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Exploring the Antidiabetic Effect of Lupenone in Rats with Type 1 Diabetes and Its Underlying Mechanism Based on Network Pharmacology

(Mengkaji Kesan Antidiabetes Lupenon pada Tikus dengan Diabetes Jenis 1 dan Mekanisme Asasnya Berdasarkan Farmakologi Rangkaian)

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ABSTRACT

Lupenone has been reported to possess numerous medicinal values and gives a positive antidiabetic effect. But the mechanism of preventing and treating type 1 diabetes has not been elucidated in type 1 diabetic rats. This study investigated the effects and mechanism of action of lupenone in preventing and treating type 1 diabetes by network pharmacology and diabetic rats. The blood glucose, glycosylated hemoglobin (HbA1c), insulin, and inflammatory factors in the pancreas of rats with type 1 diabetes were measured, and histopathological changes were observed after treatment with lupenone. The pharmacological network of 'component-target-disease' was constructed on diabetic rats. Gene function enrichment, the Kyoto Encyclopedia of Genes and Genomes pathway analysis, and molecular docking were performed. The results showed that lupenone can decrease fasting blood glucose and HbA1c levels, increase insulin content and interleukin (IL)-4, IL-10, and decrease IL-6, transforming growth factor β and tumor necrosis factor α levels in the pancreas. Furthermore, ten targets were identified, and 50 signal pathways closely related to type 1 diabetes and inflammation were screened by network pharmacology, including insulin resistance, type II diabetes, type I diabetes, insulin signal pathway, mitogen activated protein kinase (MAPK) signal pathway, and tumor necrosis factor (TNF) signal pathway. The docking affinity of potential targets and lupenone were between -3.3 and -9.8, among which caspase-3 (CASP3), cyclin-dependent kinase 4 (CDK4), inhibitor of kappaB kinase beta (IKBKB), transforming growth factor beta-1 (TGFB1), and TNF had high binding abilities. Thus, lupenone has the potential to be developed as a new drug for treating type 1 diabetes.

Keywords: Inflammatory factors; lupenone; mechanism; network pharmacology; type 1 diabetes

ABSTRAK

Lupenon telah dilaporkan mempunyai banyak nilai perubatan dan memberikan kesan antidiabetes yang positif. Tetapi mekanisme mencegah dan merawat diabetes jenis 1 belum dijelaskan dalam tikus diabetes jenis 1. Penyelidikan ini mengkaji kesan dan mekanisme tindakan lupenone dalam mencegah dan merawat diabetes jenis 1 oleh farmakologi rangkaian dan tikus diabetes. Glukosa darah, hemoglobin glikosilasi (HbA1c), insulin dan faktor keradangan dalam pankreas tikus dengan diabetes jenis 1 diukur dan perubahan histopatologi diperhatikan selepas rawatan dengan lupenone. Rangkaian farmakologi 'komponen-sasaran-penyakit' telah dibina pada tikus diabetes. Pengayaan fungsi gen, analisis laluan Ensiklopedia Gen dan Genom Kyoto serta dok molekul telah dilakukan. Keputusan menunjukkan bahawa lupenon boleh mengurangkan tahap glukosa darah puasa dan HbA1c, meningkatkan kandungan insulin dan interleukin (IL)-4, IL-10 serta mengurangkan IL-6, mengubah faktor pertumbuhan β dan tahap tumor nekrosis faktor α dalam pankreas. Tambahan pula, sepuluh sasaran telah dikenal pasti dan 50 laluan isyarat yang berkait rapat dengan diabetes jenis 1 dan keradangan telah disaring oleh farmakologi rangkaian, termasuk rintangan insulin, diabetes jenis II, diabetes jenis I, laluan isyarat insulin, laluan isyarat mitogen diaktifkan protein kinase (MAPK) dan laluan isyarat tumor nekrosis faktor (TNF). Perkaitan dok sasaran berpotensi dan lupenone adalah antara -3.3 dan -9.8, antaranya caspase-3 (CASP3), kinase 4 (CDK4 yang bergantung kepada cyclin), perencat kappaB kinase beta (IKBKB), mengubah faktor pertumbuhan beta-1 (TGFB1) dan TNF mempunyai kebolehan mengikat yang tinggi. Oleh itu, lupenone mempunyai potensi untuk dibangunkan sebagai ubat baharu untuk merawat diabetes jenis 1.

Kata kunci: Diabetes jenis 1; faktor keradangan; farmakologi rangkaian; lupenon; mekanisme

Introduction

Diabetes is a metabolic disease characterized by hyperglycemia due to defects in insulin secretion or impaired biological action. In the classification standards established by the World Health Organization (WHO) and the International Diabetes Federation (IDF), diabetes can be classified into four types: type 1, type 2, diabetes during pregnancy, and special types of diabetes. Type 1 diabetes, also known as insulin-dependent diabetes, is an autoimmune disease mostly mediated by T lymphocytes, in which islet β cells are attacked and destroyed, causing inflammation, and leading to insulin deficiency. It requires lifelong treatment with exogenous insulin injections, and the side effects include hypoglycemia, weight gain, and pain. In recent years, there have been many studies on the treatment of type 1 diabetes by stem cells, which are expected to possibly cure type 1 diabetes. However, immune rejection problems have appeared in both research and clinical studies (Harjutsalo, Sjöberg & Tuomilehto 2008; Liu & Liu 2019; Wang et al. 2017; Zou Hu & Xie 2019). Therefore, anti-diabetes drugs with low side effects have become a hot research topic worldwide.

The *Brugera gymnorrhiza* (L.) Savigny., *Euonymus alatus* (Thunb.) Sieb., *Pueraria lobata* (Willd) Ohwi, *Butea monosperma* (Lam.), *Phyllodium elegans* (Lour.) Desv., *Musa basjoo* Sied. Et Zucc, and other traditional Chinese medicines, contain the pentacyclic triterpene compound lupenone with protein tyrosine phosphatase 1 B activity (Ajay et al. 2011; Li, Li & Wang 2010; Li, Zhang & Wang 2010; Li et al. 2010; Liu, Zhou & Gong 2009; Shang, Chen & Tang 2000; Wang et al. 2012; Zhu et al. 2002). Our previous study found that lupenone could decrease fasting blood glucose (FBG) and glycated hemoglobin (HbA1c) in mice with alloxan diabetes (similar to type 1 diabetes) and obese mice with alloxan diabetes (similar to type 2 diabetes), and improve insulin resistance in rats with type 2 diabetes. Hence, these results confirmed that lupenone has a good antidiabetic effect (Wang et al. 2012; Wu et al. 2017; Xu et al. 2018). Lupenone could also significantly inhibit auricle swelling and cotton ball granuloma in mice induced by xylene, reduce the acute and subacute inflammation in mice and the expression levels of IL-1β and IFN-γ in the pancreas of rats with diabetes, which suggested that lupenone has anti-inflammatory effects, and inflammation is a key link in the development of diabetes (Helal et al. 2012; Xu et al. 2020, 2018). According to studies, the pathogenesis of diabetes is closely related to many inflammatory cytokines, such as the interleukins and tumor necrosis factor, which are involved in the occurrence and development of diabetes (Babar et al. 2019; Böni-Schnetzler et al. 2019; Duncan et al. 2003; Pechlivani & Ajjan 2019; Pouvreau et al. 2018; Yang & Wang 2014). As a result, regulating the level of inflammatory cytokines is an important way to prevent and treat diabetes. Therefore, exploring lupenone for the prevention and treatment of diabetes from the perspective of inflammation is relevant to current studies.

