Acta Pharm. Sci. Vol 63:(1), 2025
Original article
DOI: 10.23893/1307-2080.APS6312

Bioactivity of hexokinase II inhibitor ikarugamycin and relation with tissue factor in breast cancer cell lines

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ABSTRACT

Despite the understanding gained from scientific studies regarding Hexokinase II (HK2)'s involvement in cancer cell metabolism, there are no reports of directly inhibiting HK-2 enzyme to affect tissue factor (TF) activity in cancer cells. This study primarily investigates the complex mechanisms triggering neoplastic cell formation by examining the in vitro bioactivation of the Ikarugamycin (IKA) molecule, commonly used as an antibiotic. The IC50 values for MDA-MB-231 (TNBC) and MCF-7 (TPBC) cell lines are 24.1 μM and 19.25 μM, respectively. Furthermore, TF activation in breast cancer cell lines was demonstrated through Prothrombin Time (PT) analysis, showing that IKA effectively prolongs TF activation compared to Sodium Oxamate and Paclitaxel (Ptx), commonly used as a chemotherapeutic agent. Additionally, it was observed to be more effective in hormone-dependent MCF-7 breast cancer cell lines. Future studies should focus on investigating the changes in protein, enzyme, and gene levels of TF following treatment with IKA.

Keywords: hexokinase-2, tissue factor, breast cancer, glycolysis, anticancer-antibiotics

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(Received 5 Feb 2024, Accepted 16 Apr 2024)

INTRODUCTION

Breast cancer is a prevalent malignancy typified by unregulated cellular proliferation within the breast tissue. According to the latest data, it is the most diagnosed cancer in women[1-2]. Various therapeutic modalities, including surgical interventions, radiotherapy, chemotherapy, and hormone therapy, may induce significant adverse effects that detrimentally impact the overall quality of life[3]. In this sense, it is important to search for new treatments that provide better results than current breast cancer treatment methods and at the same time reduce side effects.

Hexokinases (HKs) represent evolutionarily conserved enzymes pivotal in phosphorylating hexoses, six-carbon sugars. Their significance lies in mediating essential processes within cellular metabolism and play crucial roles in Adenosine Triphosphate (ATP) synthesis, glucose storage, NADH pool enrichment, and protein glycosylation. Among mammals, the well-characterized HK isozymes include HK1 through HK4[4-6]. Recent investigations have brought to light a fifth isozyme known as HKDC1 (HexoKinase Domain Containing protein 1)[7]. Noteworthy is the cytosolic localization of all HKs, except HK-1 and HK-2, which possess a distinctive N-terminal motif facilitating binding to mitochondria—an attribute absent in other isoforms.

The binding of HK-1 and HK-2 to mitochondria manifests cytoprotective effects in both healthy and neoplastic cells, enhancing their proficiency in glucose utilization. This unique feature underscores the intricate regulatory mechanisms through which hexokinases contribute to cellular homeostasis and function. These insights contribute to a comprehensive understanding of the diverse roles played by hexokinases in cellular processes critical for metabolic regulation and overall cellular health[5,8-10].

A key feature of many cancers, especially the most aggressive, is the ability to metabolize glucose at a high rate, a phenotype that is clinically detected using positron emission tomography (PET). This phenotype gives cancer cells, including those involved in metastasis, a competitive advantage over normal cells[8].

