

Original Article

C16:0 ceramide effect on melanoma malignant behavior and glycolysis depends on its intracellular or exogenous location

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Abstract: To investigate the role of C16:0 ceramide in melanoma metastatic behavior and glycolysis, five common long-chain ceramides (C16:0, C18:0, C20:0, C22:0, C24:0) were tested in melanocyte and melanoma cell lines by LC-MS. We then treated non-metastatic and metastatic melanoma cells with PDMP and exogenous C16:0 to explore their effects on proliferation, migration, and glycolysis. The long-chain ceramide was also analyzed by LC-MS after treatment. C16:0 ceramide showed the highest levels in melanocyte and melanoma cells, with all melanomas higher than melanocytes. PDMP inhibited malignant behavior and glycolysis in melanoma, and caused the accumulation of intracellular C16:0. Exogenous C16:0 promoted melanoma glycolysis, but not malignant behavior, and decreased intracellular C16:0. Finally, pyruvate kinase (PK), hexokinase (HK), and lactic acid dehydrogenase (LDH) activity, key enzymes in glycolysis, were altered after treatment with PDMP and exogenous C16:0.

Keywords: Melanoma, long-chain ceramide, metastasis, glycolysis, intracellular ceramide, exogenous ceramide

Introduction

Ceramides are important components of cell membranes. They are a lipid component of sphingomyelin, which is involved in multiple signaling pathways related to apoptosis, autophagy, and the cell cycle in various tumors and is considered a target for cancer therapy [1]. The CERS6 gene encoding ceramide synthase 6, which catalyzes C16:0 ceramide synthesis, was found to promote metastasis in lung cancer cells [2].

In the clinic, malignant melanoma is frequently diagnosed in a metastatic state when first diagnosed. It is therefore crucial to find targets for malignant melanoma early detection and metastasis inhibition. Our previous study found that the CERS6 gene could inhibit melanoma invasion and glycolysis through regulation of the WNT5A pathway via GLUT1 [3]. Some stud-

ies reported that short chain ceramides such as C2:0 and C6:0 could inhibit melanoma [4, 5], while C16:0 levels were increased during apoptosis in cells treated by ionizing radiation [6]. The effects of changing C16:0 levels induced by CERS6 are unclear. Our study aims to further explore the role of C16:0 and ceramides in melanoma because of their effects on melanoma metastatic behavior and glycolysis.

Materials and methods

Cell culture and reagents

Primary human melanocytes (HM) and melanoma cell lines WM35, WM451, SK-MEL-28, and A375 details can be found in our previous publication [3, 7]. All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and then in-

C16:0 ceramide's role in melanoma malignant behavior and glycolysis

cubated at 37°C in a humidified incubator with 5% CO₂. Culture media, FBS, and 1% penicillin/streptomycin were purchased from Gibco (NY, USA). C16:0 ceramide (BML-SL115-0005) and PDMP (BML-SL210-0010) were purchased from Enzo (NY, USA). Before adding C16:0 ceramide and PDMP to cells, cells were cultured in media without serum for 24 h.

MTT and colony formation assays

For MTT proliferation assays, cells were seeded into 96-well plates (3,000 cells/well), then treated with media containing PDMP (5, 10, 20, 30 and 40 μM), C16:0 ceramide (20 μM), and control media (DMSO). MTT (5 mg/mL) was added to cells followed by 150 μL DMSO (4 h after MTT application) at 24, 48, and 72 h. OD values were measured at 490 nm. Time-OD growth curves were generated to monitor cell proliferation.

For colony formation assays, cells were seeded in 10 cm dishes (1,000 cells/plate), then treated with media containing PDMP, C16:0 ceramide, and control media and maintained for 21 days. Cells were then fixed in 4% paraformaldehyde for 15 min and stained with Gimsa dye. Cells were imaged and the number of colonies counted.

Scratch migration and transwell invasion assays

Cells were seeded into 6-well plates (5 × 10⁵ cells/well) and cultured for 48 h to 90% confluence. A straight scratch was made using a sterile 1-μL tip, and then the cells were treated with media containing PDMP, C16:0 ceramide, and control media. Cells were continuously cultured and imaged at 0 and 24 h (Olympus, Japan).

To measure invasive capacity, transwell invasion assays were performed using cells plated on 8 μm pore size Matrigel-coated membranes at 1 × 10⁵ cells/well in serum-free media, which were placed in the top chamber of 24-well transwell plates. DMEM containing 10% FBS with PDMP, C16:0 ceramide, or control media was added into bottom chambers as a chemoattractant. After 24 h, cells on the upper surface were removed, and cells attached on the bottom of membranes were fixed in 4% paraformaldehyde for 20 min and stained with Gimsa dye. Cells were imaged and counted under microscope (Olympus, Japan).

Long-chain ceramide testing

Liquid chromatography-mass spectrometry was used to test five common endogenous long-chain ceramide (C16:0, C18:0, C20:0, C22:0, C24:0) content in cells. Cell density was calculated for each group, and 5 × 10⁶ cells were collected and centrifuged at 1,000 rpm for 10 min. Supernatant was discarded, 1 mL saline was added, and cells were resuspended and lysed by ultrasound (300W). Then, 1.2 mL N-hexane was added for 30 min to extract lipids, then centrifuged at 14,000 × g for 5 min. The upper phase containing lipids was collected and evaporated to dryness under a nitrogen stream. Dry lipids were dissolved in 50 μL mobile phase, centrifuged, and 5 μL supernatant was injected into the chromatographic column (Thermo Hypersil-HyPURITY C18, 150 nm × 2.1 nm, 5 μm), with a column temperature of 35°C. Intracellular ceramides were separated from byproducts with the mobile phase, which were made up of methyl alcohol, acetonitrile, and water at a rate of 89.5:10:0.5. The flow rate was 0.22 mL/min. The SIM of m/z was 538.5 (C16:0), 564.70 (C18:0), 594.75 (C20:0), 622.80 (C22:0), and 650.80 (C24:0). We recorded the chromatogram and used the peak area to determine ceramide content expressed as ng/10⁶ cell number.

Detection of glucose uptake and lactic acid production

WM35 and WM451 cells were collected at a density of 1 × 10⁶ cells/mL. 1 × 10⁶ cells in 10 mL culture media were seeded per dish, whereas 10 mL culture media without cells were also kept in the same environment (blank group). Then, 24 h after seeding, PDMP, C16:0, and NS (control group) were added. 1 mL culture media was collected at 0 and 72 h after treatment in each group (including blank group), and concentrations determined according to manufacturer's protocols using the Glucose Detection Kit (F006) and Lactic Acid Assay Kit (A019-2) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Cells were collected at 72 h, and cell numbers were counted. Cell ratio (N) was calculated as treatment group cell number divided by control group cell number. Glucose concentration was tested in each group and relative glucose uptake calculated as below. Relative glucose uptake = [(blank group - treatment group) ÷ N]/(blank group - control group).

