

Original Research Article

Annexin A1 mimetic peptide Ac2-26 alleviates renal inflammatory injury in diabetic mouse model by suppressing p38MAPK/NF- κ B

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Abstract

Purpose: To investigate the protective role of mimetic peptide Ac2-26 of annexin A1 (ANXA1) in regulating p38MAPK/NF- κ B for renal inflammatory injury in a diabetic mouse model.

Methods: The mice were divided into three groups (control, model and Ac2-26; $n = 8$). Control was untreated, normal mice, while Ac2-26 was treated with mimetic peptide Ac2-26 after induction of type I diabetes with streptozotocin (60 mg/kg). The model group was not further treated after induction of diabetes. The fasting blood glucose (FBG), blood lipid, and renal function were evaluated. Serum inflammatory factors in renal tissue were also assessed.

Results: Compared with the model group, there was a significant decrease in the levels of FBG, blood lipids (TG, TC, LDL and ox-LDL), KI, 24 h urinary protein, Cr and BUN, and significant increase in body weight in AC2-26 group ($p < 0.001$). There was a marked decrease in TNF- α , IL-6, IL-1 β , and IL-18 levels, as well as levels of mRNA and protein expressions of p38MAPK, NF- κ B and ANXA1 in AC2-26 group when compared with the model group ($p < 0.001$).

Conclusion: ANXA1 is the target gene of p38MAPK, and mimetic peptide Ac2-26 alleviates renal inflammation by suppressing p38MAPK/NF- κ B pathway, thus improving renal function in diabetic mice models. This finding suggests a probable approach to developing an effective treatment for renal inflammation in diabetic renal injury.

Keywords: Renal inflammatory injury, Annexin A1, p38, Mitogen-activated protein kinases, Nuclear factor-kappa B

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INTRODUCTION

A growing number of children and adolescents are on record to be suffering from type I diabetes in recent years. Diabetic nephropathy (DN) is a chronic inflammatory disease resulting from the activation of natural immune responses induced by glucose and lipid metabolism disorders [1].

The inflammatory factors are highly expressed in the serum of DN patients, positively correlating with the degree of proteinuria, and directly contributing to renal damage DN [2]. Therefore, controlling the inflammatory response might be crucial in the treatment of childhood DN.

Annexin A1 (ANXA1) is a natural endogenous inflammation-regulatory protein with core and N-terminal domains [3]. Specifically binding to the relevant receptor, the N-terminal domain inhibits body tissue inflammation and exerts a protective effect on body tissues. Studies have shown that ANXA1 can control inflammation in rat pneumonia, as well as inhibit the expression of phospholipase A2 (PLA2) from the formation of inflammatory complexes, thereby resulting in the inhibition of inflammatory cytokine secretion such as TNF- α IL-1 β and alleviation of inflammatory response [4]. However, the effect of ANXA1 on renal inflammation in a diabetic mouse model has not been reported yet.

After activation by various inflammatory stimuli, p38 mitogen-activated protein kinase (p38MAPK) transmits the extracellular stimulus signals to the cell, promotes the secretion of inflammatory factors, and induces inflammatory injury on tissues and cells [5]. The nuclear factor-kappa B (NF- κ B) p65 is a nuclear transcription factor whose phosphorylation can also promote the transcription of inflammatory factors, with p38MAPK as the upstream kinase. Activating p38MAPK stimulates and induces NF- κ B p65 phosphorylation, and promotes an inflammatory response process [6].

The objective of this work was to determine the protective role of mimetic peptide Ac2-26 of ANXA1 in renal inflammation and its impact on p38MAPK/NF- κ B signaling pathway with aim of offering new therapeutic approaches to DN.

EXPERIMENTAL

Model preparation and grouping

Twenty-four male SPF BALB/c mice (6-week-old, 18 – 22 g) were supplied by Animal Experiment Center, West China Hospital, Sichuan University, and raised in a clean environment at room temperature (25 ± 2 °C), with a light-dark cycle of 12h-12 h, access to commercial feed and water *ad libitum*. The Ethics Committee of Chongqing Public Health Medical Center reviewed and approved all experimental procedures of the Medical School (approval no. 20220519013). Each experimental procedure was implemented in line with Guide for the Care and Use of Laboratory Animals [7].

