# Histone deacetylase-mediated silencing of PSTPIP2 expression contributes to AAI-induced PANoptosis

Xu Chuanting[1], Wang Qi[1], Changlin Du[1], Jiahui Dong[1], Zhenming Zhang[1], Na Cai[1], Jun Li[1], Cheng Huang[1], and Taotao Ma[1]

[1]Anhui Medical University

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**Abstract**

Background and Purpose: Aristolochic acid nephropathy (AAN) is a progressive kidney disease caused by using herbal medicines. Currently, no therapies are available to treat or prevent AAN. Histone deacetylase (HDAC) plays a crucial role in the development and progression of renal disease. We tested whether HDAC inhibitors could prevent AAN and determined the underlying mechanism. Experimental Approach: HDACs expression in the kidneys was examined. Mouse kidney and renal tubular epithelial cell damage were assessed after exposure to HDAC1 and HDAC2 blockade (FK-228). Conditional knock-in of Proline-serine-threonine-phosphatase-interacting protein 2 (PSTPIP2) in the kidney and knockdown of PSTPIP2 expression in PSTPIP2-knockin mice, pathological parameters, and kidney injuries were assessed. Key Results: Aristolochic acid upregulated the expression of HDAC1 and HDAC2 in the kidneys. Notably, the HDAC1 and -2 specific inhibitor, romidepsin (FK228, Depsipeptide), suppressed aristolochic acid-induced kidney injury, epithelial cell pyroptosis, apoptosis, and necroptosis (PANoptosis). Moreover, romidepsin upregulated PSTPIP2 in renal tubular epithelial cells, which was enhanced by aristolochic acid treatment. Conditional knock-in of PSTPIP2 in the kidney protected against AAN. In contrast, the knockdown of PSTPIP2 expression in PSTPIP2-knockin mice restored kidney damage and PANoptosis. PSTPIP2 function was determined in vitro using PSTPIP2 knockdown or overexpression in mTEC. Additionally, PSTPIP2 was found to regulate Caspase-8 in Aristolochic acid nephropathy. Conclusion and Implications: HDAC-mediated silencing of PSTPIP2 may contribute to aristolochic acid nephropathy. Hence, HDAC1 and -2 specific inhibitors or PSTPIP2 could be valuable therapeutic agents for the prevention of aristolochic acid nephropathy.

## Histone deacetylase-mediated silencing of PSTPIP2 expression contributes to AAI-induced PANoptosis

**Short title:** PSTPIP2 regulates AAI-induced PANoptosis

Chuanting Xu[1,2], Qi Wang[1], Changlin Du[1], Jiahui Dong[1], Zhenming Zhang[1], Na Cai[1], Jun Li[1], Cheng Huang[1,3*], Taotao Ma[1,2,3*]

1. Inflammation and Immune Mediated Diseases Laboratory of Anhui Province, Anhui Institute of Innovative Drugs, School of Pharmacy, Anhui Medical University
2. Center of Traditional Chinese medicine formula granule, Anhui Medical University
3. Anhui Provincial Institute of Translational Medicine

**Correspondence:**

Cheng Huang, PhD, Inflammation and Immune Mediated Diseases Laboratory of Anhui Province; Anhui Institute of Innovative Drugs; School of Pharmacy, Anhui Medical University, Hefei, Anhui 230032, China.

E-mail: huangcheng@ahmu.edu.cn

Taotao Ma, PhD, Inflammation and Immune Mediated Diseases Laboratory of Anhui Province; Anhui Institute of Innovative Drugs; School of Pharmacy, Anhui Medical University, Hefei, Anhui 230032, China.

E-mail: mataotao@ahmu.edu.cn

**Abbreviations:** AAN, Aristolochic acid nephropathy; HDAC, Histone deacetylase; PSTPIP2, Proline-serine-threonine-phosphatase-interacting protein 2; mTECs, Mouse renal tubular epithelial cells; GSDMD, gasdermin D; MLKL, mixed lineage kinase-like domain.

**What is already known?**

Programmed cell death, such as pyroptosis, apoptosis and necroptosis, are involved in participating in the pathogenesis of AKI.

HDAC is widely involved in the occurrence of programmed cell death, such as pyroptosis, apoptosis and necroptosis.

**What this study adds?**

HDACi targets PSTPIP2, inhibiting the occurrence of PANoptosis, thus alleviating kidney damage.

**Clinical significance?**

HDACi or PSTPIP2 could be valuable therapeutic agents for the prevention of nephropathy.

## ABSTRACT

**Background and Purpose:** Aristolochic acid nephropathy (AAN) is a progressive kidney disease caused by using herbal medicines. Currently, no therapies are available to treat or prevent AAN. Histone deacetylase (HDAC) plays a crucial role in the development and progression of renal disease. We tested whether HDAC inhibitors could prevent AAN and determined the underlying mechanism.

**Experimental Approach:** HDACs expression in the kidneys was examined. Mouse kidney and renal tubular epithelial cell damage were assessed after exposure to HDAC1 and HDAC2 blockade (FK-228). Conditional knock-in of Proline-serine-threonine-phosphatase-interacting protein 2 (PSTPIP2) in the kidney and knockdown of PSTPIP2 expression in PSTPIP2-knockin mice, pathological parameters, and kidney injuries were assessed.

**Key Results:** Aristolochic acid upregulated the expression of HDAC1 and HDAC2 in the kidneys. Notably, the HDAC1 and -2 specific inhibitor, romidepsin (FK228, Depsipeptide), suppressed aristolochic acid-induced kidney injury, epithelial cell pyroptosis, apoptosis, and necroptosis (PANoptosis). Moreover, romidepsin upregulated PSTPIP2 in renal tubular epithelial cells, which was enhanced by aristolochic acid treatment. Conditional knock-in of PSTPIP2 in the kidney protected against AAN. In contrast, the knockdown of PSTPIP2 expression in PSTPIP2-knockin mice restored kidney damage and PANoptosis. PSTPIP2 function was determined in vitro using PSTPIP2 knockdown or overexpression in mTEC. Additionally, PSTPIP2 was found to regulate Caspase-8 in Aristolochic acid nephropathy.

