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Breaking the metabolic code in triple-negative breast cancer: Mechanistic insights into glycolytic enzyme inhibitors for suppressing metastasis and tumor growth

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive and metastatic form of breast cancer with limited treatment options. Glycolytic enzymes such as hexokinase 2 (HK2), pyruvate kinase M2 (PKM2), and lactate dehydrogenase A (LDHA) serve as effective targets for metabolic reprogramming in suppressing tumor growth. This study investigates the effects of glycolytic enzyme inhibitors, particularly phytochemicals, on TNBC metastasis and tumor growth using a 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat model. Following TNBC induction, these rats were treated with phytochemical inhibitors: 2-deoxy-*D*-glucose (2-DG), shikonin, oxamate, kaempferol, quercetin, or luteolin. Molecular docking analysis was conducted to characterize the interactions between these compounds and the glycolytic enzymes HK2, PKM2, and LDHA. Glycolytic enzyme inhibition was inferred from the protein expression levels of HK2, PKM2, and LDHA in different treatment groups. Among the compounds tested, kaempferol showed the highest binding affinity toward glycolytic enzymes HK2 and PKM. In addition, shikonin and kaempferol exhibited cell-line-specific antiglycolytic properties by inhibiting glycolytic enzymes HK2, PKM2, and LDHA, thereby suppressing TNBC growth and metastasis. These results suggest that glycolytic enzymes could serve as potential therapeutic targets for improving TNBC treatment, with possible clinical application alongside primary conventional therapies.

Keywords: Shikonin; Kaempferol; Pyruvate kinase M2; Hexokinase 2; Molecular docking; Phytochemicals; Metabolic reprogramming

1. Introduction

Triple-negative breast carcinoma (TNBC) is one of the most aggressive and difficult-to-treat forms of breast cancer. TNBC is defined by the absence of estrogen receptor (ER) and progesterone receptor (PR), along with low or no expression of human epidermal growth factor receptor (HER2). It accounts for approximately 15 – 20% of all breast cancer cases and is associated with an aggressive clinical course, rapid progression, and a high risk of metastasis.[1] Due to the absence of TNBC-specific treatments, there is a gap in targeted strategies capable of managing tumor formation, invasion, and metastatic effectively. One promising strategy focuses on targeting the altered glycolytic pathway of cancer cells, often referred to as the "Warburg effect."[2,3] Like many other cancer types, TNBC undergoes a metabolic shift toward aerobic glycolysis, relying on glycolysis for energy production even under normoxic conditions. This metabolic reprogramming not only satisfies the energy requirements of the proliferating cancer cells but also contributes to oncogenic processes such as apoptosis evasion and increased invasiveness.[2,4] Glycolytic enzymes such as hexokinase-2 (HK2), pyruvate kinase-2 (PKM2), and lactate dehydrogenase A (LDHA) have been identified as key factors of metabolic shift and reported in this study, as well as in other research to support tumor growth, metastasis, and chemoresistance in TNBC.[5]

Overexpression of these glycolytic enzymes in TNBC is associated with poor clinical prognosis and thus may serve as potential therapeutic targets. HK2 catalyzes the rate-limiting first step of glycolysis and has been implicated in promoting tumor cell survival and metastasis.[6] PKM2, the M2 isoform of pyruvate kinase, functions in the final checkpoint of glycolysis and promotes tumor cell growth and metastasis through metabolic reprogramming and enhanced lactate synthesis.[7] Conversion of pyruvate to lactate in the final step of glycolysis by LDHA is necessary to maintain the glycolytic phenotype and has been shown to increase metastatic potential in various types of cancer models.[8] Given the critical role of these glycolytic enzymes in cancer metabolism, their inhibition can be a promising therapeutic approach. Multiple studies have demonstrated that small molecules and natural compounds targeting glycolytic enzymes can effectively suppress tumor growth and metastasis.[6,7] For example, 2-deoxy-*D*-glucose (2-DG), an HK2 inhibitor, has been shown to inhibit tumor development and enhance chemosensitivity in various cancers, including breast cancer, both *in vitro* and *in vivo*.[9,10] Similarly, shikonin, a naturally derived compound, has been confirmed to target PKM2 and effectively inhibit tumor growth and metastasis in multiple tumor models.[10] Oxamate, a competitive LDHA inhibitor, has also been proposed as an agent capable of reducing glycolytic flux and suppressing cancer-promoting activities.[5,8]

However, despite the potential of glycolytic enzyme inhibitors, their clinical application is limited due to challenges such as low bioavailability and side effects. Therefore, additional studies on glycolytic enzyme-inhibiting phytochemicals and their unique combinations can develop more effective methods for TNBC treatment.[6,8] Phytochemicals such as kaempferol, quercetin, and luteolin are well recognized for their favorable pharmacokinetic properties and their modulatory effects on signaling pathways critical to cancer metabolic reprogramming. [9,11] These compounds inhibit glycolytic enzyme activity and metastasis while inducing apoptosis in several breast cancer models.[12,13] The current study aimed to investigate the potential application of glycolytic enzyme inhibitors – synthetic and plant-derived – to arrest TNBC growth and metastasis. Investigation of key glycolytic enzymes (HK2, PKM2, and LDHA) enabled us to assess whether targeting metabolic reprogramming in TNBC cells is a feasible therapeutic approach for this challenging disease.[4,14]

The absence of ER, PR, and HER2 receptors in TNBC limits therapeutic choices, thus increasing the chance of metastasis.[10,13] Recently, several studies have highlighted the implication of changes in cellular metabolism, and more specifically, the Warburg effect, in the development of TNBC. Specifically, tumor cells exhibit a high glycolysis rate even in the presence of oxygen, a phenomenon that accelerates growth and metastasis.[2,3] Inhibition of glycolytic enzymes is considered a promising strategy for the development of novel anticancer therapeutics to suppress tumor growth and metastasis in TNBC.[14] Glycolysis is a central metabolic pathway that converts glucose into pyruvate, producing ATP and essential intermediates for the biosynthesis of daughter cells. In TNBC, this pathway is notably upregulated and contributes to the Warburg effect, wherein glycolysis predominates despite the availability of oxidative phosphorylation.[4,5] This metabolic transition allows TNBC to address the high energy demand for uncontrolled proliferation and metastasis. There are only limited antitumor agents currently available that selectively target mitochondria in tumor cells, and HK-2 inhibitors are among them. Potent inhibitors of HK-2 have been well characterized by the ability to suppress glycolytic flux and tumor growth while inducing cell death in both *in vitro* and *in vivo* models.[8,15] These outcomes imply that the development of HK-2 inhibitors may be a promising approach toward the treatment of TNBC.

Phosphofructokinase-1 (PFK-1) is one of the key rate-limiting enzymes in the glycolysis pathway and is crucial

in managing metabolic shifts for glycolysis in cancer cells.[16] PFK-1 is another potential target that has been thoroughly investigated to prevent tumor growth of glycolysis-dependent cancers, including TNBC. Treatment with PFK-15, a selective inhibitor of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) that indirectly suppresses PFK-1 activity, inhibited glycolysis and tumor growth in TNBC xenograft models.[17] In addition, it was observed that PFK-1 inhibition led to cell cycle arrest and apoptosis, which may inhibit tumor proliferation.[14,17]

A small molecule inhibitor of PKM2, Trans-6-(4-Chlorophenyl)-2-(4-hydroxyphenyl)-6,7-dihydro-5H-thieno[3,2-c]pyridine-5-1, has been found to inhibit glycolysis, tumor growth, and metastasis in TNBC cell lines. Besides, glycolytic enzyme inhibitors may also alter the tumor microenvironment (TME) through suppression of pro-metastatic factors, including lactic acid and extracellular matrix remodeling enzymes.[6,10] Lactate produced during glycolysis is also pro-tumorigenic as it suppresses the immune effector functions of immune cells and stimulates angiogenesis.[5,18] Suppression of glycolysis may decrease lactate production, thereby altering the TME to enhance immune responses against tumor cells. Although encouraging preclinical data were reported, clinical application of glycolytic enzyme inhibitors in the context of TNBC is still challenging. A major concern is the possibility of "aberrations" as glycolysis is essential for normal cellular function.[11,14]

Approaches to enhance the specificity and selectivity of these inhibitors – such as functional drug delivery systems or combination therapies with established therapeutic agents, may help to overcome issues related to off-target effects and therapeutic resistance. In addition, metabolic inhibitors may be bypassed due to the activation of other metabolic pathways or mutations in genes encoding the enzymes. Biomarkers need to be developed for patient classification and to enhance the efficacy of treatment using glycolytic enzyme inhibitors. There are renewed hopes in understanding the molecular basis of the response and identifying potential therapeutic targets through innovative proteomics and metabolomics research.

2. Data and methods

2.1. In silico study

2.1.1. Molecular docking analysis

The interaction studies of several potent compounds: 2-DG, shikonin, oxamate, kaempferol, quercetin, and luteolin with key glycolytic enzymes and their roles in TNBC were predicted using molecular docking. The Structure Data File structures of these compounds were obtained from PubChem and the crystal structures of the recipient proteins of glycolytic enzymes Hexosaminidase B (Protein data bank ID [PDB ID]: 4CPB), HK2 (PDB ID: 3U6O), PKM2 (PDB ID: 3TVF), and LDHA (PDB ID: 2D8T) from the PDB. Actual docking was performed using AutoDock Vina software to obtain the binding modes, affinities, and probable inhibitory effects of the compounds.

2.1.2. Docking procedure

AutoDock Vina was used for docking simulations with the receptor proteins. All co-crystallized ligands, water molecules, heteroatoms, and other impurities were removed. The active sites of the enzymes were determined based on the literature reports, and molecular docking was subsequently performed using a flexible ligand dock and a rigid receptor dock to evaluate the binding profiles. Only the best docking conformations compliant with minimal binding energies were considered for additional analysis.

2.1.3. Bonding interactions

The interactions between the ligands and enzymes were described to identify the type of bonds included in those interactions, such as hydrogen bonds, hydrophobic, hydrophilic, and electrostatic interactions. These interactions were visualized with PyMOL (Schrödinger, USA) and LigPlot (European Bioinformatics Institute, UK).

2.1.4. Absorption, distribution, metabolism, and excretion (ADME) analysis

In this case, the compounds' ADME profiles were analyzed using the SwissADME online tool. Hence, solubility, permeability, lipophilicity, as well as drug-likeness value were assessed to establish the pharmacokinetic characteristics of the identified compounds.

