Heliyon 9 (2023) e15777

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Effect of Dan-Lou tablets on coronary heart disease revealed by microarray analysis integrated with molecular mechanism studies

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ARTICLE INFO

Keywords:
Dan-Lou tablets
Coronary heart disease
Affymetrix
Molecular docking
Molecular biology

ABSTRACT

Dan-Lou tablets (DLT) effectively treat coronary heart disease (CHD). However, its pharmacological mechanism in CHD treatment requires further investigation. This study aimed to elucidate the underlying pharmacological mechanisms of DLT in the treatment of CHD through clinical trials, microarray research, bioinformatics analysis, and molecular mechanism research. In this study, DLT improved coagulation function, endothelial injury, and levels of lipids, metalloproteases, adhesion molecules, inflammatory mediators, and homocysteine. The results of molecular biology research demonstrated that DLT can increase the gene and protein expressions of meningioma expressed antigen 5 (MGEA5) and mouse doubleminute 2 (MDM2) and inhibited the gene and protein expressions of signal transcription and transcription activator 5 B (STAT5B), tropomyosin-1 (TPM1), and aromatic hydrocarbon receptor nuclear transpose (ARNT). The results indicate that DLT reduced the extent of vascular endothelial damage in CHD rats by reducing the expressions of STAT5B, TPM1, and MDM2; inhibiting the inflammatory reaction; and increasing the expressions of ARNT and MGEA5.

1. Introduction

Cardiovascular disease is a common condition that seriously threatens human health and is associated with high morbidity and mortality rates [1]. Dan-Lou tablets (DLT), a patented Chinese medicine, effectively treat angina pectoris with *phlegm and blood stasis* [2]. It has beneficial cardioprotective effects including anti-atherosclerosis and vascular endothelial protection [3]. DLT consists of *Trichosanthes kirilowii* Maxim, *Allium macrostemon* Bge, *Pueraria lobata*, *Ligusticum chuanxiong* Hort, *Salvia miltiorrhiza* Bge, *Paeonia*

Abbreviations: PT, Prothrombin time; TT, Thrombin time; APTT, Activated partial thromboplastin time; INR, International normalized ratio; FIB, Fibrinogen; TC, Total cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; ET-1, Endothelin 1; ANG-I, Angiotonin I; MMP2, Metalloprotease 2; ICAM-1, Intercellular cell adhesion; NF-κB, Nuclear factor-kappa B; GM-CSF, Granulocyte-macrophage colony stimulating factor; sCD40L, Soluble CD40 ligand; IL-1, Interleukin-1; IL-6, Interleukin-6; MCP-1, Monocyte chemotactic protein-1; TNF-α, Tumor necrosis factor-α; MGEA5, Meningioma expressed antigen 5; ARNT, Aromatic hydrocarbon receptor nuclear transpose; STAT5B, Signal transcription and transcription activator 5B; TPM1, Tropomyosin-1; MDM2, Mouse doubleminute 2.

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https://doi.org/10.1016/j.heliyon.2023.e15777
Received 11 July 2022; Received in revised form 18 April 2023; Accepted 20 April 2023
Available online 25 April 2023

lactiflora Pall, and *Alisma orientale* (Sam.) Juzep, *Astragalus membranaceus* (Fisch.), Bge. var. mongholicus (Bge.) Hsiao, *Drynaria fortunei* (Kunze) J. Sm, and *Curcuma wenyujin* Y. H. Chen et C. Ling [4]. Clinical studies demonstrated that DLT can reverse adverse left ventricular remodeling following myocardial infarction based on standard pharmacological treatment and improve the clinical outcomes of patients undergoing selective percutaneous coronary intervention with non-ST-elevation acute coronary syndrome [5]. Moreover, the ethanolic extract of DLT exhibited a good anti-inflammatory effect [6,7]. Pharmacological studies demonstrated that DLT intervention in coronary heart disease (CHD) may have antioxidant and anti-inflammatory effects [3,7].

Our previous multi-center, and fineness clinical study found that the mechanism of DLT treatment in CHD may be related to the regulation of endothelial injury, lipid metabolism, inflammatory factors, coagulation function, and metalloproteinases [8]. Although anti-inflammatory effects were initially proposed as a mechanism for DLT in the treatment of CHD, the underlying mechanisms require further exploration. In previous studies, the main chemical components of DLT were qualitatively analyzed, and flavonoids, glycosides, phenolic acids, and other compounds were reported [3].

As a new tool in molecular biology, microarray technology can simultaneously quantify hundreds or thousands of gene transcripts in cell or tissue samples. It can detect specific biomarkers and predict potential pharmacological mechanisms of action [9]. Network pharmacology is a methodology of systems biology that includes disease network construction, drug target networks, and drug–disease network screening [10]. Therefore, the biological foundation of DLT for CHD treatment was investigated using this method.

Taken together, clinical trials, microarray studies, bioinformatic analysis, molecular docking, and in *vivo* trials were performed to elucidate the pharmacological mechanisms of DLT treatment for CHD.

2. Materials and methods

2.1. Drug preparation

DLT was obtained from Jilin Cornell Pharmaceutical Co. Ltd. (20150112). In previous studies, the chemical characterization chromatogram of DLT was tested using ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry. A total of 45 compounds were analyzed, and it was determined that the chemical composition of DLT mainly included flavonoids, tanshinones, phenolic acids, lactone esters, and triterpenoids [3].

2.2. Study population

The trial was conducted in 7 clinical medical units in China from May 2016 to April 2017. Eighty-two subjects were recruited, including 38 patients with CHD and 40 healthy individuals, 4 patients were lost, each of whom provided written informed consent. Patients with CHD were selected based on refined diagnostic criteria [11,12]. The detailed inclusion criteria for patients were provided in the Supplementary Materials. The healthy participants also met strict inclusion criteria. The critical exclusion criteria were unstable angina, current pregnancy, mental illness, or cognitive dysfunction.

