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Multiplatform Metabolomics Investigation of Antiadipogenic Effects on 3T3-L1 Adipocytes by a Potent Diarylheptanoid

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Abstract

Obesity is fast becoming a serious health problem worldwide. Of the many possible antiobesity strategies, one interesting approach focuses on blocking adipocyte differentiation and lipid accumulation to counteract the rise in fat storage. However, there is currently no drug available for the treatment of obesity that works by inhibiting adipocyte differentiation. Here we use a broad-based metabolomics approach to interrogate and better understand metabolic changes that occur during adipocyte differentiation. In particular, we focus on changes induced by the antiadipogenic diarylheptanoid, which was isolated from a traditional Chinese medicine *Dioscorea zingiberensis* and identified as (3*R*,5*R*)-3,5-dihydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptane (1). Targeted aqueous metabolic profiling indicated that a total of 14 metabolites involved in the TCA cycle, glycolysis, amino acid metabolism, and purine catabolism participate in regulating energy metabolism, lipogenesis, and lipolysis in adipocyte differentiation and can be modulated by diarylheptanoid 1. As indicated by lipidomics analysis, diarylheptanoid 1 restored the quantity and

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00028.

Figure S-1: Illustration of ¹³C₆-glucose substrate labeling study. Figure S-2: Effects of diarylheptanoids 1–4 and curcumin on lipid accumulation during differentiation. Figure S-3: Distribution of coefficient of variation (CV) values of all measured aqueous metabolites from targeted metabolomics. Figure S-4: Volcano plots of the detected aqueous metabolites from cell samples. Figure S-5: PLSDA score plots of aqueous metabolites in cell samples obtained from LC–QQQ/MS in both positive and negative ion modes. Figure S-6: Heat maps and dendrogram visualization of the normalized peak areas of the significantly different lipids including TG, DG, and MG between control and model group in cell sample groups. Figure S-7: Heat maps and dendrogram visualization of the normalized peak areas of the significantly different lipids including PC, PE, PS, SM, Cer, CL, lysoPC, lysoPE, and cholesterol ester between control and model group in cell sample groups. Table S-1: Mobile phase used for the LC–QQQ/MS. Table S-2: LC gradient conditions for the LC–QQQ/MS. Table S-3: Parameters for triple quadrupole mass spectrometry. Table S-5: Mobile phase used for the LC–QTOF/MS. Table S-6: LC gradient conditions for the LC–QTOF/MS. Table S-7: Parameters for QTOF mass spectrometry. Table S-8: Significantly changed aqueous metabolites in medium samples for antiadipogenic effect of diarylheptanoid 1. (PDF) Table S-4: MRM transitions for ABSciex 5500. (XLSX)

The authors declare no competing financial interest.

degree of unsaturation of long-chain free fatty acids and restored the levels of 171 lipids mainly from 10 lipid classes in adipocytes. In addition, carbohydrate metabolism in diarylheptanoid-1-treated adipocytes further demonstrated the delayed differentiation process by flux analysis. Our results provide valuable information for further understanding the metabolic adjustment in adipocytes subjected to diarylheptanoid 1 treatment. Moreover, this study offers new insight into developing antiadipogenic leading compounds based on metabolomics.

Graphical Abstract

Keywords

3T3-L1 adipocyte; metabolomics; antiadipogenic; diarylheptanoids; LC-MS; GC-MS; metabolic flux; *Dioscorea zingiberensis*

INTRODUCTION

Obesity is a major risk factor for many disorders, including the development of type 2 diabetes, cardiovascular diseases, cancer, and sleep-disordered breathing.¹ At the cellular level, excessive accumulation of adipocytes can result in obesity; both an increased number and size of adipocytes have been shown to participate in the growth of adipose tissue.² To model this process, the 3T3-L1 cell line has been successfully used as an in vitro model of obesity for monitoring preadipocytes differentiated into adipocytes in response to the hormonal cocktail stimulation consisting of insulin, dexamethasone, and isobutylmethylxanthine (IBMX).³

There is currently no effective drug used to decrease obesity that works by inhibiting adipocyte differentiation. Recently, interest has increased in the development of suitable drug candidates or leads based on natural sources with fewer adverse effects for preventing and ameliorating obesity.⁴ Diarylheptanoids are a class of plant-based phenolic compounds that share the 1,7-diphenylheptane skeleton. These compounds are increasingly recognized as potential therapeutic agents for antiobesity treatment,^{5,6} with curcumin as one of the main representatives.⁷ In our previous studies, some bioactive diarylheptanoids have been isolated from *Dioscorea zingiberensis*.⁸ Notably, the extract obtained from the roots of *D. zingiberensis* is commercially available as an oral formulation (Dun-Ye-Guan-Xin-Ning) and is used for hyperlipidemia in China.⁹