2.1.5. Toxicity analysis

In silico programs Toxtree and ProTox-II were employed to evaluate the toxicity of the selected compounds and assess their carcinogenic activity, mutagenicity, and acute toxicity.

2.2. *In vitro* and *in vivo* study

2.2.1. Study design and animal model

The *in vivo* study was carried out using rat models with 7,12-dimethylbenz(a)anthracene (DMBA)-induced TNBC. In particular, DMBA was administered to the female Sprague-Dawley rats (n = 11) to induce TNBC. These rats were housed under standard laboratory conditions (a 12/12-h light/dark cycle, controlled temperature, and regulated humidity). The experiment aimed to test the ability of some glycolytic enzyme inhibitors in suppressing tumor growth and metastatic potential in the TNBC model.

2.2.2. Animal selection

The *in vivo* study utilized female Sprague-Dawley rats, which are commonly used to establish DMBA-induced TNBC models. The female Sprague–Dawley rats used in experimental research typically fall within the range of 200 – 250 g for young adult rats (6 – 8 weeks old). This range ensures that the rats are mature enough for reliable and reproducible experimental outcomes, especially in cancer models like DMBA-induced TNBC. Consistency in weight helps reduce variability in drug metabolism, immune response, and overall tumor progression.

2.2.3. Preparation and administration of carcinogens

The compound DMBA was dissolved in corn oil (1 mg/mL) and administered to rats via oral gavage (dose: An intraperitoneal dose of 50 mg/kg of azoxymethane was used for tumor induction). Tumor development in DMBA-treated rats was monitored, wherein palpable tumors typically appear within 2 weeks.

2.2.4. Preparation of DMBA

The compound DMBA was dissolved in corn oil to prepare a solution of 1 mg/mL, which was then aliquotted and stored at −20°C. The solution was thawed before the experiment and infused into the rats in the same manner stated above.

2.2.5. Injection protocol

Following tumor induction, treatment with the selected phytochemicals or standard drugs commenced once tumors were first observed and continued daily for 3 – 4 weeks.

2.2.6. Number of rats in each group

To achieve this goal, each experimental group comprised 10 rats to ensure the validity of the study's results.

2.2.7. Compound selection

The selected compounds include glycolytic inhibitors: 2-DG, shikonin, and oxamate (standard drugs); phytochemicals: kaempferol, quercetin, and luteolin. These compounds were selected for their ability to modulate glycolytic enzymes crucial for tumor metabolism and proliferative activities in TNBC.

2.2.8. Experimental design

The rats were divided into eight groups according to the treatments applied (Table 1). The assigned standard drugs and phytochemicals were administered intraperitoneally (IP) once daily for 4 weeks following tumor establishment. The dosages of each standard drug and phytochemical are shown in Figure 1.

Table 1. The grouping of rats and their respective treatment

Groups	Treatments (*n*=10)
G-1	Control (no treatment)
G-2	DMBA (alone)
G-3	DMBA + 2-DG
G-4	DMBA + shikonin
G-5	DMBA + oxamate
G-6	DMBA + kaempferol
G-7	DMBA + quercetin
G-8	DMBA + luteolin

Abbreviations: DMBA: 7,12-dimethylbenz (a) anthracene; 2-DG: 2-deoxy-*D*-glucose.

2.2.9. Flowchart of dosage and route

In order to investigate the effect of standard drugs and phytochemicals on TNBC, different standard drugs and phytochemicals were assigned to each group of 10 rats (Table 1). The flowchart of the experimental design is shown in Figure 1 below.

2.2.10. In vitro assays: Cell proliferation and metastasis

(a) Assay 1: Cell proliferation assay with MTT assay or Cell Counting Kit-8 (CCK-8)
The cell proliferation assay was performed to assess the results of glycolytic enzyme inhibitors, particularly 2-DG, using 3-bromopyruvate (3-BP) as the positive control. The TNBC cell lines, MDA-MB-231 and BT-20 were treated with different concentrations of glycolytic enzyme inhibitors. Cell viability was assessed using MTT assay or CCK-8 assay after 24 h, 48 h, and 72 h of incubation, with the absorbance measured at 570 nm and compared to untreated control cells.

(b) Assay 2: Invasion and migration assay (Transwell assay)
The invasion and migration assay was conducted to evaluate the inhibitory effects of glycolytic enzyme inhibitors, particularly 2-deoxy-D-glucose (2-DG), with 3-bromopyruvate (3-BP) serving as a positive control. The Transwell assay was performed using chambers with and without matrigel coating to differentiate between migration and invasion. TNBC cells were pre-treated with 2-DG or 3-BP and then seeded into the upper chamber. The chemoattractant were used in this study is Fetal Bovine Serum (FBS), was added to the lower chamber. After 24 – 48 h of incubation, cells that had migrated or invaded through the membrane were fixed, stained, and counted under a microscope.

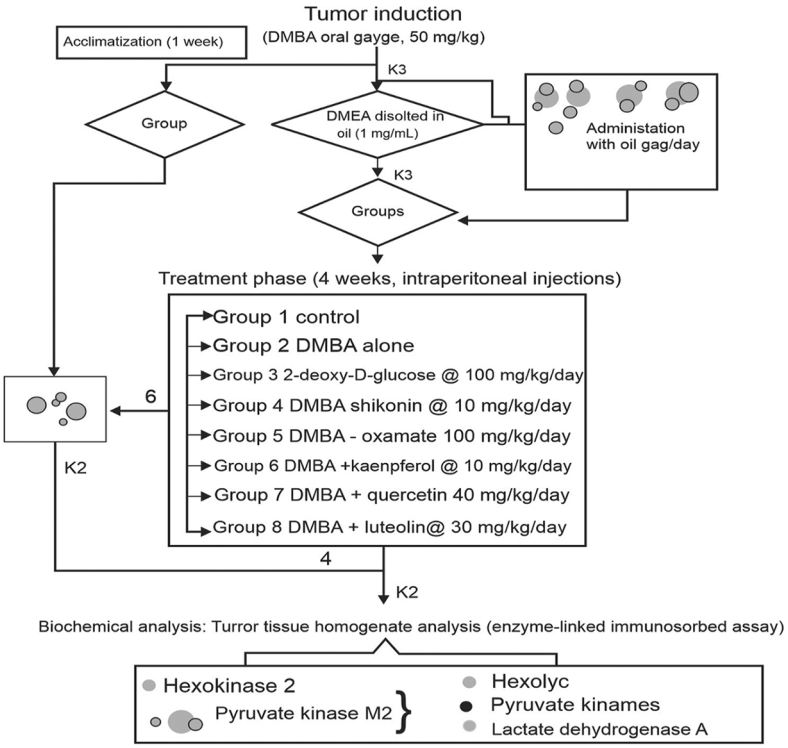

Figure 1. Flowchart of dosage and route of administration in the experimental protocol

2.2.11. In vivo assays: tumor growth and metastasis

(a) Assay 1: Tumor growth inhibition (Xenograft model)
The xenograft model was employed to evaluate the effects of glycolytic enzyme inhibitors on tumor growth in a heterotopic mouse model. MDA-MB-231 and 4T1 cells were subcutaneously injected into nude mice and Balb/c mice, divided into three groups: control (vehicle-treated), 2-deoxy-D-glucose (2-DG)-treated, and 3-bromopyruvate (3-BP)-treated. Tumor volume (TV) was measured using digital calipers every 3 days, and tumor growth curves were plotted. Tumor volume was calculated using the formula:

$$TV = (Length \times Width^2)/2 \quad (I)$$

(b) Assay 2: Metastasis assay (lung and liver metastasis)
Metastasis assay was conducted to evaluate the impact of glycolytic enzyme inhibitors on *in vivo* metastatic progression. MDA-MB-231 cells were injected into the tail vein of nude mice to induce experimental metastasis. Mice were then treated with glycolytic enzyme inhibitors such as 2-DG or 3-BP. After the treatment period, lungs and livers were extracted, and metastatic lesions were assessed by counting the number of visible metastatic nodules. Histological confirmation was performed where necessary.

Statistical analyses were conducted using GraphPad Prism software (Dotmatics, Boston, MA) unless specified otherwise. Data were expressed as mean ± standard deviation (SD). Statistical significance between groups was determined using one-way analysis of variance (ANOVA) or Student's t-test, with $p<0.05$ considered statistically significant.

2.2.12. Biochemical analysis

Analytical grade biochemical reagents and phytocompounds were purchased from Sigma-Aldrich (Merck, Germany). The glycolytic enzymes used in this study were purchased from MyBioSource (USA) (HK2 mU/L - MBS3808021, PKM2 ng/mL - MBS452938, LDHA - MBS269777). A strip plate enzyme-linked immunosorbent assay (ELISA) kit was used for the analysis of glycolytic enzymes in rat TNBC tumor tissue homogenates.

2.2.13. Preparation of tissue homogenate from TNBC rat

Tissue homogenate from rat TNBC tissue was prepared with extreme care and treatment to minimize sample degradation. After obtaining ethical approval and performing appropriate euthanasia of the rat, the TNBC tissue was excised and washed in ice-cold PBS solution to eliminate blood and debris, followed by weight measurement to ensure the weight of the tissues was in the range of 100–200 mg. The tissue was then homogenized in 1 mL homogenization buffer (50 mM Tris-HCl 7.4,

0.15 M NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM ethylene glycol-bis[beta-aminoethyl ether]-N,N,N',N'-tetraacetic acid [EGTA], 1% Triton X-100 with protease and phosphatase inhibitors) per 100 mg of tissue. Homogenization was done on ice with a mechanical homogenizer (Polytron PT 1600 E, Kinematica AG, Switzerland) or pestle and mortar (Corning Inc., USA). The homogenate was then centrifuged at 12,000 × g for 12 min at 4°C using an Eppendorf 5810R centrifuge (Eppendorf AG, Germany), followed by transferring the clear supernatant to a new tube without disturbing the pellet. The homogenate was assayed for total protein content using the BCA or Bradford method. After quantification, these samples were aliquoted to minimize freeze – thaw cycles. Aliquots were stored at –80 °C for long-term use and at –20 °C for short-term use. It was recommended to keep the samples on ice during the procedure to avoid degradation, and the wastes were then disinfected according to institutional biosafety procedures. The aliquoted homogenate was prepared for further analyses, including the measurement of cytokine and protein concentrations, as well as enzyme activity assay.

