ISOTOPICALLY MODIFIED ^{64}ZINC DIASPARTATE RENDERS ANTIMETASTATIC EFFECT ON MELANOMA *IN VITRO* AND *IN VIVO*

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ABSTRACT

Intracellular zinc homeostasis plays a critical role in melanoma development, with its dysregulation contributing to various aspects of cancer progression. These processes are accompanied by disruptions in zinc-regulated metabolic processes, which potentially affect the isotopic composition of the microelement in the cells and can significantly influence their malignant properties, in particular metastatic activity.

This study investigated the effect of isotopically modified structural analog of zinc aspartate, ^{64}Zn-Diaspartate in which ^{64}Zn atoms were enriched to exceed 99%, on the metastatic activity of melanoma B16 cells *in vitro* and *in vivo*, and the possible mechanisms of the ^{64}Zn effect on the biology of these malignant cells. In this paper, we refer to the investigational compound, the isotopically modified zinc diaspartate, as ^{64}Zn diaspartate.

Mouse melanoma MB16 cells and ^{64}Zn diaspartate chelate complex were used as the study subjects. In this study, we employed an isotopically modified structural analogue of zinc-aspartate, in which non-radioactive light isotope ^{64}Zn was enriched to a mass fraction of 99.2% - a significant enhancement compared to the natural isotopic ratio of 48.6% commonly used in the zinc compounds discussed in the literature. The migratory activity of the cells was determined using the scratch assay method. The invasive activity of melanoma cells was investigated using the agar colony formation assay. The expressions of Bcl-2, Bax, and p53 were analyzed by immunocytochemistry. The activities of MMP-2 and MMP-9 were evaluated using the zymographic method. Sequentially, the effect of ^{64}Zn diaspartate on the metastatic activity of MB16 cells was evaluated *in vivo* using an experimental hematogenous metastasis model in C_{57}Bl/6 mice.

In the *in vitro* investigations, ^{64}Zn diaspartate suppressed the migratory activity of MB16 cells and their CFA by 29-67% relative to the untreated melanoma cells. The changes in the

biology of malignant cells were accompanied by an increase in cytoplasmic expression of p53 and a 10-fold increase in the Bax/Bcl-2 ratio compared to the intact cells. Additionally, treating MB16 cells with ^{64}Zn diaspartate led to a significant decrease in the activity of MMP-2 and MMP-9 within MB16 cells.

In vivo, we have monitored that a series of intravenous injections of ^{64}Zn diaspartate lead to a 64-78% reduction in the number and 79-84% reduction in the volume of melanoma metastases in the lungs of C_{57}Bl/6 mice, compared to the control.

Administering ^{64}Zn-diaspartate suppressed the migratory and invasive activity of MB16 cells *in vitro* and *in vivo*. We conclude that the compounds inclusive of enriched isotopically light zinc-64 as an active ingredient may effectively activate apoptosis in MB16 cells *in vitro* through Bax-dependent mitochondrial pathway and inhibit MMP-2 and MMP-9 gelatinases activity, providing an anti-metastatic effect in the MB16 mouse melanoma.

1. Introduction

Tumor metastases are the leading cause of death in cancer patients, and melanoma is one of the most aggressive and metastatic types of malignant neoplasms [1]. Historically, scientists believed that tumor cell dissemination occurs in the late stages of cancer progression [2]. However, the development of new modern reliable methods for detecting circulating tumor cells has made it possible to discover that melanoma cells can metastasize to various organs and tissues long before the primary tumor reaches a clinically detectable size [3].

Metastasis is a complex process consisting of several key stages, in particular, the infiltration of tumor cells into stromal tissues, their migration, invasion, adhesion and proliferation at the site of metastasis [4].

This is a multi-stage process caused by genetic and epigenetic modifications not only in the tumor cell, but also in its microenvironment, which significantly complicates the selection of highly effective antimetastatic therapy regimens for patients with melanoma.

The modern melanoma treatment strategies are aimed at preventing the proliferation of primary tumor cells and the formation of metastases, and include chemotherapy, radiation and targeted therapy, as well as immunotherapy [5-7]. Chemotherapy and radiotherapy are not considered good options for inhibiting the metastatic activity of melanoma due to the non-specificity of these methods and the resistance of malignant cells [8]. The advances in treating advanced melanoma over the past few years have been astounding, thanks to immunotherapy and targeted therapy [9].

However, immunotherapy can lead to the development of autoimmune diseases [10] and resistance to treatment as a side effect of using targeted drugs [11]. Therefore, researchers are still searching for new methods of antimetastatic therapy for melanoma. Promising therapeutic agents

today are substances that can inhibit different stages of the metastatic process [12]. One of the recent directions in such search is the development of new compounds based on zinc as an important microelement characterized by a wide range of biological effects. Zinc regulates the functional activity of enzymes and transcription factors of key signaling pathways of cellular homeostasis [13].

Various studies have shown a high degree of correlation between zinc deficiency and the development or progression of certain types of tumors, indicating the microelement's antitumor effect [14]. Zinc has been shown to inhibit migration, invasion, and consequently, metastasis from prostate [15], breast [16], ovarian [17], and skin [18] cancer cells, *in vitro and in vivo*. Possible anti-migration and anti-invasive effects of zinc-containing substances may be realized by reducing the activity of metalloproteinases (MMPs), for example, MMP-2 [19]. Furthermore, zinc can inhibit melanoma cell proliferation by blocking glycolysis and the Krebs cycle, which leads to impaired ATP production [20]. In addition to these metabolic effects, zinc accumulation in melanoma cells leads to activation of mitochondrial apoptosis and modulation of the expression of apoptosis regulatory proteins p53 and FASL [21]. The skin is the third largest “depot” of zinc, storing about 5% of the total zinc reserve in the body [22].

Zinc comprises five stable isotopes: ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, and ^{70}Zn, with average natural abundances of 48.6%, 27.9%, 4.1%, 18.8%, and 0.6%, respectively. Normally, the dominant zinc isotope in human skin cells is ^{64}Zn [23]. Still, malignant transformation of skin cells is accompanied by disruptions in zinc-regulated metabolic processes, which changes the isotopic composition of the microelement in the cells and can significantly influence their malignant properties [24]. Due to coordination chemistry, different ligands preferentially bind isotopically different zinc, which influences zinc’s isotopic homeostasis and the biological properties of cells.

Zinc complexes of proteinogenic amino acids deserve special attention, as they are characterized by safety in relation to normal cells and high bioavailability. Zinc aspartate is a natural, bioavailable form of zinc microelement, which is also an essential regulatory molecule of signaling and biochemical metabolic pathways in cells [25]. The isotope effects were shown to improve cellular membrane permeability (ref) and reaction rates (ref). For these reasons and also based on our other experiments with enriched ^{64}Zn, we hypothesized that a compound consisting of L-aspartic acid and zinc with enriched light stable isotope (^{64}Zn) can affect the biological properties of melanoma cells by reducing their migratory and invasive activity – the key properties of malignant cells that ensure their metastasis.

Our previous studies investigating the effect of ^{64}Zn monoaspartate on the viability of malignant cells showed that mouse melanoma MB-16 cells are among the most sensitive to the cytotoxic/cytostatic effects of the test compound. [26]. Assuming that the bioavailability of ^{64}Zn monoaspartate is mainly due to aspartic acid, a new compound, ^{64}Zn diaspartate, was synthesized

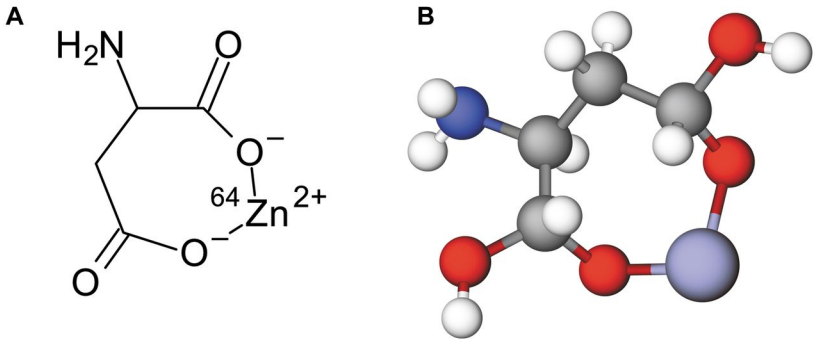
for the present study. Consequently, we have assessed the effects of ^{64}Zn monoaspartate, ^{64}Zn diaspartate and isotopically natural Zn diaspartate on melanoma cell viability in a comparative manner. Next, we studied the effects of exogenous ^{64}Zn diaspartate on the migratory and invasive activity of melanoma cells *in vitro and in vivo*. The expression of pro- and anti-apoptotic proteins, as well as the activity of MMP-2 and MMP-9 in melanoma cells after their treatment with ^{64}Zn disparate, were studied to find possible molecular mechanisms of action of the test compound on their metastatic activity.

