scientific reports

Check for updates

OPEN

An in- vitro measurement for the toxicity of peptides inhibit hexokinase II in breast cancer cell lines

Faranak Karamifard[1], Ali Dadbinpour[1,2] & Mahta Mazaheri[1,3]✉

The role of the enzyme hexokinase 2 in many cancers has been identified through increased glycolysis or binding to the pro-apoptotic channel located in the outer mitochondrial membrane, (VDCA1) and protein kinase (MTOR). To prevent the cancer-causing pathways of this enzyme, it is possible to disrupt the interaction of hexokinase subunits. Peptides can be utilized to interfere with the interaction of subunits by binding to amino acids that contribute to enzyme dimerization. Nowadays, peptides have become a suitable option for the treatment of various diseases, especially cancer, due to their small size, ease of synthesis, and ability to penetrate the tumor. This study examined the toxic effect of peptides that inhibit enzyme interaction on tumorigenic MCF-7 and MDA-MB-231 and non-tumorigenic MCF10A cell lines through MTT analysis and flow cytometry to determine cell apoptosis. The MCF-7 line experienced a significant decrease in cell proliferation with both peptides. The RYALFSS peptide caused a decrease in the number of MDA-MB-231 cells, but the EKGLGATTHPTAAVKML peptide caused a significant increase. There was no significant increase or decrease in the MCF10A cell line. The study's finding indicate that peptides can serve as a tool to prevent the proliferation of carcinogenic cells.

Keywords Hexokinase II enzyme, Inhibitory peptides, Glycolysis, Cell lines

The World Health Organization's data implies that cancer incidence will rise from 11 per 100,000 people in 2018 to 14 per 100,000 people in 2030 Cancer is the leading cause of death in both developed and developing societies today. Given the increasing prevalence of cancer and its significant impact of this disease on all aspects of the lives of affected individuals and their families, cancer is one of the problems that human societies are struggling with[1]. Breast cancer is the most prevalent malignancy in women in 140 to 180 countries worldwide, resulting in approximately 522,000 deaths every year and 6.4% of all cancer deaths[2]. Cancer is a group of diseases characterized by unregulated cell growth, invasion, and spread of cells from their original or primary site to other parts of the body[3]. The metabolic profile of cancer cells is different from that of normal cells because they rely more on anaerobic glycolysis, fatty acid synthesis, and glutaminolysis for proliferation [4]. Otto Warburg and his colleagues were the first to introduce cancer as a metabolic disease in 1924. They suggested that glucose metabolism in cancer cells proceeds via anaerobic glycolysis and hypothesized that cancer cells must depend on anaerobic glycolysis for ATP production due to mitochondrial defects[5]. According to the Warburg effect, cancer cells generate more ATP through the anaerobic pathway, which provides the necessary energy for their exceptional growth. The Warburg effect has been shown to affect intracellular pathways, and in fact, changes in these pathways can lead to tumor growth[6]. The Warburg effect is attributed to drug resistance in certain cancers, such as acute lymphoblastic leukemia (ALL), colon cancer, and breast cancer. Cancer can have high glycolysis rates as a sign. Glucose is phosphorylated by the human hexokinase II enzyme during the first step of the anaerobic glycolysis pathway in the cell cytoplasm to make glucose 6-phosphate[7]. Increased activity of the enzyme hexokinase II is a significant factor in causing the Warburg effect, as glucose-6-phosphate is produced, glycolysis continues, and ATP is produced. According to the statement, blocking glycolysis by inhibiting the hexokinase enzyme and reducing glucose-6-phosphate in cancer cells will definitely increases oxidative stress and leads to decreased tumor growth by reducing energy production[8]. Hexokinase II expression is increased in several types of cancer, and its high expression has been shown by POSITRON EMISSION TOMOGRAPHY (PET) methods

[1]Department of Medical Genetics, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. [2]Genetic and Environmental Adventures Research Center, School of Abarkouh Paramedicin, Shahid Sadoughi University of Medical Science, Yazd, Iran. [3]Mother and Newborn, Health Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. ✉email: Mazaheri54@yahoo.com

in breast tumors[9]. Increased hexokinase II associated with high glycolysis rates in breast cancer cells leads to poor prognosis and short survival of these patients, and resistance to chemotherapy, especially in patients treated with tamoxifen[10]. Targeting cancer metabolic pathways has become a treatment option for researchers because studies have revealed that the metabolic profile of tumor cells differs significantly from that of healthy cells, even when they are found in the same tissue. Extensive studies on glycolysis have demonstrated that increased glucose metabolism lowers oxidative stress in cancer cells, and the Warburg effect could be a defense mechanism that protects cancer cells from oxidative stress[11]. Treatment and prevention of cancer metastasis has always been one of the topics that has attracted the attention of researchers. Providing effective drugs with minimal side effects and reasonable prices is essential for doctors to deal with this disease. A wide range of chemical drugs are currently used to treat cancer, but efforts are still underway to design useful drugs with the shortest duration of action and side effects. In today's world, there are many treatments available to treat breast cancer, including hormone therapy, chemotherapy, and targeted therapies[12]. The evaluation of cancer patients is greatly impacted by resistance to chemotherapy agents, which is one of the most important obstacles to the successful treatment of breast cancer patients. It is a major challenge in drug design to selectively inhibit enzymes involved in the glycolysis pathway, especially kinases. One of the new and powerful strategies for inhibiting protein kinases is the design of peptides as non-toxic compounds to combat cancer, diabetes, and cardiovascular diseases in recent years[13]. The anticancer activity of different peptides depends on various mechanisms that stop tumor growth. These mechanisms include inhibition of angiogenesis, protein–protein interactions, enzymes of signaling pathways, or gene expression[14]. Protein–protein interactions (PPIs) are involved in many cellular processes and affect many cellular activities, such as enzymatic activity and the positioning of cellular components by inducing changes in protein structure. The importance of this topic in the medical and pharmaceutical sectors has caused it to receive a lot of attention, and the new generation of treatments is shifting towards designing drugs for protein–protein interaction regions[15]. Unlike chemical molecules that bind to the protein envelope, PPIs extend the protein's surface area by up to 800 nm to 2,000 angstroms. Studies using Gibbs free energy between interacting proteins have identified sensitive points involved in the interaction. PPIs are crucial in demonstrating biological processes and are utilized in drug design[16]. These peptides have an impact on the various enzymes and growth pathways of cancer cells and inhibit cell growth. In a study, we examined the crystal structure of hexokinase 2 and found out which amino acids contribute to the dimerization of the enzyme subunits. The molecular docking method was used to calculate binding mode and energy for peptides with different amino acid lengths based on this. The effect of selected peptides on inhibiting hexokinase 2 enzyme dimerization was investigated at the molecular level[17]. We considered preventing enzyme dimerization with designed peptides as inhibition of a protein–protein reaction. In this study, we named the peptide EKGLGATTHPTAAVKML (HEXOKINASE) and the peptide RYALFSS as (HEX) and investigated their toxicity effects on MCF-7, MDA-MB-231and MCF10A cells using the MTT assay.

Results

Cytotoxic effects of peptides on cell lines

The effect of 24 and 48-h treatment with peptides inhibiting the structure of the hexokinase enzyme (HEX and HEXOKINASE) and their shuffled peptides AVAGLTHLKETPTAMKG (HEXSHUF) and ARSFSLY(HEXOKINAZSHUF) as negative control peptides and cisplatin as positive control on the rate of cell death of tumorigenic lines MCF7 and MDA-MB-231 and non-tumorigenic line MCF10-A was investigated. In Fig. 1 It can be seen that there was no significant difference in survival compared to the control group after 24 h of HEX peptide treatment at the mentioned concentrations on MCF7. Within 24 h of hexokinase treatment at concentrations of 200, and 300 µM on MCF7 cell line, there was a significant decrease compared to the control group ($p < 0.001$). The MCF7 cell line was treated with HEX for 48 h at 200 and 300 Mm, which resulted in a significant decrease compared to the control group ($p < 0.05$). Finally, the results obtained after 48 h of HEXOKINASE treatment at the concentrations 200 and 300 µM on MCF7 cell line show a significant decrease compared to the control group ($p < 0.001^{***}$). No significant decrease was observed in any of the concentrations after treating the MCF-7 line with shuffled peptides (Fig. 2). Display the effects of HEX and HEXOKINASE peptides on the cell death rate of the MDA-MB-231 strain for 24 and 48-h. Treatment with HEX and HEXOKINASE for 24 h at the mentioned concentrations on the MDAMB231 cell line did not result in a significant difference in survival compared to the control group (TCP). Also, according to the results, 48-h treatment with HEX at concentration of 200 µM showed a significant decrease compared to the control group ($p < 0.01^{**}$). After 48 h, treatment of MDA-MB-231 cell line with hexokinase peptide at concentrations of 200 and 300 µM had a significant growth rate when compared to the control group ($p < 0.001$). This strain and the MCF-7 strain were both unaffected by shuffled peptides. Standard deviation and mean are presented in Tables 1 and 2 for data on the 48-h treatment of cell lines with peptides (Fig. 3). No significant decrease or increase was observed in the evaluation of inhibitory peptides on the MCF10A line (Fig. 1).The anti-cancer drug cisplatin showed a significant reduction in the tumorigenic MCF-7 cell lines after 24 h at a concentration of 300, and 48 h at 200 and 300 µM concentrations respectively (Fig. 2). At concentrations of 200 and 300 µM, cisplatin exposure decreased significantly in the mdamb231 strain after 48 h. The statistical values obtained are based on the mean ± standard deviation, and the difference between the means is significant at the level ($^{*}p < 0.05$) which is given in Tables 1 and 2. The statistical test employed is one-way variance.

