Cutaneous melanoma is one of the most aggressive types of skin cancer. It is insensitive to chemotherapy and radiotherapy and has an extremely poor prognosis due to metastasis or recurrence.[1] Recently, the incidence of melanoma has escalated to make it one of the most rapidly growing malignant tumors.[2] Although a variety of immune or targeted therapy drugs have emerged, most of them remain unavailable and unable to cure patients.

Melanoma-initiating cells (MICs) play important roles in melanoma progression.[3, 4] The MICs are a group of cells with stem cell-like self-renewal ability and heterogeneity. It is suggested that MICs act in the proliferation, invasion, and metastasis of melanoma.[6, 7] The proportion of MICs in melanoma cells is less than 0.5%.[8] However, they are important for the metastasis of melanoma cells due to their movement and migration ability. Moreover, MICs are
resistant to chemotherapy and radiochemotherapy due to their long-term dormancy.\textsuperscript{[6, 9]} Thus, targeted deletion of the MICs is very important for treating melanoma.

Studies have shown that the structure of the mitochondria in MICs is defective. Thus, aerobic metabolism is suppressed in the MICs.\textsuperscript{[10, 11]} Because of this, glycolysis is one of the main energy sources required for melanoma cell growth and self-renewal of the MICs.\textsuperscript{[10]} Furthermore, melanoma and MICs achieve rapid capacity acquisition through glycolysis either in aerobic or hypoxic conditions via the “Warburg effect.”\textsuperscript{[12, 13]}

Pyruvate kinase (PKM) and lactate dehydrogenase (LDH) are important catalytic enzymes involved in glycolysis.\textsuperscript{[14, 15]} The cellular functions of PKM2 and LDH in cancer include accelerating cell proliferation, migration, invasion, autophagy, aerobic glycolysis, and other biological processes of cancer development.\textsuperscript{[16-18]} Tumor-specific PKM2 is essential for the Warburg effect because of its well-established role in aerobic glycolysis.\textsuperscript{[16]} Therefore, PKM2 is closely correlated with glycolysis in various cancer types like colorectal cancer\textsuperscript{[19]} and ovarian cancer.\textsuperscript{[20]} PKM2 expression is regulated by signaling pathways, tumor hypoxia, and the microenvironment.\textsuperscript{[17]} Tumor LDH (LDHA) is an LDH isoenzyme that is highly expressed in tumor cells and catalyzes the redox reaction between pyruvate and lactate during glycolysis.\textsuperscript{[18]} The high expression of LDHA in patients with metastatic melanoma is an adverse prognostic factor in patients with advanced melanoma.\textsuperscript{[21]} LDHA blockade was reported to inhibit the invasion and metastasis of melanoma\textsuperscript{[22]} and improve the efficacy of anti-programmed cell death–1 (PD-1) therapy in melanoma.\textsuperscript{[23]} In conclusion, PKM2 and LDHA are involved in melanoma glycolysis and play roles in melanoma treatment.

Recent studies have pointed to the important role of artemsinin (ART) and its derivatives in cancer.\textsuperscript{[24-26]} Dihydroartemisinin (DHA), a derivative of ART that is extracted from Artemisia annua, is involved in the regulation of tumor metabolism.\textsuperscript{[27]} The water solubility of DHA is better than that of ART.\textsuperscript{[24]} DHA exhibits antitumor activity against lung cancer,\textsuperscript{[28]} liver cancer,\textsuperscript{[29]} and colorectal cancer.\textsuperscript{[30]} However, its role in MICs is unclear.

In this study, we demonstrated that DHA significantly deregulates the self-renewal of MICs. It inhibits the proliferation and invasion of melanoma cells and inhibits carcinogenesis in vivo. Furthermore, DHA treatment could reduce ATP production. Moreover, DHA treatment downregulated the activity of PKM2 and LDHA in melanoma cells, but it did not regulate the expression of PKM2 and LDHA proteins. Moreover, DHA could covalently bind to the protein skeleton of PKM2 and LDHA through its sesquiterpene lactone structure. Therefore, we hypothesize that DHA regulates self-renewal of human MICs by targeting PKM2/LDHA-related glycolysis.

Methods

Cell Lines and Culture

The human melanoma cancer cell lines A2058 (ATCC® CRL-11147) and G361 (ATCC® CRL-1424) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These two cell lines were grown in DMEM/F-12 (11320-033, Gibco™, Thermo Fisher Scientific, Shanghai, China) and DMEM high glucose (11965-092, Gibco™, Thermo Fisher Scientific, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, 10082-139, Invitrogen) in a humidified 5% CO\textsubscript{2} atmosphere at 37°C.