Twenty-four mice were randomized into three groups, with eight mice per group. The mice in the three groups had unrestricted access to feed and water. Control group (CG) was not given any treatment, while the model group (MG) received intraperitoneal injection of streptozotocin (60

mg/kg, Sigma-Aldrich), in order to establish type I diabetic model. Fasting blood glucose (FBG) and urine glucose were determined 72 h after modeling. If blood glucose was above 16.7 mmol/L and urine glucose (+++), the model was considered successful [8]. During modeling, 2 mL of normal saline injection was given through the tail vein seven times, once every three days. In the meantime, AC2-26 group (AG) was modeled by injecting AC2-26 1 mg/kg of 2 mL through the tail vein seven times, once every three days, as with the other groups. FBG and body weight were measured weekly.

Bodyweight, fasting blood glucose, and lipid metabolism indexes

Collection of urine and measurement of weight was done before the end of the experiment. Blood samples were taken from the heart, and FBG, triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and oxidized LDL (ox-LDL) were determined by Sysmex-180 Biochemistry Analyzer (Sysmex, Japan).

Assessment of renal function indices

The kidney was weighed with the kidney weight index (kidney weight/body weight, KI) calculated. The urine was collected after 24h, and centrifuged for 15 min (3000 rpm, 4 °C) to obtain the supernatant. Blood (5 mL) was collected from the abdominal aorta, left to stand for 30 min at room temperature, and subsequently centrifuged at 3000 rpm for 15 min. Up test kit (Beijing Leadman Biochemistry Co. Ltd., Beijing, China) was used to determine the 24 h urinary protein, then immunoturbidimetric assay was implemented to identify creatinine (Cr), and Sysmex-180 biochemistry analyzer (SYSMEX, Kobe, Japan) was used to measure blood urea nitrogen (BUN).

H & E staining and PAS staining

Kidney tissue was fixed with 4 % paraformaldehyde for two days, then dehydrated with gradient ethanol, embedded into paraffin, and sectioned into 5 μ m slices. The tissue was dewaxed, put into water, and then dried to remove extra water. (1) Hematoxylin-eosin (H&E) stain: The sample was put in hematoxylin and 1 % ammonia to stain for 3 min, and then rinsed with water. Afterwards, it was stained with eosin for 2 min, dehydrated with gradient ethanol, cleared with xylene, and sealed with mount. (2) Periodic Acid-Schiff (PAS) stain: The sample was stained with Schiff reagent for 10 min and rinsed in running water for 5 min. The sample was

stained with Mayer hematoxylin, then rinsed, blueed, washed, and dehydrated with gradient ethanol. It was then cleared with xylene, and then sealed with mount. The samples were observed under an optical microscope.

Enzyme linked immunosorbent assay (ELISA)

Blood samples were collected for centrifugation (3000 rpm, 10 min) after removing eyeballs, with serum stored at -80 °C. Fresh kidney tissue (50 mg) was homogenized on ice using a tissue homogenizer, and the ratio of homogenization was chosen to be 10%, equivalent to 1 g of tissue and 9 mL of homogenization solution, and PBS was selected as the homogenization solution (pH 7.2 - 7.4, concentration of 0.01 mol/L) to centrifuge at 5000 rpm for 15 min and extract the supernatant. With the serum or renal tissue supernatant sample collected, ELISA kits (R & D Systems, Minneapolis, USA) were applied to measure ox-LDL, TNF- α , IL-6, IL-1 β , and IL-18. In detail, 0.1 mL diluted test sample was added to the coated reaction wells separately, set the sample standing at 37 °C for one hour.

Then the sample was added with the HRP-conjugated antibody after washing, set to stand for another hour, washed again, added with the substrate, and set to stand for 30 min at room temperature. Afterwards, the reaction was terminated by adding stopping solution. The absorbance of samples was recorded at 450 nm with a micro-plate reader (Bio-Tek ELX800, Bio-Tek, Biotek Winooski, VT, USA). The absorbance values of the control tube and test tube were measured separately, and the ratio was calculated to obtain the sample content of the test antigen.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