Checking the penetration of peptides into cells

After obtaining the effective concentration of the peptides in the cytotoxicity test, they were labeled with FITC and removed from the chromatography column due to their larger size in the early stages. 25 fractions were obtained from the moving buffer. Figure 4 Shows that peptides isolated at 490 nm with the highest absorbance value have a higher concentration of conjugated FITC.

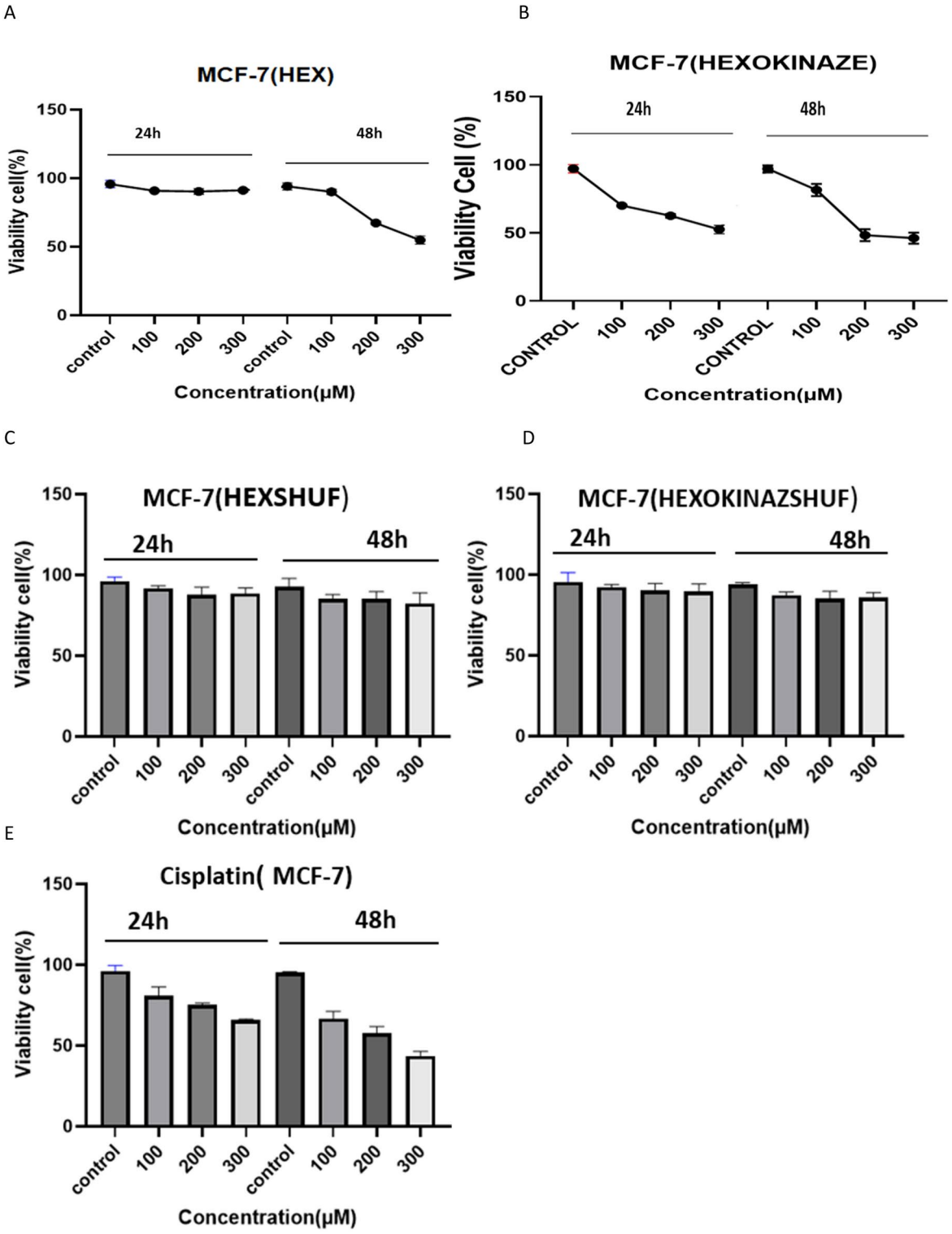

Fig. 1. Analyzing the survival of MCF-7 cell line after being exposed to peptides. Graph A demonstrates the outcome of MCF-7 treatment with HEX peptide after 24 and 48 h, demonstrating a 45% decrease in cell number over the control at 300 μM after 48 h. Graph B shows the exposure of HEXIKINASE peptide to MCF-7 cells after 24 and 48 h. The results showed a decrease in cell survival by 45% in the presence of HEXIKINASE peptide at concentrations of 200 and 300 μM with 48-h treatment ($^*p < 0.05$). Concentrations of 100 μM of both peptides showed a lesser reduction. Graphs C and D show that MCF-7 was exposed to shuffled peptides at concentrations equal to those of inhibitory peptides, but no significant reduction was observed at any concentration for either peptide. Graph E depicts the impact of cisplatin on MCF-7 cells after 24 and 48 h. The number of cells decreased significantly at Concentrations of 200 and 300 μM.

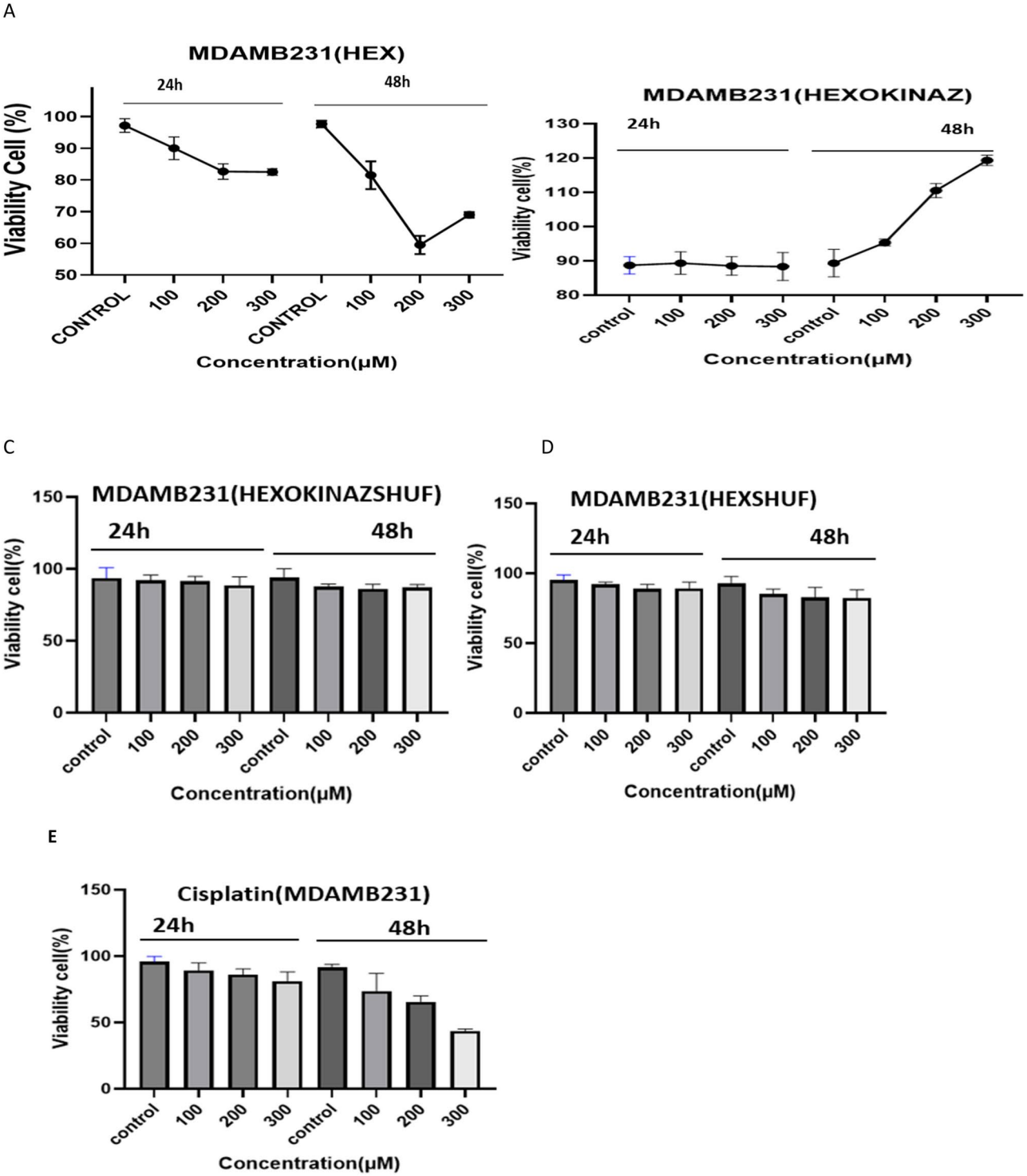

Fig. 2. Evaluation of MDAMB231 cell line survival after peptide treatment. The results of the 24 and 48-h effects of HEX peptide with MDAMB231 in graph A show that no significant decrease was seen after 24 h, but after 48 h, there was a significant decrease in the number of cells compared to the control group at a concentration of 200 (**$p < 0.01$). Graph B shows a significant increase in the effects of the peptide HEXIKINASE after 48 h at concentrations of 200 and 300 μM on the MDAMB231 cell line compared to the control group. MDAMB231 treatment with shuffled peptides did not result in any significant decrease or increase in either peptide, as indicated by graphs C and D. The number of MDAMB231 decreased significantly after 48 h' treatment with cisplatin at concentrations of 200 and 300 μM, as shown in graph E.