**Conclusion and Implications:** HDAC-mediated silencing of PSTPIP2 may contribute to aristolochic acid nephropathy. Hence, HDAC1 and -2 specific inhibitors or PSTPIP2 could be valuable therapeutic agents for the prevention of aristolochic acid nephropathy.

## KEYWORDS

aristolochic acid nephropathy; histone deacetylases; PSTPIP2; PANoptosis; Caspase-8.

## 1 | INTRODUCTION

Acute kidney injury (AKI) is the sudden loss of excretory kidney function. AKI is one of the various functional kidney conditions, which are summarized as acute kidney disease and disorders (AKD), in which slow deterioration of kidney function or persistent kidney dysfunction is associated with an irreversible

loss of kidney cells and nephrons, which can lead to chronic kidney disease (CKD) (Kellum, Romagnani, Ashuntantang, Ronco, Zarbock & Anders, 2021). Various types of abuse, such as ischemia, toxin exposure, and infection, can provoke kidney injury. Aristolochic acid (AA), an important cause of drug-related renal injury, has been reported to cause a range of serious health problems, including tubular necrosis, renal failure, and urothelial carcinoma (Debelle, Vanherweghem & Nortier, 2008; Vanherweghem et al., 1993). Aristolochic acid nephropathy (AAN) is characterized by extensive tubular epithelial cell (TEC) injury in both patients and animal models of AAN (Pozdzik et al., 2008; Wang, Xue, Zhao, Shi, Ding & Fang, 2020). However, the molecular mechanisms underlying tubular epithelial cell injury in AA-related renal injury remain unclear.

Several studies have indicated that various types of programmed cell death, such as apoptosis, necroptosis, and pyroptosis, are involved in maintaining the homeostasis of kidney tissue and participating in the pathogenesis of AKI (Bonventre & Yang, 2011). We previously reported that aristolochic acid-induced tubular damage and apoptosis are the primary insults in AAN. Baudoux et al. reported that human AAN is a tubulointerstitial (TI) nephritis reported after consuming herbal remedies containing aristolochic acid (Baudoux et al., 2022). In the early acute phase, tubular necrosis in proximal tubular epithelial cells (PTECs) is observed. Deng et al. demonstrated that aristolochic acid causes ferroptosis in HK-2 cells, providing new insights into the toxic mechanisms underlying aristolochic acid-triggered nephrotoxicity (Deng et al., 2020). Cells carry out multiple regulated cell death programs via extensive crosstalk, which can be activated simultaneously under specific conditions. This is consistent with the recently proposed concept of "PANoptosis." However, the role of PANoptosis in AAN has not been elucidated.

Recent studies in several animal models suggest that HDAC inhibitors can protect against diabetic nephropathy (Christensen et al., 2011), suppress kidney fibrosis in a unilateral ureteral ligation model (Liu et al., 2013), enhance kidney recovery from AKI (Cianciolo Cosentino et al., 2013), and suppress inflammation and kidney injury in the MRL-lpr/lpr mouse(Mishra, Reilly, Brown, Ruiz & Gilkeson, 2003). Yao et al. provided evidence that HDAC11 promotes NLRP3/caspase-1/GSDMD and caspase-3/GSDME pathways, leading to vascular endothelial cell pyroptosis (Yao et al., 2022). Wang et al. reported that HDAC inhibitors could initiate cell autophagy by inhibiting Akt and mTOR, which play important roles in protecting cells from necroptosis (Wang et al., 2013). Histone deacetylases (HDACs) are enzymes that remove acetyl groups from specific lysine residues on cellular and DNA binding proteins, such as histones, to regulate protein function, chromatin architecture, and gene expression (Chen et al., 2011).

In this study, we found that administration of the HDAC inhibitor FK-228 suppressed the tubular epithelial cell PANoptosis. The protective activity of the HDAC inhibitor was mediated through the upregulation of PSTPIP2 in tubular epithelial cells, which may provide some evidence for potential complement treatments in AAN therapy that target PSTPIP2.

## 2 | METHODS

### 2.1 | Mice

PSTPIP2-KI C57BL/6 and wild-type (WT) C57BL/6 mice were provided by Cyagen Biosciences (Guangzhou, China). C57BL/6 mice were purchased from the Experimental Animal Center of the Anhui Medical University. The animals were maintained following the guidelines of the Center for Developmental Biology, Anhui Medical University for the Care and Use of Laboratory Animals. All animal experiments used protocols approved by the institution's subcommittees on animal care (Approval No. LLSC20190682). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

### 2.2 | AAI Administration to Mice

Six- to eight-week-old male C57BL/6 mice were randomly divided into two groups (n = 5 per group): Mice were administered AAI (10 mg/kg, i.p.) or isotonic sodium chloride solution as a single intraperitoneal injection. Mice were euthanized 72 h after AAI administration. Tissue and blood were collected for further analysis. Kidney function and injury were assessed using urine protein, serum creatinine, and histology.

### 2.3 | Histone Deacetylase Inhibitors Mouse Model

Briefly, Six- to eight-week-old male C57BL/6 mice were randomly divided into four groups (n = 5 per group): the vehicle group mice were administered isotonic sodium chloride solution in a single i.p. injection. The AAI group mice were administered AAI at 10 mg/kg in a single i.p. injection. The AAI+FK-228 group mice were treated with FK-228 (2 mg/kg body weight) via gavage. The FK-228 group mice were treated with FK-228 alone (2 mg/kg body weight) via gavage. The last two groups were treated every 24 h for two days after administering AAI at 10 mg/kg in a single i.p. injection.