Metabolomics is an emerging “omics” science involving the comprehensive characterization of metabolites and metabolism in biological systems.¹⁰ In particular, metabolomics is increasingly being used to diagnose disease, understand disease mechanisms, identify novel

drug targets, customize drug treatments and monitor therapeutic outcomes.¹¹ Recently, a few global metabolic profiling studies have focused on the changes in metabolism required for differentiation of preadipocytes into mature adipocytes.^{12–15} A diverse number of pathways essential to differentiation have been highlighted as being important to 3T3-L1 maturation, including the tricarboxylic acid (TCA) cycle; glycolysis; fatty acid α -oxidation, synthesis, and desaturation; polyamine biosynthesis; purine degradation; and amino acid metabolism.^{12–18}

In the present study, the antiadipogenic effect of diarylheptanoid has been confirmed, and the metabolic changes induced by diarylheptanoid 1 associated with differentiation of the 3T3-L1 cell line have been studied using a combination of four LC–MS and GC–MS-based metabolomics platforms, including targeted aqueous profiling, global lipidomics, and fatty acid and metabolic flux analysis. This broad-based metabolic analysis provides a detailed interrogation and a number of findings on the altered metabolism involved in fat accumulation in this model system.

EXPERIMENTAL SECTION

Materials

3T3-L1 cells were purchased from ATCC (Manassas, VA). Dexamethasone, insulin, IBMX, and Oil Red O (ORO) were purchased from Sigma (St. Louis, MO). Dulbecco's modified calf Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT). LC–MS-grade CH_2Cl_2 , acetonitrile (ACN), MeOH, and isopropanol (IPA) were purchased from Fisher Scientific (Pittsburgh, PA). HPLC grade acetic acid and ammonia acetate, anhydrous pyridine, *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA), methoxyamine hydrochloride, U- ^{13}C glucose, and all of the standard compounds corresponding to the measured metabolites were obtained from Sigma. *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) was purchased from Thermo-Fisher Scientific (Waltham, MA). Fatty acid methyl esters (FAMES) were purchased from Agilent technologies (Santa Clara, CA). The internal standards $^{13}\text{C}_6$ -glucose, $^{13}\text{C}_2$ -glutamic acid, $^{13}\text{C}_2$ -L-tyrosine, and $^{13}\text{C}_1$ -L-lactate were obtained from Cambridge Isotope Laboratory (Tewksbury, MA). Lipids standards, triglyceride (TG, 17:0/17:0/17:0) and ceramide (Cer, d18:1/17:0) were purchased from Avanti Polar Lipids (Alabaster, AL). The diarylheptanoids 1–4 were isolated from *D. zingiberensis* according to our previous method.⁸

Cell Culture, Diarylheptanoids Treatment, ORO Staining, and Metabolites Extraction

The details of cell culture and ORO staining are illustrated in Figure 1, and the experimental methods were the same as previously reported.¹⁹ At 2 days postconfluence (day 0), cell differentiation was induced with an adipogenic cocktail inducer, which is a mixture of IBMX (0.5 mM), dexamethasone (Dex, 1 μM), and insulin (Ins, 10 $\mu\text{g}/\text{mL}$) in DMEM containing 10% FBS. After 48 h (day 2), the induction medium was removed and replaced with DMEM containing 10% FBS supplemented with insulin (10 $\mu\text{g}/\text{mL}$). At day 4, this medium was changed with DMEM for another 4 days. For the diarylheptanoids treatment groups, the cultures were mixed with test compounds for the whole culture period (days 0–8). The preadipocytes (Control) were stained with ORO and measured at day 0, and the

metabolites including aqueous, lipids, and fatty acids were extracted at the same day. The adipocytes (Model) and the diarylheptanoid-treated adipocytes (Treatment) were stained with ORO and measured at day 8, and their metabolites were also extracted according to the corresponding protocol at day 8. Only diarylheptanoid 1 was used for further metabolomics investigations. To quantify intracellular lipids, spectrophotometric quantification of the ORO staining was performed by dissolving the stained lipid droplets in isopropanol for 10 min. The absorbance was measured at 545 nm. The extraction steps for aqueous, lipids, and fatty acids are described in detail as follows.

