Simiao Wan alleviates obesity-associated insulin resistance via PKCε/IRS-1/PI3K/Akt signaling pathway based on network pharmacology analysis and experimental validation

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Abstract

Background: The purpose of the study was to investigate the active ingredients and potential biochemical mechanisms of Simiao Wan (SMW) in obesity-associated insulin resistance. Methods: An integrated network pharmacology method to screen the active compounds and candidate targets, construct the protein-protein-interaction network, and ingredients-targets-pathways network was constructed for topological analysis to identify core targets and main ingredients. To find the possible signaling pathways, enrichment analysis was performed. Further, a model of insulin resistance in HL-7702 cells was established to verify the impact of SMW and the regulatory processes. Results: An overall of 63 active components and 151 candidate targets were obtained, in which flavonoids were the main ingredients. Enrichment analysis indicated that the PI3K-Akt signaling pathway was the potential pathway regulated by SMW in obesity-associated insulin resistance treatment. The result showed that SMW could significantly ameliorate insulin sensitivity, increase glucose synthesis and glucose utilization and reduce intracellular lipids accumulation in hepatocytes. Also, SMW inhibited diacylglycerols accumulation-induced PKCε activity and decreased its translocation to the membrane. Conclusion: SMW ameliorated obesity-associated insulin resistance through PKCε/IRS-1/PI3K/Akt signaling axis in hepatocytes, providing a new strategy for metabolic disease treatment.

Keywords: Simiao Wan; insulin resistance; PKCε/IRS-1/PI3K/Akt signaling pathway; network pharmacology; DAG
Highlights
An integrated network pharmacology strategy and experimental evidence were taken to confirm that Simiao Wan (SMW), a traditional Chinese patent medicine, could ameliorate obesity-associated insulin resistance. SMW could reduce PKCε activation induced by hepatic diacylglycerols accumulation and activate IRS-1/PI3K/Akt signaling axis to increase glucose synthesis and glucose utilization, thereby increasing insulin sensitivity. We found a new molecular mechanism regulated by SMW to modulate intracellular lipid accumulation and insulin sensitivity, providing a potential strategy for treating obesity-associated insulin resistance and other metabolic disorders.

Medical history of objective
SMW, a traditional Chinese patent medicine, is derived from Zhang Bingcheng's "Cheng Fang Bian Du" (1904 C.E.) in the Qing Dynasty. SMW is bitter and cold in nature. Traditionally, it is used for paralysis caused by damp-heat infusion, which is characterized by redness and swelling of the feet and knees, and pain in the tendons and bones. Nowadays, it has been used in clinical to cure gout, hyperuricemia, arthritis, abnormal glucose tolerance, cervices, and eczema. It has been reported that SMW participated in the regulation of inflammatory response and ameliorated insulin sensitivity in hepatocytes.

Background
Several disorders, including central obesity, glucose intolerance, hypertension, insulin resistance, and dyslipidemia, are part of the metabolic syndrome [1]. Insulin resistance, which is the parallel pandemic of obesity and a key risk factor for developing type 2 diabetes, is defined as reduced insulin-mediated glucose absorption and decreased suppression of glucose output. It has been proved in rodent and human studies that hepatic accumulation of diacylglycerols (DAGs) is closely associated with insulin resistance, in which activation of protein kinase C epsilon (PKCε) impairs insulin signaling [2]. In addition, insulin resistance is clinically associated with cardiovascular disease, polycystic ovary syndrome, non-alcoholic fatty liver disease, and cancer. However, there is no drug specifically approved to treat insulin resistance [3]. It is urgent to identify effective medication to treat these metabolic disorders.

Simiao Wan (SMW), a traditional Chinese patent medicine, is a representative prescription of dissipating heat and moistening aridity in traditional Chinese medicine (TCM), consisting of Achyranthis Bidentata Radix, Atractylodis Rhizoma, Coicis Semen, and Phellodendri Chinensis Cortex. Traditionally, it is used for paralysis caused by damp-heat infusion, which is characterized by redness and swelling of the feet and knees, and pain in the tendons and bones. Nowadays, it has been used in clinical to cure gout, hyperuricemia, arthritis, abnormal glucose tolerance, cervices, and eczema [4, 5]. According to the TCM theory, the retention of damp-heat is closely related to diabetes and individuals with phlegm-damp and damp-heat TCM body constitution (characterized by abdominal fatness, chest tightness, excessive sputum, easy sleepiness and fatigue, and a preference for fat and sweet) have a greater chance of developing diabetes mellitus [6]. It has been reported that SMW participated in the regulation of inflammatory response and ameliorated insulin sensitivity in hepatocytes [7, 8]. Modern pharmacological studies have revealed that the main compounds of SMW include alkaloids, organic acids, lactones, and terpenoids [9]. However, the bioactive ingredients and the fundamental molecular biological mechanisms of SMW in obesity-associated insulin resistance treatment remain unknown.

