animals were multiply housed and fed Altromin® laboratory chow and water *ad libitum*. At the age of 50 days mammary tumors were induced by gastric intubation with a single dose of 20 mg DMBA in 1 ml olive oil. After three weeks the rats were palpated for tumors at weekly intervals. The tumor area was recorded by means of a caliper as the product of two perpendicular diameters, one measured across the greatest width.

Treatment of animals

When at least one tumor had covered an area of 140 mm^2 the animals were divided into experimental groups of 10 animals according to number of tumors and total tumor area. Animals had 1–4 tumors at the beginning of the treatment. Under light ether anesthesia the ovaries of tumor-bearing hosts were removed through incisions in the lumbar region of the back. The treatments employed were subcutaneous administration of olive-oil solutions of the test compounds. The intact control rats and the ovariectomized control animals received only olive-oil injections. The animals were injected 6 times a week with 1 ml/kg body wt. Treatment was given for 4 weeks and tumor size was measured twice weekly. Based on the tumor area at the end of the therapy, every single tumor was classified as CR, PR, NC or P tumor (CR = complete remission, tumor not palpable; PR = partial remission, reduction in tumor size $\geqslant$50% of initial tumor size; NC = no change, tumor size 51–150% of initial tumor size; P = progressing tumor, tumor size >150% of initial tumor size). At the 28th day after initiation of the therapy the animals were killed and completeness of castration was checked.

Statistical analysis

The significance of the results was determined by the *U* test according to Wilcoxon, Mann and Whitney.

RESULTS

Effect of diethylstilbestrol (DES) on the tumor growth of ovariectomized, DMBA tumor-bearing rats

SD rats bearing DMBA-induced mammary tumors were ovariectomized and treated with the estrogen DES (Fig. 2, Table 1). In the smallest dose tested (0.1 μg/kg/day) (this dose is insufficient to reach a physiological estrogen level) a slight stimulation of tumor growth compared to the untreated, ovariectomized control was observed. A maximum stimulation of tumor growth was obtained with the 1 μg/kg/day dose. The effect of this dose (i.e. percentage change of tumor area at the end of therapy) was not significantly different from that of the untreated, intact control. A further increase of the DES dose (10 μg/kg/day) led again to a significant inhibition of tumor growth compared to the untreated, intact control.

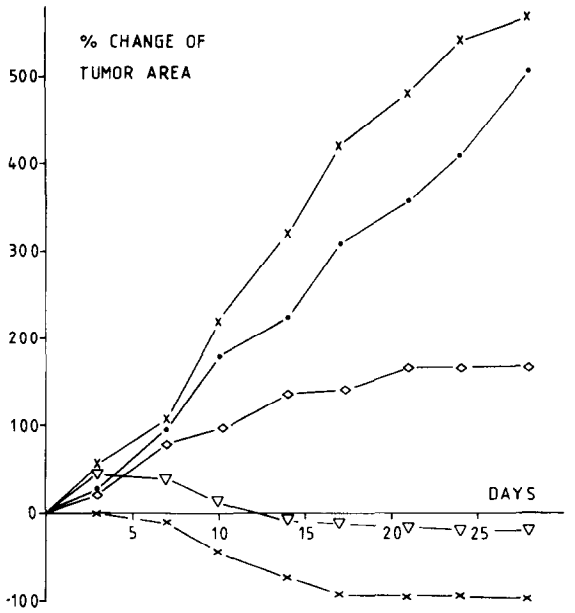

Fig. 2. The effect of DES on tumor area of ovariectomized SD rats bearing DMBA-induced mammary tumors. Intact control (X - X); *control* (x - x); *DES,* 0.1 μg/kg, *s.c., daily* (▽-▽); *DES,* 1 μg/kg, *s.c., daily* (•-•); *DES,* 10 μg/kg, *s.c., daily* (◇-◇).

The estrogen level obtained by this dose is certainly above the physiological value.

