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In Vitro Anticancer Activity of the Light Stable Zinc Isotope (^{64}Zn) Compounds

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Abstract. *Background/Aim: Today, stable isotopes of zinc are actively used for diagnostic purposes in oncology. However, there is extremely limited data on the attempts to apply stable zinc isotopes in cancer therapy or about the molecular mechanisms of their effects on the biology of tumor cells. Therefore, in this in vitro research, we evaluated the cytotoxic activity of stable zinc isotope (^{64}Zn) enriched compounds against malignant cells and determined the mechanisms of their action. Materials and Methods: Malignant and non-malignant cells of different histogenesis were used as objects of the study. The effect of the Zn^{64}aspartate, Zn^{64}glutamate, and Zn^{64}sulfate on cell viability in a comparative aspect was evaluated. Compounds containing ^{64}Zn stable isotope enriched to 99.2%. Western blot analysis was used to determine the expression level of apoptosis regulatory proteins. Results: Salts of ^{64}Zn with amino acids had the most significant cytotoxic effect on malignant cells. The studied tumor cells, and especially MB16 melanoma cells were the most sensitive to the cytotoxic effects of Zn^{64}aspartate. Zn^{64}aspartate showed more significant cytotoxic activity than Zn aspartate (with natural isotope distribution) in the studied cell models. Zn^{64}aspartate induced caspase-dependent cell death in A-549 cells and the p53-mediated apoptosis in melanoma cells. Conclusion: Malignant cells were more sensitive to the cytotoxic effect of the Zn^{64}aspartate than normal cells. An increase in the intracellular concentration of ^{64}Zn, and hence isotope mass balance changes, may lead to the suppression of the viability and proliferation of malignant cells. These results can become the basis for developing a new generation of anticancer drugs.*

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Key Words: Zinc, light isotope, stable isotope, anticancer effect, cytotoxicity, apoptosis.

Increased incidence of cancer and cancer-related mortality are among the most urgent and growing worldwide problems in modern medicine (1). Despite significant advances in modern clinical oncology, development of new methods for cancer pathology treatment still remains relevant. Today clinical oncology uses a wide range of cancer treatment methods that include surgery, chemotherapy, and radiotherapy; however, their effectiveness is still insufficient, indicating the need for alternative treatments with lower toxicity and increased effectiveness (2). It is important that cancer treatment should be targeted at the selective pathophysiological mechanisms that are inherent in malignant cells and should not disrupt homeostasis in the patient.

The use of stable isotopes of various elements is one of the new and extremely promising ways to solve the topical issues of oncology. According to the available data, stable isotopes have already been used for effective and highly sensitive diagnostics of various types of malignant neoplasms. A study carried out by Tea *et al*. showed that an isotope signature of ^{13}C and ^{15}N characteristic of the breast cancer tissue differed significantly from that of normal breast tissue (3).

At the same time, natural fractionation of stable isotopes of the main elements such as C, H, O and N has not yet been applied widely in medical practice. However, the use of stable isotopes of metals, such as zinc, may be more promising for medical purposes for several reasons: the number of their functional roles in cell biology and biology of the human body as a whole is much smaller than that of the main elements; their circulation rate in the body is relatively low; zinc is a cofactor for hundreds of important enzymes and it plays a considerable role in nucleic acid metabolism, transcription processes, stabilization of nucleic acids, proteins, and especially components of biological membranes (4). It is also known that imbalanced zinc homeostasis in the human body is associated with the development and progression of breast, lung, prostate, liver and pancreatic cancers, melanoma and myeloproliferative neoplasm (5-9).

The isotopic compositions of zinc have been previously examined in plasma from patients with hematological malignancies (HM), and their prognostic capabilities have been assessed (10). Zinc isotope ratios and their concentrations in

the peripheral blood of HM patients significantly differed from those in the control group. The group of patients with increased $\delta^{66}Zn$ values showed poorer survival rates. It should be noted that widely used prognostic factors for HM, such as the creatinine level, and anemia-related values were highly correlated with the $\delta^{66}Zn$ value in plasma from HM patients (10). Analysis of zinc isotopic composition in blood and tissues in breast cancer patients and healthy donors has shown a statistically significant increase in the levels of ^{64}Zn and ^{66}Zn only in tumor tissues compared with the controls (11). Moreover, a significant increase in the levels of light zinc isotopes ($^{66}Zn/^{64}Zn$) has been observed in urine samples from patients with pancreatic cancer (12). The data obtained allow us to conclude that disturbances in the ratio of stable zinc isotopes significantly affect cell biology, which is reflected in changes in homeostasis and, as a result, can lead to the emergence of a malignant neoplasm.

There are few available data on the attempts to apply stable zinc isotopes in therapy (13). It is known that zinc significantly affects the lipid metabolism, glycolysis and redox reactions in malignant cells (14). It is also known that changes in the isotopic composition of zinc may have a considerable effect on its metabolic activity in the cell (15). These facts suggest that compounds enriched in light stable isotopes of zinc may have significant anticancer potential.

Today we know that zinc may have an antitumor effect by suppressing the processes of angiogenesis and production of pro-inflammatory cytokines in tumors against the background of the apoptosis program activation in malignant cells. In addition, zinc may enhance cell-mediated antitumor immunity (16).

In view of the foregoing, studies of the anticancer activity of zinc compounds enriched in the light stable isotope ^{64}Zn are extremely promising and may become the basis for creating a new group of anticancer agents. Therefore, the aim of our study was to assess *in vitro* cytotoxic and anti-proliferative effects of ^{64}Zn-enriched compounds on malignant cells and to find possible mechanisms of action of these substances.

Materials and Methods

Experimental compounds. Zn^{64} aspartate (Zn^{64} asp). The compound molecule is built of one molecule of L-aspartic acid (NeoFroxx, Einhausen, Germany) and an atom of ^{64}Zn isotope (PAEP, Zelenodolsk, Russian Federation) bound to form a chelate complex (Figure 1 and Figure 2). The chemical name of the finished solution is zinc^{64} L-aspartate (1:1). Gross formula is $C_4H_5NO_4Zn^{64}$ and molar mass is 195.016 g/mol. The percentage of zinc in the compound is 32.78%. Zinc compound containing stable light isotope ^{64}Zn enriched to 99.2% mass fraction of total zinc.

Zn^{64} glutamate (Zn^{64} glu). The compound molecule is built of one molecule of L-glutamic acid (Merck Life Science LLC, Ontario, Canada) and an atom of ^{64}Zn isotope bound to form a chelate complex (Figure 3 and Figure 4). The chemical name of the finished solution: zinc 2-aminopentanedioate. Gross formula is $C_5H_7NO_4Zn^{64}$ and its molar mass is 209.042 g/mol. The percentage of zinc in the compound is 30.58%. Zinc compound containing stable light isotope ^{64}Zn enriched to 99.2% mass fraction of total zinc.

Zn^{64} sulfate (Zn^{64} sulf) (PAEP). Gross formula: $Zn^{64}SO_4$. Molar mass=159.991 g/mol. The percentage of zinc in the compound is 39.96%. Zinc compound containing stable light isotope ^{64}Zn enriched to 99.2% mass fraction of total zinc.

Zn aspartate with natural isotope distribution (Zn asp). The compound molecule is built of one molecule of L-aspartic acid and an atom of Zn with a natural distribution of isotopes (Zinza Industrials Nacionales S.A., Callao, Peru) bound to form a chelate complex. The chemical name of the finished solution is zinc L-aspartate (1:1). Gross formula is $C_4H_5NO_4Zn$ and its molar mass is 196.496 g/mol. The percentage of zinc in the compound is 39.96%. Synthesis scheme of Zn aspartate where Zn has a natural isotopic distribution is the same as for Zn^{64} aspartate.

Aspartic acid. L-aspartic acid (NeoFroxx) solution was used in this study as a negative control to assess the effect of aspartic acid on tumor cell viability. Molar mass=133.11 g/mol.

