Electronic Supplementary Material (ESI) for

Integrated metabolomics revealed the photothermal therapy of melanoma by Mo_2C nanosheets: toward rehabilitated homeostasis in metabolome combined lipidome

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Chemicals and materials

Multilayer Mo_2C was purchased from Xinxi Technology Co., Ltd. (Foshan, China). The suspension of the above multilayer Mo_2C was freeze-dried to obtain the dry powder, stored in a sealed bottle with N_2 and put in a dark place at the earliest, which facilitated long-term preservation and prevention of unnecessary oxidation and decomposition. Such a treatment could guarantee the chemical stability of pristine multilayer Mo_2C.

C57 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mouse melanoma (B16F1) cells were obtained from the American Tissue Culture Collection (ATCC). Dulbecco's modified Eagle's medium (DMEM) and cell culture plates were bought from Corning Co. (NY, USA). Heat inactivated fetal bovine serum (FBS), penicillin-streptomycin solution, phosphate buffer saline (PBS), and trypsin were purchased from Gibco (Thermo Fisher, USA). Methyl tert-butyl ether (MTBE) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The MitoSOX™ Red mitochondrial superoxide indicator, MitoTracker red CMXRos kit (Invitrogen, USA), mitochondrial membrane potential assay kit with JC-1. ATP assay kit were obtained from Beyotime Co. (Beijing, China). An Annexin V-FITC/propidium iodide (PI) double-staining kit was purchased from 4A Biotech Co., Ltd. (Beijing, China). Cell counting kit-8 (CCK-8) was obtained from BOSTER Biological Technology co.ltd. (USA). Reactive oxygen species (ROS) assay kits was bought from Solarbio (Beijing, China). Double deionized water was used throughout the experiment. All chemical reagents in this experiment were analytical grade and utilized without further purification.

Fabrication and characterization of the Mo_2C nanosheets

To fabricate the Mo_2C nanosheets, 1 g of pristine Mo_2C was dispersed in 20 ml of tetrabutylammonium hydroxide (TBAOH, 10% in water) under magnetic stirring for 48 h at room temperature. Then, TBAOH was eliminated through centrifugation (8500 rpm, 5 min, 22°C). The obtained precipitate was treated with a 0.22 μm filter and freeze-dried to obtain the dry powder, stored in a sealed bottle with N_2 and put in a dark place at the earliest, which facilitated long-term preservation and prevention of unnecessary oxidation and decomposition. Such a treatment could guarantee the chemical stability of the obtained Mo_2C nanosheets.

The morphology of the Mo_2C nanosheets was characterized by scanning electron microscopy (SEM, Sigma 500, Zeiss, Germany). Transmission electron microscopy (TEM, Tecnai G2 F20 S-TWIN, FEI, America) was applied under a 200 kV accelerating voltage. These micrographs were obtained by fully dispersing in water and drying in air on a copper grid. The X-ray diffraction (XRD) patterns of Mo_2C were obtained by using a Cu-Kα-irradiated Empyrean EMPYREAN diffractometer under the conditions of 40 kV voltage, 35 mA current, 14.79 s/d scanning speed, and 5° to 80° scanning angle. UV-Vis diffuse reflectance of Mo_2C was measured by UV-vis spectrophotometry (UV-1800, Shimadzu, Japan). Thermal detector (FLIR One Pro, USA) was used to monitor the change of temperature.

Mice feeding and establishment of mice model

Eighteen mature male mice weighing between 22 and 25 g were used in this study. The mice were raised in standard cages (6 mice per cage) at room temperature (22-24°C) with adequate ventilation, a 12 h light-dark cycle, and approximately 50 ± 5% humidity. Commercial mouse pellets and water were provided *ad libitum*. The mice were acclimated to the housing conditions for 7 days

before starting the treatment. Subsequently, the mice were randomly divided into three groups of 6 mice each. After the establishment of melanoma (12 mice in two groups) or control (6 mice in the control group), later treatment was set as follows: control group was given only normal diet and plain water; melanoma group was induced by subcutaneous injection of B16F1 cells with normal diet and plain water; Mo_2C nanosheets+melanoma (Mo_2C+melanoma) group was induced by subcutaneous injection of B16F1 cells and tail vein injection of Mo_2C nanosheets with near-infrared (NIR) irradiation, normal diet and plain water. Experimental melanoma was established through the subcutaneous injection of 100 µl, 2×10^5 B16F1 cells. Dilution of B16F1 cells was prepared in PBS. The control group was treated with PBS without B16F1 cells. Such dosage was selected to achieve the optimum effect of melanoma oncogenesis based on published dosage-response results [1].

