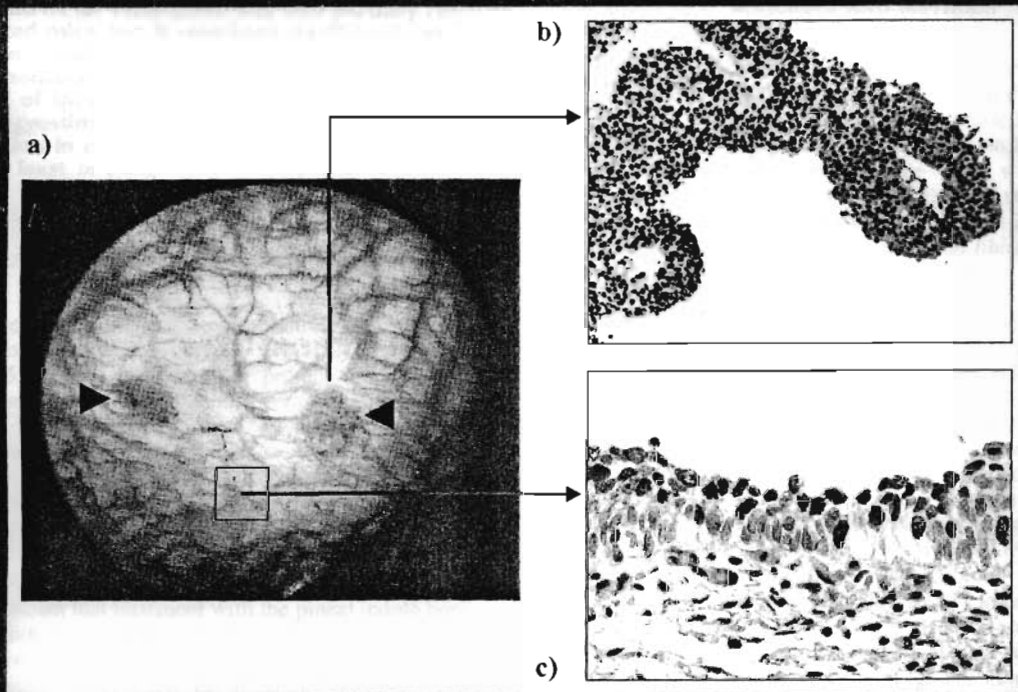


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INHIBITORY EFFECT OF THE PEPTIDE EPITALON ON THE DEVELOPMENT OF SPONTANEOUS MAMMARY TUMORS IN HER-2/NEU TRANSGENIC MICE

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Female FVB/N HER-2/neu transgenic mice from the age of 2 months were subcutaneously injected with saline, the peptide Epitalon® (Ala-Glu-Asp-Gly) or with the peptide Vilon® (Lys-Glu) in a single dose of 1 µg/mouse for 5 consecutive days every month. Epitalon treatment reduced the cumulative number and the maximum size of tumors ($p < 0.05$). Furthermore, the number of mice bearing 1 mammary tumor was increased, whereas the number of mice bearing 2 or more mammary tumors was reduced in Epitalon-treated in comparison to saline-treated animals ($p < 0.05$). The size but not the number of lung metastases was reduced in Epitalon-treated compared to saline-treated mice ($p < 0.05$). The treatment with Vilon produced significant negative effects when compared to the control group, with an increased incidence of mammary cancer development ($p < 0.05$), a shorter mean latent period of tumors ($p < 0.05$) and an increased cumulative number of tumors ($p < 0.05$). A 3.7-fold reduction in the expression of HER-2/neu mRNA was found in mammary tumors from HER-2/neu transgenic mice treated with Epitalon compared to control animals. The expression of mRNA for HER-2/neu was also partially reduced in Vilon-treated mice, but it remained significantly higher in Vilon- than in Epitalon-treated animals (1.9-fold increase). The data demonstrate the inhibitory effect of Epitalon in the development of spontaneous mammary tumors in HER-2/neu mice, suggesting that a downregulation of HER-2/neu gene expression in mammary adenocarcinoma may be responsible, at least in part, for the antitumor effect of the peptide.