Development of a test model to evaluate estrogenic and antiestrogenic activity of mammary tumor-inhibiting antiestrogens

Based on that biphasic DES effect on tumor growth of ovariectomized rats, i.e. stimulation of growth with small doses, inhibition with high doses, a test model was developed to evaluate whether mammary tumor-inhibiting compounds act by means of their estrogenic or antiestrogenic potency. In this ovariectomized, tumor-bearing rats receive the test compound together with DES. Two different DES-doses are applied (0.1 and 10 μg/kg/day). If the test compound unfolds its mammary tumor-inhibiting activity by means of its estrogenic potency, the combination test compound plus DES (0.1 μg/kg/day) should stimulate tumor growth to a larger extent than DES (0.1 μg/kg/day) alone does. In the case of the 10 μg/kg/day dose of DES the inhibition of tumor growth obtained with the combination therapy should be stronger than that of DES alone. If the

Table 1. Effect of DES, DES + antiestrogens and tetramethylHES on ovariectomized SD rats bearing DMBA-induced mammary tumors

Treatment groups				No. of tumors		Effect¶ % of tumors with				% change of	
Drug 1	Dose*	Drug 2	Dose†	B‡	NT§	CR	PR	NC	P‖	Body weight	Tumor area
DES	0.1			25	7	69	13	3	15	12	- 18
DES	1			27	38	3	1	28	68	7	507
DES	10			23	12	17	14	26	43	4	168††
control				23	8	74	23	1	0	14	- 96
intact control				22	53	0	5	21	74	5	570
DES	0.1			25	9	73	6	12	9	18	- 69
DES	0.1	tetramethylHES	1	23	5	54	21	14	11	16	- 70
DES	0.1	3,3'HES	0.5	23	17	12	10	33	45	2	58‡‡
DES	0.1	tamoxifen	0.5	28	7	66	17	6	11	2	- 53
DES	10			23	17	17	15	18	50	0	88
DES	10	tetramethylHES	10	28	7	9	11	29	51	0	135§§
DES	10	3,3'HES	5	28	6	41	24	21	14	-4	- 51§§
DES	10	tamoxifen	5	26	9	29	17	34	20	-5	11§§
		tetramethylHES	0.1	26	1	85	15	0	0	19	- 97
		tetramethylHES	1	21	2	83	17	0	0	12	- 96
		tetramethylHES	10	22	7	31	38	21	10	5	- 51**
control				24	3	89	7	4	0	21	- 98
intact control				25	25	4	6	12	78	6	315

*μg/kg, s.c., daily.
†mg/kg, s.c., daily.
‡At the beginning of the therapy.
§Occurring during the therapy.
‖CR = Complete remission, tumor not palpable; PR = partial remission, reduction in tumor size ⩾50% of initial tumor size; NC = no change, tumor size 51–150% of initial tumor size; P = progression, tumor size >150% of initial tumor size.
¶At the end of therapy.
**Significantly different ($P < 0.05$) from control animals.
††Significantly different ($P < 0.05$) from intact control animals.
‡‡Significantly different ($P < 0.05$) from DES, 0.1 μg/kg, s.c., daily-treated animals.
§§Significantly different ($P < 0.05$) from DES, 10 μg/kg, s.c., daily-treated animals.

antitumor activity of the test compound is due to its antiestrogenic activity reverse effects should be expected.

Estrogenic or antiestrogenic activity of the mammary tumor-inhibiting compounds 3,3'HES, tetramethylHES and tamoxifen?

It is important to apply the test compound in the combination experiment with 0.1 μg DES in a dose in which it shows only a moderate mammary tumor-inhibiting activity on intact DMBA tumor-bearing rats. Otherwise the maximum stimulation might be exceeded—provided that the test compound has an estrogenic mode of action. The tumor growth inhibition under those experimental conditions might be so strong that an antagonistic mode of action could be simulated by obtaining tumor area values below that of 0.1 μg DES.

TetramethylHES was tested in a 1 mg/kg/day dose (change of tumor area obtained with intact tumor bearing rats: control, +573%; TetramethylHES, 1 mg, +411% [1]). Since 3,3'HES unfolds a much stronger tumor-inhibiting activity in this dose (change of tumor area: control, +247%; 3,3'HES, 1 mg, +47% [2]), the latter compound was tested in the 0.5 mg dose. Tamoxifen was applied in the same dose, though the tumor growth-inhibiting effects of this compound were not as strong as those of 3,3'HES [8], but stronger than those of tetramethylHES [1].