MATERIALS AND METHODS

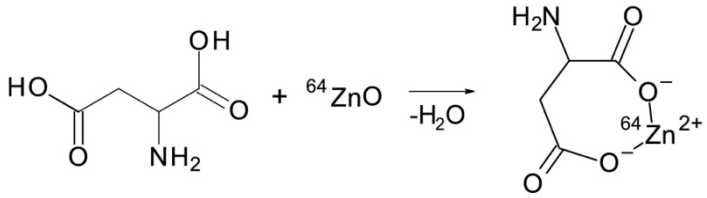
Test substances.

Three different test substances of zinc aspartate were investigated in this study:

1. Isotopically modified ^{64}Zn monoaspartate complex ("OTAVA" Company, Kyiv, Ukraine) chelated with 1 molecule of L-aspartic amino acid (NeoFroxx, Einhausen, Germany), having a molecular formula $C_4H_5NO_4{}^{64}Zn$ wherein isotopically light ^{64}Zn is enriched to >99% (Neonest AB, Solna, Sweden), molar mass is 196.0 g/mol, and the percentage of ^{64}Zn in the chelate complex is 19.85%. The structural formula of $C_4H_5NO_4{}^{64}Zn$ is:

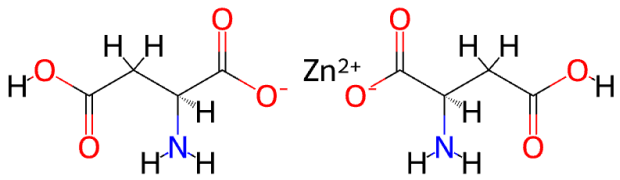


and the synthesis scheme is:



2. Isotopically modified ^{64}Zn diaspartate complex ("OTAVA" Company, Kyiv, Ukraine) chelated with 2 molecules of L-aspartic amino acid, having a molecular formula $C_8H_{12}N_2O_8{}^{64}Zn$ wherein isotopically light ^{64}Zn is enriched to >99%, molar mass is 328.1 g/mol, and the percentage of ^{64}Zn in the chelate complex is 19.85%.

The structural formula of the $C_8H_{12}N_2O_8{}^{64}Zn$:



and the synthesis scheme is:

$$2H_2NCH(CH_2COOH)COOH + {}^{64}ZnO \longrightarrow {}^{64}Zn(H_2NCH(CH_2COOH)COO)_2$$

3. Isotopically natural Zn diaspartate complex ("OTAVA" Company, Kyiv, Ukraine) chelated with 2 molecules of L-aspartic amino acid having a molecular formula $C_8H_{12}N_2O_8Zn$ and its molar mass is 328.2 g/mol. The percentage of Zn (Zinza Industrials Nacionales S.A., Callao, Peru) in the chelate complex is 19.85%.

4. L-aspartic amino acid.

Cell Culture. MB16 cells were derived from the tissue of B16 mouse melanoma strain. Melanoma cells were cultured in a DMEM medium (Biowest, France) with 10% fetal bovine serum (FBS) (Biowest, France) and 1x penicillin-streptomycin (Biowest, France), in plastic dishes (SPL, Korea), at 37°C, in a humidified atmosphere supplied with 5% CO_2. MB16 cells were kindly provided by Prof. Yu. Kudryavets (Bank of cell lines from human and animal tissues, Kyiv, Ukraine).

Determination of Cell Viability. Melanoma cells were seeded in a number 1×10^4 cells per well in the 96-well plate (TPP, Switzerland) in DMEM medium with 10% FBS and 1x penicillin-streptomycin. The next day ^{64}Zn monoaspartate, ^{64}Zn diaspartate and Zn diaspartate were added to the cells at 1-75 µg/ml concentration. The cells were incubated at 5 % CO_2 and 37°C for another 48 hours. The viability of cells were evaluated colorimetrically by staining with crystal violet dye (5 mg/ml of 70% methyl alcohol) (Sigma-Aldrich, USA). The dye was eluted from cells by 96° ethyl alcohol. The results were recorded using a multi-well spectrophotometer (Labsystems Multiskan PLUS, Finland) at wavelength of 540 nm. The percentage of viable cells was calculated relative to the number of control intact cells. Non-linear regression analysis was used to determine the IC50 values for the test compounds on melanoma cells.

In Vitro Migration Assay. The migration activity of melanoma cells was assessed using the scratch assay method. Cells ($5x10^4$/well) were seeded into wells of a 12-well plate in DMEM supplemented with 10% FBS, 1x penicillin-streptomycin and incubated under standard conditions for 24 hours. Sequentially, ^{64}Zn diaspartate (1 and 3 µg/ml zinc) was added to the cells, and cells

were cultured for 48 hours. MB-16 cells incubated without ^{64}Zn diaspartate were used as a control. Next, using a sampler tip (with a volume of up to 200 µl), the monolayer was damaged in all wells, and the medium was changed. Half of the wells where cells had previously been treated with ^{64}Zn diaspartate were re-treated with the test compound at the same concentrations. Migration activity of cells was analyzed using an inverted microscope and cells in the injury zone were photographed at several time points after monolayer damage. In the control and experimental groups, the cell incubation time required for complete restoration of the monolayer was determined.

Semisolid Agar Colony Assay. Melanoma cells ($2x10^4$/well) were seeded into wells of a 24-well plate in DMEM supplemented with 10% FBS and 1x penicillin-streptomycin and cultured under standard conditions for 24 hours. Sequentially, ^{64}Zn diaspartate (3 µg/ml zinc) was added to the cells, and they were incubated in a humidified atmosphere at 37^0C with 5% CO2 for another 72 hours. MB-16 cells not treated with ^{64}Zn diaspartate were used as a control. To study the colony-forming activity (CFA) of melanoma cells in semi-solid agar in vitro, 1 ml of a suspension of control and ^{64}Zn diaspartate-treated cells (2000 cells/well) in a nutrient medium with 0.33% agar were added to parallel wells of a 6-well plate containing a supporting layer of complete nutrient medium with 0.5% agar. Throughout the experiment, nutrient medium with 0.33% agar was added to all wells every 3 days, but nutrient agar containing ^{64}Zn diaspartate (3 µg/ml zinc) was added to half of the experimental wells. The plates were incubated for 14 days in a CO_2 incubator in a humidified atmosphere at 37^0C in the presence of 5% CO2, after which the colonies were stained for 3 hours with 0.2% MTT solution at 37^0C. Next, the number of colonies in each well was determined using a binocular (S850 Stereo Zoom, Richter Optica, USA.) The KOA of the studied cells in semi-liquid agar was determined by the formula and expressed as a percentage: KOA = (A/B) x 100%, where KOA is the colony-forming activity of the cells; A is the number of formed colonies (pcs); B is the number of cells planted in semi-liquid agar (pcs).

Immunocytochemical Analysis. The expression level of apoptosis-regulating proteins in melanoma cells was determined by immunohistochemical assay. MB16 cells were seeded at coverslips in the number of $0,5\times10^6$ cells/Petri dish (SPL, Korea) in a DMEM medium with 10% FBS and 1x penicillin-streptomycin and cultured at 37°C and 5% CO_2 for 24 hours. After that, ^{64}Zn diaspartate (4.5 µg/ml for zinc) were added to the cells and incubated under the same conditions an additional 48 hours. Melanoma cells incubated without ^{64}Zn diaspartate were used as control. MB16 cells were washed with phosphate-buffer saline (PBS) (Sigma, USA) and placed in methanol/acetone (Sigma, USA) solution (1:1) for 2 hours at a temperature of -20°C. An ICC reaction was performed using an Ultra Vision Quanto Detection System (Thermo Scientific, USA) following the manufacturer's instructions. Primary monoclonal antibodies against Bcl-2 clone 100/D5, Bax clone 6A7, p53 clone DO-7 (Thermo Fisher Scientific, USA) were used. Cells treated with mAbs were incubated at room temperature for 60 minutes. An imaging system conjugated

with peroxidase was then used and enzyme activity was detected using diaminobenzidine (Thermo Scientific, USA) as a substrate. When the immunocytochemical reaction was completed, the specimens were stained with hematoxylin (Thermo Scientific, USA). The results were analyzed by counting cells with marker expression (brown stained cells) using an Axiostar Plus light microscope (Carl Zeiss, Germany) and evaluated using the classical H-Score method: S = 1xA+2xB+3xC, where S is the H-Score index having the values within a range from 0 (no protein detected) to 300 (intense expression in 100% of cells), *A* is the percentage of weakly stained cells, *B* is the percentage of moderately stained cells, *C* is the percentage of strongly stained cells. The observation results were fixed using a Canon PowerShot G5 digital camera (Canon PowerShot G5, United Kingdom) at x400 magnification.