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Key words: peptide; HER-2/neu; transgenic mice; mammary cancer

Breast cancer is one of the most common cancers and is a leading cause of mortality in women.^{1,2} The HER-2/neu oncogene encodes a 185 kDa (p 185) receptor protein belonging to the epidermal growth factor receptor family involved in organogenesis and epithelial differentiation.³ Amplification and mutation of HER-2/neu plays a pathogenetic role in several malignancies, including carcinoma of the breast, ovary and uterus.^{4,5} Overexpression of ErbB-2/HER-2/neu occurs in 15–40% of human breast cancers.⁶ Its appearance correlates with poor prognosis, and it is, therefore, an important target for physiologic investigation and therapeutic intervention.⁵

It has been shown that treatment with the pineal indole hormone melatonin inhibits the development of mammary gland tumorigenesis both *in vitro* and *in vivo*.^{7–10} It was also shown that the polypeptide pineal preparation Epithalamin® inhibits both the growth of transplantable mammary tumors and the development of spontaneous mammary tumors in female C3H/Sn and SHR mice and mammary carcinogenesis induced by 7,12-dimethylbenzanthracene mammary tumors or by total-body X-ray irradiation in female rats.^{11–13} Clinical use of Epithalamin was shown to be effective for the treatment of ovarian disturbances and of some types of cancer, breast cancer included.¹⁴ However, the *in vivo* use of Epithalamin has been rather restricted due to difficulties in the availability of calf pineal gland for its production. The synthesis of the pineal tetrapeptide Ala-Glu-Asp-Gly (Epitalon) with high biologic activity¹⁵ gives a new opportunity for its implementation in clinical practice. The pineal tetrapeptide has been structured on the

basis of the amino acid analysis of pineal peptide preparation Epithalamin,¹⁴ and on the basis of the tetrapeptide the medicine Epitalon® has been designed. It was recently shown that epitalon increased the life span in 2 strains of fruit flies and in female CBA mice and inhibits the spontaneous tumorigenesis in mice.^{15–17} Thymic dipeptide Lys-Glu (Vilon) has been structured on the basis of the amino acid analysis of thymus preparation Thymalin®, amino acid sequences of thymic peptides and cytokines.¹⁸

The purpose of our study was to evaluate the influence of Epitalon on mammary tumor development in transgenic mice carrying a mammary cancer gene HER-2/neu. We hypothesised that exposure to the peptide exerts a protective effect. The mechanism of the pineal hormone action was investigated studying the HER-2/neu mRNA expression in tumors from the pineal peptide-treated animals. The thymic dipeptide was used as comparative preparation.

MATERIAL AND METHODS

Animals

Homozygous HER-2/neu transgenic mice originally obtained from Charles River (Hollister, CA) by the Italian National Research Center for Aging (INRCA) were housed and bred in the Department of Carcinogenesis and Oncogerontology, N.N. Petrov Research Institute of Oncology. The mice were kept 5–7 in polypropylene cages (30 × 21 × 10 cm) under standard light/dark regimen (12 hr light:12 hr darkness) at 22 ± 2°C and received standard laboratory chow and tap water *ad libitum*.

Experimental design

Ninety female FVB/N HER-2/neu mice at the age of 2 months were randomly divided into 3 groups. Mice of the control group were subcutaneously injected with 0.1 ml of 0.9% normal saline for 5 consecutive days every month, whereas the mice of the second and third group received subcutaneously 1.0 µg of either Vilon or Epitalon dissolved in 0.1 ml of saline. This treatment dosage and regimen was effective for the inhibition of spontaneous tumorigenesis in female CBA mice.¹⁷ Vilon and Epitalon were synthesised in St. Petersburg, Institute of Bioregulation and Gerontology by E.I. Grigoriev, and both were 99.8% purity. Once a week all mice were palpated for detection of mammary tumor appearance. The localisation and the size of tumors were registered on the special charts. Once a month all mice were weighed and, simultaneously, the amount of consumed food was measured and the rate of the consumed food mass (g) per mouse and per body

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weight unit were calculated. Once every 3 months, vaginal smears of the animals were cytologically examined daily for 2 weeks to estimate the estrus function. The time of appearance of mammary tumors was evaluated by palpation, and the neoplastic masses were measured with calipers in the 2 perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Because some treated mice did not display carcinomas in all mammary glands, the mean number of palpable mammary carcinomas/mouse was calculated as the cumulative number of incident tumors/number of tumor-bearing mice.