Especially, after the rapid entry of glucose into cancer cells through molecular glucose transporters, the overexpression of hexokinases, primarily HK-2, leads to high glycolytic activity through the interaction of numerous complex mechanisms[5]. This situation contributes to the continued proliferation of cancer cells (Figure 1). Numerous complex mechanisms facilitate the activation of tissue factor (TF). The initiation of TF activation is instigated by adenosine triphos-

phate (ATP), prompting the translocation of acid sphingomyelinase (A-SMase) to the plasma membrane. ATP stimulation augments the hydrolysis of sphingomyelin (SM) within the outer leaflet. Impeding the expression or activity of A-SMase not only diminishes ATP-induced sphingomyelin hydrolysis but also impedes the ATP-triggered decryption of the transcription factor[11-12]. Additionally, integrin mechanisms that trigger TF activation, particularly in breast cancers, are present. In breast cancer cells, TF plays a significant role in regulating the TF-VIIa-Protease-Activated Receptor 2 (PAR2) signaling pathway. TF is continually associated with β1 integrins on this pathway and participates in regulating the TF-VIIa-PAR2 signal. This signaling process is responsible for regulating the tumor microenvironment in breast cancer, supporting tumor cell migration, and metastasis[13] (Figure 1). It is believed that targeting TF signaling could be particularly important for the treatment of Triple-Negative Breast Cancer (TNBC) and disrupting TF/β1 interactions may help prevent recurrence and improve overall survival[14] (Figure 1).

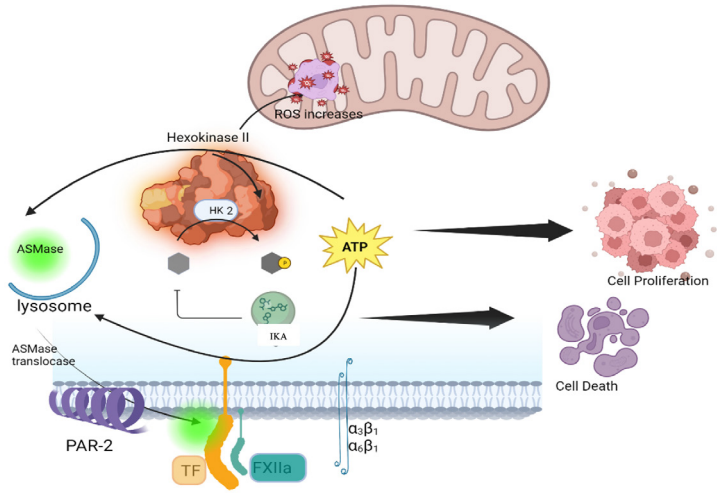

Figure 1. The Hexokinase II enzyme's role in the proliferation of neoplastic cells and its association with TF. HK-2 inhibition leads to cell death and inhibits ATP production. ATP activates tissue factor (TF), initiating its decryption and triggering sphingomyelin hydrolysis through acid sphingomyelinase. In breast cancer, TF, regulated by integrin mechanisms, plays a crucial role in the TF-VIIa-PAR2 signaling pathway, impacting the tumor microenvironment and metastasis.

ROS: Reactive Oxygen Species; PAR-2: Protease-Activated Receptor 2; ASmase: Acid sphingomyelinase

Cancer cells exhibit a distinctive metabolic phenotype wherein they preferentially employ aerobic glycolysis over mitochondrial oxidative phosphorylation for glucose metabolism, in contrast to the normative metabolic patterns observed in normal cells. The sustained elevation in lactate production by cancer cells under oxygen-rich conditions, a phenomenon recognized as aerobic glycolysis, was originally identified more than 75 years ago by Otto Warburg (Figure 2)[15-19]. Since cancer cells require energy to sustain cell growth and proliferation, the high glycolytic activity must provide sufficient ATP levels to meet the demands of rapidly proliferating tumor cells within a hypoxic microenvironment[20,21]. This condition affects various mechanisms in the body abnormally; one of these mechanisms is angiogenesis, which can be triggered directly or indirectly.