Eight representative patients with CHD (CHD_0 group) and healthy individuals (Normal group) were selected from previous clinical samples. Detailed selection criteria and demographic information were shown in Supplementary Tables 1–3. During this study, patients with CHD were treated with five tablets three times (0.3 g/tablets) a day for 8 weeks (CHD_8 group). The control group received no treatment. On the day of registration and 8 weeks after DLT administration, blood samples were collected from the anterior elbow vein for further study. In addition to the four coagulation items, the patients' samples were uniformly collected, transported, stored, and tested by Tianjin Jinyu Medical Institute Co., Ltd. representatives to ensure the accuracy and consistency of the testing methods of each clinical research center.

This study was approved by the ethics committee of the Tianjin University of Traditional Chinese Medicine (approval number: TJUTCM-EC20150001). The study was registered with the US Clinical Trial Registration Center (registration number: NCT02526381) and China Clinical Trial Registration Center (registration number: ChiCTR-OOC-15006765).

2.3. General information

The changes in serum biochemical indicators of eight CHD patients were measured, including prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT), fibrinogen (FIB), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), apolipoprotein A (ApoA1), apolipoprotein B (ApoB) and ratio of ApoA1/ApoB), endothelial injury [endothelin 1, angiotonin I (ANG-I) and angiotonin II (ANG-II), metalloprotease 2 (MMP2) and metalloprotease 9 (MMP9), adhesion molecule intercellular cell adhesion molecule-1 (ICAM-1), E-selection and P-selection, nuclear factor-kappa B (NF-κB), granulocyte-macrophage colony stimulating factor (GM-CSF), soluble CD40 ligand (sCD40L), interleukin-1 (IL-1), interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α, and homocysteine (Hcy)]. The serum was separated from the peripheral venous blood by centrifugation at 4 °C and 3000 rpm for 10 min.

2.4. Microarray gene expression analysis

Peripheral blood samples were collected from each participant in the morning after an overnight fast. The samples were immediately stored at 20 °C for 24 h and then stored at −80 °C until the RNA was separated. A microarray gene expression analysis was used to detect differentially expressed genes (DEGs) before and after DLT treatment in patients with CHD, The samples with RNA integrity

number above 7 were accepted and undertaken by Shanghai Ouyi Biomedical Technology Co., Ltd. as follows: synthesizing the double strand cDNA, in *vitro* transcription to synthesize labelled Poly-A RNA, Poly-A RNA purification, fragmentation of labelled Poly-A RNA, hybridization, washing, staining and scanning with GeneChip Scanner 3000 (Affymetrix). Data were acquired from GCOS 1.2 software (Affymetrix). For the microarray analysis, the raw data were filtered by flag signal and then processed and normalized. The raw *P*-values were adjusted by false discovery rate of 1% through the Benjamin and Hochberg method. DEGs were screened using the *P*-value and fold-change (FC) values of the *t*-test, with the screening criteria being an up-or downregulated fold-change FC $\geq$ 1.5 and *P*-value $\leq$ 0.05.

2.5. Ingenuity pathway analysis

DEGs caused by DLT after 8 weeks of treatment in CHD patients were analyzed using bioinformatic analysis. An Ingenuity Pathway Analysis (IPA) system (http://www.ingenuity.com) was used to explore upstream differential genes, typical pathways, and key targets that were most closely related to the microarray results. A Venn analysis was performed using OmicShare tools (https://www.omicshare.com/tools).

2.6. Molecular docking studies

Gallic acid, salvianic acid, puerarin, daidzin, paeoniflorin, salvianolic acid B, cryptotanshinone, and tanshinone IIA were selected as the core components of DLT [2] and docked with the key genes to verify the accuracy of the main components and forecast indicators. The main composition and the target crystal structure were downloaded from the RCSB protein data (https://www.rcsb.org/), and the crystals were dehydrated, hydrogenated, and ligand separated using Pymol 1.7.2.1 software (https://pymol.org/2/). A docking grid box was then constructed for each target, and molecular docking was performed using AutoDockTools 1.5.6. The docking interaction pattern diagram was established using binding energy ≤ -5.0 kJ/mol as the screening standard.

2.7. Molecular biology validation in CHD rats

2.7.1. Establish model of CHD rats

In this study, 84 male Wistar rats (weight, 180 $\pm$ 20 g) were assigned to seven groups: Normal, Sham, Model, At (atorvastatin 10 mg/kg), DLT-L (250 mg/kg), DLT-M (500 mg/kg), and DLT-H (1000 mg/kg). CHD rats were fed a high-fat diet and underwent left anterior coronary artery ligation (LADCA). A high-fat diet was used in the Model, At, and DLT groups. The Model and At groups were administered LADCA at week 5. The rats in the sham group were operated on similarly but the coronary artery was not ligated. The drug was administered from week 7. At the end of week 12, blood and heart tissues were collected and then preserved at -80 °C. Previous studies confirmed the successful establishment of DLT treatment models for CHD in rats [3]. This study was approved by the Ethics Committee of Tianjin University of Traditional Chinese Medicine. According to the clinical biochemical indicators and genomics research results, key upstream regulators and gene targets were selected to detect the relevant gene and protein expression levels in CHD rats.

2.7.2. Western blot

Heart tissue proteins of CHD rats were extracted according to the manufacturer's instructions. The protein concentration was determined using the BCA assay (Thermo Fisher Scientific). The primary antibodies (Abcam, UK; Cell Signal Technology Co. Ltd., US) included anti-glyceraldehyde phosphate dehydrogenase (GAPDH), anti-experiment, meningioma expressed antigen 5 (MGEA5), anti-aromatic hydrocarbon receptor nuclear transpose (ARNT), anti-signal transcription and transcription activator 5 B (STAT5B), anti-tropomyosin-1 (TPM1), and anti-mouse doubleminute 2 (MDM2) were added to the extracted portions and incubated overnight at 4 °C. The secondary antibody (horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG) (Sangon Biotech Co. Ltd.) was incubated at room temperature for 1 h the next day, and chemiluminescence was observed. Enhanced chemiluminescence reagent (Beyotime

Table 1
The primer sequence and reaction parameters.

