The polymethoxyflavone Sudachitin modulates the circadian clock and improves liver physiology

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Keywords

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ABSTRACT

Scope: Polymethoxylated flavones (PMFs) are a group of natural compounds known to display a wide array of beneficial effects to promote physiological fitness. Recent studies revealed circadian clocks as an important cellular mechanism mediating preventive efficacy of the major PMF Nobiletin against metabolic disorders. Sudachitin is a PMF enriched in *Citrus sudachi*, and its functions and mechanism of action are poorly understood.

Methods and Results: Using circadian reporter cells, we showed that Sudachitin modulated circadian amplitude and period of *Bmal1* promoter-driven reporter rhythms, and real-time qPCR analysis showed that Sudachitin altered expression of core clock genes, notably *Bmal1*, at both transcript and protein levels. Mass-spec analysis revealed systemic exposure *in vivo*. In mice fed with high-fat diet with or without Sudachitin, we observed increased nighttime activity and daytime sleep, accompanied by significant metabolic improvements in a circadian time-dependent manner, including respiratory quotient, blood lipid and glucose profiles and liver physiology. Focusing on liver, RNA-sequencing and metabolomic analyses revealed prevalent diurnal alteration in both gene expression and metabolite accumulation.

Conclusion: Our study elucidates Sudachitin as a new clock-modulating PMF with beneficial effects to to improve diurnal metabolic homeostasis and liver physiology, suggesting the circadian clock as a fundamental mechanism to safeguard physiological well-being.

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1. Introduction

Natural flavonoids are ubiquitously present in plants and known to display a wide spectrum of biological activities. Of particular interest are polymethoxylated flavones (PMFs) that have shown favorable pharmacokinetic profiles and robust efficacies [1-3]. PMFs are primarily enriched in citrus peels, and various components or extracts have demonstrated anti-inflammatory, metabolic, chemopreventive and neuroprotective activities in cell culture, rodent models and human studies. Nobiletin (NOB, hexa-methoxylated) and Tangeretin (penta-methoxylated) are two well-studied PMFs [1, 3-5]. Compared with hydroxylated analogs lacking the methoxyl groups, these PMFs are more lipophilic and display greater bioavailability due to enhanced transport and metabolic stability, contributing to a superior bioactivity [2]. For example, NOB has been found to effectively improve cholesterol/lipid metabolism in atherosclerogenic *Ldlr-/-* mice when incorporated in the diet at 0.1% (w/w) [6], whereas Naringenin, its analog lacking methoxyl groups, was used in similar mouse experiments at 1% (w/w) or higher [7, 8]. Sudachitin is a PMF enriched in the peels of *Citrus sudachi* widely grown in Tokushima, Japan, and has been found to promote energy metabolism and skeletal muscle mitochondria in mice [9-11]. However, unlike other better-known PMFs, biological activities of Sudachitin, especially *in vivo*, remain poorly characterized.

Given the excellent bioactivity and safety characteristics of PMFs as nutritional supplements and potential intervention molecules, it is important to delineate their mechanisms of action in disease models. Recent studies suggest the circadian clock as a possible cellular mechanism underlying efficacies of certain PMFs, notably NOB [12]. The circadian clock is an internal

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timing device that governs the daily life of cells and organisms [13]. In mammals, synchronized by the master pacemaker in the hypothalamus, cells throughout the body harbor a ubiquitous cellular oscillator consisting of positive and negative components forming negative feedback loops [14-16]. These cell-autonomous oscillators orchestrate cell- and tissue-specific gene expression programs, supporting specific physiological functions at individual organs [17-19]. Extensive previous research has revealed that one of the most fundamental functions of the circadian clock is metabolic regulation [20-22]. Metabolic gene expression, activities of enzymes/nutrient sensors/transporters, and metabolites have all been shown to undergo robust circadian oscillation in various tissues, cells and organelles [18, 23]. On the other hand, such metabolic rhythms were largely abolished in genetic models where circadian rhythms were disrupted or severely dampened due to core clock gene deficiency. In these circadian mutant mice, a number of metabolic disorders have been demonstrated; for example, the *ClockΔ19* mutant mice were found to be obesity-prone when fed with high-fat diet or at older ages [24]. The correlation of circadian dysregulation and metabolic disease is of particular interest because modernization of human societies has led to prevalent circadian disruptions including shiftwork, light pollution and other facets of 24/7 lifestyles, which coincides with the escalating epidemic of the metabolic syndrome (e.g., obesity and insulin resistance).

Recently we reported a circadian regulatory function in the physiological efficacy of NOB [12]. In a high-throughput screen to identify clock-targeting molecules, NOB and its close analog Tangeretin were found to enhance circadian amplitude in reporter cells [25]. More detailed analyses focusing on NOB showed that NOB directly targets circadian oscillators, which markedly improves systemic energy homeostasis in diet-induced obese mice and diabetic mice.

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Through transcriptomics/metabolomics coupled with functional assays, NOB was found to exert systemic effects to reshape circadian gene oscillations in both liver and skeletal muscle tissues [25, 26], promoting metabolic health in young and aged mice. The link between circadian and metabolic functions of NOB has also been demonstrated in other organ systems and pathophysiological conditions [12, 27-31]. Together, these studies support the notion that the systemic effects of NOB, and perhaps other PMFs, are in part mediated by the circadian clock system.

In the current study, we investigated whether Sudachitin is a novel clock-modulating PMF that functions to promote metabolic health. Using circadian reporter cell lines, we found that Sudachitin strongly activated the circadian amplitude of *Bmal1* promoter-directed reporter expression *in vitro*, and modulated core clock gene expression, particularly *Bmal1*, as revealed by qPCR and immunoblotting. Mass-spec analysis revealed systemic exposure. In mice fed with high-fat diet with or without Sudachitin, we observed enhanced wheel-running activity, lengthened free-running periods, and elevated daytime sleep, accompanied by significant metabolic improvements in both systemic and liver metabolism. Focusing on liver, RNA-sequencing and metabolomic analyses revealed diurnal circadian and metabolic alterations in response to Sudachitin. Therefore, these results elucidate Sudachitin as a new clock-modulating PMF with strong systemic and hepatic efficacies to improve metabolic homeostasis. These studies also highlight the circadian clock as a fundamental mechanism to safeguard physiological wellbeing.