		Control	100 µM	200 µM	300 µM
HEX	S.D	100 ± 17.3	59.10 ± 3.1	68.17 ± 2.09	63.94 ± 7.2
	P.Value	–	0.63	0.024	0.032
HEXOKINAZ	S.D	100 ± 19.1	72.43 ± 5.5	67.22 ± 5.1	62.38 ± 4.2
	P.Value	–	0.041	0.0007	0.0009

Table 1. Mean and standard deviation of MCF-7 cell line viability after treatment with HEX and HEXOKINAZ peptides for 48 h using the MTT method.

		Control	100 µM	200 µM	300 µM
HEX	S.D	100 ± 18.7	86.49 ± 5.43	66.21 ± 3.9	52.17 ± 4.5
	P.Value	–	0.0962	0.004	0.055
HEXOKINAZ	S.D	100 ± 15.3	57.14 ± 11.6	69.46 ± 2.7	61.12 ± 3.8
	P.Value	–	0.074	0.0006	0.0008

Table 2. Mean and standard deviation of MDA-MB-231 cell line viability after treatment with HEX and HEXOKINAZ peptides for 48 h using the MTT method.

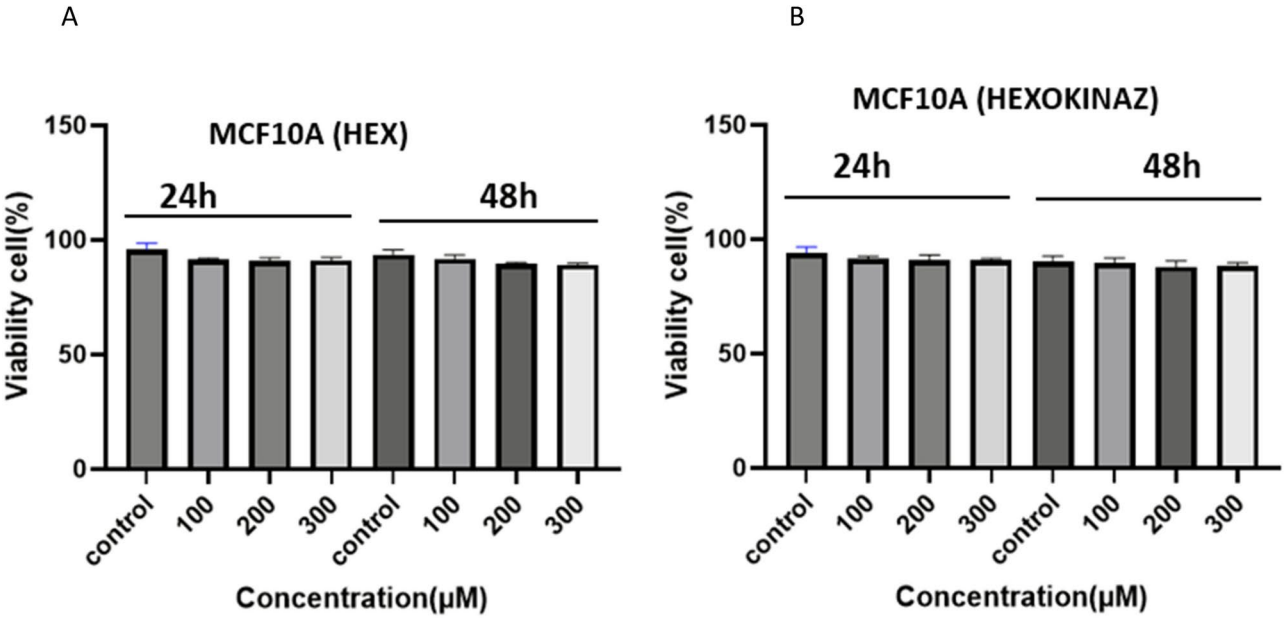

Fig. 3. Measurement of MCF10A cell viability after peptide treatment. According to graphs A and B, no significant reduction effect was observed in the MCF10A cell line after 24 and 48 h after treatment with HEX and HEXOKINAZ peptides at all concentrations.

Confirmation of peptide binding to FITC by method FTIR

To confirm the connection between the peptide and FITC, the FTIR spectrum was used at a frequency range of 500–4000 frequencies of transmitted light, and the results were recorded in the form of a graph (Fig. 5). The FTIR of pure FITC shows a specific peak in the frequency range of 2100–2150, which belongs to the N=C=S structure of FITC. This peak is absent from the peptide spectrum. When the covalent bond between the peptide and FITC is established, the mentioned structure enters the covalent bond with the amino end of the peptide and leads to the formation of thiourea. The binding of peptide and FITC is confirmed by the absence of this peak in the FTIR spectrum.

Studying the penetration of peptides into cells with fluorescence microscopy

The cells were imaged once using a blue filter (DAPI dye) and again with a green filter to detect FITC (cell surface staining). DAPI staining indicates the number of cells in a specific section, while green filter imaging shows the number of cells that are bound to the FITC-conjugated peptide. In the end, the photos were merged to see and recognize how the cells are connected. Figure 6 Displays the results.

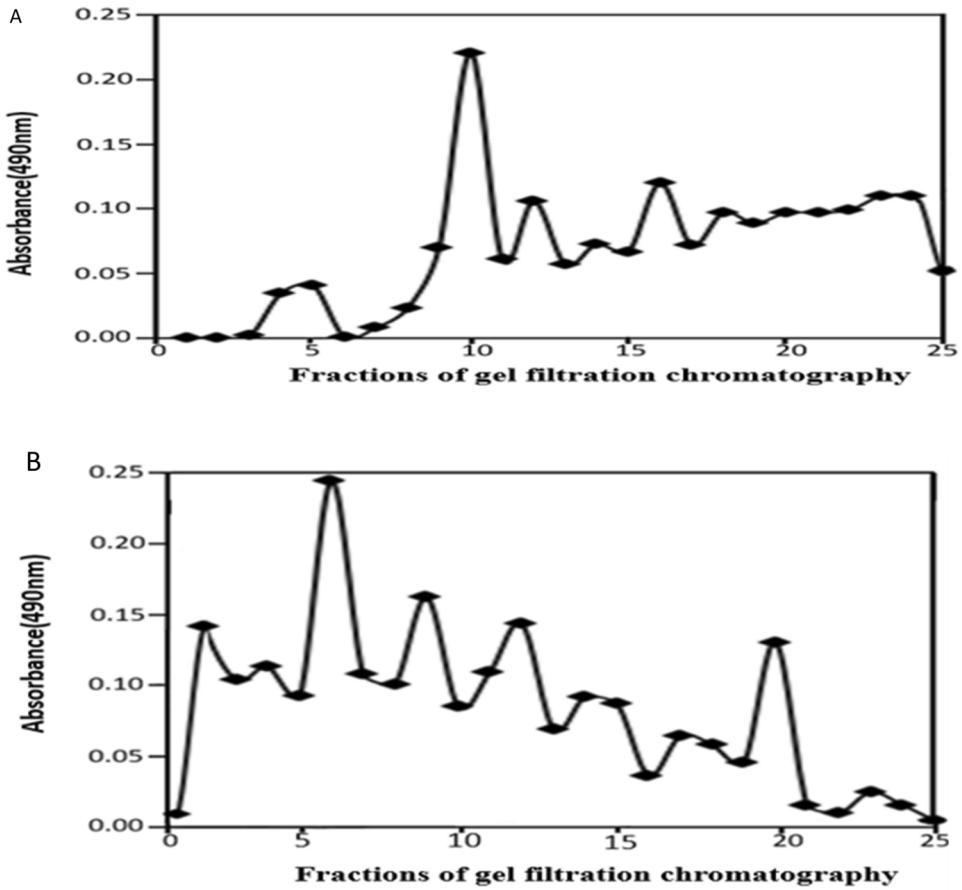

Fig. 4. Chromatography gel filtration chart. Absorption charts at 490 nm wavelength for 25 samples obtained from gel filtration chromatography column. The HEX peptide solution (diagram A) and the HEXOKINAZ peptide (diagram B) are bound to FITC.

Examination of cell apoptosis

The results indicate that Q1 necrotic cells in the treatment groups of both cell lines are not significantly different from the control group. In the control groups of both lines, cells in the early stages of apoptosis Q3 were seen. Despite being lower than the treated groups, the cells in the final stages of Q2 apoptosis were not observed in the control groups of both groups. The treated groups saw a significant increase in the amount of these cells. According to Fig. 7 The peptides can induce apoptosis in the MCF7 cell line by up to 60% compared to the control group. Around 50% of the MDA-MB-231 cell line's apoptosis rate was caused by HEX peptide. The apoptosis induced on the MCF7 cell line by two types of peptides was not significantly different, and both peptides were able to induce apoptosis in the cell to almost the same extent.

The effect of peptides on the amount of glucose-6-phosphate produced in cells

Based on the results in Fig. 8. Glucose-6-phosphate production in MCF7 and mda-mb-231 cell lines treated with HEX and hexokinase peptides were decreases compared to the control group (P value$>$0.05). The culture medium was measured photo metrically and by an ELISA device.