Pathomorphologic examination

All of the animals were autopsied. Site, number and size of mammary tumors and their metastases in lungs were checked. All of the tumors as well as the tissues and organs with suspected tumor development were excised and fixed in 10% neutral formalin. After the routine histologic processing, the tissues were embedded into paraffin. 5–7 μ m thin histologic sections were stained with haematoxylin and eosine and were microscopically examined. Tumors were classified according to International Agency for Research on Cancer recommendations.¹⁹

RNA extraction and RT-PCR

The expression of mRNA for HER-2/neu was evaluated in mammary tumor from HER-2/neu transgenic mice by RT-PCR. After homogenisation of tissue sample, RNA was extracted using TRI-REAGENT™ according to the manufacturer's instructions (Sigma Chemical, St. Louis, MO).

RNA concentrations were determined using the spectrophotometer (scientific instruments UV1601 Scimadzu, Columbia, MD, USA). cDNA was synthesised from 0.1 μ g RNA incubating RNA with dNTP (0.5 mM), Oligo dT (12.5 ng/ μ l), First Strand Buffer (1 \times), M-MLV Reverse Transcriptase (10 U/ μ l), Rnase Inhibitor (1 U/ μ l) and DTT (0.01 M) all from Gibco BRL (Life Technologies srl, Milano, Italy), in a final volume of 20 μ l.

The samples were incubated at 37°C for 1 hr and 95°C for 10 min; subsequently cDNA was frozen at -20°C until use. PCR was

performed by incubating 5 μ l cDNA with a reaction mixture containing: PCR Buffer (1 \times), MgCl₂ (1.5 mM), dNTP (200 μ M), specific forward and reverse primers (0.8 μ M of each), Taq DNA Polymerase (1 U/ μ l) in a total volume of 50 μ l (all from Roche Diagnostics, Mannheim, Germany). The samples were incubated in a GeneAmp PCR System 9700 (Perkin Elmer, Shelton, Connecticut) for a total of 35 cycles for HER-2/neu and 30 cycles for β -actin. Each cycle consisted of: 1 min at 94°C, 1 min at 65°C, 1 min at 72°C for HER-2/neu; 1 min at 94°C, 2 min at 63°C, 1 min at 72°C for β -actin.

The primers for HER-2/neu and β -actin were purchased from Roche Diagnostics using DNA published cDNA sequences. The HER-2/neu fragment of 239 bp was defined by the forward primer 5'-GATCGAATTCCTGGAGGACGTGCCGCTTGTA and the reverse primer 5'-GATCAAGCTTATAGCTCCACACATCACTCTG. β -actin fragment of 349 bp by the forward primer 5'-TGGAATCCTGTGGCATCCATGAAAC and the reverse primer 5'-TAAAACGCAGCTCAGTAACAGTCCG. The PCR products and a molecular weight standard (DNA molecular weight marker VIII, Roche Diagnostics) were visualised after electrophoresis in a 1.5% agarose gel containing 1 μ g/ μ l ethidium bromide (EtBr). Densitometric analysis was performed using the GelDoc 2000 (Biorad Laboratories, Milano, Italy).

Statistics

Experimental results were statistically processed by the methods of variation statistics with the use of STATGRAPH statistic program kit. The significance of the discrepancies was defined according to the Student *t*-criterion, Fischer exact method, χ^2 , non-parametric Wilcoxon-Mann-Whitney and Friedman RM anova on ranks. Student-Newman-Keuls method was used for all pairwise multiple comparisons.

RESULTS

The body weight gain and the food consumption were similar in the Epitalon-, Vilon- and saline-treated groups. There were no significant differences in the length or regularity of the estrus

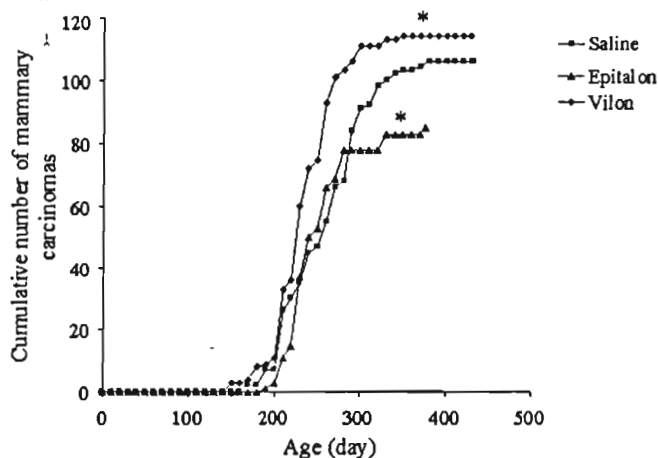


FIGURE 1 – Cumulative number of tumors in HER2/neu transgenic mice supplemented with Epitalon or Vilon. Mice were supplemented with Epitalon or Vilon as reported in Material and Methods. The cumulative number of mammary carcinomas yielded in each supplemented group in relationship to the age of mice is shown. * $p < 0.05$ vs. saline-treated group of animals.

