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Antitumor activity of three new azoloazine derivatives in orthotopic transplantation model of human breast cancer cells into mice

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Breast cancer (BC) is one of the most common types of malignant tumors, which makes scientific research in this area extremely relevant. The difficulties of breast cancer chemotherapy stimulate the search for new drugs to treat this nosology. Derivatives of imidazotriazine and imidazotetrazine, which are analogues of the antitumor drug temozolamide, can be ones of the promising drugs in this regard.

The aim of the work was to evaluate the antitumor activity of three new azoloazine derivatives in a xenogeneic breast cancer model in mice *in vivo*.

Materials and methods. A study was conducted on a xenogeneic model of BC. After the immunosuppression with azathioprine, 48 white BALB/c mice were injected with MCF-7 cells, test derivatives, and the reference drug epirubicin at doses of 1/2 IC_{50} and 1/10 IC_{50}, into the base of the mammary gland once. The body weight of the mice was monitored; on the 15th day, at the end of the experiment, the relative volume was assessed.

Results and discussion. Among the three compounds studied, imidazotetrazine 1 showed the most encouraging results: stopping the loss of body weight in the mice caused by the administration of tumor cells, and reducing the tumor volume on the 15th day of the experiment to 50.6% of that in the control when using a dose of 1/10 IC_{50}, up to 39.2% – when using a dose of 1/2 IC_{50}, which significantly exceeded the values of the reference drug epirubicin and the values in the control group. In the histological examination, the signs of spread and preservation of tumor cells viability of the MCF-7 line after its administration were minimal, the value of the histological malignancy index decreased by 9.3% of the control value.

Conclusion. Among the tested azoloazine derivatives, 3-cyclohexyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazine-8-N-piperidinylcarboxamide is the undisputed leader, causing inhibition of the tumor growth in a xenogeneic model *in vivo*.

Keywords: imidazotriazine; imidazotetrazine; breast cancer; MCF-7 cell line; mouse models

Abbreviations: BC – breast cancer; NMR – nuclear magnetic resonance; MCF-7 (Michigan Cancer Foundation-7) – human breast cancer cell line; BALB/c – genetic line of white laboratory mice; IC_{50} – concentration of half-maximal inhibition; G1-G3 – system of morphological criteria for determining the degree of malignancy.

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RESEARCH ARTICLE

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Противоопухолевая активность трех новых производных азолоазинов на модели ортотопической трансплантации клеток рака молочной железы человека мышам

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Рак молочной железы (РМЖ) относится к наиболее распространенным видам злокачественных опухолей, что делает чрезвычайно актуальными научные исследования в этой области. Трудности химиотерапии РМЖ стимулируют поиск новых препаратов для лечения этой нозологии. Одними из перспективных в этом плане могут быть производные имидазотриазина и имидазотетразина, которые являются аналогами противоопухолевого препарата темозоламида.

Цель. Оценить противоопухолевую активность трех новых производных азолоазинов в ксеногенной модели рака молочной железы на мышах *in vivo*.

Материалы и методы. Проведено исследование на ксеногенной модели РМЖ. После иммуносупрессии азатиоприном 48 белым мышам BALB/c вводили в основание соска молочной железы клетки линии MCF-7, тестируемые производные и препарат сравнения эпирубицин в дозах 1/2 IC_{50} и 1/10 IC_{50} однократно. Проводили мониторинг массы тела мышей, по завершении эксперимента на 15-е сут оценивали относительный объем опухолевой ткани и проводили гистологическое исследование.

Результаты и обсуждение. Среди трех исследуемых соединений имидазотетразин 1 продемонстрировал наиболее обнадеживающие результаты: прекращение потери массы тела мышей, вызванное введением опухолевых клеток, и сокращение объема опухоли на 15-е сут эксперимента до 50,6% от аналогичного в контроле при использовании дозы 1/10 IC_{50}, до 39,2% – при использовании дозы 1/2 IC_{50}, что значительно превышало значения препарата сравнения эпирубицина и значения в контрольной группе. При гистологическом исследовании признаки распространения и сохранения жизнеспособности опухолевых клеток линии MCF-7 после его введения были минимальными, величина гистологического индекса злокачественности снижалась на 9,3% от значения в контроле.