The pathogenesis of type 1 diabetes is relatively complex and has not yet been elucidated. It is currently clear that T1DM is related to various factors, such as genetics, glucose metabolism disorders, islet β cell damage mechanism, insulin resistance, and inflammatory response (Adeyemi & Olayaki 2018; Lu et al. 2018; Yue et al. 2017). Although lupenone can decrease FBG and anti-inflammatory effects in alloxan-induced diabetic mice (similar to those with type 1 diabetes), the mechanism of preventing and treating type 1 diabetes has not been elucidated in animals with type 1 diabetes (Wang et al. 2012b; Xu et al. 2020). There is also a lack of explanation on whether the mechanism of action of lupenone is related to its anti-inflammatory effect and how it might be used for the prevention and treatment of type 1 diabetes. In recent years, network pharmacology has provided a new perspective for studying the complex system of the traditional Chinese medicine (Hopkins 2007; Li & Zhang 2013; Zou et al. 2019). Therefore, it is important to study the effects of lupenone on the prevention and treatment of type 1 diabetes using animal models and to discuss its underlying mechanism using network pharmacology methods.

Methods

Reagent

Streptozotocin (STZ, Sigma) was purchased from Beijing Boao Biotechnology Co., Ltd. Rat IL-4, IL-6, IL-10, TGF-β, TNF-α, and ELISA Kit (96-well/box) were produced by Abcam and imported by Xinle Biology. Lupenone was prepared by the Pharmacognostical Laboratory of Guizhou University of Traditional Chinese Medicine (purity ≥ 98%). The 2D structure of Lupenone is shown in Figure 1.

Animals

Healthy male Sprague-Dawley rats were reared in the specific pathogen-free laboratory, weighing 180-200 g, and were purchased from the Chongqing Tengxin

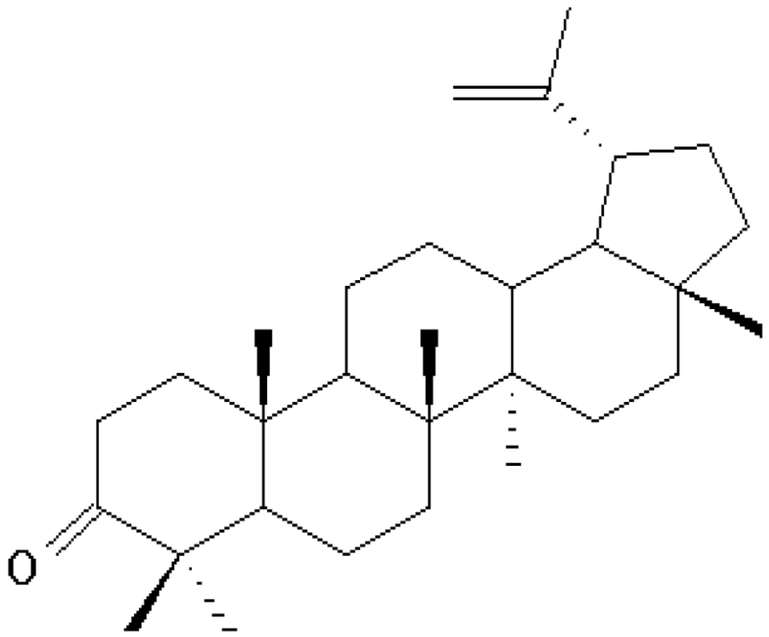

FIGURE 1. The 2D structure of lupenone

Biotechnology Co., Ltd. Qualification number: SCXK-(Jun) 2012-0011. The study was approved by the Experimental Animal Ethics Committee of Guizhou University of Traditional Chinese Medicine and followed the guidelines for the ethical review of the Laboratory Animal Welfare People' s Republic of China National Standard GB/T 35892-2018.

THE EFFECT OF LUPENONE ON RATS WITH TYPE 1 DIABETES

Establishment of type 1 diabetes model (T1DM) (Cheng 2011; Rees & Alcolado 2005)

Healthy male Sprague-Dawley rats (180-200 g) were adaptively fed for 7 days in the feeding observation room. After fasting for 12 h on the 8th day, blood samples were collected from the tail of the rats' veins using a needle. Fasting blood glucose was measured using a blood glucose meter. Before modeling, the blood glucose values were within the normal range, indicating that the basic blood glucose level was qualified. Ten rats were randomly selected as a control group, whereas the remaining forty rats fasted for 12 h and were then injected with streptozotocin solution intraperitoneally for two consecutive days with a total dose of 80 mg/kg, and the injection dose was 40 mg/kg per day. After a week, the rats fasted for 12 h, blood was drawn from the tail vein, and the FBG was measured using a blood glucose meter. Rats with blood glucose value ≥ 11.1 mmol/L were selected as model rats.

Grouping and administration

Based on the blood glucose values, forty rats with constructed type 1 diabetes were equally divided into four groups: Model group (type 1 diabetes), positive drug group (104.16 mg/kg metformin), lupenone high-dose group (8.0 mg/kg), and lupenone low-dose group (2.0 mg/kg). Other normal rats formed the control group. The control and model groups were given the same volume of distilled water by gavage, while the positive drug group was administered metformin and the lupenone groups were administered the corresponding dose of lupenone by gavage. The weight of the rats was measured once every three days, and the results were recorded. The FBG level was measured and recorded once a week. After 4 weeks of administration, blood was collected from the femoral artery after fasting for 12 h, the rats were anesthetized, and sacrificed by removing the cervical vertebra. The blood was placed in a common blood collection tube, and the serum was centrifuged and separated, which was used for the detection of

HbA1c and insulin (INS). The isolated pancreatic tissues were fixed with 4% paraformaldehyde solution, and the remaining pancreas was stored in a refrigerator at -80 °C.