Gene (Rats)	Primer Sequence (5′→3′)	PCR product (bp)
STAT5B	Forward : GGGCAGAACGAGGTTGTAAG	20
	Reverse : TGGGATAAATAATGTCGCACC	21
TPM1	Forward : GCTGACCCAGGTTTCTTTC	19
	Reverse : ACAGGACTAGCGTACAGTGTTTA	23
EGFR	Forward : CCATAGCAATGCGGTGAG	18
	Reverse : AAAGAAGTCCTGCTGGTAGTCA	22
MDM2	Forward : TCGGGTCACAGTCTATCAG	19
	Reverse : CTTTCCAGTTTGGCTTTT	18
ARNT	Forward : GGGTCCCACCATTGCTTC	18
	Reverse : CCGCCGCTCTATTTCACTA	19
MGEA5	Forward : GCAGTGGTTAGGGTGTCG	18
	Reverse : GGAGGTAGGAGTCAGTGGG	19

Biotechnology, China) was used to develop the protein band, a gel imaging system (Tanon, China) was used for imaging, and NIH ImageJ (NIH, United States) was used for analysis and calculation.

2.7.3. RT-PCR

The specific forward and reverse primers of MGEA5, ARNT, STAT5B, TPM1, and MDM2 were designed by Primer Express Software (Table 1). Heart tissue mRNA of CHD rats were extracted using trizol reagent according to the manufacturer's protocol. RNA concentration was quantified with NanoDrop 1000. RNA was transcribed into cDNA by using Improm-II Reverse Transcription System. Then the cDNA was used as a template and FastStart Universal SYBR Green Master kit was used for Real-time PCR in real-time fluorescence quantitative PCR instrument (Bio-Rad, United States). The annealing primers extend along the (5′→3′) direction and in the presence of DNA polymerase and dNTPs and Mg^{2+}. The fluorescence signal was detected at the end of each cycle as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and extension at 95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s. Each mRNA expression level was normalized to its GAPDH signal using the $2^{-\triangle\triangle CT}$ method.

2.8. Statistical analysis

Normally distributed continuous variables are expressed as mean ± standard deviation, while non-normally distributed data are shown as median (upper quartile, lower quartile). Intergroup differences were analyzed using a paired *t*-test, independent sample *t*-test, or one-way analysis of variance. All statistical analyses were performed using SPSS 24.0 (IBM Corp., New York, NY, USA).

3. Results

3.1. Biochemical indicator information

Biochemical indicator information for the eight CHD patients and eight healthy individuals is shown in Table 2. There were no

Table 2
Biochemical index information.

Variable median (interquartile)	Normal	CHD_0	CHD_8
	($n = 8$)	($n = 8$)	($n = 8$)
Coagulation function			
PT, s	13.35 (2.45)	11.90 (2.45)	12.10 (1.93)
TT, s	17.75 (1.80)	16.55 (2.67)	16.35 (2.38)
APTT, s	32.90 (10.26)	36.48 (11.77)	37.93 (13.82)
INR	1.09 (0.09)	0.90 (0.12)*	0.93 (0.11)
FIB, g/L	2.80 (0.30)	2.97 (1.43)	2.98 (1.19)
Lipid			
TC, mmol/L	4.63 (2.06)	4.88 (1.52)	5.02 (2.07)
TG, mmol/L	1.15 (1.16)	1.86 (0.70)	2.37 (1.06)
HDL, mmol/L	3.01 (1.64)	3.26 (1.43)	2.84 (1.72)
LDL, mmol/L	1.18 (0.66)	1.12 (0.47)	0.97 (0.46)
ApoA1, g/L	1.36 (0.35)	1.42 (0.36)	1.34 (0.44)
ApoB, g/L	0.86 (0.46)	1.15 (0.52)	1.19 (0.50)
ApoA1/ApoB	1.67 (0.90)	1.20 (0.79)	1.27 (0.84)
Endothelial injury			
Human ET-1, ng/L	17.39 (34.22)	19.91 (32.07)	20.63 (50.05)
Human ANG-I, ng/L	589.78 (963.60)	799.20 (1096.81)	757.28 (1850.20)
Human ANG-II, ng/L	113.56 (196.11)	123.62 (145.92)	101.80 (114.95)
Metalloprotease			
Human MMP2, ng/mL	7.12 (11.66)	5.18 (5.88)	5.92 (5.61)
Human MMP9, μg/L	16.71 (30.11)	12.68 (5.07)	13.01 (14.89)
Adhesion molecule			
Human ICAM-1, ng/L	67.28 (100.05)	75.58 (61.02)	89.25 (496.07)
Human E-Slection, ng/mL	6.42 (10.37)	9.99 (7.19)	7.20 (7.41)
Human P-Slection, ng/L	3.21 (6.01)	5.78 (8.88)	4.65 (10.43)
Inflammatory mediator			
Human NF-κB, pg/mL	1585.11 (5154.06)	1422.48 (1442.72)	1599.28 (1716.98)
Human GM-CSF, pg/mL	24.50 (18.63)	23.50 (8.50)	20.50 (5.75)
Human sCD40L, pg/mL	10942.50 (3924.50)	11442.50 (2752.87)	9496.25 (6287.63)
Human IL-1, pg/mL	54.50 (69.13)	50.00 (29.50)	31.00 (63.25)
Human IL-6, pg/mL	53.75 (33.25)	39.50 (29.75)	41.00 (18.75)
Human MCP-1, pg/mL	5146.50 (2445.50)	6342.00 (3179.00)	5069.75 (2935.37)
Human TNF-α, pg/mL	181.50 (101.75)	174.00 (83.00)	154.50 (66.50)
Risk factor of heart disease			
Homocysteine, μmol/L	15.95 (7.25)	14.98 (3.55)	14.60 (2.57)

Data are presented as median (interquartile), compared with Normal group, $^*P < 0.05$.

statistically significant differences in biochemical indicators, although they improved to some extent, including coagulation function (PT, TT, APTT, FIB), lipids (TC, TG, LDL, HDL, ApoA1, ApoB, ApoA1/ApoB), endothelial injury (ANG-I, ANG-II), metalloprotease (MMP2), adhesion molecule (ICAM-1, E-selection, P-selection), inflammatory mediators (NF-κB, GM-CSF, sCD40L, IL-1, IL-6, MCP-1), and Hcy ($P > 0.05$).

