In the name of god

Flavonoid GL-V9 induces apoptosis and inhibits glycolysis of breast cancer via disrupting GSK-3β-modulated mitochondrial binding of HKII

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Original article

Flavonoid GL-V9 induces apoptosis and inhibits glycolysis of breast cancer via disrupting GSK-3β-modulated mitochondrial binding of HKII

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ABSTRACT

Energy metabolism plays important roles in the growth and survival of cancer cells. Here, we find a newly synthesized flavonoid named GL-V9, which inhibits glycolysis and induces apoptosis of human breast cancer cell lines, and investigate the underlying mechanism. Results show that hexokinase II (HKII) plays important roles in the anticancer effects of GL-V9. GL-V9 not only downregulates the expression of HKII in MDA-MB-231 and MCF-7 cells, but also induces dissociation of HKII from voltage-dependent anion channel (VDAC) in mitochondria, resulting in glycolytic inhibition and mitochondrial-mediated apoptosis. The dissociation of mitochondrial HKII is attributed to GSK-3β-induced phosphorylation of mitochondrial VDAC. Our *in vivo* experiments also show that GL-V9 significantly inhibits the growth of human breast cancer due to activation of GSK-3β and inactivation of AKT. Thus, GL-V9 induces cytotoxicity in breast cancer cells via disrupting the mitochondrial binding of HKII. Our works demonstrate the significance of metabolic regulators in cancer growth and offer a fresh insight into the molecular basis for the development of GL-V9 as a candidate for breast carcinoma treatment.

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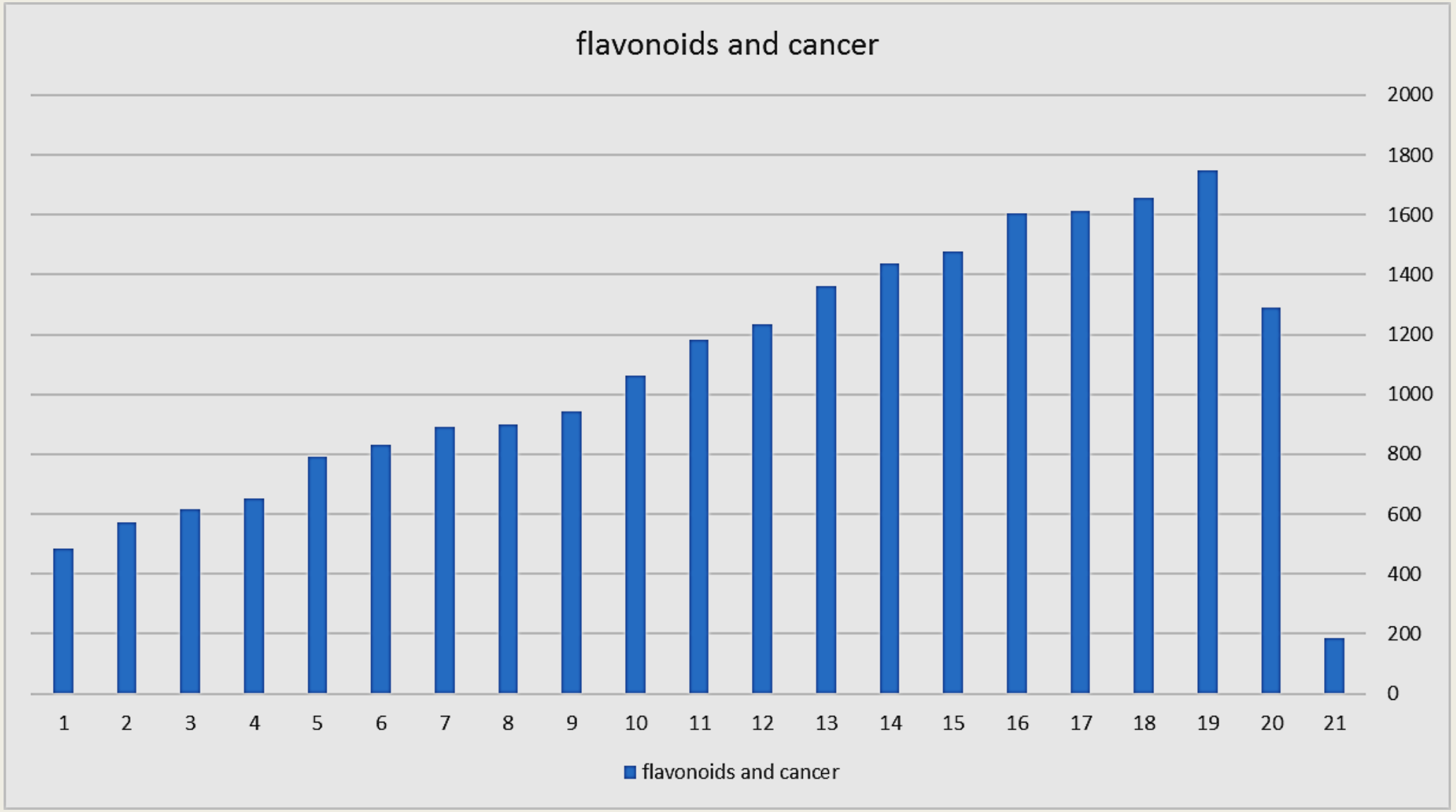
Research trend



Introduction

- Breast cancer → most common cancer in women , 252,710 new cases and 40,610 deaths in 2017. (1)
- the molecular mechanisms of breast cancer initiation and progression is unclear.
- Warburg thesis → shift in energy production from mitochondrial oxidative phosphorylation to aerobic glycolysis is a hallmark of cancer development. (2)
- Aerobic glycolysis → supports rapid growth, cancer cells less dependent on oxygen, suitable micro-environment. (3)
- high glycolytic rate → increased expression of hexokinase II (HK II), mitochondrial binding with voltage-dependent anionic channel (VDAC) . (4)
- VDAC → porin in outer mitochondrial membrane, exchanges of metabolites and ions (5)

Introduction

- binding of HK II to VDAC → inhibits apoptosis by alterations in the permeability of OMM (6) , drug resistance (7)
- activation of PKB or AKT :
  - ✓ enhances the binding of HK II to mitochondria,
  - ✓ augmenting the uptake and metabolism of glucose,
  - ✓ affecting the phosphorylation of VDAC and/or HK II, (8-9)
  - ✓ Inhibits glycogen synthase kinase 3β (GSK-3β)
- GSK3β :
  - ✓ mitochondrial dysfunction and apoptosis (10-11)
  - ✓ catalyzes the phosphorylation of VDAC
  - ✓ detachment of HKII from VDAC
  - ✓ vulnerability of the mitochondria to pro-apoptotic conditions (12)

Introduction

- GL-V9 (5-hydroxy-8-methoxy-7-(4-(pyrrolidin-1-yl) butoxy)-4 Hchromen-4-one) :
  - ✓ newly synthesized flavonoid
  - ✓ inhibits tumor invasion and metastasis via inhibition of matrix metalloproteinase-2/9 (13)
  - ✓ triggers mitochondria mediated apoptosis,
  - ✓ reverses hypoxia–drug resistance (14-15)

Methods & materials

- **Reagents :**
- **GL-V9** → synthesize from wogonin, both of them dissolved in dimethyl sulfoxide
- **2-Deoxy-D-glucose (2-DG)** → purchased from MCE (MedChemExpress, NJ, USA) and dissolved in distilled water
- **LiCl** → purchased from Sigma-Aldrich (Merck Life Science (Shanghai), dissolved in distilled water
- **Human recombinant IGF-1** → lyophilized powder, purchased from Merck Millipore (Millipore, Ireland), reconstituted to 100 μg/mL using ddH2O
- **MK-2206 dihydrochloride (MK)** → purchased from Santa Cruz (Santa Cruz Biotechnology Inc, CA) anddissolved in DMSO

Methods & materials

- **Cell culture :**
  - ✓ MDA-MB-231 and MCF-7 were purchased from Cell Bank of Shanghai Institute of Biochemistry & Cell Biology
  - ✓ cultured in PRMI-1640 and Dulbecco's Modified Eagle Medium
  - ✓ 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37 °C with 5% CO2.

