Inhibition of Tumor Progression by N^6^-Nitro-L-arginine Methyl Ester in 7,12- dimethylbenz(a)anthracene Induced Breast Cancer: Nitric Oxide Synthase Inhibition as an Antitumor Prevention

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Abstract
Breast cancer has high mortality rates among the women in the Republic of Armenia. It is now evident that nitric oxide plays important roles in various stages of carcinogenesis such as oncogene activation, tumor suppressor genes, modulation of apoptosis and metastasis. Advances in our understanding of the metabolism and molecular functions of arginine alterations in cancer have led to resurgence in the interest of targeting arginine catabolism, as an anticancer strategy. NOS inhibitors have been proposed, as a way to treat cancer. We have been researching the anti-tumor potential of the NOS inhibition by N^6^-Nitro-L-arginine methyl ester treatment (30 mg/kg/day, i.p.), administered for 5 weeks (parallelly to tumors development, every 3th day) against 7,12-dimethylbenz(a)anthracene induced mammary carcinogenesis in rats. Our treatment model results have shown the inhibition of NOS activity has influenced on development of carcinogenesis, which have been reflected in changes of rats' tumors size and quantity, mortality rate, in alteration of breast histopathology, in decrease of polyamines, NO and malondialdeide concentrations in blood. Understanding dichotomy of NO has been a great challenge for researchers working in the field of cancer therapy.

Keywords Breast cancer · NO-synthase · L-NAME · Arginase · 7,12-dimethylbenz(a)anthracene induced, antitumor prevention

Introduction
It is well established that, during development of malignancies, metabolic changes occur, including alterations of enzyme activities. Arginase and nitric oxide (NO) synthase (NOS) are two of those enzymes considered to be involved in tumorigenesis [1, 2]. Arginine, a semi-essential amino acid in humans, is critical for the growth of human cancers, particularly those...
marked by de novo chemoresistance and a poor clinical outcome [3–5]. Arginine is involved in diverse aspects of tumour metabolism, including the synthesis of nitric oxide, polyamines, nucleotides, proline and glutamate [6, 7]. Macrophages, granulocytes, or MDSC suppressed T cell–mediated immune responses by regulation of L-arginine metabolism via enzymatic mechanisms involving arginase and NOS [8, 9].

The NOS family, comprised of nNOS (NOS1), iNOS (NOS2), and eNOS (NOS3), convert arginine to nitric oxide (NO) and citruline [10]. NO is a ubiquitous free radical signaling molecule that regulates many cellular processes including angiogenesis, smooth muscle tone, immune response, apoptosis and synaptic communication [11–13]. In addition to the many normal physiologic functions, it has been implicated in the etiology and progression of many disease processes including cancer. NO have both tumoricidal as well as tumor promoting effects which depend on its timing, location, and concentration [14, 15]. NO has been suggested to modulate different cancer-related events including angiogenesis, apoptosis, cell cycle, invasion, and metastasis. On the other hand, it is also emerging as a potential anti-oncogenic agent [12, 16].