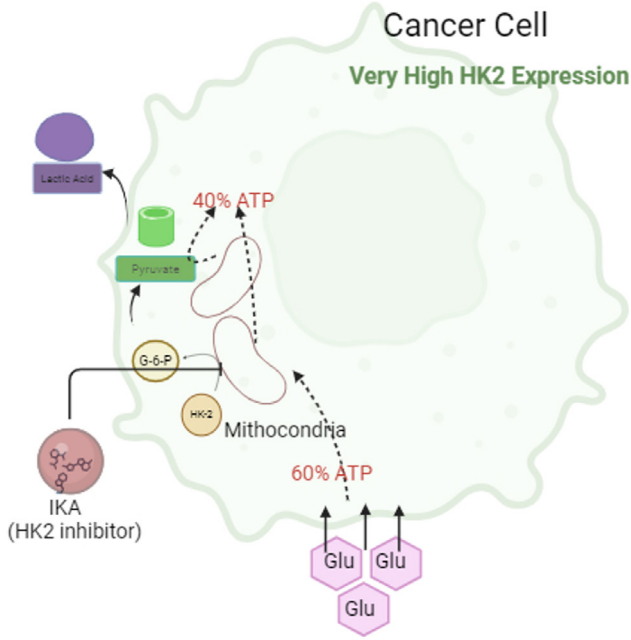

Figure 2. The mechanism by which IKA quickly and efficiently kills cancer cells through rapid energy depletion. While these percentages may vary significantly based on growth rates and cancer types, it's crucial to highlight that both glycolysis and mitochondrial ATP production are essential contributors to fueling cancer growth and facilitating metastasis. Malignant cells' energy source is ATP and about 60% of the ATP is produced by glycolysis and the other 40% by the mitochondria[22].

TF, the third protein of the coagulation system, is found as a transmembrane protein in all tissues. Due to its structural feature, TF possesses a distinct architecture and function compared to other coagulation proteins. Molecularly, it comprises three distinct domains, and it is also known as CD142 due to its signaling transmission effect. Present in the blood in micro-particles at concentrations ranging from 100 to 150 pg/mL, TF initiates the coagulation process by forming the TF/VIIa complex. In an environment containing ionized calcium and the TF/VIIa complex, citrated plasma coagulates within 2-15 seconds[23].

The deficiency of TF is not observed in vivo, given its presence in varying proportions as a structural and functional molecule in all tissues. This is attributed to its involvement in vascularization since the embryogenic period, and its absence has been reported to result in fatality[24-28].

TF serves as the primary precursor within the extrinsic coagulation cascade, exerting a notable influence on the advancement and metastatic phases across various cancer types[22]. Research has shown that TF is involved in cancer invasion, particularly through a unique pathway, independent of the coagulation pathway, via PARs, which mediate intracellular signaling. These findings demonstrate that TF is both an initiator of the coagulation pathway and deeply involved in tumor progression and tumor angiogenesis (Figure 3)[29-32].

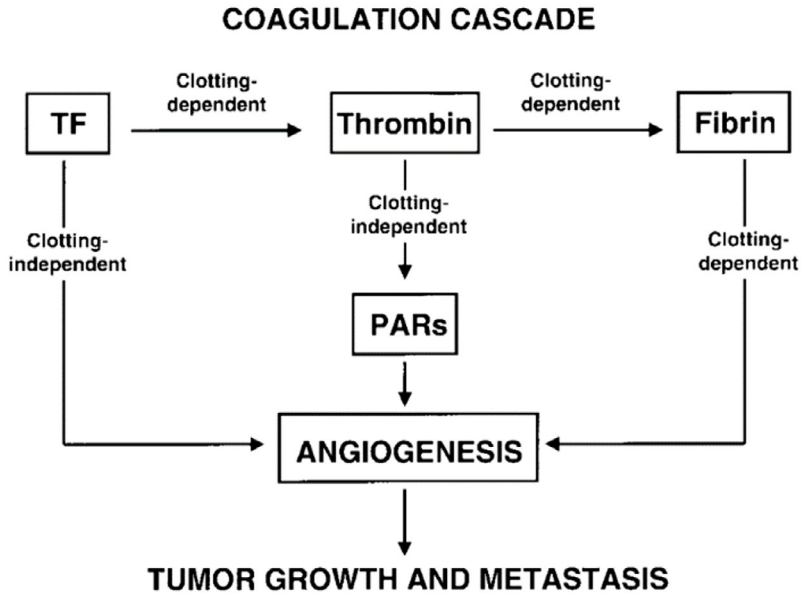

Figure 3. Diagram of the direct and indirect effects of tissue factor on angiogenesis[33-34]