2. Materials and methods

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2.1. Cell culture studies

Bmal1-luc-expressing U2OS cells expressing Bmal1-dGluc [32] and *Per2::LucSV* mouse

fibroblast cells established from adult *Per2::LucSV* knock-in mice [33, 34] were used for real-

time bioluminescence monitoring. Cells were grown to confluency on 35 mm dishes in DMEM

supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, and 100 μg/mL streptomycin

(GenDepot, Katy, TX, USA). Cells were synchronized with 200 nM dexamethasone (Sigma–

Aldrich, St. Louis, MO, USA) for 1 hr, and then the media were replaced by luciferin-containing

recording media [33, 34] supplemented with vehicle (demethyl sulfoxide, DMSO) or 1–10 μM

Sudachitin (Fujifilm WAKO chemicals, Osaka, Japan). The dishes were tightly sealed with

vacuum grease and placed in a luminometer (LumiCycle 32, Actimetrics, Wilmette, IL, USA) for

continuous bioluminescence monitoring over 5-7 days. The data were detrended and fitted to a

sine wave for measurement of circadian parameters in the LumiCycle data analysis software

(Actimetrics). For measurements of mRNA and protein levels of core clock genes, cells were

synchronized with 200 nM dexamethasone for 1 hr, and incubated in fresh media containing

vehicle (DMSO) or 10 μM Sudachitin. Cells were harvested every 4 hrs for 28 hrs for total RNA

and protein extraction.

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2.2. Purification of Sudachitin for animal treatment

The green peels of *Citrus sudachi* Hort. ex Shirai were collected in Tokushima Prefecture, Japan. The dried peer extracts with 40% ethanol were dissolved with water and fractioned using a synthetic adsorbent DIAION™ HP20 (Mitsubishi Chemical, Tokyo, Japan). The fractions eluted with 30-50% ethanol were dried-up, hydrolized by hydrochloric acid, and mixed with ethyl acetate. The hydrophobic fractions were dried and dissolved with 80% ethanol, and fractionated by using the Sephadex® LH-20 column (Cytiva, Tokyo, Japan). The highly concentrated fraction was dried and crystallized under room temperature. The purity of the purified Sudachitin was measured by using the Agilent 1260 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA).

2.3. Animal Studies

A schematic diagram to indicate the timeline for animal studies is shown in Fig. S1. Four-week-old male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). After four weeks of acclimation, the mice were fed with 45 cal% high-fat diet (HFD) (Research Diets, New Brunswick, NJ, USA) *ad libitum* and treated with either vehicle (PBS, n = 11) or Sudachitin (100 mg/kg body weight, n = 12) *via* oral gavage during ZT8-10 every other day. Note that ZT refers to zeitgeber time, with ZT0 and ZT12 corresponding to light on and off respectively. Body weight and food intake were monitored weekly. Tissue samples were collected from 30-week-old mice at the indicated circadian times (ZT6 and ZT18). All animal studies were approved by the Center for Laboratory Animal Medicine and Care

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(CLAMC) in the University of Texas Health Science Center at Houston, protocol number: AWC-17-0043, and were conducted in compliance with CLAMC-designated guidelines. The mouse facility is specific-pathogen free and is tested with sentinel mice monthly.

2.4. Dosage regimen of Sudachitin for mice

The HFD-fed mice were treated with either vehicle or Sudachitin (100 mg/kg body weight) *via* oral gavage in late afternoon (ZT8-10) every other day throughout the experimental period and the behavior analyses. There are two main reasons why we chose this dosing scheme. First, in our previous study [25] where we investigated the efficacy of another natural clock-modulating compound, NOB, we used a similar dosing design (oral gavage every other day) which revealed significant efficacies. The half-life of Nobiletin, at least in the plasma, was similar to of Sudachitin (1.8 hr vs. 1.7 hr) [35]. Second, given the limited amount of the compound which is not commercially available in large quantities, this dosing frequency was designed to optimize exposure throughout the entire study.

2.5. Pharmacokinetic studies in mice

Sudachitin was administered orally at ZT2 at 100 mg/kg in PBS suspension. Eight-week-old wild-type male mice (n = 4 per time point) were sacrificed at 0, 1, 2, 4, 8 and 24 hrs after oral gavage. Sudachitin and the derivatives in plasma, brain, liver, white adipose tissue (epididymal fat), brown adipose tissue, and skeletal (soleus) muscle were determined by LC-TOFMS (Agilent Technologies), as described in Section. 2.15. Similarly, Sudachitin was administered

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at ZT2 to mice either fed under normal conditions or overnight fasted and its brain accumulation was measured at 0.5 and 1.0 hr after oral gavage.

2.6. Mouse wheel-running behavior

Twenty-four-week-old mice were acclimated in individual cages equipped with running wheels for 3 weeks under the normal 12 hr light/12 hr dark (LD) cycle, followed by 3 weeks in constant darkness (DD). Activity data were recorded continuously and analyzed using the CLOCKLAB software (Actimetrics). Free-running periods were calculated using a periodogram with 6 min resolution. Wheel-running activity levels under both LD and DD conditions were quantified as a summation every 20 min for each mouse and averaged during the 2-week data collection.

2.7. Noninvasive piezoelectric transducer sleep/wake recording

Sleep/wake recording was carried out by using a noninvasive piezoelectric transducer sleep/wake recording system (Signal Solutions, Lexington, KY, USA) as previously described [36]. Briefly, mice were single-housed in the Piezo system with free access to food and water under the normal LD cycles. The initial 48 hr acclimation period was followed by data recording for 2 days.

2.8. Measurement of energy expenditure

Energy expenditure was examined by measuring oxygen consumption with indirect calorimetry as previously described [25, 26]. Sixteen-week-old mice were placed at room temperature (22°C–24°C) in Comprehensive Lab Animal Monitoring System (CLAMS,

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Columbus Instruments, Columbus, OH, USA) metabolic chambers. Volume of O_2 consumption (VO_2) and CO_2 production (VCO_2) was continuously recorded over a 24-hr period. Energy expenditure was calculated by average O_2 consumption. Respiratory exchange ratio (RER) was calculated as the VCO_2/ VO_2 ratio.

2.9. Glucose tolerance test

Glucose tolerance test was conducted as previously reported [25, 26]. After overnight fasting, twenty-week-old mice were weighed prior to glucose injection (1 mg/kg body weight) at ZT2. Blood glucose was measured at 0, 15, 30, 60, and 120 min after glucose challenge.

2.10. Plasma Content Assays

Plasma samples were collected from tail vein of twenty-week-old mice after overnight fasting or without fasting. Plasma insulin was measured by ELISA (Morinaga Institute of Biological Science, Yokohama, Japan). Plasma triglyceride (TG), total cholesterol, and non-esterified fatty acid (NEFA) were measured by colorimetric assays (Fujifilm Wako chemicals). Plasma alanine transaminase (ALT) was measured by a colorimetric assay (Cayman chemicals, Ann Arbor, MI, USA).