***In vivo* photothermal therapy (PTT) of melanoma with Mo_2C nanosheets**

The photothermal therapy (PTT) of melanoma by Mo_2C nanosheets was established by tail vein injection of 100 µl, 1 mg/ml Mo_2C nanosheets with NIR irradiation. The concentration of Mo_2C nanosheets was chosen to obtain optimum activity toward PTT based on published Mo_2C-based research [2] without considering a series of concentrations through a dosage-response study, while such dosage could also facilitate sufficient delivery and therapy of mice with nonsignificant nanotoxicity. The melanoma bearing mice under general anesthesia were exposed to near-infrared laser (wavelength: 808 nm) with a power density of 1.5 W/cm^2 for 3 min. The control group received H_2O with the same process of administration. The highest temperature and real-time thermal images were captured and recorded by an infrared thermal detector at the melanoma site by FLIR One Pro camera (USA). During therapy, the volume of the melanoma was measured and calculated. A vernier caliper was used to measure the length and width of melanoma. The melanoma volume was calculated by following equation:

$$\text{Melanoma volume } (\text{mm}^3) = \frac{1}{2} \times a \times b^2$$

where a refers the maximum length (mm) of and b indicates the minimum width (mm) of melanoma. After treatment, the tissue and melanoma were collected, paraffined and stained to conduct later morphological and pathological analysis. All animal experiments were performed in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Aqueous metabolomics study

Plasma samples were collected from the jugular vein in all experimental groups under general anesthesia in blood collection tubes with ethylene diamine tetraacetic acid (EDTA) and centrifuged (2000 rpm, 10 min, 8˚C) to collect the plasma. For sample preparation, 50 µl of plasma (n = 6) was quenched with 250 µl CH_3OH spiked with two internal standards (^{13}C6-L-Glucose and ^{13}C5-^{15}N-L-Glutamic acid) at -20˚C for 20 min. Subsequent vortexing 30 s at 4˚C, 1500 rpm. After centrifugation (13000 rpm, 15 min, 4°C), 150 µl of supernatant were processed with vacuum drying for 2 h. Before LC-MS/MS detection, the above dried aqueous-soluble samples were re-dissolved in 500 µl of HILIC solution (10 mM ammonium acetate in 30% water/70% ACN + 0.2% acetic acid containing ^{13}C2-L-tyrosine and ^{13}C1-L-lactate) followed by sonicating 3 min and vortexing at 1500 rpm for 30 min. Finally, sonicating 3 min and centrifugation (133000 rpm, 4˚C, 10 min). The 100 µl supernatant obtained was kept aside for later analysis. The quality control (QC) sample was a

mixture containing 20 µl of each sample.

A LC-MS/MS system, AB Sciex triple quadrupole 6500 mass spectrometer mass spectrometer coupled with Nexera LC-30A UPLC system (AB Sciex, Framingham, MA), was used for analysis by multiple reaction monitoring (MRM) mode. The 228 metabolites (99 in positive mode and 128 in negative mode) chosen for such analysis represented major metabolic pathways. UPLC conditions contain a flow rate of 300 µl/min with a mobile phase of (A) 10% acetonitrile in water (including 10 mM ammonium acetate and 0.2% acetic acid) and (B) 90% acetonitrile in water (containing 10 mM ammonium acetate and 0.2% acetic acid). The ACQUITY UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 µm, Waters, UK) was heated to 40˚C. Injection volume was 5 µl (positive modes) or 10 µl (negative modes). The MS was composed of a heating electrospray ionization (H-ESI) source. For positive mode, the interface capillary was kept at 500˚C with a ion source gas flow (Gas1) of 50 psi and ion source gas flow (Gas2) of 40 psi. The ionspray voltage was 5.5 kV. For negative mode, the interface capillary was kept at 550˚C with a ion source gas flow (Gas1) of 50 psi and ion source gas flow (Gas2) of 40 psi. The spray voltage was -4.5 kV. All the samples were injected at random.

Lipidomics study

Plasma samples were collected from the jugular vein in all experimental groups under general anesthesia in blood collection tubes with ethylene diamine tetraacetic acid (EDTA) and centrifuged (2000 rpm, 10 min, 8°C) to collect the plasma. For sample preparation, 25 µl of plasma (n = 6) was mixed with 225 µl CH_3OH and 5 µl SPLASH® LIPIDOMIX® Mass Spec Standard (Avanti, USA). After 1 min of swirl at 1500 rpm, the suspension was mixed and shaken gently with 750 µl MTBE (1500 rpm, 4˚C) for 30 min. Soon after, 188 µl H_2O was added and gently shaken for 10 min. The supernatant was then collected by centrifugation (13300 rpm, 10 min, 4˚C) and treated with a nitrogen stream for 30 min as a concentrate. Before LC-MS/MS detection, the above dried lipids were re-dissolved in 200 µl 50% CH_2Cl_2/50% CH_3OH with 10 mM ammonium acetate followed by vortexing (1500 rpm, 10°C, 30 s) and centrifugation (14,000 rpm, 10 min, 4°C). The 65 µl supernatant obtained was kept aside for later analysis. The QC sample was a mixture containing 40 µl of each sample.