- **Cell viability assays :**
  - ✓ Using MTT assay
  - ✓ Treatment with various concentrations of GL-V9 for 36 h
  - ✓ Formazan absorbance was measured spectrophotometrically at 570 nm using a Universal Microplate Reader EL800

Methods & materials

- **Annexin-V/PI double-staining assay :**
  - ✓ Cells were treated for 36 h with GL-V9 or wogonin
  - ✓ Apoptotic cells were identified by double supravital staining with recombinant FITC conjugated Annexin-V and PI
  - ✓ Apoptotic cell death was examined by FACSCalibur flow cytometry

- **Lactic acid production :**
  - ✓ the Lactic Acid Detection kit (KeyGen, Nanjing, China)
  - ✓ The assay was monitored spectrophotometrically at 570 nm using the Thermo Scientific Varioskan Flash spectral scanning multimode reader
  - ✓ The absorbance was normalized as follows:

$$OD_{normalized} = Od_{measured} / living\ cell\ number_{treated} \times living\ cell\ number_{control}.$$

Methods & materials

- **ATP Assessment :**
  - ✓ ATP Bioluminescent Somatic Cell Assay Kit from Sigma
  - ✓ Cells were lysed in an ice-cold ATP releasing buffer
  - ✓ Following the addition of 100 µl luciferin and luciferase, luminescence was monitored on a luminometer Orion II

- **Glucose uptake assay :**
  - ✓ Amplex Red Glucose Assay Kit (Invitrogen, Eugene, OR)
  - ✓ The amount of glucose was detected using a fluorometer at Ex./Em.=530 nm/590 nm
  - ✓ The living cells were counted by trypan blue staining of collected cells
  - ✓ Glucose uptake was determined by subtracting the amount of glucose in each sample from the total amount of glucose in the media

Methods & materials

- **Measurements of oxygen consumption :**
  - ✓ Cells were seeded, After drug treatments, Plates were scanned in a temperature-controlled (37 °C) plate reader with EX: 485 nm and EM: 630 nm
  - ✓ Slopes of fluorescence signal were calculated

- **Mitochondrial membrane potential determination :**
  - ✓ Quantitative changes of mitochondrial membrane potential (MMP) at the early stage of cell apoptosis were measured by the Mitochondrial membrane potential Detection kit
  - ✓ FACSCalibur flow cytometry
- **Analysis of intracellular superoxide anions ($O_2^{\bullet-}$) level :**
  - ✓ Cells were treated for 36 h, harvested and incubated with the dye, and were then detected on flowcytometer
  - ✓ Detection by DHE kit

Methods & materials

- **Hydrogen peroxide (H2O2) assay :**
  - ✓ Hydrogen peroxide assay kit

- **Preparation of mitochondrial extracts :**
  - ✓ The fractionation of the mitochondrial protein was extracted with the Mitochondria/Cytosol Fractionation Kit

- **Immunoprecipitation :**
  - ✓ 1 mg VDAC antibody and 20 ml protein A/G-conjugated beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight
  - ✓ The immunocomplexes were analyzed by western blotting and probed with antibody against GSK3β or HKII
  - ✓ Same processes for VDAC or HKII

Methods & materials

- **In vivo tumor growth assay :**
  - ✓ Female athymic BALB/c nude mice (35–40 days old), body weight ranging from 18 to 22 g
  - ✓ Forty nude mice were inoculated subcutaneously with 1×107 MDA-MB-231 or MCF7 cells into the right axilla
  - ✓ randomly divided into saline control, GL-V9 (20 mg/kg, i.v.,every 2 days), wogonin (60 mg/kg, i.v., every 2 days), and Taxol (8 mg/kg, i.v., twice a week) groups
  - ✓ Tumor sizes were measured every 3 days using micrometer calipers
  - ✓ tumor volume was calculated with TV (mm3) =d2×D/2 formula
  - ✓ d and D were the shortest and the longest diameter

Methods & materials

- **Immunohistochemistry :**
  - ✓ The expressions of GSK3β, p-GSK3β, AKT, p-AKT in the tumor tissue assessed by the SP immunohistochemical method using a rabbit antihuman monoclonal antibody and an Ultra-Sensitive SP kit
  - ✓ primary antibodies added to tissue sections and incubated at at 4 °C overnight
  - ✓ Tissues were then incubated with the secondary biotinylated antispecies antibody, labeled using a modified staining procedure

- **Statistical evaluation :**
  - ✓ Statistical analysis was performed using one-way ANOVA
  - ✓ Least Significant Difference test and Tukey’s HSD test were used for the one-way ANOVA analyses

Results

- **GL-V9 has potent anticancer activity in breast cancer cells via inducing mitochondrial-mediated apoptosis :**

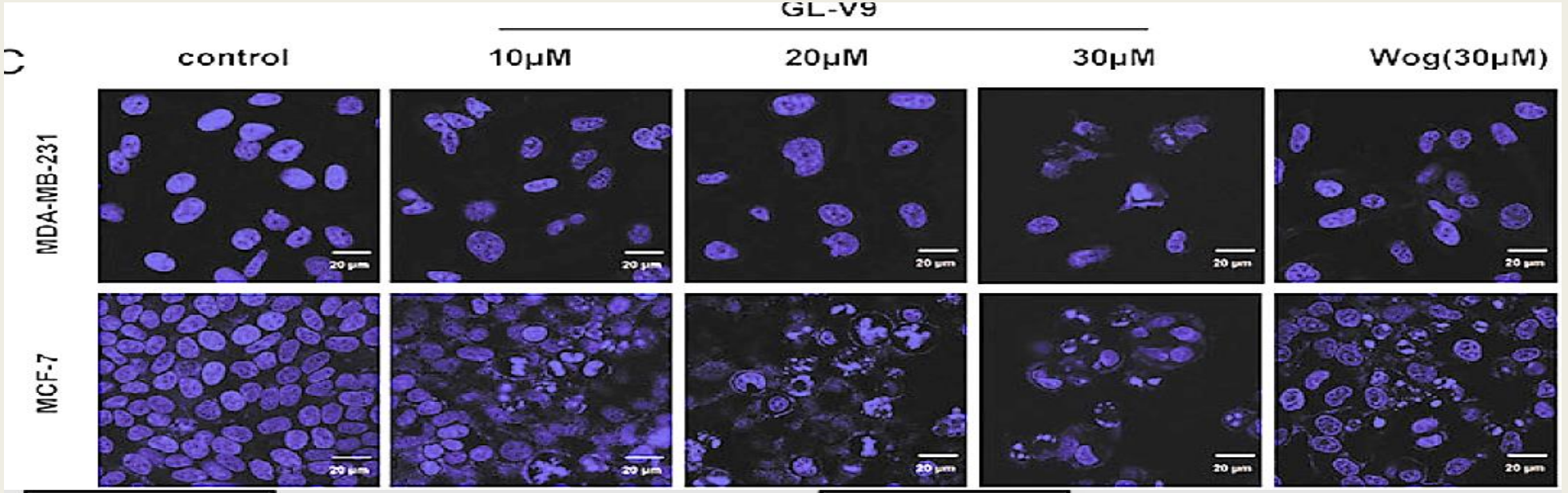
  - ✓ GL-V9ˈs IC50 for growth inhibition : 14.90 ± 1.26 μM for MDA-MB-231 and 17.81 ± 2.08 μM for MCF7
  - ✓ wogoninˈs IC50 for growth inhibition : 109.22 ± 4.08μM for MDA-MB-231 and 98.53 ± 2.23μM for MCF7
  - ✓ Untreated cells stained equably with blue fluorescence
  - ✓ treatment with 10 μMGL-V9 → early symptom of apoptosis, bright fluorescence
  - ✓ 20 and 30 μMGL-V9 → cellular nucleus disintegrated and formed many nuclear fragments