All the test compounds were diluted in water for injection.

Cell culture. Human breast cancer cells (MCF-7 and MDA-MB-231 cell lines) and murine B16 melanoma cells (MB16 cell line) were cultured in DMEM medium (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 40 μg/ml gentamicin (Sigma-Aldrich). Human lung cancer cells (A-549 cell line) and human acute leukemia cells (HL-60 cell line) were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS and 40 μg/ml gentamicin.

Human diploid fibroblasts and Madin-Darby bovine kidney cells (MDBK cell line) were cultured in DMEM medium supplemented with 10% FBS and 40 μg/ml of gentamicin. Human keratinocytes (HaCaT cell line) and porcine aortic endothelial cells (PAE cell line) were cultured in RPMI 1640 medium supplemented with 10% FBS and 40 μg/ml gentamicin. The cells were placed in plastic dishes (TPP, Trasadingen Schaffhausen, Switzerland) and cultured at 37˚C in a humidified atmosphere supplied with 5% CO_2. The medium was changed and the cells were passaged according to a standard procedure (17). All cell lines were purchased from ATCC (Manassas, VA, USA) except for MB16, HaCaT, PAE cell lines and diploid fibroblasts, which were kindly provided by Prof. Y. Kudryavets (bank of cell lines from human and animal tissues, Kyiv, Ukraine). Cells in the exponential phase of growth were used in the experiments.

Assessment of cell viability. Twenty-four to 48 h after the last passage, the test cells were seeded at a concentration of 1×10^4 cells/well in 96-well plates (TPP) in DMEM/RPMI 1640 medium supplemented with 10% FBS and 40 μg/ml gentamicin. Over the next 24 h, the cells were incubated in a humid atmosphere at 5% CO_2 and 37˚C. Further, solutions of the studied compounds were diluted to the working concentrations in DMEM or RPMI 1640 complete medium. The experimental compounds were loaded in respective wells at different concentration levels in triplicate. Immediately after adding the compounds, the cells were placed in a CO_2 incubator and cultured for another 48 h at 5% CO_2 and 37˚C.

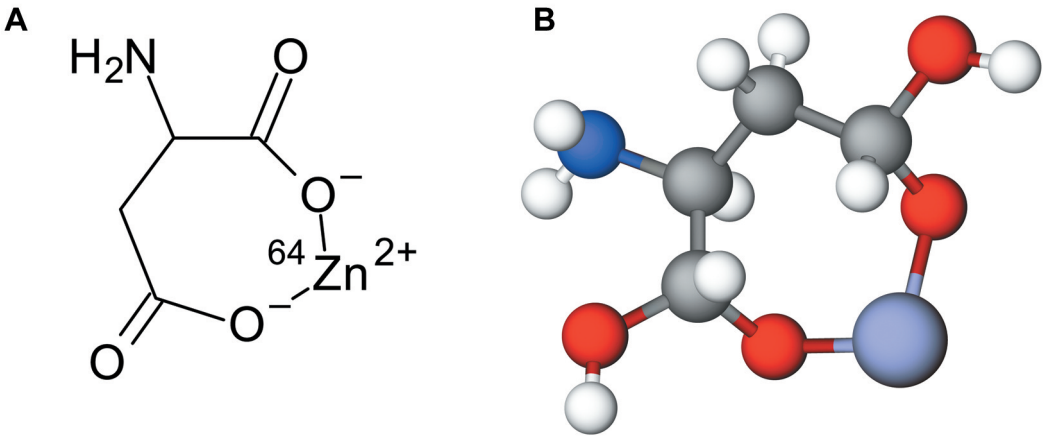

Figure 1. *Graphic image of a Zn^{64} aspartate molecule. Schematic representation (A) and 3D model (B).*

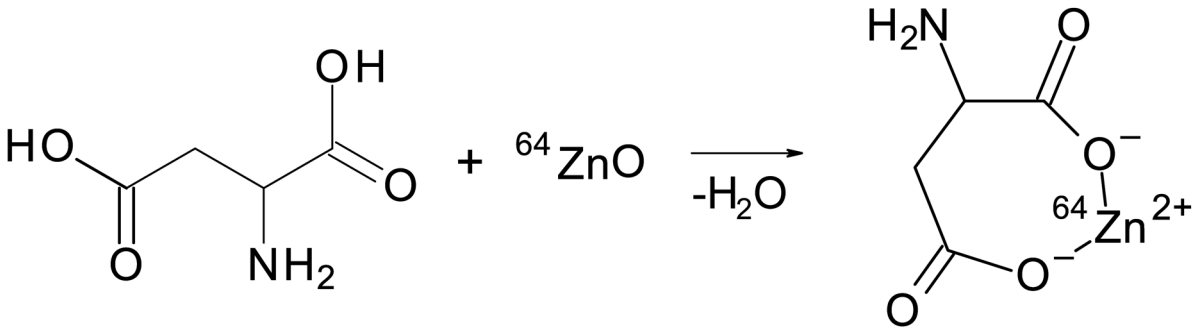

Figure 2. *Synthesis scheme of Zn^{64} aspartate from L-aspartic acid and ^{64}Zn oxide enriched stable light isotope to 99.2% mass fraction of total zinc.*

The viability of cells was assessed by their staining with crystal violet (assessment of the total number of living cells by protein and DNA content) (18) or trypan blue.

Crystal violet colorimetric assay. After incubation of cells (MCF-7, MDA-MB-231, MB16, A-549, MDBK, HaCat, PAE, fibroblasts) with the studied substances the medium was removed from all wells. The concentration of crystal violet was 5 mg/ml of 70% methyl alcohol. Crystal violet (Sigma-Aldrich) staining solution was added to each well (50 µl) and the plates were incubated for 10 min at room temperature. The dye was then removed and the plates were washed under running water. The dye was eluted by adding 100 µl of 96% ethyl alcohol to all wells of the plate. The results were recorded using a multi-well spectrophotometer (ThermoLabsystems Multiskan PLUS, Vantaa, Finland) at wavelength of 540 nm. The percentage of viable cells was calculated by the following formula:

$X=[A_{540}$ (experiment)/$_{A540}$(control)] ×100%, where A is absorbance of test and control samples at 540 nm. Untreated cells were used as control samples.

Trypan blue dye exclusion test. The viability of suspension cells (HL-60 cell line) was determined in the experiment, so the dye solution was added directly to the culture medium. Fifty µl of 0.4% trypan blue solution (HyClon, Logan, Cache County, UT, USA) and 50 µl of cell suspension of the test samples were mixed in an Eppendorf tube. The number of viable and non-viable (blue) cells was calculated at low magnification (eyepiece ×10, lens ×10) in five large squares of the hemocytometer. The viability of cells in the experiment was calculated using the following formula: $X=(a/b) \times 10{,}000 \times c$, where X is the number of live or dead cells in 1 ml; a is the number of cells counted in large squares; b is the number of squares counted cells in; c is a dilution factor. The number of viable and dead cells in each sample was counted 3 times.

Nonlinear regression analysis was used to determine the IC_{50} values for the experimental compounds against normal and malignant mammalian cells.

Western blot analysis. Cells of A-549 and MB16 cell lines were seeded at a concentration of 5.0×10^6 cells/well in 100 mm diameter Petri dishes (TPP) in RPMI 1640 or DMEM medium supplemented with 10% FBS and 40 µg/ml gentamicin. The cells were placed in a CO_2 incubator and cultured at 37˚C in a humidified atmosphere supplied with 5% CO_2 for 24 h and then Zn^{64} aspartate was added (11 µg/ml for A-549 cells and 6.5 µg/ml for MB16 cells by the concentration of zinc).