3.2. Transcriptional responses of CHD patients

All parameters of gene expression microarray hybridization experiment meet Affymetrix quality control standard (Supplementary Figs. 1–3). The Normal, CHD_0, and CHD_8 groups were analyzed to reveal the global transcriptional response (Fig. 1A,C). Compared to the Normal group, 1896 genes were found in the CHD_0 group, of which 883 and 1013 genes were upregulated and downregulated, respectively, as shown in the volcano map (Fig. 1B). Compared with the CHD_0 group, 125 DEGs were identified in the CHD_8 group, of which 15 were upregulated and 110 were downregulated as shown in the volcano map (Fig. 1D). These results suggest that CHD patients had a distinct gene expression pattern (GEO accession GSE179789).

3.3. Analysis of repeated DEGs

There were 46 common genes before and after DLT treatment (Fig. 2A). Compared with the CHD_0 group, 7 genes were upregulated

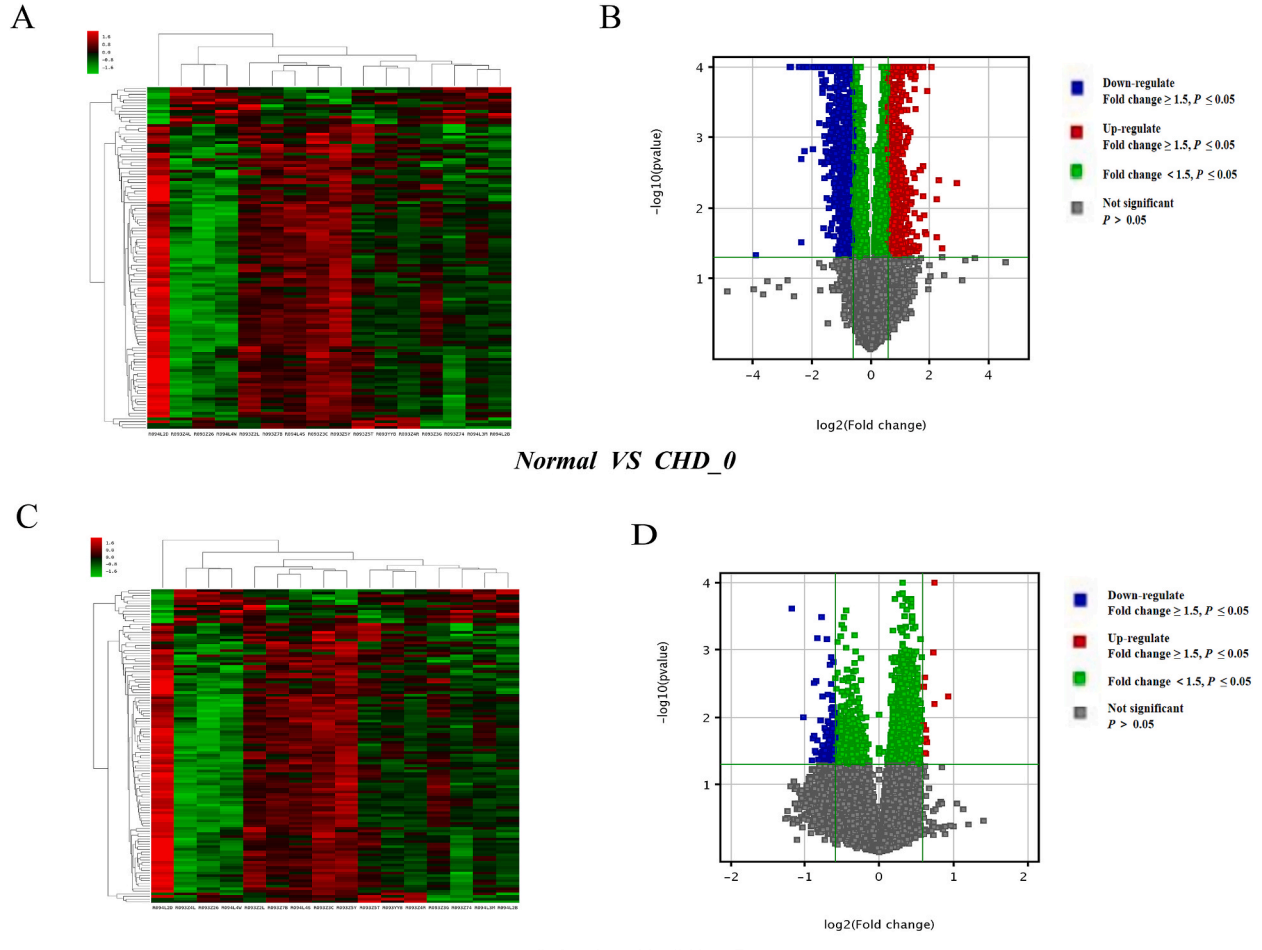

Fig. 1. Microarray gene expression analysis. A: Hierarchical cluster analysis of Normal *vs* CHD_0; B: Volcano plot of Normal *vs* CHD_0; C: Hierarchical cluster analysis of CHD_0 *vs* CHD_8; D: Volcano plot of CHD_0 *vs* CHD_8. (A and C) Chromatic order indicates the change in gene expression from relatively low (green) to relatively high (red), the horizontal abscissa names represent the sample number. (B and D) Volcano plot: The X-axis is the logarithm of the difference multiple with base 2, while the Y-axis is the *P*-value with base 10 as the negative logarithm. Red indicates upregulated genes, and green indicates downregulated genes. Gray: genes with $P > 0.05$; Green: genes with $FC < 1.5$ and $P \leq 0.05$; Red: upregulated genes with $FC \geq 1.5$ and $P \leq 0.05$; Blue: downregulated genes with $FC \leq 1.5$ and $P \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and 39 genes were downregulated, as shown in Supplementary Table 4. The study subjected 46 DEGs to IPA core analysis to identify the key targets for DLT to treat CHD. The GO analysis showed that the top 10 enriched targets were carbohydrate metabolism, molecular transport, nucleic acid metabolism, small-molecule biochemistry, protein synthesis, cellular movement, hematological system development and function, immune cell trafficking, and inflammation (Fig. 2B). Analysis of differential gene pathway results before and after DLT treatment for CHD patients showed a total of 24 differential pathways, with the top 10 being mitochondrial dysfunction, sirtuin signaling pathway, diphthamide biosynthesis, NADH repair, hypusine biosynthesis, NF-κB signaling, estrogen-dependent breast cancer signaling, ErbB signaling, oxidative phosphorylation and role of MAPK signaling in promoting the pathogenesis of influenza (Fig. 2C). An IPA analysis of the upstream regulators showed that epidermal growth factor receptor (EGFR) may be the key upstream regulatory regulator (Fig. 2D). Combined with the results of previous clinical research and metabolomics research, EGFR is the key protein of DLT intervention in CHD [3]. In order to further study, the meaningful indexes of immunity, inflammation and apoptosis obtained from clinical research of CHD syndrome were selected for verification. A protein–protein interaction network analysis showed that the interaction of five target genes, TPM1, MDM2, STAT5B, ARNT and MGEA5 (Fig. 2E).