2.3. Statistical analysis

The data were analyzed using Statistical Product and Service Solutions (SPSS; version 25.0, IBM, United States). ANOVA was performed, and the results were presented as mean ± standard deviation. Duncan multiple range (DMR) test, based on the least significant difference (LSD) approach, was used to compare the means of each group. Tukey's honestly significant difference (HSD) test was used for post hoc analysis between the treatment and control groups. The significance level used in this study was set at $p \leq 0.05$.

3. Results

3.1. Docking analysis

The docking results of the selected phytochemicals and the standard drugs with respect to the glycolytic enzymes HK2, PKM2, and LDHA in a TNBC rat model show varying binding affinity. The phytochemical 2-DG demonstrated relatively strong binding to all three target enzymes, with docking energy equal to −6.5, −7.2, and −6.9 kcal/mol, respectively, for HK2, PKM2, and LDHA. Shikonin demonstrated the highest binding affinities to all three target enzymes, with normalized binding scores of −8.1 for HK2, −7.8 for PKM2, and −8.5 for LDHA. Kaempferol and luteolin demonstrated high affinity for all three targets, with scores of −9.2, −8.5, −8.7 (kaempferol) and −9.0, −8.7, −8.8 (luteolin), suggesting their potential as strong inhibitors. Quercetin also showed considerable binding potential with scores of −8.9 (HK2), −8.6 (PKM2), and −8.4 (LDHA), while oxamate displayed moderate affinity across the targets. The docking scores of these compounds are presented in Table 2.

3.2. Bonding interactions

Table 3 summarizes the bonding interaction of the chosen ligands with the targeted proteins. Shikonin interacted most significantly with LDHA, providing the largest number of five hydrogen bonds and three hydrophobic interactions, as well as a strong salt bridge. Kaempferol showed π-π stacking with both HK2 and PKM2, especially on the residues Tyr215 and Arg520. Luteolin also showed good hydrogen binding and hydrophobic contact, especially with PKM2 and LDHA. Among the tested ligands, 2-DG, oxamate, and quercetin also demonstrated various bonding interactions with the targeted proteins.

3.3. ADME toxicity analysis

The pharmacokinetic profile of the drug candidates in this study is outlined in ADME properties, which is presented in Table 4. Among the analyzed compounds, shikonin exhibited the highest blood–brain barrier penetration potential (classified as moderate), surpassing both kaempferol and quercetin. Duration of stability was defined as half-life (t½) in the current study. Kaempferol was recorded with a t½ of 12 h, while quercetin was 10 h. The toxicity prediction also revealed that all the compounds in this study were of low toxicity except shikonin and quercetin.

3.4. Toxicity class and safety

The PROTOX-II toxicity analysis in Table 5 shows the toxicity of the selected compounds. Compounds such as 2-DG, oxamate, kaempferol, and luteolin belonged to class IV (low toxicity), suggesting a low toxicity level and favorable safety profile. In contrast, shikonin and quercetin were categorized as class III (moderate

Table 2. Docking score (kcal/mol) of phytochemicals and standard drug against hexokinase 2, pyruvate kinase M2, and lactate dehydrogenase A in triple-negative breast cancer rat model

Ligand	Hexokinase 2	Pyruvate kinase M2	Lactate dehydrogenase A
2-Deoxy-*D*-glucose	−6.5	−7.2	−6.9
Shikonin	−8.1	−7.8	−8.5
Oxamate	−7.0	−7.3	−6.8
Kaempferol	−9.2	−8.5	−8.7
Quercetin	−8.9	−8.6	−8.4
Luteolin	−9.0	−8.7	−8.8

Table 3. Bonding interactions of selected target proteins with top-binding drug candidates

Ligand	Target protein	Hydrogen bonds	Hydrophobic interactions	Salt bridges/π-π stacking	Key residues involved
2-Deoxy-*D*-glucose	Hexokinase 2	2	1	None	Thr197, Asp283
	Pyruvate kinase M2	3	2	None	Lys294, Arg330
	Lactate dehydrogenase A	2	1	None	His193, Glu229
Shikonin	Hexokinase 2	3	4	π-π Stacking	Phe385, Arg520
	Pyruvate kinase M2	4	3	None	Glu396, Lys305
	Lactate dehydrogenase A	5	3	Salt bridge	Asp160, His193, Arg169
Oxamate	Hexokinase 2	2	1	None	Thr197, Glu283
	Pyruvate kinase M2	3	1	None	Arg294, His329
	Lactate dehydrogenase A	2	2	None	Glu230, His193
Kaempferol	Hexokinase 2	4	2	π-π Stacking	Tyr215, Arg520
	Pyruvate kinase M2	3	3	Salt bridge	Arg330, Glu396
	Lactate dehydrogenase A	4	2	None	His193, Glu229
Quercetin	Hexokinase 2	3	3	None	Arg383, Thr197
	Pyruvate kinase M2	4	3	π-π Stacking	Tyr105, Glu396
	Lactate dehydrogenase A	3	2	Salt bridge	Asp160, His193
Luteolin	Hexokinase 2	3	4	None	Tyr383, Arg520
	Pyruvate kinase M2	4	2	π-π Stacking	Arg294, Lys305
	Lactate dehydrogenase A	3	3	Salt bridge	His193, Glu229

Table 4. Absorption, distribution, metabolism, and excretion analysis for top-binding drug candidates and standard compounds in a triple-negative breast cancer rat model

Ligand	Absorption (% F)	Blood–brain barrier penetration	CYP450 enzyme inhibition	Half-life ($t_{1/2}$)	LogP	Toxicity prediction
2-Deoxy-*D*-glucose	75	No	No	~2 h	−1.3	Low toxicity
Shikonin	~40	Yes (moderate)	CYP3A4 inhibitor	~8 h	3.2	Moderate toxicity
Oxamate	~50	No	No	~3 h	−0.6	Low toxicity
Kaempferol	~30	Yes (moderate)	CYP2C19 inhibitor	~12 h	2.1	Low toxicity
Quercetin	~20	Yes (high)	CYP3A4, CYP2D6 inhibitor	~10 h	1.8	Low to moderate toxicity
Luteolin	~25	Yes (high)	CYP1A2, CYP3A4 inhibitor	~10 h	2.5	Low toxicity

Table 5. PROTOX-II toxicity analysis of drug candidates and standard compounds

Ligand	LD_{50} (mg/kg)	Toxicity class	Hepatotoxicity	Immunotoxicity	Mutagenicity	Carcinogenicity
2-Deoxy-*D*-glucose	2,000	Class IV (low)	No	No	No	No
Shikonin	500	Class III (moderate)	Yes	Yes	No	Yes
Oxamate	2,000	Class IV (low)	No	No	No	No
Kaempferol	2,000	Class IV (low)	No	No	No	No
Quercetin	300	Class III (moderate)	Yes	Yes	No	Yes
Luteolin	2,000	Class IV (low)	No	No	No	No

toxicity). Specifically, shikonin was associated with hepatotoxicity and immunotoxicity, while quercetin was flagged for moderate levels of immunotoxicity and hepatotoxicity.

3.5. Expression levels of glycolytic enzymes in serum

Glycolytic enzymes are enzymes involved in glycolysis, and the expression levels of HK2, PKM2, and LDHA in the

serum of the DMBA-induced TNBC rat model are shown in Table 6 and Figures 2-4. To compare the expression profiles of HK2, PKM2, and LDHA between various treatment groups, Tukey's HSD test was applied (Table 6). The baseline expressivity of HK2 in the control group was 3.56 ± 0.23 ng/mL. The DMBA-alone group significantly increased HK2 expression to 8.23 ± 2.14 ng/mL ($p \leq 0.05$), suggesting the enhancement of glycolysis in the tumorigenic state. Treatment with 2-DG downregulated HK2 expression to 4.61 ± 1.33 ng/mL ($p \leq 0.05$), suggesting inhibition of HK2 expression. Other treatments, including shikonin (4.07 ± 1.47 ng/mL), oxamate (5.09 ± 1.43 ng/mL), kaempferol (5.27 ± 1.26 ng/mL), quercetin (4.78 ± 0,91 ng/mL), and luteolin (4.28 ± 1.57 ng/mL), were found to inhibit HK2 expression compared to the DMBA-alone group. Shikonin showed the strongest inhibition among all the treatment groups. According to the LSD post hoc test, changes in HK2 expression level between different treatments are significant at $p \leq 0.05$.

Pyruvate kinase M2 expression was elevated to 7.53 ± 1.72 ng/mL from 4.57 ± 0.93 ng/mL in the control group, with $p \leq 0.05$. Treatment with 2-DG, oxamate, and quercetin significantly reduced PKM2 levels to 3.78 ± 0.84 ng/mL, 3.93 ± 0.48 ng/mL, and 4.09 ± 0.28 ng/mL, respectively, indicating PKM2 inactivation ($p \leq 0.05$). Shikonin, kaempferol, and luteolin also decreased PKM2 levels to 5.24 ± 1.29 ng/mL, 5.26 ± 1.21 ng/mL, and 5.04 ± 1.43 ng/mL, respectively, which were slightly above the control level. Overall, 2-DG has the most potent inhibitory potential ($p \leq 0.05$). For LDHA, the DMBA-alone group had significantly higher expression of 9.03 ± 3.33 ng/mL, compared to control at 4.17 ± 1.25 ng/mL ($p \leq 0.05$), indicating an increased metabolic profile of glycolysis. Treatment with 2-DG reduced LDHA expression to 6.44 ± 2.35 ng/mL, while

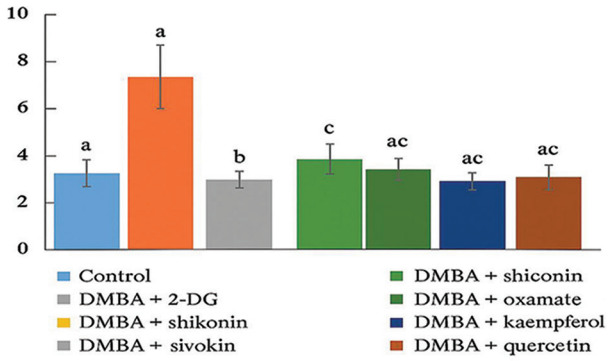

Figure 2. Expression profiles of hexokinase 2 (HK2) in various treatment groups. The control group exhibited HK2 expression level consistent with the normal glycolytic function. In contrast, the DMBA group showed a significant increase in HK2 level. This indicates an enhance of the glycolytic pathway, which is one of the hallmarks of aggressive TNBC. The administration of the glycolytic enzyme inhibitors, including 2-DG, shikonin, and oxamate, led to a significant decrease in HK2 expression level compared with the DMBA-alone group. This decrease is likely to suppress TNBC proliferation and metastasis. Furthermore, moderate but significant inhibition of the HK2 expression level has also been demonstrated by phytochemical agents such as kaempferol, quercetin, and luteolin. This observation implies that these compounds may have anti-glycolytic effects, although weaker than other glycolytic enzyme inhibitors. A statistically significant reduction was observed ($p \leq 0.01$) compared with the DMBA-alone group, with a least significant difference of 0.623. This observation confirms that the decrease in HK2 expression level is significant. The consistent decline in HK2 expression in the treatment group supports targeting the glycolytic pathway as a feasible therapeutic approach for controlling TNBC progression. A statistically significant reduction was observed ($p \leq 0.01$) compared with the DMBA-alone group, with a least significant difference of 0.623. This observation confirms that the decrease in HK2 expression level is significant.