TRIzol reagent was used for total RNA extraction from frozen renal tissues, which was quantified using ultraviolet spectrophotometry, and reverse transcribed through a reverse transcription kit (Invitrogen, Carlsbad, USA). Reaction condition: 37 °C for 15 min, 85 °C for 5 s. After reverse transcription, GenBank showed the required sequence, and the primers were designed by Premier 5.0 software. The synthesis and validation of the primers were conducted by Shanghai Boya Biotechnology Co. Ltd. The same quantity of RNA was taken from each sample in a qRT-PCR device, and β -actin served as internal reference for amplification. Primer sequences: p38MAPK: 5'-GCGTGaATGATGGACTGaAA-3' and 5'-

CCCGaACGATACCAGaACC-3'; NF- κ B p65: 5'-cacTGTCACcTGGaagcaga-3' and 5'-GacctGGagCAAGCCattag-3'; ANXA1: 5'-TCGCAATGaagGACGATag-3' and 5'-ATATCCTCTTACAGTC-3'; β -actin: 5'-CGTTGACATCCGTAAAGACC-3' and 5'-AACagtCCGCCTAGaAgcac-3'; the PCR reaction mixture consisted of SYBR 10 μ L, 2 μ L of each primer (5 μ mol/L), cDNA 2 μ L, and PCR was undertaken at 95 °C for 5 min, then at 95 °C for 5 s and at 60 °C for 30 s for 45 cycles, and the temperature increased at the rate of 1 °C/s from 65 to 90 °C. The calculation of target gene expression was performed using the formula $2^{-\Delta\Delta C_t}$.

Western blot assay

Renal tissue (100 mg) was added with 0.25 mL radioimmunoprecipitation assay (RIPA) lysate (Beyotime Biotechnology Co. Ltd., Shanghai, China) for homogenization, followed by centrifugation (12000 \times g, 2 min, 4 °C) (Centifuge 5702, Eppendorf, Hamburg, Germany), with the supernatant retained. BCA test kit (Beyotime Biotechnology Co. Ltd.) was applied for protein concentration analysis. The protein (30 mg) was boiled for 5 min and deformed. Separation gel (10 %) and concentrated gel (5 %) were prepared, and the sample (50 μ g) was added to each well. SDS-PAGE was performed to separate proteins, then electrotransferred onto a nitrocellulose (NC) membrane for 2 h, and then blocked with buffer containing 5 % skimmed milk powder for a further 2 h. Rabbit anti-rat p38MAPK, p-p38MAPK, NF- κ B p65, p-NF- κ B p65 antibodies (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA) were added to the NC membrane and then kept overnight at 4 °C, rinsed and incubated at 37 °C with horse radish peroxidase (HRP) conjugated secondary antibody (1:20000, Beyotime Biotechnology Co. Ltd.) for an hour. After washing the membrane, protein bands were measured using electrochemiluminescence (ECL) (Beyotime Biotechnology Co. Ltd). Image1.43 software was used for semi-quantitative analysis, and β -actin was regarded as internal reference to calculate the relative protein expression.

Dual-luciferase reporter gene assay

Wild type p38MAPK WT 3' non-coding region (UTR) and mutant type p38MAPK MUT 3' UTR were constructed and inserted into the plasmids. ANXA1 mimic, NC mimic, and plasmids containing p38MAPK WT 3'UTR and p38MAPK MUT 3'UTR were transfected into human kidney epithelial cell line 293T cells (Shanghai Yiyan Biotechnology Co. Ltd, Shanghai, China) for 48-h

incubation. Dual-luciferase kit was used to analyze the fluorescence intensity of the cells, as well as to calculate the relative luciferase activity.

Statistical analysis

Statistical Package for the Social Sciences (SPSS, version 19.0), was used for statistical analysis. Measurement data are presented as (mean ± SD), while ANOVA and Student t-test were used to compare multiple groups and two groups with normal distribution, respectively. Differences were deemed statistically significant at $p < 0.05$.

RESULTS

AC2-26 relieved hyperglycemia and lipid metabolism disorder

No mouse in any of the groups died during the experiment. CG, FBG, TG, TC, LDL, and ox-LDL increased significantly, while body weight reduced significantly ($p < 0.001$) in MG. There was significant decrease in FBG, TG, TC, LDL, and ox-LDL levels, but increase in body weight ($p < 0.001$) in AG when compared with MG. None of the groups exhibited significant difference in HDL level ($p > 0.05$). Thus, AC2-26 effectively improve hyperglycemia and lipid metabolism disorders in the diabetic mice, as shown in Figure 1.