3.4. Molecular docking verification

Previous studies have found that the eight chemical components of DLT (Gallic acid, puerarin, daidzin, salvianic acid, salvianolic acid B, cryptotanshinone, paeoniflorin, and tanshinone IIA) have clear efficacy on CHD [2]. The affinities of the eight major active components of DLT for the five key targets were analyzed by molecular docking. The results showed that cryptotanshinone and tanshinone IIA could fully bind to the five targets (TPM1, MDM2, STAT5B, ARNT, and MGEA5) with an affinity of less than −5 kcal/mol (Table 3). A molecular docking interaction pattern map showed TPM1 (Fig. 3A and B), MDM2 (Fig. 3C and D), STAT5B (Fig. 3E and F), ARNT (Fig. 3G and H), and MGEA5 (Fig. 3I and J) could spontaneously bind to the cryptotanshinone and tanshinone IIA to form a relatively stable conformation, indicating that the key active components of DLT have good binding activity with the key active targets of CHD treatment.

3.5. Molecular biology verification with RT-PCR and western blotting

Differentially expressed target genes identified from microarray analysis were validated using RT-PCR and western blotting in a new set of experiments. Based on previous studies, we selected key upstream regulators and gene targets to further study the indicators related to inflammation and apoptosis in CHD rats, including TPM1 (Fig. 4A, Fig. 5A), MDM2 (Fig. 4B and 5B), STAT5B (Fig. 4C and 5C), ARNT (Fig. 4D and 5D), and MGEA5 (Fig. 4E and 5E).

Studies have shown that, compared with the sham group, the levels of MGEA5 mRNA and MDM2 mRNA were significantly reduced in the Model group ($P < 0.05$ or $P < 0.01$). The levels of TPM1 mRNA, STAT5B mRNA, and ARNT mRNA were significantly increased ($P < 0.05$ or $P < 0.01$), and there were varying degrees of recovery after DLT administration ($P < 0.05$ or $P < 0.01$). Compared with the Model group, TPM1 mRNA, STAT5B mRNA, and ARNT mRNA expression levels decreased significantly after DLT administration, MDM2 mRNA was significantly increased after treatment with low-dose DLT, and MGEA5 mRNA was significantly increased after treatment with high DLT doses ($P < 0.05$ or $P < 0.01$) (Fig. 4A–E).

Studies have shown that, compared with the sham group, the levels of MGEA5 and MDM2 were significantly reduced in the Model

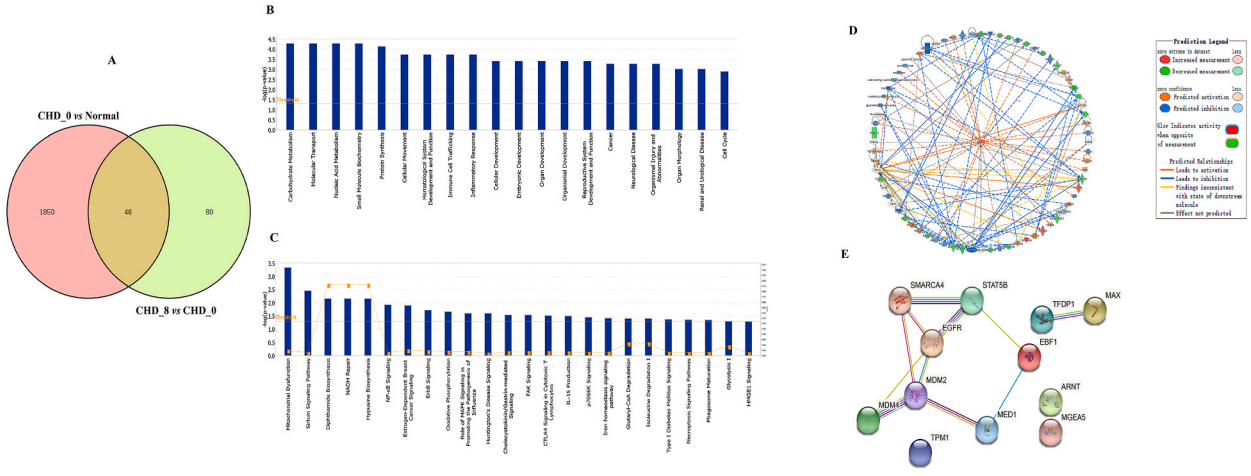

Fig. 2. Analysis of repeated differential genes. A: Venn diagram of 46 differential repetitive genes. The pink circle represents the targets of Normal *vs* CHD_0, the green are CHD_0 *vs* CHD_8, and the overlapping part is the 46 common targets; B: GO enrichment analysis of gene targets. The top 20 enrichment terms were identified for each functional category; C: KEGG pathway enrichment analysis of gene targets; D: Expression of upstream regulators of the 46 common targets; E: Protein–protein interaction network analysis of the key upstream regulators. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Information on molecular docking.