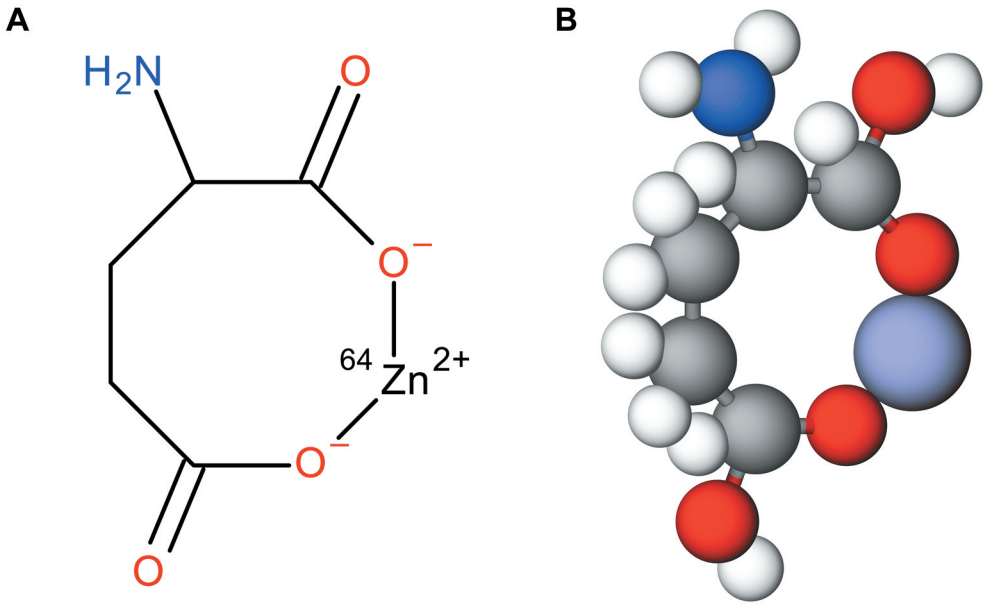

Figure 3. *Graphic image of a Zn^{64} glutamate molecule. Schematic representation (A) and 3D model (B).*

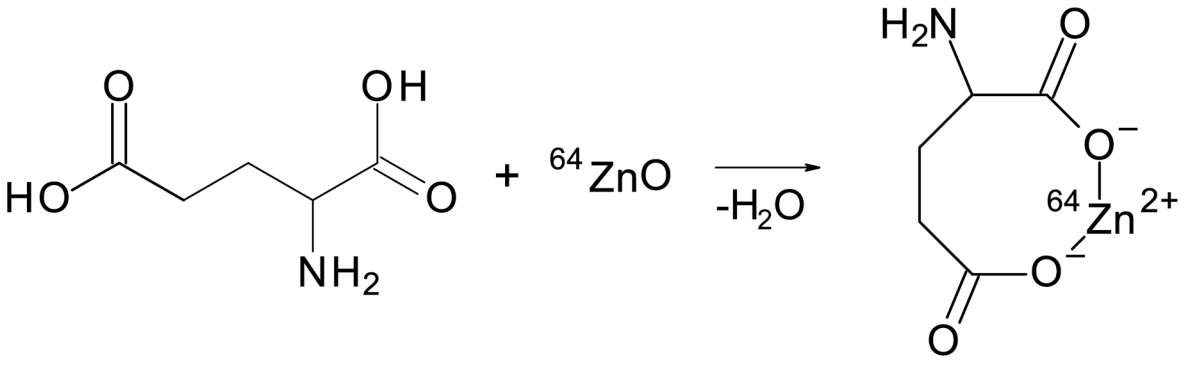

Figure 4. *Synthesis scheme of Zn^{64} aspartate from L-glutamic acid and ^{64}Zn oxide enriched stable light isotope to 99.2% mass fraction of total zinc.*

Cells that were cultured without the addition of experimental compounds but in the presence of 10% of the incubation medium volume of 0.9% sodium chloride were used as a cell control. After the compounds were added to the cells, they were placed in a CO_2 incubator and cultured at 37˚C and 5% CO_2 for another 24 h.

To separate proteins by electrophoresis, the samples were lysed in RIPA buffer with protease and phosphatase inhibitors cocktails (Sigma-Aldrich) and heated at 95˚C for 5 min in Laemmli buffer. Electrophoresis was performed in Tris-glycine buffer, pH 8.3 (Sigma-Aldrich), using a Mini-PROTEAN II electrophoresis chamber (BIO-RAD, Solna, Stockholm, Sweden). Protein electrophoresis was performed by loading samples onto a 12% sodium dodecyl sulfate polyacrylamide gel (Sigma-Aldrich), 50 μg/well, in the Laemmli buffer system. To determine molecular weights of proteins on electrophoregrams, protein standards (Thermo Scientific, Waltham, MA, USA) covering a molecular weight range of 10-250 kDa were used. Proteins were transferred from polyacrylamide gel onto a nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA) in a Mini Trans-Blot Cell unit (BIO-RAD), in transfer buffer containing 2.5 mmol/l Tris-HCl pH 8.3 (Sigma-Aldrich), 20% methanol, 192 mmol/l glycine (Sigma-Aldrich) and 0.1% SDS. The membranes were then washed with distilled water for 10 min and stained with 1% Ponceau S dye (Abcam, Cambridge, MA, USA) solution prepared in 3% trichloroacetic acid. Free binding sites on the membrane were then blocked for 1 h with 5% skim milk (Genesee Scientific Inc., El Cajon, CA, USA) in phosphate-buffered saline (Sigma-Aldrich) supplemented with 0.1% Tween 20 detergent (Sigma-Aldrich). The membranes were incubated by turn with primary antibodies overnight at +4˚C, followed by their washing with phosphate-buffered saline. The primary antibodies used were against

PARP-1, Nf-kB, p53, p-p53, p38, p-p38 and Bax (Thermo Scientific), and against β-tubulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). β-tubulin was used as loading control. Antispecies horseradish peroxidase-conjugated antibodies (Thermo Scientific) were used as secondary antibodies. Immunoreactive bands were detected using a solution containing 1.25 mmol/l luminol (Sigma-Aldrich), 2.72 mmol/l coumaric acid (Sigma-Aldrich) and 0.01% hydrogen peroxide in 0.1 M Tris-HCl pH 8.5. Exposure time of the treated membranes on radiographic film depended on the intensity of chemiluminescence reaction. Carestream Kodak reagents (Sigma-Aldrich) were used for the film development. The density of the protein bands normalized to the loading control. Densitometric analysis of the bands was performed using TotalLab TL120 v2009 software (Nonlinear USA Inc, Durham, NC, USA).

Statistical analysis. Studies of the experimental compounds cytotoxic effect were repeated three times. Western blot analysis was replicated twice. IC_{50} for test compounds was determined by nonlinear regression analysis. The results are presented as the mean values±standard deviation. Differences between the group means were determined by Student *t*-test (cytotoxic activity test) and one-way analysis of variance (one-way ANOVA) followed by the post-hoc Tukey test (western blot analysis). Differences between groups were considered statistically significant at $p<0.05$. Statistical data processing was performed using MS Excel and Origin 7.5 (OriginLab Corporation, Northampton, MA, USA).

Results

This study has examined the *in vitro* effects of three experimental compounds containing ^{64}Zn enriched to 99.2% on the viability and proliferative activity of malignant cells of different tumor histogenesis in a comparative aspect. Since the percentage of zinc in the test compounds is different, the IC_{50} values of the substances were analyzed and presented by the concentration of zinc.

The cytotoxic activity of experimental compounds was examined using the following human breast cancer cells that differ in molecular subtype and degree of malignancy: luminal MCF-7 cells and basal MDA-MB-231 cells. Analysis of IC_{50} values of the studied compounds against breast cancer cells showed that high-grade malignant MDA-MB-231 cells were more sensitive to the cytotoxic effects of the studied substances than MCF-7 cells. In addition, the obtained results indicated that salts of ^{64}Zn with amino acids suppressed the viability of breast cancer cells most effectively ($p<0.05$) compared to Zn^{64} sulfate (Figure 5). Moreover, Zn^{64} aspartate exhibited the highest cytotoxic activity against breast cancer cells of both types.