Determination of Metalloproteinases Activity. Activities of gelatinases A and B (MMP-2 and MMP-9, respectively) in MB-16 cells were analyzed using gelatin zymography and SDS-Polyacrylamide gel electrophoresis. Melanoma cells were seeded at a number of $0{,}5\times10^6$ cells/Petri dish in a standard medium and cultured at 37°C and 5% CO_2 for 24 hours. After that, ^{64}Zn diaspartate (4.5 and 3 μg/ml for zinc) were added to the cells and incubated at 37°C and 5% CO_2 for an additional 48 hours. Cells incubated without ^{64}Zn diaspartate were used as control. Thereafter, MB-16 cells were removed from the substrate and the alive and dead cells were counted with trypan blue (HyClon, USA) exclusion test. To obtain the protein fraction, a 1% SDS (Sigma-Aldrich**,** Germany) solution was added to the cellular precipitate ($1x10^6$ cells). Vertical electrophoresis of samples was carried out in a 7.5% polyacrylamide gel (Applichem, Spain) containing 0.1% SDS and 1% gelatin (Sigma-Aldrich**,** Germany). Electrophoresis conditions: 170 W for 4 hours at +4°C. Tris-glycine (Sigma-Aldrich**,** Germany) buffer was used as a buffer solution for electrophoresis. After electrophoresis was completed, the gel was washed in a Triton X-100 (Sigma-Aldrich**,** Germany) solution and incubated in 1x PBS at 37°C for 12 hours. The gels were then stained at room temperature with Coomassie blue G250 (Sigma-Aldrich**,** Germany) dissolved in a mixture of methanol/acetic acid/water. In zymograms, regions of gelatinase activities appeared as clear regions against a blue background. Correspondence of the MMP-2 and MMP-9 lysis zones was evaluated using a SigmaMarker™ molecular weight marker (Sigma-Aldrich, Germany). Zymograms were digitized. The results were processed using the Videodensitometer Sorbfil 2.0 software. The proteolytic activity of enzymes was determined using a standard set of MMP-2 and MMP-9 (Sigma-Aldrich**,** Germany). The activity of 1 ng enzyme in $1x10^6$ cells of the control sample was taken as a conditional unit (cu).

In Vivo Metastatic Activity. Experiments were conducted on C_{57}Bl/6 male mice aged 10 – 12 weeks, weighing 22-25 g. Before the experiment, all animals were healthy, with natural behavior and activity profile. All animals were maintained in accordance with the standards set forth in The Guide for Care and Use of Laboratory Animals (ILAR publication, 1996, National Academy Press,

1996). During the experiment, animals were maintained in the vivarium of the R.E. Kavetsky IEPOR NAS of Ukraine in plastic cages in a room with a natural light/dark cycle and provided standard diet with ad libitum access to food and water. Animal protocols were approved by the Bioethics Committee at R.E. Kavetsky IEPOR NAS of Ukraine. All studies were conducted in compliance with the rules of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes "General principles of animal experimentation" approved by the National Congress on bioethics (Kyiv, 2001–2007) and in concordance with Council directive of November 24, 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC).

The animals were injected intravenously (IV) with B16 melanoma cells (experimental model of hematogenous metastasis), $0.2x10^6$ cells per mouse (0.2 ml of the suspension) into the lateral tail vein. The animals were allocated randomly into 3 groups: group I - MB16 cells, control (n=8); group II - MB16 cells + ^{64}Zn diaspartate 45 minutes later after the tumor cells inoculation (n=8); group III - MB16 cells + ^{64} Zn diaspartate 24 hours later after the tumor cells inoculation (n=8). Zn^{64} diaspartate was administered IV at a dose of 3 mg/kg for zinc. The test substance was administered every other day (for 10 days), 5 administrations in total. The first injection of the Zn^{64} diaspartate was provided 45 minutes or 24 hours later after the tumor cells were inoculated. Day 26 after the start of IV injections of tumor cells all animals were euthanized via cerebral dislocation method. The lungs of all animals were excised to determine the number and volume of metastases using the formula for the volume of a sphere.

Statistical Data Processing. Calculations of the means of the investigated parameters (M), the standard deviation (SD) and the standard error of the mean (m) were performed using the Excel 2016 software package. Student's t-test was applied to assess the significance levels of differences in mean values between groups. Calculations were performed using the STATISTICA 6.0 software package. The values were considered as statistically significant if the p-value was less than 0.05.

RESULTS

The effect evaluation of different concentrations of ^{64}Zn monoaspartate and ^{64}Zn diaspartate on the viability of melanoma cells in vitro allowed us to determine the IC50 of these substances at 7.9±0.3 μg/ml and 3.1±0.4 μg/ml, respectively. The IC50 of isotopically natural Zn diaspartate for MB16 cells was 11.3±0.5 μg/ml, which was 1.4 higher than IC50 for ^{64}Zn monoaspartate and 3.6 times higher than IC50 for ^{64}Zn diaspartate, respectively (Figure 1.) Conclusively, ^{64}Zn diaspartate was determined to be the most effective in inhibiting MB16 cell viability *in vitro*. Aspartic acid in the concentrations studied did not have any cytotoxic or

antiproliferative effect on melanoma cells, leading us to assume that zinc was the active ingredient in the investigational compounds.

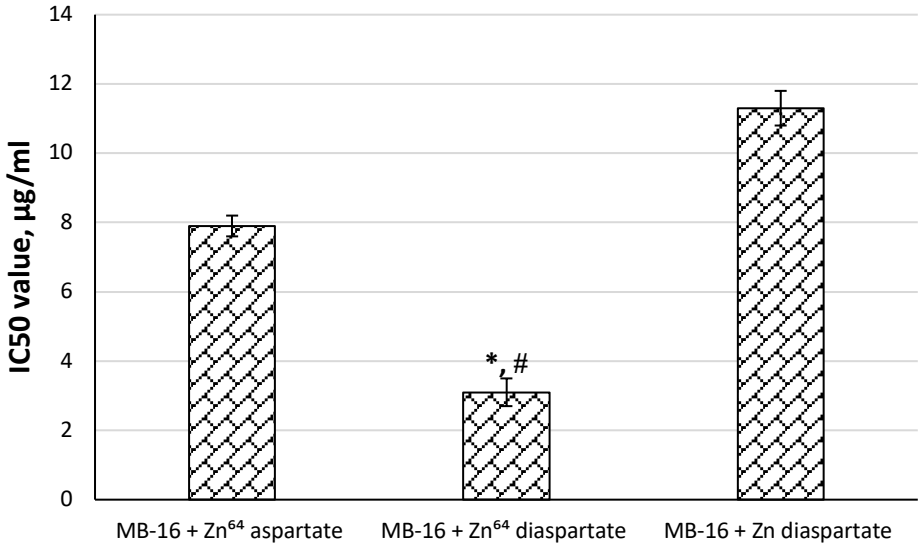


Figure 1. Comparative analysis of IC_{50} for experimental compounds containing isotopically modified (enriched >99%) ^{64}Zn and isotopically natural Zn in mouse melanoma cells MB16 *in vitro.* *$p<0.05$ ^{64}Zn diaspartate compared to ^{64}Zn monoaspartate; # - $p<0.05$ ^{64}Zn diaspartate compared to Zn diaspartate.

Given that ^{64}Zn disparate demonstrated superior performance compared to ^{64}Zn mono aspartate and isotopically natural Zn disparate, we have elected to continue this study with ^{64}Zn disparate and have excluded the other two compounds. Our further studies to evaluate the effect of ^{64}Zn diaspartate on the biological characteristics of melanoma cells associated with their metastatic activity were performed using the test compound concentrations corresponding to IC25 and IC50.