Results

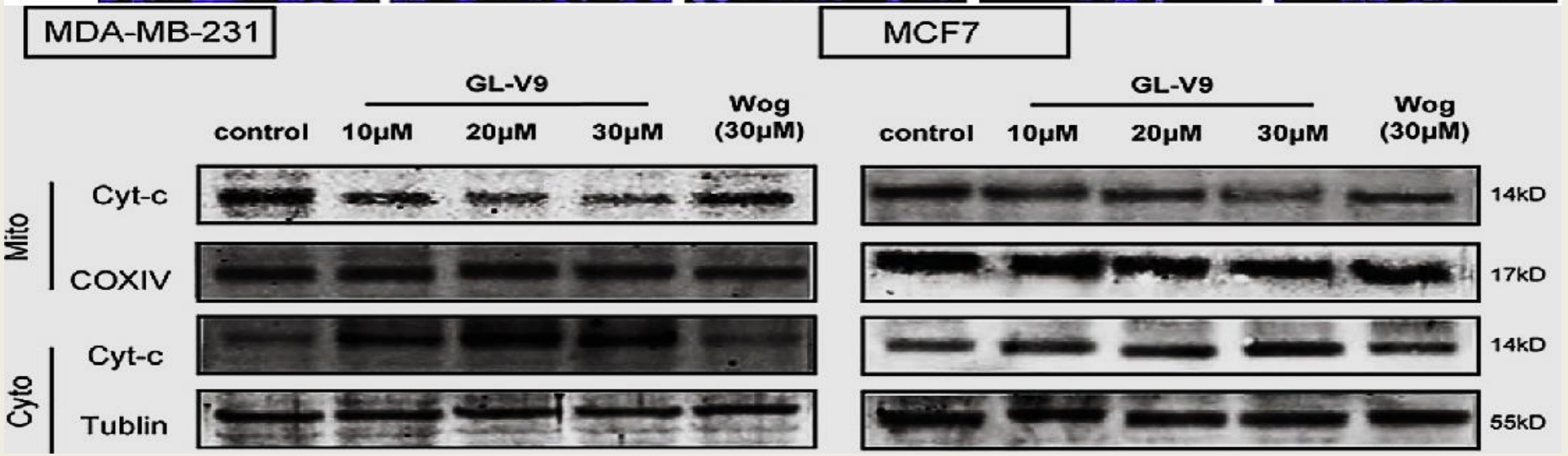
- ✓ Annexin V/PI double staining assay → GL-V9 was 3 times more efficient in promoting apoptosis than wogonin

- upon treatment with GLV9 :
  - ✓ caspase 3 was proteolytically activated
  - ✓ expressions of caspase 9, Bcl-2 and Bcl-xl were decreased
  - ✓ Expression of Bax was increased
  - ✓ loss of mitochondrial membrane potential
  - ✓ amount of Cyt c decreased in mitochondria but increased in the cytosol
  - ✓ intracellular level of O2 was increased