The physiological function of NO is primarily dependent on its concentration [17–19]. At low concentrations, NO acts as a signaling molecule regulating smooth muscle relaxation and blood flow, neurotransmission, platelet activity, iron homeostasis, cell survival and proliferation whereas at high concentrations it is believed to modulate immune-mediated anti-tumor activities [1, 10, 20–22]. NO produced by macrophages can mediate antibacterial and antitumor functions; however, chronic induction of NO and NO synthase may contribute to many pathologic processes, including inflammation and cancer. At the molecular level, high concentrations of NO derived from iNOS in macrophages induce p53 phosphorylation resulting in endothelial cell growth arrest, and higher concentrations and prolonged exposure time induce cell death [20, 23]. Prolonged production of NO has been associated with the release of cytochrome C from the mitochondria, activation of caspase, modulation of anti-apoptotic Bcl-2 proteins, and increase in p53 expression. Endogenous NO promotes tumor blood flow via dilatation of arteriolar vessels [24, 25]. Studies have shown that VEGF released as a purified protein or produced by tumor cells requires a functional NO/cGMP pathway within the end compartment to promote neovascular growth [26]. NO also has an invasion stimulating effect which is mediated by upregulation of MMP-2 and MMP-9 (matrix metalloproteinases), and downregulation of TIMP-2 and possibly TIMP-3 (tissue inhibitors of MMP). Studies have indicated that NO limits leukocyte cell proliferation which has adverse consequences on the antitumor response of the host. In this way NO may be involved in the growth and spread of tumors [7, 27]. Increased amounts of NO have been observed in blood of breast cancer patients and higher NOS activity has been found in invasive breast tumors when compared with benign or normal breast tissue [13, 28, 29]. iNOS positivity related with angiogenesis of tumors, accumulations of p53 mutations and EGFR activation [30]. Studies have showed that NOS2 expression in human breast tumors is functionally linked to poor patient survival [14]. The feature of the NOS family that makes them attractive as therapeutic targets is that there are a host of small molecules that inhibit these enzymes. Of these, the drug NG-nitro-L-arginine methyl ester (L-NAME) is one of the most clinically developed pan-NOS inhibitors; evaluated for the treatment of septic and cardiogenic shock, as well as in non-disease settings. L-NAME has also been shown inhibit tumorigenesis in various in vivo cancer models [29, 31]. Collectively, these observations support the preclinical evaluation of L-NAME different models for the treatment of 7,12-DMBA-induced breast cancer. The primary objectives of the present study were to assess the activity of NOS in 7, 12-DMBA induced breast cancer rats blood and to study the possible relationship between arginase activity, rats tumors size, numbers, weight, mortality rate, cancer histopathology, blood polyamines, NO and MDA (malondialdehyde) quantity. Our previous results have shown that human serum arginase activity and NO (resp., and NOS activity) and polyamines quantities increased in parallel with cancer stage progression and decreased after neoadjuvant chemotherapy [32]. Now we suggest that NOS inhibition may have antitumor effects on breast cancer development as it inhibits angiogenesis. In this article we have explored the anti-tumor potential of the NOS inhibition by L-NAME (30 mg/kg/day, i.p.), administered for 5 weeks (parallelly tumors development, from 2 to 8 weeks, every 3th day) against 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary carcinogenesis in rats.

Materials and Methods

Animals

The animals were housed 7 and 10 (DMBA control group) to a cage, with a base surface of 3000 cm², in a well-ventilated room at 25 °C and left one week for acclimation. The animals were kept at constant environmental and nutritional conditions throughout the experimental period with room humidity (50–55%) with 12 h light/12 h dark cycle and were fed a standard pellet diet and with water ad libitum (Animal care house, Faculty of Biology, YSU, Yerevan, Armenia). A total of 38 adult female Wistar rats weighing 90–120 g were used (8 weeks old). All handling and maintenance conditions were in accordance with the rules of the principles of the National Center of Bioethics (Armenia) [33, 34]. Rats were divided into five groups (7 rats per group): group I was untreated and served as Control. Group II and IV served as Saline and L-NAME, respectively. Rats in group III (DMBA, 10 rats in the
group) and group V (DMBA+L-NAME) were administrated intragastrically by gavage each with a single dose of 20 mg/ml DMBA (Sigma-Aldrich, USA), dissolved in 0.5 ml olive oil and 0.5 ml Saline given in a volume of 1 ml [35–38]. Rats in DMBA+L-NAME group were injected by L-NAME intraperitoneally for 5 weeks (after 10 days of DMBA administration, every 3rd day, from 2 to 8 weeks after DMBA) in dose of 30 mg/kg/day body-weight in 0.25 ml saline. Rats were palpated weekly to check for tumour appearance (detected first approximately 95 days (the 13th week) after the DMBA administration).

**Reagents**

L-NAME, chemicals for breast cancer induction, histopathological evaluation, determination of arginase activity, MDA, proteins and nitrite anions quantity, TLC of polyamines were obtained from Sigma-Aldrich Co. Ltd. (Germany) and Carl Roth GmbH + Co. KG (Germany).

**Intraperitoneal Injection into the Laboratory Rat**

Intraperitoneal injections of L-NAME into the laboratory rat were performed by rat care protocols [33, 39].

**Sampling Blood from the Lateral Tail Vein of the Rat**

Blood samples were sampling according to protocol of Lee and Goosens [40].