### 2.4 | AAV9-mediated PSTPIP2 inhibition in PSTPIP2-KI mice

Adeno-associated viruses were developed and obtained from the HANBIO Company (Shanghai, China). Six- to eight-week-old male C57BL/6 mice were divided into four groups, with five animals in each group. Mice in the wild-type control group were administered isotonic sodium chloride solution in a single i.p. injection. Wild-type AAI mice were administered AAI at 10 mg/kg via a single i.p. injection. PSTPIP2-KI group mice were injected with an empty adeno-associated virus 9(AAV9) vector using tail vein injection and administered AAI at 10 mg/kg in a single i.p. injection. The PSTPIP2-KI group mice were injected with an AAV9-PSTPIP2 vector via tail vein injections. Mice were imaged after injection with 150 mg/kg d-luciferin. AAI was administered at 10 mg/kg in a single i.p. injection. Animals were euthanized 72 h after AAI injection, and blood and kidney tissues were collected.

### 2.5 | Cell culture

Mouse renal tubular epithelial cells (mTECs) were kindly provided by Huiyao Lan. TECs were cultured in DMEM/F-12 (HyClone, Logan, UT, USA) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (Merck Millipore, Darmstadt, Germany) at 37 °C in a humidified incubator under 5% CO2. After overnight starvation in DME/F12 medium with 0.5% FBS, mTECs were treated with AAI (10 μM) for 24 h.

### 2.6 | Transfection

PSTPIP2 siRNAs were purchased from HANBIO for siRNA transfection (Shanghai, China). Cells were transfected with siRNAs using Lipofectamine-3000 (HANBIO). For plasmid transfection, cells were transfected with 1,000 ng/ml pEX-2-PSTPIP2 or pEX-2-control plasmids using Lipofectamine 3000 transfection reagent and Opti-MEM.

### 2.7 | Western Blotting

Proteins were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, China), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride (PVDF) membrane. PVDF membranes were blocked in TBST (pH 7.4, containing 0.05% Tween 20 and 5% non-fat milk) for 1.5 h at room temperature before adding specific primary antibodies. Subsequently, the PVDF membranes were incubated with primary antibodies overnight at 4 °C followed by incubation with secondary antibodies (1:10,000) for 1 h at room temperature.Protein bands were visualized using an ECL kit (ECL-plus, Thermo Scientific, Pittsburgh, PA, USA).

### 2.8 | Chromatin Immunoprecipitation Assay

ChIP assays were performed using a SimpleChIP® enzymatic chromatin IP kit with magnetic beads (#9004; Cell Signaling Technology, USA) according to the manufacturer's instructions. Chromatin DNA was immunoprecipitated using rabbit anti-H3K27Ac and normal rabbit IgG. The immunoprecipitated DNA was purified using a DNA purification column from the kit and analyzed by PCR using the forward primer 5'-GGAGAGGAGAGGAGAGGAGAGGAG-3' and reverse primer 5'-ACTACGAGTACGAGTGACACTGAGG-3' specific for the PSTPIP2 proximal promoter region.

### 2.9 | Creatinine (Cr) and blood urea nitrogen (BUN) assay kits

Blood samples were collected for creatinine and BUN measurements according to the manufacturer's instructions. Creatinine clearance (CCr) was calculated using the following equation: CCr (ml/min kg) = UCr x

24 h UV/ (SCr x body weight x 24 x 60).

### 2.10 | Kidney Histology

Renal tissues of the mice were fixed in 4% paraformaldehyde at room temperature for 24 h and embedded in paraffin. Coronal sections of the kidney tissue were stained with hematoxylin and eosin (H&E) and assessed using light microscopy.

### 2.11 | Immunohistochemical and Immunofluorescence Staining

Immunohistochemical staining was performed for cleaved-caspase-3, pMLKL, and GSDMD-N. Briefly, paraffin sections (4 mm) were fixed in 3% formaldehyde and stained with cleaved-caspase-3 (BF0711, 1:50; Affinity), pMLKL (AF7420, 1:50; Affinity), and GSDMD-N (DF13758, 1:50; Affinity) primary antibodies. Goat anti-rabbit IgG and goat anti-mouse IgG were used as secondary antibodies. All sections were examined under a light microscope (Pannoramic Scan, 3DHISTECH) and digitized using a high-resolution camera.

The sections were blocked with a 10% bovine serum albumin (BSA) solution to avoid non-specific staining. The sections were incubated with cleaved-caspase-3 (BF0711, 1:200; Affinity), pMLKL (AF7420, 1:200; Affinity), and GSDMD-N (DF13758, 1:200; Affinity). Sections were incubated overnight at 4 degC, followed by incubation with goat anti-rabbit IgG (M21014; Abcam) and goat anti-mouse IgG (M21013; Abcam) antibodies and nuclear staining with 4',6-diamidino-2-phenylindole (DAPI; Beyotime Biotechnology, Shanghai, China). The stained sections were examined using an inverted fluorescence microscope (CYTATION 5, BioTek, USA).

### 2.12 | Real-Time Reverse Transcriptase-PCR

Real-Time Reverse Transcriptase-PCR Total RNA was collected from kidney tissues and mTECs, using TRIzol reagents (Invitrogen). cDNA was synthesized using the Thermoscript RT-PCR synthesis kit (Fermentas, Pittsburgh, PA, USA) according to the manufacturer's protocol. Real-time quantitative PCR analyses for mRNA were performed by using Thermoscript RT-qPCR kits (Fermentas, Pittsburgh, PA, USA) in an ABI PRISM StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The products were used as templates for amplification using the SYBR Green PCR amplification reagent (Qiagen, Valencia, CA, USA) and gene-specific primers. Primers of genes in mice and mTECs were listed in Table S1.