2.11. RNA extraction and real-time RT-qPCR analysis

RNA extraction and RT-qPCR analysis were carried out as previously described [25, 26].

Total RNA from *Bmal1-luc* U2OS cells and mouse liver was isolated using PureXtract

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RNAsol® reagent (GenDEPOT). RT reactions were performed with 200 ng purified RNAs.

Gapdh and *act-b* were used as controls. The primer sets used are shown in Table S1.

2.12. *Western blotting*

Western blotting using 15 μg of total protein lysates was performed as previously described

[25, 26]. The following specific antibodies were used, including anti-REV-ERBα (Cell

Signaling Technology, Danvers, MA, USA), anti-PER2 (Sigma–Aldrich), and home-made

antibodies against the other clock proteins [25, 26].

2.13. Histological analysis

Mouse liver tissues were fixed in 10% neutral buffered formalin and embedded in paraffin.

Sections at 5-μm thickness were stained with hematoxylin–eosin. Histological features were

scored according to the nonalcoholic fatty liver disease (NAFLD) activity scores [37].

2.14. *RNA-sequencing*

Two micrograms of extracted RNA from liver (RNA Integrity Number > 9.0, measured by an

Agilent 2100 Bioanalyzer) was used for library construction and RNA sequencing analysis.

PolyA enriched non-stranded RNA sequencing was carried out by Novogene (Sacramento, CA,

USA) on Illumina NovaSeq 6000 with 150-bp paired-end reads, as previously reported [38].

From all sequences, sequence adaptors were removed using Trimmomatic version 0.39 [39].

The sequence reads were mapped to the mouse genome (GRCm38/mm10) using STAR

version 2.7.2a [40]. To obtain reliable alignments, the reads with a mapping quality of less

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than 10 were removed by SAM tools version 1.7 [41]. The University of California, Santa

Cruz (UCSC) known canonical gene set (55,421) was used for annotation, and the reads

mapped to the exons were quantified using the analyzeRNA.pl script in the Homer software

with an option of –rpkm or -noadj for FPKM or raw read counts, respectively. Since the

Homer treat each half of the read separately and count each as 0.5 reads for paired-end reads,

the raw read counts were rounded to the nearest integer before analyzing differential expressed

genes using DESeq2 [42]. For gene level analysis, the highest expressed isoform was chosen

to report one isoform per locus. We assumed that a gene was expressed if there were more than

20 reads mapped on average in the exon of the gene. per million reads mapped on average in

the exon of the gene. The expression level cutoff, average FPKM > 0.5, was used for

downstream data analysis. Differentially expressed genes, with threshold of p < 0.05 and fold

change of 1.5, were determined by using DESeq2 [43]. Functional enrichment analysis was

carried out by using Metascape [44].

2.15. Metabolomic analysis

The hydrophilic and lipophilic metabolites of liver homogenates were isolated using the Bligh

and Dyer method [45] and measured by CE- and LC-TOFMS, respectively, as previously

described [46]. The m/z values, retention times or migration time, and ion counts of

metabolites were extracted from the total ion chromatogram using the Molecular Future

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Extraction method of MassHunter software (Agilent Technologies). The metabolites that

significantly differed between ZT6 and ZT18 in the control and Sudachitin groups ($P < 0.05$

by one-way analysis of variance) were selected by a multivariate analysis using Mass Profiler

Professional software (Agilent Technologies). The selected metabolites were identified by

using the METLIN Personal Metabolite Database (Agilent Technologies) and LIPID MAPS®

(Wellcome Trust, London, GB). Enrichment analysis of KEGG pathway was carried out by

using MetaboAnalyst 5.0 software [47].

2.16. Statistical analysis

Using the CircaCompare rhythmicity software [48, 49], three rhythmic parameters of

endogenous clock genes expression were determined: mesor (mean level), amplitude (half the

range of oscillation), and peak time. Statistical analysis of differences was performed using

ANOVA with Bonferroni's multiple comparison tests using Statview 5.0 software (SAS

Institute Inc., Cary, NC, USA). Student's t-test was used for paired data where appropriate. $P <$

0.05 was considered statistically significant.

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3. Results

3.1. Sudachitin is a clock-modulating compound in cells

To investigate whether Sudachitin functions to modulate circadian clocks, we first tested its efficacy *in vitro*. Two circadian reporter cells were used, including Bmal1-luc U2OS cells [32] and Per2::LucSV fibroblast cells [33, 34]. The former expresses luciferase transcripts from the *Bmal1* promoter whereas in the latter, the PER2:LUC fusion proteins are driven by the endogenous *Per2* promoter. Interestingly, whereas Sudachitin showed moderate effects in Per2::LucSV cells (supplementary Fig. S2), it strongly enhanced the circadian amplitude of the luciferase reporter directed by the *Bmal1* promoter in U2OS cells in a dose-dependent manner (Fig. 1A). In comparison, a period-lengthening effect was detected at 10 µM, but not at lower doses (Fig. 1A).

We therefore conducted further molecular analyses using Bmal1-luc U2OS cells treated with 10 µM Sudachitin to determine core clock gene expression. As shown in Fig. 1B, real-time qPCR analysis revealed that *Bmal1* transcript level was elevated by Sudachitin. The rhythmic parameters, mesor and peak time, of *Bmal1* and *Per2* transcript expression were increased by Sudachitin treatment (supplementary Table S2). In comparison, Sudachitin showed distinct effects on other core clock genes.

Consistent with reporter and qPCR results, BMAL1 protein accumulated to higher levels throughout the circadian cycle and the rhythmic parameters were increased by Sudachitin (Fig. 1C, Table S2). While REV-ERB protein levels were reduced at CT18 and 22, PER2 and CRY1 protein

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levels were increased by Sudachitin (Fig. 1C). Taken together, Sudachitin shows strong effects on *Bmal1* expression at both transcript and protein levels, and differentially affects other core clock components.

3.2 Pharmacokinetics of Sudachitin

To determine the concentrations of Sudachitin and its derivatives in mouse plasma and tissues, we collected plasma, brain, liver, epididymal fat, brown adipose tissue, and soleus muscle from mice after oral administration (100 mg/kg body weight) and performed LC-TOFMS. The Sudachitin compound used was originally purified from *Citrus sudachi* and consisted of approximately 85% Sudachitin and 15% Demethoxysudachitin (Fig. 2A). Concentrations of Sudachitin in the plasma and all tissues were significantly increased at 1 hr after oral administration and higher than the glucuronide derivative and Demethoxysudachitin (Fig. 2C), suggesting a predominantly aglycon form in mouse tissues. In addition, it accumulated in the liver to a much higher level than in other tissues (Fig. 2B), suggesting that the liver is a key target tissue.

