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Cyclin-dependent kinase 5 as a potential therapeutic target to alleviate high glucose-induced podocyte apoptosis and hyperglycemia-induced renal injury in mice

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Summary. Background. Hyperglycemia is a risk factor for impaired renal function, including cellular metabolic disturbance, apoptosis, inflammation, and histologic lesion. This study aims to investigate the potential therapeutic targeting of cyclin-dependent kinase 5 (Cdk5) in hyperglycemia-induced podocyte dysfunction and renal damage.

Methods. Cell viability and apoptosis of podocytes were assessed through CCK-8 and TUNEL staining, respectively, following exposure to normal glucose (NG; 5 mM), high glucose (HG; 30 mM), or treatment with Cdk5 inhibitors (trans-resveratrol, myricetin, salvianolic acid A, and BML-259). Diabetic mice were established by intraperitoneal injection of freshly streptozotocin (STZ), which was given at a dose of 35 mg/kg in five successive injections. Additionally, histochemical staining was employed to evaluate the morphologic lesion of the kidney.

Results. Cdk5 was found to be activated by HG stimulation both *in vitro* and *in vivo*. Notably, the inhibition of Cdk5 effectively mitigated the podocyte dysfunction induced by HG, including growth inhibition, membrane damage, and apoptosis. The compounds Trans-resveratrol, myricetin, salvianolic acid A, and BML-259 exhibited low binding energy values of -8.032 kcal/mol, -8.693 kcal/mol, -8.743 kcal/mol, and -10.952 kcal/mol, respectively, indicating strong and stable binding affinity between these candidates and Cdk5. The results of *in vivo* experimental analysis demonstrate that Cdk5 inhibitors, namely trans-resveratrol, myricetin, salvianolic acid A, and BML-259, confer protection against tubular and glomerular lesions induced by hyperglycemia.

Conclusion. Both myricetin and BML-259 exhibit comparable protective effects on renal injury by inhibiting Cdk5.

Key words: Cyclin-dependent kinase 5, Diabetic nephropathy, Podocyte, Apoptosis, Therapeutic regimen

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Introduction

Hyperglycemia is the principal etiological factor that expedites the advancement of diabetic nephropathy (DN) by eliciting a low-grade inflammatory response and inducing cell apoptosis in renal cells (Volpe et al., 2018; Cheng et al., 2021; DeFronzo et al., 2021; Petrazzuolo et al., 2023). Molecular and genetic investigations have provided evidence highlighting the pivotal involvement of podocytes and endothelial cells in the pathogenesis of albuminuria and the onset of early kidney disease in individuals with diabetes (Mohandes et al., 2023). A growing body of evidence indicates that dysfunction of the glomerular endothelial cell (GEC) manifests early in DN, potentially preceding podocyte injury (Zambrano et al., 2022). This dysfunction is strongly associated with the decline in renal function and glomerular filtration rate in DN induced by hyperglycemia (Menon et al., 2022). Hence, the potential therapeutic approaches for mitigating diabetic kidney diseases may involve the prevention of apoptosis in podocyte and tubule epithelial cells.

Cyclin-dependent kinase 5 (Cdk5), a proline-directed serine/threonine kinase and member of the CDK family (Batra et al., 2023), serves as a multifunctional kinase primarily involved in neuronal regulation (Batra et al., 2023). Recent studies have revealed the presence of Cdk5 activity in podocytes, where it plays a

significant role in the development of podocyte injury (Brinkkoetter et al., 2010; Wang et al., 2021). Within the kidney, Cdk5 is predominantly present in podocytes during the capillary loop stage through the mature stage, as well as in the podocytes of adult glomeruli (Griffin et al., 2004). It is crucial in facilitating the proliferation, differentiation, and preservation of the typical morphology of podocytes (Griffin et al., 2004; Zhang et al., 2017). Previous research has demonstrated that under pathological circumstances, elevated glucose levels and TGF-β1 can trigger podocyte apoptosis by upregulating the expression of Cdk5 (Brinkkoetter et al., 2010). The aberrantly activated Cdk5 can further aggravate podocyte damage by stimulating oxidative stress reactions and inducing dysfunction in mitochondria (Wang et al., 2021; Yu et al., 2022). However, the precise mechanism underlying the role of Cdk5 in podocyte injury and DN remains unclear, and there is a paucity of literature on the pharmacological impact of targeting Cdk5 in the context of DN.

Based on the analysis of the GEO database (GSE20636 dataset), it was determined that Cdk5 serves as a major regulator in the modulation of differentially expressed genes in mouse models of diabetes. Through the utilization of the HERB database (http://herb.ac.cn/) (Fang et al., 2021), three natural products (trans-resveratrol, myricetin, and salvianolic acid A) were identified, along with a small molecule compound (BML-259), all of which exhibit potential as drugs for targeting Cdk5. Consequently, a comprehensive investigation was conducted to assess whether these four compounds mediate Cdk5 to regulate HG-induced podocyte apoptosis and hyperglycemia-induced renal injury in mice.

Materials and methods

Differentially expressed gene (DEG) screening

The Gene Expression Omnibus (GEO) database with the GSE20636 dataset was utilized to screen DEGs based on |Log2(fold change)|>0.58 and p.adj<0.05. R software (version 4.2.1) with the GEOquery package (version 2.64.2), limma package (version 3.52.2), ggplot2 package (version 3.3.6), and ComplexHeatmap package (version 2.13.1) were performed to analyze the GEO dataset, as described previously (Gu et al., 2016). Gene Ontology (GO), (including BP, biological process; CC, cellular component; MF, molecular function) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis were predicted by the DAVID online database with DN-related genes (https://david.ncifcrf.gov/). To explore the potential upstream regulators responsible for the observed gene expression patterns, we utilized the X2K Appyter (Expression2Kinases) database (https://appyters.maayanlab.cloud/#/X2K_Appyter) to analyze the kinases associated with DN-related genes, as described previously (Clarke et al., 2018). Through the utilization of the HERB database (http://herb.ac.cn/) (Fang et al., 2021), three natural products (trans-resveratrol, myricetin, and salvianolic acid A) were identified as potential Cdk5 inhibitors.