Table 6. Serum hexokinase 2, pyruvate kinase M2, and lactate dehydrogenase A expression levels in DMBA-induced triple-negative breast cancer rat model

Groups	Hexokinase 2 (mU/mL)	Pyruvate kinase M2 (mU/mL)	Lactate dehydrogenase A (mU/mL)
Control (normal)	3.56±0.23^{a}	4.57±0.93^{a}	4.17±1.25^{a}
DMBA (alone)	8.23±2.14^{b}	7.53±1.72^{b}	9.03±3.33^{b}
DMBA+2-DG	4.61±1.33^{c}	3.78±0.84^{c}	6.44±2.35^{c}
DMBA+shikonin	4.07±1.47^{c}	5.24±1.29^{d}	5.42±1.36^{d}
DMBA+oxamate	5.09±1.43^{d}	3.93±0.48^{c}	7.13±2.47^{e}
DMBA+kaempferol	5.27±1.26^{d}	5.26±1.21^{d}	5.01±1.30^{d}
DMBA+quercetin	4.78±0.91^{c}	4.09±0.28^{e}	6.79±0.36^{e}
DMBA+luteolin	4.28±1.57^{c}	5.04±1.43^{d}	4.98±0.26^{a}
p-value (≤0.05)	0.001	0.037	0.021
LSD (≤0.05)	0.623	0.957	1.823

Note: Statistical comparisons between the expression levels of glycolytic enzymes were performed using a one-way ANOVA followed by Tukey's honestly significant difference test, and the results were denoted by superscript lowercase letters. Values with different superscript letters indicate a statistically significant difference between the means, whereas values with the same letter were not significantly different ($p \leq 0.05$).
Abbreviations: 2-DG: 2-deoxy-*D*-glucose; DMBA: 7,12-dimethylbenz (a) anthracene; LSD: least significant difference.

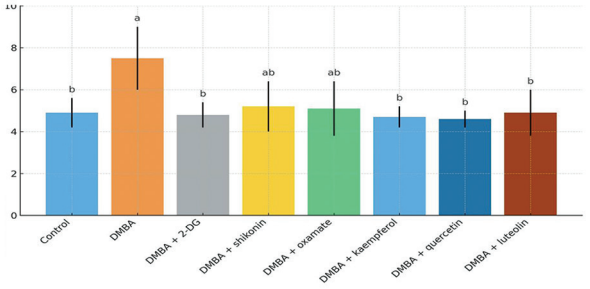

Figure 3. Expression profiles of pyruvate kinase M2 (PKM2) in various treatment groups. The control group has a baseline PKM2 level consistent with normal cellular metabolism of approximately 5 ng/mL. In contrast, the 7,12-dimethylbenz(a)anthracene (DMBA)-alone group shows a significant increase in PKM2 level to approximately 7.5 ng/mL, with a larger standard deviation, suggesting increased variability in tumor glycolytic activity. The increase aligns with the established metabolic alterations in cancer cells, where PKM2 promotes aerobic glycolysis (the Warburg effect), thereby enhancing tumor proliferation. PKM2 level was reduced when the rats were treated with the glycolytic inhibitor. In particular, 2-DG treatment markedly reduced PKM2 expression level to approximately 4 ng/mL, demonstrating strong inhibition of glycolysis. Similarly, shikonin and oxamate reduced PKM2 expression level compared to the DMBA-alone group, although weaker than 2-DG. The results demonstrate their potential in targeting the glycolytic pathway for triple-negative breast cancer (TNBC) treatment. The phytochemicals kaempferol, quercetin, and luteolin treatment showed moderate inhibition of PKM2 expression, reducing it to between 4 – 5 ng/mL. Although not as potent as the glycolytic inhibitor, these compounds suggest a promising therapeutic role in TNBC treatment. A statistically significant reduction was observed ($p \leq 0.037$) compared with the DMBA-alone group, with a least significant difference of 0.957. This observation confirms that the decrease in PKM2 expression level is significant, with low variability in treatment groups. This result suggests that suppression of PKM2 expression level can be a feasible therapeutic strategy to prevent TNBC proliferation.

shikonin and kaempferol reduced the expression to 5.42 ± 1.36 ng/mL and 5.01 ± 1.30 ng/mL, respectively, with a similar significant difference compared to the DMBA-alone group ($p \leq 0.05$). The quercetin- and oxamate-treated group showed LDHA expression levels of 6.79 ± 0.36 ng/mL and 7.13 ± 2.47 ng/mL, respectively. Despite being only slightly lower than the DMBA-alone group, the results are still significantly different, with $p \leq 0.05$. Specifically, the luteolin-treated group had the lowest LDHA expression of 4.98 ± 0.26 ng/mL ($p \leq 0.05$), indicating the strongest suppression on LDHA expression.

Altogether, glycolytic enzyme inhibitors 2-DG, shikonin, oxamate, kaempferol, quercetin, and luteolin downregulated the protein levels of HK2, PKM2, and LDHA in the DMBA-induced TNBC rat model. These findings suggest that glycolytic inhibitors can be potential therapeutic agents in preventing metastasis and tumor development in TNBC. The effectiveness of these inhibitors varies, and the most significant impact is observed in

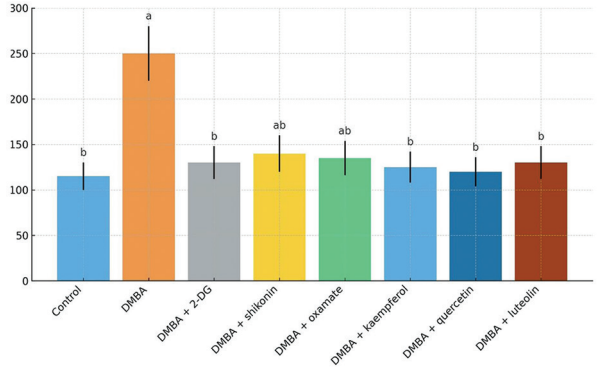

Figure 4. Expression profiles of lactate dehydrogenase A (LDHA) in various treatment groups. A baseline level of approximately 5 ng/mL of LDHA is detected in the control group, aligned with normal lactate production. In contrast, the 7,12-dimethylbenz(a)anthracene (DMBA)-alone group shows a significant increase in LDHA expression level to around 9 ng/mL, together with a larger standard deviation indicating high variability of enhanced glycolysis (Warburg effect) in triple-negative breast cancer (TNBC) cells. The increase in LDHA activity demonstrates the aggressive metabolic reprogramming observed in DMBA-induced carcinogenesis, where lactate production supports tumor progression and immune escape. A significant decrease in LDHA expression level was observed in rats treated with the glycolytic inhibitor. Specifically, 2-DG significantly reduced LDHA expression level to 6 ng/mL, indicating a strong inhibition of lactate production by the glycolytic pathway. Similarly, shikonin and oxamate showed a significant decrease in LDHA expression levels compared to the DMBA-alone group, demonstrating their potential in reducing lactate accumulation and possibly disrupting tumor microenvironment acidification. Treatment with kaempferol, quercetin, and luteolin resulted in a moderate yet significant reduction in LDHA expression levels, ranging between 6–7 ng/mL, which supported their potential role as metabolic modulators. Interestingly, the reduction of LDHA expression level by luteolin appears to be stronger than expected, suggesting its capability in disrupting the lactate-driven oncogenic signaling pathway more effectively. A statistically significant reduction was observed ($p \leq 0.021$) compared with the DMBA-alone group, with a least significant difference of 1.823. This observation confirms that the decrease in LDHA expression level is significant, with a relatively low standard deviation in the treatment group. Taken together, these findings suggest that targeting LDHA with glycolytic inhibitors or phytochemicals could offer a promising therapeutic strategy for limiting TNBC progression by disrupting the metabolic flexibility of cancer cells.

shikonin for the tested enzymes. Subsequent researches are needed to elucidate the mechanisms by which these inhibitors affect glycolytic enzyme activities.

3.6. Tumor size of DMBA-induced TNBC rat model

The *in vivo* results reveal a significant reduction in tumor size across all treatment groups compared to the DMBA-alone group, demonstrating the tumor-suppressing potential of glycolytic enzyme inhibitors and phytochemicals in DMBA-induced TNBC rat models (Table 7 and Figure 5). No tumor was observed in the control group, confirming the low rate of spontaneous tumor development. The

DMBA-alone group had a significantly larger tumor size of 284.19 ± 14.33 mm^3, which confirmed the effectiveness of DMBA-induction in the animal model. Treatment with 2-DG reduced the size of the tumor to 146.67 ± 11.58 mm^3, demonstrating a significant inhibitory effect of TNBC. Shikonin treatment resulted in a further reduction of tumor size down to 136.28 ± 13.04 mm^3, whereas oxamate exhibited the strongest anti-tumor activity, reducing the tumor size to 127.47 ± 8.54 mm^3.

The average tumor size of kaempferol-treated rats was 163.59 ± 12.05 mm^3, while quercetin and luteolin resulted in comparable anti-tumor effects, with tumor sizes of 169.70 ± 16.70 mm^3 and 154.19 ± 21.85 mm^3, respectively. Although these phytochemicals are less effective than the standard glycolytic inhibitor, their ability to reduce tumor size suggests a promising therapeutic approach through modulation of the glycolytic pathway. The observed differences in tumor size among the experimental groups are statistically significant, with a *p*-value of 0.016 and an LSD of 8.19, indicating the reliability and consistency of the results. The results indicate that standard glycolytic enzyme inhibitors as well as naturally occurring phytochemicals can effectively diminish tumor growth in a DMBA-induced TNBC model. These results suggest a feasible therapeutic strategy targeting the glycolytic pathway for TNBC and provide a novel approach to regulate cancer metabolism during aggressive breast cancer treatment.