Melanoma cells' migration and colony-forming activity *in vitro* were studied post-treatment with ^{64}Zn diaspartate. The migration activity of malignant cells was assessed using the scratch assay method. Two variants of treating melanoma cells with a test compound were used: (1) for 48 hours before the scratch was applied, and (2) for 48 hours before and 48 hours after the damage to the monolayer.

In variant 1, treatment of MB16 cells with ^{64}Zn diaspartate at a concentration of IC25 did not affect their migration activity – cells of the experimental and control groups completely restored the damaged monolayer 24 hours after the scratch was applied. Increasing the concentration of ^{64}Zn diaspartate to IC50 resulted in the suppression of melanoma cell migration

by 50% compared to the control. Complete restoration of the cell monolayer was monitored 36 hours after its damage (Figure 2c).

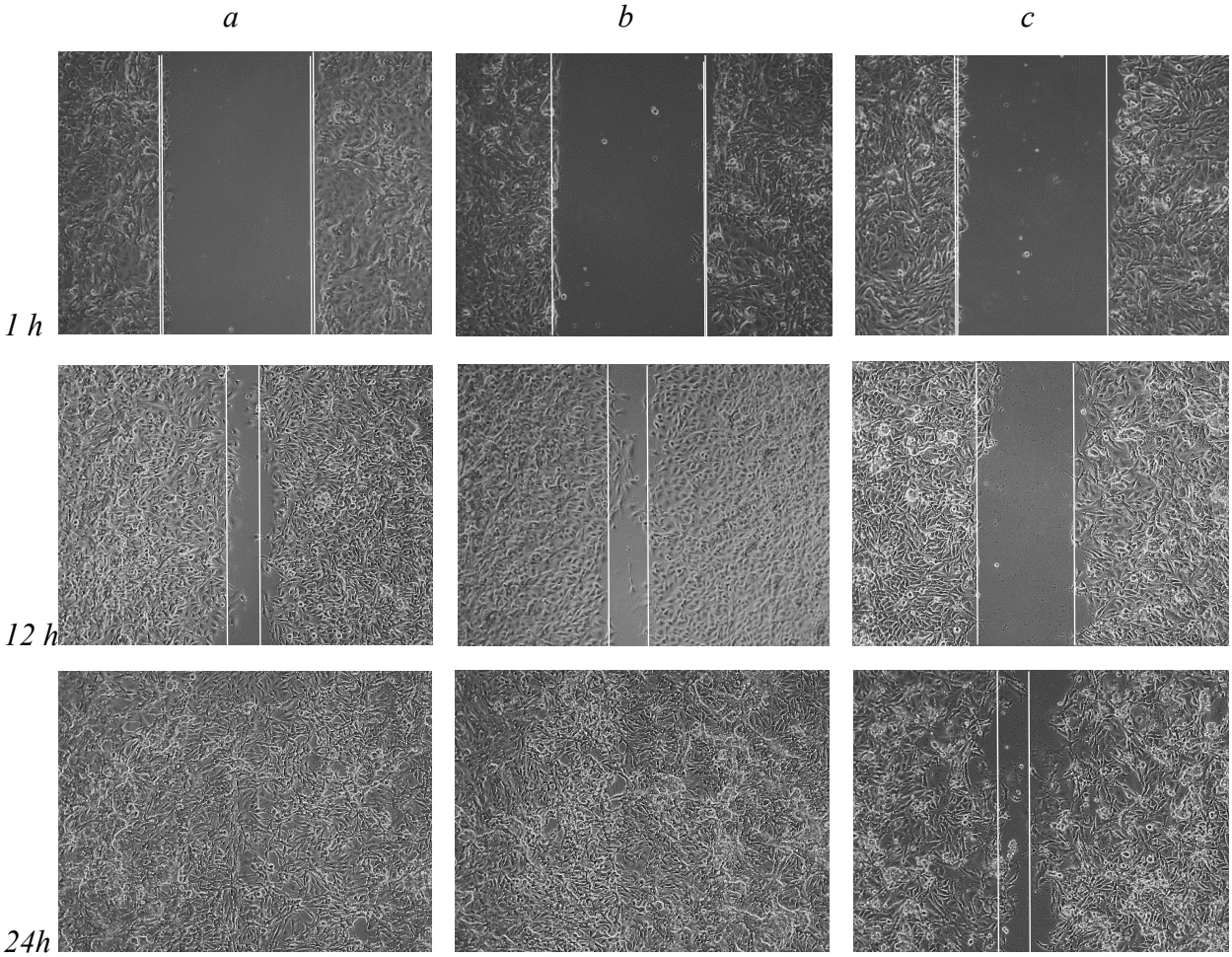


Figure 2. Migration activity of MB16 cells *in vitro* after their pretreatment with ^{64}Zn diaspartate. *(a)* - MB16 control, *(b)* - MB16 + ^{64}Zn diaspartate IC_{25}, *(c)* - MB16 + ^{64}Zn diaspartate IC_{50}. (1, 12, 24 hours after disruption of cell monolayer integrity).

При обработке клеток MB16 ^{64}Zn diaspartate до и после нанесения царапины обнаружили подавление их миграции в 1.5 – 3 раза по сравнению с контролем клеток (Figure 3). В клетках меланомы обработанных ^{64}Zn diaspartate в концентрации IC_{25} наблюдали полное восстановление клеточного монослоя через 36 часов после его повреждения, а при увеличении концентрации ^{64}Zn diaspartate до IC_{50} – через 72 часа. This shows that ^{64}Zn diaspartate suppresses the MB16 cell migratory activity in dose-dependent manner. As migration is one of the earliest and essential stages of metastasis, this indirectly demonstrates the antimetastatic activity of ^{64}Zn diaspartate, which is explored and described below in greater details.