RT-qPCR

The RT-qPCR procedures were conducted according to previously described standard protocols (Zhang et al., 2010). The PCR primers are listed as follows: *Mus musculus* cyclin-dependent kinase 7 (Cdk7): forward primer 5'-GACACCATCCCACATTAAAGCC-3' and reverse primer 5'-CACCATACATCCTAGCTCCAAAC-3'; *Mus musculus* mitogen-activated protein kinase 1 (Mapk1): forward primer 5'-GGTTGTTCCCAAATGCTGACT-3' and reverse primer 5'-CAACTTCAATCCTCTTGTGAGGG-3'; *Mus musculus* thymoma viral proto-oncogene 1 (Akt1): forward primer 5'-ATGAACGACGTAGCCATTGTG-3' and reverse primer 5'-TTGTAGCCAATAAAGGTGCCAT-3'; *Mus musculus* Rous sarcoma oncogene (Src): forward primer 5'-GAACCCGAGAGGGACCTTC-3' and reverse primer 5'-GAGGCAGTAGGCACCTTTTGT-3'; *Mus musculus* p21 protein (Cdc42/Rac)-activated kinase 6 (Pak6): forward primer 5'-CCCCACAATGGCAGAACATTC-3' and reverse primer 5'-ACCCGTGTGATTCGAGAAGGA-3'; *Mus musculus* Fyn proto-oncogene (Fyn): forward primer 5'-ACCTCCATCCCGAACTACAAC-3' and reverse primer 5'-CATAAAGCGCCACAAACAGTG-3'; *Mus musculus* lymphocyte protein tyrosine kinase (Lck): forward primer 5'-ATTGACGTGTGTGAAAACTGCC-3' and reverse primer 5'-ATCCCTCATAGGTGACCAGTG-3'; *Mus musculus* Cdk5: forward primer 5'-GGGAAGGCACCTATGGAACTG-3' and reverse primer 5'-CAGCCTGACACGCTTCAGAG-3'; *Mus musculus* c-abl oncogene 1, non-receptor tyrosine kinase (Abl1): forward primer 5'-AATGACCCCAACCTTTTTGTGG-3' and reverse primer 5'-ATACAGGGCCATGATACCAGG-3'; M*Mus musculus* male germ cell-associated kinase (Mak): forward primer 5'-ATGAACCGATACACAACCATGAA-3' and reverse primer 5'-CTCCCCAGACTCATTGCTCT-3'.

Molecular docking

To analyze the binding affinities and modes of interaction between trans-resveratrol, myricetin, salvianolic acid A, and BML-259 and Cdk5, AutodockVina 1.2.2, a silico protein-ligand docking software was employed, as described previously (Morris et al., 2008; Wang et al., 2017). Molecular docking studies were performed with Autodock Vina 1.2.2 (http://autodock.scripps.edu/).

Cell culture

The mouse podocyte cell line (MPC5, 1×10^4 cells/well) was seeded in 96-well plates and cultured as

described previously (Dan Hu et al., 2023). The conditionally immortalized MPC5 was kindly provided by Prof. San Tao Ou (Department of Nephrology of Southwest Medical University). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 10 IU mL^{-1} recombinant IFN-γ at 33°C. To induce differentiation, MPC5 cells were cultured at 37°C for 14 days. Trans-resveratrol, myricetin, salvianolic acid A, and BML-259 (0~80 μM) were dissolved in 10% dimethyl sulfoxide to stimulate normal or HG (30 mM)-treated podocytes for 48h to perform *in vitro* measurements.

Cell viability

After podocytes with or without HG incubation were treated with trans-resveratrol (MedChemExpress), myricetin (Sigma-Aldrich), salvianolic acid A (Sigma-Aldrich), and BML-259 (MedChemExpress) for 48h, cell viability was evaluated using CCK8 kits (Dojindo, Japan), according to the manufacturer's instructions. Absorbance was measured at 450 nm with a SpectraMax M5 ELISA plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Measurement of malonaldehyde (MDA) and cell apoptosis

MDA (Cat. no: A003-1-2) in the supernatant liquid and cells was detected using the commercial kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The cell apoptosis detection TUNEL kit (Roche) was used to analyze apoptotic cells with TUNEL-positive staining (red) with fluorescence microscopy, according to the manufacturer's instructions. Blood urea nitrogen (BUN), serum creatinine (Cr), and urinary total protein (BIOSINO, Beijing, China) were measured according to the manufacturer's instructions. In brief, BUN was measured by a SpectraMax M5 ELISA plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at 630 nm with an indophenol blue colorimetry method. Cr is converted to creatine by creatinine hydrolase, which is then further metabolized by creatine amidinohydrolase to produce sarcosine and urea. Sarcosine is subsequently converted to glycine, formaldehyde, and hydrogen peroxide by sarcosine oxidase. The hydrogen peroxide generated reacts with 2,4-(6-triiodo3-hydroxybenzoic acid) and 4-amino-antipyrine in the presence of peroxidase, resulting in the formation of a purplish red compound. This compound can be quantified using colorimetry at a wavelength of 546 nm. Urinary total protein was measured with the Coomassie Brilliant Blue method and quantified using colorimetry at a wavelength of 595 nm.

Animal model

C57BL/6J mice (6-8 weeks old; 20±2 g; n=36) were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China. After one week of adaptive feeding, mice were made diabetic by intraperitoneal injection of freshly prepared streptozotocin (STZ: dissolved in 10 mM citrate buffer, pH 4.2), which was given at a dose of 35 mg/kg in five successive injections. One week after the final injection, blood samples were collected from the tip of the tail vein and analyzed for random blood glucose levels using a glucose meter. The model was deemed successful if the blood glucose level was consistently ≥16.7 mmol/L for three consecutive days. Animal feeding, anesthesia, sacrifice, and specimen collection were performed according to ARRIVE guidelines 2.0 (Percie du Sert and Ahluwalia, 2020). Diabetic mice were treated with trans-resveratrol, myricetin, salvianolic acid A, and BML-259 for eight weeks (10 mg/kg/day) by intragastric administration. The animal experiment was approved by the Ethics Committee of the Zhejiang Provincial People's Hospital (Affiliated People's Hospital), Hangzhou Medical College (Hangzhou, China). Mice were divided into six groups (n=6 in each group), including Con, STZ, STZ+TR, STZ+MYR, STZ+SAA, and STZ+BML-259. Whole blood was obtained via ocular puncture, serum was separated and maintained at -20°C, and kidney tissue was fixed in 4% formaldehyde for histomorphological examination. Gene and protein specimens are maintained at -80°C.

Histologic examination

Hematein and eosin (H&E) staining and Masson's Trichrome staining were performed as described previously (Han et al., 2020).

Western blotting

Western blotting procedures were performed as described previously (Yu et al., 2018). The primary antibodies for Cdk5 (cat. no: sc-6247; dilution: 1: 1,000) and nephrin (cat. no: sc-376522; dilution: 1:1,000) were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated secondary antibody (cat. no: sc-516102; dilution: 1: 10,000) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-β-actin (cat. no. sc-130065; dilution: 1: 2,000; Santa Cruz Biotechnology) was used as the control antibody.

Statistical analysis

Data were expressed as mean and standard deviation. Statistical analysis was performed with GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA, USA). A Student's t-test was used to analyze differences in the top 10 kinases (Cdk7, Mapk1, Akt1, Src, Pak6, Fyn, Lck, Cdk5, Abl1, and Mak) in NG or HG-treated podocytes. Inter-group comparisons were calculated using a one-way analysis of variance. A *p*-value of less than 0.05 indicated a significant difference.

Results

Differentially expressed genes in Diabetes Mellitus (DM) mice

To investigate DEGs in the progression of DN, we conducted a differential expression analysis, comparing normal kidney samples to diabetic kidney samples. We applied a threshold of |LogFC|>0.58 and *p.adj*<0.05. In dataset GSE20636, we identified a total of 121 DEGs at two months (Fig. 1A), 77 DEGs at four months (Fig. 1B), and 679 DEGs at eight months (Fig. 1C) in the diabetic kidney compared with the normal kidney. Furthermore, we found that nine genes (*Slc9a8*, *Mdk*, *BC040756*, *Hmgcr*, *Dusp15*, *Lpl*, *Cbr2*, *Slc15a2*, and *Ephx1*) were consistently detected in all three stages of DN (Fig. 1D). The analysis conducted using GO and KEGG methodologies demonstrated that upregulation of the ERK1/2 cascade, MAPK cascade, phospholipase A1 activity, and protein tyrosine/serine/threonine phosphatase activity may potentially be linked to the progression of DN, as indicated in Figure 1E.