A LC-MS/MS system, Q-trap 5500 mass spectrometer coupled with Shimadzu LC-30A liquid chromatograph (AB SCIEX, USA), was used for analysis by MRM mode. The 1251 lipids (635 in positive mode and 616 in negative mode) chosen for such analysis represented major metabolic pathways. HPLC conditions included a flow rate of 500 µl/min with a mobile phase of (A) 95% acetonitrile in water (containing 10 mM ammonium acetate, pH = 8) and (B) 50% isopropanol acetonitrile in water (including 10 mM ammonium acetate, pH = 8). The Kinetex C18 column (2.6 µm, 2.1 × 100 mm, Phenomenex, USA) and Luna NH_2 column (3 µm, 2.0 × 100 mm, Phenomenex, USA) for positive and negative mode at 35°C, respectively. The injection volume was 2 µl. The MS was composed of a heating electrospray ionization (H-ESI) source. For positive mode, the MS interface capillary was kept at 550°C with a sheath gas flow of 50 psi and aux gas flow of 60 psi. The spray voltage was 5.3 kV. For negative mode, the MS interface capillary was kept at 500°C with a sheath gas flow of 50 psi and aux gas flow of 60 psi. The spray voltage was -4.5 kV. All the samples were injected at random.

Statistical analysis

The raw data used for statistical analysis was obtained from 208 aqueous metabolites (including amino acids, organic acids, saccharides, amines, alkalines, etc) in aqueous metabolomics analysis and 1192 lipids (including phospholipids, lysophospholipids, sphingolipids, glycerolipids, free fatty acids, etc) in lipidomics analysis. For the aqueous metabolomics study, the raw data were imported into Analyst (v1.7.2) for pretreatment. For the lipidomics study, the obtained raw data were imported into MultiQuant (v3.0.3) for integrated processing. The pretreated data, including sample information, metabolite identity, and peak intensities, were further processed in R (v3.5.1). During this process, the interpolation of missing values was accomplished by the k-nearest neighbor (KNN) algorithm [3], and QC-based robust locally estimated scatterplot smoothing (LOESS) signal correction (QC-RLSC) was normalized [4]. Next, principal component analysis (PCA), partial least squares discrimination analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were applied to discriminate Mo_2C+melanoma group, melanoma group and control. The p values ($p < 0.05$) in the t tests and variable importance of the obtained data in the projection (VIP) value (VIP > 1) in the OPLS-DA model were set for all metabolites. The metabolic network and pathway analysis of the lipidome was performed according to Cytoscape (v3.8.2).

Western blot (WB) analysis was utilized to investigate the protein expression. Briefly, the plasma in Mo_2C+melanoma, melanoma group and control were lysed in buffer containing protease inhibitor cocktail (10 ×) (Roche Diagnostics, Indianapolis, India)，PMSF (100 ×) and RIPA. Then, lyse the cocktail by ultrasonication at 100 W for 5 s on ice and repeat for three times. Next, the lysate was washed by centrifugation at 13000 g for 10 min at 4˚C. The concentration of protein was detected by BCA method (Thermo Scientific, USA) to achieve equal concentrations in each sample for 10% SDS-polyacrylamide gel electrophoresis with 20 μg protein per lane and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Richmond, CA). Subsequently, the membranes were blocked with QuickBlock (Beyotime, China) for 30 min and incubated for 8 h with 1:1000 dilutions of various antibodies (CS, ALDOA, PDHA1, PDHB, BHMT, DDC, L2HGDH rabbit polyclonal antibody, ABclonal, Wuhan, China). After washing with tris buffered saline tween (TBST, Cell Signaling Technologies, America), the membrane was stained with HRP-conjugated goat anti-rabbit IgG (1:10000 dilution) (Cell Signaling Technologies, America) for 1 h. Finally, WB images were obtained by a ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, Canada). Ponceau S (Beyotime, China) is used as loading control.

In vitro PTT of melanoma with Mo_2C nanosheets

B16F1 cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin solution and incubated in an atmosphere of 5% CO_2 at 37°C in a humidified incubator. The medium was changed every two days to maintain cell viability, whereas all other conditions remained the same. The cells were subcultured with 0.25% trypsin. B16F1 cells with exponential growth were utilized in all experiments. Before PTT with Mo_2C nanosheets, B16F1 cells were first rinsed with PBS, and then 1 ml 0.25% trypsin was added and resuspended in fresh medium at 5×10^5 cells/ml. Then, 2 ml B16F1 cells were finely dispersed and divided into six parts on average and inoculated into each hole of the 6-well plates. Adherent B16F1 cells were then exposed to only 1 mg/ml Mo_2C nanosheets (Mo_2C nanosheets group), NIR irradiation (NIR group) or 1 mg/ml Mo_2C nanosheets with NIR irradiation (Mo_2C nanosheets-based PTT group).