Similar results of *in vitro* cytotoxic activity of Zn^{64} aspartate and Zn^{64} glutamate were also observed against human cancer cells of other histogenetic origins. In particular, it was shown that the IC_{50} value for these compounds was 2 times lower than for $Zn^{64}SO_4$ ($p<0.05$) on A-549 non-small lung cancer cells (Figure 6) and acute leukemia HL-60 cells (Figure 7).

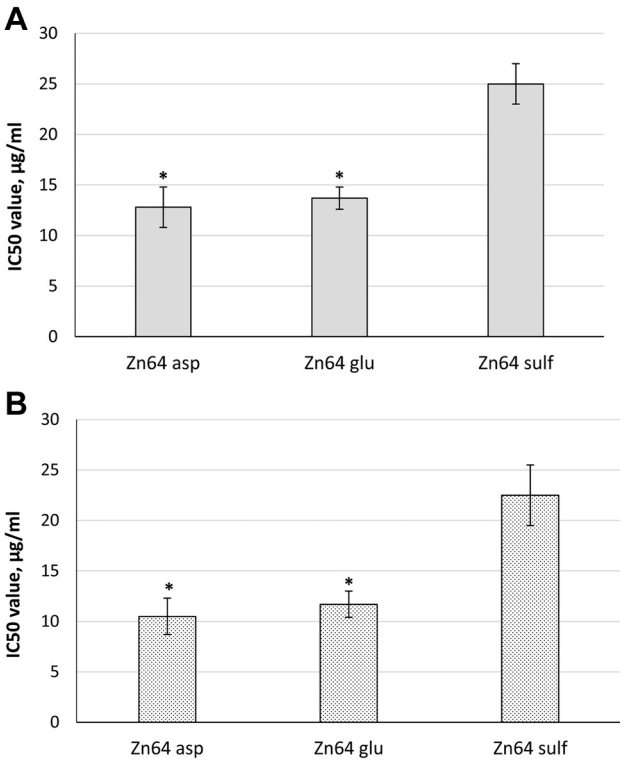

Figure 5. *In vitro cytotoxic activity of light stable ^{64}Zn isotope compounds against human breast cancer cells of the luminal MCF-7 (A) and basal MDA-MB-231 (B) cell line subtypes. *p<0.05 as compared to cells treated with Zn^{64} sulfate. Zn^{64} asp, Zn^{64} aspartate; Zn^{64} glu, Zn^{64} glutamate; Zn^{64} sulf, Zn^{64} sulfate.*

MB16 melanoma cells were found to be the most sensitive to the cytotoxic effects of salts of ^{64}Zn with the amino acids. The obtained results indicated that the IC_{50} value for Zn^{64} sulfate in MB16 cells was 3.2 times higher than for ^{64}Zn with the amino acids ($p<0.05$) (Figure 8). It should be noted that, as with the previous cell lines, Zn^{64} aspartate was more effective in suppressing the viability of melanoma cells than other experimental compounds.

Results of a comparative analysis of the cytotoxic activity of Zn^{64} aspartate and Zn aspartate with natural isotopic distribution against tumor cells of different histogenesis demonstrated that the compound enriched in stable ^{64}Zn isotope to 99.2% suppressed their viability more effectively (Figure 9). IC_{50} values for Zn aspartate against A-549 cells, breast cancer cells and HL-60 cells are 1.2-1.6 times higher than for Zn^{64} aspartate ($p<0.05$). In MB16 melanoma cells, the difference in IC_{50} values was even greater ($p<0.05$) and was equal to 2.7 times (22.4±2.7 µg/ml for Zn aspartate *vs*. 8.3±0.7 µg/ml for Zn^{64} aspartate). In this experiment, the effects of aspartic acid solution on the viability of tumor cells were also analyzed. The obtained results indicate that amino acid does not have

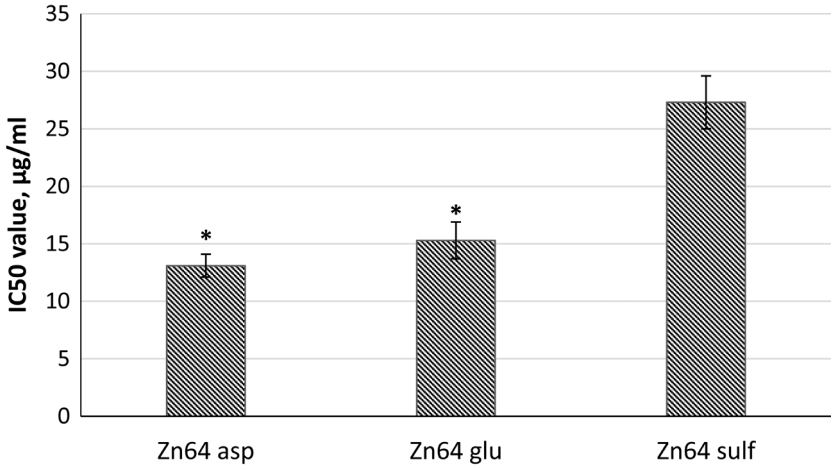

Figure 6. *In vitro cytotoxic activity of light stable ^{64}Zn isotope compounds against human lung cancer cells (A-549 cell line). *p<0.05 as compared to cells treated with Zn^{64} sulfate. Zn^{64} asp, Zn^{64} aspartate; Zn^{64} glu, Zn^{64} glutamate; Zn^{64} sulf, Zn^{64} sulfate.*

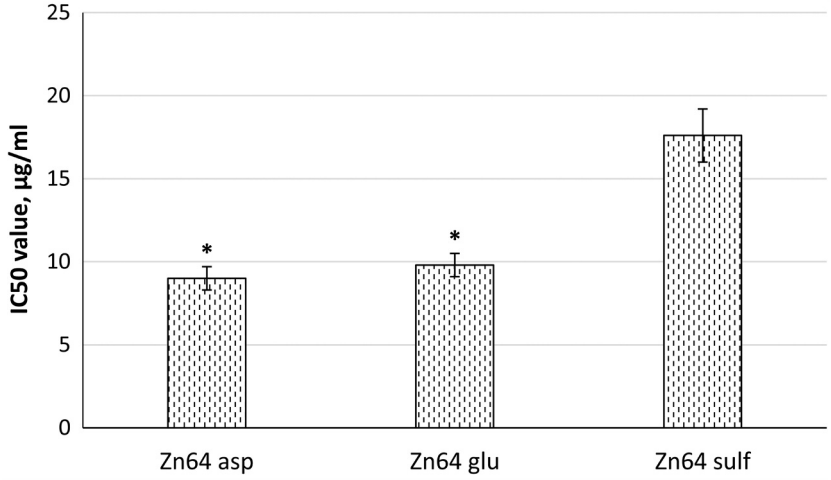

Figure 7. *In vitro cytotoxic activity of light stable ^{64}Zn isotope compounds against human acute leukemia cells (HL-60 cell line). *p<0.05 as compared to cells treated with Zn^{64} sulfate. Zn^{64} asp, Zn^{64} aspartate; Zn^{64} glu, Zn^{64} glutamate; Zn^{64} sulf, Zn^{64} sulfate.*

any cytotoxic or anti-proliferative effects on the cells used in the study.

At the next stage of our study, it was important to assess the behavior of Zn^{64} aspartate towards different types of non-malignant tissues. For this purpose, the *in vitro* effects of Zn^{64} aspartate and Zn aspartate on the viability of normal mammalian cells were studied. For such experiment, we selected a panel of cell lines of different histogenesis which represent tissues sensitive to the toxic effects of drugs, in particular, kidneys and skin, or such cell types that are present in all organs and vital systems (fibroblasts and endothelium).