FBG levels, HbA1c and INS

Blood glucose test strips were used to detect the FBG levels of rats in each group after drug administration. Then, the FBG levels of other groups were compared with the normal group. The serum of rats in each group was used for HbA1c and INS detection.

Determination of the contents of inflammatory cytokines in pancreas

The rat pancreatic tissue was homogenized, then enzyme linked immunosorbent immunoassay (ELISA) was used to determine the levels of interleukin-4 (IL-4), IL-6, IL-10, transforming growth factor β (TGF-β), and tumor necrosis factor-α (TNF-α).

Observation of HE staining sections

The fixed pancreatic tissues of each group with type 1 diabetes were collected, dehydrated, trimmed, embedded, sliced, stained, and mounted. All specimens were analyzed according to pathological examination procedures. Finally, under a microscope at ×400 magnification, specific pathological changes were observed.

NETWORK PHARMACOLOGY ANALYSIS AND RESEARCH

Molecular structure and target screening

The BATMAN-TCM (http://bionet.ncpsb.org/batman-tcm/), Swiss Target Prediction (http://www.swisstargetprediction.ch/), and Pharm Mapper Server databases (http://www.lilab-ecust.cn/pharmmapper/) were used to identify the target protein corresponding to lupenone, and to establish a drug target dataset. Through a comprehensive database of human genes and gene phenotypes (OMIM, http://www. omim.org/), the genes and protein targets related to type 1 diabetes were screened to establish a type 1 diabetes target dataset. Human target connexins were obtained from an interactive protein database (http://dip.doe-mbi.ucla.edu). Finally, all the targets obtained by the screening were converted into the UniProt ID format using the UniProt database.

Network construction and analysis

Through the analysis of the protein-protein interaction (PPI) network (http://www.genome.jp/kegg/), a complex network of 'component-target-disease' was constructed. The Cytoscape 3.6.1 software was used to visually analyze the above network and to obtain the three topology parameters of each node: degree, betweenness centrality, and closeness centrality. Then, the degree and betweenness centrality two times higher than the median value and closeness centrality one times higher than the median value of all target nodes were chosen as the key targets. Finally, the CentiScaPe 1.2 was used to evaluate the potential target protein for the prevention and treatment of type 1 diabetes by lupenone.

GO and KEGG enrichment analysis

Potential targets were imported into the DAVID (https://david.ncifcrf.gov/) database for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and gene ontology (GO) biological process analysis. STRING (https://string-db.org/) was used to analyze the protein interaction relationship of the selected potential targets.

Pathway notes

The KEGG Mapper function was used in the KEGG (https://www.genome.jp/kegg/) database to import potential target proteins, mark on the signal pathways closest to type 1 diabetes, predict lupenone to pass multiple targets, and multiple ways to play the role for the prevention and treatment of diabetes.

Molecular docking

The SailVina software was used to convert lupenone '.pdb' format to '.pdbqt' files and to perform ligand pretreatment. The protein data bank (PDB) database (http://www.rcsb.org/) downloads the crystal structure of the potential target. After processing, the crystal structure removes water molecules, polarized hydrogen atoms, force field, and conformation optimization to determine the active binding locus. AutoDock was used for molecular docking, and limited binding energy ≤ -5.0 kacl•mol^{-1} as stable connection site. The greater the absolute value of affinity, the more stable the binding ability between the ligand and receptor.

Data analysis

The experimental results were analyzed using the Statistical Package for the Socials Science SPSS 21.0, and analysis of variance was used for comparison between multiple groups, and LSD-t test was used for pairwise comparison between groups. The data were expressed

as the mean ± standard deviation (SD) using the Image-Pro Plus 6.0, (Media Cybernetics, USA) image analysis software.

Results

Effects of lupenone on FBG, HbA1c, and INS in rats with type 1 diabetes

Compared with the control group, the FBG and HbA1c values of the model group were significantly increased, and the INS content was markedly decreased (P<0.01), indicating successful experimental modeling. After administration, the positive drug and 8.0 mg/kg lupenone significantly decreased the FBG and HbA1c levels on rats (P<0.01), and the 2.0 mg/kg lupenone treatment markedly decreased the HbA1c level (P<0.05) compared with the model group. The positive drug, 2.0 mg/kg (P<0.05), and 8.0 mg/kg lupenone significantly increased the INS content (P<0.01). The levels of FBG, HbA1c, and INS content in rats in each group after drug administration are shown in Table 1.

Effects of lupenone on the levels of inflammatory factors in pancreas

Compared with the control group, the concentration of TGF-β and TNF-α in the model group was significantly increased, indicating that the pancreatic tissues had inflammation. Besides, the concentration of IL-4 and IL-10 in the model group was markedly decreased, while the concentration of IL-6 was significantly increased (P<0.01). Compared with the model group, positive drugs significantly decreased the concentration of TGF-β, TNF-α and increased the concentration of IL-4 (P<0.01), and markedly increased the concentration of IL-10 and decreased the concentration of IL-6 (P<0.05); the 8.0 mg/kg lupenone significantly decreased the concentration of TNF-α and increased the concentration of IL-4, IL-10 (P<0.01), and the high/low-dose lupenone group markedly decreased the concentration of TGF-β (P<0.05), while 2.0 mg/kg lupenone decreased the concentration of TNF-α, IL-6, increased the concentration of IL-4, IL-10 and the 8.0 mg/kg lupenone decreased the concentration of IL-6 (P>0.05), but the difference was not statistically significant. Compared with the positive group, 8.0 mg/kg lupenone significantly decreased the level of TNF-α (P<0.01), decreased the level of IL-6 (P<0.05), and 2.0 mg/kg lupenone increased the level of IL-10 (P<0.05). Maybe the physiological biomarkers in the plasma are not sensitive enough to show the dose response effects. This result also suggested that the dose of lupenone should be increased to obtain better hypoglycemic effect. The results are presented in Tables 2 and 3.