Traditional Chinese Medicine (TCM) offers a potential approach to the management of complex diseases, such as metabolic disorders, since it is multi-compound, multi-target, and multi-pathway based. However, the complexity of chemical compounds and molecular mechanisms of TCM makes it difficult to elucidate its mechanism in treatment. The emerging network pharmacology takes the strategy to screen the active ingredients and targets, discover the biological pathways, and construct drug-target-pathway networks in a high-throughput manner to reveal the mechanism of drugs at the systemic level [11]. The systematic view of network pharmacology is consistent with the holistic philosophy of TCM [12]. Introducing network pharmacology in TCM provides a new “network-target, multi-component-therapeutics” paradigm for revealing the bioactive ingredients and molecular processes of TCM.

The primary cause of DAGs accumulation in the liver is an imbalance that the rate of fatty acid delivery and transport exceeding the speed of DAGs conversion into triglyceride (TG) and mitochondrial fatty acid oxidation. Among the increase of hepatic DAG contents, it has been reported that PKCε is activated by sn-1,2 DAGs, while other isomers (sn-2,3-DAG and sn-1,3-DAG) are not involved [13]. When activated, PKCε is recruited to the cytomembrane and inhibits the insulin receptor kinase activity by phosphorylating the inhibitory threonine residue Thr1160 within the insulin receptor kinase activation loop, decreasing the activity of phosphoinositide-3-kinase (PI3K) and phosphoinositide-dependent kinase 1 (PDK1) [14]. Reduced PDK1 activation impairs PKD1-dependent serine/threonine kinase (Akt) activity, thereby inhibiting hepatic glycolgen synthesis through increasing inhibitory serine-phosphorylation of glycogen synthase kinase 3 (GSK3) and suppressing its substrate glycogen synthase activity. The impaired Akt activation also promotes nuclear translocation of FOXO1 and activation of downstream genes, including phosphenolpyruvate carboxykinase, gluconeogenic proteins pyruvate carboxylase, and glucose-6-phosphatase, resulting in increased hepatic gluconeogenesis [15]. Thus, inhibition of the DAG-PKCε signaling axis is a potential strategy for therapeutic metabolic diseases.

In this study, network pharmacology was used to identify the main ingredients, hub targets, and enriched pathways of SMW in obesity-associated insulin resistance (OIR) treatment. Further experiments in HL-7702 hepatocytes confirmed that SMW could ameliorate hepatic insulin resistance induced by lipids accumulation, especially DAGs, through the PKCε/IRS-1/PI3K/Akt signaling axis. The specific strategy is shown in Figure 1. This study provides a potential strategy for treating OIR and metabolic disorders.

Materials and methods

Materials
Insulin (INS) (1342106), Triton™ X-100 (T8787), D (+)−Glucose (G8270), palmitic acid (PA) (P5585), and Nile-Red (19123) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine™ 3000 (L3000015) and Mem-PERTM Plus Kit (B89442) were acquired from Thermo Fisher (Wallham, MA, USA). The compound preparation of SMW was acquired from Julin Zixin Pharmaceutical Industrial Co., Ltd. (Jilin, China). Anti-phospho-Akt (Ser473) (4060), anti-phospho-Akt (Thr308) (9275), anti-phospho-PI3K p85 alpha (Tyr458) (4228), anti-PI3K (4249), anti-FOXO1 (2880), anti-phospho-FOXO1 (Thr24)/FoxO3a (Thr32) (9464), anti-PKCε (2683) antibodies and cell lysis buffer (Cell Signaling Technology, 9806) were acquired from Cell Signaling Technology (Danvers, MA, USA). Anti-GSK3β (3229), anti-phospho-GSK3β (Ser9) (75814), and anti-phospho-IRS1 (Tyr632) (109543) antibodies were purchased from Abcam Technology (Cambridge, MA, USA). Anti-ACTB/beta-actin (AF0003) and GAPDH mouse monoclonal antibodies (AF0006) were purchased from Beyotime Biotechnology (Jiangsu, China). Anti-phospho-PKCε (Ser729) antibody was purchased from Immunoway Biotechnology (Beijing, China). Anti-Akt (10176-2-AP) antibodies were acquired from Proteintech Group (Rosemont, IL, USA).

Collection of active ingredients in SMW
The active compounds in SMW were collected from the Traditional
Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) database (http://tcmspw.com/tcmsp.php). It combined pharmacochemistry, ADME (absorption, distribution, metabolism, and elimination) properties, drug-likeness, drug targets, related disorders, and interaction networks, giving an opportunity for systematically studying the mechanism of herbal medicines [16]. Evaluation of ADME properties is an integral part of compound optimization in drug discovery [17]. Components with oral bioavailability ≥ 30% and drug likeness ≥ 0.18 are thought to have higher pharmacologic effects, therefore collected as potential active compounds for further analysis. In addition, literature reports in Pubmed (https://pubmed.ncbi.nlm.nih.gov/) databases were screened for functional ingredients in SMW as a supplement.