CCK-8 was used to examine cell proliferation by absorbance assay. A total of 1×10^3 cells were seeded into each well of 96-well plates. Each well, including DMEM with 10% FBS, was cultured

overnight for cell attachment. Only Mo_2C nanosheets, only NIR irradiation, or Mo_2C nanosheets-based PTT was then applied to treat with B16F1 cells. After that, 10 μl of CCK-8 solution was added to each well and incubated for 1 hour in the dark at 37°C. Cell viability was detected by absorbance at 450 nm on a Synergy MX microplate reader (BioTek, America). Cell necrosis was measured by Annexin V and propidium iodide (PI) dual staining (4A Biotech, China). Briefly, B16F1 cells were incubated with 5 μl Annexin V-FITC for 5 min at room temperature in the dark and then mixed with 10 μl PI. The necrosis rate was determined by flow cytometry analysis (Annexin V/FITC, Ex/Em: 488 nm/525 nm; PI, Ex/Em: 561 nm/575 nm). For measurement of mitochondrial superoxide production, the B16F1 cells were incubated with MitoSOX Red (2.5 μM, Thermo Fisher) at 37°C for 30 min, rinsed with PBS and resuspended in 200 μl PBS for imaging. For the assessment of intracellular superoxide production, B16F1 cells were incubated with DCFH-DA (10 μM, S0033S, Beyotime) at 37°C for 30 min. Fluorescence imaging was performed. Measurement of mitochondrial depolarization was accomplished with a mitochondrial membrane potential assay kit with JC-1. A rotating disk confocal superresolution microscope (Olympus SpinSR10, Japan) were used for fluorescence imaging.

Reference

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[3] Lee JY, Styczynski MP. NS-kNN: a modified k-nearest neighbors approach for imputing metabolomics data. Metabolomics. 2018;14.

[4] Shisheng, Wang, Xiaolei, Chen, Dan, Wen, et al. MetaboGroupS: A Group Entropy-based Web Platform for Evaluating Normalization Methods in Blood Metabolomics Data from Maintenance Hemodialysis Patients. Analytical chemistry. 2018.

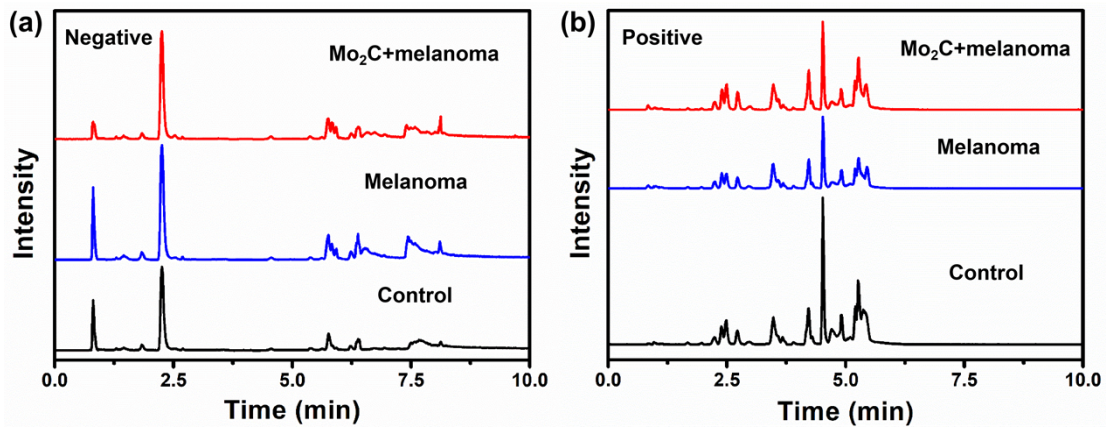

Fig. S1 Typical LC-MS/MS TIC spectra of aqueous metabolites in Mo_2C+melanoma group, Melanoma group and control. (a) negative mode, (b) positive mode.