TABLE 1. Statistical results of blood glucose, HbA1c, and INS in rats with diabetes before and after treatment ($\overline{X}$± s)

Group	No.	Blood glucose before administration（mmol/L）	Blood glucose after administration（mmol/L）	HbA1c （mmol/L）	INS （mU/L）
control group	10	4.56±0.64**	4.23±0.23**	6.21±1.18**	25.32±3.04**
model group	10	26.14±4.47	29.33±3.28	8.03±0.83	18.57±1.02
positive group	10	25.89±4.40	17.02±6.68**	6.56±0.94 **	23.50±3.99**
lupenone high-dose group	10	26.12±3.12	18.67±7.54**	6.62±0.82 **	24.81±3.21 **
lupenone low-dose group	10	25.17±3.20	18.38±8.22**	6.94±1.24 *	20.96±1.66 *

compared to model group, *P<0.05, **P<0.01

TABLE 2. Statistical results of the concentrations of inflammatory factors, TGF-β and TNF-α in the rat pancreas ($\bar{x}$ ±s)

Group	No.	TGF-β	TNF-α
control group	10	47.05±1.26 **	26.95±1.09 **
model group	10	68.53±6.36	35.02±6.39
positive group	10	54.49±7.13 **	30.86±0.65*
lupenone high-dose group	10	60.97±13.80*	28.42±0.66 **△△
lupenone low-dose group	10	59.20±12.33*	34.89±5.33

compared to model group, *P<0.05, **P<0.01; compared to positive group, △P<0.05, △△P<0.01

TABLE 3. Statistical results of the concentration of inflammatory factors, IL-4, IL-6, and IL-10 in the rat pancreas ($\bar{x}$ ±s)

Group	No.	IL-4	IL-6	IL-10
control group	10	20.18±2.94**	2.77±0.35**	30.31±1.96**
model group	10	17.27±1.16	3.76±0.51	27.07±2.10
positive group	10	19.70±1.27**	3.35±0.08*	28.89±0.72*
lupenone high-dose group	10	19.85±3.23**	3.55±0.20△	29.59±1.17**
lupenone low-dose group	10	18.26±2.03	3.54±0.49	27.91±0.87△

compared to model group, *P<0.05, **P<0.01; compared to positive group, △P<0.05, △△P<0.01

EFFECT OF LUPENONE ON PANCREAS OF TYPE 1 DIABETIC RATS

Compared with the control group, the acinar cells in the exocrine part of the pancreas in the model group were arranged irregularly. The acinar cells were degranulated to different degrees, the cell volume was reduced, more inflammatory cells were infiltrated, and the islet cells were atrophied in the endocrine part. The nuclei were agglomerated accompanied by edema, cell degeneration, and fat vacuoles and the fibrous tissue was proliferated. Metformin and 8.0 mg/kg lupenone improved the arrangement and cell volume reduction of the pancreatic exocrine acinar cells and endocrine cells, the number of islets were slightly increased, and the boundaries of islets were clear, indicating a protective effect on the physiological function of the pancreas. Additionally, 8.0 mg/kg lupenone reduced the inflammation of the exocrine cells. The 2.0 mg/kg lupenone improved the morphology and arrangement of the pancreatic islet cells of the endocrine status, but had no effect on inflammatory infiltration (Figure 2).

TARGET SCREENING RESULTS

Using the BATMAN-TCM, Swiss Target Prediction, and PharmMapper Server databases, we found that a total of 659 target proteins were corresponding to lupenone (256, 108, and 295). A total of 189 target proteins related to type 1 diabetes were identified.

NETWORK CONSTRUCTION AND ANALYSIS

The PPI network was used to construct the 'component-target-disease' network. The above networks were visualized using the Cytoscape 3.6.1 software. Different colors and shapes were used to visualize the networks, and the network relationship between lupenone and the

target of type 1 diabetes can be intuitively seen (Figure 3). In the network, the median averages of degree, betweenness centrality, and closeness centrality are 3.0, 0.0011, and 0.2786, respectively. A total of ten target proteins were obtained to meet the screening criteria and were considered as the potential targets (e.g., Caspase-3, Insulin receptor, and Cyclin-dependent kinase 4). The whole results are listed in Table 4. The potential target proteins are analyzed for protein interactions, and the results are shown in Figure 4.

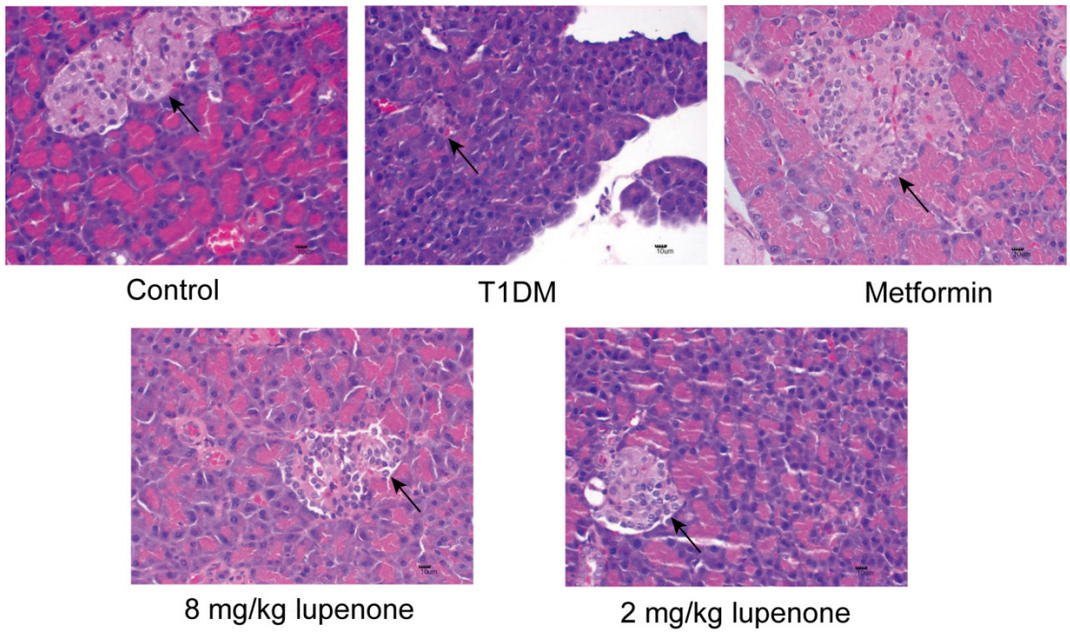

FIGURE 2. Pathological slices of the rat pancreas in each group (×400)

TABLE 4. Topological parameters of direct target of lupenone in the prevention and treatment of diabetes mellitus

Uniprot ID	Protein names	Gene names	Closeness Centrality	Degree	Betweenness Centrality
P42574	Caspase-3	CASP3	0.2799	18	0.0158
P06213	Insulin receptor	INSR	0.2794	15	0.0069
P11802	Cyclin-dependent kinase 4	CDK4	0.2796	11	0.0082
Q01094	Transcription factor E2F1	E2F1	0.2827	13	0.0060
O14920	Inhibitor of nuclear factor kappa-B kinase subunit beta	IKBKB	0.2870	15	0.0078
P01137	Transforming growth factor beta-1 proprotein	TGFB1	0.3702	9	0.0068
P01375	Tumor necrosis factor	TNF	0.3700	8	0.0026
P36956	Sterol regulatory element-binding protein 1	SREBF1	0.3707	10	0.0087
P01308	Insulin	INS	0.3715	4	0.0023
P04150	Glucocorticoid receptor	NR3C1	0.3730	9	0.0049