Заключение. Среди тестируемых производных азолоазинов, 3-циклогексил-4-оксоимидазо[5,1-*d*][1,2,3,5]тетразин-8-*N*-пиперидинилкарбоксамид является безусловным лидером, вызывающим торможение роста опухоли в ксеногенной модели *in vivo*.

Ключевые слова: имидазотриазин; имидазотетразин; рак молочной железы; клеточная линия MCF-7; мышиные модели

Список сокращений: РМЖ – рак молочной железы; ЯМР – ядерно-магнитный резонанс; MCF-7 – клеточная линия РМЖ человека; BALB/c – генетическая линия белых лабораторных мышей; IC_{50} – концентрация полумаксимального ингибирования; G1-G3 – система морфологических критериев определения степени злокачественности.

INTRODUCTION

Malignant neoplasms are currently not only one of the main causes of death in the world, but they also cause concern to specialists due to the constant increase in morbidity, extreme variability, and resistance to various types of treatment. Among all tumors, breast cancer belongs to the most common type of malignant tumors in women [1–3]. It has been detected in 1/8 of all newly diagnosed cases of tumors and determines 14% of cancer mortality in women [4]. According to global statistics, 2.3 million people were diagnosed with breast cancer in 2020, and 685 thousand died from it [5]. As a

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result, the genetic and morphological diversity of breast cancer, the difficulties of its primary diagnosis, and the high resistance of a several varieties to chemotherapy cannot satisfy clinical oncologists with a modern arsenal of chemotherapeutic agents for its treatment. The results of antitumor therapy for metastatic breast cancer are particularly pessimistic [6–9].

All these points determine the urgent need and make research into the development of new chemotherapeutic agents that would cope with breast cancer extremely relevant [10, 11].

Based on the above provisions, the authors' attention has been focused on the study of imidazotetrazine and imidazotriazine derivatives, which demonstrate a strong alkylating effect and have been used as antitumor agents, including breast cancer, for more than 40 years. Temozolomide is the most famous of them; it has proven clinical effectiveness in the treatment of lymphomas, brain tumors, and metastatic melanoma. Unfortunately, at the present stage, many tumors are resistant to this drug, or the chemoresistance develops very quickly [12, 13]. To date, more than 30 new derivatives of imidazotetrazine and imidazotriazine have been synthesized, promising to be used as antitumor agents [14–16].

The study hypothesizes a possible antitumor activity of new azoloazine derivatives in a xenogenic model of breast cancer in mice.

This *in vivo* stage is mandatory for all preclinical studies of potential antitumor compounds, since it is cancer modeling in laboratory animals that makes it possible to study the biology of malignant growth in detail and make suggestions in an evidence-based format to improve diagnostic and therapeutic strategies. Among the existing models, the orthotopic mouse is characterized by transplantation of tumor cells or fragments into the same anatomical area where the tumor usually develops. This cancer model is widely used in practice [17, 18], because it makes possible a very accurate reproduction of relationships with stromal and vascular cells, providing a microenvironment that plays a crucial role in the development of tumor cells.

THE AIM of the work was to evaluate the antitumor activity of three new azoloazine derivatives in a xenogeneic breast cancer model in mice *in vivo*.

MATERIALS AND METHODS

Test compounds

To assess the potential antitumor properties, three azoloazine derivatives: 3-cyclohexyl-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazine-8-N-piperidinylcarboxamide (imidazotetrazine **1**), diethyl ether of 4-aminoimidazo[5,1-*c*][1,2,4]triazine-3,8-dicarboxylic acid (imidazotriazine **2**) and 4-amino-8-ethoxy-carbonylimidazo[5,1-*c*][1,2,4]triazine-3-N-(p-toluyl)carboxamide (imidazotriazine **3**) synthesized at Ural Federal University named after the first President of Russia B.N. Yeltsin, have been tested in accordance with the described methods [19, 20], while NMR analysis confirmed a high chemical purity of these derivatives. Epirubicin was used as a reference drug, as a remedy widely used for the treatment of breast cancer, which toxicological effects and activities in mouse models are well known [21]. Compounds **1–3** were selected for the study from 11 candidates based on the results of an earlier determination of the minimum half inhibitory concentration of IC_{50} in an *in vitro* cytotoxicity test in MCF-7 cell culture [22].

