

IMMUNOHISTOCHEMICAL ASSESSMENT OF POSSIBLE ANTICANCER EFFECT MECHANISMS OF 2-(6,8-DIMETHYL-5-NITRO-4-CHLOROQUINOLINE-2-YL)-5,6,7-TRICHLORO-1,3-TROPOLONE IN PDX MODELS OF LUNG CANCER

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ABSTRACT

Purpose of the study. Evaluation of the expression of immunohistochemical tumor markers Ki-67, b-catenin, Bcl-2, P53, connexin 32 and connexin 43 when using 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone in mice with xenographs of squamous cell lung cancer.

Materials and methods. Subcutaneous PDX models of human squamous cell lung cancer were created in immunodeficient BALB/c Nude mice. A fragment of the patient's tumor (3 × 3 × 3 mm) was implanted subcutaneously in the right thigh of a previously anesthetized mouse. 200 µl of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone was administered orally using a probe in 12 doses once every 3 days. All animals were divided into groups depending on the tropolone doses: experimental groups 2–5 with doses of 0.0055, 0.055, 0.55 and 2.75 mg/g, respectively. The control group received 1 % starch gel which was tropolone carrier. The animals were euthanized 36 days after the start of the substance administration, and the tumor tissue was isolated and prepared for the IHC study according to the standard protocol. IHC reactions were performed using antibodies for Ki-67, b-catenin, Bcl-2, P53, connexin 32 and connexin 43.

Results. Higher tropolone doses were associated with decreased expression of Ki-67, b-catenin, and the Bcl-2 protein, but increased expression of the P53 protein. The dosage of tropolone and expression of connexin 43 were directly proportional.

Conclusion. Immunohistochemical analysis of expression of proteins in PDX models of human squamous cell lung cancer when using 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone showed the changes indicating its antitumor efficacy and suggesting a possible mechanism of action based on the activation of apoptosis.

Keywords:

2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone, squamous cell lung cancer, PDX, Ki-67, b-catenin, Bcl-2, P53, connexin 32 and connexin 43, apoptosis

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The ethical principles presented by the Helsinki Declaration of the World Medical Association (World Medical Association Declaration of Helsinki, 1964, ed. 2013) were observed in the work. The study was approved by the Ethics Committee of the National Medical Research Centre for Oncology (Protocol No. 1/61 of 02/19/2019).

Funding: this work was not funded.

Conflict of interest: authors report no conflict of interest.

For citation:

Komarova E. F., Lukbanova E. A., Dzhenkova E. A., Goncharova A. S., Zaikina E. V., Gurova S. V., Galina A. V., Kurbanova L. K., Mindar M. V., Khodakova D. V., Gusareva M. S., Zinkovich M. S. Immunohistochemical assessment of possible anticancer effect mechanisms of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone in PDX models of lung cancer. South Russian Journal of Cancer. 2023; 4(1): 6-13. (In Russ.). <https://doi.org/10.37748/2686-9039-2023-4-1-1>, <https://elibrary.ru/atehfo>

The article was submitted 18.06.2022; approved after reviewing 03.11.2022; accepted for publication 06.03.2023.

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ИММУНОГИСТОХИМИЧЕСКАЯ ОЦЕНКА ВОЗМОЖНЫХ МЕХАНИЗМОВ ПРОТИВООПУХОЛЕВОГО ДЕЙСТВИЯ 2-(6,8-ДИМЕТИЛ-5-НИТРО-4-ХЛОРХИНОЛИН-2-ИЛ)-5,6,7-ТРИХЛОР-1,3-ТРОПОЛОНА НА PDX-МОДЕЛЯХ РАКА ЛЕГКОГО

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РЕЗЮМЕ

Цель исследования. Оценить уровень экспрессии иммуногистохимических опухолевых маркеров Ki-67, b-catenin, Bcl-2, P53, коннексина 32 и коннексина 43 при применении 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-трополона у мышей с ксенографтами плоскоклеточного рака легкого.