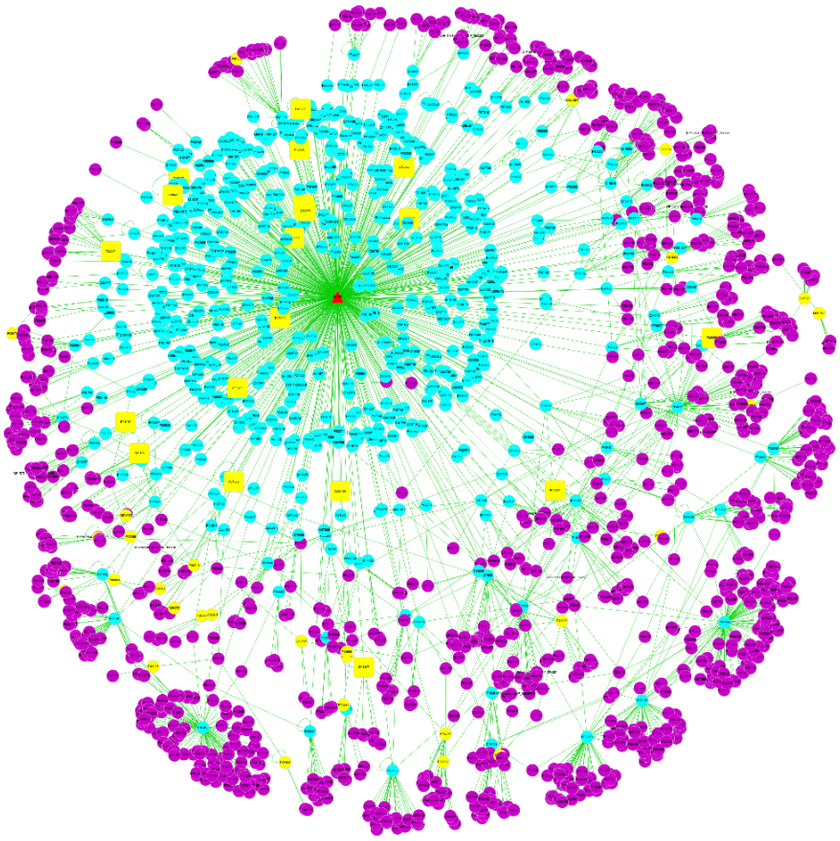

FIGURE 3. 'Component-target-disease' interaction network of lupenone (The yellow square represents the target of drugs and diseases, and it is also the most important target protein of lupenone in preventing and treating diabetes; the yellow dot represents the direct target of diabetes; the red triangle represents lupenone; the blue dots represent the direct target of lupenone; the purple dots represent the interacting protein connecting lupenone to the target of type 1 diabetes)

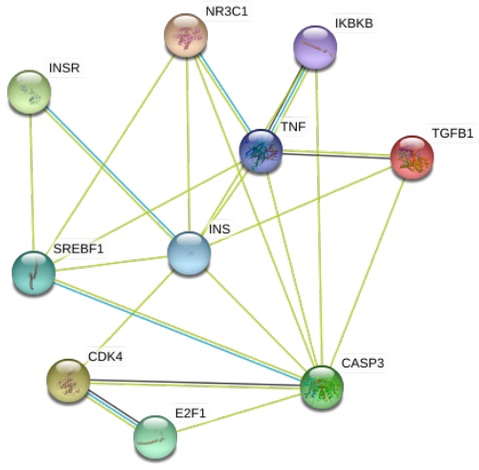

FIGURE 4. Interaction relationship diagram of lupenone's potential target protein for the prevention and treatment of type 1 diabetes

GO AND KEGG ENRICHMENT ANALYSIS RESULTS

Ten potential targets were mapped to the DAVID database for GO biological function enrichment analysis and KEGG pathway enrichment analysis. The results are presented in Tables 5 and 6. There were 69 biological processes in the GO enrichment analysis, and 27 biological processes with statistical significance of $P \leq 0.01$. The results showed that the prevention and treatment of type 1 diabetes by lupenone are related to the regulation of multiple biological processes. The main biological processes included positive regulation of nitric oxide biosynthetic process, response to glucose, insulin receptor signaling pathway, positive regulation of glycolytic process, positive regulation of cellular protein metabolic process, positive regulation of glycogen biosynthetic process, mitogen activated protein kinase (MAPK) cascade, and negative regulation of lipid catabolic process. It is suggested that the mechanism of lupenone in the prevention and treatment of type 1 diabetes is related to abnormalities in multiple biological pathways.

The enrichment analysis of KEGG pathway showed that a total of 21 signaling pathways ($P \leq 0.01$) were obtained. These pathways are closely related to the mechanism of action of type 1 diabetes, including insulin resistance, type II diabetes mellitus, pancreatic cancer, insulin signaling pathway, MAPK signaling pathway, T cell receptor signaling pathway, tumor necrosis factor (TNF) signaling pathway, phosphatidylinositol 3'–kinase (PI3K)-Akt signaling pathway, and AMP-activated protein kinase (AMPK) signaling pathway, indicating that lupenone may prevent type 1 diabetes through multiple signaling pathways.

TABLE 5. The enrichment analysis results of GO biological functions of lupenone in the prevention and treatment of type 1 diabetes mellitus

Category	Term	Count	Count (%)	P-Value
GOTERM_BP_DIRECT	positive regulation of protein kinase B signaling	4	40	0.0000099
GOTERM_BP_DIRECT	positive regulation of NF-kappaB transcription factor activity	4	40	0.000039
GOTERM_BP_DIRECT	positive regulation of transcription from RNA polymerase II promoter	6	60	0.00007
GOTERM_BP_DIRECT	positive regulation of transcription, DNA-templated	5	50	0.000097
GOTERM_BP_DIRECT	negative regulation of fat cell differentiation	3	30	0.00022
GOTERM_BP_DIRECT	positive regulation of nitric oxide biosynthetic process	3	30	0.00023
GOTERM_BP_DIRECT	positive regulation of gene expression	4	40	0.00029
GOTERM_BP_DIRECT	positive regulation of fibroblast proliferation	3	30	0.00036
GOTERM_BP_DIRECT	cellular response to organic cyclic compound	3	30	0.00043
GOTERM_BP_DIRECT	response to drug	4	40	0.00046
GOTERM_BP_DIRECT	response to glucose	3	30	0.00057
GOTERM_BP_DIRECT	insulin receptor signaling pathway	3	30	0.00075
GOTERM_BP_DIRECT	positive regulation of cell proliferation	4	40	0.0016
GOTERM_BP_DIRECT	positive regulation of protein phosphorylation	3	30	0.002
GOTERM_BP_DIRECT	positive regulation of mononuclear cell migration	2	20	0.0021
GOTERM_BP_DIRECT	negative regulation of bicellular tight junction assembly	2	20	0.0021
GOTERM_BP_DIRECT	negative regulation of myosin-light-chain-phosphatase activity	2	20	0.0027
GOTERM_BP_DIRECT	positive regulation of respiratory burst	2	20	0.0032
GOTERM_BP_DIRECT	regulation of establishment of endothelial barrier	2	20	0.0037
GOTERM_BP_DIRECT	positive regulation of cell migration	3	30	0.0041
GOTERM_BP_DIRECT	negative regulation of transcription from RNA polymerase II promoter	4	40	0.0054
GOTERM_BP_DIRECT	positive regulation of histone deacetylation	2	20	0.0059
GOTERM_BP_DIRECT	positive regulation of glycolytic process	2	20	0.0075
GOTERM_BP_DIRECT	positive regulation of cellular protein metabolic process	2	20	0.008
GOTERM_BP_DIRECT	positive regulation of glycogen biosynthetic process	2	20	0.008
GOTERM_BP_DIRECT	MAPK cascade	3	30	0.0081
GOTERM_BP_DIRECT	negative regulation of lipid catabolic process	2	20	0.0085