Discussion

One of the major causes of disease burden in the world will be cancer in the future. Cancer is the leading cause of death after cardiovascular diseases[18]. Researchers have always considered targeting the pathways and enzymes involved in cancer development as a therapeutic option in recent years. Through different pathways, the human hexokinase 2 enzyme plays an important role in the development of various cancers[19]. Considering the Warburg effect, the hexokinase enzyme initiates the glycolysis process in the cytoplasm, producing more ATP and glucose-6-phosphate. The low aerobic and mitochondrial respiration of tumor cells leads to their high tendency to take up glucose and produce lactate[20]. The inhibition of the glycolysis pathway has always been of interest to scientists to prevent cancer cell growth. Zheng et al. in 2021 showed that a selective inhibitor, Benitrobenrazide (BNBZ), with nanomolar inhibitory potency in vitro directly binds to HK2, induces apoptosis, and inhibits the proliferation of cancer cells overexpressing HK2. BNBZ has a significant effect on glycolysis by targeting HK2[21]. Shao et al. analyzed the relationship between SALL4-mediated cell death and the progression of gastric cancer via hexokinase II-mediated glycolysis[22]. In a 2024 study, Adekilekun et al. targeted ligands of the enzyme

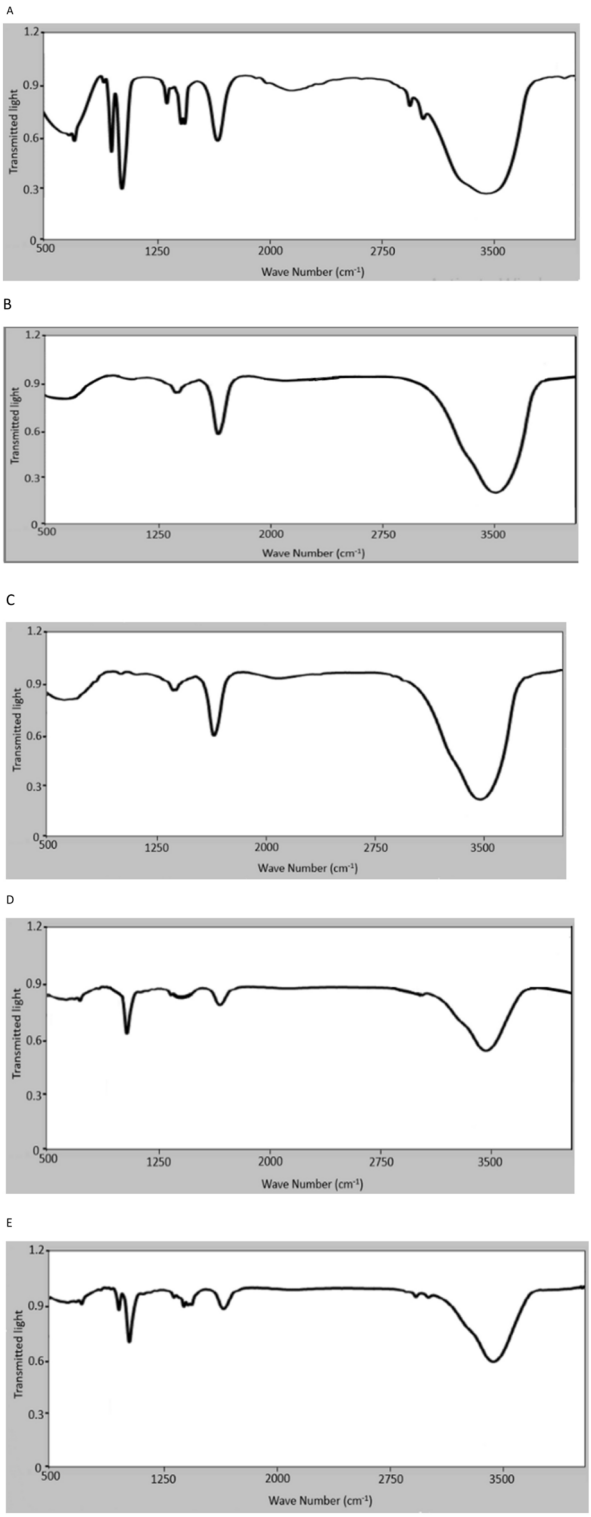

Fig. 5. FTIR spectroscopy Diagrams. pure FITC (**A**) samples conjugated with FITC. The absence of peak 2100–2150 in the conjugated sample indicates the destruction of the N=C=S group and participation in covalent bonding (**B**, **C**). The HEX peptide and HEXOKINASE peptide are both present (**D**, **E**).

hexokinase 2 with Newbouldia laevis, a phytochemical, for the treatment of liver cancer[23]. Voltage-dependent anion channels (VDAC1-3) is a proapoptotic channel responsible for permeability of the outer membrane[24]. The enzyme hexokinase II binds to the VDAC1 channel by forming a dimer, preventing the release of pro-apoptotic enzymes into the cytosol[25]. Many studies have been conducted on how the enzyme hexokinase II binds to the channel (VDAC1). Studies have been conducted to inhibit the binding of hexokinase II to the channel (VDAC1).

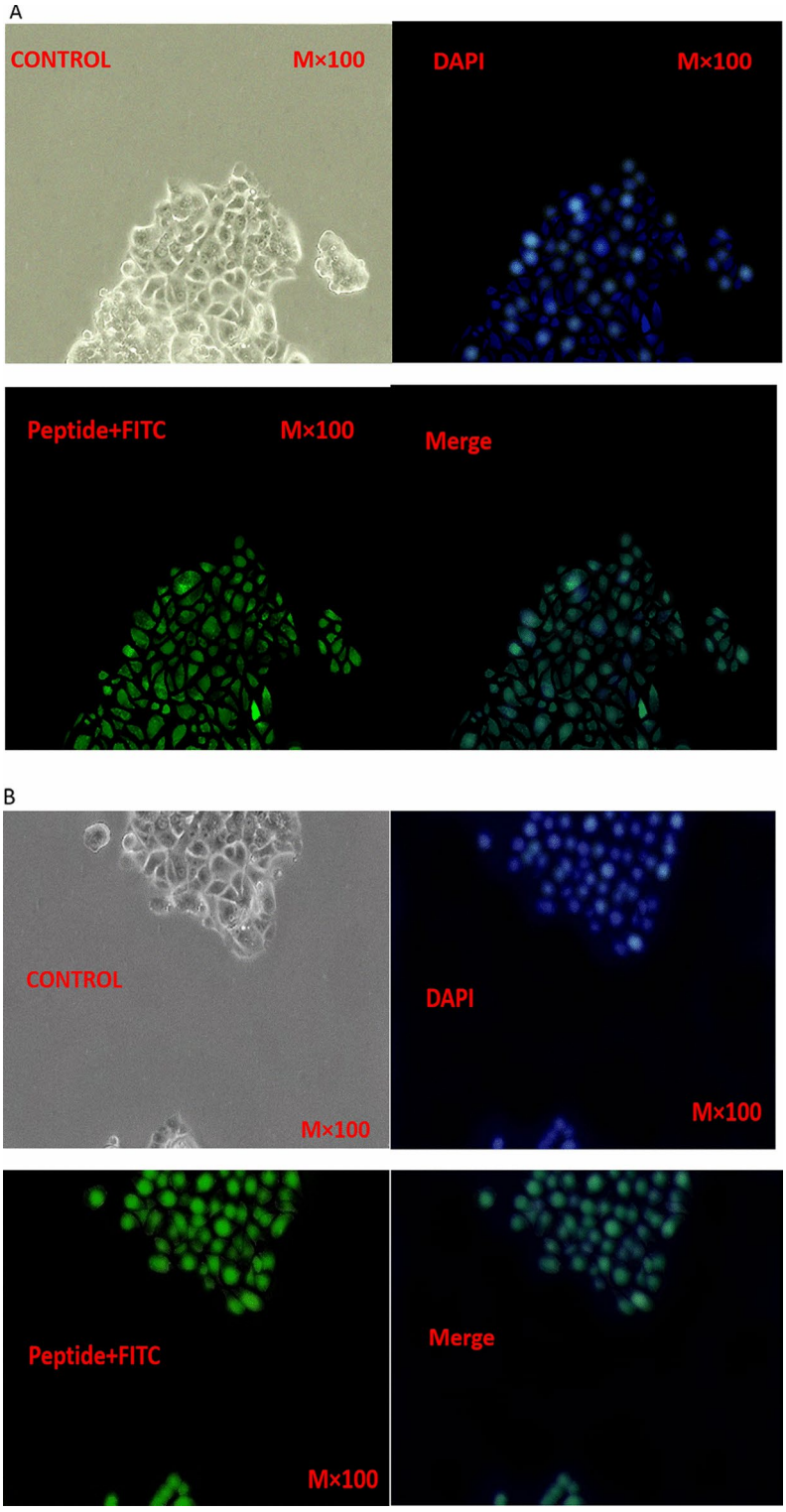

Fig. 6. The Penetration of FITC-labeled peptides into cell lines. The MCF7 cell line is represented Figures A and B, while the MDAMB231 cell line is represented by figures C and D, and the MCF10A cell line is shown in Figures E and F. DAPI (blue) staining to show the location and number of cells, and fluorescent staining to show the penetration of FITC-linked peptide into the cell. To demonstrate the overlap between the two stains, alpha blend software was used to combine two fluorescent and DAPI stains. The microscope's magnification is 100.

