

# Enhancement of Toremifene Anti-Tumor Action by Metformin and Unusual Side Effect of Toremifene in Male Transgenic Mice with HER2-Positive Breast Tumor

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## Key words

toremifene, metformin, breast cancer, FVB/N HER-2/neu mice, hernia, antitumor effect

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## ABSTRACT

HER2-positive breast tumors are found in 25–30% of patients with breast cancer and are characterized by aggressive course and reduced sensitivity to both chemotherapy and hormone therapy. The aim of the work was to study the possibilities of enhancing the therapeutic effect of anti-estrogen drug toremifene by combining it with biguanide, metformin, on the HER2-positive breast cancer model in FVB/N HER-2/neu transgenic mouse. Male FVB/N mice with intramuscularly transplanted HER2-positive mammary gland tumor from a female mouse of the same strain have been given toremifene (30 mg/kg, orally daily) or metformin (100 mg/kg, orally daily) that had a moderate antitumor effect (decrease the area under the kinetic curve of tumor growth by 1.6 and 1.5 times, respectively, when compared with intact control). Co-administration of these drugs in the same doses had a more pronounced effect (the area under the kinetic curve of tumor growth decreased by 3.1 times compared to intact control;  $p < 0.05$ ). After 10 days, in group receiving toremifene all 10 mice developed inguinal-scrotal hernias, and in group that received toremifene plus metformin - only 5 of 10 ( $p = 0.0325$ ). By the 15<sup>th</sup> day after the start of treatment, the hernias was also determined in all mice treated with the combination of toremifene and metformin, but the size of the hernial sac was significantly smaller than in those receiving only toremifene -  $537 \pm 96 \text{ mm}^3$  and  $1309 \pm 120 \text{ mm}^3$ , respectively ( $p = 0.0001$ ). A possible explanation is the manifestation of collagen-degrading effect of toremifene.

## Introduction

HER2-positive breast tumors are found in 25–30% of patients with breast cancer and are characterized by aggressive course and reduced sensitivity to both chemotherapy and hormone therapy [1, 2]. There is evidence that the use of anti-diabetic biguanide metformin can enhance the effectiveness of chemotherapy and hormone therapy of tumors, including the mammary gland [3, 4].

The aim of this study was to investigate the anti-tumor activity of anti-estrogen drug toremifene in transgenic HER-2/neu FVB/N mice with a HER2-positive breast tumors and to evaluate the modifying influence of metformin on the effect of toremifene.

## Materials and Methods

### Animals

In experiments, we used FVB/N strain mice, carrying *erbB2* (HER2) gene, which are characterized by a high incidence of the development of spontaneous HER2-positive mammary adenocarcinomas (in females up to 100%, in males - in rare cases) [5].

These FVB/N mice were obtained from the National Institute of Aging (Ancona, Italy) and are maintained as local breeding stock in vivarium of N.N. Petrov National Medical Research Center of Oncology (St. Petersburg, Russia). All animals were kept under standard conditions (12/12 h light/dark regimen and at 21–23 °C). They received standard pellet laboratory diet (PK-120; Laboratorkorm, Moscow, Russia) and tap water *ad libitum*.

### Drugs

The following drugs were used in the experiments: toremifene (Fareston, Orion Corporation, Finland) and metformin (HEMOPARM A.D., Serbia).

### Experimental design

Studies were conducted on 42 adult male mice of the FVB/N strain weighing 22–25 g. All mice were inoculated intramuscularly with a 20% suspension of tumor tissue taken from the female mouse of same strain with spontaneous breast cancer. On the 5<sup>th</sup> day after tumor inoculation, the mice were randomized and drug treatment was started.

- Group I (n = 12) – Control,
- Group II (n = 10) – Toremifene (60 mg/kg daily),
- Group III (n = 10) – Metformin (200 mg/kg daily),
- Group IV (n = 10) – Toremifene (60 mg/kg daily) + Metformin (200 mg/kg daily).