3.3 Sudachitin modulates mouse wheel-running behavior and energy metabolism

Previously we showed that NOB was able to counteract metabolic challenge by high-fat diet to improve energy homeostasis in mice [25]. We therefore treated HFD-fed wild-type C57BL/6J mice with Sudachitin *via* oral gavage during the inactive phase as previously performed [25] (supplementary Fig. S3A) and investigated its effects on circadian behavior and energy

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metabolism. Sudachitin treatment was able to increase wheel-running activity in the middle of nighttime (Fig. 3A-B) and interestingly also lengthened the free-running period under DD conditions (Fig. 3C). In the non-invasive Piezo sleep recording experiment, we found that Sudachitin increased daytime and total sleep amounts (Fig. 3D). Together, these results reveal *in vivo* efficacies of Sudachitin in clock-directed activity and sleep behaviors.

We next examined energy metabolism in these mice. Sudachitin treatment did not lead to significant changes in body weight or food intake (supplementary Fig. S3B-C). In metabolic chambers, sudachitin-treated mice show reduced respiratory exchange rates (RER) (Fig. 3F), suggesting upregulation of lipid utilization, whereas oxygen consumption was not significantly altered (Fig. 3E). We next examined circulating lipid and glucose profiles. Plasma triglyceride and NEFA levels were significantly reduced by Sudachitin under the fed, but not fasted, condition (Fig. 4, A-B), and plasma cholesterol levels remained unchanged (Fig. 4C). Likewise, blood glucose and insulin levels were reduced by Sudachitin only in the fed condition (Fig. 4, D-E). Glucose tolerance test revealed a trend of improvement in Sudachitin-treated mice relative to control mice (supplementary Fig. S3D-E).

Consistent with largely constant body weight, weights of individual tissues, including liver, epididymal and subcutaneous fat, brown fat and skeletal muscle, were not altered by Sudachitin (supplementary Fig. S3F). On the other hand, H&E staining of the liver showed that both steatosis and hepatocellular ballooning [37] were reduced by Sudachitin (Fig. 4F). In accordance, the NAFLD activity score showed a trend of reduction in response to Sudachitin, and liver triglyceride content was reduced in Sudachitin-treated mice at ZT18 (Fig. 4, G-H). On the other hand, NEFA and cholesterol levels in the liver were not changed (supplementary Fig. S3G-H). As a marker of

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liver damage, plasma ALT activity was alleviated by Sudachitin (Fig. 4I). Additionally, mRNA levels of the genes involved in fibrosis (*Timp1*, *Tgf1b*) and inflammation (*Mcp1*, *Tnfa*) were lowered at ZT18 by Sudachitin (Fig. 4J). Together, these results support a beneficial role of Sudachitin to improve energy metabolism in HFD-fed WT mice.

3.4 Transcriptomic alteration in the liver by Sudachitin

Given the observed beneficial effects of Sudachitin in the liver, we focused on this central metabolic organ and applied omics approaches to globally survey Sudachitin-induced molecular alteration. First we conducted transcriptomics by RNA-sequencing using RNA samples from livers isolated from control and sudachitin treated mice at ZT6 and ZT18 (daytime and nighttime, respectively) (NCBI GEO accession number: GSE211612). Gene expression comparison between the two time points revealed 632 and 651 diurnal DEGs (differential expressed genes) in the control and Sudachitin groups respectively ($p < 0.05$, fold change >1.5) (Fig. 5, A-B, Table S3); among them, 243 genes were shared between the groups, suggesting an intrinsic, Sudachitin-independent oscillatory pattern (Fig. 5B). Metascape analysis [44] revealed enrichment of functional pathways, including lipid/fatty acid metabolism in the control group, PPAR signaling, circadian rhythm, and lipid catabolism in the sudachitin group, and acylglycerol metabolism shared between the groups (Fig. 5C). These results highlight a modulatory role of Sudachitin in hepatic circadian and lipid metabolic pathways.

To validate the profiling results, we examined mRNA levels of selected circadian and lipid metabolic genes by real-time qPCR (Fig. 5D-F). We found diurnal expression of core clock genes

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in both groups including *Per2*, *Rev-erba*, *Cry1*, and *Rorc* (Fig. 5D). In comparison, *Per1* showed altered phase in response to Sudachitin, and the levels of *Per1* and *Per2* at ZT6 and *Rev-erba* at ZT18 were statistically different between the two groups (Fig. 5D). Furthermore, several genes involved in lipid metabolism including *Insig1*, *Srebf2*, *Hmgcs2*, *Elvol6*, and *Acot3* displayed diurnal expression in both groups (supplementary Fig. S4). Group-specific diurnal expressions were also observed, including *Cyp7a1*, *Ppard*, *Ppara*, and *Acot3* in the control group (Fig. 5E) and *Fasn*, *Fads*, and *Acly* in the sudachitin group (Fig. 5F). In addition, the levels of *Cyp7a1*, *Acly*, and *Acaca* at ZT6 and *Ppard* at ZT18 were statistically different between the two groups (Figs. 5E-F, S4). These results together indicate transcriptomic modulation in the liver by Sudachitin, notably in circadian and lipid metabolic pathways.

3.5. Metabolomic alteration in the liver by Sudachitin

To investigate how Sudachitin alters the metabolomic landscape in the liver, we prepared both hydrophilic and lipophilic fractions from the liver. First, the hydrophilic fractions were subjected to CE-TOFMS analysis and 90 metabolites were identified by a standard metabolites reference kit (HMT, Tsuruoka, Japan). Between the two time points 31 and 14 metabolites showed diurnal alteration in the control and Sudachitin groups respectively ($p < 0.05$) (Fig. 6A-B, Table S4), including 5 metabolites shared between the groups (Fig. 6B). PCA analysis revealed significant changes at ZT6 in response to Sudachitin (Fig. 6C). Enrichment analysis by MetaboAnalyst 5.0 software [47] showed greater enrichment of citrate cycle, pyruvate metabolism, and amino acid metabolism pathways in the control group compared with the Sudachitin group (Fig. 6D, S5A-B). More specifically, most metabolites associated with the tricarboxylic acid (TCA) cycle (Figs. 6E,

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S5C), pyruvate metabolism (Fig. S5C), branched-chain amino acid metabolism (Fig. 6F), aromatic amino acid metabolism (Fig. 6G), and cystein and methionine metabolism (Fig. S5D) showed diurnal changes in the control group, and at significantly lower concentrations ($p < 0.05$, in mmol/g tissue) than in the Sudachitin group at ZT6. These results highlight a modulatory role of Sudachitin in hepatic glucose and amino acid metabolism pathways.