Aqueous Metabolites

Cell samples were prepared following very similar procedures to those described in our previous papers.^{20,21} Cells were collected by removing the media and washing with PBS. The cells were quenched with a 1 mL solution of cold MeOH/H₂O (4:1) that included 0.096 mM ¹³C₆-glucose and 0.021 mM ¹³C₂-glutamic acid in the aqueous fraction, which were used as internal standards. The cell mixtures were incubated on dry ice for 30 min and then collected with a cell scraper. The cell extract was transferred into another tube. The MeOH/H₂O solution (0.4 mL) was added again to the plate to rinse off any remaining cells or their metabolites. The combined cell extracts were vortexed for 2 min and stored at -20 °C for 20 min. Next, the cell extract was sonicated for 15 min, and then centrifuged at 14 000 rpm/min for 10 min. The supernatant was collected into a new tube and dried in a SpeedVac (Eppendorf, Fisher Scientific, Pittsburgh, PA). The dried samples were reconstituted in 1 mL of 10 mM ammonium acetate in 40% water/60% ACN + 0.2% acetic acid containing 5.13 μM ¹³C₂-L-tyrosine and 22.5 μM ¹³C₁-L-lactate. The four isotope-labeled internal standards added to each sample served to monitor the analytical system performance. After centrifugation and filtration, 5 and 15 μL sample aliquots were injected into the HPLC before LC-MS/MS analysis under positive and negative ion mode, respectively. The detailed protocol for the preparation of the medium for MS analysis was the same as for our previous biofluid samples.^{22,23} A pooled sample, which was a mixture of all the cell and medium samples, was used as the quality control (QC) sample.

Lipids

Cells were collected by removing the media and washing with PBS. A CH₂Cl₂/MeOH solution (2:1 v/v, 1.5 mL) was added to the cells to quench the metabolism. The CH₂Cl₂/MeOH solution contained two lipid standards, TG (17:0/17:0/17:0) at 4.2 μM and Cer (d18:1/17:0) at 6.46 μM. The mixture was transferred to a glass vial and vortexed. Water (0.5 mL) was then added to the mixture, followed by vortexing for 2 min, incubation for 30 min at room temperature, and then centrifugation at 5000 rpm/min for 10 min. The lower organic phase was collected and dried. The dried sample was reconstituted in 100 μL of ACN/IPA/H₂O (65:30:5 v/v/v) and loaded into a glass vial for lipidomics analysis.

Long-Chain Free Fatty Acids

The extraction procedure for long-chain free fatty acids was the same as that for lipid extraction described above. Dried organic-phase samples were derivatized by adding 30 μL of methoxyamine hydrochloride solution (20 mg/mL in pyridine), mixed, and then incubated at 37 °C for 90 min. Samples were silylated with 70 μL of MSTFA at 70 °C for 90 min. Two

uL of FAMEs solution, used for retention time indexing, was added to each sample before injection.

METABOLOMICS DATA ACQUISITION

HPLC–QQQ/MS

Targeted MS-based aqueous metabolomics was performed on an Agilent 1260 LC (Agilent Technologies, Santa Clara, CA) coupled to an AB Sciex Qtrap 5500 MS (AB Sciex, Toronto, Canada) system, controlled by Analyst 1.5 software (AB Sciex, Toronto, Canada) as described previously.^{20,22,24} Replicates of the QC sample were injected after every 10 study samples. Chromatographic separations were performed by hydrophilic interaction chromatography (HILIC) using a BEH amide column (2.1 × 150 mm, 2.5 μm, Waters, Milford, MA). The mobile phase and gradient conditions are listed in Tables S-1 and S-2, respectively. The column temperature was set to 40 °C. The parameters for the mass spectrometer are listed in Table S-3. Multiple reaction monitoring (MRM) (Table S-4) mode was used to detect metabolites of interest. The 215 metabolites selected for targeted analysis represented numerous major metabolic pathways in adipogenesis.

HPLC–QTOF/MS

Global MS-based lipidomics was performed using an Agilent 1200 LC system coupled to an Agilent 6520 Q-TOF mass spectrometer. Five μL of each prepared sample for positive ESI ionization and 10 μL for negative ESI ionization were injected onto an Agilent Zorbax 300SB-C8 column (2.1 × 50 mm, 1.8 μm), which was heated to 35 °C. The mobile phase and its gradient as well as the MS parameters are listed in Tables S-5–S-7, which were performed according to previously described methods.^{23,25,26} The mass accuracy of our LC–MS system is generally better than 5 ppm, the Q-TOF/MS spectrometer was calibrated prior to each batch run, and a mass accuracy of <1 ppm was often achieved using the standard tuning mixture (G1969–85000, Agilent Technologies). The *m/z* scan range was 100–2000, and the acquisition rate was 1.0 spectra/s.

GC–MS

Fatty acid derivatives were analyzed on a GC–MS system (Agilent 7890/5975C) by injecting 1 μL of the prepared samples onto the instrument using splitless mode. Helium was used as the carrier gas with a constant flow rate 1.2 mL/min. Separation was performed using an Agilent DB5-MS + 10 m Duraguard capillary column (30 m × 250 μm × 0.25 μm). The column temperature was maintained at 60 °C for 1.00 min, then increased at a rate of 10 °C/min to 325 °C and held at this temperature for 10 min. Mass spectral signals were recorded after a 4.90 min solvent delay. Peaks were first converted to the appropriate format and then analyzed by Agilent MassHunter Quantitative Analysis software. Intensities and elution times for the fatty acid derivatives were defined with standards and verified by *m/z* values after each experiment.