Results

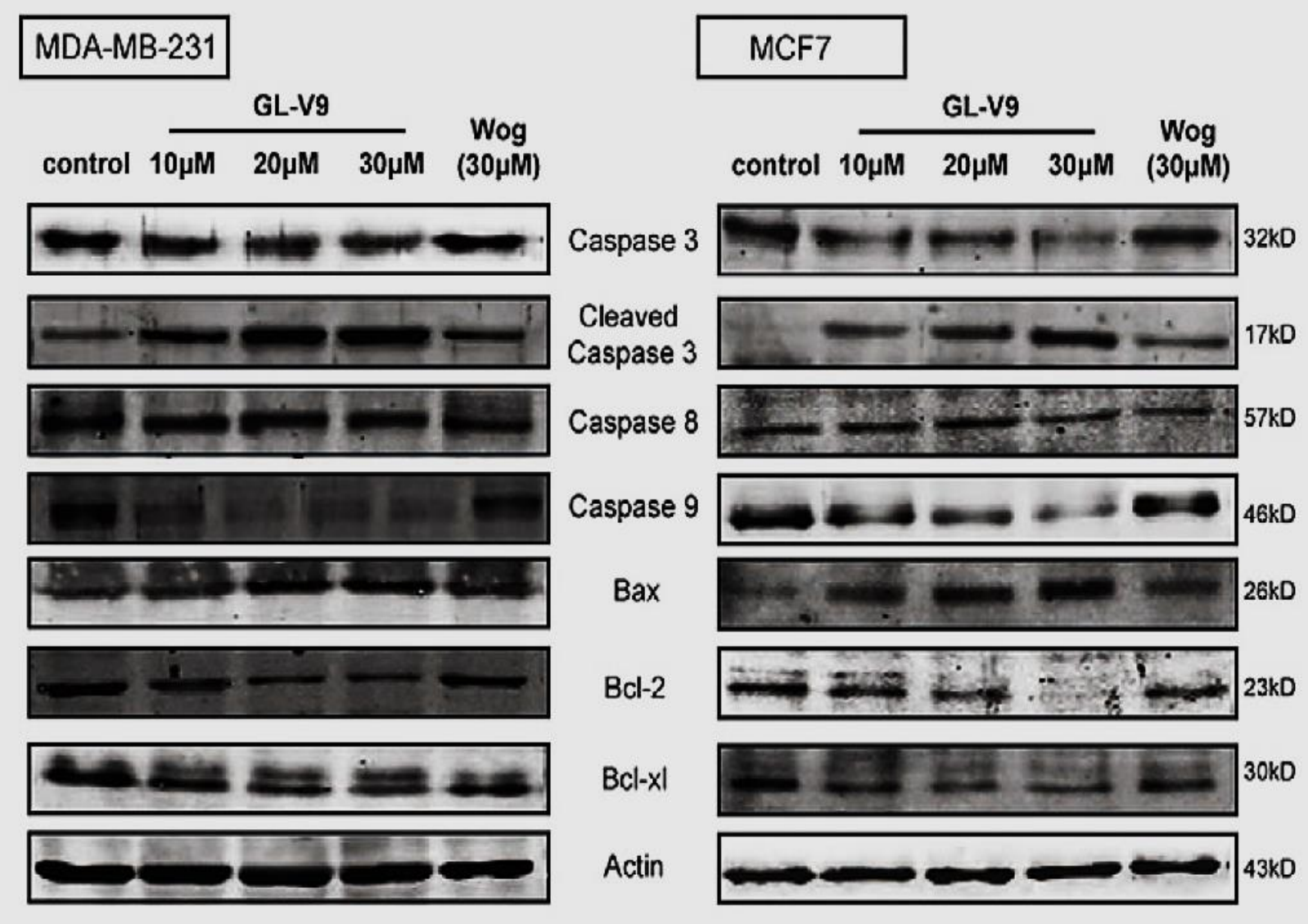


Nucleolus morphologic changes induced by wogonin observed under fluorescent microscope



Western blot assays were used to examine the expressions of cyt-c

Results



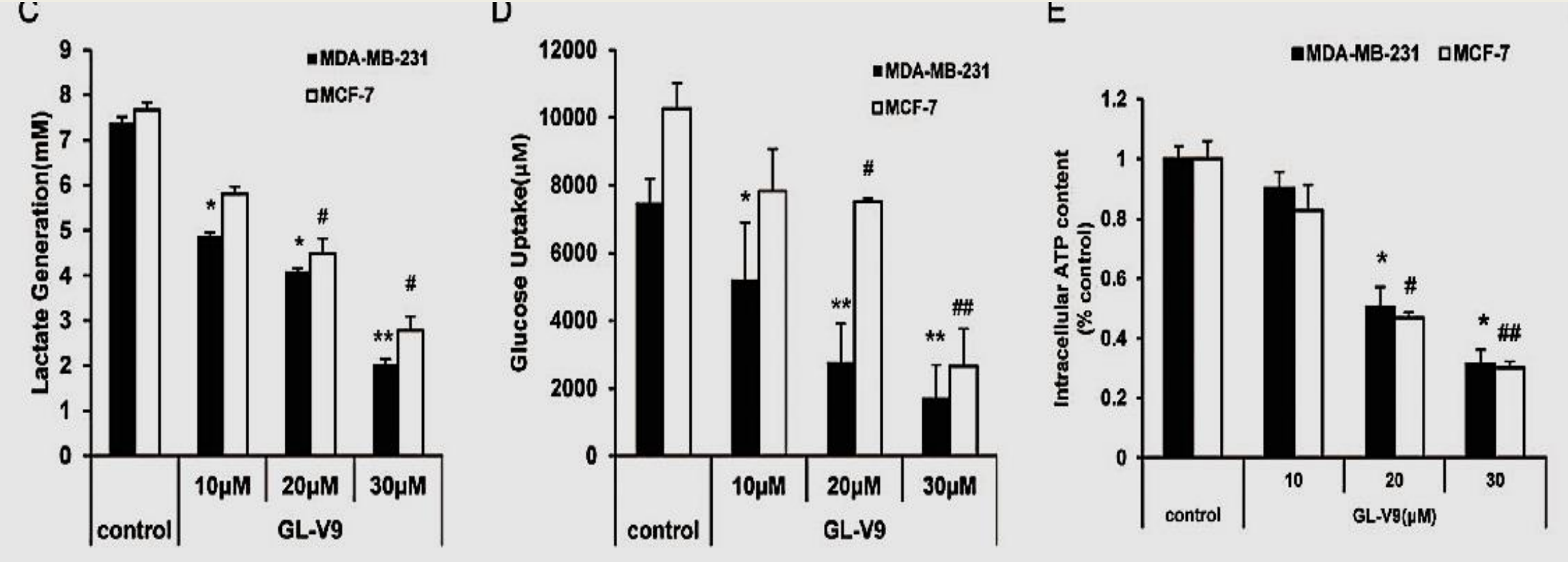
Western blot assays for the expressions of apoptosis-associated proteins

Results

- **GL-V9 suppresses aerobic glycolysis of human breast cancer cells :**

  - ✓ 24 h treatment of GL-V9 upon 10 μM, 20μM and 30 μM:
    - ✓ apoptosis rates were all less than 10%
    - ✓ cell growth were inhibited but apoptosis didn’t induced
    - ✓ lactate generation, glucose uptake and ATP production were decreased
    - ✓ oxygen consumption were promoted