Материалы и методы. На иммунодефицитных мышах линии BALB/c Nude были получены подкожные PDX-модели плоскоклеточного рака легкого человека. Фрагмент опухоли пациента размером 3 × 3 × 3 мм имплантировали подкожно в область правого бедра мыши, предварительно наркотизировав животное при помощи ксилазина концентрацией 20 мг/мл и зоветила (тилетамин, золазепам основание) концентрацией 22,57 мг/мл. Введение субстанции 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-трополона, производили перорально при помощи зонда в объеме 200 мкл в 12 приемов с кратностью 1 раз в 3 дня. Все животные были распределены по группам в зависимости от примененной дозы трополона: опытные группы № 2–5 в дозах 0,0055, 0,055, 0,55 и 2,75 мг/г соответственно. Контрольной группе животных вводили 1 % крахмальный гель, который являлся носителем для трополона. После 36 дней от начала введения субстанции животных эвтаназировали, выделяли опухолевую ткань, которую готовили к ИГХ исследованию по стандартному протоколу. Проводили ИГХ реакции с использованием антител для Ki-67, b-catenin, Bcl-2, P53, коннексина 32 и коннексина 43.

Результаты. Было обнаружено, что с повышением дозы трополона уменьшается экспрессия Ki-67, b-catenin, а также происходит снижение уровня экспрессии белка Bcl-2. При этом уровень экспрессии белка P53 нарастает при увеличении дозы примененного вещества. При исследовании влияния трополона на уровень экспрессии коннексина 43 была обнаружена прямо пропорциональная зависимость его экспрессии при повышении дозы трополона.

Заключение. Проведенный иммуногистохимический анализ уровня экспрессии белков в PDX-моделях плоскоклеточного рака легкого человека при применении 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-трополона обнаружил их изменения, указывающие на его противоопухолевую эффективность и позволяющие предполагать возможный механизм действия изученной субстанции за счет активации апоптоза.

Ключевые слова:

2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-трополон, плоскоклеточный рак легкого, PDX, Ki-67, b-catenin, Bcl-2, P53, коннексин 32 и коннексин 43, апоптоз

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В работе соблюдались этические принципы, предъявляемые Хельсинкской декларацией Всемирной медицинской ассоциации (World Medical Association Declaration of Helsinki, 1964, ред. 2013). Исследование одобрено этическим комитетом ФГБУ «НМИЦ онкологии» Минздрава России (протокол № 1/61 от 19.02.2019 г.).

Финансирование: финансирование данной работы не проводилось.

Конфликт интересов: авторы заявляют об отсутствии конфликта интересов.

Для цитирования:

Комарова Е. Ф., Лукбанова Е. А., Дженкова Е. А., Гончарова А. С., Заикина Е. В., Гурова С. В., Галина А. В., Курбанова Л. К., Миндарь М. В., Ходакова Д. В., Гусарева М. С., Зинькович М. С. Иммуногистохимическая оценка возможных механизмов противоопухолевого действия 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-трополона на PDX-моделях рака легкого. Южно-Российский онкологический журнал. 2023; 4(1): 6-13. <https://doi.org/10.37748/2686-9039-2023-4-1-1>, <https://elibrary.ru/atehfo>

Статья поступила в редакцию 18.06.2022; одобрена после рецензирования 03.11.2022; принята к публикации 06.03.2023.

INTRODUCTION

Platinum preparations are still used as a chemotherapeutic treatment for lung cancer, and therefore the search for chemical substances with an effective antitumor effect in this nosology continues [1; 2]. In recent years, tropolone alkaloids, which have demonstrated selective toxicity against malignant tumor cells, have been considered as such [3]. The most well-known tropolones of natural origin – colchicine, colchamine and β -tuplicin (quinocithiol) demonstrate pronounced antitumor properties due to various mechanisms [4].