Ethical approval

The study was conducted in accordance with bioethical standards set out in the "The Code of Practice for Care and Use of Animals for Experimental Purposes" and Directive 2010/63/EU of the European Parliament and of the Council of the European Union on the protection of animals used for scientific purposes. After the examination, the design of the experimental study was approved by the Local Ethics Committee of Volgograd State Medical University (Protocol No. 2021/049 dated May 27, 2021). All manipulations with laboratory animals were carried out exclusively by persons with higher biological or health education.

Laboratory animals and their maintenance

The work was performed on 48 female BALB/c mice obtained from the nursery of laboratory animals of Pushchino Scientific Center for Biological Research of the Russian Academy of Sciences (Russia). Before modeling metastatic breast cancer, the animals had undergone the necessary certification procedures and introductory quarantine. The animals lived in standard conditions of the vivarium of the Center for Innovative Medicines of Volgograd State Medical University (Russia) with Resolution No. 51 of the Chief State Sanitary Doctor of the Russian Federation dated Aug 29, 2014 "On approval of SP 2.2.1.3218-14 "Sanitary and epidemiological requirements for the device, equipment and maintenance of experimental biological clinics (vivariums)"[1].

The animals were kept in plastic cages for 5 individuals on a litter of sawdust with free access to water and feed, with the replacement of both daily. The air temperature in the vivarium was maintained in

[1] Resolution of the Chief State Sanitary Doctor of the Russian Federation dated August 29, 2014 No. 51 "On approval of SP 2.2.1.3218-14 "Sanitary and epidemiological requirements for the design, equipment and maintenance of experimental biological clinics (vivariums)" (together with "SP 2.2.1.3218-14. Sanitary and epidemiological rules..."); registered with the Ministry of Justice of Russia on October 31, 2014 No. 34547). Available from: https://www.consultant.ru/document/cons_doc_LAW_155631/. Russian

the range of 20–26°C, the relative humidity was in the range of 30–70%, a 12-hour light cycle was realized with a combined (natural+fluorescent) type of lighting. Bactericidal air treatment was carried out continuously by stationary bactericidal plants.

Experimental study design

The main experimental model of this study is orthotopic implantation of cancer cells subcutaneously under the nipple into the fatty pad of the breast, which simulates metastatic stages and decays breast cancer in the clinic. The injection site and technique, together with the specific properties of the selected breast cancer cell line, largely determine the dynamics of the behavior of transplanted cells in mouse tissues [18].

To induce temporary immunosuppression, white BALB/c mice were injected intraperitoneally with azathioprine at the dose of 1 mg/kg body weight three times: 3 h before the injection of tumor cells, 24 and 48 h after it. At the main stage, orthotopic tumor transplantation was performed, for which 48 mice (6 animals in each group) were injected into the base of the breast nipple with 106 human breast cancer cells of the MCF-7 line in 0.2 ml of a sterile saline solution. To reduce the vaccination dose, Matrigel (Corning, USA) was used as a carrier [23]. All the animals vaccinated with MCF-7 cells were divided into 8 groups (Table 1).

Two doses of the substance (1/2 C_{50}, and 1/10 IC_{50}) were used to study the antitumor activity spectrum. The choice of doses of the tested derivatives was justified by the authors' previous studies of their cytotoxic activity and genotoxic activity. The substances were administered once intraperitoneally in 0.2 ml of a saline solution 72 h after the tumor cells injection, in the control group, only a sterile saline solution was used.

Every three days, the mice were weighed and the injection area was visually monitored.

The animals were removed from the experiment on the 15th day with an overdose of Tiletamine at a dose of 200 mg/kg of the intraperitoneal weight. The euthanasia of mice was carried out in a room where no other animals were kept, and the disposal of corpses was carried out by a responsible person.

After euthanasia, the sections of the subcutaneous tissue of the abdominal wall where tumor cells had been injected, as well as similar areas on the opposite side were taken away. When cutting out the material, the total volume of the papilla and its base (mm^3) was calculated. The difference between the described value for intact and injected mammary glands was taken as the relative volume of the tumor tissue.

The material was placed in a buffered 10% formalin solution for a subsequent histological examination. The following histological equipment was used for the study – Microm STP 420 wiring apparatus (Microm, Germany), Leica EG 1160 modular filling system (Leica, Germany), Leica RM 2255 rotary microtome (Leica, Germany), Dako coverstainer (Dako, Denmark), Dako Link 48 immunohistostainer (Dako, Denmark), microscope Leica DM 6000B with Leica $DFC_{50}C$ digital camera (Leica, Germany). The possibilities of classical light and fluorescence microscopy were used for the visualization and morphometric examination of cells and tissues. The image analysis was performed using the ImageJ program (USA).