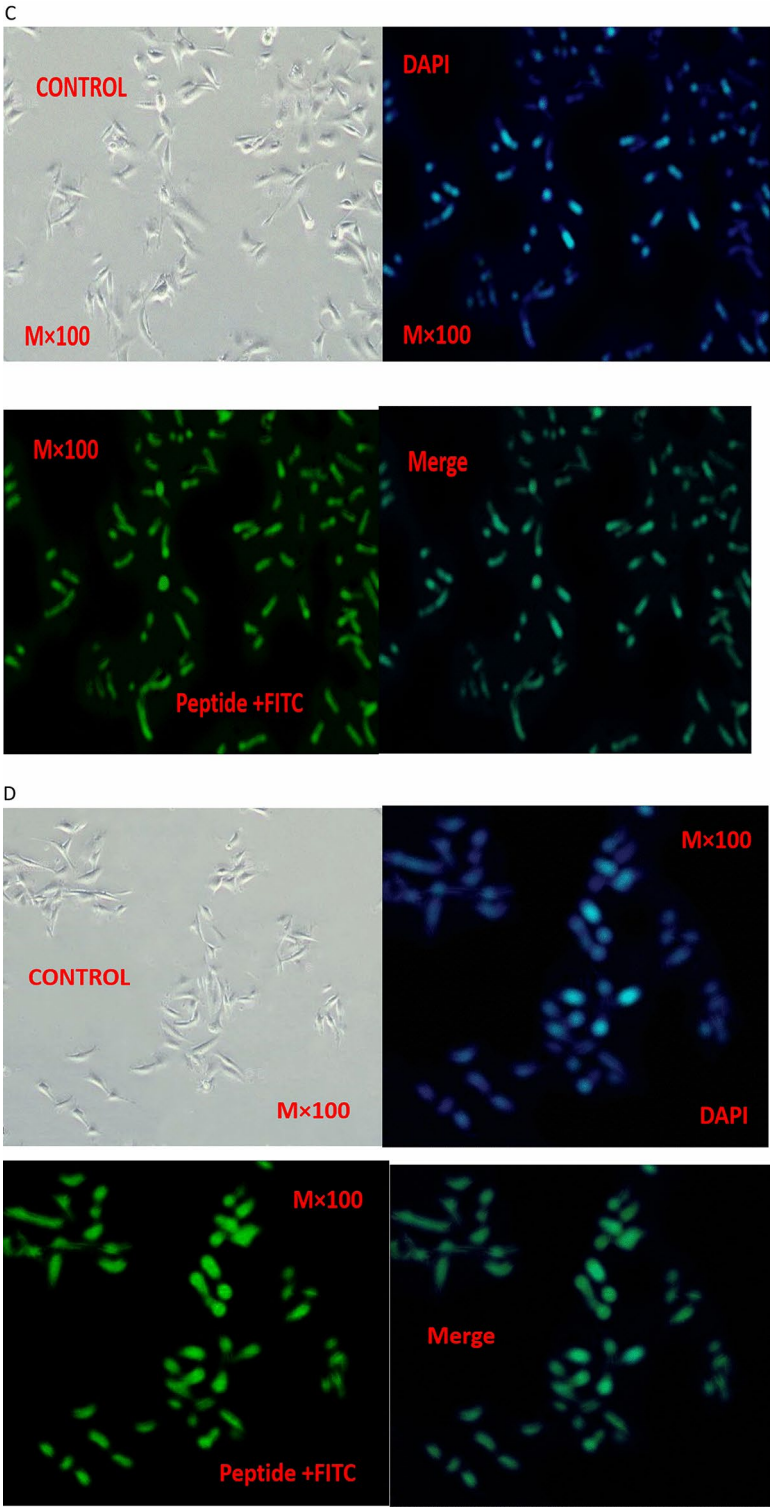

Figure 6. (continued)

In 2019, Fan et al. used a co-precipitation method to show that gemcitabine resistance in pancreatic cancer cells results in increased hexokinase II dimerization, binding to the VDAC channel, and inhibition of apoptosis. They attributed this drug resistance to increased expression of hexokinase II[26]. In 2019, Liu et al. employed a peptide derived from the N-terminal 15 amino acids to bind to the mitochondrial VDAC channel and inhibits the binding of hexokinase 2 to it, which led to a boost in apoptosis-initiating enzymes in multiple cancer cell lines[27]. In 2021, Zhang et al. designed peptides to inhibit the binding of hexokinase 2 to the VDAC1 channel, which caused the death of melanoma cells. In 2022, they also used kaempferol to impede hexokinase's interaction with

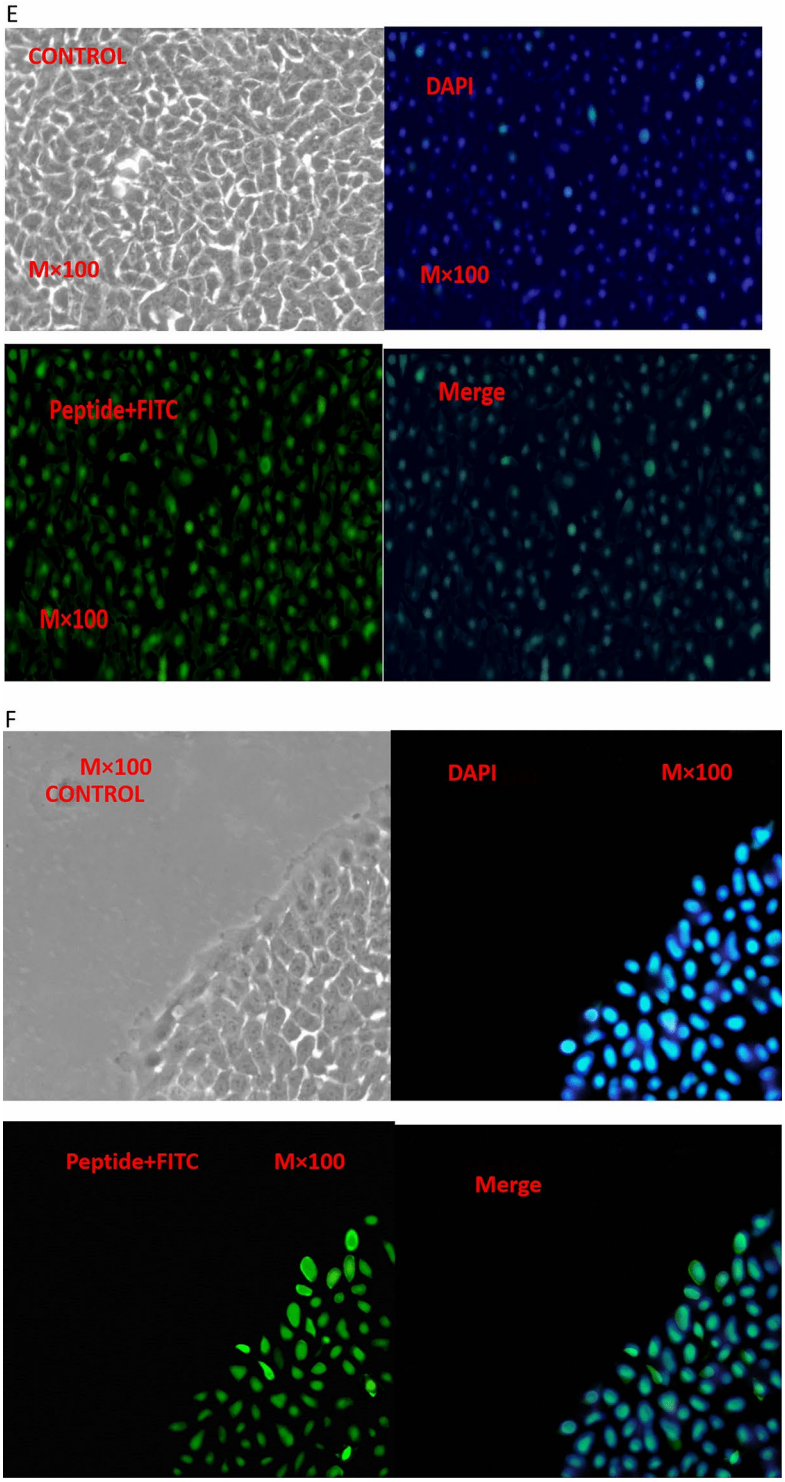

Figure 6. (continued)

the VDAC1 channel and prevent the metastasis of melanoma cells[28]. Mechanistic target of rapamycin (MTOR) is a phosphatidylinositol 3-kinase from the protein kinase family that regulates various cellular processes in humans, including cell growth and proliferation, cell death and survival, protein synthesis, autophagy, and transcription. In cancer cells, MTOR blocks the autophagy pathway of the drug tamoxifen by phosphorylation of S6K[29]. Hexokinase II levels are increased in tamoxifen-resistant breast cancer cells and interact with MTOR to inhibit the MTOR-S6K pathway, thereby increasing autophagy and chemotherapeutic drug degradation, and tamoxifen resistance. Hexokinase II Inhibition results in an increase in MTOR activity, impaired autophagy, and cancer cell sensitivity to the drug[30]. Gremke et al. proved that MTOR-mediated cancer drug resistance inhibits