Prediction of DEG-related kinases

According to the aforementioned findings, it was determined that DEGs associated with DN may exhibit a strong correlation with kinase-related genes and signaling pathways. To investigate the potential regulatory networks upstream of these DEGs,

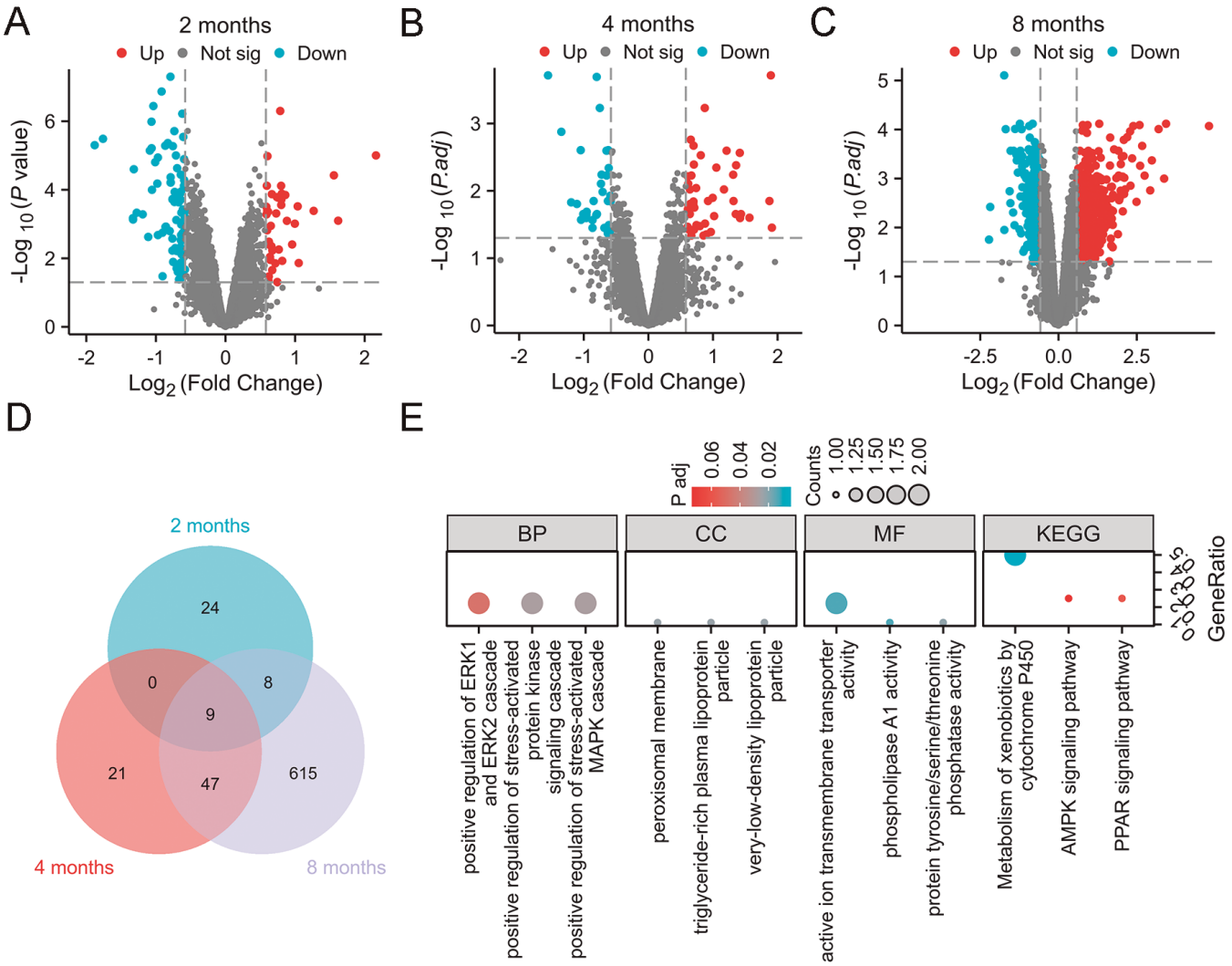

Fig. 1. DEGs in DM mice. In dataset GSE20636, a total of 121 DEGs at 2 months **(A)**, 77 DEGs at 4 months **(B)**, and 679 DEGs at 8 months **(C)** were identified in the diabetic kidney compared with the normal kidney with a threshold of |LogFC|>0.58 and *p.adj*<0.05. The Venn diagram illustrates DEGs at three distinct time points with diabetic mice **(D)**. GO and KEGG methodologies were used to enrich the functions of nine genes in the progression of diabetic mice **(E)**. Not sig, not significant.

encompassing kinases (Fig. 2A) and intermediate proteins/transcription factor interactors (Fig. 2B), we employed the X2K Appyter (Expression2Kinases) database, accessible at https://appyters.maayanlab.cloud/#/X2K_Appyter. This analysis predicted the potential association between the top 10 kinases (Cdk7, Mapk1, Akt1, Src, Pak6, Fyn, Lck, Cdk5, Abl1, and Mak) and DEGs related to DN. To verify the expression of these top 10 kinases, RT-qPCR was employed to measure the gene expression levels in podocytes. Figure 2C illustrates that seven kinases exhibited significant upregulation in podocytes treated with HG compared with the NG group. Notably, Cdk5 displayed the highest level of expression among these genes.

The anticipation of potential drugs targeting Cdk5

Through the utilization of the HERB database (http://herb.ac.cn/), three natural products (trans-resveratrol, myricetin, and salvianolic acid A) were identified, along with a small molecule compound (BML-259), all of which exhibit potential as drugs for targeting Cdk5. To assess the affinity of the candidate drugs for Cdk5, a molecular docking analysis was conducted. Autodock Vina v.1.2.2 software was utilized to obtain the binding poses and interactions of four drug candidates with Cdk5. Subsequently, the binding energy for each interaction was calculated. Among the four candidates, namely trans-resveratrol, myricetin, salvianolic acid A, and BML-259, it was observed that they exhibited low binding energy values of -8.032 kcal/mol (Fig. 3A), -8.693 kcal/mol (Fig. 3B), -8.743 kcal/mol (Fig. 3C), and -10.952 kcal/mol (Fig. 3D), respectively. This indicates a high level of stability in the binding between these candidates and Cdk5.