To prove the presence of human MCF-7 cells in mouse tissues, immunohistochemical detection of marker cytokeratin 8/18, clone EP 17+EP30 using Dako Cytomation kits (Denmark), was used. Imaging was performed using an indirect immunoperoxidase method, using positive antigen controls, a negative antigen, and antibody controls[2].

The dynamics of an animal body weight, the estimated volume of tumor tissue, and histological characteristics of the injection site were selected as indicators determining the antitumor effects of the test compounds.

For a general conclusion about the tumor condition, a histological index was calculated based on a breast cancer gradation from 0 for normal non-tumor tissue to 10 for an undifferentiated tumor[3] with a slight difference – due to a relatively small amount of tumor tissue, the maximum values of the mitotic index were reduced to 4 (Table 2).

Statistical analysis

Statistical processing of experimental data was carried out using the Statistica 10.0 program (Dell USA). After checking the samples according to the Shapiro-Wilk criterion for compliance with the normal distribution and establishing its absence, the results were presented as the median, the boundaries of the first and third quartiles – Me [Q1÷Q3]. An intra-group comparative analysis was performed using the Kraskel–Wallis criterion, and a comparison between unrelated samples was performed using the Mann-Whitney criterion. The differences were considered significant at a confidence level of p <0.05.

RESULTS

Dynamics of body weight and volume of tumor tissue

The administration of tumor cells to mice was accompanied by the cessation of their growth and loss of body weight, which amounted from 6 to 11% of the initial value in all experimental groups during the period from the injection of MCF-7 to the administration of an antitumor drug (Table 3).

[2] Frank GA. Mammary cancer. Practical guide for doctors. Ed. GA Frank, LE Zavalishina and KM Pozharisky. M.: RMAPO, 2014. 197 p. Russian
[3] Ibid.

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Table 1 – Characteristics of experimental animals groups

Group	Compound	Dose	Number of heads
Control	Saline solution	–	6
1а	Imidazotetrazine **1**	1/10 IC_{50}	6
1б		1/2 IC_{50}	6
2а	Imidazotetrazine **2**	1/10 IC_{50}	6
2б		1/2 IC_{50}	6
3а	Imidazotetrazine **3**	1/10 IC_{50}	6
3б		1/2 IC_{50}	6
Reference	Epirubicin	1/2 IC_{50}	6

Table 2 – Histological gradation of breast cancer to determine histological index in experiments on immunodeficient mice

Criteria	Score in points
Structure formation	
Glandular/tubular structures occupy: – more than 75% of the tumor area	1
– from 10 to 75% of the tumor area	2
– less than 10% of the tumor area	3
Nuclear polymorphism	
Monomorphic small nuclei with a clear contour, uniform chromatin, similar to the nuclei of normal epithelium	1
Enlarged moderately polymorphic vesicular nuclei with prominent nucleoli	2
Polymorphic vesicular nuclei, variable in size, with noticeable nucleoli, often of atypical shape	3
Mitoses and invasions	
Single mitoses	1
Multiple mitoses	2
Multiple mitoses and invasion G1	2
Multiple mitoses and invasion G2-G3	4

Table 3 – Antitumor effects of azoloazines 1–3 and epirubicin during orthotopic transplantation of MCF-7 cells to immunodeficient mice

Group (all *n*=6)	Weight of mice, g		Tumor volume on day 15, mm^2
	Initial	Final	
Control	48.8 [46.9÷50.7]	40.7 [37.9÷43.1]	24.5 [19.9÷27.8]
Epirubicin 1/2 IC_{50}	48.3 [46.2÷50.0]	43.1 [40.5÷46.2]*	18.5 [15.6÷20.9]*
Imidazotetrazine **1**			
1/10 IC_{50}	48.1 [46.0÷49.6]	47.5 [46.1÷48.8]*	12.4 [9.9÷15.1*#
1/2 IC_{50}	48.1 [46.0÷49.6]	48.6 [46.5÷50.5]*#	9.6 [7.7÷11.8]*#
Imidazotetrazine **2**			
1/10 IC_{50}	49.0 [47.2÷51.0]	45.1 [43.4÷46.9]*	18.7 [15.1÷20.4]*
1/2 IC_{50}	49.0 [47.2÷51.0]	46.3 [44.5÷47.4]*	16.5 [13.3÷19.0]*
Imidazotetrazine **3**			
1/10 IC_{50}	48.4 [47.1÷50.2]	45.6 [43.3÷48.0]*	17.3 [14.8÷20.5]*
1/2 IC_{50}	48.4 [47.1÷50.2]	46.9 [43.7÷48.2]*	15.0 [12.9÷18.1]*#

Note: statistically significant differences * – between the use of azoloazine derivative and control; # – between the use of azoloazine derivative and epirubicin (p <0.05).