3.7. *In vitro* and *in vivo* study

The efficacy of glycolytic enzymatic inhibitors in reducing TNBC proliferation, invasion, migration, and metastasis was demonstrated *in vivo* and *in vitro*. The MTT assay showed a significant decrease in cell viability after 2-DG or 3-BP treatment, where higher concentrations (5 mM and 5 μM, respectively) resulted in a stronger inhibitory effect (Table 8). The Transwell assay reported a significant decrease in cell migration, with 2-DG at 5 mM and 3-BP at 5 μM, indicating their ability in suppressing TNBC metastasis (Table 9). In a xenograft model, TV was significantly reduced by 2-DG (200 mg/kg), achieving a 58 % decrease, and by 3-BP (20 mg/kg), with a 50 % decrease compared to the vehicle, highlighting their *in vivo* anti-tumor potential (Table 10). The *in vivo* metastasis assay results demonstrated a significant reduction in the number of metastatic lesions in both lung and liver tissues across treatment groups compared to control (Table 11). Notably, higher doses of glycolytic inhibitors 2-DG and 3-BP showed a marked decrease in metastatic burden, with p-values indicating statistical significance. Treatment with 2-DG (200 mg/kg) showed a remarkable 76 % reduction in lung lesions and a

Table 7. Tumor size, lactate production, and ATP production in DMBA-induced triple-negative breast cancer rat model

Groups	Tumor size (mm^3)	Lactate production (mmol/L)	ATP production (μmol/g tissue)
Control (normal)	0.00±0.00^g	2.10±0.23^g	0.95±0.08^g
DMBA (alone)	284.19±14.33^a	7.82±0.45^a	3.85±0.22^a
DMBA+2-DG	146.67±11.58^b	4.90±0.39^b	2.10±0.18^b
DMBA+shikonin	136.28±13.04^c	4.50±0.30^c	1.90±0.15^c
DMBA+oxamate	127.47±8.54^d	4.20±0.28^d	1.70±0.12^d
DMBA+kaempferol	163.59±12.05^e	5.50±0.35^e	2.40±0.20^e
DMBA+quercetin	169.70±16.70^e	5.70±0.40^e	2.50±0.22^e
DMBA+luteolin	154.19±21.85^f	5.30±0.38^f	2.30±0.19^f
p-value (≤0.05)	0.016	0.018	0.021
LSD (≤0.05)	8.191	0.549	0.253

Note: Statistical comparisons between the tumor sizes, lactate productions, and ATP productions were performed using a one-way ANOVA followed by Tukey's honestly significant difference test, and the results were denoted by superscript lowercase letters. Values with different superscript letters indicate a statistically significant difference between the means, whereas values with the same letter were not significantly different ($p \leq 0.05$).
Abbreviations: 2-DG: 2-deoxy-*D*-glucose; DMBA: 7,12-dimethylbenz (a) anthracene; LSD: least significant difference.

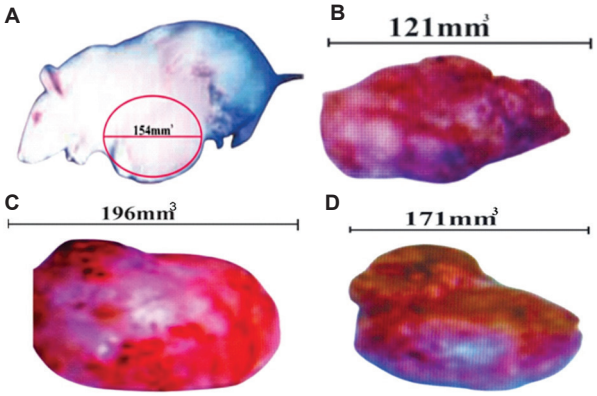

Figure 5. The size of the tumors observed in different treatment groups. (A) The 7,12-dimethylbenz(a)anthracene (DMBA)-alone group developed significantly larger tumors, indicating aggressive tumor growth. (B) The administration of glycolysis inhibitors led to a substantial reduction in tumor size, emphasizing the importance of glycolysis in triple-negative breast cancer (TNBC) progression. Treatment with shikonin, oxamate, kaempferol, quercetin, or luteolin significantly reduced tumor size, with varying degrees of effectiveness compared to the DMBA-alone group (C and D). These results suggest that phytochemicals and glycolytic enzyme inhibitors effectively hinder TNBC growth by disrupting glycolytic pathways crucial for cancer cell survival and proliferation. The observed reductions in tumor size indicate the potential of these compounds in targeting the glycolytic pathway in TNBC, supporting their potential as therapeutic agents in combination with existing treatments for TNBC.

67 % reduction in liver lesions. These findings suggest that glycolytic enzyme inhibitors effectively suppress TNBC growth and metastasis by targeting glycolytic pathways, offering promising therapeutic strategies in preventing TNBC progression. Results of Tukey's HSD test for PKM2, LDHA, and HK2 expression across treatment groups are presented in Tables 12-14, respectively.

The results of lactate production in the experimental groups show significant differences, highlighting the role of glycolytic enzyme inhibitors in tumor progression in the DMBA-induced TNBC rat model (Table 7). The control group (normal), with an average value of 2.10 ± 0.23 mmol/L, is consistent with normal glycolytic activity. In contrast, the DMBA-alone group shows a significant increase in lactate production (7.82 ± 0.45 mmol/L), which is characteristic of aggressive tumor progression. The treatment of glycolytic inhibitors led to a significant decrease in lactate production compared to the DMBA-alone group. In particular, oxamate had the strongest inhibitory effect, reducing lactate production to 4.20 ± 0.28 mmol/L, followed by shikonin and 2-DG (4.50 ± 0.30 mmol/L and 4.90 ± 0.39 mmol/L, respectively). This reduction suggests inhibition of the glycolytic enzymes, thereby diminishing the Warburg effect on TNBC. Although phytochemical treatment groups show lower inhibition than glycolytic inhibitor treatment groups, the reductions in lactate production are significant as well. The lactate productions of kaempferol, quercetin, and luteolin treatments were 5.50 ± 0.35 mmol/L, 5.70 ± 0.40 mmol/L, and 5.30 ± 0.38 mmol/L, respectively. This result highlights the potential of these compounds in tumor treatment, although to a lesser extent. The statistical study confirms the significance of these differences, with a *p*-value of 0.018 and an LSD of 0.549. Together, these results suggest that combining standard drugs with phytochemicals may provide a promising therapeutic approach to controlling TNBC progression by disrupting its glycolytic pathway.

The ATP production levels (μmol/g tissue) show significant metabolic changes in the treatment groups. The control group showed a final ATP production of 0.95 ± 0.08 μmol/g tissue, corresponding to the normal glycolytic process. The DMBA-alone group exhibited a significant increase in ATP production, with a value of 3.85 ±.22 μmol/g tissue ($p \leq 0.05$), indicating the upregulation of the glycolytic pathway associated with the TNBC progression. Among the inhibitors, oxamate showed the greatest reduction of ATP levels at 1.70 ± 0.12 μmol/g tissue, followed by shikonin (1.70 ± 0.12 μmol/g tissue) and 2-DG (2.10 ± 0.18 μmol/g tissue). These results highlight the potent suppressive effect

Table 8. *In vitro* results of cell proliferation assay

Treatment group	Cell viability (%)	Mean±SD	*p*-value (vs. control)
Control (DMSO)	100	100±5.23	-
2-DG (1 mM)	65	65±3.14	0.013
2-DG (5 mM)	45	45±4.66	0.001
3-BP (1 μM)	60	60±6.91	0.038
3-BP (5 μM)	40	40±3.18	0.001

Abbreviations: 2-DG: 2-deoxy-*D*-glucose; 3-BP: 3-bromopyruvate; DMSO: dimethyl sulfoxide; SD: Standard deviation.

Table 9. *In vitro* results of invasion and migration assay (transwell)

Treatment group	Number of cells migrated (mean±SD)	*p*-value (vs. control)
Control (DMSO)	500±30.88	-
2-DG (1 mM)	300±25.14	0.023
2-DG (5 mM)	200±15.16	0.001
3-BP (1 μM)	350±28.24	0.052
3-BP (5 μM)	150±10.44	0.001

Abbreviations: 2-DG: 2-deoxy-*D*-glucose; 3-BP: 3-bromopyruvate; DMSO: dimethyl sulfoxide; SD: Standard deviation.

Table 10. *In vivo* results of tumor growth inhibition (xenograft model)

Treatment group	Tumor volume (mm^3)	Mean±SD	*p*-value (vs. control)
Control (vehicle)	1200±100	1200±100	-
2-DG (100 mg/kg)	800±80	800±80.23	0.011
2-DG (200 mg/kg)	500±50	500±50.56	0.001
3-BP (10 mg/kg)	850±90	850±90.15	0.032
3-BP (20 mg/kg)	600±70	600±70.22	0.002

Abbreviations: 2-DG: 2-deoxy-*D*-glucose; 3-BP: 3-bromopyruvate; SD: Standard deviation.

Table 11. *In vivo* results of metastasis assay (lung and liver)

Treatment group	Number of metastatic lesions (lung)	Number of metastatic lesions (liver)	*p*-value (vs. control)
Control (Vehicle)	50±5.29	30±3.44	-
2-DG (100 mg/kg)	25±3.66	18±2.44	0.019
2-DG (200 mg/kg)	12±2.17	10±1.5	0.001
3-BP (10 mg/kg)	35±4.33	20±3.77	0.024
3-BP (20 mg/kg)	18±327	12±2.44	0.001

Abbreviations: 2-DG: 2-deoxy-*D*-glucose; 3-BP: 3-bromopyruvate.