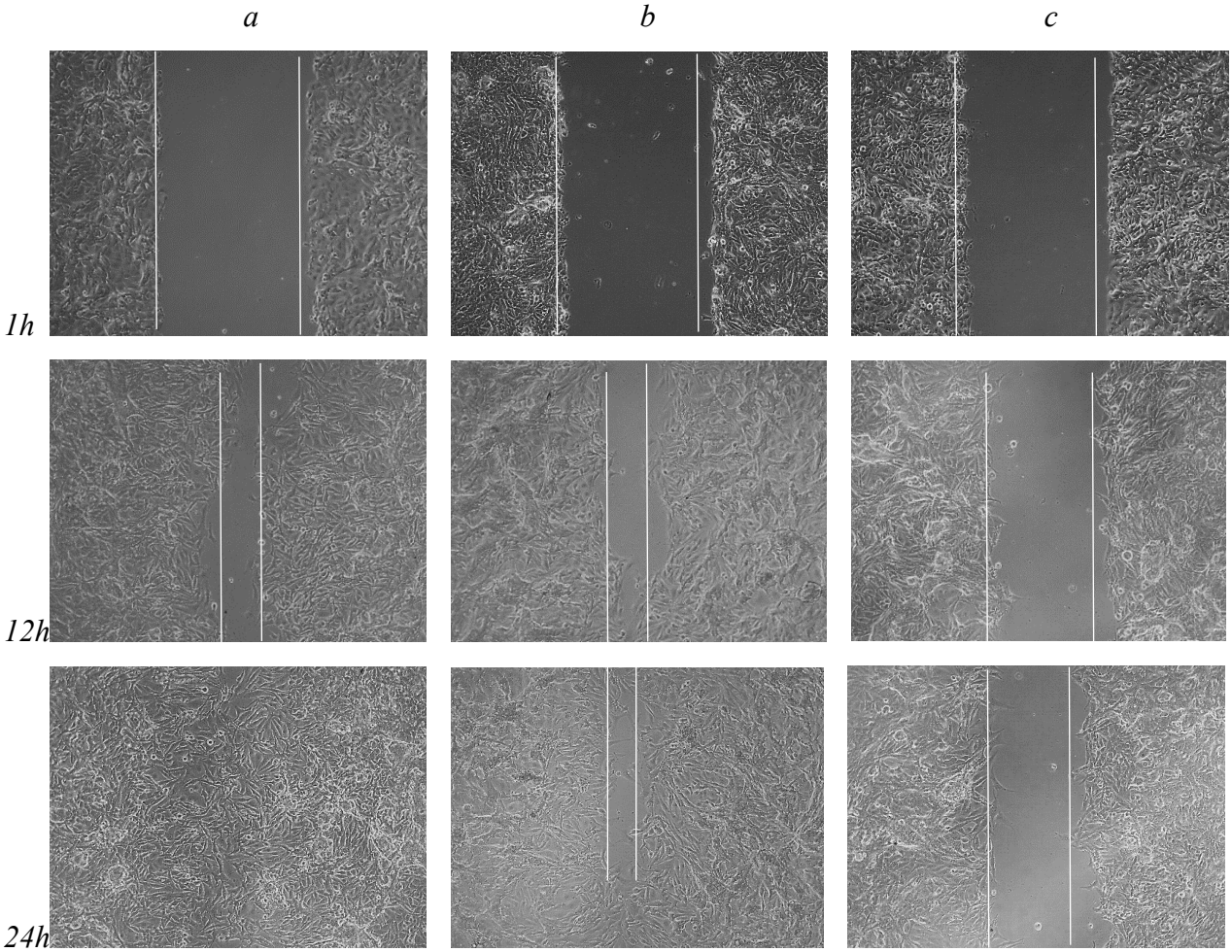
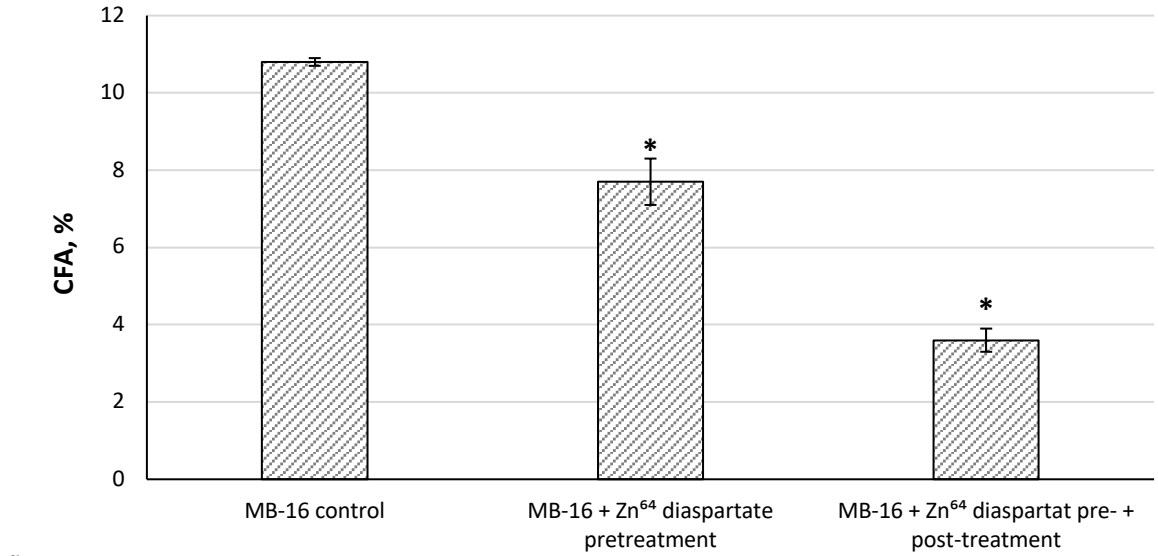


Figure 3. Migration activity of MB16 cells *in vitro* after their pretreatment and post-treatment with ^{64}Zn diaspartate. *(a)* - MB16 control, *(b)* - MB16 + ^{64}Zn diaspartate IC_{25}, *(c)* - MB16 + ^{64}Zn diaspartate IC_{50}. (1, 12, 24 hours after disruption of cell monolayer integrity).

The colony forming activity (CFA) assay of MB16 cells in semisolid agar after their treatment with ^{64}Zn diaspartate was used to evaluate its effects on the invasive activity of malignant cells [27]. The melanoma cells were treated with ^{64}Zn diaspartate in 2 ways: 1) pre-treatment (once for 72 hours, and the cells were planted in semi-liquid agar;) 2) pre-treatment and post-treatment (first for 72 hours, and the cells were planted in semi-liquid agar, and then ^{64}Zn diaspartate was added to the agar daily for 14 days.)

After the single pretreatment, ^{64}Zn diaspartate statistically significantly suppressed CFA of MB16 cells by 29% compared with control (Figures 4a and 4b2.) A continuous (pre-treatment + post-treatment, 14 days) exposure of MB16 cells to ^{64}Zn diasparate significantly enhanced its anti-invasive effect, showing a decrease in CFA of MB16 cells by 67% relative to the control (Figures 4a and 4b3).



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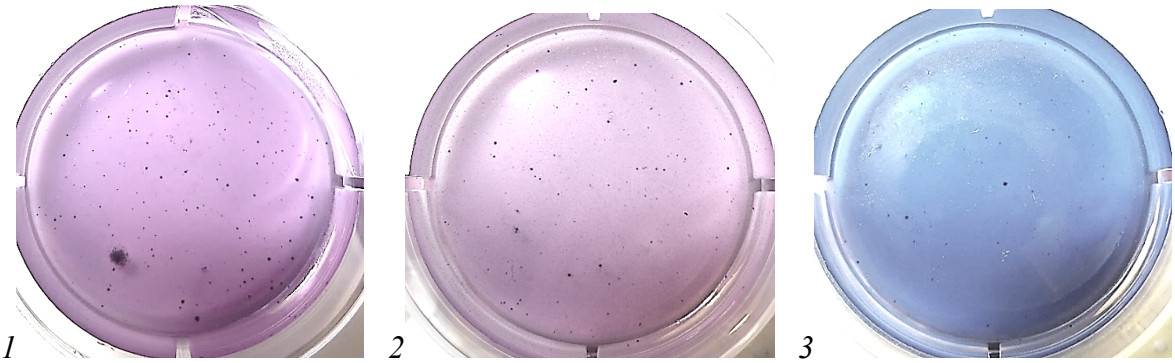

b 1 *2* *3*

Figure 4. (*a*) Evaluation of MB16 cells colony-forming activity in semi-solid agar after their pretreatment and post-treatment with ^{64}Zn diaspartate. *p<0.05 compared to control. (*b*) Photo of melanoma cell colonies in semi- solid agar. Cell colonies are stained with MTT. *(b1)* - MB16 control, *(b2)* - MB16 + ^{64}Zn diaspartate pretreatment, *(b3)* - MB16 + ^{64}Zn diaspartate pretreatment and posttreatment.

To study possible mechanisms responsible for the monotored anti-migration and anti-invasive effects of ^{64}Zn diaspartate on melanoma metastases, we investigated the influence of ^{64}Zn diaspartate on the activation of the apoptosis program in melanoma cells (expression of p53, Bax, and Bcl-2), and the production and activity of matrix metalloproteinases (MMP-2 and MMP-9) in the MB16 cells.

In our *in vitro* study, the effects of ^{64}Zn diaspartate on the expression of anti-apoptotic protein Bcl-2 and pro-apoptotic proteins Bax and p53 in mouse melanoma cells were analyzed using immunocytochemistry. The treatment of MB16 cells with ^{64}Zn diaspartate at the concentration IC50 led to an increase in the cytoplasmic expression of p53(clone DO-7) by 9.4 times and Bax by 9.0 times vs. control, and a decrease in the Bcl-2 expression by 18.2% compared to control (Table 1, Figure 5).

The literature informs that baseline Bax/Bcl-2 ratios vary among cell types but are often around 0.5 to 1. The mean increase in Bax/Bcl-2 ratios associated with administering approved anticancer pharmaceuticals ranges from 2 to 5 times vs. controls [28]. In our experiments, the treatment of melanoma MB16 cells with ^{64}Zn diaspartate resulted in 10.6 times increase of Bax/Bcl-2 vs. control (Table 1), indicating strong shift towards proapoptotic action associated with ^{64}Zn diaspartate treatment.