Antibodies and Reagents

Primary antibodies used were anti-CDK1 (ab133327, Abcam, Cambridge UK), anti-CDK2 (ab232753, Abcam, Cambridge UK), anti-cyclin A1 (ab53699, Abcam, Cambridge UK), anti-cyclin B1(ab32053, Abcam, Cambridge UK), anti-cyclin B2 (ab185622, Abcam, Cambridge UK), anti-p53 (ab26, Abcam, Cambridge UK), anti-p27 (ab32034, Abcam, Cambridge UK), anti-cyclin D1 (ab134175, Abcam, Cambridge UK), anti-α-tubulin (66031-1-Ig, Proteintech, China), anti-cyclin D1 (ab53699, Abcam, Cambridge UK), anti-p27 (ab32034, Abcam, Cambridge UK), anti-cyclin D1 (ab134175, Abcam, Cambridge UK), anti-α-tubulin (66031-1-Ig, Proteintech, China), and anti-GAPDH (181602, Abcam, Cambridge UK) antibodies. The compounds used were DHA (D7439, Sigma-Aldrich) and DMSO (D2650, Sigma-Aldrich).

Cell Viability Assay

The cell viability of melanoma cells following DHA treatment was measured using colorimetric MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay) (Promega (Beijing) Biotech Co., Ltd.). The A2058 and G361 cells were seeded in a 96-well plate at a density of 2000 cells/well. After cell attachment, the cells were treated using DHA at varying concentrations (0, 3, 6, 12, and 24 μM) in medium and were kept for incubation for 72 h. Detection regent was added to each well, and the cells were incubated for another 2 h at 37°C. Thereafter, the absorbance values of samples from each well were determined using a multi-well plate reader (Varioskan Flash, Thermo Fisher, USA) at 490 nm. The DMSO-treated wells were used as the vehicle control. Similarly, data were obtained for all replicates, and the percentage viability was calculated compared to the control groups.

EdU Cell Proliferation Assay

Cells were inoculated in 96-well plates with 1 × 10\textsuperscript{4} cells/well. Following cell attachment, DHA was added at vary-
ing concentrations (0, 6, 12, and 24 μM) to A2058 and G361 cells for 48 h. Dilute EdU solution (reagent A) was used at a ratio of 1000:1 to prepare 50 μM EdU medium. After cell fixation and apollo staining, image acquisition and analysis were performed.

**Nude Mouse Xenograft Assay**

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kunming Medical University. Female BALB/c nude mice were obtained from the Beijing Vital River Laboratory Animal, Inc. Mice were four to five weeks old and randomly divided into two groups (n=5/group). Mouse health was monitored daily, and mice were fed with water and standard food. A2058 was subcutaneously inoculated into generated mouse tumor models. Cells (1 × 10⁶) were suspended in 50 μL PBS in a 1-mL syringe before injecting into the right flank of the mice. The length and width of the xenograft tumors were measured every three days with a Vernier caliper. Mice were sacrificed seven weeks after inoculation, and the xenograft tumors were excised and weighed. Tumor volumes (cm³) were calculated based on the length and width.

**Primary and Secondary Sphere Forming Assays**

All sphere assays were performed with Ultra Low Attachment Multiple Well Plates (3471, Corning, NY, USA) in stem cell medium. Cells were seeded at a density of one to four viable cells/well for melanoma cell lines for the sphere assays. DMEM/F12 (Hyclone, Thermofisher, USA) supplemented with 20 ng/mL bFGF (BD Biosciences, NY, USA), 20 ng/mL EGF, and B27 (Invitrogen, NY, USA) was used for sphere culture.

**Primary Sphere Assay**

Cells were plated with one to four viable cells/well. Medium was replaced with fresh medium every three to five days. The cells of the experimental group were treated with DHA on day 0, while cells of the control group were treated with DMSO. The spheres were allowed to grow to a reasonable size until day 14 after seeding.

**Secondary Sphere Assay**

The procedures were the same as the primary sphere culture, except that cells were plated with three to five viable cells from primary spheres per well.

**Protein Extraction and Western Blotting**

Total cellular protein was extracted using a lysis buffer containing protease and phosphatase inhibitors. After quantification with a standard protein quantification method, 50 μg protein samples were separated using SDS-PAGE before transferring onto a 0.45-μm pore size PVDF membrane. The membranes were incubated with 8% skim milk at room temperature for 1 h and with the primary antibody overnight at 4°C. The next day, after washing with PBS-T three times, the membranes were incubated with the secondary antibody at room temperature for 30 min. Finally, the ECL kit was added, and the membranes were exposed to X-ray film.

**Measurements of Lactate Production**

Cell medium obtained from wells was pelleted at high speed to remove cell debris. Next, the pH value was adjusted to 7.0–7.5, and lactate generation was analyzed using a kit (93-K627-100, BioVision, USA) according to the manufacturer’s instructions. The result was normalized to that of the control group.