Protein	PDB ID	Docking compound	Affinity (kcal/mol)
TPM1	3MUD	Cryptotanshinone	−6.48
		Tanshinone IIA	−5.61
MDM2	6SQR	Cryptotanshinone	−8.26
		Tanshinone IIA	−8.19
STAT5B	6MBZ	Cryptotanshinone	−7.38
		Tanshinone IIA	−7.00
ARNT	2K7S	Cryptotanshinone	−5.27
		Tanshinone IIA	−5.36
MGEA5	6HKI	Cryptotanshinone	−6.51
		Tanshinone IIA	−5.96

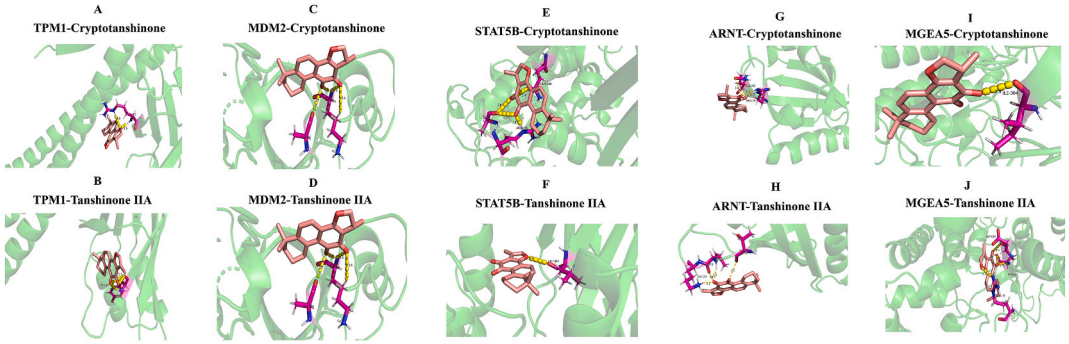

Fig. 3. The detailed simulations of the molecular docking. Cryptotanshinone and tanshinone IIA docked with TPM1, MDM2, STAT5B, ARNT, and MGEA5.

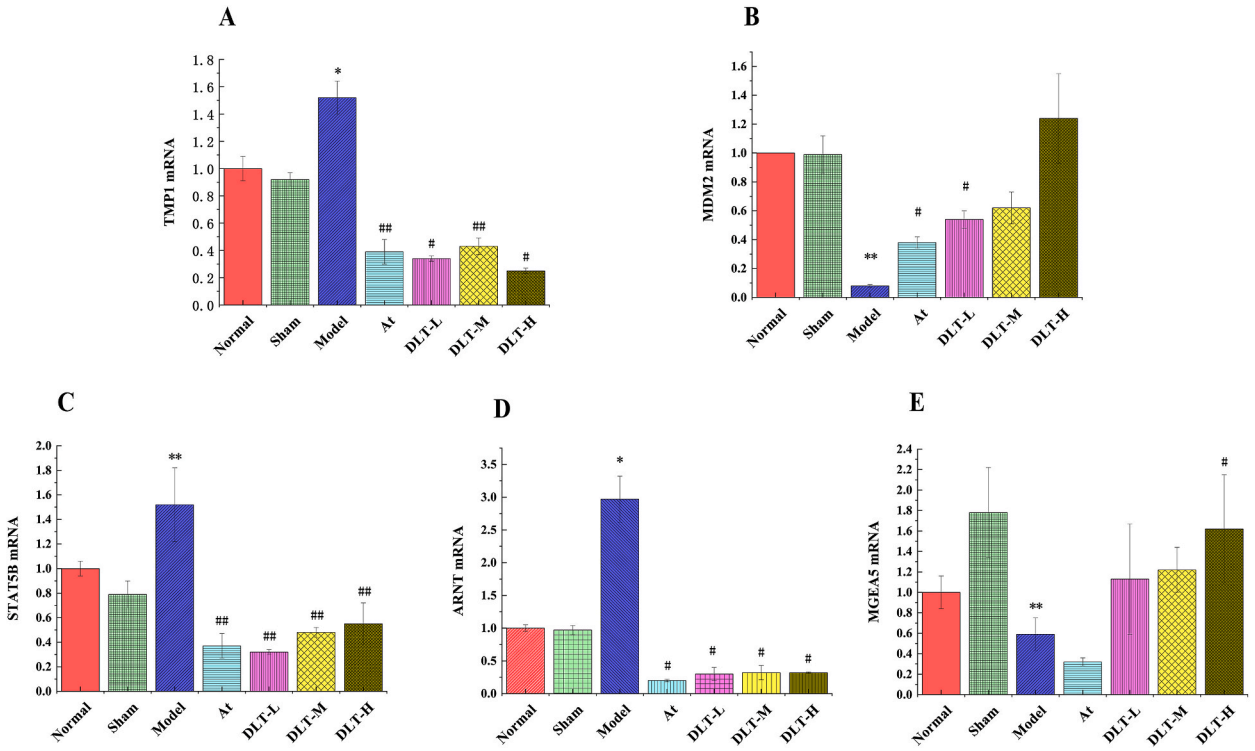

Fig. 4. Changes in real-time polymerase chain reaction expression in rat heart tissue at the end of week 12. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs.* Sham group; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ *vs.* Model group.