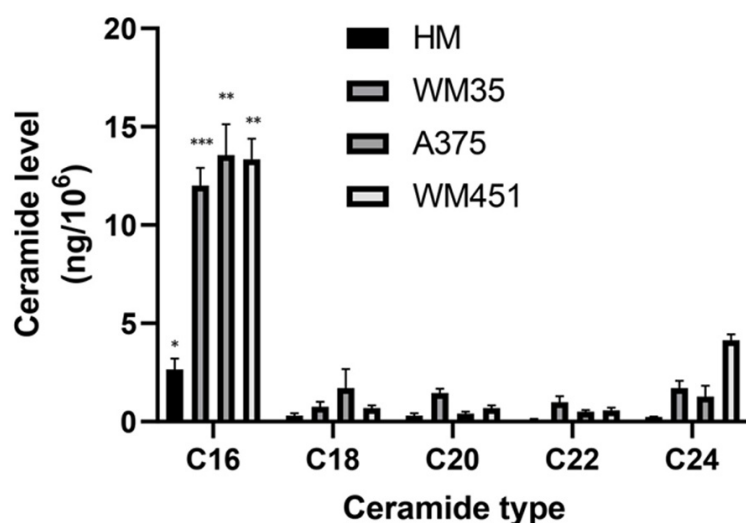


Figure 1. Long-chain ceramide content in melanoma cells (WM35, WM451, A375) and melanocytes (HM). Levels of the five most common long-chain ceramides (C16:0, C18:0, C20:0, C22:0, C24:0). C16:0 is significantly higher in all cell lines compared to other types of long-chain ceramides. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Lactate concentration was also tested in each group and relative glucose uptake calculated as below. Relative Lactate production = [(treatment group - blank group) ÷ N]/(control group - blank group).

Detection of hexokinase, pyruvate kinase, and lactate dehydrogenase activity

To detect hexokinase (HK), pyruvate kinase (PK), and lactate dehydrogenase (LDH) activity at 0 and 72 h after PDMP and C16:0 ceramide application, 5×10^6 cells were collected and centrifuged at 1,000 rpm for 10 min. Supernatant was discarded, 1 mL 0.1% Triton X-100 was added at 4°C, and cells were resuspended for lysis by ultrasound (45W). Cells were kept on ice for 30 min, then centrifuged at $14,000 \times g$ for 5 min. Supernatant was collected and protein concentration was determined by BCA kit. After normalizing protein concentrations, activity measurements were taken according to manufacturer's protocols using the Hexokinase Assay Kit (A077-2), Pyruvate Kinase Assay Kit (A076-2), and Lactate Dehydrogenase Assay Kit (A020-1) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

Experiments were repeated three times, and data shown as mean \pm SD. Either a t-test or

ANOVA test was used to compare data between groups. Data were analyzed using SPSS 19.0 software, with $P < 0.05$ considered as statistically significant.

Results

C16:0 ceramide levels in melanocyte and melanoma cells

Although C16:0 is the most common fatty acid in mammalian cells, its levels compared to other long-chain ceramides in melanocytes and melanoma cells is unknown. We selected the five most common long-chain ceramides (C16:0, C18:0, C20:0, C22:0, and C24:0), and measured their levels in human melanocytes (HM) and three melanoma cell lines (WM-

35, A375, WM451) by LC-MS. C16:0 was the most abundant of the five long-chain ceramides in both melanocytes and the melanoma cells tested (unpaired t-test to each group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) (Figure 1). Compared to HM, C16:0 levels were higher in the melanoma cells and C22:0 in HM is also significantly lower than WM35, A375, and WM451 (unpaired t-test, $P < 0.05$) (*Table 1).

Intracellular C16:0 ceramide accumulation is induced by PDMP

After confirming that C16:0 is the major component of long-chain ceramides in melanocytes and melanoma cells, we considered the ability of the CERS6 gene to inhibit melanoma invasion and glycolysis, as C16:0 levels may play a role in this inhibition [3]. To investigate the effects of C16:0 on melanoma cells and exclude the effects of the CERS6 gene, we used PDMP to inhibit glucosylceramide synthase, reducing ceramide catabolism and increasing intracellular ceramide accumulation [8]. To confirm its accumulating effect on ceramides, the five long-chain ceramides (C16:0, C18:0, C20:0, C22:0, and C24:0) were measured by LC-MS in WM35 and WM451 melanoma cells after treatment with 20 μ M PDMP. C16:0 increased significantly in WM35 cells (unpaired t test, * $P < 0.05$), but not WM451 cells (unpaired t test, $P > 0.05$) (Figure 2).

Table 1. Long Chain Ceramides Volume in different cell line (ng/106)

Cell Line	Quantification of 5 most common long chain ceramides				
	C16	C18	C20	C22	C24
HM	2.657 ± 0.5570*	0.3100 ± 0.1193	0.3100 ± 0.1193	0.0900 ± 0.05292*	0.2400 ± 0.02517
WM35	12.01 ± 0.8967	0.7567 ± 0.2483	1.454 ± 0.2284	0.9900 ± 0.3051	1.715 ± 0.3626
A375	13.56 ± 1.565	1.710 ± 0.9600	0.4057 ± 0.1091	0.5100 ± 0.07572	1.267 ± 0.5629
WM451	13.34 ± 1.051	0.6900 ± 0.1320	0.6900 ± 0.1320	0.5767 ± 0.1372	4.136 ± 0.3104

Quantification of the five most common long-chain ceramides (C16:0, C18:0, C20:0, C22:0, C24:0) in human melanocytes (HM) and three melanoma cell lines (WM35, A375, WM451) by mass spectrometry. *Volume of C16-cer and C22-cer in HM is always significantly lower than WM35, A375, and WM451 (unpaired t-test, *P < 0.05).