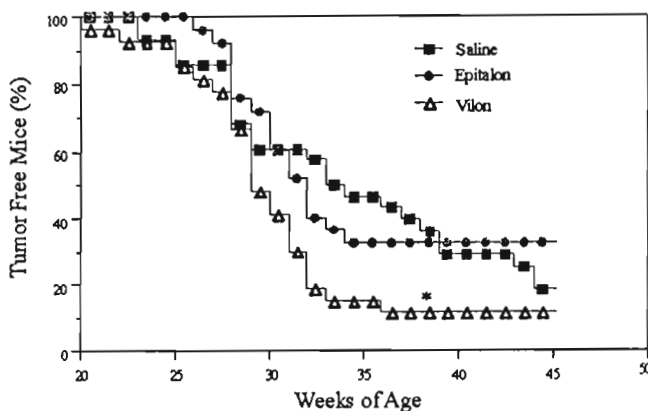


FIGURE 2 – Progression of mammary carcinogenesis in HER-2/neu transgenic mice supplemented with Epitalon or Vilon. The percentage of tumor-free mice supplemented with Epitalon or Vilon in relationship to the age of mice is shown. The number of mice for each treatment was 28 (saline), 25 (Epitalon), 27 (Vilon). * $p < 0.05$ vs. saline-treated group of animals.

TABLE 1 – EFFECT OF EXPOSURE TO VILON AND EPITALON ON MAMMARY TUMORIGENESIS IN HER-2/NEU TRANSGENIC FEMALE MICE

Group	No. of mice	No. of tumor-bearing mice (%)	Mean latent period of tumors (days)	Maximum size of tumors (cm)	No. of tumors per mouse
Saline	28	23 (82.1%)	257 \pm 4.4	2.1 \pm 0.20	4.6 \pm 0.5
Vilon	27	24 (88.9%)	235 \pm 3.3 ¹	1.9 \pm 0.19	4.8 \pm 0.4
Epitalon	25	18 (72.0%)	246 \pm 3.9	1.4 \pm 0.18 ¹	4.7 \pm 0.6

¹Difference with mice treated with saline is significant ($p < 0.05$).

cycles between these groups (data not shown). At the microscopic examination, all tumors of a mammary gland were classified as adenocarcinomas type B¹⁹ and revealed a solid, lobular and cribriform structure with multiple haemorrhagic cysts. There was no difference in the morphologic structure of mammary carcinomas between these groups.

As shown in Figure 1, the cumulative number of mammary tumors that spontaneously developed in HER-2/neu mice was significantly reduced in the Epitalon-treated group compared to the saline-treated group ($p < 0.05$). Epitalon treatment was not able to modify the appearance of mammary tumors in a significant way: The first mammary tumor was revealed at the 168th day of life in a mouse given saline and at the 188th day of life in a mouse treated with Epitalon. The treatment of mice with Epitalon failed to significantly change the incidence of mammary adenocarcinoma development. The mean latent period of tumors and the number of tumors per mouse were also unaffected by Epitalon treatment (Table I), whereas the maximum size of tumors was reduced in Epitalon-treated compared to saline-treated groups (Table I, $p < 0.05$). The treatment with Vilon produced significant negative effects when compared to the control group, with an increased incidence of mammary cancer development (Fig. 2, $p < 0.05$), a shorter mean latent period of tumors (Table I, $p < 0.05$) and an increased cumulative number of tumors (Fig. 1, $p < 0.05$).