Anticancer antibiotics are a class of chemicals that effectively fight against various types of cancer. These antibiotics exert their anticancer effects by interfering with various cellular processes involved in tumor growth and survival[27]. These groups of medicines are used to prevent and treat the spread of cancer by inhibiting DNA synthesis and repair through other mechanisms. Disrupting their structure by entering between DNA chains and/or causing stabilization of the microtubule, which prevents its depolymerization by binding to β-tubulin, are just some of these mechanisms[35]. Anticancer antibiotics can be classified according to their mechanism of action. These classes can be specified as; Aromatic Polyketides (Anthracyclines), Glycopeptides, Non-ribosomal Peptides, Mitosanes, Enediynes, Indolocarbazoles, Epothilones and Other Agents[36].

In the treatment of breast cancer, anticancer antibiotics are often used as part of chemotherapy protocols. These antibiotics act by preventing the growth and division of cancer cells or by causing the cancer cells to undergo apoptosis in different ways. Certain anticancer antibiotics used to treat breast cancer include anthracyclines (e.g., Doxorubicin), Mitomycin C, and Bleomycin[37,38].

Anthracycline derivatives cause DNA damage by increasing the formation of free radicals that damage breast cancer cells. Ikarugamycin is also an anthracycline derivative, and its hexokinase-2 inhibitory effect was discovered recently and showed therapeutic effect in pancreatic cancer[39,40].

The present study aimed to demonstrate the in vitro bioactivation of IKA, which had been hypothesized to be biologically active in breast cancer treatment manner, in MDA-MB-231 and MCF-7 cell lines. Additionally, the effect of TF and HK-2 inhibitor on cancer cell metabolism was investigated, and a comparison was made with the commonly used LDH-A inhibitor Sodium Oxamate in cancer studies and the chemotherapeutic agent Paclitaxel, widely employed in clinical settings.

METHODOLOGY

Chemicals and reagents

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to determine bioactivity, hepes-buffered DMEM (Dulbecco's Modified Eagle Medium/High Glucose), fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin-streptomycin (10,000 U/mL), L-glutamine, and ethanol for basic cell culture protocols. The Hexokinase 2 inhibitor Ikarugamycin (CAYMAN;15386), to compare therapeutic efficacy; Paclitaxel (Ptx) as a commonly used chemotherapeutic agent and Sodium Oxamate (NaOx), widely used in preclinical studies, (Figure 4) were also utilized.