### 2.13 | Statistical Analyses

The data and statistical analysis comply with the recommendations of the*British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022). All tests were treated as independent values rather than technical replicates. Data are expressed as the means +- SEM and represent at least five independent experiments. Two-group differences were analyzed using independent sample t test. One-way ANOVA followed by Tukey's post hoc test was used to compare more than two groups of data. Post hoc tests were conducted only if F in ANOVA (or equivalent) achieved the 'chosen' necessary level of statistical significance and there was no significant variance inhomogeneity. Statistical significance was set at $P < 0.05$. All analyses were performed using the GraphPad Prism 8 (GraphPad; San Diego, CA, USA).

### 2.14 | Materials

Aristolochic acid I (AAI; Cat. HY-N0510), Romidepsin (FK-228; Cat. HY-15149) were bought from MedChemExpress (Shanghai, China). Anti-MLKL (Abcam, Cat. ab184718, RRID: AB_2755030), anti-H3K27Ac (Abcam, Cat. ab4729, RRID: AB_2118291) and anti-pMLKL (Abcam, Cat. ab187091, RRID: AB_2619685) antibodies were purchased from Abcam (Cambridge, UK). Anti-caspase8 (Proteintech, Cat. 13423-1-AP, RRID: AB_2068463) antibody was bought from Proteintech (Rosemont, IL, USA). Anti-PSTPIP2 (Bioss, Cat. bs-19580R) and Anti-β-actin (Bioss, Cat. bs-10966R) antibodies were obtained from Bioss (Beijing, China). Anti-GSDMD-N (Huabio, Cat. ER1901-37) antibody was bought from Huabio (Hangzhou,China). Anti-caspase-3 (Abmart, Cat. T40044) antibody was purchased from Abmart (Shanghai, China).

## 3 | RESULTS

### 3.1 | AAI-induced PANoptosis in vivo and in vitro

To determine the renal response to aristolochic acid treatment, kidney tissues were harvested 1, 2, and 3 days after AA administration (Figure 1A). AAI administration caused time-dependent kidney dysfunction, as evidenced by increased BUN and Cr levels over time (Figure 1B). The progression of the lesions in the kidney after AAI administration was observed by histopathological examination. Tubular dilation, necrosis, cast formation, and preservation of the brush border in the kidney were observed three days after the AAI injection (Figure 1C). We found that AAI triggers multiple forms of cell death (PANoptosis). For apoptosis, we found markedly increased levels of cleaved forms of executioner caspase-3 after the AAI injection. AAI treatment resulted in robust phosphorylation of the pseudokinase mixed lineage kinase-like domain (MLKL), an inducer of necroptosis. Next, we explored the effect of the treatments on pyroptosis markers. GSDMD is the primary executioner that forms the membrane pores under specific conditions. The results revealed significant activation and cleavage of GSDMD in the treatment group (Figure 1D, E). In vitro, mouse kidney tubular epithelial cells (mTECs) were treated with AAI (10 μM) for 24 h and harvested to explore the role of AAI on nephrotoxicity. This was consistent with the in vivo results that AAI treatment could cause increased activation of pyroptotic, apoptotic, and necroptotic molecules (Figure 1F–G).

### 3.2 | Histone acetylation inhibitors inhibited AAI-induced PANopotosis

To determine which isoforms of HDAC were induced in response to AAI treatment, kidney tissue was harvested at 24, 48, and 72 h after AAI administration. The expression of HDACs was determined using real-time qPCR We found that AAI induced a large increase in HDAC1 and HDAC2 expression, whereas expression of HDAC 3, 4, 7, 8, and 11 was downregulated. (Figure 2A). To determine whether the AAI-induced increase in HDAC expression mediated the AAI-induced nephrotoxicity, FK-228 or vehicle was administered with AAI (Figure 2B). FK-228 administration significantly suppressed kidney dysfunction at 72 h after AAI administration. Saline or FK-228 administration alone did not alter kidney function (Figure 2C). Consistent with the improved kidney function with FK-228 administration, histopathological examinations showed less tubular necrosis, cast formation, and preservation of a brush border in the AAI+FK-228 administered group compared with the AAI+ vehicle-treated group (Figure 2D). In addition, FK-228 treatment impaired the activation of caspase-3 phosphorylated MLKL and GSDMD induced by AAI (Figure 2E, 3A). AAI-induced mTECs were then incubated with FK228. The levels of GSDMD-N, pMLKL, and cleaved caspase-3 expression in AAI-induced mTECs were significantly increased, whereas these levels were sharply decreased in AAI-induced mTECs treated with FK-228 (Figure 3B, C).

### 3.3 | HDAC inhibitor upregulated PSTPIP2 expression in vivo

PSTPIP2 plays a vital role in tumors, autoimmune diseases, and other diseases by influencing cell proliferation, apoptosis, and the secretion of inflammatory factors. In the AAN model, we observed that AAI treatment decreased the expression of PSTPIP2 in tubular epithelial cells; PSTPIP2 expression increased in the presence of FK-228 in vivo and in vitro (Figure 4A–D). Additionally, the H3K27Ac protein level was downregulated in vivo and in vitro, and FK-228 increased the protein level (Figure 4F, G). Chromatin immunoprecipitation (ChIP) assays revealed that AAI treatment reduced histone acetylation in the promoter region of PSTPIP2. Notably, the FK-228 treatment increased histone acetylation in the promoter region of the PSTPIP2 gene (Figure 4E).