As shown in Figure 3, the number of mice that developed only 1 mammary tumor was 7% in the control group, 4% in a group of mice given Vilon and 16% in the group given Epitalon ($p < 0.05$). The number of mice that developed 2 or more mammary tumors was 75%, 85% and 56% ($p < 0.05$) in the same groups (Fig. 3). As shown in Table II, the number of mice with lung metastases was not significantly different in the groups given Epitalon or saline, but in the group of mice given Epitalon it was 2.5-fold less than in the group of mice treated with Vilon ($p < 0.05$). The size of lung metastases in the group of mice given Epitalon was smaller than in the other ones (Table II, $p < 0.05$).

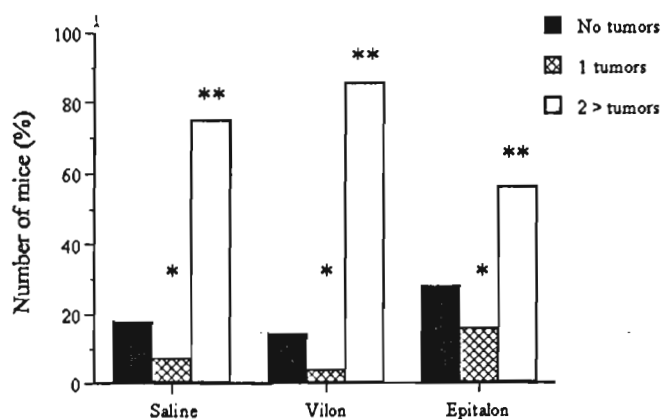


FIGURE 3 – Number of mammary carcinomas in HER-2/neu transgenic mice supplemented with Epitalon or Vilon. The percentage of mice having 1 tumor or >2 tumors is shown. Epitalon supplementation reduced the number of mice with more than 2 tumors ($p < 0.05$). The number of mice for each treatment was 28 (saline), 25 (Epitalon), 27 (Vilon). * and **: $p < 0.05$ among groups.

To evaluate whether the inhibition of mammary tumorigenesis observed in mice treated with Epitalon was due to its effect on mammary gland, we performed RT-PCR analysis for HER-2/neu gene expression in the mammary tumors from mice treated with saline, Vilon or Epitalon. As shown in Figure 4, mRNA for HER-2/neu gene was greatly expressed in saline-treated mice, whereas it was significantly decreased (3.7-fold reduction) in animals chronically treated with Epitalon. Surprisingly, mRNA for HER-2/neu also showed a decreased expression in Vilon-treated compared to saline-treated mice (1.9-fold reduction). However, the expression of mRNA for HER-2/neu was higher in Vilon- than in Epitalon-treated animals (1.9-fold increase). The mean relative expression of the HER-2/neu gene as determined by densitometric analysis was 1.637 ± 0.039 , 0.445 ± 0.212 and 0.829 ± 0.096 for saline-, Epitalon- or Vilon-treated tumor cells, respectively.

DISCUSSION

The role of the pineal gland in tumor development has been under intensive study during recent years.^{7,10} In several experimental models, it has been shown that the treatment with melatonin inhibits the development of carcinogen-induced or transplantable tumors in mammary gland, uterine cervix and colon.^{8-10,20} In other studies, long-term treatment with melatonin was followed by an increase of tumor incidence in some mouse strains.²¹⁻²³

The synthesis of Epitalon with high biologic activity¹⁵ gives a new opportunity for its implementation in clinical practice. It was recently shown that Epitalon increased the life span in 2 strains of fruit flies and in female CBA mice and inhibits the spontaneous tumorigenesis in mice.¹⁵⁻¹⁷ The results reported in our study demonstrate that the pineal tetrapeptide Epitalon exerts some inhibitory effects on the development of spontaneous mammary adenocarcinomas occurring in HER-2/neu transgenic mice at an early age.

Epitalon treatment reduced the cumulative number and the maximum size of tumors. Furthermore, in Epitalon-treated mice bearing tumors, the number of mice with 1 mammary tumor was increased, whereas the number of mice bearing 2 or more mammary tumors was reduced compared to saline-treated animals. Finally, Epitalon reduced the maximum size of lung metastasis. These data confirm results recently obtained in experiments with CBA mice, which showed that Epitalon inhibited spontaneous tumorigenesis.¹⁷ Furthermore, they are in agreement with observations showing an inhibiting effect of Epithalamin on the development of spontaneous and transplanted tumors or on tumors induced by carcinogens.^{11,12,17} Our data suggest that the primary effect of Epitalon is not to influence the incidence of mammary tumors but to reduce the tumor burden in tumor-bearing mice. This is shown by the reduced cumulative tumor number, the smaller maximum size of mammary tumors and lung metastases and the reduced number of mice with 2 or more tumors.