As shown in Fig. 3, 3,3'HES led to a significantly strong increase of the tumor growth-stimulating effect of 0.1 μg DES on ovariectomized rats, whereas the effects of tetramethylHES and tamoxifen on this DES dose (decrease and increase respectively) were not significant (Fig. 3, Table 1).

In the combination experiment with 10 μg DES the test compounds were applied in doses in which they had shown strong tumor growth-inhibitory activity in intact DMBA tumor-bearing rats [1, 2]. These relatively high doses are necessary to obtain significant effects. TetramethylHES, 3,3'HES and tamoxifen were given in 10, 5 and 5 mg/kg/day doses respectively.

As expected for an estrogen antagonist, tetramethylHES diminishes the tumor growth-inhibitory activity of 10 μg DES (Fig. 4, Table 1). On the other hand, 3,3'HES and tamoxifen increase the mammary tumor-inhibiting activity of that high DES dose (Fig. 4, Table 1).

Tumor growth-stimulating effects of tetramethylHES on ovariectomized DMBA tumor-bearing rats

As tetramethylHES had not shown any ability to increase the tumor growth-stimulating and inhibiting effects of DES, respectively, but had rather exhibited DES-antagonizing activity, this antiestrogen was given alone to ovariectomized DMBA tumor-bearing SD rats (Fig. 5, Table 1).

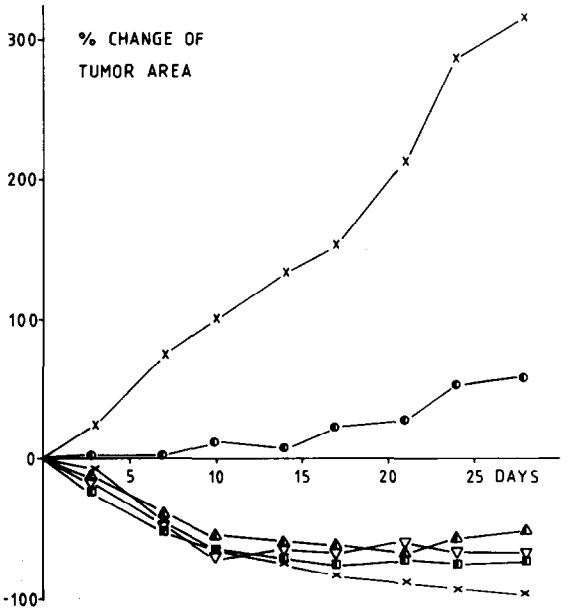

Fig. 3. The effect of antiestrogens on tumor area of ovariectomized, small DES dose-treated SD rats bearing DMBA-induced mammary tumors. Intact control (X - X); *control* (x - x); *DES*, 0.1 μg/kg, *s.c., daily* (▽-▽); *DES*, 0.1 μg/kg, *s.c., daily + tetramethylHES*, 1 mg/kg, *s.c., daily* (◧- ◧) + *3,3'HES*, 0.5 mg/kg, *s.c., daily* (◐ - ◐) + *tamoxifen*, 0.5 mg/kg, *s.c., daily* (▲ - ▲).