Table 12. Tukey's honest significant difference test results for pyruvate kinase M2 expression levels across different treatment groups in DMBA-induced triple-negative breast cancer rat model

Comparison	Mean difference	Standard error	*p*-value	Significance
Control (normal) versus DMBA (alone)	−2.96	1.07	0.003	*
Control (normal) versus DMBA+2-DG	0.79	1.09	0.645	
Control (normal) versus DMBA+shikonin	−0.67	1.11	0.512	
Control (normal) versus DMBA+oxamate	0.64	1.09	0.777	
Control (normal) versus DMBA+kaempferol	−0.02	1.09	1.000	
Control (normal) versus DMBA+quercetin	0.48	1.09	0.927	
Control (normal) versus DMBA+luteolin	−0.53	1.09	0.853	
DMBA (alone) versus DMBA+2-DG	3.75	1.23	0.003	*
DMBA (alone) versus DMBA+shikonin	2.29	1.25	0.081	
DMBA (alone) versus DMBA+oxamate	3.30	1.23	0.008	*
DMBA (alone) versus DMBA+kaempferol	2.94	1.23	0.016	*
DMBA (alone) versus DMBA+quercetin	3.44	1.23	0.003	*
DMBA (alone) versus DMBA+luteolin	2.49	1.23	0.049	*
DMBA+2-DG versus DMBA+shikonin	−1.46	1.29	0.406	
DMBA+2-DG versus DMBA+oxamate	−0.45	1.29	0.970	
DMBA+2-DG versus DMBA+kaempferol	0.81	1.29	0.728	
DMBA+2-DG versus DMBA+quercetin	−0.31	1.29	0.993	
DMBA+2-DG versus DMBA+luteolin	−1.27	1.29	0.457	
DMBA+shikonin versus DMBA+oxamate	1.01	1.36	0.884	
DMBA+shikonin versus DMBA+kaempferol	2.27	1.36	0.126	
DMBA+shikonin versus DMBA+quercetin	1.15	1.36	0.857	
DMBA+shikonin versus DMBA+luteolin	−0.19	1.36	1.000	
DMBA+oxamate versus DMBA+kaempferol	1.26	1.36	0.706	
DMBA+oxamate versus DMBA+quercetin	0.90	1.36	0.929	
DMBA+oxamate versus DMBA+luteolin	−0.48	1.36	1.000	
DMBA+kaempferol versus DMBA+quercetin	−0.36	1.36	0.998	
DMBA+kaempferol versus DMBA+luteolin	−1.52	1.36	0.517	
DMBA+quercetin versus DMBA+luteolin	−1.16	1.36	0.852	

Note: * indicates significant difference observed in compared groups ($p \leq 0.05$).
Abbreviations: 2-DG: 2-deoxy-*D*-glucose; DMBA: 7,12-dimethylbenz (a) anthracene.

of these compounds on the glycolytic flux. Compared with the DMBA-alone group, the phytochemical-treated groups showed a significant but relatively moderate decrease in ATP production. Kaempferol and quercetin treatment resulted in ATP levels of 2.40 ± 0.20 and 2.50 ± 0.22 μmol/g tissue, respectively, while luteolin treatment resulted in slightly reduced ATP levels at 2.30 ± 0.19 μmol/g tissue. The statistical analysis confirms the significance of these results with a *p*-value of 0.021 and an LSD value of 0.253, indicating that the observed differences in ATP production across the treatment group are highly significant and not due to random variation. These findings strongly suggest that glycolytic inhibition through standard drugs and organic phytochemicals can effectively suppress ATP overproduction, which is a hallmark of aggressive tumor progression in TNBC. Therefore, these findings offer promising insights into controlling TNBC progression and metastasis.

4. Discussion

TNBC is one of the most life-threatening and difficult-to-treat subtypes of breast cancer, mainly due to the absence of specific therapy and the highly invasive nature of the disease.[18,19] Recent studies have emphasized the role of metabolic shift in cancer cells, particularly the Warburg effect – a tendency of cancer cells to rely on glycolysis even in the presence of oxygen. This change in

Table 13. Tukey's honest significant difference test results for lactate dehydrogenase A expression levels across different treatment groups in DMBA-induced triple-negative breast cancer rat model

Comparison	Mean difference	Standard error	*p*-value	significance
Control (normal) versus DMBA (alone)	−4.87	1.35	0.004	*
Control (normal) versus DMBA+2-DG	−2.27	1.35	0.256	
Control (normal) versus DMBA+shikonin	−0.92	1.42	0.938	
Control (normal) versus DMBA+oxamate	−2.96	1.35	0.050	*
Control (normal) versus DMBA+kaempferol	−1.83	1.35	0.421	
Control (normal) versus DMBA+quercetin	−0.83	1.35	0.957	
Control (normal) versus DMBA+luteolin	−0.18	1.35	1.000	
DMBA (alone) versus DMBA+2-DG	2.60	1.56	0.251	
DMBA (alone) versus DMBA+shikonin	3.95	1.58	0.021	*
DMBA (alone) versus DMBA+oxamate	1.91	1.56	0.474	
DMBA (alone) versus DMBA+kaempferol	3.04	1.56	0.076	
DMBA (alone) versus DMBA+quercetin	4.04	1.56	0.014	*
DMBA (alone) versus DMBA+luteolin	4.69	1.56	0.004	*
DMBA+2-DG versus DMBA+shikonin	1.35	1.70	0.973	
DMBA+2-DG versus DMBA+oxamate	−0.69	1.70	0.991	
DMBA+2-DG versus DMBA+kaempferol	0.44	1.70	0.999	
DMBA+2-DG versus DMBA+quercetin	1.44	1.70	0.912	
DMBA+2-DG versus DMBA+luteolin	2.09	1.70	0.582	
DMBA+shikonin versus DMBA+oxamate	−2.04	1.83	0.748	
DMBA+shikonin versus DMBA+kaempferol	−0.91	1.83	0.999	
DMBA+shikonin versus DMBA+quercetin	0.09	1.83	1.000	
DMBA+shikonin versus DMBA+luteolin	0.74	1.83	0.999	
DMBA+oxamate versus DMBA+kaempferol	1.13	1.83	0.927	
DMBA+oxamate versus DMBA+quercetin	2.13	1.83	0.726	
DMBA+oxamate versus DMBA+luteolin	2.78	1.83	0.554	
DMBA+kaempferol versus DMBA+quercetin	1.00	1.83	0.999	
DMBA+kaempferol versus DMBA+luteolin	1.65	1.83	0.946	
DMBA+quercetin versus DMBA+luteolin	0.65	1.83	0.999	

Note: *Indicates significant difference observed in compared groups ($p \leq 0.05$).
Abbreviations: 2-DG: 2-deoxy-*D*-glucose; DMBA: 7,12-dimethylbenz (a) anthracene.

metabolism is crucial for tumor progression, metastasis, and treatment resistance.[19] This study aimed to investigate how glycolytic enzyme inhibitors selectively target and deactivate enzymes, including HK2, PKM2, and LDHA that are crucial to cell metabolic pathways and cancerous phenotypes in TNBC.[20]

The results of this study illustrate that the inhibition of these glycolytic enzymes significantly affects tumor growth and metastasis in the DMBA-induced TNBC rat model. In particular, treatment with inhibitors 2-DG, shikonin, oxamate, kaempferol, quercetin, and luteolin suppressed the expression of the essential glycolytic enzymes such as HK2, PKM2, and LDHA, which were overexpressed in the context of TNBC cells.[7,20] HK2 has been identified as an important marker that initiates glycolysis and is overexpressed in TNBC.[12,18] Thus, comparable to hexokinase I (HK1), HK2 also accelerates glucose metabolism, resulting in the production of ATP and lactate even under aerobic conditions. This metabolic shift results in enhanced cell survival, proliferation, and metastasis, which are the features of most cancer cells.[21] Our findings are in concordance with previous research, demonstrating that both 2-DG and shikonin have strong potential to inhibit HK2 expression.[9]

Similarly, PKM2 is overexpressed in cancer cells to support aerobic glycolysis.[7,22] While PKM2 is involved in

Table 14. Tukey's honest significant difference test results for hexokinase 2 expression levels across different treatment groups in DMBA-induced triple-negative breast cancer rat model

Comparison	Mean difference	Standard error	*p*-value	Significance
Control (normal) versus DMBA (alone)	−4.67	1.22	0.001	*
Control (normal) versus DMBA+2-DG	−1.05	1.22	0.593	
Control (normal) versus DMBA+shikonin	−0.51	1.29	0.990	
Control (normal) versus DMBA+oxamate	−1.53	1.22	0.184	
Control (normal) versus DMBA+kaempferol	−1.71	1.22	0.134	
Control (normal) versus DMBA+quercetin	−0.99	1.22	0.594	
Control (normal) versus DMBA+luteolin	−0.72	1.22	0.854	
DMBA (alone) versus DMBA+2-DG	3.62	1.46	0.026	*
DMBA (alone) versus DMBA+shikonin	4.16	1.48	0.012	*
DMBA (alone) versus DMBA+oxamate	3.14	1.46	0.070	
DMBA (alone) versus DMBA+kaempferol	2.96	1.46	0.095	
DMBA (alone) versus DMBA+quercetin	3.68	1.46	0.025	*
DMBA (alone) versus DMBA+luteolin	3.95	1.46	0.016	*
DMBA+2-DG versus DMBA+shikonin	0.54	1.57	0.994	
DMBA+2-DG versus DMBA+oxamate	−0.48	1.57	0.999	
DMBA+2-DG versus DMBA+kaempferol	−0.66	1.57	0.993	
DMBA+2-DG versus DMBA+quercetin	0.06	1.57	1.000	
DMBA+2-DG versus DMBA+luteolin	0.33	1.57	0.997	
DMBA+shikonin versus DMBA+oxamate	−1.02	1.65	0.895	
DMBA+shikonin versus DMBA+kaempferol	−1.20	1.65	0.866	
DMBA+shikonin versus DMBA+quercetin	−0.48	1.65	0.999	
DMBA+shikonin versus DMBA+luteolin	−0.21	1.65	1.000	
DMBA+oxamate versus DMBA+kaempferol	−0.18	1.65	1.000	
DMBA+oxamate versus DMBA+quercetin	0.54	1.65	0.991	
DMBA+oxamate versus DMBA+luteolin	0.81	1.65	0.986	
DMBA+kaempferol versus DMBA+quercetin	0.72	1.65	0.993	
DMBA+kaempferol versus DMBA+luteolin	0.99	1.65	0.986	
DMBA+quercetin versus DMBA+luteolin	0.27	1.65	1.000	

Note: *Indicates significant difference observed in compared groups ($p \leq 0.05$).
Abbreviations: 2-DG: 2-deoxy-*D*-glucose; DMBA: 7,12-dimethylbenz (a) anthracene.