Table 4 – Histological index after administration of azoloazines 1–3 and epirubicin in orthotopic transplantation model of human breast cancer cells MCF-7 to mice

Test compound	Dose	
	1/10 IC_{50}	1/2 IC_{50}
Control	8.8 [7.7÷9.6]	
Epirubicin	–	8.5 [7.2÷9.4]
Imidazotetrazine **1**	8.9 [7.7÷9.7]	8.2 [7.3÷9.1]
Imidazotetrazine **2**	8.5 [7.4÷9.3]	9.1 [7.9÷9.8]
Imidazotetrazine **3**	8.4 [7.5÷9.2]	8.8 [7.7÷9.6]

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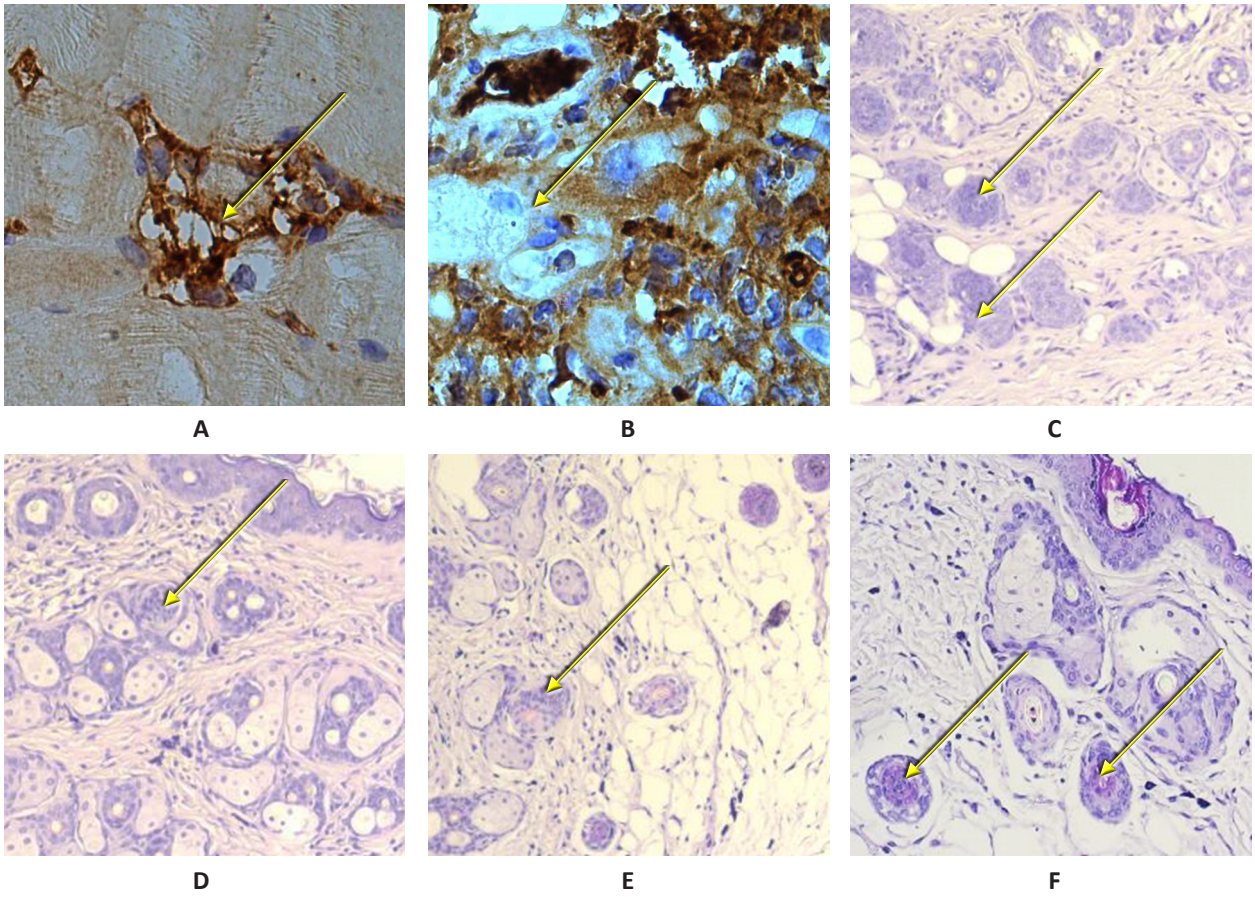