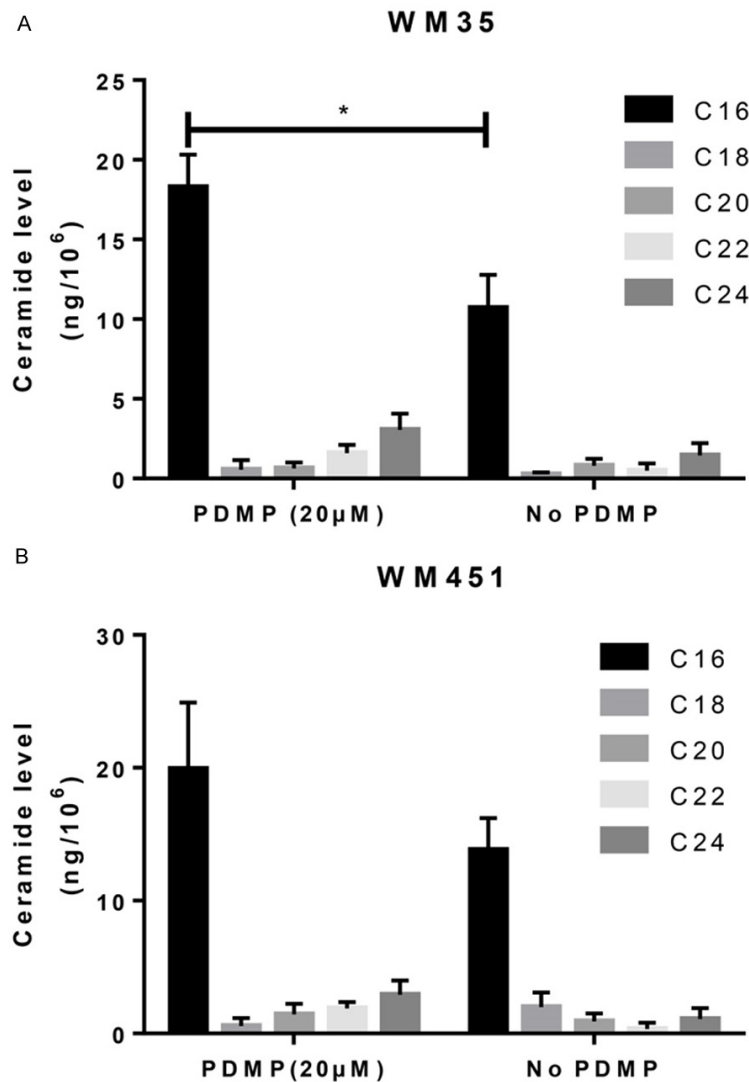


Figure 2. The effect of PDMP on WM35 and WM451 long-chain ceramide levels. The WM35 and WM451 melanoma cell lines were treated with 20 μM PDMP. The five long-chain ceramides (C16:0, C18:0, C20:0, C22:0, C24:0) were measured by LC-MS. A significant change in C16:0 was observed in WM35 (*P < 0.05), whereas the increase in C16:0 in WM451 was not significant (P > 0.05).

PDMP inhibits malignant behavior and glycolysis in melanoma cell

Proliferation and migration are the most important malignant phenotypes of cancer progression. MTT and colony formation assays were performed to assess melanoma cell proliferation after PDMP application. In MTT assays, the OD value of 24, 48 and 72 hours was recorded and shown by line graph, PDMP had an obvious concentration-based inhibitory effect on WM35 cell proliferation. For WM451 cells, PDMP had some concentration-based inhibition, but not as obvious as in WM35. But the concentration of each group did not have significant differences both in WM35 and WM451 (2-way ANOVA, P > 0.05) (Figure 3A). Colony formation assays also showed significantly higher inhibitory effects for 20 μM PDMP in WM35 cells (unpaired t test, P < 0.05), while WM451 showed limited inhibition (unpaired t test, P > 0.05) (Figure 3B). Overall, PDMP's inhibitory effect on proliferation in melanoma cell, indicating that intracellular C16:0 may play a role in preventing melanoma cell growth.