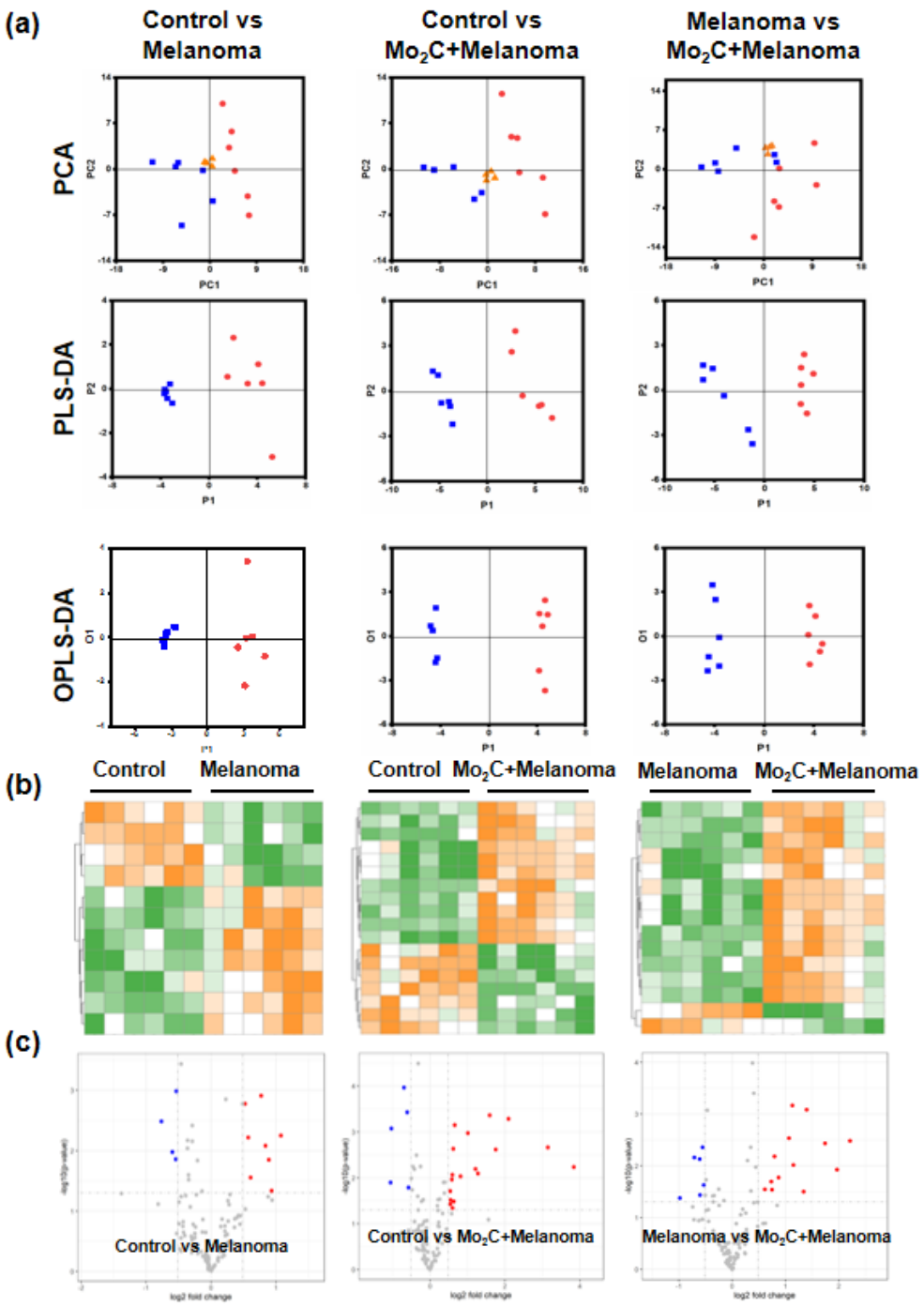

Fig. S2 Aqueous metabolomics study. (a) PCA score plots of PC1 vs PC2, PLS-DA score plots of p1 vs p2, OPLS-DA score plots of p1 vs o1. In Control vs Melanoma group, blue dots: control, red dots: melanoma group, orange dots: QC; In Control vs Mo_2C+Melanoma group, blue dots: control, red dots: Mo_2C+Melanoma group; In Melanoma group vs Mo_2C+Melanoma group, blue dots: Melanoma group, red dots: Mo_2C+Melanoma group. Permutation test: $pR^2 = 0.01$, $pQ^2 = 0.01$. (b) The extracted heatmaps of differential aqueous metabolites. The color scale illustrates the relative abundances across the samples. Correspondingly, orange indicates significantly upregulated metabolites, while green indicates metabolites that were significantly downregulated metabolites. (c) Volcano plots of all detected and quantified aqueous metabolites.