Table 1

Effects of ^{64}Zn Diaspartate on the Expression of Apoptosis-Regulating Proteins in MB16 cells

Apoptosis-regulating proteins	Expression level (M±m), H-Score points	
	MB16 control	MB16 + ^{64}Zn diaspartate
Bcl-2	120.7±12.4	98.7±8.1
Bax	15.3±2.9	136.7±14.7*#
Bax/Bcl-2 ratio	0.13	1.39
p53 (clone DO-7, cytoplasm)	18.7±3.5	176.7±16.5*#

***p<0.05 compared to control; # indicates a statistically significant difference in values between 2 experimental groups (p<0.05).*

We hypothesize that because ^{64}Zn diaspartate exerts a toxic effect on MB16 cells by activating apoptosis, the MB16 cells attempt to protect themselves through various mechanisms, including the expression of the anti-apoptotic protein Bcl-2. Typically, in such cases, an increase in Bcl-2 expression is observed in various tumor cells in response to cytotoxic effects. In this study, however, Bcl-2 expression decreased by approximately 18%, which aligns with results observed in the industry when using potent chemo- and immunotherapy drugs [29].

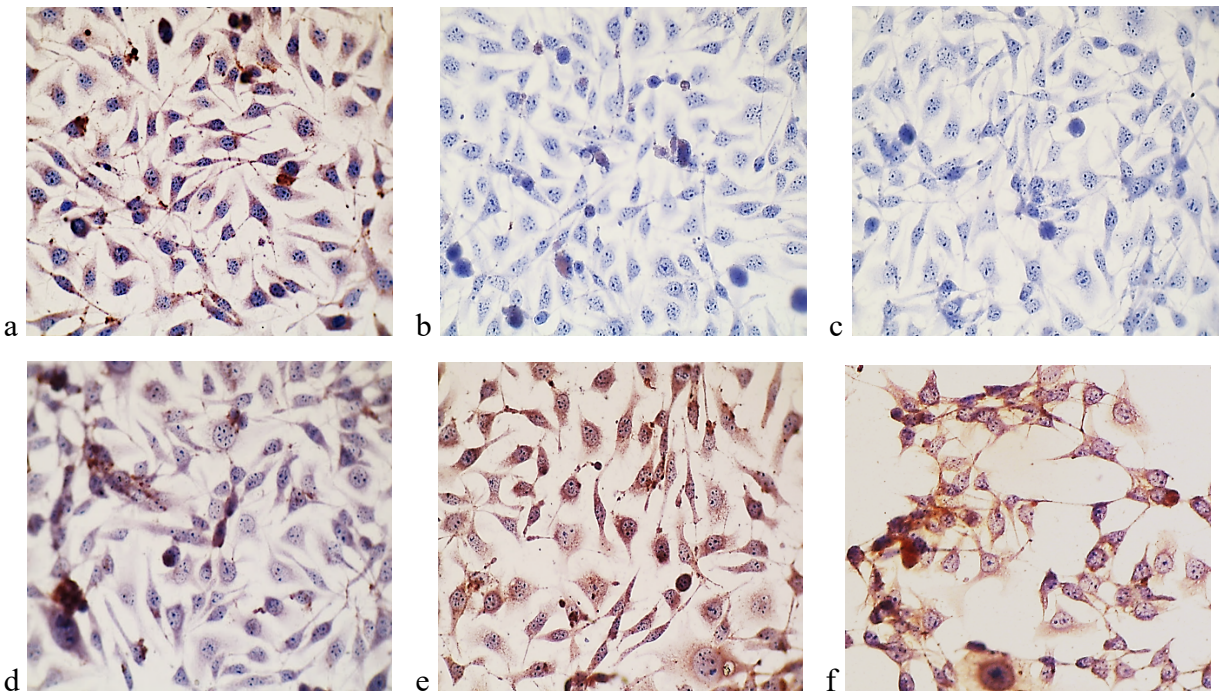

Figure 5. Effects of ^{64}Zn diaspartate on the apoptosis-regulating proteins expression in MB16 cells, ICC, x400 magnification. (a-d) - Bcl-2 expression, (b-e) - Bax expression, (c-f) – p53 expression; (a,b,c) - MB16 control, (d,e,f) - MB16 + ^{64}Zn diaspartate IC_{50}.

Matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, serve as critical indicators of cancer growth and progression across various malignancies [30]. In our study, we analyzed the activity of latent (pro-MMP-2 and pro-MMP-9) and active forms (MMP-2 and MMP-9) of MMPs in MB16 cells after they were treated with ^{64}Zn diaspartate. The results indicate that the treatment with ^{64}Zn diaspartate significantly inhibited the activities of these gelatinases in MB16 melanoma cells (Figure 6). Specifically, the treatment of MB16 cells with ^{64}Zn diaspartate in IC25 caused a statistically significant decrease in the activities of both MMPs forms: pro-MMP-2 decreased by 76.5% and pro-MMP-9 decreased by 61.2%, and a decrease of active MMP-2 and MMP-9 forms of gelatinases by 66.8% and 23.8%, respectively, compared to the control. Treating melanoma cells with ^{64}Zn diaspartate at the IC50 concentration resulted in a statistically significant reduction in the MMP-2 activity by 76% and MMP-9 activity by 18.8% compared to the control (Figure 6). We did not detect any activities of latent forms of MMPs in this group of cells (0 cu).

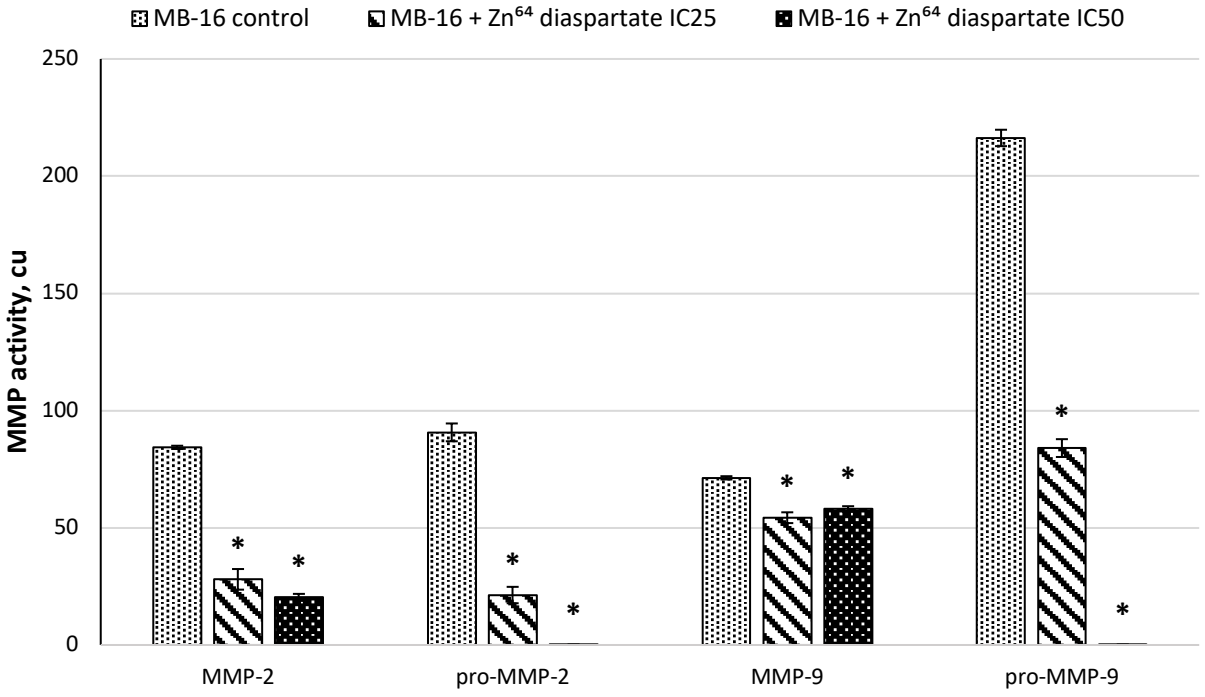

*Figure 6. Activities of MMP-2 and MMP-9 gelatinases (active and latent forms) in ^{64}Zn diaspartate treated MB16 cells. * $p < 0.05$ compared to control.*

The results of in vitro studies indicate that treatment of MB16 cells with ^{64}Zn diaspartate was accompanied by modification of a complex of phenotypic characteristics of the malignant cells towards suppression of their migratory and invasive activities, likely due to ^{64}Zn enrichment and isotope effects.

Our *in vitro* findings led us to investigate the effects of reducing the metastatic potential of melanoma cells associated with administering ^{64}Zn diaspartate *in vivo*. Table 2 summarizes the results of counting the number and volume of MB16 metastases in the lungs of mice after a series of intravenous injections of ^{64}Zn diaspartate.