TABLE 6. The enrichment analysis of the KEGG pathway for lupenone in preventing and treating type 1 diabetes

Category	Term	Count	Count（%）	P-Value
KEGG_PATHWAY	Non-alcoholic fatty liver disease (NAFLD)	7	70	8.1E-09
KEGG_PATHWAY	Hepatitis B	6	60	0.00000046
KEGG_PATHWAY	Insulin resistance	5	50	0.0000068
KEGG_PATHWAY	Type II diabetes mellitus	4	40	0.000026
KEGG_PATHWAY	Pancreatic cancer	4	40	0.000065
KEGG_PATHWAY	Chronic myeloid leukemia	4	40	0.000088
KEGG_PATHWAY	HTLV-I infection	5	50	0.0002
KEGG_PATHWAY	Toxoplasmosis	4	40	0.00031
KEGG_PATHWAY	FoxO signaling pathway	4	40	0.00056
KEGG_PATHWAY	Insulin signaling pathway	4	40	0.00061
KEGG_PATHWAY	Pathways in cancer	5	50	0.0011
KEGG_PATHWAY	mTOR signaling pathway	3	30	0.0024
KEGG_PATHWAY	Apoptosis	3	30	0.0028
KEGG_PATHWAY	MAPK signaling pathway	4	40	0.0035
KEGG_PATHWAY	Small cell lung cancer	3	30	0.0051
KEGG_PATHWAY	Prostate cancer	3	30	0.0055
KEGG_PATHWAY	T cell receptor signaling pathway	3	30	0.007
KEGG_PATHWAY	Chagas disease (American trypanosomiasis)	3	30	0.0076
KEGG_PATHWAY	Amoebiasis	3	30	0.0079
KEGG_PATHWAY	TNF signaling pathway	3	30	0.008
KEGG_PATHWAY	PI3K-Akt signaling pathway	4	40	0.0084
KEGG_PATHWAY	AMPK signaling pathway	3	30	0.011
KEGG_PATHWAY	Cell cycle	3	30	0.011
KEGG_PATHWAY	Osteoclast differentiation	3	30	0.012
KEGG_PATHWAY	Tuberculosis	3	30	0.021
KEGG_PATHWAY	Herpes simplex infection	3	30	0.022
KEGG_PATHWAY	Proteoglycans in cancer	3	30	0.026
KEGG_PATHWAY	Ras signaling pathway	3	30	0.033
KEGG_PATHWAY	Aldosterone-regulated sodium reabsorption	2	20	0.05
KEGG_PATHWAY	MicroRNAs in cancer	3	30	0.051
KEGG_PATHWAY	Bladder cancer	2	20	0.052
KEGG_PATHWAY	Type I diabetes mellitus	2	20	0.054

PATHWAY ANNOTATION DIAGRAM

Ten potential targets were input into the KEGG Mapper function of the KEGG database, and the number of potential targets in each relevant signaling pathway was marked. We combined the study report (type 1 diabetes signaling pathway) and the number of potential targets in the main signaling pathway to draw a type I diabetes mellitus signaling pathway map. The results are shown in Figure 5.

MOLECULAR DOCKING RESULTS

The pdb format of lupenone was converted to pdbqt format using AutoDock. To search for the active sites of proteins, x, y, and z coordinate distances in the grid box module were set. The docking module was used for molecular docking to obtain the lumbar lupenone docking binding affinity (affinity, kcal/mol) with potential targets. The greater the absolute value of affinity, the more stable the binding capacity between the ligand and receptor. The results showed that the affinity between these ten potential targets and lupenone ranged from -3.3 to -9.8, among which caspase-3 (CASP3), cyclin-dependent kinase 4 (CDK4), inhibitor of kappaB kinase beta (IKBKB), transforming growth factor beta-1 (TGFB1), and TNF had relatively high binding capacity with lupenone, which was consistent with the screening results of the network topological parameters, as shown in Table 7 and Figure 6.