Results

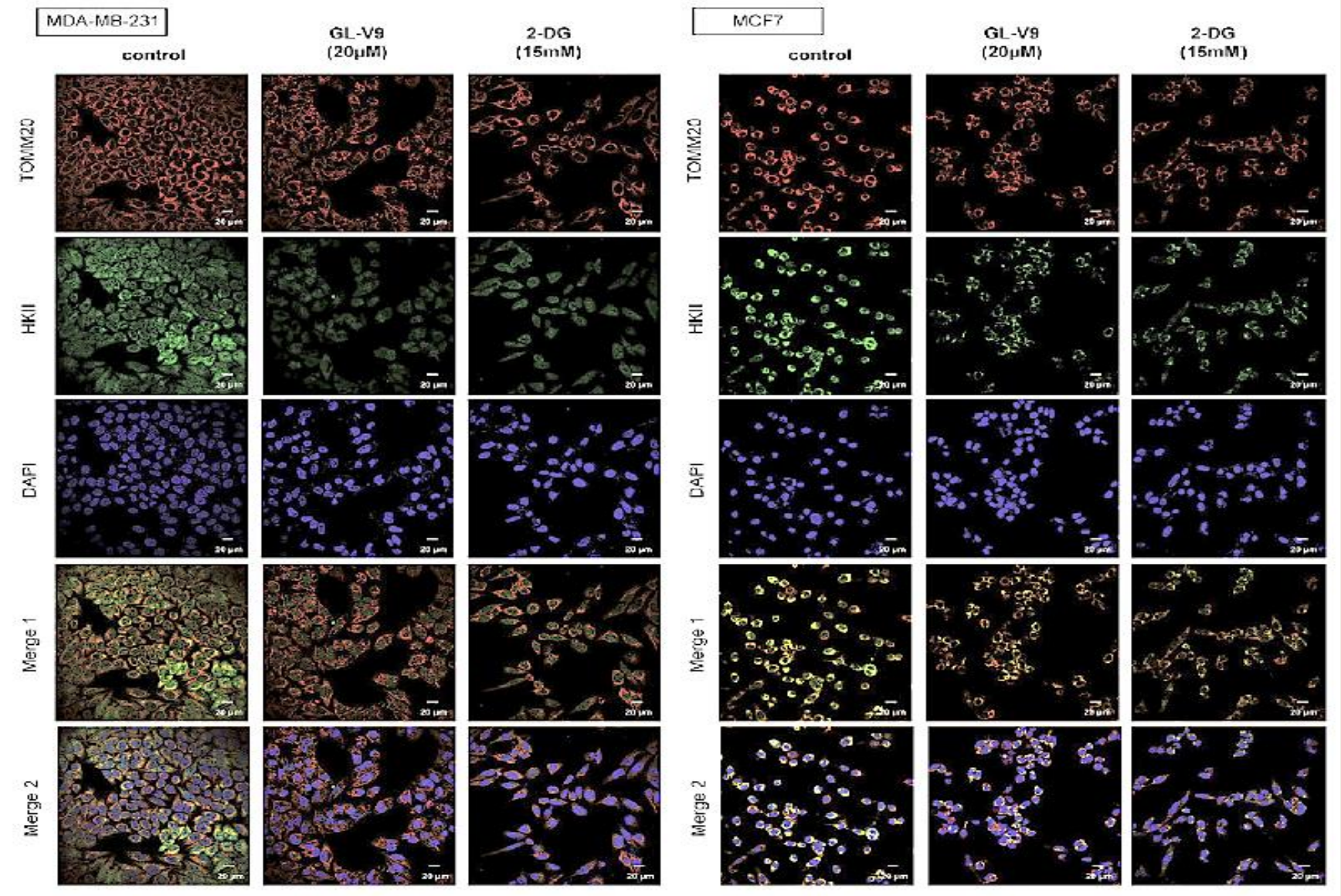


- Production of lactic acid was assayed by a Lactic Acid production Detection kit
- Glucose uptake was measured using the Amplex Red assay kit
- Quantification of ATP generation was detected by the luminometer Orion II

Results

- **The dissociation of HK II from mitochondria accounts for GL-V9-induced mitochondrial dysfunction and apoptosis :**
  - ✓ 2-deoxy-D-glucose (2-DG) for positive control as a HKII inhibitor
  - ✓ 15mM 2-DG for 36 h induced apoptosis in MDA-MB-231 and MCF-7 cells
  - ✓ 20 and 30 μM GL-V9 treatment for 36 h decreased total and mitochondrial HKII
  - ✓ After GL-V9 and 2-DG treatment, faint diffuse staining of HKII was detected in the cytosol → weaker co-localization of HKII with mitochondria
  - ✓ immunoprecipitation assay for binding of HKII and VDAC in mitochondria → binding of mitochondrial HKII with VDAC was suppressed

Results

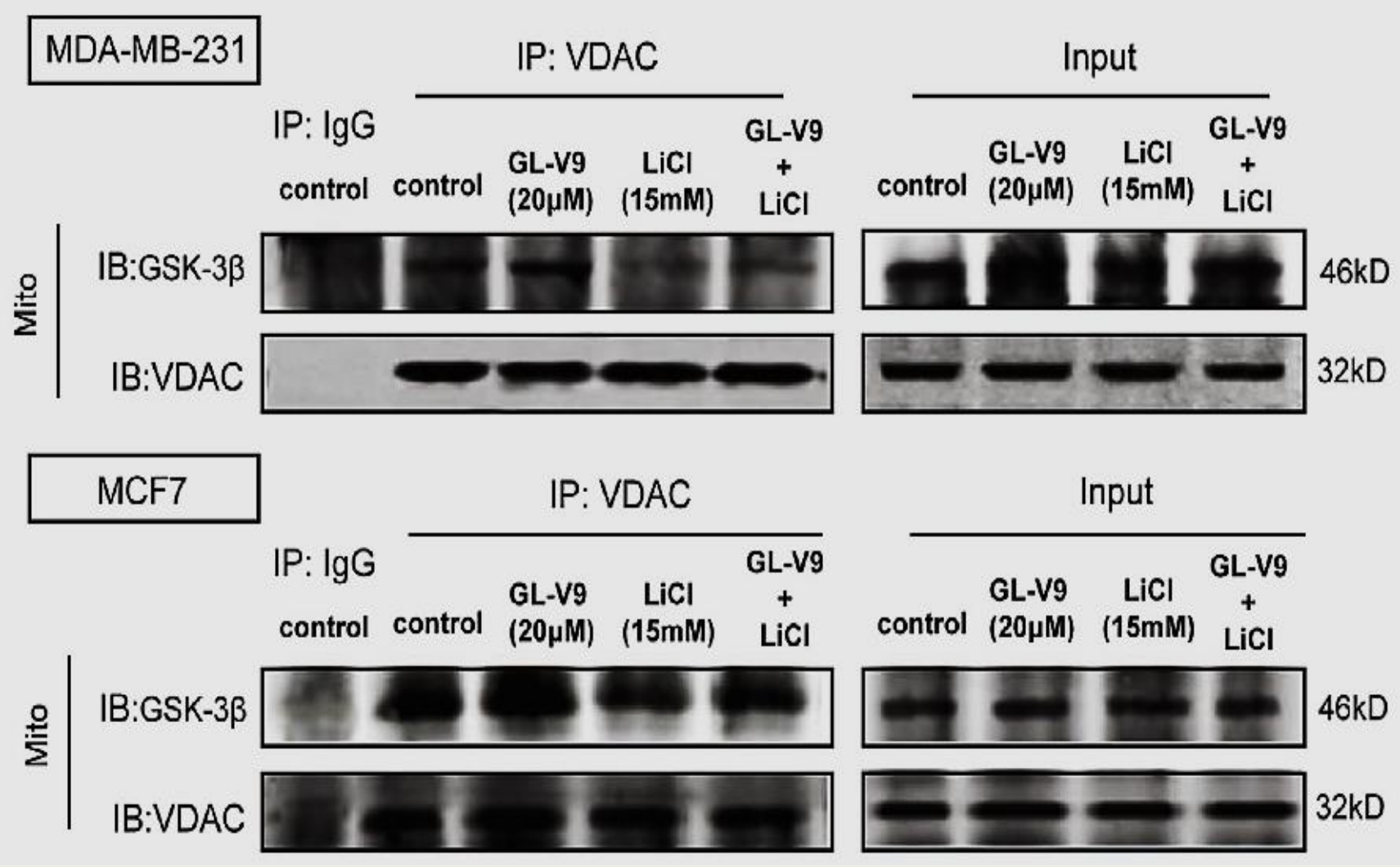


Immunofluorescence using antibodies specific to HKII and mitochondrial marker TOMM20

Results

- **The GSK-3β-mediated phosphorylation of VDAC is involved in GL-V9induced dissociation of mitochondrial HK II :**
  - ✓ To investigate the roles of GSK-3β in the binding of HKII to mitochondria, LiCl , a GSK-3β inhibitor was used
  - ✓ LiCl inhibited the interaction between HKII and VDAC
  - ✓ GL-V9 treatment → total and mitochondrial GSK-3β were increased, phosphorylated GSK-3β was decreased
  - ✓ GSK-3β could bind with VDAC in mitochondria instead of HKII
  - ✓ GL-V9 promoted the binding of mitochondrial GSK-3β with VDAC, facilitating the phosphorylation of VDAC, dissociation of HK II from VDCA