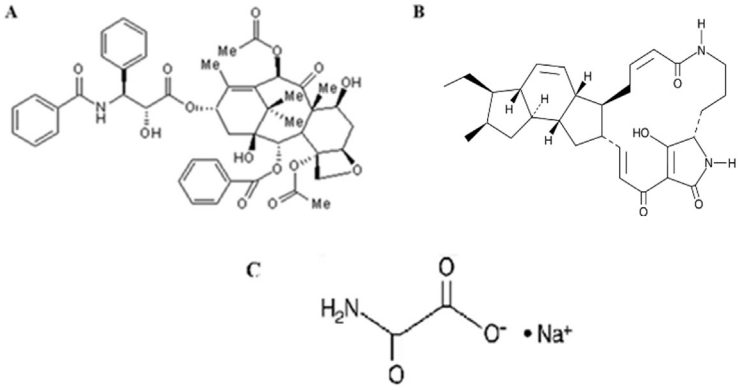

Figure 4. (A) Paclitaxel molecule(2α,4α,5β,7β,10β,13α)-4,10-Bis(acetyloxy)-13-{[(2R,3S)-3-(benzoylamino)-2-hydroxy-3-phenylpropanoyl]oxy}-1,7-dihydroxy-9-oxo-5,20-epoxytax-11-en-2-yl benzoate (B) Ikarugamycin molecule; (2R,3R,7Z,14S,19E)-3-ethyl-2,3,3aS,5aR,5bS,6,10,11,12,13,14,15,20aS,21,21aR,21bR-hexadecahydro-22-hydroxy-2-methyl-14,17-metheno-17H-as-indaceno[3,2-k][1,6]diazacycloheptadecine-9,16,18(1H)-trione (C) Sodium Oxamate molecule (Oxalic Acid monoamide, Oxamic Acid, CAS Number: 565-73-1) | Cayman Chemical)

Cell culture

The MDA-MB-231 and MCF-7 (ATCC) breast cancer cell lines were cultured in Dulbecco's Modified Eagle Medium/High Glucose (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin, and 1% (v/v) L-glutamine. The cells were incubated at 37°C in a humidified incubator with 5% CO_2. Subsequently, upon reaching confluency, the cells were subcultured into T-25 cell culture flasks to maintain uniform growth conditions. Once a sufficient population was obtained, T-75 flasks were used for the experiments, and standardized cell passage techniques were employed by our team[41].

Cell viability assay

Cell seeding was conducted in a 96-well plate at a density of 1.0 x 10^4 cells per well, with each well containing 200 µL of the specified medium and incubated for 24 hours. To determine the IC_{50} values of IKA, a dose range varying from 0.1 to 1000 µM was applied to the cells, followed by incubation for the optimal incubation times (24 and 48 hours). After incubation, 5 mg/ml of MTT was added to each well according to the manufacturer's instructions and incubated for 3 hours[42]. Subsequent to the removal of the medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 100 µL of dimethyl sulfoxide (DMSO) was introduced into each well, and a gentle agitation period of 5 minutes ensued to facilitate reaction completion. Ab-

sorbance readings at 570 nm were subsequently obtained using a plate reader (Synergy|HTX, BioTek, Agilent, USA). Each experiment was performed with 3 replicates. IC_{50} values were calculated by log[IC_{50}] using GraphPad Prism, and the experimental groups were formed accordingly: Control/ IKA (IC_{50})/ NaOx(IC_{50}) Paclitaxel (IC_{50}) (Table 1).

Table 1. Experimental Groups for both cell lines; MDA-MB-231 and MCF-7

	Group I MCF-7	**Group II MDA-MB-231**
Section A	Negative Control	Negative Control
Section B	HK-2 Inhibitor [IKA (IC_{50})]	HK-2 Inhibitor [IKA (IC_{50})]
Section C	LDH-A Inhibitor [NaOx(IC_{50})]	LDH-A Inhibitor [NaOx(IC_{50})]
Section D	Chemotherapeutic [Ptx(IC_{50})]	Chemotherapeutic [Ptx(IC_{50})]

Ptx: Paclitaxel; NaOx: Sodium Oxamate; $IC_{50:}$: the concentration of a particular drug that is needed to inhibit a given biological process to half of the maximum

Preparation of cell lysates

MCF-7 and MDA-MB-231 cell lines were collected by trypsinization and centrifuged at 1500 g for 5 minutes. The cell pellets were washed twice with cold PBS and then homogenized for 5 minutes with the addition of metal beads. Subsequently, a second centrifugation step was performed. The supernatants containing the cell lysates were collected and prepared for further experimentation[41,43,44].

TF activation - PT test / quick method

A total of 100 µL of plasma and 100 µL of cell lysate were incubated in a test tube at 37°C. After a 2-minute incubation, 100 µL of 0.025 M $CaCl_2$ was added to the mixture. The thromboplastic activities were observed, and the time at which the first appearance was noted[43-44]. The clotting time demonstrates an inverse relationship with tissue factor (TF) activity, hence, elongation of clotting time indicates a reduction in TF activity.