Cdk5 inhibitors effectively alleviate podocyte injury induced by HG

To assess the potential protective effects of trans-resveratrol, myricetin, salvianolic acid A, and BML-259 against HG-induced podocyte dysfunction, the cytotoxicity of these compounds was examined in podocytes exposed to NG (5 mM) or HG (30 mM) for 48 hours. It was observed that concentrations of trans-resveratrol (Fig. 4A), myricetin (Fig. 4B), and salvianolic acid A (Fig. 4C) below 80 μM did not significantly impact cell viability. When the concentration of BML-259 was below 40 μM, there was no apparent inhibitory effect on cell viability. However,

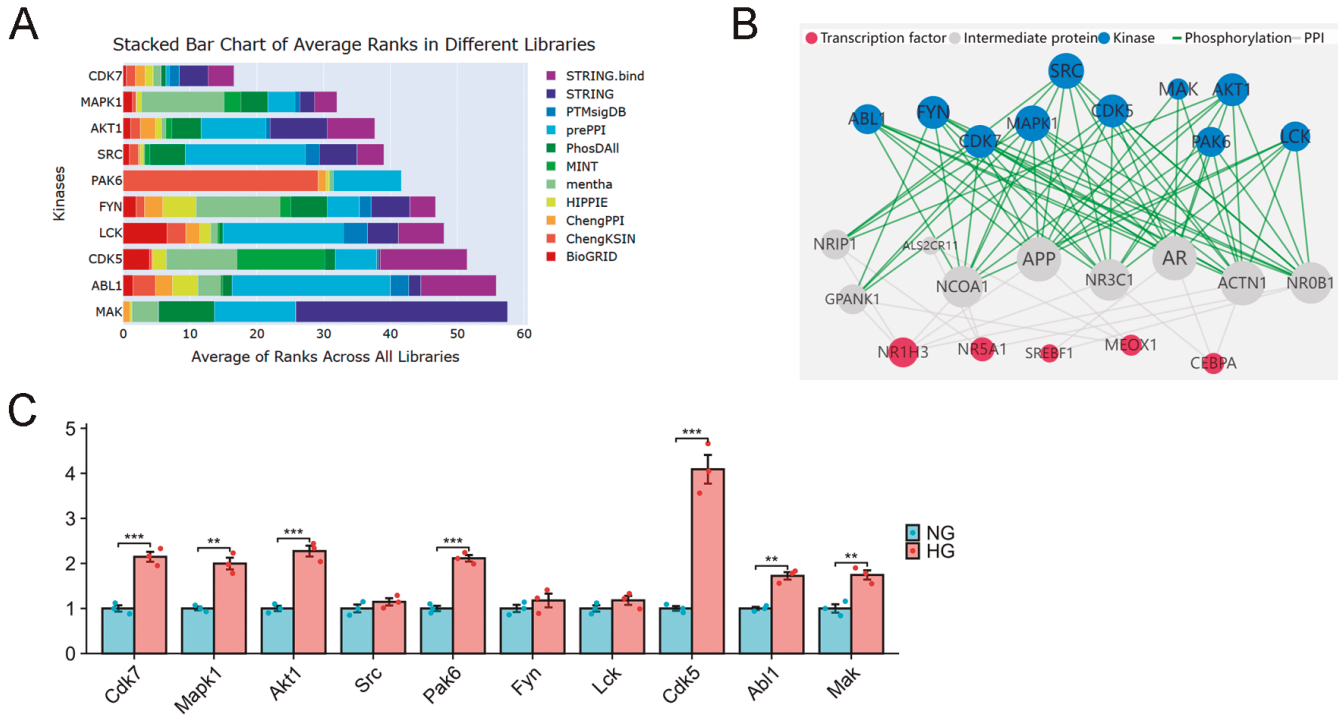

Fig. 2. Prediction of DEG-related kinases. The bar chart above is a mean rank bar chart for the top-ranked kinases. The y-axis displays the different kinases, and the x-axis displays the mean rank of a specific kinase across all the available libraries **(A)**. Red nodes represent transcription factors (tfs), white notes represent intermediate proteins/transcription factor interactors, and blue nodes represent kinases. Connecting the nodes, white lines represent a protein-protein interaction (PPI) between tfs and their interactors, and green lines represent the phosphorylation of kinases **(B)**. After podocyte exposure to HG or NG for 48h, the mRNA expression levels of the top 10 kinases were detected using RT-qPCR **(C)**. **p<0.01; ***p<0.001; n=3 in each group.

at a concentration of 80 μM, BML-259 significantly inhibited the growth of podocytes (Fig. 4D). In comparison with the NG (5 mM) group, the HG group exhibited a significant inhibition of cell viability, approximately 50%. Nevertheless, the growth inhibition of podocytes induced by HG was reversed by treatment with trans-resveratrol (Fig. 4A), myricetin (Fig. 4B), salvianolic acid A (Fig. 4C), and BML-259 (Fig. 4D). The application of HG treatment resulted in a significant increase in the levels of MDA, a recognized indicator of membrane damage, in both the supernatant (Fig. 5A) and podocyte (Fig. 5B) when compared with the NG group. Nevertheless, the administration of trans-resveratrol, myricetin, salvianolic acid A, and BML-259, which inhibit Cdk5, led to a notable decrease in MDA production, suggesting a reduction in membrane damage. Additionally, the TUNEL assay was employed to assess cellular apoptosis, with red staining indicating the presence of apoptotic cells. A higher intensity of red staining correlates with a higher percentage of apoptotic cells. Our investigation revealed that the administration of trans-resveratrol, myricetin, salvianolic acid A, and BML-259 induced a significant reduction in the proportion of apoptosis in cells exposed to HG conditions. Notably, both myricetin and BML-259 exhibited a similar protective effect against HG-induced podocyte apoptosis (Fig. 5C).

Cdk5 inhibitors effectively improve renal injury in vivo

The administration route for trans-resveratrol, myricetin, salvianolic acid A, and BML-259 in STZ-induced diabetic mice is depicted in Figure 6A. To further investigate the nephroprotective effects of trans-resveratrol, myricetin, salvianolic acid A, and BML-259, a pharmacological experiment was conducted in diabetic mice with or without their administration. Physiological parameters, including body weight and fasting blood glucose (FBG), revealed a significant decrease in body weight and an increase in FBG during the experimental period following STZ injection. However, the administration of trans-resveratrol, myricetin, salvianolic acid A, and BML-259 for eight weeks did not result in any significant changes in body weight and FBG levels in diabetic mice (Fig. 6B). Conversely, diabetic mice exhibited significantly elevated levels of BUN (Fig. 6C), serum Cr (Fig. 6D), and urinary total protein (Fig. 6E) compared with normal mice. Remarkably, the administration of trans-resveratrol, myricetin, salvianolic acid A, and BML-259 for eight weeks effectively ameliorated these renal damage parameters in diabetic mice. Notably, both myricetin and BML-259