Screening of SMW-related targets and obesity-associated insulin resistance targets


Construction of a protein-protein interaction (PPI) network
All SWM active ingredients targets and OIR targets disease targets were introduced into Omicschip (https://www.omicschip.com/) platform to generate a Venn diagram and overlap targets as candidate targets. Protein-protein interaction network was created by the STRING (https://string-db.org/, version. 11.0) database, using the candidate targets. To build the PPI network, all data were then loaded into Cytoscape [22].

Construction of a drug-ingredients-targets-disease (D–I–T–D) Network
The complex relationships between SWM, active ingredients, target genes, and OIR were visualized and analyzed by Cytoscape 3.9.1. A biological network consist with many topological parameters, such as node, edge, degree, etc. Nodes can symbolize herbs, ingredients, targets, and diseases. Edges can describe the interaction of different nodes. The amount of connections between one node and other nodes is reflected in the degree value [23].

Enrichment analysis of gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway
Metscape (http://metscape.org/, updated in 2021), incorporating functional enrichment, interactome analysis, gene annotation, and membership search, was chosen for GO and KEGG pathway enrichment analysis. Statistics were considered significant for P values under 0.05 [24].

Cell culture
HL-7702 cells (KG063) were obtained from Keygen Biotechnology and cultured in DME with 10% FBS, 100 µg/ml streptomycin sulfate, and 100 IU/ml penicillin at 37 °C and 5% CO₂.

Preparation of SWM extract
The SWM was ground into uniform powder and weighed (0.2 g) accurately. The powder was sonicated in 10 ml of 50% methanol for 45 min. After centrifugation at 13,000 r/min for 10 min, the supernatant was analyzed using a liquid chromatography quadruple-time-of-flight mass spectrometry method and 48 components were identified [25]. The supernatant was dried under vacuum and weighed. The extraction rate of SWM was 25.8% relative to the pill. The extract was dissolved with dimethyl sulfoxide to a concentration of 200 mg/ml and used for subsequent experiments.

PKCα activity rapid evaluated system
Fürster/fluorescence resonance energy transfer (FRET) analysis: The N- and C-terminal regions of PKCα are each tagged with CFP and YFP in a FRET probe. Hepatocyes were seeded on confocal dishes and transiently transfected with FRET probe vector. Following a 24-hour transfection, cells were co-cultured with PA or/and SWM extract at the indicated concentration for another 24 h. Using a high-intensity image analysis system (Opera Phenix), the ratios of YFP signal (474 nm) to CFP signal (506 nm) were tracked. Intracellular localization of PKCα: HL-7702 cells were seeded on confocal dishes and transiently transfected with the EGFP-PKCc vector for 24 h, followed by treatment with PA or/and SWM extract at the indicated concentration for 24 h. The optical signal of the plasmid was captured with a high-resolution intent imaging analysis system (Opera Phenix).

Glucose consumption
The HL-7702 cells were inoculated in culture dishes, walled and then treated with PA or/and SMW at the indicated concentration for 24 h. Then, the culture medium was gathered and assayed according to the instructions of the glucose content kit (Solarbio, BCZ2500). Glucose was detected at 505 nm. Glucose consumption equals the gap between glucose supplied and detected within themedium.

Glucose uptake
Hepatocytes were inoculated in culture dishes. After co-cultured with PA or/and SMW extract, cells were treated by two washes with KRPH buffer and incubated with 2-DG for 20 min. Then cells were lysed after being washed three times, and the cell lysate was incubated for 30 minutes with the 2-DG uptake assay working solution. The fluorescence intensity ratio of each group at 540 nm to 590 nm represents glucose uptake. All operations followed kit instructions (Abnova, KA4085).

Glucose production analysis
Hepatocytes were inoculated in culture dishes. After co-cultured with PA or/and SMW extract, cells were treated by two washes with phosphate-buffered saline (PBS) and cultured with non-glucose DMEM replenished with sodium lactate and sodium pyruvate. After 3 h, the supernatant and cells were assayed with a glucose assay kit (Nanjingjiangcheng Bioengineering Institute, F006-1-1).

Glycogen content
Hepatocytes were inoculated in culture dishes. After co-cultured with PA or/and SMW extract for 24 h. The glycogen assayed according to the instructions of the glucogen content assay kit (Solarbio, BC0340). The glycogen content was detected at 620 nm.