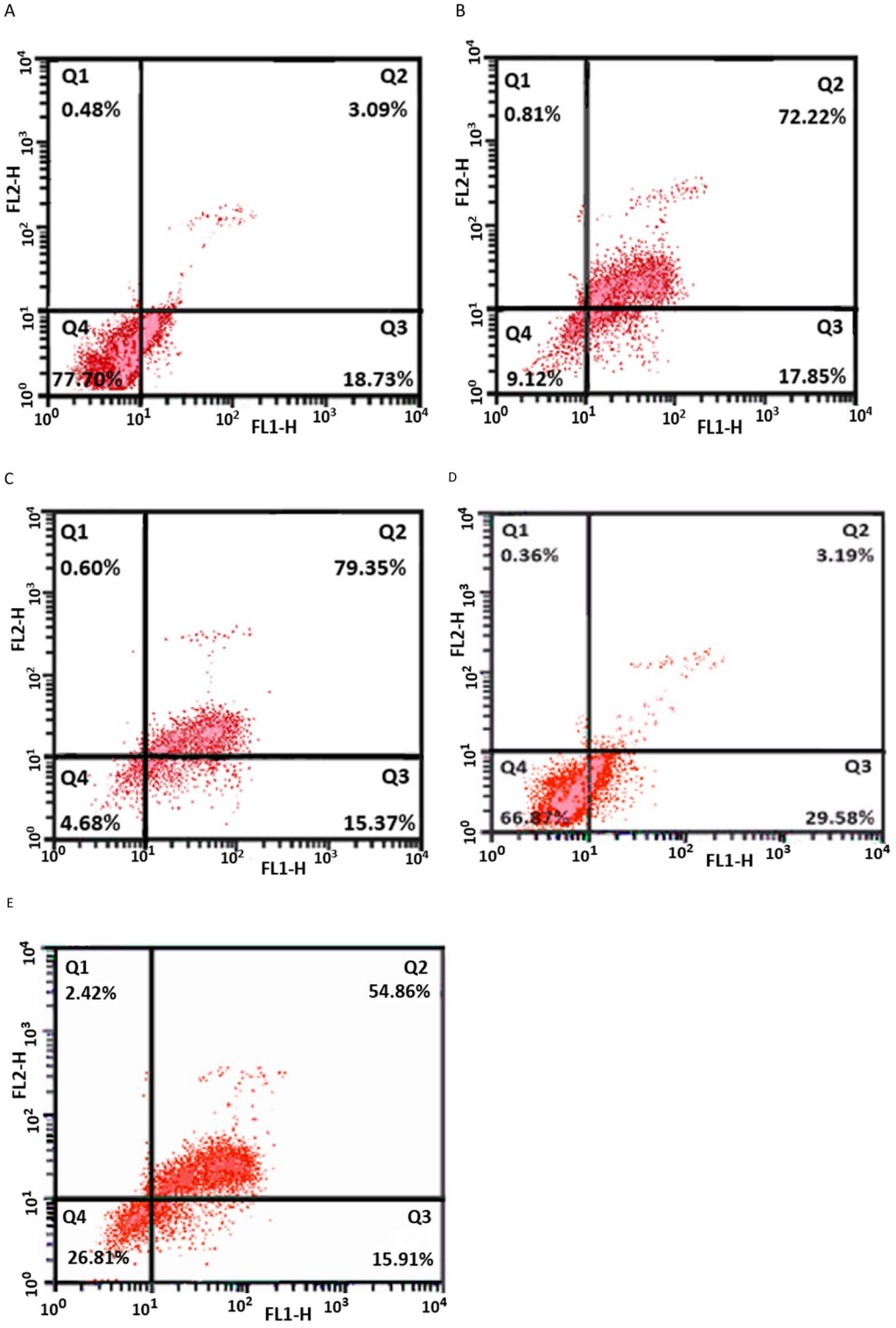

Fig. 7. Increased apoptosis in two cell lines, MCF7 and MDA-MB-231, in the presence of peptides. Comparison of the results of an apoptosis assay on untreated MCF7 cells (**A**) and cells treated with HEX peptide (**B**) and HEXOKINAZ peptide (**C**) shows increased cell apoptosis the comparison of untreated. (**D**) depicts MDA-MB-231 cells without treatment, and (**E**) displays cells that were treated with HEX peptide. The apoptosis rate with HEX peptide was approximately 50%.

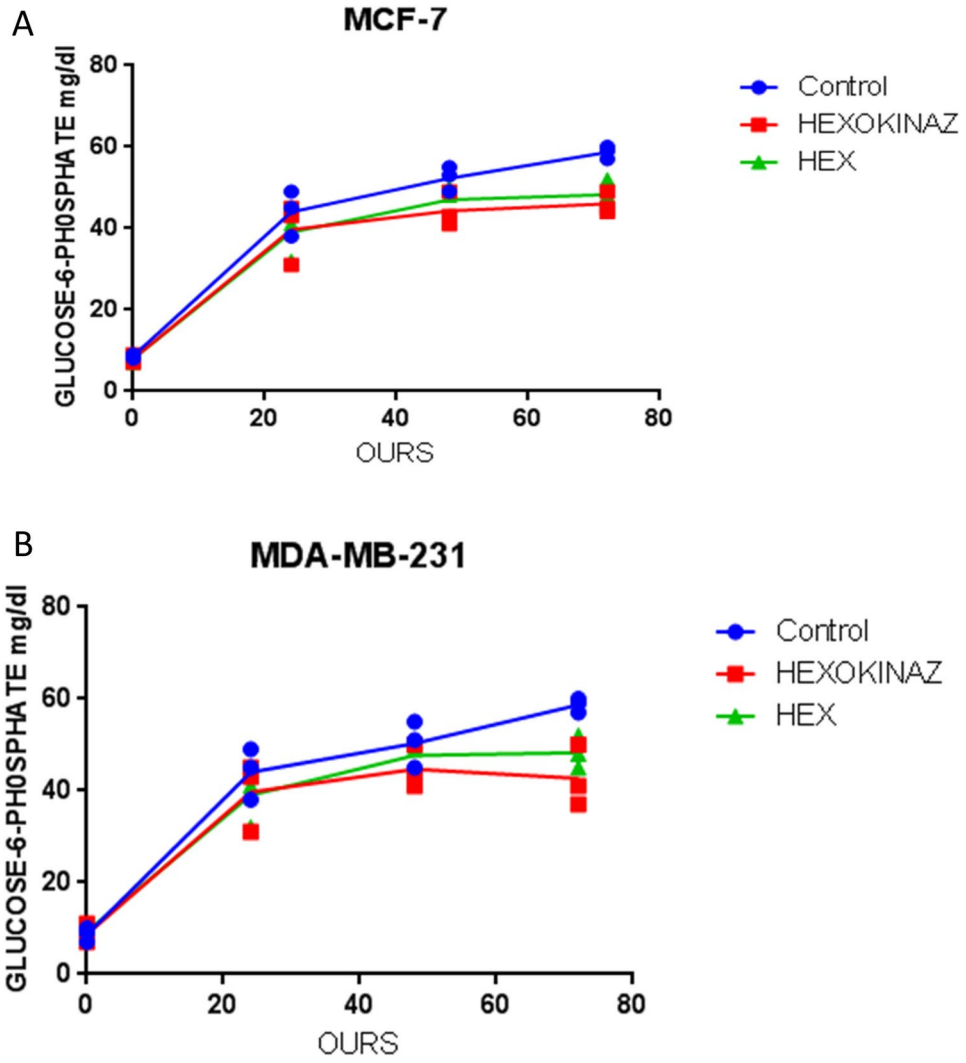

Fig. 8. The effect of peptides on the production of glucose-6-phosphate in cell lines. The results indicate that the production of glucose-6-phosphate decreased in the MCF7 cell line (**A**) and MDA-MB-231 cell line (**B**) when treated with HEX and HEXOKINAZ peptides, compared to the control group (*P* value˃0.05).

autophagy and creates metabolic vulnerability that can be treated[31]. Mehta and colleagues demonstrated that MTOR signaling is involved in cancer progression and that its inhibitors can be used as cancer therapeutic strategies[32]. Given the role of the hexokinase enzyme in the metabolic pathway and its binding to the VDAC1 and MTOR channels in causing cancer, it seems essential to dissociate the enzyme subunits to prevent access to these channels and also to decrease glucose 6-phosphate production to continue the glycolysis pathway. Liu and colleagues successfully inhibited the enzyme hexokinase II in cancer cells using small chemical molecules[33]. The use of peptides has increased because of their unique properties. Features such as small size, ease of synthesis, absence of secondary structures, ability to penetrate tumors, as well as side effects and low toxicity on organs, are unique features of peptides that make them suitable for treatment. Peptides can be a good alternative to chemicals in cancer treatment to reduce their side effects. No peptide has been designed yet that can prevent the binding of hexokinase 2 enzyme subunits and disrupt the enzyme structure. The focus of the Studies so far has been on peptides that inhibit the binding of the hexokinase enzyme to the VDCA1 channel[34]. In one study, we made peptides that prevent the formation of hexokinase enzyme subunits. Peptides were chosen for their binding energy to subunits. In this study, we investigated the toxicity of the designed peptides using the MTT test on tumorigenic breast cancer cell lines MCF7 and MDA-MB-231, as well as non-tumorigenic breast epithelial cell line MCF10A. The rate of MCF7 cell death was significantly reduced by treatment with HEX and HEXOKINASE peptides for 48 h compared to the control group. HEX peptides at a 200 µM treatment 48-h resulted in a substantial reduction of MDA-MB-231 cells. The MDAMB231 cell line underwent a significant increase in survival compared to the control group (TCP) after 48 h of treatment with HEXOKINAZE peptide at 200 and 300 µM. It was considered that the peptide may have activated other growth signaling pathways, which suggests that it should be investigated in other studies. No toxic effects were observed in any concentration of the peptides on the MCF10A cell line. Fluorescent microscopy and FITC-labeled peptides were used to confirm