**Tumour Inhibition Study**

The effect of L-NAME treatment model on DMBA-induced tumours were determined at 5th, 8th, 13th, 16th and 20th weeks after DMBA administration. The experimental design and treatment scheme have been shown in Table 1. The concentration of L-NAME was chosen based on literature data [41–44]. The experimental rats were regularly monitored for food and water consumption, the apparent signs of toxicity, weight loss, or mortality. At the end of the 145 days (the 20th week, after 7,12-DMBA administration), rats in all groups were killed by cervical dislocation under anesthesia [33, 39, 45].

**Determination of Arginase Activity**

Arginase activity in blood was determined by the colorimetric method of Van Slyke and Archibald with some modifications [32]. Activity of enzyme was evaluated with the received urea in micromoles in 1 s (kat).

**Dansylation and Thin Layer Chromatography (TLC) Analysis**

The method of Seiler was used with some modifications, as follows [46]. Blood samples were extracted in 0.2 M cold HClO₄ at a ratio of about 100 mg/ml. Up to 50 μl of dansylated extract was loaded on the preabsorbing zone of silica gel plates, and was developed for about 2 h with chloroform-triethylamine (25:2 v/v) solvent system. The dansyl polyamine bands were scraped, eluted in 2 ml ethyl acetate, and quantified in 505 nm. The quantity of polyamines is presented in nM polyamines in 1 ml of blood.

**Griess Assay for NO Quantity**

Nitrite was measured by the Griess assay, as described [44]. Briefly, 100 μl Griess reagent was added to 100 μl each of the above supernatants. The plates were read at 550 nm against a standard curve of NaNO₂. The values were corrected for the NO₂⁻ + NO₃⁻ content of water, and the recovery of NO₂⁻ was calculated [47].

**Estimation of Lipid Peroxidation**

Estimation of lipid peroxidation was assayed spectrophotometrically using thiobarbituric acid-malondialdehyde assay (TBA-MDA) with some modification according to Ohkawa method [48].

<table>
<thead>
<tr>
<th>Table 1 Experimental design and treatment</th>
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<tr>
<td><strong>Grouping</strong></td>
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<td>Group III</td>
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<td>Group IV</td>
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<td>Group V</td>
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Determination of Ammonia in Blood Serum

Ammonia in blood plasma was determined with the indophenol direct method according to Huizenga with some modification for blood [49].

Histopathological Examination

The tissue pieces were spread on glass slides, stained with hematoxylin and eosin viewed under light microscope and photographed. All palpable tumours were excised and fixed in 10% buffered formalin and processed for histopathological evaluation. Histopathological examination, tumours types and microscopic reporting was established by the recently published protocols [50, 51].

Data Processing

Data are presented as mean ± standard error of the mean. Tumor growth, body weight, mortality, serum biochemical analyses evaluated by statistical ANOVA analysis with multiple comparisons using Statistica software.

Fig. 1 Survival percentage (%; a) in DMBA and DMBA+L-NAME groups and the change of animal weight (b) in all experimental groups at 5th, 8th, 13th, 16th and 20th (x) weeks after 7,12-DMBA administration (x + 8 weeks old rats), * - 7 rats in each group (p < 0.05), *** - n = 10 rats at 5th week (p < 0.05), n = 9 at 8th week (p < 0.05), n = 7 at 13th and 16th weeks (p < 0.05), n = 6 at 20th week (p < 0.05), *** - n = 7 at 5th and 8th weeks (p < 0.05), n = 6 at 13th, 16th and 20th week (p < 0.05)
(StatSoft 10.0). A p value of less than 0.05 was considered significant. All parameters (arginase activity, polyamines, nitrite anions, MDA, $\text{NH}_4^+$ have been analyzed in blood plasma and calculated in 1 ml blood.

**Results**

**Arginase Activity, NO and Polyamines Quantities Downstream by L-NAME Attenuate Tumor Growth, Numbers and Mortality Rate**

During the course of the experiments, animal died in each of the two treatment groups (DMBA and DMBA+L-NAME groups). There was a significant difference in the timings of the deaths in DMBA and DMBA+L-NAME group (Fig.1a). Death in DMBA group occurred at 8-9th, 12-13th and 19-20th weeks, and in contrast, in the DMBA + L-NAME group, death occurred at 12-13th week after DMBA administration. In contrast to Control group in DMBA group is observed decrease of rat body mass during 8–20 weeks (Fig.1b). Comparing with L-NAME group in DMBA group is seen body mass gain during all the weeks, except 20th weeks, where is observed weight loss. In DMBA + L-NAME group comparing to DMBA group is watched loss of weight at 5th, 8th, 13th and body mass gain at 13th and 20th weeks. In conclusion, in Fig.1b is obvious, that rats’ weight in treatment group DMBA+L-NAME is close to rats in Saline group (Fig. 1b, lines).