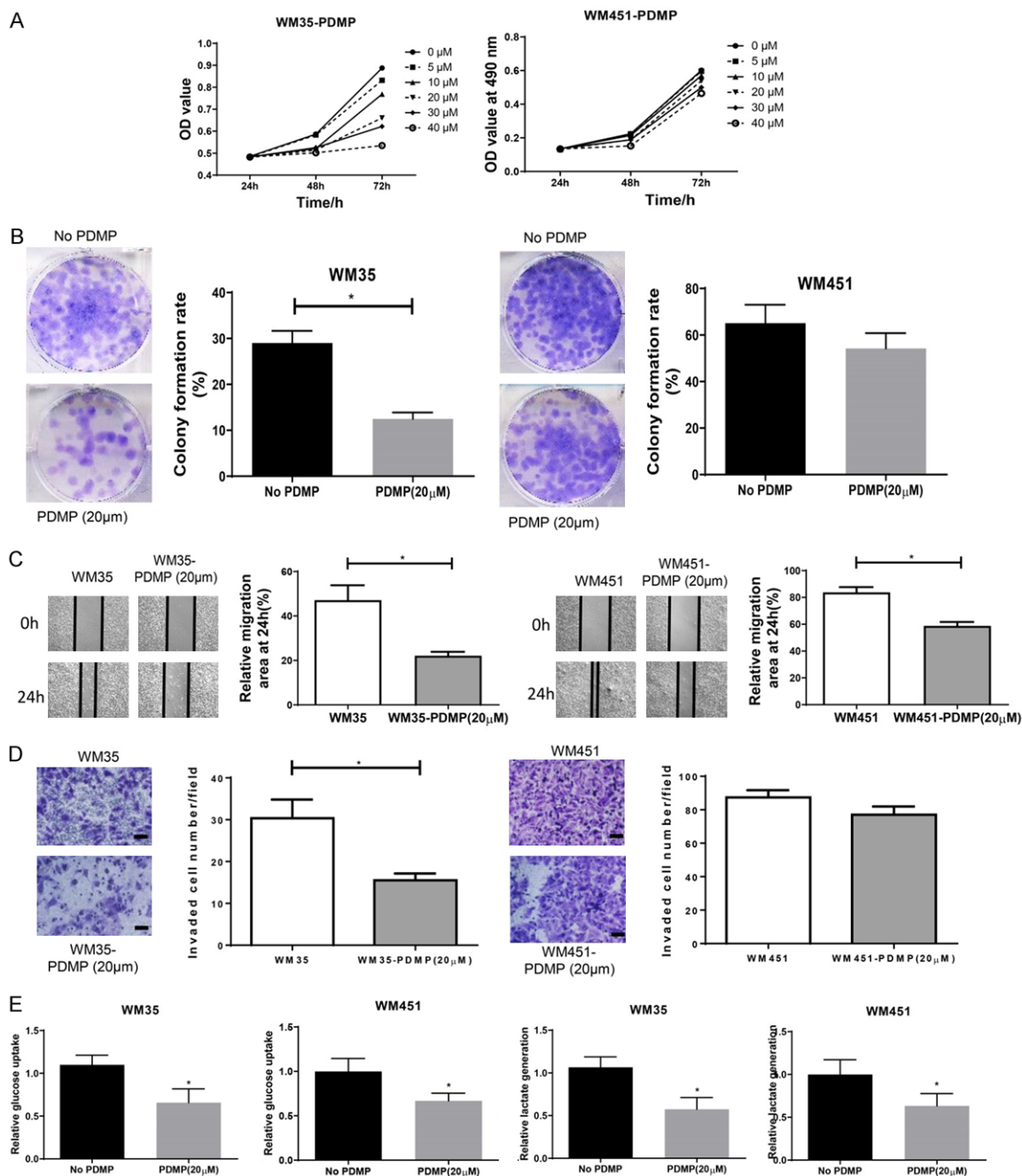


Figure 3. Cellular malignancy and glycolysis changes in WM35 and WM451 after PDMP treatment. WM35 and WM451 cells were treated with different concentrations of PDMP, then tested by MTT assay at 24, 48, and 72 h (A). Colony numbers were counted after 21 days of seeding with or without PDMP treatment. The number was divided by 1000 and presented as a percentage with a representative picture shown. The percentage of WM35 colonies after 20 μ M PDMP treatment was significantly less than the control group ($*P < 0.05$), whereas WM451 did not reach significance ($P > 0.05$) (B). The relative migration area was calculated by migration area, obtained by original scratch area minus remaining area, divided by the original scratch area and shown as a percentage. A representative picture of the scratch area at 0 and 24 h is shown. Both WM35 and WM451 cells showed significantly decreased migration after 20 μ M PDMP treatment ($*P < 0.05$) (C). The low chamber average cell number in microscopic random fields was counted 24 h after treatment with or without 20 μ M PDMP. Representative pictures show a typical microscope field. Bar = 50 μ m. WM35 cells showed a significant decrease ($*P < 0.05$) in invading cell numbers after 20 μ M PDMP treatment, whereas WM451 cells showed no significant difference ($P > 0.05$) (D). Relative glucose uptake and lactic acid yield of WM35 and WM451 cells with or without 20 μ M PDMP treatment was calculated as indicated in the Methods section. Both glucose uptake and lactic acid levels significantly decreased after treatment in WM35 and WM451 cells ($*P < 0.05$) (E).

C16:0 ceramide's role in melanoma malignant behavior and glycolysis

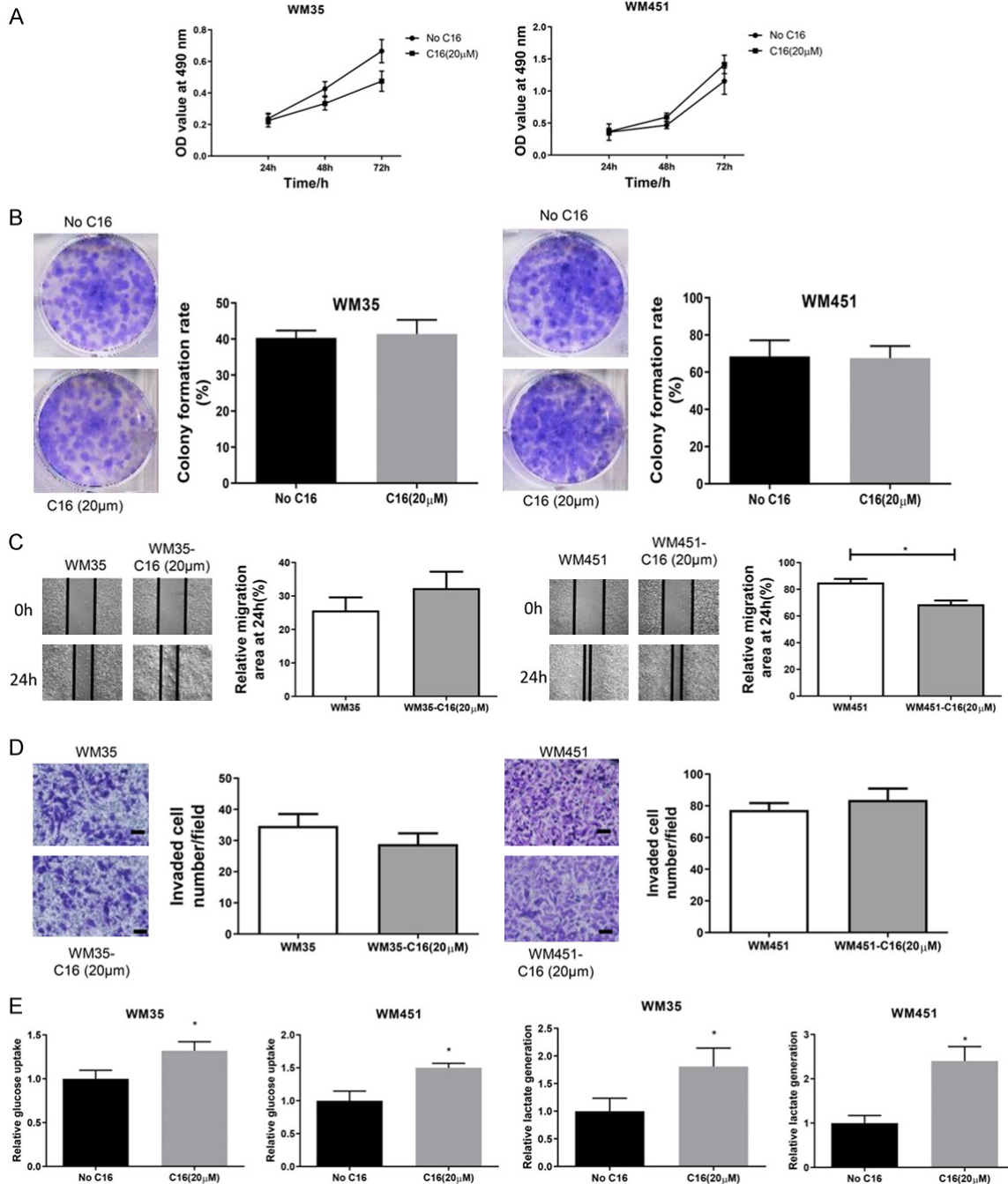


Figure 4. Cellular malignant and glycolysis changes in WM35 and WM451 cells after exogenous C16:0 ceramide application. WM35 and WM451 cells were treated with 20 μ M exogenous C16:0 with control group, then tested by MTT assay at 24, 48, and 72 h (A). Colony number was counted after 21 days of seeding with or without exogenous C16:0 treatment; the number was divided by 1000 and presented as a percentage. A representative picture is shown. The percentage of WM35 and WM451 colonies after exogenous C16:0 treatment showed no significant difference compared to the control group ($P > 0.05$) (B). The relative migration area was calculated by migration area, obtained by original scratch area minus remaining area, divided by the original scratch area and shown as a percentage. A representative picture of the scratch area at 0 and 24 h is shown. WM451 cells showed significantly decreased migration after 20 μ M PDMP treatment ($*P < 0.05$), whereas WM35 cells showed no significant difference ($P > 0.05$) (C). The low chamber average cell number in random microscopic fields was counted 24 h after treatment with or without exogenous C16:0. Representative pictures show a typical microscope field. Bar = 50 μ m. Neither WM35 nor WM451 cells showed significant changes ($P > 0.05$) (D). Relative glucose uptake and lactic acid yield of WM35 and WM451 cells with or without 20 μ M exogenous C16:0 treatment was calculated as indicated in the Methods section. Both glucose uptake and lactic acid yields were significantly increased after 20 μ M PDMP treatment in WM35 and WM451 cells ($*P < 0.05$) (E).