the entry of peptides into the cell, and flow cytometry was used to examine the effect of peptides on growth inhibition and apoptosis induction in cancer cell lines. Up to 60% of MCF7 cell lines can experience apoptosis when subjected to Peptides, compared to the control group. The MDA-MB-231 cell line has an apoptosis rate that is about 50% influenced by the HEX peptide. Inhibiting hexokinase II dimerization by don't binding to the VDAC1 channel and MTOR is expected to result in apoptosis in the tested cell lines. To determine the cell growth arrest stage of the cell cycle, a complementary flow cytometry test can be performed. We finally investigated the level of glucose-6-phosphate produced by hexokinase II before and after being treatment with peptides. The concentration of Glucose-6-phosphate in MCF7 and MDA-MB-231 cell lines treated with HEX peptides and hexokinase was significantly reduced compared to the control group (Pvalue˃0.05). The peptides in this study do not interact with the substrate and cofactor sites of the hexokinase enzyme, but rather react with amino acid sequences that are involved in enzyme dimerization and have a lower impact on other enzymes, resulting in their specificity being higher than that of other inhibitors. Research has indicated that peptides may be a viable option for cancer treatment due to their low side effects compared to chemical drugs. Studying the impact of the peptide on other cell signaling pathways, the way it crosses the membrane and binds to the target protein. To complement the cellular studies and investigate the effects of the peptides on other cancer types, it is suggested to conduct further experiments.

Methods

Cell proliferation and survival assay using the MTT method

The study of cell proliferation and survival is considered a crucial and fundamental technique in cell laboratories. This study requires precise measurement of the number of viable cells in cell culture medium. To optimize cell culture conditions and evaluate routine anticancer drugs, it is essential to have methods for calculating cell viability. Although there are several methods that can be utilized for these purposes, indirect methods using fluorescent or chromogenic markers offer very rapid methods on a large scale. One of the most widely used methods is the measurement of cell viability using soluble tetrazolium salts, and MTT is considered one of the most important of these method. This method is rapid, sensitive, simple and reproducible. Consequently, it was an appropriate method for cytotoxicity in this study[35]. To investigate the toxicity effect of peptides in this study, the MTT Assay Kit ab211091 was used. Cell lines were purchased from the Pasteur Institute of Iran. The steps involved in conducting the test will be reviewed below. First step: Flasks containing MCF7 (Cell NO: IRBC C10082), MDA-MB231 (Cell NO: IRBC C10684) and MCF10-A (IBC C10788) cell lines were counted under a class II hood and under completely sterile conditions using trypsin enzyme. In addition, 5000 cells were cultured per well of a 96-well plate. Cell culture was performed using DMEM high glucose medium that contained 10% fetal bovine serum (FBS). At 37 °C and 5% CO2, the cells were kept in an incubator for 24 h. In the second step, cells were exposed to inhibitory peptides HEX (Lot No: C0513GK300-1/PE9608) and hexokinase (Lot No: C0513GK300-1/PE9604) and shuffled peptides ARSFSLY (Lot No: C0513GK300-7/PE9610) and AVAGLTHLKETPTAMKG (Lot No: C0513GK300-3/PE9606). Gene Script synthesized the peptides.The concentration of 300 micromolar was considered effective in inhibiting the enzyme dimer in the DLS test from previous research, and lower concentrations of 200 and 100 micromolar were considered for the experiment[36]. Cisplatin (cas-number 15663-27-1) served as a positive control with concentrations that were identical to that of the peptides. The third step: After 24 and 48 h, the cell supernatant was removed and then a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-(diphenyltetrazolium bromide) (5 mg/ml) MTT was poured onto the cells. The fourth stage involved dissolving the product or formazan crystals inside the cells using dimethyl sulfoxide (DMSO), and then placing the plates in an incubator in the dark for three hours. Step 5: Cells were washed with PBS after the indicated time to remove unreacted MTT. Finally, the colored solution was analyzed at a wavelength of 570 nm.

In this experiment, cells cultured in two-dimensional conditions without drugs were used as controls. Cytotoxicity testing was performed in triplicate according to ISO-10993-5 standards.

Examining peptide entry into the cell

Fluorescent microscope was employed for the purpose of studying and investigating the entry of peptides into cells. A fluorescent microscope is a type of light microscope that uses ultraviolet rays as its light source. To view the sample through this microscope, fluorescent substances are used to stain specific parts or molecules inside the cell. Fluorescent materials can absorb light in the ultraviolet wavelength range and produce light in the visible spectrum at a longer wavelength. The light emitted by the sample creates the image you see. In order to observe a sample under a fluorescence microscope, the sample must be fluorescent. This necessitates the use of fluorescent dyes to label the peptides. The most common dyes used to label peptides include fluorescein isothiocyanate (FITC), fluorescein (FAM), carboxytetramethylrhodamine (TAMRA), and aminomethylcoumarin (AMC). The N-terminal end is where the dyes attach to the peptide, either directly or through a linker. The side chain of the amino acids lysine or cysteine at the C-terminal end can receive fluorophores as well. The peptides were labeled with FITC based on what was said. The test steps are listed below. The peptide solution was made in a buffer that contains 0.1 M sodium carbonate at a pH of 9 and a concentration of 1 mg/ml. A solution of FITC (cat number: F7250 Sigma Aldrich) in DMSO was obtained with a concentration of 3 mg/ml. 100 µl of FITC solution was added to each ml of peptide solution and gradually dissolved. A stirrer was used to conjugated the prepared solution for 24 h in a dark environment in at room temperature[37].

Gel filtration chromatography

A method for separating molecules based on their different sizes. This separation method is one of the gentlest types of chromatography, as it does not alter the structure of molecules. At this stage of the investigation, the sample contained three populations of molecules: unbound peptides, free FITC, and FITC-conjugated peptides.

Gel filtration chromatography was used to remove free FITC from the reaction solution[38]. Given the molar mass of FITC, which is 398 Daltons, Sephadex G10 (Cat number: G10120 Sigma Aldrich) was chosen for the separation of free FITC by gel filtration chromatography. The mobile phase was PBS buffer at a concentration of 20 mM, which passed through the column at an approximate rate of 500–800 µL/min. The column collected approximately 500 µl per minute. Until the mobile buffer's absorption reached zero, a total of 25 particles were obtained.

FTIR Test

Fourier-transform infrared spectroscopy, or FTIR for short, is a technique used to determine a substance's infrared absorption spectrum based on wavelength or, more specifically, wavenumber. The infrared range of (700–1 mm) is where most materials absorb light, and their absorption spectra can be used to determine their molecular components and structures. For this reason, the FTIR spectra of selected samples from the gel filtration, as well as the peptide and pure FITC, were prepared and recorded in the form of a graph in the range of 500–4000 CM^{-1}. The concentration prepared for FITC was 169×10^{-5} and for the peptide 1364×10^{-5}[39].

Cell culture

The subsequent procedure involved culture of MCF7 and MDA-MB-231 cell lines in 6-well plates with RPMI1640 medium containing 10% serum and incubation for 24 h at 37 °C and 5% CO2.

Cells were treated with FITC-labeled peptides at a concentration of 300 µM/ML (effective dose obtained from MTT test) and then incubation continued for 4 h. After that, the cell culture medium was removed, washed with phosphate buffer, and observed under a microscope with a 100× magnification[40].

Investigating the level of apoptosis in cell lines

During the early stages of apoptosis, phosphatidylserine, which is naturally present in the inner half of the membrane, is transferred to the outer half, which is due to disruption of the ATP-dependent translocase enzyme and activation of other enzyme systems such as scramblase. The presence of phosphatidylserine in the outer layer of the cell membrane is a natural signal for phagocytosis and recognition of apoptotic cells by macrophages and surrounding solutions. Annexin v, an anticoagulant protein with has a molecular weight of 35 kDa, has been discovered to bind to phosphatidylserine with high affinity when calcium is present. Flow cytometry is a common method for studying apoptosis using the conjugate of this protein with fluorescein isothiocyanate (FITC). Cell shrinkage causes a decrease in direct scattered light during apoptosis. Annexin v/PI dual staining was utilized to assess apoptosis. The fluorochromes used are FITC, which is recorded and evaluated in the green channel or FL1, and propidium iodide (PI), which is a DNA-specific dye, and is recorded and evaluated in the red channel FL2 or orange channel FL3. In this study, the Annexin v/PI kit (cat.556547) was used to detect and distinguish apoptotic cells from necrotic cells and determine the percentage of each. The following is a summary of the steps. Cells were cultured in 6-well plates in RPMI1640 medium containing 10% serum and incubated for 24 h at 37 °C and 5% CO2. Cells were treated with peptides at a concentration of ML/300µM (the effective dose obtained from MTT test), and then incubated for 48 h. The cells were collected and washed twice with phosphate buffer. After this step, the cells were suspended in a binding buffer and counted using neobar slide. The number of 10×10^4 cells was added to each sample, which consisted of 4 tubes of 5 ml for flow cytometry. Of the 4 tubes, one was the control tube, to which no color was added. Annexin v/PI dye was added to three tubes according to the kit protocol to the cell suspension in binding buffer. Thus, there were two tube with 5 mL of PI dye, another with 5 mL of Annexin dye, and a third with both dyes. The cells were analyzed using a flow cytometry device after 15 min of incubating in darkness at 37 °C[41].