Next, LC-TOFMS analysis of the lipophilic fractions identified 706 metabolites, significantly more than those in the hydrophilic fractions. Between the two time points 75 and 14 metabolites showed diurnal fluctuation in the control and Sudachitin groups respectively ($p < 0.05$) (Fig. 7, A-B, Table S5); including 3 metabolites shared between the groups (Fig. 7B). In addition, PCA analysis revealed significant changes at both ZT6 and ZT18 in response to Sudachitin (Fig. 7C). Cross-referencing the METLIN and LIPID MAPS® databases identified distinct metabolites between the control and Sudachitin groups including peptides, phosphoglycerolipids, and sphingolipids. For example, tetra/penta-peptides (Fig. 7E, S6A) and phosphoglycerolipids including phosphatidylcholine (PC) and phosphatidylinositol (PI) (Fig. 7F, S6B) showed diurnal changes in the control group and significantly lower levels at ZT18 than in the Sudachitin group. At ZT6, sphingolipids including sphingosine and sphinganine (Fig. 7F) and other phosphoglycerolipids (Fig. S6C) showed diurnal changes in the control group and significantly lower levels than the Sudachitin group. These results highlight a modulatory role of Sudachitin in hepatic phospholipids and peptide metabolisms.

4. Discussion

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The circadian clock constitutes a key regulatory mechanism of energy metabolism, and previous studies demonstrate a clock-targeting efficacy of the major PMF Nobiletin [12]. In the current study, we explored a potential circadian and metabolic efficacy of Sudachitin, a natural PMF enriched in the peels of *Citrus sudachi* that is highly abundant in the Tokushima region. Interestingly, our results reveal novel circadian and metabolic activities of this under-studied PMF, providing valuable mechanistic and functional insights into this bioactive PMF.

Our *in vitro* and *in vivo* studies provide strong evidence for the clock-altering activities of Sudachitin. Interestingly, compared with NOB [25], Sudachitin showed moderate effects on PER2::LUC rhythms; rather, it enhanced *Bmal1* promoter-driven transcription as revealed by *Bmal1-luc* reporter cells and BMAL1 protein expression analysis. Consistent with these *in vitro* effects, in HFD-fed WT mice, Sudachitin was also found to increase nighttime wheel-running activity as well as daytime sleep, supporting a role of Sudachitin to potentiate circadian behavioral rhythms. The molecular mechanism and target of Sudachitin underlying the clock-modulatory activities remain to be investigated. Previously NOB was found to bind to and activate ROR receptors, core clock components in the secondary loop known to promote circadian robustness and stability [25]. However, given the distinct effects of

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Sudachitin on reporter rhythms and core clock gene expression, it is likely that Sudachitin may

act through a different mechanism in the circadian oscillator. Both qPCR and immunoblotting

results appear to underscore a strong effect of Sudachitin on *Bmal1* expression, at both

transcript and protein levels. *Bmal1* is tightly controlled by the secondary loop including both

RORs and REV-ERBs [15]. Interestingly, our results show that Sudachitin reduces the

transcript and protein levels of REV-ERBs, providing a possible explanation for the increased

Bmal1 expression. Finally, we adopted a similar alternate-day administration of Sudachitin as

previous described for NOB [25]. The half-life of NOB, at least in the plasma, is similar to that

of Sudachitin under the acute PK study conditions (1.8 hr vs. 1.7 hr) [35]. The reason

underlying the circadian and physiological effects despite the relatively short half-life is

unclear, but could involve sustained downstream action after the initial binding of compounds

to their cellular targets and/or activity of their metabolic derivatives as reported previously for

NOB [50, 51]. Furthermore, while a single dose was administered in the PK study, our *in vivo*

experiments entailed a chronic treatment regimen. Another variable is the feeding condition.

The time of gavage at ZT8-10 for our *in vivo* studies corresponds to a fasting condition, and

our PK study (supplementary Fig. S3A) showed that Sudachitin accumulates in the brain at

higher levels in fasted mice compared with fed mice. Follow-up studies should further

characterize *in vivo* effects of Sudachitin in physiological settings over a full circadian time

course under different feeding and treatment conditions.

Circadian regulation of energy metabolism is well established [17]. In a pioneering study, it

has been shown that circadian disruption exacerbates the metabolic syndrome induced by

nutrient excess in HFD-fed mice [24]. We previously showed that circadian enhancement by

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NOB was able to counteract the adverse effects of HFD, markedly improving systemic energy homeostasis and tissue metabolism in liver and skeletal muscle [25, 26]. The metabolic effects of Sudachitin have not been well characterized. Notably, in one study where Sudachitin was administered to mice suffering from metabolic syndrome, energy homeostasis was found to be improved, accompanied by enhanced mitochondrial biogenesis in skeletal muscle [9]. To further examine the effects of Sudachitin against metabolic challenge, especially in light of its clock-enhancing activities, we treated WT mice fed with HFD with Sudachitin. While we did not find changes in body weight, several systemic metabolic parameters were improved, including reduced respiratory exchange rate and lower lipid levels in circulation. Both findings suggest diminished lipid utilization under the HFD conditions as a result of Sudachitin treatment. We also found that plasma glucose and insulin levels were reduced under fed conditions, consistent with a trend of improved glucose tolerance. While liver, adipose and muscle tissue weights were largely unchanged in accordance with the body weight pattern, several aspects of liver physiologies were improved, including lipid accumulation and liver damage. This is consistent with our previous results that liver is a key target organ for clock regulation and NOB strongly improve liver health under HFD conditions [25].