The natural troponoid colchicine has the effect of inhibiting tumor growth due to binding to tubulin, which disrupts cell division, as well as due to the ability to limit mitochondrial metabolism in malignant cells by inhibiting potential-dependent anion channels of the mitochondrial membrane [5; 6]. The antitumor effect of the modern natural troponoid quinocithiol due to the induction of caspase-dependent apoptosis, autophagy, blocking of the S-phase of the cell cycle, DNA damage and its demethylation was noted in relation to colon cancer cells, lung adenocarcinoma, breast cancer, multiple myeloma, hepatocellular cancer [7; 8].

Unlike natural and synthetic β -substituted tropolones, the mechanisms of antitumor action of α -substituted analogues have been little studied. Some studies have shown the ability of synthesized α -substituted tropolones by induced caspase-dependent apoptosis to suppress the growth of lymphocytic leukemia cells (but not healthy blood cells), as well as cell culture of multiple myeloma [9]. *In vitro* studies have shown a connection between the mechanisms of cytotoxic action of the compounds studied with the induction of apoptosis and changes in the activity of the ERK signaling pathway in ovarian and colon cancer cells [10].

Purpose of the study: to evaluate the expression level of immunohistochemical tumor markers Ki-67, b-catenin, Bcl-2, P53, connexin 32 and connexin 43 when using 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone in mice with xenografts of squamous cell lung cancer.

MATERIALS AND METHODS

The study used 50 BALB/c Nude mice of both sexes, which were obtained from the nursery of the

Russian Academy of Sciences (Novosibirsk) and were kept in standard conditions of the SPF vivarium of the National Medical Research Centre for Oncology Testing Laboratory Center. The study was approved by the Ethical Committee National Medical Research Centre for Oncology (Protocol No. 1/61 of 02/19/2019). Manipulations with animals were carried out in accordance with the ethical principles established by the European Convention for the Protection of Vertebrates Used for Experimental and Other Scientific Purposes.

Subcutaneous PDX models (Patient-Derived Xenograft) of human squamous cell lung cancer in the 4th passage were obtained on immunodeficient BALB/c Nude mice [11]. The donor of the tumor material was patient T., diagnosed with: C34.3 Central cancer of the lower lobe of the right lung, pT3N0M0, st IIB. The histological type of the patient's tumor is squamous cell lung cancer. A fragment of the patient's tumor measuring $3 \times 3 \times 3$ mm was implanted subcutaneously into the right thigh of the mouse, having previously anesthetized the animal with xylazine at a concentration of 20 mg/ml and zoletil (tiletamine, zolazepamine) at a concentration of 22.57 mg/ml [12].

In the experiment, the substance 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone (hereinafter tropolone) synthesized by the expansion of the o-quinone cycle in SRI of Physical and Organic Chemistry of the Southern Federal University was introduced [13]. The administration of substances began after the tumor nodes reached volumes of 100 mm^3 . 1 % starch gel was used as a carrier for the introduction of tropolone. The substances were administered orally using a probe in the volume of 200 μl in 12 doses with a multiplicity of 1 every 3 days, regardless of food and water intake. All animals were divided into groups depending on the applied dose of tropolone (Table 1).

The duration of the experiment was 36 days, starting from the first administration of the substance. After euthanasia of animals by dislocation of the cervical vertebrae, tumor material was isolated and, after standard preparation, enclosed in paraffin blocks. For the IHC study, sections were made from paraffin blocks using a rotary microtome, which were subsequently dewaxed according to a standard protocol. All stages of the immunohistochemical reaction were carried out in the VENTANA BenchMark ULTRA immunohistostainer of Roche (Switzerland), according

to the protocols of manufacturers attached to the mono- and polyclonal antibodies used. UltraView Universal DAB Detection, manufactured by Ventana Medical Systems, was used as a primary antibody detection system. Antibodies were used for IHC reactions: Ki-67 – Cell Marque, USA, p53, b-catenin, Bcl-2, connexin 32 and –43 – Ventana Medical Systems, USA. The intensity of immuno-staining was assessed using light microscopy (Leica DM3000 microscope).

The normality of the distribution of signs was assessed using the Shapiro-Wilk criterion. Median and interquartile range were calculated for quantitative data. The statistical significance of the differences between the groups was assessed using a pairwise comparative analysis using the Mann-Whitney criterion. The significance level for the methods used was set as $p \leq 0.05$.