### 3.4 | Epithelial cell-specific overexpression of PSTPIP2 ameliorates AAI-induced AKI in mice

To understand the function of PSTPIP2, we established a mouse model of PSTPIP2-KI, specifically in the renal proximal tubules (Figure 5A). The mice were genotyped using PCR (Figure 5C). We treated PSTPIP2-KI mice for follow-up experiments (Figure 5B). AAI induced a significant increase in BUN and Cr levels, markers of kidney injury, in wild-type mice. However, in PSTPIP2-KI mice, there was no alteration in kidney function (Figure 5D). Macroscopically, PSTPIP2 can significantly improve renal function, which is related to the improved preservation of kidney morphology. Histological analysis with HE staining revealed a

significant decrease in tissue damage in PSTPIP2-KI mice compared with that in wild-type mice (Figure 5E). Therefore, we biochemically characterized the activation of key PANoptotic proteins (GSDMD-N, pMLKL, and cleaved caspase-3) in the kidneys of AAN mice. We found decreased activation of these molecules in the kidneys of PSTPIP2-KI mice compared with that of wild-type mice treated with AAI (Figure 5F, 6A). To confirm whether the downregulation of PANoptosis is driven by the renoprotection of PSTPIP2, an overexpressed PSTPIP2 cell culture model was established. This indicates that pstpip2 overexpression downregulates AAI-induced PANoptosis in AAN. In support of this hypothesis, we found that the robust activation of PANoptotic markers was reduced in the overexpressing PSTPIP2 mTECs compared with that in control cells during AAI treatment (Figures 6B, C).

### 3.5 | AAV9-mediated restoration of PSTPIP2 increased renal dysfunction while promoting renal injury and PANoptosis in AAI-treated PSTPIP2-KI mice

To further elucidate the function of PSTPIP2, PSTPIP2 depression was rescued in PSTPIP2-KI mice using an AAV9-packaged PSTPIP2 depression plasmid (Figure 7A, B). We observed that restoring PSTPIP2 expression increased BUN levels, serum CRE levels, and renal damage (Figure 7C, D). Furthermore, IHC staining and western blotting analyses showed that restoration of PSTPIP2 induced GSDMD-N, pMLKL, and cleaved caspase-3 production and programmed cell death in AAI-induced PSTPIP2-KI mice (Figure 7E, 8A). These data indicate that PSTPIP2 plays a key role in the occurrence and development of AKI.

To confirm our hypothesis that PSTPIP2 mediates FK-228-mediated suppression of AAI-induced PANoptosis, PSTPIP2 was knocked down by transfection of small interfering RNA targeting PSTPIP2 into mTECs. FK-228-mediated suppression of AAI-induced epithelial cell PANoptosis was abolished by PSTPIP2 knockdown (Figure 8B, C). Hence, PSTPIP2 primarily functions by inhibiting the PANoptosis of mTECs in AKI. Therefore, the FK-228-mediated induction of PSTPIP2 and PSTPIP2-mediated suppression of AAI-induced epithelial cell PANoptosis suggests that FK-228 mediates its effect through PSTPIP2.

### 3.6 | PSTPIP2 regulates PANoptosis through Caspase-8

Cysteinyl aspartate specific proteinase (Caspase)-8 has long been considered a promoter of apoptosis and part of the mechanism by which cytotoxic drugs kill cancer cells (Jiang, Qi, Li, Wu, Song & Li, 2021). Caspase-8 controls the interactions between apoptosis, necroptosis, and pyroptosis and determines the type of cell death induced by the activation of cell death signaling (Schwarzer, Laurien & Pasparakis, 2020). A previous study reported a significantly higher number of caspase-8 in PSTPIP2 knockout mice than in the control group (Gurung, Burton & Kanneganti, 2016). Thus, to determine whether PSTPIP2 decreases PANoptosis expression by inhibiting caspase-8, we examined the effect of PSTPIP2 on caspase-8 expression. Notably, we observed a significantly lower number of caspase-8 in PSTPIP2 overexpression groups than in the WT controls (Figure 9A–D). To further elucidate the relationship between PSTPIP2 and Caspase-8, we also verified that Caspase-8 expression increases with the inhibition of PSTPIP2 after PSTPIP2 was inhibited (Figure 10A–D).

## 4 | DISCUSSION

Despite recent advances in understanding the basis of acute renal injury (Nakazawa et al., 2017; Tran et al., 2016), refractory nephrotic syndrome in patients with AKI continues to be a challenge for clinical practice. This study suggests that HDAC inhibitors can alleviate AKI by regulating PSTPIP2 to inhibit the occurrence of AAI-induced PANoptosis.

Aristolochic acid nephropathy (AAN) is a rapidly progressing tubulointerstitial disease caused by AA. The major nephrotoxic constituent of aristolochic acid (AA) is 8-methoxy-6-nitrophenanthrene-(3,4-d)- 1,3-dioxo-5-carboxylic acid (aristolochic acid I, AAI) (Shibutani, Dong, Suzuki, Ueda, Miller & Grollman, 2007). There is evidence that oxidative stress injury is induced in the kidneys of mice after AAI exposure, resulting in tubular epithelial cell apoptosis(Wang et al., 2022) and histopathological features of tubular necrosis in a short-term mouse model (Baudoux et al., 2012). The assembled NLRP3 inflammasome can activate caspase-1 to induce gasdermin D-dependent pyroptosis (Huang, Xu & Zhou, 2021; Shi et al., 2015). NLRP3 or caspase-

1 deficiency protects against renal injury in a mouse model of acute AAN(Wang et al., 2019). Therefore, we investigated whether programmed cell death occurred in AAI-induced AKI.