¹³C-Glucose Substrate Labeling To Analyze Metabolic Flux

For preadipocytes, at 2 days before day 0, the medium was removed from the plate and replaced with DMEM containing 10% FBS, penicillin, streptomycin, and 4.5 g/L U–¹³C

labeled glucose. After 2 days, cells were collected and aqueous metabolites were extracted as described above. For adipocytes and diarylheptaonid-treated adipocytes, at 6 days postdifferentiation, the medium was removed from the plate and replaced with DMEM contained 10% FBS, penicillin, streptomycin, and 4.5 g/L U-¹³C-labeled glucose with or without diarylheptaonid. After 2 days, cells were collected and aqueous metabolites were extracted (Figure S-1). Dried aqueous-phase samples were derivatized by adding 45 μ L of methoxyamine hydrochloride solution (20 mg/mL in pyridine), mixed, and then incubated at 37 °C for 90 min. Samples were silylated with 100 μ L of MTBSTFA at 70 °C for 90 min. Derivatized samples (1 μ L) were injected into the GC–MS. The parameters for GC–MS were the same as described before.^{27–29} Peaks were analyzed using Agilent MassHunter software. The measured distributions of mass isotopomers were corrected for ¹³C natural abundance using Isocor software.³⁰ Metabolite intensity and elution times were defined with standards and verified by *m/z* after each experiment.

Data Analysis

Multivariate data analysis was performed using SIMCA-P⁺11.0 (Umetrics, Umeå, Sweden). Different normalization methods were used for the four data sets. After exporting targeted aqueous metabolites data sets from MultiQuant software, the distribution of the coefficient of variation (CV) values of all measured metabolites were calculated. Metabolites with CV < 15% were defined as reliably detected. The targeted aqueous metabolites data were normalized using protein count. Data sets were scaled to unit variance (UV) and first analyzed using principal component analysis (PCA). The fold change (FC) and *p* value were calculated for each metabolite between control and the model group as well as between the model and treatment groups, and volcano plots were depicted. In addition, partial least-squares discriminant analysis (PLS-DA) was applied to analyze the data from control and model groups. Metabolites with FC > 1.5 or <0.6, *p* < 0.05, and variable importance in projection (VIP) > 1 were recognized as significantly changed metabolites during the differentiation process. FC, *p*, and VIP values were also calculated between the model and treatment groups. The metabolites with a restorative tendency (*p* < 0.05) were regarded as significantly changed metabolites for an antiadipogenic effect of diaryl-heptaonid 1. On the basis of these important metabolites, Metaboanalyst software (v 3.0)³¹ was employed to calculate a heat map to visualize the relative number of potential biomarkers in different groups and to perform pathway analysis.

The data processing method for the lipidomics data set was almost the same as that for targeted data, except that the screening criterion for lipidomics peak values was CV < 20%. The untargeted lipidomic data were then normalized using protein count, and then Pareto-scaled prior to PCA and PLS-DA analysis. After identifying the most significantly changed lipids between the control and model groups, the lipids were classified into classes, and the distribution of the lipids classes in three groups was depicted in heat maps and box plots. The free fatty acids were also normalized using protein count as well as the average integrated peak intensity values from the two adjacent pooled QC injections. The flux data required no normalization; however, we used cell counts to assess their consistency. Comparisons between two groups of other GC–MS, flux data, or biological data were made

using the Student's *t* test. The differences were considered statistically significant for $p < 0.05$.

RESULTS

Antiadipogenic Effects of Diarylheptanoids

Four diarylheptanoids were isolated from *D. zingiberensis*,⁸ and their antiadipogenic effects were measured using differentiating 3T3-L1 adipocytes (Figure S-2). As shown in Figure 2A, 3T3-L1 adipocytes developed visible lipid droplets, with (3*R*,5*R*)-3,5-dihydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptane (1) showing a maximal and dose-dependent decrease in intracellular fat accumulation compared with curcumin and other diarylheptanoids, as determined by morphological and quantitative analysis of intracellular lipids using ORO staining.

Metabolite Profiling of 3T3-L1 Cells Treated with Diarylheptanoid 1

Targeted aqueous metabolite profiling using a robust LC–QQQ/MS platform and global lipidomic profiling conducted on an optimized LC–QTOF/MS platform were used to measure metabolic changes in 3T3-L1 adipocytes exposed to diarylheptanoid 1. In total, we reliably detected 125 metabolites in cell samples out of 215 targeted MRM transitions on the LC–QQQ/MS platform. The coefficients of variation (CVs) of the four stable isotope-labeled internal standards were <3% in all samples. The average CV for all metabolites in the QC samples was 6.5%. (Figure S-3) The LC–QTOF/MS system provided high sensitivity, resolution, and mass accuracy for a large number of lipids. A total of 2433 features were measured using both positive and negative ion mode.