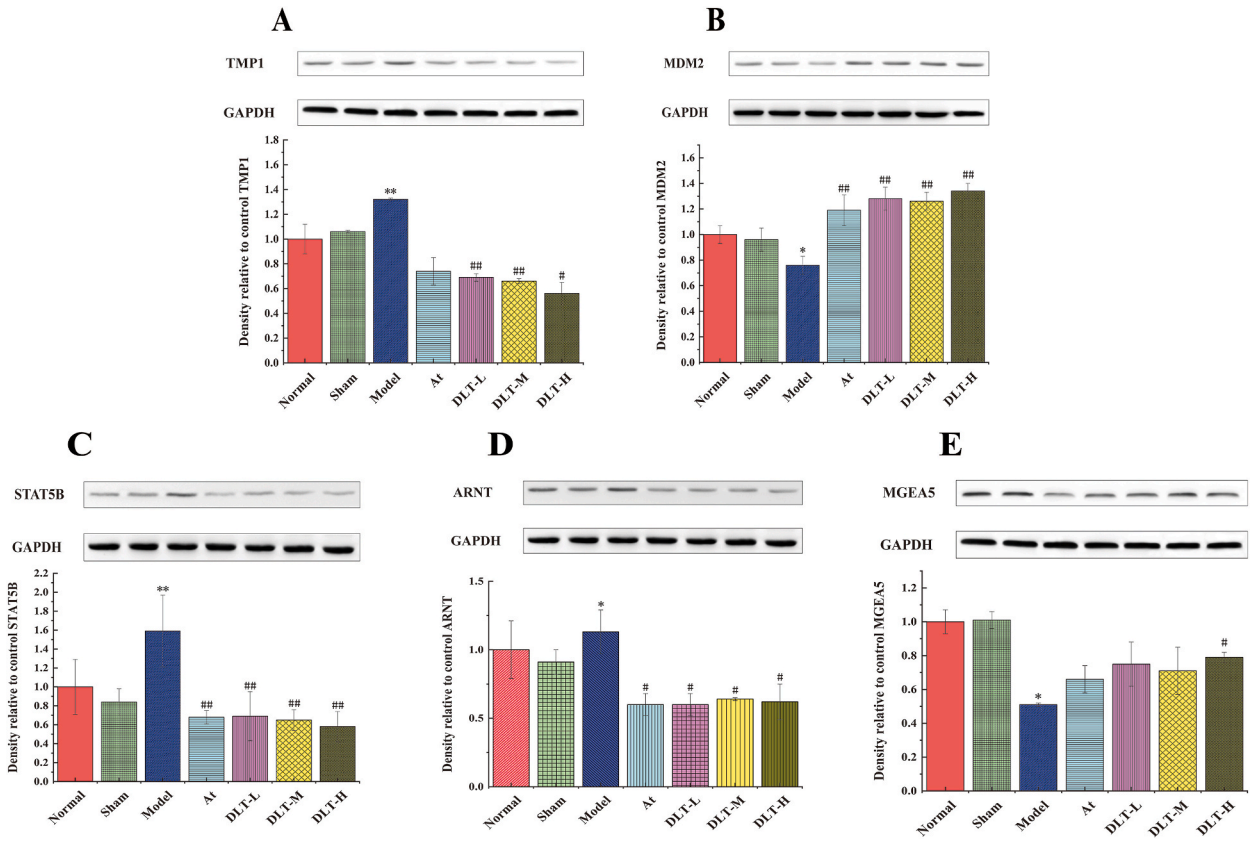

Fig. 5. Changes in western blotting expression in rat heart tissue at the end of week 12. $^{**}P < 0.01$, $^{*}P < 0.05$, *vs.* Sham group; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$, *vs.* Model group.

group ($P < 0.05$ or $P < 0.01$). The levels of TPM1, STAT5B, and ARNT were significantly increased ($P < 0.05$ or $P < 0.01$), and there were varying degrees of recovery after DLT administration ($P < 0.05$ or $P < 0.01$). Compared with the Model group, TPM1, STAT5B, and ARNT expression levels decreased significantly after DLT administration, MDM2 was significantly increased after treatment with low-dose DLT, and MGEA5 was significantly increased after treatment with high DLT doses ($P < 0.05$ or $P < 0.01$) (Fig. 5A–E).

4. Discussion

Previous studies demonstrated that DLT treatment can reduce the levels of inflammatory factors in patients with CHD and improve vascular endothelial injury. This was a small multi-center clinical study, and patients have strong individual differences in clinical practice. These results indicate that DLT plays a key role in regulating matrix protease activity, adhesion factors, and inflammatory responses. The results of many recent studies are consistent with these [13–16].

In this study, IPA core analysis of DEGs was carried out. The results of Go enrichment and KEGG analysis showed that DLT treatment of CHD, mainly involves cell movement, immunity and inflammation, and related pathways include mitochondrial dysfunction, NADH repair, NF-κB signaling and MAPK signaling. Atherosclerosis (AS) is the basis of CHD, characterized by increased apoptosis of vascular smooth muscle cells (VSMCs) and vascular wall remodeling. Studies have confirmed that AS is a chronic inflammatory and autoimmune disease, and its occurrence process includes oxidation of low density lipoprotein (LDL), inflammation, cell adhesion, vascular wall fibrosis and calcification [17]. It is involved in lipid metabolism processes such AS mitochondrial fatty acid oxidation and steroid synthesis, so mitochondrial dysfunction plays an important role in the pathogenesis of AS. When mitochondrial dysfunction occurs, the decomposition of triacylglycerol is accelerated, and the free fatty acids and glycerol in serum are increased, which promotes the formation of AS. A large number of ROS produced by damaged mitochondria cause oxidative damage to cellular proteins, lipids and mitochondrial DNA. Mitochondrial DNA is in a state of continuous replication and weak repair ability, so it is vulnerable to oxidative damage. The damage of mtDNA will further stimulate the production of ROS, forming a vicious circle and aggravating the damage of AS blood vessels [18,19]. Therefore, mitochondrial dysfunction is closely related to myocardial ischemic injury, and it is an important mitochondrial mechanism of myocardial cell death and tissue injury in the process of myocardial ischemic injury. Studies have shown that the imbalance of NAD oxidation and reduction leads to the hyperacetylation of mitochondrial proteins, which leads to pathological changes in the heart. Therefore, maintaining the redox balance of NAD can treat cardiovascular and cerebrovascular diseases such as heart failure, while NADH can effectively increase the mitochondrial ATP (energy) of human

body, which can be converted into NAD, maintain the redox balance of NAD, remove excess free radicals, and achieve the effect of preventing and relieving cardiovascular and cerebrovascular diseases [20]. Inflammation runs through all stages of AS, and p38 MAPK signaling pathway can activate NF-κB signaling pathway, causing local inflammation to develop into systemic inflammation. Previous studies have shown that DLT's intervention in the anti-inflammatory activity of ox-LDL-induced RAW 264.7 macrophages may be related to MyD88/p38 MAPK/NF-κB signaling pathway [21]. Previous studies have shown that inhibition of MAPK pathway can reverse cryptotanshinone-mediated effect on cardiomyocyte apoptosis [22]. Another study showed that ethanol extract of DLT played a key anti-inflammatory role by inhibiting NF-κB signaling pathway, triggering PPARα/ABCA1 signaling pathway, and inhibiting lipid deposition in AS macrophages [23]. To sum up, this study reveals the mechanism of DLT acting on CHD through multiple target genes and pathways, and reflects the curative effect of multiple targets and pathways of traditional Chinese medicine (TCM) in the treatment of diseases by TCM. Combined with the previous research of our research group and the results of Go enrichment and KEGG analysis in this study, it shows that DLT is closely related to immunity, inflammation and apoptosis in the treatment of CHD.