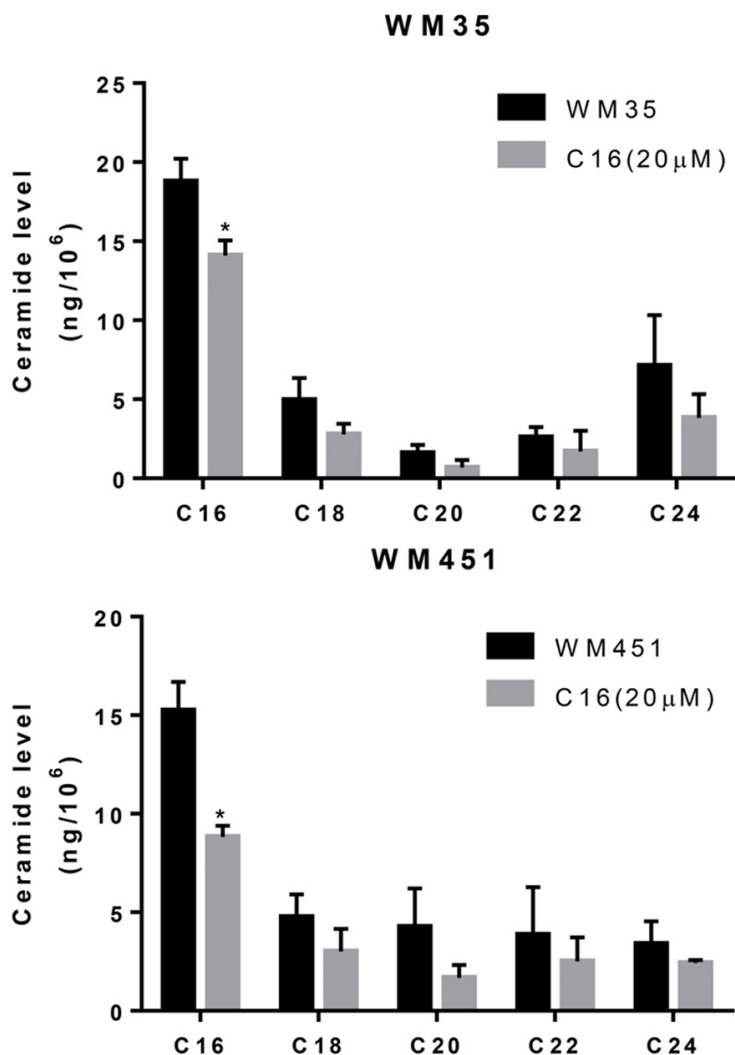


Figure 5. The effect of exogenous C16:0 ceramide treatment on long-chain ceramide levels in WM35 and WM451 cells. The non-metastatic WM35 and highly metastatic WM451 melanoma cell lines were treated with 20 μM exogenous C16:0. The five long-chain ceramides (C16:0, C18:0, C20:0, C22:0, C24:0) were tested by LC-MS. There was a significant decrease in C16:0 in both WM35 and WM451 cells (* $P < 0.05$).

Scratch assays and transwell assays are performed to assess melanoma cell migration. PDMP treatment reduced relative 24 h cell migration area for both WM35 and WM451 cells in scratch assays (unpaired t test, * $P < 0.05$) (Figure 3C). Transwell invasive assays showed that PDMP reduced WM35 invading cell numbers (unpaired t test, * $P < 0.05$), but there was no apparent reduction of invading WM451 cells (unpaired t test, $P > 0.05$) (Figure 3D). These results indicate that PDMP could inhibit migration in both WM35 and WM451

cells, but only inhibited invasion capabilities in WM35 cells.

With PDMP treatment, both WM35 and WM451 cells showed decreased glucose uptake, and the corresponding decrease in lactate, a product of glycolysis, confirmed that the consumed glucose was used for glycolysis (unpaired t test, * $P < 0.05$) (Figure 3E). These findings indicate that PDMP treatment decreased glycolysis in WM35 and WM451 cells.

Exogenous C16:0 ceramide promotes melanoma glycolysis but not malignant behavior

Since PDMP inhibited invasion in the cell line WM35 with limited inhibition in the cell line WM451, and only significantly increased C16:0 levels in WM35 with limited increase in WM451, it seemed that C16:0 would be most relevant for further studies. We therefore selected exogenous C16:0 to determine its effects on melanoma cell malignant behavior and glycolysis. In the MTT assay, 20 μM exogenous C16:0 ceramide did not have significant differences both in WM35 and WM451 (2 way ANOVA, $P > 0.05$) (Figure 4A). In colony forming assay, the percentage of WM35 and WM451 colony

treated by exogenous C16:0 ceramide showed no significant difference to control group (unpaired t test, * $P > 0.05$) (Figure 4B).

For scratch assays and transwell assays, WM451 have significant decreased migration area after 20 μM PDMP treatment (unpaired t test, * $P < 0.05$) whereas WM35 showed no significant differences (unpaired t test, $P > 0.05$) (Figure 4C). Transwell invasive assays showed that C16 didn't reduced WM35 and WM451 invading cell numbers (unpaired t test, $P > 0.05$) (Figure 4D).