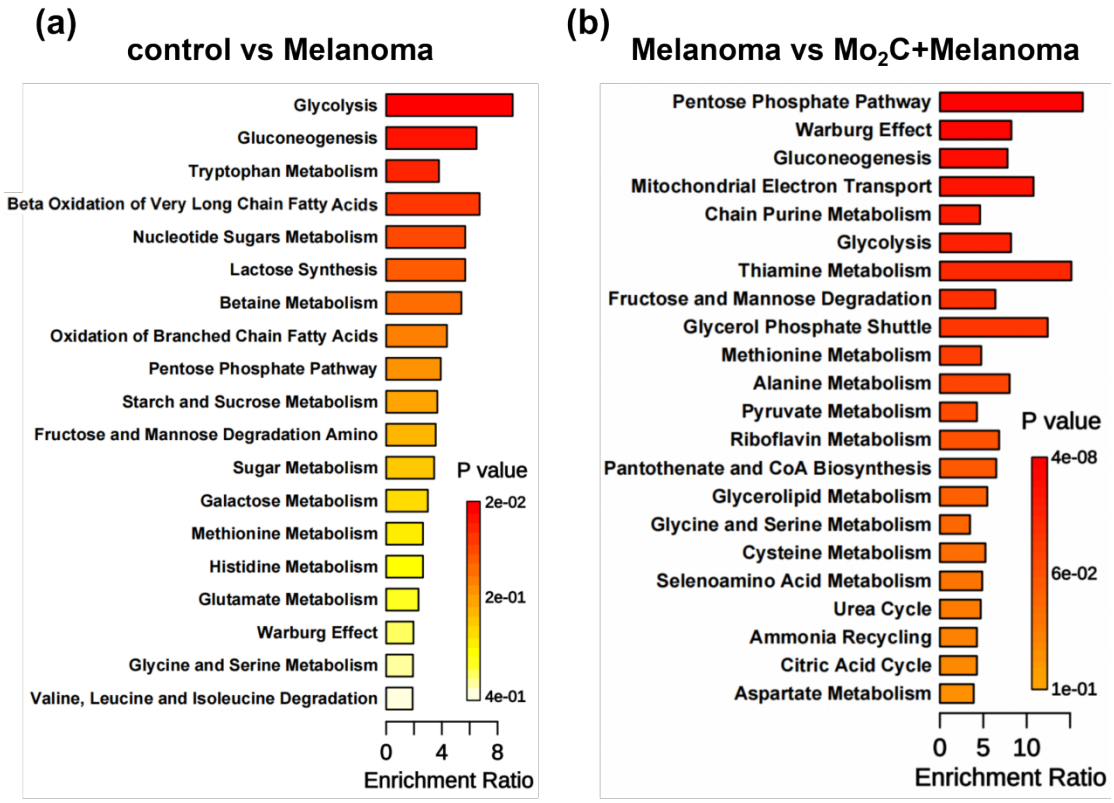

Fig. S3 Differential metabolic pathways upon comparing (a) control with melanoma group or (b) melanoma group with Mo_2C+melanoma group.

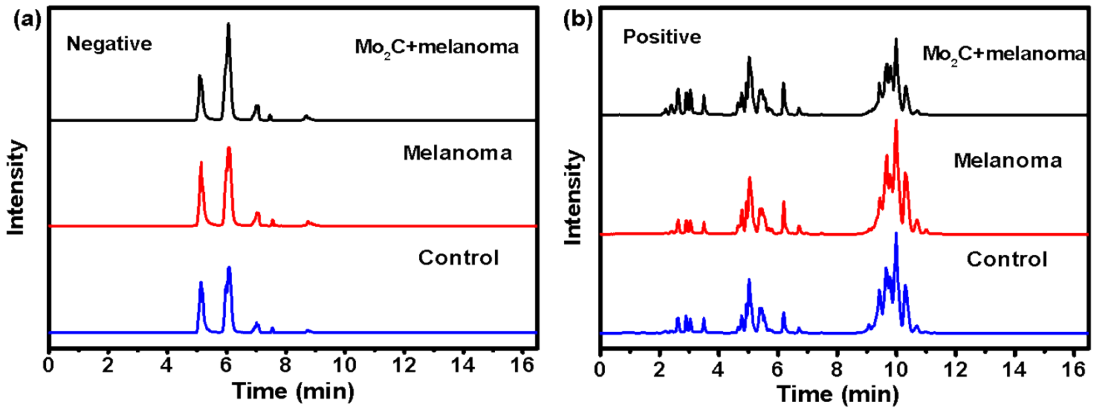

Fig. S4 Typical LC-MS/MS TIC spectra of lipids in Mo_2C+melanoma group, Melanoma group and control. (a) negative mode, (b) positive mode.