In this study, expression microarray technology and network pharmacology were used to predict the therapeutic mechanisms of DLT in CHD. Treatment may involve the regulation of EGFR, TPM1, MDM2, STAT5B, ARNT and MGEA5. In the early stage, the research group verified the EGFR protein expression by western blotting. The results showed that the expression level of EGFR in the Model group was significantly higher than that in the Sham group ($P < 0.01$), and after DLT administration, different degrees of recovery were observed ($P < 0.01$) [3]. EGFR is widely distributed in normal tissues, including the heart and blood vessels, and exists as a receptor-type protein tyrosine kinase. Studies have indicated that activated EGFR plays an important regulatory role in cell signal transmission, cell proliferation and differentiation, tumor angiogenesis, and tumor metastasis [24–26]. Excessive stimulation of EGFR can lead to cell growth disorders and tissue remodeling [27]. Here we found that DLT decreased the expression level of EGFR in the myocardium of CHD rats, which may reduce cellular proliferation and differentiation. STAT5B, a member of the STATs protein family, is mainly involved in immune–inflammatory reactions and regulates cell proliferation, differentiation, and apoptosis [28]. Studies have shown that active STAT5B can activate the differentiation and generation of adipocytes through the mediation of peroxisome proliferator-activated receptor γ [29,30], thereby slowing the blood flow rate and accelerating plaque formation. The binding ability of STAT5B to interleukin (IL)-2β receptor DNA is weakened, which decreases its protein expression, thus affecting the normal cellular immunoregulatory function [31]. Studies have shown that cryptotanshinone strongly inhibits STAT3 transcription. DLT can reduce STAT5B level in the myocardium of CHD rats and may reduce adipogenesis and inflammatory reactions. MDM2, an oncogene with transcriptional regulation function, is an antagonist gene of p53 that interacts with p53 to form a feedback regulation loop and regulate cell growth. The increase in apoptosis is attributed to a decrease in MDM2 and increase in p53 signal transduction [32]. Disruption of this regulatory mechanism can lead to excessive cell growth [33]. Studies demonstrated that MDM2 inhibition can reduce cardiac dysfunction and fibrosis following myocardial infarction. Therefore, this is an inflammatory reaction [34]. Therefore, DLT may regulate the apoptosis of myocardial cells by reducing MDM2 expression in CHD rats.

TPM1 encodes α-tropomyosin, an important component of sarcomere thin filaments, and its role is mainly to enhance the rigidity of thin filaments and influence the role of troponin I [35]. Previous studies reported that 1–5% of cases of hypertrophic cardiomyopathy is caused by this gene mutation, which is characterized by disordered arrangement of muscle cells and muscle fibers, increased myocardial weight, and variation of various myocardial fibrin [36,37]. ARNT is a nuclear transporter of the polycyclic aromatic hydrocarbon receptor (AhR) and an important part of the AhR-related signaling pathway. Many genes are regulated by AhR/ARNT [38]. AhR is involved in cell differentiation, tissue remodeling, and immune regulation and may be involved in atrial fibrosis by mediating ARNT [39,40]. O-linked *N*-acetylglucosamine hydrolase (OGA) encoded by MGEA5 can reverse the glycosylation process of various proteins in *vivo* [41]. OGA catalyzes the clearance of *O*-acetylglucosamine (*O*-GIcNac) and reverses this modification, which is encoded by the MGEA5 gene. Islet β-cells, the only cells rich in OGA in *vivo,* are very sensitive to metabolic changes in *O*-GIcNac. Glycosylated proteins are deposited in islet β-cells, leading to their death, which may lead to diabetes [42]. Diabetes can cause obesity, increase lipid content, slow blood flow, and easily induce cardiovascular diseases. Reducing the incidence of diabetes is another way to protect the cardiovascular system. Therefore, DLT may reduce lipid deposition by increasing MGEA5 expression in CHD rats.

5. Conclusions

In this study, DLT improved the levels of biochemical indicators, including coagulation function, lipids, endothelial injury, metalloproteases, adhesion molecules, inflammatory mediators, and Hcy. DLT can reduce the degree of vascular endothelial damage in CHD rats by reducing the expression of STAT5B, TPM1, and MDM2 and increasing the expression of ARNT and MGEA5.

Author contribution statement

Yu Chunquan, Gao Shan, Li Lin: Conceived and designed the experiments.
Li Zhu: Wrote the paper; Performed the experiments; Analyzed and interpreted the data; Conceived and designed the experiments.
Cheng Qi, He Yuanyuan: Performed the experiments; Analyzed and interpreted the data.
Wang Shuo, Xie Jing, Zheng Yanchao, Liu Yijia:Contributed reagents, materials, analysis tools or data.

Additional information

Supplementary content related to this article has been published online at [URL].

Data availability statement

Data associated with this study has been deposited at GEO under the accession number GSE179789.

Ethics approval and consent to participate

All procedures described in this study were approved by the Ethics Committee of Tianjin University of Traditional Chinese Medicine. And this study was approved by the ethics committee of the Tianjin University of Traditional Chinese Medicine (approval number:TJUTCM-EC20150001). Which registered with the US Clinical Trial Registration Center (registration number NCT02526381) and the China Clinical Trial Registration Center (registration number ChiCTR-OOC-15006765).

Competing interests

All authors declare that they have no competing interests for this study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Basic Research Program of China (973 project, grant numbers: 2014CB542902) and National Natural Science Foundation of China Project (grant numbers: 82074140).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15777.

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