The quantitative analysis of tumors after 20 weeks showed that in DMBA + L-NAME group rats’ tumors numbers and size were significantly decreased comparing to DMBA group (Table 2). Particularly, 20 weeks after the administration of DMBA, in DMBA + L-NAME group total tumors’ numbers and dimension were decreased by 30–40%, comparing to DMBA group.

In blood at 5th (during treatment), 8th (tumours development and after treatment), 13th (tumours stabilization), 16th (during tumours progression) and 20th (before sacrificed) weeks after DMBA administration were found to have high nitrite anions quantity (Fig. 2a). In group III (DMBA) increased blood $\text{NO}_2^-$ level at 5th (4.9%), 8th (34.9%), 13th (78.2%), 16th (69.1%) and 20th (63.5%) weeks after DMBA administration ($p<0.01$ for all) comparing to the Control group. Treatment with L-NAME in group V (DMBA+L-NAME) decreased blood $\text{NO}_2^-$ quantity (as Control and Saline groups) at 5th (27.6%) 8th (33.6%), 13th (52.2%), 16th (47.3%) and 20th (40.6%) weeks after DMBA administration ($p<0.05$ for all) comparing to the DMBA group (Fig. 2a).

In DMBA group increased blood arginase activity 98.7% at 13th and 90.7% at 20th weeks comparing to the Control group ($p<0.01$). Co-treatment with the L-NAME stopped these increases at 13th, 16th and 20th weeks, resulting in mean values similar to those of the Control and Saline groups at 20th weeks (Fig. 2b). After injection of L-NAME, in DMBA+L-NAME group is shown significant increase of arginase activity, in contrast to DMBA group by 39.1% and 5.9% at 5th and 8th weeks (during treatment), and decrease by 26.8%, 34.2% and 39.2% at 13th, 16th and 20th weeks, correspondingly ($p<0.05$ for all) (Fig. 2b). Studies using a 7,12-DMBA-induced rat model showed that treatment of these animals with 30 mg/kg/day dose L-NAME increases arginase activity in the blood of in L-NAME animals, in contrast to Control and Saline groups at 5-20th key weeks. The importance of the obtained results is that L-NAME is activated arginase activity in L-NAME and DMBA+L-NAME groups, which emphasizes the unique interaction and relationship between these two enzymes in L-arginine metabolic pathway.

**Table 2** Numbers, size, allocation and histological classification of tumors in each rat at 20th week after 7,12-DMBA administration and treatment by L-NAME (28 weeks old rats)

<table>
<thead>
<tr>
<th>Rats</th>
<th>DMBA</th>
<th>DMBA + L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Tumors quantity/size (cm²)</td>
<td>Allocation, breasts</td>
</tr>
<tr>
<td>1</td>
<td>4/0.4–0.54</td>
<td>1st, 2nd, 5th and 6th pair</td>
</tr>
<tr>
<td>2</td>
<td>3/0.48–0.64</td>
<td>2nd, 3rd and 4th pair</td>
</tr>
<tr>
<td>3</td>
<td>1/0.26</td>
<td>2nd pair</td>
</tr>
<tr>
<td>4</td>
<td>3/0.9–1.25</td>
<td>2nd and 4th (both sides) pair</td>
</tr>
<tr>
<td>5</td>
<td>1/0.72</td>
<td>2nd pair breasts</td>
</tr>
<tr>
<td>6</td>
<td>2/1.04</td>
<td>4th pair, both sides</td>
</tr>
</tbody>
</table>

*DPC*, ductal papillary carcinoma (DCIS-Ductal Carcinoma in Situ); *IDP*, Intraductal Proliferation; *DCC*, Ductal Comedocarcinoma; *IDC*, intraductal carcinoma; *PC* – *G1* - papillary carcinoma grade 1; *DSC*, ductal solid carcinoma.
Polyamines Quantity Is Decreased under Influence of Arginase Activity

Polyamines are subsequently synthesized from ornithine, the second product of arginase reaction. The polyamine metabolic pathway is, therefore, a rational target for therapeutic intervention, and inhibitors of essentially all the polyamine metabolic enzymes have been identified [52]. At this point arginase is one of those enzymes considered to be involved in tumorigenesis. In order to meet their huge metabolic needs, most tumors have a greatly increased need for polyamines compared to normal cells and, consequently, polyamines are potent modifiers of tumor development [53].