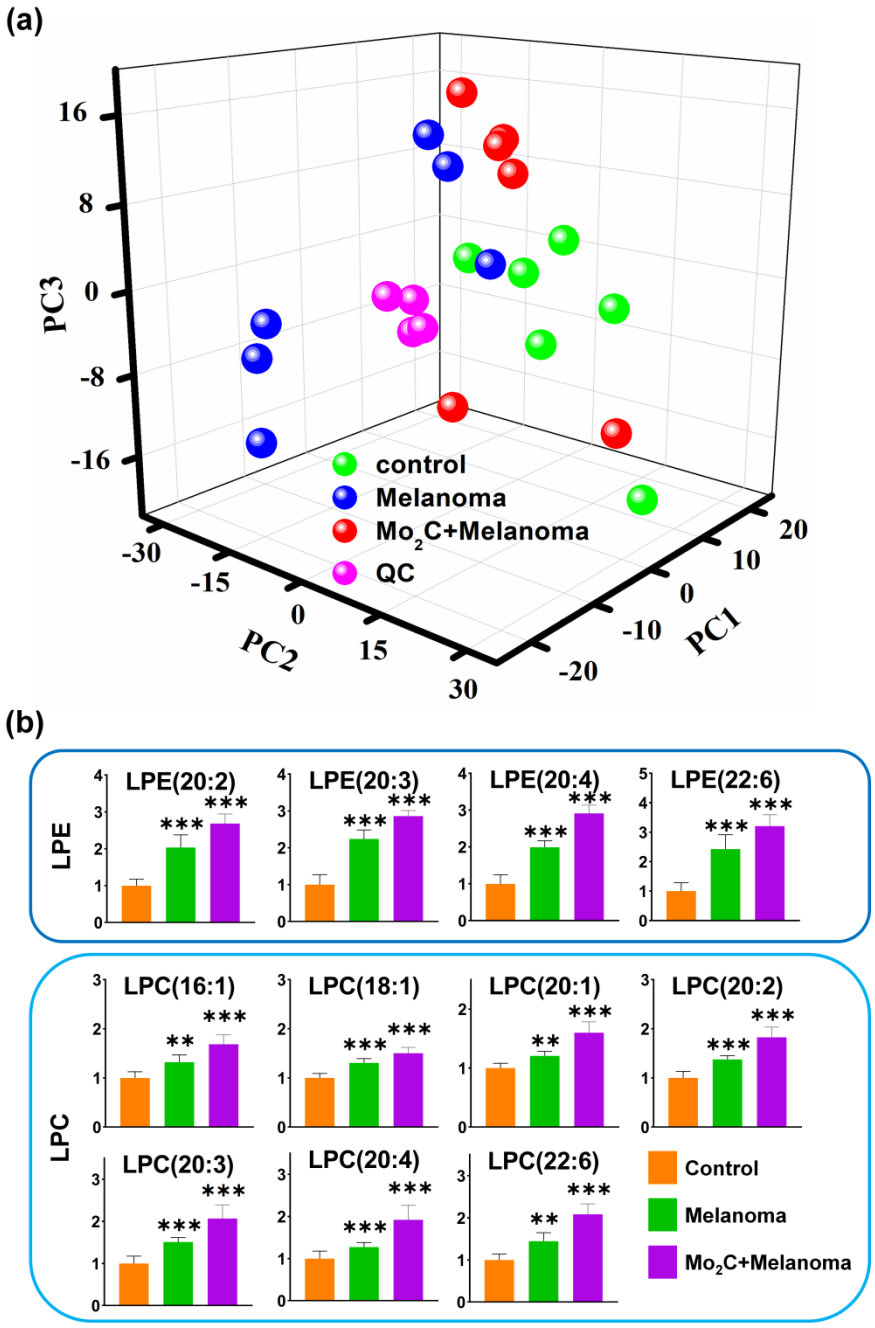

Fig. S5 Representative lipids and abundance of lipid classes. (a) PCA score plots of PC1, PC2 vs. PC3. (b) Abundance of lipids in LPC and LPE classes. The Y axis represents the abundance of MS signals in the melanoma group or Mo_2C+melanoma group compared with the control. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