It was found that kidney cells (MDBK cell line) are the most sensitive to cytotoxic effects of the Zn^{64} aspartate ($p<0.05$) (Figure 10). However, the IC_{50} values for Zn^{64} aspartate-treated keratinocytes, fibroblasts, or endothelial cells were, on average, 2 times higher than for most of the tumor cells used in the experiment. Zn^{64} aspartate significantly suppresses the viability of non-malignant cells than Zn aspartate with the natural isotopic distribution ($p<0.05$) (Figure 10), as in the case of tumor cells.

To find possible molecular mechanisms of the cytotoxic effects of Zn^{64} aspartate, the levels of expression of apoptosis regulatory proteins in cancer cells were measured by Western blot analysis. A-549 cells and MB16 cells were chosen for such study, as they were characterized by the lowest (IC_{50} 13.1±0.7 µg/ml) and the highest (IC_{50} 8.3±0.7 µg/ml) sensitivity to the cytotoxic action of Zn^{64} aspartate, respectively.

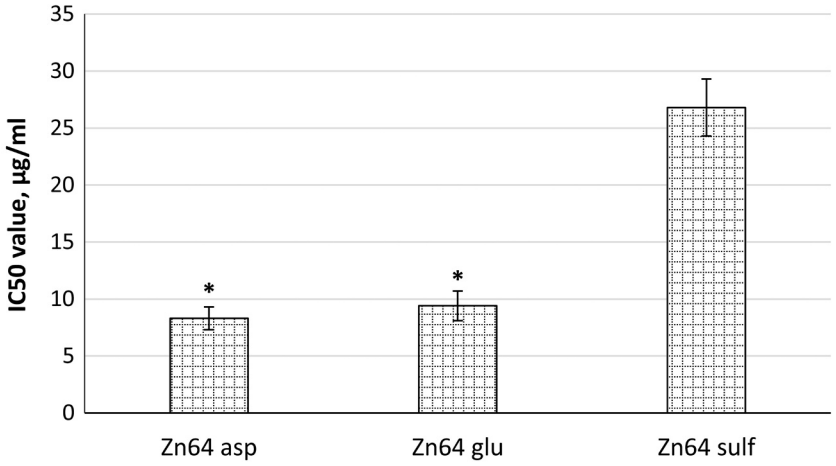

Figure 8. *In vitro cytotoxic activity of light stable ^{64}Zn isotope compounds against murine melanoma cells (MB16 cell line). *p<0.05 as compared to cells treated with Zn^{64} sulfate. Zn^{64} asp, Zn^{64} aspartate; Zn^{64} glu, Zn^{64} glutamate; Zn^{64} sulf, Zn^{64} sulfate.*

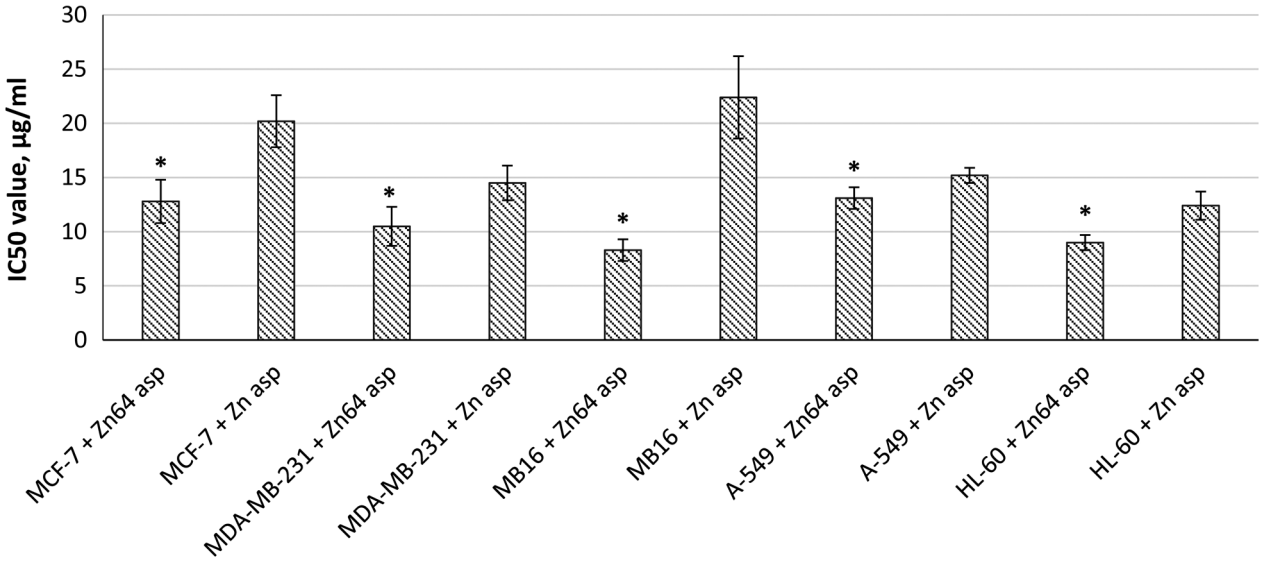

Figure 9. *In vitro comparative analysis of cytotoxic activity of ^{64}Zn aspartate (Zn^{64} asp) and Zn aspartate (Zn asp) with natural isotopic distribution against tumor cells of different histogenetic patterns. *p<0.05 as compared to cells the same cell line treated with Zn aspartate with natural isotopic distribution (Zn asp).*

Analysis of the expression levels of apoptosis regulatory proteins in A-549 cells after their treatment with Zn^{64} aspartate showed that the experimental compound caused a statistically significant decrease in the expression of cleaved PARP-1 by 53.8%, compared to the control (Figure 11). Treatment of A-549 cells with Zn^{64} aspartate also led to a statistically significant increase in the expression of NF-kB by 4.8 times compared to the control. In addition, Zn^{64} aspartate-treated A-549 cells showed statistically significant changes in the expression of p38 kinase and its phosphorylated, active form (p-p38): a decrease in p38 expression by 72% and a significant increase in p-p38 expression by 12.8 times compared with the control cells were observed (Figure 11).

The results of Western blot analysis conducted on cell lysates derived from melanoma cells showed that treatment of MB16 cells with Zn^{64} aspartate caused a substantial and statistically significant increase in the expression of cleaved PARP-1. The obtained data also indicated that treatment of MB16 cells with Zn^{64} aspartate resulted in a statistically significant increase of phosphorylated p53 expression by 6.4 times, compared to the control (Figure 12).

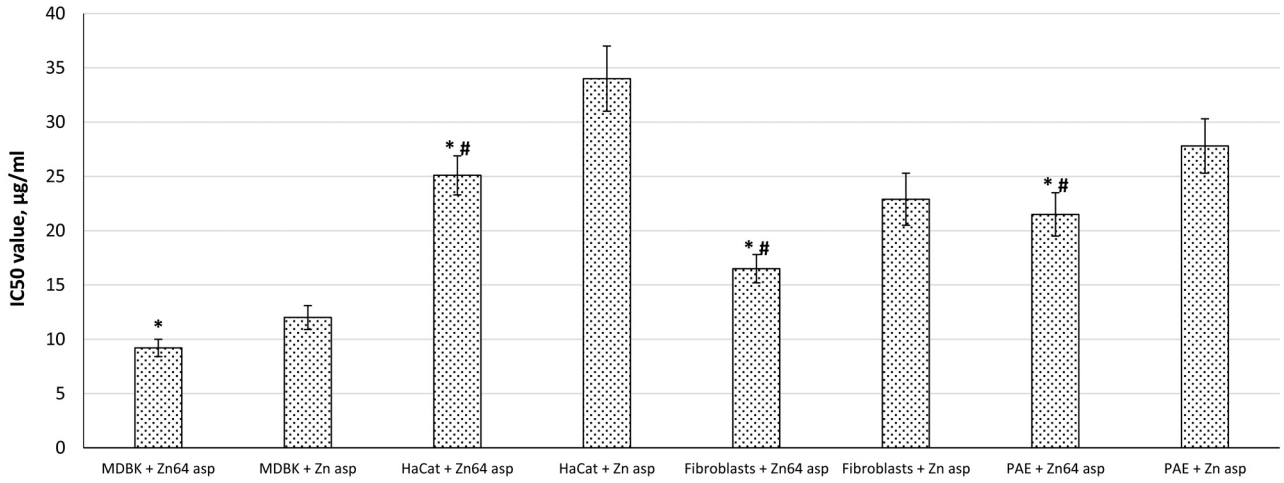

Figure 10. *In vitro comparative analysis of cytotoxic activity of Zn^{64} (Zn^{64} asp) aspartate and Zn aspartate (Zn asp) against normal cells of different histogenetic origins. *p<0.05 as compared to cells the same cell line treated with Zn aspartate with natural isotopic distribution (Zn asp). #p<0.05 as compared to MDBK cells treated with Zn^{64} aspartate.*