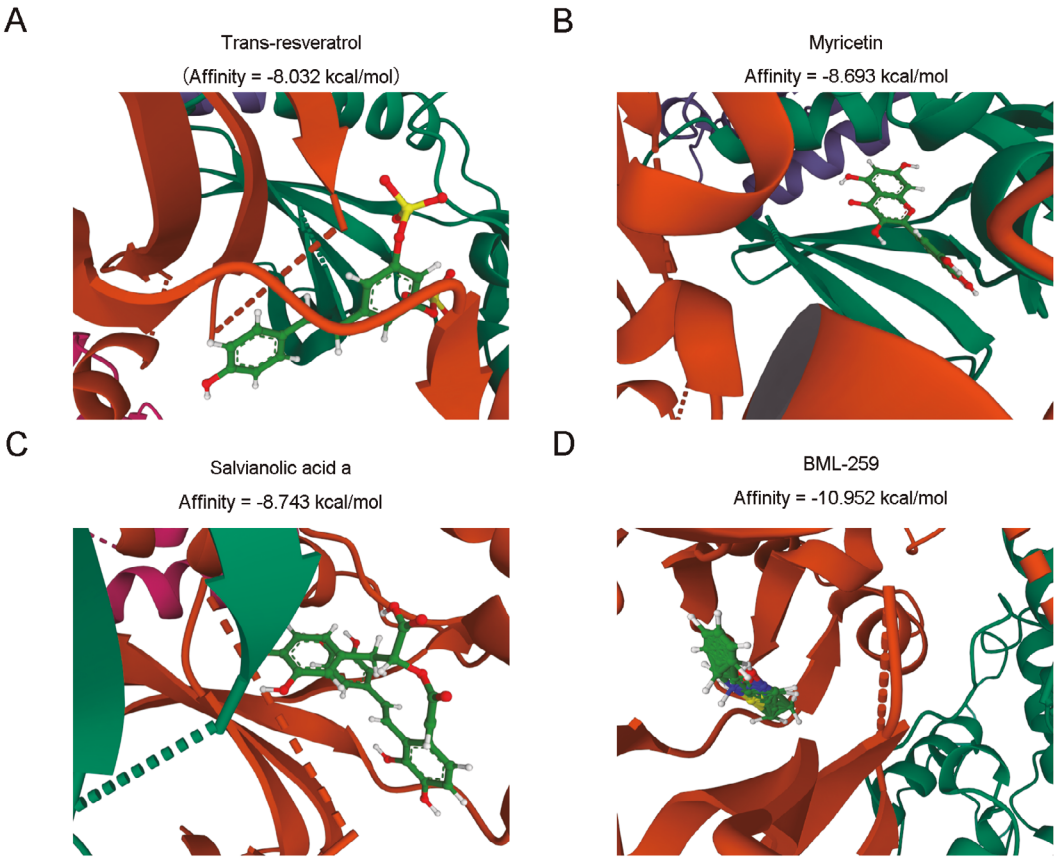

Fig. 3. Three-dimensional structures of the binding pockets are shown by PyMOL software. Binding mode of trans-resveratrol **(A)**, myricetin **(B)**, salvianolic acid A **(C)**, and BML-259 **(D)** to Cdk5 by molecular docking.

demonstrated a similar protective effect against STZ-induced renal injury. Interestingly, the utilization of H&E and Masson's trichrome staining techniques in this study unveiled a noteworthy observation. Specifically, the administration of myricetin and BML-259 to diabetic mice over eight weeks resulted in a substantial improvement in various pathological characteristics, such as tubular atrophy (Fig. 7A), glomerular pyknosis (Fig. 7A), and collagen deposition with blue staining (Fig. 7B). It is worth noting that nephrin, a crucial protein involved in renal damage repair and the filtration barrier (Li et al., 2015), exhibited a significant reduction in protein levels within the kidneys of diabetic mice when compared with those of normal mice (Fig. 8A). However, the administration of trans-resveratrol, myricetin, salvianolic acid A, and BML-259 resulted in an elevation of nephrin protein levels in diabetic mice (Fig. 8A). Additionally, the upregulation of Cdk5 protein expression induced by hyperglycemia was significantly attenuated by the treatment with trans-resveratrol, myricetin, salvianolic acid A, and BML-259 in diabetic mice (Fig. 8B). Notably, both myricetin and BML-259 exhibited similar regulatory effects on the expression of nephrin and Cdk5 proteins.

Discussion

The increasing body of evidence demonstrates a strong association between podocyte dysfunction and the development and progression of DN (Cheng and Harris, 2014; Fu et al., 2015). Hyperglycemia or HG is believed to enhance the impairment of podocytes (Cheng and Harris, 2014). In addition, the impaired activity of endothelial nitric oxide synthase and decreased availability of nitric oxide lead to the activation of oxidative stress, which further exacerbates the damage to podocytes (Cheng and Harris, 2014). Podocyte loss leads to a reduced glomerular filtration rate and albuminuria, which evoke glomerulosclerosis and tubulointerstitial fibrosis in diabetes (Brosius and Coward, 2014; Fu et al., 2015). However, the generalization of the molecular mechanisms underlying podocyte apoptosis induced by HG and renal injury induced by hyperglycemia is challenging. Consequently, the discovery of novel regulators will facilitate a more comprehensive understanding of the pathogenesis of DN.

In our study, we found that Cdk5 was activated by HG stimulation *in vitro* and *in vivo*. Inhibition of Cdk5 notably counteracted HG-evoked podocyte dysfunction, including growth inhibition, membrane damage, and apoptosis. *In vivo* experimental analysis also revealed that Cdk5 inhibitors, trans-resveratrol, myricetin, salvianolic acid A, and BML-259, protect against hyperglycemia-induced tubular and glomerular lesions. These findings suggest that inhibition of Cdk5 has a nephroprotective action under hyperglycemia conditions.