Morphological analysis of cells

MDA-MB-231 and MCF-7 cell lines were seeded at a density of 2 x 10^5 and 3 x 10^5 cells, respectively, in 6-well plates. After 24 hours, the cells were treated with the required drugs at predetermined doses and incubated for an additional 24 hours. The control groups were washed twice with PBS and observed under a light microscope for morphology and colonization status.

Statistical analysis

The study employed GraphPad Prism software for data analysis, with variables characterized by their standard deviation. A Student's t-test was employed for the comparison of means exhibiting a normal distribution. Two-way Analysis of Variance (ANOVA) was utilized to compare groups and elucidate differences among subgroups in variables demonstrating variations. P values<0.05 (*) were accepted as significant.

RESULTS and DISCUSSION

The inhibition of HK-2 is known to halt cell proliferation and glycolytic activity, leading to apoptosis in neoplastic cells[45,46]. IKA, which is used to treat bacterial infection primarily, has been found to exhibit in vitro bioactivation. The determined IC_{50} values for the MDA-MB-231 and MCF-7 breast cancer cell lines were 24.1 μM and 19.25 μM, respectively (Figure 5).

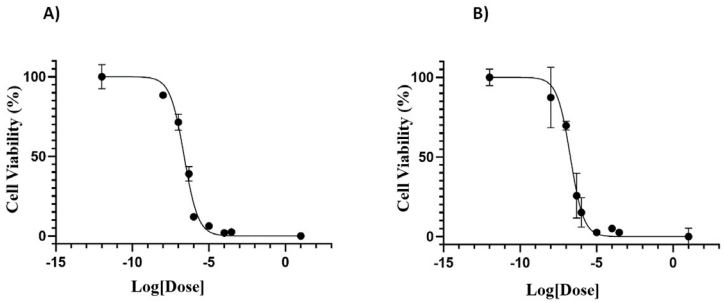

Figure 5. A) MDA-MB-231 cell viability of IKA application analyzed by MTT assay. B) MCF-7 cell viability of IKA application analyzed by MTT assay.

Table 2. Tissue Factor Activation by using Prothrombin Time (PT) Analysis

MCF-7	TF Activation (s)	MDA-MB-231	TF Activation (s)
Control	*51.11 ± 4.08	Control	66.50 ± 3.00
IKA (IC_{50})	*113.28 ± 9.53	IKA (IC_{50})	87.35 ± 6.45
NaOx (IC_{50})	*60.45 ± 5.27	NaOx (IC_{50})	70.37 ± 3.67
Ptx (IC_{50})	*98.52 ± 8.17	Ptx (IC_{50})	*91.00 ± 2.50

In the PT test conducted to determine tissue factor activation, it was found that the clot formation times were longer in both cell lines compared to the group treated with IKA, when compared to Paclitaxel, a commonly used clinical agent. Furthermore, while Paclitaxel exhibited more effective elongation in MCF-7, which is characterized by triple-positive breast carcinoma, IKA prolonged the clot formation

The data were evaluated using ANOVA and Student's t-test statistical analysis methods, and the graphs are represented as mean ± standard deviation and shown in Figure 6 (*p<0.05).