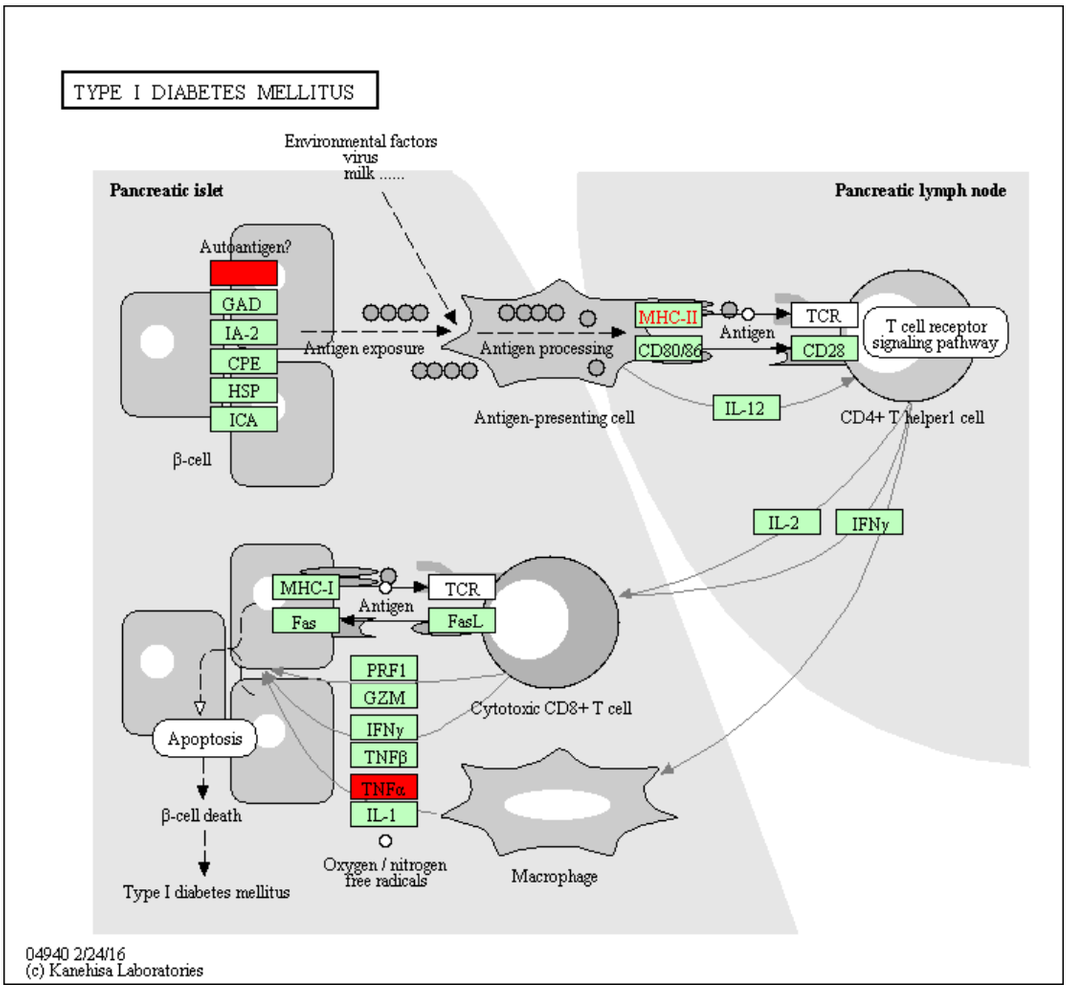

FIGURE 5. Annotation map of potential target of lupenone in type I diabetes mellitus signaling pathway

TABLE 7. Docking result of potential targets and lupenone molecule

No.	Gene name	PDB ID	Affinity (kcal/mol)
A	CASP3	6BFK	-8.1
B	INSR	5HHW	-5.6
C	CDK4	6P8E	-9.8
D	E2F1	6G0P	-5.8
E	IKBKB	4KIK	-8.0
F	TGFB1	5VQP	-7.8
G	TNF	6OP0	-6.5
H	SREBF1	1AM9	-3.3
I	INS	5MT9	-5.5
J	NR3C1	6EL7	-5.1

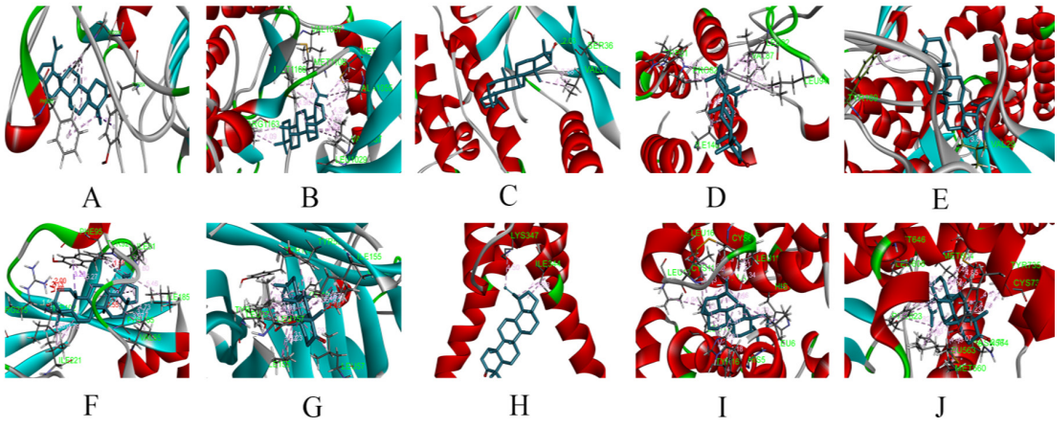

FIGURE 6. Docking diagram of potential target and lupenone molecule (A: CASP3, B: INSR, C: CDK4, D: E2F1, E: IKBKB, F: TGFB1, G: TNF, H: SREBF1, I: INS, J: NR3C1)

DISCUSSIONS

Type 1 diabetes mellitus (T1DM) is also known as insulin-dependent diabetes mellitus (DM). Insulin is a small molecule protein composed of 51 amino acids secreted by the β cells of the pancreatic tissues. The islet β cells are attacked by their own immune system, resulting in islet β cell damage or reduction and an absolute lack of insulin secretion, which leads to the occurrence of hyperglycemia causing a series of pathophysiological changes (Ferretti & La Cava 2016; Hull, Peakman & Tim 2017; Pugliese 2017). The blood glucose and glucose tolerance test (OGTT) are the internationally recognized indicator for the diagnosis of diabetes. The American Diabetes Association (ADA) has included HbA1c as a diagnostic criterion for diabetes (American Diabetes Association 2014). HbA1c is the product of a combination of hemoglobin and blood sugar in the red blood cells. The method of combining

is an irreversible reaction that is proportional to the concentration of blood glucose. Elevated HbA1c level is a dangerous cause of the occurrence and development of chronic complications (Hunag et al. 2017). According to previous studies, lupenone can decrease the blood glucose level in mice with alloxan-induced T1DM, increase the glucose load in normal mice, and improve glucose tolerance (Wang et al. 2012a; Wu et al. 2015). Our results indicated that lupenone decreases the serum HbA1c levels in streptozotocin-induced rats with type 1 diabetes and stimulates the secretion of insulin, thus, achieving a hypoglycemic effect.