All drugs were given with drinking water, calculated on the basis of daily consumption of about 4 ml per mouse. Drug solutions were prepared daily. The drugs were given to mice daily for 23 days until the end of the experiment.

### Quantitative evaluation of anti-tumor effect

Antitumor activity was assessed by calculating the percentage of inhibition of tumor growth, as well as by the area under the kinetic curve of tumor growth and tumor growth index.

The percentage of inhibition of tumor growth (TI%) was determined by the formula:

$$TI\% = \frac{V_C - V_T}{V_C} \times 100$$

where  $V_C$  is the average tumor volume in mice of the control group, and  $V_T$  is the average tumor volume in mice of the experimental group receiving the drug.

The tumor volume was calculated using the modified ellipsoidal formula [6]:

$$V = \frac{A \times B^2}{2}$$

where  $V$  – tumor volume, mm<sup>3</sup>;  $A$  – the greatest longitudinal diameter (length), mm;  $B$  – the greatest transverse diameter (width), mm.

The same formula was used to measure the volume of the hernial sac in mice with inguinal-scrotal hernias that complicated treatment with toremifene.

Due to intramuscular tumor inoculation, the volume of the whole thigh with the tumor was measured, therefore, to more accurately determine the actual volume of the tumor, the volume of the healthy contralateral thigh was subtracted from the obtained value.

The tumor growth index (TGI) was determined in percent by the formula:

$$TGI\% = \frac{S_E}{S_C} \times 100$$

where  $S_E$  is the area under the kinetic curve of tumor growth in the experimental group of mice, and  $S_C$  is the area under the kinetic curve of tumor growth in mice of the control group. It is obvious that an TGI in the control will always be equal to 100%, whereas in the experimental groups, with regression or slowing down of tumor growth, the TGI will be less than 100%, and when stimulating tumor growth, it will be more than 100%. It is important to note that the TGI is an integral criterion for the antitumor activity of the drug, which makes it possible to evaluate not only the degree of expression of the antitumor action, but also its duration.

The trapezoidal method was used to measure the area under the kinetic curve of tumor growth.

### Statistic analysis

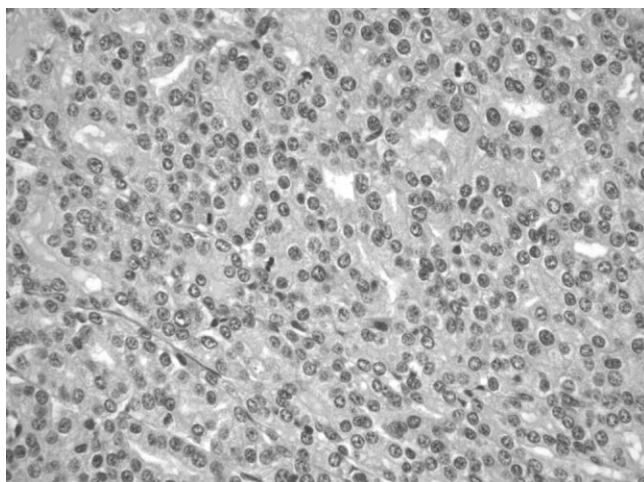
Statistical analysis was performed using programs GraphPad® Prism 6, SPSS® Statistics version 17.0. Student's t-test was used.  $P < 0.05$  was considered statistically significant.

### Ethical approval

All experimental procedure and also the design of these experiments was approved by the Ethics Committee of the N.N. Petrov National Medical Research Center of Oncology (St. Petersburg, Russia), following international guidelines for the care and use of animals.