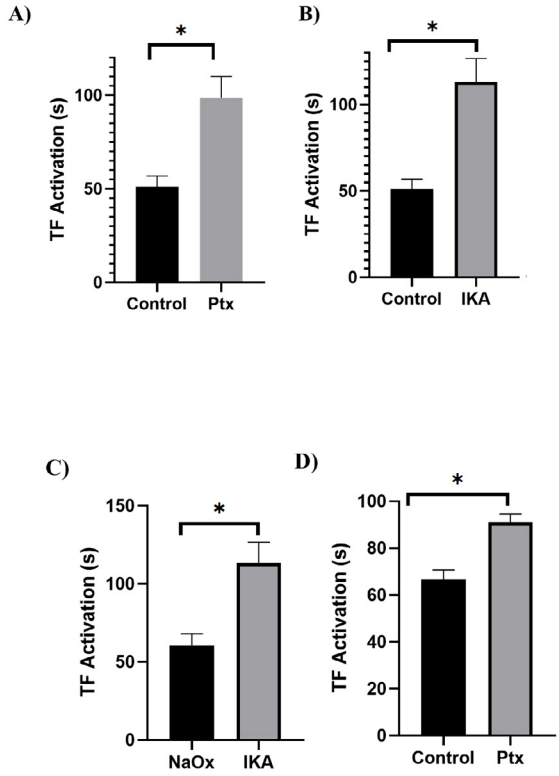

Figure 6. Comparison of MDA-MB-231 and MCF-7 cell lines' sections. A) Comparison of the control group and Ptx treatment in the MCF-7 breast cancer cell line (p<0.05) B) Comparison of the control group and IKA treatment in the MCF-7breast cancer cell line (p<0.05) C) Comparison of the NaOx and IKA treatment in the MCF-7 breast cancer cell line (p<0.05) D) Comparison of the control group and Ptx treatment in the MDA-MB-231 breast cancer cell line (p<0.05)

When all the data were individually examined, for the MDA-MB-231 cell line, except for Ptx, the other two drugs were not statistically significant when compared to the control group. The relationship between IKA and both the control group and NaOx, which is used in clinical treatments, was not found to be statistically significant. The MCF-7 cell line is a cell type with a higher chance of response to treatment and a higher survival rate compared to MDA-MB-231. When compared to the control group, all treatment groups were found to be statistically significant ($p<0.05$).

In the determined groups, predetermined doses of drug molecules were applied to MDA-MB-231 and MCF-7 cell lines. After the optimum incubation period of 24 hours, images were captured using a light microscope (ZEISS, Germany). The obtained images revealed not only a quantitative decrease in cell viability but also qualitative changes in their morphological structures. Additionally, the ability of cancer cells to proliferate by clustering/colonizing, which is a distinctive characteristic of cancer, was observed to be diminished (Figure 7).

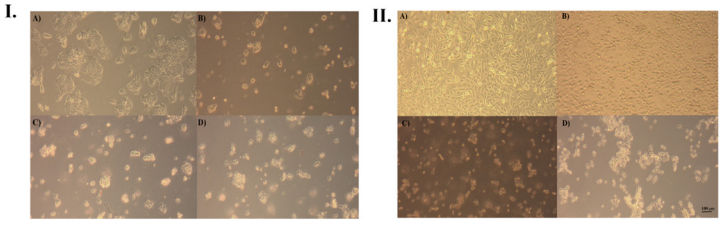

Figure 7. I) Light microscope images of MCF-7 cell line A. Control group B. Ptx (IC_{50}) group C. NaOx (IC_{50}) group D. IKA (IC_{50}) group II) Light microscope images of MDA-MB-231 cell line A. Control group B. Ptx (IC_{50}) group C. NaOx(IC_{50}) group D. IKA (IC_{50}) group (10x).

The inhibition mechanism of Hexokinase 2 (HK-2) was first noticed in cancer treatment in the 1950s[47], leading to research efforts in this area. However, the search for a more potent HK-2 molecule is ongoing, aiming to reduce undesirable effects and improve the patient's quality of life compared to current clinical agents. Sodium Oxamate, especially used in the treatment of breast cancer, ovarian cancer, and small-cell lung cancer, inhibits LDH-A, thereby halting the formation of lactate, the final product of glycolysis in cancer cells. It is also known that Taxol-resistant breast cancer cells can emerge in breast cancer patients. In this context, the unique molecule IKA suggests a potential advantage over Paclitaxel and Sodium Oxamate for cancer treatment[48].