A decrease in both studied parameters the number and the volume of metastatic melanoma nodes was observed in the group where the animals received their first injection of ^{64}Zn diaspartate 24 hours after introducing melanoma cells. However, only the volume of metastases decreased statistically significantly (by 4.8 times) compared to the control group (3.3 ± 2 mm vs 15.8 ± 5.4 mm3 in the control.) In the group where animals were administered ^{64}Zn diaspartate 45 minutes after introducing MB16 tumor cells, a statistically significant decrease in the number and the volume of metastatic melanoma nodes was observed, relative to the indicators of the control group.

Importantly, we observed that in both groups where animals received therapeutic injections of ^{64}Zn diaspartate, in 25% of animals the metastases were eradicated completely (Figure 7).

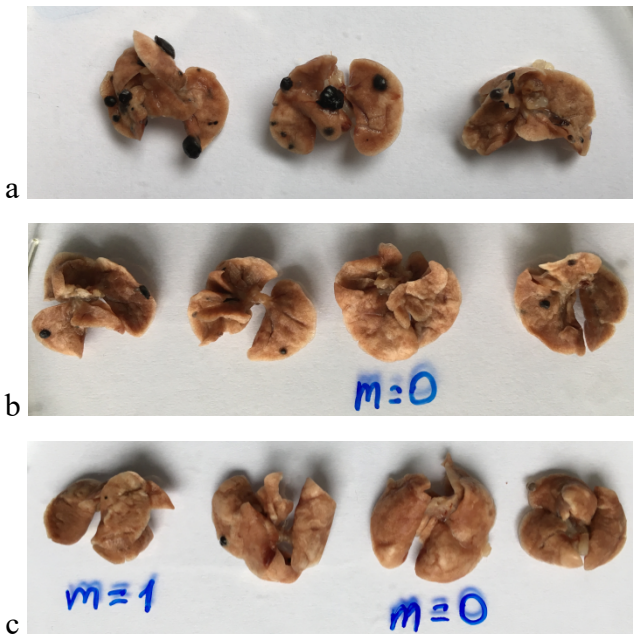

Figure 7. Representative photographs of mouse lungs.

Suppression of the growth of B16 melanoma metastases in the lungs of C_{57}Bl/6 mice with therapeutic intravenous administration of ^{64}Zn diaspartate (c) 45 minutes after, or (b) 24 hours after intravenous administration of tumor cells, compared to the control group (a). "***m***" represents the number of metastases.

Table 2

In vivo characteristics of MB16 cells metastatic activity under the effect of Zn^{64} diaspartate

Experimental groups	Number of metastases/mouse (M±m)	Volume of metastases/ mouse, mm^3 (M±m)	Number of animals in a group with metastases, %
MB16 control	18.0 ± 4.8	15.8 ± 5.4	100.0
MB16 + IV injection of Zn^{64} diaspartate 24 hours after the tumor cells were inoculated	6.4 ± 2.5	3.3 ± 2.0*	75.0
MB16 + IV injection of Zn^{64} diaspartate 45 min after the tumor cells were inoculated	4.0 ± 2.0*	2.5 ± 1.7*	75.0

*$p<0.05$ compared with the control group

Conclusively, five intravenous injections of ^{64}Zn diaspartate showed a significant reduction in melanoma cell metastasis in the experimental hematogenous metastasis system, as expressed in a decrease in the number of metastases by 78%, and the volume of metastatic nodes by 85%, compared to the control. The first injection of ^{64}Zn diaspartate was made 45 minutes after the introduction of MB16 cells.

DISCUSSION

Melanoma is the most aggressive and life-threatening form of skin cancer, characterized by high invasiveness and the ability to metastasize. It is more dangerous due to its potential to spread rapidly if not treated early [31]. Approximately 30% to 40% of patients who die from melanoma did not have metastases at the initial diagnosis, indicating that a significant portion of early-stage melanomas can progress to metastatic disease despite treatment [32]. It is estimated that nearly one-third of all melanoma patients will experience disease recurrence, which often involves metastasis [33]. Despite the significant progress made in treating melanoma oncologies, the search for new drugs with antimetastatic action against melanoma is still relevant today.

A novel experimental compound containing isotopically modified zinc (enriched with the light stable isotope ^{64}Zn>99%) as an active ingredient was synthesized in the form of ^{64}Zn diaspartate to improve zinc's bioavailability and enhance zinc's biochemical functioning. The novel compound was studied to assess its effects on the antimetastatic activity in the MB16 mouse melanoma model *in vitro* and *in vivo*.

Traditionally, the industry opined that changes in the rate of biochemical reactions and other physicochemical parameters between isotopes of chemical elements vary within the range of variations in their atomic masses. It was generally accepted that since the difference between the atomic masses of different isotopes is insignificant, then isotopic effects on the biochemical and biophysical properties of chemical elements involved in physiological processes should not be significant.

For example, a 0.5% mass difference between normal and ultralight enzymes (those depleted of heavy isotopes ^{13}C, ^{2}H, ^{15}N, and ^{18}O) was predicted to produce a kinetic effect of about 1% or less. However, recent research shows that the actual effect can actually be >2 times stronger, representing a significant differentiation from the previous understanding. This discovery highlights the need to reassess the industry's knowledge of isotopic influences on biochemical processes and their potential implications for various fields of study [34].

In this study, a comparative evaluation of the cytotoxic effects of ^{64}Zn diaspartate to ^{64}Zn monoaspartate and to Zn diaspartate (by IC50) showed that ^{64}Zn diaspartate inhibited the viability of MB16 cells in vitro 3.6 times more effectively than isotopically natural Zn diaspartate and 1.4 times more effectively than isotopically modified ^{64}Zn monoaspartate.

The migration and invasion of malignant cells are integral components of the metastatic process. Therefore, the study of the effect of ^{64}Zn diaspartate on the migration and invasion of melanoma cells was aimed at evaluating the antimetastatic activity of ^{64}Zn diaspartate *in vitro*.

The results showed that ^{64}Zn diaspartate significantly and dose-dependently suppressed the migratory activity of MB16 cells in the scratch assay, as evidenced by the time required for the

recovery of the cellular monolayer increasing by 12 hours compared to the control. Notably, the duration of treatment of MB16 cells by ^{64}Zn diaspartate also matters – pretreatment by ^{64}Zn diaspartate in addition to post-treatment led to a significantly enhanced anti-migration effect. Moreover, the time of recovery from damage to the melanoma cell monolayer increased by 12-48 hours compared to the control cells. Building on this, we posit a hypothesis that initiating ^{64}Zn diaspartate treatment for melanoma in the early phase (as soon as practical following diagnosis) may significantly reduce the likelihood of metastasis development.

The effect of ^{64}Zn diaspartate on the invasive potential of melanoma cells was assessed *in vitro* by determining the colony-forming activity (CFA) of these cells in semisolid agar [35]. Analysis of the tumor cells' ability to grow under such conditions is considered the most accurate and reliable *in vitro* assessment of the degree of malignancy of cells, correlating with their metastatic potential [36]. The results of the study showed that ^{64}Zn diaspartate could significantly suppress CFA in MB16 cell agar *in vitro* – both short-term (pre-treatment) and long-term (pre-treatment+post-treatment) effects of ^{64}Zn diaspartate administration to melanoma cells resulted in a statistically significant decrease in CFA by 29% and 67%, respectively, compared to the control. The most pronounced CFA-inhibitory effect was observed under the condition of long-term presence of ^{64}Zn diaspartate in semi-liquid agar.

The results of our experiments indicate that ^{64}Zn diaspartate suppresses the migratory and invasive activity of MB16 cells *in vitro*, and therefore can also inhibit the metastasis of melanoma cells. Implementing such an effect in melanoma cells in vivo is associated with a cascade of complex and multi-stage molecular biological processes that affect the biology of malignant cells. Therefore, we investigated some possible mechanisms underlying the observed antimetastatic effect. In particular, we analyzed the effect of ^{64}Zn diaspartate on the expression of apoptosis-regulating proteins and activities of latent and active forms of MMP-2 and MMP-9 in MB16 cells. The studied parameters are relevant specifically because zinc is located in the active centers of MMPs and the proapoptotic protein p53, regulating their functional activity [37, 38]. Although the proapoptotic protein Bax and the antiapoptotic Bcl-2 do not contain zinc ions in their structure, their activity is regulated through various zinc-mediated signaling pathways and zinc finger proteins [39], which makes these proteins an interesting object for our studies.