Liver is the central metabolic organ, orchestrating myriads of metabolic pathways important for organismal health [52, 53]. We therefore applied powerful omics approaches to survey hepatic alteration by Sudachitin in a comprehensive and unbiased manner. Transcriptomic analysis by RNA-seq revealed that Sudachitin modulated diurnal expression of lipid metabolism-related genes in the liver, including downregulation of *Ppard* known to play a key role in diurnal regulation of lipogenic gene expression [54]. Patients with nonalcoholic steatohepatitis (NASH) and NAFLD have been found to display decreased levels of hepatic

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phospholipids including phosphatidylcholines (PCs) associated with reduced activity of fatty acid desaturase (FADS) and consequently impaired phospholipids synthesis [55, 56]. In our study, Sudachitin modulates diurnal patterns of both *fads* gene expression (Fig. 5F) and several main PCs (Fig. 7F), which may contribute to the improved hepatic steatosis following Sudachitin treatment. In addition to phospholipids, the Sudachitin group showed higher hepatic contents of branched-chain amino acids (BCAAs) at ZT6 than the control group (Fig. 6, F-G). Enrichment of BCAAs could activate the mTOR pathway, inhibiting fatty acid conversion to triglycerides and therefore lipid accumulation [57]. BCAAs have also been shown to ameliorate dysfunction of the mitochondrial TCA cycle in a NASH model [58]. In accordance, Sudachitin treatment led to higher levels of mitochondrial and the intermediate metabolites of the TCA cycle at ZT6 compared with the control group (Fig. 6E), analogous to our previously reported effects of Nobiletin which promotes mitochondrial respiration in skeletal muscle of aged mice [25, 26].

Circadian rhythms are increasingly appreciated as crucially important for health and healthy aging. Whereas mounting epidemiological and genetic evidence has established a causal role of circadian disruption in chronic diseases and age-related decline [59-62], how to exploit the clock as a therapeutic target require further investigations. In addition to chronotherapy that times the administration of treatment in order to maximize the therapeutic index (efficacy vs toxicity), a distinct approach is to directly manipulate the intrinsic clocks or clock components in disease settings [63, 64]. For example, time-restricted feeding, limiting food availability to a

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restricted time window during the day (~8-10 hrs), has been shown to enhance circadian

metabolic rhythms and show preventive and therapeutic efficacies in preclinical and clinical

studies [65, 66]. A large number of small molecules have also been shown to modulate the

clock and clock-controlled cellular and physiological functions in cells and mouse models [64,

67]. The newly identified clock-modulatory activity of Sudachitin, coupled with its abundant

source and excellent PK and safety profiles, serve as a strong foundation for further research

and development of Sudachitin as a bioactive molecule with promising applications in various

fields ranging from nutrition, disease intervention and healthy aging.

Acknowledgements

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Tokushima University, and Support Center for Advanced Medical Sciences, Tokushima University Graduate School of Biomedical Sciences.

Author Contributions

& editing, Funding acquisition. **Zheng Chen**: Supervision, Conceptualization, Writing –

review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Funding

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Data Availability Statement

The RNAseq data were deposited to NCBI GEO (GEO accession number: GSE211612). The

DESeq2 results and the metabolomic measurement results with statistical analysis were also

listed in the supplementary tables S3-5. The other data that support the findings of this study

are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1. **Sudachitin is a clock-modulating compound.** (**A**) Left: Dose-dependent effects of Sudachitin on reporter rhythms in *Bmal1-luc* U2OS cells. Right: quantification of amplitude or period response to Sudachitin doses. Box plot shows the median (bar), values to the 1.5 interquartile ranges (whiskers), and the 25 to 75 percentile range (box). (**B**) mRNA levels of *Bmal1*, *Per2*, *Rev-erbα*, and *Cry1* in U2OS cells by RT-qPCR. (**C**) Representative images of immunoblot analysis for BMAL1, PER2, REV-ERBα, and CRY1 in U2OS cells. Densimetric analysis of protein levels by immunoblot analysis (lower panels). Values are shown as means ± SE (*n* = 3-4, *n* = number of independent replicates). # indicates *p* < 0.05 vs. vehicle.

Figure 2. Concentrations of Sudachitin were elevated in mouse plasma and tissues after oral administration. (**A**) Purity of the Sudachitin compound for oral administration to mice by LC-TOFMS. (**B**) Concentration of total Sudachitin in mice plasma and tissues at 1 h after oral administration of 100 mg/g body weight (BW) purified Sudachtin compound. (**C**) Pharmacokinetic study of oral administration of 100 mg/g BW Sudachitin. Sudachitin glucuronide was not detected in adipose tissues, skeletal muscle, and brain. The half-life period (t1/2) of Sudachitin and the derivatives are indicated in the graph. Values are shown as means ± SE (n = 4-5). WAT, white adipose tissue; BAT, Brown adipose tissue; SM, skeletal muscle.

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Figure 3. Sudachitin modulates circadian rhythms, sleep/wake cycle, and respiratory exchange (RER) ratio in high-fat diet (HFD)-fed mice. (**A**) Representative circadian wheel-running actograms illustrating the effect of vehicle (control) or Sudachitin on circadian activities in HFD-fed WT mice. (**B**) Left: average wave plots summarizing wheel running activity under LD (12 hr light, 12 hr dark, upper panels) or DD (constant darkness, bottom panels) for the indicated groups. Right: daily wheel-running activity during LD or DD conditions for the indicated groups (n = 9-10). (**C**) Daily wheel-running period during LD or DD conditions for the indicated groups (n = 9-10). (**D**) Left: average wave plots of sleep/wake cycle (% sleep) under LD condition. Right: daily % sleep under LD conditions for the indicated groups (n = 11-12). (**E**) Left: The diurnal rhythms of oxygen consumption (VO_2) in the four groups of mice under LD condition. Right: daily resting energy expenditure under LD conditions for the indicated groups (n = 11-12). (**F**) Left: average wave plots of RER under LD condition. Right: daily RER under LD conditions for the indicated groups. Values are shown as means ± SE (n = 11-12 per group). Each box plot shows the median (bar), values to the 1.5 interquartile ranges (whiskers), and the 25 to 75 percentile range (box). C, Control; S, Sudachitin.

Figure 4. Sudachitin modulates plasma lipids, and reduces hepatic lipid accumulation in HFD-fed mice. (**A**) Plasma triglyceride. (**B**) Plasma non-esterified fatty acid (NEFA). (**C**) Plasma total cholesterol. (**D**) Blood glucose. (**E**) Plasma insulin. (**F**) Representative images of hematoxylin and eosin staining of paraffin embedded-liver sections of 30-week-old mice. Scale bar = 0.2 mm. (**G**) NAFLD activity scores on liver tissue. (**H**) Liver triglyceride. (**I**) Plasma alanine aminotransferase (ALT). (**J**) mRNA level measurements of key genes between the control and Sudachitin groups by RT-qPCR. Blood samples were corrected from 15 h-fasted (Fasted) or non-fasted (Fed) 20-week-old mice. Values are shown as means ± SE (n = 11-12 per group). Cont, Control; Sud, Sudachitin; NAFLD, Non-alcoholic fatty liver disease.