After data acquisition and pretreatment, PCA score plots of aqueous metabolites and lipids both showed an obvious separation among preadipocytes, adipocytes, and diarylheptanoid-1-treated adipocytes (Figure 2B). This observation indicated that diarylheptanoid 1 disturbed both aqueous metabolites and lipids metabolism of 3T3-L1 adipocytes.

From the aqueous metabolite profiling, 42 of 125 metabolites exhibited a significant change ($FC > 1.5$ or < 0.6 ; $p < 0.05$; and $VIP > 1$) when comparing adipocytes to preadipocytes, with a number of very large FC values (Figures S-4 and S-5). Sixteen of these 42 metabolites showed an opposite trend after treating with diarylheptanoid 1, which was again statistically significant (Table 1). These 16 differentiated metabolites, which are closely related to diarylheptanoid 1 treatment, are mainly involved in purine metabolism, amino acid metabolism, TCA cycle, and glycolysis pathways, as indicated by the results of pathway analysis using Metaboanalyst³¹ (Figure 2C). Some of the FC values for the individual metabolites are quite large, as high as 26.4.

From the lipidomic profiling, 282 lipids showed significant changes in their levels in adipocytes as compared with preadipocytes. All of these 282 lipids, which included a number of glycerolipids (triglyceride (TG), diglyceride (DG), and monoglyceride (MG)) and glycerophospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), Cer, lysoPC, and lysoPE), were found to be increased in abundance in adipocytes relative to preadipocytes, and a few cholesteryl esters

were found to be decreased in abundance in adipocytes. Among the 282 lipids, 171 metabolites showed a significant restoration after diarylheptanoid 1 treatment (Figures S-6 and S-7). Combining the lipid signals by class, most of the major lipid classes such as TG, DG, PC, and PE increased in adipocytes, while the level of these lipids decreased after diarylheptanoid 1-treatment. (Figure 3A). After differentiation, the adipocytes accumulated more lipids, while diarylheptanoid 1 reduced lipogenesis, as demonstrated by these lipidomics results.

Free fatty acids (FFAs) and cholesterol are important components of lipid droplets in adipocytes. To analyze the changes for FFAs and cholesterol, the free long-chain fatty acid and cholesterol concentrations were measured using GC–MS analysis of the organic phase cell extracts. At 8 days postdifferentiation, there was an increase in lauric acid, palmitic acid, linoleic acid, and linolenic acid and a decrease in stearic acid and oleic acid in adipocytes compared with preadipocytes (Figure 3B). In terms of C18 chain fatty acids, more unsaturated fatty acids were produced in adipocytes, while diarylheptanoid 1 treatment resulted in a return of their concentrations to higher levels. Diarylheptanoid 1 also returned the levels of C12:0 fatty acids to those of the preadipocytes; however, the concentration of C16:0 fatty acids further increased after diarylheptanoid 1 treatment. There was also a significant increase in the concentration of cholesterol in adipocytes, which was decreased by diarylheptanoid 1. Hence, diarylheptanoid 1 decreased the synthesis of polyunsaturated long chain fatty acid and cholesterol and partly decreased the synthesis of midchain fatty acids.

¹³C Glucose Labeling and Carbohydrate Metabolism

¹³C enrichment of a number of key TCA metabolites and amino acids was measured by adding U–¹³C glucose to the media and performing GC–MS isotopic analysis. Compared with preadipocytes, there was a reduced incorporation of ¹³C into most of the TCA metabolites and amino acids except for oxaloacetate (OAA). The incorporation pattern also pointed to a significant inhibition of the TCA cycle as the adipocytes entered into a relative steady mature state. After diarylheptanoid 1 treatment, many of the metabolites showed a restored ¹³C isotope incorporation compared with adipocytes, except for pyruvate, lactate, citrate, and OAA (Figure 4), which are closely related to acetyl-CoA used for fatty acid synthesis. Thus diarylheptanoid-1-treated adipocytes showed a different carbohydrate utilization from adipocytes during the last 2 days.

DISCUSSION

In this investigation, we explored the combination of targeted aqueous profiling, global lipidomics, targeted fatty acids, and flux analysis to discover sensitive metabolic changes modulated by antiadipogenic diarylheptanoid 1 in the well-studied 3T3-L1 cell line.^{12–14} On the basis of aqueous metabolic profiling, we first built in a map of altered metabolic pathways using information obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and based on the differential metabolites measured in this study (see Figure 5). The regulation of the TCA cycle and glycolysis is important for the differentiation process. A previous study indicated that cultured adipocytes enter a state of metabolic

overdrive where increased flux through the TCA cycle occurs.¹² In our study, the intermediates detected in the TCA cycle, such as fumarate, succinate, α -ketoglutarate, and OAA, were all upregulated in 3T3-L1 adipocytes, possibly to increase ATP production for differentiation, which is consistent with the previous report. Many of these intermediates were restored to normal levels after diarylheptanoid 1 treatment, which indicated that diarylheptanoid 1 probably helped to balance energy metabolism.