Results

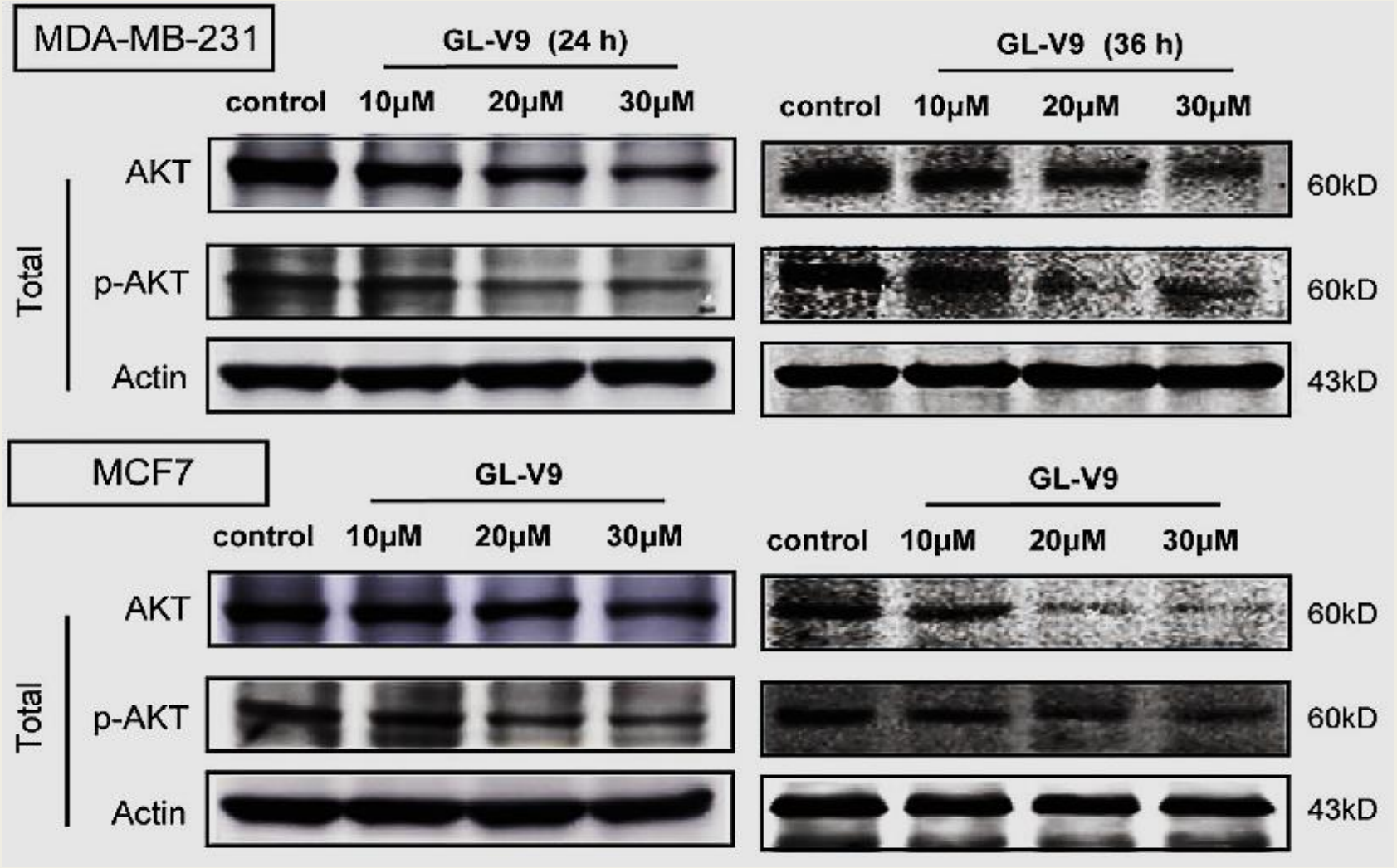


Mitochondria were isolated upon 20 μMGL-V9 with/without 15mM LiCl treatment. VDAC was immunoprecipitated using GSK-3β antibody. Western blot assays were performed for VDAC and GSK3β

Results

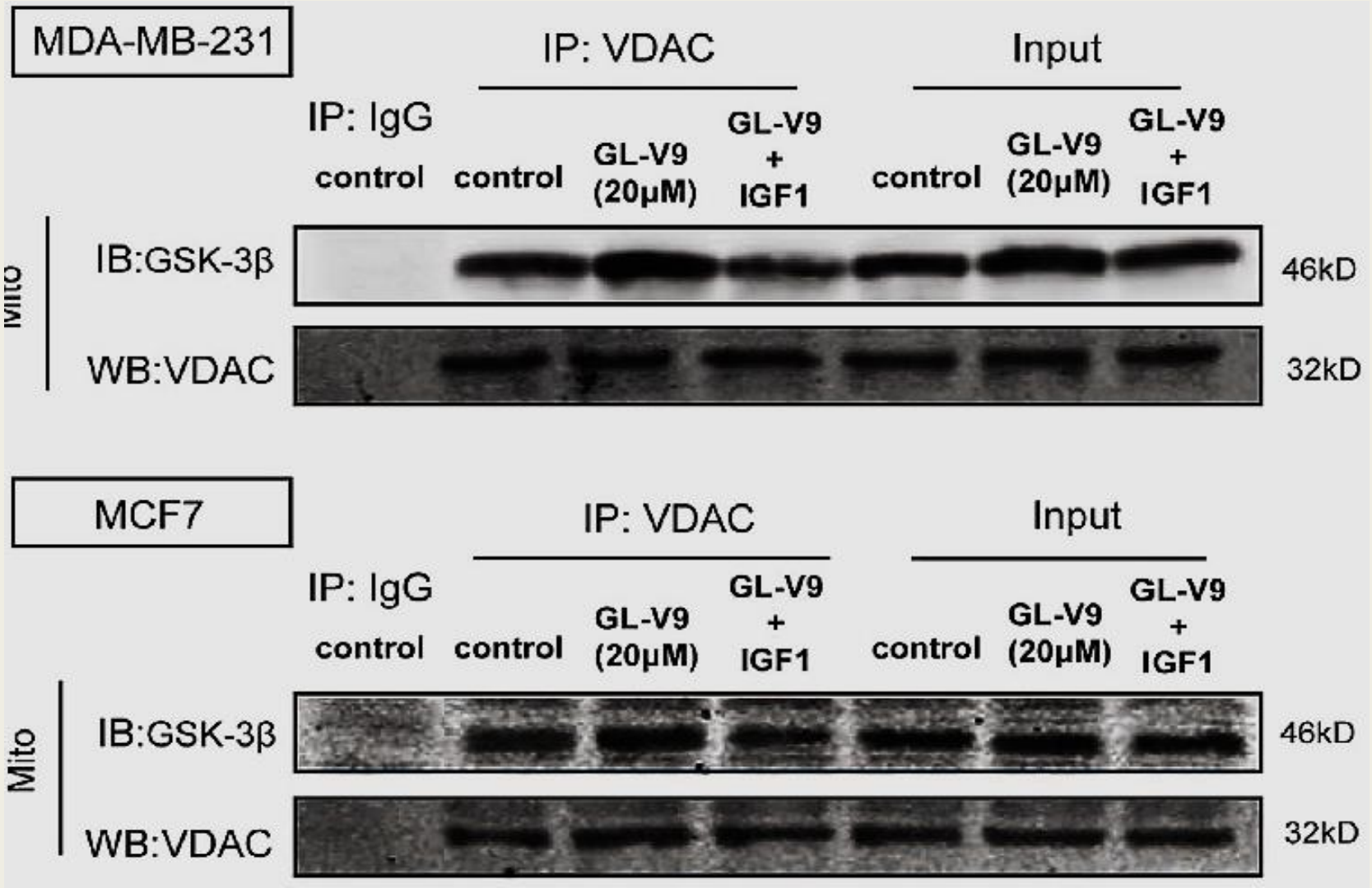
- **GL-V9 activates GSK-3β in mitochondria via inhibiting AKT :**
  - ✓ AKT inhibits the activity of GSK-3β in human neuroblastoma cells (16)
  - ✓ insulin-like growth factor 1 (IGF-1, an AKT activator) promoted the phosphorylation of GSK-3β
  - ✓ MK-2206 (MK, an AKT inhibitor) inhibited the phosphorylation of GSK-3β
  - ✓ GL-V9 decreased the protein expression of AKT, suppressed its activation
  - ✓ IGF + GL-V9 ⟶ enhanced binding of GSK-3β to VDAC in mitochondria was disturbed
  - ✓ inactivation of AKT induced by GL-V9 played important roles in the activation of GSK-3β and the dissociation of HKII from mitochondria