**Measurements of Intracellular ATP Levels**

Cells were cultured at 2.0 × 10⁵ cells/mL. After incubation with DHA (0, 3, 6, 12, and 24 μM) for 72 h, A2058 and G361 cell lysates were collected. Intracellular ATP levels were measured using an ATP Assay Kit (S0026, Beyotime, China). Luminescence was detected using a fluorescence plate reader (Varioskan Flash, Thermo Fisher, USA), and the result was normalized to that of the DMSO control group.

**Determination for PKM2 Activity**

PKM2 activity detection requires cell lysate (2g), 1 mol/L KCl, 1 mol/L Tris-HCl (pH 8.0), 0.1 mol/L MgCl2, 30 mmol/L ADP, 2 mmol/L LDH, NADH, and 50 mmol/L PEP. The absorbance wavelength at 340 nm was determined using a fluorosence plate reader. The activity of PKM2 in each DHA-treated group was normalized to that of the DMSO control.

**Statistical Analysis**

All statistical analyses were performed using the SPSS software (version 13.0. SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to evaluate statistically significant differences. Statistical analysis for mouse tumor growth data was conducted using a simple effect test for pairwise comparisons of average fold change in tumor volume between treatment groups. The analyses with p-value of 0.05 or less were considered statistically significant.

**Results**

**DHA Reduced Melanoma Cell Proliferation**

To determine the effect of DHA on melanoma cell line viability, we first treated the melanoma cell lines A2058, G361, and Hs852.T and the immortalized keratinocytes Hacat with different concentrations of DHA for 72 h before calculating the IC50 of the four DHA-treated cell lines using the Bliss method. The results showed that the IC50 values of DHA-
treated A2058, G361, and Hs852.T were 39.58±7.57 μM, 42.41±1.01 μM, and 81.5±7.89 μM, respectively. The IC50 of Hacat was more than 800 μM (Fig. 1a). Thus, we selected the A2058 and G361 cell lines and drug concentrations of 3, 6, 12, and 24 μM to treat the A2058 and G361 cell lines in follow-up experiments.

Using the MTS and Colon assays, we found that DHA inhibited the proliferation of A2058 and G361 cells in a time- and dose-dependent manner, and DHA significantly inhibited melanoma cell proliferation (Fig. 1b). Moreover, EdU staining indicated that DHA treatment reduced the proliferation of melanoma cells (Fig. 1c). Furthermore, DHA blocked A2058 cells in the S and G2/M phases and G361 cells in the G0/G1 phase (Fig. 1d). The expression of CDK1 and CDK2 in DHA-treated A2058 cells was suppressed (Fig. 1e). Collectively, these data indicate that DHA plays a vital role in the regulation of the G2/M phase and G0/G1 phase of the cell cycle in human A2058 and G361 cell lines, and it could suppress the proliferation of cells via cell cycle arrest.

**DHA Inhibited Melanoma Carcinogenesis in Mice**

The cancer-preventive effects of DHA were evaluated in mice bearing A2058 melanoma cells. Mice were injected intraperitoneally with DHA (40 mg/kg) or vehicle control

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**Figure 1.** DHA inhibited melanoma cell proliferation and induced cell cycle arrest of melanoma cells.

(a) The IC50 of DHA was evaluated in the Hs582.T, G361, and A2058 melanoma cell lines. The Hacat (human immortalized keratinocytes) cells were used as benign contrast. The IC50 values were calculated from three independent experiments and represented the mean±SD (n=3). (b) Cell viability was measured using the MTS assay and clone formation experiment. A2058 and G361 cells were treated with various concentrations of DHA for 0–72 h, respectively. DHA inhibited the proliferation of A2058 and G361 cells in a time- and dose-dependent manner, and DHA significantly inhibited the proliferation of melanoma cells at 48 h. (c) Cell proliferation was measured using EdU with various concentrations of DHA (6, 12, and 24 μM). EdU staining indicated that DHA treatment reduced melanoma cell proliferation. (d) Flow cytometric analysis and quantitation of cell cycle in A2058 and G361 treated with control or DHA. DHA blocked A2058 cells in S phase and G2/M phase and G361 cells in G0/G1 phase. (*p<0.05, **p<0.01, ***p<0.001 vs. vehicle).
(DMSO at 2 mL/kg) for 10 days and examined for tumor volume and tumor weight. As shown in Figure 2a and b, DHA significantly inhibited the progression of tumor formation. After DHA treatment, the volume and weight of tumor nodules were significantly reduced (Fig. 2c and d). These data suggest that DHA exerts an anticancer effect on mice containing melanoma cells.