Increased glycolysis is also proposed to be associated with cell differentiation and lipid synthesis in adipocytes.³² G6P is converted into 6-phosphoglucono- δ -lactone by glucose-6-phosphate dehydrogenase (G6PD). G6PD is required in lipogenesis, and its increase is tightly associated with dysregulation of lipid metabolism and insulin resistance in obesity.³² In 3T3-L1 cells, G6PD overexpression stimulated the expression of most adipocyte marker genes and elevated the levels of cellular free fatty acids, triglyceride, and FFA release.³² In this study, the intermediates G6P and 3-phosphoglyceric acid (3PG) were significantly upregulated in adipocytes and were restored to their normal levels by diarylheptanoid 1 treatment. Nicotinic acid and nicotinamide are essential for organisms as the precursors for the generation of coenzymes NAD⁺ and NADP⁺, which are essential for redox reactions. These coenzymes are also crucial for the TCA cycle and glycolysis.³³ The level of niacinamide was significantly increased in adipocytes, which was also returned to its normal level after diarylheptanoid 1 treatment. These results further indicate that diarylheptanoid 1 treatment can maintain normal TCA cycle and glycolysis levels and can inhibit excess production of ATP for cell differentiation.

Amino acid metabolism was significantly impacted in a number of ways by diarylheptanoid 1 treatment (Figure 5). First, the level of threonine was significantly increased in adipocytes and reversed to normal levels in the treatment group. Serine and threonine are precursors to pyruvate. Branched chain amino acids (BCAAs) have been reported to be implicated in obesity, insulin resistance, and type 2 diabetes mellitus. BCAA can be derived from pyruvate, which can be produced by from glycine, serine, and threonine metabolisms.¹⁸ Catabolism of BCAAs contributes significantly to synthesis of fatty acids in 3T3-L1 adipocytes. Previous research showed that leucine and isoleucine may contribute ~25% to the lipogenic acetyl-CoA pool.¹³ In this study, the concentrations of leucine and isoleucine were both significantly increased by diarylheptanoid 1 treatment, which suggests that diarylheptanoid 1 inhibits the catabolism of BCAAs to acetyl-CoA pools. In addition, glutamine metabolism is also required for 3T3-L1 cell differentiation.³⁴ Mature adipocytes are known to release glutamine. As anticipated, the concentration of glutamine increased in adipocytes, while diarylheptanoid 1 reversed the increased glutamine level. It is apparent from the observed changes in amino acid levels that the adjustment of amino acid metabolism by diarylheptanoid 1 can restrain lipogenesis.

Purines perform many important functions in energy metabolism and signal transduction.³⁵ Fatty acid synthesis is closely associated with de novo purine synthesis through the activation of the pentose phosphate pathway.³⁶ An increase in the level of ribose-5-phosphate, which is involved in the pentose phosphate pathway, was observed in adipocytes, and it was restored to normal levels after treated with diarylheptanoid 1. On the contrary, accelerated purine catabolism and uric acid production have been found in adipose tissue in

a previous study.¹⁷ Altered purine metabolism was observed in our study. (Figure 6). There were obvious increases in AMP, GMP, IMP, adenosine, guanosine, and adenine and decreases in xanthine, inosine, hypoxanthine, xanthosine, urate, and allantoin in adipocytes. The restorative impact of diarylheptanoid 1 on these purine metabolites was also observed. Conversely, we did not find any overproduction of uric acid or allantoin in the adipocytes, so we checked the levels of these metabolites in the media. (Table S-8) An obvious increase in allantoin was observed in the media of adipocytes, and it was returned to normal levels in the media of treatment group.

Obesity is associated with up-regulated lipolysis in adipose tissue.³⁷ AMP-activated protein kinase (AMPK) is an intracellular energy sensor that has been implicated as a major regulator of glucose and lipid metabolism. It is activated by increases in intracellular AMP/ATP ratio when cells are stressed. Activated AMPK accelerates ATP-producing pathways such as fatty acid oxidation in adipocytes.³⁸ In our study, there were no significant changes for ATP among the three cell groups. Instead, there was an eight-fold increase in AMP in adipocytes compared with preadipocytes, which was reversed to about four-fold in the treated group. This result suggests more accumulated adipocytes need lipolysis, and the produced ATP can compensate the ATP depletion. On the basis of the above results, we can conclude that diarylheptanoid 1 can inhibit fatty acid synthesis by regulating the pentose phosphate pathway, repair the dysfunction of adipocytes through decreasing purine catabolism, and adjust lipolysis via the AMPK-mediated pathway.