Figure 5. Sudachitin modulates diurnal transcriptome in the liver. (**A-C**) RNA-sequencing analysis of liver in 30-week-old HFD-fed mice (n = 2 per group). (**A**) Heatmap showing differential expression genes between ZT6 and ZT18 ($p < 0.05$, fold change > 1.5) in each group. (**B**) Venn diagram showing the relationship of transcriptome changes between ZT6 and ZT18 in control and Sudachitin group. (**C**) Gene ontology (GO) analysis of up-and down-regulated transcripts between control and Sudachitin group. (**D**) mRNA levels of the differential genes between control and Sudachitin group by RT-qPCR. Values are shown as

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means ± SE (n = 5-6 per group). # indicates significant difference ($p < 0.05$) under ANOVA

with Bonferroni's multiple comparison tests. Cont, Control; Sud, Sudachitin; ZT, zeitgeber

time.

Figure 6. Hydrophilic metabolite profiling in the liver of control or Sudachitin-treated mice.

(**A**) Heat map showing differential hydrophilic metabolites in the liver between ZT6 and ZT18

($p < 0.05$) in each group of 30-week-old mice. (**B**) Venn diagram showing the relationship of

metabolite changes between ZT6 and ZT18 in the control and Sudachitin groups. (**C**) Principal

component analysis score plot of metabolite profiles in the control and Sudachitin groups. (**D**)

Enrichment analysis of KEGG pathways of distinct hydrophilic metabolites between ZT6 and

ZT18 in the control and Sudachitin groups. (**E-G**) The concentrations of metabolites

associated with the citrate cycle (**E**), branched-chain amino acid metabolism (**F**), and aromatic

amino acid metabolism (**G**). The hydrophilic metabolites were measured by CE-TOFMS, as

described in the Material and Methods section. Values are shown as means ± SE (n = 5-6 per

group). # indicates significantly difference ($p < 0.05$) under ANOVA with Bonferroni's

multiple comparison tests. Cont, Control; Sud, Sudachitin; ZT, zeitgeber time.

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Figure 7. Lipophilic metabolite profiling in the liver of control and sudachitin-treated mice.

(**A**) Heatmap showing differential lipophilic metabolites in the liver between ZT6 and ZT18 (p

< 0.05) in each group of 30-week-old mice. (**B**) Venn diagram showing the relationship of

metabolite changes between ZT6 and ZT18 in the control and Sudachitin groups. (**C**) Principal

component analysis score plot of metabolite profiles of the control and Sudachitin groups. (**D**)

Categories of differentiate lipophilic metabolites between ZT6 and ZT18 in the control and

Sudachitin groups. (**E-G**) The levels of peptides (**E**), glycerophospholipids (**F**), and

sphingolipids (**G**) in the liver. The lipophilic metabolites were extracted from liver tissues by

the Bligh and Dyer method and measured by LC-TOFMS. Values are shown as means ± SE (n

= 5-6 per group). # indicates significantly difference ($p < 0.05$) under ANOVA with

Bonferroni's multiple comparison tests. Cont, Control; Sud, Sudachitin; ZT, zeitgeber time;

PC, phosphatidylcholine; PI, phosphatidylinositol; Cer, ceramide.

Molecular Nutrition and Food Research

Mawatari K, *et al.*, Fig. 1-R3

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Mawatari K, *et al*., Fig. 4-R3

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A

ZT6 vs ZT18
($p < 0.05$, FC > 1.5)

Control (632)   Sudachitin (651)

Log2FC

ZT6 ZT18 ZT6 ZT18
Cont   Sud

ZT6 ZT18 ZT6 ZT18
Cont   Sud

B

Control (632)   Sudachitin (651)

389   243   408

C

Log10 (p)

-14 -12 -10 -8 -6 -4 -2 0

GO:0006639: acylglycerol metabolic process
mmu03320: PPAR signaling pathway
GO:0006631: fatty acid metabolic process
GO:0007623: circadian rhythm
R-MMU-556833: Metabolism of lipids
GO:0016042: lipid catabolic process
GO:0046439: L-cysteine metabolic process
GO:0045444: fat cell differentiation
GO:0090207: regulation of triglyceride metabolic process
GO:0009725: response to hormone
GO:0051186: cofactor metabolic process
GO:0006066: alcohol metabolic process
GO:0014070: response to organic cyclic compound
CORUM:413: (ER)-localized multiprotein complex, Ig HC
mmu04141: Protein processing in endoplasmic reticulum
GO:0035634: response to stilbenoid

Cont
Sud

D

Per2   *Rev-erba*   *Cry1*   *Rora*

Relative abundance

#

#   #   #

#   #

#   #

#   #

ZT 6 18 6 18
Cont   Sud

ZT 6 18 6 18
Cont   Sud

ZT 6 18 6 18
Cont   Sud

ZT 6 18 6 18
Cont   Sud

Per1   *Bmal1*   *Rorc*

#

$p = 0.071$ $p = 0.095$

$p = 0.096$ $p = 0.073$

ZT 6 18 6 18
Cont   Sud

ZT 6 18 6 18
Cont   Sud

ZT 6 18 6 18
Cont   Sud

E

Cyp7a1   *Ppard*   *Ppara*   *Insig2*

$p = 0.056$

#   #   #

ZT 6 18 6 18
Cont   Sud

ZT 6 18 6 18
Cont   Sud

ZT 6 18 6 18
Cont   Sud

ZT 6 18 6 18
Cont   Sud

F

Fasn   *Fads*   *Acly*

$p = 0.052$ #   #

#   #   #

ZT 6 18 6 18
Cont   Sud

ZT 6 18 6 18
Cont   Sud

ZT 6 18 6 18
Cont   Sud

Wiley-VCH

A ZT6 vs ZT18 ($p < 0.05$)

Control (26)

Betaine
Thymidine
Citric acid
Pyruvic acid
cis-Aconitic acid
Phosphoenolpyruvic acid
3-Phosphoglyceric acid
Leu
Spermine
Pro
Anthranilic acid
Acetyl CoA_divalent
Thr
Tyr
Creatine
Spermidine
Phe
Trp
Citrulline
Ile
CoA_divalent
Hydroxyproline
Val
Lys
Ala
Lactic acid

Sudachitin (9)

S-Adenosylmethionine
CDP
ATP
2-Hydroxybutyric acid
Gluconic acid
Uridine
Inosine
Uracil
IMP