Apoptosis is the main method of malignant cell death, through which anticancer therapy eliminates tumor or metastatic cells. Several clinical and experimental studies indicate that melanoma is often associated with increased resistance of malignant cells to apoptosis induced by various therapeutic methods. Pro- and anti-apoptotic proteins are able to combine with each other, forming homo- and heterodimers, and the sensitivity of cells to apoptotic stimuli may depend on the balance of these proteins [40, 41].

In our studies, we observed a 9-fold increase in p53 expression in the cytoplasm of MB16 cells treated with ^{64}Zn diaspartate, compared to intact cells. It is traditionally believed that the functional activity of the p53 gene transcription regulator is realized in the cell nucleus [42]. However, numerous studies show that cytoplasmic p53 can also induce apoptosis through a transactivation-independent mechanism [43].

Under various conditions that cause cell death, p53 quickly moves to the mitochondria, where part of the protein interacts with the outer membrane of the organelle, inducing its permeabilization, ensuring the release of pro-apoptotic factors, in particular Bax, from the intermembrane space of the mitochondria. In this case, cytoplasmic p53 can enter into molecular interaction with Bax, activating it [44]. Importantly, in our studies, treatment of melanoma cells with ^{64}Zn diaspartate resulted in a 9-fold increase in Bax expression compared to MB16 control cells and, consequently, a 10-fold increase in the Bax/Bcl-2 ratio relative to intact cells.

The Bax/Bcl-2 ratio functions as a cellular rheostat that determines whether a cell lives or dies. The Bax/Bcl-2 ratio represents a critical molecular balance in cancer biology, serving as both a prognostic indicator and a therapeutic target. A balanced Bax/Bcl-2 ratio maintains cellular homeostasis in normal non-malignant cells, allowing for appropriate cell death when necessary. However, this balance is frequently disrupted in cancer, typically favoring cancer cell survival through decreased Bax expression, increased Bcl-2 expression, or both [45]. When the Bax/Bcl-2 ratio shifts in favor of the pro-apoptotic protein Bax, the apoptotic program is activated in cells, which leads to increased Bax translocation into mitochondria, the formation of pores in the mitochondrial membrane, and the release of cytochrome C [46]. *Feng P* (2008) and coauthors opine that zinc not only stimulates Bax translocalization in the cell, but also increases its expression by inducing transcription factors [47] containing the zinc finger domain [48]. We further opine, yet hypothetically, that an isotopic enrichment of ^{64}Zn atoms enhances these reported effects by about 4 times (judging by the enhancement in cytotoxicity.) Conclusively, administering ^{64}Zn diaspartate at the concentration IC50 have activated the Bax-dependent mitochondrial apoptotic pathway of MB16 melanoma cells, which is one of the mechanisms of its antimetastatic action.

Another important result that confirms the antimetastatic effect of ^{64}Zn diaspartate is a significant decrease in the activity of latent and active forms of MMP-2 and MMP-9. These MMPs (gelatinases A and B, respectively) are a family of zinc-containing endopeptidases. Participation of MMPs in tumor transformation and in the processes of invasion and metastasis has been proven using both *in vitro* and *in vivo* [49]. In particular, MMP-2 and MMP-9 are critical molecules for melanoma metastasis, as they induce degradation of the extracellular matrix and proteolytic activation of pro-tumor cytokines and growth factors at each stage of tumor cell dissemination [50]. MMP-2 expression

is generally associated with tumor invasion and angiogenesis, while MMP-9 is believed to play a key role in remodeling associated with neovascularization [51].

Our studies show that treatment of MB16 melanoma cells with 64Zn diaspartate results in a decrease in the activity of both MMP-2 and MMP-9. The most pronounced dose-dependent inhibitory effect of ^{64}Zn diaspartate was observed for latent forms of endopeptidases – a decrease in activity by 60-75%, relative to the control at an IC25 concentration of ^{64}Zn diaspartate, and the absence of pro-MMP-2 and pro-MMP-9 activity with an increase in the concentration of ^{64}Zn diaspartate to IC50. The presence of a dose-dependent inhibitory effect of ^{64}Zn diaspartate on proteinase activity suggests that zinc may inhibit the activity of MMPs through direct interaction with the catalytic domain of gelatinases [52]. In this case, the excess zinc binding may change the conformation of MMP proteins or form a Zn-hydroxide bridge, which blocks the catalytic center of proteinases [53]. In addition, the metal binding can alter protein consolidation in the catalytic domain and inactivate the enzyme [54]. A significant reduction in MMP activity may be interpreted as evidence of anti-invasive and anti-metastatic effects of ^{64}Zn diaspartate. This aligns with the mechanisms in anti-cancer therapies targeting tumor microenvironment remodeling.

Since this was an in vitro experiment, interpreting our results into clinical practice was theoretical. These findings suggested that the isotopically modified ^{64}Zn-diaspartate may have suppressed the growth of metastatic nodules and possibly inhibited the ability of metastatic melanoma cells to anchor themselves at metastatic sites. Notably, this aligned with the results we observed *in vivo*.

To confirm the antimetastatic effect of ^{64}Zn diaspartate in vivo, we assessed the effect of ^{64}Zn diaspartate on the formation and growth of B16 melanoma metastases in the lung tissue of C_{57}Bl/6 mice in a model of experimental hematogenous metastasis. The melanoma cells were injected into mice intravenously. ^{64}Zn diaspartate was also administered intravenously. To assess the ability of ^{64}Zn diaspartate to suppress the viability and metastatic activity of malignant cells circulating in the blood (simulating minimal residual disease) in vivo, one experimental group of animals was started to receive ^{64}Zn diaspartate 45 minutes after the injection of MB16 cells [55].

Mice in another experimental group were administered ^{64}Zn diaspartate for the first time 24 hours after the injection of melanoma cells, i.e. after the MB16 cells had entered the lung tissue and were retained there, which corresponded to the classical scheme of administering a therapeutic agent to assess its antimetastatic activity [56, 57]. Such models simulate the hematogenous stage of metastasis and are relevant for studying the antimetastatic effects of melanoma, since it belongs to those types of dangerous tumors in which metastasis often occurs much earlier than the primary tumor can be diagnosed [58].

A statistically significant decrease in the number and volume of metastases was observed after administering ^{64}Zn diaspartate. In the group of animals that received the first single injection of ^{64}Zn diaspartate 24 hours after the introduction of melanoma cells, a decrease in the number of visible metastatic nodes in the lungs of mice by 64% and their volume by 79% ($p \leq 0.05$) was observed compared to the control group. We opine that the antimetastatic effect of administering ^{64}Zn diaspartate was the result of the anti-invasive and pro-apoptotic effect of ^{64}Zn diaspartate on B16 melanoma cells, which was observed in our *in vitro* tests (suppression of colony formation in agar, MMP activity, and increased expression of pro-apoptotic proteins Bax and p53.) The observed therapeutic effect was enhanced if the first injection of ^{64}Zn diaspartate was given 45 minutes after the introduction of MB16 cells – the number of metastases in this animal group was 77.8% ($p \leq 0.05$) less, and their volume was 84% ($p \leq 0.05$) less than in the control group.

The obtained results suggest that the antimetastatic activity of ^{64}Zn diaspartate is realized not only due to the activation of apoptosis in melanoma cells and the suppression of their invasiveness but is also by an enhancement of the anti-migration effect by ^{64}Zn diaspartate, which has also been shown in our *in vitro* studies.

CONCLUSIONS

Isotopically modified structural analogue ^{64}Zn diaspartate has shown a strong ability to suppress the migratory, invasive and metastatic activity of MB16 cells *in vitro* by activating Bax-mediated mitochondrial mechanism of apoptosis, as well as inhibiting the activity of latent and active forms of MMP-2 and MMP-9 in melanoma cells. *In vivo* studies have confirmed the antimetastatic effect of ^{64}Zn diaspartate. Our data on the ability of ^{64}Zn diaspartate to modulate Bax/Bcl-2 ratio, the expression of p53 and MMPs activity indicate an important novel and functional role of enriched stable zinc isotopes in the elemental dyshomeostasis in malignant cells, which makes the substances synthesized based on isotopically modified zinc ions competitive candidates for the role of new therapeutic agents with anticancer and antimetastatic activity.

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