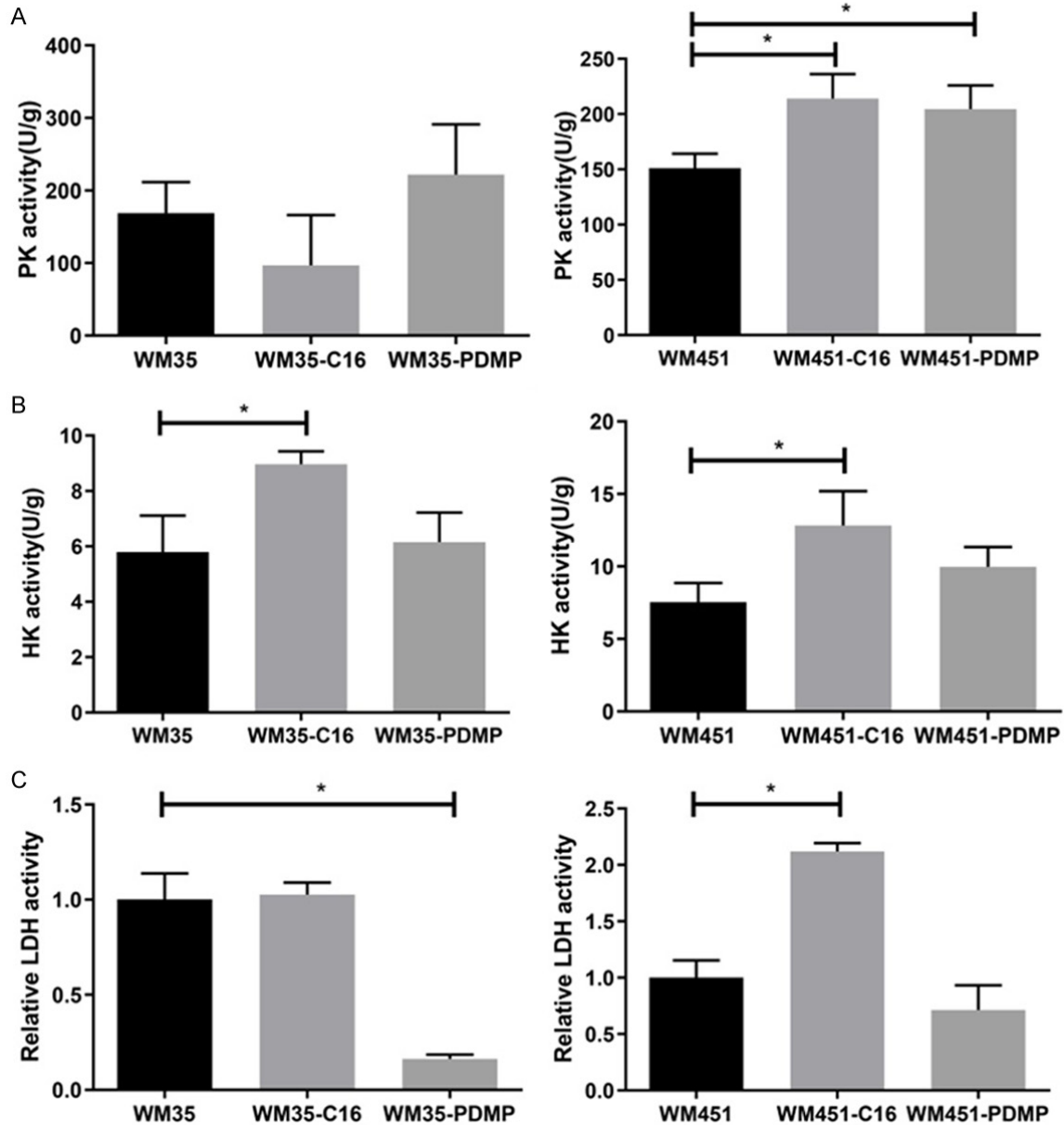


Figure 6. Hexokinase (HK), pyruvate kinase (PK), and relative lactate dehydrogenase (LDH) activity in WM35 and WM451 cells after treatment with PDMP and exogenous C16:0 ceramide. PK activity at 0 and 72 h after PDMP and exogenous C16:0 treatment. WM35 cells did not show significant changes to PK activity either by PDMP or exogenous C16:0 treatment ($P > 0.05$), whereas PDMP and exogenous C16:0 both significantly increased PK activity in WM451 cells ($*P < 0.05$) (A). HK activity at 0 and 72 h after PDMP and exogenous C16:0 treatment. Exogenous C16:0 significantly increased HK activity in both WM35 and WM451 cells, whereas PDMP showed no significant difference ($P > 0.05$) (B). LDH activity relative to control sample at 0 and 72 h. PDMP significantly decreased LDH activity ($*P < 0.05$) in WM35 cells but had no significant effect in WM451 cells ($P > 0.05$). Exogenous C16:0 increased LDH activity significantly ($*P < 0.05$) in WM451 cells but had no significant effect in WM35 cells ($P > 0.05$) (C).

To our surprise, treatment with exogenous C16:0 did not have a significant inhibitory effect on proliferation or invasion in WM35 and WM451 cells, and only limited migratory inhibition in WM451. However, increases in glucose uptake and lactate production in both WM35 and WM451 cells suggested an upregulation of

glycolysis by exogenous C16 application (unpaired t test, $*P < 0.05$) (Figure 4E).

Intracellular C16:0 ceramide is downregulated after exogenous C16:0 ceramide treatment

The inhibitory effects over melanoma malignant behavior and decreased glycolysis was

C16:0 ceramide's role in melanoma malignant behavior and glycolysis

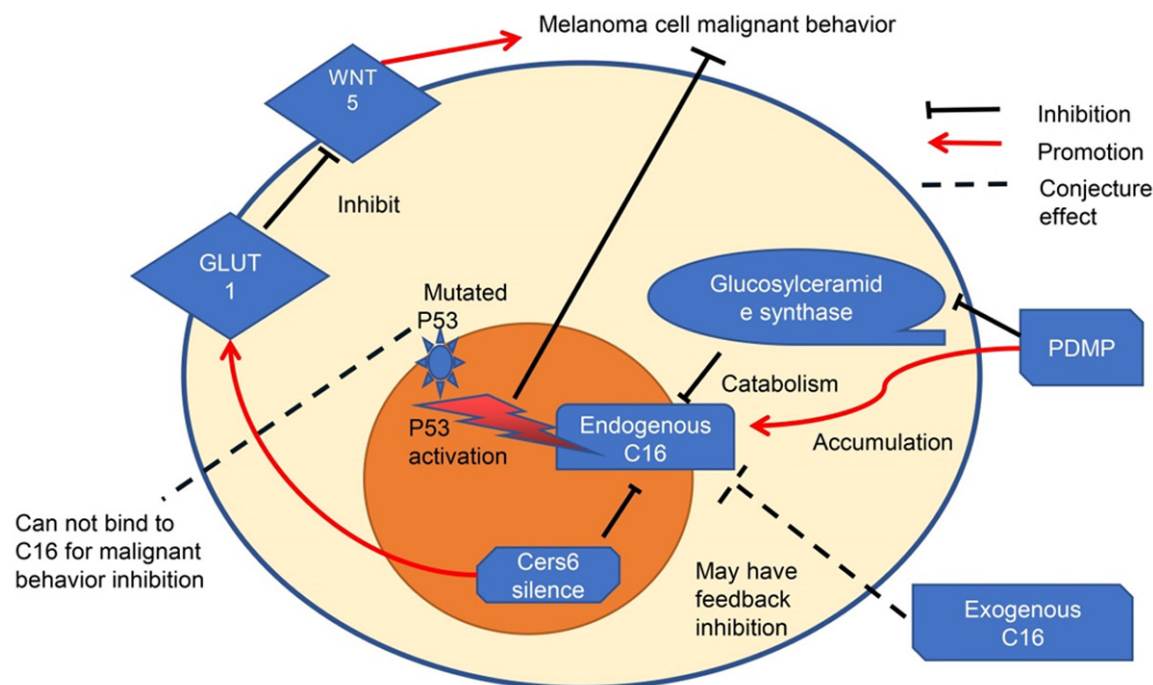


Figure 7. Graphical summary. Conjecture mechanism of PDMP and exogenous C16 on melanoma cell malignant behavior.