Complex FFAs and lipids synthesis are required for lipid accumulation,^{12,15,39} and dysregulation of fatty acids and lipid metabolism in adipocytes is a significant problem in obesity. The de novo FA synthesis is activated in adipocytes, and the composition of saturated and unsaturated FAs is important for energy homeostasis. The level of biotin, which is important for FA synthesis, was significantly upregulated in adipocytes, and it was restored to normal levels after diarylheptanoid 1 treatment (Figure 5). In agreement with previous work,¹² there was an increase in even-chain fatty acids (C12:0) and polyunsaturated even chain fatty acids (C18:2 and C18:3). After treatment with diarylheptanoid 1, the levels of lauric acid, linoleic acid, and linolenic acid were down-regulated (Figure 3B).

Lipid synthesis was also upregulated in adipocytes. Previous research indicated that droplets of the adipocytes contain a large number of neutral lipids such as triacylglycerol and cholesteryl esters and a small number of phospholipids.¹⁵ The phospholipid composition included a diverse set of lipid classes: PC, PE, PI, ePC, ePE, PS, and SM. In our study, the significantly changed lipids between preadipocytes and adipocytes were primarily from the TG, DG, PC, and PE classes and partly from MG, SM, Cer, lysoPC, lysoPE, and CL lipids and cholesterol esters. To form large droplets in adipocytes, most of the significant lipids were upregulated, except for cholesterol esters. Treatment with diarylheptanoid 1 results in 171 of these lipids being restored to their preadipocyte levels. We also found that there was a significant increase in cholesterol and a significant decrease in cholesterol esters in adipocytes compared with preadipocytes based on the lipidomics and fatty acids data analysis. This result indicates that more cholesterol esters were hydrolyzed into cholesterol upon adipogenesis, and diarylheptanoid 1 reduced the cholesterol production. Taken

together, these results indicate that diarylheptanoid 1 can inhibit fatty acid and lipid synthesis in adipocytes.

Finally, diarylheptanoid 1 could also affect carbohydrate metabolism, which has previously been shown to be affected in different stages of differentiation.¹² During the earliest stages, the TCA cycle is upregulated, as represented by an increase in the pool sizes of TCA intermediates, possibly to increase ATP in preparation for differentiation. The key intermediates continue to increase during the middle and late differentiating stages. At the end of differentiation, the TCA cycle and glycolysis enter into a relatively decreased level of activity. In our study, carbohydrate metabolism in adipocytes during the last differentiation process (days 6–8 postinduction) showed a decreased TCA flux, in agreement with previous work.¹² Not surprisingly, diaryl-heptanoid 1 treatment of the adipocytes reversed this effect and showed relatively higher TCA flux. Adipocytes that were entering into the last differentiation period were delayed by diaryl-heptanoid-1-treatment.

CONCLUSIONS

To the best of our knowledge, this is the first report in which a broad-based multiplatform metabolomics analysis has been applied to study the changes in metabolism required to bring about differentiation of 3T3-L1 cells that were reversed by (3*R*,5*R*)-3,5-dihydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptane (1). Our results demonstrated that diarylheptanoid 1 inhibited the differentiation and lipid accumulation of 3T3-L1 cells by balancing energy metabolism and regulating lipogenesis and lipolysis, which involve multiple aspects of metabolism, including adjustment of the TCA cycle flux and glycolysis, disruption of amino acid catabolism, inhibition of purine catabolism, and decreased de novo fatty acid and lipid synthesis. These data provide new information and potential targets for future mechanistic studies with respect to the anti adipogenic effects of diarylheptanoid 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Schematic protocol for the metabolomics investigation of the diarylheptanoid intervening mouse 3T3-L1 preadipocytes differentiation. See the text for a description of the procedure.

Figure 2. Antiadipogenic effect of diarylheptanoid 1 interrupts metabolism of 3T3-L1 adipocytes. (A) Antiadipogenic effect of diarylheptanoid 1 on lipid accumulation during differentiation of 3T3-L1 cells. (i) Structure of diarylheptanoid 1. Photos of preadipocytes (ii) taken at day 0, adipocytes (iii), and adipocytes treated with diarylheptanoid 1 at 25 μ M (iv) taken at day 8 after ORO staining. (B) PCA score plots showing clustering of aqueous metabolites analyzed by LC–QQQ/MS (i) and lipids analyzed by LC–QTOF/MS (ii) extracted from preadipocytes (control), adipocytes (model), and diarylheptanoid 1-treated adipocytes (Treatment). (C) Pathway analysis showing an overview of main pathways related to the differentiated aqueous metabolites in 3T3-L1 adipocytes intervened by diarylheptanoid 1.