Figure 1 – Transplantation area of MCF-7 cells to mice in xenogenic model of human breast cancer

Note: A – control series without the use of antitumor drugs. Accumulation of immunopositive material in the area of the neurovascular bundle of the adjacent muscle. B – application of imidazotriazine 2. Accumulation of tumor cells with invasion into the lumen of the duct of the breast. C – application of epirubicin. Perry and intraductal clusters of tumor cells. D – use of imidazotetrazine 1. Single clusters of tumor cells around the ducts of the breast and in the adjacent fatty tissue. E – use of imidazotriazine 2. Accumulation of tumor cells with invasion into the lumen of the duct of the breast. F – use of imidazotriazine 3. The histological picture is similar to the use of imidazotriazine 3. A, B – immunohistochemical detection of human cytokeratin 8/18. C–F is stained with hematoxylin and eosin. Magnification: ×200.

During the same period, the body weight of intact mice increased by an average of 2%. The administration of the tested compounds changed the dynamics of animal body weight to a certain extent, but these changes were different depending on the compound and its dose.

The administration of the reference drug epirubicin was accompanied by a slowdown in the decrease in body weight in mice, which eventually amounted to 89.2% of the initial *vs.* 83.4% in the control. The tumor volume calculated on the 15th day of the autopsy experiment was 75.5% of the analogous one without the administration of an antitumor drug.

Imidazotetrazine **1** caused the cessation of the body weight loss in mice and its increase over 15 days led to the restoration of this indicator to the initial one (when using a dose of 1/10 IC_{50} – 98.8%, when using a dose of 1/2 IC_{50} – 101.6%). The mass recovery was especially intense in the first three days after the injection of derivative 4: during this time, the mass increased by more than 10%. The tumor volume calculated on the 15th day of the autopsy experiment was 50.6% of the same in the control when using a dose of 1/10 IC_{50}, and 39.2% when using a dose of 1/2 IC_{50} (p <0.05).

When imidazotriazine **2** was administered to the experimental mice with transplanted MCF-7 tumor cells, the body weight dynamics similar to that of epirubicin, was observed. Stabilization of the mass and its slight increase by the 15th day of the experiment led to a final mass when using a dose of 1/10 IC_{50} in 92.0% of the initial, when using a dose of 1/2 IC_{50} – 94.5%. The tumor volume calculated on the 15th day of the autopsy experiment was 76.3% of the same in the control when using a dose of 1/10 IC_{50}, and 67.3% when using a dose of 1/2 IC_{50}, which corresponded to the inhibition of tumor growth by 23.7 and 32.7%, respectively (p <0.05).

The administration of imidazotriazine **3** caused the cessation of body weight loss and its increase by 11.5% in the first three days. Subsequently, the dynamics of body weight stabilized, and by the 15th day of the experiment

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it amounted to 94.2% of the initial when using a dose of 1/10 IC_{50}, and 96.9% when using a dose of 1/2 IC_{50}. The tumor volume calculated on the 15th day of the autopsy experiment was 70.6% of the analogous one without the administration of an antitumor drug when using a dose of 1/10 IC_{50} and 61.2% when using a dose of 1/2 IC_{50}. This corresponded to the inhibition of tumor growth by 29.4 and 38.8%, respectively (p <0.05).

Results of histological examination

The histological examination revealed changes in the transplantation area, as well as in the adjacent fatty tissue and lymph nodes (Fig. 1).

Staining for human cytokeratin 8/18 revealed the presence of this protein in mouse tissues, which indicated the validity of the applied technique. In cases where MCF-7 cells were lyzed, the marker protein was detected in the intercellular substance, since it is quite difficult to proteolysis (Fig. 1A, 1B).