Assessing glucose 6-phosphate levels in cells

Glucose is converted to glucose-6-phosphate when ATP is present and the hexokinase II enzyme is nearby. The amount of glucose-6-phosphate in cells was measured using an ELISA device. The Glucose-6-Phosphate Assay Kit Colorimetric (ab83426) was used for all steps, as we will discuss below. The cells were incubated at 37 °C and 5% CO2 after being cultured in RPMI1640 medium, 10% serum was added to a 6-well plate for 24 h. Cells were treated with peptides at a concentration of ML/300µM (effective dose obtained from MTT test), and then incubated for 48 h. Then next steps involved collecting and using the supernatant solution as a sample. 1000 µl of the test solution were added to the micro tube, and 100 µl of cell surface medium were subsequently added. It was incubated for 5 min at 37 degrees Celsius after being mixed. Finally, the standard optical absorbance and the samples were measured against the blank at a wavelength of 340 nm[42].

Data availability

Datasets during the current study are available from the corresponding author on reasonable request.

Received: 29 August 2024; Accepted: 17 March 2025
Published online: 27 March 2025

References

1. Hacker, K. The burden of chronic disease. *Lifestyle Med.* **8**, 112–119 (2024).
2. Pereira, A. C. P., Koifman, R. J. & Bergmann, A. Incidence and risk factors of lymphedema after breast cancer treatment: 10 years of follow-up. *The Breast* **36**, 67–73 (2017).
3. Suresh, D. S. & Chandrasekharan, G. Molecular principles of tissue invasion and metastasis. *Am. J. Physiol. Cell Physiol.* **324**, 971–991 (2023).
4. Finley, L. W. What is cancer metabolism?. *A Cell Press J.* **186**(8), 1670–1688 (2023).

5. Fanchon, E. & Stéphanou, A. Is cancer metabolism an atavism?. *Cancers* **16**(13), 2415 (2024).
6. Barba, I., Carrillo-Bosch, L. & Seoane, J. Targeting the Warburg effect in cancer: Where do we stand?. *Int. J. Mol. Sci.* **25**(6), 3142 (2024).
7. Simon-Molas, H., Del Prete, R. & Kabanova, A. Glucose metabolism in B cell malignancies: A focus on glycolysis branching pathways. *Mol. Oncol.* **18**(7), 1777–1794 (2024).
8. Marcucci, F. & Rumio, C. On the role of glycolysis in early tumorigenesis—permissive and executioner effects. *Cells* **12**(8), 1124 (2023).
9. Lei, P. et al. Role of glucose metabolic reprogramming in breast cancer progression and drug resistance. *Cancers* **15**(13), 3390 (2023).
10. Cunha, A. et al. Targeting glucose metabolism in cancer cells as an approach to overcoming drug resistance. *Pharmaceutics* **15**(11), 2610 (2023).
11. Farahzadi, R. et al. Oxidative stress regulation and related metabolic pathways in epithelial–mesenchymal transition of breast cancer stem cells. *Stem Cell Res. Ther.* **14**, 342 (2023).
12. Chaurasia, M., Singh, M. & Flora, S. J. S. A review of FDA approved drugs and their formulations for the treatment of breast cancer. *Front. Pharmacol.* **14**, 1184472 (2023).
13. Alexa, A. et al. Peptide based inhibitors of protein binding to the mitogen-activated protein kinase docking groove. *Front. Mol. Biosci.* **8**, 690429 (2021).
14. Dulic, M. et al. Protein-protein interactions of Seryl-tRNA synthetases with emphasis on human counterparts and their connection to health and disease. *Life* **14**(1), 124 (2024).
15. Rehman, A. U., Khurshid, B. & Wadood, A. Computational approaches for the design of modulators targeting protein-protein interactions. *Exp. Opin. Drug Discov.* **18**(3), 315–333 (2023).
16. Nandi, S. et al. Deciphering the lexicon of protein targets: A review on multifaceted drug discovery in the era of artificial intelligence. *Mol. Pharmac.* **21**(4), 1563–1590 (2024).
17. Karamifard, F., Mazaheri, M. & Dadbinpour, A. Abatement of the binding of human hexokinase II enzyme monomers by in-silico method with the design of inhibitory peptides. *In Silico Pharmacol.* **12**(1), 30 (2024).
18. Gaidai, O. et al. Prediction of death rates for cardiovascular diseases and cancers. *Cancer Innov.* **2**(2), 140–147 (2023).
19. Li, R. et al. A pan-cancer analysis of the role of hexokinase II (HK2) in human tumors. *Sci. Rep.* **12**, 2305662 (2022).
20. Cheng, Q. et al. Current advances on nanomaterials interfering with lactate metabolism for tumor therapy. *Adv. Sci.* **11**(3), 2305662 (2024).
21. Zheng, M. et al. Novel selective hexokinase 2 inhibitor Benitrobenrazide blocks cancer cells growth by targeting glycolysis. *Pharmacol. Res.* **164**, 105367 (2021).
22. Shao, M. et al. SALL4 promotes gastric cancer progression via hexokinase II mediated glycolysis. *Cancer Cell Int.* **20**, 1–13 (2020).
23. Adekilekun, H. A. et al. A computational discovery of hexokinase 2 inhibitors from Newbouldia laevis for Hepatocellular carcinoma (HCC) treatment. *South Afr. J. Bot.* **169**, 12–26 (2024).
24. Belosludtseva, N. V., Dubinin, M. V. & Belosludtsev, K. N. Pore-forming VDAC proteins of the outer mitochondrial membrane: regulation and pathophysiological role. *Biochem. Moscow.* **89**, 1061–1078 (2024).
25. Shteinfer-Kuzmine, A. et al. VDAC1-based peptides as potential modulators of VDAC1 interactions with its partners and as a therapeutic for cancer, NASH, and diabetes. *Biomolecules* **14**(9), 1139 (2024).
26. Fan, K. et al. Hexokinase 2 dimerization and interaction with voltage-dependent anion channel promoted resistance to cell apoptosis induced by gemcitabine in pancreatic cancer. *Cancer Med.* **8**(13), 5903–5915 (2019).
27. Liu, D. et al. Self-assembly of mitochondria-specific peptide amphiphiles amplifying lung cancer cell death through targeting the VDAC1–hexokinase-II complex. *J. Mater. Chem. B.* **30**, 4706–4716 (2019).
28. Zheng, X. et al. Kaempferol impairs aerobic glycolysis against melanoma metastasis via inhibiting the mitochondrial binding of HK2 and VDAC1. *Eur. J. Pharmacol.* **931**(15), 175226 (2022).
29. Liu, J., Yue, W. & Chen, H. The correlation between autophagy and tamoxifen resistance in breast cancer. *Int. J. Clin. Exp. Patho.* **12**(6), 2066–2074 (2019).
30. Panwar, V., Singh, A. & Bhatt, M. Multifaceted role of mTOR (mammalian target of rapamycin) signaling pathway in human health and disease. *Sig. Transduct. Target.* **8**(1), 375 (2023).
31. Gremke, N. et al. mTOR-mediated cancer drug resistance suppresses autophagy and generates a druggable metabolic vulnerability. *Nat. Commun.* **11**, 4684 (2020).
32. Mehta, D., Baja, A. & Rajput, K. Unveiling the role of mechanistic target of rapamycin kinase (MTOR) signaling in cancer progression and the emergence of MTOR inhibitors as therapeutic strategies. *ACS Pharmacol. Transl. Sci.* **7**(12), 3758–3779 (2024).
33. Liu, B. et al. The promoting role of HK II in tumor development and the research progress of its inhibitors. *Molecules* **29**(1), 75 (2024).
34. Ciscato, F. et al. Hexokinase 2 displacement from mitochondria-associated membranes prompts Ca2+-dependent death of cancer cells. *EMBO Rep.* **21**(7), e49117 (2020).
35. Kamiloglu, S. et al. Guidelines for cell viability assays. *Food Front.* **1**(3), 332–349 (2020).
36. Komeri, R., Kasoju, N. and Kumar, P.A., *13 - In vitro Cytotoxicity and Cytocompatibility Assays for Biomaterial Testing under Regulatory Platform.* Biomedical Product and Materials Evaluation. p. 329–353 (2022).
37. Zorko, M. & Langel, Ü. Studies of cell-penetrating peptides by biophysical methods. *Quart. Rev. Biophys.* **55**(3), e3 (2022).
38. Striegel, A. M. Method development in interaction polymer chromatography. *TrAC Trends Anal. Chem.* **130**, 115990 (2020).
39. Dutta, A., Chapter 4 - Fourier transform infrared spectroscopy. Micro and nano technologies: 73–93 (2017).
40. Bayram, D., Özgöçmen, M. & Çelik, D. A. Does boric acid inhibit cell proliferation on MCF-7 and MDA-MB-231 cells in monolayer and spheroid cultures by using apoptosis pathways?. *Biol. Trace Elem. Res.* **202**(5), 2008–2021 (2024).
41. Nolan, A. et al. Fluorescent characterization of differentiated myotubes using flow cytometry. *Cytometry* **105**(5), 332–344 (2024).
42. Peng, P. et al. Emerging ELISA derived technologies for in vitro diagnostics. *TrAC Trends Anal. Chem.* **152**, 116605 (2022).

Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors

Author contributions

1. Faranak Karamifard 2. Ali Dadbinpour 3. Mahta Mazaheri All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to M.M.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.