DAg assay
DAg levels were measured utilizing the double antibody sandwich ELISA technique. The detection antibody is a biotinylated polyclonal antibody, whereas the pre-coated antibody is an anti-Human DAg monoclonal antibody. Hepatocytes were inoculated in culture dishes. After co-cultured with PA or/and SMW extract, cells were diluted to about 1 × 10⁶ cells per 1 ml PBS, and lysed by repeated freeze-thaw treatment. After centrifugation, the supernatant and biotinylated antibodies were subsequently introduced to ELISA plate wells and incubated at 37 °C for 1 h. After washed out, avidin-peroxidase conjugates were introduced to the wells and incubated at 37 °C for 30 min. The enzyme conjugate was carefully rinsed out of the wells by PBS before the addition of TMB substrate. With the addition of the stop solution, TMB eventually became yellow after reacting to produce a blue product due to the peroxidase activity. The amount of the target analyte in the sample and the color intensity are positively associated when measured at 450 nm. All operations followed the DAg assay kit instructions (Mbio, ml062442).

Triglyceride and cholesterol measurement
Hepatocytes were inoculated in culture dishes. After co-cultured with PA or/and SMW extract, cells were gathered and suspended in 1 ml PBS. A proportion of cells was lysed for protein quantification. After removing PBS, chloroform/methanol (2:1, v/v) was introduced to the remaining cells and shaken for 3 h at 24 °C to extract intracellular lipids to extract intracellular lipids. Then sodium chloride (0.1 M) was added and mixed thoroughly. After centrifugation, the bottom organic phase was taken and volatilized till dry. The residue was dissolved in 1% Triton-X 100 diluted with ethanol, and determined the amount of TG and total cholesterol in accordance with instructions of the triglyceride/cholesterol assay kit (Wako Pure Chemical Industries, 635-50981; 632-50991).

Membrane protein analysis
Membrane protein of hepatocytes was extracted with the Mem-PERTM
Plus Kit (Thermo Fisher, 89842). Approximate $5 \times 10^5$ cells were collected and washed twice with cell wash solution. After discarding the supernatant, permeabilization buffer was introduced to the cell pellet and incubated at 4 °C for 10 min. Cells that had been permeabilized were centrifuged at 16,000 g for 15 min. Solubilization buffer was used to resuspend the residue, which was then incubated at 4 °C for 30 min. After centrifugation, the supernatant involving membrane-associated proteins and solubilized membrane was collected for further western blotting.

Western blotting analysis
Hepatocytes were collected and lysed using a lysis buffer containing phosphatase inhibitor (Roche, 4906845001), loading buffer and protease inhibitor. After heated at 99 °C for 10 min, proteins were electrophoresed on SDS-PAGE gels and shifted onto nitrocellulose membranes (Millipore, HATF00010). Blocking was done by incubating with 5% skim milk dissolved in TBST (Sangon Biotech, A510025-0001) containing 0.075% Tween-20 (Merck, 93773) for 1 h. Blots were incubated with primary antibodies overnight at 4 °C, washed with TBST, and then stained with a secondary antibody coupled to horseradish peroxidase (HRP). Western blotting brands were monitored by a chemiluminescence detection kit (Tanon Science & Technology, 180-501) and quantified with Quality One software (Bio-Rad).

Statistical analysis
GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, CA, USA) for Windows was used for analysis. One-way analysis of variance was used for the statistical analysis (Dunnett’s post-test). The mean ± SEM is used to indicate error bars. P value less than 0.05 was considered as significant.

Results
Active ingredients in SMW
Based on TCMP, a total of 63 compounds were retrieved from SMW after redundancy removal, 12 from Atractylodis Rhizoma, 44 from Phellodendri Chinensis Cortex, 22 from Achyranthis Bidentatae Radix, and 19 from Coicus Semen. Moreover, according to the literature, another 15 active ingredients were considered due to their obvious pharmacological activities, such as Magnoflorine, Phellodendrine, and Atractylenolide I [26]. Therefore, 78 active ingredients of SMW were collected (Table S1).

Candidate targets of SMW in OIR treatment
An overall of 543 targets of active components in SMW were identified using Symmap, and 996 targets were obtained as OIR-related targets according to Genecards, OMIM, and DigSee databases. Further, 151 intersection genes between SMW targets and OIR-related targets were identified as candidate targets for the therapy OIR, indicating that SMW might ameliorate OIR through these targets (Figure 2, Table S2).