Programmed cell death (PCD) is an evolutionarily conserved process that plays a central role in maintaining organismal homeostasis. Three key PCD pathways have been studied in detail: pyroptosis (inflammasome-dependent PCD executed by gasdermin family members), apoptosis (PCD mediated by the apoptosome and executioner caspases), and necroptosis (PCD mediated by RIPK3 and the downstream effector MLKL)(Samir, Malireddi & Kanneganti, 2020). Apoptosis is a form of programmed cell death that is executed by a family of proteases called caspases that can be activated by cell surface death receptors (extrinsic pathway) or perturbation of the mitochondrial membrane (intrinsic pathway) (Lewis et al., 2005). The released apoptotic factors can activate the caspase family, eventually resulting in apoptosis (Hsu & Hsueh, 2000). Apoptosis played a pivotal role in the progression of nephrotoxicity and AKI (Chen et al., 2010; Pozdzik et al., 2008; Romanov, Whyard, Bonala, Johnson & Grollman, 2011). Necroptosis, a necrotic type of cell death that follows a signaling pathway closely related to apoptosis, is the most thoroughly examined form of regulated necrosis, executed by RIPK3 and its substrate, pseudokinase mixed lineage kinase domain-like protein (MLKL) (Belavgeni, Meyer, Stumpf, Hugo & Linkermann, 2020; Muller et al., 2017). Recently, necroptosis has been implicated in acute kidney injury (AKI), acute tubular necrosis (ATN), generation of necrotic casts, and reduction of renal blood flow (Huang et al., 2019; Liu et al., 2018; Martin-Sanchez et al., 2018; Schreiber, Rousselle, Becker, von Massenhausen, Linkermann & Kettritz, 2017). Necroptosis is characterized by cellular swelling, rapid membrane permeabilization, and the concomitant release of damage-associated molecular patterns (DAMPs) into the extracellular space. Necroptosis has been implicated in the development of autoimmune, neurodegenerative, and inflammatory diseases, such as acute pancreatitis and ischemic injury (Pasparakis & Vandenabeele, 2015). AKI is characterized by the damage or death of tubular epithelial cells, in which pyroptosis has been reported to play a role in the progression of AKI (Zhang et al., 2018). Pyroptosis can resist intracellular infections by eliminating the damaged cells. It is an important natural immune response that plays a crucial role in antagonizing infection and endogenous danger signals (Miao et al., 2010). Pyroptosis is activated by inflammatory caspases, leading to cell swelling, pore formation, cell membrane disruption, and consequent release of inflammatory cytokines (Xia et al., 2021). Pyroptosis participates in various kidney diseases, as evidenced by the activation of inflammatory caspases, massive release of IL-1β, and increased cleavage of GSDMD (Li et al., 2020; Ye et al., 2019). We found that in vivo, the kidneys had increased cleaved-caspase-3, GSDMD-N, and pMLKL in response to AAI treatment compared with the control group. In vitro, mTECs cells also showed increased cleaved-caspase-3, GSDMD-N, and pMLKL following AAI treatment compared with mock treatment. Studies have revealed that various cell death programs play alternating roles and exhibit extensive crosstalk (Chen et al., 2016; Wang et al., 2018). Cells carry out multiple regulated cell death programs via extensive crosstalk, which can be activated simultaneously under specific conditions. This is consistent with the recently proposed concept of "PANoptosis" (Karki et al., 2021). Whether induced in response to infection, during organismal homeostasis, or in the context of autoinflammation, PANoptosis is executed by a molecular complex called the PANoptosome that integrates apoptotic, necroptotic, and inflammasome components (Samir, Malireddi & Kanneganti, 2020). Our results demonstrate that AAI could co-use PANoptosis in the kidney.

However, in recent years, there has been significant growth in our knowledge about the involvement of histone modifications in gene regulation, which are known to play a role in normal cell physiology and pathology. HDACs play essential roles in cellular physiology and gene regulation (Iizuka & Smith, 2003). Histone acetylation plays a crucial role in chromatin remodeling and regulation of gene transcription. The presence of acetylated lysine in histone tails is associated with a more relaxed chromatin state and activation of gene transcription, whereas deacetylation of lysine residues is associated with a more condensed chromatin state and transcriptional gene silencing(Iizuka & Smith, 2003; Ropero & Esteller, 2007). In our study, AAI induced a large increase in HDAC1 and HDAC2 expression, whereas expression of HDAC 3, 4, 7, 8, and 11 was downregulated. To determine whether the AAI-induced increase in HDAC expression mediated AAI-induced nephrotoxicity, HDAC1 and -2 specific inhibitors (romidepsin or FK-228), or vehicle were administered with AAI. AAI administration significantly increased tubular epithelial cell PANoptosis in the

kidneys, which was largely suppressed by FK-228 treatment. These results indicated that HDAC inhibitors can inhibit AAI-induced PANoptosis.

Zhu et al. reported PSTPIP2 inhibits acute kidney injury (Zhu et al., 2020). PSTPIP2 was silenced by the deacetylase activity. Our chromatin immunoprecipitation assay supports the view that increased acetylation of histones in the PSTPIP2 promoter region may enhance PSTPIP2 transcription. Thus, it appears that inhibition of deacetylase activity promotes PSTPIP2 gene transcription. PSTPIP2 belongs to the F-BAR family and contains a conserved Fes CIP4 homology (FCH) domain in the N-terminal (Liu et al., 2014). PSTPIP2 participates in macrophage activation, neutrophil migration, cytokine production, and osteoclast differentiation (Xu et al., 2021). Most tissue macrophages and osteoclasts are regulated by colony-stimulating factor-1 (CSF-1, also known as macrophage CSF). PSTPIP2 regulates the morphology and motility of macrophages downstream of the CSF-1R PSTPIP2 deficiency and causes both an expansion of macrophage progenitors and increased responsiveness of mature macrophages to stimulating stimuli, which together prime the organism for exaggerated and sustained responses leading to autoinflammatory disease (Chitu et al., 2009; Chitu et al., 2005; Pixley & Stanley, 2004). Our results prove that the HDAC inhibitor FK-228 can block apoptosis, pyroptosis, and necroptosis by regulating PSTPIP2 in AAN, a complex disorder involving insulin resistance, lipid metabolism dysfunction, oxidative stress, inflammation, and various types of cell death. We demonstrated that AAI induces kidney injury and PANoptosis in mice. However, AAI did not induce glomerulonephritis in PSTPIP2-KI mice. These results are consistent with those of previous studies. However, the regulation of AAI-mediated cell death by PSTPIP2 requires further investigation.