tumor progression, it assists in the conversion of pyruvate into lactate, thereby supporting the survival and metastasis of cancer cells.[23] This study shows that 2-DG and oxamate significantly suppress PKM2 expression, which is consistent with previous findings that the inhibition of PKM2 blocks the glycolysis pathway and suppresses tumor growth.[24] LDHA is the final enzyme in the anaerobic glycolysis pathway, which catalyzes the production of lactate from pyruvate, forming NAD+ to support the glycolysis process. LDHA has also been identified to upregulate in several cancers, including TNBC, and is linked with adverse outcomes.[22] This study shows that shikonin and kaempferol downregulate LDHA expression, which supports the idea that both compounds can be potential agents for metabolic intervention in TNBC treatment. The findings in this study align with recent studies reporting that LDHA suppression inhibits tumorigenesis, metastasis, and chemotherapy resistance in TNBC models.[25]

Our results support the hypothesis that glycolytic enzyme inhibitors can enhance treatment efficacy in combination with traditional therapies. Specifically, shikonin substantially suppressed various glycolytic enzymes, which led to the inhibition of cancer cell proliferation. These findings are in line with previous research reporting strong tumor-suppressive effects of shikonin across TNBC and other cancers.[20,23] However, the combination of glycolytic inhibitors with other current chemotherapeutic

drugs may complement their therapeutic effects in TNBC treatment. Additionally, phytochemical compounds such as kaempferol, quercetin, and luteolin have been shown to exhibit anticancer activity in various cancer models by altering glycolytic pathways and reducing the expression of glycolytic enzymes.[26] Therefore, the suppression of enzyme expression from the treatments in this study underlines the potential of glycolytic pathways in TNBC as a therapeutic target. Moreover, luteolin has been found to suppress glycolysis and promote apoptotic cell death in cancer cells.[19-21,23] However, among the glycolytic enzyme inhibitors, kaempferol is the most promising candidate as it is reported to block several signaling pathways crucial for TNBC development, such as phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt or PI3K/PKB) and mitogen-activated protein kinase (MAPK).

Despite these findings, it is crucial to consider the potential problems in utilizing glycolytic enzyme inhibitors for clinical purposes. These inhibitors could also cause unwanted effects or toxicities at higher concentrations, which may impact their therapeutic potential. Moreover, metabolic adaptability of cancer cells may change the primary energy source from glycolysis to glutamine metabolism or fatty acid oxidation (FAO).[21,27] Subsequent investigations are required to consider the chronic impact of these inhibitors and their interactions with other metabolic regulators or standard antiviral agents to enhance basic therapeutic outcomes and minimize the emergence of resistant.[12,23]

TNBC is notable for its aggressive behavior and poor prognosis due to the absence of targeted therapy. Depending on its microenvironment, TNBC can switch between several energy-producing pathways, including glutaminolysis and FAO, which are some of the essential elements contributing to the aggressive nature of TNBC. Such metabolic shifts enable cancer cells to evade the effects of glycolytic enzyme inhibitors targeting the Warburg effect (aerobic glycolysis).[24,28] Therefore, alternative metabolic pathways should be considered while investigating the effectiveness of glycolytic inhibitors. Glutamine metabolism can be a major alternative pathway in TNBC cells to support their growth and survival. Glutaminolysis (the conversion of glutamine to α-ketoglutarate in TNBC) contributes to biosynthesis and energy production.[22,25] In this process, glutamine is converted to α-ketoglutarate, which enters the tricarboxylic acid (TCA) cycle, an essential pathway for ATP production as well as the synthesis of nucleotides and amino acids.[25,27]

Glutamate metabolism helps cancer cells to sustain energy and biosynthetic demands by reducing the effectiveness of glycolytic enzyme inhibitors such as 2-DG and 3-BP.[24,25] Recent studies have demonstrated that glutaminase inhibitor CB-839 shows promising potential as an adjunct to glycolytic inhibitors, particularly due to its synergistic effects observed in preclinical models of TNBC.[20] The combination of glycolytic and glutaminase inhibitors may offer a potential solution to overcome metabolic resilience in TNBC, suggesting the importance of investigating these pathways. In addition to glutaminolysis, TNBC cells also enhance FAO under conditions of dietary restriction or metabolic stress.[17,28] A crucial alternative energy source for cancer cells is the generation of acetyl-CoA, which enters the TCA cycle. The reliance on FAO allows TNBC cells to overcome glycolytic restriction, particularly during the later stages of tumor progression, when the microenvironment is often hypoxic or nutrient-deprived.[18]

Recent research has shown that inhibiting FAO with a compound similar to etomoxir effectively reduces TNBC cell proliferation and metastasis in a preclinical model.[29] The need for compound therapy is emphasized by TNBC cells' ability to switch to an alternative oxidation pathway in response to glycolytic inhibition. Double suppression of glycolysis and FAO may be a capable approach in blocking TNBC cell metabolic flexibility to prevent tumor progression and metastasis.[30] The ability of TNBC cells to switch between glycolysis, glutaminolysis, and FAO hinders therapeutic effectiveness when only one metabolic pathway is targeted. Glycolytic inhibitors, such as 2-DG, while effective in certain contexts, might be insufficient on their own to suppress tumor progression, as cancer cells can adapt by upregulating alternative pathways such as glutaminolysis or FAO.[20,21,31]

Studies have shown that suppression of glycolysis likely drives metabolic reprogramming, causing TNBC cells to rely on glutaminolysis or FAO for proliferation.[15] It is essential to consider the interaction between glycolysis and other metabolic pathways in TNBC cells. Investigating the transition of the TNBC metabolic pathway into glutaminolysis or FAO when glycolysis is suppressed can be achieved using mass spectrometry-based metabolomics.[29,32] This will provide insights into metabolic reprogramming and mechanisms affecting drug efficacy. It is necessary to investigate glycolytic inhibitors (e.g., 2-DG), glutaminase inhibitors (e.g., CB-839), and FAO inhibitors (e.g., etomoxir) for their synergistic potential to improve therapeutic outcomes in preclinical TNBC models.[18,26] Moreover, *in vivo* studies using heterogeneous or patient-derived tumor models could be conducted to investigate the effectiveness of dual metabolic inhibition on TNBC proliferation and metastasis. Such studies may reveal tumor responses to inhibitors under hypoxic conditions, where

selecting the appropriate metabolic pathway target will be critical for optimizing therapeutic strategies.[27,30] Treatment response in TNBC can be assessed using biomarkers that target specific metabolic pathways, particularly enzymes related to glutaminolysis or FAO.[22,33]

Glycolytic enzyme inhibitors designed to disrupt metabolic reprogramming of TNBC have shown promising results in preclinical models, despite limitations such as low bioavailability, poor solubility, and rapid clearance from the body.[29,34] These limitations restrict the therapeutic potential of the inhibitors in clinical settings. It is crucial to develop strategies that enhance the bioavailability of these compounds, such as through nanotechnology or liposome packaging. Limitations related to bioavailability, stability, and controlled release of glycolytic enzyme inhibitors can be addressed using conventional nanocarrier formulations.[30,35] These approaches help ensure that glycolytic enzyme inhibitors reach the tumor site at therapeutically effective concentrations. These nanocarriers are widely used for the distribution of hydrophobic compounds and glycolytic enzyme inhibitors.[23,36]

Polymeric nanoparticles derived from biodegradable components such as poly(lactic-co-glycolic acid) (PLGA) can encapsulate and protect a drug from deterioration while providing controlled release of the drug.[24] PLGA-based nanoparticles can increase the solubility and bioavailability of glycolytic inhibitors. For instance, 3-BP, a promising glycolytic inhibitor, exhibited limited bioavailability when administered in its free form. Recent research has shown that PLGA nanoparticles can enhance the anti-tumor activities of 3-BP by prolonging drug release and improving its penetration into the tumor cells, while reducing side effects.[15,37] This method significantly enhances the therapeutic effect of the compound. Furthermore, nanocarriers can be functionalized with target ligands (e.g., antibodies or peptides) to specifically deliver a glycolytic inhibitor into TNBC cells. Polymeric micelles and lipid nanoparticles functionalized with targeting agents have been developed to enhance selective delivery of drugs to tumors, thus reducing off-target effects and improving therapeutic efficacy.[9,11,38]

Liposomes, lipid-based vesicles capable of encapsulating both hydrophilic and hydrophobic compounds, provide an efficient approach for improving the bioavailability of the glycolytic enzyme inhibitor. Liposome encapsulation enhances the stability of the compound, preventing rapid degradation and enabling sustained release.[34,39] In addition, polyethylene glycol (PEG)-coated liposomes significantly enhance the drug's pharmacokinetics by increasing the circulation time in the bloodstream, thus increasing the drug concentration at the tumor site. Liposomes can also be modified to improve their tumor-targeting properties by altering the outer layer with PEGylation or incorporating tumor-specific ligands. Liposomes are particularly advantageous for delivering glycolytic inhibitors such as 2-DG, which has poor solubility and stability.[11,29] In preclinical models, PEGylated liposomes containing 2-DG demonstrated enhanced tumor targeting and reduced off-target distribution.[37,40] Cationic liposomes are capable of incorporating negatively charged molecules, such as glycolytic enzyme inhibitors, due to their positive surface charge. Further modification can be made to cationic liposomes to specifically target TNBC cells that overexpress receptors such as the folate receptor. These liposomes have been demonstrated to enhance the bioavailability of glycolytic enzyme inhibitors and gene therapy, thereby improving therapeutic outcomes.[21]

The nanostructured lipid carrier (NLC) is a hybrid system combining the advantages of both liposomes and stable lipid nanoparticles (SLNs). NLCs have enhanced drug encapsulation capacity, greater stability, and controlled drug release, making them ideal for delivering glycolytic inhibitors. NLCs can be loaded with glycolytic inhibitors, such as oxamate, to ensure sustained drug release and prolonged therapeutic effects. The hard matrix of the NLCs provides high drug loading capacity and stability, while the lipid shell enhances absorption and cell membrane permeability.[35,41] Furthermore, the specificity of drug delivery to TNBC cells could be improved by functionalizing NLCs with ligands such as folic acid or HER2 antibodies. Exosomes, tiny extracellular vesicles derived from cells, have gained attention as drug delivery vehicles due to their ability to target natural tissues, biocompatibility, and capacity to encapsulate a wide range of therapeutic agents. Exosomes have been used in research to deliver glycolytic inhibitors in TNBC, as they can be modified to carry small molecules and RNA therapeutics such as siRNA.[42] Exosome-based delivery systems can provide a novel method to increase the bioavailability and efficacy of glycolytic enzyme inhibitors by exploiting their inherent ability to target tumors and modulate cancer cell metabolism.[10,19]