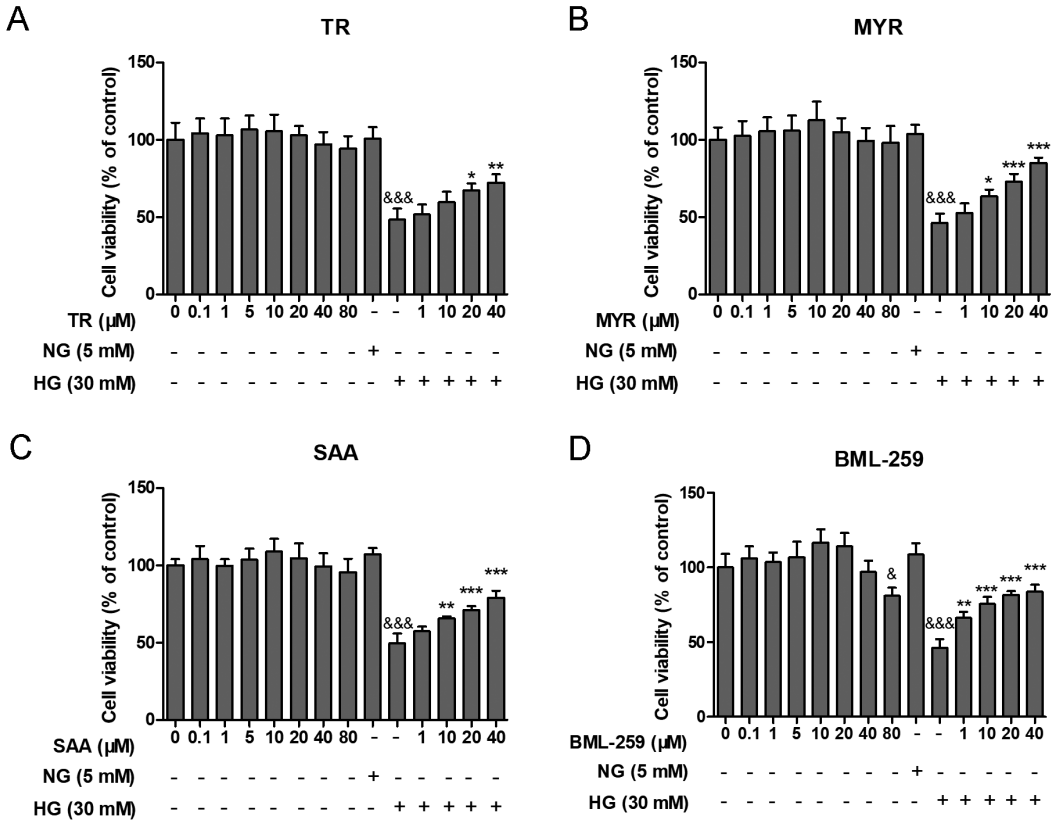

Fig. 4. Cdk5 inhibitors effectively alleviate podocyte growth inhibition induced by HG. After podocyte exposure to HG or NG for 48h, cell growth and cytotoxicity were evaluated using CCK-8 assays in the presence of trans-resveratrol **(A)**, myricetin **(B)**, salvianolic acid A **(C)**, and BML-259 **(D)**. $^{\&}p<0.05$, $^{\&\&\&}p<0.001$ compared with the NG group; $^{*}p<0.05$, $^{**}p<0.01$; $^{***}p<0.001$ compared with the HG group; n=3 in each group.

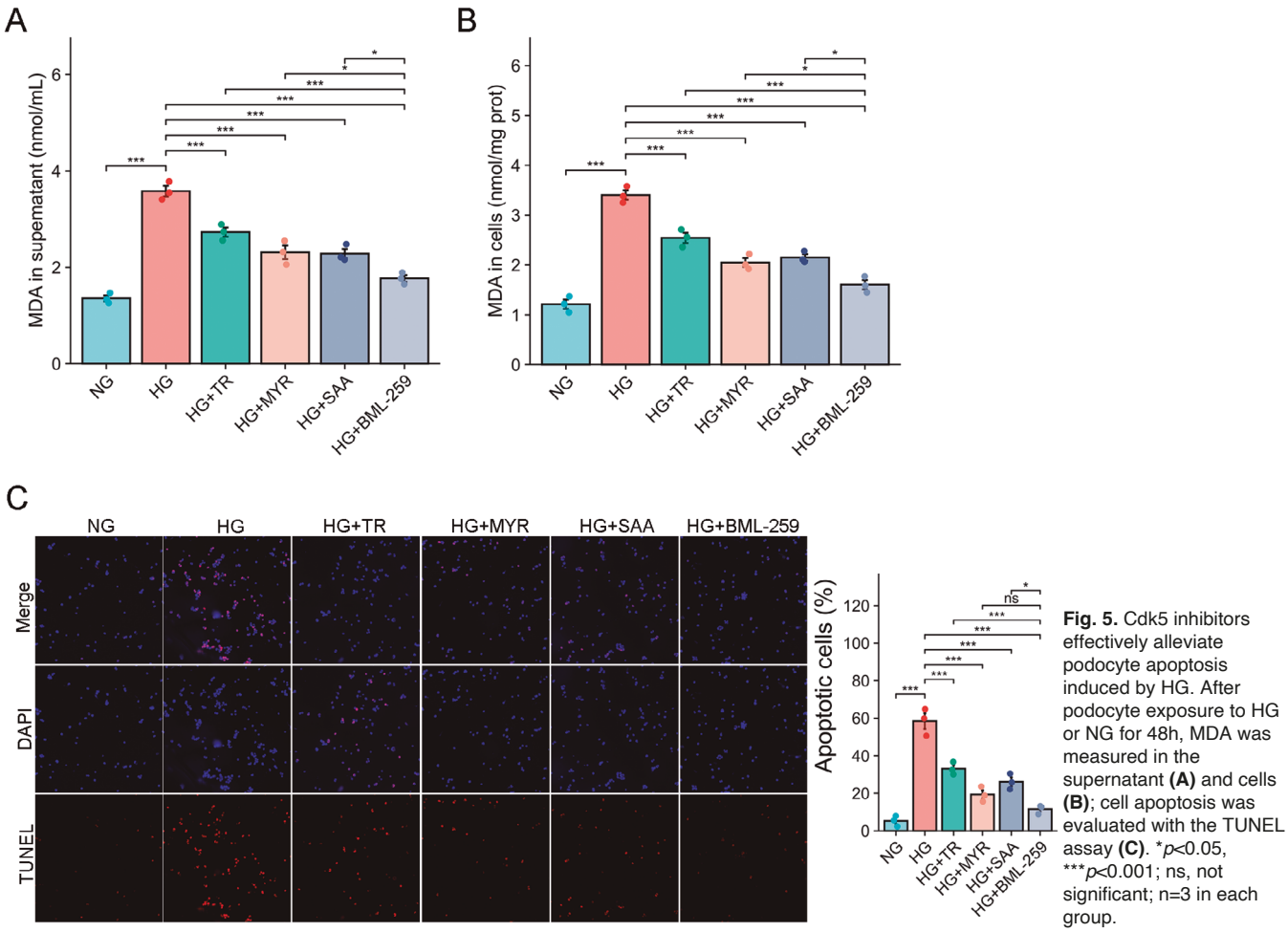

Fig. 5. Cdk5 inhibitors effectively alleviate podocyte apoptosis induced by HG. After podocyte exposure to HG or NG for 48h, MDA was measured in the supernatant **(A)** and cells **(B)**; cell apoptosis was evaluated with the TUNEL assay **(C)**. **p*<0.05, ****p*<0.001; ns, not significant; n=3 in each group.