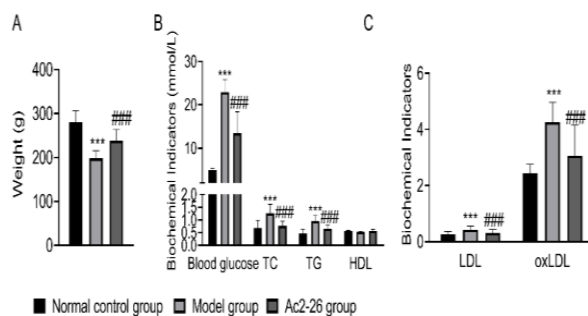


Figure 1: Comparison of general status and serological indices of mice in each group. Figure 1 displayed that AC2-26 substantially increase (A) body weight and reduce (B) FBG, TC, TG (C) LDL, and ox-LDL levels in diabetic mice, without obvious effect on (B) HDL. $***P < 0.001$ versus CG, $###p < 0.001$ versus MG

AC2-26 alleviated kidney tissue damage and improved renal function

As shown in Figure 2, MG showed balloon adhesion, mesangial matrix expansion, hyperplasia of the basement in glomeruli, vacuolar and granular degeneration of epithelial cell, and capillary luminal stenosis in the renal

tubule when compared with CG. Ac2-26 significantly ameliorated renal tissue damage compared with MG. KI, 24 h urinary protein, Cr, and BUN levels in MG were elevated ($p < 0.001$) in comparison with CG, but the levels were reduced in AG when compared with MG ($p < 0.001$). These results suggested that Ac2-26 can substantially reduce kidney tissue damage and improve renal function in diabetic mice.

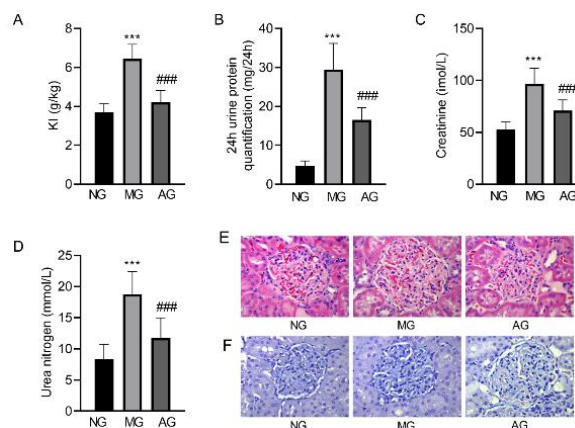


Figure 2: Comparison of renal function indices. $***P < 0.001$ versus CG, $###p < 0.001$ versus MG

AC2-26 lowered the levels of inflammatory cytokines

There was significant increase in TNF- α , IL-6, IL-1 β , and IL-18 levels in serum and renal tissue of MG ($p < 0.001$) in contrast to CG. AG showed significant decrease in TNF- α , IL-6, IL-1 β , and IL-18 levels ($p < 0.001$) when compared to MG, indicating that AC2-26 lowered inflammatory response in serum and renal tissue of diabetic mice (Figure 3).

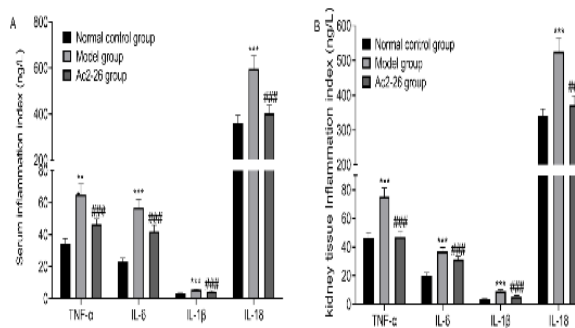


Figure 3: Comparison of inflammatory cytokines in serum and kidney tissue of mice. $**P < 0.001$ versus CG, $###p < 0.001$ versus MG

AC2-26 downregulated the expression of p38MAPK/NF- κ B p65 pathway-related factors

As shown in Figure 4, there was a marked increase in p38MAPK, NF- κ B p65, and ANXA1

mRNA expression levels in the renal tissues of mice in MG ($p < 0.001$) compared to CG, but decreased in AG in comparison with MG ($p < 0.001$). The increase in relative protein expression levels of p-p38MAPK, p-NF- κ B p65, and ANXA1 in the renal tissues of mice in MG was greater than in CG ($p < 0.001$), but decreased significantly in AG compared to MG ($p < 0.001$). Thus, AC2-26 inhibit the activation of p38MAPK/ NF- κ B p65 pathway in the renal tissues of diabetic mice.