RESEARCH RESULTS AND DISCUSSION

In the immunohistochemical study of the expression of the Ki-67 proliferation marker in human

lung cancer xenograft tissues, the proportion of immunopositive cells in the 1st control group was 66.3 [61.5–69.3] %, in the 2nd, 3rd, 4th and 5th experimental groups – 64.7 [62.3–65.3] %, 61.4 [60.3–62.3] %, 59.3 [58.1–60.2] % and 55.2 [53.2–57.4] %, respectively, that is, there was a statistically significant decrease in the level of Ki-67 expression as the dose of tropolone increased and compared with the control group ($p < 0.01$) (Fig. 1).

The percentage of positively stained cells when assessing the expression of b-catenin in human lung cancer xenographs was in the experimental groups: in the 2nd – 32.7 [31.2–33.1] %, in the 3rd – 24.5 [23.4–26.5] %, 4-th – 22.3 [20.7–23.6] % and in 5-th – 8.4 [7.2–9.3] %, which was statistically significantly lower at $p < 0.05$ relative to the control group (33.7 ± 0.3 %) (Fig. 2). When assessing Bcl-2 expression in human lung cancer xenographs, a statistically significant ($p < 0.01$) decrease in Bcl-2 expression was observed with an increase in the tropolone dose, and the proportion of immunopositive cells was on average in group 1 (control) – 61.1 [57.9–62.4] %, in

Table 1. Study design

Group number	Group naming	Number of animals involved	Injected agent	Agent dose, mg/g
1	Control	10	Starch gel	1 %
2	Study	10	Tropolon	0.0055
3		10		0.055
4		10		0.55
5		10		2.75

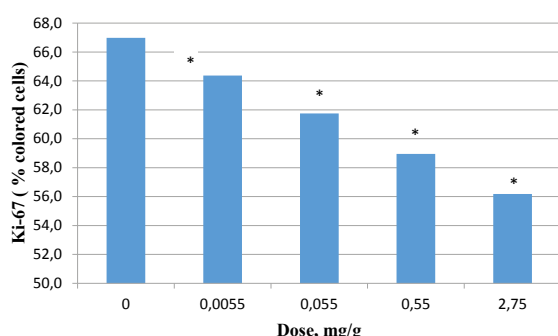


Fig. 1. The relationship between the dose of tropolone and the proportion of immunopositive cells in assessing the expression of Ki-67 in PDF models of human lung cancer in immunodeficient BALB/c Nude mice. 0 – control group, * – statistically significant differences in comparison with the control group ($p < 0.05$).

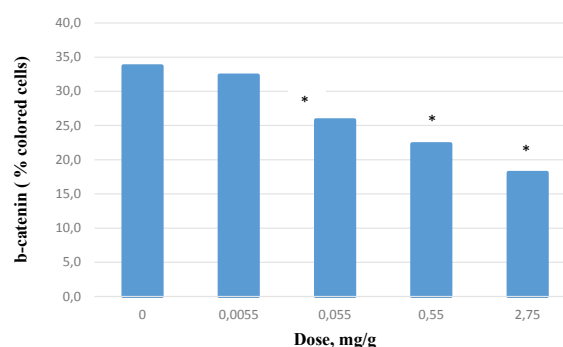


Fig. 2. The relationship between the dose of tropolone and the proportion of immunopositive cells when evaluating the expression of i-catenin in PDF models of human lung cancer in immunodeficient BALB/c Nude mice. 0 – control group, * – statistically significant differences in comparison with the control group ($p < 0.05$).

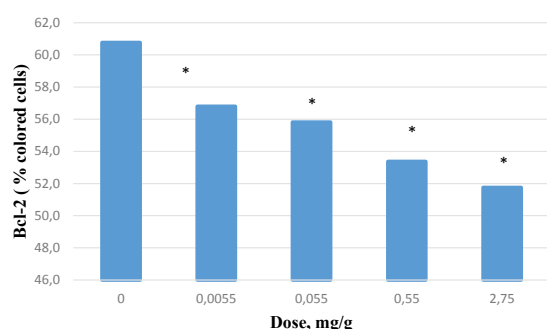


Fig. 3. The relationship between the dose of tropolone and the proportion of immunopositive cells in assessing Bcl-2 expression in PDX models of human lung cancer in immunodeficient BALB/c Nude mice. 0 – control group, * – statistically significant differences in comparison with the control group ($p < 0.05$).