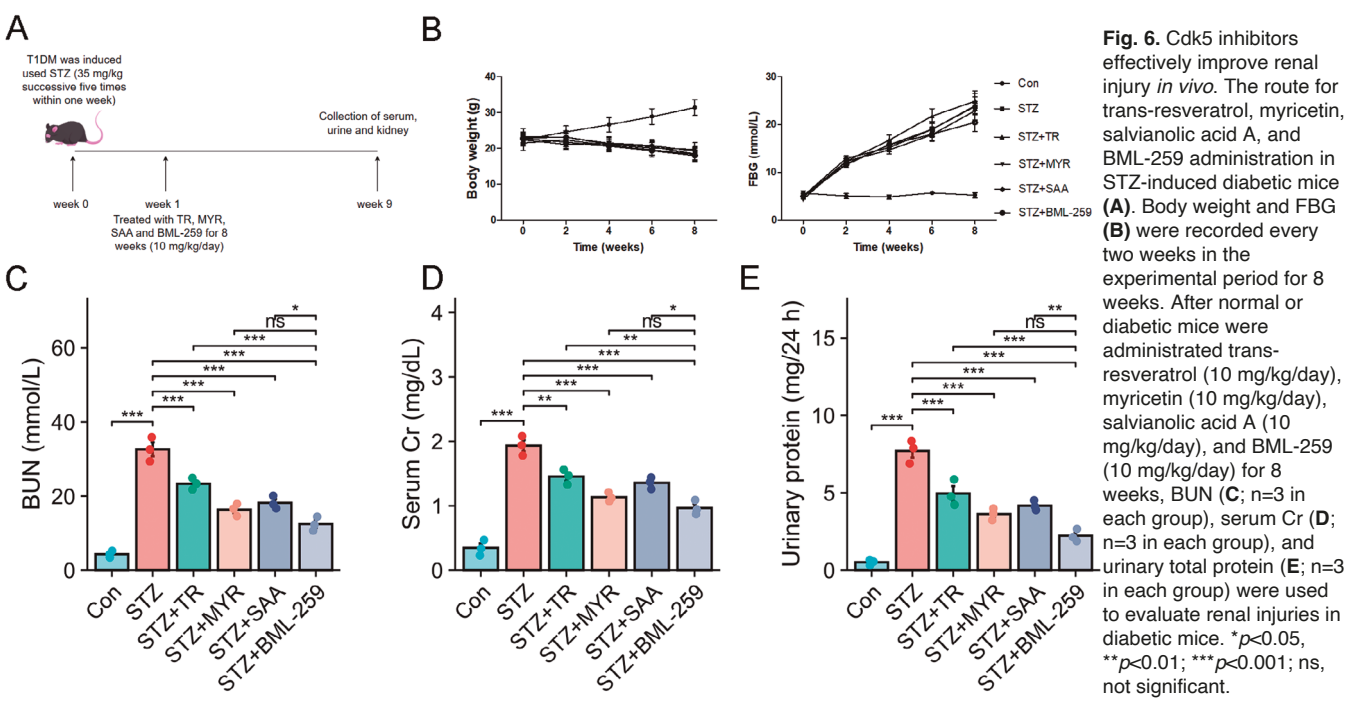

Fig. 6. Cdk5 inhibitors effectively improve renal injury *in vivo*. The route for trans-resveratrol, myricetin, salvianolic acid A, and BML-259 administration in STZ-induced diabetic mice **(A)**. Body weight and FBG **(B)** were recorded every two weeks in the experimental period for 8 weeks. After normal or diabetic mice were administrated trans-resveratrol (10 mg/kg/day), myricetin (10 mg/kg/day), salvianolic acid A (10 mg/kg/day), and BML-259 (10 mg/kg/day) for 8 weeks, BUN (**C**; n=3 in each group), serum Cr (**D**; n=3 in each group), and urinary total protein (**E**; n=3 in each group) were used to evaluate renal injuries in diabetic mice. **p*<0.05, ***p*<0.01; ****p*<0.001; ns, not significant.

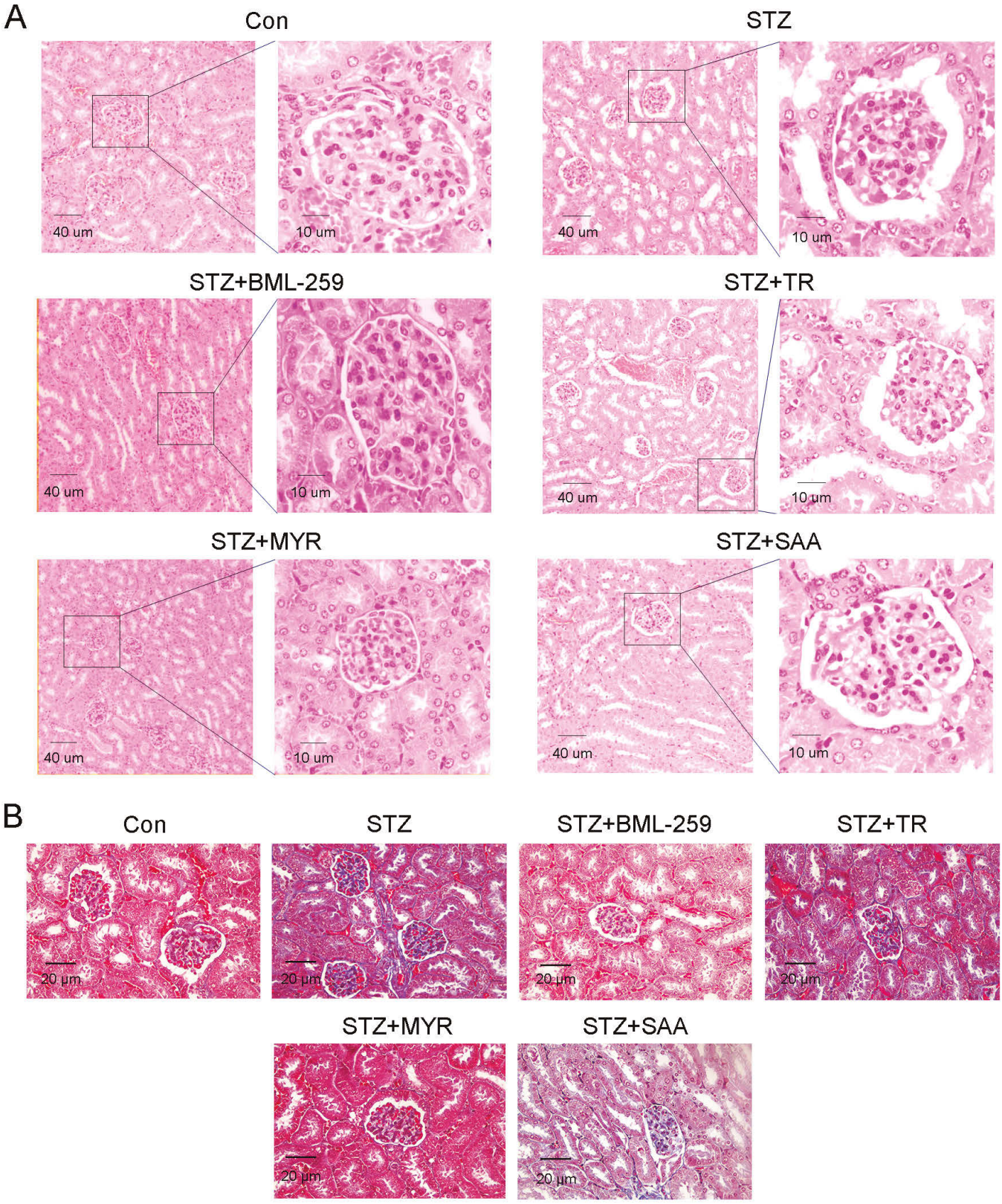

Fig. 7. Cdk5 inhibitors mitigate histologic damage in diabetic mice. After normal or diabetic mice were administrated trans-resveratrol (10 mg/kg/day), myricetin (10 mg/kg/day), salvianolic acid A (10 mg/kg/day), and BML-259 (10 mg/kg/day) for 8 weeks, H&E staining (**A**; n=6 in each group) and Masson's trichrome staining (**B**; n=6 in each group) were performed to analyze the protective effect of Cdk5 inhibitors on STZ-induced renal injury.