Prajwal et al. found a significantly higher number of caspase-8 in Pstpip2cmo neutrophils than in WT controls (Gurung, Burton & Kanneganti, 2016). In "PANoptosis," the traditional promoter of apoptosis, caspase-8, plays a pivotal role. Researchers have found that caspase-8 can switch the modes of cell death. Even when the caspase-8-dependent apoptotic pathway and MLKL-dependent necroptotic pathway are inhibited, the catalytically inactive caspase-8 (C362A) can mediate pyroptosis (Newton et al., 2019). Thus, caspase-8 has attracted significant attention as a molecular switch between these three types of cell death. Our studies show that this suppression of PANoptosis is mediated through the downregulation of caspase-8 expression in epithelial cells.

In conclusion, our results showed that administration of the HDAC1 and HDAC2 inhibitor FK-228 inhibited PANoptosis of renal tubular epithelial cells. The protective activity of HDAC inhibitors is mediated by the upregulation of a novel anti-inflammatory and anti-apoptotic protein, PSTPIP2. This study highlights that PSTPIP2 alleviates kidney injury in response to AAI treatment by suppressing PANoptosis, which may guide the design of a better therapeutic strategy for treating patients with AKI.

## AUTHOR CONTRIBUTIONS

**Chuanting Xu:** Conceptualization, Data curation, formal analysis, Visualization, Writing – original draft. **Qi Wang:**Data curation, Formal analysis, Visualization. **Changlin Du:**Data curation, Formal analysis, Visualization. **Jiahui Dong:**Data curation, Formal analysis, Visualization. **Zhenming Zhang:**Validation. **Na Cai:** Validation. **Cheng Huang:**Conceptualization, Data curation, project administration, Supervision, Writing – original draft, writing – review, and editing.**Taotao Ma:** Conceptualization, Data curation, project administration, Supervision, Writing – original draft, writing – review, and editing.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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## FIGURE LEGENDS

**FIGURE 1. Aristolochic acid I (AAI) induces kidney tissue PANoptosis and damage.**

(A) C57BL/6 mice aged 6–8 weeks were treated with AAI and euthanized three days after AAI treatment. (B) Creatinine (Cr) and blood urea nitrogen (BUN) were assessed three days after AAI treatment. (C) Representative hematoxylin-eosin (H&E)-stained sections of kidney from wild-type (WT) and AAI-treated mice (Scale bar = 40 μm). (D) Western blotting analyses of cleaved-caspase-3, pMLKL, and GSDMD-N in AAI-induced AKI mice. (E) Photomicrographs of immunohistochemical sections from WT and AAI-treated mice with cleaved-caspase-3, pMLKL, and GSDMD-N in both AAI-treated mice and WT mice (Scale bar = 40 μm). (F) Western blotting analyses of cleaved-caspase-3, pMLKL, and GSDMD-N in AAI-treated mTECs. (G) Immunofluorescence staining of cleaved-caspase-3, pMLKL, and GSDMD-N in AAI-treated mTECs (Scale bar = 100 μm). Data are presented as the mean ± SEM, n = 5. ns, not significant; *P < 0.05, significantly different, as indicated.

**FIGURE 2. Effects of histone deacetylase (HDAC) inhibitor on AAI-induced nephrotoxicity and PANoptosis.**

(A) Expression level of different isoforms of HDAC at 24, 48, and 72 h after AAI administration. (B) The schematic diagram depicts the animal experimental design. C57BL/6 mice aged 6–8 weeks were treated with AAI and FK-228 every 24 h for two days after AAI administration. (C) Creatinine (Cr) and blood urea nitrogen (BUN) after different treatments. (D) Hematoxylin-eosin (H&E)-stained kidney tissue section (Scale bar = 40 μm). (E) Photomicrographs of immunohistochemical of cleaved-caspase-3, pMLKL, and GSDMD-N in AAI-treated AKI mice administered with FK-228 (Scale bar = 40 μm). Data are presented as the mean ± SEM, n = 5. ns, not significant; *P < 0.05, significantly different, as indicated.

**FIGURE 3. Histone deacetylase (HDAC) inhibitor romidepsin (FK228, Depsipeptide) treatment reduced the kidney's AAI-induced tubular epithelial cell PANoptosis.**

(A) Cleaved-Caspase-3, pMLKL, and GSDMD-N protein levels in AAI-treated AKI mice administered with FK-228 analyses using western blot. (B) Immunofluorescence staining of cleaved-caspase-3, pMLKL, and GSDMD-N in AAI-treated mTECs treated with FK-228 (Scale bar = 100 μm). (C) Western blot analyses of cleaved-caspase-3, pMLKL, and GSDMD-N in AAI-treated mTECs treated with FK-228. Data are presented as the mean ± SEM, n = 5. ns, not significant; *P < 0.05, significantly different, as indicated.