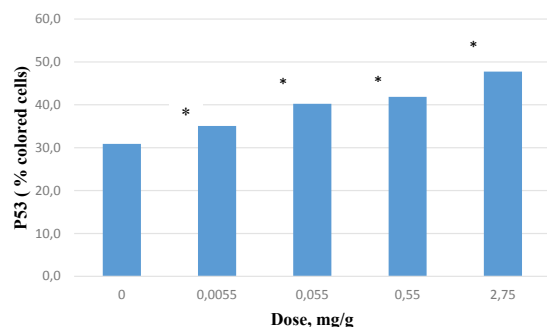


Fig. 4. The relationship between the dose of tropolone and the proportion of immunopositive cells when evaluating the expression of P53 in PDX models of human lung cancer in immunodeficient BALB/c Nude mice. 0 – control group, * – statistically significant differences in comparison with the control group ($p < 0.05$).

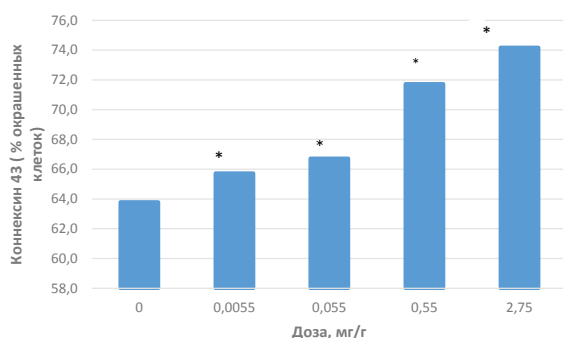


Fig. 5. The relationship between the dose of tropolone and the proportion of immunopositive cells in assessing the expression of connexin 43 in PDX models of human lung cancer in immunodeficient BALB/c Nude mice. 0 – control group, * – statistically significant differences in comparison with the control group ($p < 0.05$).

the 2nd (0.0055 mg/g), 3rd (0.055 mg/g), 4th (0.55 mg/g) and 5th (2.75 mg/g) groups – 56.8 [55.4–58.6] %, 55.5 [53.1–56.3] %, 52.5 [52.9–55.2] % and 50.9* [50.1–53.2] %, respectively (Fig. 3).

When evaluating the expression of the oncosuppressor protein P53, the proportion of immunopositive cells in human lung cancer xenographs averaged 30.9 ± 0.5 % in group 1, in groups 2, 3, 4 and 5 – 35.8 ± 0.4 [32.3–37.5] %, 41.4 ± 1 [39.9–43.1] %, 42.9 ± 1.3 [41.2–44.3] % and 48.1 ± 0.4 [45.9–51.3] %, respectively (Fig. 4). At the same time, a statistically significant ($p < 0.01$) increase in the expression level of P53 was observed with an increase in the dose of tropolone (in 2, 3, 4 and 5 groups compared with the control group), which indicates an increase in the processes of apoptosis in the tumor tissue (Fig. 4).

When analyzing the expression of connexin 32 in the tissues of PDX models of lung cancer in all experimental groups of animals, no statistically significant differences ($p > 0.05$) were found with the control group. The percentage of positively colored cells was 62.1 [60.2–63.5] %, 61.1 [59.9–62.9] %, 61.6 [60.1–63.1] %, 61.3 [59.8–62.1] % and 61.7 [60.4–62.9] % for 1–5 groups, respectively.