It should be noted that the experimental compound had a statistically significant effect on the expression levels of p38 kinase and its phosphorylated form (p-p38), compared to the control cells: treatment of melanoma cells with Zn^{64} aspartate caused a decrease in the expression levels of both forms of p38 by 67.5% and 77%, respectively (Figure 12).

Discussion

Zinc is an essential trace element involved in many cellular processes, which include the processes of cell growth, regulation of gene expression, signal transmission, stabilization of the cell redox balance, as well as cell death. In addition, zinc is an important cofactor for many enzymes, such as superoxide dismutase and metallothionein, which are involved in the control of cellular responses to hypoxia and reactive oxygen species production, and therefore play a role in cancer development (14).

An extensive network of zinc transporters and metallothioneins keep intracellular zinc content at levels required to maintain cell homeostasis. At the same time, only a small amount of unbound zinc is normally present in the cell cytoplasm. Abnormal fluctuations in intracellular free zinc levels are very often associated with the development of various pathologies, including malignant neoplasms (7). According to the literature data, such fluctuations were recorded in patients with breast, prostate, lung, intestine, stomach, and skin cancers (19). Therefore, due to the important role of zinc in biological systems and its unique properties, it has become a potential anticancer agent.

A study carried out by Eskiler and Kani demonstrated that Zn(II) complex can significantly suppress the viability of human breast cancer cells of the MCF-7 line by inducing apoptosis through multi-caspase activity in breast cancer cells (20).

Studies of Zn sulfate antitumor activity on human non-small lung cancer cells have found that the compound has a cytotoxic effect on A-549 and H1299 cells. The occurrence of oxidative stress due to a significant increase in ROS production was observed in lung cancer cells treated with Zn (21). In addition, the study by Scheiermann *et al*. showed that cultivation of A-549 cells under conditions of zinc deficiency led to decreased E-cadherin expression and increased EGFR cell surface expression, which may indicate an increase in the malignancy of lung cancer cells. An increase in the zinc contents in the culture medium was accompanied by a decrease in the metabolic activity of A-549 cells (8).

Measurement of zinc contents in the serum samples and biopsy specimens from melanoma patients showed low levels of the element in the serum and its accumulation in the tumor tissue. At the same time, an *in vitro* study of the cytotoxic activity of zinc against human melanoma cells found that increased intracellular content of this element in malignant cells led to changes in their autographic activity *via* mitochondria and lysosomes, which finally led to autophagic cell death (7). An *in vivo* study found that zinc deficiency in the diet of mice led to an increase in the survival and metastatic activity of melanoma cells (22).

A comparative analysis of intracellular zinc levels revealed a significant difference between the mean zinc concentration in normal lymphocytes and those from patients with chronic lymphocytic leukemia; in lymphocytes from healthy donors this index was 2 times higher than in cancer patients. Moreover, patients with late-stage disease had

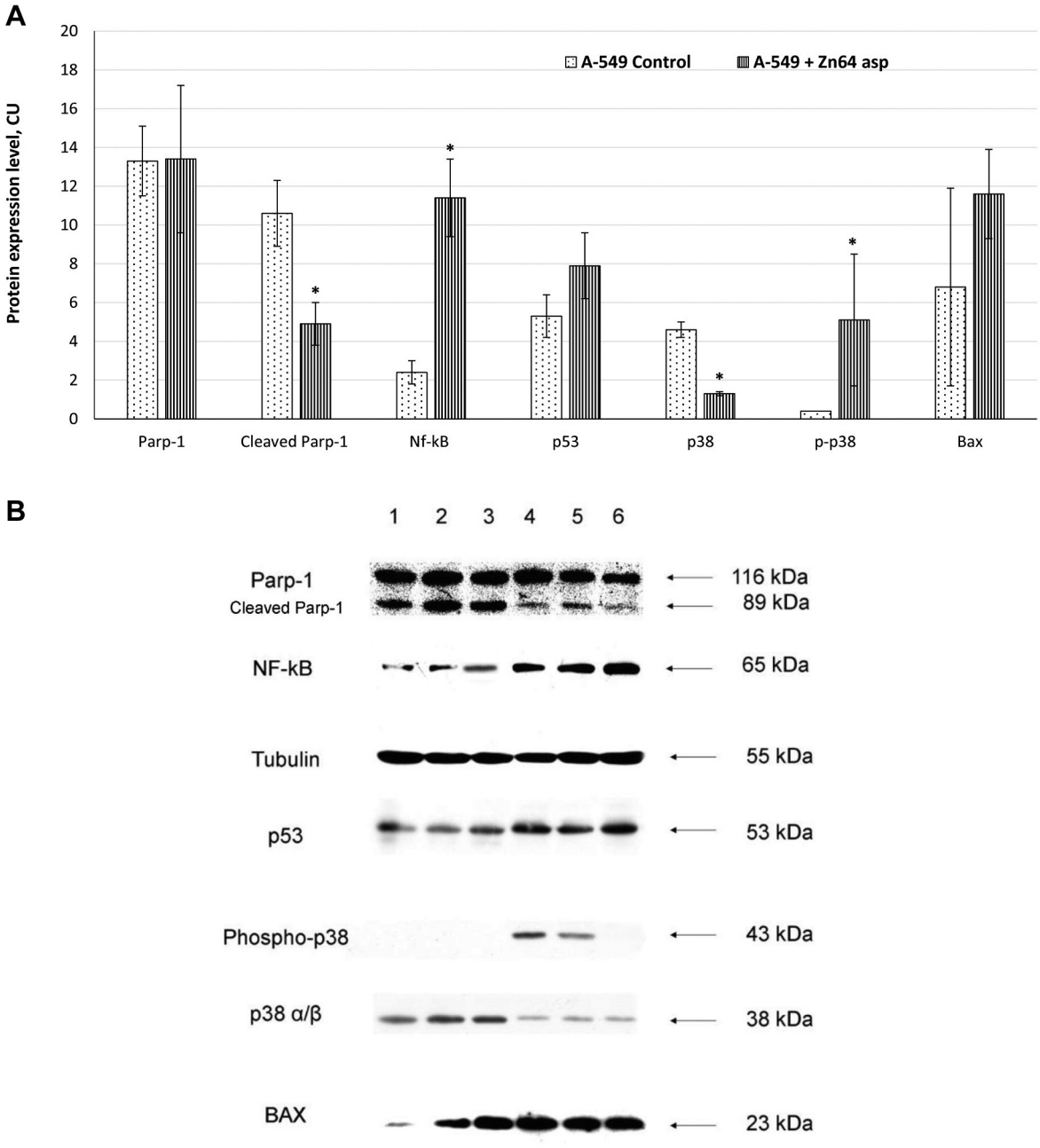

Figure 11. *Changes in the expression of apoptosis regulatory proteins in A-549 cells after their treatment with Zn^{64} aspartate (Zn^{64} asp). The bar chart shows the densitometry results of apoptosis-associated protein bands (A). *p<0.05 as compared to control cells. Western blot images are representative of two independent experiments. Lanes 1-3 correspond to A-549 control (cells + physiological saline) and lanes 4-6 to A-549 + Zn^{64} aspartate (B).*

significantly lower zinc concentration in their lymphocytes than early-stage patients (23). An *in vitro* study conducted by Zou *et al*. on the antitumor activity of zinc nanoparticles against myeloid and T-cell leukemia cells showed that zinc nanoparticles were able to suppress the viability of malignant cells in a dose-dependent manner (24).