**FIGURE 4. Histone deacetylase (HDAC) inhibitor Romidepsin (FK228, Depsipeptide) upregulates Proline-serine-threonine-phosphatase-interacting protein 2 (PSTPIP2) expression in kidney epithelial cells in vivo and in vitro.**

(A) The PSTPIP2 protein levels in AAI-treated AKI mice administered with FK-228 analyses using western blot. (B) PSTPIP2 mRNA was assessed using real-time quantitative polymerase chain reaction (real-time qPCR) in AAI-treated AKI mice administered with FK-228. (C) The PSTPIP2 protein levels in AAI-treated mTECs treated with FK-228 analyses using western blot. (D) PSTPIP2 mRNA was assessed using real-time qPCR in AAI-treated mTECs treated with FK-228. (E) Chromatin immunoprecipitation assay of acetylated histone in the PSTPIP2 promoter region. (F) The H3K27Ac protein levels in AAI-treated AKI mice administered with FK-228 were analyzed using a western blot. (G) The H3K27Ac protein levels in AAI-treated mTECs treated with FK-228 analyses using western blot. Data are presented as the mean ± SEM, n = 5. ns, not significant; *P < 0.05, significantly different, as indicated.

**FIGURE 5. Proline-serine-threonine-phosphatase-interacting protein 2 (PSTPIP2) overexpression suppressed AAI-induced kidney injury and PANoptosis.**

(A) Schematic illustrating the genetic approach used for generating PSTPIP2-KI mice. (B) The schematic diagram depicts the animal experimental design. PSTPIP2-KI mice aged 6–8 weeks were treated with AAI and were euthanized three days after AAI treatment. (C) PSTPIP2 mRNA was assessed using PCR in PSTPIP2-KI mice compared with WT. (D) Creatinine (Cr) and blood urea nitrogen (BUN) were assessed in WT, AAI-treated WT mice, untreated PSTPIP2-KI, and AAI-treated PSTPIP2-KI mice three days after AAI treatment. (E) Hematoxylin-eosin (HE)-stained kidney tissue section (Scale bar = 40 μm). (F) Photomicrographs of immunohistochemical of cleaved-caspase-3, pMLKL, and GSDMD-N in WT, AAI-treated WT mice, untreated PSTPIP2-KI, and AAI-treated PSTPIP2-KI mice (Scale bar = 40 μm). Data are presented as the mean ± SEM, n = 5. ns, not significant; *P < 0.05, significantly different, as indicated.

**FIGURE 6. Proline-serine-threonine-phosphatase-interacting protein 2 (PSTPIP2) overexpression suppressed AAI-induced tubular epithelial cell PANoptosis.**

(A) Western blotting of cleaved-caspase-3, pMLKL, and GSDMD-N. (B) Western blotting of cleaved-caspase-3, pMLKL, and GSDMD-N in mTECs treated with pEX-2-PSTPIP2. (C) Immunofluorescence staining of cleaved-caspase-3, pMLKL, and GSDMD-N in mTECs treated with pEX-2-PSTPIP2 (Scale bar = 100 μm). Data are presented as the mean ± SEM, n = 5. ns, not significant; *P < 0.05, significantly different, as indicated.

**FIGURE 7. AAV9-mediated PSTPIP2 restoration re-induces renal injury and PANoptosis in PSTPIP2-KI mice.**

(A) Schematic illustrating the rescue experiment in PSTPIP2-KI mice. (B) Imaging of LUC-labeled AAV9 in mice. (C) Creatinine (Cr) and blood urea nitrogen (BUN) were assessed. (D) Hematoxylin-eosin (H&E)-stained kidney tissue section (Scale bar = 40 μm). (E) Photomicrographs of immunohistochemical of cleaved-caspase-3, pMLKL, and GSDMD-N (Scale bar = 40 μm). Data are presented as the mean ± SEM, n = 5. ns, not significant; *P < 0.05, significantly different, as indicated.

**FIGURE 8. FK-228 does not affect AAI-induced renal injury and PANopsis under PSTPIP2 silencing.**

(A) Western blotting of cleaved-caspase-3, pMLKL, and GSDMD-N. (B) Western blotting of cleaved-caspase-3, pMLKL, and GSDMD-N in mTECs treated with siRNA-PSTPIP2 and FK-228. (C) Immunofluorescence

staining of cleaved-caspase-3, pMLKL, and GSDMD-N in mTECs treated with siRNA-PSTPIP2 and FK-228. (Scale bar = 100 μm). Data are presented as the mean ± SEM, n = 5. ns, not significant; *P < 0.05, significantly different, as indicated.

**FIGURE 9. PSTPIP2 regulates caspase-8 and inhibits kidney injury by suppressing PANoptosis.**

(A) Protein level of caspase-8 in WT, AAI-treated WT mice, untreated PSTPIP2-KI, and AAI-treated PSTPIP2-KI mice analyses using western blot. (B) The protein level of caspase-8 in mTECs treated with pEX-2-PSTPIP2 analyses using western blot. (C) Photomicrographs of immunohistochemical of Caspase-8 in WT, AAI-treated WT mice, untreated PSTPIP2-KI, and AAI-treated PSTPIP2-KI mice (Scale bar = 40 μm). (D) Immunofluorescence staining of caspase-8 in mTECs treated with pEX-2-PSTPIP2 (Scale bar = 100 μm). Data are presented as the mean ± SEM, n = 5. ns, not significant; *P < 0.05, significantly different, as indicated.

**FIGURE 10. Caspase-8 level increases in AAV9-mediated PSTPIP2 inhibition mice.**

(A) Protein level of caspase-8 using western blot. (B) The protein level of caspase-8 in mTECs treated with siRNA-PSTPIP2 and FK-228 analyses by western blot. (C) Photomicrographs of immunohistochemical of caspase-8 in WT, AAI-treated WT mice, AAI-treated PSTPIP2-KI mice, and AAI-treated PSTPIP2-KI mice with AAV9 (Scale bar = 40 μm). (D) Immunofluorescence staining of caspase-8 in mTECs treated with siRNA-PSTPIP2 and FK-228 (Scale bar = 100 μm). Data are presented as the mean ± SEM, n = 5. ns, not significant; *P < 0.05, significantly different, as indicated.

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