the opposite of our expected results. Having only applied exogenous C16:0 to cell culture media, we were not sure whether exogenous C16:0 was entering cells and increasing intracellular C16:0 levels. Therefore, WM35 and WM451 cells were treated with exogenous C16:0 and tested by LC-MS to evaluate intracellular levels of C16:0. Again to our surprise, we observed a decrease in intracellular long-chain ceramides including C16:0 ceramide (unpaired t test, $*P < 0.05$) (**Figure 5**), which may explain why exogenous C16:0 did not have the anticipated inhibitory effect on melanoma malignant behavior and an opposite glycolysis trend from PDMP application.

PDMP and exogenous C16:0 ceramide change the activity of key glycolytic enzymes in melanoma

PDMP and exogenous C16:0 had opposing effects on melanoma glycolysis levels, so we sought to explore the mechanism of this phenomenon. Because enzymatic activity is crucial for regulating glycolysis, we tested the activity of three important glycolysis enzymes: pyruvate kinase (PK), hexokinase (HK) and lactic acid dehydrogenase (LDH). As a result, none of

PDMP or exogenous C16:0 change PK activity in WM35 treatment (unpaired t test, $P > 0.05$) whereas PDMP and exogenous C16:0 both increase PK activity significantly in WM451 (unpaired t test, $*P < 0.05$) (**Figure 6A**). Exogenous C16:0 could increase HK activity both in WM35 and WM451 significantly but PDMP showed no significant difference. (unpaired t test, $P > 0.05$) (**Figure 6B**). PDMP decrease LDH activity significantly at WM35 cell (unpaired t test, $*P < 0.05$) but not in WM451 cell ($P > 0.05$). Exogenous C16:0 increase LDH activity significantly (unpaired t test, $*P < 0.05$) at WM451 cell but have no significant effect to WM35 cell (unpaired t test, $P > 0.05$) (**Figure 6C**). Overall, Exogenous C16:0 increased PK, HK, and LDH activity in WM451 cells, but only increased HK activity in WM35 cells. PDMP only increased PK activity in WM451 cells while inhibiting LDH activity in WM35 cells.

Discussion

Malignant melanoma has the highest mortality rate among skin cancers and is commonly metastatic. Ceramide synthesis and metabolism are considered new targets in cancer therapy [1]. The ceramide synthase gene family (CERS

C16:0 ceramide's role in melanoma malignant behavior and glycolysis

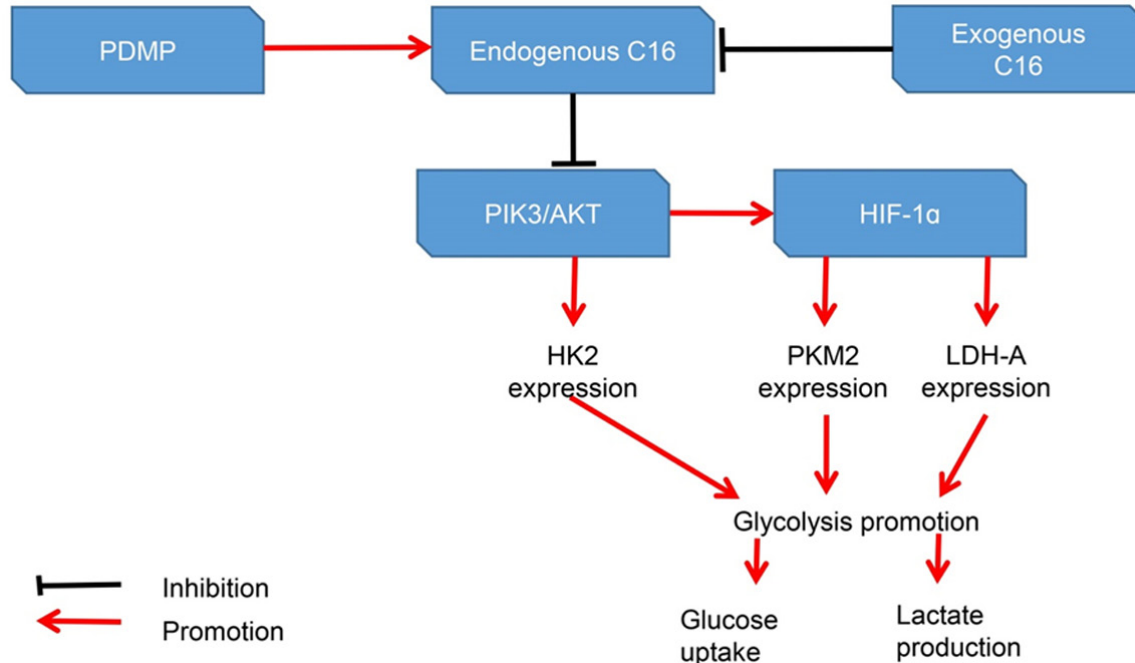


Figure 8. Graphical summary. Conjecture mechanism of PDMP and exogenous C16 on melanoma glycolysis.

genes, formerly known as Lass) regulates the synthesis of a variety of ceramide subsets. Ceramide synthase 6, which is encoded by the CERS6 gene, is the major contributor to C16:0 ceramide synthesis [2]. Our previous study found that the CERS6 gene could inhibit invasion and glycolysis in melanoma [3]. Short chain ceramides such as C2 [4] and C6 [5, 9] were found to inhibit melanoma cell lines. But as specific production of CERS6 Thomas et al. found increased levels of C16:0 during apoptosis [6], with few reports about the effects of C16:0 on melanoma metastasis and invasion. Since C16:0 is the most abundant fatty acid in mammalian cells [10], it is crucial to investigate the role of C16:0 in melanoma invasion and glycolysis.

We first confirmed C16:0 as the majority long-chain ceramide in human melanoma cells and melanocytes (**Figure 1**). We also found lower levels of C16:0 in melanocytes than in melanoma cells. This result agrees with Koyanagi et al. [11], which focused on rodent melanoma. However, there were no significant differences in C16:0 levels between WM35 and WM451 cells.