Results showed that after 5, 8, 13, 16 and 20 weeks later of DMBA administration, quantity of blood polyamines (nM/ml blood) is increased in DMBA group in contrast to Control group (Fig. 3). In DMBA group increased blood total polyamines levels at 13th (11.9%), 16th (22.5%) and 20th (35.7%) weeks (increased parallel to tumor growth) comparing to the Control and Saline group animals was detected. In DMBA + L-NAME group blood putrescine quantity in contrast to DMBA group is only decreased by 3.1%, 6.9% and 15.8% at 13th, 16th and 20th weeks, correspondingly. Spermidine amount reduction in contrast to DMBA group is by 3.4% and 16.3% only after 16 and 20 weeks correspondingly, and spermine amount reduction is by

![Graph showing the change of nitrite anions quantity and arginase activity and NH₄⁺ concentration in rat blood in all experimental groups at different key weeks after 7, 12 - DMBA administration.](image-url)
3.8%, 12.1%, 9.3%, 13.9%, 15.1% at 5th, 8th, 13th, 16th and 20th weeks after DMBA administration (\( p < 0.05 \) for all) comparing to the DMBA group. Studies showed that treatment of these animals with 30 mg/kg/day dose L-NAME did not change polyamine quantity in the blood of in L-NAME group animals, in contrast to Control and Saline groups rat during all key weeks (Fig. 3). Our results were shown arginase activity is in correlation with the change of polyamine quantity.

**NOS Activity Inhibition by L-NAME**

**In Vivo Does Not Cause Oxidative Stress and Hyperammonemia**

The risk of hyperammonemia and cell membrane damage by L-NAME in both control and treatment groups was evaluated in blood after 5, 8, 13, 16 and 20 weeks DMBA administration in all experimental groups of rats. According to different studies, it has shown that DMBA can be used to induce experimental breast carcinomas in rats and that this process involves disruption of tissue redox balance; in turn, this suggests that biochemical and pathophysiological disturbances may result from oxidative damage [45]. Treatment with DMBA in group III significantly increased blood MDA and \( \text{NH}_4^+ \) levels at 5th (29.3% and 45.1%, respectively), 8th (98.7% and 48.7%), 13th (115.1% and 40.8%), 16th (100.3% and 21.9%) and 20th (96.8% and 29.1%) weeks after DMBA administration (\( p < 0.05 \) for all) comparing to the Control group rats (Figs. 2b and 4). Importantly, subsequent co-treatment with the NOS inhibitor L-NAME blocked these increases in group V, resulting in mean values close to those of the Control and Saline groups for MDA (only increased in DMBA + L-NAME by 30.5% at 5th week, comparing to DMBA group (\( p < 0.05 \)) and in L-NAME group animals in all weeks, comparing to Control and Saline groups rats) (Fig. 4). In contrast to DMBA group in DMBA + L-NAME group is shown MDA amount reduction by 8.15%, 38.9%, 45% and 48.3% at 8th, 13th, 16th and 20th weeks, correspondingly is shown. At 5th week (the influence of injection stress) is obtained up-regulation of MDA by 23.3% (Fig. 4). The results show decrease of blood ammonia quantity in DMBA+L-NAME group comparing to DMBA group by 6.3%, 6.5%, 20.2%, 18.2% and 24.3% at the 5th, 8th, 13th, 16th and 20th weeks, correspondingly (Fig. 2b).
Histopathological Alteration of Breast Tissues was Stopped by L-NAME Treatment

Rat mammary tumors may be composed of a single histologic type or of combinations of several patterns. The histopathological examination in mammary gland samplings at the 20th week in all experimental groups of rats has been done [36, 50, 51] (Fig. 5).