Figure 3.

Changes of lipid classes and fatty acids in the three cell groups. (A) Box plots of significantly changed lipid classes among control, model, and treatment groups. Normalized values of lipids were grouped into each lipid class. Values of each lipid class from the minimum to the maximum are indicated by the lower and upper box boundaries, respectively, with the vertical solid lines marking the distribution of each single lipid. Median metabolite values are indicated by the middle horizontal lines. (B) Comparative levels of free fatty acids (FFAs) measured by GC-MS extracted from control, model, and treatment groups. * $p < 0.05$ and ** $p < 0.01$, control versus model group. # $p < 0.05$ and ## $p < 0.01$, model versus treatment group.

Figure 4.

Incorporation of ^{13}C from glucose into TCA metabolites and some amino acid metabolites. Bar charts left to right: control (green), model (blue), and treatment (red) groups; the Y axis represents mean enrichment of ^{13}C in the metabolites. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, control versus model group. # $p < 0.05$ and ## $p < 0.01$, model versus treatment group.

Figure 5.

Metabolic network of the significantly changed metabolites involved in central carbon metabolism (glycolysis and TCA) and amino acid metabolism. Bar charts left to right: control (green), model (blue), and treatment (red) groups; the *Y* axis represents relative abundance of MS signals (normalized to the control in comparison). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, control versus model group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$, model versus treatment group. Compound not in bold means no value was measured for the metabolite.

Figure 6.

Metabolic network of the purine metabolism metabolites with significantly altered levels. Bar charts left to right: control (green), model (blue), and treatment (red) groups; the Y axis represents relative abundance of MS signals (normalized to the control in comparison). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, control versus model group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$, model versus treatment group. Compound not in bold means no value was measured for the metabolite.

Table 1.

Metabolites Displaying Significant Changes in Response to the Anti-Adipogenic Effect of Diarylheptanoid 1^a

no.	metabolite name	metabolic pathway	M/C ^b	FC ^b	VIP ^b	P ^b	T/M ^c	FC ^c	P ^c
1	adenosine	purine metabolism	(↑)	26.40	1.33	4.85×10^{-6}	(↓)	0.21	6.60×10^{-8}
2	GMP	purine metabolism	(↑)	9.65	1.32	3.24×10^{-9}	(↓)	0.45	2.80×10^{-6}
3	AMP	purine metabolism	(↑)	8.24	1.34	5.50×10^{-12}	(↓)	0.54	1.53×10^{-7}
4	pyroglutamic acid	amino acid	(↑)	3.22	1.23	5.26×10^{-4}	(↓)	0.56	3.54×10^{-3}
5	IMP	purine metabolism	(↑)	2.92	1.31	1.15×10^{-7}	(↓)	0.54	6.85×10^{-6}
6	2-aminoadipate	amino acid	(↑)	2.92	1.30	1.45×10^{-7}	(↓)	0.38	3.66×10^{-7}
7	3-phosphoglyceric acid	glycolysis	(↑)	2.28	1.27	3.07×10^{-6}	(↓)	0.64	9.14×10^{-4}
8	α-ketoglutaric acid	TCA cycle	(↑)	2.05	1.29	4.23×10^{-7}	(↓)	0.74	1.66×10^{-3}
9	biotin	biotin metabolism	(↑)	1.86	1.19	1.15×10^{-4}	(↓)	0.51	1.41×10^{-4}
10	UDP	pyrimidine metabolism	(↑)	1.84	1.07	1.95×10^{-3}	(↓)	0.67	2.64×10^{-3}
11	phenylpyruvic acid	amino acid	(↑)	1.81	1.28	1.41×10^{-4}	(↓)	0.0011	2.72×10^{-6}
12	glutamine	amino acid	(↑)	1.78	1.28	1.56×10^{-6}	(↓)	0.82	1.35×10^{-3}
13	niacinamide	nicotinate and nicotinamide	(↑)	1.56	1.20	8.29×10^{-5}	(↓)	0.70	1.00×10^{-3}
14	guanosine	purine metabolism	(↑)	1.52	1.23	2.45×10^{-5}	(↓)	0.33	4.56×10^{-9}
15	iso-leucine	amino acid	(↓)	0.53	1.30	9.33×10^{-6}	(↑)	1.21	2.40×10^{-2}
16	leucine	amino acid	(↓)	0.45	1.30	6.09×10^{-6}	(↑)	1.47	9.03×10^{-4}

^a (↑), up-regulated; (↓), down-regulated.^b Represents the changes in the model (M) group compared with control (C) group.^c Represents the changes in the diarylheptanoid-1-treated (T) group compared with model (M) group.