The proportion of immunopositive cells in assessing the expression of connexin 43 in human lung cancer xenographs was on average in group 1 (control) – 62.8 [58.3–63.2] %, in the 2nd, 3rd, 4th and 5th groups – 65.3 [64.1–66.5] %, 66.9 [65.3–69.5] %, 71.8 [69.4–72.9] % and 74.8 [72.3–76.4] %, respectively, that is, when exposed to tropolone, there was a statistically significant increase in the level of connexin 43 expression in the experimental groups compared with the control group. ($P_{1-2,1-3} \leq 0.05$; $P_{1-4,1-5} < 0.01$) (Fig. 5).

The immunohistochemical analysis of the expression level of proteins Ki-67, b-catenin, Bcl-2, P53 and connexins 43 and 32 in PDX models of squamous cell lung cancer with the use of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone found their changes indicating dose-dependent antitumor activity and suggesting possible mechanisms of action of the studied substance. Thus, it was found that with an increase in the dose of tropolone, the expression of Ki-67, b-catenin decreases, and there is also a decrease in the expression level of the anti-apoptotic protein Bcl-2, while the expression level of the oncosuppressor protein P53 increases with an increase in

the dose of the substance used. The Ki-67 protein is currently considered as the most reliable marker of proliferation, including for lung cancer, and its decrease observed in the experimental groups relative to the indicators in the control group indicates a decrease in the proliferative activity of tumor cells of squamous cell lung cancer when using tropolone [14]. It is believed that b-catenin exhibits various effects in tumor cells, in particular, it activates proliferation, being one of the key molecules of the tumorigenic signaling pathway Wnt/b-catenin and, due to the intersection with other signaling pathways, regulates apoptosis, angiogenesis and cell invasion, and also participates in the shift of cellular metabolism towards oxygen-free glucose oxidation [15]. A decrease in the expression of b-catenin by lung cancer tumor cells in PDX models with the use of tropolone confirms the antitumor effect of this substance, obviously mediated through the Wnt/b-catenin signaling pathway. Studies have shown that overexpression of Bcl-2 protein blocks apoptosis and thus promotes tumor progression [16]. Lee Y.-S. and co-authors (2013) showed a decrease in Bcl-2 expression when exposed to natural tropolone – quinokithiol on subcutaneous xenografts obtained by transplantation of human colon cancer cell cultures HCT-116 and SW-620 [17]. Suppression of the expression of anti-apoptotic protein Bcl-2 in subcutaneous xenograft cells of squamous cell lung cancer in combination with increased expression of oncosuppressor P53, known for its activating effect on pro-apoptotic proteins Bax and Bid, indicates stimulation of apoptosis with the use of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone.

When studying the effect of tropolone on the expression level of connexin 43, a dependence of its expression was found with an increase in the dose

of tropolone. It is known that connexins are tumor suppressors that regulate cell proliferation, apoptosis, chemoresistance, migration and invasion with the help of intercellular communication of the slit junction [18]. Overexpression of connexin 43 in the nucleus correlates with increased aggressiveness of lung tumors, which is associated with its ability to recruit E-cadherin, providing tumor cells with a more invasive phenotype, increasing their ability to migrate, survival, and contributing to the development of distant metastases [19]. However, connexin 43 with its cytoplasmic localization is able to inhibit tumorigenesis [20]. Assuming the overall antitumor efficacy of the tropolone studied, we can talk about the induction of the suppressor function of connexin 43 relative to lung cancer in the PDX model. Some studies show that connexin 32 is overexpressed in lung cancer cells [21]. Knockout or knockdown of the connexin 32 gene leads to an increase in the frequency of chemical and radiation-induced lung tumors, and also enhances epithelial-mesenchymal transition, migration and invasion of tumor cells [22], probably partly due to activation of the MAPK pathway. However, we have not detected the effect of tropolone on the expression of connexin 32, the reason for this is not clear and requires further study.

CONCLUSION

The detected changes in the studied immunohistochemical markers on PDX models, indicating a decrease in the proliferative activity of lung tumor cells, as well as activation of apoptosis processes, indicate the manifestation of the antitumor effect of tropolone. Based on the results obtained, we assume that a possible mechanism of antitumor action of the studied tropolone is activation of apoptosis.

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