CDK5 is recognized for its role in controlling cell cycle cessation in fully differentiated cells, including podocytes. Although typically absent in renal tubular cells, CDK5 exhibits increased expression in kidney tubule epithelial cells post-injury (Mao and Hinds, 2010; Taguchi et al., 2022). Previous studies in the kidney have focused on the role of Cdk5 in terminally differentiated podocytes, suggesting that Cdk5 acts primarily as a master regulator of podocyte survival during glomerular disease and, in contrast to neurons, does not impact glomerular development or maintenance (Mangold et al., 2021). In addition, the Cdk5 pathway serves as a distinctive mechanism that governs maladaptive dedifferentiation; the ablation of Cdk5 in renal tubule cells effectively suppresses dedifferentiation and fibrosis, indicating Cdk5 as a therapeutic target for chronic kidney disease (Taguchi et al., 2022). The research conducted in this study centered on small molecule compounds and natural products that target Cdk5, with a specific focus on elucidating the potential mechanism of inhibiting Cdk5 to mitigate kidney injury from a pharmacological standpoint.

In alternative therapeutic regimens for DN, the administration of myricetin exhibits varied protective effects, such as diminishing proteinuria and podocyte loss, enhancing oxidative stress management, and ameliorating inflammatory responses in an animal model of diabetes (Niisato and Marunaka, 2023). Myricetin has demonstrated its potential as a multifaceted bioactive compound in the prevention and/or suppression of hyperglycemia. It achieves this by inhibiting the digestion and uptake of saccharides, as well as enhancing insulin secretion through its potential as a GLP-1 receptor agonist. Additionally, myricetin exhibits the ability to ameliorate complications associated with type 2 DM by safeguarding endothelial cells against oxidative stress, inflammation, and autophagy induced by hyperglycemia (Hu et al., 2021; Niisato and Marunaka, 2023). The administration of myricetin at a dosage of 1.0 mg/kg body weight for 12 weeks resulted in the normalization of urinary albumin and lipid profiles (Kandasamy and Ashokkumar, 2014). Histopathological analysis of kidney samples revealed that myricetin treatment effectively inhibited extracellular mesangial matrix expansion, glomerulosclerosis, and interstitial fibrosis in rats with DN (Kandasamy and Ashokkumar, 2014). In this study, the administration of myricetin at a dosage of 10 mg/kg for eight weeks demonstrated an improvement in the upregulation of renal damage biomarkers, including BUN, serum Cr, urinary total protein, as well as tubular and glomerular lesions induced by STZ. Furthermore, the binding energy values of myricetin targeting Cdk5 were found to be -8.693 kcal/mol, indicating a potential therapeutic application of myricetin in alleviating HG-induced podocyte apoptosis and hyperglycemia-induced renal injury in mice through the suppression of Cdk5.

A previous study demonstrated that the application of a specific Cdk5 inhibitor (BML-259, 10 μM) effectively safeguarded a substantial number of cells

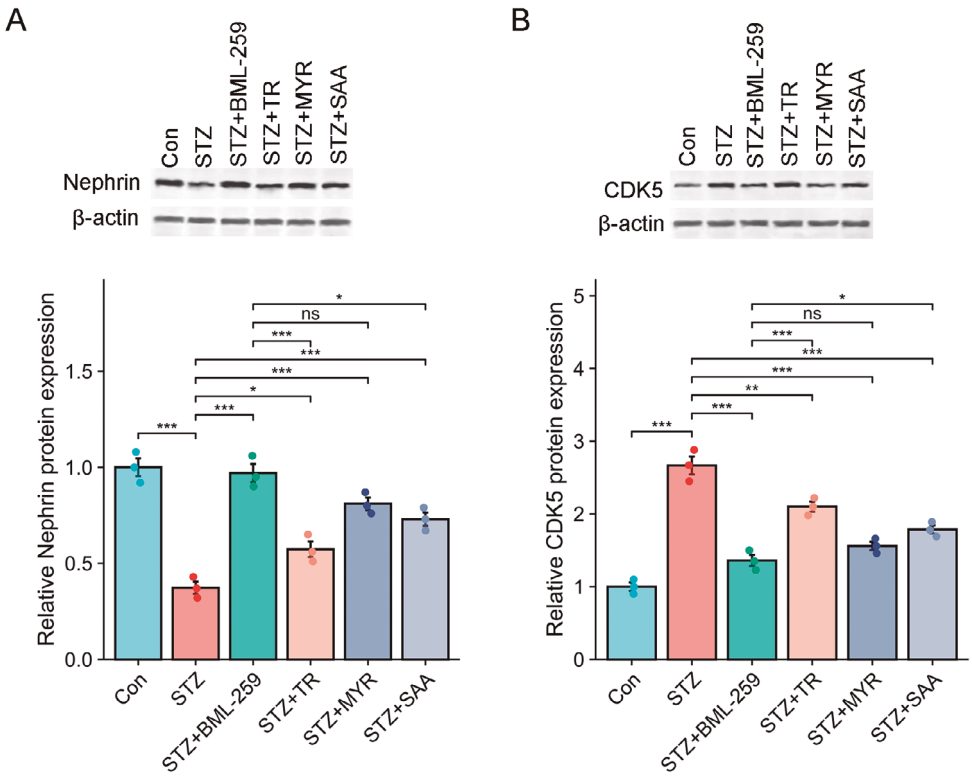

Fig. 8. Cdk5 inhibitors upregulate nephrin and inhibit Cdk5 protein expression in diabetic mice. Nephrin (**A**; n=6 in each group) and Cdk5 (**B**; n=6 in each group) protein levels were measured using western blot. *p<0.05, **p<0.01; ***p<0.001; ns, not significant.

from NAC-induced cell death in PC12 cells via inhibiting p53/Cdk5 and the Bax-dependent apoptotic signaling pathway (Kaźmierczak et al., 2011). The findings of our study indicate that concentrations exceeding 1 μM of BML-259 exhibit a positive effect on mitigating cell death induced by HG. The concentration of BML-259 at 80 μM demonstrated notable cytotoxicity. Conversely, the three natural products tested did not exhibit cytotoxic effects at the concentration of 80 μM. The findings of this study indicate that a more precise targeting approach may result in enhanced cytotoxic effects. Our study also observed that BML-259 exhibited a significant ameliorative effect on STZ-induced renal injury at a dosage of 10 mg/kg in diabetic mice.

In summary, the results of our study demonstrate that trans-resveratrol, myricetin, salvianolic acid A, and BML-259 effectively regulate the inhibition of Cdk5, thereby mitigating podocyte apoptosis induced by HG levels and preventing renal injury caused by hyperglycemia in mice. Furthermore, both myricetin and BML-259 exhibit comparable protective effects against renal damage. Cdk5 has the potential to serve as a promising therapeutic target for treating DN.

Ethics approval. This research was approved by the Ethics Committee of the Zhejiang Provincial People's Hospital of Hangzhou Medical College (Hangzhou, China).

Consent for publication. Not applicable.

Availability of data and material. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Competing interests. The authors declare that they have no competing interests.

Authors' contributions. Study design: L-Z and Y-J; Technical assistance: W-G, W-H, K-Y, and N-Y, Literature research, Data acquisition, and Data analysis: L-Z, W-G, W-H, K-Y, N-Y, and Y-J; Manuscript preparation and Manuscript editing: L-Z, W-G, W-H, K-Y, and N-Y; Manuscript review: L-Z and Y-J; Cell and animal experiments: L-Z, W-G, W-H, K-Y, N-Y, and Y-J. All authors read and approved the final manuscript.

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