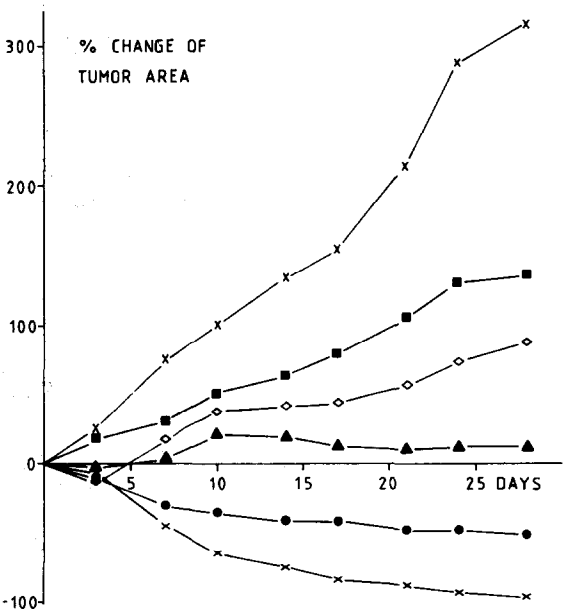

Fig. 4. The effect of antiestrogens on tumor area of ovariectomized, high DES dose-treated SD rats bearing DMBA-induced mammary tumors. Intact control (X - X); *control* (x - x); *DES*, 10 μg/kg, *s.c., daily* (◇-◇); *DES*, 10 μg/kg, *s.c., daily + tetramethylHES*, 10 mg/kg, *s.c., daily* (■ - ■) + *3,3'HES*, 5 mg/kg, *s.c., daily* (• - •) + *tamoxifen*, 5 mg/kg, *s.c., daily* (▲ - ▲).

As expected, the biphasic DES effect (Fig. 2, Table 1) was not obtained with tetramethylHES (Table 1). In the highest dose (10 mg/kg/day), however, a slight stimulation of tumor growth was observed (Fig. 5).

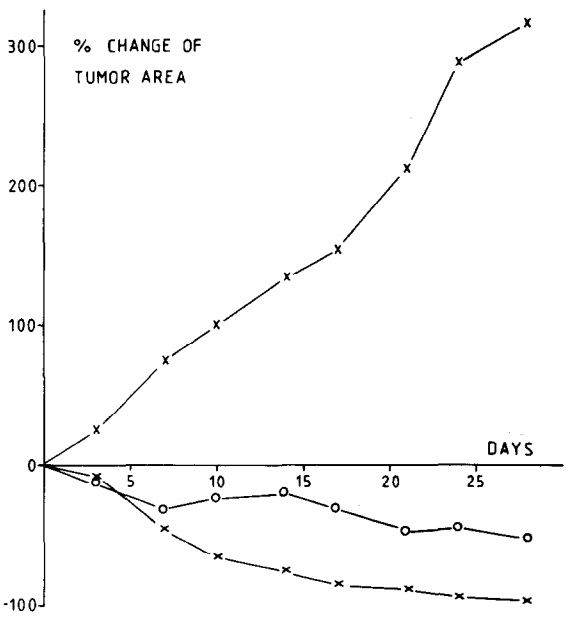

Fig. 5. The effect of tetramethylHES on tumor area of ovariectomized SD rats bearing DMBA-induced mammary tumors. Intact control (X - X); *control* (x - x); *tetramethylHES*, 10 mg/kg, *s.c., daily* (O - O).

DISCUSSION

It has been known for a long time that antiestrogens diminish the tumor-inhibiting effect of ovariectomy [9, 10]. This has been explained by the residual estrogenicity of these antiestrogens [9, 10]. Recently we put forward the hypothesis that antiestrogens might unfold their mammary tumor-inhibiting activity because of their estrogenic side-effects [8, 11].

In our first attempt to elucidate whether the antitumor activity of antiestrogens is due to their estrogenic or their antiestrogenic activity we determined the activities of carbohydrate metabolism-linked enzymes and histological characteristics of regressing DMBA-induced tumors after ovariectomy and treatment of the host with hexestrol or an antiestrogen (3,3'HES and tamoxifen respectively [8, 11]), since it is described that there are significant differences between regressing tumors due to ovariectomy and estrogen treatment [12]. We did not find differences and with that destroyed the validity of Hilf's hypothesis that estrogens inhibit mammary tumor growth by directing neoplastic cell metabolism towards secretion rather than cell proliferation [13].

The results of this work indicate that some mammary tumor-inhibiting compounds—described in the literature as antiestrogens—indeed seem to unfold their antitumor activity by means of their estrogenic potency (3,3'HES and tamoxifen). In contrast to this, the antiestrogen tetramethylHES might act as an estrogen antagonist inhibiting tumor growth, though this compound seems to possess a slight estrogenic activity on the tumor as well.

In this investigation it was also shown that a definite estrogen level is essential for maximum growth of the DMBA-induced mammary tumor of the SD rat. Supraphysiological or pharmacological estrogen doses lead to a decrease of tumor growth, as does the removal of estrogens, such as in ovariectomy. The definite estrogen level might be the physiological level, for very small DES doses (0.1 and 1 μg/kg/day) did not show any stimulation of tumor growth but caused a decrease of tumor area in intact DMBA tumor-bearing SD rats [11].

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