Results



The expressions of AKT
and p-AKT in total proteins upon GL-V9 treatment were assayed by Western blot

Results

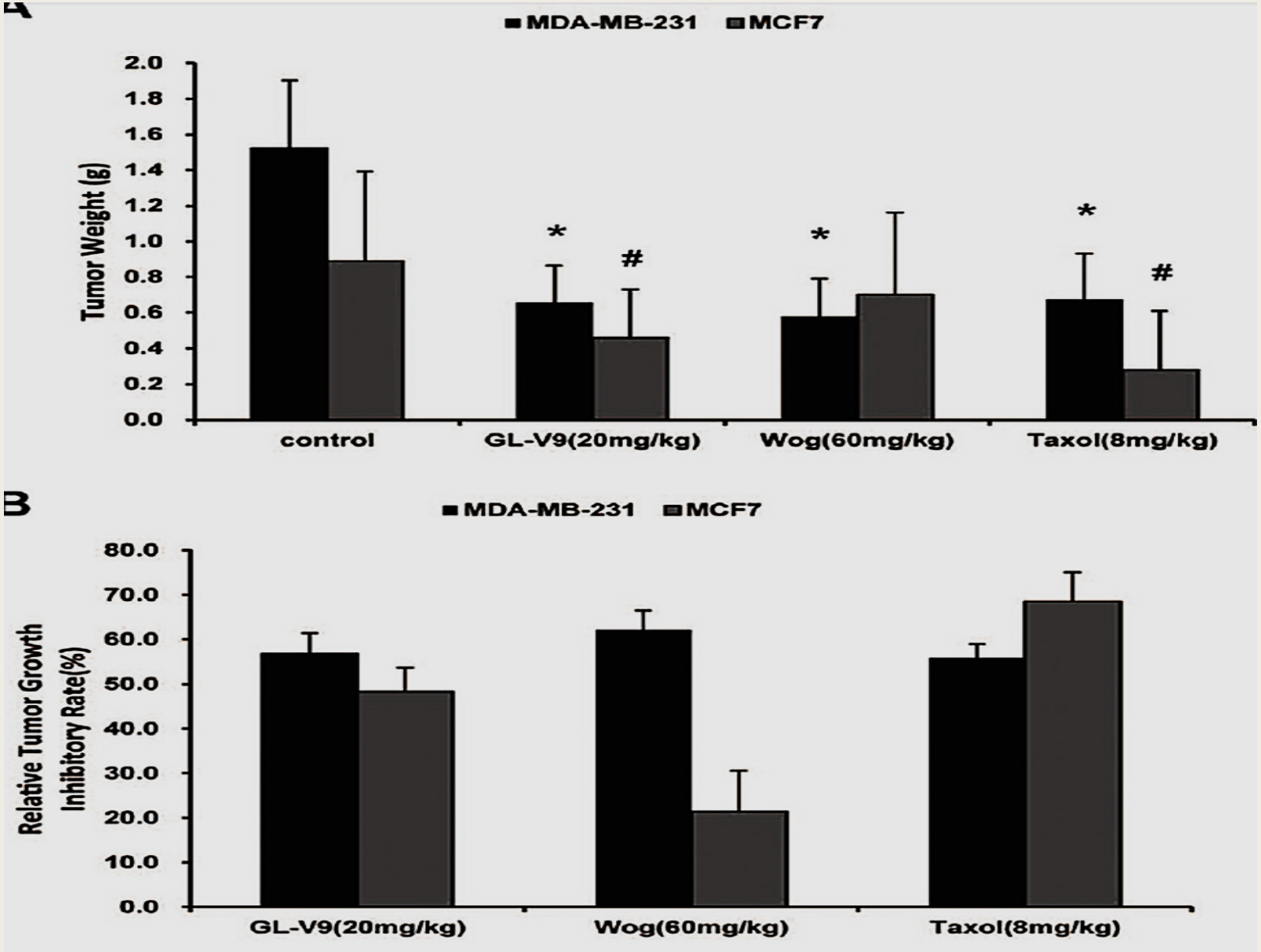


Mitochondria were isolated upon 20 μMGL-V9 treatment with/without 100ng/mL IGF-1, and GSK-3β was immunoprecipitated using VDCA antibody. Western blot were performed for VDAC and GSK-3β