TNBC is an aggressive type of breast cancer that lacks estrogen, progesterone, and the HER2 receptor, making it particularly difficult to treat. A promising approach to TNBC treatment involves metabolic reprogramming by targeting the glycolytic pathway, which is frequently upregulated in cancer cells (a phenomenon known as the Warburg effect). Glycolytic enzyme inhibitors such as 2-DG, 3-BP, and oxamate disrupt glycolysis, thereby eliminating the energy and biosynthetic precursors necessary for cancer cell survival and proliferation.[38,43] While these inhibitors have shown promising results in preclinical models, their

clinical translation is hindered by challenges such as low bioavailability and rapid metabolic degradation. Therefore, glycolytic suppression alone is insufficient to overcome the complex resistance mechanisms of TNBC cells. A combination of chemotherapy and immunotherapy could produce a particularly strong therapeutic effect.[23,44] While chemotherapy remains the cornerstone of treatment for TNBC, resistance to chemotherapy is common, and tumor recurrence frequently occurs. In this context, glycolytic suppression can induce the formation of reactive oxygen species (ROS) in cancer cells, causing oxidative stress and increasing tumor susceptibility to chemotherapeutic damage. Research has shown that 3-BP increases ROS by suppressing glycolysis, thereby amplifying the cytotoxic effects of chemotherapeutic agents such as doxorubicin.[45]

The increased ROS production could synergize with the oxidative damage caused by chemotherapy agents, a major factor contributing to tumor cell recurrence. Glycolysis plays a major role in cellular ATP production, which is crucial for DNA repair and contributes to cancer cell resilience during chemotherapy. Glycolytic enzyme inhibitors may disrupt energy production and DNA repair mechanisms in cancer cells by suppressing glycolysis. This enhances cell susceptibility to chemotherapy agents, such as cisplatin and paclitaxel.[46] Several chemotherapeutic agents exploit the metabolic reprogramming of cancer cells, particularly their reliance on glycolysis.[21,27] To overcome chemotherapeutic resistance, targeting the metabolic flexibility of TNBC cells with glycolytic inhibitors may limit the tumor's ability to adapt. For example, 2-DG was demonstrated to modify TNBC cell resistance to paclitaxel and doxorubicin *in vitro*, thereby providing a potential strategy in overcoming drug resistance.[14,30] Therefore, the combination of glycolytic enzyme inhibitors with chemotherapy may increase TNBC cell susceptibility to chemotherapy, leading to enhanced treatment efficacy and reduced drug resistance.

Immunotherapy has emerged as a promising treatment option for multiple malignancies, including TNBC. However, immunosuppressive TME has hindered its achievement. The glycolytic pathway is crucial to both cancer cells and immune cells, regulating the immune response. Tumor cells may use metabolic reprogramming to suppress immune response. Therefore, targeting glycolysis may increase the efficiency of immunotherapy.[13,34,47] The inhibition of glycolysis in cancer cells has been demonstrated to affect the conformation of the major histocompatibility complex molecule and tumor antigen. This may enhance the immune system's ability to recognize and eliminate cancer cells. In particular, glycolytic suppression may reduce the production of lactate, a by-product that contributes to immunosuppressive TME while increasing the expression of tumor-associated antigen, thereby increasing the efficacy of immune checkpoint inhibitors, such as anti-PD-1 and anti-CTLA-4.[11,17,39]

A large number of immunosuppressive cells, including Tregs and M2 macrophages, are commonly reprogrammed to promote tumor growth and are key components of TME in TNBC. To promote the antitumor phenotype, glycolytic inhibitors can reprogram cancer cells. For instance, 3-BP has been reported to change the tumor-associated macrophage from pro-tumoral (M2) to anti-tumoral (M1) phenotype, increasing the efficiency of immunotherapy.[48] For the immune system to respond effectively, suppressing glycolysis in the tumor is likely to restore its capacity for a robust immune response. Studies have shown that combining a glycolytic inhibitor with an immune checkpoint inhibitor may enhance T-cell activation and cytotoxicity against tumors. For example, 2-DG has been used in combination with an immune checkpoint inhibitor to enhance anti-tumor resistance by modulating T-cell metabolism.[14,47] Glycolytic inhibitors may alter the vascular structure of the tumor, potentially facilitating the infiltration of immune cells at the tumor site. This could increase the efficacy of immune checkpoint inhibitors by increasing tumor vascular permeability.[19,37]

TNBC has been recognized as a key example of metabolic reprogramming in cancer, including glycolysis upregulation (Warburg effect). As glycolytic inhibition is a promising therapeutic strategy, patient selection for glycolytic enzyme inhibitors is crucial. Several molecular and metabolic features can serve as biomarkers in such approaches. Lactate, a byproduct of glycolysis, is often elevated in cancer cells and TME, particularly in TNBC.[45,48] Lactate dehydrogenase (LDH), an enzyme involved in the conversion of pyruvate to lactate, has been widely studied as a biomarker of metabolic processes. Increased levels of LDH correlate with increased glycolytic activity and poor prognosis in various malignancies, including TNBC. Patients with high LDH levels and increased lactate production are likely to benefit from targeted glycolysis, as their tumor relies heavily on the glycolytic pathway.[47] Therefore, monitoring LDH levels can help identify patients who are more likely to respond to glycolytic enzyme inhibitors, such as 2-DG and 3-BP.[33,43]

A study shows that serum LDH levels in TNBC are associated with poor prognosis and proposes that LDH inhibition could be a therapeutic strategy for glycolytic targeting in patients with high LDH expression. The expression levels of crucial glycolytic enzymes, such as HK, PKM2, and PFK, are often increased in cancer cells, including TNBC. Such enzymes are necessary for regulating

glycolysis, and their overexpression is often associated with aggressive tumor types and unfavorable clinical outcomes.[29,49] Aerobic glycolysis for energy production is more likely to be utilized in patients with tumors that have high glycolytic enzyme activity. Enzymatic profiling may serve as a potential predictive biomarker for the response to glycolytic inhibitors. These investigations have shown that PKM2 expression in TNBC cells correlates with increased tumor aggressiveness and is involved in pathways that promote glycolytic activity and tumor progression.[50] HK2 expression has been associated with poor prognosis and may serve as a prognostic biomarker for glycolysis targeting strategies.[11,24]

The hypoxia-inducible factor (HIF-1) pathway plays a central role in metabolic reprogramming in response to hypoxia, a condition commonly observed in solid tumors. HIF-1α, the active subunit of HIF-1, promotes the transcription of glycolytic genes, including glucose transporter 1 (GLUT1) and pyruvate dehydrogenase kinase 1 (PDK1).[51] HIF-1 expression may serve as a predictive biomarker for glycolytic enzymatic inhibitors if its upregulation correlates with increased glycolytic activity in tumors. Patients with tumors expressing high levels of HIF-1α could benefit from treatments targeting key glycolytic enzymes involved in metabolic reprogramming. Radiotherapy (RT) signals reveal that HIF-1 overexpression in TNBC is associated with poor prognosis and resistance to treatment. Furthermore, inhibition of HIF-1 itself, or its target genes beyond those involved in glycolysis, may reduce tumor progression and enhance chemosensitivity.[52] Therefore, HIF-1 may serve as a biomarker to identify TNBC patients who could benefit from glycolysis targeting therapy.

The pyruvate dehydrogenase (PDH) complex plays an important role in the regulation of the metabolic switch during glycolysis and oxidative phosphorylation.[50] The PDH pathway is suppressed in various types of cancer, including TNBC, to promote glycolysis over mitochondrial oxidative metabolism. Measurement of PDH levels and overall mitochondrial function of the tumor could help to predict which patients respond better to glycolytic inhibition.[14,26] A glycolytic phenotype is characterized by low PDH activity and mitochondrial dysfunction, suggesting that a glycolytic enzyme inhibitor may have clinical potential. In a TNBC model, PDH inhibition and subsequent reliance on glycolysis have been demonstrated to promote tumorigenesis. Tumor growth has been suppressed by increasing PDH activity or inhibiting glycolysis.[51] Thus, evaluating mitochondrial and PDH activity may reveal metabolic reprogramming linked to therapeutic efficacy.[20,51]

Recent studies suggest that immune cells in the TME of TNBC undergo a transformation, which affects the immune response. T cells and macrophages depend on glycolysis for activation and function. Therefore, the metabolic reprogramming of TNBC influences tumor progression and response to therapy.[52] A patient with altered immune metabolism, characterized by increased glycolytic activity in T cells and tumor-associated macrophages, may show a better response to glycolytic enzyme inhibitors, which can reprogram the immune response toward a more anti-tumoral phenotype.[53,54] Glycolytic inhibition may modify TME immune cells to fight cancer. Therefore, immunometabolic biomarkers could be used to identify individuals who may benefit from glycolytic restriction and immunotherapy.

5. Conclusion

Inhibition of glycolytic enzymes shows another potential strategy for treating TNBC, as the glycolytic pathway in these tumors is reprogrammed to support tumor growth and invasion. Several enzymes, including HK2, PFK-1, and PKM2, have shown promising results in preclinical models; however, the clinical applicability of these results is still an issue. Further studies are needed to optimize these inhibitors, elucidate their molecular targets, and evaluate their use in combination with other treatments to enhance efficacy and reduce resistance. While cancer metabolism is not yet fully understood, glycolytic enzyme inhibitors are emerging as an increasingly promising treatment of TNBC. Taken together, our findings provide strong evidence that glycolytic enzyme inhibitors could be a promising therapeutic approach for TNBC. Inhibition of the glycolytic pathway in TNBC cells through targeting HK2, PKM2, and LDHA may inhibit tumor progression and invasion. However, additional investigations are required to optimize their efficacy for clinical applications. Our study provides preliminary evidence that targeting glycolytic metabolism could be a potential strategy to overcome the tenacity of TNBC. The present study also identifies kaempferol, luteolin, and shikonin as potential glycolytic inhibitors capable of suppressing TNBC proliferation and metastasis. These compounds demonstrated strong and selective binding to key glycolytic enzymes, along with favorable ADME profiles, and low to moderate toxicity. Therefore, they should be considered for further preclinical and clinical evaluation as glycolytic gene inhibitors. The significant reduction in glycolytic enzyme expression suggests their involvement in metabolic reprogramming, which is a hallmark of TNBC malignancy and could be potential therapeutic targets.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

All the experiments described herein were performed with ethical approval from the Institution Review Board (IRB), School of Pain and Regenerative Medicine (SPRM/UOL/MOCT/D01/0106). The collected data for the current study were under permission granted by the appropriate ethical committee before the beginning of the research study.

Consent for publication

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Availability of data

All data from the study are presented in the article.

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