Discussion

Mechanisms by which NO induce inflammation-associated carcinogenesis include induction of DNA damage, suppression of DNA repair enzymes, posttranslational modification of proteins, enhancement of cell proliferation, angiogenesis, metastasis, inhibition of apoptosis and antitumor immunity [12, 20, 54]. Whereas Armenia is a high-altitude locality, it was interesting to study the quantity change of total nitrite anions in blood serum during the diseases and after L-NAME treatment, for revealing the specific regional phenomena. Moreover, according to the World Health Organization, Armenia has a high smoking percent, cancer, stress level, and coronary heart disease which also can be responsible for the triggering of NO production (there is no clear data for the increase in nitric oxide concentration yet). It is important to examine and understand that different actions of NO in breast cancer at the molecular level can help in providing NO based diagnostic or prognostic markers and also in devising potential strategies for prevention and treatment. Strategies for manipulating in vivo production and exogenous delivery of NO for therapeutic gain are being investigated. It should be mentioned that anti-tumor therapeutic effect of L-NAME is still being studied, and the main research is directed to the promoting normal functioning of vessels endothelium and immune response [2, 13, 21].

Our results have shown NO quantity downstream by L-NAME attenuated tumor growth, numbers and cancer progression. We conclude that NOS activity inhibition by L-NAME in vivo does not cause oxidative stress and hyperammonemia in DMBA-induced mammary cancer and L-NAME groups. In DMBA+L-NAME group the histopathological examination has revealed only precancerous lesions and DCIS. We conclude that 30 mg/kg/day i.p. injection

Fig. 4 The change of MDA quantity in rats’ blood in all experimental groups (n = 7 for Control, Saline and L-NAME groups, p < 0.05, * – p < 0.01, n = 10 at 5th week, n = 9 at 8th week, n = 7 at 13th and 16th weeks, n = 6 at 20th week, ** – n = 7 at 5th and 8th weeks, n = 6 at 13th, 16th and 20th weeks)
(5 weeks, 12 times) of L-NAME slows the growth of DMBA-induced mammary tumours.

L-Arginine is the common substrate for two enzymes, arginase and nitric oxide synthase (NOS) [5]. According to literature data arginase and NOS can cooperate to restrain T lymphocyte functions in tumor-bearing hosts by altering the production of reactive nitrogen and oxygen species (RNS and ROS, respectively) [7]. The dual co-expression of arginase and NOS might be a singular property of MDSCs, or at least a part of this heterogeneous population of myeloid suppressors induced by tumors. The molecular bases for the synergism between these enzymes are still not yet entirely known [9]. Low extracellular L-arginine concentration, overexpression of arginase, or reduction of L-arginine uptake can decrease intracellular L-arginine concentration and halt translation of NOS2 mRNA, a phenomenon known as the arginine paradox [7, 13]. NOHA (N^G^-hydroxy-L-Arginine), the intermediate product in NO synthesis, is a potent inhibitor of both arginase isoforms. Taking into account the above mentioned and our results, it can be noted, that the activation of arginase activity after the administration of NOS inhibitor, can be a reason of low extracellular NOHA concentration after inhibition of NOS activity during treatment. The reason that treatment with the L-NAME stopped arginase activity increases at 13th, 16th and 20th weeks is also the regression of breast cancer. We conclude these enzymes can cooperate to restrain polyamines and NO function for cancer progression. Our obtained results can serve as a base to use this model for determination of productive, noncytotoxic antitumor and immune modulating concentration of anticancer agents. Increasing knowledge of the interplay between arginase and NOS, suggests potential combination therapies that will have considerable clinical promise. In addition, arginase blocking therapy, a strategy that combines the inhibition of NO biosynthesis, can be more effective than therapies based on NOS activity inhibition alone and may involve an antitumour immune response.
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Authors’ Contributions Nikolay Avtandilyan and Hayarpi Javrushyan performed the experiments and analyzed the results. Nikolay Avtandilyan wrote the manuscript. Hayarpi Javrushyan edited the manuscript. The histopathological examination in mammary gland samplings in all experimental groups of rats have been done by Anna Karapetyan. Armen Tchouiant directed the research and revised and reedited the manuscript. All authors read and approved the final version of the manuscript.

Data Availability The data supporting the conclusions of this article are included within this article.

Compliance with Ethical Standards

Conflicts of Interest The authors declare that there are no conflicts of interest regarding the publication of this article.

Ethical Approval All experiments with animals were conducted according to the principles of the National Center of Bioethics (Armenia).

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