Based on this literature evidence, we selected cells of the breast cancer MCF-7 and MDA-MB-231 cell lines, lung cancer A-549 cell line, melanoma MB16 cell line, and leukemia HL-60 cell line as experimental models for our study.

Today it is known that zinc has five stable isotopes: ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, and ^{70}Zn. Zinc isotopes have uneven atom

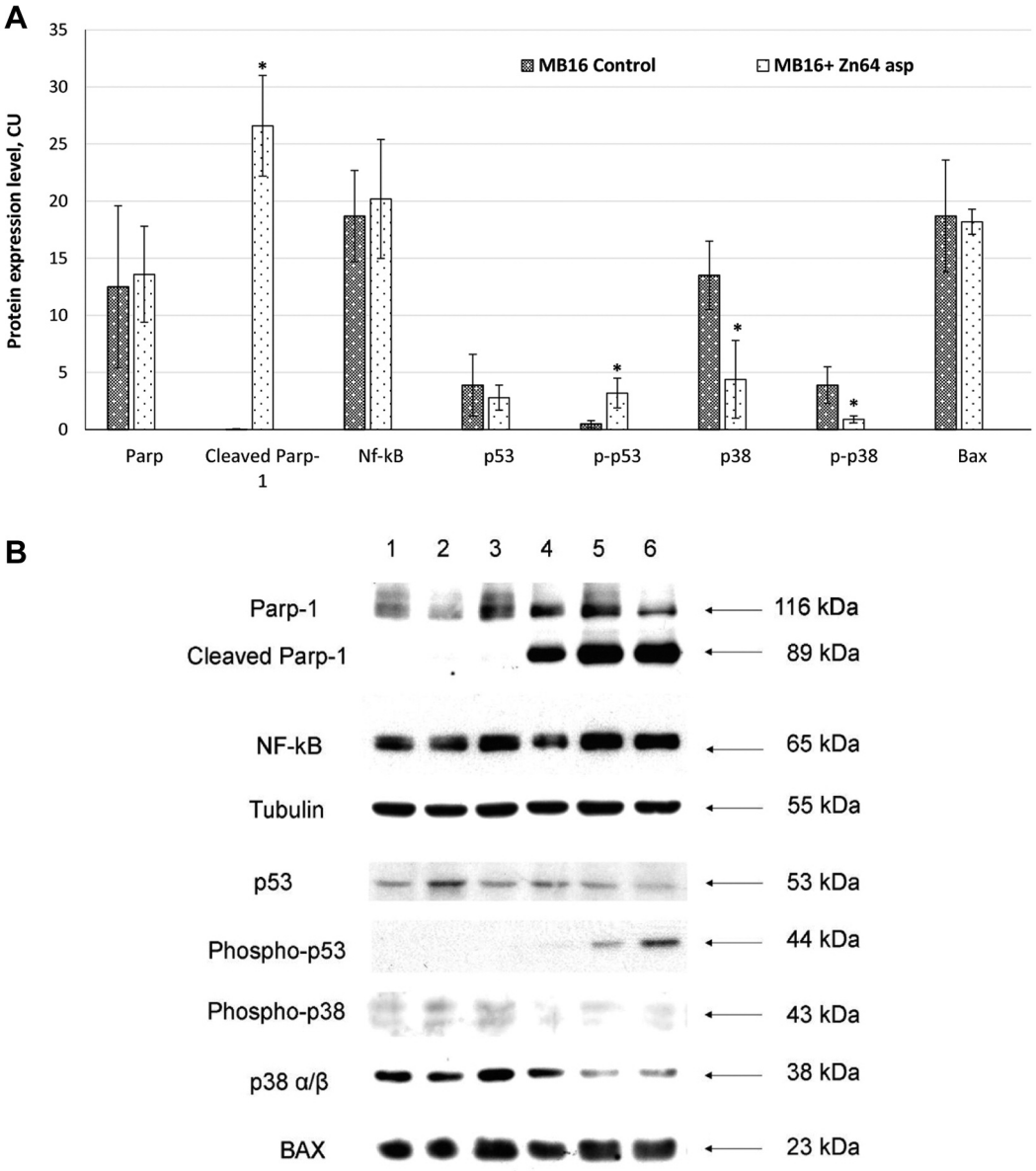

Figure 12. *Changes in the expression of apoptosis regulatory proteins in MB16 cells after their treatment with Zn^{64} aspartate (Zn^{64} asp). The bar chart shows the densitometry results of apoptosis-associated protein bands (A). *p<0.05 as compared to control cells. Western blot images are representative of two performed independent experiments. Lanes 1-3 correspond to MB16 control (cells + physiological saline) and lanes 4-6 to MB16 + Zn^{64} aspartate.*

percent natural abundances, being ^{64}Zn the most abundant with a fraction of 48.63% (25). Such data suggest that ^{64}Zn plays a significant role in ensuring the functional activity of cellular enzymes, of which this element is a cofactor, the metabolic activity of cells, and homeostasis of cells and tissues of the body as a whole. Therefore, from our point of view, compounds based on zinc enriched in ^{64}Zn isotope may have the most significant antitumor potential.

At the first stage of our study, we assessed the cytotoxic activity of Zn^{64} aspartate, Zn^{64} glutamate, and Zn^{64} sulfate. Today, Zn sulfate is known to be used in therapy as a drug that increases the effectiveness of treatment or reduces the clinical manifestations of side effects of cancer chemotherapy and radiotherapy (26-28), as well as suppresses the viability of lung cancer cells (21). However, a comparative analysis of the results of *in vitro* study has shown that Zn^{64} aspartate and Zn^{64} glutamate are more effective than Zn^{64} sulfate in suppressing the viability of malignant cells of various histogenetic origins. There is scientific evidence that the pharmacokinetics of organic and inorganic zinc compounds is different in various

animal species (29-32). Several scientific works indicate that organic chelate zinc complexes with amino acids, aspartic and glutamic acids, are characterized by high bioavailability since such soluble coordination complexes of the element ensure the main mobile functional pool of zinc in the cell (33). At the cellular level, the main ligand of zinc is citrate which requires oxaloacetate, a derivative of aspartate, for its synthesis. This is probably why Zn^{64} aspartate had the most significant cytotoxic effect on malignant cells in our experiment.

The results obtained in our study also indicate that sensitivity of tumor cells (IC_{50} values) to cytotoxic/anti-proliferative effects of Zn^{64} aspartate and Zn^{64} glutamate is different, and it increases as follows: A-549 cells < MCF-7 cells < MDA- MB-231 cells < HL-60 cells < MB16 cells.

One of the main factors providing cytotoxic/anti-proliferative effects of zinc is the functional activity of various proteins involved in zinc uptake, excretion, and trafficking. Today, two protein Zn transporter families are well-known, ZIP and ZnT. ZIP-family proteins mediate the uptake of zinc from the intercellular space and its release from organelles into the cytoplasm. ZnT-family proteins are responsible for the release of zinc from the cell and its transport from the cytoplasm to organelles. Thus, these proteins regulate zinc enzyme activation and maturation and mediate zinc signaling. Therefore, it is zinc transporters that have a significant effect on metabolism (34, 35) and the sensitivity of malignant cells to cytotoxic agents. It should be noted that, according to the literature data, breast and lung cancer cells have high expression levels of ZIP proteins and low expression levels of ZnT proteins (36, 37), *i.e.*, accumulation of zinc in the cytoplasm of these cells is a physiological process, and only a significant increase in the intracellular concentration of zinc can provoke the cell death.