PPI network
The 151 candidate targets were submitted to the STRING database to acquire their interaction relationship, and they were uploaded into Cytoscape for analysis and construction of the PPI network. Analyze Network tool was taken to calculate degree value. The node gains significance as the degree increases. As shown in Figure 3, 151 nodes and 3,457 edges were acquired for intersection genes. Then, 36 core targets were obtained by extracting nodes whose degree values were equal to or above 45, the median degree. Furthermore, 15 targets with degree values higher than 90, the two-fold median of degree, were identified as hub targets, including AKT1, INS, IL6, TNF, VEGFA, TPS3, FAPG, IL1B, CASP3, MAPK3, JUN, CTNNB1, SRC, HIF1A, MMP9. PKC was established as a widely expressed calcium-and DAG-dependent serine/threonine kinase activity [27]. PKCs is the predominant PKC isoform activated by hepatic sn-1,2-DAG in lipid-induced insulin resistance [14]. Notably, PRKCA and PRKCB (highlighted in yellow) among candidate genes, isoforms of the PKC family, are of great significance, informing potential targets of SMW in the treatment of OIR.

![Figure 2](https://www.tmrjournals.com/tmr)

**Figure 2** The candidate targets of SMW in OIR treatment. The Venn diagram demonstrates the number of intersected and specific targets between SMW and OIR. OIR, obesity-associated insulin resistance; SMW, Simiao Wan.

![Figure 3](https://www.tmrjournals.com/tmr)

**Figure 3** Topological analysis of the protein-protein interaction network. Hub targets of SMW in treating OIR were screened by degree values, in which red nodes have higher degree values. The degree value is proportional to the size of the target node. OIR, obesity-associated insulin resistance; SMW, Simiao Wan.

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D-I-T-D network
The interrelationship between herbs, active ingredients, candidate targets, and OIR was introduced into Cytoscape, concretely a D-I-T-D Network with 235 nodes and 1,017 edges (Figure 4). Five active ingredients with top degree value include quercetin, wogonin, berberine, kaempferol, and baicalein, playing an essential role of SMW in treating OIR.

GO and KEGG pathway
To further elucidate the molecular mechanisms of SMW in OIR treatment, 151 candidate targets were entered into the Metascape online platform for KEGG and GO pathway enrichment analysis, as shown in Table S3. GO analysis is based on Gene Ontology, which illustrates biological gene function from molecular function, biological process, and cellular component. The outcomes of GO analysis reveals that the candidate gene set was enriched into 2,124 biological process entries, which were primarily related to cell motility, cell response to hormone, lipid, inflammation, and extracellular stimulus (Figure 5A). The set of candidate genes was enriched into 160 molecular function entries, such as ligand-activated transcription.

Figure 4 A network of SMW-ingredients-targets-OIR. The green square represents herbs of SMW, while the yellow triangle symbolizes component, the blue rhombus symbolizes candidate targets, and the orange octagon symbolizes obesity-associated insulin resistance. OIR, obesity-associated insulin resistance; SMW, Simiao Wan.

Figure 5 Enriched GO and KEGG signaling pathways of candidate targets. (A–C) GO analysis of candidate targets, including molecular functions, cellular components, and biological processes. (D) KEGG analysis of signaling pathways enriched by candidate genes. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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factor activity, nuclear receptor activity, and lipid binding (Figure 5B). The set of candidate genes was enriched into 122 cellular component entries, including membrane raft, membrane microdomain, caveola, etc (Figure 5C).

According to the P-value < 0.05 and the number of genes ≥ 25, the most significantly enriched 20 KEGG pathways of SMW on OIR were shown in Figure 5D, mainly involving pathways related to insulin, cancer, lipid, diabetes, and inflammation. The relationship between these enriched pathways and related targets is visualized in Figure 6A. Insulin, once bound to the insulin receptor, could activate multiple signaling pathways within cells, which include both the PI3K and MAPK signaling pathways [28]. A strong relationship between SMW and the PI3K-Akt signaling pathway suggested that the PI3K-Akt signaling pathway is a potential pathway of SMW in OIR treatment (Figure 6B).

SMW inhibits PA-induced insulin resistance in HL-7702 cells To explore the effect of SMW on insulin resistance induced by PA, HL-7702 cells were treated with 200 μM PA or PA plus SMW with 100 and 200 μg/ml for 24 h. Stimulation of HL-7702 cells with 10 nM insulin for 10 min induced an evident Akt phosphorylation at Ser473 and Thr308 residues, an effect that was inhibited by PA. It’s interesting to note that SMW raised Akt’s phosphorylation status at Ser473 and Thr308 dose-dependently. Since total Akt showed no discernable decrease during stimulation with PA or SWM, this result cannot be attributed to Akt protein degradation. To determine whether SMW reversed the impaired insulin signaling by PA at the level of IRS-1 and PI3K activation, we examined the insulin-induced phosphorylation of two proteins at Tyr621 and Tyr458. Insulin-induced phosphorylation of IRS-1 at Tyr621 and PI3K at Tyr458 was impaired by 200 μM PA, while treatment of HL-7702 cells with 100 or 200 μM SMW for 24 h increased IRS-1 and PI3K activation (Figure 7A, 7B). These observations suggested that SMW increases the PA-induced insulin resistance through the IRS-1-PI3K-Akt pathway.