Cytokines are mediators and regulators of immune responses. Several studies have indicated that inflammatory factors play a leading role in the pathogenesis of diabetes, and inflammatory factors are divided into pro-inflammatory and anti-inflammatory factors (Eguchi & Nagai 2017; Khodabandehloo et al. 2016). Therefore, the use of drugs that downregulate the expression of pro-inflammatory factors, upregulate the levels of anti-inflammatory factors, improve the inflammatory damage of the tissue cells, and treat diabetes from the perspective of regulating the levels of inflammatory factors has become a hot topic. TNF-α is one of the earliest initiating factors in inflammatory mediator response. It activates the cytokine cascade in the body, inducing a series of secondary inflammatory mediators, forming a chain reaction that results in tissue cell damage (Zelová & Hošek 2013). In addition, TNF-α has a cytotoxic effect on islet β cells and directly act on islet β cells, causing cell damage and triggering an autoimmune response, which may induce type 1 diabetes (Soldevila et al. 1991). TGF-β is a pro-fibrotic growth factor that causes cell damage and necrosis, epithelial mesenchymal transformation, and an increase in the extracellular matrix, which is closely related to renal fibrosis in diabetic nephropathy (Huang et al. 2017). Islet cells can secrete IL-6; high blood sugar can promote islet cells to secrete IL-6, while low concentrations of IL-6 can promote insulin secretion, and high concentrations can inhibit insulin secretion (Campbell et al. 1989; Choi et al. 2004). Thus, it suggests that reducing the concentration of IL-6 in the pancreas can promote insulin secretion, thereby achieving the effect of improving type 1 diabetes. IL-4 is a cytokine mainly produced by T cells, it stimulates B cell activation, proliferation, and differentiation. Besides, IL-4 can alleviate the occurrence of T1DM and has good protective effect on pancreatic islet cell by inhibiting Th1 cells related to autoimmune reactions (Nakanishi et al. 1996; Zhang et al. 2005).

Previous reports had confirmed that lupenone had a good anti-inflammatory effect, and the anti-infammatory mechanism of lupenone was closely related to the Toll-like receptor, PI3K-Akt, NF-kappa B and NOD-like receptor signaling pathways by network pharmacology (Xu et al. 2020, 2018). We observed the pancreatic pathological slices of diabetic rats and found that the pancreatic islets in the model group became smaller in volume, decreased in number, and cells aggregated into nuclei. In addition, edema, cell degeneration, fat vacuoles, fibrous tissue hyperplasia, and islet atrophy were obvious. After lupenone administration, the number of islets increased slightly, and the islet boundaries were clear, which inhibited the infiltration of inflammatory cells. The results indicated that lupenone could protect and repair the islet tissue structure in diabetic rats; hence, to realize the purpose of diabetes prevention and treatment. At the same time, our study found that lupenone could downregulate the levels of TGF-β, TNF-α, IL-6, which could decrease damage and necrosis of the islet cell, inhibit inflammatory response, and promote insulin secretion. Our study also found that lupenone could upregulate the levels of anti-inflammatory factors including IL-4 and IL-10 in the pancreatic tissue of diabetic rats. These results suggested that lupenone can inhibit the inflammatory response in the pancreatic islets by downregulating the level of pro-inflammatory factors and increasing the level of anti-inflammatory factors in the pancreas of diabetic rats, thereby protecting and repairing islet cells and promoting the secretion of insulin. It can also increase insulin concentration in the serum to decrease blood sugar and prevent diabetes.

In recent years, as single-target, highly selective drugs continue to show low safety and poor effectiveness in the treatment of complex diseases, people begin to realize the limitations of research and the 'one drug, one target, and one disease' developmental model. However, network pharmacology is based on the rapid development of omics and big data. It integrates multiple emerging interdisciplinary subjects, such as systems biology and bioinformatics. And it may be crucial to improve or restore the balance of biological networks, to understand the relationship between drugs and the body, and to conduct an overall analysis of the biological system network, which is consistent with the synergistic characteristics of 'multiple components, multiple pathways, and multiple targets' of the traditional Chinese

medicine. Network pharmacology has a unique advantage in the treatment of complex diseases, such as cancer, diabetes, cardiovascular and cerebrovascular diseases, and it provides new ideas and methods for the study of the basis and mechanism of action of the traditional Chinese medicine (Li & Zhang 2013).

Based on the prediction and analysis of the network pharmacology of lupenone, ten potential targets were screened and found to play an important role in the prevention and treatment of type 1 diabetes, including CASP3, insulin receptor (INSR), CDK4, E2F1, IKBKB, TGFB1, TNF, SREBF1, INS, and NR3C1. The results of molecular docking showed that these potential targets had a high affinity for lupenone, among which CASP3, CDK4, IKBKB, TGFB1, and TNF had a relatively high binding capacity, which was verified the reliability of the previous targets screened by network pharmacology. At the same time, the targets with the closest correlation with lupenone were screened by dock binding free energies. Studies have confirmed that genes such as Insulin receptor and INS are closely related to the occurrence and development of diabetes. At the same time, genes such as IKBKB, TGFB1, TNF, and nuclear receptor subfamily 3, group C, member 1 (NR3C1) are also closely related to the occurrence and development of inflammation (Abid 2017; Romero-Kusabara et al. 2017). We used network pharmacology to predict the target and signal pathway of lupenone in the prevention and treatment of type 1 diabetes, and by constructing the 'component-target-disease' interactive network diagram. Ultimately, we found that the signals closely related to diabetes include insulin resistance, type II diabetes mellitus, type I diabetes mellitus, and insulin signaling pathway, and the signals closely related to inflammation include MAPK signaling pathway, TNF signaling pathway, and PI3K-Akt signaling pathway.

To sum up, we inferred that lupenone may act on targets such as CASP3, CDK4, IKBKB, TGFB1, and TNF, and regulate insulin resistance, type II diabetes mellitus, type I diabetes mellitus, and insulin signaling pathway, MAPK signaling pathway, TNF signaling pathway, and PI3K-Akt signaling pathway to achieve anti-type 1 diabetes effects.

However, this study leaves an unanswered question. the physiological biomarkers in the pancreas tissues are not sensitive enough to show the dose response effects. This result also suggested that the dose of lupenone should be increased to obtain better effect of treating type 1 diabetes. In addition, the analysis results of network pharmacology still need further experimental verification, which will be our next step.

Conclusions

In summary, the animal experiments found that lupenone has the effect of lowering blood sugar, its mechanism may be related to downregulate the level of TGF-β, TNF-α, and IL-6, upregulate the level of IL-4 and IL-10. Thus, it can inhibit the islet inflammatory response, protect the islet cell inflammatory damage, and exert the biological effects of multi-target and multi-pathway prevention and treatment of type 1 diabetes. In addition, 10 antidiabetic targets of lupenone were screened by network pharmacology, and 5 of them had high affinity for molecule docking with lupenone. The antidiabetic mechanism of lupenone was closely related to insulin resistance, type II diabetes mellitus, type I diabetes mellitus, and insulin signaling pathway. The anti-infammatory mechanism of lupenone was closely related to MAPK signaling pathway, TNF signaling pathway, and PI3K-Akt signaling pathway. Therefore, the network pharmacology framework integrating computational prediction, experimental validation and literature reported clinical results analysis provides a novel approach for analyzing lupenone for treating and preventing type 1 diabetes. At the same time, we will further study the mechanism of lupenone according to the prediction results of the network pharmacology. It provides a reference for the development and utilization of lupenone and the development of new drugs for the treatment of diabetes.

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