## Results

Comparative results of the inhibitory effect (TI%) of toremifene and metformin on the growth of an intramuscularly implanted HER2-positive breast tumor (► **Fig. 1**) in male FVB/N mice are presented in ► **Table 1**. The course administration of toremifene in the daily dose of 200 mg/kg had a pronounced antitumor effect. At 12–18 days tumor growth inhibition reached 54–81% and was statistically significant. Another drug, metformin, caused a slight but statistically significant inhibition of tumor growth by 12<sup>th</sup> day. With the



► **Fig. 1** The histological picture of mammary tumor transplanted intramuscularly to male FVB/N mouse from female mouse with HER2-positive spontaneous mammary adenocarcinoma. Hematoxylin-eosin. X 400.

combination of toremifene with metformin, inhibition of tumor growth was more pronounced than with their separate use. In addition, this antitumor effect was significantly manifested during all days of treatment (► **Table 1**).

When evaluating the antitumor activity of the drugs with indicators (*S* - the area under the kinetic curve of tumor growth and TGI - tumor growth index), a significant effect was manifested only with the combined use of toremifene and metformin (► **Table 2**).

In the course of this experiment, from the 10<sup>th</sup> day after the start of toremifene administration (group II), all 10 mice developed inguinal-scrotal hernias (► **Fig. 2**), which, as was found in autopsy at the end of experiment, contained intestinal loops. Similar hernias were diagnosed in 5 of 10 mice treated with toremifene in combination with metformin (group IV). On the 15<sup>th</sup> day after the start of the administration of toremifene, the hernial sacs were present in all mice of group IV, but their volumes were significantly smaller than in mice of group II (► **Table 3**). In groups 1 (control) and III (administration of metformin) no hernias were found in the course of experiment.

## Discussion

Toremifene as an antiestrogen drug is widely used in hormone therapy of estrogen-positive breast cancer, not only in women, but also in men [7]. The effectiveness of anti-estrogen drugs in HER2-positive breast tumors is markedly lower, even with estrogen-receptor-positive tumors [2]. In our experiments, toremifene produced a marked inhibitory activity on tumor growth of HER2-positive breast cancer in FVB/N mice transgenic for the HER2 proto-oncogene. In particular, it was established that the content of estrogen

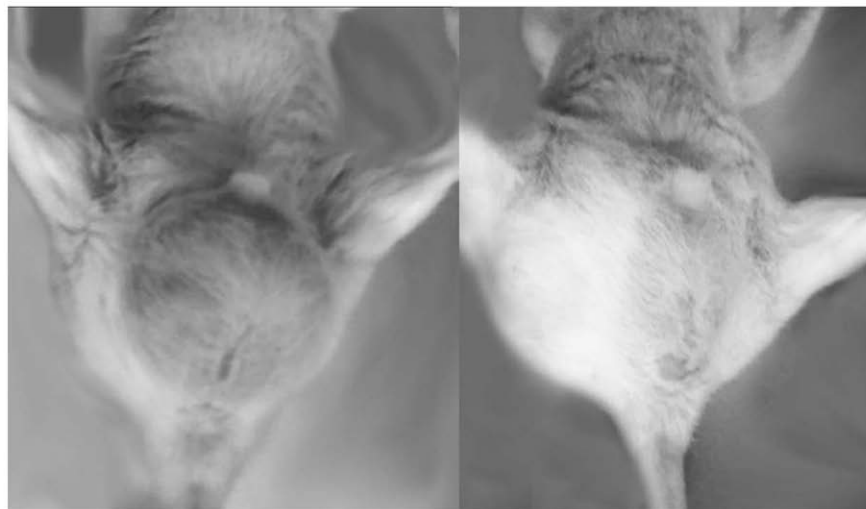
► **Table 1** The influence of toremifene and metformin on the growth HER2-positive mammary gland tumors in male FVB/N mice.