In all experimental groups on the 15th day of the experiment, the morphological picture at the site of xenotransplantation of MCF-1 cells was characterized by the presence of single tumor cells, as well as their clusters in the form of small clusters or trabeculae. The amount of stroma between tumor cells in the case of cluster formation was minimal. In some cases, the formation of chains and structures like a target or an “owl’s eye” was observed around medium-sized vessels and ducts of the mammary gland. A complete obstruction of the lumen by invasive MCF-7 cells was sometimes detected in the ducts, but more often such cells were isolated, and a part of the lumen was filled with a mucus-like secret. In case of death, the tumor cells lost their nucleus and were surrounded by lymphohistiocytic infiltrate. A small number of lymphocytic cells could also be found around clusters of viable tumor cells (Fig. 1C–1F).

The tumor cells themselves were extremely variable in structure. The cell nuclei were usually large, polymorphic, with a predominance of rounded shapes. They were located, as a rule, centrally and contained well-distinguishable nucleoli. The cytoplasm of the cells was predominantly extensive and pronounced eosinophilic. A mitotic activity on the preparations varied from single mitoses to 10–15 divisions in the field of vision.

In addition to the local invasion into the lumen of ducts, lymphatic and (much less often) blood vessels, tumor cells were able to spread to adjacent tissues and lymph nodes. In the fibrous-adipose tissue surrounding the mammary gland, both single MCF-7 cells and clusters of 7–12 cells were found, often with moderate lymphohistiocytic infiltration around them. In adipose tissue, the tumor clusters were larger; morphologically they looked like typical micrometastases closely adjacent to the feeding vessels. In lymph nodes, MCF-7 cells were usually located singly, and there was a pronounced tissue reaction from macrophages and lymphoid tissue around them.

The determination of the histological index made it possible to give a semi-quantitative assessment of histological changes in the zone of xenotransplantation of human breast cancer cells when testing azoloazine derivatives and a comparison drug (Table 4).

As can be seen from the presented data, the use of epirubicin at a dose of 1/2 IC_{50} practically did not change the histological index of tumors, in comparison with the value of the indicator in the control group of animals.

The values of the histological index in all groups practically did not differ from the control (the maximum decrease with the administration of imidazotetrazine **1** was 9.3%), which once again confirmed the fact that the cells insensitive to the applied chemotherapy retain a high ability to malignant progression in the body and can even increase it.

DISCUSSION

The development of virtual screening, chemical technologies for the targeted synthesis of organic compounds with predicted properties, as well as medical biotechnology for testing potential drugs in *in vitro* cultures and modified models on laboratory animals have become the basis for a new era of development and implementation of modern chemotherapeutic agents.

The modern paradigm implies that from the moment these compounds are presented to experimenters, their chemical structure evidently assumes an antitumor activity (docking or the closest analogues of available drugs). According to the results of preclinical tests, the drugs have clearly marked targets of exposure, proven their high antitumor activity *in vitro* and *in vivo*, have minimal toxic effects on healthy tissues and do not cause rapid development resistance [24–26].

With regard to the subject of this study, it should be emphasized that solving the problem of breast cancer in prevention cases, early diagnosis, treatment and rehabilitation of women is classified as a priority task of world and domestic healthcare [1, 5].

The class of compounds chosen (imidazotriazine and imidazotetrazine derivatives) is attractive in this regard due to a sufficient number of well-established representatives, including mitozolamide and temozolomide, as well as the emergence of protocols for the synthesis of new compounds with potentially promising properties [12, 19]. According to the results of evidence-based studies, compounds of this class exhibit antitumor, anti-inflammatory, antioxidant, and antibacterial activity [27, 28].

Orthotopic implantation of cancer cells into animal breast tissue allows very accurate reproduction of the tumor microenvironment and in many ways resembles the multiple stages involved in the development of breast cancer in patients [23, 29].

The study showed that in a xenogenic breast cancer model obtained by orthotopic injection of MCF-7 cells into mice, all azoloazine derivatives included in the work showed antitumor activity, which has signs of dose dependence and decreases in the series of imidazotetrazine **1** (leader) >imidazotriazine **3** > imidazotriazine **2** ≥epirubicin. The maximum inhibition of tumor growth with the introduction of the most active derivative of temozolomide 1 was 60.8%, which significantly exceeded the activity of the comparison drug epirubicin – 24.5%. The drug significantly suppressed the proliferation and spread of tumor cells in the model of xenotransplantation of human breast cancer MCF-7 cells to mice. The administration of the drug was accompanied by a decrease in the histological malignancy index for preserved cells by 9.3%.