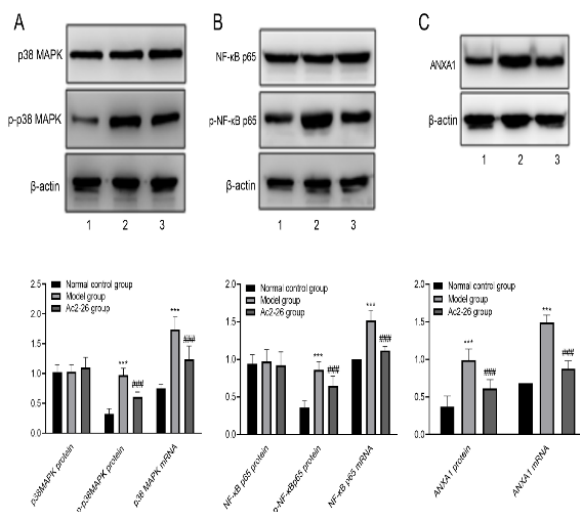


Figure 4: Expression of p38MAPK/NF- κ B p65 proteins pathway-related factors. (A) AC2-26 suppressed p-p38MAPK expression; (B) AC2-26 suppressed p-NF- κ B p65 expression; (C) AC2-26 suppressed ANXA1 expression. **Note:** *** $P < 0.001$ versus CG, ### $p < 0.001$ versus MG

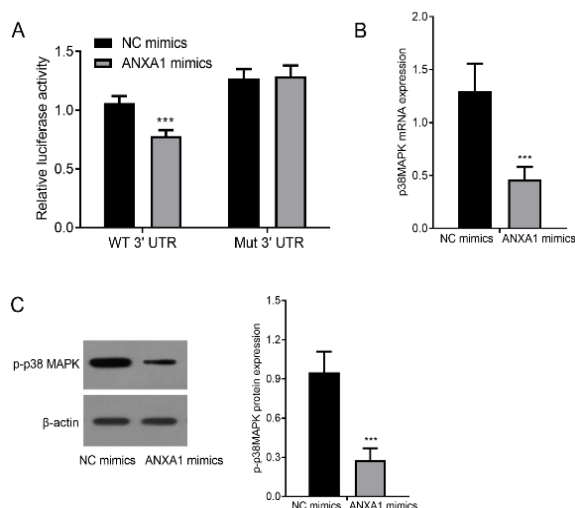


Figure 5: Dual luciferase report results. (A) ANXA1 mimics suppressed the luciferase intensity of p38MAPK 3' UTR WT; (B) ANXA1 mimics suppressed p38MAPK mRNA expression; (C) ANXA1 mimics suppressed p-p38MAPK protein expression. **Note:** *** $P < 0.001$ when compared with NC mimics

p38 MAPK was the target gene of ANXA1

Compared with NC mimics, ANXA1 mimics had a considerable inhibition on the luciferase intensity of p38 MAPK 3' UTR WT ($p < 0.001$) but had no noticeable impact on that of p38MAPK 3' UTR MUT ($p > 0.05$, Figure 5 A). The mRNA expression of p38MAPK in the ANXA1 mimics decreased in contrast to NC mimics ($p < 0.001$, Figure 5 B). The p-p38MAPK protein expression in the ANXA1 mimics was noticeably inhibited in contrast to NC mimics ($p < 0.001$, Figure 5 C). It suggested that ANXA influence p38MAPK expression, as shown in Figure 5.

DISCUSSION

DN is the most common diabetic complication, rapidly leading to ESRD [9] if active prevention and control measures are not taken, thus hurting the patient's health. Therefore, actively investigating approaches to prevent or delay DN has become the focus of medical research. It has been pointed out that ANXA1 mimetic peptide AC2-26 can effectively alleviate myocardial injury in diabetic mice [10]. This study reported that ANXA1 might substantially slow down the development of renal injury through regulation of p38MAPK/NF- κ B p65 pathway in mice with type I diabetes.