No. group, treatment	Tumor volume, mm <sup>3</sup> (M ± m); TI% to control				
	Days after tumor inoculation				
	12	15	18	20	23
I. Control (n = 12)	52 ± 8	108 ± 17	262 ± 52	619 ± 167	885 ± 190
II. Toremifene (n = 10)	10 ± 5	31 ± 10	119 ± 30	387 ± 202	677 ± 286
	81 %	71 %	54 %	37 %	24 %
	p = 0,0007	p = 0,0021	p = 0,0377	p = 0,2646	p = 0,4753
III. Metformin (n = 10)	29 ± 4	79 ± 10	141 ± 29	452 ± 106	566 ± 151
	44 %	27 %	46 %	27 %	36 %
	p = 0,0231	p = 0,1669	p = 0,0637	p = 0,4046	p = 0,1991
IV. Toremifene + metformin (n = 10)	3,5 ± 3,5	26 ± 8	96 ± 22	228 ± 44	250 ± 55
	93 %	76 %	63 %	64 %	72 %
	p < 0,0001	p = 0,0009	p = 0,0142	p = 0,0495	p = 0,0064

TI – tumor inhibition, %; p - in comparison with group I.

► **Table 2** Antitumor activity of toremifene and metformin on the model of HER2-positive mammary gland tumors in male FVB/N mice, according estimate of the effect on the area under the kinetic curve of tumor growth (*S*) and tumor growth index (TGI).

No. group, treatment	Number of mice	<i>S</i>	TGI	p in comparison with group I
I. Control	12	3932 ± 896	100 %	
II. Toremifene	10	2388 ± 1046	59 %	>0.05
III. Metformin	10	2612 ± 570	66 %	>0.05
IV. Toremifene + metformin	10	1268 ± 292	32 %	<0.05



► **Fig. 2** An inguinal-scrotal hernia in a male FVB/N mouse (left) that developed on the 10th day after the administration of toremifene (60 mg/kg orally daily). On the right - the mouse from the control group.

► **Table 3** Influence of toremifene and metformin on the volumes of hernia sac inguinal-scrotal hernias in male FVB/N mice

No. group, treatment	Number of mice	Volume of hernia sac, mm <sup>3</sup>	%% in comparison with group II	pin comparison with group II
I. Control	12	0 ± 0 mm <sup>3</sup>	0	<0.0001
II. Toremifene	10	1309 ± 120 mm <sup>3</sup>	100	
III. Metformin	10	0 ± 0 mm <sup>3</sup>	0	<0.0001
IV. Toremifene + metformin	10	573 ± 96 mm <sup>3</sup>	44	0.0001

Note: the measurement was made on the 15th day after start of the administration of toremifene and metformin.

receptors in these tumors does not exceed 10 fM/mg protein, and the content of progesterone receptors was below this level [8]. In our experiments, metformin had a slight inhibitory effect on the growth of HER2-positive tumors in mice. The combination of toremifene with metformin contributed to the mutual enhancement of their antitumor action.

Toremifene is one of non-steroidal selective estrogen receptor modulators (SERMs), which are both antagonists and estrogen receptor agonists. SERMs have an anti-estrogenic effect mainly on the mammary gland, and at the same time, its estrogenic effect can occur to other organs such as bones, liver and uterus [9]. In clinical use, toremifene causes numerous side effects, the most frequent of which are hot flashes (20%), increased sweating (14%), nausea (8%), dizziness (4%), peripheral edema (3%) [10]. In our experiments, an unusual side effect of toremifene in the form of an inguinal hernia repair was established for the first time. All mice of the FVB/N male strain, after 10 days of daily oral administration of toremifene in a dose of 60 mg/kg, developed significant inguinal-scrotal hernia. The administration of metformin simultaneously with toremifene slowed down hernia development.

A possible explanation for the occurrence of inguinal-scrotal hernias in FVB/N male mice under the influence of the antiestrogen toremifene is its estrogen-like side effect on connective tissue. According to S. Jackson, et al. [11], estrogen along with the stimula-

tion of the formation of collagen in the connective tissue can cause its degradation. There is evidence of pluripotent effect of estrogen on collagen metabolism in the pelvic fascia, which is associated with both an increase in its synthesis and degradation [11].

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## Conflict of Interest

The authors declare that there is no conflict of interest.

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