In all series of the experiments, the histological picture indicated a decrease and disintegration of a part of the tumor structures in the tissues of mice, but the preserved cells showed pronounced atypism, a tendency to mitosis and invasion, and formed fresh accumulations in breast tissue, adjacent fatty tissue and lymph nodes.

As can be seen, all the three drugs selected based on previous cytotoxicity studies on human breast cancer cells and non-tumor cells *in vitro,* showed results exceeding those for the reference drug epirubicin. The pharmacodynamic properties of epirubicin have been well studied and are similar to those of other anthracycline antitumor antibiotics. Epirubicin is most active in the phases of the cell cycle, accompanied by the most intensive DNA synthesis. After the intercalation between DNA base pairs, it stabilizes the topoisomerase II-DNA complex, which leads to an irreversible rupture of the DNA strand. Cytotoxicity has been proven for epirubicin both *in vitro* and *in vivo* against breast cancer and a number of other human tumor cells, and cell death increases with increasing drug concentration [30]. Nevertheless, the activity of the drug is quite moderate, as demonstrated by the present study. Therefore, epirubicin can be considered a classic reference drug for new preclinical trials, which are designed to find new, more effective drugs for tumor chemotherapy.

A comparison of the antitumor activity in an *in vivo* model of three azoloazine derivatives showed that, despite the similar structure and physico-chemical properties, these compounds exhibit an unequal activity in relation to breast cancer cells. The known reasons that cause differences in the antitumor effects of homologous molecules are, first of all, differences in the nature of distribution in the body during a parenteral administration and the ability to penetrate target cells. The experimental values of tumor cell survival obtained are consistent with earlier results of studying other azoloazine derivatives [12, 31, 32].

Implantation of human breast cancer cell lines into mice is relatively simple and makes the evidence-based genetic or pharmacological manipulation of implanted cells possible. However, it is now fairly believed that xenografts of tumors in immunodeficient mice cannot fully simulate tumors in humans, including due to a significant genetic polymorphism and the fact that human cells are apparently not fully adapted to growth in mouse tissues [33]. It is no coincidence that recently the attention of researchers has been increasingly focused on creating three-dimensional models of cancerous tumors *in vitro.* Modeling 3D microenvironments that maximally simulate the microenvironment of cells in human breast cancer makes them a valuable tool as an alternative for replacing tumor models in animals and for predicting a risk assessment in humans [34].

CONCLUSION

Using a xenogenic model of human breast cancer (NCF-7 cell culture), all three new azoloazine derivatives included in the study showed an antitumor activity exceeding that of the reference drug drug epirudbicin. Among the tested new azoloazine derivatives, 3-cyclohexyl-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazine-8-N-piperidinylcarboxamide is the undisputed leader causing the inhibition of tumor growth in the xenogenic model *in vivo.*

The high sensitivity of the MCF-7 cell lines to the tested substances makes it possible to conclude that further preclinical tests of their effectiveness and safety are promising. According to the results of the study of new azoloazine derivatives, it is possible to expect the inhibition of the growth of transplanted tumors in the experimental animals in relation to a wider range of simulated tumors.

The conclusion obtained should be interpreted as preliminary, since only one approach (xenogenic transplantation in mice) and one breast cancer cell line, MCF–7, were used in the work to assess the antitumor activity. The specific mechanisms of action of azoloazine derivatives, as well as the comparison of their activity with the structural features of molecules, require a separate further study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Ahmed Hamid Al-Humairi – development of the basic concept, hypothesis and design of the study, conducting research, processing research data, writing and editing text; Dmitry L. Speransky – development of the basic concept and design of the study; Valery V. Novochadov – development of the concept and design of the study, statistical analysis; Sergey V. Poroysky – conducting research, implementation of the experimental part of the study, research data processing, text editing; Nadezhda V. Cherdyntseva – development of the research concept; Vladimir V. Udut – development of the research concept. All authors confirm that their authorship meets the international ICMJE criteria (all authors have made a significant contribution to the development of the concept, research, and preparation of the article, read and approved the final version before the publication).

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