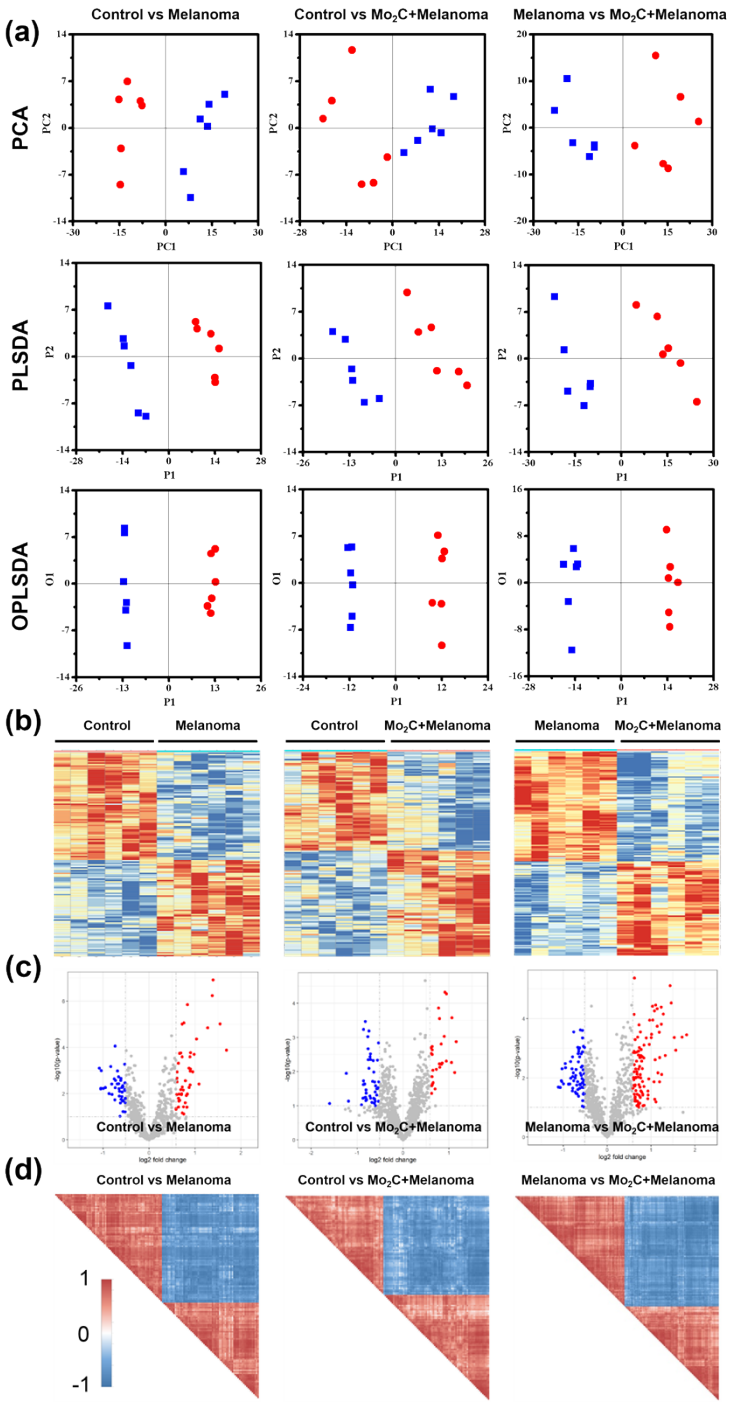

Fig. S6 Lipidomics study. (a) PCA score plots of PC1 vs PC2, PLS-DA score plots of p1 vs p2, OPLS-DA score plots of p1 vs o1. In Control vs Melanoma group, blue dots: control, red dots: melanoma group; In Control vs Mo_2C+Melanoma group, blue dots: control, red dots: Mo_2C+Melanoma group; In Melanoma group vs Mo_2C+Melanoma group, blue dots: Melanoma group, red dots: Mo_2C+Melanoma group. Permutation test: pR^2 = 0.01, pQ^2 = 0.01. (b) The extracted heatmaps of differential lipids. The color scale illustrates the relative abundances across the samples. Correspondingly, red indicates significantly upregulated lipids, while blue indicates lipids that were significantly downregulated lipids. (c) Volcano plots of all detected and quantified lipids. (d) Correlation plots of differential lipids.

Table S1 Experimental and characterization data of the Mo_2C nanosheets

	Mo_2C nanosheets
Dimension	2
Morphology	nanosheets
Lattice planes	(100), (002), (101), (102), (110), (112), and (201)
Elemental mapping	coexistence of Mo and C elements
UV-vis curves	Dose-dependent typical absorption curves
Photothermal effect	Optimun photothermal effect with 1 mg/ml Mo_2C nanosheets and NIR

Table S2 Differential metabolic pathways and metabolites in aqueous metabolomics study

Metabolic pathway	Metabolites
Amino acid metabolism	Histidine, Phenylalanine, Isoleucine, Leucine, Dimethylglycine, Kynurenic acid, 3-Methylhistidine, Kynurenine, Betaine, Serotonin
Glucose metabolism	Glucose, 2-Hydroxyglutarate, Fructose 6-phosphate, Glucose 1-phosphate, Glyceraldehyde 3-phosphate, Dihydroxyacetone phosphate, Xylulose-5P
Purine metabolism	Xanthosine, Adenosine 5'-diphosphate, Guanosine diphosphate, Adenosine monophosphate
Organic acid metabolism	Isobutyric acid, Pyruvic acid
Others	13S-hydroxyoctadecadienoic acid, 12S-HETE, Acetylcarnitine,