To further explore C16:0 without interference by the CERS6 gene, we utilized PDMP and exog-

enous C16:0 and tested their effects on malignant behavior in melanoma. PDMP treatment caused intracellular C16:0 accumulation in WM35 cells, but not as much in WM451 cells (**Figure 2**). We found that malignant behavior in WM35 cells was inhibited by PDMP, but not obviously in WM451 cells (**Figure 3**). Since WM35 is from early stage of melanoma (https://web.expasy.org/cellosaurus/CVCL_05-80) and WM451 are from metastatic lymph node (https://web.expasy.org/cellosaurus/CVCL_6357), they are considered with different metastatic potential type of melanoma [12], and WM35 was considered as non-metastasis melanoma cell line and WM451 was considered as metastasis cell line. PDMP may not perform inhibition and C16 ceramide accumulation in metastasis melanoma but could in non-metastasis melanoma at early stage. Our results indicate that intracellular C16:0 accumulation is related to inhibition of melanoma growth and migration, indicating that increased intracellular C16:0 may be a sign of reduced melanoma metastasis. It is possible that metastatic WM451 melanoma cells could be resistant to PDMP, which could be related to its lower levels of C16:0 accumulation.

Exogenous C16:0 ceramide was also included in this study for further investigation. Exogenous

C16:0 only inhibited metastatic WM451 cell migration without obvious effects in non-metastatic WM35 cells (**Figure 4**). These results indicate that exogenous C16:0 does not inhibit melanoma, which is opposite from PDMP treatment. Carpinteiro et al. found that C16:0 could restore B16F10 tumor cell metastasis *in vivo* in Asm-deficient mice [13], while our experiments found C16:0 could not inhibit human melanoma cell growth. This conflict between increased intracellular C16:0 relating to melanoma inhibition led us to test intracellular C16:0 levels after exogenous C16:0 treatment. LC-MS experiments showed a decrease in intracellular C16:0 (**Figure 5**), which could explain the conflicting results between exogenous and intracellular C16:0. Elevation of intracellular C16:0 is a common feature of several apoptosis-inducing triggers, and exogenous ceramide analogs can induce apoptosis, in turn modifying the composition/concentrations of intracellular ceramide species and associated signaling. For example, exogenous C6-ceramide and the synthetic ceramide analogs HPL-39N and HPL-1R36N induced apoptosis and C16:0 ceramide concentration [14]. Our experiments further found exogenous C16:0 could reduce intracellular C16:0 concentration, which may be because the long carbon chain of exogenous C16:0 cannot easily pass through into the cell. However, ceramides and their receptors are situated on the cell membrane as second messengers. The generation of intracellular C16:0 may be the result of negative feedback by exogenous C16:0 combined with receptors on the cell membrane. Nanoliposomal delivery may solve this problem [15]. Check the mutation gene bank of WM451 and WM35, there have 5 mutations on TP53 gene in WM451 (<https://cansarblack.icr.ac.uk/cell-line/451Lu/mutations>) but no TP53 mutation in WM35 (<https://cansarblack.icr.ac.uk/cell-line/WM-35/mutations>). Fekry et al. also confirmed that C16:0 is a natural regulatory ligand of p53, regardless of exogenous or intracellular origin [16]. The latest study from Jeffries et al. showed C16 ceramide can directly binds and activates p53. And p53 is mainly distributed in the nucleus, if exogenous C16:0 cannot pass through the cell membrane, it will not interact with p53 and inhibit tumor behavior. Watson et al. reported hotspot mutation in melanoma could change its pharmacological resistance [17]. As conjecture, WM451 have hotspot mu-

tated p53 gene, which means it may disturb the binding of C16 ceramide and interfere activation of p53 gene, with not triggering the melanoma inhibition. Our previous study found that silencing CERS6 could bypass p53 activation through WNT5, that could explain why activation of CERS6 have different affect of PDMP application and exogenous C16:0 in WM451 cell (**Figure 7**).

Our previous study also found that silencing CERS6 increased glycolysis. In this study, exogenous C16:0 treatment increased glucose uptake and lactate production in both WM35 and WM451 cells (**Figure 4E**). PIK3/AKT pathway play important role in PKM2 expression which could promote glycolysis in the manner of glucose uptake and lactate production increase [18]. PDMP could inhibit PIK3/AKT pathway through ceramide accumulation [8]. This explain PDMP inhibition to WM35 and WM451 glycolysis, LDH activity. In our study confirmed exogenous C16-ceramide could inhibit endogenous C16 which could indirectly active PIK3/AKT pathway. PI3K could promote LDH-A expression through HIF-1 α [19] and could promote HK2 expression [20], result in glycolysis increase. These could explain exogenous C16-ceramide promotion on glycolysis and key enzyme's activity's increase (**Figure 8**). However, in the melanoma cells, it is difficult to determine whether the increased glycolysis was caused by increased intracellular C16:0. PDMP treatment led to decreased glucose uptake and lactate production, indicative of decreased glycolysis (**Figure 3E**), which could be a consequence of creased intracellular C16:0 after exogenous C16:0 treatment.

The Warburg effect is a unique phenomenon involving glucose metabolism in tumor cells. Our findings indicate that melanoma cells consume more glucose and produce more lactic acid with lower intracellular levels of C16:0. We also evaluated key enzymes in the glycolytic pathway, including PK, hexokinase (HK), and lactic acid dehydrogenase (LDH) activity after treatment with PDMP and exogenous C16:0. PDMP and C16:0 had no significant effect on PK activity in WM35 cells, but elevated PK activity in WM451 cells. PDMP ceramide had no significant effect on HK activity in WM35 and WM451 cells, while exogenous C16:0 increased HK activity in both cell types. These

cell line differences may relate to the mutant of p53 and insulin resistance, which could link glucose phosphate and changing glucose uptake, but it is hard to define a potential role in tumor progression [21]. Our experiments indicate HK activity may be stable related to decreased intracellular C16:0. Glycolysis in melanoma is associated with malignancy. In the clinic, increased serum LDH, which is as an indicator for elevated glycolysis, is a sign of poor prognosis in stage IV melanoma [22]. The differing effects of PDMP on LDH activity in melanoma could offer clues about the role of C16:0 in melanoma glycolysis for future investigations.

Conclusion

C16:0 ceramide is the major long-chain ceramide in melanoma cells and melanocytes. It has differing effects on melanoma malignant behavior and glycolysis depending on its exogenous or intracellular localization. PK, HK, and LDH activities can be altered by intracellular C16:0 levels and thereby change cellular glycolysis.

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Disclosure of conflict of interest

None.

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C16:0 ceramide's role in melanoma malignant behavior and glycolysis

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