With regard to the mechanisms involved in the inhibitory effect of Epitalon on mammary carcinogenesis, we demonstrate here the possible involvement of a direct effect of the pineal tetrapeptide on tumor cells. In the past, pineal hormones and particularly melatonin have been reported to have either antiproliferative and antiestrogen capacities, as well as to act through inhibition of the prolactin level or antioxidative potential and, finally, through a stimulation of the immune system.^{10,24,25} In this study, we describe a new mechanism by which Epitalon may bring about its anticancer action preventing the development of spontaneous tumors in HER-2/neu transgenic mice. This mechanism is based

TABLE II – EFFECT OF EXPOSURE TO VILON AND EPITALON ON LUNG METASTASIZING OF MAMMARY TUMOR IN HER-2/NEU TRANSGENIC FEMALE MICE

Group	No. of mice	No. of metastases (MTS) bearing mice (%)	Maximum diameter of MTS (cm)	Minimal diameter of MTS (cm)	Maximum size of tumors (cm)
Saline	28	7 (25.0%)	0.34 ± 0.06	0.30 ± 0.07	2.1 ± 0.2
Vilon	27	11 (40.0%)	0.28 ± 0.06	0.28 ± 0.06	1.9 ± 0.19
Epitalon	25	4 (16.0%)	0.22 ± 0.02^1	0.13 ± 0.02^1	1.4 ± 0.18^1

¹Difference with mice treated with saline is significant ($p < 0.05$).

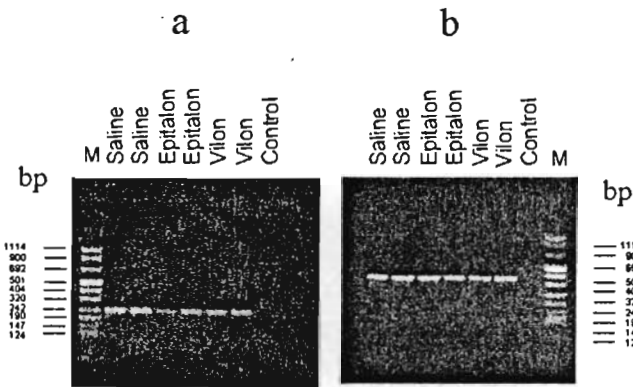


FIGURE 4 – Expression of rat HER-2/neu mRNA (a) and β -actin (b) in mammary tumors of FVB/N female mice transgenic for the activated rat HER-2/neu after chronic supplementation with Epitalon or Vilon by RT-PCR. The RNA reverse transcribed and amplified was normalized for β -actin expression in individual samples. The mean relative expression of HER-2/neu gene, as determined by densitometric analysis, was 1.637 ± 0.039 , 0.445 ± 0.212 and 0.829 ± 0.096 for saline-, Epitalon- or Vilon-treated tumor cells, respectively. Data relative to 2 mice for each group of treatment are presented.

on the downregulation of the HER-2/neu gene transcription. In fact, as shown in the RT-PCR results, the mRNA for HER-2/neu was expressed at lower levels in melatonin-treated mice compared to control mice. The evidence that the downregulation of HER-2/neu expression induces apoptosis in HER-2/neu-overexpressing cells supports the antitumoral effect of epitalon mediated by a HER2/neu modulation.²⁶ Whether melatonin may directly act on tumor cells inhibiting HER-2/neu expression or its effect is indirectly due to the *in vivo* modulation of other factors^{25,27} remains to be demonstrated. The fact that the thymic dipeptide Vilon, which failed to positively influence the mammary carcinogenesis in the transgenic HER-2/neu mice, also partially reduced HER2/neu mRNA expression may imply that the HER2/neu downregulation does not represent the only mechanism involved in the antitumoral action of Epitalon. However, the HER2/neu reduction induced by Vilon was lower than that one determined by Epitalon, thus suggesting that the effect of the thymic dipeptide was not sufficient to determine biologic effects in mammary cancer cells.

In conclusion, the data reported here demonstrate that Epitalon may exert some inhibitory effect on mammary tumor development and show that the pineal peptide may modulate the expression of the HER2/neu oncogene.

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