The significance of invasive cancer cells communicating with the tumor microenvironment is that it enables them to overcome environmental challenges, establish themselves, and colonize. In many cases, glucocorticoids are used to treat complications associated with cancer. The progression of breast cancer begins with an increase in stress hormones and glucocorticoid levels, which subsequently activate consecutive glucocorticoid receptors, enhancing cancer colonization and reducing survival rates. This suggests the need for caution when treating cancer patients with glucocorticoid therapy. Beyond this, while cytotoxic chemotherapy has been shown to be effective in breast cancer treatment, it has also exhibited prometastatic effects. Paclitaxel and doxorubicin trigger tumor-derived extracellular vesicle production in chemoresistant breast cancer models. These vesicles facilitate tumor colonization in metastatic sites, particularly in the lungs. Therefore, caution is required when treating cancer patients with glucocorticoid therapy and certain chemotherapy drugs[49,50].

Differences in cell morphology were observed under a light microscope in MDA-MB-231 and MCF-7 cell groups consisting of control and treatment groups, without staining. As shown in Figure 7, pronounced cell retraction and detachment of cells from the cell culture base were observed in all treated cell groups. These changes, which were not observed in control cells and are characteristic of apoptotic cell death, became visible in all treatment groups 24 hours after treatment[51]. Morphological changes were more prominent in experimental groups created with IKA and Ptx drug treatment.

Apart from its role in the coagulation system, tissue factor (TF) plays a crucial role in embryogenesis, wound healing, inflammatory response, tumor growth, metastasis, and angiogenesis. TF is involved in various cellular functions on the surfaces of cells such as endothelial cells and monocytes. Therefore, TF is implicated in the pathophysiology of inflammation, atherogenesis, and carcinogenesis. Studies have shown the co-localization of TF and VEGF in tumor cells. Investigations suggest that abnormal vascular structure is associated with TF, and vascularization during the embryonic period is linked to TF[24-28].

Bozkaya et al. propose that these findings indicate the potential utility of TF and VEGF levels in predicting thromboembolic complications in atherosclerotic diabetic patients.TF and VEGF levels showed significant variations between diabetic and non-diabetic groups ($p<0.001$)[52]. The association between thrombosis and cancer was first noted in 1865 by Professor Armand Trousseau through his observations. Trousseau reported that a significant number of patients with idiopathic venous thromboembolism were subsequently diagnosed with cancer[33].

Another remarkable aspect of our study is that, while the individual effects of HK-2 and TF on tumoral formations were previously known in the cancer mechanism, their direct relationship has not been investigated in the literature before. In one of the studies where TF and HK-2 were examined together, histological analyses were conducted on tissues obtained from autopsies of patients who died due to acute myocardial infarction. The results indicated that the extent of plaque disruption and the expression of TF and HK-2 are crucial vascular factors in the onset of acute myocardial events[53].

Overall, our research sheds light on the potential advantages of IKA in cancer treatment when compared to both Paclitaxel and Sodium Oxamate, and it explores the previously unexplored direct relationship between TF and HK-2 in tumorigenesis, offering novel insights into the mechanisms of vascular factors in disease progression. To further investigate the mechanism of action of breast cancer treatment candidate molecule, IKA, advanced techniques will be employed. The continuation of positive results may lead to preclinical studies, opening the floodgates for in vivo experiments and clinical studies.

STATEMENTS OF ETHICS

This study does not require any ethical permission.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication and dissemination of the information provided herein.

AUTHOR CONTRIBUTIONS

All authors contributed to the design and implementation of the research, to the analysis of the results, and to the writing of the manuscript.

FUNDING SOURCES

The authors declare that there are no funding sources of interest regarding the publication.

ACKNOWLEDGMENTS

This article is based on the doctoral thesis prepared at Istanbul Medipol University, Institute of Health Sciences, Department of Biochemistry.

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