The type-1 DN model constructed by streptozotocin administration is a classic model [11], that has been extensively used to screen drugs and explore the mechanisms of human DN. This study demonstrated that streptozotocin-treated diabetic mice had lower body weight, higher FBG, and higher lipometabolic disturbance of TG, TC, LDL, and ox-LDL. In addition, there was increase in the levels of KI, 24 h urinary protein, Cr, and BUN in the MG when compared to CG, suggesting that the ANXA1 mimetic peptide, AC2-26, effectively reduced renal tissue injury and improved renal function in diabetic mice. Wu *et al* [12] showed that AC2-26 has the potential to reduce renal injury in db/db mice and diabetic ANXA1 knockout mice. However, this study aimed to investigate the pathogenesis of diabetic kidney injury.

DN is a significant microvascular complication of diabetes, whose occurrence and development of inflammatory response plays a vital role. The pro-inflammatory cytokines of TNF- α , IL-6, IL-1 β , and IL-18 are released by an inflammatory response, and they participate in the immune responses [13]. The P38MAPK/NF- κ B-mediated immune-inflammatory signaling promotes an increase in TNF- α , IL-6, IL-1 β , and IL-18

production after activation [14], which further exacerbates diabetic kidney injury. This study showed that TNF- α , IL-6, IL-1 β and IL-18 levels in serum and renal tissue of diabetic mice increased significantly, possibly accounting for the renal inflammation and impaired renal function in diabetic mice.

A previous report [15] found a remarkable elevation in the serum levels of IL-6, IL-18, and TNF- α in DN patients at the early-stage, similar to the findings obtained in the present study. In contrast, administration of ANXA1 mimetic peptide AC2-26 decreased the levels of inflammation-related indicators in the renal tissue of the diabetic mice, and renal function impairment also reduced significantly, which indicates that mimetic peptide AC2-26 ameliorated renal tissue inflammation in diabetic mice.

Activated p38MAPK stimulates the production and release of various pro-inflammatory factors by activating the relevant signaling pathways, thus inducing inflammatory responses and causing inflammatory tissue damage [16].

The NF- κ B is a reverse transcriptional regulator that binds to promoters of pro-inflammatory genes after activation, up-regulates pro-inflammatory gene expression, enhances inflammatory response, and causes inflammatory tissue damage. Inhibiting the transcriptional activity of NF- κ B is a critical way to inhibit inflammatory response [17]. Studies have demonstrated that p38MAPK is an upstream kinase of NF- κ B, which regulates NF- κ B's phosphorylation of NF- κ B, facilitates its nuclear translocation, and stimulates the transcription of inflammatory factors such as IL-6, thereby exacerbating inflammation. Studies using animal models indicate that inhibiting p38MAPK/NF- κ B pathway alleviated the renal injury and inflammatory response in mice with DN induced by streptozotocin [18]. In the present study, gene and protein expression in p38MAPK/NF- κ B p65 pathway increased in the kidneys of diabetic rat models, resulting in the activation of p38MAPK/NF- κ B pathway. Ac2-26 significantly decreased the gene and protein expression of p38MAPK/NF- κ B p65 pathway, as well as exerted a protective effect by suppressing its activation, reducing renal inflammation, improving renal function and slowing down pathological changes in the kidney.

CONCLUSION

The mimetic peptide Ac2-26 of ANXA1 alleviates disorder of fasting glucose and lipid metabolism,

improves renal function and ameliorates pathological changes in mouse kidneys. Furthermore, it reduces expressions of NF- κ B p65, p38MAPK protein and mRNA in diabetic mouse. Thus, Ac2-26 may inhibit p38MAPK protein pathway that leads to renal inflammation in diabetic mice, thereby playing a protective role. Therefore, Ac2-26 may be a new therapeutic target for diabetic renal injury. Nevertheless, some limitations are also evident in this study. The sample size of the study is small and should be expanded in a further study. Cell assays should also be carried out to further validate the inconsistent characteristics of the pathway. The cell signaling pathway is intricate, and the mechanism by which Ac2-26 regulates the p38MAPK/NF- κ B-p65 pathway needs additional investigation.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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