Results

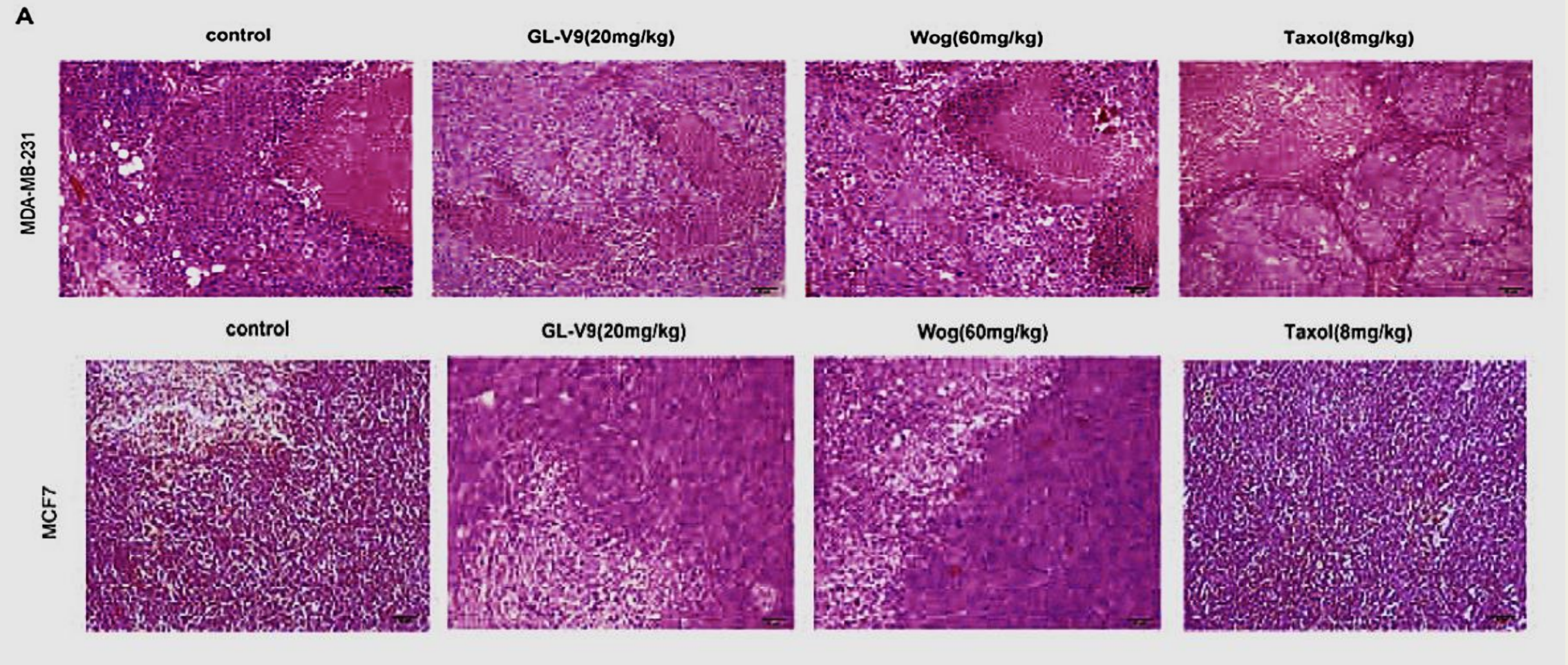
- **GL-V9 inhibits the growth of breast tumor in vivo via activating GSK-3β and inactivating AKT :**
  - ✓ Both 20mg/kg GL-V9 and 60mg/kg wogonin were as effective as positive drug Taxol (8mg/kg) for tumor inoculated with MDA-MB-231 cells
  - ✓ Hematoxylin and eosin (HE) staining → GL-V9-treated group had stronger infiltration and fewer vessels than the control
  - ✓ Immunohistochemistry (IHC) assay → GL-V9 inhibited the phosphorylation of GSK-3β and the activation of AKT in breast tumor tissue

Results



Tumor weight and Tumor inhibitory rates

Results



The influences of GL-V9 in expression and activity of AKT and GSK3β in the transplanted human breast tumor tissue

discussion

- Cancer cells satisfy energy needs through aerobic glycolysis even in the presence of oxygen (17,18) :
  - ✓ Rapid proliferation
  - ✓ Provides the necessary building materials for cancer cells
  - ✓ Reduces the threat of oxidative stress (19)
- HKII have transcriptional regulation and apoptosis induction (20,21).
- FDA approved glycolysis inhibitors drugs:
  - ✓ Enasidenib
  - ✓ Jasmonate (22)
- The expression level of HKII is 10 fold higher in malignant cells (23)
- Stable binding of HKII to mitochondria promotes aerobic glycolysis, impairs mitochondrial respiration

discussion

- Binding of HKII to VDAC with its N-terminal domain and BH4 domain compete with the binding of VDAC to Bcl-xL (24) .
- HKII binding displaces Bcl-xL from VDAC, it may interacts with Bax or Bak, protects against OMM permeabilization
- HKII detachment → prevention of Bcl-xL-Bax binding → release of cytochrome c and apoptosis
- AKT phosphorylates HKII and promoting its binding to VDAC as anti-apoptotic effect (10)
- Inhibition of AKT resulted in dephosphorylation and activation of GSk3ß
- GSk3ß → induction of mitochondrial disfunction, its inhibition blocks caspase activity and apoptosis (25)

discussion

Article

The Synthetic Flavonoid Derivative GL-V9 Induces Apoptosis and Autophagy in Cutaneous Squamous Cell Carcinoma via Suppressing AKT-Regulated HK2 and mTOR Signals

Abstract: Cutaneous squamous-cell carcinoma (cSCC) is one of most common type of non-black skin cancer. The malignancy degree and the death risk of cSCC patients are significantly higher than basal cell carcinoma patients. GL-V9 is a synthesized flavonoid derived from natural active ingredient wogonin and shows potent growth inhibitory effects in liver and breast cancer cells. In this study, we investigated the anti-cSCC effect and the underlying mechanism of GL-V9. The results showed that GL-V9 induced both apoptosis and autophagy in human cSCC cell line A431 cells, and prevented the growth progression of chemical induced primary skin cancer in mice. Metabolomics assay showed that GL-V9 potentially affected mitochondrial function, inhibiting glucose metabolism and Warburg effect. Further mechanism studies demonstrated that AKT played important roles in the anti-cSCC effect of GL-V9. On one hand, GL-V9 suppressed AKT-modulated mitochondrial localization of HK2 and promoted the protein degradation of HK2, resulting in cell apoptosis and glycolytic inhibition. On the other hand, GL-V9 induced autophagy via inhibiting Akt/mTOR pathway. Interestingly, though the autophagy induced by GL-V9 potentially antagonized its effect of apoptosis induction, the anti-cSCC effect of GL-V9 was not diluted. All above, our studies suggest that GL-V9 is a potent candidate for cSCC treatment.

discussion

GL-V9 exerts anti-T cell malignancies effects via promoting lysosome-dependent AKT1 degradation and activating AKT1/FOXO3A/BIM axis

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BIM

ABSTRACT

T-cell malignancies are characterized by the excessive proliferation of hematopoietic precursor cells of T-cell lineage lymphocytes in the bone marrow. Previous studies suggest that T-cell malignancies are usually accompanied by highly activated PI3K/AKT signaling which confers the ability of cancer cells to proliferate and survive. Here, we found that GL-V9, a newly synthesized flavonoid compound, had a potent to inhibit the activation of AKT1 and induce the cell apoptosis in T-cell malignancies including cell lines and primary lymphoblastic leukemia. Results showed that GL-V9-induced degradation of AKT1 blocked PI3K/AKT1 signaling and the degradation of AKT1 could be reversed by NH_4Cl, an inhibitor of lysosomal function. Inhibiting AKT1 promoted dephosphorylation of FOXO3A and its nuclear translocation. We further demonstrated that GL-V9-induced apoptosis effects were dependent on the binding of FOXO3A to the *BIM* promoter, resulting in the production of BH3-only protein BIM. Moreover, GL-V9 showed a more persistent and stronger apoptosis induction effects than pharmacologic PI3K inhibitor. The *in vivo* studies also verified that GL-V9 possessed the antitumor effects by reducing the leukemic burden in T-ALL-bearing *BALB/c* nude mice. In conclusion, our study provides a new insight into the mechanism of GL-V9-induced apoptosis, suggesting the potency of GL-V9 to be a promising agent against T-cell malignancies.

Conclusion

- Mitochondrial binding of HKII promotes cell survival (26).

- Anti-cancer mechanism of GL-V9:
  - ✓ Reduction the activity and expression of AKT → activation of GSk3ß
  - ✓ Promotion in binding of GSk3ß to VDAC instead of HKII
  - ✓ Dissociation of HKII from mitochondria
  - ✓ Inhibition of aerobic glycolysis, induction of mitochondrial apoptosis
  - ✓ In-vivo experiments confirmed the results

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