Common (5)

Cysteine
Sarcosine
Carnosine
2-aminoisobutyric acid
Met

ZT6 ZT18   ZT6 ZT18
Control   Sudachitin

log2FC
−2 −1 0 1 2

B

Control (31)
Sudachitin (14)

26 5 9

C Component 2 (14.4%)

Control (ZT6) Sudachitin (ZT6)

Control (ZT18)

Component 1 (34.8%) Sudachitin (ZT18) Component 3 (12.1%)

○ Control (ZT6)
● Control (ZT18)
● Sudachitin (ZT6)
● Sudachitin (ZT18)

Mawatari K, *et al*., Fig. 6_R3

D Log10 (p)

−6 −5 −4 −3 −2 −1 0

Citrate cycle (TCA cycle)
Aminoacyl-tRNA biosynthesis
Pyruvate metabolism
Cysteine and methionine metabolism
Glyoxylate and dicarboxylate metabolism
Glycolysis / Gluconeogenesis
Pantothenate and CoA biosynthesis
Glycine, serine and threonine metabolism
Glutathione metabolism
Valine, leucine and isoleucine biosynthesis
Phenylalanine, tyrosine and tryptophan biosynthesis
Fatty acid degradation
Arginine and proline metabolism
Lysine degradation
beta-Alanine metabolism
Inositol phosphate metabolism
Pyrimidine metabolism

■ Control
■ Sudachitin

E

Acetyl-Coenzyme A

Coenzyme A

cis-Aconitic acid

Citric acid

F

Valine

Leucine

Isoleucine

Threonine

p = 0.08

G

Tyrosine

Phenylalanine

Tryptophan

Anthranilic acid

A

ZT6 vs ZT18 ($p < 0.05$)

Control (72)

Sudachitin (11)

common (3)

ZT6 ZT18   ZT6 ZT18

Control   Sudachitin

log2FC
−2 −1 0 1 2

B

Control (75)

72   3   Sudachitin (14)   11

C

Component 2 (16.2%)

Y-Axis

Sudachitin (ZT18)

Sudachitin (ZT6)

Control (ZT18)

Control (ZT6)

X-Axis

Z-Axis

Component 1 (43.9%)

Component 3 (9.9%)

○ Control (ZT6)
● Control (ZT18)
● Sudachitin (ZT6)
● Sudachitin (ZT18)

D

Number of compound
12 10 8 6 4 2 0

Peptides
Glycerophospholipids
Sphingolipids
Fatty Acyls
Polyketides
Sterol Lipids
Prenol Lipids
Glycerolipids

■ Control
■ Sudachitin

E

Trp-Pro-Pro-Lys

10^4 Counts/mg tissue
25
20
15
10
5
ZT 6 18   6 18
Cont   Sud
# #

Val-Pro-Cys-Asp

10^4 Counts/mg tissue
12
10
8
6
4
2
ZT 6 18   6 18
Cont   Sud
# #

His-Ala-Phe-Pro

10^4 Counts/mg tissue
12
10
8
6
4
2
ZT 6 18   6 18
Cont   Sud
# #

Trp-Glu-Leu-Arg-Lys

10^4 Counts/mg tissue
6
5
4
3
2
1
ZT 6 18   6 18
Cont   Sud
# #

F

PC(30:2)

10^4 Counts/mg tissue
60
40
20
0
ZT 6 18   6 18
Cont   Sud
# #

PC(29:2)

10^4 Counts/mg tissue
30
20
10
0
ZT 6 18   6 18
Cont   Sud
# #

PC(28:2)

10^4 Counts/mg tissue
60
40
20
0
ZT 6 18   6 18
Cont   Sud
# #

PI(42:6)

10^4 Counts/mg tissue
50
40
30
20
10
ZT 6 18   6 18
Cont   Sud
# #

G

Sphingosine

10^4 Counts/mg tissue
5
4
3
2
1
ZT 6 18   6 18
Cont   Sud
# # #

(d18:3) sphingosine

10^4 Counts/mg tissue
10
8
6
4
2
ZT 6 18   6 18
Cont   Sud
# #

Sphinganine

10^4 Counts/mg tissue
15
10
5
ZT 6 18   6 18
Cont   Sud
# #

Cer(m42:2)

10^4 Counts/mg tissue
40
30
20
10
ZT 6 18   6 18
Cont   Sud
# #

Molecular Nutrition and Food Research

Sudachitin

Citrus sudachi
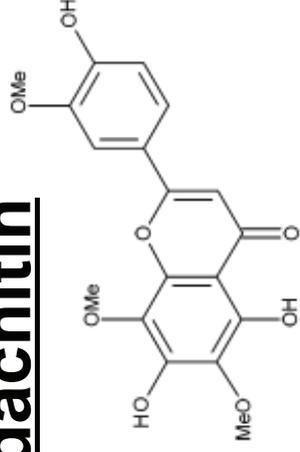
High fat diet-fed mice

Improved liver physiology
(lipid accumulation,
fibrosis, inflammation)
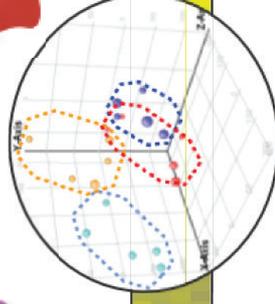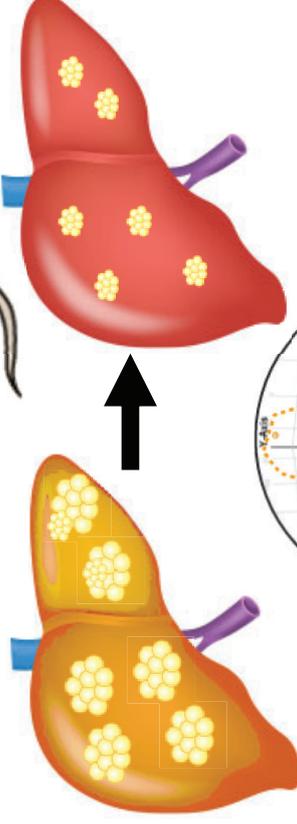
Diurnal transcriptomic and
metabolomic alteration

Circadian reporter cells

Modulation of circadian
gene expression

- Sudachitin is a polymethoxylated flavone (PMF) enriched in *Citrus sudachi*.

- Sudachitin modulates circadian rhythms *in vitro* and *in vivo*

- Sudachitin improves clock-associated systemic and liver physiology

- Integrative omics revealed altered expression and metabolite landscapes