Figure 6 Distribution of candidate targets on predicated pathways. (A) The network of top 20 enriched KEGG entries and included targets. (B) Pathway mapping of PI3K-Akt signaling pathway. Red tags represent candidate targets in the SMW treatment of OIR, while green tags represent other molecules involved in PI3K-Akt pathways. Among candidate targets, yellow tags indicate critical nodes in the insulin pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes; OIR, obesity-associated insulin resistance; SMW, Simiao Wan.

Figure 7 SMW inhibits PA-induced insulin resistance in HL-7702 cells. (A) HL-7702 cells were treated with DMSO, PA (200 μM), and/or SMW at the indicated concentration for 24 h in serum-free medium, and then stimulated with 10 nM insulin for 0.5 h. The indicated protein level in the treated cells was monitored by Western Blotting. (B) Statistical results of the protein expression of the indicated proteins. The mean ± SEM is used to indicate error bars. One-way ANOVA (Dunnett’s post-test) was used for the statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001 vs. DMSO group; P < 0.05, *P < 0.01, **P < 0.001 vs. PA model group. SMW, Simiao Wan; PA, palmitic acid; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.

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SWM inhibits PA-impaired glycogen synthesis and glucose utilization in HL-7702 cells
In response to insulin, the PI3K-Akt pathway lowers the level of glucose in the liver through the increase of glycogen synthesis and inhibition of gluconeogenesis by the GSK3β and FOXO1 pathways, which are the downstream substrate of Akt [14]. To investigate the effect of SWM on the GSK3β and FOXO1 pathway, we examined the phosphorylation of GSK3β and FOXO1. As shown in Figure 8A, 8B, pretreatment with PA decreased the insulin-induced phosphorylation of GSK3 at Ser9 in HL-7702 cells. SWM dose-dependently up-regulated the phosphorylation level of GSK3β at Ser9 significantly.

As a result, the glycogen content was reversed to the normal cellular level in HL-7702 cells co-cultured with SWM in a concentration-dependent manner (Figure 8C). Under the same conditions, we detected the phosphorylation level of FOXO1 in HL-7702 cells. PA significantly disrupted the phosphorylation of FOXO1 at Thr24 induced by insulin, which was totally reversed by SWM treatment (Figure 8A and 8B). One characteristic manifestation of insulin resistance is the impairment of glucose utilization [29]. As shown in Figure 8D, 8E, PA stimulation significantly decreased glucose uptake and consumption, which conversely increased with SWM treatment (Figure 8D, 8E). These results demonstrated that SWM reverses the impaired glycogen synthesis and glucose utilization induced by PA in HL-7702 hepatocytes.

SWM inhibits PA-induced lipids accumulation in HL-7702 cells
DAGs accumulation has been reported to associated with hepatic insulin resistance in various animal models via activation of PKCε, which may directly interfere with hepatic insulin signaling. Regarding this, it was recently shown that PKC phosphorylated the threonine 1160 of the insulin receptor, inhibiting insulin receptor kinase activity [14]. We then evaluated the hepatic lipid levels in PA-induced HL-7702 cells treated with SWM (50, 100, 200 μM). As shown in Figure 9A–9C, 200 μM PA obviously increased the TG and TC levels in hepatocytes. The increased lipid levels induced by PA was downregulated by SWM dose-dependently in hepatocytes, of which viability was not significantly changed (Figure 9A–9C). The PA-induced hepatocytes were stained with nile red dye for neutral lipids staining. In these cells, neutral lipids were accumulated (Figure 8D, 8E). SWM dose-dependently decreased the neutral lipids in PA-induced HL-7702 cells (Figure 9D, 9E). Moreover, we also assayed the level of DAGs in hepatocytes by ELISA. As expected for a true coacervation, the PA-induced high DAGs level was largely reversed by SWM in a concentration-dependent manner (Figure 9F). Altogether, these results indicated that SWM inhibits PA-induced lipids accumulation in hepatocytes.