**DHA Reduces Self-Renewal of Melanoma Stem Cells**

MICs are key factors in the invasion and metastasis of melanoma and resistance to radiotherapy and chemotherapy. Because the tumor sphere assay allows the enrichment of potential MICs, we enriched MICs using tumor sphere culture and treated them with DHA in the primary and secondary generation of spheres. As shown in Figure 3a, the DHA-treated melanoma spheres were significantly fewer and smaller than those of the controls (Fig. 3a). Moreover, DHA treatment inhibited the self-renewal of A2058 and G361 secondary spheres (Fig. 3b). Indeed, treatment with DHA directly destroyed the spheres of A2018 and G361 and induced the maturation of MICs (Fig. 3c). Protein analysis showed that DHA reduced the expression of SOX2, C-MYC, N-cadherin and KLF4 in both sphere types (Fig. 3d). Taken together, these results indicate that DHA suppresses self-renewal and inhibits survival of MICs.

**DHA Interferes with PKM2 and LDHA Activities and Suppresses ATP Production in Melanoma Cells**

To detect the effect of DHA on melanoma cell glycolysis, we measured ATP and lactic acid production in melanoma cells (A2058 and G361) treated with DHA. Interestingly, DHA treatment inhibited ATP production, but it did not suppress lactic acid synthesis (Fig. 4a). Since PKM and LDH are important catalytic enzymes in glycolysis, we detected the effect of PKM2 and LDHA after DHA treatment. According to the BIACore analysis, DHA could covalently bind to the protein skeleton of PKM2 and LDHA through its sesquiterpene lactone structure (Fig. 4b). DHA downregulates the activity of PKM2 and LDHA in melanoma cells (Fig. 4c), but it did not regulate the expression of PKM2 and LDHA proteins (Fig. 4d). This suggests that DHA might directly affect the active structure of PKM2 and LDHA and inhibit its activity.
Discussion

In this study, we identified DHA as an inhibitor of the self-renewal of MICs. Our results indicate that DHA reduces the synthesis of lactate and ATP in melanoma cells. DHA treatment strongly inhibits the proliferation of melanoma cells and restricts the self-renewal of MICs. In vivo, DHA inhibits the tumorigenic activity of melanomas in nude mice. Furthermore, we demonstrated that DHA downregulates the activity of PKM2 and LDHA in melanoma cells. DHA does not regulate the expression of PKM2 and LDHA proteins (*p < 0.05 vs. vehicle).

Figure 4. DHA interferes with PKM2 and LDHA activities and suppresses ATP production in melanoma cells. (a) DHA treatment inhibited ATP production but did not suppress lactic acid synthesis. (b) DHA could covalently bind to the protein skeleton of PKM2 and LDHA through its sesquiterpene lactone structure. (c) DHA downregulates the activity of PKM2 and LDHA in melanoma cells. (d) DHA does not regulate the expression of PKM2 and LDHA proteins (*p < 0.05 vs. vehicle).

LDHA, another key target of DHA, is an LDH isoenzyme that is highly expressed in tumor cells and catalyzes the redox reaction between pyruvate and lactate during glycolysis. The high expression of LDHA in metastatic melanoma is an adverse prognostic factor in patients with advanced melanoma. LDHA blockade reportedly inhibits melanoma invasion and metastasis. Moreover, LDHA promotes cell stemness and antitumor immunity. Thus, it is believed that PKM2 and LDHA play an important role in MIC self-renewal. By targeting PKM2/LDHA-related glycolysis, DHA regulates the self-renewal of human MICs.

Although DHA has been used clinically to treat malaria, its antitumor effect still deals with the preclinical stage, and the effective concentration is relatively high. The adverse reactions in antitumor applications are still unknown. However, with the improvement of dosage form and effective blood concentration, as well as effective monitoring of adverse reactions, DHA still has good prospects in the clinical treatment of melanoma.

In summary, these findings showed that DHA targeted the key enzymes PKM2 and LDHA, which are important enzymes for glycolysis in melanoma cells, without regulating their protein expression. Using structural analysis, we found that DHA could covalently bind to the protein skeleton of PKM2 and LDHA through its sesquiterpene lactone structure. Thus, DHA is thought to regulate self-renewal of human MICs by targeting PKM2/LDHA-related glycolysis.

Disclosures

Ethics Committee Approval: All experimental procedures were approved by the Ethics Committees of the Kunming Medical University. Human-related clinical study was not performed in this report.
Availability of data and materials: We declare that the materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

Competing interests: All authors declare that they have no competing interests.

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Authors’ contributions: ZL conceived the study, conducted the experiments, wrote the manuscript; SD supervised the study, conceived the study; BZ performed cellular and animal studies and helped to draft the manuscript; YX and XM analysed the data, software and conducted the experiments; BW conceived the study; ZL participated in the study design and coordination, funding acquisition, and project administration.

All authors read and approved the final manuscript.

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