Another important factor influencing the sensitivity of tumor cells to Zn^{64} aspartate and Zn^{64} glutamate is the isotope effects of zinc and the role of the isotopic composition of this element in the formation of metabolic phenotype of the studied cells. Isotope effects in cell metabolism are mostly caused by enzymatic reactions that preferentially consume substrates containing either the light or the heavy isotope (14). Therefore, the natural isotope abundance in metabolites depends on metabolic fluxes and source substrates, and a change in the mass balance of isotopes of an element leads to a significant change in its bioavailability and functional activity. Such changes in cell biology will undoubtedly affect cell viability and proliferative activity in the presence of cytotoxic/cytostatic agents.

The results of our comparative analysis of the cytotoxic activity of Zn^{64} aspartate and Zn aspartate with a natural abundance of isotopes can also be explained by the isotope effects of zinc. *In vitro*, it was shown that the compound enriched in ^{64}Zn isotope was on average 1.5 times more effective in suppressing the viability of breast and lung cancer cells and leukemia cells compared to Zn aspartate. Particular attention should be paid to the MB16 melanoma cells results. According to IC_{50} values, MB16 cells had the highest sensitivity towards Zn^{64} aspartate and were the least sensitive to the cytotoxic effect of Zn with natural isotopic abundance. This indicates that it is the change in the intracellular isotope composition of zinc, and hence the mass balance of isotopes, that most significantly affects the viability and proliferative activity of MB16 cells. This experimental model may be very useful for further studies of the molecular mechanisms of antitumor effects of Zn^{64} aspartate.

To assess the therapeutic prospects of a potential drug, its toxicity to normal cells of various histogenesis was determined. This phase is necessary to find out possible negative effects of the test compound on the organism at the initial stages of the study and prevent its possible side effects.

Our study has found that MDBK bovine kidney cells are the most sensitive to cytotoxic effects of Zn^{64} aspartate and Zn aspartate. An analysis of changes in zinc and zinc isotope homeostasis in the human body will help explain the obtained results. Literature data indicate that normally a large amount of zinc that enters the body is excreted *via* urine, which prevents significant accumulation of this element in the tissues (38). In addition, an analysis of zinc isotopic composition in renal tissues and urine indicates that kidney cells normally accumulate heavy zinc isotopes, while light isotopes of the element, ^{64}Zn and ^{66}Zn, are predominantly excreted *via* urine (39). Such data suggest that a significantly increased intracellular concentration of zinc or a serious change in its isotopic composition in kidney cells resulting from the addition of exogenous Zn aspartate or Zn^{64} aspartate may cause substantial deviation in the cell physiology and lead to cell death.

At the same time, topical application of the experimental compound may be promising due to its relatively low toxicity to keratinocytes and fibroblasts. In addition, the relatively low toxicity of Zn^{64} aspartate to endothelial cells suggests that intravenous therapeutic administration of the compound is possible.

The mechanisms of the cytotoxic effect of Zn^{64} aspartate on tumor cells were studied by analyzing the expression of apoptosis regulatory proteins in A-549 and MB16 cells. The results of Western blot analysis showed that Zn^{64} aspartate induced both melanoma and lung cancer cells death by activating apoptosis through the mitochondrial pathway. However, significant differences in the list of expressed proteins and the levels of their expression suggest that the pathways for initiating apoptosis in these cells may differ.

A-549 cells (the least sensitive to the cytotoxic effects of Zn^{64} aspartate) treated with Zn^{64} aspartate were found to have significantly increased levels of p38 MAP kinase, by 12.8 times compared to the control. In addition, Zn^{64}aspartate-treated A-549 cells had increased levels of

transcription factor NF-kB. The results obtained suggest that oxidative stress developed in A-549 cells after their treatment with Zn^{64} aspartate causes activation of p-38 MAP kinase, which in turn activates NF-kB (40). The active form of NF-kB is translocated to the cell nucleus where it induces the transcription of apoptosis regulatory protein genes that stimulate the release of cytochrome C from mitochondria. This is followed by activation of caspases, caspase-3 and caspase-6 in particular, which leads to caspase-dependent A-549 cell death (41, 42).

In MB16 melanoma cells, which were the most sensitive to the cytotoxic effects of Zn^{64} aspartate, treatment with Zn^{64} aspartate resulted in increased expression of cleaved, and therefore inactivated, PARP-1 DNA repair protein. The increased amount of fragmented PARP-1 with a molecular weight of 89 kDa in cells indicates significant DNA damage (43) caused by the cytotoxic effect of Zn^{64} aspartate. In response to DNA damage, the p53-mediated apoptosis was activated in melanoma cells, as evidenced by a statistically significant increase in the level of the active phosphorylated form of this protein, by 6.4 times compared to untreated MB16 cells. Activated p53, in turn, induces a network of proteins that regulate apoptosis, which leads to disruption of the mitochondrial membrane potential, chromatin condensation, and nuclear fragmentation (44).

A specific DNA-binding domain of p53 has a complex tertiary structure stabilized by the Zn atom. Nowadays, it is known that zinc in tumor cells may cause conformational changes in p53 protein, which lead to restoration of wild-type p53 function in these cells (45). This means the resumption of biological activity of this tumor suppressor protein: inhibition of mitosis and induction of apoptosis in tumor cells. Therefore, modulation of the interaction of p53 with DNA by changing the intracellular concentration (46) or isotopic composition of zinc can be an effective method for regulating p53 activity in malignant cells.

In the presented manuscript, we described only one of the possible mechanisms of Zn^{64} aspartate effect on malignant cells *in vitro* - apoptosis through the mitochondrial pathway. However, considering the extremely important role of zinc in the key metabolic processes of the cell, it is clear that it is necessary to study the effect of the test compound on the proliferative activity, molecular-biological characteristics, and invasive and metastatic properties of tumor cells. Also, additional studies are required to determine the factors that provide different sensitivity of malignant cells to the cytotoxic effect of Zn^{64} aspartate. A logical extension of our work is the evaluation of Zn^{64} aspartate antitumor activity *in vivo*. Such studies will allow us to answer several important questions regarding the capability of the test compound to suppress the experimental tumor growth, the method of Zn^{64} aspartate administration that will provide the best therapeutic effect, and the prediction of side effects from the action of the substance. All these studies will allow us to evaluate the antitumor efficacy of a new experimental compound containing a stable light isotope ^{64}Zn and determine the mechanisms of its action on malignant cells of different histogenesis.

Conclusion

Organic chelate ^{64}Zn stable isotope complexes with amino acids suppressed the viability of malignant cells of various histogenetic origins more effectively than Zn^{64} sulfate, while ^{64}Zn aspartate isotope suppressed tumor cell viability more effectively. Zn^{64} aspartate was found to induce cancer cell death by activating apoptosis through the mitochondrial pathway; however, Zn^{64} aspartate induced caspase-dependent cell death in A-549 cells and the p53-mediated apoptosis in melanoma cells. The obtained results suggest that the control of mechanisms of zinc delivery to the cell and regulation of the intracellular isotopic composition of this element can become the basis for the development of new effective drugs and methods for the treatment of malignant neoplasms of various histogenesis and degree of malignancy.

Conflicts of Interest

The Authors report that there are no conflicts of interest regarding this research.

Authors' Contributions

Research design: Peter Novak, Max Temnik; experimental work: Alexandr Balakin; statistical analysis: Peter Novak; article writing: Peter Novak, Max Temnik.

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