Figure 8 SMW inhibits PA-impaired glycogen synthesis and gluconeogenesis in HL-7702 cells. (A) HL-7702 cells were treated with DMSO, PA (200 μM), and/or SMW at the indicated concentration for 24 h in serum-free medium, and then stimulated with 10 nM insulin for 0.5 h. The indicated protein level in the cells was monitored by Western Blotting. (B) Statistical results of the protein expression of the indicated proteins. (C-E) HL-7702 cells were incubated with DMSO, PA (200 μM), and/or SMW at the indicated concentration for 24 h, and the glycogen content (C), glucose uptake (D), and consumption (E) were detected. The mean ± SEM is used to indicate error bars. One-way ANOVA (Dunnett’s post-test) was used for the statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001 vs. DMSO group; P < 0.05, **P < 0.01, ***P < 0.001 vs. PA model group. SMW, Simiao Wan; SEM, standard error of mean; PA, palmitic acid; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.
the active state results in a predominant signal change from the YFP signal intensity to the CFP signal intensity. (Figure 10A). In HL-7702 cells pretreated with 100 or 200 μM SMW, the ratio of FRET was dose-dependently decreased by SMW (Figure 10A, 10B), suggesting that SMW inhibits the activity of PKCε induced by PA. We further examined phosphorylation of PKCε at Ser729, which has been linked to PA-mediated activation. HL-7702 Cells exposed to PA showed a high level of PKCε phosphorylation after 24 h stimulation. As expected, SMW could inhibit the increased phosphorylation level of PKCε in a concentration-dependent manner (Figure 10C, 10D). GFP fusion proteins are a valuable tool for studying the intracellular localization of PKCε. As shown in Figure 10G, notably, a 3-fold increase in PKCε translocation from the cytosol to the membrane was an indicator of hepatic DAG-associated insulin resistance. SMW largely decreased the PA-induced membrane translocation of PKCε in HL-7702 cells (Figure 10E). We stimulated HL-7702 cells with PA and then isolated the membrane and cytosolic subcellular fractions. The membrane and cytosolic fractions were separated as indicated in the materials and methods section. The content of PKCε in cytosolic and membrane fraction was measured by western blotting assay.

Consistent with the results of the EGFP-PKCε fluorescence experiment, the PKCε protein significantly translated membrane from the cytoplasm in HL-7702 treated with PA, which was reversed by SMW dose-dependently (Figure 10F, 10G). These results indicated that SMW inhibits the PA-induced activation of PKCε in hepatocytes. In conclusion, SMW alleviates obesity-associated insulin resistance via PKG2/IRS-1/P13K/Akt signaling pathway (Figure 11).

Discussion

Insulin resistance, which can be induced by lipids accumulation, has been recognized as an danger for various metabolic disorders, involving type 2 diabetes, non-alcoholic fatty liver disease, and cardiovascular disease, greatly threatening the global population health. SMW has been proved to modulate insulin sensitivity in hepatocytes [30]. However, the underlying mechanism remains unknown. Here, we took the strategy of integrated network pharmacology to predicate the bioactive ingredients and potential targets of SMW for OIR therapy. Apart from the ingredients met with the criteria “oral bioavailability ≥ 30% and drug likeness ≥ 0.18”,

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Figure 10 SWM inhibits PA-induced activation of PKCε in HL-7702 cells. HL-7702 cells were treated with DMSO, PA (200 μM), and/or SMW at the indicated concentration for 24 h. (A) The YFP and CFP signals were detected in HL-7702 cells transfected with the FRET probe after being treated with DMSO, PA (200 μM), and/or SMW (100 or 200 μM). (B) Data represents the mean (± SEM) FRET ratio (CFP signal intensity/YFP signal intensity) (n = 12). (C) The indicated protein level in the treated cells was monitored by Western Blotting. (D) Statistical results of the expression of p-PKC. (E-G) the protein level of PKCε in cytosol and membranel fraction was monitored by fluorescent and WB. The mean ± SEM is used to indicate error bars. One-way ANOVA (Dunnett’s post-test) was used for the statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001 vs. DMSO group; *P < 0.05, **P < 0.01, ***P < 0.001 vs PA model group. SMW, Simiao Wan; PKCε, protein kinase C epsilon; PA, palmitic acid; DMSO, dimethyl sulfoxide; FRET, Förster/fluorescence resonance energy transfer; WB, western blotting; ANOVA, analysis of variance.

Figure 11 The molecular mechanisms of Simiao Wan in obesity-associated insulin resistance. SMW, Simiao Wan.
other ingredients with strong pharmacological activity were also taken into consideration, such as inokosterone, chlorogenic acid, and atracylenolide III. By reviewing previous literature reports, we found that atracylenolide III has been reported to activate the PI3K/Akt pathway, the main insulin signaling pathway [31]. Ferulic acid and metformin have been shown to have a synergistic impact on anti-diabetics, in which ferulic acid and metformin promote glucose uptake through the PI3K pathway and AMPK pathway, respectively [32]. Also, some ingredients have not yet been confirmed to have a therapeutic effect on diabetes or obesity, such as inokosterone and chlorogenic acid, but have shown anti-oxidative stress or anti-inflammatory activity [33, 34]. Combined with ADMV simulation parameters, database screening of active ingredients is an effective screening method, but reference to previous experimental validation would be a vital supplement. According to the analysis of the interrelationship between active ingredients and candidate targets, the five components with the highest degree value are queretin, wogonin, berberine, kaempferol, and baicaline, which four of five compounds are flavonoids, indicating that flavonoids play a significant role in SMW for the treatment of OIR.

According to KEGG pathway analysis, the most enriched pathways include the PI3K-Akt signaling pathway, MAPK signaling pathway, TNF signaling pathway, lipid and atherosclerosis signaling pathway, and pathways in cancer. Inflammation and lipid accumulation are closely associated with insulin resistance and contribute to each other. Evidence has shown that chronic inflammation within insulin target cells is an crucial cause of obesity-related insulin resistance [35]. TNFα, a cytokine related to cancer cachexia, was raised in the obese adipose tissue of rodents. Glucose tolerance and insulin sensitivity were ameliorated after the inhibition of this cytokine [36]. Obesity can lead to an increase in tissue macrophase numbers, especially in adipose tissue, and induce a tilt toward an M1-like macrophage polarization state. These polarized M1-like macrophages secrete multiple cytokines, such as TNFα, IL-1β, and activate stress kinases, such as PKCα, IKKβ, S6K, and JNK1, which suppress the activity of IRS1 by phosphorylating inhibitory serine residues and lead to impaired insulin signaling pathway [37]. Saturated fatty acids are lipid compounds that significantly activate inflammatory signaling in hepatocytes, macrophages, adipocytes, etc., leading to IKKβ/NF-κB and JNK1/AP1 stimulation and a release of cytokine [38, 39]. In addition, saturated fatty acids are precursors of other lipid products such as DAGs, and the level of intracellular DAGs can be elevated, resulting in activation of PKCα and impairment of insulin signaling [40]. Insulin resistance can change systemic lipid metabolism, causing the appearance of small dense low-density lipoproteins, low levels of high-density lipoprotein, and high levels of plasma triglycerides, promoting atherosclerotic plaque formation [41]. Targeting inflammation and lipid signaling is a potential strategy to ameliorate insulin resistance, along with the other metabolic syndrome.

In addition to regulating metabolic homeostasis, insulin is a growth factor that promotes the proliferation of all cells, especially the malignant cells that usually overexpress the insulin receptor. The insulin receptor plays a special role in the mediation of cancer progression, supported by the knockdown of either the IR or the IGF-1R in transgenic mouse models [42]. Also, genetic alterations within tumor, such as PIK3CA mutations or amplifications, increase glucose flux and the acquisition of adequate nutrients for biomass and energy production [28]. Pathways in cancer significantly enriched indicates that the mechanisms underlying the pathogenesis of insulin resistance and tumorigenesis show some similarities such as the involvement of IRS, PI3K, and Akt. Further study need to be done to explore whether SMW could ameliorate cancer.

PI3K/Akt signaling pathway plays an essential role in biological activities by mediating growth factor signaling in the cell cycle and critical cellular functions, such as cell proliferation and survival, protein synthesis, lipid metabolism, and glucose homeostasis [43]. Network analysis of the interaction between candidate targets revealed that Akt with the highest degree value is the most important target of SMW in OIR treatment. Akt is a key node with more than one hundred substrates in the insulin signaling pathway that mediates most of the biological metabolic actions of insulin [30]. The insulin response is initiated by proximal insulin signaling events, including IR activation, recruitment, and phosphorylation of signaling proteins (most prominently IRS, PI3K, and Akt isoforms) at the plasma membrane [14]. Considering the essential role of PI3K and Akt in insulin signaling transduction, the PI3K-Akt signaling pathway was chosen to investigate the mechanisms of SMW on OIR treatment. In this study, insulin resistance was induced in HL-7702 cells by triggering DAGs accumulation with PA, and SMW was found to reverse the reduced phosphorylation of IRS, PI3K, Akt, FOXO1, and GSK3β in insulin signaling, improving glucose utilization and glycogen synthesis. Furthermore, SMW was found to reduce intracellular lipids accumulation, including TC, TG, DAGs, and neutral lipids content. It has been reported that DAGs accumulation in the liver is closely related to insulin resistance, impairing insulin signaling through reducing tyrosine phosphorylation of IRS-1 and activating PKCε [27]. Consist with previous studies, target interaction network analysis reveals that PKC is a core target playing an important role of SMW in treating OIR. To further explore the activity and subcellular localization of PKCε, we constructed a rapid PKCε activity evaluated system with FRET and PKCε-EGFP. We found that SMW ameliorates insulin resistance by inhibiting PKCε activation and translocation to the membrane.

In summary, the pathogenesis of OIR is complex, involving multiple signaling pathways which are cross-linked and show mutual modulation. The active ingredients of SMW can simultaneously modulate a set of targets and pathways, showing the multi-component-multi-target-multi-pathway action pattern of TC. Here, we identified a novel mechanism of SMW in obesity-associated insulin resistance treatment through moderulating the PKCζ/IRS-1/PI3K/Akt signaling axis. Further validation is needed for the role of other pathways enriched in KEGG analysis, as well as targets of different active ingredients.

References

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